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The Effect of Chemotherapy on Stromal Components of the Bone Marrow

Ian Woodrow Hare

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The Effect of Chemotherapy on Stromal Components of the Bone Marrow

Ian Woodrow Hare

Dissertation Submitted
to the College of Medicine
at West Virginia University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in
Immunology and Microbial Pathogenesis

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ABSTRACT

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The cells comprising the stromal compartment of the bone marrow microenvironment are critical to the maintenance of several homeostatic processes of the body. For example, mesenchymal stem cells (MSCs) and osteoblasts are vital to the regulation of differentiation and quiescence of hematopoietic cells, maintaining the skeletal system, and regulating tumor microenvironments. In addition to contributing to these processes, MSCs display several properties that make them favorable for the use in transplantation therapies. The work described herein summarizes the means by which chemotherapy damages these cells, and potential consequences of such damage with regard to their function.

Previously, our laboratory has investigated chemotherapy induced damage of osteoblast potential to support hematopoiesis, describing an increased presence of tumor growth factor beta (TGF- β) and interleukin-6 as contributing factors. We have expanded upon these observations to describe damage elicited by etoposide (VP16) and melphalan on the expression of extracellular matrix and hematopoietic support proteins in the murine pre-osteoblast cell lines MC3T3E1 and 7F2. We showed that chemotherapy dysregulates extracellular matrix (ECM), resulting in reduced type I collagen expression in cell lines, as well as altered morphology of the endosteum in VP16 treated mice. In addition, chemotherapy reduced the abundance of hematopoietic support proteins CXCL12 and osteopontin. Chemotherapy exposure also reduced the expression of osteogenic differentiation associated transcription factors, coincident with reduced differentiation potential of pre-osteoblast cells. These observations highlight the vulnerability of osteoblasts to dysregulation of both hematopoietic support and osteogenic functions following chemotherapy exposure.

In the second study, we expanded our evaluation of stromal cell vulnerability to chemotherapy to mesenchymal stem cells (MSCs). MSCs are beginning to be utilized clinically for transplantation therapies, a process which requires *in vitro* expansion of cells prior to patient administration. To determine whether *in vitro* expansion affects the susceptibility of MSCs to chemotherapy stress, we evaluated the cellular response to etoposide (VP16) at various passages *in vitro*. Although passaging did not influence the susceptibility of MSCs to VP16, we found the repair of VP16 induced DNA damage was altered with extended passage. Exposure of MSCs to VP16 reduced homologous recombination (HR) associated transcripts, a phenomenon that was augmented with passage *in vitro*. Using plasmid based reporter assays, we found that HR mediated repair was reduced in untreated cells, and MSCs were less able to increase non-homologous end joining (NHEJ) following VP16 after extended passage. These results indicated an alteration of the ability of passaged MSCs to perform DNA repair following VP16 stress, indicating that MSCs should not be passaged too extensively prior to utilization for transplantation.

In contrast to the beneficial aspects of MSC function during MSC transplantation, MSCs can display pathogenic interactions with their surrounding environments, such as during cancer progression. In the case of the tumor microenvironment, MSCs have been shown to regulate tumor phenotype through the secretion of various signaling molecules. For example, the Wnt signaling

pathway has been shown to regulate the phenotype of certain tumors. We investigated the expression of Wnt signaling molecules by MSCs after chemotherapy exposure and found that Dkk-1, a secreted inhibitor of Wnt signaling, was increased following exposure to VP16, melphalan, and 5-fluorouracil. Through the use of chemical inhibitors and activators of p53, as well as siRNA silencing, we showed that Dkk-1 elevations in MSCs after chemotherapy were mediated by p53, consistent with the published presence of a p53 promoter element within the DKK1 promoter. These results suggested the potential for MSC derived Dkk-1 to elicit negative effects on patients harboring Dkk-1 responsive tumors, and the possible approach of targeting Dkk-1 pharmacologically in these patients.

Collectively, these findings highlight the dynamic nature of bone marrow derived stromal cells, and illustrate that they are responsive to stress in ways that could negatively impact the health of patients receiving chemotherapy treatment. Given the numerous processes that are regulated by these cells, it is important to identify mechanisms by which chemotherapy elicits damage, providing a conceptual framework for developing means of reducing toxicities associated with chemotherapy treatment by maintaining the ability of bone marrow derived stromal cells to perform critical functions.

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TABLE OF CONTENTS

The Effect of Chemotherapy on Stromal Components of the Bone Marrow.....	i
Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	v
List of Abbreviations.....	vii
Chapter I - The Effect of Chemotherapy on Stromal Components of the Bone Marrow:	
Introduction and Literature Review.....	1
Stromal Components of the Bone Marrow.....	2
Bone Marrow Stromal Cell Function and Clinical Application.....	8
Effects of Chemotherapy on Stromal Cell Function.....	17
References.....	21
Figures.....	30
Chapter II - Bone Marrow Osteoblast Vulnerability to Chemotherapy.....	32
Abstract.....	33
Introduction.....	34
Materials and Methods.....	35
Results.....	39
Discussion.....	43
Acknowledgements.....	46
References.....	47
Figure Legends.....	50
Figures.....	53

Chapter III - <i>In Vitro</i> Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break Repair of Etoposide Induced DNA Damage.....	59
Abstract.....	60
Introduction.....	61
Materials and Methods.....	62
Results.....	67
Discussion.....	71
Conclusions.....	76
Acknowledgements.....	76
References.....	77
Figure Legends.....	81
Figures.....	84
Chapter IV - Chemotherapy Induced Dkk-1 Expression by Primary Human Mesenchymal Stem Cells is p53 Dependent.....	92
Abstract.....	93
Materials and Methods.....	94
Introduction.....	95
Results.....	96
Discussion.....	98
Acknowledgements.....	100
References.....	100
Figure Legends.....	103
Figures.....	106
Chapter V – General Discussion.....	111
References.....	120

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMP-2	Bone Morphogenic Protein-2
BMSC	Bone Marrow Stromal Cell
BSP	Bone Sialoprotein
CAF	Cancer Associated Fibroblast
CFU-F	Colony Forming Unit Fibroblast
CLP	Common Lymphoid Progenitor
Col1A1	Collagen Type 1 α 1
Col2A1	Collagen Type 2 α 1
CXCL12	C-X-C motif Chemokine 12
Dkk-1	Dickkopf-related Protein 1
DMP1	Dentin Matrix Acidic Phosphoprotein 1
ECM	Extra Cellular Matrix
Flt-3	Fms-like Tyrosine Kinase 3
FSP-1	Fibroblast Specific Protein-1
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GVHD	Graft versus Host Disease
HA	Hyaluronic Acid
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
ICAM-1	Intercellular Adhesion Molecule-1
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IL-3	Interleukin-3
Lin-	Lineage Negative
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
M-CSF	Macrophage Colony Stimulating Factor
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
MyoD1	Myogenic Differentiation 1
OCN	Osteocalcin
OI	Osteogenesis Imperfecta
OPN	Osteopontin
OSE2	Osteocalcin-Specific Element 2
OSM	Oncostatin M
OSX	Osterix
PDGFR β	Platelet-Derived Growth Factor Receptor β
Pdx1	Pancreatic Duodenal Homeobox 1
PGE2	Prostaglandin E2
RunX2	Runt-related Transcription Factor 2

SCF
SOX9
Thy-1
TNF- α
VCAM-1
 α -SMA

Stem Cell Factor
SRY (Sex Determining Region Y)-Box 9
Thymocyte Antigen-1
Tumor Necrosis Factor- α
Vascular Cell Adhesion Molecule 1
 α -Smooth Muscle Actin

Chapter I

The Effect of Chemotherapy on Stromal Components of the Bone Marrow

Literature Review

The bone marrow is a complex environment that supports several homeostatic processes within the body. Although hematopoietic cells that arise from the bone marrow have been extensively studied, the stromal cells that share the bone marrow niche are less understood. The following addresses literature related to the stromal cells which comprise the bone marrow, outlines their functions, and addresses what is known regarding how these functions are hampered by chemotherapy treatment.

i. Stromal Components of the Bone Marrow

The bone marrow is an environment that fosters several critical homeostatic functions in mammals. Serving as a primary lymphoid organ for immune cell development, the bone marrow provides an environment permissive for the generation immune cells in the human body¹. In addition, the marrow plays a critical role in the generation of cells necessary for bone development, a constitutively active process which assembles and maintains the skeletal system². The regulation of hematopoietic and skeletal activities within the bone marrow is reliant on the presence of a multipotent population of mesenchymal derived stromal cells, termed mesenchymal stem cells (MSCs), and their progeny. The following provides a partial summary of what is currently understood regarding these stromal populations of the bone marrow, and how their plasticity is regulated within the bone marrow microenvironment.

General Architecture of the Bone Marrow

Bone marrow is a tissue comprised of hematopoietic, mesenchymal, endothelial, and neural derived cells encased by cortical bone (Figure 1). The marrow can be defined as the contents of this ring of cortical bone, and is heavily vascularized. The center of the marrow contains a medullary artery and central sinus, with branching vasculature that travels throughout the marrow and bone regions³. Gaps between the cortical bone and vasculature are filled by

various populations of bone marrow stromal cells, providing a cellular framework which secretes extracellular matrix consisting of, but not limited to, collagen, laminin, and fibronectin⁴ which provides a structural framework. Hematopoietic cells comprise the majority of the cellularity of the bone marrow, and are almost always within proximity of a stromal or endothelial cell¹. Sympathetic nerve fibers are also present within the marrow space, and are known to regulate hematopoietic functions such as cell egress from the marrow during stem cell mobilization⁵.

Mesenchymal Stem Cells

Stromal cells of the bone marrow are derived from a multipotent precursor population, termed mesenchymal stem cells (MSCs)⁶. MSCs are fibroblastic cells that are defined functionally by their ability to differentiate into osteoblasts, adipocytes, and chondrocytes *in vivo*, and have been documented to form other cell types *in vitro*, including neurons, muscle cells, and beta cells⁷. While present in adipose, perivascular, dermal, heart, and muscle tissues⁸, MSCs were first discovered in the bone marrow. In 1976, Friedenstein et al. were the first to describe stromal cells of the bone marrow when they observed colonies of adherent cells displaying a fibroblast morphology following *in vitro* culture of murine bone marrow⁹. These cells displayed clonal growth characteristics, and displayed the ability to differentiate into osteoblasts following transplantation into mice¹⁰. In 1999, Pittenger et al. were the first to comprehensively identify MSCs within human bone marrow which were capable of osteogenic, adipogenic, and chondrogenic differentiation *in vitro*, setting the current standard by which MSCs are recognized and functionally described⁶.

Although much has been learned recently regarding the biology of MSCs, they are less understood and characterized than their hematopoietic counterparts. Many MSC surface molecules have been described including CD44, CD90, CD105, c-Kit, and PDGFR α . However, these molecules are not specific to MSCs, and share expression with closely associated

hematopoietic cell populations of the bone marrow. Combinations of surface markers have been useful in isolating stromal cells that have an MSC phenotype. PDGFR α ⁺Sca-1⁺CD45⁻TER119⁻ cells isolated from murine bone marrow (termed PaS cells)¹¹, PDGFR α ⁺CD51⁺CD45⁻Ter119⁻CD31⁻ cells¹², and CD105⁺CD140a⁺ CD45⁻Ter119⁻CD31⁻¹³ cells have been shown to have the ability to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*. However, only PDGFR α ⁺CD51⁺CD45⁻Ter119⁻CD31⁻ cells have been described in human marrow, and CD105⁺CD140a⁺ CD45⁻Ter119⁻CD31⁻ cells can only differentiate into osteoblasts when observed *in vivo*. These observations highlight the functional heterogeneity displayed by MSCs, and the necessity for better characterization at a surface marker and functional level.

Given the challenges inherent to identifying MSCs by surface phenotype, recent advances have been made using genetic labeling strategies. Nestin is an intermediate filament protein which was first discovered in neural stem cell populations of the brain¹⁴. While investigating mechanisms of neural crest development, Mendez-Ferrer et al. observed a small percentage of stromal cells (< 1%) in the bone marrow of Nestin-GFP mice which were green fluorescent protein (GFP) positive. Further evaluation of these cells showed that they displayed an MSC phenotype, and were necessary for support of hematopoietic stem cells¹⁵. Further evaluation of Nestin⁺ stromal cells has shown that although MSC populations generally are enriched for Nestin, this is not unique to MSCs and osteogenic subsets are Nestin⁺ as well. Wong et al. has shown that *in vitro* osteogenic differentiation of MSCs retains Nestin expression, suggesting that the marker is not MSC specific¹⁶. In an attempt to find a novel mesoderm expressed gene for MSC enumeration, Greenbaum et al. utilized Prx1, a transcription factor which is activated during limb bud development¹⁷. Prx1-ROSA26 mice displayed fluorescence in bone marrow stromal cells with an MSC phenotype. However, ROSA26⁺ osteoblasts and adipocytes were observed as well, showing that Prx1 was a marker

of MSCs as well as more differentiated MSC derived stromal subsets within the marrow¹⁷.

Although genetic markers have been found to evaluate MSCs *in vivo*, there is a great interest in identifying genes which are expressed specifically by MSCs, and do not display expression in more differentiated MSC derived stromal cells.

Osteoblasts

Osteoblasts are derived from MSCs, and generate bone by the formation and mineralization of extracellular matrix (ECM). Osteoblasts are located primarily at bone surfaces and arise from MSC precursors that often occupy a similar niche⁶. Early evaluations of CFU-F (Colony Forming Unit – Fibroblast) cells from cultured bone marrow, later understood to contain MSCs, showed that the presence of bone morphogenic protein (BMP) could induce cells to mineralize ECM¹⁸. The heterogeneity of CFU-F was later appreciated when a population of CFU-F shown to express bone associated proteins (osteocalcin and osteonectin) was observed to differ in size, response to osteogenic stimuli, and proliferative capacity, corresponding to osteoprogenitor cells of varying degrees of differentiation¹⁹. The genetic regulation of osteogenic differentiation was poorly understood until Komori et al. showed that mice deficient in *Cbfa1*, the gene that encodes Runt-related transcription factor (RunX2), died shortly after birth and displayed reduced skeletal development. Stromal cells isolated from *Cbfa1*^{+/-} bone marrow displayed elevated alkaline phosphatase (ALP) activity and increased osteocalcin expression relative to *Cbfa1*^{-/-} marrow, indicative of reduced osteogenic potential in stromal cells with decreased *Cbfa1*²⁰. RunX2 is therefore necessary for osteogenic differentiation of MSCs, defining the step from an MSC to pre-osteoblast phenotype by initiating the expression of proteins containing an osteoblast-specific cis-acting element (OSE2) in their promoter regions. The promoter of many proteins vital to osteogenesis contain an OSE2, such as type 1 collagen alpha 1 (*Col1A1*), osteopontin (OPN), osteocalcin (OCN), and osterix (OSX)²¹. *Col1A1*, OPN, and OCN are necessary for the formation and mineralization of ECM during osteogenesis

(described in section ii of this literature review). OSX is a zinc finger transcription factor that has been shown to be expressed following RunX2 in the progression towards a mature osteoblast phenotype²². OSX^{-/-} mice have similar skeletal deformities observed in Cbfa1^{-/-} mice. RunX2 can still be detected in mesenchymal tissues of OSX^{-/-} mice, however, OSX cannot be detected in Cbfa1^{-/-} mice, indicating that OSX is regulated downstream of RunX2 and is required for differentiation to a mature osteoblast phenotype²³.

Mature osteoblasts differentiate into osteocytes, terminally differentiated stromal cells that reside deeper within bone tissue than osteoblasts and MSCs²⁴. Osteocytes are encased by bone matrix, a process thought to be a result of osteoblasts immobilizing the osteocyte within layers of ECM²⁵. However, others have postulated that osteocyte burrowing is a more active process, involving the cleavage of bone matrix by osteocyte matrix metalloproteinase-14 (MMP-14) and migration deep within bone tissue²⁶. The progression from an osteoblast to osteocyte phenotype is less understood than the mechanisms by which less-differentiated osteoprogenitors mature, however, osteocyte gene expression has been described and is distinct from mature osteoblasts²⁴. Although osteocytes express osteocalcin similarly to mature osteoblasts, RunX2, OSX, OPN, Col1A1, and ALP expression is reduced relative to osteoblasts²².

Adipocytes

Adipocytes are the primary cellular component of adipose tissue, storing glucose as triglycerides for release into the bloodstream in the form of fatty acids when the body is in need of energy²⁷. The cellular morphology of adipocytes is different from MSCs, appearing slightly larger, round in shape, and containing cytosolic lipid droplets which are visible under brightfield microscopy. Ulf Smith was the first to document the presence of stromal cells in adipose tissue, capable of differentiating into adipocytes *in vitro* in the presence of human serum²⁸.

Adipogenic differentiation is dependent on the expression of peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (C/EBP α)²⁹. PPAR γ and C/EBP α are transcription factors that activate the expression of genes necessary for adipocyte function, such as glucose transporter 4, lipoprotein lipase, and fatty acid synthase³⁰. Insulin and glucocorticoids have been shown to induce PPAR γ and C/EBP α expression in human MSCs³¹. Embryonic stem cells from PPAR γ ^{-/-} mice are unable to differentiate into adipocytes³², and although C/EBP α ^{-/-} stromal cells are capable of differentiating into adipocytes, they are not as robust as wild type cells³³. These results indicate that both transcription factors are necessary for normal adipocyte function; however, it is PPAR γ that predominantly drives adipogenic differentiation of MSCs.

Chondrocytes

Similar to bone, cartilage serves as an organic matrix with supportive properties that are conducive to loading and bearing weight; however, the ECM components that comprise cartilage are more flexible than bone, and are generally less mineralized. Chondrocytes are responsible for the generation of cartilage, forming ECM in a similar manner as osteoblasts, only secreting different molecules which are ultimately less rigid³⁴. Chondrocyte ECM is primarily composed of collagen type 2A (Col2A1) and aggrecan, a proteoglycan that has a high affinity for hyaluronic acid (HA)³⁵. High density culture of MSCs with dexamethasone and transforming growth factor β (TGF- β) has been shown to induce chondrogenic differentiation *in vitro*³⁶. SRY-type high mobility group box 9 (SOX9), which has been shown to be expressed in MSCs following TGF- β exposure, has been described as an early transcription factor driving chondrogenic differentiation, promoting the expression of Col2A1³⁷.

Other Mesenchymal Stem Cell Derived Cell Types

Although not as well described in the literature as osteoblasts, adipocytes, and chondrocytes, MSCs have been observed to differentiate into other cell types as well. Toma et al. presented evidence of MSC differentiation into cardiomyocytes *in vivo*, following transplantation of human LacZ⁺ MSCs into immunodeficient mice³⁸. *In vitro* culture of MSCs with 5-azacytidine has been shown to alter cellular morphology and induce the expression of Myogenic Differentiation 1 (MyoD1), a myogenic differentiation marker³⁹. In addition, retroviral expression of pancreatic duodenal homeobox 1 (Pdx1) in human MSCs was shown to activate the expression of insulin following glucose exposure, similar to the function of pancreatic beta cells⁴⁰. These findings indicate that MSCs are a multipotent population of cells, potentially capable of differentiating into numerous clinically beneficial cell types (described in more detail in part ii of this literature review).

ii. Bone Marrow Stromal Cell Function and Clinical Application

Hematopoiesis

Hematopoiesis is the process by which stem and progenitor cells of the immune system differentiate into the cellular constituents of blood. The hematopoietic stem cell (HSC) is the most immature of hematopoietic cells, and is capable of differentiation into all hematopoietic cell types and performing asymmetric division (Figure 2). Vertebrates harbor a deeply quiescent pool of HSCs that are capable of differentiating into myeloid and lymphoid progenitor cell populations that lose the ability to self-renew, but are more proliferative than the primitive HSC⁴¹. There is a finite number of HSCs in the bone marrow of vertebrates. When proliferation of this stem cell pool is not properly regulated, the pool can be depleted, a phenomenon known as exhaustion⁴².

The differentiation and proliferation of HSC and other hematopoietic progenitor cells are regulated in part by the bone marrow microenvironment. The concept of a niche that regulates the function of stem cells was first hypothesized by Schofield⁴³, built upon the observations that hematopoietic cells occupy distinct regions of the bone marrow⁴⁴ and the requirement of bone marrow stromal cells for *ex vivo* maintenance of hematopoietic cells⁴⁵. In 2004, Visnjic et al. suggested that osteoblasts were the stromal population responsible for supporting HSC when they observed a great reduction in HSCs as well as more differentiated lymphoid and myeloid cells following *in vivo* ablation of osteoblasts⁴⁶. This notion was revisited by Greenbaum and Ding et al., performing similar studies that involved evaluating the abundance of hematopoietic cell populations in mice where C-X-C motif chemokine 12 (CXCL12) was ablated from various stromal populations *in vivo*. CXCL12 is a chemokine that is expressed by bone marrow stromal cells and plays a critical role in the recruitment and retention of HSCs and progenitor cells to the bone marrow niche⁴⁷. Ablation of CXCL12 from MSC populations resulted in a release of HSCs from the bone marrow niche¹⁷, while ablation of CXCL12 from osteoblasts only dispersed common lymphoid progenitor (CLP) cells⁴⁸, suggesting that MSCs contribute to the maintenance of HSC support while osteoblasts are only responsible for supporting CLPs.

Stromal cells of the bone marrow secrete many factors that regulate the quiescence and differentiation of HSCs. TGF- β and OPN have both been shown to reduce the proliferation of HSCs^{49,50}. When there is a necessity for blood cell generation, MSC and osteoblast derived stem cell factor (SCF), interleukin-3 (IL-3) and Fms-like tyrosine kinase 3 (Flt-3) are some of the factors that support the proliferation and survival of HSCs and committed progenitor cells⁵¹. In addition, granulocyte, granulocyte-macrophage, and macrophage colony stimulating factors (G-CSF, GM-CSF, and M-CSF) have been shown to promote the differentiation of HSCs into neutrophils, neutrophils and macrophages, or macrophages (respectively)⁵². The balance between proliferation and differentiation is absolutely critical to steady state hematopoiesis and

highlights the potential of any dysfunction of elements of the supportive marrow microenvironment to contribute to dysregulated immune system function.

The ECM that is secreted by MSCs and osteoblasts plays an indirect role in influencing the bioavailability of cytokines to (such as TGF- β ⁵³ and OPN⁵⁰) to HSCs in the bone marrow microenvironment⁵⁴. MSCs and osteoblasts also secrete proteoglycans into ECM which can modulate the stability of cytokine gradients. For example, stromal derived heparin sulfate has been shown to be necessary for the stabilization of GM-CSF⁵⁵, IL-3⁵⁶, and CXCL12⁵⁷ in the bone marrow. Saez et al. recently utilized the CXCL12 modulating potential of heparin sulfate to mobilize HSCs by inhibiting the biosynthesis of heparin sulfate specifically in osteoprogenitor cells⁵⁸. HA is another proteoglycan expressed by MSCs and osteoblasts, and facilitates binding of hematopoietic cells to the bone marrow niche via CD44 expressed on the surface of hematopoietic cells⁵⁹. Other MSC and osteoblast expressed surface proteins that mediate direct binding to hematopoietic cells include vascular cell adhesion protein 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and thymocyte antigen 1 (Thy-1)⁵⁹, and are necessary for the anchoring of hematopoietic cells to supportive stromal cells within the bone marrow niche. Many studies have indicated that this physical interaction is absolutely essential for hematopoietic cell development, and cannot be substituted for by any combination of cytokines or growth factors¹.

Osteogenesis

Prior to the understanding that bone marrow osteoblasts and osteoprogenitor cells contribute to hematopoietic support, their primary described function was osteogenesis. Osteogenesis is a process that involves the differentiation of MSCs (described in section i of this literature review) to osteoblasts, secreting an abundance of ECM which is subsequently mineralized, resulting in the dense bone tissue which comprises our skeletal system².

The organic component of bone is referred to as osteoid, and consists of osteoblast secreted ECM and ECM binding proteins. Osteoid accounts for fifty percent of bone volume⁶⁰. The predominant component of osteoblast ECM is Col1A1, contributing to ninety percent of osteoid². The high tensile strength of collagen confers strength to bone tissue, while remaining permissive to a small degree of flexibility⁶¹. Although less abundant than Col1A1 in bone, osteoblast secreted ECM proteins are essential for several components of bone function. OCN is a non-collagenous protein produced by mature osteoblasts and osteocytes, and has been shown to play a structural role by binding tightly to Col1A1 and hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), a crystallized form of calcium and phosphate that comprises the majority of bone mineral density⁶². OPN is a highly negatively charged ECM protein that has also been described as playing a structural role in osteoblast ECM through interactions with OCN⁶³. OCN has also been shown to sequester calcium, serving as a negative regulator of bone mineralization⁶⁴.

Following the formation of osteoid, osteoblasts regulate the process by which osteoid is mineralized. The mineralization of osteoid provides compression strength and the hardness that is characteristic of bone tissue⁶⁵. The majority of the inorganic portion of bone is hydroxyapatite⁶². The formation of hydroxyapatite is regulated by various secreted osteoblast proteins which can promote its formation or generate the calcium and inorganic phosphate necessary for its synthesis. Dentin matrix protein 1 (DMP1) and bone sialoprotein (BSP) are nucleating factors, structurally ideal for initiating the crystallization process that forms hydroxyapatite in bone⁶⁶. Alkaline phosphatase (ALP) is abundantly expressed by osteoblasts, and cleaves phosphate from various molecular sources such that it can be incorporated into hydroxyapatite⁶⁷. Dietary ascorbic acid also plays a role in maintaining the presence of minerals that comprise hydroxyapatite by stabilizing levels of calcium and phosphorus in the blood⁶⁸.

The formation of bone is tightly regulated by various signaling pathways that influence the differentiation of MSCs to an osteoblast phenotype and induce the expression of osteoblast specific genes. Wnt signaling is known to be a critical regulator of bone development through the regulation of β -catenin mediated gene expression activated by various Wnt activating ligands binding to Frizzled and low-density lipoprotein receptor-related proteins (LRPs) at the surface of the osteoblast⁶⁹. The first evidence of Wnt signaling regulating bone development was described by Gong et al., showing that mutations in the LRP5 gene correlated with patient diagnosis of osteoporosis-pseudoglioma syndrome, a genetically acquired disease that is associated with very low bone mass and frequent fractures⁷⁰. This finding was later confirmed by Kato et al. in a LRP5^{-/-} mouse model⁷¹. Of the 19 Wnt activating ligands expressed by humans⁷², several positively regulate osteogenic differentiation, including Wnt3a⁷³ and Wnt10b⁷⁴. Secreted inhibitors of Wnt signaling also play a role in regulating this response, such as dickkopf 1 (Dkk-1), a steric inhibitor of LRP5/6 which is expressed by osteoblasts and prevents Wnt induced osteogenic differentiation *in vivo*⁷⁵. Soluble frizzled receptor 1 (SFRP1), a soluble form of the Frizzled Wnt receptor, quenches Wnt activating ligands in the extracellular environment. Bodine et al. observed increased bone mass and osteoblast differentiation in SFRP1^{-/-} mice, highlighting the importance of SFRP1 in the regulation of osteogenesis *in vivo*⁷⁶.

Tumor Microenvironment Regulation

MSCs were initially discovered in the bone marrow, but are now appreciated to be more widely distributed⁸. Although generally beneficial, the activities of MSCs and MSC derived stromal populations can contribute to disease states, such as in the setting of diverse tumor microenvironments. The tumor microenvironment can be defined broadly as the cellular environment surrounding a tumor. This environment is comprised of tumor cells surrounded by normal tissue which often contains MSCs, as well as fibroblasts, vasculature, and immune cells⁷⁷. Similar to the ways that MSCs regulate the hematopoietic niche, MSCs play a role in

regulating the characteristics of a tumor through the expression of cytokines or other signaling molecules. For example, co-culture of breast cancer cell lines with MSCs was shown to increase the expression of miR-199a, which decreased FOXOP2 and increased metastasis when tumor cells were implanted subcutaneously into nude mice⁷⁸. Conversely, Wang et al. showed that MSC derived Oncostatin M (OSM) inhibited tumor growth and metastasis in a murine model of lung adenocarcinoma⁷⁹. These observations highlight the ability of MSCs to both positively and negatively regulate the tumor microenvironment.

Literature describing the tumor microenvironment often focuses on the activity of cancer associated fibroblasts (CAFs), a population of fibroblasts that is considered quite heterogeneous, similar to MSCs⁸⁰. It is believed that CAFs are derived from tissue resident MSCs or fibroblasts⁷⁷, however, some consider MSCs and CAFs as similar cell types that are defined by their proximity and their propensity to regulate tumor activity⁸¹. Defined qualitatively by fibroblast morphology and adjacency to a tumor, CAFs have also been described as expressing α -smooth muscle actin (α -SMA), platelet-derived growth factor β (PDGFR β), and fibroblast-specific protein 1 (FSP-1)⁸². However, these surface markers are expressed on MSCs, as well as other cell types within the tumor microenvironment⁸³⁻⁸⁵. These observations show that MSCs and CAFs are both heterogeneous populations of stromal cells which often contribute to the regulation of the tumor microenvironment in similar ways. In addition to MSCs and CAFs, osteoblasts have been reported to regulate tumor activity in cancers that originate in the bone or marrow (such as acute lymphoblastic leukemia, multiple myeloma, and osteosarcoma), or commonly metastasize to them (breast and prostate)⁷⁹.

Although MSCs are distributed in tissues surrounding tumors, it is often observed that MSCs actively home from distant anatomical sites to the tumor. This phenomenon has been attributed, in part, to the expression of chemotactic cytokines by the tumor, such as CXCL12⁸⁶ and interleukin-6⁸⁷. Quante et al. showed that some CAFs surrounding tumors in a murine

model of gastric cancer were derived from bone marrow MSCs. Mice were administered GFP⁺ MSCs that efficiently engrafted the bone marrow prior to developing gastric cancer. Once gastric tumors developed, over 20% of the cells were α -SMA⁺GFP⁺ indicating that MSCs home to the marrow to distant tumors, even though tissue resident MSCs were already present⁸⁸. Once attracted to the site of a tumor, MSCs can change gene expression in ways that anchor them to the tumor site. For example, Uchibori et al. observed that high concentrations of tumor necrosis factor- α (TNF- α) within the tumor microenvironment induced VCAM-1 expression by MSCs, anchoring them around nearby endothelial cells⁸⁹.

Once present within the tumor microenvironment, MSCs can promote tumor growth by various mechanisms. MSCs have been shown to secrete cytokines that create a generally immune suppressive environment. For example, Patel et al. showed that MSC derived TGF- β increased the presence of regulatory T cells that resulted in the inhibition of immune mediated tumor clearance of breast cancer cells⁸⁹. MSCs have also been shown to suppress the proliferation of activated CD8⁺ T cells through the expression of prostaglandin E2 (PGE2), indoleamine 2, 3-dioxygenase (IDO) and TGF- β ⁹⁰. In addition, MSCs have been described as promoting an anti-inflammatory alternatively activated macrophage (M2) phenotype in a model of myocardial infarction⁹¹. In addition to suppressing the ability of the immune system to target cancerous cells, MSCs can promote angiogenesis. Suzuki reported increased expression of angiogenic cytokines in B16 melanoma cells co-cultured with MSCs, as well as increased tumor vessel area when B16 cells were co-injected into mice with MSCs (relative to B16 cells alone)⁹².

In vivo work investigating the role of osteoblasts in regulating hematological cancers has provided insight into ways osteoblasts can prevent malignancy, and how the initiation of disease can be influenced by niche cues. Raaijmakers et al. have shown that deletion of dicer in osteoblasts results in the generation of myelodysplastic syndrome⁹³. In addition, the constitutive activation of β -catenin in osteoblasts has been shown to initiate acute myeloid leukemia in

mice⁹⁴. These observations serve as evidence for the importance of stromal cells in the maintenance of stem cell niches, and the necessity of understanding how perturbations within the niche can influence malignancy.

Mesenchymal Stem Cell Transplantation

MSCs display several attributes that have drawn attention to their clinical potential for transplantation. MSCs represent a population of cells that can be obtained from bone marrow, adipose, and placental specimens⁶, then expanded *in vitro* to generate cells for transplantation into humans or animals experiencing diseases caused by dysfunctional mesenchymal tissues or inflammation. The well described ability of MSCs to differentiate into osteoblasts, chondrocytes, and other stromal cells of the body (discussed earlier in this literature review) led researchers and clinicians to investigate the potential utilization of MSCs to treat diseases that involve deficiencies of these cell types. Orlic et al. was the first to show that transplanted bone marrow depleted of committed hematopoietic lineage marker positive cells (Lin⁺) homed to murine necrotic tissue following myocardial infarction, subsequently repairing the affected ventricle and forming almost 70 percent of the tissue after recovery⁹⁵. Dysfunctional pancreatic beta cells in a murine model of diabetes myelitis were rescued by intravenous infusion of MSCs, resulting in increased insulin production by pancreatic cells and glucose homeostasis⁹⁶.

These findings inspired the first attempt to utilize MSC transplantation in humans. Osteogenesis imperfecta (OI) is a severe congenital disease that results in brittle and undeveloped bone due to various mutations in the COL1A1 gene. In 2002, Horwitz et al. administered allogenic MSCs to six children with OI in an attempt to repopulate their bone marrows with MSCs capable of differentiating into functional osteoblasts. The authors found evidence of MSC homing to bone and skin, and reported 60-94 percent increases in bone growth velocity (relative to age matched controls) six months following transplantation⁹⁷.

Although all of these studies documented the dissemination of transplanted MSCs throughout the body, the abundance of MSCs and their progeny were generally limited, indicating a technical difficulty regarding the use of this technique clinically. For example, Dominici et al. observed 30% of murine bone consisting of GFP⁺ MSCs shortly after transplantation, decreasing to 10% by 52 weeks of age and undetectable at subsequent time points⁹⁸. This observation suggests that multiple transplants of MSCs may be necessary to provide lasting treatment effects. Consistent with this notion, Götherström et al. found that prenatal administration of allogenic MSCs for the treatment of OI followed by re-transplantation at 19 months and 8 years of age prolonged the positive effects of treatment in two patients⁹⁹. These results highlight the clinical potential of MSC transplantation, and the necessity of efficient ex vivo expansion of cells that maintain critical functions to acquire adequate cell numbers for long term clinical benefits.

As discussed previously in this literature review, MSCs have been documented to have immune suppressive properties. MSCs were shown to reduce the proliferative potential of activated T-cells and delay the rejection of grafted skin tissue in baboons¹⁰⁰. Zappia et al. were the first to show that the anti-inflammatory properties of MSCs were capable of reducing the severity of disease in a murine model of experimental autoimmune encephalitis when administered at the onset of disease¹⁰¹. Transplanted MSCs have also benefited the motor function of rats 14 days following stroke, believed in part to be a consequence of reduced neuroinflammation by MSCs¹⁰². The immune suppressive capabilities of MSCs have been utilized to prevent graft vs. host disease (GVHD) in human patients; a Swedish patient experiencing severe treatment-resistant grade IV GVHD from an allogenic bone marrow transplant for the treatment of acute lymphoblastic leukemia was administered MSCs twice intravenously, resulting in a full recovery one year out from a disease that has a median survival of two months¹⁰³. These results were corroborated by a larger study showing a 77.7% two year

survival-rate for patients with refractory GVHD treated with two administrations of donor MSCs¹⁰⁴.

Although favorable results have been observed in animal models and some clinical trials of MSC transplantation, no MSC based therapy has currently been approved by the United States Food and Drug Administration. Prochymal®, a MSC based stem cell therapy developed by Osiris Therapeutics has currently been approved as a first line treatment of acute GVHD in Canada, and is currently under Phase III clinical trials for GVHD in the United States (clinicaltrials.gov identifier NCT00562497). Clinical trials are also currently active for the treatment of Crohn's disease, type-1 diabetes, and myocardial infarction (clinicaltrials.gov identifiers NCT00543374, NCT00690066, and NCT00877903). The technically involved process of acquiring MSCs from autologous or allogenic donors, expansion *ex vivo*, and multiple administrations to a patient has hampered the progression of MSC transplantation towards first line clinical use¹⁰⁵. However, the lack of cures (and often treatments) for the diseases that benefit from MSC transplantation have maintained interest in the process since the early 2000's. This unique necessity for *ex vivo* expansion of MSCs prior to use in stem cell therapies highlights the need for a better understanding of how to optimally expand MSCs without altering phenotype; it also illustrates the necessity of better understanding this process to ensure the optimization of good manufacturing practice during MSC expansion.

iii. Effects of Chemotherapy on Stromal Cell Function

Regulation of Immune Regeneration

Cytotoxic chemotherapy regimens preferentially target proliferative cells, a characteristic that enables the killing of tumor cells, but at the expense of healthy proliferative tissues. The bone marrow is an example of such a tissue, displaying rapid cellular proliferation to supply the

high cellular demand of continuous blood generation. In the context of hematopoietic malignancies, this immune cell toxicity can be the intent of therapy, such as during myeloablative chemotherapy regimens. Myeloablation serves to eliminate as many of the hematopoietic cells of the marrow as possible, providing space for marrow repopulation by transplanted hematopoietic cells from an autologous or allogenic source. Although hematopoietic donor cells are capable of repopulating the marrow, the process is often slow. Following myeloablative chemotherapy and transplantation, deficiencies in immune cell counts often persist for more than a year after transplantation¹⁰⁶, leaving patients susceptible to anemia and infection for an extended period of time. Immune deficiencies can persist in patients for years following treatment. For example, women treated for breast cancer have displayed deficiencies in vaccine efficacy years after the treatment of their cancers¹⁰⁷.

Although it is possible that these deficits are due to a shortcoming of the transplanted hematopoietic cells, it is also possible that the phenomenon is regulated by dysfunction of the bone marrow niche. Unlike the transplanted hematopoietic cells, stromal cells of the bone marrow experience chemotherapy damage that negatively influences their ability to support hematopoietic function. Kemp et al. showed that MSCs isolated from patients treated with high dose chemotherapy for hematological malignancies displayed reduced abilities to expand *in vitro*, as well as reduced CD44 expression¹⁰⁸. Dominici et al. observed osteoblasts in the endosteal niche becoming highly proliferative shortly after chemotherapy treatment to repair damage inflicted upon the anatomical architecture of the bone marrow¹⁰⁹. Although damaged stromal cells of the bone marrow are quickly replenished, their function has been shown to be reduced for a long period of time. For example, Galotto et al. have shown that CFU-F frequencies are diminished in patients who have received bone marrow transplantation relative to normal controls for up to 12 years following transplantation, and cultured stromal cells from these patients are less able to support the long term persistence of HSCs *in vitro*¹¹⁰. These

observations highlight the susceptibility of bone marrow stromal cells to damage that impedes their hematopoietic support functions and reminds us of the fact that this population of cells is not simply a stable support system but a dynamic, responsive population.

Evidence for stromal dysregulation is also present in the context of myelosuppressive chemotherapy regimens. When less intensive chemotherapy treatment is needed for the treatment of a malignancy, peripheral blood cell counts will drop following therapy, followed by a recovery which lasts on the order weeks to months. This phenomenon is referred to as myelosuppression, and is more clinically prevalent than myeloablative therapies¹¹¹. Myelosuppressive chemotherapy has been associated with damage of sympathetic nerves of the bone marrow, resulting in attenuated hematopoietic recovery¹¹². In addition, Brenet et al. showed that 5-fluorouracil treatment of mice resulted in an increase in TGF- β signaling in the bone marrow. By inhibiting TGF- β with neutralizing antibodies, the authors were able to promote the rate of granulocyte and erythrocyte recovery after chemotherapy treatment¹¹³. This observation is consistent with several lines of evidence from our laboratory and others outlining the suppressive activity of TGF- β on HSCs, as well as the fact that chemotherapy exposure increases the expression of active TGF- β by bone marrow stromal cells and osteoblasts^{114–116}. Our observations show the likelihood that the increased TGF- β observed by Brenet et al. after chemotherapy treatment was in part derived from stromal cells of the bone marrow. This observation indicates the potential of pharmacologically targeting niche factors that regulate HSCs for chemotherapy associated leukopenia, as opposed to HSCs themselves.

The prospect of modulating bone marrow stromal cell derived molecules following chemotherapy exposure is a new but developing field. Acceleron has developed Sotatercept®, a peptide drug consisting of an activin receptor type IIA domain fused to the Fc portion of human IgG1, serving to broadly inhibit TGF- β family extracellular signaling molecules¹¹⁷.

Sotatercept has shown clinical efficacy for the treatment of chemotherapy induced anemia in

Phase II clinical trials¹¹⁸. A better understanding of the signaling pathways that modulate hematopoietic regeneration following bone marrow stress is required to determine other potential ways to promote immune regeneration in patients who have undergone chemotherapy treatment.

Bone Development

Impaired osteogenesis is often overlooked as a toxicity associated with chemotherapy treatment. However, deficits in bone formation have been documented across a spectrum of cancers treated with various chemotherapeutic agents. Observations in Chapter II of this dissertation provide evidence for the alteration of osteoblast function *in vitro*. Consistent with these observations, Georgiou and Davies et al. have shown deficits in the function of osteoblasts isolated from rats and humans, respectively, following chemotherapy treatment^{119,120}.

Attenuated osteogenesis following chemotherapy has been documented *in vivo* as well. Tillmann et al. evaluated bone mineral density in children who have been treated with high dose chemotherapy for acute lymphoblastic leukemia, showing that chemotherapy treated children displayed a decrease in bone mineral density relative to age matched controls, correlating with an increased incidence in fractures¹²¹. Women treated with carboplatin, paclitaxel, or cisplatin for various gynecological cancers have experienced reduced lumbar bone mineral density one year after treatment¹²². Similar results were observed in men treated for Hodgkin's lymphoma, displaying attenuated bone mineral density in the lumbar spine and forearms as long as 6.8 years following treatment¹²³. Little is known regarding the mechanism behind chemotherapy induced osteogenic deficiencies; more studies are required to understand this phenomenon well enough to design therapies that prevent chemotherapy induced toxicities of the skeletal system.

Summary

The findings described in this literature review illustrate the dynamic nature of stromal cells of the bone marrow microenvironment. Playing a role in several homeostatic processes within the body, this clinically significant group of cells displays several characteristics that justify the need to better understand their biology. For example, the necessity of MSCs to regulate the characteristics of other cell populations (such as hematopoietic and tumor populations, described earlier in this literature review) underlies the importance in understanding how they operate in both normal and stressed conditions. Chapters II, III and IV of this dissertation outline distinct changes in MSC and osteoblast cells that are a consequence of chemotherapy exposure, a clinically relevant example of systemic stress *in vivo*. Furthermore, Chapter III describes the effects of *ex vivo* expansion of MSCs, and how this could potentially affect MSC function following transplantation into a host for stem cell therapy. Collectively, the following will contribute to better understanding mechanisms regarding the response of MSCs to chemotherapy stress, providing insight into means by which these responses can be modulated to better manage sequelae experienced by patients as a consequence of damaged stromal components of the bone marrow microenvironment.

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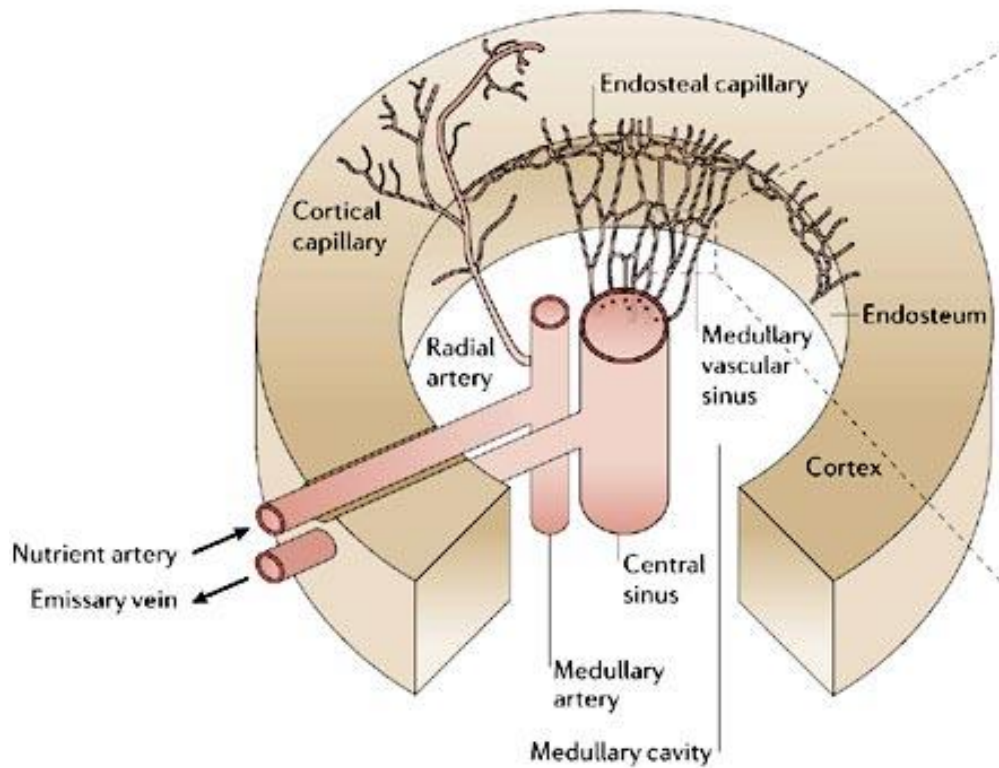
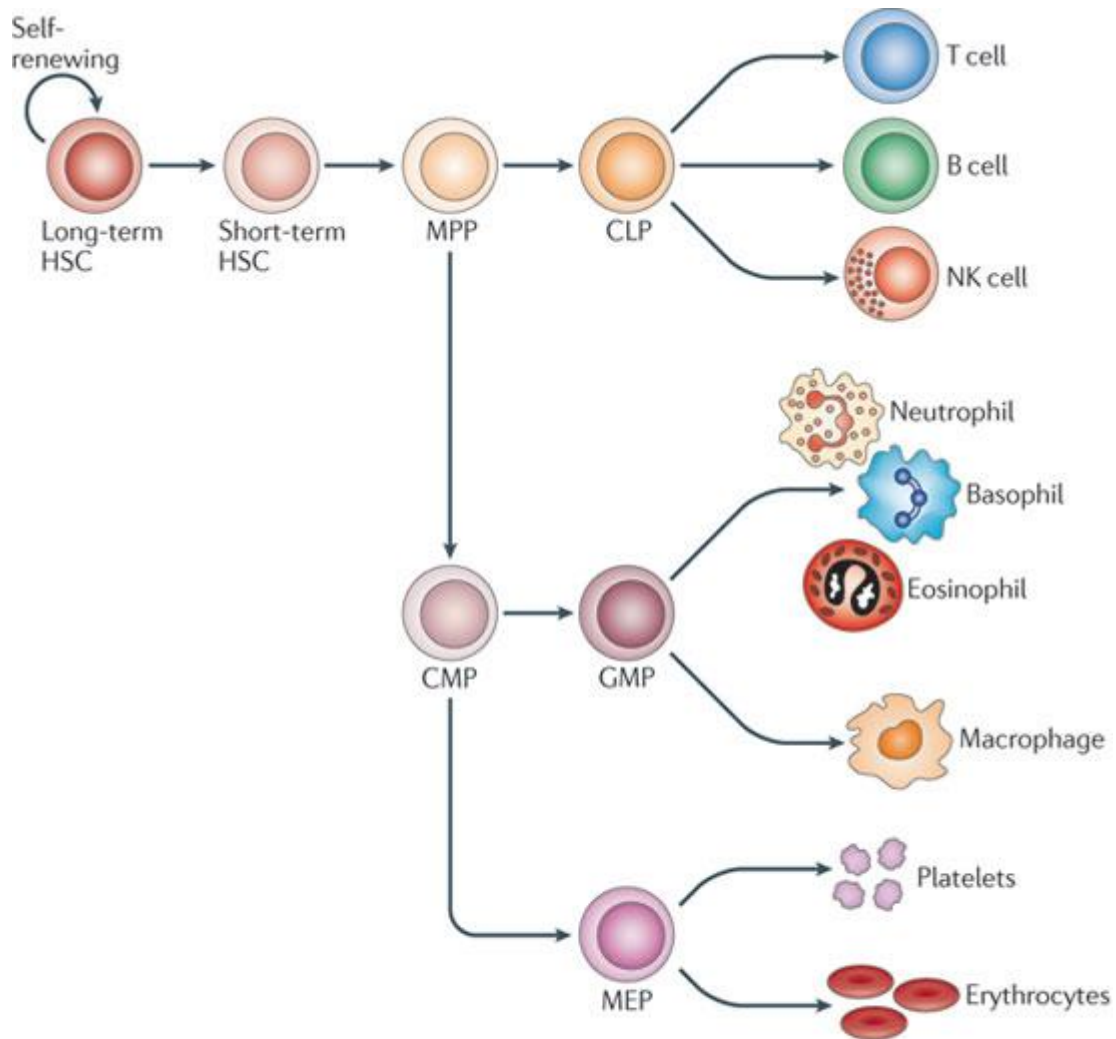


Figure 1. Structure of the mammalian femur.

Image adapted from Nagasawa T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nature Reviews Immunology*. Permission Number: 3835720017612.



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Figure 2. Differentiation schema of hematopoietic cells.

King K and Goodall M. 2010. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. Nature Reviews Immunology. Permission Number: 3835730219427.

Chapter II

Bone Marrow Osteoblast Vulnerability to Chemotherapy

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Abstract

Osteoblasts are a major component of the bone marrow microenvironment which provide support for hematopoietic cell development. Functional disruption of any element of the bone marrow niche, including osteoblasts, can potentially impair hematopoiesis. We have studied the effect of two widely used drugs with different mechanisms of action, etoposide (VP16) and melphalan, on murine osteoblasts at distinct stages of maturation. VP16 and melphalan delayed maturation of preosteoblasts and altered CXCL12 protein levels, a key regulator of hematopoietic cell homing to the bone marrow. Sublethal concentrations of VP16 and melphalan also decreased the levels of several transcripts which contribute to the composition of the extracellular matrix (ECM) including osteopontin (OPN), osteocalcin (OCN) and collagen 1A1 (Col1a1). The impact of chemotherapy on message and protein levels for some targets was not always aligned, suggesting differential responses at the transcription and translation or protein stability levels. Since one of the main functions of a mature osteoblast is to synthesize ECM of a defined composition, disruption of the ratio of its components may be one mechanism by which chemotherapy affects the ability of osteoblasts to support hematopoietic recovery coincident with altered marrow architecture. Collectively, these observations suggest that the osteoblast compartment of the marrow hematopoietic niche is vulnerable to functional dysregulation by damage imposed by agents frequently used in clinical settings. Understanding the mechanistic underpinning of chemotherapy-induced changes on the hematopoietic support capacity of the marrow microenvironment may contribute to improved strategies to optimize patient recovery post-transplantation.

Keywords: bone marrow microenvironment, osteoblast, extracellular matrix, hematopoietic stem cell, chemotherapy

Introduction

Hematopoietic stem (HSC) and progenitor cells require interaction with their niche in the bone marrow microenvironment for regulation of quiescence, cell cycle progression and differentiation [1–3]. Several types of cells in the bone marrow provide support to HSC and their lineage committed progenitors, including endothelial cells [4,5], CXCL12-abundant reticular cells (CAR) [6,7], nestin-positive mesenchymal stem cells (MSC) [8], CD146-positive subendothelial osteoprogenitors [9], and osteoblasts [10–12]. There is evidence to suggest that osteoblasts, as a defining component of the endosteal space, support HSC and regulate the fate of more differentiated hematopoietic progenitors [10,11]. Depletion of osteoblasts in mice profoundly affects hematopoiesis, manifested by decreases in HSC as well as lymphoid and myeloid progenitors [12], and B-cell commitment and maturation [13].

Osteoblasts are derived from MSCs through several somewhat poorly defined maturation stages. MSCs differentiate to osteoprogenitor cells through the activation of osteoblast-specific transcription factors such as Runx2 and subsequently Osterix (SP7) [14]. One of the earliest markers of preosteoblast cells is alkaline phosphatase (ALP) and its expression persists in subsequent maturation stages. Preosteoblasts also express Col1a1 protein, while at later stages they also start to produce OPN and further differentiate to mature osteoblasts which synthesize bone matrix [15]. These various markers provide tools to characterize specific stages of osteoblast differentiation.

In the current study we investigated how osteoblasts are affected by chemotherapy by utilizing an *in vitro* model to include drugs from two distinct classes used in clinical settings; Etoposide (VP16) which induces double strand DNA breaks by inhibition of topoisomerase II [16] and melphalan as an alkylating agent that damages DNA through crosslinking and the addition of adducts [17]. A number of chemotherapy drugs have been documented to functionally impair stromal cells in the bone marrow, including 1,3-bis(2-chloroethyl)-1-

nitrosourea, busulfan, doxorubicin, VP16, methotrexate, and vincristine [18,19] suggesting their potential to impair hematopoietic support capacity. Bone density and colony forming unit fibroblasts (CFU-F) were shown to decrease in patients following allogeneic stem cell transplant [20]. Earlier work from our laboratory indicated that treatment of primary human osteoblasts with VP16 and melphalan activated the TGF- β 1 pathway [21], consistent with the finding that bone marrow stromal cells established from leukemia patients treated with chemotherapy have elevated levels of TGF- β 1 [22]. Chemotherapy exposure was also reported to affect osteoblast-specific proteins including type I collagen and alkaline phosphatase in human primary osteoblasts, as well as the ability of mature osteoblasts to mineralize bone [23].

In the current study we have demonstrated that chemotherapy exposure decreases expression of CXCL12, a key factor mediating homing and hematopoietic cell adhesion in the bone marrow niche, while also decreasing differentiation stage-specific synthesis of osteoblast components of the ECM including OCN, OPN and Col1a1. Treatment of preosteoblasts with VP16 or melphalan impaired their differentiation potential and decreased transcripts associated with osteoblast differentiation (Runx2, SP7, and OCN). VP16 and melphalan also altered hematopoietic cell support provided by osteoblasts, demonstrated by an increased proportion of Lin- Sca1+c-kit+ stem cells and an increased number of viable Sca1-c-kit+IL7R- myeloid progenitor cells following co-culture with chemotherapy damaged osteoblasts. Taken together, these data indicate that functional dysregulation of the osteoblast component of the bone marrow microenvironment might include both chemokine gradient changes as well as altered ECM deposition.

Materials and Methods

Cell lines, reagents and drug treatment

Murine pre-osteoblast cell line MC3T3E1, subclone 4, was purchased from ATCC (ATCC CRL-2593). Both MC3T3E1 and 7F2 cell lines were cultured in α -MEM supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine, 1% sodium pyruvate, and penicillin/streptomycin, at 37°C in 6 % CO₂. VP16 (Bristol Myers Squibb, New York, NY) was used at 50-100 μ M for both MC3T3E1 and 7F2 cells; melphalan (Sigma) was dissolved in diluent containing 2% sodium citrate, 60 % Propylene Glycol, and 5.2 % EtOH, pH 1.1 immediately prior to use.

Differentiation of pre-osteoblast cells to mature osteoblasts

MC3T3E1 and 7F2 cells were plated in 24 well plates as confluent monolayers. To induce osteoblast differentiation medium was supplemented with 100 μ g/ml Ascorbic acid and 10 mM β -glycerol phosphate. Medium was exchanged every 3 days. 7F2 cells were assayed for differentiation after 7 days in culture and MC3T3E1 cells after 21 days. Cells were stained for alkaline phosphatase according to the manufacturer's protocol (SigmaFast BCIP/NBT kit or Leukocyte Alkaline Phosphatase kit, Sigma). Calcium deposition was monitored by Alizarin Red S staining as previously described [24].

Isolation of RNA and RT-PCR

RNA was isolated from osteoblasts using the RNeasy Mini kit with on-column DNase I digestion (Qiagen). One-step RT-PCR reactions were performed in triplicate using 50 ng of RNA per well, with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on an Applied Biosystems 7500 Real Time PCR machine. GUS B, GAPDH or B2M were used as loading controls. The following primer sets were purchased from Invitrogen: OCN (F: ACCCTGGCTGCGCTCTGTCTCT R: GATGCGTTTGTAGGCGGTCTTCA) Runx2 (F: TTTAGGGCGCATTCTCATC

R:TGTCCTTGTGGATTAAGGACTTG) SP7 (F: ACTCATCCCTATGGCTCGTG R: GGTAGGGAGCTGGGTTAAGG) Col1a1 (F: TGTGTGCGATGACGTGCAAT R:GGGTCCCTCGACTCCTACA) PPARG2 (F: TTTATGCTGTTATGGGTGAAACTC R:AGAGGTCCACAGAGCTGATTCC) Adipoq (F:TGTTCTCTTAATCCTGCCCA R:CCAACCTGCACAAGTTCCCTT) CEBPA (F: TGGACAAGAACAGCAACGAG R:TCACTGGTCAACTCCAGCAC) OPN (F: GTGAAAGTGAAGTACTGATTCTGGCA R:TTTTCTTCAGAGGACACAGCATT) Primers for CXCL12, TGF- β 1, B2M, GUS B, BMP4, and GAPDH were purchased from Real Time Primers. Fold change was calculated by the $\Delta\Delta C_t$ method [25].

ELISA

ELISAs for CXCL12 (R&D) and OPN (R&D) were performed according to the manufacturer's instructions. Cellular supernatants were evaluated, from osteoblasts that were either untreated, or treated for 24h with 100 μ M VP16 or 25 μ g/ml melphalan. For CXCL12 ELISAs medium was diluted 1:4 for 7F2 cells or used undiluted for MC3T3E1 cells. For OPN ELISA supernatants were diluted 1: 200.

Scanning electron microscopy (SEM)

Adult 20 week old Balb/c mice were treated either with VP16 diluent (65% polyethylene glycol 300, 8% Tween 80, 30% ethanol, 0.2% citric acid, and 3% benzyl alcohol) or 20 mg/kg VP16 once a day, for 72h. Twenty four hours after the final treatment the mice were sacrificed and marrow was dislodged by rinsing femurs with PBS at 37° C to expose the endosteal surface. Femurs were subsequently washed in 37° C PBS prior to immersion fixation in 1% formaldehyde, 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer pH 7.2 for 48 hours at

4° C. Samples were washed in sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the cacodylate buffer for 30 min. Bones were rapidly dehydrated in graded steps of acetone (25%-100%) and critically point dried using a Tousimis 815a Critical Point Dryer. Samples were mounted onto aluminum stubs and coated with a 40 nm-thick layer of Platinum using a Temescal BJD 200 E-Beam Evaporator. Samples were examined with a JEOL JSM-7600-F scanning electron microscope.

Isolation of murine hematopoietic stem and progenitor cells

Adult Balb/c mice were sacrificed with bone marrow collected from femurs and tibia. Bone marrow cells were labeled with biotinylated antibodies specific for CD5, CD45R, CD11b, Gr-1 (Ly6G/C), 7-4 and Ter119 according to the manufacturer's protocol (Lineage cell depletion kit, Miltenyi Biotec). Lineage-negative (Lin-) cells were isolated on an AutoMacs column using the Deplete S program (purity > 90 %). To assay the effect of drugs on the capacity of osteoblasts to support survival and differentiation of HSC and progenitor cells, 40,000 Lin- cells were co-cultured with a monolayer of MC3T3E1 osteoblasts (170,000 cells per 24-well) for 5 days in RPMI containing 10 % FBS and 10 ng/ml IL-3 (murine rIL-3, R&D Systems). MC3T3E1 were either untreated or pre-treated with 50 μ M VP16 or 25 μ g/ml melphalan for 24h and washed thoroughly before hematopoietic cells were added to the culture. Hematopoietic cells were collected at the termination of the experiment by collecting both the supernatant and the monolayer to include the hematopoietic cells attached to it. The two fractions of cells were combined and labeled with the following ratamouse antibodies (BD Biosciences): CD45-PE-Cy7, CD45R (B220)-Alexa Fluor 700, CD16/32-FITC, Sca1-APC, CD117-PerCP-Cy5.5 and CD127-PE. The viability of the cells was monitored by staining with Violet Live/Dead fixable dead cell stain (Invitrogen). The samples were run on a FACS Aria flow cytometer (BD Biosciences) and data were analyzed by FCS Express software (De Novo Software).

Osteoblasts were excluded from analysis by gating out live/CD45- cells.

Statistical analysis

One-Way ANOVA with Holm-Sidak post hoc multiple comparison was performed using SigmaPlot Version 11.0 to detect differences in RT-PCR and ELISA data. Differences were considered statistically significant when p-value was < 0.05.

Results

To study how osteoblasts at varied differentiation stages react to drug exposure, we used two mouse pre-osteoblast cell lines, MC3T3E1 (subclone 4) and 7F2. 7F2 cells were clonally derived from p53^{-/-} mice, while MC3T3E1 are spontaneously immortalized mouse calvarial osteoblasts [26,27]. Both were reported to differentiate to mature osteoblasts in medium containing ascorbic acid and β -glycerol phosphate [26,27]. While both control and differentiated cells stained strongly for alkaline phosphatase, consistent with the appearance of strong ALP staining in early preosteoblast cells, only differentiated cells exhibited calcium deposits (Fig. 1 A, B). We further verified the differentiation to mature osteoblasts by monitoring the levels of OCN. Both 7F2 and MC3T3E1 cell lines exhibited induction of OCN mRNA with differentiation (Fig. 2). These observations suggest that the 7F2 and MC3T3E1 cells faithfully recapitulate osteoblast differentiation and are an appropriate model for pre-osteoblast development.

To determine the effect of VP16 and melphalan on the ability of osteoblasts to support hematopoietic cells, we evaluated the changes in osteoblast-specific transcripts and several factors important for hematopoietic support. Based on the rationale of the question, clinically relevant drugs and sub-lethal concentrations were chosen for evaluation to allow investigation of

functional alterations of the microenvironment. Importantly, no significant reduction in osteoblast viability was observed, evaluated by PrestoBlue, MTT assay, and Trypan blue, in cultures treated at the concentrations of drugs utilized in this study (data not shown). In MC3T3E1 cells we detected an approximate 5 fold increase in the transcript levels of OCN, both in undifferentiated and differentiated cells after VP16 treatment, while melphalan treatment increased OCN levels by 3 fold in undifferentiated cells and 1.5 fold in differentiated cells, respectively (Fig. 2A). OPN levels, which decrease with differentiation, were also elevated after VP16 and melphalan treatment in both control and differentiated MC3T3E1 cells. In contrast, the levels of Runx2, SP7 and Col1a1 decreased in both undifferentiated and differentiated MC3T3E1 cells with treatment. RunX2, SP7, and Col1a1 were affected similarly in 7F2 cells (Fig. 2B). For 7F2 cells, OCN levels were affected differently by VP16 and melphalan. While VP16 induced an increase in OCN transcript levels similar to MC3T3E1 cells, melphalan treatment resulted in decreased OCN levels.

We also evaluated the effect of VP16 and melphalan on the mRNA levels of CXCL12 and BMP4 (Fig. 2 and data not shown). The level BMP4 transcript was not affected by either differentiation or treatment with VP16 or melphalan in both 7F2 and MC3T3E1 cells (data not shown). VP16 treatment resulted in an approximate 2 fold increase in CXCL12, while melphalan decreased CXCL12 transcript levels, by 7.7 fold and 5.6 fold in undifferentiated and differentiated MC3T3E1 cells, respectively (Fig. 2A). 7F2 cells exhibited a decrease in CXCL12 after treatment with either VP16 or melphalan (Fig. 2B).

To investigate the changes in CXCL12 and OPN at a protein level, ELISA of culture supernatants was performed (Fig. 3). CXCL12 and OPN protein response following treatment differed from the transcriptional responses shown in Fig. 2. Secreted CXCL12 levels were significantly lower than untreated controls in undifferentiated and differentiated cells for both MC3T3E1 and 7F2 (Fig. 3 A and B). OPN was affected similarly in MC3T3E1 and 7F2 as well,

showing a decrease in undifferentiated cells treated with melphalan and also differentiated compared to undifferentiated controls (Fig. 3C and data not shown). Since sublethal doses of chemotherapy altered the composition of extracellular matrix proteins and delayed osteoblast maturation we sought to determine what effect these agents had on the ultrastructure of the endosteum. To observe gross morphology of the endosteum following treatment with VP16 *in vivo* we performed SEM on diluent controls (Fig. 4, A and B) and VP16 treated (Fig. 4, C and D) long bones. The endosteal surface is composed of cord/rope like structures covered by a smooth surface coat (arrows) in diluent controls. Lacunae vary in size and number but were found to be present over the entire endosteal surface (Fig. 4A). In addition, smooth electron dense patches (*, Fig. 4A) were also identified on the endosteal surface (Fig. 4B). Overall, the endosteal surface was intact and exhibited a uniform surface coat. VP16 treatment caused disruption of the endosteal surface lining (Fig. 4C). Material composing the electron dense patches condensed into clumps (*, Fig. 4C) exposing the underlying matrix. The surface coat covering the cord/rope structures was gone (Fig. 4D) exposing collagen fibers (arrows, inset, Fig. 4D). The appearance of the endosteum following treatment with VP16 provides only a general visual representation of the damage caused by VP16 in the osteoblastic niche but further characterization is required to come to specific conclusions beyond that.

We next determined whether treatment of 7F2 preosteoblasts with VP16 or melphalan would affect their ability to differentiate to mature osteoblasts, utilizing ALP as an indicator. Overall staining for ALP was reduced by drug treatment in cells cultured in both control and differentiation medium (Fig. 5B). At the same time, microscopic examination of the cultures revealed a population of cells staining intensely for ALP (Fig. 5A), which would correspond to cells at an earlier stage of osteoblast differentiation. We further determined the OCN, Runx2, SP7, Col1a1 and CXCL12 transcript levels of cells which were treated with drugs and then allowed to differentiate for 7 days (Fig. 5C). CXCL12 mRNA abundance did not change with

differentiation or after drug treatment, but OCN mRNA decreased approximately 20 fold after VP16 exposure and 10 fold after melphalan exposure. Similarly, a statistically significant decrease in transcript levels was detected for RUNX2, SP7 and Col1a1. These data are consistent with delayed osteoblast maturation after exposure to VP16 and melphalan. MC3T3E1 cells had similar results (data not shown).

Both 7F2 cells and MC3T3E1 cells treated with VP16 or melphalan exhibited vacuoles in the cells, reminiscent of lipid droplets. Based on reports of adipocyte differentiation after chemotherapy treatment of osteoblasts [28] we stained the cultures for the presence of lipids with Oil Red and confirmed that the observed vacuoles indeed contained lipids (Fig. 5D, top and data not shown). However, there was no upregulation of adipocyte-specific transcripts and instead PPARG2, CEBPA, and Adipoq decreased significantly after drug treatment of 7F2 cells, both in control and differentiation medium (Fig. 5E, bottom). Thus, it is unlikely that drug-treated cells, in this model, undergo adipocyte differentiation.

We next determined the effect of VP16 and melphalan on the ability of MC3T3E1 cells to support HSC utilizing Lineage-negative cells co-cultured with a monolayer of MC3T3E1 cells (untreated or treated with VP16 or melphalan as described in materials and methods). To follow HSC support *in vivo*, we observed Lin-Sca1+c-kit+ cells (LSK), a cellular subset which would mark about 25 % of the hematopoietic stem/progenitor cells (HSPC) in Balb/c mice [29]. In the Lin- fraction of cells we determined the relative percentage of HSPCs, myeloid progenitors (Sca1-c-kit+IL7R-), lymphoid progenitors (Sca1+c-kit+IL7R+), as well as mature myeloid cells (CD16/CD32+) at the day of plating and after 5 days in culture with chemotherapy pre-treated or matched untreated control MC3T3E1 cells. After 5 days in culture about 80% of the Lin- cells incubated in media alone were viable, while Lin- cells in co-culture with untreated “healthy” osteoblasts were approximately 94% viable (Fig. 6A). Osteoblasts pre-treated with either VP16 or melphalan also supported the Lin- cell survival to a degree higher than media alone.

Hematopoietic cell death in co-cultures of chemotherapy pre-treated osteoblasts was noted to be slightly higher than in cultures with control osteoblasts, at approximately 8.5% and 12.0%, respectively, compared to 6% for untreated osteoblasts. Osteoblasts generally supported differentiation of Lin⁻ cells to CD16/CD32⁺ mature myeloid cells (Fig. 6B). Melphalan-treated osteoblasts supported marginally less myeloid cell differentiation compared to untreated cells (88.8% vs. 95.3%), while VP16 had no effect on MC3T3E1 ability to support myeloid differentiation.

Because the Lin⁻ compartment contains not only stem cells but also committed hematopoietic progenitors, we further evaluated the percentage of LSK cells which are IL7R⁺ (lymphoid progenitors) and the Lin⁻Sca1⁻c-kit⁺IL7R⁻ as myeloid progenitors in contrast to mature CD16/CD32⁺ myeloid cells noted earlier [30]. We detected a higher percentage of both lymphoid and myeloid progenitors co-cultured with VP16 or melphalan pre-treated osteoblasts compared to untreated osteoblast layers after 5 days in culture (Fig. 6 B and D). Our results indicate that VP16 and melphalan modulate the capacity of osteoblasts to support hematopoiesis and alter the ratio of stem and progenitor cells in this *in vitro* model, although the quantitative differences were modest.

Discussion

The current study focused on the effects of VP16 and melphalan on osteoblast cells at different stages of maturation. Cell lines utilized have been confirmed to be able to differentiate to mature osteoblasts by calcium deposition detected by Alizarin Red staining and upregulation of osteocalcin (Fig. 1). Both preosteoblast cells and mature osteoblasts expressed ALP which is consistent with its onset of detection in early preosteoblast cells [31]. We also detected similar amounts of osteoblast-specific transcription factors Runx2 and SP7 in preosteoblasts and

mature osteoblasts, consistent with the MC3T3E1 and 7F2 cells being downstream of the MSC stage [14]. Collectively these observations suggested a reasonable *in vitro* model in which to evaluate whether chemotherapy vulnerability varies with stage of differentiation.

VP16 and melphalan altered the expression of several osteoblast-specific transcripts (Fig. 2). The Runx2 and SP7 transcription factors, and Col1a1 were uniformly downregulated in both MC3T3E1 and 7F2 cells regardless of differentiation stage following treatment. On the other hand, OCN is differentially regulated in 7F2 and MC3T3E1 cells; treatment with drugs increases OCN levels in MC3T3E1 cells, while melphalan decreases OCN levels in 7F2 cells. This might result from different basal OCN levels in 7F2 and MC3T3E1 cells, since the lack of p53 in 7F2 cells contributes to faster growth and osteoblast differentiation in this cell line. Since Col1a1 is a major component of the ECM, and OCN and OPN are immobilized on the ECM, these chemotherapy drugs have the potential of altering the adhesion properties of the ECM of both pre- and mature osteoblasts. Consistent with this, we detected disruption of the integrity of the “architecture” of the endosteal region of femurs from VP16-treated mice by SEM (Fig. 4).

More specific analyses will be required to characterize the exact anatomical alterations, and the scope of specific cellular elements that are impacted on, but the observation is consistent with a general vulnerability of this anatomical site to chemotherapy induced stress. Melphalan exposure yielded similar results (data not shown).

We have also observed that VP16 and melphalan impair factors (including CXCL12 and OPN) which are important for hematopoietic cell differentiation (Figs. 2 and 3). We observed that although VP16 treatment of MC3T3E1 cells increased CXCL12 transcripts, secreted protein was decreased in both 7F2 and MC3T3E1 with treatment of either drug relative to untreated controls (Fig. 2 and 3). The differences in CXCL12 transcriptional regulation between 7F2 and MC3T3E1 might be linked to the fact that the cell lines have different basal expressions of CXCL12, produced at much higher levels in 7F2 cells. Again, this could be explained by the fact

that 7F2 cells lack p53, which has been reported to downregulate secreted CXCL12 in fibroblast cells [32]. Our results are also consistent with a previous report that in human osteoblasts CXCL12 levels are sustainably reduced following treatment with VP16 and melphalan, resulting in diminished adhesion of CXCR4+ hematopoietic cells [21]. An additional report has linked the absence of CXCL12 with HSC quiescence, but also with increased myeloid differentiation [7]. Since in this model CXCL12 was reduced by ablation of CXCL12 abundant reticular cells, which also secrete other cytokines and factors important for HSC [7], we feel that the distinct observations related to the role of CXCL12 in hematopoietic cell support could be explained by differences in the experimental settings and interplay between several factors.

We further evaluated the levels of OPN protein and detected a decrease after melphalan treatment in undifferentiated MC3T3E1 cells (Fig. 3). It has been shown that OPN inhibits HSC proliferation and reduces differentiation of HSCs to myeloid cells *in vitro* [33]. We have also detected altered Lin- support and hematopoietic cell differentiation of chemotherapy pre-treated MC3T3E1 cells (Fig. 6), consistent with the above observation. Deregulation of OPN in our model provides a potential mechanism by which hematopoiesis can be altered by chemotherapeutics.

Finally, we determined that VP16 and melphalan also disrupt the ability of preosteoblast cells to differentiate to mature osteoblasts. Drug treatment induced delayed osteoblast maturation, as evidenced by a significant decrease in a number of transcripts required for establishment of the mature osteoblast, including OCN, Runx2, SP7, and Col1a1. We also detected the appearance of a population of cells staining intensely for ALP, consistent with ALP being expressed at the highest level in preosteoblast cells (Fig. 5). ALP increases, and Col1a1 decreases, have been noted before in primary human osteoblasts from patients undergoing chemotherapy with various drugs [23], and a preferential drug effect on viability of preosteoblast cell lines compared to cultures containing mature osteoblasts was also reported [19].

Additionally, VP16 and melphalan treated osteoblasts exhibited increased lipid content but no coincident upregulation of adipocyte-specific markers (Fig. 5D and E). Increased lipid staining after *in vivo* treatment with 5-fluorouracil has been interpreted before to indicate preferential adipocyte differentiation of murine CXCL12-expressing cells [6], but we did not see a correlation between the lipid increase and appearance of adipocyte-specific differentiation markers. Thus, the increased lipid content of the chemotherapy treated osteoblasts could be explained by an alternative mechanism, with one possibility being autophagosome formation. Results from the current study contribute to better understanding the scope of effects of genotoxic stress and DNA damage on the bone marrow microenvironment. DNA double strand breaks have been shown to promote cell differentiation in normal B-cell development [34], in neuronal stem and progenitor cells [35], and in melanocyte stem cells [36]. Conversely, DNA damage impairs proper differentiation of myoblasts when applied before induction of differentiation [37]. Our data in a model of pre- and mature osteoblasts are consistent with the assertion that DNA damage can influence differentiation in a component of the marrow microenvironment. This damage is also associated with alteration of ECM gene expression profiles, disrupted structural integrity, and dysregulated support of HSC differentiation. A better understanding of the diversity of vulnerability of populations of cells in the marrow to drugs with distinct mechanisms of action may support improved approaches to pre-transplant conditioning and subsequent restoration of steady state hematopoiesis.

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Figure Legends

Figure 1. Differentiation of MC3T3E1 and 7F2 cells to mature osteoblasts.

7F2 (A) or MC3T3E1 (B) cells were incubated either with regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 7 days (7F2 cells) or 21 days (MC3T3E1 cells), respectively. Cells were stained for Alkaline phosphatase (ALP) and Alizarin Red as a measure of osteoblast differentiation.

Figure 2. Effect of VP16 and melphalan on the expression of osteoblast-specific transcripts.

MC3T3E1 cells were incubated either in regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 21 days, after which the cells were treated with 100 μ M VP16 or 25 μ g/ml melphalan (A). Total RNA was isolated and qPCR was performed with primers specific for OCN, Runx2, SP7, Col1a1, OPN and CXCL12. Relative expression was normalized to the expression in undifferentiated untreated cells (undifferentiated untr). (B) 7F2 cells were differentiated for 7 days and treated with 100 μ M VP16 or 25 μ g/ml melphalan (B) and then processed as described for (A). Error bars denote standard deviation from triplicate measurements in a representative experiment. * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA.

Figure 3. Effect of VP16 and melphalan on the levels of secreted CXCL12 and OPN. MC3T3E1

cells were incubated either in regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 21 days, after which medium was removed and replaced with fresh medium (untreated) or medium containing 100 μ M VP16 or 25 μ g/ml

melphalan. After 24 hours, medium was removed and ELISAs were performed for CXCL12 (A) and OPN (C). 7F2 cells were differentiated for 7 days then treated as in A prior to ELISA for CXCL12 (B). * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA, n=3.

Figure 4. Scanning electron microscopy of murine bone. Representative scanning electron micrographs of mouse control and VP16-treated long bones. In diluent-treated controls (A, B) the endosteum consists of an intact uniform surface coat covering the underlying ECM. (B). Enlarged view of smooth electron dense patches (*, Fig. 3A) found dispersed throughout the endosteum. VP16 treatment (C, D) caused disruption of the endosteal surface coat (*) exposing the underlying extracellular fibrillar matrix (arrows inset). Bar, 10 μ M.

Figure 5. Effect of VP16 and melphalan on the differentiation potential of preosteoblast cells. 7F2 cells were exposed for 24 h to 50 μ M VP16, 25 μ g/ml melphalan, VP16 diluent (VP16 dil) or melphalan diluent (Mel dil), respectively. Following treatment cells were washed and incubated with regular growth medium (undifferentiated) or osteoblast differentiation medium (differentiated) for 7 days. Cells were stained for Alkaline phosphatase (ALP) and images were taken with (A) or without (B) 20x magnification. (C). Total RNA was isolated from cells treated as in A and qPCR was performed with primers specific for OCN, Runx2, SP7, Col1a1 and CXCL12. Relative expression of the genes was normalized to the expression in untreated undifferentiated cells. (D). Oil Red staining for lipids in cells treated as in (A). Real time PCR for the adipocyte-specific transcripts PPARG2, CEBPA, and Adipoq (E). Error bars denote standard deviation from triplicate measurements. * indicates significantly greater or lesser than untreated control (for drug treatments) or undifferentiated untreated control, p-value < 0.05, One-Way ANOVA.

Figure 6. Support of murine hematopoietic stem and progenitor cells by osteoblasts treated with VP16 or melphalan. MC3T3E1 cells were seeded as monolayers in 24-well plates. Cells were treated with 50 μ M VP16, 25 μ g/ml melphalan, or were left untreated for 24h. After several washes of the osteoblasts, 40,000 freshly isolated lineage negative cells were added to the osteoblasts in RPMI medium containing 10 % FBS and 10 ng/ml IL-3. Cultures were incubated for 5 days, after which cells were trypsinized and stained with Violet Live/Dead fixable dead cell stain and antibodies to CD45, Sca-1, c-kit, CD16/32, B220 and CD127. Number of dead cells (A), Sca+c-kit+ (LSK) and lymphoid progenitors (CLP) (C), myeloid progenitors (D) and CD16/CD32+ myeloid cells (B) was determined by flow cytometry.

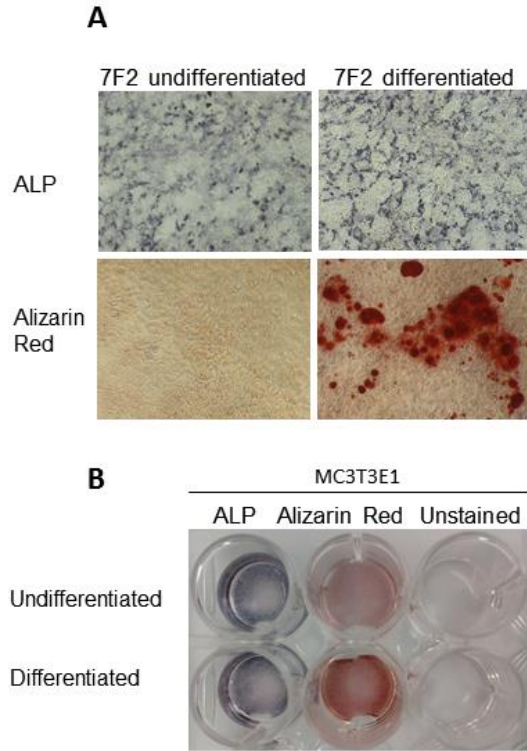


Figure 1. Differentiation of MC3T3E1 and 7F2 cells to mature osteoblasts.

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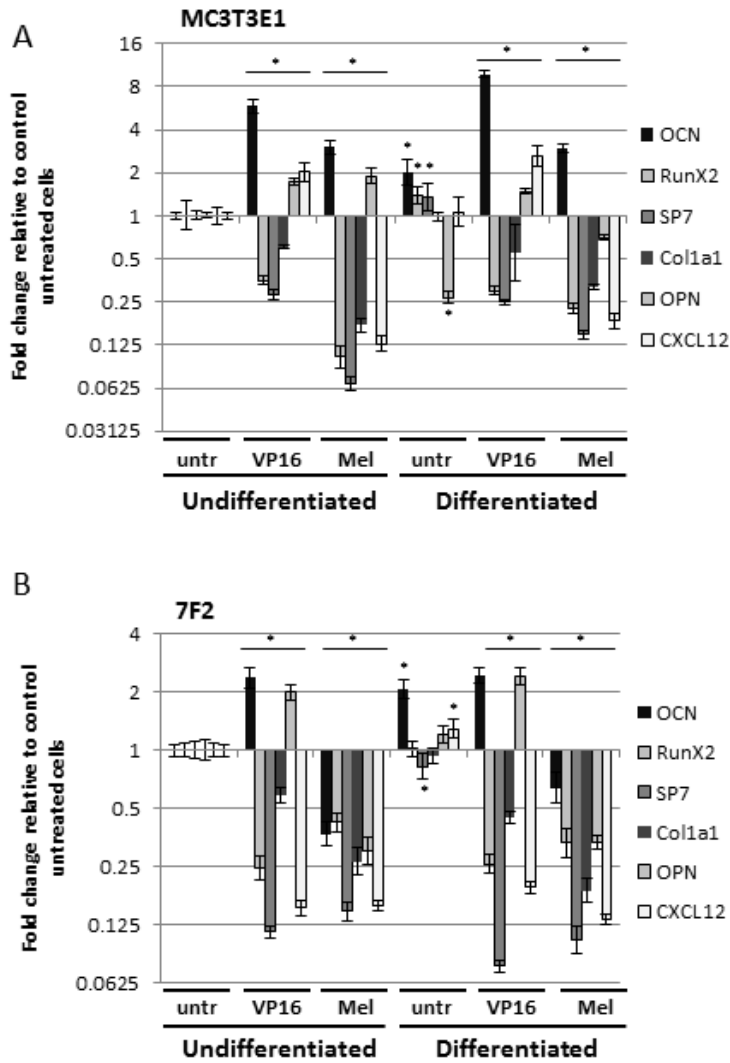


Figure 2. Effect of VP16 and melphalan on the expression of osteoblast-specific transcripts.

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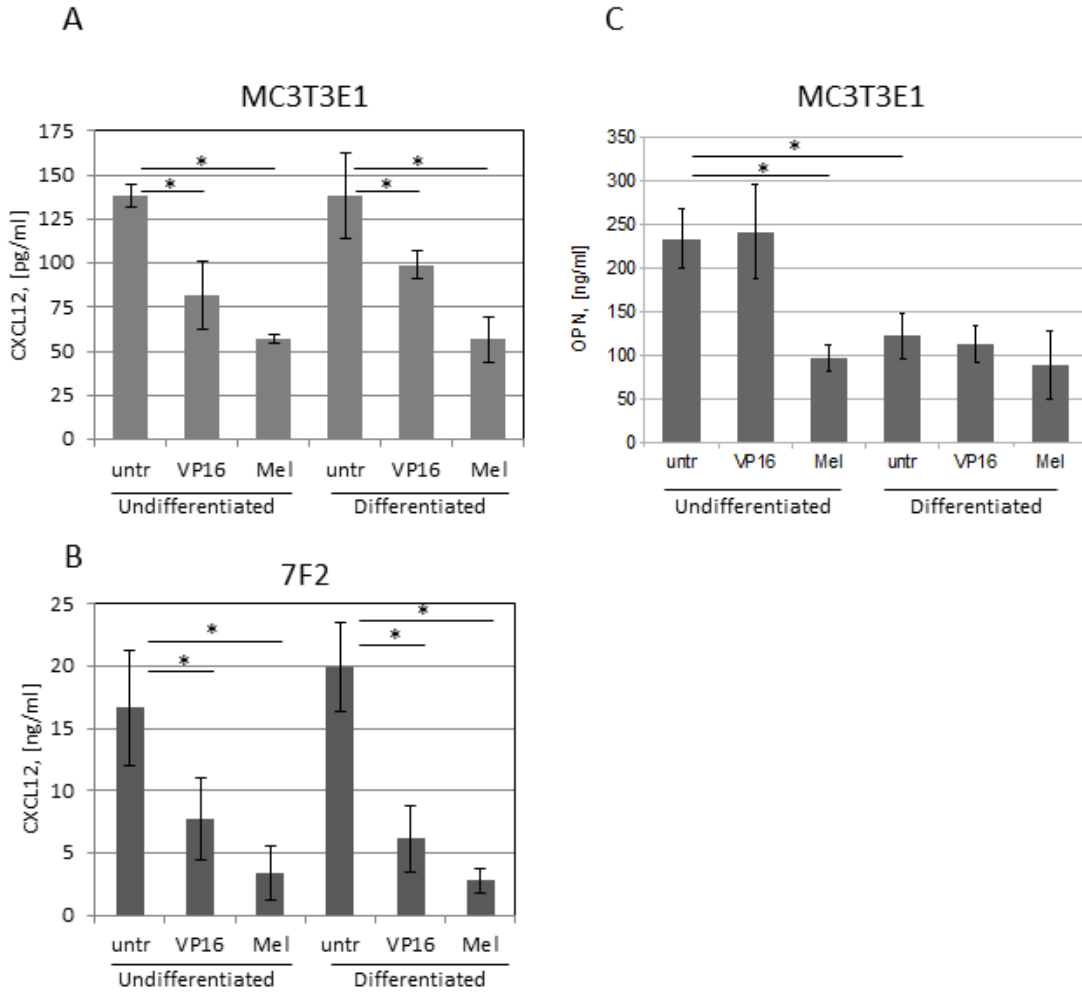


Figure 3. Effect of VP16 and melphalan on the levels of secreted CXCL12 and OPN.

Gencheva and Hare et al. 2013. Bone marrow osteoblast vulnerability to chemotherapy. European Journal of Haematology. Permission Number: 3835731013613.

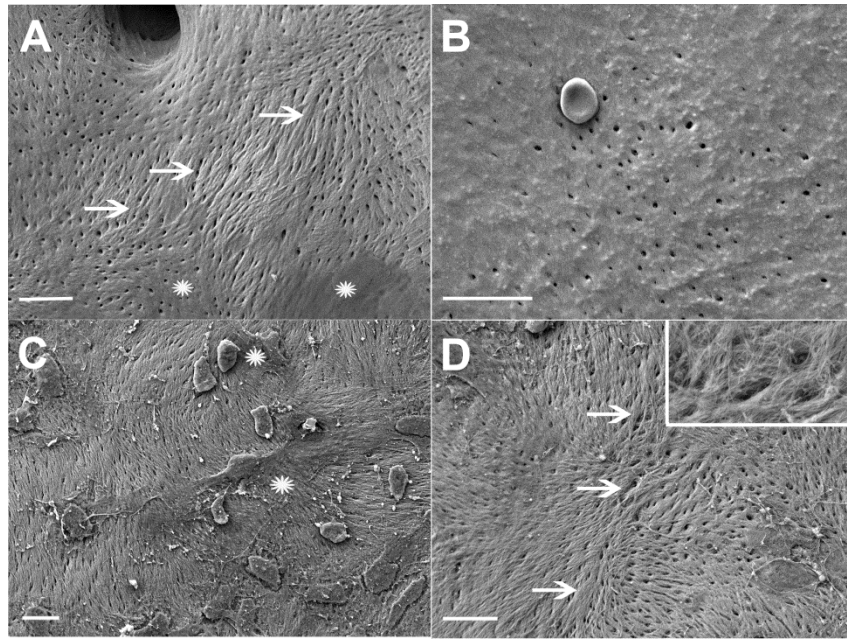


Figure 4. Scanning electron microscopy of murine bone.

Gencheva and Hare et al. 2013. Bone marrow osteoblast vulnerability to chemotherapy. European Journal of Haematology. Permission Number: 3835731013613.

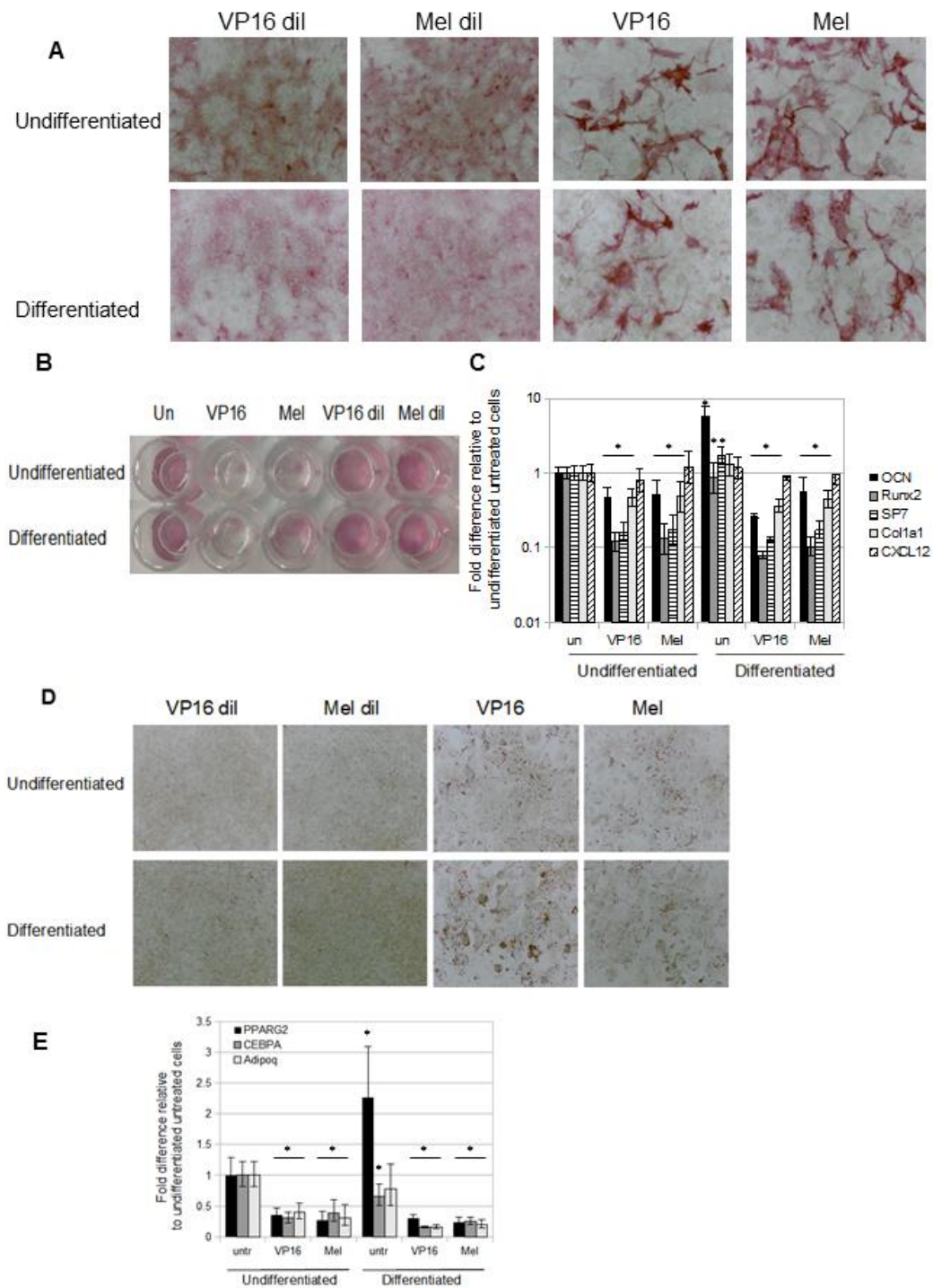


Figure 5. Effect of VP16 and melphalan on the differentiation potential of preosteoblast cells.

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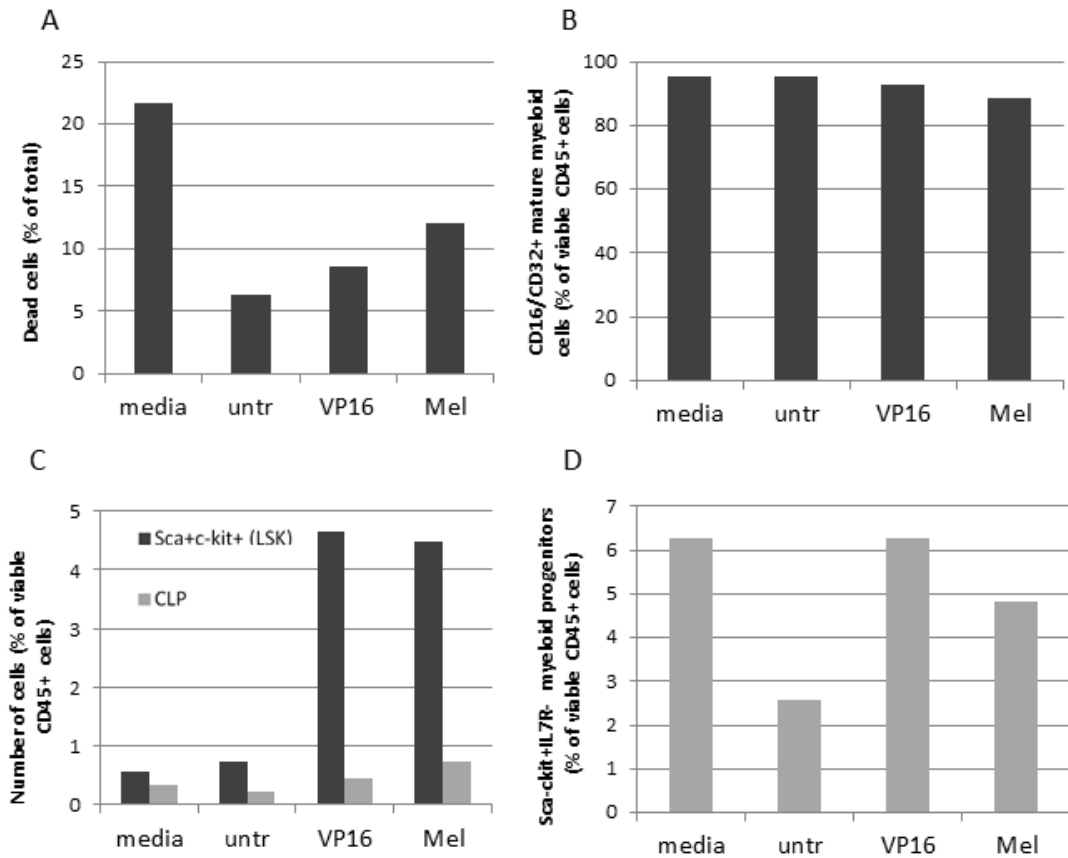


Figure 6. Support of murine hematopoietic stem and progenitor cells by osteoblasts treated with VP16 or melphalan.

Gencheva and Hare et al. 2013. Bone marrow osteoblast vulnerability to chemotherapy. European Journal of Haematology. Permission Number: 3835731013613.

Chapter III

In Vitro Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break Repair of Etoposide Induced DNA Damage

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Abstract

Mesenchymal stem cells (MSCs) are of interest for use in diverse cellular therapies. *Ex vivo* expansion of MSCs intended for transplantation must result in generation of cells that maintain fidelity of critical functions. Previous investigations have identified genetic and phenotypic alterations of MSCs with *in vitro* passage but little is known regarding how culturing influences the ability of MSCs to repair double strand DNA breaks (DSBs), the most severe of DNA lesions. To investigate the response to DSB stress with passage *in vitro*, primary human MSCs were exposed to etoposide (VP16) at various passages with subsequent evaluation of cellular damage responses and DNA repair. Passage number did not affect susceptibility to VP16 or the incidence and repair kinetics of DSBs. Non-homologous end joining (NHEJ) transcripts showed little alteration with VP16 exposure or passage, however, homologous recombination (HR) transcripts were reduced following VP16 exposure with this decrease amplified as MSCs were passaged *in vitro*. Functional evaluations of NHEJ and HR showed that MSCs were unable to activate NHEJ repair following VP16 stress in cells after successive passage. These results indicate that *ex vivo* expansion of MSCs alters their ability to perform DSB repair, a necessary function for cells intended for transplantation.

Introduction

MSCs are a mesoderm derived stromal population defined functionally by their ability to differentiate into various cell types *in vitro*. Osteoblasts, adipocytes, and chondrocytes have been shown to arise from MSC precursors under various culture conditions [1]. MSCs have also been shown to have immunomodulatory properties, displaying the ability to suppress adaptive and innate immune responses through the secretion of anti-inflammatory cytokines [2]. In addition to these functional characteristics MSCs are able to persist in culture, making it possible to alter gene expression of the cells through various transfection techniques. Such an approach has been utilized in a rat model of myocardial infarction to deliver HIF-1 α expressing MSCs to damaged heart tissue [3]. These cellular attributes have made MSCs an attractive candidate for the development of stem cell therapies in humans. MSCs can be acquired from various tissues of the body including the bone marrow [4]. The injection of bone marrow derived MSCs is currently undergoing Phase III clinical trials in the United States for the treatment of Crohn's disease and myocardial infarction (clinicaltrial.gov identifiers NCT00482092 and NCT01394432, respectively). In addition, several pre-clinical applications have been described in animal models of disease, such as autoimmune encephalitis, graft vs. host disease, rheumatoid arthritis, type I diabetes, and inflammatory bowel disease [5].

The use of MSCs for cellular therapies requires the ability of *ex vivo* expansion generating adequate numbers of cells for treatment. Although there is significant evidence documenting the clinical utility of MSCs, they are a heterogeneous population of cells that differ based on the means by which they are acquired and how they are cultured *in vitro* [6]. Given their utility, it is important to understand how they are altered during the necessary *ex vivo* expansion prior to patient administration. It has been shown previously that *ex vivo* expansion of MSCs results in alterations in genome stability [7, 8], epigenetics [9, 10], and functional abilities to differentiate into osteogenic cells [11, 12]. However, these studies have been performed in

non-stressed conditions, and little has been shown regarding how the activity of MSCs may be altered once administered to a patient where they may encounter cellular stresses, such as DNA damage. Eukaryotes have evolved means to recover from many types of DNA damage, the most lethal of which are double strand breaks (DSBs). DSBs are repaired by two major repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) [13]. NHEJ involves the resection of nucleotides from both sides of a DSB, followed by the binding of Ku70 and Ku80 proteins with DNA-PK_{cs}, which recruits DNA ligase IV and initiates ligation of the break [14]. NHEJ is capable of repairing incompatible ends regardless of cell cycle status. When a sister chromatid is present (during S/G2/M phases of cell cycle), HR is capable of repairing a break by resecting both ends of a break, followed by insertion of the 3' resected end into the homologous sequence thereby using it as a template for repair. Due to the presence of a homologous sequence, HR is less error prone than NHEJ, however, the necessity of a reference template limits the utilization of HR to S/G2/M phases of cell cycle [15].

To determine how *ex vivo* expansion alters the response of MSCs to stress, we utilized etoposide (VP16), a DNA type-II topoisomerase inhibitor that specifically induces DSBs following DNA replication [16]. DSBs are the most lethal of DNA lesions, resulting in a larger degree of somatic mutation or apoptosis than other DNA lesions [17]. To determine how *ex vivo* expansion alters the response of MSCs to DSB stress, we passaged bone marrow derived MSCs *in vitro*, evaluating their responses to cellular stress and DSB repair following VP16 exposure.

Materials and Methods

Human MSC Isolation and Cell Culture

Bone marrow aspirates were de-identified samples from patients treated at the West Virginia University Healthcare System. Cells were cultured in α -MEM supplemented with 10% fetal bovine serum (BSA), 2mM L-glutamine, 100U/mL penicillin, 100 μ g/mL streptomycin, and housed at 37°C, 6% CO₂. MSCs were derived from donors who had no previous exposure to chemotherapy or irradiation, and no history of malignancy.

Surface Staining of MSCs

Cells were trypsinized and washed in 1x phosphate buffered saline (PBS). Primary antibodies (1ug) specific for human CD45, CD44, CD105, and CD166 (BD Pharmingen, San Jose, California, United States) were added and incubated on ice for 20 minutes in PBS/3% BSA. Cells were washed in PBS/3% BSA, then incubated with 1ug donkey anti-mouse-AF488 (BD Pharmingen, San Jose, California, United States) for 20 minutes on ice. Cells were then washed in PBS/3% BSA, resuspended in 400uL PBS, and immediately analyzed using a FACS Fortessa (BD Biosciences, Franklin Lakes, New Jersey, United States).

Differentiation of MSCs

To induce osteogenic differentiation in human MSC cultures, cells were plated at 90% confluence then cultured in Stempro® Osteogenesis Differentiation Kit Medium (Life Technologies, Carlsbad, California, United States) for 21 days. Differentiation medium was changed every 3 days. To induce adipogenic differentiation, cells were treated similarly only cultured in Stempro® Adipogenesis Differentiation Kit Medium (Life Technologies, Carlsbad, California, United States) for 10 days. Differentiated cells were compared to undifferentiated controls, cultured in normal MSC medium (see “Human MSC Isolation and Cell Culture”).

Alizarin Red and Oil Red Staining

Human MSCs were fixed in 4% paraformaldehyde (Sigma, St. Louis, Missouri, United States) for 4 hours prior to staining. Alizarin red and oil red staining were performed as described previously [18, 19]. Cells were washed in deionized water and imaged immediately at 100x magnification using a Leica DMIL LED Inverted Microscope and Leica DFC 295 Digital Microscope Color Camera (Leica Microsystems, Wetzlar, Germany).

Presto Blue Viability Assay

Human MSCs were plated at 90% confluence in 96-well plates, each plate containing unexposed and VP16 exposed cells, 5 wells per group. Cells were exposed to 25 μ M VP16 or medium only control for 24 hours. After incubation, cells were washed 3 times and allowed 0, 6, or 48 hours to recover. At each time point, viability was assessed using Presto Blue® Cell Viability Reagent (Life Technologies, Carlsbad, California, United States) as per manufacturer's recommendation. Well ODs were blanked to wells containing medium and Presto Blue® Cell Viability Reagent alone prior to analysis.

Etoposide Exposure

VP16 (Bristol-Myers Squibb, New York, New York, United States) was stored in 33.98mM aliquots at -20°C and diluted to 25 μ M immediately prior to use in MSC medium (see "Human MSC Isolation and Cell Culture").

Fluorescent Microscopy

Cells were plated at 90% confluence on glass coverslips in 24-well plates, then exposed to 25 μ M VP16 or medium alone for 24 hours. Following incubation, cells were washed three times, then allowed 0, 6, or 48 hours to recover. At each recovery time, coverslips were washed with 1x PBS, then fixed for 6 hours in 4% paraformaldehyde. Following fixing, cells were permeabilized with 0.5% Triton X-100 then treated with Image-iT™ FX Signal Enhancer (Life

Technologies, Carlsbad, California, United States) for 20 minutes. Following a 20 minute block with 5% BSA, cells were incubated with γ -H2AX antibody (1:400 Dilution, Millipore, Billerica, Massachusetts, United States) overnight at 4°C. Following incubation, the cells were washed in 1x PBS then incubated for 1 hour in Goat anti-Rabbit-FITC secondary antibody (1:200, Cell Signaling, Danvers, Massachusetts, United States) prior to mounting on coverslips with ProLong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, California, United States). Antibodies were diluted in 5% BSA. Following staining, cells were imaged on a Zeiss LSM 510 Laser Scanning Confocal Microscope (Zeiss, Jena, Germany). γ -H2AX was quantitated as cells expressing 10 or more foci, or manually counted at the 6 hour recovery time point to determine γ -H2AX on a per cell basis. The 6 hour recovery time point was utilized for counting because foci were too abundant at 0 hours recovery to discern individual foci.

RNA Isolation and qPCR

RNA was isolated using Qiagen RNeasy Minikit (Qiagen, Valencia, California, United States) and stored at -80°C prior to qPCR. RNA stock solutions were diluted to 50ng/11 μ L concentration prior to qPCR analysis. One step qPCR reactions were performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, California, United States) and a 7500 Applied Biosystems Thermalcycler (Applied Biosystems, Foster City, California, United States). Primers sequences are indicated in Supplementary Table 1. PCR data were analyzed using the $\Delta\Delta$ Ct Method [20].

Cell Cycle Analysis

Following trypsinization, cells were fixed in 70% ethanol for 24 hours prior to cell cycle analysis. Once fixed, cells were washed in 1x PBS, then stained with propidium iodide staining solution (0.1% Triton X-100, 0.2mg/mL RNaseA, 0.02mg/mL propidium iodide) for 30 minutes at room temperature. Cells were then resuspended in 1x PBS and ran on a Beckman FACS

Calibur (BD Biosciences, Franklin Lakes, New Jersey, United States). Analysis of cell cycle data was performed using FCS Express 4 Software (De Novo Software, Glendale, California, United States).

Western Blot Analysis

Protein was isolated from whole cell lysates prior to Western analysis using reducing conditions. Blots were probed using Rad51 (Cell Signaling, Danvers, Massachusetts, United States) and XRCC3 (Novus Biologicals, Littleton, Colorado, United States) antibodies, and anti-rabbit-HRP (Cell Signaling, Danvers, Massachusetts, United States). Immobilon Western ECL reagents (EMD Millipore, Billerica, Massachusetts, United States) were used to develop membranes.

NHEJ and HR Reporter Assays

Functional contributions of NHEJ and HR to DSB repair was evaluated using reporter assays developed and kindly provided by Dr. Vera Gorbunova (University of Rochester, Rochester, NY). NHEJ and HR constructs are designed with a Pem1 adenoviral intron interrupting the reading frame of GFP. The Pem1 intron is flanked by I-SceI restriction sites that when repaired by NHEJ or HR restores the reading frame of GFP, resulting in expression which can be quantitated by flow cytometry along with a DsRed loading control to evaluate transfection efficiency. The total %GFP⁺/%DsRed⁺ gives the repair efficiency, a number that quantitatively reflects the degree to which MSCs have utilized NHEJ or HR to repair the reporter plasmid [21]. Cells were exposed to 25µM VP16 for 24 hours prior to isolation of cells for analysis as previously described [22]. Cells were nucleofected using an Amaxa Nucleofector (Lonza, Basel, Switzerland), program U-23. Cells were nucleofected with 2µg HR or 0.5µg NHEJ constructs and 0.5µg DsRed loading control. Prior to nucleofection NHEJ and HR constructs were linearized with I-SceI (New England Biolabs, Ipswich, Massachusetts, United

States) as previously described [22]. 10µg of EGFP-N1 or DsRed-Express-DR (Clontech, Mountain View, California, United States) plasmids were included as positive controls. Following nucleofection, cells were cultured for 72 hours then evaluated for GFP and DsRed expression using a FACS Fortessa flow cytometer.

Statistical Analysis

For Presto Blue® viability analysis, two-way Student's T-tests were performed comparing untreated to treated cells at each recovery time. Y-H2AX quantitation and NHEJ/HR reporter assay data were evaluated by One-Way ANOVA with Holm-Sidak Post Hoc. Y-H2AX data in Supplementary Figure 2 were evaluated using the Kruskal-Wallis test. Statistical significance was defined as p-value ≤ 0.05 using SigmaPlot Version 11 (Systat Software Inc., San Jose, California, United States). Experiments have been repeated at least twice using cell lines derived from different patients, and three times for surface marker phenotyping, Presto Blue®, cell cycle analysis, Y-H2AX microscopy, and NHEJ/HR qPCR experiments. Error bars in Figures indicate standard error.

Results

MSC Characteristics with Passage *ex vivo*

Consistent with an MSC phenotype [23, 24], bone marrow derived MSCs showed an ability to perform both osteogenic and adipogenic differentiation, (Figure 1A and B) and were CD45⁻CD44⁺CD105⁺CD166⁺ (Figure 1C). The adipogenic differentiation potential of MSCs appeared consistent up to passage 12 (Figure 1B). Previously, MSCs have been shown to display reduced osteogenic differentiation potential following prolonged passage *in vitro* [11, 12]. In addition, extensive culturing of MSCs has been shown to reduce the proportion of cells in S-

phase [25]. Consistent with these observations, we found that the osteogenic differentiation potential of passage 12 MSCs was reduced relative to less passaged cells (Figure 1A). The cell cycle distribution of MSCs was similar in Passage 6 to Passage 10 cells, however, the proportion of cells in S-phase was reduced at Passage 12 (Figure 3A, Untreated). These results indicate that our cells were characteristic of MSCs, and displayed a functional phenotype consistent with extended passage *ex vivo* by passage 12.

In addition to differentiating into various mesenchymal lineages, MSCs functionally contribute to the regulation of the differentiation and proliferation of hematopoietic cells [26]. MSCs also provide chemotactic gradients that enable hematopoietic cell homing to the bone marrow [27], a process that we have previously shown to be negatively affected by chemotherapy exposure of bone marrow stromal cells and osteoblasts [28, 29]. A preliminary set of experiments did not provide evidence for passage related alterations of the ability of VP16 exposed MSCs to regulate the proliferation or chemotaxis of a stromal cell dependent murine pro-B cell clone, or the differentiation of normal human CD34⁺ hematopoietic progenitor cells (data not shown).

Sub-Lethal Concentrations of VP16 Induce Cell Cycle Arrest in MSCs Regardless of Passage

To determine how MSCs respond to stress with successive passage *in vitro*, cells were exposed to VP16 for 24 hours at 90% confluence, then washed three times in complete medium and allowed 0, 6, or 48 hours to recover prior to analyses. Cells were exposed to VP16 at 90% confluence due to the cell cycle specific nature of both VP16 and HR mediated repair of DSBs. VP16 specifically induces DSBs during mitotic events [16], therefore sub-confluent cells were used in our model. 25 μ M VP16 was utilized because it was the highest concentration of VP16 that did not result in overt cell death up to 48 hours recovery time (Figure 2 and data not shown)

and therefore represented sub-lethal stress in our model. Exposure of MSCs to VP16 resulted in occasional statistically significant drops in viability, however no passage related trends were present. Consistent with the absence of passage related trends in the viability of MSCs following VP16 exposure, the abundance of pro-apoptotic (PUMA and NOXA) and anti-apoptotic (BCL-XL and BCL-2) transcripts were similar with passage following VP16 stress (Figure S1). These results suggest that MSCs do not display alterations in susceptibility to VP16 up to passage 12 *in vitro*.

Exposure of MSCs to sub-lethal concentrations of VP16 resulted in a transcriptional induction of p21 which was similar among all passages (Figure 3B). p21 was elevated approximately 4 to 6 fold after 24 hours exposure to VP16 and remained elevated following 48 hours recovery. Consistent with increased p21, VP16 exposure resulted in a reduction in the proportion of cells in S/G2/M phase of cell cycle (Figure 3A). The transcriptional abundance of p16 and p53 were gradually decreased following exposure to VP16 (Figure 3B), suggesting that the observed G1 arrest was due to the earlier and relatively more robust induction of p21 following VP16 exposure. These results suggest that VP16 induces cell cycle arrest at all passages in *ex vivo* expanded MSCs.

***Ex vivo* Passage of MSCs Does Not Alter Incidence or Repair Kinetics of DSBs Following VP16 Exposure**

To visualize DSBs in MSCs following VP16 exposure immunofluorescent staining of Y-H2AX was utilized, as described previously [30]. MSCs displayed a large number of DSBs following 24 hours exposure to VP16 (Figure 4A). After 48 hours recovery time, the abundance of DSBs decreased, resulting in less than 20% of cells displaying 10 or more Y-H2AX foci (Figure 4A and B). The proportion of cells with 10 or more Y-H2AX foci did not appear influenced by passage number at any time point, suggesting that the repair kinetics of DSBs are

similar among MSCs of all passages. To more accurately quantitate the number of DSBs on a per cell basis, γ -H2AX foci were manually counted at 6 hours recovery, showing no significant change with passage (Figure 4C). Based on these findings, it appears that the incidence and repair kinetics of DSBs following VP16 induced stress do not change with passage *in vitro*.

Alterations in NHEJ and HR Mediated Repair of DSBs with Passage and VP16 Exposure

To determine which repair pathways MSCs utilize to repair DSBs, as well as whether pathway dependence is altered by *in vitro* passage, transcriptional responses of NHEJ and HR associated genes were evaluated in our model. KU70, KU80 and DNA-PK were used to evaluate NHEJ, while XRCC2, XRCC3, and RAD51 were used to evaluate HR. Following VP16 stress, NHEJ associated transcripts remained relatively unaltered (less than 2 fold changes from baseline) and displayed no passage associated trends in abundance (Figure 5A). However, VP16 exposure reduced the abundance of HR associated transcripts, and this reduction was augmented with passage *in vitro* (Figure 5B). The decreased presence of HR transcripts following VP16 is consistent with the induction of cell cycle arrest displayed by our cells, given that HR can only be performed during S/G2 phases of the cell cycle [15]. The augmented decrease in HR associated transcripts with passage (following VP16 exposure) does not appear to be due to the reduced cycling-status of MSCs with extended passage, as the HR transcript decreases are present immediately following Passage 6 while extended culture associated decreases in S/G2/M are not present until Passage 12 (Figure 3A, Untreated). Consistent with qPCR results, protein abundance of Rad51 was decreased following VP16 stress at all passages. However, the abundance of XRCC3 was unaltered (Figure 5C). These data suggest that the reliance of MSCs on HR is reduced following VP16 stress, and that there is altered double strand DNA repair pathway utilization with *in vitro* passaging of MSCs that precedes the changes in cell cycle distribution associated with *in vitro* culture.

To evaluate the functional contribution of NHEJ and HR to DSB repair in MSCs following VP16 exposure, NHEJ and HR plasmid reporter assays were utilized. NHEJ and HR reporter assays serve as a means to quantitatively evaluate the functional ability of cells to perform NHEJ or HR, reported as repair efficiency [22]. The average repair efficiency of NHEJ in untreated Passage 6 cells was approximately 0.165, compared to 0.03 for HR, indicating that NHEJ is the predominant repair pathway of DSBs in MSCs, consistent with other cell types of the body [31] (Figure 5D). The repair efficiency of NHEJ in untreated cells remained consistent with passage of MSCs, however, the repair efficiency of HR was significantly reduced by passage 12 (Figure 5D). Coincident with reduced HR repair efficiency, untreated cells displayed reduced Rad51 protein abundance with passage (Figure 5E). Following VP16 exposure, Passage 6 and 9 cells displayed significantly elevated repair efficiency for NHEJ, while the efficiency of HR was significantly reduced (Figure 5D). Interestingly, passage 12 cells did not display significant changes in repair efficiency following VP16 exposure relative to untreated controls (Figure 5D). These results indicate that NHEJ is primarily used to repair DSBs in MSCs, and that NHEJ is increased in the context of VP16 stress while HR is decreased. However, once cells have undergone extended passage *in vitro*, MSCs are less able to utilize HR for repair, and DSB repair using NHEJ is not functionally increased following VP16 exposure. These data suggest that prolonged passage of MSCs *in vitro* can alter the ability to utilize NHEJ and HR following exposure to sub-lethal VP16 induced stress.

Discussion

The plasticity and immunomodulatory potential of MSCs have attracted attention regarding their application in cellular therapies for numerous diseases. The ability of MSCs to expand *in vitro* has further increased enthusiasm for clinical application, circumventing potential

problems with the acquisition of sufficient cell numbers for transplantation. In addition, MSCs can be acquired from various tissues of the body [4], making it possible to utilize cells from a patient autologously, nullifying the risks of graft vs. host disease. The ability of MSCs to expand *ex vivo* is beneficial towards their use clinically, highlighting the necessity of understanding how cells are changed during culture *in vitro*.

In vitro culture has been shown to epigenetically regulate gene expression in MSCs. Passage related increases in HDAC activity correlate with increased expression of HDAC4, HDAC5, and HDAC6, resulting in reduced H3 and H4 acetylation and reduced OCT4 expression [9]. Another study documented passage associated alterations in methylation at specific CpG sites, many of which regulate differentiation associated genes including RUNX3 [10], which has been implicated in osteogenesis [32]. In addition to alterations in gene regulation, *in vitro* passaging of MSCs reduces osteogenic differentiation capacity [11, 12]. Consistent with these observations, we showed a reduced ability of passage 12 MSCs to differentiate under osteogenic conditions relative to lower passages (Figure 1A), indicating that our cells had been cultured sufficiently to elicit changes in MSC function. However, we did not observe reduced adipogenic differentiation potential up to passage 12 (Figure 1B) which defined the endpoint of our model. These studies display important cellular characteristics that are altered with *ex vivo* passage, but little has been done to address how passaging alters their response to stresses resulting in DSB formation. Part of the reason DSBs are the most damaging of DNA lesions is the result of increased likelihood of erroneous repair, especially following NHEJ [33]. Erroneous repair is associated with increased cellular transformation, a phenomenon that has been documented with *in vitro* passage of MSCs [7, 8], alluding to the importance of maintaining not only survival, but genomic integrity of *ex vivo* expanded MSCs. The specific induction of DSBs by VP16 enabled us to specifically evaluate MSC responses regarding DSB repair. Although any type of DNA lesion can be harmful to a cell, DSBs are

considered the most serious and potentially mutagenic [13], hence their focus in our investigation.

To model cellular stress as a consequence of DSB presence, we utilized sub-lethal concentrations of VP16 that displayed no passage associated changes in the susceptibility of MSCs to VP16 induced cell death (Figure 2). VP16 did not result in overt death, but did increase p21 mRNA and elicited a reduction in the proportion of cells in S/G2/M phases of cell cycle indicating cell cycle arrest at all passages (Figures 3A and B). Although capable of initiating G1 arrest following cellular stress [34], we found p16 and p53 transcripts to be reduced by 6 hours recovery time (Figure 3B). The earlier and more robust presence of p21 may have chiefly contributed to the induction of cell cycle arrest, consistent with previous observations in stressed MSCs [35]. These observations show that MSCs display signs of cellular stress in our model of VP16 exposure in a manner that is sub-lethal. The absence of cellular death in our model enabled us to evaluate the incidence, repair kinetics, and cellular repair pathways of DSBs within MSCs with passage.

Of the numerous types of DNA lesions, VP16 specifically generates DSBs [36]. Within minutes following the formation of a DSB within the nucleus, various kinases (including DNA-PK, ATM and ATR) detect the presence of the break and phosphorylate histone 2A moieties. Phosphorylated histone 2A (Y-H2AX) present as nuclear puncta corresponding to an individual DSB that can be visualized by immunofluorescence [30]. When evaluating Y-H2AX foci at each time point after VP16 exposure, changes were not observed between passages (Figure 4B) suggesting that the rate by which MSCs repair VP16 induced DNA damage is not affected by passage *in vitro*. Notably, fewer than 20% of cells contained 10 or more Y-H2AX foci by 48 hours recovery (Figure 4B). The greatly reduced presence of DSBs following 48 hours recovery time suggests that MSCs are capable of recovering from 25 μ M VP16 exposure, consistent with

the sub-lethal nature of the model. These results indicate that the incidence and repair kinetics of DSBs following VP16 exposure were similar across passage number *in vitro*.

The resolution of DSBs following 48 hours recovery from VP16 exposure suggests the intact presence of double strand DNA repair pathways within MSCs for all passages observed. DSBs are repaired through two major pathways, NHEJ and HR [15]. To elucidate the contribution of these pathways to repair with passage in the context of VP16 stress, transcriptional responses of a panel of NHEJ and HR genes were evaluated. Although NHEJ transcripts displayed little change, HR associated transcripts were reduced with VP16 exposure (Figure 5A and B). Evaluations of Rad51 and XRCC3 protein showed that although XRCC3 was unaltered by passage or VP16 exposure, Rad51 was reduced by VP16, consistent with qPCR results (Figure 5C). Although these results served as evidence for VP16 exposure and passage playing a role in the means by which MSCs repair DNA, qPCR cannot describe which pathways are being utilized functionally by MSCs, and to what extent. To functionally and quantitatively evaluate the contribution of NHEJ and HR to DSB repair in VP16 stressed MSCs, plasmid based reporter assays were utilized. We showed that at baseline, NHEJ was the primary DSB repair pathway utilized by MSCs (Figure 5D), consistent with most cell types of the body [17]. In lower passage cells (Passages 6 and 9) NHEJ is increased while HR is decreased following VP16 stress (Figure 5D). However, in Passage 12 cells, there was a reduced presence of HR at baseline, possibly due to the reduced abundance of Rad51 (Figure 5E), and VP16 induced increases or decreases in NHEJ or HR (respectively) were not present (Figure 5D). These results suggested that in lower passage cells, NHEJ is increased to repair DNA damage while HR is decreased, however, later passage cells are unable to increase NHEJ mediated repair following VP16 stress. Passaging and irradiation of fibroblasts have been shown to alter the abundance and localization of Ku70/80 [37], possibly playing a role in the inability of late passage MSCs to increase NHEJ efficiency following VP16 stress (Figure 5D). Quantitation of

Y-H2AX foci in untreated cells with passage did not show significant changes (Figure S2), suggesting that the genomic integrity of untreated cells is not affected by defects in HR repair with *ex vivo* expansion. In addition, the inability of Passage 12 MSCs to increase NHEJ following VP16 exposure did not result in alterations of DSB presence or viability when compared to lesser passaged MSCs (Figures 2 and 4). These results are most likely due to the sub-lethal nature of our model. It is possible that at higher concentrations of VP16, the consequences of less efficient DNA repair would affect the presence or repair kinetics of DSBs. However, due to the nature of our assays only evaluating living cells, the phenomenon would likely not be detected (a problem evaded by our use of non-lethal concentrations of VP16). Nevertheless, our results suggest a defective ability of MSCs to increase NHEJ, their primary repair pathway of DSBs, in the context of VP16 stress following successive passage *in vitro*.

Our results showing reduction in the efficiency of HR following *in vitro* culture of MSCs are consistent with previous findings in fibroblasts [38]. However, when Seluanov et al. evaluated NHEJ efficiency in fibroblasts with successive passaging (up to 70 population doublings), NHEJ efficiency was found to be decreased [39]. Our results with normal primary human MSCs did not show significant changes in NHEJ efficiency in untreated cells regardless of passage, possibly due to the fact that our cells were passaged to a lesser extent (Passage 12). The results presented in this report contribute unique information by evaluating passage related changes in DSB repair in the context of cellular stress, a common circumstance that will be encountered when the ability to repair DNA is crucial. In addition, DSB repair being quantitatively evaluated in MSCs adds to our understanding of the characteristics of these clinically valuable stem cells. Given recent observations that NHEJ utilization varies by tissue type *in vivo* [31] the specific evaluation of DNA repair in human primary marrow derived MSCs is relevant to developing optimal models to expand cells *ex vivo* for diverse cellular therapies.

Conclusions

In conclusion, we have determined that extended culture of human primary bone marrow derived MSCs results in an inability to functionally increase NHEJ when encountering sub-lethal VP16 stress, and reduced utilization of HR in the absence of stress. Given the necessity of *ex vivo* expansion of MSCs for use in cellular therapies, these results serve as a guideline for improving strategies to sufficiently expand MSCs without inducing culture associated alterations that could have negative effects on the ability of the cells to persist following transplantation. Both the inability of MSCs to increase NHEJ following VP16 stress and reduced osteogenic differentiation capacity were detected at Passage 12 (Figures 5D and 1A, respectively). Although we do not propose that these events are directly related, they highlight the possibility of utilizing a biomarker to determine when *ex vivo* expanded MSCs display impaired DSB repair abilities in the context of stress. The discovery of such biomarkers would enable screening for DNA repair deficiencies without the time, cell, and labor intensive requirements of performing plasmid based DSB repair assays prior to utilization of *ex vivo* expanded MSCs for cellular therapy.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure Legends

Figure 1. Differentiation and surface phenotype of bone marrow derived mesenchymal stem cell (MSCs). (A) Osteogenic and (B) adipogenic differentiation at various passages evaluated by alizarin red and oil red staining, respectively. (C) Flow cytometry detecting surface expression of MSC surface markers. Black lines represent isotype controls, red lines represent indicated surface markers.

Figure 2. Viability of MSCs with passage following 24 hours etoposide (VP16) exposure. MSCs at various passages were exposed to 25 μ M VP16 for 24 hours, then allowed 0, 6, or 48 hours to recover in fresh medium before evaluation of viability by Presto Blue viability reagent. Values are reported relative to untreated controls. * Indicates a significant decrease in viability relative to untreated control, Student's T-test, p-value < 0.05.

Figure 3. Effects of VP16 on cell cycle distribution and cell cycle inhibitor transcript abundance with passage. (A) Cell cycle distribution of MSCs at various passages exposed to 25 μ M VP16 or medium only control for 24 hours. (B) qPCR evaluation of cell cycle inhibitor mRNA expression following exposure of MSCs to 25 μ M VP16 for 24 hours followed by 0, 6, or 48 hours recovery in fresh medium. Values are indicated as fold change relative to untreated control.

Figure 4. DNA double strand break incidence and repair kinetics in MSCs exposed to 25 μ M VP16. (A) Representative γ -H2AX staining (Yellow) of Passage 6 MSCs exposed to VP16 for 24 hours followed by 0, 6, or 48 hours recovery in fresh medium. γ -H2AX (Yellow) displays nuclear co-localization with DAPI (blue). (B) Percentage of MSCs displaying 10 or greater γ -

H2AX foci at each time point at various passages. (C) Average number of γ -H2AX foci per cell at 6 hours recovery time for passages 6 through 12. "NS" indicates no significant difference between any passage by One-Way ANOVA with Holm-Sidak Post Hoc, p -value > 0.05 .

Figure 5. Non-homologous end joining (NHEJ) and homologous recombination (HR) transcriptional, protein, and functional repair responses to 25 μ M VP16 with passage. qPCR evaluation of (A) NHEJ and (B) HR associated mRNA expression following exposure of MSCs to VP16 for 24 hours followed by 0 or 48 hours recovery in fresh medium. Fold changes are indicated relative to untreated controls. (C) Western analysis of Rad51 and XRCC3 after VP16 exposure with passage. 'Untr' indicates untreated cells. '0', '6', or '48' indicates recovery time (in hours) following 24 hours exposure to 25 μ M VP16. (D) NHEJ and HR plasmid reporter assays for Passage 6, 9, and 12 MSCs exposed to VP16 or medium only control for 24 hours. (E) Western analysis of Rad51 in untreated MSCs with passage. # Indicates significant change relative to untreated control, * represents significant change relative to indicated group, One-Way ANOVA with Holm-Sidak Post Hoc, p -value < 0.05 .

Supplementary Figure 1. Apoptosis associated transcriptional responses of MSCs following VP16 exposure with passage. (A) Pro-apoptotic and (B) anti-apoptotic mRNA expression evaluated by qPCR in MSCs exposed to 25 μ M VP16 for 24 hours followed by 0, 6, or 48 hours recovery in fresh medium. Fold changes are indicated relative to untreated controls.

Supplementary Figure 2. Average number of γ -H2AX foci per cell in untreated MSCs for passages 6 through 12. "NS" indicates no significant difference between any passage by Kruskal-Wallis test, p -value > 0.05 .

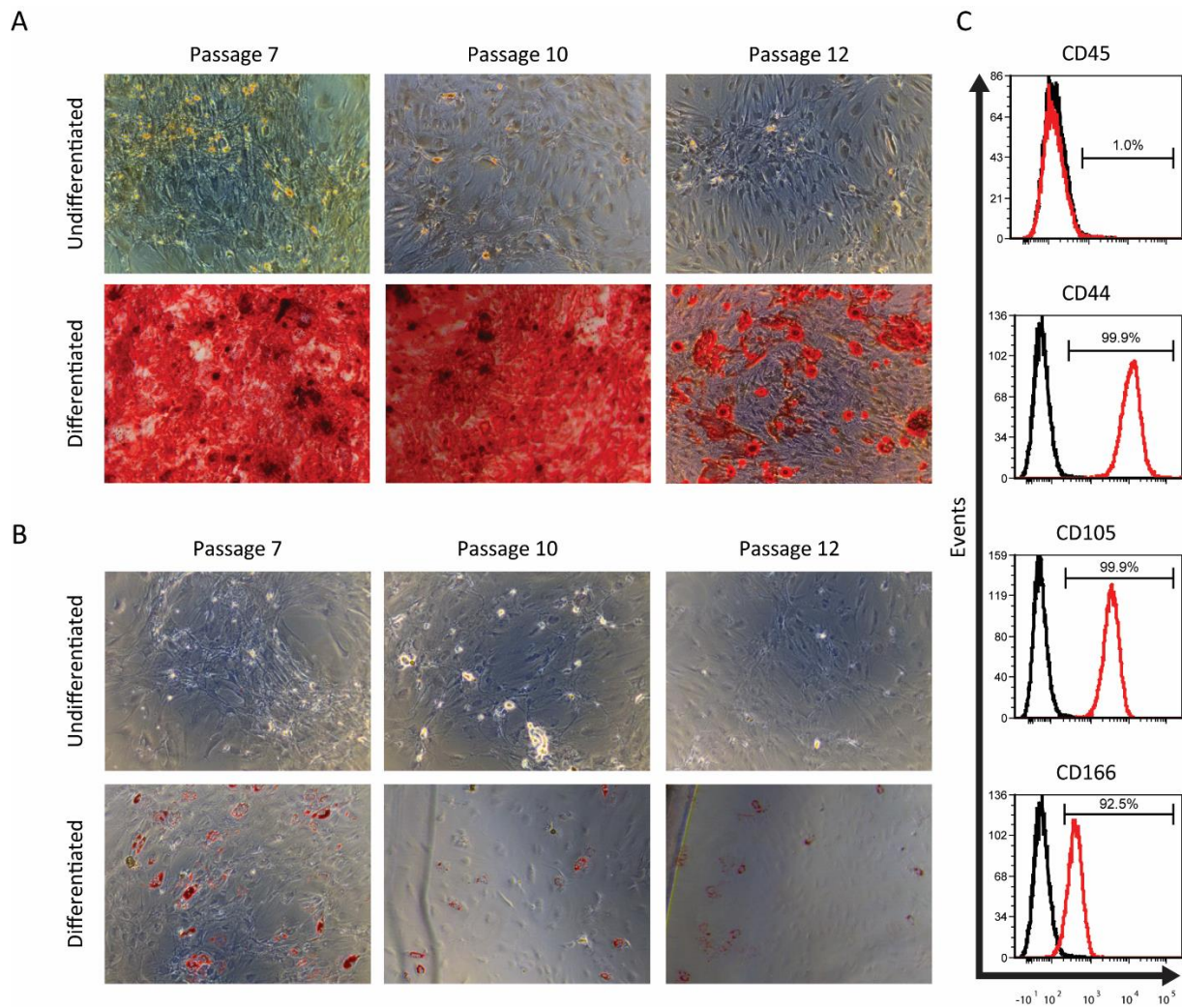


Figure 1. Differentiation and surface phenotype of bone marrow derived mesenchymal stem cell (MSCs).

Hare et al. 2016. In Vitro Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break Repair of Etoposide Induced DNA Damage. Stem Cells International. DOI: 10.1155/2016/8270464.

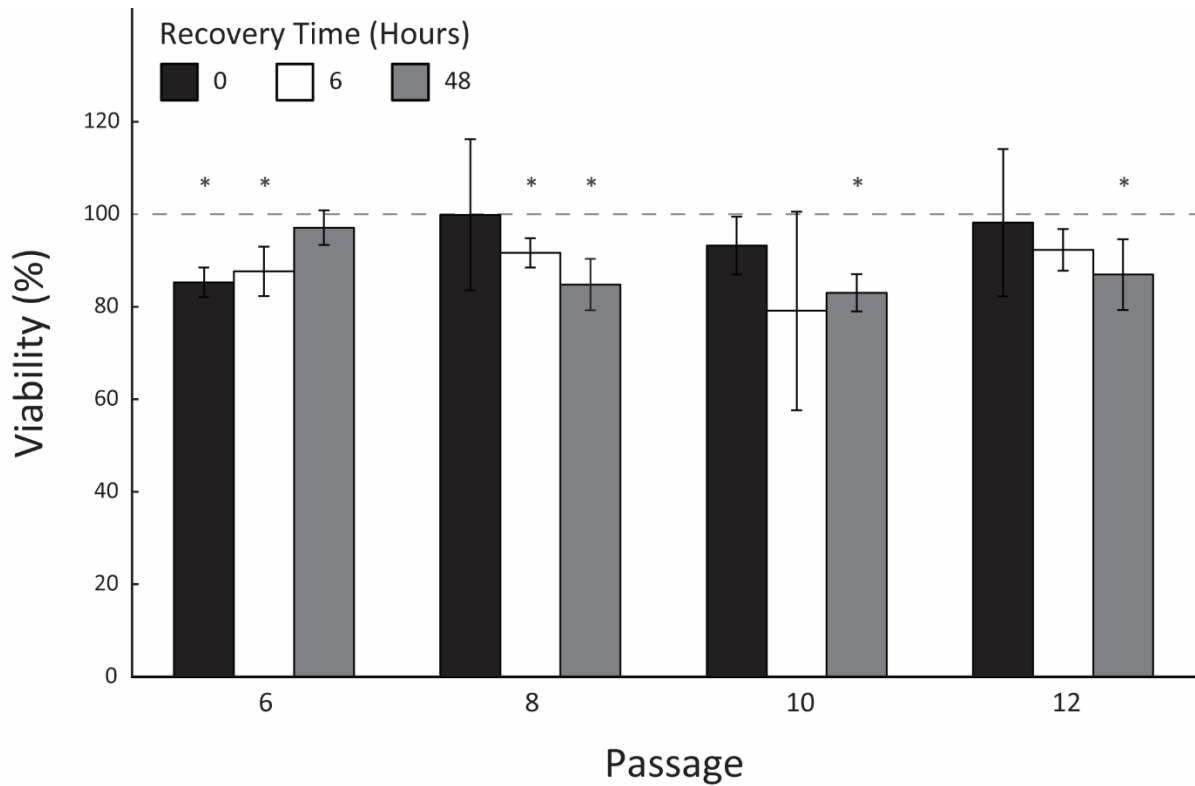


Figure 2. Viability of MSCs with passage following 24 hours etoposide (VP16) exposure.

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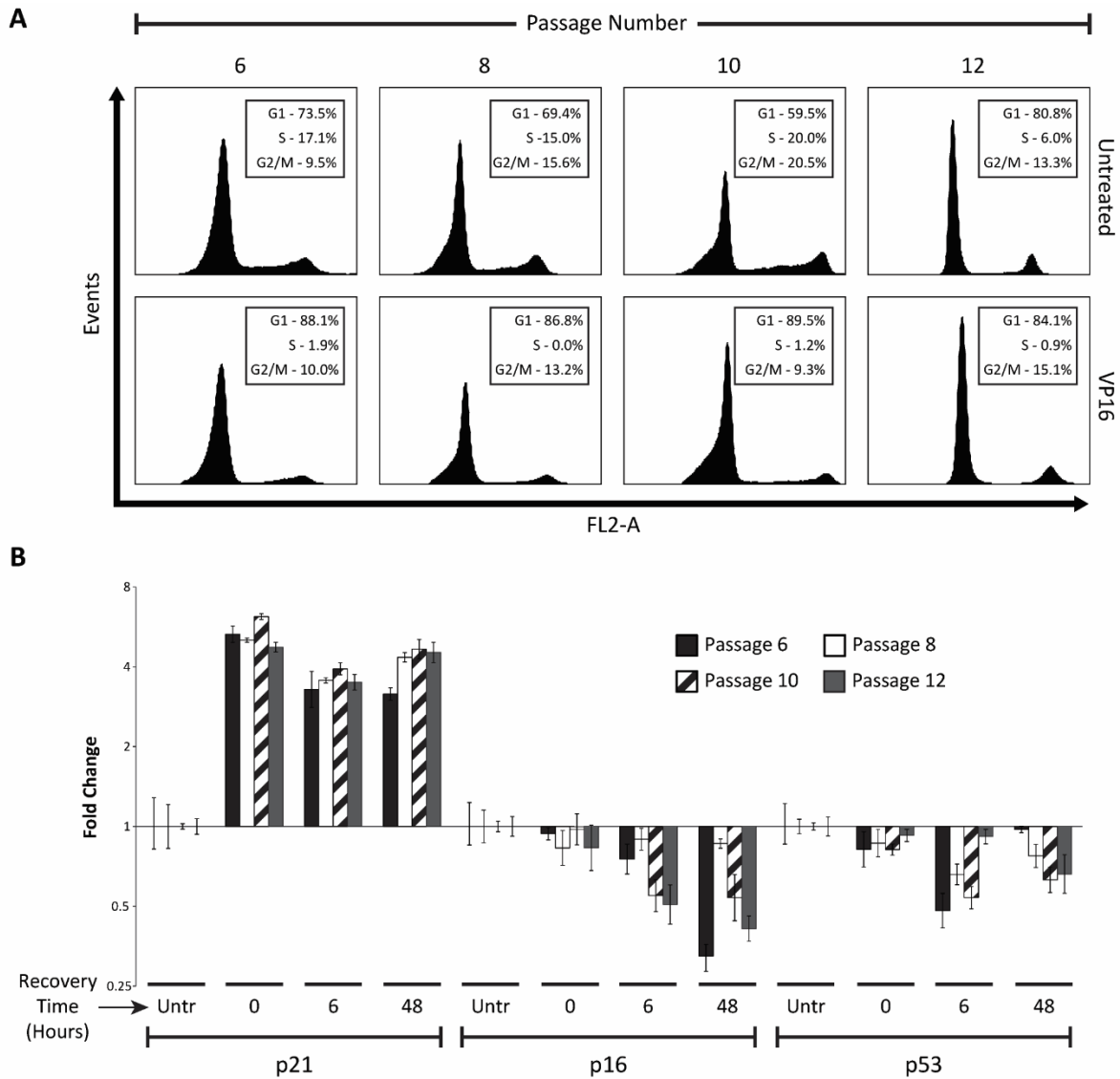


Figure 3. Effects of VP16 on cell cycle distribution and cell cycle inhibitor transcript abundance with passage.

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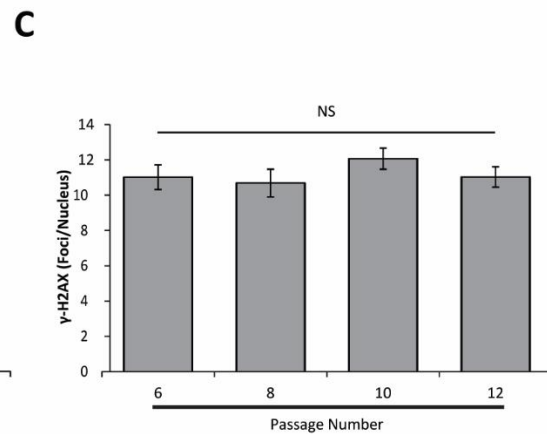
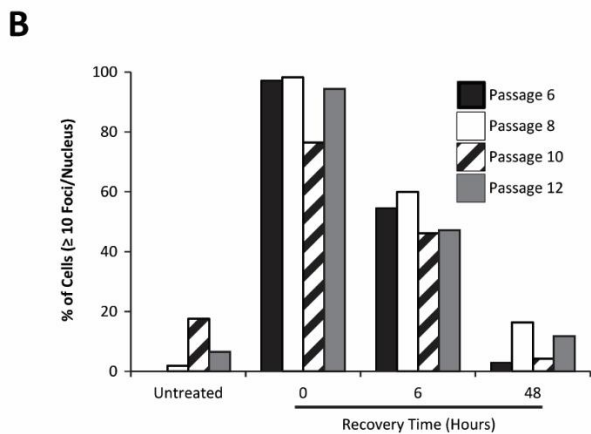
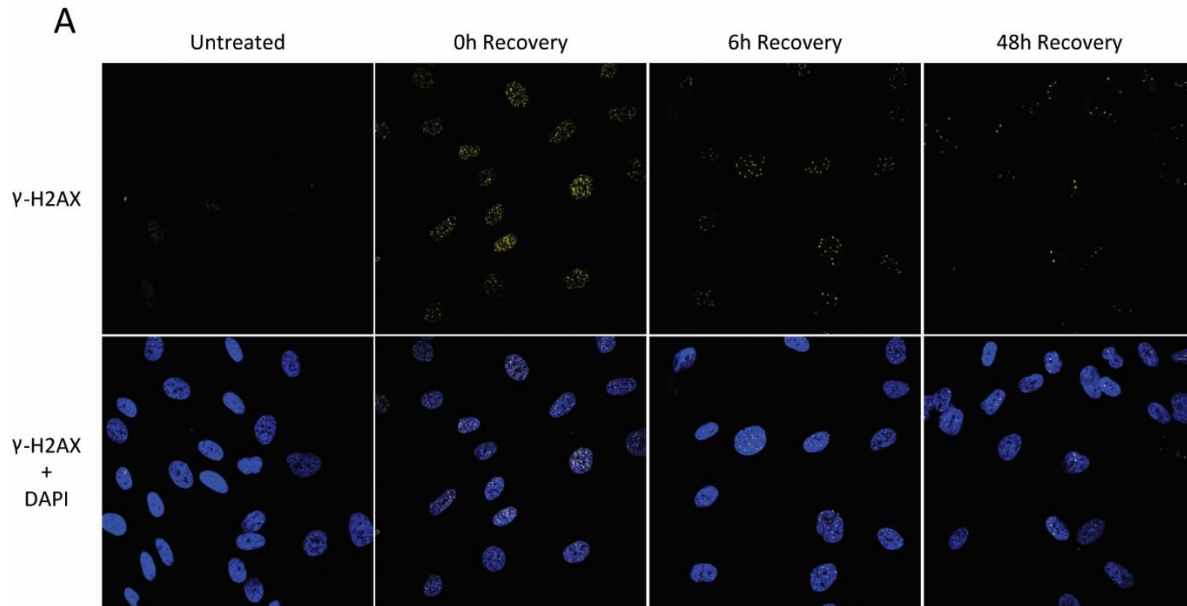


Figure 4. DNA double strand break incidence and repair kinetics in MSCs exposed to 25μM VP16.

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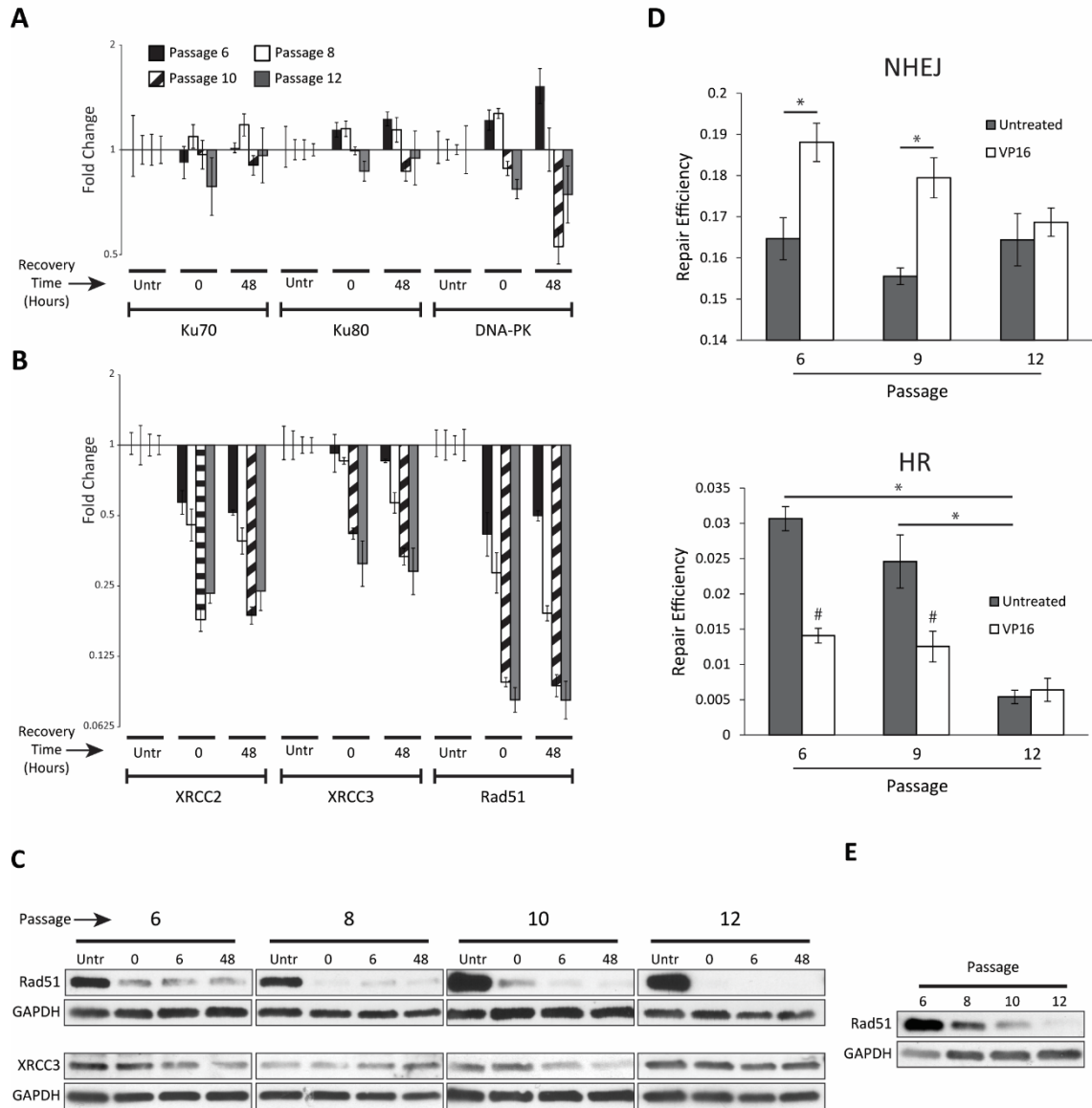
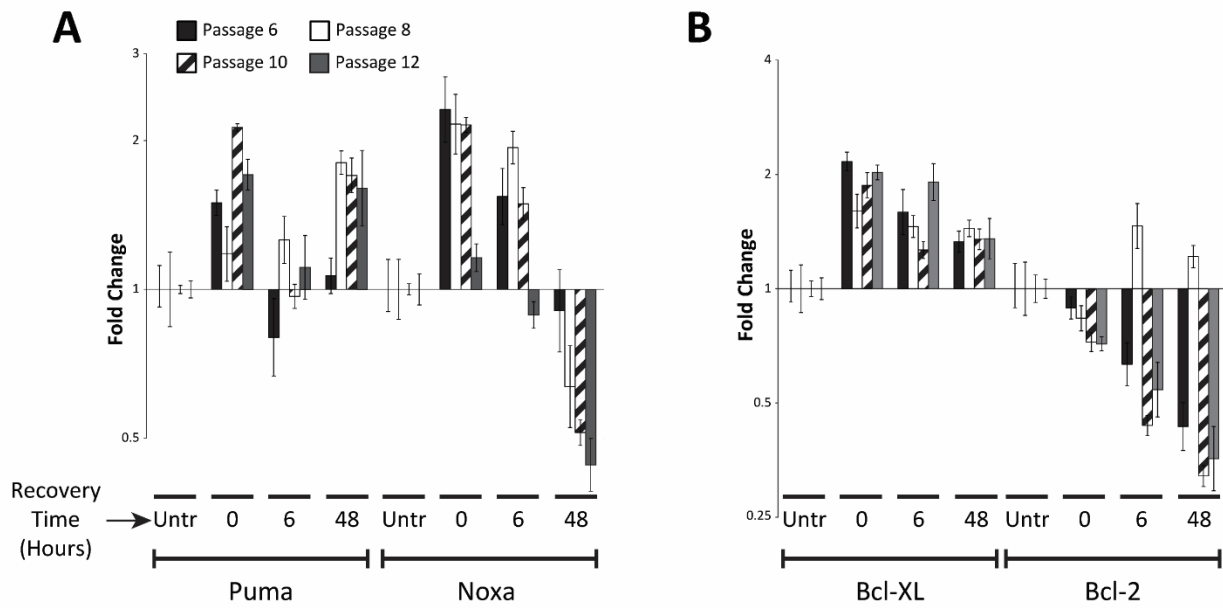


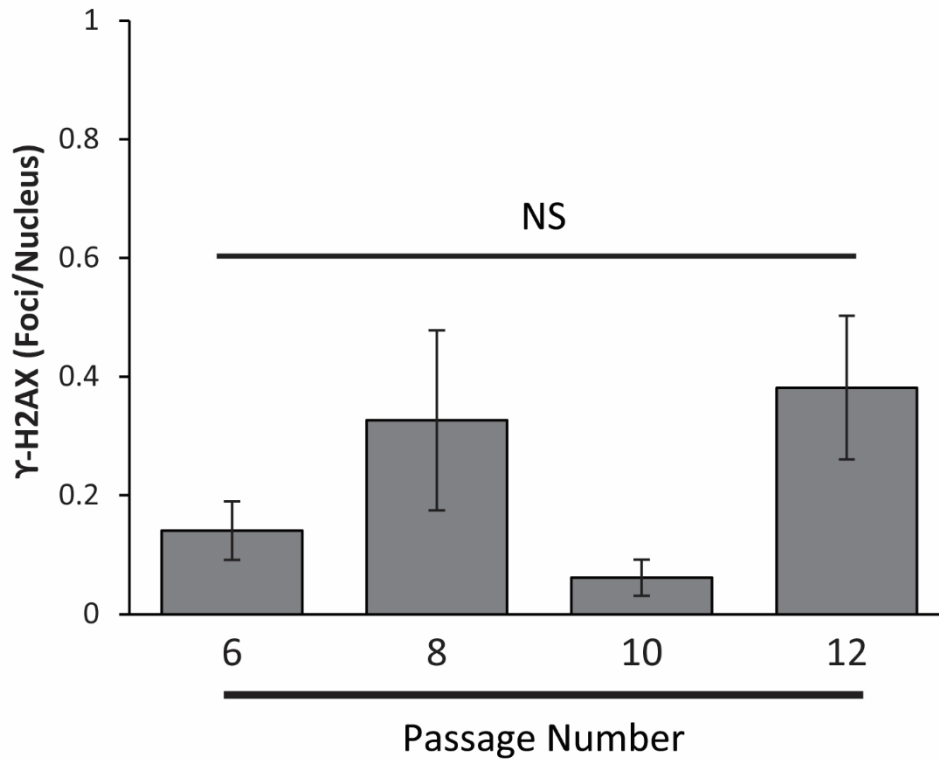
Figure 5. Non-homologous end joining (NHEJ) and homologous recombination (HR) transcriptional, protein, and functional repair responses to 25µM VP16 with passage.

Hare et al. 2016. In Vitro Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break Repair of Etoposide Induced DNA Damage. Stem Cells International. DOI: 10.1155/2016/8270464.



Supplementary Figure 1. Apoptosis associated transcriptional responses of MSCs following VP16 exposure with passage.

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Supplementary Figure 2. Average number of Y-H2AX foci per cell in untreated MSCs for passages 6 through 12.

Hare et al. 2016. In Vitro Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break Repair of Etoposide Induced DNA Damage. Stem Cells International. DOI: 10.1155/2016/8270464.

Gene	Forward	Reverse
GUSB	AAACGATTGCAGGGTTTCAC	CTCTCGTCGGTGACTGTTCA
CDKN1A (p21)	CCTGGCACCTCACCTGCTC	CGGCGTTTGGAGTGGTAGA
CDKN2A (p16)	GGGGTCGGGTAGAGGAGGT	CCGTGGAGCAGCAGCAGC
TP53 (p53)	GCACATGACGGAGGTTGTGAG	ATGGTGGTACAGTCAGAGCCAAC
KU70	CCAATAAAGCTCTATCGGGAAAC	TTTCTCCAGTATAATCTGACGACTCC
KU80	AAAATTAAGACTGAGCAAGGGG	TAGAACACGGAAGTTTTTCAGCAG
PRKDC (DNA-PK)	GCGAAGCACTGGCTTAGC	CTAACACTTCATCTTTAGGGACCC
XRCC2	GCAGTTGGTGAATGGCGTT	GCACAGGTGAATCTTCATCAGC
XRCC3	CGGCATCACTGAGCTGGC	AGCTCTCCTGGAACGTCAGTG
RAD51	GTCTCTCTGGCAGTGATGTCCT	TCTGTAAAGGGCGGTGGC
BBC3 (Puma)	AGACAAGAGGAGCAGCAGCG	CTGGGTAAGGGCAGGAGTCC
PMAIP1 (Noxa)	AGCTGGAAGTCGAGTGTGCTACT	GCAAGTTTTTGGATGCAGTCAGG
BCL2 (Bcl-2)	TGTCGCAGAGGGGCTACG	GGATGCGGCTGGATGGG
BCL2L1 (Bcl-XL)	TCCAGGAGAACGGCGGC	GAGCCCAGCAGAACCACG

Supplementary Table 1. qPCR Primer sequences (5' to 3').

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Chapter IV

Chemotherapy Induced Dkk-1 Expression by Primary Human Mesenchymal Stem Cells is p53 Dependent

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Abstract

Mesenchymal stem cells (MSCs) are abundant throughout the body, and regulate signaling within tumor microenvironments. Wnt signaling is an extrinsically regulated pathway that has been shown to regulate tumorigenesis in many types of cancer. After evaluating a panel of Wnt activating and inhibiting molecules, we show that primary human MSCs increase the expression of Dkk-1, an inhibitor of Wnt signaling, into the extracellular environment following chemotherapy exposure in a p53 dependent manner. Dkk-1 has been shown to promote tumor growth in several models of malignancy, suggesting that MSC derived Dkk-1 could counteract the intent of cytotoxic chemotherapy, and that pharmacologic inhibition of Dkk-1 in patients receiving chemotherapy treatment for certain malignancies may be warranted.

Materials and methods

MSC acquisition and cell culture

MSCs cultures were derived from de-identified bone marrow specimens from patients at the West Virginia University Cancer Institute and cultured as previously described [1]. Patients had no history of exposure to chemotherapy, radiation, or malignancy. HS-5 and HS-27A cell lines were purchased from ATCC (Manassas, VA, USA) and cultured as suggested by the supplier.

Chemotherapy and drug exposures

Chemotherapy exposure was at sub-lethal concentrations for all experiments. Etoposide (VP16) and melphalan were prepared as previously described [2] and 5-FU (Selleck Chemicals, Boston, MA, USA) was diluted to 10µg/mL in DMSO prior to use. GolgiStop (BD Biosciences, San Jose, CA, USA) was used following manufacturer guidelines. pifithrin-α, nutlin-3, and RU-486 (Selleck Chemicals, Boston, MA, USA) were diluted in DMSO, and recombinant Wnt3a (R&D Systems, Minneapolis, MN, USA) was dissolved in phosphate buffered saline prior to use.

RNA isolation and qPCR

RNA was isolated and analyzed by qPCR as previously described [1]. The following primer sequences were used (5' to 3'), DKK1 (F-CGTCACGCTATGTGCTGCC, R-GCTTTCAGTGATGGTTTCCTCA), SFRP1(F-AGTTCTTCGGCTTCTACTGGC, R-AACTCGTTGTACAGGGAGGAC), WNT2B (F-TTGACAACCTCTCCAGATTACTGTGT, R-ATTTCAACCGTCTGTTCCCTT), WNT3A (F-GATGGTGTCTCGGGAGTTTCG, R-GTGGCACTTGCACTTGAGGT), WNT4 (F-GGTCACGCACTGAAGGAGAAG, R-CAAGTACACCAGGTCCTCATCTGT), WNT5A (F-CTCTGTTTTTGGCAGGGTGA, R-GCAGCCGCAGGTGGACA), WNT10B (F-GGTCCACGAGTGTCAGCAC, R-

CAGCCAGCATGGAGAAGGA), GUSB (F-AAACGATTGCAGGGTTTCAC, R-CTCTCGTCCGGTGACTIONGTTCA).

Western blotting

Western blotting was performed as previously described [1], using the following antibodies from Cell Signaling Technologies (Danvers, MA, USA), Dkk-1 (#4687), p53 (#2524), and Phosphorylated-p53 (#9286). Antibodies were diluted as directed by manufacturer.

Dkk-1 ELISA

Dkk-1 ELISA was performed using Human DuoSet Dkk-1 ELISA (R&D Systems, Minneapolis, MN, USA) following manufacturer recommendations. Cellular supernatants were diluted 1:4 prior to analysis.

p53 siRNA Knockdown

p53 specific siRNA and scramble controls were utilized obtained GE Dharmacon (Lafayette, CO, USA), and used following manufacturer protocol. Knockdowns were performed overnight, followed by 48 hours recovery in basal culture medium prior to chemotherapy exposure.

Introduction

MSCs are a mesenchymal derived stromal population that is capable of differentiating into osteoblasts, adipocytes, and chondrocytes [3]. Although abundant in bone and adipose tissues, MSCs have been shown to be actively recruited specifically to sites of the body where they regulate angiogenesis, inflammation, and invasion of tumor cells [4]. Once within this functionally distinct tumor niche, MSCs influence the behavior of a tumor through the expression

of various proteins which regulate signaling pathways that influence survival and proliferation [5]. The Wnt signaling pathway has been described as influencing malignancy in certain tumor microenvironments, extrinsically regulated by the abundance of secreted activating and inhibitory molecules into the extracellular milieu [6]. Humans express 19 Wnt activating ligands, and several inhibitory proteins including soluble Frizzled proteins (SFRP) and Dickkopf proteins (Dkk), which inhibit Wnt signaling by quenching extracellular Wnt ligands and preventing the assembly of the Wnt surface receptor complex, respectively [7]. MSC derived Wnt ligands have been shown to initiate an epithelial-mesenchymal transition phenotype of colon cancer cells [8] and activation of Wnt signaling with lithium chloride reduced tumor burden in a murine model of myeloma [9], highlighting the importance of Wnt signaling in the tumor microenvironment.

We have reported previously that stromal cells of the bone marrow have a reduced ability to support optimal hematopoietic cell function following exposure to chemotherapy [2,10,11]. Given the observation that MSCs express Wnt activating and inhibiting molecules [12], and the importance of Wnt signaling within the tumor microenvironment, we evaluated the expression of Wnt regulating molecules by primary human MSCs following chemotherapy exposure. In the current study, we show that Dkk-1, an inhibitor of Wnt signaling that has been implicated in the progression of various tumors, is elevated in primary human MSCs after exposure to various chemotherapeutic agents.

Results

Dkk-1 expression is elevated in primary human MSCs after chemotherapy exposure

To determine whether chemotherapy exposure alters the expression of MSC derived Wnt regulating molecules, MSCs were exposed to etoposide (VP16) for 24 hours followed by qPCR analysis. Of a panel of Wnt activating and inhibitory molecules, VP16 significantly

increased the abundance of DKK1 mRNA (Figure 1a). DKK1 transcripts were elevated following exposure to VP16, melphalan, and fluorouracil (5-FU), indicating that the response was consistent among chemotherapeutics with various mechanisms of action (Figure 1b). Dkk-1 protein was also elevated, evaluated by Western blot analysis (Figure 1c), following inhibition of secretion using GolgiStop (BD Biosciences). The requirement for inhibition of secretion for detectable protein accumulation suggests that Dkk-1 is quickly secreted following transcription (Figure 1c). Finally, VP16, melphalan, and 5-FU elevated the abundance of Dkk-1 protein in culture supernatants (Figure 1d). These results indicate that primary human MSCs display elevated Dkk-1 expression following exposure to cytotoxic stress by drugs with various mechanisms of action.

Elevated Dkk-1 expression is regulated by p53

The necessity of GolgiStop to visualize intracellular Dkk-1 by Western (Figure 1c) suggested that the protein is very rapidly secreted and that elevated expression of Dkk-1 following chemotherapy exposure was potentially regulated at the mRNA level. To determine the mechanism by which Dkk-1 is elevated following chemotherapy induced stress, we investigated the potential influence of signaling pathways with promoting elements within the DKK1 promoter. The promoter region of DKK1 has been shown to contain several TCF/LEF response elements, enabling β -catenin mediated gene transcription following activation of the Wnt signaling pathway [13]. Given that exogenous activation of Wnt signaling by rWnt3a does not increase the transcriptional abundance of DKK1, and the nuclear abundance of β -catenin is not increased following VP16 exposure (Supplemental Figure 1), it is unlikely that Wnt signaling regulates DKK1 expression in MSCs following chemotherapy exposure. Glucocorticoid response elements (GRE) are also present within the DKK1 promoter [14], consistent with the elevation of DKK1 mRNA following dexamethasone exposure (Supplemental Figure 2a). However, inhibition of GRE signaling using RU-486 does not diminish chemotherapy induced

DKK1 elevations following chemotherapy exposure (Supplemental Figure 2b), suggesting that glucocorticoid signaling does not regulate DKK1 in MSCs after chemotherapy.

Previously, the promoter region of DKK1 was shown to contain a p53 response element [15]. Exposure of MSCs to VP16, melphalan, or 5-FU resulted in increased levels of phosphorylated p53 (Figure 2a). Inhibition of the transcriptional function of p53 with pifithrin- α resulted in a dose-dependent decrease in DKK1 abundance when combined with melphalan exposure (Figure 2b). In addition, inhibition of p53 by targeted siRNA resulted in decreased abundance of soluble Dkk-1 from control, as well as chemotherapy exposed, MSCs (Figure 2e). Conversely, activation of p53 using nutlin-3, an MDM2 inhibitor, increased DKK1 abundance in a dose-dependent manner when combined with melphalan (Figure 2c), as well as when used with VP16, melphalan, or 5-FU (Figure 2d). Contrary to primary MSCs, HS-5 and HS-27A human cell lines display deregulated p53 function due to the expression of human papillomavirus virus E6 and E7 proteins [16]. Consistent with the necessity of p53 to elevate Dkk-1 expression following chemotherapy in primary MSCs, exposure of HS-5 and HS-27A cells to sub-lethal concentrations of chemotherapy or nutlin-3 did not elevate Dkk-1 expression (Supplemental Figure 3), unlike primary human MSCs (Figures 1d and 2c). These results suggest that p53 regulates the elevated expression of Dkk-1 following exposure of MSCs to various chemotherapeutics.

Discussion

Our results show that MSC derived Dkk-1 is elevated in cells following cytotoxic drug exposure suggesting that it could be elevated in the tumor microenvironment following treatment with chemotherapy. VP16, a topoisomerase II inhibitor, melphalan, a DNA alkylating agent, and 5-FU, a pyrimidine analog that inhibits thymidine synthase [17–19] were all capable of

increasing Dkk-1 expression, indicating the broad spectrum of stressors which can elicit such an effect. Elevated Dkk-1 could have negative consequences for patients suffering from certain tumors. For example, Dkk-1 has been shown to promote the migration and invasion of hepatocellular carcinoma cells [20], and to promote the growth of certain esophageal, non-small cell lung cancers, and myeloma cells [21–23]. The effects of Dkk-1 appear tumor specific, since Dkk-1 has been shown to act as a tumor suppressor in certain cancers, such as melanoma and colon [24,25]. Nevertheless, these findings suggest that chemotherapy could be having an undesired effect in these cancers, potentially leading to unsuccessful treatment or relapse of disease.

To our knowledge, this is the first time Dkk-1 has been shown to be elevated in MSCs following exposure to chemotherapy. However, it has been shown previously that certain cancer cell lines with functional p53 react similarly in response to chemotherapy induced stress [26]. Importantly, our observations are the first to include primary human MSCs which have been shown to be a component of the tumor microenvironment. Wang et al. and Shou et al. have also described the role of p53 in chemotherapy induced Dkk-1 expression, supporting and refuting the notion in tumor cell lines, respectively [15,27]. Our data are consistent with the assertion that p53 is playing a role in this regulation, however, it is likely that the phenomenon is regulated differently between cell types. The necessity of functional p53 for normal cellular function makes targeting the p53 pathway unfavorable in conjunction with cytotoxic therapies, suggesting that pharmacologic targeting of this response would be best directed at soluble Dkk-1 protein.

These observations align with a recent publication describing increased Dkk-1 protein in the serum of patients who have received chemotherapy [28], indicating that MSCs could be one potential source of this response. BHQ-880, a monoclonal anti-Dkk-1 antibody which has cleared phase Ib clinical trials [29], could theoretically be used in conjunction with chemotherapy to prevent the undesired effects of Dkk-1 on tumor phenotype in patients being treated with

chemotherapy for certain malignancies. A more in depth analysis needs to be performed to understand which tumors respond to Dkk-1 as an oncoprotein and which respond to Dkk-1 as a tumor suppressor prior to use *in vivo*. Overall, our findings show that chemotherapy treatment may have an undesirable effect on the tumor microenvironment with future work required to evaluate whether MSC derived Dkk-1 negatively influences tumor behavior in the context of certain malignancies.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interest regarding the publication of this manuscript.

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Figure Legends

Figure 1 Dkk-1 is elevated in MSCs following chemotherapy exposure. **(A)** qPCR evaluating Wnt inhibitory and activating molecules in MSCs exposed to 100 μ M etoposide (VP16) or diluent control for 24 hours. **(B)** qPCR evaluating DKK1 expression in MSCs exposed to 100 μ M VP16, 25 μ g/mL melphalan (Mel), 300 μ g/mL fluorouracil (5-FU), or medium-only control. VP16 and melphalan exposures were 24 hours, 5-FU was incubated with MSCs for 1 hour followed by 23 hours incubation in normal growth medium. These chemotherapy conditions were kept consistent throughout the remaining experiments unless otherwise specified. **(C)** Western blot analysis of Dkk-1 in MSCs exposed to chemotherapy or medium-only control, in the presence of GolgiStop or diluent alone for 24 hours. Recombinant human Dkk-1 (rDkk-1) was included as a control. **(D)** Dkk-1 ELISA analysis of supernatants from MSCs exposed to chemotherapy or medium-only control for 72 hours. 5-FU was administered for 1 hour followed by 72 hours incubation in basal medium. *Indicates p-value < 0.05, using Student's T-test (A) or One-Way ANOVA with Dunnett's Post Hoc (B and D).

Figure 2 p53 regulates Dkk-1 following chemotherapy exposure of MSCs. **(A)** Western blot analysis of phosphorylated (phospho) and total p53 protein in MSCs following 6 hours exposure to chemotherapy or medium-only control. **(B)** qPCR evaluation of DKK1 in MSCs exposed to melphalan in combination with increasing concentrations of pifithrin- α (PTH α) for 24 hours. Cells were pre-treated with PTH α for 20 minutes prior to chemotherapy, and PTH α was spiked into culture vessels every 8 hours. **(C)** qPCR evaluation of DKK1 in MSCs exposed to melphalan in combination with increasing concentrations of nutlin-3 for 24 hours. Cells were pre-treated with nutlin-3 for 20 minutes prior to chemotherapy. **(D)** qPCR analysis of MSCs exposed to

chemotherapy in the presence of 25 μ M nutlin-3 or diluent control for 24 hours. **(E)** Dkk-1 ELISA analysis of MSCs transfected with two separate siRNA targeting p53 mRNA (or scramble control), then exposed to chemotherapy or medium-only control for 24 hours. Cells conditioned medium for 72 hours prior to analysis. *Indicates p-value < 0.05, One-Way ANOVA with Holm-Sidak Post Hoc.

Supplementary Figure 1 β -catenin mediated signaling does not regulate chemotherapy induced DKK1 expression in MSCs. **(A)** rWnt3a was cultured with MSCs for 24 hours at indicated concentrations prior to RNA isolation and qPCR evaluation of DKK1. **(B)** MSCs were exposed to 100uM VP16 for 24 hours, followed by nuclear fractionation and evaluation of β -catenin by Western blotting. HDAC1 and GAPDH were used as nuclear and cytosolic loading controls (respectively).

Supplementary Figure 2 Glucocorticoid signaling does not regulate chemotherapy induced DKK1 expression in MSCs. **(A)** MSCs were cultured with indicated concentrations of dexamethasone for 24 hours prior to RNA isolation and qPCR evaluation of DKK1. **(B)** MSCs were exposed to 100uM VP16 or 25 μ g/mL melphalan (Mel) for 24 hours in the presence of 10 μ M RU-486 (glucocorticoid receptor antagonist) or DMSO control followed by qPCR analysis of DKK1.

Supplementary Figure 3 Dkk-1 is not elevated in p53 deregulated cell lines following chemotherapy or nutlin-3 exposure. HS-5 **(A)** and HS-27A **(B)** cells were exposed to sub-lethal concentrations of VP16 or melphalan (Mel) for 72 hours, or 5-FU for 1 hour followed by 72 hours

incubation in basal medium, then evaluated by Dkk-1 ELISA. **(C)** HS-5 and HS-27A cells were exposed to indicated concentrations of nutlin-3 for 24 hours, followed by DKK1 evaluation by qPCR.

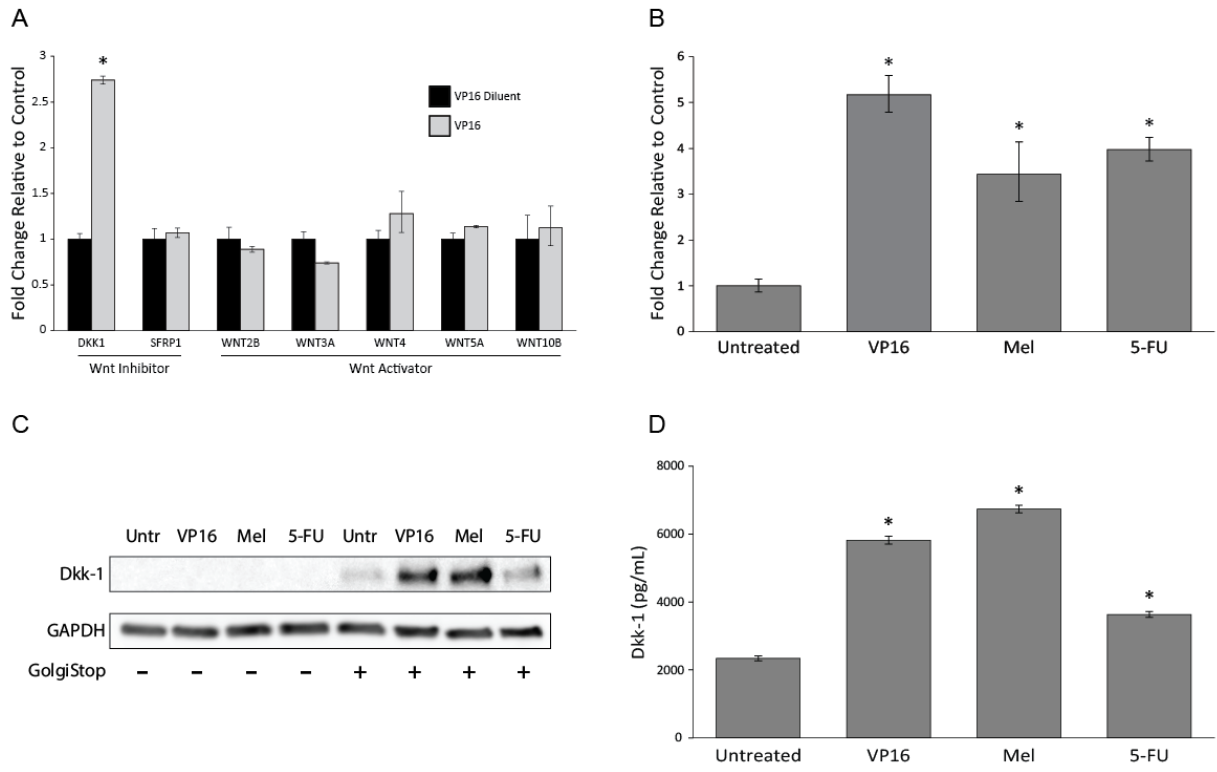


Figure 1. Dkk-1 is elevated in MSCs following chemotherapy exposure.

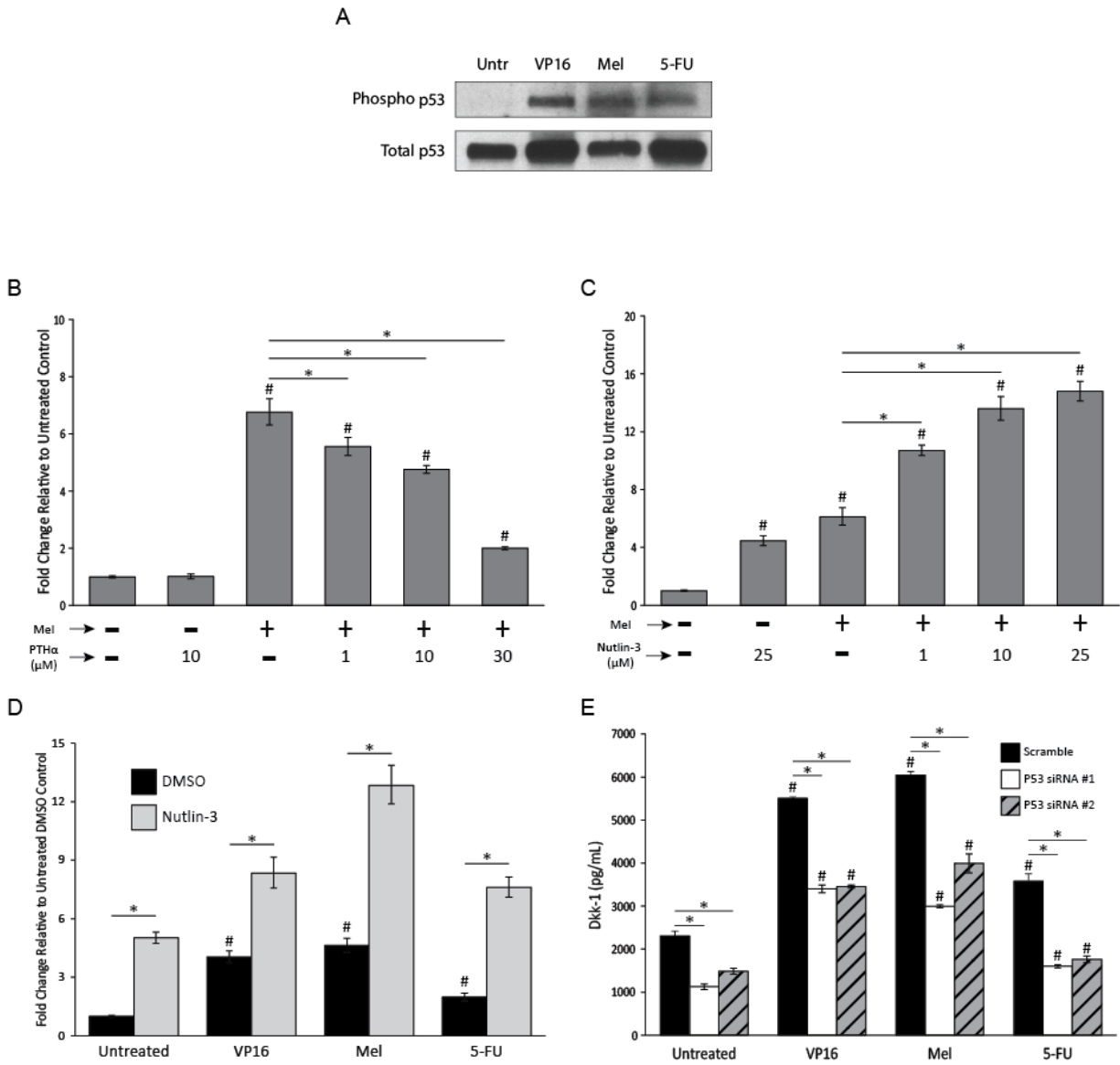
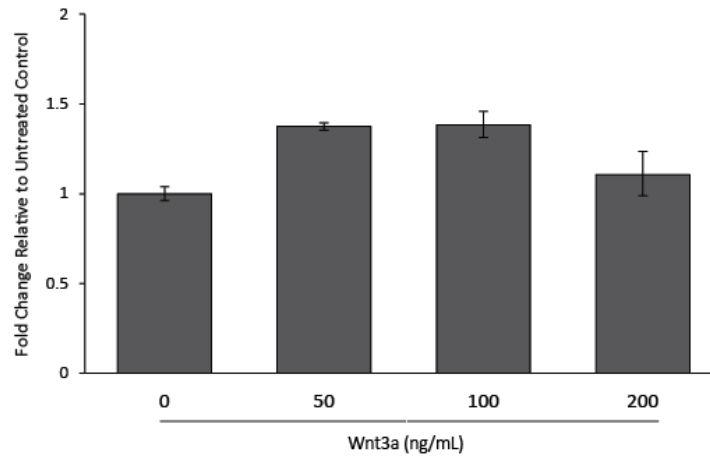
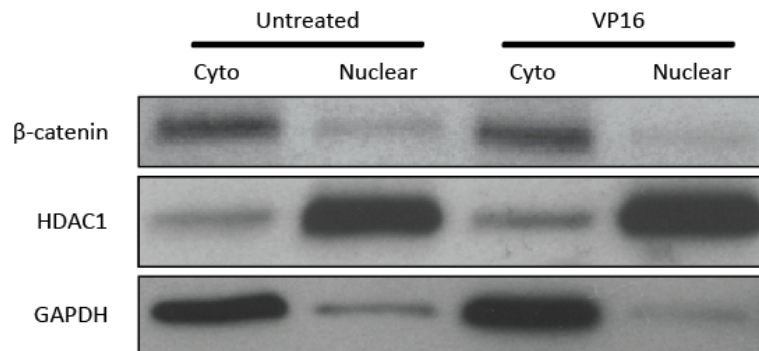


Figure 2. p53 regulates Dkk-1 following chemotherapy exposure of MSCs.

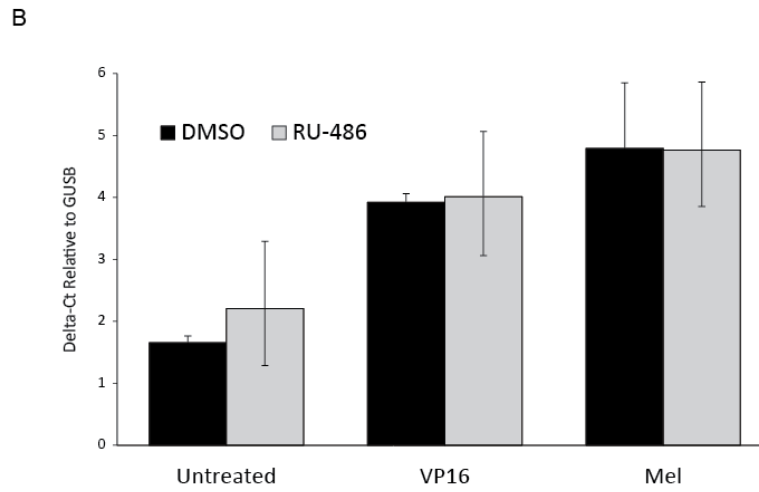
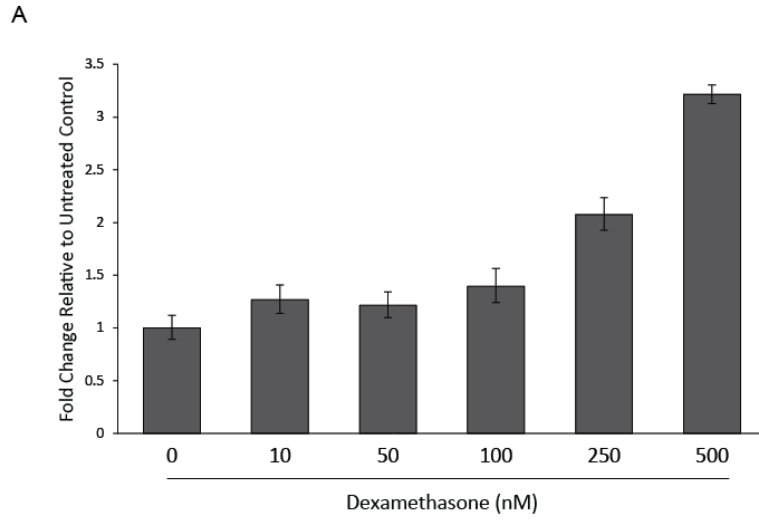
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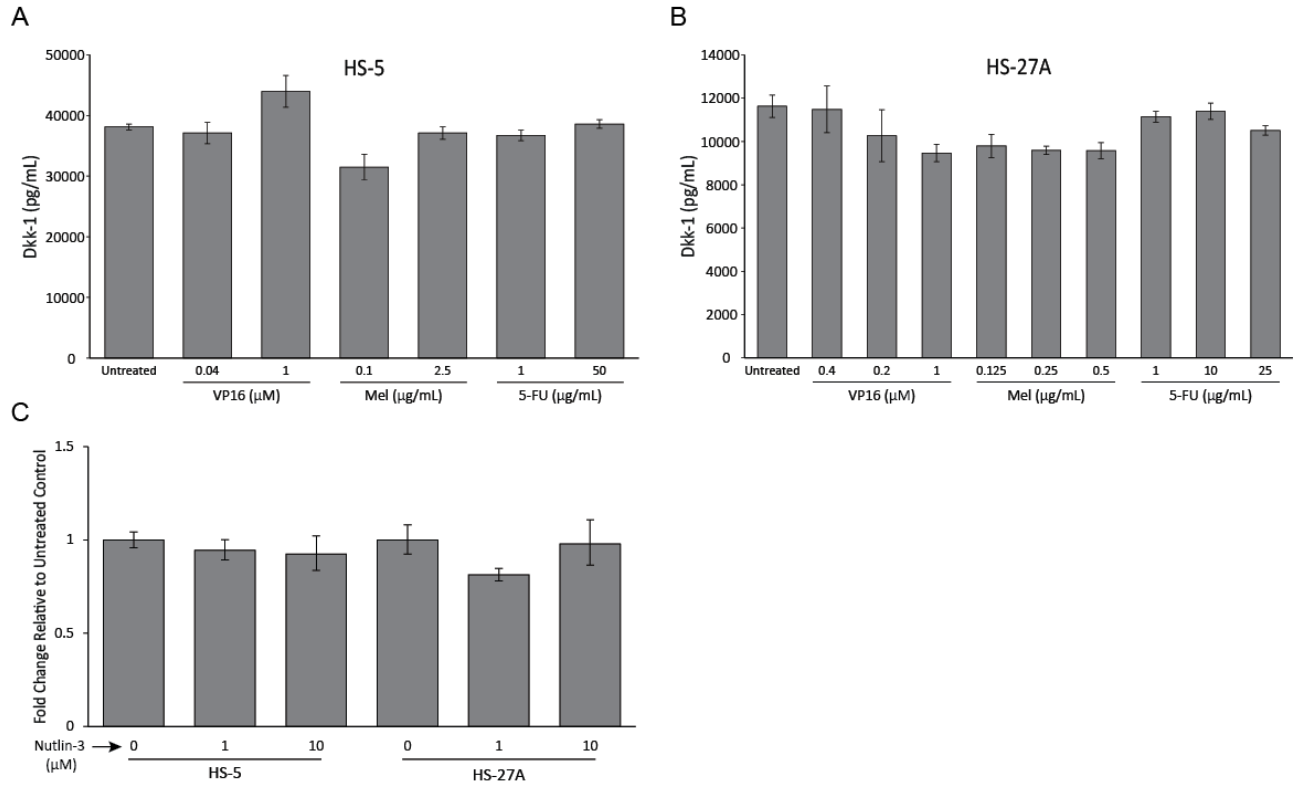
B



Supplementary Figure 1. β -catenin mediated signaling does not regulate chemotherapy induced DKK1 expression in MSCs.



Supplementary Figure 2. Glucocorticoid signaling does not regulate chemotherapy induced DKK1 expression in MSCs.



Supplementary Figure 3. Dkk-1 is not elevated in p53 deregulated cell lines following chemotherapy or nutlin-3 exposure.

Chapter V

General Discussion

The Friedenstein laboratory observed the presence of stromal cells within the bone marrow in 1976¹. It was not until 1987 that the ability of these cells to undergo osteogenic differentiation was appreciated, following observations of mineralized ECM after expansion *ex vivo*². Bone marrow stromal support of hematopoietic cells was addressed by Michael Dexter after developing the first *in vitro* culture system of HSCs, using bone marrow stromal cell feeder layers^{3,4}. Once the supportive nature of bone marrow stromal cells was understood, work by David Scadden, Paul Frenette, Daniel Link, and Sean Morrison laboratories, among others, contributed greatly to the understanding of their heterogeneity reflected as the various cellular phenotypes described in Chapter I. Although these findings displayed the plasticity of various bone marrow stromal cell populations, it was Pittenger et al. who first characterized a population of mesenchymal precursor cells capable of differentiating into osteoblasts, chondrocytes, and adipocytes, coining the name “MSCs”⁵. In addition to supporting homeostatic functions of the body, MSCs have been observed to influence the activities of malignant cells. Evidence for this phenomenon was proposed by Stephen Paget in 1889. In a report outlining his “seed and soil” hypothesis, Paget postulated that the propensity of metastatic cells to induce secondary tumors is influenced by the environment in which they reside⁶. This early observation later cumulated into an understanding of what is known as the tumor microenvironment, shown to be influenced, in part, by actively recruited MSCs from local environments as well as more distant anatomical sites⁷.

As the understanding of the biology of MSCs grew, their attributes beyond stem cell regeneration and support became apparent. MSCs have been shown to produce an immune suppressive environment, in part, through the secretion of anti-inflammatory cytokines⁸. These attributes, in combination with their plasticity and ability to support hematopoietic stem and progenitor cells, led to investigations focused on potential application in MSC transplantation therapies. Edwin Horwitz was among the first to conceptualize this idea, pioneering the use of

MSC transplantation for the treatment of osteogenesis imperfecta⁹. These initial successes led to the development of current MSC therapies utilized for the treatment of GVHD, and in pre-clinical animal models of systemic lupus erythematosus and rheumatoid arthritis¹⁰. These attributes of MSCs highlight their importance *in vivo*, as well as for use in cellular therapies. Collectively, these observations underpin the necessity to better understand the biology of these cells in a variety of contexts.

Previous studies in our laboratory have focused on MSCs and osteoblasts in their ability to support hematopoiesis following exposure to high dose chemotherapy regimens, such as those prior to bone marrow transplantation^{11,12}. Patients who have undergone high dose chemotherapy treatment for bone marrow transplantation as well as the treatment of numerous malignancies have been shown to display hematopoietic cell deficits for years following treatment. For example, Corre et al. described deficiencies in CD4⁺ T cell counts in patients two years following myeloablative chemotherapy and allogenic HSC transplantation¹³. This observation suggests an inability of transplanted HSCs to adequately repopulate blood and lymphoid tissues, or sustained damage to the supportive bone marrow microenvironment as a consequence of chemotherapy treatment. The latter of these two possibilities has been previously investigated by our laboratory. For example, Clutter et al. showed the protein abundance of CXCL12 to be decreased by chemotherapy exposed bone marrow stromal cells, resulting in the reduced ability to chemoattract a murine pro-B cell line towards the supportive stromal monolayer¹⁴. Previous members of our laboratory have also shown that the expression of cytokines relevant to the regulation of hematopoietic support, such as interleukin-6, neurotrophins, and TGF- β ^{11,15,16} are dysregulated by the exposure of stromal components of the bone marrow microenvironment to chemotherapy. These observations illustrate the vulnerability of cells comprising this critical environment to damage that alters their ability to regulate hematopoiesis. The work described in this dissertation expands upon these observations, as

well as looking more broadly into ways by which the function of bone marrow stromal cells is affected by chemotherapy beyond the realm of hematopoietic support.

As discussed in Chapter I, osteoblasts contribute to the support of hematopoietic cell development within the bone marrow niche. Rellick et al. evaluated the effects of chemotherapy on TGF- β and interleukin-6 expression by osteoblasts, showing increased and decreased expression, respectively, following exposure to a variety of chemotherapeutic agents^{11,15}. The findings described in Chapter II of this dissertation further investigated the alteration of osteoblast cells by chemotherapy exposure, expanding observations to both hematopoietic support factors and effects on cellular plasticity. Utilizing murine pre-osteoblast cell lines at previously described states of differentiation^{17,18}, we showed that chemotherapy induced reductions in the mRNA abundance of RUNX2 and SP7 (the gene that encodes osterix). Consistent with this observation, exposure of MC3T3E1 and 7F2 cell lines to VP16 or melphalan reduced osteogenic differentiation potential¹⁹. These results suggested a reduced ability of pre-osteoblasts to differentiate into a more mature osteogenic phenotype following chemotherapy exposure. A potential consequence of reduced osteogenic differentiation within the bone marrow would include a deficit in mature osteoblast cells, which has been shown by Ding and Morrison to regulate the proliferation and differentiation of common lymphoid progenitors²⁰. Bone is continually degraded by osteoclasts, then subsequently regenerated by osteoblasts in a process known as bone remodeling²¹. A deficiency in osteoblast progenitor differentiation could lead to an imbalance in the process, favoring bone degeneration and ultimately resulting in a reduced number of osteoblasts within the bone marrow microenvironment. Given the necessity of osteoblasts in the support of lymphoid progenitors, chemotherapy induced reduction in osteogenic differentiation could potentially account for lymphoid mediated immune deficits documented in patients who have received chemotherapy treatment. For example, Wiser et al. observed reduced immunoglobulin concentrations in

women years after receiving chemotherapy treatment for breast cancer²². Chemotherapy induced deficits in osteogenesis could also have negative effects on the skeletal system. Reduced bone mineral density and increased fracture incidence are a common side effect of patients who have been treated with high doses of chemotherapy. For example, a study by Tillmann et al. described these deficiencies in children who had been treated with high dose chemotherapy for acute lymphoblastic leukemia²³ and Hui et al. observed a similar phenomenon in women who had been previously treated for various gynecologic cancers²⁴. More work needs to be done to understand the events that mediate chemotherapy induced deficiencies in osteogenesis *in vivo*, and how these can be altered to prevent such toxicities during chemotherapy treatment.

In addition to reduced osteogenic differentiation potential, observations discussed in Chapter II described deficiencies in the ability of osteoblasts to generate ECM and ECM-associated proteins that have been shown to regulate hematopoiesis¹⁹. We showed that in pre-osteoblast and differentiated osteoblast cells Col1 α 1 mRNA was decreased following exposure to chemotherapy¹⁹. Consistent with this decrease, visible deficits in endosteal ultrastructure were observed by scanning electron microscopy when evaluating femurs from mice treated with VP16¹⁹. These results suggested that the ECM surrounding osteoblast cells is damaged following chemotherapy stress both *in vitro* and *in vivo*, a phenomenon that could have negative consequences for hematopoietic cell regulation. As discussed in Chapter I, various hematopoietic regulating molecules depend on ECM within the hematopoietic niche to regulate cytokine concentration gradients and promote the retention of necessary molecules near the niche. One such molecule is OPN, which we showed to also be reduced in pre-osteoblasts exposed to melphalan¹⁹. Interestingly, the promoter regions of Col1 α 1 and OPN both contain response elements for RUNX2, which we showed was decreased in both pre- and mature osteoblast cells¹⁹, suggesting that reduced RUNX2 expression following chemotherapy

exposure could potentially mediate deficits in Col1 α 1 and OPN following chemotherapy exposure. More work needs to be done to determine whether actionable targets mediating RUNX2 abundance after chemotherapy exposure exist, and means by which they could be pharmacologically modulated to prevent chemotherapy induced alterations in osteoblast function following chemotherapy treatment *in vivo*. In addition to ECM related deficits, we showed that CXCL12 protein was reduced in both pre-osteoblasts and mature osteoblast cells following VP16 or melphalan exposure¹⁹. This is consistent with our previous observations in lesser differentiated bone marrow stromal cells¹⁴ as well as primary human osteoblasts¹¹, highlighting the breadth of this response between varying stromal differentiation states and species models. CXCL12 has been shown to be vital to the support of hematopoietic cells within the bone marrow²⁵, and chemotherapy-induced decreases in its expression could hamper hematopoietic regeneration following insult such as chemotherapy treatment. Liang et al. showed that co-transplantation of MSCs overexpressing CXCL12 with CD34⁺ cells after myeloablative chemotherapy promoted hematopoietic reconstitution in a murine transplant model²⁶. These results show the potential promise of MSC transplantation to promote hematopoietic regeneration following chemotherapy treatment.

Given the potential therapeutic applications of MSCs, including the promotion of hematopoietic regeneration as well as treating many other diseases (discussed in Chapter I), it is important to better understand their biology so they can be optimally utilized for transplantation therapies. In a recent clinical trial, MSC transplantation was utilized to treat osteogenesis imperfecta²⁷ and only showed efficacy when 1x10⁷ MSCs per kilogram were administered to patients. Similar cell numbers have been needed to treat GVHD and myocardial infarction¹⁰, displaying the difficulty in acquiring enough cells for human therapies. To isolate enough MSCs for use clinically, *ex vivo* expansion is required, highlighting the necessity of understanding how cells are altered during expansion and the consequences such expansion

could have once cells are administered *in vivo*. In Chapter III, we evaluated phenotypical changes of MSCs following *ex vivo* expansion, as well as changes in the means by which they responded to DNA double strand break stress, using VP16 as a way of eliciting such damage. Consistent with the presence of sub-lethal DNA damage, 25µM VP16 induced cell cycle arrest, coincident with elevated p21 and γ-H2AX, without eliciting overt cell death²⁸. Although the cellular response and repair kinetics of DNA damage were consistent with passage, the means by which the cells repaired DNA was altered over time. VP16 reduced the mRNA and protein abundance of HR associated genes, consistent with functional repair along the HR pathway being reduced as evaluated by a plasmid based reporter assay²⁸. Although gene expression for NHEJ associated proteins was not altered by passage or VP16 exposure, the ability of MSCs to enact NHEJ repair was reduced with passage *in vitro*²⁸. These results illustrate the potential for extensively passaged MSCs to demonstrate impaired NHEJ in the context of genotoxic stress, a phenomenon that would not be desirable when transplanting cells into a human being. These findings indicate that MSCs should not be expanded extensively *in vitro* prior to patient administration. Efforts have been contributed towards the optimization of *in vitro* culture conditions during MSC expansion. For example, Doucet et al. have shown that human platelet lysate was favorable to fetal bovine serum for culture supplementation, resulting in increased MSC expansion potential²⁹. Whether the addition of culture supplements can prevent culture associated phenotypic changes (such as in DNA repair capabilities) needs to be addressed further. Another approach to lessen culture associated changes in MSC phenotype would be optimizing MSC isolation techniques to maximize cellular yield. Starting with an increased number of MSCs would lessen the number of population doublings necessary to reach clinically relevant cell numbers for transplantation. An attractive means of achieving this goal would be to use adipose derived MSCs. The majority of clinical trials utilizing MSCs have used cells derived from the bone marrow due to the fact that they are very well characterized³⁰. In addition, MSCs derived from adipose tissue have been shown to have a reduced differentiation potential

towards the osteogenic lineage³¹. Nevertheless, adipose derived MSCs are generally easier to acquire given the less invasive nature of their isolation (liposuction), and the willingness of patients to part with the specimen, providing an abundant source of MSCs for use in clinical therapies. The clinical potential of adipose derived MSCs needs to be better characterized in the context of transplantation to provide confidence towards their use in human therapies.

Findings outlined in Chapters II and III have described dysregulations of the beneficial and homeostatic functions of MSCs within our bodies following chemotherapy exposure. However, damaged MSCs can also elicit pathogenic effects in certain scenarios. A potential example of this phenomenon is described in Chapter IV, where Dkk-1, a negative regulator of certain tumor activities, is described to be elevated following chemotherapy exposure of MSCs in a p53 dependent manner. Through the binding to LRP5/6 co-receptors, Dkk-1 sterically hinders aggregation of the Wnt surface receptor complex, resulting in inhibition of downstream signaling³². Dkk-1 overexpression has been documented in several types of cancer such as prostate³³, esophageal³⁴, myeloma³⁵, and non-small cell lung cancers³⁶. Over expression of Dkk-1 has had negative consequences on tumor phenotype in these diseases. For example, Thudi et al. showed that overexpression of Dkk-1 in prostate tumor cells led to increased tumor mass and metastasis in a murine model of prostate cancer³⁷, and Nagato et al. showed that anti-Dkk-1 antibody treatment reduced invasiveness *in vitro* and tumor growth in a murine model of lung cancer³⁸. Given observations of MSCs homing to the tumor microenvironment (discussed in Chapter I of this dissertation), our finding that the exposure of MSCs to various chemotherapeutics increases the expression of Dkk-1 shows the potential for chemotherapy treatment to result in increased abundance of Dkk-1 protein in the tumor microenvironment. In cancers which have been shown to be more aggressive in the presence of elevated Dkk-1 (such as prostate and lung), Dkk-1 could potentially be pharmacologically regulated to prevent this outcome. BHQ880, an anti-Dkk-1 neutralizing antibody described in the Discussion section of

Chapter IV, has been used in clinical trials³⁹; however, the necessity of intravenous administration and the costs inherent to antibody therapies could limit clinical use as well as patient access to the drug. While clinical trials regarding the application of anti-Dkk-1 therapies should continue, investigations into the clinical use of chemical inhibitors of Dkk-1 should also be explored. Pelletier et al. have developed a small molecule (WAY-262611) which inhibits Dkk-1 mediated inhibition of Wnt signaling *in vitro* as well as in mice⁴⁰, and future work should focus on determining the safety and efficacy of this compound in humans as well as developing other structures that elicit similar effects. As mentioned above, Dkk-1 has only shown a pathological role in certain tumors. Some cancers do not respond in this manner to Dkk-1, such as colorectal cancers, which are promoted by Wnt signaling. For example, Qi et al. showed that overexpression of Dkk-1 by colon cancer cells reduced tumor growth in a nude mouse model of colon cancer⁴¹, suggesting that elevated Dkk-1 in the tumor microenvironment could have positive impacts for patients with certain malignancies. This observation highlights the necessity to better understand how different tumors respond to Dkk-1. A better understanding of which cancers elicit aggressive or invasive phenotypes in the presence of Dkk-1 would help guide the design of clinical trials to pharmacologically target MSC derived Dkk-1 following chemotherapy treatment, thereby preventing effects of MSC derived Dkk-1 on tumor phenotype. Given the known overexpression of Dkk-1 in various cancers, the novelty of our findings lies in the observation that MSCs can also express this protein, and to a greater extent in the context of chemotherapy treatment. Future studies will need to be performed to determine whether Dkk-1 is elevated by MSCs within the tumor microenvironment *in vivo* and whether chemotherapy elicits the expression of other molecules derived from MSCs that could have negative outcomes in the tumors of patients undergoing cytotoxic cancer therapies.

In summary, the findings discussed in Chapters II through IV illustrate the dynamic and responsive nature of stromal cells of the bone marrow microenvironment to physiologic stimuli,

including chemotherapy induced stress. MSCs and osteoblasts react to cellular stress in ways that can impact the health and homeostasis of various tissues within our bodies which regulate necessary functions such as hematopoiesis and bone development. The supportive nature of MSCs to various processes *in vivo* have prompted their use in transplantation, with their inclusion needing to be further optimized prior to a broader implementation clinically.

Specifically, an increased mechanistic understanding of how the properties of MSCs are altered during *in vitro* expansion needs to be obtained, for the practical purpose of efficient cell generation without negatively altering supportive MSC properties. Furthermore, MSCs have been shown to regulate the activity of malignant cells *in vivo*, a process that could be targeted pharmacologically. This process requires further investigation in various contexts, such as during chemotherapy treatment. Such an understanding could lead to new approaches to treat tumors, initiated by a hypothesis regarding the contribution of stromal cells to regulating tumor phenotype following chemotherapy treatment. More studies need to be performed to understand how chemotherapy induced stress alters the ability of bone marrow stromal cells to perform their homeostatic functions, and how these events can be modulated in a clinical setting.

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