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Sphingosine 1-phosphate Receptor Signaling in Mesenchymal Stromal Cells

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By

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies

Departments of Microbiology and Immunology

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List of Abbreviations (in order of appearance):

MSC: Mesenchymal stromal cell

S1P: Sphingosine-1 phosphate

S1PR: Sphingosine-1 phosphate receptor

CD: Cluster of differentiation

Runx2: Runt related transcriptiona factor 2

BMP2: Bone morphogenic protein 2

CAF: Carcinoma associated fibroblasts

PIN: Prostate intraepithelial neoplasia

CSC: Cancer stem cell

PCa: Prostate Cancer

MAPK: Mitogen activated protein kinase

Spns2: Spinster-2

ABC: ATP binding cassette

Edg1: Endothelial differentiation gene 1

NK: Natural Killer

ESC: Embryonic stem cells

HSC: hematopoietic stem cells

ALDH: aldehyde dehydrogenase

Acknowledgments

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Abstract

Mesenchymal stromal cells (MSCs) are a multipotent cell population acquired most prominently from bone marrow with the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, cardiomyoctes, fibroblasts and other cell types. The immunoprivileged nature of these cells combined with their ability to home to sites of injury enhances therapeutic interest in this stem cell population. Phase I-III clinical trials are being conducted evaluating the therapeutic potential of MSCs in graft vs. host disease, following acute myocardial infarction, multiple sclerosis, and bone and cartilage diseases. Sphingosine 1-phosphate (S1P) is a biologically active sphingolipid impacting proliferation, apoptosis, inflammation, and angiogenesis. Interactions with 5 G-protein coupled S1P receptors (S1PR1-5) mediate in part these functions. Whereas S1PR1-R3 are ubiquitously expressed, S1PR4 and S1PR5 have more limited expression. This project seeks to assess the role of the S1PRs in the maintenance of a multipotent MSC population and the impact of modulation of S1PR2 on the progression of prostate cancer. Inhibition of S1PR2 results in increased MSC clonogenicity, migration, and proliferation. The increased Erk phosphorylation observed with S1PR2 inhibition is required for these increases in migration and proliferation. Furthermore, decreased S1PR2 expression decreases the differentiation of MSCs into adipocytes and mature osteoblasts that may be the result of increased expression of MSC pluripotency factors including Nanog, Sox9, and Oct4. Inhibition of S1PR1 and S1PR3 in contrast does not impact MSC migration or Erk activation although increased proliferation is observed. In the study, we describe the essential role of S1PR2 in MSC differentiation pathways through modification of pluripotency factors. We propose a MAPK dependent mechanism through S1PR2 inhibition that promotes equally pluripotent MSC proliferation in a way that can be exploited for better ex vivo MSC expansion in autologous MSC transplant. When MSCs are co-cultured with murine prostate cancer cells, an increased stem cell population is observed with greater proliferation of cancer cells following inhibition of S1PR2. We therefore proposed that S1PR2 in MSCs within the tumor microenvironment enhances the metastatic potential of tumors.

INTRODUCTION

MSC History

Mesenchymal stromal cells (MSCs) were first identified and preliminarily characterized by Friedenstein et al in 1966. The subset of the stromal bone marrow fraction they identified was an adherent cell population capable both of self-renewal in vitro and differentiation into an osteogenic phenotype. Originally termed osteogenic stem cells, the cell population was described as having a spindle-like or fibroblast like appearance ranging in size from 10-30 μM (1-6). At higher passages, when the MSCs approach senescence, MSCs appear increasingly flatter and multinucleated (7). The onset of senescence in MSCs with culture in vitro due to limitations in the cell culture environment emphasizes the need to improve ex vivo expansion for clinical usage and the necessity of using MSCs at a low passage number for in vitro experimental evaluation.

Further work published in 1987, expanded on this initial characterization of MSCs citing their capacity for colony formation and chondrogenic differentiation in addition to the already recognized osteogenic differentiation potential. In 1991, Caplan et al. coined the term mesenchymal stem cells that has persisted through subsequent literature despite the ongoing debate on whether mesenchymal stem

cells are true stem cells or stromal progenitor cells (8, 9). Throughout the literature the terms mesenchymal stromal cells and bone marrow derived stromal cells have been used to represent an MSC population although careful attention must be paid to the isolation and characterization techniques employed.

The heterogeneous nature of MSCs and the frequent contamination of adherent cell cultures with macrophages and adherent hematopoietic cells prompted further investigation on proper identification of MSCS. Positive and negative surface marker expression, colony forming capacity, and multi-lineage differentiation therefore became essential to the proper characterization of MSCs. Kasseme et al. identified CD44, CD63, CD105, and CD146 as positive markers of MSCs in 1993. Pittenger et al. further expanded the gold standard for differentiation of MSCs by describing the adipogenic capacity of MSCs (1). Despite ongoing work seeking to better characterize and define a uniform MSC population, the cell of origin for MSCs remains in question. Many reports have proposed a neural cell origin for MSCs deriving either from the neuroepithelium or the neural crest, although other reports suggest a hematopoietic stem cell origin for MSCs (10-16).

Stems cells are broadly defined as an undifferentiated cell type capable of self-renewal and further cell differentiation (17, 18). Despite increasing consensus that MSCs can be considered a stem cell, some argument remains as to whether they should be called mesenchymal stromal cells or skeletal stem cells. Agreement is

higher with regard to the term skeletal stem cells as MSCs have been shown to form heterotopic bone in mice in serial transplantation assays. Sacchetti et al. conducted the first in vivo serial transplantation assays evaluating bone formation in 2007(19). In vivo differentiation following serial transplantation has not been demonstrated for adipogenic and chondrogenic lineages. The basis for the stem cell controversy is based on the heterogeneous nature of MSCs and the lack of a single definitive marker for the population. The lack of additional in vivo transplantation assays demonstrating ectodermal, endodermal, and mesodermal tissue formation adds to this controversy (20). Bianco et al. additionally argue that MSCs unlike other stem cell populations have functions beyond solely serving as a progenitor population and that MSCs from different organ sources produce cells with varying differentiation capacity. MSCs have been shown to function in vascular niche organization, peripheral injury repair, and immune modulation (11, 20, 21). MSCs have clear documented roles as progenitor cells for a number of different populations both in the bone marrow and in the periphery with critical physiological functions in these environments.

Differences between human and murine MSC populations in isolation procedures and markers have been identified. Murine MSC populations are without proper sorting or enrichment often contaminated by hematopoietic progenitors capable of plastic adherence. Murine strains display marker specific differences and differ in their optimized media for growth (22). Recently, Stro-1 has further been identified

as a strong candidate for a single positive marker of human MSCs with Stro-1 expression critical to colony formation in human MSCs. This marker is unfortunately not expressed in murine cell populations (21, 23, 24). Although differences exist between human and murine MSC populations, murine MSCs have demonstrated consistent behavior with human MSCs leading to significant contributions to understanding human MSCs both physiologically and therapeutically. Many of these discoveries are further expanded upon later in this introduction.

As a consequence of different operating definitions used for MSCs in publications, the International Society for Cell Therapy convened in 2006 to establish a consensus definition of MSCs to be used in future literature (25). Four factors were established as critical to defining an MSC population including colony formation, tri-lineage differentiation, and a specific marker expression. Positive markers for MSC expression reaching consensus included CD73, CD90, and CD105 in conjunction with the absence of hematopoietic markers CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA DR (17, 18). The establishment of a consensus definition for MSCs greatly advanced the field and created a uniform framework for comparison and replication of MSC research.

MSCs can be isolated from adipose tissue, peripheral blood, cord blood, umbilical cord tissue, muscle, pancreas, fetal tissue, heart, bone, and skin. Bone marrow isolated MSCs are the most common source of MSCs due to the lack of surgical

intervention required for harvesting (26). Furthermore, bone marrow-derived MSCs have the highest concentration of MSCs per number of cells composing only 0.01-0.0001% of bone marrow cells (17). Bone marrow and spleen derived MSCs in mice demonstrate the greatest capacity for differentiation as compared to those isolated from alternate sources (27). The incidence of MSCs decreases with increasing age. A ten-fold decrease in MSC incidence is observed between birth and the teenage years. A second ten-fold decrease is observed between the teenage years and late adulthood. When considerations for autologous as compared to allogeneic stem cell transplant are given, the age of the cell donor remains an important consideration (28, 29).

Consistent with their stem cell nature, MSCs express many transcription factor markers common with embryonic stem cells (ESCs) and other adult stem cell populations. These markers are often used as indicators of the undifferentiated status of the cells. Pluripotency factors described for MSCs include Nanog, Oct-4, SSEA-4, and Sox-2. First identified in 2002 in ESCs, Nanog overexpression maintains ESC self-renewal in part through regulation of Id1 expression (17). Oct-4, a second pluripotency factor, functions in a complex with Sox2 on the DNA. The two factors in concert program a transcriptional network of genes promoting pluripotency. Rex-1 can also function as stem cell factor that is regulated by expression of Nanog, Oct4, and Sox2 (30). Pluripotency factors as in other stem cell populations represent a

critical avenue of investigation in MSC research in determining the balance between self-renewal and differentiation.

MSC Function

MSCs function both in the bone marrow and the periphery through cell-cell interactions, differentiation, and autocrine and paracrine mechanisms. In the bone marrow, MSCs maintain the bone marrow microenvironment and in particular in the maintenance of the hematopoietic stem cell compartment (28). MSCs reside in the low oxygen regions of the bone marrow and are especially prevalent lining the bone and serving as osteoblastic precursors (31, 32). It is therefore not surprising that MSCs maintained ex vivo under hypoxic conditions comparable to that of the bone marrow display increased stem cell characteristics, improved self-renewal, and delayed senescence. The role of MSCs in the periphery differs from that of the bone marrow with the bone marrow cells having the unique capacity to home to sites of injury and contribute to repair. Although MSCs can differentiate at the site to which they are recruited, differentiation only occurs to a limited capacity (28, 33-36). MSC secretion of cytokines and chemokines contributes to local tissue repair and recruits other cell types responsible for tissue repair. The role of tissue residing MSCs has not yet been actively investigated in peripheral tissue repair (28).

MSC contribution to tissue repair is especially important in the context of chronic wounds, especially those resulting from diabetes, trauma, or vascular insufficiency (37). Depending on the stage of wound healing, the MSC contribution to healing changes based on the requirements of this stage of repair. During the initial inflammatory phase MSCs mitigate the inflammatory reaction by secretion of TNF and decreased T cell proliferation. In the secondary proliferative phase of wound healing, MSC secretion of VEGF, HGF, and FGF mediates cell recruitment of the appropriate progenitor cells and fibroblasts to repopulate the region. Finally, in the remodeling phase, MSCs regulate collagen and ECM deposition through TBFβ and MMPs/TIMPS (37). MSCs have been readily identified in the perivascular region of peripheral tissues often located ensheathing the vasculature. MSCs are a critical component to wound healing in both acute and chronic injuries.

MSC immune function

MSCs are unique in their immunoprivileged nature making their clinical use highly attractive due to their potential for allogeneic transplantation. The decreased immune recognition is mediated in part by their low MHC I expression and lack of MHC II expression. MSCs further lack expression of co-stimulatory molecules including CD40, CD80, and CD86. Furthermore, MSCs produce cytokines that downregulate immune and inflammatory responses (1, 38). Secreted factors released from MSCs that may contribute to this reduced inflammatory response

include Nitric oxide, indolamine 2,3 dioxygenase, and heme oxygenase, prostaglandin E2 and IL-10 (28, 39). Beyond their capacity to evade immune surveillance, MSCs exert critical alterations in the immune system.

MSCs impact immune function through interactions with both the innate immune system and the adaptive immune system. Within the innate immune system MSCs can inhibit the proliferation and function of NK cells. MSCs further hinder macrophage and dendritic cell proliferation while simultaneously enhancing the tolerance of dendridic cells (40). Additional interactions between MSCs and the innate immune system include research investigating MSC functions in complement, toll like receptor signaling, neutrophil function, and mast cell function.

Within the adaptive immune system, MSCs can shift the balance of T cell differentiation promoting increased Th2 response rather than a Th1 response. MSCs can additionally increase Th17 and Treg differentiation. Decreased T cell proliferation is observed following MSC secretion of IDO and PGE2. MSC modulation of B cell proliferation and function has been observed (41, 42). MSCs are recruited to sites of injury by the action of CXCL12, CXCL10, CXCL9, and CCL2. MSCs interact with many aspects of the immune system under the appropriate conditions and ongoing research seeks to analyze the individual interactions with different immune mediators given that both MSC secretory factors and direct cell interactions are involved pending on the interaction being characterized.

MSC Self-renewal

Despite ongoing research seeking to understand the processes that govern MSC self-renewal, many of these factors remain poorly understand. The pluripotency factors expressed by MSCs have been the best-characterized factors associated with the promotion of MSC self renewal. Among these factors, the most extensive research that has been conducted has investigated the role of Nanog in MSC self-renewal. Nanog expression diminishes in differentiating cells and whereas it is highly expressed in proliferating ones. Nanog has been shown in MSCs to increase expression of cell cycle genes, promote DNA replication, increase DNA damage repair, improve proliferation capacity, increase clonogenicity, and improve differentiation potential overcoming many of the effects of aging on MSCS (43). One downstream target of Nanog thought to contribute to these changes is Dnmt1, activation of which leads to changes in DNA methylation resulting in modification of cell cycle proteins including p16 and p21 (44). Some reports indicate that Nanog expression is induced by the in vitro culture environment and is not expressed in freshly isolated cells (45). Although one of the best-characterized transcription factors impacting self-renewal, Nanog does not function independently and cooperates in a network of pluripotency factors.

Oct-4 and Sox2 are two additional pluripotency factors initially identified in ESCs have also been identified as critical factors in MSCs self-renewal. Oct4 directly inhibits bmp4 leading to decreased ectodermal and endodermal differentiation. Oct-4 knockout MSCs, unlike Nanog and Sox2 knockout cells, are still capable of differentiation and colony formation but knockout cells express decreased levels of both Nanog and Sox2 (46). Additional factors have been identified as functioning in the self-renewal pathways of MSCs. These pathways include leukemia inhibitory factor (LIF), fibroblast growth factor 2, and the Wnt signaling pathway (21). These are putative factors and more work needs to be done to further characterize their contribution.

Environmental conditions can additionally impact the potential for MSC self-renewal and differentiation. One such environmental factor thought to contribute to MSC self-renewal is the culturing of MSCs under low oxygen conditions. Hypoxia can mimic the 1-7% O_2 conditions characteristic of the bone marrow niche in which MSCs reside. Decreased ex vivo expansion time and increased populations of Sca-1+ CD44+ cells have been observed under 1% hypoxic conditions as compared to cells cultured under standard cell culture conditions (47, 48). Decreased adipogenic differentiation has been observed under hypoxia that is reversible with the restoration of oxygen rich environment (49). Further exploration on the role of hypoxia in MSC function and ex vivo expansion has significant potential to impact clinical use of MSCs.

MSC differentiation

Although classified based on their tri-lineage differentiation into osteocytes, chondrocytes, and adipocytes, MSC are increasingly being reported to differentiate into a number of different cell types within the mesenchymal lineage as well as transdifferentiation into endodermal and ectodermal lineages. These cell types include but are not limited to myocytes, endothelial cells, cardiomyocytes, ectoderm, smooth muscle cells, neuronal cells, and tumor associated fibroblasts. Diminished cell differentiation has been observed with an increasing number of passages and increased MSC senescence. Twenty to forty population doublings have been reported prior to expression of senescence markers (26).

Osteogenic differentiation is controlled by its master regulator Runx2 (Runt related transcription factor 2;CBFA1). Runx2 has two known isoforms and can be repressed under hypoxic conditions by Twist following HIF-1α induction. Studies in murine MSCs using siRNA and overexpression systems have demonstrated that Runx2 is both necessary and sufficient for osteogenic differentiation (50-52). The critical function of Runx2 in osteogenic differentiation is further supported by the embryonic lethality of Runx2 knock out mice, which die at birth due to respiratory failure and lack of calcified bone. Transcription factors that modify Runx2 in MSCs include B-catenin, Msx2, and Dlx5. Runx programs this osteogenic downstream

signaling pathway through activation of alkaline phosphatase, bone sialoprotein, collagen type I alpha I, osteopontin, and osteocalcin (51). Osterix, a transcription factor impacted by Runx2 function, can be activated by MAPK to promote increased MSC osteogenic differentiation.(53). A constellation of factors primarily controlled by Runx2 but with contributions from a number of other factors influence MSC differentiation.

Expression of Runx2 results in a repression of the critical adipogenic differentiation regulator PPARγ (Peroxisome proliferation activated receptor gamma). PPARγ is sufficient for adipogenic differentiation with C/EBPalpha also functioning as a critical adipogenic factor by binding the PPAR-gamma promoter. Chondrocyte differentiation is mediated by Sox9 and the least is known about this differentiation pathway. (Sry box containing gene 9) (30)(31, 32). Sox 9 is a critical transcription factor promoting expression of and acting synergistically with bone morphogenic protein 2 (BMP-2). This functions both in the promotion of chondrogenic differentiation and in the inhibition of osteogenic differentiation. MSC differentiation is controlled by a set of pluripotency genes as well as pathway specific factors. Given the information currently understood about MSC self-renewal and differentiation and the balance of these factors, it is reasonable to determine the factors that promoted MSC maintenance. Maintenance is defined as the capacity for MSCs to proliferate in an equipotent fashion with comparable expression of pluripotency factors and differentiation capacity.

MSC Therapy

MSCs have been explored as therapy for a variety of acute and chronic disease conditions. MSCs are an attractive stem cell population due to their ease of isolation, ability to modulate the immune response, and diminished immune recognition. However, the clinical use of MSCs is limited by the number of cells that can be acquired both due to their low concentration in the bone marrow and their limited ex vivo proliferation and differentiation capacity. Reports have indicated that only approximately 2000 MSCs can be obtained from a human bone marrow sample (30). Most therapies have used bone marrow isolated MSCs as other MSC sources, such as from the spleen or adipose tissue, require surgical intervention for isolation. MSCs can be delivered either systemically by IV treatment or locally in the interstitium or vasculature. When delivered systemically MSCs were found to prominently accumulate in the lungs and spleen in the first 72 hours, prior to clearance of non-engrafted cells (26). Mechanisms for improved disease control include MSC differentiation at the site of injury and secretion of soluble factors essential for the recruitment of cell populations required for healing or that themselves promote the healing process (36, 54-56). Although limited differentiation and engraftment occurs, secreted factors have been identified as primarily responsible for the enhanced tissue regeneration. In one model, it was estimated that 99% of transplanted cells died within 4 days of delivery (57). Clinical evaluation of both

allogeneic and autologous MSC transplant has demonstrated some promising initial results with ongoing Phase I-III clinical trials in the treatment of graft-versus-host disease, Crohn's disease, multiple sclerosis, Type I diabetes, chronic obstructive pulmonary disease, bone conditions, arthritis, autoimmune conditions, and following myocardial infarction or solid organ transplant (10, 11, 30, 58-61).

In four clinical trials for heart disease, no side effects or toxicity were reported with improved cardiac function. Safety concerns associated with MSC therapy include the risk of increased arrhythmias when used for cardiac disease and tumor formation. At present, there is no evidence of these toxicities with MSC treatment in clinical trial patients, however observation is ongoing. MSCs have been shown to have the potential for spontaneous in vitro malignant transformation and this is the reason identified for the neoplastic safety concerns (62). MSCs have shown initial promise in clinical trials although technical difficulties need to be overcome in delivery and retention.

MSCs and Cancer

The tumor microenvironment is composed of a number of different factors including cancer cells, carcinoma associated fibroblasts (CAF), vasculature, smooth muscle cells, adipocytes, immune cells, and MSCs (63, 64). MSCs impacting the tumor microenvironment are derived both from tissue resident MSCs and through

bone marrow derived MSCs. MSCs have been shown to home to breast, lung, pancreatic, colon, ovarian, prostatic, and melanoma tumors (65). MSCs are recruited to tumors by TGF-ss1, IL-8, neurotropin 3, EGF, HGF, bFGF, PDGF, MCP-1, CXCL12/CCR4, VEGF, and SCK/ckit (1). MSCs within tumors increased cancer cell proliferation, motility, invasion, metastasis, angiogenesis, and desmoplasia. MSCs have also been associated with decreased tumor immune recognition. These effects are thought to be achieved through several proposed mechanisms including paracrine factors, proangiogenic cell recruitment, MSC differentiation into CAFs, increased metastatic potential, chemotherapy resistance, and immunomodulation. Tumors containing MSCs have an increased cancer stem cell content and a greater tumor initiating capacity (66).

The paracrine factors secreted by MSCs serve both to influence the local tumor microenvironment and to recruit cells for the tumor associated stroma (64). MSC secretion in co-culture with tumor cells upregulates gene clusters associated with metastasis, proliferation, and chemoresistance (67). In the bone marrow, MSCs can attract tumor cells creating a metastatic niche for tumor cells and encouraging osteolysis and drug resistance (34). A diagram outlining the mechanism of tumor promotion by MSCs is shown in Figure 1 indicating the signaling pathways and mechanisms involved in MSC influence on tumor vasculature and tumor cells survival as the metastatic potential of the cells the downregulation of immune components is also addressed.

In some cases, although much less frequently reported , MSCs recruited to tumors can inhibit tumor progression. This effect may be dependent on the time at which MSCs are delivered in cancer development. The mechanism for tumor inhibition has been proposed as a diminished immune reaction. This effect has been reported in selected publications for leukemia, melanoma, and breast cancer (62, 68, 69).

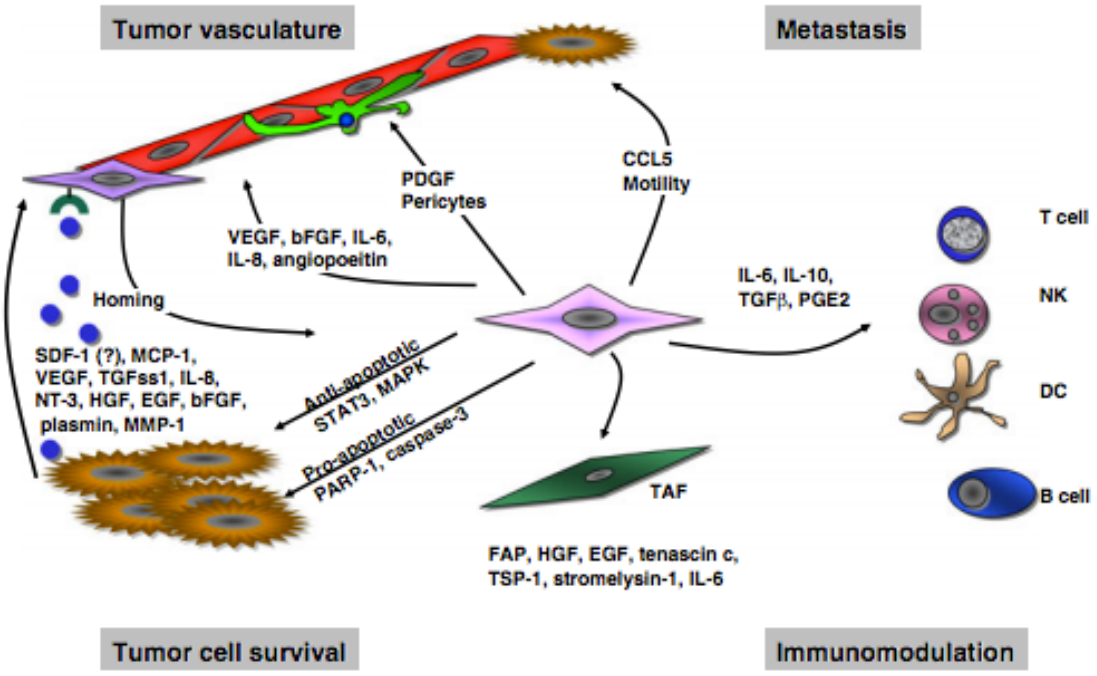


Figure 1: Role of MSCs in the tumor microenvironment (Bergfield et al. 2010): MSCs impacts tumor growth and protection through their multi-faceted interaction with the tumor microenvironment. These effects include interactions with the tumor vasculature, tumor cells themselves, and the immune system by causing immunomodulation through interactions between both the innate and adaptive immune system.. These mechanisms are further reviewed in the article by Bergfield et al. 2010.

CAFs express their pro-tumorigenic effect through a number of mechanisms. The first of these is secretion of CXCL12/SDF-1 secretion and expression that results in

the recruitment of pro-inflammatory cells to the tumor microenvironment and increases CCL5 expression that promotes the metastatic ability of the cancer. Additionally CAFs secrete a number of additional growth factors including EGF, TGFβ, and HGF. CAFs are frequently defined by their expression of alpha smooth muscle actin, fibroblast activating protein, FSP1, NG2, and PDGF beta (70). Many of these CAFs are though to derive from bone marrow precursors with one report indicating that 20% of CAFS are derived from bone marrow MSCs in the gastric tumor microenvironment (71). Once differentiated, these MSC derived CAFs can secrete factors that recruit additional MSCs to the tumor microenvironment. Beyond their role in differentiating into CAFs or their paracrine effects MSCs have been identified as the tumor-initiating cell in Ewings Sarcoma and osteosarcoma (63) (5, 6, 65).

Cancer therapies have been proposed and tested with potent anti-tumor effects include using MSCs as a vector for delivery of gene therapy including interferon beta, cytosine deaminase, and TRAIL (72-76). The timing of MSC introduction to the tumor may be the critical factor determining the impact of MSCs on the tumor microenvironment with other differences attributed to the tumor model and tumor heterogeneity (3-6). The source of MSCs used for delivery does not appear to impact this. Much of this relates to MSC recruitment to wounds and tumors acting as an inflammatory wound that never heals (77-79). MSCs have been shown to promoted tumor growth in vivo in colon cancer, lymphoma, and melanoma (4, 80).

Prostate Cancer

Prostate cancer is a cancer with a high incidence in older men with variable prognosis depending on the local or metastatic state of the disease has a significant. Prostate cancer is diagnosed more frequently with increasing age. Forty percent of men over the age of 50 are diagnosed with prostate cancer and among this population the disease only a small percentage have prostate cancer as their cause of mortality. In 2012, there were 241,740 new cases of prostate cancer with 28,170 deaths (American Cancer Society). High grade prostatic intraepithelial neoplasia (PIN) characterized by cell proliferations within the ducts and acini of the prostate gland with prominent nuclear and nucleolar enlargement without disruption of the basal cell layer (81, 82). Prostate cancer develops over the course of decades moving from normal prostate tissue to a precancerous histology call prostatic intraepithelial neoplasia (PIN) before progressing to prostate cancer and metastatic prostate cancer. Current evidence does not suggest that either low grade PIN and adenomas do not progress to prostatic adenocarcinomas. Common sites of metastasis for prostate cancer include the bone, lungs, and liver. Although prostate cancer has been studied for many decades, the cell of origin for prostate cancer remains under debate and both basal cell and luminal cells of the prostate remain potential sources of malignant transformation. Treatment includes radical prostatectomy, radiation, or watchful waiting for local disease and androgen

ablation therapy for more advanced disease. Androgen ablation long term often results in resistance to therapy commonly starting after 2 years of treatment (83).

Although prostate cancer has a very good prognosis for localized disease, the prognosis is substantially worse for metastatic or disseminated disease with only 33% five-year survival. Identification of markers or mechanisms of tumor progression are therefore critical to understanding differences between localized and metastatic disease (83). As MSCs have a role in cancer progression, invasiveness, and metastasis, understanding the role of MSCs within the prostate cancer microenvironment could be critically important in understanding the transition from localized to metastatic disease.

MSC and prostate cancer

Comparable to the role of MSCs in other cancer settings, MSCs home to prostate cancer tumors and increase tumor invasion and metastasis (84-88). This effect is accomplished through a number of different mechanisms. Recent work has identified the PCa-CxCl16 ligand and its interaction with Sxcr6 as facilitating MSC recruitment to prostate tumors (89, 90). Following MSC recruitment, increases in the PCa stem cell population were observed. This increase in the stem cell population is associated with increased CCL5, decreased androgen receptor nuclear translocation, and increased metastatic gene expression and metastasis in vivo (91).

These effects are accomplished in part by MSC secretion of Il-6, Il-10 and increased MMP expression in the tumor microenvironment (33, 34).

Prostate cancer stem cells

Somatic stem cells are a heterogeneous population of self-renewing stem cells (89). Cancer stem cells (CSCs) are similar in that they have similar phenotypes and functional heterogeneity. Comparable to other stem cell populations, cancer stem cells have been characterized as mostly quiescent cells. CSCs are thought to be responsible for relapse following therapy although they may not be the cell of origin for original tumor (92). Two ways to trace stem cell populations are either by marker expression or lineage tracing and serial transplantation assays (93). Cancer stem cells differ from typical stem cells in that they lack the multi-lineage differentiation that embryonic stem cells possess. In PCa, markers of prostate cancer stem cells include CD44+, α2β1 integrin+, CD166+, Tra-1-60+, CD151+, and Aldh+. These markers were selected in part based on the high clonogenicity of the CD44+ α2β1 integrin+, CD133+ population and the high tumorigenicity of the aldh+CD44+alpha2b1+ cells that compose 0.1% of the tumor population of human prostate cancer (93-95). Prostate cancer stem cells are a critical avenue of investigation to better understand disease progression and to develop curative therapies.

Erk/MAPK Signaling/DUSP-1

Mitogen activated protein kinases (MAPKs) are a family of serine/threonine kinases that mediate signal transduction through modification of nuclear transcription factors thereby connecting the plasma membrane and the extracellular environment to nuclear and cytoplasmic events (96). MAPKs are involved in proliferation, differentiation, migration and apoptosis (97). The MAPKs are composed on Erk, JNK, and p38 proteins that differ in their activation by a dual specificity Threonine-x-tyrosine tripeptide motif. The Erk family members are best characterized for responses to growth factor stimulation whereas p38 and JNK proteins are involved more in cell stress response pathways. JNK has been shown to be upregulated following UV radiation, growth factor deprivation, and DNA damage whereas p38 is upregulated following environment stress and inflammatory cytokines (98). MAPKs are activated by MAPK kinases that are in turn activated by MAPK kinase kinases. For example, Erk1/2 a MAPK, is activated by Mek 1/2, MAPK kinases, which are activated by three Raf isoforms, MAPK kinase kinases. This pathway is frequently downstream of Ras-GTPase signaling (97).

Erk 1/2 proteins constitute one class of mitogen activated protein kinases (MAPK) with signaling downstream of Ras. Erk 1/2 also known as p44 and p42 MAPK were first identified in 1989 with 83% sequence homology between the two isoforms (97). It can be phosphorylated at two sites with dual specificity protein kinases

(Mek ½) responsible for complete activation of Erk. Over 200 downstream signaling substrates of Erk have been identified with Erk activation promoting proliferation, differentiation, survival, apoptosis, and stress response (99-101). Commonly investigated transcription factors regulated by Erk include Elk-1, c-fos, and c-jun. Regulation of Erk has been described through a number of mechanisms including duration and intensity of activation, scaffold interactions, compartmentalization, and substrate availability (100). Erk activation can be mediated by GαI and Gαq with deficiency of Gα12 and Gα13 GTPase resulting in increased focal formation in 3T3 cells (102).

Deactivation of MAPKs is mediated by the MAPK phosphatases or MKPs with the best-described family member being MKP-1/DUSP-1. MAPK phostphastase (MKP-1), first isolated twenty years ago, is phosphorylated following Erk activation to protect the protein from proteosomal degradation (102). MKP-1 can dephosphorylate all 3 classes of MAPKs with knockout mice demonstrating a normal phenotype and increased innate immune responses (103, 104). Sphingosine 1-phosphate (S1P) has been shown to activate all 3 MAPK, but has also been shown to activate MKP-1 on an mRNA and protein level by a p38 dependent mechanism. S1P stimulation therefore simultaneously upregulates the pathway and its negative feedback (105). Given the pleiotropic effects of S1P stimulation on cells, the differential activation of different MAPK pathways is therefore important with each cell type response influenced by its receptor composition.

Sphingosine 1-phosphate

Sphingolipids are a class of membrane lipids providing critical structural roles in the lipid bilayer and impacting membrane fluidity (106). Originally named by J.L.W. Thudichum for their "enigmatic" nature in 1884, the structural role of these lipids was identified before the prominent signaling pathways were identified. Sphingolipids are amphipathic molecules composed of a hydrophobic sphingoid long chain base combined with a fatty acid with a hydrophilic head group in some classes. Sphingolipds levels are regulated on several tiers by control of enzyme expression, subcellular location, post translational modification, and allosteric isomers (107). S1P was first identified by Sarah Spiegel in the early 1990's (108). S1P is synthesized from ceramide by the actions of ceramidases and sphingosine kinases. Ceramide is the central molecule in sphingolipid signaling promoting general anti-proliferative functions with growth inhibition, apoptosis, differentiation, and senescence.

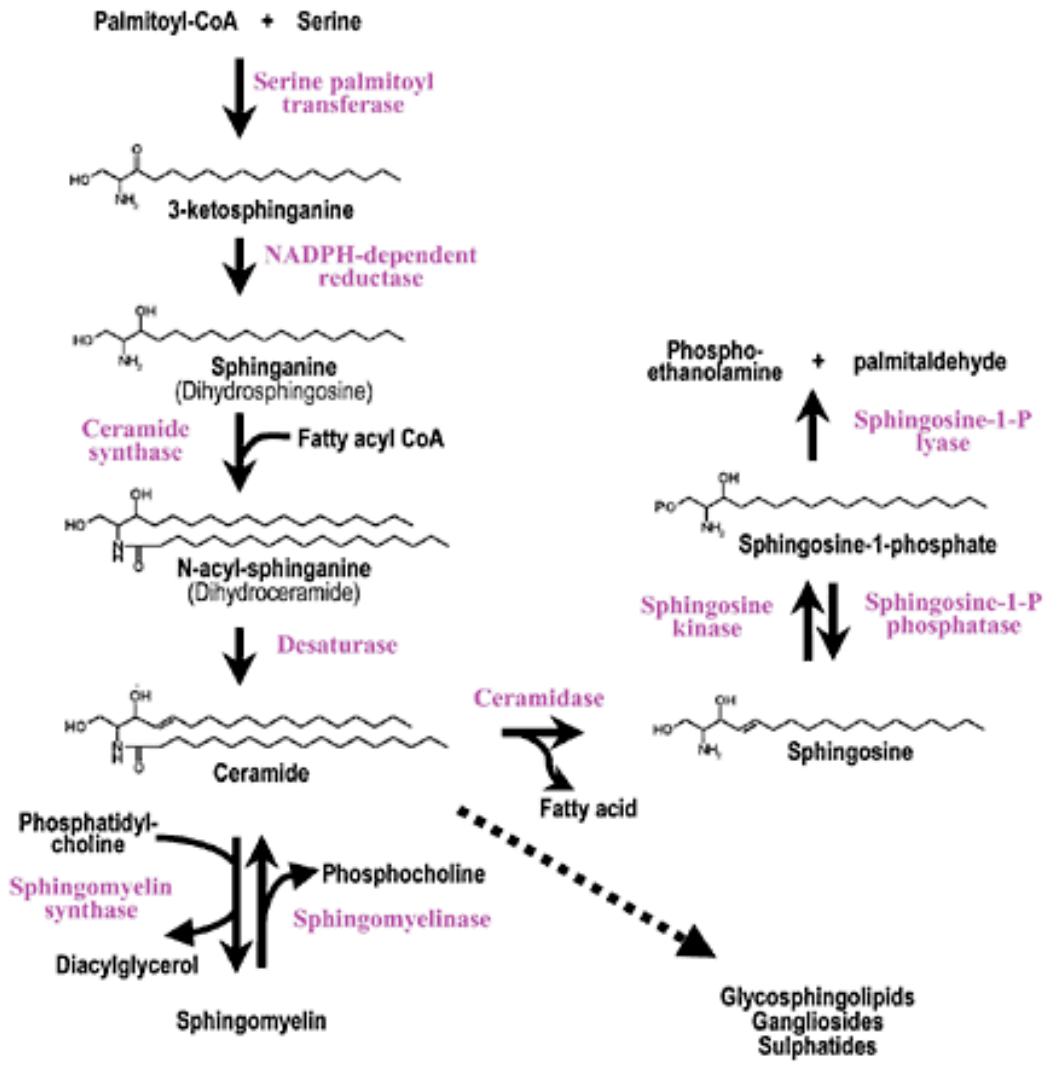


Figure 2: Diagram of Sphingolipid metabolism (Waeber et al. 2004): The central molecule of sphingolipid metabolism ceramide. Ceramide can be synthesized from a number of pathways including de novo synthesis of ceramide from palmitoyl CoA and Serine. It can additionally be formed from the salvage pathway from sphingomyelin. Once formed ceramide can be converted to sphingosine and sphingosine 1-phosphate by the action of ceramidases and sphingosine kinases. Sphingosine kinase can be further irreversible broken down by S1P lyase.

Ceramide is formed from three pathways including the de novo pathway, sphingomyelin hydrolysis, and cerebrosides. Once formed ceramide can generate S1P reversibly through the activity of ceramidases and sphingosine kinases. Both of these pathways are shown in Figure 2 above showing sphingolipid metabolism and the structure of the lipids involved. Lipids at each level in this pathway are highly interconnected as small changes in sphingomyelin result in large changes in ceramide due to the ten-fold decrease in basal concentrations. Further downstream of this metabolic pathway, 3-10% increases in ceramide result in a doubling of sphingosine and 1-3% increases in sphingosine result in a doubling of S1P concentrations. At each enzymatic level, the concentration of each lipid is highly regulated in its synthesis and degradation because small perturbations can have significant impact on the signaling of downstream molecules (109).

At the other side of this ceramide/S1P rheostat, S1P provides proliferative, inflammatory, vasculogenic, apoptotic resistant, and motility functions. It mediates these actions primarily through autocrine and paracrine functions although intracellular S1P signaling is documented (42, 106, 110, 111). S1P is composed of a polar head group and hydrophobic tail as shown in Figure 2 (112). Regulation of S1P signaling can be mediated by changes in expression and the subcellular localization of the lipids composing this pathway (106). S1P can be reversibly degraded back to sphingosine by phosphatases or irreversibly degraded by S1P lyase.

Following synthesis S1P is secreted from the cell to provide an S1P source for paracrine and autocrine signaling through membrane G-protein coupled receptors. Spinster-2 (Spns2) is the only true transporter of S1P with a unique capacity to transport FTY720. Mice deficient in Spinster-2 expression have 50% less extracellular S1P (113). Additional transportation of S1P is through the ATP binding cassettes (ABC) transporters including ABCC1, ABCA, and ABCG2. Intracellular S1P signaling is mediated through inhibition of HDAC1 and 2, TRAF2 mediated stimulation of E3 lipase and R1P, and cytoskeleton regulation through NfkB regulated dynamics (114-116).

The S1P gradient between the plasma and lymph and tissue levels has a significant impact on physiological function especially with regard to the mobilization of circulating cells. Following injury such as cardiac and hepatic injury, it has been demonstrated that tissue levels and plasma levels rise following the initial insult. Plasma S1P levels are 0.1-0.6 μM S1P levels whereas tissue levels are in nM levels (115-117). The high level of S1P in the serum is provided by the platelet and erythrocyte production of S1P due to the absence of S1P degrading enzymes in these cells. Vascular endothelial cells also commonly secrete S1P. Due to the hydrophilic nature of the protein, S1P is primarily transported in the plasma through lipid carriers. Thirty-five percent of S1P in the plasma uses albumin as a transporter and 65 binds to ApoM in HDL (42). S1P is a critical lipid mediator of

migratory, vasculogenic, and inflammatory processes although the variability in these processes is explained by the simultaneous stimulation of S1P receptors with differing downstream signaling. Within the circulation S1P has a critical role in endothelial cell barrier function by promoting increased endothelial cell permeability (118).

S1P receptors

More prominent than the signaling of S1P through intracellular mechanisms is the signaling through 5 g-protein coupled receptors called S1PR1-S1PR5. Originally identified as Edg receptors (endothelial differentiation genes), this is one of the most heavily researched areas of S1P signaling. All of the receptors have similar kd values in response to S1P with the Kd, a dissociation constant reflecting the concentration at which S1P binds in the nM range (119). (Brock TG, Sphingolipids: The sphinx of lipids)

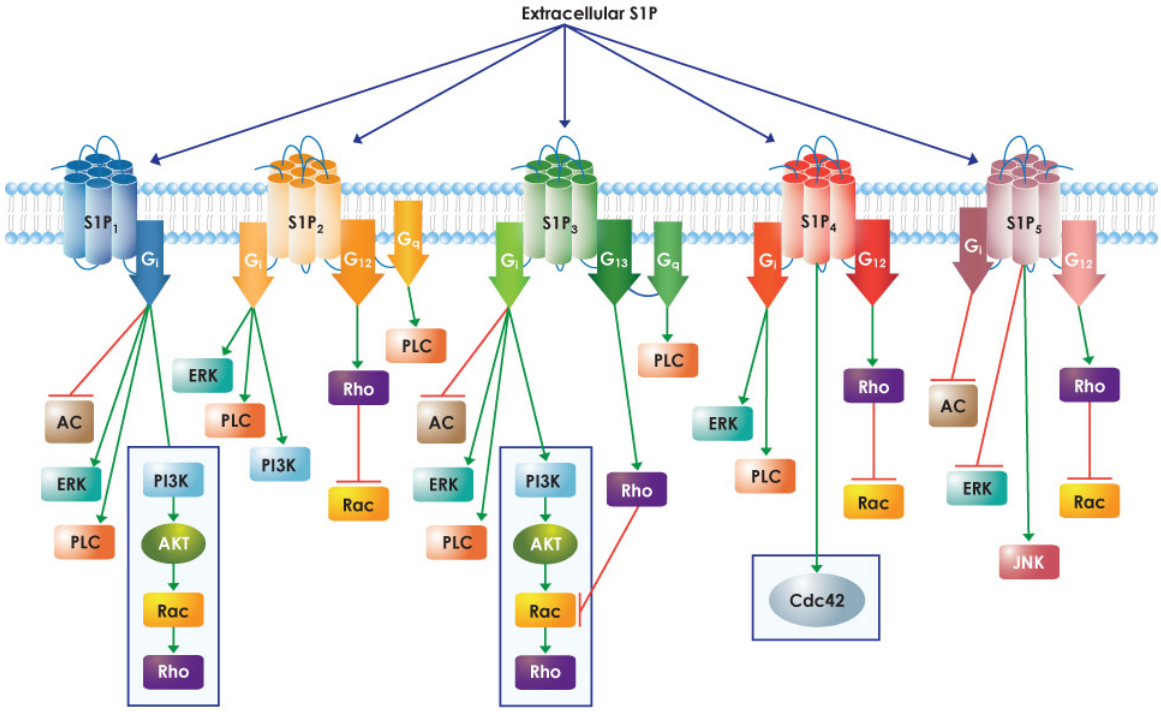


Figure 3: Diagram of S1P receptor signaling: S1P can signal through S1PR1-S1PR5. These receptors vary in their signaling through G-proteins and therefore in their down steam signaling. Different cell types have different expression levels of S1P receptors. Each S1PR has characteristic and unique downstream signaling pathway based on the G proteins and other proteins with which it interacts.

The receptors vary in their downstream signaling and in their coupling with GTPases. Whereas S1PR1 signals solely through Gi, S1PR2 and 3 signal through Gi, Gq, and G12/13. These receptors are ubiquitously expressed whereas S1PR4 and 5 have limited expression restricted primarily to immune and natural killer (NK) and neuronal expression respectively. S1PR4 and R5 couple with Gi and G12/13 (120). Each cell type has its own unique distribution of receptor expression thereby giving each cell a unique response to S1P stimulation.

S1PR1 is formerly called Edg1 (endothelial differentiation gene 1). S1PR1 signals through Gi with increased cell survival mediated by Akt activation and Ras-Erk activation (113). Knock out (KO) animals for S1PR1 die at embryonic day 12.5-14.5 by hemorrhage caused by deficient vascular development especially in the aorta (42). S1PR1 is required for endothelial cell vascular barrier functions by promoting the formation of adherens junctions (42). Another critical role for S1PR1 is in the immune system where the receptor regulates lymphocyte migration out of secondary lymphoid organs into blood and lymph counteracting the retention signals of CCR7. S1PR1 is also required for B cell trafficking between the follicle and marginal zone of the spleen by a CD69 dependent mechanism (108). S1PR1 is additionally involved in neutrophil and dendritic cell (DC) recruitment, activation, and migration (10-13). Decreased S1PR1 expression results in decreased tumor melanoma growth (113).

The immunologic role of S1PR1 in the regulating lymphocyte egress has been harnessed for treatment of multiple sclerosis and other autoimmune conditions. FTY720 (Gilenya) received FDA approval is a functional antagonist of S1PR1, R3, R4, and R5 although much of its function has been attributed to the role of S1PR1. This (42)is the basis for FTY720 treatment of MS and autoimmune conditions, where FTY720 is the first FDA approved oral therapy for MS (42). FTY720 treatment results in both lymphopenia and a decreased TH17 cell response dependent upon

S1PR1 receptor internalization (42). The immunosuppressive effect of FTY720 has put it as a prime candidate for FDA approval following solid organ transplant with a Phase III clinical trial currently in progress (108).

S1PR3 knockout mice have a normal phenotype with decreased litter sized (42). Most published studies involving S1PR3 function are tied to redundant functions with S1PR1 or S1PR2. Independent S1PR3 functions are in DCs where it is critical to switching the immune response to T helper cell expression (121). S1PR3 plays a critical role in sepsis where there are decreases in vascular resistance, and permeability (113). S1PR3 primarily has a role in B cell migration but most work has been conducted in vitro and additional in vivo characterization is required (42).

S1PR4 has a more limited expression than S1PR1, R2, and R3 with primary expression on lymphocytes. S1PR5 demonstrates the highest expression of neuronal cells and natural killer cells (NK). In NK cells, S1PR5 permits cell egress through inhibition of the CXCR4 dependent retention signals (122, 123).

S1PR2 has more complex signaling and functions than that of S1PR1 due to the multiple small GTPases that it is capable of interacting with. Signaling through Gi results in increased Erk activation and PI3K/Akt activation and downstream Rac activation. Gq primarily signals through Phospholipase C. G12/13 signals both through Rho and Adenylate cyclase. Rho is antagonized both Akt and Rac. Additional

S1P actions involve increased PLD, JNK, and P38 activation (117). Rho GTPases have a critical role in cytoskeletal signaling and actin stress fiber formation by coordinating assembly of paxillin and alpha-actinin (26). The preferential signaling of this receptor through G12/13 allows S1PR2 to function both in parallel with and antagonistic to the role of the other receptors. G12/13 activation promotes activation of Rho GTPase and VE Cadherin resulting in both a pro-apoptotic and prosurvival functions (117).

The study and characterization of S1PR2 knockout mice has greatly enhanced our knowledge about S1PR2 function physiologically and developmentally. Although these mice are viable, they have a number of defects consistent with proposed roles of S1PR2. As mentioned previously, S1PR2 knockout mice have an increased risk of seizures perinatally and are deaf perinatally due to defective cell migration. Female mice may have hidden reproductive defects that result in reduced litter size (120). Another tool used in the evaluation of S1PR2 signaling and functional roles is that of JTE013. JTE013 is a competitive antagonist of S1PR2 at an IC50 of 20 nM. Recent work has suggested that JTE013 treatment may impact other S1PRs at higher doses with changes in S1PR4 at higher concentrations (42).

In the vascular system, many of the antagonistic effects of S1PR2 to S1PR1 are observed. S1PR2 increases paracellular permeability by disruption of endothelial cell adherens junctions through the Rho/ROCK pathway. Decreased vascular tone

has been observed in the S1PR2 knockout mice (124). S1P increases the senescence of endothelial cells contributing to this function and increases neovascularization following hypoxic retinal damage (42, 113, 117). Within the innate immune system, mast cell degranulation requires S1PR2 (112, 113). S1PR2 is increased with kidney ischemia reperfusion injury and knockout mice protected promotes increased vascular permeability through VCAM/ICAM1 and increased angiogenic sprouting through RhoC and LARG (42). S1PR2 promotes mouse skeletal muscle regeneration (2). S1PR2 decreases chemotactic migration and increased membrane ruffling with S1PR1 and S1PR3 promote increased chemotactic migration (125). S1PR2 is involved in intracellular calcium release and muscle cell contraction (113). S1PR2 KO mice are protected from diabetes with decreased islet cell apoptosis, increased insulin release and decreased blood glucose. These mice further have a decreased fibrotic response following hepatic injury (126).

There is conflicting data on the role of S1PR2 in neoplasia with variations in the effect of S1PR2 inhibition depending on the type of cancer being evaluated. Anticancer effects of S1PR2 include decreased melanoma migration through RhoA and Rac, decreased lung metastasis, and decreased glioblastoma progression. B cell lymphomas occur in S1PR2 knockout mice with increasing age with over half of mice developing tumors by 1.5-2 years (126). A correlation has been observed between higher S1PR2 expression and increased breast cancer survival (114, 127,

128). To date, no studies have addressed the role of S1PR2 in MSCs and its impact on cancer promotion and development.

In contrast, S1PR2 promotes cancer in a variety of cancer types. In gliomas, S1PR2 increased tumor cell proliferation, but more importantly increased the invasiveness of the cancer by upregulating cell adhesion molecules CCN1/Cyr61 (129). In Hela cells, the ERM proteins (exrin radixin, moesin) that link actin with the plasma membrane and coordinate cytoskeletal rearrangement in response to EGF treatment activates the SK/S1P pathway through S1PR2 resulting in cell polarization, lammellipodia formation and increased cell invasion (130, 131). Increased tumor progression is observed in hepatoma models, neuroblastoma, melanoma, wilms tumor, bladder cancer and breast cancer (132-135). In chronic myelogenous leukemia increased drug resistance is observed by promoting the stabilization of bcr-able (113, 136-138).

The work on the role of S1PR2 in prostate cancer has been limited but a pro-cancerous role has been reported in the available literature. S1PR2 is part of a critical oncogenic pathway in prostate cancer through acid ceramidase and Sk1 (139, 140). Increased work evaluating the role of S1PR2 in prostate cancer specifically is essential to determining its role in cancer progression and the tumor microenvironment.

Targeting S1P in the clinical setting

Many chemical compounds are in the drug discovery spectrum aimed at treating various inflammatory and neoplastic conditions. The most prominent of these is FTY720 or Gilenya, the first available FDA approved oral treatment for MS. Following treatment, FTY720 is phosphorylated by SK2. This results in functional antagonism of all receptors except for S1PR2 through receptor internalization. S1PR2 is not impacted by FTY720 based on binding affinity studies of P-FTY720(141). Inhibition of S1PR1 results in lymphopenia and therefore decreased MS symptoms and inflammation. Recent literature has suggested additional interactions with the T cell polarization and response. Another prominent therapy in the pipeline is monoclonal antibodies against S1P. Anti-S1P antibodies are currently in Phase I/II clinical trials as iSONEP and aSONEP for age related macular degeneration and solid tumors respectively (114-116, 127, 128, 142).

S1P in Embryonic stem cells

Embryonic stem cells (ESCs) have been shown to express S1PR1-R3 with murine ESCs expressing S1PR5 as well. The role of S1P in promoting self-renewal and a stem cell phenotype has been documented primarily in its role in ESCs. Strategies designed to culture ESCs in vitro under serum free conditions have demonstrated that the combination of S1P and PDGF supplementation results in increased

proliferation of ESCs in an undifferentiated state with decreased apoptosis(143-145). In hESCs, S1P is critical to proliferation and survival with Erk1/2 activation increased following S1P treatment resulting in increased cell cycle proliferation and decreased apoptosis. However, these changes were accompanied by decreased expression of pluripotency factors including Nanog and Oct4 with increasing passages. Other studies have demonstrated that S1P regulates ESC self-renewal through Erk 1/2 with decreased spontaneous cell differentiation (142). ESCs highly express S1P lyase and knock down increases the stem cell phenotype with increased SSEA and OCT4 expression and increased proliferation. In this system, a ten fold increase in S1PR2 expression was observed (143). S1P has been shown to be important in stem cell self-renewal and maintenance in other stem cell populations. Further work evaluating the role of S1P and more specifically individual receptor contributions in ESCs will further inform characterization and ex vivo proliferation of this stem cell population.

S1P in Hematopoietic stem cells

Hematopoietic stem cells (HSCs) reside in a quiescent state within the bone marrow with a basal level of circulating cells within the peripheral blood. Following stress or injury, often the result of infection, tissue damage, or exercise, HSCs can be mobilized to the peripheral blood and site of injury (146-151). Historical understanding of this mobilization relied on retention signaling from the SDF-

1/CXCR4 and VLA-4/VCAM-1 axes (150). Based on this research, treatment with AMD3100, an inhibitor of CXCR4, results in HSC mobilization for harvesting of HSCs. HSCs are known to express S1PR1-R4 but lack S1PR5. Recently, S1P has been shown to provide a counter signal to the SDF-1/CXCR4 retention signaling promoting HSC mobilization and egress from the bone marrow. These egress signals are thought to utilize S1PR1 as expression of this receptor is required for mobilization of cells in response to an S1P gradient. S1P however is limited in its egress capacity as complementary inhibition of CXCR4 signaling is required for increased cell mobilization (150, 152). Ongoing research suggests that in cases of bone marrow depletion such as following irradiation prior to transplant, S1P may promote homing of MSCs to the bone marrow (153). Although S1P has been best characterized for this mobilization process ceramide 1-phosphate released from damaged cells may also contribute to HSC mobilization. Although the role of S1P in HSCs has been documented, HSCs actively interact with MSCs in the bone marrow and MSC behavior in response to S1P impacts the function of HSCs. Within the bone marrow, S1P signaling in MSCs is essential for the crosstalk with HSCs and maintenance of the hematopoietic compartment (151, 154-156).

S1P and Mesenchymal Stem Cells

S1P and signaling downstream of the S1P receptors has been shown to impact MSC migration, differentiation, and capacity to respond to injury. S1P has been shown to

increase and promote cell migration in MSCs (157). S1P induces MSC migration through an Erk, Rho Kinases, and MMP dependent pathways. Activation of this pathway results in rapid cytoskeletal remodeling starting as early as one minute after treatment and this effect was sustained 6 hours later with increased cellular lammellipodia and filipodia formation. S1PR1 and S1PR2 are the most prominent receptors involved in this chemotactic effect through their effect on the family of Rho GTPase. S1PR2 in particular stimulated RhoA through G12/13 with the result being stress fiber formation and a migratory and invasive phenotype (158). Within the bone marrow environment, osteoclasts can secrete S1P that stimulates MSC chemotaxis. RhoA was activated but did not contribute to migration. S1PR1/S1PR2 however contributed to this chemotaxis using downstream Jak/Stat3 and PI3K signaling (154). These functions are contrary to the canonical role of S1PR2 in inhibition of cell migration.

Alteration in the sphingolipid balance can influence MSC differentiation. S1P dose dependently stimulates adipose derived smooth muscle cells based on protein expression and actin organization (159). S1P similarly promotes cardiomyocyte differentiation (160, 161). C6 ceramide treatment in contrast decreases adipogenic differentiation through decreases in PPARγ and C/EBP expression. Maintenance of low ceramide levels through the addition of exogenous acid ceramidase allows for increased MSC chondrogenesis (162). In combination, this suggests that a shift in the sphingolipid balance to favor S1P generation promotes increased MSC

differentiation and processes that work antagonistically to this result in decreased MSC differentiation. Additionally ceramide treatment in adipose derived MSCs increases cell apoptosis and decreases differentiation (163).

S1P signaling is critical to the physiological function of MSCs both in their peripheral role and in the bone marrow. S1P and the S1P gradient are critical to MSC mobilization through interactions with the Rho, MMP and Erk dependent pathways (57, 132). Li et al. demonstrated in their 2009 and 2013 publications with a carbon tetrachloride liver injury model that MSCs migrate to the liver injury was dependent on an S1P gradient and once at the liver they are capable of differentiating into myofibroblasts through S1PR1 and S1PR3 (57, 132, 164). Treatment with the S1P receptor agonist FTY720 results in decreased severity of liver injury in this model with decreased MSC migration to the site of injury (165).

S1P and Neural progenitor cells

Neural progenitor cells are a cell population required for the development of the embryonic nervous system and for nervous system repair. The neural progenitors highly express both S1PR1 and S1PR2. Cell mobilization to sites of ischemia is associated with increased S1P and decreased CXCR4 signaling. Inhibition of S1PR2 using both shRNA and JTE013 resulted in increased progenitor cell migration.

Ongoing work is further characterizing neural progenitor migration and response to injury (153).

Sphingolipids and sphingolipid metabolism have been shown to have multiple intersections with stem cell signaling and function. To date, modulation of sphingolipids pathways has been shown to impact stem cell self-renewal pathways, differentiation, and mobilization in embryonic stem cells, hematopoietic stem cells, neural progenitor cells, and mesenchymal stem cells. Much of this work has focused on the role of S1P and the S1P and ceramide rheostat.

Conclusion

MSCs are increasingly being investigated in clinical use either through autologous or allogeneic transplant for a number of conditions resulting from its impact on the inflammatory environment, cell differentiation, and cell recruitment potential. The role of S1P and its receptors have been shown to have a strong impact on a wide range of biological functions including many of the functions critical to stem cell function and regeneration. Further evaluation of the role of S1P in mediating the functions of MSCs both in self-renewal and in the periphery will elucidate the clinical and translational potential of MSCs. S1PR2 is perhaps the most interesting of

these signaling pathways due to the potential of this receptor to act both with and against the canonical function of S1PR depending on cell type. In this project, we seek to assess the role of the S1P receptors in mesenchymal stem cell function and signaling. The impact of MSCs in the prostate cancer microenvironment is also evaluated using both in vivo and in vitro co-culture conditions.

Materials and Methods:

Cell Culture and Reagents

Animal models

Murine MSCs were isolated from C57Bl/6 mice ordered from the National Cancer Institute or Charles River Laboratories. GFP transgenic C57Bl/6 mice were generously provided by Dr. Okabe at Osaka University in Osaka, Japan (5, 6, 166). S1PR2KO mice were a gift from Dr. Richard Proia at the NIDDK (167, 168). DUSP-1 knock out mice were kindly provided by Dr. Kirkwood at the Medical University of South Carolina in Charleston, SC(169). S1PR3 KO mice were a gift from the COBRE Lipidomics and Pathobiology Animal Core at the Medical University of South Carolina in Charleston, South Carolina (167, 168). All animal work was conducted with IACUC approval.

Chemical inhibitors and substrates

JTE013, an S1PR2 inhibitor dissolved in dimethylsulfoxide, and VPC, an S1PR1 and 3 inhibitor dissolved in ethanol, were purchased from Cayman Chemicals and FR180204 from Fisher Scientific. The Erk inhibitor U0126 was purchased from Cell Signaling and dissolved in DMSO. S1P was synthesized by the Lipidomics Synthetic Core and delivered in a 2% BSA solution.

Cell isolation protocols and culturing

Whole bone marrow was harvested from the femurs of mice sacrificed by CO_2 inhalation. Bones were placed a phosphate buffered saline solution containing 2% FBS and bone marrow was released using a mortar and pestle using the "flush and crush method" (170). Additional digestion with 3 mg/mL Collagenase I (Sigma) was conducted with cell shaking at 300 rpm at 37 degrees cells. Cells were filtered using a 70 μM cell strainer (Fisher Scientific). Red blood cells were lysed in ACK buffer containing ammonium chloride, potassium bicarbonate, and EDTA. Cells were plated in Alpha Modified Eagles Medium (Invitrogen) supplemented with 20% fetal bovine serum (Atlas Biologicals), 2 mM L-glutamine (R&D), and pen-strep amphotericin (Lonza). All cells were incubated in 5% CO_2 at 37°F. When indicated, hTERT MSCs were cultured under low oxygen conditions (171). MSCs were stored in a vacuum-sealed plastic container at 2% oxygen and 37 degrees Celsius for the indicated times. Relevant normoxic controls were stored in the same incubator under standard conditions.

After 3 days, non-adherent cells were discarded, plates washed with PBS, and adherent cells cultured for 4 passages. Cells were sorted on a BD FACS ARIA II cell sorter. Data was analyzed using FlowJo vX (TreeStar inc.). Gating strategy included isolating CD45 and CD11b double negative cells and selecting for Sca1+ and CD105+ cells or CD105+ CD73+ cells using flow Cytometry antibodies provided by BD bioscience. Cells were plated immediately following cell sorting and allowed to recover from the cell sorting process. Twenty-four to forty-eight hours following cell-sorting cells were trypsinized and used for the experimental procedures outlined below.

Cell lines

HTERT immortalized MSCs were a gift of Dr. Frank Marini at Wake Forest Medical Center and were cultured in Alpha Modified Eagles Medium containing 10% Bovine growth serum (Atlas Biologicals). Tramp C2 cells were obtained from the laboratory of Dr. Jennifer Wu at the Medical University of South Carolina. Cells were cultured in high glucose Dulbecco's modified eagle's medium containing 10% FBS and Penstrep/Amphotericin. C4-2 LnCap cells were cultured in RPMI containing 10% FBS and were obtained from the ATCC.

Lipidomics analysis

Ceramides species, sphingosine, and S1P from cell pellets, culture supernatants, and xenograft homogenates were collected and analyzed with LC-MS/MS by the Lipidomics Shared Resource, MUSC, as previously described (172). Briefly, mass spectrometric analysis of lipids was performed using electrospray ionization MS/MS analysis on a Thermo Finnigan TSQ 7000 triple quadruple mass spectrometer, operating in multiple reaction-monitoring positive ionization mode as described previously. About 2-3 × 10^6 cells were fortified with the internal standards (ISs; C17 base D-erythrosphingosine, N-palmitoyl-D-erythro-C13-sphingosine, and heptade-canoyl-D-erythrosphingosine). Calibration curves were constructed by plotting peak area ratios of synthetic standards corresponding to each target analyte with respect to the appropriate internal standard. The target analyte peak areas from the samples were similarly normalized to their respective

internal standard and then compared with the calibration curves using a linear regression model. Results were normalized to total protein levels.

Clonogenic Survival Assay

Clonogenic survival was assessed as previously described (172). To conduct the clonogenic cell assays, 24-48 hours following sorting cells were plated in triplicate in 6-well cell culture plates (Corning, NY). Treatment was delivered 2 hours prior to initiation of clonogenic assay when indicated. Following 10-14 days in culture, cells were fixed in 3.7% formaldehyde and stained with 1% crystal violet. For analysis, 200-1000 cells per plate were plated depending on the treatment group. Colonies were counted when containing > 50 cells using a dissecting microscope. Colony formation was calculated as the ratio of colonies formed compared to the number of cells plated normalized to untreated or wild type controls.

Quantitative Real-time PCR Analysis and genotyping

RNA was synthesized by RNEASY (Qiagen) from cultured cells according to manufacturer's instructions or by trizol extraction procedures. RNA was quantified based on the ratio of A260/280. 2ug of RNA was used for cDNA synthesis using the Biorad iScript cDNA synthesis kit according to manufacturers instructions using a Biorad thermocyler. Real-time RT-PCR was performed with primers in Sybr Green Supermix (Biorad) was used for thermocycling reactions. Cycling conditions were as follows: pre-incubation, 50º C for 10 minutes, 95º C for 3 minutes, followed by 25-35 cycles of denaturation at 95º C, 30 seconds; annealing/extension between 52 and 60º C, 45

seconds. Relative mRNA concentration was calculated as 2^-(CtTarget-CtCalibrator). All primers are shown below in Table 1.

Genotyping was conducted following tail clipping of ear-tagged mice just prior to weaning. Tails were digested and DNA isolated according to the qiagen DNEASY kit manufacturers instructions. PCR reactions using genotyping primers 1-3 listed below were conducted as directed by Kono et al (2004)(167, 168). PCR reactions were conducted using DNA master mix at 40 cycles of 1 min each of 94 degree and 72 degree temperatures. Wild type expression resulted in 170 base

Table 1: Table of primers used for genotyping and qPCR analysis: The first five primers listed are the mouse primers used for qRT PCR. The remainder of the primers listed are murine specific primers except those primers designated with an "h" following them. Genotyping primers 1, 2, and 3 are from Kono et al. 2004.

Gene	*Forward Primer*	*Reverse Primer*
S1PR1	CTCCACCGTGCTCCCGCTCTA	GGAGATGTTCTTGCGGAAGGTCAGG
S1PR2	GCGTGGTCACCATCTTCTCC	CGTCTGAGGACCAGCAACATC
S1PR3	CATCGCCTTCCTCATCAGTATCTTC	CACAATCACTACGGTCCGCA
S1PR4	GCACCTTGAGCATAACAGGA	CGGGGACAGACTGAGAGAGG
S1PR5	ACTGCTTAGGACGCCTGGAA	CCGCACCTGACAGTAAATCCTT
Oct4	ACACCTGGCTTCGGATTTCG	GGCGATGTGGCTGATCTGCT
Nanog	GGTTGAAGACTAGCAATGGTCTGA	TGCAATGGATGCTGGGATACTC
Sox2	GCACATGAACGGCTGGAGCAACG	TGCTGCGAGTAGGACATGCTGTAGG
GAPDH	CAATGACCCCTTCATTGACC	GATCTCGCTCCTGGAAGATG
Acid Ceramidase	TTACCCTTGGGTCCTTGGCCATAA	TCTGCCACGATGTTGAAGTAGCCT-
SK1	TGAGCAGGTCACCAATGAAG	TGTGCAGAGACAGCAGGTTC
SK2	GGAGGAAGCTGTGAAGATGC	GCAACAGTGAGCAGTTGAGC
S1PR1h	GAAGGGGGAGAATACGAACA	GCCAAATGAACCCTTTAGGA
S1PR2 h	CACCTGGAAAGGCCAGATAA	CAGTGCAAGATTCCGTCTCA
S1PR3 h	GCCGACGGAGGAGCCCTTTTTC	ATGCTCCCGCAGGGTCTCGTT
S1PR4 h	CCAAGCGCTACATCCTCTTC	CAGAGGTTGGAGCCAAAGAC
Genotypying 1	GCAGTGACAAAAGCTGCCGAATGCTGATG	
Genotyping 2	AGATGGTGACCACGCAGAGCACGTAGTG	
Genotyping 3	TGACCGCTTCCTCGTGCTTTACGGTATCG	

pair product whereas knockout gene expression resulted in a 220 base pair product.

Proliferation Assays

MSCs were plated in 96 well plates at a concentration of 5,000 cells/well in at least triplicate. Absorbance was measured using a Fluostar Optima (BMG labtech) plate reader at 490 nm following MTS substrate delivery (Promega) starting the day prior to treatment and following treatment. Absorbance was measured 1 hr and 2 hours after addition of 20 uL of substrate. Proliferation was assessed according to the Incucyte Zoom kinetic proliferation assay protocol. Using the microscope contained within the incubator, an algorithm was developed to specifically detect both MSC cell number and confluence on the plate. Following cell plating in 96-well plates, images were taken and analyzed every 15 minutes for the duration of the experiment. Additional proliferation assays were conducted using crystal violet staining. Cells were set up and cultured for the indicated period of time. At the completion of the experiment cells were fixed in formaldehyde and stained with 5 mg/ml crystal violet in PBS. Stained cells were washed with PBS and water then the stain lifted using 2% SDS. Absorbance was measured from vortexed aliquots of the crystal violet solution and normalized to untreated controls.

Migration Assays

Two experimental approaches to migration were taken. In the first, 80-120,000 cells/well were plated to confluence in 24 well collagen coated plates. Cell scratches were initiated following cell attachment using 10-100 uL pipette tips and media changed to remove any

detached cells immediately prior to treatment. Scratches were imaged every 6 hours until 24 hours using a Zeiss axiovert 200 microscope examining at the same field of cells at each time point. Scratch distance and percent closure were quantified using Image J. Further validation of MSC migration findings was conducted using Incucyte Zoom evaluation of migration. Cells were plated to confluence in image locked 96-well plates coated with collagen I. Wound delivery was given using the 96 pin wound maker equipped with PFTE pin tips creating a 730-750 uM wound width. High definition phase contrast images were taken every fifteen minutes with cells identified using MSC specific algorithm. Analysis was conducted using Essen Incucyte software to determine scratch width and wound confluence. Analysis was conducted using three types of analysis. The first is wound width and evaluated the distance between each side of the scratch. The second is wound confluence that evaluates the area occupied by the cells as compared to initial wound area. The final analysis is relative wound density in which the spatial cell density of the wound area is compared to the cell density of unwounded areas. The final analysis allows for some compensation for cell proliferation in addition to using time limited evaluation to limit the influence of proliferation.

Differentiation Assay

Differentiation was induced as directed in chamber slides by the R&D Systems Mesenchymal Stem Cell Functional Identification Kit. Cells were plated to confluence at 30,000 cells per chamber in millicell ez slide 4 chamber slides. Following attachment

cells were switched to induction media. Adipogenic supplementation included hydrocortisone, isobutylmethylxanthine, and indomethacin and osteogenic supplement included dexamethasone, ascorbate-phosphate, proline, pyruvate and recombinant human TGFβ3. Concentrations of these agents are proprietary information. Induction media was changed every 2-3 days for 14 days for adipogenic media and up to 21 days for osteogenic media. Following completion of this time, cells were fixed, permeabilized with Triton x-100, and blocked. Osteogenic induction was evaluated by goat anti-mouse osteopontin and adipogenic induction was evaluated by goat anti-mouse FABP4. Secondary antibody stain to visualize included Alexafluor 555 donkey anti-goat for GFP+ cells or Alexafluor 488 donkey anti-goat for non-GFP transgenic cells. Cells were co-stained with nuclear stain To-pro-3. Imaging of stained cells was conducted using Zeiss LSM 510 META confocal microscope. A minimum of 10 images at 63x magnification were taken and cells counted from each image obtained. Differentiation was also assessed as described in the absence of induction media.

Western Blotting

Immunoblot analyses of cell lysates were performed as previously described (172) using antibodies to detect p-Erk, total Erk, p-Akt (Ser 473), total Akt, Rac 1/2/3, RhoA, RhoB, Rho C, CD44, Nanog (Cell Signaling), GAPDH (Santa Cruz Biotechnology sc-32233), and P38 (Gift of Dr. K. Kirkwood). Acid ceramidase antibody used for western blotting was provided by BD 612302. Whole cell lysates were obtained using RIPA buffer

(ThermoScientific, Waltham, MA) and protease inhibitors (Sigma). 25- 50 ug of protein quantified by BCA assay was run on a 12.5% polyacrylamide gel before being transferred to a nitrocellulose membrane. Blocking of non-specific binding was conducted using 5% non-fat milk prior to overnight exposure to primary antibody at 4 degrees Celsius. Secondary anti-mouse antibody (Santa Cruz) was used at 1:15,000 and anti-rabbit at 1:30,000. Visualization of expression was conducted using Chemoluminescent HRP substrate (Fisher).

In vitro co-culture experiments

Tramp C2 cells were seeded at the base of either 6 or 12 well BD transwell plates in high glucose DMEM with MSCs seeded to confluence at the top inserts in alpha MEM on the plate. For vehicle controls, media with the appropriate vehicle or chemical inhibitor was given in the top insert. The next morning, cell adhesion treatment was administered as indicated. Cells were harvested for the appropriate analysis described previously for western blotting, flow Cytometry, or realtime analysis.

Flow Cytometry

Analysis of cell populations was conducted with BD antibodies listed previously during cell sorting. Further evaluation of cell populations was conducted by flow Cytometry using either the BD LSR Fortessa or BD FACS Calibur. Staining for cell trace violet

(Life Technologies) was conducted according to manufacturer's instructions with a 5 minute 37 degrees Celsius staining time for the cell suspension. Aldefluor expression analysis was conducted according to manufacturer's instructions (Stem Cell). Briefly cells are suspended and substrate added for 30 to 60 minutes with a negative control provided by the DEAB inhibitor. Visualization of Aldefluor expression was conducted using flow cytometry on the GFP channel of a BD Fortessa flow cytometer. Cell cycle analysis was conducted on treated cells trypsinized to a single cell suspension following treatment then fixed with cold ethanol. Cells were stained with 40 ug/ml propidium iodide with 100 uL/ml of Rnase. Data analysis was conducted using Modfit LT software.

Nanostring

RNA was analyzed for RNA copy number using Nanostring analysis conducted by murine code set -1 developed my the Medical University of South Carolina Center for Oral Health Research (COHR). 100 ng of RNA isolated using RNEASY from primary murine MSCs and quantified by spectrophotometry was provided to the COHR for analysis. Briefly nanostring technology begins with an initial hybrization phase using mRNA probes followed by a purification and immobilization phase. Following immobilization, fluorescent barcodes associated with each probe can be identified and individual mRNA levels of each product counted. For data analysis, data was normalized to positive controls using nSolver Analysis software with background reduced to accurately depict RNA copy number.

Tumor initiation and in vivo tumor analysis

Tumors were initiated in 6 to 8 week old C57/Bl6 mice using TRAMP C-2 cells by subcutaneous injection 2 million cells in the right flank of mice delivered in 200 μL of 1:1 matrigel:phosphate buffered saline. Tumor volume and animal weight were monitored three times a week. Treatment with 3 mg/kg of JTE013 was given intraperitoneally as two injections 72 hours apart. These injections were delivered on day 30 and day 33 following tumor initiation. JTE013 was dissolved in ethanol prior to dilution to delivery dose in sterile PBS. A comparable solution was delivered for the vehicle treatment group. Animals were sacrificed when tumor volumes reached 1500 mm^2. All animals were sacrificed at the same time for consistency of tumor digestion and flow cytometry analysis. Following extraction of tumors, tumors were digested using Collagenase I overnight, digested cells pelleted, and subsequently analyzed by flow cytometry. Flow cytometry analysis consisted of evaluation for expression of CD45, Aldefluor, and CD133. Desired cells were CD45 negative cell based on prior analysis of TRAMP-C2 expression.

Statistical Analysis

Unless otherwise indicated, data represent mean $\pm$ standard error for 3 independent experiments and were tested for statistical significance by one-sided student t-test. * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

RESULTS AND DISCUSSION

Chapter 1: S1PR2 signaling in human immortalized hTERT MSCs

Based on the available information suggesting a role of S1P in stem cells, we sought to initially examine the role of S1P and S1P receptor signaling in MSCs. To initiate preliminary evaluation of this interaction, we employed the use of hTERT immortalized human MSCs. These cells were a gift of Dr. Frank Marini out of Wake Forest University. HTERT MSCs express markers characteristic human MSCs with demonstrated retention of differentiation capacity and colony formation (173). These markers include positive expression of CD19, CD73, CD44, CD90, CD105, CD105, CD166 and negative expression of CD14, CD19, CD34, CD45. HTERT MSCs

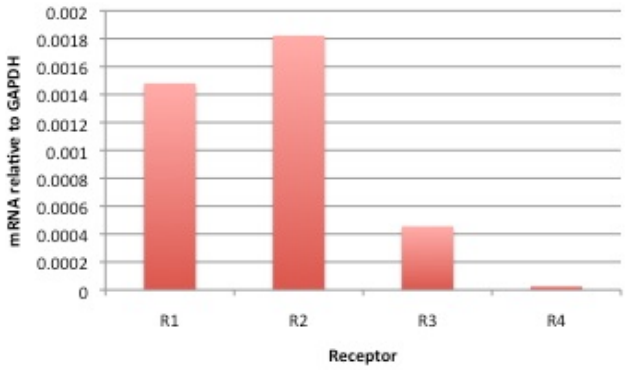


Figure 5: MSC receptor expression in hTERT immortalized MSCs: S1PR1-4 are shown are evaluated in this experiment with magnitude of expression based on relative mRNA expression. Relative mRNA expression was calculated as compared to GAPDH expression by the formula provided in the materials and methods section. Image shown is a representative experiment from two independent experiments.

have an extended lifespan with an increased number of passages as compared to non-immortalized MSCs. When the cells are injected subcutaneously into mice, no tumors formed indicating that the cells were immortalized but not transformed. Comparable to MSC populations in mice shown previously, hTERT MSCs express S1PR 1-4 with the highest expression of S1PR1 and S1PR2 as shown in Figure 4.

Treatment of MSCs with increasing doses of S1P resulted in decreased cell proliferation as assessed by cell number as shown in Figure 5a. Consistent with these results we found that alterations in the cell cycle distribution of MSCs with a bias toward an increased percent of S phase cells and diminished Go/G1 cells as shown in Figure 5b. For evaluation of

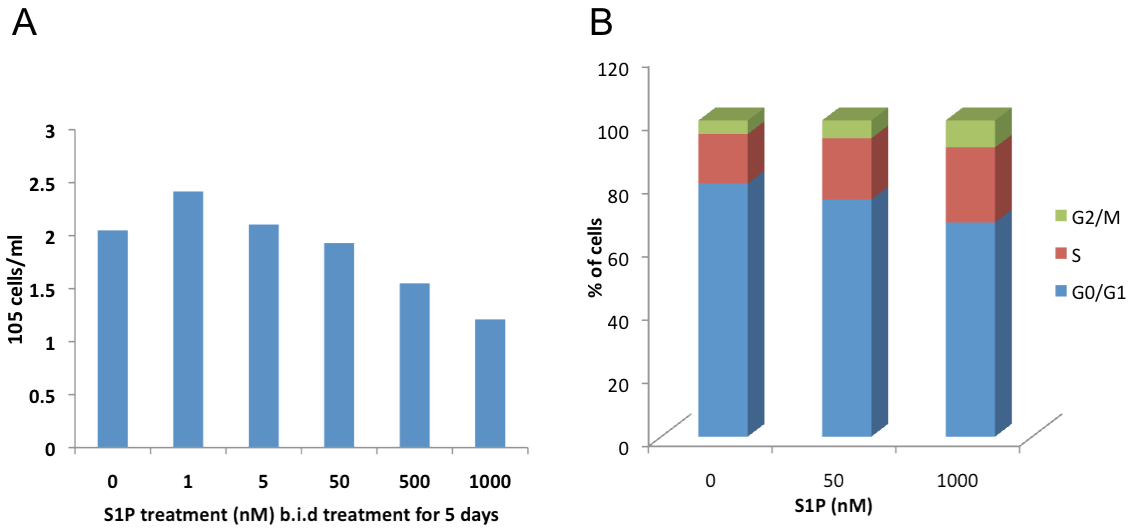


Figure 6: Impact of S1P treatment on MSC proliferation (a) Evaluation of cell proliferation by cell counts at the indicated days. S1P concentrations between 0 nM and 1000 nM were evaluated with the values reported in increments of 10^5 cells (b) Cell cycle analysis of MSCs following S1P treatment in hTERT MSCs. The percent of cells in each phase of the cell cycle is reported for S1P concentrations between 0-1000 nM. Experiment shown is representative of two independent experiments.

proliferation, cells were plated in 6 well plates and allowed to attach in standard and lifted at the indicated time points for counting with a Millipore sceptor. The Sceptor has the capacity to calculate cell number and size with error of less than 5%

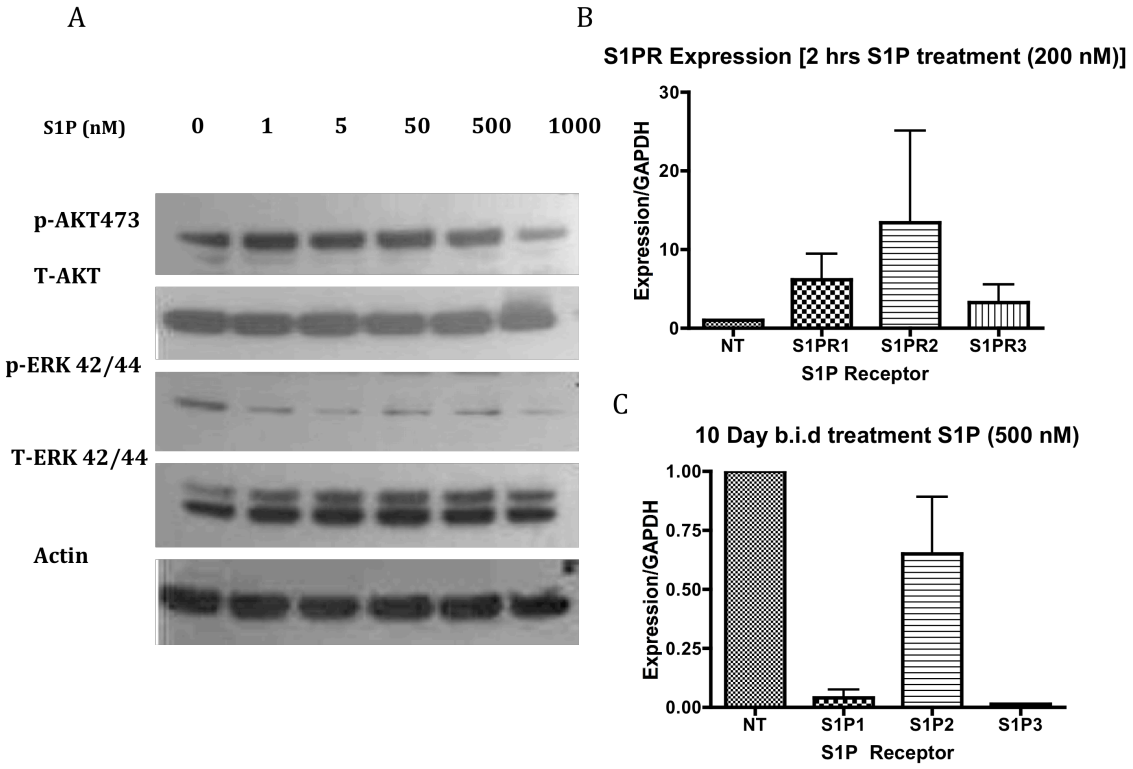


Figure 7: Impact of S1P treatment on MSCs and S1P receptor distribution. a) Western blot analysis of S1P treated hTERT MSCs after 2 hour treatment with S1P at the indicated doses. S1P concentrations between 0 nM and 1000 nM were evaluated. (b) Relative mRNA expression of hTert MSCs for S1PR1, R2, and R3 following 2 hour treatment with 200 nM S1P in PBS. (c) Transcriptional expression of S1PR1, R2, and R3 following b.i.d S1P treatment for 10 days. S1P was prepared freshly for each treatment. Statistical analysis using a Student's T-test demonstrate that S1PR1 has significantly decreased expression for S1PR1 with $p<0.01$ for S1PR3 for $p<0.05$. $n \geq 3$ for all graphs shown

when used properly. Cell cycle analysis was conducted with cells plated and treated overnight with S1P at the indicated concentrations before trypsinization to a single cell suspension. These cells were fixed in cold ethanol, stained with propidium iodine, and analyzed by flow cytometry. Following fluorophore detection, cell cycle

distribution was determined by Modfit analysis. S1P treatment in MSCs therefore decreases MSC proliferation and impacts cell cycle distribution.

Consistent with the reduction in MSC proliferation observed following S1P treatment there was a reduction in MAPK activation following S1P treatment. Treatment with increasing doses of S1P resulted in diminished expression of phosphorylated Erk as shown in Figure 6. Changes in the Akt activation relative to actin expression were only observed at the highest concentrations of S1P treatment. Changes in MAPK activation appear to parallel proliferative changes following S1P treatment as shown in Figure 4. We sought to investigate the changes in the S1P receptors following S1P stimulation in MSCs.

To accomplish this, S1PR2 was evaluated following realtime quantitative PCR. Treatment with 200 nM of S1P for resulted in a trend toward increased expression of S1PR1-R3. When S1P treatment was given twice daily for ten days, a significant decrease in S1PR1 and R3 was observed. In contrast to these two receptors, the mRNA transcript levels of S1PR2 were maintained. In combination, this suggests that following treatment with S1P mRNA levels of the S1P receptors increase acutely followed by a subsequent decline in mRNA transcript levels of S1R1 and S1PR3 suggestive of receptor internalization. Receptor internalization has been best characterized for S1PR1 although it has been demonstrated for S1PR2 as well (174-177). Long term treatment with S1P resulting in decreased receptor expression is consistent with the decreased proliferation observed following 5 days of b.i.d S1P

treatment whereas short term increases in transcriptional RNA expression for the S1PR is consistent with the increased number of cycling cells following overnight S1P treatment. Figure 7 shows the images of hTERT MSCs treated with S1P at the indicated concentrations b.i.d for ten days. At the higher concentrations of S1P, a population of cells indicated by arrows results that has a distinct morphology separate from that of the remainder of the MSC population. This morphology appears more characteristic of a differentiated cell. This suggests that S1P may promote MSC differentiation although further characterization of the cells with this morphology needs to be addressed. The retention of S1PR2 expression following long term S1P treatment suggests that S1PR2 may have a critical function in MSC maintenance. Maintenance of MSCs in this context is defined as the capacity for undifferentiated proliferation without a loss of differentiation capacity.

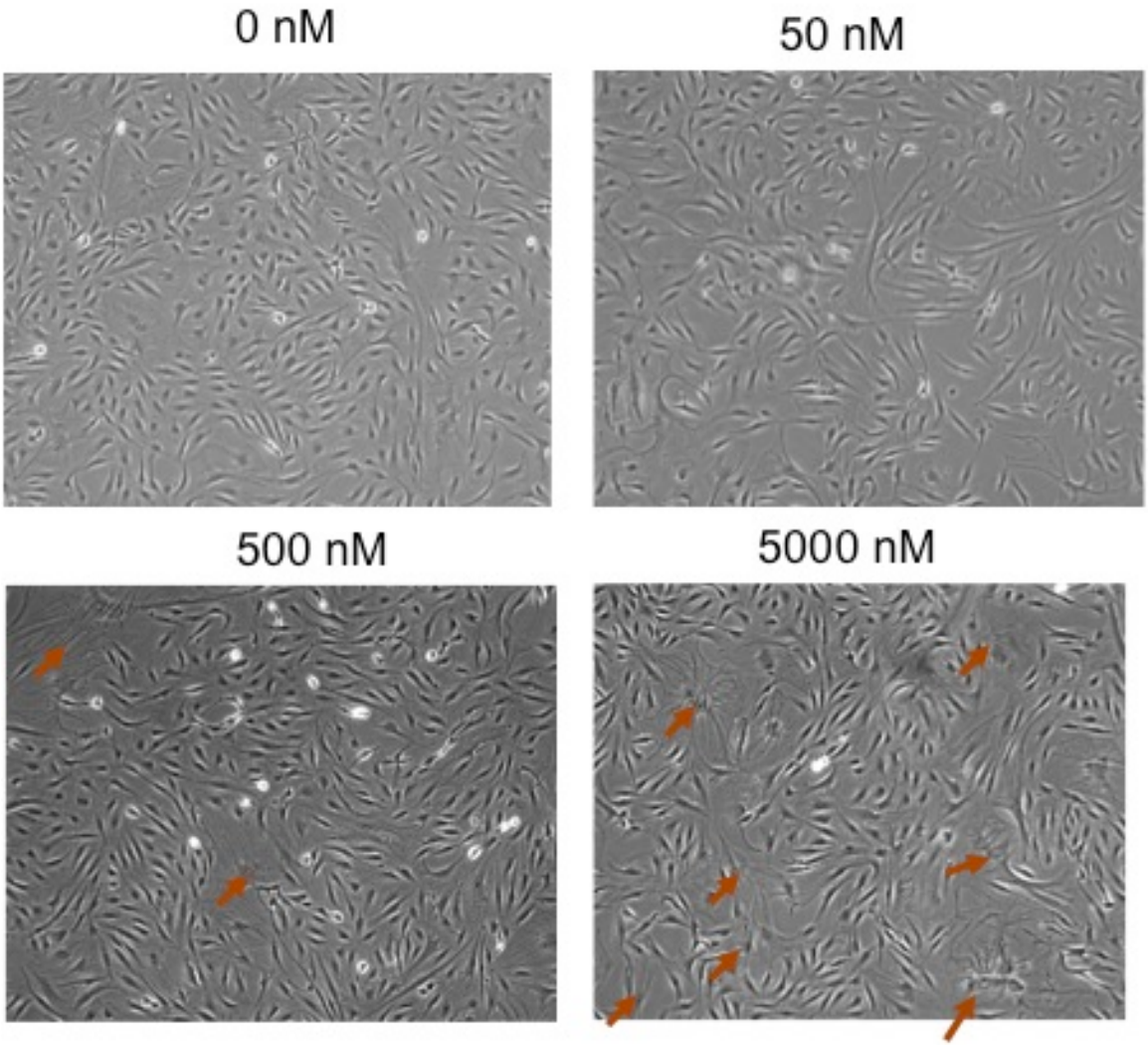


Figure 8: Images of hTERT MSCs treated with increasing concentrations of S1P. Cells were treated with 0-5000 nM S1P b.id for 10 days. Arrows in the higher treatment groups indicate cells with an increased level of differentiation. Pictures were taken using a Zeiss Axiovert cell culture microscope using the 10x lens. An increased number of differentiated cells were observed with increasing concentration of S1P in all fields examined. Representative images are shown for 3 or more independently treated cells.

Based on our previous findings, we sought to further explore the role of S1PR2 through the use of JTE013 as a chemical antagonist of S1PR2. Due to the presence of S1P in the serum, S1P stimulation was not provided in future experiments in which JTE013 treatment was given. Serum levels of S1P are shown in Figure 8 as

evaluated by the Lipidomics Analytic Core of the Medical University of South Carolina. Alpha-MEM media containing 5 or 10 percent FBS serum contained over 8 pmol/ml of S1P. The same lot of FBS was used throughout the experiments presented in this dissertation.

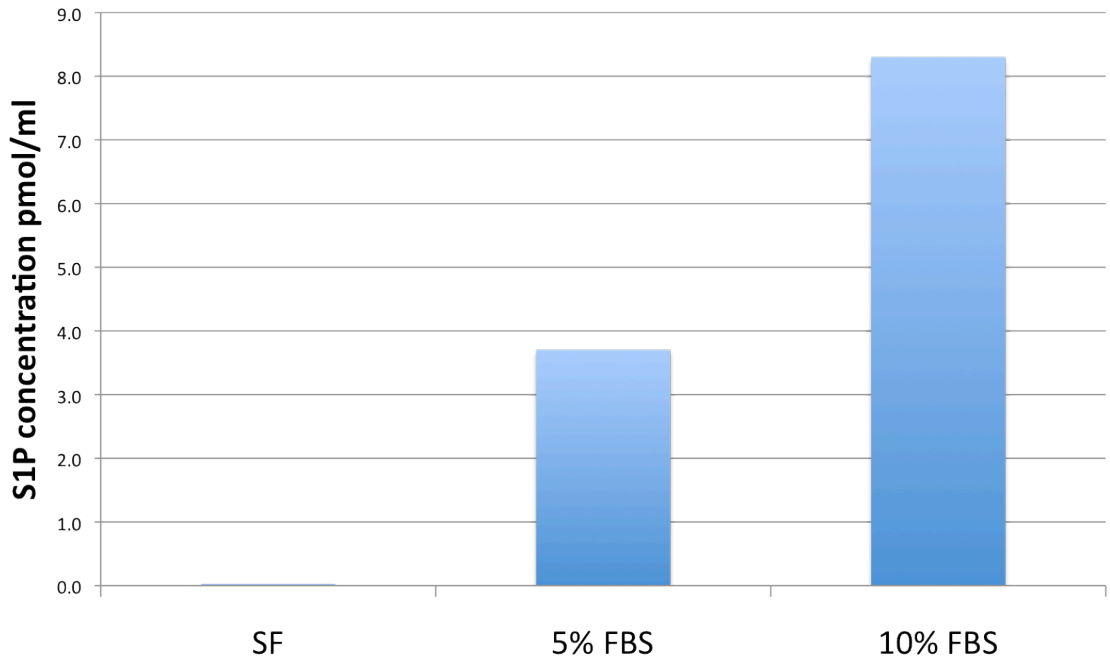


Figure 9: Lipidomics analysis of media concentration of S1P with different serum concentrations. S1P concentration was evaluated by the Lipidomics Core as described in the materials and methods section. Percentages reflect the percent of Atlas Biologicals Tet free serum in Invitrogen Alpha-MEM cell culture media. Consistency between S1P levels throughout evaluation was provided by consistency of batch number for Serum.

Following the identification of basal S1P as important for MSC maintenance, we sought to investigate the role of S1PR2 within MSCs using JTE013. Treatment of hTERT MSCs with 1 uM JTE resulted in increased MSC migration across a scratch wound and increased clonogenicity as shown in Figure 9. For scratch wound

analysis, cells were plated in 6-well plates to confluence. A scratch was made with a 1000 µL pipette tip and treatment of JTE013 or DMSO vehicle followed. Images below represent migration 24 hours after initiation of the scratch wound. For the clonogenic assay, treatment with 1 µM JTE013 was initiated at the time of cell plating. Colonies were fixed and stained 10-14 days following treatment when colonies were grossly visible. Colonies were counted using a dissecting microscope when plates contained between 10-75 colonies. Colony counts were normalized to both the cell plating number and the untreated control.

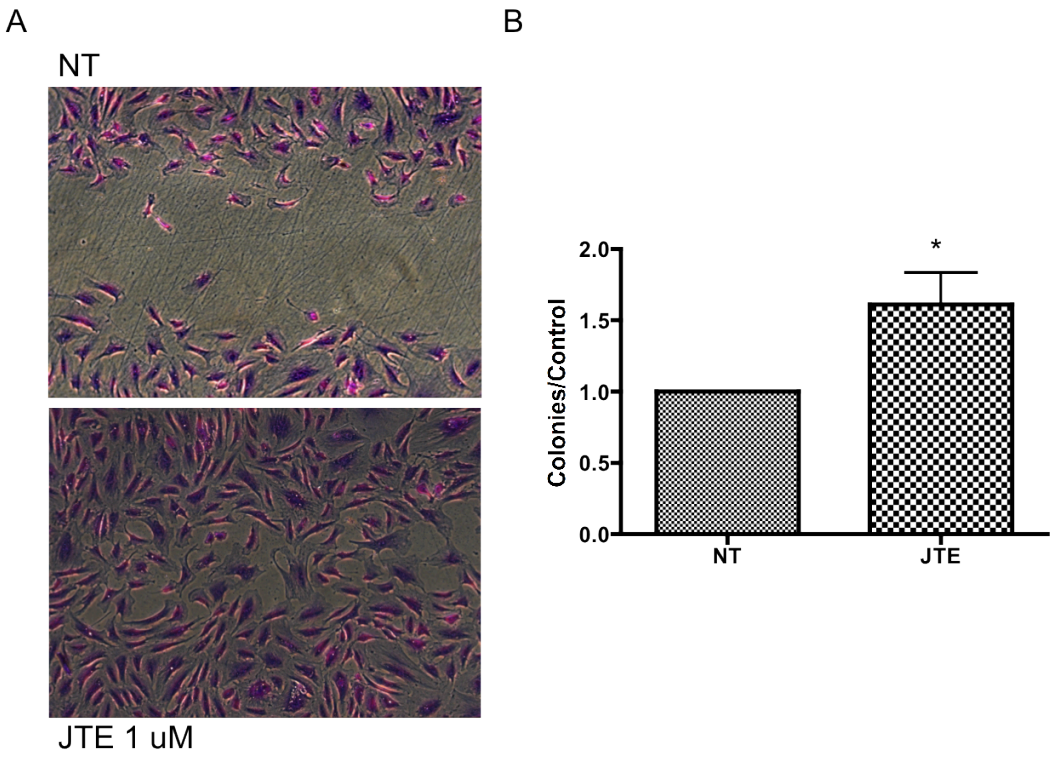


Figure 10: Effect of JTE013 treatment on hTERT MSCs (a) Scratch assay evaluation of hTERT cells treated with either vehicle or 1 µM JTE013 for 18 hours. Cells were stained with crystal violet for easier identification (b) Clonogenic evaluation of JTE013 treated hTERT MSCs. Error bars represent mean ± SEM for each condition. * indicates $p<0.05$ from Student's T-test. $n\geq3$.

The role of S1PR2 is critical in the maintenance of hTERT MSCs with S1PR2 functioning as inhibitory to cell migration and clonogenic growth.

Further evidence of the role of sphingolipids in the maintenance MSC self-renewal and proliferation was obtained through evaluation of the role of hypoxia on MSC proliferation. It has previously been demonstrated that hypoxia promotes the MSC self-renewal as evidenced by the increased undifferentiated cell proliferation of MSCs as compared to standard cell culture controls (49, 178). Hypoxia was generated by introduction of cell culture plates into a vacuum-sealed chamber perfused with a gas mixture containing 2% oxygen in a standard cell culture incubator. Comparable to previously published findings, we observed increased cell proliferation of MSCs under low oxygen conditions. The low oxygen conditions tested approximate the oxygen concentration of the bone marrow, which has been reported to be at 1-7% oxygen. Increased proliferation was observed in hTERT MSCs as evaluated by MTS assay as shown in Figure 10a with fold change evaluated as compared to when the cells were introduced to the hypoxic chamber. Due to concerns regarding the potential of low oxygen to impact the mitochondria of the MSCs and therefore the MTS assay, we evaluated proliferation using crystal violet staining with staining evaluation assessed by absorbance at OD620. Cells were washed, fixed in 5% formaldehyde, and stained with crystal violet. Following staining and washing of the wells to remove excess crystal violet, a 2% SDS solution was added to each well and cells placed on a shaker at room temperature for 30

minutes. Absorbance was measured by triplicate samples of the solution. By crystal violet proliferation analysis, hypoxic cells demonstrate increased proliferation as compared to normoxic controls. Using both methods for proliferation analysis, increased MSC proliferation was observed with inhibition of S1PR2. This change was accompanied by increases in the expression of the 13 kd a band of acid ceramidase (AC) as shown in figure 10c. Increased AC results in increased metabolism of ceramide to sphingosine and ultimately

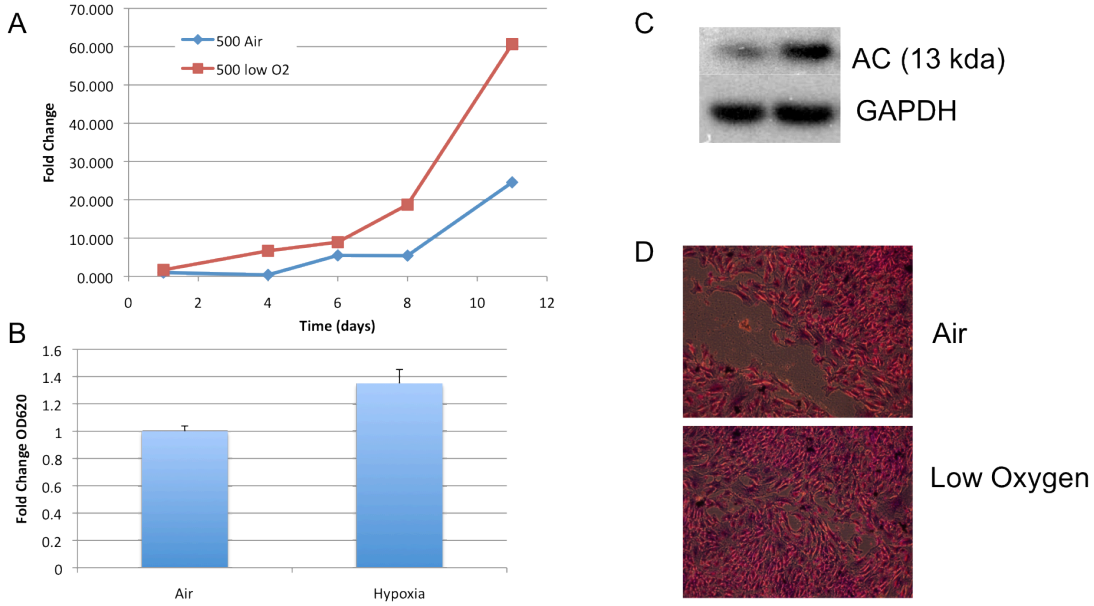


Figure 11: Evaluation of hTERT MSCs under low oxygen conditions (a) MTS proliferation assay of hTERT MSCs cultures in standard cell culture conditions at 20% oxygen and under 2% oxygen conditions. Blue lines indicated standard cell culture conditions and red lines indicated low oxygen conditions (b) Crystal violet proliferation analysis of Day 9. Cells were cultured under the indicated conditions and cells were fixed, stained, lifted, and absorbance measured to evaluate cell concentrations (c) Western blot analysis of the active band of AC Lane 1 represents the normoxic control and the second represents hypoxic conditions (d) Scratch assay of crystal violet stained cells under both normoxic controls and under hypoxic conditions at 18 hours. Representative images are shown for a minimum of 3 experiments.

increased S1P generation. Increased MSC migration was observed in cells cultured under low oxygen conditions as compared to those under standard cell culture oxygen conditions as shown in figure 10d.

Our initial evaluation of the effect of S1P in hTERT MSCs revealed that S1P treatment results in decreased cell proliferation with a corresponding decrease in phosphorylation of Erk that likely impacts the proliferative capacity of these cells. Similar to our evaluation of primary murine MSCs, hTERT MSCs have high S1PR2 expression that increases in transcriptional expression with S1P treatment. Unlike receptors 1 and 3 that decrease in expression following long-term treatment, S1PR2 expression is maintained suggesting that it has a critical function to the maintenance of an MSC population. Inhibition of S1PR2 by treatment with JTE013 results in increased MSC migration and clonogenicity. Based on this evaluation, we conclude that S1P is critical to impacting the function of MSCs and furthermore that S1PR2 expression and function is critical to the maintenance of MSC populations. Further evaluation of these criteria and evaluation of their impact on MSC self-renewal will need to be conducted in primary cells as the immortalization of cells can impact the behavior of the cell population.

Chapter 2: MSC isolation and characterization

The previous chapter addressed the impact of S1P and S1P receptor signaling in human immortalized MSCs. In this chapter, the data presented demonstrates changes in cell proliferation and MAPK activation with S1P treatment and increased clonogenicity and migration with inhibition of S1PR2. These findings suggest a role of S1P and S1P2 on the propagation of MSCs. For further evaluation of these findings and to better assess the role of S1PRs on MSC stem cell characteristics a switch was made to primary murine MSCs. Cells were isolated in accordance with the guidelines established by the stem cell community for MSCs. The criteria for evaluation are discussed further in the proceeding paragraphs.

The Tissue Stem Cell Committee of the International Society for cell therapy in 2006 proposed a consensus definition for human MSCs. This definition sets forth the central criteria defining MSCs. These criteria include plastic adherence, colony formation, a characteristic surface marker expression, and multipotency. The characteristic surface marker composition includes cells lacking expression of CD45, CD34, CD14 and positively expressing CD105, CD90, and CD73 (25, 179). This surface marker definition eliminates hematopoietic and immune cell precursors. Recent work conducted after the release of this report a population of Sca-1

expressing cells as a subpopulation of these human cells demonstrating enhanced clonogenicity as compared to cells lacking expression.

Classical criteria used to demonstrate multipotency in MSCs includes differentiation into adipogenic, chondrogenic, osteogenic lineages. Although differentiation into other lineages has been reported, there remains some controversy on the functionality of the cells that are produced with different degrees of acceptance depending the quality of functional performance markers that has been presented. The scientific community is increasingly demanding not only marker expression but also functional studies to correspond with differentiation reports when new lineages for differentiation are being proposed. Adipogenic lineages can be evaluated by immunofluorescent staining with Fatty acid binding protein 4 or by staining with Oil Red O. Osteopontin staining can be used to evaluate osteogenic differentiation or this can be accomplished by alizirin red staining. Collagen II staining can be used to evaluate chondrocytic differentiation by immuofluorescence or more classically Alcain blue staining has been used. The criteria for evaluation of human MSCs is shown in Figure 12 showing the differentiation capacity, surface marker expression, and minimum criteria as established in 2006.

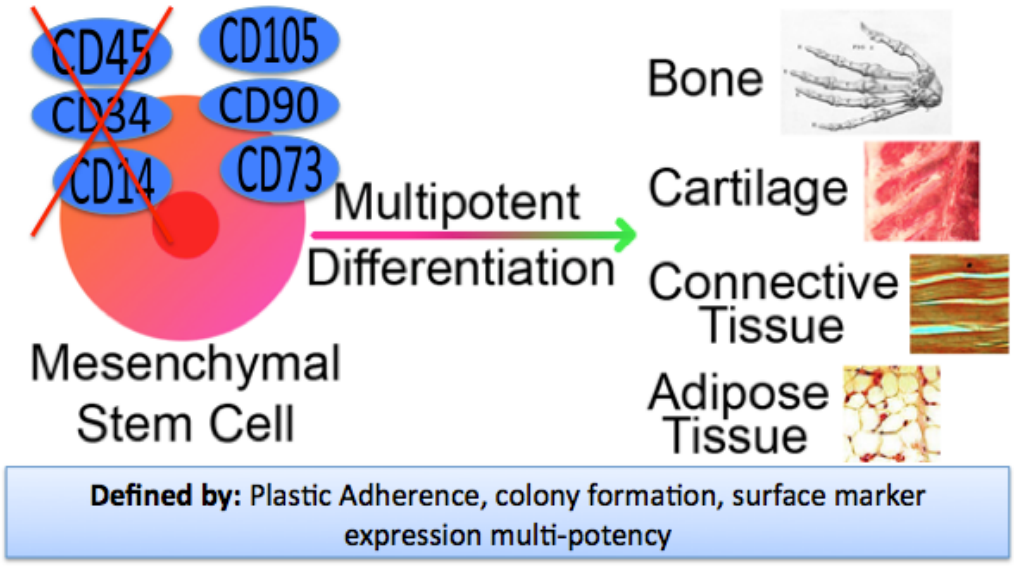


Figure 12: Criteria for the identification and characterization of mesenchymal stem cells. Image partially derived from www.stemcell.org.

Although this definition for MSCs derived from human sources has been well established, there remains ongoing debate and controversy on isolation protocols and characterizations for murine derived MSCs. Increased variation in marker expression is observed in murine derived MSCs isolated from different sources and strains of mice (180). Murine strains have been shown to differ in their optimal media, proliferation rate, and expression of certain surface markers. Part of the variation between MSC populations results from strain differences, however, additional variation can result from different isolation strategies. MSC isolation protocols from bone marrow vary in the degree of stringency of cell isolation. The most basic isolation protocols have used selection based on plastic adherence

whereas more selective protocols include mechanisms of cell sorting either through magnetic bead selection and enrichment or flow cytometric sorting (170, 181-185).

Marker	Expression
CD45	-
CD11b	-
CD44	+
CD73	+
Sca-1	+

Table 2: Murine MSC marker profile. Positive and negative markers of MSCS are indicated.

Based on these existing isolation protocols, we present a cell isolation scheme utilizing a flush and crush method of bone marrow isolation followed by a collagenase digestion. MSCs were selected from passage four bone marrow preparations based on their selective plastic adherence. Based on insufficient cell purity of this population, cell sorting is conducted for two positive and two negative markers as described in Table 2. Macrophages and hematopoietic lineage cells are excluded by eliminating cells expressing CD45 and CD11b. Cells expressing CD44+ and CD73+ cells were positively selected. Further evaluation of Sca-1+ cells is conducted and found to be positive given the gating strategy previously described. Figure 13 demonstrates this cell isolation strategy. The impact of cell sorting is shown with a more heterogeneous cell population in Figure 13a as compared to a more homogenous cell population shown in Figure 13c. The appearance of these cells is shown in Figure 13. This cell isolation strategy results in selection for elongated spindle shaped fibroblast cells characteristic of an MSC population. Less than 20% of the cells enriched for an MSC population using plastic adherence are positively sorted for. Cell expression profiles are shown in Table 2 as previously described.

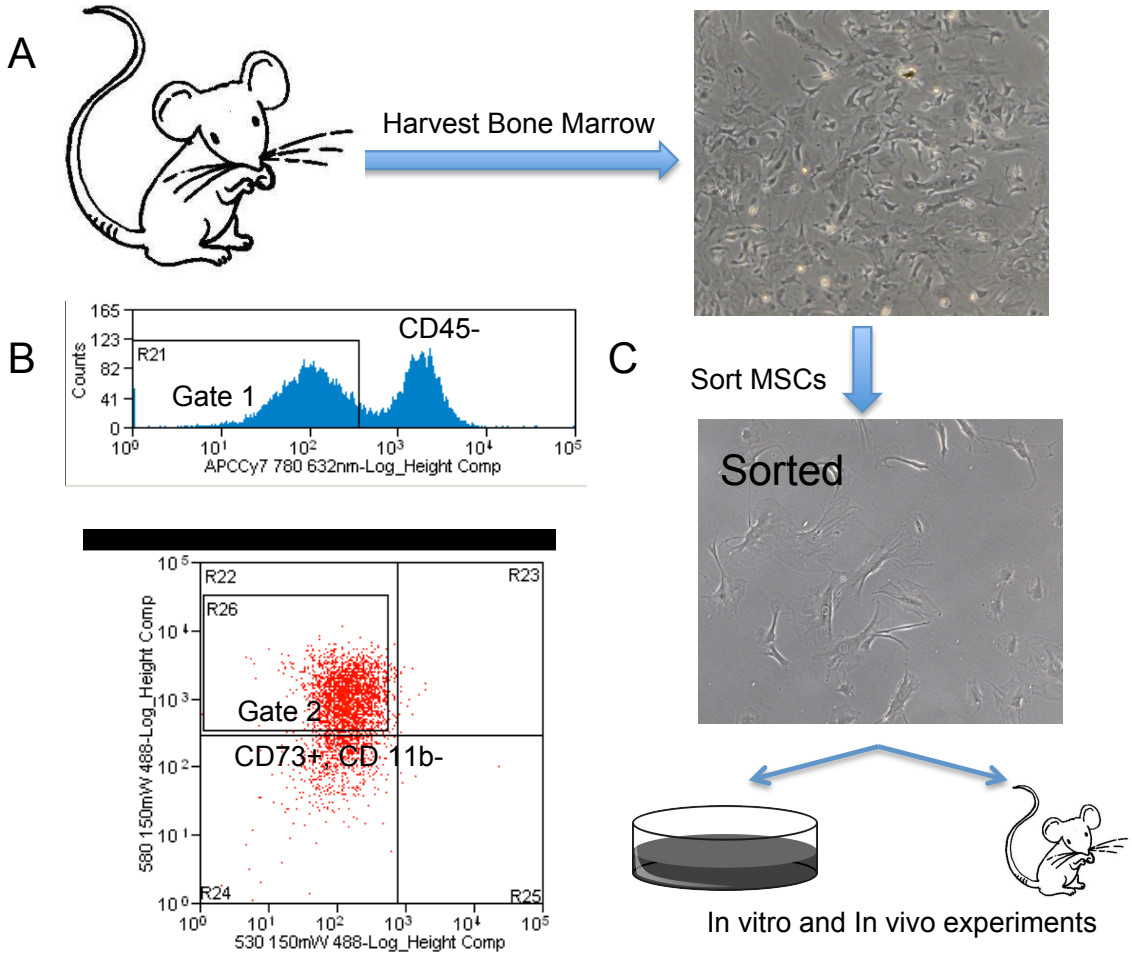


Figure 13: MSC isolation scheme (A) indicates the initial harvesting phase for the cells from the murine bones and the resulting heterogeneous population of adherent cells from which it was derived. Following initial selection by adherence, cells were sorted as shown in panel (B) using the markers shown in table 1. Sorted cells are shown in (C). This represents a more homogenous spindle-like population of cells that can subsequently be used for both in vitro and in vivo experiments.

Following cell sorting, MSCs are cultured in vitro for 24 to 48 hours to allow cells to recover from the cell sorting process. Many of the resulting cells from the sort are unable to survive the stress of the cell sorting process. These cells do not attach and are washed from the plate using PBS for trypsinization of the remained of the cells.

The sorted cells are then further evaluated for differentiation ability to ensure that an appropriately multipotent population of cells has been identified.

Differentiation of the isolated MSC population into adipogenic and osteogenic lineages. was evaluated. Differentiation assays were conducted in accordance with the R&D Mouse Mesenchymal Stem Cell Functional Identification manufacturers instructions. Cells are plated at a concentration of 30,000 cells per well in 4-well Millicell chamber slides. Following cell adherence overnight, cell culture media was changed to induction media. Media was subsequently changed every 3 days for 14 days for adipogenic differentiation and 21 days for osteogenic differentiation. Adipogenic media is composed of invitrogen αMEM with Tet-free FBS and penstrep. Additives to the media for the promotion of differentiation include hydrocortisone, isobutylmethylxanthine, and indomethacin. Osteogenic differentiation media contained the additives dexamethasone, ascorbate-phosphate, proline, pyruvate, and recombinant human TGFβ3 to promote differentiation. After induction for the allotted time, cells were fixed, permeabilized and stained for immunofluorescent evaluation of differentiation using osteopontin and Fatty acid binding protein 4. Secondary antibodies for immunofluorescent staining included donkey anti-goat Alexafluor 566 or 488. Cells were counterstained for identification of nuclei prior to visualization by confocal microscopy. MSCs under these induction conditions demonstrated differentiation into adipogenic and osteogenic lineages as compared to uninduced control. Successful differentiation into these lineages supports the

conclusion of a successful MSC isolation procedure. Differentiation is shown in Figure 14 below. Based on the data presented thus far, we assert that we have successfully isolated an MSC population. Differentiation into chondrogenic lineages was investigated, however due to difficulty with cell pellet maintenance and in consultation with the laboratory of Dr. Frank Marini, an expert in the MSC field, we opted against further evaluation of chondrogenic differentiation. Evaluation of MSCs 72-96 hours following sorting indicates that 11% of cells retain CD45+ expression the abundant majority of which positively co-stain for CD11b indicating that removal of the contaminating macrophage population is largely effectively even when examining extended time point analysis following JTE013 treatment.

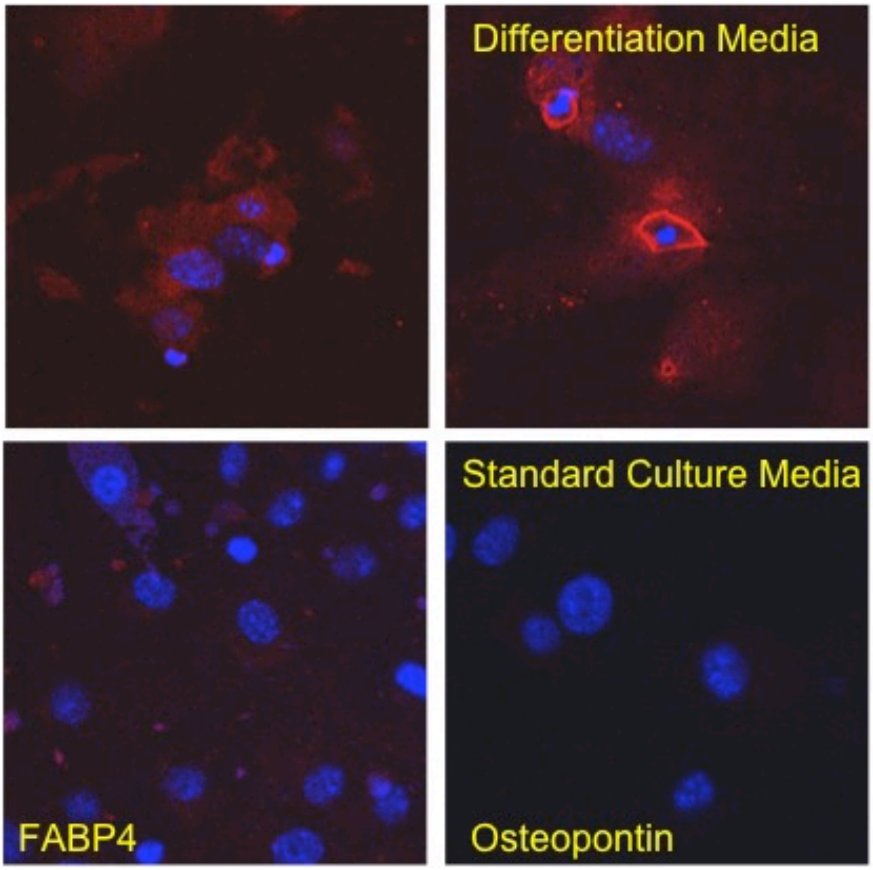


Figure 14: Evaluation of MSC functional identity by immunofluorescent staining. Evaluation of adipogenic staining was conducted by FABP4 expression and osteogenic differentiation by osteopontin staining as shown by the staining in red. Nuclear staining was conducted using Topro-1 and is shown in blue.The top image indicates cells cultured with induction media and the bottom images represent cells cultures in standard cell culture media.

Some of the controversy regarding the impact of S1P stimulation on different cell populations stems from variable S1P receptor expression and corresponding downstream signaling. All five S1P receptors are highly sensitive to S1P stimulation Kd values in the low nanomolar range. Current research does not appear to suggest that S1P concentration differentially favors certain receptor activation. As a result of the sequence and structural similarity between these receptors, it has been difficult to generate quality antibodies that effectively recognize individual

receptors. In light of the absence of good antibodies to evaluate receptor expression, quantitative real time PCR has been established as a central technique to evaluate cell type specific receptor expression. We therefore calculated the mRNA expression of each receptor relative to GAPDH expression for MSCs derived from C57Bl/6 mice. The results from this are shown in Figure 15 below. Interestingly, there is an abundance of S1PR2 expression relative to the other four receptors with all 5 receptors demonstrating some level of expression. S1PR1 and S1PR3 have the next most abundant mRNA expression after S1PR2 consistent with previous reports suggesting their ubiquity of expression.

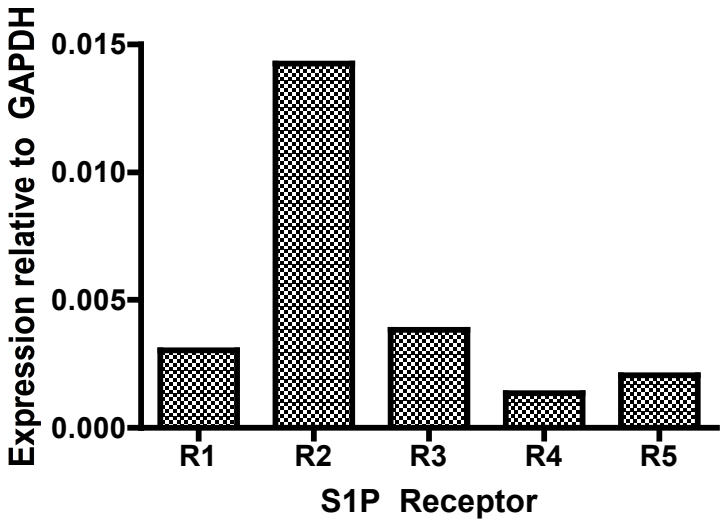


Figure 15: Relative mRNA S1P receptor expression in MSCs derived from C57BL/6 mice as compared to GAPDH expression. Primers used for evaluation are shown in the materials and methods section. This is a representative experiment demonstrating the results of 2 independent trials. Experiment shown is representative of 3 independent experiments.

Following successful verification of MSC identity and S1PR transcriptional expression, we sought to characterize changes in expression of these receptors

following inhibition of S1PR2 either subsequent to JTE013 treatment or in S1PR2 KO cells.

JTE013, a chemical antagonist of S1PR2, inhibits downstream signaling through this receptor with a Kd of antagonism in the nM range and marked specificity into the high μM range of treatment. JTE013 treatment in these cells did not result in marked changes in S1PR distribution following treatment as shown in Figure 16a. The mechanism of action of JTE013 would not suggest that transcriptional changes in receptor expression would be expected although changes in its activation and cell signaling would nonetheless be expected. Furthermore, 1 μM JTE013 treatment during clonogenic evaluation of MSCs resulted in increased colony formation as compared to vehicle treated cells as shown in Figure 16b. The clonogenic assay was conducted with JTE013 or DMSO vehicle delivered at the time of cell plating with 500 cells plated in each well of a 6-well plate. Colonies were counted after 10-14 days in cell culture following cell fixation and staining with crystal violet. Increased clonogenicity indicates a greater capacity of MSCs to self-renew and to regenerate an MSC population. Inhibition of S1PR2 results in increased clonogenicity of MSCs without impacting the receptor distribution of S1PRs in MSCs.

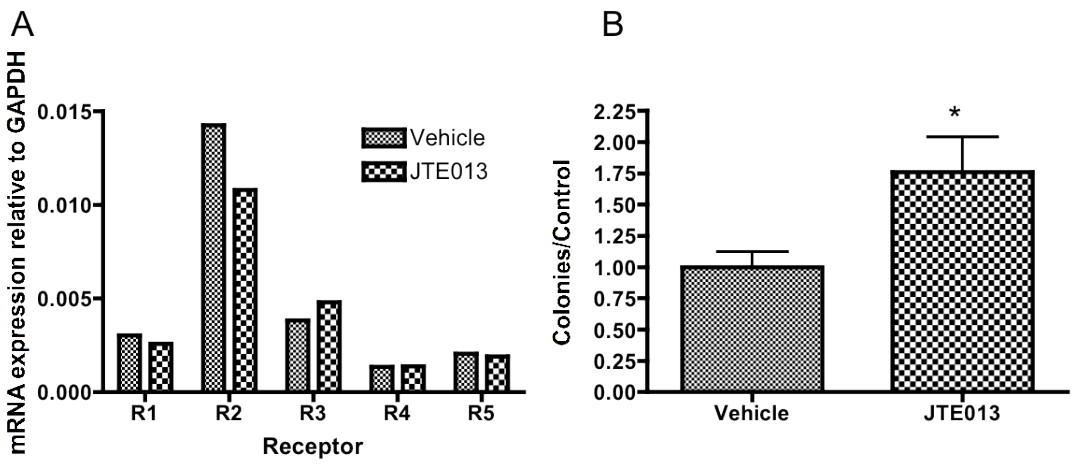


Figure 16: Impact of JTE013 treatment on MSC expression of S1P receptors and clonogenicity (a) Evaluation of relative mRNA expression for the S1P receptors from MSCs derived from vehicle treated MSCs and JTE013 treated MSCs from C57BL/6 mice. mRNA expression is shown relative to GAPDH (b) Clonogenic evaluation of vehicle treated and JTE013 treated MSCs from C57Bl/6 mice.* indicates $p<0.05$

The other mechanism of evaluating the absence of S1PR2 in MSCS and its impact on receptor distribution is the use of S1PR2 knockout mice. S1PR2 KO mice were

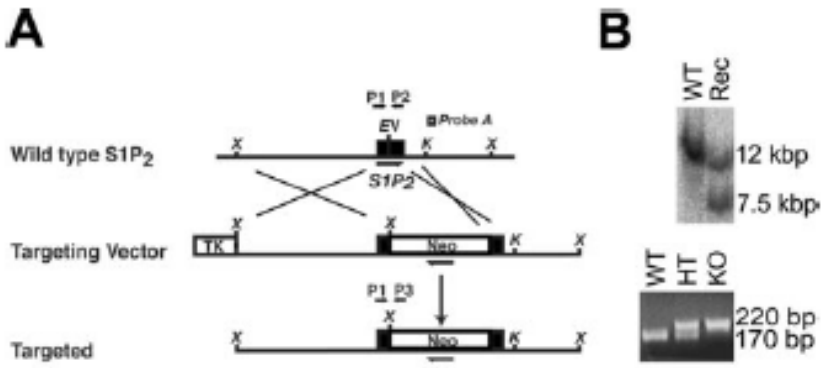
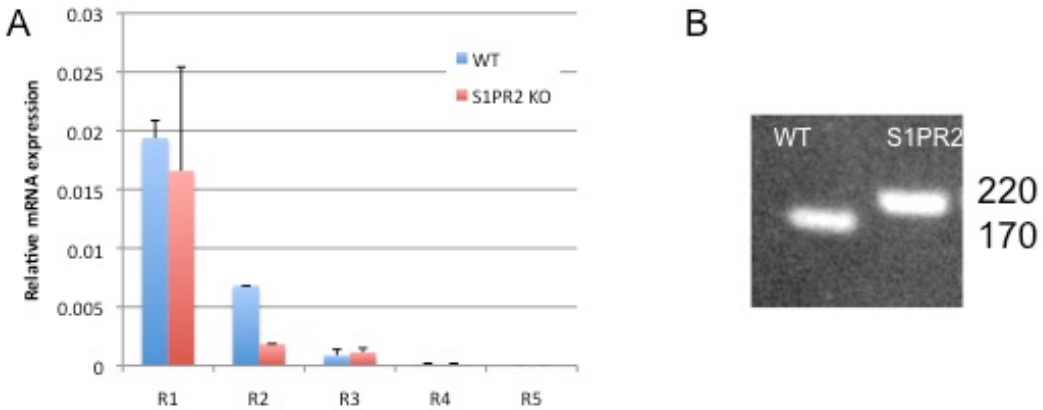


Figure 17: Generation of S1PR2 knockout mice as described by Kono et al. 2004. Figure B shows the genotyping strategy employed in this project.

kindly provided by Dr. Richard Proia of the NIDDK (167, 168). The mice were generated by insertion of 4.8 kb neomycin insertion through an EcoRV open reading frame of the S1PR2 murine gene on mice of the 129/Fv background as shown in figure 11 (167, 168). Genotyping of these mice can be conducted using primers 1 and 2 to detect the 170 bp segment of the wild type allele and primers 1 and 3 to detect the 220 bp knockout truncated MSC product. S1PR2 knockout mice have perinatal defects in their vestibular system resulting in deafness by 1 month of age. Some mice, especially on the C57Bl/6 background are prone to seizures in the first couple weeks following birth. Female knockout mice have been demonstrated to produce decreased litter sizes suggesting a need for further characterization of the female reproductive system. The mice are otherwise phenotypically normal. Figure 17 demonstrates the verification of the knockout allele and the mRNA expression of

the S1P receptors relative to GAPDH. The relative mRNA concentration of S1PR2 relative to GAPDH remains highly expressed in FVB mice. In S1PR2 KO cells, decreased transcriptional expression of the wild type form of S1PR2 is expressed whereas significantly higher expression of the mutant allele is expressed as shown in Figure 12a. S1PR1 is more highly expressed than that of the C57Bl/6 mice with diminished S1PR3-5 expression. This transcriptional difference between strains of mice in not inconsistent with previously published works indicating MSC RNA and protein differences dependent on the strain of mouse from



	WT	S1PR2 KO
S1PR2 WT	1.000	1.000
S1PR2 KO	0.022	406.375

Figure 18: A) Relative mRNA of wild type and S1PR2 knockout MSCs relative to GAPDH expression. Blue bars indicate WT expression and red bars indicate KO expression. MSCs were derived from FVB/129 mice consistent with the genotype of the KO mice (b) Genotyping of MSC knockout mice from tail vein DNA following the genotyping strategy shown by Kono et al.

which they were derived. Importantly from Figure 18a we demonstrate that the knockout mice have diminished expression of the wild type S1PR2 allele and other transcriptional changes are not observed in these knockout mice as there is no

evidence of receptor compensation in the knockout animals. This analysis allows us to proceed to further analysis of MSCs derived from S1PR2 KO animals.

It can therefore be concluded that MSCs highly express S1PR2 and inhibition of this receptor results in increased clonogenicity. This work further characterizes the S1P receptor expression of MSCs in a murine population following S1PR2 inhibition using JTE013 and in S1PR2 knockout MSCs. Although some strain variations have been observed consistent with the literature, expression S1PR2 in MSCs is present.

This chapter establishes both that MSCs have successfully been isolated and further the mechanisms for inhibition of S1PR2 that will be employed in the proceeding chapters. This therefore establishes the critical framework from which the future results can be established. To better understand the role of S1PR2 and the other S1P receptors in MSCs, further characterization of this population is necessary focusing on the capacity for proliferation, differentiation, bone marrow maintenance, and tissue repair. These processes are essential for understanding the physiological function of these cells and to be able to evaluate their clinical potential.

Chapter 3: Inhibition of S1PR2 increases MSC proliferation and migration

S1P has critical roles in promoting migration and proliferation including in MSCs. These effects are critically impacted by S1P receptor signaling. S1P receptor 2 (S1PR2) is more complex than the other receptors based on its signaling through both Gi, Gq, G12/13. Our initial analysis in Figure 9 demonstrates that inhibition of S1PR2 promotes increased MSC migration and proliferation in human Htert MSCs. It is imperative that proliferation and migration be assessed in non-immortalized MSCs.

Previous literature has suggested that inhibition of S1PR2 has been shown to both promote and inhibit migration depending on the cell type investigated. S1PR2 has been shown to negatively impact migration in satellite skeletal cells, vascular smooth muscle cells, glioma cells, murine embryonic fibroblasts, mast cells, and melanoma cells however it promotes migration in human lung fibroblasts(125, 186-191). The controversial role of S1PR2 in MSC migration is not limited to physiologic cell types and additionally has been investigated in cancer cells. Similar antagonizing roles have been proposed in cancer models with S1PR2 promoting increased invasion and metastasis in Hela cells, and esophageal cancer cells (130, 131, 192) whereas it demonstrates inhibitory roles in glioma, melanoma, gastric cancer, and CHO cells lines (129, 190, 193-195). The available literature therefore

more prominently endorses the role of S1PR2 in inhibiting cell migration. The differential impact in lung fibroblast may be the result of differences in S1PR distribution favoring signaling through S1PR1. Differential expression of the S1PRs within a cell type may be the critical factor dictating a cellular response to S1P stimulation. No evidence for differential effect has been observed for S1P receptor response based on different S1P concentrations.

As this the response of cell to modification of S1PR2 may be modulated by the type of cell under consideration, the role of S1P2 in MSCs may therefore be better predictor of the role of S1PR2 in MSCs. In Htert immortalized human MSCs, S1PR1 and S1PR2 act jointly in the promotion of migration in a transwell assay (154). Quint et al. in their 2013 publication reported that osteoclasts secrete S1P and that this S1P can stimulate MSC chemotaxis by a RhoA dependent mechanism through activation of protein kinase pathways including JAK/STAT3 and FAK/P13K/AKT dependent pathways. This study by Quint et al., utilized a transwell assay as compared to other studies generated using scratch assay a low dose of JTE013 at only 20 nM (154). The authors of this paper do not investigate the S1P concentration within the conditioned media nor is S1P treatment delivered. Further evaluation of S1PR expression and S1P concentration in the cell types and experiments conducted would provide a greater context in which to understand some of the apparently contradictory results regarding the role of S1PR2 in migration.

The role of S1PR2 in proliferation is similarly contentious with reports of S1PR2 inhibiting cell growth and promoting growth in alternate settings. S1PR2 inhibits cell proliferation in keratinocytes by decreasing Akt activation and inhibits smooth muscle cell proliferation as well (196, 197). Evaluation of cancer cell proliferation in vitro shows that S1PR2 promotes cancer cell proliferation in glioma cells and prostate cancer cells by Stat3 and Akt activation (138, 198-201). However in primary non-carcinogenic cell types S1PR2 appears to inhibit the Gi mediated pro-proliferative effects of S1P.

We sought to better understanding the competing roles of S1PR2, as both synergistic and antagonistic to S1PR1, in MSC proliferation and migration. We had previously observed that in hTERT immortalized human MSCs increased migration with inhibition of S1PR2 using JTE013. Both JTE013 as a competitive antagonist of S1PR2 and S1PR2 KO cells were employed as models for evaluation of these effects. JTE013 provides highly specific inhibition of S1PR2 but based on its mechanism of action this may not be as complete of an inhibition as in knockout cells. Knockout animals, however, despite the complete inhibition of S1PR2 may have altered S1P signaling or compensation based on creation of a constitutive knock out animal. A complete approach to evaluation of S1PR2 signaling would employ both genetic and chemical mechanisms to inhibit S1PR2 to evaluate its function within MSCs.

Evaluation of the growth of S1PR2 KO cells and wild type cells with 3 uM JTE013 by MTS assay demonstrated significantly increased proliferation as compared to the vehicle treated wild type cells as shown in Figure 19a. We further observed this effect to be dose dependent with increased proliferation at 24 hours in cells treated with higher doses of JTE013 as shown in Figure 19b.

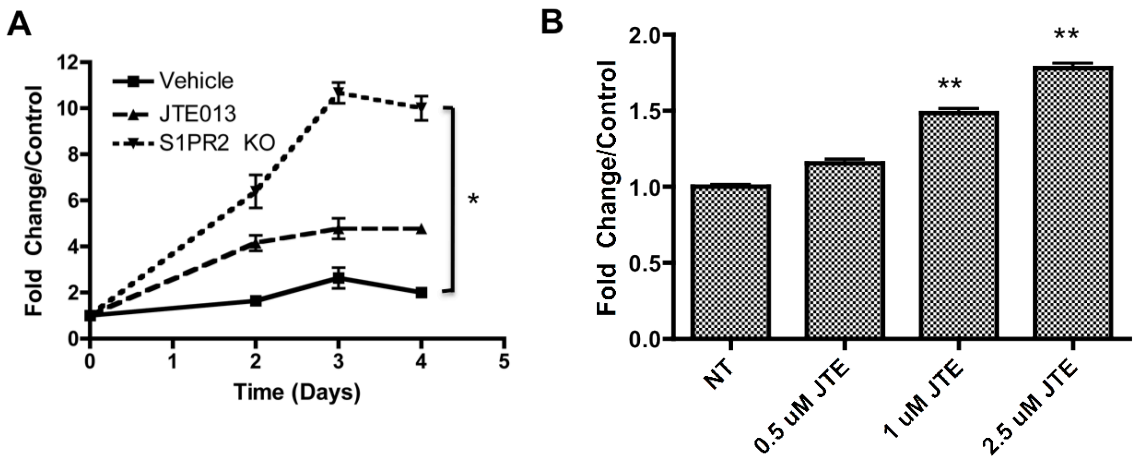


Figure 19: Proliferation of MSCs with S1PR inhibition (a) Proliferation of MSCs from day 0 to day 4 evaluated by MTS assay . Squares indicate vehicle treatment, triangles JTE013 treatment, and dashed triangles indicate S1PR2 KO cells (b) evaluation of the effect of increasing concentrations of JTE013 on MSC proliferation at 24 hours. Proliferation was evaluated by MTS assay with fold change calculated relative to t=0 absorbance. * indicates p,0.05 ** indicates p<0.01 based on student's t-test analysis.

Following the evaluation of the proliferation of cells following S1P inhibition, we evaluated migration by scratch assay, as described previously. In S1PR2 knockout cells as compared to controls cells from age matched wild type mice, increased migration and a decreased scratch wound width was observed in the knockout cells as shown in Figure 20.

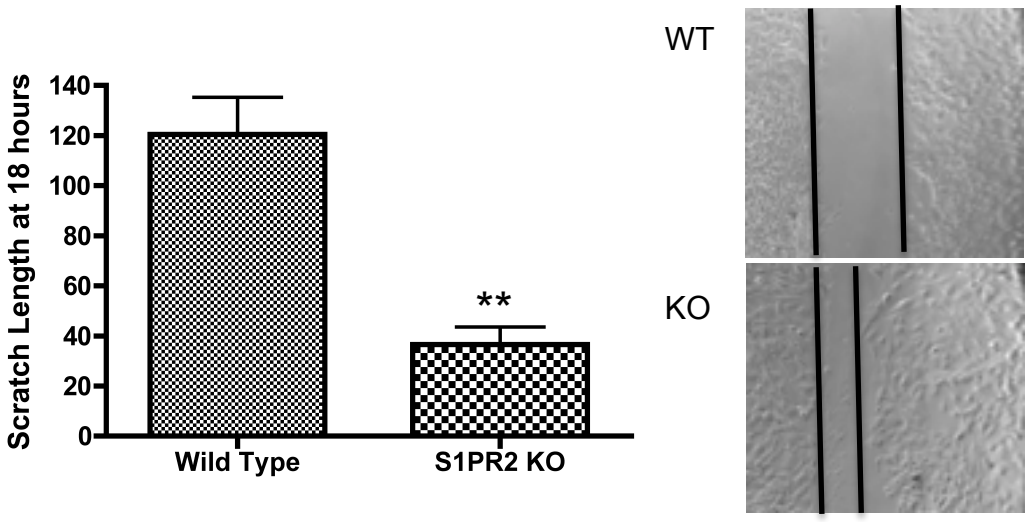


Figure 20: Evaluation of S1PR2 knockout MSC migration by scratch assay. S1PR2 KO mice were evaluated for scratch length based on the length of the scratch following treatment. Representative pictures of WT and KO scratches are shown above with quantification of scratch length. ** indicates $p<0.01$ by Student's T-test

Similarly, Essen analysis as described in materials and methods, was conducted to determine MSC migration on 96 well plates. This analysis was conducted using the image locked plates. Analysis of confluence and wound width was conducted with pictures taken every 15 minutes without disruption of the cell plate within the incubator. Scratches were made using a 96-pin Essen scratch wound technology to generate equivalent scratch wounds on collagen-coated plates. Analysis of wound confluence and wound density both indicate increased cell migration with MSC treatment with JTE013 at all concentrations evaluated in Figure 21. Importantly increased migration was observed by Essen analysis at 500 nM JTE013 at a concentration at which no increased proliferation was observed. This is critical in

differentiating migratory function from that of proliferation during that same time period.

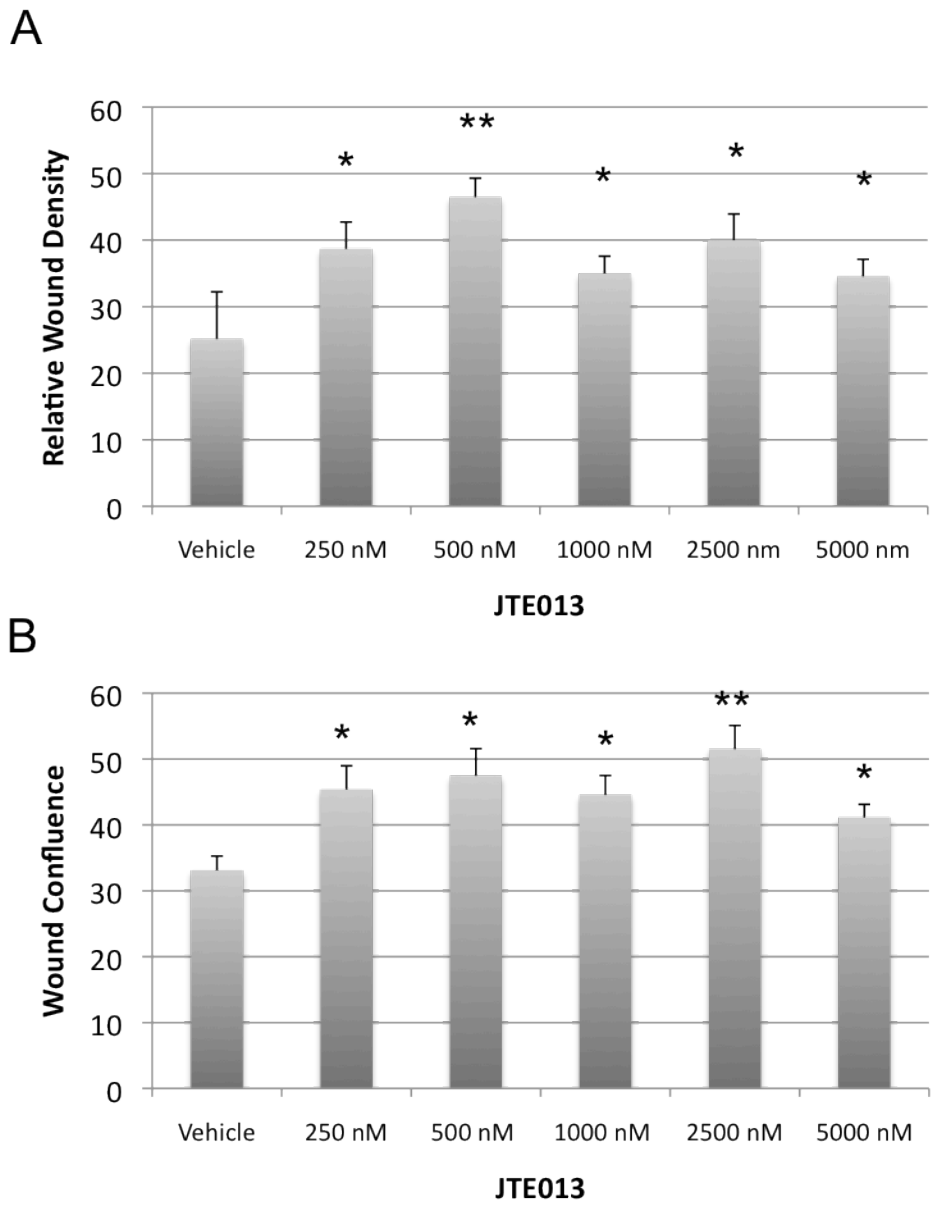


Figure 20: MSC migration as evaluated by Essen analysis following treatment with increasing JTE013 dosages shown . Relative wound density is shown in (a) and wound confluence (b). * indicates $p<0.05$ ** indicated $p<0.01$

Based on consistent data between both JTE013 treatment and S1PR2 KO cells, we conclude that S1PR2 is inhibitory to murine and human MSC migration and therefore inhibition of this receptor results in increased migration. This has significant implications for the in vivo mobilization of MSCs as this feature is critical to their physiological function. This is not further evaluated within this body of research but merits additional research especially given the findings on the impact of reduction of S1P levels on MSC mobilization to sites of injury in a carbon tetrachloride liver injury model (132).

S1PR2 is a critical inhibitory factor for MSC mobilization and proliferation. Further evaluation of the role of S1PR2 in MSCs mobilization is therefore essential to the capacity of cells to function within the bone marrow and tissue injury environment (132, 151, 157, 164). In vivo, the balance between S1PR1 in promoting cell egress and S1PR2 in inhibiting cell may be essential in maintaining cell concentrations within the bone marrow to maintain the stem cell niche and allowing peripheral mobilization of MSCs under injury situations. In vivo analysis of S1PR2 in MSC mobilization is the critical next step in evaluating S1PR2 in mobilization.

Chapter 4: Role of Erk on S1PR mediated migration and proliferation

Extracellular signal related kinase (Erk) is one of the MAPK (Mitogen activated protein kinases) proteins along with P38 and JNK. Activation of Erk is downstream of Ras activation with phosphorylation of Erk mediated by MEK1/2 at two different phosphorylation sites. Erk activation promotes proliferation, differentiation, survival, apoptosis, and stress response. Transcription factors downstream of Erk include many factors although Elk-1, c-fos, and c-jun have been most extensively explored (100). Deactivation of Erk and the other MAPK proteins is mediated by MAPK phosphatases the prominent of which is MKP-1. MKP-1 is capable of deactivating Erk, p38, and JNK although in vitro preference is given to p38 and JNK over Erk (202, 203).

Erk is a critical signaling molecule in cellular functions including proliferation and migration. It has the capacity to phosphorylate and activate hundreds of cytoplasmic and nuclear substrates (204, 205). Downstream signaling pathways of Erk promote G1/S progression. This is accomplished in part through the phosphorylation of Elk-1 and the stabilization of c-fos leading to AP-1 and cyclin D1 activation promoting G1/S progression (206). The pro-proliferative function of Erk is however not limited to these pathways. Erk has been shown to promote cell migration through the phosphorylation of proteins such as calpain, focal adhesion kinase, and paxillin

(207). Increased migration and invasion is also accomplished by the degradation of the extracellular matrix through MMP induction.

S1P is classically known to activate Erk through Gi activation of Ras and ultimately Erk activation (113, 129, 199, 208, 209). This activation of Gi has been demonstrated for both S1P delivery in BSA and on HDL (210). Erk activation importantly results in increased cell proliferation and survival. For this reason Erk activation has been implicated in the promotion of stem cell self renewal in embryonic stem cells following S1P delivery (142). Analysis of S1P stimulated Erk within MSCs was conducted by Meriane et al in 2006 (158). In this publication, S1P delivery to bone marrow derived MSCs results in increased migration with corresponding morphological changes in actin stress fiber formation mediated by a Rho Kinase (ROCK), Erk, and MMP dependent pathway. Although S1PR1, R2, and R3 are capable of Gi activation, S1PR1 and S1PR3 rely most heavily on this mechanism of signaling. S1PR2 has been shown to activate Erk in cardiomyocytes with a 60% decrease in Erk activation in S1PR2 KO cardiomyoctes as compared to wild type controls (211). We sought to evaluate the role of MAPK activation in mediating the functional changes in MSC migration and proliferation following S1PR2 inhibition.

We conducted western blot analysis of the downstream pathways impacted by S1PR2 inhibition. MSCs were treated with either 3 μM JTE013 for 30 minutes or by the use of S1PR2 knockout cells. U0126 is an Mek 1 and 2 inhibitor that prevents

phosphorylation of Erk. The figure below shows that treatment of MSCs with U0126 diminishes Erk activation as expected based on previously published literature.

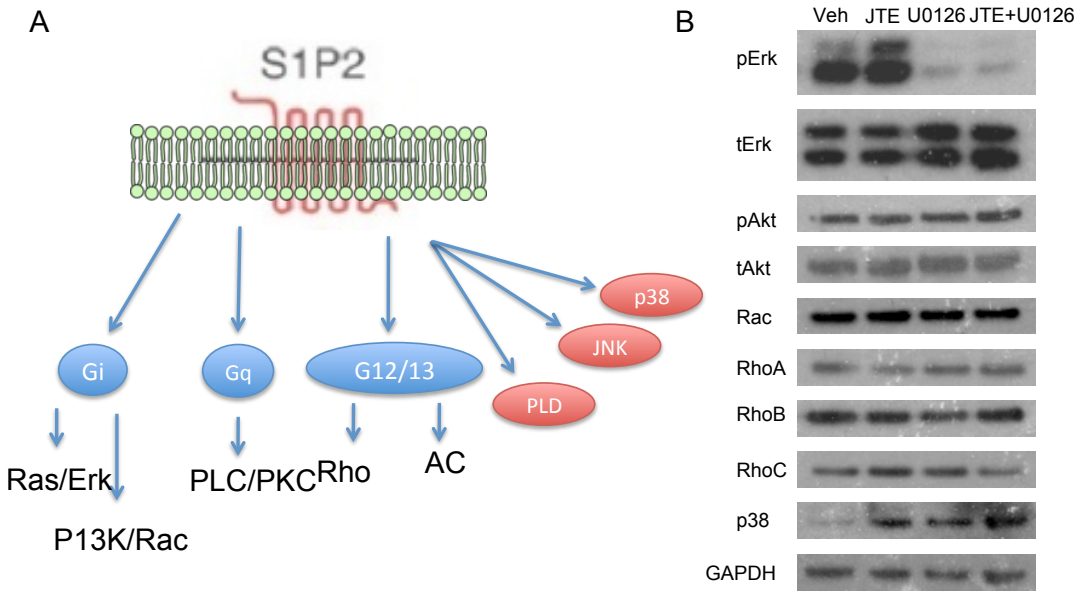


Figure 21: (a) Diagram of S1PR2 signaling. Blue circles indicate G proteins with which the receptor interacts and red circles indicate direct interactions. Downstream pathway interactions are shown. (b) Western blot expression evaluating signaling downstream of S1PR2. U0126 is an inhibitor of MEK1/2 preventing phosphorylation of Erk 1/2.

Based on this analysis, we found a marked upregulation of Erk activation following S1PR2 inhibition using JTE013 as shown in Figure 22. Akt activation remains unchanged with S1PR2 inhibition and no changes in Rac or Rho isoform expression were observed. There was a marked increase in p38 expression with S1PR2 inhibition. Having observed this in MSCs with JTE013 treatment, we evaluated whether this same change in Erk activation was present in our other systems using JTE013 treatment in hTERT human MSCs and in our S1PR2 knockout cells.

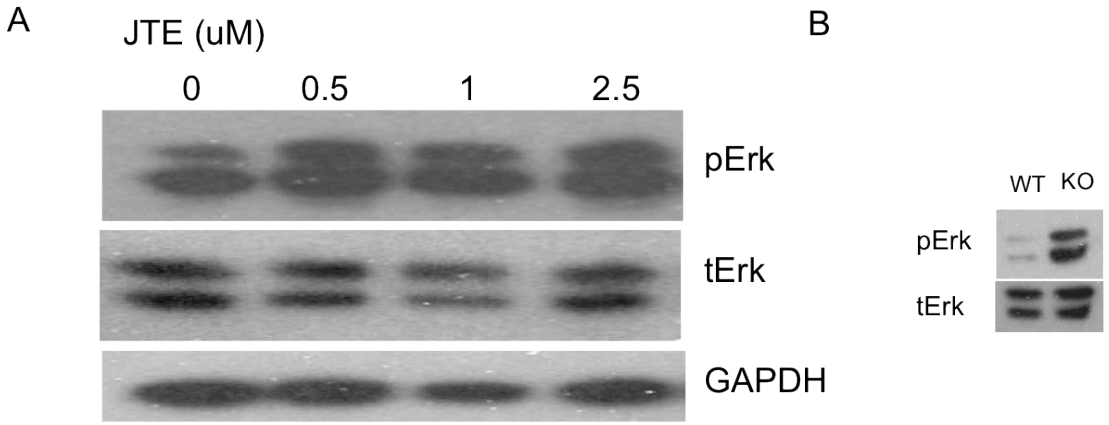


Figure 22: Western blot evaluation of Erk activation in hTERT cells 2 hours following JTE013 treatment. Phosphorylated ant total Erk expression are shown following 0-2.5 µM JTE013 treatment (a) Erk analysis in wild type and S1PR2 KO cells (b)

In both our hTERT treated cells and our S1PR2 KO cells, increased Erk activation was observed following inhibition of S1PR2 as shown in Figure 23. This suggests that contrary to the canonical role of S1P on Erk activation, S1PR2 is inhibitory to this activation in MSCs that have a strong transcriptional expression of this receptor. We propose that the mechanism of this activation may be mediated by changes in the gene expression of DUSP-1 known as the protein MKP-1. MKP-1 can be induced in stress response pathways and can catalytically inactivate p38, JNK, and Erk at tyrosine and threonine residues although an in vivo preference is given for p38 and JNK (203, 212, 213). In airway smooth muscle cells, S1P can activate all three MAPK proteins but can also simultaneously activate its negative feedback controller MKP-1 through a pathway involving adenylate cyclase, PKA, and P38 (105). We evaluated changes in DUSP-1 gene expression and its impact on Erk activation in MSC cells following S1PR2 inhibition to determine if this was a potential mechanism for

changes in Erk phosphorylation. Thirty minute treatment with 1 μM JTE013 resulted in decreased DUSP-1 gene expression as shown in Figure 24. When MSCs were isolated from MKP-1 KO animals, a give of Dr. Keith Kirkwood at the Medical University of South Carolina, and were subsequently treated with JTE013, no increases in Erk phosphorylation were observed. This change in activation of Erk suggests that diminished MKP-1 induction following inhibition of S1PR2 might result in decreased Erk dephosporylation and therefore increased Erk activation after S1PR2 inhibition. This possible explanation for the changes in Erk phosphorylation bears further investigation and other factors may be responsible for changes in Erk phosphorylation.

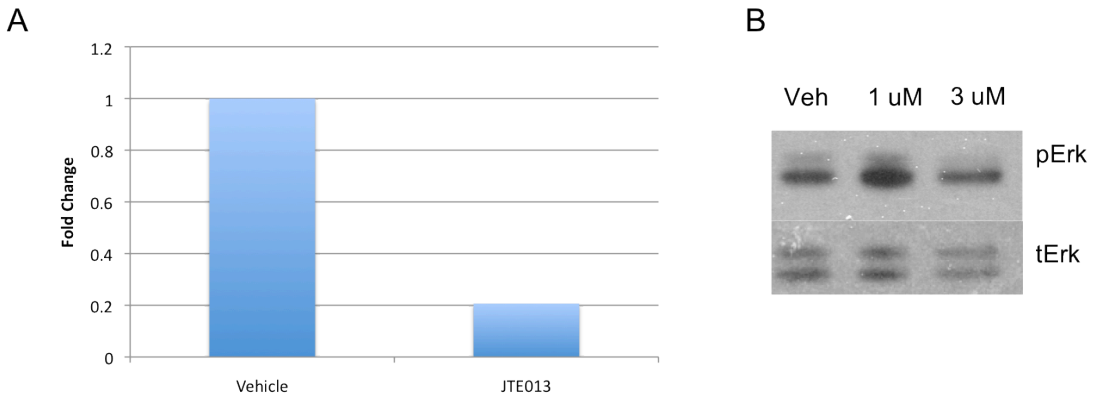


Figure 23: Evaluation of DUSP-1 treatment in MSCs (a) qPCR evaluation of DUSP-1 expression following JTE013 treatment DUSP-1 expression was normalized to GAPDH expression in the cells (b) Western Blot analysis of Phospho and total Erk expression in MKP-1 KO cells

Although we observed increased Erk phosphorylation following treatment with JTE013, we sought to determine whether this change might impact the functional changes observed in MSCs following S1PR2 inhibition. U0126 is a potent inhibitor of

Mek 1 and 2 with an IC50 value of 0.5 μM. Inhibition of Mek 1 and 2 results in decreased activation of Erk 1 and 2 (Promega)(214). FR180204 is an Erk inhibitor with an IC50 of 0.14 to 0.31 μM with no impact on P38 until 10 μM concentrations (Tocris). The mechanism of action for FR180204 is by competing with ATP for active site(215).

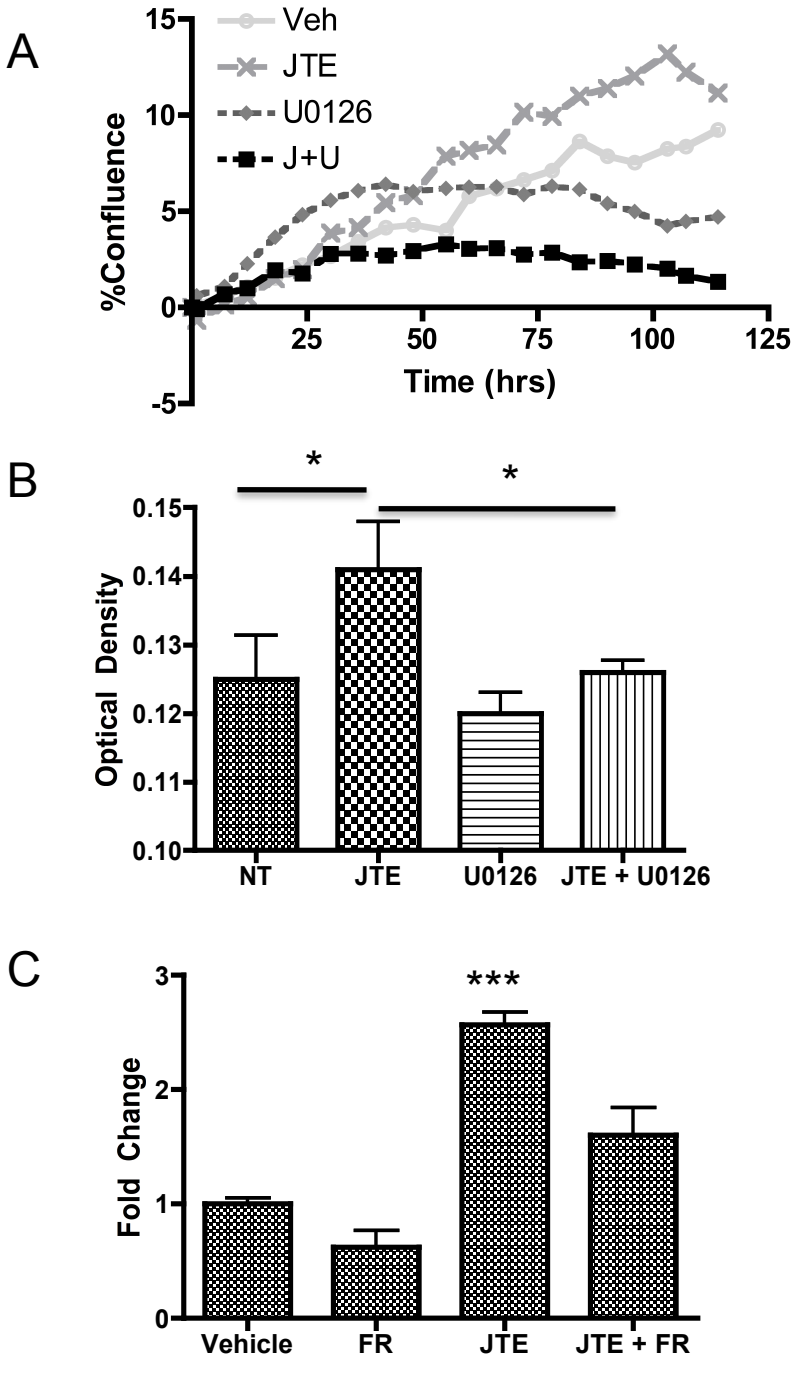


Figure 24: Evaluation of the role of Erk on JTE013 mediated increases in MSC proliferation using U0126 by Essen analysis following MSC proliferation up until 125 hours (a) MTS analysis of MSC proliferation at 24 hours using U0126 (b) or using the FR inhibitor (c). * indicates $p<0.05$ and * indicates $p< 0.001$.**

In part of A of Figure 25, cell confluence was analyzed using the Essen machine whereby an MSC specific set of cell detection parameters was established to assess cell number and confluence in 6-well cell culture dishes. MSCs were plated and their proliferation determined by pictures taken every 15 minutes for the time indicated. The graph shown depicts time points from every 6 hours indicating that as shown previously increased cell proliferation is observed following 3 uM JTE treatment. U0126 inhibition of Erk results in decreased cell proliferation as compared to vehicle treated cells and proliferation is further reduced following combined JTE and U0126 treatment. When proliferation assays were conducted using MTS in Figure 2b, a similar result was observed indicating that S1PR2 inhibition results in Erk dependent increases in cell proliferation. These results were further verified by the use of a second Erk inhibitor labeled FR and evaluated by MTS assay 48 hours following treatment as shown in Figure 26c.

Following the observed proliferative changes with Erk inhibition in combination with S1PR2 inhibition, we further explored the role Erk in migration following S1PR2 inhibition as shown in Figure 26. Inhibition of Erk activation results in decreased MSC migration as compared to wild type cells, which is maintained with combination treatment with JTE013 using both U0126 and FR180204.

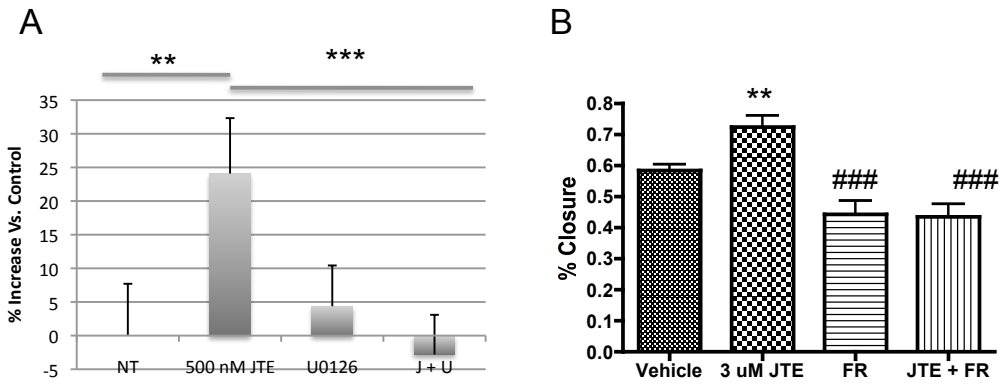


Figure 25: The role of Erk in JTE013 mediated increases in MSC migration using 1 µM U0126 treatment. Migration was assessed by Essen analysis of wound density following U0126 treatment (a) and FR treatment (b) where migration was assessed by scratch assay. ** indicates p<0.01 relative to vehicle control, * indicates p<0.001 relative to vehicle control, ### indicates p<0.001 relative to JTE013 treatment**

Based on the findings presented in this chapter, we conclude that S1PR2 acts antagonistically to S1PR1 in downregulating Erk activation potentially through an MKP-1 dependent mechanism. Inhibition of S1PR2 therefore results in increased Erk phosphorylation and corresponding increases in proliferation and migration. These conclusions while demonstrating the role of Erk in MSC migration and proliferation without identification of other downstream signaling factors that might contribute to this process. These conclusions do not exclude the involvement of other pathways in this process. Furthermore, more complete analysis of this pathway would include a G protein pull down assay for Gi, Gq, and G12/13. Nonetheless we have identified a critical component to the pathway controlling MSC migration and proliferation.

Chapter 5: The role of the remaining S1PRs in MSC behavior

The previous chapters focused on the role of S1PR2 in moderating MSC behavior and self-renewal. As S1PR2 does not function in isolation, it is critical to assess the role of the remainder of these receptors. In this chapter, we therefore look to focus on the role of the other receptors in impacting MSC signaling, proliferation, and migration. Limited focus was placed in this body of research on S1PR4 and S1PR5 due to the low levels of expression in MSCs and their documented roles primarily in lymphocytes and neuronal cells and natural killer cells, respectively. Unlike with S1PR2, both S1PR1 and S1PR3 demonstrate more dominant signaling through Gi with the functional impact of these receptors highly aligned with the canonical proinflammatory, pro-migratory, and proliferative effects of S1P signaling. S1PR3 due to its capacity for signaling through Gq and G12/13 does however have more complex potential pathways.

S1PR1 was the first S1P receptor identified with knock out animals embryonic lethal between E11.5 and E14.5 as a result of defective blood vessel development. S1PR1 has been best characterized for its roles in T and B lymphocyte egress that is the basis for FTY720 treatment in multiple sclerosis (168, 216). FTY720 is an orally available agent used in the treatment of multiple sclerosis through its actions as a functional antagonist of S1PR1, R3, R4, and R5 (217). Beyond this role in

lymphocytes, S1PR1 has critical angiogenic functions (42). S1PR3 is most often characterized for its functions in collaboration for S1PR1 and S1PR2. Knockout mice for S1PR3 are viable although decreased litter size has been notes (167, 168). S1PR3 has critical functions in dendridic cell mediated switch to a TH1 response. S1PR3 has been observed to be induced in astrocytes within multiple sclerosis suggesting a proinflammatory function of this receptor. S1PR3 has also recently been proposed as a biomarker for the inflammation associated with acute lung injury (113).

In this chapter, we employ both genetic and chemical methods to antagonize S1P receptor function. VPC23019 is a pharmacological antagonist of S1PR1 and R3 (Tocris/Avanti). We further explore the individual role of S1PR3 through the use of knockout mice that were kindly provided by the laboratory of Dr. Kelley Argraves from the Medical University of South Carolina. S1PR3 knockout mice are phenotypically normal although a smaller litter size is produced (113, 168).

The final pharmacologic agent that we use in this study is FTY720 or fingolimod marketed by Novartis under the trade name Gilenya. FTY720 is phosphorylated by sphingosine kinase 2 within the cell to produce the active metabolite of the drug. It is an agonist at a picomolar range for S1PR1 and S1PR3-5 resulting in significant functional antagonism of these receptors (42). The drug is FDA approved as an immunosuppressant for relapsing and remitting multiple sclerosis and is the first orally available approved therapy for this condition (42). Although demonstrating

effectiveness as a disease-modifying agent in MS with an acceptable safety profile, adverse effects commonly reported from the drug include bradycardia and hypertension (218). Treatment with FTY720 results in lymphopenia as a result of decreased lymphocyte egress into peripheral circulation with an increased regulatory T cell population (42). These effects are largely mediated by S1PR1 receptor endocytosis and degradation decreasing lymphocyte egress signaling.

We sought to initially investigate the role of S1PR1 and S1PR3 treatment on MSCs through the use of the pharmacologic antagonist VPC. Thirty-minute treatment of primary murine MSCs with VPC did not impact Erk activation as shown in Figure 27a. Evaluation of migration with increasing doses of VPC delivered at the start of the migration assay was conducted by Essen analysis as previously described. No changes in migration was observed between 0 nM and 5 μM VPC treatment as shown in Figure 27b. Treatment with 3 μM VPC initiated at the time 0 results in increased MSC proliferation as evaluated by percent confluence. Inhibition of S1PR1 and R3 therefore does not impact MSC Erk activation or migration but promotes cell proliferation.

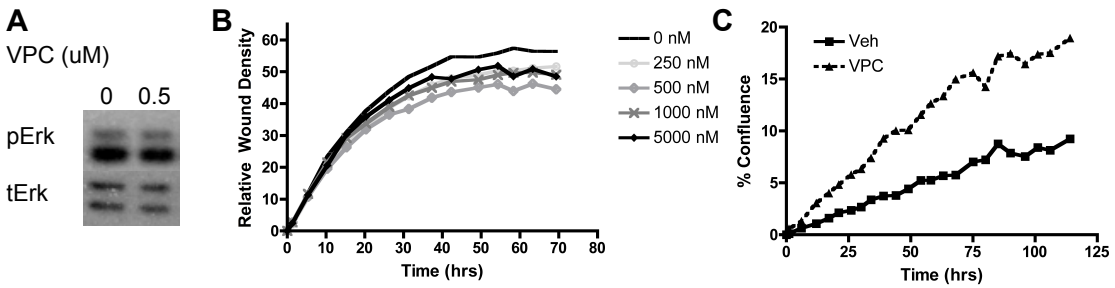


Figure 26: Evaluation of VPC treatment of MSCs (a) Western blot analysis of Phospho and total Erk expression following VPC treated MSCs (b) Essen migration analysis of VPC treated MSCs from concentrations of 250 nM to 5 µM. No significant differences in relative wound density were observed. (c) Essen proliferation analysis of VPC treated MSCs P<0.001 for VPC vs. Vehicle.

We next sought to determine whether this effect might be mediated by either inhibition of S1PR1 or S1PR3. We isolated MSCs from S1PR3 knockout animals and from age and gender matched wild type controls as shown in Figure 29. Evaluation of S1PR expression by quantitative real time analysis demonstrated a marked absence in S1PR3 in S1PR3 KO mice with concurrent diminished transcriptional expression of S1PR4 and R5. S1PR1 expression was maintained at comparable levels. There was potentially a decline in S1PR2 levels in S1PR2 knockout animals. Unlike what was seen in S1PR2 knockout mice without receptor compensation, changes in transcriptional expression of non-targeted receptors were seen in S1P3 KO mice. However, the most dominantly expressed receptors shown comparable expression levels.

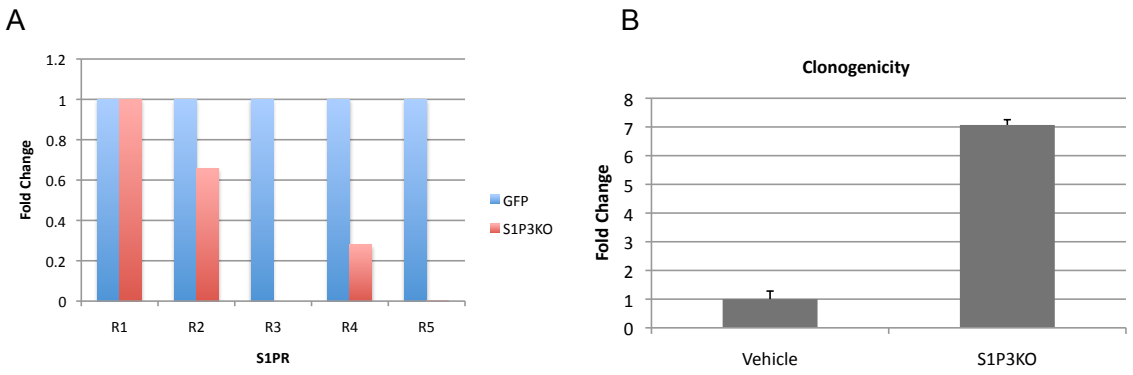


Figure 27: qPCR analysis of S1P receptor expression in wild type and S1PR3 KO MSCs. Fold change is shown as relative to GAPDH expression. Blue bars indicate control cell and red bars indicate S1PR3 KO cells (b) Clonogenic evaluation of S1PR3 KO and wild type cells. Increased cell clonogenicity is observed in S1PR3 KO cells as compared to wild type control cells.

Clonogenic evaluation of these cells demonstrates that S1PR3 knock out cells display increased clonogenicity as compared to wild type controls as shown in Figure 29b. When migration was assessed using scratch assay analysis followed by Image j quantification of initial scratch width and scratch width at 16 hours, increased migration was observed in S1PR3 knockout cells as compared to wild type controls as shown in Figure 29a. An additional control of VPC treatment in wild type cells was included demonstrating no change in migration as previously demonstrated by Essen analysis. Proliferation analysis by MTS assay was consistent with VPC treatment with KO cells proliferating more rapidly than the wild type controls suggesting that inhibition of S1PR3 may contribute more prominently to the VPC induced differences in proliferation.

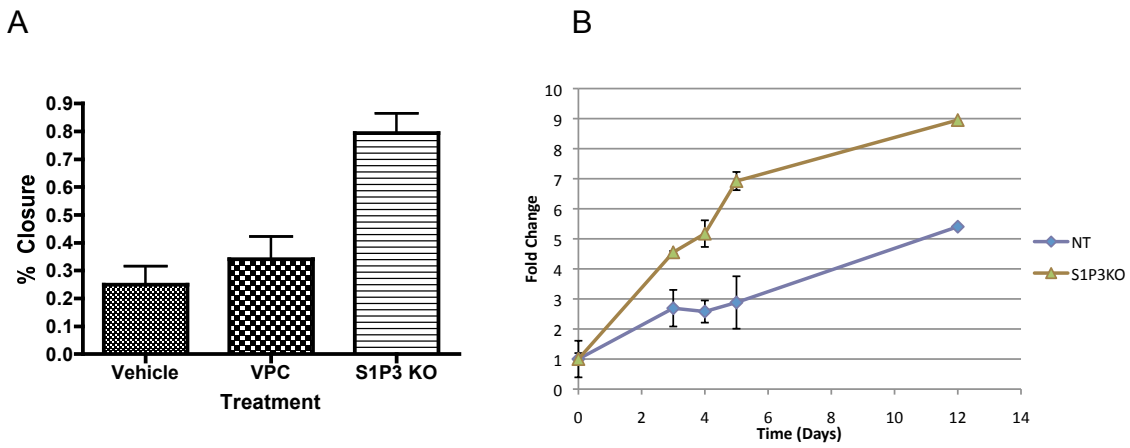


Figure 28: Evaluation of wild type, VPC treated, and S1PR3 KO cells for migration using % closure to evaluate . P< 0.001 for S1P3KO vs. vehicle control and no significant difference was observed for Vehicle vs VPC treatment (a) and proliferation analysis of control and S1PR3 KO cells with p<0.001 for wild type controls as compared to S1P3KO animals. Proliferation was assessed using MTS assay with fold change calculated based on cells prior to initiation of treatment.

Finally, we evaluated the effects of functional antagonism of all of the S1P receptors to the exclusion of S1PR2 with increasing doses of FTY720 treatment as shown in Figure 30. No changes in proliferation were observed with a dose range of 10 nM to 1 μM FTY720 as assessed by MTS assay as shown in Figure 30a. However, at this same dose range, there were no increases in migration at all concentrations of FTY720.

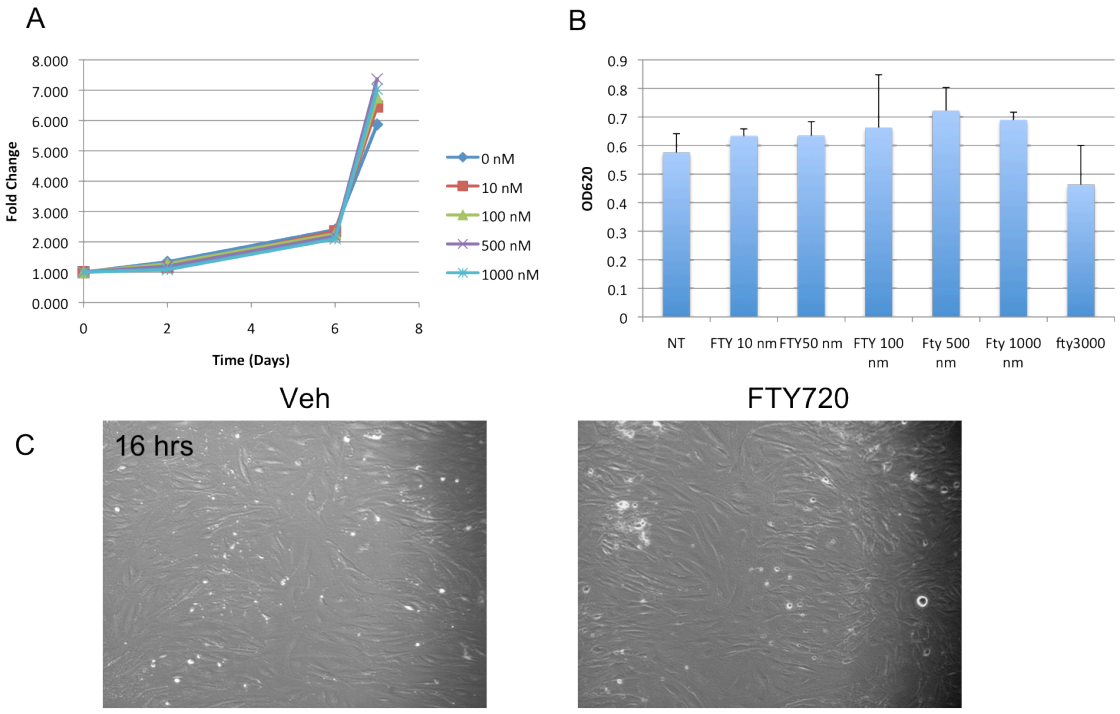


Figure 29: Proliferation analysis using MTS analysis following treatment with increasing doses of FTY720 from 0 to 1 µM (a) Proliferation analysis of FTY720 using 50 nm-3000 nM treatment as assess by crystal violet proliferation assay. Migration analysis following treatment with increasing doses of FTY720 using scratch assay(b) No significant differences in proliferation of migration were observed with FTY720 treatment in these dose ranges.

Although we have characterized the role of S1PR2 in impacting MSC differentiation, proliferation, and migration in previous chapters, S1PR1 and S1PR3 have documented roles in promoting MSC proliferation and migration often functioning antagonistically to S1PR2. When S1PR1 and R3 were inhibited by treatment with VPC in MSCs from C57Bl/6 mice, no changes in migration were observed, but an increase in proliferation was observed. The increased proliferation observed following VPC treatment is likely the result of S1PR3 as S1PR3 KO cells have

increased proliferation as compared to wild type counterparts. No changes in Erk expression were observed following VPC treatment indicating that the mechanism of these proliferative changes is not based on Erk activation as has been described for S1PR2 knockout cells. Based on this analysis, increased focus was placed on S1PR2 although increased research certainly needs to be conducted further dissecting the pathways of S1PR3 in MSCs.

Chapter 6: Role of S1PR2 in MSC adipogenic and osteogenic differentiation

Previous chapters have examined the role of S1PR2 in migration and proliferation of MSCs. However, critical to the function of a stem cell is its ability to differentiate into its derivative lineages. For MSCs, differentiation into adipogenic and osteogenic lineages is essential to the definition of an MSC. In the first chapter, we demonstrated the capacity of MSCs to differentiate into adipocytes expressing FABP4 and mature osteoblasts expressing osteopontin. I followed up on this finding in this chapter by addressing the capacity of MSCs to differentiate following modulation of S1PR2. Due to experimental limitations chondrogenic lineage was not addressed in this chapter.

Differentiation into adipocytes is controlled by master regulator PPARY whereas induction of osteogenesis is controlled by master regulator Runx2. In either case a variety of factors impact the expression of these master regulators. Evaluation of the role of MAPKs in this process has suggested that although JNK activation is critical to extracellular matrix synthesis in the osteogenic process, Erk is upregulated during days 7-11 of induction by osteogenic differentiation media during the critical differentiation time with a return to basal expression status following differentiation (219). Overexpression of a dominant negative Mek1 results in the diminished osteogenic activation (219). In contrast, Erk is not required for

MSC adipogenic differentiation and Erk phosphorylation of PPARγ diminishes its transcriptional activity (220).

Induction of osteogenesis in vitro has been achieved through a variety of protocols and agents. Most commonly osteogenic differentiation media includes a combination of dexamethasone, ascorbic acid, and B-glycerophosphate (221). Osteogenic differentiation can be evaluated by osteopontin staining, alkaline phosphatase activity, or alizirin red staining (219, 222). Adipogenic differentiation media commonly contains isobutylmethylxanthine which increases PPARγ by acting as a nonselective phosphodiesterase inhibitor and insulin which a promotes proliferation and differentiation of MSCs (223). Adipogenic differentiation can be evaluated by oil red O staining or FABP4 expression (224).

Differentiation of wild type and S1PR2 knockout MSCs was evaluated following induction into osteogenic and adipogenic differentiation lineages according to the instructions provided by the manufacturer for the R&D systems murine MSC functional identification kit. Adipogenic differentiation media contained hydrocortisone, isobutylmethylxanthine, and indomethacin and osteogenic supplementation media contained dexamethasone, ascobate-phosphate, proline, pyruvate and TGFβ. Exact concentrations of these agents are proprietary. Induction media was changed every 2-3 days for 2-3 weeks and evaluated by osteopontin immunofluorescent staining for osteogenesis and FABP4 staining for adipogenesis.

Cells were evaluated by confocal microscopy with cells counted in at least 10 fields for positive or negative staining as shown in Figure 31. Cells lacking expression of S1PR2 had decreased induction of differentiation for both adipogenic and osteogenic lineages, suggesting a more central role for S1PR2 in MSC differentiation rather than a lineage specific role. However, these MSCs still demonstrated the capacity to differentiate into both lineages. Representative images of the staining are shown in Figure 31c.

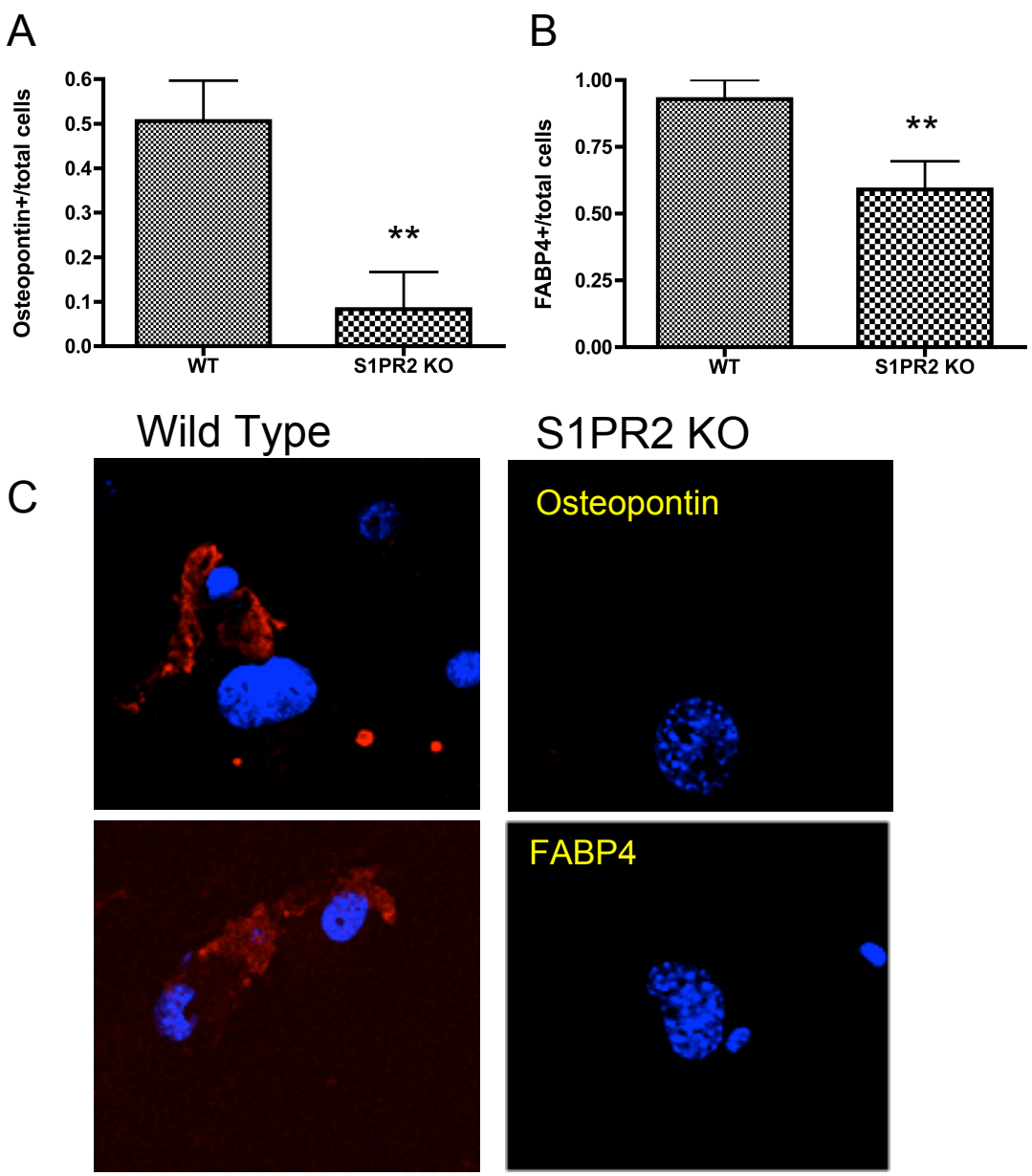


Figure 30: Evaluation of S1PR2 KO cell differentiation. Osteogenic differentiation was assessed by the number of cells expressing Osteopontin relative to the total number of cells per each high powered field (A). Adipogenic differentiation was similarly assessed using FABP4 as a marker (b) Represenative images of wild type and KO cells are shown in (C).

Following evaluation of differentiation of MSCs in knockout cells, we treated cells with JTE013 in standard culture media during each media change for MSCs.

However, given concern for the strong capacity of the induction media to promote differentiation, we sought to examine differentiation of MSCs following long-term culture in the absence of induction media and cultures in standard cell culture media. Similar to the results seen with S1PR knockout cells following induction, inhibition of S1PR2 resulted in decreased MSC differentiation into both adipogenic and osteogenic lineages as shown in Figure 32.

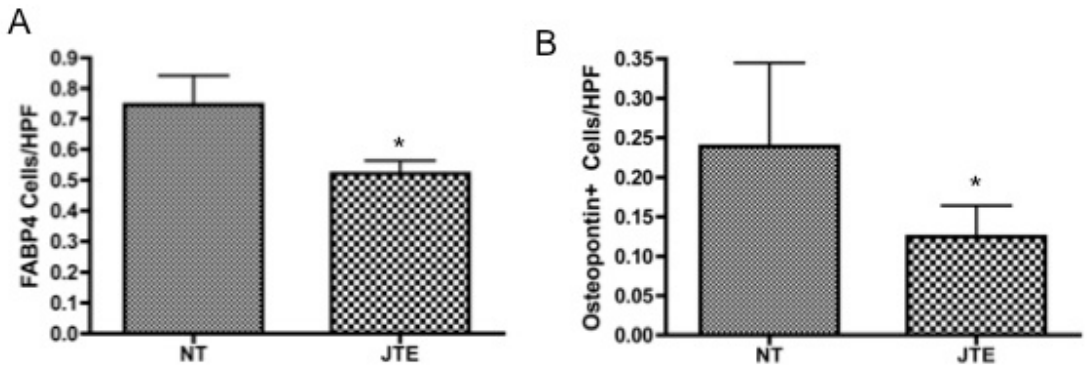


Figure 31: MSC differentiation into osteocytes and adipocytes with S1PR2 inhibition. Evaluation of JTE013 treated MSCs for differentiation into adipogenic lineages. Differentiation was calculated as the number of cells expressing FABP4 per high powered field (a). Similar analysis was conducted for osteogenic differentiation (b) using Osteopontin as a marker for differentiation. * indicates $p<0.05$ based on a student t-test.

Following identification of decreased differentiation of MSCs following inhibition of S1PR2 we sought to determine the mechanism by which this decreased differentiation was occurring. We evaluated this both on the pathway specific level and at the level of MSC self-renewal. MSCs were treated in 100 mm dishes with 1 μM JTE in duplicate for 30 minutes. RNA was synthesized using Qiagen RNEASY and quantified based on the Beckman spectrophotometer 260 nm absorbance values. RNA was analyzed for RNA copy number using Nanostring analysis conducted by

murine code set -1 developed my the Medical University of South Carolina Center for Oral Health Research. Data was normalized to positive controls using nSolver Analysis software with background reduced to accurately depict RNA copy number. Bone morphogenic protein (BMP4) promotes MSC differentiation into both adipogenic and osteogenic lineages. Sox9 is upregulated and dictates chondrogenic differentiation whereas αSMA is upregulated in fibroblast differentiation pathways. No changes in expression were observed in any of these pathway specific differentiation genes following inhibition for S1PR2 as shown in Figure 33. However, for a better analysis of these factors using a dose range and time course including longer time points will need to be evaluated to properly evaluate changes following the differentiation process. At a more central level, MSC differentiation is controlled by pluripotency factors that promote MSC self-renewal and inhibit MSC differentiation. Figure 33b shows that Nanog is prominently expressed both in MSCs have not been passaged and MSCs at passage 4. This conclusions supports the conclusion that Nanog plays a critical function in MSC differentiation and is not an artifact of cell culture processes.

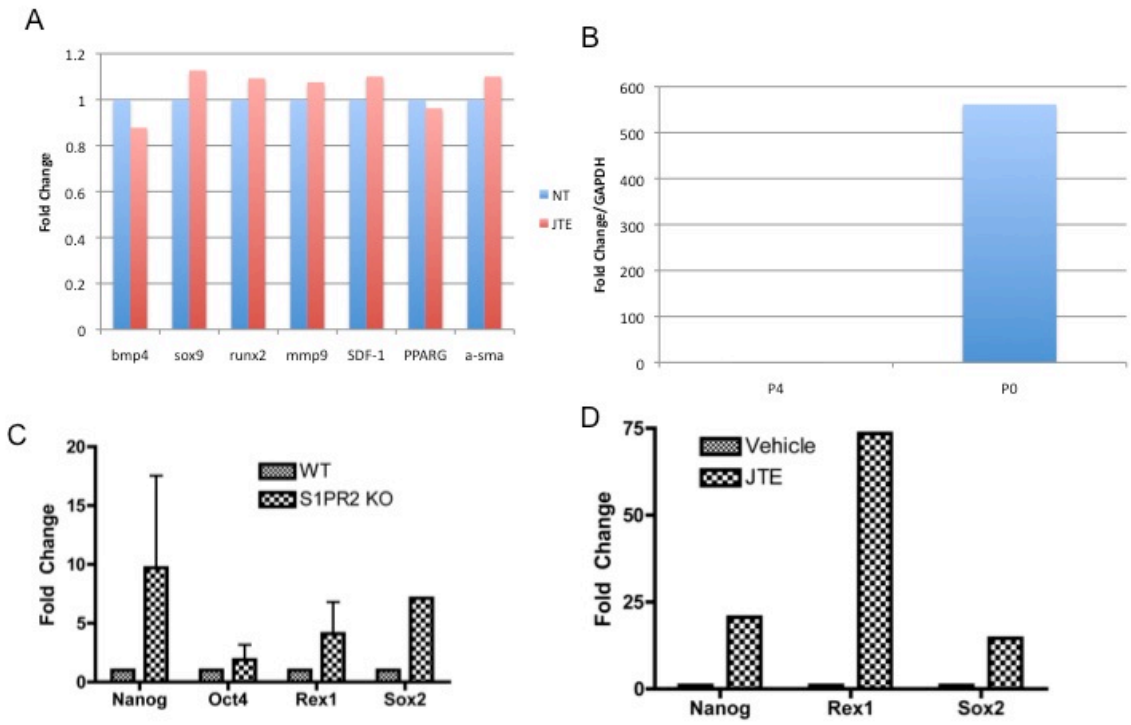


Figure 32: Mechanism of S1PR2 impact on MSC differentiation into adipogenic lineages (a) Nanostring analysis of JTE013 treated MSCs for number of transcripts of the indicated genes (b) qPCR evaluation of Nanog expression in uncultured cells as compared to P4 cultured cells after cell sorting (c) qPCR expression of MSCs in wild type and KO cells for pluripotency factors (d) qPCR expression of JTE013 treated MSCS for pluripotency factors.

In MSCs, there is variation in the pluripotency factors that are reported to be expressed. However, reports consistently implicate Nanog, Oct4, Rex1, and Sox2 as being expressed and critical to MSC self-renewal. Literature reports some controversy as to whether Nanog is expressed in vivo or whether the culture conditions of MSCs cause its upregulation. To evaluate whether Nanog was expressed in MSCs in vivo we compared sorted passage 4 cells used for experiments as compared to cells sorted directly from bone marrow and pelleted by centrifugation without culturing as shown in Figure 33C and D. These cells strongly

expressed Nanog with a 561-fold increase in Nanog expression as compared to passage 4 cultured cells. To evaluate pluripotency factors expressed in MSCs, qPCR was conducted following S1P inhibition. RNA was isolated, cDNA was synthesized, and quantitative real time PCR performed.

Increases in the transcriptional activity of pluripotency factors were observed for all pluripotency factors as evaluated in Figure 33 C and D. The diagrams shown demonstrate fold change for representative quantitative realtime PCR expression of one of three independent experiments. Consistent results were reported for both inhibition of S1PR2 using JTE013 and in the knockout cells as compared to wild type cells. The changes in pluripotency factors suggest a parallel increase consistent with the changes in the differentiation of MSCs. Further evaluation of differentiation with Nanog inhibition using siRNA or overexpression would elucidate the role of the pluripotency factors in impacting the reduction of differentiation follow S1PR2 inhibition.

The capacity of MSCs for differentiation is critical to MSC self-renewal and the maintenance of a pluripotent MSC population. Our results indicate that S1PR2 is critical to mediating MSC differentiation into adipogenic and osteogenic lineages. Inhibition of S1PR2 results in diminished MSC differentiation with associated increases in MSC pluripotency factor qPCR expression. Differentiation of osteogenic and adipogenic differentiation is mediated by a balance of factors expressed that

either promote differentiation or maintenance of the undifferentiated state. If a differentiated state is promoted the exact factors expressed will determine the lineage of differentiation into the three classical pathways osteogenic, adipogenic, or chondrogenic or the additional pathways that have been characterized.

Chapter 7: Inhibition of S1PR2 in prostate cancer

S1PR2 may have a critical function in the maintenance of other stem cell populations not limited to MSCs. Cancer stem cells are tumors cells within the malignant clonal population that can repopulate a cancer. Identification and elimination or these cells is therefore necessary to consider in strategies designed for tumor control and elimination. Serial transplantation assays and lineage tracing assays have been conducted examine which cell populations might be considered a tumor stem cell (225). In prostate cancer, stem cells have been identified and characterized to have a number of different marker characterizations. Most commonly prostate cancer stem cells have been shown to express CD44+, α2β1 integrin, and CD133+ marker expression with enzymatic activity of aldehyde dehydrogenase (93). Previous work has identified that S1P stimulated increases in Erk phosphorylation result in increased CD44+ expression in human colon cancer cells (209, 226).

Aldehyde dehydrogenase (ALDH) can be evaluated by Aldefluor assays, which provide aldefluor substrate that when metabolized results in fluorescent expression that can be detected by standard Flow Cytometry. ALDH has been shown in prostate cancer to be highly expressed in a small subset of the population with 0.5 to 6% of cells in human prostate cancer cell lines expressing ALDH and 0.6 to 4% in tumor

clinical samples (227). Cells expressing ALDH have been shown to have increased clonogenicity, migration, sphere formation, metastatic in vivo, and invasion (228-230). These assays have suggested that ALDH may be able to be used to identify tumor initiating or metastasis initiating tumor stem cells.

CD133 or prominin-1 is a peptaspan membrane glycoprotein associated with cells having a high proliferative potential (231). In prostate cancer biopsies, CD133+, when expressed, represented less than 1% of cells. Expression was identified in 50% of prostate cancers with increases in CD133 expression in matched metastatic samples as compared to the primary tumor (231). When CD133+ cells are transplanted from primary xenografts, less than 10 cells are required for tumor formation. Hypoxia, common in solid tumors, results in increased CD133+ expression through Oct4 and Sox2 upregulation (232).

CD44 is a homing adhesion molecule that acts as a transmembrane glycoprotein that interacts primarily with hyaluronin and other extracellular matrix components (233, 234). These components are highly upregulated with the desmoplastic environment of prostate cancer. Desmoplasia is the expansion of tumor stroma within the inflammatory tumor microenvironment associated with increased ECM deposition including collagens, fibronectin, proteoglycans, and hyaluronin (235, 236). The formation of this desmoplastic environment provides a significant barrier to solid tumor treatment due to decreased drug delivery. Pellacani et al. have shown

that CD44+, α2β1 integrin, and CD133+ prostate cancer cells have increased self-renewal capacity in addition to a tumor initiating capacity suggesting this combination as a important cell marker combination for prostate cancer stem cell identification(231).

In this chapter, we evaluate the impact of S1PR2 on prostate cancer stem cell formation. The role of S1PR2 in prostate cancer has only been characterized to a limited capacity with Beckham et al. demonstrating that increased S1PR2 expression results from increases in acid ceramidase. This increase is associated with an increased oncogenic phenotype as evaluated by soft agar colony formation and by MTS proliferation analysis (237). S1PR2 has been preliminarily characterized as promoting prostate cancer progression in vitro.

Following the work of Beckham et al. we examined the transcriptional expression of acid ceramidase and the sphingosine kinases following treatment with 3 μM JTE013. Overnight treatment with 3 μM JTE013 was conducted in TRAMP-C2 murine prostate cancer cells. RNA was isolated by trizol extraction and cDNA synthesized according to manufacturer's instruction using the Biorad iScript cDNA synthesis kit. Quantitative realtime pcr was conducted as previously described. JTE013 treatment results in the transcriptional upregulation of acid ceramidase as well as a moderate increase in sphingosine kinase 1 and 2 suggesting upregulation of the oncogenic pathway characterized by Beckham et al. in prostate cancer as shown in Figure 34.

Further evaluation of the transcriptional expression of pluripotency factors commonly expressed in prostate cancer tumor initiating stem cell populations suggest an increase in Nanog expression consistent with a more stem cell line population as shown in Figure 34B. JTE013 may promote increased oncogenesis and an increased stem-like population in Tramp-C2 prostate cancer cells. This discovery prompted further evaluation of the stem-like characteristics of this population.

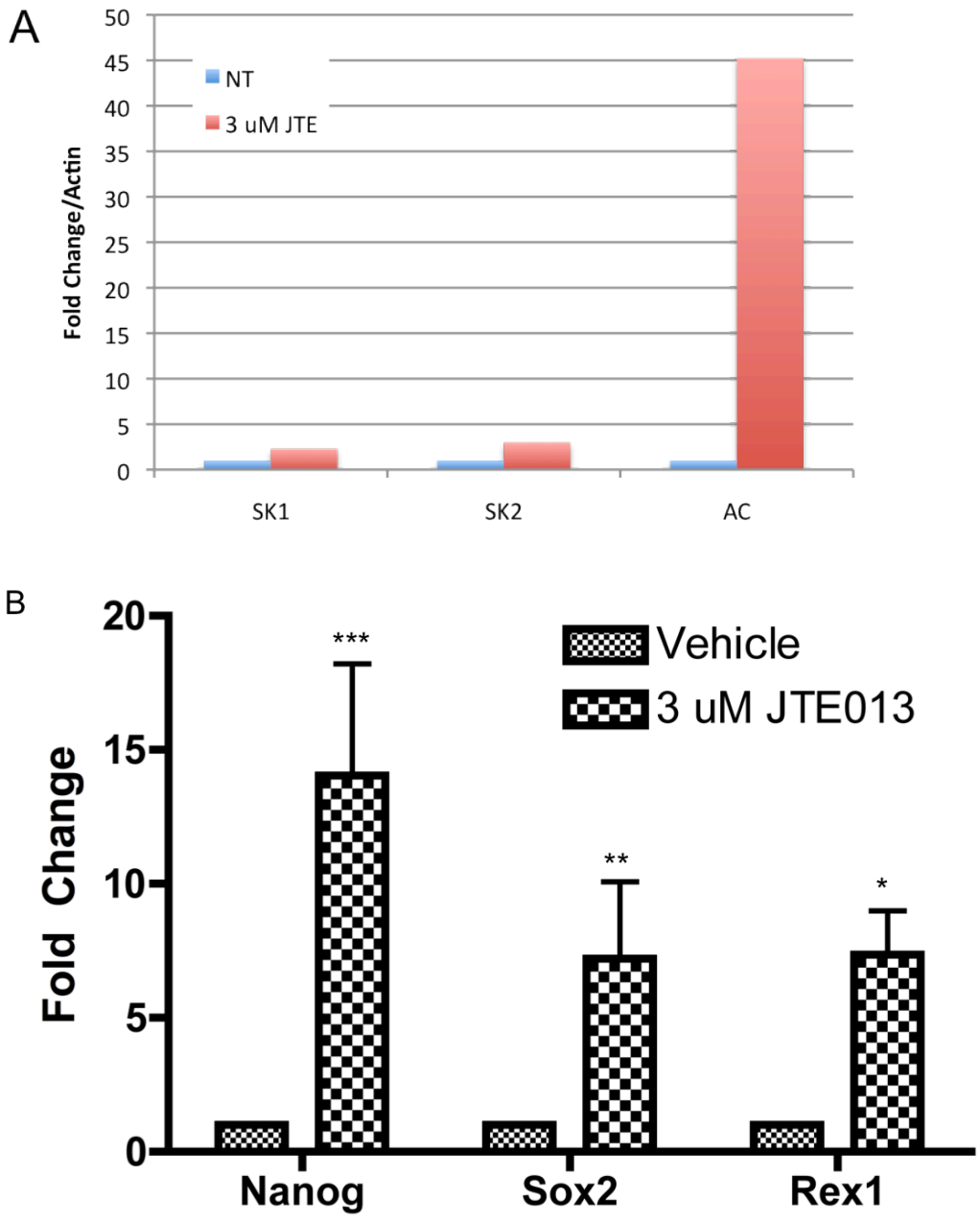


Figure 33: Transcriptional analysis of TRAMP-C2 cells following JTE013 treatment. qPCR analysis of S1P generating enzymes in TRAMP-C2 cells normalized to actin controls (a) qPCR analysis of pluripotency genes in TRAMP-C2 cells following JTE treatment (b).* indicate $p<0.001$, ** indicates $p<0.01$ and * indicates $p<0.05$**

Based on these findings, and the suggestion of a more stem-like population within JTE013 treated prostate cancer cells, we evaluated the marker expression of cell surface markers of prostate cancer stem cells for CD44+, CD133+ and the enzymatic activity of ALDH. Cells were plated in cell culture dishes treated overnight with the indicated doses of JTE013. Cells were lifted by trysinization, stained for 20 minutes with the indicated antibody or staining kit according to manufacturers instructions for the Aldefluor aldehyde dehydrogenase expression kit. CD44+ cells composed over 99% of the TRAMP-C2 cell population and as such further evaluation of this marker in differentiating tumor initiating cells was not used. Aldefluor analysis of TRAMPC2 cells yielded more informative results in the evaluation of JTE013 treatment and its impact on the stem cell population as shown in Figure 35. For aldefluor treatment, DEAB, an inhibitor of aldehyde dehydrogenase, was used as a negative control. Vehicle treatment included an equivalent volume of DMSO. Both ALDH and CD133+ have increased expression following JTE013 treatment. Representative plots of flow Cytometry are shown in figure 35a and 35b with quantitative evaluation shown in 35c. Flow cytometry was conducted using a BD FACS Calibur with subsequent analysis of cell populations evaluated by Flo Jo.

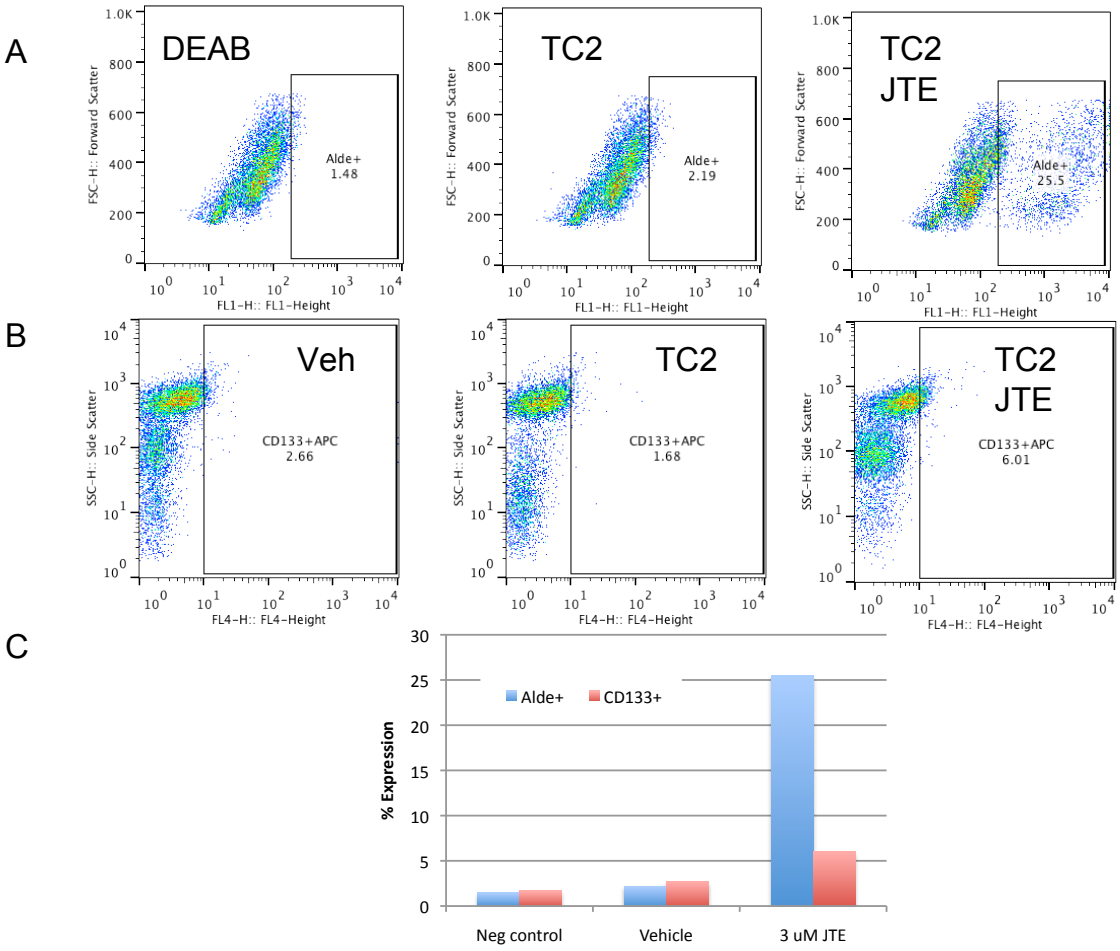


Figure 34: Flow Cytometry analysis of TRAMP-C2 cells for stem cell characteristics. Aldefluor expression analysis of TRAMP-C2 following JTE013 treatment. DEAB is an inhibitor of aldefluor dehydrogenase used as a negative control (a) CD133+ analysis of JTE013 treated TRAMP-C2 cells. Cell population summary for ALDH and CD133 expressions summary (c).Representative images are shown for two independent experiments.

From this analysis, increased expression of PCa stem markers was observed following JTE013 treatment in TRAMP-C2 murine prostate cancer cells. Expression levels of untreated ALDH were consistent with that of the previously published results for prostate cancer cell lines. This data suggests that S1PR2 inhibition results in an increased stem cell like population based on marker expression.

An interesting dichotomy proposed by the following analysis is in the dual and somewhat antagonistic effect in which S1PR2 inhibition promotes increased MSC proliferation but inhibition in PCa promotes an increased prostate cancer cell proliferation as shown by Beckham et al. S1PR2 inhibition simultaneously promotes an increased stem cell like phenotype in PCa. As stem cells are largely thought to be quiescent, we sought to analyze whether the small subpopulation of cells expressing CD133 and Alde was itself demonstrating changes in proliferation consistent with the remainder of the cell population. To evaluate this phenomenon, cells were stained with cell trace violet for 5 minutes at 37 degrees Celsius. Baseline fluorescence was assessed in this cell population using the FACS ARIA II flow Cytometry and this analysis was considered the day 0 samples. The remainder of the treatment groups were either plated with vehicle treatment or JTE013 treatment as indicated. Cells were trypsinized and analyzed 48 hours following treatment as shown in Figure 36. An increased left shift indicates further dilution of the dye and therefore increased proliferation. In these cells, no increase in cell proliferation was observed following 7.5 μM JTE013 treatment.

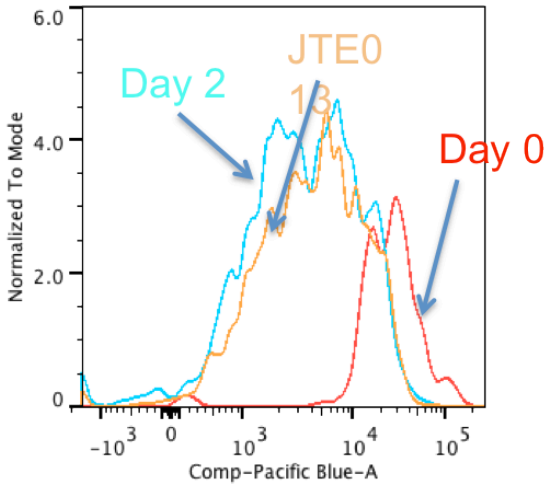


Figure 35: Proliferation analysis of JTE013 treated TRAMP-C2 prostate cancer cells by cell trace violet expression. Red indicates staining at t=0, yellow indicates JTE013 treatment at Day 2, and green indicates vehicle treated cells at Day 2.

To further evaluate whether subpopulations of JTE013 might be proliferating at different rates, prostate cancer cells were additionally stained at day 2 with aldefluor as previously described with simultaneous proliferation analysis as shown in Figure 37.

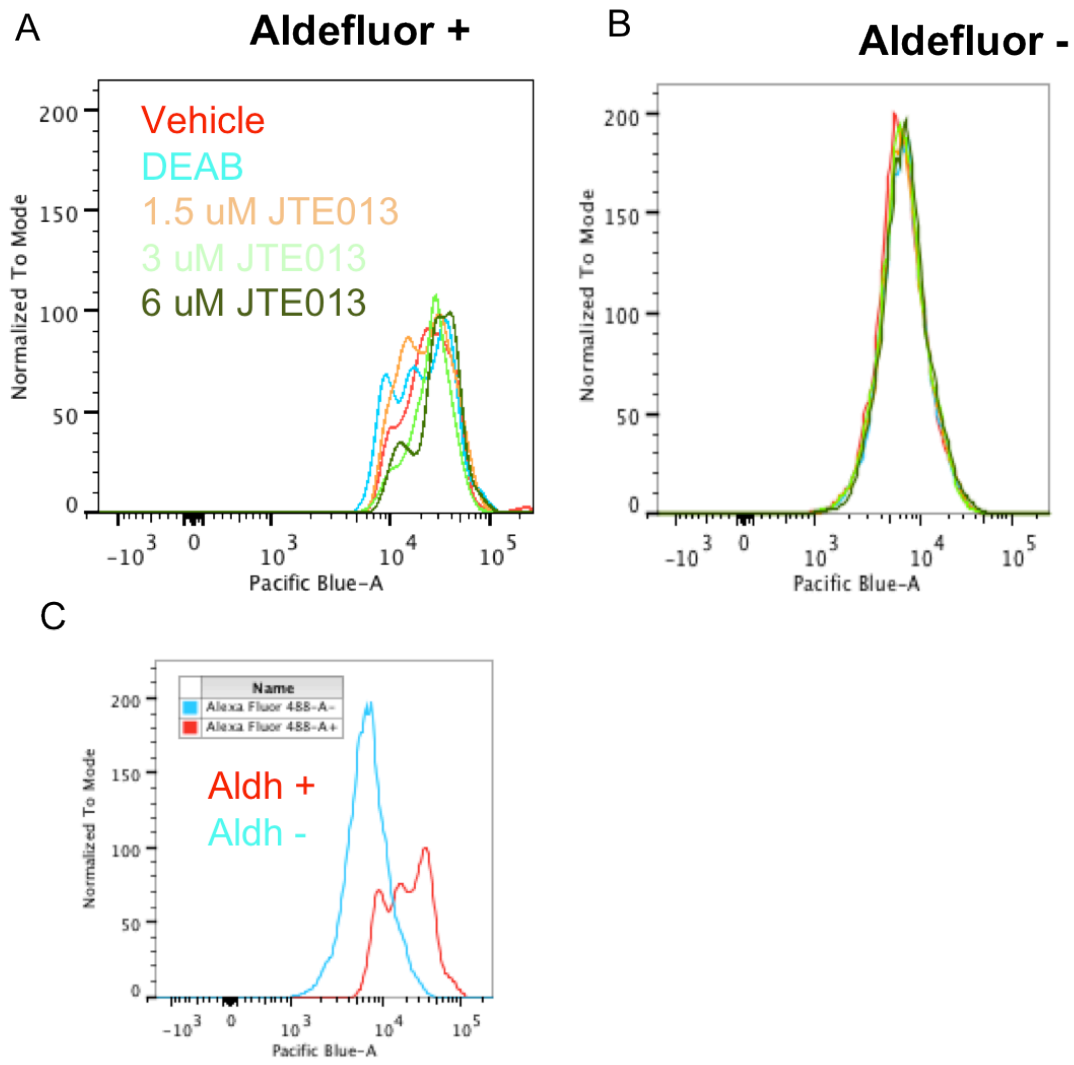


Figure 36: Evaluation of cell trace violet expression of aldefluor expression in TRAMP-C2 aldefluor positive prostate cancer cells. Cells were treated with JTEO13 from 1.5 -6 µM JTE013 with negative Vehicle controls and DEAB, an inhibitor of Aldehyde dehydrogenase (a) Similar analysis of cell trace violet staining was conducted in TRAMP-C2 aldefluor negative cells (b) Comparison between cell trace violet staining of TRAMP-C2 cells treated with vehicle control for Aldefluor positive and negative cell populations (c).

Similar to the previous evaluation of JTE013 on TRAMP-C2 prostate cancer cells, no impact on proliferation overall was observed following JTE013 treated protaste cancer cells as shown in Figure 37. JTE013 treatment between 1.5 and 6 µM JTE013

demonstrated no differences in proliferation as assessed by cell trace violet. This same result was observed in the aldefluor positive TRAMP-C2 population, the aldefluor negative population, and the total cell populations. While no differences were observed between the non-treated and treated cell populations, one interesting observation was noted. There was a distinct difference in proliferation between the aldefluor negative and the aldefluor positive cells. Aldefluor positive cells have had decreased left-ward shift and therefore decreased proliferation as compared to aldefluor negative cells. We therefore conclude that JTE013 promotes an increased stem cell like phenotype in prostate cancer cells consistent with that of previous reports for prostate cancer cells following S1PR2 inhibition.

To assess the role of S1PR2 inhibition on TRAMP-C2 cells, in vivo analysis of this system was conducted. TRAMP-C2 tumors were grown in C57BL/6 mice. Following tumor presentation in these mice, tumors were randomized to vehicle treatment or JTE013 treatment. No differences were observed in initial tumor volume between vehicle and JTE013 randomize group. Throughout the experiment, no change in animal weight was observed following JTE013 treatment. JTE013 was delivered intraperitoneally at a 3 mg/kg dose to mice as 2 doses 72 hours apart. Following treatment with JTE013, no differences in tumor proliferation were observed as shown in Figure 38B. On flow cytometry analysis no differences were observed between the mean fluorescence intensity of CD133+, Aldh+, or dual positive cell

populations as shown in Figure 38a, c, or d. This indicates that no differences in the stem cell population were observed following JTE013 treatment in these cells.

This experiment demonstrated that the JTE013 enhanced changes in the stem cell population that were observed in vitro were not recapitulated in our in vivo experiment. Limitations of this experiment were the lack of indicators of successful S1PR2 inhibition. Experimental difficulty in assessing

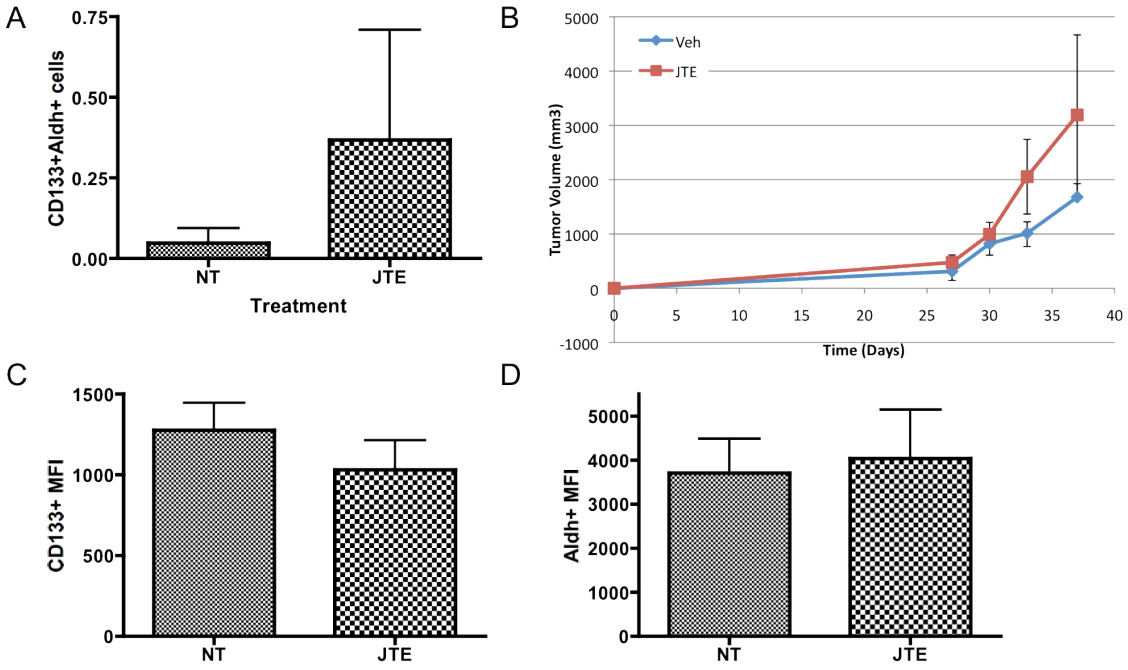


Figure 37: In vivo tumor analysis of JTE013 treated TRAMP-C2 prostate cancer tumors in C57Bl/6 mice. Tumors were grown until the average tumor volume was greater than 200 mm^3. Treatment was initiation with JTE013 as two 3 mg/kg doses of JTE013 72 hours apart. Tumor digestion was conducted following tumor removal with analysis conducted for vehicle treated and JTE013 treated tumors for expression of ALDH+ CD133+ cells (a). Tumor volume was evaluated following JTE treatment as shown (B). The MFI for CD133+ cells (c) and Aldh+ cells (c) for each treatment group is shown. N=4 for each treatment group.

this function results from the lack of quality antibodies to S1PR2 protein and additionally lack of S1PR2 activity assays. The timing of the delivery of the JTE may be a critical factor in impacting the tumor response to JTE013 treatment.

Cancer stem cells have been described in a number of different solid cancer types and represent a cell population critical for treatment approaches as the failure to eradicate cancer stem cells allows for tumor recurrence and metastasis following treatment of the primary tumor. It is therefore important to understand the mechanisms by which these cancer stem cells are generated. S1P concentrations are high within a tumor environment due to the high inflammation of the tumor and the often hypoxic nature of solid tumors resulting from the poor vascular perfusion. Although many of the contributions of this high S1P concentration have been evaluated, further understanding of the functions of S1P in prostate cancer stem cells is required. Inhibition of S1PR2 in prostate cancer cells results in an increased stem cell phenotype in this population as assessed by marker expression using CD133+ expression with further validation of expression of Nanog and aldehyde dehydrogenase. Additional characterization of these cells reveals that the prostate cancer cells expressing aldefluor have decreased proliferation as compared to their aldefluor negative counterparts. This relative quiescence suggests that these cells could represent a cancer stem cell population consistent with the marker expression.

Chapter 8: MSC co-culture with TRAMP-C2 prostate cancer cells

MSCs are actively recruited to prostate cancer tumors and once there increase the invasive and metastatic potential of the tumor (86). This is accomplished through a number of mechanisms including increasing the stem cell population of tumor cells and through the action of secreted factors that increase the motility and the epithelial to mesenchymal transition. One of the key secreted factors that mediates these changes is SDF-1, although a number of other contributing factors have also been identified (238). Based on these findings we investigated the impact of S1PR2 inhibition on MSC co-culture with Tramp C2 cells.

Evaluation of a number of proteins with expression changes that impact prostate cancer cell progression was evaluated. Nanog, although originally identified as critical to embryonic stem cell self-renewal is expressed in prostate cancer tumors and impacts the tumorigenicity of the cells (239). Increased Nanog expression results in increased drug resistance and increased tumor regeneration whereas knockdown of Nanog diminishes the tumorigenicity and clonogenic growth of the cells (240-242). B-catenin is a protein mutated in 5% of prostate cancers and that demonstrates 20-30% increased nuclear and cytoplasmic staining in prostate cancer as compared to normal prostatic epithelium (243, 244). This increased B-catenin expression results in increased growth and tumor invasion. Overexpression

of B-catenin in mouse models has been shown to promote murine PCa progression through increased invasive potential (245). Survivin in an inhibitor of apoptosis with increased expression associated with cancer progression and drug resistance. Prostate cancer cell lines highly express survivin (246). Finally, cyclin D1 is a regulatory of G1 phase progression in the cell cycle and may contribute to androgen receptor independence in prostate cancer (247).

The genes mentioned above were evaluated following delivery of either conditioned media generated from MSCs that were vehicle treated or MSCs that were treated with JTE013 overnight. This media was given for 24 hours to Tramp C2 cells and lysates collected for western analysis (244). Increased nanog, B-catenin, CD44, and cyclin D1 were observed when cells were treated with JTE013 conditioned MSC medium as shown in Figure 38.

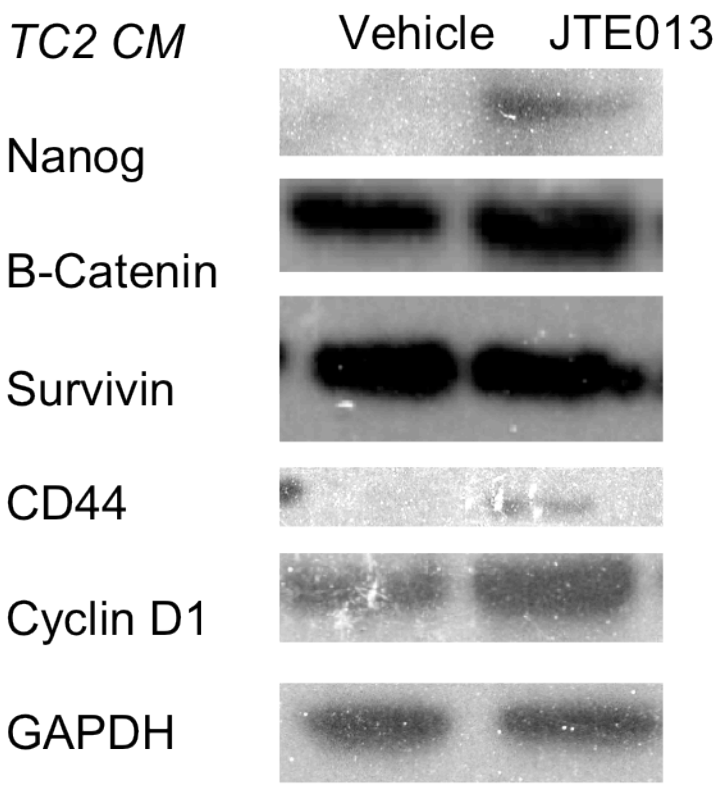


Figure 38: Western blot evaluation of TRAMP-C2 cells treated with JTE013 conditioned media on MSCs. Conditioned media from MSCs with either vehicle treatment or JTE013 treatment. Media was centrifuged down and transferred to TC2 cells for 24 hours prior to collection of the cells for western blot analysis.

Having observed that conditioned media with decreased S1PR2 expression predisposes MSCs to express genes consistent with increased cancer cell progression and proliferation, further investigation using a transwell cell culture system was merited. Tramp C2 cells were plated on the bottom well of trans-well plate with MSCs plated in the top well. Treatment with 3 μM JTE013 was delivered in the top well of the plate. Proliferation was assessed in aldefluor + cells as previously described using cell trace violet staining as shown in Figure 39. Increased proliferation was observed in aldefluor positive prostate cancer cells but

no change was observed in aldefluor negative cells. This change in proliferation was reflected in the leftward shift in cell trace violet fluorescent expression.

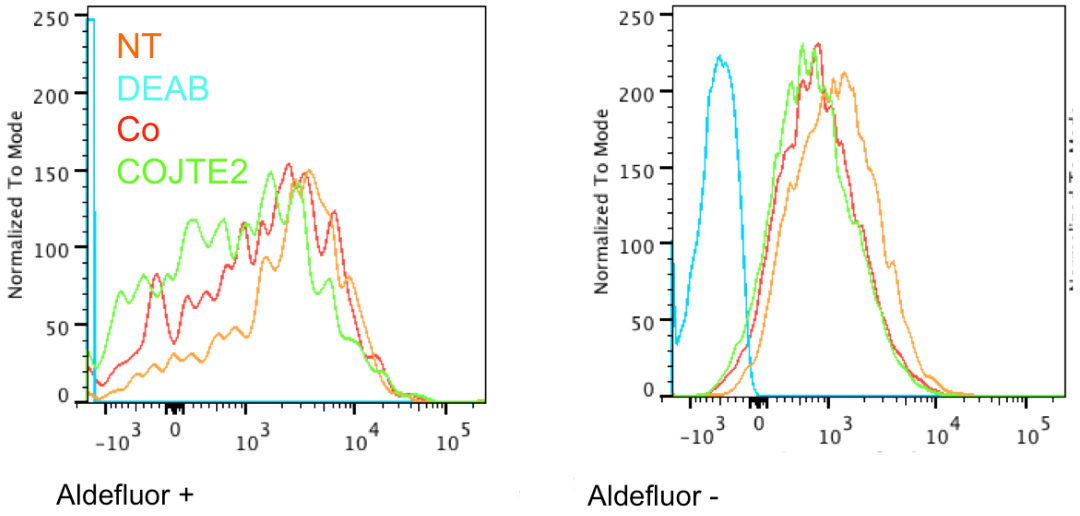


Figure 39: Cell trace violet evaluation of co-cultured MSC and TRAMP-C2 cells. Orange indicates no treatment, teal indicates DEAB, Red indicates co-culture and green co-culture with JTE treatment. JTE treatment was given to the well containing MSCs and analysis was separated by aldefluor expression as shown with aldh+ positive cells on the left and negative cells on the right for each condition.

Transwell co-culture system was also utilized to evaluate transcriptional expression of genes associated with epithelial to mesenchymal transition and metastasis. Increased JTE013 expression is associated with decreases in EMT gene expression following JTE treatment and JTE treatment of MSCs in co-culture.

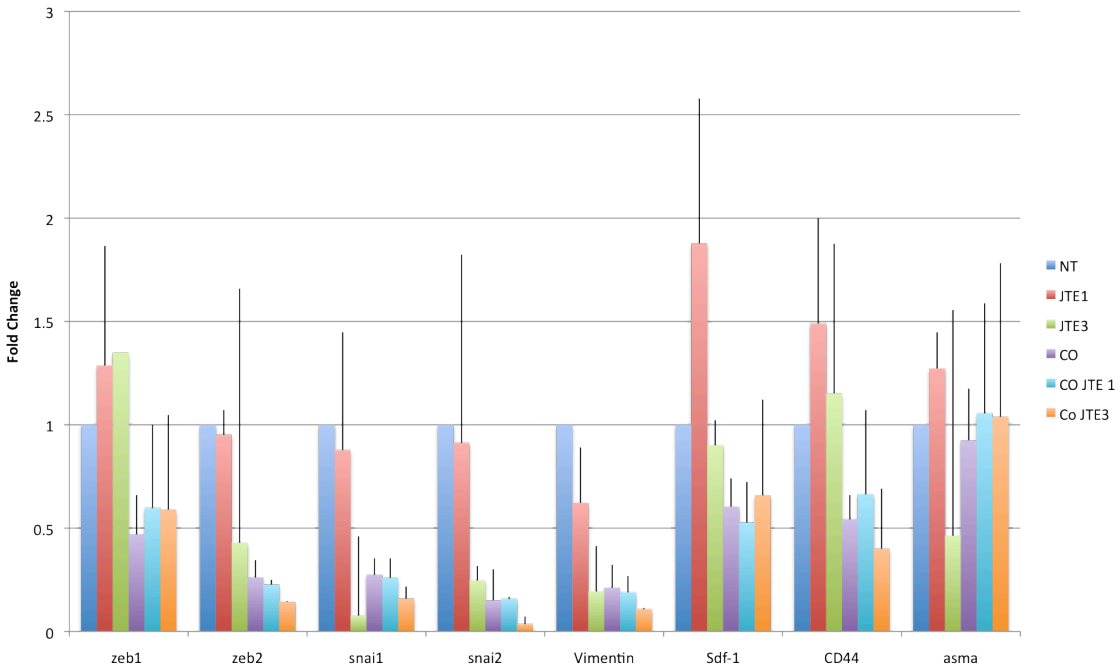


Figure 40: qPCR analysis TRAMP-C2 prostate cancer cells in a co-culture system composed of TRAMP C2 cells and MSCs for genes associated with EMT and metastasis. JTE 1 indicates 1μM JTE013 treatment and JTE3 indicates 3 μM JTE013 treatment. JTE013 treatment was given to MSC containing wells. Co indicates that a co-culture system was employed either in the presence or absence of JTE013 treatment.

Protein analysis of these factors is shown in figure 41 below. Consistent with the qPCR results Snail, Vimentin, αSMA, and E-Cadherin are all down-regulated with co-culture and further downregulated following JTE013 treatment in a co-culture setting. This provides a further confirmatory step to our transcriptional analysis.

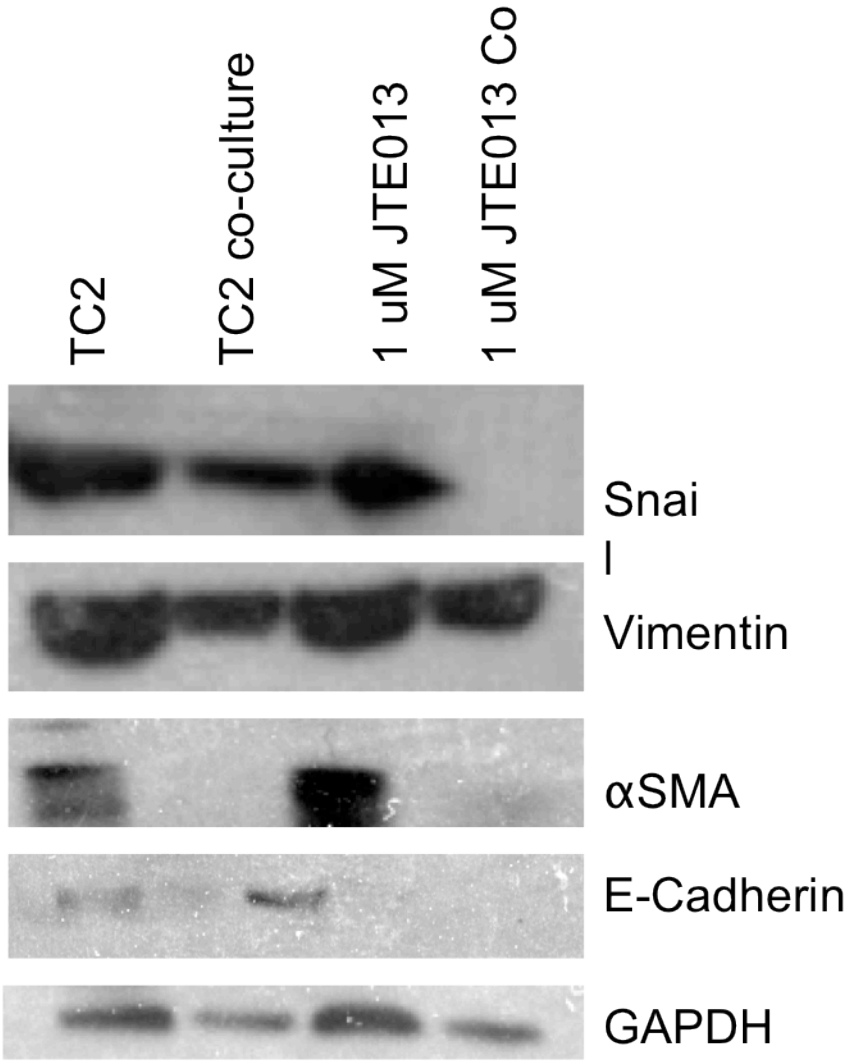


Figure 41: Western blot analysis of EMT genes. TRAMP-C2 cells were analyzed following co-culture with MSCs given either vehicle treatment or JTE013 treatment. Collection of protein lysates was conducted 24 hours after JTE013 treatment and 48 hours following cell plating.

Based on these transwell co-culture conditions co-culture of the two cell types at a 10:1 ratio of MSCs:TRAMP-C2 resulted in the formation of MSCs forming a capsule like structure surrounding the prostate cancer cells with invasive lammellipodia type projections on the exterior. MSCs for this experiment express GFP based on isolation from transgenic GFP mice.

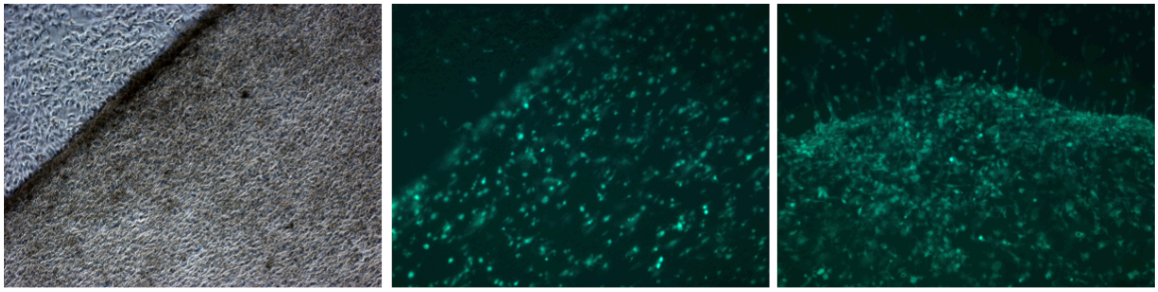


Figure 42: Images of in vitro co-culture of MSCs (GFP) and TRAMP-C2 cells. The leftmost panel shows the light microscopy view of the co-culure with the right panels showing two views of the co-culture environment.

MSCs co-cultured with prostate cancer cells require S1PR2 expression for the maintenance of the expression of genes affiliated with EMT and metastasis. Although further work will need to be conducted evaluating the protein expression and activation status of these markers, initial evaluation suggests that consistent with previous reports, S1PR2 expression in MSCs is important in mitigating their oncogenic function. Follow up on this finding using an in vivo co-culture system will be important in further investigating this phenomenon in a setting that better mimics that of a tumor microenvironment.

Discussion and Future Directions:

Discussion

MSCS are gaining increasing importance both in our understanding of their physiologic function in immunomodulation and injury repair and in a clinical setting where these properties can be harnessed to help improve injury repair and to modulate immune responses in conditions of autoimmunity (20). Research on MSCs has steadily increased following their initial isolation and characterization with an acceleration in interest and increased work following the definition of MSCs by the 2006 International Society for Cell Therapy. The capacity of MSCs to be easily isolated from bone marrow and their capacity for adherence in culture in vitro makes them ideally situated for use in adult stem cell therapy (108). This interest has been reflected in the multiplicity of clinical trials for which MSC therapy, both allogeneic and autologous, has been conducted. Although still predominantly Phase I/II clinical trials, an increasing number of Phase III trials has shown promise for MSCs in Graft vs. Host Disease, following cardiac infarction, and in bone and cartilage diseases (248). The method of the delivery of the cells, capacity for ex vivo expansion, and limited engraftment remain issues of further investigation as the clinical potential of MSCs evolves.

Ongoing clinical interest in the MSCs has made further understanding of MSC renewal, differentiation status, and physiologic function in different tissue microenvironments

critically important to both understanding the function and modulation thereof of MSCs and in further assessing the potential clinical use of the cells. Although some self-renewal factors have been identified including environmental factors and pluripotency factors including Nanog, Oct-4, Sox2, and Rex-1, further evaluation as to what additional factors might contribute to self-renewal or what factors modulate expression of these is required. Furthermore, additional understanding of the mechanism of differentiation of MSCs into the classical pathways of osteogenesis, adipogenesis, and chondrogenic signaling is critical as well as the auxillliary pathways of differentiation that are gaining increased interest.

In this dissertation, we purport that S1P has a critical function in stem cells and more specifically in MSCs. This conclusion is based on previously published data and the new data presented here in this dissertation. S1P has been shown to promote increased MSC migration, mobilization, and differentiation into cardiomyocytes and smooth muscle cells (154, 158, 159, 161, 164). At the start of the project, I was particularly interested in further investigating the effect of S1P and more specifically the S1P receptors on MSC function. Increasing our understand of how MSCs self renew in their reservoir areas including the bone marrow will expand our knowledge on the physiologic function and maintenance as well as providing information on improved *ex vivo* culture to improve their clinical utility.

Preliminary evaluation was conducted using human hTert immortalized MSCs due to their ease of expansion which express many of the characteristics of the primary MSCs but are somewhat limited due to their immortalization. We observed decreased Erk phosphorylation with increasing doses of S1P treatment following a two hour exposure with increased transcriptional expression of S1PR1, R2, and R3 in this same time frame. With continuous bid treatment with S1P transcriptional expression of S1PR1 and S1PR3 was decreased. Treatment with JTE013, a chemical antagonist of S1PR2 resulted in increased MSC clonogenicity and cell migration. Based on these experiments, it was concluded that S1P was critical to both MSC signaling and basic cell functioning. Due to the limitations of the hTERT MSCs, primary murine MSCs were used for future analysis. Primary questions addressed included understanding what the role of the S1P receptors was in the maintenance of a multipotent MSC population and what impact S1P receptors had in prostate cancer progression.

Examination of the impact of S1PR2 inhibition of primary murine MSCs both by chemical and genetic mechanisms revealed that inhibition of S1PR2 promotes increased cell clonogenicity, proliferation, and migration. The increased MSC proliferation and migration in response to S1P treatment can be inhibited by inhibition of Erk phosphorylation. S1P stimulation canonically results in an enhancement of Erk phosphorylation through the Ras and Erk signaling pathway downstream of Gi, a target g protein of S1PR1-3(142, 249, 250). Inhibition of S1PR2 in MSCs, a cell type we have shown to have high S1PR2 expression relative to S1P1, results in increased Erk1

phosphorylation. The mechanism behind this increased phosphorylation could be the reduction of decreased inhibitory signaling through G12/G13 or through changes in the MAPK regulation as MKP-1 can also be upregulated following S1P stimulation(105) Receptor compensation through increased Gi signaling in S1PR1 could also account for the increased Erk signaling in the condition of S1PR2 inhibition. Erk inhibition results in abrogation of the increases in proliferation and migration mediated by inhibition of S1PR2. Changes in protein expression and activation in the other common downstream signaling pathways downstream of S1PR2 are not impacted by genetic or chemical inhibition of S1PR2. S1PR1 and S1PR3 do not appear to be involved in regulation of MSC proliferation or migration, as inhibition of these receptors does not impact these parameters.

Evaluation of differentiation reveals that S1PR2 promotes osteogenic and adipogenic differentiation and inhibition of this receptor results in decreased differentiation into these lineages. This is likely the result of changes in pluripotency factors as MSCs that have inhibited or absent S1PR2 function demonstrate higher transcriptional expression of pluripotency factors critical central mechanisms of MSC differentiation including Nanog, Oct4, and Rex1 without significant impact of pathway specific gene expression including Runx2, Bmp4, PPARλ, or MMP9.

We therefore in the first section of this project identify S1PR2 as a receptor that promotes cell differentiation and inhibits cell proliferation therefore acting

antagonistically to the culture conditions required for ex vivo cell culture expansion of MSCS. Inhibition of S1PR2 promotes self-renewal of MSCs and enhances MSC proliferation. S1P is a critical lipid signaling molecule that promotes to cell proliferation and migration in a variety of cell types. Current research is starting to address receptor specific responses to S1P stimulation in cells of different origin. We have shown that inhibition of S1PR2 in bone marrow derived murine MSCs using genetic and pharmacological means results in increased MSC clonogenicity, proliferation, pluripotency and migration.

MSCs enable tissue repair and regeneration though a combination of factors including their immunomodulatory role, cytokine secretion and differentiation into cells required for the location (10, 11, 37). MSC can differentiate into osteocytes, adipocytes, smooth muscle cells, cardiomyocytes, fibroblasts, chondrocytes, neuronal cells, and many other cell types(251). In this dissertation, we have shown that S1P is critical to MSC differentiation into osteocytes and adipocytes. In the absence of S1PR2 signaling MSCs, there is a significant reduction in MSC differentiation.

Previously published work has examined some of the signaling pathways and factors maintaining MSCs in an undifferentiated state (17). In MSCs, increases in transcriptional and protein expression of Nanog, Oct4, Sox2, and Rex1 direct a downstream signaling network promoting maintenance of a pluripotent state although there remains some controversy over which pluripotency factors are involved (17, 44, 45, 252). Furthermore,

increased CD44 expression has been demonstrated in MSCs in a less differentiated state (6). The increases in these pluripotency factors following S1PR2 inhibition parallels the changes in differentiation observed in MSCs. The impact of S1PR2 inhibition on differentiation in both adipogenesis and osteogenesis combined with the impact on critical universal self-renewal markers places S1PR2 at a central role in MSC differentiation that would likely impact other cells types. These changes in differentiation may have significant implications in the capacity of MSCs to migrate to promote wound recovery at sites of injury and suggest an involvement of S1P and the S1P gradient in controlling MSC differentiation status. S1PR2 promotes proliferation while simultaneously promoting an increased stem cell phenotype. Although this may initially seem contradictory, it is likely that the complexity of S1P signaling and receptor compensation or local signaling from growth factors may contribute to this dual effect. In this paper we have identified S1PR2 as a critical promoter of MSC differentiation through alteration transcriptional pluripotency factors and propose a modification in cell culture conditions for expanding ex vivo pluripotent murine MSCs.

In the second part of this work, we evaluate the oncogenic function of MSCs within prostate cancer cells. Due to the high incidence of prostate cancer in the general population and the difficulty in predicting the aggressiveness of prostate cancer tumors, ongoing prostate cancer research is critical to improving patient care by both preventing the overtreatment of prostate cancer and identifying effective ways to control the progression of malignant cancers. One differentiating factor that has been explained to

classify tumors based on their malignant potential has been the stem cell content of the tumor. Using the TRAMP-C2 prostate cancer model we demonstrate that there is an increased stem cell population of prostate cancer cells following treatment with JTE013 in vitro as assessed by Aldehyde dehydrogenase expression and CD133 expression. These cell populations transcriptionally express increased factors associated with a stem cell population and that have been previously described for prostate cancer stem cells including Nanog, Sox2, and Rex1. JTE treatment in vivo on TRAMP-C2 tumors grown in vivo resulted in no changes in tumor growth or stem cell population as compared to vehicle controls. The discrepancy between this result and the results obtained in vitro could result from poor coordination of the timing, concentration, or mechanism of the drug delivery in vivo based on the more complex nature of the system. Additionally analysis on the success of S1PR2 inhibition and transcriptional and translation expression of proteins associated with prostate cancer stem cells will be necessary to further evaluate which mechanism might explain the different results observed in vitro as compared to in vivo.

In the final part of this dissertation, the impact of co-culturing MSCs with modified S1PR2 expression with prostate cancer is evaluated. The rationale for this examination is based on the recruitment of MSCs to prostate cancer tumors in vivo and their promotion of increased invasion, metastasis, and stem cell population of the tumor following their recruitment to the tumor microenvironment.

Inhibition of S1PR2 inhibition results in an increased stem cell population as assessed by CD133 expression, aldefluor expression, and increased Nanog transcription. Co-culture of MSCs with JTE013 inhibition results in a decrease in gene expression associated with EMT and metastasis. This suggests that S1PR2 expression in MSCs in the tumor environment is critical to the invasiveness and metastatic ability of the tumor. Better understanding the factors involved in this pathway is critical to our understanding of the tumor microenvironment and the role that accessory non-cancerous cells provide within this environment. Inhibition of S1PR2 using JTE013 in MSCs co-cultured with TRAMP-C2 cells resulted in decreased gene expression both transcriptionally and translationally associated with the epithelial-mesenchymal transition and with metastasis. Genes demonstrated to be downregulated following co-culture and S1PR2 inhibition include Snail, Vimentin, alpha-sma, and E-cadherin. Therefore S1PR2 provides critical stimulation to the tumor cells enabling the increased invasion and metastasis observed following MSC and prostate cancer cell co-culture. This observation requires further follow up onto what impact this might have on cell migration in a co-culture situation and other functional assays both in vivo and in vitro to follow up on this observation. This represents a promising avenue for ongoing research in this process as the mechanism by which MSCs increase tumor cell invasion, metastasis, and stem-like characteristics of adjacent prostate cancer cells is largely unknown. A summary of the findings presented in this dissertation is provided below.

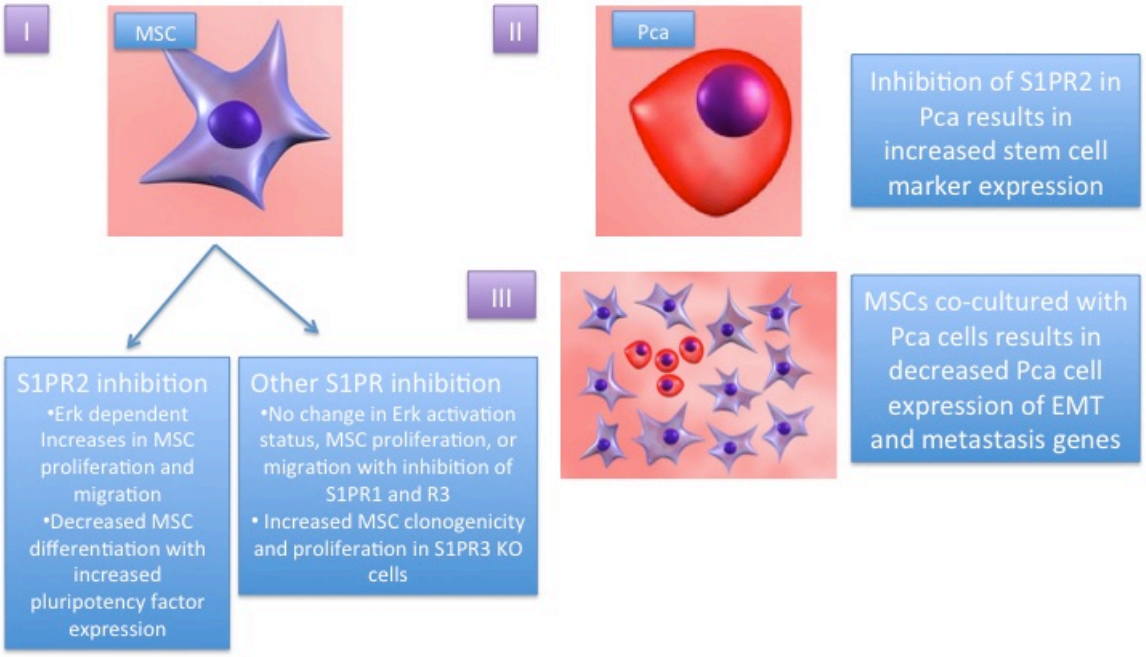


Figure 43: Summary diagram of dissertation including part I addressing the role of the S1PRs in MSC function, part II addressing the role of the S1PR2 in prostate cancer cells, and part III addressing MSCs and prostate cancer cells in co-culture with inhibition of S1PR3. Images purchased from canstock photos.com.

From this dissertation, four novel and central conclusions can be drawn that further the field of MSC research as it relates to sphingolipids and cancer. The first conclusion that can be reaches is that inhibition of S1PR2 increases proliferation of undifferentiated MSCs. This observation is critical to providing a new approach for ex vivo expansion of MSCS for clinical trial production of cells that has been a long-standing limitation of MSC cell therapy. Secondly, we identify S1PR2 as a novel factor in MSC differentiation likely impacting multiple pathways rather than the osteogenic and adipogenic pathways analyzed in this work. In the realm of cancer research, this work supports the role for S1PR2 in promoting an increased prostate cancer stem cell population. Finally, within MSCs co-cultured with prostate cancer cells S1PR2 expression on MSCs promotes

increased invasion and metastasis. It is these four conclusions that can best guide the future work conducted on this project to have a maximum impact current MSC knowledge and literature.

Future Directions

Although this research has evaluated a number of pathways involved in S1PR2 signaling within MSCs, it also opens many questions and avenues for future research to better understand and contextualize the conclusions presented from this research. This highlights the broad applicability of the research and its potential to have greater impact than has been presented currently. The future direction addressed here address the four main novel findings of the project. With regard to the role of S1PR2 on MSC proliferation, additional analysis of MSC markers is required to fully characterize the cells both following manipulation and JTE013 treatment. Additional proliferation analysis with modification of the oxygen levels and serum conditions to reflect different bone marrow and tissue microenvironments may further reveal the differing function of MSCs within different physiologic environments.

Further investigation on the role of S1PR2 on differentiation can further develop our observations regarding its role in differentiation and how it might both impact MSCs and other stem cell pathways. Additional characterization of alternate differentiation pathways including that of fibroblast lineages impacted by S1PR2 inhibition is critical in

better characterizing this differentiation ability of MSCs. Further characterization of the mRNA and protein expression characteristic of adipogenic and osteogenic lineages will better cement our understanding of the impact of S1PR2 on this pathway. JTE013 treatment in S1PR2 knockout cells will further clarify the specificity of this receptor in impacting these functions. Additional rescue experiments using S1PR2 rescue in the context of S1PR2 inhibition would also further support the conclusions presented by the differentiation studies conducted in the research presented here.

The role of S1PR2 in prostate cancer stem cell function requires further follow up to the pathways that might be involved in this effect. Further evaluation of $\alpha 2\beta 1$ integrin marker expression of JTE013 treated prostate cancer cells will further contribute to the stem cell identity of S1PR2 treated MSCs. Beyond evaluation of the individual marker, co-staining for CD133, CD44 and Aldefluor will more effectively identify a stem cell population than individual evaluation of expression. Evaluation of the pathway involvement based on the work by Beckham et al. might highlight some of the mechanisms by which this occurs focusing on inhibition of the sphingosine kinases and acid ceramidase using previously published inhibitors and siRNA. Serial transplantation assays conducted with these proposed stem cell populations for tumor initiation capacity will further evaluate the progenitor cell function of this population as compared to cells lacking this expression.

Finally the role of MSCs in a co-culture setting requires further investigation into the pathways that might be involved. Although we have examined the transcriptional involvement of genes association and EMT and metastasis, western blotting examination of proteins expression involved will further elucidate the EMT and metastatic potential of these cells. Co-culture of S1PR2 knockout cells within an in vivo tumor as compared to vehicle treated cells will further examine the potential for metastasis and would greatly contribute to the impact of this work. The further experiments proposed for the project derived from the four main novel findings presented in this dissertation reflect both on the exciting nature of these findings and their potential clinical and scientific implications.

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BIOGRAPHY

Tucker Marrison was born Brewster, NY in July 1986 and raised there prior to attending the Taft School in 2000. She received her Bachelor of Science in Molecular, Cellular, and Developmental Biology from Yale University in 2008. In 2008, Tucker joined the Medical Scientist Training Program at the Medical University of South Carolina. She completed her preclinical years of the medical school program prior to entering the laboratory of Dr. Norris in 2010. In the Norris lab, her research focused on sphingolipid metabolism in cancer and in mesenchymal stromal cells. Her awards include being a competitor on the of the winning team in the Student Venture Open Competition at the WBT Innovation Conference, a Hollings Cancer Center Scholarship Recipient, and a Abney Foundation Scholarship Recipient.

Selected Publications:

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