Application of In Vitro Erythropoiesis from Bone Marrow Derived Progenitors to Detect and Study Genotoxicity

by

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ABSTRACT

Assays that predict toxicity are an essential part of drug development and there is a demand for efficient models to better predict human responses. The in vivo micronucleus (MN) assay is a robust toxicity test that assesses the genotoxic effect of drugs on adult bone marrow (BM) using the metric of genotoxic damage to the reticulocyte population in mice. An in vitro correlate to this assay might facilitate extension to human cells and thus provide a highly predictive genotoxicity assay. As first steps in developing a toxicity assay, this thesis work (a) adapted a fetal liver-based in vitro erythropoietic culture system to induce optimized erythropoietic growth from the lineage-marker-negative (Lin') population in adult BM, as adult hematopoietic tissue is ultimately a feasible source of cells; and (b) demonstrated that exposure to alkylating agents induces physiological MN-formation in erythroid populations derived in vitro. The potential for increased efficiency in this in vitro model depends on the ability to stimulate terminal erythroid differentiation at an optimal level from adult BM. With this goal in mind, this thesis work employed experimental design strategies, erythroid-specific growth measurements, and multi-linear regression to model erythropoietic growth in this system and thus estimate the relative sensitivity of Lin' BM to erythropoietic growth parameters, including Erythropoietin, Stem Cell Factor, pO2, and Fibronectin, among others. From these erythroid-specific growth measurements, it is estimated that >1500 MN assays can be conducted using the BM of a single mouse. This throughput represents a significant improvement over the current in vivo test, which assays a single condition per mouse. This thesis work then quantified the genotoxic response to three alkylating agents (1,3-bis(2-chloroethyl)-1-nitrosourea [BCNU], N-methyl-N'-nitro-N-nitrosoguanidine [MNNG], and methylmethane sulfonate [MMS]) in this culture system and detected a significant cytotoxic response and concomitant increase in MN incidence in reticulocytes. This increase in MN frequency provides a clear signal of the genotoxic events that likely lead to global toxicity, and thus mimics the physiological hematopoietic response to alkylating chemotherapeutics. In addition, this thesis work determined that DNA repair-deficient (MGMT') BM displayed sensitivity to genotoxic exposure in vivo compared with wild-type (WT) BM, and that this phenotypic response was reflected in erythropoietic cultures. These findings suggest that this in vitro erythroid MN assay is capable of screening for genotoxicity on BM in a physiologically reflective manner. Finally, responses to genotoxicants during erythroid differentiation varied with exposure time, facilitating the study of genotoxic effects at specific developmental stages.

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Chapter One

Introduction
1.1 GENERAL INTRODUCTION

This thesis describes the application of blood cell growth (hematopoiesis), and specifically red blood cell growth (erythropoiesis), to test for and study genotoxicity. It is fitting that the study of genotoxicity and hematopoiesis are joined in this thesis, as the field of hematopoiesis has long depended on genotoxic stresses (normally delivered in the form of radiation) to perturb and prime the hematopoietic compartment for donor-derived hematopoiesis. In what is largely considered to be the first report on hematopoietic repopulating activity, Lorenz et al. (1951) rescued lethally-irradiated mice by injecting bone marrow cells from a syngenic donor (1). Then, in 1956, Ford et al. used repopulation after irradiation to demonstrate, based on karyotype differences between donor and recipient cells, that animals protected using bone marrow transplants are hematopoietic chimeras, proving that long-term hematopoietic growth is directly derived from donor marrow (2). Finally, in 1961, Till and McCulloch again used irradiation and repopulation to describe the multi-lineage spleen colony formation that has since been associated with the pluripotent repopulating activity of hematopoietic stem cells (HSCs) (3). In the decades that followed, HSC research (founded on radiation-based studies) has made HSCs the most clearly-defined and well-understood adult stem cells; and bone marrow transplantation has become the most well-established and effective form of stem cell therapy. Today, the complete differentiation hierarchy of hematopoiesis, starting with the HSC, which is contained in the bone marrow at a frequency of approximately one in \(10^5\) cells, has been described (summarized in Fig. 1-1).

![Figure 1-1. Hematopoiesis. All the blood cell lineages in the body are derived from a single stem cell precursor (the HSC). The work in this thesis focused on the erythropoietic branch of hematopoiesis (far left). Image adapted from http://daley.med.harvard.edu/assets/Willy/Willy_noFrames.htm.](http://daley.med.harvard.edu/assets/Willy/Willy_noFrames.htm)
The reason that genotoxic insult, delivered via radiation, has proven useful for perturbing normal hematopoiesis is that the hematopoietic compartment, and HSCs in particular, are sensitive to genotoxic damage. In typical HSC repopulation experiments, a rodent is given a lethal dose of radiation, and is then rescued with a bone marrow transplant. In the absence of rescue by HSC transplant, the animal succumbs to anemia and cytopenia less than a week after irradiation as terminally differentiated blood cells are lost and the compromised stem cells and progenitors in the marrow fail to produce sufficient replacement cells. Part of the reason that the hematopoietic tissue is intolerant of DNA damage is that this tissue is one of the most rapidly dividing tissues in the body, with approximately $10^{11}$ blood cells formed each day in the adult human. Radiotherapy and chemotherapy are effective at treating cancers because cancerous cells divide rapidly, but the rapidly dividing bone marrow is also sensitive to these types of genotoxic damage, and hematopoietic side effects are common.

The erythropoietic (red cell) lineage in the hematopoietic compartment is particularly useful for detecting certain types of genotoxic damage. After the final erythropoietic cell division, erythroblasts extrude their nucleus to form enucleated reticulocytes (nascent red blood cells). If sufficient damage to erythroid progenitors has occurred prior to enucleation, then a small "micronucleus," will be left behind after enucleation. These micronuclei are composed of broken DNA and entire chromosomes that have been excluded from the daughter nucleus due to excessive DNA damage, and they are easily detected and quantified in reticulocytes because the remainder of the daughter nucleus has been extruded. That is, sub-2n particles left behind in reticulocytes are clearly detected because no other DNA is present to confound the signal provided by these particles. A mouse-based assay, based on this phenomenon, is a well-established part of modern drug development. This test, known as the *in vivo* micronucleus assay, is one of the most robust genotoxicity assays available (4-6).

1.2 MOTIVATION
Toxicity assays are an essential part of modern drug development. The therapeutic benefit of a new drug may be eclipsed by its toxic side-effects, and both the patient and the manufacturer are protected by mandatory screens in model systems. The cost of these tests is significant; accordingly, there is a demand for more efficient toxicity tests to screen new drugs during early research and development. However, the most crucial quality of a toxicity test is its predictive ability. Therefore, modifications that increase efficiency should not decrease the sensitivity or relevance of the assay. The overarching goal of this thesis was to develop and characterize a
culture system that stimulates erythroid differentiation and micronucleus formation \textit{in vitro}, using adult hematopoietic tissue, to provide an optimized corollary to the \textit{in vivo} micronucleus (MN) genotoxicity assay. Further, this thesis aimed to apply this novel system to study the basic biology of the DNA damage-response in erythropoietic micronucleus formation and genotoxicity. Greater efficiency, compared with the \textit{in vivo} assay, is possible because the bone marrow (BM) is harvested prior to treatment and used to initiate multiple erythroid-differentiation cultures, each of which then serves as a model for the \textit{in vivo} erythropoietic system. In comparison to whole-animal dosage, this system provides a high-throughput system that might be further extended to conduct preclinical tests on human primary hematopoietic tissue.

Potential therapeutic agents are screened for toxic and carcinogenic effects before initiating clinical trials. This initial risk-assessment through toxicity tests is an essential part of modern drug development, and this step both protects patients and provides early data regarding the agent's biological effect. Studies in animals and \textit{in vitro} are required by regulatory agencies around the globe to assess possible hazards prior to human exposure (7). However, there is a motive to apply toxicity screens early, to provide warning that a promising lead might test positive in a key regulatory test. Positive results in early toxicity screens identify problems that could ultimately lead to failure in clinical trials. Further, there is an interest in conducting preclinical toxicity tests on primary human tissue, based on the premise that \textit{in vitro} human might be closer than \textit{in vivo} mouse to \textit{in vivo} human. However, for this promise to be fulfilled, more \textit{in vitro} toxicity screens based on primary mammalian tissue must first be established.

1.3 PRECLINICAL GENOTOXICITY SCREENS

Key assays that hold a prominent place in regulatory considerations and genetic toxicology include the Ames test in \textit{Salmonella}, the cytokinesis-block MN (CBMN) assay in human lymphocytes, the analysis of chromosome aberrations in Chinese hamster ovary (CHO) cells, and the \textit{in vivo} MN assay in rodent BM (7). The Ames assay screens mutagens using \textit{Salmonella} strains that can only form colonies if mutation converts the strain from histidine-dependency back to prototrophy (8, 9). The Ames test is extremely well characterized, and liver microsomes can be added to the assay to screen for promutagens (latent mutagens that require metabolism by mammalian-specific pathways). The CBMN assay employs cytochalasin-B (Cyt-B) \textit{in vitro} to interrupt cell division after telophase (10, 11). Cyt-B allows cells that have undergone a cell division to be identified by their binucleate appearance, and the presence of a nuclear body excluded from the daughter nuclei clearly indicates prior DNA damage. The CBMN test is
typically applied to cultured human lymphocytes or mammalian cell lines. Another common cytogenetic technique consists of exposing cells to a test agent during the DNA synthesis (S) phase of the cell cycle and then scoring chromosome aberrations during mitosis (7, 12). CHO cells have a stable, well-defined karyotype, a low number of large chromosomes, and a short cell cycle, making them ideal for visualization of chromosome aberrations in this last test.

Although each of these assays is capable of detecting some genotoxic agents, none of them is completely analogous to normal mammalian cellular biology. Mammalian cells and bacterial cells, such as those used in the Ames test, are known to differ in their response to genetic damage (13). Not surprisingly, then, model organisms and cell lines offer only modest predictive power for hematopoietic genotoxicity, and human genotoxicity in general (8-11, 13-15). Some bacterial repair enzymes, such as photolyase, do not function at all in placental mammals, and others enzymes, such as the adaptive (ada) gene-product, share only some functions with their mammalian homologs. The CBMN test, on the other hand, can be applied to human tissue. However, CBMN test results are difficult to interpret because the test compound is always administered along with Cyt-B, which is also a toxin capable of fragmenting DNA (16). Finally, any test conducted in CHO cells or in other immortalized cell lines is imperfect because these cells carry mutations in genes that normally monitor genetic fidelity and regulate cell proliferation. Therefore, some compounds yield anomalous results, testing negative in all of these in vitro systems before yielding a positive response in vivo (17).

The in vivo MN assay, however, is conducted on somatic mammalian tissue. Therefore, it is perhaps the most descriptive of the pivotal toxicity tests mentioned above. Micronuclei (MNs) were first described in erythrocytes by W.H. Howell and J.M. Jolly in the early 20th century; therefore, they are known to hematologists as Howell-Jolly bodies. Erythrocytes containing MNs are normally removed by the spleen, and their presence in the peripheral blood (PB) is an indication that the spleen is either stressed beyond capacity, damaged, or absent. The in vivo MN assay is presently conducted in a manner very similar to that first described by Schmid and coworkers in the early 1970s (5, 6). Typically, the animal is exposed to the test substance by intraperitoneal injection and then sacrificed (24-48 h later) to harvest the BM. Conducting the analysis on newly-synthesized erythrocytes in the BM, rather than on those circulating in the PB, eliminates the complex influence that spleen function can have on the assay. The current work sought to conduct the initial treatment step of this assay with greater efficiency. That is,
delivering the dose to a whole animal to score only 2000 erythrocytes is inefficient, and significant improvement can be provided by dosing \textit{in vitro} erythropoietic cultures instead.

The next steps of the \textit{in vivo} test are fixation and staining of the BM. Finally, the slide is scored to determine the frequency of micronucleated polychromatic erythrocytes (MN-PCEs) within the PCE population. Newly-synthesized erythrocytes are described as “polychromatic” because they stain bluish, rather than pink, in May-Grünwald solution. Two main differences exist between PCEs and mature erythrocytes, which are termed “normochromatic” erythrocytes (NCEs). First, PCEs still contain ribosomes and mitochondria, which both give them their bluish tint after May-Grünwald staining; in addition to rRNA, they contain also mRNA, which both stain orange after staining with acridine orange (AO). Since the original description of the MN test, technological advances have improved its sensitivity, and these technologies have been employed in this thesis work. One of the primary difficulties in scoring slides stained with May-Grünwald or Giemsa solutions is the inability to distinguish MN (consisting of nucleic acid) from artifacts of slide preparation, such as granular inclusions. The application of AO fluorescent staining in the MN test allows the scorer to clearly distinguish DNA from other debris; therefore, this technique provides greater confidence in data (18, 19). PCEs can be definitively identified using AO staining because they contain single-stranded nucleic acid (RNA) which stains bright orange. AO stains double-stranded nucleic acid (DNA), which is found in nuclei or MN, bright green. The RNA in PCEs is translated and degraded over the course of three to five days to produce NCEs, which stain a dull khaki/green color (please see Fig. 3-6 for representative micrographs).

There are at least three recognized mechanisms by which MNs can develop in PCEs: 1) loss of acentric fragments during mitosis, 2) chromosome breakage, and 3) loss of entire chromosomes during mitosis (20). Therefore, detection of increased MN-PCE frequency over normal levels is an indication that the test substance is either genotoxic or a mitotic spindle poison. The \textit{in vivo} MN assay is established as an extremely reliable genotoxicity assay. A review conducted by the Collaborative Study Group of the Micronucleus Test (CSGMT) compiled MN assay results from several laboratories that collaborated to examine approximately 100 test substances. The CSGMT study examined compounds from International Agency for Research on Cancer (IARC) Groups 1 (human carcinogen), 2A (probable human carcinogen), and 2B (possible human carcinogen). They found that the positive rates in the MN test were 68.6%, 54.5%, and 45.6% for Groups 1, 2A, and 2B, respectively (4). The test is so robust that independent requirements for
the MN test have been established by regulatory authorities in Canada, the United States, the European Economic Community, and Japan (20).

Other technologies provide further improvements to the original MN assay. A high density of nucleated cells obscures effective scoring of MN-PCE frequency. To address this issue, erythrocytes can be purified from BM using a cellulose column that retains adherent, nucleated cells in the solid phase (21). It has also been demonstrated that the MN test can be conducted on PB without a loss of sensitivity (22). The use of PB rather than BM permits repeated sampling from a single animal, though bleeding the animal perturbs erythropoiesis and may affect test results. Finally, automated methods for slide scoring have been developed to provide two main improvements: 1) higher-throughput analysis, and 2) elimination of scorer-subjectivity from the test (23). Flow cytometric analysis based on erythrocyte markers and DNA stains is one approach to automated scoring (24, 25). A second approach employs computerized image analysis to score MN-PCEs on slides by searching for regions with low integral, but high peak, DNA-fluorescence intensity (21, 26). In the current work, cellulose column separations were used for \textit{in vivo} studies, but it was found that erythropoietic populations derived \textit{in vitro} were already sufficiently pure for unobscured scoring. The use of laser-scanning cytometry and flow cytometry to score MN-PCEs was investigated in this thesis, but microscopic examination proved more robust; accordingly, all slides were scored by differential cell counts (microscopy) after blind coding.

Although the MN assay is relatively robust, an incomplete understanding of the organism- and tissue-specific cellular responses to DNA damage can lead to unexpected genotoxic outcomes. Studies in a specific DNA-repair activity context have yielded anomalous results and provide evidence of an incomplete understanding of DNA-repair mechanisms. For example, Aag\textsuperscript{−} mice displayed an unexpected resistance to alkylation in the bone marrow (27). A yeast strain expressing a mutant version of the Mec3 gene was found to only display defects in the G1 checkpoint, rather than the expected defects in both the G1 and G2 checkpoints (28). Another report, comparing BRCA2-deficient and control cells, exposed an unanticipated role for BRCA2 in stabilizing DNA structures at stalled replication forks (29). As illustrated by these examples, it is difficult to predict the specific response to DNA damage in a particular cell-type and genotype, in part, because the function of repair-related genes in a given cellular context may not be completely understood.
The prediction of hematopoietic genotoxicity is further complicated by the fact that gene expression varies during hematopoietic differentiation (30, 31), and during the course of developmental programs, in general. In this thesis, the data show that MGMT activity influences the erythropoietic response to BCNU, and it has been shown that MGMT activity varies between different hematopoietic stages (31). Therefore, it could be difficult to predict the erythroid response to BCNU using lymphocytes, which are often employed in the CBMN assay. Using the mature (CD34), progenitor (CD34+38÷), and stem cell (CD34+38-) fractions of human cord blood, Bracker and colleagues found that the expression and functional capacity of DNA damage response genes varied both between hematopoietic stages and between individuals (30). The assay system described here provides phenotypic results from mouse tissue (Chapter Three); and, thus, might capture some of the differences between individuals if extended to incorporate primary tissue from individual patients. Assays conducted in CHO cells or Salmonella cannot be adapted to reflect individual patient responses.

Other phenotypic differences that are dependent on developmental stage have been observed. In one report, unexpected differences were observed between kidney tubular epithelium and peripheral blood T lymphocytes, illustrating that mutation spectra and cellular responses can vary significantly depending on the cell type that is examined (32). In another study, it was found that the ATR protein was undetectable in peripheral blood mononuclear cells, which was an unexpected finding because ATR was thought to be essential for the viability of somatic cells and for normal human and murine embryonic development (33). The authors of this study then go on to show that the ATR-p53 pathway is suppressed in noncycling lymphocytes via ATR downregulation, and they hypothesize that this suppressed response to DNA damage may have evolved to protect quiescent lymphocytes from the potential for p53-dependent apoptosis in the face of some forms of tolerable genotoxic stress. These lymphocyte-specific characteristics, and cell-type specific differences, in general, may skew the results obtained in the CBMN assay. Of course, the assay described in this thesis is subject to those same limitations: it reflects the cellular biology of erythropoietic BM. However, erythropoietic BM may be a useful model of general marrow toxicity because a large portion of steady-state marrow growth is erythropoietic and because the in vivo MN assay is established as a useful indicator of genotoxicity.

As the studies described above illustrate, it is difficult to predict the cell type and genotype specific response to a given genotoxic agent. Assay systems, such as the one described in this thesis, which can reflect a physiologic DNA-repair activity context might provide more predictive
power in toxicity testing. While this thesis work did not test a complete panel of genotoxic agents, and thus does not establish the assay system described here as being more predictive than the Ames or CBMN assays; this work did use three model alkylating agents that differ in their chemical reactivity. The assay system described here detected all three, and more importantly, it provided an in vitro response that reflected a phenotype observed in vivo. The Salmonella strains used in the Ames assay differ significantly from differentiating BM, or other mammalian tissues that contribute to adverse effects in the clinic. Therefore, by modeling erythropoietic development and defining specific treatment protocols for generating a MN response in vitro, this system has the potential to provide more physiologically-relevant results regarding BM toxicity.

1.4 IN VITRO ERYTHROPOIESIS
Recent advances in the stimulation and analysis of in vitro erythropoiesis provided the opportunity to create an in vitro correlate to the in vivo MN assay using primary hematopoietic tissue. The work of Zhang, Socolovsky and coworkers established a culture environment for ex vivo erythropoiesis and developed a flow cytometric technique to quantitatively analyze erythroid differentiation (34, 35). Erythropoietin (Epo) is the primary hormone responsible for erythropoietic growth, and it is a key component of the media formulation. Epo promotes the survival of colony-forming units erythrocyte (CFU-Es), providing the opportunity for these CFU-Es to differentiate into enucleated reticulocytes (36). Holo-transferrin is also included in the media, and carries iron into the cell via the transferrin receptor (CD71) where the iron serves as a cofactor for hemoglobin. Finally, a coat of Fibronectin (Fn) on the culture surface promotes adhesion of CFU-Es before they differentiate and lose the Fn receptor (37, 38).

Flow cytometric analysis of erythroid differentiation is achieved by immunostaining for CD71 and Ter-119 (Fig. 1-2). Ter-119 is a surface protein associated with glycophorin A, and Ter-119 is specifically expressed on the surface of cells in the late stages of erythropoietic differentiation (39). CD71 is only transiently expressed during late erythropoiesis. Therefore, Ter-119/CD71− cells represent the most primitive erythroid cells (CFU-Es and more primitive erythroid cells), along with some non-erythroid cells. As these cells differentiate, they first express CD71, and then Ter-119, to briefly become Ter-119+/CD71− before gradually losing CD71 expression as reticulocytes become mature red blood cells. When this analysis is applied to fetal liver (FL) cells, harvested near embryonic day 14 (E14), Ter-119/CD71− cells comprise a relatively homogeneous population of CFU-Es (35). During a two-day culture, the staining characteristics and flow cytometric qualities of this Ter-119− population change as they undergo three to five terminal cell
divisions and differentiate into reticulocytes (36). In FL, Ter-119\(^{-}/\)CD71\(^{-}\) (also known as “R1”) cells comprise a highly enriched population of CFU-Es (approximately 41% of R1 cells are CFU-Es) (35). It should be noted that the boundaries of the R1 to R5 populations, defining regions of Ter-119 and CD71 expression, as applied by Zhang and colleagues during flow cytometry, do not exactly correspond to phenotypic changes occurring during exactly one cell division. Nor do these boundaries define a completely homogeneous population of cells. However, the flow cytometric technique used by these authors to distinguish the phases of erythroid differentiation serves as a useful model, dividing the continuum of expression levels into discrete stages of late-erythropoiesis (34, 35).

![Figure 1-2. Erythropoiesis tracked by flow cytometry](image)

The culture and analytical technologies described above allow stimulation and quantification of erythropoiesis in vitro. However, these technologies were developed using highly-erythropoietic FL tissue. The BM is a more relevant target tissue for toxicity assays. Further, extending a system based on fetal tissue to primary human populations is ethically complex. Developing an analogous culture system from BM or other adult tissue presented two main challenges. First, although approximately half of the Ter-119\(^{-}/\)CD71\(^{-}\) cells in FL are CFU-Es, a population with
these surface characteristics in the BM contains both the progenitors for a variety of hematopoietic lineages as well as their fully-differentiated progeny (B-cells, T-cells, etc.); therefore, an appropriate starting population in the BM needed to be identified. The ideal starting population for the development of a high-throughput toxicity test provides the maximum number of PCEs per animal. Separation of the BM population to enrich the starting population for CFU-Es and removal of reticulocytes was essential to optimize erythrocyte production in vitro and to ensure that assayed reticulocytes were formed in vitro. Fortunately, many markers for CFU-Es in BM and PB have been identified (40, 41). Although complex phenotypic definitions can be used to isolate relatively pure CFU-Es from BM, the simplest isolation strategy was preferred so that the developed technology could be applied in an industrial setting. Therefore, the potential of Ter-119 BM and lineage-marker-negative (Lin-) BM to yield PCEs in culture was examined (Chapter Two). Although these heterogeneous starting populations yield a variety of cell-types, including many nucleated cells, the culture conditions select for erythropoietic cells, and the harvested populations contain a sufficient frequency of PCEs for cytological studies (42).

The specific media formulation used by Zhang and coworkers was simply a liquid version of the commercially available, methylcellulose-containing formulation used in erythroid colony assays. Although this formulation provides sufficient stimulation to induce some measure of terminal erythroid differentiation, one goal of this work was to maximize the production of reticulocytes, which then serve as indicators of genotoxic damage. Parameters that influence erythropoiesis were varied to define a growth condition that provided an improved PCE yield during culture. Although the ultimate metric for culture optimization is PCEs produced per primary BM cell, the number of Ter-119/CD71- cells in the post-culture population proved to be a useful surrogate marker for PCE content in preliminary studies. Flow cytometric analysis of cultured populations was preferred to slide preparation and scoring because it is faster and less subjective. Some of the most well-established erythropoietic modulators, examined in Chapter Two, include: 1) Epo concentration, 2) Stem Cell Factor (SCF) concentration (43, 44), 3) pH (45), and 4) oxygen tension (46-48). The timing of media changes and culture harvest were also considered. These quantitative studies identified Lin- BM as a sufficiently pure starting population, established a culture environment that provides a high yield of PCEs (per Lin- BM cell), and provided a reasonable estimate on the throughput of an in vitro correlate to the in vivo MN assay.

In mouse hematopoietic populations, a detailed understanding of which surface phenotypes and physical characteristics correspond to various stages of erythroid development exists. For
example, a BM subpopulation was recently identified that generates CFU-E colonies at an efficiency of approximately 70% (41). The investigators who identified this population refer to this CFU-E population as the erythroid progenitor (EP) population. This EP population is characterized as having a surface protein phenotype that is Lin-, c-Kit+, Sca-1-, IL7-Rα+, IL-3Rα-, CD41-, and CD71+ and it comprises 0.41% of nucleated BM cells. Incorporation of this highly-enriched CFU-E population into the in vitro genotoxicity screen described here might provide an improved erythroid-specific model of the genotoxic response.

1.5 MODEL GENOTOXICANT: BCNU (Carmustine)

In the body, damage to cellular DNA is the combined result of environmental exposure, endogenous compounds, and foreign agents. The cell responds to these events either with damage tolerance, attempts at DNA repair, or apoptosis. Tolerance and repair are potentially mutagenic events, whereas apoptosis results in destruction of the damaged cell and its genome. Mechanisms of DNA repair include damage reversal, tolerance, and excision. Damage reversal, in placental mammals, mainly refers to the removal of alkyl adducts from monosubstituted bases or the ligation of strand breaks (49). Tolerance refers either to replicative bypass of template damage (resulting in recombination) or to translesion DNA synthesis. Finally, excision refers to a variety of mechanisms including base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Any attempt at excision repair can result in either an abasic site or a transient single-strand DNA break, and abasic sites can be converted into DNA strand breaks either due to their alkali lability or through the function of an apurinic endonuclease (50). Clastogenic events, whether arising due to excision repair or direct scission, can result in MN formation (20). Given the complexity of these varied cellular responses, it was important to choose a model genotoxicant that had been well-characterized.

The primary model genotoxicant used in this study, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), is a clinically-relevant chemotherapy drug. Damage by BCNU induces responses that are well-understood and that involve repair proteins for which transgenic mouse models were available. In particular, O⁶-methylGuanine-DNA methyltransferase (MGMT) had been studied extensively in the Samson lab, and MGMT-knockout mice had been previously derived. BCNU is a DNA alkylating agent, like many other chemotherapeutic drugs, and MGMT acts to directly reverse alkylation at the O⁶ position of Guanine. Other examples of alkylating drugs include nitrogen mustards, nitrosoureas, aziridines, alkane sulfonates, platinum compounds, and methylating agents. Studies of BCNU examining its degradation in solution, pharmacokinetics in the body,
reactivity with DNA, and behavior in toxicity assays made BCNU an attractive model genotoxicant for the present work.

1.6 EFFECTS OF BCNU: REACTIVITY AND PHARMACOKINETICS
BCNU is a chemotherapy drug that is both clinically relevant and mechanistically understood. These qualities make it an ideal model compound for use in the present work. Existing reports on BCNU provide information regarding its behavior in solution, deposition in the body, and reactivity with DNA. BCNU is a lipid-soluble alkylating agent that forms reactive species without metabolism, i.e. it is "direct-acting." Furthermore, BCNU is "bifunctional," meaning two reactive species are formed with each decomposition of the parent compound (51). Haloethylnitrosoureas (HENUs), like BCNU, are hydrolyzed in aqueous media to form a variety of reactive products, three of which are depicted below in Fig. 1-3 (52). The first of these products is a haloethyldiazenium hydroxide that reacts with nucleophilic sites in DNA to give haloethyl adducts. Hydroxyethyl adducts are also produced when HENUs react with DNA, and decomposition via the second and third routes yields the probable intermediates that hydroxyethylate DNA (52-57). Colorimetric analysis reveals that only 10% of the drug remains intact after five hours in solution at a pH of 7.8; however, at acidic pH (3.5), BCNU is relatively stable in aqueous solution (58).

![Figure 1-3. Reactive species generated by haloethylnitrosoureas in aqueous solution](image)

Although these simple chemical decompositions do occur in vivo, there is also evidence that metabolism, particularly by the liver, can contribute to the generation of reactive species from HENUs. BCNU acts as a substrate for enzymes in liver microsomes when incubated under conditions that prevent chemical decompositions (59). Furthermore, the authors of this study found that the rate of metabolism by these enzymes is sufficiently rapid for this mechanism to compete with chemical decomposition in generating reactive species. A more detailed study of HENU decomposition via enzymatic metabolism reveals that cytochrome P450 hydroxylates the parent compound (60). This finding is of some significance because hydroxylation of HENUs
during degradation has been shown to influence their alkylating activity (61). In addition, when applied in the Ames functional assay, incubation of BCNU with liver microsomes prior to dosing was shown to increase its mutagenic ability to generate revertants to prototrophy (62). Finally, a study using thin layer chromatography (TLC) to analyze the in vivo decomposition products of BCNU in Wistar rats identified several other decomposition products in addition to the ones shown in Fig. 1-3 (63).

Nucleophilic sites on DNA bases or phosphodiester linkages displace leaving groups on HENU-derived cationic intermediates to form DNA adducts. The most common site for DNA adduct formation by HENUs is the N7 position of Guanine, though additions may occur at any of the nucleophilic sites in DNA (52). For example, analysis of modified DNA following exposure of a rat gliosarcoma (9L) cells to HENU revealed that DNA adducts were composed of 40.2% phosphortriesters (PTEs), 21.7% N7-(2-hydroxyethyl)Guanine (N7-HOEtG), 19.9% N7-(2-chloroethyl)Guanine (N7-CIEtG), 2.2% O6-(2-hydroxyethyl)deoxyguanosine (O6-HOEtG), along with several other minor products (64). Another study, using calf-thymus DNA in a cell-free system, found that HENU formed thirteen distinct alkylation products including 57.8% N7-HOEtG, 13.5% PTEs, 7.9% N7-CIEtG, 1.3% O6-HOEtG, as well as several other minor products (65). Although additions to Guanine were predominant in the studies discussed above, it should be noted that modifications to all four DNA bases have been detected following exposure to HENUs (52).

Following an initial chloroethylation at a nucleophilic site, a second step involving the displacement of Cl− by a nucleophilic site on an opposite DNA strand results in an ethyl bridge between strands (66). It has been shown that these interstrand crosslinks are formed over a period of hours and that they are derived from an initial alkylation at the O6 position of Guanine (67, 68). This haloethyl adduct then rearranges to form an exocyclic ring before finally forming an ethyl bridge between bases. Therefore, though it was not detected directly, the presence of O6-(2-chloroethyl)deoxyguanosine (O6-CIEtG) adducts was indicated by detection of its downstream products, including both deoxyguanosine-deoxycytosine (dG-dC) crosslinks and N1-(2-hydroxyethyl)-2-deoxyguanosine (N1-HOEtG) (65). If dG-dC and N1-HOEtG levels are summed to estimate the amount of O6-CIEtG initially formed, it is found that the ratio of O6-CIEtG to N1-CIEtG is 0.51. Furthermore, these authors also found that the ionic strength in the buffer influenced both the total amount of adduct formation and the product ratio of various adducts, providing insight into the reaction mechanism by which they are formed. In a clinical
application based on the relative abundance of N²-HOEtG following exposure of DNA to HENU, high performance liquid chromatography with electrochemical detection (HPLC-ED) of this adduct has been used as an analytical method to quantify the delivery and effect of BCNU in patients (69, 70).

The MN assay, as performed in vivo, introduces the test compound into an environment of higher complexity than that of the cell culture or cell-free nucleic acid experiments described above. As discussed above, liver metabolism produces modified reactive species, but systemic metabolism and distribution might have led to difficulties in reproducing BCNU’s induction of MN-PCEs in vitro. General studies of the pharmacokinetics of BCNU, inspired by BCNU’s clinical relevance, have been published (58, 63, 71-81). However, despite the high-sensitivity of BM to alkylating agents, none of the studies cited have examined the BM specifically. In the current work, the in vitro BCNU concentrations that stimulated erythroid MN formation (~20μM, Chapter Three) were found to be similar to the initial plasma levels (~27 μM) following intravenous bolus injection (10mg/kg BCNU) in rats (81).

Though the pharmacokinetic studies previously mentioned did not consider the BM directly, they did provide information about the physiological deposition and clearance of BCNU. Using radiolabeled BCNU in mice, DeVita and colleagues found that 36% of the label was recovered in urine within one hour and up to 62% was recovered by four hours, indicating rapid excretion (58). Furthermore, these authors found that, both at one hour and at 24 hours, more radiolabel was found in the liver and small intestine than in the nine other organs analyzed. However, at both of these times the “residual carcass,” which contained the BM, contained approximately the same radioactivity as that found in the liver and small intestine. A study in rats using the Gliadel® wafer, a biodegradable [¹⁴C]BCNU-loaded polymer matrix used to treat malignant glioma, again found that BCNU was primarily excreted through the urine and that BCNU was primarily distributed to the brain (site of implant), liver, and residual carcass (73, 82). Several other studies also show rapid clearance of the drug from rats and a predominant distribution to the liver, small intestine, and kidney, but they are of limited relevance due to the use of intravenous (IV) injection, which allows more rapid distribution than the use of IP or Gliadel®-mediated deliveries (74-77, 79). In consideration of this information, it seemed likely that in vitro dosing of erythropoietic tissue must be brief (on the order of hours) and, again, that an initial incubation of BCNU with liver microsomes might lead to increased genotoxicity in vitro.
Although the N7-position of Guanine is the most common site for adduct formation by BCNU, enzymatic studies have emphasized the biological importance of alkylation at the O6-position of Guanine. A study by Erickson and colleagues revealed the involvement of MGMT in preventing interstrand crosslink formation following exposure to HENUs (67). Specifically, these authors found that cells possessing MGMT activity contain fewer DNA crosslinks and are resistant to cell killing. Samson and colleagues examined the effect of the E. coli homolog to the MGMT gene, ada, when expressed (along with the alkB gene) in a human cell line (Mer HeLa) (83). This cell line is deficient in alkyltransferase activity, and these authors found that exogenous expression of the bacterial enzyme resulted in resistance to both cell killing and induction of sister chromatid exchange (SCE) by BCNU. Finally, competitive irreversible inhibition of MGMT activity by O6-benzylGuanine (O6-BeG) potentiates both the in vivo toxicity (measured by colony assay and cell count) and the clastogenicity (assayed via the MN test) of BCNU in mouse BM (84, 85). These reports serve to establish the ability of direct repair by MGMT to provide protection against BCNU’s effects. Further, they suggest a role for MGMT in MN formation.

Though direct repair is one of the cellular responses to damage by BCNU, it is also known that cellular responses include repair by recombination, a variety of repair mechanisms leading to strand breaks, and apoptosis. It has been found that cellular responses including alkylation frequency, SCE induction, and cell killing, are linearly correlated to HENU dose (64). This evidence suggests that the cellular responses of SCE and cytotoxicity are highly correlated events that are direct responses to alkylation. Another report, which examined the time-courses of intracellular BCNU concentration, interstrand crosslinks, and cell death also suggested a relationship between these events in a murine cell line (86). Finally, it has been established that treatment with BCNU can lead to DNA strand breaks, which indicates a clastogenic effect that is essential to MN formation (87). These studies show that cellular exposure to BCNU results in recombination (resulting in SCEs), cleavage of damaged regions (resulting in strand breaks), and apoptosis (resulting in cytotoxicity).

There is also information indicating that MMR may be involved in the clastogenic effect of BCNU. O6-methylGuanine (O6-MeG) can be misread by DNA polymerases during replication to yield incorrect O6-MeG-Thymine (T) pairing. It has been suggested that both O6-MeG:T and O6-MeG:C, which is correct pairing, are identified as errors, ensuring that attempted MMR is futile (50). Although BCNU does not produce O6-MeG adducts, there is indirect evidence that MMR mechanisms mediate MN induction by BCNU. An investigation examining the kinetics of MN
induction and chromosome breakage revealed that rates of effect by BCNU differed depending on the dose used (88). At higher doses, induction of MN-PCEs was less efficient and began after a longer latency period, suggesting that the mechanism generating strand breaks at these doses is indirect and involves repair enzymes. Finally, a more detailed study of MN-induction showed that bifunctional alkylating agents not only induce MN-PCEs during the first cell division, but that they also induce MN formation after the third division (89). Due to differences in the kinetics observed between different damaging agents, these authors conclude that MMR may be involved in the late induction of MN-PCEs by BCNU.

BER and translesion DNA synthesis (damage tolerance) have also been implicated in cellular response to BCNU. A report by Allen and colleagues found that cells bearing homozygous null mutations in the gene for 3-methyladenine DNA glycosylase (Aag) were more sensitive to BCNU (90). These authors showed that Aag protects against the cytotoxic and clastogenic effects of BCNU as measured by cell killing, SCE, and chromosome aberration frequency. Roth and Samson observed a similar, yet more subtle, effect in Aag<sup>−/−</sup> mice (27). Their results show that Aag null mice, in comparison to WT animals, showed slight increases both in ex vivo BM cell-killing and in in vivo MN-induction when treated with BCNU. Finally, the capacity for translesion synthesis was demonstrated using a synthetic oligonucleotide containing 1, N<sup>6</sup>-ethanoadenine (EA), which is an exocyclic adduct formed from the reaction of DNA with BCNU (91). This report found that pols α, β, and ι were primarily blocked by EA with only minor extension, but that pol η incorporated all four nucleotides opposite EA in an error-prone manner.

In conclusion, cells respond to the covalent DNA modification made by BCNU via several repair mechanisms. There is evidence for the involvement of direct repair by MGMT, Aag-mediated BER, SCE by homologous recombination, induction of apoptosis, translesion synthesis, and MMR. However, the tendency for a given adduct to induce a certain repair response is not clearly established in all cases. Furthermore, the direct involvement of particular repair enzymes in effecting erythroid MN-induction has rarely been demonstrated. The simplified in vitro erythropoietic environment and flow cytometric techniques established here provided a unique tool that can be used to elucidate the process of MN-induction. Following the development of an in vitro corollary to the in vivo MN assay, the current work focused on clarifying the role of repair enzymes in the clastogenic response to alkylation damage by using DNA repair-deficient mouse models (Chapter Three).
Chapter Two

Identification of a suitable hematopoietic starting population and optimization of erythropoietic growth for increased assay throughput
2.1 INTRODUCTION

In $10^5$ nucleated Ter-119$^+$ CD71$^-$ cells from E14.5 fetal liver there are approximately $4.1 \times 10^4$ colony-forming units erythroid (CFU-Es), making this relatively-pure starting population ideal for short-term terminal erythropoietic culture (35). However, fetal liver may not be as predictive of adult hematopoietic responses as bone marrow (BM), the physiological site of normal adult erythropoiesis. Isolating erythroid progenitors from other adult murine tissues, such as peripheral blood or spleen, or from natal tissues, such as cord blood, is possible, but the isolation of erythropoietic subpopulations from these alternative murine tissues is technically challenging and not common practice. Therefore, the first aim of this thesis was to identify a suitable BM subpopulation and corresponding culture methodology for enhanced erythropoietic growth over short-term culture.

Accordingly, the ex vivo erythropoietic potentials of Ter-119$^+$ BM and lineage-marker negative (Lin$^-$) BM, obtained from C57BL/6J mice, were examined when cultured under a variety of conditions. These BM subpopulations were chosen to serve as model populations because they are adult erythropoietic populations that are readily available and easily isolated. It was found that some measure of erythropoietic growth, complete with terminal division and enucleation, can be stimulated from either of these simply-defined populations. However, Lin$^-$ BM displayed a larger erythropoietic response to various erythropoietic growth factors, including Erythropoietin (Epo) and Stem Cell Factor (SCF); thus, future growth studies, as well as the studies that established this culture system's ability to detect genotoxicity (Chapter Three), were conducted using Lin$^-$ BM.

The flow cytometric analysis of late-stage in vitro erythropoiesis, developed by Zhang, Socolovsky and coworkers, provided a method to quantify the dynamics of erythropoiesis. Thus, flow cytometry served as a metric to guide the rational modification of culture conditions to facilitate high-throughput genotoxicity testing using the hematopoietic tissue of a single animal (34, 35). As discussed further in Chapter One, flow cytometric analysis of erythrocyte differentiation is achieved by double-staining for CD71 and Ter-119. Ter-119 is a molecule that is associated with Glycophorin A, and antibodies against Ter-119 specifically bind the surface of cells in late stages of erythroid differentiation (39). Late-stage erythropoietic cells also express CD71 briefly during differentiation (see Fig. 2-1). This flow cytometric technique was originally developed for E14.5 fetal liver, and was used here to identify a suitable BM-derived starting population for an analogous, adult tissue-based culture system. As described above, the flow
cytometric staining characteristics of a Ter-119− murine erythroid progenitor population change in a predictable manner as its members undergo three to five terminal cell divisions and differentiate into reticulocytes, which are also known as polychromatic erythrocytes (PCEs) (36). The ability to track these last developmental divisions by flow cytometry allowed the net production of erythroid cells, at various stages of differentiation, to be quantified in a dynamic manner. Briefly, total cell counts were conducted on each population at harvest, and then the percentage of the population that fell within a given erythroid flow cytometric region, as depicted in Fig. 2-1, was determined. The combination of these two quantities allowed the net production of cells in a given, late-stage of erythroid development to be calculated. Through these quantitative studies, erythropoietic yield in different growth environments was estimated.

While approximately 41% of Ter-119+/−CD71− cells in fetal liver are CFU-Es, this surface phenotype in BM cells also identifies the committed progenitors and differentiated progeny of a variety of hematopoietic lineages. Fortunately, a detailed knowledge of the cell-surface markers of murine CFU-Es exists (41). To develop improved culture technology, the potential of Ter-119− BM and Lin− BM, obtained from C57BL/6J mice, to yield reticulocytes in culture was examined (Fig. 2-2). Initial studies revealed that either of these populations, when cultured in the presence of Epo for approximately 72 hours, could be induced to undergo some degree of terminal erythropoiesis. However, Lin− mouse BM displayed greater sensitivity to erythropoietic stimulatory factors and was thus used as a model erythropoietic tissue for the development of improved culture methodologies.

Epo, which is an essential component of the erythropoietic media formulation, must be added to the media during the first day of short-term culture. Epo promotes the survival of CFU-Es, thus facilitating their differentiation into reticulocytes (36). Flow cytometric analyses conducted on cultured Lin− BM found that the erythropoietic stimulatory effect of Epo reached a maximum at a concentration of 10U/mL (Fig. 2-2). There was also prior evidence that SCF has a stimulatory effect on erythropoietic growth; and the SCF receptor, c-Kit, is even used as a marker for the isolation of CFU-Es (41, 43, 44). Again, flow cytometric analyses revealed that SCF did increase erythropoietic growth up to the maximum SCF concentration tested (100ng/mL). Furthermore, coating the culture surface with Fibronectin (Fn) promotes stimulatory adhesion of CFU-Es before they differentiate and decrease expression of the Fn receptor (37, 38). In the case of Fn, erythropoietic flow cytometry revealed a small effect when coated plates were compared with
uncoated plates, but this effect could not be enhanced with increasing Fn concentration (Tbl. 2-1). That is, a coating of 2μg/cm² was found to provide approximately all of Fn’s stimulatory effect.

Another stimulatory factor that is sometimes added to erythropoietic liquid culture is Dexamethasone (Dex) (92). Dex has been found to have erythropoietic activity in vivo, and it is known that Dex stimulates the glucocorticoid receptor to induce a cooperative erythropoietic response stemming from simultaneous stimulation with Dex, Epo, and SCF (93, 94). Furthermore, Insulin-like Growth Factor-I (IGF-I) has been found to have an erythropoietic stimulatory effect both in vivo and in vitro, and an in vitro study by Sawada and Krantz suggests that IGF-I stimulates erythroid progenitors directly, rather than through the action of accessory cells (95, 96). In addition, a multifactor analysis found that the stimulatory effects of SCF, Epo, Dex, and IGF-I could be employed, in concert, to provide an increasingly proliferative erythropoietic environment (97). Therefore, flow cytometric methods were used to quantify the erythropoietic effect of Dex and IGF-I on Lin⁻ BM from C57BL/6J mice, and these preliminary studies found that these factors also had an effect on erythroid cell numbers in the present experimental system.

Further, it is known that basic chemical properties, such as media pH and dissolved oxygen content, which depends partially on atmospheric oxygen content, can have an effect on erythropoietic growth. In the literature, pH is often controlled by the addition of NaOH or HCl to culture media, and in this manner it was found that a pH near 7.6 induces greater erythropoietic growth than a pH of 7.35 or 7.1 (45). However, in this work it was found that the CO₂ content of the incubation atmosphere had a dominant effect on pH over extended culture, and that control of the atmospheric CO₂ can be used to tune pH in a more direct and robust manner. Experiments conducted at pH 7.6 (2.5% CO₂) and pH 7.4 (5% CO₂) revealed that pH, in the present experimental system, induces a response contrary to that observed by McAdams, Miller et al. That is, more erythropoietic growth, as quantified by erythropoietic flow cytometry, was observed at the lower pH. In the case of atmospheric O₂, it was found that a hypoxic ambient condition in the incubator (5-10% O₂, 5% CO₂, balance N₂) can enhance erythropoietic growth, as measured by erythropoietic flow cytometry in the present experimental system. This finding regarding hypoxic culture is consistent with previous research reports (46-48). Finally, the duration of culture was found to have an effect on erythropoietic growth, with maximal cell numbers reaching the late stages of erythropoietic growth after 72-96 hours in culture.
The Lin- and Ter-119- BM subpopulations were cultured using various levels of the factors described above, and both the level and dynamics of the resulting erythropoietic growth were quantified. In these early studies, flow cytometry, combined with total cell counts, was used to quantify erythroid (Ter-119+) cells; further, these Ter-119+ erythroid cells were subdivided based on their CD71 expression into approximate erythroblasts (CD71+) and approximate reticulocytes (CD71-). It was found that, while both the Ter-119- and the Lin- BM subpopulations undergo some measure of erythropoietic growth in this cultures system, the response of Lin- BM was more pronounced, and provided a better signal-to-noise ratio for further optimization studies. Further, these initial flow cytometry studies revealed that several parameters had a significant erythropoietic growth effect in this culture system.

These studies were conducted by stimulating Lin- mouse BM with specifically defined media formulations for 24 hours before then replacing the medium to a minimal erythroid-differentiation medium (EDM) and culturing for an additional two to three days. This EDM contains 20% fetal bovine serum (FBS), 2mM L-glutamine, and 0.1mM β-mercaptoethanol in Iscove’s Modified Dulbecco’s Medium (IMDM), whereas the “Day One” medium consists of IMDM with 15% FBS, 2mM L-glutamine, 0.1mM β-mercaptoethanol, 1% bovine serum albumin (BSA), 200μg holo-transferrin, and 10μg/mL insulin as well as further supplements (specific media formulations examined are listed in Tbl. 2-1 and Fig. 2-3). Upon harvest, the numbers of late-stage erythroid cells and enucleated erythrocytes produced over the culture period were analyzed. Flow cytometric methods, as described above, were used in initial effect screening studies to identify culture factors that had a pro-erythropoietic effect and to gauge the relative size of these effects. Although the most relevant endpoint for these cultures is histologically-distinguishable PCEs-produced per primary BM cell, the number of Ter-119+/CD71+ and Ter-119+/CD71- cells in the post-culture population, as measured by flow cytometry, provided an approximate metric of PCE-content in these early screening studies.

However, a study that compared erythropoietic flow cytometry region statistics with PCE fractions, as determined by histological examination of population samples, revealed that flow cytometry does not provide a rigorous metric of PCE production. Furthermore, some variability between various Lin- BM isolations was observed over the course of preliminary flow cytometric studies. Therefore, a definitive study was conducted that used aliquots of a large, well-mixed Lin- population that was isolated from the combined BM of fifteen mice. Furthermore, in this study flow cytometry was replaced by histological analysis to provide more rigorous quantification of a
population’s PCE content. The purpose of this histology-based study was to estimate the relative stimulatory effect of various, previously-identified erythroid growth factors on culture performance. Specifically a two-level, minimum-aberration, fractional-factorial experimental design (a $2^{6-2}$ minimum-aberration design) was executed to estimate all the main (primary) interactions between the culture response, which was PCEs produced (as assessed by histology combined with total cell counts), and the individual parameters included in the experimental design, which composes the abscissa of Fig. 2-3A. In addition, many of the secondary interactions between the culture response and various parameter-pairs were estimable by implementing this experimental design.

In this approach, more rigorous quantification, via microscopy and differential cell counting, was to provide a more detailed analysis. This fractional-factorial experiment used cytology (microscopy) and differential cell counting to quantify reticulocytes, and this broad parameter-space experiment defined a culture condition for use in later genotoxicity studies (Chapter Three) and also provided a reliable estimate of the in vitro assay throughput on a per mouse basis. Further, multi-linear regression revealed that, as expected, Epo had the largest primary effect on reticulocyte production from cells near the CFU-E stage of development while Epo*SCF had the largest secondary effect on reticulocyte production from CFU-Es. Unexpectedly, the effect of Fn-coating was found to be minor, and it is possible that mesenchymal cells, which are also contained in Lin- BM, are producing endogenous Fn in these cultures.

The main goal of this optimization of erythropoietic growth was to obtain a robust estimate of in vitro erythroid MN assay throughput during short-term erythropoietic culture. However, it should be noted that, in doing so, the erythropoietic effect of various growth parameters on CFU-Es was measured. The majority of the previously described studies on erythroid growth effects have focused on improved production of erythroid progenitors (CFU-Es and burst-forming units-erythroid) from more primitive populations. Therefore, the measurements made here are unique in that they quantify growth effects on the differentiating CFU-E directly rather than measuring the effect of various culture parameters on the production of CFU-Es. Thanks to Sophia Kamran and Wes Overton, excellent undergraduate researchers, who provided technical assistance for much of the work described in this chapter.

2.2 RESULTS

2.2.1 Stimulating and Tracking Terminal Erythropoiesis in Erythroid Progenitors
Flow cytometric analysis of erythroid differentiation is achieved by double-staining for the transferrin receptor (CD71), which is dramatically upregulated during the first two divisions of CFU-Es, and Ter-119, which is stably incorporated into the membrane of cells in late stages of erythroid differentiation (39). Erythropoietic progenitors (CD71/-Ter-119-) undergo several divisions to produce progeny that first express CD71, and then Ter-119, before CD71 is lost from the cell surface during the final stages of erythropoiesis and reticulocyte maturation. It was previously shown that this sequence of Epo-induced events can occur in vitro using Ter-119' fetal liver cells (35). Here it is shown that Lin' BM cells from adult mice also undergo Epo-induced differentiation. Thanks to Glenn Paradis for expert advice and help in flow cytometry studies.

Figure 2-1. Terminal erythropoiesis stimulated in Lin' bone marrow cells over three days in culture. Bone marrow cells were stained with biotinylated α-lineage marker (α-Lin) mAbs (α-CD3ε, α-CD11b, α-CD45R/B220, α-Ly6G/Ly6C, and α-TER-119), and the Lin' fraction of the population was subsequently removed to obtain a progenitor-rich population (Lin' bone marrow). These Lin' cells were then cultured in vitro for three days on Fibronectin-coated plates in medium containing serum. Epo was included in the medium for the first day of culture, and then the medium was changed and Epo was removed. The differentiation profile of the cultured cells was examined by both flow cytometry and benzidine-Giemsa stain after each day in culture. Two representative micrographs of these stained populations are shown from each day of erythropoietic culture. After the third day of culture, flow cytometry indicated that the majority of the resulting population had acquired a late erythroid surface phenotype (Ter-119'). Furthermore, benzidine-Giemsa stain revealed that many cells in the harvested population were enucleated and expressing hemoglobin. The arrowhead indicates a hemoglobin+ normoblast, and the arrow indicates an enucleated reticulocyte. Scale bars: 20μm.

The Lin' fraction of mouse BM was isolated by immunomagnetic negative selection, plated on Fn-coated culture dishes, and stimulated with Epo for the first 24h of culture. Lin' BM represents
approximately 1.25% of cells in marrow and comprises a mixture of hematopoietic and mesenchymal stem and progenitor cells. Analysis by flow cytometry indicated that a significant fraction of Lin' cells had begun erythroid differentiation (i.e. upregulated CD71) after one day of culture with Epo (Fig. 2-1). By day two, the majority of cells exhibited erythroid differentiation markers (i.e. CD71 and Ter-119) and, by day three, the characteristic late-erythroid loss of CD71, reported for both in vivo fetal liver and cultured Ter-119' fetal liver, had begun (34, 35). Cytology further confirmed that a substantial fraction of the Lin' BM population (nucleated cells lacking hemoglobin) had differentiated into enucleated, hemoglobinized reticulocytes over the course of two-three days (Fig. 2-1). A fraction of cells isolated as Lin' were unresponsive to Epo (Fig. 2-1), and it is likely that most of these cells do not survive under erythropoietic conditions. Ter-119' BM was also used in similar experiments, but only a small fraction of the initial population underwent erythropoietic growth; i.e., a large fraction of cells in these cultures remained Ter-119/CD71' throughout the two-four day culture.

2.2.2 The PCE Yield from Lin' BM Depends on Multiple Environmental Cues

The finding that short-term erythropoietic cultures, initiated with adult Lin' BM, undergo complete erythropoiesis and appear to recapitulate many of the in vivo features of physiologic MN formation (Chapter Three) suggested that this in vitro MN assay might substantially reduce the number of animals required to conduct genotoxicity screens. This system also facilitates genotoxic-treatments and response-measurements at specific cell proliferation and differentiation steps of erythropoiesis, and could thus provide new insights about the basic biology of the erythroid response to DNA damage. Accordingly, quantitative studies on the response of Lin' BM cultures after stimulus with Epo and other erythropoietic factors were conducted to define the PCE yield per Lin' BM cell and to investigate whether adjustments to the culture protocol could provide increased yield without sacrificing biological information. Fn and Epo (original fetal liver conditions) are sufficient to induce erythropoiesis; but, as discussed in the introduction of this chapter, there are many other well-established erythroid growth factors (37, 41, 43-48, 93-97). A preliminary screen of several individual factors was conducted using flow cytometry to enumerate the total number of cells at each differentiation stage shown in Fig. 2-1 and thus estimate erythroid yields. A typical experiment is represented in Fig. 2-2, where the effect of Epo on erythropoietic growth form Lin' and Ter-119' BM is quantified. It was found that Lin' BM was much more sensitive to adjustments in culture conditions (Fig. 2-2), and that the stimulatory effect of Epo on the maturation of CFU-Es reached a maximum at an Epo concentration of
Similar studies revealed a significant effect on culture yield in response to several other stimuli (Tbl. 2-1).

![Graph showing the effect of Epo on erythroid cell yield from Lin and Ter-119 BM. BM-derived cells were cultured for one day in media containing serum along with Epo at variable concentrations. Epo was removed from the culture media at the end of one day, and culture was continued for two more days in media with serum. At the end of the third day, the resulting populations were removed from culture and erythroid cell yields were quantified as the product of total cell counts and erythroid-specific flow cytometric region fractions. Specifically, the yield of cells at two late stages of erythropoiesis was calculated for each culture by taking the product of the total cell count and the fraction of the population found to have the indicated surface phenotype (determined by flow cytometry). These erythroid cell yields were then normalized to the number of BM-derived cells seeded. Data are presented as the mean from three independent cultures +/- the standard deviation. ** indicates a significant difference (P<0.01) from Zhang’s original culture conditions (2U/mL Epo) as determined by the two-tailed t test.]

Many of the factors identified as having significant effects (listed in Tbl. 2-1) were then incorporated into a two-level, orthogonal, fractional-factorial experiment designed to quantify primary and secondary parameter effects in this culture system. The PCE yields for these conditions (Fig. 2-3A) were assessed by acridine orange cytology (microscopy), which provides a more robust estimate of the PCE fraction in a culture than flow cytometry (data not shown). Lin-BM cells cultured under the original conditions used for fetal liver erythropoiesis yielded 0.67 +/- 0.16 PCEs per Lin-BM cell (Fig. 2-3A, “Original” condition) (35). This cytology-based optimization study revealed that an ~eight-fold increase in PCE yield could be achieved (up to 5.8 +/- 0.9 PCEs per Lin-BM cell, Fig. 2-3A), and that several conditions resulted in PCE yields within 20% of
this maximum. The hind-legs of male C57BL/6J mice, aged six-eight weeks, yield approximately 6.3x10^5 Lin^- BM cells (data not shown). Using 2000 PCEs, the number used to quantify the genotoxic response in Chapter Three, as a benchmark, it is approximated that 1850 in vitro erythroid MN assays can be conducted from a single mouse using the highest-yield culture condition.

<table>
<thead>
<tr>
<th>BACKGROUND CULTURE CONDITIONS</th>
<th>MEASURED EFFECT ON ERYTHROID CELL YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td>([Ep] [ng/ml]) [SCF] (ng/ml)</td>
<td>(&quot;X&quot; indicates the variable parameter found to affect erythroid yield)</td>
</tr>
<tr>
<td>harvest time (h)</td>
<td>CO2 (%)</td>
</tr>
<tr>
<td>X 0 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>X 0 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>X 0 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>X 0 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>3.5 X 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>3.5 X 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 X 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 X 72 5 20</td>
<td>2</td>
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<tr>
<td>5 X 72 5 20</td>
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<td>2</td>
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<tr>
<td>5 X 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 X 72 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 X 96 5 20</td>
<td>2</td>
</tr>
<tr>
<td>2 0 X 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 0 X 5 20</td>
<td>2</td>
</tr>
<tr>
<td>5 10 X 5 20</td>
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<td>2</td>
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<tr>
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</tr>
<tr>
<td>5 0 72 X 20</td>
<td>0</td>
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<td>5 0 72 X 20</td>
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<td>X 0</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>5 0 72 0.2 20</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2-1. Flow cytometry shows that erythroid cell yield in vitro is modulated by physiologic erythropoietic stimuli. The culture conditions listed on the left-hand side of the table specify the environment in which a cell yield measurement was made. All soluble growth factors examined in these experiments were removed from culture after one day. An "X" in this first section of the table indicates the
variable parameter found to have a significant erythropoietic yield-effect in this study. The measured effect of that parameter on erythroid cell yield is then given on the right-hand side of the table. In this right-hand section of the table, the first item listed is the defining surface phenotype of the erythropoietic population. The surface phenotype is followed by the high-yield condition and the resulting average yield (per Lin- cell seeded) that was observed at that condition. Next are the comparison (low-yield) condition and the average yield that was observed at that condition. Finally, the level of significance (P value) is given. Note that the first four entries to this table were derived from Fig. 2-2; other entries were obtained from similar data sets (data not shown). P values were determined by the two-tailed t test, except for the three italicized P values, which were determined using the one-tailed t test.

Figure 2-3. PCE yield from Lin' BM culture is modulated by several physiologic erythropoietic stimuli. (A) Lin' BM cells were cultured for one day in media containing serum and various combinations of soluble growth factors as listed on the abscissa; these specific six-factor combinations constitute an orthogonal, fractional-factorial design (excluding the “Original” condition). After one day, all soluble erythropoietic growth factors were removed and the cells were cultured for one, two, or three additional days in medium with serum. At harvest, the cells were removed from culture and the number of terminally differentiated erythrocytes was quantified. For each culture, PCE yields were calculated by taking the product of the total cell count and the PCE fraction of the population as determined by differential cell counting after staining with acridine orange (>2000 cells scored per culture). Erythroid yields were normalized to input cell numbers and are presented as the mean of three cultures +/- the standard deviation. The “Original” culture condition and the corresponding PCE yield represent the performance of standard culture conditions in use prior to this optimization study. All in vitro genotoxic treatment studies (Chapter Three) were conducted using a variation of the condition labeled as the “High-Yield Normoxic Condition.” (B) Culture methods that produced similar populations at harvest were grouped into subsets (α, β, γ, and δ); population characteristics for these subsets were quantified and representative micrographs were acquired. It was found that cultures not exposed to Epo or SCF (subset α) yielded populations with few total cells and which contained < 3% PCEs. Cultures exposed to SCF but not Epo (subset γ) expanded approximately 2.5-fold in total cell numbers, but again contained < 3% PCEs. Cultures exposed to both Epo and SCF (subset β) expanded approximately 13-fold in total cell numbers and yielded populations in which approximately 31% of all cells are PCEs. Finally, cultures exposed to both Epo and Dex but not SCF (subset δ) expanded approximately four-fold while producing populations in which approximately 51% of all cells are PCEs. Relative PCE yield calculations are presented as the mean of all cultures in a subset +/- the standard deviation. Scale bars: 20 μm.
Populations cultured under various conditions fell into four general classes (Fig. 2-3B) based on their exposure to Epo, SCF, and Dex. Populations exposed to both Epo and SCF (subset \( f_3 \)) underwent the greatest expansion, but populations exposed to Epo and Dex without SCF yielded populations containing a high PCE fraction (subset \( f_5 \)), which might facilitate high-throughput image analysis and scoring (Fig. 2-3B).

2.2.3 Multi-Linear Regression to Model the Late-Erythropoietic Response

With the data from Figure 2-3A, multi-linear regression was used to predict the resulting PCE yields from the experimental design matrix, and the model predicted the data with \( R^2 = 0.95 \) and thus quantified the relative importance of each factor. I must thank professors Ken Beers and Kevin Janes along with Dr. Neil Kumar for many helpful discussions regarding this analysis.

Let \( \mathcal{X} \) be the design matrix before scaling, with size \( n_1 = 144 \times n_2 = 8 \). \( n_1 \) is the number of experimental measurements that were made, while \( n_2 \) is the number of independent parameters (covariates) that were tested. For example, the first condition listed in Fig. 2-3A is represented thus:

\[
\mathcal{X}_{1,j} = [0,0,0,0,0,72,0.05,0.1995]
\]

Each element in the original design matrix was then normalized such that each parameter ranges from 0 to 1, as shown below:

\[
X_{ij} = \frac{\mathcal{X}_{ij} - \min_{i \in [1,n_1]} \mathcal{X}_{ij}}{\max_{i \in [1,n_1]} \mathcal{X}_{ij} - \min_{i \in [1,n_1]} \mathcal{X}_{ij}}
\]

In this scaled design matrix, the first condition presented in Fig. 2-3A appears as:

\[
\bar{X}_{1,j} = [0,0,0,0,0,0,0,1]
\]

Alternative scaling strategies were also used (e.g. data ranging from -1 to +1), and the MATLAB® script provided in Appendix I will conduct the following analyses using either scaling. However, it was found that scaling from 0 to 1 provided a more biologically-relevant estimate of parameter effects. After scaling, secondary interactions that increased the column rank of the design matrix were incorporated as additional columns. For example, the elements...
representing the secondary interaction between Epo \((j = 1)\) and SCF \((j = 2)\) are added to the scaled design matrix as shown below:

\[
X_{i,n_2+1} = X_{i,1} \ast X_{i,2}
\]

Ultimately, a scaled design matrix of size \(n_1 = 144 \times n_3 = 27\) is obtained, where \(n_3\) is the sum of the number of primary interaction \((n_2)\) and the number of linearly-independent secondary interactions (for this experimental design, \(n_1-n_2 = 19\)). The vector of parameter estimates \((\bar{b})\) was then calculated, using multi-linear regression, to provide the best possible prediction of the experimentally measured growth-response vector \((\bar{y})\) using the scaled design matrix \((\bar{X})\):

\[
\bar{b} = (\bar{X}' \bar{X})^{-1} \bar{X}' \bar{y}
\]

These parameter estimates were then used to predict the growth response \((\bar{\hat{y}})\):

\[
\bar{\hat{X}}\bar{b} = \bar{\hat{y}}
\]

The root mean squared error (RMSE) and \(R^2\) for the model were then calculated. Below, \(y_{avg}\) is the mean of the measured growth-response vector \((\bar{y})\).

\[
RMSE = \frac{\sum_{i=1}^{144} [y_i - \hat{y}_i]^2}{n_1 - n_3}
\]

\[
R^2 = 1 - \frac{\sum_{i=1}^{144} [y_i - \hat{y}_i]^2}{\sum_{i=1}^{144} [y_i - y_{avg}]^2}
\]

The error \(\epsilon_j\) and the t-ratio \(t_j\) for each parameter estimate were then calculated as:

\[
\epsilon_j = RMSE \ast \sqrt{(X'X)^{-1}}_{j,j}
\]

\[
t_j = \frac{b_j}{\epsilon_j}
\]

Finally, the upper and lower bound of the 95% confidence intervals for all parameters are calculated using the relevant t value \((\alpha = 0.05, \text{degrees of freedom} = n_1-n_3)\) thus:
\begin{align*}
upper_j &= b_j + t(n_1 - n_3)^{\alpha/2} \cdot \varepsilon_j \\
lower_j &= b_j - t(n_1 - n_3)^{\alpha/2} \cdot \varepsilon_j
\end{align*}

A summary of the results obtained from this complete model \((n_3=27)\) is shown in Table 2-2, below.

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Scaled Estimate</th>
<th>Standard Error</th>
<th>95% Confidence Interval</th>
<th>t Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>3.136</td>
<td>0.178</td>
<td>2.783 - 3.489</td>
<td>17.576</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*SCF</td>
<td>1.648</td>
<td>0.147</td>
<td>1.358 - 1.938</td>
<td>11.242</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*O2</td>
<td>-1.079</td>
<td>0.178</td>
<td>-1.431 - 0.727</td>
<td>-6.067</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*CO2</td>
<td>-0.845</td>
<td>0.173</td>
<td>-1.187 - 0.520</td>
<td>-4.883</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SCF*Dex</td>
<td>0.451</td>
<td>0.147</td>
<td>0.160 - 0.741</td>
<td>3.073</td>
<td>0.003</td>
</tr>
<tr>
<td>time*CO2</td>
<td>0.392</td>
<td>0.173</td>
<td>0.049 - 0.734</td>
<td>2.263</td>
<td>0.026</td>
</tr>
<tr>
<td>SCF*O2</td>
<td>-0.366</td>
<td>0.178</td>
<td>-0.719 - 0.014</td>
<td>-2.059</td>
<td>0.042</td>
</tr>
<tr>
<td>SCF*CO2</td>
<td>-0.351</td>
<td>0.173</td>
<td>-0.694 - 0.009</td>
<td>-2.031</td>
<td>0.045</td>
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<tr>
<td>IGF*O2</td>
<td>0.349</td>
<td>0.178</td>
<td>0.003 - 0.701</td>
<td>1.962</td>
<td>0.052</td>
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<tr>
<td>Dex</td>
<td>-0.298</td>
<td>0.151</td>
<td>-0.597 - 0.002</td>
<td>-1.967</td>
<td>0.052</td>
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<tr>
<td>Dex*O2</td>
<td>0.272</td>
<td>0.178</td>
<td>-0.080 - 0.625</td>
<td>1.531</td>
<td>0.129</td>
</tr>
<tr>
<td>Epo*Fn</td>
<td>0.244</td>
<td>0.147</td>
<td>-0.046 - 0.535</td>
<td>1.667</td>
<td>0.098</td>
</tr>
<tr>
<td>IGF-I</td>
<td>-0.231</td>
<td>0.151</td>
<td>0.530 - 0.069</td>
<td>-1.526</td>
<td>0.129</td>
</tr>
<tr>
<td>SCF*IGF-I</td>
<td>0.186</td>
<td>0.147</td>
<td>-0.105 - 0.476</td>
<td>1.267</td>
<td>0.208</td>
</tr>
<tr>
<td>CO2</td>
<td>-0.166</td>
<td>0.228</td>
<td>-0.618 - 0.286</td>
<td>-0.727</td>
<td>0.469</td>
</tr>
<tr>
<td>SCF</td>
<td>0.166</td>
<td>0.162</td>
<td>0.155 - 0.487</td>
<td>1.023</td>
<td>0.308</td>
</tr>
<tr>
<td>CO2*IGF-I</td>
<td>0.096</td>
<td>0.173</td>
<td>-0.247 - 0.438</td>
<td>0.552</td>
<td>0.582</td>
</tr>
<tr>
<td>time</td>
<td>-0.090</td>
<td>0.138</td>
<td>-0.364 - 0.183</td>
<td>-0.654</td>
<td>0.514</td>
</tr>
<tr>
<td>O2*Fn</td>
<td>0.081</td>
<td>0.178</td>
<td>-0.272 - 0.433</td>
<td>0.453</td>
<td>0.651</td>
</tr>
<tr>
<td>O2*time</td>
<td>-0.077</td>
<td>0.178</td>
<td>-0.429 - 0.275</td>
<td>-0.434</td>
<td>0.665</td>
</tr>
<tr>
<td>Epo*IGF-I</td>
<td>0.072</td>
<td>0.147</td>
<td>-0.218 - 0.362</td>
<td>0.491</td>
<td>0.624</td>
</tr>
<tr>
<td>Fn</td>
<td>0.053</td>
<td>0.138</td>
<td>-0.221 - 0.326</td>
<td>0.380</td>
<td>0.705</td>
</tr>
<tr>
<td>Epo*Dex</td>
<td>-0.049</td>
<td>0.147</td>
<td>-0.339 - 0.242</td>
<td>0.331</td>
<td>0.741</td>
</tr>
<tr>
<td>CO2*Fn</td>
<td>0.038</td>
<td>0.173</td>
<td>-0.304 - 0.381</td>
<td>0.221</td>
<td>0.826</td>
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<tr>
<td>O2</td>
<td>0.010</td>
<td>0.191</td>
<td>-0.368 - 0.389</td>
<td>0.055</td>
<td>0.956</td>
</tr>
<tr>
<td>CO2*Dex</td>
<td>-0.004</td>
<td>0.173</td>
<td>-0.347 - 0.338</td>
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<td>0.980</td>
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<tr>
<td>Epo*time</td>
<td>0.002</td>
<td>0.147</td>
<td>-0.287 - 0.292</td>
<td>0.012</td>
<td>0.990</td>
</tr>
</tbody>
</table>

\(R^2 = 0.9508\)
\(R^2_{\text{adj}} = 0.9398\)
\(RMSE = 0.4478\)

Table 2-2. Parameter estimates derived from the complete model, including all primary and all estimable secondary interaction parameters.

Some of the less significant factors (those highlighted above) were then removed from the design matrix in order to simplify the model, and thus emphasize the more important parameter effects.

Specifically, all effects with \(|t|<1\) were removed from the final version of the model (represented in Tbl. 2-3 and Fig. 2-4) except for the time and Fn effects. The time and Fn
primary effects were retained, despite their low t-ratios, because they were key effects in the original experimental design. In this manner, the adjusted $R^2$ value was slightly improved and the model was simplified. A summary of the results obtained from this reduced model ($n=17$) is provided below:

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Scaled Estimate</th>
<th>Standard Error</th>
<th>95% Confidence Interval</th>
<th>t Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>3.130</td>
<td>0.141</td>
<td>2.852 - 3.409</td>
<td>22.259</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*SCF</td>
<td>1.668</td>
<td>0.138</td>
<td>1.396 - 1.940</td>
<td>12.122</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*O2</td>
<td>-1.075</td>
<td>0.156</td>
<td>-1.384 - -0.766</td>
<td>-6.883</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Epo*CO2</td>
<td>-0.888</td>
<td>0.144</td>
<td>-1.173 - -0.604</td>
<td>-6.183</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SCF*Dex</td>
<td>0.471</td>
<td>0.138</td>
<td>0.198 - 0.743</td>
<td>3.420</td>
<td>0.007</td>
</tr>
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<td>SCF*CO2</td>
<td>-0.395</td>
<td>0.144</td>
<td>-0.679 - -0.111</td>
<td>-2.749</td>
<td>0.007</td>
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<td>IGF*O2</td>
<td>0.376</td>
<td>0.145</td>
<td>0.089 - 0.663</td>
<td>2.589</td>
<td>0.011</td>
</tr>
<tr>
<td>SCF*O2</td>
<td>-0.362</td>
<td>0.156</td>
<td>-0.671 - -0.053</td>
<td>-2.317</td>
<td>0.022</td>
</tr>
<tr>
<td>Dex</td>
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<td>0.130</td>
<td>-0.590 - -0.077</td>
<td>-2.571</td>
<td>0.011</td>
</tr>
<tr>
<td>time*CO2</td>
<td>0.320</td>
<td>0.134</td>
<td>0.056 - 0.585</td>
<td>2.395</td>
<td>0.018</td>
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<tr>
<td>Epo*Fn</td>
<td>0.264</td>
<td>0.138</td>
<td>-0.008 - 0.537</td>
<td>1.921</td>
<td>0.057</td>
</tr>
<tr>
<td>Dex*O2</td>
<td>0.256</td>
<td>0.145</td>
<td>-0.031 - 0.544</td>
<td>1.767</td>
<td>0.080</td>
</tr>
<tr>
<td>SCF*IGF-I</td>
<td>0.206</td>
<td>0.138</td>
<td>-0.067 - 0.478</td>
<td>1.495</td>
<td>0.137</td>
</tr>
<tr>
<td>IGF-I</td>
<td>-0.200</td>
<td>0.130</td>
<td>-0.456 - 0.057</td>
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<td>0.126</td>
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<tr>
<td>SCF</td>
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<td>0.147</td>
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<td>0.934</td>
<td>0.352</td>
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<tr>
<td>time</td>
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<td>0.082</td>
<td>-0.286 - 0.038</td>
<td>-1.515</td>
<td>0.132</td>
</tr>
<tr>
<td>Fn</td>
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<td>0.092</td>
<td>-0.087 - 0.278</td>
<td>1.037</td>
<td>0.302</td>
</tr>
</tbody>
</table>

$R^2 = 0.9501$

$R^2_{adj} = 0.9438$

$RMSE = 0.4330$

Table 2-3. Refined model, including only the most significant parameter effects.

The Matlab® code used to perform all matrix algebra is provided in Appendix I, and the final results of this analysis are summarized below in Fig. 2-4.

### 2.2.4 Differences Between Epo-only and Improved Culture

In order to better understand how the modifications to culture conditions affected growth dynamics in this system, cultures containing only Epo (10U/mL) along with basal supplements were compared with "improved" cultures that also contained SCF (10ng/mL), IGF-I (100ng/mL), and Dex (10μM). Populations from each of these culture systems were harvested various times after initiation of culture and the viable cell numbers were measured using trypan blue exclusion (Fig. 2-5).
Figure 2-4. Multi-linear regression to predict PCE yield and quantify relative parameter effects. Multi-linear least-squares regression was used to generate the linear model that best predicts PCE yield from the experimental design matrix. (A) The calculated PCE yields from each culture (data points) are plotted vs. the response predicted by the regression model (abscissa/solid line). The regression model is capable of predicting PCE yield in this culture system ($R^2 = 0.95$) from information about the key culture parameters listed in Figure 2-4B. (B) The scaled parameter estimates that constitute the regression model are given. The parameters are listed in order of decreasing impact on PCE yield, with the most significant growth factor (Epo) at the top. The error bars indicate the span of the 95% confidence intervals for each parameter estimate.
Figure 2-5. Growth dynamics during improved and Epo-only (10U/mL) erythropoietic cultures. Erythropoietic cultures were initiated with Lin BM and then harvested at various times to assess growth and cell cycle status. (A) Viable cell numbers were measured using a ViCellXR viable cell counter, and were then normalized to input cell numbers. The log of this ratio was then taken to estimate the total number of doublings that had occurred at a given culture time. The data are presented as the mean of four biologically-independent samples, obtained over the course of two experiments, +/- the standard deviation. While growth on days one and three resulted in a single doubling of cell numbers, growth on day two resulted in more than two doublings.

As illustrated by these data, growth is actually more rapid during the first two days of culture for Epo-only cultures. However, between day two and harvest at day three, growth in the improved culture system continues while viable cell numbers actually decrease in Epo-only cultures. Although these data seem to imply that there is very little difference between Epo-only and improved cultures, it should be noted that Epo-only culture here included Epo at a concentration of 10U/mL, whereas the original media formulation used by Zhang and coworkers included Epo at 2U/mL. Consistent with the data of Fig. 2-4, which show that Epo is responsible for the largest growth effect, there is not a large difference between Epo-only cultures and those that include SCF and other growth factors.

Next, I sought to determine whether culture with SCF, Dex, and IGF-I (in addition to Epo) leads to maintenance of CFU-E numbers in these cultures. In all experiments, media containing erythroid-specific growth factors was replaced with EDM after one day in culture. Therefore, it seemed possible that some mature burst-forming units-erythroid may from CFU-Es during the first day of culture, and that these CFU-Es might then arrest when Epo was withdrawn. Consistent with this hypothesis, it was found that CFU-Es remained present in detectable numbers if improved cultures were harvested after the first day and then seeded into methylcellulose-based colony assays (Fig. 2-6). Culture under Epo-only conditions during the first day yielded a much lower frequency of CFU-Es at late culture times. These data suggest that
the erythropoietic potential of improved cultures has not been fully exploited using the culture methodology described here, and that prolonged exposure to Epo, beyond the first day, or reintroduction of Epo at later culture times might provide an additional burst of erythropoietic growth. If so, a modified version of this culture protocol could be used to examine the MN effect of genotoxic exposure to earlier stages of erythropoietic growth, or to examine the effects of chronic exposure during long-term culture.

Figure 2-6. Erythropoietic populations cultured under improved conditions contain CFU-Es well beyond the point at which Epo is withdrawn (24h). Cells were introduced into erythropoietic culture and were then harvested at various times after seeding. These harvested populations were then used to seed semi-solid, methylcellulose-based colony plates. CFU-Es were scored 48h after seeding methylcellulose cultures.

2.3 DISCUSSION

Erythroid progenitors contained in both Lin- and Ter-119 BM respond to several physiologic stimuli in this culture system. Epo exerts the largest primary effect on PCE yield, and the next largest effects were observed for the Epo*SCF and Epo*pO₂ synergy effects. These results indicate that PCE yield in this system is largely controlled by these well-established physiologic erythropoietic growth factors. For multi-linear regression, the parameters were scaled to range from zero to one, rather than the more-commonly used normalization and centering from negative one to one; however, the MATLAB® script provided in Appendix I conducts the analysis using
either scaling. These alternate normalizations were found to provide comparable fits of the data, but scaling from zero to one provided more biologically-relevant estimates of the parameter effects. For example, when negative one to one scaling was used, the primary effect of SCF on PCE production was a negative value with a magnitude close to that seen for the Epo*SCF synergy effect in Fig. 2-4. Under the negative one to one scaling, the Epo*SCF synergy effect became a large positive value (approximately twice the size of the Epo*SCF effect in Fig. 2-4) to compensate for the negative SCF primary effect and fit the measured data. However, experiments in cell biology have found that Epo is necessary for the completion of terminal erythropoiesis and that SCF enhances this effect. Therefore, the zero to one scaling, which puts the SCF primary effect near zero and the Epo*SCF synergy effect as an intermediately-sized positive factor, is more in keeping with the current understanding of erythroid development. That is, it is not that SCF alone has a negative effect on erythropoietic growth, but rather that Epo is necessary for this effect to be realized; the zero to one scaling used to generate Fig. 2-4 reflects this understanding.

Through quantitative measurements of reticulocyte yields the throughput of the in vitro assay was estimated to be >1000 conditions per animal. The frequency of CFU-Es in Lin^− BM was measured to be approximately 60 CFU-Es in 5000 Lin^− BM cells. If each of these 60 CFU-Es were to undergo 5 divisions, 60x2^5 = 1920 reticulocytes would be formed. On a per Lin^− BM cell basis, this is ~0.4 PCEs per Lin^− BM cell. However, it was found in this culture system that approximately 5.8 PCEs could be obtained per Lin^− BM cell (Fig. 2-3A). These data, along with the continued presence of CFU-Es in improved erythropoietic cultures, suggest that either some mature burst-forming units-erythroid form CFU-Es that then go on to form PCEs. Alternatively, some limited degree of CFU-E “self-renewal” in this culture system could explain this large PCE yield. Furthermore, differences in the erythropoietic profile (as assessed by flow cytometry) were observed between Epo-only cultures and those that were simulated with SCF, Dex, and IGF-1, along with Epo (Chapter Three, Fig. 3-2). These differences are consistent with the proliferation of mature burst-forming units-erythroid to yield CFU-Es, some of which arrest near the CFU-E stage after Epo is withdrawn at 24h.

The in vitro erythroid MN assay (Chapter Three) was conducted using a variation of the “High-Yield Normoxic Condition” indicated in Figure 2-3A. This condition, referred to throughout this thesis as “improved,” differs from the “High-Yield Normoxic Condition” in that SCF was used at 10ng/mL (rather than 100ng/mL), and cells were harvested at 72h (rather than 96h). These
changes, and the selection of normoxic culture over hypoxic culture, were necessary to decrease cost and technical difficulty. Erythropoiesis continued normally using this improved culture method (Fig. 3-2), and this condition afforded reasonably high PCE yields while reducing the cost, time, and complexity of culture compared to conditions associated with the highest observed PCE yield.

2.4 METHODS

2.4.1 Cells

BM cells were isolated from the hind legs of C57BL/6J mice aged six-eight weeks (Jackson Laboratory, Bar Harbor, ME) and were mechanically dissociated by pipetting in Iscove’s modified Dulbecco’s medium (IMDM) containing 4% FBS. Single-cell suspensions were prepared by passing the dissociated cells through 70μm cell strainers. BM cells with diameter larger than six μm were counted using a Coulter particle counter Z1 (Beckman Coulter, Fullerton, CA). The animals used in this study were treated and housed in accordance with approved guidelines and supervised by MIT’s animal care committee.

2.4.2 Erythropoietic Culture

Total BM cells were labeled with biotin-conjugated α-lineage-marker (α-Lin) antibodies, consisting of α-CD3ε, α-CD11b, α-CD45R/B220, α-Ly6G/Ly6C, and α-TER-119 antibodies (2μL each Ab: 10⁶ cells) (BD Pharmingen, San Diego, CA), and Lin- cells were purified through a 0.3-in StemSep negative selection column as per the manufacturer’s instructions (StemCell Technologies, Vancouver, BC, Canada). Purified cells were seeded in either Fibronectin-coated (2μg/cm²) or uncoated tissue-culture treated polystyrene wells (BD Discovery Labware, Bedford, MA), as indicated in the text, at a cell density of 10⁵/mL. On the first day, the purified cells were cultured in IMDM containing basal supplements consisting of 15% FBS, 1% detoxified bovine serum albumin (BSA), 200μg/mL holo-transferrin (Sigma, St Louis, MO), 10μg/mL recombinant human insulin (Sigma), 2mM L-glutamine, 10⁻⁴ M β-mercaptoethanol, as well as various combinations of soluble erythropoietic growth factors according to the experiment. These erythropoietic growth factors included Epo (Amgen, Thousand Oaks, CA), SCF (R&D systems, Minneapolis, MN), IGF-I (R&D systems), and Dex (Sigma); these factors were added in various combinations in order to quantify their relative erythropoietic growth effects (see text, Fig. 2-2, Fig. 2-3, and Tbl. 2-1). “Original” erythropoietic culture conditions ([Epo] at 2U/mL without additional soluble growth factors, see Fig. 2-3) were used for cultures presented in Fig. 2-1. Improved culture conditions (Epo at 10U/mL, SCF at 10ng/mL, Dex at 10μM, and IGF-I at
100ng/mL) were used for all genotoxicity studies (Chapter Three). In all experiments, the media were replaced with erythroid-differentiation medium (EDM) (IMDM containing 20% FBS, 2 mM L-glutamine, and 10⁻⁴ M β-mercaptoethanol) after one day of culture. At harvest, suspended cells were removed from culture wells by pipetting and then the culture well was incubating in phosphate-buffered saline (PBS)/10% FBS/5mM EDTA (ethylenediaminetetraacetic acid) at 37°C for 5 minutes to dissociate adherent cells. Dissociated cells were then removed by pipetting and combined with the suspended cell fraction from the same culture well for analysis of the total population.

2.4.3 Immunostaining and Flow Cytometry to Analyze Erythroid Differentiation
BM-derived cells were immunostained at 4°C in PBS/4% FBS. Cells were incubated with phycoerythrin (PE)-conjugated α-Ter119 (1:200) (BD Pharmingen) and fluorescein isothiocyanate (FITC)-conjugated α-CD71 (1:200) (BD Pharmingen) antibodies for 15 minutes and were then washed in PBS/4% FBS. Flow cytometry was carried out on a Becton Dickinson FACS Calibur (Franklin Lakes, NJ). Flow cytometry plots and region statistics were acquired using CellQuest Pro™ (BD Biosciences, San Jose, CA).

2.4.4 Cytospin Preparation and Cytological Staining
Approximately 2x10⁴ cells per culture were centrifuged onto slides for 3 minutes at 800 rpm (Cytospin 3; Thermo Shandon, Pittsburgh, PA) and air dried. For benzidine-Giemsa staining, cells were fixed in -20°C methanol for 2 minutes and stained with 3,3’-diaminobenzidine and Giemsa stains according to the manufacturer’s recommendations (Sigma). For acridine orange staining, cells were fixed in room-temperature methanol for 10 minutes and stained in acridine orange (Fisher Scientific, Hanover Park, IL) at a concentration of 20 μg/mL in staining buffer (19 mM NaH₂PO₄ and 81 mM Na₂HPO₄) for 10 minutes at 4°C. After acridine orange staining, slides were protected from light, washed for 10 minutes in 4°C staining buffer, air dried, and stored at 4°C until microscopic examination and scoring was complete.

2.4.5 Viable Cell Counting, Histological Imaging and Quantification
Viable cell counts, based on Trypan blue exclusion, were conducted using a ViCellXR viable cell counter (Beckman Coulter, Miami, FL) according to the manufacturer’s instructions. The instrument was set to include all cells with diameter between 6μM and 50μM in analyses. Slides were examined blind using a Labophot microscope (Nikon, Garden City, NY) and representative micrographs were acquired using a Sony DSC-P93A Cyber-Shot digital camera. Micrographs of
benzidine-Giemsa stained cells were acquired using a 100X oil-immersion objective and brightfield illumination, while micrographs of acridine orange stained cells were acquired using a 40X oil-immersion objective and fluorescence (100W Hg lamp excitation). Cytological slides were examined blind and differential cell counting was used to enumerate relevant cell types and thus quantify the frequency of PCEs among total cells (> 2000 cells scored per slide), the frequency of MN-PCEs among PCEs (> 2000 cells scored per slide), and the frequency of PCEs among RBCs (>1000 cells scored per slide).

2.4.6 Methylcellulose Colony Assays

All methylcellulose colony assays were conducted in MethoCult 3334 as per manufacturer’s instructions (StemCell Technologies). Briefly, Lin’ BM cells or cultured populations were harvested as described above and were washed to remove soluble growth factors and EDTA. The cells were suspended such that the targeted number of cells or fraction of the culture was contained in 400μL of EDM, and then this suspension was delivered into a 4mL aliquot of MethoCult M3334. This methylcellulose medium was then vortexed. Three 35mm petri dishes were each then seeded with approximately 1.1mL of this semi-solid suspension through a 16-gauge needle. CFU-Es were counted after two days incubation at 37°C (5% CO2).

2.4.7 Statistics, Design of Experiments, and Multi-Linear Regression

To determine the statistical significance of mean comparisons, distributions were first checked for normality using the Shapiro-Wilk test (using JMP5 [SAS Institute, Inc., Cary, NC]). Normal data sets were then subjected to independent sample Welch’s t-tests to determine P values using the data analysis tool in Microsoft® Excel. The design of experiments feature in JMP 5 was used to generate the resolution IV, minimum-aberration, 2-level, 6-factor ($2^{6-2}$) fractional-factorial design used to estimate primary and secondary parameter effects. This $2^{6-2}$ fractional-factorial design was conducted in three different atmospheres using a single well-mixed Lin’ BM population to estimate the primary and secondary effects of eight factors (six orthogonal parameters + full-factorial analysis off pO2 and pCO2) simultaneously and thus minimize the inherent variability of primary cell isolations. A model to predict the measured erythropoietic growth was generated using multi-linear regression (see corresponding section in Chapter Two text), and MATLAB® 6.5 was used for to perform all matrix algebra, statistical calculations, and to generate Figure 2-4 (see Appendix I online).
Chapter Three

Genotoxic responses during erythropoiesis: detection of model genotoxic agents and study of DNA repair’s effect on erythroid micronucleus formation
3.1 INTRODUCTION

Normal erythropoiesis in the adult human generates $\sim 10^{11}$ new red blood cells (RBCs) in the bone marrow (BM) each day through the proliferation and differentiation of erythroid progenitors that descend from hematopoietic stem cells (98). Erythropoietin (Epo) is the principal hormone that stimulates erythropoiesis; and, in Epo’s presence, a single erythroid progenitor (known as a colony-forming unit erythroid or CFU-E) divides $\sim$ five times over two to three days to produce $\sim 30$ progeny that enucleate and form nascent erythrocytes. These newly-formed erythrocytes are also known as reticulocytes or polychromatic erythrocytes (PCEs) (36).

If a cell suffers DNA damage while proliferating and differentiating, the biological outcomes are cell death, cell survival with mutations, or cell survival without mutations, depending upon whether and how the cell repairs the damage. Some genotoxic agents, such as ionizing radiation, are direct-acting clastogens that create double-strand DNA breaks by direct scission. Other DNA damaging agents generate DNA strand breaks as replication and repair intermediates, and these breaks can induce homologous or non-homologous DNA recombination events; in addition, damaged centromeres or spindle components can trigger aneugenic events. Whether genetic fragmentation occurs due to clastogenic or aneugenic mechanisms, the result after erythropoietic growth is the same: cells that suffer sufficient DNA damage undergo enucleation but form PCEs that contain “micronuclei”- small fragments of nuclear membrane-encapsulated DNA (20). The spleen clears damaged erythrocytes, so normally less than 1% of circulating erythrocytes contain spontaneous micronuclei arising from background levels of DNA damage.

The hematopoietic system is highly sensitive to genotoxic agents in part because hematopoietic cells undergo rapid division. The BM is often the dose-limiting tissue in chemotherapy and radiotherapy regimens, and therapy-related anemia, cytopenia, and leukemia are common side effects. These serious morbidities have prompted efforts to increase hematopoietic DNA repair capacity via gene therapy in patients receiving chemotherapeutic agents and to improve pre-clinical screening of new drugs for genotoxic effects (99-104). Although leukemias and cytopenias arise in white cell populations, erythrocyte populations serve as a useful system to measure DNA damage because normal and micronucleated PCEs are easily recognized and scored by microscopy, flow cytometry, and laser scanning cytometry (21, 24-26). The assay system described here tests agents using terminally differentiating erythrocyte populations to indicate general hematopoietic genotoxicity.
Efforts to predict the effect of drugs on human hematopoiesis, or to develop therapeutic approaches to mitigate these effects, are hindered by a lack of readily-accessible model systems that duplicate the highly orchestrated set of cellular sensing, signaling, and repair responses that DNA damage triggers in exposed hematopoietic cells. Some repair pathways require sequential activity by multiple enzymes in a series of reactions that transiently decrease the stability of the double helix before the completion of repair. Reducing the level of one repair enzyme within the pathway can thus, paradoxically, increase resistance to genotoxic agents (27). The situation is further complicated by the diversity of expression levels of various DNA repair-related genes during different stages of hematopoietic differentiation (30, 31). Not surprisingly, then, model organisms and cell lines offer only modest predictive power for hematopoietic genotoxicity, and human genotoxicity in general (8-11, 13-15).

Currently, one of the most robust tests for genotoxicity is the in vivo micronucleus (MN) assay, which is conducted using an improved version of the method first described by Schmid and colleagues (5, 6). Close to 70% of known human carcinogens are detected by the in vivo MN test; accordingly, regulatory authorities around the world require a preclinical in vivo cytogenetic test, such as the MN assay (4, 20). Twenty-four to 48 hours after injection of a test substance into a rodent, the BM is harvested and stained to distinguish PCEs from older normochromatic erythrocytes (NCEs) and to reveal the presence of micronuclei; a total of 2000 PCEs from each animal are sampled to score MN frequency. Despite recent advances in the automated detection and enumeration of micronucleated PCEs that eliminate microscopy and increase assay throughput, the assay is still applied late in preclinical drug development due to the large numbers of animals required to encompass multiple doses and replicates for each agent tested (21, 24-26).

M. Socolovsky, J. Zhang and colleagues recently developed a flow cytometry protocol for analyzing erythropoietic differentiation in vivo in a quantitative and step-by-step fashion (34, 35). This analytical procedure was used to establish in vitro conditions that stimulate Ter-119 fetal liver erythroid progenitor cells to undergo terminal proliferation, erythroid differentiation, and enucleation in a physiologic manner. This system has also been used to analyze the role of several signal transduction pathways activated by the Epo receptor (35, 105). Fetal erythropoiesis in the liver is very similar to adult erythropoiesis in the BM, though the CFU-E frequency in BM is much lower than that in fetal liver. Although adult BM-derived cells can form erythroid colonies in vitro, the fidelity of the in vitro process compared to physiological erythropoiesis in adult BM had not been examined.
The overarching goal of the work in this chapter was to determine whether erythropoietic cultures, derived from adult BM, can capture essential features of the in vivo MN assay, thus enabling step-by-step examination of the effects of genotoxic agents at different developmental stages. Furthermore, an in vitro correlate to the in vivo assay can provide a platform for high-throughput screening of drugs and toxic environmental agents using the BM of a single animal, whereas the in vivo assay tests only a single condition per animal. To this end, this work demonstrates that the lineage-marker-negative (Lin⁻) population of adult mouse BM undergoes erythropoietic differentiation according to established in vivo patterns, and that erythrocytes generated in vitro undergo MN formation when exposed to direct-acting genotoxic agents. Specifically, these data demonstrate that adult BM cultures respond to Epo with physiological erythropoietic proliferation, differentiation, and enucleation, and they demonstrate that this in vitro erythropoietic system clearly signals exposure to genotoxicants through erythroid MN formation. Further, this work shows that this in vitro system reflects a phenotypic sensitivity observed in DNA repair-deficient mice in vivo. Specifically, this work established that DNA repair deficient (MGMT⁻/⁻) BM displayed sensitivity to genotoxic exposure in vivo compared with wild-type (WT) BM, and that this phenotypic response was reflected in erythropoietic cultures. These findings suggest that this in vitro erythroid MN assay is capable of screening for genotoxicity on BM in a physiologically reflective manner. Finally, responses to genotoxicants during erythroid differentiation varied with exposure time, demonstrating that this novel system can be used to study the effect of DNA damage at specific developmental stages. Although this system removes the erythroid MN assay from the whole animal, and thus isolates promutagens from liver metabolism, it is well-established that incubation with liver microsomes can restore some measure of metabolic activation to in vitro genotoxicity screens (62). Thanks to Sophia Kamran and Catherine Moroski for technical assistance, their efforts contributed greatly to the work in this chapter.

3.2 RESULTS

3.2.1 Stimulation of Terminal Erythropoiesis in Lin⁻ BM Cells

Flow cytometric analysis of erythroid differentiation is achieved by double-staining for the transferrin receptor (CD71), which is dramatically upregulated during the first two divisions of CFU-Es, and Ter-119, which is stably incorporated into the membrane of cells during late stages of erythroid differentiation (39). Erythropoietic progenitors (CD71⁺/Ter-119⁻) undergo several divisions to produce progeny that first express CD71, and then Ter-119, before CD71 is lost from
the cell surface during reticulocyte maturation. It was previously shown that this sequence of Epo-induced events can occur in vitro using Ter-119<sup>+</sup> fetal liver cells (35). Here the data show that Lin<sup>-</sup> BM cells from adult mice also undergo Epo-induced differentiation according to this pattern. Thanks to Glenn Paradis for expert technical assistance in all flow cytometry studies.

Figure 3-1. Terminal erythropoiesis stimulated in Lin<sup>-</sup> BM cells over three days in culture. BM cells were stained with biotinylated α-Lin mAbs, and the Lin<sup>-</sup> fraction of the population was subsequently removed to obtain a progenitor-rich population (Lin<sup>-</sup> BM). These Lin<sup>-</sup> cells were then cultured in vitro for three days on Fibronectin-coated plates in medium containing serum. Epo was included in the medium for the first day of culture, and then the medium was changed and Epo was removed. The differentiation profile of the cultured cells was examined by both flow cytometry and benzidine-Giemsa stain after each day in culture, and a representative micrograph of these stained populations is presented from each day of erythropoietic culture. After the third day of culture, flow cytometry indicated that the majority of the resulting population had acquired a late erythroid surface phenotype (Ter-119<sup>-</sup>). Furthermore, benzidine-Giemsa staining revealed that many cells in the harvested population had enucleated and expressed hemoglobin. The arrowhead indicates a hemoglobin<sup>+</sup> normoblast, and the arrow indicates an enucleated reticulocyte. Scale bars: 20μm.

The Lin<sup>-</sup> fraction of mouse BM was isolated by immunomagnetic negative selection, plated on Fibronectin-coated culture dishes, and stimulated with Epo for the first 24h of culture. Lin<sup>-</sup> BM cells represent ~1.25% of cells in the marrow and comprise a mixture of hematopoietic and mesenchymal stem and progenitor cells. Analysis by flow cytometry indicated that a significant fraction of Lin<sup>-</sup> cells had begun erythroid differentiation (i.e. upregulated CD71) after one day of culture with Epo (Fig. 3-1). By day two, the majority of cells express CD71 and Ter-119, and, by day three, the characteristic late-erythroid loss of CD71, reported for both in vivo fetal liver and
cultured Ter-119' fetal liver, had begun (34, 35). Cytology confirmed that a substantial fraction of the Lin' BM population (nucleated cells lacking hemoglobin) had differentiated into enucleated, hemoglobinized reticulocytes over the course of two to three days (Fig. 3-1). A fraction of Lin' BM cells were unresponsive to Epo (Fig. 3-1), and it is likely that most of these cells are not erythropoietic and do not survive under these culture conditions.

Figure 3-2. Dynamic analysis of Lin' bone marrow cultured for three days under improved erythropoietic conditions: flow cytometry and benzidine-Giemsa staining. Purified Lin' cells were cultured in vitro for three days on Fibronectin-coated plates in medium containing serum. Epo, SCF, Dex, and IGF-I were included in the medium for the first day of culture, and then the medium was changed to remove soluble growth factors. The differentiation profile of the cultured cells was examined by both flow cytometry and benzidine-Giemsa stain after each day in culture. Two representative micrographs of these stained populations are presented from each day of erythropoietic culture. After the third day, flow cytometry indicated that much of the cultured population expressed Ter-119 and CD71. Furthermore, benzidine-Giemsa stain revealed that many cells in the harvested population were enucleated and expressing hemoglobin. The arrowhead indicates a hemoglobin normoblast, and the arrow indicates an enucleated reticulocyte. Scale bars: 20µm.

Quantitative studies on the response of Lin' BM cultures after stimulus with Epo and other erythropoietic factors were conducted to define the PCE yield per Lin' BM cell and to investigate whether adjustments to the culture protocol could provide increased yield while maintaining physiologic terminal erythropoiesis (Chapter Two). A quantitative, cytology-based study revealed that a ~eight-fold increase in PCE yield could be achieved (up to 5.8 +/- 0.9 PCEs per
Lin' cell, please see Fig. 2-3). Accordingly, all genotoxicity studies were conducted using this improved erythropoietic culture protocol, which includes Epo, SCF, Dexamethasone, and IGF-I in the culture medium for the first 24h of culture. Erythropoiesis continued normally using the improved culture method, although expression of Ter-119 was slightly delayed (compare Fig. 3-1 with Fig. 3-2), and resulting PCE yields were sufficient to conduct hundreds of genotoxicity assays on the BM of a single mouse (Fig. 3-3).

Figure 3-3. Growth and cell cycle dynamics during improved erythropoietic culture. Erythropoietic cultures were initiated with WT Lin' BM and then harvested at various times to assess growth and cell cycle status. (A) Viable cell numbers were measured using a ViCellXR viable cell counter, and were then normalized to input cell numbers. The log2 of this ratio was then taken to estimate the total number of doublings that had occurred at a given culture time. The data are presented as the mean of four biologically-independent samples, obtained over the course of two experiments, +/- the standard deviation. While growth on days one and three resulted in a single doubling of cell numbers, growth on day two resulted in more than two doublings. At harvest, approximately 30% of viable cells are PCEs (Fig. 2-3) and viable cell numbers have increased by a factor of ~2^4.2. Given that approximately 5x10^5 Lin' BM cells are obtained per mouse, and that 2000 PCEs are sufficient to detect genotoxicity by in vitro erythroid MN formation, it is estimated that over 1000 in vitro erythroid MN assays can be conducted using the hind-leg BM of a single mouse. (B) Biologically-independent samples were harvested at various points in erythropoietic culture (please see the table for n). The harvested populations were washed, fixed in cold ethanol for over 1h, washed again, and then stained for cell cycle analysis using propidium iodide (50μg/mL) in the presence of RNaseA (500μg/mL). DNA content was measured for each cell using flow cytometry, and then the ModFit LT diploid model was used to fit the resulting flow cytometry histograms. These results from cell cycle analysis are tabulated here for various times in erythropoietic culture. Consistent with the rapid increase in viable cell numbers during the second day of cell culture (see part A of this figure), most cells were found to be in S phase during this period.

3.3.2 Detection of Genotoxic Exposure: in vitro Erythroid MN Assay

Three mutagenic alkylating agents that test positive in the in vivo MN assay were used as model genotoxicants: BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea), MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), and MMS (methylmethane sulfonate). Cultures treated with genotoxicant for 1h starting 23h after seeding progressed normally through erythropoiesis (Fig. 3-4). Micronuclei
appeared in both treated cultures and controls (Fig. 3-5A), but with greater frequency in treated cultures (Fig. 3-5A,B,C,D), as expected based on the known ability of these agents to increase MN frequency above basal levels in vivo. Both the control and treated MN frequencies observed in vitro were higher than the levels typically observed in vivo, which might be related to the relatively high rate of erythropoietic growth. Indeed, increasing erythropoietic rates in vivo through bleeding or exogenous Epo expression increases erythroid MN frequencies, and can sensitize the in vivo assay to genotoxic exposure (106, 107). Cell viability at harvest was >90% for all cultures, but viable cell yields were lower for treated vs. control controls (Fig 3-5B,C,D). Cell proliferation and death were not explicitly measured, thus reductions in viable cell yields cannot be specifically attributed to cytotoxic or cytostatic mechanisms. However, these data demonstrate that genotoxic exposure can be detected using this erythropoietic culture system.

Thanks to Drs. Dharini Shah and Lisi Meira for expert guidance in genotoxicity studies.

Figure 3-4. in vitro erythropoiesis continues after treatment with alkylating agents: flow cytometry and benzidine-Giemsa cytology. The cultured populations analyzed for Fig. 3-5 were also examined by flow cytometry and benzidine-Giemsa staining. Two representative micrographs of populations treated with each model genotoxicant are presented. Cytology revealed that large fractions of the treated populations were hemoglobin⁺ and fully enucleated. In addition, large fractions of the treated populations were found by flow cytometry to express the characteristic late-erythroid surface markers Ter-119 and CD71. The arrowheads indicate hemoglobin⁺ cells containing micronuclei. Scale bars: 20 μm.
Figure 3-5. Detection of genotoxicity through *in vitro* erythropoiesis. Purified Lin- cells were cultured *in vitro* for one day on Fibronectin-coated plates in medium containing serum, Epo, SCF, Dex, and IGF-I. Genotoxic alkylating agents were introduced into the culture media 23h after seeding. One hour later, the media were changed and all soluble growth factors and alkylating agents were removed. Populations were then cultured for two additional days in media with serum. At harvest, the cells were removed from culture, and genotoxic effects were quantified through viable cell counts and differential cell counts. (A) Representative micrographs are provided from both treated and untreated cultures. The arrow indicates a normally enucleated PCE and the arrowheads indicate micronucleated PCEs. Scale bars: 20 μm. (B) The response of this culture system to BCNU treatment is quantified in terms of relative viable cell numbers and MN frequencies. On the left, relative viable cell numbers are plotted vs. BCNU concentration. On the right, MN frequency is plotted vs. BCNU concentration. (C) The response of this culture system to MNNG treatment is quantified in terms of relative viable cell numbers and MN frequencies. (D) The response of this culture system to MMS treatment is quantified in terms of relative viable cell numbers and MN frequencies. Data are presented as the mean of three independent cultures +/- the standard deviation. * indicates a significant difference (P<0.05) from untreated control cultures. ** indicates a significant difference (P<0.0001) from the untreated control cultures.

### 3.2.3 MGMT-/- Mice Display Sensitivity to BCNU Exposure by the in vivo MN Assay

BCNU is an Sn1 bifunctional alkylating agent that can form adducts at nucleophilic sites on DNA, including the O^6^ position of Guanine (52, 65, 108). After this initial addition reaction, the alkyl group on O^6^ of Guanine can react with the opposite Cytosine to form an interstrand crosslink; importantly, the O^6^ alkyl group can be removed by a DNA repair protein known as O^6^-methylGuanine DNA methyltransferase (MGMT) (66, 109). The BM of MGMT-/- mice (on a C57BL/6J background) was previously shown to display sensitivity to BCNU-induced cytotoxicity (110).
Here, MGMT\textsuperscript{−/−} mice were treated with BCNU doses by intraperitoneal injection and examined by the \textit{in vivo} MN test. Counter to our expectations, the MN frequency in the MGMT\textsuperscript{−/−} BM was lower than that observed in WT C57BL/6J BM 24h after treatment at some BCNU doses (Fig. 3-6A). It seemed likely that the genotoxic response in the MGMT\textsuperscript{−/−} marrow was delayed, possibly because proliferation slows in the presence of increased genomic instability. Therefore, the marrow of MGMT\textsuperscript{−/−} and WT mice was examined 48h after exposure and, as initially expected, the MN frequency in the MGMT\textsuperscript{−/−} marrow was drastically higher than that observed in WT marrow (Fig. 3-6B). Moreover, a decrease in the ratio of PCEs to total RBCs was also observed in MGMT\textsuperscript{−/−} marrow 48h after exposure (Fig. 3-6D,E). The increased sensitivity of MGMT\textsuperscript{−/−} BM to BCNU-induced MN formation and inhibition of PCE growth remained evident 72h after exposure (Fig. 3-6C,D,E). Taken together, these data indicate that MGMT\textsuperscript{−/−} marrow is more sensitive to BCNU than WT marrow, which had largely recovered to resemble untreated marrow.
48h after treatment, both in terms of MN-formation and as measured by erythroid cytotoxic/cytostatic effects (Fig. 3-6).

Near the completion of these studies, it was discovered that a mouse lacking the gene for 3-Methyladenine DNA glycosylase (Aag<sup>+/−</sup>) had been inadvertently crossed with an MGMT<sup>−/−</sup> mouse. As a result, the MGMT<sup>−/−</sup> mice used in these experiments varied in their Aag genotype. To determine whether this variable Aag expression affected the conclusions drawn from the data in Fig. 3-6, genotyping was performed on preserved tissue from each animal, and the data were grouped to reflect the Aag genotype of the animals (Fig. 3-7).

The data in Fig. 3-7, taken together with a previously published report that showed no difference in MN formation between Aag<sup>+/−</sup> and Aag<sup>+/+</sup> mice 24h after a 7.0mg/kg dose of BCNU (27), suggest that Aag does not play a role in erythroid MN formation in response to BCNU. These findings confirm the conclusion drawn from Fig 3-6, that MGMT plays a role in protecting erythropoietic cells from genetic fragmentation following exposure to BCNU. Further, these data suggest that defects in base-excision repair of 3-Methyladenine neither protect nor sensitize erythroid progenitors to MN formation after treatment with BCNU.
3.2.4 *in vitro* Erythroid MN Assay Reflects the *in vivo* Phenotype of MGMT⁻/⁻ Mice

MGMT⁺ Lin⁻ BM was used in our erythropoietic culture system to test whether an *in vitro* genotoxicity screen could reflect the DNA repair capacity of primary BM. In the absence of genotoxic exposure, erythropoietic differentiation in MGMT⁺ cultures was indistinguishable from that of WT cultures (Fig. 3-2 vs. Fig. 3-8). Following BCNU addition, erythropoiesis in MGMT⁻ cultures continued normally before leading to significant increases in MN frequency as compared with WT cultures (Fig. 3-9A). Furthermore, reduced PCE yields were observed in the MGMT⁻ cultures, meaning that this assay can detect the increased sensitivity of MGMT⁻ erythroid progenitors to BCNU both by cytotoxic/cytostatic signals and by increased production of micronucleated progeny (Fig. 3-9B).

**Figure 3-8.** Dynamic analysis of MGMT⁻/⁻ Lin⁻ bone marrow cultured for three days under improved erythropoietic conditions: flow cytometry and benzidine-Giemsa staining. Purified Lin⁻ cells from MGMT⁻/⁻ mice were cultured *in vitro* for three days on Fibronectin-coated plates in medium containing serum. Epo, SCF, Dex, and IGF-I were included in the medium for the first day of culture, and then the medium was changed to remove soluble growth factors. The differentiation profile of the cultured cells was examined by both flow cytometry and benzidine-Giemsa stain after each day in culture. Two representative micrographs of these stained populations are presented from each day of erythropoietic culture. After the third day, flow cytometry indicated that much of the cultured population had acquired a late erythroid surface phenotype during culture. Furthermore, benzidine-Giemsa stain revealed that many cells in the harvested population were enucleated and expressing hemoglobin. The arrowhead indicates a hemoglobin+ normoblast, and the arrow indicates an enucleated reticulocyte. Scale bars: 20µm.
Figure 3-9. Similar to in vivo responses, Lin’ BM from MGMT⁻/⁻ mice exhibits sensitivity to MN-formation and decreased PCE yields when treated with BCNU during erythropoietic culture. (A) Purified Lin’ cells from MGMT⁻/⁻ and WT mice were cultured in vitro for one day on Fibronectin-coated plates in medium containing serum, Epo, SCF, Dex, and IGF-I. After one day, the medium was changed to one containing serum without other hormones. Populations were then cultured for two additional days before harvest at 72h. BCNU was introduced into the culture media at various times (10h, 23h, or 30h) after seeding. At harvest, the cells were removed from culture, and genotoxic effects were quantified through viable cell counts and MN enumeration. (B) The MN response of Lin’ bone marrow from WT and MGMT⁻/⁻ mice to BCNU in this erythropoietic culture system is quantified. MN frequency is plotted vs. BCNU concentration, and data are presented as the mean of three independent cultures +/- the standard deviation. + indicates a significant difference (P<0.05) between WT and MGMT⁻/⁻ cultures. ++ indicates a significant difference (P<0.01) between WT and MGMT⁻/⁻ cultures. * indicates a significant difference (P<0.05) from the vehicle control. ** indicates a significant difference (P<0.01) from the vehicle control. *** indicates a significant difference (P<0.001) from the vehicle control. (C) The effect of BCNU exposure on the viable cell number of Lin’ bone marrow from WT and MGMT⁻/⁻ mice after erythropoietic culture is quantified. Relative viable cell number is plotted vs. BCNU concentration, and data are presented as the mean of three independent cultures +/- the standard deviation.

In the study shown in Fig. 3-9, BCNU was introduced into erythropoietic cultures at various times after seeding to determine the time at which these differentiating populations are most sensitive to MN formation in response to genotoxic exposure. In MGMT⁻/⁻ BM cultures, BCNU treatment at earlier times exacerbated decreases in viable cell numbers, whereas BCNU treatment
at later times resulted in further increases in micronucleated PCE frequencies; when WT BM cultures were treated 4h after culture initiation, a similar trend was observed (compare Fig. 3-10 to the WT data in Fig. 3-9). These observations lead to the hypothesis that unrepaired genetic damage to Epo-responsive progenitors (--CFU-E stage) during the first day of culture results in a greater degree of cell death/senescence, whereas genetic damage to the erythroid cells present later in culture, if not repaired, is more likely to yield micronucleated PCEs.

Figure 3-10. Early BCNU treatment of WT Lin` BM cultures leads to decreased viable cell numbers and diminished MN formation. Erythropoietic cultures initiated with WT Lin` BM were treated with BCNU 4h after culture was initiated. 72h later, the cultures were harvested and genotoxic effects were quantified both by: (A) cytological scoring of MN frequency and (B) viable cell counts. The methods used to generate these data were identical to those used to generate Fig. 3-9, except that treatment of BCNU was delivered 4h after cultures were initiated. By comparison with Fig. 3-9, these data further support the noted trend that treatment earlier in the erythropoietic culture period leads to a lower frequency of micronucleated PCEs and a lower number of viable cells upon harvest. * Significant difference from untreated control (P<0.05).

BCNU-derived chloroethyl adducts at the O6 position of Guanine, when not repaired by MGMT, can go on either to form interstrand crosslinks or to generate mispairs during the next round of DNA synthesis (66, 111, 112). It is known that interstrand crosslinks can lead to double strand breaks and cytotoxicity, and it is possible that this variety of BCNU-induced damage contributes to the genotoxic response observed during erythropoiesis. Although interstrand crosslink repair is not completely understood, it is known that stalled replication is a signature of crosslink repair; and, more-specifically, it is known that an S-phase checkpoint is activated during such repair (113, 114). If, on the other hand, O6 adduction leads to mispairing, a Thymine residue will be inserted opposite of the adducted Guanine residue during DNA synthesis. During long-patch mismatch repair (which is often replication-associated), the mismatch repair pathway can remove the mispaired Thymine along with up to ~3 kilobases of the daughter DNA sequence. Portions of the DNA are removed in both directions of the mismatch, and the genomic stability of the DNA is
thus compromised. Even in the case of short-patch mismatch repair, approximately ten nucleotides are removed in the daughter strand, and the attempts by mismatch repair to correct the damage are in vain: during the next synthesis across the O\(^6\) lesion, Thymine will again be inserted opposite of the O\(^6\)-adducted Guanine. A futile cycle of mismatch repair and DNA synthesis ensues that ultimately leads to delayed progression through the S-phase of the cell cycle. Given that MGMT protects erythropoietic cells from genetic fragmentation and cytotoxicity following treatment with BCNU, the O\(^6\) alkylGuanine adducts presumably play a role in the erythroid response. Therefore, cell cycling was examined at various times after treatment with BCNU to determine whether progression through S-phase is affected (Fig 3-11). Such an effect was observed, consistent with crosslink repair, mismatch repair, or another form of replication-linked repair playing a role during the erythropoietic response to BCNU.

Counter to expectations, less cells were observed to be in S-phase following treatment with BCNU (Fig. 3-11, 48h analysis times). Although no S-phase delay was detected at the times examined, differences in the cell cycle profile were observed and it is possible that some form of replicative arrest occurs during erythroid MN formation. Furthermore, it should be noted that the
resulting population is heterogeneous, and contains >10% non-erythroid cells at harvest. Therefore, the observed differences might not be erythroid-specific. However, these effects on cell cycle dynamics were measured in a rapidly cycling primary-hematopoietic population, and thus might be a clinically relevant response. It should also be noted that the effect of BCNU on cell cycle was measured in WT Lin- marrow, meaning that endogenous levels of MGMT might be masking the effect of O6 adducts. Although efforts to detect MGMT activity in these cultures, or in Lin- BM, have been unsuccessful, the MGMT activity assay is technically challenging, especially given that primary Lin- BM cells are a rare population (~5x10^5 cells per animal) yielding little protein for enzymatic experiments. Therefore, it is possible that endogenous levels of MGMT in WT erythroid progenitors are sufficient to mask the effects of O6 adducts during replication at these treatment levels. Performing a comparable set of experiments in MGMT-/- Lin- BM may provide further evidence regarding the direct effect of unrepaired O6 Guanine adducts. That is, presumably the effect of these adducts will be exacerbated and more easily detected in MGMT-/- BM.

3.3 DISCUSSION

Many drugs and environmental agents can damage DNA, and understanding and predicting the outcomes of exposure to DNA-damaging agents remains an important challenge in pharmaceutical development and in environmental health sciences. The complex interplay between the numerous cellular pathways that influence damage and repair determines the final biological consequences of exposure. The outcomes observed for MN formation in mice treated with BCNU (Fig. 3-6) underscore this complexity: mice lacking the DNA repair enzyme MGMT appear to be slightly less sensitive than WT mice 24h after exposure, but exhibit a clear sensitivity to genetic alkylation 48h and 72h post-exposure. Chromosome aberrations arising through the processing of the O6 alkyl Guanine lesion require a second round of DNA replication to be expressed, and this might contribute to the late appearance of micronuclei in MGMT-/- BM (115, 116). Thus, this late aberration yield of micronucleated PCEs might not simply result from cell cycle delays, although the data in Fig. 3-11 suggest that erythropoietic cell cycling is affected by BCNU treatment. Furthermore, our dynamic treatment studies (Fig. 3-9) and in vitro growth measurements show that differentiating erythroid populations are extremely sensitive to MN formation while they undergo the final two to three divisions before enucleation (Fig. 3-3 and Fig. 3-9).
Although agents that require metabolic activation (e.g. by cytochrome P450s) to produce a genotoxic effect would escape detection if introduced directly into the assay system that is described here, this same limitation applies to all existing in vitro genotoxicity assays (e.g. the Ames assay in Salmonella and the chromosome aberration assay in CHO cells). It is well established that this limitation can be overcome, to some extent, by incubating test agents with S9 microsomes from hepatocyte lysates prior to introduction into a test culture (60, 62). These S9 microsomes lend some of the normal metabolic functions of the liver to the in vitro screen. Though the work described here did not make use of genotoxic agents requiring metabolic activation, incubation with S9 microsomes provides a sufficient degree of mammalian metabolism to sensitize test systems that are quite far removed from normal mammalian physiology (e.g. Salmonella cultures) to the genotoxic metabolites of promutagens. Therefore, this same approach can surely be used to incorporate some metabolic activity to the assay system described here.

As a first step towards capturing a complex physiological response in vitro, this work establishes that Lin- cells from adult mouse BM undergo normal erythropoiesis in culture, as assessed by flow cytometry and cytology. In this culture system all cells are Epo-stimulated during the first day of culture, and erythrocytes near the CFU-E stage of development then undergo metasynchronous development. The trends that were observed in vivo and in vitro suggest that MGMT functions in erythroid progenitors to mitigate the effects of BCNU, and responses in this in vitro assay system lead to the hypothesis that unrepaired damage leads to different outcomes (decreased reticulocyte yield vs. increased MN frequency) depending on the stage of erythropoiesis. Cell death was not explicitly measured in this work; therefore, decreases in viable cell yield at harvest can not be specifically attributed to decreased survival (cell killing) or to replicative arrest. However, it is known that a cellular response to BCNU is cell cycle arrest, and it seems likely that both death and growth arrest occur to certain extents in this culture system (117, 118). The data in Fig 3-11 support the hypothesis that erythropoietic cell cycling is altered in response to BCNU treatment, but the effect is slight and occurred at only a single analysis time (48h); accordingly, further studies are required to verify this effect.

When SCF, Dexamethasone, and IGF-I were added along with Epo, the fraction of cells at each stage of differentiation (as assessed by flow cytometry) was different than that in Epo-only cultures (Fig. 3-1 vs. Fig. 3-2 and Fig. 3-8), in a manner consistent with proliferation of mature burst-forming units erythroid (BFU-Es) stimulated by SCF and Epo. Since SCF and Epo are
withdrawn 24h after seeding, these cells might then arrest near the CFU-E stage. Therefore, it might be possible to examine the effect of chronic or early genotoxic exposure during erythropoiesis by reintroducing Epo to cultures late in the culture period. Indeed, colony assays conducted on populations harvested from these cultures at day three revealed that these populations contain CFU-Es (Chapter Two). The background level of micronucleated PCE in these in vitro experiments is very high compared with the typical two to five micronucleated PCEs per 1000 PCEs found in vivo in BM. This is likely related to the rapid turnover, as it is known that high rates of erythropoiesis in vivo lead to increased error rates. These additional aberrations are thought to be due to chromosome loss, and this effect is observed in vivo after Epo treatment, for example, or after anemia induced by bleeding or hemolysis (106, 107).

Fig. 3-1 gives a clear picture of late-erythropoiesis as monitored by flow cytometry, whereas the data in Fig. 3-2 and Fig. 3-8 reflect the more heterogeneous population that arises from culture under conditions developed for increased throughput. In mouse hematopoietic populations, a detailed understanding of which surface phenotypes and physical characteristics correspond to various stages of erythroid development exists: a BM subpopulation was recently identified that generates CFU-E colonies at an efficiency of approximately 70% (41). The investigators who identified this population refer to this highly-enriched CFU-E population as the erythroid progenitor (EP) population. This EP population is characterized as having a surface protein phenotype that is Lin⁻, c-Kit⁺, Sca-1⁻, IL7-Rα⁻, IL-3Rα⁻, CD41⁻, and CD71' and it comprises 0.41% of nucleated BM cells. Use of the EP population would provide the opportunity to directly measure the DNA modifications made to erythroid progenitors by BCNU with less background noise arising from the heterogeneity of the Lin⁻ BM population.

As the correlation between the in vivo and in vitro responses of MGMT⁻ marrow illustrates, this in vitro erythropoietic system is capable of detecting the DNA-repair capacity of primary BM donors, and thus, if extended to human cells, may allow individual patient testing, or testing panels of human cells both to predict average human responses and to better understand the basic biology of the human hematopoietic response to genotoxic agents. Further, the levels of key molecules in DNA damage-response pathways can be manipulated more easily in culture than in vivo (eg, via RNAi), allowing the effects of pathway components to be explored. Finally, this genotoxicity screen conducts the erythroid MN assay on adult tissue in vitro to provide increased throughput over the existing in vivo erythroid MN assay, which tests a single agent (at a single dose) per animal.
3.4 MATERIALS AND METHODS

3.4.1 Cells
BM cells were isolated from the hind legs of C57BL/6J mice aged six-eight weeks (Jackson Laboratory, Bar Harbor, ME) and were mechanically dissociated by pipetting in Iscove’s modified Dulbecco’s medium (IMDM)/4% FBS. Single-cell suspensions were prepared by passing dissociated cells through 70μm cell strainers. BM cells with diameter larger than 6μm were counted using a Coulter particle counter Z1 (Beckman Coulter, Fullerton, CA). The animals used in these studies were treated and housed in accordance with approved guidelines and supervised by MIT’s animal care committee.

3.4.2 Erythropoietic Culture
Total BM cells were labeled with biotin-conjugated α-Lin Abs, consisting of α-CD3e, α-CD11b, α-CD45R/B220, α-Ly6G/Ly6C, and α-TER-119 Abs (2μL each Ab: 10^6 cells; BD Pharmingen, San Diego, CA), and Lin^- cells were purified through a 0.3-in StemSep negative selection column as per the manufacturer’s instructions (StemCell Technologies, Vancouver, BC, Canada). Purified cells were seeded in Fibronectin-coated (2μg/cm^2) tissue culture treated polystyrene wells (BD Discovery Labware, Bedford, MA) at a cell density of 10^5/mL. On the first day, the purified cells were cultured in IMDM containing basal supplements consisting of: 15% FBS, 1% detoxified bovine serum albumin, 200μg/mL holo-transferrin (Sigma, St Louis, MO), 10μg/mL recombinant human insulin (Sigma), 2mM L-glutamine, 10^-4 M β-mercaptoethanol, 50U/mL penicillin G, and 50μg/mL streptomycin; as well as soluble erythropoietic factors. The previously described erythropoietic medium, including Epo (Amgen, Thousand Oaks, CA) at 2U/mL along with the basal supplements, was used in the cultures presented in Fig. 3-1 (35). An improved erythropoietic medium formulation, including Epo at 10U/mL, SCF (R&D systems, Minneapolis, MN) at 10ng/mL, Dexamethasone (Sigma) at 10μM, and IGF-I (R&D systems) at 100ng/mL, along with basal supplements, was used in all other cultures. For all cultures, the media was replaced with erythroid-differentiation medium (EDM) (IMDM with 20% FBS, 2 mM L-glutamine, and 10^-4 M β-mercaptoethanol) after one day of culture. At harvest, suspended cells were removed from culture wells by pipetting and then the culture well was incubated in PBS/10% FBS/5mM EDTA at 37°C for 5min to dissociate adherent cells. Dissociated cells were then removed from a culture well by pipetting and combined with the suspended cell fraction from the same culture well for analysis.
3.4.3 Immunostaining and Flow Cytometry

BM-derived cells were immunostained at 4°C in PBS/4% FBS. Cells were incubated with phycoerythrin-conjugated α-Ter119 (1:200) (BD Pharmingen) and fluorescein isothiocyanante-conjugated α-CD71 (1:200) (BD Pharmingen) Abs for 15 min and were then washed in PBS/4% FBS. Flow cytometry was carried out on a Becton Dickinson FACSCalibur (Franklin Lakes, NJ). Flow cytometry plots and region statistics were acquired using CellQuest Pro™ (BD Biosciences, San Jose, CA).

3.4.4 Cytospin Preparation and Cytological Staining

Approximately 2x10⁴ cells per culture were centrifuged onto slides for 3 min at 800 rpm (Cytospin 3; Thermo Shandon, Pittsburgh, PA) and air dried. For benzidine-Giemsa staining, cells were fixed in -20°C methanol for 2 min and stained with 3,3’-diaminobenzidine and Giemsa stains according to the manufacturer’s recommendations (Sigma). For acridine orange staining, cells were fixed in 25°C methanol for 10 min and stained in acridine orange (Fisher Scientific, Hanover Park, IL) at a concentration of 20μg/mL in staining buffer (19mM NaH₂PO₄ and 81mM Na₂HPO₄) for 10 min at 4°C. After acridine orange staining, slides were protected from light, washed for 10 min in 4°C staining buffer, air dried, and stored at 4°C until microscopic examination and scoring was complete.

3.4.5 in vivo Genotoxic Treatment and Cytology

BCNU (Sigma) was first dissolved in cold absolute ethanol (EtOH), and mice were treated with BCNU by intraperitoneal injection using a vehicle of PBS/10% EtOH. At the appropriate time following dosage (24h, 48h, or 72h), the mice were sacrificed using CO₂, and the BM was removed from the femurs and tibiae of the hind legs. A single cell suspension was generated by mechanical dissociation and this suspension was then passed through a cellulose column to enrich erythrocytes. This enriched erythroid population was then spread on a slide, fixed in methanol, and stained with acridine orange for cytological examination. The animals used in this study were treated and housed in accordance with approved guidelines and supervised by MIT’s animal care committee.

3.4.6 Genotoxic Treatment of Erythropoietic Cultures

Lin− BM was cultured in 500μL of medium per culture well according to the method described for erythropoietic culture. Alkylating agents were added to the culture medium at various times after seeding and, if added during the first day of culture, were removed when the medium was
exchanged for EDM. When treating 30h after seeding, BCNU was not removed from the cultures. BCNU was first dissolved in 4°C EtOH to make a 10mM solution. This 10mM solution was diluted in 4°C IMDM to produce an IMDM/0.4mM BCNU/4% EtOH solution. The required volume of this 0.4mM BCNU solution (0-25μL) was then added to each culture, along with a compensatory volume of vehicle (IMDM/4% EtOH), such that each culture was exposed to the targeted concentration of BCNU (0-20μM) and an equal concentration of EtOH (0.2% by volume). A MNNG (Aldrich, Milwaukee, WI) stock solution (1mg/mL in 0.1M sodium acetate buffer [pH=5.1]) was mixed with 4°C IMDM to yield two solutions of different concentration (2μg/mL and 10μg/mL). To deliver 0.02μg/mL treatments to cultures, 5μL of 2μg/mL MNNG was added to the medium; to deliver 0.10μg/mL or 0.20μg/mL treatments to cultures, 5μL or 10μL of 10μg/mL MNNG was added to the medium, respectively. MMS (Aldrich) was mixed with 4°C IMDM to yield two solutions of different concentration (0.5mM and 5mM). To deliver 10μM treatments to cultures, 10μL of 0.5mM MMS was added to the medium; to deliver 50μM or 100μM treatments to cultures, 5μL or 10μL of 5mM MMS were added to the medium, respectively. All alkylating agent solutions were prepared immediately before treatment to minimize degradation and decreased reactivity.

### 3.4.7 Viable Cell Counting

Viable cell counts, based on Trypan blue exclusion, were conducted using a ViCellXR viable cell counter (Beckman Coulter, Miami, FL) according to the manufacturer’s instructions. The instrument was set to include cells with diameters from 6-50μM in analyses. The relative number of viable cells in treated cultures was determined by normalizing the mean of viable cell counts from a set of treated cultures to the mean of viable cell counts from the set of untreated cultures.

### 3.4.8 Histological Imaging and Quantification

Slides were examined using a Labophot microscope (Nikon, Garden City, NY) and representative micrographs were acquired using a Sony DSC-P93A Cyber-Shot digital camera. Micrographs of benzidine-Giemsa stained cells were acquired using a 100X oil-immersion objective and brightfield illumination, while micrographs of acridine orange stained cells were acquired using a 40X oil-immersion objective and fluorescence (100W Hg lamp excitation). Cytological slides were examined blind and differential cell counting was used to enumerate relevant cell types and thus quantify the frequency of MN-PCEs among PCEs (> 2000 PCEs scored per slide), and the frequency of PCEs among RBCs (>1000 RBCs scored per slide).
3.4.9 Cell Cycle Analysis

Cells were harvested from culture as previously described and were then washed twice in PBS/4%FBS and resuspended at a density of ~10^6/mL. If insufficient cells were available then the population was resuspended in a minimal volume (~100µL). Three-times the suspension volume of 4°C ethanol was then added, and the cells were fixed at 4°C for >1h. The fixed suspension was then stored at -20°C until analysis. At analysis, the fixed suspension was washed twice in PBS/4%FBS and the stained with propidium iodide (50µg/mL) in the presence of RNaseA A (500µg/mL) for >4h. Cells were kept at 4°C until analysis by flow cytometry, which was achieved using the FL2-A signal as a representation of DNA content. The raw FACS data from CellQuest was analyzed using ModFitLT after gating based on side-scatter and forward-scatter to eliminate some aggregates and debris. The ModFitLT diploid model, based on Gaussian statistics, was used to identify G1, S, G2, sub-G1 (debris) and aggregates.

3.4.10 Statistics

To determine the statistical significance of mean comparisons, distributions were first checked for normality using the Shapiro-Wilk test (using JMP5 [SAS Institute, Inc., Cary, NC]). Normal data sets were then subjected to two-tailed Welch’s t-tests to determine P values using the data analysis tool in Microsoft® Excel.
Chapter Four

Conclusions and Future Work
4.1 CONCLUSIONS

CFU-E progenitors, when stimulated with Epo and exposed to DNA damage, can arrive at several biological outcomes including: (1) clonal production of normally enucleated reticulocytes; (2) clonal production of reticulocytes containing micronuclei; and (3) death/senescence (Fig. 4-1). Death and senescence are mentioned together, here and throughout this thesis, because they both lead to decreases in the relative viable cell yield at harvest, and thus cannot be distinguished by the techniques employed in this work. It is possible that, within a cell, the decision-state depends on the cell cycle phase and replication rate at the time of exposure, and this question will be addressed in future studies. At this time, however, this work has produced no specific evidence that relates the cell cycle phase or replication rate in a cell to its fate in erythropoietic culture; the chief technical obstacle arising because it is extremely difficult to measure these properties without fixing or otherwise disturbing a cell's progress through erythropoietic development in this culture system. That is, if a cell is harvested from culture and assayed for these properties, it will not arrive at its erythropoietic endpoint, making it difficult to firmly tie the erythropoietic endpoint to cell cycle status or replication rate. Therefore, the interpretations of erythropoietic responses following genotoxic insult described in this thesis remain hypotheses.

Figure 4-1. Model of erythropoietic growth and genotoxic responses as observed in this culture system. Key receptors for normal erythropoiesis and surface markers of late-erythroid cells are indicated. Based on the data in Chapter Three, unrepaired genotoxic damage to primitive cells (mature BFU-Es and CFU-Es) is likely to stimulate apoptosis, whereas unrepaired genotoxic damage to later erythroid cells is likely to result in the growth of micronucleated reticulocytes.
Consistent with the data in this thesis, the model in Fig. 4-1 depicts cells at later stages of erythropoiesis as “sensitive” to the formation of micronucleated progeny, whereas cells at earlier stages of erythropoiesis are likely to die or senesce. These two different fates are signaled by the production of micronucleated progeny or by reductions in viable cell yields, respectively. However, even cultures treated at 4h or 10h yield a detectable MN response (that is, some cells near the CFU-E stage still go on to form micronucleated progeny). This MN response remains detectable, despite reduced MN frequencies and exacerbated reductions in viable cell yield as compared with dosing at later culture times (23h or 30h). These observations lead to the hypothesis that cells at an early erythropoietic stage (those present at early culture times) are more likely to die/senesce, while those at later erythropoietic stages (i.e. those present at later culture times) are more likely to complete differentiation and yield micronucleated progeny (Fig. 4-1). Although the work in this thesis suggests that the probability that a given cell will arrive at a given biological endpoint depends on the stage of erythropoiesis, some members of large populations will still arrive at low-probability fates.

The molecular basis of the cellular decision-state and the DNA damage detection and repair pathways that mediate death/senescence and MN formation were only partially explored in this thesis. However, the system established here provides a useful in vitro model for future studies in these areas. Specifically, this in vitro assay facilitates direct inhibition of pathway components at the protein level or RNAi directed towards pathway components at the message level, and thus complements in vivo studies in repair-deficient transgenic mice. In this manner, research may be conducted to further explore the repair enzymes that mediate the erythroid response to genotoxic agents. Knockout or knockdown of the initiating enzyme in a repair pathway (such as Aag^+, in the case of the base-excision repair response to N^3^-methylAdenine) can give insight about whether a repair pathway is involved in the normal response of hematopoietic progenitors to the damage inflicted by a given genotoxicant. Here, using BCNU as a model genotoxic agent, it was found that a substantial increase in genetic fragmentation (MN production) in erythroid progenitors results when the MGMT repair protein is knocked out. In contrast, it was found that knocking out the enzyme that initiates base-excision repair by glycosylation at N^3^-methylAdenine had no detectable effect on the production of micronucleated PCEs from erythroid progenitors treated with BCNU. By comparing both in vivo and in vitro data from WT and MGMT^{-/-} BM following treatment with BCNU, it can be estimated that erythroid MN formation is approximately doubled if O^6^-alkylGuanine cannot repaired. For example, this trend was observed at all in vivo doses investigated when the marrow was examined 48h or 72h after dosing (Fig. 3-
6). Furthermore, when 20μM BCNU was introduced into erythropoietic cultures 23h or 30h after seeding, it was observed upon harvest at 72h that MN production in MGMT−/− cultures was approximately double that observed in WT cultures (Fig. 3-9).

However, while it may be true that O6-alkylGuanine in erythroid progenitors can lead to the production of micronuclei, many questions remain regarding erythroid MN formation during WT erythropoiesis. Although an increased MN response is observed in MGMT−/− BM, it is not known what fraction of genetic fragmentation arises from this adduct in WT erythroid progenitors, which might repair either some or all of the O6-Guanine adduct. Indeed, SN2 alkylating agents, which form negligible amounts of O6-alkylGuanine, still stimulate erythroid MN formation in WT progenitors. That is, while O6-alkylGuanine contributes to MN formation in MGMT−/− BM, the role this adduct plays during MN formation in WT marrow remains unknown. Furthermore, if WT Lin− BM cells are protected by MGMT, then MGMT activity should be detectable in these cells. However, attempts to detect MGMT activity in Lin− BM, or in erythropoietic cultures derived from these cells, via transfer of radiolabel from an alkylated DNA substrate to the protein fraction of cellular extracts, have been unsuccessful. This activity assay is technically challenging in primitive hematopoietic cells because very low cell numbers (and thus low amounts of protein extract) are available (there are approximately 5x10^5 Lin− cells per mouse). Therefore, accelerator mass spectroscopy, rather than typical scintillation counting, might provide the extra sensitivity needed to measure the transfer of radiolabeled DNA adducts to protein. Alternatively, MGMT expression (rather than activity) could be assayed using RT-PCR (message level detection) or Western blotting (protein level detection).

Further insight regarding the mechanisms by which DNA damage and repair lead to erythroid MN formation can be gleaned by comparing the WT erythroid MN response to alkylating agents with varying reactivity. In this thesis, an SN1 crosslinking compound (BCNU), an SN1 nonfunctional compound (MNNG), and an SN2 alkylating compound (MMS) were used. While exposure to all three alkylating agents led to erythroid MN formation in this system, the MN-response was much more dramatic in response to SN1 alkylating agents (Fig. 3-5). SN2 alkylating agents mainly form adducts at the most nucleophilic sites on DNA (the N7 and N3 positions of the purine bases), while SN1 alkylating agents are more reactive and form adducts at a variety of sites, including the O6 position of Guanine. Therefore, these data suggest that adducts formed at the less nucleophilic sites on DNA play an important role in erythroid MN formation. Furthermore, when SN1 alkylating agents were introduced into erythropoietic culture at various
times after seeding, it was found that the erythroid MN response was enhanced by treatment at later stages of culture (23h or 30h) vs. earlier treatment times (4h or 10h). In contrast, MMS (an $S_N$1 alkylating agent) induced a constant MN response, regardless of treatment time (data not shown). These data suggest that $O^6$ alkylGuanine, and other adducts formed at less nucleophilic sites on DNA, contribute to erythroid MN formation and that sensitivity to MN formation in response to these adducts may increase as erythropoiesis progresses and replication rate increases. Furthermore, the differences in reactivity between MNNG and BCNU, which are both $S_N$1 alkylating agents generating a similar spectrum of primary DNA adducts, could be used in future studies to determine the relative contributions of mismatch repair (triggered by both alkylating agents) and crosslink repair (triggered only by BCNU) to erythroid MN formation. In such studies, MGMT$^{-/-}$ BM could be used to increase the number of $O^6$ adducts formed and thus increase the signal generated by such damage.

Twenty-three hours to 30h after seeding, $>70\%$ of all cells in these erythropoietic cultures are in the DNA synthesis (S) phase of the cell cycle (Fig. 3-3). This population’s cell cycle profile is similar to that observed in embryonic stem (ES) cells, which do not express G1-S checkpoint regulation (119). S-phase arrest following treatment with BCNU is a typical response during mismatch or crosslink repair, and so the cell cycle status of treated cultures was compared to that of untreated cultures in an attempt to detect an erythroid DNA repair response (Fig. 3-11). Contrary to expectations, no increase in S-phase population percentages was observed following treatment with BCNU. In fact, it was found that treatment with 20μM BCNU 23h or 30h after seeding led to slight decreases in the fraction of erythropoietic populations found to be S-phase 48h after seeding. As S-phase population fractions decreased in treated cultures, the number of cells in both the G1 and G2 phases of the cell cycle increased. This effect, observed 48h after seeding, was most dramatic in cultures treated with BCNU 30h after seeding. These observations suggest that repair pathways that delay progress through S-phase are not involved in erythroid MN formation. However, measurements were only made at certain discrete times following treatment, and an S-phase arrest could have occurred between measurements. That is, populations that arrest in S-phase might eventually yield a decreased fraction of S-phase members as these damaged cells complete genomic replication and move out of S-phase together. One experiment that might provide data to further explain growth and MN-formation in this system is the use of a vital dye to track number of cell divisions. In this manner, the number of divisions between Lin$^+$ BM and both normal and micronucleated PCEs could be determined. Further, the
number of divisions between the MN-sensitive populations (present at 23h and 30h) and their micronucleated progeny (observed at 72h) could be determined.

4.2 EXTENSION TO HUMAN TISSUE
Although all studies conducted in this thesis were conducted on murine tissue, much is known about human erythropoiesis, and much is conserved between the two species. Ideally, a hematopoietic population could be used in this system that was genetically matched with a targeted epidemiological cohort, in the case of a candidate therapeutic, or with a target market, in the case of a cosmetic, in order to best predict adverse side-effects. One example of a human hematopoietic population containing CFU-Es, but not reticulocytes, is the glycophorin A+ transferrin receptor1 population (120). Such a population can be obtained from adult human BM or from human peripheral blood, as some erythroid progenitors leave the marrow and enter circulation.

Cells near the CFU-E stage of erythropoiesis are useful for in vitro MN testing because they are at a stage of development that will provide a sufficient number of developmental divisions and, consequently, enough time for most test substances to take effect, while still yielding PCEs in two to three days. In addition, it is preferred for the erythropoietic starting population to be devoid of preexisting, enucleated PCEs. The presence of these cells in the initial population of short-term test cultures might confound test results because some PCEs that were formed in vivo, prior to introduction of a test agent, may still remain at time of harvest and analysis and may thus confound final cell counts.

Fully-differentiated hematopoietic cells of non-erythroid lineages, such as megakaryocytes, cannot undergo erythropoietic growth, and the presence of these cells in the starting population may actually hinder the sensitivity and accuracy of test results. Culture of excessive non-erythroid cells may confound the results of this test system for three main reasons. First, such cells can deplete media supplements that support erythropoietic growth. Second, non-erythroid cells can act as unintended targets of test substances and could blunt the substance’s effect on erythroid progenitor cells, which are the preferred target because they provide a metric of genotoxic damage in their MN-PCE progeny. For example, a blunting effect could arise from irreversible reactions between biomolecular components within non-erythroid cells and the active molecular components of the test agent and its derivatives. Third, apoptotic or necrotic decay of non-erythroid populations, which may not be supplied with necessary survival cues, can lead to...
excessive debris and “bystander” toxicity in a test culture. Depletion of initial hematopoietic tissue of such non-erythroid, differentiated cell types consequently leads to the enrichment of erythroid progenitor content mentioned above.

Human tissue types that can provide primary hematopoietic cell populations containing erythroid progenitors include bone marrow, peripheral blood, fetal liver, splenic tissue, and umbilical cord blood and tissue. In spite of the high erythropoietic potential of fetal liver, the use of fetal tissue is ethically complex and questionably predictive of adult responses. When testing a therapeutic substance to be used in adults, the most preferable source of hematopoietic test populations is human BM, which is the normal site of adult erythropoiesis. If the test is being used to predict clinical outcomes, this human BM would most preferably be genetically matched with the targeted epidemiological cohort. Much information is available in the literature regarding the isolation of erythroid progenitors from human BM, peripheral blood and cord blood.

It is known that the BM subpopulation that shares side-scatter and forward-scatter characteristics with blood monocytes is comprised of immature cells of various lineages, and that populations within this side-scatter, forward-scatter region contain nearly all of the marrow’s colony-forming cells. This region is thus referred to as the “blast” region of the BM. In one study, cells in this blast region were then subdivided based on expression of the glycophorin A (Gly A) cell surface protein and based on expression of the transferrin receptor (CD71), to obtain a population containing most of the CFU-Es in human BM. Specifically, this work found that a promising test population is the subpopulation of the BM blast region that expresses Gly A at an intermediate level and CD71 at a level in the high to intermediate range (120). In another study, two antibodies were generated (5F1 and CLB-Ery-3) that bound specifically to antigens on CFU-Es and erythrocytes, and a third antibody (an α-Gly-A antibody, termed VIE-G4) was generated that only bound to erythrocytes (121). These antibodies offer another promising combination of specificities for the enrichment of CFU-Es from human BM. Furthermore, a simple alternative is offered by use of the CD34 human hematopoietic stem and primitive progenitor cell marker. In fact, a recent study has shown that CD34+ hematopoietic cells obtained using immunomagnetic techniques from human BM can be induced to undergo terminal erythropoiesis in culture (122).

Many phenotypic characteristics of erythropoietic populations within human cord blood have also been elucidated and these will be briefly mentioned here as possible selectable characteristics defining populations for extension to human tissue. A detailed analysis of cell surface antigen
expression during erythropoiesis from cord blood mononuclear cells found that CD34, CD41, and human leukocyte antigen (HLA)-DR disappear as erythropoiesis progresses while CD71, CD36, and Gly A appear during erythropoietic differentiation (123). To be exact, this work, which is representative of several other studies, suggests that CD71⁺, CD36⁺, Gly A⁻ mononuclear cells from human cord blood constitute an appropriate target population. Furthermore, it has been shown that the easily-isolated CD34⁺ hematopoietic cells in human cord blood can be induced to undergo terminal erythropoiesis in culture (122).

Cell surface marker and physical characteristics of erythroid progenitors in human peripheral blood have also been identified, and it has been demonstrated that these unique characteristics can be exploited to effect the isolation of these progenitors from peripheral blood (40). Specifically, it has been demonstrated that erythroid progenitors are contained within the mononuclear cell (MNC) fraction of human peripheral blood, which is a population that can be isolated by density gradient centrifugation (92). Although these progenitors mostly consist of BFU-Es, rather than the preferred CFU-Es, Fibach and Rachmilewitz, along with other authors, have demonstrated that these BFU-Es can then be induced to yield CFU-Es in culture. It has also been demonstrated that simply isolating CD34⁺ hematopoietic cells from granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood provides a population that can be induced to undergo terminal erythropoiesis in culture (122). In fact, it has been found that granulocyte colony stimulating factor (G-CSF) mobilized PB contains a higher frequency of BFU-Es in the MNC population than that contained in the MNC population of either BM or cord blood (124).

Not only have distinguishing characteristics of human erythropoietic populations been identified, erythropoietic culture conditions, based on human tissue, have also been defined. Liquid erythropoietic culture of human PB-MNCs in α minimum-essential medium (αMEM) supplemented with FBS, Epo, and conditioned medium from cultures of the 5637 bladder-carcinoma cell line induces some degree of erythropoietic growth, although terminal division and enucleation were only rarely observed using these growth conditions (92, 125). Specifically, these authors used a two-step liquid culture, with a first phase that is Epo-independent. The media used in the phase I culture, which is five to seven days in length, is αMEM supplemented with 10% conditioned media from cultures of 5637 cells and 10% FBS; during this phase BFU-Es differentiate into CFU-Es. In the second phase, αMEM containing 1U/mL Epo, 1µM Dex, 30% FBS, 1% BSA, and 10µM β-mercaptoethanol is used to induce the proliferation and further maturation of CFU-Es. After four days of phase II culture, centrifugation at 1000g (for 20
minutes in Percoll with \( p = 1.0585 \text{ g/mL} \) is used to effect the removal of lymphocytes from the population, yielding a supernatant that contains the proerythroblasts. These proerythroblasts are then returned to phase II culture for another ten to twelve days.

Furthermore, a three-step culture system has been used to produce fully mature human RBCs from CD34\(^+\) cells collected from normal BM, peripheral blood mobilized with G-CSF, and umbilical cord blood (122). These CD34\(^+\) cells were cultured in a serum-free base medium supplemented with 1\% BSA, 120\(\mu\)g/mL holotransferrin, 900\(\text{ng/mL}\) ferrous sulfate, 90\(\text{ng/mL}\) ferric nitrate, and 10\(\mu\)g/mL insulin. In the first step (eight days in duration, split after four days), these cells were cultured in the presence of 10\(\mu\)M hydrocortisone, 100\(\text{ng/mL}\) SCF, 5\(\text{ng/mL}\) interleukin-3 (IL-3) and 31\(\text{IU/mL}\) Epo. In the second step, the cells were cocultured with an adherent stromal cell line and stimulated with additional Epo for three days. In the third step, all exogenous factors are withdrawn and cells are incubated on a simple stroma for up to ten days. In the second and third steps, the stromal feeder layers consisted of either the MS-5 murine cell line or mesenchymal stromal derived from normal adult whole BM. Overall, these authors report an expansion of CD34\(^+\) HSCs of over 10\(^6\)-fold and a conversion to mature RBC near 100\%.

Finally, erythropoietic growth, in culture, from umbilical cord blood has been demonstrated. Light-density MNCs were first isolated from umbilical cord blood samples using Ficoll-Paque density-gradient centrifugation, and then incubated at 37\(\text{°C}\) for two hours (126). Then, the nonadherent MNC fraction was removed and red cells were lysed in 0.75M \(\text{NH}_4\text{Cl}\). Culture of the resulting population in IMDM supplemented with 20\% FBS, 1\(\text{U/mL}\) Epo, and 10\(\text{ng/mL}\) SCF resulted in late-stage erythroid development as determined using flow cytometric indicators such as the disappearance of CD34, CD41, and HLA-DR and the appearance of CD71, CD36, and Gly A (123). The representative culture methodologies mentioned above are provided only as exemplary protocols for the induction of terminal erythropoiesis from primary human tissue. As stated previously, any stimulatory ex vivo culture is adequate for extension of the present work if the culture induces a signal state, within erythroid progenitors, that yields terminal division and enucleation.

### 4.3 AUTOMATED HIGH-THROUGHPUT ANALYSIS

Hand scoring microscope slides to detect and enumerate PCEs and micronucleated cells is a time-consuming and tedious process that can lend itself to experimenter bias. Both flow cytometry and laser scanning cytometry (LSC) analyses can be used to automatically detect and enumerate
micronucleated PCEs among total PCEs (24-26). In order to save time and remove bias, attempts were made in this thesis work to use a laser scanning cytometer (LSC) to automatically analyze cells and score slides, thanks to Wes Overton for his efforts in this area. Each slide was loaded onto the LSC platform, brought into focus, and then the laser was set to scan the entire area of the slide that contained cells. Each cell that the LSC detected was then reported as an event with several different measured properties. These events could then be plotted on multiple graphs, some of the more useful graphical representations included plots of Area vs. PI Max Pixel, FITC Max Pixel vs. FITC Integral, FITC Integral vs. Red Integral, and Area vs. Red Max Pixel.

The first of these, Area vs. PI Max Pixel, was used to define which events would be counted, and instrument settings were changed to yield a linear distribution of events along the left side and a slightly less dense linear distribution of events along the top of the graph. The FITC Max Pixel vs. FITC Integral graph was used to identify the micronucleated region; and, although it was the most successful of all tested regions, it was not 100% accurate. For best accuracy in counting, it was determined that events should be spread out along a line with a slope of approximately one. Some events should fall slightly below the line and in the gated region; these are the micronucleated PCEs. Further, events should be denser towards the origin and less dense further from the origin. Next, the FITC Integral vs. Red Integral graph was used to detect PCEs. For best scoring, instrument settings should be adjusted such that there is a fairly straight line of events on the left side of the graph with very few events forming a line with a slope of approximately one. The event density should be higher on the lower part of the graph and should taper off as the y value increases; a gated region can then be defined that contains all PCEs. Finally, the Area vs. Red Max Pixel graph is used to filter out large clumps or events that do not have a high red maximum pixel value. Only the cells in the gated region of this graph are plotted on the FITC Integral vs. Red Integral graph for consideration as PCEs. Instrument settings should be adjusted such that this last plot is fairly spread out with no large clusters of events. Occasionally, there will be a large group along the bottom of the graph, but usually most events will be evenly distributed.

As the LSC is scanning the slide, the windows being counted by the LSC can be viewed and the instrument settings can be adjusted so that the inherent variability in staining among slides is accounted for. After the LSC finishes scanning the slide, a gallery can be produced that shows representative pictures of cells in a particular region—in this case the PCE gated region. Figure 4-2 is a typical gallery of sixteen PCEs:
The events in the PCE gated region were counted, and a percentage of PCEs on each slide was obtained. The following graph is a correlation between the PCE percentage as counted by hand and by the LSC.
When the percent PCE data from the LSC was graphed with the percent PCE data from hand scoring, a fairly linear trend was observed, but the line had a negative slope (Fig. 4-3). There was only obvious outlier (hipHhiIns2Fn.1), and the LSC data from that slide is somewhat questionable because the slide contained very few cells.

The negatively sloping trend observed among the data points is disappointing, and it is due to this result that hand-scoring was used throughout this work. A positively sloping line would be expected after applying this method and would imply that the two methods of counting gave similar results. One possible explanation for the observed data trends involves staining differences among slides. When a well-stained slide is put on the LSC and scanned, the bright red signal needs to be reduced using the photomultiplier tube settings. While decreasing this setting reduces some of the red signal, it does not eliminate all of the bright red fluorescence. Therefore, when the slide is scanned, PCEs have a slightly higher red integral than normal, and the added fluorescence increases the perceived area of the cell when it is contoured by the LSC. This could lead to the event being excluded from the PCE region, which was defined using a slide of average stain intensity- one that was neither stained extremely brightly nor very faintly, by excluding it from the gate on the Area vs. Red Max Pixel graph. Thus, the percentage of PCEs on a brightly stained slide, as counted by the LSC, would be lower than that of a “normal” slide.

Another possible explanation for the deviation from the expected results involves the LSC’s inability to distinguish a large clump of PCEs as separate events. While a technician can recognize a clump of PCEs and enumerate the approximate number of cells in the clump, the LSC contours the group of PCEs as a single event, thus making the area far too large to be allowed into the gated region on the Area vs. Red Max Pixel graph. By excluding such clumps from the PCE region, the LSC counts fewer PCEs than a technician, thus reducing the PCE percentage on the slide. Additional work is required to identify a region defining micronucleated cells using the LSC. All attempts at enumerated micronucleated PCEs were unsuccessful, mainly due to poor control over the focus and image quality from individual cells (see Fig. 4-2). Further work is required to improve the correlation between hand-scoring and automated scoring using the LSC.
Appendix I

MATLAB® Script for Multi-Linear Regression
clear all; close all;

germ=zeros(144,8);

% 'germ' is the initial design matrix, still containing % actual numerical values for the covariates

-----------------------------------------
% the format is [Epo,SCF,IGF-I,Dex,Fn,t,CO2,O2]  
% and the corresponding 
% units are [U/mL,ng/mL,ng/mL,uM,ug/sq.cm.,h,atm,atm]
-----------------------------------------

germ=[
    [0,0,0,0,0,72,0.05,0.1995];
    [0,0,0,0,0,72,0.05,0.1995];
    [10,100,100,0,0,72,0.05,0.1995];
    [10,100,100,0,0,72,0.05,0.1995];
    [10,100,0,10,0,72,0.05,0.1995];
    [10,100,0,10,0,72,0.05,0.1995];
    [10,100,0,10,0,72,0.05,0.1995];
    [10,100,0,10,0,72,0.05,0.1995];
    [0,0,100,10,0,72,0.05,0.1995];
    [0,0,100,10,0,72,0.05,0.1995];
    [0,0,100,10,0,72,0.05,0.1995];
    [0,100,0,0,2,72,0.05,0.1995];
    [0,100,0,0,2,72,0.05,0.1995];
    [0,100,0,0,2,72,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [0,100,100,0,0,96,0.05,0.1995];
    [0,100,100,0,0,96,0.05,0.1995];
    [0,100,100,0,0,96,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [10,0,0,0,0,96,0.05,0.1995];
    [10,100,0,0,2,96,0.05,0.1995];
    [10,100,0,0,2,96,0.05,0.1995];
    [10,100,0,0,2,96,0.05,0.1995];
];
scale the design matrix

% jad=1 centers each column around
% the mid-point for that covariate and then scales
% by the variable's range/2... here "range" = max-min
% i.e. lowest value will go to -1 and
% highest value will go to 1

% jad=2 will zero each column at the
% minimum value for that covariate
% and then will scale by the variable's range (max-min)
% i.e. lowest value will go to 0 and
% highest value will go to 1

% jad=3 will simply scale all members of a column
% by the max for that column
% i.e. the max will be 1 using this method, but the min
% will not always be 0 (for CO2 and O2 here, it will be
% greater than 0

jad=2;

if jad==1,
    mid=zeros(8,1);
    for i=1:8,
        mid(i,1)=(max(germ(:,i))+min(germ(:,i)))/2;
    end;
    for i=1:8,
        unigerm(:,i)=[(germ(:,i)-mid(i,1)*ones(144,1))/(max(germ(:,i))-mid(i,1))];
    end;
elseif jad==2,
    for i=1:8,
        unigerm(:,i)=[(germ(:,i)-min(germ(:,i))*ones(144,1))/(max(germ(:,i))-min(germ(:,i)))];
    end;
elseif jad==3,
    for i=1:8,
        unigerm(:,i)=germ(:,i)/max(germ(:,i));
    end;
end;

%%%%%%%%% done scaling design matrix %%%%%%%%%%%%%

%%%%%%%%%%%%%%%%%%%%%%%% Now add columns to the design matrix %%%%%%%%%%%%%%%%%%

%%% For the full version of the model (including all linearly-
%%% independent secondary interactions) let mjs = 1,
%%% for the refined version, let mjs = 2

mjs = 1;

if mjs==1,
    biggie=[unigerm, unigerm(:,1).*unigerm(:,2),...
unigerm(:,1).*unigerm(:,3), unigerm(:,1).*unigerm(:,4),...
unigerm(:,1).*unigerm(:,5), unigerm(:,1).*unigerm(:,6),...
unigerm(:,2).*unigerm(:,3), unigerm(:,2).*unigerm(:,4),...
unigerm(:,7).*unigerm(:,1), unigerm(:,7).*unigerm(:,2),...
unigerm(:,7).*unigerm(:,3), unigerm(:,7).*unigerm(:,4),...
unigerm(:,7).*unigerm(:,5), unigerm(:,7).*unigerm(:,6),...
unigerm(:,8).*unigerm(:,1), unigerm(:,8).*unigerm(:,2),...
unigerm(:,8).*unigerm(:,3), unigerm(:,8).*unigerm(:,4),...
unigerm(:,8).*unigerm(:,5), unigerm(:,8).*unigerm(:,6));

elseif mjs==2,
    biggie=[unigerm(:,1:6), unigerm(:,1).*unigerm(:,2),...
         unigerm(:,1).*unigerm(:,5),...
         unigerm(:,2).*unigerm(:,3), unigerm(:,2).*unigerm(:,4),...
         unigerm(:,7).*unigerm(:,1), unigerm(:,7).*unigerm(:,2),...
         unigerm(:,7).*unigerm(:,3), unigerm(:,7).*unigerm(:,4),...
         unigerm(:,7).*unigerm(:,6),...
         unigerm(:,8).*unigerm(:,1), unigerm(:,8).*unigerm(:,2),...
         unigerm(:,8).*unigerm(:,3), unigerm(:,8).*unigerm(:,4)];
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% y is the vector of measured (dependent) variables  %
% the units are (PCEs produced)/input cell          %
% in this expt, input cells are lineage negative    %
% mouse BM from male B6 mice aged 6-8 weeks        %
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

y=zeros(144,1);
y=[1.5639E-02;
  2.1833E-02;
  1.9622E-02;
  4.5675E+00;
  4.0582E+00;
  3.6555E+00;
  3.4470E+00;
  4.5539E+00;
  2.9897E+00;
  3.0224E-02;
 -7.2679E-03;
  1.6470E-02;
  4.3651E-02;
  5.6381E-02;
  2.3537E-02;
  2.1335E+00;
  2.0780E+00;
  3.1194E+00;
  2.4955E+00;
  2.4544E+00;
  2.8685E+00;
  7.3135E-01;...
1.1324E+00; 5.4415E-02; 2.2004E+00; 1.7794E+00; 2.1273E+00; 1.0802E-02; 2.5108E-02; 4.2348E-02; 3.5185E-02; 1.1524E-02; 4.8842E-02; 2.1369E+00; 1.9215E+00; 1.7897E+00; 2.6903E+00; 4.4534E+00; 3.2634E+00; -3.0683E-03; 1.6534E-02; 5.0599E-02; -5.4893E-03; -3.0672E-03; 2.7195E-03; 5.4337E+00; 4.4451E+00; 4.0772E+00; 2.2636E-02; 3.3430E-02; 1.4088E-01; 4.6718E+00; 5.3943E+00; 4.3801E+00; 6.6917E+00; 5.2960E+00; 5.5057E+00; 2.3195E-02; 9.1450E-03; 3.8402E-02; 1.5804E-01; 1.1285E-01; 1.2761E-01; 3.8012E+00; 3.4329E+00; 3.4298E+00; 2.6435E+00; 2.8257E+00; 2.4459E+00; 1.0269E-01; 6.4135E-02; 8.8578E-02; 3.2717E+00; 2.9968E+00; 3.1435E+00; 7.4880E-02; 7.2846E-02; 7.4802E-02; 2.3842E-03;
```
1.9315E-02;
2.8503E-02;
2.1589E+00;
2.8503E-02;
1.9549E+00;
4.8223E+00;
5.5239E+00;
5.7899E+00;
-7.2679E-03;
4.1257E-02;
1.3865E-02;
-9.9652E-04;
-5.0621E-03;
5.3215E+00;
5.5294E+00;
3.9897E+00;
-7.2679E-03;
1.0732E-02;
-4.0382E-03;
2.3349E+00;
2.4993E+00;
2.7104E+00;
2.1749E+00;
1.9529E+00;
2.8019E+00;
7.8284E-03;
-1.4641E-03;
4.0379E-03;
-7.2679E-03;
-4.2723E-03;
-4.8702E-03;
1.8819E+00;
1.6847E+00;
2.2539E+00;
1.5423E+00;
1.4003E+00;
1.5453E+00;
1.3040E-02;
7.2207E-03;
-1.3789E-03;
1.8804E+00;
1.5957E+00;
1.6373E+00;
4.4356E-03;
-4.0867E-03;
-5.6043E-03;
1.2352E-02;
3.4688E-03;
7.9416E-03;
1.5003E+00;
1.4255E+00;
1.6530E+00;
2.2722E+00;
2.2288E+00;
2.9651E+00;
-4.0617E-03;
```
-3.1789E-03;
1.5134E-02;
-6.5316E-03;
-7.2679E-03;
-4.7442E-03;
5.4120E+00;
4.1244E+00;
3.7056E+00];

%%%%9%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%% Solve for parameter estimates %%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%%%%%%%%%%%%%%%%%%%%%%
% (X'X)b=X'y -> b=inv(X'X)X'y %%%%%%
%%%%%%%%%%%%%%%%%%%%%%
estimates=inv(biggie'*biggie)*biggie'*y;

%%%%%%%%%% Use param estimates to predict y %%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
prediction=biggie*estimates;

%%%%%%%%%%%%%%%%%%%%%% plot measured v. predicted %%%%%
plot(prediction,y,'k.',y,y,'k-');
xlabel('Predicted Response');ylabel('Measured Response');title('Measured Response v. Model Prediction');

%%%%%%%%%%%%%%%%%%%%%% Parameter estimation complete, %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
statistics below %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

rawerror=(y-prediction);
mse=sum(rawerror.*rawerror)/(length(rawerror)-length(estimates));
rmse=sqrt(mse);

mcb=2; % specify method for calculating R^2, all are
% essentially equivalent

if mcb==1,
diffy=y-ones(144,1)*mean(y);
diffpred=prediction-ones(144,1)*mean(prediction);
c0=sum(diffy.*diffpred)/(length(y)-1);
r=c0/(std(y)*std(prediction));
rsquare=r^2;
elseif mcb==2,
sse = sum(rawerror.*rawerror);
diffy = y - ones(144,1) * mean(y);
sst = sum(diffy.*diffy);
rsquare = 1 - sse/sst;
rsquadj = 1 - sse*(144-1)/(sst*(144-length(estimates)));
elseif mcb == 3,
    rsquare = 1 - cov(rawerror)/cov(y);
end;

ssfact = zeros(size(biggie,2),1);
facterr = ssfact;
twistdesign = inv(biggie'*biggie);

for i = 1:size(biggie,2),
    facterr(i,1) = rmse * sqrt(twistdesign(i,i));
end;

trats = estimates./facterr; % calculate t ratios

bmc = 95; % input desired % confidence intervals
alpha = 1 - bmc/100; % compute corresponding alpha
nu = length(y) - size(biggie,2); % calc dof
T_val = tinv((1-alpha/2),nu); % obtain T value

lowers = estimates - T_val * facterr; % calc lower bound
% ("bmc" % confidence intervals)
uppers = estimates + T_val * facterr; % calc upper bound

rsquare
rsquadj
rmse
display ('estimate, error, t-ratio, lower-bound, upper-bound:')
output = [estimates, facterr, trats, lowers, uppers]
Appendix II

References