**Name of Journal:** *World Journal of Stem Cells*

**Manuscript NO:** 55027

**Manuscript Type:** ORIGINAL ARTICLE

***Basic Study***

**How old is too old? *In vivo* engraftment of human peripheral blood stem cells cryopreserved for up to 18 years - implications for clinical transplantation and stability programs**

Underwood J *et al.* Engraftment of long-term cryopreserved PBSC

John Underwood, Mahvish Rahim, Carijo West, Rebecca Britton, Elaine Skipworth, Vicki Graves, Steven Sexton, Hillary Harris, Dave Schwering, Anthony Sinn, Karen E Pollok, Kent A Robertson, W Scott Goebel, Kerry M Hege

**John Underwood**, Departments of Internal Medicine and Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Mahvish Rahim, Karen E Pollok, Kent A Robertson, W Scott Goebel**, **Kerry M Hege,** Department of Pediatrics, Division of Pediatric Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Carijo West**, **Rebecca Britton, Elaine Skipworth, Vicki Graves, Steven Sexton, Hillary Harris, Dave Schwering,** Cellular Therapy Laboratory, Indiana University Health, Indianapolis, IN 46202, United States

**Anthony Sinn**, **Karen E Pollok,** Department of Pediatrics, Herman B Wells Center for Pediatric Research, In Vivo Therapeutics Core and Angio Biocore Shared Resource Facilities for the Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Author contributions:** Skipworth E, Pollok KE, Robertson KA, Goebel WS and Hege KM designed and coordinated the study; West C, Britton R, Graves V, Sexton S, Harris H, Schwering D, Sinn A, Goebel WS and Hege KM performed the experiments, acquired and analyzed data; Underwood J, Rahim M, West C, Britton R, Graves V, Sexton S, Harris H, Schwering D, Pollok KE, Robertson KA, Goebel WS and Hege KM interpreted the data; Underwood J, Rahim M, Goebel WS and Hege KM wrote the manuscript; all authors approved the final version of the manuscript.

**Supported by** a pilot grant from the Indiana University Center of Excellence in Molecular Hematology, NIDDK, No. P30DK090948 (to Hege KM and Goebel WS); the NIH/NCI Cancer Center, No. P30CA082709 awarded to the Indiana University Simon Comprehensive Cancer Center (to Sinn A and Pollok KE).

**Corresponding author**: **W Scott Goebel, MD, PhD, Medical Director of the Cellular Therapy Laboratory,** **Associate Professor of Clinical Pediatrics,** Division of Pediatric Hematology/Oncology, Indiana University School of Medicine, Room 2626, 705 Riley Hospital Drive, Indianapolis, IN 46202, United States. sgoebel2@iu.edu

**Received:** February 28, 2020

**Revised:** April 14, 2020

**Accepted:**April 28, 2020

**Published online:**

**Abstract**

BACKGROUND

Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant. Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. Clinical programs, stem cell banks, and regulatory and accrediting agencies interested in product stability would benefit from such data. Thus, we assessed recovery and colony forming ability of PBSC following long-term cryopreservation as well as their ability to engraft in NOD/SCID/IL-2Rγnull (NSG) mice.

AIM

To investigate the in vivo engraftment potential of long-term cryopreserved PBSC units.

METHODS

PBSC units which were collected and frozen using validated clinical protocols were obtained for research use from the Cellular Therapy Laboratory at Indiana University Health. These units were thawed in the Cellular Therapy Laboratory using clinical standards of practice, and the pre-freeze and post-thaw characteristics of the units were compared. Progenitor function was assessed using standard colony-forming assays. CD34-selected cells were transplanted into immunodeficient mice to assess stem cell function.

RESULTS

Ten PBSC units with mean of 17 years in cryopreservation (range 13.6-18.3 years) demonstrated a mean total cell recovery of 88% ± 12% (range 68%-110%) and post-thaw viability of 69% ± 17% (range 34%-86%). BFU-E growth was shown in 9 of 10 units and CFU-GM growth in 7 of 10 units post-thaw. Immunodeficient mice were transplanted with CD34-selected cells from four randomly chosen PBSC units. All mice demonstrated long-term engraftment at 12 wk with mean 34% ± 24% human CD45+ cells, and differentiation with presence of human CD19+, CD3+ and CD33+ cells. Harvested bone marrow from all mice demonstrated growth of erythroid and myeloid colonies.

CONCLUSION

We demonstrated engraftment of clinically-collected and thawed PBSC following cryopreservation up to 18 years in NSG mice, signifying likely successful clinical transplantation of PBSC following long-term cryopreservation.

**Key words:** Colony-forming units assay; Cryopreservation; Hematopoietic stem cells; Hematopoietic stem cell transplantation; *In vitro* techniques; Peripheral blood stem cell; Viability; Transplant; Long-term storage

Underwood J, Rahim M, West C, Britton R, Skipworth E, Graves V, Sexton S, Harris H, Schwering D, Sinn A, Pollok KE, Robertson KA, Goebel WS, Hege KM. How old is too old? *In vivo* engraftment of human peripheral blood stem cells cryopreserved for up to 18 years - implications for clinical transplantation and stability programs. *World J Stem Cells* 2020; In press

**Core tip:** Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant. Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. We demonstrated engraftment of clinically-collected and thawed PBSC following cryopreservation up to 18 years in NSG mice, signifying likely successful clinical transplantation of PBSC following long-term cryopreservation.

**INTRODUCTION**

Peripheral blood stem cells (PBSC) are the most common source of stem cells for hematopoietic stem cell transplantation (HSCT), being used for about two-thirds of all transplants[1]. PBSC are the standard of care for adult HSCT and are often used for pediatric autologous HSCT as well. PBSC are widely used due to the ease of collection from donors, the high numbers and quality of the hematopoietic stem cells (HSC), flexibility of timing for collection, and faster engraftment time compared to marrow. Another reason for this popularity is the relative ease of storage of PBSC. This allows for many centers to initially harvest enough PBSC for multiple transplants and cryopreserve them for future use in the setting of tandem/multiple transplants, or for use after relapse. The potential for long term storage is especially helpful due to the added difficulty of collecting adequate stem cells in a relapse setting after a first transplant[2].

Peripherally collected hematopoietic stem cell (HSC) units are held in cryopreservation around the world waiting to be used for the treatment of malignant and nonmalignant conditions in both children and adults. It is assumed that these cryopreserved cells can be stored for long periods and used with no negative impact on the patient receiving the cells for transplant[3,4]. Clinically, most PBSC units are used within a few months to a year from collection. It is currently not well delineated how long-term cryopreservation (commonly defined as > 60 mo) affects stem cell recovery, viability, and stem cell function during transplantation.

Whereas the ability to cryopreserve HSC for extended periods has been appreciated for over 25 years[5-8], data on PBSC viability after long-term cryopreservation is limited. Although decreased viability by trypan blue dye exclusion, decreased colony forming ability and/or decreased CD34+ cell content have been described for PBSC units cryopreserved for more than 10 years[9,10],other authors have reported that these in vitro parameters largely remain stable for up to 19 years, at least after initial losses due to freezing[11-13].Furthermore, several studies report successful clinical engraftment of PBSC cryopreserved for at least 2 up to 11 years[4,12,14,15]. However, there is insufficient data about successful engraftment of PBSC units cryopreserved beyond 11 years.

Our aim in this study was to determine if long-term cryopreserved PBSC units could exhibit hematopoietic reconstitution after transplantation into immunodeficient mice. Such functional studies in mice document the engraftment capability of long term cryopreserved PBSC units and permit analysis of progenitor and mature cell subtypes present in engrafted mice. We hypothesized that long-term cryopreservation and thawing performed in a clinical Cellular Therapy Lab using validated protocols, would not negatively impact recovery of PBSC nor the ability for engraftment in NOD/SCID/IL-2R**γ**null (NSG) mice. Knowledge of successful engraftment of long term cryopreserved PBSC units is valuable information for cryopreservation facilities, as it demonstrates clinical use of stored PBSC units. Successful long-term cryopreservation of PBSC units also has implications for banking of HSC from other sources, and the banking of other cellular therapy products.

**MATERIALS AND METHODS**

***PBSC collection, cryopreservation, thawing and separation***

PBSC units used for this study were collected and cryopreserved for clinical use by the Indiana University Health Cellular Therapy Laboratory following standard operating procedures in place at the time of collection. These PBSC units were scheduled for discard once no further clinical needs were identified (reasons include death of patient or clinical practice no longer utilizing HSCT for the indicated disease). PBSC use for research purposes was included in patient consent obtained at the time of PBSC collection. The Institutional Review Board of the Indiana University School of Medicine approved this study.

Briefly, in a biological safety cabinet, a sample was removed from the apheresis collection bag for required clinical testing (*e.g.*, WBC count, ABO typing, sterility). The product volume was calculated by weighing the bag, and the total WBC/mL was calculated. The minimum freeze volume was next calculated, which is the minimum total volume of cells and freeze solution needed to ensure that the cell concentration was a minimum of 0.50 × 108/mL and a maximum of 5.0 × 108/mL. Plasma depletion was performed if the product volume was significantly greater than the minimum freeze volume. The product was then distributed into the desired number of freezing bags, and cell viability was determined by trypan blue staining prior to the addition of freeze solution. Final freeze volume was calculated by dividing actual volume by 0.8; freeze solution volume was calculated as 20% of the final freeze volume. Freeze solution consisting of a final concentration of donor plasma (if available) or 5% human serum albumin plus 10% DMSO was prepared in a 1:1 ratio, and chilled on ice for at least 15 min before adding to the freezing bags. Freezing mix was slowly added to the cells, and freezing bags were chilled if warming due to DMSO addition occurred. Following removal of samples for preparation of two cryovials for quality assurance and final sterility testing, freezing bags were placed into freezing canisters which were in turn placed into a controlled rate freezer and frozen to -80 °C over approximately 50 min. The final frozen product was transferred to the vapor phase of liquid nitrogen for storage.

Total nucleated cell count, viability and CD34+ cell content were determined at the time of PBSC collection, and these data were available for comparison at the time of thawing. Products were thawed using a standard clinical thaw and wash procedure. A water bath filled with sterile normal saline was warmed to 39-41 °C. The product bag was removed from the freeze canister and, after identity was confirmed, the bag was submerged into the warmed saline and gently agitated until thawed. After thawing, the bag was transferred to a biological safety cabinet, the port covers removed and swabbed with 70% isopropanol, and the product was washed with a 1:1 ratio of 6% hetastarch and 5% human serum albumin to remove the DMSO prior to beginning experiments. HSC recovery was measured by the following: post-thaw cellular counts and viability as compared to pre-freeze, in vitro colony forming assays, and in vivo transplantation into NSG mice. Cellular measurements included: total nucleated cell counts, CD34+ cell count *via* flow cytometry, and percent viable cells by trypan blue dye exclusion.

***In vitro colony assays***

Thawed cells were plated for colony forming assays to assess burst-forming units-erythroid and colony forming units-granulocyte/macrophage (BFU-E/CFU-GM) utilizing standardized clinical assays. Normal donor PBSC previously validated in the Cellular Therapy Lab were used as a positive control for the BFU/CFU assay. Test cells were plated at concentrations to achieve approximately 100-150 total CFUs per 1mL dish (to yield high enough colony numbers to get accurate colony counts with minimal colony overlap). Cells used in this assay were free of DMSO. Colonies were scored after 14 d incubation in a humidified chamber.

***In vivo NSG mouse assay***

Most importantly, stem cell function was evaluated in vivo by transplantation into NSG mice. McDermott *et al* established NSG mice as supporting greater engraftment of human hematopoietic stem cells than all other strains[12]. Four randomly chosen thawed PBSC units, as a representative sample from the 10 PBSC units studied, were transplanted into NSG mice (*n* = 6-7 recipients for each thawed unit) to investigate engraftment potential. Prior to transplantation into immunodeficient mice, CD34+ cells were selected to deplete T cells and prevent recipient mice from developing graft-vs-host disease during the post-transplant period. CD34+ cells were isolated from the PBSC units by incubation with anti-CD34 antibody directly conjugated to magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany). Positive CD34+ cell selection was performed using the AutoMACS device (Miltenyi) according to the manufacturer’s instructions, and yielded a purity of at least 93% CD34+ cells. NSG mice were conditioned with a single dose of sublethal 300-cGy total-body irradiation using a GammaCell 40 (Nordion International Inc., Ontario Canada). Transplantation was performed as we previously described[15] with 2 × 105 CD34+ cells per mouse in 400 μL of IMDM, 0.1% BSA given by tail vein injection. Controls were age-matched NSG mice that received no irradiation or transplanted human cells. Peripheral blood evaluations at 4 wk represented short-term engraftment, and bone marrow evaluations at 12 wk represented long-term engraftment of human HSC. Evaluations at these time points included determination of the percentage of human CD45+, CD19+, CD3+, and CD33+ cells from the blood and/or bone marrow, and were performed as previously described[16]. All animal experiments were performed under supervision of the In Vivo Therapeutics Core using procedures approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

**RESULTS**

PBSC units were analyzed pre-freeze (*i.e.*, at the time of initial cryopreservation) and again following validated, standardized clinical thaw procedures (post-thaw). The total nucleated cell (TNC) count and percent viability were measured post-thaw and compared to pre-freeze values. TNC recovery (TNC post-thaw as a percentage of pre-freeze TNC) was calculated. BFU-E and CFU-GM were quantified using standardized colony assays. These data regarding the PBSC units are summarized in Table 1.

The PBSC units tested were cryopreserved for a mean of 17 years (range 13.6-18.3 years). The mean donor age at the time of collection was 47 years-old (range of 24-66 years). Diseases for which PBSC were clinically collected included glioblastoma multiforme, chronic myelogenous leukemia, multiple myeloma, and non-Hodgkin’s lymphoma. The mean TNC recovery from the 10 units was 88% ± 12% (range 68%-110%). Variable post-thaw viability was evident with a mean of 69% ± 17% viability (range 34%-86%). Eight of the 10 units had a post-thaw viability of > 50%. Of the ten PBSC units, nine exhibited BFU-E growth and seven showed CFU-GM growth.

Analysis of PBSC post-CD34+ selection and engraftment data in NSG mice were obtained for four random units from patients with four different diagnoses. The four units included the youngest (13.6 years) and second oldest (18.1 years) cryopreserved units. The CD34+ selected cell characteristics and engraftment findings are summarized in Table 2*.* CD34+ selected cells demonstrated vigorous growth of erythroid and myeloid colonies. Following transplantation, all mice demonstrated short- and long-term engraftment at 4 and 12 wk, respectively. Marrow from transplanted mice harvested at 12 wk post-transplant demonstrated a mean of 34% ± 24% human CD45+ cells, indicating substantial levels of long-term human cell engraftment. PBSC Unit “B” had the highest average human CD45 levels at 63.6%, despite being one of the older cryopreserved units with cryopreservation time of 17.7 years. All mice demonstrated multilineage differentiation on bone marrow at 12 wk with the presence of human CD19+ (B lymphocytes), CD3+ (T lymphocytes) and CD33+ (myeloid) cells by flow cytometry. This transplantation study demonstrates that PBSC can be cryopreserved for up to 18 years (*i.e.*, the age of the oldest cryopreserved unit) while retaining colony forming ability and the capability to engraft into NSG mice.

**DISCUSSION**

PBSC units are held in cryopreservation around the world awaiting clinical use. The ability to successfully store PBSC units is critical for patients who require multiple transplants or for storing PBSC units for future use if a patient relapses. Whereas frozen cord blood units are entirely infused for a transplant, sufficient PBSC for multiple transplants can be collected from a single donor and used over time. Indeed, tandem (or even triple) autologous transplants are currently used for patients with multiple myeloma, germ cell tumors, neuroblastoma, and pediatric brain tumors, among others. Most of these tandem transplants are performed within a year of PBSC collection; however, under certain circumstances, PBSC will be stored for later transplantation. Many institutions collect sufficient PBSC for at least two transplants for multiple myeloma patients, which may occur years apart. In addition, additional allogeneic PBSC may be collected and stored should the patient relapse and require a second transplant, need a stem cell boost for poor engraftment, or benefit from donor lymphocyte infusions for relapse or declining donor chimerism. Again, these infusions may occur years after the initial PBSC collection. The ability to successfully store and thaw long-term cryopreserved products is particularly important for autologous HSCT patients who may not successfully mobilize PBSC after relapse and salvage therapy and for allogeneic recipients whose donors may no longer be available for subsequent PBSC collections.

Regulatory agencies and accreditation bodies do not currently have specific guidelines or limitations on the duration of storage for cryopreserved cell therapies. The guidelines that are available state that cells used for hematopoietic or immunologic reconstitution must be preserved in a manner that is “appropriate for long-term storage” and that caution should be used for cell units cryopreserved for longer than 5 years[17,18]. Furthermore, clinical transplantation programs as well as stem cell banking facilities are required to develop stability programs to demonstrate proficiency in processing, freezing, storing and thawing clinically-relevant cellular therapy products. At the present, programs must devise their own protocols for thawing frozen products, in part due to the lack of long-term data.

Several groups have demonstrated that viable CD34+ cells and/or colony-forming cells can be isolated in vitro from PBSC units cryopreserved for up to 19 years[3,9,11,12,19]. However, only a few reports exist demonstrating that long-term (defined in these studies as 2-11 years) cryopreserved PBSC can successfully engraft in vivo[2,4,7,8,14]. Two interesting paired studies in particular are enlightening, in which multiple myeloma patients had sufficient PBSC collected for multiple transplants. Patients had one transplant within months of collection, then had a second transplant years later. In this manner, the quality and function of a single PBSC collection could be compared over time. Pavlu *et al*[4] found no differences in the time to neutrophil and platelet engraftment in 50 myeloma patients who received a second autologous PBSC transplant 2-9 years after the first. Similarly, Liseth *et al*[14] reported a one day delay in both neutrophil and platelet recovery in 17 myeloma patients receiving a second transplant a mean of 3.5 years after the first; this finding was modest but significant in this small patient population. These studies indicate that PBSC can successfully engraft after cryopreservation for up to 11 years. Unfortunately, essentially no data exists for PBSC units stored beyond 11 years.

The paucity of data on long-term cryopreserved PBSC is somewhat surprising, especially since PBSC are by far the most common stem cell source for HSCT[1]. With limited data on long-term cryopreservation of PBSCs, investigations into the properties of human cord blood may be informative. More data on cryopreserved cord blood may exist since public cord blood banking is stringently regulated, in part because, unlike PBSC, cord blood units are typically used years after processing and freezing; thus, stability programs are necessary to monitor the quality of cord blood units between banks over time. Compounding this issue is the fact that, also unlike PBSC, cord blood units are usually processed and cryopreserved at a different institution, often in a different country, than where the transplant occurs. Efforts are underway to create international standards governing the processing, storage, and distribution of human cells and tissues, especially cord blood, harmonizing regulations from government agencies and adopting selected standards from accrediting bodies such as NetCord-FACT (Federation for the Accreditation of Cellular Therapies) and The Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) to develop a set of minimal criteria for cord blood banking[20].

Yamamoto *et al*[21] evaluated 18 cord blood units in storage for over 10 years and found 84% viability, with CD34+ counts and in vitro colony forming unit activity (CFU) similar to controls. In the most extensive non-clinical studies to date, Broxmeyer *et al*[22-24], through a series of publications, evaluated cord blood units in cryopreservation for 9-10 years, 15 years and 21-23.5 years. In the 15-year study, this group found highly efficient cell recovery in 9 cord blood units, with post-thaw CD34+ counts similar to pre-cryopreservation counts. Furthermore, 3 of 4 units tested were successfully transplanted into NSG mice with evidence of engraftment. In the 21-year study, this group again found highly efficient cell recovery in 23 cord blood units with post-thaw CD34+ counts similar to pre-cryopreservation counts, cell recovery with colony formation, as well as successful engraftment into NSG mice with secondary repopulation.

Several groups examined engraftment of long-term cryopreserved cord blood in patients[25-27]. Parmar *et al*[25] described engraftment of 15 cord blood units cryopreserved for 5-12 years. The only factors identified as significant for recipient survival were myeloablative conditioning and HLA mismatch, not the age of the cord blood unit. Two other groups transplanted 62 cord blood units cryopreserved from 5 to 11 years[27] and 22 cord blood units cryopreserved for 10-13.4 years[26], respectively, and found no difference in engraftment compared to younger units. In sum, these clinical studies indicate that cryopreserved cord blood retains its engraftment potential in vivo for at least 11 years.

Our study evaluated PBSC after an average of 17 years in cryopreservation. We focused upon PBSC units from patients which were collected, frozen and thawed using validated clinical protocols for human transplantation in place at the time of freezing and thawing, respectively; an evaluation of PBSC frozen using experimental procedures, such as uncontrolled freezing methods and/or storing frozen cells in mechanical freezers rather in vapor phase of liquid nitrogen[10,28] is beyond the scope of this study. We assessed the in vitro proliferative abilities of these cells, and, most importantly, assessed the in vivo capacity of these recovered cells to engraft and repopulate the hematopoietic system of sublethally irradiated NSG mice. The post-thaw viability of the 10 older units (mean 69 ± 17% viability, range 34%-86%) was similar to that obtained in the Cellular Therapy Lab for PBSC units frozen for < 7 wk and transplanted in quarter 4 of 2019 (73% ± 6% viability, range: 61%-84%, *n* = 55). Of the ten different PBSC units, nine exhibited BFU-E growth and seven showed CFU-GM growth. Interestingly, PBSC unit “F” that lacked both BFU-E and CFU-GM growth was the unit with the lowest post thaw percent viability of 34%, but this was not one of the oldest or youngest cryopreserved units. PBSC unit “F” was, however, obtained from the oldest patient in our cohort of samples. Moreover, the two units (units “E” and “I”) which had no CFU-GM growth, and the unit which had no CFU-GM or BFU-E growth (unit “F”), were all from multiple myeloma patients. Of note, mobilization and collection of PBSC from patients with multiple myeloma often presents a challenge[29], and thawed PBSC products from myeloma patients may exhibit lower viability and TNC recovery than products from patients with other diagnoses[30]. Furthermore, colony formation is known to be reduced in myeloma patients relative to healthy donors[31]; however, PBSC from myeloma patients can still successfully engraft in immunodeficient mice[31] and patients[32] despite reduced or even absent colony-forming ability. Overall, these data indicate a highly efficient total cell recovery and viability, which is an important factor in determining the likelihood of engraftment.

Most importantly, both short- and long-term engraftment was demonstrated in NSG mice with differentiation into multilineage phenotypes. Although the ultimate success for PBSC following long-term cryopreservation would be long-term engraftment in humans, our data build upon the pre-clinical and clinical experiences with PBSC and cord blood detailed above, and imply that older PBSC units could also successfully be used for clinical applications. Based on these data, our institution increased the time of “safe storage” from 5 years to 12 years without requiring additional testing of the unit for viability (*e.g.*, thawing of a cryovial for testing), and will continue to increase the duration of safe storage based on ongoing stability data with longer cryopreservation times.

**ARTICLE HIGHLIGHTS**

***Research background***

Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant (HSCT). Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. Our study examines the engraftment potential of long-term cryopreserved PBSC units. This could allow for PBSC units to be stored for a longer time without repeated viability testing and for these units to be utilized in clinical HSCT.

***Research motivation***

We investigated the viability and colony-forming unit capacity in vitro, and the in vivo engraftment potential of long-term cryopreserved PBSC units. This was done to gain an understanding of the viability of long-term cryopreserved PBSC units so that these long-term cryopreserved units could be used for clinical HSCT.

***Research objectives***

Our intention was to investigate if long-term cryopreserved PBSC units, which are being preserved in stem cell banks for many years, can be utilized with successful in vivo engraftment. This will help with gaining insight to the potential use of long-term cryopreserved PBSC units.

***Research methods***

PBSC units were collected and frozen as per validated clinical protocols. The units were then thawed as per clinical standards of practice. Progenitor function was assessed with standard colony-forming assays. CD34-selected cells were transplanted into NOD/ SCID/IL-2Rγnull (NSG) mice and stem cell function was assessed.

***Research results***

Ten long-term cryopreserved PBSC units (mean of 17 years) demonstrated appropriate post-thaw viability of which nine had BFU-E growth and seven showed CFU-GM growth. Immunodeficient NSG mice (6-7 recipient mice/PBSC unit) were transplanted with 4 randomly selected PBSC units that were cryopreserved for up to 18 years, and all mice showed short-term and long-term engraftment and reconstitution of human myeloid and lymphoid cells. Moving forward it will be important to analyze the engraftment of long-term cryopreserved PBSC units *in vivo* on a larger scale.

***Research conclusions***

This study demonstrates the appropriate long term engraftment of clinically collected and thawed PBSC units follow cryopreservation up to 17 years in immunodeficient mice. This is one of few studies that analyzes the *in vivo* engraftment potential of long-term cryopreserved PBSC units. This can allow institutions to safely increase the time of safe storage for PBSC units, without further viability testing of the units. These findings are beneficial for clinical programs, stem cell banks, and regulatory and accrediting agencies interested in product stability.

***Research perspectives***

In summary, this study demonstrates that long-term cryopreserved PBSC can exhibit short- and long-term engraftment in immunodeficient mice with differentiation into multilineage phenotypes. Future research would be to expand studies to look at *in vivo* engraftment on a larger scale and ultimately to apply this to clinical transplantation in humans.

**ACKNOWLEDGEMENTS**

The authors thank John Delph, RN, for assistance in compiling patient transplant data and for discussions on regulatory requirements for cryopreserved HSC.

**REFERENCES**

1 **National Marrow Donor Program**. Transplant Therapy and Donor Matching: Cell Sources: Be The Match; [Transplant Therapy and Donor Matching]. Available from: <https://bethematchclinical.org/transplant-therapy-and-donor-matching/cell-sources/>

2 **Lisenko K**, Pavel P, Kriegsmann M, Bruckner T, Hillengass J, Goldschmidt H, Witzens-Harig M, Ho AD, Wuchter P. Storage Duration of Autologous Stem Cell Preparations Has No Impact on Hematopoietic Recovery after Transplantation. *Biol Blood Marrow Transplant* 2017; **23**: 684-690 [PMID: 28013016 DOI: 10.1016/j.bbmt.2016.12.631]

3 **Vosganian GS**, Waalen J, Kim K, Jhatakia S, Schram E, Lee T, Riddell D, Mason JR. Effects of long-term cryopreservation on peripheral blood progenitor cells. *Cytotherapy* 2012; **14**: 1228-1234 [PMID: 22900962 DOI: 10.3109/14653249.2012.706707]

4 **Pavlů J**, Auner HW, Szydlo RM, Sevillano B, Palani R, O'Boyle F, Chaidos A, Jakob C, Kanfer E, MacDonald D, Milojkovic D, Rahemtulla A, Bradshaw A, Olavarria E, Apperley JF, Pello OM. Analysis of hematopoietic recovery after autologous transplantation as method of quality control for long-term progenitor cell cryopreservation. *Bone Marrow Transplant* 2017; **52**: 1599-1601 [PMID: 28650454 DOI: 10.1038/bmt.2017.113]

5 **Parker LM**, Binder N, Gelman R, Richman CM, Weiner RS, Yankee RA. Prolonged cryopreservation of human bone marrow. *Transplantation* 1981; **31**: 454-457 [PMID: 7256827 DOI: 10.1097/00007890-198106000-00012]

6 **Attarian H**, Feng Z, Buckner CD, MacLeod B, Rowley SD. Long-term cryopreservation of bone marrow for autologous transplantation. *Bone Marrow Transplant* 1996; **17**: 425-430 [PMID: 8704699 DOI: N/A]

7 **Cameron G**, Tantiworawit A, Halpenny M, Letcher B, Berrigan S, Hindmarsh K, Giftakis A, Fortier J, O'Hoski P, Hogge D. Cryopreserved mobilized autologous blood progenitors stored for more than 2 years successfully support blood count recovery after high-dose chemotherapy. *Cytotherapy* 2011; **13**: 856-863 [PMID: 21385094 DOI: 10.3109/14653249.2011.563293]

8 **Aird W**, Labopin M, Gorin NC, Antin JH. Long-term cryopreservation of human stem cells. *Bone Marrow Transplant* 1992; **9**: 487-490 [PMID: 1628134]

9 **Spurr EE**, Wiggins NE, Marsden KA, Lowenthal RM, Ragg SJ. Cryopreserved human haematopoietic stem cells retain engraftment potential after extended (5-14 years) cryostorage. *Cryobiology* 2002; **44**: 210-217 [PMID: 12237086 DOI: 10.1016/s0011-2240(02)00027-5]

10 **Muramaki M**, Hara I, Miyake H, Yamada Y, Okada H, Kamidono S. Long-term cryopreservation of peripheral blood stem cells in patients with advanced germ cell tumors using the dump-freezing method at -80 degrees C. *Oncol Rep* 2003; **10**: 1993-1998 [PMID: 14534732]

11 **Winter JM**, Jacobson P, Bullough B, Christensen AP, Boyer M, Reems JA. Long-term effects of cryopreservation on clinically prepared hematopoietic progenitor cell products. *Cytotherapy* 2014; **16**: 965-975 [PMID: 24910385 DOI: 10.1016/j.jcyt.2014.02.005]

12 **McDermott SP**, Eppert K, Lechman ER, Doedens M, Dick JE. Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood* 2010; **116**: 193-200 [PMID: 20404133 DOI: 10.1182/blood-2010-02-271841]

13 **Fernyhough LJ**, Buchan VA, McArthur LT, Hock BD. Relative recovery of haematopoietic stem cell products after cryogenic storage of up to 19 years. *Bone Marrow Transplant* 2013; **48**: 32-35 [PMID: 22659683 DOI: 10.1038/bmt.2012.97]

14 **Liseth K**, Ersvær E, Abrahamsen JF, Nesthus I, Ryningen A, Bruserud Ø. Long-term cryopreservation of autologous stem cell grafts: a clinical and experimental study of hematopoietic and immunocompetent cells. *Transfusion* 2009; **49**: 1709-1719 [PMID: 19392777 DOI: 10.1111/j.1537-2995.2009.02180.x]

15 **Cai S**, Wang H, Bailey B, Hartwell JR, Silver JM, Juliar BE, Sinn AL, Baluyut AR, Pollok KE. Differential Secondary Reconstitution of In Vivo-Selected Human SCID-Repopulating Cells in NOD/SCID versus NOD/SCID/γ chain Mice. *Bone Marrow Res* 2011; **2011**: 252953 [PMID: 22046557 DOI: 10.1155/2011/252953]

16 **Cai S**, Wang H, Bailey B, Ernstberger A, Juliar BE, Sinn AL, Chan RJ, Jones DR, Mayo LD, Baluyut AR, Goebel WS, Pollok KE. Humanized bone marrow mouse model as a preclinical tool to assess therapy-mediated hematotoxicity. *Clin Cancer Res* 2011; **17**: 2195-2206 [PMID: 21487065 DOI: 10.1158/1078-0432.CCR-10-1959]

17 **World Health Organization**. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. 2010. Available from: <https://www.who.int/immunization_standards/vaccine_regulation/en/>

18 **US Food and Drug Administration**. Guidance for Industry: Biologics License Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System. 2014. Available from: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bla-minimally-manipulated-unrelated-allogeneic-placentalumbilical-cord-blood-intended-hematopoietic>

19 **McCullough J**, Haley R, Clay M, Hubel A, Lindgren B, Moroff G. Long-term storage of peripheral blood stem cells frozen and stored with a conventional liquid nitrogen technique compared with cells frozen and stored in a mechanical freezer. *Transfusion* 2010; **50**: 808-819 [PMID: 19912586 DOI: 10.1111/j.1537-2995.2009.02482.x]

20 Model Criteria for Regulation of Cord Blood Banks and Cord Blood Banking: Adopted by the Cord Blood Association, Board of Directors, January 29, 2019. *Stem Cells Transl Med* 2019; **8**: 340-343 [PMID: 30843653 DOI: 10.1002/sctm.cbmc]

21 **Yamamoto S**, Ikeda H, Toyama D, Hayashi M, Akiyama K, Suzuki M, Tanaka Y, Watanabe T, Fujimoto Y, Hosaki I, Nishihira H, Isoyama K. Quality of long-term cryopreserved umbilical cord blood units for hematopoietic cell transplantation. *Int J Hematol* 2011; **93**: 99-105 [PMID: 21207212 DOI: 10.1007/s12185-010-0755-x]

22 **Broxmeyer HE**, Lee MR, Hangoc G, Cooper S, Prasain N, Kim YJ, Mallett C, Ye Z, Witting S, Cornetta K, Cheng L, Yoder MC. Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21- to 23.5-year cryopreserved cord blood. *Blood* 2011; **117**: 4773-4777 [PMID: 21393480 DOI: 10.1182/blood-2011-01-330514]

23 **Broxmeyer HE**, Srour EF, Hangoc G, Cooper S, Anderson SA, Bodine DM. High-efficiency recovery of functional hematopoietic progenitor and stem cells from human cord blood cryopreserved for 15 years. *Proc Natl Acad Sci USA* 2003; **100**: 645-650 [PMID: 12518050 DOI: 10.1073/pnas.0237086100]

24 **Broxmeyer HE**, Cooper S. High-efficiency recovery of immature haematopoietic progenitor cells with extensive proliferative capacity from human cord blood cryopreserved for 10 years. *Clin Exp Immunol* 1997; **107 Suppl 1**: 45-53 [PMID: 9020936]

25 **Parmar S**, de Lima M, Worth L, Petropoulos D, Lee D, Cooper L, Kongtim P, Alousi A, Hosing C, Popat U, Kebriaei P, McNiece I, Shpall E, Rondon G, Champlin R. Is there an expiration date for a cord blood unit in storage? *Bone Marrow Transplant* 2014; **49**: 1109-1112 [PMID: 24797184 DOI: 10.1038/bmt.2014.92]

26 **Jaing TH**, Chen SH, Tsai MH, Yang CP, Hung IJ, Tsay PK. Transplantation of unrelated donor umbilical cord blood for nonmalignant diseases: a single institution's experience with 45 patients. *Biol Blood Marrow Transplant* 2010; **16**: 102-107 [PMID: 19772946 DOI: 10.1016/j.bbmt.2009.09.009]

27 **Mitchell R**, Wagner JE, Brunstein CG, Cao Q, McKenna DH, Lund TC, Verneris MR. Impact of long-term cryopreservation on single umbilical cord blood transplantation outcomes. *Biol Blood Marrow Transplant* 2015; **21**: 50-54 [PMID: 25262882 DOI: 10.1016/j.bbmt.2014.09.002]

28 **Detry G**, Calvet L, Straetmans N, Cabrespine A, Ravoet C, Bay JO, Petre H, Paillard C, Husson B, Merlin E, Boon-Falleur L, Tournilhac O, Delannoy A, Halle P. Impact of uncontrolled freezing and long-term storage of peripheral blood stem cells at - 80 °C on haematopoietic recovery after autologous transplantation. Report from two centres. *Bone Marrow Transplant* 2014; **49**: 780-785 [PMID: 24686987 DOI: 10.1038/bmt.2014.53]

29 **Musto P**, Simeon V, Grossi A, Gay F, Bringhen S, Larocca A, Guariglia R, Pietrantuono G, Villani O, D'Arena G, Cuomo C, Musto C, Morabito F, Petrucci MT, Offidani M, Zamagni E, Tacchetti P, Conticello C, Milone G, Palumbo A, Cavo M, Boccadoro M. Predicting poor peripheral blood stem cell collection in patients with multiple myeloma receiving pre-transplant induction therapy with novel agents and mobilized with cyclophosphamide plus granulocyte-colony stimulating factor: results from a Gruppo Italiano Malattie EMatologiche dell'Adulto Multiple Myeloma Working Party study. *Stem Cell Res Ther* 2015; **6**: 64 [PMID: 25889496 DOI: 10.1186/s13287-015-0033-1]

30 **Foïs E**, Desmartin M, Benhamida S, Xavier F, Vanneaux V, Rea D, Fermand JP, Arnulf B, Mounier N, Ertault M, Lotz JP, Galicier L, Raffoux E, Benbunan M, Marolleau JP, Larghero J. Recovery, viability and clinical toxicity of thawed and washed haematopoietic progenitor cells: analysis of 952 autologous peripheral blood stem cell transplantations. *Bone Marrow Transplant* 2007; **40**: 831-835 [PMID: 17724443 DOI: 10.1038/sj.bmt.1705830]

31 **Bruns I**, Cadeddu RP, Brueckmann I, Fröbel J, Geyh S, Büst S, Fischer JC, Roels F, Wilk CM, Schildberg FA, Hünerlitürkoglu AN, Zilkens C, Jäger M, Steidl U, Zohren F, Fenk R, Kobbe G, Brors B, Czibere A, Schroeder T, Trumpp A, Haas R. Multiple myeloma-related deregulation of bone marrow-derived CD34(+) hematopoietic stem and progenitor cells. *Blood* 2012; **120**: 2620-2630 [PMID: 22517906 DOI: 10.1182/blood-2011-04-347484]

32 **Moreb JS**, Salmasinia D, Hsu J, Hou W, Cline C, Rosenau E. Long-Term Outcome after Autologous Stem Cell Transplantation with Adequate Peripheral Blood Stem Cell Mobilization Using Plerixafor and G-CSF in Poor Mobilizer Lymphoma and Myeloma Patients. *Adv Hematol* 2011; **2011**: 517561 [PMID: 22190942 DOI: 10.1155/2011/517561]

**Footnotes**

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board of Indiana University School of Medicine.

**Institutional animal care and use committee statement:** All animal experiments were performed under supervision of the In Vivo Therapeutics Core using procedures approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (protocol 0000002985).

**Conflict-of-interest statement:** Goebel WS receives fees as a consulting medical director for Cook Regentec, LLC, and serves as medical director for Ossium Health, Inc. All other authors report no potential conflicts of interest.

**Data sharing statement**: No additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

**Manuscript source:** Invited manuscript

**Peer-review started:** February 28, 2020

**First decision:** April 2, 2020

**Article in press:**

**Specialty type:** Cell and tissue engineering

**Country/Territory of origin:** United States

**Peer-review report’s scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): B

Grade C (Good): C

Grade D (Fair): 0

Grade E (Poor): 0

**P- Reviewer:** Gonzalez FM, Li YG **S- Editor:** Gong ZM **L- Editor:** **E- Editor:**

**Table 1 Cryopreserved peripheral blood stem cells unit characteristics**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **PBSC unit** | **Unit characteristics** | **Pre-freeze characteristics** | **Post-thaw characteristics** | **Colony forming ability** |
| **Donor age (yr)** | **Disease** | **Cryopreservation (yr)** | **TNC × 103/µL** | **%CD34** | **TNC × 103/µL** | **%TNC Recovery** | **%Via-bility** | **BFU-E/105** | **CFU-GM/106** |
| **A** | 25 | GLIO | 18.1 | 110 | 0.2 | 121 | 110 | 84 | 2 | 2 |
| **B** | 58 | CML | 17.7 | 120 | 0.5 | 111 | 103 | 86 | 6 | 10 |
| **C** | 46 | MM | 13.6 | 120 | 3.9 | 116 | 81 | 68 | ++ | ++ |
| **D** | 24 | NHL | 14.6 | 116 | 3.8 | 110 | 68 | 67 | ++ | ++ |
| **E** | 52 | MM | 17.9 | 127 | 0.2 | 164 | 90 | 86 | 0.3 | 0 |
| **F** | 66 | MM | 17 | 102 | 0.5 | 145 | 95 | 34 | 0 | 0 |
| **G** | 54 | MM | 18.3 | 134 | 0.5 | 114 | 79 | 81 | 4 | 6 |
| **H** | 52 | MM | 17.9 | 120 | 0.2 | 150 | 90 | 70 | 9 | 6 |
| **I** | 47 | MM | 17.2 | 114 | 1.5 | 120 | 80 | 62 | 0.3 | 0 |
| **J** | 47 | MM | 17.1 | 114 | 1.5 | 124 | 83 | 49 | ++ | ++ |

TNC: Total nucleated cells; BFU: Burst forming units; PBSC: Peripheral blood stem cells; CFU: Colony forming units; GLIO: Glioblastoma multiforme; CML: Chronic myelogenous leukemia; MM: Multiple myeloma; NHL: Non-Hodgkin's lymphoma; ++: Overgrowth too numerous to count.

**Table 2 Post-CD34 selection characteristics and engraftment data**

|  |  |  |  |
| --- | --- | --- | --- |
| **PBSC unit** | **Cryopreservation (yr)** | **Post-CD34 selection characteristics** | **Mouse bone marrow engraftment** |
| **TNC × 103/µL** | **%Viability** | **%Purity** | **BFU-E/105** | **CFU-GM/106** | **Mean %CD45** | **Mean %CD19** | **Mean %CD3** | **Mean %CD33** |
| **A** | 18.1 | 0.3 | 96 | 95 | 19 | 56 | 43.4 | 14.8 | 1.1 | 12.6 |
| **B** | 17.7 | 0.3 | 94 | 93 | 54 | 85 | 63.6 | 20.8 | 0.4 | 13.1 |
| **C** | 13.6 | 2.5 | 96 | 98 | ++ | ++ | 10.3 | 2.8 | 1 | 5.6 |
| **D** | 14.6 | 2.4 | 95 | 98 | ++ | ++ | 20.3 | 6.8 | 0.5 | 7.8 |

TNC: Total nucleated cells; PBSC: Peripheral blood stem cells; BFU: Burst forming units; CFU: Colony forming units; ++: Overgrowth too numerous to count.