In Vivo Assessment of Hematopoietic Cell Homing and Engraftment in Zebrafish Using Bioluminescence Imaging

A THESIS

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Finally, I would like to thank my thesis committee members for their time and advice.

Dedication

This thesis is dedicated to the memory of my beloved maternal grandmother, who passed away on May 3, 2013.

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Abbreviations

AGM	aorta-gonad-mesonephros
ALM	anterior lateral mesoderm
BLI	bioluminescence imaging
BSA	bovine serum albumin
CCD	charge-coupled device
CHT	caudal hematopoietic tissue
DAPI	4',6-diamidino-2-phenylindole
dmPGE2	12,16-dimethyl-PGE2
GVHD	graft versus host disease
HCT	hematopoietic cell transplantation
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
ICM	intermediate cell mass
LT-HSC	long-term repopulating hematopoietic stem cell
MHC	major histocompatibility complex
MLC-2	myosin light chain 2
MPP	multipotent progenitor
PBS	phosphate buffered saline
PLM	posterior lateral mesoderm
ROI	region of interest
SEM	standard error of mean
ST-HSC	short-term repopulating hematopoietic stem cell
WKM	whole kidney marrow

INTRODUCTION

Hematopoietic cell transplantation (HCT) has been increasingly used as treatment for malignant and non-malignant diseases over the past 40 years. This procedure was conceived from the studies on the biological effect of irradiation during World War II. It was observed during these studies that the hematopoietic system is the most radiosensitive tissue in the body, and that infusion of spleen or bone marrow cells could protect the animals from lethal effect of irradiation (Jacobson, Marks, Robson, & Zirkle, 1949; Lorenz, Uphoff, Reid, & Shelton, 1951). The protective effect was initially thought to be caused by humoral factor in the bone marrow or spleen, but later investigators showed that it was due to the transplanted donor cells (Barnes & Loutit, 1954; Food, Hamerton, Barnes, & Loutit, 1956). This discovery encouraged clinicians to translate this proof of concept to the clinic. In 1957, Thomas reported the first clinical use of total body irradiation followed by allogeneic marrow transplantation in patients with leukemia (Thomas, Lochte Jr, Lu, & Ferrebee, 1957). This first attempt was largely unsuccessful and similar failures were also reported in many other trials in the late 1950s and early 1960s (Bortin, 1970), except for transplantation in identical twins (Thomas, Lochte Jr, Cannon, Sahler, & Ferrebee, 1959). Better understanding of human leukocyte antigen (HLA) and prevention and treatment of HCT complications, especially infection and graft *versus* host disease (GVHD), eventually led to the first successful allogeneic transplant in 1968 (Gatti, Meuwissen, Allen, Hong, & Good, 1968).

The basis of HCT relies on the ability of hematopoietic stem cells (HSCs) to migrate to the bone marrow and reconstitute the hematopoietic system. Homing of HSCs occurs within few hours following transplantation and is a multistep process that involves adhesion of HSCs to endothelial cells, transendothelial migration, and anchoring of the HSCs to the bone marrow microenvironment, where they subsequently self renew and differentiate (Sahin &

Buitenhuis). Attachment of hematopoietic cells to the endothelial cells is mediated by adhesion molecules, including selectins expressed on the endothelial cells as well as hematopoietic cell's major integrins and their ligands (Frenette, Subbarao, Mazo, Von Andrian, & Wagner, 1998; Mazo et al., 1998; Williams, Rios, Stephens, & Patel, 1991). The chemoattractant SDF-1 is released by bone marrow niche cells and the main factor that directs migration of HSC to the bone marrow by interacting with its receptor, CXCR4, expressed on the surface of HSCs (Peled et al., 1999). In addition, HCT conditioning by TBI is thought to induce activation of the complement cascade that can increase the sensitivity of HSCs to SDF-1 and promote secretion of SDF-1 and other chemoattractants, such as sphingosine-1-phosphate and ceramide-1-phosphate by the bone marrow microenvironment (Kim et al., 2011). Pre-transplant conditioning, however, does not seem to be a critical requirement for homing (Lapidot, Dar, & Kollet, 2005). By contrast, myelosuppresive conditioning by chemotherapy or TBI facilitates hematopoietic reconstitution by potentially creating niche space in the bone marrow as well as providing immunosuppression to avoid graft rejection, and is necessary to achieve durable engraftment. Short-term engraftment occurs transiently for few weeks after transplant and is established by multipotent progenitors (MPP) and short-term repopulating HSC (ST-HSC), whereas long-term repopulating HSCs (LT-HSC) are responsible for durable engraftment that can last for life and are able to reconstitute multilineage hematopoiesis in secondary and tertiary recipients in serial transplantation, indicating their extensive self renewal capacity (Iscove & Nawa, 1997; Jones, Celano, Sharkis, & Sensenbrenner, 1989; Morrison & Weissman, 1994). Mouse LT-HSC can be prospectively isolated in high purity based on their $CD150^{+}CD244^{-}CD48^{-}Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ phenotype (Kiel et al., 2005). Human LT-HSC are found in $Lin^{-}CD34^{+}CD38^{-}$ populations of cells in bone marrow (Majeti, Park, & Weissman, 2007).

Many advancements have been made to improve HCT outcome, such as development of non-myeloablative conditioning for older patients or individuals with severe conditions whom otherwise would not tolerate a fully ablative regimen (Giralt et al., 1997; Kasamon et al., 2010; Khouri et al., 1998), GVHD control using immunosuppressive agents or T cell depletion (Bethge et al., 2006; Luznik et al., 2008), and the use of other stem cell sources, such as peripheral blood and umbilical cord blood. Nevertheless, this procedure still faces major challenges, especially the limited donor availability and significant morbidity and mortality, mainly due to GVHD. The use of umbilical cord blood as donor source offers a potential solution for these problems, as it is associated with lower incidence of GVHD despite the HLA disparity (Barker et al., 2001), potentially due to its naïve lymphocytes (L. Chen, Cohen, & Lewis, 2006) and higher number of regulatory T cells (Lee, Lin, Cheng, & Kuo, 2009; Wing, Ekmark, Karlsson, Rudin, & Suri-Payer, 2002). However, the numbers of HSCs in the umbilical cord blood are ten times less than that in bone marrow, resulting in engraftment failure and delayed immune reconstitution. Consequently, this therapy option has been limited for pediatric patients. To improve the outcome of cord blood transplant, current researches are directed at expanding the number of UCB stem cells *ex vivo* and improving the HSC homing (Broxmeyer, 2010; Dahlberg, Delaney, & Bernstein, 2011; Schuster et al., 2012).

Much of what is known about transplant and HSC biology comes from the experimental studies in animals. The mouse has been the most commonly used model organism due to advantages, such as physiology and pathology similarities to human and short generation time. However, mice have some limitations, including the difficulty to perform forward genetic screens as well as the high maintenance cost. Over the past decade, the zebrafish has been increasingly appreciated as a versatile model to complement murine and human studies.

Zebrafish are small freshwater fish originally found in the rivers of South Asia (Figure 1a). Although zebrafish had been used in research since 1930s, only in the past 20 years have they become prominent as a model organism. In 1981, Streisinger published his pioneering work demonstrating zebrafish as genetically tractable system (Streisinger, Walker, Dower, Knauber, & Singer, 1981). Two large-scale genetic screens were done in Tubingen and Boston in 1990s, identifying around 4,000 mutants with developmental defects, including more than 50 blood mutants (Driever et al., 1996; Haffter et al., 1996). The availability of these mutants has propelled the eminence of zebrafish in developmental biology studies. Due to the increasing interest in zebrafish research, Sanger institute initiated the zebrafish genome sequencing project in 2001, which is currently near its completion (http://www.sanger.ac.uk/Projects/D_rerio/).

Zebrafish offer several advantages that make them a powerful animal model. Unlike mammals, zebrafish embryos are fertilized and develop externally, which makes the embryos accessible for observation and technical manipulation. The optical clarity of the embryos allows the developmental processes to be easily and directly observed under the microscope and makes them amenable for forward genetic screening in which observation plays a role. Zebrafish display a rapid embryonic development, and major organs are formed by 24 hours post fertilization (hpf) (Santoriello & Zon, 2012). This quick process creates more simple and straightforward system to study embryonic development and genetic questions. The fish reach sexual maturity by 3-4 months, by which time they are able to breed and a pair of zebrafish can lay hundreds of eggs every week (Westerfield, 1993). Zebrafish adults are small, with body length around 4-5 cm, allowing them to be kept in large number in a small space. The maintenance of zebrafish is easy and the cost is cheaper than that of mice. Although the use of zebrafish has traditionally been in developmental biology studies, the remarkable attributes and the ability to perform diverse techniques in zebrafish, including

forward and reverse genetics, has expanded the application of zebrafish to more areas of biomedical research, such as system for drug screening and models for human diseases (Lieschke & Currie, 2007; Santoriello & Zon, 2012).

As vertebrates, zebrafish share high degree of genetic conservation with humans. Similar to mammals, zebrafish have two successive waves of primitive and definitive hematopoiesis. In zebrafish, primitive hematopoiesis occurs at two distinct intraembryonic sites, as indicated by expression of the *stem cell leukemia* (*scl*) gene: the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM) that later converge at the midline forming the intermediate cell mass (ICM), which is functionally analogous to the extraembryonic yolk sac in mammals and birds (Davidson et al., 2003). ALM cells generate mainly primitive myeloid cells (*pu.1*+) whereas ICM cells generate primitive erythroid (*gata1*+) and endothelial precursors (Bennett et al., 2001; Davidson et al., 2003; Detrich et al., 1995). When the heart starts to contract around 24 hpf, the proerythroblasts formed in the ICM enter the circulation and develop into mature erythrocytes. Although the primitive hematopoiesis halts at approximately 24 hpf, the erythrocytes from primitive hematopoiesis stay in the circulation until around 6 days post fertilization (dpf), when the blood cells from the definitive wave start to circulate (A. T. Chen & Zon, 2009). Definitive hematopoiesis is marked by the formation adult HSCs capable of self-renewing and generating all blood cell lineages. In mammals, these HSCs are first produced in the region called aorta-gonad-mesonephros (AGM) (Medvinsky & Dzierzak, 1996). The site of hematopoiesis then shifts to the fetal liver and finally to the bone marrow, which function as the primary hematopoietic organ. Similarly, definitive hematopoiesis in zebrafish initially takes place in the ventral wall of the dorsal aorta in the AGM region, as identified by *runx1* transcript between 24-48 hpf in this location (Kalev-Zylinska et al., 2002). At 3 dpf, the HSCs are found in the caudal hematopoietic tissue (CHT) and later seed the kidney, which serves as primary hematopoietic organ in adult zebrafish, at 4 dpf (Martin, Moriyama, & Zon, 2011) (Figure 1b &

c). Adult zebrafish produce all major blood lineages: erythrocytes, graculocytes, monocytes, lymphocytes, and thrombocytes (Davidson & Zon, 2004) (Figure 1d).

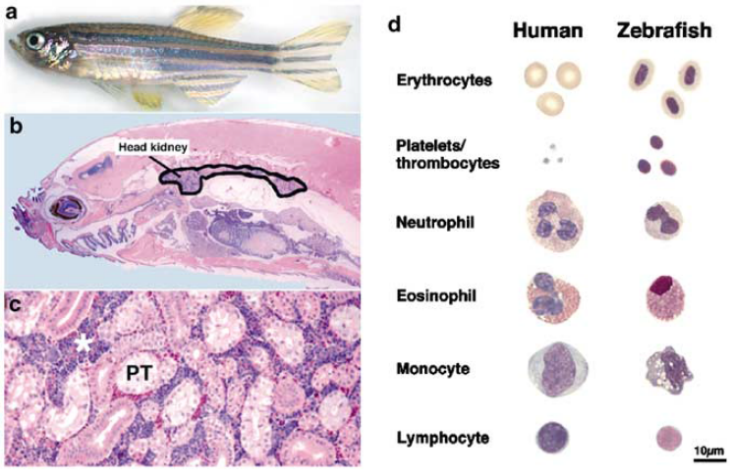

Figure 1. Zebrafish hematopoietic system. (a) Adult male zebrafish. (b) Parasagittal section of adult zebrafish. (c) Tubules (PT) and hematopoietic cells (asterisk) in the kidney. (d) Comparison of human and zebrafish peripheral blood cells (Davidson & Zon, 2004).

The ability to do HCT has further established zebrafish as a versatile model in hematologic research. In 2003, Traver et al. reported the first HCT experiment in zebrafish, in which they injected whole kidney marrow (WKM) cells (refers to cells collected from kidney where the marrow cells reside) from adult zebrafish to rescue otherwise lethal gata1$^{-/-}$ embryos (Traver et al., 2003). In addition, they demonstrated the ability to separate each of the major blood lineages (erythrocytes, lymphocytes, myelomonocytes, and precursor cells) in WKM based on their light scatter characteristics on flow cytometry, which was then used as a standard method to assess engraftment (Figure 2). Following this experiment, they subsequently performed HCT in adult zebrafish (Traver et al., 2004). Like in mammals, donor engraftment is not achievable when WKM cells are transplanted to unconditioned adult recipients. The effect of ionizing radiation on the hematopoietic system was then studied to determine the dose for pre-transplant conditioning in adult zebrafish. They reported 40 Gy as the minimal

lethal dose, which was defined as the radiation dose resulting in 10% survival within 30 days, and 20 Gy as threshold where effect on marrow cellularity could be seen. The greatest decrease in marrow cellularity was observed on day 8 post irradiation and while zebrafish receiving 20 Gy eventually recovered their marrow after this point, 40-Gy-irradiated animals did not. They subsequently showed that intracardiac injection of WKM cells on day 2 after transplantation rescued 40-Gy-irradiated recipients from lethal effect of the radiation. Multilineage engraftment by donor cells was confirmed by flow cytometry. Following these first reports, HCT assay has facilitated more studies in the field. Upon transplantation, it was determined that the frequency of repopulating cells in zebrafish WKM is ~1/40,000 (Hess, Iwanami, Schorpp, & Boehm, 2013). Moreover, like in mammals, a zebrafish homolog of SDF-1 is also found to have important functions in the homing process of WKM cells following HCT (Glass et al., 2011).

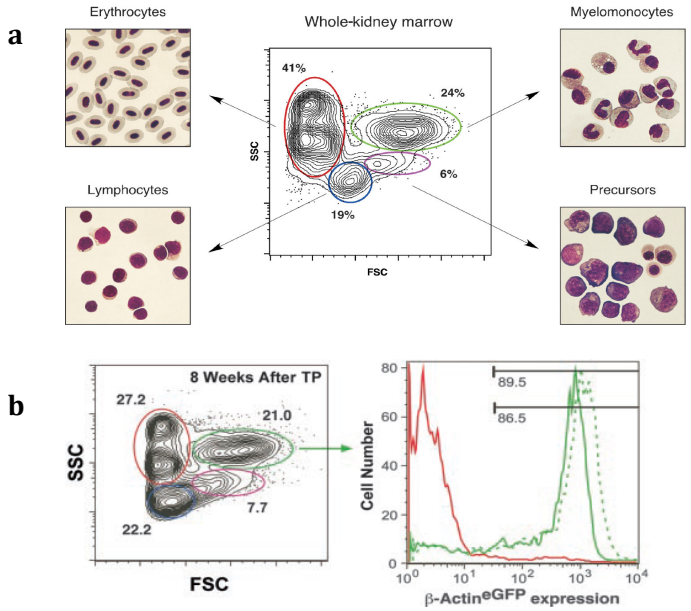

Figure 2. HCT in zebrafish. (a) Separation of zebrafish major blood lineages based on side and forward scatters. (b) Engraftment of β actin:GFP donor cells in the recipient kidney 8 weeks after transplantation (Traver et al., 2003; Traver et al., 2004).

As HCT in zebrafish is still in its early development, further refinements are needed. Several modifications have been made to improve the survival of recipients. A radiation dose of 40 Gy many times results in very poor survival despite the rescue with WKM cells injection (de Jong et al., 2011). Retroorbital injection was developed as an alternative to intracardiac injection procedure which is thought to be more deadly for the fish (Pugach, Li, White, & Zon, 2009). Administration of antibiotics have been difficult, as it requires the fish to live in stagnant water, which causes even higher death. Maintaining the water quality might compensate the necessity for antibiotics to prevent infection. Unlike in the murine system, HCT in zebrafish currently cannot be easily done in isogenic or congenic animals due to the difficulty of generating truly homogenous inbred strains. Consequently, immune mismatch between donor and recipient will likely cause rejection of the donor cells or GVHD that may affect the engraftment or the recipient survival. Indeed, HCT using MHC-matched donors and recipients showed better engraftment and survival (de Jong et al., 2011). Recently, Hess et al. reported successful allogeneic transplantation of WKM cells into *c-myb* mutants without pretransplant conditioning (Hess et al., 2013). These mutants lack definitive hematopoiesis and display loss of all adult blood lineages, thus can serve as immunodeficient zebrafish for transplantation.

The assessment of engraftment by harvesting the hematopoietic organs gives an accurate measurement of engraftment level and multilineage reconstitution by donor cells. However, postmortem analysis does not provide a complete picture of the biological process, and the dynamic of long-term engraftment within each individual fish cannot be observed. On the other hand, *in vivo* imaging techniques, including optical imaging using bioluminescence or fluorescence, allow the engraftment process in the same animal to be continuously examined.

Bioluminescence is emission of light produced by chemical reaction naturally found in some living organisms, where enzyme called luciferase

catalyzes oxidation of its substrate (luciferin). Bioluminescence imaging (BLI) techniques are based on detection of the photons that result from this reaction using an ultra light-sensitive charge coupled device (CCD). Since its development more than a decade ago, BLI has been widely used in biomedical research, including in transplantation experiments. In the murine HCT assay, BLI was able to reveal engraftment kinetics and patterns by different populations of human and mice HSCs (Cao et al., 2004; Wang et al., 2003). Firefly luciferase (Fluc) is so far the most commonly used luciferase for *in vivo* optical imaging, as it emits green light (562nm) that penetrates tissue better than blue light produced by sea pansy (*Renilla*) luciferase (Rluc) or *Gaussia* luciferase (Gluc) (Badr & Tannous, 2011). Compared to fluorescence imaging, BLI can yield much higher sensitivity. Furthermore, unlike fluorescence imaging, BLI does not require an external light source to provide excitation energy, which can result in high background noise due to excitation photons or autofluorescence in the tissues. For this reason, BLI yields high signal-to-noise ratio with almost no background (Lin, Molter, Lee, & Gerson, 2008).

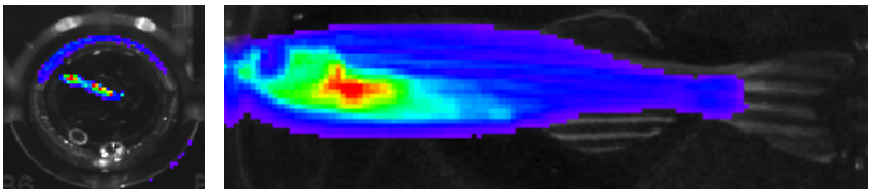

Figure 3. *Ubi:luc* embryo (left) and adult (right).

Our lab has generated transgenic a zebrafish that ubiquitously expresses luciferase, that can serve as a donor for HCT experiments (Figure 3). The *ubiquitin* promoter was used to drive the expression of firefly luciferase and the transgenesis was performed using a tol2 transposon system (Kawakami, Shima, & Kawakami, 2000; Mosimann et al., 2011). To aid transgenic screening, this fish also expresses the GFP fluorophore under the control of cardiac myosin light

chain 2 (MLC-2) promoter, and thus displays GFP^+ myocardial cells. This study aimed to develop BLI as analysis tool for HCT assay in zebrafish and to perform *in vivo* longitudinal assessment of homing and engraftment following HCT in adult zebrafish. **We hypothesized that BLI can be used to track the transplanted donor hematopoietic cells in the living recipient animals.**

METHODS

Fish husbandry

Zebrafish were bred and maintained at the University of Minnesota Zebrafish Core Facility according to standard guideline (Westerfield, 1993) and with the approval of the Institutional Animal Care and Use Committee, University of Minnesota. The following lines were used: Segrest wild-type, *ubi:luc*, *ubi:luc/h2afv:gfp*, and *casper* mutant.

WKM cells isolation

Fish were euthanized by immersion in ice water until the heart stopped beating. The aorta was flushed with phosphate buffer saline (PBS) to remove the peripheral blood. WKM was then harvested, triturated using P1000 pipette, filtered through 40-μm filter, and centrifuged at 2000 g for 6 minutes. Supernatant was removed and WKM cells were resuspended in PBS.

Peripheral blood draw

Fish were anesthetized by immersion in 0.01% tricaine. The operculum was gently poked using 20-μl pipet tip until minor bleeding occurred. Blood was then drawn (~1 μl) into a heparin-coated tip and expelled into 96-well plate containing 5 U/ml heparin.

Total body irradiation

Irradiation was given using X-Rad320 irradiator (Precision X-Ray, Inc.). During irradiation, fish were put in large petri dish containing fish water. The irradiation doses were given at a rate of 2.7 Gy/min. The dose used throughout the study was 20 Gy, unless specified otherwise.

Hematopoietic cell transplantation

Transplantation was performed 2 days after fish were conditioned with irradiation. Recipient fish were anesthetized with 0.01% tricaine methanesulfonate and placed on a sponge. Donor cells were intracardially injected in a 5 μl volume using a 30 GA Hamilton syringe. Each recipient fish was transplanted with 200,000 cells, unless specified otherwise.

Bioluminescence imaging

For *in vitro* BLI, cells were suspended in PBS containing 150 μg/ml D-luciferin (Gold Biotechnology) and immediately imaged at 1- to 5-minute exposure. For *in vivo* BLI in zebrafish embryo, embryos were placed in fish water containing 150 μg/ml D-luciferin. For *in vivo* BLI in adult zebrafish, prior to imaging, fish were intraperitoneally injected with 5 μl of 15 mg/ml D-luciferin and then imaged 10-15 minutes after injection. At the time of imaging, fish were anesthetized with 65 ppm isoflurane and 65 ppm tricaine in hyper-oxygenated fish water. The hyper-oxygenated fish water was prepared by immersing oxygen bubbler in the fish water until the dissolved oxygen was higher than 20 mg/l. Images were taken at 1-minute exposure. After imaging, fish were allowed to recover in hyper-oxygenated fish water. Optical images were acquired with CCD camera (Xenogen IVIS50 system, Caliper Life Sciences) and analyzed with Living Image (Caliper Life Sciences) software. Region of interest (ROI) was manually drawn around the fish body. The average radiance (in photons/s/cm^2/steradian) and total flux (in photons/s) were used as measurement of luminescent signal.

Immunofluorescence

WKM cells were harvested, dropped on to slide, and air-dried. Cells were then fixed with cold 100% methanol for 5 minutes, permeabilized with 0.4% Triton X-100 for 10 minutes, and blocked with 1% bovine serum albumin (BSA) in

PBS + 0.1% Tween 20 for 1 hour at room temperature. Cells were probed with mouse monoclonal antiluciferase antibody (Novus Biologicals) or mouse IgG1 isotype control (Novus Biologicals) 1:100 overnight at 4^0C, followed by donkey polyclonal antimouse IgG1-Cy3 1:300 for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

PGE2 pulse exposure

Harvested 200,000 WKM cells were incubated with zebrafish media containing 5 mM dmPGE2 (Cayman Chemical) in dimethyl sulfoxide (DMSO) for 2 hours on ice. DMSO was used as vehicle control. Cells were then washed twice with media and resuspended in PBS before injected to recipient fish.

Statistical analysis

Quantitative data were presented as mean plus or minus SEM and compared using unpaired t test. Statistical significance was determined as P less than or equal to .05. Survival was analyzed using Wilcoxon test.

RESULTS

Generation of *ubi:luc* lines and screening method

As our *ubi:luc* fish was generated using transposon vector, we expected some variability in the transgene expression among the fish. To minimize this variability, we generated independent lines from separate founders. The WKM cells were pooled from six fish of each line and tested for their luciferase activity by BLI. Three lines were positive (Figure 4a) and we used line 1 in this study. The luciferase expression was comparable among the line 1 fish (Figure 4b).

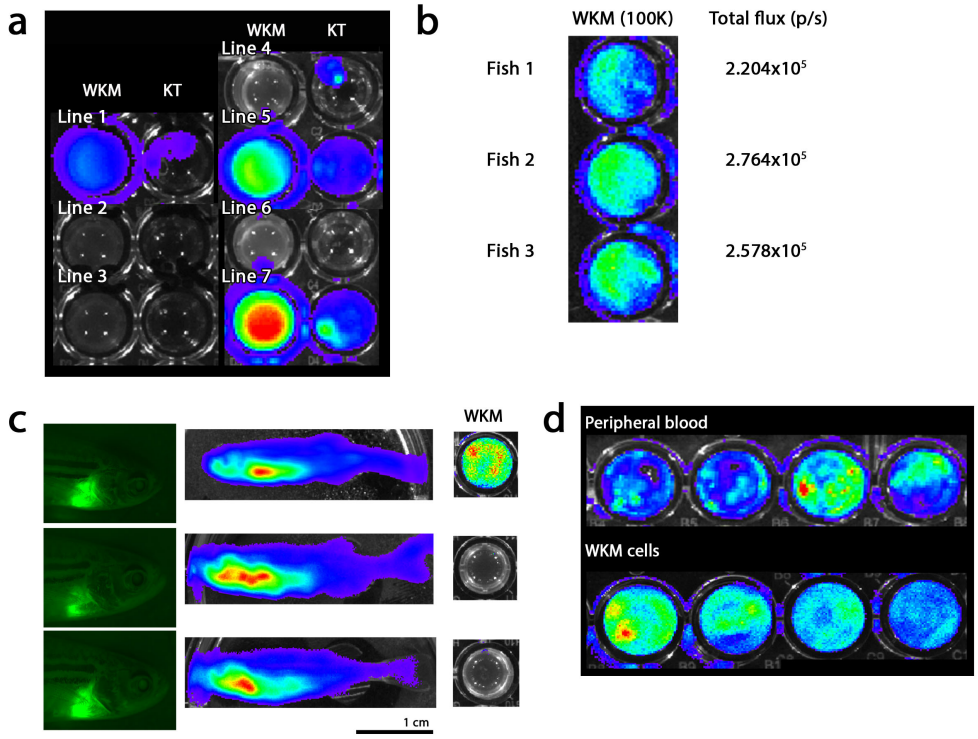

Figure 4. Generation of *ubi:luc* lines and screening method. (a) Seven different *ubi:luc* lines tested for their luciferase activity in the WKM cells (WKM, left well) and kidney tubules (KT, right well) by BLI. (b) BLI on the WKM cells from three *ubi:luc fish* in line 1, showing minimal variability in the luminescent signal. (c) BLI on *ubi:luc* adult fish with GFP+ hearts and their WKM cells, displaying discrepancy between whole-body and WKM cell luminescence. (d) BLI on peripheral blood and WKM cells, showing consistent luciferase activity in these two samples.

We initially screened the *ubi:luc* fish based on their GFP expression in the cardiomyocytes. However, we found some fish that expressed GFP in their hearts, but not luciferase in the WKM cells. Moreover, upon *in vivo* BLI, these fish showed luciferase expression in their body (Figure 4c). This discrepancy in the luciferase expression may be caused by the insertion of the transgenes into the regions that are transcriptionally inactive in the WKM cells. To more accurately screen the fish with positive luciferase expression in the WKM cells, we then did *in vitro* BLI on the peripheral blood cells, which luciferase expression was consistent with that in WKM cells (Figure 4d).

Luciferase expression in *ubi:luc* WKM cells

Immunostaining of the *ubi:luc* WKM cells showed luciferase expression in majority of the cells (Figure 5a). To examine the luciferase expression in the hematopoietic lineages, lymphoid, myeloid, and precursor cell populations of *ubi:luc* WKM cells were isolated by flow cytometry and the luciferase activity was assayed by BLI. Consistent with previous report (Mosimann et al., 2011), *ubi*-driven transgene expression was observed in all cell populations (Figure 5b).

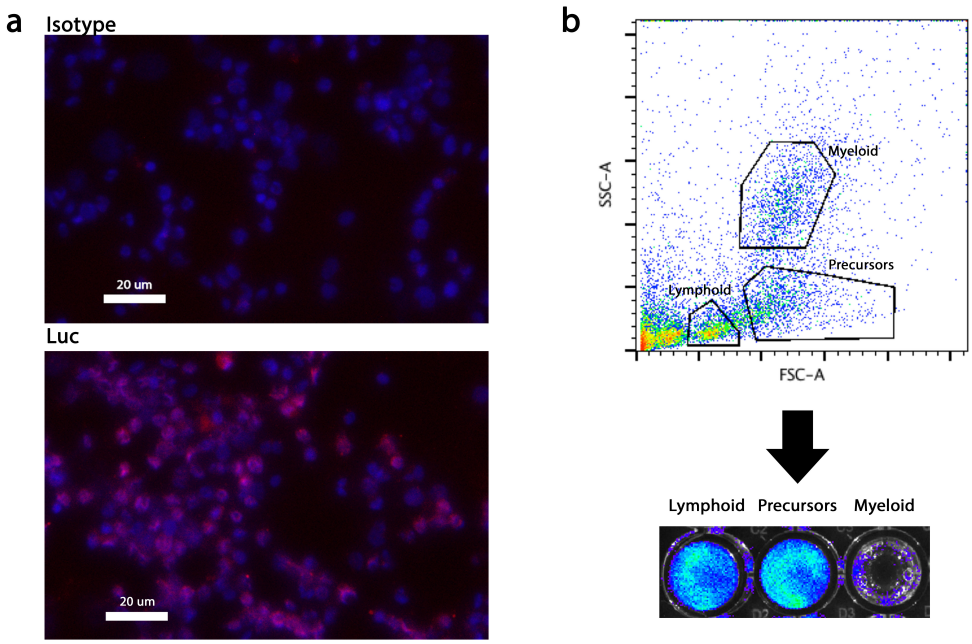

Figure 5. Luciferase expression in *ubi:luc* WKM cells. (a) Luciferase immunostaining of *ubi:luc* WKM cells, showing luciferase in red and DAPI in blue. (b) Sorting gates (top) for lymphoid, precursor, and myeloid populations of *ubi:luc* WKM cells (top) and BLI on these populations (bottom).

Improving survival of transplant recipients subjected to bioluminescence imaging

We found decreased survival in the recipient fish that were subjected to BLI, typically with high mortality on week 1 and no surviving fish by week 2. However, this procedure did not affect the survival in non-irradiated transplant recipients. BLI procedure, including substrate injection and long-exposure imaging, might impart additional stress to the recipient fish that were already in distress due to pre-transplant irradiation. Therefore, we sought to optimize the BLI protocol to improve the recipient survival in this system.

The duration of BLI performed in this study was 3-4 minutes (lateral and dorsal images were taken with 1-minute exposure each), during which fish were kept anesthetized. Early in this study, we used 0.01% tricaine to anesthetize the fish during imaging. Induction time was rapid, with loss of equilibrium and

minimal response to external stimuli reached in less than 1 minute. However, at this stage, the fish also showed decreased gill movement accompanied with hypoxia-induced behavior, such as surface breathing, and reduced heart rate. This condition rapidly deteriorated in the next few minutes that often times, fish failed to recover from anesthesia when returned to fresh water and eventually died. We therefore reduced tricaine dose to 0.005-0.007% to lower the toxicity. However, this dose range showed long induction time and the muscular tone remained intact after 10 minutes of anesthesia. This was especially true for recipient fish imaged within first week after transplantation that showed higher resistance to anesthesia. As the imaging required the fish to be kept still, the fish were positioned in small closed compartment. This treatment caused the fish to be highly agitated and required extra handling leading to increased stress response. Consequently, the recipient survival was not improved. We subsequently used hyper-oxygenated water containing combination anesthesia with tricaine and isoflurane, which was reported to be suitable for lengthy procedure (Huang et al., 2010). At a dose of 130 ppm (65 ppm tricaine + 65 ppm isoflurane), anesthetic induction was achieved in 2-3 minutes. Decreased respiratory and heart rate were not observed in anesthetized fish and deep anesthesia could be maintained for at least 20 minutes without inducing respiratory arrest. Most importantly, with this anesthesia regimen we found improvement in survival (from zero recipients surviving past day 10 to 25-50% surviving at week 8-12).

Administration of luciferin was done by intraperitoneal injection. As this procedure was quite unpleasant for the fish, we tested less stressful delivery method by adding the substrate to the fish water. At 1.5 mg/mL dose of luciferin, only minimal luminescent signal around the gill area was detected during 20-minute exposure (Figure 6a). We therefore continued using the i.p. injection method to deliver the luciferin before imaging.

In addition to modification of the BLI protocol to improve recipient survival, we also examined the optimal transplant condition using different injection routes. As mentioned earlier, retroorbital injection was reported to be associated with less procedure-related mortality. Therefore, we tested and compared the survival in recipients transplanted via cardiac, retroorbital, and intraperitoneal injections. We found no significant difference in survival among the groups (Figure 6b).

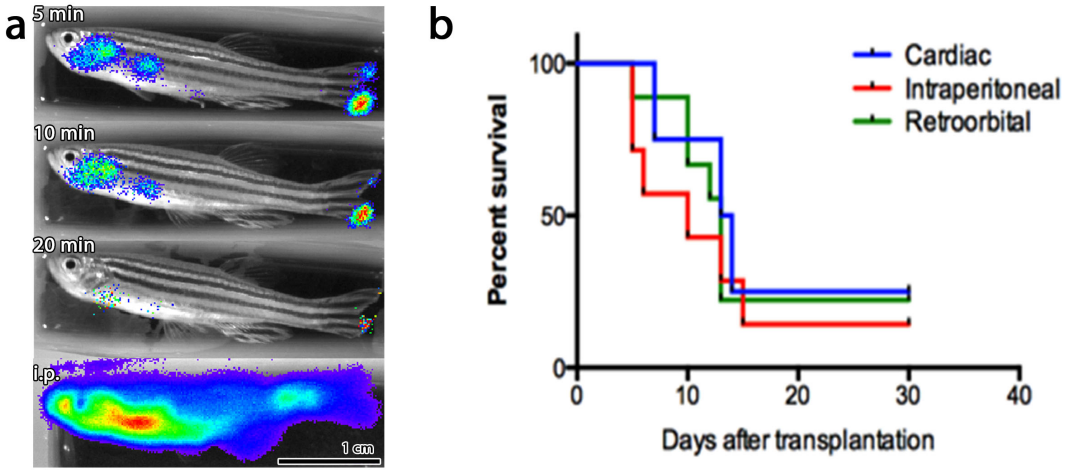

Figure 6. Attempted BLI and HCT protocol modifications to improve recipient survival. (a) BLI on transplant recipient following substrate delivery via fish water and i.p. injection, showing substantial difference in the luminescent signal resulted from these two methods. (b) Comparable survival of recipients transplanted with 100,000 *ubi:luc* WKM cells via cardiac, intraperitoneal, and retroorbital injection routes.

In vitro and in vivo luminescent signal of *ubi:luc* WKM cells

The luminescent signal of *ubi:luc* WKM cells increased with increasing cell number (Figure 7). In our experiments, we used 1-minute exposure, 4-cm field of view (FOV), 4x4 binning, and 1 f/stop. With this setting, signals were detected in 50,000 or more cells *in vitro* and in 300,000 or more cells *in vivo* (Figure 7a). Although increasing the exposure time and binning (the FOV and f/stop were already at maximum setting) could enhance the sensitivity (Figure 7b), longer exposure was more harmful for the fish and higher binning resulted in low spatial resolution, which could hinder the anatomic visualization.

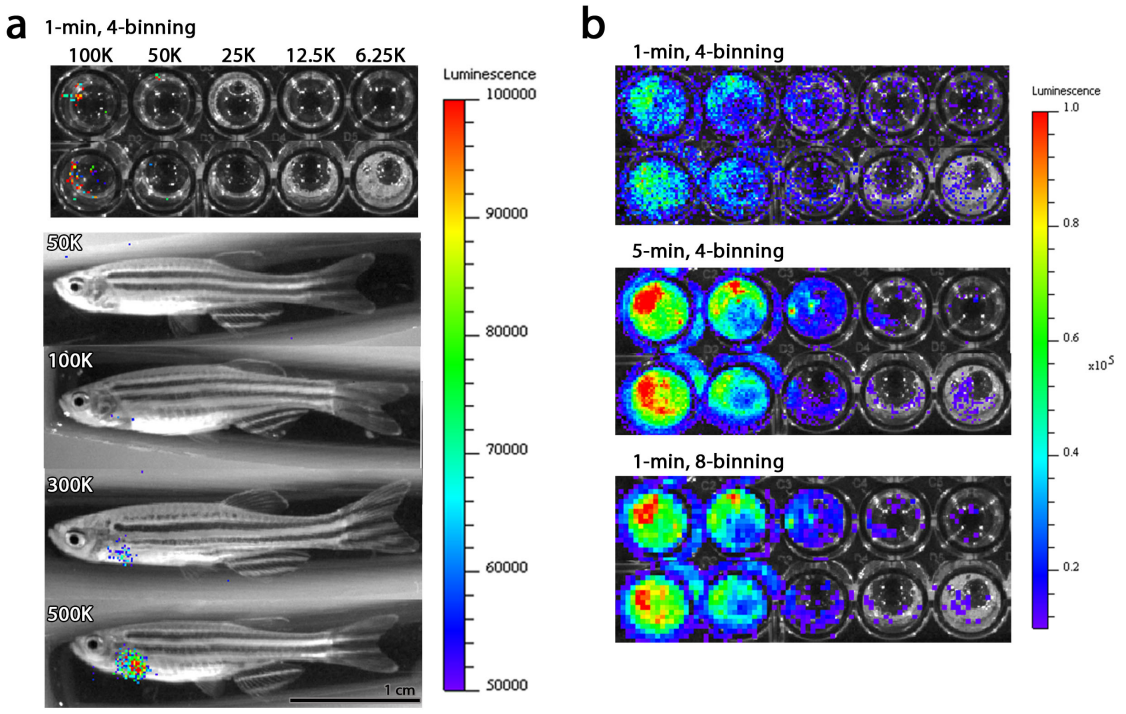

Figure 7. *In vitro* and *in vivo* bioluminescence in *ubi:luc* WKM cells. (a) *In vitro* and *in vivo* BLI taken at 1-minute exposure and 4x4 binning. The images were shown with optimal scale ($5x10^4$-$1x10^5$ p/s/cm^2/sr), which minimized the appearance of background noise. Least detectable signal was observed in 50,000 or more cells *in vitro* and in 300,000 or more cells *in vivo*. (b) Increased exposure time and binning improved the sensitivity. The *in vitro* BLI image from (a) with different scale ($1x10^4$-$1x10^5$ p/s/cm^2/sr) revealed more signals from the cells along with background noise (top). The sensitivity increased with longer exposure time (middle) and higher binning (bottom).

Time course of bioluminescent signal following luciferin injection

To determine the optimal time to acquire a bioluminescence image, we examined the time course of light emission following luciferin administration. After the substrate was injected intraperitoneally, we serially imaged the fish every 2-5 minutes. Luminescent signal reached plateau phase starting from 10 minutes after luciferin injection (Figure 8). This phase was maintained until at least 45 minutes after the injection. For the subsequent experiments, we therefore imaged the fish 10-15 minutes after substrate injection.

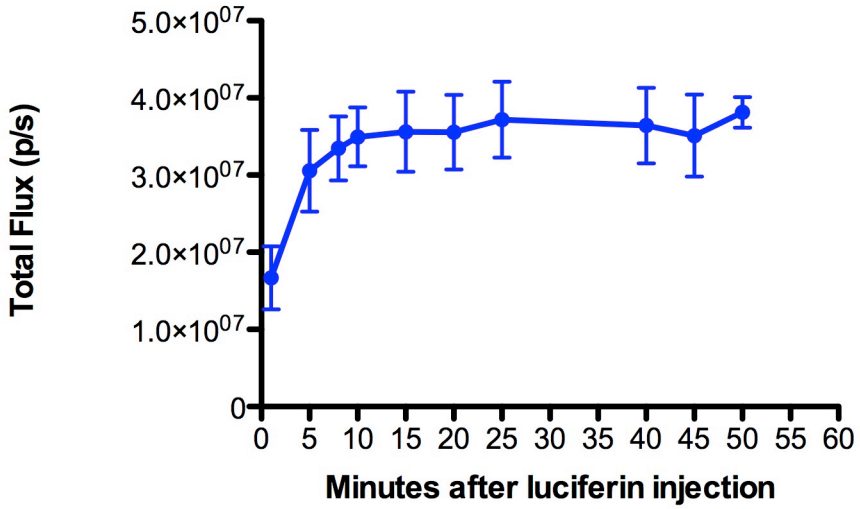

Figure 8. Time course of light emission following luciferin injection. Fish were injected with 5 µl of 15 mg/ml luciferin and then serially imaged. Data represents measurement from 3 fish.

Validation of bioluminescence imaging of donor-derived cells

To test the reliability of BLI data, we transplanted double-labeled cells expressing luciferase and GFP from *ubi:luc*/*h2afv:gfp* donors into 20-Gy irradiated wild-type zebrafish. After the fish were imaged, we harvested the WKM cells and measured the donor cell engraftment by flow cytometry. We observed a linear correlation between the intensity of the luminescent signals in the kidney or in the whole body with the donor cell contribution/engraftment data from flow cytometry (Figure 9).

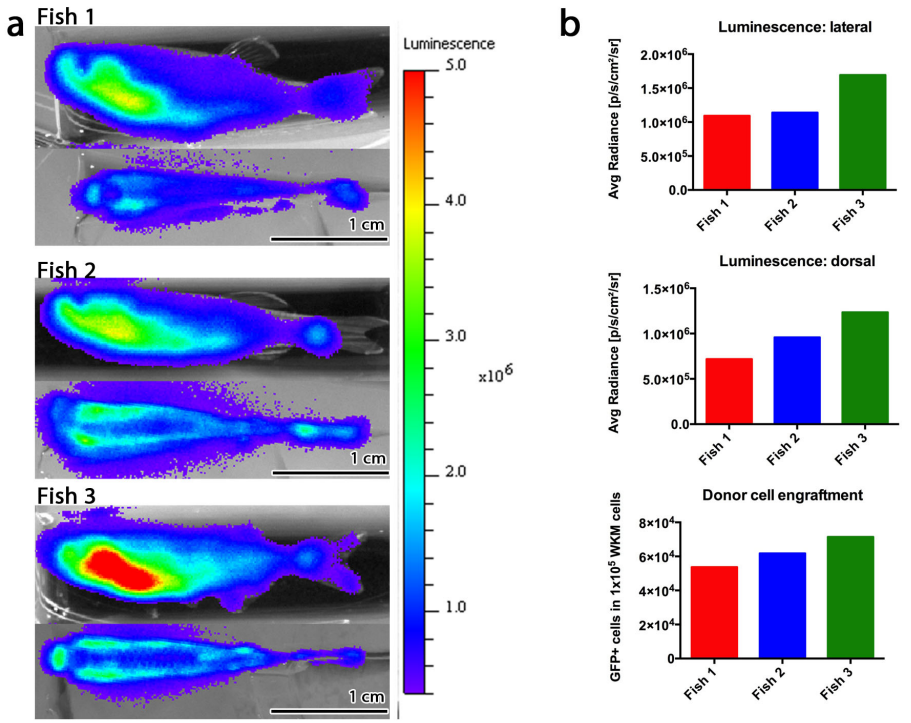

Figure 9. Correlation of luminescent signal and donor cell quantification by flow cytometry. (a) BLI on wild-type fish transplanted with 200,000 *ubi:luc*/*h2afv:gfp* WKM cells on day 13 post transplant from lateral and dorsal views. (b) Average radiance from lateral (top) and dorsal (middle) images in figure (a) and corresponding donor cell quantification obtained from flow cytometry (bottom).

Detection of homing and engraftment of donor cells following transplantation

To study the dynamics of homing and engraftment in adult zebrafish, we performed BLI on the recipient fish at multiple time points beginning from 1 day post transplantation (dpt) on non-irradiated and 20-Gy-irradiated recipients transplanted with 500,000 *ubi:luc* WKM cells. On day 1 the luminescent signals were detectable in the head kidney, the main site of hematopoietic tissue, in both non- and 20-Gy-irradiated recipients (Figure 10c and 10d). This finding indicated that, like in mammals, the migration of donor hematopoietic cells to the kidney did not require preconditioning of the host (Lapidot et al., 2005). In some fish, most of the signals at this time point were still located around the injection site, which may be emitted from the cells in the pericardial space that did not get into

the heart chambers. While donor cells were no longer detected in non-irradiated recipients by 7 dpt, more dispersed signals were observed in 20-Gy-irradiated recipients at this time, indicating donor cell engraftment. The whole-body signal in irradiated recipients continued to increase until 4 weeks post-transplant, after which it decreased to a lower, more steady level of luminescence (Figure 10b and 10d). This decline may be due to rejection of the donor cells by the recipients' immune system that had recovered from the sublethal 20-Gy irradiation.

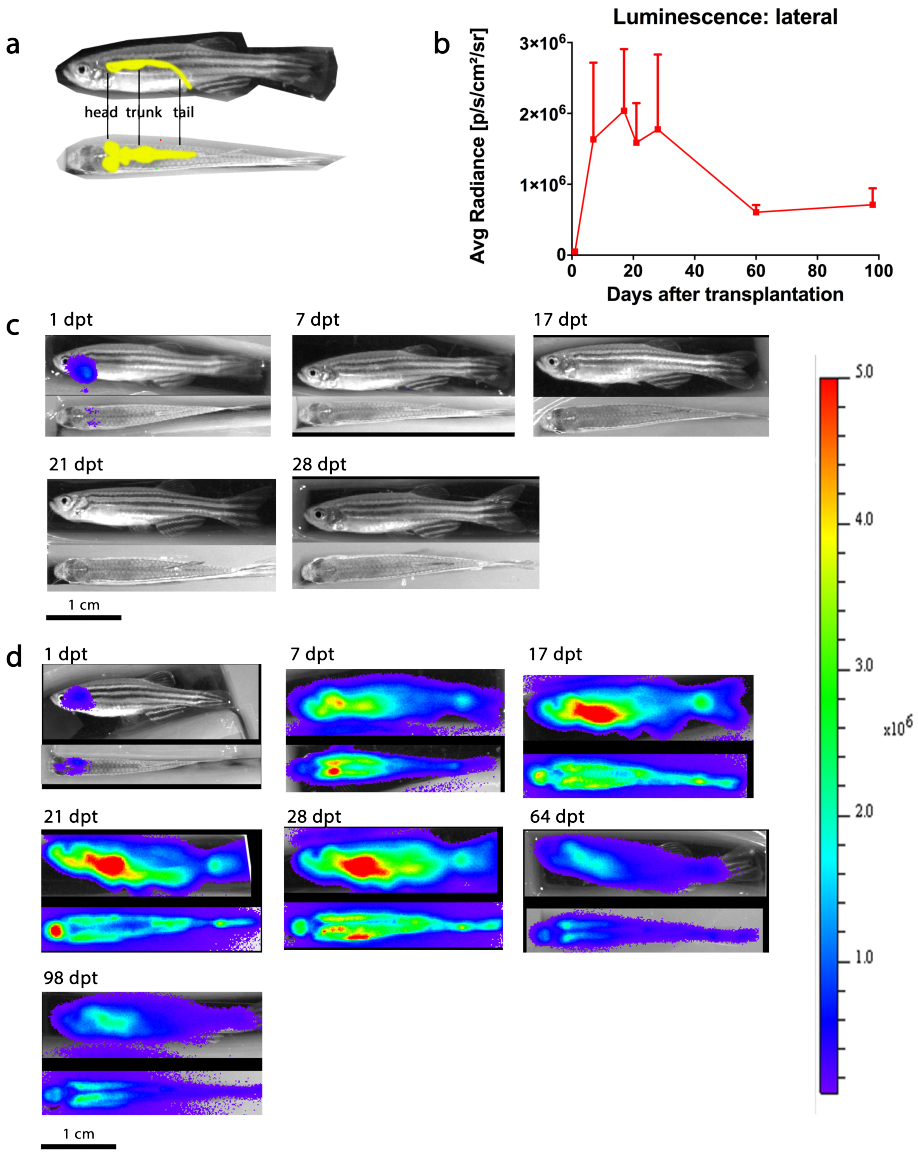

Figure 10. Homing and engraftment donor cells in adult zebrafish recipients. (a) Location of kidney in adult zebrafish (depicted in yellow). (b) Quantification of luminescence from lateral view of 20-Gy-irradiated recipients receiving 500,000 *ubi:luc* WKM cells at 1-98 dpt. (c) Serial BLI of non-irradiated recipient receiving 500,000 *ubi:luc* WKM cells from lateral (top) and dorsal (bottom) view. (d) Serial BLI of 20-Gy-irradiated recipient receiving 500,000 *ubi:luc* WKM cells from lateral (top) and dorsal (bottom) view.

Improvement of survival and early engraftment by prostaglandin

Prostaglandin E2 (PGE2) is an eicosanoid compound derived from arachidonic acid that has been known for its major role in inflammation, immune response, and more recently, in hematopoiesis. The effect of PGE2 on HSC was initially recognized through chemical screen in zebrafish, where exposure of its long acting derivative, 16,16-dimethyl-PGE2 (dmPGE2), enhanced the generation of definitive HSC (*runx1*$^{+}$/*cmyb*$^{+}$) in the AGM region of zebrafish embryos (North et al., 2007). This compound was also later shown to improve the hematopoietic recovery following radiation injury in zebrafish and mice, enhance the homing, survival, and engraftment of transplanted murine HSCs, and expand human UCB-derived HSCs (Goessling et al., 2011; Hoggatt, Singh, Sampath, & Pelus, 2009; North et al., 2007; Porter et al., 2013).

To test whether PGE2 has the same effect on engraftment in zebrafish as it does in mice, we treated the WKM cells with dmPGE2 before transplantation and analyzed the engraftment using BLI. We found significant increase of luminescent signal in recipients transplanted with dmPGE2-treated WKM cells in first week after transplant (Figure 11a and 11b). Furthermore, this finding coincided with significant decrease in mortality ($p = 0.03$, Wilcoxon test), which, as mentioned earlier, mostly occurred within first week after transplant (Figure 11c). At later time points, we found no significant difference between the two groups. This result was different from what was previously reported in mice transplanted with *ex vivo* dmPGE2-treated whole bone marrow (WBM) cells, where PGE2 treatment significantly enhanced long-term engraftment in primary and secondary recipients, indicating sustained PGE2 effect (Hoggatt et al., 2009). This may be due to the difference transplantation setting that we used in zebrafish, for example our experiment was not a competitive transplantation assay. Interestingly, *in vivo* exposure of PGE2 in mice was shown to affect MPPs/ST-HSCs, but not LT-HSCs, and consequently, transplantation with WBM cells from these mice only displayed improvement in short-term engraftment

(Frisch et al., 2009). The discrepancy between the effect of *in vivo* and *ex vivo* PGE2 exposure on HSC engraftment is still unclear.

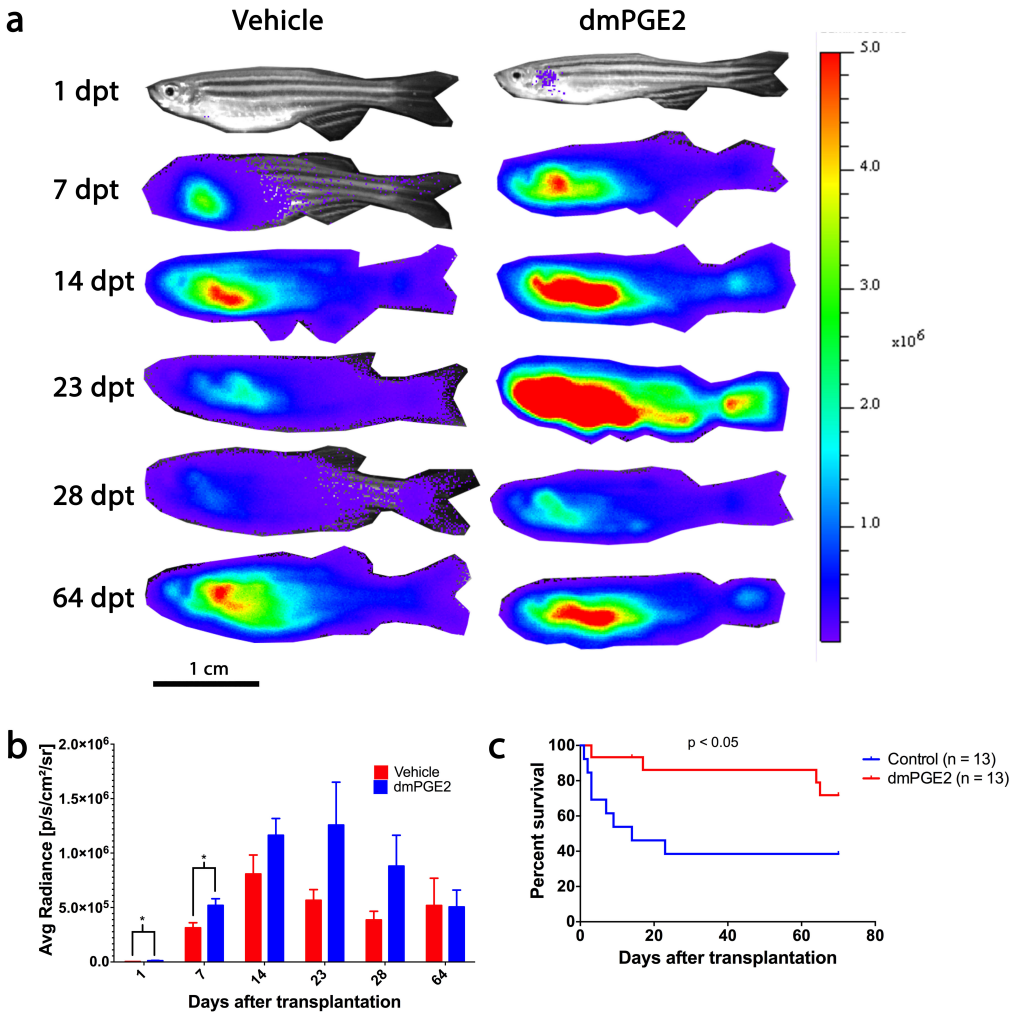

Figure 11. Engraftment and survival improvement by PGE2. (a) Serial BLI of recipients transplanted with vehicle- and dmPGE2-treated *ubi:luc* WKM cells. (b) Light emission quantification of recipients in control and treatment groups. (c) Survival graph of recipients in control and treatment groups.

DISCUSSION

The development of new techniques together with inherent beneficial attributes of the zebrafish model have been the major driving forces behind the emergence of this vertebrate as prominent model organism. Likewise, the ability to perform HCT assays has expanded the possibility to study stem cell and transplant biology. Furthermore, as a small organism with high fecundity and low maintenance cost, zebrafish serve as a cost-effective organism to use in screening studies that prefer large sample sizes. To facilitate these studies, *in vivo* optical imaging that offers rapid analysis of donor cell homing and engraftment is needed. This approach is currently achievable in adult zebrafish by using fluorescence imaging in *casper*, a transparent mutant zebrafish (White et al., 2008). Although this technique has important advantages, such as high resolution and rapid image acquisition, fluorescence imaging is commonly hampered by high background noise, preventing the detection of low signals.

In this study, we demonstrated BLI as useful tool to *in vivo* track the transplanted donor cells in the adult zebrafish that allowed the engraftment process to be observed continuously. The optical imaging data were consistent with results from flow cytometry analysis. In addition, using this imaging method, we were also able to observe the effect of PGE2 in improving short-term engraftment and survival in zebrafish, further supporting the suitability of BLI as analysis tool in drug screening studies.

We found that the BLI procedure increased the mortality of the recipient fish. Modification of the anesthesia regimen using combination of tricaine and isoflurane, together with the use of hyper-oxygenated water during imaging, increased the survival to 25-50%. The low survival of recipient fish has actually been a problem in zebrafish HCT as this procedure is still in its early development. In addition, the damaging effect of BLI on the fish was not greater than that caused by irradiation, and thus, the mortality rate still reflected the

biological process of radiation injury and hematopoietic reconstitution by donor cells as the survival was improved in fish transplanted with dmPGE2-treated WKM cells. Therefore, refinements in the BLI technique, along with further improvements in HCT procedures as well as the supportive treatments (better water quality, isolated environment, the ability to administer antibiotic to fish, etc.) will greatly enhance the survival in this system.

In summary, our study showed that BLI can be used to track the transplanted hematopoietic cell in living recipient fish. Using this technique, we were able to demonstrate the homing and engraftment processes following HCT as well as the effect of PGE2 on short-term engraftment and survival in zebrafish. Future study will be done to apply this system for screening of compounds that could increase the speed of engraftment and/or hematopoietic cell recovery.

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