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Joo Hyun Lee April 10, 2015

# Infectious Leukemogenesis: Role of Lateral Gene Transfer in Bone Marrow to Create Clonal Diversity in Leukemia

**By**

**Joo Hyun Lee**

**Dr. Edmund Waller, M.D., Ph.D.**
**Adviser**

**Department of Biology**

**Dr. Edmund Waller, M.D., Ph.D.**

**Adviser**

**Gregg Orloff, Ph.D.**

**Committee Member**

**Victor Corces, Ph.D.**

**Committee Member**

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## Abstract

### Infectious Leukemogenesis: Role of Lateral Gene Transfer in Bone Marrow to Create Clonal Diversity in Leukemia

**By Joo Hyun Lee**

The uncontrolled proliferation of genetically mutated cells is a mechanism for cancer growth and invasion. The accumulation of spontaneous mutations in a malignancy contributes to clonal diversity of cancer derived from a single founding event. We hypothesized that lateral gene transfer between malignant cells and adjacent normal stem cells may provide an alternative mechanism for the accumulation of mutated genes and the multiplicity of distinct clones in leukemia cells. To test this hypothesis we injected C1498 into GFP transgenic murine recipients and cultured leukemia cells (C1498) grown with normal GFP+ (green fluorescent protein). We used *in vitro* and *in vivo* model systems to test whether the presence of C1498 induces a survival advantage to GFP+ bone marrow cells. We sorted GFP+ cells from C1498 bearing mice or co-cultures of C1498 with GFP+ bone marrow onto stroma and resorted for GFP+ progeny into secondary, tertiary, and quaternary cultures. Serial transplantation of sorted GFP+ cells from mice initially inoculated with C1498 resulted in an increase, from 1/140 to 1/47, in the precursor frequency of clonogenic GFP+ cells when cells were sorted on to stromal monolayers. The frequency of clonogenic cells progressively increased from 1/170 to 1/54 following co-culture of C4198 with bone marrow from GFP+ mice followed by 5 successive fluorescent activated cell sorting procedures to re-isolate GFP+ cells over a 2 month period. In contrast, parallel experiments sorting GFP+ cells from stromal cultures without C1498 or serial transplantation of GFP+ cells from mice without leukemia cells showed loss of clonogenic activity *in vitro* after the second sort. These data are consistent with a model in which growth-promoting or transforming genes from leukemia cells become incorporated within a healthy hematopoietic stem or progenitor cell, contributing to the genetic diversity of leukemia through the initiation a new leukemic clone. Genetic analysis must be done to compare the DNA sequences between the parental leukemia cell line, sorted populations of clonogenic GFP+ cells obtained from the *in vitro* and *in vivo* experiments and C57BL/6 mice to confirm the transformation of healthy bone marrow hematopoietic stem cells with genetic sequences derived from the leukemia cells.

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## Introduction

Cancer is generally thought to grow through uncontrolled proliferation of genetically transformed neoplastic cells. The cells undergo genetic alterations that can accelerate cell division or suppress normal cellular functions that control growth, such as programmed cell death. The genetic mutations arise *de novo* and are passed on to daughter cells. Clonal expansion of these daughter cells occurs through mitosis (vertical gene transfer), which involves division into two cells with identical sets of chromosomes (NCBI, 2010). Cancer cells can then metastasize from their origin to other tissues in the body. As additional mutations occur within the genome of a transformed cell and are passed on to its progeny, genetic heterogeneity of the cancer develops with multiple subclones of distinct sub-populations that share a common set of founder mutations.

An alternative mechanism to explain clonal diversity, the possibility of lateral gene transfer between a cancer cell and normal stem cell, challenges the standard model of cancer initiation and vertical gene transfer. The transfer of DNA sequences encoding oncogenes provides recipient normal cells with dynamic genomes, similar to the development of antibiotic resistance in bacteria (Groisman 2000). In addition, lateral gene transfer is often used to transfect organisms to induce selective advantages (Higgs 2011). The routine transfer of genes from one cell to another in laboratory settings such as the creation of transgenic mice and transformation of mouse fibroblast by carcinoma transforming genes raises the questions of whether lateral gene transfer occurs spontaneously between normal stem cells and cancer cells (Nishimune 1997) (Chiago 1981).

In leukemic cell lines, lateral gene transmission and the dynamic genome are predicted to yield a heterogeneous cell population (Yuan 2010). This rapid alteration of genome is more often found in high-risk patients, with heterogeneous clonal mixture consisting of multiple dominant clones being one of the three causes (Keats 2012). Among these subclones in leukemia, the genome is nonlinear and comes from multiple divergent lineages, indicating that it does not follow an evolutionary pattern (Anderson 2011). These independent alterations obtained by leukemic subclones in patients, leading to clonal heterogeneity in leukemia, indicates the transfer of leukemic-promoting genes from a malignant cell to a non-malignant stem cell.

The discovery of a murine malignant mesenchymal cell type after engraftment of human tumor cells in nude mice brought new light onto the possibility and importance of lateral gene transfer (Goldenberg 1981). The transmission of DNA from human cancer cells to murine mesenchymal cells resulted in the mesenchymal cells acquiring tumorigenic characteristics. The transformed and tumorigenic mesenchymal cells introduced an alternative mode of malignant transformation. Similar processes of lateral gene transfer have been described in the transformation of cells by oncogenic viruses and/or cell-cell fusion mechanisms. Even with these mechanisms to explain the development and proliferation of tumor cells, its ability to metastasize was uncertain. The introduction of cell fusion theory in 1911 provided a possible mechanism. White blood cells move around the body in response to chemotactic signals but have a limited life span. Cancer cells are limited in mobility but are immortal. Therefore, a hybrid, due to improper phagocytosis of a cancer cell, could display characteristics of both cells: high mobility and unlimited divisions (Aichel 1911) (Pawelek, 2000). Immunohistochemistry and genetic analysis of cells that

underwent heterospecific fusion reveals the genetic makeup of both tumor and host cells. Lateral gene transfer offers an explanation for the genetic diversity of cancer, even among the same cancer types, and how it is capable of overriding the host immunity (Goldenberg 2014).

Here, we investigated lateral gene transfer in bone marrow cells as a viable mechanism for creating a polyclonal population of cells transformed with genes from an acute myeloid leukemia cell line, also known as C1498 (Sauer 2004). The increase in clonogenic frequency of GFP+ progeny that was grown with leukemic cells supports the hypothesis that lateral transfer of oncogenic genes conferred a growth advantage upon healthy stem/progenitor cells present in normal bone marrow. The isolation of single clonogenic cells through serial fluorescent-activated cell sorting from co-cultures of bone marrow exposed to C1498 but not in culture of bone marrow without C1498 is indicative of a transmissible alteration in the DNA sequence of normal bone marrow cells. Confronting the standard model of cancer initiation and proliferation with lateral gene transfer can enhance current therapeutic approaches to cancer and improve early diagnostic tests in patients.

## Materials and Methods

### *Cell Lines and Mouse Strains*

The C1498 (luciferase and DSRed (TexasRed)) transduced murine acute myeloid leukemia cell line) was generous provided by Bruce Blazar. B6 green fluorescent protein (C57BL/6-Tg (CAG-EGFP)) mouse, referred to hereafter as B6 GFP+ mouse, was derived from the eGFP strain (Nishimune 1997). Stromal monolayers of mesenchymal stem cell

(MSC) were derived from harvested B6 bone marrow cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 37°C in 5% $CO_2$. Apart from the MSC stromal cultures, all other cells were cultured in RPMI 1640 media without phenol red.

C57BL/6 (WTB6) mice, 7-8 weeks old at study, were obtained from the Jackson Laboratory. FVB x B6 albino mice, 14-16 weeks old at study, were bred at Emory University from mice provided from Jackson.

***In vitro co-culture of C1498 with normal GFP+ bone marrow***

20 x $10^6$ bone marrow cells from B6 GFP+ mice were co-cultured at 37°C in 5% $CO_2$ with 1.6 x $10^6$ leukemia cell line (C1498) in 15ml of RPMI media on an nearly confluent irradiated (6Gy) MSC stromal layer in a T75 flask for four days. The MSC stromal layers were prepared four days prior to the start of the co-culture and irradiated to 6Gy with a cesium source (.77Gy per min) 2 hours in advance. After four days of co-culture, non-adherent cells were removed from the flask and stained with an antibody cocktail containing lymphoid markers (CD3, CD19, and NK1.1) and stem cell progenitor markers (c-kit, Sca-1, CD90) for phenotypic characterization and isolation of GFP+ cells by flow cytometry.

***Poisson analysis of clonogenic frequency of bone marrow cells***

GFP+ cells from cultured bone marrow or from GFP+ mice inoculated with C4198 were sorted to select for GFP+ cells using a fluorescence activated cell sorter (FACSAria, Beckton Dickinson) equipped with the FACSAria ACDU to deposit defined number of GFP+

cells in individual wells (2000, 1000, 500, 100, or 10 cells per well). The sorted cells were re-cultured into 96-well plates with nearly confluent irradiated MSC stromal layers. After four days of culture, individual wells were scored (using an EVOS® FL microscope) for the presence of cells that were expressing either GFP+ or dual GFP+ and DSRed+ (fluorescence of C1498) cells. The percentage of wells lacking GFP+ cells was used to calculate the clonogenic precursor frequency of GFP+ cells using the method of Poisson (Weisstein).

***PEG-mediated fusion of leukemia and normal bone marrow cells***

36 million bone marrow and 12million C1498 cells were mixed in RPMI media containing 10% FCS and centrifuged at 500g for 5 minutes. One ml of PEG solution re-warmed to 37°C was added to the cell pellet in a drop wise manner and the cell suspension incubated for 3 minutes. Then, an additional 5mL of warm PEG solution was added. The volume of the tube holding the cell was then doubled with RPMI media containing L-glutamine, 10% autologous plasma, and 1% gentamicin 10ug/ml. The cell suspension was re-centrifuged as above and the cell pellet washed twice using the RPMI media to remove remaining PEG solution. These cells were cultured in an irradiated MSC layer flask for one week, while refreshing the media on the fourth day. Afterwards, these cells were observed by EVOS®FL microscopy for indications of fusion through dual GFP+/DSRed+ cells.

***Monitoring growth of luciferase+ cells in vivo by bio-luminesence***

Bioluminescence imaging (BLI) using Xenogen IVIS® Spectrum (bioluminescence detection of luciferase activity) of WTB6 injected with C1498 (control group)were obtained

following subcutaneous injection of luciferin and anesthetized with isoflurane. The fur on the ventral side of the WTB6 mice was removed with clippers prior to imaging.

***Bone marrow transplantation***

Bone marrow was harvested from the tibia and femurs of B6 GFP+ mice with C1498 injections and from B6 GFP+ control groups. The bone marrow was then filtered to remove cell clumps and debris. Recipient mice were transplanted one day following irradiation to 5.5Gy or 11Gy with a cesium source (WTB6 recipients received 0.77 Gy per minute and FVBxB6 albino recipients received 0.77Gy per minute twice with 3 hours apart, respectively) or without prior irradiation.

## Results

### Evidence for *in vivo* lateral gene transfer

Figure 1 represents the schematic diagram of the *in vivo* experiment. The B6 GFP+ mice were each intravenously injected with 2 x $10^5$ leukemia cells (C1498-Luc). Then bone marrow from B6 GFP+ mice injected with C1498 was harvested after 2 weeks. Leukemic growth was confirmed in the WTB6 (control group) by BLI shown in figure 2. Following inoculation with leukemia, 5 x $10^6$ sorted GFP+ cells from leukemia-bearing mice were injected into non-irradiated WTB6 mice and irradiated WTB6 mice or irradiated FVB x B6 albino mice ($2^{nd}$/$3^{rd}$ recipients). In parallel, 5 x $10^6$ unsorted GFP+ cells from C1498-free mice were injected into non-irradiated WTB6 mice and/or irradiated WTB6 mice or irradiated FVB x B6 albino mice ($2^{nd}$/ $3^{rd}$ recipients). Regardless of whether irradiated recipients were or were not inoculated with leukemia had a mixture of bone marrow

harvested from mice that received GFP+ cells showed that irradiated 2nd and 3rd recipients had a mixture of GFP+/DSRed- cells or GFP-/DSRed- cells (figure 3, 4, 5A). Notably, figure 5 indicates that bone marrow of irradiated WTB6 that were 2nd recipients of GFP+ marrow cells from mice original injected with C1498 had more GFP+ cells (86.6% of total cell count) than bone marrow of irradiated WTB6 2nd recipients with no C1498 injection (67.7% of total cell count). Unsurprisingly, the analysis of bone marrow and spleen from all non-irradiated recipients had few GFP+ cells in the GFP+ sorting gate. This implies a failed engraftment of transplanted bone marrow due to a lack of hematopoietic niche, or the ability of non GFP+ cells in the host to outgrow the transformed GFP+ cells..

To further test the provocative results from the *in vivo* experiment, FVB x B6 albino GFP+ bone marrow from mice (2nd recipients) with and without inoculation of C1498 were observed under the EVOS® FL microscopy (figure 5B). Although not visible on figure 5B, some of the bone marrow in FVB x B6 albino GFP+ mice (with C1498 injection) expressed GFP fluorescence as bright as those of FVB x B6 albino GFP+ bone marrow (without C1498 injection), though not as abundant. Figure 5C demonstrates the sort gate for GFP+ bone marrow with stem cell markers using FACSARia ACDU onto irradiated MSC 96-well plates (10 or 1 cell per well). Clonogenic precursor frequencies were determined through a Poisson distribution analysis (figure 6). The clonogenic frequency of stem cells for both cell types increased, but a greater escalation for B6 GFP+ bone marrow stem cells (with C1498 injections) was shown.

**Evidence for emergence of clonogenic cells with tumorigenic properties *in vitro***

The sorts for GFP+ progeny cells in 96-well plates were performed to generate single cell cultures that are GFP+ with growth advantage induced by lateral gene transfer from the leukemia cell line. To calculate the precursor frequency of clonogenic cells, plates with either 2000, 1000, 500, 100 or 10 cells per well were observed by EVOS® FL microscopy for presence of GFP+ cells. A positive well indicated the proliferation of GFP+ cells, while a negative well represented no GFP+ cells. The presence of DSRed+ cells was taken into consideration only when it was dual GFP+ and DSRed+ for this calculation, since single DSRed+ cells may represent C1498 cells rather than an occurrence lateral gene transfer. The percentage of negative wells was calculated and a Poisson distribution was created per day of observation. The frequency was calculated by finding $x$ in $Y = Ae^{-bx}$. The Y indicates frequency of coming up empty handed when it is expected to sort 1 cell. The Poisson distribution provides the values of *A* and *b.* In figure 9, the serial cultures of sorted GFP+ cells that were co-cultured with C1498 showed increase frequency of clonogenic cells. By day 57, the precursor frequency of clonogenic GFP+ cells was about 1 cell per 54 cells sorted. The increase in precursor frequency during serial resorts and *in* vitro culture indicates promising results for isolating a single clonogenic GFP+ cell after additional sorts. The decline in precursory frequency and its ultimate death of the GFP+ BM, grown individually in culture, by day 15 (figure 8) supports the idea that without a successful leukemic gene transfer that provides growth advantage, normal bone marrow would have difficulty proliferating *in vitro.*

## Comparison of PEG fusion with *in vitro* co-cultured GFP+ bone marrow

To assess lateral gene transfer *in vitro*, 96-well plates with GFP+ progeny and PEG-fusion hybrid cells were observed by EVOS®FL microscopy. Fluorescent images showed brighter GFP+ and DSRed+ expression in more cells on day 57 than those taken on day 46 of GFP+ bone marrow previously co-cultured with C1498 (figure8). In addition, the brightest GFP+ and DSRed+ expressions suggest a hybrid cell due to the slight yellow fluorescence given off in the overlay, as displayed similarly on the PEG-fusion image. The bright yellow fluorescence given off by a cell in the PEG-fusion image indicates the success and supports the possibility of forming a hybrid cell. This gives significance to the phenotypic similarity of a PEG-fusion hybrid cell and GFP+ sorted bone marrow once co-cultured with C1498 as a representation of a successful lateral transfer of leukemic-promoting genes from the C1498 cell line to the GFP+ marrow cells.

**Discussion**

The development of an oncogene can alter the mechanisms within a cell. One consequence confers the cell with a growth advantage (Croce 2003). An increase in clonogenic frequency was observed after normal bone marrow from GFP+ mice was exposed to C1498 cells in both the *in vitro* and *in vivo* experiments. These data support the hypothesis that lateral gene transfer may introduce oncogenic genes derived from leukemia cells into normal stem/progenitor cells in the bone marrow conferring upon them enhanced capacity for proliferation and survival. However, lateral gene transfer between a bone marrow cell and C1498 is highly complex (Papke 2012); therefore, the accumulation of genetic alterations that define characteristics of cancer is not dependent on a single explanation.

To continue these studies, GFP+ and GFP+/DSRed+ cells were frozen in liquid nitrogen from various time points during the *in* vivo and *in vitro* experiments for subsequent genetic analysis to test for the incorporation of possible new genetic material. These cells can be screened for SNP analysis. Lateral gene transfer can be confirmed by analyzing the DNA from frozen samples to identify concordance of specific mutations between C1498 and the putative transformed GFP+ cells. Previously identified genetic mutations within a clonal population of leukemia, such as FLT3, NPM1, IDH1[1], and IDH1[2], can facilitate the comparison process. Mutations of FLT3 and NPM1 are often indicative of a complex clonal diversity within a clonal population (Paguirigan 2015). IDH1[1]and IDH1[2] mutations are present as drivers in 8% of myeloid neoplasms with increasing frequency dependent on the stage of the disease (Molenaar 2015). Consequently, mutations in IDH1[1] may associate with mutations of the NPM1 gene. Thus, mutated myeloid neoplasm driver genes could be laterally transferred to serve an initiating role in accumulation of genetic alterations within a non-malignant cell. If these mutations or other related genetic alterations were identified in the frozen samples, it would provide positive evidence for the transfer of genes. Different concentration of leukemia cells and GFP+ bone marrow mixtures, not co-cultures, should also be analyzed to accurately correlate mutated genes within a sample to lateral gene transfer, not an imprecise or contaminated sort on GFP+ gate. Analysis of the cells from the polyethylene glycol (PEG)- mediated cell fusion can serve as a positive control and provide an alternative to understanding the outcome of a hybrid cell.

Furthermore, cells from peripheral blood and spleen of future recipient mice should be analyzed to provide accurate characteristics of a naturally transformed bone marrow

cell (Miller 2014). In addition, high percentage of acute myeloid leukemia subclones migrates to the peripheral blood (Klco 2014); consequently, the isolation of a GFP+ bone marrow with leukemic phenotypes within the peripheral blood can strengthen the occurrence of lateral gene transfer.

Proliferation of cells is initiated by an accumulation of genetic mutations, which causes uncontrolled division of cells, a common characteristic of cancer (Bishop 1991). However, there is a heterogeneous cell population among the leukemia cells. We propose that the accumulation and heterogeneity of genetic mutations within cells may be, in part, due to transformation of healthy cells into malignant cells through lateral gene transfer. Challenging the current understanding of cancer can develop novel treatments to leukemia and control its complex, heterogeneous cell population.

**Acknowledgments**

I would like to acknowledge Dr. Edmund Waller and Dr. Cindy Giver for providing me with training and guidance throughout this project. I would also like to acknowledge Dr. Gregg Orloff, Dr. Victor Corces, and Mr. Wayne Harris for their input and assistance in this project.

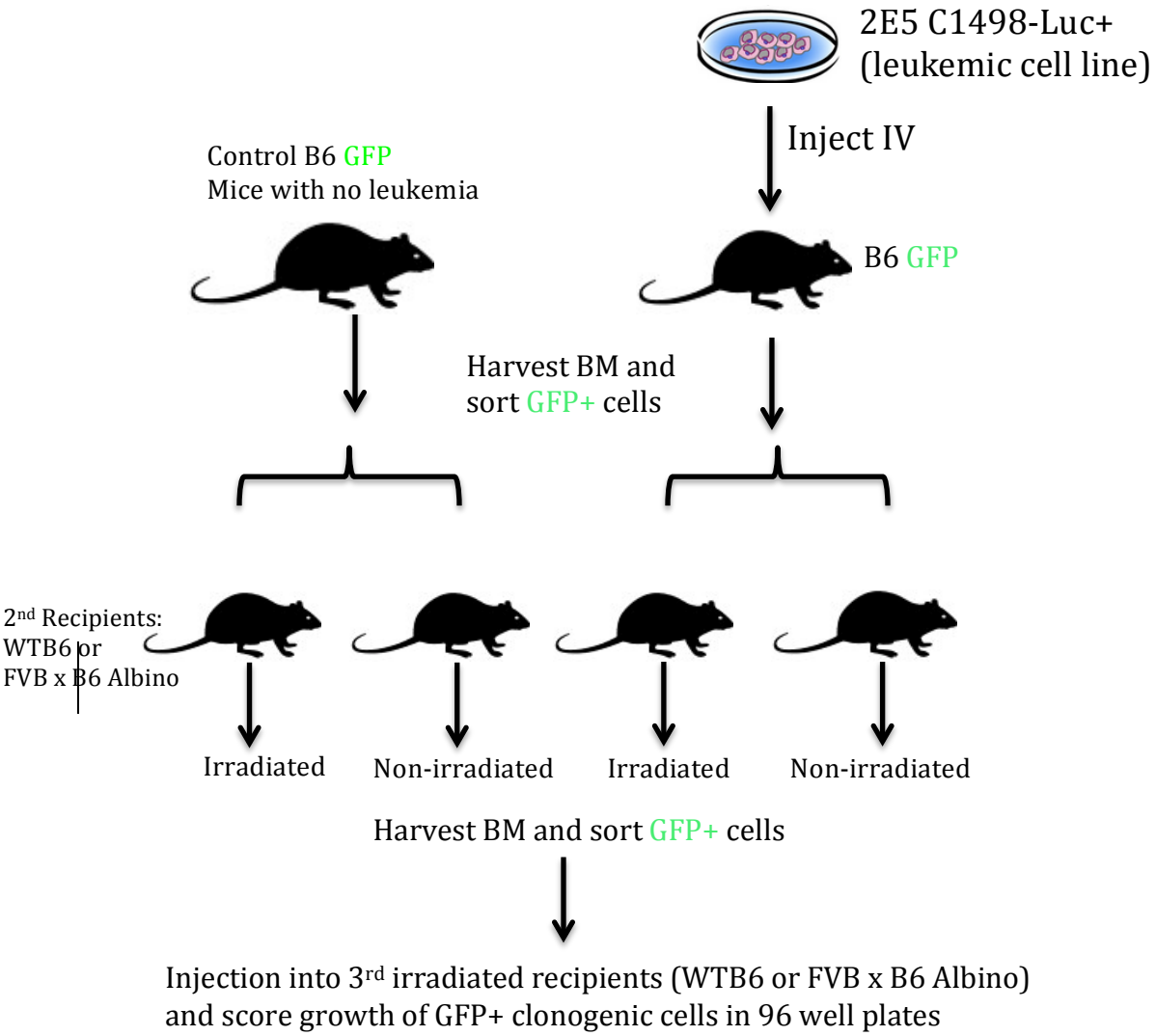



**Figure 1.** A schematic diagram of the *in vivo* experiment. Ten B6 GFP mice received I.V. injection of C1498. Bone marrow from five of the B6 GFP+ mice was harvested 2 weeks post-C1498 injections, and bone marrow from the remaining five B6 GFP mice was harvested 4 weeks post-C1498 injections. A total of two control B6 GFP mice without C1498 injections were sacrificed for bone marrow. The 2nd recipients for bone marrow two weeks after C1498 injections were twenty irradiated and non-irradiated WTB6. The 2nd recipients for bone marrow four weeks after C1498 injections were twenty irradiated FVB x B6 Albino. Irradiation for 2nd recipients were done a day before transplants (5.5Gy for WTB6 and 11Gy for FVB x B6 albino). Identical sorts were performed for GFP+ bone marrow injections into 3rd recipients, except that all the recipients were irradiated. The gating strategy through FACSAria ACDU of harvested bone marrow was for any cell that fluoresced GFP+, whether it was single GFP+ or dual GFP+/DSRed+. Cells after each sort was frozen in liquid nitrogen for future DNA analysis.

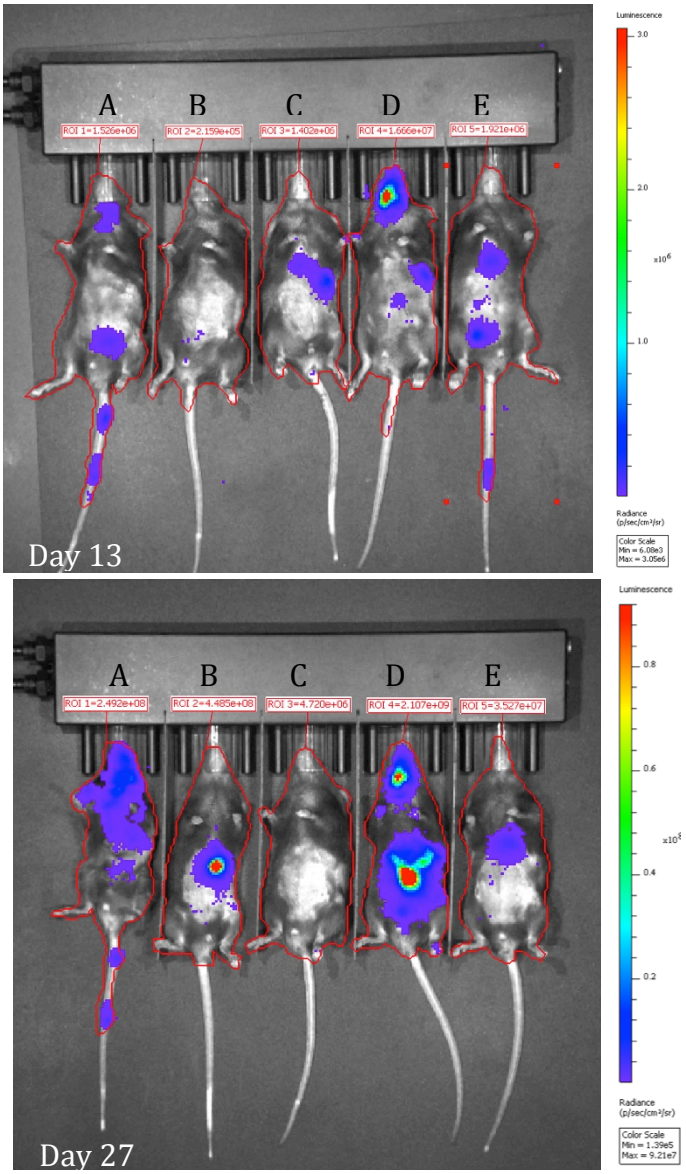



**Figure 2**. Bioluminescence imaging of WTB6 mice (control group 3) previously injected with C1498 on day 13 and 27. Subcutaneous injection of luciferin given 8 minutes prior to a 2-minute exposure to bioluminescence inside the Xenogen IVIS® spectrum. Radiance per second is given per mouse (indicated by ROI) indicating intensity of luciferase activity. On day 13, the recipeients averaged $1.2x10^4$ radiance per second (rad/s) with a range from $6.07x10^2$ to $4.61x10^4$ rad/s (figure 3). On day 27, the same recipients averaged $1.75x10^6$ rad/s, ranging from $1.35x10^4$ to $6.44x10^6$ rad/s. Although mouse "C" showed a reduction in radiance, the overall increase in the other 4 mice indicated the proliferation of the leukemic cells inside the recipients.

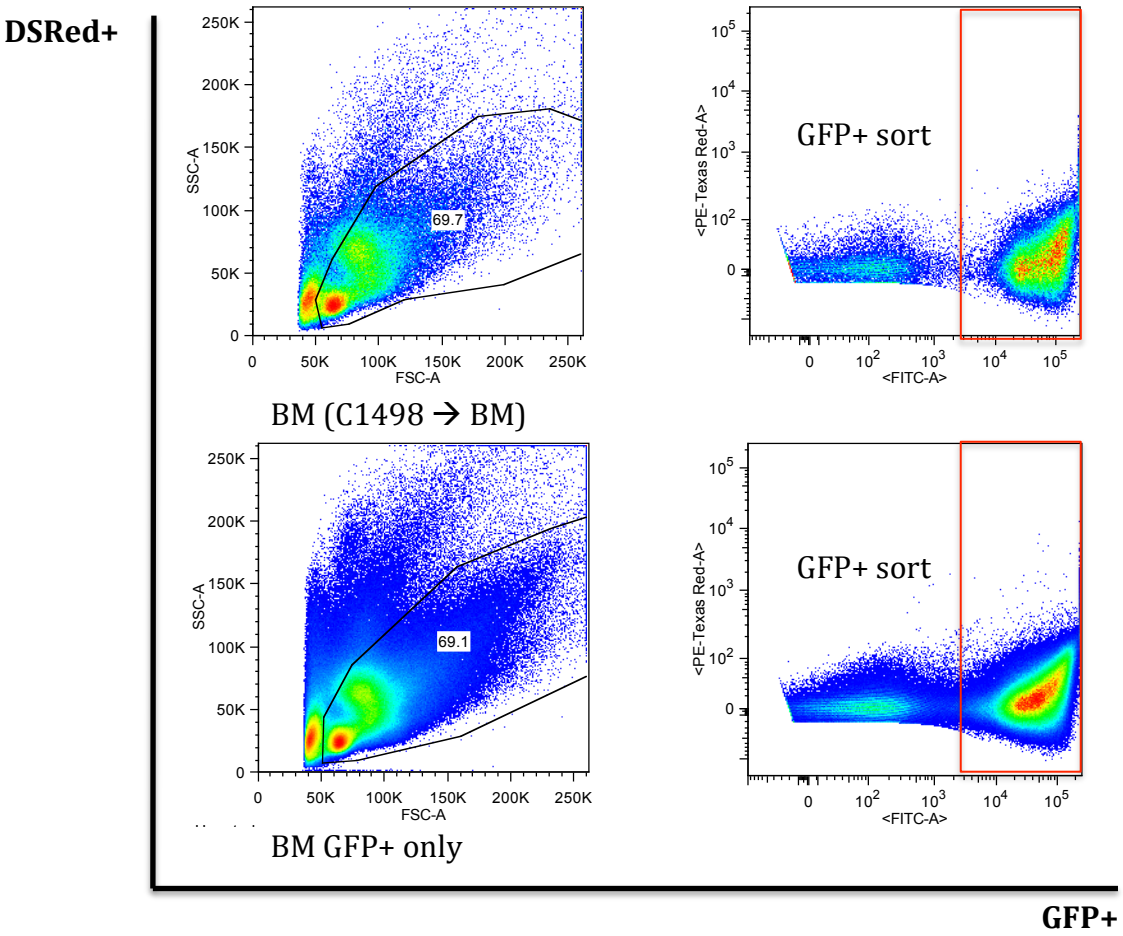



**Figure 3**. Sort strategy for experimental group 2 and control group 2 of GFP+ mice on day 17. Harvest bone marrow from 5 GFP+ mice with C1498 injection was sorted through FACSAria. Two mice were used for GFP+ mice without C1498 injections. These cells were only analyzed through FACSAria but not sorted for injection. Initially gated with a forward and side scatter to reduce background noise and dead cells. An additional gate was generated to isolate for GFP+ bone marrow cells for injection.

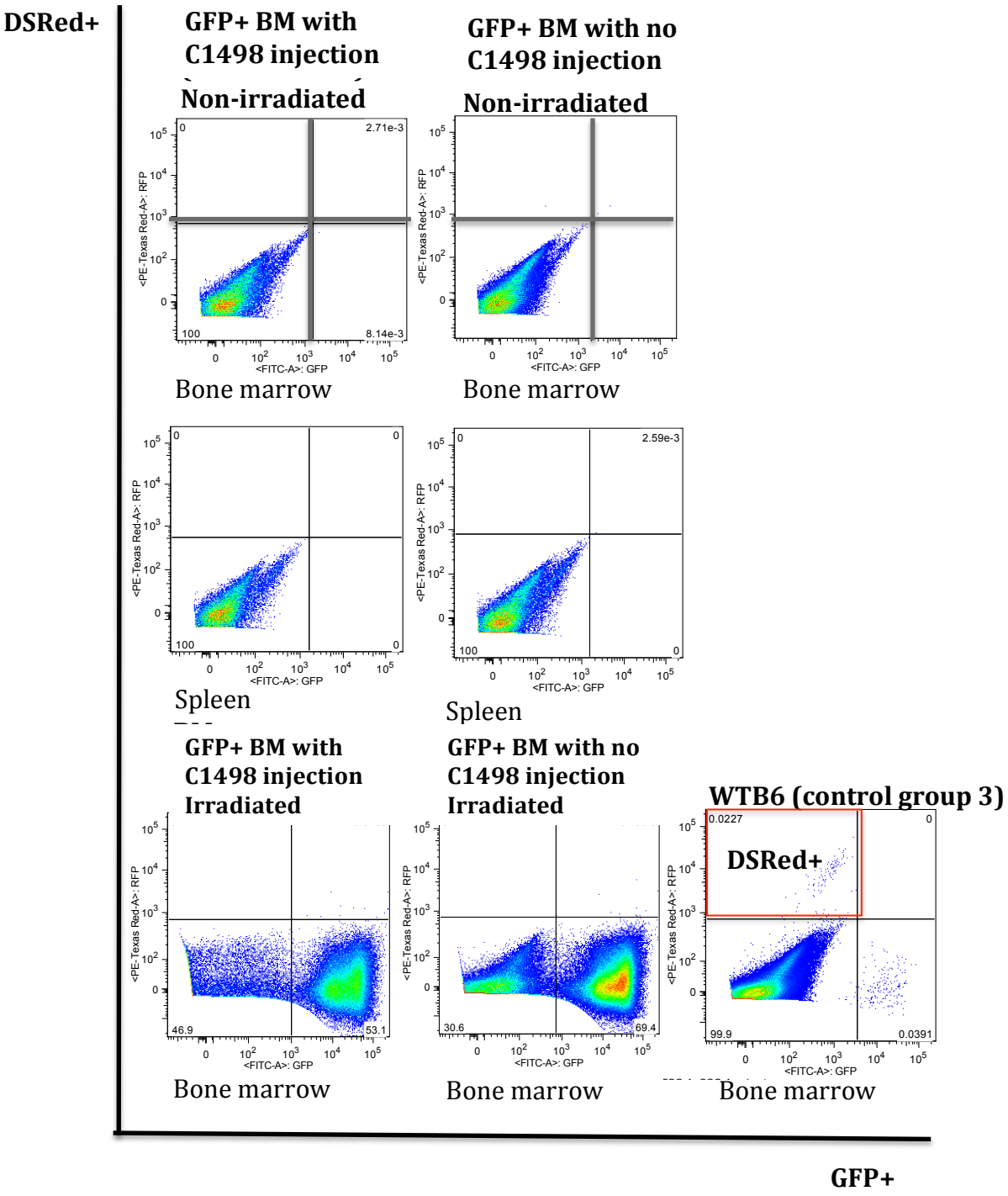



**Figure 4**. Analysis of bone marrow and spleen from WTB6 mice with sorts for GFP+ progeny for second transplant of bone marrow into recipients. Forward and side scatter gate purified the WTB6 recipients' bone marrow for GFP+ sort gates, but GFP+ were also DSRed-. Bone marrow of irradiated mice and WTB6 of control group 3 were harvested on day 46. Non-irradiated recipients' bone marrow was harvested on day 51. WTB6 (control group 3) provided proper DSRed+ gate.

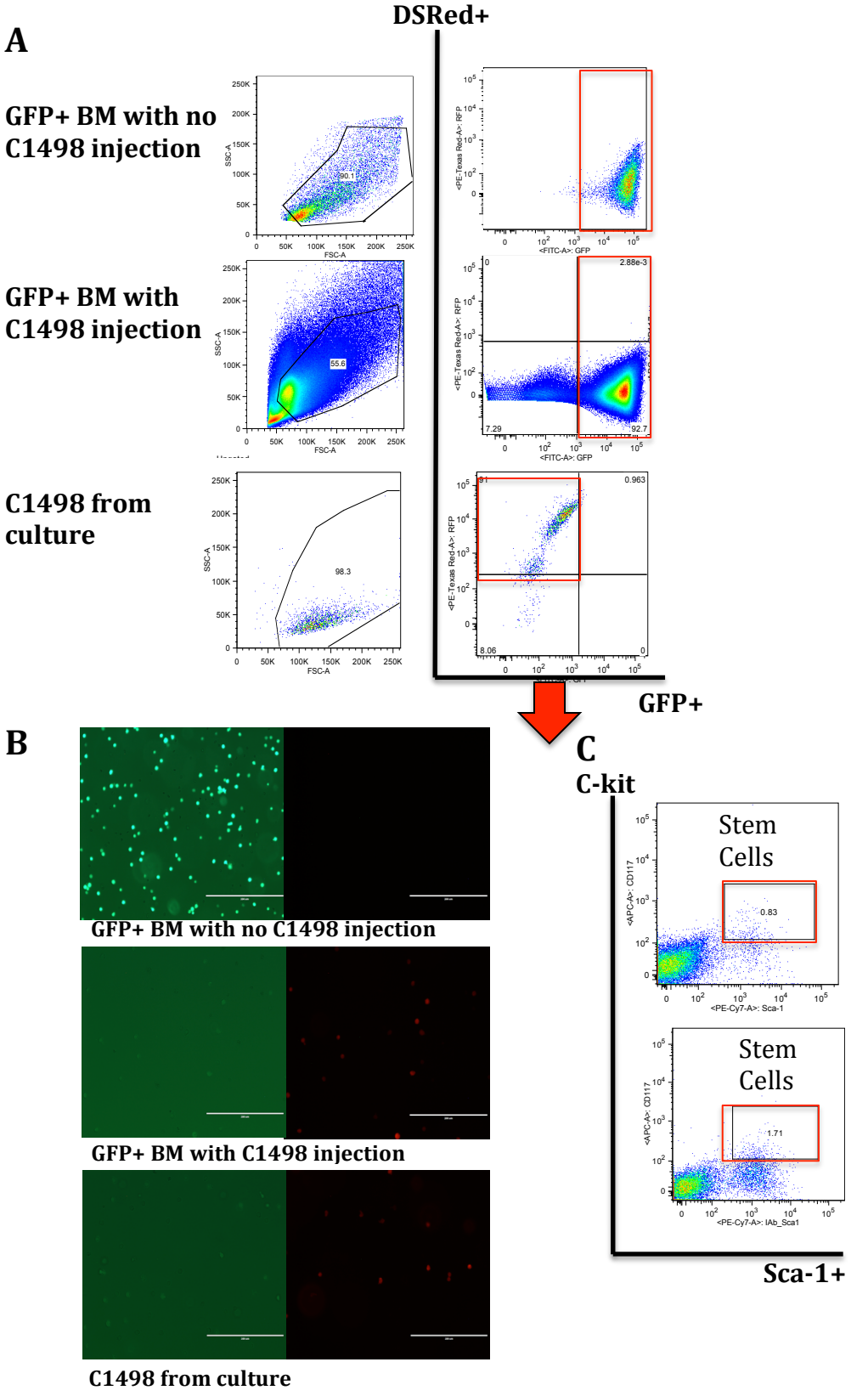
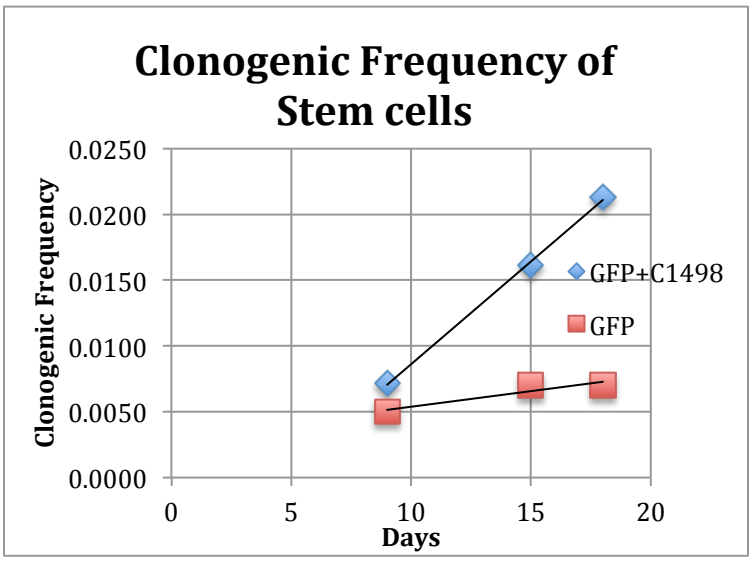



**Figure 5.** (A) Gate strategy to sort for GFP + progeny cells from FVB x B6 albino recipients for injection and immediate observation under EVOS ® FL microscopy. C1498 grown in culture analyzed to provide DSRed+ boundary. (B) Fluorescent images at 40x magnification of GFP+ sorted cells or DSRed+ cells observed immediately after sort. Not used for culture. (C) GFP+ sorted cells from GFP BM (with C1498) and GFP BM (without C1498) went through secondary sort for stem cell markers (Sca-1 and Ckit). These stem cells were grown in irradiated MSC layer 96-well plates. Second sort done 10 days later.

**A**




**B**

| | Clonogenic Frequency | |
|---|---|---|
| Day sorted/ Day observed | GFP+ BM w/ C1498 | GFP BM w/o C1498 |
| 1/9 | 0.0071 | 0.005 |
| 10/15 | 0.0161 | 0.007 |
| 10/18 | 0.0213 | 0.007 |

**Figure 6.** (A) Clonogenic precursory frequency of GFP+ bone marrow stem cells (with C1498) and GFP+ bone marrow stem cells (without C1498) per day of observation. Poisson distribution was used to calculate the clonogenic precursor frequency by indicating the number of GFP+ wells per day of observation. (B) Clonogenic precursory frequency of the same groups indicated in the graph. First sort was done on day 1 and the second sort was done on day 10. Plates were observed and resorted onto new irradiated stromal layer until not enough cells could be pooled for FACSAria ACDU.

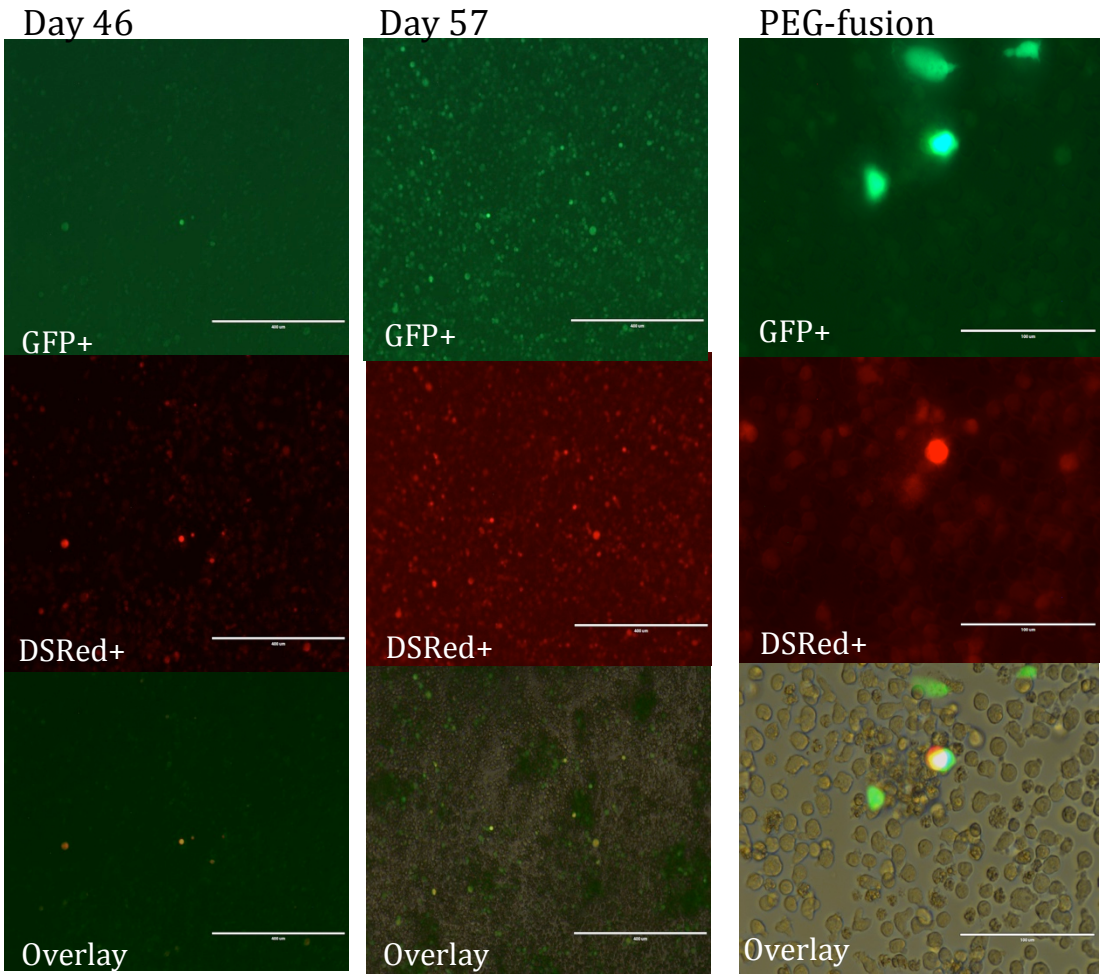
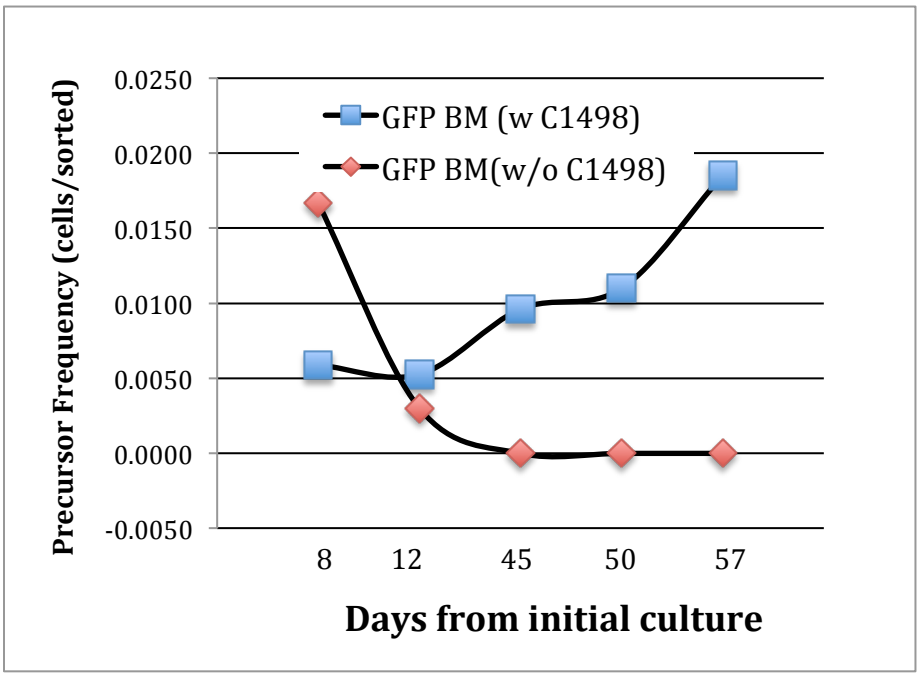



**Figure 7.** Fluorescent microscopy of GFP+, DSRed+, and overlay images GFP+ bone marrow with C1498 injections and PEG-fusion cells. Images obtained using EVOS ® FL microscopy. Each column of fluorescent images indicates cells from a consistent location on a well of a 96-well plate. Images of GFP+ bone marrow cells were taken at x20 magnification. Images of cells from the PEG-fusion, taken at x40 magnification 7 days after initial co-culture, provided a control to compare possible transformed bone marrow. Yellow fluorescence emitted in overlay indicates the combination of both GFP+ and DSRed+ fluorescence.

**A**




**B**

| | Clonogenic frequency | |
|---|---|---|
| Day sorted/ Day observed | GFP+ BM w/C1498 | GFP BM w/o C1498 |
| 4/8 | 0.00588 | 0.01667 |
| 9/12 | 0.00526 | 0.00313 |
| 40/45 | 0.00962 | 0 |
| 40/50 | 0.01099 | 0 |
| 54/57 | 0.01852 | 0 |

**Figure 8.** (A) Serial cultures of 96-well plates from GFP+ bone marrow (with C1498) and GFP+ bone marrow (without C1498) were observed at least 4 days post-sort for presences of GFP+ bone marrow. Poisson distribution was used to calculate the clonogenic precursor frequency by indicating the number of GFP+ wells per day of observation. (B) Clonogenic precursory frequency of the same groups indicated in the graph. Plates were observed and resorted onto new irradiated stromal layer until not enough cells could be pooled for FACSAria ACDU, which is indicated by "0."

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