Altering Fate Determination of Human Bone Marrow-Derived Mesenchymal Stem Cells through Environmental and Transcriptional Cues

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Abstract

Preservation of adult stem cells pools is critical for maintaining tissue homeostasis into old age. Exhaustion of adult stem cell pools as a result of deranged metabolic signaling and premature senescence as a response to oncogenic insults to the somatic genome both contribute to tissue degeneration with age. Both progeria, an extreme example of early-onset aging, and heritable longevity have provided avenues to study regulation of the aging program and its impact on adult stem cell compartments.

Stem cells are promising candidate cells for regenerative applications because they possess high proliferative capacity and the potential to differentiate into other cell types. Mesenchymal stem cells (MSCs) are easily sourced but do not retain their proliferative and multi-lineage differentiative capabilities after prolonged *ex vivo* propagation. We investigated the use of hypoxia as a preconditioning agent and in differentiating cultures to enhance MSC function. Culture in 5% ambient O_2 consistently enhanced clonogenic potential of primary MSCs from all donors tested. We determined that enhanced clonogenicity was attributable to increased proliferation, increased vascular endothelial growth factor (VEGF) secretion, and increased matrix turnover. Hypoxia did not impact the incidence of cell death. Application of hypoxia to osteogenic cultures resulted in enhanced total mineral deposition, although this effect was only detected in MSCs preconditioned in normoxic conditions. Osteogenesis-associated genes were up-regulated in hypoxia, and alkaline phosphatase activity was enhanced. Adipogenic differentiation was inhibited by exposure to hypoxia during differentiation. Chondrogenesis in 3-dimensional (3D) pellet cultures was inhibited by preconditioning with hypoxia. However, in cultures expanded under normoxia, hypoxia

applied during subsequent pellet culture enhanced chondrogenesis. While hypoxic preconditioning appears to be an excellent way to expand a highly clonogenic progenitor pool, our findings suggest that it may blunt the differentiation potential of MSCs, compromising their utility for regenerative tissue engineering. Exposure to hypoxia during differentiation (post normoxic expansion), however, appears to result in a greater quantity of functional osteoblasts and chondrocytes and ultimately a larger quantity of high quality differentiated tissue.

Media formulations for MSC culture and differentiation vary between labs; two notable variables addressed inconsistently in the field are ascorbate supplementation and glucose concentration in MSC expansion medium. Glucose and ascorbate effects are coupled to the Hif1α pathway. Therefore, we examined the effects of ascorbate supplementation and low versus high glucose medium in the settings of hypoxia and normoxia on colony formation, colony morphology, proliferation, VEGF secretion, and senescence of MSCs. We found that there is no one medium formulation that maximizes MSC performance for all of these assays. Depending on the desired outcome of the culture, the medium and other culture conditions can be adjusted to optimize for a particular aspect of MSC biology, such as colony formation when clonal selection is the desired end application or delayed senescence if MSCs are to be expanded extensively.

Not only do MSCs not retain their proliferative and multi-lineage differentiative capabilities after prolonged ex vivo propagation, there is also evidence to suggest MSCs in aging humans exhibit functional decline. Modifying MSCs to resist aging during ex vivo propagation would yield a favorable cell source for regenerative medicine applications. We developed and tested novel methods for partial transcriptional reprogramming of bulk MSC

populations and investigated whether this technique is effective for enhancing function in MSCs subjected to *in vitro* aging.

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This dissertation is dedicated to the memory of Jack Wicker, who would have enjoyed seeing it, and to all of the people who remind us of why we do what we do.

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Abbreviations/Nomenclature

2D	two-dimensional
3D	three-dimensional
20-HETE	20-hydroxyeicosatetraenoic acid
ΔMFI	delta mean fluorescence intensity, calculated as MFI of experimental condition minus MFI of control
μg	microgram
μL	microliter
μM	micromolar
μm	micrometer
ab	antibody
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AF	AlexaFluor
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AKT	survival/metabolism-regulating serine/threonine-specific protein kinase
alk phos	alkaline phosphatase
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
Asc-2-P	L-ascorbate-2-phosphate
ASCs	adipose-derived mesenchymal stem cells
ATF4	activating transcription factor 4
BMI1	B lymphoma Mo-MLV insertion region 1 homolog oncogene
BM-MSCs	bone marrow-derived mesenchymal stem cells
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CD8	co-receptor for T cell receptor; primarily marker of cytotoxic T cells
CD34	hematopoietic progenitor and endothelial cell marker
CD44	receptor for hyaluronic acid; MSC marker

CD45	hematopoietic stem cell marker
CD73	ecto-5-prime-nucleotidase; MSC and lymphocyte marker
CD90	stem cell marker; also Thy-1
CD105	endoglin; TGFβ co-receptor; mesenchymal marker
CD146	endothelial and MSC marker; also MCAM
cDNA	complementary deoxyribonucleic acid
C/EBPα	CCAAT/enhancer-binding protein alpha
CFU-F	colony forming unit-fibroblast
CFU-O	colony forming unit-osteoblast
c-Myc	avian myelocytomatosis virus oncogene cellular homolog
Col	collagen
CSF	colony stimulating factor
Ct	threshold cycle
CTSK	cathepsin K
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DHA	dehydroascorbic acid
DHEA	dehydroepiandrosterone
diff	differentiation oxygen tension
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EETs	epoxyeicosatrienoic acids
ELISA	enzyme linked immunosorbant assay
EMT	epithelial-to-mesenchymal transition
EdU	5-ethynyl-2´-deoxyuridine, BrdU alternative
eNOS	endothelial nitric oxide synthase
ESCs	embryonic stem cells
F	female

Fas	Fas cell surface death receptor
FBS	fetal bovine serum
FBX	F-box-containing protein
FDA	Food and Drug Administration
FFP	fresh frozen plasma
FGF	fibroblast growth factor
FGF-2	fibroblast growth factor 2, also known as bFGF (basic fibroblast growth factor)
FITC	fluoresceinisothyocyanate
FLT-3	Fms-like tyrosine kinase 3; hematopoietic progenitor proto-oncogene
FMO	fluorescence minus one, inclusion of all but antibody of interest as a flow cytometry control, antibody of interest is sometimes replaced with isotype
FN	fibronectin
FoxO	forkhead box transcription factor O
FSC	forward scatter
GAG	glycosaminoglycan
GFP	green fluorescent protein
GH	growth hormone
h	human
H	histone
H+ATPase	proton pump
H3k4me3	trimethylation of lysine 4 on histone 3
H3k9me3	trimethylation of lysine 9 on histone 3
H3k27me3	trimethylation of lysine 27 on histone 3
H4k16	lysine 16 of histone 4
HCl	hydrochloride
HDAC	histone deacetylase
HGNC	HUGO Gene Nomenclature Committee
HGPS	Hutchinson-Gilford Progeria Syndrome

HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSCs	hematopoietic stem cells
ICAM-1	intercellular adhesion molecule 1
ICC	immunocytochemistry
IFNα	interferon alpha
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
iPSCs	induced pluripotent stem cells
IRB	institutional review board
Iso	isotype
ITS	insulin-transferrin-selenous acid
JNK	c-Jun N-terminal kinase
kb	kilobase
Ki-67	marker of proliferation
Klf4	kruppel-like factor 4
L	ligand
LAA	L-ascorbic acid
LEF	lymphoid enhancer-binding factor
M	male
mAb	monoclonal antibody
MFI	mean fluorescence intensity
miRNA	micro ribonucleic acid
mL	milliliter
mM	millimolar
MMP	matrix metalloproteinase

Mof	mammalian ortholog of Drosophila MOF (males absent on the first); histone H4 lysine 16-specific acetyltransferase
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stem cells
mTOR	mammalian target of rapamycin
N	sample number
Na+K+ATPase	sodium-potassium adenosine triphosphatase; sodium-potassium pump
NAD+	aldehyde dehydrogenase
Nanog	homeobox protein NANOG
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nM	nanomolar
O_2	molecular oxygen
Oct4	octamer-binding transcription factor 4; also Oct3/4, POU5F1
Osc	osteocalcin; also BGLAP (bone gamma-carboxyglutamic acid-containing protein)
P	passage number
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phycoerythrin
pg	picogram
PGE2	prostaglandin E2
PI3K	phosphatidylinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
pre	preconditioning oxygen tension
PTEN	phosphatase and tensin homolog tumor suppressor
PTHrP	parathyroid hormone related peptide

qRT-PCR	quantitative real time PCR
r	recombinant
R	receptor
Rag	recombination activating gene
RNA	ribonucleic acid
RBC	red blood cell
ROS	reactive oxygen species
ROX	carboxy-X-rhodamine reference dye
RT	reverse transcription
Runx2	runt-related transcription factor 2
SA-β-gal	senescence-associated beta-galactosidase
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean
sGAG	sulfated glycosaminoglycan
siRNA	small interfering ribonucleic acid
SIRT	sirtuin
SMAD	homolog of Drosophila MAD and C. elegans SMA; transcription factors that transduce TGF-β signaling
SNP	single nucleotide polymorphism
Sox	sex-determining region Y-related HMG box transcription factor
SSC	side scatter
STAT	signal transducer and activator of transcription
Stro-1	endothelial and mesenchymal stem cell marker
SUV39H1	histone-lysine N-methyltransferase suppressor of variegation 3-9 homolog 1
TCF	T cell factor
TERT	telomerase reverse transcriptase

TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TNFα	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UTX-1	ubiquitously-transcribed X chromosome tetratricopeptide repeat protein
VEGF	vascular endothelial growth factor
Wnt	wingless-related integration site signaling pathway
WRNp	Werner syndrome helicase
Zmpste24	zinc metallopeptidase STE24

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Chapter 1: Introduction

Maintaining the Adult Stem Cell to Combat Diseases of Aging

1.1 Abstract

Preservation of adult stem cells pools is critical for maintaining tissue homeostasis into old age. Exhaustion of adult stem cell pools as a result of deranged metabolic signaling and premature senescence as a response to oncogenic insults to the somatic genome both contribute to tissue degeneration with age. Both progeria, an extreme example of early-onset aging, and heritable longevity have provided avenues to study regulation of the aging program and its impact on adult stem cell compartments. In this chapter I discuss recent findings concerning the effects of aging on stem cells, contributions of stem cells to age-related pathologies, key signaling pathways at work in these processes, and lessons about cellular aging gleaned from the development and refinement of cellular reprogramming technologies. I highlight emerging therapeutic approaches to manipulation of key signaling pathways corrupting or exhausting adult stem cells, as well as other approaches targeted at maintaining robust stem cell pools to extend not only lifespan but healthspan.

1.2 Introduction

Diseases of aging constitute a huge burden for society, both in terms of economic cost and quality life-years of the population. The need for therapies to prevent and/or correct age-related molecular events leading to these diseases is growing. These include metabolic syndrome and diabetes, atherosclerosis, neurodegenerative diseases, osteoporosis, and cancer (Table 1.1).[1] Onset of these diseases is highly correlated to advancing age across organ systems. Though molecular mechanisms contributing to cancer formation have been extensively studied, the mechanisms underlying age-related disease on the whole have only begun to be elucidated.[2] Molecular changes associated with age include telomere

dysfunction, oxidative stress and mitochondrial metabolism, inflammation, cellular senescence, and altered signaling of sirtuins, insulin and IGF-1, and the mTOR pathway.[3, 4]

Phenotype	***H. sapiens***	***M. musculus***	***D. melanogaster***	**C. *elegans***
Decreased cardiac function	Yes	Yes	Yes	NA
Apoptosis, senescence (somatic cells)	Yes	Yes	Yes	Unknown
Cancer, hyperplasia	Yes	Yes	No	No
Genome instability	Yes	Yes	Yes	Yes
Macromolecular aggregates	Yes	Yes	Yes	Yes
Reduced memory and learning	Yes	Yes	Yes	NA
Decline in GH, DHEA, testosterone, IGF	Yes	Yes	Unknown	Unknown
Increase in gonadotropins, insulin	Yes	Yes	Unknown	Unknown
Decreased thyroid function	Yes	Yes	NA	NA
Decrease in innate immunity	Yes	Yes	Yes	Yes
Increase in inflammation	Yes	Yes	No	No
Skin/cuticle morphology changes	Yes	Yes	Unknown	Yes
Decreased mitochondrial function	Yes	Yes	Yes	Yes
Sarcopenia	Yes	Yes	Yes	Yes
Osteoporosis	Yes	Yes	NA	NA
Abnormal sleep/rest patterns	Yes	Yes	Yes	Unknown
Decrease in vision	Yes	Yes	Unknown	NA
Demyelination	Yes	Yes	Unknown	No
Decreased fitness	Yes	Yes	Yes	Yes
Arteriosclerosis	Yes	No	NA	NA
Changes in fat*	Yes	Yes	Unknown	Unknown

Table 1.1 Conserved aging phenotypes. Adapted with permission from Macmillan Publishers Ltd: Nature,[1] copyright 2008.

A three-pronged approach exists to combatting diseases of aging in the clinic, and further research can improve all three areas: the first arm is prevention of age-related disease through better understanding of the molecular causes of systemic aging and age-related disease; the second is pharmacologic intervention to reverse, correct, or prevent age-related disease; and the third, in lieu of therapies that prevent and correct age-related molecular changes, is to surgically repair degenerated tissues, which sometimes includes engineering cells and tissues *ex vivo* to replace or augment regeneration of those in diseased and injured solid organs, nervous system components, and musculoskeletal structures.[5-9] The use of adult stem cells for this third approach, as well as the suspected regenerative functions of adult stem cells *in vivo*, have led researchers to closely consider the effects of systemic aging on the stem cell pools of an individual. Stem or progenitor cells are generally considered highly promising candidate cells for regenerative applications not only because they possess a high proliferative capacity and the potential to differentiate into other cell types,[10-13] but also because they can be sourced autologously, eliminating any concerns about rejection or need for immunosuppressive therapy.[7, 14-18] They possess innate immunomodulatory properties, home to sites of injury or inflammation, and direct the cells around them to begin a repair process via the production of bioactive factors and signaling molecules. Stromal stem cells are currently in clinical use as gene delivery agents to enhance tissue regeneration, to destroy cancer cells, and to regenerate cartilage and bone, and hematopoietic stem cells (HSCs) have been in clinical use for many years to reconstitute the immune system in cancer and other illnesses. However, studies of adult stem cells show that they do not fully retain their proliferative and multi-lineage differentiative capabilities in aging humans or after prolonged *ex vivo* propagation. Genetically or epigenetically modifying adult stem cells either to

rejuvenate those of an elderly individual or to confer resistance to cellular aging during *ex vivo* propagation would yield a favorable cell source for regenerative medicine applications. Understanding the events that contribute to stem cell aging and developing methods to reverse those changes will also facilitate development of therapies to maintain *in vivo* adult stem cell pools as people age.

1.3 Adult Stem Cells and Diseases of Aging

Adult stem cells are thought to reside *in vivo* as self-renewing pools and facilitate repair/replacement of damaged tissues over the lifespan of the organism. Stem cell quiescence lies on one end of a spectrum of self-renewal potential spanning from quiescence, to robust proliferation, to senescence, and death. Maintaining stem cell quiescence is essential for preserving the long-term self-renewal potential of the stem cell pool in a number of organ systems, such as the brain, bone marrow, musculoskeletal system, and skin [19, 20]. There is an emerging body of evidence that altered and decreased function of adult stem cells *in vivo* secondary to accumulated metabolic stress plays an important role in the initiation of diseases of aging.[21, 22] Studies on the osteoblastic versus osteoclastic differentiation of progenitors in aging mouse models have shown that over time osteoblastic potential of stromal progenitors decreases while osteoclastic differentiation of hematopoietic progenitors increases, suggesting an organismal aging program that results in common diseases of aging, including decreased bone quality.[23] Clonal diseases of the myeloid system occur more frequently with increasing age and become more resistant to therapy,[24] and the hypothesis is now being investigated that this is caused by age-related genomic instability, causing a defective DNA damage response that results in abnormal differentiation of HSCs.[25]

Studies on the molecular causes/effects of aging in adult stem cells have shown that in aged subjects these cells display an altered proteome, with proteins involved in cytoskeletal organization and anti-oxidant defense being age-dependent and associated with functional impairment of the cell, including decreased responsiveness to physical environmental cues and decreased resistance to oxidative stress.[26] Mesenchymal stem cells (MSCs) from both bone marrow and adipose tissue have been shown to have reduced capacity for oxidative stress with increasing donor age.[27-29] Similarly, gene expression profiling has been done in adult stem cells to examine the effects of age in the setting of osteoarthritis and vice versa, revealing that different sets of genes were differentially regulated in association with either aging or osteoarthritis.[30, 31] The pathways associated with aging were closely associated with glycan metabolism, in contrast to osteoarthritis, which was heavily associated with aberrations in immune signaling genes and regulators of self-renewal and differentiation, such as Wnt-related transcripts. Another gene expression profiling study looking specifically in human skin showed sex-specific age-related changes, with females displaying increased expression of pro-inflammatory genes that was not observed in males.[32] A recent meta-analysis of genome-wide association studies performed to identify polymorphisms associated with diseases of aging revealed that genes associated with multiple diseases known to occur in elderly individuals are generally associated with pathways regulating either inflammation or cell senescence, with the most highly significant susceptibility locations mapping to regulators of senescence, leading the authors to conclude that germline genetic heterogeneity in regulators of these pathways contributes significantly to onset of age-related disease.[3]

It would appear that the primary hit to adult stem cells during aging is to their proliferative/self-renewal potential more than their ability to undergo terminal differentiation

effectively, although this is somewhat lineage-dependent. HSC populations in mice have been shown to actually increase in number and frequency with age, but with reduced ability to divide, delayed cell cycle progression, and age-related genetic changes in cell cycle regulators such as p21 and p18.[33] In humans lower numbers of neuronal progenitor cells have been found in aged brains compared to young brains, but this population is still responsive and proliferates in response to ischemic injury.[34] Circulating hematopoietic progenitors were shown to increase more dramatically in younger patients after cardiopulmonary bypass graft than in older patients, and advanced age was associated with impaired coronary microvascular response to VEGF.[35] Conversely, advanced age has been associated with a higher S-phase fraction of circulating HSCs in patients with aplastic anemia, but this predisposed them to dysplasia and conversion to acute myeloid leukemia, indicative of abnormal HSC function.[36] Studies of adult stem cell isolation yield in elderly individuals have shown that equivalent numbers of adipose-derived MSCs can be isolated from older individuals undergoing vascular surgical procedures as from young, healthy individuals,[37, 38] but the question remains whether those cells can be adequately expanded in tissue culture and whether they are able to mobilize, proliferate, and effect tissue repair *in vivo* when they are needed. In fact these same studies have shown that while advanced age does not impact availability of stem cells, fat from patients with diabetes yielded significantly fewer stem cells than fat from non-diabetic patients, suggesting that stem cells in the context of aging should probably be considered distinctly from stem cells in the context of *diseases* of aging. Advanced glycation endproducts, which accumulate in the setting of advanced age or diabetes, have been shown to directly impair HSC function and induce MSC apoptosis.[39, 40] Other studies have shown that patients of increasing age yield adipose-derived MSCs that

can be differentiated, but frequently these studies do not compare the quality of differentiation to that achieved with cells from younger patients, leaving the question of whether differentiation potential has declined unanswered. [41] In one study looking at the efficacy of MSC transplantation following myocardial infarction, cells from aged donors did not perform as well as cells from younger donors.[42] Similarly, MSCs obtained from young individuals have been induced to undergo neuroectodermal differentiation *in vitro*, but this effect cannot be replicated in MSCs from elderly individuals.[43] A study demonstrating that lineage fate of MSCs from human donors was unaffected by donor age also found that activation from quiescence, including replicative function and quality of differentiation, was negatively impacted by advanced donor age.[44]

A further complication in teasing apart the effects of aging on adult stem cell compartments is the changing interaction between stem cell types and with an aging immune system.[45] For example, the health and age of marrow-derived stromal stem cells has been shown to have an impact on the quality of HSCs, both *in vivo* and upon co-culture *ex vivo.*[46, 47] Chronic pro-inflammatory cues, such as circulating inflammatory cytokines, which are upregulated in aged individuals,[48] may both dysregulate the differentiation of stromal cells, and in turn negatively impact their ability to support hematopoietic progenitors, resulting in further dysregulation of the immune compartment. Mouse models of premature aging have demonstrated induction of NF-κB signaling and secretion of high levels of pro-inflammatory cytokines as a causative factor in the accelerated aging phenotype.[49]

Some of the most pertinent research to understanding the molecular mechanisms underlying aging, rather than the molecular effects resulting from aging, is in the area of heritable longevity and premature aging in humans. Many genetic variants, the value of

which is unknown, have been identified in areas associated with longevity and disease resistance, including dietary restriction, metabolism, autophagy, stem cell activation, tumor suppression, DNA methylation, progeroid diseases, stress response, and neural processes.[50, 51] One of these variants, a single nucleotide polymorphism (SNP) in the gene *TOMM40*, was found not to be directly linked to decreased longevity, but instead reflects a linkage disequilibrium with multiple isoforms of the *APOE* gene that are deleterious to longevity and have been strongly associated with elevated cholesterol, cardiovascular disease, Alzheimer's disease, and cognitive decline and dementia, as well as serum IGF-1 levels in women.[52] Several genetic variants in the insulin/IGF-1 pathway have been associated with longevity or increased healthspan and include multiple SNPs from nine different genes along this signaling axis: *AKT1*, *FOXO1A*, *FOXO3A*, *GHR*, *GHRHR*, *IGF1R*, *IGFBP3*, *IGFBP4*, and *PTEN*. Indeed, common SNPs in *AKT1* and *FOXO3A* have consistently been associated with longevity in three independent cohorts,[53] as well as a SNP in the *CAMKIV* gene, which *in vitro* has been shown to activate *AKT*, *SIRT1*, and *FOXO3A*.[51] A SNP in the *MNPP1* gene, which codes for an enzyme similar to PTEN that regulates intracellular levels of polyphosphates, critical for determining the rate of cell proliferation, has also been associated with longevity in meta-analyses of large-scale genome-wide association studies.

Progeria, or premature aging, reflects an opposite outcome from long lifespan or long healthspan. Individuals with Hutchinson-Gilford progeria syndrome (HGPS), caused by a point mutation in *LMNA*, the gene for the lamin A nuclear envelope protein, experience premature aging as a result of nuclear defects that lead to impaired cell division and transcriptional deregulation.[54] This point mutation activates a cryptic splice donor site, leading to production of a dominant negative form of the lamin A protein which has been

named progerin; this splice variant is also expressed at low levels in normal individuals, accumulates in some cell types with normal aging, and is expressed at higher levels in several human cancer cell lines.[55] Similarly, individuals with Werner's syndrome, who display adult-onset progeria, have a defect in the WRNp protein, which is critical for DNA replication and repair. In both aging syndromes, telomere shortening and DNA damage synergistically destabilize the genome, leading to accelerated p53-dependent senescence and apoptosis; this phenotype has been rescued in experimental models by over-expression of hTERT or p53 inactivation,[56-58] and this process has also been documented in normal human fibroblasts.[59] Increased rates of nuclear DNA damage in all cell types, in combination with impaired stem cell regeneration of damaged tissues, are thought to be directly responsible for the accelerated aging phenotype that is observed. As a result this disease has inspired the generation of several aging models, both transgenic animal models and *in vitro* systems employing induced pluripotent stem (iPS) cells derived from fibroblasts of HGPS patients.[60-67] From these models, it has been learned that high rates of cellular senescence and apoptosis due to increased nuclear DNA damage correlate very well with decreased lifespan, independent of increased rates of cancer, whereas models with comparatively low rates of cellular senescence and apoptosis display increased lifespan.[58, 68] Comparison of tissue phenotypes observed in normal aging and HGPS suggests that lamin A may play an important role in sensing and transducing stress response signals critical for adult stem cell and niche maintenance in all individuals.[69] Progerin has been demonstrated to accumulate in MSCs, vascular smooth muscle cells, and fibroblasts, both in *in vitro* disease models and in human subjects, in association with disease and other signs of aging in the skin, musculoskeletal, and cardiovascular systems.[62, 70, 71] MSCs have been shown not only to be most susceptible to

progerin accumulation and failed cell division, but also more susceptible to oxidative and other kinds of stress in the context of progerin acculumation both *in vitro* and *in vivo*. In the absence of normal lamin A or abundance of progerin, mild oxidative stress is sufficient to induce nuclear disorganization and premature senescence, confirming the importance of this protein for maintaining tolerance to reactive oxygen species.[72] These factors combine to effectively wipe out this adult stem cell pool in HPGS patients, leaving them with a critical deficit in tissue regeneration,[62, 73] and it is likely this same process plays a role in progressively declining MSC function with normal aging.

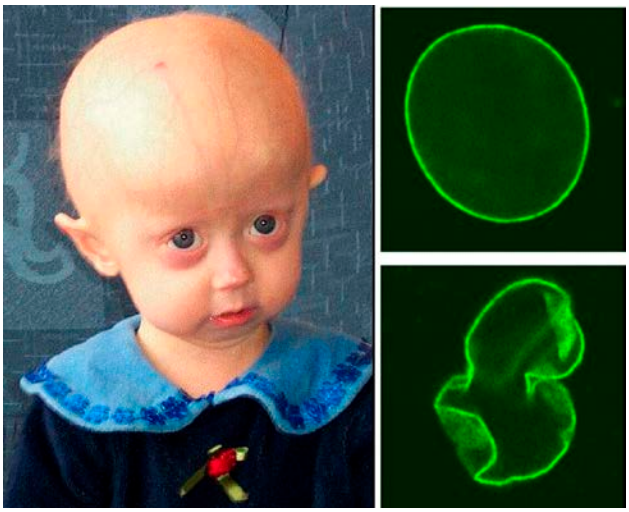

Figure 1.1 Hutchinson-Gilford Progeria Syndrome. HGPS is a childhood disorder caused by mutations in one of the major architectural proteins of the cell nucleus. In HGPS patients the cell nucleus has dramatically aberrant morphology (bottom, right) rather than the uniform shape typically found in healthy individuals (top, right). Reprinted from Scaffidi et al, The Cell Nucleus and Aging: Tantalizing Clues and Hopeful Promises, PLOS Biology, 2005.[74]

A wholly separate but potentially instructive area of research involves those studies focused on *ex vivo* aging of adult stem cells. *Ex vivo* stem cell aging has been shown to be very

similar to *in vivo* stem cell aging in rodent models, but this has not held true on a molecular level in every study done with human cells. For example, telomere shortening, which drives cellular senescence in cultured human cells, is not observed in rodent cells clearly undergoing replicative senescence.[75] Despite this finding, there does appear to be an association between *ex vivo* cell senescence and organismal lifespan, and studies of telomerase mutations in humans have revealed an association with diseases of aging in which tissue compartments require a high degree of cell self-renewal.[76] Similarly, short telomeres have been linked with some tissue-specific degenerative diseases, and telomere length is evaluated as a clinical parameter in determining therapeutic approaches.[77] Ablation of senescent cells in progeroid mice has been shown to delay or rescue the aging phenotype at the organismal level, implicating senescent cells in the pathogenesis of age-related disease *in vivo.*[78] Taken together, these findings suggest that the study of *ex vivo* senescence could yield information pertinent to *in vivo* aging. Examination of adult stem cell proliferation in *ex vivo* tissue culture have shown that MSC proliferation declines precipitously after repeated passaging.[79] Studies of differential gene expression between early and late passage MSCs showed progressive down-regulation of genes associated with self-renewal, such as *OCT4*, *SOX2*, and *TERT* and up-regulation of genes associated with osteogenic potential; this was accompanied by an increased propensity for spontaneous osteogenic differentiation and decreased proliferation over time.[80] The authors of this work noted a concomitant increase of epigenetic dysregulation of histone H3 acetylation in association with these differentially regulated genes, and correction of this dysregulation with bFGF administration during culture, resulting in promotion of proliferation and suppression of spontaneous osteogenesis.

1.4 Role of Metabolic Stress

Adult stem cells experience many stressful insults in the course of a lifetime of tissue repair. Regulation of energy metabolism is critical to withstanding stress, which comes in the form of nutrient deprivation, oxidative stress, DNA damage, pathogens, and other stressors. Blood gas levels in the marrow niche of patients sustaining a myocardical infarction have been correlated with maintenance of the self-renewal capacity and therapeutic efficacy of autologous bone marrow-derived MSCs after percutaneous coronary intervention, indicating that the function of these cells is highly dependent on their redox status.[81] Oxidative stress is increasingly being recognized as a fundamental underlying component of the aging process, leading to hyperactivity of pro-growth pathways like insulin/IGF-1 and mTOR, subsequent accumulation of toxic aggregates and cellular debris, and ultimately activation of cell death/survival pathways leading to apoptosis, necrosis, or autophagy.[82] Insulin/IGF-1, mTOR, FoxO, AMPK, and the sirtuin pathways all play a role in stem cell maintenance and differentiation through their sensing and regulation of energy availability in times of stress,[83-85] and these same pathways have been associated with advancing age in humans (Figure 1.2).[86] Studies of two independent cohorts testing the expression of mTOR-related transcripts in aging found robust associations for genes involved in insulin signaling (*PTEN*, *PI3K*, *PDK1*), ribosomal biogenesis (*S6K*), lipid metabolism (*SREBF1*), cellular apoptosis (*SGK1*), angiogenesis (*VEGFB*), insulin production and sensitivity (*FOXO*), cellular stress response (*HIF1A*) and cytoskeletal remodeling (*PKC*), all of which were negatively correlated with advancing age, and for genes involved in inhibition of ribosomal components (*4EBP1*) and inflammatory mediators (*STAT3*), which were positively correlated with advancing age.[87]

In response to sublethal insults, cells must remove or repair damaged components in order to reestablish homeostasis. Autophagy is one of the processes by which cells accomplish stress-induced metabolic adaption, and it has been identified as a critical mechanism for maintenance of stem cell function with aging.[88] Basal levels of autophagy are higher in adult stem cells from many tissue types compared to terminally differentiated cells, and autophagy is down-regulated during differentiation of adult stem cells. mTORC1, AMPK, and the sirtuins have all been shown to induce autophagy in response to nutrient stress, suggesting one possible connection between starvation and resistance to aging.[89]

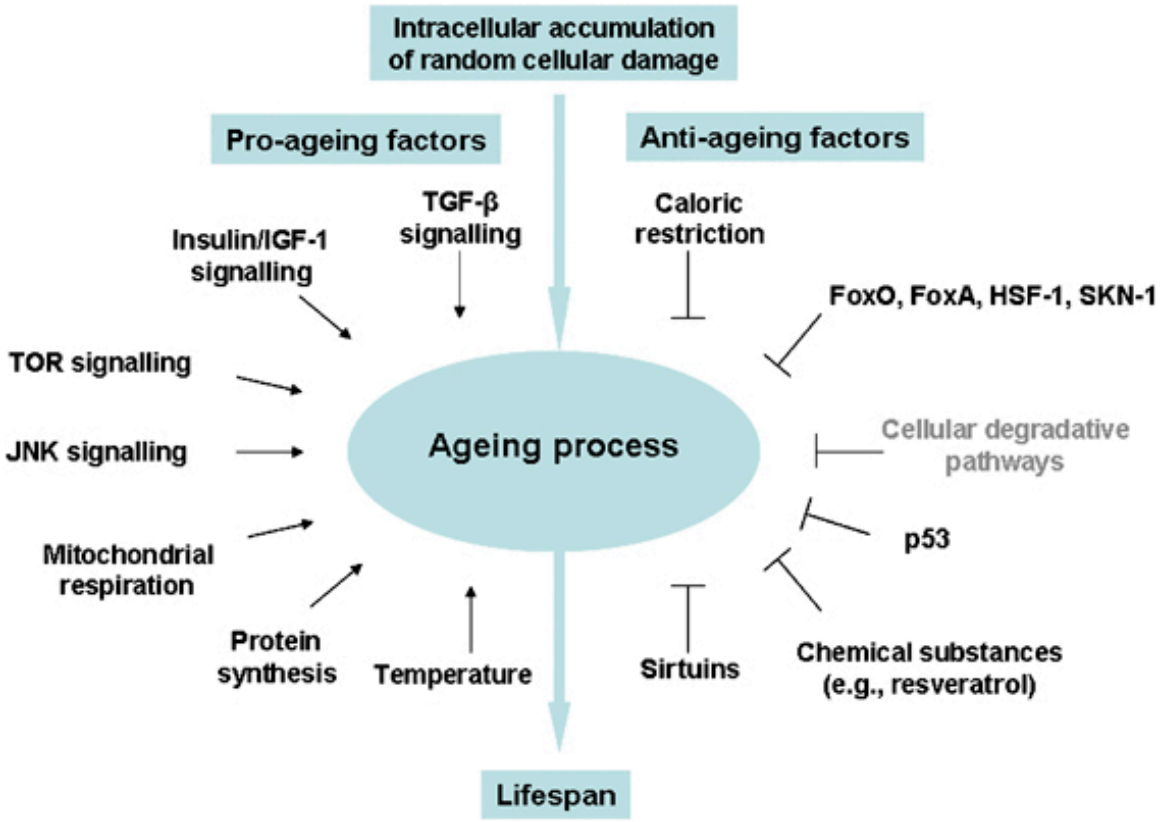

Figure 1.2 Environmental cues and evolutionarily conserved pathways that regulate the aging process in diverse eukaryotic phyla. Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation,[86] copyright 2008.

The sirtuins are a family of NAD^+-dependent deacetylases which are critical for maintaining cellular homeostasis in the face of age-related metabolic and other stressors, helping to prevent diseases of aging, but they are not thought to be involved in regulation of organismal lifespan.[90] Sirt1 coordinates stress responses and cell metabolism and regulates replicative senescence, is found in much higher levels in stem cells than differentiated cells, and is down-regulated upon differentiation of stem cells.[91] HSCs are dependent upon Sirt1 for maintenance of their undifferentiated state through elimination of reactive oxygen species, FOXO activation, and p53 inhibition.[92] In the case of embryonic development or tissue revascularization following ischemic injury, Sirt1 promotes endothelial progenitor branching and proliferation, although it is not required for endothelial cell differentiation.[84] These effects of Sirt1 are a result of its negative regulation of downstream effectors such as FOXO and Notch proteins. Resveratrol, a known Sirt1 agonist, has been shown to enhance osteogenic differentiation over adipogenic differentiation of MSCs, thereby conferring bone-protective effects and highlighting the importance of Sirt1 and its downstream target FOXO3 in preventing age-related osteoporosis.[93] Sirt1 confers sensitivity to insulin when over-expressed, and has been shown to be significantly down-regulated in cells resistant to insulin.[94] Given the critical function of this enzyme for maintaining robust adult stem cell pools and regulating their differentiation in multiple organ systems, its down-regulation in the context of insulin resistance provides one clue as to why metabolic disease is so damaging for regenerative processes in aging individuals.

1.4.1 Adult Stem Cells: Caught in the Balance Between Cancer and Metabolic Disease

Regulation of organismal longevity is coordinated through many intersecting signaling pathways that maintain a tight balance between carcinogenesis and apoptosis in individual cells.[95, 96] For a stem cell, which over the course of its existence travels on the self-renewal spectrum from unlimited proliferative potential to senescence and ultimately death, many of these pathways are at work in opposition to each other all the time (Figure 1.3).[97] A number of the well characterized pathways that control cell proliferation in cancer are now being examined for their role in regulating stem cell renewal and aging. One group took advantage of this similarity in regulatory networks between cancer cells and stem cells to study the effect of anti-aging reagents on induction and maintenance of self- renewal behavior and underlying mechanisms in stable cancer cell lines. They found that *BMI1*, a well known proto-oncogene and critical regulator of self-renewal in multiple adult stem cells populations, took over the epigenetic program in cells retrogressing to a more primitive state as a result of the anti-aging treatments.[98] This proto-oncogene has also been identified as a critical promoter of osteogenesis through its coordinated stimulation of *SIRT1* expression and inhibition of p16, p19, and p27 in response to pararthyroid hormone related peptide (PTHrP) signaling,[99] resulting in enhanced proliferation, decreased apoptosis, and decreased adipogenic differentiation of MSCs.[100]

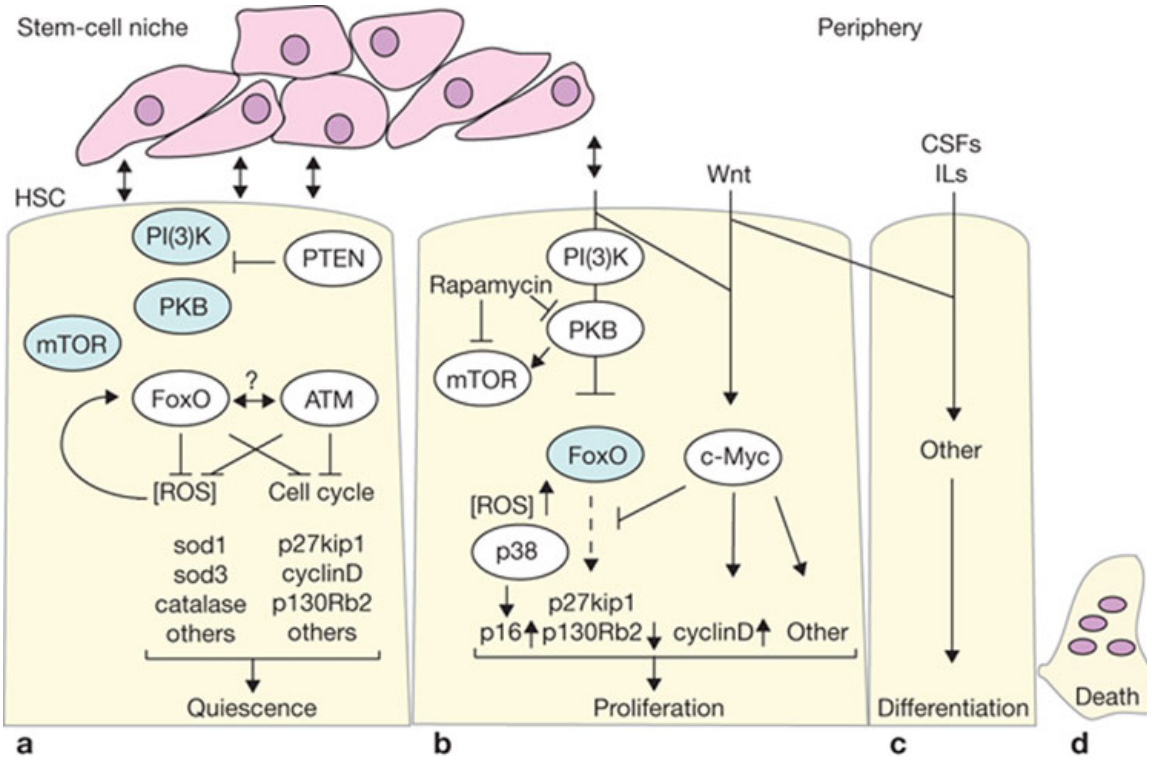

Figure 1.3 Regulation of HSC potential. (**a**) PTEN is active in quiescent HSCs and represses signaling towards downstream components such as mTOR and FoxO. Active FoxO programs cells to remain quiescent by cell-cycle repression and induction of quiescence, but also allows survival by switching to a metabolic program of gluconeogenesis and fatty acid metabolism, together with elimination of ROS. (**b**) HSCs are driven to proliferate after loss of PTEN or FoxO. Loss of FoxO results in increased intracellular ROS levels, which in turn activates p38. If PI(3)K signalling is required to drive HSCs into proliferation under normal conditions, what external niche signals would do so are unknown. However, PI(3)K signalling may function in cooperation with other signalling pathways, and this is illustrated by c-Myc — a downstream target of Wnt signalling. Myc represses FoxO, and may also independently regulate control of proliferation. (**c**) Differentiation of lineage-restricted cells further continues and is guided by extracellular signals such as interleukins (ILs) and various colony stimulating factor (CSFs). (**d**) After executing their function, all hematopoietic cells die and regeneration begins. Adapted with permission from Macmillan Publishers Ltd: Nature Cell Biology,[97] copyright 2007.

Network analyses of signaling pathways differentially regulated in aging suggest that, rather than being a tightly regulated, well-defined program, aging may reflect a destabilization of other programs over time, although extreme differences in lifespan between evolutionarily closely related species would argue that there is a dominant central aging program that determines organismal lifespan, as would research indicating that survival to old age is not correlated with absence of risk alleles for common age-related diseases, such as cancer, coronary artery disease, and type 2 diabetes.[51] Ultimately aging at the cellular level is suspected to result from a disruption of the balance between alternative cellular states,[101] with proto-oncogenes that promote stem cell function, such as *BMI1* and Wnt/β-catenin, operating in opposition to tumor suppressor genes that induce death or senescence in stem cells, such as *INK4A* ($p16^{Ink4a}$) and *ARF* ($p19^{Arf}$).[102] In the hematopoietic system aging has been closely linked with impaired repair response to DNA damage, leading to increased propensity for dysplastic syndromes and ultimately cancer.[25] The occurrence of cellular senescence, in contrast to quiescence or proliferation, is thought to be a protective response against oncogenic insults. Expression of *INK4A* has been shown to increase with age, and this progressive increase in tumor suppressor activity independent of levels of proto-oncogene expression may account for reduced stem cell activity with aging.[102] The tumor suppressor AIMP3/p18, endogenous levels of which increase in aged human tissues, drives cells to senescence when overexpressed and in transgenic mice promotes a progeroid phenotype through selective degradation of normal lamin A.[103]

A frequently raised concern about the therapeutic use of autologous adult stem cells from aged individuals is precisely that with the life-long accumulation of potentially mutagenic insults to their DNA, if they are still capable of robust self-renewal, they might pose an

increased risk for cancer upon mobilization or exogenous activation.[104] That said, several studies have indicated that over the course of human development, as the need for growth decreases and the risk of oncogenesis increases, requirements for critical tumor suppressor mechanisms change, with adult stem cells displaying dependence on self-renewal regulatory signaling pathways that are not necessary in embryonic or even fetal stem cells.[105]

Just like the constant struggle between carcinogenesis and apoptosis at the cellular level, growth versus somatic preservation is also balanced at the organismal level throughout life. Insulin/IGF-1 signaling, turned on at the systemic level in response to glucose or growth hormone, is a potent stimulator of cell growth and proliferation via the AKT-TOR pathway, regulating organismal growth in childhood and anabolic metabolism in adulthood. As discussed above, gene variants along this pathway have been associated with longevity (age ≥92 years) in clinical cohorts.[53] Interestingly, reduced IGF-1 levels are present not only in extremely long-lived individuals, but also in progeroid individuals, illustrating that suppression of this axis is not a causative factor in increasing or decreasing lifespan and healthspan, but rather an adaptive response against accumulating DNA damage at the expense of growth and other metabolically expensive processes.[106-108] Adult stem cells are highly responsive to insulin/IGF-1 signaling and programmed to replicate and repair, both metabolically costly activities. As somatic DNA is exposed to a lifetime of potentially mutagenic hits, these metabolically active cells are increasingly caught in the balance between cancer and metabolic disease, the hallmark of which is insulin resistance.

Pharmacologic agents targeting the insulin/IGF-1 axis – both neutralizing monoclonal antibodies against IGF-1/IGF-1R and tyrosine kinase inhibitors which target the insulin receptor and IGF-1R – have been developed to treat cancer, but in clinical trials to evaluate

these agents, a common side effect was hyperglycemia due to inhibition of insulin signaling.[109] Similarly, metformin, an agent already in use for the treatment of type 2 diabetes, has been shown to reduce the incidence of cancer, in part through AMPK-dependent inhibition of mTOR (thus cell growth) and AMPK-independent cell cycle arrest,[110] but also in part through decreased levels of insulin and insulin resistance.[111-113]

Manipulating this pathway to combat disease in aging humans is fraught with complications, and creative, highly specific approaches are required to avoid trading one disease for another. This is especially important to consider in the case of stem cells; chemotherapy-resistant cancer stem cells, characterized by a high degree of metabolic flexibility, have been shown to be very sensitive to metformin.[114, 115] One of the proposed mechanisms of action of metformin on cancer stem cells is interference with TGFβ-induced epithelial-to-mesenchymal transition,[116] and metformin has been shown to prevent transcriptional activation of *OCT4* through AMPK activation.[117] These properties of metformin should generate concern about the effects on adult stem cell populations during its use for treatment of diabetes and cancer, but studies have also shown that while metformin-induced AMPK activation interferes with mechanisms critical for cancer stem-cell related tumorigenesis, adult stem cells may be less susceptible to disruption by metformin.[118]

Accumulating evidence suggests that pathways governing self-renewal have distinct effects on normal stem cells and cancer stem cells even within the same tissue. In their study demonstrating adult stem cell dependence on PTEN in the hematopoietic compartment, in contrast to cancer stem cells, Yilmaz et al discussed several mechanisms through which maintenance of normal adult stem cells may be different from that of cancer stem cells. They suggested that persistent activation of PI3K in the absence of PTEN inhibition may lead to

the accelerated exit of normal HSCs from the progenitor pool,[119] and later showed that PTEN deficiency induces senescence and apoptosis in normal HSCs via increased expression of the cell cycle-regulating tumor suppressors p16 and p53,[120] in contrast to other cells from the hematopoietic compartment. Effects of PTEN deficiency on the HSC pool could be rescued by rapamycin, indicating events downstream of mTOR are responsible, for example changes in AKT signaling. It is known that mTOR inhibition activates FOXO signaling, resulting in increased stress resistance and longevity in invertebrate models.[121] It was hypothesized that prolonged rapamycin treatment might actually be inhibiting AKT signaling through mTORC2 rather than activating AKT through mTORC1, leading to loss of FOXO function, an attractive explanation for the accelerated stem cell aging observed with PTEN deficiency.[122] However, thus far this has not been shown to be the case in PTEN-deficient HSCs.[105, 120] Cancer stem cells are able to escape this process through secondary mutations that attenuate mTOR-dependent tumor suppressive mechanisms.

Similar results have been obtained in HSCs with deletion of the cell cycle regulator p21, showing that control of cell cycle entry under conditions of stress is crucial for maintenance of stem cell quiescence and prevention of premature deletion of an entire adult stem cell pool.[123] Indeed, even in the case of pluripotent cells, metformin appears to have split effects: when administered to mice after iPS cell transplantation, metformin prevented teratoma formation but did not interfere with tissue formation from all three germ layers.[124] The findings are controversial regarding the effect of metformin on the differentiation of adult stem cells. In one study on rat marrow-derived MSCs, metformin enhanced osteogenesis at the expense of adipogenesis, presumably through modulation of PPARγ activity,[125] opposite of the effect observed with glitazones, which activate PPARγ and can lead to bone loss.[126] However in

another study of human and rabbit MSCs, metformin did not induce osteogenesis, while 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), a small molecule activator of AMPK, induced robust osteogenic differentiation even in the absence of induction medium.[127] In studies of primary osteoblasts, activation of AMPK signaling was observed during early differentiation events, but chemical induction of AMPK with metformin blocked terminal differentiation and matrix mineralization.[128]

Even in the absence of pharmacologic intervention targeting these pathways, metabolic disease is frequently the trade-off associated with oncogenic resistance. Perturbation of the aforementioned lifespan determinant pathways, such as SIRT1, insulin/IGF-1, FOXO, and mTOR, leads to the development of metabolic syndrome features in mice.[129] Metabolic syndrome – characterized by the triumvirate of high cholesterol, high blood pressure, and high fasting blood glucose – and type 2 diabetes in turn lead to accelerated aging. In the case of full-blown type 2 diabetes, this accelerated aging is evidenced at the cellular level by slower DNA unwinding, increased collagen cross-linking, capillary basement membrane thickening, increased oxidative damage, and decreased Na^+K^+-ATPase activity, and at the organismal level by an increased incidence of cataracts, vascular disease and associated events (myocardial infarction, stroke, and pressure ulcers), cognitive decline, hip fracture, pain, incontinence, infections, and depression.[130]

A vicious cycle then evolves whereupon metabolic disease can in turn increase oxidative stress and associated dysregulation of adult stem cell function. An expanded adipose compartment produces higher levels of free radicals, leading to oxidative stress, one of the effects of which is to disrupt adipocytokine production. Adiponectin, an adipocytokine down-regulated in obesity and metabolic syndrome, is an important regulator of glucose and fatty

acid metabolism, and in combination with other adipose-derived hormones, such as leptin, prevents insulin resistance.[131] One of the critical functions of adiponectin is to oppose the actions of angiotensin II, local (adipose) over-production of which also contributes to a pro-inflammatory state and increases oxidative stress *in vivo*.[132] The resultant disruption to homeostasis has many downstream effects that further increase inflammation and co-opt adult stem cells in worsening the situation. First, trafficking of multiple types of adult stem cells is likely altered in response to the inflammatory adipocytokines (IFNα, TNFα, IL-6) up-regulated during this process, leading some to speculate about adult stem cell exhaustion and the resulting impairment of tissue repair as the primary mechanism underlying long-term effects of metabolic disease, and in a less fulminant way the aging process in general.[22] Additionally, fate of adult stem cells is differentially regulated in this environment. For example, enhanced production in metabolic syndrome of 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) by the cytochrome P450 system and its cyclooxygenase-2-derived product 20-OH-PGE_2 act to bias MSCs toward adipogenic differentiation through up-regulation of PPARγ and β-catenin, resulting in compounded inflammation-driven adipogenesis and impaired peripheral tissue maintenance through loss of otherwise uncommitted progenitors in patients with these disorders.[133-135] Activation of PPARγ has been shown to impair IGF-1 signaling in the marrow microenvironment,[135] further contributing to skeletal loss, disruption of metabolic homeostasis, and potentially altering organismal lifespan.[136] In this way adult stem cells contribute to the pathogenesis of metabolic disease and also are impaired in their physiologic function by the presence of metabolic disease.

Despite the occurrence of insulin resistance and other attempts by cells to thwart oncogenic transformation in an aging metabolic system, metabolic disease is frequently associated with a higher incidence of cancers,[137] particularly in sites with a high degree of cell metabolism and/or turnover. In part this reflects not a causal relationship but twin manifestations of a stressed system struggling and failing to restore homeostasis. However, the peripheral insulin resistance of metabolic disease also drives cancer growth through a decrease in hormone binding globulins (thus higher free steroid hormone levels), dysregulation of inflammatory cytokine and steroid and peptide hormone levels, and most importantly compensatory hyperinsulinemia, with many cancer cell types expressing high levels of insulin receptors and IGF receptors.[109, 138] Large chromosomal clonal mosaic events, the incidence of which has been shown to increase with age,[139] have been associated both with type 2 diabetes and with an increased risk of blood and solid organ cancers.[140, 141] Clonal mosaicism in the blood compartment in particular further contributes to cancer formation, as well as increased susceptibility to disease in general, by leading to a reduced number of immune cell clones in circulation and resultant immunosenescence with age.[139]

1.4.2 Canonical Wnt Signaling

As the MSC pool is skewed away from an osteogenic fate toward an adipogenic one in metabolic disease, there is massive disruption of signaling pathways that have implications for adult stem cells beyond differentiation. One of these pathways is the Wnt pathway, which is not only critical during development for axial patterning, but is also critical in stem cell fate determination. Many developmental events in stem cells are regulated by Wnt signaling,[142-145] including self-renewal,[146-149] differentiation,[150-152] aging,[153, 154] and senescence.[155-157]

Extensive crosstalk has been documented between Wnt signaling and FGF,[158-160] PGE2,[161, 162] BMP,[144, 160, 163, 164] Notch,[144, 160] TGF-β,[165] and SMAD[166-168] signaling pathways, with the common downstream target of this crosstalk being β-catenin.[169, 170] In canonical Wnt signaling, β-catenin interacts with members of the TCF/LEF transcription factor family to enhance expression of their target genes,[154] which in turn regulate cell proliferation, carcinogenesis, differentiation, embryonic patterning, and stem cell maintenance.[171-173] The effects of Wnt signaling, however, have been shown to be highly tissue-specific and Wnt-specific. In the case of hair follicle stem cells in the skin, signaling through Wnt1 activated stem cell hyperproliferation via an mTOR-dependent mechanism, but long-term this activation of mTOR led to stem cell exhaustion and senescence.[155] This finding led the authors to conclude that while Wnt signaling can be a potent stimulus for stem cell proliferation, prolonged mTOR activation may serve as a protective mechanism to prevent tumor formation. The cost of this, of course, is exhaustion and depletion of that stem cell pool, ultimately resulting in impaired regeneration and aging of the tissue. In patients with acute myeloid leukemia, aberrant Wnt/β-catenin signaling, which controls self-renewal in the HSC pool, was higher in patients with unfavorable karyotypes and predicted a shortened survival.[174]

In HGPS the tissue-specific patterns of accelerated aging point to a defect in MSC function. The defect in this adult stem cell compartment has been shown to be the result of both impaired self renewal and dysregulated differentiation resulting from aberrant Notch and Wnt signaling.[175] Disruption of the nuclear lamina by progerin was shown to be directly responsible for downstream activation of Notch signaling effectors, spurring uncontrolled sporadic differentiation of MSCs along all three germ layers and enhanced osteogenesis at the

expense of adipogenesis when differentiation was directed.[176] Wnt signaling was found to be severely disrupted in the progeroid *Zmpste24*$^{-/-}$ mouse model (deletion of this enzyme causes restrictive dermopathy in humans), where the absence of normal lamin A resulted in an absence of active nuclear β-catenin in follicular stem cells, leading to down-regulation of cyclin D1 and repression of AKT and mTOR activation.[177] Further work has shown that *LEF1* is down-regulated as a result of this impaired nuclear translocation/retention of β-catenin; the absence of this transcription factor-activator complex in adult cells markedly reduced activation of canonical Wnt targets.[178] In this setting of severe Wnt inhibition, stem cells were not reduced in number, but instead entered a senescent state earlier and failed to proliferate, resulting in exhaustion of the functional stem cell compartment. The authors of this study also discovered increased apoptosis of the support cells in the stem cell microenvironment, which they suggested is another negative impact of defects in critical fate-determining signaling pathways that enable communication between tissue-resident stem cells and their niches.

Another mouse model of accelerated aging also points to aberrant Wnt signaling as a causative factor in degeneration due to stem cell defects, but from a different perspective. The Klotho mouse, which lacks klotho, a transmembrane and secreted β-glucuronidase involved in regulating insulin sensitivity among other functions, displays an accelerated aging phenotype, including short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema.[179] Analysis of tissue-resident stem cells from multiple organs in Klotho mice revealed that they were reduced in number and displayed abundant senescence-associated markers prematurely.[180] Klotho was found to be a secreted Wnt antagonist capable of binding Wnts1, 3, 4, and 5a, and over-activation of Wnt signaling in Klotho mice drove

tissue-resident stem cells into an early senescent phenotype, resulting in lack of self-renewal and stem cell compartment exhaustion. Taken together with findings regarding aberrant Wnt signaling in the *Zmpste24*$^{-/-}$ mouse, these studies reveal the exquisite sensitivity of adult stem cell pools to the fate-determining effects of Wnt signaling, too much or too little of which results in failed maintenance of quiescent progenitors in adulthood. Perturbation of this pathway one way or the other has been demonstrated to result in adult stem cell aging and exhaustion, along with impaired differentiation, in the muscle compartment,[181] the hematopoietic compartment,[182, 183] the vasculature,[178] and the skeletal system[178, 184] in addition to the skin, the gut, and the kidney. It is suspected that these processes unfold in normal aging as well as accelerated aging phenotypes, especially given the accumulation of low levels of progerin over time in normal individuals and declining serum levels of klotho in human aging.[185]

Another mechanism leading to altered Wnt signaling in aging is a shift in β-catenin binding to favor FOXO transcription factor signaling over the canonical Wnt pathway, mediated by TCF/LEF transcription factor signaling, in response to increasing oxidative stress.

1.4.3 The FOXO Family

The FOXOs are a family of transcription factors regulating several of the previously discussed intersecting pathways, and have been shown to coordinate cell response in tumor suppression, metabolism, and organismal longevity.[2, 186, 187] In this capacity FOXO signaling acts to resist cellular stress opposite the growth-promoting signaling of mTOR.[188] FOXO transcription factors are downstream targets of insulin, growth factors, and nutrient and

oxidative stress stimuli and in turn regulate several fundamental processes, depending on the cell type, including gluconeogenesis, neuropeptide secretion, cell cycle arrest, atrophy, autophagy, apoptosis, and stress resistance[186, 189, 190] (Figure 1.4).[190] Furthermore, the FOXOs are an interesting family of transcription factors in that several are ubiquitously expressed, but display both highly specialized and universal functions in distinct cell types, as well as distinct and redundant functions which can be attributed to different FOXOs within the same cell type.[2]

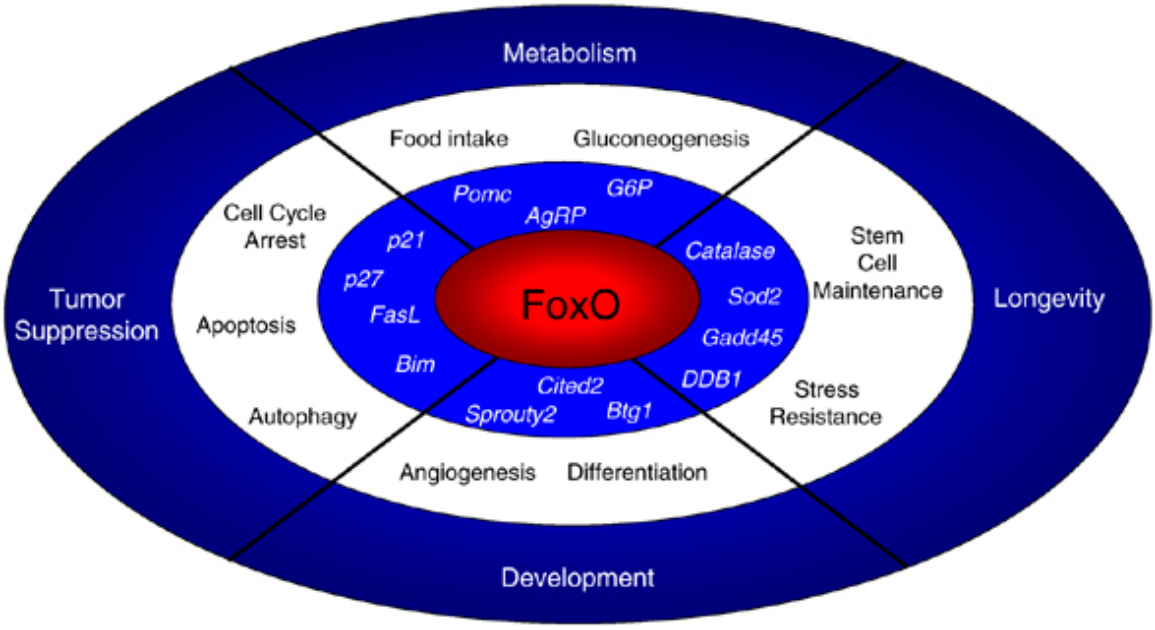

Figure 1.4 Roles of FoxO transcription factors in cells and in the organism. FoxO transcription factors trigger a variety of cellular processes by upregulating a series of target genes (in italics). The cellular responses elicited by FoxO affect a variety of organismal processes, including tumor suppression, longevity, development and metabolism. Note that some cellular processes may not be exclusive to one organismal function (e.g. cell cycle arrest). Reprinted by permission from Macmillan Publishers Ltd: Oncogene,[190] copyright 2008.

Studies on the effect of oxidative stress on adult stem cells suggest that it drives a shift in binding of the cellular β-catenin pool from the TCF/LEF family to the FOXO family.[2, 153, 191] As a cofactor in FOXO-mediated transcription, β-catenin facilitates defense against oxidative and other cellular stresses.[192-194] Diversion of β-catenin binding from TCF/LEF to FOXO, combined with inhibitory feedback to β-catenin/TCF signaling, serves to diminish canonical Wnt signaling.[153, 154, 195, 196] This redirection of β-catenin function secondary to oxidative stress and resulting change in the cell's transcriptional program have been implicated in several aging-related disease processes and cell senescence.[97, 154, 157] This transcriptional shift is particularly important in osteoporosis, where canonical Wnt signaling in osteoblasts is critical for skeletal homeostasis. This is one example of how increased activation of FOXO signaling by oxidative stress accumulated with age serves to directly undermine maintenance of an entire organ system, independent of the more broadly discussed "oxidative hypothesis of senescence," wherein reactive oxygen species drive cells into arrest by activating FOXO signaling.

FOXO1 is of particular importance in age-associated skeletal disease. Osteoporosis, for example, is characterized by decreasing bone mass, which is attributed both to declining numbers of osteoblasts and declining function of osteoblasts. FOXO1 is critical for regulating osteoblast proliferation in the face of age-related oxidative stress and declining resistance to the effects of oxidative stress, both through its regulation of protein synthesis via interaction with the osteoblast-specific transcription factor ATF4 and through suppression of stress-induced p53 signaling which would otherwise lead to cell cycle arrest.[197]

Several FOXOs regulate organismal metabolic function and thus play a role in metabolic diseases of aging and resistance to those diseases. FOXO1, through its regulation of

osteocalcin secretion by osteoblasts, impacts pancreatic β-cell proliferation, insulin secretion, and insulin sensitivity.[198] In the case of atherosclerosis and vascular diseases of aging, FOXOs are important downstream effectors of PI3K, AMPK, and JNK signaling. In the endothelium FOXO1 serves as a negative regulator of angiogenic behavior; this suppressive function, which is enhanced by activities of FOXO3 and FOXO4, is critical for organized vessel growth during development and repair.[84] FOXO1, FOXO3, and FOXO4 are also required to maintain endothelial quiescence within healthy vessels, and formation and remodeling of the endothelial barrier function is regulated by FOXO1 in a β-catenin-dependent manner.

FOXO transcription factors also play a role in diseases of aging through their regulation of the immune system and its progenitor pools.[199] In general, FOXOs regulate survival, cell cycle progression, and resistance to stress in immune progenitors and differentiated immune cells, as they do in many other cells types, consistent with a decline in overall immune function and proliferative capacity of the HSC pool with aging. FOXO transcription factors also regulate immune activity via specialized functions in different cell types. FOXO1 specifically regulates development and trafficking of B and T lymphocytes through several mechanisms, including survival and homing of Pre-B cell and naïve T cells in response to growth factor receptor regulation, pre-B cell maturation by induction of Rag genes and B cell receptor recombination, and B cell class-switch recombination and somatic hypermutation in response to germinal center formation.

FOXO3 has been extensively studied in the immune system, and regulates not only lymphocyte function, but also innate immunity. Like FOXO1, FOXO3 regulates T and B lymphocyte survival and cell cycle progression, but FOXO3 additionally controls survival

and entry of memory T cells into a quiescent state, critical for later response to infection. This particular function of FOXO3 is of great interest in the human immunodeficiency virus (HIV) field, as inhibitors of FOXO3 might be used to prolong survival of memory T cells during chronic HIV infection. FOXO3 also has similar roles in B cells, and is likely important for terminating immune responses to infection, and possibly for controlling lymphocyte responses that would result in autoimmunity. FOXO3 also has critical specific regulatory functions in innate immune cells: controlling the number of neutrophils, monocyte/macrophages, dendritic cells (DCs), and erythrocyte progenitors (in opposition to erythropoietin signaling); directing neutrophil migration; and regulating inflammatory cytokine secretion by DCs in response to coinhibitory molecules.[2, 199] Immune-specific functions of FOXO transcription factors, including the examples outlined here, are not well understood in the context of aging and increased metabolic stress, but may also contribute to the overall decline of cell-specific immunity observed in elderly individuals, including decreased maintenance of the HSC pool, increased susceptibility to infection, reactivation of latent viruses, and decreased immune surveillance with respect to cancer.

1.5 Lessons from Transcriptional Reprogramming

In addition to their place on a spectrum of proliferative capacity, stem cells also exist on a spectrum of differentiation bounded by terminally differentiated unipotent effector cells at one extreme and pluripotent embryonic stem cells (ESCs) at the other.[200] Pluripotent cells are thought to be extremely rare in adult mammals. Much work has investigated directed epigenetic manipulation of cell fate, inducing a cell to follow a completely different transcriptional program and as a result shift to an entirely different phenotype and spectrum

of activity. The recent development of nuclear reprogramming methods used to generate iPS cells has created new opportunities for regenerative medicine using stem cells, but the mechanisms underpinning cell reprogramming remain incompletely understood, and many areas where stem cell manipulation can enhance regenerative medicine have yet to be explored.[201, 202] Introduction of a cocktail of pluripotency-maintaining transcription factors, likely in combination with a series of stochastic epigenetic events, can direct terminally differentiated cells to revert to a state similar to that of an ESC,[201, 203-206] resulting in iPS cells that are pluripotent and germ-line competent, and exhibit the capacity for chimerism and teratoma formation and a gene expression profile characteristic of ESCs.[207] The exact nature of the pluripotency induction steps that take place during and subsequent to the expression of the exogenously transduced reprogramming transcription factors is an active area of research. Whether the two key "stemness" features of iPS cells, proliferative capacity and multi-lineage differentiation potency, arise from specific epigenetic events during the reprogramming process and result from the synergistic action of more than one of the reprogramming factors is unknown. Full reprogramming, i.e., the production of iPS cells, requires a minimal period of expression of these reprogramming transcription factors; this period was discovered by Yamanaka's group to be significantly longer for induction of pluripotency in human cells compared to mouse cells, taking approximately three weeks.[206] Recent studies have also focused on the screening of small molecules capable of reprogramming.[208, 209] However, iPS cells do not make ideal starting material for regenerative medicine or cell therapy.[210] Like ESCs, it is technically challenging to direct them to undergo exclusive differentiation along a specific cellular lineage, and they exhibit a shift in the self-renewal spectrum that confers a high risk of carcinogenesis, frequently

forming tumors in animals.[207, 211] It is possible that in the future we may be able to achieve partial reprogramming, resulting in the acquisition of renewed proliferative capacity and an increased differentiation lineage potential, but without other characteristics of ESCs and fully reprogrammed iPS cells, such as the capacity for chimerism and teratoma formation.

Partial reprogramming is the process of moving an adult cell on the spectrum of differentiation from limited multipotency toward pluripotency, without returning it to the completely pluripotent state of an ESC. Several groups are engaged in studying how partial reprogramming can most effectively be induced in adult stem cells, how it alters the transcriptional program and phenotype of adult stem cells, and how this approach may be used to preserve the potency, proliferative capacity, and regenerative utility of adult stem cells as they are cultured *in vitro*.[212] Observations from Yamanaka's work in mouse cells suggests that selection based on expression of FBX15 yields partially reprogrammed iPS cells.[213] The definition of partial reprogramming described in that work was that partially reprogrammed iPS cells formed teratomas but lacked the ability to generate adult chimeric mice. A lesser degree of partial reprogramming has been described in umbilical cord blood cells cultured in medium supplemented with FGF4, SCF, and FLT-3 ligand.[214] These cells exhibited increased binding of acetylated histones H3 and H4 at the *OCT4* promoter and up-regulation of OCT4 and Nanog expression, but their reprogramming was considered a partial event because they exhibited DNA hypermethylation in the *OCT4* gene region, and continued H3 and H4 acetylation at promoter regions for markers of terminal differentiation. Other studies have achieved partial reprogramming by administration of growth factors or transcription factors to redirect a non-pluripotent progenitor cell to a pluripotent phenotype.[214-216] Growth factor-induced partial reprogramming has been used to enhance plasticity in peripheral

blood monocytes and subsequently to convert them to immature β endocrine cells.[215] During the observed limited life span of increased plasticity, these cells exhibited up-regulation of pluripotency markers. Recently some groups have undertaken to "directly reprogram" or transdifferentiate cells from one terminally differentiated phenotype to another using both developmental and lineage-specific transcription factors for therapeutic application in specific organ systems.[217-225] Partial reprogramming is a potentially promising approach to confer some of the desirable properties of ESCs onto adult stem cells or terminally differentiated effector cells, but it is evident that controlling partial reprogramming and resulting changes in potency requires a more complete understanding of underlying regulatory mechanisms.

Discovery of mechanisms by which reprogramming events redefine the transcriptional program in adult cells, particularly signaling related not only to potency, but to telomere maintenance, oxidative stress, and senescence, will aid in generating techniques to increase the longevity of the adult stem cell in culture and preserve those cells *in vivo*.[226-231] Regulation of stem cell pluripotency and differentiation has been studied at the transcriptional and epigenetic level in ESCs, particularly mouse ESCs.[232-237] High-throughput sequencing methodologies are now used to characterize whole networks of regulation in ESCs[238, 239] and analyze the roles of overlapping and interactive regulatory networks in determining stem cell fate, including the role of microRNAs[240] and epigenetic marks.[241] Regulatory networks in reprogrammed cells are also now being studied using genome-wide analytical tools, and initial results from studies of iPS cells derived from aged individuals suggests that reprogramming can undo many, though not all, effects of age.[242] SIRT1, critical for maintenance of stemness in multiple types of adult stem cells, is post-transcriptionally up-

regulated during the reprogramming process.[91] Reprogramming of aged HSCs to iPS cells with subsequent re-derivation of HSCs showed comparable function to endogenous blastocyst-derived HSCs in marrow reconstitution assays.[243] Perhaps the most critical lesson regarding stem cell aging and loss of self-renewal gleaned from reprogramming research has been that cell aging as we know it is a largely reversible process, characterized not by permanent genetic mutations so much as progressive epigenetic inflexibility.

Studies of transcriptional reprogramming efficiency have proven very instructive in the area of methods for enhancing cell stemness and overcoming senescence. Several pathways controlling onset of cellular senescence must be differentially regulated to achieve reprogramming, including telomerase, p53, and mitochondrial/oxidative stress pathways.[244] Telomere length as a measure of cellular aging has revealed interesting differences between reprogrammed pluripotent cells and their embryonic counterparts. Many widely used human iPS cell lines derived from somatic cells display prematurely aged telomeres compared to hESCs with accompanying differential regulation of genes regulating telomere length; iPS cell clones derived from an hESC-derived mortal clone (for isogenic comparison) largely followed the same pattern with the exception of one clone spontaneously displaying levels of telomerase activity comparable to the parent hESC line, with maintenance of longer telomere length in culture.[245] From this finding we have learned that current reprogramming methods do not always result in iPS cells where the aging process has been fully reversed, but that further – likely stochastic – epigenetic events can enable full reversal of cell aging. Discovery of those specific events that result in maintenance of long telomeres is a relatively focused research problem that is likely solvable with the massive generation of transcriptional network data currently underway. Comparative studies of reprogramming in aged cells from

multiple organs in mice have thus far demonstrated that age is an impediment to efficient reprogramming; however, many groups have successfully generated *bona fide* iPS cells from somatic cells of aged human subjects,[242] and with ever-improving techniques have even demonstrated comparable reprogramming efficiency in fibroblasts from young versus old patients.[246]

With respect to oxidative stress and mitochondrial function, the observation has been made that iPS cells rely on a Warburg-type switch to glycolytic metabolism. During reprogramming of fibroblasts to iPS cells, repression of H^+-ATPase and up-regulation of the lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase is observed, as is the case in cells from many types of cancer, and inhibition of these lipogenic enzymes greatly decreases reprogramming efficiency.[247] Studies of mitochondria within human iPS cells have revealed that they revert to an immature state similar to those of an ESC, complete with reduced oxidative damage, contributing significantly to rejuvenation of the cell.[242, 244] Pharmacologic induction of autophagy has also been shown to enhance reprogramming efficiency, perhaps through elimination of older, damaged mitochondria.[88]

Metformin, an AMPK activator, has been shown to decrease reprogramming efficiency in multiple studies,[117] despite the fact that AMPK activation induces endogenous antioxidant expression and reduces intracellular reactive oxygen species.[248] When activated, AMPK, which functions as a master sensor and regulator of intracellular changes in energy status, prevents transcriptional activation of *OCT4* (though not other reprogramming transcription factors) and prevents somatic cells from making the energetic switch to glycolysis, thereby effectively blocking reprogramming.[117] This is highly instructive for two reasons. First, the malignant component of teratomas derived from implanted iPS cells are driven by OCT4,

and application of metformin to iPS cells (after reprogramming) has been used to suppress or block entirely the formation of iPS-derived teratomas,[124] suggesting that this well characterized FDA-approved drug might enable clinical application of iPS cells without risk of carcinoma. Second, studies on the effects of metformin and other AMPK activators such as AICAR on the reprogramming process have illuminated a critical path to achieving pluripotency: appropriation of energetic capital. It has long been known that many types of stem cells are able to survive in harsh, energetically unfavorable conditions such as hypoxia because of their ability to rely heavily on glycolysis (provided they are not calorically restricted), but the discovery that a particular metabolic phenotype is required for supporting the energetic requirements of the reprogramming process has resulted in the understanding that being able to readily shift to a glycolytic metabolic phenotype is a defining property of stem cells. The implications of these findings to the study of stem cells in aging are enormous, because manipulating the metabolic phenotype of a cell as a strategy to restore its function is an approachable problem.

Ascorbate, a potent antioxidant, has been shown to accelerate the kinetics of reprogramming and to alleviate cell senescence by reducing levels of p53.[249] Curcumin, another antioxidant, has been shown to have similar effects on reprogramming efficiency.[250] It is possible that ascorbate enhances reprogramming in part through reduction of reactive oxygen species, but more likely by increasing the rate of transcriptome changes through other mechanisms: it is a cofactor for several enzymes, including collagen prolyl hydroxylases, HIF (hypoxia-inducible factor) prolyl hydroxylases, and histone demethylases,[251] and may facilitate histone demethylation. Epigenetic modifiers such as valproic acid, on the other hand, have been shown to enhance reprogramming efficiency, either alone or in combination

with antioxidants, further supporting the idea that enabling histone demethylation confers epigenetic flexibility and enhances the ability of the cell to dramatically shift its transcriptional program.[249] Regulation of senescence and metabolic state through the mTOR hub seems to be of particular importance during reprogramming and is a pathway that can be fine-tuned to direct cell fate.[252] mTOR inhibitors, such as rapamycin and resveratrol (which is also a sirtuin activator), are known to slow cellular senescence in response to DNA damage by limiting the accumulation of p16 and p21, thereby enabling entry into a reversible quiescent state rather than an irreversible senescent state. These same compounds have been shown to increase the efficiency of reprogramming, in addition to other sirtuin activators, antioxidants, autophagy inducers, and PI3K inhibitors.[250] Interestingly, although it enhances reprogramming of normal somatic cells, resveratrol inhibits the stemness, epithelial-mesenchymal transition, and metabolic reprogramming of cancer stem cells to glycolysis through activation of p53, again highlighting the innate differences between the molecular circuitry of normal stem cells and cancer stem cells, a finding that can potentially be exploited for therapeutic purposes.[253]

1.6 Therapeutic Targets and Clinical Trials

Given the growing evidence that many diseases of aging may reflect adult stem cell exhaustion, it is not surprising there is great interest in restoring adult stem cell function to ameliorate these conditions and regenerate aged tissues.[22] Adoptive transfer of fetal MSCs into adult mice has been shown to extend median lifespan of the animals.[254] Adult stem cell mobilization and transplant are two obvious strategies that have achieved moderate success for certain types of injury and disease in humans, and many types of adult stem cells have

been utilized for this purpose.[255] MSC cellular therapy has proven to be safe for a number of vascular disorders, such as coronary artery disease, peripheral vascular disease, erectile dysfunction, and stroke, and is an attractive option for patients who are poor surgical candidates.[256-260]

Despite these successes, the problem remains that adult stem cells from elderly donors, the very people who most frequently require enhanced peripheral stem cell function for tissue repair, undergo changes in their functional capacity as a result of aging.[104] This decline in functional capacity, therefore therapeutic utility, has been combatted using some surprisingly simple interventions: conditioning with hypoxia prior to transplant, for example, has been extensively documented as effective for reducing reactive oxygen species production by adult stem cells and improving their therapeutic efficacy in many *in vivo* ischemia and other disease models.[261-263] This has proven sufficient to counteract the impaired oxidative stress resistance of MSCs from elderly donors.[27] Likewise, the use of naturally occurring antioxidant polyphenols, such as curcumin, has been documented to suppress inducible oxidative stress in human MSCs *ex vivo* and may prove to be a safe method for reducing oxidative damage to the *in vivo* MSC pool.[264] Rejuvenation of aged human MSCs has been achieved by seeding cell scaffolds with proangiogenic growth factors, resulting in improved functional capacity of the aged cells after implantation into an infarcted rat heart compared to aged cells seeded on untreated scaffolds.[265] Systemic administration of growth factors has also proven effective for restoring aged MSCs *in vivo*; in the case of senile osteoporosis, intraperitoneal injections of rhBMP2 were sufficient to reverse the osteoporotic phenotype, and this effect was mediated by an expanded MSC pool displaying increased proliferation and decreased apoptosis.[266] *Ex vivo* genetic modification has also been used to overexpress rejuvenating factors in aged bone

marrow- and adipose-derived MSCs prior to therapeutic delivery; transplantation of aged MSCs overexpressing telomerase and/or myocardin was more efficacious in stimulating arteriogenesis and blood flow in a limb ischemia model than transplantation of control aged MSCs.[267] A similar study achieved increased angiogenesis and less adverse matrix remodeling in a rat model of myocardial infarction using aged MSCs transfected with *TIMP3* or *VEGF*.[268]

1.6.1 Reprogramming

The idea has been raised that it might be possible to exploit reprogramming techniques for renewal of the *in vivo* stem cell pool to combat diseases of aging.[269] While full reprogramming of stem cells *in vivo* to restore tissues degenerated as a result of age is not likely to manifest clinically until highly efficient reprogramming can be achieved through delivery mechanisms other than lentiviral vectors, the idea of "direct reprogramming" of cell fate in specific tissues *in vivo* has been pursued using developmental regulators that redirect a cell's terminally differentiated state rather than returning the cell to a pluripotent state – what is known in the adult stem cell world as transdifferentiation, as opposed to dedifferentiation. This approach has been employed successfully to convert pancreatic exocrine cells to endocrine cells, rescuing the hyperglycemic phenotype in a mouse model of diabetes.[270]

Generation of stem cells resistant to the phenotypic changes that accompany replicative senescence, such as arrested proliferation and decreased differentiation potential, would create a more ideal cell type for use in stem cell-based tissue engineering and cell therapy. *Ex vivo* reprogramming to achieve a kind of cell "reset" may in the future yield this improved cell source. iPS cells generated from HGPS patient fibroblasts display no evidence of progerin

accumulation, nuclear envelope and epigenetic defects, or accelerated aging, suggesting this approach can in fact be used to reset an aged cell.[61] In the case of HGPS-iPS cells, differentiation results in the rapid accumulation of progerin and restoration of the accelerated aging phenotype;[271] however, this would not be an issue with physiologically aged donor cells. iPS cells derived from young and old non-progeroid human fibroblasts displayed no differences in mitotic activity after differentiation back to a fibroblast phenotype, suggesting that reprogramming is a successful approach to reset aged cells to a youthful phenotype in physiologically aged donors. In this study an excisable vector was used, further illustrating what might be a feasible approach to *ex vivo* rejuvenation of aged cells.[246]

Stem cell rejuvenation techniques are also needed in situations where it is preferable to use cells from a specific donor who happens to be of advanced age, since HSC donor age is correlated with adverse events after infusion.[272] Meeting this need is critical for transplants with autologous or HLA-matched sibling HSCs from elderly donors, which result in better outcomes in leukemia and lymphoma than HSCs from an HLA-matched unrelated younger donor.[273] Several groups have investigated the use of reprogramming transcription factors to restore differentiation potential and proliferative capacity of adult stem cells from aging donors. In one such study Nanog was over-expressed in adult marrow-derived MSCs, resulting in reversal of lost myogenic differentiation potential and enhancement of proliferation comparable to that observed in neonatal marrow-derived MSCs.[274] It remains to be seen if other approaches to dedifferentiation will restore an unblemished phenotype to cells to the same degree that reprogramming appears to.

1.6.2 Calorie Restriction and Pharmacologic Mimicry of Calorie Restriction

Calorie restriction as a therapeutic intervention to delay aging and extend lifespan has been extensively studied in animal models but, at levels that would confer significant clinical benefit, is unlikely to gain much traction due to low rates of adherence. Pharmacologic agents to reduce nutrient intake or absorption might be employed to this end.[275] The effects of diet and exercise to reduce body weight and correct metabolic disease on adult stem cell populations are unknown, although reduction in visceral fat has been shown to correct endocrine functions of adipocytes. Enhancement of PPARδ signaling has been suggested as an adjunct therapy to boost catabolism in visceral adipose tissue, perhaps in part through differentiation of adipose-resident MSCs to mitochondria-enriched small adipocytes.[275] To this end PPARδ agonists have been tested in clinical trials, but despite protective effects against obesity and diabetes, development was discontinued due to multi-organ cancer formation in animal models.[276, 277]

Other studies have investigated the use of pharmacologic agents to mimic the molecular benefits of calorie restriction for extending lifespan and healthspan (Figure 1.5).[278] A recent report described extension of lifespan and healthspan in male mice with administration of metformin beginning in middle age;[279] past work has established this same phenomenon in invertebrates.[280] At the cellular level, treated mice displayed increased AMPK activity, decreased oxidative damage, and a transcriptomic shift mimicking the effects of calorie restriction, and as a result the mice maintained sensitivity to insulin and low levels of systemic inflammation into old age. It should be noted that the dose of metformin used to achieve these effects resulted in serum drug levels an order of magnitude higher than what is typically achieved in patients when the drug is used as an antidiabetic therapy; a ten-fold higher dose

proved toxic rather than beneficial in this study. As is the case for many pathways regulating longevity, cellular aging, and oncogenic resistance, the degree to which AMPK signaling is altered is likely to require a fine balance between too much and too little. This, combined with concerns about the pleiotropic effects of metformin *in vivo*, which manifest differently with short-term versus long-term use, means that significant work is still needed before this potentially attractive therapy for systemic anti-aging can be safely employed.

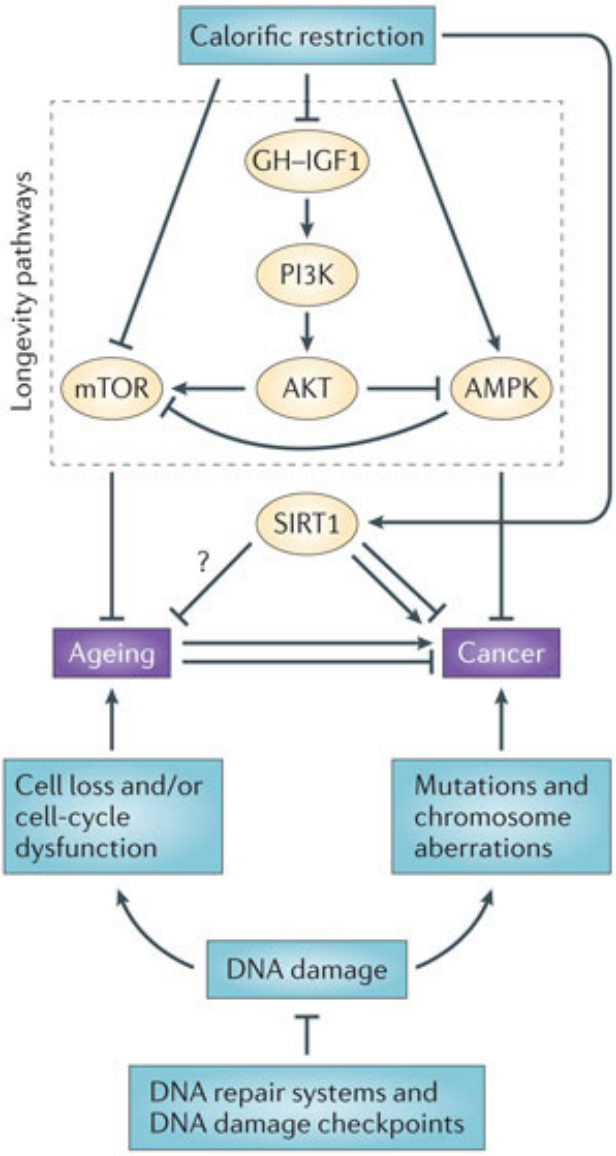

Figure 1.5 The growth hormone (GH)–insulin-like growth factor I (IGF1) pathway and its signaling cascade, which involves PI3K and AKT, can modulate longevity and cancer in model systems. Calorific restriction inhibits GH–IGF1 signalling and can also inhibit mTOR and activate AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1). Interactions between components of these pathways, and with SIRT1, remain incompletely understood. The role of SIRT1 in modulating mammalian aging has not been demonstrated, and it seems to have a dual role in cancer. DNA repair systems and DNA damage checkpoints prevent the DNA damage accumulation that contributes to cancer and aging, although possibly through different cellular mechanisms. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer,[278] copyright 2013.

1.6.3 Epigenetic Modification

The emerging field of translational epigenetics is aimed at correcting heritable but potentially reversible "epimutations" with chemical modifiers, and is gaining some traction in diseases of aging such as metabolic syndrome[281] and cancer[282] because it offers the possibility of targeting some of the same cell processes as transcriptional reprogramming without the associated risks of introducing exogenous genetic material. The treatment of epigenetically disrupted stem cells in cancer in particular may yield tremendous clinical benefit as the number of epigenetic modifiers grows, allowing for more specific targeting of known associated epimutations. In the area of metabolic disease, epigenetic modification with nucleic acids or small molecules may allow for extending the healthspan if not the lifespan of patients. The demethylase UTX-1, the activity of which increases in mid-life, removes gene inactivating marks such as histone H3 trimethylation on lysine 27 (H3K27me3) on members of the insulin/IGF-1 signaling pathway, enhancing their activity and resulting in decreased FOXO activity and age-related cellular decline. Restoration of H3K4me3 on an insulin-like receptor gene in *C. elegans* has been shown to decrease insulin/IGF-1 signaling, resetting the cell to a more naïve epigenetic state and ultimately extending the life of the animal by 30%.[136] Although this effect was achieved through the use of RNA interference, the authors expressed optimism that in the near future small molecules might be employed to target epigenetic marks and/or modifying enzymes in a similarly specific strategy. Similarly, the histone methyltransferase SUV39H1 is protected from proteasomal degradation by enhanced binding to progerin in *Zmpste24*$^{-/-}$ mice, resulting in increased H3K9me3 levels and compromised genome maintenance, which leads to accelerated senescence.[283] Targeting of SUV39H1 in this study resulted in amelioration of the progeroid phenotype in *Zmpste24*$^{-/-}$

mice, including reduction of bone loss and extension of lifespan by 60%, suggesting a similar strategy might be useful in the context of normal aging. In an analogous approach, H4K16 hypoacetylation was targeted in the same mouse model to ameliorate the progeroid phenotype, with overexpression of the histone acetyltransferase Mof or addition of the histone deacetylase inhibitor sodium butyrate to drinking water promoting repair of damaged DNA and resulting in reduced evidence of disease.[284] Hypoacetylation of this mark was also found in aged wild-type mice, suggesting that aberrant histone acetylation may play a role in physiologic aging and administration of HDAC inhibitors may have therapeutic value in disease of aging.

1.6.4 Strategies to Delay Senescence

Genetic modification strategies have specifically targeted known regulators of senescence and lifespan to combat diseases of aging. Preventing senescence, clearing senescent cells, or interfering with the senescence-associated secretory phenotype, in which cells release inflammatory mediators such as cytokines and matrix metalloproteinases, are all approaches that might lessen the contribution of cellular aging to chronic illness.[285] Given the complexity of the signaling crosstalk regulating senescence and associated events, identification of therapeutically targetable elements in this network – the "senectome" – is proceeding at multiple levels, the most recent of which includes senescence-associated micro-RNAs, which could be manipulated or used as clinical biomarkers.[286] In non-healing diabetic skin ulcers, siRNA- or Vivo-Morpholino antisense-based gene therapy targeting of *CAV1* or *PTRF*, which are both turned on by oxidative stress in diabetic fibroblasts and induce p53-dependent premature senescence, inhibited senescence and accelerated ulcer repair.[287] Atherosclerosis,

especially in the context of type 2 diabetes, is related to endothelial senescence and has been reduced using a variety of interventions targeting nitric oxide levels and bioavailability in the endothelial microenvironment, including eNOS gene therapy.[288] Gene therapy to induce telomerase activity in CD8 T cells, which undergo premature senescence in the context of HIV infection, results in enhanced proliferation and increased antiviral function.[289] While preliminary research in this area has not resulted in karyotypic changes or wildly altered growth kinetics of the CD8 compartment, the authors of this study emphasized the need for pharmacologic approaches that would mimic these effects without the need for *TERT* gene therapy due to the obvious risks.

The use of pharmacologic agents to modulate senescence-associated pathways is a promising avenue to counteract the effects of aging in the clinic. Mice of both genders treated with rapamycin starting in mid to late life display extended lifespan and reduced incidence of cancer.[290] Treatment of cells from HGPS patients with rapamycin results in enhanced clearance of the mutant protein progerin by autophagy and delayed onset of senescence.[291] Conversely, activation of AKT-mTOR signaling through inhibition of isoprenylcysteine carboxyl methyltransferase, the enzyme which processes prelamin A to lamin A and enables trafficking to the nuclear rim, in *Zmpste24*$^{-/-}$ mice also delayed onset of senescence and improved disease phenotype, suggesting the AKT-mTOR axis, like the Wnt axis, is finely tuned and must be carefully manipulated to achieve therapeutic benefit [292]. Rapamycin is unlikely to be utilized extensively as an anti-aging therapeutic due to its side effects, which include hyperlipidemia and immunosuppression; however, newer analogs of rapamycin (rapalogs) are in development and may find use as anti-aging compounds, along with other agents that inhibit mTOR.[293] Pharmacologic activation of SIRT1 in a rat model

of diabetes restored endothelial differentiation, pro-angiogenic chemokine secretion, and *in vivo* angiogenic activity of bone marrow-derived early outgrowth cells to that of cells from control animals.[294] Pharmacologic blockade of angiotensin II signaling through its type I receptor, which is used clinically to lower blood pressure and prevent insulin resistance in metabolic syndrome, also inhibits adipogenesis in adipose- and bone marrow-derived MSCs, both preventing further pathologic expansion of adipose tissue and helping to maintain an uncommitted progenitor pool for tissue homeostasis and repair.[295] In opposition to the enhanced production of 20-HETE by the cytochrome P450 system observed in metabolic syndrome, MSCs generate P450-derived epoxyeicosatrienoic acids (EETs) from arachidonic acids, and when administered exogenously these lipid mediators have been shown to decrease adipocyte differentiation of MSCs via an increase in heme oxygenase-1 and decrease in *PPARγ*, C/EBPα, and FAS and to reprogram adipocyte stem cells to a new phenotype displaying a smaller cell size, increased secretion of adiponectin, and decreased secretion of inflammatory cytokines.[296] EET agonists have also been shown to reverse a metabolic syndrome phenotype in an obese animal model, highlighting the therapeutic potential of targeting production of these molecules in the adult stem cell pool to combat this age-related disease phenotype in humans.[297]

Given the complex and sometimes unpredictable nature of these emerging pharmaceutical and genetic approaches to age-related disease therapy, sometimes the simplest approaches to maintaining health are best. In the lifelong struggle between growth-promoting signaling pathways and stress resistance pathways, sleep has a critical place in determining the balance.[188] mTOR and FOXO signaling are turned on in distinct temporal windows during early and late sleep, respectively, in response to alterations in somatotrophic signaling,

suggesting that a good night's sleep truly does have restorative powers. Adult stem cells have been demonstrated to undergo significant circadian regulation in multiple studies, with HSCs, marrow- and adipose-derived MSCs, and cancer stem cells all subject to transcriptome modulation by core circadian regulatory proteins.[298] ESCs, in contrast to adult stem cells, are not subject to circadian regulation and have been shown to acquire molecular circadian oscillation upon differentiation; subsequent transcriptional reprogramming with *Sox2*, *Klf4*, *Oct3/4*, and *c-Myc* genes was shown to suppress circadian cycling, literally resetting the internal clock in the resulting iPS cells.[299] Interestingly, action of core circadian regulatory proteins on physiologic cellular processes is opposed by SIRT1 with aging,[300] and control of central circadian cycling by SIRT1 in the brain decays over time.[301] In general circadian rhythms break down with age, coinciding with the development of metabolic derangements and potentially contributing significantly to the organismal aging process.[302] Research into the impact of macro-environmental factors, such as organismal circadian rhythms, on stem cell niches may open new therapeutic avenues for manipulating stem cell fate in diseases of aging.[303] As therapeutic approaches go, good sleep hygiene is safe and free from side effects, cost-effective, and potentially contributes more than we currently know to the long-term maintenance of adult stem cell compartments.

1.7 Conclusions

Adult stem cells serve to replenish differentiated cells and direct repair at sites of tissue injury throughout the body, and exhaustion or dysfunction of an adult stem cell population *in vivo* with age results in degenerative disease. Several finely tuned and contextually regulated pathways coordinate the activities of tissue-resident adult stem cell pools over time in

response to a host of cellular stressors in an effort to maintain the balance between growth-promoting function and oncogenic resistance. Manipulation of one or more of these pathways has the potential to prevent or reverse the impact of advancing age on adult stem cell function, but is fraught with the difficulty of tipping the balance toward metabolic derangement, or more likely toward cancer formation. Harvest and manipulation of adult stem cells *ex vivo* for use in regenerative medicine is a piecemeal approach to addressing systemic age-related chronic illnesses, but for now may prove to be a safer approach. In this regard, it is noteworthy that the clinical safety of HSCs and MSCs has been well documented, not in the least on the basis of decades of successful clinical outcomes of heterologous bone marrow transplantation. Further development of therapeutic approaches to maintain these cells *in vivo* requires that the mechanistic basis of their age-related degeneration or renewal be understood. This is an area continually being informed by studies of early-onset aging syndromes and of families exhibiting extreme longevity. Transcriptional reprogramming, which effectively wipes away all signs of age from most cell types, is also yielding valuable insights into what makes a cell young or old. Rejuvenating stem cells to stave off aging safely will require highly innovative approaches, but the results of this research will have far-reaching implications for regenerative medicine.

Chapter 2

Human Bone Marrow-Derived Mesenchymal Stem Cells Display Enhanced Clonogenicity but Impaired Differentiation with Hypoxic Preconditioning

2.1 Abstract

Stem cells are promising candidate cells for regenerative applications because they possess high proliferative capacity and the potential to differentiate into other cell types. Mesenchymal stem cells (MSCs) are easily sourced but do not retain their proliferative and multi-lineage differentiative capabilities after prolonged *ex vivo* propagation. We investigated the use of hypoxia as a preconditioning agent and in differentiating cultures to enhance MSC function. Culture in 5% ambient O_2 consistently enhanced clonogenic potential of primary MSCs from all donors tested. We determined that enhanced clonogenicity was attributable to increased proliferation, increased vascular endothelial growth factor (VEGF) secretion, and increased matrix turnover. Hypoxia did not impact the incidence of cell death. Application of hypoxia to osteogenic cultures resulted in enhanced total mineral deposition, although this effect was only detected in MSCs preconditioned in normoxic conditions. Osteogenesis-associated genes were up-regulated in hypoxia, and alkaline phosphatase activity was enhanced. Adipogenic differentiation was inhibited by exposure to hypoxia during differentiation. Chondrogenesis in 3-dimensional (3D) pellet cultures was inhibited by preconditioning with hypoxia. However, in cultures expanded under normoxia, hypoxia applied during subsequent pellet culture enhanced chondrogenesis. While hypoxic preconditioning appears to be an excellent way to expand a highly clonogenic progenitor pool, our findings suggest that it may blunt the differentiation potential of MSCs, compromising their utility for regenerative tissue engineering. Exposure to hypoxia during differentiation (post normoxic expansion), however, appears to result in a greater quantity of functional osteoblasts and chondrocytes and ultimately a larger quantity of high quality differentiated tissue.

2.2 Introduction

Adult mesenchymal stem cells (MSCs) are considered highly promising candidate cells for regenerative applications because they possess a high proliferative capacity and the potential to differentiate into other cell types.[10-13] MSCs are also actively investigated for clinical use as gene delivery agents to enhance tissue regeneration, to destroy cancer cells, and to regenerate cartilage and bone.[304]

It has been suggested that the high plasticity of adult MSCs, which makes them an attractive candidate for cell-based therapies, is due to low-level expression of a variety of gene families that characterize differentiated progeny, endowing them with a state of readiness to differentiate along one direction or another, depending on external cues.[305] Hypoxia is a critical external cue in mesenchymal progenitor fate starting as early as embryogenesis. A notable example of this is formation of the early skeleton, in which chondrogenic differentiation of limb mesenchymae is initiated by hypoxic niches formed when embryonic blood vessels regress from sites of mesenchymal cell condensation.[306-310] MSCs are hypothesized to persist in the adult for tissue repair and remodeling, among other functions, in perivascular niches throughout the body.[311]

One *in vivo* niche for MSCs is the bone marrow, where oxygen concentration has been reported to vary from 7% to less than 1%.[312-314] However, bone marrow-derived MSCs are frequently cultured at atmospheric oxygen (20-21% O_2) in the laboratory. The evolved defenses of MSCs against oxidative stress may be overwhelmed by the level of free radicals generated in these culture conditions, leading to decreased utility of these cells after expansion *in vitro*.[314] Many studies have thus explored the effects of low oxygen on MSCs relative to atmospheric oxygen, with highly variable results. Definitions of hypoxia vary in

these studies, with different groups defining hypoxia at oxygen concentrations ranging from less than 1% O_2 to 8% O_2.[315] MSCs are cultured under widely varying conditions, with different laboratories employing different isolation methods, selection markers, culture media, supplements, oxygen tensions, and differentiation induction strategies.[316] Compounding this lack of consistency in the field is the heterogeneous nature of MSCs themselves, which perhaps reflects their high plasticity, or perhaps reflects multiple related cell types. Many studies on the effect of oxygen concentration on MSC differentiation investigate only one lineage, such as adipogenesis. Given the lack of consistent methodology, it is difficult to compare these studies to one other.

Learning how to appropriately tune contextual cues for differentiation in synchrony, of which oxygen tension is only one, to recapitulate microenvironments present during development may yield a more desirable tissue engineered product than empiric manipulation of one variable in response to a single or limited set of outcome measures. With this motivation we undertook a comprehensive characterization of the effects of hypoxia on MSC differentiation along three commonly studied lineages as well as on their phenotype and other cell functions. This would allow us to observe the effects of hypoxia on various measures of "potency" and "stemness" from the same starting conditions. Since our primary interest is cartilage tissue engineering, we selected 5% ambient oxygen for our hypoxic condition, as one study which examined MSC chondrogenic differentiation at 0% to 35% O_2 found 5% O_2 to be optimal.[308]

In human MSCs the effects of hypoxia have been tested extensively with varying results. There is general agreement that hypoxia enhances proliferation of human MSCs; this has been shown at oxygen tensions varying from 1% to 5%.[317-322] However, there are studies

that have shown decreased proliferation at low oxygen levels. In one such study platelet lysate and fresh frozen plasma (FFP) were used as culture supplements in lieu of serum, and MSCs were isolated from pediatric patients, while most studies of hypoxia in human MSCs have been performed with adult-derived cells.[323]

In terms of human MSC differentiation, some studies have demonstrated inhibition of differentiation along adipogenic and osteogenic lineages under hypoxic conditions (1% - 3% O_2),[312, 323-326] and others found adipogenic and osteogenic differentiation potential in hypoxic conditions (1.5% - 3% O_2) comparable to normoxia.[323, 327] With regard to chondrogenic differentiation potential, several studies showed that hypoxia as a preconditioning agent results in preservation of chondrogenic differentiation potential or enrichment for chondrogenic progenitors during *ex vivo* expansion, but there are fewer studies detailing the isolated effects of hypoxia on MSCs during induction of chondrogenic differentiation.[328] Some studies have reported that hypoxia (1% - 5% O_2) enhanced chondrogenesis in pellet cultures of MSCs relative to 21% O_2;[324, 327, 329-331] however hypoxia did not enhance chondrogenesis in pellet cultures in other studies, with optimal chondrogenic conditions achieved at higher oxygen concentrations (15% - 20%).[332, 333] The variability in results from human MSCs is frequently attributed to inter-individual differences in patient samples.

Our study originally arose from curiosity about why colony-forming unit-fibroblasts (CFU-Fs) arise in greater numbers when MSCs are cultured under hypoxic conditions in our and other laboratories. Hypoxia-mediated increase of MSC colony formation has been shown to be HIF-independent.[334] Numerous factors contribute to the size and number of colonies in a 14-day CFU-F assay, among them (1) cell proliferation, (2) cell death, (3) cell migration, (4) growth factor secretion (which in turn affects cell proliferation), and (5) matrix

turnover (which in turn affects cell migration). In this study we sought to address each of these factors in MSCs cultured under hypoxic (5% O_2) and normoxic (21% O_2) conditions.

We then investigated the effects of steady-state hypoxia on differentiation. Many studies have addressed the use of hypoxia in preconditioning prior to differentiating MSCs under normoxic conditions. Preconditioning with hypoxia would be an easy, inexpensive, and theoretically benign approach for maximizing expansion of a progenitor pool before differentiation, expanding the clinical use of autologous MSCs, provided differentiation *potential* is preserved during hypoxic preconditioning. Our objective in this study was to address this question of whether differentiation potential is preserved if MSCs are cultured (preconditioned) under hypoxic conditions, and then compare this with MSCs cultured in normoxic conditions and to do so in osteogenic, adipogenic, and chondrogenic lineages within the same study. Additionally we wanted to address the effect of hypoxia during the differentiation stage.

Several studies have demonstrated the negative impact of hypoxia on osteogenic potential of MSCs.[323, 325, 334-337] One study employing an approach similar to ours demonstrated enhanced chondrogenesis under normoxic conditions as a result of hypoxic preconditioning (5% O_2), however this study was performed using ovine not human MSCs, and examined differentiation to only one lineage.[338] The same group published another study demonstrating this enhancement held true in 3D collagen hydrogel cultures as well as pellet cultures.[339] Similar results for chondrogenesis have been obtained in 3D micromass cultures with murine adipose-derived mesenchymal stem cells (ASCs); in that study osteogenesis was inhibited by hypoxic preconditioning.[328] Adipogenic differentiation potential has been shown to be enhanced by hypoxic preconditioning (2% O_2) in murine MSCs.[340]

2.3 Materials and Methods

2.3.1 Cell Culture

MSCs were harvested from consenting arthroplasty patients with IRB approval as described by Baksh and Tuan, 2007.[171] Adherent Passage-0 MSCs were cultured in growth medium consisting of high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen) with antibiotic-antimycotic (Gibco/Invitrogen) and 10% MSC-qualified fetal bovine serum (Gibco/Invitrogen) until they reached 80% confluence, then passaged using 0.25% trypsin and replated at a density of 6.0×10^3 cells/cm^2 as Passage-1 MSCs. At 80% confluence Passage-1 MSCs were trypsinized and cryopreserved in commercially available freeze medium (Invitrogen) and stored in liquid nitrogen. Upon recovery from cryopreservation, Passage-2 MSCs were cultured in separate closed incubators purged with either 95% air and 5% CO_2 (21% O_2 or normoxia) or 5% oxygen, 5% CO_2, and 90% nitrogen (5% O_2 or hypoxia).

2.3.2 Colony Forming Unit-Fibroblast (CFU-F) Assay

CFU-F assays were performed as described by Baksh and Tuan, 2007.[172] One hundred Passage-2 MSC's were plated on 10 cm tissue culture-treated polystyrene dishes in triplicate in growth medium and cultured for 14 days with medium changes every 3 days. At the end of 14 days colonies were rinsed with phosphate-buffered saline (PBS), fixed with crystal violet dye in methanol, and again rinsed with PBS to remove residual dye. Differences in colony number were evaluated by a two-tailed one sample t-test to block for variability between individual human subjects.

2.3.3 Cell Proliferation Assays

The Click-iT EdU Alexa Fluor 647 Cell Proliferation kit (Molecular Probes) was used according to the manufacturer's protocol. MSCs were incubated with 10 μM Click-iT EdU for 16 hours, fixed, permeabilized, labeled, and EdU was detected via flow cytometry using a FACSAria cytometer and FACSDiva software (Becton Dickinson). Data were analyzed using FlowJo (Tree Star). Differences in EdU incorporation were evaluated by a paired two-tailed t-test.

Proliferation was also assessed with Ki67 immunostaining. MSCs cultured on Lab-Tek Permanox chamber slides (Nunc) at a density of 6.0×10^3 cells/cm^2 for 48 hours were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with a mouse anti-Ki67 antibody (Abcam, clone PP-67) overnight at 4°C, a FITC-conjugated goat anti-mouse secondary (Abcam), then counterstained with Vectashield DAPI (Vector Labs). Differences in number of Ki67 posititive cells were evaluated by a paired two-tailed t-test.

2.3.4 Metabolic Activity Assay

AlamarBlue (Invitrogen) was used to quantify MSC metabolic activity according to the manufacturer's protocol. MSCs from each condition were plated in triplicate at a density of 3.0×10^3 cells/well in a 96 well plate and incubated with 10% AlamarBlue in culture medium for three hours. Fluorescence was measured on a BioTek microplate reader. AlamarBlue solution from an empty well and AlamarBlue solution incubated with cells overnight were used to determine the lower and upper bounds of the assay, respectively. Differences in metabolic activity were evaluated by a two-tailed one sample t-test at 24 and 96 hour timepoints.

2.3.5 Cell Death Assays

Cell death was measured using a fluorescent LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) according to the manufacturer's protocol. MSCs from each condition were plated in triplicate at a density of 3.0×10^3 cells/well in a 96 well plate and incubated for 48 hours before staining. Fluorescence of each population was measured on a BioTek microplate reader, and differences in live:dead ratios were determined using a paired two-tailed t-test.

The DeadEnd Fluorometric TUNEL System (Promega) was used to quantify apoptosis according to the manufacturer's protocol. Briefly, MSCs were plated on Lab-Tek Permanox Chamber Slides (Nunc), cultured under normoxic or hypoxic conditions, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. A positive control sample was prepared by applying DNase to the fixed, permeabilized cells. TdT solution was applied to fluorescently label nick-ends of DNA fragments, and differences in the number of apoptotic cells were determined using a two-tailed one sample t-test.

2.3.6 Immunophenotyping

MSCs were stained after 14 days of culture according to the methods described in Nesti et al, 2008.[341] A panel of standard positive and negative MSC markers was interrogated, including CD34 (clone 563), CD44 (clone 515), CD45 (clone TU116), CD73 (clone AD2), CD90 (clone 5E10), CD105 (clone 266), CD146 (clone P1H12) (BD Biosciences), and Stro-1 (clone STRO-1) (BioLegend), as well as the stem cell markers Oct4 (clone 40/Oct-3), Sox2 (clone 245610), Nanog (clone N31-355) (BD Biosciences), and c-Myc (clone 9E10) (Abcam).

Marker expression was measured using a FACSAria cytometer and FACSDiva software (Becton Dickinson). Isotype controls for each marker were used to calculate delta mean fluorescence intensity for the population. Data were analyzed using FlowJo (Tree Star), and two-tailed one sample t-tests were used to test for differences between hypoxic and normoxic cultures for each surface marker.

2.3.7 Gene Expression Assay

RT–PCR SuperArrays (SABiosciences/Qiagen) were used according to the manufacturer's protocol. RNA was isolated from MSCs using the RNeasy Mini-prep Kit (Qiagen), then amplified using the RT2 profiler first strand kit (SABiosciences/Qiagen). RT2 profiler SYBR-Green master mix with ROX (SABiosciences/Qiagen) was used to amplify cDNA on SuperArray plates with pre-aliquotted primers using an ABI 7900HT. All data were analyzed using SDS 2.3 software and the company's web-based PCR Array Data Analysis platform, as well as Excel (Microsoft), Prism (GraphPad Software), and SPSS (IBM). Fold regulation was determined for hypoxia, extracellular matrix and adhesion molecules, stem cells, osteogenesis, and apoptosis pathways at 2 days, and for hypoxia, extracellular matrix and adhesion molecules, cell cycle, and apoptosis pathways at 10 days, and differences in delta CTs were detected for each gene using two-tailed t-tests with blocking for patients where appropriate.

2.3.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Secreted levels of growth factors, Fibroblast Growth Factor 2 (FGF2) and VEGF in culture media, as well as Tumor Necrosis Factor alpha (TNFα), were quantified by enzyme-

linked immunosorbent assay (ELISA) according to the manufacturers' protocols. FGF2 (Abnova), and VEGF and TNFα (Pierce/ThermoFisher) ELISAs were performed on 1-day and 3-day culture supernatants applied to pre-coated microtiter plates, and read colorimetrically on a BioTek microplate reader. All samples and standards were measured in duplicate. Results were normalized to DNA content of each culture as determined by Picogreen assay (Invitrogen), and data were analyzed using repeated measures two-way ANOVA, with matched subjects serving as their own controls and Bonferroni post-tests to detect differences between group means.

2.3.9 Matrix Metalloproteinase Activity Assay

MMP activity was assayed using 520 MMP Fret Substrate XI (sequence 5-FAM-P-Cha-G-Nva-HA-Dap(QXL™ 520)-NH2) (AnaSpec). MSCs were plated in triplicate at a density of 3.0×10^3 cells/well in a black 96-well plate and cultured for three days under serum-free conditions. To measure secreted MMP activity, two experimental medium replicates were collected without pooling from independent cultures of cells for each patient sample and condition. After addition of the substrate, fluorescence was measured on a BioTek microplate reader every 5 minutes over 60 minutes, and a linear fit model was used to determine the rate of the cleavage reaction. To measure activity of retained MMPs, 520 MMP Fret Substrate XI solution was applied to the cells remaining after collection of conditioned media, and MMP activity was measured in a similar fashion, then normalized to the DNA content of each culture. Differences between hypoxic and normoxic cultures were detected using two-tailed one sample t-tests.

2.3.10 Differentiation Assays

Osteogenic differentiation was induced and assayed according to the methods described in Boland et al, 2004.[173] Osteogenesis was induced in monolayer MSC cultures plated at a density of 1.0×10^4 cells/cm^2 in 6-well and 24-well plates with osteogenic medium consisting of DMEM supplemented with 10% FBS, 50 μg/ml L-ascorbate-2-phosphate, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 10 nM 1α,25-$(OH)_2$ vitamin D_3 (Sigma). On day 14, alkaline phosphatase activity was detected histochemically in paraformaldehyde/methanol-fixed cultures using the Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manufacturer's protocol. On day 21 osteogenic cultures were rinsed with PBS, fixed in 60% isopropanol, and stained in a 1% Alizarin Red solution (Rowley) to detect matrix mineralization. For assessment of gene expression in osteogenic cultures, RNA was collected at day 21. For preconditioning assays MSCs were cultured at either 5% or 21% O_2 for two weeks in growth medium, then trypsinized, counted, replated at 1.0×10^4 cells/cm^2 in 24-well tissue culture plates, and cultured in osteogenic medium as described above at either the same or the opposite oxygen tension. This resulted in four groups: preconditioned and differentiated at 5% O_2 (5%-5%), preconditioned at 5% O_2 and differentiated at 21% O_2 (5%-21%), preconditioned at 21% O_2 and differentiated at 5% O_2 (21%-5%), or preconditioned and differentiated at 21% O_2 (21%-21%). To determine the contribution of preconditioning oxygen tension versus differentiation oxygen tension, as well as the interaction of these two variables, to the observed variance of each dependent variable tested, we employed two-way ANOVA. Bonferroni corrections were used to compare the means of the two pairs of treatment groups to one another for each dependent variable tested. This approach was used for analysis of all experiments with preconditioning.

Adipogenic and chondrogenic differentiation were induced and assayed as described previously by Baksh and Tuan, 2007.[342] Preconditioning was carried out as stated above for osteogenic differentiation. Adipogenesis was induced in monolayer MSCs plated at a density of 1.0×10^4 cells/cm^2 in 6-well and 24-well plates with adipogenic medium consisting of DMEM supplemented with 10% FBS, 1 μM dexamethasone, 1 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). On day 21, hypoxic and normoxic adipogenic cultures were stained with Oil Red O stain (Sigma), and dye content was quantified by calculating area of positive staining in each culture across 3-4 photomicrograph fields. We employed the analytical approach described in the previous paragraph to determine the contribution of preconditioning versus differentiation oxygen tension and any interaction.

To induce chondrogenesis, MSCs were grown as high-density pellets (2.5×10^5 cells) in serum-free DMEM supplemented with ITS Premix (BD Biosciences), 50 μg/ml asorbic acid (Sigma), 40 μg/ml L-proline (Sigma), 100 μg/ml sodium pyruvate (Gibco/Invitrogen), 0.1 μM dexamethasone (Sigma), and 10 ng/ml recombinant human transforming growth factor (TGF)-β3 (R&D Systems). On day 28, pellets were harvested for histology to detect total proteoglycan content via Safranin O/Fast Green staining (Rowley) and sulfated glycosaminoglycan (sGAG) specifically via Alcian Blue staining (Rowley). sGAG content was quantified using the Blyscan sGAG assay (Accurate Chemical & Scientific Corporation) and normalized to DNA content of each pellet. We developed a modified scoring system for cartilage histology (Table 1) based on the system described in Im et al.[343] Duplicate pellets from each patient and condition were independently scored by two investigators, and an average quality score was generated. We employed the two-way ANOVA approach described above to determine the contribution of preconditioning versus differentiation

oxygen tension and any interaction on cartilage quality score and sulfated GAG content of pellets.

Table 2.1 Histological and histochemical grading scale

Cartilage Quality Score = (a x b)/2 + (b x c)	
	Score
(a) Cellular morphology	
Small	0
Low hypertrophy	1
High hypertrophy	2
(b) Matrix quality	
Fibrous tissue	0
Incompletely differentiated mesenchyme	1
Hyaline Articular Cartilage	2
(c) Safranin-O staining of the matrix	
Slight to no staining	1
Moderate	2
Intense staining	3

2.4 Results

2.4.1 Hypoxia Enhances MSC Colony Formation, Proliferation, and Metabolic Activity

CFU-F assays performed on MSCs cultured in hypoxic or normoxic conditions showed that hypoxia enhanced colony formation ($p<0.01$). Hypoxic conditions increased proliferation as shown by EdU incorporation by ($p<0.05$); Ki67 staining was not significantly enhanced by hypoxic culture. Normalized AlamarBlue assays showed no difference in metabolic activity after 24 hours, but higher metabolic activity in hypoxic cultures after 96 hours (Figure 2.1) ($p<0.01$).

2.4.2 Hypoxia Does Not Alter Incidence of MSC Death

LIVE/DEAD assays to quantify nonspecific cell death showed no difference in the ratio of viable to dead cells between MSCs cultured in hypoxic conditions and MSCs cultured in normoxic conditions. To determine if there was any difference in cell death due to apoptosis specifically, we performed TUNEL staining, which showed a very low level of apoptosis in all MSC cultures, and no difference in level of apoptosis between MSCs cultured in hypoxia versus normoxia (Figure 2.2).

2.4.3 Differential Gene Expression in Hypoxic MSC Cultures

We performed PCR arrays representing a number of pathways of interest to screen for genes differentially regulated under hypoxic versus normoxic conditions both short term (2 days) and in the steady state (10 days). The results of these analyses are shown in Tables 2.2 and 2.3 and discussed below.

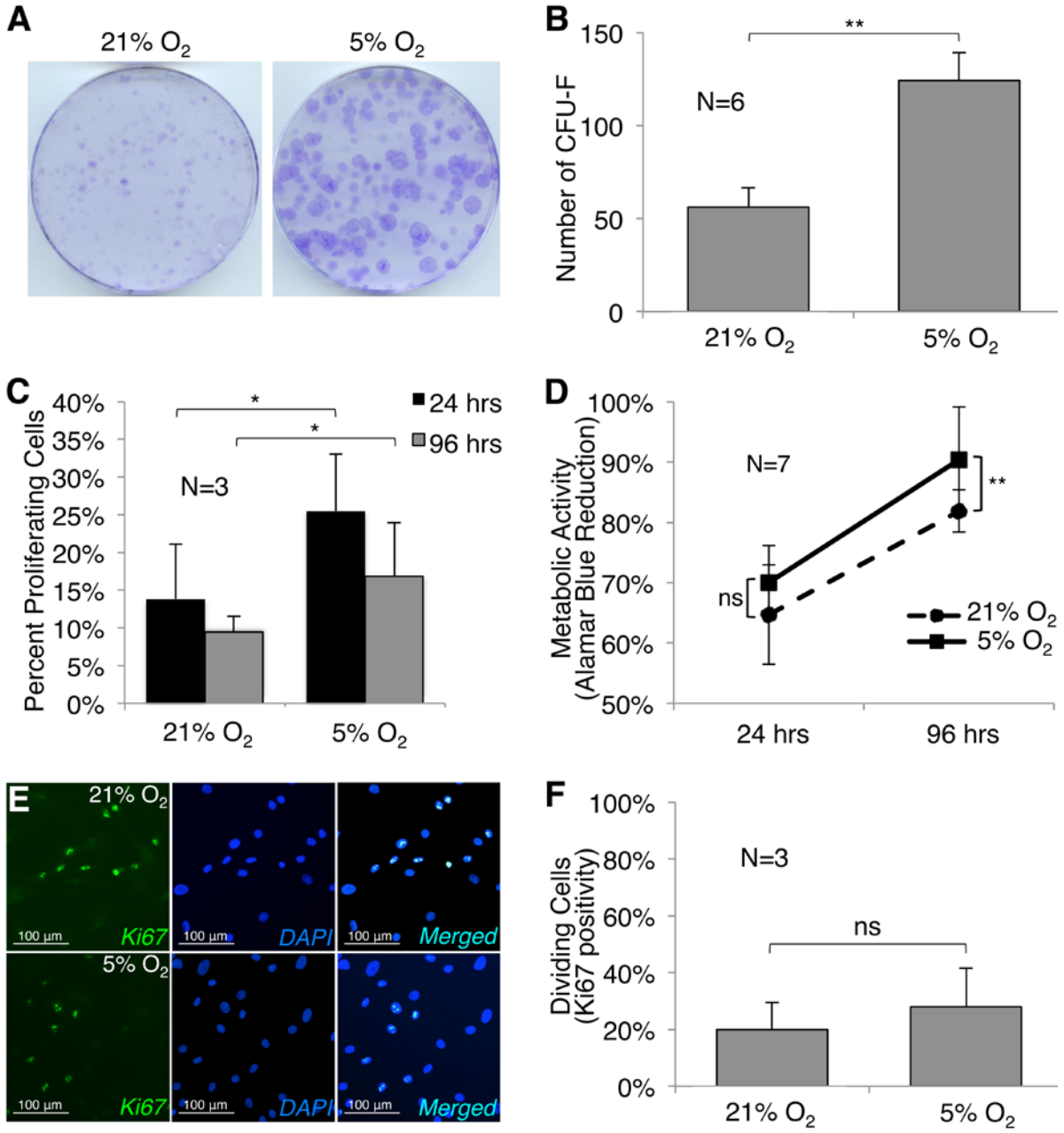

Figure 2.1 Hypoxia enhances clonogenicity, proliferation, and metabolic activity of human MSCs. MSC colony size (**A**), colony number (**B**), and cell proliferation as measured by EdU incorporation (**C**) and Ki67 labeling (**E**), and quantified (**F**). AlamarBlue staining showed increased reduction of the substrate in hypoxic cultures, with a statistically significant difference observed in the 4 day cultures (**D**). Photomicrographs captured at 10x, scale bar = 100µm. Significant differences of relevant comparisons are indicated: *, ** indicate p values of <0.05 and <0.01, respectively; ns indicates non-significance. Abbreviations: CFU-F = Colony Forming Unit-Fibroblast.

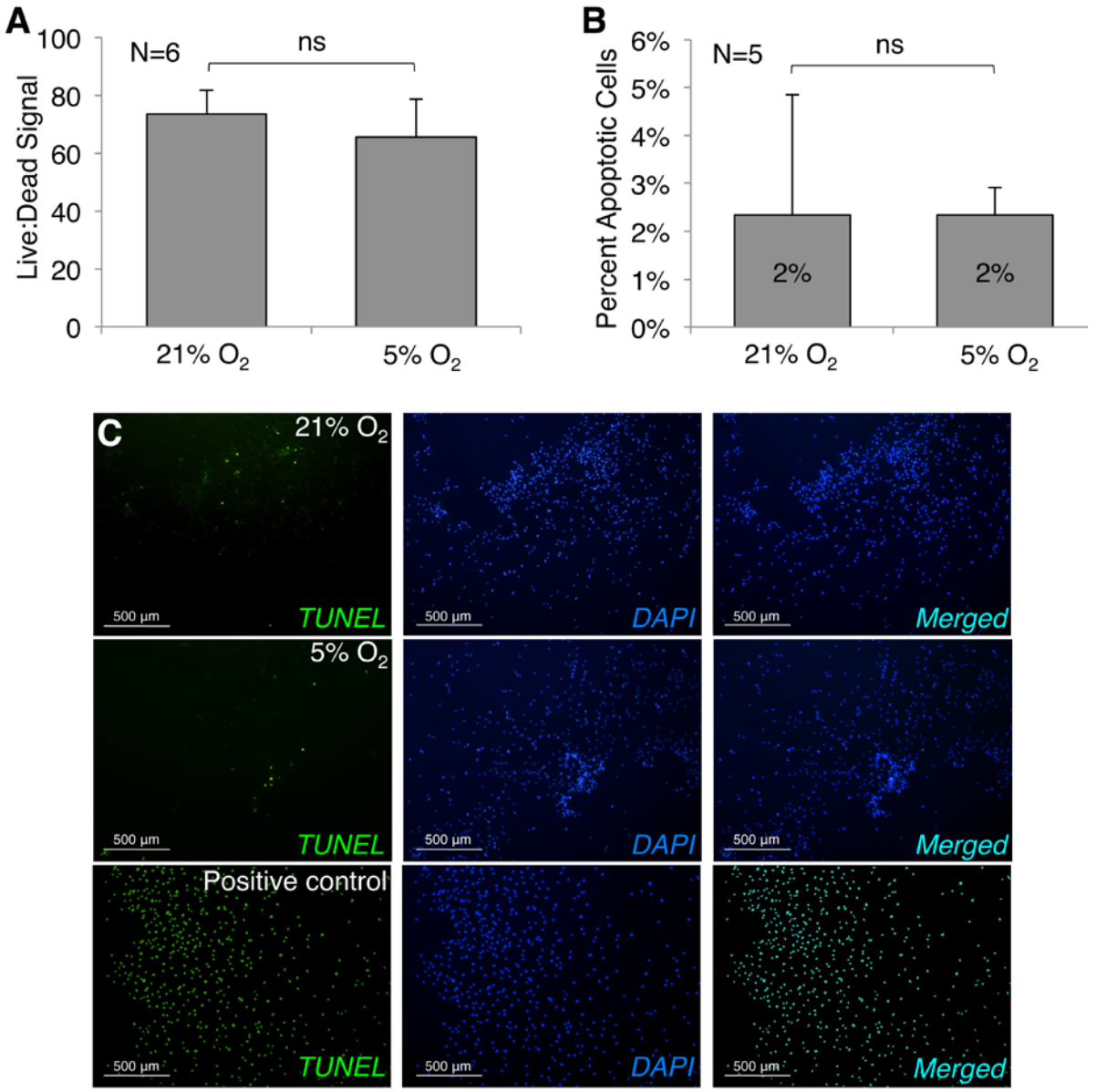

Figure 2.2 Hypoxia does not impact MSC death and apoptosis rates. Both normoxic and hypoxic conditions generated similar rates of cell death as measured by LIVE/DEAD (**A**) and apoptosis as measured by TUNEL staining (**C**), and quantified (**B**). Photomicrographs captured at 4x, scale bar = 500µm. Significant differences of relevant comparisons are indicated: ns indicates non-significance.

Table 2.2 Comparison of genes differentially expressed between hypoxic and normoxic two-day MSC cultures

Gene symbol	Gene title	Fold regulation	p value	Protein function[1]
Genes up-regulated in hypoxic compared to normoxic culture after two days				
ITGAM	Integrin, alpha M	18.60	0.033	Adhesion, migration
GPI	Glucose-6-phosphate isomerase	1.49	0.035	Glycolysis, gluconeogenesis, migration
ACAN	Aggrecan	1.35	0.027	ECM in cartilaginous tissue, withstands compression in cartilage
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	1.32	0.052	Breakdown of extracellular matrix
COL9A1	Collagen, type IX, alpha 1	1.17	0.012	Major component of hyaline cartilage
KHSRP	KH-type splicing regulatory protein	1.16	0.044	mRNA processing
PPARA	Peroxisome proliferator-activated receptor alpha	1.06	0.008	Ketogenesis, proliferation, differentiation, inflammation
Genes down-regulated in hypoxic compared to normoxic culture after two days				
IPCEF1	Interaction protein for cytohesin exchange factors 1	-8.03	0.004	Guanine exchange
FGF3	Fibroblast growth factor 3	-2.92	0.026	Embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	-2.57	0.041	Cell-cell adhesion glycoprotein, tumor suppressor
MSX1	Msh homeobox 1	-1.54	0.018	Transcriptional repressor, developmental patterning

Gene symbol	Gene title	Fold regulation	p value	Protein function[1]
CTNNA1	Catenin (cadherin-associated protein), alpha 1	-1.52	0.04	Migration
LAMA2	Laminin, alpha 2	-1.29	0.027	Basement membrane component, adhesion, migration
SMAD3	SMAD family member 3	-1.22	0.021	TGFβ signaling
APC	Adenomatous polyposis coli	-1.14	0.011	Tumor suppressor, nuclear export
EPAS1	Endothelial PAS domain protein 1	-1.14	0.038	HIF2A; hypoxia-induced signal transduction
SMAD2	SMAD family member 2	-1.13	0.022	TGFβ signaling, cell proliferation, apoptosis, differentiation

[1] Protein function: from Entrez Gene, UniProtKB/Swiss-Prot.

Table 2.3 Comparison of genes differentially expressed between hypoxic and normoxic ten-day MSC cultures

Gene symbol	Gene title	Fold regulation	p value	Protein function[1]
Genes up-regulated in hypoxic compared to normoxic culture after ten days				
LEP	Leptin	17.08	0.019	Regulation of body weight, immune and inflammatory responses, hematopoiesis, angiogenesis, wound healing
MT3	Metallothionein 3	6.77	0.0002	Binds heavy metals
IGFBP1	Insulin-like growth factor binding protein 1	2.97	0.029	Prolongs the half-life of the Insulin-like Growth Factors and alters their interaction with cell surface receptors
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	2.21	0.001	Glucose transporter
VEGFA	Vascular endothelial growth factor A	2.11	0.004	Angiogenesis, vasculogenesis, cell growth, migration, inhibition of apoptosis
DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11	2.04	0.004	ATPase and DNA helicase activities; may function to maintain chromosome transmission fidelity and genome stability
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.91	0.002	Regulation of apoptosis
BHLHE40	Basic helix-loop-helix family, member e40	1.62	0.0005	Control of cell differentiation
GTSE1	G-2 and S-phase expressed 1	1.61	0.028	Represses ability of tumor suppressor p53 to induce apoptosis
TGFBI	Transforming growth factor, beta-induced	1.59	0.001	Induced by TGFβ; acts to inhibit cell adhesion; involved in cell-collagen interactions, possibly endochondral ossification
ANGPTL4	Angiopoietin-like 4	1.59	0.021	Regulates glucose homeostasis, lipid metabolism, insulin sensitivity
CDC2	Cyclin-dependent kinase 1 (CDK1)	1.59	0.004	Cell cycle progression
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	1.5	0.046	Collagen calcification; extracellular matrix synthesis, cell shape changes

Gene symbol	Gene title	Fold regulation	p value	Protein function[1]
MAD2L1	MAD2 mitotic arrest deficient-like 1	1.45	0.016	Regulation of cell cycle progression
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.40	0.05	Cyclin-dependent kinase inhibitor; controls G1 progression; induced by TGFβ
CDKN3	Cyclin-dependent kinase inhibitor 3	1.39	0.003	Prevents activation of CDK2 kinase, thereby inhibiting G1/S phase transition
CCNB1	Cyclin B1	1.37	0.017	Regulatory protein involved in mitosis
CKS2	CDC28 protein kinase regulatory subunit 2	1.30	0.035	Binds the catalytic subunit of the cyclin dependent kinases; essential for their function
ENO1	Enolase 1, (alpha)	1.28	0.047	Glycolytic enzyme; splice variant binds c-myc promoter, acts as tumor suppressor
HPRT1	Hypoxanthine phosphoribosyltransferase 1	1.27	0.014	Transferase which plays a central role in the generation of purine nucleotides through the purine salvage pathway
Genes down-regulated in hypoxic compared to normoxic culture after ten days				
DIRAS3	DIRAS family, GTP-binding RAS-like 3	-1.88	0.034	Associated with growth suppression; putative tumor suppressor gene
EPAS1	Endothelial PAS domain protein 1	-1.64	0.005	HIF2A; hypoxia-induced signal transduction
HIF1A	Hypoxia inducible factor 1, alpha subunit	-1.39	0.048	Master regulator of cellular and systemic homeostatic response to hypoxia
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	-1.31	0.015	Activates NF-kappaB and MAPK8/JNK, induces apoptosis, mediates signal transduction of TNF-receptors
CDKN1A	Cyclin-dependent kinase inhibitor 1A	-1.29	0.028	Regulator of cell cycle progression in response to stress stimuli, DNA replication damage repair; execution of apoptosis following caspase activation
CUL1	Cullin 1	-1.11	0.016	Mediates ubiquitination of proteins involved in cell cycle progression, signal transduction, and transcription

Gene symbol	Gene title	Fold regulation	p value	Protein function[1]
HIF1AN	Hypoxia inducible factor 1, alpha subunit inhibitor	-1.09	0.036	Functions as an oxygen sensor and, under normoxic conditions, the hydroxylation prevents interaction of HIF-1 with transcriptional coactivators

[1] Protein function: from Entrez Gene, UniProtKB/Swiss-Prot.

2.4.4 Hypoxic Culture Minimally Alters MSC Immunophenotype

Flow cytometric immunophenotyping of MSCs cultured under normoxic conditions for two weeks showed the cells to be $CD34^-$, $CD44^+$, $CD45^{dim}$, $CD73^+$, $CD90^{++}$, $CD105^+$, $CD146^{dim}$, and Stro-1^{dim} relative to isotype controls. This differed slightly from the immunophenotype of MSCs cultured under hypoxic conditions, which showed no detectable CD45 expression (p=0.06) nor CD146 expression (p<0.01) (Figure 2.3). We also performed intracellular staining for the transcription factors Oct4, Sox2, c-Myc, and Nanog. Both normoxic and hypoxic cultures were negative for all four of these transcription factors in the samples tested.

2.4.5 Effects of Hypoxic Culture on MSC Growth Factor and Cytokine Secretion

We assayed hypoxic versus normoxic cultures for differences in growth factor secretion and TNFα secretion. ELISAs for VEGF showed increasing VEGF secretion from day 1 to day 3 in high-density cultures (6.0×10^3 cells/cm^2), with slightly higher overall VEGF levels in hypoxic cultures, but lower VEGF production per cell in hypoxic cultures after normalization to cell number. In low density cultures analogous to the conditions experienced by MSCs in colony-forming assays, VEGF secretion normalized for cell number was initially undetectable, quite high by day 3, and then decreased over time, but was maintained at higher levels in hypoxic cultures (oxygen tension p<0.0001; repeated measures two-way ANOVA, matched subjects serving as their own controls). ELISAs for TNFα showed the opposite: TNFα secretion was initially high in high-density cultures, and dropped by day 3, with higher TNFα secretion in normoxic cultures. In low-density cultures, there was no difference in TNFα secretion between hypoxic and normoxic cultures, and TNF levels at

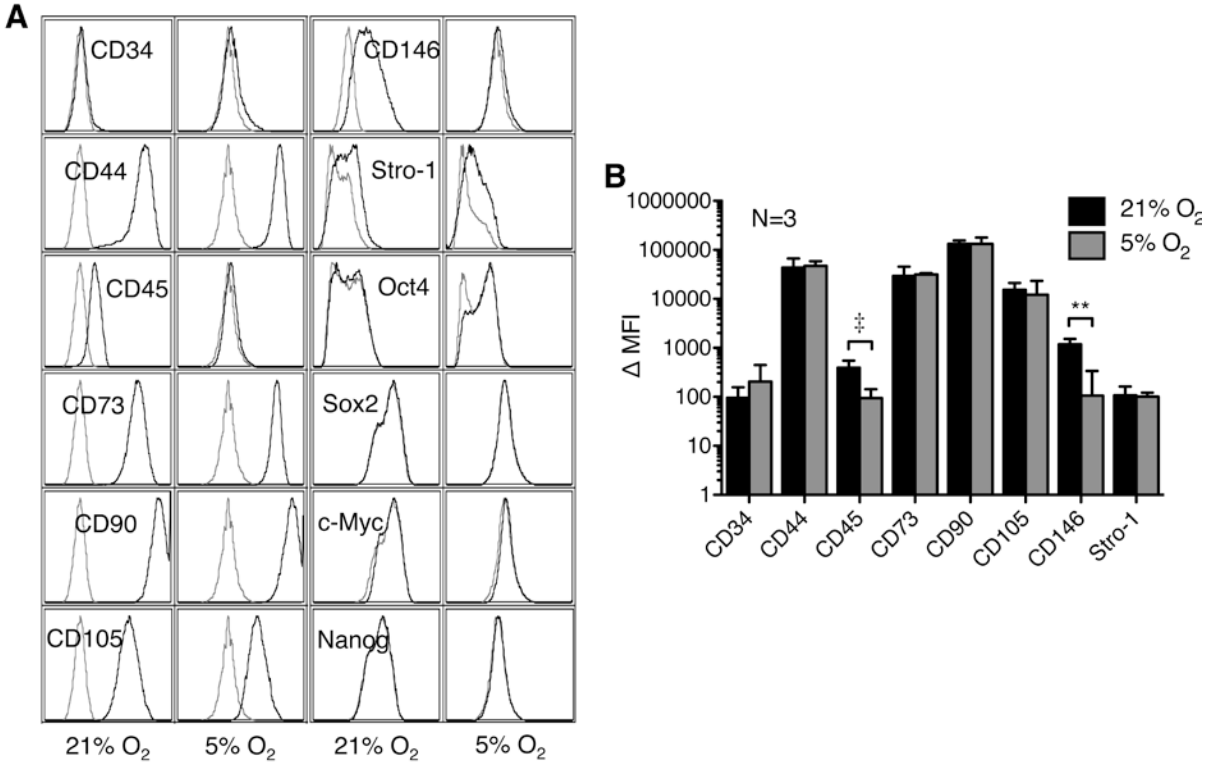

Figure 2.3 Effect of hypoxia on MSC immunophenotype. Immunophenotype under normoxic (left) and hypoxic (right) conditions (**A**). Histograms are from one representative donor; isotype controls are represented as grey curves, stained samples as black curves. Quantitation of surface marker expression (**B**), shown as delta MFI. Data plotted are from three distinct donors; error bars represent standard deviation. Significant differences of relevant comparisons are indicated: ‡, **, indicate p values of 0.06 and <0.01, respectively. Abbreviations: MFI = Mean Fluorescence Intensity. Surface marker designations are as defined by HCDM/HLDA nomenclature and intracellular markers are listed as defined by the HGNC.

day 3 were low compared to high-density cultures (when normalized to cell number), but increased as cell density increased, with a slight lead in hypoxic cultures by day 12. ELISAs for FGF2 showed that FGF2 secretion varied significantly between samples from different patients for high-density cultures, with a greater spread in levels of FGF2 secretion in hypoxic cultures. In low-density cultures FGF2 secretion decreased over time, but overall there was no observable effect of oxygen tension on FGF2 secretion (Figure 2.4).

2.4.6 Hypoxic Culture Enhances MMP Expression in MSCs

Analysis of gene expression of MMPs showed up-regulation at day 2 of several MMP genes under hypoxic conditions relative to normoxic conditions, most notably *MMP15*, with up-regulation of several others by day 10, especially *MMP9, MMP10, MMP11, and MMP12*. This was accompanied by an increase in overall TIMP gene expression in response to hypoxic culture. *TIMP1* expression in hypoxic cultures was initially the same as normoxic cultures (at day 2), but increased more than 3-fold compared to normoxic cultures by day 10. *TIMP3* expression at day 2 of culture was 24-fold higher in hypoxic cultures than normoxic cultures, and remained highly elevated at day 10. These results led us to test MMP activity in hypoxic and normoxic cultures using a broad-spectrum activity assay. There were no significant differences observed in membrane-bound or secreted MMP activity between hypoxic and normoxic cultures when the data were normalized for cell number; however, there was a non-significant trend toward greater membrane-bound MMP activity in hypoxic cultures (Figure 2.5).

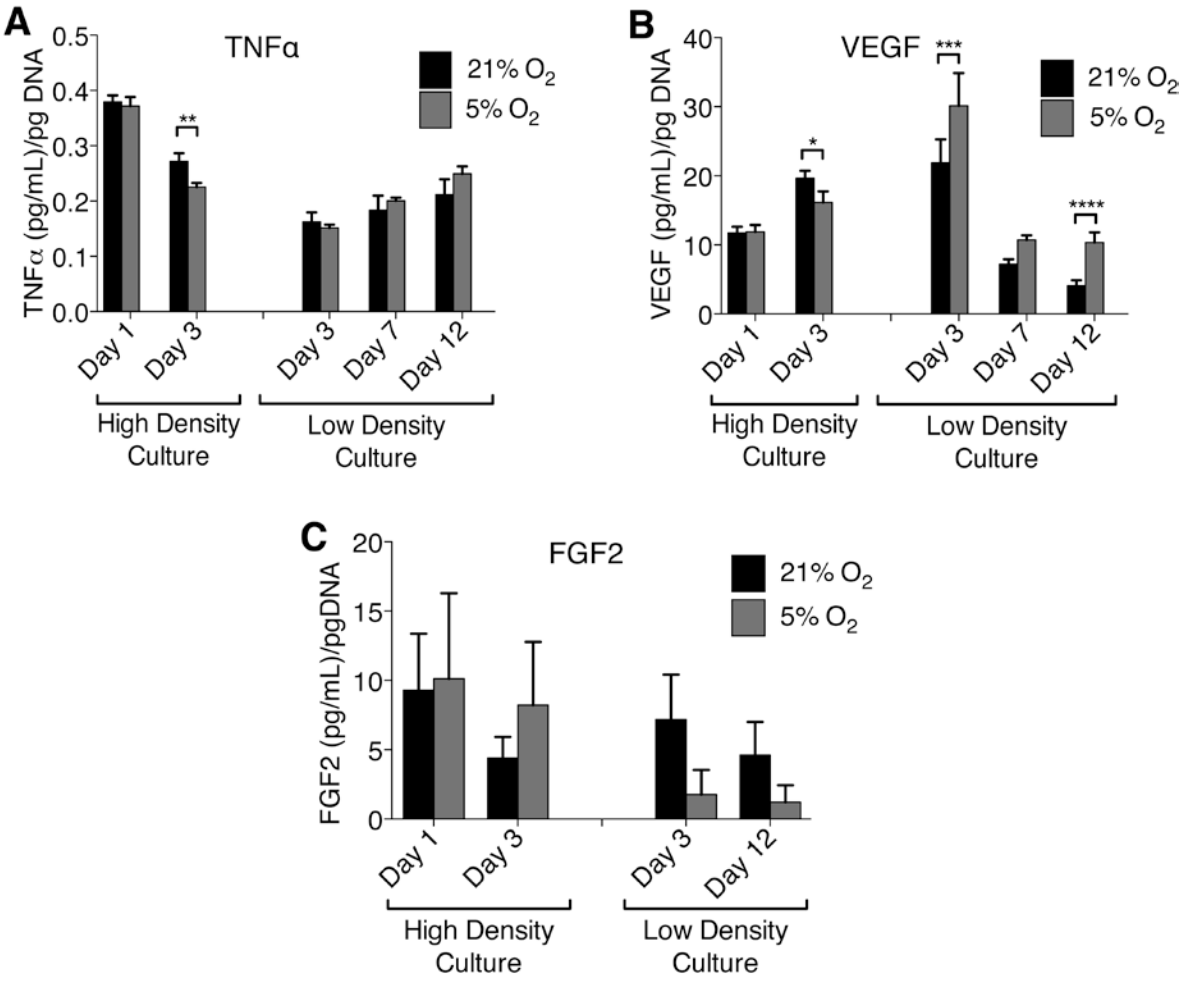

Figure 2.4 TNF and growth factor secretion by MSCs in normoxic and hypoxic cultures. TNFα secretion (**A**), VEGF secretion (**B**), and FGF2 secretion (**C**) in high and low density cultures. All data are normalized to cell number. Error bars represent standard error of the mean computed on 3 to 5 biologic replicates (distinct donors). Significant differences of relevant comparisons are indicated: *, **, ***, **** indicate p values of <0.05, <0.01, <0.001, and <0.0001, respectively. Abbreviations: TNFα = tumor necrosis factor α, VEGF = vascular endothelial growth factor, FGF2 = basic fibroblast growth factor.

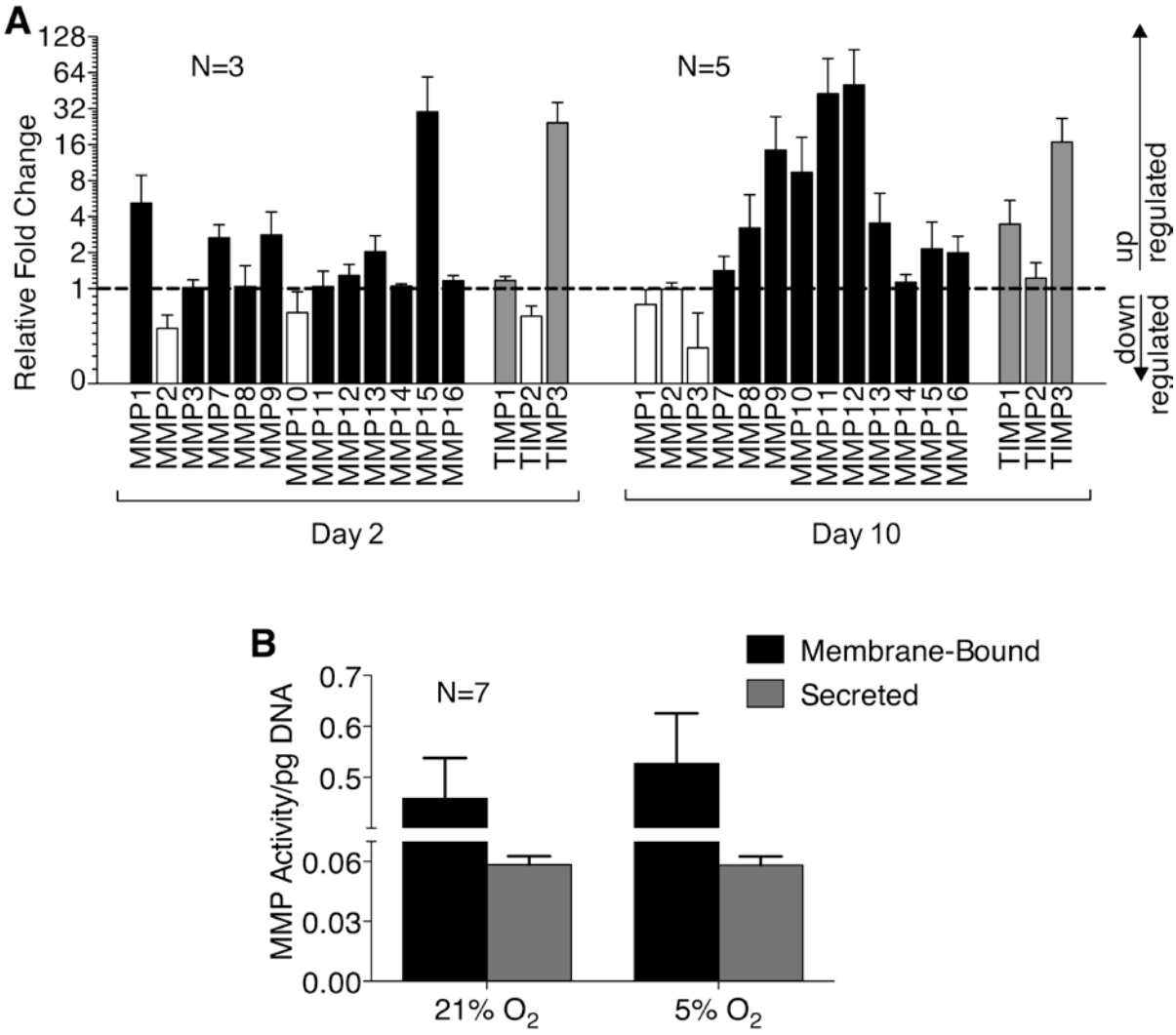

Figure 2.5 Effect of hypoxia on MMP expression and activity. Fold regulation of MMP and TIMP gene expression in MSCs under hypoxic conditions relative to normoxic conditions (**A**). Black = upregulated MMPs. Gray = up-regulated TIMPs. White = down-regulated MMPs and TIMPs. Dashed line indicates a fold change of 1. Membrane-bound and secreted MMP activity (**B**). Error bars represent standard error of the mean.

2.4.7 Hypoxia During Differentiation but not Preconditioning Enhances Osteogenic Matrix Mineralization

Hypoxia enhanced alkakine phosphatase staining at 14 days of osteogenic induction, and quantitative PCR showed increased gene expression of osteocalcin, collagen type I, and runt-related transcription factor 2 (*RUNX2*) in hypoxic osteogenic cultures relative to normoxic osteogenic cultures, in addition to many other markers of osteogenesis, with the exception of cathepsin K, which was significantly down-regulated (p=0.01) (Figure 2.6F). Two-way ANOVA analyses were performed to examine the effect of preconditioning oxygen tension and differentiation oxygen tension on both total matrix mineralization and matrix mineralization normalized to cell number. A significant interaction between the effects of preconditioning oxygen tension and differentiation oxygen tension on total matrix mineralization could not be detected (F =2.078, p=0.16). There was a significant main effect of differentiation oxygen tension on total mineralization (F=7.506, p=0.01), but not of preconditioning oxygen tension (F=0.968, p=0.333). Post-hoc tests (Bonferroni) of the differentiation oxygen tension effect showed that cultures differentiated at 5% O_2 yielded higher total mineral content (p<0.05), but this effect was only apparent in cultures preconditioned at 21% O_2 (Figure 2.6G). After normalization of mineralization to DNA content in each culture, no significant effects of oxygen tension could be detected (Figure 2.6H).

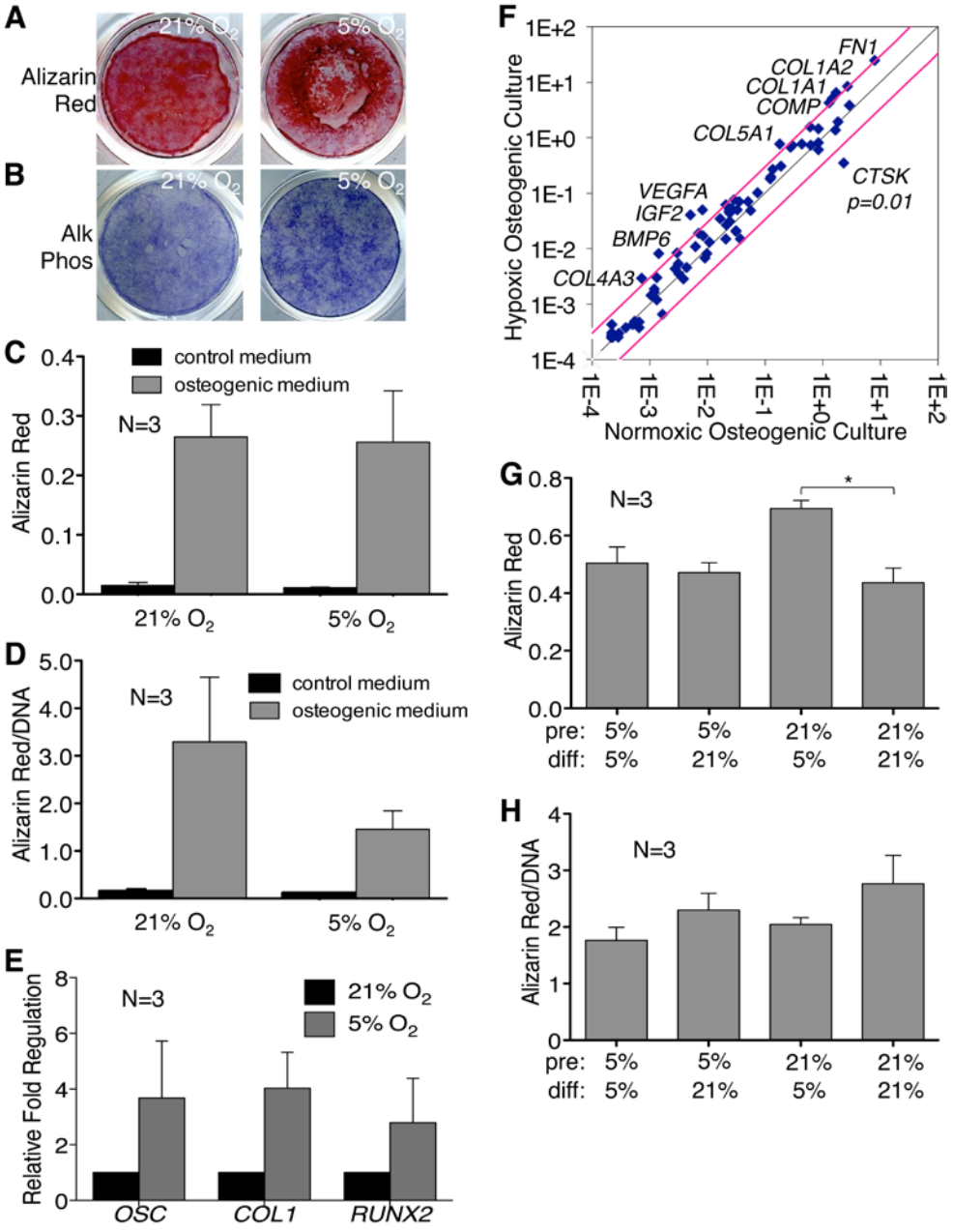

Figure 2.6 Effects of hypoxia on MSC osteogenesis. Alizarin Red (**A**), staining quantified (**C**), and normalized to DNA (**D**). Alkaline phosphatase (**B**). Expression of osteogenic markers (**E**) and osteogenesis-associated genes (**F**). Black line indicates a fold-regulation of 1; pink lines define fold-regulation of -3 to 3. Effect of hypoxic preconditioning: Alizarin Red quantitation (**G**), normalized to DNA (H). Significant differences of relevant Bonferroni comparisons are indicated: * indicates a p value of <0.05. Abbreviations: Alk Phos = alkaline phosphatase, pre = preconditioning oxygen tension, diff = differentiation culture oxygen tension; gene abbreviations are as defined by the HGNC. Images of the cultures were obtained at 1X and are representative of three donors tested. Error bars represent standard error of the mean.

2.4.8 Adipogenesis of MSCs is Inhibited by Hypoxia

A two-way ANOVA performed to determine the effects of preconditioning oxygen tension and differentiation oxygen tension on adipogenesis revealed no significant interaction or main effect of either variable, but there was a trend for hypoxia applied during differentiation to inhibit adipogenesis relative to normoxia. When we examined the data to look at the response of individual patient samples, we observed that some samples exhibited modest adipogenic differentiation under all experimental conditions (apparent differentiation relative to non-induction medium controls, but lack of responsiveness to changing oxygen tensions). If we excluded these subjects, the observed effect of differentiation oxygen tension was statistically significant in cultures preconditioned in normoxia ($p<0.05$) (Figure 2.7).

2.4.9 Chondrogenesis is Enhanced in MSCs Expanded in Normoxia Then Differentiated in Hypoxia

Overall, hypoxic preconditioning decreased chondrogenesis of MSCs in pellet cultures accompanied by lower cartilage quality scores on histologic analysis. The 5%-5% condition generated extremely low quantities of sulfated GAGs per cell number compared to other conditions tested. MSCs preconditioned in normoxic conditions differentiated robustly in pellet cultures under both hypoxic and normoxic conditions (21%-5% and 21%-21%). The 21%-5% condition showed enhanced chondrogenesis compared to the 21%-21% condition, as demonstrated by wet pellet size, Alcian Blue staining, and Safranin-O/Fast Green staining (Figure 2.7). Two-way ANOVAs were performed to examine the effect of oxygen tension during preconditioning and during differentiation on both cartilage quality score and sulfated GAG content of pellets. There was a significant interaction between the effects of preconditioning O_2 and differentiation O_2 on cartilage quality score (F =5.367, p=0.023). There was a significant main effect of preconditioning O_2 on cartilage quality score (F=4.110,

p=0.046), but not of differentiation O_2 (F=0.827, p=0.366). Post-hoc tests of the preconditioning effect showed that MSCs preconditioned at 21% O_2 generated pellets with higher cartilage quality scores when differentiated at 5% O_2 (p<0.01), but the positive effect of preconditioning at 21% O_2 was lost when the pellets were differentiated at 21% O_2 (p>0.05) (Figure 2.7F). There was also a significant interaction between the effects of preconditioning O_2 and differentiation O_2 on sulfated GAG content of the pellets (F=8.642, p=0.006). There was a significant main effect of differentiation O_2 on sulfated GAG content (F=14.50, p=0.0006), but not of preconditioning O_2 (F=2.458, p=0.126). Post-hoc tests of the differentiation O_2 effect showed that MSCs preconditioned at 5% O2 produced significantly more sulfated GAGs when subsequently differentiated at 21% O2 (p<0.0001), whereas MSCs preconditioned at 21% O2 were able to generate a large quantity of sulfated GAGs whether they were differentiated at 5% O2 or 21% O2 (p>0.05) (Figure 2.7G). In monolayer chondrogenic culture, hypoxia had no discernible effect (Figure 2.8).

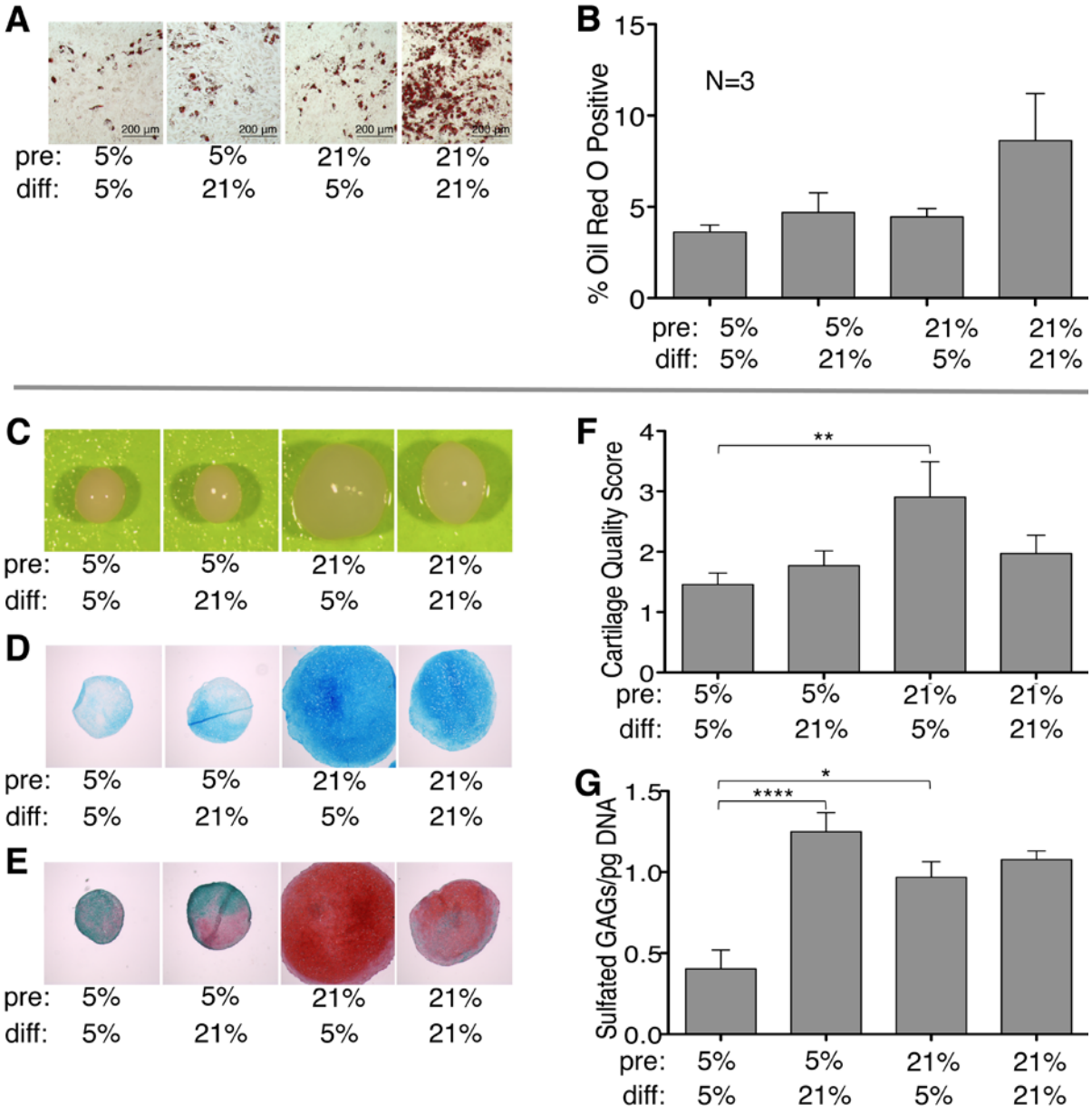

Figure 2.7 Hypoxia inhibits adipogenesis but enhances chondrogenesis. Adipogenesis: Oil Red O-stained accumulated intracellular lipid droplets (**A**), and staining quantified (**B**). Photomicrographs captured at 10x. Chondrogenesis: wet pellet (**C**), Alcian Blue (**D**), and Safranin-O/Fast Green (**E**). Photomicrographs captured at 4x, scale bar = 200µm. Effect of hypoxic preconditioning on cartilage quality score (**F**) and content of sulfated GAGs (**G**). Significant differences of relevant Bonferroni comparisons are indicated: *, **, **** indicate p values of <0.05, <0.01, and <0.0001, respectively. Abbreviations: pre = preconditioning oxygen tension, diff = differentiation culture oxygen tension.

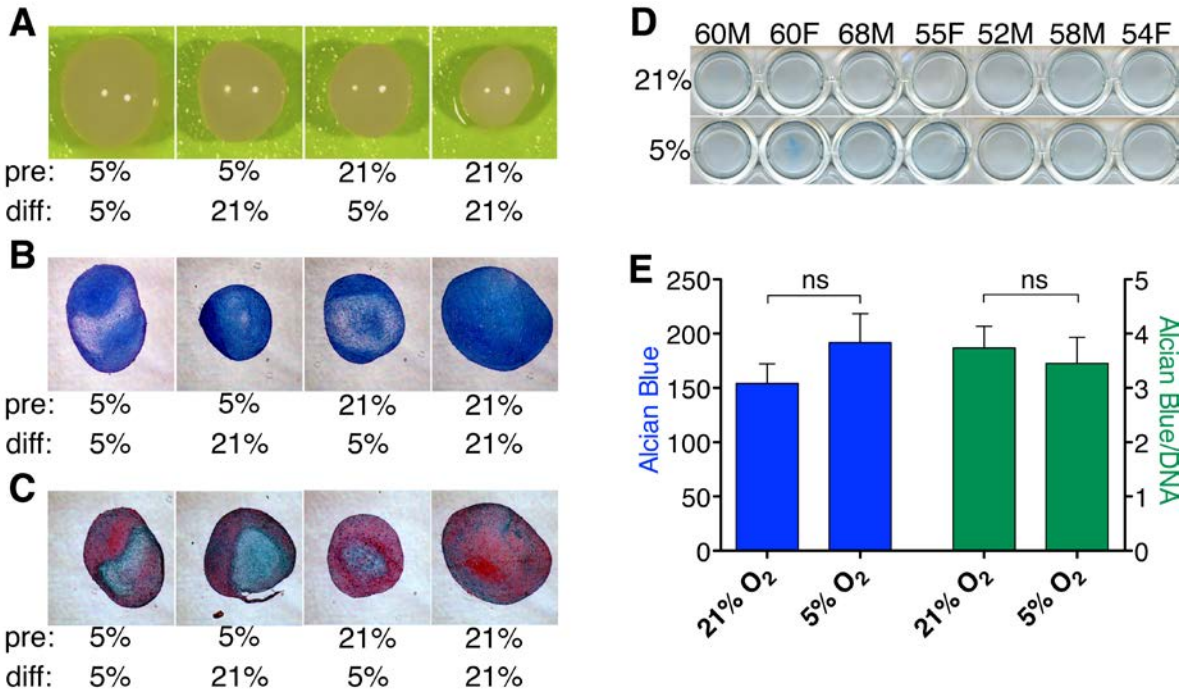

Figure 2.8 Pellet cultures from some subjects displayed hypoxic cores with exposure to hypoxia. Wet pellet (**A**), Alcian Blue staining (**B**), and Safranin-O/Fast Green staining (**C**). Photomicrographs captured at 4x. Alcian Blue staining of monolayer chondrogenic cultures (**D**) showed no difference in sGAG content in hypoxic cultures compared to normoxic cultures (**E**). Photographs captured at 1x.

2.5 Discussion

We observed hypoxia-enhanced MSC clonogenicity in all samples tested. Hypoxia-enhanced proliferation of MSCs was accompanied by no detectable change in cell death. Of note, all of our proliferation experiments were conducted in high glucose culture medium supplemented with serum. MSCs have been previously reported to be more sensitive to oxygen deprivation in the face of concurrent nutrient deprivation.[344, 345]

We employed PCR arrays to screen for signaling pathways related to colony formation, which might be perturbed by hypoxia. We observed small but significant changes in several genes, some of which regulated glycolysis and IGF signaling, and many with interesting connections to regulation of Wnt /β-catenin signaling, which is critical for maintenance of stemness in adult and embryonic stem cell populations.[346] After two days of hypoxic culture, among those transcripts differentially regulated were the following up-regulated transcripts: *GPI (*glucose-6-phosphate isomerase/autocrine motility factor) and *MMP8;* and down-regulated transcripts: *CDH1* (E-cadherin), *CTNNA1*(α-catenin), *APC, SMAD2*, and *SMAD3*, all of which are connected in regulation of epithelial-to-mesenchymal transition (EMT) and canonical Wnt signaling through modulation β-catenin trafficking and signaling, either directly or through release or increase of cell adhesions.

GPI is likely up-regulated to enhance glycolytic metabolism as the MSCs accommodate for lowered aerobic respiration in a hypoxic environment. GPI has been shown to promote degradation of β-catenin, but also to enhance its expression and nuclear translocation.[347] Down-regulation of E-cadherin, α-catenin, and *APC,* all known to inhibit nuclear translocation and signaling of β-catenin,[348-351] also suggests enhanced Wnt/β-catenin activity at this time-point. Down-regulation of SMAD2 and SMAD3, both of which enable β-

catenin co-activation in response to TGFβ signaling and vice versa,[352-356] suggests that cross-talk between TGFβ and Wnt signaling pathways is playing a less important role in directing cell fate after brief (2 day) exposure of undifferentiated MSCs to hypoxia.

After ten days of hypoxic culture, none of these specific genes continued to be significantly differentially regulated in hypoxic versus normoxic cultures, suggesting they are differentially regulated as the cell reestablishes homeostasis. In the case of MSCs this is likely achieved through metabolic conversion to a largely glycolytic phenotype; induction of this phenomenon, known in cancer biology as the Warburg effect, can be mediated through Wnt or β-catenin activation, either in combination or with other signaling partners.[357-359] At that point, however, when the cells are at or approaching a steady state, we observed differential regulation of different actors shown to impact the same signaling pathway, namely the following up-regulated transcripts: *IGFBP1*, *VEGFA*, *CDC2*, *SPARC;* and down-regulated transcripts: *HIF1A, TNFRSF21 (DR6)* , *CDKN1A, CUL1,* and *HIF1AN.*

IGFBP1 serves to stabilize IGF receptor expression at the cell surface, suggesting MSCs are ramping up IGF signaling in hypoxic conditions, which would enable them to make use of the abundant glucose in the medium for these experiments to drive cell proliferation; up-regulation of the glucose transporter *SLC2A1*, also known as *GLUT1*, further supports this. However, IGFBP1 is also an inhibitor of β-catenin and may be executing signaling functions outside of the primary IGF axis. *HIF1A* was down-regulated in long-term hypoxic culture relative to normoxia; regulation of *HIF1A* in hypoxia has been shown to occur as a result of altered redox status of the cell.[360-362] This would suggest that in the absence of caloric restriction MSCs readily adapt to hypoxic conditions and are able to establish redox homeostasis.

In longer term cultures we also observed significant up-regulation of leptin. Hypoxia has been shown to induce leptin expression in multiple cell types, including preadipocytes and MSCs.[363, 364] Leptin has a somewhat cryptic role in influencing MSC fate, due to its distinct systemic versus local activities and disparate effects on different mesenchymal cell types, but is generally thought to serve as a regulatory link between fat and bone, with secretion of leptin by fat serving to protect bone through maintenance of mineralization.[365] Leptin has been shown to maintain peripheral mesenchymal progenitors in an undifferentiated state, but to also promote mineralization in differentiated osteoblasts.[366]

Analysis of the effect of hypoxia on immunophenotype of MSCs showed low level CD45 expression on MSCs cultured in normoxic conditions, whereas hypoxia appeared to maintain MSCs in culture with no CD45 expression. MSC fractions have been shown to have low CD45 and low CD146 expression directly after harvest from bone marrow,[367, 368] and MSCs can be induced to express CD45 after epigenetic modification using 5-aza-2'-deoxycytidine.[369] MSCs cultured under normoxic conditions expressed CD146 as expected; however MSCs cultured under hypoxic conditions were negative for CD146, a known pericyte marker. This finding has been observed by others, with CD146 expression in CFU-Fs correlating with in situ localization to the endosteal versus perivascular space and induction of CD146 expression upon *ex vivo* culture in normoxic conditions.[368] Taken together, these findings regarding CD45 and CD146 expression suggest that during *in vitro* culture hypoxia maintains an MSC immunophenotype more akin to what would be observed *in vivo* in the bone marrow or in avascular cartilaginous tissues than does normoxia. CD146 negativity has been associated with increased chondrogenic potential in adult stem cells[370] and other chondrogenic progenitor populations.[371]

Differential expression of genes related to growth factor signaling and inflammatory signaling prompted our investigation of growth factor and TNF levels in the medium. In low-density cultures recapitulating the environment of a colony-forming assay, we observed higher VEGF levels in hypoxic cultures over time, which likely contributed directly to enhanced colony formation in hypoxia through its regulation of proliferation. In high-density cultures we observed slightly higher overall VEGF levels in hypoxic cultures, but lower VEGF production per cell in hypoxic cultures after normalization to cell number of the culture, suggesting that VEGF secretion in MSC culture is tightly regulated beyond a critical threshold. VEGF secretion in MSCs was enhanced by hypoxia in several studies,[372] and hypoxia has been shown both to induce expression of and increase the half-life of *VEGF* mRNA.[373] VEGF secretion has been shown to be attenuated in hypoxic cultures by the presence of high glucose concentrations,[345] which may explain why the observed effect here was not more pronounced.

While we observed increased expression of *MMP* genes and *TIMP* genes with hypoxia, there was no increase in MMP activity in supernatants from hypoxic cultures. When we looked at MMPs retained on the cell surface and in the extracellular matrix, slightly higher overall MMP activity was observed in hypoxic cultures. This would suggest that MSCs are in a matrix-stabilizing state in normoxic cultures, and that hypoxia may be a stimulus for matrix turnover, enabling cell migration and contributing to colony expansion, but with continued tight regulation of matrix metalloproteinase activity.

Application of hypoxia to osteogenic cultures during differentiation yielded more mineralized tissue, but no more mineral deposition per cell in the tissue. This would suggest that hypoxia is useful for tissue engineering of bone *ex vivo*, but from a cell biology perspective does not necessarily enhance the osteogenic potential of an individual MSC. It is possible

that alkaline phosphatase activity was higher in hypoxic cultures as an indication of preserved stemness or of enhanced osteogenic differentiation; other groups have shown that levels of alkaline phosphatase activity are not proportional to observed mineralization levels in *in vitro* osteogenesis assays.[374] When we looked closely at gene expression data from osteogenic cultures, we noticed that osteogenic induction with supplemented medium up-regulates cathepsin K expression relative to undifferentiated MSCs. This effect is nullified by the addition of hypoxia, which down-regulated cathepsin K expression in all patient samples tested to the level of undifferentiated cultures (Figure 2.6). Boskey et al reported that in a chick limb-bud mesenchymal micromass culture system, decreased mineral deposition was seen upon pharmacological inhibition of cathepsin K during early stages of matrix mineralization.[375] They proposed a mechanism whereby reduced proteoglycan catabolism in the extracellular matrix in the setting of cathepsin K inhibition results in inhibited hydroxyapatite formation.

Hypoxic preconditioning did not enhance osteogenesis, as indicated by Alizarin Red quantification of matrix mineralization, either in total or when normalized to DNA content. Analysis of the differentiation oxygen tension revealed that our hypoxic cultures were mineralized to the same degree as our normoxic cultures (per cell), but with a higher number of cells present. This would suggest that matrix mineralization either starts later in hypoxic cultures or is less efficient per cell. The fact that this effect was significant only after preconditioning in normoxia suggests that there is in fact an interaction effect of preconditioning and differentiation oxygen tensions, but that the effect was not great enough to be detected here. We conclude that expanding cells in normoxic conditions, then differentiating in hypoxia generates the highest total mineral content, although this result

must be interpreted with caution because an increased rate of cell death can also enhance mineral deposition in 2D cultures.[376]

It is likely that 2D *in vitro* assays of osteogenesis give an incomplete picture of MSC osteogenic potential. Multiple studies have demonstrated that hypoxia enhances osteogenesis in 3D *in vitro* culture, including one study where the authors directly compared 3D to 2D cultures and found no enhancement with hypoxia in 2D.[377, 378] However, another recent study evaluated the effect of hypoxia on osteogenesis in 3D culture and found no enhancement.[379] Increased oxygenation by delivery with perfluorotributylamine has been shown to enhance osteogenesis in a 3D *in vivo* model,[380] yet another study examining *in vivo* osteogenesis in hypoxic (13%), normoxic (21%), or hyperoxic (50%) conditions found that no condition significantly improved early osteogenic or chondrogenic differentiation in a fracture healing model.[381] Preconditioning MSCs in hypoxia *ex vivo* prior to implantation into a rabbit calvarial defect model resulted in improved bone formation compared to normoxic preconditioning.[382] Similar results have been achieved using hypoxic preconditioning prior to Achilles tendon repair with MSCs in a rat model.[383] It is difficult to determine the effect of *ex vivo* hypoxic preconditioning using *in vivo* studies of differentiation because of the enhanced survival and proliferation conferred on MSCs by hypoxic preconditioning, which may account for their enhanced function in critical defects and ischemia models, rather than increased differentiation potential *per se*.

It is also possible that conflicting reports exist in the literature regarding molecular events associated with hypoxia and osteogenesis due to inappropriate selection of housekeeping genes/proteins. *GAPDH*, for example, is commonly used as a housekeeping gene in these studies despite the fact that it is differentially regulated in hypoxic versus normoxic conditions.[384]

There was a trend for adipogenesis to be inhibited under hypoxic conditions, which is consistent with the findings of most other groups. The HIF-1α regulated gene *DEC1(STRA13)* has been shown to repress *PPARG* thereby transducing oxygen sensing as a signal for suppression of adipogenesis.[385, 386] Preconditioning with hypoxia also appeared to suppress adipogenesis even when MSC cultures were switched to normoxia for differentiation. However, it should be noted that the effects of oxygen tension on adipogenesis were patient sample dependent, and as a result not statistically significant in our study.

Upon histological examination of chondrogenesis in pellet culture, we observed internal heterogeneity at 5% O_2, with obvious cartilage formation in patches and fibrous matrix deposition lacking proteoglycans in other areas. Despite this patchy differentiation, sGAG content was still higher overall when normalized to DNA content of the pellet compared to normoxic pellets, suggesting that there was a higher rate of cell death in hypoxic pellets, but the surviving cells differentiated robustly. This is perhaps due to uneven oxygen diffusion within the 3D pellet culture. To further assess these results, we performed a monolayer screen for chondrogenic potential in hypoxic versus normoxic MSC cultures. Chondrogenesis was poor in all monolayer cultures, consistent with the findings of others.[387] Alcian Blue staining, quantified after guanidine-HCl extraction, was not different after normalization to DNA content of the culture. Markway et al have shown enhanced chondrogenesis with hypoxia in micropellet cultures, where oxygen concentration is more consistent throughout the pellet. While that model is excellent for addressing the problem of gas and nutrient distribution in the pellet, its obvious limitation is that for tissue engineering purposes we would like to study larger pieces of cartilage in culture rather than smaller ones.

For chondrogenic differentiation we found that the best overall results were obtained by expanding MSCs in normoxic conditions, then performing pellet culture with chondrogenic induction medium in hypoxic conditions. This approach has been practiced in many labs based on the empiric finding that it works, but pellet cultures from some patients developed severely hypoxic cores (Figure 2.8). It is possible this approach could be improved with the application of a flow-perfusion bioreactor to optimize diffusion of oxygen and nutrients throughout the pellet. In all cases preconditioning with hypoxia failed to enhance chondrogenesis, and in most subjects significantly inhibited it. When we considered these results in the context of our MMP expression data, the possibility emerged that enhanced chondrogenesis in hypoxia is mediated, at least in part, through regulation of MMP activity. It has been established that MMP production is critical for progression through chondrogenic differentiation, both in the growth plate and in pellet culture.[388-392] Dose-dependent disruption of chondrogenic differentiation of MSCs in pellet culture has been demonstrated with application of pan-MMP inhibitors.[392] While we did not directly analyze matrix turnover in cartilage pellets, a significant increase in MMP activity was not observed with hypoxia in conditioned medium, although increased *MMP* transcription and MMP accumulation in matrix were detected. We observed no differential regulation of *ADAMTS1*, *8*, or *13* with hypoxic versus normoxic culture (data not shown). Overall, this suggests hypoxia-mediated protease secretion may influence matrix accumulation by modulating growth factor activity and matrix molecule processing, particularly in early chondrogenesis, when others have shown MMPs are required for differentiation. Extremely low oxygen tension may detrimentally impact matrix accumulation through unregulated high matrix protease activity leading to matrix degradation, which becomes evident later in chondrogenic pellet culture.

Interestingly, *HIF1A* was slightly but consistently down-regulated over time in our long-term hypoxic cultures (10 days), at which time we saw more dramatic up-regulation of *MMP* genes, as well as *TIMP1* and *TIMP2*, in hypoxic cultures compared to normoxic cultures, than was observed at day 2. Thus MMP activity may be tightly controlled by the concurrent hypoxia-mediated increase in protease inhibitors. Previous data from our group indicate that MSCs overall maintain a matrix-protective MMP/TIMP secretion profile even in the context of stressors like hypoxia and inflammatory cytokines, which is consistent with the findings from this study.[393]

2.6 Conclusion

Mesenchymal stem cells display context-specific responsiveness to hypoxia as a differentiation cue during development and tissue repair. The existing conflicts in the literature regarding the effects of hypoxia on MSC differentiation potential likely stem from differences in cell culture methodology between laboratories and the specific models utilized to test effects on differentiation, as well as how differentiation is defined and measured. Oxygen tension should be carefully considered as a variable when culturing cells for tissue engineering applications. Our study conclusively demonstrates that expansion of MSCs in normoxic culture followed by application of hypoxia during chondrogenic differentiation is a useful technique for enhancing *ex vivo* chondrogenesis.

Chapter 3

Altered Colony Formation Dynamics of Adult Bone Marrow-Derived Mesenchymal Stem Cells in Response to Glucose Concentration and Ascorbate

3.1 Abstract

Media formulations for MSC culture and differentiation vary between labs; two notable variables addressed inconsistently in the field are ascorbate supplementation and glucose concentration in MSC expansion medium. Glucose and ascorbate effects are coupled to the Hif1α pathway. Therefore, we examined the effects of ascorbate supplementation and low versus high glucose medium in the settings of hypoxia and normoxia on colony formation, colony morphology, proliferation, VEGF secretion, and senescence of MSCs. We found that there is no one medium formulation that maximizes MSC performance for all of these assays. Depending on the desired outcome of the culture, the medium and other culture conditions can be adjusted to optimize for a particular aspect of MSC biology, such as colony formation when clonal selection is the desired end application or delayed senescence if MSCs are to be expanded extensively.

3.2 Introduction

MSCs are prized by the researchers who work with them for their ability to survive and function normally in conditions that would be intolerable to many cell types. This intrinsic ability to withstand stress is critical for their use in cell-based therapeutic applications *in vivo* and their postulated role as on-site architects of the tissue repair process in normal wound healing. As discussed in Chapter 2, it has been shown by many groups that MSCs proliferate and maintain or scale up their trophic functions in hypoxic environments. Whether this phenomenon reflects biomimicry of the MSC niche or superior metabolic flexibility of the cells is a topic of active interest. There is some evidence to suggest that MSCs strongly express glycolytic enzymes and transporters at baseline and are readily able to up-regulate

components of this pathway in response to hypoxia, in part through direct binding of HIF1α to promoter sequences in these genes.[394] Some members of the glycolytic pathway function not only to enable glycolysis, but also to serve as metabolic sensors and switches, protecting the cell from apoptosis during hypoxia but inducing apoptosis in response to nutrient deprivation.[395]

Metabolic flexibility has been extensively studies in several cell types, in particular cancers and cancer stem cells, beginning with Warburg's work in the 1920s, for which he was awarded the Nobel Prize in 1931. As discussed in Chapter 1, cells that are able to thrive in hypoxic environments frequently do so by switching the bulk of their metabolic dependence from mitochondria-dependent aerobic respiration to anaerobic glycolysis (Figure 3.1). While dysregulated cancer cells can ratchet their levels of glycolysis up to more than a hundred fold higher than normal cells, MSCs exhibit modest but consistent increases in genes associated with glucose transport and carbohydrate metabolism in response to hypoxia, at least at the transcriptional level (see Chapter 2). However, they have been clearly shown to become more dependent on glucose availability in hypoxic conditions, exhibiting higher values of specific consumption rate of glucose,[319] and withstanding continuous, severe hypoxia for long periods of culture in the presence but not the absence of glucose.[344] Similarly, MSCs have been shown to withstand hypoxia in the presence of FBS, but die in long-term hypoxic culture in the absence of FBS.[396]

MSCs have also been shown to consume more glucose and divert it to anaerobic respiration when availability is higher. One study examining glucose consumption of MSCs in various culture media formulations found that glucose consumption increased with the concentration supplied in the medium, and also that lactate-pyruvate ratios reflecting anaerobic versus aerobic metabolism increased as additional glucose was made available.[397]

Interestingly, addition of higher concentrations of fetal bovine serum to the medium enhanced this effect. In addition, pyruvate is routinely added to MSCs in chondrogenic culture conditions. This may enhance glucose consumption and anarobic metabolism (Figure 3.1), and thereby promote chondrogenesis. Whether this suggests that MSCs consume more nutrients, including not only glucose but the proteins provided in serum, to engage in greater anabolic activity or whether consumption simply increases proportionately to availability, is of great interest to the cell therapy and tissue engineering communities.

Despite the supportive effects of adequate glucose in culture medium, there is also evidence that high glucose levels can induce excess intracellular superoxide in MSCs, leading to decreased HIF1α expression and, as a result, decreased expression of trophic factors such as VEGF and PDGF.[345] This has been shown to occur at glucose concentrations of 30 mM, compared to the 25 mM glucose present in commercially available high-glucose media formulations such as the one tested in this study. Low-glucose media formulations have glucose concentrations that come closer to approximating glucose levels in normal serum, on the order of 5 mM.

Like high glucose, ascorbate has also been shown to contribute to HIF inhibition. Ascorbate is a potent antioxidant. The HIF hydroxylases, which mark HIF family members for ubiquitination under normoxic conditions, are ascorbate-dependent,[398] and addition of L-ascorbic acid to cultures has been shown to reduce intracellular HIF levels.[399] In MSCs ascorbate supports many cell functions, and addition to culture media at low levels has been shown to promote proliferation, extracellular matrix secretion, and mesenchymal lineage differentiation.[400] Unlike other mammals, primates lost the ability to synthesize ascorbic acid from glucose with the loss of functional L-gulonolactone oxidase, making recycling of the reduced form of ascorbic acid from dehydroascorbic acid (DHA) all the more critical for

cellular homeostasis, immune defense, and other functions of ascorbate in humans. DHA is transported by the glucose transporters, particularly GLUT1, which, as we demonstrated in Chapter 2, is up-regulated in MSCs upon exposure to hypoxia.

Given their direct role in modulating MSC metabolism and function, and their indirect role through regulation of HIF signaling, we employed a factorial analysis to discern the effects of glucose and ascorbate in MSCs under hypoxia vs. normoxia. We subjected MSCs to eight culture conditions representing all combinations of high (25 mM) vs. low (5 mM) glucose, ascorbate supplement (50 ug/ml) and hypoxia (5% pO_2). We used low density CFU cultures to investigate colony formation, size, and cell orientation, and high density cultures ($6.0x10^3$ cells/cm^2) to investigate cell proliferation, VEGF secretion, and senescence.

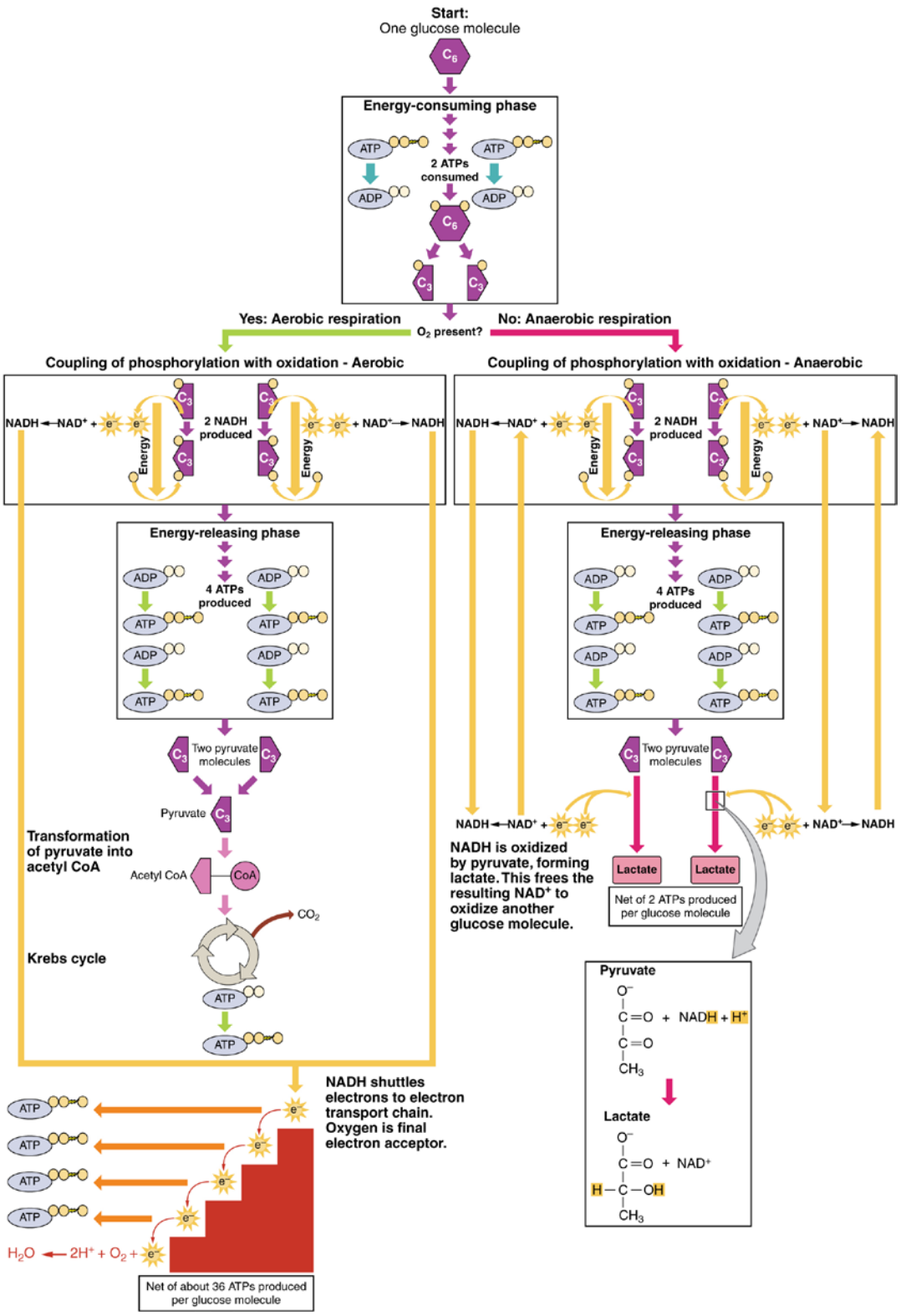

Figure 3.1 Aerobic versus Anaerobic Respiration. Reprinted from Carbohydrate Metabolism, Connexions, OpenStax College, 2013.[401]

3.3 Materials and Methods

3.3.1 Cell Culture

MSCs were harvested from consenting arthroplasty patients with IRB approval as described in Chapter 2. Adherent Passage-0 MSCs were cultured expanded in growth medium consisting of high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen) with antibiotic-antimycotic (Gibco/Invitrogen) and 10% MSC-qualified fetal bovine serum (Gibco/Invitrogen) until they reached 80% confluence, then passaged using 0.25% trypsin and replated at a density of 6.0×10^3 cells/cm^2 as Passage-1 MSCs. At 80% confluence Passage-1 MSCs were trypsinized and cryopreserved in commercially available freeze medium (Invitrogen) and stored in liquid nitrogen.

Upon recovery from cryopreservation, Passage-2 MSCs were cultured in one of the following media: (**1**) low glucose (5.55 mM) Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen) with antibiotic-antimycotic (Gibco/Invitrogen) and 10% MSC-qualified fetal bovine serum (Gibco/Invitrogen), (**2**) low glucose (5.55 mM) DMEM with antibiotic-antimycotic, 10% fetal bovine serum, and 50 μg/ml L-ascorbate-2-phosphate, (**3**) high glucose (25 mM) DMEM with antibiotic-antimycotic and 10% fetal bovine serum, or (**4**) high glucose (25 mM) DMEM) with antibiotic-antimycotic, 10% fetal bovine serum, and 50 μg/ml L-ascorbate-2-phosphate. These cultures were placed in separate closed incubators purged with either 95% air and 5% CO_2 (21% O_2 or normoxia) or 5% oxygen, 5% CO_2, and 90% nitrogen (5% O_2 or hypoxia).

3.3.2 Colony Forming Unit-Fibroblast (CFU-F) Assay

CFU-F assays were performed as described in Chapter 2. Briefly, one hundred Passage-2 MSC's were plated on 10 cm tissue culture-treated polystyrene dishes in triplicate in one of

the four growth media described above and cultured for 14 days with medium changes every 3 days. At the end of 14 days colonies were rinsed with phosphate-buffered saline (PBS), fixed with crystal violet dye in methanol, and again rinsed with PBS to remove residual dye. Differences in colony number were computed as a percent change for the variables ascorbate supplementation and glucose concentration in both hypoxic and normoxic cultures.

3.3.3 Cell Proliferation Assays

The Click-iT EdU Alexa Fluor 647 Cell Proliferation kit (Molecular Probes) was used according to the manufacturer's protocol as described in Chapter 2. Briefly, MSCs plated at $6.0x10^3$ cells/cm^2 were incubated with 10 μM Click-iT EdU for 16 hours, fixed, permeabilized, labeled, and EdU was detected via flow cytometry using a FACSAria cytometer and FACSDiva software (Becton Dickinson). Data were analyzed using FlowJo (Tree Star).

3.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Secreted levels of VEGF in culture media of CFU plates were quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol as described in Chapter 2. VEGF ELISAs (Pierce/ThermoFisher) were performed on 3-day and 12-day culture supernatants applied to pre-coated microtiter plates, and read colorimetrically on a BioTek microplate reader. All samples and standards were measured in duplicate. Results were normalized to DNA content of each culture as determined by Picogreen assay (Invitrogen).

3.3.5 Analysis of Cell Orientation within Colonies

Cells organization within MSC colonies was evaluated by calculating the degree of cell alignment with the radial axis from the colony center. Cell orientations were compiled both within the outer portion of the colony, of interest because this is the zone where cell migration is highest, and at the leading edge of the outer portion of the colony, where cell density is lowest and cells are most free to change their orientation (Figure 3.2). Absolute cell direction was defined as the direction of the major axis of the ellipse fitting a cell's shape using NIS-Elements v 4.13 imaging software (Laboratory Imaging s.r.o., Praha, Czech Republic). The radial orientation was then defined as the relative angle between the global cell orientation and the radial line passing through the cell centroid (ellipse center). Radial orientations of the cell with respect to the leading edge of the colony aare demonstrated in Figure 3.3, where cells orthogonal to the radial line measured 90 degrees, and cells oriented in the same direction as the radial line measured 0 degrees. Radial orientation was measured for every cell within the outer area and within the leading edge area. Results are reported with descriptive statistics and analyzed as described in the statistical analysis section (3.3.7).

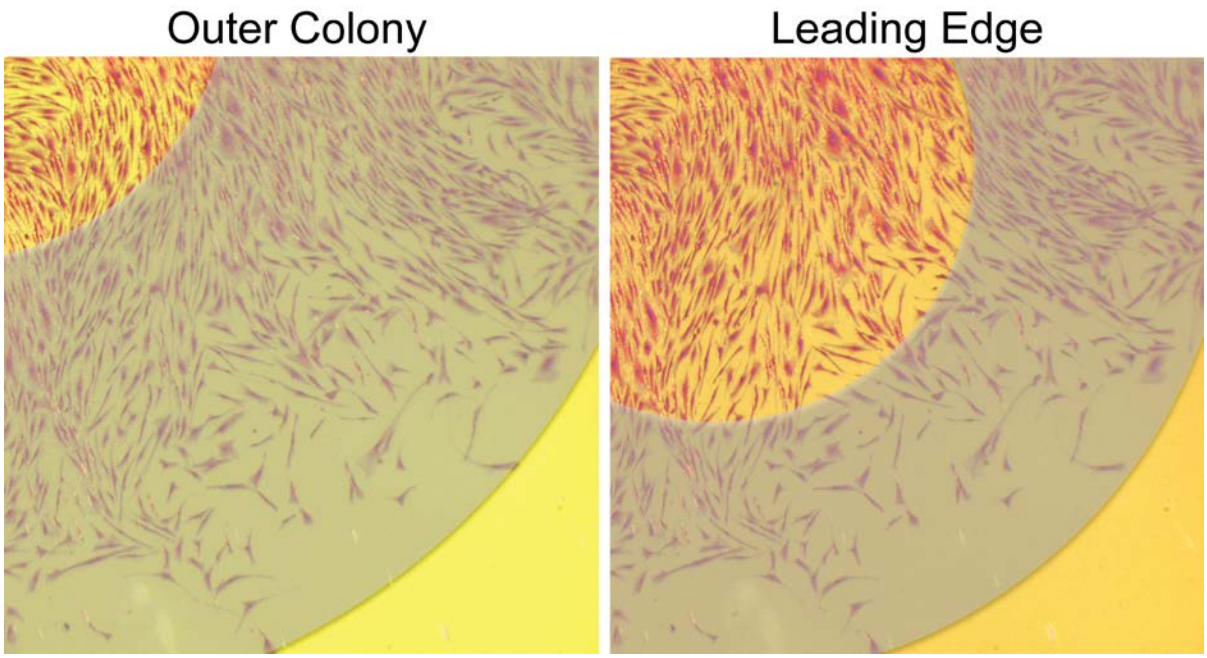

Figure 3.2 Zones of cell orientation analysis. Cells within MSC colonies were evaluated for their radial orientation with respect to the colony's leading edge. Cell orientations were compiled both within the outer portion of the colony (left), where cell migration is highest, and at the leading edge within the outer portion of the colony (right), where cell density is lowest and cells most free to change orientation.

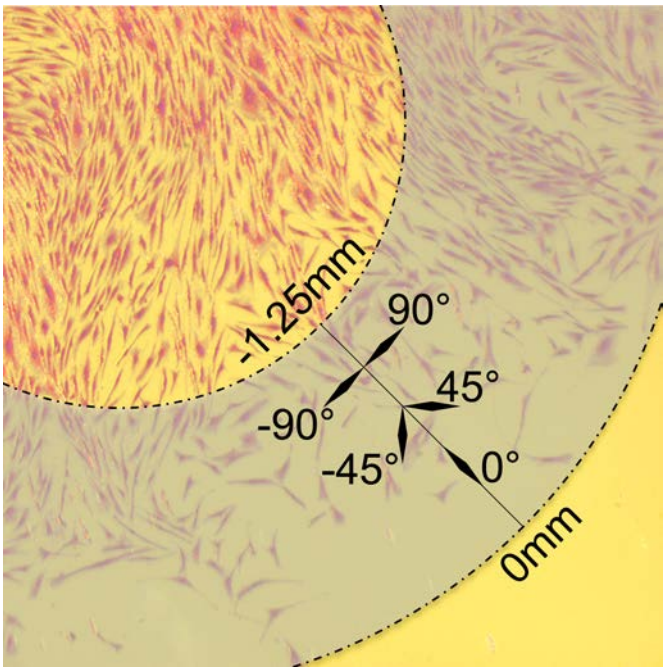

Figure 3.3 Cell orientation measurements. Radial orientation of the cell with respect to the leading edge of the colony (0mm) was measured as shown above, where cells orthogonal to the tangent edge measured 0 degrees, and cells oriented in the same direction as the tangent measured 90 degrees. Radial orientation was measured for every cell at each linear position from the edge of the colony (0mm) inward for the outer 1.25mm of the colony (leading edge figures) or the outer 3mm of the colony (outer colony figures).

3.3.6 Senescence-Associated β-Galactosidase Staining

Senescence was detected in MSC cultures plated at $6.0x10^3$ cells/cm^2 by senescence-associated β-galactosidase (SA-β-gal) staining using a commercially available kit (Cell Signaling Technologies) according to the manufacturer's protocol. MSCs were cultured in one of the four media described in method 3.3.1 above in both hypoxic and normoxic culture conditions. Cells were then rinsed with PBS, fixed with a 2% paraformaldehyde-based reagent provided in the kit, rinsed with PBS twice more, and incubated with the SA-β-gal stain overnight in a dry room air 37 degree incubator. Cultures were imaged after

counterstaining with eosin and the ration of SA-β-gal cells to total cells manually counted.

3.3.7. Statistical analysis: Significant differences in outcomes were evaluated by a repeated measures (blocking for patient effects) two-way ANOVA to examine the variables ascorbate supplementation and glucose concentration in both hypoxic and normoxic cultures, with matched subjects serving as their own controls and Bonferroni post-tests to detect differences between group means.

3.4 Results

3.4.1 Ascorbate Supplementation Does Not Enhance MSC Clonogenicity

We demonstrated in Chapter 2 that hypoxia significantly enhances colony count in colony forming assays compared to normoxia (see Chapter 2). In this chapter, we find that hypoxia also significantly enhanced colony diameter compared to normoxia ($p<0.001$) (Figure 3.4). Addition of ascorbate to CFU-F cultures had no significant effect on colony counts or colony diameter irrespective of oxygen tension. Performing CFU-F assays in high glucose culture medium significantly increased colony count (98.75% ± 2.5%, $p=0.01$) compared to low glucose medium (Fig 3.4.B)

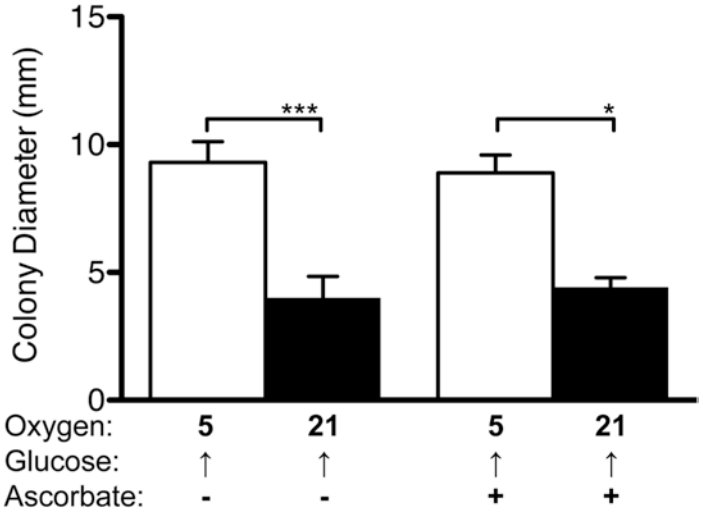

Figure 3.4.A Effect of hypoxia and ascorbate supplementation on CFU-F colony diameter in high glucose medium. Significant are indicated: *, **, *** indicate p values of <0.05, <0.01, and <0.001, respectively.

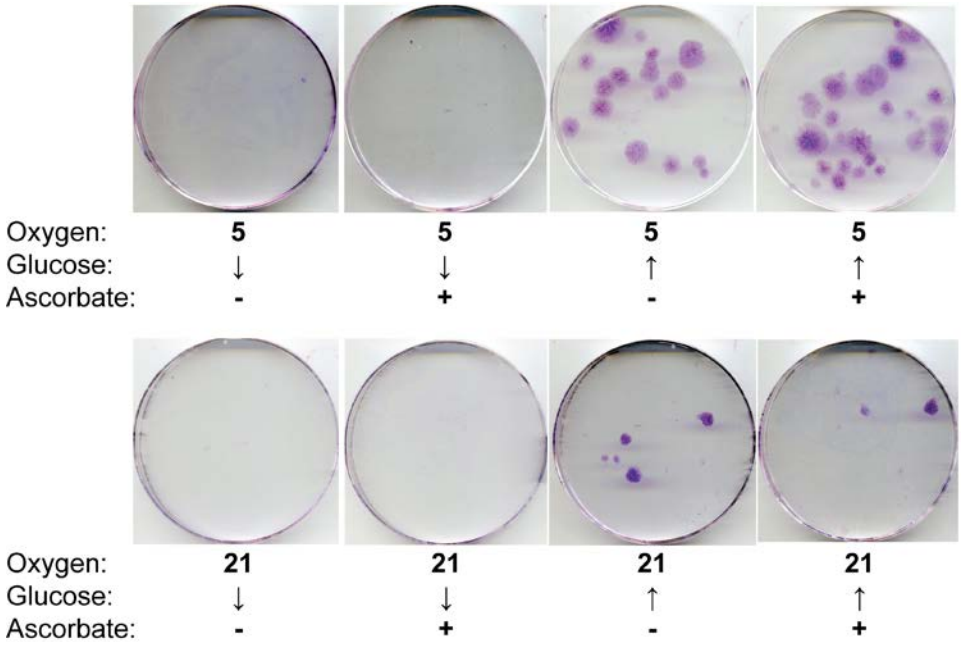

Figure 3.4B High glucose medium enables glycolytic switch and colony formation in isolated culture conditions. In some donors the manifestation of MSC reliance on caloric availability for glycolysis during times of stress manifests dramatically, as in this subject, where colony formation is near absent in low glucose medium.

3.4.2 Ascorbate Supplementation Enhances MSC Proliferation

In Chapter 2 we discussed the effect of hypoxic conditions enhancing proliferation as shown by EdU incorporation in higher density expansion cultures. Knowing oxygen tension has a significant effect, we then examined the combinatorial effects of glucose level and ascorbate supplementation in culture medium at both high and low oxygen tensions on proliferation as measured by EdU incorporation. The use of this assay, unlike the colony forming assay, enabled us to look at early versus late effects on cell growth. At day 3 we noted a significant enhancement of MSC proliferation with ascorbate supplementation regardless of medium glucose concentration or oxygen tension applied to the culture ($p<0.001$) (Figure 3.5A). By day 10, however, this effect was only apparent in cultures raised in low glucose medium and then only significant in cultures exposed to a normoxic

atmosphere ($p<0.05$) (Figure 3.5B). Glucose concentration did not have a significant effect on MSC proliferation at three days, but low glucose in the medium enhanced proliferation at ten days in the presence of ascorbate irrespective of oxygen tension ($p<0.05$). There was no significant interaction effect of glucose and ascorbate.

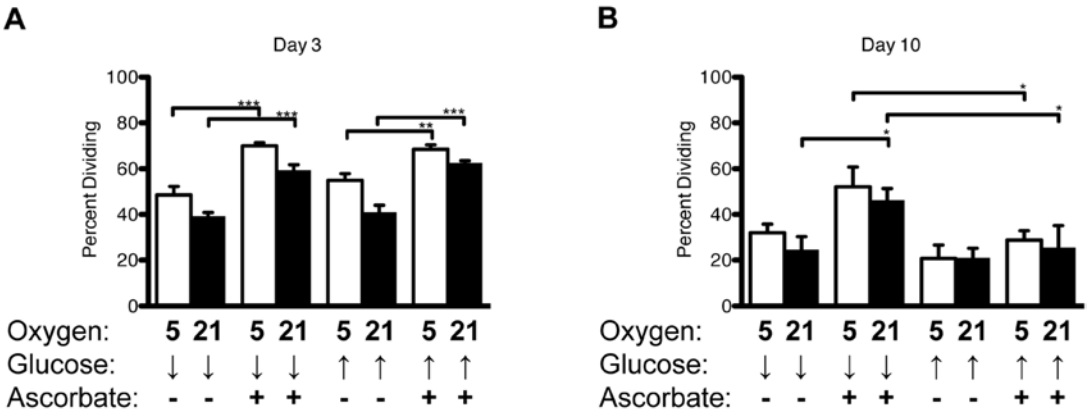

Figure 3.5 Ascorbate enhances proliferation during early expansion culture; low glucose later. Combinatorial effects of ascorbate supplementation and medium glucose concentration in hypoxic and normoxic culture conditions after 3 days (**A**) and 10 days (**B**). Significant differences are indicated: *, **, ***, **** indicate p values of <0.05, <0.01, <0.001, and <0.0001, respectively.

3.4.3 Ascorbate Supplementation Does Not Enhance MSC VEGF secretion

We recently demonstrated that hypoxia induces maintenance of higher VEGF secretion by MSCs grown in low density CFU-F cultures (see Chapter 2). In this study we examined the combined effects of medium glucose concentration and ascorbate supplementation on VEGF secretion in both hypoxic and normoxic conditions. Mean levels of VEGF secretion at days 3 and 12 were not increased by low glucose. Low glucose always increased VEGF section at day 12 except in hypoxic cultures without ascorbate supplementation. Culture in low glucose medium under this single condition decreased VEGF section, indicating a significant interaction of glucose with hypoxia and ascorbate supplement on day 12 ($p=0.02$)

(Figure 3.6). A trend was apparent of ascorbate increasing VEGF secretion at day 3 (p=0.06). At day 12, it is apparent that hypoxia increases VEGF, as also observed in Chapter 2.

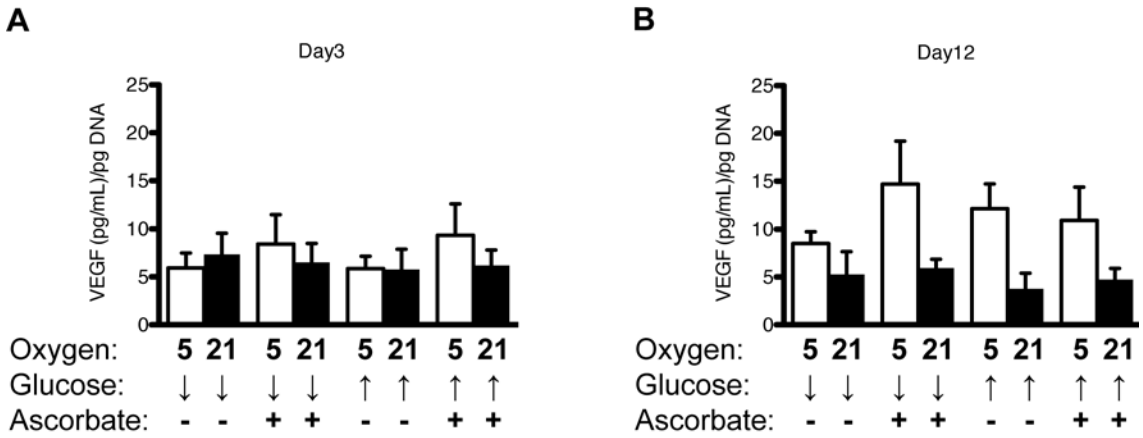

Figure 3.6 Effects of oxygen tension, glucose, and ascorbate on VEGF secretion in CFU-F cultures after 3 days (**A**) and 12 days (**B**).

3.4.4 Cell Orientation

We determined radial orientation as a function distance from the colony edge for MSCs in colonies grown in high glucose medium with or without ascorbate supplementation in hypoxic and normoxic cultures (Figure 3.7). We tabulated these results for the two zones of interest at the colony edge, the outer region and the leading edge area. Initially we examined from the outer 3mm of each colony, which we characterized as the "outer colony region", and subsequent to that we focused more closely on approximately the outer 1-1.5mm of each colony, which we termed the "leading edge zone". While addition of ascorbate to culture medium appeared to increase whorl pattern formation in the outer portion of the colony, there were no quantifiable differences in the range of cell orientations observed in the outer portion of the colony or at the leading edge. For some conditions the slope of linear regressions we performed was non-zero (Figure 3.7), suggesting that cell alignment at the

edge of the colony was different from cell alignment closer to the center of the colony. However, when we analyzed the spread of cell orientations for each condition (Figure 3.8A,C), we did not detect any patterns as a result of the individual variables we tested, though it is possible there are combinatorial effects that our study was underpowered to detect. We binned cell orientations in 5 degree intervals and plotted histograms to detect differences in the distribution of cell orientations for each condition, but again, no patterns emerged for the variables tested here due to the large variance in cell orientation (Figure 3.8B,D).

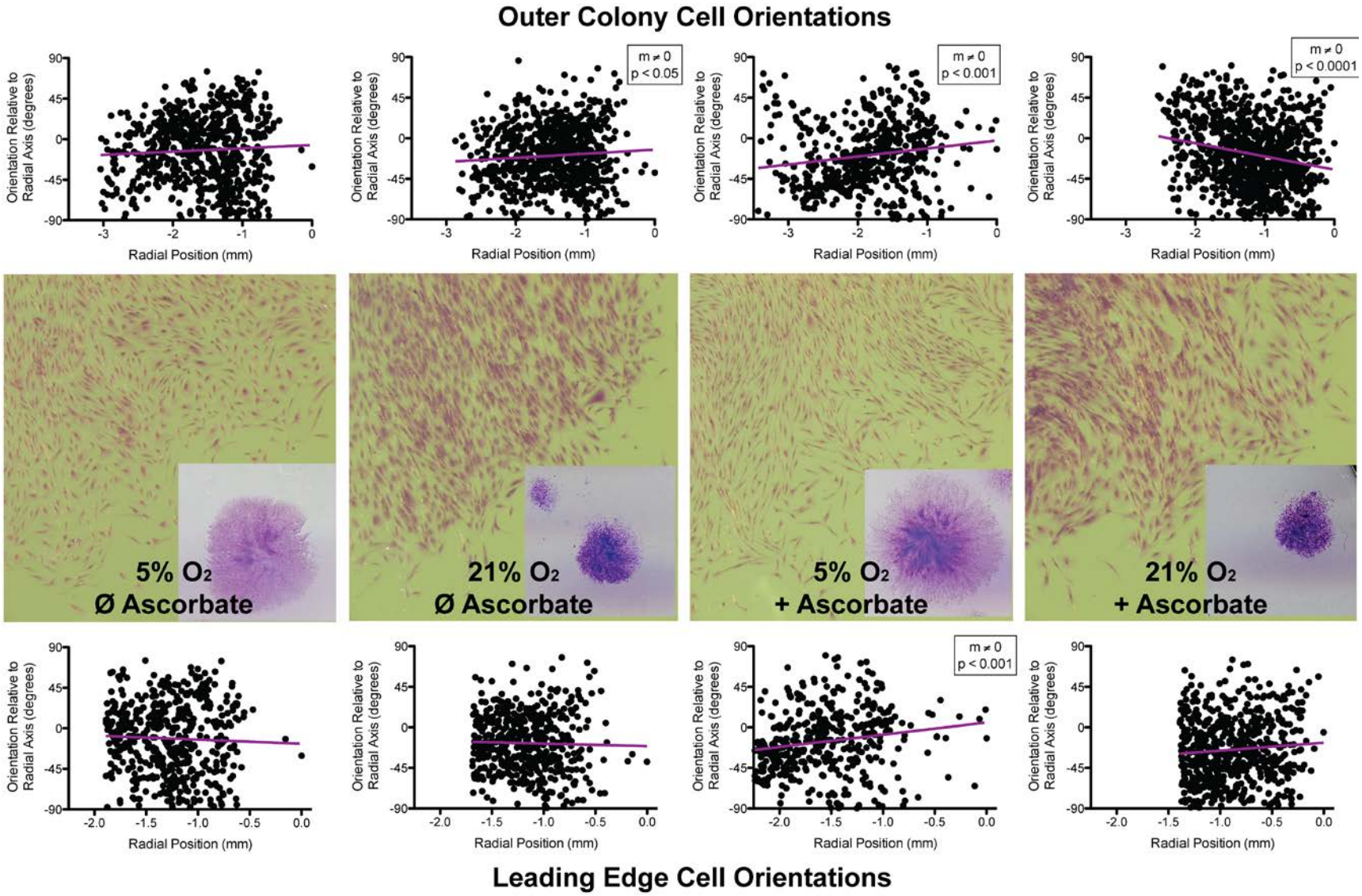

Figure 3.7 Cell orientation measurements in the outer portion (top) and at the leading edge of colony growth (bottom).

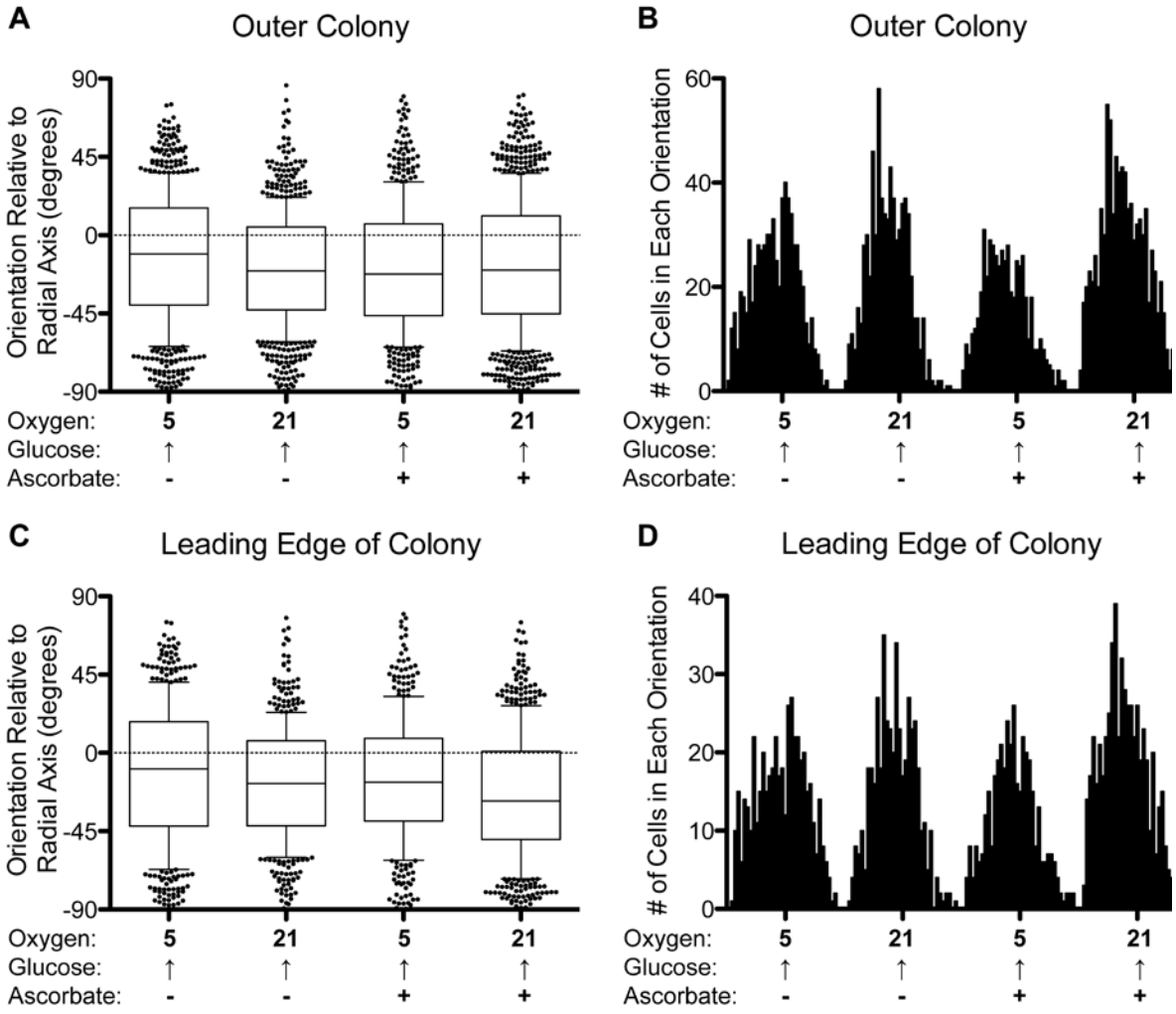

Figure 3.8 Range of cell orientation measurements. The range (box and whisker plots) of cell orientation measurements for each condition, shown for the outer colony region (**A**) and leading edge (**C**). Whiskers represent 10-90 percentiles with outliers represented as dots. The distribution (histograms) of cell orientation measurements from each condition centered at 0 degrees, shown for the outer colony region (**B**) and leading edge (**D**).

3.4.5 Ascorbate Decreases MSC Senescence Resulting from Hypoxia-High Glucose

We stained for the presence of SA-β-gal in the absence and presence of ascorbate at low and high oxygen tension in high and low glucose containing mediums (Figures 3.9 and 3.10). SA-β-gal accumulation was most evident in hypoxic cultures grown in high glucose medium

with no ascorbate. The addition of ascorbate largely eliminated the presence of SA-β-gal-stained cells (Figure 3.11). Addition of ascorbate significantly decreased senescence ($p<0.05$). In ascorbate free cultures at hypoxia, addition of high glucose significantly increased senescence ($p < 0.05$). There were significant interactive effects of these variables ($p<0.01$). Oxygen tension, evaluated in a separate analysis, also contributed to observed differences in group means, with hypoxia accelerating senescence ($p<0.05$).

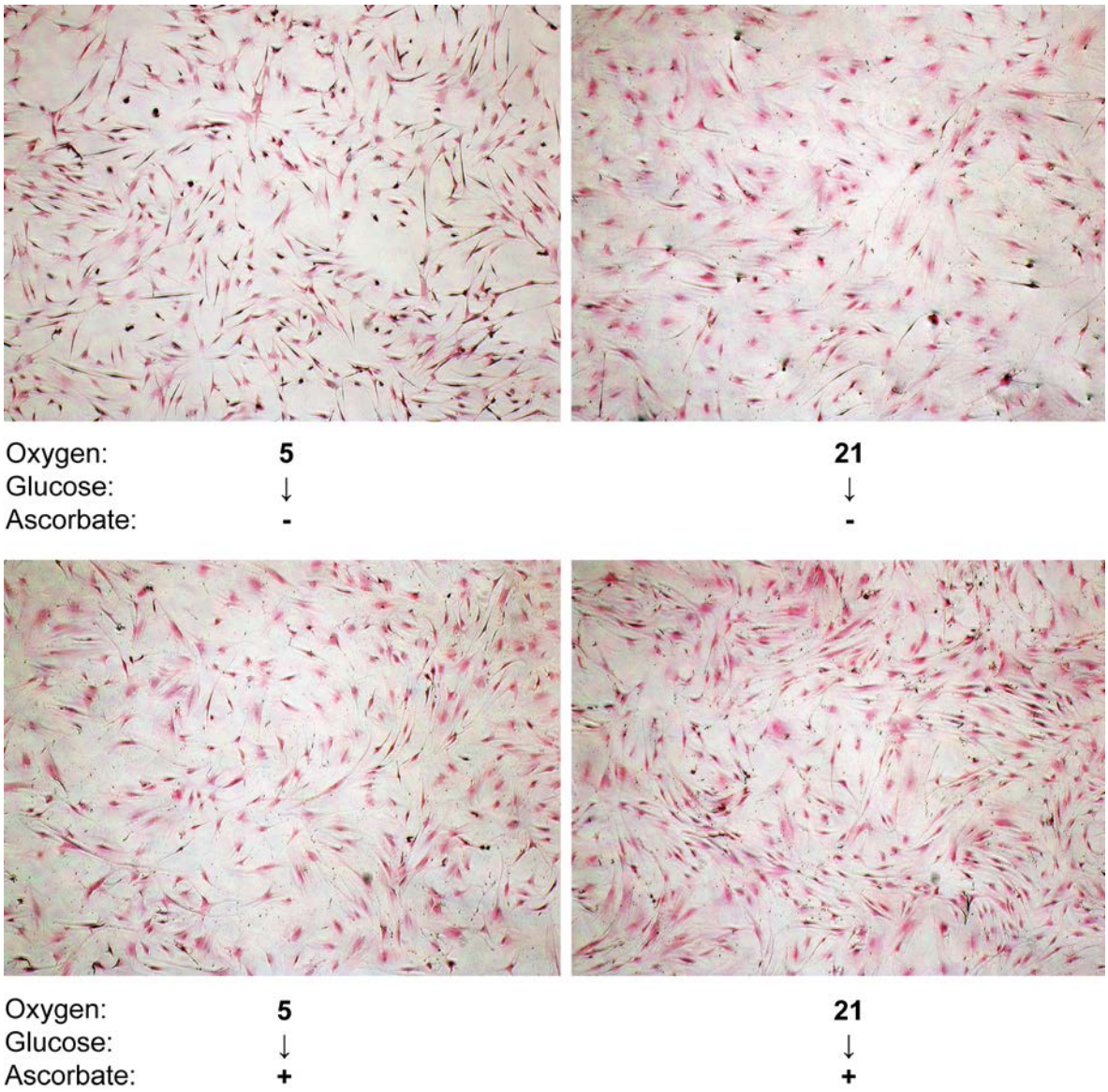

Figure 3.9 SA-β-gal staining in low glucose cultures. The top panel shows SA-β-gal staining in the absence of ascorbate at low and high oxygen tension; the bottom panel shows cultures supplemented with 50 μg/ml ascorbate. Photomicrographs were captured at 10X and are representative of three donors assayed.

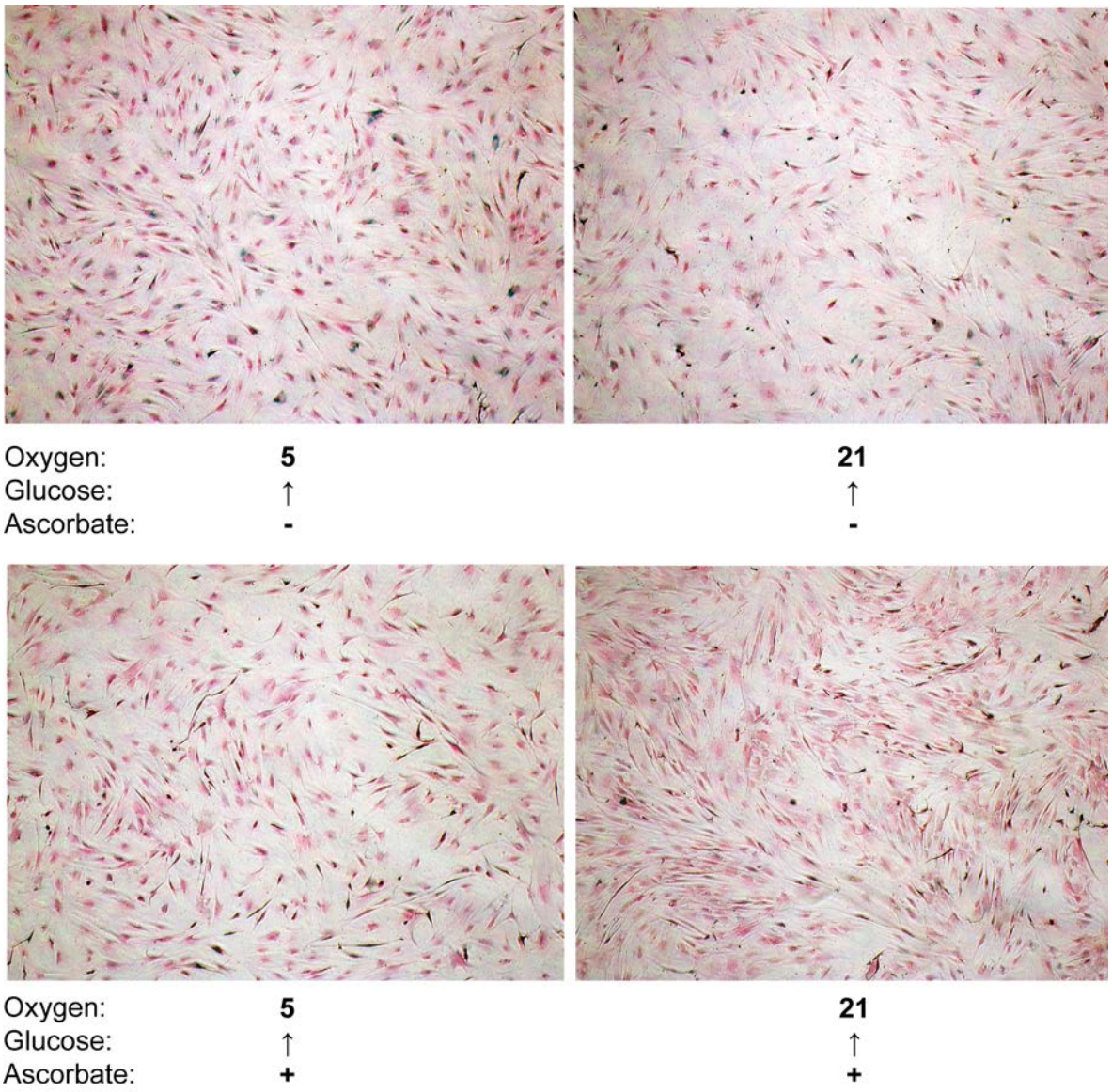

Figure 3.10 SA-β-gal staining in high glucose cultures. The top panel shows SA-β-gal staining in the absence of ascorbate at low and high oxygen tension; the bottom panel shows cultures supplemented with 50 μg/ml ascorbate. SA-β-gal accumulation is most evident in hypoxic cultures grown in high glucose medium with no ascorbate. The addition of ascorbate largely eliminates the occurrence of SA-β-gal-stained cells. Photomicrographs were captured at 10X and are representative of three donors assayed.

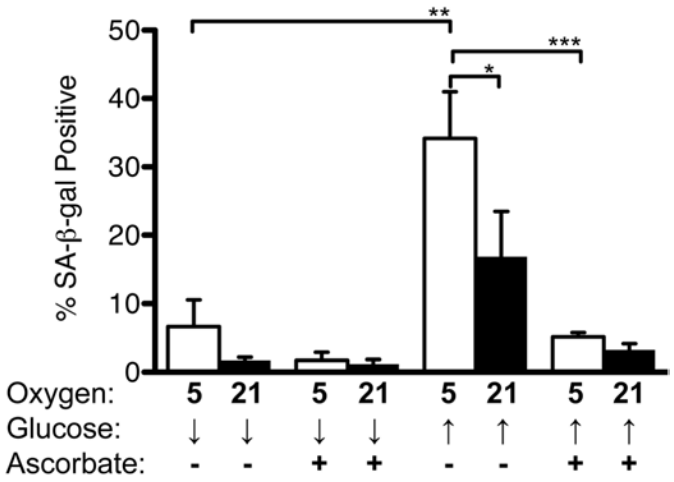

Figure 3.11 MSC senescence is more rapidly induced by culturing cells in high glucose medium under hypoxic conditions, likely due to more rapid cell cycling enabled by abundant caloric input. Increasing the oxygen tension decreases the percentage of cells entering a senescent state; addition of ascorbate to the cultures brings senescence levels back down to those observed in low glucose medium. Significant differences of relevant comparisons are indicated: *, **, *** indicate p values of <0.05, <0.01, and <0.001, respectively.

3.5 Discussion

The results of combined effects of glucose concentration and ascorbate supplementation suggest MSCs thrive in an environment with abundant antioxidant availability, when a balanced redox status allows them to engage in both aerobic and anaerobic respiration and undergo robust division, but not to the point of exhaustion. MSCs can readily switch to a glycolytic metabolic phenotype in less advantageous circumstances, but at the expense of robust division over time. Interestingly, these differences are manifested in a more extreme way in the low density conditions of a colony forming assay, where paracrine growth factors, cell-cell contact, and cell-matrix interactions play a significantly lesser role in supporting growth until the colony is well established. In those harsher conditions of isolation, MSCs rely heavily upon the presence of high glucose concentrations in the culture medium for single clones to survive and establish colonies, irrescpective of oxygen tension and ascorbate

supplement (Figure 3.4B).

The observed combinatorial effects of ascorbate supplementation and glucose concentration in culture medium on VEGF secretion by MSCs, as evidenced by statistically significant interactive effects, warrants further investigation, possible in larger numbers of human cell donors, different time points, or more stressful conditions, such as more extreme hypoxia. This would allow for more firm determination of optimal conditions to maximize colony formation in settings where large numbers of distinct MSC clones are desired. Monitoring of HIF activity under such conditions might also yield valuable insight into the balance between these variables, particularly in cultured maintained over longer time periods, as is required to grow a large number of cells for therapeutic applications.

Our observations of varying morphology in colonies formed under these different culture conditions led us to undertake a systematic, quantitative examination of cell arrangement within the colony. While we didn't detect any significant differences as a result of the culture manipulations we studied, development of this technique will prove useful for application in future studies of colony formation dynamics in response to growth factors, cytokines, genetic manipulations, and other variables, and we will continue to develop image-based colony analysis methods to approach quantitation of phenomena such as whorls and other multicellular ordering.

Comparing our results from Chapters 2 and 3, we find that across all experimental treatments (glucose, ascorbate, and hypoxia), Vegf secretion and proliferation are positively correlated with low glucose, ascorbate supplementation, and hypoxia. Regulation of Vegf expression and cell proliferation is under regulation by several intercellular pathways, some of which exhibit crosstalk. Hif1a serves as a master regulator for key mesenchymal cell events,

such as epithelial-to-mesenchymal transition. It also serves as a key transcription factor linking Vegf expression to proliferation, and our separate treatments to their observed effects. Vegf directly increases the survival and proliferative capacity of mesenchymal stem cells,[402] and inhibition of autocrine Vegf signaling suppresses the proliferative response of stem cells to growth factors.[403, 404] The Vegf promoter contains a HIF1a binding site,[405] and increased Vegf expression in response to hypoxia has been extensively documented, as discussed in Chapter 2.

Superoxide, on the other hand, down-regulates expression of Hif1a and, as a result, expression of Vegf. The formation of superoxide radicals in vitro is lower under hypoxia compared to normoxia. As reviewed in Chapter 3, ascorbate is an antioxidant that suppresses formation of superoxide radicals found in normoxic cultures. Thus, ascorbate supplementation in normoxia can mimic the lower levels of superoxide radicals present in hypoxia, resulting in Hif1a-mediated enhancement of Vegf expression and stem cell proliferation. As was also reviewed in Chapter 3, high glucose can induce excess intracellular superoxide, leading to decreased Hif1a expression. Low glucose does not generate this effect, therefore higher Hif1a-mediated Vegf expression can be maintained. Low glucose also shifts metabolism to a more anaerobic state, as does hypoxia. Thus, the three treatments and their similar effect on mesenchymal stem cell proliferation and Vegf secretion may be coupled in a fundamental manner to the cellular metabolic state, specifically redox status.

3.6 Conclusion

Addition of ascorbate to culture medium at modest doses clearly enhances MSC proliferation and decreases cell senescence in metabolically demanding conditions. In

combination with glucose and concentration and ambient oxygen tension, it also has effects on VEGF secretion by MSCs. Glucose availability in the medium is a critical factor in early colony establishment when cells have no reliance on paracrine trophic factors. MSCs subjected to ischemic conditions of hypoxia and nutrient deprivation die in long-term cultures like a CFU-F assay; in this regard our findings are in complete agreement with what has been observed by others in the field.

Chapter 4: The Next Frontier

Regulation of *Ex Vivo* Adult Stem Cell Aging with Transcriptional Reprogramming

4.1 Abstract

As discussed in Chapter 1, MSCs are generally considered highly promising candidate cells for regenerative applications because they possess a high proliferative capacity and the potential to differentiate into other cell types, but we and other have observed that MSCs do not retain their proliferative and multi-lineage differentiative capabilities after prolonged ex vivo propagation. There is also evidence to suggest MSCs in aging humans exhibit functional decline (see Chapter 1). Modifying MSCs to resist aging during ex vivo propagation would yield a favorable cell source for regenerative medicine applications. In the work described in this chapter we sought to develop and test novel methods for partial transcriptional reprogramming of bulk MSC populations and to investigate whether this technique is effective for enhancing function in MSCs subjected to *in vitro* aging.

4.2 Introduction

As discussed in Chapter 1, there is an emerging body of evidence that altered and decreased function of adult stem cells in vivo secondary to accumulated metabolic stress plays a huge role in the initiation of diseases of aging [21]. Stem cells are thought to reside in vivo as self-renewing pools and facilitate repair/replacement of damaged tissues over the lifespan of the organism. Maintaining stem cell quiescence is essential for preserving the long-term self-renewal potential of the stem cell pool in a number of organ systems, such as the brain, bone marrow, musculoskeletal system, and skin.[19, 20] Quiescence lies on one end of the *spectrum of proliferative capacity* of a stem cell (Figure 4.1). This spectrum spans from quiescence, to high proliferation, to senescence and death.

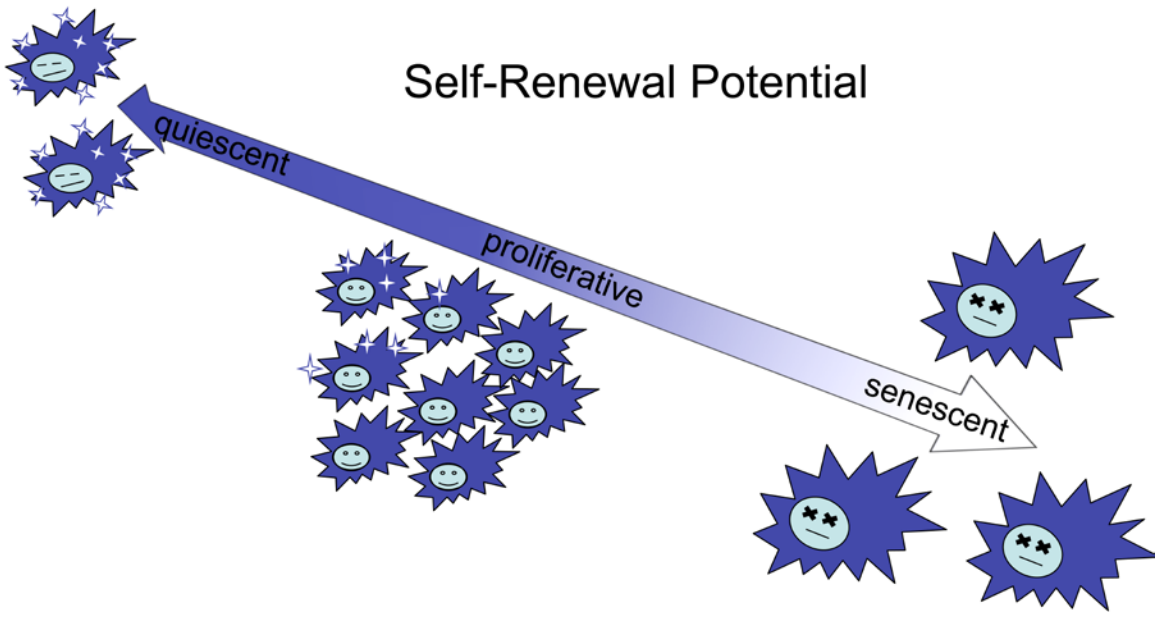

Figure 4.1 Stem cells exist on a spectrum of self-renewal/proliferative capacity. At one end of the spectrum, quiescent stem cells maintained in the niche harbor the largest potential for renewal of the stem cell pool. At the other end of the spectrum, exhausted stem cells that have undergone many cycles of division in peripheral tissue have no reserve potential for self-renewal and may enter a senescent state and cease to divide for a period before they die.

Stem cells also exist on a *spectrum of differentiation* bounded by terminally differentiated unipotent effector cells at one extreme and pluripotent embryonic stem cells at the other (Figure 4.2).

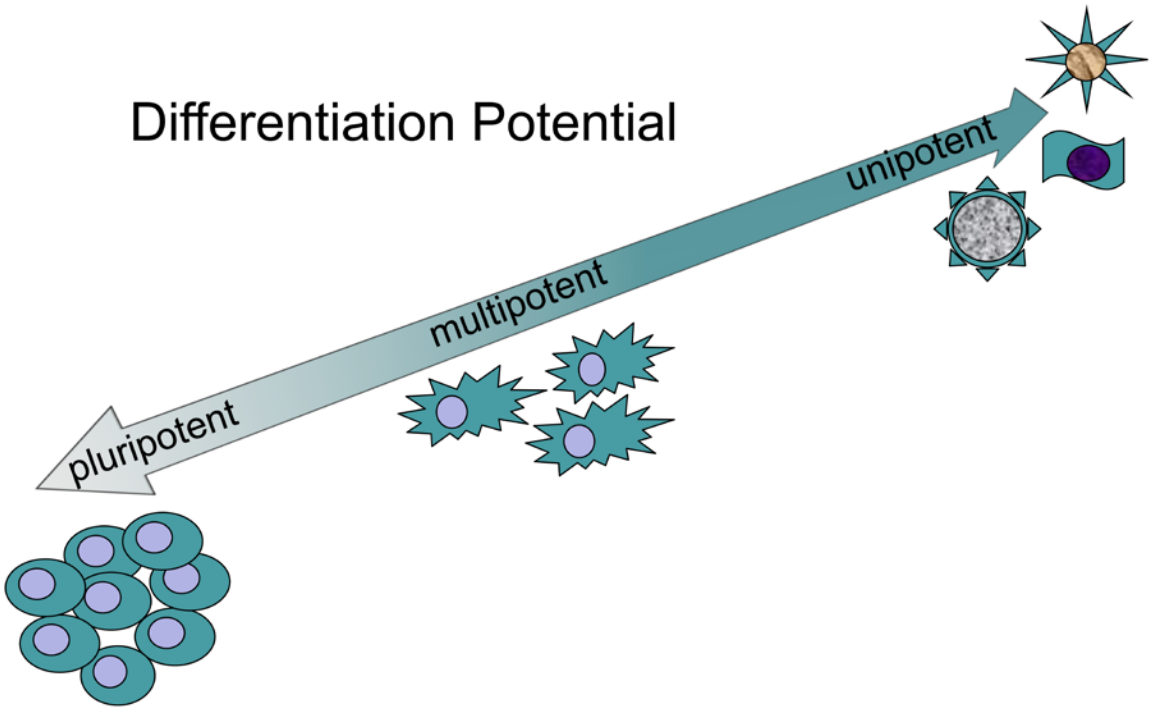

Figure 4.2 Stem cells exist on a spectrum of differentiation potential. At one end of the spectrum, pluripotent stem cells possessive theoretically unlimited differentiation potential. At the other end of the spectrum, unipotent progenitors become terminally differentiated cells are committed to a specific fate within a lineage.

In theory stem cells differentiate from a pluripotent state to a multipotent progenitor state to a unipotent progenitor to a terminally differentiated cell type (Figure 4.3). For many decades this process has been viewed as linear and one-way, but there is growing evidence that some cell types are plastic, particularly at the progenitor stage, and may transdifferentiate or dedifferentiate through switching to a new transcriptional program,

complete with regulation by a different master transcription factor. This has been shown in our own lab with MSCs transdifferentiating from one mesenchymal lineage to another,[11] and the discovery of mechanisms enabling a programmatic transcriptional shifts in terminally differentiated cells as they transdifferentiate is a subject of active investigation.[406]

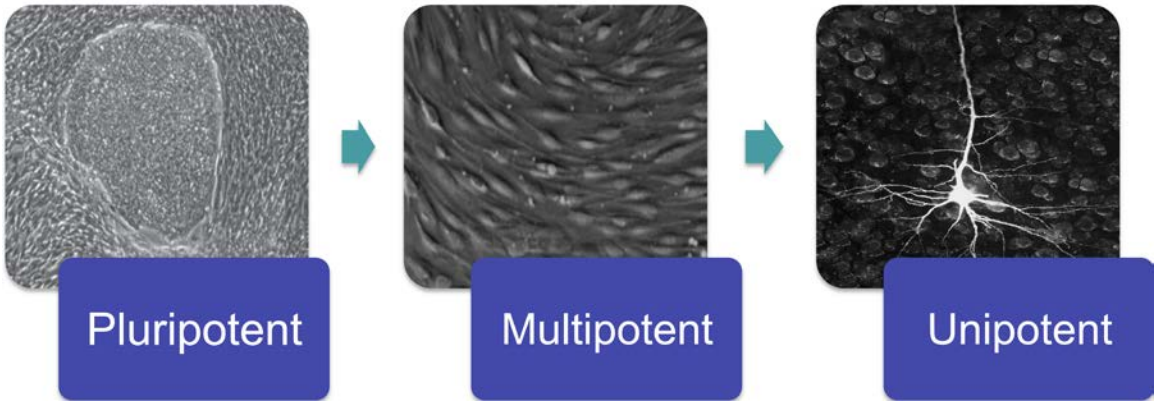

Figure 4.3 Stem cells differentiate from a pluripotent state to a multipotent progenitor state to a unipotent progenitor to a terminally differentiated cell type. For many decades this process has been viewed as linear and one-way, but there is much evidence that some cell types are plastic, particularly at the progenitor stage, and may transdifferentiate or dedifferentiate through switching to a new transcriptional program, complete with regulation by a different master transcription factor.

There is great interest in manipulating a cell's position on the spectra of self-renewal capacity and differentiation potential and the resulting cell fate for investigative and therapeutic purposes.[11] Several recent studies have examined the process of replicative senescence in adult stem cells and demonstrated that senescence occurs as a series of programmed changes over the course of *ex vivo* propagation (Figure 4.4).[21, 155, 227, 407] Our long-term goal is to exploit reprogramming techniques to render cultured adult stem cells resistant to the phenotypic changes that accompany replicative senescence, such as arrested

proliferation and decreased differentiation potential (Figure 4.5). Manipulating cellular fate has become more feasible with the advent of nuclear reprogramming methods used to induce pluripotent stem (iPS) cells, as discussed in Chapter 1.[203, 205, 206] In this study, we hypothesized that by introducing reprogramming genes to bulk populations of MSCs, it is possible to achieve partial reprogramming of the population, meaning some MSCs in the population are genetically altered, change their transcriptional program, and influence the functional fate of the culture as a whole.

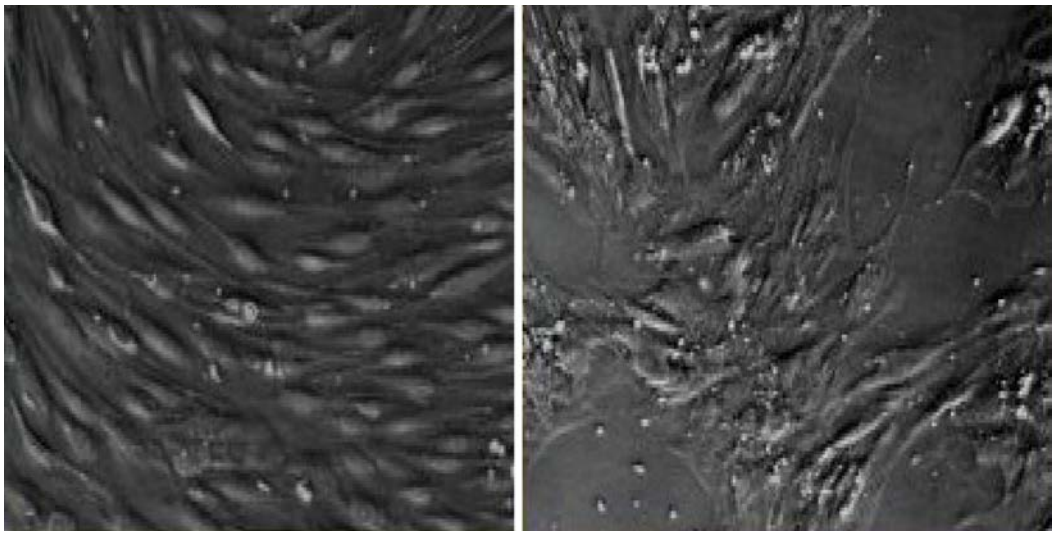

Figure 4.4 MSCs display signs of *in vitro* aging upon extended propagation. Passage 2 MSCs (l**eft panel**) exhibit a small, plump, spindle-shaped morphology, but upon expansion to Passage 8 begin to flatten and spread over larger areas of the culture surface (**right panel**).

We aimed to develop an understanding of how partial reprogramming can most effectively be induced in adult stem cells, how it alters the phenotype of adult stem cells, and how this approach may be used to preserve the potency, proliferative capacity, and regenerative utility of adult stem cells as they are cultured *in vitro*. To accomplish this we developed and tested methods for reprogramming MSCs with the transcription factors

c-Myc, Klf4, Oct4, Nanog, and Sox2 (Figure 4.6). Based on gene transfer technologies reported by other laboratories[408, 409] and developed in our own laboratory, several strategies for partially reprogramming MSCs with the transcription factors c-Myc, Oct4, Klf-4, and Sox2 exist.

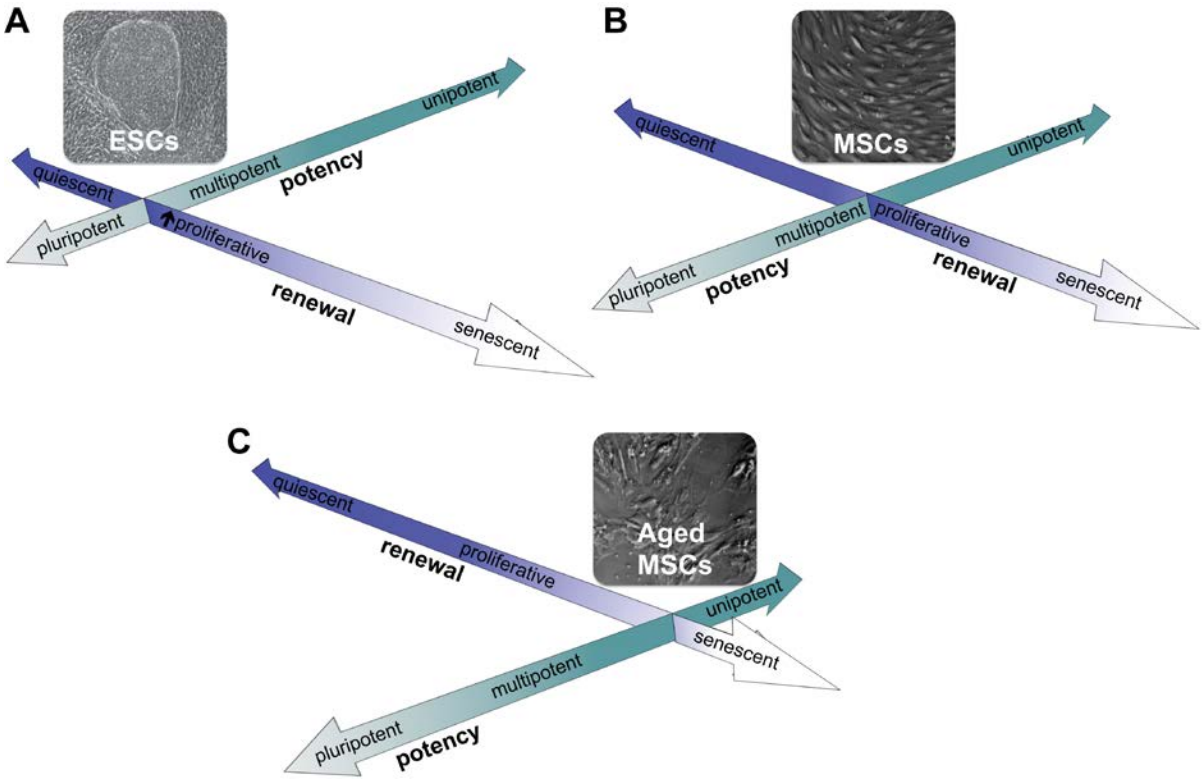

Figure 4.5 Different types of stem cells exist at different intersection points on the self-renewal and potency spectra. ESCs (**A**), which exhibit nearly limitless capacity for division, are almost the most plastic of stem cells and can readily differentiate into many more cell types than an MSC (**B**). MSCs from aging individuals or that have been aged *in vitro* by extensive passaging exhibit less self-renewal capacity than freshly harvested MSCs, particularly from young individuals (**C**). Aged MSCs also exhibit diminished potency; this is thought to be one reason for conversion of musculoskeletal tissue to fat with advancing age.

By controlling the duration of expression of these genes with transiently transfected pcDNA vectors, we sought to prompt a phenotypic shift in transduced MSCs to a more stem-

like state without inducing them to a pluripotent state. An unevenly reprogrammed population of cells was an acceptable outcome, with small subpopulations potentially becoming either fully reprogrammed or partially reprogrammed to some degree, while the remainders retain their non-reprogrammed MSC phenotype. The reprogramming process has been demonstrated to switch cells to an entirely different transcriptional program, and we hypothesize that through the partial reprogramming process, some MSC clones generated will become more resistant to senescence and able to retain their differentiation potential. These cells would then either out-compete the remaining MSCs as they are subjected to *in vitro* aging or secrete trophic factors that enhance the fate of the population. This would create a more ideal cultured cell source for tissue engineering.

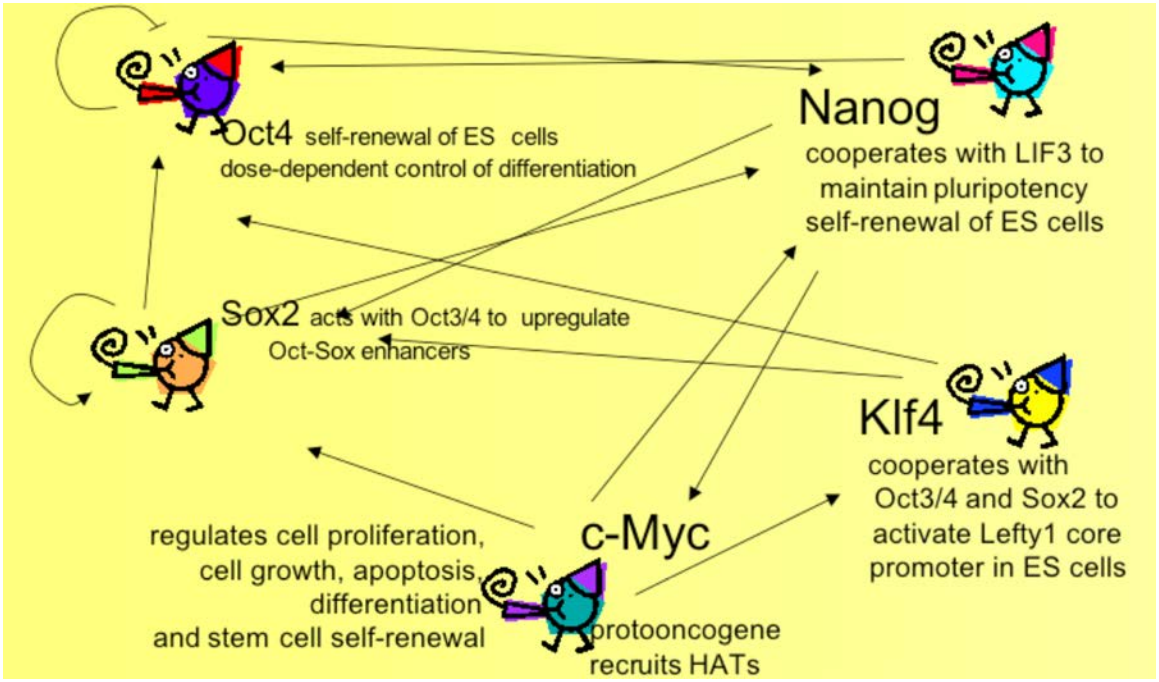

Figure 4.6 Reprogramming transcription factors regulate fundamental cell processes and each other.

4.3 Materials and Methods

4.3.1 Vector Cloning and Production

We cloned the human genes Oct4, Sox2, c-Myc, Nanog, and Klf4 from commercially available cDNA libraries (Open Biosystems) individually into pcDNA plasmid expression vectors (Invitrogen) (Figure 4.7) and performed sequencing of each construct of interest to ensure the correct insert was placed in the correct orientation for each construct. We then subcloned our genes of interest Gateway Entry clones (Invitrogen) for transfer to pLenti expression vectors (Invitrogen) (Figure 4.9).

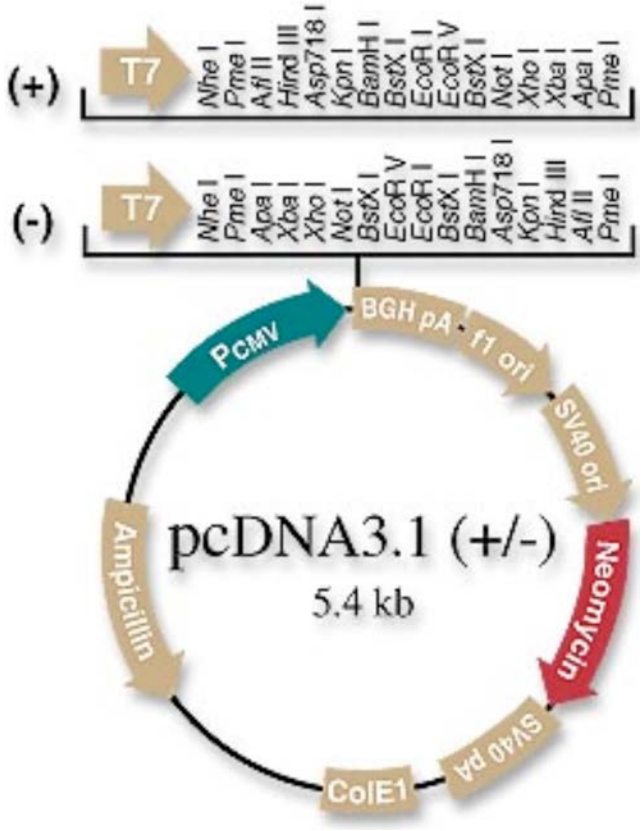

Figure 4.7 Plasmid vector pcDNA3.1. We used the plasmid vector pcDNA3.1 to transiently transfect MSCs with individual reprogramming transcription factors.

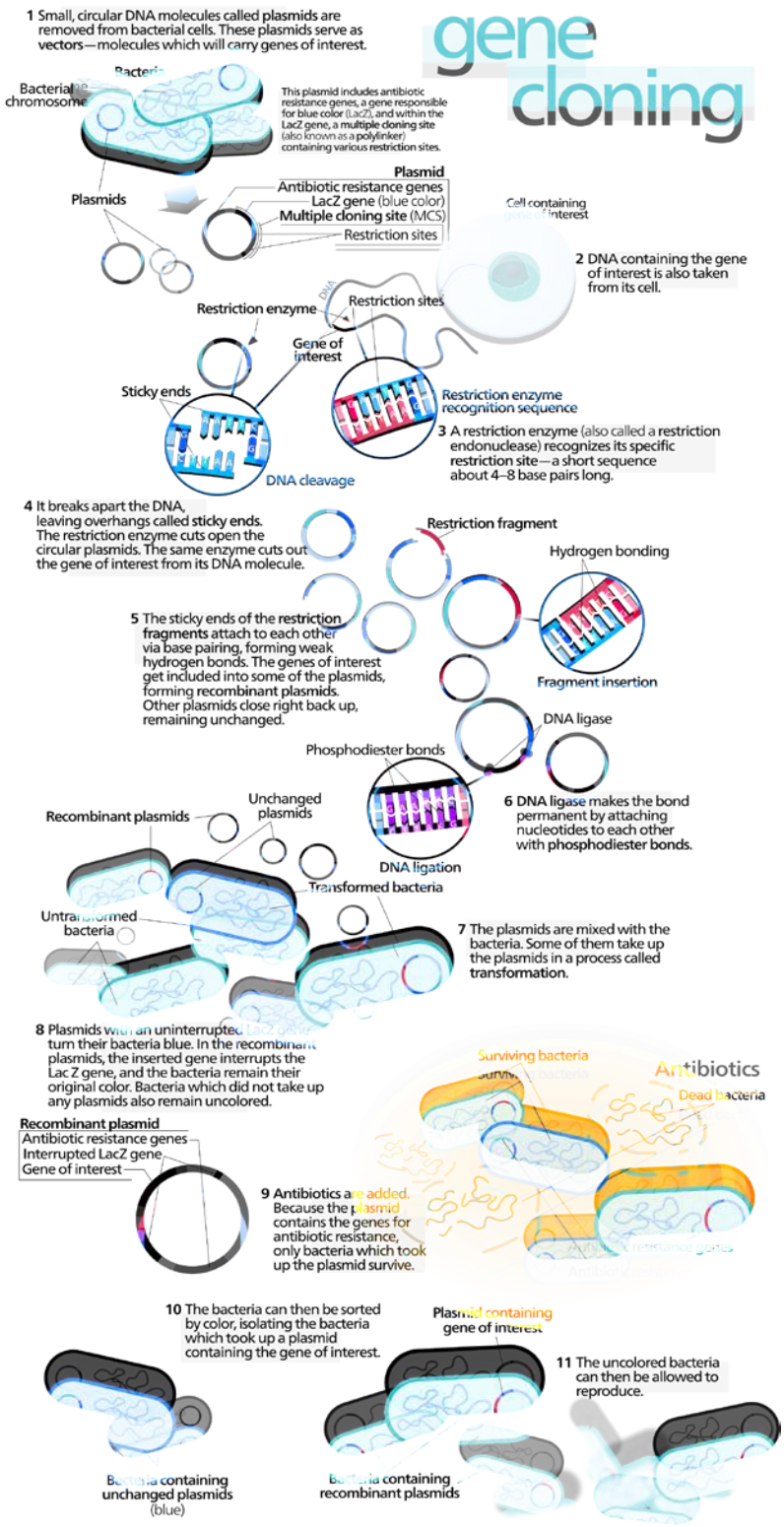

Figure 4.8 A schematic of molecular cloning. Reprinted with permission from Kelvin Song.

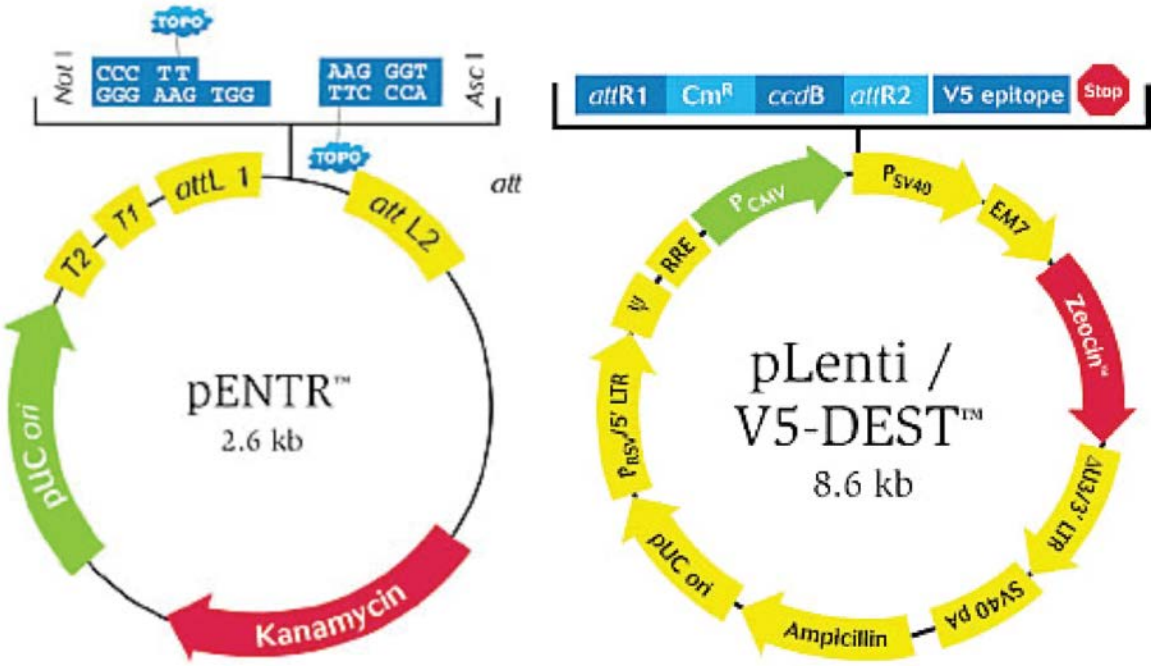

Figure 4.9 Lentiviral cloning strategy. We employed directional TOPO cloning to insert our genes of interest in Gateway Entry Clones, then transfered them to lentiviral Gateway Destination vectors.

In addition to cloning our individual transcription factors into separate lentiviral vectors, we also obtained a lentiviral cassettes containing all four genes Oct4, Sox2, c-Myc, and Klf4 from the Jaenisch lab (Addgene), one constitutively active (FUW-OSKM) and the other inducible with tetracycline (TETO-FUW-OSKM), once they became available.[410] This enabled us to deliver a higher number of transcription factor copies to our cells with a smaller quantity of vector, which we employed for some control experiments. Lentiviral particles were produced using ViraPower Lentiviral Expression System (Invitrogen 3rd generation Lenti vector) off-site by the UCLA Vector Core and the University of Pittsburgh Cancer Center Vector Core (Figure 4.10).

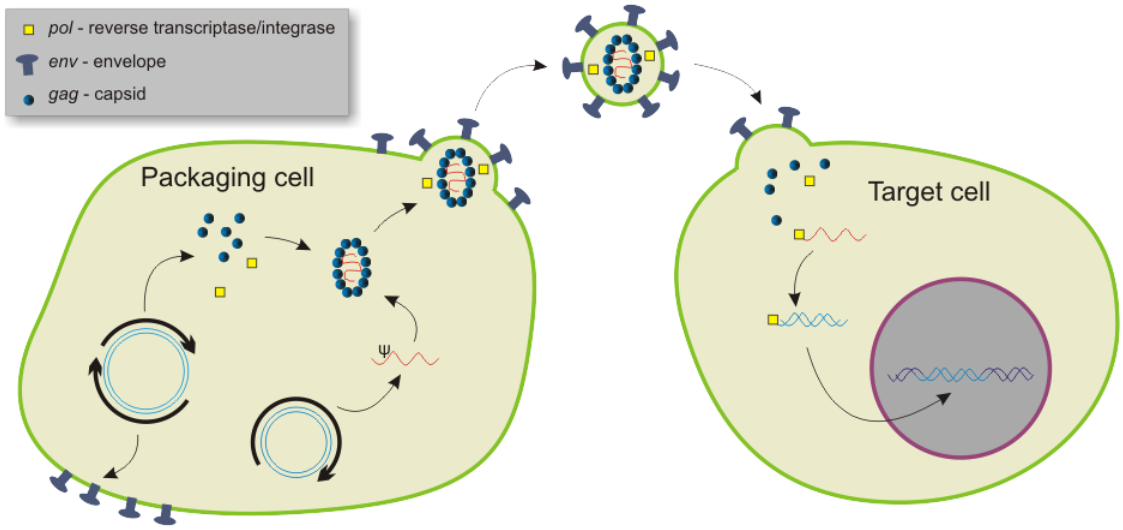

Figure 4.10 Production of virus particles by packaging cells for use to infect target cells. Reprinted with permission from Peter Znamenskiy.

4.3.2 MSC Culture

MSCs from hip arthroplasty patients were harvested from bone marrow according to published protocols and cryopreserved for a minimum of one week with approval of the Institutional Review Boards of the University of Pittsburgh and the University of Washington as described in previous chapters. A portion of the cryopreserved MSCs were plated on tissue culture plastic at a density of 6.0×10^3 cells/cm^2 and passaged at 80% confluency for no less than eight total passages. MSCs with a passage number P8 or greater were considered aged MSCs. The remaining portion of cryopreserved MSCs were thawed and passaged for no more than three total passages. MSCs with a passage number P3 or less were considered non-aged MSCs. Size and morphology of aged MSCs were documented at each passage number over the course of their expansion and compared to non-aged MSCs.

4.3.3 MSC Reprogramming

MSCs were electroporated to induce transient overexpression of Oct4, Sox2, c-Myc, and Klf4 using pcDNA vectors using the Amaxa Nucleofector transfection system (Lonza) according to the manufacturer's protocol. Briefly, 5.0×10^5 cells were combined in Nucleofector solution with linearized purified plasmid DNA and electroporated using the high-efficiency U-23 program, at which point they were transferred to pre-warmed culture medium supplemented with FBS. A control vector expressing GFP was used to quantify transfection efficiency.

4.3.4 Immunophenotyping

Non-aged, aged, and reprogrammed MSCs were stained according to the methods described in Nesti et al, 2008[341] for surface markers associated with MSCs and ESCs to generate an immunophenotype for each population. A panel of standard positive and negative MSC markers was interrogated, including CD34 (clone 563), CD44 (clone 515), CD45 (clone TU116), CD73 (clone AD2), CD90 (clone 5E10), CD146 (clone P1H12) (BD Biosciences), and Stro-1 (clone STRO-1) (BioLegend), as well as the stem cell markers SSEA-3 (clone MC631) and SSEA-4 (MC813-70) (BD Biosciences). Marker expression was measured using a FACSAria cytometer and FACSDiva software (Becton Dickinson).

4.3.5 Differentiation Assays

Non-aged, aged, and reprogrammed MSCs were induced to undergo (1) osteogenic differentiation and (2) chondrogenic differentiation according to the methods described in Chapter 2. Briefly, Osteogenesis was induced in monolayer MSC cultures plated at a density

of 1.0×10^4 cells/cm^2 in 6-well and 24-well plates with osteogenic medium consisting of DMEM supplemented with 10% FBS, 50 µg/ml L-ascorbate-2-phosphate, 0.1 µM dexamethasone, 10 mM β-glycerophosphate, and 10 nM 1α,25-$(OH)_2$ vitamin D_3 (Sigma). On day 21 osteogenic cultures were rinsed with PBS, fixed in 60% isopropanol, and stained in a 1% Alizarin Red solution (Rowley) to detect matrix mineralization. To induce chondrogenesis MSCs were grown as high-density pellets (2.5×10^5 cells) in serum-free DMEM supplemented with ITS Premix (BD Biosciences), 50 µg/ml asorbic acid (Sigma), 40 µg/ml L-proline (Sigma), 100 µg/ml sodium pyruvate (Gibco/Invitrogen), 0.1 µM dexamethasone (Sigma), and 10 ng/ml recombinant human transforming growth factor (TGF)-β3 (R&D Systems) and harvested after 28 days of culture.

4.3.6 Senescence-Associated β-Galactosidase Staining

Senescence was detected in MSC cultures as described in Chapter 3. Briefly, SA-β-gal staining was performed using a commercially available kit (Cell Signaling Technologies) according to the manufacturer's protocol. Early passage, late passage, and reprogrammed late passage cells were fixed with 2% PFA and stained overnight. Cultures were then counterstained with eosin and imaged the same day.

4.4 Results

4.4.1 Vector Cloning and Transfection of MSCs

We successfully cloned the genes into the plasmid and lentiviral vectors described and confirmed proper orientation of each construct through sequencing (Figure 4.11). We tested these vectors in human MSCs and adult dermal fibroblasts and confirmed expression of the

four transferred genes; expression of these genes was undetectable in cells transfected with a control vector or untreated (UT) cells; plasmid DNA was added to the final lane as a positive control for each construct (Figure 4.12).

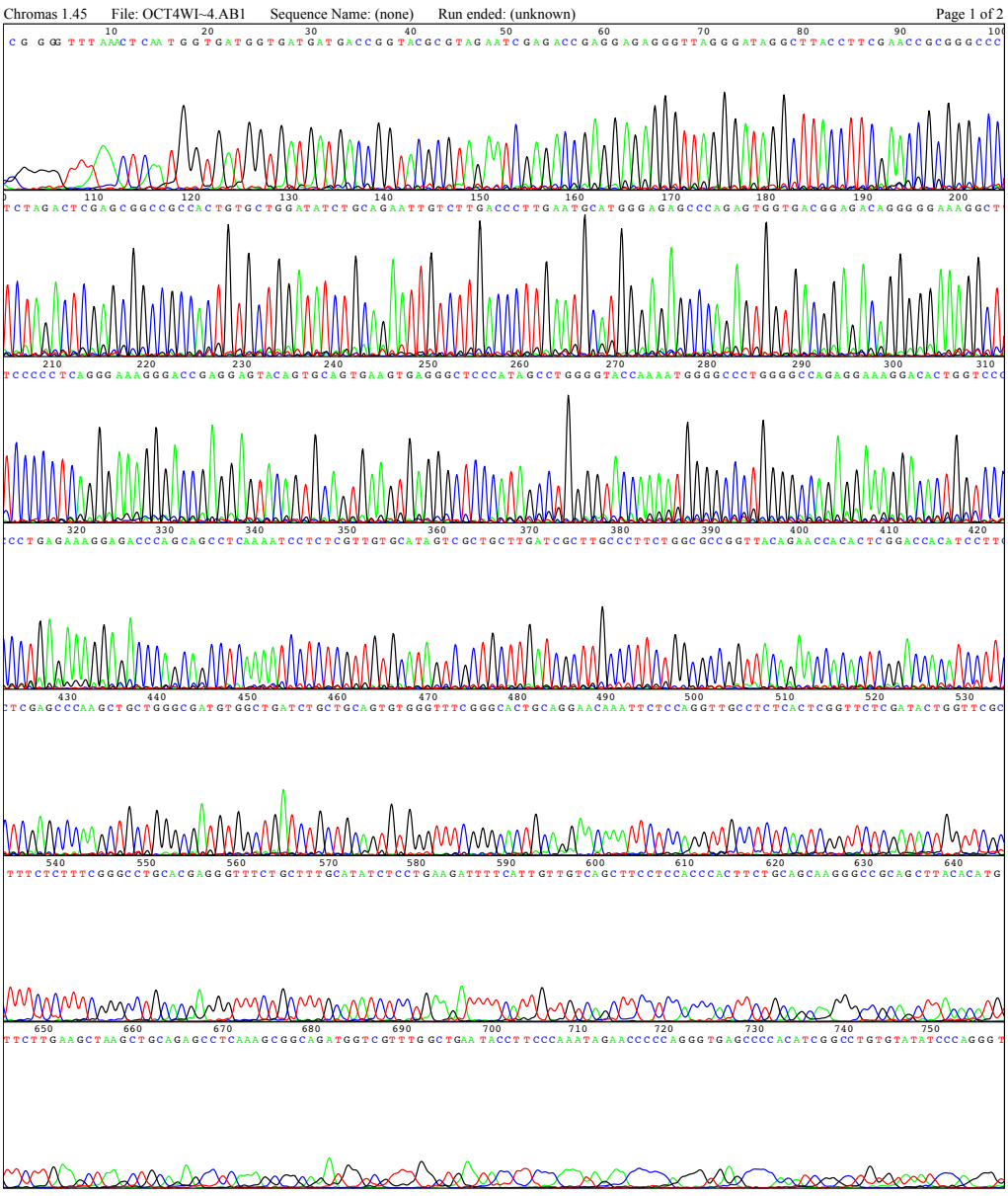

Figure 4.11 Example of construct sequencing to ensure correct orientation of the correct insert in each pcDNA clone. This sequencing report is for reverse sequencing from the BGH polyadenylation sequence engineered into the pcDNA3.1 construct (Invitrogen).

We confirmed expression of the target genes visually (Figure 4.13) and by flow cytometry (Figure 4.14) using an Oct4 promoter-reporter construct driving GFP expression (System Biosciences) which we introduced via lentiviral transduction.

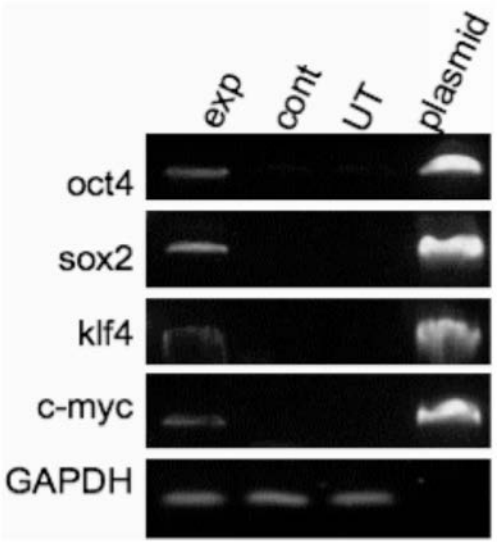

Figure 4.12 Products from RT-PCR were run on a gel to confirm expression and appropriate band size following transfection. Abbreviations: exp = MSCs transfected with pcDNA constructs containing genes of interest, cont = MSCs transfected with a control GFP vector, UT = untreated, plasmid = positive control purified plasmid.

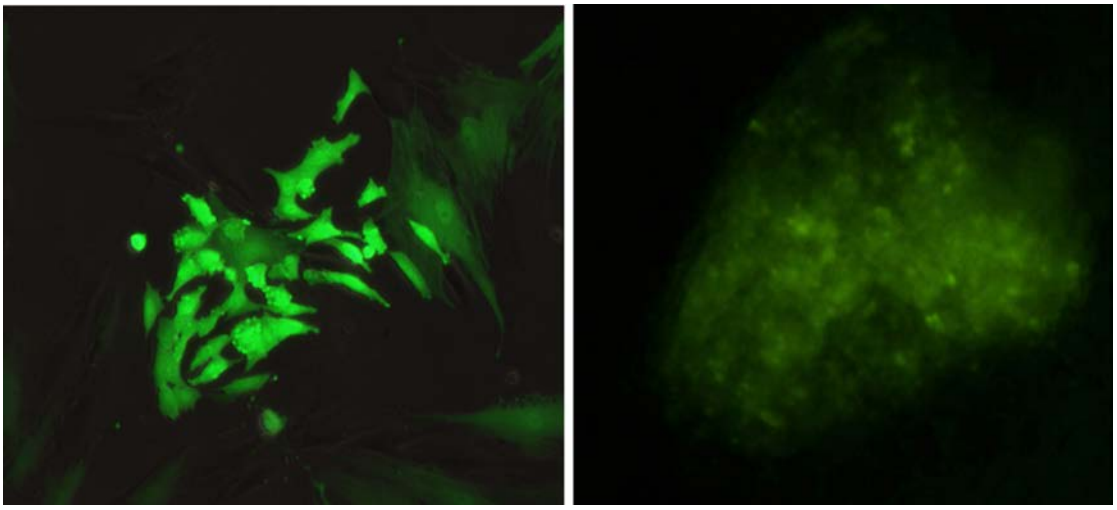

Figure 4.13 Expression of transfected factors was confirmed with an Oct4 promoter-reporter driving GFP by imaging. Transfected MSCs (**left**); after dedifferentiation to iPS cells (**right**).

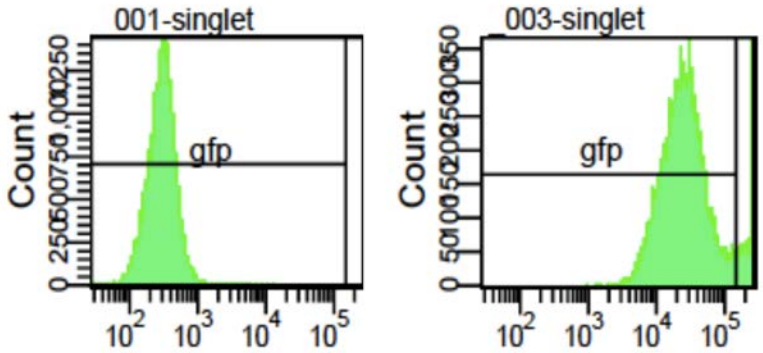

Figure 4.14 Expression of transfected factors was confirmed with an Oct4 promoter-reporter driving GFP by flow cytometry. Control cells transfected with an empty plasmid express no detectable GFP after transduction with the promoter-reporter (**left**), while MSCs transfected with reprogramming factors including Oct4 exhibit bright GFP expression (**right**).

4.4.2 Non-viral Reprogramming of Late Passage MSCs Induces Partial Restoration of Immunophenotype

Since our greatest interest is in the area of preserving stem cell-specific functions during *ex vivo* expansion, which induces a type of cellular aging, we applied this partial reprogramming approach utilizing transient over-expression of reprogramming factors to late passage MSCs to determine whether their function could be improved. We then compared their functional capacity to early passage MSCs and to late passage MSCs that were not reprogrammed, specifically their immunophenotpye, differentiation potential, and resistance to senescence. We found that partial reprogramming of late passage MSC cultures restored expression of CD90, CD146, and Stro-1 (Figure 4.16) – which was lost in late passage control cultures – to levels comparable to early passage MSC cultures. We did not observe any expression of ESC markers SSEA-3 and SSEA-4, which are present on the surface of human iPS cells, in late passage MSCs, although we did see expression of SSEA-4 on early passage MSCs (Figure 4.17).

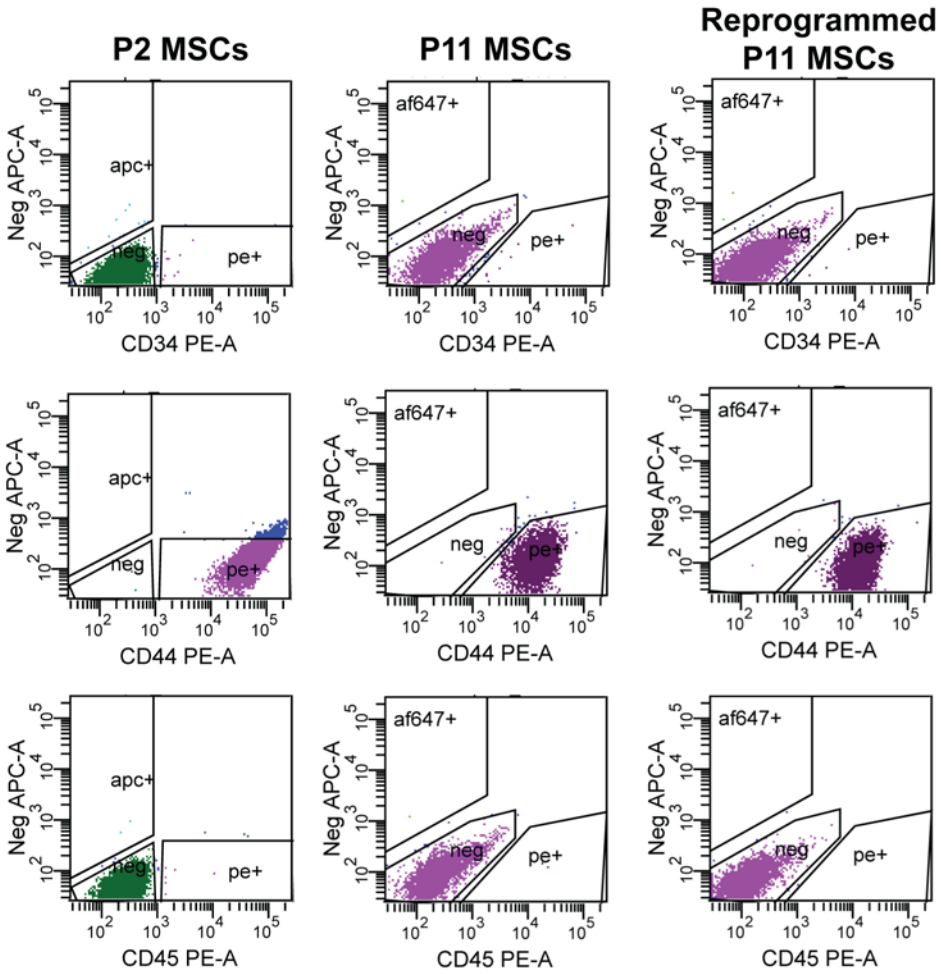

Figure 4.15 Expression of positive and negative MSC surface markers. Single color flow cytometry comparing phenotypic markers on early passage cells (**left panel**), late passage cells (**middle panel**), and reprogrammed late passage cells (**right panel**). Top row = CD34, middle row = CD44, bottom row = CD45.

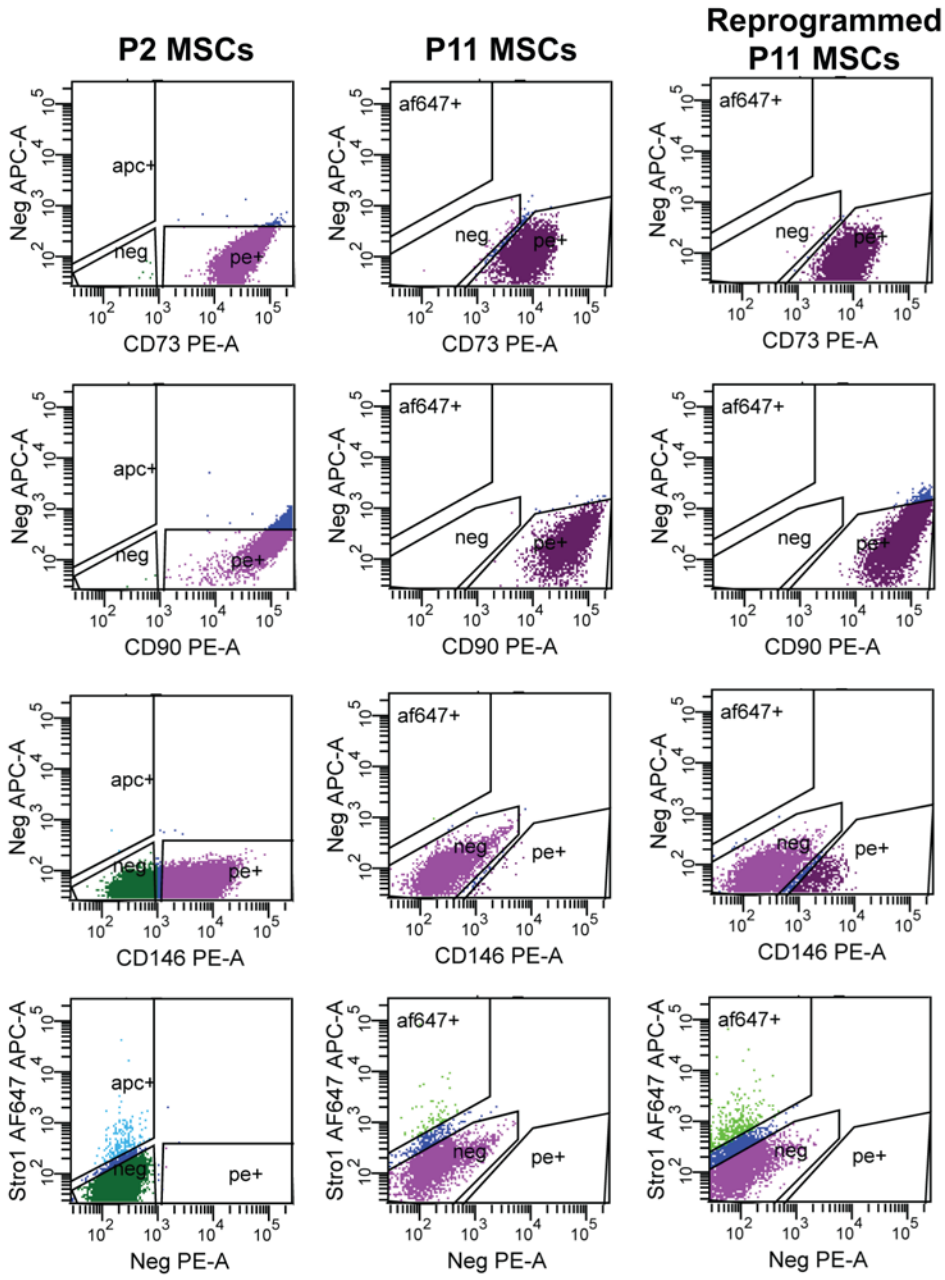

Figure 4.16 Expression of positive MSC surface markers. Single color flow cytometry comparing phenotypic markers on early passage cells (**left panel**), late passage cells (**middle panel**), and reprogrammed late passage cells (**right panel**). Top row = CD73, second row = CD90, third row = CD146, bottom row = Stro-1 (on y axis).

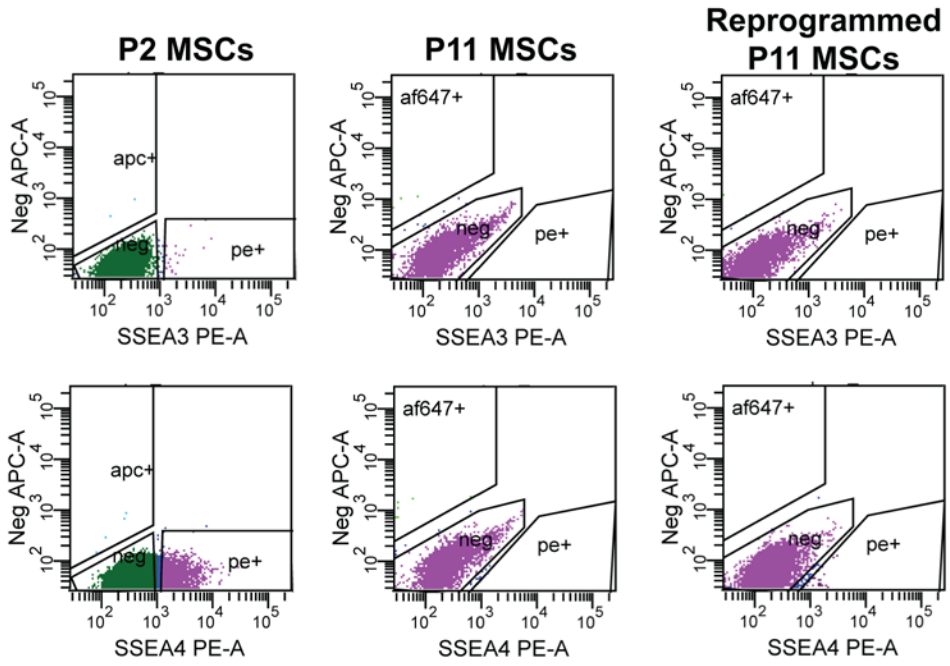

Figure 4.17 Expression of ESC surface markers. Single color flow cytometry comparing phenotypic markers on early passage cells (**left panel**), late passage cells (**middle panel**), and reprogrammed late passage cells (**right panel**). Top row = SSEA-3, bottom row = SSEA-4.

4.4.3 Non-viral Reprogramming of Late Passage MSCs Enhances Osteogenic and Chondrogenic Differentiation

We compared the extent of differentiation of reprogrammed late passage MSCs to the parent early passage MSCs and non-reprogrammed late passage MSCs (Figure 4.18). In osteogenic cultures alizarin red staining reflecting matrix mineralization was decreased in response to extensive passaging, reflecting loss of osteogenic differentiation potential. However, in late passage MSCs that were reprogrammed, we observed restoration of matrix mineralization to levels approaching early passage cells. Similarly under conditions inducing chondrogenesis, early passage cells formed large, well-integrated cartilage pellets, while late

passage MSCs exhibited almost no pelleting and had to be removed from the well with a pipette as a loose collection of cells. Late passage cells that underwent reprogramming did form pellets, reflecting partial restoration of chondrogenic differentiation potential. The pellets were more loosely held together than for early passage MSC cultures, but they could be removed from the culture medium as one unit without disaggregating and without the use of suction and displayed a clear border and three-dimensionality.

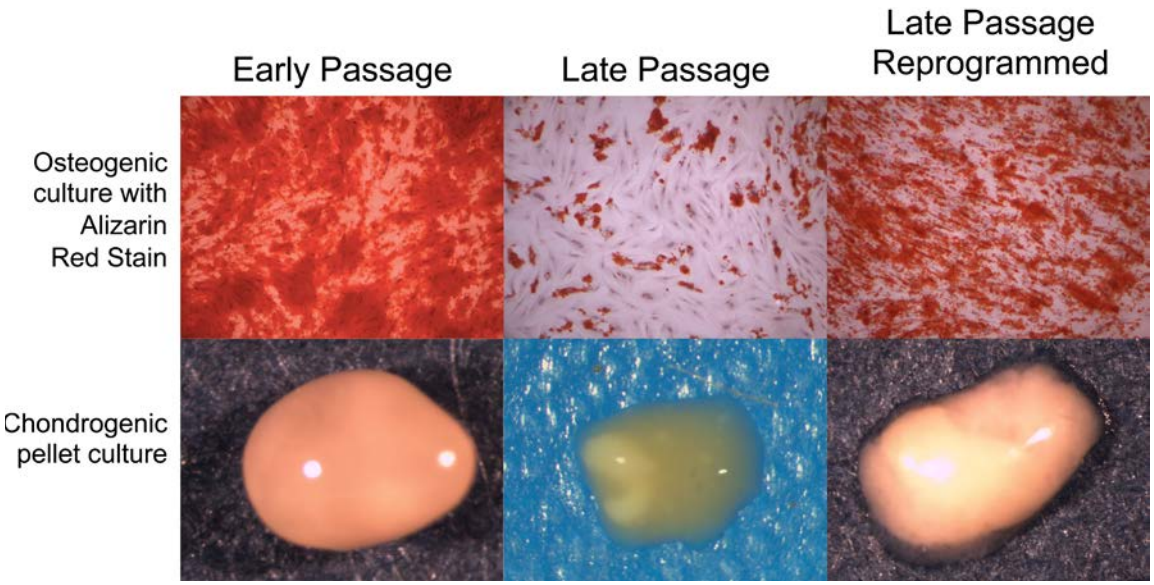

Figure 4.18 Phenotypic differences between early passage, late passage, and late passage reprogrammed MSCs upon differentiation. Alizarin red stains show matrix mineralization in osteogenic culture. Chondrogenic pellets show ability to make a cartilaginous extracellular matrix under each condition.

4.4.4 Non-viral Reprogramming of Reduces Senescence in Late Passage MSCs

Resistance to senescence improved in reprogrammed late passage MSC cultures compared to control late passage cultures, with significantly fewer cells staining positively for SA-β-gal and less disruption of normal cell morphology (Figure 4.19).

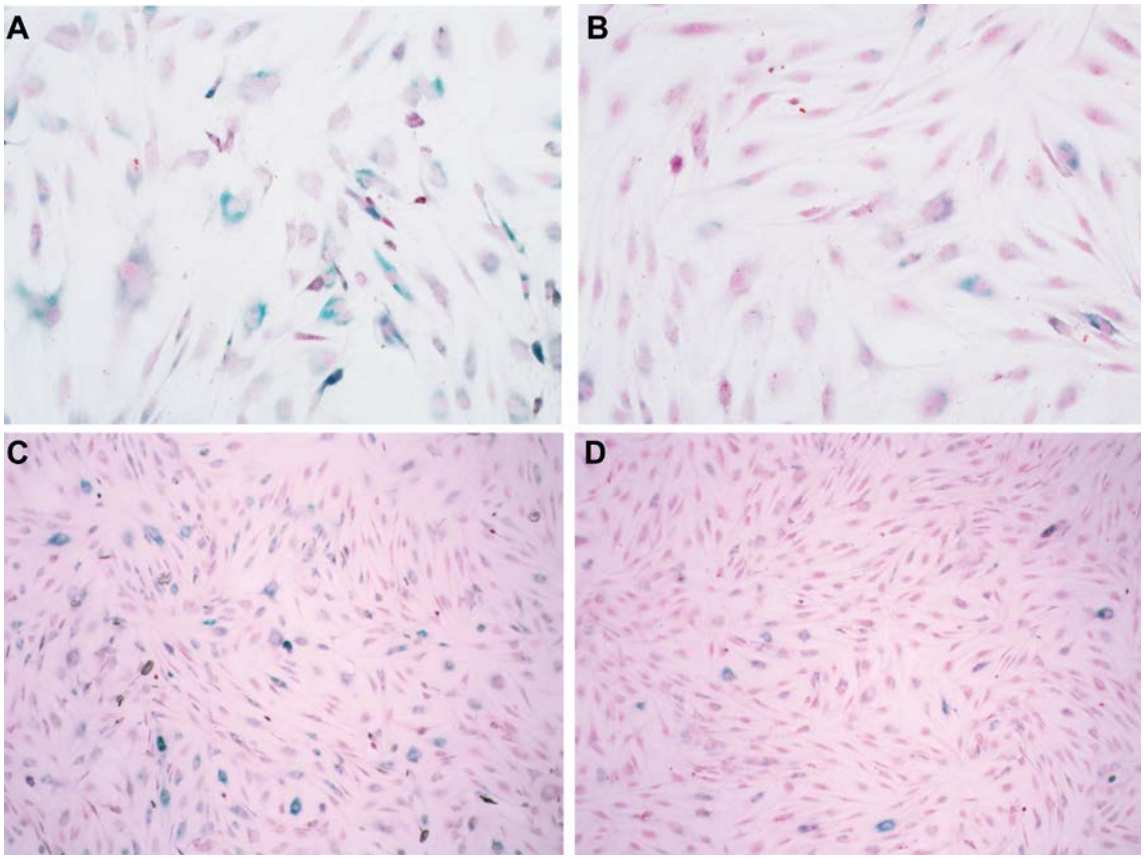

Figure 4.19 High levels of cell senescence are reduced with transcriptional reprogramming of late passage MSCs. SA-β-gal staining of P8 MSC cultures shows a high degree of senescence at 10X (**A**) and 4X (**C**). A smaller fraction of positively staining senescent cells is observed after transcriptional reprogramming of P8 MSCs at 10X (**B**) and 4X (**D**).

4.5 Discussion

The goal of this work was to exploit reprogramming techniques to render cultured adult stem cells resistant to the phenotypic changes that accompany replicative senescence, such as arrested proliferation and decreased differentiation potential, creating an ideal cell type for use in stem cell-based tissue engineering and paving the way for studies of partial reprogramming in cells directly harvested from aged donors. We first developed non-viral reprogramming techniques to induce a more youthful phenotype in human MSCs. We did this as a proof-of-concept study to demonstrate the feasibility of applying reprogramming technology to enhance translational regenerative medicine approaches, in our case for cartilage tissue engineering.

We found in these preliminary studies that we were able to restore significant osteogenic and chondrogenic differentiation potential to near-senescent MSCs using non-viral transcriptional reprogramming. This study has far-reaching implications for regenerative medicine. The art of reprogramming by over-expressing transcription factors has just been developed in the last decade, and little work has been done in the area of transient, partial reprogramming. Thus, our development of techniques for partial reprogramming and our approach to enhancing the properties of the adult stem cells that have been considered a primary candidate cell type for tissue engineering by partially reprogramming them is highly unique. Further refinement may yield a cell type with superior qualities for tissue engineering.

As part of this work, we compared several protocols for reprogramming efficiencies on MSCs, including electroporation, lipid-based transfection reagents, and viral vectors. The highest efficiencies were observed with electroporation of naked plasmid DNA using the

Amaxa Nucleofection system and with lentiviral vectors. Dosing studies of plasmid and virus particle concentration indicated that lentiviral gene transfer was more efficient for generating iPS and partially reprogrammed MSC colonies than any other technique tested, and that higher viral particle concentration yielded greater numbers of colonies with minimal cell death. We developed our own lentivirus-based vector system to enhance the efficiency of MSC reprogramming, but other groups have since generated even more efficient approaches, such as the broadly used all-in-one reprogramming cassette created in the Jaenisch lab. This affords us a system in the future in which we can study mechanisms underlying reprogramming and subsequent alterations in cell fate, particulary as we differentiate reprogrammed MSCs, in a higher throughput approach.

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