Bone Marrow Transplantation Procedures in Mice to Study Clonal Hematopoiesis

Eunbee Park¹, Megan A. Evans⁴, Heather Doviak⁴, Keita Horitani⁴, Hayato Ogawa⁴, Yoshimitsu Yura⁴, Ying Wang², Soichi Sano^{3,4}, Kenneth Walsh^{1,4}

¹Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine

²Department of Cardiology, Xinqiao Hospital, Army Medical University

³Department of Cardiology, Osaka City University Graduate School of Medicine

⁴Hematovascular Biology Center, Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine

Abstract

Clonal hematopoiesis is a prevalent age-associated condition that results from the accumulation of somatic mutations in hematopoietic stem and progenitor cells (HSPCs). Mutations in driver genes, that confer cellular fitness, can lead to the development of expanding HSPC clones that increasingly give rise to progeny leukocytes harboring the somatic mutation. Because clonal hematopoiesis has been associated with heart disease, stroke, and mortality, the development of experimental systems that model these processes is key to understanding the mechanisms that underly this new risk factor. Bone marrow transplantation procedures involving myeloablative conditioning in mice, such as total-body irradiation (TBI), are commonly employed to study the role of immune cells in cardiovascular diseases. However, simultaneous damage to the bone marrow niche and other sites of interest, such as the heart and brain, is unavoidable with these procedures. Thus, our lab has developed two alternative methods to minimize or avoid possible side effects caused by TBI: 1) bone marrow transplantation with irradiation shielding and 2) adoptive BMT to non-conditioned mice. In shielded organs, the local environment is preserved allowing for the analysis of clonal hematopoiesis while the function of resident immune cells is unperturbed. In contrast, the adoptive BMT to non-conditioned mice has the additional advantage that both the local environments of the organs and the hematopoietic niche are preserved. Here, we compare three different hematopoietic cell reconstitution approaches and discuss their strengths and limitations for studies of clonal hematopoiesis in cardiovascular disease.

Introduction

Clonal hematopoiesis (CH) is a condition which is frequently observed in elderly individuals and occurs as a result of an expanded hematopoietic stem and progenitor cell (HSPC) clone carrying a genetic mutation¹. It has been suggested that by the age of 50, most individuals

Corresponding Author: Kenneth Walsh, kw9ar@virginia.edu.

Disclosures

The authors have nothing to disclose.

will have acquired an average of five exonic mutations in each HSPC², but most of these mutations will result in little or no phenotypic consequences to the individual. However, if by chance one of these mutations confers a competitive advantage to the HSPC—such as by promoting its proliferation, self-renewal, survival, or some combination of these—this may lead to the preferential expansion of the mutant clone relative to the other HSPCs. As a result, the mutation will increasingly spread through the hematopoietic system as the mutated HSPC gives rise to mature blood cells, leading to a distinct population of mutated cells within the peripheral blood. While mutations in dozens of different candidate driver genes have been associated with clonal events within the hematopoietic system, among these, mutations in DNA methyltransferase 3 alpha (*DNMT3A*) and ten eleven translocation 2 (*TET2*) are the most prevalent³. Several epidemiological studies have found that individuals who carry these genetic mutations have a significantly higher risk of cardiovascular disease (CVD), stroke, and all-causal mortality^{3,4,5,6,7}. While these studies have identified that an association exists between CH and increased incidence of CVD and stroke, we do not know whether this relationship is causal or a shared epiphenomenon with the aging process. To gain a better understanding of this association, proper animal models that correctly recapitulate the human condition of CH are required.

Several CH animal models have been established by our group and others using zebrafish, mice, and non-human primates^{8,9,10,11,12,13,14}. These models often use hematopoietic reconstitution methods by transplantation of genetically modified cells, sometimes using Cre-lox recombination or the CRISPR system. This approach allows for the analysis of a specific gene mutation in hematopoietic cells to assess how it contributes to disease development. In addition, these models often employ congenic or reporter cells to distinguish the effects of mutant cells from normal or wild-type cells. In many cases, a pre-conditioning regimen is required to successfully engraft donor hematopoietic stem cells.

Currently, the transplantation of bone marrow to recipient mice can be divided into two main categories: 1) myeloablative conditioning and 2) non-conditioned transplantation. Myeloablative conditioning can be achieved by one of two methods, namely, total body irradiation (TBI) or chemotherapy¹⁵. TBI is carried out by subjecting the recipient to a lethal dose of gamma or X-ray irradiation, generating DNA breaks or cross-links within rapidly dividing cells, rendering them irreparable¹⁶. Busulfan and cyclophosphamide are two commonly used chemotherapy drugs that disrupt the hematopoietic niche and similarly cause DNA damage to rapidly dividing cells. The net result of myeloablative preconditioning is apoptosis of hematopoietic cells, which destroys the recipient's hematopoietic system. This strategy not only allows for the successful engraftment of the donor HSPCs, but can also prevent graft rejection by suppressing the recipient's immune system. However, myeloablative preconditioning has severe side effects such as damage to tissues and organs and their resident immune cells as well as destruction of the native bone marrow niche¹⁷. Therefore, alternative methods have been proposed to overcome these undesirable side effects, particularly in regard to damage to the organs of interest. These methods include shielded irradiation of recipient mice and the adoptive BMT to non-conditioned mice^{9,17}. Shielding the thorax, abdominal cavity, head or other regions from irradiation by the placement of a lead barriers keeps tissues of interest protected from the damaging effects of irradiation and maintains their resident immune cell

population. On the other hand, the adoptive BMT of HSPCs to non-conditioned mice has an additional advantage because it preserves the native hematopoietic niche. In this manuscript, we describe the protocols and results of HSPC engraftment after several transplantation regimens in mice, specifically the delivery of HSPC to TBI mice, to mice partially shielded from irradiation, and to non-conditioned mice. The overall goal is to help researchers understand the different physiological effects of each method as well as how they affect experimental outcomes in the setting of CH and cardiovascular disease.

Protocol

All procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia.

1. Prior to preconditioning

1. Place the recipient mice on antibiotic-supplemented water (5 mM sulfamethoxazole, 0.86 mM trimethoprim) ~24 h prior to irradiation. This is necessary to prevent infection, as the immune system will be suppressed following irradiation, and maintained for 2 weeks following irradiation. At this point, supplement mice with a nutritional/hydration gel to encourage feeding and to prevent weight loss and dehydration after irradiation.

2. Preconditioning of recipient mice (optional)

1. Total body irradiation

1. Place recipient mice into a uniformly sliced pie-cage, or a mouse cage in the reflective chamber within the calculated radius to receive same irradiation dose; however, a maximum of 8 mice per pie-cage and 5 mice per mouse cage is recommended to ensure uniform irradiation (Figure 1A,B).
2. To achieve complete myeloablation, ensure that recipient mice receive a total radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4–24 h interval.

NOTE: While optimal engraftment can be obtained by implementing a 4 h interval between fractions, this can be extended to a 24 h interval, which can be helpful when labor and/or the irradiator is unavailable.

2. Partially shielded irradiation

1. Anesthetize the recipient mice by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg). The restraint of mouse movement is critical to ensure the uniform irradiation and effective protection of the targeting organs during the shielding process.
2. For thorax and abdomen shielding, orient the radiation beam of the X-ray irradiator vertically to the mouse (Figure 1C).
 1. Position the anesthetized mice onto a flat plate, centering the radiation source from above. Place the mice inverted to each other in a supine position with arms and legs fully extended (Figure 1D,E).

NOTE: X-ray irradiator facility, for this experiment, allows that two mice at a time can be positioned within the effective radius that allows uniform irradiation. While the effective radius is calculated based on the distance between the radiation source and the tray, the number of animals that can be simultaneously irradiated will depend upon the specific irradiator.

2. Fasten the paws of the mice onto the plate using tape to ensure the mice are immobilized during the irradiation procedure. Place lead shielding so that it covers regions that require protection.
3. For thorax shielding, prepare the lead shield by measuring the length from the mouse's xiphisternum bone to the thymus and calculating the thickness that will provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns with the xiphisternum bone. The upper end of the lead barrier will fit near the thymus (Figure 1D).
4. For abdomen shielding, prepare the lead shield by measuring the length from the mouse's anus to the diaphragm and calculating the thickness that will provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns with the anus. The upper end of the lead shield will fit below the diaphragm (Figure 1E).

NOTE: Localizing the lead shield to be consistent among cohorts may reduce some variation with regard to the size of the mice.

3. For head shielding, orient the radiation beam of the Cesium irradiator horizontally to the mouse.
 1. Carefully tape the forepaws of an anesthetized mouse to the abdomen. This ensures that the arms get a full dose of irradiation and are not covered by the shield.
 2. For head shielding, place the mouse in a conical restrainer, which fits inside a lead shield. Once the mouse is inside the conical restrainer, slide the restrainer into the slot within the lead shield (Figure 1F). The lead shield should completely cover the mouse's head and ears (~3.2 cm), leaving the rest of the mouse's body exposed for irradiation. The position of the restrainer inside the shield can be adjusted to fit different sized animals by sliding it further inside or outside the shield.
 3. Place mice inside the irradiator, perpendicular to the source for irradiation.
4. Expose mice to two 5.5 Gy fractions of irradiation (total dose of 11 Gy) separated by a 4–24 h interval.
5. After each irradiation fraction, place the cages with anesthetized mice on heated mats or under red heat lamps to prevent hypothermia and aid in the recovery from anesthesia.

NOTE: Caution must be taken to not overheat the anesthetized mouse when using a lamp since they cannot escape the heat. As described above, the positioning of animals and the thickness of lead shield can differ between studies based upon the specific features of the irradiator (radiation type/direction of beam, etc.). Researchers will need to adjust their experiments accordingly.

3. Bone isolation—NOTE: Ideally, donor mice and recipient mice should be similar in age, and within 8–12 weeks old. Using at least 3 mice as donors (rather than single donor) is preferred to minimize for heterogeneity (even when using mice with the same genotype). Approximately, 40 million unfractionated bone marrow cells can be obtained from six bones (two femurs, two tibias, and two humeri) of a single mouse. Transplantation of 5 million bone marrow cells to each recipient mouse will typically ensure engraftment.

1. Euthanize donor mice by cervical dislocation without anesthesia (preferred method to avoid chemical contamination of cells) and place each mouse onto an absorbent pad.
2. Disinfect the skin using a 70% ethanol spray.
3. Make a small transverse cut in the skin below the rib cage and hold the skin tightly at either side of the incision, tear in opposite directions toward the head and feet. Peel off the skin from all the limbs.
4. Cut over the shoulders and the elbow joints, and remove the attached muscles and connective tissues with the aid of a Kimwipe to obtain the humeri.
5. Carefully dislocate the hip joints between the femur and hip bones. Use blunt scissors to cut along the femur head and detach the legs. Cut over the knee joint to separate the femur and tibia, and carefully remove the attached muscles and connective tissues with the aid of a Kimwipe to harvest the femur and tibia.

NOTE: Pay special attention to keep the bone epiphysis intact during this step. Discard any broken bones due to loss of sterility. Hip bones and spine bones can be collected in addition to the femur, tibia, and humerus. To collect spine bones, a mortar and a pestle can be used to crush the bones into pieces and harvest the bone marrow cells.

6. Place the isolated bones from mice of the same genotype into correspondingly 50 mL conical tube containing 20 mL ice-cold sterile PBS, and keep it on ice until further use. Pay special attention to correctly place the bones into tubes with matched genotypes.
7. Repeat the above steps for each donor animal changing gloves in between each mouse. Also, clean scissors and other instruments with 70% ethanol between each mouse.

4. Bone marrow cell isolation—NOTE: Perform the following steps in a biosafety class II cabinet.

1. Preparation of tube sets: Make a small hole in the bottom of a sterile 0.5 mL microcentrifuge tube using an 18 G needle and place it into a sterile 1.5 mL microcentrifuge tube, which contains 100 μ L of ice-cold sterile PBS at the bottom.

NOTE: As only six bones can fit into the 0.5 mL microcentrifuge tube, it is recommended to prepare sufficient tube sets to process all the bones at the same time.
2. Aspirate the PBS and transfer the isolated bones onto a sterile 100 mm cell culture dish. Holding each bone using fine forceps, carefully cut the epiphyses off each end using small scissors that were sterilized in an autoclave. Place the cut bones into the prepared tube sets.
3. Centrifuge the tubes at 10,000 $\times g$ for 35 s at 4 $^{\circ}$ C.
4. After centrifugation, confirm that the bone marrow has been successfully removed from the bones. Bones should appear white and translucent with a relatively large red pellet at the bottom of the 1.5 mL microcentrifuge tube. Discard the 0.5 mL microcentrifuge tube.

NOTE: If the visual inspection fails to detect bone marrow at the bottom of the 1.5 mL tube, cut the bone again and repeat step 4.3.
5. Resuspend the bone marrow in 1 mL of ice-cold PBS, then transfer the cell suspension from the same genotype to a matched 50 mL conical tube.
6. Dissociate the cells by passing them through an 18 G needle with a 10 mL syringe 10 times.
7. Filter single cell suspensions through a 70 μ m cell strainer. Add additional ice-cold PBS to a final volume of 10 mL, and resuspend the cells through the gentle use of pipette-aid.
8. Centrifuge at 310 $\times g$ for 10 min at 4 $^{\circ}$ C.
9. Aspirate the supernatant and resuspend the cell pellet with 10 mL of serum-free RPMI media. Spare 30 μ L of this material for cell counting.
10. Determine cell concentration with a cell counter, and calculate the volume of cell suspension required for the transplantation. For the example of a 100% BMT, 5 $\times 10^6$ bone marrow cells are required for each recipient mouse.

NOTE: For a competitive BMT, prepare a total of 5 $\times 10^6$ bone marrow cells comprising a mixture of donor cells (e.g., CD45.2⁺) and competitor cells (e.g., CD45.1⁺). Preparing extra bone marrow cells is highly recommended. For example, if there are 10 recipient mice per experimental group, we typically prepare enough cells for 12 recipient mice.
11. Transfer the calculated volume of cell suspension into a new 50 mL conical tube. Centrifuge at 310 $\times g$ for 10 min at 4 $^{\circ}$ C.

12. Aspirate the supernatant and resuspend the cells using the calculated amount of serum-free RPMI medium to achieve the appropriate cell density and volume. Typically, 200 μ L is the optimal volume for a retro-orbital injection.

5. Transplantation of bone marrow cells to irradiated mice

1. Anesthetize the recipient mice with 5% isoflurane.
2. While mice are anesthetized, slowly inject 200 μ L of bone marrow cells into the retro-orbital vein using a 28–30 G needle with an insulin syringe.
 1. Alternatively, perform the delivery of the donor cells by tail vein intravenous injection and femoral intramedullary injection, with a maximum volume of 0.2 mL and 25 μ L, respectively.
3. Once the cells are injected, place a drop of proparacaine-containing eye-drops onto the surface of the eye for pain relief. The animal can then be allowed to regain consciousness.

6. Transplantation of bone marrow cells to non-conditioned mice

1. Anesthetize the recipient mice by inhalation of 5% isoflurane.
2. Inject 5×10^6 unfractionated bone marrow cells from either genotype retro-orbitally into non-irradiated recipient mice with 28–30 G insulin syringe.
3. Repeat steps 6.1 and 6.2 over 3 consecutive days, such that the recipient mice will be transplanted with a total of 1.5×10^7 bone marrow cells.

NOTE: Because the adoptive BMT without pre-conditioning procedure requires bone marrow transplantation for 3 consecutive days, one should attempt to alternate eyes for each injection.
4. Post-injection, administer a drop of proparacaine-containing eye drops to the affected eye.

Representative Results

To compare the effect of three BMT/pre-conditioning methods on donor cell engraftment, the fractions of donor cells in peripheral blood and heart tissue were analyzed by flow cytometry at 1-month post-BMT. Isolated cells were stained for specific leukocyte markers to identify the different subsets of leukocytes. In these experiments, wild-type (*WT*) C57BL/6 (CD45.2) donor bone marrow cells were delivered to *WT* B6.SJL-*Ptprca*^a*Prpc*^b/BoyJ (CD45.1) recipient mice to distinguish donor cells from the recipient's cells. The flow cytometry gating strategies that were used are described previously by Wang et al.⁹ in **Supplementary Figure 1**.

The total body irradiation (TBI) treated group received 5×10^6 bone marrow cells following a total lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval. In the peripheral blood of recipient mice, monocytes, neutrophils, and B cells were largely ablated and replaced by the progeny of donor bone marrow-derived cells. In addition, the

resident cardiac monocyte and neutrophil population in hearts of recipient mice were almost completely replaced by donor-derived cells (Figure 2A).

In the partially shielded irradiation group, recipient mice were irradiated with a thorax shield and transplanted with 5×10^6 bone marrow cells following a total radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval. In contrast to the TBI group, the contribution of donor-derived cells to cardiac immune cells was modest, which probably reflects the combined effects of protecting the local immune cells in the hearts of recipient mice and the physiological repopulation of bone marrow-derived donor cells from the peripheral blood. Recipient mouse bone marrow cells in shielded regions are also likely to have contributed to peripheral blood reconstitution, which reduces the percentage of donor derived cells in peripheral blood compared to the TBI treated group (Figure 2B).

In the group without BMT pre-conditioning (adoptive BMT), recipient mice were transplanted with 5×10^6 bone marrow cells over 3 consecutive days. At 4 weeks post-BMT, the portion of donor-derived cells in peripheral blood and heart was detectable, (approximately 5%) (Figure 2C).

To illustrate how the adoptive BMT model can be applied to the study of the CH model, CD45.2⁺ donor bone marrow cells (*WT* or *Tet2*^{-/-}) were transplanted into CD45.1⁺ recipient mice. Recipients without conditioning were transplanted with 5×10^6 bone marrow cells each day for 3 consecutive days (for a total of 1.5×10^7 , $n = 5-6$ per group). Flow cytometric analysis of peripheral blood was performed 4, 8, 12, and 16 weeks post-transplantation. The *Tet2-deficient* donor cells conferred a competitive advantage and gradually expanded over time; WBCs, monocytes, Ly6C^{hi} monocytes, neutrophils, T cells, and B cells increased significantly over time. Compared to the *Tet2-deficient* donor cells engrafted into recipient mice, recipient mice engrafted with *WT* donor cells showed less significant clonal expansion of donor cells (Figure 3). Consistent with the clinical paradigm of clonal hematopoiesis, the expansion of *Tet2-deficient* cells does not impact the absolute numbers of the various blood cell types⁹ (data not shown).

Discussion

For studies of clonal hematopoiesis, we described three methods of BMT: BMT with total-body irradiation, BMT with irradiation with partial shielding, and a less commonly used BMT method that involves no pre-conditioning (adoptive BMT). These methods have been used to assess the impact of clonal hematopoiesis on cardiovascular disease. Researchers can modify these methods accordingly to suit the specific purpose of their study.

Clonal hematopoiesis models

Clonal hematopoiesis is the phenomenon in which mutant hematopoietic cells compete with wild-type cells and obtain clonal dominance over time. To create a model of this competition, mice can be administered bone marrow, which consists of a mixture of genetically different cells. Generally, this mixture will include mutant and wild-type cells, which have been labeled with a fluorescent tag or different panleukocyte markers (i.e., CD45.1 and CD45.2). For example, when creating models that mimic *Tef2*-mediated CH,

we perform a competitive bone marrow transplantation into lethally irradiated recipients that typically involves mixing 90% of cells that originate from CD45.1 *Tet2*^{+/+} donors and 10% of cells that originate from CD45.2 *Tet2*^{-/-}, *Tet2*^{+/-} or control *Tet2*^{+/+} donors⁸. Flow cytometry detection of the CD45 variants using specific monoclonal antibodies enables one to distinguish donor cells (CD45.1⁻/CD45.2⁺) from competitor cells (CD45.1⁺/CD45.2⁻) within the blood, and assess the clonal dynamics of the test cells over time.

By doing so, we have been able to observe a gradual increase in donor chimerism of *Tet2*-deficient cells, while the percentage of wild-type cells remain at approximately 10%. This experimental setting mimics the human scenario of individuals carrying a *TET2* somatic mutation, since these mutations are initially carried by a small number of HSPCs, which will gradually expand overtime. Employing this approach in cardiovascular disease models of atherosclerosis and heart failure has led to the documentation of a potential causal link between *Tet2*-mediated CH and CVD^{8,9,10}.

When employing these approaches, researchers should take into consideration the possible confounding effects generated by TBI. Although TBI prior to transplantation enables a high degree of HSPC engraftment, this pre-conditioning will lead to several undesirable effects outside of the hematopoietic system. It has been documented that TBI can lead to inflammation, injury, and fibrosis in multiple organ systems including skin, liver, kidneys, lungs, bone marrow, heart, brain, etc.^{18, 19, 20}. These side effects can negatively impact the cardiovascular organs under study, and they also alter disease pathogenesis^{21, 22}. A notable example is the effect of irradiation on resident macrophages in the heart. Irradiation of the thorax results in a marked replacement of cardiac-resident macrophages with circulating monocyte-derived macrophages. Studies have shown that cardiac-resident macrophages display distinct characteristics relative to circulating monocyte-derived macrophages that will engraft in the heart following radiation injury. In disease settings, cardiac-resident macrophages are thought to play a cardioprotective role, whereas infiltrating blood-borne macrophages have been reported to promote injury and inflammation²³. Therefore, it is conceivable that replacing resident cardiac immune cells with blood-borne cells will alter the pathological processes under study, which contribute to cardiovascular disease^{24,25,26}. Similarly, in the brain, TBI results in depletion of resident microglia and replacement by peripherally-derived macrophages^{27, 28}. While peripherally-derived macrophages can behave like microglial cells, they maintain a unique functional and transcriptional identity compared to monocyte-derived microglia²⁸. Therefore, it is possible that the disease sequela may be altered, particularly when studying diseases such as ischemic strokes. In order to avoid these confounding effects, shielding the thorax and head can be recommended. This is advantageous because it provides protection to the heart and brain, respectively; and it also maintains their resident immune cells intact, better recapitulating the human condition of CH. However, as noted previously, shielding results in a lower rate of chimerism compared to TBI pre-conditioning, which essentially eliminates all the host's hematopoietic cells.

Another important impediment in pre-conditioning is its deleterious effect on the bone marrow niche. Although irradiation-induced damage of the BM niche can be restored to a suboptimal extent, it is unclear whether naïve hematopoiesis is recovered in these damaged microenvironments. In addition, transplantation of mixed HSPCs into empty BM initiates

a race for proliferation between clones, rather than the simple “competition” for a niche that is occupied by a wild-type HSPC—which is presumably what occurs in CH. Thus, a potentially preferable approach to studying CH may be the adoptive BMT method, whereby BM cells are transferred into recipient mice without pre-conditioning. This adoptive BMT without pre-conditioning method minimally affects the ongoing naive hematopoiesis, most faithfully recapitulating CH observed in humans²⁹. Figure 2C shows the level of chimerism at 1-month post-transplantation without pre-conditioning. While the donor chimerism is low at this early timepoint, we find a progressively increasing fraction of *Tet2-deficient* clones over time, as presented in Figure 3. It should be noted that this model is most useful when the mutant cells have a competitive advantage over wild-type cells under homeostatic conditions such as *Tet2-deficient* cells. When *Tet2-deficient* cells are engrafted, there is a marked expansion in various leukocyte populations such as neutrophils, monocytes, NK cells, and B cells. A slower expansion was noted in T cells, presumably due to the longer half-life of this population.

The expansion of *Tet2-deficient* cells has been observed not only in the peripheral blood but also in several other tissues, including bone marrow, liver, and kidney, with different dynamics of hematopoietic cell reconstitution⁹. For example, our lab’s previous published paper described the bone marrow cell chimerism of *WT* and *Tet2-deficient* donor cells engrafted into CD45.1 recipient mice 8 months after adoptive BMT⁹. *Tet2-deficient* donor cells transplanted into CD45.1 recipient mice have shown a competitive advantage over immature lineage[−]Sca1⁺ c-Kit⁺ (LSK) cells, short-term and long-term HSC cells, and multipotent progenitors (MPPs) compared to that of *WT* donor cells transplanted into the CD45.1 recipient mice. In addition, as *Tet2-deficient* donor cell engrafted recipient mice, they develop an age-related cardiomyopathy phenotype without exogenous factors causing cardiac dysfunction, thereby recapitulating the effect of clonal hematopoiesis in a manner similar to that of aging humans. This observation was accompanied with increased degree of chimerism in cardiac neutrophils and Ly6C^{hi} monocytes. Collectively, this adoptive BMT regimen can be applied to future studies that could expand our understanding of the association between cardiovascular disease development and clonal hematopoiesis on a more advanced level.

In summary, we described three BMT methods and discussed their application in generating CH models. CH is associated with poorer prognoses in cardiovascular diseases such as atherosclerosis and heart failure. Although considerable progress has been made, the study of the causal links between CH and CVD is still in its infancy, and further investigations are required through the use of optimized animal models. We hope that these protocols allow researchers to select a more physiologically appropriate method of BMT, which minimizes potential confounding effects on the cardiovascular system, ultimately yielding studies that expand our understanding of how CH contributes to cardiovascular disease.

Design of lead shielding

The thickness of the lead shield will be dictated by the type of irradiation used to induce the myeloablation. X-ray or gamma-ray types of radiation are frequently used for experimental myeloablation but differ in terms of their frequency, wavelength, and photon energy. When it

comes to shielding, the photon energy, which describes the energy or speed at which the rays are traveling, is the most important parameter. Typically, radiation source X-rays have an energy of 160 kVp whereas cesium-137 sources, which emit gamma rays, have an energy of 662 KeV. The energy emitted by these radiation sources equates to their penetration power, with higher energies having a greater penetration power. Therefore, a greater thickness of lead shield is required when using cesium source-based irradiators in comparison with using X-ray-based irradiators. X-ray-based irradiation, which we use when we perform thorax and abdominal shielding, requires a 7 mm thick lead shield to provide sufficient protection. However, for cesium 137 sources, which we use when we perform head-shielding, requires lead shields to be at least 1 inch thick to provide sufficient protection.

Lead shields for use in X-ray irradiators can be purchased from commercial vendors. Alternatively, lead sheets can be cut to size to either fit around the animal's body or to fit around a restrainer (see Figure 1). When using a cesium-based irradiator, lead bricks, which are considerably thicker should be used and can be custom made by companies that specialize in making these types of shields. For instance, for the headshield, we custom designed a lead brick to hold a 50 mL conical tube restrainer (see Figure 1F). The animal is able to fit inside the restrainer, which is then slotted into a hole made in the brick, to provide 1.5 inches of protection from the irradiation. Importantly, all lead shields should be coated either with paint or tape to prevent exposure to lead dust, which can be toxic.

Based on the equipment and its parameters, researchers can design their own lead shields for their sites of interest. Here, we introduced thorax, abdominal, and head shielding; however, other sites such as limb or flank can be considered for shielding as well. In addition, while both radiation sources (Cesium-137 and X-ray) are suitable for bone marrow ablation and successful engraftment, variability in the reconstitution of lymphoid and myeloid cell populations has been observed between Cesium-137 and X-ray irradiation sources³⁰. Thus, researchers should take into account the disparate physiological responses to the radiation source for use in studies.

Dosing intervals

Dosage and dosing intervals may affect the efficiency of donor cell engraftment and survival rates. In human patients, high-dose irradiation can cause idiopathic interstitial pneumonia, gastrointestinal injury, and cataract formation. In mouse models, single high-dose irradiation followed by bone marrow transplantation can produce similar results and can also affect survival rates³¹. Therefore, fractionated irradiation is highly recommended for the mouse BMT studies. In addition, the dosing intervals of fractions can impact mouse survival rate and reconstitution rate, leading to different fractions of donor cell chimerism in the hematological organs as well as other tissues³¹. Thus, researchers should be careful in designing a fractionated irradiation dosing schedule for BMT studies.

In the context of survival rate and immune cell reconstitution, our small study showed that a group of mice receiving total body irradiation with a lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 24 h interval group had no significantly different results than the group which received the same TBI dosage at a 4 h interval (see Table 1). However, with thorax-shielding BMT, the 24 h interval group appeared to show less efficiency of donor cell

chimerism in comparison with the 4 h interval group. A possible explanation for this result is that irradiation with 24 h interval may not have been sufficient to remove the recipient's immunocompetent cells because the prolonged intervals gave recipient mice sufficient time to repair damaged cells. In addition, thorax-shielding protects partial spine bones that also contain the recipient's HSCs. Thus, the remaining and recovered immunocompetent recipient cells may have attacked the donor-derived cells and induced an outcome that showed lower engraftment efficiency.

Animal care

Multiple steps involving mice are required for the success of this experiment. Thus, extra attention is required at the following points: First, the delivery of viable donor cells is crucial for successful engraftment. One should be properly trained in the collection of intact BM cells and their injection into recipient mice. Consequences of poor delivery of cells include the failure of donor HSPCs to reconstitute recipient bone marrow leading to mortality. Second, care must be taken after transplantation to avoid infection, particularly following myeloablative therapy. Contact with pathogens can be fatal, as mice become transiently immunodeficient following irradiation. As indicated above, supplementing the drinking water with antibiotics can lower the risk of fatal infection. Moreover, providing recipient animals with nutritional/hydration gel can minimize dehydration and nutritional deficiencies which may occur following irradiation, as irradiation can disrupt the intestinal epithelium leading to diarrhea³². Cages should also be replaced frequently to reduce the risk of fecal bacteria contamination, animals should be handled in a proper animal transfer station, and the recipient mice should be monitored carefully for weight loss and any signs of distress or pain.

Acknowledgments

This work was supported by US National Institutes of Health grants to K. Walsh (HL 131006, HL 138014, and HL 132564), to S. Sano (HL 152174), American Heart Association grant to M. A. Evans (20POST35210098), and a Japan Heart Foundation grant to H. Ogawa.

References

1. Evans MA, Sano S, Walsh K Cardiovascular disease, aging, and clonal hematopoiesis. *Annual Review of Pathology: Mechanisms of Disease*. 15 (1), 419–438 (2020).
2. Welch J Set al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 150 (2), 264–278 (2012). [PubMed: 22817890]
3. Jaiswal Set al. Age-related clonal hematopoiesis associated with adverse outcomes. *New England Journal of Medicine*. 371 (26), 2488–2498 (2014).
4. Dorsheimer Let al. Association of mutations contributing to clonal hematopoiesis with prognosis in chronic ischemic heart failure. *JAMA Cardiology*. 4 (1), 25 (2019). [PubMed: 30566180]
5. Genovese Get al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *New England Journal of Medicine*. 371 (26), 2477–2487 (2014).
6. Jaiswal Set al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *New England Journal of Medicine*. 377 (2), 111–121 (2017).
7. Bick A Get al. Genetic interleukin 6 signaling deficiency attenuates cardiovascular risk in clonal hematopoiesis. *Circulation*. 141 (2), 124–131 (2020). [PubMed: 31707836]
8. Fuster J Jet al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science*. 355 (6327), 842–847 (2017). [PubMed: 28104796]

9. Wang Yet al.Tet2-mediated clonal hematopoiesis in nonconditioned mice accelerates age-associated cardiac dysfunction. *JCI Insight*. 5 (6), e135204 (2020).
10. Sano Set al.Tet2-mediated clonal hematopoiesis accelerates heart failure through a mechanism involving the IL-1 β /NLRP3 inflammasome. *Journal of the American College of Cardiology*. 71 (8), 875–886 (2018). [PubMed: 29471939]
11. Sano Set al.JAK2-mediated clonal hematopoiesis accelerates pathological remodeling in murine heart failure. *JACC: Basic to Translational Science*. 4 (6), 684–697 (2019). [PubMed: 31709318]
12. Yu KRet al.The impact of aging on primate hematopoiesis as interrogated by clonal tracking. *Blood*. 131 (11), 1195–1205 (2018). [PubMed: 29295845]
13. Sano Set al.CRISPR-mediated gene editing to assess the roles of Tet2 and Dnmt3a in clonal hematopoiesis and cardiovascular disease. *Circulation Research*. 123 (3), 335–341 (2018). [PubMed: 29728415]
14. Stachura DLet al.Clonal analysis of hematopoietic progenitor cells in the zebrafish. *Blood*. 118 (5), 1274–1282 (2011). [PubMed: 21415264]
15. Gyurkocza B, Sandmaier BMConditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood*. 124 (3), 344–353 (2014). [PubMed: 24914142]
16. Bacigalupo Aet al.Defining the intensity of conditioning regimens: working definitions. *Biology of Blood and Marrow Transplantation*. 15 (12), 1628–1633 (2009). [PubMed: 19896087]
17. Abbuehl JP, Tatarova Z, Held W, Huelsken JLong-term engraftment of primary bone marrow stromal cells repairs niche damage and improves hematopoietic stem cell transplantation. *Cell Stem Cell*. 21 (2), 241–255. e6 (2017). [PubMed: 28777945]
18. Shao Let al.Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a- and Arf-independent manner. *Blood*. 123 (20), 3105–3115 (2014). [PubMed: 24622326]
19. Cui YZet al.Optimal protocol for total body irradiation for allogeneic bone marrow transplantation in mice. *Bone Marrow Transplantation*. 30 (12), 843–849 (2002). [PubMed: 12476275]
20. Koch Aet al.Establishment of early endpoints in mouse total-body irradiation model. *PLOS One*. 11 (8), e0161079 (2016). [PubMed: 27579862]
21. Ismaiel A, Dumitraşcu DLCardiovascular risk in fatty liver disease: the liver-heart axis—literature review. *Frontiers in Medicine*. 6, 202 (2019). [PubMed: 31616668]
22. Amann K, Wanner C, Ritz ECross-talk between the kidney and the cardiovascular system. *Journal of the American Society of Nephrology*. 17 (8), 2112–2119 (2006). [PubMed: 16825329]
23. Liao Xet al.Distinct roles of resident and nonresident macrophages in nonischemic cardiomyopathy. *Proceedings of the National Academy of Sciences*. 115 (20), E4661–E4669 (2018).
24. Honold L, Nahrendorf MResident and monocyte-derived macrophages in cardiovascular disease. *Circulation Research*. 122 (1), 113–127 (2018). [PubMed: 29301844]
25. Lavine KJet al.The macrophage in cardiac homeostasis and disease. *Journal of the American College of Cardiology*. 72 (18), 2213–2230 (2018). [PubMed: 30360829]
26. Ginhoux F, Guilliams MTissue-resident macrophage ontogeny and homeostasis. *Immunity*. 44 (3), 439–449 (2016). [PubMed: 26982352]
27. Mildner Aet al.Microglia in the adult brain arise from Ly-6C hi CCR2+ monocytes only under defined host conditions. *Nature Neuroscience*. 10 (12), 1544–1453 (2007). [PubMed: 18026096]
28. Cronk JCet al.Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia. *Journal of Experimental Medicine*. 215 (6), 1627–1647 (2018).
29. Lu R, Czechowicz A, Seita J, Jiang D, Weissman ILClonal-level lineage commitment pathways of hematopoietic stem cells in vivo. *Proceedings of the National Academy of Sciences*. 116 (4), 1447–1456 (2019).
30. Gibson BWet al.Comparison of cesium-137 and X-ray irradiators by using bone marrow transplant reconstitution in C57BL/6J mice. *Comparative Medicine*. 65 (3), 165–172 (2015). [PubMed: 26141441]

31. Cui YZ et al. Optimal protocol for total body irradiation for allogeneic bone marrow transplantation in mice. *Bone Marrow Transplantation*. 30 (12), 843–849 (2002). [PubMed: 12476275]
32. Kim CK, Yang VW, Bialkowska AB The role of intestinal stem cells in epithelial regeneration following radiation-induced gut injury. *Current Stem Cell Reports*. 3 (4), 320–332 (2017). [PubMed: 29497599]

Figure 1: Images showing various preconditioning setups.

(A) Pie-cage total body irradiation setup using gamma-ray (Cesium-137): The radiation beam comes from the back of the irradiator in the y-axis direction (horizontal radiation). (B) Mouse cage total body irradiation setup using X-ray: The mouse cage is placed in the reflective chamber. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the cage is 530 mm. (C) Adjustable tray in X-ray irradiator: This setup is used for partially shielded irradiation using X-ray. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the tray is 373 mm, and the radius is 250 mm. (D) Thorax-shielding: Anesthetized mice are placed on a tray. The mice are placed inverted to each other in supine positions with arms and legs fully extended. The lower end of the lead-shield is aligned with the xiphisternum bone and the upper end with the thymus. (E) Abdominal-shielding: Anesthetized mice are placed as in the thorax-shielding set-up with the lower end of the lead-shield aligned with the anus and the upper end below the diaphragm. (F) Head-shielding irradiation setup using gamma-ray (Cesium-137): The anesthetized mouse's forepaws are taped down and the mouse is placed in a conical restrainer. The black lead-shield (marked) covers the mouse's head and ears. The radiation beam comes from the back of the irradiator in the direction of the Y-axis (horizontal radiation). [Please click here to view a larger version of this figure.](#)

Figure 2: Flow cytometric analysis of blood and heart using different methods of preconditioning.

Flow cytometric analysis of peripheral blood and heart was performed 1 month after bone marrow transplantation. To distinguish donor cells from the recipients' cells, CD45.1⁺ recipient mice were transplanted with CD45.2⁺ donor bone marrow cells. (A) 5×10^6 bone marrow cells were transplanted following two fractions of total body irradiation (2×5.5 Gy, 4 h interval, $n = 3$). (B) 5×10^6 bone marrow cells were transplanted following two fractions of total body irradiation with thorax-shielding (2×5.5 Gy, 4 h interval, $n = 10$). (C) Recipients without conditioning were transplanted with 5×10^6 bone marrow cells for 3 consecutive days (for a total of 1.5×10^7 , $n = 4$). Data are expressed as mean $\pm$ SEM. Total WBCs are defined as CD45⁺; Neutrophils as Ly6G⁺; Ly6C^{hi} monocytes as CD115⁺, Ly6G⁻, and Ly6C⁺; Ly6C^{lo} monocytes as CD115⁺, Ly6G⁻, and Ly6C⁻; B cells as CD45R⁺; CD4⁺ T cells as CD3e⁺ and CD4⁺; CD8⁺ T cells as CD3e⁺ and CD8⁺; and Macrophages as CD64⁺, Ly6G⁻, and Ly6C⁻. (WBCs: white blood cells, Neut: neutrophils, Mono: monocytes, Mac: macrophages). Figure 2A,C have been modified from Wang et al.⁹. [Please click here to view a larger version of this figure.](#)

Figure 3: Clonal expansion of *Tet2*-deficient cells using adoptive BMT to non-conditioned mice. CD45.2⁺ donor bone marrow cells (*WT* or *Tet2*^{-/-}) were transplanted into CD45.1⁺ recipient mice. Recipients without conditioning were transplanted with 5×10^6 bone marrow cells each day for 3 consecutive days (for a total of 1.5×10^7 , $n = 5-6$ per group). Flow cytometric analysis of peripheral blood was performed after 4, 8, 12, and 16 weeks post-transplantation. The fraction of CD45.2⁺ donor cells in each population is shown. (A) WBCs (B) Mono (C) Ly6C^{hi} mono (D) B cells (E) T cells. Data is expressed as mean $\pm$ SEM. WBCs are defined as CD45⁺; Neutrophils as Ly6G⁺; Ly6C^{hi} monocytes as CD115⁺, Ly6G⁻, and Ly6C⁺; Ly6C^{lo} monocytes as CD115⁺, Ly6G⁻, and Ly6C⁻; B cells as CD45R⁺; CD4⁺ T cells as CD3e⁺ (*WT*: wild-type, WBCs: white blood cells, Mono: monocytes) [Please click here to view a larger version of this figure.](#)

Table 1:
The efficiency of donor cell engraftment using different dosing intervals.

Flow cytometric analysis of peripheral blood was performed 1 month after bone marrow transplantation. *WT* CD45.2⁺ donor bone marrow cells were transplanted into CD45.1⁺ recipient mice. 5 x 10⁶ bone marrow cells were transplanted following two fractions of total body irradiation (2 x 5.5 Gy) with or without thorax-shielding separated by a 4 h interval or 24 h interval, (n = 3–4 per group).

		WBC (%)	B cell (%)	T cell (%)	Mono (%)	Ly6C ^{hi} mono (%)	Ly6C ^{lo} mono (%)	Neutrophil (%)
TBI-BMT	4h interval	97.4 ± 1.0	100 ± 0	59.1 ± 18.7	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	24h interval	97.2 ± 2.2	100 ± 0	79.0 ± 8.1	100 ± 0	100 ± 0	100 ± 0	100 ± 0
thorax-Shielding BMT	4h interval	56.2 ± 4.0	54.2 ± 6.2	0.5 ± 0.1	66.7 ± 6.1	63.8 ± 6.3	70.8 ± 5.7	82.0 ± 3.8
	24h interval	34.4 ± 3.1	34.8 ± 3.1	2.9 ± 1.7	45.0 ± 3.2	34.2 ± 3.6	56.2 ± 4.9	56 ± 10.0