Published in final edited form as:

Cell Transplant. 2010 ; 19(11): . doi:10.3727/096368910X314161.

The Assessment of Human Erythroid Output in NOD/SCID Mice Reconstituted With Human Hematopoietic Stem Cells

Jun Hayakawa^{*}, Matthew M. Hsieh^{*}, D. Eric Anderson[†], Oswald Phang^{*}, Naoya Uchida^{*}, Kareem Washington^{*}, and John F. Tisdale^{*}

^{*}Molecular and Clinical Hematology Branch, National Institutes of Diabetes and Digestive and Kidney Disorders (NIDDK) and National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health, Bethesda, MD, USA

[†]Proteomics and Mass Spectrometry Facility, National Institutes of Diabetes and Digestive and Kidney Disorders (NIDDK), National Institutes of Health, Bethesda, MD, USA

Abstract

The third-generation NOD/LtSz-*scid*/*IL2R γ ^{null}* (NOD/SCID *IL2R γ ^{null}*) mouse represents a significantly improved xenograft model allowing high levels of human leukocyte engraftment over extended follow up. One remaining limitation of this mouse model, however, is the low level of circulating human erythrocytes. We established a practical ex vivo erythroid culture system of xenograft marrow progenitors to enrich for human erythroid progeny. At various time points after transplant, erythroid cells were easily assayed after 17 days of ex vivo culture of xenograft marrow, with nearly all nucleated cells of human origin and approximately 60% human GPA or CD71 positive. We then transplanted cord blood CD34⁺ cells marked with a lentiviral vector encoding green fluorescent protein (GFP). Three months later, ex vivo culture of xenograft marrow progenitors showed 41.3% of the cultured erythroid cells were positive for GFP and human CD71, and 56.2% were positive for GFP and human GPA, similar to that of circulating leukocytes at the same time point. Next, G-CSF mobilized peripheral blood CD34⁺ cells from a sickle cell trait subject were infused in this mouse model to determine if the hemoglobin pattern could be modeled. CD34⁺ cells from the sickle cell trait subject engrafted equally compared to CD34⁺ cells from normal subjects, establishing the sickle cell trait phenotype. Lastly, a comparison of adult-derived peripheral blood CD34⁺ cells and cord blood-derived CD34⁺ cells xenografted mice was made, and long term follow-up demonstrated a recapitulation of the fetal to adult hemoglobin switch. This approach should prove a useful tool for testing strategies for genetic manipulation of erythroid progeny and the study of hemoglobin switching.

Keywords

NOD/LtSz-*scid*/*IL2R γ ^{null}* mice; Erythropoiesis; Xenograft mouse model; Sickle cell; HbF; HbS; Hemoglobin switching; Lentiviral vector; GFP

INTRODUCTION

The xenograft mouse model is an attractive tool to query the long-term repopulating potential of steady-state or modified human hematopoietic stem cells (HSC). The recently developed NOD/SCID *IL2R γ ^{null}* mouse model has several advantages including a longer life

span, a much reduced risk of developing malignancy, and very low numbers of murine T, B, NK, and mast cells. As a result, higher human cell engraftment can be accomplished than with previous NOD/SCID mouse models (9,26,31). Our group has established an alternative to irradiation conditioning regimen using parenteral busulfan that achieves high-level human cell engraftment at low mortality compared to conventional conditioning with total body irradiation (27). While sufficient numbers of human leukocyte can be achieved, circulating human erythrocytes in the xenograft peripheral blood remain low.

Ishikawa et al. previously showed that in xenografted newborn NOD/SCID *IL2R γ ^{null}* mice, human red cells were detectable in the peripheral circulation at very low levels, and human erythroid progenitors were present at 9.5% in the bone marrow (BM) 3 months posttransplant (11). In our model using 7–10-week-old mice, human glycophorin A (GPA) positive erythrocytes were only detectable in peripheral blood after intraperitoneal injection of human holo-transferrin immediately after transplantation. These human red cells were present for about 3–4 weeks with the highest level at 0.1% (7). There were no human red cells circulating long term after transplantation, after human red cell infusion, or after splenectomy. The lack of human red cell output may result from one or more of several possibilities: differences in the erythroid stress response (24), low globin gene and protein expression (15,28), lack of human-specific cytokines (19), species differences between human and murine transferrin, or other anti-human inhibitory signals.

In this report, we have built upon our prior experience with human cell output in this NOD/SCID *IL2R γ ^{null}* mouse model using ex vivo culture of xenograft marrow for human erythroid differentiation. This approach using common transplantation and cell culture techniques has allowed us to overcome the limitation of human red cell reconstitution in this model.

MATERIALS AND METHODS

Mice

Male NOD.Cg-Prkdc^{scid} IL2r^{tm1Wjl}/SzJ (NOD/LtSz-*scid*/*IL2R γ ^{null}*, NOD/SCID *IL2R γ ^{null}*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and have been reported elsewhere (10,26). Mice were 7–10 weeks old at the time of transplant. Mice were cared for according to a protocol approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) at the National Institutes of Health (NIH).

Human Hematopoietic Cell Preparation

Cord blood (CB) cells were obtained from healthy volunteers postpartum after written informed consent using a protocol approved by the NIDDK Institutional Review Board (IRB). Mononuclear cells (MNC) were obtained after density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biotech, Piscataway, NJ) and red blood cells (RBCs) were removed using ammonium chloride potassium (ACK) lysis buffer (Quality Biological Inc, Gaithersburg, MD). CD34 enrichment was performed using CD34 biotinylated antibody and streptavidin microbeads (Miltenyi Biotec, Auburn, CA). Cells were passed through the positive selection column twice, and their purity assessed by flow cytometry using phycoerythrin (PE)-conjugated anti-human CD34 antibody (clone 563) on a FACS Calibur (all BD Biosciences, San Jose, CA).

Peripheral blood CD34⁺ cells (PB CD34⁺) were obtained from healthy adult volunteers using a protocol approved by the NIDDK IRB (12). Briefly, volunteers received 5 days of 10 μ g/kg recombinant human (rHu) granulocyte colony-stimulating factor (G-CSF, Amgen,

Thousand Oaks, CA). On day 5 of mobilization, PB CD34⁺ cells were collected by one 12–15-L leukapheresis, and CD34-positive selection was performed using the Isolex 300i automated immuno-magnetic system (Nexell, Miltenyi, Auburn, CA).

NOD/SCID *IL2Ry^{null}* Mice Transplantation

Busulfan (Busulfex, Otsuka Pharmaceutical, Rockville, MD) was diluted with phosphate-buffered saline (PBS, Biofluids, Rockville, MD) to deliver 35 mg/kg in a final volume of 200–500 μ l and was injected into recipient mice intraperitoneally at least 24 h prior to the cell infusion. CB CD34⁺ cells (2×10^6) or PB CD34⁺ cells (2×10^6) were infused intravenously via the tail vein as previously described (8).

Lentiviral Vector Preparation and Transduction

Self-inactivating (SIN) human immunodeficiency virus 1 (HIV1) vector was prepared as previously described (6). We used a four-plasmid system with pCAG KGP1.1R (gag/pol), pCAG4-RTR2 (rev/tat), pCAGGS-VSVG (VSV-G envelope), and pCL20c MpGFP. This vector expresses GFP under the control of MSCV-LTR-U3 promoter. Human CB CD34⁺ cells were prestimulated in X-VIVO 10 (BioWhittaker, Walkersville, MD) containing SCF, FLT3L, and TPO (all at 100 ng/ml, R&D Systems, Minneapolis, MN) in CH-296 (RetroNectin, Takarashuzo, Japan)-coated plates for 24 h and then transduced with these vectors at a multiplicity of infection (MOI) of 50. Four days later, the GFP expression of transduced CB cells was evaluated by FACS Calibur.

Flow Cytometric Analysis for Donor Chimerism and Leukocyte Subsets

BM was harvested as previously described (8,22) and suspended in DMEM with 0.1% bovine serum albumin (BSA, Roche, Basel, Switzerland). BM and PB cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 and PE-conjugated anti-mouse CD45; human leukocyte subsets were also stained with one of the following PE-conjugated antibodies: CD3, CD14, CD16, CD20, CD41, and CD56. Red blood cells were stained with anti-mouse TER119-FITC and anti-human glycophorin A (GPA)-PE (CD235a); erythrocyte subsets were stained with human CD45-FITC and CD71-PE. All antibodies were purchased from BD Biosciences. For the evaluation of GFP-marked human BM cell engraftment for the mice, we stained with anti-human CD45-PE.

Ex Vivo Culture of Progenitors for Human Erythroid Differentiation

To obtain erythroid output in vitro, we modified previously described erythroid culture methods (3,14,18, 20,29). In the first 3 days (Fig. 1), mouse bone marrow (BM, 2×10^7 cells), human CB CD34⁺ ($1-2 \times 10^6$ cells), or PB CD34⁺ cells ($1-2 \times 10^6$ cells) were cultured in six-well plates in 5 ml of DMEM containing 10% fetal bovine serum (FCS) (Hyclone®, Thermo Scientific, UT), 2 mM glutamine with penicillin/streptomycin (Invitrogen, Carlsbad, CA), 100 ng/ml of rHu stem cell factor (SCF), and 10 ng/ml of rHu interleukin-3 (IL-3) (both R&D systems) at 37°C in 5% CO₂. This cytokine combination preferentially differentiated human cells. In the next 7 days, nonadherent cells were collected and resuspended in 20 ml of media specific for erythroid differentiation from a modified protocol containing Dulbecco's modified essential media (DMEM, Mediatech, Herdon, VA), 30% FCS, 2% BSA, 10^{-5} M β -mercaptoethanol, 10^{-6} M dexamethasone, 0.6 mg/ml human holo-transferrin (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine with penicillin/streptomycin (Invitrogen), 100 ng/ml of SCF, 1.25 ng/ml of transforming growth factor- β (TGF- β , R&D Systems) in the medium, and 5 U/ml rHu erythropoietin (EPO, Amgen), in a 75-cm² flask at 37°C in 5% CO₂. In the final 7 days, the nonadherent cells were again collected and resuspended in the same erythroid-specific media but without TGF- β .

Hemoglobin Determination by Hemoglobin Electrophoresis

Hemoglobin electrophoresis was performed according to the manufacturer's procedures (Helena Laboratories, Beaumont, TX) (4). To determine the hemoglobin content from red cells, RBC pellets were lysed with water, frozen at -20°C for 5 min, and thawed at 37°C for 5 min. Then 15 μl of the supernatant was mixed with 10 μl of Hemolysate Reagent (Helena Laboratories) and run on Titan 3 cellulose acetate membranes (Helena Laboratories) at 270 volts for 40 min. After electrophoresis, the membrane was stained in Ponceau S (Helena Laboratories) for 5 min and washed with 7.5% acetic acid (Sigma) five times and ethanol five times.

Mass Spectrometry Analysis of Hemoglobin Subtypes

The products from erythroid culture were analyzed for protein content by mass spectrometry. Human GPA-positive cells were pelleted, washed twice, and lysed with water, frozen at -20°C for 5 min, and thawed at 37°C for 5 min. Proteins were analyzed using tandem HPLC-mass spectrometry (MS). Mass from eluted peptides was queried in the Mascot database, and searched for human hemoglobin variants.

Statistics

All measures of variance are presented as standard errors of the mean (SEM). Statistical analyses were performed using Student's *t*-test with a value of $p < 0.05$ deemed significant.

RESULTS

Human Erythroid Cells Can Be Cultured From the Marrow of Humanized Mice

When murine peripheral blood was directly assayed periodically after transplantation, there was $<1\%$ human CD71⁺ or human GPA⁺ cells up to 3 months posttransplant (data not shown). We then decided to harvest BM cells from an NOD/SCID *IL2R γ ^{null}* mouse 4 months after transplant with human PB CD34⁺ cells, and culture them in human erythroid-specific media. In the beginning of the ex vivo culture, the BM cell pellet after red cell lysis appeared white or gray, became visibly hemoglobinized at day 10, and progressed to a more red color by day 18 (Fig. 2A). When we analyzed cells for human CD71 and GPA from five other mice 1–5 months after transplantation of PB CD34⁺ cells, the percentage of human erythroid cells (CD71 or GPA) in culture began to increase after day 7 and plateaued between day 14 and 18 (Fig. 2B and data not shown). Without ex vivo culture, no human GPA⁺ cells could be detected in the circulating PB (data not shown) or BM. Ex vivo culture of murine bone marrow after transplantation of human cord blood CD34⁺ cells also shows similar results (Fig. 2C). To confirm that the GPA⁺ cells were of human origin, GPA⁺ RBCs from day 18 were sorted, and the hemoglobin extracts were analyzed by tandem HPLC-MS. MS revealed the presence of human α -, β -, and γ -globin chains (data not shown).

GFP Marking Is Present Among Erythroid Progeny After Ex Vivo Culture of Humanized Murine BM

To test if this transplantation model could be employed to model gene therapy applications, human CB CD34⁺ cells were transduced by a lentiviral vector encoding GFP at a MOI of 50. The in vitro transduction efficiency rate was 51.7%. Transduced human CB CD34⁺ cells (1×10^6) were infused in each NOD/SCID *IL2R γ ^{null}* mouse after conditioning with 35 mg/kg of busulfan ($n = 6$). The human CD45⁺ percentage in the PB of the humanized mice was $3.4 \pm 1.7\%$ 3 weeks posttransplant and $33.3 \pm 14.3\%$ 3 months posttransplant. Interestingly, the percentage of GFP among human CD45⁺ cells was $42.4 \pm 5.8\%$ 3 weeks posttransplant and increased to $79.4 \pm 1.85\%$ 3 months posttransplant. CD3⁺, CD14⁺, CD16⁺, CD20⁺, CD41⁺, or CD56⁺/GFP⁺ cells could also be detected in the recipient mice (data not shown).

When murine peripheral blood was directly assayed, there were no human CD71⁺ or human GPA⁺ cells 3 months posttransplant (data not shown). However, when BM was collected 3 months posttransplant in one representative mouse, 70.2% were positive for both GFP and human CD45 (Fig. 3A). After 18 days of ex vivo culture, GFP and human CD45⁺ cells were enriched to 79.2% (Fig. 3B–D); GFP and human CD71⁺, 41.3% (Fig. 3E); and GFP and human GPA⁺, 56.3% (Fig. 3F). We then sorted human CD71⁺ or GPA⁺ cells by using the Miltenyi magnetic selection system and confirmed an almost 100% enrichment by flow (data not shown). These experiments indicated that the genetically modified human CD34⁺ cells could engraft in the murine BM, and maintain the capability to differentiate to human erythroid cells ex vivo with human-specific cytokines.

Recapitulation of Sickle Cell Trait in NOD/SCID *IL2Rγ*^{null} Mice

We next transplanted PB CD34⁺ cells from healthy adults with sickle cell trait (1×10^6 cells per mouse, $n = 12$), or without sickle cell trait (controls, 1×10^6 cells per mouse, $n = 5$), in NOD/SCID *IL2Rγ*^{null} mice after 35 mg/kg of busulfan. The percentage of human CD45⁺ cells 3 months posttransplant was similar in the PB of sickle cell trait recipients and control recipients: $13.1 \pm 8.4\%$ versus $13.9 \pm 10.0\%$, respectively. At 1 and 3 months posttransplant, BM cells were harvested from one representative mouse from each group and cultured ex vivo. After 18 days of human erythroid differentiation, the hemoglobin composition was assessed by electrophoresis (Fig. 4). Adult hemoglobin (HbA) and sickle hemoglobin (HbS) were detected both 1 and 3 months after transplant, and the pattern was similar to that derived from sickle trait product cultured pretransplant. Interestingly, a significant amount of fetal hemoglobin (HbF) was also present at both time points, likely a reflection of the culture conditions.

Recapitulation of Hemoglobin Switching

To determine if erythroid development occurs in a manner similar to that in humans, we performed additional experiments to examine the hemoglobin content with respect to infused cell type. First, we cultured a small aliquot of PB CD34⁺ or CB CD34⁺ cells ex vivo and determined the hemoglobin content after 18 days. As expected, PB CD34⁺ cells produced more HbA, whereas CB CD34⁺ cells produced more HbF. The addition of TGF-β did not increase the amount HbF from PB CD34⁺ or CB CD34⁺ culture (data not shown). We then transplanted PB CD34⁺ and CB CD34⁺ cells to two groups of mice after 35 mg/kg of busulfan conditioning, harvested BM cells at various time points, and cultured BM cells ex vivo. The hemoglobin electrophoresis of the ex vivo cultured red cells showed that transplanted PB CD34⁺ cells consistently produced more HbA than HbF, and this proportion of HbA to HbF ratio did not change from 3 weeks to 6 months posttransplant (Fig. 5A). In contrast, transplanted CB CD34⁺ cells produced mostly HbF at 3 weeks, but the HbA to HbF ratio gradually reversed, similar to the ratio from PB CD34⁺ cells, by 6–9 months (Fig. 5B). The progressive loss of HbF synthesis over 9 months closely mirrors that which is observed in the normal hemoglobin switching, which occurs in newborns from 1 to 6 months postnatally.

DISCUSSION

Humanized mice are a useful tool to assay long-term hematopoietic progenitors, and could reduce the need for the more cumbersome and expensive large-animal models. We and others have achieved high level in vivo human cell engraftment in this third-generation NOD/SCID mouse model, which is sustained beyond 4–6 months and in multiple leukocyte subsets (granulocytes, B and T lymphocytes, and NK cells). However, human erythroid cells in murine peripheral blood remained low in vivo, despite injection with human cytokines (7). In this report, we extend our prior observations and demonstrate that human erythroid

progenitors reside long term in the humanized murine bone marrow and can differentiate to sufficient numbers of mature red cells ex vivo in the presence of human-specific cytokines to allow evaluation of hemoglobin at the protein level. This straightforward approach of transplantation, marrow progenitor harvest, and ex vivo culture overcomes one limitation of the NOD/SCID *IL2R γ ^{null}* xenograft model and allows for assaying erythroid progeny of human HSCs after extended follow up.

Our ex vivo culture system employs common murine transplantation techniques, reagents, and human-specific cytokines. The first 3 days of rHu IL-3 and SCF preferentially enriched human cells. The next 7 days of SCF, EPO, and TGF- β directed the differentiation of erythroid cells: SCF increased erythroblast growth (17,21,29), EPO provided the necessary erythroid cytokine, and TGF- β accelerated this entire process (1,2,32). We have previously shown that human holo-transferrin provides the iron necessary for erythroid production (7), and have incorporated this in the culture cocktail. The last 7 days of SCF and EPO served to further expand the number of mature red cells, which were positive for human CD71 or GPA. Culturing beyond the 18 days only increased the red cell number modestly; the maximal number plateaued between 3 and 4 weeks of culture (data not shown).

We then tested whether this approach could be combined with genetic modification of progenitor cells to express GFP using a SIN-lentiviral vector as a model for gene transfer approaches targeting red blood cells. The transplantation of these marked cells yielded 33% of the circulating peripheral blood leukocytes of human origin 3 months later, consistent with our prior experience. When bone marrow cells were harvested and cultured ex vivo, GFP⁺ and human CD45⁺ leukocytes could be further enriched. Furthermore, GFP⁺ and GPA⁺ red cells can be cultured using the same system, yielding sufficient quantities of red blood cells for analysis of the success of the genetic modification strategy at the protein level. The logical next step would be to substitute GFP for a gene of interest, such as β - or γ -globin, and evaluate the amount of globin expressed per vector copy in the erythroid compartment.

The usefulness of this xenograft model and ex vivo human erythroid culture also allowed us to test whether a human erythroid phenotype, such as HbS in red cells, could be reproduced. Our transplantation of G-CSF mobilized PB CD34⁺ cells from a sickle cell trait donor demonstrated that these cells engraft equally well, and that their ability to synthesize HbS was preserved in the xenograft bone marrow. Although not a disease model, engraftment of sickle trait HSCs in transplanted NOD/SCID mice should allow testing of genetic modification strategies aimed at correction of the hemoglobinopathies such as RNA transplicing (13) or RNA interference (25). This model may prove invaluable, as collection of mobilized CD34⁺ cells from individuals with sickle cell disease is associated with prohibitive toxicity (5). Additionally, other hemoglobin variants or bone marrow diseases could be modeled without transgenic techniques (16, 23,30).

During the process of creating sickle cell trait mice, we discovered that the ability to make HbF was dependent on hematopoietic progenitor source. While HbF was detected from culturing PB CD34⁺ as shown previously, CB CD34⁺ cells retained substantially higher HbF synthetic potential when cultured in vitro (data not shown), or when cultured from xenograft mice shortly after transplantation (Fig. 5). This disparity between CB versus PB CD34⁺, however, could not be overcome even by transplantation or potent cytokine cocktails previously shown to induce HbF, such as SCF or TGF- β . The longer CB CD34⁺ cells remained in the xenograft bone marrow, the lower amount of HbF protein was synthesized. Eventually the HbA/HbF proportion in the CB CD34⁺ group approached to that of the PB CD34⁺ group, suggesting that the fetal to adult hemoglobin switch had occurred (30). The combination of the murine marrow microenvironment, murine cytokines, and/or

gene silencing was sufficiently potent to mimic the normal postnatal human hemoglobin switching process, across a major interspecies difference.

In summary, our practical method to evaluate the erythroid compartment in the humanized NOD/SCID *IL2R- γ ^{null}* mice model is an important step forward and should allow for future preclinical testing of genetic modification strategies aimed at human red cells. This ex vivo human erythroid culture system also allows recapitulation of hemoglobin variants without the need for creating transgenic mice. Finally, the combination of transplantation and ex vivo culture simulated the fetal to adult hemoglobin switch, and should allow future detailed investigation of this important human postnatal transition.

Acknowledgments

This work was supported by the Intramural Research Program of NIDDK and NHLBI at the NIH.

References

1. Bhanu NV, Trice TA, Lee YT, Gantt NM, Oneal P, Schwartz JD, Noel P, Miller JL. A sustained and pancellular reversal of gamma-globin gene silencing in adult human erythroid precursor cells. *Blood*. 2005; 105(1):387–393. [PubMed: 15367428]
2. Chen LL, Dean A, Jenkinson T, Mendelsohn J. Effect of transforming growth factor-beta 1 on proliferation and induction of hemoglobin accumulation in K-562 cells. *Blood*. 1989; 74(7):2368–2375. [PubMed: 2804370]
3. Dao MA, Pepper KA, Nolte JA. Long-term cytokine production from engineered primary human stromal cells influences human hematopoiesis in an in vivo xenograft model. *Stem Cells*. 1997; 15(6):443–454. [PubMed: 9402657]
4. Ferris TG, Easterling RE, Budd RE. Hemoglobin electrophoresis in acrylamide gel. *Blood*. 1962; 19:479–482. [PubMed: 13892595]
5. Fitzhugh CD, Hsieh MM, Bolan CD, Saenz C, Tisdale JF. Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: Time for a moratorium? *Cytotherapy*. 2009; 11(4):464–471. [PubMed: 19513902]
6. Hanawa H, Persons DA, Nienhuis AW. Mobilization and mechanism of transcription of integrated self-inactivating lentiviral vectors. *J Virol*. 2005; 79(13):8410–8421. [PubMed: 15956585]
7. Hayakawa J, Hsieh MM, Uchida N, Phang O, Tisdale JF. Busulfan produces efficient human cell engraftment in NOD/LtSz-Scid *IL2Rgamma*(null) mice. *Stem Cells*. 2009; 27(1):175–182. [PubMed: 18927475]
8. Hayakawa J, Migita M, Ueda T, Shimada T, Fukunaga Y. Generation of a chimeric mouse reconstituted with green fluorescent protein-positive bone marrow cells: A useful model for studying the behavior of bone marrow cells in regeneration in vivo. *Int J Hematol*. 2003; 77(5):456–462. [PubMed: 12841383]
9. Hiramatsu H, Nishikomori R, Heike T, Ito M, Kobayashi K, Katamura K, Nakahata T. Complete reconstitution of human lymphocytes from cord blood CD34⁺ cells using the NOD/SCID/gammacnull mice model. *Blood*. 2003; 102(3):873–880. [PubMed: 12689924]
10. Hubin F, Humblet C, Belaid Z, Greimers R, Boniver J, Thiry A, Defresne MP. Maintenance of functional human cancellous bone and human hematopoiesis in NOD/SCID mice. *Cell Transplant*. 2004; 13(7–8):823–831. [PubMed: 15690985]
11. Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, Watanabe T, Akashi K, Shultz LD, Harada M. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood*. 2005; 106(5):1565–1573. [PubMed: 15920010]
12. Kang EM, Areman EM, David-Ocampo V, Fitzhugh C, Link ME, Read EJ, Leitman SF, Rodgers GP, Tisdale JF. Mobilization, collection, and processing of peripheral blood stem cells in individuals with sickle cell trait. *Blood*. 2002; 99(3):850–855. [PubMed: 11806986]

13. Kierlin-Duncan MN, Sullenger BA. Using 5'-PTMs to repair mutant beta-globin transcripts. *RNA*. 2007; 13(8):1317–1327. [PubMed: 17556711]
14. Koestenbauer S, Zisch A, Dohr G, Zech NH. Protocols for hematopoietic stem cell expansion from umbilical cord blood. *Cell Transplant*. 2009; 18(10–11):1059–1068. [PubMed: 19523346]
15. Larochelle A, Vormoor J, Lapidot T, Sher G, Furukawa T, Li Q, Shultz LD, Olivieri NF, Stamatoyannopoulos G, Dick JE. Engraftment of immune-deficient mice with primitive hematopoietic cells from beta-thalassemia and sickle cell anemia patients: Implications for evaluating human gene therapy protocols. *Hum Mol Genet*. 1995; 4(2):163–172. [PubMed: 7757063]
16. Lu L, Ge Y, Li ZH, Freie B, Clapp DW, Broxmeyer HE. CD34 stem/progenitor cells purified from cryopreserved normal cord blood can be transduced with high efficiency by a retroviral vector and expanded ex vivo with stable integration and expression of Fanconi anemia complementation C gene. *Cell Transplant*. 1995; 4(5):493–503. [PubMed: 8520833]
17. Miller BA, Perrine SP, Bernstein A, Lyman SD, Williams DE, Bell LL, Olivieri NF. Influence of steel factor on hemoglobin synthesis in sickle cell disease. *Blood*. 1992; 79(7):1861–1868. [PubMed: 1373091]
18. Nakahata T, Kobayashi T, Ishiguro A, Tsuji K, Naganuma K, Ando O, Yagi Y, Tadokoro K, Akabane T. Extensive proliferation of mature connective-tissue type mast cells in vitro. *Nature*. 1986; 324(6092):65–67. [PubMed: 3491321]
19. Neildez-Nguyen TM, Wajcman H, Marden MC, Bensidhoum M, Moncollin V, Giarratana MC, Kobari L, Thierry D, Douay L. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol*. 2002; 20(5):467–472. [PubMed: 11981559]
20. Nolta JA, Hanley MB, Kohn DB. Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: Analysis of gene transduction of long-lived progenitors. *Blood*. 1994; 83(10):3041–3051. [PubMed: 7514050]
21. Peschle C, Gabbianelli M, Testa U, Pelosi E, Barberi T, Fossati C, Valtieri M, Leone L. c-kit ligand reactivates fetal hemoglobin synthesis in serum-free culture of stringently purified normal adult burst-forming unit-erythroid. *Blood*. 1993; 81(2):328–336. [PubMed: 7678510]
22. Powell JD, Fitzhugh C, Kang EM, Hsieh M, Schwartz RH, Tisdale JF. Low-dose radiation plus rapamycin promotes long-term bone marrow chimerism. *Transplantation*. 2005; 80(11):1541–1545. [PubMed: 16371922]
23. Puthenveetil G, Scholes J, Carbonell D, Qureshi N, Xia P, Zeng L, Li S, Yu Y, Hiti AL, Yee JK, Malik P. Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood*. 2004; 104(12):3445–3453. [PubMed: 15292064]
24. Rothman IK, Zanjani ED, Gordon AS, Silber R. Nucleoside deaminase: An enzymatic marker for stress erythropoiesis in the mouse. *J Clin Invest*. 1970; 49(11):2051–2067. [PubMed: 5475986]
25. Samakoglu S, Lisowski L, Budak-Alpdogan T, Usachenko Y, Acuto S, Di Marzo R, Maggio A, Zhu P, Tisdale JF, Riviere I, Sadelain M. A genetic strategy to treat sickle cell anemia by coregulating globin transgene expression and RNA interference. *Nat Biotechnol*. 2006; 24(1):89–94. [PubMed: 16378095]
26. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL, Handgretinger R. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol*. 2005; 174(10):6477–6489. [PubMed: 15879151]
27. Uchida N, Washington K, Hayakawa J, Hsieh M, Bonifacino AC, Krouse AE, Metzger ME, Donahue RE, Tisdale JF. Development of An HIV1-based lentiviral vector able to transduce both human and rhesus blood cells. *Blood*. 2008; 112(11):2353a. [PubMed: 18625884]
28. Ueda T, Yoshino H, Kobayashi K, Kawahata M, Ebihara Y, Ito M, Asano S, Nakahata T, Tsuji K. Hematopoietic repopulating ability of cord blood CD34(+) cells in NOD/Shi-scid mice. *Stem Cells*. 2000; 18(3):204–213. [PubMed: 10840074]
29. Wojda U, Noel P, Miller JL. Fetal and adult hemoglobin production during adult erythropoiesis: Coordinate expression correlates with cell proliferation. *Blood*. 2002; 99(8):3005–3013. [PubMed: 11929793]

30. Wood WG. Haemoglobin synthesis during human fetal development. *Br Med Bull.* 1976; 32(3): 282–287. [PubMed: 788839]
31. Yahata T, Ando K, Nakamura Y, Ueyama Y, Shimamura K, Tamaoki N, Kato S, Hotta T. Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. *J Immunol.* 2002; 169(1):204–209. [PubMed: 12077246]
32. Zermati Y, Fichelson S, Valensi F, Freyssinier JM, Rouyer-Fessard P, Cramer E, Guichard J, Varet B, Hermine O. Transforming growth factor inhibits erythropoiesis by blocking proliferation and accelerating differentiation of erythroid progenitors. *Exp Hematol.* 2000; 28(8):885–894. [PubMed: 10989189]

Figure 1.

Experimental schema of ex vivo culture of progenitors for human erythroid differentiation. NOD/SCID, nonobese diabetic/severe combined immune deficiency; DMEM, Dulbecco's modified essential media; FCS, fetal calf serum; BSA, bovine serum albumin; EPO, erythropoietin; IL, interleukin; SCF, stem cell factor; TGF, transforming growth factor.

Figure 2.

Confirmation of human-specific erythroid expansion. (A) Appearance of cell pellet before and after ex vivo culture. (B) Percent human red cells throughout ex vivo culture. The y-axis denotes the percentage of human CD71⁺ or GPA⁺ cells by flow cytometry at various time points. BM cells were collected from mice ($n = 5$) transplanted with 1×10^6 human PB CD34⁺ cells 1–6 months ago. The error bars represent SD. (C) Percent human CD45⁺ cells throughout ex vivo culture. The y-axis denotes the percentage of the human CD45⁺ cells by flow cytometry at various time points. Bone marrow cells were collected from mice transplanted with 2×10^6 human peripheral blood mobilized (PB) CD34⁺ cells ($n = 6$) or 2×10^6 cord blood (CB) CD34⁺ cells ($n = 5$) 3 months ago. The error bars represent SD.

Figure 3.

Transplantation of GFP marked progenitors in NOD/SCID *IL2R γ ^{null}* mice. Three months after transplantation, bone marrow (BM) from one representative mouse was harvested, and percent of green fluorescent protein (GFP) and human CD45⁺ cells are shown before culture (A) and after 18 days of ex vivo culture (B). GFP⁺ cells were visualized at day 18 under fluorescent microscope at low magnification (10 $\times$), under optical light (C) and UV light (D). Double positive cells for CD71 and GFP (E) and glycophorin-A (GPA) and GFP (F) are shown.

Figure 4.

Cellulose acetate hemoglobin electrophoresis gel. Lane 1 contained adult hemoglobin (HbA); lane 2, cord blood with majority of fetal hemoglobin (HbF); and lane 3, peripheral blood of sickle cell trait (HbS + HbA). Lanes 4 and 5 showed hemoglobin proteins present in the RBCs after 18 days of ex vivo culture humanized NOD/SCID *IL2R γ ^{null}* mice BM. The last lane was a sample derived from PB CD34⁺ cells of a sickle cell trait individual after 18 days of ex vivo culture, before xenograft transplantation.

Figure 5. Hemoglobin content from xenograft mice mimics postnatal hemoglobin switching. Bone marrow cells were collected and cultured ex vivo from xenograft mice at various time points after transplantation of peripheral blood mobilized (PB) CD34⁺ cells (A) or cord blood (CB) CD34⁺ cells (B). Cellulose acetate hemoglobin electrophoresis showed the same HbF amount at all time points in PB CD34⁺ group, but gradually decreasing HbF level in the CB CD34⁺ group.