TOPOISOMERASE II INHIBITORS INDUCE AN ILLEGITIMATE GENOME REARRANGEMENT COMMON IN INFANT LEUKEMIA

by

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ABSTRACT

BHAWANA BARIAR. Topoisomerase II inhibitors induce an illegitimate genome rearrangement common in infant leukemia. (Under the direction of DR. CHRISTINE RICHARDSON)

Infant acute leukemias account for ~30% of all malignancy seen in childhood across the Western world. They are aggressive and characterized by rapid onset shortly after birth. The majority of these have rearrangements involving the *MLL* (mixed lineage leukemia) gene. Although *MLL* fusion to more than 75 genes have been identified, *AF9* is one of its most common translocation partners. Since *MLL* breakpoint sequences associated with infant acute leukemia are similar to those in secondary AML following exposure to the topoisomerase II (topo II) poison etoposide, it has been hypothesized that exposure during pregnancy to biochemically similar compounds may promote infant acute leukemia. Some studies have shown an epidemiological link between bioflavonoid intake and increased incidence of *MLL*-rearranged infant leukemias. Hundreds of unregulated nutritional supplements are widely available and perceived to prevent cardiovascular disease, inflammation and cancer. However, their potential to promote leukemic translocations should be determined. My goal was to test the hypotheses that 1) *MLL* and *AF9* breakpoint cluster regions (bcrs) undergo double strand breaks (DSBs), and are highly recombinogenic independent of chromosomal context, and in the absence of a leukemic fusion protein; and 2) a number of topoisomerase II inhibitors have the potential to promote these translocations analogous to those observed in infant leukemia. To test these hypotheses, I developed transgenic reporter cell lines such that a translocation between *MLL* and *AF9* bcrs results in a full-length GFP transcript and

quantifiable green fluorescence. I show here that topo II inhibitors such as the bioflavonoids genistein, quercetin, luteolin and myricetin lead to *MLL-AF9* bcr translocations, genistein and quercetin being the most potent promoters. Notably, benzoquinone which is associated with non-*MLL*-rearranged leukemias do not promote *MLL-AF9* translocations even at a high dose. I demonstrate here the sensitivity of the *MLL* and *AF9* bcrs to topo II poisons and bioflavonoids independent of their normal chromatin context and independent of the formation of a leukemic fusion protein. In addition, this reporter gene system allows for rapid and reproducible screening of hundreds of compounds that may have the potential to promote leukemogenic translocations.

DEDICATION

I dedicate my doctoral dissertation to my parents Prof. (Dr.) Lalit Mohan Bariar and Mrs. Deepali Bariar for their unconditional love, faith, support, and encouragement. Without them, this work would not have been possible.

I would also like to dedicate this accomplishment to my husband Dr. Nilabh Srivastava and my lovely children Arjun and Anoushka for their constant love and support throughout the period of my doctoral work.

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HR	homologous recombination
NHEJ	non-homologous end joining
DSB	double strand break
IPCR	inversePCR
Topo II	topoisomerase II
ES cells	embryonic stem cells
GFP	green fluorescent protein
bcr	breakpoint cluster region
MAG	*MLL-AF9-GFP*

CHAPTER 1: INTRODUCTION

In this thesis I have tested the hypotheses that 1) MLL and AF9 bcrs undergo DSBs, and are highly recombinogenic independent of chromosomal context, and in the absence of a leukemic fusion protein; and 2) a number of topo II inhibitors have the potential to promote these translocations analogous to those observed in infant leukemia. I demonstrate here the sensitivity of the MLL and AF9 bcrs to topo II poisons and bioflavonoids independent of their normal chromatin context and independent of the formation of a leukemic fusion protein.

Chapter 3 describes the establishment of a reporter gene model system to screen for the frequency of MLL-AF9 translocation events. Chapter 4 describes the quantification of GFP+ colonies after endonuclease ISceI expression which induces directed DNA breaks in reporter cell lines. Chapter 5 describes the quantification of GFP+ colonies after treating the reporter cell lines with varying doses of different topo II inhibitors including the chemotherapeutic drug etoposide and different bioflavonoid compounds which are found in our diet and are also used as nutritional supplements.

1.1 Hematopoiesis

Hematopoiesis is the process of formation of blood cells (Figure 1). All mature and differentiated blood cells are derived from hematopoietic stem cells (HSCs).

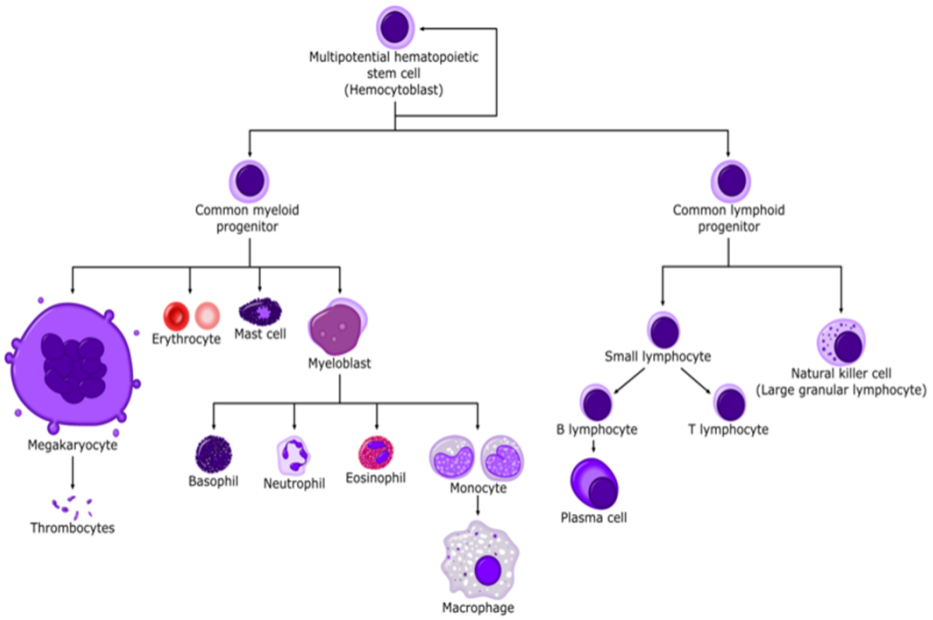


Figure 1: The process of hematopoiesis

Hematopoiesis is characterized by the constant self-renewal of a small stem cell pool, and expansion and differentiation into an array of progressively restricted myeloid and lymphoid cell types. Hematopoietic stem cells (HSC) are a rare cell population among hematopoietic progenitors present in the bone marrow, characterized by CD34+/CD38- markers and are distinguished by self-renewal, long-term multi-lineage repopulating potential, and ability to reconstitute hematopoiesis both *in vivo* and *in vitro* (Hao, Shah et al. 1995, Hao, Thiemann et al. 1996). Leukemic HSCs display properties similar to normal HSC including CD34+ expression, self-renewal and potential for propagation of a leukemic clone (Lapidot, Sirard et al. 1994, Sutherland, Blair et al. 1996, Bonnet and Dick 1997, Jamieson, Weissman et al. 2004).

1.2 Childhood and infant leukemia

Hematopoietic malignancies include leukemia and lymphoma. Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of

immature white blood cells. Lymphoma is a type of blood cancer that occurs when B or T lymphocytes that form a part of the immune system undergo uncontrolled division. Lymphoma may develop in many parts of the body, including the lymph nodes, spleen, bone marrow, blood or other organs, and eventually form a tumorous mass of cells. These tumors may grow and invade the space of surrounding tissues and organs.

Infant acute leukemias account for ~30% of all malignancy seen in childhood across the Western world. Leukemias are the most common cancer that affect children since 25% to 35% of all childhood cancers are leukemias (Lightfoot and Roman 2004). Infant leukemias which occur at less than one year of age, grow rapidly, are aggressively invasive and are characterized by rapid onset shortly after birth. The major morphological types of childhood leukemia have been documented as acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML). ALL is the more predominant form of infant leukemia, while AML accounts for around 15 – 17% of the cases. ALL is the most common form of childhood cancers. Youngest infants have the worst outcome leading to death. These leukemias are frequently characterized by genetic alterations which include chromosomal translocations and other rearrangements such as deletions (Felix and Lange 1999). The high frequency of recurring chromosomal rearrangements identified in leukemia and lymphoma suggests they result from an error in normal gene rearrangements of the hematopoietic developmental program.

1.3 *MLL* gene rearrangements and leukemia

Approximately 85% of infant ALL cases, and 50% of infant AML cases involve rearrangements of the *MLL* 11q23 gene (Cimino, Lo Coco et al. 1993, Ford, Ridge et al. 1993, Sorensen, Chen et al. 1994, Gale, Ford et al. 1997, Dimartino and Cleary 1999,

Felix and Lange 1999, Lightfoot and Roman 2004). 11q23 rearrangements are associated with a poor outcome (Chen, Sorensen et al. 1993, Taki, Ida et al. 1996, Reaman, Sposto et al. 1999). *MLL* is a transcriptional regulatory factor expressed in hematopoietic cells, and aberrant expression of *MLL* in these cells due to a translocation may give rise to leukemia.

A diverse group of karyotypic abnormalities involving the rearrangement of the *MLL* gene are found in these hematological malignancies. More than 75 fusion partner genes for *MLL* have been identified. *AF9* gene is one of its most common translocation partners (Dimartino and Cleary 1999, Ayton and Cleary 2001, Lightfoot and Roman 2004). In addition to reciprocal balanced translocations to partner genes, 11q23 rearrangements include deletions, duplications or amplifications (Bernard and Berger 1995, Rowley 1999, Ayton and Cleary 2001). There is evidence to suggest that these translocations may initially form *in utero* (Ford, Ridge et al. 1993, Taki, Ida et al. 1996, Gale, Ford et al. 1997, Megonigal, Rappaport et al. 1998, Wiemels, Cazzaniga et al. 1999, Wiemels, Xiao et al. 2002, Felix, Kolaris et al. 2006).

The human *MLL* gene is found on locus 11q23 and its translocations are typically found in mixed lineage leukemia as discussed earlier. Its normal function in the cells and the aberrant activities of *MLL* fusion proteins are well known and briefly described here.

Rearrangement of the *MLL* locus can be sufficient to promote transformation of normal blood progenitors to leukemia stem cells (Cozzio, Passegue et al. 2003, Hotfilder, Rottgers et al. 2005, Jordan 2006, Kong, Sham et al. 2006)

The *MLL* (mixed lineage leukemia) gene is a homolog of a known *Drosophila* gene named trithorax (*Trx*) (Slany 2009). Like Trx, MLL also regulates the expression of

Hox genes which play an important role in development and pattern formation in the embryo. The MLL protein contains a highly conserved SET domain (an acronym for Suppressor of variegation, Enhancer of zeste, trithorax) at the C-terminus as the site of a histone methyltransferase activity that specifically methylates histone H3 at lysine 4. MLL is post-translationally processed by proteolytic cleavage where it is cut by an aspartic protease into an N-terminal 320 kDa fragment and a C-terminal 180 kDa moiety.

The C-terminal subunit associates with proteins that help in preparing the chromatin for efficient transcription. The histone H4 lysine 16 specific acetyltransferase MOF loosens up the chromatin. The WDR5 protein recognizes the H3K4 methyl-mark introduced by MLL and ensures the processivity of histone modification. The proteins RBBP5 and ASH2L appear to be necessary for efficient methyltransferase activity.

The N-terminal domain MLL^N is essential for correct targeting of the MLL complex. It interacts with the proteins menin (the product of the tumor suppressor gene multiple endocrine neoplasia) and LEDGF (lens epithelium derived growth factor). In addition, MLL codes for several AT-hooks, which is a motif that binds to minor groove DNA, and preferentially recognizes DNA with distortions like bends or kinks. MLL also has a CxxC domain which binds specifically to unmethylated CpG dinucleotides. The N-terminal subunit also has a plant homeodomain (PHD). These domains of the MLL complex coordinate three major mechanisms of chromatin modification: methylation, acetylation and nucleosome remodeling.

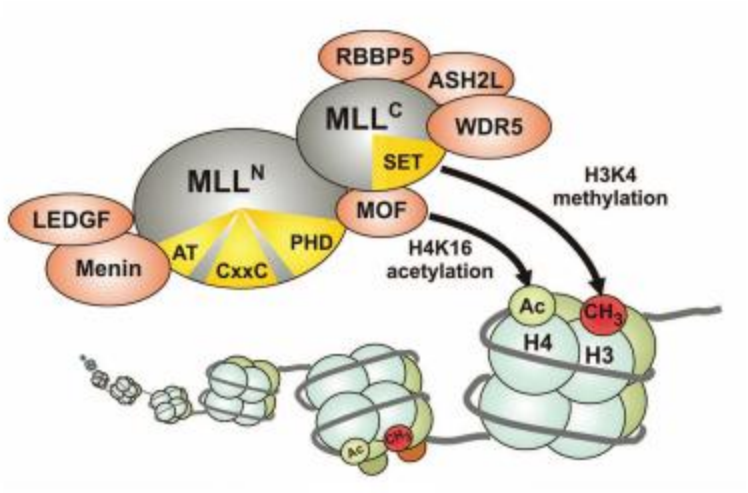
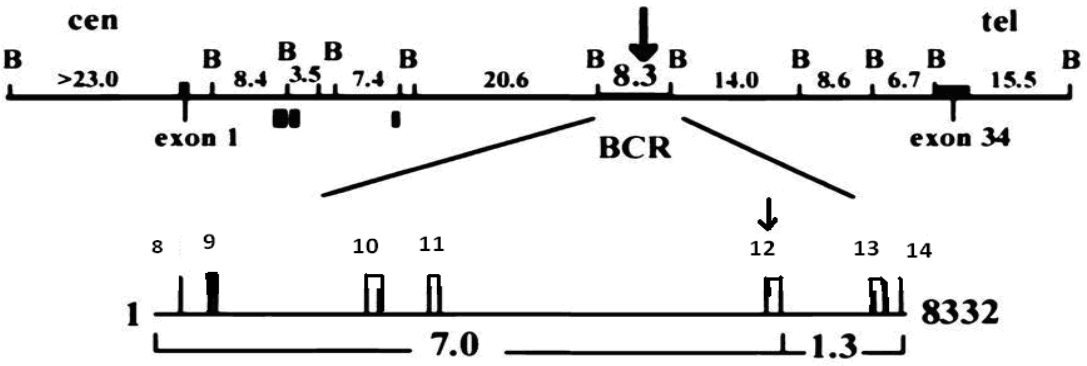


Figure 2: The MLL complex. After post-transcriptional proteolytic processing amino-terminal and carboxy-terminal portions of MLL are incorporated in a macromolecular complex with histone methyltransferase and histone acetyltransferase function. Functional domains in MLL are indicated in yellow. AT = AT-hooks, a DNA binding domain, CxxC = motif recognizing unmethylated CpG dinucleotides, PHD = plant homeodomain, SET = histone methyltransferase active site.

1.4 *MLL* gene structure

The *MLL* gene, also known as the *All-1* or *HRX* is present at chromosomal position 11q23 (Stanulla, Wang et al. 1997). The gene spans a length of ~120 kb, and is a major fusion partner of reciprocal chromosomal translocations involved in acute de novo and therapy-related leukemias affecting hematopoietic cells of lymphoid and myeloid lineages (Daser and Rabbitts 2005). The MLL protein is 500kD in size, is involved in transcriptional regulation and contains regions of high homology to the Drosophila trithorax protein. Virtually all *MLL* translocation breakpoints in patient samples occur in an 8.3 kb BamHI fragment, the bcr (Strissel, Strick et al. 2000) present between exons 8 to 14. An *in vivo* topo II cleavage site and a DNase I hypersensitive (HS) site colocalize within a high affinity scaffold associated region (SAR). Recent research has also shown the presence of two palindromic sequences, each 10 bp in size, ~101 bp apart and present

towards the 3' end of the bcr (Le, Singh et al. 2009) . A high affinity topo II binding site has shown to be present at the geometric midpoint of this palindromic DNA(Le, Singh et al. 2009). This specific region of *MLL* present at the intron 11 and exon 12 borders is also a DNase I hypersentitivity target(Le, Singh et al. 2009). It has been postulated that these in cis elements promote the cleavage and eventual rearrangements that initiate within the *MLL* bcr. In addition, the *MLL* bcr is AT rich and contains numerous Alu elements that may promote open chromatin structure and recombination events during repair of DSBs or stabilized topo II cleavage sites.



Strick R et al. PNAS 2000;97:4790-4795

Figure 3: MLL bcr with the exons indicated. The bcr is within an 8.3 kb BamHI (B) fragment and is frequently involved in translocations. The arrow at exon 12 shows the presence of a strong topo II cleavage site (Strissel, Strick et al. 2000).

1.5 Impact of 11q23 translocations and MLL fusion proteins

As mentioned earlier in the introduction, *MLL* has been found in 73 different translocations and 54 partner genes have been cloned so far. An aberrant non-homologous end joining (NHEJ) process most likely results in these illegitimate translocations. All MLL fusion proteins share a common structure where the fusion

partner is fused in frame to MLL^N after the CxxC domain but excluding the PHD fingers. Studies have suggested that the leukemogenic function of these MLL fusion proteins can be activated in four different ways (Slany 2009):

1. Transcriptional elongation and histone methylation (fusion partners ENL, AF9, AF4, ELL, and AF10)

There is a protein complex known as EAP (ENL associated protein complex) where ENL is not only linked with all members of the AF4 protein family that occur as MLL fusion partners, but also with positive transcription elongation factor b (pTEFb) and the histone methyltransferase DOT1L. pTEFb phosphorylates the carboxy-terminal repeat domain of RNA polymerase II, and this activity is essential for efficient transcriptional elongation. DOT1L methylates H3K79, which also happens during transcriptional elongation.

AF10, ENL, ELL fusion proteins with MLL have all been shown to interact or participate in these two processes of histone methylation and transcription elongation. Thus these common fusion partners with clinical importance participate in the same biological process for control of transcriptional elongation. MLL fusions might recruit EAP to genomic loci to induce ectopic target gene expression.

2. Histone acetylation (fusion partners CBP and p300)

A few cases of therapy induced secondary leukemia present MLL fusions with the histone acetyl-transferases CREB binding protein (CBP) and the related p300. The bromo- and histoneacetyltransferase domains of CBP are necessary and sufficient for the oncogenic function of the respective fusion proteins. The permanent HAT activity results in a hyperacetylation of chromatin and activated transcription.

3. MLL-EEN arginine specific histone methylation

The EEN fusion partner was cloned from a single case of mixed lineage leukemia. EEN was demonstrated to be bound to the arginine methyltransferase PRMT1 through the adaptor protein SAM68. PRMT1 is an arginine specific methyltransferase that also methylates histone H4 at arginine 3. This modification is correlated with an increased histone acetylation. This could lead to an activation of transcription in the same way as mentioned for CBP or p300 above.

4. Cytoplasmic fusion partners and dimerization

These fusions are found preferentially in older patients and seem to be more weakly transforming as compared to fusions with nuclear proteins. MLL fusions with the cytoplasmic proteins GAS7, AF1p and AF6 have been studied and the minimally necessary contributions of the respective fusion partner have been localized to coiled-coiled dimerization domain. All MLL fusion proteins are imported to the nucleus because of the strong nuclear import signals in MLL. This leads to aberrant protein-protein interactions of the fusions partners and aberrant activation of pathways.

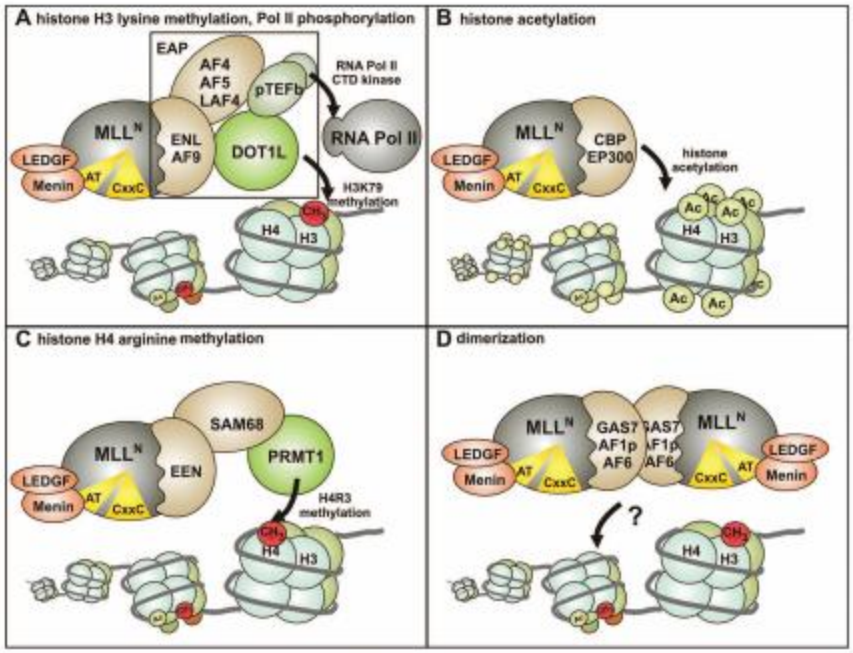


Figure 4: Molecular pathways leading to oncogenic activity of MLL fusion proteins. MLL fusions are aberrant transcription factors that activate gene expression. Four different mechanisms have been suggested as to how fusion partners might induce transcriptional activation. (A) The most frequent fusion partners of the ENL and AF4 family are members of the EAP complex that combines histone H3K79 methyltransferase activity catalyzed by DOT1L with transcriptional elongation stimulation by pTEFb (positive transcription elongation factor b, a dimer of CDK9 and a cyclinT) that phosphorylates the C-terminal repeat domain of RNA polymerase II. It is speculated that MLL fusion proteins aberrantly recruit this complex to target chromatin. (B) Active histone acetyltransferases are fused to MLL in the MLL-CBP and MLL-p300 proteins. (C) MLL-EEN indirectly recruits the histone H4R3 arginine methyltransferase through binding of the adaptor SAM68. (D) Dimerization of MLL via coiled-coiled or other dimerization domains supplied by the fusion partner activates target genes by unknown mechanisms.

These are the different mechanisms of activation of MLL fusion proteins. Deregulation of Hox genes is the most important factor for MLL fusion induced leukemogenesis. HOX proteins, especially HOXA9, and its dimerization partner MEIS1, are the major hematopoietic proteins that are overexpressed in a wide variety of different leukemias. HOX expression is high in hematopoietic stem cells and early precursor cells

and needs to be down-regulated for normal hematopoietic differentiation and maturation. Therefore, a continuous ectopic HOX expression blocks normal differentiation and creates a rapidly proliferating pre-leukemic precursor pool which can undergo secondary mutations to lead to acute leukemia.

1.6 *MLL* and *AF9* translocations

AF9 is one of the most common translocation partners for *MLL* (Dimartino and Cleary 1999, Ayton and Cleary 2001, Lightfoot and Roman 2004). Sequence analysis of *MLL-AF9* translocation breakpoints from patient-derived tumor samples has shown small deletions and insertions, as well as larger deletions, duplications, and more complex rearrangements (Gu, Alder et al. 1994, Strissel, Strick et al. 1998, Whitmarsh, Saginario et al. 2003).

1.7 *AF9* gene structure

The *AF9* gene, also called *LTG9* or *MLLT3*, spans >100kb and is present at chromosomal position 9p22. *AF9* is a common fusion partner of the *MLL* gene (Strissel, Strick et al. 2000, Alonso, Longo et al. 2008). The *MLL-AF9* fusion gene is associated with AML, ALL as well as with t-AML (Strissel, Strick et al. 2000). The translocation, denoted by t(9;11)(p22;q23), is the third most frequently reported abnormality in infant ALL cases (Alonso, Longo et al. 2008). *In vitro* and *in vivo* studies have shown that the *MLL-AF9* fusion gene plays an important role in stem cell development and leukemogenesis (Corral, Lavenir et al. 1996, Dobson, Warren et al. 1999). Leukemias that carry 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 and survival rates (Elliott and Jasin 2002). This is especially true with infant leukemias.

At present, the specific function of the AF9 gene is unknown. However, the mouse homolog of human AF9 is suggested to have a role in embryo patterning via Hoxd4 gene expression (Collins, Appert et al. 2002, Daser and Rabbitts 2005). The AF9 gene has two bcr regions identified within BamHI sites; bcr1 within intron 4 and bcr2 spanning introns 7 and 8 (Strissel, Strick et al. 2000). A topo II cleavage site and a DNaseI hypersensitivity site are located within intron 7 in the bcr2. Two SARs border both breakpoint regions (Strissel, Strick et al. 2000). These breakpoint and topo II cleavage sites were identified from clinical samples of t-AML, infant AML and adult ALL patients (Negrini, Felix et al. 1993).

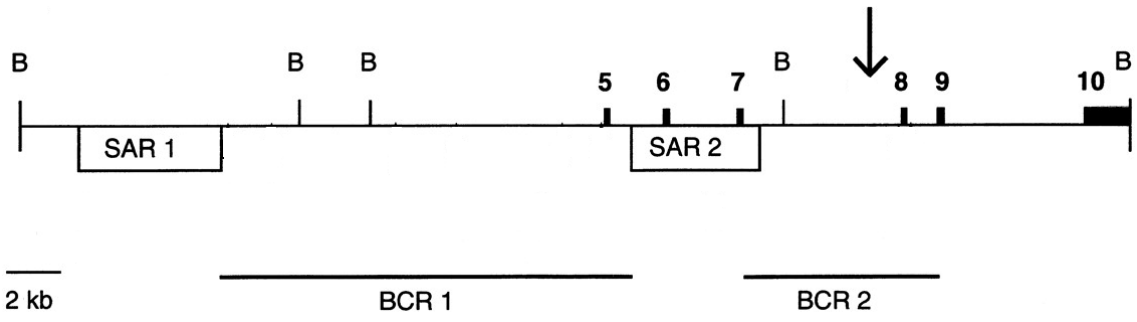


Figure 5: Human *AF9* gene with the bcrs shown. The numbered bars on the map indicate the position of exons. BamHI sites (B) and the SARs are represented. The arrow indicates the location of the topo II cleavage site.

1.8 Topo II poisons and chemotherapeutic agents lead to *MLL-AF9* translocations

I am interested in investigating the possible causes that lead to *MLL-AF9* translocations. One of the numerous possible factors that can lead to these translocations are topoisomerase II (topo II) poisons. DNA topoisomerases are essential cellular

enzymes that cause topological changes in the DNA for processes like replication, transcription etc. They are the targets of many important anticancer and antibacterial drugs. Topo II is targeted and inhibited by the widely used anticancer agents etoposide, doxorubicin, daunorubicin and mitoxantrone (Pommier, Leo et al. 2010). These topo II poisons target the DNA-protein complex. These compounds inhibit the religation of the transient DSBs made by topo II and this can lead to chromosomal abnormalities. The induction of DSBs is associated with chromosomal rearrangements, especially of the *MLL* gene which is frequently rearranged in chemotherapy-related leukemia. These breakpoints and rearrangements are similar to those that are found in infant leukemias due to exposure to topo II inhibitors such as these chemotherapeutic drugs.

The widely used anticancer drug etoposide has been reported to initiate *MLL* rearrangements in primitive hematopoietic stem cells and in human fetal hematopoietic stem cells in several studies (Libura, Slater et al. 2005, Moneypenny, Shao et al. 2006, Sung, Libura et al. 2006, Libura, Ward et al. 2008). Etoposide induces chimeric *MLL* gene fusions in mouse embryonic stem cells (Blanco, Edick et al. 2004). Other anticancer drugs including etoposide, teniposide, anthracyclines and dactinomycin are associated with *MLL* rearrangements due to topo II inhibition and enhanced DNA cleavage leading to defective DNA repair. Thus, these potent inhibitors induce topo II to cause chromosomal breakage and the repair of these breaks leads to translocations (Felix 2001).

1.9 Do other topo II inhibitors promote *MLL-AF9* translocations?

Studies have indicated that certain dietary compounds may have the potential to cause illegitimate DNA repair which can have important consequences for cancer development and other diseases. Bioflavonoids comprise a diverse group of polyphenolic

compounds. The most common sources of these bioflavonoids are fruits, vegetables, soy, tea, coffee and wine (Ross 2000). Due to their antioxidant capacity, they 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, they have been shown to potently suppress the activity of the essential cellular enzyme topo II.

High levels of genistein in maternal diet during pregnancy have been associated with the development of infant leukemia. Evidence suggests that topo II is implicated in genistein-induced infant leukemia. It has been reported that genistein induces DNA sequence rearrangements that are mediated by topo IIβ and the proteasome (Azarova, Lin et al. 2010). This study was carried out *in vitro* using purified recombinant human topo II enzymes as well as in cultured mouse myeloid progenitor cells (32Dc13) and Top2β knockout mouse embryonic fibroblasts (MEFs). It was shown that 100 μM genistein efficiently induced topo II-DNA cleavage complexes in both the systems and it was suggested that these complexes are processed by proteasome which led to chromosome rearrangements. This is evidence that both topoII and proteasome play a major role in genistein-induced infant leukemia.

Exposure of the developing fetus to such compounds during pregnancy increases the risk of infant leukemia. The first study that dietary bioflavonoids have the potential to induce cleavage in the *MLL* gene which could lead to infant leukemia tested 20 different bioflavonoids (Strick, Strissel et al. 2000). *MLL* cleavage was shown to be induced both *in vivo* in primary human progenitor hematopoietic cells from healthy newborns and adults as well as *in vitro* in hematopoietic progenitor cell lines (BV173 and K562). These

compounds caused inhibition of topo II enzyme in different doses. These results were the first to suggest that maternal ingestion of these compounds could lead to infant leukemia by inducing chromosome translocations.

Importantly, a study has shown that synthetic flavonoids are able to cross the placenta in the rat and are found in all fetal tissues (17% of the initial dose) including the fetal brain (Schroder-van der Elst, van der Heide et al. 1998). A synthetic bioflavonoid EMD-49209 was radioactively labeled and its maternal and fetal distribution was investigated in this study 1-24 h after intravenous injection in Wistar rats that were 20 days pregnant.

Genistein has been reported to induce chromosome aberrations similar to those found in infant leukemia (Abe 1999). Genistein is abundant in soybeans and is a potent topo II inhibitor. Cultured human lymphocytes treated with 50 μM genistein display chromosome abnormalities in metaphase karyotypic analyses. Topo II inhibition by different bioflavonoids was investigated in an *in vitro* plasmid DNA cleavage assay using purified recombinant wild-type human topo IIα and IIβ (Bandele and Osheroff 2007) where it was shown that these compounds were active against topo IIβ. Genistein (50 μM) was shown to be the most effective of the bioflavonoids tested and stimulated enzyme-mediated DNA cleavage ~10-fold.

Benzene and Its Metabolites

Benzene is a ubiquitous pollutant and is one of the top production chemicals in the United States. 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. It has been reported that it causes DNA damage through its metabolites,

especially 1,4-benzoquinone (Lindsey, Bromberg et al. 2004). This study reported that 1,4 benzoquinone is a strong topo II poison *in vitro* using purified human topo IIα in a plasmid DNA cleavage assay. It stimulates DNA cleavage by topo II ~ 8-fold at a concentration of 25 µM, and at sites close to defined chromosome breakpoints in leukemia. Benzene and its metabolites including benzoquinone also increase the homologous recombination in a Chinese hamster ovary (CHO) cell line due to generation of oxidative stress (Winn 2003). A CHO cell line containing a neo direct repeat recombination substrate underwent increased recombination (2.7-, 5.5-, or 6.9-fold) with exposure to 1, 10, or 30 µM benzoquinone respectively. Benzene metabolites 1,4-BQ (1-10 µM) or 1,4-HQ (10-100 µM) cause DNA damage and fragmentation, as detected by pulsed-field gel electrophoresis in cultured HL60 cells though the generation of H_2O_2 leading to apoptosis (Hiraku and Kawanishi 1996).

Other drugs and pesticides

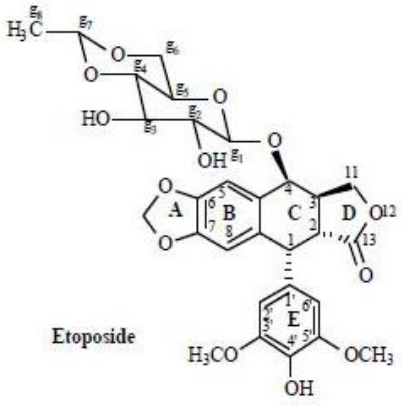
Use of certain other drugs and pesticides has also been reported to be associated with *MLL* gene fusions. A pilot case-control study (Alexander, Patheal et al. 2001) that investigated the risk of infant acute leukemia due to transplacental chemical exposure reported that several groups of drugs including herbal medicines, the nonsteroidal anti-inflammatory drug, dipyrone and exposure to pesticides like mosquitocidals including Baygon were associated with causing *MLL* gene fusions. This study was done across different countries and ethnic groups. 136 population-based cases and 266 controls were included in most centers. *MLL* rearrangement status was derived by Southern blot analysis, and maternal exposure data were obtained by interviews using a structured questionnaire.

Maternal use of dipyrone during pregnancy was shown to be associated with infant acute leukemia (IAL) in a hospital-based case-control study conducted in different cities of Brazil (Pombo-de-Oliveira, Koifman et al. 2006). A total of 202 children with newly diagnosed IAL (infant acute leukemia) were enrolled along with 440 age-matched controls. The magnitude of the odds ratio of 1.45 for maternal exposure to dipyrone suggested the occurrence of interactions between dipyrone exposure during pregnancy and *MLL* rearrangements, leading to IAL development.

I am interested in investigating the potential of the compounds mentioned in this section to induce *MLL* translocations.

1.10 Structures of topo II inhibitors used in this study

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 (Figure 6) (Baldwin and Osheroff 2005, Wilstermann, Bender et al. 2007, Bender, Jablonksy et al. 2008). The binding of etoposide to topo II is driven by interactions with the A-ring and the B-ring (Bender, Jablonksy et al. 2008). The E-ring is important for etoposide function (Wilstermann, Bender et al. 2007). Alterations in the E ring dramatically impair etoposide ability to enhance DNA cleavage mediated by topo II since every substituent on the E ring is intimately associated with the topo II protein in the binary complex (Wilstermann, Bender et al. 2007, Bender, Jablonksy et al. 2008). The glycosidic moiety of etoposide does not contact the enzyme and it is not clear if the moiety plays any role in enhancing topo II mediated DNA cleavage (Wilstermann, Bender et al. 2007, Bender, Jablonksy et al. 2008).



Baldwin and Osheroff. Curr. Med. Chem. - Anti-Cancer Agents 2005; 5: 363-372

Figure 6: Etoposide structure showing the rings A, B, C, D and E, and the glycosidic moiety at position C4.

Multiple bioflavonoid compounds such as genistein and quercetin have also been shown to inhibit topo IIα and topo IIβ activity (Bandele and Osheroff 2007). Both these compounds have been shown to induce *in vitro* cleavage in the *MLL* gene and contribute to infant leukemia (Strick, Strissel et al. 2000). Bioflavonoids are divided into three main groups: flavones, flavonols, and isoflavones. The 5-OH in genistein plays an important role in mediating genistein binding to topo II and the 4`-OH plays a significant role in genistein function (Figure 7) (Bandele and Osheroff 2007). Etoposide, genistein and quercetin contain pendant rings that feature a 4`-OH group that is essential for drug action (Figures 6 and 7) (Long, Musial et al. 1984, Elsea, McGuirk et al. 1993, Bandele and Osheroff 2007).

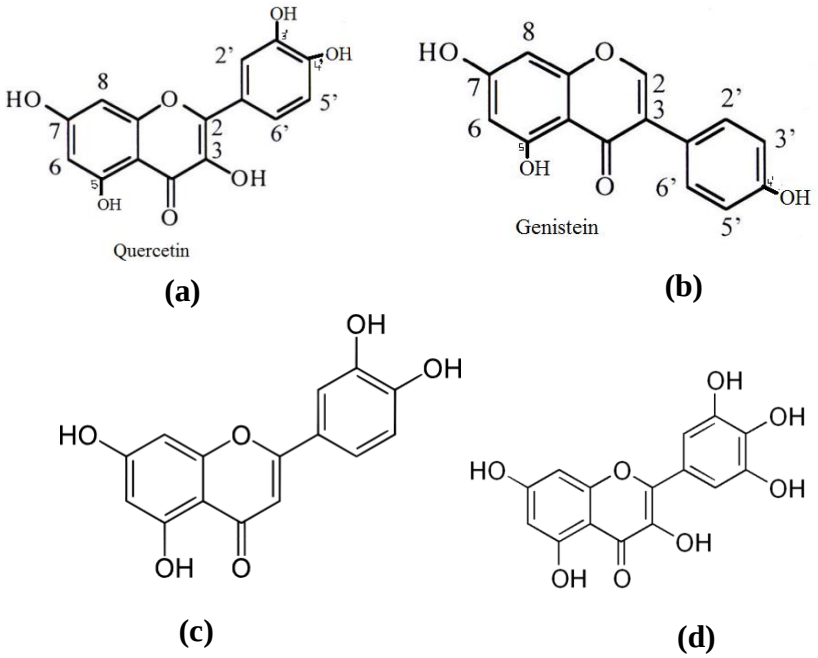


Figure 7: Quercetin (flavonol) (**a**), genistein (isoflavone) (**b**), luteolin (flavone) (**c**), and myricetin (flavonol) (**d**) structures. The 4`-OH group in the pendant ring is important in genistein function and the 5-OH mediates binding to topo II (Bandele and Osheroff 2007).

In addition to etoposide and bioflavonoids, two other compounds – dipyrone and benzoquinone were included in the study for their distinct mechanisms of action.

Dipyrone is a nonsteroidal anti-inflammatory drug and its use has been shown to be associated with *MLL* gene fusions as described above.

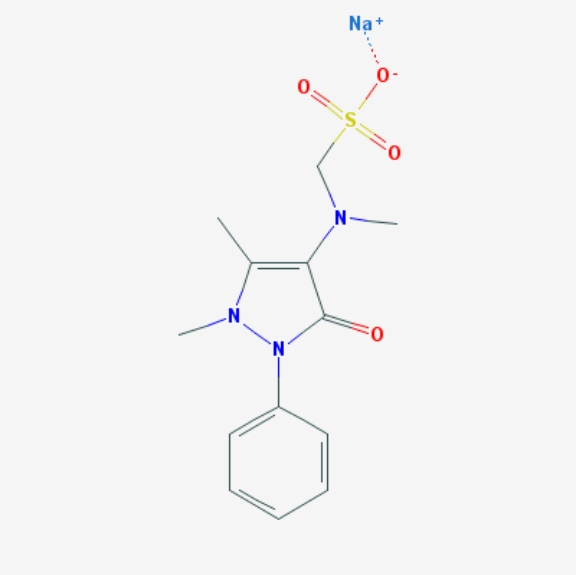


Figure 8: The structure of dipyrone

Benzoquinone has been shown to be a strong topo II inhibitor in *in vitro* studies as described above. It is a non-bioflavonoid thought to have a distinct mechanism of action, and is clinically associated with non-*MLL*-rearranged leukemias.

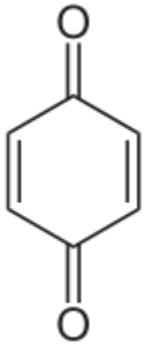


Figure 9: The structure of benzoquinone

1.11 DNA repair of DSBs

A possible mechanism by which these topo II inhibitors cause *MLL-AF9* translocations is by inducing DSBs in the DNA. DSBs are highly recombinogenic, increasing the exchange of information between two DNA duplexes by several orders of

magnitude; thus mammalian cells are at risk for rearrangements arising during DSB repair (van Gent, Hoeijmakers et al. 2001, Elliott and Jasin 2002)

The major DNA repair pathways include homologous recombination (HR) and non-homologous end joining (NHEJ). HR involves using a homologous sequence, usually on the undamaged sister chromatid or homologous chromosome as a template to repair the DNA damage (San Filippo, Sung et al. 2008). It has the highest potential for accurate repair (Essers, van Steeg et al. 2000). One of the models of HR repair is called double-strand break repair (DSBR) (Szostak, Orr-Weaver et al. 1983).This pathway involves (a) DSB creation, (b) processing of the broken ends to result in 3' single strand ends, (c) recombinase filament formation on the ssDNA ends, (d) strand invasion into a homologous sequence to form a D-loop intermediate, (e) DNA polymerase extension of the invading strand, (f) annealing of the extended strand to capture the second DSB end, (g) formation of two Holliday junctions (HJ) and (h) resolution of the HJs resulting in crossover or non-crossover products (San Filippo, Sung et al. 2008). Synthesis-dependent strand annealing (SDSA) is another model of HR in which after DNA strand invasion and repair DNA synthesis, the invading strand is displaced and anneals with the second DSB end (Strathern, Klar et al. 1982, Nassif, Penney et al. 1994, Ferguson and Holloman 1996, San Filippo, Sung et al. 2008). In the single strand annealing (SSA) method, the DSB ends are resected and the single strand resected ends basepair and anneal to each other independent (Symington 2002) of strand invasion into a homologous sequence (Lin, Sperle et al. 1984, Fishman-Lobell, Rudin et al. 1992, Symington 2002).

The HR processes are mediated by several factors. The MRN complex comprising of Mre11, Rad50 and Nbs1 is involved in DNA binding and DSB end resection (Sung

and Klein 2006). Replication Protein A (RPA) binds to the newly resected ssDNA with high affinity and removes secondary structures (Sugiyama, Zaitseva et al. 1997). The Rad 51 protein forms a complex with Rad 52, and displaces RPA from the ssDNA, forming a presynaptic filament (Milne and Weaver 1993). BRCA2 binds DNA, physically interacts with Rad51 and is needed for the formation of DNA damage induced Rad51 foci (Yuan, Lee et al. 1999, Yang, Li et al. 2005, San Filippo, Chi et al. 2006). Rad54 and Rad54B associate with Rad51, has dsDNA-dependent translocase activity, stimulates the D-loop reaction to initiate DNA repair synthesis and has the ability to remove Rad51 from dsDNA (Tanaka, Kagawa et al. 2002, Tan, Kanaar et al. 2003, Heyer, Li et al. 2006, Wesoly, Agarwal et al. 2006, Zhang, Fan et al. 2007, San Filippo, Sung et al. 2008).

NHEJ, on the other hand, requires minimal or no sequence homology to repair damage, and is usually not error free (Essers, van Steeg et al. 2000). Nucleases such as FEN-1 exonuclease are involved in end-processing of the DSB break, before ligation of the broken ends, resulting in loss of nucleotides (Pastwa and Blasiak 2003). DNA polymerases μ and λ, involved in NHEJ, can slip back on their template strand during synthesis, resulting in direct repeats (Dominguez, Ruiz et al. 2000, Jager, Bocskor et al. 2000, Welzel, Le et al. 2001, Bebenek, Garcia-Diaz et al. 2003). Template independent addition of nucleotides by pol μ sometimes folds back on itself to form a stem-loop structure that can act as a primer/template substrate (Ramadan, Shevelev et al. 2004, Gu, Lu et al. 2007, Lieber 2010). This will result in inverted repeats at the NHEJ junctions, and has been observed in chromosomal translocations in humans (Jager, Bocskor et al. 2000, Welzel, Le et al. 2001). NHEJ mode of DNA repair is presumed to occur in the formation of translocations observed in AML (Acute Myeloid Leukemia) and ALL

(Acute Lymphoid Leukemia) patients, because of the absence of significant homology at mapped breakpoint junctions (Weinstock, Elliott et al. 2006).

NHEJ is initiated by the binding of the Ku 70/80 heterodimer to the ends of the DSB (Mimori and Hardin 1986, Blier, Griffith et al. 1993, Weterings and Chen 2008). The Ku-DNA complex recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in order to form a synaptic complex bridging the DSB ends (Ma, Lu et al. 2004, Weterings and Chen 2008). Artemis, forming a complex with DNA-PKcs, can endonucleolytically cut DNA overhangs (Ma, Pannicke et al. 2002). DNA polymerases μ and λ can add in nucleotides; polymerase μ can perform template independent synthesis (Sonoda, Hochegger et al. 2006). The Ligase IV/XRCC4/XLF complex catalyses the ligation reaction of the processed DNA ends (Gu, Lu et al. 2007, Gu, Lu et al. 2007, Weterings and Chen 2008).

1.12 The connection between DSBs and epigenetic alterations

The bioflavonoid compounds that induce DSBs due to topo II inhibition are also implicated in epigenetic changes. Epigenetic changes such as chromatin remodeling and histone-specific modifications control DSB detection, signaling and repair. The factors that influence this complex process have been reviewed (Pandita and Richardson 2009). After damage to the DNA, 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 histone H1; acetylation of H2A, phosphorylation and ubiquitination of H2AX; acetylation and methylation of H3; and phosphorylation and acetylation of H4. 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.

It has been demonstrated that heterochromatic DSBs are generally repaired more slowly than euchromatic DSBs (Goodarzi, Noon et al. 2008). Studies have shown that HAT (histone acetyl transferase) complexes act with the ATP-dependent SWI/SNF and RSC-containing chromatin remodeling complexes to facilitate DNA repair (Kimura and Horikoshi 1998).

Heterochromatin (HC) is the more tightly compacted DNA structure acts as a barrier for some DNA repair processes. The role of ataxia telangiectasia (ATM) and its damage response mediator proteins in overcoming the constraints posed by heterochromatin superstructure in DNA repair has been reviewed (Goodarzi, Jeggo et al. 2010). Following DSB induction, the heterochromatin undergoes a series of events to implement the dynamic and localized changes that are necessary for DSB repair. DSB repair may be stalled within HC regions if these changes fail to take place. KAP-1 co-repressor and HP1 chromodomain protein are two HC factors that are targets of the DNA damage response. Activation of the ATM signaling pathway and the subsequent phosphorylation of KAP-1 trigger the HC modifications required for DSB-repair.

Some of the key epigenetic regulatory processes involved in DSB repair have been reviewed (Orlowski, Mah et al. 2011). This review also discusses the potentially pathological repercussions of incomplete or incorrect restoration of chromatin structure resulting in a DSB-induced epigenetic memory of damage. Phosphorylation of histone variant H2AX generates γH2AX which spreads over large chromatin domains surrounding the DSB. This leads to large-scale chromatin reorganization within the DSB

vicinity to recruit repair factors, recombination proteins and chromatin remodeling complexes involved in NHEJ and HR repair pathways. Phosphorylation of histones H2B and H4 also occur in DSB repair response. Histone acetylation, methylation and ubiquitination are the other histone modifications that take place, also mentioned above. DNA methylation, which is a significant epigenetic mark of transcriptional inactivity, also plays a role in DSB damage response. A DSB, in the course of repair, can occasionally leave a remnant in the form of an epigenetic mark. If such incorrect epigenetic modifications are mitotically stable, and if they relate to tumor suppressor silencing or the ectopic expression of an oncogene, they could play a role in carcinogenesis.

Polycomb group proteins, which have well-established roles in gene regulation, were recently found to accumulate on chromatin surrounding DNA damage (Gieni, Ismail et al. 2011). Polycomb group proteins (PcG) are a family of proteins that form complexes involved in the epigenetic regulation of gene expression. Polycomb repressive complexes (PRC) catalyze post-translational modifications critical to their gene silencing function, including histone H3K27 trimethylation and histone H2A ubiquitination. This complex may also be involved in DNA methylation. PcG and PRC components found to respond to DNA damage include BMI-1, MEL-18, EZH2 methyltransferase, EZH1, EED, SUZ12. Gene silencing activity of PcG proteins, like mono-ubiquitylation of H2A is exploited during DSB repair.

Chromatin dynamics at DSBs exert a powerful influence on the process of DSB repair (Xu and Price 2011). Heterochromatin and euchromatin utilize distinct remodeling complexes and pathways for DSB repair. 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.

The recent advances in the understanding of the interplay between chromatin remodeling and DNA damage response have been reviewed (Lai, Li et al. 2013). Signal transduction pathways in DNA damage response (DDR) communicate with chromatin-remodeling factors. The p53 signaling pathway responds to DNA damage by regulating DNA repair. In addition to DNA repair, the p53 signaling pathway is also associated with chromatin structure changes that mainly involve the histone acetyl transferase Tip60 following DNA damage. Numerous chromatin-remodeling factors that are involved in DNA methylation and demethylation also play a role in DDR. In the thymus, genotoxic stress exposure decreases DNA methylation globally by a reduction in the levels of enzymes involved in DNA methylation such as DNA methyltransferase 1 (DNMT1), DNMT3a, DNMT3b and methyl-binding proteins MeCP2 and MBD2. Several chromatin-remodeling factors form a complex with DDR-related proteins in response to DNA damage. Scaffold matrix attachment region 1 (SMAR1) binds other SMAR1 elements along with HDAC1 and p53, forming a repressor complex to downregulate transcription. The chromatin-remodeling factor Tip49 recruits Rad51 to DNA damage sites.

1.13 Long-term impact of chromatin remodeling and DNA damage in stem cells induced by environmental, dietary and therapeutic agents

In addition to the topo II inhibitors mentioned above, there are several other agents that have the potential of causing genetic and epigenetic alterations in stem/progenitor cells. These have been discussed in appendix II (Bhawana Bariar and

Christine Richardson, "Long-term impact of chromatin remodeling and DNA damage in stem cells induced by environmental, therapeutic and dietary agents" *manuscript in review*).

Commonly occurring environmental agents, and commonly used dietary and therapeutic agents can induce several different changes at the cellular and molecular levels. Some of the examples of environmental agents that have been shown to alter epigenetic modifications are metals, UV radiation, bisphenol A, alcohol, aldehydes, and cigarette smoke. Among dietary agents, many plant-derived compounds e.g. bioflavonoids are being used as dietary supplements which have the potential of causing epigenetic changes in the chromatin. Therapeutic agents such as drugs used in the treatment of cancer, estrogens used in hormone supplement therapy can induce such changes as well. Exposure to these agents can induce epigenetic alterations by histone modification, changes in cell differentiation, modulation of cellular signal transduction pathways including DNA damage repair pathways, changes in gene expression, illegitimate DNA repair leading to genome rearrangements and carcinogenesis. Multiple lines of evidence through studies in *in vitro* and *in vivo* models have shown that *in utero* exposure to environmental toxicants can cause epigenetic modifications which in turn induce alterations in gene expression that may persist throughout life as reviewed (Singh and Li 2012). These modifications include DNA methylation, histone modification, and expression of non-coding RNAs (including microRNAs) and play a role in the etiology of complex diseases such as cancer and diabetes.

1.14 My thesis work

Infant acute leukemias account for ~30% of all malignancy seen in childhood across the Western world. They are aggressive and characterized by rapid onset shortly after birth. The majority of these (~80% ALL and ~60% AML) have rearrangements involving the *MLL* gene. Although *MLL* fusion to more than 75 genes have been identified, AF9 is one of its most common translocation partners. *MLL* breakpoint sequences associated with infant acute leukemia are similar to those in secondary AML following exposure to the topo II poison etoposide. This similarity led to the hypothesis that exposure during pregnancy to biochemically similar compounds may promote infant acute leukemia. Some studies have shown an epidemiological link between bioflavonoid intake and increased incidence of *MLL*-rearranged infant leukemias. These bioflavonoids have also been shown to inhibit topoII in *in vitro* DNA cleavage assays. Hundreds of unregulated nutritional supplements are widely available and perceived to prevent cardiovascular disease, inflammation and cancer. However, their potential to promote leukemic translocations should be determined.

The influence of hematopoietic-specific developmental programs on the repair of DNA damage such as DSBs and the initial molecular events that lead to translocations, which are a hallmark of leukemia, lymphoma and soft-tissue sarcomas is not well understood. The mechanisms by which specific translocations associated with leukemias occur remain controversial. Specific gene loci may be particularly susceptible to breakage or aberrant repair only when expressed or as open chromatin within certain hematopoietic subpopulations. Alternatively, these translocations may arise with equal frequency in all cell types, but specific malignant outcome results due to preferential selection or survival.

Clinical parameters and tumor pathology of multiple tumor types has suggested that stem cells are the target of the initiating lesion. Stem cells could act as tumor initiators because they possess a greater capacity for proliferation into multiple lineages even if the initial event is no more likely in any particular cell, or because they are more susceptible than more differentiated cell types to the initial acquisition of genome alterations. The survival and stability of the cells that acquire these alterations is another important factor. It is also not known whether the genome rearrangements characteristic of leukemia and lymphoma arise as part of normal DSB repair processes or if they require signaling pathways specific to the DNA damaging agents discussed above.

A model system to directly examine the relative role of specific repair pathways to prevent or promote specific genome rearrangements has been lacking. There is preliminary association between bioflavonoids in the generation of specific infant leukemias, and multiple bioflavonoid compounds have been shown to inhibit topo II activity and promote DNA cleavage. However no genetic assay yet has been able to definitively determine the relative potential for each of these compounds to promote AML associated translocations. My goal was to test the hypotheses that 1) *MLL* and AF9 bcrs undergo DSBs, and are highly recombinogenic independent of chromosomal context, and in the absence of a leukemic fusion protein; and 2) a number of topo II inhibitors have the potential to promote these translocations analogous to those observed in infant leukemia. I demonstrate here the sensitivity of the *MLL* and *AF9* bcrs to topo II poisons and bioflavonoids independent of their normal chromatin context and independent of the formation of a leukemic fusion protein. This genetic assay does not

rely on IPCR that requires elimination of artifacts or less physiologically relevant internal deletions or intronic alterations from analysis.

Chapter 3 describes the establishment of a reporter gene model system to screen for the frequency of *MLL-AF9* translocation events. I developed reporter mouse embryonic stem cell lines that contain two transgene constructs- (1) the *MLL* bcr fragment containing a genetically-engineered GFP 5' exon, and (2) the AF9 bcr containing a genetically-engineered GFP3' exon. A translocation between the two bcrs reconstitutes the full-length GFP transcript resulting in quantifiable green fluorescence. The technical details are presented in chapter 2 which describes the materials and methods.

Chapter 4 describes the quantification of GFP+ colonies after endonuclease I-SceI expression which induces directed DNA breaks in reporter cell lines. The optimum conditions for the model system and isolation of green fluorescent colonies by microscopy was established by inducing directed DSBs in the reporter transgene constructs at the specific I-SceI restriction sites that have been engineered in to the constructs. I-SceI expression in these cell lines created DSBs at these sites, leading to a translocation between the *MLL-5'GFP* and *AF9-3'GFP* reporter constructs leading to the expression of GFP protein and green fluorescence in cells that undergo this genomic rearrangement. The results describe the comparison of frequencies of appearance of GFP+ cells among three different and independently obtained reporter cell lines and the possible factors that could lead to different frequencies. Finally, the chapter includes the DNA sequence analysis of the repair junction of a GFP+ clone that was isolated and expanded after I-SceI expression. The sequence analysis indicates the use of non-

homologous end joining (NHEJ) repair pathway to repair the DSB created by I-SceI, thus resulting in *MLL-AF9* translocation.

Chapter 5 describes the quantification of GFP+ colonies after treating the reporter cell lines with varying doses of different topo II inhibitors including the chemotherapeutic drug etoposide and different bioflavonoid compounds which are found in our diet and are also used as nutritional supplements. The results include comparison of frequencies of appearance of GFP+ colonies among three different and independently obtained reporter cell lines and among the different compounds used and the possible explanations for these differences. Cells were treated with etoposide, quercetin, genistein, luteolin, myricetin, dipyrone, or benzoquinone for 1 hour at 25 µM - 200µM concentrations then allowed to repair and proliferate in culture. GFP+ fluorescent colonies as a result of *MLL-AF9* translocations were readily scorable by 96 hours in a dose-dependent manner.

As previously demonstrated, totipotent stem cell viability was extremely impaired by multiple concentrations of etoposide, but the surviving fraction exhibited a significant number of *MLL-AF9* translocations (frequency of 1.7×10^{-4}). Bioflavonoids genistein and quercetin (75 µM) were also potent promoters of *MLL-AF9* translocations in totipotent stem cells at roughly similar frequencies as etoposide. The significance of this model system is apparent from treatment of stem cells with benzoquinone that was not sufficient to produce *MLL-AF9* translocations following exposure to concentrations up to 125 µM (frequency $< 0.1 \times 10^{-6}$). Benzoquinone is a non-bioflavonoid thought to have a distinct mechanism of action and clinically associated with non-*MLL*-rearranged leukemias. This system is a direct measure of the sensitivity of the *MLL* and *AF9* bcrs to

topo II poisons and bioflavonoids independent of their normal chromatin context and independent of formation of a leukemic fusion protein. In addition, the system allows for rapid and reproducible screening of hundreds of compounds that may have the potential to promote leukemogenic translocations in early stem cell and more differentiated cell subpopulations analogous to the events observed in infant acute leukemias.

CHAPTER 2: MATERIALS AND METHODS

The tables and figures referred to in this chapter have been included in the results chapters 3, 4 and 5. Please refer to those chapters for the tables and figures as appropriate.

2.1 Creation of transgene constructs

The green fluorescent protein (*GFP*) gene was engineered to contain an adenovirus 227 bp intron. This construct was then separated into two DSB repair substrate constructs. One construct contains the 5' half of the *GFP* gene (containing the promoter), a splice donor (SD), and an adenovirus intron sequence ligated to an I-SceI endonuclease recognition site. The second construct contains an I-SceI recognition site, and an adenovirus intron sequence, a splice acceptor (SA), and 3' half of the GFP gene. These DSB-inducible recombination reporter constructs were then engineered into human *MLL* and *AF9* bcrs. The first construct containing the 5' half of the *GFP* gene was inserted into intron 11 of the human *MLL* bcr BamHI-BamHI fragment. The locus of insertion was chosen based on its association with a high proportion of therapy-related leukemia translocation breakpoints. (Broeker, Super et al. 1996). The second construct containing the 3' half of the *GFP* gene was inserted into intron 8 of the human AF9 bcr2 region (Strissel, Strick et al. 2000). The regions where the *GFP* reporters were inserted were upstream (*5'GFP*) or downstream (*3'GFP*) of known strong topoII cleavage sites and mapped breakpoints identified in clinical samples of infant AML and t-AML cases.

Transgene constructs were prepared through standard restriction digestions and ligations using enzymes from New England Biolabs Inc. Restriction digests were performed on the backbone and insert sequences were ligated into the backbone. Cloning of these constructs was done using the TOPO TA cloning kit provided by Invitrogen according to the manufacturer's instructions and transforming competent E. coli TOP10 cells provided by Life technologies according to the manufacturer's instructions, growing the cells and harvesting plasmid DNA from them. The constructs were verified by agarose gel electrophoresis. Introduction of an I-SceI expression plasmid will lead to the formation of specific and unique DSBs at these engineered sites.

2.2 Generation of transgenic cell lines

The transgene constructs were electroporated into mouse embryonic stem (ES) cells (Richardson, Moynahan et al. 1998, Elliott, Richardson et al. 2005). The two constructs were randomly integrated into the genome of mouse ES cells through two separate rounds of electroporation. The electroporation protocol is as follows.

Mouse EtG2a ES cells growing in standard ES medium were washed with 1X PBS (phosphate buffered saline), treated with 0.25% trypsin for 10 minutes at 37^{o}C and centrifuged at 1000 rpm for 10 minutes. The cells were resuspended in 1X PBS and counted using a hemacytometer. Puromycin and neomycin resistance gene-containing plasmids were used as positive selection markers and were cotransfected with the transgene constructs. Random integration resulted in puro or neo expression selectable with the drugs puromycin or neomycin.

The *neomycin* plasmid was electroporated along with the *MLL* transgene into a single cell suspension of 20 million mouse ES cells. 24 hours post electroporation, the

media was changed to include G418. Upon growing in selection medium, surviving cells formed colonies (Richardson, Moynahan et al. 1998, Elliott, Richardson et al. 2005). The surviving colonies were identified, picked, grown and screened for the presence of a single copy of the *MLL* transgene by performing appropriate digests (BgIII/XbaI; BamHI/BglII; EcoRV) (New England Biolabs Inc.) followed by standard Southern Blots. The full length *GFP* gene was used as the probe. Single copy *MLL* clones were selected, grown and expanded (Table 1). One of the clones, *MLL*-F2, was selected and electroporated with the *AF9* transgene along with *puromycin* (Table 1). 20 million cells were used for the electroporation. 24 hours post electroporation, selection medium containing puromycin was used. Surviving colonies were picked, grown and screened for the presence of a single copy of the *AF9* transgene.

The isolated surviving clones were expanded in 96 well plates and screened by both PCR (using 5' primers GFP-bcl and GFP 5' intron 2; and 3' primers GFP-nco top and GFP-orf 3') and Southern blotting using appropriate digests (KpnI; BamHI; NcoI; BamHI/BglII; XhoI; PstI) (New England Biolabs Inc.) and the full length GFP gene as a probe to identify single copy clones. Selected clones were expanded and used for further experiments (Tables 1 and 2).

2.3 DSB induction in transgenic cell lines by I-SceI expression

To create translocation events between the two transgene constructs, DSBs were induced by ISceI expression through electroporation of the pCBASce vector which contains the *ISceI* gene in it (Richardson, Moynahan et al. 1998). Cell lines MAG 32, MAG 32A and MAG 68 were used for the electroporations (Table 3). All these reporter

cell lines have a single copy of the *MLL* and *AF9* transgene randomly integrated into the genome (Tables 1 and 2) (Figure 10).

In each experiment, three sets of electroporations were performed on 20 million cells per set. Out of the three cohorts, one had no DNA (negative control), one cohort had 40 µg of the *GFP* gene (NZE-GFP, P. Sung GFP #2) (positive control), and the third cohort with 20 µg of the I-SceI plasmid pCBASce. The electroporated cells were plated on 5 plates per cohort resulting in 4 million cells per plate. One plate per cohort was used to count the number of surviving cells 24 hours post electroporation, using a hemacytometer (Richardson, Moynahan et al. 1998, Elliott, Richardson et al. 2005). All plates from the three cohorts were examined each day for GFP expression using the fluorescent filter on an inverted microscope. Green fluorescent colonies seen were captured by Zeiss camera, isolated using a micropipette, expanded and examined each day for GFP expression. Genomic DNA from the colonies was isolated and used for PCR using primers GFP-bcl and GFP-orf 3' to check for the occurrence of a translocation between the *MLL* and *AF9* transgenes and thus amplification across the repair junction. Some of the PCR products were cloned in TOPO TA vector and sent for sequencing to Sequetech Corporation, California and the sequence was analyzed across the repair junction.

2.4 DSB induction in MAG cell lines by treatment with topo II inhibitors

10^7 cells (counted using Orflo Moxi Z automated cell counter) from the MAG cell lines were treated with different doses of the various topo II inhibitor compounds in 15 ml of ES cell medium on 100 cm^2 plates for one hour at 37^oC. The cells on each plate were washed with 1X PBS, trypsinized and replated on to three plates after centrifugation

at 1000 rpm for 10 minutes and resuspension in fresh ES cell medium. The plates from all the doses were washed with 1X PBS (twice daily on the first two days after treatment, and once every day after that), supplemented with fresh ES medium and observed every day using the fluorescent filter on the inverted microscope for any GFP expression and thus green fluorescence. Any green fluorescent colonies observed were captured by Zeiss camera fitted to the microscope, isolated using a micropipette, expanded and examined each day for GFP expression. Genomic DNA from the colonies was isolated and used for PCR using primers GFP-bcl and GFP- orf 3' to check for the occurrence of a translocation between the *MLL* and *AF9* transgenes and thus amplification across the repair junction. Some of the PCR products were cloned in TOPO TA vector and sent for sequencing to Sequetech Corporation, California and the sequence was analyzed across the repair junction.

2.5 Cell viability assays

1 X 10^6 EtG2a mouse ES cells each were plated onto 3 plates each per dose 0, 25, 50, 75, 100 and 200 µM) of a test compound. After treatment for one hour at 37^{o}C, the number of surviving cells were counted for each dose at 24, 36 and 72 hours using the automated Moxi cell counter. The cells were trypsinized for 10 min at 37^{o}C, resuspended, diluted and counted in a 75 µl volume. The percentage of surviving cells was plotted on a graph.

2.6 FACS (Fluorescence Activated Cell Sorting)

FACS was performed on the cells treated with different doses of etoposide. The FACSAria (BD Biosciences) machine was used to sort GFP+ cells. Selected plates were washed with PBS, trypsinized, centrifuged at 1000 rpm for 10 minutes and resuspended

in fresh ES cell medium. The cell suspension was filtered using sterile filter tubes before performing FACS on them. Sorted green fluorescent cells were plated onto 96 well plates at the rate of 5 events per well and allowed to expand. The survival of cells after FACS treatment was tabulated.

2.7 DNA extraction and analysis

ES cells on a plate were washed with PBS and trypsinized for 10 min. After trypsin treatment, the cells were resuspended by adding fresh medium and transferred to a conical tube. They were centrifuged at 1000 rpm for 10 min and the supernatant aspirated off. The cell pellet was treated with 0.5mg/ml of Proteinase K resuspended in DNA-PK buffer. The samples were placed at 60^{o}C overnight. The next day the volume was transferred to eppendorf tubes, an equal amount of phenol added, mixed by inverting and centrifuged at 13,000 rpm for 10 minutes. The top aqueous layer was then transferred to a new tube and the phenol wash repeated followed by a wash with phenol-chloroform mixture. DNA from the final aqueous layer was precipitated using 3% sodium acetate and 70% ethanol and the pellet was dissolved in TE buffer containing RNase. The tubes were placed at 37^{o}C for one hour and the concentration of DNA measured using a nanodrop. The extracted DNA was then used for PCR analysis and Southern blots as explained above.

CHAPTER 3: CREATION OF DOUBLE TRANSGENIC *MLL-AF9* GFP REPORTER CELL LINES

3.1 Generation of single copy double transgenic clones screened by Southern Blotting and PCR

The human *MLL* bcr-5'GFP-intron-I-SceI construct was electroporated into EtG2a mouse ES cells along with the *neomycin* reporter gene. The cells were allowed to grow in selection medium containing neomycin. A total of 123 surviving clones were identified (Table 1). A panel of restriction digests followed by Southern Blots was performed. Four clones were identified as having a single copy of the MLL transgene each. The clones were named *MLL*-13, *MLL*-G2, *MLL*-F2 and *MLL*-E8 (Table 1).

The *MLL*-F2 clone was selected, expanded and electroporations were performed using the human *AF9* bcr2-3'*GFP*-intron-*ISceI* construct along with *puromycin* as the selection marker. The cells were allowed to grow in selection medium containing puromycin. A total of 80 clones survived. A panel of restriction digests followed by Southern Blots was performed. Three clones were identified as having a single copy insert of the *AF9* transgene. The clones were named MAG 32, MAG 60 and MAG 68 (Table 1). Similar electroporations were also done on clone *MLL*-13 (Table 1).

MAG 32 did not show *MLL* on Southern reproducibly, so we electroporated *MLL* back into it using *hygromycin* as the selection marker to generate clone MAG32A (Table 1). Southern blotting was used to confirm the presence of both the transgenes (Figure 10).

Electroporations were again performed with either the human *MLL* bcr-5'*GFP*-intron-*ISceI* construct or the *AF9* bcr2-3'*GFP*-intron-*ISceI* construct on a few parental clones using the appropriate drug resistant markers for cotransfection (Table 1). This was done since these clones failed to show one of the transgenes in Southern blotting. The potential double transgenic clones were grown in selection medium, isolated and expanded for PCR analysis.

These clones were screened using PCR (using 5' primers GFP-bcl and GFP 5' intron 2; and 3' primers GFP-nco top and GFP-orf 3') for the presence of a single copy each of both the transgenes. The clones selected from the PCR screen were analyzed by Southern blotting after the appropriate restriction digests.

Table 1: **S**ingle copy clones screened by Southern blotting (SB) that contain *MLL* and *AF9* GFP reporter transgenes. MAG32 did not show *MLL* on Southern reproducibly, so *MLL* was electroporated back into it.

Transgene electroporated	Parental Cell Line	Drug Selection	# Screened by SB	Single Copy Clones
MLL-5'GFP-intron-ISceI	EtG2a	Neomycin	123	F2, G2, 13, E8
AF9-3'GFP-intron-ISceI	*MLL*-F2	Puromycin	80	MAG- 32, 60, 68
AF9-3'GFP-intron-ISceI	*MLL*-13	Puromycin	60	5, 6, 30
MLL-5'GFP-intron-ISceI	MAG 32	Hygromycin	48	MAG 32A

Table 2: Clones screened by PCR that contain *MLL* and *AF9* GFP reporter transgenes.

Transgene electroporated	Parental Cell Line	Drug Selection	# Screened by SB	Single Copy Clones
MLL-5'GFP-intron-ISceI	MAG60	Hygromycin	96	M7, M21, M25, M29, M31, M35
MLL-5'GFP-intron-ISceI	5	Hygromycin	44	M7, M6, M35
MLL-5'GFP-intron-ISceI	6	Hygromycin	12	None
MLL-5'GFP-intron-ISceI	30	Hygromycin	10	M2, M6, M8, M9, M5, M7
AF9-3'GFP-intron-ISceI	E8	Puromycin	96	5

Parental clones MAG60, 5, 6 and 30 were electroporated with both *MLL* and *AF9* using *neomycin* and *puromycin* markers but repeated Southern blots only showed the presence of AF9, so *MLL* was added back by electroporation using *hygromycin* as a selectable marker. 30M2 showed both transgenes in Southern. Clones 30M2 and 5M35 failed to grow when thawed from the frozen 96-well plates stored at -80^{o}C (Table 2).

Southern blotting was performed on MAG clones obtained after electroporations and drug selection as described above. Clones MAG 32A and MAG 32B showed both *MLL-GFP* and *AF9-GFP* transgenes in addition to MAG 32 and MAG 68 (Figure 10). However, MAG 32B was not used for future experiments since it failed to give any green fluorescent colonies after ISceI treatment.

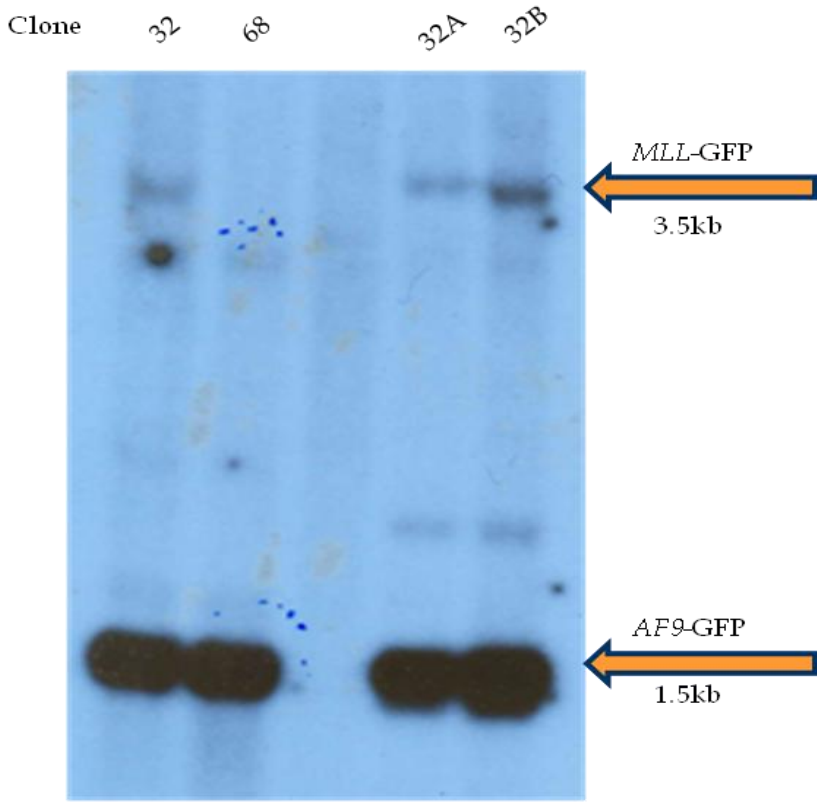


Figure 10: Southern blots of clones containing single copy inserts of the *MLL*-5'GFP reporter and the AF9-3'GFP reporter transgenes.

PCR screening as described above also confirmed the presence of *MLL* and *AF9* transgenes in some other clones mentioned in table 2 above.

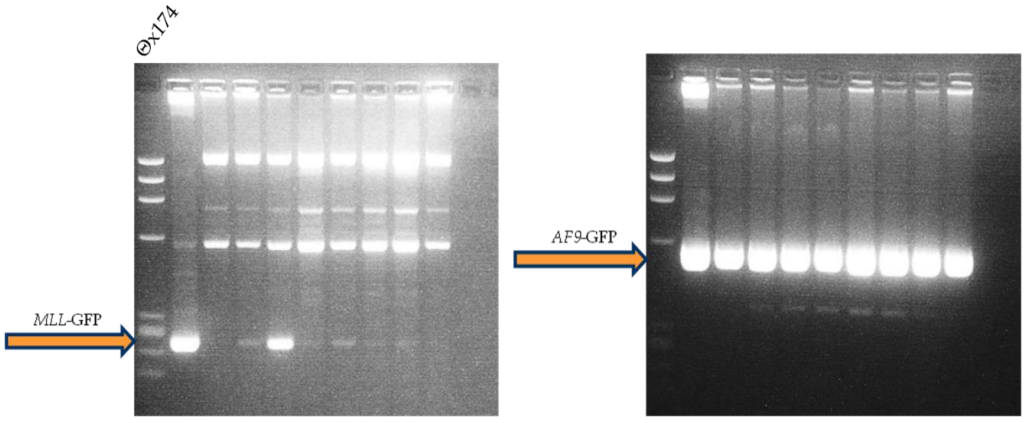


Figure 11: PCR confirming the insertion of *MLL-GFP* and *AF9-GFP* transgenes.

The resultant *MLL-AF9* double transgenic reporter stem cell lines contain *MLL-5'GFP* and *AF9-3'GFP* genetically engineered constructs as shown in figure below:

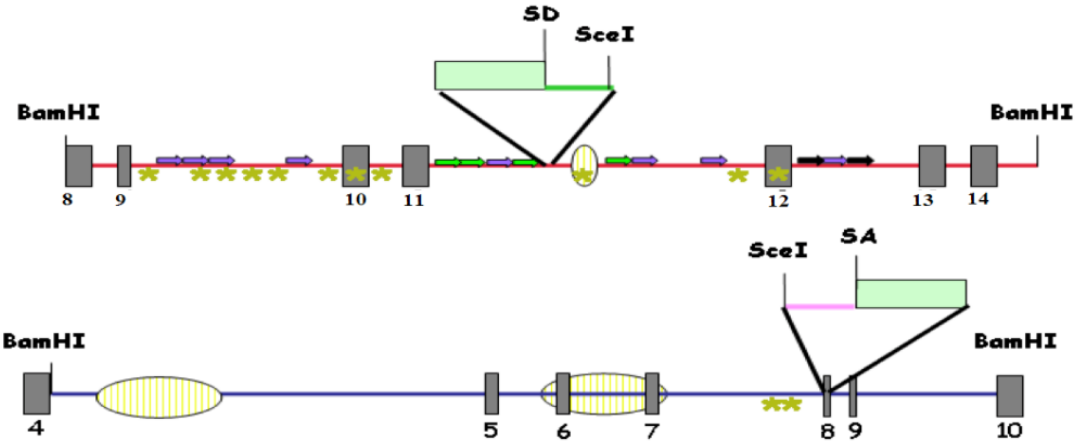


Figure 12: Map showing position of GFP-intron repair reporter constructs inserted in the human *MLL* (top) and AF9 (below) bcr regions.

SD stands for splice donor, SA stands for splice acceptor. Exons are shown as grey rectangles and numbered. MAR/SAR sites are indicated as yellow-white ovals (one in *MLL* and two in AF9). Topo II cleavage sites are indicated by green *.

3.2 Conclusion

In this chapter I have described the establishment of a reporter gene system to screen for the frequency of *MLL-AF9* bcr translocation events. A translocation between the two bcrs reconstitutes the full-length GFP transcript resulting in quantifiable green fluorescence.

CHAPTER 4: ISCE-I EXPRESSION GENERATES FLUORESCENT (GFP+) COLONIES IN MAG REPORTER CLONES FOLLOWING DSB INDUCTION

4.1 DNA repair reconstitutes GFP expression in MAG reporter clones

The reporter clones containing single copy inserts of the *MLL* as well as the *AF9* transgene constructs integrated randomly into the genome, were used for further experimentation to create DSBs in the *MLL* and *AF9* bcr constructs. NHEJ repair resulting in translocations brought the 5' *GFP* exon and the 3' *GFP* exon together to create a restored *GFP* gene. Expression of the restored gene will result in GFP+ cells (Figure 13).

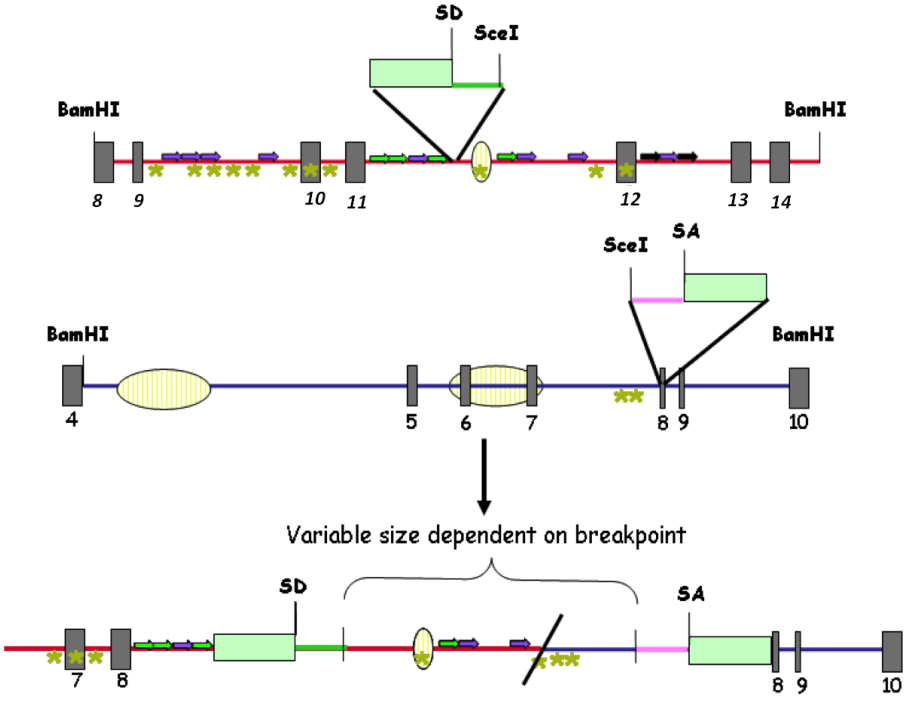


Figure 13: Map showing position of the GFP-intron reporter constructs inserted in the human *MLL* (top) and *AF9* (center) bcr regions; and NHEJ repair product (bottom). SD

stands for splice donor, SA stands for splice acceptor. Exons are indicated as grey rectangles and numbered. MAR/SAR sites are indicated as yellow-white ovals (one in *MLL* and two in *AF9*). Topo II cleavage sites are indicated by green *. In *MLL* multiple repetitive elements in the same orientation are shown with different families of dispersed repeats indicated by different colors. These elements have not been mapped in the *AF9* locus.

Each experiment included

1. A negative control with no DNA

2. A positive control with the GFP gene

3. Endonuclease I-SceI expression plasmid, pCBASce.

24 hours post electroporation, the number of surviving cells was calculated using a hemacytometer. The percentage of surviving cells was calculated.

NHEJ repair resulting in translocations can result in restoration of the GFP gene and green fluorescing cells (GFP+ cells). The electroporated cells were examined each day using the fluorescent filter on the microscope for the presence of GFP+ colonies (Figures 14 and 16). GFP+ colonies were identified on both the GFP+ control plates and the ISceI plates. These colonies were examined each day for continued GFP expression.

4.2 GFP+ green fluorescent colonies are generated by I-SceI expression in reporter cell lines.

The electroporated cells were examined each day using the fluorescent filter on the microscope for the presence of GFP+ colonies (Figures 14 and 16).

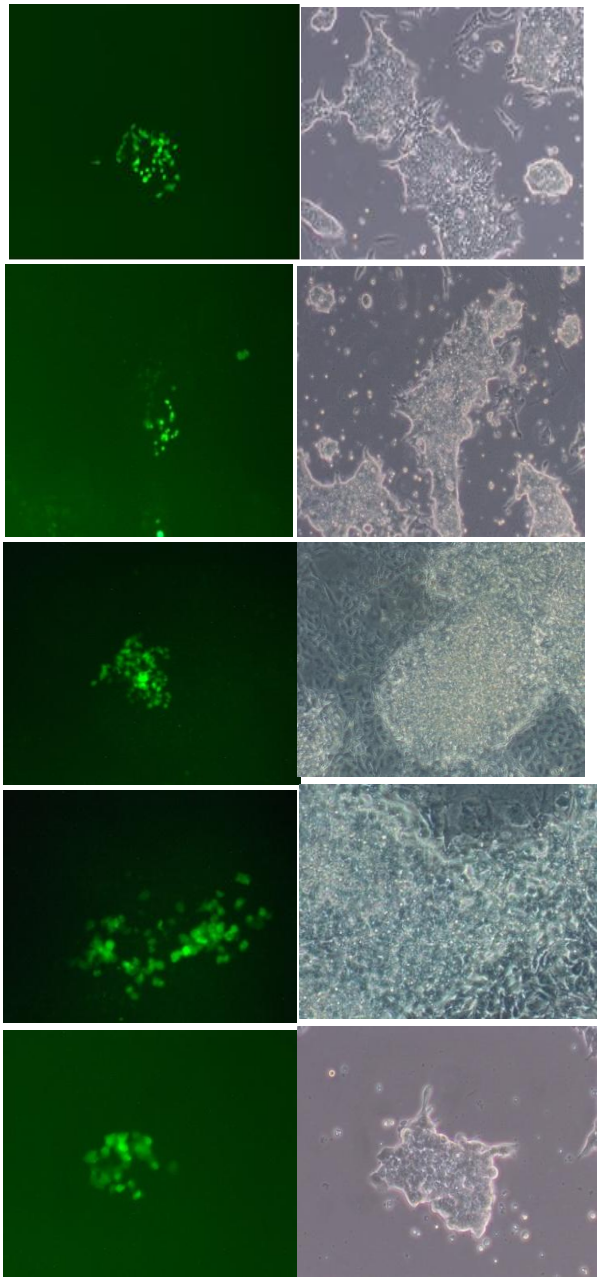


Figure 14: Confocal microscopy images of GFP+ cells (left panel) and the phase contrast images (right panel), following electroporation of MAG 32 cells with ISceI.

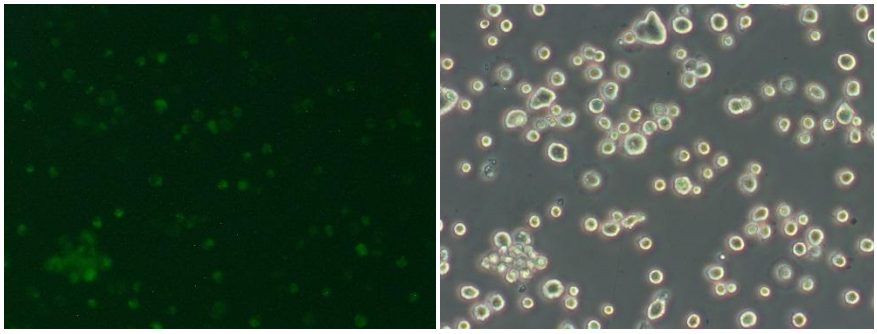


Figure 15: GFP+ cells along with the phase contrast images of MAG 32 cells electroporated with GFP, the positive control.

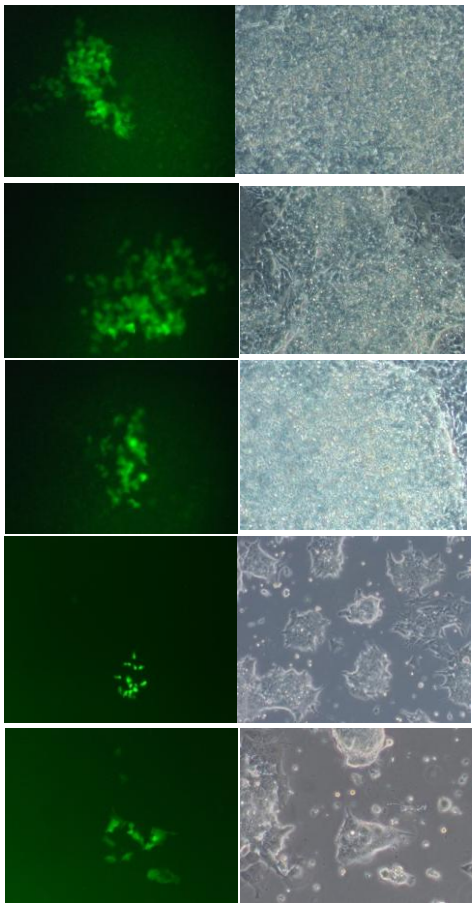


Figure 16: Confocal microscopy images of GFP+ cells (left panel) along with the phase contrast images (right panel) following electroporation of MAG 32A cells with *ISceI*.

4.3 The frequency of GFP+ colonies varies between three independently obtained MAG reporter cell lines, being the highest in MAG32A and the lowest in MAG68

The number of colonies identified was tabulated and the frequency of GFP+ colonies observed was calculated (Table 3). These data show that DSBs in the *MLL* and *AF9* bcrs is sufficient to lead to aberrant NHEJ and translocations.

Table 3. Induction of interchromsomal recombination and translocations by I-SceI-induced DSBs between *MLL* and *AF9* bcrs in three independent cell lines.

Experiment/ Cell Line		DNA	% survival post-EP	# of GFP+ colonies	Frequency *
MAG32A	expt. 1	no DNA	65%	0	$< 7.6 \times 10^{-8}$
		GFP	41%	many	not calculated
		I-SceI	38%	18	2.3×10^{-6}
	expt. 2	no DNA	73%	0	< 6.8 X 10-8
		GFP	46%	many	not calculated
		I-SceI	40%	22	2.7×10^{-6}
	expt. 3	no DNA	70%	0	$< 7.1 \times 10^{-8}$
		GFP	46%	many	not calculated
		I-SceI	42%	23	2.7×10^{-6}
		Avg. no DNA			$< 7.2\times10^{-8} \pm 0.4\times10^{-8}$
		Avg. I-SceI			$2.5\times10^{-6} \pm 0.2\times10^{-6}$^

Experiment/ Cell Line		DNA	% survival post-EP	# of GFP+ colonies	Frequency *
MAG32 expt. 1		no DNA	57%	0	$< 8.7\times10^{-8}$
		GFP	50%	many	not calculated
		I-SceI	37%	11	1.4×10^{-6}
	expt. 2	no DNA	60%	0	$< 8.3\times10^{-8}$
		GFP	50%	many	not calculated
		I-SceI	38%	9	1.1×10^{-6}
	expt. 3	no DNA	60%	0	$< 8.3\times10^{-8}$
		GFP	48%	many	not calculated
		I-SceI	38%	10	1.3×10^{-6}
		Avg. no DNA			$< 8.4\times10^{-8} \pm 0.2\times10^{-8}$
		Avg. I-SceI			$1.2\times10^{-6} \pm 0.1\times10^{-6}$$

Table 3: (continued)

Experiment/ Cell Line	DNA	% survival post-EP	# of GFP+ colonies	Frequency *
MAG68 expt. 1	no DNA	78%	0	$< 6.4\times10^{-8}$
	GFP	31%	many	not calculated
	I-SceI	24%	1	0.2×10^{-6}
expt. 2	no DNA	70%	0	$< 7.1\times10^{-8}$
	GFP	35%	many	not calculated
	I-SceI	29%	6	1.0×10^{-6}
expt. 3	no DNA	75%	0	$< 6.6\times10^{-8}$
	GFP	37%	many	not calculated
	I-SceI	32%	5	0.7×10^{-6}
	Avg. no DNA			$< 6.7\times10^{-8} \pm 0.3\times10^{-8}$
	Avg. I-SceI			$0.6\times10^{-6} \pm 0.4\times10^{-6}$#

Three independently derived MAG cell lines were electroporated with the I-SceI endonuclease expression vector pCBASce. As a positive control, cells were electroporated with the GFP+ wild-type plasmid pCBAGFP. 2.0×10^{7} cells were electroporated in each sample.
* The interchromsomal recombination and translocation frequency was calculated as the number of GFP+ clones per number of cells surviving electroporation (EP; as counted 24 hrs postelectroporation).
p values using an unpaired t-test:
^ <0.0001
$ 0.0019
0.1532

MAG32A: The two-tailed P value using an unpaired t-test is less than 0.0001. By conventional criteria, this difference is considered to be extremely statistically significant.

MAG32: The two-tailed P value equals 0.0019. By conventional criteria, this difference is considered to be very statistically significant.

MAG68: The two-tailed P value equals 0.1532. By conventional criteria, this difference is considered to be not statistically significant.

The electroporated MAG clones were examined every day for the presence of GFP+ cells. Green fluorescent cells (usually visible at 96 hours) identified were isolated manually using the fluorescent filter on the microscope. The number of isolated green fluorescent colonies was tabulated and the frequency of isolated GFP+ colonies was calculated.

The average frequency was the highest in MAG 32A and the lowest in MAG 68. This could be explained by the insertion of the reporter transgenes at different sites in the genome, causing differences in the frequencies of DNA repair leading to translocations.

4.4 Molecular analysis of a GFP+ clone suggests NHEJ at the DSB repair junction

A few green fluorescent clones were analyzed by PCR after expansion and isolation of genomic DNA. Clone 32A I-SceI G2 showed a band after amplification with GFP primers GFP 5'Intron 1F and GFP 3'Intron 2R. This PCR product was cloned in TOPO TA vector and sent for sequencing. The sequence at the breakpoint junction (Figure 17) shows that there was NHEJ without any nibbling in the introns but with the loss of ISceI sites.

ANGANNCNNATAGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCC
AGTGTGATGGATATCTGCAGAATTCGCCCTTGGGACAGGATAAGTATGACAT
CATCAAGGAAACCCTGGACTACTGCGCCCTAGACGTGCAGC**A**AGCTTGACA
ACAAAAAGATTGTCTTTTCTGACCAGATGGACGCGGCCACCCTCAAAGGCAT
CACCGCGGGCCAGGTGAATATCAAATCCTCCTCGTTTTTGGAAACTGACAATC
TTAGCGCAGAAGTCATGCCCGCTTTTGAGAGGGAGTACTCACAAGGGCGAAT
TCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTG
GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAAT
TCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAA
TGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTC
GGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGA
GGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG
CTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATA
CGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAA

GGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC
ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAANGTCAGA
GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTNNAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCG
CTTTCTCCCTNCGGGAAGCGNGNGCTTNCTCATAGCTCACGCTGNAGNANCN
CANTTCGNN

Figure 17: MAG32A-I-SceI-Green colony intron PCR sequence

The sequences in blue and red denote the 5’ and 3’ halves of the GFP intron respectively. The nucleotide “A” in large black font denotes the 5’/3’ junction of the two introns. The I-SceI restriction site at the junction has been lost during repair.

4.5 Conclusion

In this chapter I have described the quantification of GFP+ colonies after endonuclease ISceI expression which induces directed DNA breaks in reporter cell lines. The results describe the comparison of frequencies of appearance of GFP+ cells among three different and independently obtained reporter cell lines.

CHAPTER 5: TOPO II INHIBITORS GENERATE GREEN (GFP+) COLONIES IN MAG REPORTER CLONES THROUGH DSB INDUCTION

The next step after obtaining green fluorescent colonies by I-SceI expression in independently obtained reporter cell lines, was to test the potential of various topo II inhibitors to induce DSBs and lead to the formation of GFP+ colonies in these cell lines.

5.1 Etoposide is the most toxic to cells, which also exhibit considerable sensitivity to bioflavonoids genistein and quercetin.

Cell viability assays were performed on parental ETG2a cell lines to determine the sensitivity and cell survival percentages to various doses of these genotoxic compounds.

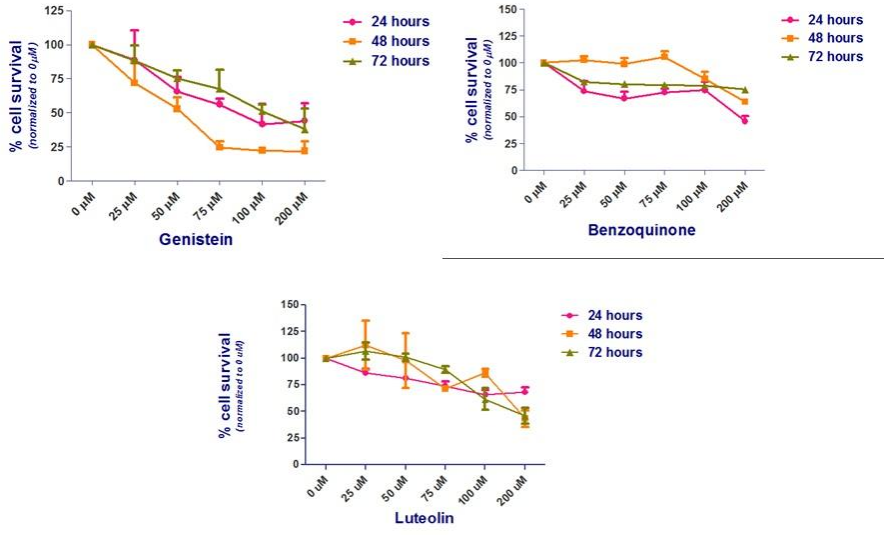


Figure 18: Cell viability plots for genistein, benzoquinone and luteolin at 24, 48 and 72-hour time points.

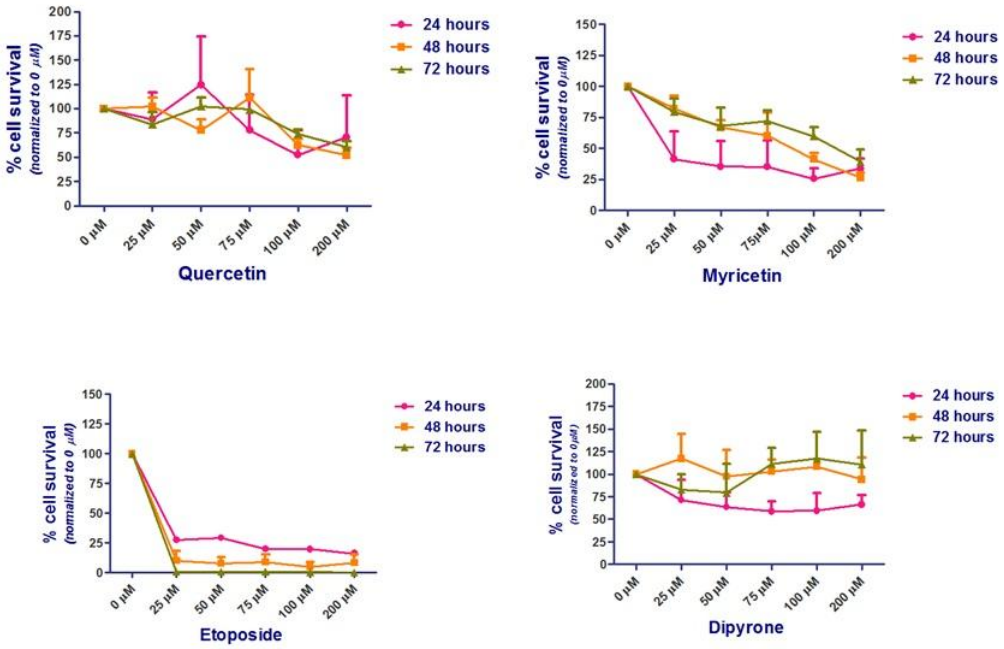


Figure 19: Cell viability plots for quercetin, myricetin, etoposide and dipyrone at 24, 48 and 72-hour time points.

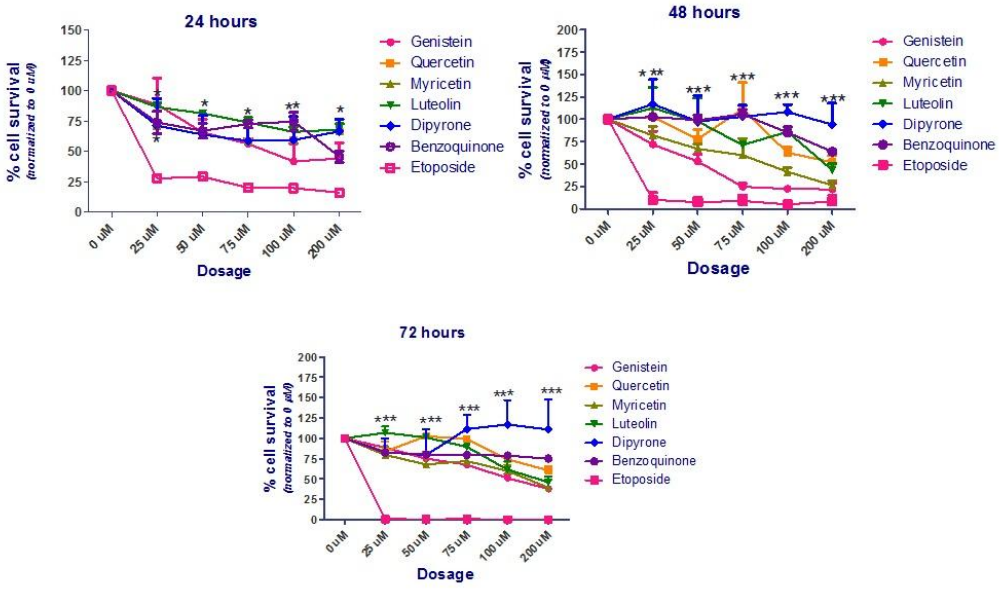


Figure 20: Comparative analysis of cell viability and sensitivity to genotoxic compounds: genistein, quercetin, myricetin, luteolin, dipyrone, benzoquinone and etoposide at 24, 48 and 72 hours.

A two-way ANOVA analysis (among compounds and doses) was performed comparing all the plots against etoposide using Bonferroni post-hoc analysis. The most significant differences as compared to the cell viability for etoposide are indicated in ***.

These experiments demonstrate the sensitivity of mouse embryonic stem cells to these compounds that are topoII inhibitors. The chemotherapeutic drug etoposide is the most toxic to cells, and the cells also exhibit considerable sensitivity to bioflavonoids genistein and quercetin, whereas luteolin and benzoquinone seem to moderately affect the cells.

These cell viability plots were used to determine the optimum dose to be used in the treatment experiments, broadly in the range of IC_{50} for each compound. The doses were also chosen based on previous studies which have described the physiological doses for these compounds. For example, three different doses of etoposide were used in the FACS analysis (Figure 25) (Libura, Ward et al. 2008). The dosage corresponds to the physiologically relevant dosage administered to patients during chemotherapy (Libura, Slater et al. 2005, Goodman, Gilman et al. 2008).

5.2 GFP+ colonies are generated in MAG reporter cell lines upon treatment with topo II inhibitors

MAG 32A cells were treated with different doses of myricetin, quercetin, genistein, luteolin, dipyrone, or benzoquinone for 1 hour at 75 µM - 200µM concentrations. They were then allowed to repair and proliferate in culture and observed every day for the appearance of green fluorescent colonies. MAG68 was treated with quercetin in the same way and allowed to repair and proliferate in culture.

Topo II inhibition results in DSB creation at various places in the genome, including the *MLL* and *AF9* bcr construct regions. DNA repair of the DSBs created at the bcrs may result in translocations that brings the 5' and 3' halves of the GFP gene from the two constructs together. Expression of the reconstituted GFP gene resulted in GFP+ (green fluorescent) cells. GFP+ fluorescent colonies as a result of *MLL-AF9* translocations were readily scorable by 96 hours in a dose-dependent manner.

Green fluorescent colonies were observed after approximately 96 hours of treatment and were followed, picked and expanded. GFP+ colonies are shown in figure 21 below.

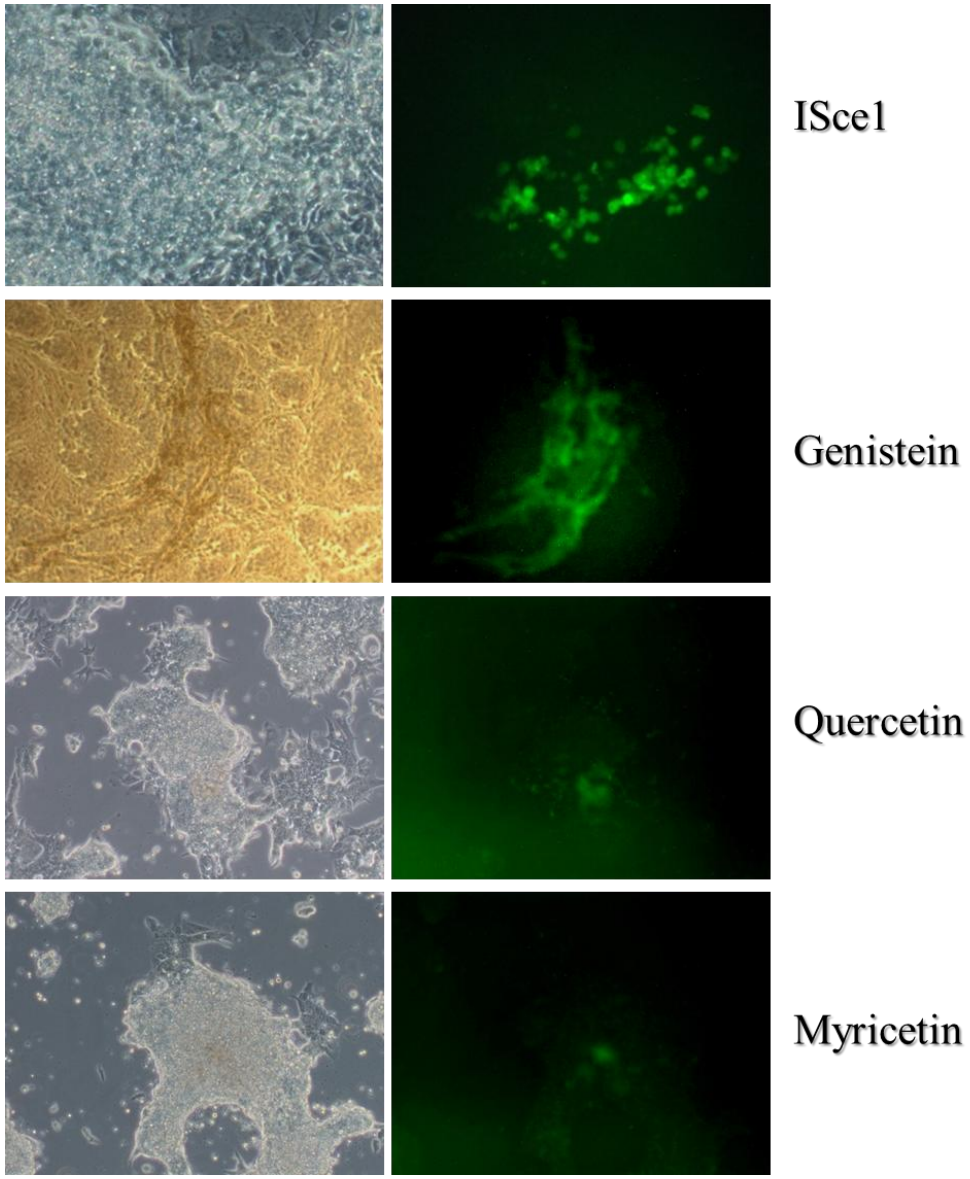


Figure 21: GFP+ ES colonies obtained after treatment of MAG 32A with topo II inhibitors

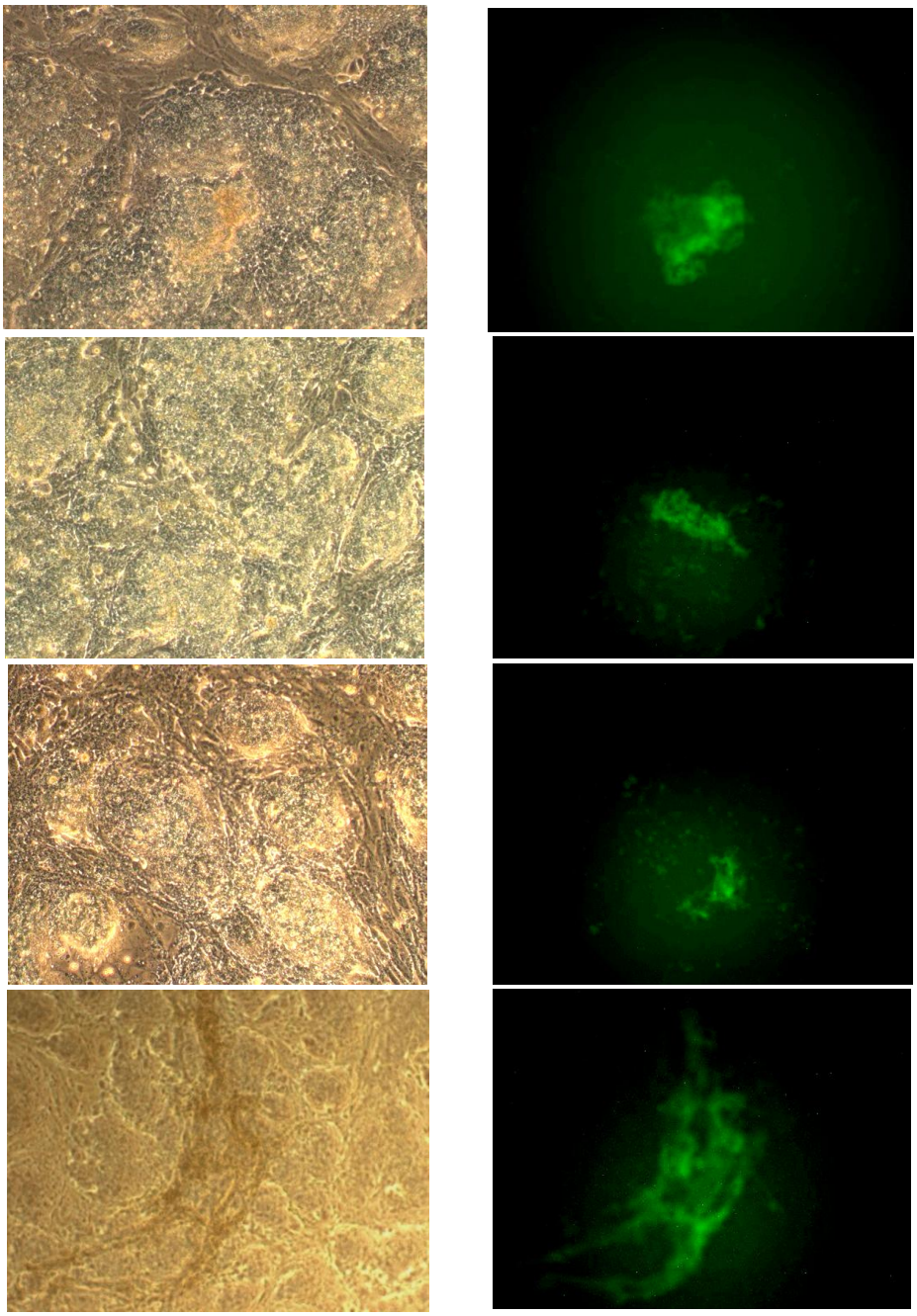


Figure 22: GFP+ ES colonies obtained after treatment of MAG 32A with genistein. The average frequency of GFP+ colonies after genistein treatment was calculated to be 1.2 X 10^{-6}.

5.3 Genistein and quercetin are the most potent promoters of *MLL-AF9* translocations

The frequencies of appearance of green fluorescent cells upon treatment of MAG 32A with various compounds in different doses are shown in table 4 below.

Table 4. Induction of interchromosomal recombination and translocations by topo II inhibitors between *MLL* and *AF9* bcrs in MAG32A cell line: a comparison of frequency of GFP+ cells generated by different compounds.

Compound	Expt	Dose	# GFP+ Colonies	Frequency*	Average frequency at highest dose
Genistein	1	75 µM	15	1.5×10^{-6}	
	2	75 µM	8	8.0×10^{-7}	
	3	75 µM	14	1.4×10^{-6}	$1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$
Quercetin	1	75 µM	8	8.0×10^{-7}	
	2	75 µM	14	1.4×10^{-6}	
	3	75 µM	11	1.1×10^{-6}	$1.1 \times 10^{-6} \pm 0.3 \times 10^{-6}$
Benzoquinone	1	75 µM	0	$<1.0 \times 10^{-7}$	
	2	125 µM	1	1.0×10^{-7}	
	3	125 µM	1	1.0×10^{-7}	
	4	125 µM	0	$<1.0 \times 10^{-7}$	$<0.06 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Myricetin	1	75 µM	1	1.0×10^{-7}	
	2	75 µM	1	1.0×10^{-7}	
	3	75 µM	2	2.0×10^{-7}	$0.1 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Dipyrone	1	75 µM	0	$<1.0 \times 10^{-7}$	
	2	100 µM	0	$<1.0 \times 10^{-7}$	
	3	100 µM	1	1.0×10^{-7}	
	4	100 µM	0	$<1.0 \times 10^{-7}$	$0.03 \times 10^{-6} \pm 0.05 \times 10^{-6}$
Luteolin	1	75 µM	0	$<1.0 \times 10^{-7}$	
	2	100 µM	0	$<1.0 \times 10^{-7}$	
	3	175 µM	0	$<1.0 \times 10^{-7}$	
	4	200 µM	1	1.0×10^{-7}	
	5	200 µM	0	$<1.0 \times 10^{-7}$	
	6	200 µM	0	$<1.0 \times 10^{-7}$	$0.03 \times 10^{-6} \pm 0.05 \times 10^{-6}$

MAG32A cell line was treated with various topo II inhibitors. 10^7 cells were treated in each experiment.

* The interchromsomal recombination and translocation frequency was calculated as the number of GFP+ clones per number of treated cells.

The relative frequencies of the appearance of GFP+ colonies from table 4 are plotted below in the form of a bar chart. It is noteworthy that genistein and quercetin are the most potent promoters of *MLL-AF9* translocations. Benzoquinone, which is associated with non-*MLL*-rearranged leukemias, and dipyone, which is not a known topo II inhibitor generated none and one GFP+ colonies respectively at a high dose. Both these compounds are believed to have distinct mechanisms of action, different from the other topo II inhibitors used in this study.

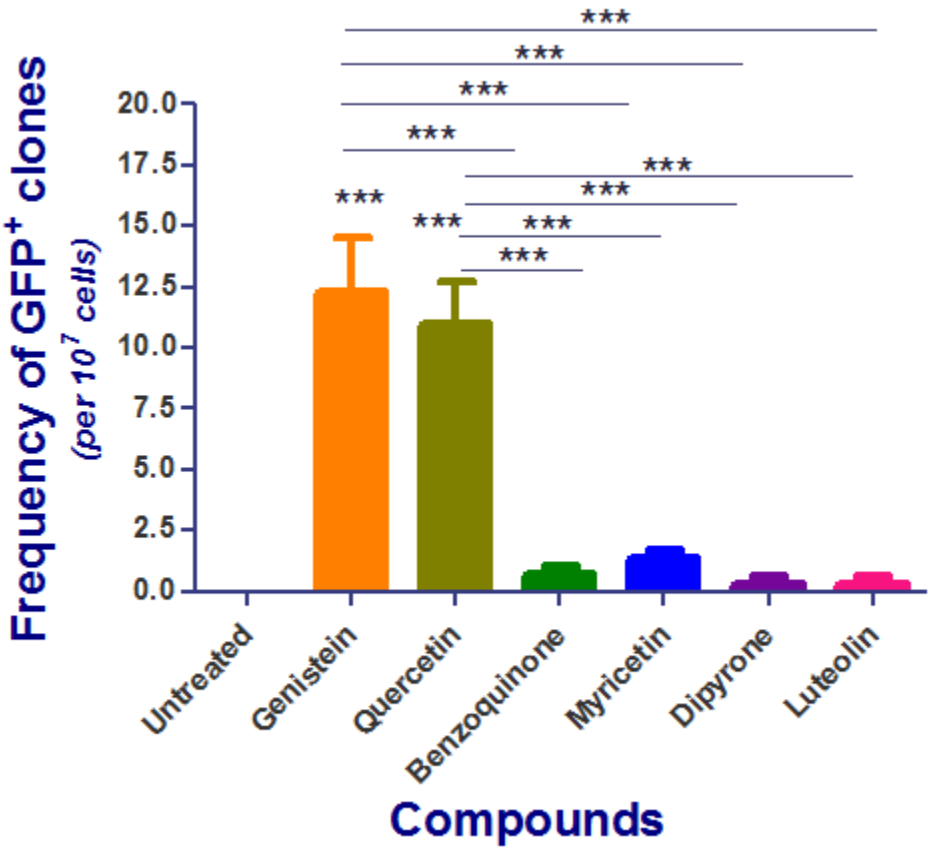


Figure 23: A bar chart representing the relative frequencies of appearance of GFP+ colonies upon treatment of MAG32A with various topo II inhibitors.

A one-way ANOVA (comparing frequencies) was performed using Bonferroni post-hoc test. Both genistein and quercetin were significantly different from the untreated group, and both these compounds were also significantly different from benzoquinone, myricetin, dipyone and luteolin.

Bioflavonoids genistein and quercetin (75 µM) were the most potent promoters of *MLL*-AF9 translocations in MAG reporter stem cell line. Treatment of stem cells with benzoquinone was not sufficient to produce *MLL-AF9* translocations following exposure to concentrations up to 125 µM (frequency < 0.06×10^{-6}).

5.4 MAG68 generates GFP+ colonies at a lower frequency than MAG32A upon quercetin treatment

An independently generated reporter cell line MAG68 was treated with 75µM quercetin and GFP+ colonies were observed for this clone too, although at a lower frequency than for MAG32A. The comparative frequencies are provided in table 5.

Table 5. Induction of interchromsomal recombination and translocations by quercetin between *MLL* and *AF9* bcrs in MAG68 cell line: a comparison of frequency of GFP+ cells generated between MAG32A and MAG68.

Compound	Expt	Dose	# GFP+ Colonies	Frequency*	Average frequency
Quercetin	1	75 µM	2	2.0×10^{-7}	
	2	75 µM	0	$<1.0 \times 10^{-7}$	$1.0 \times 10^{-7} \pm 1.0 \times 10^{-7}$
	3	75 µM	1	1.0×10^{-7}	

MAG68 cell line was treated with 75 µM quercetin. 10^7 cells were treated in each experiment.
* The interchromsomal recombination and translocation frequency was calculated as the number of GFP+ clones per number of treated cells.

MAG68 avg. frequency: $1.0 \times 10^{-7} \pm 1.0 \times 10^{-7}$
MAG32A avg. frequency: $1.1 \times 10^{-6} \pm 0.3 \times 10^{-6}$ (Table 4)

The following figure 24 shows the relative frequencies of appearance of GFP+ colonies between MAG32A and MAG68 upon treatment with quercetin.

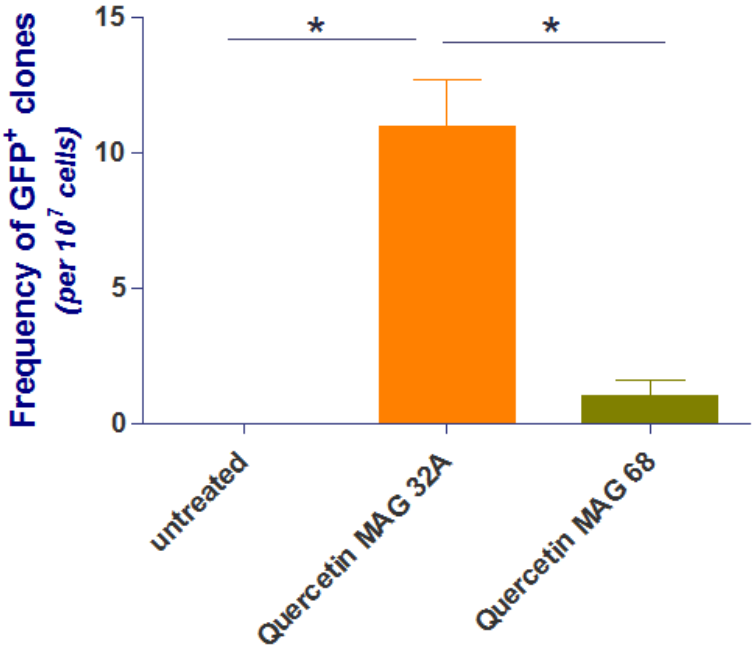


Figure 24: A bar chart representing the relative frequencies of appearance of GFP+ colonies upon treatment with quercetin between MAG32A and MAG68.

The frequencies were analyzed using a two-way ANOVA statistical analysis (both groups compared to untreated, and compared to each other) and Bonferroni post-hoc test. GFP+ frequencies for both MAG32A and MAG68 were statistically significant as compared to untreated and compared to each other.

5.5 GFP+ cells are generated in a dose-dependent manner upon treatment of MAG32 by etoposide as detected by FACS

Since no viable green fluorescent colonies could be obtained after etoposide treatment due to extensive cell death, fluorescence activated cell sorting (FACS) was performed on the etoposide treated MAG 32 cells as another approach to identify and

quantify GFP+ cells. This is a highly sensitive procedure and is able to sort individual GFP+ cells. GFP+ cells were sorted onto 96 well plates and expanded. They were observed each day. The frequency of GFP+ cells sorted by FACS after exposure to etoposide increased in a dose-dependent manner (figure 25).

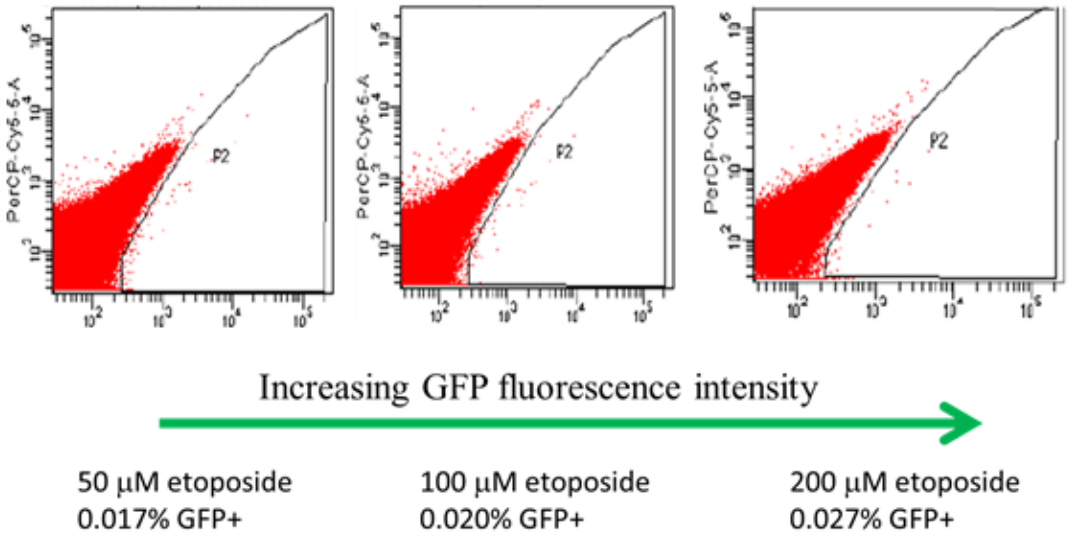


Figure 25: Etoposide exposure promotes *MLL-AF9* repair events detectable as GFP+ cells FACS data of etoposide treated MAG 32 cells sorted for GFP+ cells. (**a)** Frequency of GFP+ MAG 32 cells treated with 50 µM etoposide is 0.017% (**b**) Frequency of GFP+ MAG 32 cells treated with 100 µM etoposide is 0.02% (**c)** Frequency of GFP+ MAG 32 cells treated with 200 µM etoposide is 0.027%

The following table shows the frequency of GFP+ events sorted by FACS.

Table 6. Induction of interchromsomal recombination and translocations by etoposide between *MLL* and *AF9* bcrs in MAG32 cell line: Comparison between immediate events and long-term viable and proliferating events.

Expt	Etoposide Dose	# cells	# GFP+ cells	Frequency *	# GFP+ cells proliferate	Frequency ^
1	50 µM	2.5×10^{7}	0	$< 4.0\times10^{-8}$	0	$< 4.0\times10^{-8}$
	100 µM	2.0×10^{7}	180	9.0×10^{-6}	0	$< 5.0\times10^{-8}$
	200 µM	1.5×10^{7}	305	20.3×10^{-6}	0	$< 6.6\times10^{-8}$
2	50 µM	2.9×10^{7}	480	16.6×10^{-6}	3	0.10×10^{-6}
	100 µM	3.0×10^{7}	180	6.0×10^{-6}	3	0.10×10^{-6}
	200 µM	3.9×10^{7}	420	10.1×10^{-6}	15	0.36×10^{-6}
3	50 µM	9.8×10^{6}	20	2.0×10^{-6}	0	$< 0.10\times10^{-6}$
	100 µM	1.2×10^{7}	10	0.8×10^{-6}	3	0.24×10^{-6}
	200 µM	8.3×10^{6}	10	1.2×10^{-6}	3	0.36×10^{-6}
Avg. 50 µM				$6.2\times10^{-6}\pm 9.0\times10^{-6}$		$<0.08\times10^{-6}\pm 0.03\times10^{-6}$
Avg. 100 µM				$5.2\times10^{-6}\pm 4.1\times10^{-6}$		$<1.7\times10^{-6}\pm 2.8\times10^{-6}$
Avg. 200 µM				$10.5\times10^{-6}\pm 9.5\times10^{-6}$		$2.4\times10^{-6}\pm 3.6\times10^{-6}$

MAG32 cell line was electroporated with the I-SceI endonuclease expression vector pCBASce. 96 hrs post-electroporation, cells were analyzed by FACS. GFP+ fluorescent cells were single-cell sorted into 96 well plates and cultured for XX days post-sort.
* The initial interchromsomal recombination and translocation frequency was calculated as the number of GFP+ cells detected by FACS per number of cells analyzed by FACS.
^ The long-term viable and proliferating interchromsomal recombination and translocation frequency was calculated as the number of GFP+ sorted cells that proliferated over time per the number of cells initially sorted as a fraction of the initially sorted GFP+ calculated frequency.

In spite of detecting GFP+ cells by flow cytometry, I could not see any viable green fluorescent colonies under the microscope upon treatment of reporter cell lines by various doses of etoposide. This could be possible due to etoposide being severely lethal to the cells. Due to the high genotoxicity associated with etoposide treatment, it is possible that the colonies that undergo *MLL-AF9* translocations are relatively impaired in cell division and are outcompeted by other healthier wild type cells. This results in their

not producing enough green fluorescent cells to be detectable by microscopy. Since FACS is much more sensitive in detection of fluorescence from even a single cell, I was able to detect and sort those green fluorescent cells which probably could not produce colonies on a culture dish.

However, these etoposide-treated cells were not allowed to proliferate long term in an LTC-IC (long-term culture initiating cell) assay as has been discussed in previous studies (Libura, Ward et al. 2008). It is possible that these green fluorescent cells that were detected by FACS would have proliferated to form colonies if I had performed an LTC-IC assay on these plates.

5.6 Conclusion

In this chapter I have described the quantification of GFP+ colonies after treating the reporter cell lines with varying doses of different topo II inhibitors including the chemotherapeutic drug etoposide and different bioflavonoid compounds. The results include comparison of frequencies of appearance of GFP+ colonies among three different and independently obtained reporter cell lines and among the different compounds used.

CHAPTER 6: DISCUSSION AND FUTURE DIRECTION

In this thesis I have demonstrated that *MLL* and AF9 bcrs undergo DSBs, and are highly recombinogenic independent of normal chromosomal context, and in the absence of a leukemic fusion protein; and a number of topo II inhibitors have the potential to promote these translocations analogous to those observed in infant leukemia. In addition, this reporter gene system allows for rapid and reproducible screening of hundreds of compounds that may have the potential to promote leukemogenic translocations. The results shown here above provide the first direct evidence of induction of DSBs and translocations by dietary compounds and nutritional supplements that act as topo II inhibitors.

These bioflavonoids have been shown to inhibit topo II in *in vitro* DNA cleavage assays of the *MLL* gene (Schroder-van der Elst, van der Heide et al. 1998, Strick, Strissel et al. 2000, Bandele and Osheroff 2007, Barjesteh van Waalwijk van Doorn-Khosrovani, Janssen et al. 2007, Bandele and Osheroff 2008, Azarova, Lin et al. 2010, Kalfalah, Mielke et al. 2011, Vanhees, de Bock et al. 2011). Unregulated use of these bioflavonoids as nutritional supplements may prove to be harmful, and their potential to promote leukemic translocations should be determined.

To determine the potential of topo II inhibitors such as etoposide to lead to *MLL* rearrangements, *in vitro* studies have been performed using inverse polymerase chain reaction (IPCR) to characterize the resultant *MLL* repair products following exposure to

etoposide. This technique has been used to detect alterations to the *MLL* translocation hotspot region (Bernard and Berger 1995, Reichel, Gillert et al. 2001, Rowley and Olney 2002, Whitmarsh, Saginario et al. 2003, Blanco, Edick et al. 2004, Libura, Slater et al. 2005). However this approach requires the elimination of artifacts such as ligation of the target DNA fragment with other fragments, inefficient amplification of large digested fragments, less physiologically relevant internal deletions or intronic alterations from analysis. This approach also does not allow for the clonal expansion of the cells containing a particular translocation/rearrangement event. A genetic system to directly and rapidly examine the role of different compounds in promoting these specific genome rearrangements has been lacking. I demonstrate here the sensitivity of the *MLL* and AF9 bcrs to topo II poisons and bioflavonoids independent of their normal chromatin context and independent of the formation of a leukemic fusion protein. This genetic assay does not rely on IPCR that requires elimination of artifacts or less physiologically relevant internal deletions or intronic alterations from analysis.

6.1 Influence of Chromsomal Context:

1) Context-independent recombinatorial potential of *MLL* and *AF9* bcrs

These results have demonstrated the significant recombinatorial potential or sensitivity of the two bcr regions to these compounds independent of their normal chromosomal context. My system generated recombination leading to translocations between human *MLL* and *AF9* bcrs following their random and independent integration into the mouse genome. The *MLL* bcr is AT rich and contains repetitive Alu elements which have been shown to play a role in promoting the rearrangements in this region

even when taken out of their normal context (Elliott, Richardson et al. 2005, Libura, Slater et al. 2005).

The DSBs created in the *MLL* and *AF9* GFP reporter constructs were sufficient by themselves to result in aberrant NHEJ repair and translocations in the cells. The formation of translocations and stable viable GFP+ colonies in this system did not require the presence of a pre-existing mutation or another protein factor only expressed in more differentiated cell types. Expression of the restored exon resulted in GFP+ cells in all three MAG clones (Figure 13). Overall, these experiments with these cell lines demonstrate that aberrant NHEJ repair of the *MLL* bcr construct with the AF9 bcr construct to produce translocations occur independently of the normal chromosomal context.

2) Influence of integration site and chromosomal context on the rate of recombination between *MLL* and *AF9* bcrs

We observed variation in the frequencies of GFP+ cells generated among different independently created reporter cell lines. The frequency of appearance of GFP+ colonies generated in the MAG 68 cell line after both ISceI expression and quercetin treatment was consistently lower than the frequencies observed in MAG32A (Table 5). A possible explanation for this is that the MAG 32A and MAG 68 cell lines have the *MLL* and *AF9* bcr constructs integrated into the genome at different chromosomal locations. This trend suggests that chromosomal context can lead to very subtle differences. Alternatively, other characteristics of MAG 68 may lead to reduced fitness and increased length of the cell cycle. Such differences could result in generation of smaller colonies or

non-proliferating colonies that were not observable by microscopy although such a difference would be small since many colonies were readily detected.

In another independently obtained reporter cell line (MAG60) (Table 1), I did not see the generation of any GFP+ colonies even though the presence of *MLL* and *AF9* transgenes were confirmed by Southern blotting. This could have occurred due to two possibilities.

1) The two GFP reporter constructs may have integrated in separate chromosome domains which do not or rarely intermingle (Visser and Aten 1999). These could be regions of heterochromatin which do not undergo breakage easily.
2) The integration of the *MLL* and *AF9* GFP reporter constructs may have possibly occurred in heterochromatin where there is low or no transcription. Even if a translocation occurred, the fusion gene formed as a result of translocations may have never been expressed leading to no GFP expression. In the future, a next step could be to target these reporter transgene constructs to specific sites in the genome to address these problems. However, this reporter gene system provides valuable data on *MLL-AF9* translocations that occur independent of the context and the site of insertion of these constructs into the genome.

6.2 Comparative genotoxicity and mechanisms of action of compounds used in the study

The reporter mouse embryonic stem cells were treated with etoposide, quercetin, genistein, luteolin, dipyrone, myricetin or benzoquinone, then allowed to repair and proliferate in culture. GFP+ fluorescent colonies indicative of *MLL-AF9* bcr translocations were readily scorable by 96 hours in a dose-dependent manner.

As previously demonstrated (Libura, Slater et al. 2005), embryonic stem cell viability was significantly impaired by multiple concentrations of etoposide. My results show that etoposide treatment is sufficient to result in formation of translocations between the two bcrs, and the frequency is dose dependent. Three different doses of etoposide were used in FACS analysis (Figure 25) (Libura, Ward et al. 2008). The dosage corresponds to the physiologically relevant dosage administered to patients during chemotherapy (Libura, Slater et al. 2005, Goodman, Gilman et al. 2008).

My results demonstrate the sensitivity of mouse embryonic stem cells to these compounds that are topoII inhibitors. The chemotherapeutic drug etoposide is the most toxic to cells, and the cells also exhibit considerable sensitivity to bioflavonoids genistein and quercetin, whereas luteolin seems to moderately affect the cells (Figure 20).

Genistein and quercetin were potent promoters of *MLL-AF9* translocations in totipotent reporter stem cells in this genetic assay. It is noteworthy that GFP+ colonies were obtained after treatment with the bioflavonoids genistein and quercetin which have been hypothesized as being topo II inhibitors but have not yet been shown to directly promote translocations that involve the *MLL* gene (Bandele and Osheroff 2007). These results for the first time provide insight into the interplay between topo II poisons, topo II cleavage and religation, and the resulting DNA damage and translocations analogous to those observed in human leukemia samples.

The bioflavonoids genistein, quercetin, luteolin and myricetin are shown here for the first time to induce such leukemogenic translocations. The frequency is dose-dependent and can easily be quantified. However, myricetin and luteolin were much less potent than genistein and quercetin, and luteolin only gave one GFP+ clone at a high dose

of 200 μM (see table 4). These results could be explained based on the observations that different topo II inhibitors differ in their interaction mechanisms with the enzyme topo II. Different topo II poisons have been shown to have different sequence specificity *in vitro* e.g. clerocidin requires a guanine, VM-26 a cytosine, and dh-EPI a 5'-TA dinucleotide, respectively, at the 3' terminus of DNA break generated by topo II (Borgnetto, Tinelli et al. 1999). Also, clinical studies have shown that different chemotherapeutic agents predispose patients to different translocations associated with leukemias (Mistry, Felix et al. 2005).

6.3 Benzoquinone and dipyrone act through a different mechanism

The significance of this reporter system is apparent from treatment of stem cells with benzoquinone that was not sufficient to promote *MLL-AF9* bcr translocations following exposure to concentrations up to 125 μM. Benzoquinone is a non-bioflavonoid thought to have a distinct mechanism of action and is clinically associated with non-*MLL*-rearranged leukemias. Benzene is carcinogenic and causes primarily hematopoietic cancers in humans. It has been reported that it causes DNA damage through its metabolites, especially 1,4-benzoquinone. 1,4 benzoquinone is a strong topo II poison *in vitro* as has been shown using purified human topo IIα in a plasmid DNA cleavage assay(Lindsey, Bromberg et al. 2004). However, it has not been shown to induce rearrangements of the *MLL* bcr. It is noteworthy that in spite of benzoquinone being a topo II inhibitor, I did not observe any *MLL* bcr translocations induced by it, suggesting that it causes other genomic rearrangements by topo II inhibition, and is not associated with *MLL*-rearranged leukemias.

Maternal use of dipyrone during pregnancy has been shown to be associated with infant acute leukemia (IAL) in a hospital-based case-control study conducted in different cities of Brazil (Pombo-de-Oliveira, Koifman et al. 2006). Dipyrone is not a known topoII inhibitor but is implicated as an increasing risk in infant acute leukemia. I could observe only one GFP+ colony upon treatment with dipyrone in this reporter gene system at a high dose of 100 µM (frequency 0.03 X 10^{-6}), which correlates with the fact that it possibly acts through a different mechanism and does not inhibit topo II. In countries such as Bulgaria, Chile, China, Egypt etc, it is one of the most popular analgesics. However, it was banned in the US in 1977.

6.4 DNA Repair Mechanisms that could be involved in the generation of reporter GFP+ colonies

The two major pathways of DSB repair are HR and NHEJ. As discussed in the introduction, extensive work with ISceI model systems has demonstrated that HR or a combination of HR and NHEJ can lead to genome instability and chromosomal translocations. However, sequencing of breakpoint junction DNA from translocations between the *MLL* and *AF9* bcrs in patients with t-MDS or t-AML suggests that they arise from aberrant NHEJ repair of DNA DSBs between the two broken duplexes (Harper and Aplan 2008). It is known that DSBs can be induced by a variety of agents including topo II poisons, suggesting that the previous treatment regimens with these poisons can induce the translocations to form that lead to t-MDS and t-AML (Azarova, Lyu et al. 2007). However, it is also possible that the translocations occur in these patients by a different mechanism altogether, or that the creation of the translocations only happens in cells already mutant in other genes making them predisposed to this event.

Thus this genetic system was created to specifically mimic the aberrant repair that leads to *MLL* and AF9 translocation reported in clinical samples. The focus of this project is to directly determine if DSBs created in the *MLL* and *AF9* bcrs analogous to those seen in patient samples are sufficient to produce translocations in mouse EtG2a ES cells. In this thesis, I have shown for the first time that these compounds are sufficient to directly induce *MLL* and *AF9* translocations as have been observed in the clinical setting in wild type cells that do not harbor any other mutations. Molecular analysis of the breakpoint junctions cloned in the GFP+ cells generated by these compounds is being performed in our lab. However, I showed in a GFP+ clone generated in this reporter system by the expression of endonuclease I-SceI that the GFP gene was reconstituted due to an aberrant NHEJ repair of the DNA DSBs created in the *MLL* and *AF9* bcrs at the ISceI restriction sites (Figure 17). This is a relatively more controlled system as compared to inducing several DSBs in the same system when topo II inhibitors are used.

DSBs were created by the use of the highly specific ISceI endonuclease, a known topo II poison etoposide, certain bioflavonoids, the non-steroidal anti-inflammatory drug, dipyrone and the benzene metabolite benzoquinone. The etoposide dosage used corresponds to the physiologically relevant dosage administered to patients during chemotherapy (Libura, Slater et al. 2005, Goodman, Gilman et al. 2008). The doses for the other compounds were determined from previous studies and also determined from the cell viability plots obtained for these compounds (Figures 18, 19 and 20).

Translocation events could be identified by the fusion of GFP reporters specifically designed for this system. Translocation events, as a result of NHEJ, brought together the 5’ GFP and 3’ GFP exons as a functional intron containing gene (Figure 13).

Transcription, splicing and protein expression led to the expression of the restored GFP gene, resulting in GFP+ cells (Figures 21 and 22) (Tables 4 and 5). NHEJ DNA repair is hypothesized to occur, since it is the predominant mechanism of DSB repair in mammalian cells in the G1 phase of the cell cycle (Rothkamm, Kuhne et al. 2001). Sequencing of the breakpoint junction from a GFP+ clone produced by ISceI endonuclease in the reporter cell line MAG 32A also indicates NHEJ as the mechanism (Figure 17).

6.5 Possible alternative events not leading to the generation of a GFP+ cell

This system was designed to specifically detect and quantify NHEJ mediated translocations between *MLL* and *AF9* bcrs. Generation of a GFP+ fluorescent colony is a simple and readily identifiable readout for these events. I acknowledge that there are multiple additional events that may have occurred during repair of the DSBs that would not produce a GFP+ cell. However, overall these events would also not be expected to lead to a stable chromosomal translocation in a viable cell capable of continued long-term proliferation; thus, their occurrence would not necessarily be relevant to the questions that I addressed.

Firstly, there is a possibility that the DNA repair between the DSBs created at the *MLL* and AF9 GFP reporter constructs had DSB end resection that continued to remove nucleotides so that the splice donor site and/or the splice acceptor site in the 5' and 3' GFP exons were lost (Figure 13). The Artemis:DNA-PKcs complex during NHEJ repair can endonucleolytically cut overhangs (Ma, Schwarz et al. 2005, Yannone, Khan et al. 2008). The average nucleolytic resection from each DNA end varies from 4 to 14 bp, but there are cases of resections up to ~25 bp (Gauss and Lieber 1996, Lieber 2010). The

resection at the break sites may have continued into the GFP exons, removing nucleotides. Expression of the restored GFP exon may have resulted in a mutant or truncated protein which cannot function properly and result in green fluorescent cells. For compounds that do not produce any GFP+ cells or give a very low frequency of such cells, an alternative approach could be to screen several randomly picked colonies by PCR using primers specific for the *MLL* and *AF9* bcrs to pick any translocation that may have deleted parts of the GFP exon. New techniques are available where it is possible to extract DNA from single cells or very few cells and use it for PCR after whole genome amplification.

Secondly, there is a possibility that the breaks created were repaired without loss or gain of nucleotides to reform a viable I-SceI site. Continued expression of the pCBASce plasmid may have resulted in the DSB being formed and religated through repair. The I-SceI community has grappled with this issue for over a decade. However, these continued breaks are not physiologically significant, therefore the use of topo II inhibitors in this study is an even more relevant approach to investigate the kind of DNA damage that cells are exposed to that can lead to leukemogenic translocations.

Thirdly, the DSBs created may have been repaired by NHEJ (Gauss and Lieber 1996, Lieber 2010) independently of each other thus not generating a translocation or generating a GFP+ gene. Such an event is known to be significant and occur more frequently than an aberrant repair in wild type cells as well. The DSBs that are induced in a cell after exposure to DNA damaging agents lead to translocations in some cases, but in other cases they are normally repaired. So, the frequencies that we obtain are

representative of the net potential of these compounds to lead to aberrant DNA repair after DSB induction.

Fourthly, there is a possibility that DSB induction may have triggered a cell cycle arrest and have resulted in apoptosis and cell death due to the presence of multiple DSBs all over the genome. They may not have been able to divide and form colonies to be seen under a microscope. To determine the toxicity of each compound, I performed cell viability assays (Figures 18, 19 and 20). In addition, I noted cell death visually on plates as evidenced by colonies rounding up and detaching from the plates, although I did not definitively distinguish between the different pathways leading to cell death (apoptosis vs necrosis). There are staining procedures like TUNEL assay that can be performed to scan and visualize the apoptotic colonies on a plate that has been treated with a topo II inhibitor. However, apoptotic cells will not lead to and propagate a genome rearrangement and thus will not contribute to any leukemogenic translocations. If a cell harboring a translocation does not proliferate in culture and is not able to divide and form more cells harboring the same mutation, it probably does not have the potential to lead to leukemia. Therefore, this possibility does not affect the conclusions from this reporter system. No GFP+ colonies could be seen under the microscope after etoposide treatment possibly due to the high toxicity of this drug to the cells after acute treatment.

FACS was performed on the etoposide-treated cells to identify GFP+ cells. Etoposide was shown to generate GFP+ cells with a dose-dependent increase in frequency. FACS is a highly sensitive procedure capable of sorting individual GFP+ cells to provide a much more accurate identification of GFP+ cells as compared to scanning under a microscope by human eye using a fluorescent filter. Not all of the sorted cells

survived, as shown in (Table 6). There are two possibilities for the low survival rate. Firstly, the cells may not have been able to survive the FACS procedure and the stress it puts upon the cells. Secondly, DSB repair by NHEJ resulting in translocations may have caused the cells to become unstable, thereby undergoing apoptosis. The cells that did survive the sort were expanded and used to isolate their genomic DNA for future experiments.

The low number of GFP+ cells and colonies, isolated by FACS as well as scanning under the microscope, may be due to the possibility that the initial number of cells treated with etoposide was low. We did increase the number of cells treated with etoposide, but were not able to isolate or observe any live colonies that were GFP+. In the future, using low doses of etoposide in chronic exposure rather than an acute exposure for 1 hour may lead to survival of some GFP+ colonies.

6.6 Further molecular analysis of the repair products from GFP+ reporter colonies

DNA from expanded as well as sorted GFP+ clones isolated may be used for further molecular analysis including PCR sequencing across the breakpoint junctions. Several GFP+ colonies were picked and expanded after ISceI expression and treatment with the other compounds (Tables 3, 4 and 5). DNA from one GFP+ clone obtained after ISceI expression was sequenced and shown to possibly undergo NHEJ at the repair junction (Figure 17). In addition, metaphase spreads of the GFP+ clones can be sent for spectral karyotyping (SKY) that labels each chromosome with a unique fluorescent color which can be used to identify chromosomal translocations at the karyotypic level. This will provide more data and specific details about the type of DNA repair mechanism used to repair the induced DSBs.

6.7 Significance and future direction

I have successfully demonstrated the sensitivity of the *MLL* and *AF9* bcrs to topo II poisons and bioflavonoids independent of their normal chromatin context and independent of formation of a leukemic fusion protein. In addition, the reporter system allows for rapid and reproducible screening of hundreds of compounds that may have the potential to promote leukemogenic translocations in early stem cell and more differentiated cell subpopulations analogous to the events observed in infant acute leukemias. A wide variety of dietary compounds are being consumed and recommended widely for their presumed health benefits. This is a great system to allow for rapid screening of all those compounds and assess the potential risks associated with their unregulated use and in the development of infant leukemia due to maternal consumption of these compounds. All of these compounds can be quickly screened and the frequency of leukemogenic translocations induced by them can be readily assessed.

ISceI induction led to the appearance of green fluorescent cells due to induction of DSBs in the bcrs, whereas the negative (no DNA or empty vector) controls did not generate any GFP+ colonies.

In parallel with experiments with the reporter clone MAG 68, a control cell line (F2) that did not show the presence of both *MLL* and *AF9* transgenes by Southern blotting was also treated with ISceI or topo II inhibitors. By contrast with results with clone MAG68, F2 did not lead to any detectable GFP+ colonies by microscopy after any treatment. These results are consistent with the idea that GFP+ colonies detected in this system are specific to DSBs and the generation of translocations rather than background fluorescence.

This approach has several advantages over the use of IPCR in *in vitro* studies to study *MLL* rearrangements after exposure to topo II inhibitors. IPCR requires the elimination of artifacts such as ligation of the target DNA fragment with other fragments, inefficient amplification of large digested fragments, less physiologically relevant internal deletions or intronic alterations from analysis.

In the future, it would be significant to determine the frequency of translocations on combined treatment/exposure to multiple topo II inhibitors to investigate the effect of analogous combinatorial treatment with these compounds during chemotherapy and exposure to multiple bioflavonoids in the diet. Any increase/alterations to the frequencies on such combined exposure will provide great insights in to the possible risk due to the synergistic effect of these compounds on DNA repair mechanisms.

The next step would be to modify this system for *in vivo* analysis and generate mice that contain these reporter transgenes in their genome. Direct exposure of these mice to these compounds or their combinations would be the most physiologically relevant and informative model of an assessment of the risk of genome rearrangements due to human consumption/exposure of these compounds. The physiological aspects that can be studied in mice are drug clearance, absorption, any tissue- or cell type- specific differences in chromosomal rearrangements, and influence of the genetic background of mice. Stem cells proliferate highly in culture, which could be different from how they respond in mice. Developmental programming and *in utero* exposure to these compounds can also lead to long-term genetic and epigenetic changes (see appendix). In addition, bioflavonoids genistein and quercetin have also been shown to produce *MLL* rearrangements by IPCR in mice (Vanhees, de Bock et al. 2011). However, IPCR

requires elimination of artifacts or less physiologically relevant internal deletions or intronic alterations from analysis, and hence is not a reliable and valid technique to investigate rearrangements.

The use of the environmental, dietary and therapeutic agents is widespread today. Increasing evidence is emerging regarding the long-term implications and adverse effects of using these compounds in an unrestricted manner. Due to the beneficial effects assumed to be associated with the use of bioflavonoids, their use as dietary supplements is popular and widespread. The cytotoxic and genotoxic properties of all the agents discussed above have so far generally been ignored as far as their use for various purposes is concerned. In view of the variety of adverse effects these agents have in exposed individuals and the transgenerational nature of these effects, it is important to raise public awareness, set guidelines and regulate the use and market availability of such compounds to reduce the risk of human 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. Using my developed model system for cells at different stages of differentiation with different susceptibility to genomic damage and repair will provide useful insights into the comparative risk to the human population in terms of the different windows where exposure occurs.

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APPENDIX A: AWARDS AND ACHIEVEMENTS

PUBLICATIONS

- **Bhawana Bariar** and Christine Richardson, "Long-term impact of chromatin remodeling and DNA damage in stem cells induced by environmental, therapeutic and dietary agents" *manuscript in preparation*
- **Bhawana Bariar**, C. Greer Vestal, R. Warren Englewood, and Christine Richardson "*MLL-AF9* breakpoint cluster region translocations induced by common bioflavonoids independent of chromosomal context: a model system to rapidly screen environmental risks" *manuscript in preparation*

POSTER/PRESENTATIONS

- Carol Greer Vestal, **Bhawana Bariar** and Christine Richardson, "Identification of Dietary and Environmental Compounds that Promote *MLL-AF9* translocations", New York Academy of Sciences: The Bone Marrow Niche, Stem Cells, and Leukemia: Impact of Drugs, Chemicals, and the Environment, May 29-31, 2013, New York City, NY
- Carol Greer Vestal, **Bhawana Bariar**, Richard Engledove and Christine Richardson, " System for Rapid and Reproducible Identification of *MLL-AF9* Translocations Induced by Dietary Compounds and Nutritional Supplements", 54th American Society of Hematology Annual Meeting and Exposition, December 8-11, 2012, Atlanta, Georgia
- **Bhawana Bariar**, Christine Richardson**,** "Exposure to topoisomerase II inhibitors promotes translocations analogous to leukemia patients", Graduate Research Fair, February 25, 2012, University of North Carolina at Charlotte
- **Bhawana Bariar**, Christine Richardson, "Etoposide and an illegitimate genome rearrangement common in therapy-related leukemia", Graduate Research Fair, February 26, 2010, University of North Carolina at Charlotte

AWARDS

- *Recipient***: Second place** (oral presentation), 12th Annual Graduate Research Fair, UNC Charlotte. 2012
  Exposure to topoisomerase II inhibitors promotes translocations analogous to leukemia patients

ADDITIONAL PUBLICATIONS AND POSTERS:

- Denis A. Kiktev, Jesse C. Patterson, Susanne Müller, **Bhawana Bariar**, Tao Pan and Yury O. Chernoff, "Regulation of chaperone effects on a yeast prion by cochaperone Sgt2" *Molecular and Cellular Biology, 2012 Dec;32(24)*
- **Bariar, B.**, Patterson, J., Mueller, S., Chernoff, Y.O., "Effects of the components of the Get pathway on prion propagation", XIV Southeastern Regional Yeast Meeting, March 31- April 1, 2007, University of Alabama at Birmingham, Birmingham, Alabama

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

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Abbreviations
DNA methyltransferases (DNMTs); double-strand break response (DDR); double-strand breaks (DSBs); phosphorylated histone variant H2AX (γH2AX); ataxia telangiectasia mutated (ATM); histone acetyl transferase (HAT); Polycomb group (PcG); H3K27 monomethylation (H3K27me1); H3K27 trimethylation (H3K27me3); Scaffold matrix attachment region 1 (SMAR1); histone deacetylase (HDAC); inter-strand crosslinks (ICLs); Fanconi anemia (FNAC); Fanconi anemia D2 (FANCD); H3K9 acetylation (H3K9ac); 1,4-benzoquinone (1,4 BQ); Chinese hamster ovary (CHO); constitutive androstane receptor (CAR); H3K9 monomethylation (H3K9me1); H3K9 dimethylation (H3K9me2); chromatin immunoprecipitation (chIP); Alzheimer's disease (AD); beta-amyloid precursor protein (APP); fetal basis of adult disease (FeBAD); arsenic trioxide (As(2)O(3)); Bisphenol A (BPA); embryonic stem (ES); phosphodiesterase type 4 variant 4 (PDE4D4); topoisomerase II (topoII); multipotent hematopoietic cells (MHCs);

Keywords
Environmental toxicology, epigenetics, chromatin remodeling, *in utero* exposure, bioflavonoids

Abstract
The presence of histones acts as a barrier to protein access, and 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 the variety of agents present in the human lifestyle that have the potential to promote epigenetic

changes that impact developmental programs of specific cell types, and may be transgenerational, *e.g*. able to be transmitted through multiple cell divisions.

1. Introduction

1.1 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. 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 [1]. Modifications promote either open or closed chromatin which in turn influences multiple cellular processes transcription and replication. 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. DNA methylation is 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.

Recent literature demonstrates that commonly used dietary, environmental, and therapeutic compounds have the potential to induce both DNA genomic and also epigenetic modifications. Exposure to these agents can induce epigenetic alterations by histone modification, changes in cell differentiation, modulation of cellular signal transduction pathways including DNA damage repair pathways, changes in gene expression, illegitimate DNA repair leading to genome rearrangements and carcinogenesis. Recently, multiple lines of evidence through *in vitro* and *in vivo* studies have shown that *in utero* exposure to environmental toxicants can cause epigenetic modifications which in turn induce alterations in gene expression that persist throughout life [1, 2].

1.2 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 the 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 [3-5]. 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 [6]. 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. 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. Phosphorylation of histone variant H2AX (γH2AX) spreads over large chromatin domains 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.

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 [7], and heterochromatin and euchromatin utilize distinct remodeling complexes and pathways for DSB repair. DSB repair may be stalled within HC regions if a series of dynamic and localized changes fail to take place. 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 [8]. Activation of the ATM signaling pathway and the subsequent phosphorylation of KAP-1 trigger HC modifications required for DSB-repair. In addition, studies have shown that histone acetyl transferase (HAT) complexes act with the ATP-dependent SWI/SNF and RSC-containing chromatin remodeling complexes to facilitate DNA repair [9].

Polycomb group (PcG) proteins, which have well-established roles in gene regulation, were recently found to accumulate on chromatin surrounding DNA damage [10]. PcG proteins are a family of proteins that form complexes involved in the epigenetic regulation of gene expression. Ploycomb repressive complexes catalyze post-translational modifications critical to their gene silencing function, including histone H3K27 trimethylation (H3K27me3) and histone H2A ubiquitination. This complex may also be involved in DNA methylation. PcG and PRC components found to respond to DNA damage include BMI-1, MEL-18, EZH2 methyltransferase, EZH1, EED, SUZ12. Gene silencing activity of PcG proteins, like mono-ubiquitylation of H2A is exploited during DSB repair.

Several chromatin-remodeling factors form a complex with DDR-related proteins in response to DNA damage. Scaffold matrix attachment region 1 (SMAR1) binds other SMAR1 elements along with histone deacetylase 1 (HDAC1) and p53, forming a repressor complex to downregulate transcription. The chromatin-remodeling factor Tip49 recruits Rad51, the homolog of bacterial RecA and major homologous recombination repair protein, to DNA damage sites. Signal transduction pathways in DDR communicate with chromatin-remodeling factors. In addition to DNA repair, the p53 signaling pathway is associated with chromatin changes that mainly involve the HAT Tip60. Numerous

chromatin-remodeling factors that are involved in DNA methylation and demethylation also play a role in DDR. In the thymus, genotoxic stress exposure decreases DNA methylation globally by a reduction in DNMT1, DNMT3a, DNMT3b and methyl-binding proteins MeCP2 and MBD2.

2. Environmental Toxins

2.1 Alcohols and Aldehydes

There is sufficient evidence in the literature demonstrating the genotoxic effects of aldehydes on cells. Aldehydes produced endogenously have also been found to have genotoxic effects. Reactive aldehydes, such as acetaldehyde, are by-products of metabolism. Bone marrow failure in Fanconi anemia may result in part from aldehyde-mediated genotoxicity in the hematopoietic stem and progenitor cell pool. Mouse hematopoietic stem and progenitor cells are more susceptible to acetaldehyde toxicity as compared to mature blood precursors. 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 [11]. Another endogenous source of acetaldehyde is in 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) [12]. 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 [13]. 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 acetyladehyde in a dose range of 0.1 – 1 mM 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 [13]. 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 ccells in culture and in the midbrain of C57BL/6J mice *in vivo* [14]. FANCD2 response induced by alcohol thus plays a role in DDR in post-mitotic neurons and in neural precursor cells.

Alterations in epigenetic marks induced by alcohols and aldehydes are linked with altered cellular differentiation. In cardiac progenitor cells, although low levels of ethanol, acetaldehyde, and acetate had no effect on the proliferation of cells, they did promote a >2-fold increase in histone H3K9 acetylation (H3K9ac). High concentrations sufficient to produce a 30% reduction in cell viability also increased H3K9ac by >5-fold. In addition, high concentrations significantly elevated the expression of GATA4 and Mef2c genes related to heart development, resulting in their impaired differentiation [15]. 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.
Occupational and environmental exposures to formaldehyde are prevalent. Its production is carried out on a large scale in the manufacture of resins, particle board, 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. In addition, lysine residues in the N-terminal tail and the globular fold domain of histone have been identified as binding sites for formaldehyde in in vitro studies using purified calf thymus or human H4 [16]. The final concentration of formaldehyde varied from 5mM to 100 mM, and the reaction time ranged from 3 h to 2 weeks [16]. This study also demonstrated that formaldehyde could inhibit post-translational modifications on histone thereby affecting epigenetic regulation.

2.2 Benzene and its metabolites

Benzene is a ubiquitous pollutant and is one of the top production chemicals in the United States. 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. It has been reported that it acts through its metabolites, especially 1,4-benzoquinone (1,4 BQ), as a strong topoisomerase II poison causing DNA DSBs [17]. 25 µM 1,4BQ *in vitro* stimulates DNA cleavage by topo II 8-fold at sites close to defined chromosome breakpoints in leukemia. Benzene metabolites 1,4-BQ (1-10 µM) or 1,4-HQ (10-100 µM) cause DNA damage and fragmentation in cultured HL60 cells though the generation of H_2O_2 oxidative stress leading to apoptosis [18]. 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 in a Chinese hamster ovary (CHO) cell line containing a *neomycin* gene direct repeat recombination substrate by 2.7-fold [19]
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 [20]. These epigenetic changes were maintained in mice throughout life and resulted in a permanent change of liver drug metabolism [20]. 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.

2.3 Metals

Trace amounts of metallic compounds are pervasive in the environment. 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 [21-26], 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 [27]. 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 [27]. Similarly, animal models have shown pregnant C57BL6/J mice administered sodium arsenite in drinking water result in dose-dependent accumulation in newborn pups [28]. 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 [29]. Most importantly, these changes have been shown to occur in stem cells, potentially being transgenerational. Dimethylation of H3 has been demonstrated to lead to gene silencing, 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 [30]. Treatment of human mononuclear THP-1 cells with 50 µmol/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 [29]. Ni ion exposure at 250 µM 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, human lung carcinoma cells, human osteosarcoma cells, human embryonic kidney cells, and mouse embryo fibroblast cells [31]. Further, Ni ions induced *gpt* transgene silencing and exhibited inhibition of H3K9 de-methylation, that led to, or permitted, the observed increase in H3K9me2 [31]. 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 [32]. Low concentrations of trace metals also 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 proteins Rad-18, Top-3a, and Ogg-1, and overall decreased total histone protein production [32].

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 [33-36]. 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 (2 µM 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 100 μg/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 [37]. 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 [38]. 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 1 year, but suprisingly 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 [39-43]. 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 [44-47]. Studies associate arsenic exposure to multiple cancer types in human subjects and gene-specific DNA hypermethylation [48-52]. This direct link between arsenic, tumorigenesis, and hypermethylation was further documented by low dose (0.5 μM) 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 [53]. 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 [53].

2.4 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. Exposure to bisphenol A occurs through its use in food and drink packaging, and water pipes. 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. Genistein and other estrogen derivatives are also

available at health food stores as dietary supplements. The epigenetically toxic effects of environmental chemicals like BPA and phthalates include DNA methylation, histone modification, and changes in microRNA expression levels [2]. 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 receptoer subtypes. In the female reproductive tract, exposure is associated with alterations in morphology, hormonal response, and gene expression, and promote diverse outcomes such as obesity and cancer later in life [54-56]. 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 [57]. After 50 μM 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 [58]. The number of neural stem/progenitor cells was decreased due to promotion of neurogenesis in the dorsal telencephalon. Animal studies have also reported that postnatal exposure to BPA accelerates neurogenesis and causes neuronal migration defects which impair neocortex development in embryos [59]. BPA modulates adipogenic differentiation of cultured human primary adult stem cells [60] and suppresses adipogenic differentiation of mouse mesenchymal stem cells [61].

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 [62]. Developmental exposure to estradiol and BPA leads to an increase in the susceptibility to prostate carcinogenesis with aging through epigenetic regulation [62]. 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 (10 μg/kg) or 17β-estradiol 3-benzoate (2500 μg/kg or 0.1 μg/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 [63-65]. Sensitivity to BPA derivatives may be global as exposure of keritinocytes, skin fibroblasts, intestinal cells (line LS174T), and hepatoma cells (line HepG2) all produce marking of damaged DNA by γH2AX [66, 67].

3. Anti-cancer agents anthracyclins: daunomycin

Anthracyclins are powerful chemotherapeutic agents for the treatment of many cancers. Daunomycin has side effects of bone marrow suppression, anemia, and premature aging of the ovary. Histone acetylation increases the binding affinity of daunomycin by chromatin and enhances the DNA dissociation from nucleosomes possibly facilitating its effects through both DAN damage and suppression of transcription of active gene loci [68]. In multipotent hematopoietic cells (MHCs) of mouse bone marrow [69] daunomycin produces dose-dependent cell toxicity in parallel with time and dose-dependent decrease in the amount of histone proteins bound to the DNA, and a decrease in methylation and acetylation patterns of histone H3 (H3K9ac and H3K9me2). These modifications cause compaction and aggregation of chromatin in MHCs of mouse bone marrow and transcriptional silencing. Mouse models indicate that while doxorubicin leads to DNA breaks, accumulating γH2AX foci, and cell death in the majority of primordial follicles, oocytes, and granulosa cell, a minor proportion of oocytes and granulosa cells survive that may result in long-term impaired function [70].

4. Inhibitors of Topoisomerase II

4.1 Anti cancer agents

DNA topoisomerases are essential cellular enzymes that cause topological changes in the DNA for processes such as replication and transcription. Topoisomerase II (topoII α and topoII β in mammalian cells[71]) is targeted and inhibited by the anti-cancer agents etoposide, doxorubicin, daunorubicin and mitoxantrone [71, 72]. These compounds inhibit the religation of the transient DSBs made by topo II potentially leading to illegitimate repair and chromosomal abnormalities.
The induction of DSBs by topo II inhibitors is associated with chromosomal rearrangements, especially of the *MLL* gene that is frequently rearranged in therapy-related leukemia following initial therapy to a primary tumor with agents such as etoposide. Etoposide has been reported to initiate *MLL* rearrangements in mouse embryonic stem cells [73], primitive hematopoietic stem cells and in human fetal hematopoietic stem cells in several studies [74-77]. Other anti-cancer agents including teniposide, anthracyclines and dactinomycin also are associated with *MLL* rearrangements due to topo II inhibition and enhanced DNA cleavage leading to defective DNA repair and chromosomal translocations [78].

4.2 Bioflavonoids

Bioflavonoids comprise a diverse group of polyphenolic compounds. The most common sources of these bioflavonoids are fruits, vegetables, soy, tea, coffee and wine [79]. Genistein is abundant in soybeans. Due to their antioxidant capacity, they 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 the dietary flavonoids are potent topo II inhibitors and induce DNA cleavage (*i.e.* DSBs), genome instability and chromosomal translocations. Topo II 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 topoisomerase Iiβ [80]. Genistein (50 μM) was shown to be the most effective of the bioflavonoids tested and stimulated enzyme-mediated DNA cleavage ~10-fold [80]. It was shown that 100 μM genistein efficiently induced topo II-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 [81]. Cultured human lymphocytes treated with 50 μM genistein display chromosome abnormalities in metaphase karyotypic analyses [82]. 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) [83]. Quercetin, genistein and kaempferol induced DSBs in primary human hematopoietic CD34+ stem cell-enriched cells (at 25 μM and 50 μM doses) [84]. Besides chromosomal translocations, monosomy or trisomy of *MLL* was also reported in quercetin-exposed cells [84].

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 [85]. 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 [79]. Parallel *in vitro* studies with bone marrow cells exposed to genistein (50 μM) or quercetin (50 μM) showed 2.1-5 rearrangements/80ng genomic DNA (1 per 13,000 cells) for quercetin or genistein as compared to 0.2 translocations/80 ng 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 this 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 [86] which showed that the phytoestrogen genistein, a naturally occurring bioflavonoid, which is found in soy products leaves a permanent signature on the hematopoietic lineage. Studies have shown that exposure of progeny to genistein through maternal diet during pregnancy can have long lasting effects on the progeny. Mice (129/SvJ:C57BL/6J background), ~8 wk of age were given genistein (270 mg/kg of feed) from conception until birth. Genistein induced epigenetic changes and altered the coat color of agouti mice. In this study it was shown that mice prenatally exposed to genistein had a significantly increased hematopoiesis. It led to hypermethylation of repetitive elements and a significant down-regulation of genes involved in hematopoiesis in bone marrow cells of mice that were exposed to genistein. Thus prenatal exposure to genistein affected the process of DNA methylation of hematopoietic cells and caused long lasting alterations in gene expression while also affecting fetal erythropoiesis.

Exposure to the flavonoid quercetin during pregnancy can result in the long-term changes in iron homeostasis at adulthood [87]. 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 (302 mg/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 (~94 ng/mg for quercetin exposure versus ~62 ng/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 [88]. Mice (129/SvJ:C57BL/6J background) were given quercetin (1 mmol or 302 mg/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.

4.3 Other drugs and pesticides

Given the association of *MLL* gene rearrangements and translocations with both therapy-related leukemias as well as infant acute leukemias, analysis of the *MLL* gene structure following exposure to multiple compounds has been widely studied in hopes of determining the common etiologies of these two diseases. Use of certain drugs and pesticides has also been reported to be associated with *MLL* gene fusions. A pilot case-control study [89] that investigated the risk of infant acute leukemia due to transplacental chemical exposure reported that several groups of drugs including herbal medicines, the nonsteroidal anti-inflammatory drug, dipyrone and exposure to pesticides like mosquitocidals including Baygon were associated with *MLL* gene fusions across different countries and ethnic groups. *MLL* rearrangement status was derived by Southern blot analysis, and maternal exposure data were obtained by interviews using a structured questionnaire.

Maternal use of dipyrone during pregnancy was shown to be associated with infant acute leukemia in a hospital-based case-control study conducted in different cities of Brazil [90]. A total of 202 children with newly diagnosed infant acute leukemia were enrolled along with 440 age-matched controls. The magnitude of the odds ratio of 1.45 for maternal exposure to dipyrone suggested the occurrence of interactions between dipyrone exposure during pregnancy and *MLL* rearrangements, leading to infant acute leukemia development.

5. Concluding remarks

A growing body of evidence suggests that several environmental, dietary and therapeutic agents have the potential to cause long-term changes in multiple cellular functions. Epigenetic modulation, cell differentiation, gene expression, signal transduction and illegitimate DNA repair are all associated with human diseases and cancer [91]. The use of the environmental, dietary and therapeutic agents discussed above is widespread today. Evidence is emerging regarding the long-term implications and adverse effects of using these compounds in an unrestricted manner. Due to the beneficial effects assumed to be associated with the use of bioflavonoids, their use as dietary supplements is increasingly popular and widespread. 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. Use of model systems for cells at different stages of differentiation with different susceptibility to genomic damage will provide useful insights into the comparative risk to the human population in terms of the different stages at which exposure occurs.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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APPENDIX C: *MLL-AF9* BREAKPOINT CLUSTER REGION TRANSLOCATIONS INDUCED BY COMMON BIOFLAVONOIDS INDEPENDENT OF CHROMOSOMAL CONTEXT: A MODEL SYSTEM TO RAPIDLY SCREEN ENVIRONMENTAL RISKS

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Keywords
MLL, infant acute leukemia, environmental toxicology, epigenetics, *in utero* exposure, bioflavonoids, genome instability, double-strand breaks, topoisomerase II.

Abstract
Infant acute leukemias account for approximately 30% of all malignancy seen in childhood across the Western world. They are aggressive and characterized by rapid onset shortly after birth. The majority of these have rearrangements involving the *MLL* gene, and *AF9* is one of its most common fusion partners. Since *MLL* breakpoint sequences associated with infant acute leukemia are similar to those in secondary acute myeloid leukemia following exposure to the topoisomerase II poison etoposide, it has been hypothesized that exposure during pregnancy to biochemically similar compounds may promote infant acute leukemia. Hundreds of unregulated nutritional supplements are widely available and perceived to prevent cardiovascular disease, inflammation and cancer. However, there is epidemiological association between bioflavonoids in the generation of specific infant leukemias and multiple bioflavonoid compounds have been shown to inhibit topoisomerase II activity and promote DNA cleavage and *MLL* rearrangements. Thus, their potential to promote leukemic translocations should be directly determined. For this, we established a mouse embryonic stem cell approach to examine the potential for a large number of compounds to mediate DNA damage and repair that results in chromosomal translocations. We found that topo II inhibitors such as the bioflavonoids genistein, quercetin, luteolin and myricetin lead to *MLL-AF9* bcr translocations, genistein and quercetin being the most potent promoters. By contrast, benzoquinone and dipyrone act through a different mechanism and do not generate GFP+ colonies in a statistically significant frequency. In addition, these results demonstrate that the identified *MLL* and *AF9* breakpoint cluster regions are sensitive to these agents and recombinogenic independent of chromosomal context. This system now provides for rapid screening of hundreds of compounds, their systematic analysis of relative risk, dose dependence, and combinatorial impact.