IN VIVO IMAGING OF ENGRAFTMENT AND ENRICHMENT OF LENTIVIRAL
TRANSDUCED HEMATOPOIETIC BONE MARROW CELLS
UNDER MGMT-P140K MEDIATED SELECTION

by

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Dedication

This work is dedicated to my parents, my wife Phuong and my children, Edwin and Karina, for their continuous support, patient and sacrifice.
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List of Abbreviations

AGT: alkyguanine DNA-alkyltransferase
APC: antigen presenting cell
ATCC: American Type Culture Collection
ATP: adenosine triphosphate
BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea
BG: O\textsuperscript{6}-benzyguanine
BLI: bioluminescence imaging
CA: capsid
CCD: Charge-coupled device
CCR5: C-C chemokine receptor type 5
CD: cytidine deaminase
CFC: colony forming cells
CMV: cytomegalovirus
cPPT: central polypurine tract
CT: computed tomography
CXCR4: CXC chemokine receptor 4
DC: dendritic cell
DHFR: dihydrofolate reductase
DMEM: Dulbecco’s Modified Eagle Medium
EtOH: Ethyl Alcohol
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
FL: Flt-3 ligand
FOV: field of view
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
G-CSF: granulocyte-colony stimulating factor
GFP: green fluorescent protein
H&E: hematoxylin and eosin
HIV: human immunodeficiency virus
HSC: hematopoietic stem cell
HSV-TK: herpes simplex virus thymidine kinase
IL-3: interleukin 3
IL-6: interleukin 6
IP: intra-peritoneal
LAM-PCR: linear amplification mediated PCR
Lin-: lineage negative
LTCIC: long term culture initiating cells
LTR: long terminal repeat
LV: lentivirus
MA: matrix
MDR-1: multidrug-resistance protein 1
MGMT: methylguanine DNA-methyltransferase
MLV: murine leukemia virus
MND: MPSV promoter with negative control region deleted
MOI: multiplicity of infection
MPSV: myeloproliferative sarcoma virus

MRI: magnetic resonance imaging

MRSI: magnetic resonance spectroscopic imaging

MSC: mesenchymal stem cell

NC: nucleocapsid

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLS: nuclear localization signal

NMC: nucleated marrow cells

NOD/SCID: non-obese diabetic/severe combined immunodeficiency

OCT: optimal cutting temperature

ORF: open reading frame

PBS (1): phosphate buffered saline

PBS (2): primer binding site

PE: propidium iodide

PET: positron emission tomography

PIC: pre-integration complex

PPT: polypurine tract

RCL: replication competent lentivirus

RNA: ribonucleic acid

ROI: regions of interest

RRE: rev responsive element

SA: splice acceptor

SB: Sleeping Beauty transposon system
SD: splice donor
SCF: stem cell factor
SIN: self-inactivating
SIV: simian immunodeficiency virus
SPECT: single photon emission computed tomography
SRC: SCID repopulating cell
SU: surface unit
TAA: tumor-associated antigen
TM: transmembrane unit
TMZ: temozolomide
TPO: thrombopoietin
TP53: tumor protein p53
U3: unique 3’ region
U5: unique 5’ region
VSVG: vesicular stomatitis virus G protein
WPRE: woodchuck hepatitis virus post-transcriptional regulatory element
Today hematopoietic cell transplantation (HCT) has become routine for treating patients with hematopoietic malignancies and bone marrow failure. Despite many successful preclinical and clinical studies for treating certain genetic defects by transplanting hematopoietic stem cells (HSC) containing one or more therapeutic genes, there have been limitations and complications with this approach. In our lab, we have been working to improve the efficiency of viral gene transfer and transgene expression in HSC. Due to the difficulties of culturing and enriching HSC ex vivo, we have developed a strategy to select and enrich transplanted HSC in vivo with the help of a mutant human alkylguanine DNA-transferase (MGMT-P140K). In order to monitor the fact of transduced and transplanted cells and to understand the dynamic selection process in vivo, we utilized a novel imaging technique, bioluminescence imaging (BLI). Developed in the past few years, genetic modification using the firefly luciferase gene allows researchers to non-invasively visualize transduced HSC homing, expansion, engraftment and in vivo selection over a long period of time. Periodical bioluminescence imaging revealed dramatic spatial and temporal shifting of transgene expression in early phase of engraftment, indicating a stochastic event of homing and expansion after HSC
transplantation. Long-term monitoring of in vivo selection showed an effective MGMT-P140K mediated selection on lentiviral transduced bone marrow cells, resulting in persistent BLI foci, which represented long-lived transgene expressing HSC derived stem and progenitor cells. The results from these studies provided interesting and valuable information on the biology of bone marrow transplantation and the field of gene therapy.
Chapter 1

Hematopoietic Stem Cell Gene Therapy

1.1 Hematopoietic Stem Cells as Targets for Gene Therapy

Hematopoietic stem cells (HSCs) have the ability of extensive self-renewal and the capacity to differentiate into blood cells of all hematopoietic lineages throughout the lifetime of an animal. HSCs were first discovered by McCulloch and Till in 1963 [1, 2], and today are the most well studied adult stem cells. Even before its identification, Nobel Laureate Dr. E. Donnall Thomas had performed bone marrow derived stem cell transplant for leukemia patients in the 50s[3]. In 1968, Dr. Robert A. Good was the first to successfully perform human bone marrow transplant for a SCID patient [4, 5]. Today, bone marrow transplantation has been routinely used to treat hematological diseases, hereditary disorders, congenital immunodeficiency, and severe myelo-suppression in non-hematological cancers. The power to regenerate and differentiate gives HSC a tremendous advantage over any other somatic cells as target cells of gene therapy treatments, especially for hematopoietic and/or immune diseases, as well as providing protection and facilitating recovery from chemotherapy in patient’s bone marrows because the therapeutic gene can be passed along to progeny cells and have long-lasting impact. Other cells have more limited potential, such as lymphocytes, muscle, cardiac, and MSC.

There are four reservoirs of hematopoietic stem cells, and each reservoir can be used as HSC collection source. In adult, hematopoietic stem cells can be obtained from bone marrows and peripheral blood. A high percentage of HSC can also be found in umbilical
cord blood and fetal liver. Fetal liver is an excellent organ for studying the development of hematopoietic system and for experimental hematopoietic stem cell research, but fetal liver is not feasible for human gene therapy purpose. Cord blood can potentially be an excellent source for patient if one’s own cord blood was saved during birth. HSCs are rare cells, and based on current murine SKL (Sca+Kit+Lin-) labeling of HSC, there is an estimate of 1 in 10,000 to 15,000 hematopoietic nucleated marrow cells (NMC) in the bone marrow. The frequencies of HSCs appeared to be conserved in mammals with a frequency of HSCs in humans estimated to be only 0.7-1.5 HSCs/10^6 NMCs [6]. There are small numbers of HSCs circulating in peripheral blood. In order to recruit more HSCs in peripheral blood, granulocyte-colony stimulating factor (G-CSF) is administered. In addition, reagent that blocks the VTLA-4 integrin also releases HSCs. Either approach can be used to mobilized marrow-bound HSCs into the blood for collection.

In most cases, the identification of those primitive stem cells has been derived from functional assays for “stemness”. As a feasible approach of identification and isolation, surface proteins and antigens recognized by monoclonal antibodies have been utilized as markers to define HSCs. However, there is no specific marker that identifies the most primitive HSCs. In human, primitive HSCs are identified as CD34 positive and CD38 negative, and in mouse, murine HSCs are Sca-1 positive, c-Kit positive, and lineage negative [7, 8]. Another identification was established based on the stem cell’s ability of efflux of a dye, Hoechst 33342 [9]. The functional impact of dye efflux links to HSC by the function of ABC transporters on cellular membrane. This population isolated based
on efflux of Hoechst has 1000-fold increase in HSC frequency. However, there is still broad clonal diversity in this purified HSC population [10].

The widely accepted concept of HSC hematopoiesis is hierarchical and unidirectional (Fig. 1). Only primitive HSC is able to self-renew, and the differentiation to progenitor cells and terminal differentiated cells is irreversible. Primitive HSC can go through either symmetric or asymmetric cell divisions. It can go through asymmetric cell division to generate a more committed progenitor cells for further differentiation and a copy of original stem cell for self-renew (Fig. 2a). It has also been suggested that, under certain conditions, primitive HSC can go through symmetric division to generate two copies of primitive stem cells or two committed progenitor cells (Fig. 2b). The condition that dictates the outcome is not yet understood, and most likely, the determinant is generated from surrounding hematopoietic microenvironment. Most of the evidence was quite consistent with this model. Cell cycle studies by Quesenberry et al. suggest some plasticities to these rigid categoring with a reversible continuum based on cell cycle status [11-13]. A further refinement to the hierarchic model is the newly identified “intermediate-term” multipotent hematopoietic stem cells that undergo self-renewal and differentiate for up to 6-8 months [14].
Figure 1. Hematopoiesis.

Hematopoietic cell lineages are based on a hierarchical system. Hematopoietic stem cell is the most primitive cell, which can self renew and differentiated to progenitors that can give rise to all types of blood cells and platelets.
In order to measure the “stemness” of HSC, several in vitro and in vivo assays have been developed to measure differentiating HSCs. Primitive HSCs and their progeny can form colonies in semi-solid methylcellulose culture in the presence of different cytokines, this ability can be measured in colony forming cell (CFC) assay. Although CFC assay can only measure progenitor cells, many primitive HSCs can form colonies in this assay, and some of which will have “re-colony” formation potential. The usual assay identifies hematopoietic myeloid progenitors. T-lymphocytes can’t form cell clusters identified as colonies because T colony-forming cells are highly motile [15]. This assay is particularly useful for viral transduction analysis because we can measure the percentage of progenitor cells containing transgene that have clonal out growth potential.

A more direct in vitro assay to measure long-term repopulating cells is the long-term culture-initiating cell (LTC-IC). Irradiated human marrow adherent cells can be used as stromal cells and co-cultured with limiting diluted primitive bone marrow cells. After 5
weeks of incubation with weekly replacement of media, the cobblestone areas formed in culture can be counted as short and long term repopulating cells [16, 17]. Longer than 8 weeks may be necessary to identify long-term HSC, and only colonies formed after 8 weeks are derived from long-term repopulating cells. Replacing irradiated human marrow adherent cells with murine fibroblasts that express human steel factor (SF), interleukin-3 (IL-3), and granulocyte colony-stimulating factor (G-CSF) can greatly increase the sensitivity of LTC-IC [18]. Interestingly, the overall number of LTC-IC after 6 weeks was not changed by this modification, indicating there was no stimulation in the self-renewal of long-term repopulating cells.

An *in vivo* assay of early hematopoietic progenitor cells is the colony forming unit-spleen (CFU-S). About 1x10^4 to 1x10^5 bone marrow cells are injected into each lethally irradiated syngeneic recipient via tail-vein. Between 10-12 days after infusion, spleen of the recipient is removed. Visible nodules can be identified during this early stage of hematopoietic reconstitution. Each nodule represents a colony formed by a differentiated progenitor cell [2, 19]. Later studies identified CFU-S colonies are not long-term repopulating cells, which are much less common in frequency.

To measure a true hematopoietic stem cell, the gold standard assay has been the transplantation experiment. In humans, the HSCs are identified as a population of cells capable of long term (6 months 20 years) hematopoietic reconstitution. In order to study human HSCs *in vivo*, a xenograft model with non-obese diabetic/severe combined immunodeficiency mice (NOD/SCID) has been established to study human CD34+ cells [20, 21]. The long-term engrafted stem cells are called NOD/SCID repopulating cells (SRC). In mice, the primitive HSCs were able to sustain all hematopoietic cells for more
than 2-5 months in lethally irradiated recipients, and they can also be serially transplanted into and support secondary lethally irradiated recipients.

1.2 Gene Transfer Vectors

In order to treat genetic defect or provide marrow protection, one or more therapeutic genes have to be transfer to the target cells and to be expressed efficiently. When treating defective gene, there is a need to incorporate therapeutic gene into the genome to establish stable transmission and expression progeny. There have been several methods developed to deliver genes to target cells. Joyner et al. was the first to transfer a herpesvirus gene or a bacterial gene into hematopoietic progenitor cells with a retroviral vector [22]. Since gamma-retrovirus can transduce cells efficiently and stably integrate therapeutic gene into cellular genome, over 60% clinical gene therapy trials have used gamma-retroviral vectors. Naked DNA, adenovirus, and adeno-associated virus have also been used, and recently lentivirus made up the remaining trials. The following table shows advantages and disadvantages of those vectors:
<table>
<thead>
<tr>
<th>Gene Transfer Vectors</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked DNA</td>
<td>Easy manufacturing, increased transgene size.</td>
<td>Very low transfection efficiency, and can’t obtain sustained transgene expression.</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Easy to generate high titer viruses and easy to transfect cells.</td>
<td>Non-integrating virus, and induce host immune response.</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>Lack of pathogenicity and immunogenicity.</td>
<td>Very low percentage of genome integration and small payload size.</td>
</tr>
<tr>
<td>Gamma-retrovirus</td>
<td>Stable integration in target cells, producer cell lines to generate consistent amount of viruses, low immunogenicity.</td>
<td>Insertional mutagenesis, integration requires cell division, and small payload.</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Stable integration in target cells, transducing non-dividing cells, and low immunogenicity.</td>
<td>Lower but possible insertional mutagenesis than gamma-retrovirus, less than 10k payload, no producer cell lines.</td>
</tr>
</tbody>
</table>

Table 1. Advantages and disadvantages of gene transfer vectors.

While anticipated, based on well known possibility of insertional mutagenesis by retroviruses, it was only when leukemia was developed in a clinical trial in early 2000 that all retroviral gene therapy trials in the United States were placed on hold by the FDA [23-25]. All have been reopened with greater attention in monitoring retroviral insertion sites. However, lentiviral vector studies were still gaining momentum in the transduction of various stem cells including hematopoietic, germ cells, neural stem cells, and embryonic stem cells [26-29]. In our studies, we have been focused on lentiviral vector gene transfer because of its stable integration, transduction of non-dividing cells, and
possible reduced frequency of insertional mutagenesis. A detailed review on lentiviral vectors in cancer gene therapy is in Chapter 2.

1.3 Approaches to therapeutic stem cell treatment

1.3.1 Myeloablative vs non-myeloablative transplantation

One of the biggest challenges in hematopoietic stem cell gene therapy in clinic is to acquire enough gene-modified marrow cells to reach a therapeutic effect. However, the competition between transplanted and endogenous HSCs often results in extremely low engraftment measured by transgene expressing cells in peripheral blood. To eliminate the competition and create more stem cell niches in body before transplantation, two preconditionings, myeloablation and non-myeloablation, were developed for the preparation of recipients. Myeloablation is achieved by giving recipients high doses of chemotherapy or irradiation to eradicate as much diseased cells as possible. It also eliminates endogenous stem cells and gives an advantage for transplanted HSCs to engraft. However, myeloablation is highly toxic to the recipient, and by eliminating endogenous stem cells, recipient lacks effective immune system and will rely completely on infused stem cell to survive. Therefore, it is often used on patients with late stages of diseases. Interestingly, it has been shown that myeloablation may not be necessary to allow successful engraftment of transplanted HSCs in recipients [11, 30]. Non-myeloablation requires to give recipients low dose of chemotherapy or irradiation which will not kill a lot of bone marrow cells and will low the risk for infection and bone marrow failure. Non-myeloablation can be given to transplant patients who are not qualified for myeloablation. However, non-myeloablation still leaves the patient with
their own hematopoietic stem cells, which compete with injected hematopoietic stem cells used in treatment, leading to lower engraftment. To overcome this limitation, the expansion of HSCs with integrated transgene would be necessary.

1.3.2 Efforts at *ex vivo* expansion

*Ex vivo* culturing and expansion of hematopoietic stem cells has been developed for more than three decades with limited success. In order to expand hematopoietic stem cells, culturing media must contain cytokines stimulating HSC self-renewal and preventing differentiation. Extensive expansion of human primitive hematopoietic stem cells from cord blood has been shown with supplying of two hematopoietic growth factors, FLT-3 ligand (FL) and thrombopoietin (TPO), to the media [31]. Despite many laboratories claimed successful expansion of hematopoietic stem cells *in vitro*, a wide accepted cytokine cocktail specifically for HSC *ex vivo* expansion has not yet been achieved. Co-culturing HSCs with mesenchymal stem cells (MSCs) derived from umbilical cord blood or bone marrow can also significantly expand HSCs *ex vivo* [32, 33]. In addition, MSCs have been shown to support the gamma-retroviral transduction of hematopoietic stem cells [34]. The goal of including MSCs in the mix is to mimic the microenvironment of hematopoietic stem cell niche. However, the data is not conclusive. Recently, a lot of studies have been focused on understanding the mechanism that drives HSC self-renewal. Understanding the mechanism will greatly enhance our ability to culture and expand gene-modified HSCs *ex vivo*. Until then, alternative approach to *ex vivo* expansion is to enrich transduced HSCs *in vivo* by utilizing endogenous HSC niches for stem cell expansion.
1.3.3 Drug selection and survival mediated reconstitution

There are two ways to enrich and expand HSCs in vivo, and the underlining principle is to give them survival advantage. One approach is to give them a proliferative advantage over endogenous HSCs. The homeodomain transcription factor HOXB4 has been shown to expand hematopoietic stem cells in vitro and in vivo, as well as inducing hematopoietic differentiation from embryonic stem cells [35-38]. However, balancing the expression level of HOXB4 to induce self-renewal instead of differentiation is a delicate matter [39].

The second approach is to give HSCs a drug resistant gene to prevent them from cytotoxicity. Chemotherapy for cancer patients usually results in myelo-suppression. To protect hematopoietic stem cells, drug resistance genes can be introduced to HSCs to protect them. Several drug resistance genes have been developed over the years, such as multidrug-resistance protein 1 (MDR-1) [40, 41], cytidine deaminase (CDA) [42, 43], and dihydrofolate reductase (DHFR) [44-46] etc. Up to date, mutant forms of a DNA repair protein, alkylguanine DNA-alkyltransferase (AGT), also known as methylguanine DNA-methlytransferase (MGMT), has been shown to be the most effective for in vivo selection of hematopoietic stem cells.

When alkylating chemotherapeutic drugs, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or methylating agent, such as Temozolomide (TMZ), are in contact with cellular DNA, they will transfer alkyl or methyl group to the O-6 position of guanine on cellular DNA. This adduct on the O-6 position of guanine is toxic to cell when cell is replicating, leading to DNA damage. AGT protein can remove adduct and repair the DNA. Using the same mechanism, many human tumors have been shown to express
high level of AGT protein, rendering them more resistant to chemotherapeutic drugs [47].

Wild-type AGT inhibitor, O6-benzylguanine, has been used to combat AGT-mediated resistance to BCNU [48, 49]. In order to protect hematopoietic bone marrows from damage caused by the same chemotherapeutic drugs, two mutant forms of AGT, MGMT-P140K and MGMT-G156A, were developed in our lab and others, and both mutants have been shown to be highly resistant to BG-mediated degradation [50, 51].

By introducing those mutant forms of AGT in murine hematopoietic stem cells with gamma-retrovirus, transduced HSCs were protected against BG plus BCNU or BG plus TMZ treatments [52, 53]. From those studies, we can see that endogenous or untransduced HSCs are most likely to be killed by these chemo-drugs, and the proliferative stress caused by drug treatments will lead to the expansion of surviving gene-modified HSCs, resulting in increase in percentage among all HSCs.

Understanding this mechanism, our lab and other labs have developed an in vivo selection strategy to select for mutant MGMT expressing HSCs (Fig. 8). The published results clearly showed MGMT-P140K lentiviral transduced human HSCs were more resistant to BG plus BCNU treatments, and in vivo selection of human HSCs can enhance the repopulation of human HSCs in non-myeloablated NOD/SCID recipients [54, 55].
Chapter 2

Lentiviral Vectors for Cancer Therapy

*Portions of this chapter are from:


2.1 Introduction

It was about four decades ago when researchers began to first consider the possibility that genetic diseases could potentially be reversed through correcting defective genes [56]. As the idea developed in the early 1980s the practice of gene therapy began albeit with early misses and delays [57]. The rise to prominence was due to the notion that gene therapy could replace pharmaceuticals in medical treatments. The initial goal for gene therapy was to treat monogenic diseases by supplying patients with a healthy gene to compensate for the defective gene. However, early on, cancer seemed like an obvious target especially for proof of concept and assessments of toxicity [58, 59]. This is shown by the fact that about two-thirds of all gene therapy clinical trials measured in 2007 was targeting cancer [60].

In order to generate an effective and safe vector for gene therapy, both non-viral and viral vectors have been extensively studied and used in clinical trials. There are limitations in each of the vectors, non-viral and some viral vectors efficiently transfect the cells but do not integrate their genetic payload into the genome of targeted cells, resulting in transient expression. On the other hand, gamma-retroviruses, lentiviruses,
and adeno-associated viruses are able to integrate their transgenes into targeting cells to ensure long-term expression with various degrees of efficiency. Among those integrated viral vectors, lentiviral vectors have the largest payload capacity for packaging of 8-10k base-long, compared to 8kb and 5kb size limits in gamma-retroviral vectors and adeno-associated viral vectors, respectively. While retroviral and lentiviral vectors efficiently integrate, less than 10% adeno-associated viral vectors are integrated into targeting cells [61]. A limitation of retroviruses is that they can only transduce actively dividing cells because the integration step requires the breakdown of the nuclear membrane during infection [62, 63].

In the early days of gene therapy, lentiviruses, such as the human immunodeficiency virus (HIV-1), were not considered a possible gene transfer vector for clinical use because of their pathogenicity in humans. The risks appeared to be too great to consider the benefits. However, sophisticated interrogation of the lentiviral genome and the identification of the essential elements for biology vs integration have yielded effective and safe vectors for clinical use. Two key observations have further enabled this effort. First, contrary to gamma-retroviruses, lentiviruses are able to transduce non-dividing, quiescent cells, such as quiescent stem cells, neuronal cells, and other terminally differentiated cells [64, 65]. Second, clinical trials with gamma-retroviral vectors faced a major setback when an X-linked severe combined immunodeficiency (X-SCID) trial in France resulted in patients developing leukemia. This complication resulted in the suspension of retroviral gene therapy trials [23, 66]. The mechanism of oncogenesis in these patients was shown to be the insertion and activation of LMO2 T-cell oncogene by the gamma-retroviral vectors used in the trial [24, 67]. The explanation appears to be that
gamma-retroviruses prefer the integration in open regions of DNA, typically near promoters whereas lentiviruses have preference to integrate into active transcription without a bias for promoter regions, thus significantly reducing the risk of insertional mutagenesis [68].

In this chapter, we discuss lentiviruses, the development of lentiviral vector system, various lentiviral applications in treating cancer, and the current progress with lentiviral vector clinical trials. We focus more on the HIV-1 derived vector, which is currently the most widely used lentiviral vector.

2.2 Lentiviruses

Lentivirus belongs to the family *retroviridae* and has a characteristic of long clinical latency after initial infection. The genome of HIV-1 has been extensively studied in the past three decades because of its clinical relevance and its potential role as a gene therapy vector. Even though a vast amount of questions remain unanswered, the basic functions of HIV-1 viral proteins are known.

2.2.1 Lentiviral genome and structure

Each HIV-1 particle has two identical copies of a single positive 9kb RNA strand, and each RNA strain contains 9 open reading frames (ORFs), which encode for 15 viral proteins (Fig. 3a). Three of the nine ORFs encode for common retroviral proteins: *gag* (MA, CA, NC and p6), *pol* (protease, reverse transcriptase and integrase), and *env* (SU and TM). These are viral capsid proteins that are viral enzymes necessary for the reverse transcription and integration steps, and envelope glycoprotein to form new viral particles.
The remaining six unique lentiviral proteins are accessory and regulatory proteins: Vif, Vpr, Vpu, Nef, Tat, and Rev [69].

The function of each of these accessory proteins has been determined, and each has been shown to affect the production of new lentiviral particles and their virulence. Vif (Viral infectivity factor) plays an important role in the production of infectious viruses. Viruses with mutated Vif are unable to produce infectious viruses because of a cell factor produced in certain cells [70], and this factor has been identified as APOBEC3G, which can impair the infectivity of HIV viruses [71, 72]. Vpr (Viral protein R) contains NLS (nuclear localization signal) and direct the pre-integration complex (PIC) to the nucleus without the breakdown of nuclear membrane, allowing the infection of non-dividing cells. Vpr is also able to induce cell cycle arrest in G2 phase to increase viral transcription and protein production. Additionally, over-expression of Vpr can eliminate T-cells through apoptosis [73]. Vpu (Viral protein U) also has multiple functions. It is shown to down-regulate CD4 cells on host cells, interact with the cellular restriction factor tetherin, and play a critical role in the virion budding process [74]. Nef (Negative factor) also impairs the host immune response by down-regulating CD4 and MHC class I expression [75]. Two regulatory proteins, Tat and Rev, are critical in transcription elongation and viral RNA export from the nucleus. Rev (Regulator of expression) binds to the Rev responsive element (RRE) of the RNA to transport viral mRNA or unspliced
viral genomic RNA out of the nucleus for translating viral proteins and packaging new viral particles [76]. Tat binds to the 5’ end of all nascent viral mRNA and enhances transcription, and Tat is also released to extracellular environment [77].

Within a mature HIV-1 particle, two copies of viral genomic RNA surrounded by nucleocapsid (NC) proteins are enclosed in a conical shell formed by capsid proteins along with several viral enzymes (protease, reverse transcriptase, and integrase), p6, and
three accessory proteins, Vif, Vpr, and Nef [78]. The viral capsid is wrapped inside a lipid membrane in which viral matrix proteins cover the inner side of the lipid membrane, and viral envelope proteins localize across and outside of it (Fig. 3b).

2.2.2 Lentiviral life cycle

Once the HIV-1 viral particles bind to CD4 receptors on target cells, the viral surface protein gp120 undergoes a conformational change to reveal secondary binding sites for co-receptors CCR5 or CXCR4 [79]. After the binding of gp120 to CD4 and the co-receptor, further conformational changes result in the fusion of the viral and cell membranes, which causes the release of the viral core into the target cells. This HIV-1 entry step has recently been contested. Miyauchi et al. suggested that the fusion of the HIV-1 membrane happens at the endosome instead of at the cell surface [80].

When viral core gains entry into the cell, it uncoats itself and forms the reverse transcription complex. With the presence of viral reverse transcriptase in the uncoated viral core, single stranded RNA molecule is transcribed into double stranded cDNA, which can then be integrated into the host genome. Reverse transcription starts when tRNA\textsubscript{lys}3, presented in both the cell cytoplasm and viral core [81], binds to the primer binding site (PBS) on the 5’ end of the viral genome. After the binding, a small fragment of cDNA is synthesized, termed the negative-strand strong-stop cDNA. This cDNA fragment then dissociates from the RNA-DNA complex and undergoes first strand transfer, in which the cDNA binds to the 3’ end of the viral genome and acts as the primer for the negative strand cDNA synthesis. Once the negative strand cDNA is generated, the viral genomic RNA is degraded by RNase H, except at two polypurine tracts (PPT). One locates in the central region called the cPPT, and the other locates at
the 3’ end of viral genome. These two polypurine tracts allow for the synthesis of positive strand cDNA. The fragment of positive strand cDNA beginning from the 3’ PPT undergoes second strand transfer, in which the cDNA fragment binds to the PBS region on the negative strand cDNA. The complete synthesis of both strands results in the final desired product of double strand cDNA. At the completion of the synthesis, there remains a 99-nucleotide overlap sequence at the center of the positive strand named the central DNA flap. This central DNA flap is critical for nuclear import of the pre-integration complex (PIC) in non-dividing cells, it is eventually be removed by cellular endonuclease [82].

The PIC is moved to the nucleus through actions of the cellular cytoskeleton [83]. The unique feature of lentiviral vectors being able to transduce non-dividing cells is due to the entry of PIC into the nucleus through formation of a nucleoprotein complex without the breakdown of the nuclear membrane. Once the PIC enters the nucleus, provirus is integrated into the cellular genome by viral integrase [84]. Both spliced viral mRNAs and unspliced viral genomic RNA are regulated by the Rev protein. After viral proteins are synthesized, along with the new viral RNA genome, new viral particles are generated and released from cell surface by budding.

2.3 HIV-1 derived lentiviral vector

HIV-1 is the most studied lentivirus, and it was first considered as a gene transfer vector in 1990s. However, because of its ability to impair host immune system, lentiviral vector derived from HIV-1 required thorough examination and vigorous testing to ensure its bio-safety. Compared with gamma-retroviruses, lentiviruses are unique in that they
are able to transduce non-dividing cells. Another important feature is the non-biased proviral integration site. Lentiviruses have been shown to integrate into transcriptional hot spots [85], which naturally increases concerns about the risk of insertional mutagenesis. However further studies have shown that lentiviral vector has different integration patterns compared to gamma-retroviral vector [86]. While gamma-retroviruses like to integrate near transcription start sites and CpG islands [87], dramatically increasing the chance of insertional mutagenesis, gene activation and enhancer like function. Lentiviruses tend to integrate into active transcriptional units [85, 88]. Thus lentiviruses have less chance to activate proto-oncogenes. However, this fact does not necessarily diminish the risk of inactivating a tumor suppressor gene or disrupting another important gene function.

The general strategy for a safe lentiviral vector is similar to that followed for gamma-retroviral vector design, namely, separating cis-acting sequences (viral non-coding elements necessary for RNA synthesis, packaging, reverse transcription, and integration) from trans-acting sequences (viral enzymes, along with structural and accessory proteins).

2.3.1 Lentiviral vector packaging system

To avoid the generation of replication competent lentiviruses (RCL), trans-acting elements are put on separate plasmids. gag and pol sequences are put together in the packaging plasmid, and pseudotyping glycoprotein is put in envelope plasmid (Fig 4b). Over the course of lentiviral development, there have been three generations of packaging systems evolved with increasing safety in each generation (Fig 4a). The first generation packaging plasmid includes the entire gag and pol sequences, as well as all of
the viral accessory genes and regulatory genes. To ensure that it only expresses viral proteins and enzymes for viral packaging, the packaging signal and the primer-binding site (PBS) were removed, and viral LTR promoter was replaced with CMV promoter. Poly A tail was also added to the 3’ end of packaging plasmid. In the second-generation packaging system, with more understanding of viral genes for infectivity and virulence, four accessory genes (vif, vpr, vpu and nef) were also removed without affecting viral titer and infectivity [89, 90]. The third generation packaging system put the regulatory gene, rev, on another separate plasmid to increase bio-safety, and further remove tat by replacing the 5’ LTR with a constitutively active promoter in the transfer vector [91]. In combination with deletion in U3 region from 3’LTR in SIN (self-inactivating) vector, viral LTR can be completely eliminated, thus further reducing genotoxic potential of viral LTR [92].

2.3.2 Design and improvement of lentiviral transfer vector

The transfer vector is the only genetic material to be packaged into lentivirus and integrated into targeting cell. To avoid the possible genetic recombination with wild-type lentiviruses, minimum viral genome is included in the transfer vector plasmid. Thus the lentiviral vector only consists of an expression cassette, viral cis elements and the transgene (Fig 4c). Several improvements have been developed over the years to make the transfer vector safer and lead to more robust transgene expression. For instance, as described above, the U3 region of the 5’ long terminal repeat (LTR) was replaced with the CMV or RSV promoter, resulting in Tat-independent transcription [91, 93]. Additionally, the self-inactivating (SIN) lentiviral vector was a major breakthrough in vector design. It was first developed at the Salk Institute in 1998 [94] and was important
because it removed part of the enhancer/promoter sequence in the U3 region, thus making LTR inactive during transgene expression. The deleted portion of the U3 region included the TATA box and the binding sites for the transcription factors Sp1 and NF-κB. The deletion does not decrease viral titer but is beneficial for safety because it even further minimizes RCL generation by reducing common viral genome with wild-type HIV-1. It also decreases the chances of host gene activation around the insertional site. Other improvements that have been made are the incorporation of the cPPT element and CTS pol gene, both of which are important in forming DNA flaps and facilitating PIC entry to the nucleus [95, 96], especially when transducing non-dividing cells. These developments have been shown to dramatically increase transduction efficiency [97].

Another important improvement in transfer vector was the addition of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). WPRE is added to the 3’ side of transgene, and has been shown to enhance mRNA transcript stability and increase the overall transgene expression [98, 99].

One of the ideal goals for lentiviral gene therapy is to express the transgene only in target cells while avoiding non-specific infection. Recent developments have incorporated cellular endogenous miRNA to regulate the transgene expression from lentiviral vectors [100, 101]. MicroRNAs are small non-coding RNAs approximately 22 nucleotides in length that are being shown to play an important role in the natural cellular mechanism of down-regulating gene expression [102, 103]. Several studies have shown effective suppression of gene expression in certain cells with the lentiviral vector containing sequences matching endogenous miRNA [104, 105]. When transduced cells express endogenous miRNA, transgene expression is repressed (Fig. 5b).
Figure 4. Schematic representation of lentiviral vector system.

(a) Three generations of lentiviral packaging systems. (b) Pseudotyping envelope plasmid. (c) Improved lentiviral gene transfer vector.

Figure 5. Regulated transgene expression vectors.

(a) Four regulated lentiviral vectors: miRNA regulated transfer vector, tissue specific promoter transfer vector, Cre-LoxP transfer vector, and inducible transfer vector.

(b) miRNA regulated transfer vector can only express transgene in the cells that lack complementary endogenous miRNA. In non-targeted cells, endogenous miRNA degrades transgene mRNA, suppressing transgene expression.
However careful consideration needs to be made so as to not overwhelm the endogenous microRNA system and interfere with its physiological role in target cells. Brown et al. recently published a review on how to select candidate microRNAs and design microRNA target sites [100]. A more direct way to control transgene expression in specific tissue is through the use of a tissue-specific promoter [106, 107]. Alternative methods also include using a LoxP flanked sequence for conditional Cre-Lox regulated transgene expression [108, 109] and using inducible promoter, such as tetracycline inducible system [110-112] (Fig. 5a). Cellular antiviral mechanisms may adversely influence transgene expression. Researchers have worked around this by adding insulator, such as chicken beta-globin locus cHS4 insulator, flanking the transgene in the transfer vector. In this way transgene expression can be isolated from the influence of surrounding cellular genes [113]. By the same token, this is yet another safety mechanism designed to prevent the activation of nearby genes by proviral integration [114].

2.3.3 Non-HIV-1 derived lentiviral vectors

Because of the pathogenicity of HIV-1, non-HIV lentiviral vectors were suggested [115]. The other two primate lentiviruses, HIV-2 and SIV, have been developed as gene transfer vectors with limited success [116-118]. Non-primate lentiviruses were also considered, such as FIV [119, 120], EIAV [121], BIV [122], and CAEV [123]. Among those non-primate lentiviral vectors, FIV and EIAV were commonly used. Their payload capacity is only around 7-8kb [124]. Non-HIV lentiviral vectors are considered to be safer for human because they cause less or no pathogenic damage to human in their parental forms, and these lentiviral vectors can reduce the chance of recombination with
wild-type HIV-1. As lentiviruses, they can efficiently transduce non-dividing cells as well. However because they are not pathogenic in human, their basic biology is less studied. In order to generate safe and efficient lentiviruses, similar split-genome strategy was also used to develop non-HIV-1 lentiviral vectors inspired by HIV-1 lentiviral vector development. Viral accessory genes were moved to separate packaging vectors. Improvements, such as WPRE element and SIN vector, were also applied in non-HIV lentiviral vectors [124]. For safety concern, non-HIV lentiviral vectors are also made in human 293T cells. Successful gene transfer with non-HIV lentiviral vectors has been achieved, nevertheless, for those non-HIV lentiviral vectors to be widely used in clinical setting, more extensive understanding in their basic biology and their interaction with human is warranted.

2.3.4 Pseudotyping lentiviral vector

In order to broaden the tropism of lentiviral vectors outside of their native target cells, the viral envelope is replaced with different viral glycoproteins in a process called pseudotyping. This allows for the vector to extend its range of targets to various cell types in different species. The most widely used viral glycoprotein is vesicular stomatitis virus G glycoprotein (VSVG) because of its very broad tropism and stable pseudotyped viral particle [125, 126], which allows for further concentration of viruses by ultracentrifugation. In contrast to the traditional proposed mechanism of wild type HIV-1 entry, VSVG pseudotyped lentiviral vectors enter the cell via an endocytic pathway [127]. However VSVG is cytotoxic to most mammalian cell lines, which makes it harder to generate lentiviral producer cell lines [125, 128]. In addition, VSVG pseudotyped HIV and FIV vectors can be targeted and eliminated by host immune responses and
inactivated by human serum complement, which makes use of VSVG pseudotyped lentiviral vectors difficult for certain in vivo clinical applications, especially for direct intravenous injection [129, 130]. This inactivation can potentially be avoided by PEGylation of VSVG pseudotyped lentiviruses and can protect the virus from inactivation in the serum and also improve the transduction efficiency [131]. Alternative viral glycoproteins derived from other viruses, such as lyssavirus [132], lymphocytic choriomeningitis virus [133], hepatitis C virus [134], and sendai virus [135], can also be used for pseudotyping lentiviral vectors [126, 136].

2.3.5 Production of lentiviral vector

In most current studies lentiviral vectors are generated through transient co-transfection of three or four different plasmids in human embryonic kidney 293T cells, depending on the packaging system. The plasmids transfected include the packaging plasmid, the envelope plasmid, and the transfer vector plasmid (Fig. 6). The commonly used packaging system in both preclinical and clinical studies is the second generation packaging system with SIN lentiviral transfer vector. In order to avoid the production of empty viral particles, the plasmid ratio of packaging plasmid vs transfer vector plasmid vs envelope plasmid is 3:3:1. 48-72 hours post transfection, lentiviral particles released into the media from 293T cells are collected and filtered through a 0.22-micron filter to remove cell debris. The titer of lentiviral vector produced by transient transfection usually has a range from $10^6$-10$^7$ IU/ml.

In order to obtain clinical grade lentiviruses with reproducibility and standardization, either large-scale transient production or stable packing cell lines are needed. Herein lies a difficulty because stable packaging cell lines for lentiviruses are hard to acquire. This
is due to cytotoxicity of lentiviral proteins and the pseudotype envelope protein VSVG [137-140]. Several labs have tried to generate stable cell lines for lentiviral production [65, 141-144]. So far stable producer cell lines can only be achieved with an inducible expression system for controlled expression of viral proteins and VSVG [144, 145].

Figure 6. Lentiviral production and concentration.

Transfer vector plasmid, packaging plasmid, and envelope plasmid are transiently transfected into 293T cells, and viral supernatant is collected and concentrated with ultracentrifugation, ultra-filtration, or precipitation.
High multiplicity of infection (MOI) studies and clinical studies, requiring large quantities and high grade of viruses, may need to further concentrate lentiviruses to increase the titer. Several methods are currently available for concentrating lentiviruses depending on the production levels desired. Ultracentrifugation and ultra-filtration of viral supernatant are more common for making large-scale production and concentration\[146, 147\] while small-scale concentration through precipitation can be done with calcium phosphate or poly-L-lysine [148, 149]. Lentiviral vector supernatant can be stored in -80°C, however freeze-thaw cycle should be kept at minimum because it can decrease viral titer by 60% [130, 150].

2.4 Applications of lentiviral vector in cancer therapy

There are two reasons that cancer has been a target for lentiviral gene transfer technology. First, proof of concept gene transfer efforts can be validated with an intention to treat advanced malignancies, and second, there are a number of targets in cancer for which there is no effective agent or treatment [151]. Non-viral and viral vectors have been extensively investigated for cancer therapy with limited success. In one study, lentiviral vectors were shown to transduce human hematopoietic progenitor cells in the G1, G2/S/M phases more efficiently than those cells in the G0 phase [152]. Coincidentally, quantitative analysis of G0 and G1 phases in primary carcinomas showed that over 50% of primary cancer cells were blocked in transition in G1 and thus were not in G0 or the quiescent state [153]. Lentiviral vectors seem to have a better chance at transducing cancer cells at different cell-cycle phases and thus are susceptible to lentiviral-mediated gene transfer. Below we will discuss several strategies currently
employed using lentiviral vectors to treat cancer. These are suicide gene therapy, immunotherapy, gene replacement and down-regulation, anti-angiogenesis therapy, and myelo-protection.

2.4.1 Suicide gene therapy

The concept of suicide gene therapy is to give cancer cells a suicide gene that converts a non-toxic prodrug to a cytotoxic drug, which kills cancer cells [154]. Commonly used suicide genes are herpes simplex virus thymidine kinase (HSV-TK) and cytosine deaminase (CD). Studies have shown that systemic administration of gancyclovir can be converted to cytotoxic gancyclovir triphosphate in the presence of HSV-TK when treating glioma cells and prostate carcinoma cells [155, 156]. Small numbers of transduced tumor cells can also result in the killing of non-transduced surrounding tumor cells, a phenomenon called the “bystander effect” [157-159]. Recent developments have shown that using suicide gene therapy in stem cells can have an impact on killing surrounding cancer cells [160, 161]. Other suicide genes are also being developed. For instance, engineered human thymidylate kinase (tmpk) was introduced in cell lines with a lentiviral vector to make transduced cancer cells sensitive to 3’-azido-3’-deoxythymidine (AZT). This led to the suppression of tumor growth in NOD/SCID mice [162].

2.4.2 Lentiviral immunotherapy for cancer

One of the characteristic advantages of cancer cells is the ability to evade immune surveillance in the host [163-165]. By identifying and introducing tumor-associated antigens (TAA) to the host immune system, a specific immune response against TAA can be initiated which could recognize and eliminate tumor cells [166]. Lentiviral vector can efficiently introduce TAA to host immune system (Fig. 7). One approach is to inject
lentiviral vector expressing TAA directly to the host to induce antitumor responses in vivo, targeting antigen-presenting cells (APC). VandenDriessche et al. showed that *in vivo* injection of lentiviral vectors could efficiently transduce APC in the spleen [167]. He et al. also showed direct transfection of skin-derived dendritic cells (sDC) after cutaneous delivery of lentiviral vectors, which resulted in potent and prolonged antigen presentation [168]. However the parental HIV-1 has been shown to induce both the cellular and antibody-mediated response [169, 170]. Recent studies have also shown that there is a host immune response towards lentiviral vectors [171, 172]. It has also been shown that lentiviral vectors can efficiently transduce DC *ex vivo* and lead to the expression of transgenic protein [173-177]. Transgene-derived peptides can be efficiently expressed on the cell surface of DC and lead to activation of antigen-specific T cells [178, 179]. In vivo studies showed that lentiviral transduced DC can efficiently inhibit established tumors and are effective against subsequent tumor challenges [180, 181].
Figure 7. Lentiviral immunotherapy for cancer.

Lentivirus, encoding for tumor-associated antigen (TAA), can be directly injected into patient or transduce dendritic cells ex vivo. Transduced DC can be infused back to patient to directly present to CD8 T cells, initiating targeting and the destruction of cancer cells.

2.4.3 Gene replacement and gene silencing

Carcinogenesis occurs largely due to the loss of tumor suppressor genes and the activation of oncogenes, and great effort has been made to identify both classes of genes [182, 183]. Tumor growth can contribute to the loss of a single tumor suppressor gene but by introducing a wild type tumor suppressor gene expressed at normal levels, cellular control mechanisms can be restored and the cancer growth can be arrested.
Abnormalities in the TP53 gene family have been documented in a very large number of human cancers [184, 185], and studies have shown that restoring the wild-type p53 gene in human lung cancer cell lines with a gamma-retroviral vector led to significant growth arrest and apoptosis in cancer cell lines lacking p53 [186, 187]. In another study, stable lentiviral transduction of HCT116 colon cancer cells with wild-type p53 gene sensitized the transduced cells to chemotherapeutic agents [188].

In addition to introducing tumor suppressor gene, another strategy is to use gene transfer vectors to knockdown activated oncogenes. Today, RNAi is a vastly used mechanism for post-transcriptional gene silencing in experimental system [189, 190]. Compared to oligonucleotide siRNA, lentiviral vectors can efficiently deliver short hairpin RNA (shRNA) to cancer cells for stable integration and consistent expression of the transgene. When shRNA is released into the cytoplasm, the linker sequence is degraded by Dicer to form siRNA, which subsequently targets the appropriate mRNA for degradation [191, 192]. The ras family is among the most activated oncogenes in human cancers and thus a natural target for cancer therapeutics [193-195]. Brummelkamp et al. showed stable integration and expression of siRNA against the K-RAS<sup>v12</sup> allele in human tumor cells using retrovirus, and this resulted in loss of anchorage-independent growth and tumorigenicity [196]. Efficient RNAi silencing requires high copy numbers of siRNA. However, a drawback is that it can also lead to non-specific gene silencing [197]. To optimize the specificity for gene silencing, multiple siRNA sequences linked in one transgene targeting one mRNA have been developed [198, 199]. In addition, tissue-specific promoters can further decrease the chance of non-specific silencing by localizing the effect [200].
2.4.4 Anti-angiogenesis therapy

For tumors to grow, angiogenesis is essential and critical. New blood vessels around tumors provide routes of delivering nutrients and can also be the travelling vessels for metastatic cancer cells [201]. Endogenous inhibitors of angiogenesis, including angiostatin and endostatin, have been shown to reduce tumor size and maintain tumor dormancy without increasing drug resistance in mice [202-205]. However, the requirements for long-term administration and large amounts of protein put a limitation on this potentially effective treatment [206]. Lentiviral vectors offer a solution that allows the cells to constantly express those anti-angiogenic factors. Shichinohe et al. were the first to test third generation HIV-1 derived SIN lentiviral vectors expressing angiostatin or endostatin directly in endothelial cells. However the efficiency was low, and the inhibition on cell growth was not significant [207]. In another study, a lentiviral vector containing endostatin has been shown to decrease vascularization and inhibit tumor growth in lentiviral transduced bladder tumor cells [208].

2.4.5 Myelo-protection against chemotherapeutics

Traditional treatments for cancer include radiotherapy and chemotherapy, which have severe side effects on patients. Chemotherapeutic drugs are particular toxic to bone marrow cells, causing hematopoietic cytopenia, and limit the necessary dose of chemotherapeutics needed to kill cancer cells [47]. Instead of directly targeting and eliminating cancer cells, strategies have been developed to protect hematopoiesis in patients from cancer treatment with lentiviral mediated gene transfer of a drug resistance gene. O^6^-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein, which removes alkyl adducts on the O^6^ position of guanine. It has been discovered that
low MGMT expression is found in bone marrow cells while MGMT over-expression is found in many types of human tumors, making tumor cells more resistant to chemotherapeutics [209-212]. The mutant form of MGMT (MGMT-P140K) has been shown to be effectively resistant to O\textsuperscript{6}-benzylguanine (BG), an inhibitor to wild-type MGMT, and also methylating and alkylating agents, such as Temozolomide or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [54, 213]. By using a lentiviral vector to introduce MGMT-P140K into hematopoietic stem cells (HSC), hematopoiesis can be restored, and transduced bone marrow cells can be protected and selected in vivo in preclinical models [214, 215]. MGMT-P140K provides leverage to bone marrow cells over tumor cells expressing wild-type MGMT against BG and chemotherapeutic treatments (Fig. 8).
Wild-type MGMT repairs DNA damage caused by alkylating agents. Treatment with benzylguanine (BG) and alkylating agents inactivates wild-type MGMT, resulting in double strand break and cell apoptosis. BG and alkylating agent treatment does not affect MGMT-P140K, which can still repair damaged DNA.

2.5 Clinical trials of lentiviral vectors

In 2007, adenoviruses and gamma-retroviruses were equally weighted in the number of clinical trials, with each over 20% among all gene transfer clinical trials. During that time the number of studies involving lentiviral vectors were negligible [60]. However
with the increasing interests in research and development of lentiviral vectors, the first lentiviral clinical trial was successfully completed in 2003 with optimistic results [216]. The first clinical trial was logically designed to treat HIV infection. According to the Journal of Gene Medicine website, updated on March 2009, there are a total of 21 phase I lentiviral clinical trials, and only 4 out of those 21 trials were approved for targeting cancer, and all of the lentiviral vectors are HIV-1 derived (Table 2). Three of four trials directly treat cancer target antigen receptor and T-cell receptors while the fourth clinical trial is for myelo-protection against cancer chemotherapy (see section 2.4.5 for mechanism).

<table>
<thead>
<tr>
<th>Trial ID</th>
<th>Vector Condition</th>
<th>Target Cells</th>
<th>Phase</th>
<th>Principal Investigator</th>
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<td>HIV-1 derived Malignant melanoma</td>
<td>α &amp; β chains of T-cell receptor specific for Mart-1</td>
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<td>HIV-1 derived Glioma</td>
<td>O6-methylguanine DNA methyltransferase</td>
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<td>Gerson SL, Case Western Reserve University</td>
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Table 2: Lentiviral Clinical Trials (Journal of Experiment Medicine, updated March 2009).
2.6 Conclusion

Despite the fact that retroviruses had been shown to induce cancer through the activation of a proto-oncogene three decades ago [217], the development of a safe retroviral vector and the benefits of a stable gene transfer has resulted in the first gamma-retroviral vector gene therapy clinical trial in treating melanoma [59]. After 10 years of research and hundreds of retroviral clinic trials, the onset of leukemia in the French clinical trial was a serious setback for retroviral studies and the field of gene therapy as whole. While most of the children benefited with a restoration of immune function, the potential for insertional leukemogenesis became a reality. Other similar studies in treating SCID-X1 with retroviruses have not encountered a similar fate [218, 219]. The tremendous benefit for those successfully treated patients in these trials are truly rewarding. Because of their unique abilities to transduce both dividing and non-dividing cells and to integrate into the cell genome with a lower risk of insertional mutagenesis, lentiviral vectors are receiving considerable attention in preclinical and clinical trials. Different generations of packaging systems, greatly improved transfer vectors, pseudotyped viral envelope have made current HIV-1 derived lentiviral vectors safe but efficient. Different strategies of using lentiviral vectors in treating cancer have been developed, and the future in lentivirus-mediated gene therapy is promising. Studies on non-HIV-1 lentiviruses are being conducted with increasing numbers, and soon non-HIV-1 lentiviral vectors can be expected in clinical trials. Despite the great promise lentiviral vectors offer, constant vigilance is required in designing and applying an integrating viral vector as a gene transfer method for the treatment of either malignant or nonmalignant indications.
Chapter 3

Bioluminescence Imaging of Hematopoietic Stem Cell Repopulation

in Murine Models

*Portions of this chapter are from:

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Abstract:

Hematopoietic stem cells (HSCs) have been studied for decades in order to understand their stem cell biology and their potential as treatments in gene therapy, and those studies have resulted in tremendous advancement of understanding HSCs. However, most of the studies required the sacrifice of cohorts of the animals in order to obtain data for analysis, resulting in the use of large animal numbers along with difficult long-term studies. The dynamic engraftment and expansion of HSC are not fully observed and analyzed. Until recently, with the development of optical imaging, HSC repopulation can be continuously monitored in the same animal over a long period of time, reducing animal numbers and opening a new dimension for investigation. In this chapter, bioluminescence imaging of murine HSC is described for observing the dynamic repopulation process after transplantation. Photons emitted from transplanted murine HSCs expressing firefly luciferase within the mice can be visualized in light-sealed chamber with a highly sensitive digital camera after injection of substrate D-luciferin. Xenogen IVIS200 imaging system is used to record the process, and other similar imaging systems can also be used for this process.
3.1 Introduction

Hematopoietic stem cells (HSCs) have been an attractive target for gene therapy due to its self-renewal and pluripotency [220, 221]. After transplantation into myeloablated recipients, HSC can give rise to all lineages of blood cells. True long-term HSC currently can only be identified through functional transplantation and repopulation assay [222, 223]. About 5 years ago, most data for interpreting the engraftment and expansion of transplanted HSCs in small animal model was obtained and interpreted from post-mortem collection and analysis of the hematopoietic organs. However, the dynamic range of movement and the engraftment pattern of HSCs were not available until recently. With the development of imaging equipments and techniques, molecules and cells can be detected in vivo without sacrificing the subjects [224-226]. HSCs can be tagged with optical imaging reporters and transplanted into recipients. With the expansion of HSCs, photons emitted from the progeny of those HSCs could provide spatial and temporal information that no other techniques could provide. For the first time, the extent of hematopoiesis in murine transplantation model can be visualized.

3.1.1 Benefits and Advantages of Bioluminescence Imaging

Obvious benefit and advantage of in vivo imaging techniques including the optical imaging are non-invasiveness, less study animals, complete picture of biological process, and long-term monitoring. Optical imaging includes fluorescent imaging and bioluminescent imaging. Fluorescent imaging captures photons emitted from the fluorescent probes after being excited, such as green fluorescence protein, inside of the targeting cells to track cell migration and monitor gene expression. Fluorescent imaging, especially GFP signal, is limited by its relatively low tissue-penetrating capability and
high background due to auto-fluorescence of the animals studied. However, for superficial imaging, such as the tumor xenograft model, fluorescent imaging can provide fast, reasonably sensitive, and less expensive imaging services. To study HSC repopulation, in which cells circulate and reside deep inside the hematopoietic organs and bone marrows, bioluminescent imaging (BLI) provides higher sensitivity and almost no background signals for overall better performance [227, 228]. BLI utilizes an enzymatic reaction to give off visible light with the ability to penetrate couple centimeters. BLI requires the injection of a substrate to initiate the reaction, and no excitation light source is needed, which dramatically reduces imaging background from excitation or animal auto fluorescence, and increases the signal to noise ratio. Besides optical imaging, there are other imaging techniques, such as MRI/MRSI and nuclear medicine imaging (SPECT, PET), which can offer similar services. However, those will not be discussed this dissertation.

3.1.2 Bioluminescence proteins and their applications

There are many different bioluminescent enzymes, which have been isolated and cloned from vast and diverse organisms. The most studied and widely used luminescent enzyme is from North American firefly (*Photinus pyralis*) [229]. Firefly luciferase is a 61-kDa monomeric protein, and it interacts with its substrate D-luciferin in the present of oxygen, Mg\(^{2+}\) and ATP to release green light with peak wavelength at 562 nm (Fig. 9). At body temperature of 37°C, this peak shifts towards 600 nm. The light generated from this reaction has a broad spectrum, and only the upper 30% of the spectrum (>600nm) is able to travel through tissues with low level of scattering and absorption [230]. Other useful luciferases from bacteria, jellyfish (*Aequorea*), sea pansy (*Renilla*), and click beetle
(Pyrophorus plagiophthalamus) were also cloned and are used in various applications [231]. However the light spectrums generated from those luciferases do not have a longer wavelength component as that of firefly luciferase, thus they are less frequently used for deep tissue imaging. Nevertheless, most of luciferases have been used to study tumor growth and metastasis, viral infections, progress of infections, cellular protein activity and protein-protein interaction [232-235].

Figure 9. Bioluminescence reaction.

In the presence of ATP and oxygen, firefly luciferase converts substrate D-luciferin to oxyluciferin and emits light.

3.1.3 Bioluminescence imaging for HSC

Current knowledge of HSC transplantation and repopulation has come from decades of peripheral blood and post-mortem studies. Now the dynamic engraftment and expansion of HSC in vivo is finally able to present itself with the help of BLI. Firefly luciferase has been successfully incorporated into highly purified KTLS (Lin^Sca-1^-c-Kit^-Thy-1^-) murine HSC, visualized, and the process of repopulation can be monitored over long period of time [236, 237]. Even though transplanted HSC can home to bone marrows within 24 hours of infusion [238], BLI signals can be seen 3-5 days after transplantation. Low number of luciferase positive cells during transplantation limited
the detection of early homing process, and onset of foci from repopulation of HSC and progenitor cells happens in a cell dose dependent manner. Limitation of current BLI of HSC has been due mainly to massive diffusion and scattering/absorption of the light photons in living animals, which makes the quantitative measurement and precise localization of those transplanted HSCs and their progenies difficult. However, other imaging modalities, such as MRI, CT and PET, and certain post-mortem studies can provide spatial reference and data confirmation for BLI results.

3.2 Materials

3.2.1 Mice and Hematopoietic Stem Cells

1. Albino mice are the best normal recipients for BLI imaging because they have low absorption of light emitted from their bodies. A good strain would be Balb/c background mice. Black C57 background or brown color mice can also be used for imaging in special study model, however fur is usually shaved from imaging area to reduce photon absorption. Black and brown mice tend to have very dark skin as well which can absorb the signal (emitted light photons) more than albino.

2. Hematopoietic stem cells (KTLS in murine or CD34+CD38- in human) can be isolated with direct or indirect isolation methods.

3.2.2 Reagents

3.2.2.1 Diluted rodent anesthesia mixture

Diluted mice anesthesia solution:
<table>
<thead>
<tr>
<th>Drugs</th>
<th>Amount used</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine HCl</td>
<td>15 mg (100 mg/ml)</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Xylazine HCl</td>
<td>3 mg (20 mg/ml)</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Acepromazine</td>
<td>0.5 mg (10 mg/ml)</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Sterile H₂O or Saline</td>
<td>N/A</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td>N/A</td>
<td>1.75 ml</td>
</tr>
</tbody>
</table>

Table 3. Rodent cocktail mixture.

IP injection Dose: 0.1-0.2 ml/25-gram-mouse (recipe and working dosage obtained from Animal Resource Center at Case Western Reserve University).

Alternatively, Avertin can be used instead of rodent anesthesia mixture. Avertin (2-2-2 Tribromoethanol) can be dissolved in tert-amyl alcohol to make stock solution and stored at room temperature in the dark. Working solution is 20 mg/ml from the dilution of the stock solution with PBS. Sterilize with Nalgene 0.22 µm filter bottle and store the working solution in 4°C in the dark for up to several months.

IP injection dose: 0.3-0.6 mg/gram in mice.

3.2.2.2 D-luciferin solution

Synthetic firefly D-luciferin potassium salt (Biosynth or Xenogen) is dissolved in Dulbecco’s Phosphate Buffered Saline (Cellgro) to make injection solution at 12.5mg/ml final concentration. Dissolved D-luciferin solution is filtered through 0.22 µm Steriflip (Millipore) to ensure sterility. Aliquot into 2 ml sterile O-ring tubes (Fisher Scientific, Cat#0566957). Working solution should be kept in the dark and stored in -20°C freezer for up to three month. Working solution is best kept at -80°C. This will allow you to store it up to one year. Only thaw out the quantity you will use for that imaging session. Avoid multiple freezing and thawing cycles.
3.2.2.3 Bac/Neo Antibiotics

For 50 ml of volume, dissolve 5.0 gram of neomycin sulfate (Fisher Scientific) and 250,000 units of bacitracin zinc salt (Sigma-Aldrich) in sterile water. Antibiotic solution is stored in -20°C freezer. When use, thaw and add 2 ml of solution to each drinking-water bottle in the cage.

3.2.3 Supplies and Equipments

1. Common lab supplies: weight scale, alcohol swipes, 1 ml syringes with 26-28 gauge needles (1cc Insulin syringes with built-in needles work very well), and Contura HS-40 shaver (Weller) if needed.

2. Black paper and black photographer’s tape, mice can be imaged on top of the black paper. Photographer’s tape (3M #235 Photographic Blocking tape). You can use tape to affix the animals to the paper when they are imaged in the supine position. This holds down the legs and allows unobstructed views of the chest and abdomen.

3. Imaging system: Charge-coupled device (CCD)-camera, light-sealed imaging chamber, and computer with image analysis software, such as ImageJ or Matlab. In this protocol, we use Xenogen IVIS200 systems with XGI8 gas anesthesia system and Living Image 2.5 software (see Note 1).

3.3 Methods

3.3.1 Isolation of hematopoietic stem cells expressing firefly luciferase reporter gene

There are three different ways to obtain murine HSC expressing luciferase gene. First, luciferase plasmid can be transient transfected into HSC \textit{ex vivo}. The method provides fast and safe introduction of luciferase gene into HSC, however the expression of
luciferase protein may decrease with the repopulation after transplantation because the plasmid does not replicate with cell division. Second, luciferase gene can be stably integrated into targeted cell genome ex vivo by retroviral or lentiviral transduction (see previous chapter for viral transduction of HSC). This method could achieve long-term luciferase gene expression. Third, luciferase transgenic mice could also be generated to obtain murine HSC for long-term luciferase expression. The third method could be the best to generate ‘natural’ HSC without any ex vivo modification, which may potentially affect HSC repopulation. Human HSC can also be isolated and introduced luciferase transgene via retroviral or lentiviral transduction.

3.3.2 Bone marrow transplantation

Irradiation of recipients can facilitate the engraftment of transplanted HSCs. Balb/c mice (Charles River) were irradiated 5-24 hours before transplantation at 750-800 rad with a Cs radiation source. Viral transduced HSCs (murine or human) or murine transgenic HSCs with bioluminescence gene are infused into irradiated syngeneic recipients via tail-vein injection with 28-gauge 1 ml syringe. Antibiotics, bacitracin/neomycin sulfate, are needed in the drinking water after irradiation and transplantation to prevent bacteria infection.

3.3.3 Anesthesia of experimental animals

Two methods of anesthesia can be performed on the experimental animals: injection of diluted Ketamine/Xylazine/Acepromazine (Henry Schein Inc.) mixture or the use of isofluorane vapor and oxygen supplied by Xenogen XGI-8 gas anesthesia system. Based on the size of mice and the concentration of the rodent anesthesia mixture, about 100-150 µl of mixture is intraperitoneally injected into each mouse 3-5 minutes prior to the
injection of D-luciferin. The anesthesia affect lasts about 30-40 minutes with minimum movement, and mice will completely wake up in couple of hours after imaging (see Note 2).

XGI-8 gas anesthesia system offers better imaging flexibility. It only temporally anesthetizes studied subject, and the animal can wake up quickly after being removed from the anesthetic gas. Steady flow of anesthesia gas allows repeated imaging within a very short time and longer imaging time could be performed with this gas anesthesia system. The animal will first be placed in the induction chamber with 2.0-3.0% isoflurane with 100% oxygen with a 1 to 1.5 liter per minute flow rate. Once the animal is anesthetized, it will be quickly removed from the induction chamber and placed on the sample stage inside the imaging chamber and put its nose inside a nose-cone attached to the XGI-8 system, and adjust isoflurane level to 1.0-2.0% with 100% oxygen (see Note 3).

3.3.4 Acquisition of bioluminescence images

The imaging system should be turned on hours before imaging due to the cooling and stabilizing of the CCD camera to working temperature (see Note 4 and 5).

1. Start up the Living Image 2.5 software program by clicking the program icon, and enter user identification (user initials). The IVIS system control panel will appear.

2. Before any other information can be entered, you must click the Initialize IVIS system button in the control panel. When initialization starts, the machine will reset all of the motor-controlled components, such as the camera and stages, and all the software variables of the IVIS system. After initialization, make sure the
temperature box of system status in the control panel is green to indicate the
temperature of the camera is ready for imaging.

3. Select the appropriate imaging parameters, such as exposure time, binning, and
field of view (FOV), from the control panel. First of all, ensure the imaging mode
was set at luminescent. Select exposure time from a few seconds to 10 minutes,
depending on the intensity of the signal. Exposure time is the time period during
which photons captured by the CCD camera will be added together to increase
sensitivity of bioluminescent signal. For HSC imaging, close to 5 minutes of
exposure time should be sufficient enough. Too long of exposure time will result
in overly saturated signals, which is harder to quantify (see Note 6). Binning
increases pixel size of the camera, which delivers higher sensitivity at the expense
of spatial resolution (Xenogen manual). Medium binning (4X4) for HSC imaging
can provide decent resolution and good sensitivity. FOV indicates the distance
between the camera and the sample stage, and it is preset to 5 settings (A to E). A
represents the smallest while E represents the largest imaging area. For example,
to imaging 4 mice, position D (18.4 cm) will be a good size. f/stop, filters and
subject height use their default value at 1, open, and 1.5 cm respectively. Click
on ‘Select sequential mode’ to expand the control panel, and click ‘set’ twice to
setup obtaining two consecutive images together (see Note 7).

4. Make sure the laser-generated alignment grid box is enabled because it can show
the exact area of the image the CCD camera sees. The size of the alignment grid
changes with the change of FOV. The samples should be placed in the middle of
the grid.
5. IP injection of rodent anesthesia mixture or turn on XGI-8 gas anesthesia system to anesthetize the studied animals (see section 3.3).

6. After animal is anesthetized, inject appropriate amount of D-luciferin into each mouse based on their body weights 7 minutes prior to imaging to allow D-luciferin to circulate inside the body (see Note 8). Average amount of D-luciferin injected is 10 µl per gram of body weight. Since the stock is 12.5 mg/ml, the final concentration of D-luciferin per animal is 125 ml/kg (see Note 9).

7. Place studied animals on their back on black paper, spread out and tape down their limbs with black photographer’s tape (see Note 10). Place the black paper on the sample stage, and make sure the sample animals are in the center of the alignment grid.

8. When 7 minutes from injection of D-luciferin passes, click ‘Acquire Sequence’ button to start collecting images.

9. When the image taken process is over, the computer will automatically display luminescent pseudo-color image overlaid on grayscale photographic image. On the right side of the image, a rainbow scale bar shows the relative intensity of bioluminescent signal from the subject. Purple and blue indicate weakest while yellow and red indicate strongest signals. The unit is in photon counts.

10. A window will pop up asking for the information regarding this image upon the completion of taking each image, then save the image data by choosing ‘Save Living Image Data’ or by closing each image window, not the software window, and follow the instruction to save images in the right folder.
11. Printing of the images after collection of data may require restart of the program. Enter the user ID, but initialization of the system is not required for printing and analysis. Select ‘Load LI Data’ button from Living Image menu, and select correct images from their folders, and choose print command from the pull down menu.

12. Remove the sample animals from the imaging chamber, turn off the XGI-8 gas anesthesia system, and exit the program. Leave the imaging system on.

3.3.5 Analysis of Imaging Data

Imaging data obtained by IVIS200 system is automatically pseudo-colored based on bioluminescence intensity, and only the second images from the sequential images were compared (see Note 7).

1. Startup the Living Image 2.5 software, and enter user ID. Do not need to initialize the system for analysis.

2. Choose ‘Browse for LI Data’ or ‘Load LI Data’ from ‘Living Image’ pull-down menu, and select image folders needed to be analyzed from directory, and double-click the image file name to open the analysis window.

3. In order to measure the bioluminescence intensity, regions of interest (ROIs) are created by choosing the number and shape from the analysis window and clicking ‘Create’. To compare HSC repopulation, ROI can be defined as the whole body of the studied animal.

4. Move ROI over the area of interest, resize if necessary and click ‘Measure’ (see Note 11). The data is displayed in photons mode, and the unit is photons/s/cm²/sr.
5. Save ROIs and measurements of BLI intensity by choosing pull down menus
from ‘Tools’ and ‘Living image’.

6. For visual presentation, Images with the same BLI intensity scale from the same
animal on different days can be selected, cropped and compared side by side (see
Note 12) (Fig. 10).

7. Off-line processing can be utilized when software on the system is not adequate
for certain tasks. We have done image fusion with BLI and x-ray for better signal
source or internal organ localization. Make sure that you have registration marks
that will help you determine scale and alignment between the different imaging
modalities. Additional software such as ImageJ (free from NIH
(http://rsb.info.nih.gov/ij/) or Adobe Photoshop can be used to do image fusion.

Figure 10: Ventral images of murine hematopoietic stem cell transplant.
All of the images are from the same mouse and pseudocolor is set on the same scale.
(Imaging day 5 to 79 after bone marrow transplant.)
3.4 Notes

1. Bioluminescence imaging can be obtained by placing studied animals in light-sealed box with highly sensitive digital camera on the top. There is no preference for particular system, however for convenience in this protocol, we describe the procedures with Xenogen IVIS200 Living Imaging system we are currently using. Different systems are available for BLI, such as Roper, Kodak, Pixis and many others. Follow recommendations from the manufacture for set-up and imaging times. Important considerations are light tight chamber for imaging, CCD cooling and a proper lens that will allow the shortest lens to target distance. This increases the amount of light to the camera. Most of the protocol can be applied directly to the IVIS100 systems. Consult manuals for other manufacturers systems, both camera operations and software interface. Assure adequate cooling for the CCD camera. Routinely check for light leaks in the dark box.

2. Particular attention must be paid to keeping the animals warm. Ketamine based anesthetics reduce the mouse’s ability to regulate its body temperature. Animals will get cold very quickly and can die. Keep animals warm throughout the entire time under anesthesia. Heat lamps or warming blankets can be utilized if the imaging system does not provide heating device. IVIS200 imaging system maintains the imaging chamber at a steady 37°C, which can keep the study animals comfortable through out the imaging process.

3. Stand alone Isoflurane systems can be easily adapted to other camera systems. Many other manufacturers of camera systems will have light trap ports on their dark boxes to allow the introduction of anesthesia, oxygen and waste gas lines.
To reduce the death of study subjects, use the minimal amount of gas to keep the animal under.

4. Test any materials for auto-luminescence prior to using them with the mice. Many plastics and some papers will give off a “glow” when imaged.

5. The camera needs to be cooled to reduce the background signal (dark current) that is caused by thermal energy arising from the CCD over long exposure times. Many cameras utilize either a thermoelectric Peltier-type device or a liquid refrigerant system to maintain a low temperature. The best dark current suppression happens at -100 °C or lower. Some older systems utilize liquid Nitrogen. Please use caution when handling cryogens. For Xenogen IVIS200 system, the power should be left on all the time because the system needs to perform background measurements at night. The cooling system on the IVIS200 is a closed loop and is not user serviceable.

6. Selection of exposure time needs to be consistent during imaging of the same experimental group on different days. Selection of time longer than 5 minutes may require manually measurement of background. Exposure time can be changed if the BLI signal is saturated during the initial imaging process. However keep in mind, during later days of imaging, saturation may happen, but changing exposure time at that point makes comparison with the data from previous imaging day impossible.

7. Consecutive imaging allows the user to observe the change of BLI signal during imaging. It is very useful to detect the peak signal throughout one imaging session. Usually, only the images taken at the same time are used for
quantification and comparison. Not all images from consecutive imaging process are necessary for quantification.

8. From published data, bioluminescence signal reaches peak at 15-20 minutes after IP injection of D-luciferin, and half life of D-luciferin in vivo can be as long as 3 hours [239]. Since two consecutive images are taken in our studies, 7-minute waiting time is chosen. The starting imaging time can be flexible based on the experiment and the user.

9. D-luciferin concentration can be varied from 115 mg/kg to 150 mg/kg.

Continuous release of D-luciferin has been studied by using a micro-osmotic pumps [240].

10. Spreading the extremities of the animal can help view the body and its bone marrows more clearly. Imaging on animal back provides clear picture of their limbs, chest, and abdominal. However, a dorsal image is highly recommended to capture the foci or signal blocked by the body of the animal in two-dimensional imaging (Fig. 11).

11. In the study of HSCs engraftment and repopulation, bioluminescence signals from hematopoietic stem cells are too weak to be observed until they are expanding and undergoing hematopoiesis. Different organs of the mice can be taken out several minutes after injection of D-luciferin, each organs can be imaged, selected, and analyzed as their own ROIs.

12. To arrange images side by side, make sure the max and min values on the analysis window for each image are the same before cropping.
Figure 11: Bioluminescence imaging of murine hematopoietic bone marrow cells.

a. Ventral bioluminescence image, b. Dorsal bioluminescence image.
Chapter 4

Imaging stem cell derived persistent foci after in vivo selection of lentiviral MGMT-P140K transduced murine bone marrow cells

*Portions of this chapter are from:

Abstract

The engraftment of hematopoietic stem cells after drug resistance gene transfer and drug selection may recapitulate stress response hematopoiesis, but the processes remain elusive. Homing, trafficking, and localization of transduced cells and the impact of insertion site on focal expansion have not been well characterized. With the goal of optimizing and understanding these processes under conditions of low MOI lentiviral gene transfer, we used drug resistance gene MGMT-P140K and in vivo selection to enrich for transduced and transgene-expressing HSCs. To systemically monitor homing, trafficking, and expansion after transplantation and drug selection over time, we linked MGMT-P140K to the firefly luciferase gene in lentiviral SIN vectors. Periodic bioluminescence imaging of transplanted recipients was followed for up to 9 months after both primary and secondary transplantation. Initial dispersion and widespread early homing and engraftment were transient, followed by detection of persistent and discrete foci at stable tissue sites after in vivo drug selection. From these studies, we concluded that drug resistance gene transfer followed by early or late drug selection can result in stable gene expression and cell expansion in persistent foci of transduced bone marrow cells that often remain in fixed sites for extended periods of time.
4.1 Introduction

Lentiviral vectors are a preferred approach to introduce therapeutic genes into hematopoietic stem cells (HSC) because they transduce quiescent adult stem cells and have reduced risk for insertional mutagenesis [241-243]. Still, with these systems, little is known about engraftment and repopulation dynamics of lentiviral transduced bone marrow cells, particularly long lived stem cells. This is compounded by the observation of relatively low rates of transduced stem cells identified in vivo [244]. Recently, lentiviral vector systems have been implemented in clinical trials [216, 245], but a lot of studies typically used a relatively high multiplicity of infection (MOI) to raise clinical efficacy, and gene expression which increases the theoretical risk for insertional mutagenesis. To overcome the potential risks of insertion, which include gene disruption as well as insertional mutagenesis, conditions promoting low MOI lentiviral transduction would be safer for gene transfer, particularly for hematopoietic stem cells. Low MOI transduction, however, also reduces the efficiency of gene expression in the hematopoietic stem cell compartment. For this reason, gene transfer that enables in vivo drug selection has been developed to enrich for an in vivo population of gene-modified hematopoietic cells.

In vivo selection is an effective strategy for enriching and expanding transgene-expressing stem cells and progenitor cells [215, 246-248]. The O⁶-methylguanine-DNA-methyltransferase MGMT-P140K, contains a proline-to-lysine mutation that renders the protein resistant to the clinical inhibitor, O⁶-benzylguanine (BG). MGMT is a major factor in resistance to alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or Temozolomide (TMZ). As predicted, O⁶-benzylguanine (BG) inactivates
cellular MGMT and increases alkylating agent toxicity resulting in marrow suppression, which has limited its clinical use in anti-cancer treatments. Conversely, MGMT-P140K transduced bone marrow cells are much more resistant to BG and alkylating agent combinations. We and others have shown that MGMT-P140K expression in transduced human CD34+ cells protects them from BG plus BCNU treatment \textit{in vitro} and \textit{in vivo} and in non-myeloablative autologous and allogeneic HSC transplantation settings in mice, dogs and primates, results in significant levels of selection and enrichment [55, 247, 249-251]. Despite the observation that these transduced cells engraft and expand after drug selection, knowledge of these processes is fairly rudimentary. Studies of HSC engraftment and \textit{in vivo} selection have commonly required frequent collections of peripheral blood and sacrifice of the research animals to collect hematopoietic tissues. This precludes analysis of the time-dependent process of drug selection, which includes the periods of repeated drug treatment, stem cell expansion, repopulation and engraftment.

To assess these processes, we have developed a lentiviral vector containing the luciferase gene to monitor engraftment after transduced hematopoietic stem cells were transplanted. We coupled the luciferase gene to the MGMT-P140K drug resistance gene so that cells transduced at low multiplicity of infection (MOI) could be monitored by bioluminescence imaging and enriched by drug treatment \textit{in vivo}. Monitoring these processes with \textit{in vivo} imaging provided new data on reconstitution dynamics, stem and progenitor cell expansion and transgene expression under selection pressure.

Of the various imaging technologies available, including MRI, PET, CT, and fluorescence imaging, etc [252, 253]. The most cost-effective monitoring over extended
periods of time for small animal studies is bioluminescence imaging (BLI) [254-256]. Similar to fluorescence imaging, BLI is non-invasive and can be used repeatedly for long-term monitoring in vivo. However, compared to GFP imaging, BLI has much greater sensitivity due to low background signal, and more importantly, better tissue penetration since the emitted wavelength extends beyond 600nm [230]. BLI has been used to track purified human HSC and progenitor cells (CD34+ and CD34+CD38-) and mononuclear cells homing and engraftment [237, 257]. BLI has also been used to track regulatory T-cell trafficking and survival after allogeneic transplantation [258]. However, bioluminescence imaging of reconstituting lentiviral transduced hematopoietic stem cells after in vivo drug selection has not been done. We hypothesized that low MOI lentiviral transduction of hematopoietic cells would result in long-term engraftment after MGMT-P140K mediated selection, and that bioluminescence imaging would be an effective method to monitor this process and uncover physiologic processes of stem cell expansion and migration after drug selection over time, and that it would provide data very different from the “mean effect” analysis of gene transfer in peripheral blood cells.

4.2 Results

4.2.1 LV-mnd-P2AL transduction resulted in robust MGMT-P140K expression and provided protection against BG+BCNU treatment in vitro.

To transfer the MGMT-P140K and luciferase mini-genes into murine hematopoietic cells, we generated a bicistronic lentiviral vector with an MND promoter from myeloproliferative sarcoma virus (MPSV), the MGMT-P140K gene placed proximal and the firefly luciferase gene placed distally to the 2A sequence in LV-mnd-P2AL (Fig.
12a). The titers of lentiviruses of various preparations ranged from $1.97 \times 10^5$ to $2.04 \times 10^6$ infectious particles per milliliter, and they were used without further concentration. To confirm the consistency of transgene expression, we transduced 293T and K562 cells at MOI of 1. Every two weeks, we analyzed cells for transgene expression by flow cytometry. Over a 21-week period, MGMT-P140K positive cells remained at an average of $95 \pm 4\%$ and $69 \pm 8\%$ for 293T and K562, respectively (Fig. 12b). Stable transgene expression was also observed in murine NIH-3T3 cells, at 90% under the same MOI condition (Data not shown).

Figure 12

a. pLV-mnd-P2AL

<table>
<thead>
<tr>
<th>CMV</th>
<th>R</th>
<th>US</th>
<th>Δag</th>
<th>MPSV</th>
<th>P140K</th>
<th>2A</th>
<th>LacI</th>
<th>ΔU6</th>
<th>R</th>
<th>US</th>
</tr>
</thead>
</table>

b. MGMT-P140K expressing cells %

- K562 untransduced
- K562 transduced with LV-P2AL
- 293T untransduced
- 293T transduced with LV-P2AL

Weeks 1, 3, 5, 7, 9, 15, 17, 21

c. Survival %

- Untransduced BM cells
- LV-mnd-P2AL transduced BM cells

0 μM to 60 μM BCNU Concentration
Figure 12. Lentiviral vector construct and transgene expression in cell lines.

(a) The construct of bicistronic lentiviral vector plasmid (pLV-mnd-P2AL) contained MGMT-P140K gene and firefly luciferase gene linked by FMDV 2A cleavage site and was controlled by an MND promoter. (b) In vitro transduction experiments by transducing 293T and K562 cells with lentiviruses (LV-mnd-P2AL) made with above bicistronic lentiviral vector plasmid at MOI of 1 showed robust MGMT-P140K expression over 21 weeks. (c) C3H murine bone marrow cells were transduced with LV-mnd-P2AL and treated with BG and various concentrations of BCNU, and analysis of surviving colonies in CFU showed strong protection in lentiviral transduced primary bone marrow cells against BCNU treatment.

To test whether the expressed MGMT-P140K provided drug protection, we transduced primary bone marrow cells from 10 week-old C3H/HeNCrI BR mice with LV-mnd-P2AL at MOI of 1. After 48 hours of transduction, the cells were washed free of virus and treated with 20 µM BG and 0-60 µM BCNU for 2 hrs and plated in methylcellulose. Colony counts from the cultures showed greater survival after BCNU exposure for bone marrow cells transduced with LV-mnd-P2AL compared to untransduced bone marrow cells (Fig. 12c). PCR analysis for the MGMT transgene from CFU progenitor colonies of initial transduction and from CFU progenitor colonies receiving BG and 0 µM BCNU showed 50% transgene positivity whereas 100% colonies analyzed from cells that received 10 µM to 40 µM BCNU were positive for the transgene. (PCR data not shown)

4.2.2 BLI tracking of murine bone marrow cell homing and early engraftment.
We selected white Balb/C female mice as donors and recipients for *in vivo* imaging because of their low photon absorption and scattering [254]. We collected Balb/c bone marrow cells and transduced them with LV-mnd-P2AL at low MOI of 0.5-1. Transgene transduction was measured in CFU assay, and transgene expression was measured by flow cytometry of the whole cell cultures for MGMT-P140K before infusion. After transduction, we observed an average of 40±10% transgene positivity in progenitor cell populations in CFU by PCR whereas there was only an average of 3±1% transgene expressing cells in the cultured bulk bone marrow cells by flow cytometry. The difference is larger than might have been anticipated and is addressed in the Discussion.

To visualize homing, migration and long-term engraftment of transduced stem and progenitor cells, we infused two different doses of transduced bone marrow cells (1x10^5 and 6x10^5) into groups of lethally irradiated syngeneic Balb/C mice (n=4). Three to five days after transplantation, weak BLI signal was detected in recipients. Recipient mice that received 6x10^5 transduced cells showed a greater degree of engraftment and expansion than mice that received 1x10^5 transduced cells, suggesting a cell-dose dependent effect. Visible BLI foci first appeared at day 8 in the recipients received 6x10^5 transduced cells, and engraftment in the spleen was observed between days 12-14 in both groups. The BLI signal became stronger in both groups by day 28 (Fig. 13a). Thereafter, the BLI signal weakened and disappeared. These initial imaging studies of engraftment were made using a laboratory prototype imaging system made by Dr. David Wilson in the Department of Biomedical Engineering at CWRU. In the subsequent experiments, we transplanted 1x10^6 transduced cells per recipient and used the Xenogen IVIS200 Imaging system for BLI imaging.
In the next experiment, we transplanted 1x10^6 transduced murine bone marrow cells into each lethally irradiated recipient and observed initial engraftment as early as day 4-5 with a pattern that varied from animal to animal. The most common pattern included robust initial engraftment sites within the long bones and vertebrae. BLI signal was the strongest between days 5-13 but decreased with time, indicating a short burst of initial expansion from progenitor cells expressing transgene early after transplantation (Fig. 13b). Thereafter, between days 8-14, we began to observe luciferase expression in the spleen, suggesting the formation of CFU-S spleen progenitor colonies (Fig. 13c). When we counted luciferase-expressing colonies among total CFU-S colonies in animals harvested at days 12-14, 9% of the CFU-S progenitor colonies were luciferase positive by visual inspection using the BLI camera (n=2 mice).
Figure 13.
Figure 13. Bone marrow cell engraftment appeared in a cell-dose-dependent manner and became less active with time.

(a) 1x10^5 and 6x10^5 LV-mnd-P2AL transduced Balb/C bone marrow cells showed a dose-dependent engraftment pattern after transplantation. Green arrows indicated the locations of the foci. Images in this figure were taken with a Case BME prototype imaging system. Images were pseudo-colored and analyzed with Matlab software. The remaining images throughout this paper were taken and analyzed with Xenogen IVIS200 and its software. (b) 1x10^6 transduced bone marrow cells showed early rapid engraftment and expansion at day 5, but transgene expression started to fade after 3-4 weeks without any drug selection. (c) Lethally irradiated recipient received 5x10^5 transduced bone marrow cells and imaged on day 12. After BLI imaging, spleen was removed and imaged for bioluminescence, and then spleen was fixed and photographed for CFU-S. (d) Serial transplantation of lentiviral transduced bone marrow cells. At day 58 after primary transplantation, 3x10^6 bone marrow cells from primary recipient was transplanted into 4 secondary recipients. Identical engraftment and expansion pattern was observed in secondary recipients as seen in the primary recipients. None of the secondary recipients received any drug treatment.
While there was significant linear correlation between transduced cell numbers and BLI signal (Fig. 14a), to translate this relationship \textit{in vivo} is more complex, given the attenuation of photons through different mouse tissues. Nonetheless, we were able to distinguish distinct foci \textit{in vivo} with whole body BLI (Figs. 13c and 14b). Under periodic observation of luciferase expression from these animals for up to 146 days, we noted that by week 4 after transplantation, most foci had faded away, and by week 8, most previously observed foci disappeared without drug selection (data not shown).

![Figure 14. BLI signal correlated to cell numbers.](image)

(a) BLI imaging of transduced bone marrow cells showed linear correlation between BLI signal to cell numbers. (b) BLI image of lethally irradiated recipient received $1 \times 10^5$ cells at day 14, and spleen was removed and imaged, showing two strong distinct foci that matched whole body BLI.
4.2.3 BLI monitoring of transgene expression in long-term hematopoietic stem cells during serial transplantation.

Our results with transduction and BLI signal of CFU-S colonies indicated successful transgene expression of hematopoietic progenitor cells. However, due to low transgene expression from lentiviral transduced murine hematopoietic cells at MOI of 0.5-1 and the low BLI signal after 4-5 weeks, it was necessary to determine whether long-term engrafting cells had been transduced. To test this, we performed serial transplantation. Using the same MOI of 1 for bone marrow cells transduction, we transplanted 2x10^6 total bone marrow cells into primary recipients. In serial transplantation experiment, the primary recipient did not receive any drug treatment. After 8 weeks, we collected bone marrow cells from a primary recipient and measured transgene-expressing cells by flow cytometry. We observed only 1.9% MGMT-P140K expressing cells by flow cytometry, whereas, unexpectedly, about 72% of the marrow CFU progenitor cell colonies contained the transgene by PCR. This is further discussed in the Discussion. We transplanted 3x10^6 bone marrow cells from the primary recipient into each of four lethally irradiated secondary recipients. Two of the mice died of transplant complications. We monitored two surviving mice over 12 weeks for luciferase expression (Fig. 13d). Persistent expression of luciferase was observed in long bones, vertebrae, and spleen over 12 weeks in these secondary recipients, indicating an efficient lentiviral transduction in long-term repopulating hematopoietic stem cells. In addition, there was no evidence that expression of luciferase or MGMT-P140K altered the capacity for long-term engraftment.
4.2.4 BLI showed effective selection of transduced bone marrow cells with BG and TMZ in both lethally irradiated and non-myeloablated recipients

Monitoring limited numbers of stem cells for transduced gene expression after low MOI transduction can be challenging due to low level of transgene expression. *In vivo* selection has been shown to be an effective way to enrich for MGMT-transduced HSC. We used BLI to observe the impact of the drug selection process on hematopoietic engraftment and expansion, and the distribution of transduced cells in real time. Recipients (n=3) received 750cGy total body irradiation followed by infusion of $1 \times 10^6$ lentiviral transduced bone marrow cells. Two sample mice were shown in Figure 3a. After initial engraftment and early expansion at day 5, and hematopoietic-organ-oriented foci (marrow and spleen) of expansion at day 12, transgene expression decreased gradually for 7 weeks post cell infusion (Fig. 15a). After transgene expression started to decrease, we administered the first 3-day-injection of BG and TMZ starting on day 48 and the second 3-day-drug treatment starting on day 75 after cell infusion. The first treatment had a modest effect on transgene expressing cells in mouse #1 and no effect on mouse #2 in terms of BLI intensity (Fig. 15a). In mouse #1, a diffuse signal from the abdominal region increased, and there was a slight increase of BLI signal in spleen (Fig. 15a). The second drug treatment led to substantial transgene expression in the spleens, spine, abdomen, and lower legs of mouse #1 and #2. This expression persisted for more than 2.5 months after second drug treatment, suggesting that transduction and selection of long-term repopulating cells had occurred. In addition, the foci were persistent in discrete locations and migration of these sites did not appear to take place. At week 17, spleens and ribs were removed and imaged *ex vivo*, the BLI signal was confirmed and was focal
(Fig. 15b). In both mice, the spleens appeared normal in size, and histological examination with hematoxylin and eosin stain showed no evidence of abnormal clonal expansion or disruption of the normal splenic architecture (Fig. 16).

We also compared the engraftment of lentiviral transduced bone marrow cells in both lethally irradiated recipients and non-myeloablated recipients before and after drug treatments [for treatment schedules, see Methods] (n=15). The imaging data showed a similar early engraftment after both methods of pre-conditioning (Figs. 15a and 15c). After non-myeloablated and lethally irradiated pre-conditioning of the recipients, the BLI signal subsided within 3-4 weeks and did not return throughout the remaining observation period when the recipients did not receive any drug treatment. To determine whether transgene-expressing cells could be enriched and induced to expand and whether these progeny would express the MGMT transgene, we treated mouse #2 in Figure 3 with BG and TMZ for 3-day treatment starting on day 21. The transgene expression increased dramatically 2 weeks later in the bone marrow of the upper right limb and in the spleen (Fig. 15c). To determine whether rescue of expressing cells could occur later after transplant, when transgene expression was not detected with BLI, we gave mouse#1 the first BG and TMZ treatment starting on day 44, at the same time that we gave mouse#2 the second BG and TMZ treatment. In mouse #1, we observed persistent foci 8 days after drug treatment. Transgene expression in mouse #2 increased slightly after the second round of drug treatment and persistent foci were easy to identify. These persistent foci were visible for an additional 10 weeks. Following a third cycle of BG and TMZ treatment at day 69, we observed further enhancement of transgene expression in the foci from the chest, vertebrae, and lower limbs of mouse#2 (Fig. 15c).
Figure 15.
Figure 15: BG+TMZ treatment enhanced transgene expression in lethally irradiated and non-myeloablated recipients.

(a) Two lethally irradiated mice received $1 \times 10^6$ LV-mnd-P2AL transduced bone marrow cells and BG+TMZ treatments for three days beginning on day 48 and day 75 (indicated with red arrows). Both ventral and dorsal views are shown. (b) Spleen and chest rib were collected at the termination of the experiment, placed in a 100mm dish, and imaged to confirm bioluminescence signal in both locations. (c) In two non-myeloablated recipients, sample mouse #1 received $5 \times 10^5$ transduced bone marrow cells and $5 \times 10^5$ untransduced supporting cells and was treated with BG+TMZ for three days beginning on days 44 and 69, and sample mouse #2 received $5 \times 10^5$ transduced bone marrow cells and $5 \times 10^5$ untransduced supporting cells and three rounds of BG+TMZ treatments for three days beginning on days 21, 44 and 69 (drug treatments indicated with red arrows). Both ventral and dorsal views are presented in the figure.
Figure 16. Spleen of lethally irradiated recipient after *in vivo* selection.

Spleen of lethally irradiated recipient after 2 rounds of BG+TMZ treatments with H&E staining. Spleen morphology was normal. Red pulp and lymphoid follicles were distinct, and there was no abnormal infiltration of lymphocytes in red pulp.

The detection of luciferase expression is highly sensitive for relative small numbers of cells and can accurately present transgene expression in the whole animal. For instance, in a mouse with clearly visible BLI foci on day 32, peripheral blood analysis by flow cytometry revealed very low levels of (~2%) MGMT-P140K positive cells (Fig. 17).
4.2.5 Semi-quantitative analysis of BLI confirmed enhanced engraftment of lentiviral transduced bone marrow cells.

To semi-quantify the BLI signal in the mouse, we used the Xenogen Living Image 2.5 software to select regions of interest (ROI). We selected bones and hematopoietic and lymphoid organs for further analysis. As noted from the qualitative observations, total body BLI signals were strong during the early phase of homing and engraftment. Photon emission then decreased in most mice 3-4 weeks after transplantation. However after
receiving BG and TMZ treatments, in both lethally irradiated and non-myeloablated recipients, imaging data showed a 3 to 4-fold increase in BLI signal compared to untreated recipients (Fig. 18a). Imaging detection in regions of interest (ROI) of hematopoiesis, such as in marrows and spleen, indicated cell expansion manifested by a significant increase in transgene expression after drug selection that persisted for 90-115 days (Figs. 18b-f). ROI analysis also showed more robust transgene expression at late time points in spleen and vertebrae of lethally irradiated recipient mice compared to non-myeloablated mice (Figs. 18e, f). This suggested that long-term engraftment and cell expansion were enhanced in lethally irradiated recipients, and that in both settings, persistent expression after drug selection was observed.
Figure 18.
Figure 18. Semi-quantitative analysis of BLI signal showed increased engraftment and transgene expression after BG+TMZ treatment.

Four groups of animals are compared longitudinally for evidence of BLI positive cells based on pre-conditioning status. The groups are: a) untransduced bone marrow cells in non-myeloablated recipients (n=2), b) transduced BMC in non-myeloablated recipients without BG+TMZ treatments (n=1), c) transduced BMC in non-myeloablated recipients given BG+TMZ treatments (n=2), and d) transduced BMC in lethally irradiated recipients given BG+TMZ treatments (n=2). Image intensity was compared over time for the total body, as well as regions and organ sites. (a) Whole body comparison, (b) Comparison in bone marrows of all of the limbs, (c) Chest, (d) Abdomen, (e) Spleen, (f) Vertebral bodies. Cyan arrows (↓) in Figure 4c indicate the start of the three-day drug treatments in non-myeloablated recipients and green arrows (↓) in Figure 4d indicate the start of the three-day drug treatments in lethally irradiated recipients.
4.2.6 *In vivo* drug selection resulted in persistent BLI foci.

To analyze BLI foci more carefully, we increased the minimum imaging detection setting to 2500 p/sec/cm²/sr to eliminate background noise and used a pseudo-color image to identify and semi-quantify the number of persistent foci. Identified persistent foci were analyzed using a fixed size ROI for their intensity (Fig. 19a). 8 weeks after bone marrow transplantation, only rare foci were noted. However after two or three rounds of BG and TMZ treatment in either irradiated or non-myeloablated recipients, there was sharp increase in the numbers and signal intensity of persistent foci (Figs. 19b, c) (treated mice: n=10; untreated mice: n=8). Locations of those persistent foci were estimated based on BLI images, and the most common sites were spleen (9 out of 10 animals), vertebrae (5 out of 10), abdominal regions (4 out of 10), skull (3 out of 10), thoracic regions (2 out of 10), chest (5 out of 10), and both upper and lower limbs (6 out of 10). The organs containing the most foci were in the abdominal region (most likely mesentery), spleen, and vertebrae (Fig. 19a). Interestingly, many of those sites had appeared in bioluminescence imaging during early engraftment. The duration of transgene expression among those persistent foci varied across sites and animals. In many instances, these foci persisted in intensity and location over extended periods of time (Fig. 20b). Most notable, some of those BLI foci appeared then disappeared after transplantation, and only reappeared at the same locations after drug selection.
Figure 19: Persistent BLI foci resulted from *in vivo* drug selection.

Predefined “regions of interest” [ROI] analysis was used to measure persistent BLI foci from both ventral and dorsal sides of recipient mice. Measurements from 18 recipients of both lethally and non-myeloablated preconditioning were pooled together to measure persistent foci, stratified by drug treatment. (a) An example of persistent foci selected with predefined ROI. The black circle in the dorsal image is an example of background used in the comparative measurement of foci intensity. (b) The number of persistent foci identified averaged 0.125 per mouse in untreated mice compared to 5.1 per mouse in treated recipients, p<0.01. (c) The mean±SD of BLI intensity were 8389.43±6353.17 photons/second (data range: 5826.11-24027.67) in untreated mice and 111019.15±187641.07 photons/second (approximately 13-fold increase, data range: 24033.33-1045000) in chemotherapy treated mice, p<0.01.
We also analyzed the integration sites of CFU progenitor colonies from transduced cells before transplantation and from recipients after in vivo drug selection by LAM-PCR and direct sequencing to better understand those BLI foci. Initial analysis of CFU from lentiviral transduced cells before transplantation showed a variety of clones with a low number of insertion sites (1-3 insertions per colony) (Fig. 20c). After in vivo selection, CFU progenitor colonies from both bone marrow and spleen obtained from mouse #2 in Fig. 15a had a similar pattern of integration by LAM-PCR (Fig. 20d), indicating that a small number of transduced hematopoietic progenitors contributed to both bone marrow and spleen after drug treatment. Further sequencing was performed directly on excised DNA bands from LAM-PCR, and BLAT search showed identical insertion sites from different CFU progenitor colonies recovered from both bone marrow and spleen of the same animal, indicating progenies of a common progenitor in both bone marrow and spleen (Table 1).
<table>
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<th>Strand</th>
<th>Integration site</th>
<th>RefSeq ID</th>
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Table 4. Insertion site analysis of LAM-PCR from CFU colonies of hematopoietic stem and progenitor cells before transplantation and after \(\text{in vivo selection}\)

Transgene positive CFU colonies (Before transplantation CFU colonies; after \(\text{in vivo selection}\) bone marrow (BM) CFU; after \(\text{in vivo selection}\) spleen (Sp) CFU colonies were processed for LAM-PCR, and the bands were excised and purified. Sequencing was directly performed on purified bands, and the results were searched through BLAT (University of California Santa Cruz) against mouse genome database. Bands with genome matches were marked with black stars in Figure 6c and 6d. N/A indicates no RefSeq ID and gene available at integration sites. (Abbreviation: Chr. Chromosome band; BM. Bone marrow; Sp, spleen)
Figure 20: Distribution of persistent foci in recipient animals and insertion analysis of CFU colonies of transduced bone marrow cells in vitro and after in vivo drug selection.

Detailed analysis of persistent BLI foci was performed from ten recipients after drug treatment. (a) Tissue localization of persistent foci was enumerated based on 2D BLI images. (b) The duration of visual detection of these persistent tissue foci (n=51 foci analyzed). (c) LAM-PCR of transgene positive CFU colonies (n=19) collected from LV-mnd-P2AL transduced bone marrow cells before transplantation (without prior drug treatment). (d) LAM-PCR of transgene positive CFU colonies collected from bone marrow (lanes BM1-BM4) and spleen (lanes Sp1-Sp3) of one of the recipient mice after in vivo selection (BM:n=4 and Spleen: n=10). Black arrows in Figures 6c and 6d and labeled IC indicate the band identified by amplification of the proviral internal control sequence that simply indicates presence of the provirus. The amplicons sequenced and listed in Table 1 are marked with black stars in Figure 6.c and d. (M: 1kb plus ladder)
4.3 Discussion

This study was performed to assess long-term patterns of engraftment and expansion of lentiviral transduced murine hematopoietic cells after in vivo selection. Bioluminescence imaging allowed observation of low-level early engraftment of genetically modified stem and progenitor cells before in vivo selection as well as sequential drug selection mediated enrichment of transduced cells over time. The most important contribution to our knowledge of the engraftment of genetically altered stem cells is the observation of patterns and locations of transgene expression by long-term engrafting cells and the discovery of discrete persistent foci at various locations in the recipients after in vivo selection.

After lentiviral gene transfer of murine hematopoietic cells, we observed a discrepancy between the transduction efficiency in progenitor cell population by CFU assay and transgene expression in bulk whole bone marrow cells. This is similar to the results in our earlier retroviral study in primary murine cells [52]. It has also been observed in lentiviral transduction of murine Sca+Kit+Lin- cells and a foamy viral transduction study [259, 260],[259, 260][259, 260][259, 260][259, 260]. In these lentiviral and retroviral studies, the differences between transduction and gene expression were likely in part due to transduction mosaicism and heterogeneous gene expression in CFU colonies [260]. Another possible reason for the discrepancy, as suggested in a study of Foamy virus transduction and selection, rests in the different cell populations that were analyzed. CFU colonies include more rapidly dividing progenitor cells whereas the majority of bulk cells in whole bone marrow were non-dividing hematopoietic cells. Lentiviral transduction rates in these two populations were different. These differences
may have been exacerbated by our low MOI transduction conditions. It is of interest that
the drug selection allowed selection under these conditions, since the non-expressing
transduced cells were selected against, in favor of MGMT expressing progenitors. To
determine whether MGMT expression studies by flow underestimated the proportion of
cells expressing the transgene, we inserted GFP alone with MGMT-P140K into the same
lentiviral backbone, conducted transduction studies in 293T and K562 cell lines and
murine primary bone marrow cells, and observed similar expression pattern (Data not
shown). Due to the low MOI lentiviral transduction, we suspect that a large portion of
transduced progenitor cells did not express the transgene in primary murine
hematopoietic cells in vivo. Since none of the PCR reactions showed evidence of a
rearranged transgene, the next most likely explanation is lentiviral gene silencing,
although we have no direct evidence.

While other BLI studies have tracked purified human CD34 cells and HSCs
transplanted from luciferase transgenic mice [236, 261], our imaging study provides visual evidence of the dynamic complexity of engraftment,
transgene expression, stem cell persistence and in vivo selection of low MOI lentiviral
transduced hematopoietic bone marrow cells. The emergence late after transplantation of
gene expressing clones after in vivo selection has not been previously observed. During
the early engraftment process before drug treatment, even though transplanted cells home
to bone marrow and splenic spaces within 24 hours, detectable bioluminescence signal
only appears 5 days later, which is consistent with the proliferation of clusters of stem,
progenitor and mature cells for a short period of time post-transplantation [262-264].
Yoshimoto et al. used GFP+ hematopoietic stem cell transplantation to study niche sites
occupied by stem cells soon after transplantation in bone marrow [265]. They were able to detect individual cells resident within the endosteal niche by fluorescent stereomicroscope in ribs and vertebrae and noted that these stem cells proliferate in a “stepping-stone”-like manner. The shortcoming of these observations include that they are not able to track these niche occupying cells weeks to months later. We have also observed engraftment foci in ribs and vertebrae and cell expansion by increased transgene expression with selection over extended periods of time, indicating that these long term engrafting cells become resident at these niche sites for extended periods of time. Unlike previous studies, however, we were able to observe the sequential processes after transplantation of hematopoietic stem cell homing, CFU-S formation, and conversion to an unobserved quiescent state resulting in undetectable transgene expression three to four weeks after transplantation. Recrudescence of expression took place after drug selection, and transgene expression is more likely due to cell expansion since drug treatment does not have direct affect on gene expression. While we have no way of identifying these cells months later without selection, the appearance of gene expressing cells after selection suggests that they are also present in untreated mice.

The engraftment and expansion of transduced hematopoietic bone marrow cells after \textit{in vivo} selection was highly influenced by drug treatment. The observation that, after \textit{in vivo} selection, these visual foci appeared and were stable for over 6 months has not been previously reported. Although this pattern of engraftment after selection was specific for MGMT-P140K transduced repopulating cells augmented by the drug selection procedure, it is likely that the patterns of engraftment, expansion and persistent proliferation may be common to all dormant repopulating stem cells called stochastically over time into
proliferation due to inflammation, cytopenia or other stress. Of course, some clusters may be macrophage, lymphoid or dendritic cells that persist in marrow, splenic, hepatic or lymphoid environments. However, those clusters seem to contain stem and progenitor cells able to expand upon drug selection pressure and to undergo secondary transplantation and engraftment. These highly generalized observations represent some of the first to assess the dynamic of foci of repopulating hematopoietic cells, some or many of which may be clonal, over extended periods of time. Other types of stress – such as LPS, other chemotherapy, infection, growth factors or chemokines may be expected to also result in recruitment of small foci of tissue resident stem cells, it would be difficult to identify without a visualization strategy.

The sites of persistent foci of transduced stem cells appeared to be randomly distributed among various marrow sites and the spleen. Since expansion of the observed foci occurred almost exclusively after drug selection, drug cytotoxicity followed by hematopoietic expansion appears to be the predominant instigator of clonal expansion. In any stem cell transduction and transplant study, there needs to be an assessment of clonal expansion due to insertional mutagenesis. In these studies, there was no evidence that the lentiviral transduction led to clonal expansion in the absence of drug selection. Further, no alteration in hematopoiesis in terms of either splenic morphology, blood counts or marrow cellularity was noted in any animal. This was consistent with other studies of MGMT-P140K mediated drug selection that have documented expansion of the MGMT-P140K expressing cells but not over-expansion of individual clones [251, 266, 267].[251, 266, 267][251, 266, 267][251, 266, 267][251, 266, 267] Thus, MGMT-P140K remains the most effective method of protecting human CD34+, murine, and canine hematopoietic stem
cells against chemotherapeutic drugs \textit{in vivo}, resulting in multi-log enrichment and selection over time [249, 268, 269].

The sensitivity of bioluminescence imaging was astonishing throughout our studies. In many cohorts, there was a very low level of MGMT-P140K transgene expressing cells in peripheral blood samples and bone marrows that were detectable by flow cytometry. However, BLI signals were clearly visible within the animals, providing clues to possible stem cell niches, including bone marrow spaces of the extremities, vertebral bodies, ribs, and even lymph nodes, as found in other studies [270, 271]. In addition, BLI imaging showed that \textit{in vivo} selection resulted in cell proliferation and increased transgene expression with persistent focal expansion of engrafted cells. To further explore these sites, we will use the recently developed Case Cryo-imaging system that performs automated cryo-sections with block-face imaging and reconstitution of images of the entire mouse [272]. In initial studies, we were able to analyze a limited number of transgene positive samples collected from the abdominal region. These sites contained transgene positive cells originating from progenitors with only 2-3 different insertions. Of note, these insertions were not the same as those recovered from the peripheral blood cells of the same animal. While these results are very preliminary, the foci could represent a composite of hematopoietic stem cells that were quiescent and only started cycling after transplantation and drug selection, but were not contributing sufficient cells to be identified in the blood, which may explain the low transgene expression in peripheral blood samples. A more complete cryo-imaging sample collection from MGMT-P140K–GFP-Luc transduced and drug selected mice is underway to identify the cells at specific foci.
These studies identify a pattern of late emergence of hematopoietic progenitor and stem cells at various marrow and splenic sites in a subpopulation of transduced and drug selected HSC derived cells in the mouse. It would seem likely that this pattern of focal engraftment and expansion particularly the pattern that emerges after drug selection would also be present in hematopoietic stem cell gene therapy clinical trials. Furthermore, this pattern suggests an inhomogeneity in the HSC engraftment and expansion process after gene transduction. Since blood sampling may not reflect this pattern, other approaches to assess the full scale of engraftment of cells that remain tissue resident foci are needed.

4.4 Materials and methods

4.4.1 Vector and lentivirus production

The luciferase lentiviral plasmid, pCSO-rre-cppt-MCU3-LUC, was kindly provided by Dr. Donald B. Kohn (Children’s Hospital Los Angeles, CA), containing firefly luciferase gene under the control of MND promoter, which increases transgene expression in hematopoietic cells, in a SIN lentiviral backbone [237, 273]. MGMT-P140K gene was excised from pLV-mnd-P140K plasmid with ClaI and MluI. 2A sequence from foot and mouth disease virus (FMDV) was used as a linker for MGMT-P140K and luciferase genes because it offers one to one ratio of expression [274]. 2A sequence was inserted between MGMT-P140K and luciferase by designing overlapping MGMT-2A primer (5’-CTGCTGGCGAAACGCACCGGTGAAACAGCTTTTGAGCTTTGACCTGCTCAA
GTTGGCAGG-3’) and 2A-luc primer (5’-TCAAGTTGGCAGGGGACGTCGAGTCCAACCCTGGGCTATGGAAAGACGCCAA AAACATATA-3’). MluI and Scal sites were used to digest the MGMT-2A-luc PCR fragment, and then the fragment was ligated to P140K fragment cut out from pLV-mnd-P140K. Then MGMT-P140K-2A-luc long fragment was cloned into pCSO-re-re-cppl-MCU3-LUC with Clal and Scal to generate plasmid pLV-mnd-P140K-2A-luc (pLV-mnd-P2AL). After cloning, plasmid was digested and sequenced to ensure the correct orientation and in frame. Lentivirus, named LV-mnd-P2AL, was generated by triple transient transfection in 293T cells as described previously [54]. In brief, component plasmids included pMD.G (VSVG envelop plasmid), pCMVΔR8.91 (packaging plasmid), and pLV-mnd-P140K-2A-luc were added to 293T cells at a ratio of 1:3:3 with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco, Paisley, UK), and 12 hours later, Opti-MEM was replaced with fresh DMEM (Mediatech, Manassas, VA). After another 12 hour-incubation, Lentiviral supernatant was collected and filtered with Millipore Steriflip Filter (Millipore, Billerica, MA). The titer of lentiviral vectors was measured in K562 cells by measuring MGMT-P140K expression by flow cytometry with an anti-MGMT monoclonal antibody (Kamiya Biomedical Company, Seattle, WA).

4.4.2 Mice

Female Balb/C mice (Charles River, Wilmington, MA) were used as donors and recipients in all imaging studies. Mice were housed in pathogen-free Animal Resource Center according to approved animal protocol. In non-myeloablative pre-conditioning, two days before transplantation, recipient mice received 30 mg/kg of BG and 7.5 mg/kg
of BCNU; in lethal irradiation pre-conditioning, recipient mice received lethal dose of 750 cGy with a Cs137 source within 1-5 hours before transplantation.

4.4.3 *In vitro* transgene expression and drug selection

5x10^5 293T or K562 cells were plated in 6-well-plate in triplicates, and lentiviral vector LV-mnd-P2AL supernatant was added to each well at MOI of 1 with 8 µg/ml polybrene (Sigma-Aldrich, Milwaukee, WI) for 48 hours. Transduced 293T and K562 cells were maintained in DMEM and Iscove’s MEM respectively, and passed every 3-4 days for over 4 months. Every two to three weeks, cells were measured for MGMT-P140K expression by flow cytometry. For in vitro assessments of gene transfer, murine bone marrow cells were collected and transduced with lentiviral vector LV-mnd-P2AL at MOI of 1.5 supplemented with 20ng/ml mIL-3 (R&D Systems, Minneapolis, Minnesota), 50ng/ml mIL-6 (R&D Systems, Minneapolis, Minnesota), 50ng/ml rSCF (R&D Systems, Minneapolis, Minnesota), and 20%FBS (Invitrogen, Carlsbad, CA) with 8 µg/ml polybrene for 48 hours. Then cells were treated with 20µM O6-benzylguanine for 1 hour and followed by treating with 0-60µM of BCNU for 2 hours. After drug treatments, cells were plated in murine methylcellulose for CFU analysis. CFU colonies were counted and tested for the present of transgene by PCR.

4.4.4 Lentiviral gene transfer of murine bone marrow cells

Bone marrow cells from 6-8 week old female Balb/C mice were collected by flushing femurs, tibia, and hip bones with α-MEM (Mediatech, Manassas, VA) containing 10% Heparin (Baxter Healthcare Corp., IL). Cells were washed in fresh α-MEM and counted with a hemacytometer after mixed with 4%(v/v) acetic acid. Based on viral titer and MOI
of 0.5-1, appropriate amount of whole bone marrow cells or Lineage depleted cells were incubated in lentiviral supernatant with the same supplements described above. Lineage depleted cells were isolated by using anti-PE microbeads (Miltenyi Biotec, Auburn, CA) to separate Lin+ cells stained with PE conjugated CD3, CD4, Ter119, B220, and CD11b (BD Pharmagen, San Diego, CA). After transduction, cells were washed and re-suspended in fresh α-MEM prior to transplantation. Lin- cells were mixed back to Lin+ bone marrow cells for transplantation. Mice received transduced whole bone marrow cells or transduced Lin- cells were pooled together for BLI foci analysis and grouped based on pre-conditioning and drug treatments. 5x10⁵ total bone marrow cells were used to measured MGMT-P140K expression by flow cytometry, and 1-2x10⁵ cells were plated in CFU. Transduction efficiency was measured by testing CFU progenitor colonies for the presence of transgene by PCR.

4.4.5 Transplantation and drug treatments

Transduced bone marrow cells were washed and re-suspended in α-MEM. 1x10⁵ to 1x10⁶ bone marrow cells were transplanted into preconditioned syngeneic mice by tail-vein injection, and about 1x10⁵ bone marrow cells were saved for in vitro analysis. After pre-conditioning of recipients, animals were provided with water supplied with Bacitricin/Neomycin (Sigma-Aldrich, St. Louis, MO). Every three to four weeks after transplantation, recipient mice received three-consecutive-day treatments of 30 mg/kg of O⁶-benzylguanine (BG) and 60 mg/kg of Temozolomide (TMZ). BG was dissolved in 40% PEG (polyethylene glycol) and diluted in 60% pH 8.0 PBS, and TMZ was dissolved in 10% DMSO and 90% PBS. TMZ was administrated one hour after BG treatment. In serial transplantation experiment, at day 58 post transplantation, one primary recipient
mouse was sacrificed, and 3x10⁶ recovered whole bone marrow cells were transplanted into lethally irradiated secondary recipients.

4.4.6 CFU-S assay

6-8 week old recipient Balb/C mice were lethally irradiated (750 cGy) 1-3 hours before cell infusion. Whole bone marrow cells from donor mice were transduced with LV-mnd-P2AL at MOI of 1 two days before cell infusion. 1x10⁵ or 5x10⁵ transduced bone marrow cells were transplanted into irradiated recipients via tail-vein injection. At 12 days or 14 days later, recipient mice were imaged with IVIS 200 system, and spleens were removed and imaged again. After imaging, spleens were fixed in Bouin’s solution for 15 minutes and photographed for visible colonies.

4.4.7 Bioluminescence imaging of bone marrow cells in vivo

Bioluminescence imaging of in vivo selection studies was performed on Xenogen IVIS 200 system (Caliper LifeSciences, Alameda, CA) at Case Western Reserve University Small Animal Imaging Center. A diluted anesthesia mix (Ketamine HCl, Xylazine HCl, and Acepromazine), prepared by Case Animal Resource Center, was injected 5-7 minutes intra-peritoneally prior to another ip injection of 125 mg/kg of D-Luciferin (Biosynth AG, Switzerland) into each mouse, and the animals were taped down on black photographic paper. The imaging parameters were maintained throughout the experiments. The exposure time for each image was 5 minutes, and first image was taken 7 minutes after injecting D-luciferin. Two consecutive ventral images were followed by one dorsal image. Only the second ventral image and the dorsal image from each animal were used for analysis because the peak bioluminescence signal emitted from most
organs was reached 15-20 minutes after the injection of D-luciferin based on preliminary studies that identified the signal intensity as a function of time (data not shown). For confirmation of the exact location of foci, some mice were sacrificed after BLI imaging, and their internal organs were imaged again to confirm the bioluminescence signal.

4.4.8 BLI imaging analysis

Xenogen Living Image 2.5 software was used to quantify BLI signals. Region of interest (ROI), including the whole body, four limbs (bones of extremities), chest, the whole abdominal region, spleen and vertebrae, were created for quantification. Identified persistent BLI foci were also selected as ROIs and compared for BLI intensity. ROIs for foci were selected as circle with a diameter of 1 cm. Only visible focus that appeared at least 8 weeks after bone marrow transplantation, had twice the intensity as the background signal taken from each animal, and lasted more than 3 weeks at a specific location was defined as a persistent focus. Number of foci and signals were quantified and analyzed using paired t-test (GraphPad Software). Some locations of the foci were estimated from BLI images due to the 2D nature of BLI and compared for the occurring frequency and duration.

4.4.9 LAM-PCR and Sequencing

CFU colonies were collected and washed once with 1 ml PBS, and digested with 25 µl of protease K solution at 50°C overnight. Samples were heat-inactivated at 95°C for 10 minutes and spun down. LAM-PCR was performed according to published protocol. Linear amplification was done with 5’-biotinylated primer, LV-LTR1 (5’-(biotin)-GAGCTCTCTGGCTAACAAG-3’). Dynabeads M-280 Strepavidin magnetic beads
(Invitrogen, Carlsbad, CA) were used in the following steps to wash and pull-down PCR products. Random priming was performed to convert linear product to double stranded DNA fragments with Klenow polymerase and hexanucleotide mix (Roche, IN) at 37°C for 1 hour. Enzyme digestion was done with Tsp509I (New England Biolabs, MA) at 65°C for 1 hour. Digested DNA fragments were ligated to a linker cassette (5’-GACCCGGAGATCTGAATTCAGTGGCACAGCAGTTAGG-3’) with a complementary overhang. Then two nested PCR reactions with performed with two sets of primers. First nested PCR used LV-LTR2 (5’-GACCCGGAGATCTGAATTC-3’) and LC-1 (5’-GACCCGGAGATCTGAATTC-3’), and the second nested PCR used LV-LTR3 (5’-AGTAGTGTGTGCCCCTGTCTGT-3’) and LC2 (5’-GATCTGAATTCAGTGGCACAG-3’). The products of the second nested PCR were run on 2% agarose gels. PCR bands were then excised and purified with QIAquick gel extraction kit (QIAGEN, CA) and sent for sequencing. Sequencing results were searched with BLAT or BLAST for matching mouse genome.
Chapter 5
Cryo-Imaging of Hematopoietic Bone Marrow Transplantation

Abstract

Hematopoietic bone marrow transplantation has been routinely performed in clinics for defective blood cell diseases and leukemia patients. Successful engraftment of transplanted bone marrow cells is always analyzed with peripheral blood or bone marrow samples. However, recent murine bone marrow stem cell transplant studies with bioluminescence imaging and other imaging modalities had provided valuable insight into this dynamic and stochastic engraftment process. Analysis of these images enhanced by newly developed technologies can accurately pinpoint transplanted cells within an organ. In this study, we used a cryo-imaging system made at Case to verify BLI imaging data and study engrafted bone marrow cells in tissues through out the body after transplantation. The Case Cryo-imaging system allows us to collect bright field and fluorescence images of the whole mouse at histological resolution. At higher magnification, we were able to observe single bone marrow cell inside bone marrow cavities. While this is a single-point time analysis, a 3D model can be reconstructed from cross-section images to show the exact locations of transduced and fluorescent cells.
5.1 Introduction

*In vivo* imaging of study animals provides valuable information on adult and embryonic stem cell biology and functional benefits of cell therapies. High sensitivity, non-invasiveness, and long-term monitoring have made bioluminescence imaging (BLI) a highly desirable imaging modality for studying biological process, especially stem cells, in small animals *in vivo*. However, the two-dimensional property of BLI only allows researchers to estimate the locations of the biological process and the possible numbers of cells emitting photons. Due to the need for a true 3-D BLI imaging system, a 3-D diffuse tomographic IVIS Spectrum has been developed and used in small animal imaging studies [275, 276]. Even though other small animal imaging modalities, such as MRI, PET, CT and SPECT, have been used to validate BLI data, there are still limitations due to low-resolution and sensitivity of those imaging modalities [277-281]. In studies of gene transfer of hematopoietic cells and bone marrow transplant, frequent collection of peripheral blood sample is necessary, but this provides no data on cell localization in organs. At best, cells can be harvested at the end of the animal’s life to identify reporter gene expressing cells in bone marrow, spleen or thymus, but to perform analysis on cells from locations other than those hematopoietic organs were extremely difficult without the use of cutting-edge technology to retrieve the cells first. In addition, without high resolution imaging of the whole animal, to observe and study the exact location of all reporter gene positive stem and progenitor cells and their surrounding microenvironment are not possible.

With the increasing needs for high-resolution imaging in whole animals, Dr. David Wilson’s lab in the Department of Bioengineering at Case Western Reserve University
has developed a mouse-sized cryostat and a microscope imaging system [272, 282, 283]. The goal of this imaging system is to capture and image fluorescence protein expressing cells in their nature environment and the possible retrieval of cells if needed. The system is composed of a cryomicrotome, an imaging system, a robotic xyz positioner, and a Window-based computer control, capturing, and analysis system. The imaging system consists a stereomicroscope, low noise camera for capturing bright field and fluorescence images, illumination sources, and interchangeable optical filters for different fluorophores [272]. Sections of episcopic images of block face sample are taken and stitched together to show the whole section of the animal in histological detail (Fig. 21).

Figure 21. Cryo-imaging of a whole adult mouse.

The image was composited from 20 tiled acquisitions at 15.6 μm pixels [282].

Hematopoietic stem cell homing and engraftment and stem cell niche have been studied extensively with peripheral blood and bone marrow analysis, transplantation studies using PKH26 or GFP labeled bone marrow cells [265, 284], and by real-time
microscopic imaging of bone marrow space [271, 285]. However, there are still questions remain regarding to this dynamic process of engraftment after transplant, and no one has ever been able to show the location of all transplanted and labeled bone marrow cells in an animal at any single time point after transplant. By taking advantage of this newly established imaging modality, we can give an appreciation of those complex processes. Further, by using Case Cryo-imaging system to study bone marrow transplantation in cohorts of mice at various time points allows us to track and locate all labeled and transplanted bone marrow stem and progenitor cells. This detailed spatial distribution of transplanted hematopoietic cells throughout the animal can provide information on short-term and long-term engraftment and further our understanding on the biology of hematopoietic stem cell, stem cell niche, and bone marrow transplantation.

5.2 Results

5.2.1 In vitro transduction with LV-mnd-PAGAL results in robust firefly luciferase, GFP and AGT expression in cell lines.

In order to track transduced bone marrow cells using the cryo-imaging system, eGFP was added to the SIN lentiviral vector, containing an MND promoter from myeloproliferative sarcoma virus (MPSV), to generate a tricistronic lentiviral vector with MGMT-P140K and firefly luciferase. MGMT-P140K gene, eGFP, and firefly luciferase were linked by two 2A sequences from FMDV (Fig. 22). To test luciferase expression, Balb/C murine bone marrow cells were transduced with LV-mnd-PAGAL at MOI of 1, and 100, 500, 5000, and 10000 transduced cells were imaged with Xenogen IVIS200. BLI images showed a strong correlation ($R^2 = 0.998$) between cell numbers and BLI
signal, indicating a uniform transduction and transgene expression in transduced murine bone marrow cells.

Transgene Vectors:

![Tricistronic Lentiviral Vector Diagram](image)

Figure 22. Tricistronic lentiviral vector with MGMT-P140K, GFP, and luciferase genes. Construct of tricistronic lentiviral vector contains MGMT-P140K, GFP, and firefly luciferase gene, linking by two FMDV 2A sequences. Transduction of Balb/C primary murine bone marrow cells shows BLI signal and cell number’s correlation efficiency at 0.998.

To measure the consistency of transgene expression, 293T and K562 cells were transduced with LV-mnd-PAGAL at MOI of 1. Every two weeks, cells were analyzed for MGMT-P140K and GFP expression by flow cytometry. Over a 7-week period, MGMT-P140K positive cells remained at an average of 92±5% and 47.5±4% for 293T
and K562, respectively. GFP expression was at an average of 81±10% and 44.8±7% for 293T and K562, respectively (Fig. 23). Taken together, the tricistronic lentiviral vector was able to express all three transgenes efficiently, and the expressions of MGMT-P140K (AGT) & GFP were robust and persistent in cells. From this we conclude that constant expression of the transgenes should occur from the efficient integration and expression of lentiviral vector, which is critical for the in vivo experiments.

![Figure 23](image.png)

Figure 23. Robust transgene expression in cell line over 7 weeks.

293T and K562 cells were transduced with LV-PAGAL at MOI of 1. AGT and GFP expressions were measured by flow cytometry every other week for a period of 7 weeks, showing persistent AGT and GFP expression in both cell lines.

5.2.2 Imaging of bone marrow transplantation with Case Cryo-imaging system

Compared with the base version of the imaging system, huge improvements have been made to the new cryo-imaging system over the years by Dr. Wilson’s lab [286]. A stereomicroscope with much more improved field of view and depth of focus. Additional
bright-field and fluorescent light sources were attached to the microscopy (Fig. 24a).

Another huge advancement was the addition of a computer-controlled motorized positioner (Fig. 24b), which allows tiled acquisition for high-resolution-images of each block-face throughout the entire animal.

Figure 24. Case Cyro-imaging system.

Case Cryo-imaging system made by Dr. David Wilson’s lab at the Department of Biomedical Engineering. (a) Stereomicroscope with light sources attached; (b) Robotic XYZ positioner.

Bone marrow cells from Balb/C mice were used for tricistronic lentiviral transduction at low MOI of 1. Syngeneic recipients (n=15) were lethally irradiated and transplanted with 1x10^6 – 1.5x10^6 bone marrow cells. BLI images were monitored over the time. At various time points after bone marrow transplant, mice were sacrificed and quickly
frozen in OCT with liquid nitrogen for cryo-imaging. We were able to detect GFP positive cell cluster, single cells in epiphysis and endosteum of bone at high magnification of 63X (Fig. 25a, b), and interestingly, GFP positive vasculature was also observed at a magnification of 50X (Fig. 25c).
Figure 25.
Figure 25. GFP positive bone marrow cells after transplantation.

Different magnifications were used for cryo-imaging. (a)&(b) showed cell cluster in epiphysis and possible single cells in endosteum region; (c) showed GFP lining of a vasculature. (Magnifications are indicated, and white arrows showed GFP cell cluster, single cells and vasculature.)

At magnification of 50X, we were able to observe the localization of GFP positive cells in the liver of the recipient day 19 after bone marrow transplantation (Fig. 26). Some GFP positive cell clusters appeared to be in liver tissue, but most of GFP signal appeared to be in the lining of hepatic sinusoids. One potential limitation with taking fluorescence images in the abdominal region was largely contributed by the strong auto-fluorescence from the content in the digestive track (Fig. 26). Alfalfa-free rodent diet was used to reduce this intense background signal, however it was not used in the early stage of this study.
Figure 26. Transgene positive cells in the liver of the recipient.

Some GFP positive cells were indicated by write arrows, and most of GFP positive cells were found in the lumen of vasculature in liver sinusoids of the recipient as grouped by a window (white squares). Strong fluorescence at left bottom was due to the auto-fluorescence in the digestive tract.

5.2.3 GFP positive cells in liver were transgene positive.

One advantage of running block-face imaging with Cryo-imaging system is that we can stop the imaging process and collect tissue samples for further genetic analysis. In order to show that those GFP positive cells were transduced and transplanted bone marrow cells. We paused the imaging process and collected GFP fluorescence positive tissue with sterile syringe needles (later with micro-scoop). In the meantime, we also collected GFP negative part of liver tissue and part of intestine with strong auto-fluorescence as control. Transgene PCR was done with all of the samples along with PCR of GAPDH to show the presence of tissue. Three samples collected from GFP positive cell clusters and GFP positive lining were positive for transgene, and as
expected, GFP negative liver tissue, adjacent tissue proximate to GFP positive tissue on
the same block-face, showed no transgene, as did tissue collected from intestine (Fig. 27).

Figure 27. Cryo-imaging samples are positive for transgene.

(a) 3 samples (Lane 1-3) from GFP positive cells were collected and tested for the present
of transgene by PCR. 1 sample (Lane 4) from GFP negative portion of liver and 1
sample (Lane 5) from intestine with strong auto-fluorescence were used as control, and
they were all transgene PCR negative. (b) GAPDH PCR reaction for samples in (a).
(Lane 6: H₂O; Lane 7: 293T cells transduced with LV-mnd-PAGAL)
5.2.4 Reduction of auto-fluorescence during imaging analysis.

Due to the high auto-fluorescence in the digestive track during imaging acquisition, fluorescence detection sensitivity was reduced for weak GFP positive samples. In order to decrease the interference of auto-fluorescence in the final data analysis and 3D model reconstruction, we used a ratio reduction method. We were able to adjust a threshold of the ratio of signals from green channel vs red channel so that genuine GFP positive signal had much higher ratio. After setting a threshold, auto-fluorescence was significantly diminished (Fig. 28).

Figure 28. Adjustment of auto-fluorescence in captured images.

The threshold for the ratio of signals from green channel against red channel was set to remove a significant portion of auto-fluorescence before 3D model reconstruction.
5.2.5 Correlation of BLI with cryo-imaging.

Recipient mice were imaged by bioluminescence at various time points after bone marrow transplant (Fig. 29a). At the end of the observation period, the recipient mice were frozen and imaged with Case cryo-imaging system right after bioluminescence imaging. In order to exam if bioluminescence images and fluorescence images were correlated, we reconstructed the tiled block-face images of the section performed with cryo-imaging into a single complete image. All the images were processed with reduced auto-fluorescence setting before reconstruction. Overlapping BLI image with cryo-imaging image showed a very similar pattern of transgene expression in both imaging modalities (Fig. 29b). The data shows that cryo-imaging can be used to co-register with BLI, confirming that there was concurrent transgene expression in vivo.

Figure 29. Correlation of BLI and cryo-imaging.

(a) Bioluminescence imaging of recipient of bone marrow transplant at day 6, 12, 16, and 19 from a single mouse. The same BLI scale was used for all four pictures, and only ventral side was shown; (b) overlapping BLI image with fluorescent image from cryo-imaging showed a similar transgene expression pattern.
5.2.6 Three-dimensional reconstruction of cryo-imaging.

In order to show the spatial anatomical location of transplanted donor bone marrow cells, the recipient mouse was sacrificed, and continuous tiled fluorescence images were taken at magnification of 36X. To prevent missing data on the block-face data reconstruction, GFP positive cells were not collected from block-face sample during cryo-imaging. Tiled fluorescence images were merged, and auto-fluorescence reduction was applied. 3D reconstruction of the fluorescence images clearly showed the spatial locations of GFP positive cells in the dorsal portion of the skull and at scattered sites in the abdominal (Fig. 30). Those images showed us that bone marrow stem and progenitor cells could reside outside of long bones and vertebrae after bone marrow transplantation, more obviously in liver and abdominal region. This study also gave us a proof of principle that cryo-imaging can be very useful in identifying small clusters of cells after bone marrow transplantation.
Figure 30. 3-D reconstruction from cryo-images of BMT recipient.

A bone marrow transplant recipient was imaged with Case Cryo imaging system. The thickness between each slice was 80 µm. After auto-fluorescence signal reduction, tiled block-face images were reconstructed to form this 3D model. Yellow spots indicated the GFP positive cells. (Reconstruction was performed by David Prabhu.)

5.3 Discussion

Murine model systems provide an underpinning to the understanding of biological phenotypes, genetic mutations, pathogen-host interactions, stem cell biology, and cancer development and therapies. In vivo imaging and end-of-experiment imaging expand the scopes of these researches by providing new approaches to address those questions. For example, bioluminescence imaging was used to track and monitor Smad signaling in murine brain in response to excitatory stimulation [287]. There are several imaging modalities for small animal imaging, including CT, bioluminescence imaging,
fluorescence imaging, PET, MRI, SPECT, etc. However, each imaging modality has its pros and cons [288]. In this study, we were able to use a novel imaging system, Case Cryo-imaging system, to track hematopoietic marrow cells after bone marrow transplant with high precision and resolution. It allowed us to visualize the transplanted bone marrow cells in well-known engraftment sites, as well as discover uncommon location.

Hematopoietic marrow cell engraftment after bone marrow transplant is a dynamic and stochastic event. However, to really understand HSCs’ behavior, we have to look at them at their natural environment. Location can offer us a very important clue. Advanced microscopic imaging in vivo has been done in several studies [271, 285], showing cell-cell interactions, but advanced microscopic imaging was not applied throughout the animals. Using our cryo-imaging method, we were able to locate GFP positive cell cluster and even single cells in epiphysis and endosteum 19 days after transplant, which provides visual evidence of engraftment into an osteal niche.

Our previous studies have also shown that in vivo selection of transduced hematopoietic marrow cells could result in the enhanced engraftment and persistent foci in locations other than bone marrow (Chapter 4). With Cryo-imaging, we observed GFP positive cell clusters in liver sinusoids, and by extracting GFP positive foci from cryo-slice and processing for the present of transgene by PCR, we were able to show that those GFP positive foci were transgene positive and thus derived from transplanted murine bone marrow cells. Whether these cells homed and engrafted or lodged in the liver could not be determined by a single-point time analysis. One fact that supported the homing and engraftment in the liver came from our previous studies, showing that bioluminescent foci in chest and abdominal region responded to proliferative stress caused by in vivo
drug treatments. Since drug treatment does not induce transgene expression in single gene-modified cells, an increase in transgene expression was more likely due to the proliferation of cells. Thus, we suggested that those BLI and GFP foci were more likely to be stem or progenitor cells, which also responded to signals from their microenvironments.

In addition, images collected through cryo-imaging can be combined by imaging software to reconstruction of a 3D model, which is particularly useful for spatial analysis of labeled cells, as well as screening and discovering unforeseen phenotypes of various gene-knockout models. In our studies, we imaged several animals received low MOI lentiviral transduced murine bone marrow cells. After transplantation, gene-modified bone marrow cells were seen throughout the animals. By bioluminescence imaging, we clearly saw the luciferase expressing cells in the skull, vertebrae, chest and abdomen. 3D reconstruction gave us a more detailed spatial aspect of those transgene expressing cells, showing that they were able to reside among internal organs and only formed small clusters, which are more consistent with the idea of a vascular niche for hematopoietic stem and progenitor cells. However, in the field of HSC niche study, there is still limited data showing that stem and progenitor cells can proliferate rapidly in vascular niches. In our imaging data, we can see that BLI foci can respond relatively quickly to in vivo drug selection.

Overall, this study provided a chance to study BLI foci in great details by using cryo-imaging system. It allowed us to track, locate, and retrieve hematopoietic stem and progenitor cells throughout the animal at any time point after bone marrow transplant. In addition to tract hematopoietic stem cells, cryo-imaging system can also be very useful to
track other types of stem cells, such as the homing of mesenchymal stem cells and neuronal stem cells. Another potential application with this imaging modality is to label subpopulation of hematopoietic stem cells, and cryo-imaging can allow us to track those subpopulation individually with supporting unlabeled bone marrow cells. With the improved analysis software and algorithm, we are expected to be able to quantify the number of cells from the images accurately.

5.4 Methods and Materials

5.4.1 Vector and lentivirus production

Two bicistronic lentiviral plasmids were used to generate this triple vector. These two plasmids were pLV-mnd-P140K-2A-GFP generated by Dr. Justin Roth and pLV-mnd-GFP-2A-luc. The generation of pLV-mnd-GFP-2A-luc was similar to the method described in Chapter 4. In short, GFP-2A-luc was created by overlapping and extension PCR. PCR products of GFP-2A primer with GFP 5’ primer and 2A-luc primer with 3’mnd primer were PCR again and inserted into original plasmid pCSO-re-MCU3-LUC to create pLV-mnd-GFP-2A-luc. Both pLV-mnd-P140K-2A-GFP and pLV-mnd-P140K-2A-GFP were partial digested with BsrGI and EcoRI. Insert from pLV-mnd-GFP-2A-luc was cloned into pLV-mnd-P140K-2A-GFP to generate plasmid pLV-mnd-P140K-2A-GFP-2A-luc (pLV-PAGAL). After cloning, plasmid was digested and sequenced to ensure the correct orientation and in frame. Lentivirus, named LV-PAGAL, was generated by triple transient transfection in 293T cells as described previously [54]. In brief, component plasmids included pMD.G (VSVG envelop plasmid), pCMVΔR8.91 (packaging plasmid), and pLV-PAGAL were added to 293T cells at a ratio of 1:3:3 with
Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco, Paisley, UK), and 12 hours later, Opti-MEM was replaced with fresh DMEM (Mediatech, Manassas, VA). After another 12 hour-incubation, Lentiviral supernatant was collected and filtered with Millipore Steriflip Filter (Millipore, Billerica, MA). The titer of lentiviral vectors was measured in K562 cells by measuring GFP expression or MGMT-P140K expression by flow cytometry with an anti-MGMT monoclonal antibody (Kamiya Biomedical Company, Seattle, WA).

5.4.2 Mice
Female Balb/C mice (Charles River, Wilmington, MA) were used as donors and recipients in all imaging studies. Mice were housed in pathogen-free Animal Resource Center according to approved animal protocol. For bone marrow transplant, all recipient mice received lethal dose of 750 cGy with a Cs137 source within 1-5 hours before infusion. Special water with Bac/Neo was given to irradiated animals. Before cryo-imaging, mice were sacrificed by cervical dislocation.

5.4.3 In vitro transgene expression
5x10^5 293T or K562 cells were plated in 6-well-plate in triplicates, and lentiviral vector LV-PAGAL supernatant was added to each well at MOI of 1 with 8 µg/ml polybrene (Sigma-Aldrich, Milwaukee, WI) for 48 hours. Transduced 293T and K562 cells were maintained in DMEM and Iscove’s MEM respectively, and passed every 3-4 days for over 2 months. Every two weeks, cells were measured for AGT and GFP expression by flow cytometry.

5.4.4 Lentiviral gene transfer of murine bone marrow cells
Bone marrow cells from 6-8 week old female Balb/C mice were collected by flushing femurs, tibia, and hip bones with α-MEM (Mediatech, Manassas, VA) containing 10% Heparin (Baxter Healthcare Corp., IL). Cells were washed in fresh α-MEM and counted with a hemacytometer after mixed with 4%(v/v) acetic acid. Based on viral titer and MOI of 1, appropriate amount of whole bone marrow cells were incubated in lentiviral supernatant with the same supplements described in Chapter 4. After 48 hour-transduction, cells were washed and re-suspended in fresh α-MEM prior to transplant. Transduction efficiency was measured by testing CFU colonies for the presence of transgene by PCR.

5.4.5 Bioluminescence imaging of bone marrow cells in vivo

Bioluminescence imaging was performed on Xenogen IVIS 200 system (Caliper LifeSciences, Alameda, CA) at Case Western Reserve University Small Animal Imaging Center. A diluted anesthesia mix (Ketamine HCl, Xylazine HCl, and Acepromazine), prepared by Case Animal Resource Center, was injected 5-7 minutes intra-peritoneally prior to another IP injection of 125 mg/kg of D-Luciferin (Biosynth AG, Switzerland) into each mouse, and the animals were taped down on black photographic paper. The imaging parameters were maintained throughout the experiments. The exposure time for each image was 5 minutes, and first image was taken 7 minutes after injecting D-luciferin.

5.4.6 Cryo-imaging preparation and operation

After bioluminescence imaging and before waking up from anesthesia, mice were sacrificed by cervical dislocation. A tissue-histology compound, optimal cutting temperature (OCT) (Tissue-Tek, Terrance, CA), was thoroughly applied to the skin and
fur of the animals to minimize air bubbles. Each animal was then put in a small container made of aluminum foil and completely covered with OCT. Liquid nitrogen was used to quick freeze the animal embedded block. Since the cryo-microtome was operated in -20°C freezer, after removing foil container outside, additional OCT was used to attach the block to the cutting platform. In the continuous cutting for the 3D reconstruction study, mouse was cut at 80µm thickness. After setting up the imaging tiles and cutting thickness, the computer controlled system made a slice or several slices and then took bright field and fluorescence images.
Chapter 6

Clonal Selection and Engraftment of Hematopoietic Stem and Progenitor Cells after Bone Marrow Transplantation and In Vivo Drug Selection

Abstract

MGMT-P140K mediated selection has been shown to be one of the best in vivo selection strategies for the enrichment of HSC and the enhancement of HSC engraftment. Our previous data showed that low MOI lentiviral transduction by a MGMT-P140K/luc transgenes in murine bone marrow cells resulted in long-term integration of transgene in transduced bone marrow cells, and in vivo selection after exposure to chemotherapeutic drugs enriched for cells expressing MGMT-P140K, resulting in persistent BLI foci of cells expressing luciferase. However, the clonal identities of cells recovered from peripheral blood compared to hematopoietic cells in persistent foci have not been established. More importantly, the impact of drug treatment on clonal expansion of hematopoietic cells in peripheral blood and from persistent foci was unknown. To address those questions, we studied the clonality of peripheral blood samples during drug selections in transplanted mice with LAM-PCR. In addition, by using newly modified Case Cryo-imaging system, we were able to retrieve transgene positive samples from whole animal and analyze their clonalities. In our analysis, samples from cryo-imaging and peripheral blood showed different clonal patterns by LAM-PCR. In vivo drug selection reduced clonal diversity in peripheral blood to an oligo-clonal pattern and indicated a selection pressure in favor of a subpopulation of MGMT-P140K expressing transplanted hematopoietic cells.
6.1 Introduction

Integrating gene transfer vectors, such as gamma-retrovirus and lentivirus, have been extensively studied for their integration preferences in recent years [68, 88]. After bone marrow transplant, gamma-retroviral or lentiviral transduced hematopoietic stem cells has been shown to be polyclonal in canine and non-human primates [289, 290]. However, in the in vivo drug selection studies, the impact of drug treatment on the clonalities of lentiviral transduced hematopoietic bone marrow cells was not well understood.

We have performed in vivo selection studies with low MOI lentiviral transduction as described in detail in Chapter 4. By using highly sensitive bioluminescence imaging, we were able to observe a dynamic and stochastic homing and engraftment. After treating mice with several rounds of BG + TMZ, we saw a dramatic increase in transgene expression, especially in spleen and marrow spaces within vertebrae. For the first time, we also observed that drug selection resulted in persistent BLI foci, which could not be detected by peripheral blood analysis. However, since bioluminescence imaging is only able to generate 2D images, it was almost impossible to pinpoint and retrieve those foci outside of known hematopoietic organs and the process of doing so without disrupting the tissue localization. In order to locate and study the persistent foci in detail, we had to find an alternative way of retrieving cells. Then, by analyzing the clonality, we assigned ancestor cell lineage and studied their response to drug selection in comparison to that of peripheral blood samples.

For the past few years, Dr. David Wilson’s group has developed a Case cryo-imaging system, which consists a cryomicrotome, a stereomicroscope imaging system, a robotic
xyz positioner, and the computer control system. It is able to acquire and combine tiled images with up to single-cell resolution [283]. With the availability of this imaging system, we were able to further characterize those persistent foci discovered by \textit{in vivo} selection and described in chapter 4. In the experiments described in this chapter, we analyzed the clonal aspects of gene-modified peripheral blood cells and gene-modified hematopoietic cells collected from BLI foci to study the impact of \textit{in vivo} drug selection on their patterns. We hypothesize that drug treatment would select for a subpopulation of MGMT-P140K expressing cells in BLI foci and their progenies could be also reflected in peripheral blood samples. From the results of those experiments, we were able to show that drug treatment decreased clonal diversity in samples from peripheral blood to a greater extent than those from untreated recipients. However, we discovered that hematopoietic clones from BLI foci were more likely to be oligoclonal and, even after drug selection, were more distinct from clones of hematopoietic cells present in peripheral blood.

\textbf{6.2 Results}

6.2.1 Tricistronic lentiviral vector showed robust transgene expression in cell lines

As described in Chapter 5.2.1, we have generated a tricistronic lentiviral vector, containing MGMT-P140K, eGFP, and firefly luciferase genes linked by two FMDV 2A sequences in MND SIN vector backbone. To see if all three transgenes were able to express successfully, tricistronic viruses were used to transduce 293T and K562 cells, as well as primary murine bone marrow cells. The data showed that the tricistronic vector was able to successfully transduce primary murine bone marrow cells and maintain
MGMT-P140K and eGFP transgene expression in 293T and K562 cells for up to 7 weeks (Fig. 22-23).

6.2.2 Experimental design

In order to analyze the impact of drug selection on clonality of hematopoietic cells in peripheral blood samples, peripheral blood was collected 14 days after transplantation and collected 1 day before and 3 days after drug treatments (Fig. 31). Clonality was analyzed by LAM-PCR [291, 292]. BLI images were taken every week and also the same day immediate before the collections of peripheral blood. After BLI imaging, recipient mice were sacrificed and quick-freeze in OCT compound with liquid nitrogen. Frozen animas were then prepared for cryo-imaging.
Figure 31. Schema of clonal analysis during *in vivo* drug selection.

Donor bone marrow cells were collected and transduced with LV-PAGAL at MOI of 1. Transduced cells were infused to lethally recipients, and every three weeks, treatment-group recipients received BG+BCNU treatment. Every week, recipients were imaged with bioluminescence. Some recipients were prepared for cryo-imaging after bioluminescence imaging.
6.2.3 Transgene expression enhanced by drug selection, resulting in persistent foci

Balb/C bone marrow cells were collected and transduced with LV-PAGAL at MOI of 1. 48 hour after transduction, $1 \times 10^6$ transduced bone marrow cells were transplanted into each lethally irradiated syngeneic recipient. Every 3 weeks, recipients were treated with 30 mg/kg of BG and 7 mg/kg of BCNU. First drug treatment usually did not result in evident increase in the transgene expression (Fig. 32), which was consistent with the observation we made in Chapter 4. Also in Figure 32, the mouse on top of the figure did not receive first drug treatment until day 42 after transplantation to see the transplantation time effect on the selection. After two or three rounds of BG + BCNU treatments, transgene expression, in terms of BLI signal, was greatly increased. The overall pattern of drug selection mediated transgene expression was very similar to the data of previous experiments (see Chapter 4). It indicated that BG+TMZ or BG+BCNU inserts a similar selection pressure to lentiviral transduced hematopoietic cells in vivo. After drug treatments, persistent BLI foci were observed in all BG+BCNU treated recipients, and they located in well-known hematopoietic organs, such as bone marrows, spleen, skull, marrow cavity of vertebrae, and chest area (Fig. 32). These results showed that long-term engraftment of tricistronic lentivirus transduced hematopoietic bone marrow cells were enhanced by BG and BCNU treatments.
Figure 32. Enhanced engraftment of LV-PAGAL transduced bone marrow cell with BG+BCNU treatments.

All three lethally irradiated recipients received 1x10^6 transduced bone marrow cells. Mice were imaged on both ventral and dorsal side. Red arrows indicated the relative time point of BG + BCNU treatments in this figure.

6.2.4 BG+BCNU treatments reduced clonal diversity in peripheral blood

Fourteen days after the transplantation of lentiviral transduced bone marrow cells, peripheral blood samples from all recipients were collected by retro-orbital bleeding. Transgene PCR and LAM-PCR were performed on bulk peripheral blood cells. Based on the methodology (Fig. 33a), each band generated by LAM-PCR theoretically represents an insertion site in cellular genome in correlation to one “internal control” band,
representing a PCR product generated from sequences within the provirus itself (Fig. 33b). The internal control band indicated the presence of lentiviral vector.

Figure 33. The method of LAM-PCR.

Linear amplification mediated-PCR. (a) Method of LAM-PCR (b) Generation of internal control after first and second nested PCR with primer sets of LV-LTR2 with LC1 and LV-LTR3 with LC2, respectively.
The average number of insertion sites based on the number of bands measured in peripheral blood samples from day 14 was between 7-9 (Fig. 34b). This may due to the high number of transduced short-term hematopoietic cells in the blood after transplantation, and it’s consistent with early time points of BLI images, showing high level of transgene expression (Fig. 32). Interestingly, in both unselected and drug selected groups, the LAM-PCR analysis in mice observed after 59 days appeared to show stable clonal patterns (Fig. 34a), hinting of a stochastic stem cell engraftment process. This is consistent with the observation that initially, during the first 6-8 weeks, different clones of hematopoietic stem and progenitor cells participate in hematopoiesis until homeostasis is reached, and some early repopulating progenitor cells and short-term repopulating stem cells are lost. This shift in LAM-PCR detectable bands overtime as, for instance, in untreated mice indicated a net lost of bands that had initially been identified, suggesting that the cells harboring these proviral integrations had been lost through terminal differentiation and exhaustion after the early phase of transplantation. Only when drug treatment was given, did we observe a further decline in number of clones in peripheral blood with the added selection in favor of the MGMT-expressing sub-population and the further expansion and exhaustion of additional stem and progenitor cells (Fig. 34b). However, there were usually multiple bands still present in most peripheral blood samples, indicating the persistence of a small subpopulation of oligoclonal HSCs selected after drug treatment. A further indication that blood contained an oligoclonal population when we compared the blood sample results with the LAM-PCR analysis of CFU samples taken before transplantation. LAM-PCR of those pre-transplantation stem and progenitor cells in CFU showed only 1-2 insertion sites.
Figure 34. Clonal diversity in peripheral blood reduced with drug treatment.

Peripheral blood samples from untreated and BG+BCNU treated groups were performed LAM-PCR at different time points. (a) LAM-PCR bands before and after drug treatments. (b) Number of transgene insertion from peripheral blood samples decreased with time but toward an oligoclonal pattern. (M: 1kb plus ladder, red arrows indicated drug treatments, and IC was the internal control)
6.2.5 Cells from cryo-imaging and peripheral blood sample were different in clonality.

In the initial cryo-imaging studies, we used higher magnification (50x) to survey small regions of bone marrows (legs, vertebrate, and chest), and we were able to observe GFP positivity in various specific sites, example shown here in the tip of bone marrow (Fig. 35a). On day 59 after transplantation, about 2 weeks after the 2nd drug treatment, one recipient mouse was sacrificed for cryo-imaging. In order to increase the field of view, only 32X magnification was used to image. We collected 11 samples from the abdominal regions, three of which are shown in Figure 35b. Anatomically, GFP positive “sample #1” appeared to be in a liver sinusoid or duct. “Sample #2 & #3” were recovered between the intestines (Fig. 35b). Further analysis with LAM-PCR showed that each sample contained at least a transgene, and they appeared to either have multiple integrations in one clone or to be oligoclonal (Fig. 35c). Sequence analysis of sample #1 and sample #3 revealed one common insertion site, indicating that cells in these two locations had a possible mutual ancestor (Table 5). However, the sequenced insertion sites from those cryo-imaging samples were not found in any of the peripheral blood samples from this mouse collected on either day 14 or day 37 (Fig. 35d). This was consistent with the fact that we did not detect BLI foci with peripheral blood analysis in experiments described in Chapter 4, perhaps because the level of contribution to the blood cell pool was less than the molecular limit of detection by LAM-PCR. The overall number of insertion sites recovered from the cryo-imaging samples was higher than peripheral blood at the same time. This may indicate that transplanted bone marrow cells contributed to those cryo samples did not contribute to hematopoiesis right after bone marrow transplant.
Figure 35.
Figure 35. LAM-PCR of GFP positive cells showed different clonalities as in peripheral blood sample.

Recipient mouse sacrificed day 59 after bone marrow transplant. (a) GFP positive cells found in tip of the bone. (b) GFP positive cells in liver and abdomen. 11 samples were collected for PCR analysis and 3 samples were shown here. (c) Left panel, lane 1-3 represented GFP positive samples #1-3; right panel, LAM-PCR of peripheral blood samples collected on day 14 and day 37.

<table>
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</table>

Table 5. Analysis of integration locus of cryo samples and peripheral blood samples.

All of the cryo samples and peripheral blood samples were collected from the same mouse. LAM-PCR bands were gel extracted and sent for sequencing. Sequencing results were searched with UCSB Mouse BLAT database. Scores lower than 30 were eliminated. (N/A: no RefSeq found in insertion site; band numbers are for white stars next to the band, from top to bottom).
6.3 Discussion

Due to the concern for insertional mutagenesis, low MOI retroviral and lentiviral transductions have been tested in animal models. Effective engraftment of viral transduced hematopoietic stem cells under MGMT-P140K mediated *in vivo* selection has been shown in several studies [266, 267]. In our imaging study, after MGMT-P140K mediated selection, we were able to show persistent BLI foci, which were not easily detected in peripheral blood samples. In order to investigate this further, in this study, we combined bioluminescence imaging with Case Cryo-imaging to retrieve transgene positive foci after two rounds of drug treatment. We performed clonal analysis on peripheral blood samples at different time points after transplant and samples from cryo-imaging, and we also performed clonal analysis on initially transduced stem and progenitor cells in CFU, showing a low (1-3 band) integration pattern.

BLI imaging of selection with BG and BCNU in this study showed great similarity comparing to selection with BG and TMZ. Both kinds of treatments showed dynamic selection patterns with weak or no increase in transgene expression after 1st round of drug treatment. But subsequent treatments resulted in enhanced transgene expression and persistent BLI foci. Our data also showed a polyclonal nature of hematopoietic bone marrow cells in peripheral blood at early days after bone marrow transplant. Only after 8 weeks, clonal diversity was greatly reduced in both drug treated and untreated groups. These data are consistent with the observation that short-term repopulating cells and progenitor cells contribute mostly to early hematopoiesis after bone marrow transplant [284, 293, 294]. During late stage of hematopoiesis after bone marrow transplant, long-term hematopoietic stem cells are actively contributing to hematopoiesis. Abkowitz et al.
estimated that murine HSCs replicate about every 17 days according to her stochastic modeling on the basis of observations of the behavior of derivative cells.

Our cryo-imaging data showed that we could successfully retrieve transgene positive samples in animal cross-sections. In a number of samples GFP positive areas observed in vasculature in liver and abdomen and were found to have LAM-PCR-detectable transgenes. In addition, two of those samples contained cells with the same integration site, suggesting that they were derived from the same progenitor and stem cell. It has been described that HSCs are maintained in vascular niches near sinusoids [295]. Others have shown that bone marrow donor cells can home to vascular injury and engraft, and bone marrow progenitor cells can differentiate to endothelial cells and/or smooth muscle under specific promoters [296-298]. Our data supports these possibilities, showing hematopoietic stem and progenitor cells give rise to progenies reside in the liver sinusoid. However, LAM-PCR data showed that those foci in liver sinusoid and abdomen did not contribute greatly to hematopoiesis but responded to proliferative stress, such as drug selection pressure.

Another important fact was that the oligoclonal nature of the samples from both cryo-imaging and peripheral blood indicated that, in our studies, there was low frequency of proliferative clonal dominance with low MOI lentiviral transduction and under proliferative duress.
6.4 Materials and Methods

6.4.1 Vector and lentivirus production

Two bicistronic lentiviral plasmids were used to generate this triple vector. These two plasmids were pLV-mnd-P140K-2A-GFP, generated by Dr. Justin Roth, and pLV-mnd-GFP-2A-luc. The generation of pLV-mnd-GFP-2A-luc was similar to the method described in Chapter 4, and the method was described again in Chapter 5. The final virus was named LV-PAGAL.

6.4.2 Mice

Female Balb/C mice (Charles River, Wilmington, MA) were used as donors and recipients in all imaging studies. Mice were housed in pathogen-free Animal Resource Center according to approved animal protocol. For bone marrow transplant, all recipient mice received lethal dose of 750 cGy with a Cs137 source within 1-2 hours before infusion. Special water with Bacitricin/Neomycin (Sigma-Aldrich, St. Louis, MO) was given to irradiated animals. Before cryo-imaging, mice were sacrified by cervical dislocation according to ARC protocol.

6.4.3 Lentiviral gene transfer of murine bone marrow cells

Bone marrow cells from 6-8 week old female Balb/C mice were collected by flushing femurs, tibia, and hip bones with $\alpha$-MEM (Mediatech, Manassas, VA) containing 10% Heparin (Baxter Healthcare Corp., IL). Cells were washed in fresh $\alpha$-MEM and counted with a hemacytometer after mixed with 4%(v/v) acetic acid. Based on viral titer and MOI of 1, appropriate amount of whole bone marrow cells were incubated in lentiviral supernatant with the same supplements described in Chapter 4. After 48-hour transduction, cells were washed and re-suspended in fresh $\alpha$-MEM prior to transplant.
Transduction efficiency was measured by testing CFU colonies for the presence of transgene by PCR.

6.4.4 Transplantation and drug treatments

Transduced bone marrow cells were washed and re-suspended in α-MEM. 1x10⁶ bone marrow cells were transplanted into lethally irradiated recipients. Every three to four weeks after transplantation, recipient mice received one treatment of 30 mg/kg of O⁶-benzylguanine (BG) and 7 mg/kg of 1.3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BG was dissolved in 40% PEG (polyethylene glycol) and diluted in 60% pH 8.0 PBS, and BCNU was dissolved in 10% EtOH and 90% PBS. BCNU was administrated one hour after BG treatment.

6.4.5 Bioluminescence imaging of bone marrow cells in vivo

Bioluminescence imaging was performed on Xenogen IVIS 200 system (Caliper LifeSciences, Alameda, CA) at Case Western Reserve University Small Animal Imaging Center. A diluted anesthesia mix (Ketamine HCl, Xylazine HCl, and Acepromazine), prepared by Case Animal Resource Center, was injected 5-7 minutes intra-peritoneally prior to another IP injection of 125 mg/kg of D-Luciferin (Biosynth AG, Switzerland) into each mouse, and the animals were taped down on black photographic paper. The imaging parameters were maintained throughout the experiments. The exposure time for each image was 5 minutes, and first image was taken 7 minutes after injecting D-luciferin.

6.4.6 Cryo-imaging preparation and operation

After bioluminescence imaging and before waking up from anesthesia, mice were sacrificed by cervical dislocation. A tissue-histology compound, optimal cutting
temperature (OCT) (Tissue-Tek, Terrance, CA), was thoroughly applied to the skin and fur of the animals to minimize air bubbles. Each animal was then put in a small container made of aluminum foil and completely covered with OCT. Liquid nitrogen was used to quick freeze the animal embedded block. Since the cryo-microtome was operated in -20°C freezer, after removing foil container outside, additional OCT was used the bottom to attach the block to the cutting platform. During fluorescence image acquisition, we paused the imaging process to collect GFP positive samples with syringe needles under fluorescence microscope.

6.4.7 LAM-PCR and Sequencing

GFP positive samples were washed once with 1 ml PBS, and digested with 25 µl of protease K solution at 50°C overnight. Genomic DNA was extracted from peripheral blood with Zymo DNA kit. Samples were heat-inactivated at 95°C for 10 minutes and spun down. LAM-PCR was performed according to published protocol. Linear amplification was done with 5’-biotinylated primer, LV-LTR1 (5’-(biotin)-GAGCTCTCTGGCTAACTAGG-3’). Dynabeads M-280 Strepavidin magnetic beads (Invitrogen, Carlsbad, CA) were used in the following steps to wash and pull-down PCR products. Random priming was performed to convert linear product to double stranded DNA fragments with Klenow polymerase and hexanucleotide mix (Roche, IN) at 37°C for 1 hour. Enzyme digestion was done with Tsp509I (New England Biolabs, MA) at 65°C for 1 hour. Digested DNA fragments were ligated to a linker cassette (5’-GACCCGGGAGATCTGAATTCAGTGGCACAGCAGTTAGG-3’) with a complementary overhang. Then two nested PCR reactions with performed with two sets of primers. First nested PCR used LV-LTR2 (5’-GACCCGGGAGATCTGAATTC-3’).
and LC-1 (5’-GACCCGGAGATCTGAATTC-3’), and the second nested PCR used LV- LTR3 (5’-AGTAGTGTGTGCCCCTCCTGT-3’) and LC2 (5’-GATCTGAATTCAGTGGCACAG-3’). The products of the second nested PCR were run on 2% agarose gels. PCR bands were then excised and purified with QIAquick gel extraction kit (QIAGEN, CA) and sent for sequencing. Sequencing results were searched with BLAT for matching mouse genome.
Chapter 7
Discussion and Future Directions

7.1 Gene Therapy

Just a decade ago, gene therapy caused serious concerns from both scientific community and the public with a patient’s death caused by immune response to adenoviral vectors and childhood leukemia associated with gamma-retroviral vector [23, 24, 299]. Over the years, no additional complication has been reported while vast amount of studies have been done to improve gene transfer vectors.

In the past two to three years, the promise of gene therapy has been reignited. With the launch of a clinical trial to treat blindness in 2007, gene therapy once again showed great potential. In addition, gene therapy trials have successfully treated some severe genetic diseases with no apparent side effects [300, 301]. Late last year, Mancuso et al. successfully used adeno-associated virus, containing human L-opsin gene, to treat red-green color blindness in primates [302]. In just a month later, another landmark paper was published in Science [303]. In this study, two X-linked adrenoleukodystrophy (ALD) patients received autologous CD34+ cells transduced by a lentiviral vector, containing a wild-type \( ABCD1 \) gene. Within 24-30 months, patients had 9-14\% of their blood cells expressing ALD protein.

Despite all of those successes, improvement in gene transfer vectors and extending gene therapy treatments to other diseases, such as various types of cancer, are still the main goals of most researchers in the field. In another recent study in spotlight, gene therapy has been actively pursued in professional sports. There will be more hurdles and
unforeseen consequences in the field of gene therapy. But it will definitely be a big part of future medicine.

7.2 Gene Transfer Vectors

There are four crucial criteria for a good gene transfer vector: 1. Be able to stably integration into target-cell genomes. 2. Do not active host immune response against the vector or transgene. 3. Achieves efficient expression of transgene. 4. Do not cause unwanted side effects, such as uncontrollable proliferation of gene-modified cells or targeting and killing normal host cells. In addition, a good gene transfer vector should also allow carrying a large payload without sacrificing the efficiencies of transduction and expression. Additional control mechanisms in the vector construct would also be a good modification for a safer transfer vector, which allows a toxic transgene to be safely turned off when it is no longer needed, such as when treating cancers or infectious diseases.

Lack of evidence of insertional mutagenesis by lentiviral vectors and the ability of lentiviral vector to infect non-dividing cells have helped tremendously the approval of applications for clinical trials using lentiviral vectors. The use of lentiviral vector in clinics could surpass retroviral vector in the near future. However, in preclinical studies, especially in murine model, transgene expression by lentiviral transduction has been disappointing compared to that by retroviral vectors. This could due to a native restriction mechanism in murine cells against lentiviral vector. Since murine models are important in developing the rationale for clinical studies, it remains a common observation that lentiviral vectors are being used in murine studies with limited gene
expression. Future lentiviral studies may provide the insight for this intrinsic restriction against the vector in murine cells. Removing the fragment from lentiviral vector may increase transduction and expression efficiency in murine studies, and also possibly in human cells.

An alternative to lentiviral vector is the transposon system. The transposon systems have been developed in the past few years. Two most promising systems are the Sleeping Beauty (SB) transposon system or piggyBag transposon system, which can stably integrate therapeutic gene into target cells by transposition [304-306]. They have also been used to generate iPS cells [307, 308]. However, their transfer rates into primary cells have been low. Mátés et al. have developed a novel hyperactive Sleeping Beauty transposase, which could be 100X more efficiency and resulted in 35-50% stable gene transfer in CD34+ hematopoietic stem cells [309]. Even though transposon system is easier to control and safer than viral vector, but the integration efficiency is still low.

7.3 In Vivo Clonal Selection of Hematopoietic Stem Cells

With the invention of the iPS cells, researchers have been trying to differentiate them to adult stem cells. Hematopoietic stem cell is on top of the list, and in recent years, a number of advances have been made to elucidate the mechanisms controlling hematopoietic stem cell self-renewal and differentiation with the goal of generating hematopoietic stem cells from iPS cells. Recently, Grigoriadis et al. reported direct differentiation of human ES and iPS cells into hematopoietic precursors and functional osteoclasts [310]. In another study, Bertrand et al. showed that they generated HSCs
from aortic endothelium in zebrafish. The authors have suggested that murine HSCs could also potentially be derived from endothelia cells during development [311].

Successful gene transfer into hematopoietic stem cells requires the understanding of the underline mechanisms controlling HSC self-renewal and differentiation. A better understanding of HSC biology can lead us to better-optimize HSC culture conditions. Nevertheless, heterogeneous population of cells from lentiviral or retroviral gene transfer is still the predominant result. In vivo drug selection remains an important modality in the field of gene transfer. It has been a safe and effective way of the enrichment of HSCs expressing therapeutic gene. In term of stem cell selection, MGMT-P140K mediated selection has been shown to be the most effective. However, in clinical settings, MGMT-P140K gene transfer to primary human CD34+ cells by retroviruses or lentiviruses has been difficult because of the sheer number of transplanted cells required. In order to get a long-lasting gene transfer and more effective in vivo selection, it may be important to better define the target of long-term repopulating hematopoietic stem cells so that these cells can be specifically transduced, leading to more effective reconstitution and selection.

In addition, clonal analysis of lentiviral transduced HSC is a piece of crucial information for clinical trials. It is a safety measurement to monitor the possible onset of insertional mutagenesis. In murine studies, from clonal analysis, we can interpret the biology of hematopoietic bone marrow cells after transplant and their response to drug treatments. Harkey et al. has proposed to use a modified LAM-PCR to optimize clonal analysis [312]. With advancement of current sequencing technology, we can even use DNA barcode to label each samples and send for 454 sequencing for every insertion site.
present, which will give us a complete view of every clone after transplant. There has been another modification of LAM-PCR specifically for 454 sequencing due to the bias of enzyme digestion during the process of LAM-PCR [291].

7.4 Small animal imaging in biomedical research

Before bioluminescence imaging was used for optical small animal imaging, PET, CT, and MRI have been used to image small animals in biomedical researches. PET requires the use of radionuclides, and all three imaging modalities are expensive. Today, optical imaging has rapidly become one of the more preferred imaging modalities. The early bioluminescence imaging studies were used to measure ATP level in tissue samples [313, 314], now BLI is used for monitoring disease progression, cell tracking, protein-protein interaction, metastasis, viral infection, drug treatment, etc. It is non-invasive and does not require the sacrifice of the animals, and the researchers can monitor the biological process in the same animal for a longer period of time. However, optical imaging has its limitations. For examples, photons emitted from the luciferase reaction have poor tissue penetration, scattering and absorption by tissues, and there is only 2D image with low resolution. Several researchers have tried to shift the luciferase to red spectrum, which has a better penetration in tissue [315]. Microscopic imaging can be used for in vivo imaging of bone marrow cells, but it is restricted to a small section of the body at a time.

To increase the field of view and imaging resolution, Case Department of Biomedical Engineering has made its own advance cryo-imaging system. It can image a whole mouse with a histological resolution, and reconstruct a 3D model of that mouse. In addition, it can also take fluorescence images side by side with bright-field images. It is
ideal for tracking labeled stem cells or cancer cells. However, this imaging system
requires the sacrifice of the research animals, which sharply increases the number of
animals in each experiment.

There are many applications for which we can use cryo-imaging system: studying the
process of hematopoietic stem cell transplant, tracking labeled mesenchymal stem cells,
tracking metastatic cancer cells, etc. With the newly installed fluorescent filter system,
we can image different cell populations with different fluorescence labels. This could be
very useful to study competitive repopulation assays or analyze subpopulation of HSCs.

Overall, no matter which imaging modality to be used, knowing the limitations of
each imaging modality and asking the right questions are the key to new discovery.

7.5 What we have done and what’s lying ahead

During the course of these studies described in this dissertation, we have focused on
utilizing cutting edge in vivo imaging modalities to study lentiviral gene transfer in
murine bone marrow stem and progenitor cells. The goals of our studies are to
understand the basic biology of hematopoietic stem and progenitor cells after bone
marrow transplantation and to improve gene transfer method used in clinical gene
therapy. In order to achieve an effective therapeutic result from lentiviral gene transfer,
our lab has been using in vivo drug selection strategy to enrich lentiviral transduced bone
marrow cells. We have made several discoveries from those studies.

By using bioluminescence imaging, we have observed the dynamic homing and
engraftment processes of bone marrow transplantation. We have discovered a dramatic
expansion and migration of short-term stem and progenitor cells, starting at 4-5 days after
transplant, and this early engraftment only lasted for 2-3 weeks. In addition, through LAM-PCR analysis, we found that during early stage of engraftment, there were a lot of proviral integration clones in peripheral blood. With time, those clones in peripheral blood disappeared and moved toward a more stable oligo-clonal pattern after 7-8 weeks. Those data from in vivo imaging and PCR analysis were consistent with the understanding of short-term repopulating cells in bone marrow transplantation setting.

In the field of gene therapy, we have clearly demonstrated by in vivo imaging that MGMT-P140K mediated selection enhanced the engraftment and expansion of lentiviral transduced bone marrow cells. However, the most important finding from these studies was the discovery of persistent BLI foci derived from lentiviral transduced bone marrow cells after in vivo drug selection. Those persistent foci were found in spleen, bone marrow, as well as liver sinusoid. However, they were hard to detect in peripheral blood. This has profound clinical implication because in clinical trials, if low transgene expression was detected in peripheral blood, no engraftment was usually assumed. From our imaging studies, cluster of transgene expressing cells derived from transduced bone marrow cells after in vivo selection could still present and expand in liver sinusoid or other locations. We can imagine that similar foci could be present in human patients as well. Then an interesting question can be asked – can those foci be activated and migrate to bone marrows to initiate hematopoiesis? Future studies can be designed to use cytokines, such as GM-CSF, trying to stimulate those foci. In combination with BLI and cryo-imaging, we also used LAM-PCR to study the impact of in vivo drug selection on the clonality of transduced bone marrow cells in peripheral blood and BLI foci. We discovered a further decrease in clonality in drug treated animals compared to untreated
animals. However, in drug treated animals, clonality of transgene expressing foci was still oligoclonal. These data was still preliminary, more sample collection and analysis are needed. Another future study would be to use 454 sequencing method to analyze peripheral blood samples and BLI foci to address the impact of drug selection.

The best part of doing scientific research is to further our knowledge in the understanding of our physical world and in improving the well-being of everyone. We hope that our discoveries can be useful in the pursuit of these goals.
## Appendix:

Lentiviral insertional sites sequenced in the studies. (N/A means no RefSeq found at insertional sites)

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