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Therapeutic Modulation of DNA Damage and Repair Mechanisms in Blood Cells

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1. Introduction

Hematopoietic stem cells (HSCs) are a rare population of pluripotent cells that predominantly reside in the bone marrow. Under the appropriate microenvironmental cues, HSCs can undergo self-renewal, expansion, and differentiation into all types of progenitor and terminally differentiated blood cells required for survival of the host (Figure 1). Due to the importance of this cell population for survival, protection of its genome from endogenous and exogenous genotoxic insults is a necessity. However, the intracellular molecular signaling network in hematopoietic cells that control surveillance of the genome as well as maintain genome stability is still largely unexplored. As more is learned regarding how these cells detect a genotoxic event and seek to repair the damaged nucleotides (i.e. DNA adducts), it will become even more feasible to design strategies to protect these life-sustaining cells when the host is exposed to a genotoxic event.

Maintenance of genome stability in both the hematopoietic stem and progenitor cell (HSPC) populations is essential for the sustainment of normal hematopoiesis. For example, transient depletion of bone-marrow derived HSC induced by irradiation or chemotherapy can induce these primitive cells to expand so that the bone marrow can be fully reconstituted; blood-cell development can then continue with minimal disruption. However, once therapy-mediated DNA damage is too high, a DNA-damage threshold is reached resulting in subsequent cell death, myelosuppression, and if not treated, life-threatening bone-marrow failure (Figure 1). With the basal level of DNA repair relatively low in these cells, this does present a challenge to maintain normal hematopoiesis in individuals exposed to prolonged or high levels of genotoxic stress. The reduced ability to repair DNA damage in HSPCs that give rise to multiple mature blood-cell lineages can cause detrimental and long-lasting effects to the host resulting in abnormal cell function, cell death, cellular transformation, and eventually leukemogenesis (Figure 1). Numerous studies have shown that HSPCs are intrinsically more sensitive than other cell types and tissues mostly due to intrinsic limitations in DNA-repair capacity. Buschfort-Papewalis *et al* previously demonstrated that when human HSPCs (phenotypically defined as $CD34^+$ cells) or differentiated cells (phenotypically defined as $CD34^-$ cells) from the same donor were exposed to alkylating agents, an overall decrease in repair capacity of the more primitive $CD34^+$ cells compared the more differentiated cells $CD34^-$ cells was observed. When human $CD34^+$ cells were exposed to a variety of chemotherapeutic drugs, single-strand DNA breaks as well as DNA adducts were found at higher levels and persisted for longer time periods than in $CD34^-$ cells (Buschfort-Papewalis et al., 2002), providing evidence that the kinetics of DNA repair are slower overall in the

$CD34^+$ cells which is the most likely reason for their enhanced sensitivity to irradiation and chemotherapy.

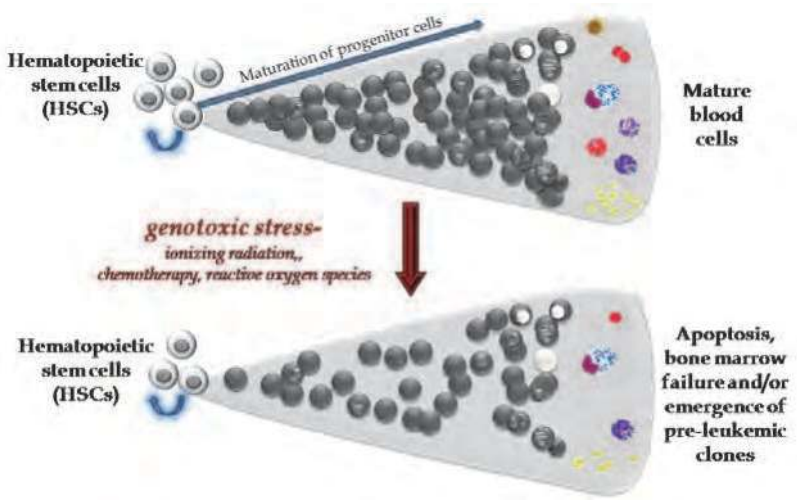

Fig. 1. **Hematopoiesis and impact of genotoxic stress.** Hematopoietic stem cells (HSCs) undergo self-renewal or differentiate into progenitor cells and ultimately into mature terminally differentiated blood cells. When HSC are exposed to exogenous genotoxic stress such as ionizing radiation or chemotherapy and intrinsic stress such as high levels of reactive oxygen species, the bone marrow can become myelosuppressed. If the stress if transient and not of high intensity, the HSC can repopulate the bone marrow. However, if the stress is of sufficient and prolonged intensity, a large number of cells will undergo apoptosis which will lead to bone marrow failure and potentially emergence of pre-leukemic cells (white cells).and ultimately transformation and full blown leukemia.

In this chapter, an overview and analysis of investigations that employ *in vitro* and *in vivo* model systems to study how hematopoietic cells in mouse and man respond to genotoxic stress is presented. In addition, we summarize new therapeutic strategies designed to protect and limit therapy-induced stress to the hematopoietic system and the animal models used to develop and test these therapies.

2. DNA repair pathways in hematopoietic stem and progenitor cells

Mammalian cells are equipped to varying degrees with multiple DNA repair pathways in which to defend against the accumulation of DNA damage caused by environmental insults such as reactive oxygen species, ionizing irradiation, and chemotherapy. The DNA repair pathways- O^6-methylguanine DNA methyltransferase (MGMT) direct repair, nucleotide-excision repair, base-excision repair, mismatch repair, non-homologous DNA end-joining, and homologous recombination- all play prominent roles in maintaining genome stability. A brief overview of the major DNA repairs pathways that are operative in the vast majority of mammalian cells including hematopoietic stem and progenitor is presented below and detailed reviews of these pathways have been previously published (Bekker-Jensen & Mailand, 2010; Niedernhofer, 2008; Seita et al., 2010)

2.1 The direct repair protein O^6-methylguanine DNA methyltransferase (MGMT)

MGMT is also referred to as O^6-methylguanine DNA methyltransferase and in contrast to the other DNA repair pathways which rely on a series of DNA repair proteins, acts singly to repair DNA damage caused by DNA adducts (Gerson, 2004). MGMT repairs DNA damage mediated by endogenous alkylation or by chloroethylating and methylating agents used in anti-cancer therapies. In a one-step stoichiometric reaction, MGMT removes adducts from the O^6 position of guanine (Gerson, 2002). Each MGMT molecule repairs one O^6 lesion; MGMT is then inactivated and degraded. For chloroethylating agents such as carmustine (BCNU) and lomustine (CCNU), if lesions at the O^6 position are not repaired by MGMT, an intramolecular rearrangement occurs over the next 18-24 hours resulting in an interstrand crosslink with the paired cytosine on the opposite DNA strand (Sorrentino, 2002). Since this covalent crosslink between guanine and cytosine is poorly repaired, DNA replication is blocked and caspase 3-mediated apoptosis ensues. In regards to methylating agents such as temozolomide (TMZ), procarbazine, streptozotocin, and dacarbazine, formation of O^6-methyl adducts disrupts hydrogen bonding between the methylated guanine and the corresponding cytosine. As DNA replication proceeds, O^6-methyl guanine pairs with thymidine instead of cytosine and this mismatch ultimately results in a guanine to adenine switch resulting in a point mutation. As a result, the mismatch repair pathway is activated. During this repair process, single-strand gaps occur in the DNA and once this occurs in the same region on the opposite DNA strand, double-strand breaks occur. This leads to erroneous mismatch repair cycles and ultimately leads to apoptosis. Chloroethylating agents are far more potent than methylating agents in terms of cellular cytotoxicity. For chloroethylating agents, formation of only 5-10 O^6-chloroethyl adducts per cell results in cell death. In contrast, for methylating agents, formation of approximately 6000 O^6-methyl adducts per cell is required for cellular toxicity.

MGMT is ubiquitously expressed in all tissues with the bone marrow express substantially lower levels(Sorrentino, 2002). The kinetics of MGMT regeneration following DNA repair also vary amongst tissues; MGMT expression in tumor cells regenerates relatively faster compared to normal tissues(Kreklau et al., 2001). The role of MGMT activity in repairing mutations that would otherwise lead to cancer is confirmed in studies using transgenic or knock-out mice. Indeed transgenic mice that overexpressed MGMT in the thymus were significantly less susceptible to thymic lymphomas caused by methylating agents compared to control littermates(Dumenco et al., 1993). MGMT knock-out mice exhibited an increased incidence of tumors compared to wild-type mice (Glassner et al., 1999). These observations set the stage for developing strategies that can selectively protect normal tissues while simultaneously disrupting DNA repair in cancer cells. MGMT removes two kinds of alkyl adducts-O^6-alkylguanine and O^4 on thymine-leaving behind an intact guanine or thymine in the DNA strand (Park & Gerson, 2005). The MGMT DNA repair protein is referred to as a "suicide" protein since after a single repair event, each molecule is ubiquitinated and ultimately degraded by the proteosome (Gerson, 2002, 2004). Inactivation of the *MGMT* gene via promoter methylation as well as over-expression of its gene product have been found in multiple types of cancer including glioma, melanoma, lymphoma, colorectal cancer and breast cancer and can lead to cell sensitivity or resistance to alkylators such as temozolomide, CCNU, and BCNU. Therefore establishing the expression level of MGMT expression in a particular cancer could help guide the selection of appropriate treatment modalities(Pegg, 2011).

2.2 Nucleotide excision repair (NER)

NER is a type of repair in which a small series of nucleotide bases in each direction from the damaged DNA adduct are removed. There are two types of pathways associated with NER-global genome and transcription-coupled repair-the specific pathway used depends on how the DNA damage is recognized (Park & Gerson, 2005). With global genome repair, xeroderma-pigmentosum complementation group C (XPC) and proteins recognize DNA damage. Many proteins are then recruited to remove the damaged bases as well as to repair the damage including XPA, RPA, transcription factor IIH, XPG, and polymerases delta or epsilon (Park & Gerson, 2005). With transcription-coupled NER, DNA damage leads to a halt in gene transcription and "sensor" proteins are rapidly recruited to the site for initiation of repair including MSH2, CSA, XBA2, XPB, XPG, BRCA1, and BRCA2 (Park & Gerson, 2005). Defects in NER genes are the underlying cause of severe genetic disease states such as Xeroderma pigmentosum and Cockayne's Syndrome.

2.3 Base excision repair (BER)

BER removes a misplaced or damaged base in the DNA strand. DNA glycosylases first remove a damaged base by creating an apurinic or apyrimidinic site within which AP endonuclease (APE-1) cuts the 5′ end of DNA so that the damaged base can be removed and replaced with the correct base. There are two pathways within BER which can be classified into "simple" and "complex." The DNA glycosylases are different between the two pathways. In the simple pathway, N-methylpurine DNA glycosylase (MPG) removes a damaged site leaving an apurinic or apyrimidinic site without nicking the DNA backbone(Park & Gerson, 2005) . With the complex BER pathway, the DNA glycosylase such as 8-oxoguanine DNA glycosylase (OGG1) removes the damaged base and also nicks the DNA backbone(Park & Gerson, 2005). β-polymerase fills in the missing base and DNA ligase seals the nick in the DNA backbone completing BER(Limp-Foster & Kelley, 2000) . Interestingly, BER protein knockout mice are embryonic lethal which in contrast to NER, could explain why no known genetic diseases have been implicated with BER protein loss.

2.4 Mismatch repair (MMR) pathway

The MMR pathway recognizes single mismatches or misaligned sequence repeats (Belcheva et al., 2010; Martin et al., 2010). Msh2 will link with either Msh6 or Msh3 when recognized sequences are detected. Following detection, the Mlh1-Pms2 complex will coordinate the DNA endonuclease removal of damage, DNA re-synthesis, and ligation to complete the repair (Schmutte et al., 2001). Many diseases have been implicated in MMR deficiencies including lymphomas and stem-cell derived leukemias(Park & Gerson, 2005).

2.5 Non-homologous DNA end-joining (NHEJ)

Double strand breaks are most often repaired by NHEJ which can be caused by free radicals or ionizing radiation. Double-strand breaks can also occur following a malfunction during V(D)J recombination in T and B lymphocytes(Park & Gerson, 2005). This pathway is quite complex and uses does not always accurately repair the DNA which could be detrimental to the cell or in some situations such as during V(D)J recombination can add sequence diversity to genes encoding the T and B lymphocyte antigen-specific receptors. The ends of the broken DNA strands are held by a protein complex comprised of Ku70, Ku80, and DNA-PKcs. The repair is completed by XRCC4-DNA ligase IV (Lieber, 2010; Nick McElhinny et

al., 2000). Seita et al have demonstrated that in human HSPCs, that cycling status can dictate selection of the repair pathway that is operative. For example, in quiescent human HSPCs, the NHEJ pathway is preferentially used (Seita et al., 2010).

2.6 Homologous recombination (HR)

Homologous recombination (HR) can also repair double-strand breaks; however, it often can lead to further DNA damage with misalignments, deletions, and rearrangements. The PI-3 kinase, ATM binds to the DNA following a double strand break acting to phosphorylate target proteins involved in repair including Mre11/Rad50/Nbs, BRCA1, BRCA2, RAD 51, BLM, and WRN(Park & Gerson, 2005). Abnormalities in homologous recombination are linked to many genetic diseases including Ataxia Telangiectasia, Werner's syndrome and Bloom's syndrome. In contrast to NHEJ, HR repair is generally considered superior in regards to ensuring that all DNA adducts are accurately repaired. As discussed below, in the study by Seita *et al*, they find that cycling HSPCs in contrast to quiescent HSPCs, utilized the HR pathway to repair DNA strand breaks (Seita et al., 2010). Abnormalities in nucleotide excision repair, telomere maintenance, or non-homologous DNA end-joining in mice have shown increased deficiencies in hematopoietic stem cells (HSCs) as they age implicating DNA damage as a driving force for stem-cell aging(Naka & Hirao, 2011). The continued research in employing gene therapy targeting increased DNA repair proteins in HSCs will continue to be an area of research as the limiting factor in anti-cancer treatments is normal tissue toxicity typically myelosuppression(Niedernhofer, 2008) and will be discussed in detail below.

3. DNA damage responses in hematopoiesis

Understanding the DNA-damage response and DNA-repair pathways that control the sensitivities of HSPCs to DNA-damaging agents will be key as the field continues to develop new pharmacological and cellular-based therapies to protect and maintain genome stability in these life-sustaining cells. The maintenance of HSC in a quiescent state *in vivo* is essential for long-term survival. Quiescence is widely considered to be an essential protective mechanism for stem cells that minimizes endogenous stress caused by cellular respiration and DNA replication. What molecular cues are essential for maintenance of "stem-ness" *in vivo* is a rapidly growing area of investigation and a complex network of proteins control the DNA-damage response and repair pathways. The tumor suppressor protein, p53, clearly plays a pivotal role in the regulation and promotion of senescence, apoptosis, and cell cycle arrest in a variety of cell types(Vousden & Lane, 2007). Several laboratories have utilized p53-deficient mice to investigate the role of this protein in hematopoiesis. Due to the dual functions of p53 in promoting survival versus cell death, results have been somewhat difficult to interpret. In transplantation studies, reconstitution of the bone marrow with p53-null murine HSC has resulted in increased, equivalent or decreased levels of engraftment in the mouse. Most likely, small nuances in experimental design are the most likely reason for these discrepancies and underscore the complexity of the p53-signaling network in hematopoiesis (Chen et al., 2008; Liu et al., 2009; Marusyk et al., 2010). In competitive repopulation assays, Dumble *et al* demonstrated that with increased p53 expression, HSCs exhibited decreased self-renewal capabilities (Dumble et al., 2007). In murine studies by Liu *et al*(Liu & Gerson, 2006; Liu et al., 2009), p53 was highly expressed in primitive Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) wild-type cells compared to the more differentiated hematopoietic myeloid cells. The maintenance of LSK frequency appeared to

be dependent on p53 expression since LSK frequency of bone marrow cells derived from p53 -/- mice was significantly decreased. *In vivo* bromodeoxyuridine assays suggested that while p53 promoted HSC quiescence, p53 absence promoted entry of HSCs into cycle(Liu et al., 2009).

The mechanisms by which HSPCs respond to DNA double-strand breaks (DSBs) induced by genotoxic stress, such as ionizing radiation (IR), is an emerging area of investigation. Two provocative studies conducted by Mohrin et al and Milyavsky et al studied IR-induced DNA damage responses of HSPC compartments derived from mice (Mohrin et al., 2010) and humans (Milyavsky et al., 2010) The molecular mechanisms by which murine and human HSPCs respond to IR appear to differ substantially(Seita et al., 2010). In the mouse model, Mohrin et al. found that murine HSPCs exposed to 2 Gy of IR were significantly more resistant than differentiated myeloid progenitors. Furthermore, significant differences in the radiosensitivity of quiescent versus proliferating murine HSPCs were observed. The role of the *ataxia telangiectasia mutated* (ATM) protein in resistance to IR was also studied. ATM is a serine-threonine protein kinase and a key sensor of DNA damage which is rapidly recruited to DNA double-strand breaks; upon activation at the strand break, ATM plays a major role in activation of the DNA-damage checkpoint. In HSCs derived from ATM-null mice, quiescent and proliferating HSCs exhibited similar radiation sensitivities, indicating the importance of this protein in monitoring DNA damage in these populations. Further studies indicated that different modes of DNA repair are used to repair IR-induced DNA damage in quiescent versus cycling HSPCs. Quiescent HSPCs preferentially utilized the NHEJ pathway, which as mentioned previously is a fairly inefficient DNA repair pathway and is much more error prone than the HR pathway. High basal levels of NHEJ activity were detected in the quiescent HSPCs and this activity increased by ~2-fold following exposure to IR. In contrast to the quiescent HSPCs, the cycling HSPCs preferentially switched to the HR pathway to repair IR-induced double-strand breaks. The HR pathway is able to repair IR-induced DNA damage more accurately than the NHEJ pathway. These data are consistent with the concept that cell-cycle status may influence the integrity of the DNA-repair processes and hence, influence long-term genome stability. For example, irradiated quiescent HSPCs gave rise to progeny that have genomic abnormalities at a higher frequency than cycling HSPCs. In particular when compared to non-irradiated resting HSPCs, greater than 30% of the cells derived from IR-treated resting HSPCs exhibited genomic rearrangements such as reciprocal translocations, interstitial deletions, and complex rearrangements. When mice were transplanted with non-irradiated or irradiated quiescent HSPCs (CD45.1+ cells) into CD45.2 lethally irradiated recipient mice, there was decreased engraftment in mice transplanted with the IR-treated versus non-treated HSPCs. While no transplanted mice developed leukemia or showed signs of abnormal hematopoiesis, further analyses demonstrated the presence of increased genome alterations in mice transplanted with IR-treated quiescent HSPC donor cells. These data suggest a DNA-repair threshold exists in which repair of DNA damage in the mouse may not be fully accomplished in the quiescent HSPCs. Collectively, these findings also suggest that mouse HSC quiescence and reliance on NHEJ to repair IR may be an important mechanism contributing to mutagenesis at the stem-cell level. (Mohrin et al., 2010) This may serve as the starting point for the emergence of certain blood-cell cancers. In addition, the accumulation of mutations in quiescent HSPCs over time may account for many of the stem-cell based hematological abnormalities observed during aging. While the upside is that the quiescent state of murine HSPCs preserves these cells for use in the future, the downside is

that at least in mice, HSPCs in the quiescent state may be intrinsically more vulnerable to mutagenesis following DNA damage, particularly if the DNA damage pathways, such as NHEJ are operative and are not as reliable in repairing the DNA damage.

Milyavsky et al. focused on the response of different hematopoietic cell populations isolated from human cord blood to IR-mediated DNA damage (Milyavsky et al., 2010). In contrast to the murine study, human HSPCs were found to have increased sensitivity to IR than the more differentiated progenitor cells. One likely reason for the increased sensitivity to IR was that the IR-mediated DNA damage led to slower repair of DNA damage in the human HSPC following IR exposure. In this study, Milyavsky et al determined the relative sensitivity of 3 human hematopoietic subpopulations isolated from cord blood and phenotypically defined as : (1) HSC- Lineage-negative (Lin^-), $CD34^+CD38^-$ or $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ fraction; (2) myeloid progenitor population (MPP)-$Lin^-CD34^+CD38^-CD90^-CD45RA^-$ population which contains fewer HSCs and many MPPs; (3) progenitor population-more mature progenitor/precursor cells, including CFCs, which are $Lin^-CD34^+CD38^+$.

To monitor the DNA damage and repair kinetics of the different hematopoietic cell populations, neutral comet assays and subnuclear foci of phosphorylated H2AX (γH2AX) were used as measures of IR-induced DNA double-strand breaks. Different cell population were treated with 15 Gy IR and damage monitored at 30-60 minutes post-IR exposure. In the progenitor cells, 19.4% of the breaks were repaired by 30 minutes and 36.4% of the breaks had been repaired by 60 minutes. In contrast, in the more primitive population-HSC/MPP-no repair of DNA-strand breaks was observed at 30 minutes post-IR; and at 60 minutes, 29.9% of the breaks had been repaired. The delay in repair of the double-strand DNA breaks indicated that the more quiescent HSC/MPP cells differed in their repair capacity from the differentiated progenitor population. Similar differences in DNA repair between the primitive and more differentiated cell populations were noted with the levels of γH2AX foci. At one hour post-exposure to IR, similar levels of γH2AX foci were evident in both the HSC/MPP and the progenitor populations. By 12 hours post-IR exposure, more γH2AX foci still remained in the HSC/MPP compared to the progenitor population (7.1 versus 2.7 foci/nucleus respectively) further confirming the differential DNA repair capabilities of the two populations. Additionally these data correlated with decreased survival in the HSC versus the more differentiated progenitor cells in clonogenic assays.

To assess the viability of irradiated HSPCs in the bone-marrow microenvironment, the SCID-repopulating assay was used(Milyavsky et al., 2010). The nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were transplanted with cord blood $CD34^+$ cells and 5-10 weeks post-transplant cohorts of mice received 3 Gy IR. The bone marrow was harvested from the mice 1.5 hours after IR delivery and the viability of the more primitive human HSC ($Lin^-CD34^+CD38^-$) versus the more differentiated ($Lin^-CD34^+CD38^+$) was determined by flow cytometry. The *in vivo* data correlated with the *in vitro* DNA damage and repair studies, for there was a 2-fold increase in IR-induced apoptosis in the more primitive $Lin^-CD34^+CD38^-$ versus the $Lin^-CD34^+CD38^+$ cells. (Milyavsky et al., 2010) The authors went on to show that the primitive versus more differentiated hematopoietic cells utilize the p53/Bcl-2 pathway but to different degrees. Both *in vitro* and *in vivo* studies indicated that decreased p53 expression or over-expression of Bcl2 in cord blood progenitors blocked IR-induced apoptosis indicating the involvement of the p53-BCL2 pathway in regulating apoptosis and conferring some degree of radioprotection. However, in the secondary transplant setting, lack of p53 expression in the

HSC resulted in higher levels of γH2AX foci in the engrafted cells and this correlated with decreased levels of engraftment. In contrast, in mice transplanted with the Bcl-2-overexpressing HSCs, engraftment was within a normal range. To begin to define in more detail, the intracellular proteins involved in regulating the DNA damage response to IR, global microarray expression profiling indicated that the apoptosis-stimulating protein of p53 (ASPP1) was preferentially expressed and found to play a major role in mediating stem-cell radiosensitivity. ASPP1 is involved in the activation of p53-dependent apoptosis. Expression levels of ASPP1 were higher in HSC versus the more differentiated populations. The critical role of ASPP1 in response of primitive hematopoietic cells to IR was further confirmed in knockdown experiments; knockdown of ASPP1 in a $CD34^{+}CD90^{+}$ primitive cell population versus knockdown in a more differentiated $CD34^{+}CD90^{-}$ cell population revealed that only in the primitive $CDC34^{+}CD90^{+}$ cells with decreased ASPP1 was there an increased resistance to IR exposure (Milyavsky et al., 2010). These studies clearly highlight the differences between these two species and underscore the importance of evaluating DNA damage responses in different models and cell types. It is becomingly increasing clear that DNA damage thresholds that dictate survival versus cell death do vary among different cell types and the mechanisms that regulate these responses will be different and need to be understood in order to develop relevant, effective, and safe strategies to protect bone marrow cells from genotoxic stresses.

4. Increasing cell cycle arrest by pharmacological intervention to improve DNA-repair activity in hematopoietic cells exposed to genotoxic stress

The use of growth-factor support such as granulocyte-colony stimulating factor, granulocyte/macrophage-colony stimulating factor, or erythropoietin can help increase the resistance to DNA-damaging agents and help facilitate recovery of the bone marrow following genotoxic therapy. However, these treatments are very expensive and can have adverse side effects. With the explosion of small-molecule development and drug discovery and as more is learned regarding how different cell types repair DNA damage, it may be possible to modulate DNA damage and repair pathways to increase protection and stability of the hematopoietic genome. These types of strategies could be used to prevent life-threatening myelosuppression in the case of radiation accidents or disasters as well as an adjuvant to aggressive cancer therapies that typically induce severe myelosuppression or complete non-irreversible myeloablation. In terms of IR exposure, the DNA double-strand DNA breaks caused by IR are cell-cycle dependent. The early G1 and late S phases are fairly resistant to IR effects, but the G1/S transition and the G2/M phases are the most sensitive to IR. It has been shown previously that extension of the G1 period following exposure to a genotoxic stress can enhance resistance; it is possible that this could allow for a longer period of time to repair the DNA before entering the cell cycle (Johnson et al., 2010). The cyclin-dependent kinases (CDK2, CDK4, and CDK6) are involved in promoting the G1 to S cell-cycle transition. Johnson et al demonstrated significant increases in the radioprotection of human cell lines and mice exposed to CDK4/6 small molecule inhibitors such as PD0332991(Pfizer, Inc.) that block entry into cell cycle. If wild-type mice were treated with the selective inhibitors, a reversible cell-cycle arrest ensued in the most primitive HSPCs but not in cells already cycling the bone marrow or in other tissues (Johnson et al., 2010). The inhibitor-mediated inhibition of CDK4/6 function abrogated the total-body irradiation whether the inhibitor was given before or even up to several hours after delivery of total

body irradiation (TBI). The authors also determined if inhibition of CDK4/6 could be used in the context of an anti-cancer therapy such as IR as to protect the mice from life-threatening hematotoxicity but also to see if inhibition of CDK4/6 would have any impact on tumor–cell kill (Johnson et al., 2010). In a genetically engineered melanoma mouse model, male *TyrRas Ink4a/Arf* $^{-/-}$ develop autochthonous melanomas due to melanocyte-specific promoter expression of mutant H-Ras. In this model, the growth of the tumors was not inhibited by CDK4/6 inhibitor treatment. When tumor-bearing mice were treated with 7.5 Gy TBI, tumor growth slowed substantially for ~20 days. When the tumor-bearing mice were treated with 1 dose of the CDK4/6 inhibitor 4 hours before TBI, the tumors still responded to the irradiation and growth slowed as in control mice but also there was a substantial decrease in irradiation-induced myelosuppression and mortality. The relative contribution of a prolonged G1 arrest versus a block in the G1 to S transition in enhancing radioprotection of murine HSPCs is not completely clear. Likewise, how these intriguing results will translate into the radioprotection of human bone-marrow cells and whether cancer-initiating mutations could permanently reside in the genome will require further investigation.

5. Human gene-therapy clinical trials that target HSPCs-safety, efficacy, and vectors

HSPCs have been studied for the past three decades as a relevant target for gene therapy due to feasibility with which they can be harvested, *ex vivo* manipulated, and transplanted back into the patient. While the field of hematopoietic-stem cell gene therapy has had set backs and successes, many hurdles still remain in moving this from an experimental to a well accepted treatment modality. Investigations originally focused on utilization of retrovirus vectors for transduction of murine HSC(Williams et al., 1984). As studies progressed and investigators moved to humanized mouse models and large animal models, the promise shown in the murine transplant models did not always hold up. The first attempts of using a gene-therapy approach in human hematopoietic cells were also not very promising. Low-gene transfer and engraftment levels of genetically modified cells in humans were noted (Brenner et al., 1993b; Dunbar et al., 1995). Recent technical advances, however, including identification of suitable cytokine cocktails that minimize stem-cell commitment and differentiation during *ex vivo* culture, use of Retronectin-enhanced gene transfer, as well as enrichment of stem and progenitor cells prior to transduction have improved the efficiency of gene transfer into human cells and resulted in substantial successes in human gene-therapy trials particularly in X-SCID (Abonour et al., 2000; Cavazzana-Calvo et al., 2000) but also some unexpected abnormal events. In terms of the success of the trial, the selective advantage of the corrected hematopoietic cells *in vivo* clearly was a determining factor in generating sufficient numbers of corrected progenitors and lymphocytes in these patients. In spite of these improvements, however, the efficiency of gene transfer into primitive human hematopoietic cells and the engraftment of sufficient numbers of these transduced cells into patients still remains a major impediment. The very early gene-therapy trials using T-lymphocyte populations or hematopoietic stem/progenitor cells into subjects with adenosine deaminase deficiency associated with SCID revealed no signs of genotoxicity(Aiuti et al., 2009) . However, in the first trial of X-SCID gene therapy, in which vector and transduction protocol were optimized, the reality

that oncoretroviral-mediated insertional mutagenesis could serve as an initiator of leukemia in some patients transplanted with genetically engineered autologous hematopoietic cells is concerning. These early success stories coupled with the adverse events reported in the X-SCID(Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b) and chronic granulomatous disease (Metais & Dunbar, 2008; Ott et al., 2006)trials demonstrate that gene-therapy based approaches have a place in the clinic, but at the same time, more research into all aspects of these approaches and the disease state to be treated require more investigation. When the first cases of insertional mutagenesis were reported in the X-SCID trials, the community responded in a very positive and aggressive way to unravel the underlying cause of the T-cell leukemia in several of X-SCID patients. Retroviral insertion into the genome near the LMO-2 and other growth-promoting loci in the transplanted hematopoietic cells was defined very early after the adverse events were reported. In addition, as preclinical studies in nonhuman primates, dogs, and mice have progressed over the past decade, development of leukemias most likely linked or initiated by vector-mediated insertional mutagenesis have been reported in these animal models(Li et al., 2005; Li et al., 2002; Modlich et al., 2005; Nienhuis et al., 2006; Seggewiss et al., 2006; Zhang et al., 2008). Since these reports, a large effort has focused on designing vectors with better safety profiles, well-defined transduction protocols, as well as improved small and large animal models for long-term evaluation of efficacy and safety testing. As the field of hematopoietic gene therapy continues to progress, it is highly likely that vector design coupled with optimal transduction protocols and better understanding of the underlying hematological disease will result in cures (Trobridge et al., 2005).

5.1 Retroviral vectors

Many improvements in vector design resulting in improved transgene expression and safe guards against generation of replication-competent viruses have been summarized in detail elsewhere(Trobridge, 2011; Yi et al., 2011). A brief summary of retroviral vector types and transduction considerations are discussed below.

5.1.1 Gamma-retroviral vectors

Gamma-retroviral vectors were the first to be used for the purpose of marking murine hematopoietic stem and progenitor cells (Williams et al., 1984). Due to their long history in development, they have been the most commonly used so far as vectors for clinical studies. It has been widely appreciated for some time now, that cell division of the retrovirally transduced cells is essential for stable integration of gamma-retroviral vectors into the host genome (Lewis & Emerman, 1994; Miller et al., 1990). Due to the highly quiescent nature of the hematopoietic cells, cytokines that promote cell division in primitive hematopoietic cells have been utilized over the years and in combination with Retronectin-coated plates-to enhance proximity of retroviral particles and cells-have resulted in fairly high levels of transfer into hematopoietic stem and progenitor cells derived from mice, canines and primates(Horn et al., 2002b; Kiem et al., 1999; Kurre et al., 2002; Rosenzweig et al., 1999). There are generally 3 disadvantages in using a gamma-retroviral vector to transduce hematopoietic cells. Silencing of the transgene expressed by the gamma-retroviral vectors can occur over time and this has been well documented in murine transplant studies (Halene et al., 1999; Klug et al., 2000; Robbins et al., 1998). Additionally, while gene transfer into primitive hematopoietic cells is fairly efficient, the cytokine-mediated entry into cell

cycle *ex vivo* can lead to a loss or primitive hematopoietic cells residing in the graft, which could compromise engraftment kinetics (Tisdale et al., 1998). The final downside of gamma-retroviral vectors is their preferential integration near transcript start sites(Wu et al., 2003), which in the proper molecular context can increase the chances of insertional mutagenesis and development of leukemia as discussed above. Alternative vector systems such as lentiviral or foamy viral vectors, are being investigated in a variety of contexts and may have better integration properties such that only minimal cytokine exposure and *ex vivo* culture time are required to achieve acceptable gene transfer into repopulating hematopoietic stem and progenitor cells (Horn et al., 2004a; Josephson et al., 2002; Miyoshi et al., 1999; Trobridge et al., 2005).

5.1.2 Lentiviral vectors

Lentiviral vectors do not necessarily require mitosis in order to enter the nucleus and integrate into the genome. This vector system has been shown to successfully transduce a variety of nondividing target cells(Case et al., 1999; Naldini et al., 1996; Reiser et al., 1996). However, lentiviral-mediated transduction was relatively higher still, when the target cells were induced to undergo cell division during the transduction period (Naldini et al., 1996; Russell & Miller, 1996; Trobridge & Russell, 2004). Lentiviral vectors can also efficiently transduce SCID-repopulating cells in the NOD/SCID mouse model(Miyoshi et al., 1999), as well as long-term repopulating cells in canines (Horn et al., 2004b) and monkeys(An et al., 2001; An et al., 2000; Horn et al., 2002a).

As more long-term repopulation studies are completed, it is becoming clear that lentiviral vectors in contrast to gamma-retroviral vectors allow for efficient transduction, optimal reconstitution, and quicker recovery following transplantation (Goerner et al., 1999; Goerner et al., 2001; Horn et al., 2002a). It is possible that lentiviral vectors can transduce larger numbers of immature hematopoietic repopulating cell compared to gamma-retroviral vectors. One obstacle to extending these observations to large animal models is that human immunodeficiency virus type 1 (HIV1)-based lentiviral vectors in nonhuman primates have a low transduction rate in Old World monkey cells(Song et al., 2005; Stremlau et al., 2004; Stremlau et al., 2005). Peter-Kiem and colleagues have found, however, that human and pigtail macaque (*Macaca nemestrina*) but not baboon (*Papio cynocephalus anubis*) $CD34^+$ cells could be efficiently transduced with lentiviral vectors (Beard et al., 2007; Trobridge et al., 2005).

5.1.3 Foamy virus vectors

Foamy (*spuma*) retroviral vectors are the newest vector system to undergo extensive development and testing for use as a gene-transfer vector for HSC gene therapy. The vector backbones clearly possess several characteristics that make this vector attractive for gene transfer into primitive hematopoietic cell lineages. The vector has the ability to allow for subcloning of larger therapeutic transgenes than the other vector systems. Importantly for use in the clinic, the foamy virus does not cause any known pathogenesis in humans which further strengthens the safety of this novel viral vector (Trobridge et al., 2002). Foamy viral vectors require mitosis for integration into the host genome. In contrast to gamma-retroviral and lentiviral vectors, foamy vectors can form a stable intermediate in quiescent cells(Trobridge & Russell, 2004) which can exist for days. Therefore, this allows for increased opportunities for the foamy viral intermediate to integrate into the genome of the

transduced cell once it divides at a later time point, presumably even after it is transplanted. This is possibly one reason why foamy viral vectors have shown promise in the transduction of SCID-repopulating cells derived from quiescent mobilized peripheral blood (Josephson et al., 2004). How well lentiviral versus foamy viral vectors can transduce canine hematopoietic stem and progenitor cells has been investigated. Equivalent levels of gene transfer were evident in both myeloid and lymphocyte lineages (Beard & Kiem, 2009). Interestingly, when the retroviral insertion sites of lentiviruses and foamy viruses were compared, different insertion profiles was found between the two viral vectors. (De Palma et al., 2005; Dunbar, 2005; Hematti et al., 2004; Laufs et al., 2004; Nowrouzi et al., 2006; Trobridge et al., 2006). Lentiviral vectors integrated more frequently within the coding region of genes compared to gamma-retroviruses which exhibited a strong preference for the 5′ end of genes most often near the enhancer/promoter regions. Another attractive feature of the foamy vectors is that they do not preferentially integrate near transcript start sites, which would predict there is less likely chance of activating near-by gene expression (Trobridge et al., 2005). Investigations continue to interrogate if the genome-insertion profile of lentiviral vectors will result in any difference in the potential mutagenic properties of these vector systems(Modlich et al., 2006; Montini et al., 2006).

5.2 Viral envelopes for pseudotyping vectors

Selection of an appropriate viral envelope for pseudotyping a particular vector is critical and can influence the transduction efficiency and engraftment capability of the target population. For example, if the levels of the host receptor that is specific for the viral envelope is high, then optimal levels of gene transfer can be expected (Kurre et al., 1999; Kurre et al., 2001b; Orlic et al., 1996; Sabatino et al., 1997). Gamma retroviral vectors were originally pseudotyped with the amphotropic envelope in human ((Brenner et al., 1993a; Dunbar et al., 1995; Kohn et al., 1995)and nonhuman primate (Bodine et al., 1993; van Beusechem et al., 1992). As studies progressed, it was discovered that hematopoietic cells in general have low levels of the amphotropic receptor on the most primitive hematopoietic cells; thus, providing a reason for the low levels of gene transfer into the primitive hematopoietic cells(Orlic et al., 1996). Other viral envelope glycoproteins have been discovered and tested(Akkina et al., 1996; Cone & Mulligan, 1984); these include the gibbon ape leukemia virus (GALV) envelope(Wilson et al., 1989), the feline endogenous retrovirus (RD114) envelope(Porter et al., 1996), and the vesicular stomatitis virus G glycoprotein (VSVG) envelope(Emi et al., 1991). When GALV- versus amphotropic-pseudotyped gamma-retroviral vectors were compared, GALV-pseudotyped vectors had higher marking than vectors with amphotropic envelopes in both human progenitors(von Kalle et al., 1994) and baboon repopulating cells(Kiem et al., 1999). The VSVG envelope has undergone extensive testing and has been attractive to the community since it can be substantially concentrated via ultracentrifugation to increase viral titers (Burns et al., 1993). Establishing stable packaging lines that also provide high titer has been problematic since the VSVG can be toxic to the packaging cells(Yang et al., 1995; Yee et al., 1994). The RD114 envelope has some advantages in that it is not toxic, and due to its stability, viral titers can be increased by ultracentrifugation. The RD114 envelope is also resistant to inactivation by human and macaque sera(Kelly et al., 2001; Sandrin et al., 2002). The disadvantage of this envelope, is that transduction efficiencies into human hematopoietic cells is variable and is due to differing levels of RD114 receptor expression on the human cells(Green et al., 2004).

5.3 Optimal conditions for retroviral-mediated transduction of HSPCs

The development of an optimal cytokine cocktail for retroviral transduction of primitive hematopoietic cells has been challenging. To optimize gene transfer into quiescent HSCs, the cytokine combination needs to promote entry into cell cycle but at the same time provide a cytokine milieu that minimizes differentiation, maintains the ability of the cells to engraft efficiently in the host and still undergo self renewal. A number of cytokine cocktails have promoted reasonable levels of gene transfer. (Dunbar et al., 1996; Hematti et al., 2004; Horn et al., 2002b; Kiem et al., 1998; Kiem et al., 1997a; Kiem et al., 2002; Kurre et al., 2001a; Tisdale et al., 1998; Wu et al., 2000). For example, 72 hr to 96 hr transduction periods for gamma-retroviral transductions and 18 hr to 24hr for lentiviral and foamy virus transductions has yielded reasonable levels of gene marking *in vitro* and *in vivo*. The immobilization of the fibronectin fragment, Retronectin (originally referred to as CH-296) on plates prior to transduction improves transduction efficiencies of numerous cell types (Donahue et al., 2001; Hanenberg et al., 1996; Kiem et al., 1998; Vassilopoulos et al., 2001; Wu et al., 2000) by co-localizing VLA-4^+ and/or VLA-5^+ target cells and viral particles on the plates, and in addition, via integrin-mediated signaling, promotes survival and improves stem-cell engraftment (Dao et al., 1998; Donahue et al., 2001).

6. Expression of drug-resistance genes in HSPCs

A strategy for protecting HSPCs from genotoxic stress caused by alkylator therapy is to overexpress DNA-repair proteins in these cells. As long as expression levels of the DNA repair protein are adequate during the treatment phase, this is an attractive approach since the DNA damage caused by the therapy could be adequately repaired in the hematopoietic cells. Depending on the doses of therapy used, one could use alkylators to preferentially expand transduced versus non-transduced cells, or if the treatment is an actual cancer therapy which would need to be administered at higher levels and more frequently, this approach could be used to kill cancer cells and protect the highly susceptible bone-marrow cells from the therapy.

In terms of utilizing a gene-therapy approach to serve as a chemoprotectant of the HSPCs, there are three central questions regarding the introduction of genes into hematopoietic cells that render them resistant to chemotherapy. First, is it possible for genetically modified cells to be amplified *in vivo* and still maintain self-renewal capacity? Second, is sufficient and sustained expression of the chemotherapy-resistant gene obtained so that low dose-induced selection of transduced cells or bone-marrow toxicity typically associated with intensive cancer chemotherapy can be significantly reduced? Third, will hematopoiesis proceed in a normal manner in the post-chemotherapy recovery period? There is a theoretical concern that HSC depletion or exhaustion may ensue. In this case, small numbers of transduced stem cells would be pushed to preferentially expand and differentiate on a continuous basis. Although self-renewal would be presumably be an integral part of this process, overtime preferential differentiation beyond normal hematopoietic limits may lead to extinction of the transduced stem-cell pool(Pollok, 2003). The expression of chemoprotective gene products such as the multidrug resistance protein (MDR1)(Abonour et al., 2000; Hanania et al., 1995; Hesdorffer et al., 1998; Hildinger et al., 1998; Schiedlmeier et al., 2000), dihydrofolate reductase (DHFR) (Allay et al., 1998; Brenner et al., 1993b; Corey et al., 1990; Persons et al., 2004; Warlick et al., 2002; Williams et al., 1987), cytidine deaminase gene(Beausejour et al., 2001; Eliopoulos et al., 2002; Momparler et al., 1996; Rattmann et al., 2006) and MGMT (Cai

et al., 2008; Cai et al., 2006; Cai et al., 2011; Cai et al., 2005; Chinnasamy et al., 1998b; Davis et al., 2000; Davis et al., 1999; Hickson et al., 1998; Jansen et al., 2001; Jansen et al., 2002; Moritz et al., 1995; Neff et al., 2003; Ragg et al., 2000; Sawai et al., 2001) have been thoroughly investigated as potential candidates to protect hematopoietic cells residing in the bone marrow. A number of retroviral vectors derived from gamma-retroviruses, lentiviruses, and foamy viruses as described above continue to be tested for delivery of drug-resistance genes to primitive hematopoietic cells.

7. *In vivo* selection and protection of chemoresistant HSPCs

Studies using small and large animal models continue to investigate the consequences and potential of using genotoxic stress to select *in vivo* for retrovirally transduced HSPCs. A powerful tool for *in-vivo* selection of HSPCs is overexpression of a mutant form of the MGMT DNA repair protein, $MGMT^{P140K}$, which is resistant to the MGMT inhibitor, O^6-benzylguanine (O^6-BG) (Figure 2). Overexpression of $MGMT^{P140K}$ in HSC derived from mice, canines, nonhuman primates, and humanized xenograft models, shows promise as a an approach for selection and protection of the $MGMT^{P140K}$-transduced HSPCs following administration of O^6-BG in combination with alkylators such as BCNU, CCNU, or TMZ (Gerson, 2002).

If $MGMT^{P140K}$ expression is adequate in the HSC, it should also protect the HSPC from high-dose alkylator therapy required in some cancer treatments and thereby prevent therapy-induced myelotoxicity (Figure 3). Generation of HSPC that efficiently repair DNA damage due to chemotherapy may protect patients from life-threatening cytopenias commonly observed following dose-intensified therapy. A case in point, in recent phase II clinical trials, patients with nitrosourea-resistant gliomas were simultaneously treated with O^6-BG to deplete MGMT in the cancer cells, followed by treatment with the DNA-damaging agents, BCNU or temozolomide(Quinn et al., 2009; Quinn et al., 2002). Although lack of tumor progression was transiently observed in some patients, effective dose-escalation therapy could not be achieved due to severe hematopoietic toxicity. These studies provide clinical proof that strategies protecting HSC during dose-intensified therapy are indeed clearly needed in relapsed patients requiring high-dose alkylator therapy. There are two ways in which expression of a chemoresistant gene such as an MGMT could be used for hematopoietic gene therapy (Figure 4). First, cells transduced with an oncoretrovirus vector that co-expresses the MGMT mutant gene as well as another therapeutic gene that does not otherwise have a selective proliferative advantage could be selected or enriched for *in vivo*. In this situation, selection of transduced cells using a low-dose chemotherapeutic regimen would be desirable so as not to cause unnecessary alkylator-mediated DNA damage to other tissues. However, the requirement for alkylators which are highly genotoxic and potentially mutagenic, has dampened enthusiasm for using overexpression of MGMT mutant proteins as an *in vivo*-selection tool. Second, as mentioned above, expression of the MGMT mutant would be utilized as a means of protecting hematopoietic cells from high-dose alkylator therapy designed to kill tumor cells. In the cancer patient setting, the use of MGMT to actually protect the hematopoietic cells from alkylator-based regimens used to treat cancers, such as glioblastoma and melanoma is a reasonable and clinically appropriate use of this chemoprotective mechanism. The use of bicistronic oncoretrovirus vectors that co-express MGMT mutant proteins and the enhanced green fluorescent protein (EGFP) have aided in the evaluation of *in vivo* selection of transduced hematopoietic cells (Figure 4).

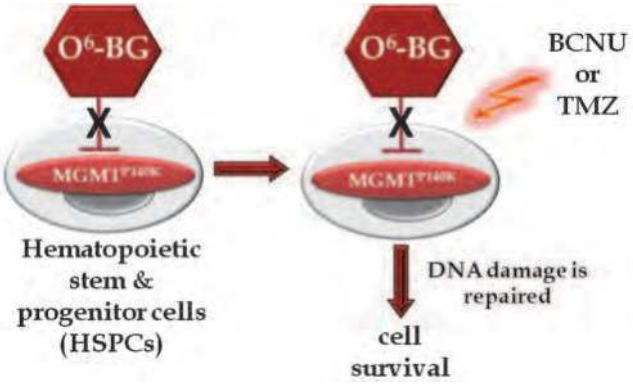

Fig. 2. **Creation of chemoresistant HSPCs by expression of a mutant MGMT protein.** HSPCs are transduced with a retroviral vector that encodes for a mutant MGMT protein such as MGMT^{P140K}. If expression levels of the MGMT^{P140K} transgene are sufficient and sustained, then the transduced cells will be able to repair the DNA damage caused by alkylators such as BCNU and TMZ. Nontransduced HSPCs will only express wild-type MGMT and therefore will not survive since wild-type activity will be inhibited by the MGMT inhibitor, O^6-benzylguanine (O^6-BG). While many mutant MGMT molecules have been tested, the MGMT^{P140K} has shown the most consistency in terms of *in vivo* selection in a variety of animal models.

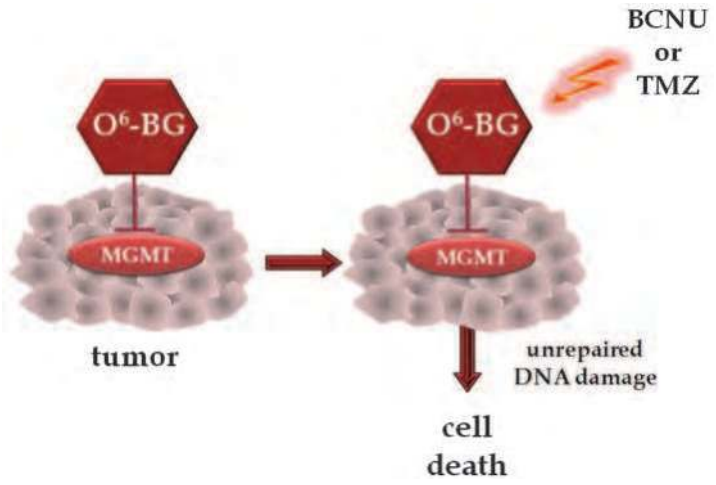

Fig. 3. **Increased tumor-cell killing by sensitizing tumor cells to standard-of-care alkylator therapy.** When tumor cells are exposed to MGMT inhibitors such as O^6-BG, wild-type MGMT is irreversibly inhibited. However, *de novo* synthesis of MGMT is not blocked and will continue. Therefore, a series of bolus injections or continuous infusion of the MGMT inhibitor may be necessary to sufficiently inhibit MGMT-mediated DNA repair protein. If MGMT inhibition is complete, subsequent exposure to alkylators such as BCNU or TMZ will lead to sustained levels of DNA damage and cell death.

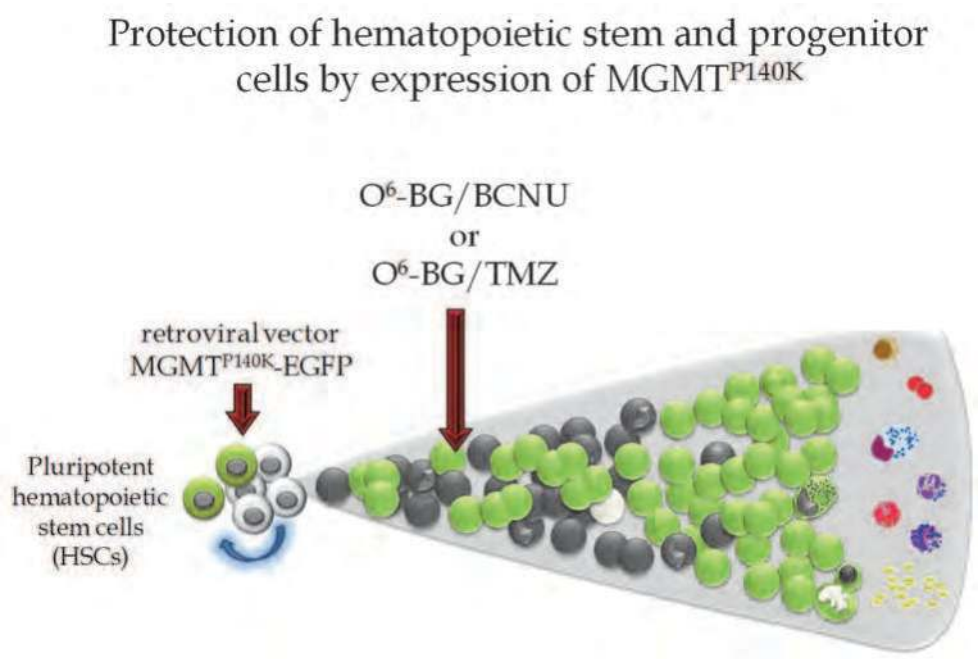

Fig. 4. *In vivo* **selection of MGMT^{P140K}-EGFP-transduced hematopoietic cells.** The enhanced green fluorescent protein (EGFP) has been used in a number of *in vivo* studies to follow the selection and emergence of MGMT^{P140K}-positive cells. In a successful *in vivo*-selection protocol, EGFP-positive cells (green cells) increase over time in multiple hematopoietic lineages following exposure to O^6-BG/BCNU or O^6-BG/TMZ.

Several groups have evaluated the efficacy of chemoprotection using different mutant forms of MGMT that are highly resistant to O^6-BG (Davis et al., 2000; Pollok, 2003; Ragg et al., 2000; Sawai et al., 2001). Ragg *et al* compared efficacy of *in vivo* selection using MGMT-P140A, P140K, and wild-type MGMT. This study showed that MGMT^{P140K} expression resulted in improved protection of the bone-marrow HSCs from an O^6-BG/BCNU chemotherapy regimen compared to the MGMT^{P140A} mutant or wild-type MGMT(Ragg et al., 2000). When compared to wild-type MGMT, the MGMT^{P140A} mutant was 40-fold more resistant to O^6-BG and the MGMT^{P140K} mutant was 1000-fold more resistant. These data support the hypothesis that the combination therapy of O^6-BG/BCNU effectively selects for higher numbers of murine HSC cells compared to BCNU treatment alone and that partial selection with BCNU was presumably due to survival of transduced differentiated progenitors, but inefficient selection of MGMT^{P140K}-transduced stem cells. This hypothesis was confirmed in secondary transplants studies.

TMZ continues to be intensely studied as an alternative to BCNU and may have several advantages over BCNU in terms of DNA repair and multi-organ toxicity. Myelosuppression is the primary toxicity associated with TMZ therapy and pulmonary and renal toxicity is less often observed. Therefore, it has been proposed that dose-intensified therapy to effectively kill tumors with high levels of MGMT may be more feasible with a combination

of O^6-BG and TMZ in contrast to O^6-BG and BCNU. Reese *et al* demonstrated *in vitro* that murine and human clonogenic cells were indeed protected from O^6-BG and TMZ by expression of $MGMT^{G156A}$ and were also protected to a significantly higher degree than a SW40 colon cancer line that expresses high levels of MGMT(Reese et al., 1999). Sawai *et al* demonstrated effective protection and sequential selection of murine stem cells *in vivo* by expression of $MGMT^{P140K}$ and repeated cycles of O^6-BG and TMZ(Sawai et al., 2001).
Koc *et al* treated mice with nonmyeloablative doses of O^6-BG and BCNU and transplanted cohorts of mice with $MGMT^{G156A}$-transduced bone marrow cells(Koc et al., 1999). Subsequently, mice were inoculated with SW480 colon cancer cells that express high levels of wild-type MGMT. In contrast to control mice, there was a 9-fold increase in resistance of clonogenic cells to BCNU and tumor growth was significantly delayed in tumor-bearing mice following multiple cycles of O^6-BG/BCNU. These studies indicated that marrow protection from high-dose chemotherapy and simultaneous decline in tumor growth was feasible even using a tumor that is resistant to standard alkylator therapy.

7.1 Disadvantages of murine transplantation models for assessing utility of transgene over-expression in HSPCs

The use of murine stem-cell transplantation modeling clearly set a solid foundation on which to initially determine the promise of genetically modifying long-term repopulating stem cells and helped move the field of hematopoietic gene therapy forward (Dick et al., 1985; Keller et al., 1985; Williams et al., 1984). Yet, inter-species differences between mice and humans do need to be taken into consideration as the field evaluates feasibility gene-therapy approaches for the clinic. The life span of mice is shorter compared to humans and the high gene-transfer levels obtained in murine HSCs has not been obtainable in human clinical trials. This could be due to relative levels of receptors on mouse versus human hematopoietic cells. For example, transduction of murine hematopoietic progenitor cells with murine ecotropic envelope pseudotyped gamma-retroviral vectors was highly efficient (Dick et al., 1985). In contrast, transduction of canine and human progenitors with a gamma-retroviral vector pseudotyped with the amphotropic envelope used in clinical trials was much less efficient than transduction of mouse progenitors with the ecotropic envelope(Brenner et al., 1993a; Stead et al., 1988). This was due at least in part to low levels of the amphotropic receptor on human hematopoietic stem and progenitor cells (Orlic et al., 1996). A variety of inter-species differences can dictate the transduction efficiency of primitive hematopoietic cells in mice and man including differences in cell-cycle status, telomerase activity, and repopulation capacity of the hematopoietic stem cells (Abkowitz et al., 1996; Abkowitz et al., 1995; Cheshier et al., 1999; Guttorp et al., 1990; Miller et al., 1996; Naldini et al., 1996; Shepherd et al., 2007; Trobridge & Russell, 2004; Trobridge & Kiem, 2010). For example, in mice, a single HSC is capable of engrafting and reconstituting the entire murine hematopoietic system (Dick et al., 1985; Osawa et al., 1996). Additionally, sources of the hematopoietic cells could yield conflicting results; for mouse studies, Lin^-, $Sca\text{-}1^+$, $c\text{-}kit^+$ (LSK) or bone-marrow derived from 5-flurouracil-treated mice are used in contrast to human $CD34^+$ cells that are the target cell population for the SCID-repopulating assay and for human gene therapy trials (Drew et al., 2002; Okuno et al., 2002). In contrast, except for studies using pre-leukemic or leukemic cells, the reconstitution capacity of HSCs in non-human primates and dogs appears to always be derived from polyclonal populations of HSCs. Importantly, in studies evaluating the *in vivo* safety profiles of gene-transfer

vectors, mouse cells tend to be more easily transformed than human cells, which could complicate decisions regarding the utility of new gene-therapy strategies(Hahn et al., 1999; Hahn & Weinberg, 2002; Land et al., 1983) The ease of transforming murine cells could also over-estimate the likelihood of an adverse mutagenic event and hence, not provide a realistic simulation of what to expect in human gene-therapy trials.

The long-term outcome of $MGMT^{P140K}$ expression in conjunction with extended alkylator dosing is a critical variable in assessing the safety of this approach and the potential for mutagenic events due to incomplete repair of DNA adducts. Recently, the long-term effect of $MGMT^{P140K}$-mediated DNA repair and the possibility for emergence of leukemic cells was followed in a murine serial bone-marrow transplant model; the clonality of hematopoiesis in animals transplanted with *in vivo* selected $MGMT^{P140K}$-expressing cells was followed all the way through tertiary transplant recipients(Giordano et al., 2011). In this study, a gamma-retroviral $MGMT^{P140K}$-IRES-EGFP vector was utilized and the target cells were from 5-fluorouracil-treated C57Bl/6 mice. At approximately one month post-transplantation, weekly cycles of alkylator therapy were administered for 5 weeks and consisted of 25 mg/kg O^6-BG followed 1 hour later by injection of either BCNU, 10 mg/kg , ACNU, 6 mg/kg, or TMZ, 70 mg/kg and followed over time. In secondary and tertiary recipients, mice received four weekly treatment doses. The bone-marrow cells were subsequently analyzed for selection of $MGMT^{P140K+}$ cells and for proviral insertion sites at 3–5 weeks following the last cycle of chemotherapy. Due to the repetitive doses of therapy, all cohorts had levels of $MGMT^{P140K}$-transduced cells that approached 100%. While polyclonal reconstitution could be found in three primary, one secondary, and three out of four tertiary transplant recipients analyzed, monoclonal or biclonal integration patterns were observed in most 2° or 3° recipients with insertions found in intron 1 of the *Usp10* gene or downstream of the *Tubb3* gene, respectively. The administration of high- versus low-dose alkylator therapy could also dictate the clonality of the *in vivo*-selected transduced cells. In earlier studies, the selection of $MGMT^{P140K}$-transduced hematopoietic cells using low doses of chemotherapy allowed for the preservation of polyclonal hematopoiesis (Ball et al., 2007; Beard et al., 2009; Gerull et al., 2007). However, the outcome of myeloablative dose of therapy in large animal models still needs to be investigated.

7.2 *In vivo* selection of HSPCs in humanized mouse models

With the development of immunodeficient mouse strains, such as the nonobese diabetic/severe combined immunodeficiency mouse (NOD/SCID), human hematopoiesis can be established and gene-therapy strategies targeting primitive human $CD34^+$ cells can be evaluated. (Ishikawa et al., 2005; Ohbo et al., 1996). Human hematopoietic cells that home and engraft in the bone marrow of the NOD/SCID mouse are called SCID-repopulating cells (SRC). These cells proliferate and undergo multi-lineage differentiation(Kamel-Reid & Dick, 1988; Larochelle et al., 1996; Lee et al., 2001; Wang et al., 1997; Wang et al., 1998).The NOD/SCID xenograft model has been used to study selection and protection of human cells. Although efficient transfer of genes into SRC derived from umbilical cord blood $CD34^+$ cells has been demonstrated(Conneally et al., 1997; Conneally et al., 1998; Demaison et al., 2000; Hennemann et al., 1999; Kelley et al., 2000; Marandin et al., 1998; van Beusechem et al., 1992), we reported that retrovirus-mediated gene transfer into SRC derived from G-CSF-mobilized peripheral blood was markedly less efficient(Pollok et al., 2001). With this in mind, we previously tested to what extent small numbers of transduced G-CSF-mobilized

peripheral blood $CD34^+$ cells could be *in vivo* selected by expression of the P140K mutant and administration of O^6-BG/BCNU. Our laboratory demonstrated effective *in vivo* selection of primitive human hematopoietic cells in NOD/SCID mice following injection of O^6-BG/BCNU. Up to 100% of human cells derived from umbilical cord blood or G-CSF-mobilized peripheral blood were resistant to O^6-BG/BCNU treatment. Nonlethal doses of chemotherapy, consisting of 20 mg/kg O^6-BG and 5 mg/kg BCNU, were used and resulted in only mild cytopenia in the NOD/SCID mice. $MGMT^{P140K}$-transduced cells underwent multi-lineage differentiation *in vivo* and hematopoietic cells were present after *in vivo* treatment that possessed clonogenic activity and expressed high levels of the $MGMT^{P140K}$ activity (Cai et al., 2006).

In regards to assessing whether HSPCs can be protected from a chemotherapy regimen used to kill cancer cells, a combination therapy consisting of an O^6-BG double bolus and BCNU has been studied by us and others. Kreklau et al showed significant regeneration of wild-type MGMT activity in tumors 24 hours post-O^6-BG injection(Kreklau et al., 2001). For optimal numbers of covalent crosslinks between guanine and cytosine to form, it is highly desirable that MGMT activity be kept to a minimum during the first 24 hours following alkylator therapy. Therefore a high-dose chemotherapy regimen consisting of an O^6-BG double-bolus injection (30 mg/kg followed by 15 mg/kg 8 hours later) and BCNU (10 mg/kg, administered one hour after first O^6-BG dose) that efficiently inhibits wild-type MGMT in tumor cells for 24 hours was utilized. Using NOD/SCID mice transplanted with $MGMT^{P140K}$-selected murine bone marrow cells and engrafted with a human glioma, SF767, that expresses high levels of wild-type MGMT, we demonstrated significant regression of a human glioma(Kreklau et al., 2003).

Utilization of the NOD/SCID xenograft as a model to study *in vivo* selection and protection of human HSPCs during chemotherapy provides a useful model for testing new vector designs for expression of chemoresistant genes and evaluating the effect of *ex vivo* manipulation of stem and progenitor cells on hematopoiesis *in vivo*. Development of strategies to protect larger numbers of primitive clonogenic cells is needed. HSCs derived from adult sources can be nonresponsive to cytokine stimulation that is needed for integration of oncoretrovirus vectors into the host genome(Veena et al., 1998) . In addition, adult HSCs lose their pluripotency due to cytokine-mediated differentiation (Gothot et al., 1998; Guenechea et al., 1999). One promising alternative to protect larger numbers of stem cells is to use other virus vectors such as lentivirus or foamy virus that are reported to more efficiently transduce nondividing stem cells. Zielske and Gerson demonstrated significant protection of human clonogenic cells *in vitro* using a lentivirus vector that expresses the $MGMT^{G156A}$ mutant (Zielske & Gerson, 2002).

Repetitive low-dose treatment for *in vivo* selection of $MGMT^{P140K}$-transduced cells has been successful in mice and large animal models. (Beard et al., 2010; Neff et al., 2005) Numerous transplant studies have convincingly proven that long-term repopulating murine HSCs could be selected *in vivo* with O^6-BG/BCNU, O^6-BG /TMZ, or O^6-BG /CCNU. (Cai et al., 2008; Davis et al., 2004; Jansen et al., 2002; Kreklau et al., 2003; Milsom et al., 2004; Persons et al., 2003; Persons et al., 2004; Ragg et al., 2000; Sawai et al., 2003; Sawai et al., 2001) In regards to modeling of this approach with human HSPCs, we and others previously demonstrated that $MGMT^{P140K}$-transduced SCID-repopulating cells and their progeny could be selected *in vivo* in NOD/SCID mice. (Pollok et al., 2003; Zielske et al., 2003) Human HSPCs derived from umbilical cord blood (UCB) or granulocyte colony-stimulating factor - mobilized peripheral blood (MPB) that expressed $MGMT^{P140K}$ could be selected *in vivo* by

nonmyeloablative doses of O^6-BG and BCNU. Gerson and colleagues also reported similar results using $MGMT^{P140K}$-transduced UCB in the NOD/SCID xenograft model. (Zielske et al., 2003) Additionally, our laboratory went on to investigate the extent to which $MGMT^{P140K}$-transduced human SCID-repopulating cells and progeny could be protected *in vivo* by $MGMT^{P140K}$expression during delivery of high-dose alkylator therapy administered that kills cancer cells. In this study, we compared the outcome of administering a low-dose O^6-BG /BCNU regimen versus a high-dose regimen in NOD/SCID mice transplanted with $MGMT^{P140K}$-transduced mobilized peripheral blood $CD34^+$ cells. We found that, at least in the NOD/SCID xenograft model, when human MPB were transduced with an oncoretroviral vector that expresses $MGMT^{P140K}$, only low numbers of human MPB cells were protected following delivery of the myeloablative regimen and that these cells were limited to mature lymphoid and myeloid cells. (Cai et al., 2006) In all these studies, NOD/SCID mice were used and analysis of long-term reconstitution in secondary recipient mice was not determined. We recently determined to what degree long-term human SCID-repopulating cells could be selected *in vivo* by alkylator therapy and to compare the levels of selection in primary and secondary NOD/SCID and NSG mice. Our data demonstrate that human hematopoietic cells of multiple lineages were capable of expressing $MGMT^{P140K}$ for at least 4 months in primary recipients and *in vivo*-selected populations while not as robust as non-selected populations, were able to home and engraft in the bone marrow of secondary recipient NSG months for at least an additional 2 months. In contrast to the NOD/SCID xenograft model, the NSG bone-marrow microenvironment appears to allow for optimal reconstitution and feasibility of long-term follow up of human hematopoiesis(Cai, 2011). The SCID-repopulating assay, while not perfect, does provide an opportunity for testing and refining clinically promising gene-transfer vectors and *ex vivo* transduction conditions. However, the downside is that while SCID-repopulating cells can be transduced with retroviral vectors, lack of correlation with the levels of gene marking in large animal studies and human gene therapy trials is the reality (Horn et al., 2003; Mezquita et al., 2008).

7.3 *In vivo* selection of chemoresistant HSPCs in large animal models

Achieving high transduction levels in HSPCs using large animal models has been challenging and hence, these may be the models that most closely simulate the low transduction rates observed in human gene-therapy trials to date (Bodine et al., 1993; Kiem et al., 1996b; Kiem et al., 1997b). While this model can be expensive, meaningful numbers of dogs can generally be studied and data obtained more closely represents what may be expected in the human situation than studies conducted in mice or humanized mouse models. (Suter et al., 2004). Additionally, since the dog leukocyte antigen type I and II loci are fully characterized, this provides the opportunity to evaluate gene-therapy based approaches in an allogeneic transplantation setting (Ladiges et al., 1990; Maris & Storb, 2002; Nyberg et al., 2004; Suter et al., 2004; Venkataraman et al., 2007; Wagner et al., 1999). Due to the clinical applicability of the results obtained in the dog model, a large amount of effort has been devoted to optimizing the conditions in the dog model which include optimizing the procedures to mobilize HSCs, culture these cells ex vivo, as well as transduce them with retroviral vectors and achieve efficient engraftment *in vivo*(Goerner et al., 1999; Goerner et al., 2001; Horn et al., 2004a; Kiem et al., 2007; Kiem et al., 1996a; Kiem et al., 1999). In terms long-term primary and secondary transplantation studies in canines, more than 80% of the granulocytes can now be marked by *in vivo* selection of cells expressing the $MGMT^{P140K}$

(Beard et al., 2009; Neff et al., 2005). However, it is critical that some studies be performed in nonhuman primates since the hematopoietic cells derived from nonhuman primates can interact and respond to many of the human cytokines used for mobilization of HSCs as well as for transduction protocols. The availability of the macaque SHIV (simian–human immunodeficiency virus) model, that can be used to test gene-therapy strategies for acquired immunodeficiency syndrome (AIDS) is a clear advantage.(Joag, 2000; Trobridge & Kiem, 2010). Additionally in terms of long-term monitoring of animals transplanted with transduced cells, the relative similarity of the human genome to the genome of the nonhuman primates is most likely a highly relevant model for investigating retroviral-insertions sites and monitoring for retroviral-mediated insertional mutagenesis (Calmels et al., 2005).

The use of nonhuman primates to simulate potential outcomes in human gene therapy trials continues to develop. It is likely that the number of transduced cells will need to be expanded in diseases in which there is not an intrinsic growth advantage in diseases such as thalassemia and other hemoglobinopathies as well as expansion of cells that express anti-HIV transgenes. A recent report of selection in rhesus macaques resulted in only transient selection *in vivo*(Larochelle et al., 2009), but efficient and stable long-term selection of >60% in the pigtailed macaque using lentiviral vectors and 80% in the baboon model using gamma-retroviral vectors have been obtained(Trobridge & Kiem, 2010) Beard and colleagues show convincing data that $MGMT^{P140K}$-trasnduced HSPCs of multiple lineages could be selected *in vivo* in both macaques and baboon nonhuman primates. Animals with different levels of base-line engraftment were all efficiently selected with a combination of O^6-BG and BCNU and selection of transduced cells was sustained over time. With over 2 years of follow up in some animals, there have been no signs of clonal emergence or hematopoietic malignancy. Detailed analysis of retroviral integration sites indicated the presence of multiple clones(Beard et al., 2010). One critical difference in these two studies showing transient versus sustained *in vivo* selection in the nonhuman primate models was that animals with the stable increases in selected cells had higher marking levels before initiating treatment with O^6-BG and the alkylating agent.

8. Genotoxicity concerns for *in-vivo* selection, retroviral-vector integration and potential mutagenicity of alkylator regimens

All retroviral vectors will stably integrate into the genome of the host. Depending on where the vector integrates, its proximal integration near promoters of genes could cause increased activation of proto-oncogenes, inactivation of tumor suppressor genes, or modulate expression of genes that do not lead to noticeable changes in cellular metabolism and growth. The risk of biologically relevant proviral-mediated insertional oncogenesis has been previously documented (Baum et al., 2003). As mentioned previously, the most publicized adverse event due to insertional mutagenesis was documented in the X-SCID trial and was predominantly caused by dysregulated expression of the LMO2 proto-oncogene (Baum et al., 2003; Baum et al., 2006; Ferguson et al., 2005; Hacein-Bey-Abina et al., 2003b; Williams & Baum, 2003). Following the reports that retroviral-insertional mutagenesis was directly linked to the clonal expansion that caused leukemia in the French X-linked SCID gene therapy trial(Hacein-Bey-Abina et al., 2003b), a comprehensive study of large animals that had been previously transplanted with gamma-retroviral-transduced HSCs was conducted

(Kiem et al., 2004). In rhesus macaques, baboons, and dogs that had high levels of marked hematopoietic cells, oligoclonal or monoclonal expansion was not evident. However, at ~5 years post-transplantation, in one rhesus macaque transplanted with gamma-retroviral vector-transduced $CD34^+$ cells acute myeloid leukemia did develop (Seggewiss et al., 2006). Analysis of the leukemic cells tumor showed two clonal retroviral-vector insertions one of which was located near the anti-apoptotic gene, BCL2-A1. It is important to note that the X-SCID disease itself could also be an underlying factor that contributed to the leukemogenesis observed in some patients in the X-SCID trial.

O^6-methylating and chloroethylating agents such as TMZ and BCNU (Debiak et al., 2004; Hong et al., 1999; Kaina et al., 2001; Roos et al., 2007; Sanada et al., 2004) place an alkyl lesion at the O^6 position of guanine. The O^6-alkylguanine adducts can be highly mutagenic and cause cell death. The mechanisms by which O^6-methylating agents, such as TMZ, is via futile cycles of mismatch repair pathway which ultimately lead to double-strand DNA breaks. For the chloroethylating agents, a variety of intracellular mechanisms can lead to cell death; the major toxicity is through the creation of interstrand DNA cross-links that block replication (Gerson, 2004; Kaina, 2004; Margison et al., 2002; Rasouli-Nia et al., 1994). The formation of chloroethyl and methyl lesions at the O^6 position of guanine by alkylating agents are highly toxic to cells. In addition, other DNA alkyl adducts formed by exposure to these drugs, actually represent the vast majority of the adducts-N3 position of adenine; N^7 position of guanine; O^2 position of thymine and O^4 position of thymine(Gerson, 2004). The long-term impact of these lesions if left unrepaired is not known but clearly has the potential to lead to genome instability and subsequent emergence of transformed cells. A malignant phenotype has been described for some of these adducts in cell lines and rodent tissues(Maher et al., 1990; Sukumar & Barbacid, 1990; Vogel et al., 1996). While this is still an area of active investigation, there is no evidence to date that overexpression of mutant forms of MGMT and exposure to alkylating agents can lead to leukemia. To this end, several groups have demonstrated that hematopoietic cells transduced with retroviral vectors that express MGMTmutant proteins do not show an increased in the frequency of mutations or chromosomal aberrations upon challenge with O^6 alkylating agents(Allay et al., 1997; Chinnasamy et al., 1998a; Chinnasamy et al., 1998b; Dumenco et al., 1993; Fairbairn et al., 2000; Liu et al., 1994; Liu et al., 1999; Reese et al., 2001). Additionally, there have been no signs of hematopoietic clonal expansion in a canine large animal model in which animals transplanted animals $MGMT^{P140K}$-transduced cells received alkylator therapy over a prolonged time period that also included some escalation in alkylator dose. There was a significant selection of the transduced cells over time, but whether dosing levels were indeed high enough to push the envelope on creating a mutagenic event in the genome and whether the regimen reached the dose range required to kill cancer cells is not clear at this time. (Neff et al., 2005). At least in the canines analyzed so far in which up to a 3-year follow up has been completed, there were no signs of mono- or oligo-clonal hematopoiesis or full blown leukemia in animals that underwent the $MGMT^{P140K}$-dependent *in vivo* chemoselection. (Neff et al., 2006; Neff et al., 2005). In a rhesus macaque that was transplanted with DHFR-transduced cells and underwent an anti-folate *in-vivo* selection regimen, an acute leukemia emerged at 5 years post-transplant and 3 years post-selection. (Seggewiss et al., 2006). Two proviral insertions were found in the leukemic clone; one of the insertion sites was located near the anti-apoptotic gene BCL2-A1. Investigation and

testing in large animal models over a prolonged time period will be able to uncover whether potential toxicities linked to a particular transduction and/or chemoselection approach will yield clinically relevant information.

9. Conclusions

The potential for *in vivo* selection-induced stem-cell exhaustion does remain a concern. The use of small molecule MGMT inhibitors in combination with alkylating agents represent the most potent *in vivo* selection regimen characterized in all animal models studied to date. (Hobin & Fairbairn, 2002; Sawai et al., 2003). Due to the alkylator-mediated death and loss of hematopoietic cells that do not express the MGMT transgene, as well as the immense pressure that is placed on the $MGMT^{P140K}$-expressing cells to repopulate the marrow, these populations are forced to undergo extreme degrees of expansion. At what point the gene-modified HSC can no longer self-renew and undergo exhaustion and depletion is not clear. While we and others have demonstrated that O^6-BG-resistant MGMT-transduced human $CD34^+$ cells can be selected *in vivo*, the dose level and number of cycles could be critical factors that will tip the balance between adequate self-renewal and re-population versus stem-cell exhaustion and subsequent bone-marrow failure. While human CD34+ cells could be selected *in vivo* using chemoselection, a high-dose O^6-BG/BCNU regimen appeared to result in a loss of transduced primitive human cells in the NOD-SCID xenograft model (Cai et al., 2006; Cai et al., 2011; Pollok et al., 2003). Our work clearly highlights the need to further define whether *in vivo* selection regimens place a detrimental proliferative stress on transduced HSPCs. (Neff et al., 2006; Neff et al., 2005). The result of using myeloablative alkylator regimens which would be required in a cancer therapy setting will require further investigations in large animal models to determine the long-term efficacy and safety of this approach.

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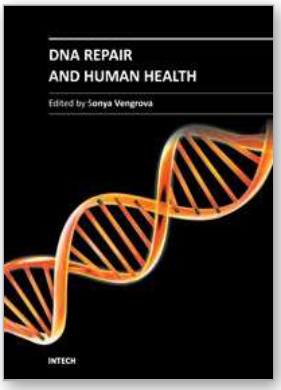

DNA Repair and Human Health

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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