ROLE OF SPY1 IN HEMATOPOIESIS: IMPLICATIONS FOR BLOOD MALIGNANCIES

by

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Abstract

In blood malignancies, the balance of hematopoietic stem cell (HSC) self-renewal and differentiation is disturbed such that HSCs produce abnormal cells with an increased capacity to proliferate; however, the exact causes of this imbalance are not known. SpeedyA1 (Spy1) is a positive cell cycle regulator known to enhance cellular proliferation by direct binding and activation of Cyclin-dependent kinase (Cdk) Cdk2 and by promoting degradation of the Cdk inhibitor (CKI) $p27^{Kip1}$. I demonstrate that Spy1 expression levels are elevated in early stem and progenitor cells during hematopoiesis, but that Spy1 may also be implicated in later stages of myeloid differentiation. Spy1 protein was elevated in human bone marrow tumour samples and Spy1 knock-down in HL-60 cells led to decreased cell growth as well as expression of leukemic stem cell (LSC) markers. Together, these findings demonstrate a potential role for Spy1 in regulating HSC fate and in the development of blood malignancies.

Dedication

I would like to dedicate this thesis to the memory of Katelyn Bedard.

I never had the pleasure of knowing Katelyn, but in the past three years I have gotten the wonderful opportunity to meet many of her family members and friends at the Katelyn Bedard Bone Marrow Association. You are all truly amazing individuals who keep the memory of Katelyn alive through reaching out to other families afflicted by blood cancers and blood diseases. Your volunteerism and generosity is incredibly inspirational.

This thesis is also dedicated to my grandmother who has very recently been diagnosed with multiple myeloma. Just like you have always shown pride in my accomplishments, I am proud to have you as my grandmother. I support you in the journey to come and hope that your strength and courage help you to live each day to the fullest.

Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Lisa A. Porter, for her continued guidance and support, without which, I would never have been able to complete this work. I would also like to thank Dr. Boffa and Dr. Swan for their valuable insight and advice.

I also acknowledge all the members of the Porter lab, both past and present, for making my research experience unforgettable! I would like to thank: Jiamila for virus production; Bre-Anne for help with mouse work; Rosa for technical advice and help with statistical analysis; Ingrid for technical assistance and for being an outstanding 420 student; and Janice, Elizabeth, Nicole, Espanta, Martin, Jessica, and Megan for technical assistance. Last, but certainly not least, I would like to thank Dorota Lubanska for her patience and mentorship; she has played a large role in helping me become a better scientist and I am forever thankful for her teaching and assistance.

I also need to thank my family, specifically my parents, for their continued support throughout my studies. They have always had faith in my abilities and encouraged me to accomplish great things. I thank them for making me the ambitious young woman I am today. I also thank my boyfriend for his unwavering belief in me and for his encouragement throughout this work.

Finally, I sincerely extend my gratitude to the Katelyn Bedard Bone Marrow Association for their generous donations which provided the funding for this project. Their enthusiasm to fight back against cancer has truly been inspirational to me while working to complete this project. I also want to acknowledge the Ontario Graduate Scholarship program for supporting this work.

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List of Abbreviations

HSC.....hematopoietic stem cell

LT-HSC.....long term repopulating hematopoietic stem cell

ST-HSC.....short term repopulating hematopoietic stem cell

MPP.....multipotent progenitor

CLP.....common lymphoid progenitor

CMP.....common myeloid progenitor

MEP.....megakaryocyte-erythroid progenitor

GMP.....granulocyte-macrophage progenitor

Cdk.....Cyclin dependent kinase

CAK.....Cyclin dependent kinase activating enzyme

CKI.....Cyclin dependent kinase inhibitor

Cdk2.....Cyclin dependent kinase 2

CSC.....cancer stem cell

AML.....acute myelogenous leukemia

LSC.....leukemic stem cell

CLL-1.....C-Type Lectin-Like Molecule-1

CD33.....Siglec-3

Spy1.....SpeedyA1

RINGO.....Rapid Inducer of G2/M Progression in Oocytes

MAPK.....Mitogen Activated Protein Kinase

shRNA.....short-hairpin ribonucleic acid

qRT-PCR.....quantitative real-time polymerase chain reaction

MOI.....multiplicity of infection

TU.....transducing units

C/EBPε.....CCAAT enhancer binding protein epsilon

IMDM.....Iscove's Modified Dulbecco's Medium

P/S.....penicillin/streptomycin

FBS.....fetal bovine serum

hiFBS.....heat-inactivated FBS

SCF.....stem cell factor

IL-3.....interleukin-3

GM-CSF.....granulocyte macrophage-colony stimulating factor

atRA.....all-trans retinoic acid

EGTA.....ethylene glycol tetraacetic acid

PMSF.....phenylmethanesulfonylfluoride

PBS.....phosphate buffered saline

BSA.....bovine serum albumin

BCA.....bicinchoninic acid

INTRODUCTION

In Canada, there are approximately 90,000 people diagnosed with, or in remission from, a variety of blood malignancies including leukemia and lymphoma[1]. Leukemia and lymphoma are the most common cancers diagnosed in children and youth under the age of twenty; these groups of cancers account for approximately one third and one fifth of childhood cancers, respectively[1-3]. While advances in treatment have drastically improved survival rates for some types of blood cancers[4-11], there are still many for which a cure has yet to be discovered[4,12-13]. Complicating the discovery of new treatments is the enormous diversity among different types of blood cancers, each presenting with a different genetic background not yet fully elucidated. Therefore, it is of utmost importance to resolve the causes of blood cancers on a cellular and molecular level so that better treatments can be implemented.

I. Hematopoiesis: Blood Cell Development

The production and development of blood cells is a process known as hematopoiesis. This process is arranged in a hierarchy such that all blood cells are derived from a primitive hematopoietic stem cell (HSC) that is responsible for maintaining homeostasis of the blood system (Figure 1)[14-15]. HSCs, like other stem cells, have the unique ability to undergo asymmetric cellular division. Thus, they not only give rise to more differentiated blood cells to produce all the many types of blood cells found in the body, but they also generate more stem cells through self-renewal to maintain the pool of adult stem cells.

Although HSCs were discovered over 50 years ago by McCulloch and Till[16-18] and first isolated over 20 years ago[19], it was not until recently that HSCs were discovered to

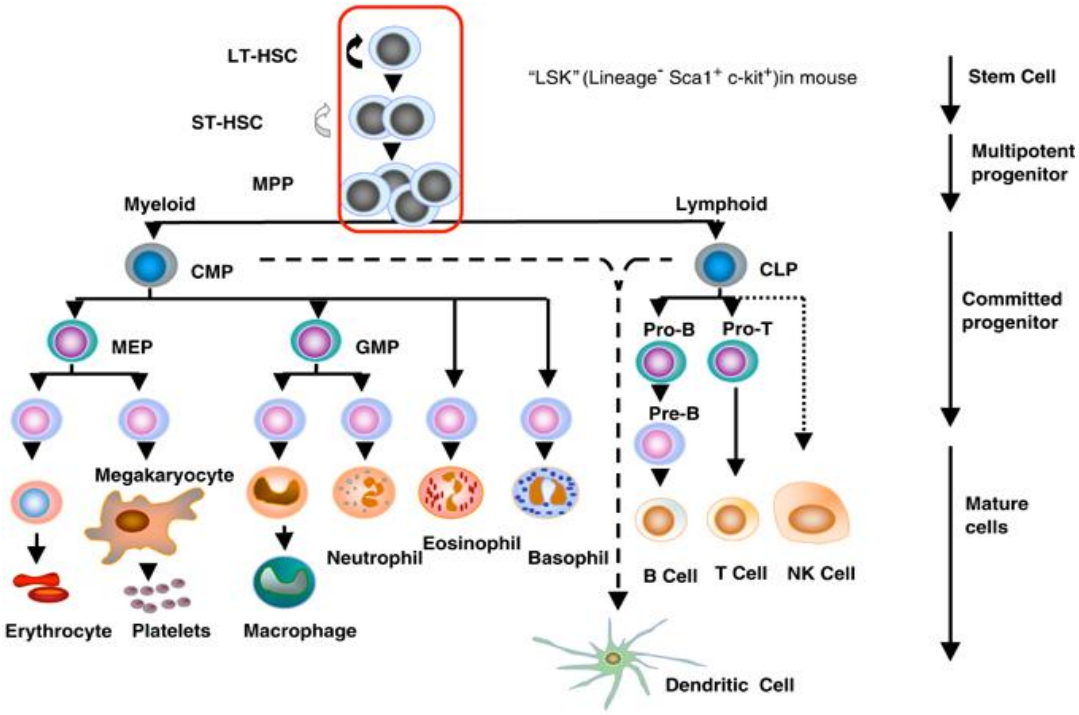


Figure 1. Hematopoietic Cell Development. Primitive HSCs give rise to all blood cells of the body through a series of steps in which cells get progressively more committed. LT-HSC, long-term repopulating HSC; ST-HSC, short-term repopulating HSC; MPP, multipotent progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte-macrophage progenitor. *(from Larsson, J. And Karlsson, S. Oncogene 2005)*[14]

be a heterogeneous population of stem cells with differential reconstitution ability[20-22,23]. In fact, different kinds of HSCs have now been characterized and isolated within the human hematopoietic system[22-29]. The long-term repopulating HSC (LT-HSC) is thought to be responsible for life-long maintenance of the adult stem cell pool[22]. LT-HSCs are relatively rare, representing <0.01% of cells found in the adult bone marrow[30-31]. These stem cells, when transplanted into irradiated mice, have been shown to be capable of retaining engraftment potential indefinitely[22]. LT-HSCs are relatively inactive, dividing infrequently to produce more rapidly proliferating short-term repopulating HSCs (ST-HSCs) which give rise to multipotent progenitors (MPPs) with a decreased ability for self-renewal[22,32]. MPPs can differentiate into the common lymphoid progenitor (CLP) and/or the common myeloid progenitor (CMP) and at this point in hematopoiesis, blood cell development is restricted to the CLP or CMP lineage[14-15].

Through a series of differentiation steps, CLPs will give rise to white blood cells important for the functioning of the lymphatic system such as B-Lymphocytes, T-Lymphocytes, and Natural Killer cells[14-15]. CMPs give rise to erythrocytes, megakaryocytes, granulocytes, and monocytes[14-15]. Erythrocytes (red blood cells) contain hemoglobin and transport oxygen throughout the circulatory system and megakaryocytes produce platelets which are important in the formation of blood clots[33-34]. Monocytes are white blood cells that produce macrophages which are important in destroying bacteria[33-34]. Granulocytes are white blood cells important to the functioning of the human immune system and include neutrophils, eosinophils, and basophils[33-34].

The fate of HSCs is highly dependent on different growth factors present in the microenvironment. While the roles of several growth factors and cytokines in hematopoietic lineage decisions have been resolved, it is important to note that

interleukin-3 (IL-3) and granulocyte-colony stimulating factor (G-CSF) are important in specifying a myeloid fate[30,35], whereas a lymphoid fate is largely controlled by the presence of interleukin-7 (IL-7)[30].

II. Mammalian Hematopoietic Development

The hematopoietic system is one of the first mammalian tissue systems to form in the developing embryo[36-37]. Primitive erythrocytes can be found in the blood islands of the extra-embryonic yolk sac early on in embryonic development[36-37]. However, definitive HSCs, capable of hematopoietic reconstitution upon engraftment in an adult irradiated mouse, are first observed in the aorta-gonad-mesonephros (AGM)[36-38]. In humans HSCs in the AGM are seen as early as embryonic day 27, indicating that the AGM is the first site of hematopoiesis in the developing embryo[37].

There are several developing organs involved in fetal hematopoiesis, including the liver, spleen, thymus, and bone marrow, that can contribute to fetal blood cell production after first being colonized by migrating blood cells[34,36-37,39-40]. Beginning as early as embryonic week 5 in humans the liver plays a distinctive role in fetal hematopoiesis as it supports proliferation and differentiation of the colonizing progenitor cells[37]. Nonetheless, it is not until later in embryonic development that the human fetal liver produces definitive HSCs that express the stem cell marker CD34 and have the capacity to undergo hematopoiesis *in vitro*[37,39]. At this point, the liver is the primary organ responsible for human fetal hematopoiesis[37].

The bone marrow is the last tissue important for hematopoietic development that is established in utero. The bone marrow microenvironment begins to form around 10 weeks during human embryonic development and primitive myeloid and erythroid cells

can be found in the developing marrow as early as week 11[37]. However, HSCs capable of hematopoietic reconstitution are not observed in the marrow until right before birth[37]. At this time, blood development is diminished in the yolk sac and occurs only to a limited extent in the liver[37]. This sequence of events has been shown to be very similar to that of hematopoietic development in mice[36].

The bone marrow will sustain hematopoiesis on a long-term basis for the remainder of the mammalian lifespan. However, other organs also serve important hematopoietic functions in the adult mammal. First, the spleen aids in red blood cell turnover as it is responsible for the destruction of damaged red blood cells; it also contributes to white blood cell synthesis[40]. In addition, the thymus plays a role in adult lymphocyte maturation[40]. Moreover, the adult liver is also involved in the turnover of red blood cells[40] and has been found to contain small populations of adult HSCs[41]. HSCs can also be found in very small numbers in the circulating peripheral blood. An understanding of the role each secondary hematopoietic tissue plays in hematopoiesis is important as both fetal and adult tissues were examined in this work.

III. HSC Quiescence

The majority of adult HSCs, like other adult stem cells, remain in a relatively inactive state with low turnover referred to as quiescence[32,42-43]. HSC quiescence is largely controlled by cell cycle inhibitors at G1 phase of the cell cycle including p21, p27, and p57 as well as by the tumour suppressor protein p53[44-47]. The infrequent division of HSCs acts as a protective mechanism to limit DNA damage and to prevent premature proliferative exhaustion of the stem cell pool[32,42-43]. However, as previously mentioned, HSCs do enter the cell cycle occasionally to give rise to ST-HSCs that will support the

continuous production of many types of blood cells[32,42-43]. Furthermore, under times of physiological stress on the hematopoietic system such as injury or severe blood loss, adult HSCs are capable of exiting quiescence and rapidly proliferating to repopulate the hematopoietic system[42]. Thus, HSCs are relatively quiescent under homeostatic conditions, but retain the ability to rapidly self-renew when necessary.

IV. Hematopoietic Malignancies: Subtypes

Leukemia is primarily a disease of the hematopoietic system affecting organs such as the bone marrow and circulating blood. There are many different subtypes of leukemia that are classified based on the type of blood cell that is affected. Lymphoblastic or lymphocytic leukemia initiates in a marrow cell that is a precursor of a lymphocyte[48]. Myelogenous or myelocytic leukemia initiates in a marrow cell that is destined to become a red blood cell, platelet, or a type of white blood cell other than a lymphocyte[48]. Leukemia can be further classified into several subtypes based on how quickly the disease develops and on the maturation stage of the cells as determined by staining and pathology analysis[48]. Chronic leukemia takes months, sometimes even years, to develop and is most common in adults[3,48]. On the other hand, acute leukemia, the most common form of leukemia in children[3], has a rapid onset and can progress much more quickly[48].

Similar to lymphocytic leukemia, lymphomas involve abnormally proliferating lymphocytes. Unlike leukemia however, lymphoma results in the formation of masses of tumourigenic cells in the lymph nodes. The cancer can also spread to the liver, spleen, and other parts of the lymphatic and immune system; metastases to the lungs, bone, and central nervous system are also known to occur[3].

Finally, a blood disorder known as myeloma also exists. Multiple myeloma is the second most common hematological malignancy and accounts for approximately 1% of all cancers[49]. This disease primarily develops in B lymphocytes as they mature in the marrow to become plasma cells[49]. Cancerous plasma cells grow very rapidly and, in doing so, they prevent normal production of healthy blood cells and platelets; the end result is the formation of tumours in the marrow of the bones affected. Myeloma cells can invade and weaken the bones causing what are known as osteolytic lesions or bone lesions[49]. A plasmacytoma refers to a patient with a bone lesion or tumour in only one site, whereas, multiple myeloma refers to a patient with tumours in the marrow of multiple bones[49].

V. Blood Malignancies: Involvement of Cell Cycle Proteins

Although there are several different types of blood cancer, each affecting different kinds of blood cells at different stages of hematopoietic development, the common hallmark of blood cancers is an abnormal proliferation of specific kinds of blood cells. While the molecular mechanisms driving this abnormal proliferation have not been fully elucidated, several cell cycle proteins have been found to be implicated in different blood malignancies.

The mammalian cell cycle is largely regulated by a family of protein kinases called Cyclin dependent kinases (Cdks) that, when bound to Cyclin proteins, can promote progression through the cell cycle. In addition to cyclin binding, Cdks are activated post-translationally via phosphorylation by Cdk activating kinase (CAK) and removal of inhibitory phosphates by the Cdc25 phosphatases. Cdks are also negatively regulated by Cdk inhibitors (CKIs), such as the Cip/Kip family of inhibitors which bind to Cdk-Cyclin

complexes and interfere with the proper folding of the catalytic cleft, thereby, rendering the complex inactive. The Cip/Kip family includes $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$.

Several blood malignancies have been found to display elevated Cdk levels and low levels of CKIs have been associated with poor prognosis in patients[50-52]. For example, leukemia patients with low levels of p27 have presented with a worse prognosis[51] and aberrant regulation of p21 has also been demonstrated in many human leukemias[44]. Furthermore, an increase in Cdk2 activity has been observed in both lymphoid and myeloid derived leukemias as well as in some lymphomas[50,52]. This increase has partly been attributed to the abnormal localization of p27 in the cytoplasm where it cannot inhibit activity of Cdk2[52].

VI. Cancer Stem Cell Hypothesis & Leukemic Stem Cells

A population of cells known to drive tumour growth has been shown to exist in many cancers[15,53-57]. These cells, termed cancer stem cells (CSCs) possess similar characteristics of normal stem cells in that they can propagate themselves and also differentiate; thereby, they can not only sustain growth of the cancer, but can also give rise to the many differentiated cell types found within a heterogeneous tumour[15,58].

The existence of CSCs in blood malignancies was originally hypothesized based on the observation that only a very small percentage of human leukemia cells had clonogenic properties[59-60]. However, the first conclusive evidence for the existence of CSCs in leukemia came from the identification of a rare population of human acute myeloid leukemia (AML) cells that could generate leukemias representative to the human disease in xenograft transplants[53,61-62]. These CSCs, called leukemic stem cells (LSCs),

were later shown to continuously generate leukemias in murine serial transplantation experiments, indicating that they possessed an indefinite potential to self-renew[58,63].

Although the discovery of LSCs in AML has prompted much research in the area, the origin of LSCs has not yet been completely resolved. LSCs may result from the transformation of normal HSCs or they may arise from mutations or epigenetic changes in more committed progenitor cells allowing for enhanced self-renewal[64-65]. Interestingly, there has been evidence to support that AML is organized in a hierarchy with distinct classes of LSCs with differential long term repopulating (self-renewal) potential similar to that of HSCs[53,58]. Resolving the ambiguity surrounding the origin of the LSC will aid in the determination of specific molecular markers that are differentially expressed in normal and leukemic stem cells. This is a necessary step in targeting LSCs to eradicate this population therapeutically. To date, a few cell surface markers have been found to be commonly deregulated in leukemias and researchers are beginning to exploit these for drug design and clinical trials[66-70]. In AML the markers CLL-1 and CD33 have been identified as potential therapeutic targets for the eradication of LSC populations[67-70].

Although possessing the ability to rapidly self-renew, many LSCs have been shown to be quiescent, similar to LT-HSCs[58,64,71-72]. This low cycling state provides the same protective advantages to LSCs as it does to normal HSCs, limiting DNA damage and preventing exhaustion of the LSC population[72]. LSCs are also known to contain an abundance of ATP-Binding Cassette Transporters (ABC Transporters)[73]. ABC-transporters are proteins known to efficiently pump chemotherapeutics out of the cell, contributing to drug resistance. The presence of ABC-transporters and the relatively low cycling rate of LSCs makes them highly resistant to conventional therapies, especially those involving chemotherapeutic drugs[55,58,64,71-73]. Thus, LSCs often persist in the patient

post-treatment and can initiate disease relapse (Figure 2)[55,64,71,73]. It is extremely important to uncover novel ways to target the LSC population in different blood malignancies to successfully induce disease remission. This requires resolving the origin(s) and mechanisms behind the development of LSCs.

VII. Spy1

SpeedyA1 (Spy1) was initially identified by two independent groups. Ferby and colleagues demonstrated that a novel protein, p33-RINGO (Rapid Inducer of G_2-M Progression in Oocytes) was sufficient to initiate oocyte maturation in *Xenopus* and that depletion of this protein inhibited progesterone-induced oocyte maturation[74]. Similarly, Lenormand and colleagues also discovered a novel protein, Spy1, that could induce *Xenopus* oocyte maturation and that this was dependent on activation of the MAPK pathway[75].

To date, Spy1 is known to be expressed in a variety of human tissues and cell lines[76]; of particular interest to this study are the thymus and the liver. Spy1 was also found to be a nuclear protein and to be expressed in a cell cycle dependent manner, peaking in G1/S phase[76]. Although Spy1 has no sequence homology to the Cyclin proteins, it has been found to be capable of binding and activating both the G2/M Cdk, Cdk1, and the G1/S Cdk, Cdk2[74-77]. Interestingly, Spy1 can activate Cdk1 and Cdk2 in the absence of phosphorylation of the respective Cdks on Thr-161 and Thr-160; a modification normally required for Cdk kinase activity[77]. In addition, Spy1-Cdk2 complexes have been found to have broader substrate specificity than other Cyclin-Cdk2 complexes, suggesting that Spy1 may alter the substrate specificity of Cdk2[78]. Taken together, the novel activation and altered substrate specificity of Cdk2 by Spy1 may result

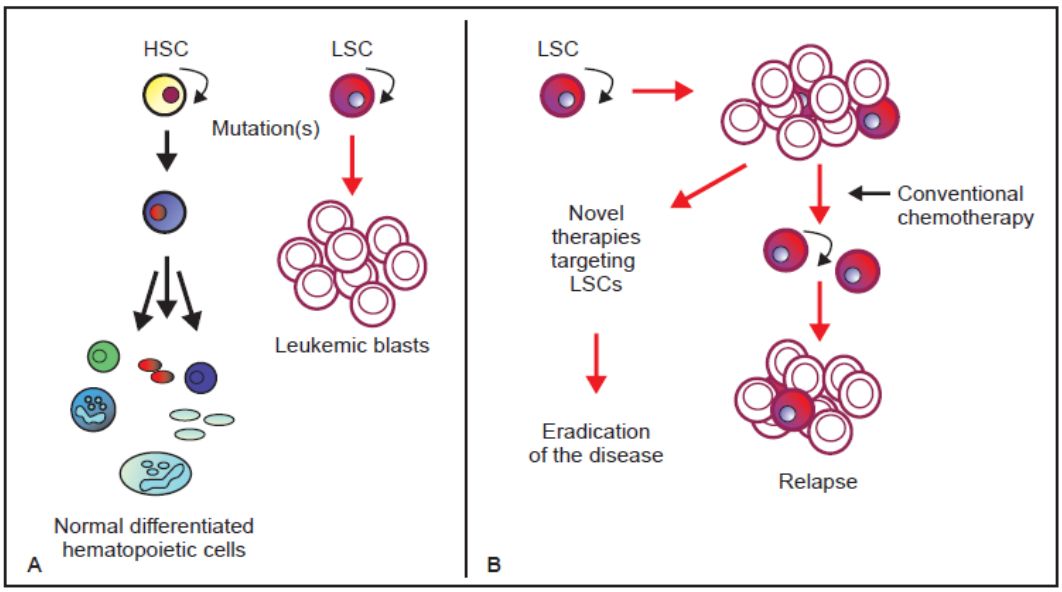


Figure 2. LSC Hypothesis. (A) The LSC hypothesis predicts that the leukemic blast cell population is maintained by a small subset of tumourigenic cells with stem cell properties. LSCs can self-renew and differentiate to maintain the heterogeneous tumour. **(B)** Current treatments do not target LSCs so they persist and can initiate relapse. Novel treatment designs that target LSCs may help eliminate the entire malignancy. *(from Guzman, M.L. and Jordan,C.T. Cancer Control 2004)*[55]

in aberrant proliferation at times when it may be inappropriate; this represents one way in which Spy1 could contribute to tumourigenesis.

Additionally, Spy1 is capable of overriding normal cellular responses to stress such as apoptosis, senescence, and DNA damage response pathways[79]. It is known that endogenous Spy1 levels increase in cells following treatment with DNA damaging agents and that the overexpression of Spy1 enhances cell survival in the presence of such agents by preventing apoptosis and interfering with repair pathways[79-81]. Specifically, the overexpression of Spy1 prevents cells from efficiently activating both G1/S and G2/M phase checkpoints following exposure to ultraviolet radiation and the activation of DNA damage response pathways increases in the absence of Spy1[79,81]. Moreover, Spy1-Cdk complexes demonstrate decreased sensitivity to inhibition by the cell cycle inhibitor, $p21^{Cip1}$ and Spy1 has been shown to be capable of overriding the $p27^{Kip1}$-mediated checkpoint at G1/S phase of the cell cycle[77,82-83]. Collectively, these findings have demonstrated that Spy1 can also contribute to tumourigenesis by promoting cell cycle progression even in the presence of cellular stressors.

Recent data from our lab have demonstrated a role for Spy1 in maintaining stemness in the breast and the brain[84]. In addition, Spy1 is known to be elevated in many human cancers and it has been recently reported as important in human non-hodgkin's lymphomas[85]. Hang and colleagues found that Spy1 protein was highly expressed in a variety of different types of human non-hodgkin's lymphoma samples and that this expression was correlated with that of proliferation markers[85]. Importantly, they found that patients with high levels of Spy1 protein had a worse prognosis and decreased overall survival compared to patients with lower levels of Spy1 protein[85].

The goals of this study were to explore the involvement of Spy1 in normal hematopoietic cell development and to determine its role in human blood malignancies. Initially, I sought to examine the expression of Spy1 in different hematopoietic tissues and to establish a role for Spy1 in hematopoietic fate decisions *in vitro*. Secondly, I aimed to discover whether Spy1 may be implicated in hematopoietic malignancies, particularly those known to contain LSC populations.

Objective 1: Determine a role for Spy1 in hematopoietic stem cell fate. EML cells were utilized as a model system of hematopoietic development. These are murine bone marrow cells representative of MPP populations that are capable of responding to differentiation stimuli to generate differentiated blood cells of the erythroid, myeloid, and lymphoid lineages[30,86]. Spy1 expression was examined during the myeloid differentiation of EMLs *in vitro*.

Objective 2: Study the role of Spy1 in the development of hematopoietic malignancies. HL-60 cells were used as a representative case of acute myeloid leukemia as Spy1 is known to be highly expressed in this line. Short hairpin RNA (shRNA)-mediated knock-down of Spy1 was performed and growth assays were used to study the effects of Spy1 depletion on leukemic cell growth. Gene expression levels of known LSC markers in AML were also studied following shRNA-mediated depletion of Spy1. Moreover, analysis of Spy1 protein levels in human bone marrow tumours was also performed.

Collectively, this study aims to broaden the understanding of novel cell cycle regulators during normal hematopoiesis and in various blood malignancies, particularly AML. The

findings of this study have identified Spy1 as important in regulating HSCs and have suggested a potential role for Spy1 in LSC populations. Overall, the outcomes of this study may have important implications in the treatment of blood malignancies.

MATERIALS AND METHODS

I. Tissue Microarray Analysis

Paraffin-embedded Tissue Microarray slides (US BioMax) were first baked at 60˚C to remove paraffin. Immersion in xylene for 10 minutes, followed by subsequent immersion in 100% ethanol, 95% ethanol, and 70% ethanol for 5 minutes each was then performed to rehydrate the slide. After being washed in 1X PBS for 5 minutes, slides were immersed in sodium citrate buffer (pH 6.0) at 95ºC for approximately 15 minutes for antigen retrieval. Slides were then stained with different antibodies. Specifically, a blocking antibody (normal goat serum; Cayman Chemical 10006577) was applied to the slides at room temperature for 20 minutes followed by application of anti-Spy1 antibody at a dilution of 1:100 (Novus Biologicals NB100-2521) for 60 minutes also at room temperature. Slides were washed twice for 5 minutes in 1X PBS prior to application of a fluorescence-conjugated secondary antibody (Alexa488-goat anti-Rabbit IgG; Invitrogen A11008) at a dilution of 1:1300 for 20 minutes at room temperature in the dark. Slides were again washed in 1X PBS before application of TOTO-3 nuclear stain (1:1300; Molecular Probes T-3600) for 20 minutes in the dark. After a final wash in 1X PBS for 5 minutes, slides were immersed subsequently in 70%, 95%, and 100% ethanol for 5 minutes each, followed by immersion in xylene for 8 minutes. Slides were mounted to coverslips and ScanArray Express software (Perkin Elmer Inc.) was used to detect and quantify fluorescence. Data were normalized to the nuclear stain, TOTO-3. To control for non-specific signals, slides were compared to previously established slides stained with secondary antibody alone.

II. Murine Hematopoietic Tissue Extraction

Balb-C mice were humanely sacrificed at post-natal day 7 and every week thereafter for 6 weeks for the extraction of tissues. All dissection tools were sterilized prior to use. Incisions were made on the ventral surface of each mouse beginning at the posterior end and moving anteriorly up the centre of the mouse to expose the body cavity and the internal organs. Samples of the liver, spleen, and thymus were cut, placed in an eppendorf tube and flash frozen in liquid nitrogen prior to storage. Peripheral blood was extracted using a syringe and needle. Bone marrow samples were obtained by flushing the bones of the hind limbs with 1X PBS; bone marrow from both left and right femurs and tibias were pooled. Red blood cells were removed by incubation with red blood cell lysis buffer (Sigma R7757) for up to 5 minutes on ice followed by centrifugation at 1200 rpm and 4ºC for 5 minutes. All samples were flash frozen in liquid nitrogen prior to storage in a -80ºC freezer. Three samples of each tissue were taken from one mouse for each time point.

III. Protein Extraction

Murine tissue samples were lysed and homogenized using a Sonic 60 Dismembrator (Fisher Scientific FM2602) followed by centrifugation for 20 minutes at 10,000 rpm and 4ºC to remove any fat or undesired tissues. For lysates in which fat was still present, a second centrifugation was necessary. Lysis buffer was composed of 0.1% Nonidet P-40, 50 mM Tris-HCl pH 7.4, 0.25% Deoxycholate acid, 1 mM EGTA pH 8.0, 0.2% SDS, and 150 mM NaCl. The following protease inhibitors were added: PMSF (10 µL/mL); Leupeptin (1 µL/mL); and Aprotinin (0.5 µL/mL). Protein lysates were stored at -80ºC.

IV. Immunoblotting

Protein concentrations of lysates were determined using the Micro-BCA protein assay kit (Thermo Scientific). Briefly, protein lysates were mixed with a working reagent in a 1:1 ratio and allowed to incubate at 60ºC for 60 minutes prior to analysis of absorbance at 562 nm in a spectrophotometer (Biomate 5, Thermo Electron Corporation BIO 145108). All protein concentrations were corrected to the reading of lysis buffer. A total of 50 to 100 µg of lysate was prepared in 4X sample buffer (10% glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.01 mg/mL bromophenol blue, and 2% β-mercaptoethanol), boiled for 5 minutes, and run on a 10% SDS gel for 3 hours at 120 volts. Gels were transferred onto PVDF membrane (Millipore IPVH00010) for 2 hours at 30 Volts and membranes were subsequently blocked in 1% milk for a minimum of 45 minutes. Membranes were blotted with either anti-Spy1 antibody (Novus Biologicals NB100-2521) or anti-Spy1 antibody (AMICUS Biotech) overnight at 4ºC for detection of Spy1 or with anti-actin monoclonal antibody (Chemicon Intl. MAB1501R) for 1 hour at room temperature for detection of a loading control protein. Secondary antibodies were either anti-rabbit IgG (Sigma A0545) or anti-goat (Santa Cruz sc-2020) for Spy1 and anti-mouse IgG (Sigma A9917) for actin. All secondary antibodies were applied at room temperature for 1 hour. Membranes were washed in between primary and secondary antibody applications with TBS-Tween. Protein was visualised and densitometry was analyzed using FluorChem HD2 imaging software (Alpha Innotech). Only results obtained with the Spy1 antibody from Novus Biologicals are shown.

V. Cell Culture

EML cells were purchased from American Type Culture Collection (CRL-11691) and cultured in Iscove's Modified Dulbecco's Medium (IMDM; ATCC 30-2005) supplemented with 20% heat-inactivated fetal bovine serum (hiFBS; Invitrogen 906532), 1% penicillin/streptomycin solution (P/S; GIBCO 991805), and 50 ng/mL murine stem cell factor (SCF; MyBioSource MBS400071). EML cells were maintained at a cell density of 2.0 x 10^5 cells/mL and 2 x 10^6 cells/mL and passaged every 2 to 3 days. HL-60 cells were a kind gift of Dr. Michael Boffa (University of Windsor, Biochemistry Department) and were cultured in IMDM supplemented with 20% FBS and 1% P/S. All cells were maintained in a 37°C humidified incubator set to 5% CO_2.

VI. Differentiation of EML Cells

To induce differentiation of EML cells into myeloid progenitor cells, 1 x 10^5 cells/mL were seeded in either 12 or 6 well plates. Differentiation media consisted of complete growth media (IMDM with 20% hiFBS, 1% P/S, 50 ng/mL SCF) with the addition of 10 ng/mL murine interleukin-3 (IL-3; Peprotech 213-13), and 10^{-5} all-trans retinoic acid (atRA; Sigma R2625). Control cells were grown in the absence of IL-3 and atRA. All plates containing atRA were stored in the dark. Cells were collected by centrifugation at 0, 24, 48, and 72 hours after the addition of differentiation media.

Myeloid progenitor cells generated by 72 hours of growth in media containing IL-3 and atRA were maintained in IMDM supplemented with 20% hiFBS, 1% P/S, and 10 ng/mL murine granulocyte macrophage-colony stimulating factor (GM-CSF; Peprotech 315-03). After 5 to 7 days of incubation with GM-CSF, myeloid progenitor cells were selected. To induce terminal differentiation, GM-CSF-dependent myeloid progenitor cells

were seeded at a density of 1 x 10^5 cells/mL in either 12 or 6 well plates in complete growth media containing 10 ng/mL murine GM-CSF and 10^{-5} M atRA. Control myeloid progenitor cells were grown in the absence of atRA. All plates with media containing atRA were stored in the dark. Cells were collected by centrifugation at 0, 24, 48, and 72 hours after the addition of atRA.

VII. EML Immunomagnetic Cell Sorting

To obtain a CD34+ population of cells, EML cells were sorted using Dynabeads M-450 Epoxy (Invitrogen 140.11) and an EasySep Magnet (Stem Cell Technologies 18000) according to the manufacturer's protocol. Briefly, magnetic beads were conjugated to anti-CD34 antibody (Santa Cruz IC0115) by incubation for 16 to 24 hours on a mutarotator at room temperature (5 µg anti-CD34 per 25 µL of Dynabeads). 25 µL of CD34-conjugated magnetic beads and 25 µL buffer (0.1% BSA in 1X PBS) were allowed to incubate with 2.5 x 10^6 cells at 4ºC for 20 minutes with gentle rotation on a mutarotator. This ratio was scaled up or down depending on the starting cell number. Following this, a magnetic field was applied for 1 minute in the EasySep Magnet causing CD34+ cells to be pulled to the inner walls of a test tube so that CD34- cells could easily be obtained by removal of the buffer. Several 2 to 3 minute washes with 0.1% BSA buffer removed the CD34+ cells. CD34- and CD34+ cells were plated in 6 well tissue culture plates for approximately 1 week prior to collection, along with a heterogeneous population of EML cells, for further analysis.

VIII. HL-60 Lenti-viral Infections

All HL-60 cell infections were performed in either 96 or 24 well plates. 5.0 x 10^4 cells/well were seeded for infections in 96 well plates and 1.0 x 10^5 cells/well were seeded for infections in 24 well plates. For all infections, HL-60 cells were seeded in serum-free and antibiotic-free IMDM containing 1 μg/mL polybrene (SantaCruz sc-134220). Cells were allowed to incubate with polybrene-containing media for approximately 20 minutes before the addition of virus. Cells were infected with a vector carrying shRNA against Spy1 (pLKO-shSpy1) and control cells were infected with pLKO-shScrambled (pLKO). Lenti-virus was used at a titre of 10^7 transducing units (TU)/mL and a multiplicity of infection (MOI) of 2 was used for all infections. Virus was removed by centrifugation and replaced with complete growth media approximately 20 hours after the addition of virus. Cells were allowed a 24 to 48 hour recovery period prior to the addition of puromycin (Sigma P9620) at a final concentration of 2 μg/mL to select for successfully infected cells. Puromycin selection was performed for a minimum of 72 hours before any subsequent assays. Non-infected control cells that were subjected to the same procedure were included in all cell infections. Quantitative real-time polymerase chain reaction (qRT-PCR) for expression of the human Spy1 gene was performed to quantify infection efficiency.

IX. HL-60 Cell Proliferation Assays

Upon successful selection in puromycin, cells expressing pLKO and pLKO-shSpy1 were counted using Trypan Blue exclusion and cell counts performed using a BioRad TC10 Automated Cell Counter. 100,000 cells were seeded in 6 well plates in

complete growth media and cell counts were performed in triplicate every 24 hours for 3 days using Trypan Blue exclusion. Wildtype HL-60 cells were also included.

X. HL-60 Cancer Stem Cell Marker Analysis

After successful selection in puromycin, cells expressing pLKO and pLKO-shSpy1 were collected along with wild-type HL-60 cells for qRT-PCR analysis of leukemic stem cell markers commonly used as diagnostic markers in acute myelogenous leukemias. Genes examined were human CD33 and CLL-1. mRNA levels of each gene in cells exhibiting Spy1 knock-down were compared to levels in pLKO-infected control cells.

XI. HL-60 Cell Differentiation

To induce granulocytic differentiation, 1 x 10^5 cells were seeded in 6 well plates. Differentiation medium consisted of complete growth media with the addition of atRA at a final concentration of 1 μM. Additional cells were also seeded in media containing 0.1% ethanol to serve as vehicle controls. Cells were allowed to differentiate over a period of 5 days; media was changed every 48 hours and cells were grown in the dark. Vehicle control and differentiated cells were collected 3 days and 5 days after the addition of atRA for further analysis.

XII. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cell pellets using an RNeasy Extraction Kit (Qiagen 74134). Briefly, samples were lysed and homogenized prior to the removal of genomic DNA. Ethanol was used to enhance binding of RNA to an RNeasy spin column

and a series of wash steps were performed before elution of RNA using RNase-free water. RNA concentrations and quality were assessed on a NanoDrop Spectrophotometer (ND-1000 software version 3.3.0 Thermo Scientific). RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen 100004925), 0.5 µg Oligo dT's (Eurofin) and 0.5 µg random nanomers (Thermo Scientific SO142). qRT-PCR was performed using Fast SYBR green detection (Applied Biosystems 4385616) on an ABI Viia7 thermocycler (Applied Biosystems 278880504). All qRT-PCR reactions were performed for 55 cycles and the stages were as follows: denaturation of cDNA was performed at 95ºC for 1 minute; annealing of primers to single stranded DNA occurred at 60ºC for 20 minutes; elongation occurred at 72ºC for 30 seconds. All gene-specific primers were used at a concentration of 5 µM with the exception of murine Spy1 and murine Sca-1 primers which were used at a concentration of 6 µM. Primer pairs are listed in Table 1. All data were normalized to GAPDH as an internal control.

XIII. qRT-PCR Calculations

All qRT-PCR reactions were analyzed using Viia7 software version 1.1.5 to generate Ct values. Briefly, the Ct of all target genes is balanced to that of the internal control, GAPDH, such that ΔCt values are calculated (example: $\Delta Ct_{Spy1\ 48hr} = Ct_{Spy1\ 48hr} - Ct_{GAPDH\ 48hr}$). The sample to which all other samples will be compared is then set to zero to generate ΔΔCt values; in this case, wildtype cells, pLKO control cells, or 0hr time points for differentiation experiments were set to zero (example: $\Delta\Delta Ct_{Spy1\ 48hr} = \Delta Ct_{Spy1\ 48hr} - \Delta Ct_{Spy1\ 0hr}$). The relative quantification (RQ) value is then calculated by taking $2^{-\Delta\Delta Ct}$ (example: $RQ_{Spy1\ 48hr} = 2^{-\Delta\Delta Ct}{}_{Spy1\ 48hr}$). All data represents $\log_{10}$ relative quantification. Error bars represent the standard error of the mean ΔCt value.

Table 1. Murine qRT-PCR Primer Pairs

Murine Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GATGCCCCCATGTTTGTGAT	GTGGTCATGAGCCCTTCCA
Spy1	GCTTTAGGGAAAAACTGGAGAAAA	AATGGCCATGACCTCTTCACA
c-kit	GGGCAAGAGTTCCGCCTTCTT	GCTGCGACCACAAAGCC
Sca-1	CTCTGAGGATGGACACTTCT	GGTCTGCAGGAGGACTGAGC
CD34	GAGAATTCTGGAATCCGAGAAGTGAGGT	ACTCTAGAACCCAGCCTTTCTCCTGTAG
Mac-1	GCCAATGCAACAGGTGCATAT	CACACATCGGTGGCTGGTAG
Gr-1	TGGACTCTCACAGAAGCAAAG	GCAGAGGTCTTCCTTCCAACA
C/EBPε	GACCTACTATGAGTGCGAGCCT	ACACCCTTGATGAGGGTAGCAG

Table 2. Human qRT-PCR Primer Pairs

Human Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	GGATCTCGCTCCTGGAAGATG
Spy1	TTGTGAGGAGGTTATGGCCATT	GCAGCTGAACTTCATCTCTGTTGTAG
CD33	AAGTACAGGAGGAGACTCAGG	GTGATTATGAGCACCGAGG
CLL-1	GTGATGATGTCCAAACATGGC	GATTGATGCCTCATGCCTCC
Mac-1	AGTTGCCGAATTGCATCGA	GGCGTTCCCACCAGAGAGA

XIV. Statistical Analysis

All statistical analyses for qRT-PCR data was performed with the use of a Student's paired *t*-test. Data were only taken as significant if the p-value was less than 0.05. For statistical analysis on the tissue microarray of human multiple myeloma samples (TMA T293) a Student's paired *t*-test was performed in Statistica software.

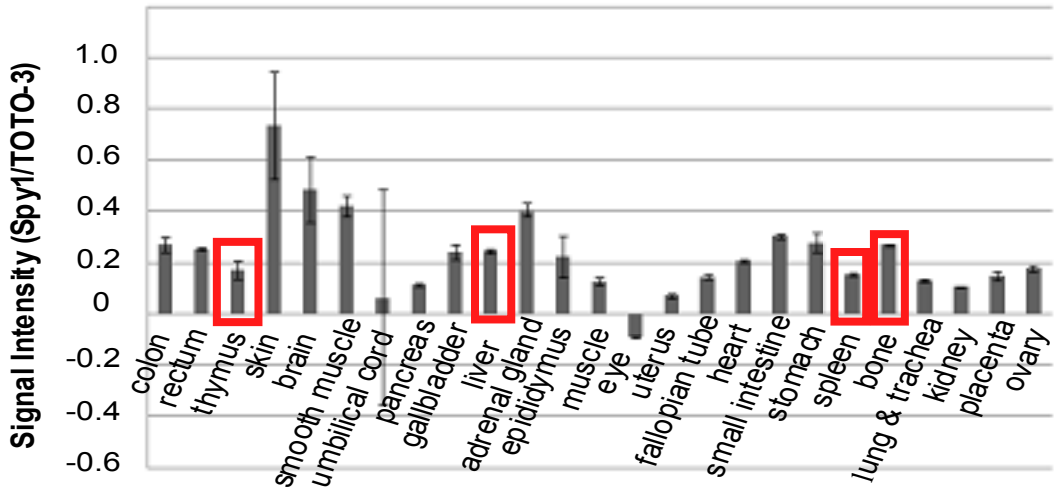
RESULTS

I. Spy1 Protein is Expressed in Mammalian Sites of Hematopoiesis

To analyse protein expression of Spy1 in tissues known to be implicated in blood cell production and maturation, tissue microarray analysis was performed. Tissue samples present on the microarray represent those of a human fetus at age 5 months gestation. Tissues were stained with anti-Spy1 and anti-rabbit-Alexa 488 antibodies as well as TOTO-3 nuclear control stain in order to quantify Spy1 protein levels. Although several human tissues were analysed, those of interest to blood cell development are the thymus, liver, spleen, and bone. Results indicate that Spy1 is expressed at the protein level in the thymus, liver, spleen, and bone and that this expression is moderate in comparison to other tissues represented on the microarray (Figure 3A). Interestingly, of the hematopoietic tissues examined, Spy1 was expressed to the greatest extent in the liver and the bone.

Further analysis on liver, spleen, and thymus samples from Balb/C mice at post-natal age 7 and 21 days was performed using SDS-PAGE and immunoblotting (Figure 3B). Results were quantified using densitometry and Spy1 protein levels were analyzed relative to an internal control protein, actin. Results indicate that Spy1 protein is expressed in all the murine hematopoietic tissues sampled thus far. Remaining consistent with the tissue microarray data, Spy1 protein was expressed to the highest extent in the liver. Furthermore, levels of Spy1 protein showed, at minimum, a 2 fold decrease at each site by day 21. Overall, these results show that Spy1 is expressed in mammalian tissues important for hematopoietic development.

A



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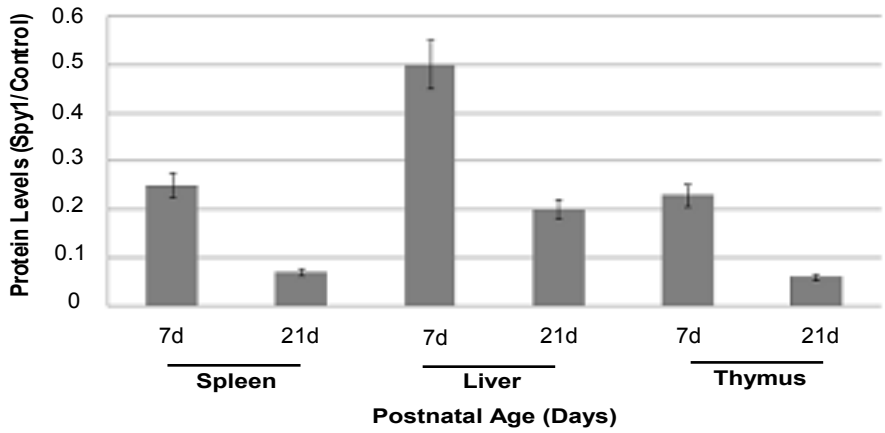


Figure 3. Spy1 protein is expressed in mammalian hematopoietic tissues. (A) Spy1 protein levels in human fetal samples as measured by tissue microarray analysis. Values are presented as relative to the nuclear control TOTO-3. Error bars represent standard error of the mean of at least 3 separate samples for each tissue. Hematopoietic tissues are highlighted in red boxes. **(B)** Levels of Spy1 protein in Balb/C spleen, liver, and thymus tissue samples extracted at post-natal days 7 and 21. Densitometry analysis depicts the ratio of Spy1 protein relative to loading control (β-Actin). Error bars represent standard error of the mean of 3 protein lysates from each tissue.

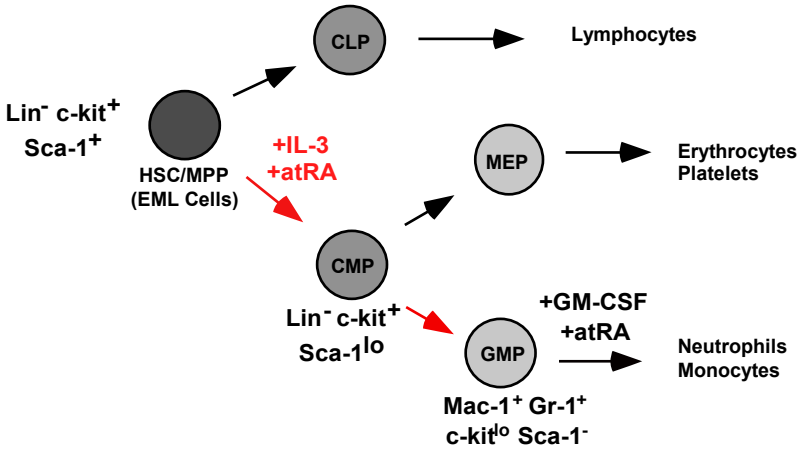
II. Spy1 Expression Decreases During EML Myeloid Differentiation

The multipotent murine bone marrow cell line, EML, was used as a model for hematopoietic myeloid differentiation. EML cells were stimulated to differentiate down the myeloid cell lineage to become more committed early progenitor cells capable of producing many myeloid cell types (Figure 4A). Specifically, EML cells were given murine IL-3 and atRA over a time period of 3 days. Control cells were grown in the absence of IL-3 and atRA. mRNA expression of Spy1 relative to EML control cells was evaluated every 24 hours using qRT-PCR. Results indicate that both of the stem cell markers c-kit and Sca-1 significantly decrease over time (Figure 4B & 4C) while the myeloid specific differentiation markers, Mac-1 and Gr-1, both increase over time (Figure 4D & 4E). Collectively, these results indicate that the EML cells were successfully undergoing myeloid differentiation. Furthermore, it was demonstrated that Spy1 mRNA consistently decreases as EML cells are forced to differentiate into myeloid progenitor cells; at least a 3-fold decrease was observed at all time points (Figure 4F). These results were found to be statistically significant.

III. Spy1 Expression is Elevated in $CD34^+$ EML Cells

It has previously been shown that EML cells are a heterogeneous population of multipotent blood cells[30,86]. Immunomagnetic sorting using anti-CD34 antibody was performed to isolate the $CD34^+$ and $CD34^-$ populations of EML cells; these populations have been shown previously to possess stem-like characteristics[26,52,86-87]. qRT-PCR analysis of CD34 mRNA levels shows successful sorting of $CD34^+$ and $CD34^-$ cells (Figure 5A). qRT-PCR analysis of Spy1 mRNA levels within each population compared to a heterogeneous population was also performed (Figure 5B). Although these data

A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Sca-1+
HSC/MPP
(EML Cells)
+IL-3
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MEP
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Platelets
CMP
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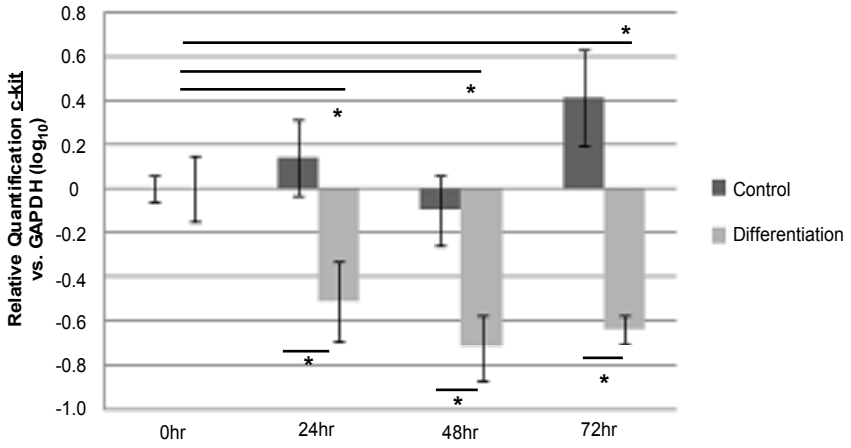
+atRA
GMP
Neutrophils
Monocytes
Mac-1+ Gr-1+
c-kitlo Sca-1-

B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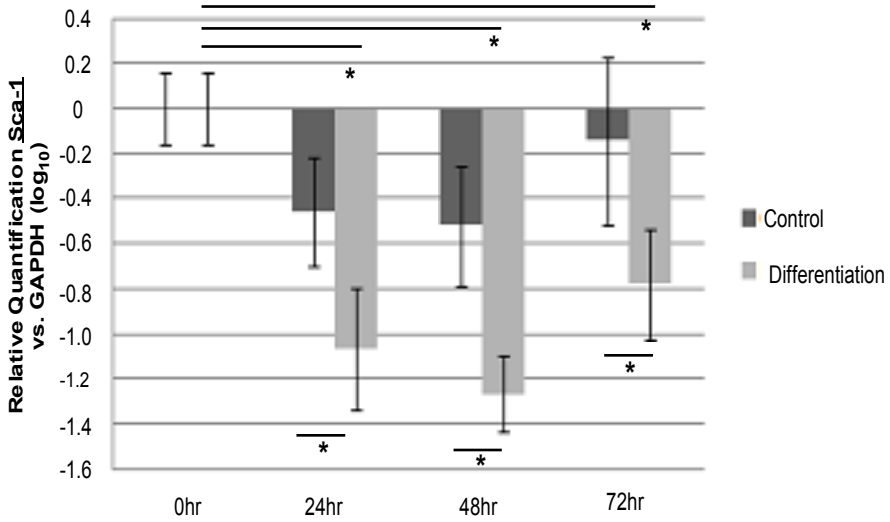
24hr
48hr
72hr
Control
Differentiation

C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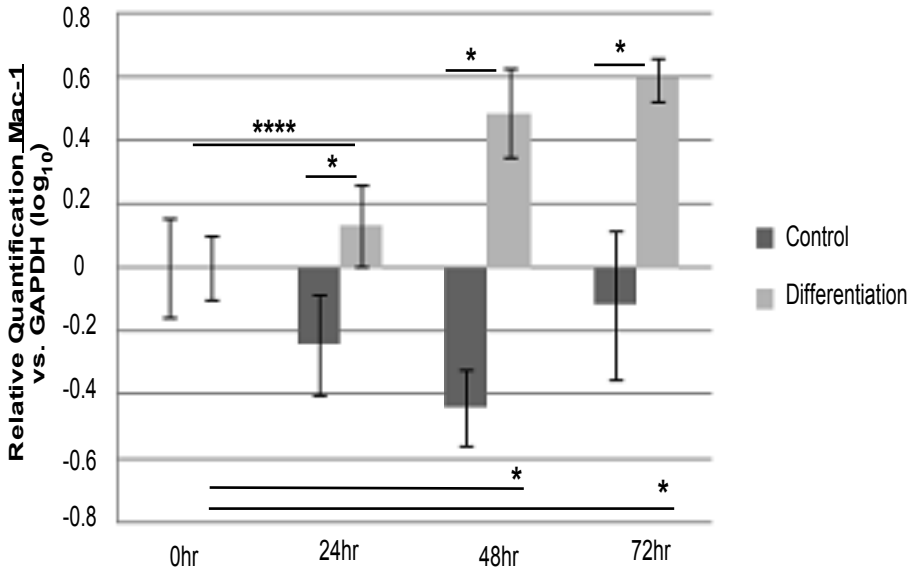
0hr
24hr
48hr
72hr
Control
Differentiation

D


Relative Quantification Mac-1
vs. GAPDH (log10)
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0.4
0.2
0
-0.2
-0.4
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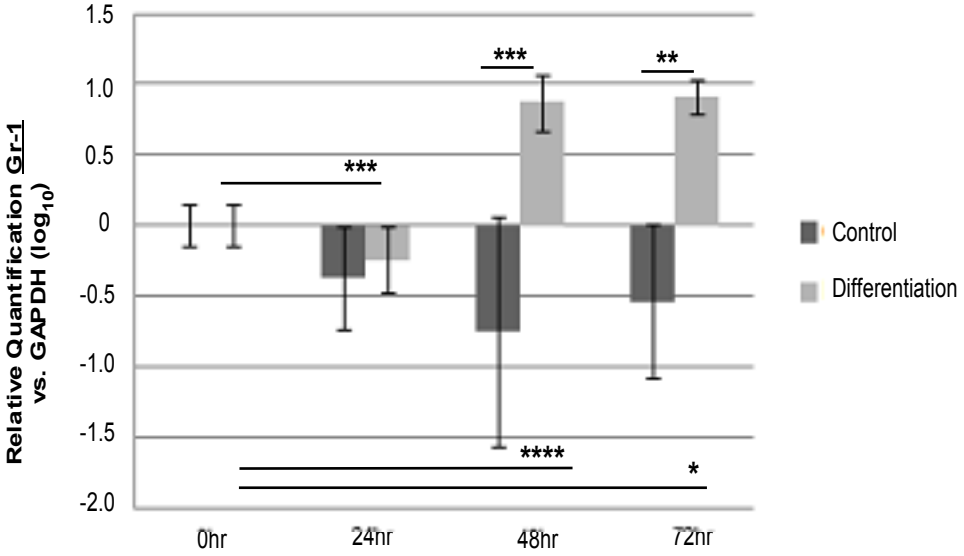
E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vs. GAPDH (log10)
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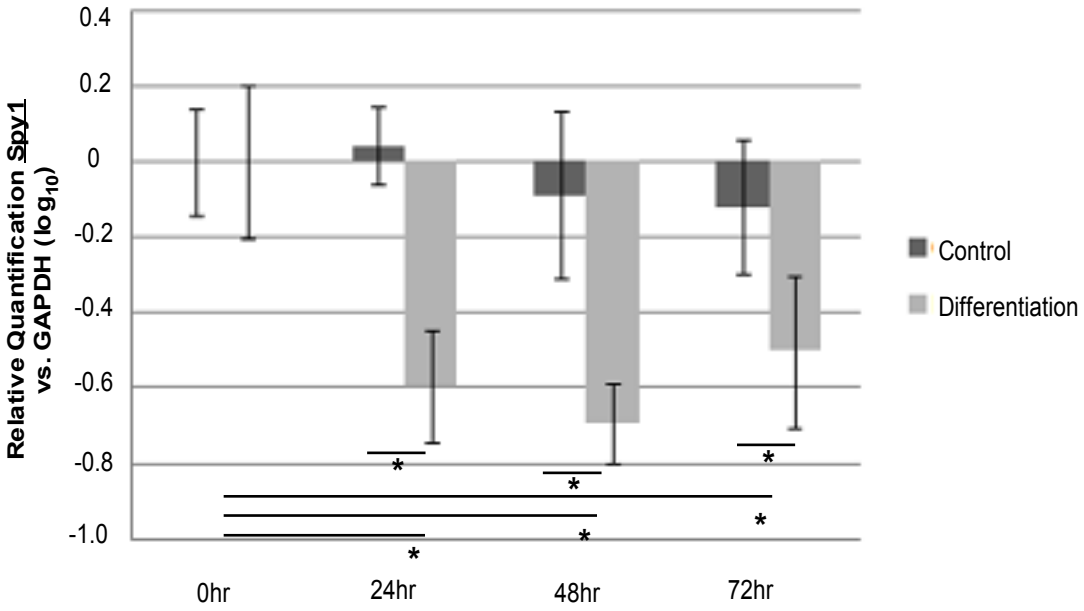


Figure 4. Spy1 expression decreases during EML myeloid differentiation. (A) A schematic representation of EML cell differentiation. Differentiation steps (arrows) and growth factors used are marked in red. **(B-F)** qRT-PCR was used to analyze mRNA levels of stem cell markers: c-kit **(B)** and Sca-1**(C)** as well as myeloid specific differentiation markers: Mac-1 **(D)** and Gr-1**(E)**, and Spy1 **(F)**. All data are normalized to GAPDH and are expressed as relative quantification (RQ) on a logarithmic scale ($\log_{10}$). Error bars represent standard error of the mean of three independent experiments each run in triplicate. Statistical analysis was performed using a Student's paired *t*-test (*p<0.001, **p<0.002, ***p<0.005, ****p<0.05)

represent only two independent experiments, these preliminary results indicate that Spy1 is elevated in the $CD34^+$ population; this is an approximately 50 fold increase over the $CD34^-$ EML cell population. Taken together, the results presented in Figures 4 and 5 demonstrate correlative expression of Spy1 in early stem and MPP cells in the hematopoietic system.

IV. Spy1 Expression Increases During EML Terminal Myeloid Differentiation

EML cells that were successfully differentiated into myeloid progenitor cells were further stimulated with murine GM-CSF and atRA to induce differentiation into terminal cells of the granulocyte and monocyte lineages (Figure 6A). It is important to note that EML-derived myeloid progenitors are absolutely dependent on GM-CSF for growth; therefore, control cells were grown in the absence of atRA and the presence of GM-CSF. Gene expression of Spy1, c-kit, and the myeloid specific differentiation markers, Mac-1 and C/EBPε, was analyzed using qRT-PCR every 24 hours for a period of 72 hours. Results show that myeloid progenitor cells were successfully differentiating into terminal myeloid cells as indicated by the decrease in c-kit expression (Figure 6B) and the increase in both Mac-1 (Figure 6C) and C/EBPε (Figure 6D) expression. However, the control cells showed a slight increase in Mac-1 and C/EBPε, indicating spontaneous differentiation potentially as a result of GM-CSF. Although results were inconsistent with regards to Spy1 expression levels between 4 independent experiments, results do suggest that Spy1 may be increased at the gene expression level during these later stages of differentiation (Figure 6E).

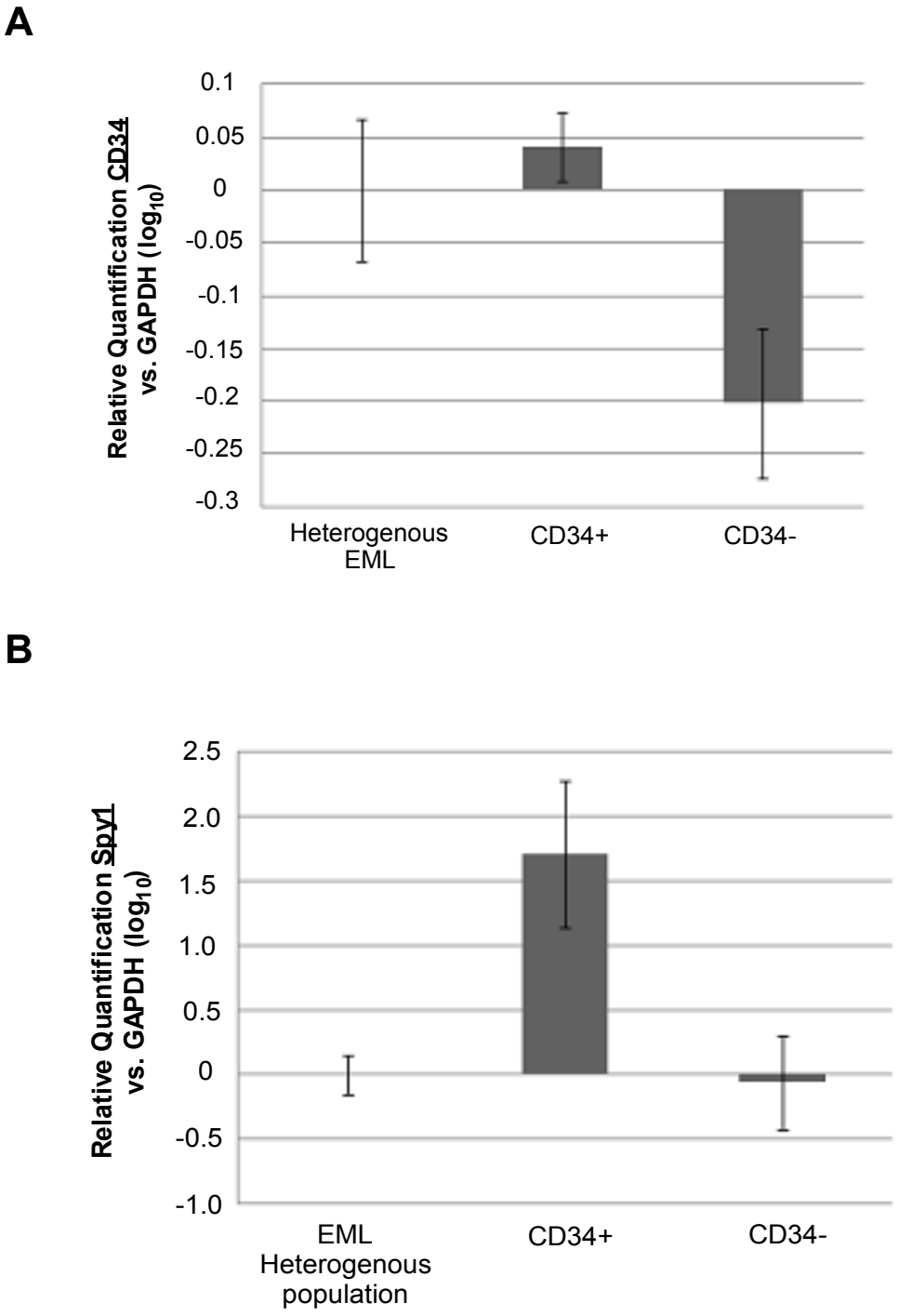
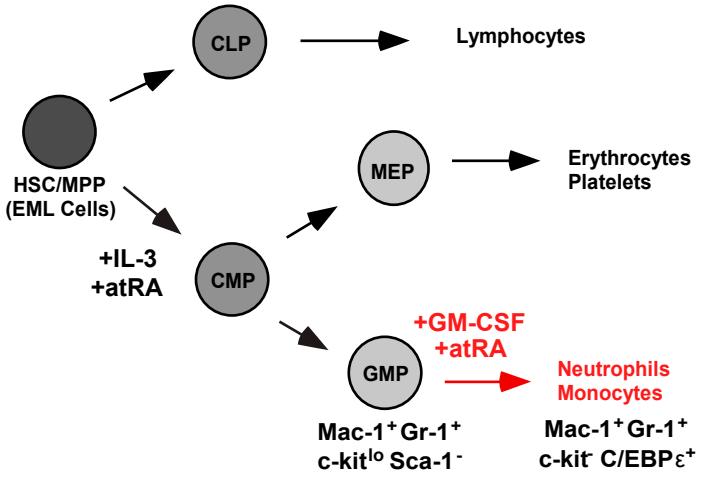


Figure 5. Spy1 expression is elevated in CD34$^+$ EML cell populations. Relative mRNA levels of CD34 **(A)** or Spy1 **(B)** were assessed by qRT-PCR in heterogenous EML populations, CD34 enriched populations (CD34$^+$), and the CD34 negative populations (CD34$^-$). Data are normalized to GAPDH and are represented as the relative quantification (RQ) depicted on a logarithmic scale ($\log_{10}$). Error bars represent the standard error of the mean of two independent sorts each run in triplicate qRT-PCR reactions.

A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(EML Cells)
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c-kit- C/EBPε+

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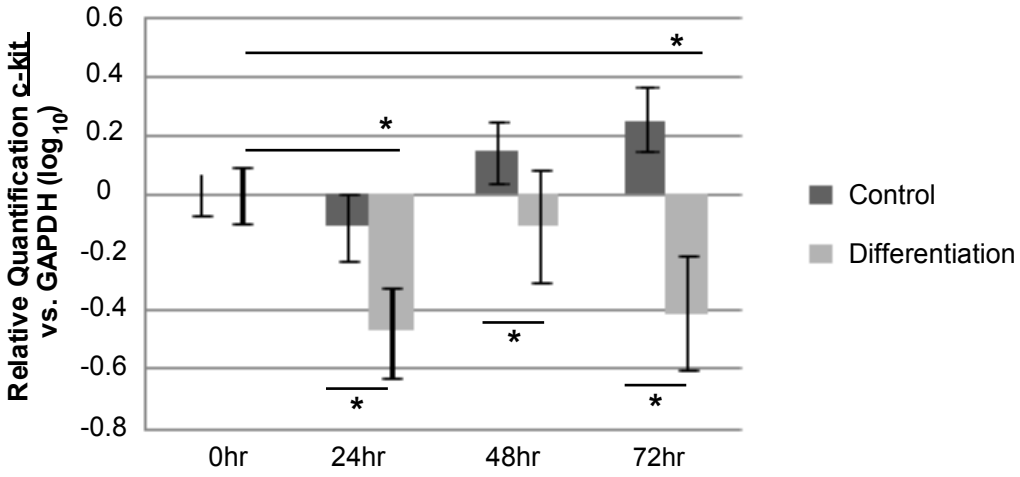
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vs. GAPDH (log10)
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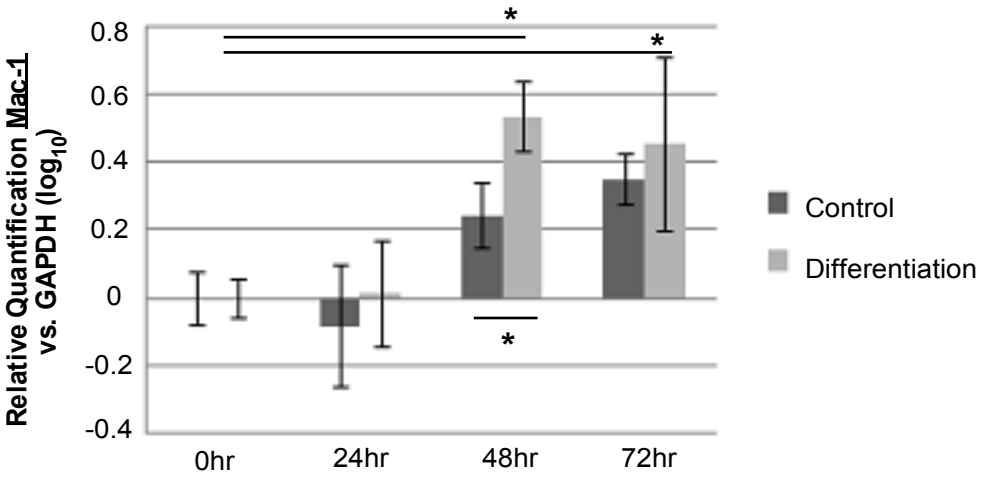
Control
Differentiation

C


Relative Quantification Mac-1
vs. GAPDH (log10)
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Differentiation

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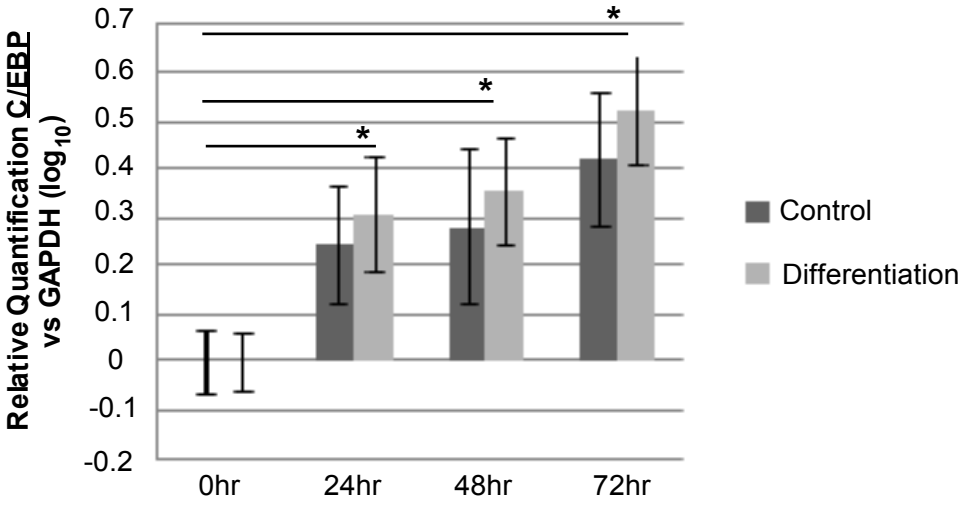

E

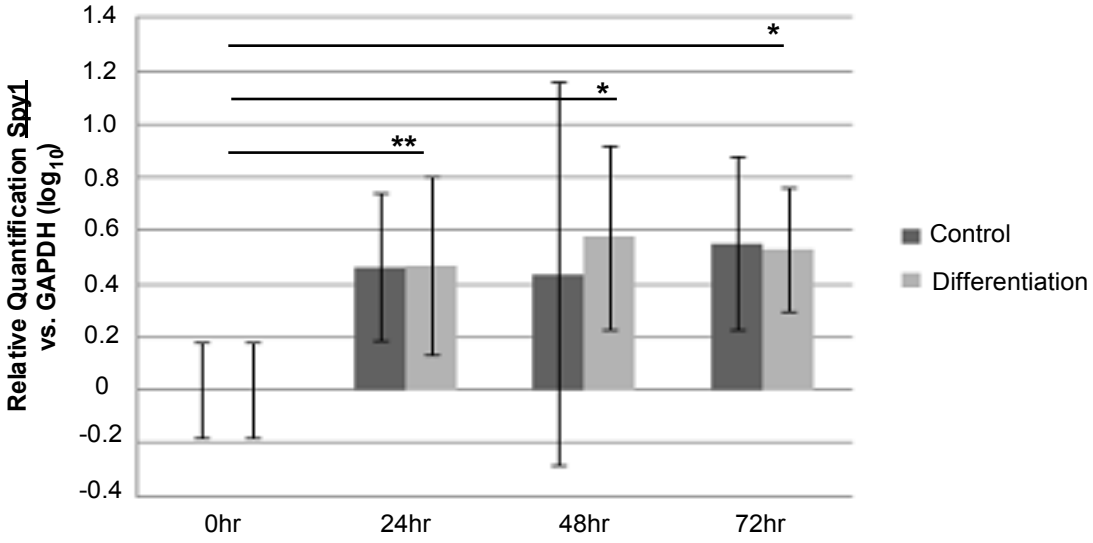

Figure 6. Spy1 mRNA levels increase during EML terminal myeloid differentiation. **(A)** Schematic representation of EML cell line differentiation. The differentiation steps performed in this experiment are shown in red. **(B-E)** qRT-PCR analysis of the mRNA levels of: the stem cell marker c-kit **(B)**, the differentiation markers Mac-1 and C/EBPε **(C & D** respectively**)** , and Spy1 **(E)**. All data are normalized to GAPDH endogenous control and data are presented as relative quantification (RQ) on a logarithmic scale ($\log_{10}$). Error bars represent standard error of the mean of four independent experiments each run in triplicate. Statistical analysis was assessed by the Student's paired *t*-test (*$p<0.001$, **$p<0.05$).

V. Leukemia Cell Growth Decreases in the Absence of Spy1

To study the role of Spy1 in leukemic stem cell populations, a human acute promyelocytic cell line was chosen as a representative case of AML. HL-60 cells were infected with lenti-virus to express pLKO-shSpy1 or with pLKO-shScrambled (pLKO) as a control. qRT-PCR analysis was used to confirm the knock-down of Spy1; results demonstrate approximately an 8 fold decrease in the expression of Spy1 in cells infected with pLKO-shSpy1 compared to control pLKO cells (Figure 7A). Cells successfully expressing pLKO and pLKO-shSpy1 were selected by growth media containing puromycin. After 72 hours of selection, 100% of wildtype HL-60 cells were dead, while 100% of pLKO and approximately 80% of pLKO-shSpy1 cells were alive, indicating they were successfully infected (Figure 7B-G). To assess the effect Spy1 depletion would have on HL-60 cell growth, 100,000 cells were seeded in 6 well plates for each construct and cell number was assessed every 24 hours using Trypan Blue Exclusion and cell counts performed in triplicate. Results indicate that HL-60 cells exhibiting Spy1 knock-down grow more slowly than both pLKO control and wildtype cells at all time points (Figure 8). After 72 hours of growth pLKO-shSpy1 cells have multiplied to produce approximately 3.5×10^5 cells, whereas pLKO control cells have nearly double the cell number (6×10^5 cells). Therefore, it is evident that HL-60 cells grow significantly slower when Spy1 is depleted. These results were found to be statistically significant.

VI. Expression of LSC Genes Decreases in the Absence of Spy1

CLL-1 and CD33 are both known to be highly expressed on leukemia cells in patients with AML, but not on normal HSCs or non-myeloid cells[66,68,70,88]. In addition,

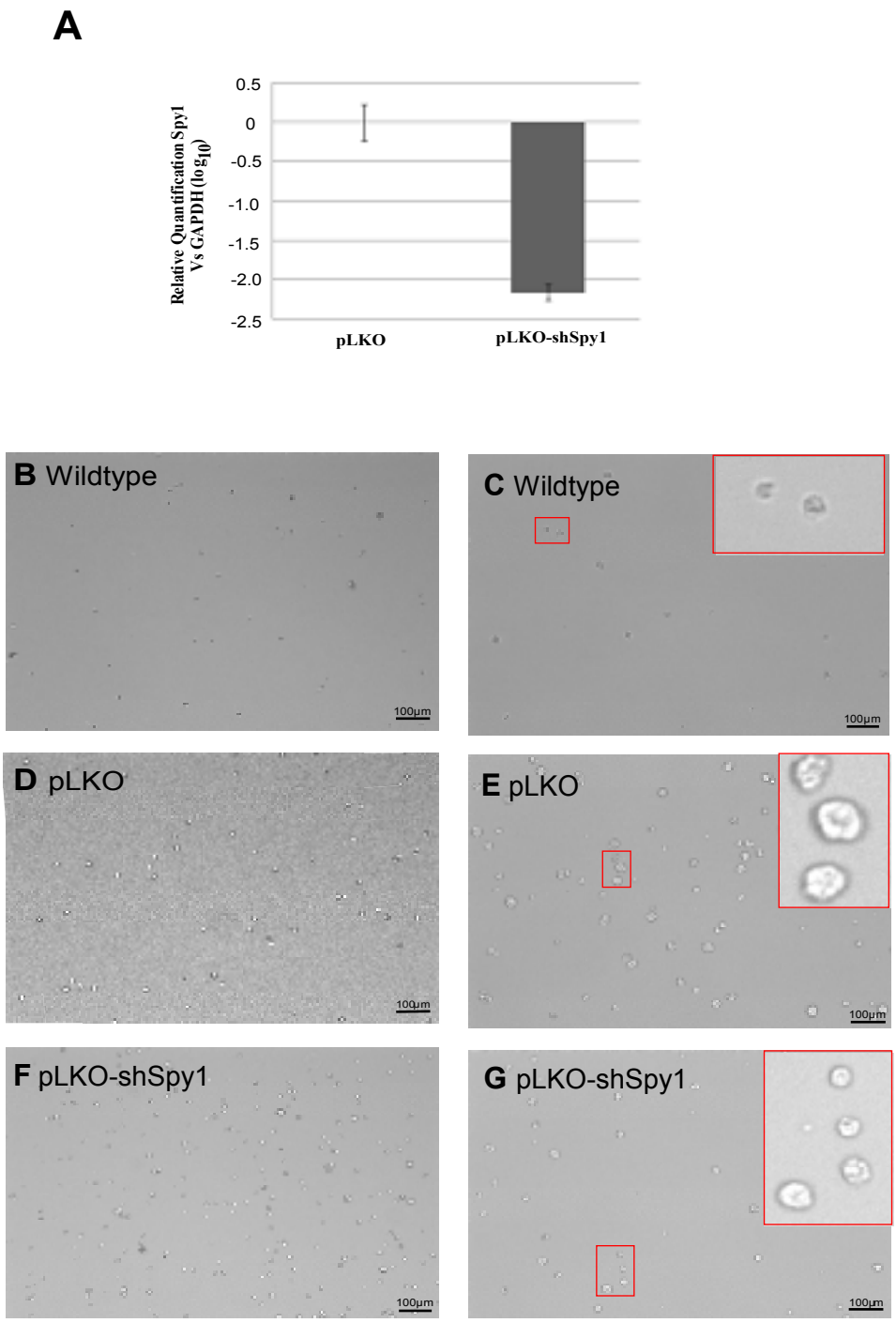

Figure 7. Successful shRNA-mediated knock-down of Spy1 in HL-60 cells. HL-60 cells were infected with lentivirus carrying shRNA against Spy1 (pLKO-shSpy1) or a scrambled control (pLKO). **(A)** qRT-PCR was used to assess knock-down efficiency. Data is normalized to GAPDH andpresented as relative quantification (RQ) on a logarithmic scale (log_{10}). **(B-G)** Microscopy images of cells treated with Puromycin. **(B, D, and F)** Total magnification of 100x. **(C, E, and G)** Total magnification of 200x.

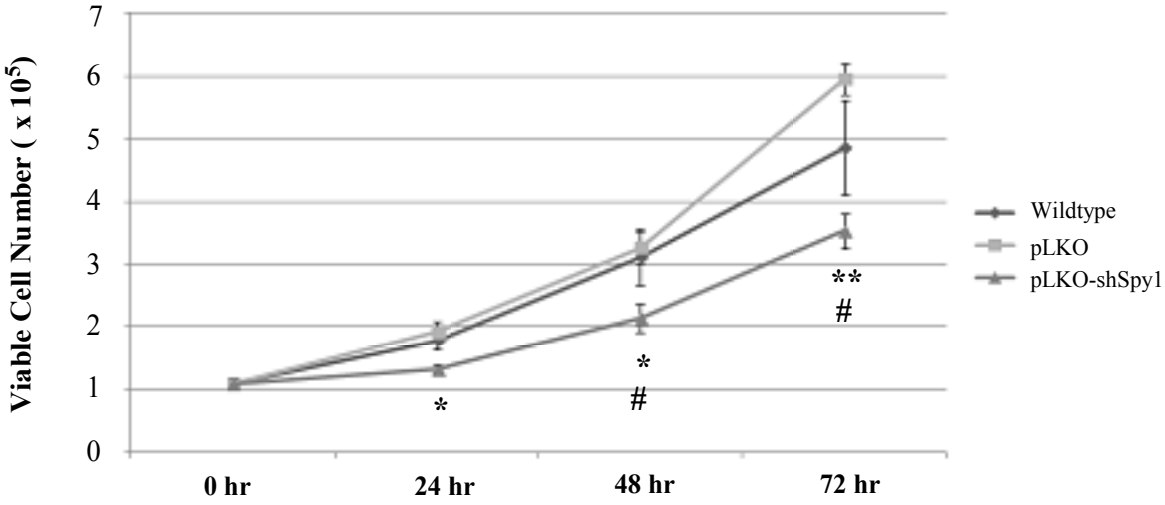

Figure 8. Spy1 knock-down slows HL-60 cell growth. Trypan blue exclusion assay was performed to assess cell proliferation of HL-60 cells successfully infected with shRNA against Spy1(pLKO-shSpy1) or scrambled control (pLKO). over a period of 72 hours. Error bars represent the standard error of the mean viable cell number of three independent experiments each counted in triplicate. Statistical analysis using a Student's paired *t*-test was performed to compare growth of pLKO to pLKO-shSpy1 cells (#$p<0.001$) and to wildtype cells (*$p<0.001$, **$p<0.005$) at all time points.

CLL-1 and CD33 have been previously studied as potential LSC targets in clinical trials for treatment of human AML[66-67,69]. Following selection in puromycin, cells exhibiting successful knock-down of Spy1 were analysed by qRT-PCR for expression of both CLL-1 and CD33. Results indicate that both CLL-1 and CD33 levels are decreased in HL-60 cells expressing shSpy1 when compared to control pLKO cells (Figure 9). More specifically, an approximate 3 fold decrease in the expression levels of CLL-1 and an approximate 4 fold decrease in the expression levels of CD33 were observed. These results indicate a potential role for Spy1 in promoting formation of LSC populations in AML.

VII. Spy1 Expression Increases During HL-60 Differentiation

To examine whether Spy1 levels may be altered by current therapeutic regimens for AML, HL-60 cells were treated with atRA. Specifically, HL-60 cells were cultured in media containing atRA for 5 days to allow for granulocytic differentiation or in media containing 0.1% ethanol as a vehicle control. Cells were analysed using qRT-PCR for expression of the human myeloid cell marker Mac-1 and for Spy1 at 3 days and 5 days after the addition of atRA (Figure 10). Preliminary results demonstrate that Mac-1 expression levels increase as HL-60 cells differentiate, demonstrating an approximate 5.6 fold increase in expression over vehicle control cells by 5 days differentiation. Spy1 gene expression also increased as HL-60 cells differentiated, demonstrating approximate 5 fold and 16 fold increases in expression over cells treated with a vehicle control at 3 and 5 days atRA differentiation, respectively. Interestingly, this result is consistent with the increase of Spy1 observed during atRA-induced terminal differentiation of EML cells.

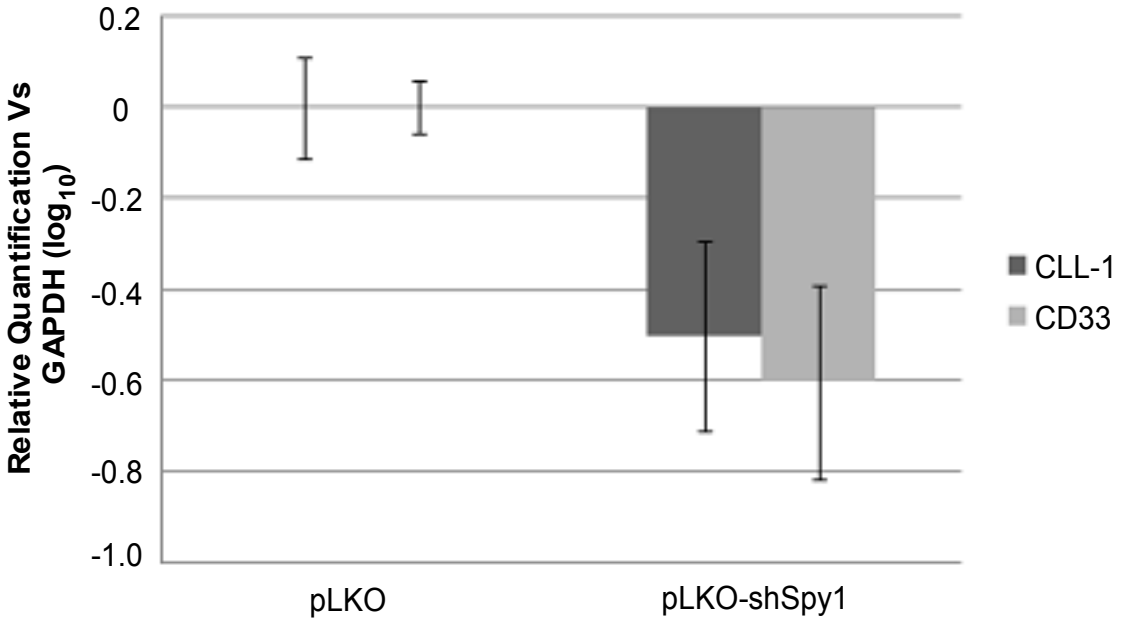

Figure 9. Expression of LSC gene decreases in the absence of Spy1. Cells successfully infected with either pLKO-shScrambled (pLKO) or pLKO-shSpy1 constructs were collected and analyzed for expression of known leukemic stem cell markers for acute myelogenous leukemia. qRT-PCR was used to analyze mRNAlevels of CLL-1 and CD33. Data was normalized to GAPDH and shown as relative quantification (RQ) on a logarithmic scale ($\log_{10}$). Error bars represent standard error of the mean of two independent experiments.

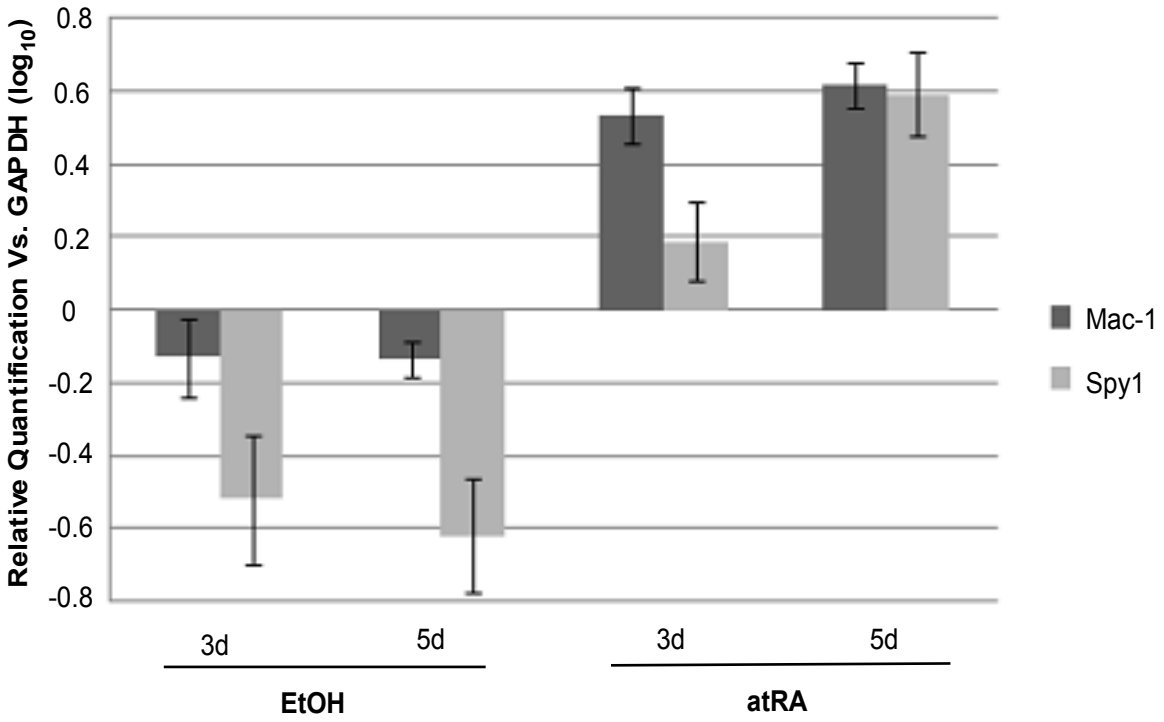

Figure 10. Spy1 expression increases during HL-60 cell differentiation. HL-60 cells were stimulated to differentiate into terminal granulocytes using atRA. Cells treated with a vehicle control (EtOH) are also shown. qRT-PCR analysis of Spy1 and of the differentiation marker Mac-1 show mRNA levels at 3 and 5 days differentiation. Data is normalized to GAPDH as an internal control and all data is represented as relative quantification (RQ) on a logarithmic scale ($\log_{10}$). Error bars represent the standard error of the mean of three replicates of the same sample.

VIII. Spy1 Protein is Significantly Elevated in Myelomas

To examine levels of Spy1 in a more differentiated blood malignancy, tissue microarray analysis of human bone marrow-derived myeloma tumours was performed. Specifically, Spy1 protein levels were examined in a variety of human myeloma samples relative to levels in normal bone marrow. Paraffin-embedded tissues were stained with anti-Spy1 antibody followed by Alexa488 fluorescent conjugated secondary antibody and a nuclear control, TOTO-3. Results indicate that Spy1 protein is expressed in normal human bone marrow and that this expression is significantly elevated in all of the tumour samples examined to date (Figure 11). For example, Spy1 protein was expressed at levels more than ten times as great as those observed in normal marrow (Figure 11; Myeloma of the Vertebrae). Spy1 protein was increased to the least extent in Ewing's sarcoma, but even this expression was three times that of normal marrow samples (Figure 11).

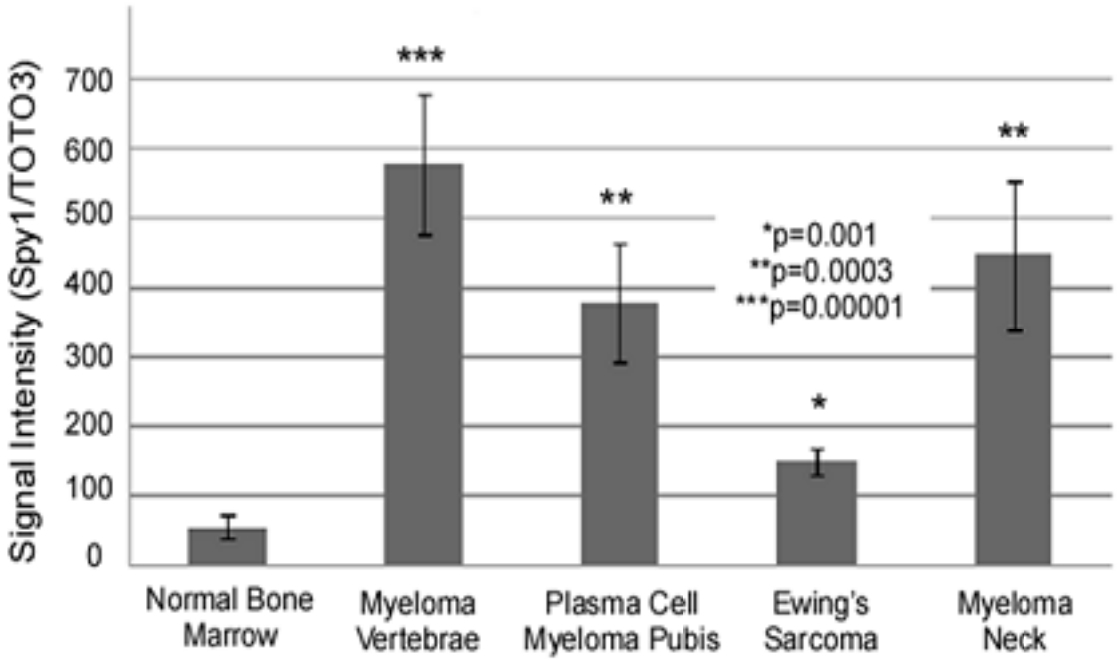

Figure 11. Spy1 protein is significantly elevated in multiple myelomas. Levels of Spy1 protein relative to a nuclear control stain, TOTO-3 are graphed. Tissues are representative of four different types of multiple myeloma (n=16) (myeloma of the vertebrae, plasma cell myeloma of the pubis, Ewing's sarcoma, and myeloma of the neck) and normal bone marrow tissue (n=8). Samples were obtained from human patients ranging in age from 10-57 years. Error bars represent the standard error of the mean. Statistical significance was assessed using a student's paired t-test in Statistica software (*p=0.001, **p=0.0003, ***p=0.00001)

DISCUSSION

Collectively blood malignancies are the 4th most common cancer diagnosed in both men and women worldwide[1]. Despite overall drops in mortality due to advances in treatment and earlier diagnosis, leukemias still have a poor prognosis with an overall five year survival rate of only 51%[1]. Blood malignancies represent a diverse group of cancers with many genetic backgrounds, not all of which have been resolved to date. The findings presented in this study have demonstrated involvement of the novel cell cycle regulator, Spy1, in normal hematopoietic cell development and suggest implications for Spy1 in the development of LSCs.

To determine whether Spy1 protein was expressed in tissues known to play important roles in the hematopoietic system, tissue microarray analysis was performed. It was found that Spy1 was expressed, at the protein level, rather ubiquitously across many human fetal tissues. Notably, Spy1 protein was found to be expressed in fetal tissues known to support hematopoietic cell development including the spleen, thymus, and liver. This is consistent with previous work which demonstrated Spy1 expression in many human tissues including the liver and thymus[76]. Of the tissues examined in this study, Spy1 protein levels were greatest in the liver. The liver plays a very prominent role in development of the hematopoietic system by supporting hematopoiesis for a large portion of fetal development[37,39,41]. It is notable that Spy1 has been found to be involved in hepatocellular carcinoma[89]; hence, whether expression is occurring in the hematopoietic cells or early hepatic cells from these data is not known.

These results were further supported by western blot analysis of murine spleen, thymus, and liver tissues where Spy1 protein was found to be expressed at 7 and 21 days post-natal development. Consistent with the tissue microarray analysis, Spy1 was

expressed to the greatest extent in the liver. The murine liver has been shown to support HSC production and development for a short period of time post-natally[39] and HSCs have recently been identified in the adult mammalian liver[41,90]. Moreover, the presence of hematopoietic progenitors capable of forming hematopoietic colonies *in vitro* has been observed in many murine adult organs including the liver and the spleen[90-91]. Therefore, this result provides further evidence that Spy1 is expressed in tissues important in early hematopoietic development. Furthermore, densitometry analysis shows that Spy1 protein expression decreased by day 21 in all tissues examined. The proportion of HSCs in hematopoietic tissues is known to be the greatest in younger mammals due to a developmental need[34]; thus, the decrease in Spy1 protein levels observed in older murine samples may be explained by a decrease in the proportion of early stem and progenitor cells in these tissues. However, due to the many cell types present in each of these tissues, future steps should involve cell sorting in order to draw any direct conclusions between Spy1 and HSC development in these tissues.

To further clarify a role for Spy1 in hematopoietic cell development, murine bone marrow cells capable of erythroid, myeloid, and lymphoid (EML) differentiation in response to different growth factors *in vitro* were utilized[30,86]. EML cells are representative of the MPP population in hematopoietic development expressing the cell surface markers c-kit, Sca-1, and some early lineage progenitor markers[30]. EML cells have previously been immortalized by the expression of a dominant negative retinoic acid receptor; this induces a block in EML myeloid differentiation that can be overcome with very high concentrations of all-trans retinoic acid[30]. For the purposes of this study, EML cells were induced to differentiate down the myeloid pathway by the addition of IL-3 and atRA to generate the more committed myeloid progenitor cells, termed GMPs. Results

show a significant decrease in Spy1 gene expression during the initial stages of EML myeloid differentiation, demonstrating that Spy1 levels correlate with stages of development focused on maintaining the early hematopoietic stem and progenitor cell populations. This is consistent with previous work in our lab showing that Spy1 levels correlate with its ability to regulate stemness decisions in both the breast and the brain[84,92]. Future experiments in which Spy1 levels are manipulated prior to differentiation will be necessary to further substantiate the functional importance of this expression pattern and provide direct evidence that Spy1 plays a role in directing HSC or MPP fate.

As previously mentioned, EML cells are known to be a heterogeneous population of MPP cells expressing early stem cell genes such as c-kit and Sca-1, but also expressing markers for early lineage progenitor cells[30]. Furthermore, EML cells contain both a $CD34^+$ and $CD34^-$ population of cells with different capacities to proliferate and differentiate[86]. To determine if Spy1 is differentially expressed in certain populations of the HSC compartment, EML cells were subject to immunomagnetic sorting to isolate $CD34^+$ and $CD34^-$ populations. Results demonstrate an approximate 50 fold increase in Spy1 gene expression levels in the $CD34^+$ EML population in comparison to both the $CD34^-$ and the heterogeneous EML populations. This finding further demonstrates that Spy1 expression correlates with the stages of hematopoietic development involving the early $CD34^+$ stem and MPP cells. Differential expression of CD34 in the murine hematopoietic stem cell compartment mirrors the proliferative status of the cell populations. Relatively quiescent LT-HSCs residing in the bone marrow niche are $CD34^-$ and capable of long-term engraftment[25-26,28,93]. In contrast, ST-HSCs and MPPs have transient engraftment ability, can be mobilized from the marrow, and are $CD34^+$ [25-26,28,93].

Despite these established correlative patterns, it is notable that differences in cell surface protein expression have not been definitively linked as playing a causative role in the functional differences between ST-HSCs and MPPs[25-26,52,87,94]. Spy1 is expressed to a greater extent in the ST-HSCs and MPP cells and, therefore, Spy1 may be important in fate decisions of these cells, however, this functional role needs to be directly assessed.

EML-derived GMP cells are dependent on GM-CSF for survival and can spontaneously produce mature monocytes and macrophages. However, EML-derived GMP cells display a defect in terminal granulocyte differentiation that can be overcome with high concentrations of atRA[30]. To determine how Spy1 levels correlate with these later stages of development, EML-derived GMP cells were induced to terminal differentiation with GM-CSF and atRA. Control cells were grown in GM-CSF containing medium. Interestingly, Spy1 mRNA levels were found to be elevated during the terminal differentiation of EML cells, although changes in Spy1 levels were not significantly greater than those observed in control cells. This may be due to the fact that EML-derived GMPs are capable of spontaneously differentiating down the monocytic pathway; therefore, differentiated cells of the monocyte lineage are likely present in the control cell populations as indicated by the increases in Mac-1 and C/EBPε in these cells.

Although LT-HSCs and ST-HSCs have the highest capacity for self-renewal of all hematopoietic cells, it has been discovered that MPPs, early and late lineage progenitor cells, and even some mature blood cells maintain a proliferative index due to the high turnover of blood cells[95-96]. Spy1 has been shown to be necessary for proliferation in a variety of mammalian cell types[76,82,84,92]; therefore, perhaps Spy1 is elevated during the differentiation of GMP cells to aid in proliferation of lineage progenitors and mature

blood cells lower in the hierarchy. Alternatively, since Spy1 levels were only analysed at the mRNA level, it is possible that Spy1 protein continues to decrease during GMP differentiation, but that this is regulated at the translational or post-translational level. Overall, this result may suggest broader implications for Spy1 in hematopoietic development as it indicates a potential need for Spy1 in later stages of blood cell differentiation.

Collectively, these *in vitro* experiments indicate that Spy1 expression correlates with proliferative stages of myeloid development including the early stem and MPP stages; therefore, subsequent experiments aimed to elucidate a role for Spy1 in AMLs known to have LSC populations. HL-60 cells are human leukocytes consisting primarily of neutrophilic promyelocytes taken from a patient with acute promyelocytic leukemia[94]. HL-60 cells were subjected to shRNA-mediated knock-down of Spy1 and effects on growth were assessed using trypan blue exclusion and cell counts. It was observed that HL-60 cells grew more slowly in the absence of Spy1 in comparison to both control pLKO cells and wildtype HL-60 cells. This demonstrates that Spy1 plays an important role in promoting cell growth and proliferation in this representative case of AML. These results are consistent with previous data demonstrating a proliferative role for Spy1 in mammalian cells[76,82,84,97]. Further exploration of these effects in a wide panel of AML cell lines is needed.

Furthermore, HL-60 cells have been found to express high levels of the LSC specific markers CLL-1[69] and CD33[66]. CLL-1 and CD33 have been extensively studied and identified as potential LSC targets important in drug development and clinical trials for the treatment of human AML[66-67,69]. Interestingly, when Spy1 was depleted using shRNA in HL-60 cells, gene expression levels of both CLL-1 and CD33 decreased as

assessed by qRT-PCR. This finding indicates a role for Spy1 in promoting the expression of known LSC markers. Taken together, these results suggest a role for Spy1 in promoting formation of LSC populations in this representative case of AML. This is consistent with previous work from our lab and others demonstrating the involvement of Spy1 in aggressive forms of breast and brain cancer and that Spy1 can promote the expression of CSC markers important in driving tumour formation[84,92,99-100]. Future experiments utilizing sorted CLL-1^{+} and CD33^{+} HL-60 cell populations are necessary to accurately examine the effects of Spy1 knock-down on growth and proliferation of the LSC compartment of HL-60 cells.

To address whether Spy1 levels are altered during therapeutic treatment of AML, HL-60 cells were differentiated with atRA to mimic current treatment of acute promyelocytic leukemia in the clinic. Promyelocytic leukemia is characterized by an accumulation of promyelocytes in the bone marrow that are unable to complete granulocytic differentiation[8]; treatment with atRA has seen much success clinically[5-8], however, cases of atRA resistant myelocytic leukemia have been reported[101]. As previously observed, knock-down of Spy1 has effects on both leukemic cell growth and LSC marker expression in this cell line. Therefore, it was my hypothesis that knock-down of Spy1 might sensitize HL-60 cells to differentiation treatment with atRA. Surprisingly, initial experiments examining endogenous levels of Spy1 during HL-60 atRA differentiation showed an increase in Spy1 gene expression as cells became mature granulocytes. Although this result is not consistent with the hypothesis for this experiment, it is similar to the observations made during atRA induced terminal EML maturation and may be explained by the proliferative potential maintained by late lineage progenitors and mature blood cells[95-96]. Furthermore, it is important to note that Spy1

levels were only examined at the mRNA level; protein analysis should be completed before drawing final conclusions as it is possible that Spy1 is being regulated differently at the translational level. Furthermore, functional effects of the knockdown of Spy1 on success of treatment will require assays measuring overall cell growth properties following treatment, including clonal assays to measure the effects on the LSC population.

Lastly, tissue microarray analysis was performed to analyse protein expression of Spy1 in human blood malignancies. Due to the endogenous expression of Spy1 observed in late stage hematopoietic cells, a more differentiated blood malignancy, multiple myeloma, was analyzed for expression of Spy1. Multiple myeloma is a plasma cell malignancy affecting B lymphocyte development within the hematopoietic system[49]. Results demonstrate that Spy1 protein is expressed in normal bone marrow, but that this expression is significantly elevated in all of the myeloma tumours examined. This finding implies that Spy1 may be important in myeloma and warrants further investigation into a role for Spy1 in myelomas. Interestingly, this result suggests a link to Spy1 in a more differentiated cancer in contrast to previous work from our lab showing implications for Spy1 in aggressive breast and brain cancers of a stem cell origin[84,92]. This may be explained by the fact that mature blood cells of the B cell lineage are highly proliferative and can undergo clonal expansion during immune reactions[34,96]. Future directions may aim to examine a role for Spy1 in EML B Cell differentiation decisions.

In summary, this study has demonstrated that Spy1 is expressed in mammalian sites important to both murine and human hematopoiesis. Taken together, the decrease in Spy1 expression during *in vitro* differentiation of EMLs and the high expression of Spy1 in $CD34^+$ EML populations demonstrate that Spy1 is endogenously expressed within the

ST-HSC and MPP compartment of the hematopoietic hierarchy. However, findings also indentified Spy1 to be expressed in more mature hematopoietic cells. Collectively, these *in vitro* experiments demonstrate a correlation between Spy1 expression and proliferative stages of the hematopoietic hierarchy. Furthermore, these findings identify Spy1 as important in promoting development of LSCs in one subtype of AML and it was revealed that Spy1 is significantly elevated in a variety of human myelomas. Overall, this study demonstrates that Spy1 may have broader implications in blood malignancies than the previously identified role in some types of lymphoma[85]. These findings may have implications into the future treatment of different blood malignancies.

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Appendix

Appendix A

Gene Manipulation in EML Cells

This appendix reviews in detail the many different techniques attempted for both transfection of plasmid DNA and infection using lenti-virus. In all cases, successful transfection was assessed using a GFP reporter and light microscopy.

I Transfections

Transfections with polyethylenimine (PEI)

EML cells were seeded in 12 well plates at densities of 0.5 x 10^5 cells/mL, 1.0 x 10^5 cells/mL, and 2.0 x 10^5 cells/mL in serum-free media on the day of transfection. Plasmid DNA for both the pEIZ control and pEIZ-Spy1 were diluted at a ratio of 1:10 in 150mM NaCl. 1 μg plasmid DNA was then mixed with 5 μL of 10 mg/mL polyethylenimine (PEI) in eppendorf tubes. This mixture was allowed to incubate at room temperature for 10 minutes prior to addition to cells. The plate was centrifuged for 5 minutes at 500 x g and 4ºC. Following this centrifugation, media was replaced with serum-free media either immediately or after approximately 16 hours.

Transfections with JetPrime Reagent

EML cells grown to approximately 75% confluency in 5 mL of complete growth media in 6 cm plates were transfected with GFP plasmid DNA using Jet Prime Reagent (VWR CA89129-922) and following the manufacturer's protocol. Briefly, plasmid DNA was added to Jet Prime buffer in an eppendorf tube and vortexed prior to the addition of Jet Prime Reagent. Following an incubation period of 10 minutes at room temperature, plasmid DNA, Jet Prime Reagent, and buffer mixture was added to cells. Different ratios

of plasmid DNA to Jet Prime Reagent and buffer can be found in table 3. Each transfection was performed in duplicate so that transfection times of both 4 hours and 20 hours could be attempted.

Table 3. EML Transfection with JetPrime, Conditions

	GFP Plasmid DNA (µg)	Amount JetPrime Reagent (µL)	Amount Buffer (µL)
Transfection 1	1.0	2	100
Transfection 2	2.0	3	200
Transfection 3	4.0	4	200

II Infections with Lenti-Virus

Lenti-viral Infection

5,000, 10,000, 25,000 or 50,000 EML cells were seeded in 96 well plates in serum-free and antibiotic-free media on the day of infection. Polybrene was added to media at final concentrations ranging from 1 µg/mL to 8 µg/mL. In some instances lenti-virus containing either the pEIZ control vector, pEIZ-Spy1, pLB-shScrambled, and pLB-shSpy1were added immediately after the addition of polybrene. In other instances, cells were allowed to incubate in media-containing polybrene for 20 to 30 minutes prior to the addition of virus. Viral titres for all constructs were 10^7 TU/mL and different MOIs ranging from 0.1 to 10 were attempted. EML cells were allowed to incubate with virus for a minimum of 6 hours to a maximum of 24 hours. Upon removal of virus-containing media, complete growth media was added.

Lenti-viral Infection – Spinoculation

50,000 EML cells were seeded in 96 well plates in serum-free media containing 4 to 8 µg/mL polybrene. After the addition of lenti-virus containing pEIZ control and pEIZ-

Spy1, plates were subject to centrifugation at either 1500 rpm or 800 x g for 10 to 30 minutes at 32ºC. Following centrifugation, media was then replaced with complete growth media. Alternatively, plates were allowed to incubate for a minimum of 6 hours and a maximum of 24 hours before the removal of virus. MOI ranged from 1 to 5.

Lenti-viral Infection with Magnetic Beads

50,000 EML cells were seeded in 96 well plates in serum-free media containing 4 μg/mL polybrene for infection with pEIZ control and pEIZ-Spy1. A mixture of magnetic beads and virus was prepared at a ratio of 1 μL to 10 μL, respectively, and allowed to incubate on ice for 30 minutes. During this incubation, bead-virus mixtures were vortexed every 5 minutes. Prior to the addition of the bead-virus mixture, plates were centrifuged for 5 minutes at 1500 rpm. Plates were then placed on a magnet and allowed to incubate for 20 minutes in a 37ºC humidified incubator. Virus-containing media was replaced with complete growth media approximately 16 hours later.

Vita Auctoris

Kaitlyn N. Matthews was born in Ajax, Ontario. She attended F.J. Brennan Catholic High school in Windsor, graduating in 2006. She then went on to the University of Windsor to study Biological Sciences and Psychology through the Interfaculty programs; she completed an undergraduate thesis project in Biology and obtained an Honours B. Arts. Sc. degree in 2010. She is currently a candidate for the Master's degree in Biological Sciences at the University of Windsor.