Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Rachel Pan April 9, 2019

Establishing a murine model for preclinical evaluation of novel erythrocyte transfusion therapies

by

Rachel Pan

Dr. John Roback
Adviser

Department of Biology

Dr. John Roback
Adviser

Dr. Arri Eisen

Committee Member

Dr. Corey Keyes

Committee Member

Dr. LaTonia Taliaferro-Smith

Committee Member

2019

Establishing a murine model for preclinical evaluation of novel erythrocyte transfusion therapies

By

Rachel Pan

Dr. John Roback

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2019

Abstract

Establishing a murine model for preclinical evaluation of novel erythrocyte transfusion therapies

By Rachel Pan

The United States' blood supply, though large, fails to meet the transfusion needs for certain patient populations, such as those with sickle cell disease and rare blood types. Induced pluripotent stem cell differentiation and expansion may provide a facile way to produce large quantities of patient-personalized, immunologically-compatible red blood cells (RBCs) to augment or replace conventional donated transfusion products. However, before clinical evaluation can take place, stem-derived RBCs must first demonstrate preclinical efficacy and an ability to deliver oxygen to tissues in a manner comparable to conventional transfusion products. This study presents and validates a novel, severely immunocompromised murine model to provide the basis for preclinical studies of novel red blood cell therapies. This model was highly human RBC-tolerant, acutely anemic, and conducive toward direct tissue pO_2 measurement. Mice bred on a NOD-SCIDγ background were treated with reagents meant to deplete the innate immune system and given human RBC transfusion. It was demonstrated that the mouse model was highly permissive to human RBCs as transfused cells were found to circulate *in vivo* up to 42 days. To determine RBCs' ability to reverse tissue hypoxia and related sequelae, Black 6 mice were made acutely anemic via isovolumic hemodilution using either serum infusion or mouse blood transfusion as fluid replacement. During cycles of bleeding and fluid replacement, changes in tissue pO_2 were detected in real time using tissue-localized phosphorescence quenching oximetry. Results indicate that this preclinical model is optimized to demonstrate stem derived-RBCs' ability to deliver oxygen to tissues and indicate future clinical development, representing a significant step toward clinical applications.

Establishing a murine model for preclinical evaluation of novel erythrocyte transfusion therapies

By

Rachel Pan

Dr. John Roback

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2019

Acknowledgements

I would like to thank my mentor, Dr. John Roback, for his invaluable guidance and expertise on in this project. Special thanks are due to Drs. Ryan Jajosky, Satheesh Chonat, and Jiusheng Deng. Finally, I thank Dr. Eisen, Dr. Smith, and Dr. Keyes for serving as members of my committee as well as for their unwavering support and encouragement.

Table of Contents

Introduction

Current state and future directions of transfusion therapy in the United States

Approximately 13.6 million erythrocyte transfusion units are collected in the United States yearly, adequately meeting the clinical needs of most anemic patients (1). However, as the US population grows older, there will be increased demand for transfusion products in the near future. Also adding to this demand are transient, unpredictable shortages in the blood supply which may arise in times of disaster. The existing blood supply is often insufficient to treat populations dependent on chronic transfusion, such as those with sickle cell disease (SCD). These patients often face a greater risk of adverse transfusion reactions due to long-term development of red blood cell (RBC) allo-antibodies, a relatively common outcome of chronic transfusion therapy which occurs in 25%-35% of all patients living with SCD (2, 3). Alloimmunization interferes with care delivery, causing long wait times to find phenotypically-matched transfusion products and delaying transfusion (4). In some cases, alloimmunization can lead to severe clinical complications such as delayed hemolytic transfusion reactions during which the recipient mounts an immune response against an antigen present in transfusion product, causing pain and shock immediately after transfusion (5). Patients with SCD and related conditions must therefore strike a balance between the risks of inducing alloimmunization and the benefits of maintaining normal hemoglobin levels from transfusions. Thus, work must be done to enhance the efficacy and availability of transfusion products to address this unmet need.

Engineering novel iPSC-derived RBCs for specialized transfusion applications

Alloimmunization can be avoided with extensive RBC antigen matching (e.g. Rh, K, C), lowering the frequency of occurrence to 0%-7% (6). However, efforts to identify a perfect phenotypically-matched donor resulted in no found match for 6.9%-8.6% of SCD patients (7). Induced pluripotent stem-cell derived red blood cells (henceforth "iPSC-RBCs") have been studied as a means to circumvent alloimmunization and matching concerns by producing personalized, immunologically-compatible RBCs. Here, the term "iPSC" is meant to include cell lines established by reprogramming mononuclear cells obtained from 1-25mL volumes of peripheral blood using lentiviral vectors or episomal plasmids expressing the Yamanaka factors (Su). Such cells can be re-engineered via genome editing to express particular phenotypes or altered functions and differentiated into mature, enucleated RBCs under xeno-free, feeder-free, and cGMP-compliant conditions (8).

Recent improvements in scaled *ex vivo* expansion and differentiation seek to address unmet needs in patient populations living with hemoglobinopathies (9). Several groups have developed protocols driving the differentiation of various stem cell types to mature, enucleated erythrocytes with similar function and morphology to conventional donor-derived RBCs (10). As a proof-of-principle, a landmark study demonstrated *ex vivo*-generated iPSC-RBCs were able to circulate in a human subject with a half-life of 28 days while performing hemoglobin-oxygen binding comparable to donated erythrocytes (12).

Regulatory obstacles

Large-scale clinical studies of cultured RBCs currently face regulatory obstacles set forth by the Food and Drug Administration (FDA). Much work has been done to improve and commercialize methods for producing various *ex vivo*-produced RBCs, but little has been directed at addressing their preclinical efficacy (13). Indication for future refinement and development of cultured RBC products requires that RBC products demonstrate an ability to deliver oxygen to tissues in a manner comparable to conventional transfusion products. Specifically, reports from the FDA detail requirements for *in vitro* hemoglobin-oxygen binding data as well as preclinical model evidence that cultured RBC products effectively deliver oxygen to peripheral tissue (8, 14). Thus, iPSC-RBCs should be tested *in vivo* in a small animal system as a proof-of-principle evaluation of RBC quality.

Proposed murine transfusion model

This investigation proposes and validates a novel mouse transfusion model which, in the future, can be used to assess the survival and functional performance of iPSC-RBCs produced under cGMP-compliant conditions in order to evaluate therapeutic efficacy. This murine model must exhibit compensatory metabolic responses in response to tissue hypoxia and sequelae and be tolerant to human RBC (henceforth "hRBC") transfusion. Previously, the FDA's Center for Biologics Evaluation and Research (CBER) designed and evaluated a mouse model similarly aimed to assess cultured RBCs *in vivo* (14). However, model parameters were not optimized to fully address preclinical efficacy. Mice were bred to be heterozygous for thalassemia, a condition adversely affecting RBC oxygen carrying capacity, creating a state of chronic anemia to which the mouse may have become acclimated. Tissue hypoxia was induced by exercise, which carries potential confounds such as hypertension. Mice were also bred on a severe combined

immunodeficiency (SCID) background which still saw significant graft rejection of cultured RBCs.

In this investigation, acute tissue hypoxia will be induced via isovolumic hemodilution in which mice with wild-type hemoglobin are bled and infused with an equal volume of 5% human serum albumin (henceforth "HSA") solution or whole blood-anticoagulant solution. Isovolumic hemodilution and subsequent decrease in oxygen delivery to tissues will result in much more noticeable physiologic compensatory changes such as lactic acidemia, metabolic shift from aerobic respiration to glycolysis, and heightened pulse in comparison to thalassemic hypoxia. A more severely immunocompromised NOD-SCIDγ mouse strain, which lacks natural killer, B, and T cells and is currently the most permissive model for hRBC transfusion in mice, will be used. To further optimize *in vivo* hRBC survival and circulation, mice will be treated with clodronate liposome to deplete dendritic cells and macrophages, cobra venom factor to deplete complement, and cyclophosphamide to deplete neutrophils (15, 16).

Tissue oxygen monitoring

In order to assess metabolic efficacy of iPSC-RBCs *in vivo,* tissue oxygen must be directly and accurately measured throughout isovolumic hemodilution and following transfusion. The FDA study indirectly examined oxygen delivery indirectly by measuring plasma lactic acid levels after exercise-induced tissue hypoxia (14). However, this approach does not provide robust preclinical data and leaves this parameter subject to several confounding variables. In contrast, this investigation seeks to apply phosphorescence quenching oximetry, a powerful, non-invasive method which can directly detect changes in local tissue oxygen partial pressure (pO_2) in real time with high accuracy (17).

This method employs Oxyphor G4© (Pd-meso-tetra-(3,5-dicarboxyphenyl)-tetrabenzo-porphyrin), a protected dendritic probe which can be injected systemically (intravenous) or locally (intramuscular, subcutaneous, intracranial, etc.). Oxygen is a highly specific quencher of this probe and, after binding, can be excited to a detectable triplet state producing an exponential phosphorescence curve with finite lifetime. Changes in phosphorescence decay lifetime and pO_2 are inversely proportional changes, meaning that longer phosphorescent half-lives indicate lower tissue oxygen levels. pO_2 changes can be monitored in a manner independent of the concentration of probe in tissue as oximetry provides measurements calculated based on the difference in phosphorescence before and after 425nm excitation pulse. Concentration independence is critical for this experimental system in which fluctuations in tissue fluid may arise due to hemodilution and subsequent transfusion.

Hypothesis, specific aims, strategy, and significance

Hypothesis: This novel murine model for preclinical studies of RBC therapies will improve upon several aspects of previous models by implementing methods to increase human RBC survival *in vivo* and more accurately measure tissue oxygen delivery.

Specific aim #1: To differentiate, expand, and yield quantities of iPSC-derived RBCs for use as transfusion products in the murine model system.

Specific aim #2: To create and validate a severely immunocompromised preclinical murine model highly permissive to hRBC transfusion.

Specific aim #3: To use this model to assess compensatory physiologic and metabolic responses to acute tissue hypoxia following isovolumic hemodilution.

Strategy: NOD-SCIDγ mice will be treated with reagents intended to further deplete the immune system in order to optimize hRBC circulatory survival. To this end, the percent of circulating hRBCs will be monitored for days or weeks following transfusion. Isovolumic hemodilution will be used to induce acute hypoxia and phosphorescence oximetry will be applied thereafter to examine physiologic compensation and hypoxia-associated sequelae.

Significance: This strategy will validate a murine model system meant to provide robust preclinical data examining the ability of human RBC transfusions to reverse hypoxia and its sequelae.

Materials and Methods

iPS cell line establishment

Small volumes of peripheral whole blood were obtained from one healthy, consenting donor at Emory University Hospital with approval from the Emory University institutional review board on research ethics and informed consent. iPSC lines were established as previously described by the Cheng group (18). Briefly, mononuclear cells (MNCs) were isolated from peripheral blood by loading 35mL of 1X PBS-diluted blood (Millipore-Sigma, St. Louis, MO #806552) onto 15mL lymphocyte separation medium (STEMCELL Technologies, Cambridge, MA #07801) in a 50mL conical tube. Buffy coat cells were harvested, washed, and immediately cultured on an untreated 12-well dish to establish erythroblast cultures in serum-free medium (SFM) composed of 50% Iscove's modified Dulbecco's medium and 50% Ham's F12 medium, insulin-transferrin-selenium supplement, and synthetic lipids (all from Life Technologies, Carlsbad, CA), 0.5% human serum albumin, 50μg/ml of L-ascorbic acid (2-phosphate sesquimagnesium salt), 200μM 1-thioglycerol, 2mMglutamine (all from Millipore-Sigma) supplemented with human stem cell factor (SCF) at 50ng/mL (R&D Systems #255-SC), interleukin-3 (IL-3) at 10ng/mL (PeproTech, Rocky Hill, NJ #200-03), human holo-transferrin at 100μg/mL (R&D Systems, Minneapolis, MN #29140HT), erythropoietin at 2U/mL (Procrit [epoetin alfa], NDC 59676-303-01), insulin-like growth factor 1 at 40ng/mL (Peprotech #100-11), and dexamethasone at 1μM (Millipore-Sigma # 265005). Cultures were established under feeder-free, xeno-free conditions.

After 10 days, erythroblasts were reprogrammed by nucleofection with 2μg plasmid episomal vectors expressing *BCL2L1* gene and the Yamanaka factors (Addgene.org plasmids 28213, 28220) using the 4D Nucleofector with CD34 cell solution on setting 'EO-100' (Lonza, Basel Switzerland #VPA-1003). Nucleofected cells were subsequently cultured in SFM for an

additional 14 days before colony selection via live TRA-1-60+ antibody staining (Millipore-Sigma, MAB4360). Positive colonies were harvested via Accutase digestion (STEMCELL Technologies #A07920) and plated onto 2 wells of an untreated 6-well plate coated with vitronectin 0.5μg/cm^2 (Life Technologies #A14701SA) and cultured in E8 medium (STEMCELL Technologies #05990). To enhance cell survival, anti-apoptotic ROCK inhibitor Y27632 at 10μM (Miltenyi Biotec, San Diego, CA #130-103-922) was added to E8 medium for the first 24 hours after seeding. iPSCs were maintained in an undifferentiated state under these conditions and routinely passaged (1:10 approximately every 4 days) as small clumps using Accutase digestion (STEMCELL Technologies #A07920).

In vitro hematopoietic differentiation

Human iPSCs were differentiated into CD34+CD45+ hematopoietic stem cells (HSCs) under feeder- and serum-free conditions following the protocol described by STEMCELL Technologies (#05310, document #29768 (28)). Briefly, iPSCs at 70%-80% confluency were passaged using Accutase (STEMCELL Technologies #A07920) digestion as previously described and allowed to recover and grow for 1-2 days. After sufficient colony growth, cells were induced to become mesodermal for the first three days of differentiation. For following 9 days, cells undergo hematopoietic differentiation and can be harvested from suspension in the supernatant. Harvested HSCs then underwent erythroid differentiation for 10 days and terminal maturation for subsequent 8 days as previously described by the Cheng group (13, 18). Enucleation rates were quantified by visually counting the number of terminally mature iPSC-RBCs in a representative sample under a light microscope.

Transfusion-permissive, acutely immunocompromised murine model development

NOD.Cg-*Prkdc*^{scid} Il2rg^{tm1Wjl}/SzJ (henceforth "NSG") mice lacking NK-cells, B-cells, T-cells, complement and serum immunoglobulin and defective in macrophages, dendritic cells, and neutrophils were purchased from Jackson Laboratory. Animal experiments were approved by the Emory University Division of Animal Resources and Institutional Animal Care and Use Committee. All animal care was in accordance with institutional guidelines. NSG mice between 5-8 weeks of age weighing 17-22g were administered clodronate liposome (henceforth "CL", Liposoma Research, Amsterdam, The Netherlands #C-005), cobra venom factor (henceforth "CVF", Quidel, San Diego, CA #A600), and cyclophosphamide (henceforth "CPD", Millipore-Sigma #C3250000) via the lateral tail vein according to a dosing regimen (Fig. 1) to deplete circulating macrophages and dendritic cells, opsonic complement, and neutrophils, respectively.

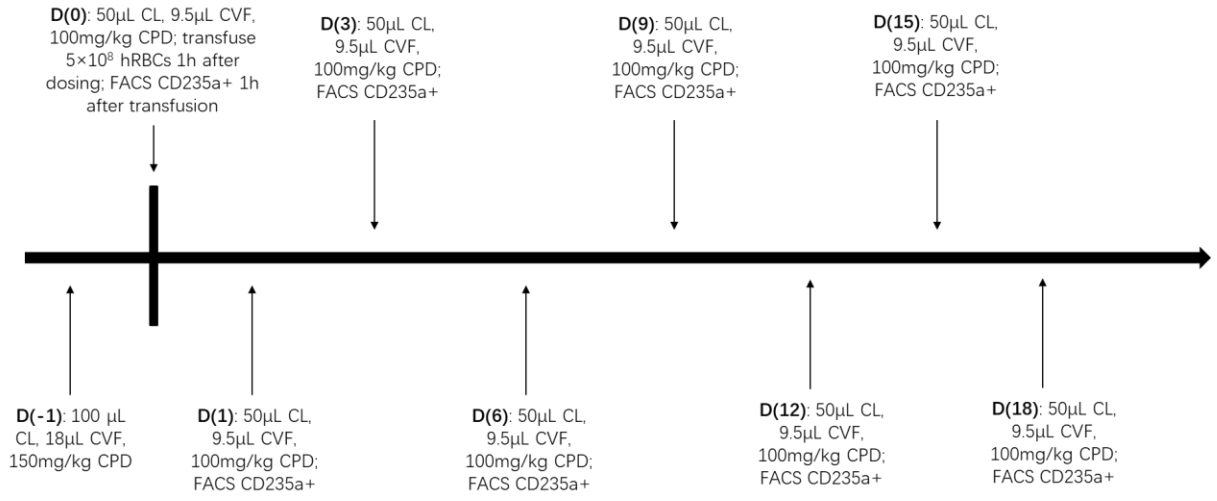

Fig. 1: Reagent dosing, administration timeline; schedule for monitoring hRBC circulatory survival in NSG mouse by flow cytometric staining (FACS) of CD235a

Small volumes of peripheral whole blood were obtained from one healthy donor at Emory University Hospital with approval from the Emory University institutional review board on research ethics and informed consent. Samples were collected in 2mL EDTA vacutainer tubes (BD Biosciences, Franklin Lakes, NJ # 367842) and washed three times in 1X PBS (Millipore-

Sigma, St. Louis, MO #806552). Subsequently, 5×108 RBCs (~50uL packed hRBC volume) were transfused into the lateral tail vein (Fig. 1). The lateral tail vein was nicked at specified intervals starting at 1 hour post-transfusion and 2μL peripheral blood samples were collected via heparinized capillary tubes (Fisher Scientific, Waltham, MA #22-362574). These samples were considered representative of systemic peripheral blood. Blood was then subjected to antibody staining and flow cytometric analysis.

Flow cytometry

Flow cytometry was used to characterize cells throughout stages of differentiation. Single-cell suspensions of Accutase-dissociated iPSCs (STEMCELL Technologies #A07920) were characterized using primary antibodies anti-human TRA-1-60 (Millipore-Sigma, #MAB4360) and detected using secondary antibodies anti-mouse IgM-Alexa Fluor 555 (ThermoFisher Scientific, Waltham, MA #A21426). Single-cell suspensions of harvested HSCs were washed in PBS supplemented with 1% FBS and 1mM EDTA (all from Millipore-Sigma, St. Louis, MO). Cells were resuspended in 200μL 1X PBS and labeled with fluorochrome-conjugated anti-human CD34-APC (Miltenyi Biotec, #130-090-954), CD45-PE (Miltenyi Biotec, #130-080-201). Background staining was determined using isotype-matched control antibodies. Flow cytometry was performed using the FACSCalibur or LSRII (both from BD Biosciences, San Jose, CA) and analyzed using FlowJo (FlowJo LLC, Ashland, OR).

To determine survival of transfused hRBCs, 2uL whole blood samples from the lateral tail vein were washed three times, resuspended in 500uL 1X PBS (Millipore-Sigma, St. Louis, MO #806552), and stained using anti-human CD235a-FITC, a surface antigen found only on human erythrocytes (glycophorin A, BioLegend, San Diego, CA # 349103). CD235a staining was performed at regular intervals (Fig. 1). To confirm macrophage/dendritic cell depletion by

CL, one CL-naïve mouse (PBS-treated) and one CL-treated mouse were sacrificed and their spleens harvested. Splenocytes were washed, resuspended in 1X PBS, and stained with anti-human F4/80-Pacific Blue (Bio-Rad, Hercules, CA #MCA497RT).

Isovolumic hemodilution

C57BL/6J ("Black 6", Jackson Laboratory, Bar Harbor, ME #000664) immune-competent mice were anesthetized by intraperitoneal ketamine-xylazine ketamine injection (120uL/20g body weight; kindly provided by Dr. A. Alam) and placed prone and spread-eagle on a warming platform heated to 37°C. A depilatory cream (Reckitt Benckiser, Slough, United Kingdom) was applied to the right hind leg for 1 minute then wiped away to remove hair. A 10μM solution of Oxyphor G4© (Pd-meso-tetra-(3,5-dicarboxyphenyl)-tetrabenzo-porphyrin; henceforth, "probe", OxyLED Instruments, Philadelphia, PA) was injected directly into the right hind quadratus femoris.

To induce tissue hypoxia, 200μL of blood was bled from the retro-orbital and submandibular veins draining the face using a 4mm lancet (Braintree Scientific, Inc., Braintree, MA) and collected in Eppendorf tubes containing 40μL acid citrate dextrose anticoagulant solution (BD Biosciences, Franklin Lakes, NJ #364606). Following a 3-minute period of real-time pO_2 measurement, 200μL of either 5% human serum albumin ("HSA", Millipore-Sigma, St. Louis, MO #12667) with no oxygen-carrying capability or 200μL whole mouse blood-anticoagulant solution obtained from the face bleed were infused via the lateral tail vein. Cycles of bleeding and fluid replacement were repeated at least three times for each mouse or until no intramuscular pO_2 was detected (Fig. 2).

Phosphorescence quenching oximetry

Detector LED was positioned 5mm above the probe injection site perpendicular to skin surface. Intramuscular pO_2 was measured at four-second intervals following scheduled cycles of bleeding followed by fluid replacement (Fig. 2). Real-time tracking was output using OxyLED software and analyzed using Microsoft Excel (Microsoft, Seattle, WA) and Biopython software.

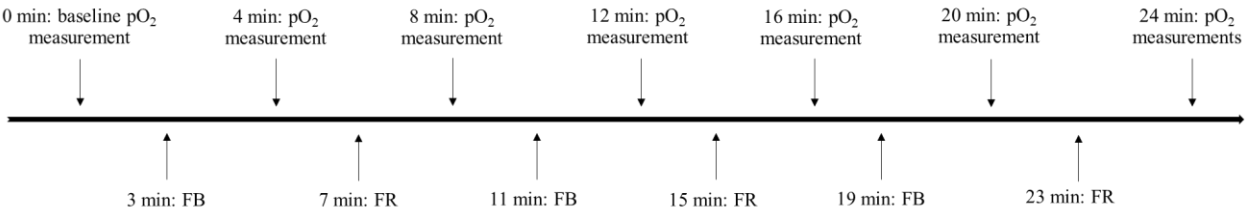

Figure 2: Experimental timeline of phosphorescence quenching oximetry and isovolumic hemodilution. FB: 200µL face bleed; FR: fluid replacement with either 200µL mouse blood-ACD solution or 5% HSA

Results

PBMC reprogramming and iPSC differentiation and expansion produced robust yields of terminally mature RBCs

iPSC-to-RBC differentiation success was determined by cell morphology and flow cytometry, both examining markers and characteristics specific to each stage of differentiation. Peripheral blood MNC reprogramming to iPSCs, driven by a plasmid episomal vector, produced several colonies expressing surface TRA-1-60, indicating successful production of iPSCs (data not shown). After expansion in E8 medium, iPSC colonies exhibited morphology similar to previously established iPSC lines in the literature (18, Fig. 3a). iPSCs could be passed 8-10 times while maintaining iPSC phenotype.

The serum-, feeder-, and xeno-free culture conditions and hematopoietic differentiation methods first produced cells with mesodermal morphology. Following mesodermal differentiation, 70%-80% of hematopoietic suspension cells expressed definitive CD34 and CD45 cell surface antigens, confirming HSC state (Fig. 3a). Purified CD34+, CD45+ cells were expanded ~50-fold over a ten-day period and developed erythroblast morphology including a reddish tint, indicating hemoglobin expression. Erythroid terminal maturation saw a ~10-fold increase in cell number after 8 days and produced a bright red pellet after collection and centrifugation (Fig. 3c). Cells were also noted to lack nuclei under light microscopy (Fig. 3d).

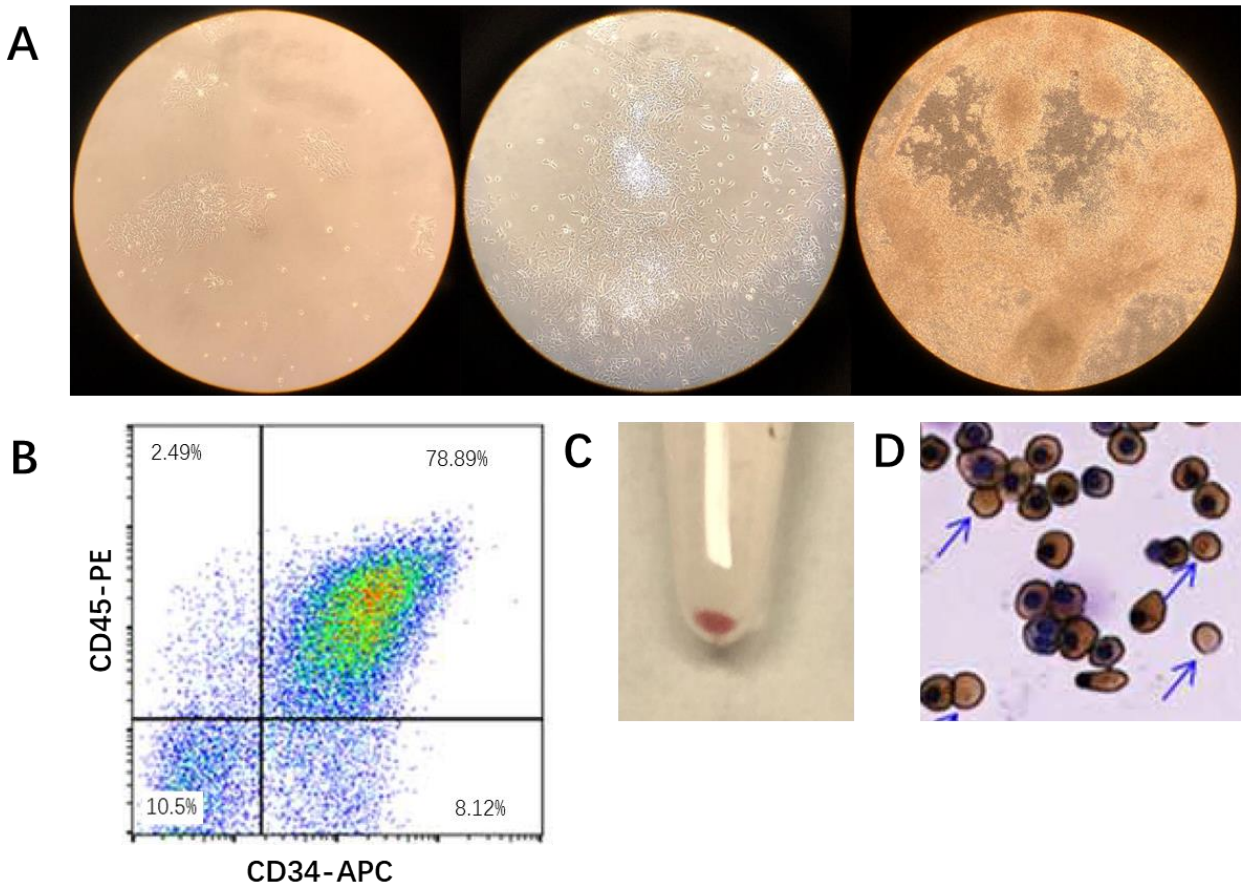

Figure 3: iPSC-RBC differentiation. (A): From left to right, Light microscopy images of iPSCs, medodermal pre-HSCs, and HSCs (×10). (B): Flow cytometry of harvested HSCs, of which a majority are CD34+ and CD45+, indicating successful differentiation. (C): Visibly red pellet of representative terminally mature ex vivo-produced erythrocytes in a 1.5mL Eppendorf tube. (D): Erythroblasts at day 6 of terminal erythroid maturation (×40) with enucleated cells labeled.

NSG mice treated with clodronate liposome, cobra venom factor, and cyclophosphamide were highly tolerant to hRBC transfusion

Permissiveness to hRBCs in mouse systemic circulation was determined by transfusing 5×10^8 RBCs (~50uL packed hRBC volume) via the lateral tail vein. Peripheral blood hRBC composition was regularly monitored via flow cytometry monitoring CD235a+ cells. hRBCs circulated through the mouse vasculature for periods upwards of nine days. Intuitively, peripheral blood hRBC composition was greatest 1 hour following transfusion and steadily decreased over time. Initial levels of hRBCs, ranged between 16.5%-20.6% of total RBCs (Fig. 4a). The largest decrease in hRBC count, 50%, occurred between 6 and 9 days after transfusion

(Fig. 4c). About 15%-20% of transfused RBCs were able to persist steadily in circulation beyond 12 days with little decrease (Fig. 4c). In some individuals, small numbers of hRBCs were able to persist in circulation beyond 42 days after transfusion (data not shown). Light microscopy showed that approximately half of all terminally mature iPSC-RBCs were enucleated (Fig. 3d).

Innate immune depletion was also examined by staining for macrophage/dendritic cell antigens and performing flow cytometry. Levels of splenic macrophages, suspected to be the largest contributor to hRBC sequestration in NSG mice, were counted before and after CL administration. There was an observed 98% drop in the number of splenic macrophages present following CL treatment (Fig. 4d).

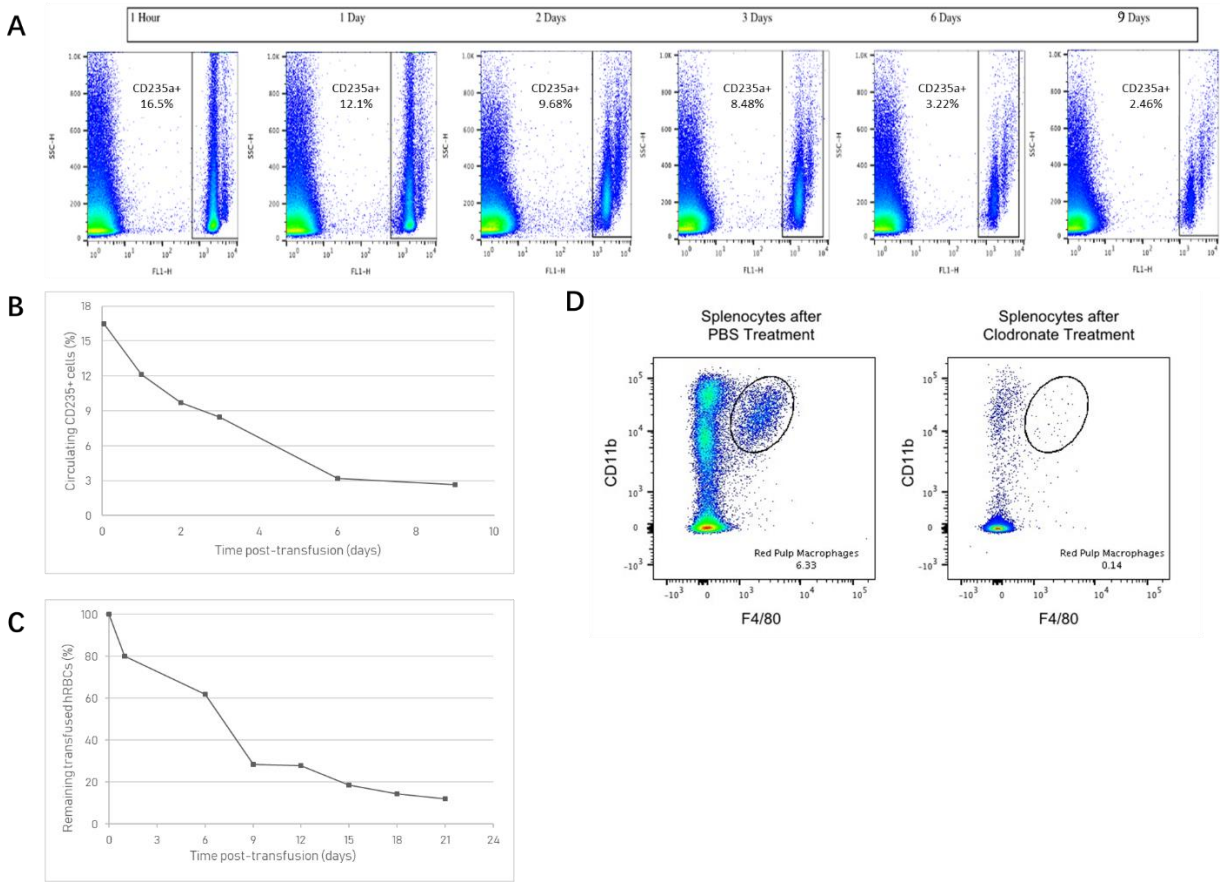

Fig. 4: Flow cytometric analyses of hRBC-permissive murine model. (A): Representative flow cytometric plots of CD235a+ hRBC circulatory survival after transfusion into CL-, CVF-, and CPD-treated NSG mice (n=33). (B): Peripheral blood CD235a+ hRBC composition over time. (C): Percent remaining transfused hRBCs over time. (D): Confirmed CL efficacy in depleting macrophage/dendritic cells marked by surface antigen F4/80.

Isovolumic hemodilution effectively induces tissue hypoxia in a facile, quantifiable manner

Transient intramuscular pO_2 levels following cycles of bleeding and fluid replacement in B6 mice were quantified in real time using phosphorescent probe and LED detector oximetry system. As expected, bleeding resulted in a decreased intramuscular pO_2 followed by a short period of physiologic compensation, a small increase in pO_2, in response to hypoxia. The magnitude of compensation decreased as total bled volume increased. This effect was observed to be independent of fluid replacement (Fig. 5a, 5b).

Without fluid replacement, repeated cycles of bleeding elicited a nearly linear decrease in tissue oxygen levels. For bleeding volumes of 400μL or smaller, both serum infusion and whole blood transfusion served to rescue or maintain tissue oxygen levels (Fig. 5c, 5d, 5e). Specifically, after the first 200μL bleed, whole blood transfusion increased tissue oxygen levels beyond baseline measurements. HSA infusion, in contrast, was only able to maintain tissue oxygen levels for this level of anemia (Fig. 5c). After 600μL was bled, tissue oxygen continued to decrease despite HSA infusion (Fig. 5c, 5d). In contrast, blood transfusion was able to maintain pO_2 at or slightly above levels after bleeding (Fig. 5e).

Whole blood-anticoagulant solution transfusion increased tissue pO_2 in a more gradual manner than serum infusion. pO_2 following transfusion also showed a consistent sinusoidal pattern which showed oxygen level at peak values greater than those following face bleed, which eventually flattened. Overall, whole blood transfusion elicited larger increases in intramuscular pO_2 following bleeding in comparison to 5% HSA infusion (Fig. 5b, 5e). Notably, the range of pO_2 values after blood transfusion was much larger than that following 5% HSA infusion (Fig. 5b).

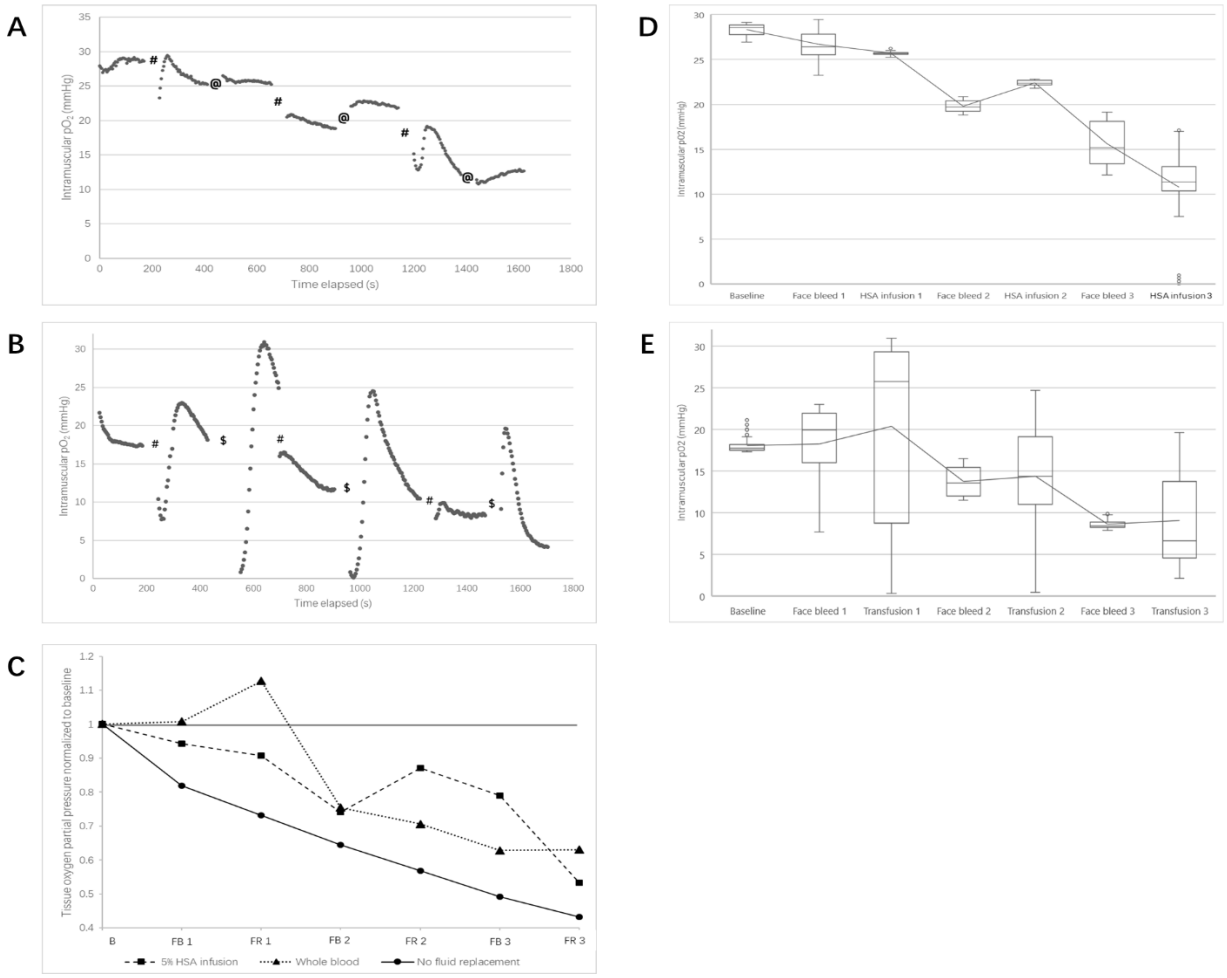

Fig. 5: Isovolumic hemodilution studies. (A): Representative time course graphs of transient changes in intramuscular pO_2 as result of isovolumic hemodilution using 5% HSA solution as fluid replacement. # = 200µL face bleed. @ = 200µL 5% HSA infusion. (B): Representative time course graphs of transient changes in intramuscular pO_2 as result of isovolumic hemodilution using whole blood-anticoagulant solution as fluid replacement. # = 200µL face bleed. $ = 200µL blood transfusion. (C): Average tissue pO_2 normalized to baseline measurements following various fluid replacement methods. FB = face bleed. FR = fluid replacement. (D): Representative plots depicting average and interquartile ranges of tissue pO_2 in the periods following bleeding and 5% HSA fluid replacement. (E): Representative plots depicting average and interquartile ranges of tissue pO_2 in the periods following bleeding and blood transfusion.

Discussion

Ex vivo-produced iPSC-derived RBCs hold immense therapeutic potential as supplements or alternatives to conventional transfusion products. Before any clinical evaluations can be performed, physiological and functional characteristics of iPSC-RBCs must be determined in a non-human animal model system. This investigation used a modified expansion and differentiation protocol from Chou et al. to successfully and consistently yield mature, enucleated human erythrocytes from reprogrammed peripheral blood MNCs. Enucleation rates, however, only reached 50%, indicating further improvements are needed for this approach to reach clinical scale. Improved iPSC-RBC manufacturing methods as well as manufacturing costs will be crucial elements of clinical feasibility as patients with greatest therapeutic need often fall within the most socioeconomically marginalized groups in the United States (19). Thus, low-cost culture conditions and methods to enhance expansion and enucleation must be implemented to produce suitable transfusion products accessible to many.

As noted by many groups studying iPSC-RBC differentiation, these methods produce erythrocytes which express fetal hemoglobin (HBF), which has a greater oxygen affinity than adult hemoglobin (14, 11). Both adult CD34+ cells and cord blood have equally greater oxygen affinity when compared to adult peripheral blood erythrocytes (20). Despite their atypical phenotype, the presence of HBF in iPSC-RBCs should not be a hindrance to their clinical applications. Areas in which thalassemia and hemoglobinopathies are common, such as the Mediterranean coast and sub-Saharan Africa, show greater incidences of hereditary persistence of fetal hemoglobin (HPFH). This benign condition is thought to be selected for to alleviate hemoglobinopathy severity as the HPFH trait is found in 1 in 12 patients with the sickle cell trait (Forget).

This study presented an innovative, non-invasive model system aimed to present preclinical efficacy data required by regulatory entities. A promising study demonstrated *ex vivo*-generated RBCs were able to circulate in one human subject with a half-life of 28 days while performing hemoglobin-oxygen binding comparable to donated erythrocytes, indicating the therapeutic potential of these RBCs. Before clinical trial validation can take place, iPSC-RBCs must demonstrate a capacity to effectively bind and distribute oxygen to tissues in a small model organism.

The FDA mouse model was an important first step toward quantifying RBC functionality in a preclinical system. However, this system was not fully optimized as the SCID mouse background was weakly hRBC-permissive, chronically anemic, and only indirectly assessed oxygen delivery and utilization. In contrast, NSG, reagent-treated mice presented in this investigation were highly permissive for hRBC transfusion with prolonged hRBC circulatory survival on the scale of weeks. One hour after transfusion, 40% of transfused hRBCs persisted (14) whereas nearly 80% remained in the CL-, CVF-, and CPD-treated NSG murine model. After 24 hours, roughly half of all transfused hRBCs were still in NSG circulation. This stable persistence of hRBCs shortly transfusion will be crucial as we anticipate that future measurements of tissue hypoxia and sequelae will take place during this period.

Isovolumic hemodilution successfully induced acute tissue hypoxia in anesthetized mice. Transient decreases in tissue oxygen levels following cycles of bleeding and fluid replacement were accurately measured using phosphorescence quenching oximetry. Notably, fluid replacement using whole blood elicited an increase in average tissue oxygen in acutely anemic mice. It can thus be expected that future experiments comparing iPSC-RBCs and standard hRBCs from healthy donors head-to-head would show similar oxygen delivery efficacy. This

method provided a more direct and accurate method to measure hypoxia-induced tissue oxygen debt which does not rely on correlations or indirect parameters such as plasma lactate (22). Phosphorescence quenching oximetry has also been used to detect tumor angiogenesis and hypoxia to identify more aggressive phenotypes as well as cerebrovascular hemodynamic oxygenation in mouse stroke models (23, 24). It is possible that ketamine-xylazine anesthesia resulted in systemic deoxygenation due to respiratory depression (25). All experimental animals received equal concentrations of ketamine-xylazine anesthesia, and, therefore, should have experienced the same degree of respiratory depression. Despite this, future investigations should use un-anesthetized animals to circumvent anesthesia-related effects on tissue oxygenation.

This study validated an optimized murine model to determine the oxygen delivery performance of iPSC-RBCs. In this model, acutely anemic mice exhibited gradually decreased tissue pO_2 which could be rescued to by blood transfusion, indicating that transfused hematocrit is capable of accepting oxygen from the pulmonary circulation and delivering oxygen to tissues. Transfused hRBCs CL-, CVF-, and CPD-treated mice were highly permissive to hRBC transfusion, especially within 24 hours of transfusion. This preclinical model is appropriate to demonstrate iPSC-RBCs' ability to deliver oxygen to tissues and indicate future clinical development.

References

1. Whitaker, B., Rajbhandary, S., Kleinman, S., Harris, A., & Kamani, N. (2016). Trends in United States blood collection and transfusion: results from the 2013 AABB blood collection, utilization, and patient blood management survey. *Transfusion*, 56(9), 2173-2183.
2. Zimring, J. C., Welniak, L., Semple, J. W., Ness, P. M., Slichter, S. J., Spitalnik, S. L., & NHLBI Alloimmunization Working Group. (2011). Current problems and future directions of transfusion-induced alloimmunization: summary of an NHLBI working group. *Transfusion*, 51(2), 435-441.
3. Garratty, G. (1997). Severe reactions associated with transfusion of patients with sickle cell disease. *Transfusion*, 37(4), 357-361.
4. Wun, T., & Hassell, K. (2009). Best practices for transfusion for patients with sickle cell disease. *Hematology Reviews*, 1(2).
5. Poole, J., & Daniels, G. (2007). Blood group antibodies and their significance in transfusion medicine. *Transfusion medicine reviews*, 21(1), 58-71.
6. LaSalle‐Williams, M., Nuss, R., Le, T., Cole, L., Hassell, K., Murphy, J. R., & Ambruso, D. R. (2011). Extended red blood cell antigen matching for transfusions in sickle cell disease: a review of a 14‐year experience from a single center (CME). *Transfusion*, 51(8), 1732-1739.
7. Wilkinson, K., Harris, S., Gaur, P., Haile, A., Armour, R., Teramura, G., & Delaney, M. (2012). Molecular blood typing augments serologic testing and allows for enhanced matching of red blood cells for transfusion in patients with sickle cell disease. *Transfusion*, 52(2), 381-388.
8. Bai, H., Liu, Y., Xie, Y., Hoyle, D. L., Brodsky, R. A., Cheng, L., Cheng, T., & Wang, Z. Z. (2016). Definitive hematopoietic multipotent progenitor cells are transiently generated from hemogenic endothelial cells in human pluripotent stem cells. *Journal of cellular physiology*, 231(5), 1065-1076.
9. Anstee, D. J., Gampel, A., & Toye, A. M. (2012). Ex-vivo generation of human red cells for transfusion. *Current opinion in hematology*, 19(3), 163-169.
10. Migliaccio, A. R., Masselli, E., Varricchio, L., & Whitsett, C. (2012). Ex-vivo expansion of red blood cells: how real for transfusion in humans?. *Blood reviews*, 26(2), 81-95.
11. Cai, L., Bai, H., Mahairaki, V., Gao, Y., He, C., Wen, Y., You-Chuan, J., Wang, Y., Pan, R.L., Qasba, A., Ye, Z., & Cheng, L. (2018). A universal approach to correct various HBB gene mutations in human stem cells for gene therapy of beta-thalassemia and sickle cell disease. *Stem cells translational medicine*, 7(1), 87-97.
12. Giarratana, M. C., Rouard, H., Dumont, A., Kiger, L., Safeukui, I., Le Pennec, P. Y., & Jolly, S. (2011). Proof of principle for transfusion of in vitro–generated red blood cells. *Blood*, 118(19), 5071-5079.
13. Dowey, S. N., Huang, X., Chou, B. K., Ye, Z., & Cheng, L. (2012). Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. *Nature protocols*, 7(11), 2013.
14. Shah, S. N., Gelderman, M. P., Lewis, E. M., Farrel, J., Wood, F., Strader, M. B., & Vostal, J. G. (2016). Evaluation of stem cell-derived red blood cells as a transfusion product using a novel animal model. *PloS one*, 11(12), e0166657.
15. Hu, Z., Van Rooijen, N., & Yang, Y. G. (2011). Macrophages prevent human red blood cell reconstitution in immunodeficient mice. *Blood*, 118(22), 5938-5946.
16. Zuluaga, A. F., Salazar, B. E., Rodriguez, C. A., Zapata, A. X., Agudelo, M., & Vesga, O. (2006). Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases. *BMC infectious diseases*, 6(1), 55.
17. Vinogradov, S., & Wilson, D. F. (2002). U.S. Patent No. 6,362,175. Washington, DC: U.S. Patent and Trademark Office.
18. Chou, B. K., Gu, H., Gao, Y., Dowey, S. N., Wang, Y., Shi, J., & Cheng, L. (2015). A Facile Method to Establish Human Induced Pluripotent Stem Cells From Adult Blood Cells Under Feeder‐Free and

Xeno‐Free Culture Conditions: A Clinically Compliant Approach. *Stem cells translational medicine*, 4(4), 320-332.

19. Haque, A., & Telfair, J. (2000). Socioeconomic distress and health status: The urban‐rural dichotomy of services utilization for people with sickle cell disorder in North Carolina. *The Journal of Rural Health*, 16(1), 43-55.
20. Jin, H., Kim, H. S., Kim, S., & Kim, H. O. (2014). Erythropoietic potential of CD34+ hematopoietic stem cells from human cord blood and G-CSF-mobilized peripheral blood. *BioMed research international*, 2014.
21. Forget, B. G. (1998). Molecular basis of hereditary persistence of fetal hemoglobin. *Annals of the New York Academy of Sciences*, 850(1), 38-44.
22. Dhabangi, A., Ainomugisha, B., Cserti-Gazdewich, C., Ddungu, H., Kyeyune, D., Musisi, E., ... & Dzik, W. H. (2015). Effect of transfusion of red blood cells with longer vs shorter storage duration on elevated blood lactate levels in children with severe anemia: the TOTAL randomized clinical trial. *JAMA*, 314(23), 2514-2523.
23. LaRochelle, E. P. M., Shell, J. R., Jia, M., Cao, X., Bruza, P., Vinogradov, S. A., & Pogue, B. W. (2019, March). Advancing optical methods for real-time non-contact pO2 estimation during external beam radiation therapy (Conference Presentation). In *Molecular-Guided Surgery: Molecules, Devices, and Applications V* (Vol. 10862, p. 108621E). International Society for Optics and Photonics.
24. Kisler, K., Lazic, D., Sweeney, M. D., Plunkett, S., El Khatib, M., Vinogradov, S. A., & Zlokovic, B. V. (2018). In vivo imaging and analysis of cerebrovascular hemodynamic responses and tissue oxygenation in the mouse brain. *Nature protocols*, 13(6), 1377.
25. Ruiz-Colón, K., Chavez-Arias, C., Díaz-Alcalá, J. E., & Martínez, M. A. (2014). Xylazine intoxication in humans and its importance as an emerging adulterant in abused drugs: a comprehensive review of the literature. *Forensic Science International*, 240, 1-8.
26. Walasek, M. A., Kardel, M., Walasek, M., Noort, R., Chen, J., Chau, C., & Thomas, T. (2017). Stemdiff™ hematopoietic kit reproducibly generates functional hematopoietic progenitor cells from human pluripotent stem cells. *Experimental Hematology*, 53, S59.