MMP1: A TARGET FOR HEMATOPOIETIC STEM CELL DISEASES

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MRP1: A TARGET FOR HEMATOPOIETIC STEM CELL DISEASES

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine, Graduate Center for Toxicology at the University of Kentucky.

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Dr. Mary Vore, Professor of Toxicology

Lexington, Kentucky

2014

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Multidrug resistance-associated protein 1 (MRP1) is a member of the adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporters. MRP1 actively effluxes a variety of endogenous and exogenous substrates from cells, ultimately, working to remove these compounds from the body. MRP1 was initially discovered based on its ability to confer resistance against a variety of chemotherapeutics when overexpressed in cancer cell lines. MRP1 function is important for a number of physiological processes, including regulating cellular and extracellular levels of the anti-inflammatory leukotriene C4 (LTC4) and the antioxidant glutathione (GSH). Our studies have focused on the role of MRP1 in regulating hematopoietic stem cell (HSC) self-renewal and differentiation and the role of CK2 as a regulator of MRP1 function. Reactive Oxygen Species (ROS) cellular levels are tightly regulated and fluctuations in ROS levels affect many cellular processes, including the self-renewal and differentiation of hematopoietic stem cells and kinase signaling pathways. MRP1 regulates ROS through the transport of reduced and oxidized GSH. MRP1 is highly expressed in HSCs, therefore we hypothesized that MRP1 regulates ROS levels in HSCs via efflux of GSH. We have shown that MRP1 regulates HSC self-renewal by modulating cellular ROS via the efflux of GSH. The decrease in ROS results in downregulation of p38 activity and altered expression of a number of redox response genes.

CK2 is a master regulator of the cell and controls cell growth, proliferation, death and survival. Yeast studies from our lab using Ycf1p (a homologue of MRP1) and Cka1p (a homologue of CK2) have found that Cka1p regulates Ycf1p function. This result suggests that CK2 regulates MRP1 function via phosphorylation. We have found that CK2 does regulate MRP1 function via phosphorylation of the N-terminal extension at Thr249. Using A549, H460, and HeLa cancer cell lines, we found that inhibition of CK2 with tetrabromobenzimidazole (TBBz) reduces MRP1 function and increases cellular toxicity to known MRP1 substrates.
Key Words: ABC Transporter, MRP1, Hematopoietic Stem Cell, ROS, Glutathione
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“Challenges are what make life interesting and overcoming them is what makes life meaningful.” - Joshua J Marine

This is dedicated to those that have both inspired me and encouraged me to set goals and strive to complete them.
ACKNOWLEDGEMENTS

The success of one cannot be accomplished without the hard work of many. For that, I am eternally grateful to those who have supported me throughout this journey of completing my PhD. First, I would like to take the time to express my gratitude to my Doctoral Advisor, Dr. Christian Paumi. By taking me on as his first doctoral student, he took a gamble, and I like to think it worked out beneficially for both of us. There were times when I stumbled during the process, but with his patience and guidance, I have developed into the scientist I am today. I would also like to thank my Dissertation Committee, Dr. Mary Vore, Dr. Xianglin Shi and Dr. Rolf Craven for their guidance and support throughout the process of undertaking the doctoral degree.

When engaging in new research endeavors, it is rare that we start as experts in our field of choice. Many researchers were kind to share with me tips on techniques, protocols, and instruments. I owe many thanks to Dr. Donna Coy, as well as Wei Zhang and Baoxiang Yan for all of their help with getting the mice necessary to do the experiments. I also need to acknowledge Dr. Van Zant’s lab, Dr. Ying Li and Yi Liu for their expertise on hematopoietic stem cells and flow cytometry. Their guidance in the beginning on how to conduct the experiments was vital to the progression of the project. I would also like to express gratitude to the former post doc in the lab, Dr. Ela Stolarzcyk, for her help on my many projects prior to settling in to my final thesis project. I would also like to thank the current lab member Dustin Carroll for his continued support, whether it be listening to me practice presentations or offering advice.
I would like to recognize my peers in the Graduate Center for Toxicology, both former and present. Specifically, I would like to acknowledge those that entered the same year as me Mo Dan, Tripti Shresha, and Fred Odago for all of their time spent studying for exams with me. I would additionally like to acknowledge some of my former IBS classmates Sarah Collier and Woodrow Friend for their support throughout the first few years of the program. Additionally, I would also like to acknowledge my college friend Gwendolyn Waddingham, who although, wasn't in the program, was always an email away whenever I needed her.

I would also like to thank my family. Shortly after being admitted into the program my dad was diagnosed with cancer. It was a very difficult decision for me to stay in the program, but my family shouldered more of the stress and never made me feel guilty for not being closer. I am also grateful that my dad pushed me to stay even though I wanted to quit. It is with a heavy heart that I acknowledge my dad, one of my biggest supporters, who didn’t make it to see the completion of my dissertation.

Finally, none of this would be possible without financial support. For this I am thankful to have received an National Institute of Environmental Health Sciences T32 training grant predoctoral fellowship and for the University of Kentucky’s support.
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Chapter 1
Introduction

ATP BINDING CASSETTE TRANSPORTERS:

Adenosine 5’-triphosphate (ATP)-binding cassette (ABC) transporters are integral membrane bound proteins that use ATP as an energy source to move endogenous and exogenous compounds across the membranes from cells in which they reside (1). There are multiple subfamilies of ABC transporters, ABCA-ABCG. Our work has focused on the ABCC subfamily, which includes multidrug resistance-associated protein 1 (MRP1). The ABCC subfamily is comprised of 13 members, 10 MRPs (MRP1-6/ABCC1-6 and MRP7-10/ABCC10-13), the cystic fibrosis transductance regulator (CFTR/ABBC7), and SUR1 (ABCC8) and SUR2 (ABCC9). The ABCC subfamily differs from all other ABC transporters in that they have an extended N-terminal region (Figure 1.1)(2). The extended N-terminus contains either an additional membrane-spanning domain (MSD0) and is referred to as long-MRPs, (MRP1-3, 6 and 7) or contains an extended cytoplasmic N-terminal domain and is referred to as short MRPs (MRP4, 5, 8 and 9) (Figure 1.1) (2). The work described in this thesis focuses on MRP1. In addition, we examine and discuss the possible overlap between MRP1 and other ABC transporters, such as MRP4.

MRP4, like MRP1, is ubiquitously expressed in the human body. In the blood, MRP4 is expressed in erythrocytes, leukocytes, and blood platelets (3-7).
MRP1 and MRP4 have overlapping substrate specificities. For example, MRP1 is thought to be the main player in glutathione (GSH) homeostasis, however, a study done by Bai et al. has shown that MRP4 is also capable of transporting GSH alone, as well as, GSH conjugates (8).

**ABC Transporters and Disease:**

Mutations in numerous transporters have been shown to result in diseases such as Cystic Fibrosis, Stargardts disease, and Dubin Johnson’s syndrome, while overexpression of many of the ABC transporters results in cancer multidrug resistance (9). Some transporters upregulated in cancer include, but aren’t limited to, ABCB1, ABCC1/MRP1, ABCC2, ABCC3, ABCC4, ABCC5, and ABCG2 (1). The ability of these ABC transporters to efflux a wide range of chemotherapeutics has resulted in extensive investigation of their roles in multidrug resistance and their potential as chemotherapeutic targets (10). It is important to note that for many of the ABC transporters the correlation between transporter expression and multidrug resistance in cancer is often small and limited to specific cancer subtypes. Despite their promise as chemotherapeutic targets for some specific cancers, development of therapeutic drugs that effectively inhibit transporter function has achieved less than the desired results. Transporter research continues to expand beyond its traditional focus on multidrug resistance as new cellular roles are being discovered (11, 12).
Regulation of ABC transporters

A critically important area of investigation is ABC transporter post-translational regulation, most commonly through phosphorylation (13, 14). Phosphorylation plays an important role in many cellular processes, including the regulation of transcription, ubiquitination, degradation, localization, and protein function (15). Due to the large size of ABC transporters and the resulting high number of potential phosphorylation sites, studying ABC transporter phosphorylation has been extremely difficult. In recent years, with the improvement in protein purification techniques and mass spectrometry, several phosphorylation sites in ABC transporters have been identified; these are reviewed in Stolarczyk et al. and are summarized in Table 1.1(14).

MRP1

The ABC transporter MRP1 was first discovered from the drug resistant cell line H69AR, in which the resistance was not due to the transporter P-gp (16). MRP1 is ubiquitously expressed in the mouse and human body especially in the lung, heart, kidney, testes, blood and skeletal muscles (3-7). In the blood MRP1 can be found in erythrocytes, leukocytes, and blood platelets (3-7). MRP1 is a long-MRP roughly 190 kDa in size and overexpression of MRP1 can decrease cellular toxicity of a broad range of compounds via cellular efflux. Substrates of
MRP1 include both xenobiotics and endogenous toxins made within the cell. Many of the substrates of MRP1 are chemical conjugates of a toxin with sulfate, GSH or glucuronate, or are co-substrates with GSH (17).

**MRP1 (GS-X transporter)**

One very important function of MRP1 is its role in GSH homeostasis. Although initially MRP1 was shown to transport GSH-chemical conjugates, it has since become evident that MRP1 can also transport GSH as a co-substrate or transport GSH alone (17). MRP1 is a low affinity and high capacity transporter of GSH (18-20). Intercellular concentrations of glutathione is around 5 mM (19-21). Interestingly, the Km for GSH (reduced) is estimated to be around 5-10 mM and the Km for GSSG (oxidized) is closer to 50-100 µM, ie ranges almost identical to cellular concentrations (18-20).

Intracellular levels of GSH are increased in MRP1 null mice, particularly in tissues that typically have high MRP1 expression, including the breast, lung, heart, kidney, muscle, colon, testes, bone marrow cells, blood mononuclear leukocytes, and blood erythrocytes (21). The increase of basal GSH levels is thought to be due to decreased GSH export in Mrp1 null mice (22). Conversely, overexpression of Mrp1 decreases cellular levels of GSH due to increased MRP1-mediated efflux of GSH (40).
Glutathione:

The cell uses protective mechanisms to deal with reactive oxygen species (ROS). These mechanisms include both non-enzymatic systems, such as GSH and enzymatic protective mechanisms, such as SOD1-3, catalase, and glutathione peroxidase (6) (Figure 1.2). For the purpose of our work, we have focused on the cellular antioxidant, GSH.

GSH is a tripeptide that consists of the amino acids L-cysteine, L-glutamic acid, and glycine (23). In the first and rate-limiting step of glutathione synthesis, L-cysteine and L-glutamate are utilized by the enzyme glutamyl cysteine ligase (GCL) to make gamma-glutamylcysteine (23). GCL is composed of two subunits: catalytic (GCLC) and modulary (GCLM) (23). After gamma-glutamylcysteine is made, the enzyme glutathione synthetase (GSS) adds glycine in the final step of GSH synthesis (23).

GSH exists in two forms, reduced glutathione (GSH) and oxidized glutathione (GSSG) (24). When in the reduced form cysteine is able to donate a H-radical to ROS, such as OH-, H2O2, and O2- thereby stabilizing its reactivity (25). In a healthy environment the majority of GSH will be in the reduced form (6). A shift in the ratio of GSH and GSSG is a sign of oxidative stress (24, 25).
Reactive Oxygen Species

Reactive Oxygen Species, ROS, are subdivided into two classes: the free radical ROS and the non-radical ROS. The free radicals have an unpaired electron in their outer orbital and examples include $O_2^-$ and HO $^\cdot$ (26, 27). The non-free radical ROS do not have unpaired electrons in their outer orbitals, but can become reactive. Examples of non-free radical ROS include hydrogen peroxide ($H_2O_2$), ozone ($O_3$), and hydroxide (OH) (26, 28).

ROS are known to play important roles in regulating cellular functions, including regulation of cellular growth arrest, increased cellular proliferation, senescence, apoptosis and necrosis. Cellular damage caused by ROS includes lipid peroxidation, DNA adduct formation, protein oxidation, and enzyme inactivation, which can lead to apoptosis (29). Excess ROS can cause irreversible peroxidation of lipids, amino acids, nucleic acids, and carbohydrates (30). Although excess ROS is associated with having negative consequences in the cell, a certain level of ROS is needed to maintain the normal physiological processes of the cell, such as defense against infectious agents and the induction of signaling pathways (31). The bone marrow (BM) microenvironment is hypoxic with levels of $O_2$ in the 0.1-4% range (32, 33). In less hypoxic tissues the physiological levels of $O_2$ are below 6% (34). In the quiescent state of hematopoietic stem cells (HSCs) the level of $O_2$ is typically the lowest at 0.1%.
(35). When ROS exceeds these physiological levels it can lead to diseases, such as cancer, cardiovascular disease, as well as aging.

**Hematopoiesis/Hematopoietic Stem Cells:**

Hematopoietic Stem Cells (HSCs) were the first tissue specific stem cells to be identified and remain the most studied of all stem cell types. Till et al. were the first to establish the components necessary for blood regeneration (36). Their definition of an HSC from 1961 still holds today: a cell that can **self-renew** and **differentiate** to all the blood cell types (36). Another important feature of stem cells is their ability to **repopulate** the bone marrow of lethally irradiated animals and people (37).

HSCs are found in the bone marrow (BM), and despite their ability to maintain the balance of blood cells, they compromise only 1:10,000 cells in the BM (38-40). The bone marrow niche, where the HSCs reside, was defined by Schofield in 1978 as “an entity in which the stem cell’s maturation is prevented and the properties of ‘stemness’ are preserved” (41). This preservation is the result of low oxygen levels that limit the production of ROS. The main sources for ROS in HSCs are NADPH oxidases (NOX) and mitochondria (42, 43). Specifically NOX2 is found to be highly expressed in murine bone marrow, and is thought to be the NOX most important in ROS regulation and subsequent HSC differentiation (42, 43).
HSCs can be separated into different categories based on their developmental potential and lineage relationships (44). The more primitive a HSC, the more self-renewal capacity it will have. HSCs start as long-term HSCs (LT-HSCs) and become more differentiated as they progress to becoming short-term HSCs (ST-HSCs), multipotent progenitors (MPP) and finally fully committed blood cells (45). See Figure 1.3 for a diagram depicting hematopoiesis and the three subpopulations of HSC’s.

**HSC Identification**

Identifying HSCs can be difficult due to their low numbers. This is further impacted by the inability of HSCs to be identified via traditional pathological microscopy. This is due to the small size of HSCs and the similar appearance of HSCs to other blood and BM cells (45). In 1988 Irving Weismann’s group at Stanford was the first to identify a set of cell surface markers that could be used to identify murine HSCs via flow cytometry. Their markers were CD34 low/−, Sca-1+, Thy+/low, CD38+, C-Kit+, and Lin- (45). See Table 1.2 for commonly used protein surface markers in HSC research, and their definition.

Prior to protein surface markers being identified, researchers would carry-out transplantations of cells they thought contained HSCs into irradiated mice. A functional hematopoietic system would be restored if HSCs were present in the transplanted cells (36). The experimental design for transplantations is depicted in Figure 1.4. Discrepancy in the best markers for use during FACS analysis for
HSCs necessitates the testing of a stem cell’s ability to self-renew via transplantation studies. Therefore, transplantation studies have remained the “gold” standard in HSC murine studies till this day (46).

ROS in HSCs

ROS have been shown to play a role in regulating HSC self-renewal and differentiation. Previous work has established that hydrogen peroxide is a major player in ROS-mediated signaling and ROS-induced cellular affects in HSCs (47). Low levels of hydrogen peroxide are needed to maintain the physiological processes of the HSCs, but excess of hydrogen peroxide can lead to increased cellular proliferation, senescence, and apoptosis (47). See Figure 1.5 for a diagram of how oxidative stress impacts the differentiation of HSCs. The low numbers of HSCs make it difficult for ROS to be directly measured in those cells. Previous studies using 2’-7’-dichlorodihydrofluorescein diacetate (DCF-DA), used to measure the redox status of the cell, have found that DCF-DA high HSC populations tend to behave like aged HSCs. Importantly, the DCF-DA low populations have high self-renewing capacity, are more quiescent in character, and are often found in the HSC niche (48).

ROS serve as a promising treatment target for cancer stem cells. ROS levels can be manipulated using xenobiotics to target and subsequently destroy cancer cells and cancer stem cells. For example, drugs that increase ROS levels, such as Niclosamide, can selectively kill leukemic stem cells (LSCs) while having
minimal cytotoxicity against normal HSCs (49). The ability of Niclosamide to target leukemic stem cells is due to the ability of Niclosamide to inhibit the NF-κB pathway, and NF-κB has been found to be activated in the primitive blast cells in AML patients (49). Additionally, Niclosamide has been found to increase ROS in AML cells resulting in apoptosis (49). Studies have shown that ROS-mediated cell senescence may be acting through the p38 mitogen-activated protein kinase (MAPK), which is known to activate NF-κB (50).

**p38 MAPK Pathway**

The p38 MAPK pathway has been found to play a role in cell cycle arrest, apoptosis, differentiation, and senescence (51). p38 MAPK was identified by Han et al. in 1994 during a pharmacological screen used to identify compounds that modulate tumor necrosis factor (TNFα) production (52). Phosphorylation, by either mitogen-activated protein kinase kinase (MKK)3 or MKK6 at Thr180 or Tyr182, is required to activate p38 MAPK (53). Once activated, p38 MAPK phosphorylates and activates downstream targets, such as mitogen-activated protein kinase activated protein (MAPKAP) Kinase 2 (54). Additionally, p38 phosphorylates and activates transcription factors such as activating transcription factor 2 (ATF), p53, and myocyte enhancer factor 2 (MEF2) (Figure 1.6)(54).
N-Acetyl Cysteine (NAC)

Cysteine is the rate-limiting amino acid in the synthesis of GSH and is required by gamma glutamyl ligase, the enzyme that catalyzes the synthesis of cysteine-gamma glutamyl. GSH synthesis is stimulated with a synthetic thiol, N-Acetyl Cysteine (NAC). NAC easily passes through cell membranes and becomes deacetylated in tissues/cells to form cysteine (55). Besides stimulating GSH synthesis by providing cysteine, NAC is a ROS scavenger, forming NAC-disulfide products (56). Additionally, NAC has the ability to restore HSC self-renewal, which is thought to be through the p38 MAPK pathway (48).

CK2

CK2 is a serine/threonine protein kinase. It forms a tetrameric complex, but its individual subunits can be active on their own (57). The complex is made of two catalytic α and two regulatory β units (57). CK2 has over 300 substrates and plays a role in a variety of cellular processes including cell growth, proliferation, death, and survival (58). It has been established that CK2 can phosphorylate ABC transporters, such as ABCA1 and ABCB1/Pgp (59, 60). The diverse roles of CK2 have led to CK2 being nicknamed a “master regulator of the cell.” Increased protein expression of CK2 is found in all cancers that have been
studied (58, 61). CK2 has gained interest as a “druggable” target for leukemia treatment due to a small number of studies that have shown that CK2 inhibitors target leukemic stem cells selectively (62, 63).

Our laboratory has previously used MRP1’s yeast homolog, Ycf1p, to demonstrate that Cka1p, the yeast homolog to CK2α, could regulate transporter function during salt stress. This regulation was due to Cka1p phosphorylating Ser251 at the L0 region (Figure 1.7) (64). Additionally, our laboratory has shown that Cka1p-mediated phosphorylation of Ycf1p results in decreased ROS due to increased GSH (65). The increase of GSH was due to a loss of Ycf1p-mediated GSH transport (65).
Research Objectives

Drug transporters such as MRP1 play an important role in both the detoxification of endogenous and exogenous toxins, efflux of cellular signaling molecules, and in drug resistance in cancer(1). In Chapter 2 we will discuss the role of MRP1 in the regulation of hematopoietic stem cell differentiation and self-renewal. Chapter 3 will discuss the role of CK2-mediated post-translational phosphorylation of MRP1 as a mechanism of regulating transporter function.

The aims of chapter 2 were as follows:

1. Characterize HSC function in MRP1+/+ and MRP1−/− C57BL6 mice.
2. Determine the role of MRP1 in regulating HSC self-renewal and differentiation
3. Determine the mechanism by which MRP1 regulates HSC self-renewal and differentiation.

Here, using flow cytometry, we found that deletion of MRP1 increases the frequency of LT-HSCs, ST-HSCs, and multipotent progenitors (MPPs)in bone marrow (BM). Further, deletion of MRP1 increases the ability of HSCs to repopulate the BM of lethally irradiated mice due to an increase in HSC self-renewal, as determined by serial dilution competitive repopulation assays. We provide strong evidence that the mechanisms by which self-renewal increases in
MRP1−/− mouse HSCs include: 1) decreased cellular ROS, 2) decreased p38 signaling, and 3) altered redox response protein expression, all due to increased GSH levels. We provide additional evidence to support our model in experiments aimed at recapitulating the MRP1−/− mouse HSC phenotype via administration of the antioxidant NAC. NAC treatment decreased cellular ROS levels and increased the number of LT-HSCs and ST-HSCs, similar to what we have seen in the MRP1−/− mouse HSCs. Overall, the data from this study strongly suggest a role for MRP1 in regulating HSC self-renewal via efflux of GSH and subsequent decreased levels of ROS.

The aims of Chapter 3 were as follows:

1. Screen several different types of cancer derived cell lines for expression of MRP1 and CK2.
2. Determine if MRP1 function is inhibited by treatment of cancer cell lines with CK2 inhibitors.
3. Determine if treatment of cell lines with CK2 inhibitors decreases MRP1-mediated drug resistance in standard cytotoxicity assays

Here, we found that A549, HeLa, and H460 cell lines expressed MRP1 and CK2. Doxorubicin (Dox) accumulation assays and cytotoxicity assays were used to measure MRP1 function. To effectively measure MRP1 function in the context of a multidrug transporter setting of each cell line, we used an MRP1 inhibitor, MK571, to block MRP1-dependent Dox transport and provide a baseline
for comparison for Dox accumulation when MRP1 is maximally inhibited. These experiments were conducted in the presence and absence of a CK2 inhibitor. We found that inhibition of CK2 reduced MRP1-dependent Dox efflux and MRP1-dependent protection against Dox cytotoxicity.
Figure 1.1: Structure of ABC transporters. A. Half-transporters, such as ABCG2, that require dimerization for full function. B) Full length transporters, such as Pg-p, MRP4, MRP5, also known as short transporters C) Extended, full length transporters, also known as long transporters, that contain an extra membrane spanning domain at the N-terminus (depicted as N in the diagrams), such as MRP1-3, MRP6.
Table 1.1. Phosphorylation sites of the ABC Transporters identified by MS analysis of tryptic derived peptides (14)

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**Figure 1.2 Model of Oxidative stress response proteins present in cells.**

Glutamate-cysteine ligase catalytic (GCLC), glutamate-cysteine ligase modulary (GCLM), glutathione synthetase (GSS) glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD) and MRP1 regulate reactive oxygen species in cells. Furthermore the complex GCLM/GCLC is important for the synthesis of GSH, GR is important for recycling glutathione, and MRP1 is important for GSH efflux.
Figure 1.3: Hematopoiesis. Hematopoietic Stem Cells are comprised of three subpopulations: LT-HSCs, ST-HSCs, and MPP’s. LT-HSCs are the most self-renewing and these self-renewing properties decrease as they progress through the HSC subpopulations into the common myeloid progenitor, common lymphoid progenitor and finally into the fully differentiated blood cell types. Common surface markers used to identify blood cells are listed on the following page in Table 1.2. Abbreviations used in the above diagram include long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitor (CMP), common lymphoid progenitor (CLP), megakaryocyte erythroid progenitor (MEP), granulocyte macrophage progenitor, Natural Killer (NK), DC (Dendritic Cell).
Table 1.2 Protein Surface Markers for HSCs in mice

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<td>Lineage Cells/Differentiated</td>
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<tr>
<td>SCA-1</td>
<td>All: SCA-1+</td>
<td>Stem Cell Antigen. Maintains the stem cell population.</td>
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<tr>
<td>C-Kit</td>
<td>All: C-Kit+</td>
<td>Cytokine receptor that binds stem cell factor which signals growth. Proliferation/Differentiation</td>
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<td>CD34</td>
<td>LT-HSCs- CD34- ST-HSC/MPP CD34+</td>
<td>Mediates attachment to bone marrow matrix</td>
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<tr>
<td>CD135</td>
<td>LT/ST-HSC CD135- MPP CD135+</td>
<td>Development of B and T Cells Survival/proliferation/differentiation</td>
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Transplants

A.

Figure 1.4

B.

Figure 1.4
**Figure 1.4 Schematic of HSC transplantations:**

**A.** Bone marrow is extracted from two different strains of mice with differing alleles CD45.1 or CD45.2. The wild-type (WT) and knock-out (KO) mouse of interest are CD45.2 positive. The recipient mice and helper cells are CD45.1 positive. This will allow us to identify the cells of interest from our WT and KO mice after transplantation. To conduct the study the bone marrow is mixed together and transplanted into a lethally irradiated CD45.1 mouse. The transplanted HSCs are allowed to expand and repopulate the bone marrow, as well as blood, for 16 weeks. Flow cytometry can be used to analyze the cells derived from the CD45.2 mouse for limiting-dilution competitive repopulation studies. These assays allow us to determine the frequency of HSCs.

**B.** For serial transplantation studies the principle of the transplants are the same, except all mice get the same number of cells and the study extends beyond the first recipient mouse. After analysis at 16 weeks we also harvest the bone marrow from the first recipient mouse and transplant it into a second irradiated mouse. The bone marrow is allowed to expand and repopulate the BM niche and the BM is harvest after 16 weeks. This assay allows us to determine the ability of the HSCs to self-renew and repopulate the bone marrow niche.
Figure 1.5: Model for oxidative stress related changes in HSCs. Increased oxidative stress has been shown to play a role in regulating HSC differentiation. An increase in oxidative stress coincides with the differentiation of HSCs from LT-HSCs to ST-HSCs and finally to MPPs, which ultimately impacts hematopoietic self-renewal capacity.
Mitogen-activated protein kinase kinase (MKK)3, MKK4, and MKK6 are activated by G-coupled receptors, cytokine receptors, reactive oxygen species (ROS), and ultra-violet (UV). Once activated they can phosphorylate p38. Once p38 is phosphorylated it becomes active and can phosphorylate a number of downstream targets. Shown are some of the downstream targets for phosphorylated-p38 (phos-p38) such as p53, activating transcription factor 2 (ATF2), myocyte enhancer factor 2 (MEF2), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), mitogen and stress activated protein kinase 1 and 2 (MSK1/2).
Figure 1.7: Ycf1p is regulated by Cka1p-mediated phosphorylation of Ser251. Ycf1p consists of 3 transmembrane spanning domains (TMDs) and 2 nucleotide binding domains (NBDs). Our laboratory has shown that Ycf1p is phosphorylated at Ser251 within the L0 domain by Cka1p (66).
Chapter 2
MRP1 Regulation of Hematopoietic Stem Cell Self-Renewal

ROS and cellular oxidative stress play an important role in regulating HSC self-renewal and differentiation (67-70). Exposure of HSCs to high levels of ROS is toxic and can lead to HSC senescence, apoptosis, and ultimately diseases, such as bone marrow failure and leukemia (71-73). Cellular ROS levels are regulated by a complex antioxidant system that includes glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD). Low levels of ROS are important for maintaining normal HSC differentiation and proliferation (73-76) and therefore, HSCs maintain a very tight regulation of cellular ROS and cellular antioxidant systems (72). GSH is a key component of the HSC redox system, however, the mechanism by which GSH cellular levels are regulated remains unclear (71, 77).

Multidrug resistant protein 1 (MRP1/ABCC1) is an ATP binding cassette (ABC) transporter that effluxes a broad range of substrates, including both reduced and oxidized GSH (19, 78, 79). MRP1-mediated efflux of GSH is an important mechanism of ROS regulation in a variety of cell types (80, 81). MRP1 is expressed at high levels in embryonic stem cells (ESCs), HSCs, bone marrow mononuclear cells (BMMC) and erythrocytes (82, 83) and deletion of MRP1 in mice increases endothelial progenitor cell function and differentiation due to loss of MRP1-mediated GSH efflux, increased GSH levels, and decreased ROS (81).
Interestingly, MRP1 expression is higher in HSCs as compared to mature blood cells suggesting a role for MRP1 in regulating hematopoiesis(84).

In the current study, we show that MRP1 plays a role in regulating HSC self-renewal. Deletion of MRP1 in mice results in increased HSC frequency and increased HSC self-renewal. The increase in HSC frequency and self-renewal is the result of decreased ROS levels due to the loss of MRP1-dependent GSH efflux. Additional evidence to support this mechanism comes from studies in which we treat normal mice with the GSH mimetic, N-acetyl-L-cysteine (NAC), which has been shown to increase HSC self-renewal properties (48, 85). NAC treatment of C57BL/6 MRP1+/- mice results in a phenotype that closely resembles that of C57BL/6 MRP1-/- mice, strongly suggesting that, indeed, increased HSC frequency and self-renewal in C57BL/6 MRP1-/- mice is the result of decreased ROS. Finally, we have evidence that suggests that the downstream mechanism by which MRP1 regulates HSC self-renewal via GSH and ROS modulation is in part through the p38 MAPK pathway.
Methods:

Animals:

C57BL/6MRP1+/+ CD45.2 (MRP1+/+), C57BL/6 MRP1−/− CD45.2 (MRP1−/−) (generous gifts from Dr. Mary Vore’s laboratory, the University of Kentucky), and B6.SJL-∗Ptprc Pepc+/BoyJ MRP1+/+ CD45.1 (MRP1+/+ CD45.1) mice (The Jackson Laboratories, Bar Harbor, ME) were maintained in the Division of Laboratory Animal Resources facility and were provided food and water ad libitum. B6.SJL-∗Ptprc Pepc+/BoyJ mice were utilized in the transplants. All assays utilized the C57BL/6 strain of mice. All mice were 8-12 weeks of age at the time of use. The Institutional Animal Care and Use Committee of the University of Kentucky approved all experiments. Transplantation recipient mice in competitive repopulation experiments were provided special water containing antibiotics (sulfamethoxazole 0.8mg/ml and trimethoprim 0.16mg/ml) 1 week prior to irradiation. During NAC treatment studies, mice were provided with 5mg/mL NAC in their drinking water for four weeks.

Flow Cytometry Analysis of Bone Marrow and Peripheral Blood

HSC and progenitor cell analysis was carried out as previously described (86, 87). Briefly, donor mice were sacrificed and bone marrow (BM) was harvested from the tibias and femurs. BM cells were suspended in phosphate buffered saline(PBS) containing 2% fetal bovine serum (FBS) (Life Technologies,
Carlsbad, CA) and subjected to red cell lysis with 0.15 M ammonium chloride solution with 0.1M sodium bicarbonate to enrich for mononuclear blood cells (MNBCs). MNBCs were stained with APC-Cy 7-conjugated lineage markers (CD45R, CD11b, CD45R/B220, CD19, CD3e, TER119) and stem cell–specific markers, PE-CY7 conjugated Ly6A/7 (Sca-1), PerCP-Cy 5.5 conjugated CD117 (c-Kit), PE conjugated CD135 (Flt-3), and FITC conjugated CD34. Stem cell cells were stained with PE-conjugated rat anti-mouse 135, Alexa Fluor 647-conjugated rat anti-mouse CD150 and FITC-conjugated rat anti-mouse CD34. Cell viability was assessed using the exclusion dyes DAPI and propidium iodide (PI) (5 μg/ml). Alternatively, peripheral blood (PB) was taken from the submandibular vein of mice and collected in a 1mL EDTA containing blood collection tube. Peripheral blood (PB) was lysed with red cell lysis buffer as described above and mononuclear blood cells (MNBCs) were collected. PB MNBCs were subsequently stained for Lineage Marker analysis (FITC conjugated CD11b, PerCP-Cy5.5 conjugated CD19, V450 conjugated Gr1, PE conjugated Ter119, PE-Cy7 conjugated CD3e). All monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes NJ). Flow cytometric analysis was carried out in the University of Kentucky Flow Cytometry Core Facility on a BD LSRII (BD Biosciences, Franklin Lakes, NJ). For the DCF-DA assay Alexa Fluor 647-conjugated rat anti-mouse CD150 was not used and Alexa Fluor 647-conjugated rat anti-mouse CD34 was used in place of FITC-conjugated rat anti-mouse CD34. For the monochlorobimane (MCB) assay 4’-6-diamidino-2-phenylindole (DAPI) was excluded due to overlap with MCB detection.
Peripheral Blood Analysis

MRP1+/+ CD45.2 and MRP1-/- CD45.2 mice were bled via submandibular vein and 250 μL of blood was collected in a 1mL, EDTA containing, collection tube. Complete blood analysis was carried out using a Hemavet 950 FS (Drew Scientific Inc Oxford, Connecticut) and by flow cytometry as described above.

Bromodeoxyuridine (BrdU) Assay

The proliferative activity of MRP1+/+ CD45.2 and MRP1-/- CD45.2 HSCs was measured using the BrdU Assay Kit (BD Bioscience, Franklin Lakes, NJ). Mice were injected intraperitoneally (IP) with FITC-BrdU (1 mg per kg body weight). After 5 hours, mice were sacrificed and BM was collected for flow cytometric analysis as described above with modifications. Since fluorescein isothiocyanate (FITC) was utilized for BrdU detection, Alexa Fluor 647-conjugated rat anti-mouse CD150 was not used and Alexa Fluor 647-conjugated rat anti-mouse CD34 was used in place of FITC-conjugated rat anti-mouse CD34.
Colony Forming Cell (CFC) Assay

BM was obtained from MRP1\(^{+/+}\) CD45.2 and MRP1\(^{/-}\) CD45.2 mice as described above. Enriched MNBCs were washed and resuspended in Iscove’s Modified Dulbecco’s Medium (IMDM) + 2% FBS to a concentration of 50,000 cells/mL. Next 0.3 mL of the resuspended cells was added to 3 mL of Methocult GF M3434 media (Stem Cell Technologies, Vancouver, BC, Canada), vortexed and allowed to sit for 20 minutes. Next, 1.1 mL of the mixture was added to a 35mm culture in duplicate. The duplicate 35 mm dishes were placed within a 100 mm dish containing a third uncovered 35 mm dish containing sterile water. Cells were allowed to grow for 8-12 days at 37° C and 5% CO\(_2\). After incubation, colonies were identified and counted under the microscope and scored as blast-forming units (BFUs), colony-forming units (CFUs), colony forming unit- granulocyte macrophage progenitor (CFU-GMs), or colony-forming unit granulocytes, erythrocyte, monocyte, megakaryocyte progenitors (CFU-GEMMs).

Annexin V- Staining for Apoptosis

The apoptotic activity of MRP1\(^{+/+}\) and MRP1\(^{/-}\) HSCs was measured using the Annexin V Assay Kit (BD Bioscience, Franklin Lakes, NJ). Briefly, HSCs were stained as previously described and additionally, cells were washed with binding buffer before staining with annexin V. Cells were washed and analyzed by fluorescence activated cell sorting (FACS). Since FITC was utilized for Annexin V
detection, Alexa Fluor 647-conjugated rat anti-mouse CD150 was excluded from the analysis to allow for Alexa Fluor 647-conjugated rat anti-mouse CD34 in place of FITC-conjugated rat anti-mouse CD34.

Transplantations:

Limiting-dilution competitive repopulation assays were carried-out to determine HSC frequency as previously described (86). Briefly graded numbers (10,000, 20,000 and 60,000 chosen based on previous studies) of test cells (MRP1+/+ or MRP1-/- MNBCs) were admixed with 200,000 competitor cells (MRP1+/+ CD45.1 MNBCs) and injected intravenously into lethally irradiated (total dose 9 Gy) MRP1+/+ CD45.1 recipient mice(86). Blood was collected from recipient mice via submandibular cheek bleeds at 4, 8, 12, and 16 weeks post transplantation and subject to linear marker analysis (B cells (PerCP-Cy5.5 CD19), T lymphocytes (PE-Cy7 conjugated CD3e), granulocytes (V450 conjugated (Ly6G/Ly6C), and macrophages (APC-CY7 conjugated CD11b)). Additionally, APC conjugated monoclonal anti-CD45.2 and PE conjugated anti-CD45.1 stains were used to identify the origin of the cells. For serial transplantation studies to study ability of HSCs to repopulate the bone marrow (BM), 1 million test cells (MRP1+/+ or MRP1-/- MNBCs) were admixed with 1 million competitor cells (MRP1+/+ CD45.1 MNBCs) and injected intravenously into lethally irradiated (total dose 9 Gy) MRP1+/+ CD45.1 recipient mice. After 16 weeks blood and bone marrow were extracted for analysis. Additionally, 1 million whole BM cells from each primary
mouse were injected into a matched lethally irradiated (total dose 9Gy) MRP1+/+ CD45.1 recipient mice. Blood and BM were analyzed again after 16 weeks.

**DCF-DA:**

MNBCs were enriched from MRP1+/+ and MRP1−/− C57BL/6 mouse BM and stained for HSC (LSK) analysis as described above in flow cytometry analysis of bone marrow and peripheral blood. After a 60 min incubation on ice, the cells were washed and resuspended in 100 μL RPMI1640/10%FBS in the presence of 50 μM MK571 (Cayman Chemical Company, Ann Arbor, MI), an MRP1 inhibitor. Cells were washed and resuspended in RPMI1640/10%FBS with 50 μM MK571 and DCF-DA to stain for levels of ROS (Sigma Aldrich, Saint Louis, MO) (1:5000 dilution using 25mM stock) for 10-15 min at 37° C. Cells were washed and resuspended in PBS containing 2% FBS containing MK571 and analyzed by flow cytometry as previously described.

**Monochlorobimane:**

MNBCs were enriched from MRP1+/+ and MRP1−/− C57BL/6 mouse BM and stained for HSC (LSK) analysis as described above in flow cytometry analysis of bone marrow and peripheral blood. After a 60 minute incubation on ice, the cells were washed and resuspended in 100 μL RPMI1640/10%FBS and incubated with 50 μM MK571 (Cayman Chemical Company, Ann Arbor, MI) an MRP1 inhibitor. Cells were washed and resuspended in RPMI1640/10%FBS with 50 μM
MK571 and subsequently incubated with monochlorobimane, which has high affinity for glutathione, but will also bind to other thiols (MCB, EMD Millipore, Merck KGaA, Darmstadt, Germany) (10 μM) 20 min 37° C. Cells were washed and resuspended in PBS containing 2% FBS and MK571. Cells were then analyzed by flow cytometry as previously described.

**Statistics:**

Unless otherwise noted, mean fluorescence was extrapolated from flow cytometry analysis using FlowJo software (Tree Star Inc., Ashland, OR USA) and plotted graphically with a minimum of n=3. Statistical analysis was performed in Prism software (GraphPad, LaJolla, CA USA) (student t-test).
Results:

Deletion of MRP1 increases HSCs, B Cells and T Cells in Mice

It has been established that ABC transporters, mainly ABCG2, play an important role in the regulation of HSC self-renewal and differentiation (88). Therefore, it is reasonable to speculate that other ABC transporters, such as MRP1, could play a similar role. To investigate the possibility that MRP1 might regulate HSC self-renewal and/or differentiation, we enriched BM MNBCs from MRP1+/+ and MRP1−/− C57BL/6 mice and determined their HSC content by flow-cytometry as described in the Methods (Figure 2.2, flow cytometry gating shown in Figure 2.1). Our analysis revealed that MRP1−/− mice have a significant increase in each subpopulation of HSCs (long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs)) as compared to MRP1+/+ mice (Figure 2.2A). In addition, we determined BM progenitor content and found that MRP1−/− mice have increased numbers of megakaryocyte erythroid progenitors (MEPs) (Figure 2.2B).

To determine how the increased number of HSCs in the MRP1−/− mice impacts overall hematopoiesis, we analyzed the peripheral blood (PB) of both the MRP1+/+ and MRP1−/− C57BL/6 mice for progenitor and fully differentiated blood cell subtypes by flow cytometry (Figure 2.2C) and hemavet (Table 2.1), determined the ability of BM derived MNBCs to give rise to more differentiated blood types by colony forming cell (CFC) assay (Figure 2.2D), and determined
HSC proliferation (Figure 2.2E). Flow cytometry analysis revealed that MRP1\(^{-/-}\) mice have more CD19 (B cells), CD3e (T cells), and Gr1 (macrophages, natural killer (NK) cells, eosinophils, monocytes and neutrophils) marked cells as compared to the MRP1\(^{+/+}\) mice (Figure 2.2C). Interestingly, no difference was seen with CD11b, which marks macrophages and NK cells, suggesting the differences seen in Gr-1 marked cells is due to eosinophils, monocytes, and neutrophils. Based on our BM studies, we reasoned that an increase in HSCs would correlate with an increase in the number of differentiated colonies measured by CFC assay however, this was not the case. CFC assay analysis showed that the number of BFU-E, CFU-E, CFU-GM, and CFU-GEMM colonies formed from BM derived from MRP1\(^{-/-}\) MNBCs was all decreased as compared to the MRP1\(^{+/+}\) mice (Figure 2.2D). Although this seemed contradictory, it is likely due to the decreased proliferation of the MRP1\(^{-/-}\) mouse derived HSCs (Figure 2.2E and 2.3). Analysis of HSC proliferation by BrdU staining (Figure 2.2E (Flow Cytometry shown in Figure 2.3)) showed that the HSCs derived from the MRP1\(^{-/-}\) mice are replicating more slowly (Figure 2.2E) than those from MRP1\(^{+/+}\) mice. Cells in the G0 phase of the cell cycle are dormant/quiescent and the G1 phase proceeds S phase where replication is starting to occur. S phase of the cell cycle is when DNA replication occurs. An increase in the cells in G0/G1 and decrease in S phase would signify less/slower replication is taking place. Analysis of apoptosis by annexin V staining in HSCs derived from MRP1\(^{+/+}\) mice and MRP1\(^{-/-}\) C57BL/6 mice showed no difference (Figure 2.4). Hemavet analysis, a full blood panel, showed no major differences except strikingly,
MRP1−/− mice have double the number of platelets. Considering that MRP1−/− mice have no adverse effects related to their physiology, viability, or fertility unless stressed, it is not surprising that the hemavet analysis of fully differentiated cells in the blood did not reveal any differences, other than a doubling of the platelets. However, it is reasonable to postulate that differences in the blood, other than platelets, could arise under stressed conditions, such as, exposure to toxins and/or naturally occurring carcinogens (21). Increased cellular stress has been known to play a role in a variety of diseases such as cancer, cardiovascular disease and aging.

**HSCs express MRP1 and MRP4 differentially**

MRP1 and MRP4 have overlapping substrates and are ubiquitously expressed in a variety of tissues, including HSCs, in both mice and humans (3, 4, 6, 21, 89, 90). Based on these findings, it is reasonable to postulate that MRP4 may compensate for deletion of MRP1 in HSCs. Therefore, we determined MRP4 expression levels in the MRP1+/+ and MRP1−/− C57BL/6 mice HSCs (Figure 2.5B). Additionally, we determined the expression levels of MRP1 in HSCs from MRP1+/+ C57BL/6 mice to establish a baseline for MRP1 expression in HSCs (Figure 2.5A). Using flow cytometry as described in the Methods, we found that all HSC subtypes in MRP1+/+ mice express MRP1, however ST-HSC and MPPs express significantly more MRP1 as compared to LT-HSCs (Figure 2.5A). Conversely, MRP4 expression was constant across all three HSC
subtypes in MRP1+/+ mice and MRP4 expression did not change upon deletion of MRP1 (Figure 2.5B). These results suggest that MRP4 does not compensate for the loss of MRP1, and therefore is not contributing to the overall increase in the number of HSCs found in the MRP1−/− mouse.

Deletion of MRP1 increases HSC self-renewal capacity.

To better understand the effect that the increased number of HSCs in the MRP1−/− mouse could have on hematopoiesis, we carried out a competitive limiting dilution assay (Figure 2.6) and a competitive serial repopulation assay (Figure 2.7). The competitive limiting dilution assay utilized MNBCs from the C57BL/6 MRP1+/+ CD45.2 and MRP1−/− CD45.2 mice as the test cells and MNBCs from B6.SJL-Ptprca Pepcb/BoyJ MRP1+/+ CD45.1 mice as competitor/donor cells, and was carried-out as described in the Methods (Figure 2.6). Our studies showed that the total CD45.2 content in the transplanted mice was higher in mice transplanted with MRP1−/− MNBCs and increased with time at all three dilutions tested, with the most pronounced effect at the 60,000 dilution (Figure 2.6). Competitive repopulation unit (CRU) values were calculated from week 16 blood samples to determine HSC frequency by scoring for all blood samples either positive or negative with regard to successful competitive growth of test cells, a positive score was given if all four blood cell types had 5% or greater CD45.2 cells. The frequencies of long-term HSCs were calculated from the proportions of negative recipients in each cell dose group using L-Calc.
software (Figure 2.6B-D). Our analysis revealed that MRP1\(^{+/+}\) MNBCs gave rise to higher frequency of CD45.2 cells in our transplants as compared to MRP1\(^{+/+}\) MNBCs, as shown by visual inspection and statistical analysis of flow data (Figure 2.6B-D). These differences were statistically significant and resulted in calculated CRU values of a HSC frequency of 1/87,163 for MRP1\(^{+/+}\) CD45.2 mouse MNBCs and a HSC frequency of 1/18,073 for MRP1\(^{-/-}\) CD45.2 mouse MNBCs, with 95% confidence interval of (50,061 – 281,839) and (13,006 – 48,844) respectively after 16 weeks.

Taken together, our competitive limiting dilution assay and phenotypic analysis of C57BL/6 MRP1\(^{+/+}\) and MRP1\(^{-/-}\) mice suggested that deletion of MRP1 increases the number of HSCs via increased self-renewal. To test this model we conducted a competitive serial transplantation assay. After 16 weeks post-transplant the CD45.2 content was analyzed, decompiled, and graphed for comparative purposes in Figure 2.7A. Figure 2.7A shows that the secondary recipients transplanted with MRP1\(^{-/-}\) MNBCs have a significantly higher percentage of CD45.2 cells as compared to secondary recipients receiving MRP1\(^{+/+}\) MNBCs. This data was further broken down into BM total lymphocytes (Figure 2.7B), LSKs (HSCs) in BM (Figure 2.7C), and peripheral blood (PB) (Figure 2.7D) for both primary and secondary transplants. The results of the competitive serial transplant assay strongly support the hypothesis that deletion of MRP1 increases the HSC self-renewal capacity.
Deletion of MRP1 increases HSC self-renewal via increased GSH and decreased ROS in the cell.

MRP1-mediated regulation of cellular ROS plays an important role in regulating redox gene expression and oxidative stress signaling pathways (81, 91-94). Additionally, studies have shown that this regulation occurs via MRP1-dependent efflux of GSH (94-96). Based upon these studies we hypothesized that deletion of MRP1 increases HSC self-renewal by decreasing cellular ROS due to the loss of MRP1-mediated GSH efflux. Therefore, we compared the cellular levels of GSH (Figure 2.8A) and ROS (Figure 2.8B) in HSCs from C57BL/6 MRP1+/+ and MRP1−/− mice. To measure GSH levels we utilized the MCB assay, a standard flow based assay that measures relative GSH content. Our analysis of HSCs from C57BL/6 MRP1+/+ and the MRP1−/−mice revealed that deletion of MRP1 significantly increases cellular GSH levels (Figure 2.8A). If our hypothesis is correct, then increased GSH, an antioxidant that directly scavenges ROS, should decrease cellular ROS. To examine the cellular ROS levels we utilized 2′-7′-dichlorodihydrofluroscein diacetate (DCF-DA), a stain that measures the redox state of the cell. Previous studies using DCF-DA have found that low DCF-DA populations have the self-renewing, quiescent characteristics of HSCs found in the HSC niche (48). Our analysis of HSCs from C57BL/6 MRP1+/+ and MRP1−/− mice revealed that deletion of MRP1 decreased cellular DCF-DA in every subpopulation of HSCs (Figure 2.8B) indicating a decrease in cellular
The data from the DCF-DA and MCB assays were in agreement and support our hypothesis that deletion of MRP1 increases HSC self-renewal via decreased ROS levels from loss of MRP1-mediated GSH efflux.

**Deletion of MRP1 alters the expression of redox response genes and p38 MAPK activity.**

ROS levels are tightly regulated and changes are associated with altered expression levels of redox response genes. Therefore, it is likely that the increase in cellular GSH and decrease in cellular ROS that we see in C57BL/6 MRP1−/− HSCs alters the expression profile of a number of redox response genes. To investigate this possibility, we measured the expression of a number of redox response genes in HSCs, specifically in LT-HSC, ST-HSC, and MPPs in C57BL/6 MRP1+/+ and MRP1−/− mice by flow cytometry (glutamate-cysteine ligase modifier subunit (GCLM) and glutamate-cysteine ligase catalytic subunit (GCLC), glutathione reductase (GSR), superoxide dismutase (SOD1 and SOD2), catalase (CAT), and glutathione peroxidase (Gpx)) (Figure 2.8C). Our analysis revealed a number of interesting changes. Deletion of MRP1 significantly reduced the expression of glutamate-cysteine ligase, modifier subunit (GCLM) and increased the catalase (CAT) expression in LT-HSCs. Furthermore, expression of GSR was significantly increased in all HSC subtypes, LT-HSCs, ST-HSCs, and multipotent progenitors (MPPs). It is important to note that the expression of GCLM and GCLC are closely linked as they form a stable heterodimer, which is
required for the first step in GSH synthesis, the conjugation of glutamate and cysteine. Since less GSH would be synthesized with decreased GCLC and GCLM, the increased glutathione is most likely the result of increased GSH recycling.

ROS and the activity of the p38 MAPK pathway in stem cells are closely linked (50, 97, 98). Upon increase of cellular ROS, p38 is activated by dual phosphorylation at the Thr180/Tyr182 residues (99-101). To evaluate if p38 phosphorylation was decreased by the deletion of MRP1, we analyzed the expression and phosphorylation status of p38 in C57BL/6 MRP1+/+ and MRP1−/− HSCs (Figure 2.8D). Our hypothesis, if true, would anticipate a decrease in phosphorylation at Thr180/Tyr182. Indeed, our analysis revealed that p38 phosphorylation at Thr180/Tyr182 was significantly decreased in LT-HSCs MRP1−/− mice as compared to MRP1+/+ mice. It is important to note that although the decrease in p38 phosphorylation was not of significance in ST-HSCs and MPPs, both subpopulations were significant by student t-test, with p values of 0.03 and 0.04 respectively before applying a Holm-Sidak correction for multiple comparisons, where the values became non-significant. Our results imply that deletion of MRP1 lowers cellular ROS by increasing cellular GSH, and the decreased cellular ROS alters the expression of several ROS response genes and importantly, downregulates the p38 MAPK pathway. These results strongly support our model that deletion of MRP1 increases HSC self-renewal via increasing cellular GSH, which decreases cellular ROS. Further, these results suggest two potential mechanisms (redox response gene expression and p38
signaling) by which deletion of MRP1 increases HSC self-renewal. Our results are in agreement with previous research finding that increased ROS in stem cells typically act through the p38 MAPK pathway to limit the lifespan of HSCs (50). These studies found that increased ROS would activate HSC specific phosphorylation of p38 MAPK, which decreased HSC quiescence(50). They could also rescue this ROS induced activation of p38 MAPK by using an inhibitor for p38 MAPK (50).

**NAC treatment can recapitulate MRP1-/- phenotype in WT**

Treatment of mice with NAC was shown to increase HSC quiescence and rescue a “normal” HSC phenotype in mice with a genetic deletion that results in increased HSC ROS (102). Treatment of cells with NAC stimulates glutathione synthesis, by providing cysteine, and upon entering the cells, NAC can act as a strong cellular antioxidant on its own (55). Based upon these findings, we hypothesized that if our model is true that the increase in HSC self-renewal in the C57BL/6 MRP1-/- mice is the result of decreased ROS due to increased cellular GSH, then treatment of MRP1+/+ mice with NAC should result in HSCs that are phenotypically similar to HSCs derived from the MRP1-/- mice. To test this hypothesis as a proof of principle, we administered NAC treated water to C57BL/6 MRP1+/+ mice over 4 weeks and subsequently carried out a complete BM and PB analysis (**Figure 2.9A and B**) and determine GSH and ROS levels in the mouse HSCs (**Figure 2.9C and D**). Our experiments revealed that after 4
weeks of NAC treatment, there were significantly more LT-HSCs and ST-HSCs in the NAC treated MRP1<sup>+/+</sup> mice as compared to non-NAC treated (Figure 2.9A). Contrary to our findings in the BM, 4 weeks of NAC treatment had no effect on progenitors in the PB (Figure 2.9B). This is not entirely surprising, as it takes 16 weeks to completely repopulate the progenitors and fully differentiate cells in the blood. NAC treatment did, however, significantly reduce HSC ROS (Figure 2.9D). Surprisingly, the MCB assay revealed that GSH was reduced in the NAC treated mice as compared to the non-treated MRP1<sup>+/+</sup> mice (Figure 2.9C). Overall, the results of the NAC study supports our model that deletion of MRP1 increases HSC self-renewal by increasing cellular GSH that in turn reduces cellular ROS. The reduction of cellular ROS then signals changes in redox response gene expression and reduces p38 MAPK activity and signaling.
In the current study we present data that strongly suggest a role for MRP1 in regulating HSC self-renewal. The key findings of our study are that deletion of MRP1 in mice results in increased cellular GSH, due to loss of MRP1-mediated GSH efflux. The increased levels of the antioxidant GSH resulted in a decrease in cellular ROS, which in turn, signals changes in the expression of redox response genes and decreases p38 signaling. Due to the decreased efflux of GSH, GSH levels are increased. GCLC and GCLM were downregulated while GSR was upregulated with the increased GSH levels. This is mostly likely due to GSH being recycled and not additional GSH being synthesized. These findings are supported by our experiments in which mice were supplemented with NAC, which showed that treatment of MRP1<sup>+/+</sup> mice with NAC increases primitive HSCs and reduces HSC ROS similar to that seen in HSCs from MRP1<sup>−/−</sup> mice. Interestingly, NAC decreased the levels of GSH while still decreasing ROS. We reasoned this is most likely due to NAC being able to scavenge ROS on its own, and doesn’t necessarily have to donate a cysteine to stimulate GSH synthesis. Together all our studies presented here strongly support our proposed model (Figure 2.10). To our knowledge the data presented in this paper are the first to demonstrate that lack of MRP1 can increase HSC frequency and self-renewal, and suggests that downregulation of MRP1 function and/or expression by the cell
or pharmaceutically will increase HSC self-renewal. Potential implications of our findings are that by increasing cellular GSH with use of MRP1 inhibitors or use of GSH synthesis inducers will enhance therapeutic uses of HSCs for bone marrow failure, bone marrow transplants, and treatments for leukemia (71).

While this is the first study to indicate that lack of MRP1 can decrease the amount of ROS present in the HSCs, there have been several studies in HSCs and the importance of low ROS for self-renewal and ability to transplant in the long term, where increased levels of ROS altered the quiescent state of HSCs and their ability to self-renew (47, 71) (48, 103). Further support for MRP1 regulating cellular ROS via GSH efflux has clearly been established for maintenance of neurons and protection of cardiomyocytes (80, 94, 104, 105). To date, there have been only a small number of studies that have mentioned a possible role for GSH in regulation HSC self-renewal and differentiation. However, there was a recent study using gastrointestinal cancer stem cells that showed CD44v (glycoprotein correlated with tumors and metastatic potential)-mediated changes in GSH directly alter cellular levels of ROS and ROS-activation of the p38MAPK pathway (97). This study showed that CD44v could control the levels of reduced GSH, where increased levels of CD44v showed enhanced GSH synthesis (97). This enhanced GSH in turn decreased ROS levels and downstream targets of ROS, such as p38MAPK (97). Downregulation of p38 MAPK has been shown to increase the cellular growth in cancer cells (97). In the present study we provide strong evidence that supports a similar role in HSCs.
HSCs from our MRP1<sup>-/-</sup> mouse appear to differentiate preferentially towards megakaryocyte erythroid progenitors (MEPs) and platelets. A recent study has found that loss of self-renewal is not required for differentiation to occur, especially with regards to megakaryocyte progenitors (106). This supports our finding that MRP1<sup>-/-</sup> HSCs occur in both a higher frequency and with a greater self-renewal capacity, and the finding that deletion of MRP1 results in an increase in MEPs in the PB. Interestingly, MRP4 is preferentially expressed in progenitors and fully differentiated cells, especially megakaryocytes and platelets (107). In our studies we found that MRP4 expression is similar in LT-HSCs, ST-HSCs, and MPPs in both MRP1<sup>-/-</sup> and MRP1<sup>+/+</sup> mice; however, we cannot rule out the possibility that MRP4 is influencing progenitor differentiation beyond early HSC differentiation to MPPs in the absence of MRP1 with our current analysis.

In our study we found that several redox proteins were altered in our MRP1<sup>-/-</sup> mouse. With an increase in glutathione and decrease in ROS we also saw a significant decrease in GCLM, while we saw a significant increase in both glutathione reductase and catalase. Due to the fact that GCLM is downregulated and glutathione reductase is upregulated it is reasonable to conclude that glutathione is being recycled and not synthesized in the absence of MRP1. It is also important to note that DCF-DA is a non-specific stain for ROS, and thus we could not conclude which type of ROS was present in our MRP1<sup>-/-</sup> mice.

Interestingly, HSCs derived from MRP1<sup>-/-</sup> mice have a similar redox profile to hepatic stellate cells, which are found to be derived from HSCs during liver injury (108). Greater glutathione and decreased catalase is found upon hepatic
stellate cell proliferation/activation out of a quiescent state (108). Similarly, our HSCs show low levels of glutathione in the LT-HSCs, with increasing levels in the ST-HSCs and MPPs. Further, the MRP1\(^{-/-}\) mouse has increased catalase in only the LT-HSCs, with significantly lower catalase levels in ST-HSCs and MPPs (Figure 2.8C). In hepatic stellate cells, catalase and not glutathione, is required to protect against oxidative stress induced apoptosis (108). In the hepatic stellate cell study they found that inhibition of glutathione increased necrosis in both the absence and presence of exogenous hydrogen peroxide (108). Additionally, inhibition of catalase and glutathione peroxidase increased apoptosis in both the absence and presence of exogenous hydrogen peroxide (108). Since both catalase and glutathione can scavenge hydrogen peroxide it would seem counterintuitive for both to upregulated in LT-HSCs; however, dual upregulation of both glutathione and catalase have been seen in other studies (109). Interestingly, a study found that NAC treatment, which can scavenge ROS on its own and stimulate glutathione synthesis, would stimulate the upregulation of catalase (109). Therefore it is possible that catalase levels are increased in LT-HSCs derived from the MRP1\(^{-/-}\) mice to protect LT-HSCs from apoptosis when glutathione synthesis is altered. Support for this comes from our finding that there is no difference in HSC apoptosis between MRP1\(^{-/-}\) and MRP1\(^{+/+}\) mice. The model in which catalase is the main protector against apoptosis, and not glutathione, in HSCs warrants further investigation.

A very recent study has found that when glutathione metabolism is altered it will impact the expression of other antioxidants, such as catalase (110). This
study, like ours, found that GCLM and GCLC were expressed at relatively low levels in hematopoietic stem cells (110). Additionally they found increased GCLM and GCLC expression in the leukemic stem cells (110). Interestingly, as the stem cells become leukemic catalase expression became downregulated (110).

Our data, suggests a role for glutathione in regulating cell growth independent of apoptosis. This model is supported by our findings in the colony forming cell assay (Figure 2.2E) that there was an unexpected decrease in our differentiated colonies that could not be attributed to apoptosis but rather as the result of decreased cell growth of the MRP1−/− mouse HSCs.

Our hypothesis is further supported by our NAC studies that showed that treatment of normal MRP1+/+ mice with NAC would result in an MRP1−/− like phenotype in the HSCs. We utilized NAC to decrease the ROS present in MRP1+/+ mice in an attempt to recapitulate the MRP1−/− HSC phenotype. We found that after 4 weeks of NAC treatment the amount of LT and ST-HSCs was increased. Such a finding strongly suggests that indeed in the MRP1−/− mouse, HSC self-renewal is increased by the reduced cellular levels of ROS that result from the loss of MRP1-mediated GSH efflux and subsequent rise in cellular GSH. It is important to note that we saw a significant decrease in the amount of GSH following NAC administration. We reason that this is due to either NAC scavenging ROS on its own, forming NAC-disulfide products, or a mechanism similar to buthionine sulfoxide (BSO) in conjunction with NAC administration showing decreased GSH (111, 112). BSO alters glutathione synthesis by inhibiting γ-glutamylcysteine synthetase so glutathione isn’t synthesized (111). If
we truly recreated our MRP1\(^{-/+}\) phenotype we would anticipate that increased glutathione would also downregulate glutathione synthesis. Alternatively, NAC treatment was found to increase levels of \(\text{H}_2\text{O}_2\) in some cells and decrease glutathione levels(113). Pertinent to our study, treatment of mice with NAC was found to restore HSC self-renewal, through what is believed to be a p38 MAPK-dependent pathway (48). Overall the data in our current study establishes MRP1 as a regulator of self-renewal and differentiation in HSCs.
Figure 2.1
Figure 2.1: MRP1\(^{+/+}\) and MRP1\(^{−/−}\) Flow Cytometry Analysis of BM

Flow-cytometric analysis of MNBC’s derived from MRP1\(^{+/+}\) and MRP1\(^{−/−}\) mice BM for HSC content. Bone marrow extractions were performed on MRP1\(^{−/−}\) and MRP1\(^{+/+}\) mice as described in methods. Enriched MNBCs were stained and analyzed for HSC content as described in methods. MRP1\(^{+/+}\) and MRP1\(^{−/−}\) lymphocytes, negative for lineage markers, were further separated by c-Kit and Sca1 expression (LSK cells, representing the HSCs) (A and C). Cells expressing both c-Kit and Sca1 were selected and further analyzed for CD34 and Flt3 expression (B and D). Here, CD34\(^−\) and Flt3\(^−\) cells represent LT-HSCs, CD34\(^+\) and Flt3\(^−\) represent ST-HSCs, and CD34\(^+\) and Flt3\(^+\) cells represent MPPs. Flow data shown here are representative of all samples analyzed for each mouse type. (n=3)
Figure 2.2
Figure 2.2 MRP1−/−mice have more HSCs than MRP1+/+. Bone marrow extractions were performed on MRP1−/− and MRP1+/+ mice. Flow cytometric analysis of MNBCs derived from MRP1+/+ and MRP1−/− mice BM for HSC content (A) and BM progenitor content (B); peripheral blood (PB) for progenitors content (C). Colony forming cell (CFC) assays were carried-out with BM derived MNBCs from MRP1+/+ and MRP1−/− mice to determine ability of progenitors to give rise to differentiated blood cells (D). Microscopy was used to identify visually distinct colonies (E). BrdU analysis of BM-derived HSCs from MRP1+/+ and MRP1−/− mice were carried out as described in methods. In brief, mice were treated with BrdU by IP injection 5 hours prior to harvesting bone marrow for analysis by flow cytometry. 10,000,000 cells were collected for each sample run on flow cytometry. All graphs are comprised of data from at least n=3 mice. Each bar represents the mean ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 vs. control (MRP1+/+).
Figure 2.3
Figure 2.3 HSC proliferation was measured by flow cytometry via BrdU assay. MRP1+/+ and MRP1-/- mice were analyzed by BrdU staining for cellular proliferation from MNBCs. Mice were injected by IP with BrdU as described in methods. After a 5 hours, MNBCs derived from MRP1+/+ and MRP1-/- BM were subjected to staining and flow cytometric analysis as described in methods. Upper panels depict the gating (selection) for HSCs in MRP1+/+ (left) and MRP1-/- (right) mice which is representative of LSK cell (Lyn- C-KIT+ SCA-1+) (A). BrdU fluorescence was then analyzed in LSK cells in histogram format to calculate the percent of cells in G0/1 and S phase for MRP1+/+ (left) and MRP1-/- (right) (lower panels). Data shown here are representative for all samples analyzed for each mouse type (n=3).
Figure 2.4. Analysis of apoptosis in MRP1\textsuperscript{+/+} and MRP1\textsuperscript{−/−} mouse HSCs by Annexin V

Apoptosis was analyzed in MRP1\textsuperscript{+/+} and MRP1\textsuperscript{−/−} mouse derived HSCs by annexin V staining and flow cytometry as described above in methods. Annexin V has high affinity for phosphatidyl serine (PS), and can detect early stages of apoptosis where PS is being translocated. HSCs from MRP1\textsuperscript{+/+} (left) and MRP1\textsuperscript{−/−} (right) show no differences in their Annexin V flow cytometry profiles. An increase in apoptosis would be marked by an increase in fluorescence of Annexin V in the upper right quadrant of histograms. Data shown here are representative for all samples analyzed for each mouse type (n=3).
Table 2.1 Murine Whole Blood Analysis

*Table 2.1. Data represented in the table is calculated from n=3 mice and includes standard error. Abbreviations used in table are for cell types white blood cells (WBC), neutrophil (NE), lymphocyte (Ly), monocyte (MO), eosinophil (EO), basophil (BA), red blood cell (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV).
**Figure 2.5**

(A) Comparison of MRP1 expression levels across LT-HSC, ST-HSC, and MPP cells. Significant differences are indicated by asterisks (* = p ≤ 0.05).

(B) Comparison of MRP4 expression levels across LT-HSC, ST-HSC, and MPP cells, showing no significant differences.
Figure 2.5: MRP1 expression increases as HSCs become more differentiated while MRP4 does not change. MRP1 (A) and MRP4 (B) expression was measured in HSCs enriched from MNBC’s derived from MRP1+/+ and MRP1−/− mouse BM via flow cytometry and 5,000,000 cells were collected for each sample. All graphs are comprised of data from at least n=3 mice. Each bar represents the mean ± S.E.M. *p<0.05; **p<0.01 vs. control. MRP1−/− mice were used as a negative control for MRP1 expression in MRP1 +/+ mice. MRP1 +/+ mice were the control for MRP4 expression in MRP1−/− mice.
CRU (95% confidence)
MRP1+/+ = 1/87,163 (50,061-281,883)
MRP1-/- = 1/18,073 (13,006-48,844)

Figure 2.6
Figure 2.6: Deletion of MRP1 increases HSC frequency in BM. Limiting-dilution competitive repopulation assays were carried out as described in the methods. Transplants were carried out using 10K (B), 20K (C), and 60K (D) MNBC test cells from either MRP1\(^{+/+}\) CD45.2 or MRP1\(^{-/-}\) CD45.2 mice and admixed with 200,000 competitor MNBC cells (MRP1\(^{+/+}\) CD45.1). Blood was extracted for analysis of CD45.2 test cell content of CD3e, CD11, CD19, and Gr1 marked blood cells at four week intervals. The results were used to calculate an overall frequency of HSCs (competitive repopulation unit (CRU)) after 16 weeks (A). Transplantations were scored for all blood samples and scored as positive or negative with the regard to successful competitive growth of test cells, a positive score was given if all four blood cell types had 5% or greater CD45.2 cells. The frequencies of long-term HSCs were calculated from the proportions of negative recipients in each cell dose group using L-Calc software (StemCell Technologies, Vancouver, BC, Canada) (n=6). *p<0.05.
Figure 2.7
Figure 2.7: Deletion of MRP1 increases HSC self-renewal. Serial transplantation studies were carried out as described in the methods. In brief, BM was extracted from recipient mice at 16-weeks post transplantation, and analyzed by flow cytometry for CD45.2 marked lymphocytes (B) and HSC (C) content in primary and secondary mice. From the primary transplant mice, 1x10^6 BM derived MNBCs were used for the secondary transplants. In addition to analyzing lymphocyte and HSC (LSK) CD45.2 percentiles, the percent of CD45.2 present in PB was measured (D). 5,000,000 cells were collected for bone marrow and 1,000,000 counts were collected for blood for each mouse sample via flow cytometry. Each bar represents the mean + S.E.M. * p<0.05 vs. MRP1+/+ (n=6).
Figure 2.8
Figure 2.8 Deletion of MRP1 reduces intracellular ROS, increases GSH, and alters redox response gene expression. MNBCs derived from MRP1<sup>−/−</sup> and MRP1<sup>+/+</sup> mouse BM were subjected to the MCB assay for intracellular GSH content (A) and the DCF-DA assay for intracellular ROS levels (B) as described in methods. Similarly, redox response gene expression is reported as relative fluorescence as measured by flow cytometry (C). MNBCs were derived from MRP1<sup>+/+</sup> and MRP1<sup>−/−</sup> mouse BM and were also subjected to p38 expression and phospho-p38 analysis; results are reported as relative fluorescence as measured by flow cytometry (D). Expression studies were carried out as described in methods. A minimum 5,000,000 cells was collected for all flow cytometry analysis. Mk571, an inhibitor for MRP1, was utilized in MCB and DCF-DA assays due to the fact they are MRP1 substrates. All graphs are comprised of data from at least n=3 mice. Each bar represents the mean + S.E.M. *p<0.05; **p<0.01, ***p<0.001 vs. MRP1<sup>+/+</sup>. 


Figure 2.9
Figure 2.9: Treatment of MRP1\(^{+/+}\) mice with NAC partially recapitulates MRP1\(^{-/-}\) mouse phenotype. MRP1\(^{+/+}\) mice were either treated with saline or NAC in the drinking water for 4 weeks as described in methods. Flow cytometric analysis was subsequently used to determine the HSC content of BM (A), the percent composition of differentiated cells in the PB (B), the intracellular GSH content of HSCs in BM (C), and intracellular ROS levels in BM HSCs (D). All experiments were done as described in methods. A minimum of 1,000,000 cell counts were collected for PB and 5,000,000 cell counts were collected for BM analysis. Each bar represents the mean + S.E.M for n=3. * p<0.05 vs. saline treated control.
Figure 2.10: Proposed model for MRP1-mediated regulation of HSC self-renewal. GSH is a substrate for MRP1-mediated efflux. Reduction of MRP1 function and/or expression decreases MRP1-mediated GSH efflux which results in increased intracellular GSH and decreased intracellular ROS. In addition, decreased intracellular ROS signals changes in redox response protein expression as well as decreases p38 activity via reducing p38 phosphorylation. This reduction in p38 activity ultimately leads to reduced MAPK signaling and decreased expression of a variety of cellular proteins sensitive to cellular redox state. Of particular interest is the decrease in GCLM expression, a protein that forms a dimer with GCLC, which results in the formation of a holoenzyme that is directly responsible for the rate-limiting step in GSH synthesis. An increase in GR, the protein responsible for GSH recycling, suggests that GSH is being recycled rather than synthesized.
Chapter 3
Post-translation regulation MRP1 by CK2α

Background

The function of Ycf1p, the yeast MRP1 homolog, is regulated via phosphorylation within the L0 region at Ser251 of the N-terminal extension by Cka1p (yeast homolog to CK2α) during salt stress (Figure 3.1A) (64). Unlike MRP1, Ycf1p is localized to yeast vacuole membranes rather than the plasma membrane. Ycf1p transports substrates, including GSH, into the vacuole where it is sequestered and ultimately broken down via gamma-glutamyl transpeptidase (GGT) (65). Phosphorylation of Ycf1p by Cka1p correlates with decreased ROS due to a decrease in Ycf1p-mediated sequestration of GSH into the yeast vacuole (65). Ycf1p is about 42.5% identical to human MRP1 (114). However, much of the shared identity is restricted to the cytoplasmic loops of the protein, while there is very little shared identity in the membrane spans. Based on a very high degree of homology in the L0 region (Figure 3.1B), we hypothesized that human CK2α regulates MRP1 via a similar mechanism at a conserved putative phosphorylation site at Thr249.

The serine/threonine protein kinase CK2 is made of two catalytic α and two regulatory β units (57). Interestingly, these subunits can be active on their own. It has been established that CK2 can phosphorylate ABC transporters,
such as ABCA1 and ABCB1/Pgp (59, 60). Increased protein expression of CK2 is found in all cancers that have been studied, which make CK2 a target for therapeutic cancer treatments (58, 61). CK2 has gained interest as a “druggable” target for leukemia treatment due a small number of studies that have shown that CK2 inhibitors target leukemic stem cells selectively (62, 63).

Previously in our lab a knockdown of CK2α in MCF7-derived cells expressing MRP1 was created. MCF7 cells served as an ideal model to study MRP1 function due to their lack of MRP1 expression, unless altered. Knockdown of CK2 resulted in increased sensitivity to a known MRP1 substrate, doxorubicin. (115). Additional substrates for MRP1, such as leukotriene C4 and estradiol-17β-D-glucuronide, were found to have decreased transport during CK2α knockdown indicating that CK2α was impacting MRP1-mediated transport (115). Previously our lab found that the yeast homolog to CK2 could phosphorylate the yeast MRP1 homolog at Ser251. A semiconserved site in human MRP1 at Thr249 was studied to see if human CK2 could control its phosphorylation. Thr249 was mutated to an alanine (Thr249A), which decreased MRP1-mediated transport (115). Another mutation Thr249E, a phosphomimicking mutation, was found to increase the MRP1-mediated transport (115). From these studies it was concluded that CK2α was regulating MRP1 by phosphorylation at Thr249 (115). For proof of principle to these studies we wanted to look at the role of CK2α on MRP1-mediated transport in cancer cell lines that naturally express both MRP1 and CK2α. Since Dox sensitivity was altered in our lab’s previous CK2 knockdown of CK2α cell-line we wanted to see if inhibitors for both CK2α and MRP1
could alter Dox sensitivity in the cell lines that expressed both MRP1 and CK2α using Dox-accumulation assays. If our previous studies were correct that CK2α could regulate MRP1 function we would anticipate that inhibitors for CK2α inhibitors would decrease MRP1-mediated export in a similar fashion as MRP1 inhibitors. The results of our Dox-accumulation assays showed that treatment with inhibitors for CK2α and/or MRP1 resulted in increased MRP1-dependent sensitivity/accumulation of intracellular Dox in all human cell lines, H460, HeLa, and A549. The decrease in MRP1-dependent efflux of Dox is supported by our findings in Dox cytotoxicity assays. In the Dox cytotoxicity assays we measured MRP1-dependent drug resistance in the presence and absence of CK2α inhibitors. We found that pretreatment of cells with CK2 inhibitors decreased MRP1-mediated protection against Dox cytotoxicity.
Materials and Methods:

Materials:

CK2 and ABC transporter inhibitors: tetrabromobenzotriazole (TBBz) was purchased from Sigma-Aldrich (St. Louis, MO), MK571 from Cayman Chemicals (Ann Arbor, MI), and PSC883 and Fumitremorgin C (FTC) from Solvo Biotechnology (Budapest, Hungary). DMEM, RPMI, F-12K, FBS, Penicillin/Streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA). Doxorubicin (Dox) was purchased from Calbiochem/ Merck KGaA (Darmstadt, Germany). PMSF and Pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture:

A549 (a human lung adenocarcinoma epithelial cell line which was a gift from Dr. Rolf Craven University of Kentucky) and cultured in F-12K with 20% FBS and Pen/Strep. H460 and HeLa cells (a gift from Dr. Vivek Rangnekar University of Kentucky) and were cultured in RPMI media with 10% FBS and Pen/Strep, and DMEM with 10% FBS and Pen/Strep, respectively.

Immunoblotting:

Standard Western blotting procedures were followed. Briefly, cells were lysed in the presence of protease and phosphatase inhibitors. Protein concentrations were determined by BCA assay (Thermo-Scientific/Pierce, Waltham, MA). Total protein (20 μg) was loaded onto a 10% SDS-Page gel for analysis. Proteins were
wet transferred to nitrocellulose membrane, blocked with 5% non-fat dry milk in TBST, and subsequently incubated with primary and secondary antibodies to detect MRP1, CK2α, ABCB1, or ABCG2 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Dox Accumulation Assay**

Cells were plated in appropriate media 24 hours prior to the assay, without antibiotics, at 3x10^5 cells/well in 24 well dishes. This cell plating density was empirically determined to result in roughly 90% cell confluency at the time of the assay (~ 24 hour incubation time). When 90% confluency was reached, cells were incubated with 50µM Dox for 1 h, followed by a 30 min efflux step without Dox. Where indicated, cells were pretreated with inhibitor 30 min to 1 h prior to the addition of Dox. Next, cells were washed with PBS, trypsinized, transferred to 1.7ml Eppendorf tubes, centrifuged and lysed in 700µl of RIPA buffer. Next, 200µl of lysate was then transferred to Optilux 96-well Microplates (BD Falcon) and fluorescence read by Synergy 2 Multi-mode Microplate Reader (BioTek Instruments, Inc.) with excitation filter wavelength 460/40 and emission wavelength 560/15.

**Cytotoxicity Assays.**

MTT assays (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) were used to measure cytotoxicity when exposed to Dox. Cells were plated in 96 well plates at 5x10^4/well to ensure proper confluence 24 hours prior to the assay. The following day, cells were plated in fresh media, media for each cell type is listed in materials and methods, containing drug or vehicle and
incubated at 37°C under 5% CO₂ for 72 hours. MTT solution in amount equal to 10% of the culture volume was added to the final concentration of 0.5ug/ml and cells were returned to the incubator. After 3 hours media was removed, DMSO was added, and plates were shaken until all MTT crystals dissolved. Absorbance was read at 560nm with Titertek Multiskan MCC/340 plate reader (Thermolabsystems, Fisher Scientific).

**Statistics:**

Statistical analysis for Dox Accumulation assays were performed by ANOVA followed by Bonferroni correction for multiple comparisons. Statistical analysis for MTT assays were done by normalizing the baseline absorbance (with no drug) and using Graphpad Prism 5 to calculate the four parameter logistic nonlinear regression curves.
Results:

Inhibition of CK2 in MRP1 expressing cancer cell lines decreases MRP1-mediated Dox efflux and increases cytotoxicity.

A549 and H460 non-small cell lung cancer (NSCLC), and HeLa cervical cancer cells were selected for our analysis based on their high levels of MRP1 and CK2 expression. In addition, we determined the expression level of ABCB1 and ABCG2, which have similar substrate specificities as MRP1. The substrate overlap between MRP1, ABCB1, and ABCG2 includes Dox; therefore, assessment of ABCB1 and ABCG2 expression and their contribution to Dox accumulation and cytotoxicity is a critical component of our studies. Figure 3.2A shows that none of the chosen cell lines express ABCB1 and only H460 cells express ABCG2.

Dox accumulation assays were performed on all three cancer cell lines, A549, H460, and HeLa. Cells were pretreated with vehicle alone, MK571 (MRP1 inhibitor), TBBz (CK2 inhibitor), FTC (ABCG2 inhibitor) and PSC833 (ABCB1 inhibitor) (Figure 3.2B). Using MK571 to inhibit the function of MRP1 we found a significant increase in Dox accumulation in all cell lines. TBBz-pretreatments resulted in Dox accumulation that was significantly higher than in untreated cells and lower than in MK571 treated cells. Co-administration of TBBz and MK571 resulted in Dox accumulation that was similar to what was achieved by treatment of the cells with MK571 alone, suggesting a MRP1-dependent mechanism of action for TBBz. Pretreatment of cells with the ABCB1 inhibitor, PSC833, and the
ABCG2 inhibitor, FTC, did not increase Dox accumulation. This result confirms that ABCB1 and ABCG2 do not play a role in Dox efflux in these cells (Figure 3.2B). This is expected due to the fact that no cell lines used expressed ABCB1 or ABCG2, except H460, which expressed ABCG2 in low levels (Figure 3.2A). It has been found that only a mutated form of ABCG2 (R482T/G) can transport doxorubicin. Since ABCG2 is expressed in H460’s but no change in Dox was found, it is reasonable to conclude that the ABCG2 expressed isn’t the mutant form that has been found to be able to transport Dox.

Based on increased accumulation of Dox in the cells when inhibited by TBBz, it would be expected there would be an increase in cellular cytotoxicity. Using 3-[4,5-dimethylthiazol-2-y]-2,5 diphenyl tetrazolium bromide (MTT) assays to measure cytotoxicity, we found that the sensitivity/cytotoxicity to Dox was increased when HeLa, H460, and A549 cells were pretreated with MK571 or TBBz (Figure 3.2C and Table 3.1). Pretreatment of cells with both MK571 and TBBz showed no additional cytotoxicity compared to MK571 treatment alone. If Dox accumulation is dependent on MRP1, then co-treatment of cells with TBBz and MK571 would not increase cytotoxicity of Dox beyond what is achieved by using MK571 alone.

The data from this study compliments additional studies from our lab that utilized a knockdown of CK2α in MCF7-derived cells expressing MRP1, where doxorubicin sensitivity was increased (115). In these studies other substrates for MRP1, such as leukotriene C4 and estradiol-17β-D-glucuronide, were found to have decreased transport during CK2α knockdown (115). Additionally, in MRP1
where Thr249 was mutated to an alanine decreased MRP1-mediated transport was found (115). Another mutation Thr249E was found to increase the MRP1-mediated transport, where Thr249E is a phosphomimicking mutation (115). From these studies using mutations in MRP1, combined with the additional data to support CK2α regulation of MRP1 transport, it was concluded that CK2α regulates MRP1 by phosphorylation at Thr249 (115).
Discussion:

In this study we present data that strongly suggest that CK2α regulates MRP1 function in humans. This finding is clinically important because MRP1 and CK2 have been found to be upregulated in a variety of cancer types, such as, leukemia, prostate, breast and lung (1, 58). The key finding of this study is that MRP1 function can be downregulated by inhibitors of CK2α. Inhibition of CK2α by TBBz resulted in decreased MRP1 function and increased cytotoxicity when cells were exposed to Dox. Additionally, inhibiting CK2α in combination with MRP1 inhibition did not result in an additive decrease in MRP1 function or cytotoxicity compared to utilizing an inhibitor for MRP1 alone. This is most likely due to the fact that the inhibitor for CK2α, TBBz, is only impacting MRP1 function and not other ABC transporters in relation to increased Dox accumulation and increased Dox cytotoxicity in these cell lines.

To our knowledge, this is the first study to show that CK2α regulates MRP1 function. A number of studies have shown that upregulation of CK2α in cancer correlates very strongly with multidrug resistance and cancer relapse. Our studies suggest that upregulation of MRP1 function is one mechanism by which overexpression of CK2α results in increased cancer multidrug resistance and a higher risk of relapse. Additional work from our lab has shown that CK2α regulates MRP1 function via phosphorylation at Thr249 (115). This work used two cell lines: a knockdown of CK2α in MRP1 expressing MCF7 cells, as well as, a mutation of Thr249 to alanine in MRP1 expressing MCF7 cells. Both the
knockdown and mutated cell lines showed increased sensitivity to Dox. In Chapter 3, using human cancer cell lines that natively express MRP1 and CK2, we found that inhibitors for CK2α could decrease MRP1 function. This finding supported the finding found in the mutant cell lines, and therefore together strongly suggest a role for CK2α regulation of MRP1 in cancer.

Overall the study presented in Chapter 3 has revealed that inhibition of CK2α can downregulate the function of MRP1 in several cancer cell lines leading to increased sensitivity to MRP1-mediated transport of chemotherapeutics. In the future we hope to further explore the therapeutic benefits of regulating MRP1 function through the use of CK2α inhibitors. Furthermore, we hope to conduct additional experiments in other cancer cell types, such as leukemia, to see if MRP1 function can be regulated by CK2α inhibitors. It would also be interesting to see if CK2α inhibitors could produce the same phenotype as our MRP1-/- mice with increased numbers and self-renewal capacity of HSCs.
Figure 3.1A Ycf1p is regulated by Cka1p-mediated phosphorylation of Ser251. Ycf1p consists of 3 MSDs and 2 NBDs. Ycf1p is phosphorylated at Ser251 by Cka1p (66).

Figure 3.1B Sequence homology in the L0 region between Ycf1p and MRP1. Close analysis of the sequences of Ycf1p and MRP1 L0 regions by sequence alignment (using ClustalW2) show that a homologous putative phosphorylation site to Ycf1p Ser251 can be found at MRP1 Thr249.
Symbols used:

- * p<0.05
- ** p<0.01
- n.s. = not significant

- Control
- MK571
- TBBz
- MK571 + TBBz

Figure 3.2(115)
Figure 3.2 CK2α can decrease MRP1 function in cancer cell lines resulting in increased sensitivity to chemotherapeutics. A) Western Blot analysis of MRP1, ABCB1, ABCG2, and CK2 in HeLa, H460 and A549 cell lines. An ABCB1-expressing cell line was loaded as a positive control for immunoblotting of ABCB-1B) Doxorubicin accumulation assay using the inhibitors MK571 (MRP1) TBBz (CK2), PSC833 (ABCB1) and FTC (ABCG2) as described in the Materials and Methods section. Statistical analysis for this assay was done using an ANOVA followed by Bonferroni. C). MTT assays measuring the cytotoxicity of Dox using the inhibitors MK571, TBBz, and MK571+TBBz as described in the Materials and Methods section. Logistic nonlinear regression curves were generated with Graphpad Prism5.
Table 3.1 Comparison of AUC, IC$_{50}$ and IC$_{90}$ of cell lines in Figure 3.2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC</th>
<th>IC$_{50}$</th>
<th>95% CI</th>
<th>IC$_{90}$</th>
<th>95% CI</th>
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<tr>
<td><strong>HeLa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2,083</td>
<td>~ 1925</td>
<td>(Very wide)</td>
<td>~ 1664</td>
<td>(Very wide)</td>
</tr>
<tr>
<td>+MK571</td>
<td>896</td>
<td>697</td>
<td>637.2 to 762.4</td>
<td>384.2</td>
<td>327.7 to 450.5</td>
</tr>
<tr>
<td>+TBBz</td>
<td>1,255</td>
<td>1057</td>
<td>903.6 to 1236</td>
<td>797.6</td>
<td>441.8 to 1440</td>
</tr>
<tr>
<td>+MK571+TBBz</td>
<td>699</td>
<td>613</td>
<td>521.1 to 721.1</td>
<td>281.4</td>
<td>203.2 to 389.7</td>
</tr>
<tr>
<td><strong>H460</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1,038</td>
<td>390.5</td>
<td>303.5 to 502.6</td>
<td>48.52</td>
<td>27.94 to 84.25</td>
</tr>
<tr>
<td>+MK571</td>
<td>575</td>
<td>116</td>
<td>105.1 to 127.9</td>
<td>53.38</td>
<td>43.35 to 65.72</td>
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<tr>
<td>+TBBz</td>
<td>929</td>
<td>243.4</td>
<td>186.9 to 317.0</td>
<td>13.93</td>
<td>6.882 to 28.18</td>
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<tr>
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<td>660</td>
<td>125.3</td>
<td>101.3 to 154.9</td>
<td>28.05</td>
<td>17.38 to 45.28</td>
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<tr>
<td><strong>A549</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2,117</td>
<td>1.634e+06</td>
<td>(Very wide)</td>
<td>~ 271.4</td>
<td>(Very wide)</td>
</tr>
<tr>
<td>+MK571</td>
<td>1,557</td>
<td>280.8</td>
<td>112.4 to 701.6</td>
<td>4.36</td>
<td>0.2873 to 66.11</td>
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<tr>
<td>+TBBz</td>
<td>1,632</td>
<td>~ 13412</td>
<td>(Very wide)</td>
<td>0.87</td>
<td>0.0024 to 317.2</td>
</tr>
<tr>
<td>+MK571+TBBz</td>
<td>1,466</td>
<td>98.81</td>
<td>42.72 to 228.5</td>
<td>1.59</td>
<td>0.0793 to 31.99</td>
</tr>
</tbody>
</table>
Hematopoiesis: The Role of MRP1

The most characterized ABC transporter in HSCs is ABCG2. ABCG2 is expressed at relatively high levels in LT-HSCs and decreases throughout the early stages of HSCs differentiation (83, 88, 116, 117). Deletion of ABCG2 in mice results in decreased HSCs self-renewal and differentiation (83, 116). MRP1 is also expressed in HSCs, however the expression pattern of MRP1 is opposite that of ABCG2 with lower levels in LT-HSCS and increasing throughout the early stages of differentiation, at both the protein and mRNA level (84, 117) (Figure 2.5A). This finding implies that, similar to ABCG2, MRP1 plays a role in regulating HSC self-renewal and differentiation. A number of studies have shown that MRP1 plays a role in regulating differentiation and self-renewal in a variety of early stem cell types, including dendritic and endothelial progenitor cells (118, 119). For example, in the endothelial progenitor cells (EPCs) it was found that changes in the redox homeostasis caused by MRP1 expression would lead to decreased function and survival of the EPCs (120). Based on these findings, we hypothesized that MRP1 regulates HSC self-renewal and differentiation. To determine if MRP1 does regulate HSC self-renewal and differentiation, we utilized C57BL6 MRP1<sup>−/−</sup> and MRP1<sup>+/+</sup> mice. C57BL6 mice have served as the research model of choice for HSC studies in hematopoiesis for over 40 years, largely due to a limiting ability for HSCs to self-renew in culture.
C57BL/6 mice (CD45.2) and B6.SJL-Ptprc<sup>a</sup> Peps<sup>b</sup>/BoyJ mice (CD45.1) are used in HSC transplants. The B6.SJL-Ptprc<sup>a</sup> Peps<sup>b</sup>/BoyJ mice were derived from C57BL/6 mice to express CD45 variant (also known as Ly5 and Ptprc) to make them easily distinguishable by flow-cytometry (121, 122).

The most exciting finding presented in Chapter 2 is that MRP1 regulates HSC self-renewal and differentiation. Using serial dilution competitive repopulation assays, we found that deletion of MRP1 increases the ability of HSCs to repopulate BM of lethally irradiated mice. Our studies strongly support that the mechanism by which MRP1 regulates HSC self-renewal is via regulation of cellular GSH levels. Here in our work, deletion of MRP1 results in increased GSH levels, decreased cellular ROS, decreased p38 signaling, and altered redox response protein expression. Using a GSH mimetic NAC, we were able to recapitulate the MRP1<sup>−/−</sup> phenotype of increased LT-HSCs and ST-HSCs, and decreased ROS. Importantly, we determined that upregulation of MRP4 expression is not a mechanism by which the HSCs compensate for the loss of MRP1 expression. Overall the data presented in Chapter 2 strongly support a role for MRP1 in regulating HSC self-renewal via efflux of GSH and subsequent reduction of ROS.

Post-translational regulation of MRP1

Our laboratory has previously established that Cka1p can phosphorylate Ycf1p at Ser251(66). Ycf1p and MRP1 are highly homologous within the L0 region. MRP1 contains a putative phosphorylation site at Thr249 that aligns with Ser251 in Ycf1p. Based on these findings in yeast and the high degree of
homology within the L0 of Ycf1p and MRP1, we hypothesized that CK2 regulates MRP1 function in humans via phosphorylation at Thr249 (Figure 3.1).

The most exciting finding, presented in Chapter 3, is that treatment of cancer cell lines with an inhibitor of CK2α decreases MRP1 function in three different cancer cell lines. Further, decreased function correlates with a loss of MRP1-mediated protection from Dox cytotoxicity. Using inhibitors to both ABCG2 and ABCB1 we have ruled out the possibility that ABCB1 and ABCG2 transport of Dox, respectively, plays a role in our study outcomes and that our results are indeed dependent on MRP1. The worked described in chapter 3 together with additional analyses provided by Dr. Ela Stolarczyk (a postdoctoral fellow in the Paumi lab) shows that MRP1 function is regulated by CK2α via phosphorylation of Thr249 (115).

**Future Directions: Targeting MRP1 to Enhance Therapeutic Outcomes**

A number of studies suggest that if ROS production is limited, then self-renewal is increased in the HSCs. These HSCs low in ROS should transplant more efficiently and better repopulate the BM niche. Drugs that alter ROS, such as Niclosamide, are currently being developed for leukemia treatment based on their abilities to target leukemic stem cells (LSCs) (49). Niclosamide is a chemotherapeutic that increases ROS levels, and has been shown to selectively kill CD34+/CD38− AML stem cells, while having minimal cytotoxicity against normal HSCs (49).
It is hypothesized that if MRP1−/− HSCs were used for bone marrow transplants that chemotherapeutics that normally spare healthy HSCs would start targeting them, as well as LSCs, due to altered levels of ROS. If this hypothesis is true, any benefit from using MRP1−/− HSCs for bone marrow transplants would be negated if transplant patients were also on chemotherapeutics, such as Niclosamide that alter ROS. Alternatively, it has been shown that high levels of cellular GSH decrease chemotherapy effectiveness (49). Since MRP1−/− HSCs have more GSH it is just as likely that those HSCs may be to a greater extent spared from chemotherapeutics.

Interestingly, GSH is elevated in a variety of cancer types, including leukemia, breast, colon, larynx, and lungs (123). This increase in GSH is thought to allow cancer cells to withstand ionizing radiation treatments and afford resistance to a variety of chemotherapeutic drugs (123). We have hypothesized that deletion or downregulation of MRP1 in leukemic stem cells (LSCs), where MRP1 downregulation is found in some subtypes of AML (that have an inversion of chromosome 16), will result in increased resistance to drug treatments due an increased cellular GSH content that would prevent redox cycling, and therefore toxicity, of many of the standard chemotherapeutics used in the clinic today that work based on increasing ROS levels.

Our lab has carried-out pilot studies using short-term 24-hour exposure of mice to Dox and found no significant differences between MRP1−/− and MRP1+/+ HSC numbers. In the clinic most patients are exposed for a longer time to chemotherapeutics and this long-term exposure might be selective for
enrichment of MRP1<sup>−/−</sup> LSCs. Longer exposure to chemotherapeutics in mice will allow us to elucidate whether there is enhanced selectivity to MRP1<sup>−/−</sup> LSCs.

Bone marrow transplants are not limited to cancer patients and are an important part of the treatments for bone marrow failure and a number of other hematological diseases, such as sickle cell anemia and autoimmune disorders. Therefore, it would be interesting to evaluate other types of drugs that are used concurrently with bone marrow transplants. This evaluation would ensure drug combinations used with transplants would not lead to increased cellular stress, which is known to lead to diseases such as cancer, cardiovascular disease, and aging.

Currently our lab is in the process of developing a leukemic mouse model to study the role of MRP1 in regulating LSC self-renewal and differentiation in acute myeloid leukemias (AMLs), due to MRP1 being downregulated in AML patients with an inversion of chromosome 16 (124). Based on the findings described in Chapter 2 that MRP1<sup>−/−</sup> mice HSCs have increased self-renewal and an increased ability to repopulate the bone marrow niche, it is anticipated that MRP1<sup>−/−</sup> LSCs would also have these characteristics. This would make MRP1<sup>−/−</sup> LSCs much more aggressive than MRP1<sup>+/+</sup> LSCs. It is also anticipated that MRP1<sup>−/−</sup> LSCs will have lower ROS levels due to the increase in GSH levels that results from deletion of MRP1. If this is true it would be important to evaluate how MRP1<sup>−/−</sup> LSCs respond to chemotherapeutics that target and/or alter ROS. Since LSCs already have low levels of ROS it may appear that MRP1<sup>−/−</sup> LSCs, with even lower levels of ROS, could be better targeted by chemotherapeutics, such as
niclosamide, that target and/or alter ROS by increasing it; however, if the
decrease in ROS is due to higher GSH levels, which are anticipated in MRP1−/−
LSCs, chemotherapeutics that target ROS would become less effective (49).

Previous studies have found that leukemia cell lines differentiate towards
megakaryocytes (107). This preferred differentiation towards megakaryocytes
was also observed in MRP1−/− mice. It should be investigated if the lack of MRP1
is causing the shift to megakaryocytes, or if there is another cause for this shift.
Alternatively, a very recent study has proposed that most HSCs will preferentially
differentiate towards megakaryocytes, without the loss of self-renewal capacity in
HSCs (106). If this is true it would be plausible that simply increasing the amount
of self-renewal in HSCs will increase megakaryocytes.

The increase in megakaryocyte progenitors in MRP1−/− mice was
accompanied by a doubling of the platelets compared to MRP1+/+ mice. It would
be interesting to investigate if the MRP1−/− platelets demonstrate any alteration in
their normal function, such as clotting ability. Phosphatidylserine (PS), which
plays a role in clotting by enhancing the activation of prothrombin to thrombin,
has been shown to increase with increased ROS exposure (125). Since MRP1−/−
mice have lower levels of ROS it would be anticipated that they have lower levels
of PS as compared to MRP1+/+ mice. A decrease in clotting ability due to lower
PS levels is a possible explanation for increased platelets to maintain normal
platelet function in the MRP1−/− mice.

In the future, the Paumi lab would like to determine if CK2α regulates
MRP1 function in HSCs. These future studies will have an immediate impact on
the treatment of multiple cancers. Currently, inhibitors of CK2 are under development for treatment of leukemia due to their ability to target leukemic cancer cells and potentially LSCs, while sparing healthy cells. HSCs and LSCs reside in a highly protective BM microenvironment and care should be taken to ensure the inhibitors can successfully penetrate the niche.

In conclusion, MRP1 plays a role in the regulation of HSC differentiation and self-renewal. Further studies are warranted to elucidate whether or not MRP1 can be targeted to enhance the therapeutic treatment of those with bone marrow disease.
Appendix A: Solutions

**1 L Red Cell Lysis Buffer:**
8.02 g NH$_4$Cl
0.84 g NaHCO$_3$
2 mL 0.5M EDTA

Directions: Combine the above ingredients and dilute to 1 L. Filter sterile before use and store at 4 degrees.

**Facs Buffer:**
PBS+2%FBS
Appendix B: FACS Bone Marrow Staining

1. Extract Bone Marrow from Femur and Tibias of mice
   a. To do this simply fill a syringe with FACs buffer and flush the contents of the bone from one end out the other into a tube.
2. Lyse Bone Marrow cells in RBC lysis buffer.
3. Spin down lysed cells at 1600 rpms for 3 minutes.
4. Wash cell pellet 2x with FACS buffer (PBS +2%FBS)
5. Resuspend pellet in 500 μL of FACS buffer and filter in blue cap Falcon FACS tube.
6. Spin cells down again at 1600 rpm for 3 min.
7. Resuspend cells in 100 μL of FACS buffer with appropriate Ab added except Dapi. See Tables below. (all antibodies are used at 1:50 dilutions except Flt-3 which is at 1:25 dilution)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Antibody</th>
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<tbody>
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<td>PE</td>
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<tr>
<td>FITC</td>
<td>CD34</td>
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<tr>
<td>PerCP-Cy5.5</td>
<td>c-Kit</td>
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<tr>
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<td>Sca-1</td>
</tr>
<tr>
<td>APC/Alexa647</td>
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</tr>
<tr>
<td>APC-Cy7</td>
<td>Lineage</td>
</tr>
<tr>
<td>DAPI</td>
<td>+</td>
</tr>
</tbody>
</table>

*Here Lin− represents B220, CD3, Ter199, and Gr1

8. Incubate on ice covered in aluminum foil for 60 minutes.
9. Spin cells down at 1600 rpm for 3 minutes
10. Wash cells 1x in 500 μL of FACS buffer
11. Resuspend cells in 100μL FACs buffer and add 5 μg/mL of Dapi
12. Flow analysis in one hour

*Please Note: When staining bone marrow used during transplantation studies the antibody for CD45.2 must be used in the panel. For APC CD45.2 simply omit CD150.
Appendix C: FACS Peripheral Blood Staining

1. Collect blood in EDTA lined collection tube
   1. Typically by submandibular cheek bleed but eye bleeds can also be used.
2. Add FACS buffer to collection tube to help avoid clotting
3. Spin down cells 1600 rpms for 3 minutes.
4. Remove the top layer from the tubes (the FACS buffer)
5. Lyse with red cell lysis buffer
6. Centrifuge 1600 rpm for 3 min
7. Wash with 500 μL of FACS buffer
8. Centrifuge at 1600 rpm for 3 min
9. Resuspend cells in 100 μL of FACS buffer with appropriate Ab. See Tables below. (all antibodies are used at 1:50 dilutions)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Antibody</th>
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</thead>
<tbody>
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<td>PerCP-Cy5.5</td>
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<td>PE-Cy7</td>
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<tr>
<td>APC-Cy7</td>
<td>CD11b</td>
</tr>
<tr>
<td>DAPI</td>
<td>V450</td>
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</table>

10. Incubate on ice covered in aluminum foil for 60 minutes.
11. Spin cells down at 1600 rpm for 3 minutes
12. Wash cells 1x in 500 μL of FACS buffer
13. Resuspend in 500 μL of FACS buffer
14. Flow analysis in one hour

*Please Note: CD45.1 and CD45.2 only used in peripheral blood staining from transplantation studies.
Appendix D: FACS MCB Staining (Glutathione)

1. Extract Bone Marrow from Femur and Tibias of mice.
2. Lyse Bone Marrow cells in RBC lysis buffer.
3. Spin down lysed cells at 1600 rpms for 3 minutes.
4. Wash cell pellet 2x with FACS buffer (PBS +2%FBS)
5. Resuspend pellet in 500 μL of FACS buffer and filter in blue cap Falcon FACS tube.
6. Spin cells down again at 1600 rpm for 3 min.
7. Resuspend cells in 100 μL of FACS buffer with appropriate Ab added except MCB. See Tables below. (all antibodies are used at 1:50 dilutions except Flt-3 which is at 1:25 dilution)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Antibody</th>
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<td>PE</td>
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<td>DAPI</td>
<td>MCB</td>
</tr>
</tbody>
</table>

*Here Lin describes B220, CD3, Ter199, and Gr1

8. Incubate on ice covered in aluminum foil for 60 minutes.
9. Spin cells down at 1600 rpm for 3 minutes.
10. Wash cells 1x in 500 μL of FACS buffer.
11. Resuspend cells in 100uL RPMI1640/10%FBS with 50uM MK571 for 60 min.
12. Resuspend cells in 100uL RPMI1640/10%FBS with 50uM MK571 + MCB (1:5000 dilution using 25mM stock) 20 min 37 degrees C.
13. Wash with 3 mL ice cold FACS buffer 1600 RPMI’s for 3 min.
14. Resuspend in 500 μL of FACS buffer containing 50uM MK571 and 5 μg/mL of DAPI.
15. Flow analysis in one hour.
Appendix E: **FACS DCF-DA Staining (Intracellular ROS)**

1. Extract Bone Marrow from Femur and Tibias of mice.
2. Lyse Bone Marrow cells in RBC lysis buffer.
3. Spin down lysed cells at 1600 rpms for 3 minutes.
4. Wash cell pellet 2x with FACS buffer (PBS +2%FBS)
5. Resuspend pellet in 500 μL of FACS buffer and filter in blue cap Falcon FACS tube.
6. Spin cells down again at 1600 rpm for 3 min.
7. Resuspend cells in 100 μL of FACS buffer with appropriate Ab added except DCF-DA. See Tables below. (all antibodies are used at 1:50 dilutions except Flt-3 which is at 1:25 dilution)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>CD135</td>
</tr>
<tr>
<td>FITC</td>
<td>DCF-DA</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>c-Kit</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>Sca-1</td>
</tr>
<tr>
<td>APC/Alexa647</td>
<td>CD34</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>Lineage</td>
</tr>
<tr>
<td>DAPI</td>
<td>+</td>
</tr>
</tbody>
</table>

*Here Lin' represents B220, CD3, Ter199, and Gr1

8. Incubate on ice covered in aluminum foil for 60 minutes.
9. Spin cells down at 1600 rpm for 3 minutes
10. Wash cells 1x in 500 μL of FACS buffer
11. Resuspend cells in 100uL RPMI1640/10%FBS with 50uM MK571 for 60 min
12. Resuspend cells in 100μL RPMI1640/10%FBS with 50uM MK571 + DCF-DA (1:5000 dilution using 25mM stock) 10-15 min 37 degrees C
13. Wash with 3 mL ice cold FACS buffer 1600 RPMI’s for 3 min
14. Resuspend in 500 μL of FACS buffer containing 50uM MK571 and 5 μg/mL of DAPI
15. Flow analysis in one hour
Appendix F: **Intracellular Protein expression via FACS**

a. Follow Bone Marrow staining as normal making sure panel doesn’t contain color that intracellular protein of interest uses.

b. After incubating with extracellular antibodies wash with 1 mL FACs buffer and centrifuge at 1600 rpm for 3 min

c. Resuspend pellet in 1mL fixation and permeabilization solution

d. Incubate for 60 min in foil covered ice

e. Rinse twice with 1 mL permeabilization buffer

f. Block with 2 uL of FBS in 100 uL permeabilization buffer on foil covered ice for 15 minutes

g. Add antibody of interest-2 uL in 100 uL permeabilization buffer and incubate on foil covered ice
   i. 4 hours for transporter proteins
   ii. Overnight for ROS proteins

h. Wash with permeabilization buffer and centrifuge at 1600 rpm for 3 min

i. Add secondary antibody for 15 minues

j. Resuspend in 500 uL FACS buffer-analyze via facs
### Appendix G: List of Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating Transcription Factor 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>Basophil</td>
</tr>
<tr>
<td>BFU</td>
<td>Blast Forming Unit</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone Marrow Mononuclear Cells</td>
</tr>
<tr>
<td>BSO</td>
<td>Buthionine sulphoximine</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CFC</td>
<td>Colony Forming Cell Assay</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transductance Regulator</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>Colony Forming Units granulocyte, erythrocyte, monocyte, megakaryocyte</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>Colony Forming Units –Granulocyte macrophage</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>CRU</td>
<td>Competitive Repopulating Unit</td>
</tr>
<tr>
<td>DCF-DA</td>
<td>Dichloro-fluorescein diacetate</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EO</td>
<td>Eosinophil</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cells</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTC</td>
<td>Fumitremorgin C</td>
</tr>
<tr>
<td>GCLC</td>
<td>Glutamyl Cysteine Ligase Catalytic</td>
</tr>
<tr>
<td>GCLM</td>
<td>Glutamyl Cysteine Ligase Modulary</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GR/GSR</td>
<td>Glutathione Reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced form)</td>
</tr>
<tr>
<td>GSS</td>
<td>Glutathione Synthetase</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide (Oxidized form)</td>
</tr>
<tr>
<td>GS-X</td>
<td>Glutathione Conjugated</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HB</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HCT</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<td>IMDM</td>
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</table>
kDA Kilodalton
KO Knockout
O3 Ozone
OH Hydroxide
LSC Leukemic Stem Cell
LSK Lin-, C-kit+, Sca-1+ (HSCs)
LT-HSC Long-term HSC
LTC4 Leukotriene C4
LY Lymphocyte
M Molar
MAPK Mitogen-activated Protein Kinase
MAPkKAP Mitogen-activated Protein Kinase
Activated Protein Kinase
MCB Monochlorbimane
MCH Mean corpuscular hemoglobin
MCHC Mean corpuscular hemoglobin
concentration
MCV Mean corpuscular volume
MDR Multi drug resistance
MEF2 Myocyte Enhancer Factor 2
MEP Megakaryocyte Erythroid Progenitor
Mg Milligram
MKK Mitogen activated protein kinase kinase
mL Milliliter
mM Millimolar
μM Micromolar
MO Monocyte
MNBC Mononuclear Blood Cells
MPP Multipotent Progenitor
MPV Mean platelet volume
MRP1 Multidrug resistance associated protein 1
Mrp1/- Mrp1 knock out
MRP4 Multidrug resistance associated protein 4
MSD Membrane spanning domain
MSK1/2 Mitogen and stress activated protein
Kinase 1 and 2
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5
NAC N-acetyl cysteine
NBD Nucleotide binding domain
NE Neutrophil
Nrf2 Nuclear factor-like 2
NK Natural Killer
NOX NADPH Oxidase
PB Peripheral Blood
PBS Phosphate Buffer Saline
PGP P-glycoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PLT</td>
<td>Platelet</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RDW</td>
<td>Red cell distribution width</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper-Zinc superoxide dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>SUR1</td>
<td>ABBC8</td>
</tr>
<tr>
<td>TBBz</td>
<td>Tetrabromobenzimidazole</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline + Tween</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YCF1</td>
<td>Yeast Cadmium Factor Protein 1</td>
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</table>
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PEER REVIEWED ARTICLES


PUBLISHED ABSTRACTS:

Cassandra J Reiling, Dianna Howard, and Christian M Paumi

MRP1 Plays a Role in Regulating Hematopoietic Stem Cell Oxidative Stress and Differentiation Via MRP1-Dependent Gsh Efflux. Blood (ASH Annual Meeting Abstracts), Nov 2012; 120: 1223
ORAL PRESENTATION

MRP1 Plays a Role in Regulating Hematopoietic Stem Cell Oxidative Stress and Self-Renewal Via MRP1-Dependent GSH efflux. Markey Cancer Center Research Day April 2013

POSTER PRESENTATIONS

Cassandra J Reiling, Dianna S. Howard, Christian M Paumi*. MRP1 Plays a Role in Regulating Hematopoietic Stem Cell Oxidative Stress and Differentiation Via MRP1-Dependent GSH Efflux. Markey Cancer Center Research Day April 2013

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Cassandra J Reiling, Dianna S. Howard, Christian M Paumi*. MRP1 Plays a Role in Regulating Hematopoietic Stem Cell Oxidative Stress and Differentiation Via MRP1-Dependent GSH Efflux. American Society of Hematology Dec 8-11 2012

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C. Reiling, J. Stumme, J. Phelps, and E. DuPre*. The Combined Effects of Atrazine and Metolachlor on survival rate, male to female sex ratio and growth rates in Poecilia reticulata. Iowa Academy of Science Poster 2005

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