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The isolation and characterization of bovine adult derived adipose stem cells for the use in nuclear transfer

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**THE ISOLATION AND CHARACTERIZATION OF
BOVINE ADULT DERIVED ADIPOSE STEM CELLS
FOR THE USE IN NUCLEAR TRANSFER**

**A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science
In
The Interdepartmental Program of
the School of Animal Sciences**

**By
Alicia A. Picou
B.S., Louisiana State University, 2007
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ABSTRACT

Since the cloning of Dolly there has been little change in the efficiency of nuclear transfer (NT). Research is beginning to investigate the characteristics of donor cells. Adipose tissue is an abundant source of adult-derived cells that have displayed “stemness” in-vitro (Gimble et al., 2003). The overall goal of this research was to define the in-vitro characteristics of bovine adipose-derived adult stem cells (ADAS) for the use in NT. Isolation methods were determined by a 3 x 3 factorial design. 1 g of subcutaneous fat was collected and subjected to 0.10%, 0.25% or 0.50% collagenase type I solution for 1, 2 and 3 h. Nucleated cells were counted using heochst stain. There was no significant difference ($P>0.05$) in number of nucleated cells released during the incubation period or collagenase concentrations. Viable cells were determined by those that remained adherent 24 h post plating. Incubation in 0.25% collagenase for 2 h had the consistently highest percentage of viable cells (45%). The lifespan and growth characteristics were determined by in a 2 x 2 factorial experiment of DMEM or DMEM:F12 supplemented or not supplemented with growth factors. DMEM with growth factors supplementation was significantly shorter lifespan ($P>0.05$) than DMEM:F12. The average lifespan was ~30 population doublings (PDs), with 1 cell cycle every two days until passage 8 (P8). Two bovine ADAS cell lines were differentiated into adipocytes, chondrocytes and osteoblasts at middle and late passages along side of adult derived skin fibroblasts. Differentiation was confirmed by histological staining resulting in early passage ADAS cells staining more intensely compared to late passage ADAS cells and skin fibroblasts. Global levels of DNA methylation and histone acetylation were analyzed from P1 to P6 in ADAS and skin fibroblasts from three animals. There was no significant difference ($P>0.05$) between cell types for DNA methylation or histone acetylation. The percentage of cleaved and developing blastocyst embryos from the ADAS cells (62% and 8%) and skin fibroblasts cells (42% and 8%) were not different ($P>0.05$). Interspecies nuclear transfer utilized eland ADAS cells into enucleated bovine oocytes. A total of 3 interspecies embryos (1%) developed to blastocyst.

CHAPTER I

INTRODUCTION

With the birth of Dolly (Wilmut et. al., 1997), it became clear that the technique of nuclear transfer utilizing fully differentiated somatic cells could be used to produce live animals. Since then, cloning or somatic cell nuclear transfer (SCNT) has been utilized for making transgenic animals and animal breeding. In many cases SCNT provides a way for limited populations to expand their genomes where other options are limited.

Over the past 11 years, the efficiency of NT has not increased dramatically. There are several reasons for the failure of nuclear transfer including asynchrony of cell-cycles between nucleus and cytoplasm or failure of the donor cell nucleus to reprogram (Dean et al., 2001). A great deal of research has focused on synchronizing the cell-cycles of oocytes and donor cells, however, researchers are shifting their focus to the type and status of donor cells used (Bosch et al., 2006; Giraldo et al. 2007). Commonly fetal fibroblasts are utilized as donor cells for NT. Embryonic stem cells (ES cells) have shown promise as karyoplasts for nuclear transfer but these cell types are not available for species other than mice. Skin fibroblasts are a viable alternative but have limited proliferative capacity and contain substantial chromosomal abnormalities when cultured in vitro (Azad and Woodruff 2006; Giraldo et al., 2007). These factors could contribute to the low numbers of successful nuclear transfer. It has been hypothesized that somatic stem cells may possess “stemness” characteristics in culture. Adipose tissue has been shown to possess some of these “stemness” characteristics (Gronthos et al., 2001). Adipose tissue can be collected readily by minimally invasive procedures and thus is an attractive target for this application. Characterizing in vitro culture of bovine adipose tissue will provide useful information that can be applied to this and other bovid species.

The overall goal of this research is define and characterize the culture conditions for bovine adult derived adipose tissue. More specifically, defining the in vitro characteristics to determine if adult derived adipose stem cells (ADAS) are a viable donor cell source for nuclear

transfer. The results of this research could potentially provide an efficient cell population that is alternative to skin fibroblasts or embryo derived tissues for donor cells for of nuclear transfer.

CHAPTER II

LITERARY REVIEW

Stem Cells “Stemness” Defined

Stem cells share the defining characteristics of unspecialized cells that self-renew for long periods of time. Under the correct induction conditions, they can be induced into specialized cell types (Roeder and Lorenz, 2006). The defining self-renewal and multi-lineage differentiation characteristics of stem cells are encoded by a shared set of genes that are expressed by all distinct stem-cell populations. This therefore represents a conserved stem-cell molecular signature (Ivanova et al., 2003). The best characterized stem cells are embryonic (ESCs), neural (NSCs) and hematopoietic (HSCs) (Ramalho-Santos et al., 2002). The common characteristics of the previously listed stem cells are abundant with JAK/STAT pathways, TGF- β pathways, chromatin-remodeling helicases, SNF2/SWI2 family (Ramalho-Santos et al., 2002) and protected from differentiation signals at most times (Blau and Blatimore, 1991). Oct4 and Nanog are crucial to the molecular signature. In the absence of Oct4, ESCs transdifferentiate into trophectodermal cells (Eckfeldt, 2005). All of these genes are in relation to maintaining pluripotency and self-renewal. The microenvironment controls expansion and differentiation of the “stem cell state”; the balance and composition of proteins that surround it require constant maintenance and regulation in order to function correctly (Blau and Blatimore, 1991). Other related genes found are Notch pathway, DNA methylases, or transcriptional repressors of the histone deacetylase but these genes are not consistently found in all stem cell types (Ramalho-Santos et al., 2002). In classic embryology, true embryonic stem cells are totipotent and can give rise to any other cell type. Stem cells in adult tissues are generally restricted to their differentiated cell type potentials (Sell, 2005). However, there is some evidence that at least some adult stem cells are highly plastic and amenable to transdifferentiation.

This adult plasticity may be due to a pool of dormant, non-proliferating stem cells formed during embryogenesis (Kay, 1965). Consequently, under differentiation conditions, individual cells from

this reserve can be activated to initiate the production of a differentiated clone. Terms such as transdifferentiation occurs when a non-stem cell transforms into a different type of cell, or when an already differentiated stem cell creates cells outside its already established differentiation path. There are many studies indicating that transdifferentiation is possible using a multitude of cell sources into many different cell types. It is widely accepted that the key to differentiation are growth factor supplementation or environmental signals (Roeder and Lorenz, 2006).

There are many inconsistencies by which true “stemness” among different cell types is defined. Some suggest that these inconsistencies are related to the methods by which stem cells are isolated from heterogeneous cell populations. Populations of stem cells are often sorted by fluorescence-activated cell sorting (FACs) to select cells based on expression of specific cell surface proteins. The differences between expression levels of the marker proteins are not always consistent (Blau et al., 2001) and the undetectable differences of surface markers result in a heterogeneous population of cells. There are the possibilities that stem cells maintain multipotency in other surface markers or “stemness” is transiently expressed (Fortunel, 2003) which also make it difficult to select cells from a heterogeneous cell population.

There is not one characteristic that can definitively defines “stemness”; the cross-validation of researchers is necessary (Fortunel, 2003). There are a multitude of stem cell sources that can participate in tissue repair or differentiate into specific lineages. Therefore, “stemness” most accurately refers to a biological function rather than a discrete cellular entity (Blau et al., 2001).

Embryonic Stem Cells

The embryo reaches the blastocyst stage during early embryonic development. The blastocyst is composed of three structures; the trophectoderm, which is the layer of cells that will make up the placenta during embryonic development. The blastocoels is a hollow cavity inside the blastocyst. The third structure consists of the inner cell mass (ICM), where the pluripotent stem cells are derived. The cells derived from the ICM give rise to the three primary germ cell layers: mesoderm, endoderm and ectoderm. As the ICM cells differentiated they

become more restricted in their development. The multipotent stem cells are what comprise the mature and specific tissue (Eckfeldt, 2005). ICM cells can be isolated and cultured in vitro and are referred to as embryonic stem cells (ESC). The ability to culture ESCs is limited to a few species, namely mice, humans and the rhesus monkey (Pain et al. 1996 and Thomson and Marshall, 1998). The first ESC were isolated and established in 1981 by Evans, et. al. Then in 1998, human embryonic stem cells (hESCs) were successfully cultured and characterized from the genital ridge of blastocysts. Since the first success of ESC culture, much of the transcriptome and proliferative characteristics have been evaluated in the murine species.

ES cells undergo symmetric self-renewal which is accomplished by the creation of two identical daughter cells (White and Dalton, 2005, Roeder and Lorenz, 2006). ESCs also have unlimited proliferative ability that is not anchorage dependent or subject to contact inhibition (Walton and Dalton, 2005). Their rapid proliferation is attributed to their unusual cell cycle. The cell cycle generally consists of four phases: the S phase, M phase and two gap phases (G_1 and G_2). In the case of murine ESCs the cell cycle lacks the gap phase cycle, where a high proportion of cells are in the S phase (White and Dalton, 2005).

There are some differences noted about the transcriptome of ESCs between species. Yet it is agreed that the expression of Oct4 and Nanog are crucial to the characterization of ESCs (Mitsui et al., 2003; Chambers et al., 2003; Nichols et al., 1998). Oct4 is a mammalian pituitary-specific octamer (POU) transcription factor expressed by early stage embryo cells and germ layer cells. If embryos lack Oct4 expression, they can develop to the blastocyst stage, but the inner cell mass cells are lacking or are no longer pluripotent (Nichols, 1998). In contrast, if Oct4 is overexpressed, ESCs differentiate into primitive endoderm and mesoderm cells (Chambers, 2004). Nanog is a divergent homeodomain protein that directs the expansion of undifferentiated ESCs. In preimplantation embryos, Nanog is restricted to the founder cells from which ES cells can be derived; however, Nanog and Oct4 expression alone are not sufficient to prevent ES cell differentiation (Niwa et al., 2000). Nanog acts in parallel with stimulation of

Stat3 to drive ES cell self-renewal. Oct4 and Stat3 also interact and with other cofactors to regulate the expression of specific target genes (Refer to Figure 2.1).

POU factors are complex multifunctional proteins that mediate protein-protein interactions that can either activate or repress transcription depending on the circumstance (Nichols, 1998).

Leukemia inhibitory factor (LIF) has been utilized to maintain the self renewal. STAT3 is the key downstream transcription factor of the LIF pathway in ES cells (Mitsui et al., 2003). There are several transcription factors that are essential for pluripotency in the ICM, but none of them act independently of LIF/Stat3 (Mitsui et al., 2003). Thus, Oct4, Nanog and Stat3 all work together in a highly orchestrated manner to either promote or prevent pluripotency.

Stat3 is expressed in many cell types and is required for differentiation (Nakajima et al., 1996). Oct4 is inactivated through a series of epigenetic modifications following uterine implantation. Oct4 is also present in a variety of somatic stem cells and tumor cells. Oct4 may maintain self-renewal and perhaps promote tumor formation in somatic cells (Lengner et al., 2007). Some somatic cells possess some of the same gene expression as seen in prolific ES cells. Oct4 is the most frequently reported in hematopoietic and mesenchymal stem cells of humans and mice as well as multipotent progenitors (Nayernia et al., 2006). Though transcription factor reprogramming of fibroblast cells, ectopic expression of the four transcription factors Oct4, Sox2, c-Myc and Klf4 can be accompanied (Maherali et al., 2007). Expression of the four transcription factors leads to global reversion of the somatic epigenome into an ES-like state (Maherali et al., 2007). Due to the limited number of species in which embryonic stem cell cultures have been accomplished and the controversy surrounding human ES cells, research has shifted to adult derived stem cells that possess some of these same stemness qualities.

Adult Derived Stem Cells

Adult stem cells are undifferentiated cells among the differentiated cells of a specialized tissue. The cells also possess many of the capabilities that resemble those of more immature, primitive, pluripotent cells, similar to ES cells. The primary objection to using ES cells, particularly human derived, is due to the fact that a live embryo must be destroyed to obtain the

cells (reviewed by Kamm, 2005) thereby avoiding this controversy, will enable researchers to advance technologies and future research.

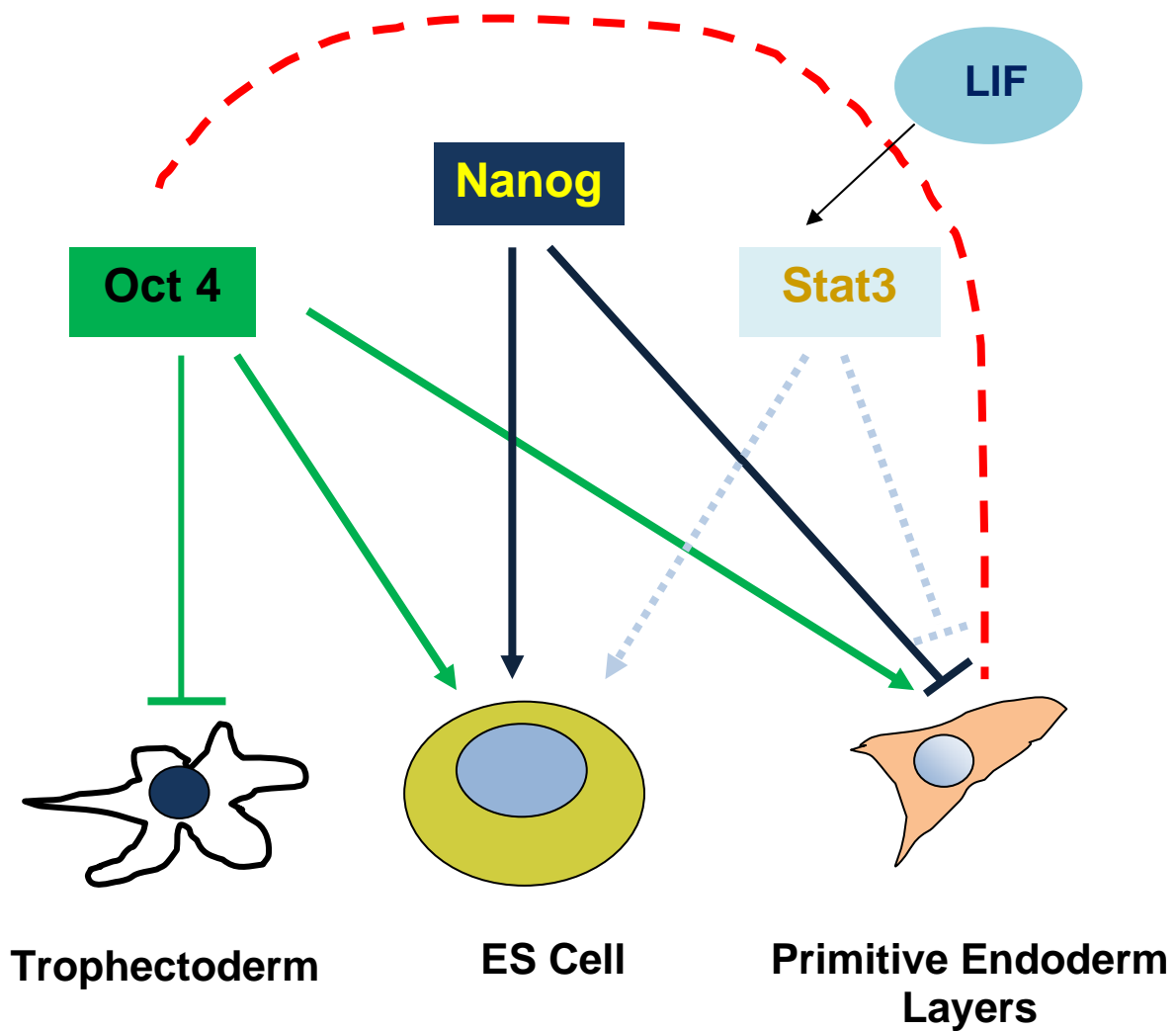
There are many questions about whether or not stem cells in adult tissues have the same properties as ES cells including the ability to differentiate into other tissues. In many cases, adult derived stem cells are restricted to differentiation into one (unipotent), two (bipotent), or several (oligopotent) cell types (Sell, 2005). Mesenchymal stem cells (MSCs) are a type of somatic or adult stem cell which have pluripotent precursors and are an important source for human cell-based therapies (Bosch et al., 2006). Gene and protein expression profiles have been defined in some species, but the information is largely limited to humans.

Equine blood-derived fibroblast-like cells have also displayed the capability to differentiate into adipogenic, chondrogenic and osteogenic lineages (Giovanni, et al., 2008). Bovine pluripotent stem cell lines derived from embryos or primordial germ cells are a plentiful source of pluripotent cells. However, placental development of NT fetuses derived from these cells as donor cells is incomplete (Saito et al., 1992). Pluripotent stem cells will become more valuable after demonstration of germ line transmission to give a full term live calf.

Bovine BMSC differentiate into various cell lineages, but there are limitations for the use of BMSCs. The collection process is painful and can lead to collection site contamination (Giovannie et al., 2008). Therefore, an adult-derived stem cell source that can be easily collected is of interest to many researchers to facilitate tissue engineering applications with adult stem cells.

Adipose Derived Stem Cells

Adipose tissue is an attractive source for adult stem cells due to its abundance, plasticity in culture and relative ease of collection. Recent reports demonstrate that adipose tissue has the potential to differentiate into other cell types, as well as have the potential for clinical applications. In nearly every tissue, there is a population of multipotent stem cells.



(Figure 2.1). Oct 4 and Nanog are essential to sustain ES cell characteristics. Oct 4 blocks trophoblast differentiation but tends to promote differentiation into primitive endoderm layers. Nanog and Stat3 block this function of Oct4 unless down regulated to commit to differentiation (red line). (Chambers et al., 2003)

Several groups have demonstrated that mesenchymal cells within the stromal-vascular portion (SVF) of subcutaneous adipose tissue display multilineage potential and plasticity in vitro and in vivo (Zuk et al., 2001, 2002; Gimble et al., 2003; Erickson et al., 2002; Safford et al., 2002 and Halvorsen et al., 2001). These cells are often referred to as “processed lipoaspirate cells” (PLA), ADAS (adipose derived adult stem cells), mesenchymal progenitor cells or preadipocytes. The various names reflect the limited information surrounding of their origin, function and phenotype (Katz, 2005).

The most common source of adult stem cells is mesenchymal stem cells (MSCs) and most studies have investigated those isolated from bone marrow (BMSCs). These cells have been studied extensively and have the capability to differentiate into multiple mesenchymal tissues such as bone, adipose and cartilage. Several lines of evidence indicate that adipose tissue contains some of the same characteristics of stemness seen in ES cells (Cao et al., 2005).

There are commonalities between ADAS cells and BMSCs. Human ADAS (hADAS) cells are adherent and have a fibroblast-like morphology, similar to BMSCs. The morphology remains consistent through subcultures and under non-stimulating conditions (Coa et al., 2005). Undifferentiated hADAS are characterized by being positive for the cell-surface markers cluster differentiation 10 (CD10), CD13, CD29, CD44, CD49e, CD59, CD90 and HLA-ABC and negative for the markers CD11b, CD45, and HLA-DR. The absence of CD45 is significant because it indicates that hADAS cells do not originate from bone marrow hematopoietic stem cells (Gimble et al., 2004). Some hADAS cells also express SH3 (Zuk et al., 2002), which is considered a marker for BMSCs (Haynesworth et al., 1992) but lack other hematopoietic markers. Some of these differences may reflect the proliferative stage of the cells in culture or the donor heterogeneity. More studies are needed to fully define the differences between bone marrow-derived stromal cells and adipose-derived stromal cells (Gronthos et al., 2001).

Many studies have investigated stem cell surface markers for ADAS cells (Table 2.1). Currently, there are no known stem cell specific markers for the identification of putative stem

cells or progenitor cells within adipose tissue, bone marrow, or any other mesodermally derived adult tissue (Katz, 2005). Nonetheless, several proteins have emerged as candidate markers associated with a primitive stem cell phenotype.

In addition to surface cell markers, other studies have included evaluation of the clonal differentiation potential of hADAS. By evaluating the multipotent potential at the clonal level it can be determined if the differentiated phenotypes originate from subsets of committed progenitor cells within a heterogeneous population.

Guilak et al. (2006) isolated clones from lipoaspirates by using cloning rings and induced adipogenesis, osteogenesis and chondrogenesis using lineage specific differentiation media. Cloning rings are small plastic rings that are used to isolate individual cell colony. Differentiation was confirmed by Oil Red O (adipocytes), Alzarin Red (osteocytes) staining and spectrophotometric readings. These confirmation techniques consisted of measuring leptin production, alkaline phosphatase and determination of the amount of sulfated glycosaminoglycan produced, respectively. The majority of hADAS cell clones can differentiate into two or more of the adipogenic, osteogenic and chondrogenic-like cells. Human ADAS are also able to undergo numerous population doublings while retaining their differentiation capabilities. Differentiation induction and continued population doublings in culture gives an indication that hADAS share similarities with ES cells.

Differentiation induction has been successful in several species. Human adipose tissue stem cells will take on cardiomyocyte properties following transient exposure to rat cardiomyocyte extracts (Gaustad et al., 2003) and expanded mesenchymal cells function as stromal cells in vitro in human MSCs (Majumdar et al., 1998). Rabbit MSCs have been shown to be an effective way to regenerate a severed rabbit tendon (Young et al., 1998). The specific cues that are needed for cell differentiation to occur are still unknown. It has been proposed that different assay conditions, basal nutrients, cell density, spatial organization, mechanical force, growth factors and cytokines all influence differentiation. Autocrine and paracrine factors may also be involved in lineage progression (Pittenger et al., 1999). Epigenetic differences may also

account for variability. Noer et al. (2006) used bisulfite sequencing analysis of CpG (cytosine phosphate guanine) methylation of adipogenic genes in freshly isolated human ADAS cells and in cultured ADAS cells to study gene expression and differentiation potential. Bisulfite sequencing enables identification of individual methylated cytosines in single DNA molecules (Grunau et al., 2001; Warnecke et al., 2002.) Noer et al. (2006) observed that mosaic DNA methylation of adipogenic DNA promoter sequence in ADAS cells remains stable in vitro and during differentiation. Conversely, non-adipogenic loci, were highly methylated, concluding that DNA methylation does not seem to be the sole determinant of differentiation potential of ADAS cells.

The differentiation potential of adipose stem cells suggests utility for “tissue engineering”. Tissue engineering consists of growing tissues in vitro for the use of in vivo repairs such as cartilage repair by transplanting cells or engineered constructs into an injured site (Brittberg et al., 1994). ADAS cells have shown chondrogenic potential by abundantly synthesizing cartilage matrix molecules including collagen type II (Zuk et al., 2002), proteoglycans (Awad et al., 2003) and chondroitin 4-sulfate (Gimble and Guilak, 2003). Differentiated cells have been injected into nude mice where they continued to produce the matrix molecules, further suggesting their potential in tissue engineering (Erickson et al., 2002). In vivo fat and bone formation has been successful in rat models as well (Parrette et al., 2003). ADAS cells show potential for pro-angiogenic therapies. Cultured and expanded hADAS cells were differentiated into endothelial cells and injected into ischemic hind limbs of mice.

The injections of the endothelial cells improved bloodstream perfusion to the limbs and contributed to angiogenesis of the mice. Results were confirmed by Doppler imaging and by RT-PCR of human endothelial cell markers CD31, CD34, CD144 and eNOS (Gaustad et al., 2004). Currently, only preliminary data exists for ADAS cells in livestock species. Porcine adipose have been isolated successfully and found to be positive for the cell surface markers CD34 (Williams et al., 2008), similar to the hADAS cells. Surface protein profiles of early passage cells (P to P4) are not similar to humans.

(Table 2.1) Summary of cell surface markers found in human ADAS cells.

Surface Proteins	Species	Authors
CD4/MHC class II co-rec., T4		Katz et al., 2009
CD9/Adhesion receptor molecule/Tretaspan	Human	Gronthos et al., 2001
CD10/metalloproteinase/CALLA	Human	Gronthos et al., 2001; Aust et al., 2004
CD13/metalloproteinase/Aminopeptidase N	Human	Gronthos et al., 2001; Aust et al., 2004
CD29/intergrin β_1 /fibronectin receptor	Human	Gronthos et al., 2001; Katz et al.2009; Coa et al., 2005; Gronthos et al., 2001; Aust et al., 2004
CD34/ Hematopoietic	Human	
CD44/Adhesion receptor molecule/Hyaluronate	Human	Gronthos et al., 2001; Coa et al., 2005; Gronthos et al., 2001; Aust et al., 2004
CD49 _d /intergrin α_4 , CD49 _c /intergrin α_3	Human	Gronthos et al., 2001; Katz et al., 2009
CD49b/intergrin α_2 /ITGA2 ^b , CD49d/intergrin α_4 /ITGA4 ^b		
CD49 _e /intergrin α_a	Human	Gronthos et al., 2001; Katz et al., 2009; Aust et al., 2004
CD49 _e /VLA-5/intergrin α_5 /fibronectin receptor		
CD49 _f /VLA-6/intergrin α_5 /lamin receptor		
CD54//Adhesion receptor molecule/ICAM-1	Human	
CD55/other/DAF	Human	
CD59/other/complement protection	Human	Katz et al., 2009; Aust et al., 2004
CD105/Adhesion receptor molecule/(SH2)Endoglin	Human	Coa et al., 2005
CD106/Adhesion receptor molecule/VCAM	Human	
CD146/Adhesion receptor molecule/Mus18	Human	
CD166/Adhesion receptor molecule/ALCAM	Human	Coa et al., 2005
CD51/Intergrin α_V (ITGA V)/vitronectin receptor	Human	Katz et al., 2009
CD61/Intergrin β_3 /ITGB3/gpIIla	Human	Katz et al., 2009
CD61/Intergrin β_5 /ITGB5		
CD18/intergrin2	Human	Katz et al., 2009
CD90	Human	Aust et al. 2004; Gronthos et al., 2001
Fik1	Human	Coa et al., 2005
HLA-ABC	Human	Aust et al. 2004; Coa et al., 2005
CD41b/Integrin α_2 b(IGTA2B) ^b	Human	Katz et al., 2009
Integrin α_X (ITGA X)b CD11c	Human	Katz et al., 2009
Cadherin 5 (CDH 5); [VE-cadherin (vascular epithelium)] CD144	Human	Katz et al., 2009
GPIV (CD36) CD36	Human	Katz et al., 2009
CEA (CEACAM 5) CD66e		Coa et al., 2005
Endothelial leukocyte adhesion molecule I (SELE); [ELAM 1]		
CD62e		
Intercellular adhesion molecule 1 (ICAM 1) CD54		
Platelet/endothelial cell adhesion molecule (PECAM 1) CD31		
Vascular cell adhesion molecule 1 (VCAM 1) CD106		
Deleted in colorectal carcinoma (DCC)b		
Neural cell adhesion molecule 1 (NCAM 1)b CD56		
Endoglin (ENG)2–3x CD105	Human	Katz et al., 2009

Adipose tissue has been isolated successfully from bovine as well as from the common eland (*Taurotragus oryx*) (Picou et al., 2009). The bovine tissue has been shown to be viable in culture for long periods of time and can be differentiated into different cell types. Equine adipose tissue-derived stromal cells have a different in vitro growth rate, adipogenic and osteogenic differentiation potential than other mammalian species (Vidal et al., 2007). Vidal et al. (2006) described the cell growth characteristics of equine BMSCs compared to ADAS cells. Cell doubling time of ~ 2 days for equine ADAS cells was more rapid than the ~ 4 days required for human ADAS (hADAS) (Mitchell et al., 2006) suggesting a difference in cell doubling time among species. Vidal et al. also documented that the primary cultures of the stromal vascular fraction of ADAS cells appeared to be heterogeneous in nature compared to the BMSCs. Interestingly; the primary culture ADAS cells had fewer cells with fibroblast morphology than did the primary BMSCs. The ADAS cells did not display a lag time for initial culture as observed with MSCs (Vidal et al. 2006). Successful isolation of adipose and bone marrow derived stem cells from these species is promising, but more information about their culture plasticity is needed.

Methodology of Nuclear Transfer

Somatic cell nuclear transfer in amphibians was first reported by Briggs and King (1952) to address the question of genetic equivalency; the nucleus of a somatic cell is genetically identical to the nucleus of a zygote. Willadsen (1986) utilized nuclear transfer with embryonic cells as donor cell nuclei for the production of identical viable offspring in domestic sheep. Rob et al. (1987) was the first to report techniques of nuclear transfer with bovine embryos, that culminated with the birth of calves (Prather et al., 1987). Campbell et al. (1997) then announced the success of Dolly, the first cloned mammal from somatic cell nuclei.

Nuclear transfer or “cloning” consists of several steps. In mammalian species, the standard protocols use mature metaphase II (MII) oocytes. The MII oocytes can be collected in a variety of ways, each with species specificity and limitations. The MII oocyte is the recipient cell of choice owing to the lack of development obtained using enucleated zygotes (Campbell, 2002).

The genetic material of the oocyte is located in the meiotic plate. The meiotic plate is removed by micromanipulation with a small beveled glass pipette or by chemical means. This step is often referred to as “enucleation”. It is important to note that the oocyte nucleus has undergone germinal vesicle breakdown prior to ovulation. As nuclear maturation is reinitiated, the membrane of the germinal vesicle disintegrates and this swollen nucleus disappears.

The location of the polar body indicates the location of the metaphase plate. In most cases, it is assumed that the genetic material is located behind the polar body. Correct focus planes can be confirmed by “tapping” on the oocyte, and the polar body should “bounce” when in the correct position. At this position, a small puncture is made into the zona pellucida. Once inside the perivitelline space, negative pressure is applied to slowly remove the genetic material and polar body. Removal of the genetic material can be confirmed by DNA-specific fluorescent, Hoechst 3332. The fluidity of the cell membranes allows the oocyte to reseal following manipulation (Campbell et al., 1998).

Once the oocytes are enucleated, reconstruction consists of injecting the donor cell or donor cell nucleus into the perivitelline space of the recipient oocyte and fusion of the donor cell with the oocyte. Electrofusion (using DC electrical pulse) is one the most common methods for cell fusion. Following fusion, a variety of techniques can be used to activate the oocyte or cause the induction of fertilization responses (Campbell, 2002). At this point the reconstructed embryos are able to begin development, but there are many factors that are involved with the successful development of cloned embryos.

Nuclear Transfer Using Stem Cells

Researchers are currently investigating a more acceptable source of donor cells for somatic cell nuclear transfer (SCNT). Currently, the qualities of a good donor cell are unknown. There are more than 200 distinct cell types in mammals that are distinguishable by morphology and more will be identified as more surface markers are discovered. Only a small fraction of these cell types have been tested as nuclear donors for SCNT (refer to Table 2.2, summarized by Obak and Wells, 2002). Of the cell types tested, they all have made it to blastocysts stage, but the

efficiency of full term development remains low (Niemann et al., 2002 and Han et al., 2003) with most embryos dying at the pre-implantation stage. Most attribute the developmental failure to the asynchrony between the donor cell nucleus and oocyte (Rideout et al., 2001) inhibiting the complete or correct epigenetic reprogramming of the donor cell nuclei upon transfer of the donor cell nuclei to the enucleated oocytes (Rideout et al., 2001). It is believed that reprogramming of the donor cell nuclei must be completed so that zygote transcription commences (Obach, et al., 2002). The cell cycle stage of donor cells play a role in the initial survival of the clones, because cells in G₀ or G₂ appear to be the most efficient donor cells for NT, resulting in a higher proportion of live births than cells in other stages of the cell cycle (Campbell et al., 1996 and Cibellie et al., 1998). However, approximately 60% of ES cells are in the S-phase of the cell cycle (Rideout et al., 2001). The S-phase of the cell cycle is an undesirable state due to the nucleus being exposed to high levels of maturation promoting factor (MPF). MPF in the MII oocytes promotes nuclear breakdown and premature chromatin condensation. As a result of MPF exposure, the embryos can develop abnormal ploidy (Guiraldo et al., 2007).

There is evidence that a less differentiated donor-cell may marginally improve the success of NT to the blastocyst stage. Embryos that are derived from ES cells are more successful than embryo clones derived from somatic cells such as cumulus cells (Rideout, et. al. 2001 and Obach, et. al., 2002). Murine karyoplasts from G₁ stage zygotes, had a 34% success rate compared to a 3% success rate of cumulus derived embryos (Takashi and Solter, 2005). Takashi and Solter (2005) have proposed that a 20% increase in the efficiency of somatic cell nuclear transfer by improving the reprogramming of donor cells. ES cell NT embryos that reach the blastocyst stage develop to term 10- to 20-fold more efficiently than those from somatic cells (Rideout et al., 2001). This may be due to less reprogramming required for the ES cell nucleus compared to that of a nucleus of a differentiated somatic cell (Hiiragi and Solter, 2005; Rideout, et. al., 2001).

Thus far, ES cells are limited to the mouse. There have been reports of embryonic cell lines derived from domestic farm species (cattle, Sato et al., 1992; sheep and pigs Notarianni et

al., 1990). The cell lines maintain normal karyotype through late passages as well as differentiate in vitro and form embryoid bodies, but there is a lack of evidence of their developmental capacity (Stice et al., 1996). In a study by Stice et al. (1996), over a thousand NT embryos were produced using bovine embryonic cell lines (either morula or blastocyst-stage) as donor cells. The embryos were capable of developing through early organogenesis, however, the embryos were unable to reach developmental capacity greater than 85 days (Stice et al., 1996). The fetal deaths were attributed to abnormal placental development. Maternal response did appear to occur, but proper placentome formation did not take place. Other bovine NT investigations had a high incidence of pregnancy loss and placental abnormalities using blastomeres as donor nuclei (Wiladsen et al., 1991). It is important to note that there have been reports of NT calves derived from inner cell mass (ICM) cells suggesting that fetuses or offspring could be produced using embryonic cells as donor cell nuclei (Keefer et al., 1994). Therefore, finding a somatic cell population that has many of the same qualities of ES cells could improve the efficiency of SCNT. Bosch et al. (2006) utilized porcine BMSCs as donor cells in nuclear transfer and embryos developed more efficiently than embryos derived from skin fibroblasts.

Interspecies Nuclear Transfer

Interspecies nuclear transfer consists of a recipient oocyte being of a different species than the donor cell species. Somatic cell nuclear transfer represents a methodology to reintroduce valuable genomes into critical populations where no other options are available. There have been several demonstrations of the utility of nuclear transfer technology for the preservation of endangered species. Many species had interspecies embryos that have developed to the blastocyst stage, for example, bovine and porcine cells into ovine oocytes (Hamilton et al., 2004), buffalo cells into bovine oocytes (Lu et al., 2005), eland (*Taurotragus oryx*), bongo (*Tragelaphus euryceros*), banteng (*Bos javanicus*) and yak (*Bos grunniens*) cells into bovine oocytes (Nel-Thematt et al., 2008; Lee et al., 2003; Sansinena et al. 2003; Murakmi

et al., 2005). Endangered animals have successfully been produced from somatic cell nuclear transfer e.g., African Wildcat (*Felis silvestris lybica* (Gomez et al., 2004) and gaur cattle (*Bos gauras* (Lanza et al., 2000) but the success rates are minimal.

Interspecies nuclear transfer is faced with additional problems. In previous studies, there was not a significant difference in cleavage and fusion between interspecies embryos and standard NT embryos, but there was always a decreased amount of embryos that progressed beyond the 8-cell stage and even less to blastocysts. This could be due to the genetic divergence between the donor nucleus and recipient cytoplasm (Hamilton et al., 2003), In addition to the genetic differences between donor and recipient on the developmental competence (Murakami et al., 2005), there are also species specificity that is unknown, such as mitochondria differences and activity of unknown factors in oocyte cytoplasm (Murakami et al., 2005). Furthermore, if two species are very distantly related, than reprogramming would become less efficient. Placentation appears to be the greatest limiting factor of cloning in the conservation effort (reviewed by Ryder and Benirschke, 1997). There are great differences seen in both structure and function among all species. At present, there has only been success when hybridization between the species in which embryo transfer is practiced (reviewed by Ryder and Benirschke, 1997).

Epigenetic Reprogramming in Mammalian Nuclear Transfer

As previously noted, various cell types have been used as nuclear donors. The success varies considerably among cell types and even among different cell lines within the same cell types (Makoulaki et al., 2008). It is likely that epigenetics plays a role in this variability. Epigenetic changes are heritable, reversible gene expression changes that occur without alterations in the DNA sequence (Wolffe and Matzke, 1999). The cell types of all higher organisms, with the exception of lymphocytes have been derived from a single totipotent fertilized oocyte. All of these cells are functionally and morphologically different due the differences in gene expression patterns arising in a temporally controlled manner (Shi et al., 2003). Genome-wide epigenetic

Table 2.2. Somatic Cells utilized for nuclear transfer (Obach and Wells, 2002)

Tissue Origin	Cell Type	Species
Embryonic/fetal/newborn	Fibroblasts	Cattle, goat, mouse pig, sheep
Embryonic Disc	Epithelial	Sheep
Testis	Immature Sertoli	Mouse
Gonadal	Fibroblast*	Cattle
Liver	Fibroblast	Cattle
Skin	Fibroblast	Cattle, Goat
Adult		
Mammary gland	Epithelial*	Cattle, Sheep
Follicle	Granulosa	Cattle, Pig
Follicle	Cumulus	Mouse
Testis	Mature Sertoli	Mouse
Oviduct	Epithelial*	Cattle
Tail-tip	Fibroblast	Mouse
Skin	Fibroblast	Cattle
Thymus	Lymphocytes	Mouse
Peritoneal Cavity	Macrophages	Mouse
Spleen	Leukocytes	Mouse
Blood	Leukocytes	Cattle
Brain	Neuron, Glia*	Mouse

*Presumptive cell type.

reprogramming in germ cells is needed for proper genomic imprinting to occur (Dean et al., 2003). Epigenetic modification ensures proper gene activation during development (Rideout et al., 2001). During normal mammalian fertilization two parental genomes must come together and reprogram in order to make a new individual. There are two phases during mammalian embryonic development that incorporate epigenetic reprogramming. The first is associated with genomic imprinting and the second occurs throughout the preimplantation period (Dean et al., 2003). These epigenetic changes are widely thought to be controlled by DNA methylation, discussed in further detail later.

The successful cloning of animals by transferring somatic nuclei into enucleated oocytes demonstrates the epigenetic reprogramming required for the somatic nuclei totipotency to be restored. During cloning, the donor nucleus must be transcriptionally silenced, have appropriate activation and support proper gene expression throughout development. Increasing experimental evidence across many species has indicated that the abnormalities of cloned animals in many species are due, at least in part, to incomplete establishment of or lack of reprogramming of epigenetic states (Young et al., 2001). After the genetic material has been inserted and fused with the enucleated oocyte, the challenges of epigenetic reprogramming consist of: DNA methylation, X chromosome inactivation, genomic imprinting, chromatin remodeling, histone modifications, maintenance of telomeres, and epigenetic inheritance (Shi et al., 2003). Failure of reprogramming suggests a possible dysregulation of epigenetic control. However, very little is known about the actual mechanisms that occur during reprogramming. It has long been speculated that reprogramming is controlled by DNA methylation.

DNA Methylation and Histone Acetylation

Methylation commonly occurs at the 5'-cytosine residues of CpG nucleotides. CpG islands are regions of high concentrations of CG (cytosine guanine) dinucleotides. The "p" in CpG notation refers to the phosphodiester bond between the cytosine and guanine. CpG methylation is associated with genomic regions with transcriptional repression (Fujita and Wade, 2004). The 5-cytosine residues are considered the best mechanism to trace global methylation patterns

during preimplantation development since DNA methylation is generally well conserved among animals. CpG islands are generally unmethylated in normal tissues and frequently span the 5' end (promoter, untranslated region and exon 1) of a number of genes (Shi et al., 2003). Methyl groups accumulate and bind at the methyl CpG. A protein exchange can result in the loss of the methyl-CpG-binding domain proteins (MBD) (Fujita and Wade, 2004). With MBD proteins absent, the chromatin becomes less compact resulting in an open chromatin structure that still contains cytosine residues. With supporting enzymes and assembly of an active transcription complex, initiation of transcription can then begin.

The most common case of CpG island methylation is X chromosome inactivation. After fertilization, female mammalian cells have two active X chromosomes. During embryonic development, one of the X chromosomes in each cell becomes condensed and genetically inactive. This inactivation takes place at implantation. At implantation both the paternal and maternal X chromosomes have equal probabilities of becoming inactive, thus, all somatic cells of a placental mammalian female display "random X inactivation" (Riggs, 1984). Other observations of DNA methylation occur in imprinted genes, germ-line and tissue-specific genes (Jaenisch and Jahner, 1984). In mammals however, a small proportion (<1%) of genes are imprinted, meaning that gene expression occurs from only one allele (Wilkinson et al., 2007).

Histone acetylation is closely related to the function of the DNA methylation and gene transcription. Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. This promotes RNA polymerase and transcription factor access to the promoter region. Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription.

Trichostatin A (TSA), a histone deacetylase inhibitor, has been investigated to aid the reprogramming of the donor cell. Treating donor cells with TSA causes an increase in acetylated levels, but has no effect on the DNA methylation levels following treatment (Enright

et al., 2003). There is speculation of TSA is toxic to embryos because treating with TSA leads to lower blastocyst development rates and offspring production (Kishigami et al., 2006).

Donor Cell DNA Methylation and Histone Acetylation

The epigenetic status of the donor cell nucleus can affect the success of SCNT. During normal development, DNA methylation and histone acetylation is a dynamic process. The main purpose of the epigenetic system is to regulate the repression of those genes not required in specific stages or cell types without DNA sequence changing (Wolffe and Matzke, 1999). It is generally agreed among researchers that the lack of success in cloning is due to the failure of reprogramming and many are turning their attention to altering the epigenetic state of the donor nucleus. Since gene expression is controlled by DNA methylation and histone modification, studies suggest that donor cell nuclei should express transcription factors Oct-4, Nanog, Sox2 and be hypomethylated to facilitate reprogramming (Boiani et al., 2005). Blomch et al. (2006) showed that the differentiation and methylation state of the donor nucleus are a critical component of the epigenetic program that influence cloning efficiency. Somatic stem cells could reprogram more efficiently than their differentiated counterparts by increased genomic hypomethylation. Using donor fibroblasts carrying a hypomorphic allele of the DNA methyltransferase Dnmt1, which results in the global hypomethylation, there is a significant increase in efficiency of cloning (Blomch et al. 2006).

The cell cycle stage of the donor cell seems to play a critical role in cleavage development. It has been reported that cell cycle stage should be in G₀, G₁ or G₂ for successful NT (Campbell et al., 1996, Cibelli et al., 1998). Serum starvation is a common method to synchronize the donor cell cycle to the G₀ stage. The rate of blastocysts development is reported as high as 70% using this technique, versus using rapidly dividing ES cells which results in blastocysts rates of 10% to 15% (Blomch et al., 2006). In addition to cell cycle stage, the passage number of cultured cells affects the developmental potential of cloned embryos (Enright et al., 2003; Giraldo, 2007). There is a difference in cell type as well. When cumulus cells and fibroblasts cells are compared, more of the cumulus cells are in the G₀/G₁ phase

compared to fibroblast cells (88% vs. 82%, respectively). This is consistent with that fact that fewer cumulus cells were in the G₂/M phase than fibroblast cells (Enright et al., 2003). The most dramatic difference between cell cycles is the levels of histone acetylation, specifically H1 acetylation.

H1 histone is a linker histone that associates with internucleosomal DNA and helps regulate chromatin structure and transcriptional activity (Spencer and Davie, 1999). Enright (2003) reported significantly higher levels of acetylation from passage 5 to passage 15, and this is called “culture effect”. Based on these observations, it may help to use cells from a later passage that they may be more easily reprogrammed.

Wilson and Jones (1983) demonstrated that late population doublings of diploid cells became hypomethylated. This point is of interest because donor cells are often transfected for nuclear transfer which requires numerous passages. Giraldo et al. (2007) also demonstrated the mRNA content of chromatin remodeling proteins and the level of DNA methylation of in vitro cultured cells is altered during culture. In this study, the fetal bovine fibroblasts cells displayed constant levels of methylation during the proliferative stage of culture and increased during the last divisions. However, there were no significant changes that corresponded with the population doublings and levels of global DNA methylation and histone acetylation present. The previous studies confirm it is important to note that culture conditions can play a role in the levels of DNA methylation and histone acetylation. When investigating specific gene sequences for DNA methylation, it is difficult to make correlations with passage numbers and differentiation (Noer et al., 2007). Noer et al. (2007) examined adipogenic differentiation potential of human ADAS cells in early passage and upon senescence, and determined whether senescence was the associated with changes in adipogenic promoter methylation. There was reduced in vitro differentiation ability in late passage cells on the basis of lipid synthesis and reduced transcriptional activation of adipogenic genes (FABP4 and LPL) compared to the ADAS cells from earlier cultures. In contrast, two other adipogenic genes (LEP and PPAR gene2-master regulator of adipogenesis) (Farmer, 2006) remained unaffected or moderately enhanced

(FABP4, LEP) in undifferentiated cells in late passages. Thus, these results could not be attributed to specific changes in DNA methylation at adipogenic promoters in the undifferentiated state.

These changes seen during in vitro culture undeniably affect the efficiency of NT. Currently, the mechanisms behind DNA methylation and histone acetylation are poorly understood. A better understanding of these mechanisms and how to alter these mechanisms may increase the ability to reprogram donor cells.

DNA Methylation Patterns of Preimplantation Embryos

The DNA methylation patterns of the murine preimplantation embryos have been studied extensively. During preimplantation development, dynamic reprogramming of global methylation is taking place. Prior to fertilization, the DNA of both the oocyte and the sperm are highly methylated (Dean et al., 2002). Sperm chromatin is highly compacted by protamines, whereas oocyte chromosomes have a normal nuclear structure (Perreault, 1992). The maternal and paternal gametes must undergo extreme chromosomal reprogramming in order for the zygote to develop into a normal embryo and a functioning placenta.

During fertilization the paternal genome undergoes active demethylation while the maternal genome is passively demethylated. The active demethylation of the paternal genome occurs within a few hours after fertilization and before the first cell division (Monk et al., 1987). The asymmetric differences between the maternal and paternal pronuclei during the first cell cycle, the ICM and trophectoderm lay the foundation for development. There are series of check points that must be successfully negotiated in order for full term development to occur (Dean et al., 2003).

At the morula stage of development, both parental genomes are hypomethylated (Reik et al., 2001). (Figure 2.2) Later in the development, de novo methylation occurs. De novo methylation are posttranscriptional events that occur after the maternal to zygotic gene transfer occurs. During de novo methylation methyl groups are added to unmodified cytosine residues in DNA. The methyl groups result in a rapid increase in genomic methylation levels during

postimplantation development (Lei, 1996). De novo methylation preferentially occurs at the ICM, resulting in a uniform pattern of methylation on the homologous chromosomes by gastrulation (Shi et al., 2003). Methylation continues postgastrulation and hence could be a mechanism that initiates, or signals, differential programming in the definitive germ layers (Monk, 1987). The consistent correlation between gene activity and hypomethylation strongly suggests a role for DNA modification in regulation of gene expression during development (Cedar, 1984).

The methylation patterns among the previous scenarios are species and tissue-specific (Bird, 1986). A dramatic loss of cytosine methylation from the male pronucleus has been observed in mice, swine and cattle (Dean et. al., 2001). In contrast, there is not an observed loss of methylation from either the maternal or paternal pronucleus in in-vivo derived ovine zygotes (Beajean et. al., 2004). At pronuclear stages demethylation in the human zygote takes place but is absent in rabbit zygotes. The expression of certain cell-surface antigens also differs during early embryonic development between mouse and human preimplantation-stage embryos (Henderson et al., 2002).

Beajean et. al. (2004) suggested that variations in the DNA methylation patterns indicate that demethylation of the paternal genome is not an obligate requirement for early mammalian development. In contrast to the mouse and cow, (Dean et al., 2001) there is no passive demethylation throughout sheep implantation but an increase in methylation between the 8-cell and morula stage. Only at the blastocyst stage is demethylation visible in the sheep trophectoderm; whereas, the cells of the ICM (inner cell mass) remain methylated (Beaujean, 2004). The demethylation of the trophectoderm may be due to the trophectoderm cells being the first differentiated cell type to form during development and trophoblast-specific gene expression is essential for embryonic nutrition and implantation.

Preimplantation embryos demonstrate enormous flexibility to compensate for suboptimal environments, but when the capacity for compensation is overloaded development is arrested. Suboptimal conditions may result in abnormal embryonic, fetal, perinatal and postnatal

development that are collectively referred to as LOS (large offspring syndrome) (Wrenzycki et al., 2004).

Aberrant DNA Methylation Patterns of Cloned Embryos

It has been recently demonstrated by Santos et al. (2003) and Dean et al. (2001) in cloned embryos, that the frequency of the normal/abnormal demethylation pattern closely reflects the developmental ability of reconstructed embryos. These distinct methylation patterns may reflect further differences occurring much later for the process of implantation and placenta formation (Malassine et. al., 2003). There is also evidence indicating that cloned animals have a variety of abnormal symptoms such as increased birth weight, pulmonary hypertension, placental over-growth and respiratory problems, suggesting these abnormalities are due to aberrant gene expression (reviewed by Shoita and Yanagimachi, 2002).

Aberrant DNA methylation patterns are what ultimately lead to embryo demise. Several sources report that the inefficiency in cloning is due to the failure to reprogram the somatic nucleus of the donor cell (Shi et al., 2003). It is important to mention that each cloned animal has different aberration patterns. It was once thought that cloned animals were exact copies of the original animals, but due to aberrant methylation occurring randomly, they cannot be considered perfect genetic copies of the original animals (Ohgane et al., 2001).

Characterization of DNA methylation patterns of embryos can be achieved through techniques such as antibodies to 5-methyl-cytosine (5-MeC) and to the methylated and acetylated modification of lysine 9 of histone (H3-K9), as well as bisulphate sequencing. Characterization of DNA methylation patterns of embryos can be achieved through techniques such as using antibodies to 5-methyl-cytosine (5-MeC) and to the methylated and acetylated modification of lysine 9 of histone (H3-K9), as well as bisulphate sequencing. Dean et al. (2003) documented heterogeneous labeling of nuclei observed in normal embryos compared to cloned embryos. There was a highly homogenous population with a methylation pattern strongly reminiscent of the donor cell somatic nuclei.

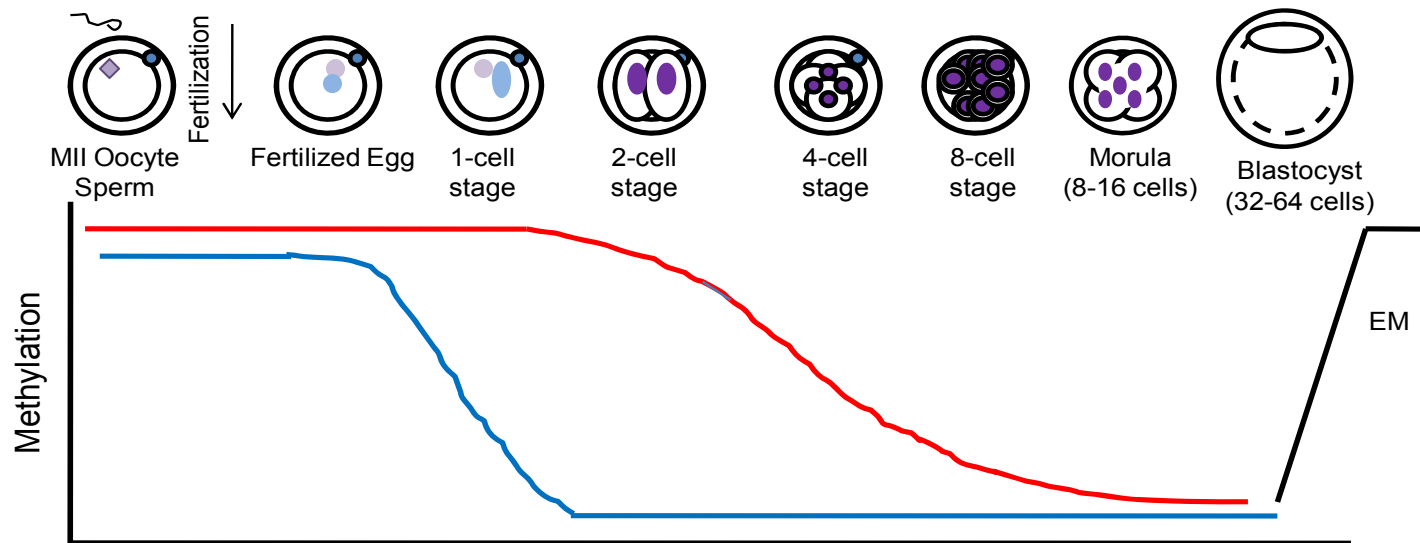


Figure 2.2. Dean et al. 2003 Seminars in Cell & Developmental Biology (Maternal (red), Paternal (blue) (EM) embryonic. (Edited by Alicia Picou, 2009)

By the blastocyst stage a marked difference in fluorescence intensity of DNA methylation between ICM and the trophectoderm was observed in the normal embryo population. The cloned embryos processed more homogenous labeling, indicating that aberrant trophectoderm hypermethylation took place.

In general, SCNT embryos are characterized by abnormal cleavage and the timely offset of embryonic genome transcription, deficits that may explain their reduced pre- and postimplantation developmental capacity (Niu et al., 2008). It is certain that the methylation pattern is incorrect in all or almost all cloned animals. Since aberrant methylation occurs randomly in the placenta and other tissues of term cloned fetuses, it is difficult to evaluate the extent of epigenetic abnormality before birth (Ohgane et al., 2001). However, animals that survive to birth and beyond should have normal DNA methylation patterns in order to survive. Healthy cloned mice have similar methylation patterns to normal mice (Shiota and Yanagimachi, 2002). There is evidence that healthy offspring can be produced (reviewed by Cibelli et al., 2002) even after extensive culture time, freezing, thawing and clonal expansion from single cells (Bondioli et al., 2001).

CHAPTER III

THE ISOLATION AND CHARACTERIZATION OF BOVINE ADULT DERIVED ADIPOSE STEM CELLS FOR THE USE OF NUCLEAR TRANSFER

Introduction

Adipose tissue has been shown to be a rich and abundant source of adult stem cells that are capable of differentiating into mesodermal lineages (Noer et al., 2006). Stromal cells from adipose tissue have been cultured extensively to senescence, but little is known about the epigenetic alterations associated with extended culture (Noer et al. 2007). Most of the characterization of ADAS cells has been conducted with human cells that demonstrate a variety of stem cell markers (Gronthos et al., 2001). These studies suggest that adipose stem cells have the ability to dedifferentiate from their original cell source type and be differentiated into new cell types (Guilak et al., 2004). Improved culture conditions and the characteristics of bovine adipose stem cells will create an important animal model and may provide an alternative cell type for nuclear transfer.

Currently, it is unknown what makes a good donor cell for the use in nuclear transfer (Oback and Wells, 2002). Less than 5% of cell types have been tested as nuclear donors and support development to blastocyst; however, even fewer have been able to generate offspring (Kato et al., 2000; Wakayma and Yangimachi, 2001). There are many hypotheses as to why the efficiency of nuclear transfer still remains low including: asynchrony between the donor genome and the recipient oocyte (Wilmut and Campbell, 1998), chromosomal abnormalities of the donor cell (Giraldo et al., 2006) or failure of the oocyte to reprogram the donor chromatin (Dean et al., 2001; Cezar et al., 2003). Recently the emphasis has shifted to the type and status of donor cells used (Bosch et al., 2006; Giraldo et al., 2007). Since the cloning of 'Dolly' most experiments with large animals have utilized fetal fibroblasts as the primary source of donor cells. This is primarily because of the perception that these cells can undergo more cell doublings before becoming senescent than cells from adult animals. However, in many cases,

fetal derived cells are unavailable. Skin fibroblasts from adult animals are a viable alternative but have limited proliferative capacity and accumulate chromosomal abnormalities when cultured in vitro (Azad and Woodruff, 2006; Giraldo et al., 2007). Skin fibroblasts recovered from aged animals likely have numerous chromosomal abnormalities prior to being cultured (Azad and Woodruff, 2006). These factors likely contribute to the low efficiency of NT and the poor viability of cloned offspring (Gomez et al., 2004).

The reprogramming process includes alteration of epigenetic modifications such as DNA methylation, imprinting, histone acetylation, and X chromosome inactivation (Shi et al., 2003; Kono, 1997). During NT, the DNA methylation patterns must be reorganized to resemble that of undifferentiated nuclei (Rideout, et al., 2001). Differences in the nature of epigenetic modifications may affect the ability to reprogram a certain cell for NT. A cell with low levels of DNA methylation and high levels of acetylated histones would be expected to have a less compacted chromatin and may be more amenable to nuclear reprogramming during NT (Adams et al., 2005). We and others hypothesize that somatic stem cells may possess some of the same undifferentiated characteristics as ES cells making them attractive candidates for use in nuclear transfer. Adipose tissue from adults is abundant in nature and has demonstrated plasticity in culture, adult derived adipose stem cells (ADAS) are an attractive source for donor cells in nuclear transfer.

Materials and Methods

Experimental Design

Experiment 1

Subcutaneous fat was collected from the brisket of 9 adult cows immediately post mortem at the local abattoir. Tissue was enzymatically digested in Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 1% bovine serum albumin (BSA) and collagenase concentrations of 0.10%, 0.25% or 0.50% each incubated for 1, 2 and 3 hours in a 3 x 3 factorial design. The number of nucleated cells was recorded per gram of tissue.

Experiment 2

Two cell lines were divided into a 2 x 2 factorial to evaluate culture conditions for the ability to support proliferation. Two basal media high glucose DMEM or a 1:1 mixture of DMEM and Hams F12 with each medium plus or minus growth factor supplements. At each passage, the cell cycle length was determined and the number of population doublings till senescence or until passage 20 were determined.

Experiment 3

Two bovine ADAS cell lines and one adult skin fibroblast cell line at passages 3, 6 and 11 were tested for their ability to differentiate into osteoblasts, chondrocytes and adipocytes. At the end of a 21-day induction period, differentiation was confirmed by histological staining. Controls consisted of the same cell lines at passages 4, 6, 11 that did not receive differentiation treatments. Controls were also stained by the same methods to confirm that spontaneous differentiation did not occur.

Experiment 4

Three sets of cell lines consisting of ADAS cells and skin cells from the same animal were used to evaluate the differences in global DNA methylation and histone acetylation of the two different cell types. Cells were fixed, permeabilized and labeled with rabbit anti-Acetylated histone H3 IgG, amino acids 1-20 (Upstate, Cat No. 06-599) and mouse anti-5-methylcytidine IgG (Serotec, cat No. MCA2201) and appropriate Alexa Flour 488[®] labeled secondary antibodies. Flow cytometric analysis was used to quantify global levels of DNA methylation and histone acetylation in bovine ADAS and adult skin fibroblasts for passages 1 to 6. Appropriate controls for nonspecific binding by the primary and secondary antibody were included.

Experiment 5

Commercially supplied mature metaphase II bovine oocytes were enucleated and were randomly divided into two groups. One group was reconstructed with bovine ADAS cells and the other with adult derived skin fibroblasts. Two different bovine ADAS cell lines and 1 adult skin fibroblast cell line were utilized and replicated 3 times for each cloning experiment. The number

of oocytes that fused, cleaved and developed into blastocysts were recorded on days 3 and 7 of in vitro culture.

Experiment 6

Interspecies somatic cell nuclear transfer was performed using eland ADAS cells as donor cells into enucleated bovine oocytes. Two different eland cell lines were used as donor cell nuclei and NT was replicated three times for each cell line. Controls consisted of bovine ADAS cells that have produced blastocysts previously. The number of oocytes that fused, cleaved and developed in blastocysts were recorded on days 3 and 7 of in vitro culture.

Establishment of Primary Cultures

Primary cultures were established from subcutaneous adipose tissue of adult bovine collected by surgical means or post-mortem from a local abattoir. The surgical procedure was performed with the animal standing. An area located about 12.70 cm behind the shoulder and about 15.24 cm from the backbone was clipped and prepared surgically. Local anesthesia was administered using an inverted L block. A 3 cm incision was made through the skin until fat pad was visible. Using tissue forceps, an estimated gram of adipose tissue was removed by blunt excision. Tissue was placed in DBPBS supplemented with 1% penicillin streptomycin (P/S) (Gibco, No. 15140) and kept on ice until processing could begin. A simple interrupted suture was used to close the incision and antibiotics were administered post operatively as precaution to infection. Sutures were removed 9 to 15 days later.

Post-mortem tissue collection consisted of trimming an estimated 5 g of adipose tissue from the brisket of adult cattle. The tissue was held on ice in normal saline until processing could begin. The tissue was minced and washed two times in normal saline containing 2% P/S and 1% Fungizone® (Gibco, 15290-018). For enzymatic dissociation, tissue samples were placed in an Erlenmeyer flask with DPBS containing either 0.10%, 0.25% or 0.50% Collagenase Type I (Gibco, 17100-017), 1% bovine serum albumin (BSA), 1% P/S and 1% Fungizone and incubated for 1, 2 and 3 hours in a continuous shake incubator. After enzymatic dissociation cell

mixtures were transferred into 50 ml conical tubes and centrifuged at 1200 rpm for 5 minutes. Suspended adipose tissue was removed, and the pellet resuspended and passed through a Millipore double filter consisting of 80 μm and 120 μm nylon filters. The cell mixture was centrifuged again at 1200 rpm for 5 minutes, washed in 5 ml of DPBS with 1% BSA and centrifuged again. Cells were washed and resuspended in Dulbecco's Modified Eagle Medium with high glucose (DMEM) plus 10% fetal bovine serum (FBS), 1% P/S and 1 % Fungizone. Cells were cultured in 12.5-cm² tissue culture flasks with 2 ml of DMEM, 10% FBS, 1% P/S and 1% Fungizone under 5% CO₂ and 90% humidity at 39° C for 48 hours. At 48 hours, non-adherent cells were removed by washing with DPBS. To determine the number of viable cells, some cultures were trypsinized at 24 hours and cells counted. The adipose cells were passaged when they reached 90% confluence.

Primary skin fibroblasts cultures were established using pieces of minced skin collected at slaughter. Skin pieces were enzymatically dissociated in 5 ml of a collagenase solution of DPBS containing 0.25% collagenase, 1% BSA, 2% P/S, 1% Fungizone[®] and 5 ml of DMEM with 10% FBS. Post dissociation, cell mixtures were centrifuged at 1200 rpm for 5 minutes then washed in 5 ml of DMEM medium and centrifuged again. The pellet was resuspended in stromal medium and placed in the bottom of 12.5-cm² tissue culture flasks. At 48 hours tissue pieces were removed and 2 ml of new medium was added. Culture conditions were the same as previously described. The cells were passaged at 90% confluence.

Cell Culture Maintenance

Medium was changed every 3 to 4 days as needed with the exception of the primary culture when the media was changed after 48 h of initial plating. The cells were passaged at 90% confluence. Cultures were passaged by releasing cells with trypsin (0.25%), counted using a hemacytometer and re-seeded at an initial concentration of 300,000 cells per 75-cm² tissue culture flask.

Population doublings (PDs) and cell cycle length were calculated at every passage. PDs were calculated by the following equation: $\log (\text{final concentration} / \text{initial concentration}) \times 3.33$

(Arat et al., 2002). Cell cycle length for every passage was calculated by dividing the number of days a cell culture required to reach confluence by the number of PDs. These parameters were calculated in continuously proliferating cells without intermediate freeze/thaw procedures. Cells were passaged until they reached senescence or until passage 20 was achieved.

Cell Cryopreservation

Bovine ADAS and skin fibroblast cells were frozen and thawed as needed. For freezing, cells were removed by trypsinization and were resuspended in calf serum (CS) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma, No. D2650). Cells were cooled at 1°C/minute until reaching -80°C before being transferred into liquid nitrogen. Approximately 300,000 cells were frozen in 1 ml of freezing medium. Cells were thawed by being placed in a 38°C water bath then resuspended in culture medium prior to culture.

Cell Differentiation

Three cells lines were represented, from three separate adult cows. Two ADAS cell lines were differentiated at Passage 3 (P3), P6 and P11 respectively and one adult skin fibroblast line was differentiated at P3 and P7. Cells were seeded in a 35 mm tissue culture Petri dish with 50,000 cells. Culture medium was changed every 3 to 4 days for 21 days. Adipogenesis was induced by culturing in DMEM supplemented with 3% FBS, 1% P/S and 1% Fungizone, 10 ng/ml Insulin, 10^{-9} M dexamethasone. The first four days of culture, 250 μ M of isobutyl methylxanthine (IBMX) and 0.2mM indomethacin were also added. After day 4 of culture, IBMX and indomethacin were no longer added. An adapted protocol from (Mackay et al., 1998) was utilized for chondrogenesis. 15 ml conical tubes were seeded with approximately 50,000 cells and centrifuged at 1200rpm for 5 minutes to form a cell pellet. DMEM with 10% FBS, 1% P/S, 1% Fungizone, 6.25 μ g/ml insulin, 10 ng/ml transforming growth factor ($\text{TGF}_{\beta 1}$) and 10^{-9} M dexamethasone was added to the cell pellets. Osteogenesis was induced by culturing in DMEM with 10% FBS, 1% P/S, 1% Fungizone, 10 mM β -glycerophosphate, 50 ng/ml ascorbic acid and 10^{-9} M dexamethasone.

Histological Staining

Cells were washed with calcium and magnesium free DPBS then fixed in 4% paraformaldehyde at 4°C for 24 h. After fixation all cells were washed four times with calcium and magnesium free DPBS prior to staining. Adipocytes were stained with 1 ml of Nile red to detect intracellular lipid accumulation (Williams et al., 2007). Chondrocytes were stained with Safran O (Sigma, No. S2255) stain to detect glycosaminoglycans in the intracellular matrix (Williams et al., 2007). Osteocyte induction was confirmed by 2% alizarin red S (Sigma, No. A5533) to verify mineralized calcium deposit (Williams et al., 2007).

DNA Methylation and Histone H3 Acetylation Immunolabeling

A protocol modified by Giraldo et al. (2007) from (Habit et al., 1999) was utilized for intracellular immunolabeling. DNA methylation and histone acetylation levels were quantified by flow cytometry (Accuri C6 Flow Cytometer). Cells were labeled with mouse anti-5-methylcytidine and rabbit anti-acetyl-histone H3 primary antibodies at 1:200 and 1:100 dilution factors respectively. Secondary antibodies were Alexa Fluor® 488 labeled anti-mouse and –rabbit IgG (Molecular Probes, No. A11029 and A11008, respectively) with a dilution of 1:100. At each passage cells were trypsinized then washed in a tween solution consisting of Ca⁺⁺ and Mg⁺⁺ free dPBS, 1% BSA and 0.1% Tween20 to allow permeabilization to occur. Cells were fixed in 0.25% paraformaldehyde in dPBS at 37°C for 10 min and cooled to 4°C by the addition of 1.8 ml cold methanol. Cells were held in 88% methanol in the freezer for a minimum of 48 h. Following fixation, cells were washed in tween solution, 2N HCL was added to the pellet and incubated for 30 min at 37°C, then neutralized with 0.1 M borate buffer (pH 8.5) for 5 min at room temperature. Cells were washed twice and incubated with dPBS 1% BSA for 30 minutes at room temperature to block non-specific binding. Primary antibodies were added (10 µ/ml) for 30 min at room temperature then labeling with secondary antibodies, anti-mouse IgG conjugated with Alexa Fluor 488® or anti-rabbit IgG conjugated with Alexa Fluor 488® for 30 min at room temperature. Between antibody labeling, cells were washed two times with dPBS with containing 1% BSA. Cells were then counter stained in a sodium citrate solution (0.112%)

containing 50 µg/ml propidium iodide (PI) and RNase (10 µg/ml) for 30 minutes at room temperature. Cells were washed and resuspended in dPBS plus 1% BSA for cytometric analysis.

Labeled cells were excited at 488 nm and Alexa Flour 488® fluorescence detected in FL-1 channel. PI levels were detected in FL-3 channel to gate for cells that were in G₁ of the cell cycle. A minimum of 10,000 events were collected at cell passages 1 through 6. Data generated was converted to Microsoft Excel spread sheet for calculations of arbitrary fluorescence units (AFU).

Somatic Cell Nuclear Transfer

Bovine oocytes were ordered from commercial suppliers (Ovitra, Amarillo, TX, USA; Mini Tube, Madison, WI, USA; Applied Reproductive Technologies., WI, USA) and matured overnight in maturation medium in a shipping incubator. At ~ 18 hour after initiation of maturation oocytes were denuded by vortexing for 4 minutes in a 0.2% hyaluronidase (Sigma, No. H3506-1G) solution and mature MII oocytes were selected by excursion of the first polar body. Polar bodies and metaphase plates were removed by micromanipulation. Removal of genetic material was confirmed by staining oocytes in 1 mg/ml of Hoechst for 15 minutes prior to enucleation and viewing the aspirated portion under UV light. Enucleated oocytes were pooled into two groups randomly then reconstructed with bovine ADAS cells or adult derived skin fibroblasts or eland ADAS cells. Following reconstruction cells were then fused with two DC pluses of 2.25 kV/cm for 15 µsec and incubated in post fusion medium consisting of KSOM culture media (Millipore MR-020P-5F) and cytochalasin B for 1 hour. Cells that were fused were set aside and non-fused cells were pulsed again and incubated for an additional 15 minutes prior to chemical activation. Oocytes were activated by exposure to 5 mM inositol for 4 minutes followed by a washing for 5 minutes in TL Hepes and 4 hours incubation in DMAP. Nuclear transfer embryos were cultured in KSOM culture media for 7 days. Cell division was observed at day 3 of culture. At day 7 and 8 the number of blastocysts and morulas were recorded.

Statistical Analysis

All data were analyzed using Sigma Stat Statistical Software Version 3.5 (Systat Software, Richmond, CA, USA). Two-way ANOVA was used to detect significant differences for collagenase concentrations and exposure times in experiment 1. Two-way ANOVA, followed by multiple pair-wise comparisons using Tukey's test when applicable, was used to detect differences in population doublings until senescence for media treatments and among growth factor supplementation. A Chi square test was used to detect differences in the number of fused oocytes, cleavage and blastocysts rates between embryos made from adult skin fibroblast donor cells and bovine ADAS donor cells. Differences of $P < 0.05$ were considered significant. It should be noted that different commercial oocyte suppliers were used in each of the experiments and embryos were produced at separate times for each experiment. As a consequence, no comparisons were made between experiments.

Results

Experiment I: Cell Isolation Methods

Cell morphology was documented throughout cell culture. Primary culture cell morphology of bovine ADAS cells was heterogeneous (Figure 3.1 A) then developed into a uniform fibroblast morphology. Adult skin fibroblast cell morphology remained homogenous as fibroblast morphology (Figure 3.1 C) until cultures approached senescence. As cell cultures approached senescence, the number of viable cells significantly decreased and a visible change in cell morphology in both cell types was evident (Figures 3.1 B and D). Senescent cells were defined as cells with irreversible growth arrest, flat-cell morphology, enlarged cytoplasm and heterogeneous shapes. Irreversible growth arrest was determined by cells unable to reach 90% confluence after 2 weeks of culture or having a negative population doubling.

On average 23.4% of cells were viable post-plating. The average number of nucleated cells that were released per gram of tissue (Table 3.3 and Figure 3.2) was no difference between treatments ($P > 0.05$). The collagenase concentration 0.25% for 2 hour incubation period had the highest total viable and adherent cells 24 hours post plating (Figure 3.3 and

Table 3.3). This treatment was utilized the remainder of the study and was applied successfully to 4 common eland (*Tragelaphus oryx*).

Experiment 2: Culture Media and Growth Factor Supplementation

Cells were cultured until senescence or until passage 20 was reached. The population doublings and cell cycle length were calculated for every passage and total population doublings until senescence was determined. Two cell lines, Line #1 and Line #2, were studied. Each cell line was used in a 2 x 2 factorial design consisting of high glucose DMEM or a 1:1 mixture of DMEM and Hams F12 as a basal medium plus or minus growth factor supplements. Growth factor supplements consisted of basic fibroblast growth factor (bFGF) at 1 ng/ml, transforming growth factor ($TGF_{\beta 1}$) at 10 ng/ml and epidermal growth factor (EGF) at 5 ng/ml.

Cell cycle length remained shorter for a longer period of time with growth factor supplementation in both media types and in both cell lines (Tables 3.1 and 3.2), however, late passage cells in all treatments did have an increase in cell cycle length compared to early passage cells. There was a significant interaction ($P < 0.05$) between medium and growth factors for Line #1 for PDs but no interaction between medium and growth factors for Line #2 for PDs (Table 3.4). DMEM with growth factor supplementation had significantly ($P < 0.05$) shorter lifespan than all other media treatments.

The cell cultures completed approximately one cell cycle every two days until ~passage 8. At passage 8 there was an increase of cell cycle length (Figures 3.4 A and B) and inconsistencies in the cell cycle length as the cells began to reach senescence. Abnormal spikes in the cell cycle lengths are characteristic of cells that approaching replicative senescence. There was not a significant interaction between medium and growth factors for the average cell cycle lengths for cell Lines #1 and #2 (Table 3.5). The average cell cycle length and lifespan of bovine ADAS cells for each medium type and growth factor supplementation did not differ significantly with the exception of F12GF in Line #1 (Table 3.5). F12GF in Line #1 reached ~105 PDs by passage 20 compared with an average of 31 PDs. The abnormally long

lifespan maybe a result of the cells transforming into another cell type which is an artifact of in vitro culture.

Experiment 3: Differentiation of Bovine ADAS and Adult Derived Skin Fibroblasts

Two bovine ADAS cell lines, Line #1 and Line #2, and one adult skin fibroblast cell line at passages 3, 6 and 11 were differentiated into adipocytes, chondrocytes and osteoblasts. Differentiation was confirmed by histological staining, using Nile Red, Safrin O and Alizarin S. Nile Red stains lipid droplets, Safrin O stains glycosaminoglycans (GAGs) in extracellular matrix and Alizarin S is specific for mineralized Ca^{++} deposits.

Adipocyte controls (Figures 3.5 A, B, C and D) had less staining, indicating limited adipogenesis. Cells that received differentiation medium (Figure 3.6 A, B, C, D, E and F) had a consistently more intense staining of Nile Red staining than controls. There was a marked decrease in intensity of staining of the cells in later passage with cells in P3 differentiating most efficiently. Cells induced to differentiate into chondrocytes by pellet culture (Figure 3.7 A, B, C, D) stained more intensely than controls (Figure 3.7 A and B). Mild GAG staining was notable in controls but there was a marked difference in intensity between controls and pellet cultures. Cell at P3 differentiated the most efficiently (Figure 3.8 A). The mild staining that was evident in the controls indicates that limited spontaneous differentiation did occur, but differentiation was more efficient in cells that received differentiation media.

Cells induced to differentiate into osteocytes (Figures 3.10 A, B, C, D, E, F) stained more intensely than the controls (Figures 3.9 A, B, C, D, E, F). Earlier passages had a more intense staining than later passages indicating induced differentiation occurred more efficiently in early passages than later passage cells.

Adult derived skin fibroblasts were utilized as a cell type control. When skin fibroblasts cells were differentiated into adipocytes they stained with similar intensity to the ADAS cells (Figure 3.11 A). Comparing chondrocyte differentiation between ADAS and skin fibroblasts, ADAS cells stained with greater intensity than skin fibroblasts (Figures 3.11 B, E). Osteogenesis did not appear to occur to either early or late passage skin fibroblast cells (Figures 3.11 C, F).

Table 3.1 Average Cell Cycle Length Through Lifespan of Line #1 Bovine ADAS Cells

Average Cell Cycle Length (d) \pm SEM				
Medium	P1 to P5	P6 to P10	P11 to P15	P16 to P20
DMEM	1.97 \pm 0.35	4.112 \pm 1.54	7.49 \pm 1.25	5.58 \pm 0.85
DMEMGF	1.53 \pm 0.35	2.58 \pm 0.67	*	*
DMEM:F12	3.82 \pm 1.42	5.41 \pm 2.44	7.39 \pm 0.79	11.57 \pm 5.50 [†]
DMEM:F12GF	1.46 \pm 0.14	3.28 \pm 0.28	7.27 \pm 2.51	9.00 \pm 2.68

* Reached replicative senescence at P11.

[†] Reached replicative senescence at P17.

Table 3.2 Average Cell Cycle Length Through Lifespan of Line #2 Bovine ADAS Cells

Average Cell Cycle Length (d) \pm SEM				
Medium	P1 to P5	P6 to P10	P11 to P15	P16 to P20
DMEM	2.11 \pm 0.15	3.01 \pm 1.00	11.40 \pm 7.27	15.17 \pm 2.79 [†]
DMEMGF	1.49 \pm 0.21	2.49 \pm 0.52	5.37 \pm 1.49	9.04 \pm 2.79 [†]
DMEM:F12	3.85 \pm 1.47	2.90 \pm 0.45	3.88 \pm 0.59	5.14 \pm 1.53 [♦]
DMEM:F12GF	2.19 \pm 0.62	3.62 \pm 0.811	5.62 \pm 1.07	4.03 \pm 0.08 [†]

[†] Reached replicative senescence at P19.

[♦] Reached replicative senescence at P18.

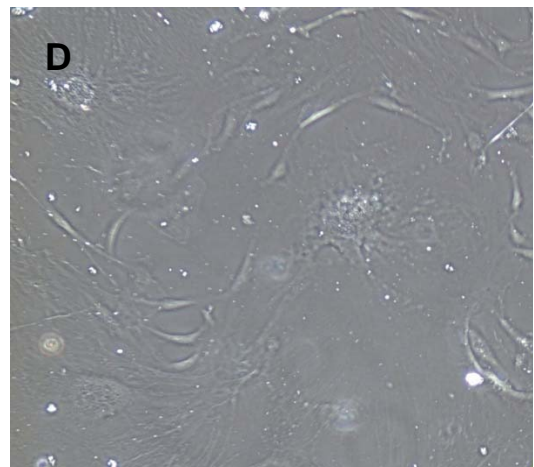
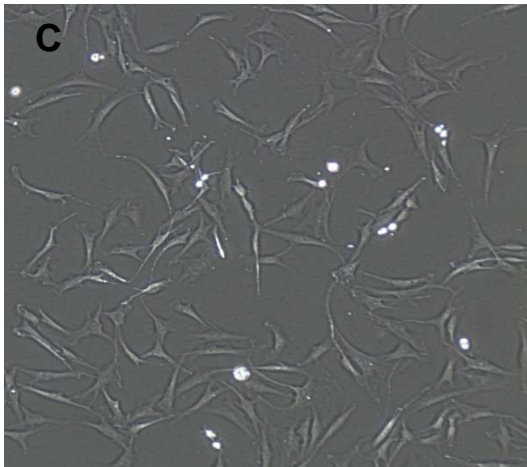
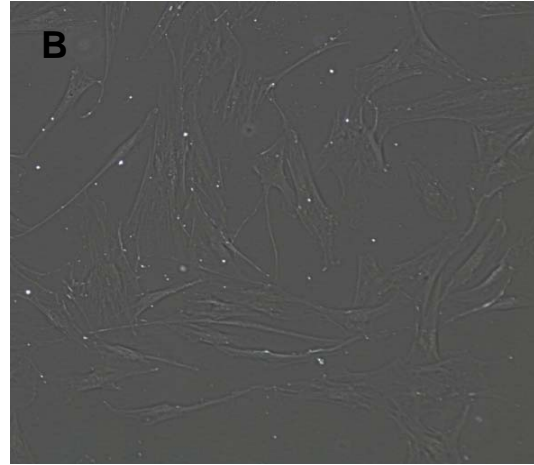
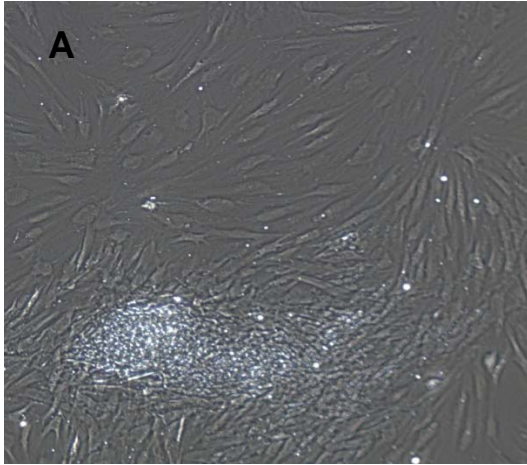


Figure 3.1. Morphology of bovine ADAS and skin cells primary passage and late passage (A) Morphology of primary culture bovine ADAS cells (B) Morphology of late passage (P15) bovine ADAS cells approaching senescence (C) Homogenous fibroblast morphology of primary culture adult derived bovine skin fibroblast cells and (D) morphology of late passage (P11) adult derived skin fibroblasts cells

Table 3.3. Average Number of Cells Released per gram of Adipose Tissue and Viable 24 hours Post-Plating

Collagenase Concentration	0.10%		0.25%		0.50%	
Incubation Time	Total Cells ±SEM x10 ⁴	No. Viable 24h ± SEM x10 ⁴	Total Cells ±SEM x 10 ⁵	No.Viable 24 h ± SEM x10 ⁴	total cells ±SEM x10 ⁵	No.Viable 24 h ± SEM x10 ⁴
1 h	3.81±1.16 ^a	1.63 ±315 ^a	3.83±1.55 ^a	2.25±190 ^a	5.00±1.89 ^a	4.42±1.64 ^a
2 h	4.85±2.73 ^a	2.33±1.09 ^a	5.54±2.89 ^a	5.17±2.36 ^b	6.38±2.57 ^a	4.29±2.14 ^a
3 h	3.63±1.07 ^a	2.21±.62 ^a	3.17±.52 ^a	3.88±1.19 ^a	4.33±1.11 ^a	5.82±3.36 ^a

*Change in superscript indicates significant difference (P<0.05).
(9 replicates).

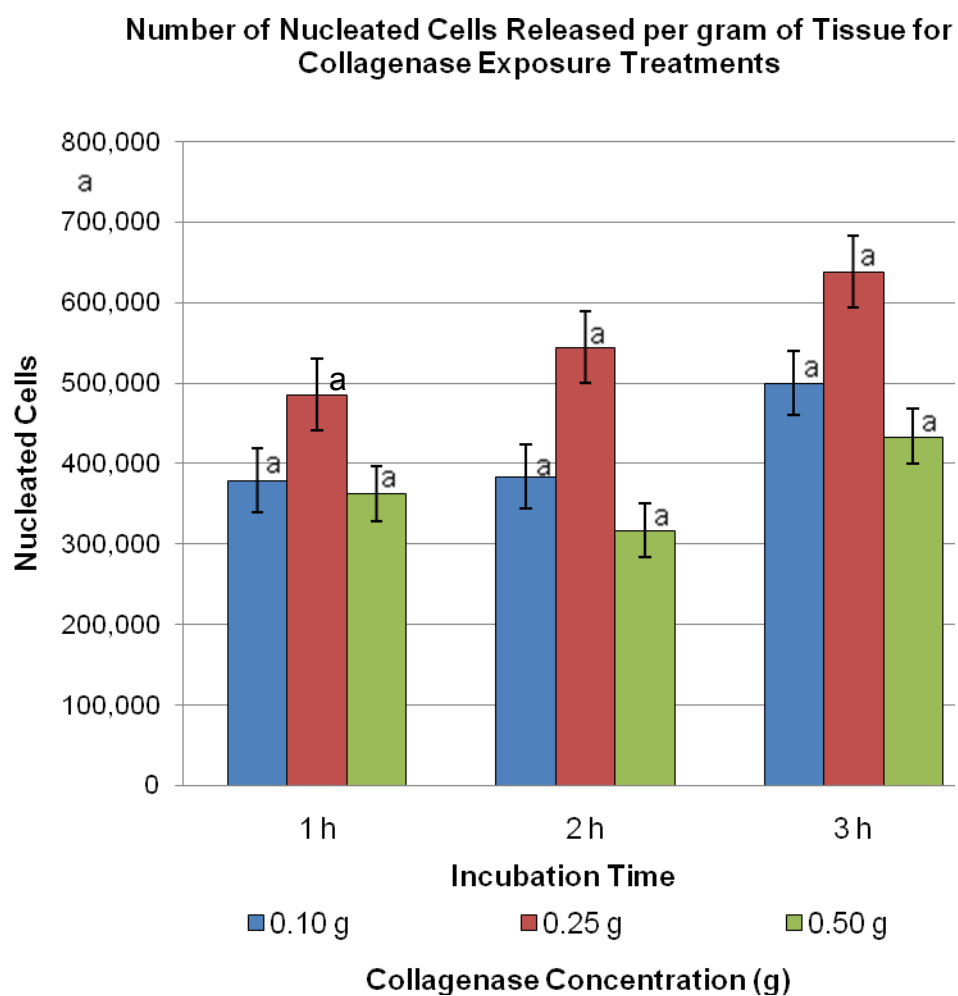


Figure 3.2. The number of nucleated cells released per gram of tissue for collagenase digestions. No significant differences among treatments ($P > 0.05$).

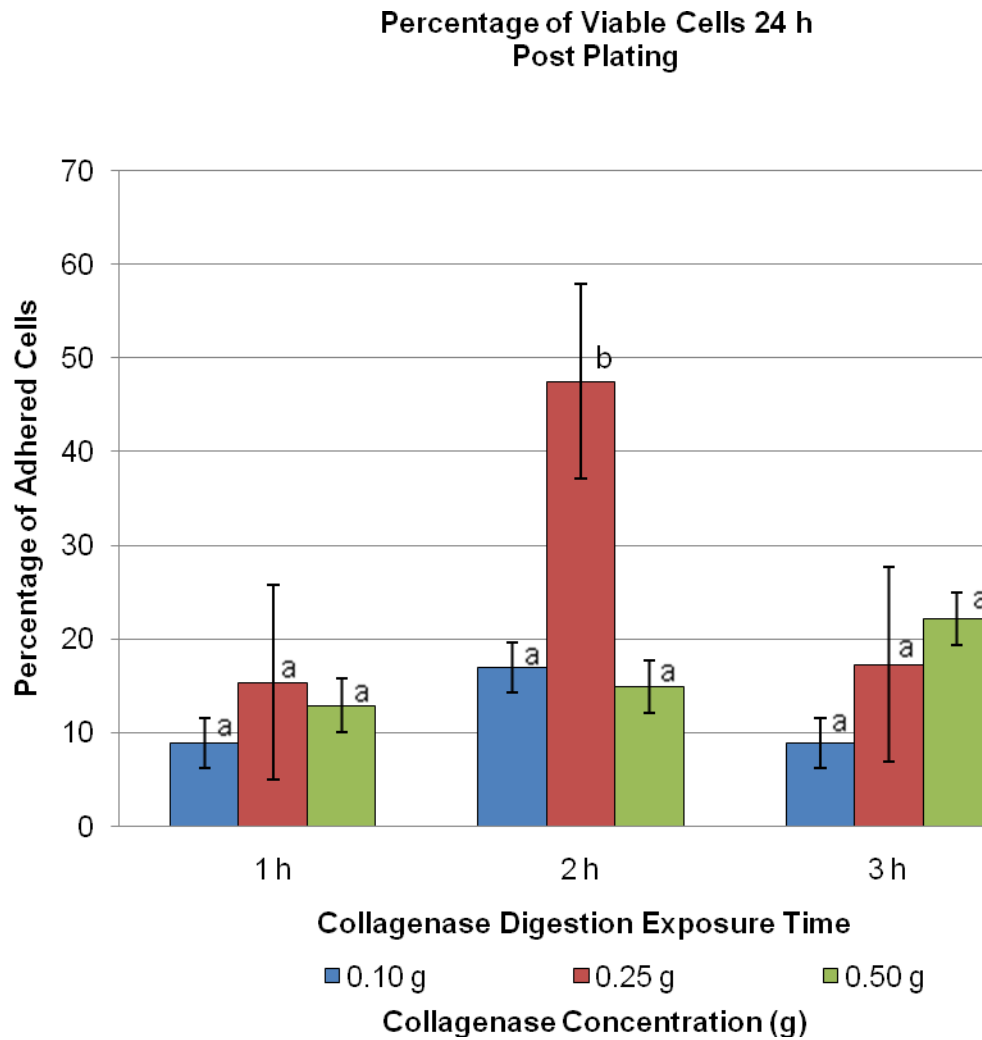


Figure 3.3. The percentage of viable adherent cells 24 hours post plating. Change in superscript indicates significant difference ($P < 0.05$).

Table 3.4. Population Doublings for Bovine ADAS Cells Line #1 and Line #2 Until Senescence or Passage 20 Reached

Line # 1		Line #2	
Media	Population Doubling (\pm SEM)	Media	Population Doubling (\pm SEM)
DMEM	1.59 \pm 0.16 ^a	DMEM	1.46 \pm 0.18 ^a
DMEMGF	2.64 \pm 0.30 ^c	DMEMGF	1.92 \pm 0.23 ^a
F12	1.48 \pm 0.15 ^a	F12	1.85 \pm 0.12 ^a
F12GF	1.67 \pm 0.19 ^a	F12GF	1.91 \pm 0.16 ^a

Change in superscript indicates significant difference (P<0.05).

Standard error of the mean (SEM).

Dulbecco's Modified Eagle Medium (DMEM).

Ham's F12 (F12).

Growth factor supplementation (GF).

Table 3.5. Average Cell Cycle Length per Passage Until Senescence and Lifespan of Bovine Adult Derived Adipose stem cells

Cell Line	Media	CLL \pm SEM	Lifespan (PD)
Line #1	DMEM	4.78 \pm 0.69	31.90
	DMEMGF	4.35 \pm 1.90	28.99
	F12	6.52 \pm 1.25	25.59
	F12GF	5.26 \pm 1.09	105.09*
Line #2	DMEM	7.95 \pm 2.20	29.23
	DMEMGF	4.36 \pm 0.93	36.44
	F12	3.81 \pm 0.50	33.25
	F12GF	3.85 \pm 0.47	36.38

*Significant Difference.
Cell Cycle Length (CCL).
Population Doublings (PD).
Standard error of the mean (SEM).
Dublecco's Modified Eagle Medium (DMEM).
Ham's F12 (F12).
Growth factor supplementation (GF).

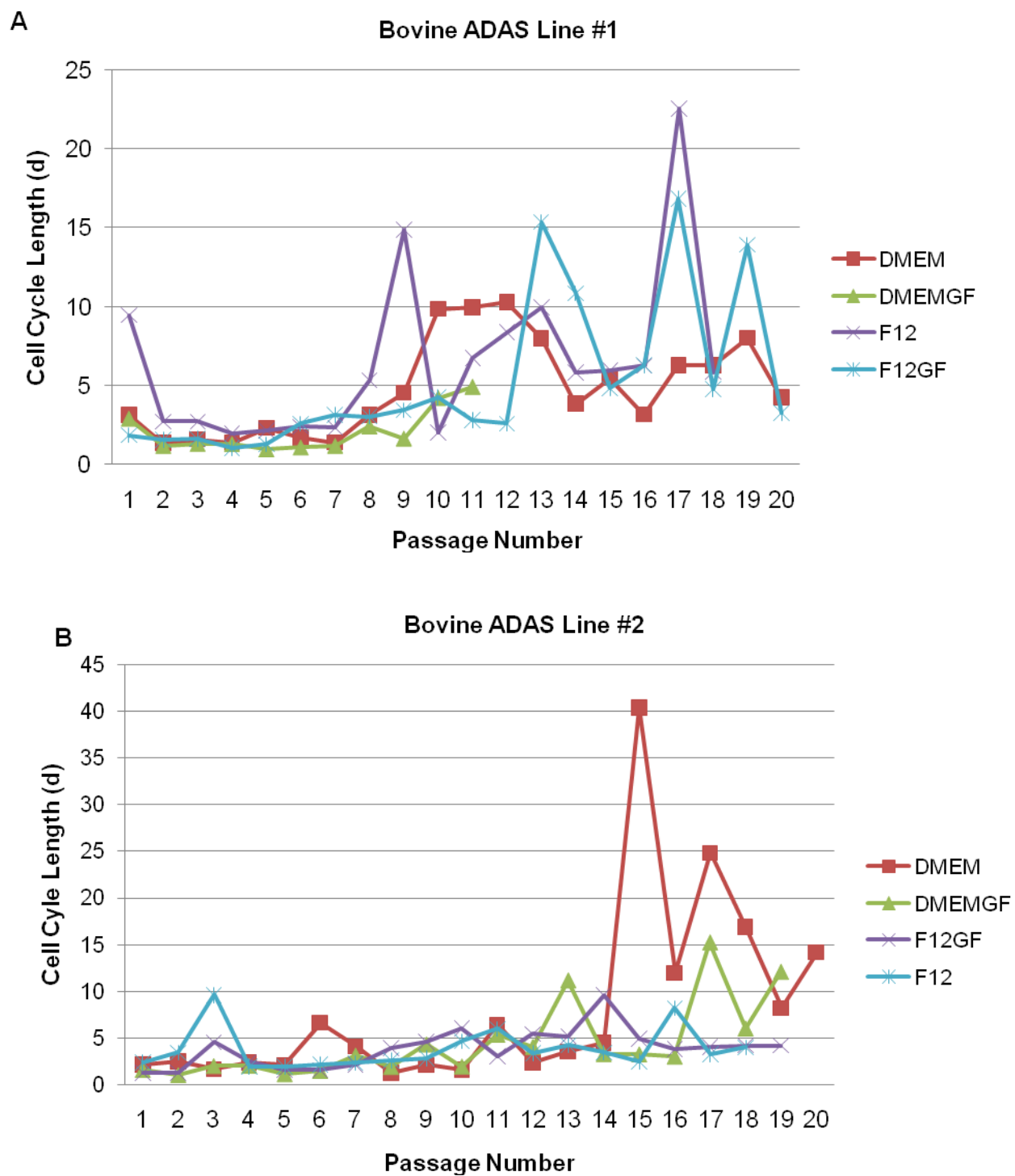


Figure 3.4. Cell cycle length per passage until senescence or passage 20 (P20) was reached for bovine ADAS cell lines #1 (A) and #2 (B).

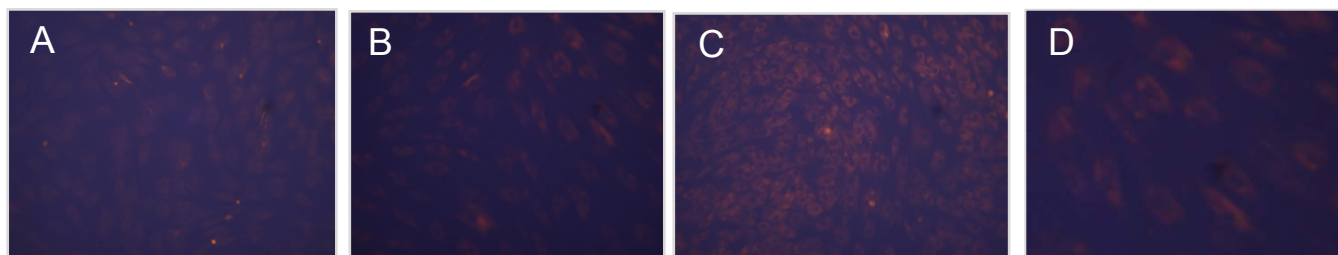


Figure 3.5. Adipogenesis Controls (A) Line #1 passage 3 .(B) Line #1 passage 12. (C) Line #2 passage 3. (D) Line #2 passage 11.

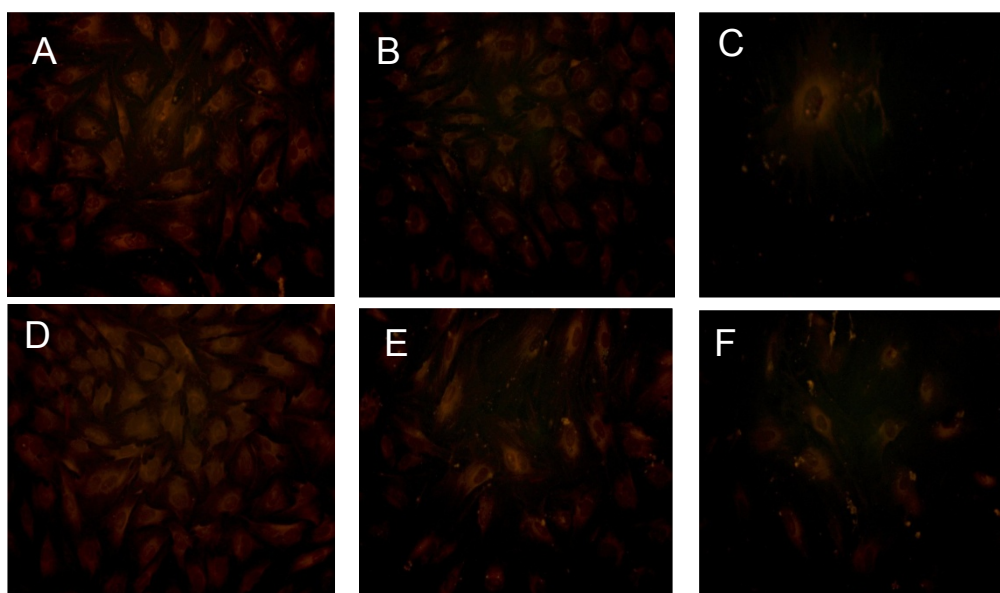


Figure 3.6 .Adipogenesis (A) Line #1 passage 3. (B) Line #1 passage 6 .(C) Line #1 passage 12. (D) Line # 2 passage 3. (E) Line #2 passage 6. (F) Line # 2 passage 11.

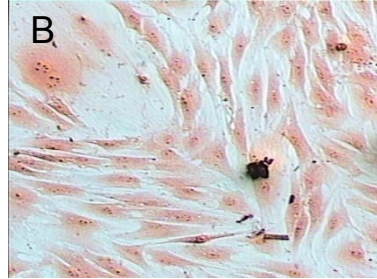
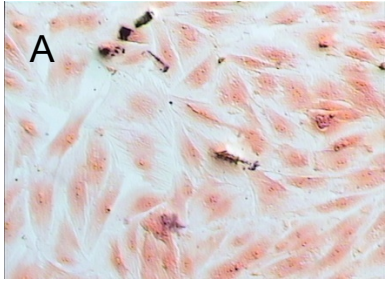


Figure 3.7. Differentiation controls into chondrocytes. (A) Line #1 passage 3. (B) Line #2 passage 6. (C) Line #2 passage 11. (D) Line #1 passage 12.

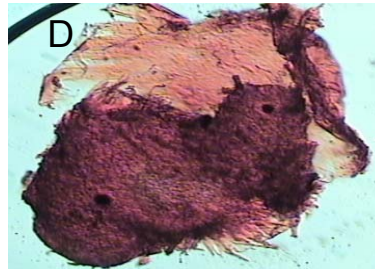
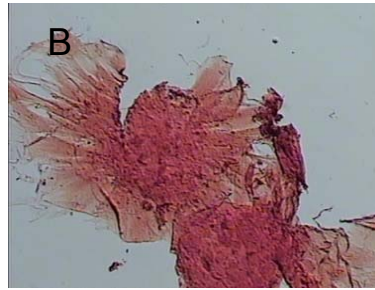
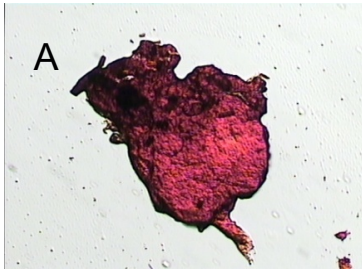


Figure 3.8. Induced differentiation into chondrocytes. (A) Line #1 passage 3. (B) Line #1 passage 6. (C) Line #2 passage 3. (D) Line #2 passage 6.

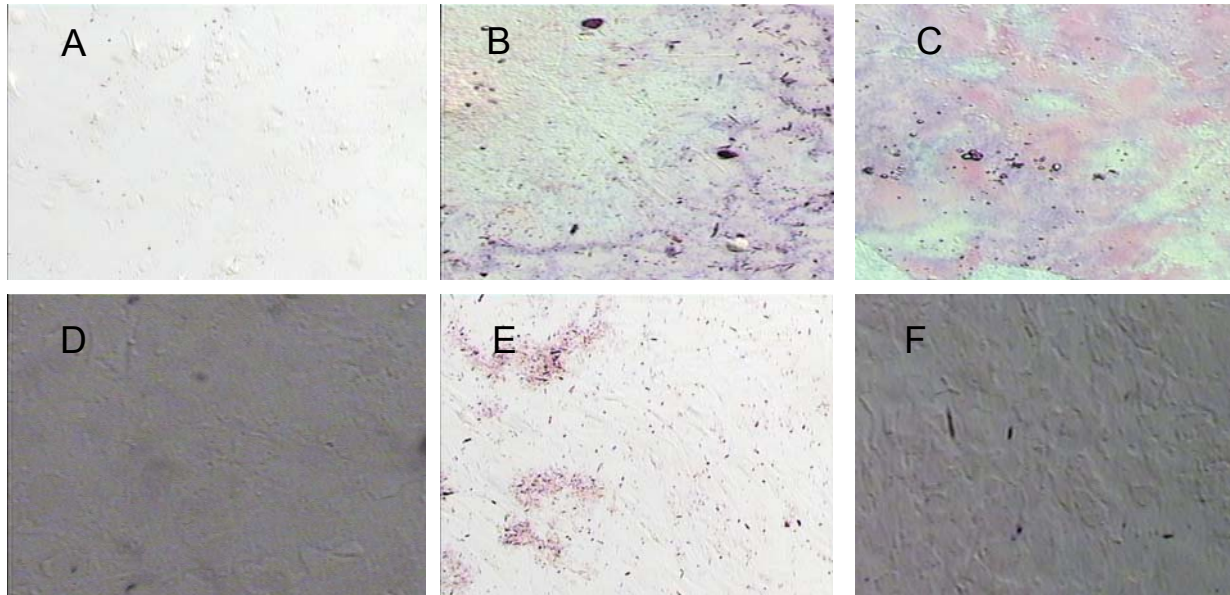


Figure 3.9. Osteogenesis Controls. (A) Line #1 passage 3 (B) Line #1 passage 6 (C) Line # 1 passage 12 (D) Line #2 passage 3 (E) Line #2 passage 6 (F) Line #2 passage 11

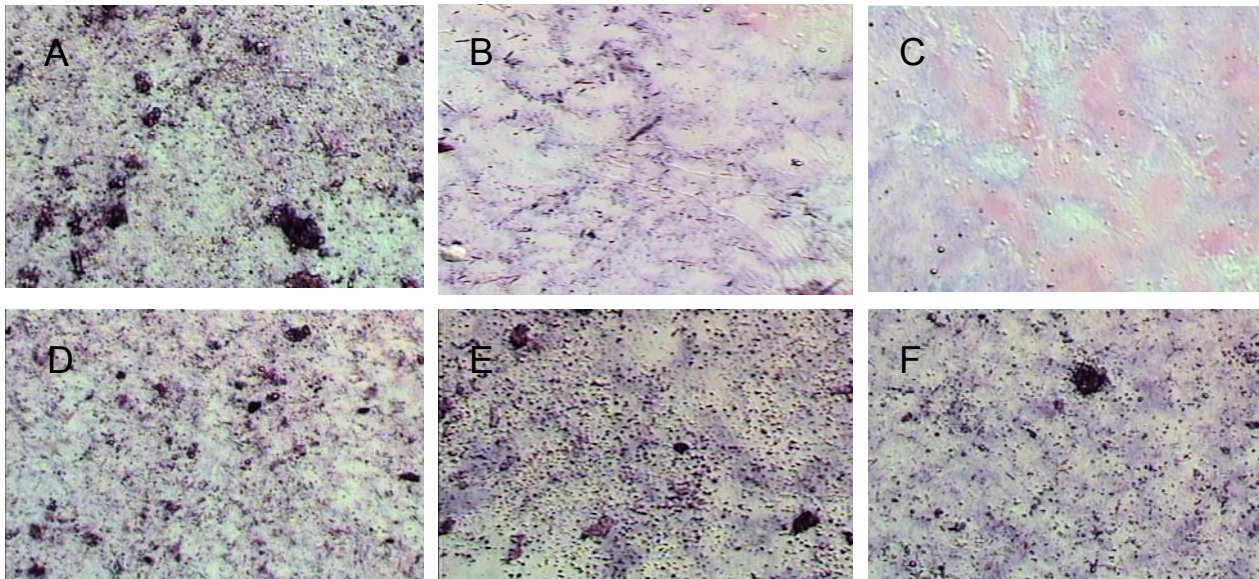


Figure 3.10 Differentiated Osteogenesis (A) Line #1 passage 3 (B) Line #1 passage 6 (C) Line # 1 passage 12 (D) Line #2 passage 3 (E) Line #2 passage 6 (F) Line #2 passage 11

Experiment 4: Global Levels of DNA Methylation and Histone Acetylation of Bovine ADAS and Skin Fibroblasts Cells

Three different cell pairs were analyzed from P1 until P6. A cell pair consists of a cell population isolated and cultured from adipose tissue and skin of the same animal. The three cell lines were combined in order to determine an average level of global DNA methylation and histone H3 acetylation per passage that was expressed in arbitrary fluorescence units (AFUs).

There was not a significant difference ($P>0.05$) of global methylation or histone acetylation between ADAS cells and adult derived skin (Table 3.6 and 3.7). The average levels of global DNA methylation and acetylated histone H3 for adult skin fibroblasts were: $\sim 13,931 \pm 2,926$ and $25,636 \pm 7,721$, respectively and the average levels of global DNA methylation and acetylated histone H3 of bovine ADAS cells were $15,928 \pm 2424$ and 15462 ± 3333 , respectively. BC lines #1 and #3 had higher levels of DNA methylation and histone acetylation in early passages (Figure 3.12 A, B and Figure 2.14 A, B). There was a decrease in DNA methylation and histone acetylation levels in P3 and later. BC line #2 had no differences in global methylation. The levels of histone acetylation for BC line #2 remained low throughout passage, with one outlier, P6 skin cells. In cell lines BC #2 and BC#3, P3 did not have data due isolated cell counts being too low.

Experiment 5: Comparing the Efficiency of Bovine ADAS Cells to Skin Fibroblast Cells as Donor Cells in Nuclear Transfer

Nuclear transfer results are represented in Table 3.8. The fusion efficiency of bovine ADAS cells (56%) and (58%) for adult skin fibroblasts were not significantly different. The percentage of cleaved and developing blastocyst embryos from the ADAS cells (62% and 8%) and skin fibroblasts cells (42% and 8%) were not different ($P<0.05$).

Experiment 6: Interspecies Nuclear Transfer with Eland ADAS Cells and Enucleated Bovine Oocytes

Nuclear transfer results are represented in Table 3.9. The average cleavage rates for embryos derived from eland ADAS cells and bovine ADAS cells were 39% and 37%

respectively. The number of embryos derived from each donor cell type were not different significantly ($P>0.05$). A total of 3 interspecies embryos (1%) developed to the blastocyst stage.

Discussion

SCNT is a valuable tool for production of transgenic animals or genetically superior animals and biomedical research. The number of species for which somatic cell nuclear transfer (SCNT) has been successful has grown dramatically since the birth of Dolly (Wilmut et al., 1997). However, the efficiency of SCNT has not improved dramatically from the reported 0.4% of reconstructed embryos that produce healthy embryos (Wilmut, 1997). The use of the technique in livestock production is very limited to a small number of cloned animals that are commercially produced (Wilmut et al., 2009) because of the failure of embryo development. Some of these developmental arrests are attributed to inappropriate reprogramming of the donor cell nuclei (reviewed by Fujita and Wade, 2004), cell cycle asynchrony between the donor cell and oocyte (Wilmut and Campbell, 1998) and non-optimal cells used as donor cells (reviewed by Oback and Wells, 2002). Most investigators concentrate on synchrony of cell cycles between the donor cell and oocyte. Recently, the emphasis has now shifted to concentrating on the type and status of the donor cells used (Bosch et al., 2006; and Giraldo et al., 2007). Data concerning the status of donor cells has been poorly analyzed to date.

The most common source of NT donor cells are fetal fibroblast cells primarily because of the perception that these cells will undergo more cell doublings before becoming senescent than adult-derived cells. Fibroblasts from adult skin are a viable alternative but have limited replicative capacity, contain extensive epigenetic changes and have been shown to rapidly accumulate chromosomal abnormalities in culture (Giraldo et al., 2007; Azad and Woodruff, 2006).

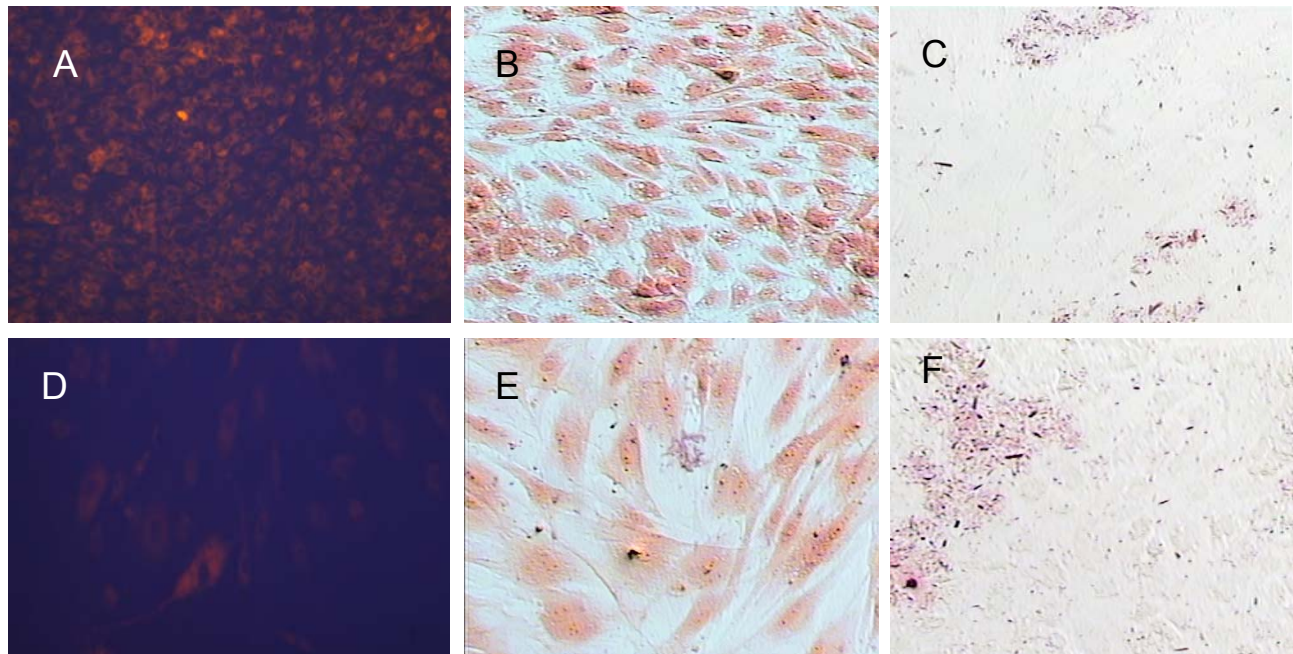


Figure 3.11. Adult skin fibroblasts. (A) Adipogenesis passage 3. (B) Chondrogenesis passage 3. (C) osteogenesis passage 3. Bottom row: (D) adipogenesis passage 7. (E) chondrogenesis passage 7. (F) osteogenesis passage 7.

Table 3.6 Global Levels of Methylated DNA at Different Passages in Bovine ADAS Cells and Skin Fibroblasts Cells

Global Levels of Methylated DNA (AFUs) $10 \times 10^4 \pm 10 \times 10^4$ SEM						
Cell Type	P1	P2	P3	P4	P5	P6
ADAS ^a	2.67 ± .80	1.59 ± .92	1.61 ± .98	1.08 ± .35	1.00 ± .06	1.59 ± .39
Skin ^a	2.23 ± .56	1.74 ± .53	.70 ± .15	.46 ± .19	2.00 ± .73	1.23 ± .33

P (passage number).

Change in subscript indicates significant difference (P<0.05).

Arbitrary fluorescence units (AFUs).

Table 3.7 Global Levels of Acetylated Histone H3 at Different Passages in Bovine ADAS Cells and Skin Fibroblasts Cells

Global Levels of Acetylated Histone H3 (AFUs) $10 \times 10^4 \pm 10 \times 10^4$ SEM						
Cell Type	P1	P2	P3	P4	P5	P6
ADAS ^a	2.10 ± 1.11	2.58 ± .28	1.78 ± 1.06	.65 ± .17	.51 ± .34	1.61 ± 1.22
Skin ^a	3.82 ± 1.99	2.50 ± .89	1.20 ± .40	.77 ± .21	1.39 ± 1.20	5.70 ± 5.62

P (passage number).

Change in subscript indicates significant difference (P<0.05).

Arbitrary fluorescence units (AFUs).

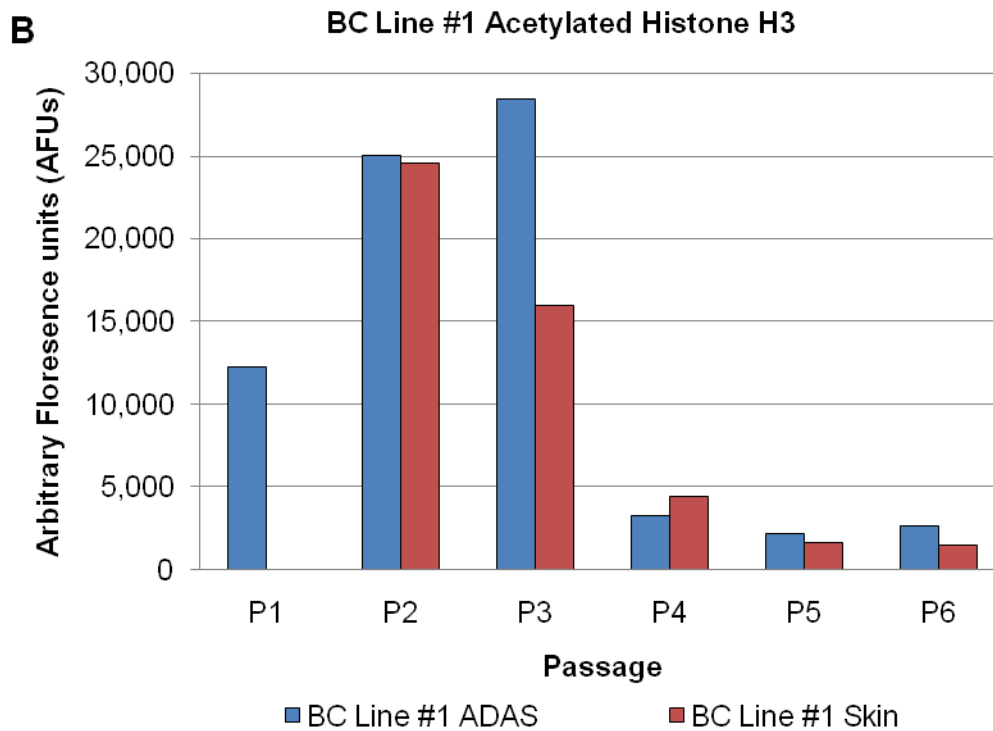
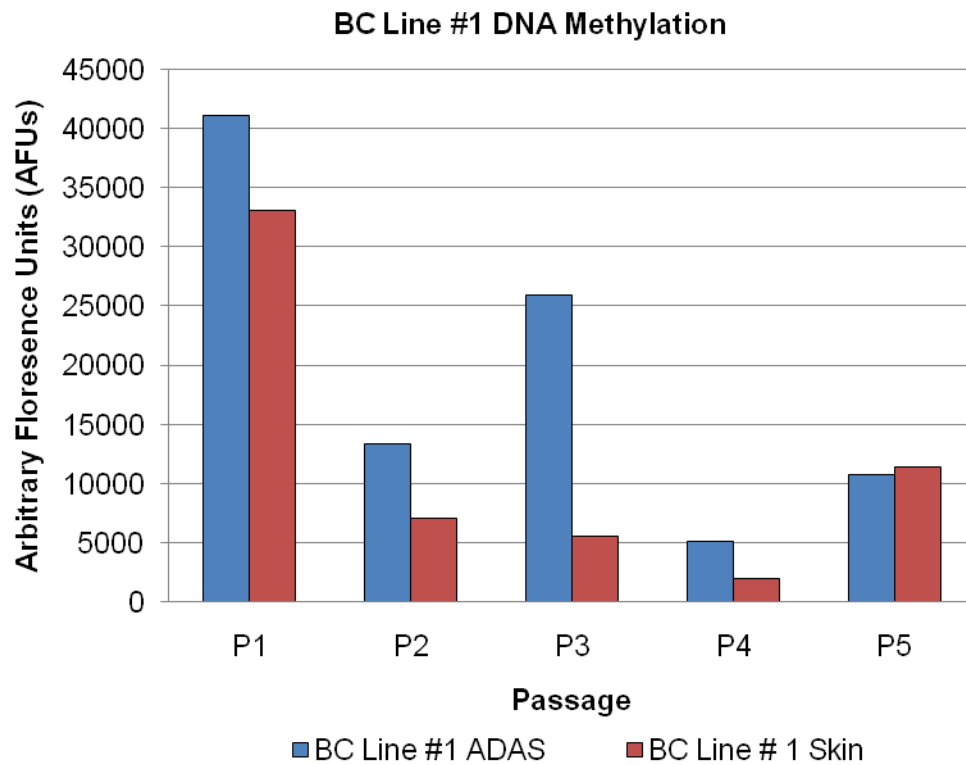


Figure 3.12 Global levels of methylated DNA (A) and acetylated histone H3 (B) in bovine ADAS (blue) and adult skin fibroblasts (red) for BC Line #1 expressed in arbitrary fluorescence units

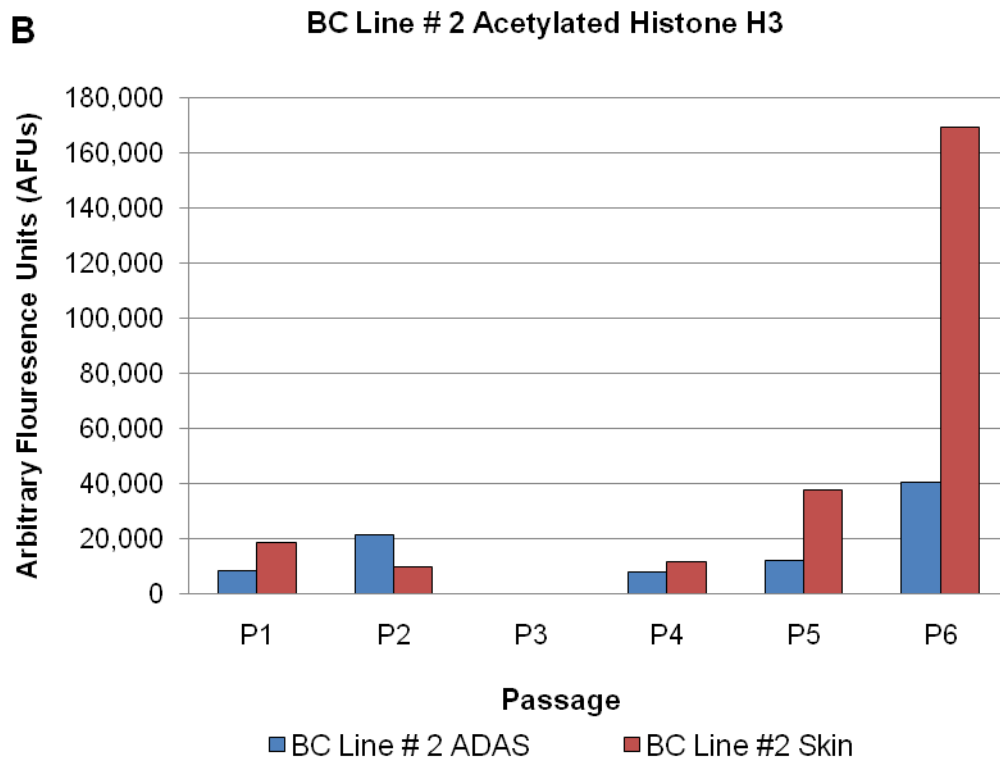
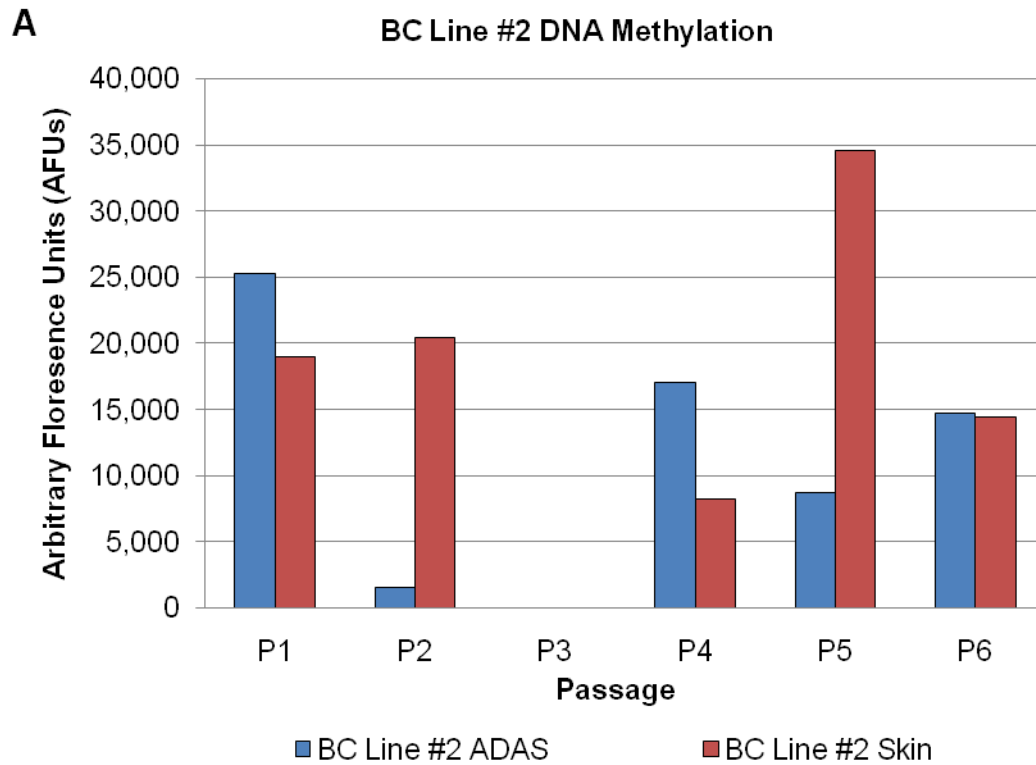


Figure 3.13 Global levels of methylated DNA (A) and acetylated histone H3 (B) in bovine ADAS cells (blue) and skin fibroblasts (red) for BC Line #2 expressed in arbitrary fluorescence units

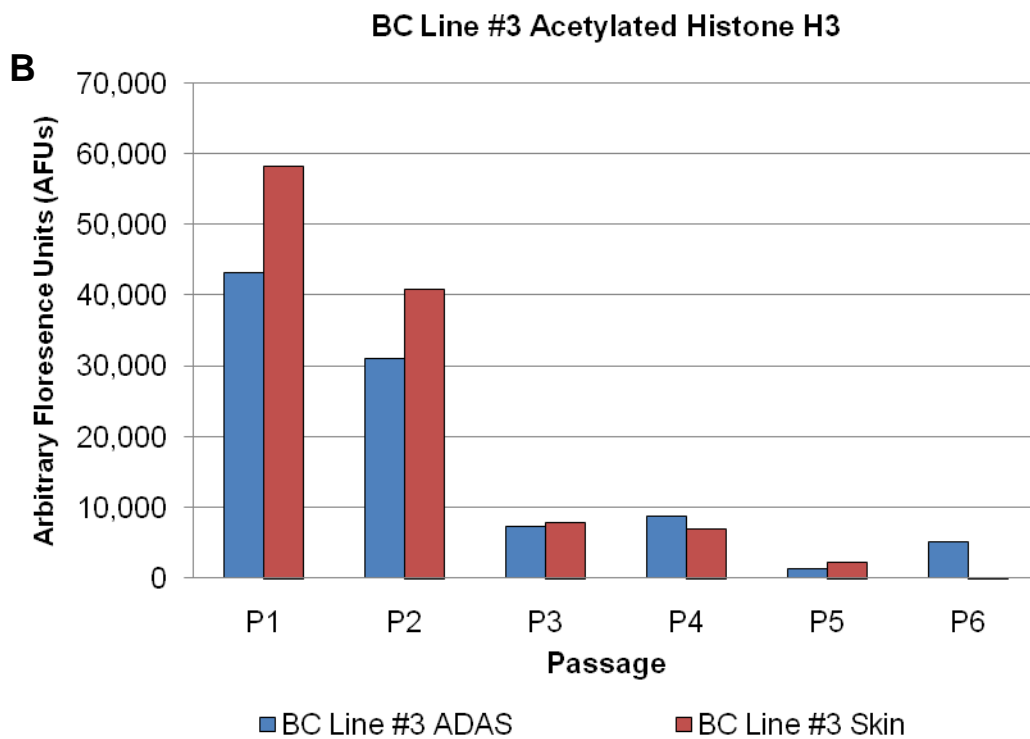
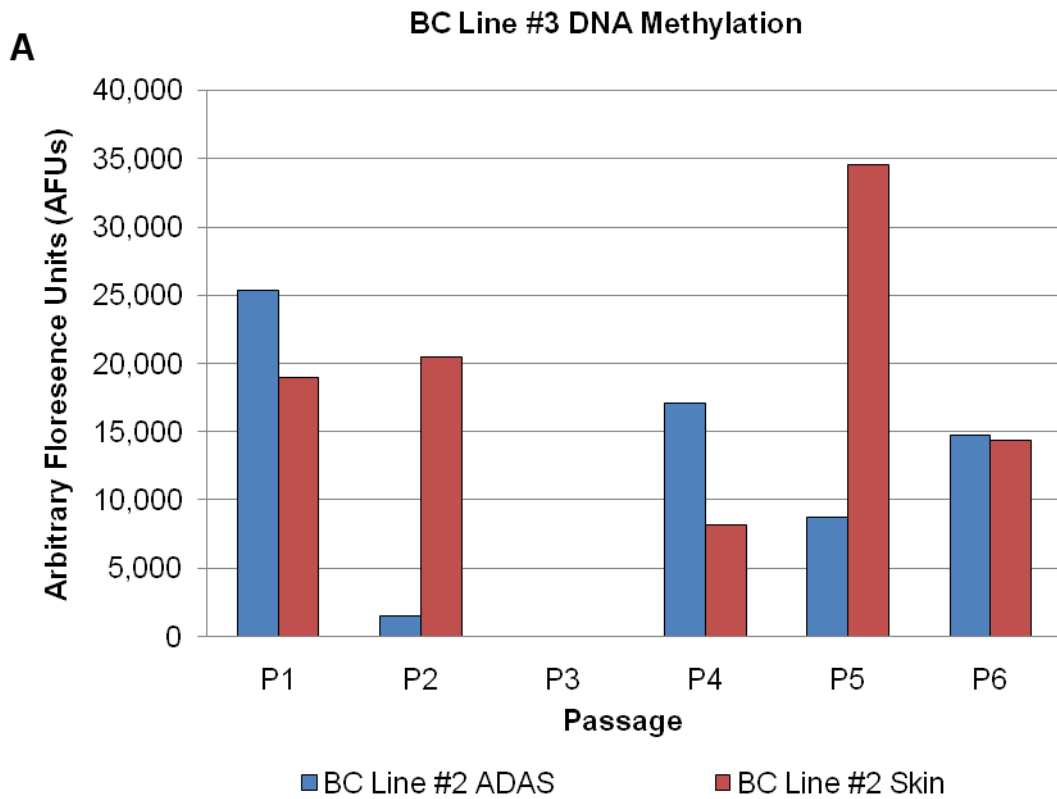


Figure 3.14 Global levels of methylated DNA (A) and acetylated histone H3 (B) in bovine ADAS cells (blue) and skin fibroblasts (red) for BC Line #3 expressed in arbitrary fluorescence units

Table 3.8 Development of Embryos Produced by Nuclear Transfer of Bovine ADAS Cells and Bovine Adult Skin Fibroblasts Cells into Enucleated Bovine Oocytes.

Donor Cell	Enucleated Oocytes (replicates)	Fused Couplets	Cleaved D3 N (%) ^a	Blastocysts D7-9 N (%) ^b
Line #1 bovine ADAS [†]	81 (3)	40	23 (56)	2 (8%)
Adult skin fibroblasts [†]	85 (3)	40	16 (40)	0
Line #2 bovine ADAS [†]	82 (3)	51	33 (67)	5 (15%)
Adult skin fibroblasts [†]	85 (3)	58	25 (29)	5 (20%)

^a Percentage from total fused couplets.

^b Percentage from total cleaved embryos.

[†] No difference between cell types (P> 0.05).

Table 3.9 Development of Embryos Produced by Nuclear Transfer of Eland ADAS cells and Bovine ADAS Cells into Enucleated Bovine Oocytes.

Donor Cell	Enucleated Oocytes (replicates)	Fused Couplets	Cleaved D3 N (%) ^a	Morula D7 N	Blastocysts D7-9 N (%)
Eland 258 [†]	153 (3)	128	66 (52)	0	1 (1%)
Cow 2147 [†] ADAS	102 (2)	87	39 (45)	0	0
Eland 129 [†]	137 (3)	123	32 (26)	2	2 (6%)
Cow 970 [†] ADAS	132 (3)	114	35 (31)	1	5 (14%)

^a Percentage from total fused couplets.

^b Percentage from total cleaved embryos.

[†] No difference between cell types (P> 0.05).

These abnormalities could be present prior to being cultured in vitro (Azad and Woodruff, 2006). Cells cultured in vitro rarely retain the same gene expression patterns as in vivo-derived cells (Enright et al., 2003). In this study we compared and evaluated characteristics of adipose derived cells for characteristics important for use in nuclear transfer.

In the first experiment, isolation and culture of these cells was done with minimally invasive procedures. In human studies, the common method of obtaining adipose tissue is by liposuction. The lipoaspirate waste is usually finely minced, compared to a solid piece of fat that was utilized in this study. The minced fat tissue forms an excellent starting material for ADAS cell isolation and the shear forces during the suction process do not significantly alter cell viability (Moore et al., 1995). Aust et al. (2004) obtained an average of $404,000 \pm 206,000$ cells per milliliter of lipoaspirate which is comparable to the number of cells released per gram of adipose tissue that were adherent and viable 24 hours post plating (Table 3.2). The isolation methods utilized in this study are very similar to the original methods of isolation of rat adipocytes and progenitors. Rodbell (1964) minced tissue into small fragments, digested at 37°C with collagenase type I and separation of cell types by differential centrifugation. The supernatant contains the mature adipocytes due their high lipid content; these cells float and the pellet that accumulates post centrifugation are the cells of interest as reported in previous studies (Robell, 1964). Utilizing these methods, others have modified their approach of isolating cells such as adding inductive growth factors (Hauner et al., 1989), but we have found that there is no need to add growth factors to primary cultures in order to establish cultures. These isolation and culture procedures have also been successfully applied to 3 common eland (Picou et al., 2009).

ADAS cells in this study were able sustain in culture for extended periods of time in DMEM and DMEM:F12 with or without the addition of growth factors as reported in human studies. Human ADAS cells are able to maintain culture in Eagle's Ham's F-10 medium (Katz et al., 2005), DMEM:F12 or DMEM (Ugarte et al., 2003), and bovine and human ADAS cells both

require 10 to 15% fetal bovine serum (FBS) supplementation and the use of antibiotics to prevent contamination of samples.

Human adipose tissue has some of the same surface proteins (Gronthos et al., 2001) and differentiation potential (Guilak et al., 2005) as ES cells. Adipose tissue from livestock animals has also been shown to have some of the same stemness characteristics (porcine, Williams et al., 2008) and (equine, Vidal et al., 2007). The morphology and population doublings were similar to those reported for equine (Vidal et al., 2007), porcine (Williams et al., 2007) and human (Gronthos et al., 2001, Guilak et al., 2005). The morphology of the primary cultured cells displayed fibroblastic characteristics and upon further culture, the cells displayed small vacuoles within the cytoplasm which is similarly to that reported for human cells (Gimble and Guilak, 2003).

In the third experiment, the differentiation potential of bovine adipose tissue was comparable to previous studies. The bovine ADAS cells appeared to lose their differentiation potential in later passages (passages greater than 6) indicating a loss of pluripotency. Growth factors that were utilized to induce differentiation in humans varied only slightly in this study. Interestingly, both species require pellet culture in order for induced differentiation into chondrocytes to occur. Bovine and human ADAS cells will remain in a monolayer under the same chondrogenic culture conditions, but expression of chondrogenic markers or stain intensity is reduced (Erickson et al., 2002). However, more testing is needed to confirm differentiation of cell types by other methods other than histological staining.

It is also interesting to note the seeding density required for induced differentiation in humans is significantly higher (0.25 million mL) compared to bovine cells (0.05 million mL) for induced chondrogenesis (Awad et al., 2003). For osteogenic and adipogenic conditions, seeding densities of human (30,000 cells) and bovine (50,000 cells) derived cells were similar as well as growth factor supplementation for induced differentiation (Gronthos, et al., 2001). In this study, it was found that ADAS cells appeared to differentiate more efficiently than skin fibroblasts, especially in osteogenesis and in later passages (> P6) according to histological

staining. The ability of these cells to differentiate in vitro indicates a potential use as an animal model for tissue engineering.

Few studies have investigated the global levels of DNA methylation and histone acetylation of in culture cells. In a study done by Giraldo et al. (2007), levels of methylated DNA in fetal derived skin fibroblasts cells increased with time with the exception of one cell line. The acetylated H3 levels remained constant among cell lines and culture time for the fetal fibroblast cells. In addition, skin fibroblasts have shown to have an increased level of histone acetylation, in either H3 or H4 or both, after long term culture (P15 vs. P5) (Enright, 2003). In our results, cell lines BC #1 and BC #3, had an increase in the levels of acetylated H3 in early passages P1 and P2, with a dramatic decrease from P3 on. Cell Line #2, histone acetylation levels remained constant, until late passage (P6), where there was a marked increase in acetylation levels for skin fibroblasts. This increase in histone acetylation levels supports the hypothesis that later passage cells may have higher developmental rates than early passage cells (Kubota et al., 2000). Giraldo (2007) also observed better embryo development when late passage cells were used compared to early passage cells. This is notable because it is standard for NT to use fibroblasts that have been passaged a limited number of times, therefore further studies are needed to confirm the effect of passage number on the outcome of NT.

The ploidy abnormalities seen in skin fibroblast cells (Giraldo, 2007), are likely the leading cause of the low rates of development to the blastocysts stage. If the donor cells are aneuploid when utilized for NT, the abnormalities appear in the NT embryos and inhibit appropriate embryo development. It is proposed that using a less differentiated donor cell may alleviate some of the abnormalities seen in culture and allow improved development (Rideout, et al., 2001). Preliminary data using porcine BMSCs suggest that this may hold true for somatic stem cells (Bosch et al, 2006). Bosch et al. (2006) used porcine BMSCs as donor cells and produced blastocysts at rates higher than when fibroblasts cells were used as donor cells. In the present study, there was not a significant difference between donor cells types (ADAS vs. skin fibroblasts) for cleavage rates and blastocysts development, indicating that ADAS cells are just

as efficient donor cell source as skin fibroblasts. ADAS cells may be a better candidate for the use of NT because of their extended plasticity in culture compared to skin fibroblasts. The ADAS cells cultured did not reach replicative senescence that was seen in the skin fibroblasts when exceeding passage 10. This is a valuable application because in the production of transgenic animals, cells are passaged several times before they are utilized as donor cells for NT.

Since NT could be a valuable tool for the conservation of endangered species it is important to investigate appropriate donor cells for these applications. Generally, oocytes from endangered species are scarce; therefore, the oocytes from domestic species are utilized. In this study, we applied the same isolation methods to obtain eland ADAS cells. The eland ADAS cells were utilized as donor cells for interspecies NT into enucleated bovine oocytes. The cleavage rates and embryo development rates were similar for eland ADAS cells and bovine ADAS cells. In a previous study, (Nel-Themaat, et al., 2007) semen-derived epithelial cells were used as donor cells for interspecies NT. They also reported similar cleavage rates of bovine NT and eland interspecies embryos; however development to the morula and blastocysts stage was higher for bovine NT embryos than for eland interspecies embryos reported by Nel-Themaat (2007). In addition, no interspecies embryos developed to the blastocysts stage using semen-derived epithelial cells. Using embryo cell counts, the highest total number of cells obtained in eland IgNT embryos derived from semen epithelial cells was 23 cells. In our study, cell counts were not performed on the embryos. However, there were a total of 3 blastocysts produced compared to the 6 bovine NT embryos. In all NT embryos produced there was a marked developmental arrest at the 3-to 4-cell stage. This coincides with embryonic genome activation that limits embryos to reach the blastocysts stage (Ty et al., 2003; Murakami et al., 2005). These development limitations could be caused by a variety of factors, but inappropriate genome activation seen in interspecies embryos is associated with improper early reprogramming due to the genetic distance between donor nucleus and recipient oocyte (Hamilton et al., 2004).

CHAPTER IV

SUMMARY AND CONCLUSIONS

Nuclear transfer (NT) is a valuable methodology for animal production. Since the announcement of 'Dolly', (Campbell et al., 1997) very little success in has been made in increasing the efficiency of live animal production. Many hypothesize that the failure of embryo development is due to the lack of reprogramming of the donor cell nuclei (reviewed by Fujita and Wade, 2004). It is suggested that finding a cell that has stem cell characteristics maybe more amendable for the reprogramming in nuclear transfer. Embryonic stem cells (ES cells) are a population of stem cells that have defined the characteristics of 'stemness'. They are pluripotent, undifferentiated cells that are capable of replicating for long periods of time and being transdifferentiated into other cell types. However, ES cells are namely limited to mice (Pain et al., 1996). Therefore, there is a need to find an adult-derived population of cells that possess stem cell qualities. Adipose tissue is an abundant source of cells that has demonstrated multipotent properties (Gronthos et al., 2001; and Guilak et al., 2005). Adult derived adipose stem (ADAS) cells are an attractive source of donor cells in nuclear transfer because of their reported 'stemness' qualities.

The present study focused on the isolation and characterization of bovine ADAS cells for the use in somatic cell nuclear transfer (SCNT). Experiment 1 optimized isolation and culture techniques for the expansion of ADAS cells. Experiment 1 resulted in a reliable technique of cell isolation than can be utilized across species. Experiment 2 studied the lifespan and growth characteristics of bovine ADAS cells in vitro among different media and growth factor supplementation. Results from experiment 2 showed that growth factor supplementation was not necessary to maintain viability of cells in culture and bovine ADAS can be successfully cultured in DMEM or Ham's F12/ DMEM (1:1) supplemented with fetal bovine serum, penicillin streptomycin and Fungizone[®]. Experiment 3 compared in vitro plasticity of bovine ADAS cells to adult derived skin fibroblasts at various times in culture to indicate when the cells lose their differentiation potential. Bovine ADAS cells are capable of induced differentiation during early

passages (P3) and differentiate more efficiently than adult derived skin fibroblasts. Experiment 4 characterized and compared the global levels of DNA methylation and histone H3 methylation throughout passages 1 to 6 between ADAS cells and skin fibroblast within the same animal. There was no significant difference between cell types. Both cell types in early passage (<P3) had higher levels of DNA methylation and histone acetylation than cells in later passage suggesting that utilizing cells in later passage may lead to increased embryo development. However, more testing is needed to validate this point. Experiments 4 and 5 tested the efficiency and feasibility of using bovine ADAS cells as donor cells for SCNT. ADAS and skin fibroblasts cells did not differ in efficiency of embryo development, but ADAS cells are capable of producing interspecies blastocysts. Eland ADAS were used as donor cells into enucleated bovine oocytes resulting in 3 interspecies blastocysts.

In conclusion, our results indicate that bovine ADAS cells are a viable source of somatic cells that can be cultured easily. ADAS cells also have better differentiation potential than adult skin fibroblasts because of their extended viability in culture and ability to differentiate into chondrocytes, osteoblasts and adipocytes. The global DNA methylation and histone acetylation patterns were similar to skin fibroblasts, and their patterns suggests that later passage cells maybe more conducive for use in NT. Their success in culture indicates that these cells are a more suitable donor cell source for nuclear transfer. In addition, ADAS cells are a viable source of donor cells and can be applied in the case of interspecies NT. While, the low success rates of NT are still present using ADAS cells, they are a viable alternative to adult skin fibroblasts.

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VITA

Alicia Ann Picou is the second child born to Rudy and Juanita Picou in Lafayette, Louisiana. Alicia has one older sister, Karrie McMoy and one younger sister Laura Picou. Karrie is a registered nurse, and married to Todd McCoy with one child, Madeline Grace McCoy. Laura is currently attending Louisiana State University to obtain a bachelor's degree in biological engineering. Alicia was born in Houma, Louisiana, and raised in Lafayette, Louisiana. She attended high school at St. Thomas More in Lafayette, Louisiana.

After graduating in 2003, she attended Louisiana State University where she earned her bachelor's of science degree in Animal, Dairy and Poultry Science in May of 2007. Alicia participated in undergraduate research projects under the supervision of Dr. Ken Bondioli. While pursuing her degree, Alicia worked as a student worker at the Louisiana State University Reproductive Biology Center and as a fitness instructor at Spectrum Fitness, in Baton Rouge, Louisiana.

Alicia entered graduate school in the fall of 2007 under the direction of Dr. Ken Bondioli. Now she is a candidate for the degree of Master of Science in reproductive physiology in the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.