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REGULATION OF TELOMERASE EXPRESSION IN STEM CELL
REPROGRAMMING

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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Table of Contents

	Page
Acknowledgements	iii
List of Tables	vii
List of Figures	viii
List of Abbreviations.....	xii
Abstract.....	xiv
Chapter	
1 Background and Review of Literature.....	1
Aging, Disease and the Telomere	1
Telomerase.....	8
hTERT regulatory networks	12
Bone Marrow Mesenchymal Stem Cells.....	18
Adipose-derived Mesenchymal Stem Cell	22
Embryonic and Induced Pluripotent Stem Cells.....	25
Study Rationale	32
2 Functional Stem Cell Classification of Adipose-derived Stromal Cells.....	36
Introduction.....	37
Materials and methods.....	38
Results.....	45
ASCs can be isolated from many sources, have normal growth and are karyotypically stable.....	45

ASCs lack telomerase, and show telomere erosion with serial passaging	50
Immunophenotyping.....	53
ASCs have high multipotency.....	60
ASCs express Oct-4, SOX2, and NANOG.....	63
Discussion.....	67
3 hTERT Regulation During the Process of Induced Pluripotent Stem Cell	
Generation.....	70
Introduction.....	71
Materials and Methods.....	73
Results.....	80
Stable Oct-4 expression does not activate hTERT.....	80
Adenoviral Oct-4, SOX2, NANOG, and Lin28 expression does not activate hTERT in ASCs or BJ cells.....	83
Adenoviral Oct-4, SOX2, NANOG, and Lin28 expression with Trichostatin A or C-MYC activates hTERT expression.....	96
Oct-4, SOX2, NANOG, and Lin28 infections upregulate C-MYC expression.....	105
hTERT promoter in ASCs and BJ fibroblasts is in a closed, inactivated chromatin state.....	109
Discussion.....	114
4 Discussion.....	121

References	131
Vita.....	142

List of Tables

	Page
Table 1: Summary of ASC karyotypic analysis.	49
Table 2: Summary of cell surface markers from literature	54
Table 3: Summary of Infections and hTERT Activation.	104
Table 4: RT-PCR and qRT-PCR Primers.....	120

List of Figures

	Page
Figure 1: “Aging kills”	2
Figure 2: The end replication problem.....	4
Figure 3: Telomere attrition leads to cellular crisis	5
Figure 4: Telomerase extension mechanism	9
Figure 5: The hTERT promoter.....	13
Figure 6: The histone code	16
Figure 7: Mesenchymal stem cell multipotential.....	19
Figure 8: ASC primary isolation procedure	23
Figure 9: Pluripotent gene regulatory network.....	30
Figure 10: Growth chart of representative populations of ASCs	47
Figure 11: ASC karyotype analysis	48
Figure 12: ASCs lack telomerase activity in all isolated samples	51
Figure 13: ASCs experience telomere attrition with continued passage.....	52
Figure 14: iPS cell surface immunophenotype.....	55
Figure 15: ASC 8 cell surface immunophenotype.....	56
Figure 16: ASC 9 cell surface immunophenotype.....	57

Figure 17: ASC12s cell surface immunophenotype	58
Figure 18: BJ fibroblast cell surface immunophenotype	59
Figure 19: HL-60 cell surface immunophenotype	61
Figure 20: ASCs differentiate into adipocytes, compared to controls	62
Figure 21: ASCs differentiate into osteoblasts, as compared to controls.....	64
Figure 22: ASCs differentiate into chondrocytes, as compared to controls.....	65
Figure 23: qRT-PCR of Oct-4, SOX2 and NANOG in ASCs show a significant increase in expression over BJ fibroblasts	66
Figure 24: ESC and iPS colony morphology.....	72
Figure 25: ASC and BJ lentiviral stable Oct-4 over-expression as compared to iPS levels	81
Figure 26: Lentiviral Oct-4 expression in ASCs and BJs does not activate hTERT	82
Figure 27: Elevated hTERT expression using adenoviral hTERT in ASCs and BJ.....	84
Figure 28: Adenoviral Oct-4 does not activate hTERT expression.....	85
Figure 29: BJ and ASC Adenoviral Oct-4 does not upregulate hTERT expression, Adenoviral hTERT telomerase expression	86
Figure 30: ASC and BJ Adenoviral SOX2 over-expression does not activate hTERT....	88
Figure 31: ASC and BJ Adenoviral NANOG over-expression does not activate hTERT	89
Figure 32: ASC and BJ Adenoviral Lin28 over-expression does not activate hTERT	90
Figure 33: ASC and BJ Adenoviral Oct-4 over-expression	91
Figure 34: ASC and BJ Adenoviral SOX2 over-expression	92
Figure 35: ASC and BJ Adenoviral NANOG over-expression.....	93

Figure 36: ASC and BJ Adenoviral Lin28 over-expression.....	94
Figure 37: Adenoviral Oct-4, SOX2, NANOG and Lin28 expression does not activate hTERT	95
Figure 38: TSA treatment has no effect on telomerase activity in BJ and ASC	97
Figure 39: Adenoviral Oct-4, SOX2, NANOG, Lin28 + TSA activates hTERT in ASC and BJ.....	98
Figure 40: hTERT activation in ASCs upon Adenoviral C-MYC and/or TSA treatment....	99
Figure 41: hTERT activation in BJ upon Adenoviral C-MYC and/or TSA treatment...	100
Figure 42: Representative telomerase activity gel for ASCs and BJs infected with Adenoviral C-MYC, Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment	102
Figure 43: Telomerase activity in ASC and BJ following adenoviral infection with C- MYC, Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment.....	103
Figure 44: Western blot analysis of adenoviral Oct-4, SOX2, NANOG, Lin28 and C-MYC protein over-expression.....	106
Figure 45: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment	107
Figure 46: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28, C- MYC and/or TSA treatment.....	108
Figure 47: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment	110

Figure 48: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28, C-MYC and/or TSA treatment.....	111
Figure 49: Optimization of sonication conditions for ChIP	112
Figure 50: Histone modifications indicative of a closed chromatin state at the hTERT promoter in BJ fibroblasts.....	113
Figure 50: Chromatin state of the hTERT promoter in BJ, ASC, IMR90 and iPS cells.....	115
Figure 52: Working model for ASC hTERT regulation	119

List of Abbreviations

ASC	Adipose Derived Stromal Cell
BMMSC	Bone Marrow Mesenchymal Stem Cells
CFU	Colony Forming Unit
ChIP	Chromatin Immunoprecipitation
CMV	Cytomegalovirus
c-MYC	Myelocytomatosis Viral Oncogene Homolog
DKC	Dyskeratosis Congenita
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cell
H3	Histone 3
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase Complex
HMT	Histone Methyltransferase
HP1	Heterochromatin Protein 1
hTERT	Human Telomerase Reverse Transcriptase
hTR	Human Telomerase RNA
iPS	Induced Pluripotent Stem Cell
K9	Lysine 9
KLF4	Kruppel-Like Factor 4
Lin28	Lin-28 Homolog (<i>C. Elegans</i>)
LSD1	Lysine-Specific Demethylase

M1	Mortality Stage 1
M2	Mortality Stage 2
Mad1L	Mitotic Arrest Deficient-like 1
MHC	Major Histocompatible Complex
MSC	Mesenchymal Stem Cell
NANOG	Homeobox Protein
Oct-4	POU class 5 homeobox 1
PD	Population Doubling
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCID	Severe Compromised Immune Deficient
SCNT	Somatic Cell Nuclear Transfer
SOX2	Sex Determining Region Y-box 2
SSEA	Stage Specific Embryonic Antigen
SVF	Stromal Vascular Fraction
TSA	Trichostatin A
TRAP	Telomere Repeat Amplification Protocol
TRF	Telomere Restriction Fragment
USF	Upstream Stimulating Factor
VPA	Valproic Acid

Abstract

REGULATION OF TELOMERASE EXPRESSION IN STEM CELL REPROGRAMMING

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Virginia Commonwealth University, 2002

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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A great need exists for an abundant, easily accessible source of patient-specific cells that will function for use in regenerative medicine. One promising source is the adult stem cell derived from adipose tissue (ASCs). Isolated from waste lipoaspiration, these cells could serve as a readily available source for the regeneration of damaged tissues. To further define the biology of ASCs, we have isolated multiple cell strains from different adipose tissue sources, indicating wide-spread distribution in the body. We find that a widely used set of cell surface markers fail to distinguish ASCs from normal fibroblasts. However, our ASC isolations are multipotent while fibroblasts show no differentiation potential. In further contrast to fibroblasts, these cells also show expression of genes

associated with pluripotent cells, Oct-4, SOX2, and NANOG. Together, our data suggest that while the cell surface profile of ASCs do not distinguish them from normal fibroblasts and their lack of telomerase shows their limited proliferation capacity, the expression of genes closely linked to pluripotency and their differentiation capacity clearly define ASCs as multipotent stem cells.

iPS cells are another promising cell type for tissue regeneration, due to their expression of hTERT and their capacity to differentiate into all three germ layers. Interestingly, telomerase is activated during the induction process, accomplished by the exogenous expression of four genes in normal, non-hTERT-expressing fibroblasts. To elucidate the mechanisms behind this activation, we examined the overexpression of these four factors in BJ fibroblasts and ASCs, which resulted in undetectable hTERT expression. We then demonstrated a lack of an acetylated histone H3K9 with the opposing dimethylation, indicative of a closed chromatin state at the hTERT promoter. Subsequent treatment of cells with TSA alone showed an upregulation of hTERT mRNA without telomerase activity. However, telomerase activity was found when ASCs, but not BJs were treated with TSA and all four factors, indicating differential regulation of hTERT in cells of similar mesenchymal origins. Our data suggest that while hTERT's expression is universally dependent on the presence of a relaxed chromatin state and sufficient transactivating factors, other cell to cell differences can prevent its expression.

Chapter 1

Background and Review of Literature

Aging, Disease and the Telomere

There are approximately 2.5 million deaths each year in the United States, and 1.7 million of these deaths are in individuals sixty-five and older. With the average life span in the USA at approximately seventy-eight years of age, this places roughly 70% of all deaths in America in the final 16% of our average available life (Heron *et al.*, 2009). This strong correlation between aging and the increased incidence of death and disease has an exponential growth relationship causing the statistical probability of death to increase significantly each year (Heron *et al.*, 2009, Figure 1). The underlying causes of these late-in-life deaths are diverse and complicated; however, there are two specific diseases in the forefront: heart disease, comprising approximately 40% of deaths in the US and malignant neoplasms with 23% of deaths, which equates to ~63% of elderly deaths. The relationship between the increased incidence of these two diseases with ongoing organismal aging creates a strong need to understand the mechanism relating these two phenomena. This particularly holds true for cancer, which is defined as an accumulation of acquired or inherited DNA mutations leading to the genetic dysfunction and resulting phenotypic neoplasias seen in human disease. These genetic aberrations occur with certain statistical probability over time at an approximate mutation rate of 2.2×10^{-9} per base pair per year (Kumar & Subramanian, 2002). Further, due to the statistical guarantee of base pair mutations, the culmination of these events and subsequent disease seem to be an almost inevitable event if overall life is long enough.

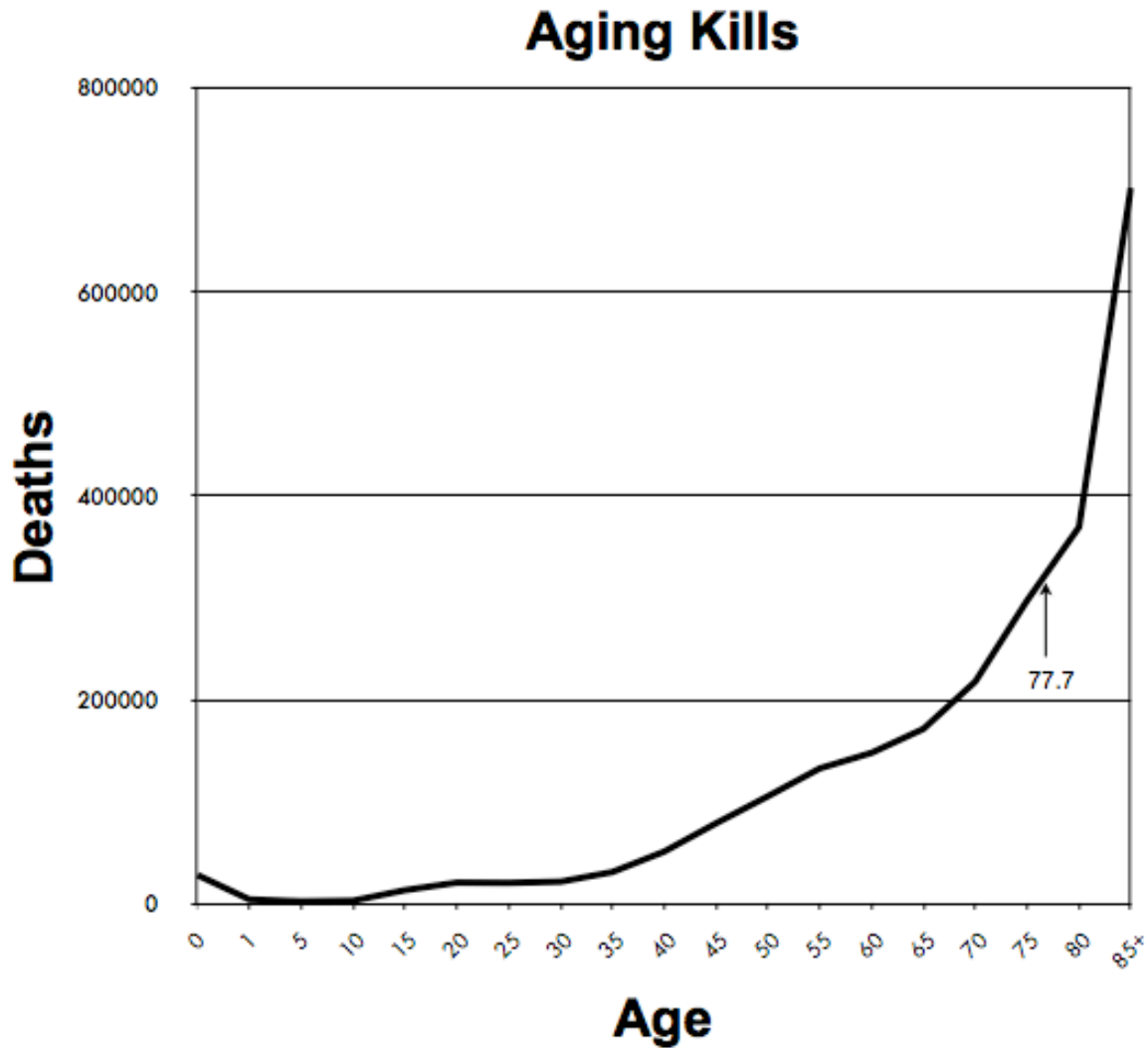


Figure 1: “Aging Kills.” As humans age, the likelihood of death increases exponentially. Of the sum 2.5 million deaths every year, 70% occur after the age of 65 with a sharp increase after passing the average life expectancy of 77.7 years. An obvious sentiment, “aging kills,” summarizes how the many diseases that cause death are linked to the deterioration of the body as we grow older. Data adapted from CDC 2006 Annual death rate report (Heron *et al*, 2009).

When considering DNA damage and accumulative mutation, another important process must also be taken into account: the relation of the gradual loss of telomeres with age. The ends of linear chromosomes, termed telomeres, are constructed of a hexameric repeat sequence TTAGGG (Watson 1978, Moyzis *et. al.* 1988, Olovnikov 1971) Due to the nature of DNA replication machinery, these tracts are lost progressively due to the mechanism known as the “end replication problem” (Figure 2) (Watson 1978, Olovnikov 1971). As DNA is replicated, the leading strand is continuously synthesized from 5’ to 3’, while the lagging strand is discontinuously replicated from multiple RNA primers (Watson 1978, Olovnikov 1971). The resulting DNA segments of the newly synthesized lagging strand, termed Okazaki fragments, have their primers removed and the gaps are then filled in with DNA. Problems with this process arise when the most terminal RNA primers are removed. Due to the nature of RNA priming, the last RNA initiation sites typically are not annealed at the very end of the telomere. When removed, DNA ligase is incapable of filling in the gap as there is no flanking DNA to bridge to, which results in incomplete synthesis and a shortened 5’ strand with a 3’ overhang. This 3’ overhang is typically degraded resulting in a loss of DNA every time the cell divides (Moyzis *et. al.* 1988, Blackburn 1991). These shortened telomeres can be relatively innocuous for a cell, functioning as a proliferative “clock,” which turns on very strict signaling for a senescent non-dividing state once a certain shortened length is achieved.

This limit to the proliferative capacity was first described in 1961 by Leonard Hayflick, thus the point at which cells undergo proliferative senescence is termed the “Hayflick limit” (Hayflick *et. al.* 1961). The inactivation of cell checkpoint molecules that signal senescence, such as pRB and p53, typically through gained gene mutation, allow for continued cellular proliferation past the Hayflick limit resulting in further telomere erosion (Figure 3)

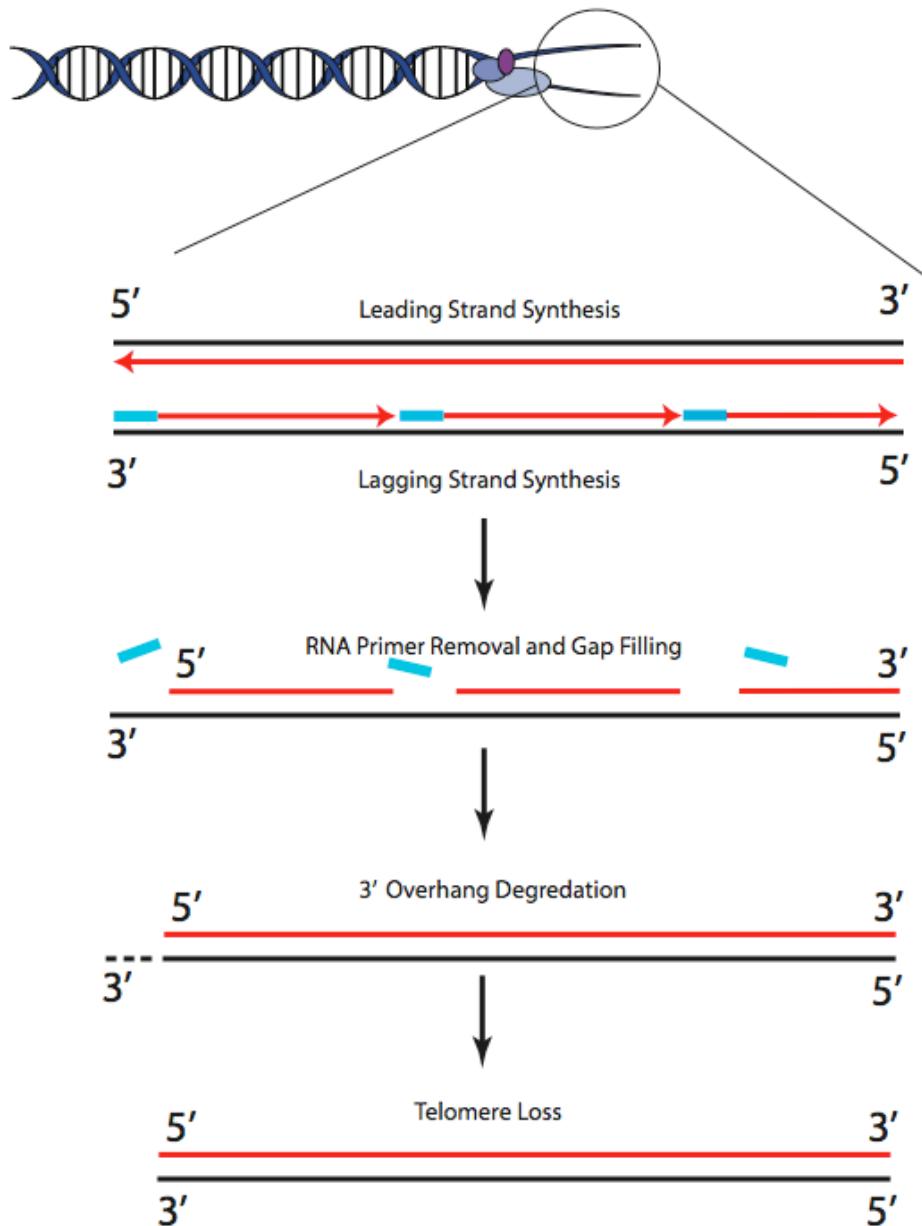


Figure 2: “The End Replication Problem.” During normal eukaryotic linear DNA replication, the synthesis of the lagging strand results in multiple pieces of new DNA called Okazaki fragments. The replication of these fragments is initiated via multiple RNA primers, which are removed after synthesis is completed. When the primer bound on the most terminal end of the telomere is removed, due to its position, it is not replaced, resulting in a gap on the 5' portion of the newly synthesized strand.

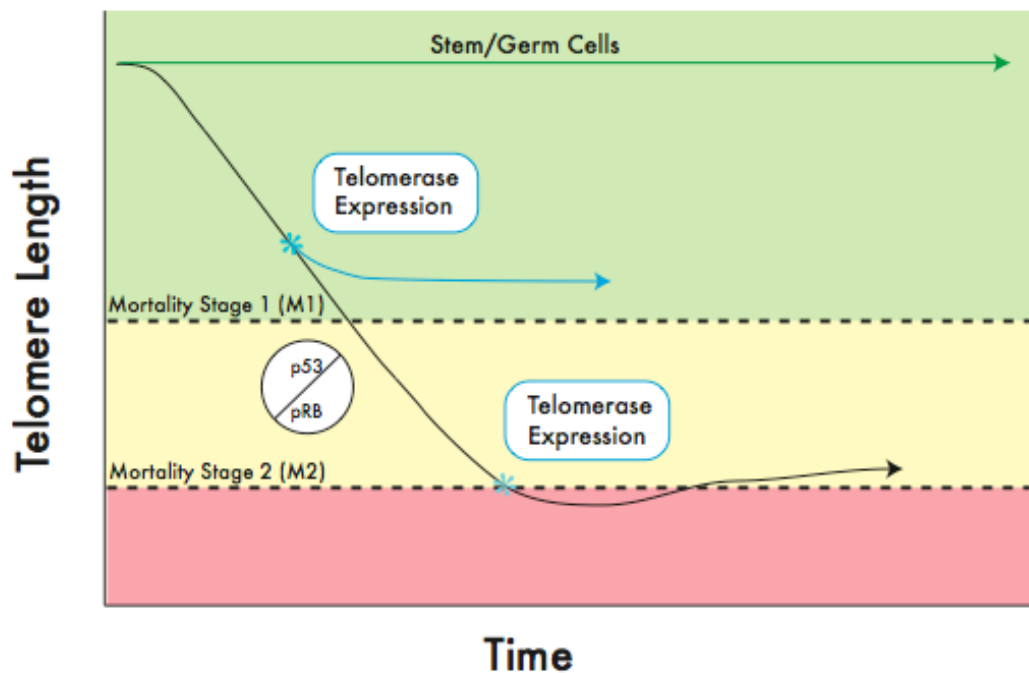


Figure 3: Telomere attrition leads to cellular crisis. As normal somatic cells proliferate, they lose telomeric tracts of DNA due to the “end replication problem”. Germ and some stem cells however, maintain their telomeres using the ribonucleoprotein enzyme telomerase. If telomerase is activated ectopically in normal somatic cells before mortality stage 1 (M1; a.k.a senescence), cells begin to maintain and stabilize their telomeres allowing for an infinite proliferative lifespan (blue arrow). Once telomeres reach a certain length, cells will undergo a senescent growth arrested state (M1). If cells are able to inactivate cell checkpoint molecules such as p53 and pRB, they will continue to divide until telomeres become critically short and enter into mortality stage 2 (M2; a.k.a. crisis). This is typically characterized by cell death, but some cancer cells manage to immortalize and activate endogenous telomerase, allowing for continued proliferation.

As the telomeres continue to erode past mortality stage 1 (M1), they will reach a critically short state that typically results in the formation of chromosomal aberrations such as end to end fusions, anaphase bridges, and chromosomal breakage resulting in the induction of mortality stage 2 (M2; crisis) (Holt *et al.*, 1999). All of these chromosomal abnormalities lead to acquired genetic deficiencies and allow cells to eventually progress to a tumorigenic state.

Along with tumor formation, the telomere is involved in many other human diseases as well, most of which interestingly mimic an aged human state. One such disease is Werner Syndrome, a type of progeria that was first described in 1904. The symptoms of Werner Syndrome include a phenotypic premature aging characterized by graying hair, substantially increased risk for tumor formation, heart disease, atherosclerosis, diabetes mellitus, thickening skin, and cataracts (Epstein *e. al.*, 1966). The molecular mechanisms have been linked to a mutation in the WRN helicase gene on the short arm of chromosome 8. The WRN Helicase's function is of paramount importance during DNA replication allowing for the proper unwinding of the template strand. The dysfunctional helicase causes large amounts of telomeric DNA to be left out during synthesis causing quicker than usual telomere erosion (Crabbe *el al.*, 2007). This causes many of the proliferating cells in the body to undergo premature senescence, explaining many of the traits associated with this disease.

Another disease associated with telomere dysfunction is Dyskeratosis Congenita (DKC) or Zinsser-Cole-Engman syndrome. This disease is also characterized by an advanced aging phenotype and an increased incidence of tumor formation (Georgouras 1965). The mechanism of this disease is hypothesized to be due to either a mutation or indirect inhibition of the RNA component of telomerase (hTR or TERC), which helps to extend the telomeric tracts in human non-somatic cells (discussed later) (Armanios *et al.*, 2009). This mutation was also mimicked in

mice, where the mTERC (mouse telomerase RNA component) gene was knocked out. At first, these mice showed no phenotype, but following many subsequent generations, the progeny began to show severe age-related degeneration (Blasco *et al.*, 1997). This delay was thought to be due to the extremely long telomeres of inbred mice. Once the aged phenotype began presenting itself, these mice also demonstrated an increased incidence of chromosome abnormalities. This genomic instability further demonstrates the necessity of telomere maintenance for normal function in an *in vivo* environment.

In addition to these diseases another interesting discovery linking telomere biology to aging became apparent with the first successful cloning of a mammal. Dolly the sheep was cloned in 1997 using a technique called somatic cell nuclear transfer (SCNT), a process that involves the fusion of an enucleated egg with a somatic cell, discussed in further detail later. Dolly was a healthy sheep, but ended up only living about half of the average 12 year lifespan of her species and died due to age related complications (Ritchie *et al.*, 1996). It was later hypothesized that this was due to the donor somatic cell being extracted from a sheep of six years of age. Thus, the sheep's telomeres had eroded down to about half of their total available length giving Dolly only six years of viable life until her cells began to senesce (Shiels *et al.*, 1999). These data demonstrate that the loss of telomeres is not only a cause of *in vitro* cellular senescence but is also strongly correlated with the onset of detrimental changes during organismal aging. Further, these syndromes show that specific diseases, such as tumor formation, are associated with the onset of old age and are linked to telomere erosion and dysfunction. While all cells of the body are subject to telomere attrition during normal division, some cells of the body have to circumvent this to fulfill their normal function, typically by expressing the enzyme telomerase.

Telomerase

Telomerase is a ribonucleoprotein enzyme, which is composed of two primary components: hTERT (human Telomerase Reverse Transcriptase), the catalytic portion of telomerase, which facilitates the physical addition of the telomeric tracts of TTAGGG, and hTR (human Telomerase RNA), an associated RNA that serves as the template for telomerase's reverse transcriptase activity (Blackburn, 1992; Bodnar *et al.*, 1998). hTERT is located on the short arm of chromosome 5 at 5p15, while the hTR gene is located on chromosome band 3q26 (Cong *et al.*, 1998; Wick *et al.* 1999). hTERT uses hTR as its RNA template, which allows telomerase to recognize and attach to the 3' overhang at the telomere and then processively reverse transcribe tracts of the hexameric repeat sequence to the end of the DNA (Figure 4, Feng *et al.*, 1995). This action provides additional telomeric DNA for replication to occur further out on the end of the lagging strand, thus preventing any loss with continued replication. This elongation prevents many of the deficits seen during continue proliferation due to telomere attrition and allows for a state of cellular "immortality."

In normal somatic cells, telomerase activity is not detected, as the hTERT gene is silenced through currently unknown mechanisms, while the hTR gene is constitutively expressed in almost all cell types (Bodnar *et al.*, 1998). This is in contrast to telomerase activity seen in most (~90%) cancer cells, germ line cells, and some stem cells (Holt *et al.*, 1996; Avilion *et al.*, 1996). These cell types are capable of maintaining their telomeric tracts through telomerase activity and thus have a potentially infinite replicative life span (Elmore *et al.*, 1999). To examine the specificity of hTERT's correlation with this immortal phenotype, normal fibroblasts were stably transfected with a vector containing only the hTERT gene coupled to a CMV

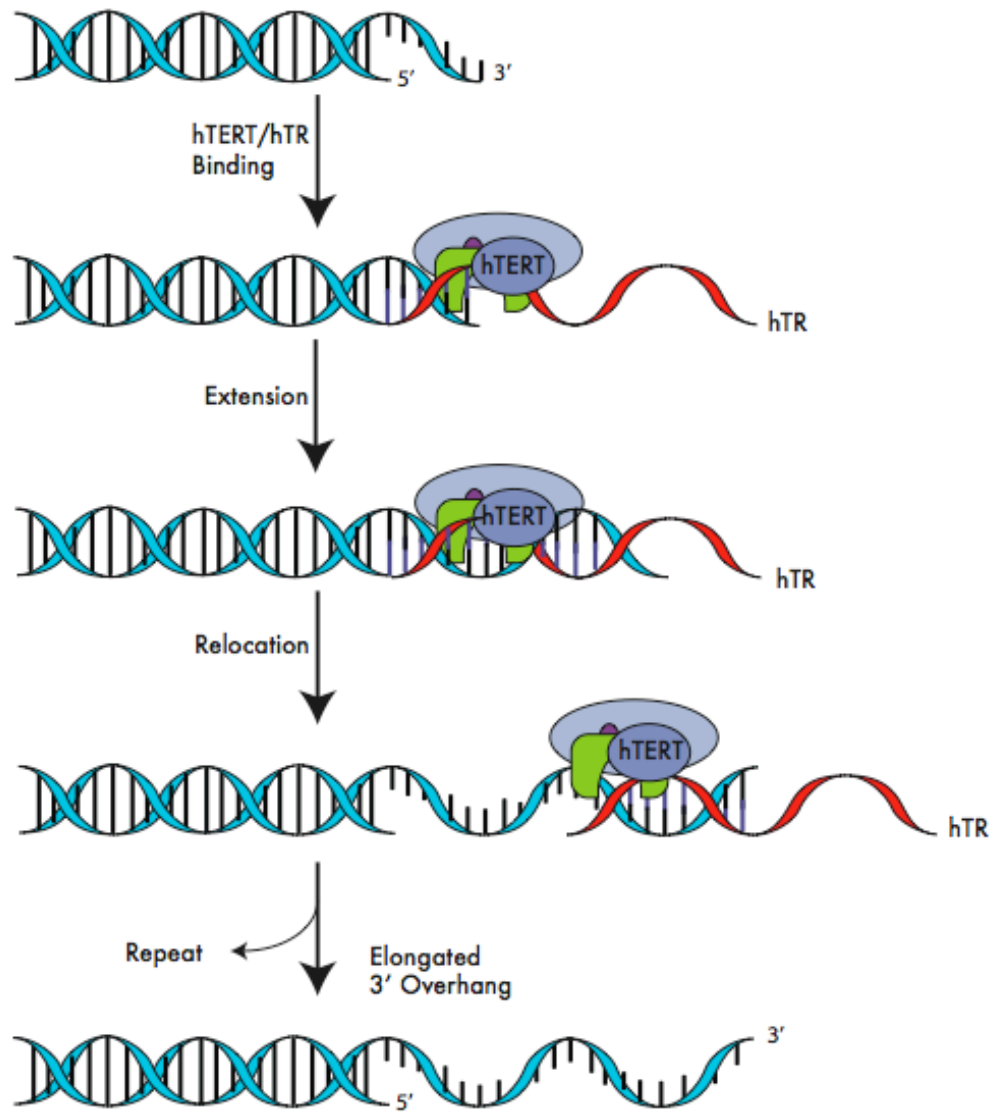


Figure 4: Telomerase extension mechanism. Telomerase operates in a processive manner adding telomeric hexamer tracts of TTAGGG to the 3' ends of DNA. hTERT first associates to the 3' overhang using its complementary RNA template (hTR) sequence. Telomerase then adds the six base pair sequence to the DNA end, translocates to the next available six base pairs, and elongates again. This process continues until a larger 3' overhang is achieved, allowing for DNA replication to occur further out on the telomere.

promoter (Counter *et al.*, 1998). Once cells were selected, the resulting population of fibroblasts exhibited the same immortal characteristics of cancer cells without any transformative capacity (Bodnar *et al.*, 1998). This demonstrated that hTERT is the only component in normal cells missing for proper telomerase re-constitution. This also showed that telomerase, although necessary for cancer progression, is not alone sufficient for malignant transformation, as the stable fibroblasts were propagated for many passages past their normal Hayflick limit with no tumorigenic properties detected, besides unlimited proliferation potential (Morales *et al.*, 1999). Due to telomerase's near universal expression in cancers and its capability for proliferative immortalization, it has been studied extensively as both a marker of tumor formation and as a target for anti-tumor therapy (Norton *et al.*, 1996; Shay & Wright 1996; Taylor *et al.*, 1996). Many researchers have approached telomerase inhibition primarily as an adjuvant therapy to standard cancer treatment modalities. After traditional cancer treatment using chemotherapy/radiation and/or surgery, telomerase would be inhibited allowing for a continued protection against tumor recurrence by limiting the proliferative capacity of any remaining neoplastic cells. Telomerase inhibition has also been suggested as a means to sensitize cancer to standard treatment, by preventing its recovery following standard methods of treatment (Huminiacki, 1996; Poynter *et al.*, 2007).

While telomerase poses a very attractive means at tumor prevention, its use in normal cells cannot be overlooked. When individuals undergo chemotherapy/radiation treatment, many cells that depend on high levels of proliferation such as hair follicles, crypt cells of the gut, and germ line cells are heavily affected as well. This is evident by the many negative side effects such as hair loss and continued GI tract irritation manifested post-treatment. Following treatment, many of these stem cell niches are permanently affected, causing premature grey hair

and possibly disease of the gut and gonads (Dekaney, 2009; Aulmann *et al.*, 2009; Kamil *et al.*, 2010). These effects are typically related to the senescence of the cellular source of replenishing tissues. The point has been argued that a therapy of telomerase activation could function as a restorative means of proliferation (Yudoh & Nishioka, 2004; Harley, 2005). This sort of telomere rejuvenation could be a mechanism of treatment for cells affected following cancer treatment. It could also be applied during disease progression of syndromes such as Werners, DKC and possibly during the normal process of aging (Davis *et al.*, 2006; Ariyoshi *et al.*, 2009; Rowe-Rendleman, 2004). This has been met with skepticism as many camps argue that this would cause more harm via a presumed increase in cancer susceptibility (Noble *et al.*, 2004). While this is certainly a cause for concern, studies have been done showing that telomerase expression and the subsequent telomere stabilization could have a protective effect, preventing tumor formation (Varadi *et al.*, 2009; Kassem *et al.*, 2004; Elmore *et al.*, 2002). One study in particular involved cells from patients afflicted with Li-Fraumeni syndrome, a disease characterized by a high rate of tumor formation due to a germline mutation in the tumor suppressor gene p53 (Elmore *et al.*, 2002). Due to p53's heavy involvement in the signaling of DNA damage including critically short telomeres, it was demonstrated that telomerase expression prevented spontaneous immortalization via telomere stabilization. This model indicates that the dysfunctional p53 present in Li-Fraumeni cells was incapable of inducing a senescent state, allowing for continued telomere erosion. This erosion (Figure 3) eventually leads to telomere dysfunction, chromosomal abnormalities and subsequent immortalization. By expressing telomerase and stabilizing telomeres before the cells were subject to genetic alterations, these cells were protected from tumor formation. These data support the idea that telomerase and telomeres are in fact primarily protective in nature. The expression of

telomerase, even in cells taken from people predisposed to premature tumor formation, abrogates, not causes, tumor progression.

hTERT regulatory networks

The promoter region of hTERT was characterized and independently published by a few groups in 1999 and the results hinted at multiple possible mechanisms of control, which could vary depending on the cell or tissue type (Cong *et al.*, 1999; Wick *et al.*, 1999). The promoter was found to contain a TATA binding box, being primarily composed of heavy GC rich regions making up large CpG islands surrounding the ATG start site (Cong *et al.*, 1999). To discover which regions encompassing the core promoter were most influential on hTERT expression deletion analysis was performed, showing that the largest reduction of activity was seen when the deletions fell into regions approximately ~300 bp upstream of the ATG start site (Cong *et al.*, 1999; Wick *et al.*, 1999). Further analysis of this “core promoter region” revealed two E box binding regions at -34 and -242, which were found to associate with a few different transcription factors (Takakura *et al.*, 1999) (Figure 5) and included direct repressive activities from transcription factors such as Mitotic arrest deficient-like 1 (Mad11) and Upstream activating factor (USF). Indirect mechanisms of repression were also found through factors such as Tax and Receptor CK (Sikand *et al.*, 2006; Gabet *et al.*, 2002). Activation through interaction with the E boxes was also associated with the binding of transcription factors such as the myelocytomatosis viral oncogene homolog (C-MYC) (Latil *et al.*, 2000; Günes *et al.*, 2000), and USF1/2 (Horikawa *et al.*, 2002) (Figure 5). C-MYC was of particular interest as it is a potent oncogene over expressed in many cancers (Suárez, 1989). Its role at the hTERT promoter is still

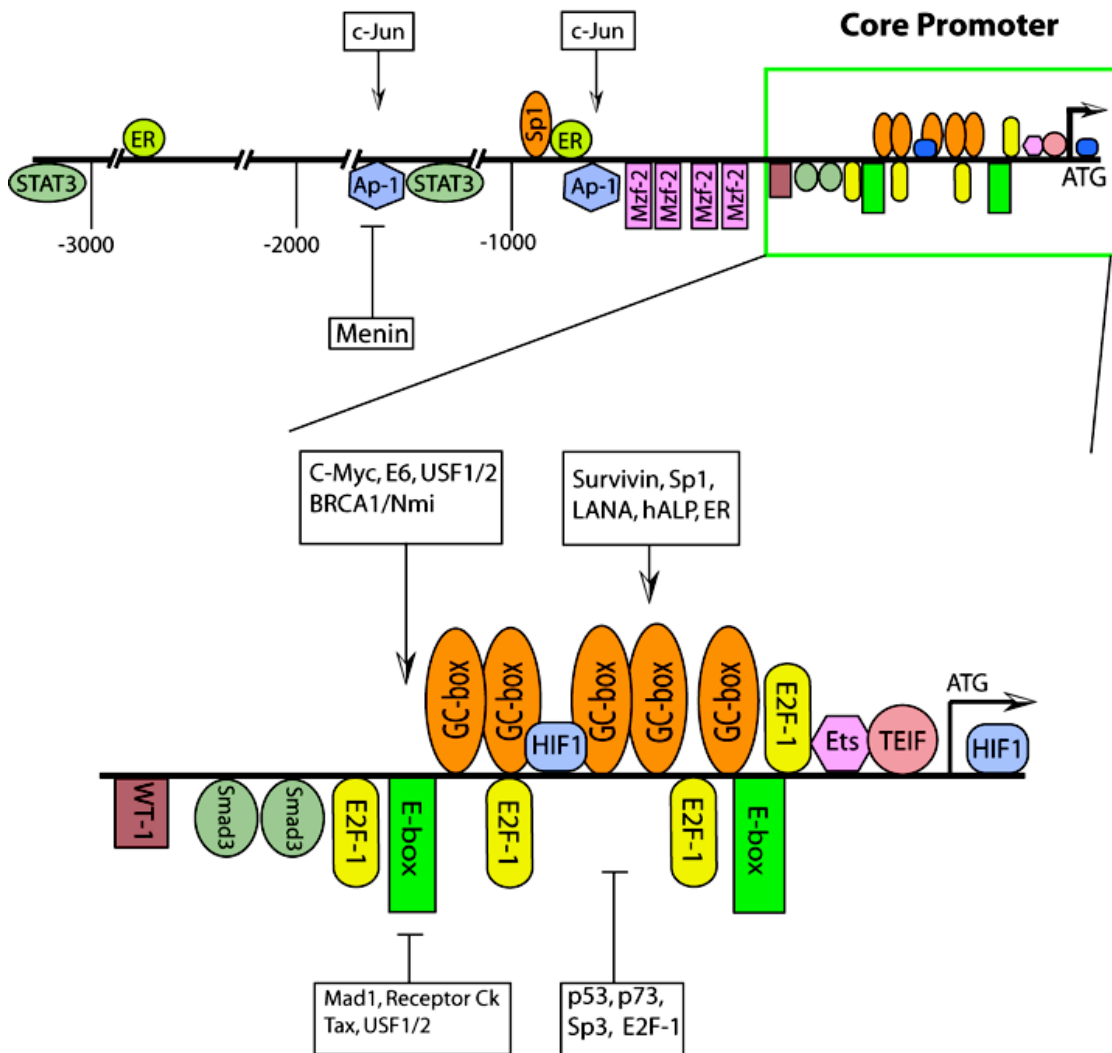


Figure 5: The hTERT promoter. The hTERT promoter has many sites for different mechanisms of activation and repression. Illustrated here is a selection of factors shown to have some direct binding and activity. Listed above each line are known activators, while those listed below the line are known repressors. The core promoter region encompassing approximately -300 to +25 of the ATG start site is highlighted and expanded for better resolution (Deprynski et al., 2008)

controversial as some studies have shown a strong activation effect when C-MYC is exogenously expressed in certain cell types, while others showed no such activation (Oh *et al.*, 1999). This activation was in contrast to that of Mad1, whose activity was shown to primarily act as an antagonist of C-MYC, competing for both heterodimerization with Max and binding to the E-boxes to down-regulate telomerase activity (Cerni *et al.*, 2002; Oh *et al.* 2000).

The E box was also shown to have an indirect interaction with a few other factors, such as the breast cancer susceptibility gene BRCA1. Its ability to activate telomerase was focused around some mutant forms of the BRCA1 protein, these mutations abrogate its binding to C-MYC, perhaps allowing for it to have more bioavailability and thus more activation of hTERT transcription in tumors (Li *et al.*, 2002; Xiong *et al.*, 2003). The core promoter was further shown to carry five GC boxes. These binding sites were shown to interact with a variety of different factors including Sp1, p53 and E2F-1 (Figure 5) (Cong *et al.*, 1999; Wick *et al.*, 1999). Sp1 was found to act primarily as a repressor, as it binds and recruits both histone de-acetylase complexes (HDAC) and p53 to the hTERT promoter (Won *et al.*, 2002). In contrast, Sp1 was also found to have the ability to upregulate expression through interactions with C-MYC (Oh *et al.*, 2001; Kyo *et al.*, 2000). One study showed that through mutational analysis of regions of the promoter, C-MYC activation was dependent on the presence of functioning GC boxes (Drissi *et al.*, 2001). Other control mechanisms were also seen at the epigenetic level, including methylation of the CpG islands surrounding the ATG start site and the modification of histone tail residues. CpG islands are stretches of DNA with a high guanine and cytosine nucleotide content. These CpG residues are susceptible to modification of the cytosines 5' carbon of its pyrimidine ring via the addition of a methyl group (Sharma *et al.*, 2009). DNA methylation usually has a repressive effect, preventing the binding of activating factors or allowing the binding of methylation-specific

transcriptional repressors (Lafon-Hughes *et al.*, 2008). On the hTERT promoter however, sites could be either hypermethylated or hypomethylated largely dependent upon the cellular environment (Dessain *et al.*, 2000). Some repression appeared to be due to the methylation of transactivating factor's binding sites (Guilleret *et al.*, 2004), while activation was seen due to the methylation of a putative repressor's binding site (Iliopoulos *et al.*, 2009). Both mechanisms proved to be highly variable and inconsistent however, as the distinction between an active and repressive state did not correlate with any discernable methylation pattern (Guilleret *et al.*, 2004; Iliopoulos *et al.*, 2009). It was also found that modifications of histones, specifically the acetylation, methylation and phosphorylation patterns on exposed N-terminal tails, plays a role in the modulation of hTERT gene expression in some cancer cells (Cong *et al.*, 2000; Won *et al.*, 2002; Lv *et al.*, 2003). Histones are multi-protein, heterodimerized complexes found in nucleosomes, which are primarily comprised of two histone H3 proteins, two histone H4 proteins, 1 histone H2a protein, 1 histone H2b, a histone H1 linker, and approximately 200bp of coiled DNA. In total this makes the entire protein complex approximately 130 KDa in size. The N-terminal residues of histones are typically exposed to the nuclear environment, and many studies have shown that particular patterns of modifications on these residues can alter the protein's overall conformation (Hon *et al.*, 2009). The shift in conformation is dependent upon the particular residues being modified and the type of modification, causing the histone to either physically loosen or tighten its binding to the DNA (Hadnagy *et al.*, 2008). This physical alteration hinders transcriptional machinery from interacting with the nucleosomes DNA. These tail residue modifications create what is called the "histone code" regulating the ability for genes to be expressed simply based on the ability for factors to gain access to the promoter region (Berger, 2007) (Figure 6). This "histone code" was examined during the differentiation of the

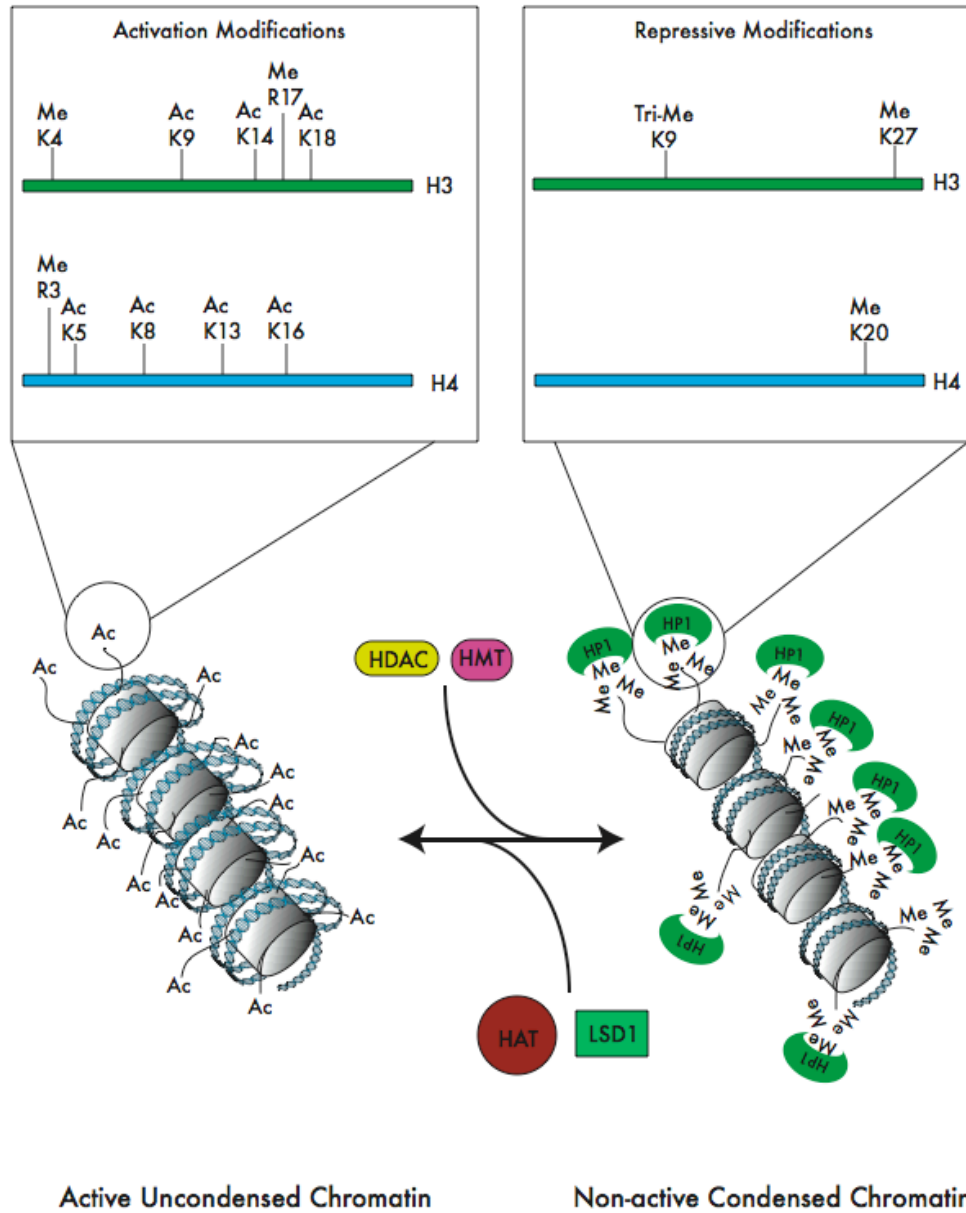


Figure 6: “The Histone Code.” Histone modification and the subsequent condensation or relaxation of chromatin is one epigenetic modification that regulates gene expression. The patterns highlighted above are modifications most often observed during histone relaxation and gene activation or condensation and gene repression. Highlighted are a few proteins involved in the process of a chromatin repressive (condensed) state, including histone deacetylase complexes (HDAC) and histone methyl transferase (HMT), as well as during the process of an active chromatin (relaxed) state, including histone acetyl transferase (HAT) and lysine specific demethylase (LSD1).

hTERT-expressing promyelocytic leukemia cell line HL-60 (Love *et al.*, 2007; Xu *et al.*, 1999). Upon differentiation of these cells by exposure to compounds such as dimethyl sulfoxide (DMSO) or retinoic acid causes the down-regulation of hTERT. During differentiation, the lysine tail residue K9 on histone H3 was deacetylated and di- or tri-methylated at the hTERT promoter, which indicated that the chromatin was in a condensed state (Love *et al.*, 2007; Xu *et al.*, 1999) (Figure 6). This histone modification correlated with hTERT's progressive deactivation during differentiation of HL-60 cells (Ge *et al.*, 2006). Once this heterochromatin state was achieved, the phosphorylation of the nearby tail residue serine 10 on H3 was further shown to allow for the binding of heterochromatic protein 1 (HP1) (Ge *et al.*, 2006), which has been implicated in maintaining heterochromatic state (Gwen *et al.*, 2006; Dormann *et al.*, 2006) (Figure 6). Of the three isoforms of HP1 (alpha, beta and gamma) HP1 α and HP1 β seem to be the causative members in chromatin binding in telomeric regions where the hTERT promoter is located (5p15.33) (Gwen *et al.*, 2006; Dormann *et al.*, 2006). The opposite "histone code" was also seen in HL-60 cells. When in an undifferentiated state, the hTERT promoter was associated with acetylated H3K9, a modification indicative of an open conformation (Figure 6). This modification correlated with the continued expression of telomerase (Cong *et al.*, 2000).

While the hTERT promoter has been cloned and sequenced, its regulation through transcriptional activation and repression is still underdeveloped. Many studies done to elucidate its activation used primarily artificial reporter systems (Ahmed *et al.*, 2003; Janknecht, 2004). Most have been conducted in cancerous lines as well, which largely ignores hTERT's regulation during the normal aspects of development and in normal adult tissues. To examine the regulation of hTERT in a non-cancer setting poses a challenge as most cells, which express telomerase are derived from cancerous growths. Of the non cancerous cells one of the most

promising cells for the study of hTERT are stem cells of various origins. Some adult stem cells have been shown to express low levels of hTERT while many others expression status is still debatable (Serakinci *et al.*, 2007).

Bone Marrow Mesenchymal Stem Cells

One of the first examples of stem cell research was attempted in the early 1960's by Till *et al.* at the University of Ontario, Canada (Till *et al.*, 1964), where they described a population of cells taken from the bone marrow of mice. These cells, when injected into the tail vein of another mouse, would form colonies in the spleen correlating to the exact number of cells injected. These colonies were shown to come from a single cell and, as they hypothesized, were progenitor cells of the hematopoietic compartment. Due to the nonproliferative nature of many cells such as erythrocytes and mature granulocytes, they theorized that these cells existed for the replacement of lost or damaged hematopoietic tissues (Siminovitch *et al.*, 1964; Till *et al.*, 1964). They further began to define the characteristics of a stem cell by making guidelines to highlight their capacity: they must have high proliferative capacity, must retain the ability to differentiate into other cell types, and must be capable of self-renewal (Till *et al.*, 1964). These guidelines persist today as the two defining features of stem cell populations, the ability to divide symmetrically (into identical daughter cells) and the capacity for asymmetric division (into differentiated cells) (Till *et al.*, 1964; Figure 7). In 1974, Friedenstein *et al.* further examined these same cells using an *in vitro* mechanism to assess colony formation (Friedenstein *et al.*, 1966; Friedenstein *et al.*, 1974; Luria *et al.*, 1971). They determined that following irradiation, the cellular viability and regeneration of cells in the bone marrow were due to these CFU (colony forming unit) cells, and as a result of their studies, they began referring to these

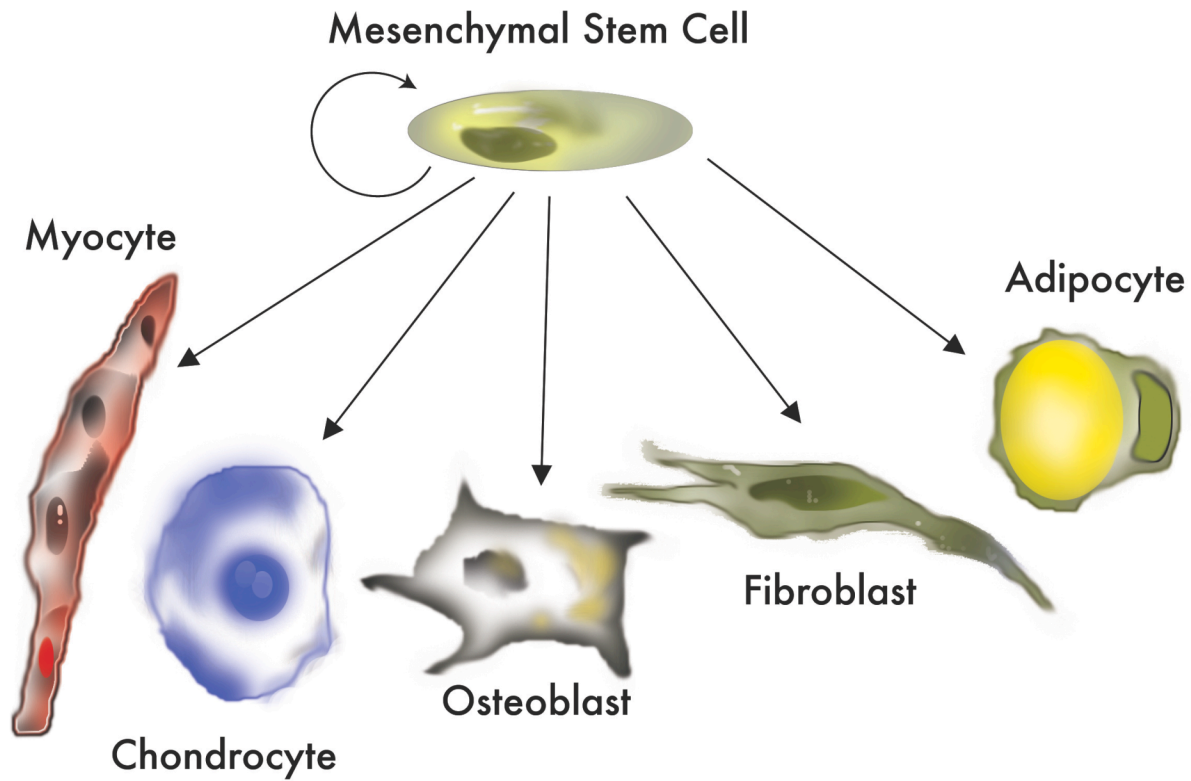


Figure 7: Mesenchymal Stem Cell Multipotential. MSCs are capable of differentiation into multiple lineages. They also retain the ability to divide both symmetrically, to produce identical daughter cells and asymmetrically (to produce differentiated lineages). MSCs, to date have been shown to differentiate into tendon, adipocyte, fibroblast, osteocyte, chondrocyte, myocyte, and some less frequent reports of non-mesenchymal cells such as neuronal cell lineages and pancreatic β -islet cells.

cells as colony forming unit-fibroblasts (CFU-F). Many names would follow including marrow stromal cells, multipotent adult progenitor cells and finally in 1991 by Caplan *et al.* the term mesenchymal stem cell (MSC) (Caplan *et al.*, 1991). This term was eventually adopted by most in the field due to growing evidence that the cells origins were from the mesodermal germ layer. One of the main adult stem cells studied in modern science was found, bone marrow mesenchymal stem cell or BMMSC. These cells, which Caplan and Friedenstein had been studying, were subsequently shown to have a multipotent ability to differentiate (Wakitani *et al.*, 1994; Vilamitjana-Amedee *et al.*, 1993). These cells primarily had the capacity to turn into osteocytes, chondrocytes, adipocytes, and myocytes (Wakitani *et al.*, 1995; Muraglia *et al.*, 2000) and with some reports surfacing indicating some non-mesodermal cell fates as well (Woodbury *et al.*, 2000; Chen *et al.*, 2004; Figure 7). Following this, the definition for the differentiation capacity of a stem cell centered around the number of lineages it was capable of following. The cells present in the zygote were described as totipotent, having the capacity to differentiate into all cell lineages including the extra-embryonic tissues. The cells of the blastocyst (discovered and isolated later) were described as pluripotent, capable of differentiating into all three germ layers. The last three categories typically describe the more limited potential adult stem cell, multipotent being capable of differentiating down more then two lineages, bipotent down two and unipotent directed into a single lineage. These differentiation capacities distinguish these cells from another adult stem cell, hematopoietic stem cells. Hematopoietic stem cells are also found in the bone marrow. However, their capacity to form cells is limited solely to blood-related lineages, which will not be covered extensively here.

Following a surge in research interest in the late 1990's and into the new millennia, there exist many groups generating very intriguing data in diverse fields related to adult stem cell

biology. Much of these studies have been focused around the use and description of the BMMSC that were originally described in the 1960's (Till *et al.*, 1964; Friedenstein *et al.*, 1966). These cells are primarily extracted and isolated from the iliac crest of marrow donors. Briefly, a general or local anesthesia is used, dependent on the quantity being extracted, a large coring needle is used to suction out the bone marrow, typically at multiple puncture sites for a complete transplantation. This is then filtered, and if used therapeutically, it is given to a recipient following an ablative treatment of chemotherapy or radiation therapy to remove all traces of the patient's dysfunctional stem cells. The infusion is then given through an IV drip placed inside the patient's chest cavity into the *superior vena cava* where the cells are allowed to migrate through the body and take up residence in the now vacant bone marrow cavity (Smiler *et al.*, 2006). If the cells are to be used for research purposes, the aspirate is filtered and plated on culture dishes in serum supplemented media. Cells will then adhere to the plate and begin to grow (Tocci *et al.*, 2003). Much of what is known about these cells comes from bone marrow use as a means to cure many diseases.

It is interesting to note that the bone marrow compartment has only in recent years become more thoroughly understood, even though the first successful treatment of a disease through bone marrow transplantation was performed in the 1960's (Kersey *et al.*, 1968). These transplantations have since been used to treat a diverse spectrum of diseases, including leukemia, aplastic anemia, lymphomas, multiple myeloma, immune deficiency disorders, as well as to replenish cells after toxic chemotherapies in some breast and ovarian cancer (Battiwalla & Hematti, 2009). *In vitro*, BMMSC have been shown to be relatively stable, maintaining good chromosomal stability with continued passaging and retaining a normal cellular phenotype (Garayoa *et al.*, 2009). With this lack of transformation ability, these cells have been justified as

a cellular source for regenerative medicine applications. One difficulty with BMMSCs is the method by which they are obtained, being relatively painful and not a procedure people do on an elective basis. It has also been shown that these cells lack a mechanism for the maintenance of telomeres, and any culturing of these cells, ultimately limits their *in vivo* proliferative potential.

Adipose-derived Mesenchymal Stem Cell

Following the discovery of the resident population of mesenchymal stem cells in the bone marrow, many groups began searching for other adult stem cell niches in the body. Many sources began appearing including stem cells described in the cord blood (Jäger *et al.*, 2009), in peripheral blood (Herbein *et al.*, 1994), in human dental pulp (Jo *et al.*, 2007), amniotic fluid (You *et al.*, 2008), and even from menstrual blood (Patel *et al.*, 2008). In 2001 another resident tissue type was discovered by Zuk *et al.* 2001, Adipose-derived Stem Cells (ASC). These cells are primarily isolated from discarded post-operative waste from individuals undergoing liposuction or abdominoplasty. The liposuction procedure, typically focused on the sections around the abdomen and buttocks, yields on average a few liters of adipose tissue. Following the initial liposuction, the tissue is processed via a few centrifugation steps and a collagenase digestion into what is called the stromal vascular fraction (SVF) (Figure 8; Zuk *et al.*, 2001). This portion of the tissue is primarily comprised of any remaining blood cells and mononuclear fibroblastic-like cells. The SVF is then subject to a red blood cell lysis buffer and the remaining stromal cells are then allowed to adhere to tissue culture plastic overnight. This step allows for any remaining buoyant cell types such as adipocytes and red blood cells to be washed away (Figure 8; Zuk *et al.*, 2001).

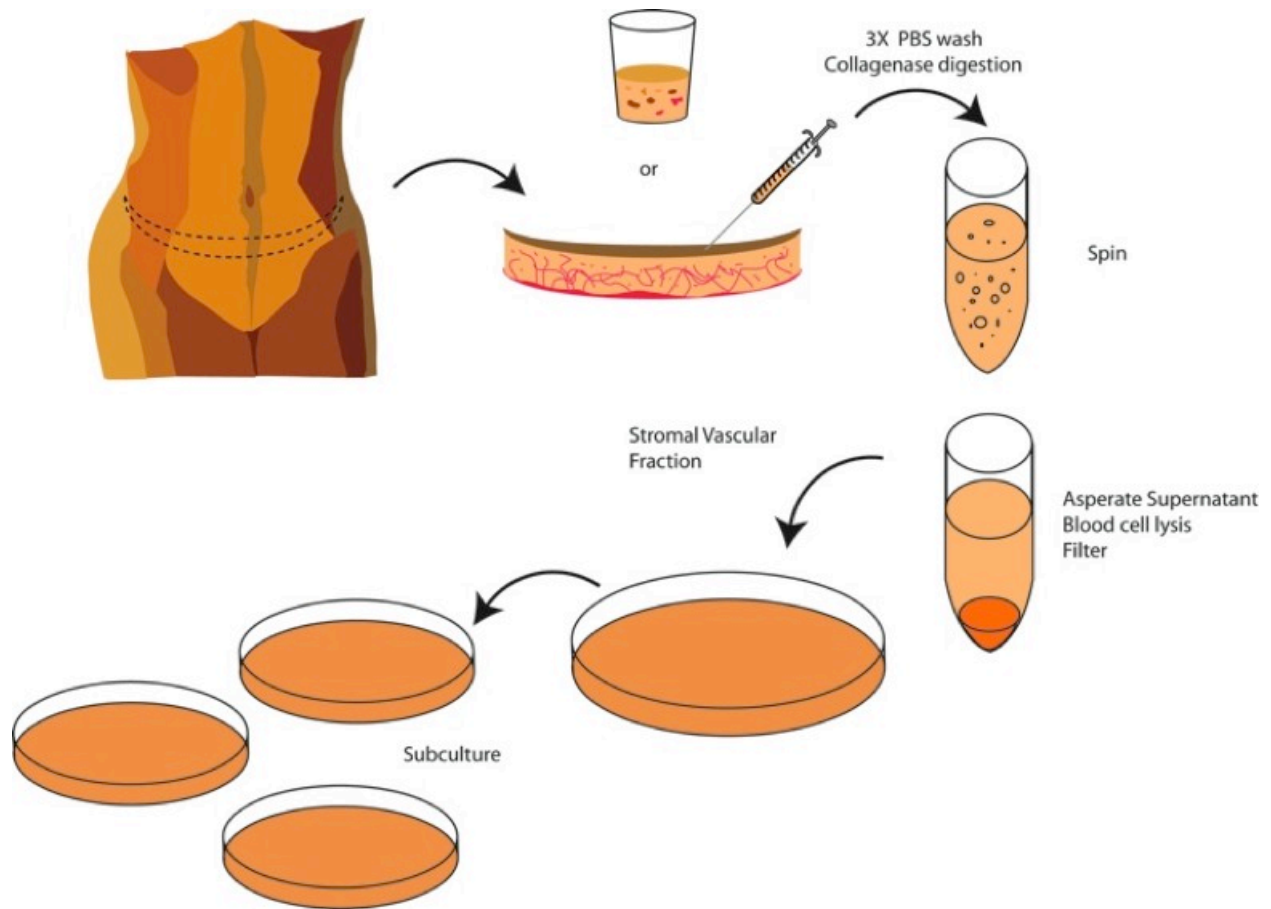


Figure 8: ASC primary isolation procedure. Tissues are harvested from either abdominoplasty sections or from liposuction waste. These unprocessed tissues are washed and subjected to a collagenase digestion, which results in a mixture with a soup-like consistency. This cell suspension is then centrifuged to separate the adipocyte layer from the stromal vasculature fraction (SVF). The SVF is then subjected to a red blood cell lysis, filtered and the mononuclear cells are isolated via a Percoll gradient. Finally, these mononuclear cells are plated in high serum media overnight to allow for attachment and subsequent subculturing.

Following plating and a few days of washing what remains was described as a population of cells closely mimicking the morphologies of bone marrow-derived mesenchymal stem cells. Their abundance, however, was on the order of 100 fold higher than those isolated from a similar volume of bone marrow tissue. These cells were subsequently shown to differentiate into osteocytes, chondrocytes, adipocytes and myocytes, proving their multipotency. These ASCs also grew at a much faster rate, and with a more robust ability to differentiate at older population doublings (Zuk *et al.*, 2001). With the discovery of ASCs, many believe the use of BMMSC should be replaced (Zhu *et al.*, 2008). Their obvious advantages being: their acquisition through an elective outpatient procedure, which occurs on a daily if not hourly basis in the US, with most of the removed tissue being waste; these procedures yield a high volume of cell/tissue; and the cells have the ability to differentiate better at higher passages than BMMSC (Zhu *et al.*, 2008; Peng *et al.*, 2008). These facts should allow ASCs to become a primary source of adult stem cell therapies, however, these cells have been shown to have a few negative properties, which infer caution. ASCs have been shown by some labs to undergo spontaneously transformation at higher passages (Rubio *et al.*, 2005) and have also been shown to express telomerase (Lin *et al.*, 2008; Peng *et al.*, 2008). These observations are somewhat controversial as many groups have reported no such transformation (Kang *et al.*, 2004; Kassem *et al.*, 2004), claiming these cells do senesce similar to BMMSC, just at a higher passage. While the presence of telomerase is viewed as somewhat of a negative due to its involvement in cancer progression, its expression from a tissue engineering standpoint would be a benefit (Kang *et al.*, 2004; Kassem *et al.*, 2004). For *in vitro* or *in vivo* tissue regeneration, there is a need for the cells to divide multiple times in order to fully replace or form new tissue. Following so many divisions, the cells may undergo

telomere-based senescence, making their viability after transplantation extremely limited (Garcia-Olmo *et al.*, 2009).

Adult stem cells present a unique opportunity for autologous transplant therapies. This strategy of rejuvenation therapy circumvents host vs graft disease and is capable of supplementing many damaged tissues if necessary. The limitations of this strategy is also stunted by a few negative aspects including the cells inability to differentiate into all types of tissues. These cells are also subject to culturing sensitivity and will senesce due to their lack of a telomere maintenance mechanism. For a universal solution, it would be necessary to further elucidate the mechanisms of hTERT regulation in an attempt to maintain cells for extended periods of time and to discover mechanisms to create more cellular lineages.

Embryonic and Induced Pluripotent Stem Cells

One of the first observations of pluripotent ability was made in reference to a type of cancer, teratocarcinoma (Beck *et al.*, 1969). These tumors were found to grow all three of the germ layers when removed from patients. These cancerous cells laid the foundation for many culturing techniques and importantly set the standard for pluripotent potential with the ability to turn into all three germ layers *in vitro* (Lehtonen *et al.*, 1989). These studies led to what has become a more common test of a cell's *in vivo* pluripotent ability, the injection of cells into athymic nude mice. Once injected, cells are allowed to grow, and then the mass is assessed for different lineages. The first embryonic stem cell (ESC) isolation was from mouse embryos, described in a paper published in 1981 by Gail Martin (Martin, 1981). This isolation involved the removal of the inner cell mass of late stage blasocysts from early implantation mouse embryos. These first embryonic stem cells were dependent on a feeder layer culturing system

using inactivated fibroblast cells (Pantazis *et al.*, 1985), which allowed for the ESCs to adhere to the plate and maintain an undifferentiated state, theoretically due to the extracellular matrix and growth factors the fibroblasts secrete.

Following the successful study of mouse ESCs, there were many attempts to isolate similar cells from human blastocysts. There was little success until 1997, when Dr. James Thomson from the University of Wisconsin in Madison, WI reported that he had successfully generated human ESCs (Thomson *et al.*, 1998). These cells acted very similarly to the mouse embryo derived ESCs, with the capacity to maintain an undifferentiated state *in vitro* when cultured continuously while retaining some hallmarks of pluripotency. These hallmarks included the expression of alkaline phosphatase, stage specific embryonic antigen (SSEA) 3 and 4, TRA-1-60 and TRA-1-81 (Thomson *et al.*, 1998). The cells also expressed the crucial enzyme telomerase in quantities sufficient to maintain their telomeres, and allow them to be continuously cultured without cellular senescence. These cells demonstrated the ability to differentiate into all three germ layers through the teratoma test via injection into severe immune combined deficient (SCID) mice. This discovery created enormous interest and opened up the possibility of generating any tissue type that was needed. Therapies for many different diseases would benefit from this work, such as spinal cord damage, Parkinson disease, diabetes, and many more.

To progress into therapeutics using these cells as a tissue substrate, there are many obstacles to overcome. One is the xenobiotic contamination of ESCs due to the necessity of a mouse feeder layer to maintain a proliferative undifferentiated state (Mallon *et al.*, 2006). Another serious scientific hurdle is the difficulty of immune rejection if these cells were to be used therapeutically, as harvested *in vitro* fertilized blastocysts would not be autologous when used for a variety of patients (Findikli *et al.*, 2006; Rippon & Bishop, 2004). To get ESCs to this

therapeutic, homogeneously differentiated end point is also difficult, as injecting any undifferentiated ESCs could pose a potential for teratoma formation (Rippon & Bishop, 2004). And finally, one of the biggest problems occurred in 2001, when then President George Bush in a national broadcast claimed any newly generated ESCs would be amorally obtained, and signed legislation limiting publicly funded research on ESCs to strains that had already been isolated. ESC usage suddenly came under extreme scrutiny and their utilization became a subject of debate as fevered as abortion. This legislation stunted ESC research for the next eight years in the public sector.

To address the difficulty of immune rejection, many studies were conducted to describe the cells immuno-reactivity (Lui *et al.*, 2009). Reports initially emerged that embryonic stem cells could be immune privileged due to their reduced expression of major histocompatibility complexes (MHC) class I and an absent expression of MHC class II expression on their surface (Drukker *et al.*, 2006; Drukker & Benvenisty, 2004). This led to studies indicating that undifferentiated cells had not only an anti-inflammatory response when injected into immune competent mice (Li *et al.*, 2004), but also seemed to be compatible upon a brief differentiation and subsequent implantation (Drukker *et al.*, 2006). Much about the immune reactive nature of ESCs is still open for debate and study, although it seems unlikely that these cells once fully differentiated will function without some sort of host immune response (Drukker *et al.*, 2006; Drukker & Benvenisty, 2004). A better solution to the immune responsive dilemma is a long hypothesized process that had originated in studies done on amphibians, Somatic Cell Nuclear Transfer (SCNT). SCNT was first realized in a mammal in 1997 by a group in Scotland led by Dr. Ian Wilmut (Ritchie *et al.*, 1996). The now famous sheep Dolly was created using a somatic cell from the udder of a six year old ewe. This process begins with the enucleation of a donated

egg cell or ovum and this genome-free egg is then electrically stimulated in the presence of an adult somatic cell causing the two to fuse (Ritchie *et al.*, 1996). Following this false fertilization, the egg began to divide and was shown initially to turn into a viable embryo with all the necessary cellular lineages (Ritchie *et al.*, 1996). This process, although seemingly simple sounding, is extremely labor intensive as there was no means for automation, meaning that each egg has to be individually fused (Ritchie *et al.*, 1996). Also, the process was very inefficient, as 277 eggs were fused in an effort to clone Dolly, which resulted in only 29 viable blastocysts. Of these, only one came to term and produced an adult sheep (Dolly). This process seemed at first to be a viable solution to generate isogenic lines capable of realizing the promise of ESCs potential as a therapeutic substrate.

Unfortunately, this process also had its own caveats, mainly owing to the public's perception on cloning, the need of large quantities of donor eggs and the difficulty encountered when cloning of primates was attempted (Fulka & Fulka, 2007). The process by which human eggs are harvested involves the treatment of the donor with high levels of hormones, which leads to a state of hyperovulation in which a woman produces as many as 5 eggs at a time (Bodri *et al.*, 2007). These hormone treatments have been met with much skepticism in terms of their degree of safety, as well as the prospect the demand might create an illicit market for harvesting human tissues as it is illegal to purchase human ovum for research purposes (Isasi & Knoppers, 2007). The field of SCNT research took a large blow when in 2006, Dr. Woo Suk Hwang, from Seoul National University, fabricated data indicating that he had created the long sought after human embryonic stem cell from a SCNT procedure (Hwang *et al.*, 2006; Hall *et al.*, 2006). The cells were eventually shown to actually be derived from the process of parthenogenesis, or the development of an embryo from an unfertilized egg. This notwithstanding many in the field

including Ian Wilmut, have discontinued their research on SCNT due to the discovery of induced pluripotent stem cells (iPS).

The discovery of iPS cells began with research conducted on embryonic cells to elucidate the necessary genetic factors to maintain a state of pluripotency (Rao *et al.*, 2004; Buehr *et al.*, 2003; Chambers *et al.*, 2003). Many studies found that there exists a complement of genes that were crucial for maintaining a pluripotent state. Of these genes, three were discovered to be essential, Oct-4, Sox2, and NANOG (Loh *et al.*, 2006). Oct-4, or Pou5f-1, is a homeodomain transcription factor of the POU family, which binds to the octamer motif on DNA (5'-ATTTGCAT-3') and has been shown to regulate many genes that are critically involved in the maintenance of ESCs in an undifferentiated state (Berrill *et al.*, 2004). SOX2, or sex determining region Y box -2, is a transcription factor and a key regulator of embryonic development and cellular differentiation. Finally, NANOG is also a homeodomain transcription factor that has been shown to be pivotal in embryonic development and in the maintenance of a dedifferentiated state (Cavaleri & Schöler, 2003). These factors were revealed to co-regulate one another (Figure 9), as a heterodimer Oct-4 and SOX2 have been reported to bind to the NANOG promoter, while the Oct-4/SOX2 heterodimer also has a self regulatory mechanism by binding to both promoter regions (Boyer *et al.*, 2005; Wang *et al.*, 2006; Figure 9). This interconnected regulatory network is thought to function as a transcriptional loop, maintaining the necessary levels of all of the genes to allow for the undifferentiated state to persist (Boyer *et al.*, 2005). While these genes regulatory mechanisms have been studied extensively, hTERTs regulation has yet to be studied in detail in ESCs. Its role in the maintenance of a pluripotent state is pivotal as ESCs were shown to undergo differentiation upon inhibition of hTERT

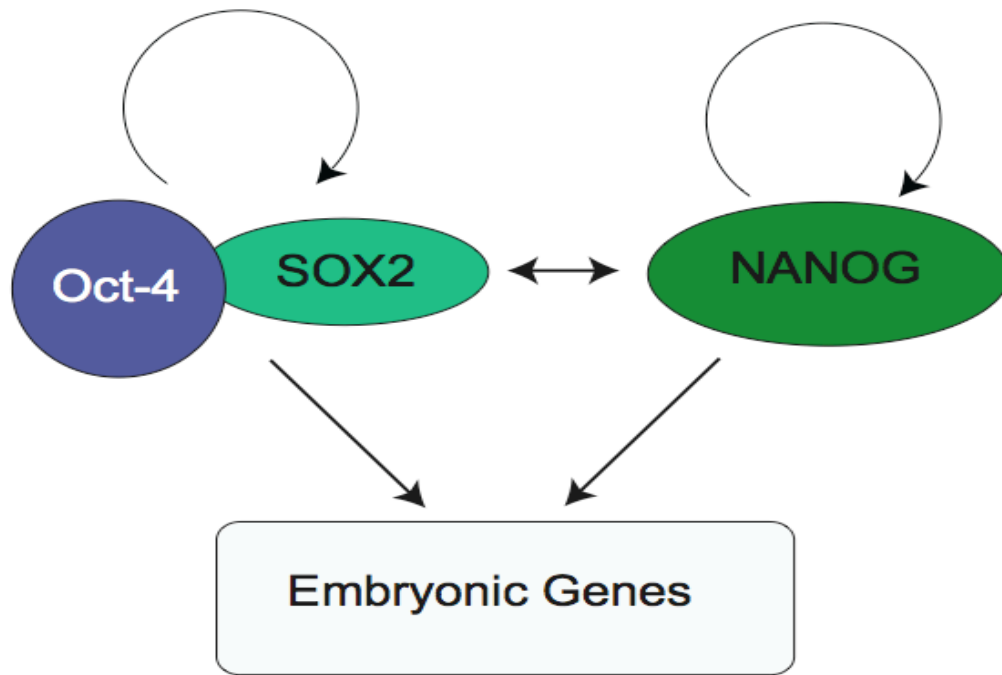


Figure 9: Pluripotent Gene Regulatory Network. Oct-4 and SOX2 heterodimerize and regulate both their own transcription as well as NANOGs. NANOG binds to both the Oct-4 and SOX2 promoters also increasing their transcriptional levels. Together these genes create a genetic network up-regulating embryonic specific-genes, allowing for the pluripotent stem cell phenotype.

(Yang *et al.*, 2008). This indicated that beyond hTERT's ability to maintain an immortal state, it also may have a more direct involvement in the necessary cellular environment for pluripotency.

In 2006, Dr. Shinya Yamanaka and Takahashi made a pivotal discovery when they stably infected both embryonic and adult fibroblasts with Oct4, Sox2, Klf4 and C-MYC (Takahashi & Yamanaka, 2006). They showed that with the stable integration and up-regulation of these genes in normal mouse fibroblast cells, they could generate colonies of pluripotent cells, termed induced pluripotent stem cells (iPS). These iPS cells were subsequently shown to be capable of differentiating into all 3 germ layers using the same teratoma forming assay, used to demonstrate ESCs pluripotency. And more importantly, when injected into blastocysts and implanted back into the womb, these cells were able to create chimeric mice with gametes capable of generating offspring entirely comprised of the iPS cells. One major caveat in creating these cells was the fact that overexpression of C-MYC, a potent oncogene was used for the process. As anticipated, it was shown that mice created from iPS cells generated using the C-MYC oncogene, had extremely elevated incidence of cancer, with all of the mice generated developing tumors. Fortunately C-MYC was shown to be dispensable for iPS generation, as the Yamanaka group subsequently showed iPS formation, albeit with lower efficiency, using just Oct-4, Sox2, and KLF4 (Nakagawa *et al.*, 2008). The initial discovery of iPS cells was quickly followed by other groups performing similar induction procedures on human adult fibroblasts, with the Thomson lab using Oct-4, Sox-2, Nanog, and Lin-28 (Yu *et al.*, 2007). These discoveries led to a veritable onslaught of new data, with many labs analyzing different mechanisms to induce pluripotency, including transient viral expression systems (Stadtfield *et al.*, 2008) and non-viral mechanisms ((Yu *et al.*, 2009; Kaji *et al.* 2009). Of particular interest is the status of hTERT during this induction process. Derived from normal, non-telomerase expressing cells, these cells undergo

many changes to allow for the cell to shift to a more primordial state, including the activation of hTERT. While many studies have addressed the fact that this enzyme exists and some studies going so far as to induce its expression as a means to make cells more susceptible to iPS formation (Park *et al.*, 2008), there is still much to discover about its role in this process and its regulation. Significant research has been dedicated to examining which of the factors are necessary for iPS formation, including some showing a very minimal set of genes as necessary (Nakagawa *et al.*, 2008; Huangfu *et al.*, 2008). One such study used only two factors successfully, Oct-4 and SOX2, this coupled with a histone deacetylase complex (HDAC) inhibitor, valproic acid (VPA) (Huangfu *et al.*, 2008). VPA is one of a family of compounds capable of preventing the action of HDACs, causing a general relaxation of the chromatin. VPA specifically inhibits HDAC1, while many other compounds of its type inhibit a broader range of HDACs such as trichostatin A (TSA), which potently inhibits almost all of the HDAC families.

Study Rationale

Telomerase expression has been a subject of interest to many research groups, with many advances in our understanding of how it is regulated occurring in the last decade (Lv *et al.*, 2003; Won *et al.*, 2002; Xu *et al.*, 1999; Cong *et al.*, 1999; Drissi *et al.*, 2001; Latil *et al.*, 2000). The vast majority of these studies focus on cancer due to hTERT's expression in over 90% of tumors. These studies have since led to many theories about the use of hTERT as a therapeutic target to combat tumorigenicity (Chen & Tollefsbol, 2009). While telomere maintenance is the primary source of immortalization in cancers, hTERT expression is still only a necessary but not causative. In fact, studies indicate that telomere elongation and stabilization through hTERT expression preventing telomere dysfunction, could serve as a means of protection against tumor

formation (Elmore *et al.*, 2002). Telomerase activity is strictly regulated in most somatic cells, yet hTERT becomes active again in immortalized cells and seems to remain active in some proliferating adult stem cell populations. While there have been reports in the literature that some adult stem cell populations do express hTERT, this finding is controversial and if present may be insufficient to maintain telomere lengths with continued proliferation (Lin *et al.*, 2008; Peng *et al.*, 2008). With the lack of a telomere maintenance mechanism, adult stem cells are subject to a limited proliferative life span. Thus, during the process of aging, slow erosion of telomeres over time within the stem cell populations will eventually lead to a senescent state. Once a critical amount of resident stem cells reach the end of their replicative life span, many tissues would go without cellular components to replace lost or damaged tissues. In particular, this sort of degradation can be seen in the premature aging syndromes, described above, such as Werners and DKC, which leads to the conclusion that the phenotype observed during the normal process of aging is at minimum partially due to the prevention of tissue renewal due to telomere dysfunction in replenishing cells. Clearly, telomerase's function in these stem cells is critical for the continued proliferation and their contribution to the constant rejuvenation necessary from normal cell turnover.

In adult stem cells, such as BMSC and ASCs, hTERT expression status is still controversial (Kang *et al.*, 2004; Kassem *et al.*, 2004; Lin *et al.*, 2008; Peng *et al.*, 2008). While some groups claim that hTERT can be used as an efficient marker of stem cell identity, many groups have attempted to use immunophenotyping of cell surface markers with variable results (Izadpanah *et al.*, 2006; Zuk *et al.*, 2001; Astori *et al.*, 2007; Katz *et al.*, 2005; Kern *et al.*, 2006; Leong *et al.*, 2005; Lin *et al.*, 2008; Noël *et al.*, 2008; Rebelatto *et al.*, 2008; Wagner *et al.*, 2005; Yoshimura *et al.*, 2006; Lin *et al.*, 2008; Zhu *et al.*, 2008). Due to the undefined stem cell

immunophenotype and the underdeveloped stem cell gene expression profile, it is still debatable how to properly identify stem cells.

We hypothesize that ASCs stem cell-like properties are based upon their multipotential abilities and associated gene expression rather than any specific cell surface marker profile.

During the process of iPS generation, two primary sets of stem cell genes were employed. Dr. Yamanaka's group demonstrated that Oct-4, Sox2, KLF4 and C-MYC are capable of dedifferentiation adult cells into an embryonic-like state. At the same time, Dr. Thomson's group demonstrated that a set of genes including Oct-4, Sox2, NANOG, and Lin28 were capable of performing the same process. In both studies, these groups demonstrated that these four factors were capable of generating embryonic-like cells that importantly expressed the catalytic component of telomerase, hTERT. The mechanism behind this activation has yet to be studied. The genes used by the Yamanaka group at first included the oncogene C-MYC. Its involvement in many cellular processes has been examined including direct targeting of the hTERT promoter by C-MYC in some cell types (Drissi *et al.*, 2001; Kyo *et al.*, 2001). However, the Thomson group showed that their factors are capable of creating iPS cells that express hTERT without the infection of C-MYC.

We hypothesize that the introduction of the iPS genes Oct4, SOX2, NANOG and Lin28 create changes in transcriptional regulators and the chromatin structure at the hTERT promoter. These epigenetic changes cause the acetylation/de-methylation of specific

histone tail residues resulting in the relaxation of the hTERT promoter, increased access for transcription factors and the subsequent expression of the hTERT gene.

The data generated by these studies will lead to a better understanding of the many complicated mechanisms by which hTERT is regulated. These studies will allow for the advancement of important knowledge relating to the proper identification and functional analysis of the diverse population of the differing stem cells, both adult and embryonic. The results obtained could potentially lead to improved stem cell-related treatment for many diseases related to the natural process of aging. Also these studies will advance the use of telomerase for regenerative therapies for diseases that currently use non-hTERT expressing adult stem cells as cellular substrates.

Chapter 2

Functional Stem Cell Classification of Adipose-Derived Stromal Cells

Abstract

The discovery of ASC has created many opportunities for the development of patient-specific cell-based replacement therapies with a higher efficiency than the standard adult stem cell, BM-MSC. To further define these cells, we have isolated multiple cell strains from different adipose tissue sources, indicating wide-spread distribution in the body. Unfortunately, there exists a general lack of agreement in the literature as to their surface characteristics. We find that a widely used set of cell surface markers fail to distinguish ASCs from normal fibroblasts, as both are positive for CD29, CD73, and CD105 and negative for CD45, CD31, and CD14. All of the ASC isolates are multipotent and capable of differentiating into osteocytes, chondrocytes and adipocytes, while fibroblasts show no differentiation potential. In further contrast to fibroblasts, these cells also show expression of genes associated with pluripotent cells, Oct-4, SOX2, and NANOG. These pluripotent factors are expressed at significantly higher levels than in fibroblasts and BM-MSC, yet are still lower than induced pluripotent stem cells (iPS). Finally, we find undetectable levels of the catalytic component of telomerase, hTERT, in the ASCs independent of age and culturing conditions. ASCs undergo telomere attrition and eventually senesce, while maintaining a stable karyotype without the development of any spontaneous tumor-associated abnormalities. Together, our data suggest that while the cell surface profile of ASCs does not distinguish them from normal fibroblasts, the expression of genes closely linked to pluripotency, and their differentiation capacity clearly define ASCs as multipotent stem cells.

Introduction

The utility of adult stem cells presents a unique opportunity for the development of low cost, low risk methods to treat diseases using patient-specific cells. Current techniques employ a generally low yield isolation protocol for BM-MSCs from the iliac crest of the pelvis (Izadpanah *et al.*, 2006), which is an invasive and painful procedure. More recently, a cell type similar to BM-MSCs was discovered residing in fatty deposits removed during elective lipoaspiration surgeries. These cells, appropriately named adipose-derived stromal cells (ASC), can be isolated in much higher efficiency relative to the BM-MSCs isolations (Zuk *et al.*, 2001), have a greater expansion capacity, and appear to differentiate efficiently into the same cell lineages as BM-MSCs (Zuk *et al.*, 2001). Following their discovery, the stem cell-like qualities of ASCs came under scrutiny as their identity and characteristics were still being determined. Many groups have attempted to define a specific set of cell surface markers to purify ASCs from the complex mix of cells in the stromal vascular fraction (SVF) isolated from lipoaspirate. A variety of combinations have been attempted (Izadpanah *et al.*, 2006; Zuk *et al.*, 2001; Astori *et al.*, 2007; Katz *et al.*, 2005; Kern *et al.*, 2006; Leong *et al.*, 2005; Lin *et al.*, 2008; Noël *et al.*, 2008; Rebelatto *et al.*, 2008; Wagner *et al.*, 2005; Yoshimura *et al.*, 2006; Lin *et al.*, 2008; Zhu *et al.*, 2008) with varying results, but a general lack of agreement exists as to which of these factors are the most relevant.

Accumulating evidence has been generated in an attempt to identify more of ASC's properties, including their resident niche, their stability in culture, and their gene expression profile (Lin *et al.*, 2008; Izadpanah *et al.*, 2006). A few investigators have shown that some SVF-derived cells undergo spontaneous transformation and/or aneuploidy with continuous culture, (Rubio *et al.*, 2005) while others show no such potential (Abdallah *et al.*, 2005; Fu *et al.*,

2001; Zhu *et al.*, 2008). Interest also exists in determining a set of genes such as Oct-4 and hTERT that would suggest these cells as more embryonic-like than the morphologically similar fibroblast cells,. Very few investigators have reported that ASCs express hTERT and some show expression of genes associated with pluripotency such as Oct-4. (Pochampally *et al.*, 2004; Lin *et al.*, 2008; Fu *et al.*, 2001; Zhu *et al.*, 2008). The indication that ASCs express markers of pluripotency suggests that these cells have characteristics similar to ESCs while maintaining the mesenchymal state of normal fibroblasts.

Using multiple ASC isolations, we demonstrate that the expression of a set of cell surface markers is similar for both ASCs and fibroblasts, indicating that this primary experimental method for defining stem cells likely does not depend on its cell surface characteristics. In order to more accurately define the stemness of our ASC cultures, we assessed differentiation capacity and the expression profile of a subset of genes associated with pluripotency, which provided evidence of their multipotency and stem cell qualities.

Materials and Methods

ASC isolation

Isolation was carried out as described previously (Zuk *et al.*, 2001) except where noted. Freshly harvested fat was obtained and immediately subject to isolation. Any separated oil was removed from the top of the lipoaspirate followed by the removal of the saline/blood fraction. For ASC 12s and 4s strains, we modified the protocol by removing the saline fraction to a separate conical and pelleting the cells as previously described (Francis *et al.*, 2010). Briefly, the pellet was washed once in 1X PBS and then subjected it to red blood cell lysis using 160mM NH₄Cl incubated at room temperature for 10 minutes. The treated saline fraction was filtered

through a 100µm mesh (BD Bioscience, Mississauga, ON, CAN), pelleted and resuspended and plated in 25ml DMEM/F12 supplemented with 50% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA) and 1% antibiotic/antimycotic (ABAM) (Invitrogen), 10ng/mL epidermal growth factor (EGF) (BD Bioscience, Mississauga, ON, CAN). For the remaining strains, the fat was separated into 200ml aliquots washed 3-4X with equal volume 1X PBS, and then resuspended in 50% volume 150µg/mL collagenase (Sigma, St. Louis, MO) with 1% antibiotic/antimycotic. This solution was incubated for 60 minutes in a 37°C shaker set at 250rpm. When the fat was digested sufficiently into a soup-like consistency, it was removed, and the collagenase was inactivated with 10% FBS. The digestion was then distributed into 50ml conicals and centrifuged at 1000xg for 10 minute to separate the oil and remaining fat lobules from the stromal vascular fraction (SVF). Once the SVF has been isolated, it was treated with 160 mM NH₄Cl to lyse red blood cells and applied to a Percoll gradient to purify the mononucleated cells. This fraction of mononuclear cells was then resuspended in 25ml DMEM/F12 supplemented with 50% FBS, 1% ABAM, and 10ng/mL EGF, and allowed to incubate overnight to select for adherence. The remaining floating cells were aspirated off the following day, and the plate is washed to remove any remaining red blood cells or fat lobules.

Cell Culture

Adipose stem cells were maintained in DMEM low glucose supplemented with 10% FBS, 1% antibiotic/antimycotic, and 10ng/ml at 5% CO₂ at 37°C. IMR90, a fetal lung fibroblast (CCL-186, ATCC Manassas, VA) and BJ fibroblasts cells (CRL-2522, ATCC) post natal foreskin fibroblasts, were cultured in DMEM high glucose supplemented with 10% cosmic calf

serum (Thermo Scientific, Waltham, MA), 3% Media 199 (Invitrogen, Carlsbad, CA) and 1% ABAM (Invitrogen).

Induced pluripotent stem cells and embryonic stem cells were maintained on an mytomycin C (Sigma) inactivated mouse embryonic fibroblasts monolayer, in knock out replacement media containing DMEM/F12, 20% KoSR (Invitrogen), 1% Penicillin/streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β -Mercaptoethanol (Sigma), and 4ng/ml basic Fibroblast Growth Factor (bFGF) (Invitrogen).

Osteocyte Differentiation

Cells were plated and grown until approximately 75% confluent, and then osteogenic differentiation media was added. The differentiation media consisted of DMEM high glucose, 1% FBS, 0.01 μ M 1,25-dihydroxyvitamin D3 (Sigma), 50 μ M ascorbate-2-phosphate (Sigma), 10mM β -glycerophosphate (Sigma), and 1% ABAM. The cells were cultured for 2 weeks until significant calcium deposits were observed. The cells were then washed and fixed using 4% paraformaldehyde solution and stained using Alizaran red S (Sigma), which specifically stains calcified deposits in the extracellular matrix. Whole field light microscopy images were captured at 10x magnification.

Adipocyte Differentiation

Cells were plated and grown until 100% confluent. Adipogenic media containing DMEM low glucose, 1% FBS, 0.5mM isobutyl-methylxanthine (Sigma), 1 μ M dexamethasone (Sigma), 10 μ M insulin (Sigma), 200 μ M indomethacin (Sigma), and 1% ABAM was then added. The plates were maintained for 2 weeks until lipid droplet formation was observed. The

cells were then washed and fixed using 4% paraformaldehyde and stained with Oil Red O (Sigma), which specifically stains lipid droplets. Whole field light microscopy images were captured at 10x magnification.

Chondrocyte Differentiation

Cells were collected and pelleted at 300xg in a 2ml V bottomed tube at 2×10^5 cells per pellet. These tubes were placed in the incubator at 37°C in 5% CO₂ in chondrogenic differentiation media containing DMEM low glucose, 1% FBS, 6.25 µg/ml insulin, 10ng/ml recombinant TGFβ3 (R&D systems Minneapolis, MN), 50nM ascorbate-2-phosphate, and 1% ABAM. After 1 week, the pellets were transferred to a 10cm² dish and cultured in the same media formulation for another 2wks. The pellets were then removed, and the remaining cells on the plate were fixed in 4% paraformaldehyde and stained with Safranin O (Sigma), which specifically detects GAG proteins present in high concentrations in chondrocyte extracellular matrix. The micromass pellets were imaged with a 1-mm scale bar to indicate gross anatomy and size.

Terminal Restriction Fragment Analysis (TRF)

Genomic DNA from 2×10^7 cells was isolated using the Qiagen genomic extraction kit, following the provided protocol (Qiagen, Valencia, CA). Then, strictly following radioactive handling safety, the telomere specific oligonucleotide G-rich probe (5'-TTAGGGTTAGGGTTAGGG-3') HindIII and 1kb ladder (Invitrogen) were labeled using $\gamma^{32}\text{P}$ -ATP via T4-polynucleotide kinase (Invitrogen) at 37°C for 15 minutes. The isolated genomic DNA was then digested using a cocktail of *AluI*, *HinfI*, and *RsaI* for overnight at 37°C and

electrophoresed on a 0.7% agarose gel at 80V overnight with the radio labeled 1kb and HindIII ladders. The gel was then denatured for 30 minutes in 0.5M NaOH and 1.5M NaCl, dried for 2 hours at 80°C, and neutralized for 15 minutes in 0.5M Tris and 1.5M NaCl. The gel was then placed in a hybridization chamber, prehybridized in un-labeled G-rich probe solution for 30 minutes, and hybridized to the radio-labeled telomere specific probe overnight at 42°C. The next day the gel was exposed to a phosphorimager cassette and imaged using a phosphorimager and analyzed using ImageQuant software (Molecular Dynamics, Sunnvale, CA).

Flow Cytometry

Cells were first trypsinized and counted, and for each sample to be assayed, 5×10^5 cells were used for the primary antibody, while 2×10^5 were used for both secondary only and no antibody controls. These cells were centrifuged at 1000xg for 1 minute at 4°C and resuspended in 75µl of FACS buffer (PBS, 2% FBS) and incubated on ice for 10 minutes. The primary antibody was then added at 13µg/µL (CD14, CD29, CD34, CD45, CD73, or CD105) (Millipore, Billerica, MA) and incubated with rotation at 4°C for 30 minutes followed by centrifugation and 3 washes with FACS buffer. The secondary antibody (Alexa 488 anti-mouse) was then added at a dilution of 1:400 and incubated in the dark at 4°C with rotation for 30 minutes. These washes were then repeated, and the resulting pellet was resuspended in 500µl 1x PBS and then filtered through a 35µm nylon mesh to remove any cell clumps. These cell suspensions were kept on ice until cytometric analysis, which was performed using a Coulter Epics XL-MCL (BeckmanCoulter, Brea, CA).

Telomeric Repeat Amplification Protocol (TRAP)

For the detection of telomerase activity, the TRAPeze kit (Millipore) protocol was followed. Briefly, cells were trypsinized and counted, and 100,000 cells were removed, pelleted and lysed in 200 μ l of CHAPS lysis buffer supplied by the manufacturer (Millipore) for 30 minutes on ice with protease inhibitors. The sample at a concentration of 500 cells/ μ l was then centrifuged at 12,000xg to remove cell debris, and the subsequent lysate was collected and frozen at -80°C until analyzed. Radioactive handling procedures being strictly followed, γ^{32} P-ATP was used to label the TS-primer using T4-polynucleotide kinase (Invitrogen) at 37°C for 30 minutes. The telomerase extension reaction was then carried out using the labeled TS-primer mixed with 1000 cells (2 μ l) per sample at room temperature for 25 minutes, followed by PCR amplification of elongated samples. PCR products were visualized by electrophoresis on a 10% polyacrylamide gel at 300V for 2 hours, followed by a brief fixation and exposure to a phosphoimager screen overnight. The subsequent radiographic image was captured and quantified using a Molecular Dynamics phosphoimager and ImageQuant software (Molecular Dynamics, Sunnyvale CA).

RT-PCR

Cells were grown in 10cm² dishes, trypsinized, and total RNA was extracted using TRIzol (Invitrogen) reagent following the manufacturer's protocol. After resuspension in TRIzol for 5 minutes, 200 μ l of chloroform was added, vortexed, and then the phases were separated by centrifugation at 12000xg for 15 minutes at 4°C. The aqueous phase was then carefully removed and transferred into a new 1.5ml tube. The RNA was precipitated, the supernatant was removed, and the pellet was then washed with 75% ethanol, mixed and centrifuged at 7500xg for 5 minutes at 4°C. The ethanol was removed, and the pellet was air dried for 5 minutes and

resuspended in 30µl of dH₂O. The RNA purity and concentration was then analyzed using a Nanodrop 1000 UV/Spectrophotometer (Thermo Scientific, Waltham, MA). The sample was then treated with DNase to remove any DNA contamination that may have come from the TRIzol extraction. RT-PCR was then carried out using the RETROscript kit protocol (Ambion, Austin, TX). Briefly, 2µg of total RNA was subjected to a reverse transcription reaction using random decamers as primers and MMLV-Reverse transcriptase. This mixture was incubated at 44°C for 1 hour, deactivated at 92°C for 10 minutes, and the resulting cDNA was either stored at -20°C or used immediately for PCR or qPCR analysis. The PCR conditions were 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and then 72°C for 5 minutes. Primers used were as follows: Oct-4 Fwd 5'-CAGTGCCCGAAACCCACAC-3', Rev 5'-GGAGACCCAGCAGCCTCAAA-3'; SOX2 Fwd 5'-TACCTCTTCCTCCCACTCCA-3', Rev 5'-GGTAGTGCTGGGACATGTGA-3'; NANOG Fwd 5'-TTTGGGAAGCTGCTGGGGAAG 3', Rev 5'-GATGGGAGGAGGGGAGAGGA 3'; Lin28 Fwd 5'-AAGCGCAGATCAAAAGGAGA-3', Rev 5'-CTGATGCTCTGGCAGAAGTG-3'; C-MYC Fwd 5'-GCGTCCTGGGAAGGGAGATCCGGAGC-3', Rev 5'-TTGAGGGGCATCGTCGCGGGAGGCTG-3'; hTERT Fwd 5'-CGGAAGAGTGTCTGGAGCAA-3', Rev 5'-GGATGAAGCGGAGTCTGGA-3'. The resulting PCR products were then visualized after electrophoresis on a 1.5% agarose gel and staining with EtBr.

qPCR

Total RNA was isolated and reverse transcribed as described above. The resulting cDNA was then used to create a standard curve for optimized cDNA amplification and primer disassociation

or each primer set using the SYBR-greener qPCR Supermix kit (Invitrogen). Primers used for the genes of interest were as described above. Relative quantitative polymerase chain reaction (qPCR) was then carried out using an Applied Bioscience 7900HT instrument. The resulting data was analyzed using the $2^{-\Delta\Delta C_t}$ method with ABI SDS 2.2.2 software (Applied Bioscience) where ribosomal 18S (Ambion, Austin, TX) gene expression was used as the endogenous gene control, and baseline gene expression was set based on the signal present from reverse transcribed RNA from BJ fibroblasts or ASC.

Karyotypic Analysis

The chromosomes from cell strains ASC 8, 9, and 12s were analyzed using GTG-banding. Each cell line was harvested and slides were prepared according to standard procedures (Rooney, 1992). Briefly, actively dividing cells were blocked in metaphase with 0.1 g/mL of Colcemid for 2-4 hours. After a 10 minute incubation in a 0.075 M KCl hypotonic solution, the cells were fixed by serial washes in a methanol–glacial acetic acid solution (3:1). GTG-banding was performed using standard procedures (Barch, 1997). Representative images were captured using a Cytovision image analysis system (Applied Imaging, Santa Clara, CA)

Results

ASCs can be isolated from many sources, have normal growth and are karyotypically stable. For the initial isolation of ASCs, we followed the guidelines set forth in a previous report. (Zuk, *et al.*, 2001) Our isolations were similar to these studies showing an abundance of adherent cells from each isolation and a 100-fold increase in viable cells compared to those isolated from a bone marrow aspiration. (Izadpanah *et al.*, 2006; Zhu *et al.*, 2008) Upon further expansion, we

noticed a moderately elevated doubling time (~50 hours) with an appropriate proliferative lifespan for our ASC cell strains (Figure 10) similar to other reports.(Izadpanah *et al.*, 2006; Zuk *et al.*, 2001; Astori *et al.*, 2007; Katz *et al.*, 2005; Kern *et al.*, 2006; Leong *et al.*, 2005; Lin *et al.*, 2008; Noël *et al.*, 2008; Rebelatto *et al.*, 2008; Wagner *et al.*, 2005; Yoshimura *et al.*, 2006; Lin *et al.*, 2008; Zhu *et al.*, 2008). We also examined karyotypic stability with continued passage, using metaphase G-banding techniques. In contrast to previous reports (Rubio *et al.*, 2005), our ASCs were karyotypically stable at both early and late population doubling times (Figure 11, Table 1), none of which spontaneously immortalized. Because prior studies suggested that ASCs reside in a niche located in the perivascularity of capillaries and arterioles, (Lin *et al.*, 2008) we hypothesized that the blood/saline fraction of lipoaspiration procedures might contain a subset of ASCs, released due in part to the disruptive nature of the lipoaspiration procedure. To test this hypothesis, we modified the existing extensive 8-10 hour protocol into a simple 30 minute procedure, as previously reported (Francis *e. al.*, 2009). Briefly, the saline fraction was removed from the lipoaspirate, pelleted, treated with a blood cell lysis buffer, filtered, and plated with high FBS. After adherent cells were allowed to attach overnight, the plates were washed, and the adherent cells were subsequently maintained and cultured. We successfully isolated two strains using this rapid, saline isolation from two separate patients designated ASC4s and ASC12s and tested these cells for their multipotential capacity. Both rapid isolation cell strains show the ability to differentiate into mesenchymal lineages, including osteocytes, adipocytes, and chondrocytes (Figures 20-22), similar to our ASC cultures obtained via the standard isolation procedure. We hypothesized that if these cells reside in the capillary beds interspersed throughout fatty deposits, then it should be possible to obtain ASCs from many other localities in the body. To test this theory, we next

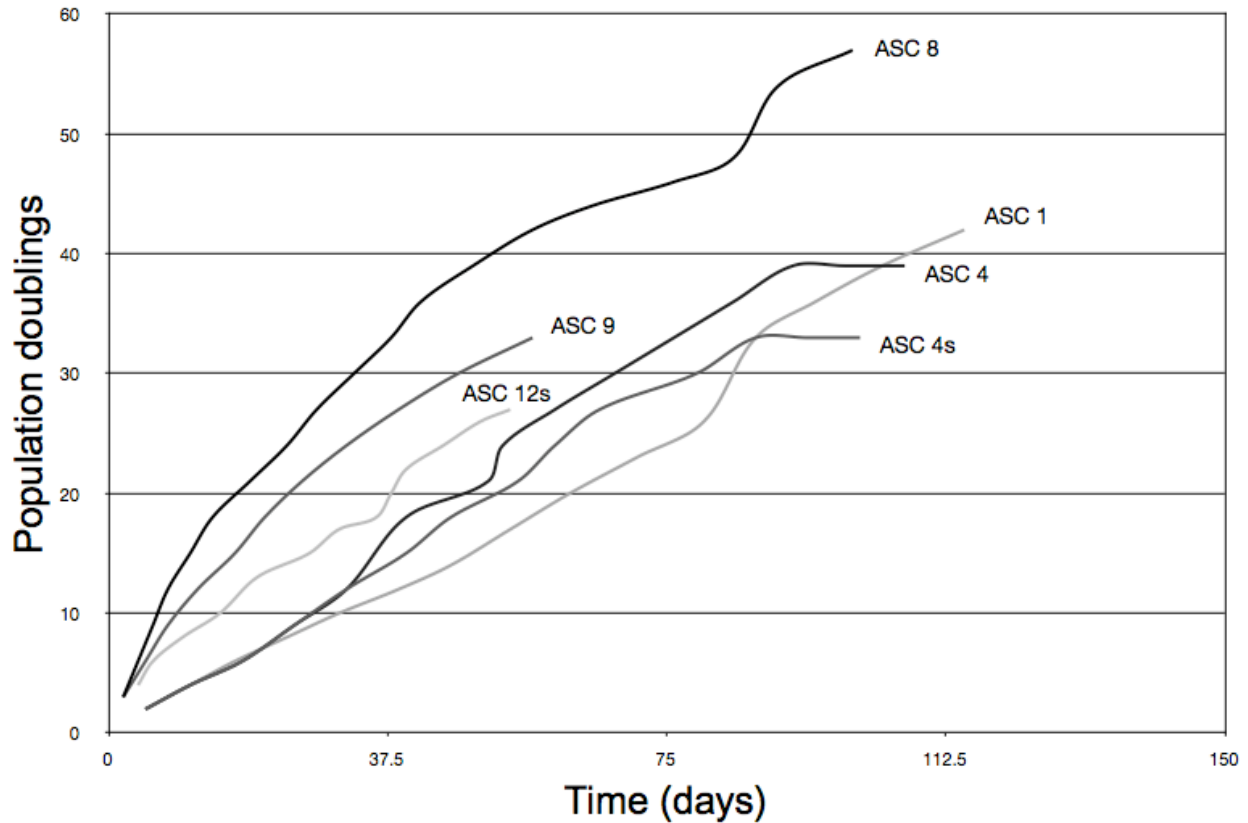
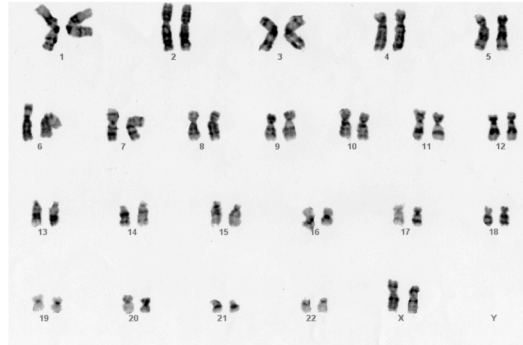


Figure 10: Growth chart of representative populations of ASCs. Cells (~2 Population doubling) were counted and seeded at a density of $\sim 2.5 \times 10^5$ cells/100mm dish. Cells were then cultured until near confluency and then passed at a ratio of 1:4 or 1:8. All relevant passage dates/ratios were recorded and population doubling time was assessed. The number of population doublings were plotted vs. time in culture. ASC4, 4s, and 1 were cultured continuously in EGF free media, while ASC 8, 9 and 12s were supplemented with 10ng/ml EGF.

A



B



C

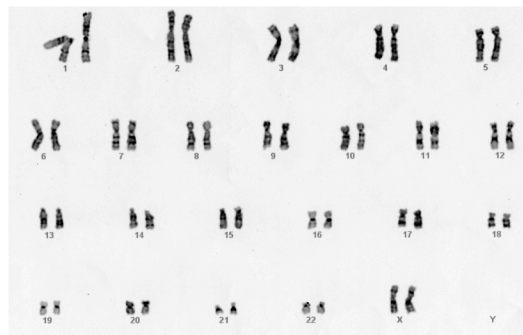


Figure 11: ASC karyotype analysis. Representative G-banded karyotype analysis of ASC 8 PD 2 (A), ASC 9 PD 20 (B) and ASC 12s PD 4 (C). ASC 9 cell strain was found to have a chromosomal inversion of $inv(9)(p11q13)$, which is representative of 1-2% of African Americans, and 0.1% of caucasians and is considered a normal “variant” that is not clinically significant. Note that all patients were female as evident from XX complement. PD = population doublings

Table 1: Summary of ASC karyotypic analysis.

Cell Strain	Population Doubling	Modal Number	Karyotype	Number Cells Scored
ASC 8	2	46	46, XX	8
	21	46	46, XX	4
ASC 9	13	46	46, XX inv(9)(p11q13)	18
	20	46	46, XX inv(9)(p11q13)	8
ASC 12s	4	46	46, XX	6

isolated cells obtained from fat tissue throughout the abdomen (ASC8) , legs and arms (ASC9), and three successful isolations from breast reduction mammoplasties (bASC1-3). (Dr. ? Zhao and Dr. Lynn Elmore personal communication). All of these cells showed a similar differentiation potential for adipocytic (Figure 20), osteocytic (Figure 21), and chondrocytic (Figure 22) lineages.

ASCs lack telomerase, and show telomere erosion with serial passaging. Due to the finite lifespan of our ASCs (Figure 10) undergoing the typical “fried-egg” morphology following continued passage, we wanted to determine the potential mechanism for the induction of this senescence. A few studies show telomerase activity in ASCs (Rubio *et al.*, 2005; Lin *et al.*, 2008; Peng *et al.*, 2008), while the majority report undetectable telomerase expression (Kang *et al.*, 2004; Kassem *et al.*, 2004). In order to determine if our ASCs express telomerase, we tested for activity under a variety of culture conditions: day 0 cultures, low population doubling (PD 2), actively dividing ASCs, and ASCs in ESC media. In none of these conditions did ASCs exhibit or upregulate telomerase activity (Figure 12). There are also reports of elevated telomerase expression in BM-MSCs upon culturing after serum deprivation (Zhao *et al.*, 2008). However, repeated attempts to stimulate cells after serum starvation failed to show any telomerase activity (Figure 12). To determine if our ASCs erode telomeres over time in the absence of telomerase we performed a TRF analysis to examine the global telomeric lengths with continued culturing (Figure 13). As expected, we find that with higher population doublings, ASC’s overall telomere lengths decrease with continued culture, correlating the progressive loss of proliferative capacity to the slow erosion of the telomeres (Figure 13).

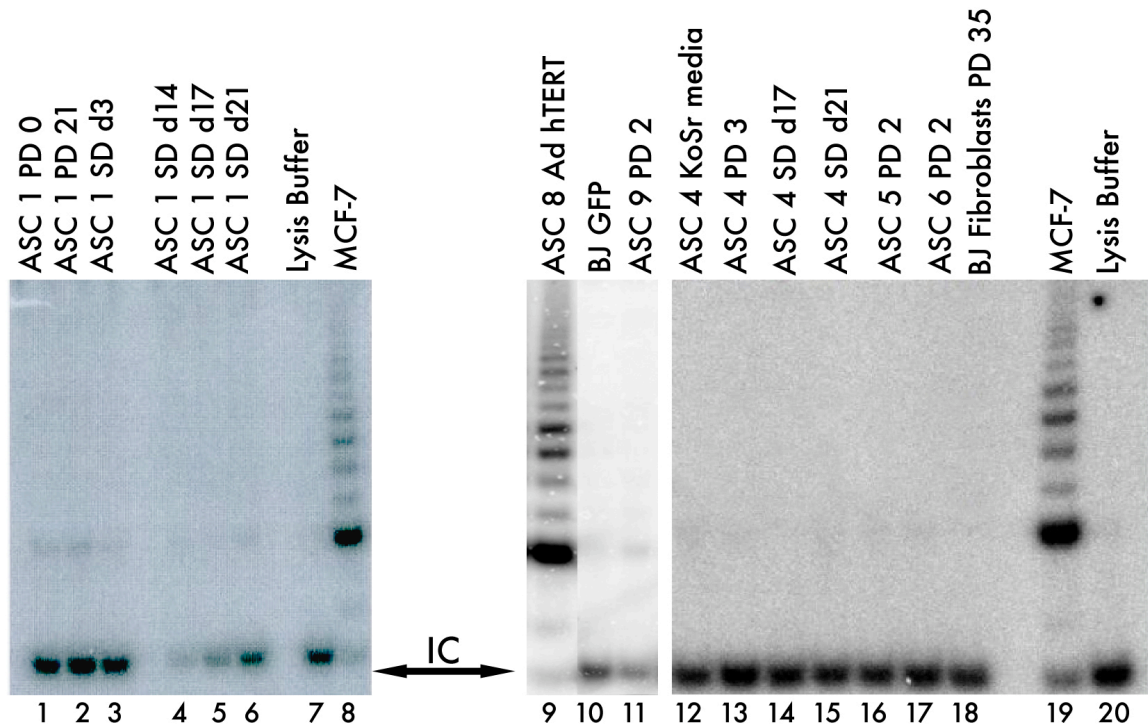


Figure 12: ASCs lack telomerase activity in all isolated samples. As assessed by TRAP activity. ASC 1 was taken at a PD 0 (lane 1) and PD 21 (lane 2). ASC 1 and 4 during a serum deprived (SD) period ranging from day 3 (lane 3) to 2 weeks (lane 4), 2.5 weeks (lane 5, 14) to 3 weeks (lane 6, 15). Negative control was lysis buffer alone (lane 7, 19), and the positive control was lysis of MCF7 cells (lane 8, 19) or Adenoviral infected hTERT ASC 8 (lane 9). ASC 9, 4, 5, and 6 also show no activity (lane 11, 13, 16, 17). ASCs grown in knock out replacement serum (KoSr) embryonic media formulation (lane 12). IC = 36bp Internal control

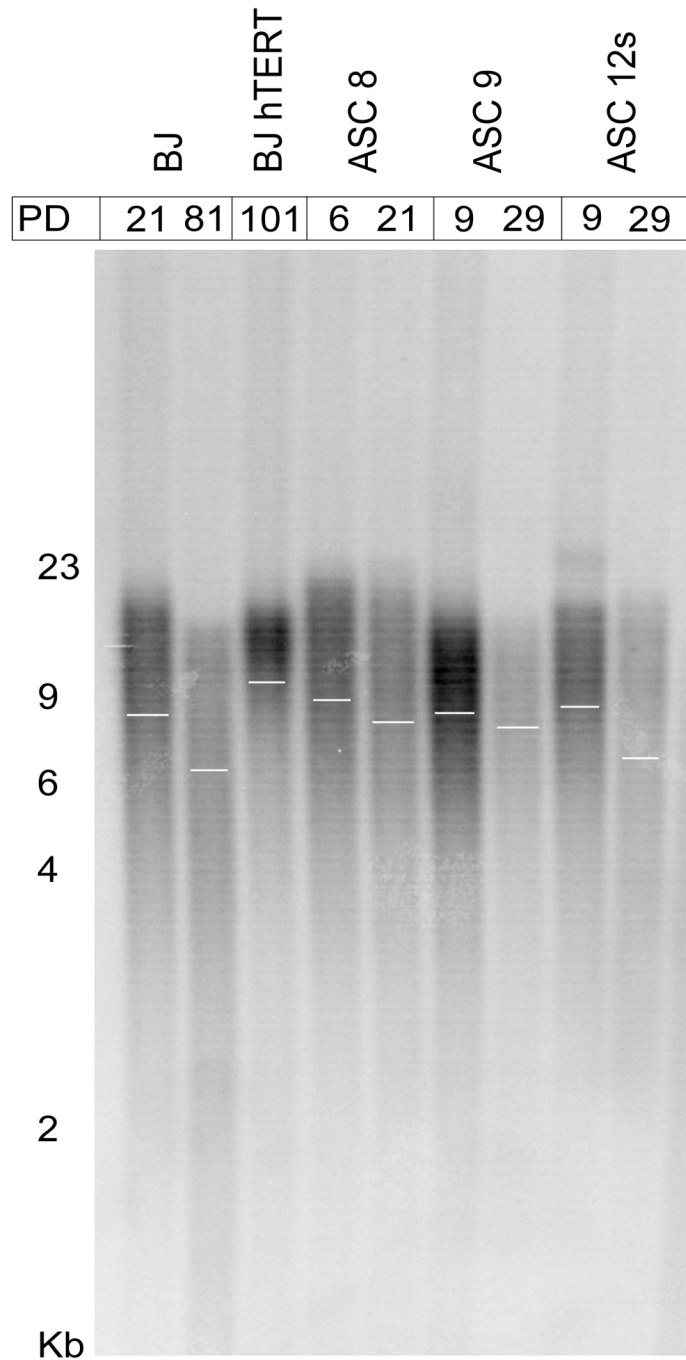


Figure 13: ASCs experience telomere attrition with continued passage. The TRF is a specialized southern blot assay to examine specific telomere lengths. Mean telomere length is calculated by creating densitometric curve of lane, mean is $.5 \times$ total area under curve. BJ fibroblast were used as a control, PD 21 to PD 81 show telomeric loss, BJ hTERT at PD 101 shows a typical telomerized cells telomere length with a tight band of long telomeres. ASC 8, 9, and 12s show erosion with continued passage.

Immunophenotyping. To clarify the combination of markers most appropriate for distinguishing our ASCs as stem cells, we tested a panel of antibodies most commonly reported in recent literature (Izadpanah *et al.*, 2006; Zuk *et al.*, 2001; Astori *et al.*, 2007; Katz *et al.*, 2005; Kern *et al.*, 2006; Leong *et al.*, 2005; Lin *et al.*, 2008; Noël *et al.*, 2008; Rebelatto *et al.*, 2008; Wagner *et al.*, 2005; Yoshimura *et al.*, 2006; Lin *et al.*, 2008; Zhu *et al.*, 2008). While there are many conflicting reports some commonly used markers do exist (Table 2). We focused on a consensus set of markers derived from current literature and that were promoted by commercial biotechnology companies to be stem cell markers. The positive markers included: CD29, (stains a diverse number of cells including endothelial cells, monocytes and platelets), CD73 (found on epithelium, endothelial and some mature lymphocytes), CD105 (a marker of endothelium and perhaps some stem/progenitors), and the more controversial marker, CD34, which is primarily seen on hematopoietic stem cells. For negative cell surface markers, we also used a panel of antibodies generally accepted as negative for a cell of mesenchymal lineage including: CD45 (a general marker of leukocyte presence), CD31 (a marker of endothelial cells), and CD14 to distinguish the cells from macrophages/monocytes.

We first tested the iPS line derived from BJ fibroblasts to determine if these cells were similar to our stem cells and found a complete lack of expression for all markers tested (Figure 14). We also analyzed our ASC strains, which consistently stained positive for CD29, CD73, CD105 and negative for CD45, CD31, and CD14 (Figures 15-17 A-F). However, we found a small population of cells in the ASC cultures that express CD34 (Figure 15-17 G), whereas the CD34 marker was absent from BJ fibroblast cells (Figure 18 G). The expression of CD34 was low enough (< 8%) that ASCs could easily be called negative, but were classified as CD34^{low}. Other than the low expression of CD34, BJ fibroblasts showed an almost indistinguishable

Table 2: Summary of cell surface markers from literature.

Cell Isolation	CD14	CD29	CD31	CD34	CD45	CD73	CD90	CD105
Adipose Stromal Cell	-	+	-	-/+	-	+	+	+
Adipose Stromal Cell P0	n/a	+	-	+	-	n/a	+	-
Bone Marrow Stem Cell	-	+	-	-	-	+	+	+
Stromal Vascular Fraction	n/a	n/a	-	+	-	+	+	+
Umbilical Cord Blood	-	+	-	-	-	+	+	+
Dermal Fibroblast	-	+	-	-	-	+	+	+
BJ Fibroblast	-	+	-	-	-	+	+	+
Vascular Smooth Muscle Cell	n/a	n/a	-	+	-	n/a	+	-
ASC Consensus Profile*	-	+	-	+/-	-	+	+	+

*average of all qualitative acknowledgement of presence or absence

iPS

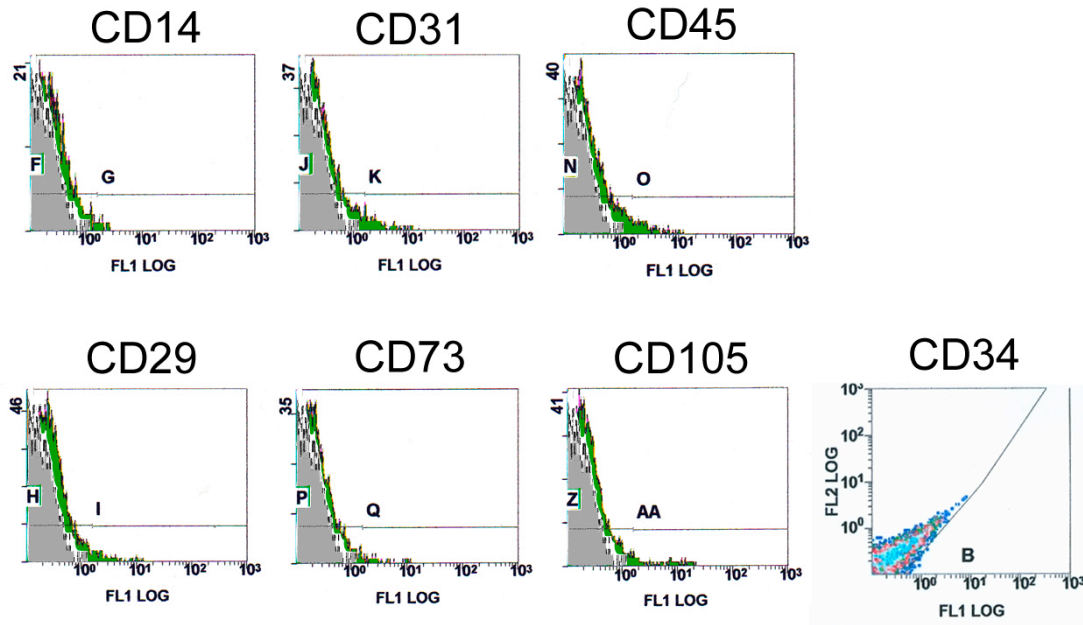


Figure 14: iPS cell surface immunophenotype. Flow cytometry was performed on iPS cells, which were negative for CD14, CD31, CD45, CD29, CD73, CD105. Highlighted grey overlay represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells. Note CD34 shown to highlight individual populations, any positive fluorescent cells are seen as a shift to the right of the gate line, showing a lack of CD34+ cells.

ASC 8

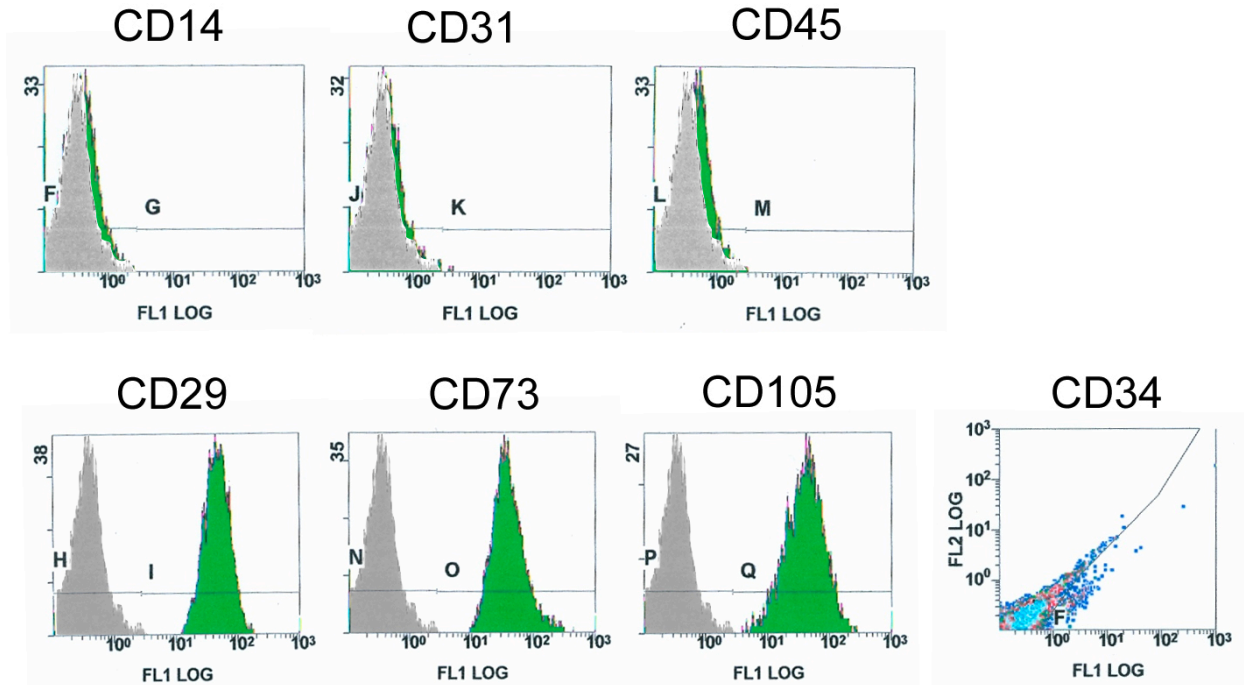


Figure 15: ASC 8 cell surface immunophenotype. Flow cytometry was performed on ASC 8, which were negative for CD14, CD31, CD45 and positive for CD29, CD73, CD105. Highlighted grey overlay represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells. Note CD34 shown to highlight individual populations, any positive fluorescent cells are seen as a shift to the right of the gate line, suggesting a population of CD34+ cells.

ASC 9

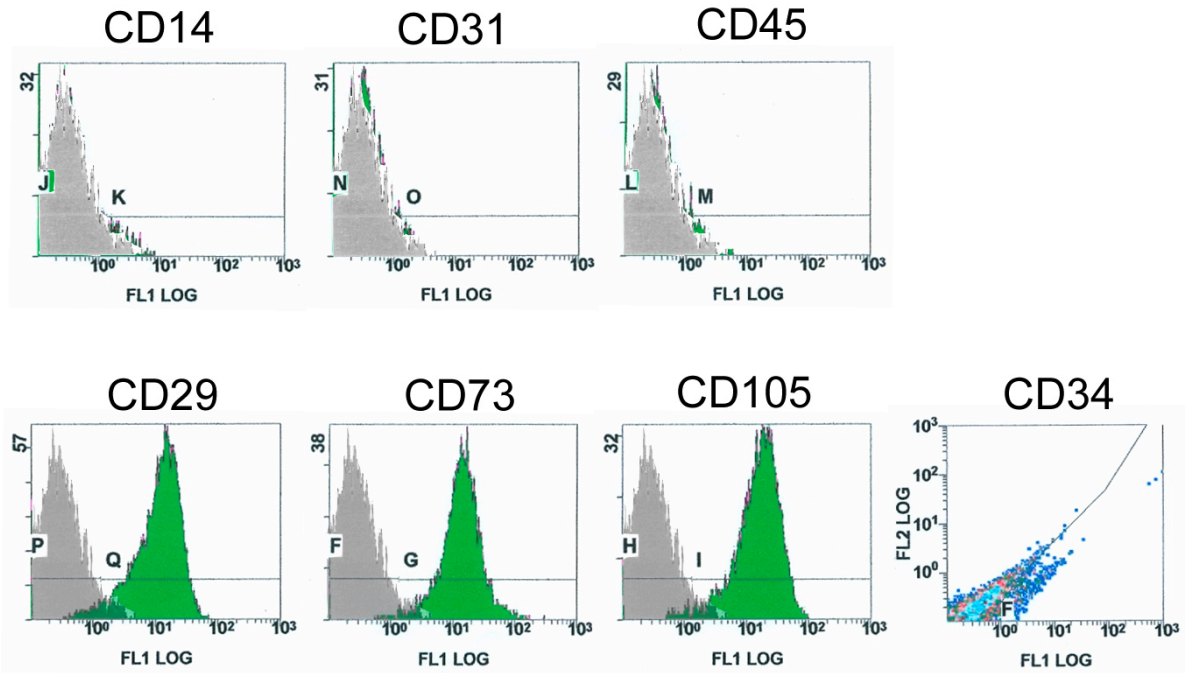


Figure 16: ASC 9 cell surface immunophenotype. Flow cytometry was performed on ASC 9, which were negative for CD14, CD31, CD45 and positive for CD29, CD73, CD105. Highlighted grey overlay represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells. Note CD34 shown to highlight individual populations, any positive fluorescent cells are seen as a shift to the right of the gate line, suggesting a population of CD34+ cells.

ASC 12s

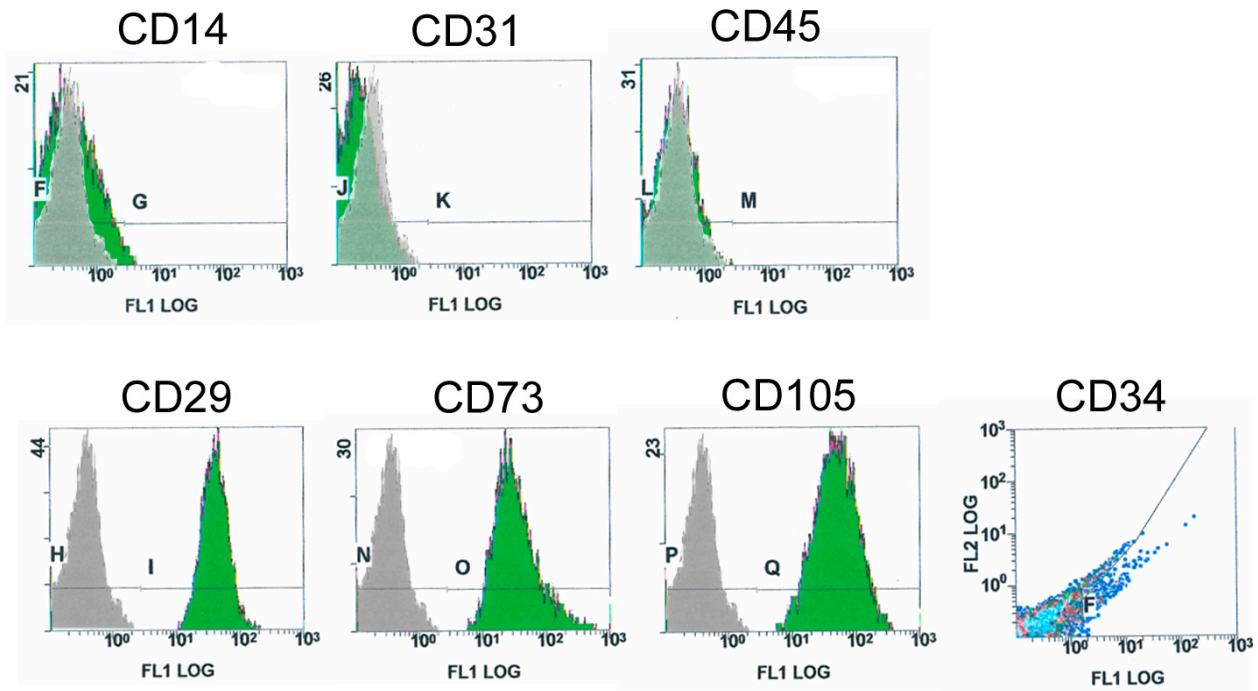


Figure 17: ASC12s cell surface immunophenotype. Flow cytometry was performed on ASC 12s, which were negative for CD14, 31, 45 and positive for CD29, 73, 105. Highlighted grey over lay represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells. Note CD34 shown to highlight individual populations, any positive fluorescent cells are seen as a shift to the right of the gate line, suggesting a CD34+ population.

BJ fibroblasts

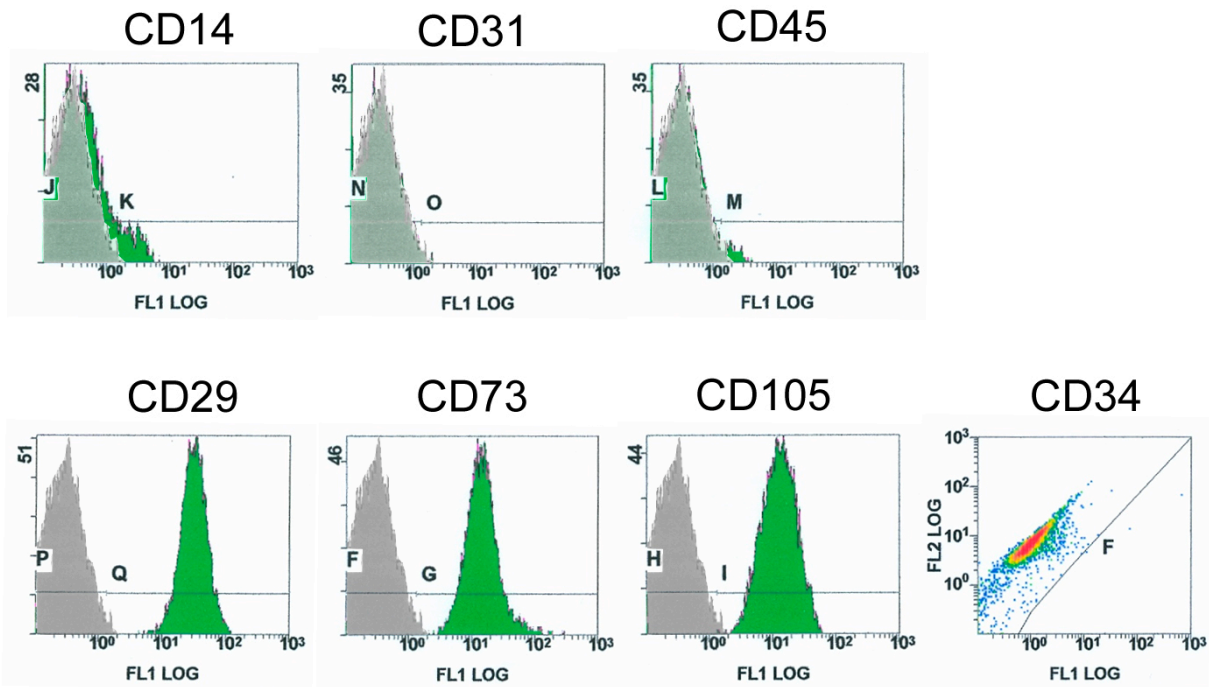


Figure 18:BJ fibroblast cell surface immunophenotype. Flow cytometry was performed on BJ fibroblasts, which were negative for CD14, CD31, CD45, CD34, Positive for CD29, CD73, CD105. Highlighted grey area represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells. Note CD34 shown to highlight individual populations, any positive fluorescent cells are seen as a shift to the right of the gate line.

HL60

similarity to the ASC strains, suggesting that the immunophenotype of ASCs does not classify them as stem cells. Further, to test for positive staining with the antibodies for CD45, CD31, and CD14 we used HL60 cells subject to differentiation with Vitamin D exposure. This indicated that our negative markers were functioning properly, just undetectable in our ASCs (Figure 19).

ASCs have high multipotency. To determine the differentiation capacity of all of our isolated ASC strains, we examined their multipotential capabilities by differentiating cells down various mesenchymal lineages reported in the literature with the highest frequency. (Izadpanah *et al.*, 2006; Zuk *et al.*, 2001; Astori *et al.*, 2007; Katz *et al.*, 2005; Kern *et al.*, 2006; Leong *et al.*, 2005; Lin *et al.*, 2008; Noël *et al.*, 2008; Rebelatto *et al.*, 2008; Wagner *et al.*, 2005; Yoshimura *et al.*, 2006; Lin *et al.*, 2008; Zhu *et al.*, 2008). First, we tested their ability to differentiate into their source tissue, adipose. For this differentiation procedure we used an adipogenic cocktail media on all of our strains once they achieved ~100% confluence with BJ fibroblast as our negative mesenchymal control, and BM-MSCs as our positive control. Whether isolated from the saline fraction, abdomen, arms, legs, breasts, or bone marrow, all ASCs successfully differentiated into adipocytes (Figure 20A-G). There was no differentiation seen in our BJ fibroblasts controls (Figure 20G, J), nor from ASCs and BMMSCs maintained for the same length of time at confluence with regular growth media (Figure 20F-J). The successful differentiation into adipocytes was initially characterized morphologically by the formation of round lipid droplets within the cell, then confirmed by their staining with the lipid and neutral triglyceride-specific stain Oil red O (Figure 20A-G).

HL-60

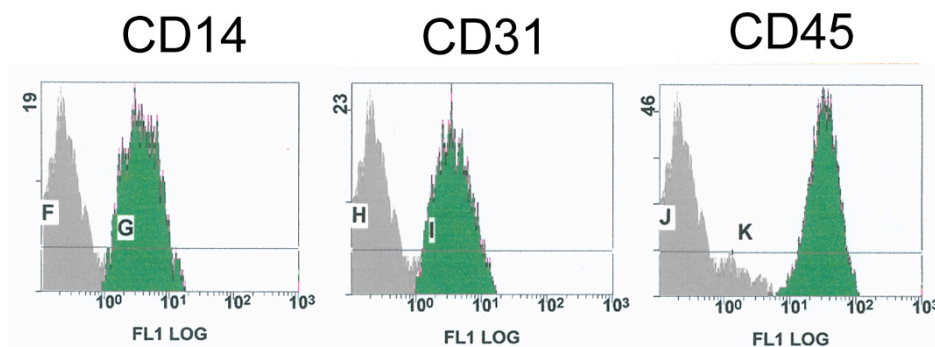


Figure 19: HL-60 cell surface immunophenotype. Flow cytometry was performed on HL-60 cells as a positive control for CD14, CD31, and CD45. Cells were treated with Vitamin D to induce differentiation, upon differentiation cells became positive for CD14 and CD31, while retaining CD45 expression. Highlighted grey overlay represents negative antibody control, which is cytometric analysis on a no-antibody portion of cells.

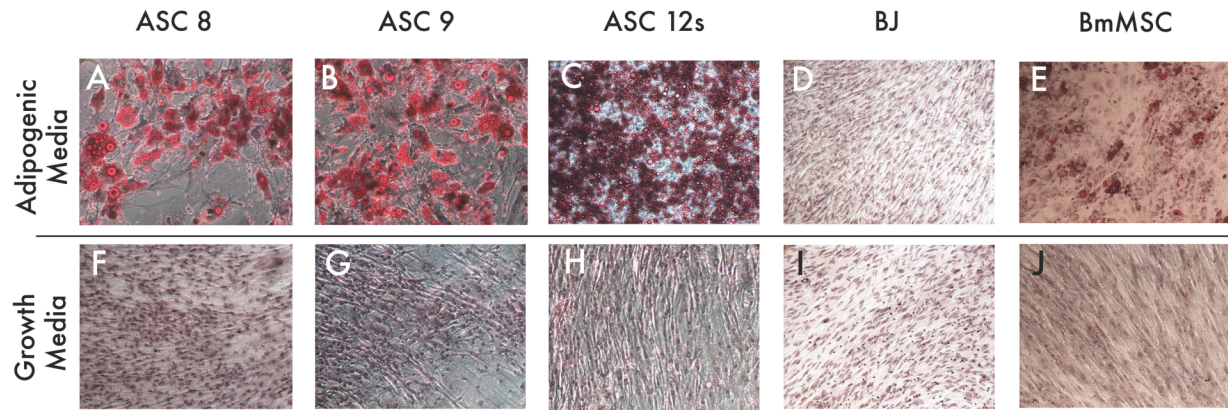


Figure 20: ASCs differentiate into adipocytes, compared to controls. All cells were plated and grown to 100% confluency, following which cells were maintained in either adipogenic media (A-E) or standard growth media (F-J) for 14 days. Following visualization of lipid droplet formation in a significant proportion of cells (~70%) after ~ 2 weeks, all cells were fixed and stained with Oil Red O to specifically highlight lipid formation representative of adipocytes.

We next analyzed the cells' ability to differentiate into osteocytes. The same panel of cells was grown to ~70% confluence and then subject to a cocktail media containing elements to specifically cause osteogenic differentiation. We saw similar results, as morphologically the cells began to group together and form "bone nodules", a characteristic of osteogenesis *in vitro*. These nodules are the primary source of calcification in the surrounding extracellular matrix, visualized via staining with Alizaran Red S (Figure 21A-E). All of our ASC strains and the BM-MSCs were positively histochemically stained, while BJ fibroblasts failed to differentiate along an osteogenic lineage in induction media or in normal media (Figure 21E, J).

To unequivocally confirm these cells as multipotent, we tested their capacity to differentiate down a third, chondrogenic, pathway. The cells were collected in a V-bottom 2-ml tube into a micromass pellet, which was maintained in a chondrogenic differentiation cocktail for 30 days. Again, all ASC cell types and the BM-MSCs successfully differentiated into solid, white, chondrogenic micromass pellets (Figure 22K), and this morphology was further analyzed by staining with the GAG protein-specific Safranin O stain (Figure 22A-E), which was absent in BJ fibroblasts. In our hands, normal BJ fibroblasts failed to differentiate down any of the three mesenchymal lineages tested, despite some reports to the contrary (Kern *et al.*, 2006) (Figure 22E, J). The BM-MSCs showed varying degrees of differentiation down the mesenchymal lineages tested, although they did always differentiate.

ASCs express Oct-4, SOX2, and NANOG. The same studies that indicated elevated telomerase expression in ASCs after serum deprivation also reported the expression of a marker for pluripotency, Oct-4 (Pochampally *et al.*, 2004). Since our ASC strains have no distinct immunophenotype and lack telomerase expression, our goal was to determine if these cells

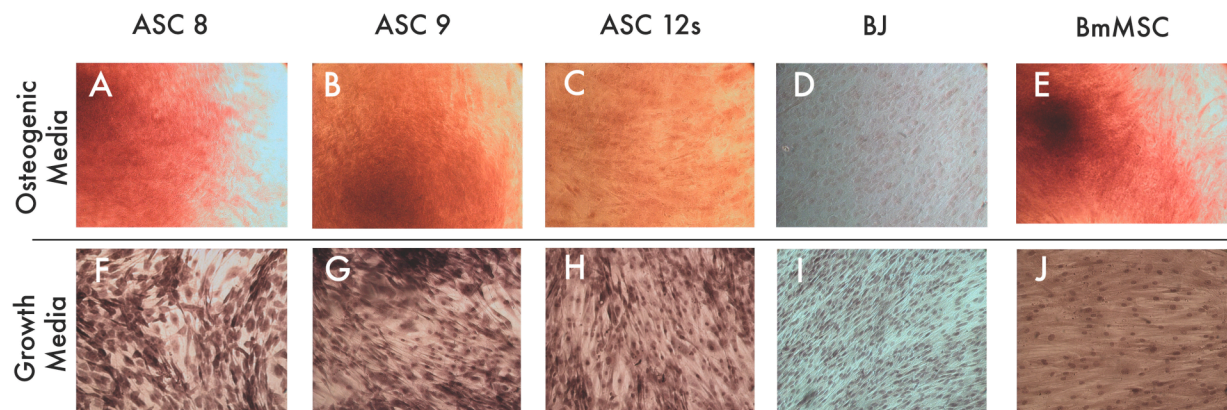


Figure 21: ASCs differentiate into osteoblasts, as compared to controls. Continuous culture was done in either osteogenic induction media (A-E) or regular growth media (F-J) for 14 days. Once cells begin to form bone nodules, and produce significant amounts of calcium deposits (~ 2 weeks), all cells were fixed and stained with Alizarin red S to specifically highlight calcium production, which is representative of osteoblasts.

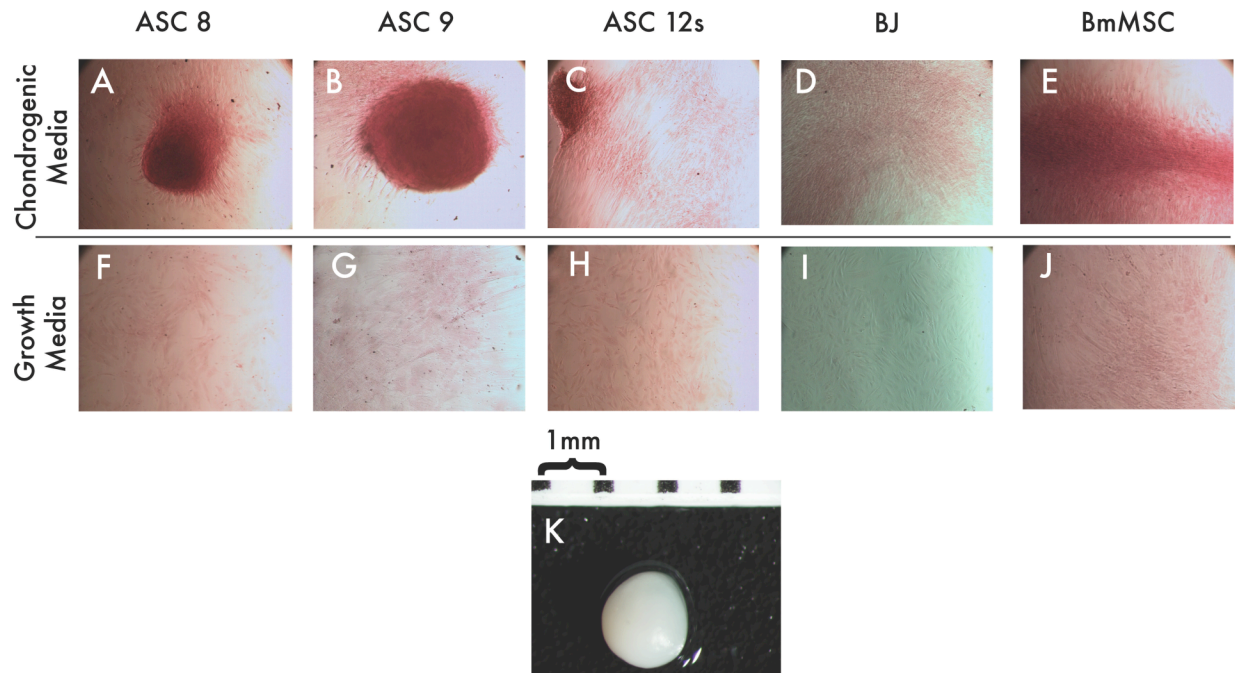


Figure 22: ASCs differentiate into chondrocytes, as compared to controls. Following micromass pellet formation, cells were continuously cultured in either induction chondrogenic media (A-G) or normal growth media (F-J) for 30 days. The whole pellet is then imaged (K), processed and stained with Safarin O to specifically highlight GAG protein production representative of chondrogenic cells.

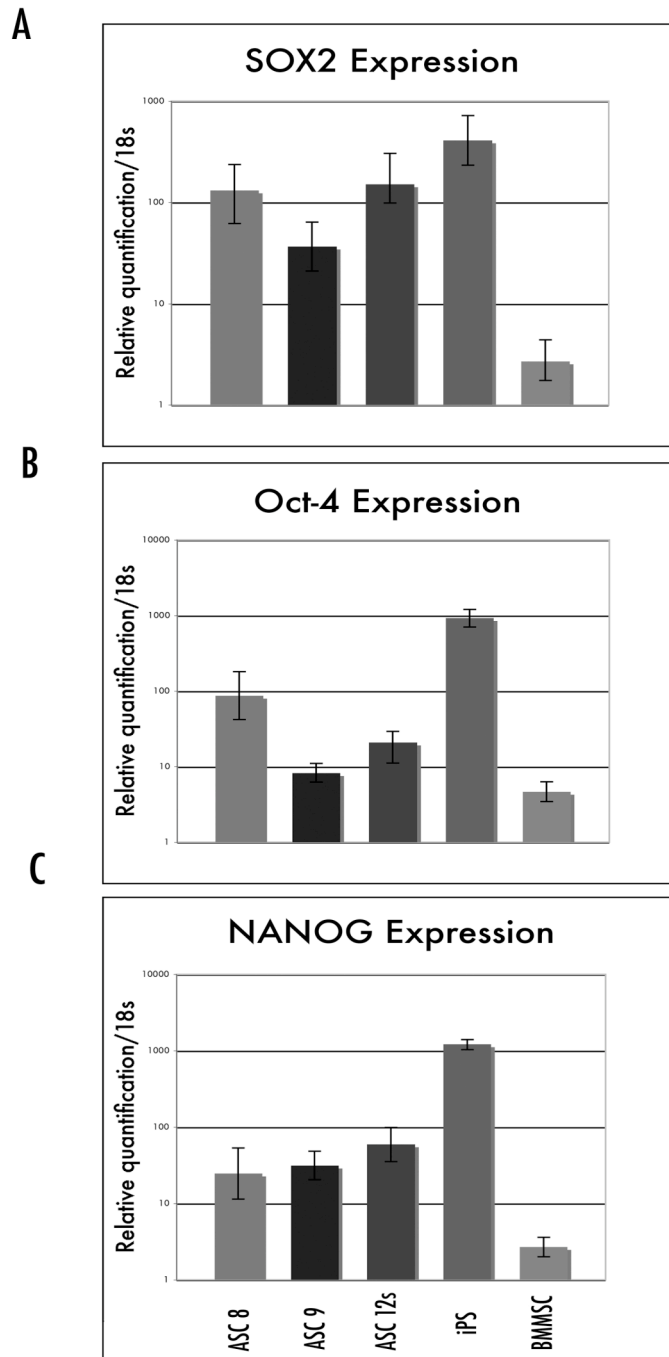


Figure 23 qRT-PCR of Oct-4, SOX2 and NANOG in ASCs show a significant increase in expression over BJ fibroblasts. Relative expression levels for SOX2 (a), Oct-4 (b) and NANOG were assessed using $\Delta\Delta C_t$ method in ASC 8, 9, 12s, BM-MSC and iPS. Data shown relative to endogenous control RNA 18s, with fold increase over expression levels in BJ fibroblasts. All lanes shown at $p < 0.05$ as compared to BJ Fibroblasts.

express any markers of pluripotency, making them more similar to stem cells than fibroblasts. We performed qRT-PCR, to determine the quantitative levels of Oct-4, SOX2 and NANOG mRNA transcripts (Figure 23 A-C). Our baseline expression was set relative to expression found in BJ fibroblasts, which express extremely low levels of the respective genes. iPS cells created from the same BJ fibroblasts served as our positive control. We found that our ASC cell strains express all three markers of pluripotency at higher levels than BM-MSCs (Figure 23). SOX2 is expressed at an average of 100-fold higher levels than BJ fibroblasts, while expressing 10-fold less than their pluripotent iPS counterparts (Figure 23A). Oct-4 Levels were ~10-100 fold below iPS cells, while significantly higher than BJ fibroblasts (Figure 23B). NANOG approached 100-fold higher levels in ASCs than BJ fibroblasts while being ~20-fold below iPS cells (Figure 23C).

Discussion

With the onset of many diseases, either from injury or from the inevitable aging of our organs over time, there is a need to replenish and repair these damaged tissues. Many problems currently with advancing medicine hinge upon a lack of useable replacement tissues. We show here adipose-derived adult stem cell populations with the capacity to differentiate in a similar fashion to mesenchymal stem cells from bone marrow. Obtaining ASCs, however, does not require a painful aspiration from the iliac crest and circumvents the low yield associated with the isolation of BMMSCs, lending to a much more accessible source of stem cells to develop regenerative techniques. In addition, we identified a fraction of ASCs that can be readily isolated from the saline fraction of lipoaspirate without the previous lengthy isolation procedure, making them even more easily attainable for stem cell-related studies (Francis *et al.*, 2009). We also show that ASCs are found in fat depots throughout the body and could be located in capillary beds in association with adipose tissue. Our hypothesis that sonication used during liposuction

would cause a greater disruption of tissues releasing more ASCs from the capillary and arteriole beds in the adipose tissue, proved to be the case. This allowed for the establishment of a much more streamlined isolation procedure for ASCs without compromising any of their stem cell-like features. While contrary to studies that suggest sonication as a source of cellular damage and loss of proliferative capacity (Zuk *et al.*, 2001), we demonstrate that the ASCs remain viable and differentiate down a variety of mesenchymal lineages.

We also sought to define a subset of cell surface markers as a means to identify ASCs as true stem cells and to clarify the inconsistency in the literature and from commercial sources. We find no definitive immunophenotype for our ASCs that would distinguish them from either normal fibroblasts or BM-MSCs. Consistent with these observations, we find that our ASCs lack telomerase expression and gradually shorten their telomeres with advancing age, indicating a telomere-based senescence mechanism. Together, these data suggest that ASCs, in many ways, behave like normal diploid fibroblasts.

While lacking the ability to proliferate indefinitely, ASCs and to a lesser extent BM-MSCs, express markers of embryonic stem cell pluripotency, Oct-4, SOX2 and NANOG, at significantly higher levels than fibroblasts. In stark contrast to BJ fibroblasts' inability to differentiate, our ASCs have multipotent capacity and differentiate into multiple cell types, consistent with a stem cell phenotype. Our data suggests that ASCs exist in the body as a more primitive cell type, with elevated pluripotent gene levels and increased ability to differentiate than other mesenchymal cell types. However, the lack of telomerase in our ASCs suggests that if these cells do play a critical role in the continued replacement of lost or damaged tissues, the body's ability to signal and use these cells would be reduced as they undergo age-related senescence.

We suggest here that perhaps future therapies could isolate ASCs in a non-intrusive manner, expand with telomerase activity if necessary, and re-implant cells in damaged areas as needed. Such therapies could lead to cures for many diseases such as osteoporosis, arthritis, and possibly diseases of the brain and nervous system such as spinal cord injuries and Parkinson's disease (Picinich *et al.*, 2007). Importantly, these procedures would all be autologous, avoiding any graft *vs.* host disease and circumventing much of the controversy surrounding embryonic stem cells.

In conclusion, the common cell surface markers and telomerase measurements used to identify ASCs succeed in only identifying them as similar to fibroblasts. Our results indicate that's ASC stemness should be defined by their ability to differentiate into multiple lineages coupled with their expression of Oct-4, SOX2 and NANOG in order to distinguish ASCs as more stem cell-like.

Chapter 3

hTERT Regulation During the Induction of Pluripotent Stem Cells

Abstract

iPS cells express high levels of hTERT, the catalytic component of the ribonucleoprotein telomerase. Expression of telomerase is activated during the induction process, which is accomplished by the exogenous expression of the genes Oct-4, SOX2, NANOG, and Lin28 from normal, non-hTERT expressing fibroblasts. To elucidate the mechanisms behind this activation, we examined the effect of expression of these four genes in BJ fibroblasts and ASCs. By expressing each gene individually and in various combinations using a transient adenoviral expression system, we show that these genes are incapable of activating hTERT alone, even though these stem cell factors were upregulated to levels observed in iPS cells. We hypothesized that the negative regulation of hTERT in the BJ and ASC strains is associated with an inactive chromatin state at the promoter. This hypothesis was supported by our observation of a lack of acetylated and the presence of di-methylated histone H3 in ASCs and BJs. This finding is in contrast to the observations noted in the hTERT-expressing iPS cells. Subsequent treatment of cells with Trichostatin A (TSA) alone showed an upregulation of hTERT mRNA without the commensurate telomerase activity. However, telomerase activity was found when ASCs, but not BJs, were treated with both TSA and all four induction factors, indicating differential regulation of hTERT in cells of similar mesenchymal origins. As expected, over-expression of C-MYC in these cells is also capable of activating telomerase. These data suggest that while the expression of hTERT is universally dependent on the presence of a relaxed chromatin state and sufficient transactivating factors, other cell to cell differences can ultimately prevent its expression.

Introduction

Immortalization, defined as the process by which the number of divisions a cell can complete are not limited, is almost always coupled to the expression of the telomerase enzyme. For pluripotent cells, such as ESC and the morphologically indistinguishable iPS cells (Figure 24), expression of the catalytic component of telomerase, hTERT, is found in high abundance. Telomerase expression allows these cells to avoid telomere-based senescence and facilitates the cell's ability to differentiate following continuous passage. ESCs express hTERT as an inherent feature of its pluripotent state, present from the point of derivation from the inner cell mass of a blastocyst stage embryo, while hTERT expression in iPS cells is a function of the induction process from normal cells that lack hTERT. Four ESC-associated factors are typically used to shift a cell from a normal somatic cell to a pluripotent cell: Oct-4, SOX2, NANOG, and Lin28 and alternatively Oct-4, SOX2, C-MYC, and KLF4 (Yu *et al.*, 2007; Takahashi & Yamanka, 2006s). While the expression of hTERT following the induction process has been well documented, the mechanisms underlying this activation have not (Yu *et al.*, 2007; Takahashi & Yamanka, 2006). Of the factors used, only one, C-MYC, has been shown to have a direct effect on hTERT activation (Kyo *et al.*, 2000), although it appears to be at least partially cell type-specific (Zou *et al.*, 2005). This ability has been attributed to C-MYC's direct binding to either of the E-box regions in the hTERT promoter at -34 and -242 upstream of the ATG start site. While C-MYC is the only factor of the ESC-associated genes previously shown to activate hTERT, both sets of genes used for iPS formation exhibit hTERT upregulation (Yu *et al.*, 2007, Takahashi *et al.*, 2007), even though only one set uses C-MYC.

To further understand the mechanisms underlying hTERT upregulation during iPS formation, we overexpressed each factor individually and in various combinations using an

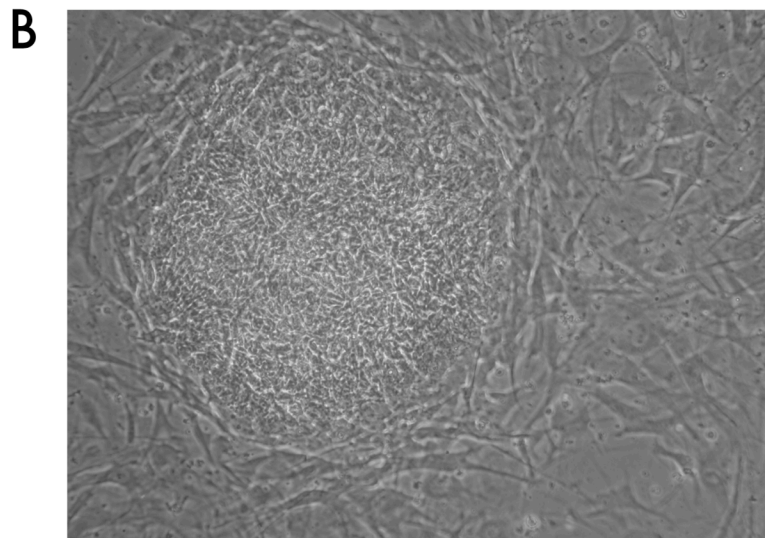
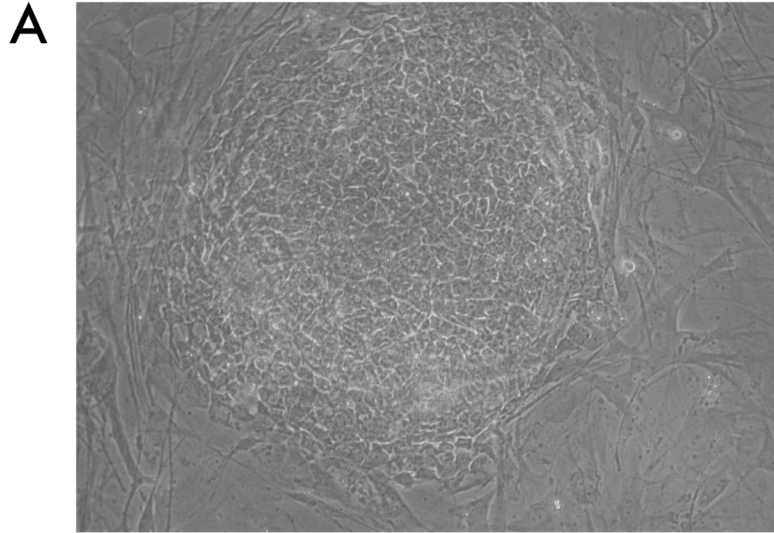


Figure 24: ESC and iPS colony morphology. Two representative images showing morphological similarities between an ESC colony (A) and an iPS colony (B), including high nucleus to cytoplasm ratio. Cells are shown on MEF feeder layer at 10x magnification.

transient adenoviral expression system in normal fibroblasts and ASCs. We have shown previously that ASCs have basal levels of the iPS induction genes, lending to a more susceptible gene environment, and our BJ fibroblasts were the cell type used to create the iPS cells. We show that all of the iPS factors are incapable of activating hTERT without the assistance of either the histone deacetylase inhibitor Trichostatin A (TSA) or the expression of C-MYC, suggesting the potential for alternative activation mechanisms. We also find that ASCs and BJ fibroblasts have a condensed hTERT promoter as indicated by the lack of acetylated H3K9 but a methylated H3K9, which is a pattern that is opposite of the hTERT-expressing iPS cells. Together, these data suggest that during the process of iPS generation, it is necessary to overcome key regulatory checkpoints for hTERT activation such as the epigenetic state. Furthermore, expression of iPS factors, Oct-4, SOX2, NANOG, and Lin28, appear to not have direct hTERT activation ability.

Materials and Methods

Cell Culture

Adipose stem cells were maintained in DMEM low glucose supplemented with 10% FBS, 1% antibiotic/antimycotic (ABAM), and 10ng/ml at 5% CO₂ at 37°C. IMR90, a fetal lung fibroblast (CCL-186, ATCC Manassas, VA) and BJ fibroblasts cells (CRL-2522, ATCC) post natal foreskin fibroblasts, were cultured in DMEM high glucose supplemented with 10% cosmic calf serum (Thermo Scientific, Waltham, MA), 3% Media 199 (Invitrogen, Carlsbad, CA), and 1% ABAM (Invitrogen).

Induced pluripotent stem cells and embryonic stem cells were maintained on an mytomycin C (Sigma) inactivated mouse embryonic fibroblasts monolayer, in knock out

replacement media containing DMEM/F12, 20% KoSR (Invitrogen), 1% Penicillin/streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β -Mercaptoethanol (Sigma), and 4ng/ml basic Fibroblast Growth Factor (bFGF) (Invitrogen).

Adenovirus Construction and Amplification.

The adenovirus vector was provided by the Massy Cancer Center shared resource viral core facility at Virginia Commonwealth University. Expression vectors for pGEM-Oct4, pGEM-Sox2, pGEM-NANOG, and pGEM-Lin28 were obtained from Addgene (Cambridge, MA). All four of the pGEM vectors were transformed into DH5 α cells (Invitrogen), clones were selected, and vector DNA was extracted through column-based plasmid prep systems (Qiagen, Valencia, CA). The resulting vectors were digested with *NotI* (New England Biolabs, Ipswich, MA) to remove the inserted gene, separated on a 1% agarose gel, purified, and then ligated into the multiple cloning site of the *NotI* linearized shuttle vector pZeroTg-CMV. To create the final adenoviral construct, the shuttle vector was co-transformed into bacterial cells with *Cla I*-linearized Adenoviral vector *Ad5dl324*. The viral vectors were then transfected into 70% confluent HEK-293B cells (ATCC). After 24hrs of infection, cells were collected and lysed. The lysate was then subject to centrifugation to clear the sample of any cellular debris, then concentrated via ultracentrifugation, and the resulting crude extract pellet was then resuspended in minimal DMEM/F12 media. The crude extract was then analyzed using a plaque forming assay to determine viral concentration.

Lentivirus Production, Infection, and Selection

The lentiviral pSin iPS gene vectors were obtained from Addgene and transformed and selected as above (Adenoviral). For virus production, pSin-Oct4, pSin-Sox2, pSin-Nanog, or pSin-Lin28 were individually transfected into 30-50% confluent 293T HEK cells, together with the remaining viral components including PsPax2 and pMD2.g at a ratio of 4:3:1 using Fugene 6 reagent (Invitrogen). Following a 24 hour incubation period at 37°C and 5% CO₂, the media was changed and the cells incubated for another 24hrs. After 24hrs and 48hrs, the media containing active virus was carefully removed and filtered using a 0.45µ syringe filter. The resulting viral supernatant was then directly added to the cells and allowed to incubate overnight. The following day, viral media was removed and replaced with normal growth media, followed by selection using appropriate antibiotics. IMR90, BJ and ASC 8 were selected for 3 days with puromycin (Invitrogen) at a concentration of 500ng/ml. Following selection, cells were maintained in media containing 1ng/ml puromycin for continued selection pressure.

Telomeric Repeat Amplification Protocol

For the detection of telomerase activity, the TRAPeze kit (Millipore) protocol was followed. Briefly, cells were trypsinized and counted, and 100,000 cells were removed, pelleted and lysed in 200µl of CHAPS lysis buffer supplied by the manufacturer (Millipore) for 30 minutes on ice with protease inhibitors. The sample at a concentration of 500cells/µl was then centrifuged at 12,000xg to clear out cell debris, and the subsequent supernatant was collected and frozen at -80°C until used. With radioactive handling procedures being strictly followed, $\gamma^{32}\text{P}$ -ATP was used to label the TS-primer using T4-polynucleotide kinase (Invitrogen) at 37°C for 30 minutes. The telomerase extension reaction was then carried out using the labeled TS-primer mixed with 1000 cells (2µl) per sample at RT for 25 minutes, followed by PCR amplification of

elongated samples. PCR products were visualized by electrophoresis on a 10% polyacrylamide gel at 300V for 2 hours, followed by a brief fixation and exposure to a phosphorimager screen overnight. The subsequent radiographic image was captured and quantified using a Molecular Dynamics phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale CA).

RT-PCR

Cells were grown in 10cm² dishes, trypsinized, and total RNA was extracted using TRIzol (Invitrogen) reagent following the manufacturer's protocol. The RNA purity and concentration was then analyzed using a Nanodrop 1000 UV/Spectrophotometer (Thermo Scientific). The sample was subsequently treated with DNase to remove any genomic DNA contamination that may have come from the TRIzol extraction. RT-PCR was then carried out using the RETROscript kit protocol (Ambion, Austin, TX). Briefly, 2µg of total RNA was subjected to a reverse transcription reaction using random decamers as primers and MMLV-Reverse transcriptase. This mixture was incubated at 44°C for 1 hour, deactivated at 92°C for 10 minutes, and the resulting cDNA was either stored at -20°C or used immediately for PCR or qPCR analysis. The PCR conditions were 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and then 72°C for 5 minutes. Primers used were as follows: Oct-4 Fwd 5'-CAGTGCCCGAAACCCACAC-3', Rev 5'-GGAGACCCAGCAGCCTCAAA-3'; SOX2 Fwd 5'-TACCTCTTCCTCCCACTCCA-3', Rev 5'-GGTAGTGCTGGGACATGTGA-3'; NANOG Fwd 5' TTTGGAAGCTGCTGGGGAAG 3', Rev 5' GATGGGAGGAGGGGAGAGGA 3'; Lin28 Fwd 5'-AAGCGCAGATCAAAAGGAGA-3', Rev 5'-CTGATGCTCTGGCAGAAGTG-3'; C-MYC Fwd 5'-GCGTCCTGGGAAGGGAGATCCGGAGC-3', Rev 5'-

TTGAGGGGCATCGTCGCGGGAGGCTG-3'; hTERT Fwd 5'-CGGAAGAGTGTCTGGAGCAA-3', Rev 5'-GGATGAAGCGGAGTCTGGA-3'. The resulting PCR products were then visualized after electrophoresis on a 1.5% agarose gel and staining with EtBr.

qPCR

Total RNA was isolated and reverse transcribed as described above. The resulting cDNA was then used to create both a standard curve for optimized cDNA amplification and primer dissociation for each primer set using the SYBR-greener qPCR Supermix kit (Invitrogen,). Primers used for the genes of interest were as described above. Relative quantitative polymerase chain reaction (qPCR) was then carried out using an Applied Bioscience 7900HT machine. The resulting data was analyzed using the $2^{-\Delta\Delta Ct}$ method where ribosomal 18S (Ambion) gene expression served as the endogenous gene control, and baseline gene expression was set based on the signal present from reverse transcribed RNA from BJ fibroblasts or ASC.

Western Blot Analysis

ASCs and BJ fibroblasts were infected with genes of interest and select samples were treated with TSA. Cells were washed with PBS, trypsinized and collected from 10cm² dishes, centrifuged at 300xg for 4 minutes, resuspended in PBS, and the centrifugation was performed again. The cells were resuspended in radio immunoprecipitation buffer (RIPA, 50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 100mM DTT) and incubated on ice for 30 minutes with Protease inhibitor cocktail IV (Calbiochem, San Diego, CA). The lysate was passed through a 22 gauge syringe 10 times and

centrifuged at 11,000xg at 4°C for 20 minutes, and the supernatant was transferred to a fresh tube. The protein contents was quantified using the Biorad (Hercules, CA) Protein reagents kit. 40µg of total protein was resolved using SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blot was blocked with 5% non-fat milk (Biorad) followed by incubation with the primary antibody of interest [Anti-Oct-4 100ng/ml, Anti-SOX2 200ng/ml, Anti-NANOG 200ng/ml, Anti-Lin28 100ng/ml, Anti-C-MYC 200 ng/ml (Millipore) and Anti-β-actin (Sigma)] for 1 hour with rocking at room temperature. The blot was washed and secondary antibody was applied (Goat anti-rabbit-HRP at 1/2500, Goat anti-mouse-HRP at 1/2500; Biorad) and incubated for 1 hour with rocking at room temperature. The blot was then washed, treated Supersignal West Pico Chemi-luminescent kit (Thermo Scientific), and exposed to Kodak autoradiographic film.

Chromatin Immunoprecipitation

ChIP was performed using the Millipore (Billerica, MA) protocol with some modification. Briefly, one 10cm² dish of cells was cross linked by the addition of 1% formaldehyde at room temperature for 10 minutes, followed by a glycine soak at a concentration of 125mM for 5 minutes at room temperature. The cells were then washed twice using ice cold PBS containing protease inhibitors and scraped into a 1.5ml tube. The cells were centrifuged at 2000xg for 4 minutes at 4°C and resuspended in 300µl/10⁷ cells RIPA (Invitrogen) and incubated on ice for 30 minutes. Cell lysates were then subjected to sonication using a Missonex 3000 sonicator with microtip at 30% power 10 cycles at 10 sec each with a one minute on ice in-between sonications. To assess the size of fragmented DNA an aliquot of each sample was centrifuged, phenol:chloroform extracted, and ethanol precipitated. Sonicated DNA was then run

on a 1% agarose gel and compared to a 1Kb ladder. Once efficient sonication was confirmed, resulting in DNA shearing of 300-1000bp, the remaining lysate was centrifuged and the supernatant transferred to a new tube.

The lysate was precleared using 60 μ l 50% agarose A/G beads (Invitrogen) and Salmon Sperm DNA (Invitrogen) mixture, centrifuged, and transferred to a new tube. The precleared lysate was incubated with the antibody of interest [either H3AcK9 1/500 (Millipore), H3k9dime 1/250 (Millipore), Anti-C-MYC 1/250 (Millipore), or IgG control (Millipore)] overnight at 4°C with rotation. The following day, agarose A/G beads were added to the lysate/antibody mix, allowed to incubate at 4°C with rotation for 1 hour, and the histone DNA complexes were retrieved by centrifugation. The supernatant was removed, and the pelleted beads were washed first with a low salt concentration buffer containing: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl; then with a high salt concentration buffer containing: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl; then with a lithium chloride wash buffer containing: 250 mM LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1, and finally twice with TE buffer: 10mM Tris-HCl, 1mM EDTA pH 8.0. The beads were then washed in an elution buffer twice and the supernatant containing the immunoprecipitated complexes was collected. The salt concentration of the supernatant was then adjusted to a final 200mM NaCl and incubated overnight at 65°C to reverse the crosslinks. The resulting suspension was subjected to phenol:chloroform extraction and ethanol precipitation, and then used for PCR amplification of the hTERT promoter. The PCR conditions were as follows: 2.5 μ l of DNA was amplified using the primers specific for a 275bp fragment of the hTERT promoter within the core region encompassing ~300bp upstream of the ATG start site. hTERT core Forward primer 5'-CCAGGCCGGGCTCCCAGTGGAT-3', hTERT core

Reverse primer 5'-GGCTTCCCACGTGCGCAGCAGGA-3'. The products were electrophoresed and visualized on a 1.5% agarose gel with Ethidium Bromide (EtBr) staining.

Results

Stable Oct-4 expression does not activate hTERT. Oct-4 is extensively involved in many of the processes involved in pluripotency (Berrill *et al.*, 2004) and is also one of the primary elements in the pluripotent regulatory network, as it has been shown to bind and activate both SOX2 and NANOG, two of the other main factors used in reprogramming differentiated cells (Boyer *et al.*, 2005; Wang *et al.*, 2006). In order to quantify the levels of expression from Oct4 infection in both ASCs and BJs, cells stably infected with Oct-4, NANOG, and Lin28 were allowed to grow for 6 passages or approximately 14 population doublings. These later passage cells, along with early passage infected cells, were used to compare the levels of Oct-4 expression to uninfected BJ cells and to the pluripotent hTERT-expressing iPS cells (Figure 25). In both ASC and BJ infected cells, the overall Oct-4 expression was elevated following selection, while Oct-4 was also elevated at similar levels to iPS following multiple passages. We next examined stable lentiviral infection of Oct-4 into both BJ fibroblasts and ASC 8 cells. After selection with puromycin and 3-5 population doublings, total RNA was isolated for RT-PCR to determine expression levels of hTERT and Oct-4 (Figure 26). As shown in both types of infection methods in ASCs and the BJ fibroblasts, Oct-4 was expressed to the same levels as in iPS cells; however, there was no hTERT transcript detected.

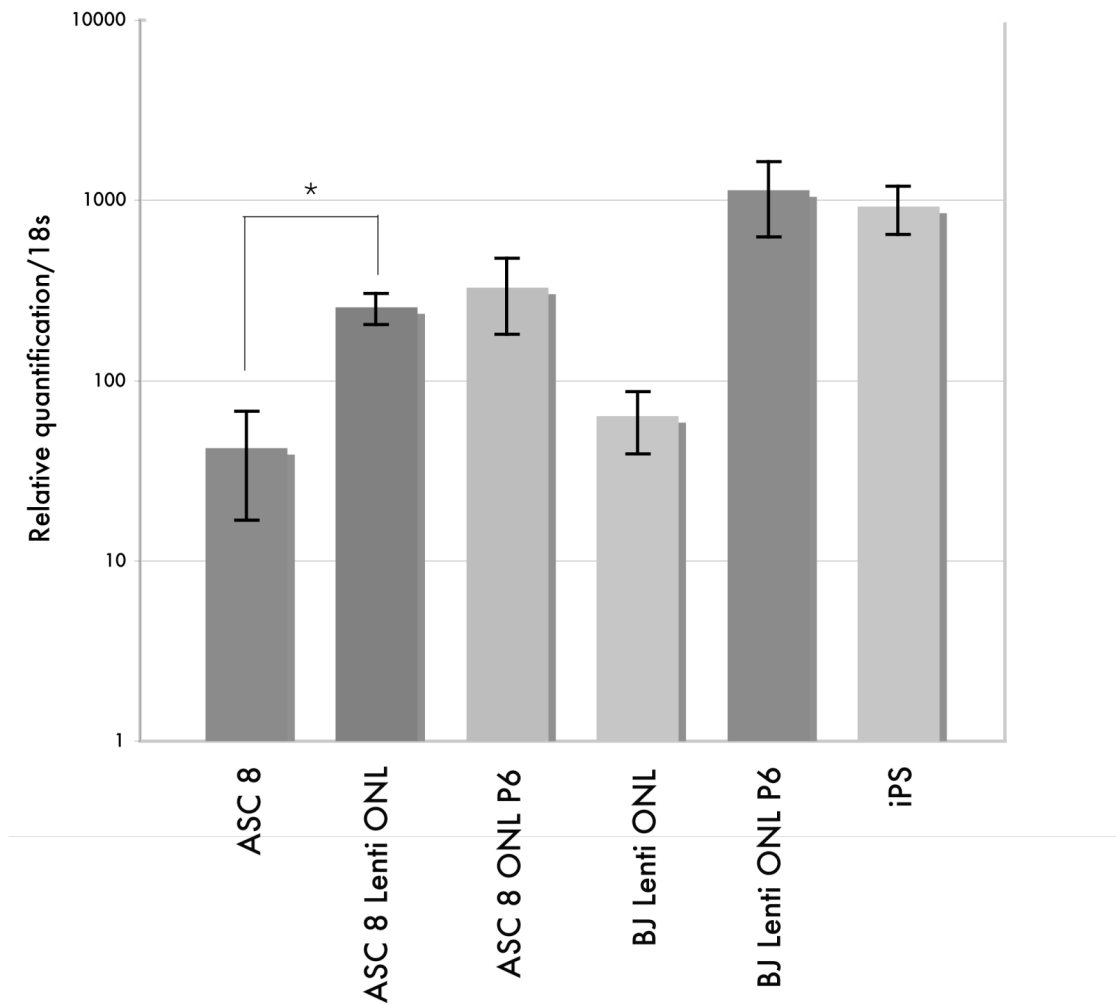


Figure 25: ASC and BJ lentiviral stable Oct-4 over-expression as compared to iPS levels. Quantitative PCR assessment of stable lentiviral Oct-4 expression following 6 passages in both ASCs and BJ fibroblasts. All levels are shown at a significant ($p < 0.05$) expression over baseline BJ fibroblast Oct-4 levels which was set at 1. Lenti ONL = lentiviral infection with Oct-4, NANOG, and Lin28 and P6 = passage 6, while other cells were harvested at 3 days post infection. (* $P < 0.05$)

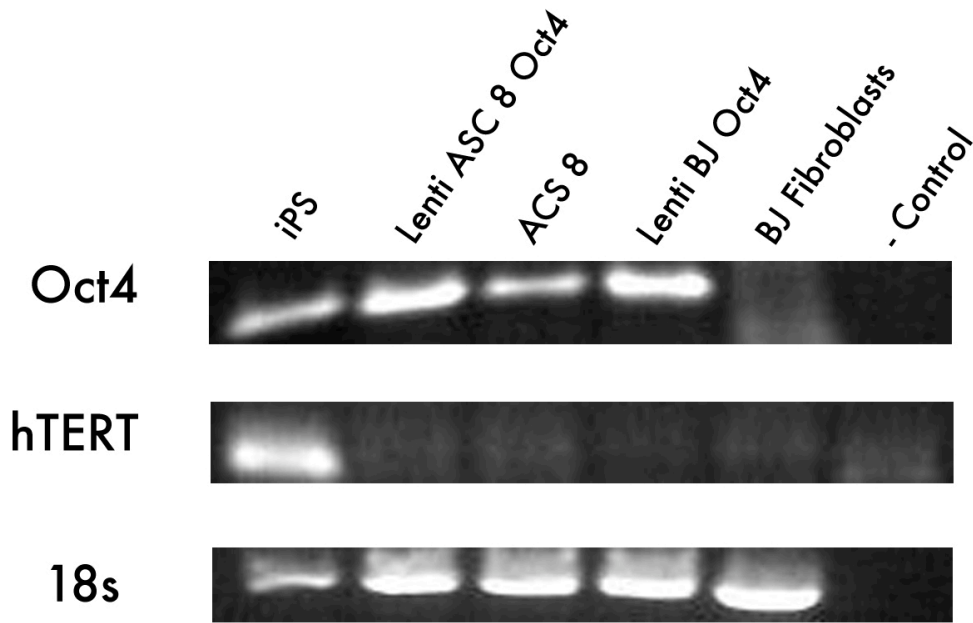


Figure 26: Lentiviral Oct-4 expression in ASCs and BJs does not activate hTERT. RT-PCR analysis of Oct-4 stably infected ASCs and BJ fibroblasts show expression of Oct-4, but no upregulation of endogenous hTERT expression. Negative control is a template-free PCR.

Adenoviral Oct-4, SOX2, NANOG, and Lin28 expression does not activate hTERT in ASCs or BJ cells. We next determined if the expression of iPS factors using transient adenoviral was capable of activating hTERT. First, we wanted to establish the expression kinetics of the adenoviral infection in both ASCs and BJs. Because it takes approximately 3 weeks to induce pluripotency, we assessed hTERT expression following two infections spaced by 2 weeks. To do this, we infected hTERT into ASCs and BJ fibroblasts and extracted total RNA 2, 6, 10 and 14 days for qRT-PCR analysis (Figure 27). As a positive control, the adenoviral hTERT infections were capable of expressing hTERT mRNA at consistently elevated levels for the entire 2 week period. We then transiently infected BJ fibroblasts with Oct-4 and hTERT twice over a 2 week period in order to test if adenoviral Oct-4 was capable of activating hTERT (Figure 28). hTERT was expressed in the cells that were infected with the adenoviral hTERT, yet while Oct-4 alone? was overexpressed, no hTERT expression was found in the adenoviral Oct-4 infections (Figure 28). Similarly, telomerase activity was found in only those cells that received the adenoviral hTERT infections as evident from a TRAP assay performed following various adenoviral infections (Figure 29).

We next tested if the other iPS factors were capable of activating hTERT expression. To do this, we performed similar adenoviral infections on both early passage ASC and BJ fibroblasts. We first infected cells with either adenoviral SOX2, NANOG, or Lin28 as described above for adenoviral Oct-4 infections. The initial infection was followed after 6 days with another infection, then cells were allowed to rest for 3-6 days, harvested, and total RNA

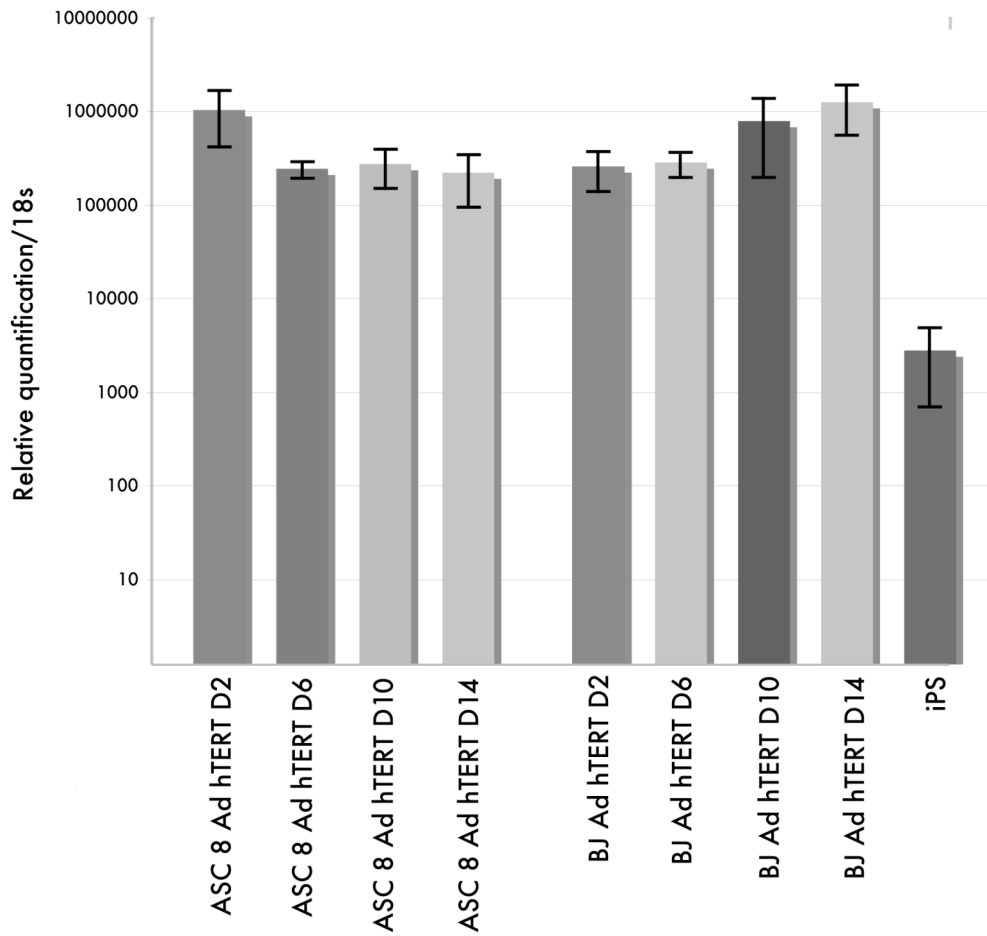


Figure 27: Elevated hTERT expression using adenoviral hTERT in ASCs and BJ cells.

qPCR analysis of hTERT expression of Ad hTERT infected BJ fibroblasts and ASCs. Cells were allowed to grow unpassaged for two weeks then collected for analysis following initial adenoviral infection. Both BJ and ASC adenoviral hTERT infections showed expression at all time points as significant ($p < 0.05$) over baseline uninfected BJ and ASC expression, showing the robust expression capabilities of the adenoviral vectors. iPS = induced pluripotent stem cells.

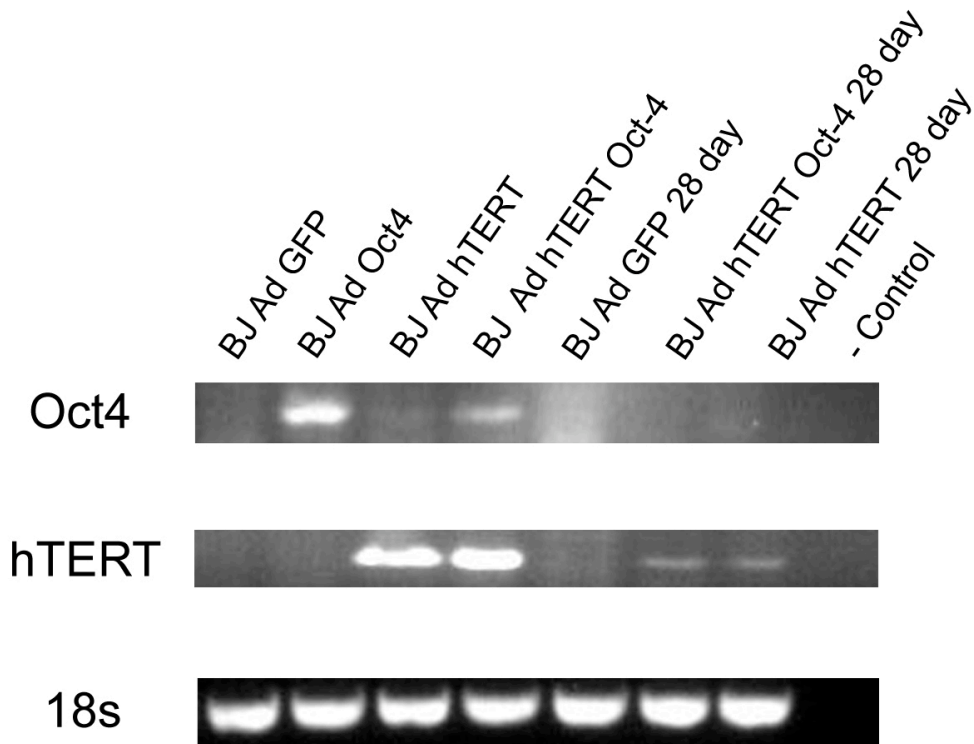


Figure 28: Adenoviral Oct-4 does not activate hTERT expression. RT-PCR for Oct-4 and hTERT expression following Ad Oct-4 infection over 2 weeks with 2 infections in ASC and BJ fibroblasts. Ad Oct-4 infections show undetectable upregulation in hTERT mRNA expression. Ad hTERT mRNA following infection can be seen upregulated for approximately 4 weeks with continued passage. Negative control is a template-free PCR reaction.

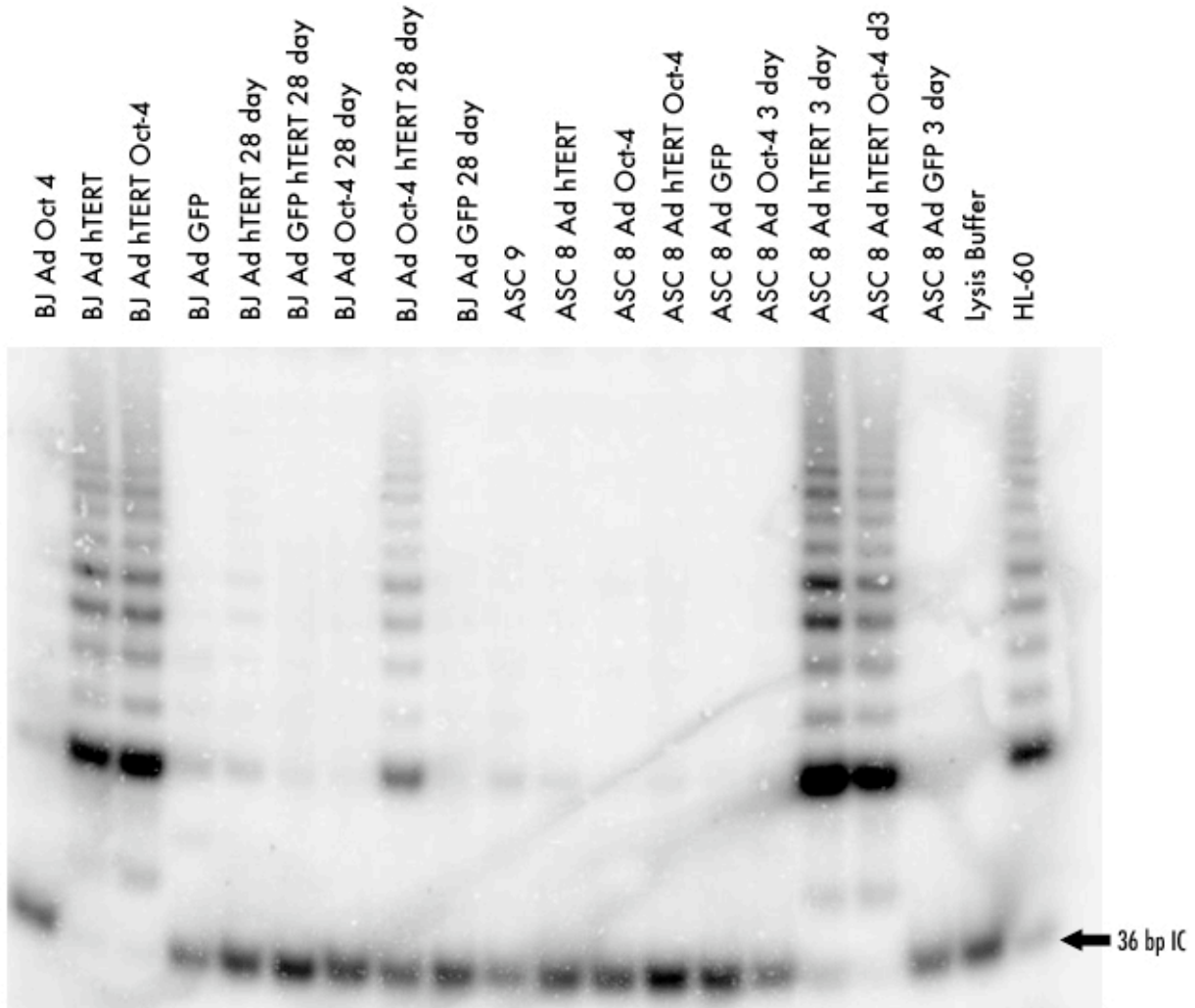


Figure 29: BJ and ASC Adenoviral Oct-4 does not upregulate hTERT activity, Adenoviral hTERT telomerase activity. TRAP assay for telomerase activity following adenoviral infections of both Oct-4 and hTERT into ASC and BJ fibroblasts. Ad Oct-4 infections alone did not induce hTERT activity and Ad hTERT infection shows activity with the dual Oct-4/hTERT infection even after 28 days. 36bp Internal control shown on bottom of gel, marked with an arrow.

was extracted and used for RT-PCR (Figure 30-32). Similar to the results with the Oct-4 adenoviral infections, SOX2, NANOG, and Lin28 were expressed at higher levels as compared to uninfected cells, yet no hTERT expression was observed. In order to ensure that the adenoviral factors were being expressed following infection at sufficient levels, we used qPCR and comparisons to both a baseline uninfected BJ fibroblast expression (negative) and the over-expressing iPS cells (positive) as our standards. Adenoviral Oct-4 was expressed at a significant ($p < 0.05$) level over BJ fibroblasts and at levels similar to those seen in iPS cells (Figure 33). Likewise, adenoviral SOX2, NANOG, and Lin28 were significantly overexpressed at levels comparable to iPS cells (Figure 34-36). Interestingly, we observed significant levels of SOX2 and NANOG by qPCR in uninfected ASCs and BJs, and essentially no expression observed using a gel based RT-PCR, suggesting that ASCs do express these iPS factors at insufficient levels to induce pluripotency.

Having found that none of the factors alone were capable of activating hTERT even though their expression was similar to iPS cells, we asked if there was a combination of iPS-related genes that would induce hTERT expression. Since many groups have shown that the core required factors for iPS formation is the expression of both Oct-4 and SOX2, we first infected both ASCs and BJ fibroblasts with adenoviral Oct-4 and SOX2, showing the successful overexpression without the upregulation of hTERT (Figure 37). We then infected ASC and BJs with all four iPS factors (OSNL) in an attempt to determine if all of these factors together were capable of activating hTERT (Figure 37). Again, following 2 successive infections over the span of 14 days, we found successful overexpression of all four infected factors, but no induction of hTERT.

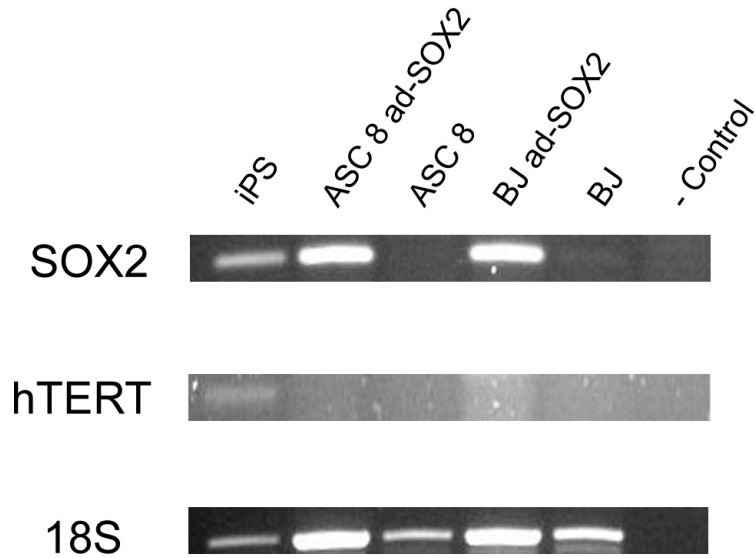


Figure 30: ASC and BJ Adenoviral SOX2 over-expression does not activate hTERT.
 Traditional RT-PCR for Ad SOX2 infections show over-expression in both ASC and BJ without an increase in hTERT message. Negative control is a template-free PCR reaction.

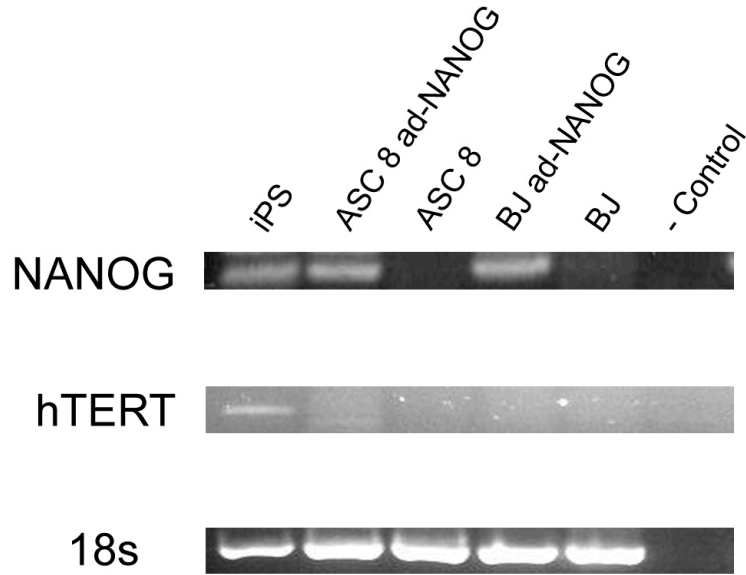


Figure 31: ASC and BJ Adenoviral NANOG over-expression does not activate hTERT. Traditional RT-PCR for Ad NANOG infections show over-expression in both ASC and BJ without an increase in hTERT message. Negative control is a template-free PCR reaction.

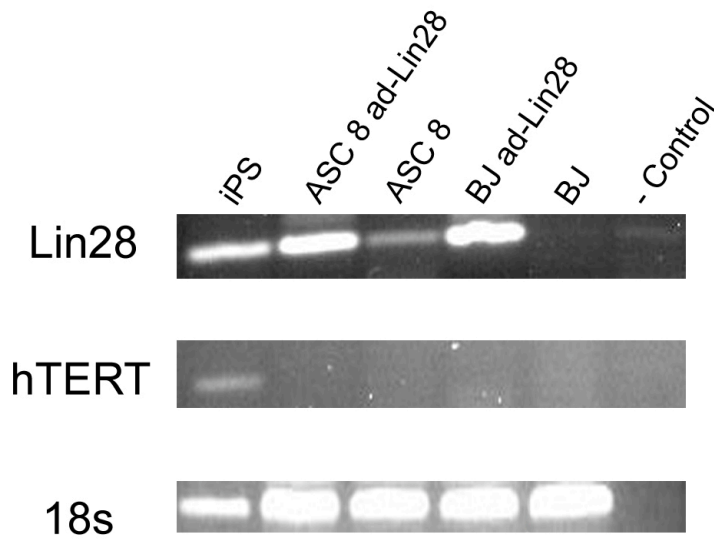


Figure 32: ASC and BJ Adenoviral Lin28 over-expression does not activate hTERT.
 Traditional RT-PCR Ad Lin28 infections show over-expression in both ASC and BJ without an increase in hTERT message. Negative control is a template free-PCR reaction.

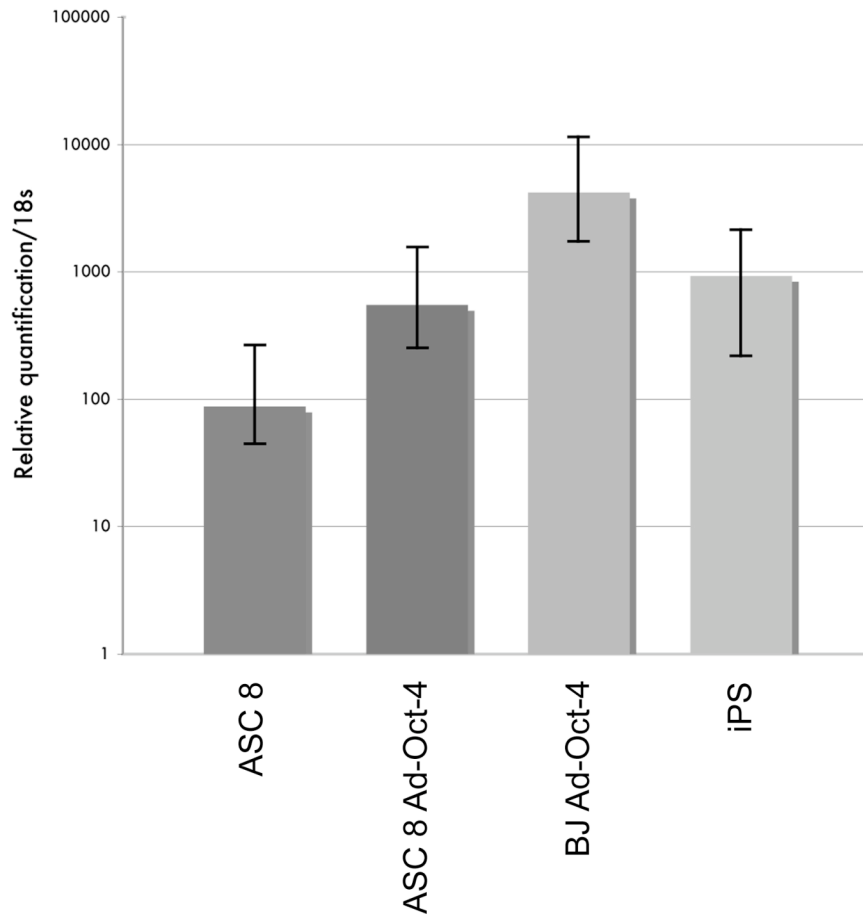


Figure 33: ASC and BJ Adenoviral Oct-4 over-expression. qPCR for Ad Oct-4 infections show over-expression in both ASCs and BJs to levels comparable to those seen in the hTERT expressing iPS cells. All samples are significant ($p < 0.05$) over baseline BJ fibroblast expression.

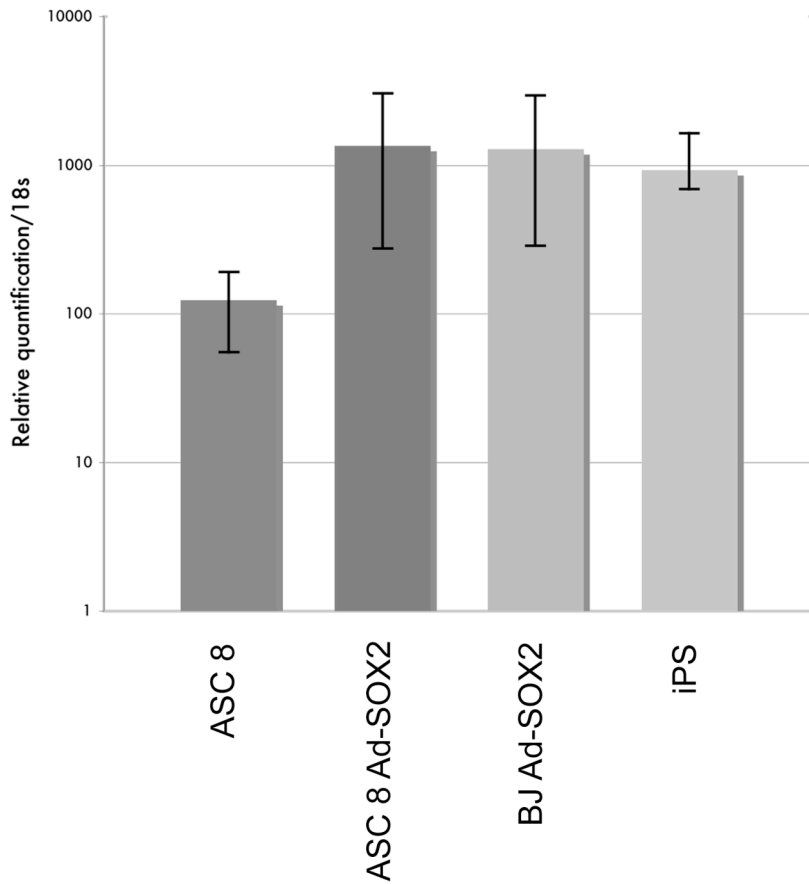


Figure 34: ASC and BJ Adenoviral SOX2 over-expression qPCR for Ad SOX2 infections show over-expression in both ASCs and BJs to levels similar to those seen in the hTERT expressing iPS cells. All samples are significant ($p < 0.05$) over baseline BJ fibroblast expression.

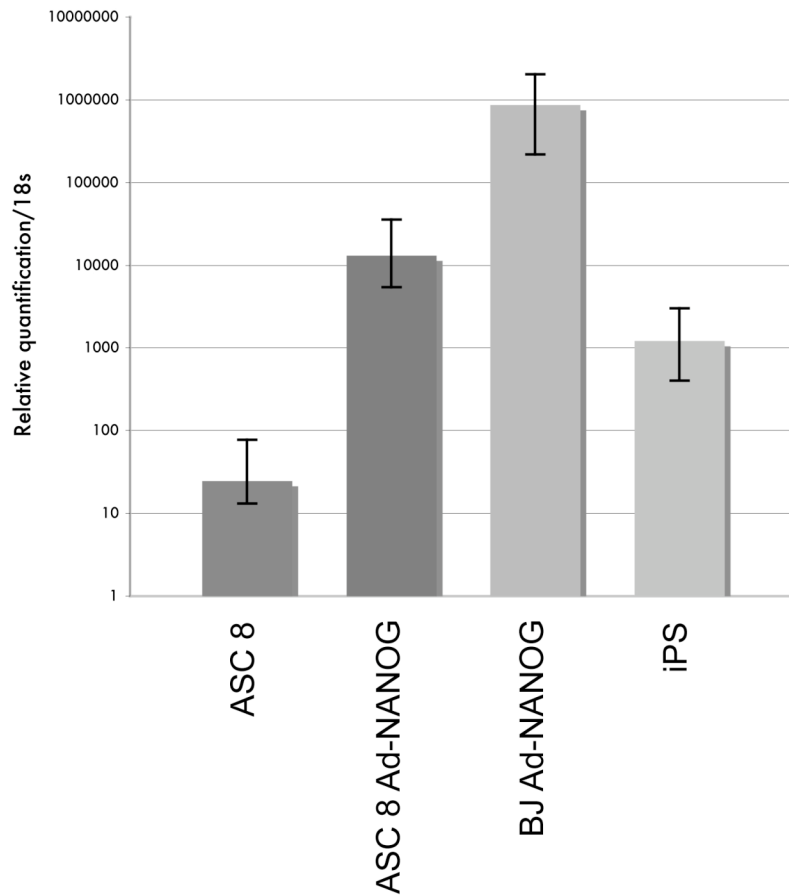


Figure 35 ASC and BJ Adenoviral NANOG over-expression. qPCR for Ad NANOG infections show over-expression in both ASCs and BJs to levels similar to those seen in the hTERT expressing iPS cells. All samples are significant ($p < 0.05$) over baseline BJ fibroblast expression.

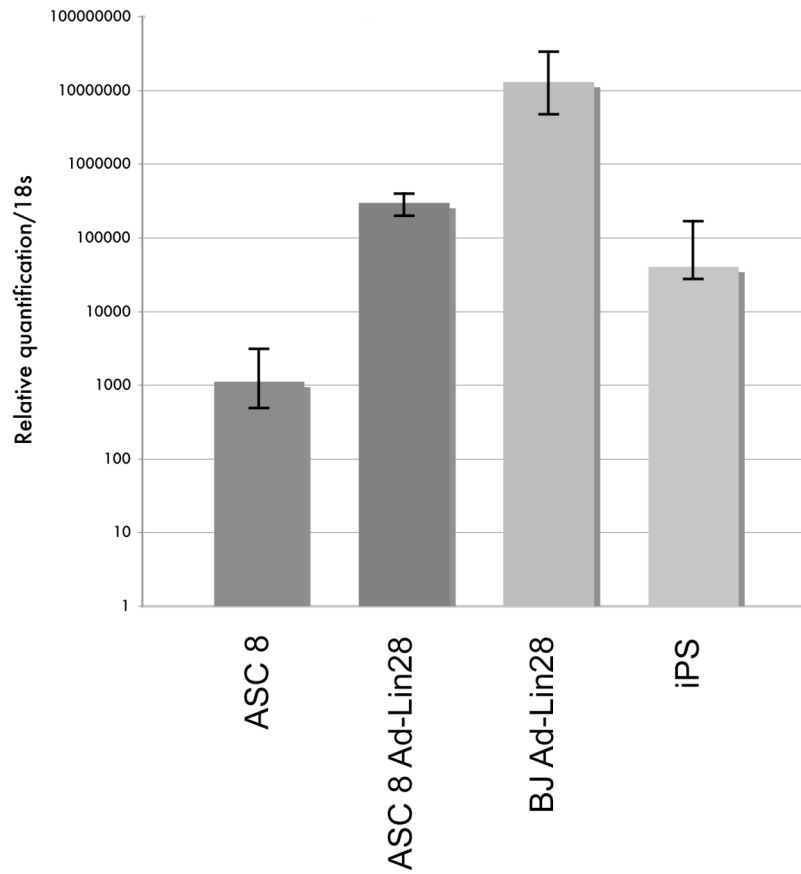


Figure 36: ASC and BJ Adenoviral Lin28 over-expression. qPCR for Ad Lin28 infections show over-expression in both ASCs and BJs to levels similar to those seen in the hTERT expressing iPS cells. All samples are significant ($p < 0.05$) over baseline BJ fibroblast expression.

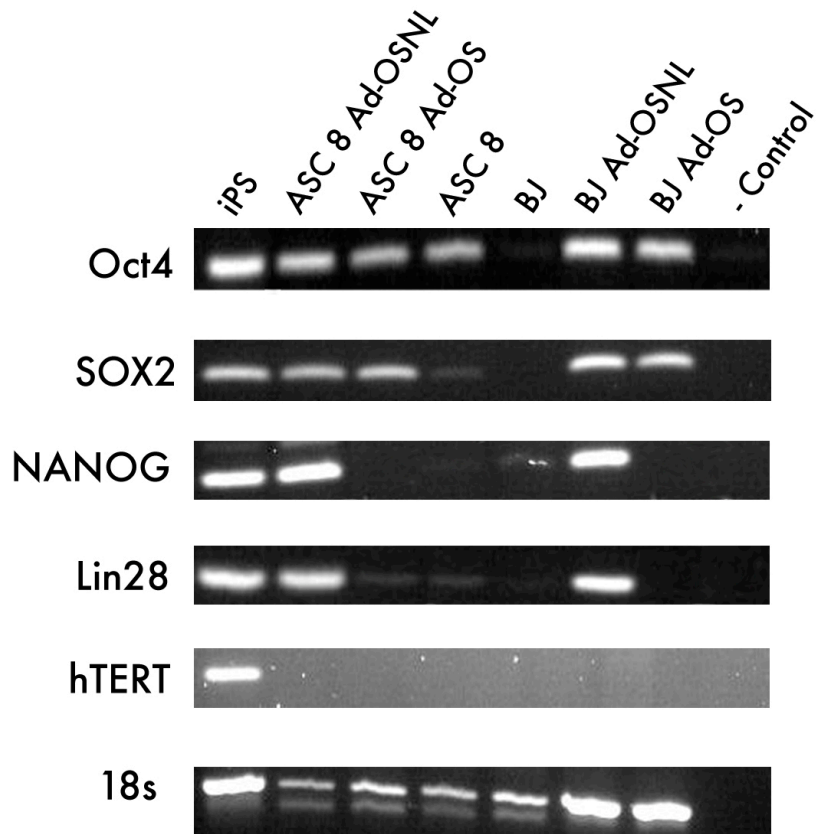


Figure 37: Adenoviral Oct-4, SOX2, NANOG and Lin28 expression does not activate hTERT. Infections of Ad-Oct4/SOX2/NANOG/Lin28 (OSNL) show over-expression of all genes in both ASCs and BJs without an increase in hTERT expression.

Adenoviral Oct-4, SOX2, NANOG, and Lin28 expression with Trichostatin A or C-MYC activates hTERT expression. Trichostatin A (TSA) is a potent histone deacetylase inhibitor, where treated cells undergo extensive histone modifications, likely allowing for a more relaxed and active chromatin state. It has been reported that TSA is capable of activating hTERT expression in BJ fibroblasts (Cong & Bacchetti, 1999; Takakura *et al.*, 2001), suggesting that one negative regulatory element is the state of the histones at the hTERT promoter. To test if TSA is capable of upregulating hTERT expression in our cells, we performed several treatments of differing concentrations for 24 hours as previously reported (Cong & Bacchetti, 1999). A TRAP assay was performed to test for telomerase activity, and we found that regardless of the TSA concentration, telomerase activity was not upregulated (Figure 38). To determine if the expression of Oct-4, SOX2, NANOG, and Lin28 coupled with TSA treatment was capable of upregulating hTERT activity, we initially infected cells with adenoviral Oct-4, SOX2, NANOG, and Lin28 as before and treated the cells with 1 μ M TSA for 24 hours. RT-PCR analysis showed that all four genes were upregulated in infected cells, but hTERT was elevated in only the samples treated with TSA (Figure 39).

Because TSA is a global histone modifier with targets spanning the entire genome, we sought to determine if hTERT activation after TSA treatment was dependent on any single iPS factor. Accordingly, we infected both ASCs and BJ fibroblasts with Oct-4, SOX2, NANOG, or Lin28 and the combination of Oct-4 and SOX2, followed by treatment for 24 hours with 1 μ M TSA. Standard RT-PCR for hTERT and 18S (Figure 40-41) suggested that each factor, when coupled with TSA, was capable of activating hTERT. However, we saw a similar increase in hTERT activation in both ASC and to a smaller degree in BJs when treated with TSA alone

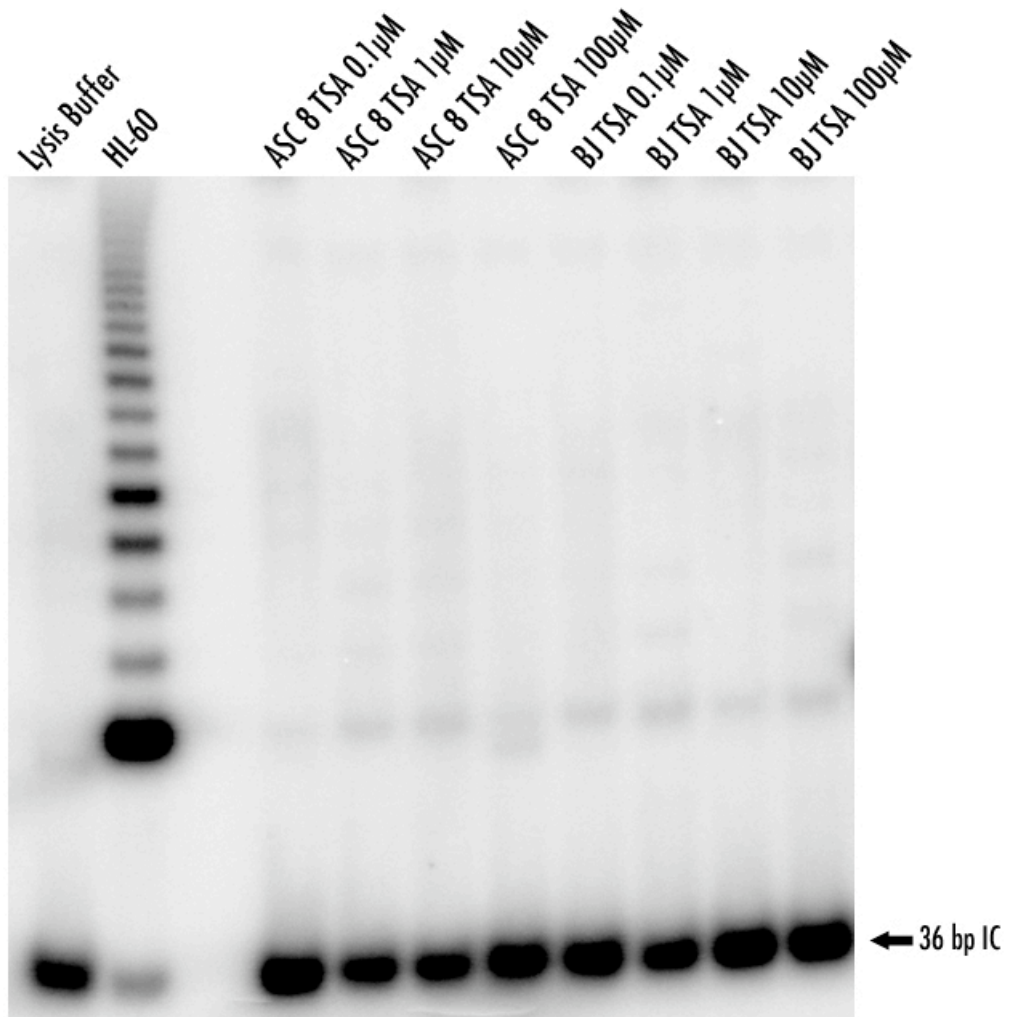


Figure 38: TSA treatment has no effect on telomerase activity in BJ and ASC. TSA treatments at varying dosages were performed on BJs and ASCs for 24 hours, followed by a TRAP assay. No telomerase activity was seen for any of the different concentration dosages. Positive control is the promyelocytic leukemia cell line, HL-60, negative control is lysis buffer alone. 36bp Internal control shown on bottom of gel marked with an arrow.

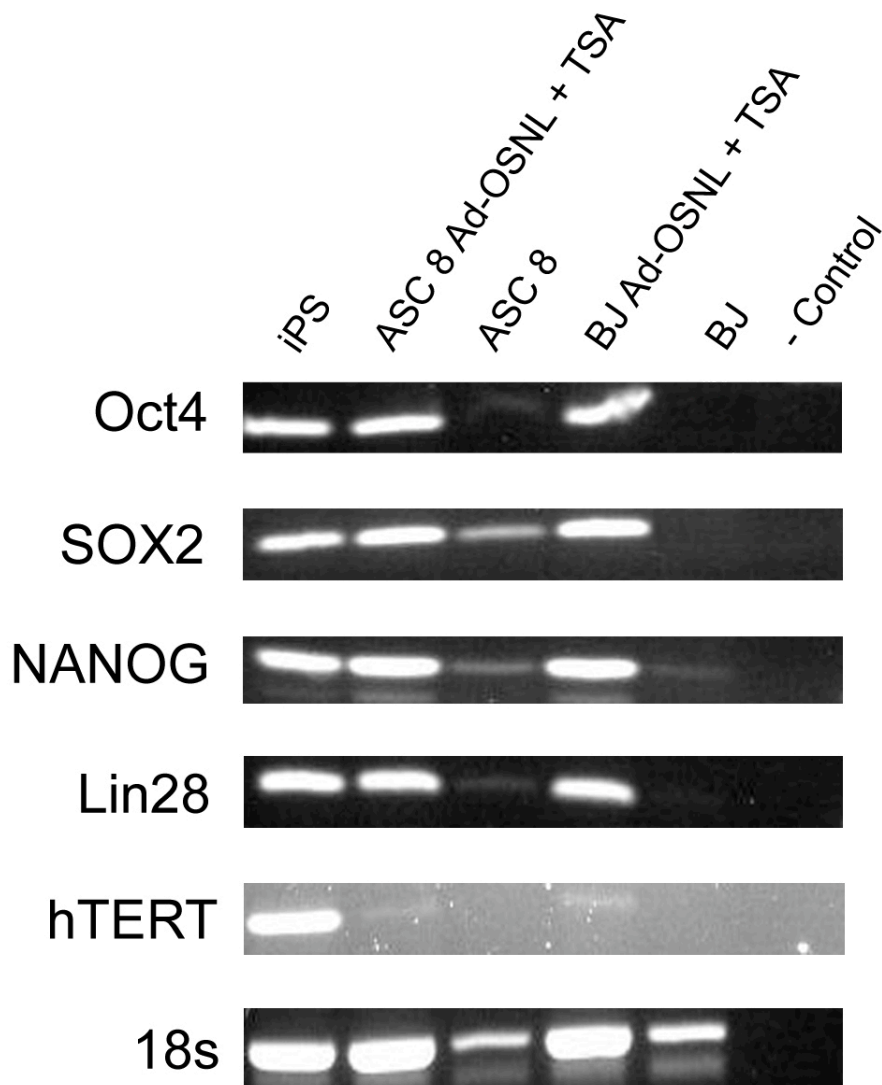


Figure 39: Adenoviral Oct-4, SOX2, NANOG, Lin28 + TSA activates hTERT in ASC and BJ. Cells were infected with Ad Oct-4/SOX2/NANOG/Lin28 (OSNL) together with 1 μ M TSA treatment for 24 hours showed hTERT transcriptional activation. Negative control is a no template PCR reaction.

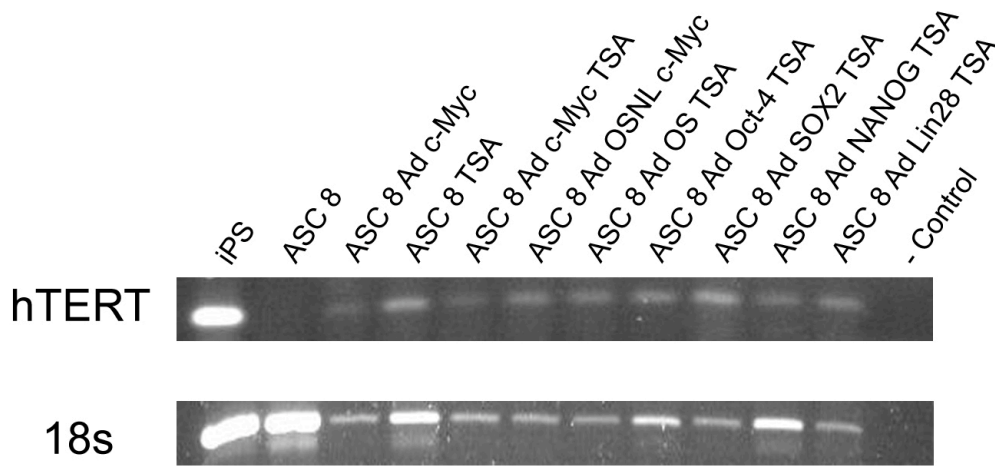


Figure 40: hTERT activation in ASCs upon Adenoviral C-MYC and/or TSA treatment.

Cells were infected with Ad Oct-4, SOX2, NANOG, Lin28 (OSNL), Oct-4 and SOX2 (OS) or individually, with/without C-MYC then with/without 1 μ M TSA for 24 hours. Infections were done twice, spaced six days apart, samples were collected on the 14th day. All infections with C-MYC showed increased hTERT expression, while all treatments with TSA showed an increase as well. Negative control is a no template PCR reaction.

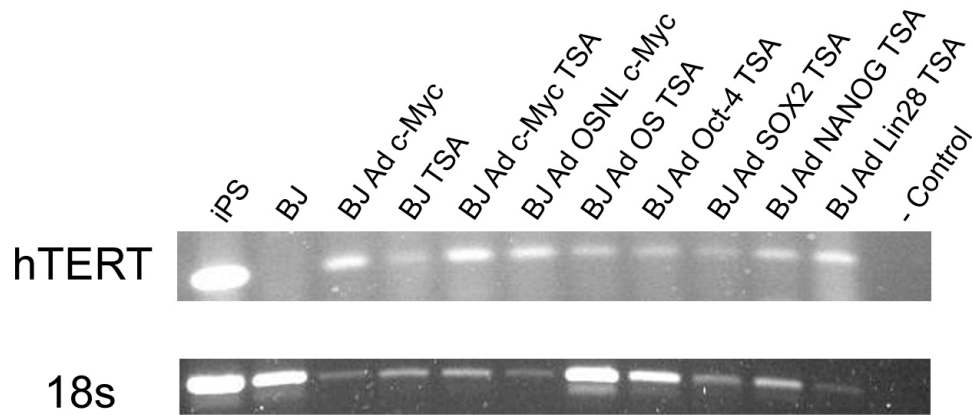


Figure 41: hTERT activation in BJ upon Adenoviral C-MYC and/or TSA treatment. Cells were infected with Ad Oct-4, SOX2, NANOG, Lin28 (OSNL), Oct-4 and SOX2 (OS) or individually, with/without C-MYC then with/without 1 μ M TSA for 24 hours. Infections were done twice, spaced six days apart, samples were collected on the 14th day. All infections with C-MYC showed increased hTERT expression. Negative control is a no template PCR reaction.

(Figure 40-41), which was not translated into telomerase activity as shown earlier (Figure 38). To examine the effect of C-MYC infection on hTERT expression, we used an adenovirus to infect cells with C-MYC and found an upregulation of hTERT expression for all C-MYC infections for both ASCs and BJ cells (Figure 40-41).

Since the end goal for all hTERT expression is the enzymatic activity of the ribonucleoprotein complex that is telomerase, we tested the effect of these treatments on its activity. The telomerase activity levels were highly variable, as shown by the representative TRAP gel (Figure 42). To further quantify which infections had the most substantial increase in telomerase activity, we repeated this assay three separate times and used densitometry standardized to the internal 36bp control to obtain semi-quantified telomerase activity (Figure 43). We found that in ASC 8, there was a significant increase in telomerase activity when cells were infected with C-MYC alone or in combination with TSA treatment. Oct-4, SOX2, NANOG, and Lin28 infection together with TSA showed the highest levels of telomerase activity of all the tested samples, levels even surpassing those of ESC and iPS cells. Similar results were observed with BJ fibroblasts infected with only C-MYC or C-MYC in combination with Oct-4, SOX2, NANOG, and Lin28. In contrast to the ASCs infected with Oct-4, SOX2, NANOG, and Lin28 treated with TSA, BJ cells showed minimal telomerase activity in the absence of C-MYC, indicating that these cells regulate hTERT differently than ASCs. We also performed numerous other combinations of infections using both lentiviral and adenoviral infections in an attempt to upregulate hTERT and telomerase activity, as well as to find the optimal combination for iPS formation (Table 3).

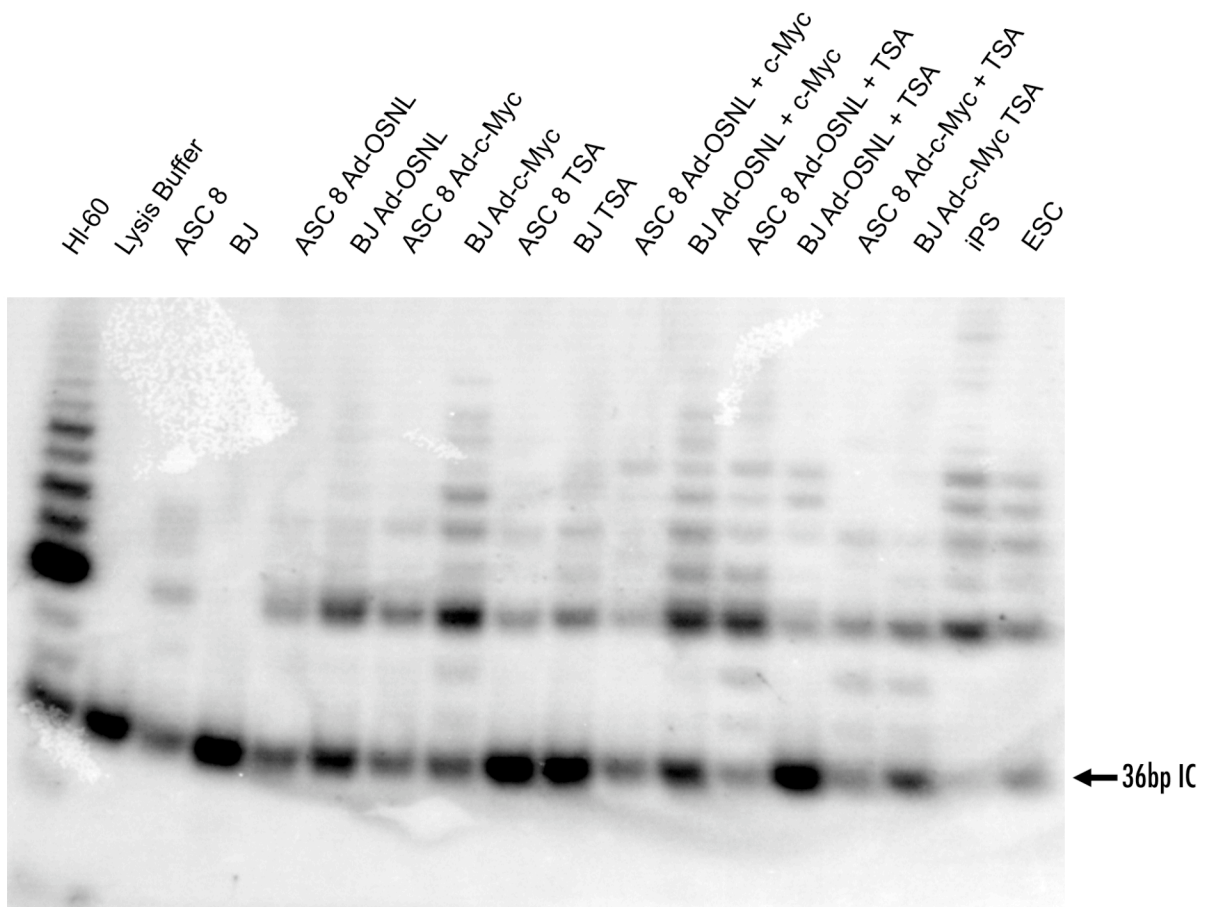


Figure 42: Representative telomerase activity gel for ASCs and BJs infected with Adenoviral C-MYC, Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment. Cells were infected with Ad Oct-4, SOX2, NANOG, Lin28 (OSNL), or Oct-4 and SOX2 (OS), or individually, with/without C-MYC and with/without 1 μ M TSA for 24 hours. Infections were done twice, spaced six days apart, and samples were collected on the 14th day. Ad C-MYC and TSA with combinations of Ad (OSNL) show varying levels of telomerase activity. 36bp internal control shown at bottom of gel marked with an arrow.

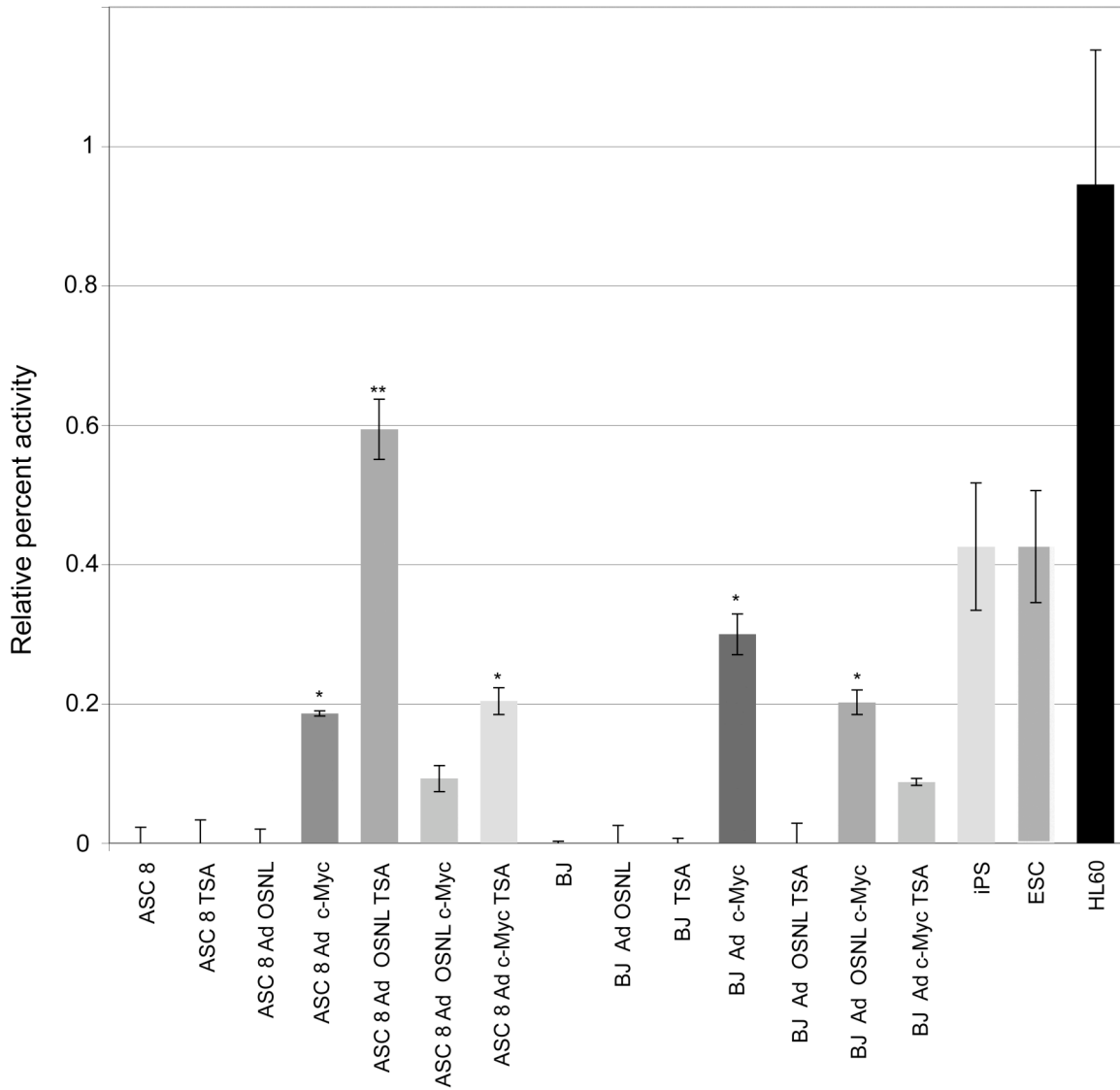


Figure 43: Telomerase activity in ASC and BJ following adenoviral infection with C-MYC, Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment. Cells were infected with Ad Oct-4, SOX2, NANOG, Lin28 (OSNL), Oct-4 and SOX2 (OS) or individually, with/without C-MYC then with/without 1µM TSA for 24 hours. Infections were done twice, spaced six days apart, samples were collected on the 14th day. TRAP assay was performed in triplicate and gels were quantified using densitometry, standardized to 36 bp internal control. Relative baseline was set to uninfected cell telomerase activity, experiments with significant increase over baseline, $p < 0.05$ (*) or $p < 0.001$ (**).

Table 3: Summary of Infections and hTERT Activation

Gene used	iPS plating	Cell Strain Infected	iPS Induction	hTERT Activation
Lentiviral Infections				
Oct4	Gelitin, MEF	BJ, IMR90, ASC	-	-
Oct4, SOX2	N/A	N/A	-	-
Oct4, NANOG, Lin28	Gelitin, MEF	BJ, IMR90, ASC	-	-
Oct4, SOX2, NANOG, Lin28	N/A	N/A	-	-
Adenoviral Infections				
GFP	N/A	BJ	-	-
hTERT	N/A	BJ, ASC	-	+
Oct4	MEF	BJ, ASC	-	-
SOX2	N/A	BJ, ASC	-	-
NANOG	N/A	BJ, ASC	-	-
Lin28	N/A	BJ, ASC	-	-
Oct4, hTERT	Gelitin, MEF	BJ, ASC	-	+
Oct4, SOX2, hTERT	Gelitin, MEF	BJ, ASC	-	+
Oct4, SOX2	Gelitin, MEF	BJ, ASC	-	-
Oct4, SOX2, NANOG, Lin28	Gelitin, MEF	BJ, ASC, BJ hTERT	-	-
Oct4, SOX2, NANOG, Lin28 c-Myc	N/A	BJ, ASC	N/A	+
Oct4, SOX2, NANOG, Lin28 + TSA	N/A	BJ, ASC	N/A	+
Adeno-Lentiviral Infections				
Lenti-Oct4, Nanog, Lin28, Adeno-Sox2	Gelitin, MEF	BJ, ASC	-	-

Oct-4, SOX2, NANOG, and Lin28 infections upregulate C-MYC expression. We next wanted to determine if that the infections of ASC and BJ with Oct-4, SOX2, NANOG, and Lin28 could be affecting the upregulation of genes such as C-MYC, which as demonstrated above, can positively regulate hTERT. We hypothesized that C-MYC levels would be detectable, but in insufficient quantities to overcome the levels of Mad1 (C-MYC's opposing counterpart) or the chromatin state of the hTERT promoter, only allowing hTERT activation when TSA alleviates the closed chromatin state. To test this, we initially examined the protein levels of C-MYC following the infection of Oct-4, SOX2, NANOG, and Lin28, as well as after treatment with TSA. We performed a Western blot analysis and probed for all five factors with actin as our loading control (Figure 44), showing that we were getting sufficiently high protein levels for all of the induction factors following adenoviral infection, comparable to the levels in iPS cells. Additionally, we showed that upon treatment with Oct-4, SOX2, NANOG, and Lin28 in ASC 8, there was an upregulation of C-MYC expression with or without TSA. However, BJ fibroblasts did not show an increased protein product until the cells were treated with TSA, indicating a difference in the inherent hTERT regulation for ASCs and BJs. hTERT protein levels were not examined due to the available antibodies incapacity to detect protein at moderate or low levels.

Since we found a relationship between the infection of the iPS factors and C-MYC upregulation, we next wanted to examine more specifically the effect of Oct-4, SOX2, NANOG, and Lin28 infection on C-MYC mRNA expression. We infected ASCs and BJ fibroblasts individually and various combinations of Oct-4, SOX2, NANOG, Lin28, and/or C-MYC with or without TSA treatment, then harvested cells for gel-based RT-PCR and qPCR analysis. Our

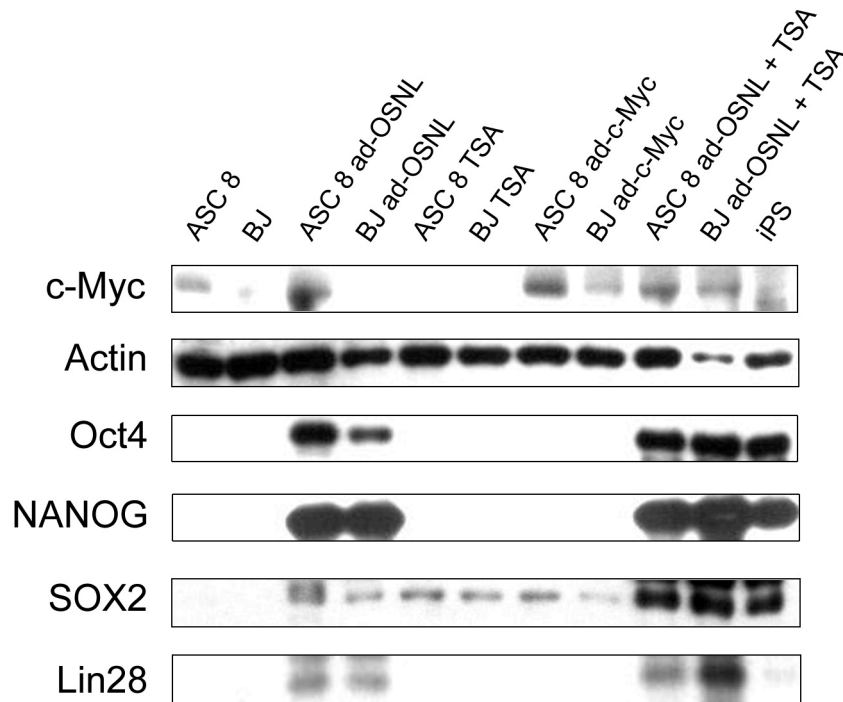


Figure 44: Western blot analysis of adenoviral Oct-4, SOX2, NANOG, Lin28 and C-MYC protein overexpression Cells were infected with Ad Oct-4, SOX2, NANOG, Lin28 (OSNL), or C-MYC then with/without 1 μ M TSA for 24 hours. Infections were done twice, spaced six days apart, samples were collected on the 14th day. All overexpressed genes showed an increased protein signal as detected by Western.

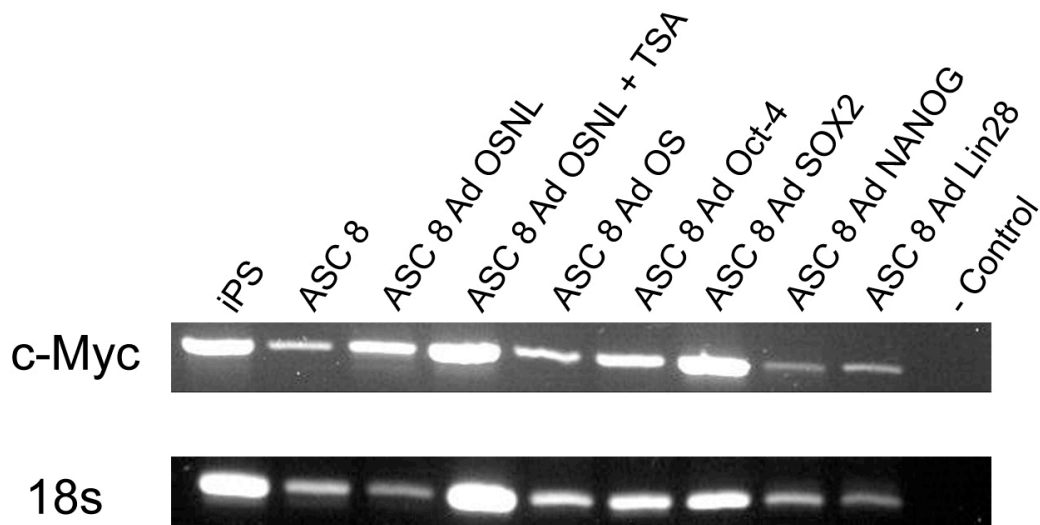


Figure 45: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment. Gel-based RT-PCR for ASCs showing levels of C-MYC expression following infection with Oct-4, SOX2, NANOG and Lin28 (OSNL) alone or in combination. Negative control is template negative PCR.

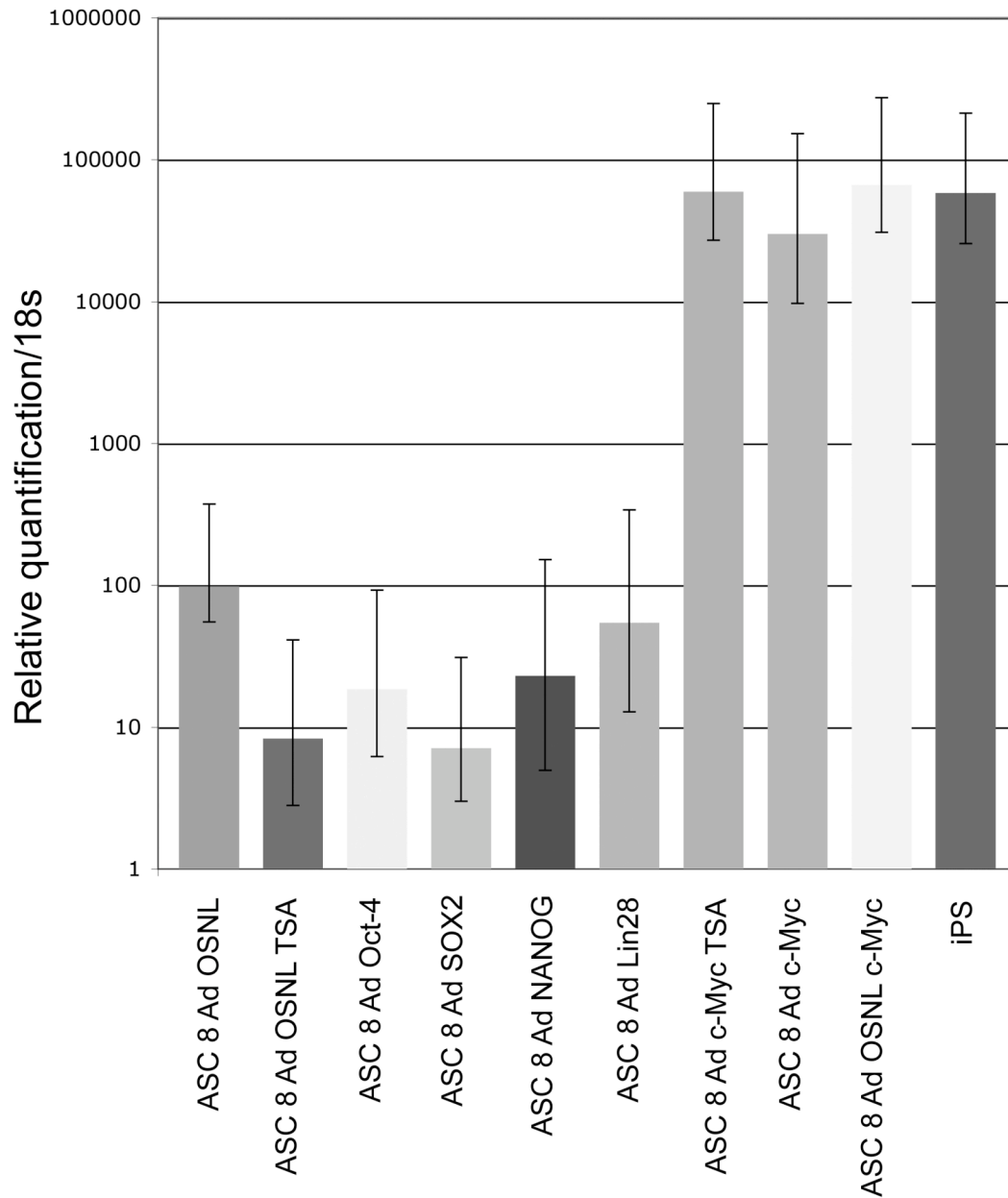


Figure 46: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28, C-MYC and/or TSA treatment. qPCR of ASCs showing levels of C-MYC expression following infection with Oct-4, SOX2, NANOG, Lin28 (OSNL), and C-MYC alone or in combination. Base level was normalized to expression in ASCs alone, and all expression is significantly ($p < 0.05$) increased when compared to baseline values. Relative expression normalized to the levels of 18S endogenous expression.

initial RT-PCR showed an upregulation of C-MYC following infection with all of the four factors (Figure 45), further quantified with qPCR (Figure 46). Each factor, individually and in combination, showed a similar significant upregulation of C-MYC over the baseline, uninfected ASC. However, this increase was not comparable to levels in iPS cells, while the adenoviral C-MYC infections showed levels as high as those in the pluripotent stem cells. We also saw the upregulation of C-MYC in a similar manner when the same infections were performed in BJ fibroblasts, with the adenoviral C-MYC infections showing levels similar to those seen in iPS (Figure 47-48).

hTERT promoter in ASCs and BJ fibroblasts have a closed, inactivated chromatin state. To further define the mechanism behind the activation of hTERT observed for both BJ fibroblasts and ASCs, we wanted to examine the chromatin state of the hTERT core promoter. To do this, we first optimized the sonication conditions to generate chromatin at a size small enough (300-1000bp) to ensure specificity for the DNA region of interest during the ChIP assay; too large of a fragment and the specificity of the assay decreases significantly. We found in most of our cell strains the DNA was sheared to relevant sizes with 10 pulses at 30% power output of our sonicator (Figure 49). Using this protocol, our ChIP analysis initially revealed that BJ fibroblasts had an elevated level of dimethylated lysine 9 on the exposed N-terminal tail of histone H3, while the same residue was lacking the presence of an acetyl group at the core 300bp hTERT promoter (Figure 50). Our data indicates that elements of the hTERT promoter are in a closed, inaccessible chromatin conformation, providing a likely explanation as to why hTERT is not activated in BJ and ASC cell strains. We hypothesized that if this is a marker of expression, then the hTERT expressing iPS cells should have the opposite complement of histone

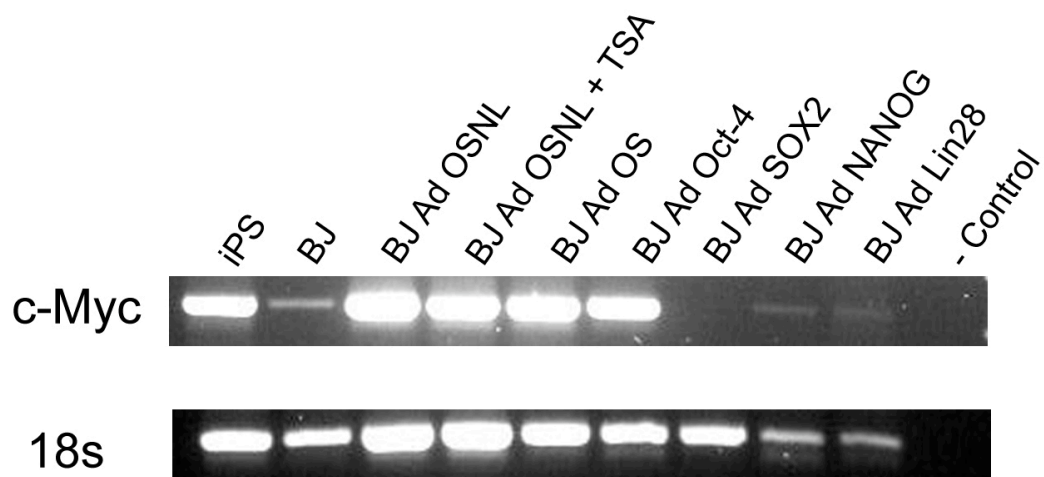


Figure 47: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28 and/or TSA treatment. Gel-based RT-PCR for BJ cells showing levels of C-MYC expression following infection with Oct-4, SOX2, NANOG, and Lin28 (OSNL) alone or in combination. Negative control is template negative PCR.

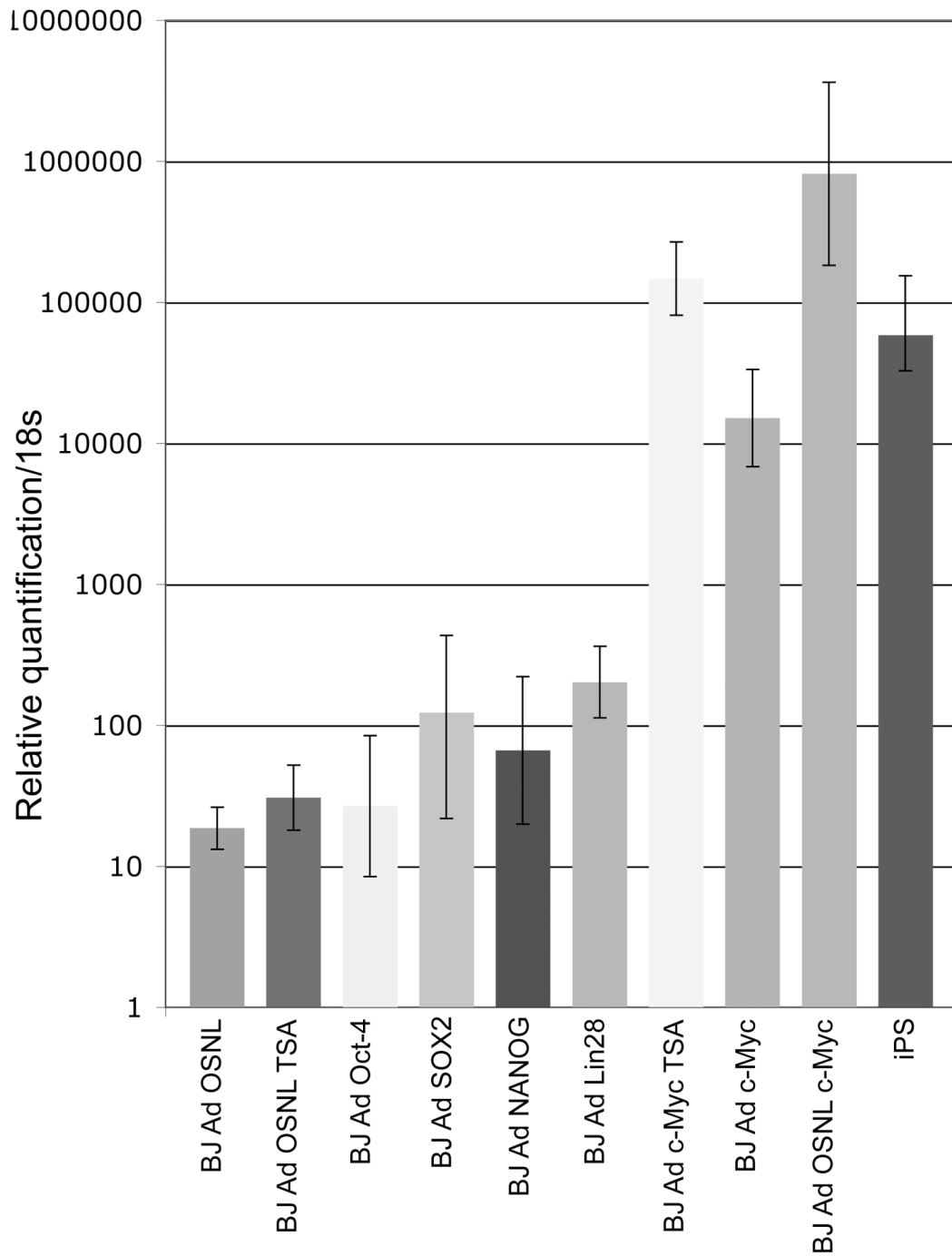


Figure 48: C-MYC Activation following Adenoviral Oct-4, SOX2, NANOG, Lin28, C-MYC and/or TSA treatment. qPCR of BJ fibroblasts showing levels of C-MYC expression following infection with Oct-4, SOX2, NANOG, Lin28 (OSNL), and C-MYC alone or in combination. Base level was normalized to expression in BJ alone, and all expression is significant ($p < 0.05$) over baseline. Relative expression set to the levels of 18S endogenous expression.

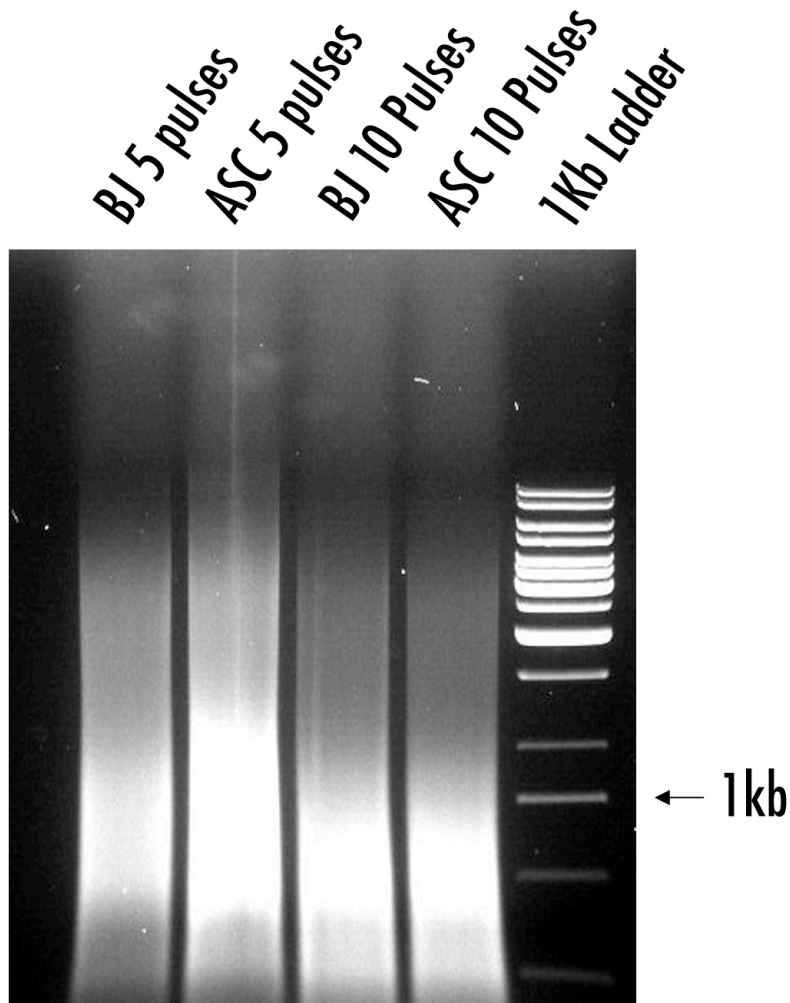


Figure 49: Optimization of sonication conditions for ChIP. Genomic DNA from ASCs and BJs were crosslinked with formaldehyde then subjected to sonication at a set power output for either 5 x 10 second pulses or for 10 x 10 second pulses. The samples sonicated at 10 x 10 second pulses showed DNA sheared into sizes of approximately 300-1000 bp, which is an appropriate size for chromatin immunoprecipitation.

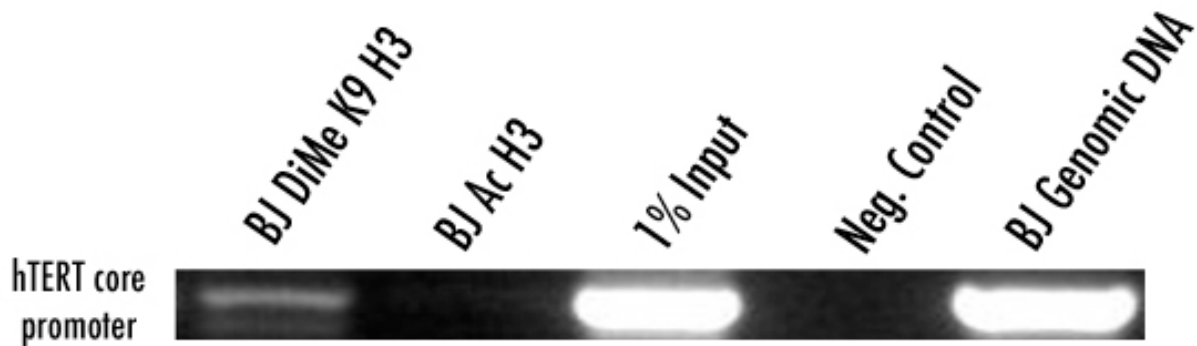


Figure 50: Histone modifications indicative of a closed chromatin state at the hTERT promoter in BJ fibroblasts. BJ fibroblasts were fixed and the resulting genomic DNA was sonicated and immunoprecipitated with an antibody for either acetylated lysine 9 histone H3 or dimethylated lysine 9 histone H3. Lanes 3 represents 1% input of BJ and lane 5 is a BJ genomic DNA positive control, while a template-negative PCR control (lane 4). PCR amplification was achieved using primers specific for a 300bp region of the hTERT core promoter.

modifications. Thus, we next extended our analysis to examine both ASCs, another hTERT-negative fibroblast line of fetal origin IMR90, and iPS cells. Our ChIP analysis revealed that all of the telomerase-negative cell strains, BJFs, ASCs, and IMR90s, had similar H3 patterns, with very low acetylated H3 lysine 9 and substantial dimethylated H3 lysine 9, indicating closed chromatin conformation. As hypothesized, the iPS cells had a reduced complement of dimethylated H3 lysine 9 and a substantial increase in acetylated H3 lysine 9 residue at the core hTERT promoter, suggesting an open chromatin conformation and expression at the hTERT promoter. Together, these data support chromatin condensation as an important mechanism for hTERT regulation in normal fibroblasts and adipose-derived stem cells (Figure 51).

Discussion

The recent creation of pluripotent populations of cells from normal somatic cells has generated much excitement about the possibilities for a viable option for autologous cellular therapies. Many therapeutic approaches have been theorized for the use of these iPS cells in a variety of applications including the creation of disease-specific cell types for tissue engineering. Importantly, these therapies would be patient-specific, eliminating immune response and tissue rejection associated with graft *vs.* host disease. Another important feature is that these iPS cells are essentially indistinguishable from their native, pluripotent counterparts, ESCs, with the capacity to differentiate into all three germ layers and theoretically the ability to create any cell type in the human body. Pluripotent cells continuously divide without the telomere attrition-based senescence found in most normal cells, primarily due to their expression of telomerase, which stabilizes the telomere lengths. iPS cells are typically created from normal non-hTERT

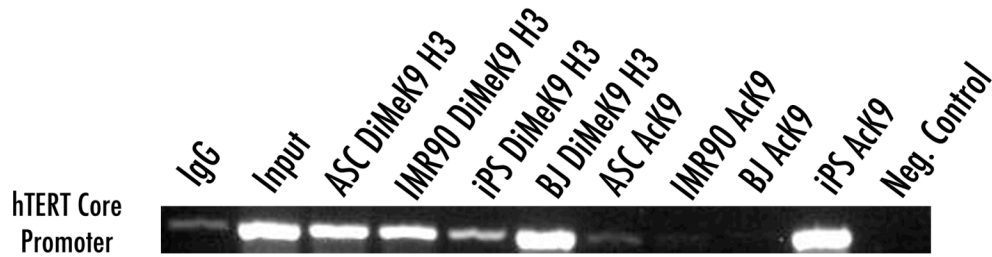


Figure 51: Chromatin state of the hTERT promoter in BJ, ASC, IMR90 and iPS cells. BJ, ASC, IMR90s, and iPS cells were fixed, sonicated, and immunoprecipitated with an antibody for either acetylated histone lysine 9 H3 or dimethylated lysine 9 histone H3. Shown is the background precipitated hTERT core promoter from non-specific interaction with IgG, a 1% input, and a PCR no-template negative control. PCR amplification was achieved using primers specific for a 300bp region of the hTERT core promoter.

expressing cells such as BJ fibroblasts, indicating that during the process of iPS induction the factors used are either directly or indirectly regulating expression of telomerase.

We show here that BJ fibroblasts and ASCs both have a closed chromatin state at the hTERT core promoter, which is one mechanism of hTERT regulation in ASCs and BJs. Both cell types are capable of upregulating hTERT mRNA following treatment with the histone deacetylase inhibitor Trichostatin A, which causes the relaxation of the hTERT core promoter. However, neither cell type showed enzymatic telomerase expression with TSA treatment alone, regardless of the concentration of TSA used, which is possibly due to insufficient hTERT expression or a lack of appropriate hTERT posttranslational modifications. We also show that the four iPS factors, Oct-4, SOX2, NANOG, and Lin28 from the Thomson group (Yu *et al.*, 2007) do not individually have a direct hTERT regulatory ability, even when expressed at levels similar to iPS cells. Because the process of iPS formation is very inefficient with only 0.001% of cells undergoing iPS formation, the necessary mechanisms are dependent not only on the expression of these factors but also on their ability to properly activate downstream genes in the appropriate combinations and sequence to achieve pluripotency. This is likely the reason why we do not detect hTERT expression until treatment with TSA. However, we show also that the overexpression of these iPS factors changes the expression of C-MYC, which was used to induce pluripotent cells by the Yamanaka group (Takahashi *et al.*, 2007), although not to the levels of iPS cells. Although ectopic C-MYC overexpression does have the ability to upregulate hTERT at both RNA and enzyme levels in ASCs, the activity is still present at lower levels than iPS cells. This shows that even with a closed chromatin state at the hTERT promoter, the overexpression of C-MYC causes upregulation of hTERT expression. Thus, C-MYC seems to have a very similar effect in both cell types, upregulating hTERT to low levels, which

corresponds with most published data describing the regulatory Myc/Mad/Max network. Mad1 and C-MYC compete for their mutual binding partner Max, and once bound, these factors exhibit HDAC (causing a closed chromatin state) or HAT (causing a relaxed chromatin state) recruitment respectively at their target genes. By upregulating C-MYC, the balance of competition would be shifted in favor of its binding to Max and the activation of the hTERT promoter by HAT-induced histone acetylation.

ASCs and BJ fibroblasts have many similarities including their morphology and their mesenchymal origin; yet as we have shown previously ASCs have a multipotent capacity and a baseline expression of the various iPS factors at significantly higher levels than BJ cells, suggesting these cells are more stem-like. We show here that ASCs and BJs also have alternative mechanisms of hTERT regulation even though they have a similarly condensed chromatin state. These regulatory differences were related to the levels of telomerase activation observed when the cells were infected with Oct-4, SOX2, NANOG, and Lin28 and then treated with TSA. BJ fibroblasts exhibited minimal telomerase activity with all four iPS factors and TSA, which was similar to that found with TSA alone, while ASCs showed a significant increase of telomerase activity to levels even higher than those found in iPS cells. This difference could be due to ASCs more primordial state, where appropriate hTERT transcriptional activators are expressed in the ASCs to allow for the upregulation of hTERT.

In conclusion, during the generation of iPS cells, hTERT is an important step in the pathway to pluripotency and cellular immortality. Because groups have shown that it is possible to induce adult fibroblasts such as BJs and ASCs to pluripotency, albeit at extremely low efficiencies (Takahashi *et al.*, 2007; Yu *et al.*, 2007), we wished to elucidate the mechanism underlying the activation of hTERT during this process. While the chromatin state of the hTERT

promoter in ASCs and BJ is similar and iPS cells have the opposite chromatin complement, this appears to be the one aspect of regulation that both of these cell strains share. Our data suggest that the hTERT promoter has a very diverse number of regulatory sites, starting with chromatin structure and ending with transcriptional activators and repressors. BJ fibroblasts seem to have a tighter regulation of hTERT than ASCs, allowing ASCs to express sufficient levels of hTERT to give iPS levels of activity. Our working model depicts the regulation of the hTERT core promoter in ASCs, showing that TSA blocks the effects of HDACs and allows for the relaxation of the hTERT promoter (Figure 52). However, there is still an obvious need for a transcriptional activator, which may be the C-MYC oncogene. Yet as we have shown, there may be alternative transcription factors, which may be either constitutively present in ASCs or activated following infection with Oct-4, SOX2, NANOG, and lin28 coupled with the TSA treatment in ASCs alone.

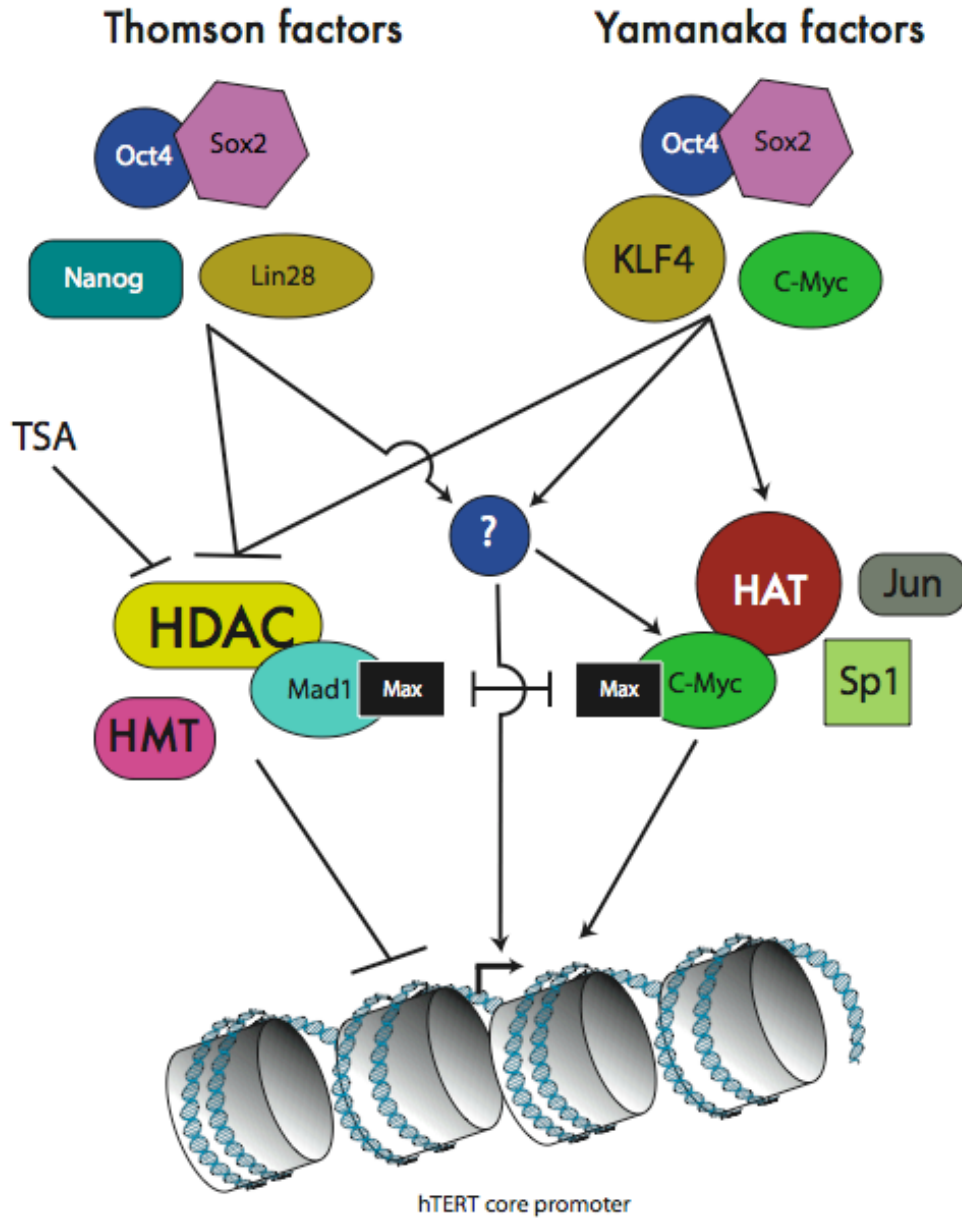


Figure 52: Working model for ASC hTERT regulation. The hTERT core promoter is normally in a closed chromatin conformation. This model highlights some of the hypothetical mechanisms employed by both the Yamanaka and the Thomson iPS factors. They both share very similar concepts, and while the Yamanaka group uses C-MYC, a factor implicated in direct activation of hTERT, the Thomson group does not.

Table 4: RT-PCR and qRT-PCR Primers

Gene	Forward Primer	Reverse Primer
Oct4	5'-CAGTGCCCGAAACCCACAC-3'	5'-GGAGACCCAGCAGCCTCAA-3'
SOX2	5'-TACCTCTCCTCCCACTCCA-3'	5'-GGTAGTGCTGGGACATGTGA-3'
NANOG	5' TTTGGAAGCTGCTGGGAAG 3'	5' GATGGGAGGAGGGGAGAGGA 3'
lin28	5'-AAGCGCAGATCAAAGGAGA-3'	5-CTGATGCTCTGGCAGAAGTG-3'
hTERT	5'-CGGAAGAGTGCTGGAGCAA-3'	5'-GGATGAAGCGGAGTCTGGA-3'
c-Myc	5'-GCGTCCTGGGAAGGGAGATCCGGAGC-3'	5'-TTGAGGGGCATCGTCGCGGGAGGCTG-3

Chapter 5

Discussion

The medical paradigm of healing today prescribes that following trauma or disease, the quality and speed of rejuvenation is primarily dictated by the slow and sometimes incomplete methods the body has to employ, with doctors largely playing an observatory role during the process ensuring that healing happens as cleanly and smoothly as possible. When intervention is necessary, many of the procedures are invasive, yet while correcting the original error, often the procedure itself can cause many more problems of its own. This is sometimes due to a general lack of useable replacement tissues during corrective surgery and the dependence on synthetic prosthetics that have only basic organic simulation ability. For example, total knee joint replacements are typically extremely invasive procedures, leaving patients with months if not years of recovery, and importantly, their new body parts generally under-perform (Bremander *et al.*, 2005; Robertsson & Dunbar, 2001). While the problem this procedure is designed to repair is in itself extremely painful, the solution employed usually involves the removal of large portions of bone, only to be replaced by plastic and metal parts, which is less than optimal. However, without an alternative solution, total or partial knee replacement is the only thing that functions well enough for extended periods of time.

One solution for this problem, which has started to gain momentum, is the replacement of the lost cartilage with the patient's own cells, which could circumvent months of pain and discomfort and would allow for a true reversion to a more youthful, usable knee. The difficulty found with this solution is a lack of readily available, autologous replacement tissues. Many recent studies have searched for this source of replacement tissues with focus on the adult stem

cell niche, with bone marrow mesenchymal stem cells as the primary adult stem cell type being studied. These cells have an extensive history behind them, and were first successfully used in the 60's and 70's, via a total bone marrow transplantation following ablative chemotherapy of the patient's own cells. To obtain these cells, a painful harvesting procedure involving the coring and aspiration of bone marrow from the iliac crest of the pelvis is necessary (Abdallah & Kassem, 2008), only producing low cell yields that require further expansion and culturing to achieve the required volume of cells for use in patients (Abdallah & Kassem, 2008). Even with these shortcomings, some studies have examined the possibility of using these cells as a source for replacement tissues for therapies, such as alternatives for those suffering from knee joint degradation with promising results (Chen & Tuan, 2008; Centeno *et al.*, 2009).

Another promising mesenchymal stem cell type is the adipose derived mesenchymal stem cell (ASCs). We show that these cells are readily available, with isolations possible from the many fat deposits distributed throughout the body. We have successfully isolated these cells from the fat surrounding the abdomen, thighs, arms and breasts (Francis *et al.*, 2010; Elmore, Zhao unpublished observations). These cells can also be isolated in a number of different ways, directly from the processed fat lipoaspirate or from whole tissue obtained following abdominoplasty or reduction mammoplasty. We have also shown these cells can be isolated from a simple, 20 minute process from the saline/blood fraction of the lipoaspirate (Francis *et al.*, 2010), which is possibly due to the disruptive nature of the suction and sonication that occurs during the lipoaspiration procedure. Further, these procedures are typically elective, out-patient operations that are performed on a daily basis in hospitals around the world, making ASCs one of the most readily available sources of adult stem cells.

Regardless of the process by which the cells are obtained and isolated from the adipose tissue, the cellular yield is far greater than that obtained from bone marrow, a feature that would allow for immediate processing and potentially a quick return to the patient's body. We also show that our ASCs have multipotent capability from varying isolation procedures, successfully differentiating into bone, cartilage, and adipose cells as efficiently as BMMSCs, indicating their functional similarities. Further, we found that our cell strains have an immunophenotype identical to those seen in many other publications. Interestingly, this cell surface characterization successfully aligns them with BMMSCs, but fails to distinguish these cells from normal BJ fibroblasts which, presents a difficulty with identification during processing but in no way diminishes ASCs capabilities as BJ fibroblasts showed no differentiation capacity. To resolve a method to successfully discern ASC population via cell surface markers, it would be necessary to find either a complement that is more specific or to find a particular marker that is unique to these cells, which has yet to be accomplished.

We also found that ASCs do not express the enzyme telomerase and are subject to telomere attrition, in contrast to other ASC studies (Lin *et al.*, 2008; Fu *et al.*, 2001). The lack of telomerase poses a problem if it becomes necessary to significantly expand these cells, as their available proliferative capacity may be exhausted after implantation into the patient's body and the subsequent *in vivo* expansion. If the quantity of cells obtained from the original isolation is insufficient to function in transplantation or other therapeutics, we propose that these cells could be transiently infected with hTERT in an effort to stabilize the cell's telomeres until all necessary expansion has been achieved. The cells could then be reimplanted in the body without fear of the possibility that the cells would retain telomerase activity and any potential, albeit unlikely, negative tumorigenic properties from its expression. Experiments could also be performed to

further define these cells. Constitutively expressing hTERT would allow us to determine if telomerase can enhance differentiation after cellular proliferation past the normal limit of these cells. These studies would also be used to determine if these cells have any transformative capacity following immortalization and continuous passage.

In an attempt to further define stem cell features of ASCs, we successfully show a set of genes that could prove to be key identifiers of a stem cell phenotype. Oct-4, SOX2 and NANOG are genes largely associated with the features of pluripotent cells (Wang *et al.*, 2006). These genes are key to many of the processes that govern the embryonic stages of development and allow for many of the differentiation capacities seen in pluripotent cells. These genes also play a crucial role as regulators in the generation of somatic cells induced into pluripotent stem cells. We successfully show that these genes are expressed at significantly higher levels than those found in normal fibroblasts, further distinguishing them as adult stem cells.

To further understand the relationship of BJ, ASCs, and their pluripotent counterparts iPS cells, we wanted to examine the regulation of the key marker for cellular immortalization, hTERT. We have shown that the expression of hTERT is undetectable in both BJ fibroblasts and also from our ASCs isolations; however, once BJ fibroblasts are infected with the four induction factors (Oct-4, SOX2, NANOG and Lin28) other groups have shown that a very small subset of infected cells (0.001%) undergo a process of de-differentiation into pluripotent cells (iPS), which express hTERT (Yu *et al.*, 2007). Interestingly, ASCs do express these pluripotent factors at higher levels than in BJ fibroblasts but lower than the levels found in iPS cells. This low level expression in ASCs could be sufficient to allow these cells their multipotent ability, while not being high enough to allow them to fully become pluripotent.

Due to ASCs expression of these iPS genes, we further hypothesized ASCs could also possess a more pluripotent stem cell-like epigenetic/regulatory state, which would allow for a more efficient upregulation of hTERT following infection. To examine this, we overexpressed each of these genes individually using a transient adenoviral expression system, which showed undetectable hTERT expression. This lack of hTERT upregulation could be due to the inefficiency of the iPS generation process. It has been reported previously that only a very small fraction of cells typically undergo iPS colony formation following stable infection and overexpression of these genes (Yu *et al.*, 2007; Takahashi *et al.*, 2007). The small subset of cells that appear to be more susceptible to pluripotency could have a particular epigenetic state that would be more favorable for the induction process to occur. If this is the case, then any cells that may be expressing hTERT could be in such a small proportion making the signal undetectable in our ASC and BJ cell population.

To further examine the epigenetic state at the hTERT promoter, we used TSA, a potent histone deacetylase inhibitor, to treat both our ASCs and BJ fibroblasts. With multiple TSA treatments, no detectable telomerase activity was observed, which is in contrast to studies suggesting TSA as the only necessary component for telomerase activation in other cell strains (Cong & Bacchetti 1999). When we examine the levels of RNA however, we found that TSA treatment alone increased hTERT mRNA expression in both BJ and ASCs, as well as when we used Oct-4, SOX2, NANOG, and Lin28 infected cells with TSA treatment. This effect corresponded to our examinations of the hTERT promoter chromatin state, which showed characteristics of a closed chromatin conformation, including dimethylated lysine 9 at the n-terminal tail of histone H3 with a lack of acetylation at the same residue. When we examined the same modifications in hTERT expressing iPS cells, we found the opposite, lysine 9 histone H3

modification, indicating an open conformation. These data together indicate that there likely exists the means for transcriptional activation, but not the means to relieve the repression due to the closed chromatin state at the hTERT promoter. However, when the epigenetic repression is relieved via the treatment with TSA there still exists a post-transcriptional mechanism limiting its expression, possibly with mRNA processing or with post-translational degradation.

We hypothesized that one possible latent transcriptional activator could be a low level expression of the oncogene C-MYC. C-MYC's ability to activate target genes is dependent on a few different factors. When C-MYC binds to Max, this dimerization allows the transcription factor to bind to its target promoters, where it has been shown to further recruit histone acetyl transferases (HAT) allowing for the relaxation of the bound chromatin (Amati *et al.*, 2001). In competition with this, Mad1 will also bind to the ubiquitously expressed Max, and in an antagonistic manner will bind the same locations on promoters, instead recruiting HDAC to deacetylate the histones and allow for a closed chromatin state (Laherty *et al.*, 1997). If there is a balance between C-MYC and Mad1 expression within the cell, there would be a threshold level of expression necessary for one to overcome the effects of the other, i.e. just a small change in one would be buffered by the other, thus the outcome would be minimal. Importantly, these transcription factors have also been shown in the literature to bind directly to the hTERT core promoter at two separate E-box locations. These E-boxes are contained within the area of the hTERT promoter that we found exhibiting a closed chromatin conformation. Interestingly, C-MYC is also one of the alternative genes (Oct-4, SOX2, Klf4 and C-MYC) used by the Yamanaka group to generate induced pluripotent stem cells (Takahashi *et al.*, 2007), which could result in immediate hTERT activation that could lend a growth advantage to the newly

generated, rapidly dividing, colony forming iPS cells. However, mice formed from these cells were shown to form premature tumors (Takahashi *et al.*, 2007).

To examine the effect of C-MYC overexpression on hTERT upregulation in BJ cells and ASCs, we first looked at the basal level of C-MYC expression before and after infections with the Thomson iPS factors. Interestingly, there was a significant increase in expression of C-MYC with the introduction of Oct-4, SOX2, NANOG, and Lin28, but at a much lower level than in iPS cells. We observed an upregulation of C-MYC to iPS levels when overexpressed alone. When we examined the mRNA expression of hTERT with the overexpression of C-MYC, we saw a similar activation to those seen with our TSA treatments. The most significant increase in telomerase activation was found when Oct-4, SOX2, NANOG, and Lin28 were coupled with TSA treatment, where hTERT activation in ASCs was at levels above those observed in both ESCs and iPS cells. This was in direct contrast to same infections/treatments in BJ fibroblasts, which showed telomerase activity similar to the treatments with TSA alone, which suggests that there are further differences between these two cell strains with regards to their individual gene expression environments. Specifically, ASCs require only the four factors and TSA treatment to show full activation, while lacking a post transcriptional/post-translational mechanisms, found in BJ fibroblasts. C-MYC's activation functions in a more universal, but lower manner could be attributed to not only C-MYC's well documented binding to the core promoter, but also to the regulation of one of its many other targets. To further define the regulation of hTERT during the generation of pluripotent cells, analysis of alternative binding factors such as SP1, and USF1 could lead to a better understanding of hTERT activation. Also, examination of levels of hTERT transcriptional (i.e. MAD1, WT1, and SP3) and post translational repressors (i.e. MKRN1 and CHIP) would also prove insightful. Since the efficiency of reprogramming is very minute,

studies examining the specific cellular population which might activate telomerase would also be informative. To do this we would express a reporter system (GFP, Luciferase) driven by the hTERT promoter. If there is a small population of cells, which are not detectable by TRAP or PCR, we can sort or image cells for individual cellular activation, isolate those populations and continue our analysis of hTERT regulation.

In summation, there has been promising advancements in the field of regenerative medicine. A crucial missing element for this field is a readily available source of cells for tissue reconstruction. ASCs could fill a portion of this requirement due to their ease of access in quantities sufficient for use immediately in many cellular based therapies. While morphologically similar to BJ fibroblasts, ASCs are genetically distinct due to their ability to differentiate into multiple cell types and express genes closely associated with pluripotency. The most limiting aspect of ASCs is their relatively limited potential for expansion due to a lack of a telomere maintenance mechanism and their lineage specific differentiation capacity, which has been partially circumvented by the discovery of iPS cells, which can differentiate into all three germ layers. These iPS cells originate from normal somatic cells and using a very specific set of genes, are significantly altered into the embryonic-like cell type. An important feature of this process is the induction of hTERT expression and telomerase activity, allowing for an immortal-like proliferative capacity. We show that the Thomson factors by themselves are not capable of activating hTERT expression, due likely to the necessity of a compliant epigenetic state at the hTERT promoter and to a presumed lack of direct hTERT promoter binding ability (i.e. no binding sites within the hTERT promoter). The hTERT core promoter in both BJs and ASCs is normally in a closed inactive state, where as iPS cells show a relaxed accessible promoter. This closed state could prevent the endogenous activation by C-MYC, which shows a similar

activation ability in ASCs and BJ cells when overexpressed. Similarly, it has previously been shown that one can activate hTERT expression with the use of HDAC inhibitors, but in our hands no telomerase activation was found after TSA treatment, indicating a possible regulatory mechanism at the post-transcriptional/translational level. This regulation is removed when ASCs, but not BJ cells, are treated with TSA following infection with Oct-4, SOX2, NANOG, and Lin28, highlighting another important distinction between ASCs and BJ fibroblasts. These observations further validate that ASCs as a more primordial cell type with a gene profile somewhere inbetween the pluiipotent embryonic cell and the fully committed fibroblast, giving these cells the unique ability to function with fibroblast-like growth while retaining a limited ability to differentiate into other cell types. If this understanding could be further expanded, the difference that create these cell types could be teased apart, allowing manipulation of cells into whatever form is clinically needed. This would allow us to be able to grow new tissues *in vitro*, with the strong differentiation capacity of embryonic cells, while retaining the ease of culture that ASCs possess as a means of filling the ever growing need for replacement cellular therapies.

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Vita

Patrick Christian Sachs was born in Milwaukee, Wisconsin on May 30, 1978. Moving around the world he found himself attending much of elementary school in Frankfurt and Heidelberg Germany, where his father worked for the United States Office (USO). He and his family returned to the USA in the late 80's where Patrick continued his education in northern Virginia. He received his high school diploma in 1996 from Woodbridge Senior High School, in Lakeridge VA. Following in his brothers footsteps, Patrick was admitted to Virginia Commonwealth University in 1998 with the intentions of pursuing a degree in the arts. He soon discovered that most of his thoughts and ambitions lay more in the sciences and he quickly switched his degree to Biology. He graduated with his bachelors degree in biology in 2002 and quickly decided that he must further pursue his understanding of this field in graduate school. He intends to continue his career in academic sciences with a post-doctoral position to further advance his knowledge of aging and stem cells biology.