COMMON DIETARY AND ENVIRONMENTAL COMPOUNDS PROMOTE A LEUKEMIC TRANSLOCATION IN HEMATOPOIETIC STEM CELLS AND DIFFERENTIATED CELLS

by

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ABSTRACT

C. GREER VESTAL. Common dietary bioflavonoids and environmental compounds promote a leukemic translocation in hematopoietic stem cells and differentiated cells. (Under the direction of DR. CHRISTINE RICHARDSON)

Maternal exposure to topoisomerase II (topoII) inhibitors that are biochemically similar to the chemotherapeutic drug etoposide may promote infant acute leukemia. Several studies have shown that common dietary bioflavonoids as well as benzene metabolites can induce DNA double strand breaks (DSB) and inhibit topoII. The goal of this study was to determine the relative potential for bioflavonoids genistein and quercetin, and possible leukemogenic compounds p-benzoquinone and dipyrone to promote rearrangements between the *MLL* and *AF9* breakpoint cluster regions within specific hematopoietic cell subpopulations. A murine embryonic stem (ES) cell line containing two Green Fluorescent Protein (GFP) DSB-inducible recombination reporter substrates was differentiated in vitro into embryoid bodies (EB) containing hematopoietic stem cells (HSC) followed by differentiation into myeloid progenitor subpopulations. Following exposure to genistein, quercetin, p-benzoquinone, or dipyrone at each of three developmental stages, the frequency of interchromosomal translocations between the *MLL-AF9* bcrs was determined as well as the mechanism used for repair. Bioflavonoids genistein and quercetin were the strongest inducers of translocations during the earliest stage of differentiation. The frequency of translocations induced by each of these compounds at later stages of differentiation was dramatically reduced. The results demonstrate that all four compounds promote translocations between the *MLL* and *AF9* bcrs at different stages of differentiation and ES cells are particularly susceptible.

DEDICATION

I dedicate this doctoral dissertation to my sister Barbara Elizabeth Parman and to my parents John Greer Vestal and Barbara Corden for their unconditional love, faith, support and encouragement throughout this period of my doctoral work. This accomplishment would not have been possible without them.

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CHAPTER 1: INTRODUCTION

1.1 DNA Double Strand Break Repair

Maintaining genome integrity requires efficient repair of damaged DNA in order to prevent propagation of mutant or damaged cells. DNA double strand breaks (DSB) are the most detrimental type of DNA damage because the continuity of the DNA strand, which is essential for replication and transcription, is disrupted. Free ends of a DSBs can separate from each other increasing the likelihood of aberrant repair or exchange of information between non-homologous chromosomes. DSBs occur regularly from endogenous processes such as variable (diversity) joining (V(D)J) rearrangement in lymphoid cells, DNA replication and meiosis; they also occur from exposure to exogenous agents such as ionizing radiation, alkylating agents, and topoisomerase II (topoII) inhibitors. It has been estimated that there are 10 DSBs formed daily per cell, or more accurately, 50 DSBs per cell per replication cycle [1, 2]. Any one of these DSBs has the potential to be inaccurately repaired which could result in chromosome rearrangements in the form of deletions, duplications, inversions, or translocations. These types of chromosomal rearrangements could lead to mutagenesis or cancer.

Chromosomal translocations are a hallmark of many hematopoietic malignancies including acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) as well as some soft tissue sarcomas [3, 4]. Additionally, defects in the DNA DSB repair mechanisms leads to detrimental syndromes, such as ataxia telangiectasia (AT) and

Nijmegan breakage syndrome (NBS), which effect the nervous system as well as confer a predisposition for cancer [5]. Although DNA DSB repair processes and the different proteins involved have been well established, there are still many questions that remain to be answered in terms of mutation risk and repair capacity in different cell types and at different stages of differentiation.

Although many DNA lesions occur each day, only a small fraction leads to carcinogenesis; this is due to the activity of efficient DNA repair mechanisms that allow for the maintenance of genome integrity. DSBs are repaired in one of two ways: homologous recombination (HR) or non-homologous end joining (NHEJ) (Figure 1). With homologous recombination, the damaged DNA is repaired by use of a homologous template DNA strand whereas with NHEJ a homologous template is not required.

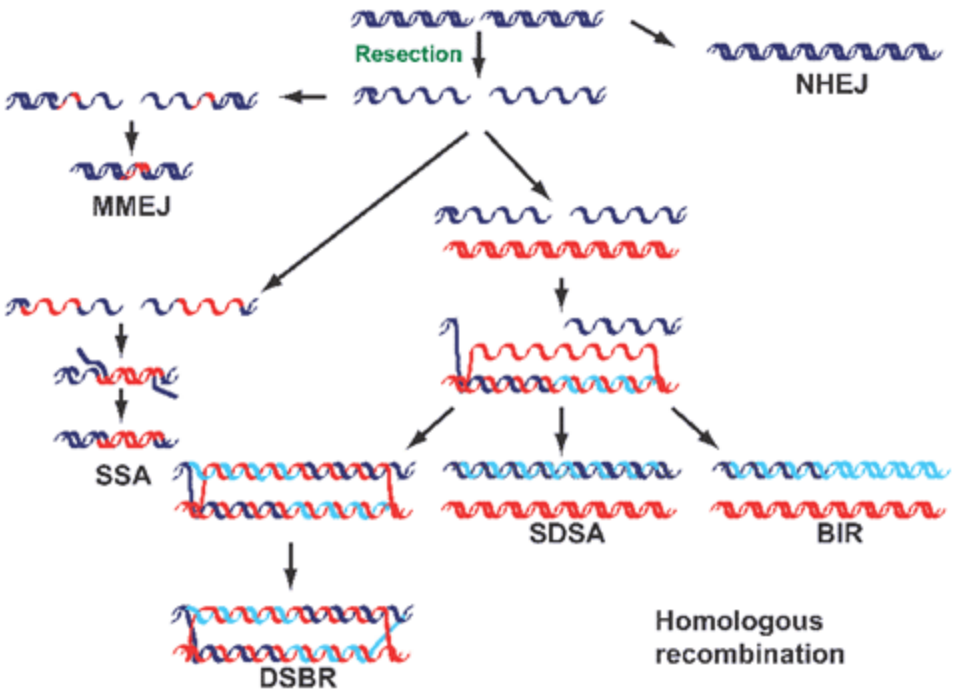

Figure 1: Processes of DNA double strand break repair [6]

1.2 Homologous Recombination and Non-Homologous End Joining

HR has the highest potential for accurate repair. DSB repair by HR involves (a) initiation by a DSB, (b) processing of DSB ends by nucleases resulting in 3' single stranded DNA overhangs, (c) formation of recombinase filament on single stranded DNA ends, (d) strand invasion into a homologous DNA sequence to form a D-loop intermediate, (e) DNA polymerase extension of the invading DNA strand, (f) capture and annealing of the second DNA end to the extended strand, (g) formation of two Holliday junctions (HJ), and (h) resolution of the Holliday junctions into crossover or non-crossover products [7]

HR is generally an error-free repair process and includes three subtypes of DSB repair; gene conversion, single-strand annealing (SSA), and break-induced replication (BIR) [7, 8]. Gene conversion is the nonreciprocal transfer of genetic information from one DNA molecule to a homolog. With this type of repair, the 3' ends of DNA at the DSB site invade an undamaged homologous template and initiate new DNA synthesis. This results in the restoration of genetic information that may have been lost at the DSB site. This type of DSB repair has been shown to occur during meiosis both with and without cross-over [9]. By contrast, in somatic cells, gene conversion with cross-over would result in translocations and generally does not occur [10]. A model for gene conversion without crossover, called synthesis dependent strand annealing (SDSA), allows for full restoration of sequence information at double strand break sites in mitotic cells [11, 12]. In this model, only one 3' end of the double strand break invades the intact homologous DNA molecule. This creates a D-loop structure which serves as a replication bubble in which synthesis of new DNA takes place. The newly synthesized DNA is

displaced from the replication bubble and rejoined to the non-invading end of the double strand break. This gives a gene conversion on the repaired DNA molecule but not the template molecule [13]. BIR is a form of SDSA when the non-invading end of the DSB is lost. In this case, the replication bubble must proceed to the end of the repair template for accurate repair to occur.

Single strand annealing is an alternative to gene conversion that results in the deletion of genetic material. This type of repair occurs when there is a double strand break between repeated sequences. Resection of the 5' ends of the double strand break leaves the 3' tails, each containing the repeated sequence, which can then anneal, resulting in the deletion of one of the repeats [14, 15]. It has been suggested that the gene conversion pathway and the single strand annealing pathway compete with one another for repair substrates, thus the inhibition of one will lead to an increase in activity of the other. Considering that SSA between DSBs on two different chromosomes can lead to translocations, the inhibition of the gene conversion pathway would cause an increase in the overall frequency of genome rearrangements [14, 16].

Homologous recombination ensures accurate DNA repair due to the requirement of a homologous template. This makes it far less likely for HR to be involved in chromosomal translocations than NHEJ. However, these two pathways are not mutually exclusive in that repair events can occur that involve the activity of both repair pathways [17]. It has been previously shown in a genetic model designed for HR that the ends produced from DSBs in two chromosomes in mouse ES cells can be readily joined by SSA and NHEJ to form reciprocal translocations at a frequency of 1×10^{-4} [16].

End-joining, unlike homologous recombination, does not require any homology between the ends of a DSB, but occurs by aligning and rejoining the broken ends. Microhomologies of 1-7 base pairs may aid in aligning the broken ends [18, 19] and nucleotides may be added or lost before rejoining occurs [20, 21]. The process of NHEJ can also result in deletions, duplications, inversions and translocations. In this manner, NHEJ can lead to mutations in the genetic sequence and with the occurrence of multiple double strand breaks can also lead to translocations [22, 23].

The potential to cause genome rearrangements is linked to the mechanism that is used [24]. The processes of both end-joining and homologous recombination can result in the loss or addition of nucleotides during the joining of broken DNA ends, and with the occurrence of multiple double strand breaks simultaneously, can lead to chromosomal translocations. The contribution that each of these pathways has in repairing a DSB depends on factors other than template homology including cell cycle and the developmental stage of the cell [25]. The HR pathway appears most prevalent in the S and G2 phases of the cell cycle due to the availability of a sister chromatid whereas NHEJ is most likely to occur in the G1 phase of the cell cycle [17].

1.2.1 HR and NHEJ Repair Proteins

The eukaryotic proteins involved in repair of DSBs by HR were identified in *S. cerevisiae* by genetic screening of radiation sensitive and meiotic recombination deficient mutants. The proteins that were identified are known as the RAD52 epistasis group [26]. HR proteins are evolutionarily conserved and mammalian homologues have been identified by homology searches, cloning of genes that abrogate recombination deficiency in cell lines, and by creating HR gene knockout mutants in the cell line DT40

[27, 28]. The mammalian HR proteins include RAD 50, RAD51, RAD 52, RAD54, Mre11, Nbs1, Xrcc2, and Xrcc3[27]. Mre11, RAD50, and Nbs1 form a protein complex in mammalian cells known as the MRN complex [29].

The initial cellular response to a DNA DSB is mediated through the ataxia telangiectasia mutated (ATM) protein and the MRN complex. ATM is a serine threonine kinase and is associated with DNA damage surveillance and activation of cell cycle check points. In response to DSBs, ATM phosphorylates many downstream targets including p53 and H2AX and effectively halts the cell cycle bringing DNA replication to a stop. ATM then either triggers apoptosis or repair depending on the severity of the damage [30]. The MRN complex is an early responder to DNA DSBs and mutations lead to cancer predisposition syndromes Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder (ATLD). The MRN complex senses DSBs and quickly establishes a protein-DNA complex in order to stabilize and process the free DNA ends. MRN also initiates cell cycle signaling cascades via ATM and regulates chromatin remodeling in the vicinity of the DSB [31]. Subsequently, MRN is responsible for the regulation of exonucleases CtIP and EXO1 and helicase BLM which interact to process the DNA ends in the 5'-3' direction leaving 3' overhanging single stranded DNA ends [32, 33]. DNA resection is followed by the recruitment of other HR proteins including replication protein A (RPA), BRCA1, BRCA2, RAD51, RAD52, RAD54.

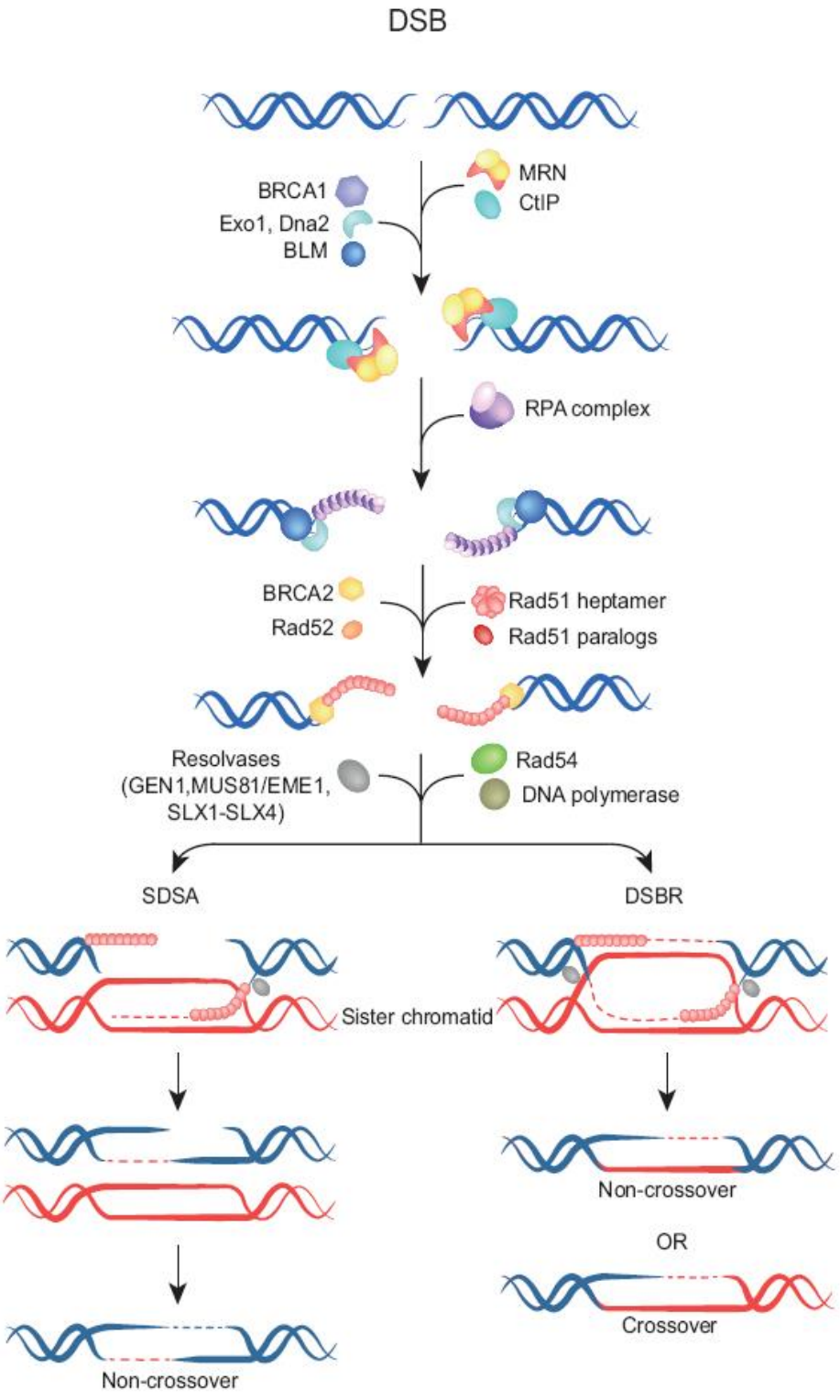

Figure 2: The mechanism of homologous recombination [34]

RPA binds to the single stranded DNA preventing endonuclease activity and removing any secondary structure. RPA is then replaced by RAD51 which is the main protein involved in HR. RAD51 is a recombinase that catalyzes the homology search and strand exchange with a homologous sequence ensuring accurate repair of the DSB. RAD51 strand exchange activity is inhibited *in vitro* by the addition of RPA to the reaction before or at the same time as RAD51. However, addition of RAD51 prior to RPA allows for efficient strand exchange [35]. This indicates that another protein is required to remove RPA from the single stranded DNA to facilitate the binding of RAD51. Addition of the protein RAD52 *in vitro* allows for this replacement to occur and promotes DNA strand exchange by RAD51 [36, 37]. Thus, RAD52 binds to RPA and RAD51 facilitates the replacement of RPA with RAD51 on single stranded DNA forming the RAD51 nucleoprotein filament for strand invasion.

The RAD51 nucleoprotein filament is assembled onto single stranded DNA to form a polymer that can extend up to thousands of base pairs. DNA paired with RAD51 is highly extended and is approximately 50% more extended than B form DNA duplexes. Studies have shown that only the RAD51-single stranded DNA nucleofilament is able to join DNA ends [38]. RAD51 is the eukaryotic homolog to the bacterial RecA protein and similar to RecA, RAD51 is known to catalyze strand exchange activity [39, 40]. BRCA2 also binds to DNA and physically interacts with RAD51 and is required for the formation of DNA damage induced RAD51 foci [41]. The RAD51 nucleoprotein filament is required for strand invasion, homologous pairing, and D-loop formation. The RAD54 protein, an ATPase and RAD51 dependent helicase, promotes RAD51 mediated strand invasion into duplex template DNA [42, 43]. RAD54 promotes strand invasion and D-

loop formation by transiently opening the template DNA duplex, allowing invasion of the RAD51 nucleoprotein filament and binding to homologous sequence.

Homologous pairing of the incoming strand with a template provides a primer for new DNA synthesis to take place and restore information lost at the DSB. In mammalian cells, DNA polymerase η interacts with RAD51 and extends DNA synthesis from the D-loop recombination intermediates [44]. The non-invading end of the DSB is then captured and ligated to the D-loop by DNA ligase-1 forming the hetero-duplexed HJs. These DNA structures are resolved by a resolvase enzyme, possibly by RAD51C in complex with XRCC3 which has been associated with nuclease activity capable of resolving HJ [45]. The RAD51-DNA complex is removed from the repaired DNA possibly by RAD54 which has been shown to remove RAD51 from duplex DNA by ATP hydrolysis [46]. The proteins involved in HR are shown in figure 2.

NHEJ repair involves a group of enzymes that first capture both ends of the DSB, then form a molecular bridge that brings the two ends together, and finally re-ligate the broken DNA ends. Proteins required for NHEJ are DNA dependent protein kinase catalytic subunit (DNA-PK_{CS}), Ku70, Ku80, DNA Ligase IV, and XRCC4. The NHEJ process is initiated by the binding of Ku70/80 heterodimer to both ends of the DSB. Ku70/80 has very high affinity for DNA and is abundant in the nucleus [47]. The association of the Ku70/80 heterodimer with DNA ends acts as a scaffold for the assembly of other NHEJ enzymes. DNA-PK_{CS} is recruited to the DSB and forms a synaptic bridge between the two ends bringing them together in close proximity [48, 49]. Once the DNA ends are tethered together in this Ku70/80 and DNA-PK_{CS} complex, DNA ends are processed. Non-compatible DNA termini are processed by either the addition of

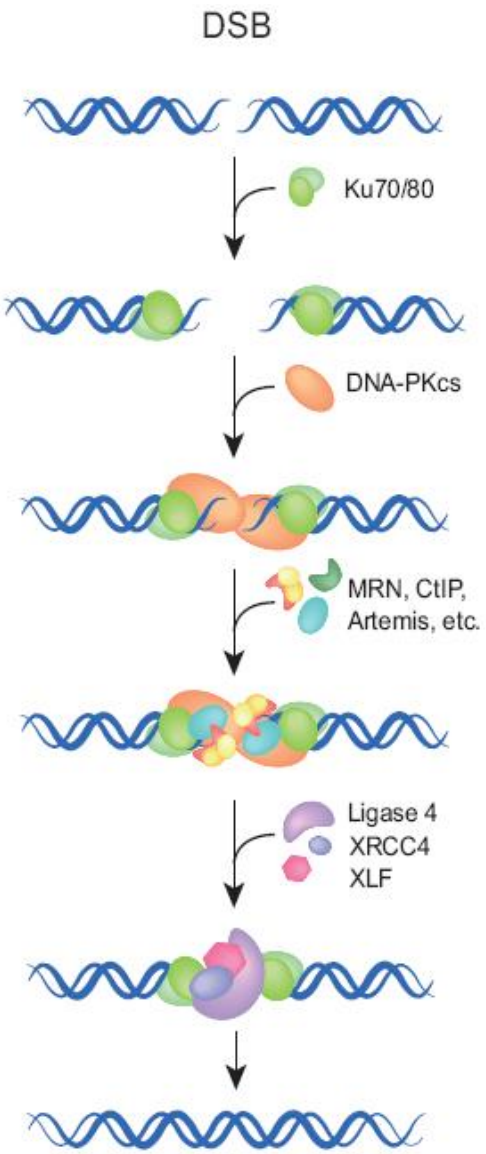

Figure 3: The mechanism of non-homologous end joining [34]

nucleotides or removal of necleotides. DNA polymerase μ and λ can add nucleotides to DNA ends and DNA polymerase μ can add nucleotides in the absence of a template [50]. The enzyme Artemis forms a complex with DNA-PK_{CS} and endonucleolytically cuts single stranded DNA overhangs [50]. The processed DNA ends are ligated by the ligase IV/XRCC4 complex in “classic” processing. A subset of DSBs that have partially

complementary overhangs can be joined together by the ligase IV/XRCC4 complex in conjunction with the XLF/Cernunnos complex. Together, these complexes mediate the joining of one single stranded overhang with the other DNA end followed by gap filling by DNA polymerase [51, 52]. The proteins involved in NHEJ are shown in figure 3.

NHEJ is usually not error free. Nucleases such as FEN-1 exonuclease are involved in end-processing of the DBS before re-ligation which results in the loss of nucleotides [53]. DNA polymerases μ and λ can potentially slip backwards on their template during synthesis which results in direct repeats [54, 55]. The activity of DNA polymerase μ can result in template independent addition of nucleotides. These structures can fold back on themselves and form a step-loop structure and act as a primer/template substrate [50]. This results in inverted repeats which have been observed with chromosomal translocations in humans [56, 57].

Additionally, there is evidence for an even more error prone mechanism of NHEJ termed alternative NHEJ (Alt-NHEJ). There is evidence that alternative NHEJ is mechanistically distinct from classical NHEJ. One study found that alt-NHEJ shares Ku/CtIP-mediated end processing in common with SSA and and HR. Alt-NHEJ is Ku70 independent and is also inhibited by Ku70 [58]. CtIP is essential for chromosomal translocation formation through alt-NHEJ [59]. Alt-NHEJ is significantly reduced in CtIP-depleted cells whereas classical NHEJ is increased. Another study found that alt-NHEJ, independent of the DNA-PKcs/XRCC4/DNA ligase IV complex, required synapsis activity of PARP-1 and ligase activity of XRCC1/DNA ligase III, proteins in common with BER [60]. The requirement for PARP-1 for alt-NHEJ is dependent on the absence of Ku but not DNA-PKcs and does not require microhomologies. PARP-1 and

Ku compete for repair of DSBs by classical or alternative pathways [61]. In a study with Ku-deficient hamster cells, 85% of alt-NHEJ repair junctions in CHOK1 cells were formed upon using either no or one overlapping base and only 15% by longer microhomologies. This study also showed that Ku-independent alt-NHEJ is slow and highly error-prone as DSB ends are subject to continuous degradation [62]. DNA ligase I and III cooperate and are required for MMEJ [63, 64], and it has also been shown that they mediate ligation in alt-NHEJ.

Aberrant NHEJ, by classical or alternative repair, is the assumed mechanism involved in the formation of translocations as no significant sequence homology has been found at mapped translocation breakpoint junctions from AML and ALL patients [22, 23].

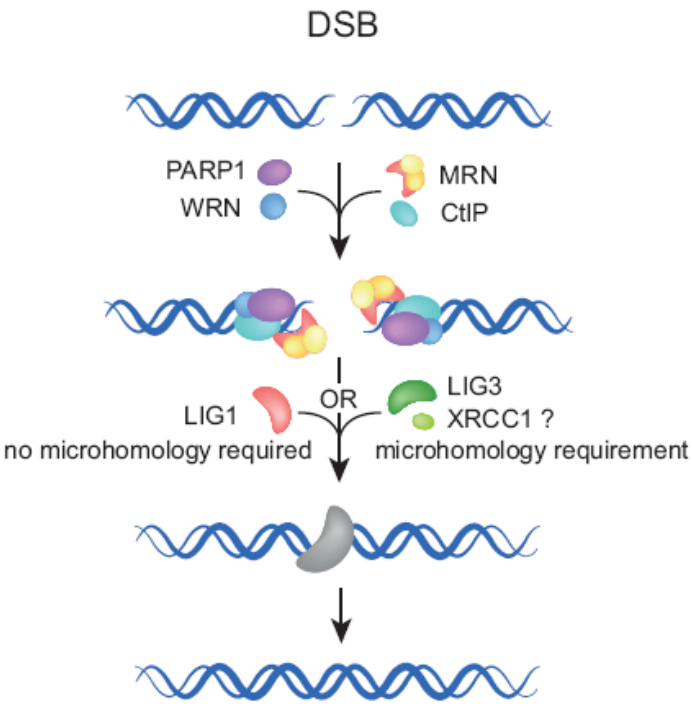

Figure 4: The mechanism of alternative non-homologous end joining [34]

1.3 DNA Repair and the Cell Cycle

The stage of the cell cycle influences the type of DSB repair that occurs. DSBs commonly occur during DNA replication when the replication fork collapses upon encountering an unrepaired DNA single-strand break (SSB). These DSBs stimulate HR activity resulting in the repair and restarting of the stalled replication fork [65]. It is estimated that about 10 collapsed and stalled replication forks result in HR during each round of DNA replication where the sister chromatid is used as the homologous template. NHEJ is necessary to repair DSBs in the absence of a homologous template and it has been shown that cells defective in NHEJ show severe defects in the ability to repair DSBs as well as high radiation sensitivity throughout the cell cycle [65]. Given the absence of homologous templates during the G1 phase of the cell cycle, cells in this stage may be more susceptible to chromosomal rearrangements by NHEJ repair mechanisms following multiple DSBs. Cells that proliferate and thus replicate DNA rapidly are at an increased risk of DNA damage as genotoxins have increased access to DNA strands undergoing transcription or replication [66]. In addition, a shorter cell cycle increases the chance that damaged DNA will be aberrantly repaired potentially resulting in cancer.

1.4 Hematopoiesis and Hematopoietic Stem Cells

Stem cells are considered the guardians of the genome in that they are responsible for continuously replenishing damaged or apoptotic cells. There are multiple pathways that protect the integrity of the genome including the DNA damage response (DDR), cell-cycle checkpoints, and DNA repair mechanisms; however, inaccurate repair of damaged DNA, especially DNA double strand breaks (DSBs) can ultimately lead to genome rearrangements and tumorigenesis.

Hematopoietic stem cells (HSCs) are a unique and very small population of cells that are responsible for generating myeloid and lymphoid cells of all lineages. They are multipotent and self-renewing by means of asymmetric cell division. Asymmetry in cell division ensures self-renewal as one daughter cell is identical to the original HSC and one daughter cell is slightly more differentiated. The more differentiated cell progresses to generate more committed progenitors and ultimately one of eight types of mature, terminally differentiated cells of either myeloid or lymphoid lineage [67]. This process of differentiation and proliferation is called hematopoiesis (Figure 5). Considering that

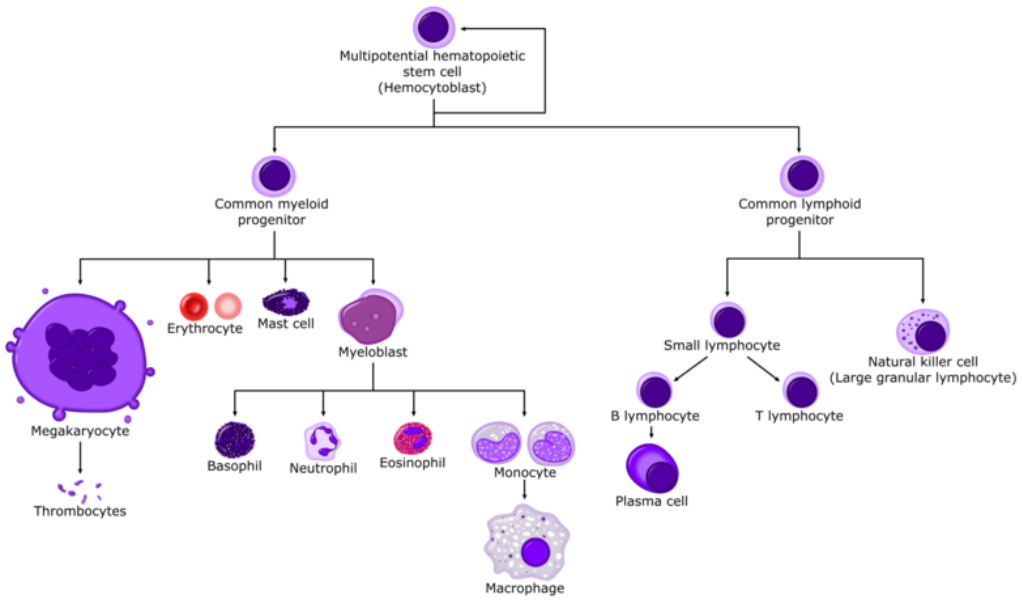

Figure 5: The process of hematopoiesis

mature terminally differentiated hematopoietic cells have a limited lifespan ranging from 1 day for neutrophils to 120 days for erythrocytes, these populations must be continuously maintained by the very small population of HSC clones. Less than 0.1% of all hematopoietic cells in the bone marrow are HSCs and it has been estimated that 10^{11}-

10^{12} mature hematopoietic cells are generated each day by this very small population [68].

The ability of embryonic stem cells (ESC) to be committed to developing into specific cell types is dependent on gene regulatory networks which are dependent on transcription factors and biological signals [69, 70]. The hematopoietic system is ideal for studying these regulatory networks as such a large number of hematopoietic cells from all lineages are produced daily from the small pool of HSCs [71, 72]. The formation of each cell type must be highly regulated and responsive to external environmental stimuli. It is likely that the processes leading to each terminally differentiated cell type requires the expression of distinct lineage specific factors, and it has been shown that different stages of hematopoiesis as well as the different terminally differentiated cell types have unique expression profiles [71]. Disruption of this highly regulated process by aberrant repair of DNA damage or changes in epigenetic regulatory processes can lead to hematopoietic malignancies such as leukemia and lymphoma.

1.5 Differentiation and Hematopoiesis *in vitro*

Development is a modular and hierarchical process in which a common template is categorically refined to have various and distinct properties [69]. Hematopoiesis is the developmental process during which undifferentiated stem cells serve as a common template and give rise to proliferating progenitor cells that progress into differentiated cells of both the myeloid and lymphoid phenotypes. Over the past several years, protocols for *in vitro* differentiation of mouse ESCs into a variety of different lineages including hematopoietic, neural, muscular, skeletal, melanocyte, keratinocyte, follicle and epithelial cell types have been successful. There have also been some successes in the

development of pancreatic and hepatic cell types. Overall this provides a vast framework for studying the entire developmental process from ESCs to populations of differentiated cell types which has vast potential for future developments in drug discovery and cell replacement therapy [73, 74]. The hematopoietic system is an ideal *in vitro* model for studying differentiation in that the earliest stages of hematopoietic development occur within embryoid bodies (EBs) without the addition of other growth factors and follow a sequence of events similar to the developing embryo [75].

A measure of HSC activity is the *in vivo* long-term repopulation assay in which HSCs are injected into lethally irradiated mice followed by determination of reconstituted donor blood after 6 months [76]. *In vitro* assays can also measure HSC activity and detect hematopoietic progenitor populations. The long-term culture initiating cell (LTCIC) is the earliest hematopoietic progenitor cell measured *in vitro*. These cells are present at a frequency that closely correlates with the frequency of HSCs, 1 in 10,000 to 100,000 total blood cells [77, 78]. LTCICs, can initiate long-term hematopoietic cultures for 5-10 weeks while being maintained on preformed stromal feeder layers. In this assay, committed progenitor cells known as colony forming units (CFU) can be quantified by stimulating differentiation and proliferation into mature hematopoietic colonies in semi-solid media followed by quantification of the resulting colonies [79, 80]. Colonies derived from LTCICs or CFUs can be detected after 7-14 days in culture. Mature colony types include colony forming unit- granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM), granulocyte-monocyte (CFU-GM), granulocyte (CFU-G), monocyte (CFU-M), erythrocyte (CFU-E), megakaryocyte (CFU-Mk), and mast (CFU-Mast). Colonies that contain multiple cell types such as CFU-GEMM and CFU-GM are

presumably initiated by immature progenitor cells that are capable of differentiation into multiple cell types, indicating LTCICs or HSCs. Colonies that contain only a single population of mature cells such as CFU-M, CFU-E, CFU-G are presumably derived from more differentiated progenitor cells that are lineage committed and expand into a single cell type. A schematic of *in vitro* differentiation and examples of differentiated cells are depicted in figure 6.

Murine ESCs *in vitro* either self-renew or differentiate based on culture conditions. Their pluripotent state can be maintained when grown on a layer of feeder cells or on gelatin coated culture dishes in the presence of leukemia inhibitory factor (LIF) and serum [81-83]. The nuclear protein transcription factors Sox2, Oct3/4, and Nanog are also critical for ESC maintenance in both murine and human ESCs [84-86]. Removal of ESCs from this culture environment and subsequent culture in suspension media or on stromal layers allows for differentiation of ESCs into EBs. EBs are aggregates of various types of cells including endothelial, neuronal, muscle, and HSCs [87-90]. It has been noted that EBs in culture form cystic structures that that contain blood islands that are similar to those found in the yolk sac [91]. Other studies have previously determined that myeloid and erythroid lineages begin to develop in EBs [92, 93]. The emergence of hematopoietic progenitors at this stage of differentiation can be determined by the presence of lineage specific markers via gene expression analysis, immunohistochemistry, and fluorescence activated cell sorting (FACS) [94-96].

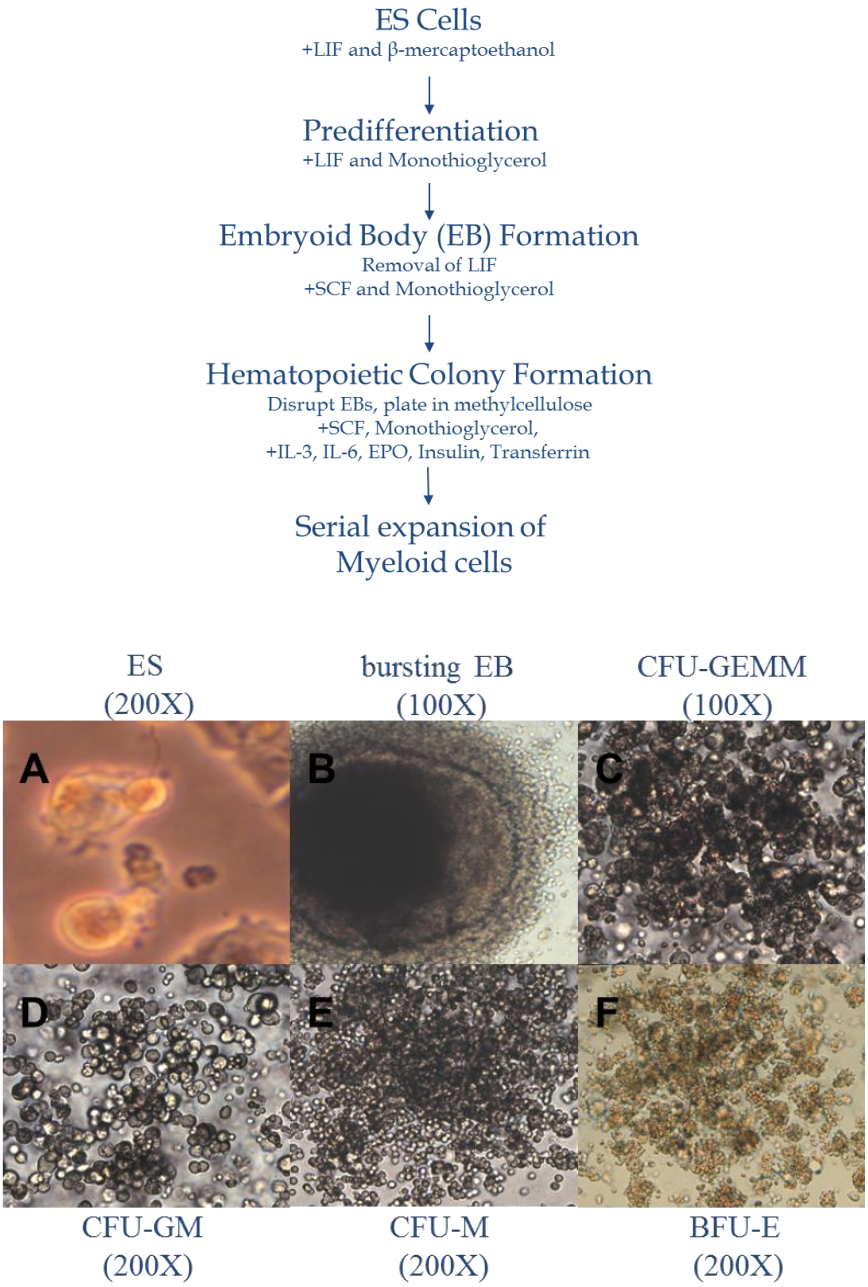

Figure 6: *In vitro* myeloid differentiation of embryonic stem cells

A method for *in vitro* differentiation of murine embryonic stem cells is described above with the necessary cytokines and signaling molecules required for myeloid differentiation.
Pictures: (A) Embryonic stem cells; (B) bursting embryoid body; (C) colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte; (D) colony forming unit-granulocyte, monocyte; (E) colony forming unit-monocyte; (F) blast forming unit-erythroid.

HSCs are negative for lymphocyte, monocyte, granulocyte, and erythroid lineage markers which has been described as lineage negative (Lin^-) [97]. Murine HSC populations have been determined to express the cell surface differentiation markers Sca-1, Thy-1, c-kit, and the stem cell factor (SCF) receptor [98, 99]. Cells expressing these markers are the only cells from the mouse bone marrow capable of reconstituting all blood cell lineages in lethally irradiated mice [100]. The common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) represent the earliest detectible commitment decision between myeloid and lymphoid pathways in hematopoiesis [101, 102]. CMPs and CLPs can also be distinguished by specific cell surface markers. CMPs express the granulocyte macrophage colony stimulating factor receptor (GM-CSFR) and do not express the cell surface interleukin -7 (IL-7) receptor; CLPs however express the IL-7 receptor but do not express GM-CSFR [102].

Cells within EBs produce various cytokines in the absence of growth factors other than serum initiating the first stages of hematopoiesis [103, 104]. However, the addition of specific combinations of cytokines can induce the production of specific mature hematopoietic populations. Following the development of EBs, a secondary culture process promotes differentiation of HSCs into distinct hematopoietic colonies. Different techniques have been developed to promote hematopoietic differentiation including culture on stromal layers [90, 105, 106], culture in chemically defined media with hematopoietic inducers [107], and culture in semisolid methylcellulose media containing cytokines [75, 104]. CFU assays performed by plating cells from EBs in methylcellulose have demonstrated that all myeloid progenitors that are found in the bone marrow are also present in EBs [93, 104], validating *in vitro* differentiation methods. *In vitro*

differentiation of ESCs has the potential to be useful in treating hematopoietic diseases. Methods for the *in* vitro generation of specific hematopoietic lineages have been determined.

1.6 Childhood and Infant Leukemia

Leukemia is a hematopoietic malignancy of the bone marrow and blood that is characterized by an abnormal increase of immature white blood cells. Leukemia initiates in the bone marrow when a hematopoietic progenitor cell undergoes a leukemic change. This leukemic cell can then rapidly proliferate and self-renew leading to much higher white blood cell counts. Leukemia cells eventually crowd out the normal hematopoietic cells suppressing their development and function. This suppression of normal, healthy hematopoietic cells leads to anemia, low erythrocyte counts; neutropenia, low neutrophil counts; and thrombocytopenia, low platelet counts. Anemia leads to fatigue and shortness of breath due to a lack of circulating oxygen, neutropenia causes the immune system to be essentially ineffective against invading pathogens and thrombocytopenia leads to bleeding and bruising without any apparent cause.

Childhood acute leukemia accounts for approximately 30 percent of all malignancy in childhood [108]. Infant acute leukemia (IAL) is rare and accounts for 4.5%-19% of all pediatric acute leukemia. IAL occurs at less than one year of age, is typically very aggressive with rapid onset of disease shortly after birth, and generally has very poor prognosis. There are two major morphological types of IAL; acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). ALL accounts for the majority of IAL cases, approximately 80 percent, and AML accounts for

approximately 15-17% of cases. Infant leukemia is typically characterized by genetic abnormalities including duplications, deletions, inversions, and translocations.

Leukemia is primarily a stem cell disease. Leukemic cells mimic the phenotypes of HSCs, and like HSCs these leukemic stem cells (LSCs) produce progeny of heterogeneous populations [109-111]. It has been shown that both chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) can be initiated by a small population of leukemic stem cells [112, 113]. Interestingly, it has also been shown that LSCs can potentially arise from progenitor cells that have developed abnormal phenotypes, such as the ability to proliferate or self-renew [114]

The majority of both infant ALL and infant AML cases have chromosomal translocations involving a specific gene known as the mixed lineage leukemia gene (*MLL*) on chromosome 11q23; ~80% of infant ALL cases and ~60% of infant AML cases [115-117].

1.7 *MLL*-Associated Leukemia

Rearrangements involving the *MLL* gene are considered recurring genetic abnormalities, found in ~10% of patients with acute leukemia overall. *MLL* is involved in recurrent translocations with more than 75 different partner genes, only 9 of which account for ~90% of all *MLL* rearrangements. These include the ALL-fused gene from chromosome 4 (*AF4*), *AF9,* eleven nineteen leukemia (*ENL*), *AF10, AF6,* RNA polymerase elongation factor (*ELL*), *AF1p, AF17,* and *SEPT6* [117].

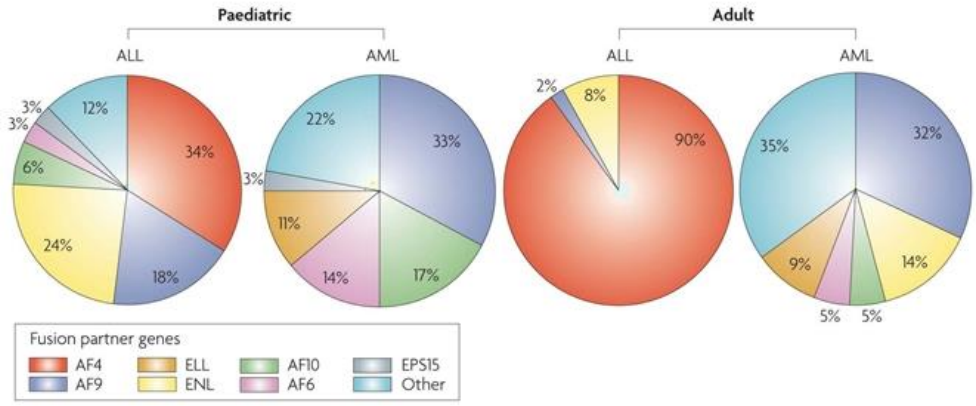

Figure 7: Distribution of *MLL* fusion partner genes in pediatric and adult acute leukemias [117]

Reciprocal translocations, as shown above, result in an exchange of chromosomal segments from the two chromosomes leading to the formation of two derivative chromosomes each containing part of each original chromosome. This results in the deregulation of a gene located at or near the breakpoint by disrupting the coding sequence or transcriptional regulatory elements [118, 119]. Reciprocal translocations can also result in the creation of a new coding sequence by fusing genes from the two original chromosomes. The creation of fusion proteins is commonly seen with reciprocal translocations involving the *MLL* gene and a variety of its fusion partners [108, 118, 120-122]. These recurring rearrangements with the *MLL* gene imply that there is an association between gene expression during hematopoiesis and leukemogenesis. These fusions of *MLL* with partner genes are all "gain-of-function" mutations and result in the expression of chimeric proteins that have altered function [121, 123, 124].

The translocation partner with the *MLL* gene along with the stage of activation likely determines the leukemia phenotype [122]. One common fusion partner with *MLL* is the *AF9* gene located on chromosome band 9p21-22. Fusion of these two genes is

found most commonly in AML whereas fusion of *MLL* with the *AF4* gene is found almost exclusively in acute ALL. This indicates that certain translocations are either more likely to occur at certain stages of development and in specific cell types or the specific translocations determine the phenotype of the cell. The function of the *AF9* gene product is largely unknown, however it has been demonstrated that it has similarities to the *MLL* gene product and may also regulate *Hox* gene expression [125]. AF9 also has a serine and proline rich domain as well as a nuclear localization sequence which are typical of transcription factors.

The proper function of the MLL protein is required for maintenance of gene expression early in embryogenesis [126]. MLL is expressed in most tissues and positively regulates expression of *Hox* genes through histone 3 lysine 4 (H3K4) methyltransferase activity. MLL functions as part of a large macromolecular complex that incorporates methyltransferase and histone acetyltransferase activity. *Hox* genes are essential for accurate spatial development of the embryo due to their patterned expression [126]. It has been determined that the proper temporal expression of *Hox* genes during development is also necessary for regulation of hematopoietic differentiation [120]. *Hox* genes are essential for tissue development and expression is regulated in a spatiotemporal manner by the MLL complex. *MLL*-rearranged acute leukemia typically show high expression levels of *Hox* genes, and it has been strongly suggested that MLL-fusion proteins initiate leukemia through direct upregulation of the A-cluster *Hox* genes. Thus, *MLL* rearrangements lead to leukemia by interfering with the proper transcriptional patterns which typically lead to terminal differentiation of hematopoietic cells.

The *MLL* gene is approximately 120 kb in length, contains 34 exons over a 100kb region of chromosome 11, and it is ubiquitously expressed. The 430kD MLL protein has 4 distinct domains which are shown in figure 8 [127]), two of which are deleted with the occurrence of *MLL* translocations. The SET domain on the carboxyl terminus of the protein which serves to recruit chromatin remodeling proteins for either the activation or repression of chromatin complexes is lost with *MLL* translocations [120]. Thus, the ability for MLL to correctly activate or repress the expression of specific genes is lost. The PHD domain, also known as the activation domain, may bind to tri-methylated H3K4 and aid in transcriptional repression. This domain is also deleted with *MLL* translocations. The remaining two domains of MLL, the AT hook domain and the methyltransferase domain, are both DNA binding domains and constitute the amino terminal half of the protein. AT hook domains bind to AT rich regions of DNA based on homology and the methyltransferase domain recognizes methylated DNA and is capable of non-specific binding [120]. These two domains are located 5' of the breakpoint cluster region (bcr) of the *MLL* gene and are retained as the amino portion of the fusion proteins that result from *MLL* translocations. These two DNA binding domains of the MLL protein are likely sufficient to target the derivative fusion proteins to the correct binding sites, however the expression of target genes becomes deregulated due to the new function of the chimeric protein.

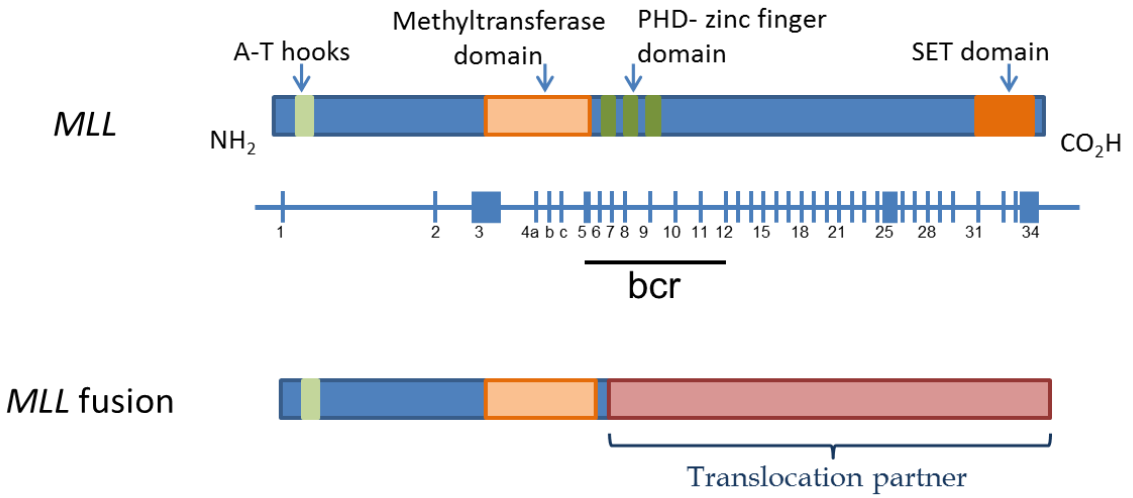

Figure 8: *MLL* and *MLL*-fusion partner structure

MLL translocation breakpoints in patient samples have nearly all been shown to occur in an 8.3 kb BamHI fragment known as the breakpoint cluster region. Certain elements such as topo II cleavage sites, Alu elements, scaffold-associated regions (SARs) and DNase I hypersensitive sites have been found in bcrs of various genes and are predicted to play a role in translocations [25, 128]. The *MLL* gene contains all of these elements within its bcr. The *AF9* gene contains three of them within its two separate bcrs (Figure 9). These elements may play a role in promoting the illegitimate recombination that occurs between these two genes. The risk for certain translocations to occur most likely changes during the developmental process due to changes in gene expression. This may also indicate that hematopoietic cells can become altered in their ability to faithfully repair DNA DSBs as is evidenced by the occurrence of chemotherapy-related leukemia in patients who have been exposed to DNA damaging agents as treatment for cancer as well as the high incidence of the specific *MLL-AF9* translocation in infant AML [129].

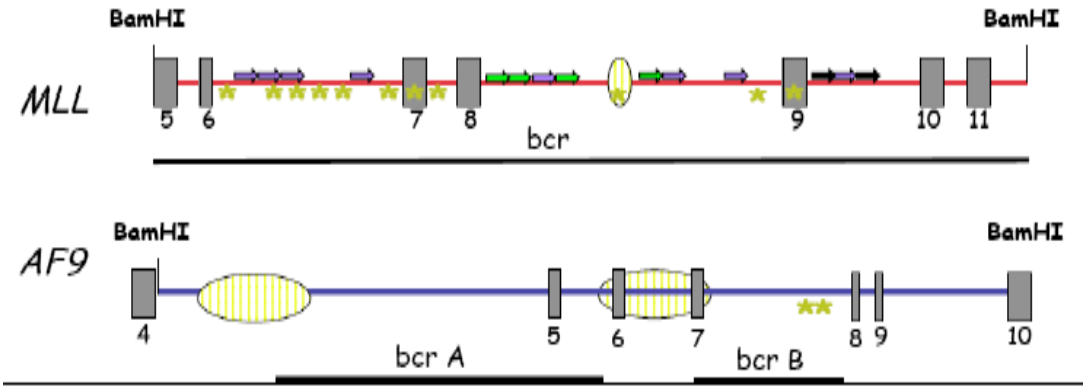

Figure 9: Breakpoint cluster regions of *MLL* and *AF9*

The *MLL* bcr is an 8.3 kb region flanked by BamHI restriction sites. The *AF9* bcr region is a 70 kb region also flanked by BamHI restriction sites. Unlike the *MLL* bcr though, the *AF9* bcr has two peaks of mapped breakpoints (bcrA and bcrB). Grey rectangles are exons; yellow ovals are MAR/SAR sites; gold stars are topo II cleavage sites; and different colored arrows are multiple repetitive elements in the same orientation in *MLL* (these elements have not been mapped in *AF9*). *Intron/exon boundaries have been redefined and consequently, 4 more exons have been characterized. The exons above are actually numbered with an addition of 3 to what is shown [130].

1.8 Mechanisms of MLL and MLL-Fusion Protein Function

MLL is post-translationally processed by proteolytic cleavage. It is cleaved by the threonine-aspartase TASPASE1 into an N-terminal 320 kDa fragment (MLL^N) and a C-terminal 180 kDa fragment (MLL^C) [131, 132]. After cleavage, the two fragment associate with each other via non-covalent interaction between phenylalanine and tyrosine rich domains termed FYRN and FYRC on MLL^N and MLL^C respectively. Subnuclear localization signals in MLL^N translocate MLL to the nucleus.

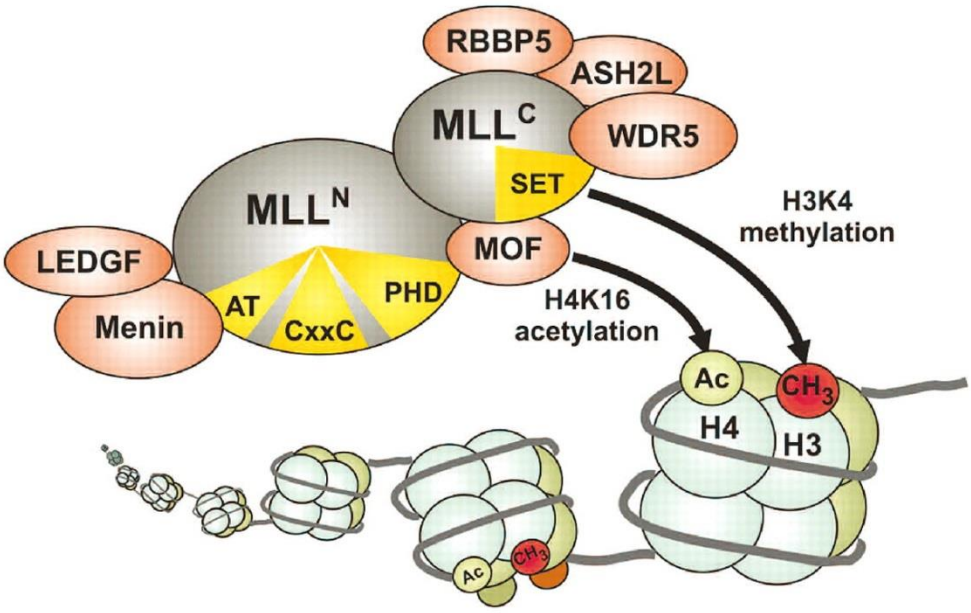

Figure 10: The MLL protein complex [118]

MLL functions as part of a large protein complex that includes menin (the product of the tumor suppressor gene multiple endocrine neoplasia) and LEDGF (lens epithelium derived growth factor) interacting with MLL^N and MOF (mammalian ortholog of drosophila "males absent on the first"), WDR5 (WD repeat domain protein 5), RbBP5 (retinoblastoma binding protein 5), and ASH2L (absent, small, or homeotic 2 like) interacting with MLL^C [133-135]. The N-terminal trimolecular complex of MLL^N, menin, and LEDGF is essential for the proper targeting of MLL or MLL fusion proteins to specific target genes such as *HoxA9* for transcriptional up-regulation [136, 137] The WDR5 protein mediates interaction between the MLL^C catalytic subunit and the core complex and also mediates interaction with the histone H3 substrate [138]. Additionally, MOF acetyltransferase activity is required for full transcriptional activation of target genes in conjunction with MLL methyltransferase activity. MLL-mediated methylation of

H3K4 and MOF-mediated acetylation of H4K16 have been determined to be correlated by the distribution of these marks on active genes [135].

The protein-protein interactions described with the MLL^C catalytic subunit are invariably lost in MLL fusion proteins and do not contribute to aberrant activity and leukemogenesis. The function of the MLL fusion protein is dependent on the activity of the fusion partner. Studies have determined that the leukemogenic function of MLL fusion proteins may be activated and initiate leukemogenesis in one of four different ways [118].

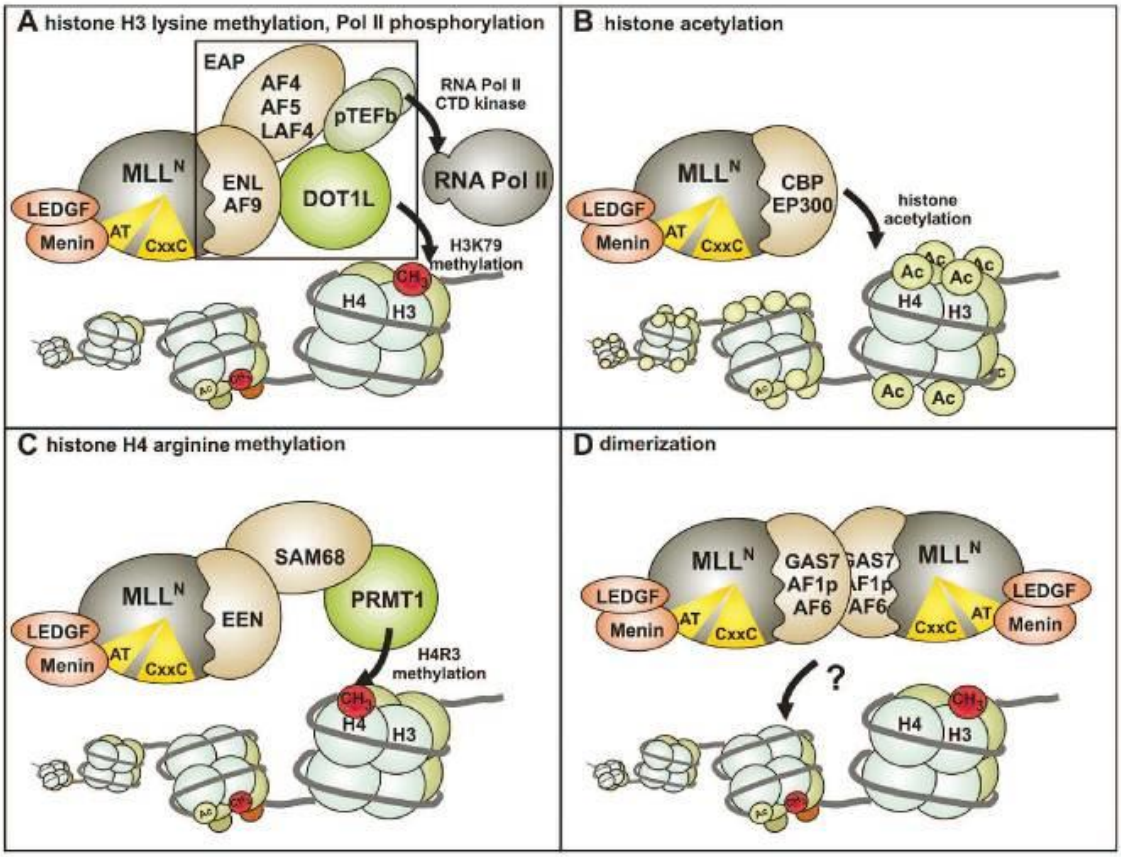

Figure 11: Molecular pathways leading to oncogenic activity of MLL fusion proteins [118]

1. Transcriptional elongation and histone methylation likely accounts for the leukemogenic activity of common fusion partners *ENL*, *AF9*, *AF4*, *ELL*, and *AF10*. The protein complex known as *ENL* associated protein complex (EAP) associates *ENL* with all members of the AF4 protein family that are known fusion partners with *MLL* [139]. The EAP complex also associates with positive transcription elongation factor b (pTEFb) and histone methyltransferase DOT1L. Phosphorylation of the carboxyl terminal repeat domain of RNA polymerase by pTEFb results in transcriptional elongation. *MLL* fused with a pTEFb associated protein could up-regulate the expression of MLL target genes. DOT1L methylates H3K79, an activity that also happens during transcriptional elongation [140]. AF4, AF9, AF10, and ENL have been shown to associate either directly or indirectly with DOT1L [141-143]. Association of the MLL fusion protein with DOT1L would confer positive regulation of transcriptional activity which could induce leukemogenesis via ectopic target gene expression.

2. Histone acetylation likely accounts for the leukemogenic activity of fusion partners CBP and p300. A few cases of therapy related secondary acute leukemia have shown MLL fusions with the histone acetyl-transferase CREB binding protein (CBP) and the related p300 protein. Activity of the bromo- and histone- acetyltransferase domains confer oncogenic function to the MLL fusion protein via hyperacetylation of chromatin and activated transcription of target gene.

3. Arginine specific histone methylation accounts for the leukemogenic activity of fusion protein MLL-EEN [144]. The *MLL-EEN* fusion is rare and has been cloned from a single case of *MLL* leukemia. EEN has been shown to bind to the arginine methyltransferase PRMT1 through an adapter protein known as SAM68. PRMT1 is an

arginine specific methyltransferase that may activate transcription through H4R3 di-methylation. This specific histone modification has been associated with an increase in histone acetylation which is known to activate transcription.

4. Dimerization of cytoplasmic fusion partners accounts for the leukemogenic activity of *MLL* fused with *GAS7*, *AF1p*, and *AF6*. These fusions are typically found in older patients and are weakly transforming compared to *MLL* fusions with genes coding nuclear proteins. Each of these fusion partners has a coiled-coiled dimerization domain which is the minimal necessary contribution in order to induce leukemogenesis. The nuclear localization signal of MLL transports these cytoplasmic fusions to the nucleus. This leads to aberrant activation of molecular signaling pathways by sequestering these cytoplasmic proteins [145]. Dimerization of the fusion proteins leads to aberrant transcriptional activation of MLL target genes and initiation of leukemogenesis.

1.8.1 *MLL* and *AF9* Translocations

The *AF9* gene, also known as *LTG9* and *MLL*T3 spans >100kb and is located on chromosomal position 9p22 and is one of the most common translocation partners with *MLL*. It has been shown [115, 120, 121]. Sequence analysis of *MLL-AF9* translocation junctions from patient-derived samples has shown evidence of small deletions and insertions, large deletions, duplications, and some complex rearrangements [146]. The *MLL-AF9* translocation has been associated with ALL, AML, as well as therapy related AML (t-AML), also known as secondary AML, after treatment with etoposide [147]. The *MLL-AF9* translocation, written as t(9;11)(p22;q23), is the third most common genetic abnormality reported in infant ALL cases and is the most common genetic abnormality reported in infant AML cases. Studies with *in vitro* and *in vivo* models have shown that

MLL fusion genes play an important role in the initiation of leukemogenesis and that the *MLL-AF9* gene fusion is sufficient for leukemia stem cell transformation [114, 148, 149]. Leukemias that carry the *MLL-AF9* translocation are typically aggressive, difficult to treat, are often resistant to traditional therapy regimens, and this is especially true with infant AML [25, 150]. The five-year overall survival rate of patients with the *MLL-AF9* translocation is approximately 40% [151, 152]; however a considerable number of patients have relapses and end up dying of the disease [153].

The mechanism of AF9 function is currently not fully elucidated. AF9 has been shown to interact with AF4 at subnuclear loci which suggests that AF9 acts as part of the EAP complex as well [141]. The murine homolog to human AF9 has been shown to play a role in embryo patterning and proper development via Hoxd4 expression, similar to the role of human MLL [125]. It has also been shown that AF9 may play a distinct role in myeloproliferation [149]. The *AF9* gene has two bcr regions that have been identified between two BamHI sites. Bcr1 is located within intron 4 and bcr2 spans introns 7 and 8. Both bcr regions are bordered by two SARs and a topoII cleavage site and a DNAseI hypersensitive site are located in intron 7 of bcr2 [154]. The two bcrs and topoII cleavage sites were initially identified from clinical samples of infant AML, t-AML, and adult ALL patients [155]. The topoII cleavage sites and SARs located in both the *MLL* and *AF9* bcr regions likely contribute to the formation of *MLL-AF9* recurring genetic abnormalities.

1.9 Topoisomerase II and Chromosomal Translocations

There are several possible causes of DNA DSBs that could lead to *MLL-AF9* translocations. One possible factor leading to these translocation is the inhibition of the

topoII enzyme by topoII poisons. TopoII is an essential and ubiquitous enzyme that is required for the survival of all eukaryotic organisms [156-158]. TopoII functions to regulate under- and over-winding of DNA and remove knots and tangles in the genome by passing an intact double helix through a transient DSB that it generates in a separate double helix [159-161]. TopoII is required for transcription and replication and also plays a role in chromosome organization and segregation [162-164].

Previous studies have elucidated the topoII reaction and have determined that the catalytic cycle of double stranded DNA passage has six distinct steps in one round of enzyme activity [165].

1. DNA binding

TopoII interaction with DNA is determined by nucleotide sequence and the topological structure of DNA substrates. TopoII binds to DNA at preferred nucleic acid sequences that are within its binding/recognition sites [166-169]. The stringency of topoII sequence recognition however, is low reflecting its ability to function in a global manner. TopoII binding is also regulated by the topological structure of DNA and preferentially interacts with negatively or positively supercoiled DNA over relaxed DNA [170]. Unusual DNA structures are also recognized by topoII such as hairpins[171], Z-form DNA [172], and DNA tetraplexes [173].

2. Pre-strand passage DNA cleavage/relegation

TopoII generates a DNA cleavage/relegation equilibrium prior to the passage of the second double-stranded DNA molecule [174, 175]. The presence of a divalent cation is necessary for this step and every subsequent step in the catalytic cycle [176, 177]. TopoII generates a DSB by creating nicks on each strand and leaving 4 base 5’ overhangs

[178-180]. During this scission reaction, topoII forms covalent bonds between the 5' termini of the DNA and its active site tyrosyl residues [178, 181].

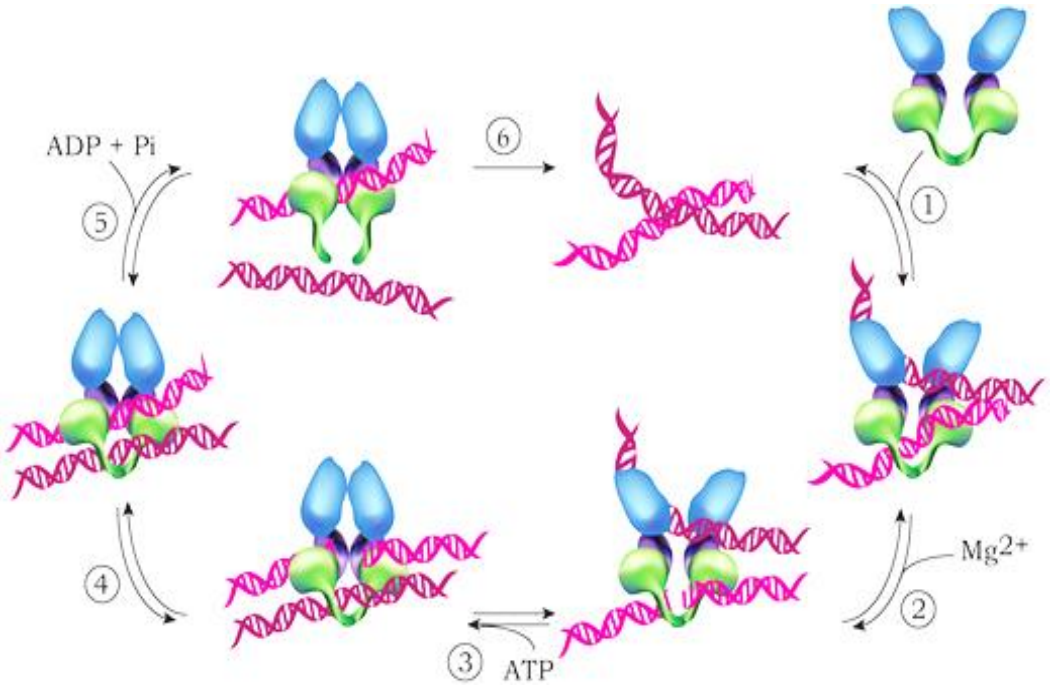

Figure 12: The catalytic cycle of topoisomerase II [182]

3. DNA strand passage

TopoII undergoes a conformational change upon binding of ATP that triggers DNA strand passage [183-185]. ATP is not required for either cleavage or relegation, although its binding triggers the translocation of the intact DNA strand through the DSB in the other DNA molecule [186]. It has also been determined that ATP hydrolysis is required for enzyme recycling [184]. ATP hydrolysis is not required for the strand passage event; however, this step proceeds more quickly with hydrolysis of bound ATP [186].

4. Post-strand passage DNA cleavage/relegation

Following the DNA strand passage event, topoII establishes DNA cleavage and religation equilibrium as in the second step of the catalytic cycle [184]. This topoII-DNA complex is similar to the pre-strand passage topoII-DNA complex. The post-strand passage complex is 2-4 fold more stable than its pre-strand passage counterpart due to the binding of ATP [185, 187].

5. ATP hydrolysis

ATP hydrolysis triggers a conformational change and consequently the opening of the protein clamp and release of the intact DNA products [185, 187, 188]. Phosphorylation of topoII by protein kinase C stimulates the rate of ATP hydrolysis by approximately 2-3 fold and may enhance the overall catalytic activity of topoII[189].

6. Enzyme recycling

TopoII regains the ability to initiate a new catalytic cycle following the hydrolysis of ATP. TopoII either remains bound to the same DNA substrate and initiates a new round of catalysis in a processive fashion or dissociates and initiates a new catalytic cycle on a new DNA substrate in a distributive fashion. Whether the enzyme behaves in a processive or distributive fashion likely depends on reaction conditions and the presence of positive or negative supercoils in the original DNA substrates [190].

Cellular concentration of topoII is tightly regulated. Typically, topoII is present at low steady-state levels *in vivo* and the topoII-mediated DNA cleavage is tolerated without deleterious effects [191, 192]. It has been shown that cells with lower levels of topoII are unable to undergo chromosome segregation and die of mitotic failure [193-196]. TopoII-DNA cleavage complexes are required for completion of the catalytic cycle; however, they can also lead to DSB and be potentially deleterious to the cell. While transcription or

translation proteins traverse along DNA and collide with a covalently bound topoII-DNA cleavage complex, this leads to a permanent DSB [197, 198]. The DSB then initiates the DNA damage response and recombination and repair pathways [195, 199]. This stimulates sister chromatid exchange, the generation of deletions, insertions, translocations, and other abnormalities [165, 200, 201]. Conditions that increase the physiological concentration or the lifetime of topoII-DNA cleavage complexes, significantly increase the generation of mutagenic events [202, 203].

1.10 Topoisomerase II Inhibitors

Given the importance of topoII enzymatic activity for cellular function, drugs that target this enzyme are some of the most active drugs available for the treatment of cancer. Approximately half of all chemotherapy regimens include a drug targeted to topoII. [199, 204, 205]. These drugs function by increasing the cellular levels of topoII-DNA cleavage complexes and are termed topoII "poisons" because they convert the enzyme into a detrimental cellular toxin [204, 206, 207]. TopoII poisons function by two mechanisms which are not mutually exclusive [165, 199, 208]. Some compounds impair the ability of topoII to religate cleaved DNA ends, while others increase the forward rate of DNA scission. TopoII poisons do not act as simple catalytic inhibitors by blocking the overall catalytic activity of the enzyme [209, 210].

TopoII poisons can be classified into three categories by mechanism of action. The first increases cellular levels of topoII-DNA cleavage complexes by non-covalent interaction at the topoII-DNA interface; the second acts by covalent modification of topoII; the third acts by covalent modification of DNA structure [211]. All topoII targeting drugs currently in clinical use such as etoposide, mitoxantrone, and doxorubicin

act by non-covalent interaction with the enzyme bound to DNA. Several naturally occurring compounds such as bioflavonoids and catechins have also been shown to be topoII poisons by non-covalent interaction. These topoII poisons are all polyphenolic compounds with variations in their ring structures [212]. The best characterized topoII poisons that covalently modify the enzyme are quinones which are thought to cross-link the N-terminal protein gate and block activity [213, 214]. Covalent modifications of DNA that distort the double helix also act as topoII poisons. These poisons can be in the form of abasic sites or ethano-bases [215-217].

Drugs that target topoII are also implicated in the initiation of leukemia-associated chromosomal translocations [208]. Secondary, or therapy related acute leukemia, first became acknowledged in the late 1980s by the observance of balanced chromosomal translocations that coincided with the introduction of epipodophyllotoxins such as etoposide and teniposide into clinical use. These epipodophyllotoxins were associated with leukemia after combination chemotherapy for treatment of non-small cell lung cancer [218] and adult and pediatric primary leukemias and solid tumors [219-222]. Treatment regimens for ALL that incorporated teniposide had a 6-year cumulative risk of developing AML of >12% [219]. These drugs function by disrupting the cleavage/religation equilibrium of topoII, stabilizing the cleavage complex on DNA leading to DSBs and aberrant repair.

Over 50% of therapy related leukemias have translocations within the *MLL* bcr [223-226]. These therapy-related *MLL* rearrangements are unique to chemotherapy regimens that use topoII-targeted drugs. DNA breakpoints found in patients with these leukemias have been shown to be in close proximity to topoII cleavage sites [227-229].

Several studies have reported that the widely used chemotherapeutic drug etoposide can initiate *MLL* rearrangements in primitive hematopoietic stem cells and in human fetal hematopoietic stem cells [129, 230-232]. It has also been shown that etoposide induces chimeric *MLL* gene fusions in mouse embryonic stem cells [233]. Other chemotherapeutic agents are associated with *MLL* rearrangements by topoII inhibition including anthracyclines and dactinomycin. Thus, these topoII targeted drugs are potent inhibitors of the enzyme that induce chromosomal breakage and aberrant repair of these breaks leads to leukemic translocations [224].

The involvement of topoII in DNA cleavage and *MLL* translocation events is widely accepted; however, the mechanism by which these translocations occur is still not fully elucidated. There is evidence that breaks induced by topoII play a direct role in inducing *MLL* translocations [225, 234, 235]. An alternate theory is that apoptotic nucleases play a major role in the translocation process while topoII has a more indirect role [236, 237]. In fact, a site of apoptotic cleavage has been located in the bcr of the *MLL* gene [236, 238]. Thus, the molecular pathways and mechanisms that lead to *MLL* translocations are likely very complex and involve elements of DNA repair proteins as well as apoptotic proteins.

Given that a high percentage of infant leukemias as well as therapy related leukemias display translocations involving the *MLL* gene, it has been hypothesized that exposure to topoII inhibiting agents *in utero* may play an important role in the initiation of infant leukemia. TopoII is more prevalent in cells that are proliferating rapidly. This indicates there may be a critical interval when exposure to topoII poisons in the form of chemotherapeutic drugs, high levels of bioflavonoids, or other inhibiting compounds

along with increased cell proliferation could lead to illegitimate repair events and oncogenic translocations. Also, given the timing of hematopoietic development with rapidly proliferating and differentiating cells, exposure to these compounds likely carries a higher risk earlier in fetal development [239, 240].

Considering the evidence and strong association between topoII inhibitors and leukemia exhibiting *MLL* translocation, dietary or environmental compounds that play a role as topoII inhibitors and may lead to leukemia development need to be identified. Maternal intake and exposure to any of these compounds would certainly be a risk for the fetus developing infant leukemia according to current epidemiological evidence [241-245].

1.10.1 Dietary Bioflavonoids

Bioflavoniods make up a large and diverse group of compounds and are categorized as flavones, flavonols, or isoflavones. They are polyphenolic compounds that are abundant in nature and an integral component of the human diet [246-248]. Common sources of dietary flavonoids are fruits, vegetables, nuts, legumes, plant leaves, tea, coffee, wine and some fungi. Bioflavonoids are the most abundant source of antioxidants and some potential health benefits of these compounds include their use as anticarcinogens, cardioprotectants, and hormone replacement alternatives in menopause [249-251]. The isoflavone genistein is an estrogen derivative and is available over the counter as an estrogen supplement at health food stores. It is also a soy phytoestrogen and is prominent in foods containing soy as well as infant soy formulas [252, 253]. The activity of genistein in cells is likely pleiotropic acting through two different mechanisms. Other bioflavonoids including quercetin, luteolin, and myrecetin are

available as individual health supplements or in complex with multiple bioflavonoids. These bioflavonoid supplements are currently unregulated and widely commercially available in doses as high as 100mg.

Despite their many health benefits, bioflavonoids have cytotoxic and genotoxic properties. Ingestion of these compounds during pregnancy has been linked to the development of infant leukemia, the majority of which feature aberrations in the *MLL* gene [244, 245, 254]. They also been shown to have an adverse effect on the activity of topo II at increased concentrations [255]. Some *in vitro* biochemical cleavage assays have reported that exposure to the bioflavonoid genistein causes topoII mediated DSBs similar to etoposide exposure at the same dose indicating that they act as topoII poisons as well [256, 257]. TopoII inhibition by different bioflavonoids was investigated using human recombinant wild-type topoIIα and topoIIβ and plasmid DNA. It was shown that bioflavonoids were active against topoIIβ with genistein (50 μM) being the most effective at stimulating enzyme-mediated DNA cleavage [258]

One previous study tested 20 different bioflavonoids and demonstrated direct potential for some of these compounds including genistein and quercetin to induce cleavage of the *MLL* gene. Cleavage of *MLL* was was induced *in vitro* in human primary hematopoietic progenitor cells as well as two hematopoietic progenitor cell lines (BV173 and K562) [259]. Other studies have shown that genistein and quercetin are also inhibitors of cell proliferation and may induce differentiation [260-262].

Genistein has been reported to induce DNA sequence rearrangements that are mediated by topoIIβ and the proteasome [256]. This study showed that 100μM genistein was able to induce topoII-DNA cleavage complexes and chromosome rearrangements in

cultured mouse myeloid progenitor cells as well as topoIIβ knockout mouse embryonic fibroblasts. Evidence from this study suggests that genistein-induced infant leukemia involves topoII and processing by the proteasome which leads to chromosomal translocations.

Another important study had demonstrated that synthetic bioflavonoids have the ability to cross the placenta in rats [263]. For this study, a synthetic bioflavonoid EMD-49209 was radioactively labeled and injected intravenously into Wistar rats that were 20 days pregnant. The maternal and fetal distribution of EMD-49209 was then evaluated 1-24 hours after injection. This study demonstrated that bioflavonoids not only cross the placenta but they were also found in all fetal tissues including the fetal brain at approximately 17% of the initial dose. The previously described studies support the hypothesis that bioflavonoid compounds may be linked to the *in utero* development of infant AML as a result of maternal exposure and the fact that these compounds can cross the placenta [263].

1.10.2 Environmental Compounds

Benzene is a ubiquitous environmental pollutant and is ranked among the top 20 chemicals for production in the US. It is commonly used in industry as a solvent or in the production of plastics, dyes, detergents, pesticides, and rubbers. Benzene is also a component of cigarette smoke and is a known human carcinogen. It has been recognized for more than a century that exposure to benzene is toxic especially to the hematopoietic system. The first laboratory experiments demonstrating the toxicity of benzene to hematopoietic organs were in the early 1900's [264]. Association of benzene exposure specifically with leukemia has been noted in many studies since [265-268]. The most

common malignancies related to benzene exposure are AML and acute non-lymphocytic leukemia (ANLL) [269, 270].

Many epidemiological studies, some including large numbers of workers exposed to benzene or benzene-containing mixtures have also linked benzene exposure to the development of leukemia [266, 267, 271]. Collaborative studies with the Chinese Academy of Preventive Medicine (CAPM) and the National Cancer Institute (NCI) confirmed an increased risk of AML as well as non-Hodgkin lymphoma and myelodysplastic syndrome in workers exposed to benzene. These studies also revealed an excessive risk at relatively low levels of exposure (<10 ppm average and <40ppm-years cumulative) [272]. A nested case-control study in Australia reported that exposure to even low levels of benzene can be carcinogenic. Benzene exposure was estimated by occupational history, local site information, and the Australian petroleum industry monitoring data for 79 cases. It was determined that there is an increased risk of leukemia associated with cumulative benzene exposure >8ppm-years. They also indicated that there is no threshold of cumulative exposure below which there is no risk.

Benzene itself does not initiate leukemogenesis directly but causes DNA DSBs through one of its metabolites, namely p-benzoquinone [273-276]. The chemical metabolic processing of benzene to p-benzoquinone is depicted in figure 13. Benzene is metabolized in the liver to benzene oxide by cytochrome P450 2E1. A small portion of benzene oxide is converted to phenol by non-enzymatic rearrangement. Some of the phenol can then be oxidized by cytochrome P450 2E1 to p-hydroquinone which is circulated in the blood. It is converted to p-benzoquinone in the marrow due to a high concentration of MPO. P-benzoquinone is reduced back to p-hydroquinone by NADH:

quinine oxidoreductase (NQO1). It is of note that individuals who are heterozygous or homozygous for the C609T polymorphism of the NQO1 gene, meaning that they cannot reduce p-benzoquinone back to p-hydroquinone, have a much higher risk for developing leukemia with *MLL* rearrangements [277-279].

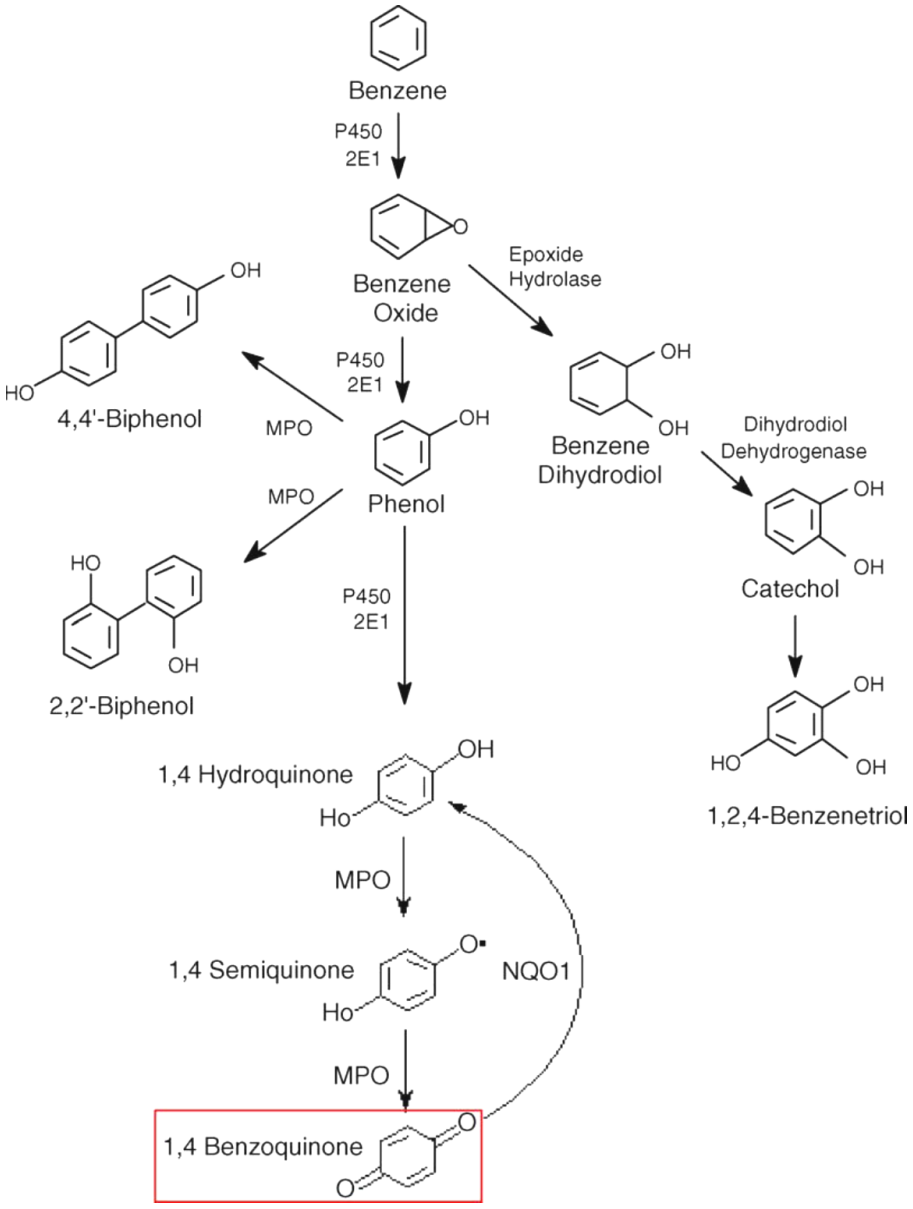

Figure 13: Metabolism of benzene to 1,4-benzoquinone (p-benzoquinone) [280]

Exposure of mammalian cells to p-benzoquinone has been shown to cause DNA mutations and strand breaks, and induce DNA recombination, sister chromatid exchange, and apoptosis [281-283]. Benzene and its metabolites have been shown to increase HR activity in a Chinese hamster ovary (CHO) cell line due to generation of oxidative stress [283]. Exposure to 1, 10, or 30μM p-benzoquinone caused increased recombination of 2.7-, 5.5-, and 6.9-fold respectively within a neo direct repeat recombination substrate. The genotoxic activity of p-benzoquinone has not been fully elucidated. It has been shown to trigger oxidative stress and form adducts with both DNA and proteins, therefore multiple pathways may be involved [284-286] including its effect on topoII [287-289].

It has been determined that p-benzoquinone is a strong topoII poison that is more potent *in vitro* than etoposide due to the fact that it inhibits topoII by binding covalently to the enzyme in a redox-dependent manner thus "freezing" the enzyme/DNA cleavage complex [290]. This study used purified human topoIIα in a plasmid DNA cleavage assay. P-benzoquinone stimulated plasmid DNA cleavage by 8-fold at a concentration of 25μM. Cleavage sites were close to a defined chromosome breakpoint in a case of *MLL* related infant AML.

It has also been shown that p-benzoquinone is a potent topoII poison by covalently binding to the enzyme and inhibiting function [280]. In this study, no DNA cleavage was observed when p-benzoquinone was incubated with reducing agent dithiothreitol (DTT) at 250μM or glutathione at 500μM prior to being added to the topoII-DNA complex. In contrast, addition of DTT or glutathione to the reaction after p-benzoquinone incubation with the topoII-DNA complex did not have any effect on DNA

scission. This indicates covalent attachment in that once a cleavage complex is formed with topoII and p-benzoquinone, reducing agents have no effect. This is further supported by the fact that while incubating p-benzoquinone with topoII-DNA complexes enhances DNA cleavage, incubation of p-benzoquinone with topoII prior to the addition of DNA completely inhibits catalytic function [291].

Effects of exposure to benzene varies from person to person depending on the dose, frequency, route of exposure, genetics, age, gender, and personal dietary and and physical habits [292]. From all of the previous studies on benzene exposure, it is reasonable to presume that benzene and its metabolites have multiple effects on hematopoietic cellular function that lead to hematotoxicity and malignancy. The previously described studies support the hypothesis that exposure to benzene or the metabolite p-hydroquinone may initiate *MLL* translocations and leukemia *in utero*.

Other environmental compounds have also been implicated in causing infant leukemia. These include pesticides, flame retardants, and the common non-steroidal anti-inflammatory drug (NSAID) dipyrone. Some herbal medicines, dipyrone, and the pesticide Baygon have, like genistein and quercetin, been shown to cross the placental barrier and have been associated with *MLL* leukemia [240, 293].

Halogenated compounds in household and baby products [294] are possible risk factors for childhood leukemia. Polychlorinated biphenyls (PCBs) are banned in the United States but detectable in indoor carpets [295] and linked to non-Hodgkin lymphoma [296] and childhood ALL [295]. Polybrominated diphenyl ethers (PBDEs), structurally similar to PCBs, are used as flame retardants [297], and exposure to specific PBDE congeners has been associated with childhood ALL [297]. In support of

mechanistic action, certain PCB quinone metabolites have been shown to increase DNA cleavage by topoIIα *in vitro* and in cultured human cells [214].

Halogenated organic compounds, are of interest because they are widely produced, bioaccumulative, many are alkylating agents and brominated aromatic compounds have been implicated as hormone disruptors [298]. Tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant (BFR) with approximately 200,000 metric tons produced annually worldwide [298]. TBBPA has been found in air, sediment, soil, and human blood samples [299]. Several studies on mammalian cells *in vitro* have shown that TBBPA acts as a cytotoxicant, neurotoxicant, thyroid hormone disruptor, and has anti-estrogenic activity [300]. Several studies have demonstrated that exposure to TBBPA *in vivo* significantly reduces levels of serum thyroxine (T_4) [301-303] which could lead to developmental abnormalities. TBBPA has been shown to have an inhibitory effect on estrogenic activity [304]. Bisphenol A (BPA), a metabolite of TBBPA, has been shown to have estrogenic activity and endocrine-disruptive effects [305, 306]. This indicates that TBBPA and its metabolites have multiple disruptive cellular activities and should be tested for their potential risk in initiating leukemia.

Dipyrone is commercially available in many countries around the world. It was once available worldwide until the 1970s when it was discovered that it carries a risk of causing agranulocytoma, though the level of risk is controversial. Since then, it has been banned in the US, UK and Sweden but is still widely in use in other countries. At low concentrations, dipyrone has been shown to be cytoprotective against UV induced cell death in Jurkat and Raji cell lines; however, it becomes cytotoxic at higher concentrations, inducing apoptosis [307]. Dipyrone use in pregnant women has been

associated with Wilms' tumor in Brazil and several etiological studies have been done that associate maternal use of dipyrone with infant acute leukemia specifically with *MLL* gene fusions [240, 293]Dipyrone is not known to be a topoII inhibitor, although its association with infant acute leukemia indicates that it may induce *MLL* rearrangements.

1.11 Structures of Compounds Used in this Study

The chemotherapeutic drug etoposide is an epipodophyllotoxin that is composed of a polycyclic ring system (rings A-D), a pendant E-ring at the C1 position and a glycosidic moiety at the C4 position [308, 309]. It has been demonstrated that protons of the A-ring and B-ring are responsible for etoposide binding to topoII and that protons and methoxyl protons of the E-ring are required for drug activity. The E-ring substituents have been demonstrated to be directly associated with topoII and DNA in the active site binary complex of the enzyme as any addition to this ring dramatically reduces drug function [309]. The glycosidic moiety of etoposide does not appear to interact with the enzyme or enhance topoII mediated DNA cleavage [308]. The structure of etoposide is shown in figure 14.

The bioflavonoids genistein and quercetin, which have also been shown to inhibit topoII function, were used in this study. Bioflavonoids are biochemically similar to etoposide in that they are also are comprised of multiple phenolic rings. Genistein is an isoflavone and quercetin is a flavonol, and both have structures that mimic etoposide. The 4'-OH group of genistein and quercetin are essential for topoII-mediated drug activity [310, 311]. The 5-OH group of each compound plays an important role in mediating binding to topoII [312]. The structures of both genistein and quercetin are shown in figure 15.

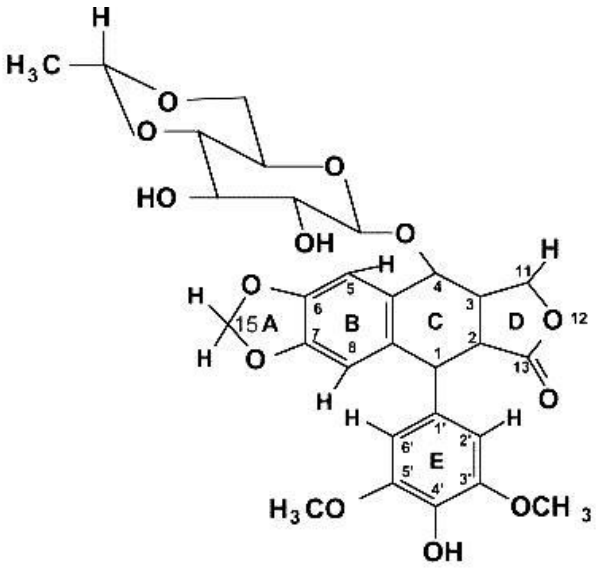

Figure 14: The structure of etoposide with labeled rings A-E and the glycosidic moiety at position C4

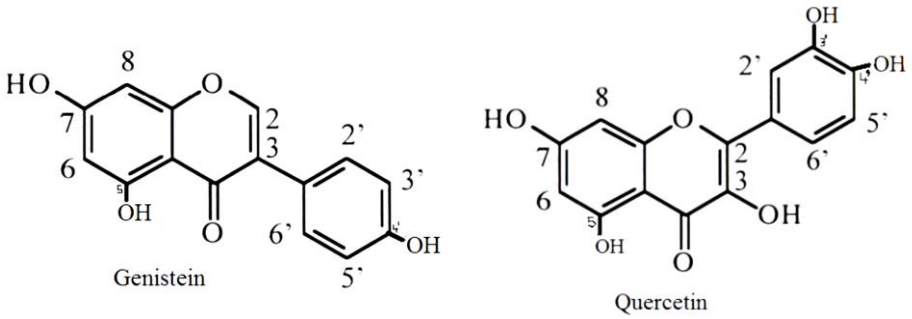

Figure 15: The structures of genistein and quercetin: the 4'-OH group is required for activity and the 5-OH is required for binding to topoII [258]

In addition, the compounds p-benzoquinone and dipyrone were included in this study. Both of these compounds have been associated with *MLL* leukemia as described above. These two compounds are distinctly different in their mechanism of action from etoposide and bioflavonoids. The structures of p-benzoquinone and dipyrone are shown in figure 16.

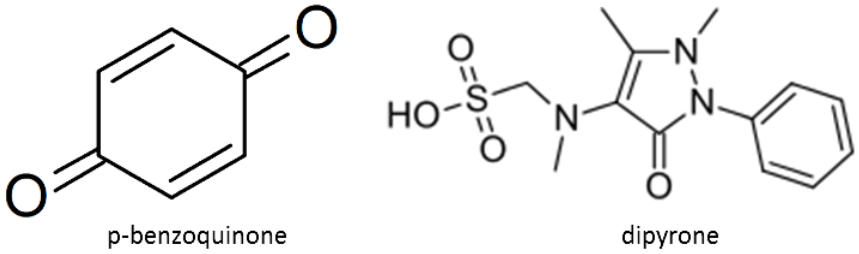

Figure 16: The structures of p-benzoquinone and dipyrone

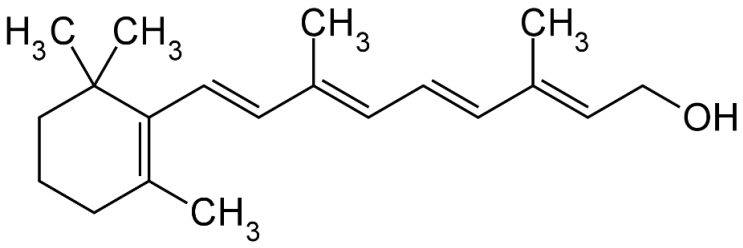

Figure 17: The structure of Vitamin A (retinoic acid)

Vitamin A, which also contains a phenolic ring was also used in this study as a control. Vitamin A is not associated with *MLL* leukemia and has not been shown to be a topoII inhibitor. In fact, it has been shown to promote resistance to topoII-mediated drug activity [313]. All-trans retinoic acid, the bioactive metabolite of vitamin A, has been shown to promote differentiation [314] and downregulate the expression of topoII [315]. It has recently been shown that all-trans retinoic acid-induced downregulation of topoII is through proteasome-mediated degradation [316]. The structure of vitamin A is shown in figure 17. All compounds used in this study were provided by LKT Laboratories and certified for 99.9% purity.

1.12 The Role of Oxidative Stress in DNA Damage

Genomes of all organisms are exposed to endogenous and exogenous stresses such as oxidative stress (OS). OS refers to an imbalance between antioxidant defenses

and production of reactive oxygen species (ROS) [317-320]. ROS are generated endogenously from cellular metabolism such as oxidative phosphorylation in mitochondria and long-chain fatty acids oxidation in peroxisomes, or exogenously by environmental toxins such as ionizing radiation (IR), ultraviolet (UV) radiation, and DNA damaging/chemotherapeutic agents [321-326]. When ROS generation exceeds antioxidant defense capacity in cells, ROS may react with almost all macromolecules including DNA, RNA, proteins, and lipids. Notably, oxidative DNA damage may represent the major type of DNA damage, evidenced that approximately 10,000 DNA alterations are generated per mammalian cell per day [327-330].

Oxidative DNA damage includes oxidized base (purine and pyrimidine) damage, oxidized sugar moiety damage, apurinic/apyrimidinic *(*AP) sites, DNA single-strand breaks (SSBs), DNA intrastrand crosslinks, DNA interstrand crosslinks (ICLs), protein-DNA crosslinks, mismatched pairs with damaged bases, stalled DNA replication forks, oxidatively-generated clustered DNA lesions (OCDLs), and most notably DNA DSBs [327-331]. Elevated intracellular ROS promotes DNA DSBs and altered NHEJ repair leading to chromosomal deletions, translocations, and tumorigenesis [332-334]. OS has been implicated in the pathogeneses of multiple diseases such as bone marrow failure, cancer, and neurodegenerative disorders [335-337]. OS may also play a role in mutation accumulation and progression of myelodysplastic syndromes (MDS) to AML as suggested by studies in a mouse model of MDS that displays increased ROS in Lin- bone marrow cells, increased DNA breaks, and increased mutation frequency over time [338].

Bone marrow failure is characterized by the progressive absence of one or more hematopoietic cell lineages. These syndromes are characterized as inherited or acquired.

The best-characterized inherited syndromes include Fanconi anemia (FA), as well as the telomere diseases dyskeratosis conegenitia (DC), Diamond-Blackfin anemia (DBA), and Shwachman-Diamond syndrome, while the most prevalent acquired syndrome is aplastic anemia (AA). There is increasing evidence linking elevated ROS levels and impaired DNA repair mechanisms to contribute to bone marrow failure observed in these diseases. The hematopoietic system appears particularly sensitive to the control of intracellular ROS and OS, and altered responses to these leading to DNA damage have been implicated in the pathogeneses of both bone marrow failure and neurodegenerative disorders [335-337].

ROS are important for fate determination of normal stem cells [339]. Normal HSCs are primarily in a quiescent state within the BM niche [340]. HSC exposed to elevated ROS exhibit altered characteristics and undergo both proliferation and differentiation, typically after mobilization to the oxygen rich bloodstream [341], but also to senescence and apoptosis in a dose-dependent manner [339]. This impact of ROS is evolutionarily conserved and observable in analogous *Drosophila* systems [342]. Control of ROS in ES cells is now understood to be critical to maintenance of self-renewal phenotype. It has been shown that differentiation of ES cells leads to increased intracellular ROS [343, 344]. Interestingly, during differentiation of ES cells, superoxide production, cellular levels of intracellular ROS, and DNA damage levels increase although expression of major antioxidant genes and genes involved in multiple DNA repair pathways are downregulated [343], and DNA repair by HR is reduced [24]. This indicates that differentiating HSCs may be more at risk for abberant NHEJ repair to occur potentially leading to leukemogenic translocations as a result of OS.

1.13 The Purpose of my Research

DNA DSBs are the most detrimental type of DNA damage. DSBs have the potential to be inaccurately repaired and can lead to the generation of deletions, duplications, insertions, inversions, and translocations that are a hallmark of hematopoietic malignancies such as leukemia and lymphoma. Pediatric acute leukemia accounts for ~30% of all malignancy seen in childhood. Infant acute leukemia is rare and account for 4.5%-19% of all pediatric acute leukemia and have poor prognosis. The majority of these patients have rearrangements involving the *MLL* gene; ~80% among infant ALL patients and ~60% among infant AML patients. *MLL* breakpoint sequences found in infant acute leukemia are similar to those found in secondary AML resulting from exposure to chemotherapeutic topoII inhibitors. It has been hypothesized that maternal exposure to biochemically similar topoII inhibitors may promote infant acute leukemia *in utero*. Several studies have shown that common dietary bioflavonoids as well as benzene metabolites can induce DNA DSB and inhibit topoII; however, a direct link between these compounds and *MLL* rearrangements analogous to clinical samples has not been shown. Maternal exposure to the NSAID dipyrone has also been implicated by several epidemiological studies to increase risk of infant acute leukemia although there is no direct evidence.

Hematopoietic-specific developmental programs may influence the repair of DNA damage such as DSBs and the initial molecular events that lead to translocations and leukemogenesis. The mechanisms by which recurring and specific *MLL* translocations occur is not well understood. Specific gene loci may be particularly susceptible to DNA damage, breakage, and aberrant repair when expressed or as open chromatin within

hematopoietic subpopulations. ESC and HSC may be more at risk for aberrant repair to occur than their more differentiated counterparts due to differences in gene expression and chromatin remodeling.

OS could play an important role in the pathogenesis of leukemia as well. The hematopoietic system appears to be uniquely susceptible to ROS as is evidenced by the sensitivity to ROS and OS in bone marrow failure syndromes. A direct link between OS and genetic instability in HSCs had not been previously determined. Additionally, considering that ROS can generate DNA DSBs, it may also be sufficient to generate leukemic translocations between the *MLL* and *AF9* bcrs.

The goal of this study was to determine the relative potential for bioflavonoids genistein and quercetin, and possible leukemogenic compounds p-benzoquinone and dipyrone to promote rearrangements between the *MLL* and *AF9* bcrs, independent of their chromatin context and independent of the formation of a chimeric fusion protein, within specific hematopoietic cell subpopulations. The effect on the proliferative potential of primary human umbilical cord blood $CD34^+$ cells from exposure to this panel of compounds was also determined. In addition, genetic instability induced by OS was investigated to determine a direct link between OS and the HSC population. These studies provide a direct link between exposure to these compounds and leukemogenesis, specifically with *MLL* rearrangements analogous to clinical samples.

Chapter two describes the generation of double transgenic *MLL-AF9* GFP reporter cell lines used in this study to identify chromosomal translocations between the *MLL* and *AF9* bcrs. Reporter murine embryonic stem cell lines that contain two transgene constructs (1) the *MLL* bcr fragment with a genetically engineered GFP 5' exon and an I-

SceI recognition site and (2) the *AF9* bcr with a genetically-engineered GFP 3' exon and an I-SceI recognition site. Translocations between these two gene constructs leads to reconstitution of the full-length GFP transcript resulting in detectible green fluorescence.

Chapter 3 describes the quantification of GFP^+ colonies after treatment with genistein, quercetin, p-benzoquinone, dipyrone, vitamin A, or the expression of I-SceI endonuclease in different hematopoietic cell subpopulations. Results describe the overall frequencies of translocations in each cell population following treatment with each compound and the possible factors that may account for differences between populations. This chapter also includes the DNA sequence analysis of repair junctions of a GFP^+ clone that was isolated and expanded after treatment.

Chapter 4 describes the potential for repeated and biologically relevant concentrations of genistein, quercetin, a combination of both genistein and quercetin, benzoquinone, and dipyrone to induce long-term repopulation of primary human $CD34^+$ cells. Results demonstrate similarities or differences between exposure to this panel of compounds and the known leukemogenic drug etoposide on the proliferative potential of these cells.

Chapter 5 describes the detection and quantification of GFP^+ colonies after the generation of OS in one of the engineered ES cell lines by H_2O_2. The results of these experiment demonstrate a direct link between ROS and OS and genome integrity in HSCs. Conclusions made from this study and future directions for my research using other experimental models are described in chapter 6.

CHAPTER II: CELL LINES CREATED FOR THIS STUDY

2.1 Materials and Methods

2.1.1 Creation of Transgene Constructs

Two green fluorescent protein (GFP) gene reporters were generated (Figure 18) NZE-GFP fragment of GFP open reading frame (orf) was inserted into pCAGGs [345] to engineer GFP into two exons, adenovirus intron sequence 246 nucleotides [346, 347] was inserted into the GFP orf between the GG doublet (nucleotides 241/242). This GFP-intron plasmid was used to generate two separate constructs as follows: GFPe1: one construct contained the chicken β-actin promoter, 5' engineered exon 1 with the first 233 nucleotides of the GFP orf, splice donor (sd), 160 nucleotides of adenovirus intron to the HindIII site [346, 347], an I-*Sce*I endonuclease recognition site within an inserted HindIII-I*Sce*I-XhoI linker (H3-*S*-X). GFPe2: second construct contained an I-*Sce*I endonuclease recognition site within a XhoI-I*Sce*I-HindIII linker (X-*S*-H3), 76 nucleotides of adenovirus intron from the HindIII site to a splice acceptor (sa) [346, 347], 3' engineered exon 2 of the GFP gene, and β-globin polyA sequence. A blunted Xho1-XhoI fragment of GFPe1 was inserted into intron 11 in the blunted XbaI site of the human *MLL* bcr 8.2 kb BamHI-BamHI fragment [348] (numbering based on Nilson *et al* [349]) (Figure 18). A blunted XhoI-PstI fragment of GFPe2 was inserted into intron 7 of the human *AF9* bcr2 region 8.9 kb fragment between mapped topoII cleavage site and exon 8 [154] (Figure 18). Constructs were verified by sequencing (Sequetech

Corporation, CA, USA). Single copy clones MAG1 and MAG2 were used for subsequent experiments.

2.1.2 Generation of Transgenic Cell Lines.

Transgene constructs were transfected by electroporation and randomly integrated into the genome of EtG2a mouse embryonic stem (ES) cells [3, 346]. Puromycin and neomycin resistance gene-containing plasmids were co-transfected with the transgene constructs to allow for selection. ES cells in standard medium (cite) were washed with 1X PBS (phosphate buffered saline), treated with 0.25% trypsin for 10 minutes at 37ºC and centrifuged at 1000 rpm for 10 minutes. Cells were resuspended in 1X PBS and 2 x 10^7 cells electroporated with the *MLL*-GFPe1 transgene along with *neomycin* gene plasmid. 24 hours post electroporation, the media was changed to selection for 10 days (200ug/mL neomycin). [3, 346]. Surviving colonies were identified and screened for the presence of a single copy of the *MLL*-GFPe1 transgene by performing appropriate enzymatic digests (BgIII/XbaI; BamHI/BglII; EcoRV) (New England Biolabs Inc.) followed by Southern blotting using a full length GFP gene as the P32 labelled probe. Single copy *MLL*-GFPe1 clones were selected, grown and expanded. Cells in standard ES medium were washed with 1X PBS (phosphate buffered saline), treated with 0.25% trypsin for 10 minutes at 37ºC and centrifuged at 1000 rpm for 10 minutes. Cells were resuspended in 1X PBS and counted using a hemacytometer. 2 x 10^6 cells were electroporated with the *AF9*-GFPe2 transgene along with *puromycin* gene plasmid. 24 hours post electroporation, the media was changed to selection for 10 days (120ug/mL puromycin). [3, 346]. Surviving colonies were identified and screened for the presence of a single copy of the *AF9* transgene. Surviving colonies were identified and screened for

the presence of a single copy of the *AF9*-GFPe2 transgene by performing appropriate enzymatic digests and Southern blotting using appropriate digests (KpnI; BamHI; NcoI; BamHI/BglII; XhoI; PstI) (New England Biolabs Inc.) and the full length GFP gene as the P32 labelled probe. Clones were also confirmed by PCR (5' primers GFP-bcl and GFP 5' intron 2; and 3' primers GFP-nco top and GFP-orf 3'). Selected clones MAG1, MAG2 and MAG3 were expanded and used for further experiments.

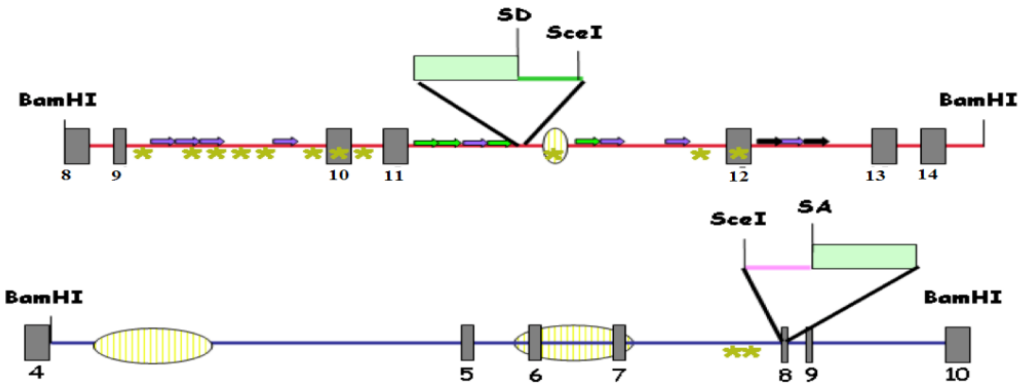

Figure 18: Schematic of transgene constructs.

The positions of GFP reporter constructs were inserted into the human *MLL* (top) and *AF9* (bottom) bcr regions. SD indicates a splice donor, SA indicates a splice acceptor, exons are shown as grey rectangles, MAR/SAR sites are indicated by yellow ovals, and topoisomerase II cleavage sites are indicated by gold stars.

2.1.3 FACS (fluorescence Activated Cell Sorting)

MAG1 cells were treated with 50, 100, or 200 µM etoposide for 1 hour and allowed to recover overnight. Cells were washed with PBS, trypsinized, centrifuged at 1000rpm for 10 minutes and resuspended in fresh ES cell medium. The cell suspension was then filtered and FACS was performed using the FACSAria machine (BD Biosciences).

2.2 Results

It was determined that MAG clones contained both genetic reporter constructs by Southern blotting. MAG 1, MAG2 and MAG3 showed the presence of both *MLL*-GFPe1 and *AF9*-GFPe2 (Figure 19). MAG3, however was not used for further experiments as it failed to produce any fluorescent colonies following expression of ISceI endonuclease.

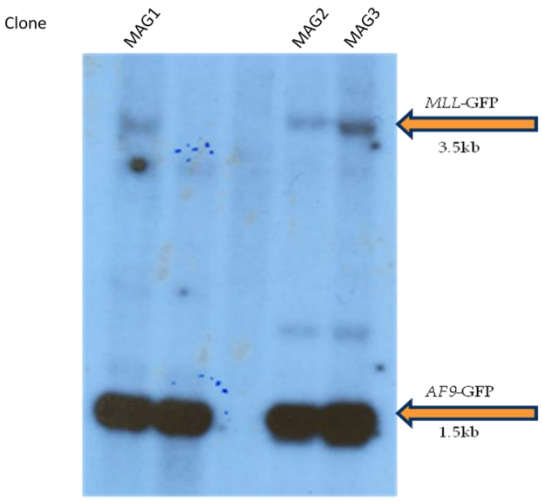

Figure 19: Southern blot of clones containing single copy inserts of *MLL*-GFPe1 and *AF9*-GFPe2 reporter constructs

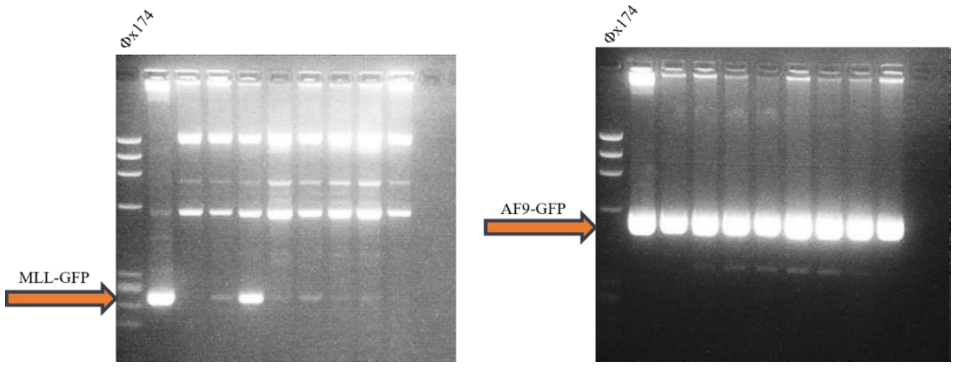

Figure 20: PCR confirming the insertion of *MLL*-GFPe1 and *AF9*-GFPe2 transgenes

PCR screening was also performed to confirm the presence of both genetic constructs in MAG1, MAG2 and MAG3. Results of PCR are shown in figure 20.

In order to determine that this model system would generate detectable GFP^+ fluorescent cells, fluorescent activated cell sorting (FACS) was performed on etoposide treated MAG1 cells. FACS is a highly sensitive procedure that is able to sort individual GFP^+ cells. GFP^+ cells were detected and their frequency increased in a dose dependent manner (figure 21). The frequency of GFP^+ cells treated with 50, 100, or 200 µM etoposide was 0.017%, 0.020%, and 0.027% respectively.

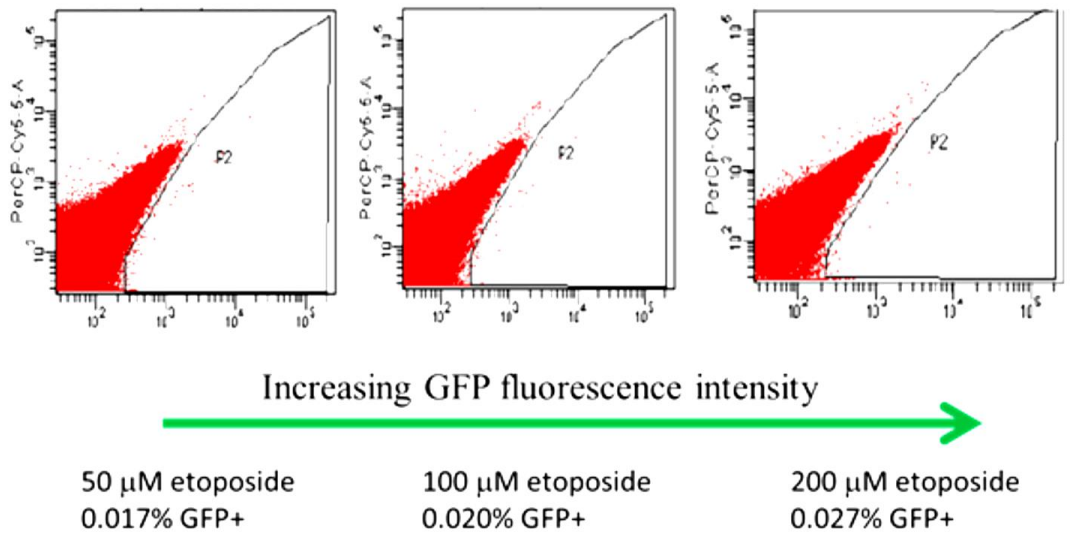

Figure 21: Etoposide exposure promotes *MLL-AF9* repair events detectable as GFP^+ cells by FACS

*The X-axis represents the intensity of fluorescence emitted in a single channel (FITC) on a log scale and the Y-axis represents the intensity of fluorescence emitted in a separate channel (PerCP-Cy5.5) to indicate the presence of different cell populations and detect any auto-fluorescence.

2.3 Conclusion

This chapter describes the development of a reporter gene model system to screen for the frequency of *MLL-AF9* translocation events in the MAG cell lines. A translocation between the two bcrs reconstitutes a full length GFP transcript resulting in quantifiable green fluorescence. The efficacy of this model was confirmed by treatment of MAG1 cells with etoposide, which is known to promote DSBs and translocations, followed by FACS analysis. This model provides an elegant system for screening potentially thousands of compounds for their risk of promoting the development of leukemia. This system can identify compounds that promote DNA DSBs and a chromosomal rearrangement associated with infant and therapy related AML.

CHAPTER III: COMMON DIETARY BIOFLAVONOIDS AND ENVIRONMENTAL COMPOUNDS PROMOTE A LEUKEMIC TRANSLOCATION IN HEMATOPOIETIC STEM CELLS AND DIFFERENTIATED CELLS

The potential for common dietary bioflavonoids, genistein and quercetin, environmental compound, p-benzoquinone, and NSAID dipyrone to promote the leukemic *MLL-AF9* translocation in differentiating hematopoietic cells was determined. Cell viability assays were performed to determine the sensitivity of ES cells to these compounds at various doses. Transgenic cell lines were then electroporated with ISceI endonuclease or treated with a test compound at one of three different stages of differentiation (ES, EB, and bursting EB). Cells were allowed to differentiate and were screened for the formation of fluorescent colonies and translocation events.

3.1 Materials and Methods

3.1.1 ES Cell Culture

All ES cell cultures were maintained in a humidified incubator at 37˚C and 5% CO_2. The *MLL-AF9* GFP (MAG) cell lines 32A and 68 were maintained at $5x10^6$-$2x10^7$ cells per 10cm culture dish coated with 0.2% gelatin. Cell culture medium was changed at least every other day with ES-maintenance medium containing Dulbecco's Modified Eagle's Medium (DMEM), 15% ES-qualified fetal bovine serum (FBS), 100U/ml penicillin/streptomycin, 2mM L-glutamine, 0.1mM non-essential amino acids, 1000U/ml ESGRO® Leukemia Inhibitory Factor (LIF), And 100µM 2-mercaptoethanol (β-ME).

Cells were passaged every 2-3 days by trypsinization and re-plated in ES maintenance medium.

3.1.2 Cell Viability Assays

1×10^6 EtG2a mouse ES cells were plated onto 3 plates each per dose (0, 25, 50, 75, 100, and 200 µM) of a test compound. Cells were exposed to a test compound for 1 hour at 37ºC. The number of surviving cells were counted for each dose at 24, 36, and 72 hours using the automated Moxi cell counter. Cells were trypsinized for 10 minutes at 37ºC, resuspended, and counted in a 75µM volume. The overall percentage of surviving cells was calculated.

3.1.3 *In vitro* Hematopoietic Differentiation

ESC treatment

Cells were plated at a density of $1x10^6$ and treated with 75µM each etoposide, genistein, quercetin, p-benzoquinone, or dipyrone for one hour or were electroporated with 20µg CBAS or GFP-NZE expression plasmid and allowed to recover overnight. Surviving cells were plated in predifferentiation medium the following day.

Predifferentiation

Undifferentiated ES cells were harvested via trypsinization and re-plated at a density of $2.85x10^5$ (for untreated cohorts) or $2x10^6$ (for treated cohorts) cells per 10cm gelatinized tissue culture dish in pre-differentiation medium containing Iscoves Modified Dulbecco's Medium (IMDM), 15% ES qualified FBS, 1mM sodium pyruvate, 100U/ml penicillin/streptomycin, 2mM L-glutamine, 0.1 mM non-essential amino acids, 1000U/ml ESGRO® LIF, and 100µM monothioglycerol (MTG) for 48 hours at 37˚C and 5% CO_2.

Primary differentiation

Following the 48hr incubation in predifferentiation medium, cells were harvested by trypsinization and re-plated at a density of $5x10^2$ (for untreated cohorts) or between $1x10^3$ and $3x10^3$(for treated cohorts) in 3.5cm low adherence tissue culture dishes in primary differentiation medium containing IMDM, 1% basic methylcellulose (Stemcell Technologies #M3120), 15% non-ES-qualified FBS, 2mM L-glutamine, 100U/ml penicillin/streptomycin, 150µM MTG, and 40ng/ml murine stem cell factor (mSCF) (Stemcell Technologies #02731) for 14 days, during which time cells form embryoid bodies (EBs). On day 7 of primary differentiation, EBs were fed with medium containing IMDM, 0.5% methylcellulose, 15% non-ES-qualified FBS,100U/ml penicillin/streptomycin, 150µM MTG, 160ng/ml mSCF, 30ng/ml murine interleukin-3 (mIL-3) (Stemcell Technologies #02733), 30ng/ml human interleukin-6 (hIL-6) (Stemcell Technologies #02506), and 3U/ml human erythropoietin (hEpo) (Stemcell Technologies #02625), added in a drop-wise fashion evenly on top of methylcellulose. Cultures to be treated as bursting EBs were fed this same medium with cytokine levels increased by 50 percent. On day 14 of primary differentiation, EBs derived from ES-treated cohorts were scored and plates were analyzed for the presence of fluorescent colonies. For cells to be treated as EBs or bursting EBs, colonies were harvested and disrupted on day 14 and day 19 respectively.

Harvesting EBs

Each culture was harvested by diluting the methylcellulose with IMDM and transferring to a 50ml conical tube. EBs were washed once with phosphate buffered saline (PBS) and then disrupted with 0.25% collagenase (Gibco 17100-017) for 60 minutes at 37˚C. Colonies were further disrupted by passage through a 21G 1.5” needle

three times and collagenase was neutralized by adding an equal volume of IMDM supplemented with 5% FBS.

EB and bursting EB treatments

Following disruption with collagenase, cells were counted and treated with 75µM each etoposide, genistein, quercetin, p-benzoquinone, or dipyrone for one hour in IMDM supplemented with 5% FBS or were electroporated with 20µg CBAS or GFP-NZE expression plasmid

Hematopoietic differentiation

EB cells were plated at a density of 1.0×10^5 (for untreated cohorts) or at a minimum of 5×10^5 (for treated cohorts) per 3.5cm low adherence tissue culture dishes in hematopoietic differentiation medium. Hematopoietic differentiation medium contained 1% basic methylcellulose (Stemcell Technologies #M3120), 15% non-ES-qualified FBS, 2mM L-glutamine, 100U/ml penicillin/streptomycin, 150µM MTG, 1% bovine serum albumin (BSA), 10µg/ml insulin, 200µg/ml transferrin, 150ng/ml mSCF, 30ng/ml mIL-3, 30ng/ml hIL-6, and 3 U/ml hEpo. Cells were allowed to differentiate for an additional 12-14 days at which point hematopoietic colonies were scored and isolated for further analysis.

3.1.4 DNA Extraction and Molecular Analysis

Whole genome amplification

EBs and hematopoietic colonies were harvested by picking individual colonies of interest by pipetting into microcentrifuge tubes containing PBS. Colonies were then pelleted by centrifugation and flash frozen. DNA extractions and whole genome amplification (WGA) was performed using the Qiagen Repli-G kit (Qiagen Cat.#

150035). Buffer DLB was reconstituted with 500µl nuclease free water. Buffer D2 (denaturation buffer) is a 1:10 dilution of 1M DTT (supplied with kit) in reconstituted buffer DLB. To denature one EB or hematopoietic colony, 3µl of PBS was added and pipetted up and down in order to disrupt the colony, 3µl of buffer D2 was added to the cells, mixed by pipetting, and incubated on ice for 10min. To stop denaturation, 3µl of Stop solution (supplied with kit) was added and mixed by pipetting. This reaction was then added to 16µl of master mix containing REPLI-g UltraFast DNA polymerase and REPLI-g Ultrafast reaction buffer. Samples were incubated at 30˚C overnight followed by incubation for 3 minutes at 65˚C in order to inactivate the DNA polymerase. Following amplification, DNA was purified by ethanol precipitation and concentrations were determined by spectrophotometric measurement.

Nested PCR

1.0 µg of amplified DNA was used for PCR amplification. For nested PCR, 1.0 µg of DNA template was used for the first round of amplification and 1µl of the first round product was used as template for the second round of nested PCR amplification. Each 25 µl PCR reaction contained template DNA, 17.0 µl of Millipore H_2O, 3.0 µl of each primer, and a PuReTaq Ready-To-Go PCR bead (GE Healthcare Cat# 27-9559001). PCR primers are shown as 1F, 1R 2F and 2R in figure 22. Amplification was performed by denaturation at 94˚C for 5 minutes; followed by 40 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 30 seconds; and extension at 72˚C for 7 minutes.

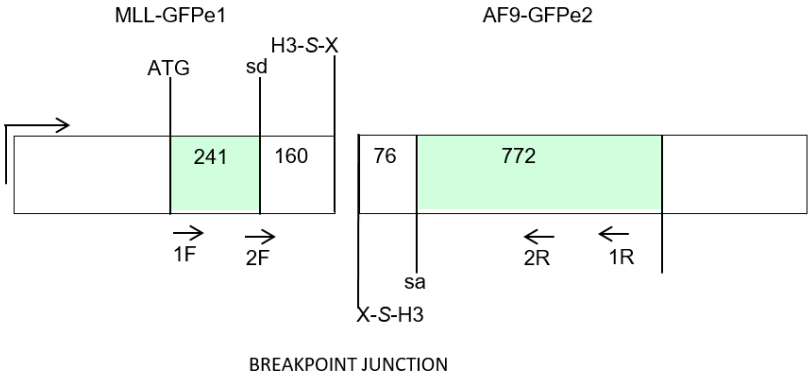

Figure 22: Schematic of translocation breakpoint junctions between the *MLL*-GFPe1 and *AF9*-GFPe2 genetic constructs.

Primers for nested PCR across breakpoint junctions are 1F and 1R for the first reaction and 2F and 2R for the second reaction.

Sequencing Analysis

PCR products were cleaned using DNA Clean and Concentrator^{TM}-5 (Zymo Research Cat# D4014) and eluted in 10µl elution buffer (supplied). DNA sequences across the repair junction were determined (Sequetech Corporation, California) and sequence analysis was done using CLC Sequence Viewer software.

3.2 Results

3.2.1 Murine ES Cells Exhibit Considerable Sensitivity to Bioflavonoids Genistein and Quercetin; and Moderate Sensitivity to P-Benzoquinone and Dipyrone.

Cell viability assays were performed on parental EtG2a murine ES cell line to determine the sensitivity of these cells to various doses of these potentially leukemogenic compounds. Compounds tested were bioflavonoids genistein, quercetin, myricetin, luteolin, p-benzoquinone, dipyrone, and etoposide.

A two-way ANOVA was performed in order to determine any significant differences of compounds and doses compared to etoposide at each of the time points. Significant differences are indicated by *, with the most significant differences indicated by ***, which were determined using Tukey's multiple comparison test. These experiments demonstrate the sensitivity of ES cells to these compounds. The chemotherapeutic drug etoposide is the most toxic to cells with less than 30% surviving after 24 hours even at the lowest dose. Cells also exhibited sensitivity to the bioflavonoids genistein and quercetin, whereas myricetin, luteolin, p-benzoquinone and dipyrone seemed to have a moderate effect.

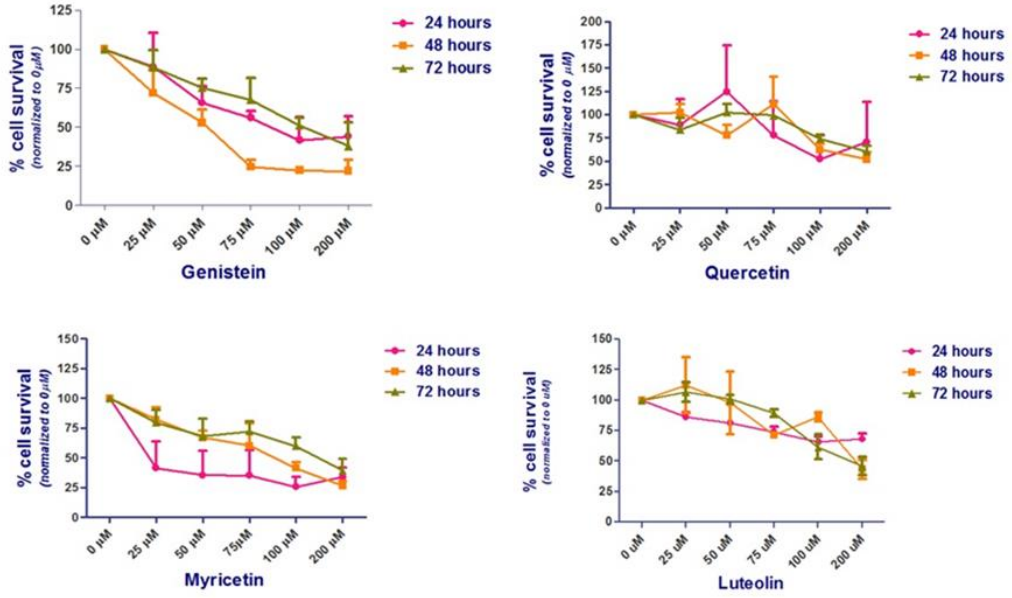

Figure 23: Cell viability plots for genistein, quercetin, myricitin, and luteolin at 24, 48, and 72-hour time points

Treatment of MAG1 cells with bioflavonoids at different concentrations demonstrated a dose dependent response at all time points. Three replicates were performed for each treatment at each dose. Genistein treatment was the most cytotoxic followed by quercetin, myrecetin and luteolin.

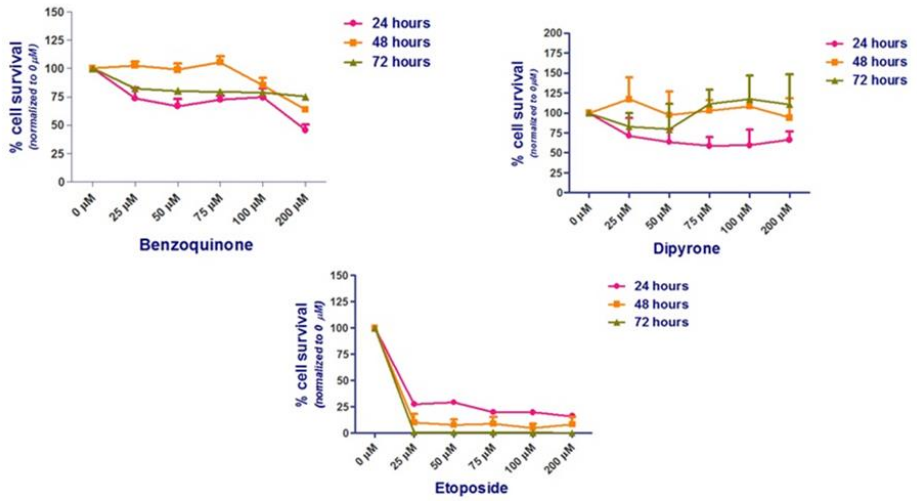

Figure 24: Cell viability plots for p-benzoquinone, dipyrone, and etoposide at 24, 48, and 72 hour time points

Treatment of MAG1 cells with benzoquinone, dipyrone and etoposide at different concentrations demonstrated a dose dependent response at all time points. Three replicates were performed for each treatment at each dose. Etoposide is the most cytotoxic; and benzoquinone and dipyrone are moderately cytotoxic.

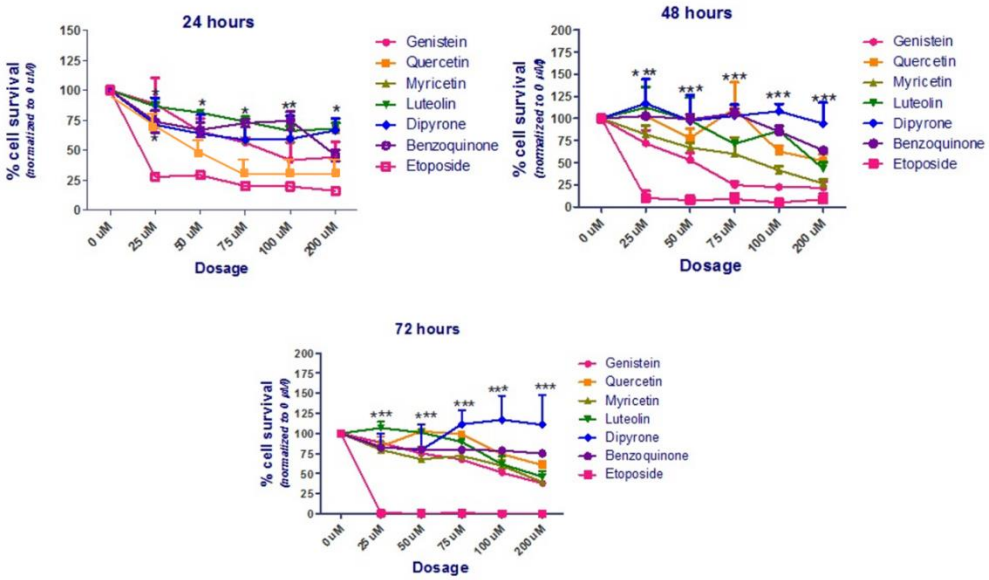

Figure 25: Analysis of cell viability and sensitivity to genistein, quercetin, myricetin, luteolin, p-benzoquinone, dipyrone and etoposide at 24, 48, and 72 hours

Tukey's multiple comparison tests showed there were significantly more cells following treatment with luteolin and dipyrone compared to treatment with etoposide. * indicates $p < .05$, ** indicates $p < .005$, and *** indicates $p < .0005$.

3.2.2 *MLL-AF9* bcr Translocations Induced by Targeted DSBs in Myeloid Progenitors and Differentiated Cells.

Each of the gene constructs, described in chapter 2, contains an ISceI recognition site for the induction of DBSs within the bcrs. Following ISceI-induced DSBs, aberrant repair that results in a translocation joins the 5'GFP exon and the 3'GFP exon onto the same DNA duplex allowing for expression of full length GFP. In the absence of ISceI-induced DSBs, the formation of stable translocations without a promoting event is rare with the overall frequency calculated at $<7.2\times10^{-8}$ in nondifferentiated ESCs (previously published data). ISceI-induction of DSBs significantly stimulated aberrant repair between the *MLL* and *AF9* bcrs at all three stages of differentiation (ES, EB, and bursting EB myeloid progenitor) resulting in detectable GFP^+ fluorescent colonies 14 days after electroporation. The average frequencies of translocations were calculated at each of the three stages of differentiation. Average frequency observed following treatments were $85.8\times10^{-4}\pm2.66$ in ESCs, and $0.113\times10^{-4}\pm4.7$ in bursting EB myeloid cells. An average frequency could not be determined for ISceI expression in EB cells due to zero fluorescent colonies observed in some replicates. The NHEJ recombination frequency is approximately 100 times higher than HR frequencies observed in a previous study in ES cells, approximately 10 times lower in EB/HP cells and approximately 2.5 times lower in bursting EB myeloid cells [24] . This suggests that the *MLL* and *AF9* bcrs are highly recombinogenic by NHEJ at early stages of myeloid differentiation.

3.2.3 *MLL-AF9* bcr Translocations are Induced by Common Bioflavonoids and Potentially Leukemogenic Compounds.

MAG cells were differentiated *in vitro* into EBs containing HSCs followed by differentiation into myeloid progenitor subpopulations and terminally differentiated myeloid cells. Cohorts of cells at three different stages of differentiation were exposed to etoposide, genistein, quercetin, p-benzoquinone, dipyrone for 1hr at 75μM. These concentrations were determined from previously published reports to be biologically relevant [350-352]. Following treatment, cells were plated in methylcellulose media and allowed to differentiate and proliferate for 12-14 days. Aberrant repair resulting in a translocation between the *MLL* and *AF9* bcrs resulted in detectable GFP^+ fluorescent colonies that were readily scored and isolated (Figure 26). All compounds induced translocation events at each stage of differentiation. Translocation frequencies were calculated as the number of GFP^+ colonies observed per number of treated and plated cells.

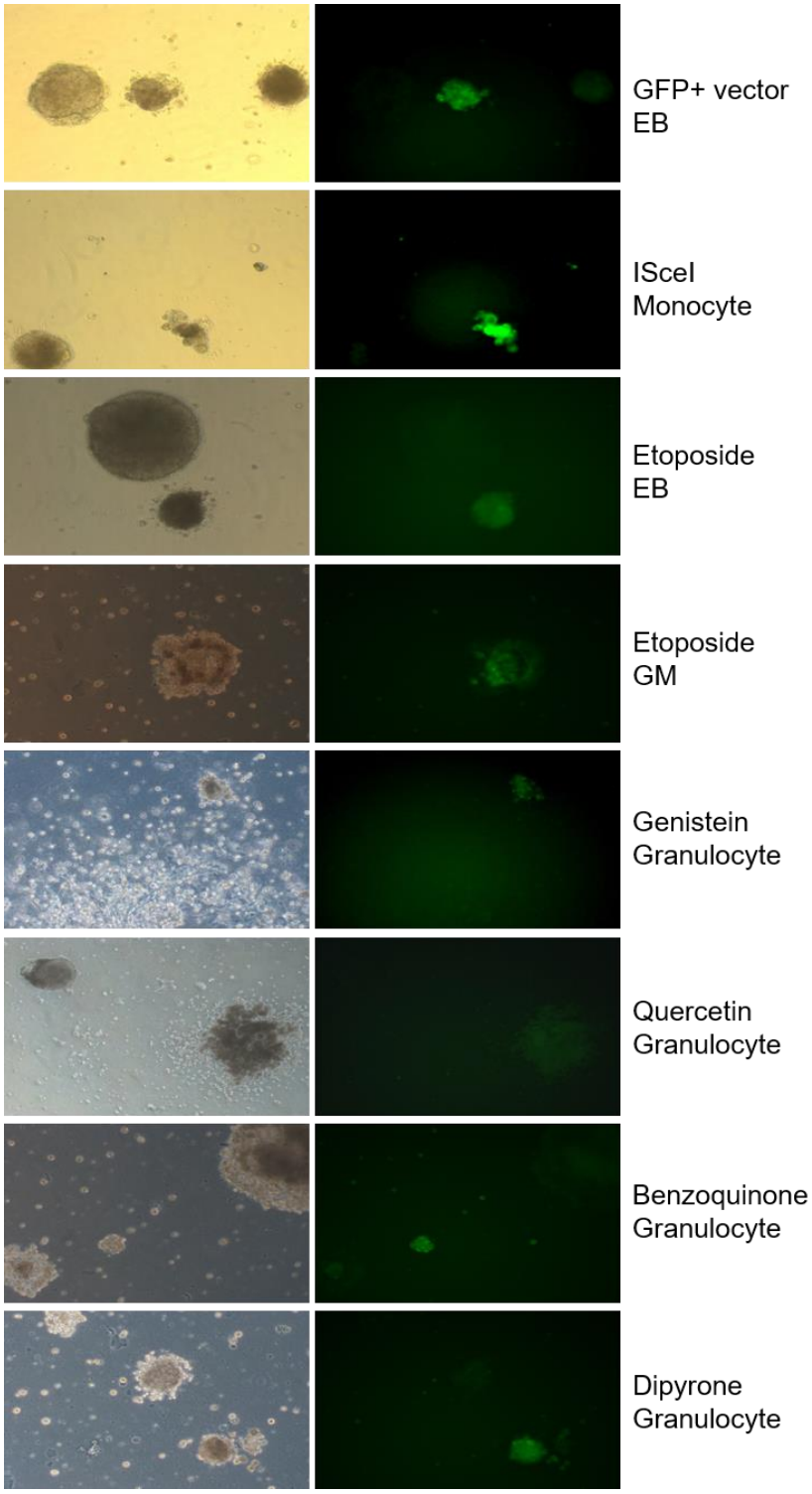

Figure 26: Fluorescent GFP^+ colonies detected after treatment and differentiation into EBs or terminally differentiated myeloid colonies

*All images were taken with a contrast and a fluorescent filter at 200x magnification.

Bioflavonoids genistein and quercetin were the strongest inducers of translocations between the *MLL* and *AF9* bcrs in ESCs resulting in GFP^+ EB colonies (Table 1). Genistein treatment produced an average of 16 GFP^+ colonies per replicate treatment and an average frequency of translocations was calculated as $38.7\times10^{-4}\pm25$. Quercetin treatment produced a total of 23 GFP^+ colonies over 3 replicate treatments and an average frequency of translocations was calculated as $39.5\times10^{-4}\pm28$. One replicate treatment with quercetin resulted in zero detectable GFP^+ colonies, so the average frequency was calculated for the other two replicates. Dipyrone, an NSAID associated with infant leukemia with *MLL* rearrangements though not a topoII inhibitor [353] generated an average of 8.67 GFP^+ EB colonies per replicate treatment with an average frequency of translocations calculated as $26.7\times10^{-4}\pm16.7$. P-Benzoquinone, a by-product of manufacturing that is also associated with childhood leukemia with *MLL* rearrangements through epidemiological studies [354, 355] generated an average of 3.3 GFP^+ EB colonies over 3 replicate treatments with an average frequency of translocations calculated as $8.89\times10^{-4}\pm1.9$. This is consistent with the average recombination frequency seen in pZK1 fetal liver cells after treatment with 50µM p-benzoquinone [356]. Similar to treatment with p-benzoquinone, etoposide treatment generated an average of 7.6 GFP^+ EB colonies per replicate treatment with an average frequency of translocations calculated as $2.4\times10^{-4}\pm1.7$, indicating similar levels of genotoxicity.

Table 1**:** Induction of interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 ES cells: a comparison of frequency of GFP^+ colonies generated

Compound	**Expt**	**# GFP+ colonies**	**Frequency ($\times 10^{-4}$)**	**Avg. Frequency ($\times 10^{-4}$)**
Etoposide	1	4	1.6	
	2	9	1.8	
	4	6	1.2	
	5	12	4.8	
	6	0	< 0.2	2.4 ± 1.73*
Genistein	1	20	33.3	
	2	20	66.7	
	3	8	16.0	38.7 ± 25.7
Quercetin	1	11	55.0	
	2	12	24.0	
	3	0	< 3.33	39.5 ± 27.6*
P-benzoquinone	1	5	10.0	
	2	3	10.0	
	3	2	6.67	8.89 ± 1.92
Dipyrone	1	5	10.0	
	2	13	43.3	
	3	8	26.7	26.7 ± 16.7
ISceI	1	13	52.0	
	2	11	73.0	
	3	19	120.0	
	4	16	80.0	
	5	26	104.0	85.8 ± 26.6

MAG1 ES cells were treated with various compounds, differentiated, and analyzed for the generation of fluorescent colonies at the formation of EBs.
*The interchromosomal recombination and translocation frequency was calculated as the number of GFP^+ clones per number of plated cells.
*Average frequency calculations excluded experiments with zero GFP^+ colonies.

EB cells treated with this panel of compounds produced few GFP^+ colonies though at least one replicate experiment for each compound generated a translocation and a GFP^+ colony (Table 2). Average frequencies of translocations could not be calculated due to zero GFP^+ colonies in some replicate experiments. Genistein treatment produced 1

GFP^+ colony over three replicate treatments. Quercetin treatment produced 5 GFP^+ colonies over five replicate treatments. Dipyrone treatment produced a total of 13 GFP^+ colonies over four replicate treatments. P-Benzoquinone produced 3 GFP^+ colonies over four replicate treatments. Treatment with etoposide resulted in 4 GFP^+ colonies over three replicate treatments. These data demonstrate that EB/HP cells retain the potential to repair DNA DSBs via NHEJ although the frequency of translocations between *MLL* and *AF9* bcrs is approximately 700 fold lower for geistein and quercetin, 400 fold lower for dipyrone, and 100 fold lower for p-benzoquinone and etoposide compared to ES cell treatments. This is likely due to epigenetic factors, chromatin remodeling, or changes in gene expression patterns associated with hematopoiesis.

Table 2**:** Induction of interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 EB cells: a comparison of frequency of GFP^+ colonies generated

Compound	**Expt**	**# GFP+ colonies**	**Frequency* ($\times 10^{-4}$)**
Etoposide	1	1	.002
	2	2	.02
	3	1	.01
Genistein	1	1	.005
	2	0	< .033
	3	0	< .067
Quercetin	1	0	< .01
	2	0	< .033
	3	0	< .016
	4	2	.013
	5	3	.02
P-benzoquinone	1	0	< .033
	2	3	.05
	3	0	< .0067
	4	0	< .0067
Dipyrone	1	3	.05
	2	0	< .0067
	3	3	.033

	4	7	.023
ISceI	1	1	.027
	2	1	.033
	3	0	< .025
	4	0	< .02

MAG1 EBs were disrupted and cells were treated with various compounds, differentiated, and analyzed for the generation of fluorescent colonies at the formation of terminally differentiated colonies.
*Average Frequency could not be determined due to the low number of GFP^+ colonies.

Cells from bursting EBs also generated few GFP^+ colonies after treatment with this panel of compounds, though all replicates (with the exception of one quercetin treatment and one p-benzoquinone treatment) generated at least one GFP^+ colony (Table 3). Genistein treatment resulted in an average of 1.3 GFP^+ colonies per replicate treatment and an average frequency of translocations was calculated as $.0094\times10^{-4}\pm.0048$. Quercetin treatment produced 5 GFP^+ colonies over three replicate treatments with an average frequency of translocations calculated as $.018\times10^{-4}\pm.007$. Dipyrone treatment produced an average of 13.3 GFP^+ colonies per replicate treatment with an average frequency of translocations calculated as $.076\times10^{-4}\pm.055$. p-Benzoquinone treatment produced 4 GFP^+ colonies over three replicate treatments and an average frequency of translocations was calculated as $.009\times10^{-4}\pm.0078$. Etoposide treatment produced an average of 11 GFP^+ colonies per replicate treatment and an average frequency of translocations was calculated as $.055\times10^{-4}\pm.058$. Both etoposide and dipyrone treatments at this stage resulted in an increased average translocation frequency compared to treatments as EB/HPs. These data demonstrate that differentiated myeloid cells also retain the ability to repair DNA DSBs via NHEJ although the frequency of translocations between *MLL* and *AF9* bcrs is >1000 fold lower for genistein and quercetin, approximately 200 fold lower for dipyrone, and 400 fold lower for p-

benzoquinone compared to ES cell treatments. Standard deviations that were higher or equal to the calculated frequencies of translocations were due to a high degree of variability between experimental replicates.

Table 3**:** Induction of interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 bursting EB cells: a comparison of frequency of GFP^+ colonies generated

Compound	**Expt**	**# GFP+ colonies**	**Frequency ($\times 10^{-4}$)**	**Avg. Frequency ($\times 10^{-4}$)**
Etoposide	1	3	.005	
	2	24	.12	
	3	6	.04	.055 ± .058
Genistein	1	1	.0067	
	2	2	.015	
	3	1	.0067	.0094 ± .0048
Quercetin	1	2	.013	
	2	3	.023	
	3	0	< .0066	.018 ± .007*
P-benzoquinone	1	2	.0033	
	2	2	.015	
	3	0	< .0066	.009 ± .0078*
Dipyrone	1	23	.115	
	2	2	.013	
	3	15	.10	.076 ± .055
ISceI	1	6	.08	
	2	6	.091	
	3	11	.167	.113 ± .047

MAG1 bursting EBs containing myeloid progenitors were disrupted and cells were treated with various compounds, differentiated, and analyzed for the generation of fluorescent colonies at the formation of terminally differentiated colonies.
*The interchromosomal recombination and translocation frequency was calculated as the number of GFP^+ clones per number of treated cells.
*Average frequency calculations excluded experiments with zero GFP^+ colonies.

In order to compare frequencies of appearance of GFP^+ from treated groups to untreated controls, a One-way ANOVA was performed with Tukey's multiple comparison tests for the ES group of treatments as well as the bursting EB group of treatments. The frequency of genistein-, quercetin-, and dipyrone-induced GFP^+ colonies from treated ES cells was significantly different from untreated controls. The frequency of ISce-induced GFP^+ colonies from treated ES cells was significantly different from all other treatments with the exception of quercetin. The overall p-value from this analysis was <0.0001 indicating very significant variation between treatment groups. The frequency of ISceI and dipyrone-treated bursting EBs reached significance compared to untreated controls with Tukey's multiple comparison tests. ISceI treatment was also significantly different from genistein and p-benzoquinone treatments. The overall p-value was 0.0015 indiciating significant variations.

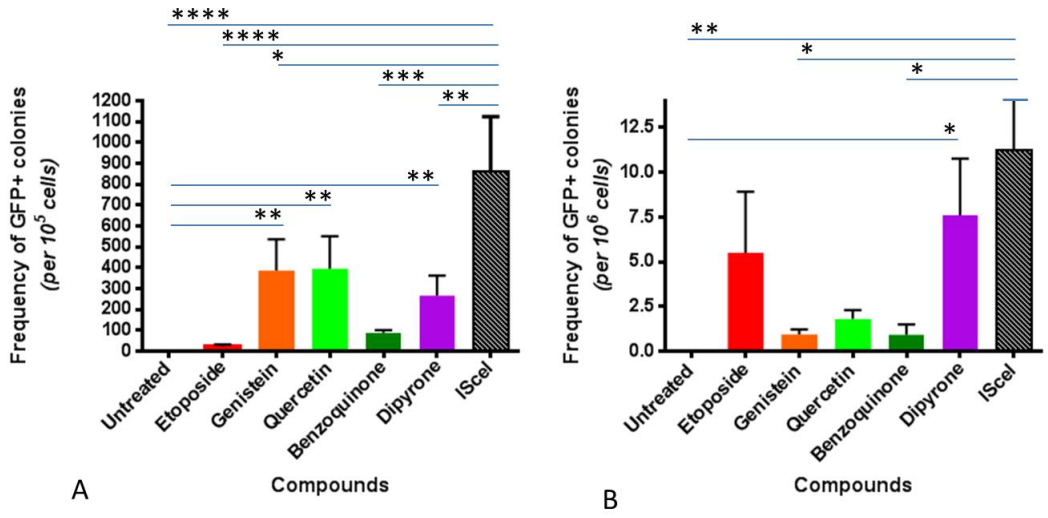

Figure 27: The frequencies of appearance of GFP^+ colonies following treatment and differentiation of MAG1 (A) ES cells and (B) bursting EB cells. Cells were treated with genistein, quercetin, p-benzoquinone, dipyrone, or electroporated with ISceI.

MAG 2 cells were treated with this panel of compounds and differentiated as well. GFP^+ colonies were observed for this clone as well although fewer than observed with clone MAG1. Results with this clone were consistent with results from clone MAG1, as the two bioflavonoids genistein and quercetin were the strongest inducers of translocations between the *MLL* and *AF9* bcrs in ESCs giving rise to GFP^+ EB colonies. Genistein treatment produced a total of 5 GFP^+ colonies over three replicate treatments and an average fruequency of translocations calculated as $8.33\times10^{-4}\pm7.0$. One replicate treatment with genistein resulted in zero detectable GFP^+ colonies, so the average frequency was calculated for the other two replicates. Quercetin treatment produced a total of 5 colonies over thee replicate treatments with an average frequency of $5.55\times10^{-4}\pm3.85$. P-benzoquinone generated a total of 2 GFP^+ colonies over three replicate treatments with an average frequency calculated as 3.33×10^{-4} (between two replicates). Dipyrone generated 3 GFP^+ colonies over three replicate treatments with an average frequency calculated as $5\times10^{-4}\pm2.35$ (Table 4).

Similar to treatments with clone MAG1, treatments with this panel of compounds at later stages of differentiation generated very few, if any, GFP^+ colonies. Induction of interchromosomal translocations in MAG2 EB cells was not observed and zero GFP^+ colonies were generated over three replicate treatments (data not shown). A few GFP^+ colonies were generated following treatment of MAG2 bursting EBs. Etoposide treatment generated only one detectable GFP^+ colony over three replicate treatments. Genistein treatment generated a total of 3 GFP^+ colonies over three replicate treatments. The Average frequency of translocation for genistein could not be calculated because two replicates gave zero GFP^+ colonies. Quercetin treatment gave 6 GFP^+ colonies over three

replicate treatments and the average frequency of translocations was calculated as $.02\times10^{-4}$. P-benzoquinone and dipyrone generated 5 and 4 GFP^+ colonies over three replicate treatments, with average frequencies calculated as $.017\times10^{-4}\pm.005$ and $.013\times10^{-4}\pm.0094$ respectively (Table 5).

Table 4: Induction of interchromosomal recombination and translocation by dietary and environmental compounds in MAG2 ES cells: a comparison of frequency of GFP^+ colonies generated

Compound	**Expt**	**# GFP+ colonies**	**Frequency ($\times10^{-4}$)**	**Avg. Frequency ($\times10^{-4}$)***
Etoposide	1	0	< .033	
	2	4	1.33	
	3	1	.33	.83 ± .70
Genistein	1	4	13.3	
	2	0	< 3.3	
	3	1	3.3	8.3 ± .70
Quercetin	1	3	10.0	
	2	1	3.3	
	3	1	3.3	5.5 ± 3.8
p-benzoquinone	1	0	< 3.3	
	2	1	3.3	
	3	1	3.3	3.3
Dipyrone	1	2	6.67	
	2	0	< 3.3	
	3	1	3.3	5.0 ± .024
ISceI	1	5	33.3	
	2	4	26.7	
	3	5	33.3	31.1 ± 3.8

MAG2 ES cells were treated with various compounds, differentiated, and analyzed for the generation of fluorescent colonies at the formation of EBs.
*The interchromosomal recombination and translocation frequency was calculated as the number of GFP^+ clones per number of plated cells.
*Average frequency calculations excluded experiments with zero GFP^+ colonies.

Table 5**:** Induction of interchromosomal recombination and translocation by dietary and environmental compounds in MAG2 bursting EB cells: a comparison of frequency of GFP^+ colonies generated

Compound	**Expt**	**# GFP+ colonies**	**Frequency ($\times 10^{-4}$)**	**Avg. Frequency ($\times 10^{-4}$)***
Etoposide	1	0	< .0067	
	2	0	< .0067	
	3	1	.0067	
Genistein	1	0	< .0067	
	2	3	.02	
	3	0	< .0067	
Quercetin	1	0	< .0067	
	2	3	.02	
	3	3	.02	.02*
p-benzoquinone	1	0	< .0067	
	2	2	.013	
	3	3	.02	.016 ± .005*
Dipyrone	1	0	< .0067	
	2	1	.0067	
	3	3	.02	.013 ± .0094*
ISceI	1	5	.10	
	2	7	.14	
	3	5	.10	.11 ± .023

MAG2 bursting EBs containing myeloid progenitors were disrupted and cells were treated with various compounds, differentiated, and analyzed for the generation of fluorescent colonies at the formation of terminally differentiated colonies.
*The interchromosomal recombination and translocation frequency was calculated as the number of GFP^+ clones per number of treated cells.
*Average frequency calculations excluded experiments with zero GFP^+ colonies.

A two-way ANOVA was performed to determine if there were significant differences in treatment outcome between the two clones when treated as ES cells. Results showed an interaction between drugs and clones with a very significant (**) p-value of 0.0041. There were highly significant differences between drug treatments and between clones ($p < 0.0001$). Using Sidak's multiple comparison tests, a more stringent

analysis that reduces the probability of detecting significance by chance, significant differences between clones were identified for genistein treatment (*) and ISceI expression (****) (Figure 28). Thus, MAG1 is much more susceptible to DSBs and induction of translocations than MAG2.

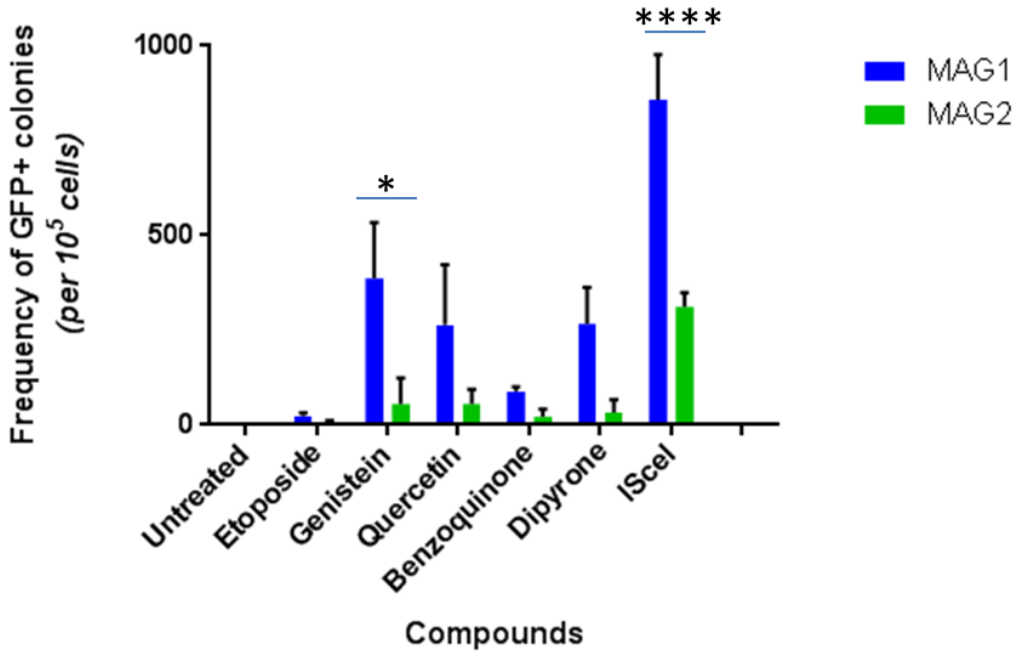

Figure 28: Frequencies of appearance of GFP^+ colonies following treatment and differentiation of ES cells: a comparison between clones MAG1 and MAG2

FACS analysis was also performed for a set of MAG1 and MAG2 treated cells. The x-axis represents the intensity of fluorescence emitted from cells in a single channel (FITC-A) and the y-axis represents the number of events detected at that intensity. Thus, a large number of events detected at a particular intensity are represented as a spike in the histogram. Here, histograms were overlaid to allow for comparison between the treated populations. MAG1 ES derived EB cells demonstrated an increase in fluorescence from etoposide, genistein, quercetin, and dipyrone treatments compared to an untreated

control. MAG2 ES derived EB cells did not demonstrate an increase in fluorescence from dipyrone treatment but there was a slight increase in fluorescence from p-benzoquinone treatment compared to the untreated control. MAG1 EB derived myeloid cells demonstrated a large increase in fluorescence from quercetin and dipyrone treatments and a moderate increase in fluorescence from genistein and p-benzoquinone treatments. MAG2 EB derived myeloid cells demonstrated only a slight increase in fluorescence from genistein, quercetin, p-benzoquinone, and dipyrone treatments compared to untreated controls. MAG1 and MAG2 bursting EB derived myeloid cells demonstrated increases in fluorescence for all treatments (figure 29). This could possibly indicate that MAG2 is more susceptible to *MLL-AF9* translocations at later stages of differentiation.

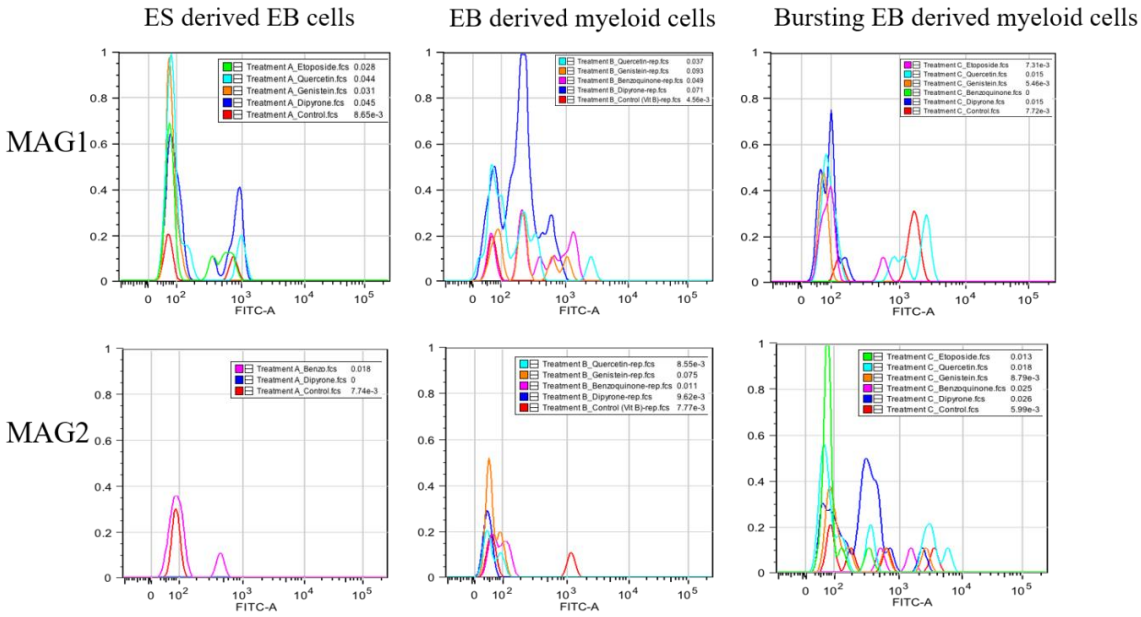

Figure 29: Histograms of fluorescence intensity from FACS analysis of treated MAG1 and MAG2 hematopoietic cells

Fluoresence intensity increases along the X-axis on a log scale. Etoposide treatment is represented by bright green, quercetin is represented by aqua, genistein is represented by orange, p-benzoquinone is represented by pink, dipyrone is represented by blue, and untreated controls are represented by red. Any peaks that are higher than untreated controls indicate an increase in the number of GFP^+ cells fluorescing at that intensity.

3.2.4 Genotoxic Effect of Common Bioflavonoids and Potentially Leukemogenic Compounds at Different Stages of Differentiation.

Differences in total numbers of surviving colonies in cohorts following treatment with each compound indicated a genotoxic effect. In order to determine a correlation between the number of *MLL-AF9* translocations and a genotoxic effect at the different stages of differentiation, the percentage of colonies that were GFP^+ out of the total number of surviving colonies was determined (Figure 30). Treatments with genistein, quercetin, and dipyrone demonstrated a higher genotoxic effect in ES cells than in more differentiated counterparts. Genistein treatment produced an average of 9.63% ±2.91 GFP^+ colonies following treatment of ES cells, 0.925% ±1.85 GFP^+ colonies following treatment of EB/HP cells, and 1.73% ±2.06 GFP+ colonies following treatment of bursting EB cells. Quercetin treatment produced an average of 2.97% ±2.95 GFP^+ colonies following treatment of ES cells, 0.31% ±0.46 GFP+ colonies following treatment of EB/HP cells, and 0.64% ±0.65 GFP^+ colonies following treatment of bursting EB cells. Dipyrone treatment produced an average of 3.5% ±1.8 GFP^+ colonies following treatment of ES cells, 1.4% ±1.6 GFP+ colonies following treatment of EB/HP cells, and 1.81% ±1.31 GFP^+ colonies following treatment of bursting EB cells. Treatment with etoposide demonstrated high genotoxic effect at all treatment stages with an average of 20.06% ±16.04 GFP^+ colonies following treatment of ES cells, 17.0% ±18.5 GFP^+ colonies following treatment of EB/HP cells, and 19.4% ±12.4 GFP^+ colonies following treatment of bursting EB cells. P-benzoquinone, unlike the other compounds demonstrated a higher genotoxic effect at later stages of differentiation. Treatment with p-benzoquinone produced an average of 1.87% ±0.51 GFP+ colonies

following treatment of ES cells, 2.75% ±5.5 GFP^+ colonies following treatment of EB/HP cells, and 14.7% ±12.7 GFP^+ colonies following treatment of bursting EB cells. This effect is not entirely conclusive though, as there were consistently very few colonies generated from p-benzoquinone indicative of a very high cytotoxicity in differentiated cell types. The presence of even very few fluorescent colonies represented a high percentage of the total number of colonies. Standard deviations that were greater than or approximately equal to the average percentage of GFP^+ colonies were due to some replicates producing zero GFP^+ colonies.

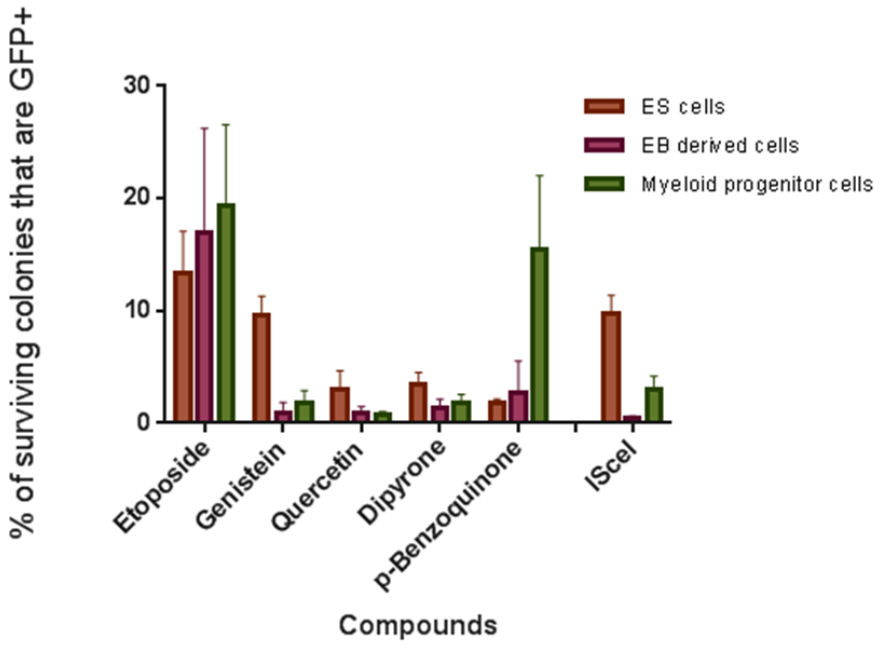

Figure 30: The percentage of surviving colonies that are GFP^+ following treatment as ES cells, EB derived cells, or bursting EB derived myeloid progenitor cells

3.2.5 *MLL-AF9* bcr Translocation Repair Products are Consistent with NHEJ.

Whole genome amplification (WGA), nested PCR amplification, and sequencing was performed across repair junctions on DNA isolated from 124 individual GFP+ hematopoietic colonies representing each treatment and each stage of differentiation. 51

ES derived EB colonies were analyzed (Table 6), 50 EB derived myeloid colonies were analyzed (Table 7), and 23 bursting EB derived terminally differentiated colonies were analyzed (Table 8). The majority (42 of 51 analyzed) of *MLL-AF9* repair products in ESC derived EB/HP colonies were consistent with NHEJ. The remaining 9 repair products did not match sequences from either gene construct and likely resulted from incorrect primer annealing. The majority (40 of 42) of the repair junctions deleted at least the original ISceI site after endonuclease expression or following treatment with any of the compounds indicating that this was consistently the site of DSB formation even in the absence of ISceI expression. Two ISceI-induced DSB repair products contained one ISceI recognition site inserted 5' to its original location. 6 of the 42 NHEJ repair products had simple deletions at the 5' or 3' end of the sequence indicating incomplete PCR reactions and 3 repair products had simple point mutations.

Repair products from EB derived myeloid progenitor colonies and bursting EB derived myeloid colonies were also consistent with NHEJ; 46 of 50 analyzed samples from EB derived myeloid colonies and 21 of 23 from bursting EB derived myeloid colonies. All repair junctions at these stages of treatment, regardless of the treatment compound had deleted at least the original ISceI site. 14 of the 47 repair products from EB derived myeloid colonies and 3 of 21 bursting EB derived myeloid colonies had simple deletions of the 5' or 3' end. 16 of the 47 NHEJ repair products from EB derived myeloid colonies had simple point mutations and 1 of 47 had a 25 bp deletion 3' of the repair junction. 7 of the 21 NHEJ repair products from bursting EB derived myeloid colonies had simple point mutations.

Table 6: Induction of NHEJ interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 ES cells: a comparison of breakpoint junctions with perfect or imperfect NHEJ repair

ES Cell Treatments		
Etoposide		
Class I perfect NHEJ	4/7	
Class II Simple Δ 3' or 5'	2/7	a. Loss of 3' end ~40bp
		b. Loss of 5' end ~40bp
Class III Point mutations	1/7	a. (2) 5'
Genistein		
Class I perfect NHEJ	3/5	
Class II Simple Δ 3' or 5'	1/5	a. Loss of 5' end ~40bp
Class III Point mutations	1/5	a. (1) 3' and (1) 5'
Quercetin		
Class I perfect NHEJ	6/7	
Class II Simple Δ 3' or 5'	1/7	a. Loss of 5' end ~40bp
Class III Point mutations	~~	
p-Benzoquinone		
Class I perfect NHEJ	6/7	
Class II Simple Δ 3' or 5'	1/7	a. Loss of 3' end ~40bp
Class III Point mutations	~~	
Dipyrone		
Class I perfect NHEJ	5/7	
Class II Simple Δ 3' or 5'	1/7	a. Loss of 3' end ~40bp
Class III Point mutations	1/7	a. (1) 5'
ISceI endonuclease		
Class I perfect NHEJ	7/9	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	~~	
Class IV Insertion/deletion	2/9	a. 29bp insertion 5'

Breakpoint junctions between *MLL*-GFPe1 and *AF9*-GFPe2 were classified as perfect NHEJ or imperfect NHEJ. Perfect NHEJ includes sequences that only have deletions of the original ISceI sites. Imperfect NHEJ includes sequences with larger deletions, insertions, and/or point mutations.

Table 7: Induction of NHEJ interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 EB cells: a comparison of breakpoint junctions with perfect or imperfect NHEJ repair

EB cell treatments		
Etoposide		
Class I perfect NHEJ	4/4	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	~~	
Genistein		
Class I perfect NHEJ	6/10	
Class II Simple Δ 3' or 5'	2/10	a. Loss of 3' end ~40bp
Class III Point mutations	2/10	a. (2) 3'
		b. (2) 3' and (2) 5'
Quercetin		
Class I perfect NHEJ	7/11	
Class II Simple Δ 3' or 5'	4/11*	a. Loss of 3' end ~40bp
Class III Point mutations	4/11*	a. (12) 3' and (2) 5'*
		b. (>20) 3' and (2) 5'*
		c. (6) 3' and (1) 5'*
		d. (13) 3' and (2) 5'*
p-Benzoquinone		
Class I perfect NHEJ	2/6	
Class II Simple Δ 3' or 5'	2/6	a. Loss of 3' end ~40bp[a]
		b. Loss of 5' end ~40bp[b]
Class III Point mutations	4/6	a. (4) 5'[b]
		b. (4) 5'
		c. (8) 3'[a]
		d. (>20) 3' and (13) 5'[x]
Class IV Insertion/deletion	1/6	a. 25 bp deletion 3'[x]
Dipyrone		
Class I perfect NHEJ	1/5	
Class II Simple Δ 3' or 5'	4/5*	a. Loss of 3' end ~40bp
Class III Point mutations	4/5*	a. (>20) 3' and (6) 5'
		b. (>20) 3' and (8) 5'
		c. (>20) 3' and (24) 5'
		d. (>30) 5'
ISceI endonuclease		
Class I perfect NHEJ	8/10	
Class II Simple Δ 3' or 5'	2/10	a. Loss of 3' end[a]
		b. Loss of 5' end[b]
Class III Point mutations	2/10	a. (>20) 3' and (1) 5'[a]
		b. (4) 3'[b]

*, [a, b] indicate and match sequences that are in two different classes of repair.

Table 8: Induction of NHEJ interchromosomal recombination and translocation by dietary and environmental compounds in MAG1 bursting EB cells: a comparison of breakpoint junctions with perfect or imperfect NHEJ repair

Bursting EB cell treatments		
Etoposide		
Class I perfect NHEJ	2/6	
Class II Simple Δ 3' or 5'	1/6	a. Loss of 3' end*
Class III Point mutations	4/6	a. (1) 5'*
		b. (1) 5' same for 2 seq.'s
		c. (2) 3' and (2) 5'
Genistein		
Class I perfect NHEJ	3/3	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	~~	
Quercetin		
Class I perfect NHEJ	2/3	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	1/3	a. (1) 5'
p-Benzoquinone		
Class I perfect NHEJ	3/3	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	~~	
Dipyrone		
Class I perfect NHEJ	3/3	
Class II Simple Δ 3' or 5'	~~	
Class III Point mutations	~~	
ISceI endonuclease		
Class I perfect NHEJ	1/3	
Class II Simple Δ 3' or 5'	2/3*	a. Loss of 3' end
Class III Point mutations	2/3*	a. (>20) 3' and (15) 5'
		b. (>20) 3' and (12) 5'

* indicate and match sequences that are in two different classes of repair.

A summary of breakpoint junction sequences in terms of perfect or imperfect NHEJ repair for each stage of differentiation is shown in table 9. ES derived EBs demonstrated the highest percentage (74%) of perfect NHEJ repair products in which there were no deletions or mutations beyond loss of the original ISceI sites. EB and bursting EB derived myeloid colonies had 61% and 67% of repair junctions that were perfect NHEJ, respectively. All three populations were able to repair DSBs by NHEJ, though it appears that ES cells may be more proficient at NHEJ repair.

Table 9: A comparison of perfect vs. imperfect NHEJ repair in MAG1 cells at three stages of differentiation

	Perfect NHEJ	Imperfect NHEJ
ES derived EBs	31 (~74%)	11 (~26%)
EB derived myeloid colonies	28 (~61%)	18 (~39%)
Bursting EB derived myeloid colonies	14 (~67%)	7 (~33%)

A one way ANOVA was also performed to compare perfect vs imperfect NHEJ at all three stages of differentiation. Using Tukey's multiple comparison test, ES cells had significantly more perfect NHEJ repair products than imperfect NHEJ repair products. ES cells also had significantly more perfect NHEJ repair products than bursting EB cells.

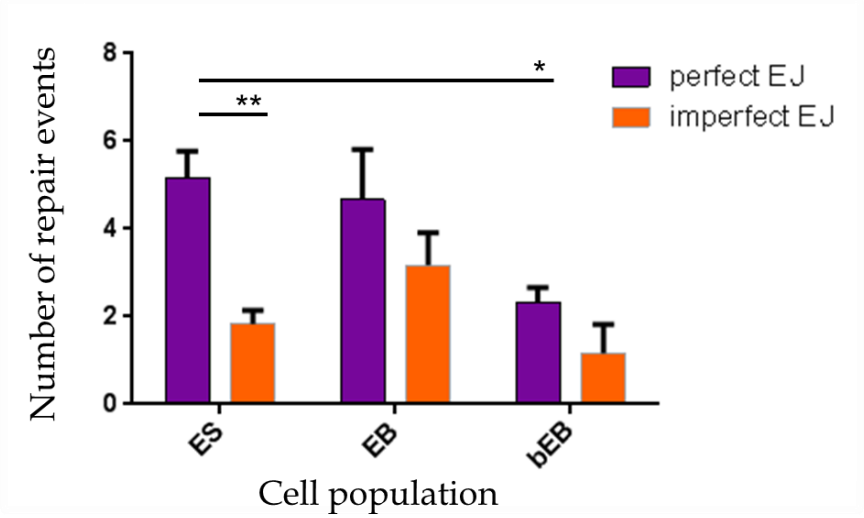

Figure 31: A comparison of perfect vs imperfect NHEJ repair in MAG1 cells at three stages of differentiation

One way ANOVA generated an interaction of p=.0256; row factor of p=.009; column factor of p=.0013.

Discussion

3.3.1 ESCs and Multipotent Hematopoietic Myeloid Progenitor Cells Can Repair DNA DSBs by Interchromosomal NHEJ Between the *MLL* and *AF9* bcrs.

The data presented in this study provides evidence that ESCs and multipotent hematopoietic myeloid progenitor cells can repair DNA DSBs by interchromosomal NHEJ specifically between the *MLL* and *AF9* bcrs. Significantly, DSB induction by ISceI or a panel of possible leukemogenic compounds in ESCs or early hematopoietic progenitors produced a large number of *MLL-AF9* translocations consistent with NHEJ. These data support the hierarchical model of leukemogenesis in which stem cells act as tumor initiators because of their ability to proliferate and differentiate into multiple lineages or because of their greater susceptibility to genome rearrangements than their differentiated counterparts [357]. These data demonstrate that differentiated hematopoietic cells also have the potential to repair DSBs via NHEJ, but erroneous NHEJ is more likely to occur in ESCs or early hematopoietic progenitors. It has been previously demonstrated that DSB repair by interchromosomal HR is significantly higher in ESCs and early hematopoietic progenitors indicating repair pathways (LTGC and SSA) that are distinct to this population and are more likely to result in genome rearrangements [24].

These data demonstrate that ES cells and early hematopoietic progenitors also have an increased capacity for error-prone NHEJ repair leading to a leukemogenic translocation compared to their differentiated counterparts. The reduced overall frequency of *MLL-AF9* chromosomal translocations in myeloid progenitors and terminally differentiated cells indicate a shift toward cell death as a likely outcome of DNA DSBs or inaccurately repaired DNA. Suppression of rearrangements in EB and

bursting EB derived cells could be due to transcriptional and epigenetic changes as part of differentiation programs or differences in the DNA damage response.

3.3.2 ESCs are Highly Susceptible to DSBs and Translocations Compared to their Differentiated Counterparts.

The calculated frequencies of translocations were 2-3-fold higher following treatment at the ES stage of differentiation for each compound when compared to treatments at later stages of differentiation (See Table 1). It is noteworthy that the number of cells plated after treatment for the first stage of differentiation from ESCs to EBs, was approximately 500 times lower than the number of cells plated at later stages of differentiation. To determine that these cells were not undergoing translocation events simply by the presence of a phenolic compound, an additional treatment with vitamin A was performed that resulted in zero GFP^+. This indicates that the population of ESCs is highly susceptible to DSBs and translocations when exposed to this panel of compounds.

The frequency of translocations with these reporters in ESC derived EB/HP cells is high compared to previously described models of DSB repair reviewed by Weinstock et al [23]. In two different studies with mammalian cells, the frequency of DSB-induced chromosomal translocations in the p5pF model was reported as $2.6\times10^{-5}\pm1.9$ with NHEJ repair accounting for 93% of translocations [346]; in the pCr15 model the frequency of DSB-induced chromosomal translocations was reported as 3.1×10^{-5} and NHEJ accounted for 100% of translocations [22]. Another previous study using mouse ES cells and targeted DSBs reported an average interchromosomal translocation frequency of 1×10^{-4} with 100% of derivative chromosomes consistent with NHEJ and SSA of the secondary derivative chromosome [16]. In this model, the absence of homology between the two

construct leaves NHEJ as the only pathway for repair following ISceI-induced DSBs. The lack of homology in these constructs can be related to leukemic translocations between two heterologous chromosomes since no consistent homology has been found at leukemic breakpoints junctions [358].

3.3.3 Differences in Translocation Frequencies due to Chromatin Structural Elements.

It has been previously shown that NHEJ predominates as the translocation pathway when there is heterology at the DNA ends [359]. The difference in translocation frequencies in this model could be due to a number of chromatin structural elements such as topoII cleavage sites, DNase hypersensitive sites, scaffold attachment regions and repetitive Alu elements in both the *MLL* and the *AF9* bcrs. This model displays chromosomal translocations in the *MLL* and *AF9* bcrs independent of their chromasomal context which suggest that these chromatin structural elements play a major role in the induction of leukemogenic *MLL* translocations. These reporter constructs have been inserted randomly into the genome so it is impossible to determine whether the chromatin structure or DNA sequence of topoII cleavage sites has more of a determining factor in the location of DSBs. If the insertion sites for the *MLL* and *AF9* bcrs have favorable chromatin structure for topoII cleavage, this could explain the elevated number of translocations seen with this model system in ESCs. TopoII appears to recognize chromatin structure instead of specific DNA sequences as has been shown by its colocalization with DNaseI hypersensitive sites in several genes involved in leukemia including *MLL, AF9, AF4, AML, ETO, BCR, and ABL* [360-363]. In the *MLL* gene a single strong topoII cleavage site has been identified near exon 12 that colocalizes with a DNase hypersensitive site [227, 361]. In *AF9*, one strong topoII cleavage site that

colocalizes with a DNase I hypersensitive site has also been identified near exon 8 [154]. In this model, the 5'-GFP reporter construct was inserted into the *MLL* bcr 5' of the strong topoII cleavage site, and the 3'-GFP reporter construct was inserted into the *AF9* bcr 3' of its strong topoII cleavage site thus enhancing the likelihood of topoII mediated DSBs in each gene construct.

Repetitive Alu elements within the *MLL* and *AF9* bcrs likely play a major role in *MLL* translocations. Alu repetitive elements comprise approximately 11% of the genome and have been implicated in the etiology of several inherited diseases as well as some cancers [364, 365]. An abundance of Alu elements are in the *MLL* and *AF9* bcrs and are associated with many translocated genes involved in cancer. For example, Alu elements have a high prevalence in the *BCR* and *ABL* genes and are also correlated with genomic breakpoints [366]. *MLL* has demonstrated both intra and interchromosomal recombination that indicate involvement of Alu elements [367]. Intrachromosomal recombination between intronic Alu elements can lead to partial tandem duplications of *MLL* which has been shown to occur in several cases of AML [368-370]. On the other hand, interchromosomal translocation between Alu elements is rare but has also been detected in tumor DNA [371]. Alu elements specific to the *MLL* gene have demonstrated reciprocal translocations *in vitro* using both homologous and heterologous Alu elements [359]. In this model, the abundance of Alu elements in each of the gene constructs contributes to the high frequency of translocations seen in ESCs following treatments with this panel of potential leukemogenic compounds. The fewer number of translocations seen with cells at later stages of differentiation could indicate differences

in chromatin conformation which does not allow for physical association of Alu elements following induction of DSB.

3.3.4 *MLL* and *AF9* Chromosomal Integration Sites Influence the Frequency of Translocations.

The influence of *MLL* and *AF9* integration sites accounts for the differences in the frequency of tranlocations between clones MAG1 and MAG2. The frequency of appearance of GFP^+ colonies generated in the MAG2 cell line was consistently lower than the frequency observed with MAG1 regardless of treatment compound or stage of differentiation. This indicates that chromatin context plays a very important role in the occurrence of chromosomal translocations. However, other characteristics of MAG2 could play a role. MAG2 could have reduced overall fitness and increased length of the cell cycle which could result in the generation of smaller colonies of cells that are not proliferating as rapidly. This difference would be slight as MAG2 colonies were readily detected. Another possibility is that the GFP reporter constructs integrated into chromosome domains that do not intermingle as often or do not break as easily [372].

3.3.5 Alternative Events Not Leading to the Generation of a GFP^+ Cell.

Generation of a GFP^+ colony using this model system was a readily detectable readout for translocation events. There are other events that could have occurred that would not lead to expression of GFP even with a *MLL-AF9* translocation. It is possible that DSBs of the reporter constructs had significant end resection resulting in loss of a splice donor, splice acceptor, or nucleotides in the GFP exon. This could potentially occur through activity of the Artemis/DNA-PKcs complex during NHEJ repair that can endonucleolytically cut single stranded overhangs [373, 374]. PCR using primers specific

for the *MLL* and *AF9* bcrs would be sufficient to screen random non-fluorescing colonies for these translocation events.

There is also a possibility that the induction of DSBs may have triggered cell cycle arrest which led to apoptosis and cell death. Toxicity of the compounds used in this study was apparent from cell viability assays as well as the presence of small, dense, non-proliferating colonies. Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay could be used to stain and visualize apoptotic colonies. Apoptotic cells, however, could not propagate a genome rearrangement and could not contribute to any leukemogenic translocations. These events would not be expected to lead to stable chromosomal translocations in a cell capable of long-term proliferation, so their occurrence is not necessarily relevant.

3.3.6 The Physiological Relevance of Dietary Bioflavonoids.

The potential leukemogenic compounds chosen for this study were two dietary bioflavonoids, genistein and quercetin that have previously been shown to cause topoII mediated DSBs. However, this is the first study to directly demonstrate that these compounds can induce translocations with the *MLL* bcr independent of its native chromatin construct and independent of a fusion protein in not only ESCs but their differentiated counterparts as well. These compounds may be linked to the *in utero* development of infant AML as a result of maternal exposure and the fact that these compounds can cross the placenta [263]. In addition, genistein along with several other bioflavonoids are currently unregulated and widely commercially available as dietary supplements in doses as high as 1200mg. There is some controversy as to what doses of these compounds *in vitro* can be considered physiologically relevant. In terms of dietary

intake, *in vitro* concentrations of genistein >5μM have been defined as non-physiological [375]. Even comparably low plasma concentrations of genistein in mice neonates, between 1-5μM, resulted in adverse reproductive effects later in life [376]. Soy-based diets have also become increasingly popular in the U.S. and Europe. Previous studies have reported average adult soy intake to be 0.15-3mg/day [377]. An evaluation in the United Kingdom reported and average adult intake of 1mg/day however, people consuming soy-based vegetarian or vegan diets consume approximately 10 times more with averages at 8.6mg/day in women and 7.5mg/day in men [378], thus it is reasonable to assume their plasma concentrations are 10 times higher as well. Infants fed soy formula also consume approximately 10 times more soy isoflavones with some plasma concentrations of genistein alone reported at 2.5μM [379]. Considering foods contain multiple bioflavonoids, the combined concentrations and topoII inhibiting effects is likely much higher. Also, it has been demonstrated that bioflavonoids are bioaccumulative [380] and levels are higher in human umbilical cord blood possibly from differences in metabolic and excretion rates of mother and fetus [381]. This is concerning considering fetal cells are proliferating rapidly, have higher levels of topoII [382], and are thus more sensitive to topoII inhibiting agents. The doses of genistein and quercetin for this study are high relative to an average diet, but still considered biologically relevant. 75μM doses were also preferred due to their biochemical similarity to etoposide and reported peak plasma concentrations in patients following etoposide treatment. An early pharmacokinetic study reported peak plasma concentrations between 45.87 and 193.69μM in 12 patients after administering 400 to 800mg etoposide [383].

3.3.7 The Physiological Relevance of P-benzoquinone and Dipyrone.

P-benzoquinone and dipyrone were chosen for this study due to their implications in causing infant AML from epidemiological studies [240, 267, 271, 353, 384-386]. Dipyrone exposure during pregnancy has been linked to *MLL* rearranged infant acute leukemia specifically, though there is no direct evidence from *in vitro* or *in vivo* studies that support this claim. Here, dipyrone has been shown to promote the specific translocation between the *MLL* and *AF9* bcrs in murine ES cells as well as their differentiated counterparts. This is the first study to show direct evidence of dipyrone-induced *MLL* translocations. Dipyrone is not known to be a topoII inhibitor and its structure is markedly different from that of etoposide and bioflavonoids that are known topoII inhibitors, thus it is likely that dipyrone acts to promote *MLL* translocations through a completely different mechanism. Doses used in this study were determined from reported plasma concentrations of dipyrone metabolite 4-methyl-amino-antipyrine (MAA) and 4-amino-antipyrine (AA) which have been reported as high as 59.1μM and 7.4μM respectively following a 1000mg oral dose [386]. In combination, these two metabolites could result in plasma concentrations as high as 75μM of total dipyrone metabolites, supporting that the concentration used in this study is biologically relevant.

Benzene exposure has been known to cause detrimental hematopoietic effects since the early 1900's with the first documented cases of its hematotoxic effect in the US dating as early as 1910 in reports from Johns Hopkins hospital. More recent studies have shown that benzene exposure results in chromosomal abnormalities [268] and is hematotoxic and genotoxic [266]. Benzene metabolites p-benzoquinone and hydroquinone have also been shown to be genotoxic yet they are not considered to be

carcinogenic, even though it is likely that benzene toxicity is due to activity of these metabolites. These two metabolites are interchangeable via oxidation/reduction *in vivo* due to the activity of NAD(P)H quinone oxidoreductase (NQO1) and myeloperoxidase (MPO) [274-276]. P-Benzoquinone has been shown to cause DNA DSBs and recombination in pKZ1 transgenic mouse fetal hematopoietic cells, cause DEL recombination in *s. cerevisiae* [356, 387], and has been shown to be a topoII inhibitor [280, 290].

Benzene and its metabolites are not specifically associated with any specific leukemogenic translocation but are known to induce a wide variety of chromosomal abnormalities leading to AML including chromosomal aneuploidy, deletions, and translocations of various chromosomes (reviewed in [388]). Though p-benzoquinone is not specifically associated with *MLL* rearrangements, one case of ALL with a translocation of t(4,11) has been reported [389]. Also, it has been determined that $CD34^{+}$ hematopoietic progenitor cells are sensitive targets for toxicity [390].

Peak plasma concentrations of p-benzoquinone from naturally occurring benzene exposure could not be determined, so the dose of p-benzoquinone used in this study were chosen based on reported plasma concentration levels of hydroquinone from previous reports [391-393]. Hydroquinone, is commonly used in dermatological creams and is also present in common foods such as tea, coffee, wheat, and pears. Peak plasma concentrations reported in a dermatological study following application of 1.0 gram of cream containing 4% hydroquinone were 8.654µg/ml or 78.3µM. Therefore, the concentrations used in this study can be considered biologically relevant. Results from p-benzoquinone exposure demonstrate for the first time that this benzene metabolite can

directly cause translocations between the *MLL* and *AF9* bcrs independent of their chromatin context and that ES cells are highly susceptible. P-Benzoquinone also resulted in an increased number of GFP^+ cells compared to the total surviving cells at later stages of differentiation. This indicates that p-benzoquinone may target the *MLL* and *AF9* bcrs and results in higher genotoxic stress than the other compounds at later stages of differentiation.

3.3.8 Conclusion

These experiments demonstrate that the generation of induced translocations following treatment with common dietary and environmental compounds occurs readily between *MLL* and *AF9* bcrs independent of sequence and chromatin context of their native loci. This is the first direct demonstration that exposure to common dietary bioflavonoids genistein and quercetin, environmental pollutant p-benzoquinone, and the NSAID dipyrone can initiate a leukemic translocation between the *MLL* and *AF9* bcrs. In addition, it was shown that the ESCs are highly susceptible to these rearrangements induced by exposure to these compounds indicating that the stem cell pool *in vivo* is responsible for initiation of leukemogenesis. The high frequency of observed translocations in ES derived EB colonies specifically in the absence of other competitive pressures that would be expected *in vivo* indicates that illegitimate NHEJ resulting in translocations can occur with high frequency not only in the highly susceptible stem cell pool but in differentiated myeloid cells as well. This model system also provides a framework for screening hundreds of potentially leukemogenic compounds for their long-term genotoxic effects, their relative risk for promoting the *MLL-AF9* translocation, and the stage of differentiation during which myeloid cells are most at risk.

CHAPTER IV: EFFECTS OF REPEATED EXPOSURE TO DIETARY AND ENVIRONMENTAL COMPOUNDS ON LONG-TERM REPOPULATING POTENTIAL OF HUMAN $CD34^+$ CELLS

4.1 Background

Leukemia is primarily a stem cell disease; leukemic cells mimic the phenotypes of HSCs, and like HSCs these leukemic stem cells (LSCs) produce progeny of heterogeneous populations [109-111]. It has been shown that both chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) can be initiated by a small population of leukemic stem cells [112, 113]. It has also been shown that LSCs can potentially arise from committed progenitor cells that have developed abnormal phenotypes, such as the ability to proliferate or self-renew [114]. These LSCs have a distinct competitive advantage over their normal counterparts which cannot be entirely explained by the proliferation of leukemic blasts. Leukemic blasts have been shown to have limited proliferative potential; however, they remain active in the cell cycle [394]. Normal blasts including colony forming cells (CFC) and the more primitive long-term culture initiating cell (LTC-IC) are mostly quiescent in normal steady state peripheral blood [395-398]. Continuous leukemic progenitor proliferation has not been completely elucidated on a molecular level. It may be related to autocrine or paracrine growth factor production by AML blasts [399, 400]. The cytokines produced by leukemic cells have been shown to promote AML-CFC growth *in vitro* and appear to facilitate proliferation

of AML cells *in vivo* in immunodeficient mice [401]. This increased proliferative capacity can be observed and quantitated by LCT-IC assays.

LTC-IC are rare progenitor cells that have many of the characteristics of bone marrow repopulating HSCs [109]. LTC-IC are defined and detected by their ability to generate committed clonogenic CFCs after five or more weeks in long-term culture. In this system, colonies can be sustained for 8-12 weeks [402]. This is due to the development of an adherent stromal cell layer that provides the microenvironment needed for stem and progenitor cells to proliferate and differentiate [403, 404]. This culture environment mimics the marrow stroma and does not require the addition of exogenous cytokines [402].

There is clear evidence that exposure to irradiation or some chemotherapeutic drugs such as etoposide and teniposide can lead to the development of therapy-related AML. It has been well established that drugs that target the essential cellular enzyme topoisomerase II (topoII), such as etoposide, promote DSBs and chromosomal rearrangements similar to those seen in leukemia patients [232]. A previous study to characterize the impact of etoposide on primary human long-term repopulating HSC determined that exposure to one dose of 20-50μM or weekly doses of .01μM etoposide significantly stimulated LTC-IC proliferation and differentiation [232]. Other initiating factors that cause LSCs to arise have yet to be entirely elucidated, although the microenvironment, stem cell niche, epigenetics, dietary and environmental compounds all may play a role. Some dietary and environmental compounds have been associated with an increased risk of leukemia development including the dietary bioflavonoids genistein and quercetin. These bioflavonoids are bioactive compounds commonly found in many

fruits and vegetables, tea leaves and coffee, and many herbs and spices [259, 405]. The environmental toxin, p-benzoquinone, has been shown to increase risk of infant acute leukemia (IAL) in several epidemiological studies [406-408]. P-benzoquinine has been shown to be a topoII poison [290]and induces genotoxicity in human $CD34^+$ hematopoietic progenitor cells [409]. The non-steroidal anti-inflammatory drug, dipyrone, has been implicated in epidemiological studies to increase risk of infant leukemia, though it is not known to be a topoII inhibitor [240, 293, 386].

It has been previously shown that etoposide can induce leukemia related *MLL* translocations in primary human $CD34^+$ cells [410, 411]. Data from chapter 3 demonstrates that genistein, quercetin, p-benzoquinone and dipyrone can promote *MLL-AF9* translocations in murine ESCs; however, whether this panel of compounds can stimulate the long-term repopulating potential of human $CD34^+$ HSCs, which represent <10% of the population, similar to etoposide, has not been determined [412]. Here, primary human umbilical cord $CD34^+$ cells were exposed to biologically relevant concentrations of genestein, quercetin, p-benzoquinone, and dipyrone. Considering the likelihood of repeated fetal exposure *in utero* to these compounds, cells were exposed on a weekly basis and analyzed for their ability to initiate hematopoiesis via long-term culture initiating cell (LTC-IC) assay. Genistein demonstrated significant stimulation of long-term repopulating potential of LTC-IC when compared to untreated counterparts. Quercetin and p-benzoquinone demonstrated a significant decrease in long-term repolulating potential when compared to untreated controls. This indicates that genistein not only initiates leukemia associated rearrangements, but also induces differentiation and proliferation, potentially making it a very potent initiator of leukemogenesis.

Quercetin, p-benzoquinone, and dipyrone, though they have been shown to promote leukemia-associated rearrangements in primary human CD34+ cells, suppress differentiation and proliferation of those cells.

4.2 Materials and Methods

4.2.1 Cell Culture of Human Umbilical Cord $CD34^+$ Cells

$CD34^+$ cells were isolated from human umbilical cord blood and were obtained from AllCells. Cells were frozen immediately upon arrival until ready for use. Once thawed, cells were cultured overnight in StemSpan medium (Stemcell Technologies) prior to treatment. The following day, cells were exposed to 5µM genistein, 5µM quercetin, 5µM genistein and quercetin, 75µM p-benzoquinone, or 75µM dipyrone, all prepared from 20mM stock solutions in Iscove's Modified Dulbecco's Medium (IMDM) for 1 hour. Untreated and treated cells were counted using Trypan Blue exclusion method and immediately seeded on supportive feeder layers for LTC-IC.

4.2.2 Long-term Culture Initiating Cell Assay (LTC-IC)

Murine Fibroblast cell line M2-10B4, obtained from ATCC, were expanded in RPMI medium supplemented with 10% FBS. Cells were irradiated (8,000cGy) and plated into confluence (~3 x 10^5 cells/well) into collagen-coated 6-well tissue culture dishes and allowed to recover overnight in LTC-IC medium (H5100 supplemented with 10^6µM hydrocortisone; StemCell Technologies). Treated and control CD34+ cells were resuspended in LTC-IC medium and plated on M2-10B4 feeder cell layers in triplicate and at three concentrations (10^5, 10^4, and 10^3) for each treatment group. LTC-IC cultures were incubated at 37˚C in a humidified incubator with 5% CO_2 for 6 weeks with treatments repeated and fresh media added weekly. After 6 weeks, cultures were

harvested and the contents of each well were plated in methylcellulose medium (Methocult GF H4435; StemCell Technologies) which contains cytokines supportive of myeloid differentiation. Cultures were incubated for 18 days at which point all CFUs were scored.

Statistical Analysis

Total numbers of CFUs were scored for each serial dilution of initially seeded $CD34^+$ cells for each treatment group and compared to untreated control groups using a two-way ANOVA via GraphPad Prism software. Significant comparisons were determined by using Dunnett's multiple comparisons test.

4.3 Results

4.3.1 Genistein Exposure Stimulates and Quercetin and P-benzoquinone Exposure Inhibit Hematopoietic Stem Cell Proliferation.

Primary human umbilical cord $CD34^+$ cells were exposed for 1hr to either 5µM genistein, 5µM quercetin, 75µM p-benzoquinone, or 75µM dipyrone. Following initial treatment, untreated control and treated cells were seeded on supportive feeder layers for LTC-IC. Additional treatments using the same concentrations of compounds for 1hr were conducted during weekly media changes. Six week LTC-IC cultures were seeded in methylcellulose media to allow for CFU colony formation. Hematopoietic colonies are shown in figure 32.

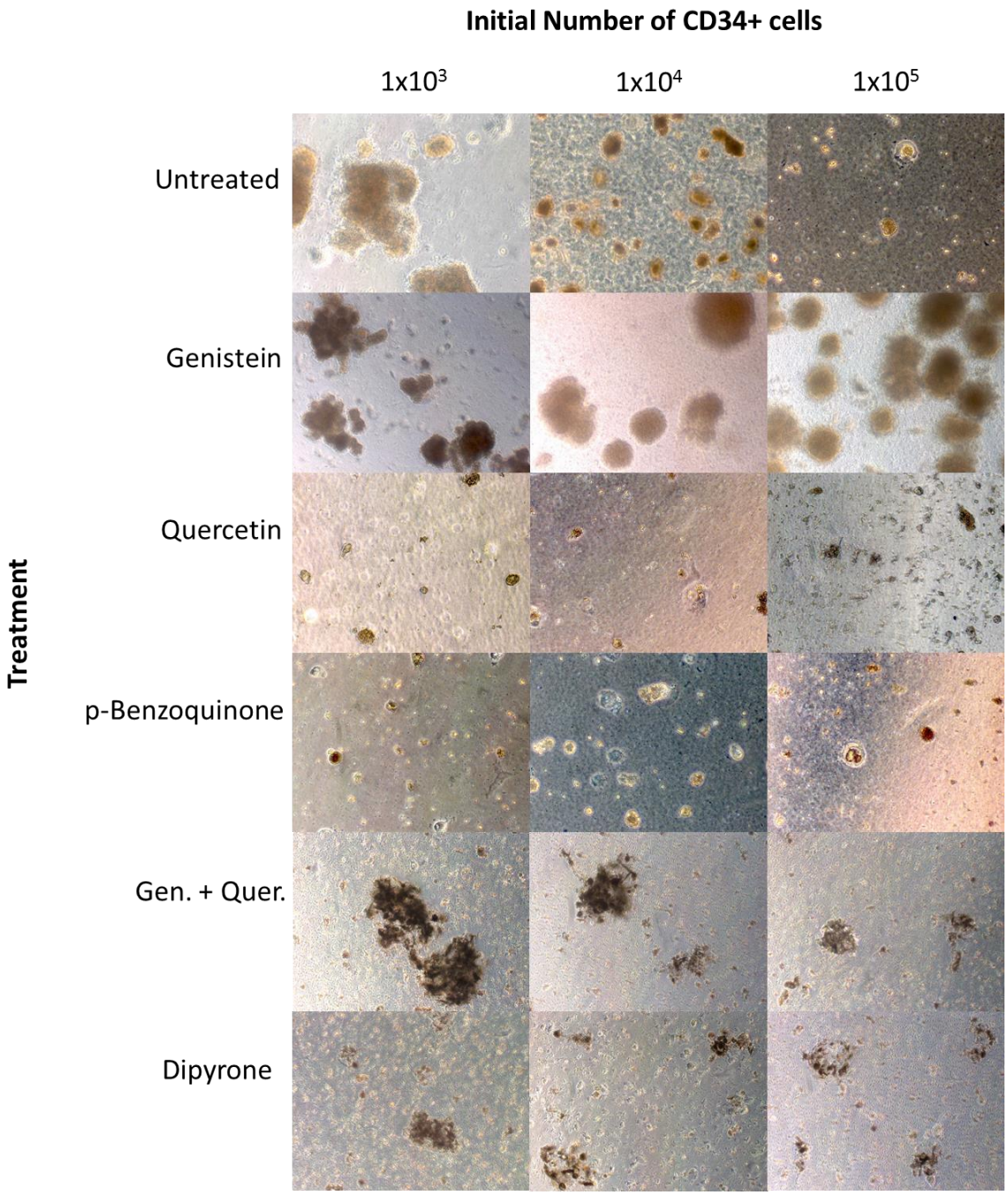

Figure 32: Hematopoietic colonies from LTC-IC assays: all images are at 100x magnification

The number of $CD34^+$ HSC-derived CFUs that survived treatment were analyzed and compared to the untreated controls. LTC-IC cultures were initiated with a serial dilution seeding of treated or untreated $CD34^+$ cells and clonogenic CFU output was

almost inversely related to the input for all three treatment groups; CFU output was higher in cultures initiated with fewer cells. Results from each of the three replicate experiments for each treatment displayed some variation; however each experiment showed a significant increase in the number of CFUs derived from HSCs following repeated genistein exposures compared to untreated controls; and each experiment showed a decrease in the number of CFUs derived from HSCs following repeated quercetin or p-benzoquinone exposures (Table 10).

Table 10: Colony-forming units in LTC-IC from genistein-, quercetin-, and p-benzoquinone-treated, combined genistein and quercetin-treated, dipyrone-treated, and control untreated $CD34^+$ cells

No of CD34+ cells seeded per well	No. of CFU Colonies					
	Control	Genistein	Quercetin	Gen +Quer	Benz.	Dipyr.
$1x10^5$	156 (±151.3)	511.3 (±64.5)	29.75 (±4.03)	318.3 (±87.4)	54.7 (±5.13)	150 (±36.12)
% Stimulation	~~~~	227.60%	-80.90%	104.04%	-64.90%	-3.85%
$1x10^4$	343.3 (±6.5)	396.3 (±17.6)	47 (±10.5)	252.3 (±91.2)	131.7 (±163.2)	67.33 (±18.15)
% Stimulation	~~~~	15.40%	-86.30%	-26.50%	-61.60%	-80.39%
$1x10^3$	209.6 (±147.2)	709.8 (±67.5)	62 (±20)	132 (±29.0)	66.7 (±24.4)	101.0 (±26.51)
% Stimulation	~~~~	238.60%	-70.40%	-37.02%	-68.20%	-51.81%
Average % Stimulation	~~~~	160.50%	-79.20%	13.50%	-64.90%	-32.80%

[1]Number of CFU colonies obtained in LTC-IC assays derived from $CD34^+$ cells were averaged from three or more replicates for all experimental condition at each cell dilution with standard deviations.
[2]% stimulation represents the percentage increase or decrease of LTC-IC average per 10^5, 10^4, or 10^3 cells in each treated population. This number was obtained by calculating the percentage of cells in treated populations relative to controls to reveal the percentage of growth stimulation in relation to controls. This was calculated as [(average # of CFUs from treatment-average # CFUs from control)/average # CFUs from control] expressed as a percentage.
[3]Average % stimulation represents the average of the three % stimulation calculations from each treatment at all three concentrations. This was calculated as [(%stimulation10^5 + % stimulation10^4 + %stimulation10^3)/3] showing the average percentage of growth stimulation in relation to controls.

Overall, untreated controls generated an average of 156 CFUs per 10^5 cells, Repeated exposure to 5µM genistein increased the number of CFUs to 511.3 per 10^5 cells, a 227% increase with an average overall stimulation of proliferation of 160.5%. Repeated exposure to 5µM quercetin decreased the number of CFUs to 29.75 per 10^5 cells, an 80.9% decrease in proliferation with an average percent inhibition of 79.2%. Repeated exposure to 75µM p-benzoquinone decreased the number of CFUs to 54.7 per 10^5 cells, a 64.9% decrease with an average inhibition of proliferation of 64.9%; and repeated exposure to 75µM dipyrone decreased the number of CFUs to 150 per 10^5 cells with an average inhibition of proliferation of 32.8%.

This data was analyzed for statistical significance with two-way ANOVA using GraphPad Prism software. The overall p-value was <0.0001 and significant comparisons were determined by Dunnett's multiple comparisons test. Significant comparisons are indicated in figure 33**.** The increase in the number of CFUs derived from genistein treated $CD34^+$ cells was highly significant in two out of three replicate treatment groups with a p-value less than 0.00005. The decrease in the number of CFUs derived from quercetin treated $CD34^+$ cells was significant in two out of three replicate treatment groups, one group with a p-value less than 0.00005 and the other group with a p-value less than 0.05. The number of CFUs derived from p-benzoquinone treated $CD34^+$ cells indicated a significant decrease in one out of three replicate treatment groups with a p-value less than 0.005. The number of CFUs derived from dipyrone treated $CD34^+$ cells indicated a significant decrease in proliferation in one of three treatment groups.

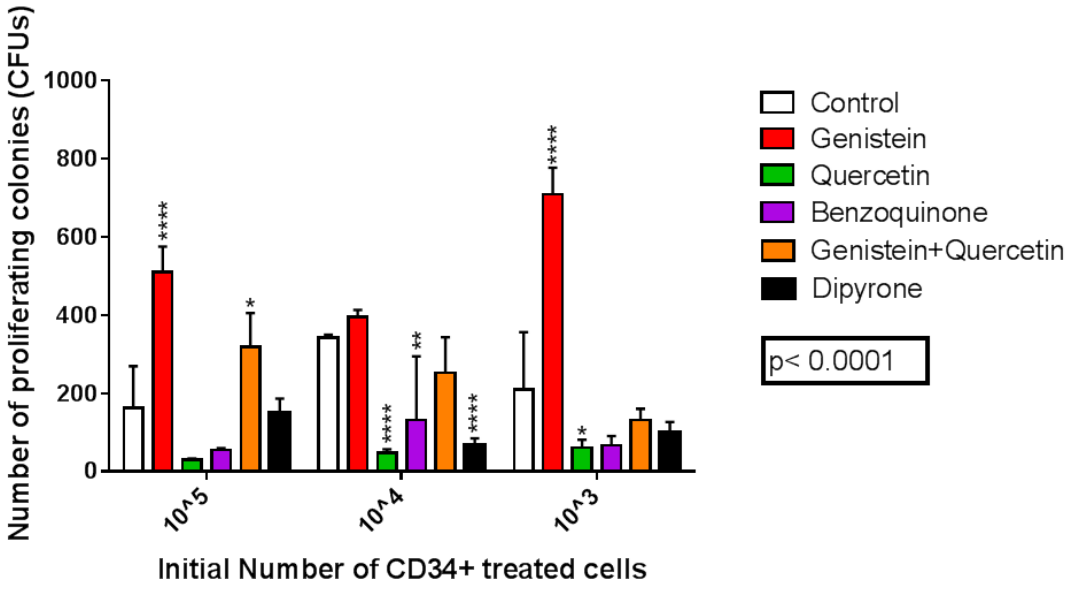

Figure 33: Two-way ANOVA and multiple comparisons of colony-forming units in LTC-IC assay: comparisons of genistein-, quercetin-, and p-benzoquinone-treated, combined genistein and quercetin-treated, dipyrone-treated, and control untreated CD34+ cells.

***** indicate a significant comparison from Dunnett's multiple comparisons test. Comparisons were made between averages of CFUs from each treatment and the untreated control for each initial number of CD34+ seeded cells. *indicates $p<0.05$, **indicates $p<.005$, ****indicates $p<.00005$.

4.4 Discussion

Leukemia is believed to be initiated in the small HSC population and most often involves chromosomal abnormalities of the *MLL* gene [235, 413, 414]. It has been demonstrated that these leukemia-associated abnormalities are initiated by inhibitors of the topoII enzyme [224, 362]. The topoII poison, etoposide, has been shown to promote *MLL* rearrangements in primary human $CD34^+$ cells as well as stimulate long-term repopulating potential of the HSC population. [230, 232]. Likewise, bioflavonoids have been shown to induce *MLL* translocations in human CD34+ cells [410], though their

effect on long-term proliferation was not demonstrated. Here we developed LTC-IC assay to characterize the effect of the topoII inhibitors genistein, quercetin, p-benzoquinone, and the NSAID dipyrone on the long-term repopulating potential of human cord blood $CD34^+$ HSCs. Results demonstrate that the dietary bioflavonoid genistein has a stimulatory effect on the proliferative capacity of HSCs whereas dietary bioflavonoid quercetin, environmental toxin p-benzoquinone, and NSAID dipyrone all have an inhibitory effect when compared to untreated control cells.

The biological effects of genistein are multifaceted. Genistein is thought to have chemopreventative properties as evidenced in epidemiological studies (reviewed in [415]). Genistein is a phytoestrogen that may modulate the activity of endogenous estrogen [416, 417]. It acts as a tyrosine kinase inhibitor and prevents cellular proliferation through this mechanism [418]. Genistein has been shown to promote terminal differentiation in human tumor cells [261, 419]. Genistein is also a known topoII inhibitor and has been shown to induce cleavage of the *MLL* gene [258, 259]. Here it is demonstrated that genistein significantly increases the proliferative potential of human cord blood $CD34^+$ cells very similar to etoposide as demonstrated by previous studies [230, 232, 420, 421]. These findings suggest that genistein consumption may have either beneficial or adverse effects depending on cell type or developmental stage. It appears that stem cells in particular may be particularly susceptible to these adverse effects; and hematopoietic stem cells exposed to genistein even in low doses could potentially become leukemogenic. Biologically relevant doses of bioflavonoids is a point of controversy and here we have chosen a low dose that is relevant to blood plasma concentrations following dietary intake of genistein containing foods [375, 422, 423].

Quercetin, like genistein is known to have chemopreventative properties. Quercetin has been shown to inhibit cellular proliferation of cancer cells by inducing expression of transforming growth factor-β (TGF-β) [262, 424]. Quercetin is also known to inhibit topoII, like genistein, and has been shown to induce cleavage in the *MLL* gene [258, 259, 312] . Here, quercetin had an inhibitory effect on the proliferative potential of human cord blood $CD34^+$ HSCs. This indicates that perhaps quercetin acts through a different mechanism than genistein. TGF-β is a bidirectional regulator of hematopoietic cell growth [425]. It either stimulates or inhibits the *in vitro* growth of normal myeloid progenitors depending on their stage of differentiation [426, 427]. Therefore, quercetin induces TGF-β-mediated suppression of proliferation and differentiation in HSCs, however it could have the opposite effect on differentiated populations. Also, considering that quercetin has been shown to promote *MLL-AF9* translocations, this data suggests that the surviving population of differentiated cells may indeed be leukemogenic even though the initial proliferative potential of HSCs was decreased. The dose of quercetin used in these experiments was also determined according to previously published literature as biologically relevant blood plasma concentration following dietary intake [380, 428, 429].

P-benzoquinone and dipyrone were chosen for this study due to their implications in causing leukemia from epidemiological studies [240, 267, 271, 353, 384-386]. P-benzoquinone is a known topoII inhibitor as has been previously demonstrated to induce DSBs and DNA recombination [430]. Additionally, it has been demonstrated that p-benzoquinone is cytotoxic and genotoxic to human $CD34^+$ cells [409]. P-benzoquinone had an inhibitory effect on the long-term repopulating potential of HSCs which indicates

high cytotoxicity and genotoxicity in this population as well. The molecular mechanism of p-benzoquinone induced leukemia has not been fully elucidated, however evidence suggests that the aryl hydrocarbon receptor (AhR) may play an important role [431]. One study demonstrated that AhR knockout mice are resistant to benzene-induced toxicity of hematopoietic cells [432]. Several studies have determined that AhR levels vary in a cell and tissue specific manner as well as with stage of development and age. Levels of AhR have also been shown to change with cell density, presence or absence of growth factors, neoplastic transformation, and state of differentiation [433-435]. This has been shown to occur in a variety of cell types suggesting that this may be true for HSCs as well. It has been suggested that AhR expression is down-regulated in proliferating and differentiating HSCs [436]. This indicates if benzene induced hematotoxicity is dependent on AhR activity [437], then the HSC pool is particularly susceptible. This is consistent with the reduced proliferative capacity of p-benzoquinone treated HSCs demonstrated in this study. Biologically relevant concentrations of p-benzoquinone could not be determined, however, plasma concentrations of hydroquinone, an interchangeable metabolite of benzene have been reported following exposure [391-393].

Dipyrone is not a known topoII inhibitor and its mechanism of myelotoxicity or as an NSAID have been elusive. Dipyrone has been shown to be a cyclooxygenase (COX) inhibitor [438] and may inhibit cell proliferation through COX-2 downstream targets. Dipyrone had an inhibitory effect on the proliferation potential of HSCs in this study although there were some large colonies indicating some proliferation did occur. Biologically relevant concentrations of dipyrone were determined from reported plasma concentration following a 1000mg dose.

All of the compounds used in this study have been associated with IAL. In this study, all of these compounds disrupted normal hematopoietic proliferation and differentiation in either a stimulatory or inhibitory fashion. Bioflavonoids genistein and quercetin are popular dietary supplements that are widely commercially available. Both of these compounds have been shown to promote leukemic rearrangements and here genistein stimulated and quercetin inhibited HSC proliferation and differentiation. As supplements, these compounds are completely unregulated and are available at very high doses. The risk of leukemia development associated with consumption of these bioflavonoids is an issue that should be addressed for regulation and safety standards.

CHAPTER V: A LINK BETWEEN ROS AND GENETIC INSTABILITY IN HEMATOPOIETIC STEM CELLS

5.1 Background

Organisms in aerobic environments are continuously exposed to reactive oxygen species (ROS). ROS include oxygen molecules (O_2), superoxide anion radicals ($^{\cdot}O_2^-$), hydroxyl free radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), and can be generated by imbalanced endogenous reduction of oxygen by cellular enzymes or in mitochondrial respiratory pathway, as well as by exogenous exposure to UV or environmental damaging agents [439]. Regulation of intracellular ROS levels and ROS-mediated signaling is central to maintaining the balance between self-renewal, proliferation, and differentiation of normal stem and progenitor cells in the hematopoietic and neuronal compartments, as well as the early embryonic stem (ES) cell compartment. Evidence indicates that the inability to regulate high levels of ROS leading to alteration of cellular homeostasis or defective repair of ROS-induced damage lies at the root of diseases characterized by both neurodegeneration and bone marrow failure [440-443]. Further, high levels of ROS appear to be a distinct feature of acute myeloid leukemia (AML) [444-446], and likely contributes to the pathogenesis of the disease. Evidence directly linking elevated ROS to genetic instability and hematopoietic stem cells is lacking. Here the MAG1 genetically engineered murine stem cell line was utilized to determine if elevated ROS could induce genetic instability and chromosomal translocations.

5.2 Materials and Methods

5.2.1 Generation of Transgenic Cell Line

A murine HSC line MAG1 was genetically engineered to contain two transgene reporter of recombination, each containing a 5' or 3' exon of GFP. This process is described in chapter 2. This cell line allows for identification of chromosomal translocations following DNA damage and repair by interchromosomal NHEJ that results in a repair event joining the two exons onto the same DNA helix to form an intact GFP transcript.

5.2.2 *In Vitro* Hematopoietic Differentiation and Treatment with H_2O_2

MAG1 ESCs were exposed to either 100 µM H_2O_2 or 5mM H_2O_2 for 30 minutes. Surviving cells were then allowed to proliferate into myeloid CFU by standard protocols [24], and those CFU expressing GFP were readily identified by fluorescent microscopy. The process of *in vitro* hematopoietic differentiation that was used for these experiments is described in chapter 3. Fluorescent GFP^+ colonies were identified and scored using contrast and fluorescent microscopy. Statistical analysis was performed using Graphpad Prism software.

5.3 Results

5.3.1 H_2O_2 Promotes Genome Rearrangements in HSCs.

This experiment demonstrated that a single 30 min exposure of HSCs to 100 µM H_2O_2 or 5mM H_2O_2 is sufficient to promote the appearance of GFP+ colonies in a dose-dependent manner at average frequencies of 6×10^{-6} and 10×10^{-6}, respectively. A representative ESC and a GFP^+ derivative myeloid CFU colonies are shown by contrast microscopy (100X), along with the same GFP^+ CFU colony shown by fluorescent microscopy in figure 34.

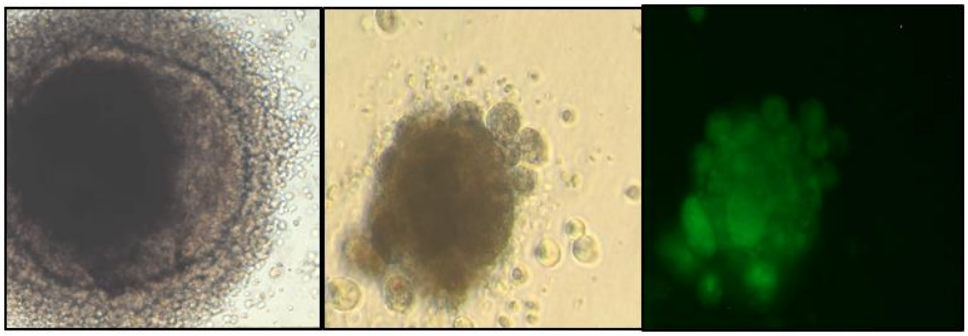

Figure 34: Confocal microscopy images following treatment of MAG1 ESCs with H_2O_2. Left image -- HSC colony by contrast microscopy (100X). Middle image -- GFP+ derivative myeloid CFU by contrast microscopy (100X). Right image -- same GFP+ CFU colony shown in middle by fluorescence microscopy (100X).

A one-way ANOVA was performed in order to determine if there was a dose-dependent effect of H_2O_2 on the appearance of GFP^+ colonies. The results of this analysis are depicted in a bar graph in figure 34. Results showed there is a dose dependent effect on the appearance of GFP^+ myeloid CFU derived from HSC exposed to 0, 100µM, or 5mM H_2O_2 for 30 min, then returned to normal conditions. 100µM resulted in an average frequency of CFU at 6.5×10^{-6}. 5mM resulted in an average frequency of CFU at 10.4×10^{-6}. ***** denotes statistically significant stimulation of chromosomal translocations by H_2O_2 (students T-test p value<0.037). A few colonies were observed in untreated samples that by contrast microscopy but appeared to be inviable and auto-fluorescent rather than bona fide GFP+ CFU; however, further analysis was not performed on them so they are reported here.

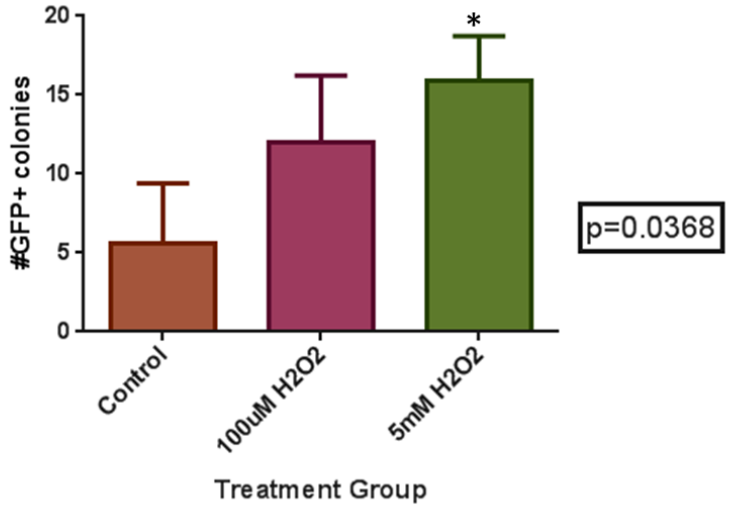

Figure 35: Dose-dependent appearance of GFP^+ myeloid CFU derived from HSCs exposed to 0, 100µM, or 5mM H_2O_2.
100µM resulted in an average frequency of CFU at 6.5 x 10^{-6}. 5mM resulted in an average frequency of CFU at 10.4 x 10^{-6}. ***** denotes statistically significant stimulation of chromosomal translocations by H_2O_2 (students T-test p value<0.037).

5.4 Discussion

These data provide a direct link between ROS and genetic instability in HSC. This approach demonstrated that a single 30 min exposure of HSC to 100 µM H_2O_2 or 5mM H_2O_2 is sufficient to promote translocations following DNA damage and repair by interchromosomal NHEJ. This was demonstrated by the appearance of GFP^+ myeloid colonies following treatment. The *in vitro* system used here is analogous to the outgrowth of transformed myeloid leukemic cells and connects ROS and genome rearrangements in HSC and leukemias.

Control of ROS in ES cells is now understood to be critical to maintenance of self-renewal phenotype. Differentiation of ES cells leads to increased intracellular ROS [343, 344]. Interestingly, during differentiation of ES cells, superoxide production, cellular levels of intracellular ROS, and DNA damage levels increase and expression of

major antioxidant genes and genes involved in multiple DNA repair pathways are downregulated [343], and DNA repair by HR is reduced [24]. Aging is also associated with reduced expression of multiple DNA repair proteins, reduced DNA repair efficiency and elevated levels of genome instability in hematopoietic cells [442, 447-449].

ROS seem well tolerated in young cells with highly proficient DNA repair, and thus have few if any long-term deleterious effects. However, older cells with less efficient repair become more sensitive to ROS levels which lead to increased genome instability. Recent work demonstrated that quiescent HSC (analogous to the ROS^{low} fraction) repair DNA damage less efficiently than progenitors leading to accumulation of DNA damage and impaired function, suggesting a mechanism for increased presentation of myeloid malignancies in aged populations [450]. HSC reactivity to ROS during aging *in vivo* combines cell-intrinsic and cell-extrinsic mechanisms [451]. A relationship between the HSC compartment and supporting cells within the bone marrow niche is central to homeostasis and changes during not only aging but also other pathophysiologic processes including atherosclerosis, hypertension, and diabetes [452, 453].

Defective signaling in response to ROS also occurs in multiple leukemic stem cell populations to promote proliferation, differentiation, genomic, and epigenetic alterations, immune evasion, and survival [454]. ROS act as signaling molecules to regulate redox-sensitive transcriptional factors, enzymes, oncogenes, and other downstream effectors, and thus AML cells are thought to be addicted to elevated ROS [454]. Extracellular ROS, but not intracellular mitochondrial ROS, have been found to be significantly elevated in both AML cell lines and cells derived from patients [444-446]. OS may also play a role in mutation accumulation and progression of myelodysplastic syndromes (MDS) to AML

as suggested by studies in a mouse model of MDS that displays increased ROS in Lin- bone marrow cells, increased DNA breaks, and increased mutation frequency over time [338].

A growing body of evidence (see appendix C) indicates that the inability to regulate high levels of ROS leading to alteration of cellular homeostasis or defective repair of ROS-induced damage lies at the root of diseases characterized by both neurodegeneration and bone marrow failure [335-337]. This study directly demonstrated that ROS can promote DNA damage and aberrant repair by NHEJ in HSCs.

CHAPTER VI: SIGNIFICANCE AND FUTURE DIRECTIONS

I have demonstrated that some common dietary and environmental compounds promote leukemic translocations between the *MLL* and *AF9* bcrs independent of their normal chromatin context and independent of the formation of a fusion protein. I have also demonstrated that the ESC population is particularly sensitive and susceptible to rearrangements induced by the dietary compounds genistein and quercetin, and the environmental compounds p-benzoquinone and dipyrone compared to their more differentiated counterparts. Genistein exposure caused an increase in the proliferative potential of primary human $CD34^+$ cells similar to etoposide, further establishing genistein as a potent leukemogenic compound. I have also demonstrated that ROS and aberrant DNA repair in HSCs are linked indicating a direct role in the pathogenesis of leukemia. A wide variety of dietary bioactive compounds and functional foods are consumed for their presumed health benefits [455]. However, many claims about the health benefits of bioactive compounds need to be supported by scientific and clinical evidence. The reporter system used in this study is a great system to allow for screening of hundreds of these compounds for potential risks associated with their unregulated use. Specifically, this system would allow for the identification of compounds or combinations of compounds that may have the potential to promote leukemic translocations in stem cells and more differentiated myeloid cell subpopulations analogous to events in development of IAL.

Sequences of *MLL-AF9* breakpoint junctions in DNA from patients with therapy related MDS or AML suggests that these translocations arise from aberrant NHEJ repair of DSBs at those sites [456]. DSBs are induced by a variety of compounds including topoII poisons, suggesting that treatment regimens including these poisons can induce the translocations that lead to therapy related MDS and AML [457, 458]. It is possible, however, that the translocation events in these patients occur by a different mechanism or that they are predisposed to the occurrence of these events because of other mutations. The genetic system used here was designed to mimic the aberrant repair that leads to *MLL* and *AF9* translocations reported in clinical samples. I have demonstrated that compounds that are biochemically similar to chemotherapeutic topoII poisons can directly induce *MLL* and *AF9* translocations and that the bcrs of these genes are sufficient for their occurrence. Molecular analysis of breakpoint junctions from GFP^+ colonies generated by these compounds or expression of ISceI is consistent with NHEJ which is also analogous to observations of clinical samples [219, 459].

Further molecular analysis to identify translocations and determine the location of the transgenes in each clone can be done by fluorescent in situ hybridization (FISH) and spectral karyotyping (SKY). The FISH technique is used to label specific sequences on a chromosome with a fluorescent probe. Probes can be designed that have a high degree of complementarity to the reporter constructs allowing for identification of the chromosomal insertion site[460]. The SKY technique is used to label each chromosome with a unique fluorescent color which allows for identification of translocations at the karyotypic level [461]. Use of both of these techniques would provide more information on DNA repair mechanisms being used following treatment with these compounds. This would also

allow for visualization of other translocation events that may have occurred as a result of exposure.

For future experiments, this system could be modified for *in vivo* analysis. Mice that contain these reporter transgenes in their genome could be exposed to these compounds individually or in various combinations in their diet. This would be a physiologically relevant and informative model for assessment of the risk of genome rearrangements from human exposure or consumption of these compounds. A wide range of physiological aspects could be studied including drug clearance, absorption, peak plasma concentrations at physiologically relevant low and high doses, tissue-specific differences in the frequency of chromosomal rearrangements, and influence of genetic background. It would be interesting to determine differences in the frequency of translocations in wild type mice deficient in HR repair containing these genetic reporters in response to these compounds. Additionally, prenatal *in utero* exposure to these compounds in wild type mice could determine if these compounds can initiate *MLL* rearrangements and AML in mouse neonates. One study has determined by inverse PCR that genistein and quercetin can produce *MLL* rearrangements in mice [405]. Inverse PCR that was used in this study however, is not a reliable technique to make this claim in that it requires digestion of DNA prior to amplification which could produce target DNA segments ligated to other fragments, large fragments that would not be efficiently amplified, and includes less relevant internal deletions or intronic alterations. This technique also does not allow for the clonal expansion of cells containing a specific translocation event.

Hematopoiesis is largely conserved between mice and humans; however, differences in body mass, metabolism, life-span, and environmental exposures has led to several differences in hematopoiesis, including the DNA damage response [462]. Murine HSCs have been shown to have high classical NHEJ activity compared to more differentiated populations [463, 464], whereas human HSCs have been shown to have decreased classical NHEJ activity [463, 465]. Irradiation-induced DNA damage in murine HSCs initiates p53 dependent NHEJ repair that promotes cell survival, whereas irradiation induced DNA damage in human HSCs initiates p53 dependent apoptotic response [464, 466]. Classical NHEJ proteins have been shown to have increased expression in murine HSCs [463]. Human HSCs have no change in expression of Ku70, Ku80, or XRCC4 compared to their more differentiated populations, however, ATM and DNA ligase IV has been shown to be down regulated [467, 468]. This could indicate that alternative NHEJ accounts for aberrant repair and chromosomal translocations in human HCS. The differences in DNA damage response between species also indicate an evolutionary difference between species with short or long lifespans. Damage in murine HSCs initiates NHEJ repair and continued survival even if repair is imperfect, and human HSCs sacrifice damaged cells to avoid persistence of potentially harmful genetic alterations thus insuring longevity. It has been recently shown that longer lived species have higher overall expression of DNA repair genes involved in base excision repair (BER), mismatch repair (MMR), HR, as well as NHEJ in liver cells [469].

Murine and human HSCs differ in their DNA repair capacity, so it may be more clinically applicable to investigate the ability of these compounds to promote leukemic translocations in human cells. For future experiments, this model system could be

modified, and the transgene reporter constructs could be inserted into human ESCs. There are over 300 human ES cell lines available and approved by the National Institutes of Health (NIH) for research use and *in vitro* maintenance of human ESC self-renewal and pluripotency has been well characterized [470-472]. hESCs have typically been maintained in co-culture with MEFs; however, they have also been successfully cultured on human fetal muscle cells [470], human foreskin fibroblasts [473], human uterine endometrial cells, and breast parenchymal cells [474]. hESCs have also been successfully cultured with hydrogels, organic or inorganic polymers that are analogous to the extracellular matrix [475, 476]. Mimicking the experiments described in this dissertation with hESCs would provide insight into the earliest stages of human embryogenesis, allow for examination of exposure risks associated with dietary and environmental compounds at different stages of differentiation, and also allow for further comparison between human and mouse ESCs.

Compounds determined to be leukemogenic using the hESC model system could then be screened for their ability to engraft and initiate a leukemia phenotype in non-obese diabetic/severe combined immunodeficient mice (NOD/SCID). Primary human umbilical cord $CD34^+$ cells could be treated *ex vivo* with a panel of dietary or environmental compounds, injected into irradiated NOD/SCID mice, and analyzed for their potential to repopulate the bone marrow and generate leukemic rearrangements analogous to observations from clinical samples. This engraftment experiment would be a logical next step for the bioflavonoid genistein considering it stimulated proliferation of primary human umbilical cord $CD34^+$ cells, similar to etoposide, using LTC-IC. Mice engrafted with treated cells would be observed for the development of leukemia by

weekly examination for changes in body weight, number of white blood cells and leukemia cells among peripheral blood. FISH analysis could also be performed to detect the presence of cytogenetic abnormalities characteristic of leukemic cells such as *MLL* rearrangements. Positive results would provide evidence directly linking genistein or any number of compounds, to leukemia initiation.

The cytotoxic and genotoxic properties of many popular dietary compounds have generally been ignored due to widespread acceptance of their beneficial effects. However, there is an increasing body of evidence highlighting potentially adverse effects of using some dietary supplements in an unrestricted manner. The dietary and environmental compounds used in this research directly link exposure to leukemia associated *MLL* rearrangements, thus the described future experiments focus on this relationship. Considering the total exposure to bioactive compounds for each individual is unique, there are likely many other relationships between dietary and environmental exposure and potentially adverse effects that have yet to be elucidated. Thus, it is important to set guidelines for the use and availability of these compounds to reduce the potential risk of disease. The experiments discussed above would provide insight into risk associated with exposure to these compounds in human cells and their relative cytotoxic and genotoxic profiles.

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APPENDIX A: PUBLICATIONS AND PRESENTATIONS

Publications:

Vestal CG, Bariar B, Deem B, Engledove W, Richardson C. MLL-AF9 bcr translocations induced by common bioflavonoids independent of chromosomal context: a model system to rapidly screen environmental risks. 2016
Submitted to: Environmental and molecular mutagenesis

Benedetto G, Vestal CG, Richardson C. Aptamer-Functionalized Nanoparticles as "Smart Bombs": The Unrealized Potential for Personalized Medicine and Targeted Cancer Treatment. Target Oncol. 2015; 10(4):467-85.

Richardson C, Yan S, Vestal CG. Oxidative stress, bone marrow failure, and genome instability in hematopoietic stem cells. Int J Mol Sci. 2015; 16(2):2366-85.

Tait DL, Bahrani-Mostafavi Z, Vestal CG, Richardson C, Mostafavi T. Down-regulation of HOXC6 in Serous Ovarian Cancer. Cancer Invest. 2015; 33(7):303-11.

Bariar B, Vestal CG, Richardson C. Long-term effects of chromatin remodeling and DNA damage in stem cells induced by environmental and dietary agents. J Environ Pathol Toxicol Oncol. 2013; 32(4):307-27.

White RR, Sung P, Vestal CG, Benedetto G, Cornelio N, Richardson C. Double-strand break repair by interchromosomal recombination: an in vivo repair mechanism utilized by multiple somatic tissues in mammals. PLoS One. 2013; 8(12): e84379.

Presentations:

American Association for Cancer Research Annual Meeting, poster presentation, 2014.
Bahrani-Mostafavi Z, Yeganeh PN, Parrott ME, Vestal CG, Richardson C, Tait DL, Mostafavi MT, *PAX8 Protein Detection in Serum of Patients with Serous Ovarian Cancer.*

New York Academy of Sciences conference; The Bone Marrow Niche, Stem Cells and Leukemia: Impact of Drugs, Chemicals, and the Environment, poster presentation, 2013.
Vestal, G; Richardson, C. *Identification of Dietary and Environmental compounds that promote MLL-AF9 Translocations.*

UNC Charlotte Graduate Research Symposium, oral presentation, 2013.
Vestal, G; Richardson, C. *The potential for dietary compounds to promote chromosomal translocations associated with leukemia.*

American Association for Cancer Research Annual Meeting, poster presentation, 2013.
Tait DL, Bahrani-Mostafavi Z, Vestal CG, Richardson C, Mostafavi T. *Down-regulation of HOXC6 in Serous Ovarian Cancer.*

UNC Charlotte Departmental Seminar, oral presentation, 2012.
Vestal, G., Richardson, C. *The potential for dietary compounds to promote a leukemic translocation at different stages of hematopoiesis.*

American Society of Hematology Annual Meeting and Exposition, poster presentation, 2012.
Vestal, G; Richardson, C. *System for Rapid and Reproducible Identification of MLL-AF9 Translocations Induced by Dietary Compounds and Nutritional Supplements.*

APPENDIX B: *MLL-AF9* BCR TRANSLOCATIONS INDUCED BY COMMON BIOFLAVONOIDS INDEPENDENT OF CHROMOSOMAL CONTEXT: A MODEL SYSTEM TO RAPIDLY SCREEN ENVIRONMENTAL RISKS

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infant acute leukemia, AML, environmental toxicology, *in utero* exposure, bioflavonoids, DNA repair, topoisomerase II, chromosomal translocations, genome instability

Abstract:
Infant acute leukemias are aggressive and characterized by rapid onset shortly after birth. The majority harbor translocations involving the *MLL* gene with *AF9* one of its most common fusion partners. The overlap of *MLL* breakpoint junction sequences associated with both infant acute leukemia and therapy-related leukemia following exposure to the topoisomerase II inhibitor etoposide led to the hypothesis that exposure during pregnancy to compounds biochemically similar to etoposide such as bioflavonoids may promote infant acute leukemia. Both *MLL* and *AF9* loci contain breakpoint cluster regions (bcrs) with sequence motifs hypothesized to be targets of topoisomerase II inhibitors that promote translocation formation. We established stem cell reporter system to systematically examine the potential for such compounds to promote chromosomal translocations between the *MLL* and *AF9* bcrs analogous to those in infant leukemia. This system demonstrated that bioflavonoids genistein and quercetin, most biochemically similar to etoposide, were potent promoters of *MLL-AF9* translocations stimulating them at least 2 logs. The system provides a clear readout of relative risk of translocation formation with genistein and quercetin showing strong association, myricetin showing weak but consistent association, but other compounds showing minimal association. The *MLL* and *AF9* bcrs were sensitive to these agents and recombinogenic independent of chromosomal context suggesting the bcr sequences themselves are drivers of illegitimate DNA repair reactions and translocations, not generation of functional oncogenic fusions. This system provides for rapid simple systematic screening of relative risk, dose dependence, and combinatorial impact of multitudes of dietary and environmental exposures on *MLL-AF9* translocations.

Introduction

Infant acute leukemias account for approximately 30% of all childhood malignancies in the Western world. Infant leukemias are characterized by rapid onset less than one year of age, progress rapidly, and are aggressively invasive [1-3]. Acute lymphoblastic leukemia (ALL) is the more predominant form of infant leukemia while acute myeloblastic leukemia (AML) accounts for about 18% of cases [4]. Approximately 85% of infant ALL and 50% of infant AML cases involve rearrangements of the *MLL* gene at chromosomal position 11q23 [5-7] that may initially form *in utero* [8-11]. More than 75 fusion partner genes for *MLL* have been identified [12]. This particularly broad spectrum of rearrangements is a hallmark of *MLL* and may reflect an inherent recombinogenic nature of the locus, or the centrality of the MLL protein in hematopoietic development and differentiation such that rearrangements often possess leukemogenic potential. *MLL-AF9* t(9;11)(p22;q23) fusion is one of the most common *MLL* translocations [5,12] associated with infant ALL [13], therapy-related AML (t-AML) [14], and a smaller portion of adult ALL and AML. Leukemias with the MLL-AF9 fusion are clinically aggressive, difficult to treat, often resistant to traditional therapy regimens with the intensive therapies generally being toxic, and have poor prognosis.

The significant majority of *MLL* translocation breakpoints in clinical samples occur in the well-characterized 8.2 kb breakpoint cluster region (bcr) fragment [11,12,14-16] containing multiple topoisomeraseII (topoII) cleavage sites, Alu repetitive sequences, DNaseI hypersensitive sites, and high affinity scaffold associated region (SAR) [14,17]. The *AF9* locus has two bcr regions identified from clinical samples of t-AML, infant AML and adult ALL patients [18]; bcr1 within intron 4 and bcr2 spanning introns 7 and 8 [14]. Elements similar to the *MLL* bcr have been identified including a topoII cleavage site and a DNaseI hypersensitivity site located within intron 7 in bcr2 and two SARs at the borders of both AF9 bcrs [14,19].

Exposure to topoII inhibitors (or poisons) such as etoposide is predominantly associated with t-*MLL*-rearranged leukemias [20-28]. TopoII is an essential cellular enzyme that catalyzes changes in DNA topology via its cleavage-religation equilibrium [29,30]. Inhibitors stabilize topoII-DNA covalent complexes, most often by decreasing the rate of religation in a dose-dependent manner, resulting in accumulation of double-strand breaks (DSBs), p53 activation, and induction of repair or apoptosis [31-33].

Since *MLL* breakpoint sequences associated with infant acute leukemia are similar to those in t-AML following etoposide-containing regimens [34,35], it has been hypothesized that exposure during pregnancy to biochemically similar compounds including bioflavonoids may promote infant acute leukemia [36-38]. The most common sources of bioflavonoids are fruits, vegetables, soy, tea, coffee, wine, and over-the-counter supplements [37,39]. Due to their antioxidant capacity, bioflavonoids are used for their presumed health benefits such as protection against cardiovascular diseases, cancer and inflammation. However, they have pleiotropic effects on cells, and their direct role in any single pathway has been less clear [38,40].

Similar to etoposide, bioflavonoids genistein and quercetin contain pendant rings that feature a 4’-OH group essential for activity. Genistein and quercetin and other

bioflavonoids inhibit topoIIα and topoIIβ activity [41-43], induce *in vitro MLL* gene cleavage, and result in detection of *MLL* rearrangements by detectable by inverse PCR [39,44] or karyotype analysis [45]. These data, along with a few epidemiological studies, suggest they may contribute to infant leukemia from *in utero* exposure from maternal diet [36,37,46-50] [39] [51,52], although the relative potency of compounds or their additive, synergistic, or even quenching effects on the potential to promote translocations remains unclear. *MLL* bcr cleavage and rearrangements have also been detected following exposure of cells to multiple non-genotoxic agents, possibly due to apoptotic DNA fragmentation [53-56] or exposure to environmental DNA damaging agents [57], underscoring the importance of screening a large number of compounds to determine their relative potential for initiating *MLL* translocation events.

We established a stem cell reporter system that can systematically examine the potential for a large panel of compounds to promote DNA DSBs and chromosomal translocations between the *MLL* and *AF9* bcrs analogous to those observed in infant leukemia. For this we generated stem cell reporter cell lines such that illegitimate DNA repair by nonhomologous end-joining (NHEJ) producing a chromosomal translocation between *MLL* and *AF9* bcrs results in a functional full-length green fluorescent protein (GFP) transcript. This system enabled us to determine (1) if *MLL* and *AF9* bcrs that undergo DSBs are recombinogenic independent of chromosomal context or fusion protein; (2) if a panel of bioflavonoids have the potential to promote these translocations analogous to those observed in infant leukemia; and (3) if a panel of bioflavonoids demonstrate quantifiably different abilities to promote such events.

These studies demonstrated that bioflavonoids genistein and quercetin are potent promoters that directly lead to *MLL-AF9* bcr translocations, while myricetin consistently but weakly leads to *MLL-AF9* bcr translocations. Notably, benzoquinone that is associated with topoII inhibition did not promote *MLL-AF9* translocations even at high doses. The sensitivity of the *MLL* and *AF9* bcrs to bioflavonoids was independent of their normal chromatin context and independent of the formation of any leukemic fusion protein. These studies provide a relative range of the susceptibility of these two bcrs to these agents and their susceptibility to illegitimate NHEJ repair that results in translocations. This reporter system provides for rapid, simple, reproducible, and systematic screening of relative risk, dose dependence, and combinatorial impact of multitudes of compounds in diet and environment that may have direct potential to promote leukemogenic translocations.

Materials and Methods

Transgene constructs: Two green fluorescent protein (*GFP*) gene reporters were generated (Figure 1A). NZE-GFP fragment of GFP open reading frame (orf) was inserted into pCAGGs [58]. To engineer GFP into two exons, adenovirus intron sequence 246 nucleotides [22,59] was inserted into the GFP orf between the GG doublet (nucleotides 241/242). This GFP-intron plasmid generated two separate constructs. GFPe1: contained the chicken □-actin promoter, 5' engineered exon 1 with the first 233 nucleotides of the *GFP* orf, splice donor (sd), 160 nucleotides of adenovirus intron to the HindIII site [22,59], an I-*Sce*I endonuclease recognition site within an inserted HindIII-I*Sce*I-XhoI linker (H3-*S*-X). GFPe2: contained an I-*Sce*I endonuclease recognition site within a XhoI-I*Sce*I-

HindIII linker (X-*S*-H3), 76 nucleotides of adenovirus intron from the HindIII site to a splice acceptor (sa) [22,59], 3' engineered exon 2 of the GFP gene, and □-globin polyA. Blunt-ended Xho1-XhoI fragment of GFPe1 was inserted into intron 11 in blunt-ended XbaI site of the hu*MLL* bcr 8.2 kb BamHI-BamHI [15] (numbering based on Nilson *et al* [16]) (Figure 1B). Blunt-ended XhoI-PstI fragment of GFPe2 was inserted into intron 7 of the hu*AF9* bcr2 region 8.94 kb (1276-10221 of AL513498GenBank) between topoII cleavage site and exon 8 (4015 1.18 kb 5' of exon 8) [14] (Figure 1B). Constructs were verified by sequencing (Sequetech Corporation, CA, USA).

Generation of cell lines: Constructs were transfected into EtG2a mouse embryonic stem (ES) cells [22,60] to generate a hu*MLL*-hu*AF9* bcr reporter system. Co-transfection with either *puromycin* or *neomycin* resistance gene-containing plasmids was used for drug selection. 2 x 10^7 cells were transfected by electroporation with the linearized GFPe1-hu*MLL* bcr construct and pMCneo containing the *neomycin* gene. 24 hours post-electroporation, cells were placed in 200□g/mL G418 10 days [22,60]. DNA extracted from $G418^R$ clones was screened for single copy GFPe1-hu*MLL* construct by Southern blotting using a GFPe1 fragment as probe on DNA digested with appropriate endonucleases (BgIII/XbaI; BamHI/BglII; EcoRV; New England Biolabs Inc.). 2 x 10^7 GFPe1-hu*MLL* cells were transfected by electroporation with the linearized GFPe2-hu*AF9* bcr construct and pgk-puro. 24 hours post-electroporation, cells were placed in 120□g/mL puromycin 10 days [22,60]. DNA extracted from $Puro^R$ clones was screened for single copy of the GFPe2-*AF9* construct by Southern blotting using a GFPe2 fragment as probe on DNA digested with appropriate endonucleases (KpnI; BamHI; NcoI; BamHI/BglII; XhoI; PstI; New England Biolabs Inc.). Inserts were confirmed by PCR and sequencing (Sequetech Corporation, California). Single copy clones MAG1, MAG2 and MAG3 were used.

DSB induction and translocation formation by I-SceI expression: DSBs were induced by I*Sce*I expression of the pCBASce expression vector [60]. 2 x 10^7 MAG cells per cohort were electroporated with (a) no DNA, (b) 40μg NZE-GFP expression plasmid, or (c) 20μg pCBASce expression plasmid. Cells were plated at 4 x 10^6 cells. Surviving cells were determined 24 hours post electroporation [22,60]. All cohorts were screened every 24hrs for GFP expression at 400X magnification on an inverted Zeiss Axiovert25 microscope with images recoded by Zeiss AxioCam MRc digital camera.

Cell viability following exposure to potential topoII inhibitors: 1 X 10^6 MAG cells each were plated at 0, 25, 50, 75, 100 and 200□M of each genistein, luteolin, myricetin, quercetin, dipyrone, or benzoquinone 1hr 37ºC. Cells were then trypsinized, washed 1X PBS, and replated. Surviving cells were determined for each dose at 24, 36 and 72 hrs. The percentage of surviving cells and standard deviations were determined. n=3

DSB induction and translocation formation by potential topoII inhibitors: 1 x 10^7 cells MAG cells per cohort were treated with each compound 1hr 37ºC. Compounds were bioflavonoids genistein (max dose 75□M), luteolin (max dose 200□M), myricetin (max dose 75□M), quercetin (max dose 75□M), as well as benzoquinone (max dose 125□M) or dipyrone (max dose 100μM). Etoposide (max dose 200□M) was used as a known potent inhibitor. Following exposure, cells were trypsinized, washed 1X PBS, and replated. All treatment cohorts were further washed with 1X PBS (twice daily on the first two days after

treatment, and once every day after that), supplemented with fresh medium and screened each 24hrs for GFP+ fluorescence at 400X magnification on an inverted Zeiss Axiovert25 microscope with images recoded by Zeiss AxioCam MRc digital camera. n=3

Results

Independently derived reporter cell lines MAG1, MAG2, and MAG3 with single copy insertions of the GFPe1-hu*MLL* bcr and GFPe2-hu*AF9* bcr constructs were utilized. *GFP* reporters were upstream (*GFPe1*) or downstream (*GFPe2*) of strong topoII cleavage sites and mapped breakpoints identified in clinical samples of infant- and t-AML [61] [14]. Construct design allowed for determining if the compounds promote DSBs in the bcrs independent of chromatin context [62,63] and promote illegitimate NHEJ to result in translocations. A translocation by this mechanism will ligate the GFPe1 exon and the GFPe2 exon onto the same DNA duplex and generate a functional *GFP* gene (GFP+)(Figure 1). To validate the system and confirm a lack of cryptic splice sites within the intervening bcr regions, I-*Sce*I endonuclease-induced DSBs were generated. DSB induction significantly stimulated illegitimate NHEJ DNA repair between the hu*MLL* and hu*AF9* bcrs in all three parental cell lines resulting in readily observable GFP+ fluorescent cells by 72 hours (Table 1; Figure 2). The frequencies of interchromosomal illegitimate NHEJ translocations were calculated to be highest in MAG1, intermediate in MAG2, and lowest in MAG3 ($2.5\times10^{-6} \pm 0.2\times10^{-6}$, $1.2\times10^{-6} \pm 0.1\times10^{-6}$, $0.6\times10^{-6} \pm 0.4\times10^{-6}$ respectively) at average calculated frequency of $1.4\times10^{-6} \pm 0.3\times10^{-6}$. While all cell lines readily produced detectable GFP+ colonies, the average number of GFP+ colonies detected was statistically different between all groups (t-test; 1vs2 p=0.0025; 2vs3 p=0.02; 1vs3 p=0.0014). The observed frequency of translocations by I-*Sce*I here was approximately 4-fold lower than a previous model system that utilized I-*Sce*I to induce NHEJ translocations promoted by the presence of homologous Alu cassettes [22].

MAG1 cells were exposed to the bioflavonoids genistein, luteolin, myricetin, or quercetin, as well as the non-steroidal anti-inflammatory drug dipyrone or the environmental toxin benzoquinone for 1hr at concentrations of 75□M–200□M (Table 2). Dose-dependent cell survival assays demonstrated 25-70% cell survival within these ranges, although consistent with other reports all compounds resulted in some cell death in a dose dependent manner (Supplemental Figure 1). The highest doses used for exposures to score for GFP+ events demonstrated approximately 75% cell survival at 24hr post-exposure (Supplemental Figure 1). Following exposure, compounds were washed away, and cells cultured to allow for DNA repair by illegitimate NHEJ and a chromosomal translocation resulting in GFP+ cells (Figure 1). GFP+ cells were scorable by 96 hrs (Figure 2, Table 2). By contrast, in the absence of DSBs, none of the parental cell lines generated GFP+ cells over 6 passages (frequency calculated $< 7.2\times10^{-8}$; Table 2) indicating that the formation of stable translocations in the absence of a promoting event or factor is extremely rare, calculated at more than 2 logs lower frequency. Etoposide is a known potent inhibitor of topoII and well-associated with the induction of *MLL* rearrangements. As expected, exposure of MAG cells to increasing doses of etoposide (50 100, and 200□M) promoted increasing numbers of GFP+ cells. The average frequencies of NHEJ repair and translocations increased from $1.2\times10^{-6} \pm 2.0\times10^{-6}$, $5.2\times10^{-6} \pm 4.1\times10^{-6}$, and $10.5\times10^{-6} \pm 9.5\times10^{-6}$ following 50 100, and 200□M, respectively. It was noted that many of the initially

identified GFP+ cells did not proliferate in culture likely due to excessive DNA damage; by contrast cells scored as GFP+ following exposure to bioflavonoids continued to proliferate over 2 weeks consistent with the reduced toxicity of these compounds (Supplemental Figure 1). It is possible that GFP+ cells initially detected would proliferate in appropriate long-term culture assays.

Genistein and quercetin were the most potent promoters of *MLL-AF9* translocations in this assay with calculated average frequencies at 75□M to be 1.2×10^{-6} and 1.1×10^{-6}, respectively (Table 2). Myricetin weakly but consistently promoted translocations with calculated average frequency of 0.1×10^{-6} (Table 2). Luteolin generated a single GFP+ colony in one replicate (calculated frequency of 0.03×10^{-6}; Table 2) suggesting at least 40-fold lower potential to promote such events. However since only a single colony across multiple experiments was observed, we cannot rule out the possibility that it was a rare spontaneous event.

Although dipyrone is not a known topoII inhibitor, it is associated with *MLL*-rearranged infant leukemia [51]. Benzoquinone is a topoII inhibitor at concentrations as low as 1–10 □M [64] stimulating DNA DSBs, oxidative stress, or fragmentation [64-66] and associated with hematopoietic malignancies [66-68]. Both compounds are believed to have distinct mechanisms of action from the other potential topoII inhibitors examined here. Dipyrone exposure of MAG1 cells up to 100□M generated a single GFP+ colony in one replicate (calculated frequency of 0.03×10^{-6}; Table 2). However, as with luteolin, we cannot rule out the possibility that it was a rare spontaneous event. Benzoquinone exposure of MAG1 cells was not sufficient to promote *MLL-AF9* translocations following exposure up to 125□M (frequency of $<0.01\times10^{-6}$; Table 2). This contrasts from other studies in which as little as 1□M benzoquinone stimulated intrachromosomal homologous recombination by 2-3 fold over undamaged cells [69]. To distinguish from the possibility that *MLL-AF9* translocations are stimulated in response to a broad spectrum of environmental agents, MAG1 cells were exposed to vitamin A that contains ring structures similar to the bioflavonoids; doses up to 75□M did not lead to GFP+ colonies (data not shown).

Similar results were observed with MAG2 and MAG3 cells although at frequencies of ½ log lower than MAG1 (data not shown); these results are consistent with results from I-*Sce*I DSB studies (Table 1) and overall suggest that the *MLL* and *AF9* bcrs are highly recombinogenic independent of chromosomal context and in the absence of generating a functional fusion protein (data not shown).

Genistein and quercetin were the most potent promoters of *MLL-AF9* translocations in this assay with calculated frequencies at 1.2×10^{-6} and 1.1×10^{-6}, respectively (Table 2). These frequencies are similar to those observed following endonuclease-induced I-*Sce*I DSBs at both loci suggesting that these particular bioflavonoids are potent promoters of DSBs within the two bcrs even at doses that allow for significant cell survival and proliferation in culture. Additionally, the established assay provides a clear readout of relative risk of illegitimate NHEJ and translocation formation in response to physiological doses of the tested compounds with genistein and quercetin showing a strong association, and myricetin a weak but consistent association. Other compounds demonstrate rare to undetectable promotion of such events, at least 100-fold lower potency. A one-way

ANOVA (comparing frequencies) was performed using Bonferroni post-hoc test. Both genistein and quercetin were statistically significantly different from untreated cells, and both these compounds were also significantly different from myricetin, luteolin benzoquinone, and dipyrone.

Discussion

This reporter system demonstrated that physiologically relevant doses of bioflavonoids genistein and quercetin are potent promoters of hu*MLL*-hu*AF9* bcr translocations stimulating them by at least 2 logs over undamaged cells. Importantly, these data suggest that specific *MLL* and *AF9* bcrs' motifs are the drivers of the illegitimate DNA repair reactions and represent a distinct mechanism of interaction of these compounds with them rather than their overall chromosomal context, specific hematopoietic developmental programs or lineages, or a survival advantage conferred by a functional oncogenic fusion. The development of genetic engineering technologies involving knock-in models, the use of customizable DNA nucleases, *MLL* TALENs, or the CRISPR/Cas9 system offers promising methods in which to model progression of malignancies characterized by chromosomal translocations but do not model the initial formation of these translocations, particularly in response to exposure to topoII inhibitors in a physiologically relevant manner.

The number of homeologous Alu repetitive elements within the bcrs of these two regions has led to the proposal that illegitimate fusions are stimulated by local alignments of homeologous sequences during the process of DNA repair. A previous study using a *neomycin* reporter system and Alu repetitive element cassettes within introns determined that I-*Sce*I induced NHEJ-NHEJ events (analogous to those observed here) were promoted at a frequency of 4.2×10^{-6} (16% of all events occurring at a frequency of 2.6×10^{-5}). The frequency of NHEJ-driven translocations induced by I-*Sce*I here is approximately one fourth (1.4×10^{-6}). Our lower frequency likely highlights the importance of sequence identity of Alu elements present nearest the DNA breakpoints. Alternatively it is possible that the potential in that system for other repair mechanisms (*e.g.* homologous recombination or single strand annealing) was sufficient to promote the alignment of potential junction partner DNA ends more frequently even if ultimately repair would occur via NHEJ. The *MLL* bcr is small and well-characterized such that a number of mapped infant leukemia breakpoints map to the 3' half of the bcr. Thus placement of the GFPe1 into intron 11 likely mimics the sites of breakage induced by the compounds tested. The *AF9* locus contains two bcr regions; bcr2 (10 kb) is flanked on the 5' side by a scaffold attachment region and contains multiple topoII cleavage sites [14,18]. Thus the placement of GFPe2 into this region likely mimics the sites of breakage induced by the compounds tested. However the broader span of the *AF9* mapped bcr may have led to DNA damage and illegitimate repair events that did not reconstitute a functional GFP gene and would not be recovered here.

Our data highlight the difference between the potential to promote DNA damage and to result in a downstream chromosomal translocation analogous to those observed in leukemias. Although these compounds have all been shown to strongly induce *MLL* cleavage *in vitro* [46], including luteolin and myricetin, the results here show a difference between relative potential to induce cleavage and the relative potential to induce *MLL-AF9*

translocation events. Although benzoquinone is a topoII inhibitor stimulating DNA DSBs, oxidative stress, or fragmentation at concentrations as low as 1–10□M [64-66], it did not lead to translocations here even at significantly higher concentrations (10–100-fold). Differences between damage and repair may lie in the number of DNA damage sites, the stability of the breaks incurred by these compounds, kinetics of removal of stabilized DNA-topoII complexes, or a favored mechanism of repair. However, the specificity of damaging agent rather than type of damage induced should be noted since direct induction of oxidative stress by H_2O_2 exposure in this assay system does lead to these translocations in a dose-dependent manner [70].

The most common sources of bioflavonoids are fruits, vegetables, soy, tea, coffee and wine. Genistein is an estrogen derivative available at health food stores as dietary and menopausal supplements [71], and a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas [72,73]. Low plasma concentrations of genistein in mice neonates (1-5μM) were sufficient to promote adverse reproductive effects later in life [74]. Previous studies reported average adult soy intake to be 0.15-3.0 mg/day [75], but daily intake averages can vary and increase to 8.6mg/day in women and 7.5mg/day in men [76]. Foods contain multiple bioflavonoids, and bioflavonoids are bioaccumulative which likely increases plasma concentrations [77]. Study of the potential for environmental or dietary compounds to induce infant leukemias is more relevant since they cross the placental barrier as shown with the synthetic bioflavonoid EMD-49209 [78], genistein [39,79] quercetin [39,80,81], herbal medicines, dipyrone, and pesticides including mosquitocidal Baygon [51,82]. Further, *in utero* exposure is likely more damaging due to differences in metabolic and excretion rates of mother and fetus [83] as well as rapidly developing and proliferating fetal cells that are more sensitive to topoII inhibiting agents [84].

Halogenated compounds in household and baby products [85] are another possible risk factor for childhood leukemia to be tested. Polychlorinated biphenyls (PCBs) are banned in the United States but detectable in indoor carpets [86] and linked to non-Hodgkin lymphoma [87] and childhood ALL [86]. Polybrominated diphenyl ethers (PBDEs), structurally similar to PCBs, are used as flame retardants [88], and exposure to specific PBDE congeners has been associated with childhood ALL [88]. In support of mechanistic action, certain PCB quinone metabolites increase DNA cleavage by topoIIα *in vitro* and in cultured human cells [89].

Genetic differences among infants exposed prenatally to topoII inhibitors likely affect susceptibility to formation of chromosomal translocations. Genome-wide association studies (GWAS) have not identified common variants of susceptibility to either *de novo* or t-AML, but incorporating exposures into GWAS may reveal specific SNPs that contribute to disease [90]. However, infants specifically with *MLL*-rearranged leukemias were 2.5-fold more likely to harbor NAD(P):quinone oxoreductase (NQO1) polymorphisms associated with reduced activity versus unselected cord blood [91]. Variant alleles of DNA damage signaling or repair proteins may also promote susceptibility to flavonoid-induced translocations. Both heterozygous and homozygous mutants of the DNA damage response gene *Atm* exposed prenatally to flavonoids exhibited detectable numbers of *MLL* rearrangements [39]. Further, inhibition or mutation of multiple DNA repair proteins potentiates cytotoxicity of topoII inhibitors [92-94], and MRE11 plays a

direct mechanistic role in removal of topoII-DNA complexes in yeast and mammals [92,95]. Our model cell system can readily be adapted to directly address the impact of such polymorphisms or mutations through genetic engineering of the parental cell lines or use of RNAi prior to exposures. Further, this model system can systematically screen a large panel of compounds, even those not yet suspected to have mutagenic potential, in combination with each other to understand their additive, synergistic, or even quenching effects on the potential to promote these events.

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Conflict of Interest

None

Table 1. Induction of interchromsomal recombination and translocations by I-*Sce*I-induced DSBs between hu*MLL* and hu*AF9* bcrs in three independent cell lines.

Experiment/ Cell Line		DNA	% survival post-EP	# of GFP+ colonies	Frequency *
MAG1	expt. 1	no DNA	65%	0	$< 7.6\times10^{-8}$
		I-*Sce*I	38%	18	2.3×10^{-6}
	expt. 2	no DNA	73%	0	$< 6.8\times10^{-8}$
		I-*Sce*I	40%	22	2.7×10^{-6}
	expt. 3	no DNA	70%	0	$< 7.1\times10^{-8}$
		I-*Sce*I	42%	23	2.7×10^{-6}
		Avg. no DNA			$< 7.2\times10^{-8}$
		Avg. I-*Sce*I			$2.6\times10^{-6}\pm 0.23\times10^{-6}$
MAG2	expt. 1	no DNA	57%	0	$< 8.7\times10^{-8}$
		I-*Sce*I	37%	11	1.4×10^{-6}
	expt. 2	no DNA	60%	0	$< 8.3\times10^{-8}$
		I-*Sce*I	38%	9	1.1×10^{-6}
	expt. 3	no DNA	60%	0	$< 8.3\times10^{-8}$
		I-*Sce*I	38%	10	1.3×10^{-6}
		Avg. no DNA			$< 8.4\times10^{-8}$
		Avg. I-*Sce*I			$1.3\times10^{-6}\pm 0.15\times10^{-6}$
MAG3	expt. 1	no DNA	78%	0	$< 6.4\times10^{-8}$
		I-*Sce*I	24%	1	0.2×10^{-6}
	expt. 2	no DNA	70%	0	$< 7.1\times10^{-8}$
		I-*Sce*I	29%	6	1.0×10^{-6}
	expt. 3	no DNA	75%	0	$< 6.6\times10^{-8}$
		I-*Sce*I	32%	5	0.7×10^{-6}
		Avg. no DNA			$< 8.4\times10^{-8}$
		Avg. I-*Sce*I			$0.63\times10^{-6}\pm 0.40\times10^{-6}$

Three independently derived MAG cell lines were transfected by electroporation with the I-*Sce*I endonuclease expression vector pCBASce. 2.0×10^{7} cells were electroporated in each experiment. * The interchromosomal NHEJ repair and translocation frequency were calculated as the number of GFP+ colonies per number of cells surviving electroporation (EP; as determined 24 hrs post-electroporation).

Table 2. Frequency of interchromsomal NHEJ repair and translocations between hu*MLL* and hu*AF9* bcrs in MAG1 cell line by potential inhibitors of topoisomerase II.

Compound	Dose	# GFP+ Colonies	Frequency*	Average frequency at highest dose
Control	----	0	----	$<0.07 \times 10^{-6}$
I-*Sce*I plasmid^	----		$2.6 \times 10^{-6} \pm 0.23 \times 10^{-6}$	
Genistein	75 □M	15	1.5×10^{-6}	
	75 □M	8	8.0×10^{-7}	
	75 □M	14	1.4×10^{-6}	$1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$
Quercetin	75 □M	8	8.0×10^{-7}	
	75 □M	14	1.4×10^{-6}	
	75 □M	11	1.1×10^{-6}	$1.1 \times 10^{-6} \pm 0.3 \times 10^{-6}$
Myricetin	75 □M	1	1.0×10^{-7}	
	75 □M	1	1.0×10^{-7}	
	75 □M	2	2.0×10^{-7}	$0.1 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Luteolin	75 □M	0	$<1.0 \times 10^{-7}$	
	100 □M	0	$<1.0 \times 10^{-7}$	
	175 □M	0	$<1.0 \times 10^{-7}$	
	200 □M	1	1.0×10^{-7}	
	200 □M	0	$<1.0 \times 10^{-7}$	
	200 □M	0	$<1.0 \times 10^{-7}$	$0.03 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Dipyrone	75 □M	0	$<1.0 \times 10^{-7}$	
	100 □M	0	$<1.0 \times 10^{-7}$	
	100 □M	1	1.0×10^{-7}	
	100 □M	0	$<1.0 \times 10^{-7}$	$0.03 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Benzoquinone	75 □M	0	$<1.0 \times 10^{-7}$	
	125 □M	0	$<1.0 \times 10^{-7}$	
	125 □M	0	$<1.0 \times 10^{-7}$	
	125 □M	0	$<1.0 \times 10^{-7}$	$<0.01 \times 10^{-6}$

MAG1 cells were exposed 1 hr 37ºC with the indicated potential topoisomerase II inhibitors then washed and plated in fresh media. Surviving fractions of cells were calculated and 1 x 10^7 cells viable cells were used in each experiment. GFP+ colonies were scored 96 hr later. *Interchromosomal NHEJ repair and translocation frequency was calculated as the number of GFP+ colonies per number of treated and surviving cells. ^ I-*Sce*I plasmid frequency as detailed Table 1.

Figure Legends

Figure 1. Structure of GFPe1 and GFPe2 constructs in hu*MLL* and hu*AF9* breakpoint cluster regions. (A) For each construct schematic, the numbers of bases are indicated within each portion. GFPe1 includes promoter and upstream region, engineered exon 1, and adenovirus intron sequence. H3-S-X indicates engineered linker containing I-SceI endonuclease site. sd indicates splice donor. GFPe2 includes adenovirus intron sequence, engineered exon 2, and polyA sequence. X-S-H3 indicates engineered linker containing I-SceI endonuclease site. sa indicates splice acceptor. (B) Schematics of *MLL* bcr 8.2 kb region and *AF9* bcr2 8.94 kb region and location of GFPe1 and GFPe2 insertion sites into constructs used. Grey bars indicate scaffold attachment sites. * indicate topoisomerase II cleavage sites. Arrows indicate Alu repetitive elements as white, grey, or black designating Alu element sub-families.

Figure 2: DSB-induced and bioflavonoid-induced GFP+ colonies resulting from illegitimate DNA repair and a chromosomal translocation. GFP+ colonies were scored by inverted fluorescent microscopy. Representative phase contrast and fluorescent microscopy images of GFP+ colonies are shown.

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APPENDIX C: OXIDATIVE STRESS, BONE MARROW FAILURE, AND GENOME INSTABILITY IN HEMATOPOIETIC STEM CELLS

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Article

Oxidative stress, bone marrow failure, and genome instability in hematopoietic stem cells

Short Title**:** Oxidative stress and bone marrow failure

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Abstract: Reactive oxygen species (ROS) can be generated by defective endogenous reduction of oxygen by cellular enzymes or in the mitochondrial respiratory pathway, as well as by exogenous exposure to UV or environmental damaging agents. Regulation of intracellular ROS levels is critical since increases above normal concentrations lead to oxidative stress and DNA damage. A growing body of evidence indicates that the inability to regulate high levels of ROS leading to alteration of cellular homeostasis or defective repair of ROS-induced damage lies at the root of diseases characterized by both neurodegeneration and bone marrow failure as well as cancer. That these diseases may be reflective of the dynamic ability of cells to respond to ROS through developmental stages and aging lies in the similarities between phenotypes at the cellular level. This review summarizes work linking the ability to regulate intracellular ROS to the hematopoietic stem cell phenotype, aging, and disease.

Keywords: Oxidative damage; reactive oxygen species; ROS; bone marrow failure; base excision repair; excision repair cross complement; aging; genome stability; cancer

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1. Introduction

Organisms in aerobic environments are continuously exposed to reactive oxygen species (ROS). ROS include oxygen molecules (O_2), superoxide anion radicals ($^{\cdot}O_2^-$), hydroxyl free radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), and can be generated by imbalanced endogenous reduction of oxygen by cellular enzymes or in mitochondrial respiratory pathway, as well as by exogenous exposure to UV or environmental damaging agents [1]. Regulation of intracellular ROS levels and ROS-mediated signaling is central maintaining the balance between self-renewal, proliferation, and differentiation of normal stem and progenitor cells in the hematopoietic and neuronal compartments, as well as the early embryonic stem (ES) cell compartment. Increases in ROS above normal concentrations can lead to oxidative stress (OS) and DNA damage [2]. OS is thought to damage approximately 10,000 bases per day per human cell and be one of the major causes of DNA damage and mutation [3]. The majority of these damaged sites are repaired through base excision repair (BER), although persistent damage or damage on two opposing strands at nearby nucleotides may result in double-strand breaks (DSBs) requiring repair by non-homologous end-joining (NHEJ) or homologous recombination (HR). OS is also known to induce higher order chromatin single strand DNA (ssDNA) nicks characteristic of those generated during programmed cell death. ROS and the DNA damage that may result are both capable of triggering multiple DNA damage response pathways. Results of signaling and cellular response to ROS range from cell proliferation and survival to growth arrest, senescence, and cell death depending on the level of ROS and cell type studied. A growing body of evidence indicates that the inability to regulate high levels of ROS leading to alteration of cellular homeostasis or defective repair of ROS-induced damage lies at the root of diseases characterized by both neurodegeneration and bone marrow failure [4-7]. Further, high levels of ROS appear to be a distinct feature of acute myeloid leukemia (AML) [8-10]. That these diseases may be reflective of the dynamic ability of cells to respond to ROS through developmental stages and aging lies in the similarities between phenotypes at the cellular level.

2. ROS Induces DNA Damage Signaling and Repair

2.1. Oxidative Stress and Oxidative DNA Damage

Genomes of all organisms are exposed to endogenous and exogenous stresses such as OS. OS refers to an imbalance between antioxidant defenses and production of ROS [11-14]. ROS are generated endogenously from cellular metabolism such as oxidative phosphorylation in mitochondria and long-chain fatty acids oxidation in peroxisomes, or exogenously by environmental toxins such as ionizing radiation (IR),

ultraviolet (UV) radiation, and DNA damaging/chemotherapeutic agents [15-20]. When ROS generation exceeds antioxidant defense capacity in cells, ROS may react with almost all macromolecules including DNA, RNA, proteins, and lipids. Notably, oxidative DNA damage may represent the major type of DNA damage, evidenced that approximately 10,000 DNA alterations are generated per mammalian cell per day [21-24]. Oxidative DNA damage includes oxidized base (purine and pyrimidine) damage, oxidized sugar moiety damage, apurinic/apyrimidinic *(*AP) sites, DNA single-strand breaks (SSBs), DSBs, DNA intrastrand crosslinks, DNA intrastrand crosslinks, DNA interstrand crosslinks (ICLs), protein-DNA crosslinks, mismatched pairs with damaged bases, stalled DNA replication forks, and oxidatively-generated clustered DNA lesions (OCDLs) [21-25]. OS has been implicated in the pathogeneses of multiple diseases such as bone marrow failure, cancer, and neurodegenerative disorders [26-28].

2.2. DNA Damage Response Pathways and DNA Repair

To maintain genomic integrity, DNA repair and DNA damage response (DDR) pathways are employed in cellular responses to oxidative DNA damage [22,23,29,30] (Figure 1). The majority of these damaged sites are repaired through BER. However, depending on the extent of oxidative DNA damage processes such as nucleotide excision repair (NER), mismatch repair (MMR), HR, and NHEJ may be employed in the repair processes to protect cells from OS [17,22]. Additionally, Ataxia-telangiectasia mutated (ATM)- Checkpoint kinase 2 (Chk2) and ATM- and Rad3-related (ATR)-Checkpoint kinase 1 (Chk1) checkpoints are the two major DDR pathways induced by oxidative DNA damage in order to coordinate DNA repair process with cell cycle progression, transcription, apoptosis, and senescence [23,30-34]. ATM modulates cell cycle in response to multiple types of cell stress and DNA damage including oxidative stress. In response to DSBs, ATM auto-phosphorylates its own Serine 1981, leading to dimer dissociation into a monomer and the full activation of ATM [35,36]. ATM is also directly activated after disulfide bond-dependent conformation change induced by OS in the absence of direct DNA damage [37-39]. In support of a critical role for ATM in protecting hematopoietic stem cells (HSC) from OS, the HSC compartment of ATM-deficient mice present with increased levels of ROS-dependent phosphorylation of the downstream effector mitogen activated protein kinase (MAPK) p38 protein that is known to activate both cell cycle signaling and apoptosis in response to multiple cellular stressors including OS, UV, and inflammatory cytokines. This loss of ATM signaling and increase in p38 phosphorylation is in turn associated with a loss of HSC quiescence [40]. Notably, ATR is activated by oxidative DNA damage after a distinct 3'-5' SSB end resection by a BER protein APE2 [41]. Functional interplay between various DNA repair programs and ATM-Chk2- and ATR-Chk1-dependent DDR pathways in response to oxidative stress is summarized in a recent review article [28]. A chronic impairment of the DDR would be expected to result in accumulation of DNA damage, a mutator effect, and genome instability as observed in normal aging, cancer, and neurodegenerative disease (Figure 1). Elevated intracellular ROS promotes DNA DSBs and altered NHEJ repair leading to chromosomal deletions, translocations, and tumorigenesis [42-44]. Interestingly, multiple studies have identified elevated ROS production in the mitochondrial compartment of apoptotic cells. However, the extent of interplay, if any,

between damage signaling pathways or DNA repair pathways in the mitochondrial and nuclear compartments is unclear.

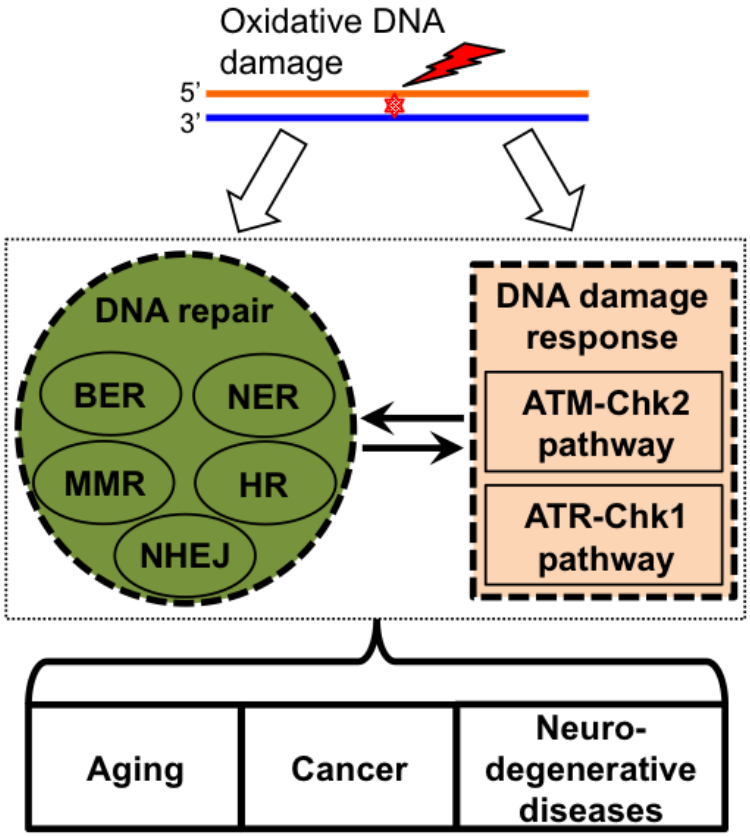

Figure 1. Cellular responses to oxidative DNA damage. DNA repair pathways (BER, NER, MMR, HR, and NHEJ) and DNA damage response pathways (ATM-Chk2 and ATR-Chk1) are integrating into an interacting network in response to OS. Defective DNA repair and response pathways are associated with aging, cancers, and neurodegenerative diseases.

3. Bone Marrow Failure Syndromes

Bone marrow failure is characterized by the progressive absence of one or more hematopoietic cell lineages. These syndromes are characterized as inherited or acquired. The best-characterized inherited syndromes include Fanconi anemia (FA), as well as the telomere diseases dyskeratosis conegenitia (DC), Diamond-Blackfin anemia (DBA), and Shwachman-Diamond syndrome, while the most prevalent acquired syndrome is aplastic anemia (AA). There is increasing evidence linking elevated ROS levels and impaired DNA repair mechanisms to contribute to bone marrow failure observed in these diseases. On the other hand, neurodegeneration corresponds to any pathological condition that primarily affects neurons and neurodegenerative disorders including Alzheimer disease, Parkinson disease, Huntington disease, and amyotrophic lateral sclerosis [45]. However,

both systems appear particularly sensitive to the control of intracellular ROS and OS, and altered responses to these leading to DNA damage have been implicated in the pathogeneses of both bone marrow failure and neurodegenerative disorders [4,6,7,26-28,46,47].

3.1. Fanconi Anemia

FA is an autosomal X-linked disease that is characterized mainly by bone marrow failure, anemia, developmental abnormalities, cancer susceptibility, and altered mitochondrial function. It is considered a rare disease affecting approximately 1 in every 100,000 people [48]. FA is a chronic and degenerative disorder with the probability of patients developing leukemia, squamous cell carcinomas, or liver tumors increasing by 40% by age 30, 50% by age 45 and 76% by age 60 [49]. Progressive bone marrow failure and myeloid malignancies account for 90% of deaths in FA patients. At the molecular level, there are 15 complementation groups that been identified, and mutations in any of these genes can lead to FA or a similar disorders. FA proteins that make up the core complex are FancA, FancB, FancC, FancE, FancF, FANCG, FANCL, and FANCM, and defects in any are known to cause FA in humans. This core complex acts to ubiquitylate downstream targets FancD2 and FancI that then act in parallel with the remaining FA proteins (FancD1, FancJ, FancN, FancO, and FancP) to repair ICLs. Several mouse models have provided insight into the pathology and etiology of the disease.

There is some discordance between humans with FA and FA mouse models, most notably the absence of bone marrow failure, anemia, and developmental abnormalities in mice that are the defining characteristics of the disease in humans. These differences, however, could be in part due to human reliance on the FA pathway for detoxification and tumor suppression to ensure normal development whereas laboratory mice lack the same genotoxic stressors that may trigger the disease [50]. Almost all FA mouse models so far have exhibited reduced fertility, and cells derived from these mice display sensitivity to ICL inducing agents [50]. *FancA*$^{-/-}$ mice, for instance, do not display hematopoietic abnormalities or congenital defects; however, they have hypergonadism and mouse embryonic fibroblasts (MEFs) derived from these mice are hypersensitive to mitomycin C (MMC) [51]. Interestingly, bone marrow-derived megakaryocyte progenitors demonstrate impaired proliferation and all bone marrow cells derived from *FancA*$^{-/-}$ mice display increased apoptosis indicating a possible HSC defect [52]. Also, both *FancA*$^{-/-}$ and *FancG*$^{-/-}$ mice have increased apoptosis in neural stem and progenitor cells that leads to microcephaly [53].

FancC$^{-/-}$ mice born do not display overt hematopoietic abnormalities, however they are born with sub-Mendelian frequency and have a significantly increased incidence of micropthalmia in C57BL/6J mice [54]. HSC derived from *FancC*$^{-/-}$ mice have impaired differentiation and self-renewal capacity [55], a defect that is rescued by retroviral-mediated gene transfer of *FancC*, indicating that FANCC is required for properly functioning HSCs. Also, *FancC* $^{-/-}$ mice exposed to low doses of MMC develop progressive bone marrow failure and chromosomal aberrations [56]. Differences in humans with FA and the mouse models may be due to the differences in environmental and dietary exposures.

FancD1/Brca2 is downstream from the FA core complex. *Brca2* is required for HR-directed repair of DNA DSBs. *FancD1* knockout in mice leads to embryonic lethality, but a mouse model has been created with homozygous deletion of exon 27, which allows expression of the protein but prevents *FancD1* from interacting with *FancD2* [57]. *FancD1*$^{\Delta 27/\Delta 27}$ mice display similar phenotypes to *FancA*$^{-/-}$ and *FancC*$^{-/-}$ mice, though there is some evidence that the hematopoietic abnormalities are more severe than with the other phenotypes. For instance, *FancD1*$^{\Delta 27/\Delta 27}$ mice are significantly more sensitive to MMC and acquire spontaneous chromosomal aberrations and early loss of colony forming hematopoietic progenitor cells compared to the other phenotypes [58]. This severity would be expected as *FancD1* is necessary not only for ICL repair, but also for DSB repair via HR. Notably, unlike the other FA mouse models, *FancD1*$^{\Delta 27/\Delta 27}$ mice do not have impaired fertility indicating that it is dispensable for meiosis unlike the other FA proteins, but is important for somatic cell HR [57].

FancD2$^{-/-}$ mice are born with sub-Mendelian frequency and also display growth retardation, a phenotype that varies based on the genetic background of the mice. These mice have an increased incidence of tumor formation similar to that of *FancD1* haploinsufficiency [59]. Also, the phenotype of these mice is more severe than those with deleted FA core proteins, indicating alternate activity outside of the FA pathway, possibly protecting against oxidative DNA damage including DSBs [60].

Cells derived from FA mutant mice are hypersensitive to OS. It has recently been shown that impaired or reduced mitochondrial function in FA cells is coupled with increased intracellular ROS, and inactivation of enzymes essential for energy production [61]. Intriguingly these defects, as well as hypersensitivity to MMC can be partially rescued by overexpression of superoxide dismutase (SOD1) [61]. Bone marrow progenitor cells derived from *FancC*$^{-/-}$ mice undergo replicative senescence following exposure to hypoxic/hyperoxic conditions [62]. Interestingly, the *FancC*$^{-/-}$/*Sod1*$^{-/-}$ mouse model was created to increase endogenous OS. These mice demonstrate loss of hematopoietic progenitor cells in the bone marrow leading to anemia and leucopenia [63], strongly indicating that OS is a key factor in the initiation of bone marrow failure in FA. Also, the *FancD2*$^{-/-}$/*Aldh2*$^{-/-}$ mice, which can no longer detoxify aldehydes by oxidizing them to carboxylic acids, present with severe phenotypes including the development of acute lymphoblastic leukemia (ALL) after 3-6 months. Exposure to ethanol *in utero* results in development of physical abnormalities and death; and surviving mutant mice have a vast reduction in bone marrow cellularity [64]. Taken together, these models demonstrate that FA phenotypes that are hallmarks of the disease may indeed require stressors such as aldehydes or ROS in order to initiate the disease phenotype, and it is the absence of these stressors in the laboratory environment that prevents the FA mouse models from accurately mimicking the disease in humans.

3.2. Additional Degenerative Excision Repair Defects

Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are characterized by defects in DNA repair of damage by crosslinking agents or elevated ROS due to mutation of any one of the excision repair pathway cross complementation (ERCC) group genes. Whereas CS patients are defective in the transcription coupled NER (TC-NER) pathway and present with neurological abnormalities, XP patients are deficient in the global

genome NER (GG-NER) pathway and are hypersensitive to sun and susceptible to carcinoma [65]. Mutant mouse models have been generated for most of the proteins involved in this pathway that mimic the genotypes and phenotypes of patients with CS or XP.

CS is caused by defects primarily in *Csa* and *Csb* which are the core proteins essential for TC-NER. $Csa^{-/-}$ or $Csb^{m/m}$ mice genetically mimic the disease, and both mutants present with UV sensitivity, loss of photoreceptors, increased sensitivity to γ-irradiation, and mild neurodegeneration [66]. Cells from $Csb^{m/m}$ mice but not $Csa^{-/-}$ mice show hypersensitivity to γ-IR and paraquat which generates superoxide anions and H_2O_2 in cells indicating that *Csb* plays a role in the repair of oxidative damage [67,68]. Although $Csa^{-/-}$ mice do not show an increased sensitivity to OS, human cells deficient in CSA are more sensitive to H_2O_2 than wild-type cells [69], and primary fibroblasts and keratinocytes from CSA patients are hypersensitive to potassium bromate, a known inducer of oxidative damage [70]. This indicates that the role of the CSA protein may differ between species and that it is likely involved in the oxidative damage response in humans but not mice [71].

XP is caused by defects in core NER proteins as well as proteins in the GG-NER pathway. *Xpc* functions primarily in the GG-NER pathway and $Xpc^{-/-}$ mice have a reduced lifespan and have a propensity for developing spontaneous tumors Also, these mice have been demonstrated to have a very large increase in lung tumor incidence compared to controls thought to be a result of chronic OS [72]. Also, fibroblasts derived from $Xpc^{-/-}$ mice have demonstrated OS sensitivity compared to controls by mutation accumulation and decreased survival [73]. Analysis of bone marrow cells from *Xpc*-/- mice shows reduced cellular viability, hypocellularity of most lineages, reduced numbers of CFU, and increased sensitivity to carboplatin [74]. Since XP patients develop skin cancers by the age of 20, and the average life expectancy is early 30s, the findings in bone marrow of the *Xpc*-/- mice suggest the possibility that if patients were to live to later age, they could exhibit myelosuppression or bone marrow failure. In contrast to the $Xpc^{-/-}$ mice, $Xpa^{-/}$ mice have a significant increase in tumors in the liver, but not in the lungs and do not have an increase in mutation load like the $Xpc^{-/-}$ mice [73]. However, cells derived from XPA patients exhibit defective repair in 8,5'-(S)-cyclo-2'-deoxyadenosine, a DNA lesion induced by ROS indicating that XPA may play a role in the repair of oxidative bulky lesions [75].

Cells from both CS and XP individuals affected by disease and from multiple mouse models have elevated ROS and defective mitochondrial function [65,75]. Interestingly, a similar set of defects are observed in lymphoblasts from DC patients, namely elevated ROS production, impaired DNA response leading to apoptosis and a proliferative defect [76], further connecting elevated ROS with bone marrow failure outcome.

Additional proteins in the ERCC complement group are now being identified as central to the etiology of other bone marrow failure syndromes. Through Next-Gen sequencing, ERCC6L2 was recently reported as deficient in two patient samples both presenting with bone marrow failure and neurodegeneration [77]. Loss of function of the same gene in both cases demonstrates the link between these two degenerative processes. Reduction of ERCC6L2 induced by siRNA knockdown in cells results in significantly increased intracellular ROS, effective DNA repair response shown by γ-H2AX foci,

reduced cell viability and increased sensitivity to a panel of DNA damaging agents [77]. The similarities in phenotypes from ERCC genes thus far suggest that additional mutations in ERCC genes will be identified as a paradigm for bone marrow failure syndromes.

4. Hematopoietic Stem Cell Response to ROS

4.1. HSC Response to ROS

ROS are important for fate determination of normal stem cells [78]. Normal HSC are primarily in a quiescent state within the BM niche [79]. HSC exposed to elevated ROS exhibit altered characteristics and undergo both proliferation and differentiation, typically after mobilization to the oxygen rich bloodstream [80], but also to senescence and apoptosis in a dose-dependent manner [78]. This impact of ROS is evolutionarily conserved and observable in analogous *Drosophila* systems [81].

A number of transcriptional microarray, exome, and proteomic approaches have been utilized in an effort to understand the signal transduction pathways activated in response to ROS [82-91]; however, few have centered on specific HSC populations. Despite this, the p53, Akt, MAPK, and ATM pathways have all been implicated in HSC response to ROS. MDM2 promotes survival of hematopoietic progenitors by promoting p53 degradation [92]. The forkhead box O (FoxO) family of transcription factors has known roles in metabolism, proliferation, and OS response and resistance (recently reviewed in [93]). Single Foxo3a deficiency in HSCs is associated with elevated intracellular ROS [94]. Triple knock-out *Foxo1*$^{-/-}$ *Foxo3a*$^{-/-}$ *Foxo4*$^{-/-}$ mice exhibit reduced numbers of both HSC and progenitor cell populations [95]. Recent molecular analysis suggests that the co-factor KDM5 activates OS resistance genes through interaction with the lysine deacetylase HDAC4 to in turn promote Foxo deacetylation and alter specificity of target gene binding [96]. *Drosophila* also demonstrates signaling paradigms with increases in ROS signaling through JNK pathway, FoxO activation, and polycomb downregulation [81]. In conjunction with elevated ROS, depletion of the Meis1 transcription factor in mice leads to apoptosis, loss of HSC quiescence, and loss of transplantation capacity [97].

Differences in the levels of ROS also impact the observed cellular response of stem cells [98]. Elevated levels of OS induced by mutations of metabolic enzyme genes, ischemia/reperfusion, chemotherapy, or chronic exposure to 10-100 μM H_2O_2 induces multiple effects ranging from cell cycle arrest to death, depending on the cell type [99,100]. At submicromolar concentrations, ROS and H_2O_2 act as proliferation and growth signaling molecules. Low concentration exposure (100 μM) of stem cells is sufficient to promote hyperphosphorylation of Akt and RAF1, activation of insulin signaling pathways, and phosphorylation of MAPK p38 within 1 hr [98]. This initial rapid growth is then temporally followed 4-24 hr later by induction of cell cycle checkpoints characterized by dephosphorylation of Rb, hyperphosphorylation of cdc2, and down-regulation of E2F targets such as *foxd3* [98].

To directly link ROS to genetic instability in HSC, we genetically engineered murine HSC cells to contain two transgene reporters at unlinked loci -- each with either a 5’ or 3’ exon of the green fluorescent protein (GFP). This scheme enables us to score for

chromosomal translocations following DNA damage and repair by interchromosomal NHEJ repair that results in a repair event joining the two exons onto the same DNA helix to form an intact GFP (Figure 2A). Following exposure of the engineered HSC to H_2O_2 (Figure 2A, Figure 2B left image), surviving cells were then allowed to proliferate into myeloid CFU by standard protocols [101] (Figure 2B middle image), and those CFU expressing GFP readily identified by fluorescent microscopy (Figure 2B right image). This approach demonstrated that a single 30 min exposure of HSC to 100 μM H_2O_2 or 5mM H_2O_2 is sufficient to promote the appearance of GFP+ colonies in a dose-dependent manner at average frequencies of 6 x 10^{-6} and 10 x 10^{-6}, respectively (Figure 2C). This *in vitro* system is analogous to the outgrowth of transformed myeloid leukemic cells and connects ROS and genome rearrangements in HSC and leukemias.

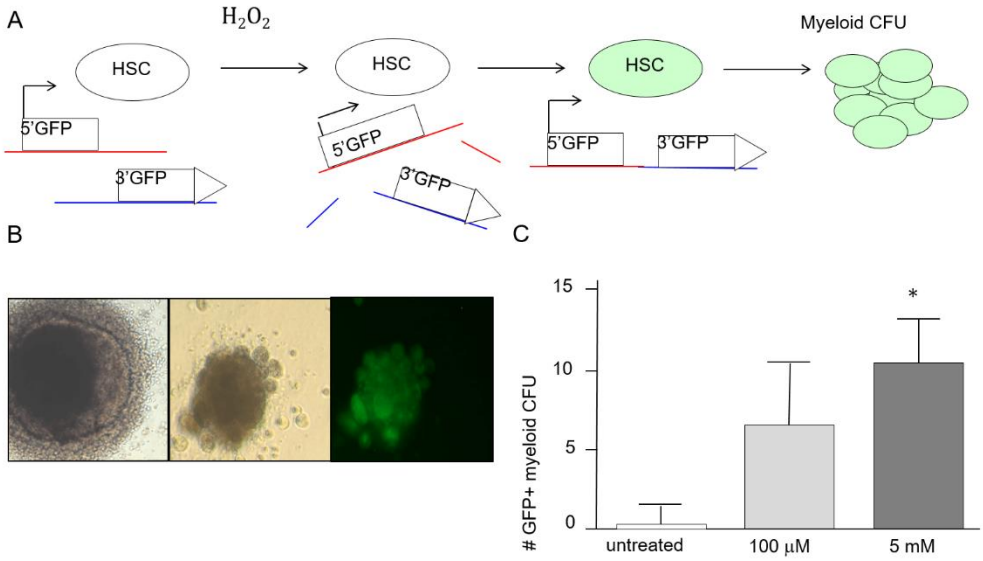

Figure 2. Direct connection between exposure of HSC to ROS and genome rearrangements. A. Scheme of exposure of genetically engineered HSC to ROS and scoring of GFP+ cells indicative of translocations. B. Left image -- HSC colony by contrast microscopy (100X). Middle image -- GFP+ derivative myeloid CFU by contrast microscopy (100X). Right image -- same GFP+ CFU colony shown in middle by fluorescence microscopy (100X). C. Bar graph showing dose dependent appearance of GFP+ myeloid CFU derived from HSC exposed to 0, 100□M, or 5mM H_2O_2 for 30 min, then returned to normal conditions. 100□M resulted in an average frequency of CFU at 6.5 x 10^{-6}. 5mM resulted in an average frequency of CFU at 10.4 x 10^{-6}. ***** denotes statistically significant stimulation of chromosomal translocations by H_2O_2 (students T-test p value<0.037). A few colonies were observed in untreated samples that by contrast microscopy but appeared to be inviable and auto-fluorescent rather than bona fide GFP+ CFU; however, further analysis was not performed on them so they are reported here.

It is now well established that IR promotes differentiation, short-term apoptosis, and long-term senescence of hematopoietic stem cells [78,102]. Several studies demonstrated that IR of CD34+ hematopoietic progenitor cells induces indirect effects on DNA damage through elevated OS [102,103]. IR of CD34+ cells induces early increase of superoxide anion radicals and hydrogen peroxide, followed by elevated MnSOD expression by 24 hr, followed by elevated persistent intracellular ROS over longer culture periods associated with reduced clonogenic potential [104,105]. By contrast γ-H2AX expression and foci associated with direct DNA DSBs are elevated in short periods following IR but return to baseline by 24 hr [105]. Presence of insulin-like growth factor 1 (IGF-1) in irradiated CD34+ cells can inhibit the mitochondria-mediated apoptosis and rescue the clonogenic potential defect [104]. This role for IGF-1 in response to ROS mirrors transcriptional and proteomic profiling of stem cells demonstrating induction of insulin regulated pathways in response to ROS [98]. Further, sublethal total body IR of mice leads to a persistent elevation of ROS in HSC associated with sustained increases in oxidative DNA damage and DSBs, inhibition of HSC clonogenic potential, and induction of HSC senescence, at least in part due to upregulation of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) [103].

4.2. HSC Response to ROS and Aging

Control of ROS in ES cells is now understood to be critical to maintenance of self-renewal phenotype. Molecular evidence suggests this is due, at least in part, to ROS activation of miR-29b that targets Sirt1 to control ES cell self-renewal [106]. Differentiation of ES cells leads to increased intracellular ROS [61,107]. Interestingly, during differentiation of ES cells, superoxide production, cellular levels of intracellular ROS, and DNA damage levels increase although expression of major antioxidant genes and genes involved in multiple DNA repair pathways are downregulated [107], and DNA repair by HR is reduced [101].

Aging is associated with reduced expression of multiple DNA repair proteins, reduced DNA repair efficiency and elevated levels of genome instability in hematopoietic cells [6,108-110]. Hematopoietic cells in older mice and humans are characterized by reduced regenerative potential, lineage bias toward myeloid cells, and increased potential for outgrowth of genetically unstable cells and malignancy. Serial transplantation of human HSC into immunodeficient mice is associated with replication stress, elevated intracellular ROS, accumulation of DNA damage, and reduced self-renewal potential that can be further aggravated by addition of a glutathione synthetase inhibitor [80]. The connection between natural HSC aging and intracellular ROS was made by the ability to separate the CD34-, Lin-, Sca-1+, c-kit+ HSC compartment into two fractions based on intracellular ROS levels [111], and the observation that the ROS^{low} fraction has higher self-renewal capacity and serial transplantation capability. Conversely, ROS^{hi} fraction has reduced capacity for self-renewal and in addition is biased toward myeloid differentiation, similar to aged mice *in vivo* [111]. Intriguingly, the observed phenotype can be partially rescued following inhibition of MAPK p38 suggesting that p38 is a potential therapeutic target [40,111].

ROS seem well tolerated in “young” cells with highly proficient DNA repair, and thus have few if any long-term deleterious effects. However, “older” cells with less

efficient repair become more sensitive to ROS levels which lead to increased genome instability. Recent work demonstrated that quiescent HSC (analogous to the ROS^{low} fraction) repair DNA damage less efficiently than progenitors leading to accumulation of DNA damage and impaired function, suggesting a mechanism for increased presentation of myeloid malignancies in aged populations [112].

HSC reactivity to ROS during aging *in vivo* combines cell-intrinsic and cell-extrinsic mechanisms [113]. A relationship between the HSC compartment and supporting cells within the bone marrow niche is central to homeostasis and changes during not only aging but also other pathophysiologic processes including atherosclerosis, hypertension, and diabetes [114,115]. HSC and progenitors are typically exposed to low levels of ROS in the bone marrow niche, and hypoxia in turn helps preserve HSC and progenitor characteristics. HIF1α, HIF2α □□□□□□□ have been shown to be major regulators in modulating the response of HSC to ROS in this microenvironment [103,116-118]. ROS derived from NAPH oxidase and mitochondria in response to injury and stress is elevated in supportive bone marrow microenvironment cells, leading to hypoxia and HIF-1α expression, and HSC progenitor expansion and differentiation [114]. Similarly, HIF2α depletion in HSC leads to increased ROS, activation of ER stress and apoptosis [119].

4.3. Leukemic Cells and ROS

Defective signaling in response to ROS occurs in multiple leukemic stem cell populations to promote proliferation, differentiation, genomic, and epigenetic alterations, immune evasion, and survival [120]. ROS act as signaling molecules to regulate redox-sensitive transcriptional factors, enzymes, oncogenes, and other downstream effectors, and thus AML cells are thought to be addicted to elevated ROS [120]. Extracellular ROS, but not intracellular mitochondrial ROS, have been found to be significantly elevated in both AML cell lines and cells derived from patients [8-10] and correlates with constitutive activation of NOX, reduced levels of glutathione, and attenuated p38 MAPK responsiveness, all postulated to promote proliferation of AML blasts [121]. AKT negatively regulates the FoxO proteins, which target expression of several anti-oxidant enzymes, including the GPx, catalase (CAT) and SODs. Akt is constituitively activated in most primary AML cells, leading to FoxO inhibition, and increased ROS levels [95,122]. Activated RAS is a feature of a portion of AML patients, and upregulates ROS levels through NOX to promote proliferation of hematopoietic cells [121]. Similarly, FLT3 plays a role in H_2O_2 production in AML cells through NOX p22 protein, while knockdown of p22 leads to immediate reduction of H_2O_2 within 24 hr [123]. Activating mutations of FLT3 by internal tandem duplications or activating mutations of the FLT3 receptor are seen in a large percentage of AML patients associated with increased DNA DSBs, genome instability, and poor prognosis [124]. Interestingly FLT3 activation in AML cells leads to inactivation of tumor suppressor PTP, DEP1, and reactivation of DEP1 is sufficient to decrease ROS. Elevated ROS DNA damage occurs both in the nuclear fraction and mitochondria. Mitochondria have more limited DNA repair functions and mitochondrial DNA genome instability has been noted in AML [125]. HIF2α activity may also promote proliferation and inhibition of apoptosis of AML blasts in response to ROS [119].

Philadelphia-negative chronic myeloproliferative neoplasms (MPNs; essential thrombocythemia, polycythemia vera, and myelofibrosis) have recently been shown to be associated with chronic inflammation, OS and accumulation of ROS. Transcriptional profiling of these revealed several OS modulating genes significantly deregulated in MPNs [126]. Interestingly the transcriptional factor Nrf2, which is upregulated in multiple AML cells, was found to be downregulated in this study [126,127]. In addition, downregulation of ATM and p53 were also observed, although the impact of these is likely to be broader than through OS signaling. OS may also play a role in mutation accumulation and progression of myelodysplastic syndromes (MDS) to AML as suggested by studies in a mouse model of MDS that displays increased ROS in Lin- bone marrow cells, increased DNA breaks, and increased mutation frequency over time, although to date these studies have been correlative and not causative [128].

Elevated ROS in in AML cells may be exploited as a therapeutic strategy. A recent report outlines the design of an H_2O_2 activatable anti-AML agent based on a conjugate addition strategy [129]. In this, a dual pharmacophore was developed in which the first is a peroxidase acceptor and the second is a pendant amine which react via conjugate addition following H_2O_2 oxidation within the tumor cell, and demonstrated 10-fold increased activity of the pendant amine agent in AML cells over normal CD34+ cells.

5. Conclusions

A growing body of evidence indicates that the inability to regulate high levels of ROS leading to alteration of cellular homeostasis or defective repair of ROS-induced damage lies at the root of diseases characterized by both neurodegeneration and bone marrow failure [4-7,26-28,46,47]. That these diseases may be reflective of the dynamic ability of cells to respond to ROS through developmental stages and aging lies in the similarities between phenotypes at the cellular level. It appears that neuronal or hematopoietic progenitors are particularly susceptible to altered ROS and mutation of any gene within these systems that alters ROS levels is more likely to trigger signaling pathways to promote cell death and hypocellularity within the tissue compartments. Natural aging in the human population is associated with multiple neurodegenerative phenotypes as well as a loss of the HSC population, genome instability, and cancer. These outcomes are likely also reflective of the sensitivity of progenitors in both these compartments to ROS homeostasis. The coupled reduction of DNA repair protein expression and DNA repair efficiency in neuronal stem and HSC compartments would also put these cells at higher risk. However, why these two organ systems appear to be particularly susceptible to ROS remains unclear and warrants further study. It is intriguing that several recent studies have demonstrated some amelioration to elevated ROS levels through add-back of DNA repair proteins to diseased cells; whether these will be harnessed for personalized medicine remains to be seen.

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Author Contributions

C.R. conceived and wrote and edited the paper and generated Figure 2. S.Y. wrote and edited the paper and generated Figure 1. C.G.V. wrote and edited the paper and generated Figure 2.

Conflicts of Interest

The authors declare no conflict of interest.

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APPENDIX D: LONG-TERM IMPACT OF CHROMATIN REMODELING AND DNA DAMAGE IN STEM CELLS INDUCED BY ENVIRONMENTAL AND DIETARY AGENTS

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Abstract
The presence of histones acts as a barrier to protein access; thus chromatin remodeling must occur for essential processes such as transcription and replication. In conjunction with histone modifications, DNA methylation plays critical roles in gene silencing through chromatin remodeling. Chromatin remodeling is also inter-connected with the DNA damage response, maintenance of stem cell properties, and cell differentiation programs. Increasingly, chromatin modifications are shown to produce long-lasting alterations in chromatin structure and transcription. Recent studies have shown environmental exposures *in utero* have the potential to alter normal developmental signaling networks, physiologic responses, and disease susceptibility later in life in a process known as developmental reprogramming. In this review we will discuss long-term impact of exposure to environmental compounds, chromatin modifications that they induce, and the differentiation and developmental programs of multiple stem and progenitor cell types altered by exposure. The main focus is to highlight agents present in the human lifestyle that have the potential to promote epigenetic changes that impact developmental programs of specific cell types, may promote tumorigenesis through altering epigenetic marks, and may be transgenerational, *e.g*. able to be transmitted through multiple cell divisions.

I. INTRODUCTION

A. Chromatin remodeling and epigenetics

Epigenetics is the study of heritable changes in gene expression without a change in the DNA sequence. Nucleosomes are composed of 147 bp of DNA wrapped around core histone proteins H2A, H2B, H3, and H4 (1-3). Histone H1 acts to link histones together and also to the nuclear scaffold. H3 and H4 termini extend out from the nucleosome and

can be modified chemically by acetylation, methylation, ubiquitination, phosphorylation, sumoylation, citrullination, and ADP-ribosylation (3). Modifications promote either open or closed chromatin (3), which in turn influence multiple cellular processes transcription and replication (4-6). Chromatin remodeling is also inter-connected with the DNA damage response, maintenance of stem cell properties, and cell differentiation programs. Patterns of histone modifications are maintained after replication and thus inherited through multiple cellular generations. DNA methylation predominantly involves the covalent addition of a methyl group (CH3) to cytosine in the context of CpG in DNA creating a significant epigenetic mark of transcriptional inactivity. Patterns of DNA methylation are generated during development involving *de novo* methylation and demethylation mediated by DNA methyltransferases (DNMTs). DNMT3 regulates *de novo* methylation during development, and DNMT1 maintains DNA methylation patterns following replication. Global genome methylation patterns are highly developmental stage- and tissue-specific.

This review focuses on commonly used environmental and dietary compounds previously known or suspected to have carcinogenic or mutagenic properties but recently identified to have the potential to disrupt chromatin remodeling and epigenetic regulation in stem cells and induce long-term changes in developmental programs and promote tumorigenesis. Further we highlight recent reports that have shown that *in utero* exposure to these can also promote epigenetic modifications which in turn induce gene expression changes that persist throughout life (7, 8).

B. The connection between epigenetic alterations and DNA double-strand breaks

The DNA double-strand break response (DDR) is facilitated by hierarchical signaling networks that orchestrate chromatin structural changes, cell cycle checkpoints, and multiple enzymatic activities to repair broken DNA ends. DNA double-strand breaks (DSBs) have the highest potential to promote illegitimate repair mechanisms, accumulation of mutations, and are considered the critical primary lesions in the formation of chromosomal rearrangements associated with disease and tumorigenesis. The recent advances in the understanding of the interplay between chromatin remodeling, epigenetics, and DDR have been reviewed (9-11). New emerging evidence extends earlier findings with the potentially pathological repercussions of restoration of chromatin structure resulting in a DSB-induced epigenetic memory of damage.

Chromatin dynamics and changes in chromatin architecture that occur for repair of DSBs include nucleosome eviction from DSBs, relaxation of heterochromatin structure, and localized chromatin destabilization at DSBs (12). After DNA damage, chromatin structure is altered by ATP-dependent chromatin remodeling, incorporation of histone variants into nucleosomes, and covalent histone modifications. These histone modifications include phosphorylation of H1; acetylation of H2A, phosphorylation and ubiquitination of H2AX; acetylation and methylation of H3; and phosphorylation and acetylation of H4 (3). Among the different histone modifications, phosphorylation of all four histones as well as the variant H2AX plays a primary role in DNA damage response by facilitating access of repair proteins to DNA breaks (13, 14). Phosphorylation of H2AX (γH2AX) spreads over large chromatin domains away from a DSB. This chromatin marking and large-scale chromatin reorganization recruits repair factors, recombination proteins and chromatin remodeling complexes involved in DNA repair pathways (15).

Heterochromatin is the tightly compacted DNA structure that acts as a barrier for DNA repair processes. As a result, heterochromatic DSBs are generally repaired more slowly than euchromatic DSBs (16), and heterochromatin and euchromatin utilize distinct remodeling complexes and pathways for DSB repair (17-21). DSB repair may be stalled within HC regions if a series of dynamic and localized changes fail to take place (18, 22, 23). The ataxia telangiectasia mutated (ATM) protein and DDR mediator proteins overcome constraints posed by heterochromatin superstructure to promote repair through modulation of two HC factors -- KAP-1 co-repressor and HP1 chromodomain protein (19). Activation of the ATM signaling pathway and the subsequent phosphorylation of KAP-1 trigger HC modifications required for DSB-repair (24). In addition, studies have shown that histone acetyltransferase (HAT) complexes act with the ATP-dependent SWI/SNF and RSC-containing chromatin remodeling complexes to facilitate DNA repair (25).

Polycomb group (PcG) proteins, which have well-established roles in gene regulation, were recently found to accumulate on chromatin surrounding DNA damage (26). PcG proteins form complexes involved in the epigenetic regulation of gene expression. PcG repressive complexes catalyze post-translational modifications critical to their gene silencing function, including histone H3K27 di- and trimethylation (H3K27me2, H3K27me3) and histone H2A ubiquitination (27-29). PcG and PRC components found to respond to DNA damage include BMI-1, MEL-18, EZH2 methyltransferase, EZH1, EED, and SUZ12 (30) suggesting that DNA methylation modifications occur as part of the DDR. Recruitment of PcG protein BMI1 promotes mono-ubiquitination of H2A and also DNA DSB repair (31).

Signal transduction pathways in DDR communicate with chromatin-remodeling factors through protein-protein interactions. The chromatin remodeling protein scaffold matrix attachment region 1 (SMAR1) binds other SMAR1 elements along with histone deacetylase 1 (HDAC1) and p53 to form a repressor complex to downregulate transcription (32, 33). The chromatin-remodeling factor Tip49 recruits Rad51, the homolog of bacterial RecA and major homologous recombination repair protein, to DNA damage sites (34). In cells depleted of Tip49, Rad51 recruitment can be restored by addition of a HDAC inhibitor underscoring the interplay of epigenetic marks and DDR. In addition to DNA repair, the p53 signaling pathway is associated with chromatin changes that mainly involve the HAT Tip60 to modulate cell fate between cell-cycle arrest and apoptosis (35). Numerous chromatin-remodeling factors involved in DNA methylation and demethylation also play a role in DDR. In the thymus, genotoxic stress decreases global DNA methylation by a reduction in DNMT1, DNMT3a, DNMT3b and methyl-binding proteins MeCP2 and MBD2 (36). That these alterations are carried into the offspring of exposed individuals led the authors to suggest profound epigenetic dysregulation, which in turn could lead to genome destabilization and possibly serve as precursor for transgenerational carcinogenesis (36).

C. Stem Cells and iPSCs

Stem cells are the focus of study in many laboratories due to their unique properties of self-renewal and pluripotency that allow for both maintenance of the stem cell pool as well as generation of various cell types through differentiation. Induced pluripotent stem cells (iPSCs) have allowed for study of developmental processes of multiple tissue and organ

systems (37-40). Stem cell models have been used for drug discovery and toxicology screens, and development of biomarker panels (41-43).

Because stem cells give rise to all of the mature cells in an organism, they have become a promising source for potential replacement therapies in the treatment of diseases (44) although both genetic integrity and a "correct" epigenome will be important factors in both their experimental and therapeutic usefulness. Extensive literature exists analyzing transcriptional and proteomic profiles as well as chromatin modification profiles of stem cells, ES cells, iPSCs, and differentiated cell types to create signatures of "stemness" (45-61).

Genomic instability of human ES cells and iPSCs has been reported *in vitro*. Common abnormalities of ES cells are gains of chromosomes 12, 17, and 20 (62-67). Chromosome 12 contains genes implicated in cell survival such as *STELLAR, GDF3,* and *NANOG* thus likely giving cells a proliferative advantage. It is interesting to note that trisomy of chromosome 12 is one of the most commonly described mosaicisms in amniocentesis (68) and is characteristic of germ cell tumors in almost all cases (69). Similarly, gain of chromosome 17 is associated with tumorigenesis, specifically breast cancer (70), and amplifications of chromosome 20 are observed in a wide variety of tumors (71, 72). Gain of chromosome X in stem cells cultured *in vitro* (70) is associated with trisomy 17 as well although it is unclear whether this provides a proliferative or survival advantage as loss of chromosome X has also been reported *in vitro* (64). The fact that these chromosomal abnormalities are common in both cultured stem cells and in multiple *in vivo* settings suggests that stem cells may be particularly susceptible to specific alterations. iPSCs have shown copy number variations, abnormal karyotype, and point mutations (42). In addition they have DNA methylation and histone modification defects, and altered X chromosome inactivation (42). Some studies have indicated that iPSCs may "remember" their previous somatic cell fate as stored within the epigenome (49, 73, 74) reducing their experimental and therapeutic potential.

Transcriptional regulation and gene expression patterns occur in the context of chromatin. In general, stem cells have epigenetic signatures that are characteristic of an active chromatin state, *e.g.* chromatin is generally de-condensed. Chromatin is reorganized during differentiation programs and ChIP-seq approaches have demonstrated that chromatin structure at promoters and regulatory regions correlates with active, repressive, or poised "bivalent" transcriptional states, that in turn, correlates with the state of cell differentiation (75-78).

Stem cell specific structures are regulated not only by epigenetic marks but also by higher order chromatin structures not discussed here (reviewed in (78)). Global levels of DNA methylation in stem cells are similar to levels in somatic cells; however, 25% of methylation sites in stem cells are in non-CpG sites, indicating a unique methylation program (79). Multiple studies demonstrate that histone acetylation is central to maintenance of pluripotency. H3K9 acetylation is overall higher in human stem cells and necessary to maintain an undifferentiated state while H3K9 de-acetylation occurs with differentiation (80). HDAC inhibitors such as butyrate and valproic acid increase overall H3K9ac levels (80) as well as expression of pluripotency genes such as Nanog, Sox, and Oct4 (81), promote stem cell and iPSC survival (82, 83). Other histone modifications

associated with transcriptionally active chromatin have also been shown to be increased in stem cells including H3K14 acetylation, H3K4 trimethylation (H3K4me3), and H3K36 di- and tri-methylation (H3K36me2, H3K36me3) (84, 85). Trimethylated H3K27 (H3K27me3) levels remain unchanged during differentiation but preferentially localize at the nuclear periphery in stem cells and this localization decreases during differentiation (86). It is interesting to note that both H3K27me2 and H3K27me3 marks are mediated by the Polycomb repressive PRC2 complex and present at Polycomb target genes known to be suppressed in stem cells (87, 88). Taken together these observations suggest that Polycomb target genes are located at the nuclear periphery in stem cells and this localization may serve as an additional epigenetically regulated repressive mechanism (79). Whole genome ChIP-seq approaches have shown that both H3K4me3 (a mark of active promoters) and H3K27me3 (a mark of silenced promoters) colocalize in embryonic stem cells at 16-22% of genes, most of which are required for developmental regulation, creating a "bivalent" mark hypothesized to poise such genes for future expression while maintaining repression in the stem cell state (75, 77, 78, 89, 90).

Thus, stem cells have specific epigenetic markers important for maintaining stem cell pluripotency and self-renewal that, when disrupted, could lead to dysregulated differentiation or tumorigenesis. One well-defined example of this is in the initiation of acute myeloid leukemia (AML) with mixed lineage leukemia (*MLL*) gene rearrangements. *MLL* is a homologue of *Drosophila melanogaster* trithorax (Trx) and is a positive regulator of Hox gene expression by H3K4 methylation. Hox gene expression is also negatively regulated by H3K27 methylation by Polycomb group proteins thus conferring a delicate balance of epigenetic marks. Disruption of these opposing epigenetic regulatory factors through *MLL* chromosomal translocation, leads to hyperactivation of Hox genes and, ultimately, to leukemogenesis (91). The mechanisms by which stem cells might transform into cancer stem cells remain widely unknown; however repeated exposure to DNA damaging agents or agents that disrupt epigenetic gene regulation may cause stem cells to become more similar to cancer stem cells and eventually initiate disease. In support of this, repeated exposure of cultured stem cells to toxic stress and metals has been shown to promote differentiation at the expense of an accumulating stem cell pool, induce abnormal cell signaling and global proteomic alterations analogous to those observed in transformed cells, acquire multiple tumor cell characteristics, and lead to an enrichment of cancer stem cells (51, 92-94).

II. ENVIRONMENTAL TOXINS

A. Aldehydes and Alcohols

Carbonyl compounds are stable intermediates of photochemical oxidation of most hydrocarbons and are the precursors to free radicals and ozone; thus environmental exposure can be pervasive. Higher levels of reactive aldehydes such as acetylaldehyde and formaldehyde have been measured in ambient air samples of urban communities and are linked to toxicity, mutagenicity, and carcinogenicity (95-99) (Figure 1). Exposure to ozone with exercise results in ozonation of lipids to produce aldehydes in the airway epithelial lining fluid of humans (100). Reactive aldehydes and acetaldehyde are also by-products of endogenous cellular metabolism and found to have genotoxic effects. Bone marrow failure in Fanconi anemia may result in part from aldehyde-mediated genotoxicity in the

hematopoietic stem and progenitor cell pool. In support of this, mouse hematopoietic stem and progenitor cells are more susceptible to acetaldehyde toxicity as compared to mature blood precursors (101). Hematopoietic stem cells from $Aldh2^{-/-}$ $Fancd2^{-/-}$ mice that are deficient in the Fanconi anemia pathway-mediated DNA repair and in endogenous acetaldehyde detoxification undergo a > 600-fold reduction in numbers, display a predisposition to leukemia, and require Aldh2 for protection against acetaldehyde toxicity (101). Another endogenous source of acetaldehyde is as the first product from breakdown of alcohol in cells. It has been previously proposed that acetaldehyde generated from alcohol metabolism reacts in cells to generate DNA lesions that form inter-strand crosslinks (ICLs) (102). Since the Fanconi anemia-breast cancer associated (FANC-BRCA) DNA damage response network plays a crucial role in protecting cells against ICLs, Marietta et al. tested the proposed role of acetaldehyde in generating ICLs (103). They exposed human lymphoblastoid cells from normal individuals, an XPA patient, an FA-G patient and an FA-A patient to acetaldehyde and studied the activation of the FANC-BRCA network. Their study reported that acetylaldehyde in a dose range of 0.1–1mM stimulates FANCD2 monoubiquitination, BRCA1 phosphorylation at Ser1524, and γH2AX at Ser139 in a dose-dependent manner. These results demonstrate interplay between multiple DDR networks and may also support differential tissue specificity of alcohol-related carcinogenesis (103). The data also support findings of alcohol and increased breast cancer risk. Chronic exposure to ethanol induces DNA damage and an induction in the levels of the Fanconi anemia D2 (FANCD) protein in both human neural precursor SH-SY5Y cells in culture and in the midbrain of C57BL/6J mice *in vivo* (104). FANCD2 response induced by alcohol thus plays a role in DDR in post-mitotic neurons and in neural precursor cells.

Alcohols and aldehydes are linked with altered histone H3K9 acetylation (H3K9ac) and altered cellular differentiation in bone marrow stem cells, cardiac progenitor cells, and hepatocytes (105-110). A genome-wide reduction in H3K9 acetylation typically occurs during human embryonic stem cell differentiation, and HDAC activity is required for embryonic stem cell differentiation (80, 111). In cardiac progenitor cells, low levels of ethanol, acetaldehyde, and acetate promoted a >2-fold increase in histone H3K9 acetylation (H3K9ac) without impacting the proliferation of cells consistent with maintenance of a progenitor state (112). High concentrations sufficient to produce a 30% reduction in cell viability also increased H3K9ac by >5-fold (112). In addition, high concentrations significantly elevated the expression of GATA4 and Mef2c genes related to heart development, resulting in their impaired differentiation (112). Consistent with these findings, the deregulation of genes that play a role in heart development has been proposed to be one of the mechanisms for the occurrence of congenital heart disease due to alcohol exposure during pregnancy (113).

Occupational and environmental exposures to formaldehyde are prevalent. Its production is carried out on a large scale in the manufacture of resins, particleboard, plywood, leather goods, paper, and pharmaceuticals. Formaldehyde is known to have genotoxic and mutagenic potential. It has been demonstrated that formaldehyde induces genotoxicity by causing DNA-protein crosslinks (114). In addition, lysine residues in the N-terminal tail and the globular fold domain of histone H4 have been identified as binding sites for formaldehyde in *in vitro* studies at concentrations from 5mM to 100mM suggesting another mechanism of formaldehyde through affecting epigenetic regulation (115).

B. Benzene and its metabolites

Benzene is a ubiquitous pollutant and is one of the top production chemicals in the United States (Figure 1). It is used in the manufacturing industry and is a combustion product of cigarette smoke. Benzene is carcinogenic and causes primarily hematopoietic cancers in humans (116, 117). It has been reported that it acts through its metabolites, especially 1,4-benzoquinone (1,4 BQ), as a strong topoII poison causing DNA DSBs (118). 25μM 1,4BQ *in vitro* stimulates DNA cleavage by topoII 8-fold at sites close to defined chromosome breakpoints in leukemia. Benzene metabolites 1,4-BQ (1-10μM) or 1,4-HQ (10-100μM) cause DNA damage and fragmentation in cultured HL60 cells though the generation of H_2O_2 oxidative stress leading to apoptosis (119). Benzene and its metabolites including benzoquinone also influence the downstream DNA repair of DSBs. As little as 1μM benzoquinone was sufficient to increase homologous recombination repair by 2.7-fold in a Chinese hamster ovary (CHO) cell line containing a *neomycin* gene direct repeat recombination substrate (120). Global genomic hypomethylation is a common event in cancer and frequently observed in hematopoietic malignancies, including leukemia. Gasoline station attendants are exposed to benzene fumes, and this exposure has been thought to lead to higher rates of lymphatic and hematopoietic cancers (121). In support of this, one study has shown a 1.6% decrease in global DNA methylation of these workers suggesting an epigenetic mechanism of benzene action and cancer (122).

Studies have extended earlier cell culture studies to *in vivo* mouse models showing alterations in epigenetic marks and developmental reprogramming. Neonatal exposure to 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene resulted in activation of constitutive androstane receptor (CAR) and a permanent increase of H3K4 mono-, di-, and trimethylation (H3K4me, H3K4me2, H3K4me3) and decrease of H3K9 trimethylation (H3K9me3) within the Cyp2B10 locus (123). These epigenetic changes were maintained in mice throughout life and resulted in a permanent change of liver drug metabolism (123). Taken together, the *in vitro* and *in vivo* studies provide further support for the interplay between the DDR, DNA repair, and long-term chromatin remodeling.

C. Trace Metals

Trace amounts of metallic compounds are pervasive in the environment (Figure 2). They are present in air, water, and food, and occupational exposure to them may occur through industrial production and waste disposal. Several studies have determined that trace metals cross the placenta (124-129), and presence of Cd, Cu, Cr, Ni, Pb, and Zn in placentas correlates with response of biomarkers metallothioneins, delta-amniolevulinic acid dehydratase, and lipid peroxidation (130). Consistent with environmental exposure to these elements, both levels of metals and biomarker responses were statistically significantly related to maternal dietary habits, consumption of canned food and bottled mineral water, as well as smoking (130). Similarly, animal models have shown pregnant C57BL6/J mice administered sodium arsenite in drinking water result in dose-dependent accumulation in newborn pups (131). Trace metals elicit pleiotropic biochemical and physiological effects such as mimicry of binding in protein active sites, oxidative changes in lipid, proteins or DNA, impaired transfer of nutrients to the fetus, low birth weight and developmental delay. The variation of chemical properties and reactive toxicities of each indicates that a uniform mechanism of action for all toxic metals is unlikely.

Recent reports have demonstrated trace quantities of metals directly promote overall histone production, specific epigenetic modifications, and heritable changes in gene expression (132). Most importantly, these changes have been shown to occur in stem cells, potentially being transgenerational. Dimethylation of H3 leads to gene silencing (133), and multiple metal exposures have been linked to this phenomenon. Zn can modulate overall histone gene expression and possibly mediate Zn effects on chromatin regulation (134). Treatment of human mononuclear THP-1 cells with 50µmol/L $ZnSO_4$ for 40 hours produced decrease of H2B transcription by 1.58 fold. Conversely, Zn deprivation by treatment with 2.5 µmol/L of the membrane permeable Zn chelator TPEN led to a 4.38-fold increase in H2B transcription. Exposure to Cd, Cr, Hg, and Ni leads to global changes in DNA methylation and histone modifications (132). Acute *in vitro* exposure of mouse embryonic stem cells to As, Cd, Hg and Ni led to a more than 50% decrease in H3K27 monomethylation (H3K27me) suggesting a global induction of transcriptional repression (135). Ni ion exposure at 250µM or higher increased global H3K9me and H3K9me2 by 2-3 fold in a time-dependent manner in cell lines of different lineages including mouse embryonic stem cells and embryonic fibroblast cells, and human lung carcinoma, osteosarcoma, and embryonic kidney cells (136). Further, Ni ions induced *gpt* transgene silencing and exhibited inhibition of H3K9 de-methylation, that led to, or permitted, the observed increase in H3K9me2 (136). Analysis of peripheral blood mononuclear cells of humans with occupational exposure to Ni suggests that chronic exposure leads to further epigenetic changes *in vivo* as these samples showed an increase in H3K9me3 (137). Interestingly, epigenetic alterations by metal exposure may be gender specific as peripheral blood mononuclear cell analysis of women exposed to As in drinking water supply demonstrated and increase in H3K9me2 and decrease in H3K9ac, both marks of repression, but led to opposite changes in similarly exposed men (137).

Low concentrations of trace metals are also sufficient to induce multiple cellular effects. Prolonged *in vitro* exposure of mouse embryonic stem cells to low concentrations ($< IC_{50}$) of As, Cd, Cu, Pb, Li, Hg and Ni led to decreased cell proliferation, altered expression of cell differentiation markers *Oct-4* and *egfr*, altered expression of DNA repair genes *Rad-18*, *Top-3a*, and *Ogg-1*, and overall decreased total histone protein production (135).

As a downstream result of transcriptional silencing by alterations in epigenetic marks, exposure leads to defects in cellular differentiation pathways. The As derivative arsenite suppresses expression of cellular differentiation markers to inhibit signaling pathways, maintain proliferative ability, and suppress differentiation of keratinocyte progenitor cells as well as transform human prostate epithelial progenitor cells to a cancer stem-cell phenotype (138-141). In one study, SCC9 human squamous carcinoma cells that exhibit a keratinocyte progenitor cell phenotype were stably transfected with constructs containing the proximal human involucrin promoter, wild-type or mutated at both AP1 sites, were examined for their transcriptional activity using luciferase reporter activities with and without treatment with arsenate. Notably, effects were detectable with a nontoxic concentration within the range of environmental exposures (2µM sodium arsenate or sodium arsenite). As one marker of inhibition of differentiation, arsenite resulted in a significant reduction of c-Fos transcription factor and of acetylated H3 at the proximal and distal AP1 response elements of the involucrin gene promoter and of coactivator p300 at the proximal element of the involucrin gene promoter, as shown by chromatin

immunoprecipitation (chIP) studies. Treatment with arsenite led to a dramatic suppression in the transcriptional activity of the involucrin gene to 2% of the level observed in the absence of any treatment.

Studies have extended cell culture studies to examine long-term impact *in vivo*. Exposure of C57Bl6/J mice to 100μg/L arsenic in drinking water from 1 week before conception until birth resulted in offspring with global H3K9 hypoacetylation, changes in functional annotation with highly significant representation of Krüppel associated box transcription factors in brain samples, and long-term memory impairment as compared to unexposed controls (142). Timed-pregnant Long–Evans hooded rats exposed to 200 ppm Pb-acetate in deionized drinking water during pregnancy delivered offspring with age-related neuropathological characteristics analogous to those seen in Alzheimer's disease (AD). These characteristics were accompanied by changes in the methylation patterns of key AD genes (143). Continued exposure to Pb during the postnatal period resulted in a transient increase in beta-amyloid precursor protein (APP). mRNA expression during the first month after birth followed by a return to basal levels by one year, but surprisingly a subsequent delayed overexpression at 20 months after exposure to Pb had ceased. These data suggest that environmental influences occurring during brain development predetermined methylation patterns, gene expression, and regulation of APP later in life, potentially altering the course of amyloidogenesis. These studies support the fetal basis of adult disease (FeBAD) hypothesis which states that many adult diseases have a fetal origin (144-148). Injury or environmental influences occurring at critical periods of organ development in the fetus at early stages of cell differentiation could lead to alterations in gene expression or gene imprinting which can result in "programmatic" changes in gene expression and functional deficits evident later in life.

Epidemiological studies have well documented metals as human carcinogens associated with skin, lung, liver, and bladder cancers; however the underlying mechanisms have not been clear. Cancer incidence increases with chronic exposure to metals such as As, Cd, Cr, and Ni (149-152). Studies associate arsenic exposure to multiple cancer types in human subjects and gene-specific DNA hypermethylation (153-157). This direct link between arsenic, tumorigenesis, and hypermethylation was further documented by low dose (0.5μM) exposure to arsenic trioxide (As(2)O(3)) that led to transformation of BALB/c 3T3 cells, and dramatic tumor growth increase of these cells in a xenograft mouse model (158). Further, these cells exhibited activated polycomb group proteins BMI1 and SUZ12, increased H3K27me3, and suppression of p16 and p19 that could be rescued by shRNA to either BMI1 or SUZ12 (158).

III. DIETARY EXPOSURES AND SUPPLEMENTS

A. Bisphenol A and other estrogens

Bisphenol A (BPA) is a hormonally active environmental xenoestrogen widely used in the production of polycarbonate plastics and resins, including some dental composites (Figure 3). Human exposure to BPA comes mainly from daily diet, as it leaches from food and drink packaging, water pipes, and dental sealants (159). BPA can cross the placenta, and has been detected in urine, amniotic fluid, maternal and fetal plasma, placental tissue, and breast milk of lactating mothers (159-162). Estrogens are both natural hormones produced

in the body and widely used in hormone supplement therapy. 17β-estradiol is an endogenous estrogen. Genistein is a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas (163, 164) (Figure 3). Genistein and other estrogen derivatives are also available at health food stores as dietary and menopausal supplements (165). The validated and widely used embryonic stem cell test for toxicity (166, 167) demonstrated that exposure to BPA or genistein significantly altered genome wide methylation patterns and decreased embryonic stem cell to cardiomyocyte differentiation (168, 169). Further, the combination of BPA and genistein had a synergistic effect at lower concentrations similar to those observed in human blood or sera (168).

The epigenetically toxic effects of environmental chemicals like BPA and phthalates include DNA methylation, histone modification, and changes in microRNA expression levels (7). Some of these effects have been found to be transgenerational. Numerous studies show that exposure to xenoestrogens can developmentally reprogram multiple organ systems. Differences in the ability of xenoestrogens to induce developmental reprogramming are likely driven by intrinsic differences in their binding to specific estrogen receptor subtypes. In the female reproductive tract, exposure is associated with alterations in morphology, hormonal response, and gene expression, and promotes diverse outcomes such as obesity and cancer later in life (170-172). BPA is an endocrine disruptor causing an adverse effect on mammalian reproduction due to impaired development of germ cells. BPA has been reported to play a role in modulating germ cell differentiation, retinoic acid signaling, and the expression of germ cell marker genes in mouse embryonic stem (ES) cells (173). After 50μM BPA, up-regulation of meiotic entry gene *Stra8* (20-fold), up-regulation of ovarian markers *Foxl2* and *Wnt4* (15-20 fold), and suppression of testicular markers *Sox9* and *Fgf9* were detected showing that in addition to germ cell differentiation, BPA also affects testicular and ovarian development. BPA dosing C57BL/6J pregnant mice from embryonic day 8.5 to 13.5 accelerated neurogenesis in the developing neurocortex and decreased the number of detectable neural stem/progenitor cells (174). Animal studies have also reported that postnatal exposure to BPA accelerates neurogenesis and causes neuronal migration defects which impair neurocortex development in embryos (175). BPA modulates adipogenic differentiation of cultured human primary adult stem cells (176) and suppresses adipogenic differentiation of mouse mesenchymal stem cells (177).

Physiologically relevant doses of BPA or estradiol have been reported to increase susceptibility to adult-onset prostate precancerous lesions and hormonal carcinogenesis. This imprinting involves epigenetic changes such as permanent alterations in the DNA methylation patterns of multiple cell signaling genes (178). Developmental exposure to estradiol and BPA leads to an increase in the susceptibility to prostate carcinogenesis with aging through epigenetic regulation (178). In normal prostates, gradual methylation occurs within the specific genomic cluster containing the gene for phosphodiesterase type 4 variant 4 (PDE4D4) which is an enzyme responsible for cyclic AMP breakdown. This methylation is associated with decreased expression. By contrast, neonatal Sprague-Dawley rat exposure to BPA (10μg/kg) or 17β-estradiol 3-benzoate (2500μg/kg or 0.1μg/kg) resulted in early and prolonged hypomethylation at this site and continued, elevated PDE4D4 gene expression throughout life, consistent with observed

hypomethylation of this gene in prostate cancer cells. Several genes showed methylation changes in response to neonatal estrogen treatments, many of which are permanent.

Estrogens have also been linked to the generation of DNA DSBs or inhibition of their repair. Exposure of primary gingival fibroblasts to dental adhesives containing BPA derivatives produced increased numbers of DNA breaks, marking of damaged chromosomes with γH2AX, alterations in cell cycle profiles, and slower kinetics of repair (179-181). Sensitivity to BPA derivatives may be global as exposure of keratinocytes, skin fibroblasts, intestinal cells (line LS174T), and hepatoma cells (line HepG2) all produce marking of damaged DNA by γH2AX (182, 183).

B. Bioflavonoids as topoisomerase II inhibitors

Bioflavonoids comprise a diverse group of polyphenolic compounds divided into three main groups: flavones, flavonols, and isoflavones (Figure 3). The most common sources of these bioflavonoids are fruits, vegetables, soy, tea, coffee and wine (184). Genistein is both an estrogen derivative (discussed above) available at health food stores as dietary and menopausal supplements (165), and a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas (163, 164). Thus, its effects in cells are thus likely pleiotropic acting through both mechanisms. Due to their antioxidant capacity, flavonoids are used for their presumed health benefits such as protection against cardiovascular diseases, cancer and inflammation. Flavonoid supplements are available worldwide over-the-counter in pharmacies and drugstores.

However, accumulating evidence indicates that dietary flavonoids are potent inhibitors of topoisomerase II (topoII α and topoII β in mammalian cells (185, 186)). DNA topoisomerases are essential cellular enzymes that cause topological changes in the DNA for processes such as replication and transcription. Inhibitors of topoII block the religation of the transient DSBs made by topoII, leading to cell death at high doses but also potentially leading to illegitimate repair, genome instability, and chromosomal abnormalities in surviving cells. The chemotherapeutic agents etoposide, doxorubicin, daunorubicin and mitoxantrone (185, 187) are well-characterized topoII inhibitors that are typically used as comparison controls in testing of other potential inhibitors. Etoposide is composed of a polycyclic ring system (rings A-D), a glycosidic moiety at the C4 position, and a pendant ring (E ring) at the C1 position (188-190). The binding of etoposide to topoII is driven by interactions with the A-ring and the B-ring (189) while the E-ring is important for etoposide function (190). Etoposide has been reported to initiate *MLL* rearrangements in mouse embryonic stem cells (191), primitive hematopoietic stem cells and in human fetal hematopoietic stem cells in several studies (192-195). Other anti-cancer agents including teniposide, anthracyclines and dactinomycin also are associated with *MLL* rearrangements due to topoII inhibition and enhanced DNA cleavage leading to defective DNA repair and chromosomal translocations (196).

Multiple bioflavonoid compounds are also polyphenolic ring structures and thus biochemically and mechanistically may act similarly to etoposide (186). Etoposide, genistein and quercetin contain pendant rings that feature a 4'-OH group that is essential for drug action (186, 197, 198). The 5'-OH group of genistein plays an important role in mediating binding to topoII, and the 4'-OH plays a significant role in function (186).

TopoII inhibition by bioflavonoids was investigated in an *in vitro* plasmid DNA cleavage assay using purified recombinant wild-type human topo IIα and IIβ where it was shown that these compounds were active against topoIIβ (186). Genistein (50μM) was shown to be the most effective of the bioflavonoids tested and stimulated enzyme-mediated DNA cleavage ~10-fold (186). 100μM genistein efficiently induced topoII-DNA cleavage complexes in both cultured mouse myeloid progenitor cells (32Dc13) and Top2β knockout mouse embryonic fibroblasts (MEFs), and it was suggested that these complexes are processed by proteasome which led to chromosome rearrangements (199). Cultured human lymphocytes treated with 50μM genistein display chromosome abnormalities in metaphase karyotypic analyses (200). DSBs with the *MLL* gene breakpoint cluster region were induced by bioflavonoid exposure both in primary human progenitor hematopoietic cells from healthy newborns and adults as well as in hematopoietic progenitor cell lines (BV173 and K562) (201). Quercetin, genistein and kaempferol induced DSBs in primary human hematopoietic CD34+ stem cell-enriched cells (at 25μM and 50μM doses) (202). Besides chromosomal translocations, monosomy or trisomy of *MLL* was also reported in quercetin-exposed cells (202).

Importantly, synthetic flavonoids are able to cross the placenta in the rat and are found in all fetal tissues (17% of the initial dose) including fetal brain (203). Maternal and fetal distributions of a synthetic radioactively labeled bioflavonoid EMD-49209 were detectable 1-24 h after intravenous injection into pregnant Wistar rats. Transplacental exposure to high but biological amounts of the flavonoids genistein and quercetin in *Atm*-ΔSRI mutant mice with an impaired capacity for DSB repair led to the Inverse PCR detection of two-fold higher number of *MLL* rearrangements compared with their wild-type siblings (184). Parallel *in vitro* studies with bone marrow cells exposed to genistein (50μM) or quercetin (50μM) showed 2.1-5.0 rearrangements/80ng genomic DNA (1 per 13,000 cells) for quercetin or genistein as compared to 0.2 translocations/80ng genomic DNA for wild-type cells. Thus, the risk of these rearrangements due to *in utero* exposure to these bioflavonoids increases in the presence of compromised DNA repair, although in that study *MLL* rearrangements were detectable in all samples regardless of diet or mutational status.

The epigenetic and transgenerational effects of these dietary compounds were addressed in a study that showed that exposure of progeny to genistein through maternal diet during pregnancy can have long lasting effects on resultant progeny (204). Mice (129/SvJ:C57BL/6J background), ~8 wk of age were given genistein (270mg/kg of feed) from conception until birth. Genistein induced epigenetic changes and altered the coat color of agouti mice. Progeny mice had a significant down-regulation of genes involved in hematopoiesis in bone marrow cells, increased erythropoiesis, and a permanent signature hypermethylation of repetitive elements in the hematopoietic lineages. Thus prenatal exposure to genistein affected epigenetic signature of hematopoietic cells and caused long lasting alterations in gene expression.

Exposure to the flavonoid quercetin during pregnancy can result in the long-term changes in iron homeostasis at adulthood (205). Quercetin is a strong iron chelator and has the ability to cross the placenta and accumulate in the fetus. In this study female mice (129/SvJ:C57BL/6J background) were given quercetin (302mg/kg feed) from 3 days before conception until the end of gestation. Mice prenatally exposed to quercetin had an

upregulated iron-associated cytokine expression and significantly increased iron storage in the liver (~94ng/mg for quercetin exposure versus ~62ng/mg for control). Quercetin exposure was associated with hypermethylation of repetitive elements and these epigenetic modifications could cause these long-term changes in cytokine gene expression. All of these changes led to a shift towards a higher expression of cytokines associated with inflammation in the liver of adult mice that were prenatally exposed to quercetin.

Quercetin has also been shown to affect xenobiotic metabolism of chemical carcinogens in mice that were prenatally exposed to this compound (206). Mice (129/SvJ:C57BL/6J background) were given quercetin (1mmol or 302mg/kg of feed) from 3 days before conception until the end of gestation. Quercetin-exposed mice showed altered biotransformation of the environmental pollutant benzo[a]pyrene. This occurred due to altered gene expression of the metabolic enzymes such as Cyp1a1, Cyb1b1, Nqo1 and Ugt1a6 which persisted into adulthood in a tissue- and gender-dependent manner. These long lasting changes were associated with epigenetic alterations since prenatal quercetin exposure led to hypomethylation of repetitive elements SINEB1. These persistent alterations in the metabolic enzymes of adult mice may affect cancer risk due to environmental chemical carcinogens.

IV. CONCLUDING REMARKS

The exposure to environmental and dietary agents discussed above is widespread today. Due to the beneficial effects assumed to be associated with the use of bioflavonoids, their use as dietary supplements is increasingly popular and widespread. However, a growing body of evidence is emerging regarding the long-term implications and adverse effects of using these compounds in an unrestricted manner. Multiple environmental toxins and dietary agents have the potential to cause long-term epigenetic changes leading to dysregulation of multiple cellular functions and developmental pathways. Epigenetic modulation, cell differentiation, gene expression, signal transduction and illegitimate DNA repair are all associated with human diseases and cancer (207). Importantly, epigenetic alterations following exposure will continue to impact cellular development long after exposure has ceased. In view of the variety of adverse effects these agents have in exposed individuals, it is important to raise public awareness, set guidelines and regulate the use and market availability of such compounds to reduce the risk of disease. Since the role of *in utero* exposures in causing long-term transgenerational effects has been demonstrated to be critical, it is important to address the susceptibility of different stages of cell differentiation to the deleterious molecular changes induced by these agents. However, a system to directly and rapidly examine the role of a large number of different compounds, both individually and in combination, in inducing the genetic/epigenetic changes discussed above has been lacking and thus testing has been limited to small isolated studies. Even the inherent genomic instability of existing stem cell lines or the memory of previous epigenetic codes of iPSCs suggest that they may not always mimic *in vivo* consequences. Development of model systems for cells at different stages of differentiation with different susceptibilities to epigenetic alterations will provide useful insights into the comparative risk to human populations and how the timing or stage of development may impact *in vivo* consequences to individuals.

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APPENDIX E: DOUBLE-STRAND BREAK REPAIR BY INTERCHROMOSOMAL RECOMBINATION: AN IN VIVO REPAIR MECHANISM UTILIZED BY MULTIPLE SOMATIC TISSUES IN MAMMALS

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Double-Strand Break Repair by Interchromosomal Recombination: An *In Vivo* Repair Mechanism Utilized by Multiple Somatic Tissues in Mammals

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Abstract

Homologous recombination (HR) is essential for accurate genome duplication and maintenance of genome stability. In eukaryotes, chromosomal double strand breaks (DSBs) are central to HR during specialized developmental programs of meiosis and antigen receptor gene rearrangements, and form at unusual DNA structures and stalled replication forks. DSBs also result from exposure to ionizing radiation, reactive oxygen species, some anti-cancer agents, or inhibitors of topoisomerase II. Literature predicts that repair of such breaks normally will occur by non-homologous end-joining (in G1), intrachromosomal HR (all phases), or sister chromatid HR (in S/G_2). However, no *in vivo* model is in place to directly determine the potential for DSB repair in somatic cells of mammals to occur by HR between repeated sequences on heterologs (i.e., interchromosomal HR). To test this, we developed a mouse model with three transgenes—two nonfunctional green fluorescent protein (GFP) transgenes each containing a recognition site for the I-*Sce*I endonuclease, and a tetracycline-inducible I-*Sce*I endonuclease transgene. If interchromosomal HR can be utilized for DSB repair in somatic cells, then I-*Sce*I expression and induction of DSBs within the GFP reporters may result in a functional GFP+ gene. Strikingly, GFP+ recombinant cells were observed in multiple organs with highest numbers in thymus, kidney, and lung. Additionally, bone marrow cultures demonstrated interchromosomal HR within multiple hematopoietic subpopulations including multi-lineage colony forming unit–granulocyte-erythrocyte-monocyte-megakaryocte (CFU-GEMM) colonies. This is a direct demonstration that somatic cells *in vivo* search genome-wide for homologous sequences suitable for DSB repair, and this type of repair can occur within early developmental populations capable of multi-lineage differentiation.

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Introduction

Faithful repair of DNA damage, including double-strand breaks (DSBs), is crucial to genome stability and normal cell survival and proliferation [1]. Chromosomal breaks can occur in a programmed manner through meiosis, immunoglobulin class-switch recombination, and V(D)J recombination [2–4]. In addition, reactive oxidative species may promote 10,000-20,000 DNA damaged sites per cell per day [5–7], and DNA replication errors or stalls may promote another 10-50 DSBs per cell [8,9]. Exposure to ionizing radiation (IR), alkylating agents, and chemotherapeutic drugs such as topoisomerase II inhibitors also promote chromosomal breaks [10–14]. Some environmental and/or dietary compounds may promote DSBs, and the recent observations that bioflavonoids can stabilize DNA DSBs and lead to illegitimate repair and genome rearrangements in cultured cells underscores the importance of understanding DSB repair processes *in vivo* [15–18].

DSBs are potent inducers of recombination and increase both homologous recombination (HR) and non-homologous end-joining (EJ) events by several orders of magnitude [19,20]. These two major DSB repair pathways differ based on their requirement for a donor DNA template with significant

sequence homology; thus, their relative activity changes with each stage of the cell cycle. Studies in multiple organisms have demonstrated that EJ is most efficient in G1 and in noncycling somatic cells while homology-directed DSB repair is favored in both S/G^2 utilizing a sister chromatid and intrachromosomal HR [19,21–26]. *In vivo* systems have been developed to detect EJ, sister chromatid, and intrachromosomal HR that arise both spontaneously and in response to induced DSBs [27–30]. Homologs are utilized for HR-directed DSB repair with lower efficiency although this is increased in organisms that exhibit a high degree of mitotic pairing, supporting the hypothesis that proximity of homologous sequences is an important factor in determining template choice [31–33]. While repair of specific DSBs by more distant homologous repeat sequences on heterologous chromosomes (i.e. interchromosomal HR) has been examined *in vivo* using mitotic yeast and tobacco [34,35], studies in mammalian cells have been limited to cultured cell assays [36–39]. Whether repair of DSBs *in vivo* in mammals occurs by interchromosomal HR at significant and detectable frequencies has not been demonstrated.

If cells are exposed to irradiation, chemotherapeutic agents, or even environmental factors and metabolites, multiple DSBs at unlinked loci will occur in the same cell at the same time. Repair of multiple breaks using interchromosomal HR *in vivo* has the potential to result in reciprocal exchanges that may be viable, inherited by daughter cells in the next cell division, or inherited through the germ line. Genome analysis of plants suggests that translocations are a regular mechanism of plant evolution [40,41]. In mammals, one third of the genome is composed of repetitive elements [42]. The presence of Alu elements elevates recombination rates [43], and Alu-Alu mediated recombination has been associated with founder mutations and evolution [44–49]. In somatic cells, translocations can be tumorigenic, and are a hallmark of human hematopoietic malignancies and some soft-tissue sarcomas [36,50–56]. Thus, such events would likely be suppressed in somatic cells *in vivo* where a selective pressure exists to maintain genome stability and avoid immortalization. Specialized cell types within mammals may preferentially utilize different pathways of repair, particularly as more differentiated cells spend less time in S phase of the cell cycle [57–60] or as proliferation rates change with age [61,62].

To directly test the potential for multiple DSBs to promote interchromosomal HR *in vivo* in mammals, we developed a mouse model with three transgenes--two nonfunctional green fluorescent protein (GFP) reporter transgenes each containing a recognition site for the I-*Sce*I endonuclease, and a tetracycline-inducible I-*Sce*I endonuclease transgene. Induced expression of I-*Sce*I and the resulting induction of DSBs within the GFP reporters may produce a functional GFP gene if interchromosomal HR is utilized for repair. In this system, GFP + recombinant cells were observed in all seven organs examined--pancreas, liver, spleen, kidney, thymus, heart, and lung–with highest numbers in thymus, kidney, and lung. Bone marrow cultures demonstrated interchromosomal HR within multiple colony types including early progenitor CFU-GEMM. This is a direct demonstration that somatic cells *in vivo* maintain the potential to search genome-wide for homologous sequences suitable for DSB repair, and this type of repair can occur within progenitor populations capable of proliferation and multi-lineage differentiation.

Results

In vivo mouse model

Constructs were designed to introduce two defective green fluorescent protein (GFP) genes and a tetracycline-responsive (TET-ON) inducible I-*Sce*I endonuclease gene construct onto heterologous chromosomes in the mouse genome. 1S-GFP and 2S-GFP reporter constructs each contain a unique 18bp restriction site for the endonuclease I-*Sce*I [63,64] in the 5' and 3' ORF regions, respectively, with 460bp homology to each other between the two restriction sites (Figure 1A). The TET-ON I-*Sce*I endonuclease gene is on a single auto-regulated bi-directional expression vector with the tet operator regulating both a TK-rtTAN repressor of the transactivator gene (vector kindly provided by Craig Strathdee) [65] and an I-*Sce*I gene (Figure 2A) [64,66]. Presence of the transgenes within mice was shown by both Southern Blotting and PCR of DNA isolated from tail tips. Founder mice containing each transgene were crossed with wild type, and those that inherited single insertion sites at Mendelian ratios and with the lowest copy number as estimated by both Southern blotting and Q-PCR as compared against a standard (Figure 1B and Methods) were maintained for further breeding. Taken together these analyses estimated 4-5 copies of 1S-GFP and 2-4 copies of 2S-GFP. Mice were screened for an intact I-*Sce*I site at both the 1S-GFP and the 2S-GFP reporters using PCR primers that flank each I-*Sce*I site and digestion of the PCR product with I-*Sce*I endonuclease (Figure 1A, 1C). Individually 1S-GFP and 2S-GFP positive lines were crossed to each other, and then crossed to the I-*Sce*I transgenic line over generations, and inheritance of the three transgenes in expected Mendelian ratios supports unlinked loci. Breeding resulted in triply positive transgenic GS lines for analysis.

DSB-induced interchromosomal HR occurs in mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were harvested at day E13.5. MEFs from each GS mouse were divided and cultured in one of 3 conditions: (1) cultured in media without DSB induction, (2) cultured in the presence of tetracycline (2 μg/mL) to induce DSBs through I-*Sce*I expression, or (3) transfected with 30μg I-*Sce*I expression vector CBAS [20] to induce DSBs through I-*Sce*I expression. I-*Sce*I RNA transcripts and protein were detectable by RT-PCR and Western blotting, respectively, following addition of tetracycline to culture media of MEFs (Figure 2B) or to H_2O provided transgenic mice in subsequent experiments (see below).

Individual GFP+ MEFs were detectable by inverted fluorescent microscopy as early as 4 days following the addition of tetracycline (Figure 3A). Cells were analyzed by fluorescent activated cell sorting (FACS) 6-10 days post-tetracycline. Untreated MEFs had an undetectable number of GFP+ cells. By contrast, intermediate/bright GFP+ cells were greater than 12% of the treated cells (compared against

Figure 1

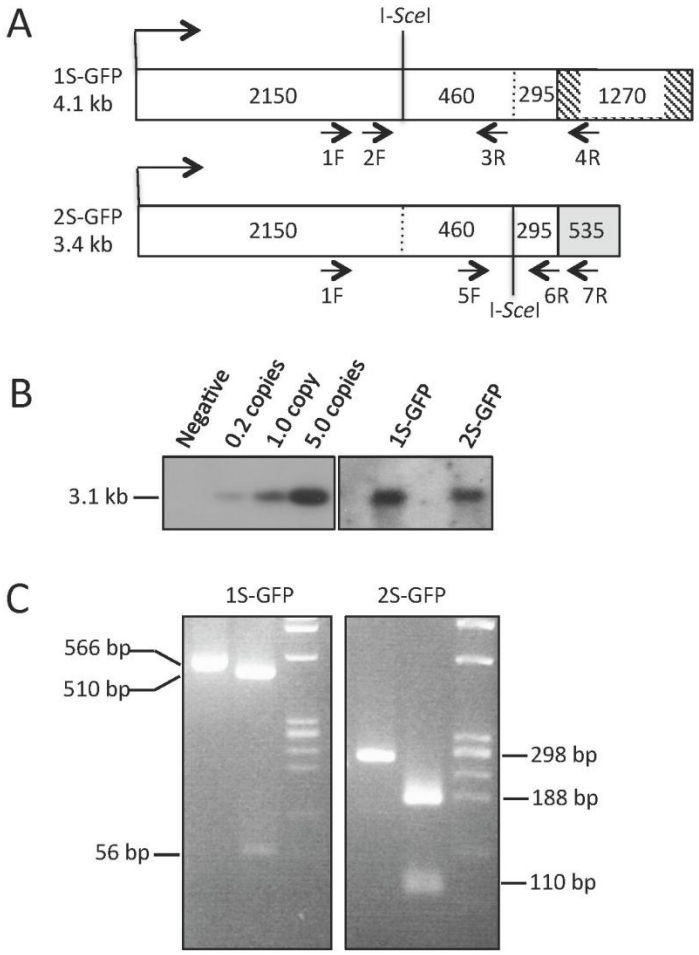

Figure 1. Structure and confirmation of the 1S and 2S GFP transgenes. (**A**) For each construct schematic, the numbers of bases are indicated to show the lengths of homology between the two as well as the relative positions of the engineered I-*Sce*I restriction sites. The 3'UTR sequences of the two constructs do not share homology and are indicated as a hatched box of 1270 bp for 1S-GFP and a grey box of 535 bp for 2S-GFP; these non-homologous sequences allow for PCR amplification specific to each transgene. Nested PCR primer pairs used for verification of intact construct sequences and for analysis of GFP+ hematopoietic colonies are indicated. Primers 1F-4R followed by 2F-3R amplify sequence flanking the I-*Sce*I site in 1S-GFP. Primers 1F-7R followed by 5F-6R amplify sequence flanking the I-*Sce*I site in 2S-GFP. (**B**) Southern blotting to estimate copy number utilized a GFP ORF DNA fragment of 3.1 kb and diluted to pg amounts that approximated 0, 0.2, 1.0, and 5.0 copies per genome spiked into 10µg non-transgenic mouse DNA. Genomic DNA from single transgenic mice (either 1S-GFP or 2S-GFP) was digested with restriction endonucleases within the GFP promoter and ORF of both transgenes to yield a 3.1 kb fragment. Band intensities are consistent with 4-5 copies of 1S-GFP and 2-4 copies of 2S-GFP, and were confirmed with Q-PCR data on the same samples (data not shown). (**C**) PCR reactions flanking each DSB site in the two GFP constructs confirm intact I-SceI recognition sites. Nested PCR as described in Materials amplified each transgene shown in the left side lane of each image. Digestion with I-*Sce*I endonuclease produced the expected sizes indicated in the middle lane of each image. Right side Marker lane PhiX.

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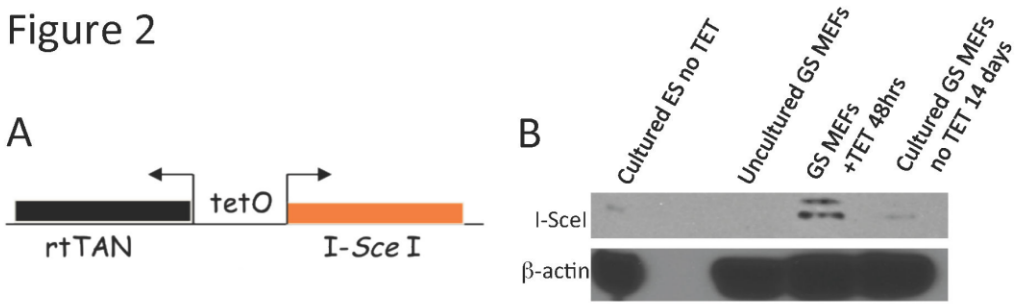

Figure 2. Structure and confirmation of the tetracycline inducible I-*Sce*I transgene. (**A**) For details of the bicistronic I-*Sce*I transgene construct refer to [65]. (**B**) MEFs derived from GS mice were cultured in media supplemented with TET at 2μg/mL for 48 hours. Total protein extracts were harvested and analyzed by Western blotting. By 48 hours post-TET, detectable quantities of I-*Sce*I endonuclease can be observed. As a negative controls, total protein extracts were harvested from cultured E14 ES cells or uncultured MEFs from GS mice. Loading control: Western blotting for β-actin.

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untreated cells with a gate set at 0.1%; n=12) (Figure 3B). Individual GFP+ cells were FACS sorted and confirmed to be GFP+ by inverted fluorescent microscopy (Figure 3C).

DSB-induced interchromosomal HR occurs *in vivo* in multiple somatic cell types

GS mice at least 3 months of age (n=47) were administered tetracycline through H_20 for 21d to allow an extended period of I-*Sce*I expression and subsequent induction of DSBs. Mice were then taken off tetracycline for 7d-21d prior to analysis. This waiting period would restrict analysis to viable GFP+ cells after cells with unstable repair structures would be cleared from the *in vivo* tissues. A total of seven organs--pancreas, liver, spleen, kidney, thymus, heart, and lung--were analyzed for GFP+ recombinants by FACS (Figure 4). Mice were analyzed in batches, and each batch included an age-matched non-transgenic mouse (n=8). Gates for determination of GFP+ cells were set such that negative controls had ≤3 events per million, and then the same gates were used to score GFP+ cells from GS tetracycline-treated mice. This analysis directly demonstrated that GFP+ cells, as determined by >3 GFP+ cells per million by FACS, were readily detectable in multiple tissues from 40 of the 47 mice treated and analyzed (Figures 4,5; Table S1). Despite variance in GFP+ numbers detected between mice, all organs had significantly increased GFP+ cells as compared to the age-matched negative controls (Figure 5). For comparison, constitutively expressing EGFP mice consistently contained >45% GFP+ cells in all tissues examined (data not shown) [67]. These data demonstrate that somatic cell types *in vivo* retain the potential to repair DSBs with a homologous sequence on a heterologous chromosome. Furthermore, the potential for interaction between sequences on heterologous chromosomes in wild-type cells has not been eliminated by epigenetic factors or chromatin remodeling associated with differentiation programs.

Additionally, age-matched GS mice that were not administered tetracycline were analyzed (n=15). 13 of 15 of these mice had undetectable levels of GFP+ cells in all organs examined, similar to the non-transgenic controls. However, two of the 15 mice contained GFP+ populations of cells in multiple tissues (data not shown). In these mice, it is possible that the I-*Sce*I transgene became activated. Alternatively, it is possible that an early progenitor cell *in utero* underwent spontaneous interchromosomal HR giving rise to a GFP+ progenitor cell that contributed to multiple tissues, or was a cell type that gave rise to cells capable of infiltrating multiple organs, e.g. circulating hematopoietic cells.

Impact of aging on DSB-induced interchromosomal HR in multiple somatic cell types

Close examination of the variance of numbers of GFP+ cells detected in tetracycline-treated GS mice indicated that 7 of the 47 mice contained no detectable GFP+ cells in any organs analyzed, similar to non-transgenic controls. All 7 mice were older. Thus, we separated analysis of the 47 mice GS mice into two age cohorts, young (≤ 5months old, n=16) and old (≥ 8 months, n=31) (Figures 6A and 6B, respectively). Regardless of age, statistically significant numbers of GFP+ cells were in most organs examined, as compared to negative control mice. Comparison of GFP+ cell numbers by age (Figure 6C) indicated that in 5 of the 7 organs examined (pancreas, kidney, spleen, lung, and thymus), overall numbers of detectable GFP+ cells were lower in the cohort of older mice (Figure 6C). The decrease in detectable number of GFP+ cells was significant in 3 of these (pancreas, lung, thymus). However, two organs (heart and liver) appeared to have an overall slight increase in numbers GFP+ cells in older mice, although the trend did not reach statistical significance. Decreases in transgene expression levels with age has been observed in multiple other models. A similar mechanism of transgene shutdown may be involved in this model, but only occur in a subset of tissue types. It is possible that certain organs contain specific cell types or progenitor cells capable of DSB-induced interchromosomal HR, even within older mice. Further

Figure 3

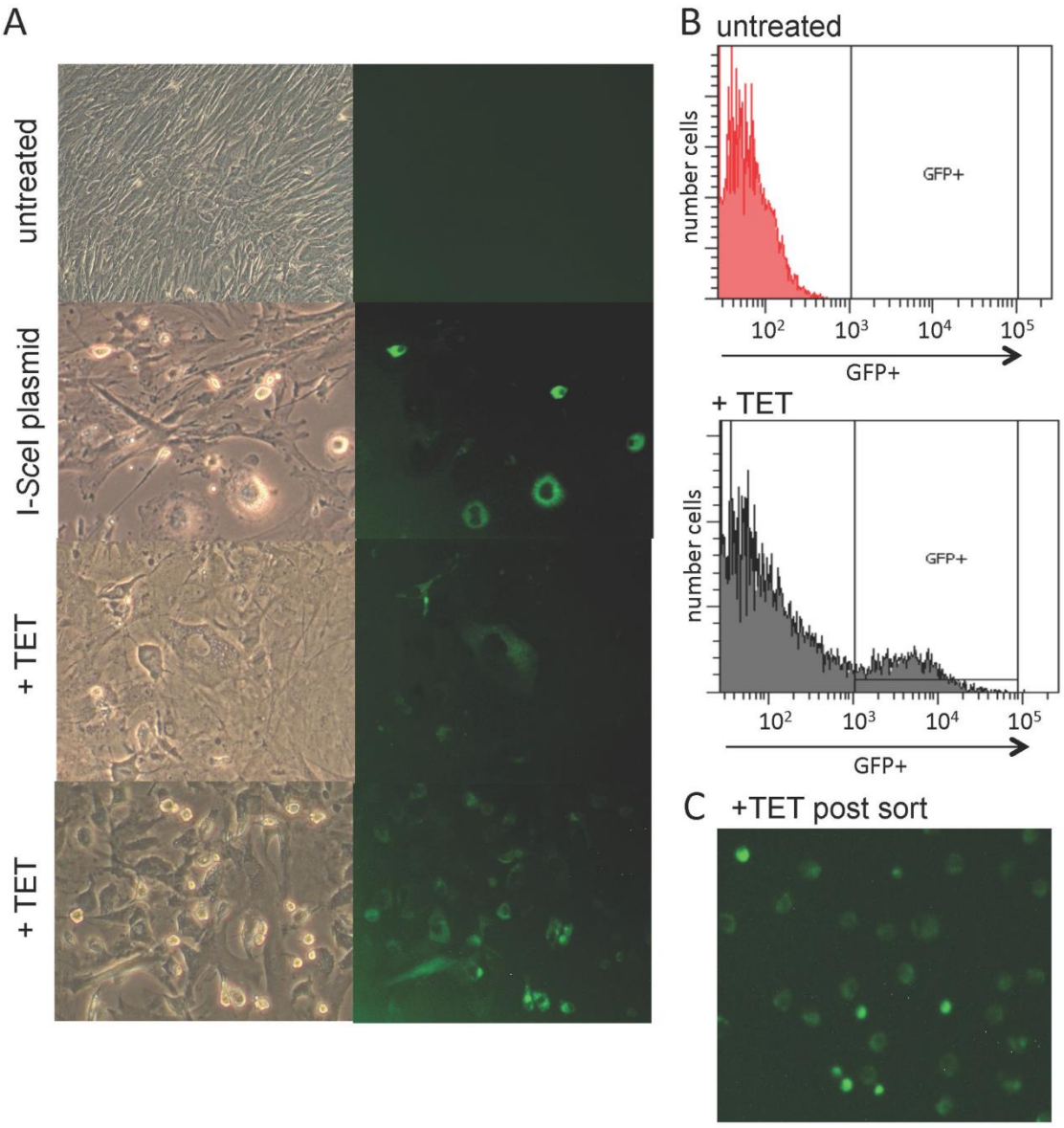

Figure 3. Analysis of GFP+ MEFs post-TET. (**A**) Phase contrast and matched fluorescent microscopy images of MEFs in culture —magnification 400X. Top row-untreated MEFs. Second row-96 hrs post-electroporation with I-*Sce*I expression plasmid. Third and fourth rows-96 hrs after addition of tetracycline to the culture medium (+TET). (**B**) Representative FACS plot of MEFs with GFP positivity in log scale on the x axis plotted against number of cells on the y axis. Upper plot--untreated MEFs. Lower plot-- +TET treated MEFs. In this sample, the GFP+ population is 12.4%. (**C**) Confirmation of GFP+ cells after FACS single cell sorting for GFP+ MEFs. Cells within the GFP+ gate indicated in B lower panel were sorted and then viewed by fluorescent microscopy—magnification 400X.

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Figure 4

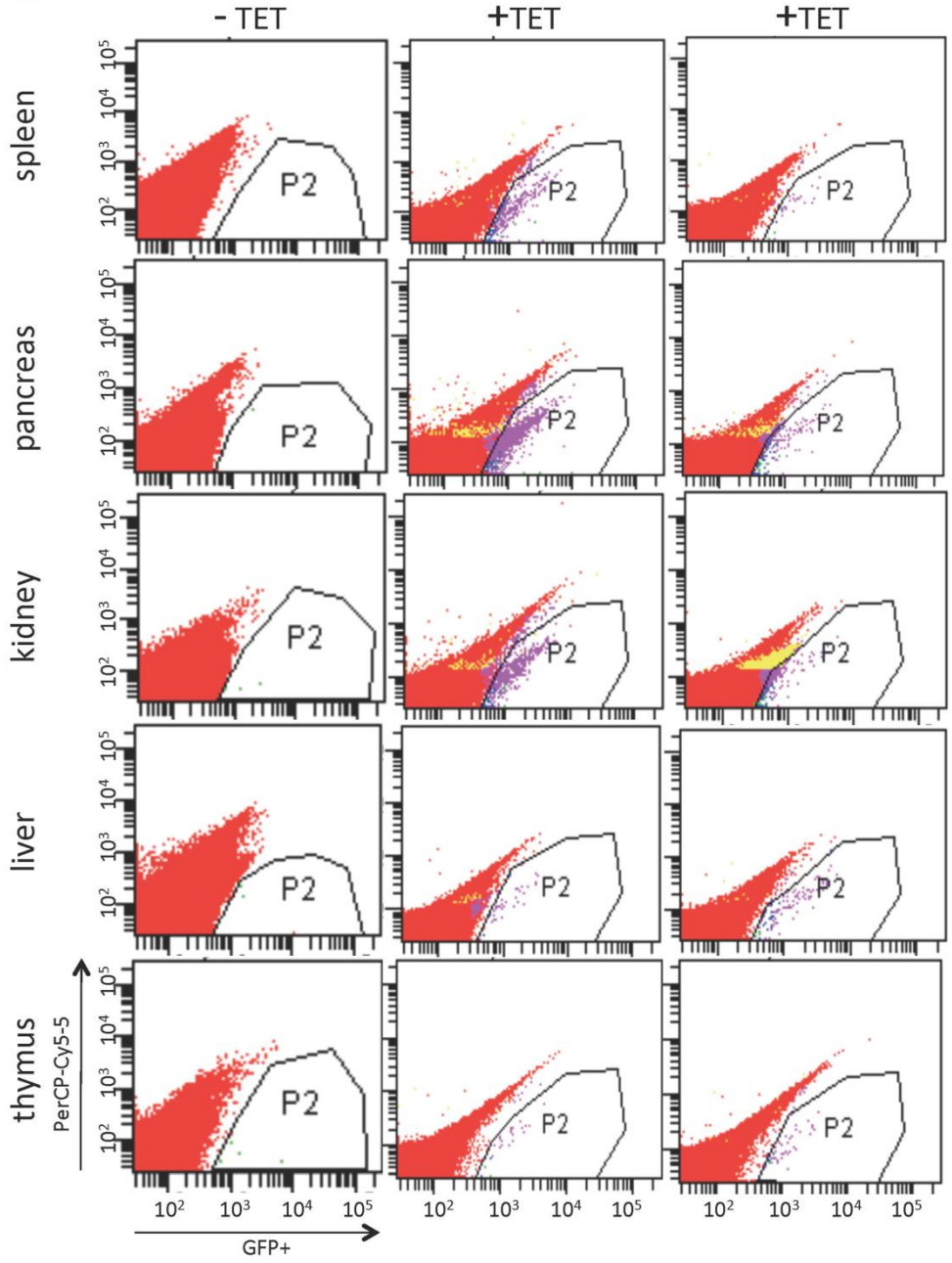

Figure 4. Representative FACS analysis plots of three GS mice for spleen, pancreas, kidney, liver, and thymus. GFP positivity is shown on log scale on the X axis plotted against nonspecific PerCP-Cy5-5 on the Y axis to visualize individual cells. Age-matched negative control mice were not provided TET (-TET). Two representative age-matched mice contain all 3 transgenes and were provided TET as described in text (+TET). Establishment of gates is described in text.

doi: 10.1371/journal.pone.0084379.g004

Figure 5

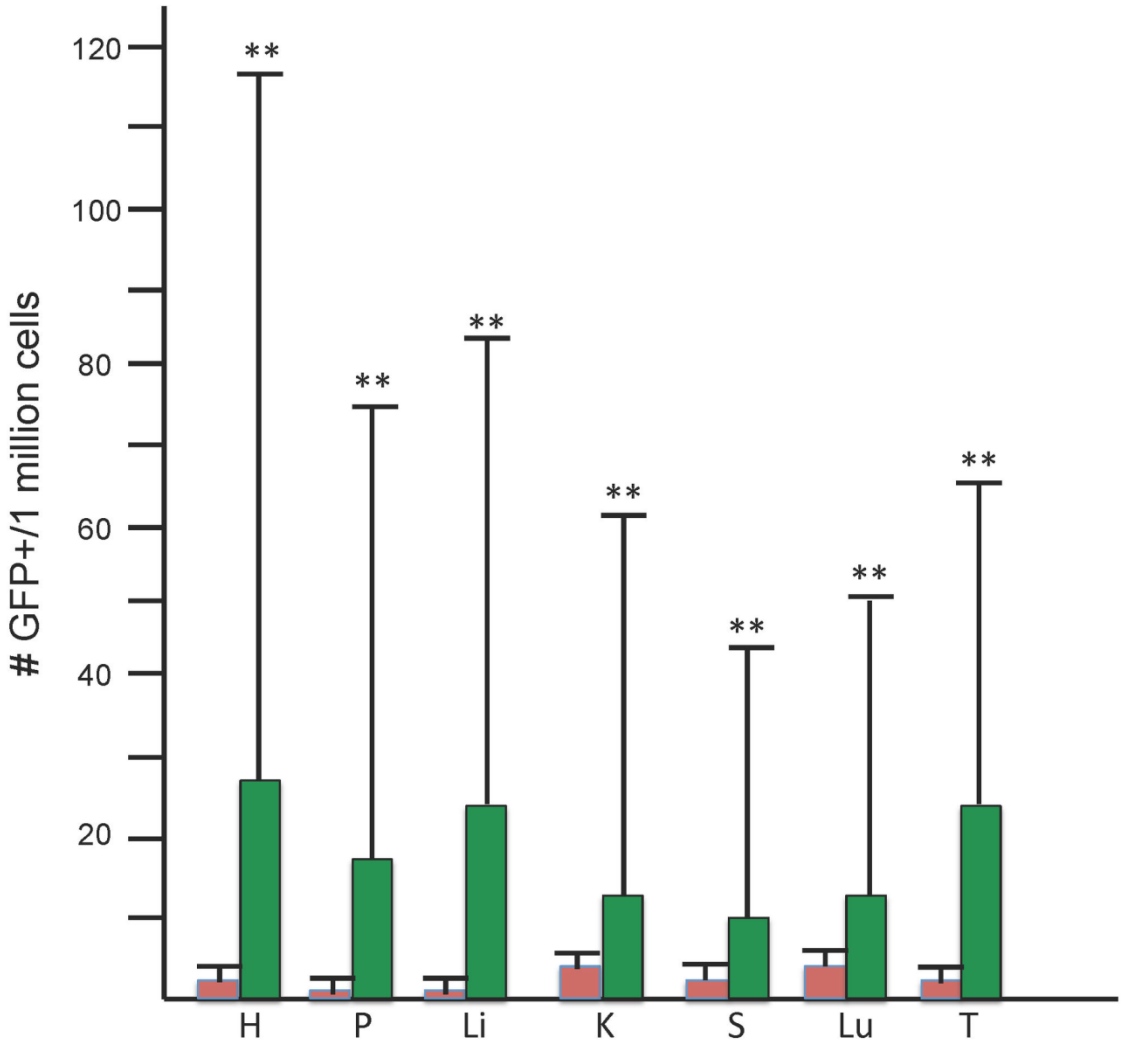

Figure 5. Quantitative analysis of GFP+ cells in all mice analyzed. The number of GFP+ cells in each organ of analyzed mice was determined. Establishment of gates is described in text. From FACS analysis, the average number of GFP+ recombinant cells per million cells and the standard deviation of each was calculated for seven organs and represented in bar graph form. Negative controls are shown in red bars (n=8), and +TET are shown in green bars (n=47). H=heart, P=pancreas, Li=liver, K=kidney, S=spleen, Lu=lung, T=thymus. Organs with statistically significant increased numbers of GFP+ cells groups are indicated by ** above the error bars.

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determination of the specific cell types that are GFP+ within each of the mice could provide this information.

Given the variance in GFP+ numbers detected between mice (Figures 5, 6; Table S1), statistical significance of the probabilities associated with covariance among the traits was calculated for each pair of traits separately in the young and old cohorts (Table S2). In the young cohort, only a single strong positive correlation of covariance between spleen and kidney

Figure 6

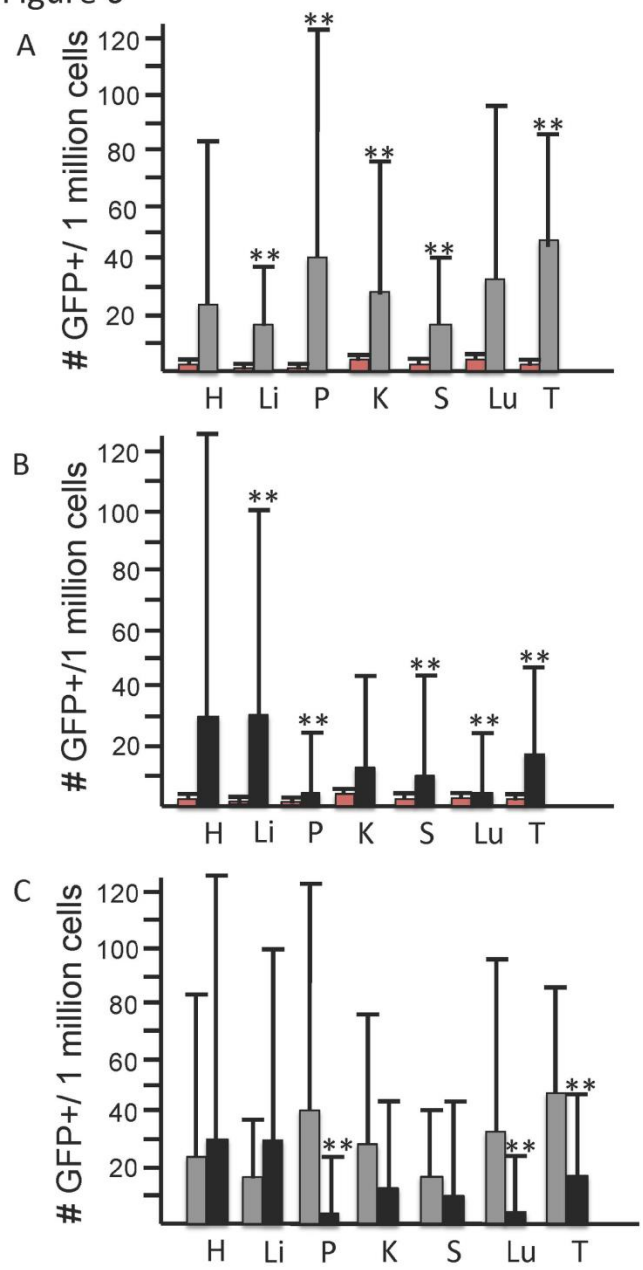

Figure 6. Quantitative analysis of GFP+ cells by age. (**A**) In the young mouse cohort (age<5.5 months), negative controls are shown in red bars (n=5), and +TET are shown in grey bars (n=16). Organs with statistically significant increased numbers of GFP+ cells groups are indicated by ** above the error bars. (**B**) In the older mouse cohort (age>8 months), negative controls are shown in red bars (n=11) and +TET are shown in black bars (n=31). (**C**) Comparison of +TET young mice (grey bars) versus +TET old mice (black bars) from A and B. Organs with statistically significant different numbers of GFP+ cells by age are indicated by ** above the error bars. For all panels, H=heart, P=pancreas, Li=liver, K=kidney, S=spleen, Lu=lung, T=thymus.

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(p=0.002) was noted. In the old cohort, a larger number, although weaker, of positive correlations of covariance were noted; these were between heart and pancreas (p=0.021) or thymus (0.021), as well as between spleen and kidney (0.021) or thymus (p=0.021).

DSB-induced interchromosomal HR occurs *in vivo* in hematopoietic multi-lineage progenitor cell types

Hematopoiesis is characterized by a hierarchy of cells, with hematopoietic stem cells (HSC) possessing the highest proliferative potential and thought to be the targets of aberrant interchromosomal DSB repair events leading to mutagenic chromosomal rearrangements. Our previous *in vitro* studies demonstrated that early stem and progenitor cells are more proficient than terminally differentiated myeloid cells in repairing DSBs by interchromosomal HR [68]. Here, we determined the potential for hematopoietic multi-lineage progenitor cells to utilize this mechanism of repair *in vivo*. GS mice (ages 3-5 months) were administered tetracycline, then bone marrow cells harvested, and subsequently seeded into methylcellulose colony forming assays that support proliferation of myeloid, erythroid, or B-cell progenitors [68–70]. Total numbers of hematopoietic CFUs were scored and classified based on their morphology, and individual GFP+ CFUs determined by inverted fluorescent microscopy (Figure 7). Mature colonies derived from individual precursors included the colony forming unit- granulocyte-erythrocyte-monocyte-megakaryocte (CFU-GEMM), granulocyte-monocyte (CFU-GM), granulocyte (CFU-G), monocyte (CFU-M), erythrocyte (CFU-E), and pre-B (CFU-pre-B). Colonies that contain mixed cell populations are presumed to derive from immature progenitor cells capable of differentiation into multiple cell types. Colonies that contain a single cell population are presumed to derive from more differentiated progenitors that only have the capacity to expand a single cell type.

Following DSBs, GFP+ recombinants were readily obtained from all sub-populations assayed. Strikingly, the results parallel observations previously made in studies of DSB-induced interchromosomal HR using genetically engineered murine ES cells differentiated *in vitro* into hematopoietic colonies [68]. The highest average number of GFP+ recombinant colonies (32 ± 15) was observed in the multi-potent CFU-GEMM cells scored by this assay (Table 1). Observed numbers of GFP+ recombinants decreased with increased differentiation status with the lowest average number of GFP+ recombinant colonies (5 ± 5) observed in the terminally differentiated but actively proliferating monocytic cells (p value = 0.02) (Table 1). The average frequency of recombination in this *in vivo* system was estimated to be 8.0×10^{-5} in CFU-GEMM cells, 5.5×10^{-5} in CFU-GM cells, 6.5×10^{-5} in CFU-G cells, and decreasing to 1.25×10^{-5} in CFU-M cells. Overall these data demonstrate that both multipotent and terminally differentiated cell types retain the potential to repair DSBs with a homologous sequence on a heterologous chromosome *in vivo*.

Because CFU represent clonal populations, the recombinant HR repair products could be verified at the sequence level. DNA was extracted from individual BM colonies, and nested PCR used to amplify across the two I-*Sce*I endonuclease DSB repair sites (Figure 1A). Because the 3' UTR ends of the transgene sequences are unique, primers could selectively amplify each of the two transgenes (Figure 1A). A total of 22 individual BM colony PCR products were cloned and sequenced. Because each transgene is inserted in multiple copies, PCR will amplify both GFP+ recombinant and parental non-recombinant copies of the transgenes. These were distinguishable following TA cloning and sequencing of multiple TA clones from each BM colony PCR. This analysis verified that all 22 BM colonies contained a repaired GFP+ wild-type sequence on at least one allele. In 6 of 22 colonies this analysis detected HR repair at only one allele (4 at 1S-GFP and 2 at 2S-GFP). In 16 of 22 colonies this analysis detected HR repair at both alleles; however given the multiple copy inserts, these likely represented independent events.

Discussion

This study presents an *in vivo* model that directly demonstrates that DSB-induced interchromosomal HR occurs at readily detectable rates. GFP+ recombinant cells were readily detectable in a broad range of somatic cell types. Variability in numbers of GFP+ recombinant cells was observed between the multiple somatic cell types and mice in all cohorts examined. Such variability could be due to differences in GFP expression, recombination rates, clonal expansion of individual GFP+ recombinants, or I-*Sce*I transgene induction, expression, or stability. This mouse model initiates I-*Sce*I expression *in vivo* using a single bicistronic TET-ON system [65]. The experiments in MEFs with this system show strong and specific induction, but *in vivo* kinetics could be different. In addition, individual mice self regulate feeding and thus vary dosage to tetracycline. However, similar inter-mouse variability in the *in vivo* mouse model of spontaneous intrachromosomal/sister chromatid HR suggests that I-*Sce*I is not the major determinant of these results [30].

Intrachromosomal HR may occur if homologous repeat sequences lie on the same chromosome in the same direct repeat orientation such as repetitive elements within several kb of each other. Several studies have used *Arabidopsis* and *N. tabacum* models to detect spontaneous and DSB-induced sister chromatid and intrachromsomal HR with spontaneous frequencies estimated at 10^{-5} to 10^{-4} [29,35,71,72] and up to 10,000X stimulation by I-*Sce*I expression [29,35]. Further, similar to *in vitro* findings, SSA was a predominant mode of DSB repair with ectopic joining contributing to a smaller subset of repair events [35]. In mice, spontaneous intrachromosomal and sister chromatid HR have been demonstrated utilizing a yellow fluorescent protein (YFP) reporter or LacZ/β-galactosidase reporter construct [27,30,73–76]. These studies demonstrated median spontaneous HR frequencies of 5 per 10^6 cells in the pancreas [30,73–76]. Ionizing radiation or the interstitial cross-linking agent mitomycin-C led to an increase of recombination suggesting that non-specific DNA damage is also sufficient to promote intrachromsomal HR, at least in pancreatic cells [75]. Although comparisons between different model systems are difficult, these results are surprisingly similar to the findings presented here suggesting that both

Figure 7

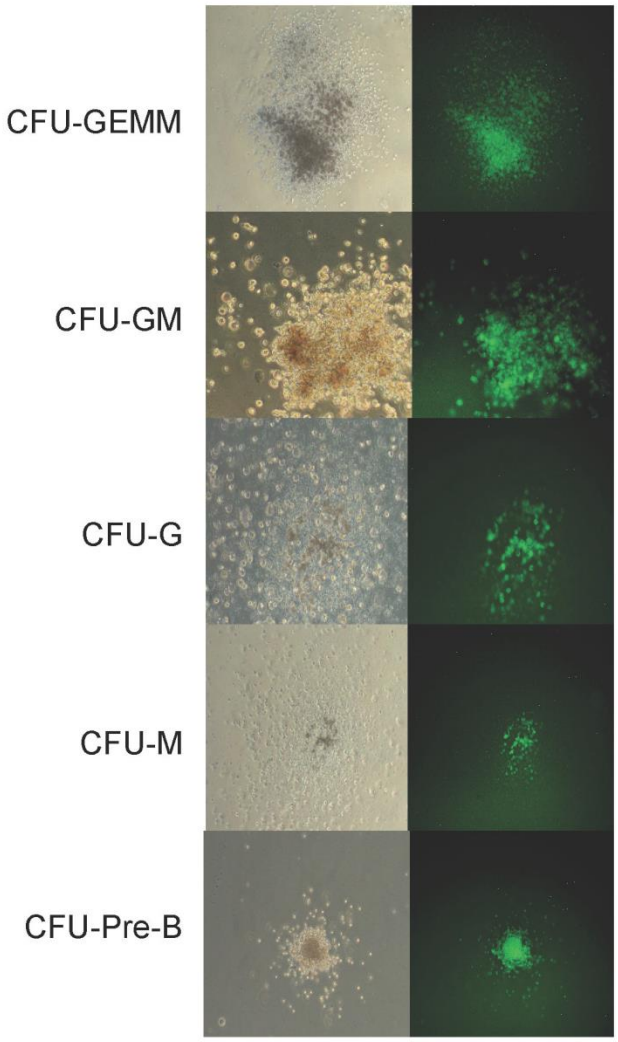

Figure 7. DSB-induced GFP+ recombinants in hematopoietic subpopulations isolated from bone marrow of GS mice. Colonies were scored by inverted fluorescent microscopy and faint background fluorescence of negative controls was subtracted out of total repair frequency. Representative phase contrast and fluorescent microscopy images of GFP+ recombinants from bone marrow CFC assay. Granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM), Granulocyte-macrophage (CFU-GM), Granulocyte (CFU-G), Macrophage (CFU-M), Pre-B cell (Pre-B), and Burst forming unit-erythroid (BFU-E). Magnification 400X.

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types of HR repair are utilized with roughly the same overall efficiency, although likely in different cell types or at different stages of the cell cycle [60].

Cytologic studies indicate that nuclei are ordered, and chromosomes/genomes generally exist within defined nuclear territories [77,78], and single DSBs remain stable in these defined regions [79,80]. Genetic studies seem to support this

Table 1. DSB-induced interchromosomal HR in hematopoietic progenitor cell populations.

	# GFP colonies[a]			
Bone Marrow CFC	**Expt 1**	**Expt 2**	**Expt 3**	**avg. GFP colonies**
CFU-GEMM	47	17	32	32 ± 15
CFU-GM	31	0	35	22 ± 19
CFU-G	23	49	7	26 ± 21
CFU-M	0	4	10	5 ± 5 p value = 0.02[b]
CFU-Pre B	37	21	27	28 ± 8
BFU-E	8	0	40	16 ± 21

GS mice were administered tetracycline via drinking H_2O for 14d. Mice were then sacrificed, and femur bone marrow cells isolated and seeded into methylcellulose colony forming assays. Cells were plated at 1.0 x 10^5 cells/plate. Each experiment included 4 technical replicates and the total number of colonies is shown.

a. GFP+ colony numbers were normalized to account for variation of overall plating efficiency (total number of CFC of each type) between mice.

b. Number of GFP+ CFU-M colonies observed was statistically significantly lower as compared to the number of GFP+ CFU-GEMM observed (student's T test).

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model as repair of a single DSB in mouse and human cells does not promote large scale genome rearrangements between heterologs, although they can be associated with regional loss of heterozygosity (LOH) and insertions with sequences of unknown origin [20,31–33,81]. Similarly, multiple DSBs on the same chromosome do not significantly promote large-scale genome rearrangements in mouse or human cell lines, although the efficiency of repair decreases as the distance between two DSBs increases (up to 9 kb apart) [82,83].

By contrast, cytological analysis indicates chromosome movement is more fluid in DNA repair deficient cells [79]. Chromosome movement has also been observed in the presence of multiple induced DSBs on heterologous chromosomes in mitotic yeast [84] or following global exposure of cells to ionizing radiation or topo II inhibitors [85–87], leading to foci suggestive of repair centers ("repairosomes" [84,88]). The steps by which such repairosomes are initiated by chromatin remodeling programs as a normal step in DNA repair or the biological understanding of how translocations are formed within the ordered nucleus remain unclear [2,89,90]. It is not clear if chromosome movement is in response to multiple breaks in different loci or after prolonged or persistent damage. The established nuclear matrix and chromatin loop structures may also influence choice of recombination partners during DSB repair [89]. In support of the cytologic data, our genetic study here indicates that *in vivo* interaction of DNA sequences and recombination is promoted by multiple DSBs.

A wider range of HR mechanisms are used to repair DSBs on heterologous chromosomes as compared to intrachromosomal HR. In addition, intrachromosomal HR is not typically associated with the genome rearrangements observed in human tumors. DSBs in cultured ES cells and multiple *in vitro* differentiated hematopoietic cell types can stimulate interchromosomal HR as a repair pathway [68]. We observed with this *in vivo* system that repair by HR in multiple hematopoietic lineages is also quite prevalent with observed GFP+ numbers decreasing with differentiation. Similarly, in most organs the number of GFP+ cells decreased with age. These data support other hypotheses that differentiation and age will determine different pathways of repair or utilize apoptotic programs with different frequencies [57–60].

Topo II is an essential cellular enzyme that catalyzes changes in DNA topology via its cleavage-religation equilibrium. Topo II inhibitors convert topo II into a DNA-damaging enzyme by disrupting the cleavage-religation equilibrium, resulting in accumulation of DSBs, activation of DNA damage sensors, cell cycle arrest, and initiation of apoptosis or repair. A wide range of agents, including some chemotherapeutic agents, are classified as topo II inhibitors, and exposure to these is associated with development of secondary leukemias [91,92]. However, they also include benzene metabolites, bioflavinoids, anthraquinone laxatives, podophyllin resins, quinolone antibiotics, pesticides, many phenolic compounds, as well as certain fruits, tea, coffee, wine, soy, and cocoa [11,12,93]. The recent observations that bioflavinoids can stabilize DNA DSBs and promote illegitimate repair and genome rearrangements in cultured cells has led to the hypothesis that exposure to these agents *in utero* or through unregulated high doses as dietary supplements may promote leukemia [15–18]. Further study of this *in vivo* system could determine the potential for exposure to such agents at early stages of development to promote HR *in vivo* and their long-term impact.

Rearrangements resulting from DSB repair that occurs in germ cells can have evolutionary implications. It has been observed that topoII has a role in DSB formation in spermatids [94], and chromatin loop organization is similar between spermatids and somatic cell types [89]. These observations have led to the suggestion that DSB repair pathways and partner choice may be more similar in meiotic and mitotic cells than appreciated and has the potential to result in rearrangements leading to genome variation [89,95]. That this may be universal across multiple kingdoms, is supported by genome analysis of plants that suggests translocations are a regular mechanism of plant evolution [40,41]. In addition, mutation fixation has been implicated during DSB repair in the first zygotic cell division in mice [96]. Our demonstration that interchromosomal HR occurs *in vivo* in response to DSBs at just two loci in a broad range of cell types, particularly progenitor cells, is a novel finding and lends further support to the idea that exposure to the growing list of environmental agents, dietary supplements, or groundwater contaminants that induce or stabilize DSBs may promote potentially tumorigenic rearrangements, accelerate genomic variation, and influence evolution.

Materials and Methods

Ethics Statement

All studies were approved by IACUC (protocol #AAAA0123 Columbia University; protocol #08-035 University of North Carolina at Charlotte). All studies were conducted under supervision of appropriate regulatory bodies and in accordance

to established NIH guidelines for ethical treatment of animals in research.

Transgenic Mice

Transgenic mice for study were generated by establishing three independent transgenic lines of mice: (1) the tetracycline-regulated I-*Sce*I expression gene, (2) 1SGFP with I-*Sce*I cut site 1, and (3) 2SGFP containing I-SceI cut site 2 (1). TET-I-*Sce*I – XbaI-PstI fragment of CBAS containing the I-*Sce*I gene [64,66] sequence was inserted into NheI-BamHI digested pBIG3i bicistronic tetracycline-regulated vector (kindly provided by Craig Strathdee) [65]. DNA was digested with BspHI and the fragment was provided to the Columbia University Transgenic Mouse facility (2). 1SGFP – SacII-HindIII fragment of pCAGGS-NZE-GFP containing the GFP sequence was sub-cloned into SacII-HindIII digested pBluescript SK+, creating SKRGFP(Sac2H3). Single-stranded oligomers BHI-Δ1-ISceI-NcoI P1 (5'-GATCTGGATCCACCGGTCGCAATTACCCTGTTATCCCTAC CATGGAGTAC-3') and BHI-Δ1-ISceI-NcoI P2 (5'-GTACTCCATGGTAGGGATAACAGGGTAATTGCGACCGGT GGATCCAGATC-3') were annealed and digested with BamHI and NcoI. This BamHI-NcoI fragment containing the I-SceI recognition site was ligated into the BamHI-NcoI digested SKRGFP, creating SKRGFP(Sac2H3)Δ1-S. The SacII-HindIII fragment of SKRGFP(Sac2H3)Δ1-S was subcloned back into the SacII-HindIII digested pCAGGS-NZE-GFP plasmid, to create pCAGGS-GFPΔ1-S. DNA was digested with SalI and PstI, and the 3433 bp fragment was provided to the Columbia University Transgenic Mouse facility (3). 2SGFP – A PvuII site was engineered in pCAGGS-NZE-GFP using annealed single-stranded oligomers GFP-Pvu2-1 (5'-CGCCGACCACTACCAGCTGAACACCCCCATCGGCGAC-3') and GFP-Pvu2-2 (5'-GTCGCCGATGGGGGTGTTCAGCTGGTAGTGGTCGGCG-3 'and QuikChangeII Site-Directed Mutagenesis Kit (Stratagene), following manufacturer's protocol, creating pGFP-Pvu2. Single-stranded oligomers SCE1 (5'-Phos-ATTACCCTGTTATCCCTA)and SCE2 (5'-Phos-TAGGGATAACAGGGTAAT-3') were annealed and ligated into the PvuII blunt end digested pGFP-Pvu2, creating pGFP-Pvu2-S. DNA was digested with SalI and PstI, and the 3433 bp fragment was provided to the Columbia University Transgenic Mouse facility. The Columbia University Transgenic Mouse facility generated transgenic mice in F1 (C57BL/6J-CBA) hybrids, and mice were transferred to University of North Carolina at Charlotte.

The two GFP lines were intercrossed and the resulting line crossed with mice containing the tetracycline regulated I-SceI expression transgene. The resultant triply positive transgenic line was denoted "GS" and used for further study. Genotyping for presence of all three transgenes was performed by PCR and Southern blotting of mouse tail tip genomic DNA and subsequent digestion of PCR products with I-*Sce*I endonuclease (New England Biolabs) to confirm intact I-*Sce*I sites. Amplification was performed by 94°C 5 min; followed by 40 cycles of 94°C 30s, 60°C 30s, 72°C 2 min; and 72°C 15 min. For nested PCR 5uL of the first PCR product was used as template for second round of PCR following the same protocol. PCR primers for each: Sce1F 5'-gtccgaactctaaactgctga-3'; Sce2R 5'-ACCAGTATGCCAGAGACATC-3'; GFP 1F 5'-aaggccaagagggccaa-3'; GFP 2F 5'-TGGACGGCGACGTAAAC-3'; GFP 3R 5'-gtgctcaggtagtggttg-3'; GFP 4R 5-CTCTGTTCCACATACACTTC-3'; GFP 5F 5'-tgaaccgcatcgagctgag-3'; GFP 6R 5'-GACCATGTGATCGCGTTC-3'; GFP 7R 5'-TTCTGATAGGCAGCCTG-3'. Southern blotting to determine copy number utilized a plasmid fragment of full length GFP ORF of 3.07 kb and diluted to pg amounts that approximated 0.2, 1.0, 5.0, 10, 20, and 100 copies per genome spiked into 10μg non-transgenic mouse DNA. Genomic DNA of transgenic mice was digested with restriction endonucleases that flank the GFP promoter and ORF of both transgenes. The GFP probe fragment was an Sph-Not I fragment homologous to both transgenes. Q-PCR for copy number estimation utilized a GFP ORF fragment diluted to pg amounts that approximated 0.2, 1.0, 5.0, 10, 20, and 100 copies per genome to amplify a 296 bp fragment of GFP DNA. Genomic DNA isolated from transgenic mice was utilized for Q-PCR. Fluorescent detection of PCR products was reported using a SYBR® Green PCR kit (Quanti Tect) in 20μL reactions established according to the manufacturer's recommended protocol. A standard curve was generated (n=3) using the control plasmid GFP ORF DNA according to the manufacturer's protocol (QuantiTect). Q-PCR analysis was simultaneously analyzed by a 96 well 7500 Fast Real-Time PCR System (Applied Biosystems) in which transgenic mouse genomic DNA was compared against the standard curve and statistical analysis performed according to the Applied Biosystems protocol for 7500 Fast Real-Time PCR System protocol.

MEFs

Mouse embryonic fibroblasts were isolated from day E13.5 of GS mice and washed with phosphate buffered saline (PBS). The head was removed from isolated embryos and used for DNA genotyping. The body was minced well and 10mL of 0.25% Trypsin-EDTA (Gibco, Grand Island, NY) added. Solution was triturated with a pipette and added to 25 mL of medium [Dulbecco's Modified Eagle Medium (Gibco), 15% FBS (Gemini Bio-Products, West Sacramento, CA), 1.2% 200mM L-Glutamine (Gemini Bio-Products), 1.2% Non-essential Amino Acids (Gibco), and 1.2% Penicillin-Streptomycin (Gibco)]. Cells were then collected by centrifugation (1000 rpm x 10 min), resuspended into 4mL medium, and cultured on a 6-well dish at 37°C with 5%CO_2. MEFs were then passaged onto 10cm dishes after initial growth. Tetracycline HCl (Barr Laboratories) was dissolved in 1x PBS to 1mg/mL and passed through a 0.2 micron filter. MEFs were given a final concentration of 2μg/mL for up to 6 days.

DSB induction in Mice

Tetracycline HCl (Barr Laboratories) was dissolved into .5X PBS/H20/ sucrose at 10mg/mL and passed through a 0.2 micron filter. Mice were administered tetracycline at 2mg/mL in a water bottle for up to 21 days.

Flow cytometry and statistical analysis

MEFs were trypsinized and collected by centrifugation (1000 rpm x 10 min). Cells were resuspended in 1x PBS at a concentration of $1.0x10^6$ cells/mL. Sections of individual organs were harvested and a single cell suspension generated in 5% Bovine Serum Albumin (Gemini Bio-Products)/1x PBS. Suspensions were passed through a 53μM nylon mesh filter (Spectrum Laboratories Inc) and analyzed on a FACSAriaII for GFP positivity. To assess statistical significance of increased numbers of GFP+ cells among organs in tetracycline treated mice (Table S1), we utilized a non-parametric t-test for all mice versus negative controls (Figure 5), and then separately in the young and old cohorts versus negative controls (Figure 6). To assess statistical significance of the probabilities associated with covariance of the number of GFP+ cells in organs of individual mice, we calculated Spearman's nonparametric correlation coefficients for each pair of organs separately in the young and old cohorts, and utilized the false discovery rate procedure to control the proportion of false positive results (Table S2).

Western Immunoblot analysis

Protein was isolated from pelleted cells using Total Protein Extraction Kit (Millipore). Cell lysate proteins were then separated on a 10% NuPage Bis-Tris SDS-Page gel (Invitrogen) and transferred to Amersham Hybond-P membrane (GE Healthcare Life Sciences). The membranes were then blocked in 5% Non-Fat dry milk in 1X tris buffered saline (Bio-Rad). Membranes were probed with a mouse monoclonal IgG anti-HA antibody to detect the HA tag within I-*Sce*I (Cell Signaling Technology) at 1:100 dilutions for 20-22 hours at 4°C or a mouse monoclonal IgG anti-β-actin antibody (Santa Cruz Biotechnology) at 1:400 dilution for 1 hour at room temperature. Blots were subsequently exposed to an anti-mouse IgG HRP-linked secondary antibody (Cell Signaling Technology) at 1:1000 dilutions for 1 hour at room temperature. Blots were washed 3x for five minutes each in a 1x TBS-.05% Tween 20 solution. Membranes were developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

Bone Marrow-CFC Assay

GS mice (ages 3-5 months) were administered tetracycline through H_2O for 14d. Mice were then sacrificed, and femur bone marrow (BM) cells isolated and seeded into methylcellulose colony forming assays [69,70]. Whole BM was flushed from femurs into IMDM supplemented with 2% FBS and disrupted into a single cell suspension by a 22G needle and syringe. Cell viability counts were performed using .05% trypan blue staining. Total viable BM cells were plated at 1.0×10^5 cells per 35mm low adherence tissue culture dishes in hematopoietic differentiation medium (STEMCELL Technologies) containing IMDM, 1% methylcellulose, 15% non-ES qualified FBS, 100U/mL penicillin, 100μg/mL streptomycin, 2mM L-glutamine, 150μM monothioglycerol, 1% bovine serum albumin, 10μg/mL insulin, 200μg/mL transferrin, 150ng/mL mSCF, 30ng/ml mIL-3, 30ng/mL mIL-6, and 3U/ml hEPO for 14 days.

DNA Sequence Analysis of HR Recombinants from BM CFCs

Individual CFU-GEMM expressing GFP were identified by inverted fluorescent microscopy and isolated. Genomic DNA from 24 individual CFU-GEMMs was extracted from each with DNeasy Tissue Kit (Qiagen) followed by whole genome amplification (WGA) with Repli-G Kit (Qiagen) as previously described [68]. 1.0μg of WGA DNA template was used for PCR. Each 25μL PCR reaction contained template DNA, 10X reaction buffer, 1.5mM $MgCl_2$, 200μM each dNTP, 0.48μM each primer, 2.5 units Taq DNA polymerase. PCR primer sets are indicated in Figure 1 and in Methods above. Amplification was performed by 94°C 5 min; followed by 40 cycles of 94°C 30s, 55°C 30s, 72°C 2 min; and 72°C 15 min. For nested PCR 5uL of the first PCR product was used as template for second round of PCR following the same protocol. PCR reaction products were cloned with the TA cloning system (Invitrogen) and blue-white screening used to determine which individual clones to amplify, isolate DNA, and sequence by Sequetech (Mountain View, CA) using M13 forward and M13 reverse primers. Sequencing of up to 10 white colonies from each PCR reaction/TA cloning reaction was sufficient to identify GFP+ recombinants among parental GFP sequences.

Supporting Information

Table S1. Number of GFP+ cells detected per million analyzed by FACS in young and old cohorts. Individual mice are noted with young cohort mice indicated by Y and old cohort mice indicated by O. Organs from which technical error led to no sample recovered for FACS analysis are noted as nd (no data). These values were the basis for the covariance of traits analysis in Table S2.
(DOCX)

Table S2. Covariance of GFP+ cells in organs of young and old cohorts. To assess statistical significance of the probabilities associated with covariance, Spearman's nonparametric correlation coefficients for each pair of traits separately in the young and old cohorts (Table S1), and utilized the false discovery rate procedure to control the proportion of false positive results. Calculated p-values in the young mouse cohort are represented within the top diagonal half of the matrix. Calculated p-values in the old mouse cohort are represented within the bottom diagonal half of the matrix. p-values <0.05 are denoted with **.
(DOCX)

Acknowledgements

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Author Contributions

Conceived and designed the experiments: RRW PS CR. Performed the experiments: RRW PS CGV GB NC CR. Analyzed the data: RRW CGV GB NC CR. Contributed reagents/materials/analysis tools: PS CR. Wrote the manuscript: RRW PS CGV CR.

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APPENDIX F: APTAMER-FUNCTIONALIZED NANOPARTICLES AS "SMART BOMBS": THE UNREALIZED POTENTIAL FOR PERSONALIZED MEDICINE AND TARGETED CANCER TREATMENT

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Abstract
Conventional delivery of chemotherapeutic agents leads to multiple systemic side effects and toxicity, limiting the doses that can be used. Development of targeted therapies to selectively deliver anti-cancer agents to tumor cells without damaging neighboring unaffected cells would lead to higher effective local doses and improved response rates. Aptamers are single-stranded oligonucleotides that bind to target molecules with both high affinity and high specificity. The high specificity exhibited by aptamers promotes localization and uptake by specific cell populations such as tumor cells, and their conjugation to anti-cancer drugs have been explored for targeted therapy. Advancements in the development of polymeric nanoparticles allow anti-cancer drugs to be encapsulated in protective nonreactive shells for controlled drug delivery with reduced toxicity. Conjugation of aptamers to nanoparticle-based therapeutics may further enhance direct targeting and personalized medicine. Here we present how the combinatorial use of aptamer and nanoparticle technologies has the potential to develop "smart bombs" for targeted cancer treatment, highlighting recent pre-clinical studies demonstrating efficacy for the direct targeting to particular tumor cell populations. However, despite these pre-clinical promising results, movement of this technology to the bedside has made little progress.

APPENDIX G: DOWNREGULATION OF HOXC6 IN SEROUS OVARIAN CANCER

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Abstract
Homeobox (HOX) genes encode transcription factors critical to morphogenesis and cell differentiation. Although dysregulation of several HOX genes in ovarian cancer has been reported, little is known about HOXC6 expression in epithelial ovarian cancer. In this report, analysis of laser capture microdissected samples determined HOXC6 expression patterns in normal versus malignant serous ovarian carcinoma tissues. HOXC6 protein was quantified by ELISA in parallel serum samples and further validated in a larger cohort of serum samples collected from women with and without serous ovarian carcinoma. These data demonstrate significant downregulation of HOXC6 in serous ovarian cancer.