52nd Annual Meeting
April 9 – April 10, 2019

Creating Customized Red Cell Reagents from iPSC Cells

Connie M. Westhoff, SBB PhD
Executive Scientific Director
Immunohematology and Genomics

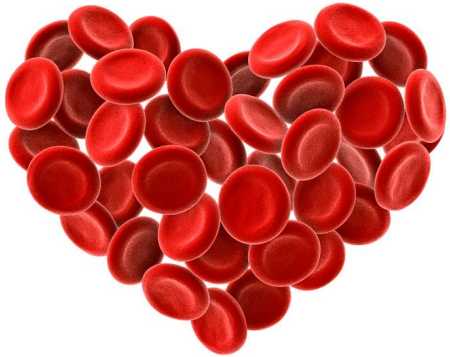
OBJECTIVES

- **Discuss the challenges of growing RBCs in culture in the laboratory for future transfusion**
- **Understand the technology of gene editing with CRISPR (clustered regularly interspaced short palindromic repeats)**
- **Describe the use of genetic tools to make “designer” RBCs and how they might be useful**

CELLS IN THE HUMAN BODY

Number of cells in the average human body

25-30 trillion = 30,000,000,000,000!



200 different types of cells

- **Red blood cells (RBCs) - by far the most abundant**
  over 80 percent of all cells in the body
  producing between 173 and 259 billion RBCs per day
  roughly the same number of RBCs are dying off
- **skin cells**
- **neurons (nerve cells)**
- **fat cells**

38 trillion bacterial cells = microbiome

January 2005 Nature Biotechnology

Douay L Laboratory, Paris

2002 Nat Biotechnol., 20:467-72. **Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo.**

2005 Nat Biotechnol. ,23:69-74. **Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells.**

Banking on red blood cells

Narla Mohandas

The bulk production of human red blood cells in culture is a first step toward an alternative source of transfusible blood.

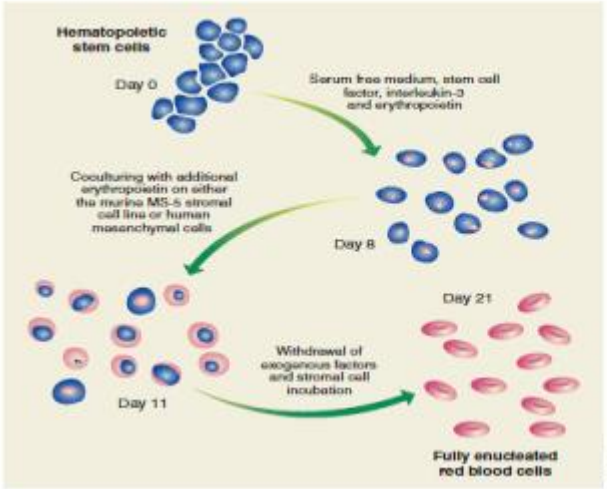


Figure 1 A schematic diagram of the three-step protocol used for bulk *ex vivo* production of cultured human red blood cells. During the first step, $CD34^+$ stem cells are cultured for 8 days in serum-free medium in the presence of stem cell factor, interleukin-3 and erythropoietin. In the second step, the cells are cultured for an additional 3 days on adherent stromal cells in the presence of erythropoietin. In the third step, the cells are cultured for up to an additional 10 days on adherent stromal layers without cytokines. At the end of these three steps, $CD34^+$ stem cells are amplified up to 2 million–fold and have differentiated into mature functional red blood cells.

3 step method – 21 days

- CD34+ stem cells from cord blood
  - culture with SCF, IL-3, Epo
- expansion ~1.95 x 10^6
  - almost 2 million fold
- mature RBCs (enucleated)
- “normal” RBCs survival
  - immunodeficient mice

DO RBCS GROWN IN LABORATORY SURVIVE IN HUMANS ?

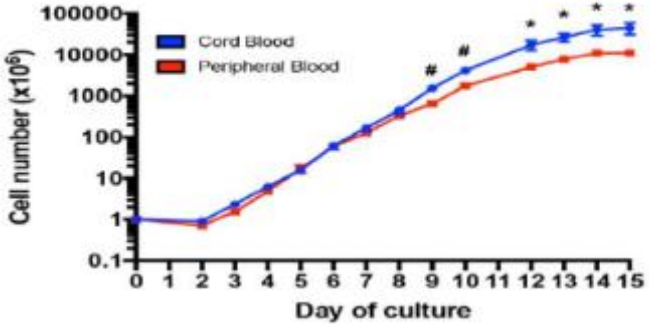
2011: Proof of principle for transfusion of in vitro generated red blood cells.

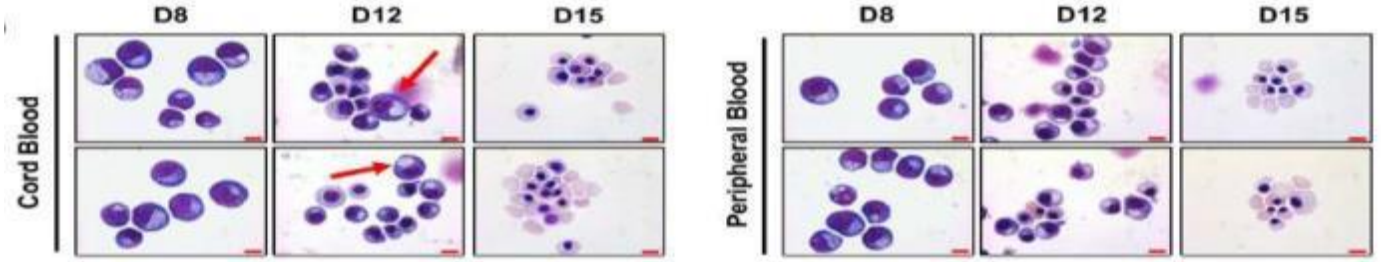
Blood,118:5071–9 Giarratana MC, Rouard H, Dumont A et al.

- **CD34^{+} cells stem cells from *adult* - mobilization with G-CSF**
  - 81% ± 2% enucleated RBCs
  - blood group antigen expression equivalent
  - O2 carrying capacity, deformability equivalent
- **injected 10^{10}** (10 billion) **cRBCs grown under GMP conditions**
  - labeled with ^{51}Cr
  - cells in circulation at 26 days - between 41% and 63%
- **“compared favorably with the reported half-life of 28 ± 2 days for native RBCs”**
- 4 weeks of storage

CULTURED RBCS:
CD34+ CORD BLOOD OR CD34+ ADULT

2018: Yan et al. Am. J Hematology 1-10

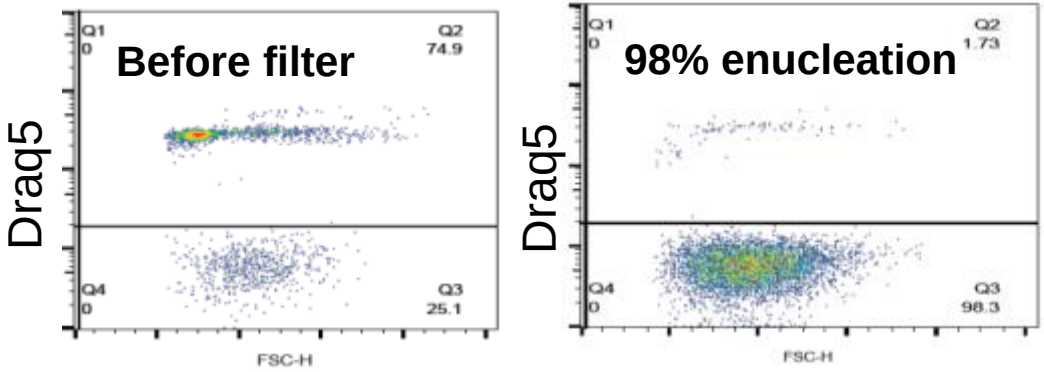


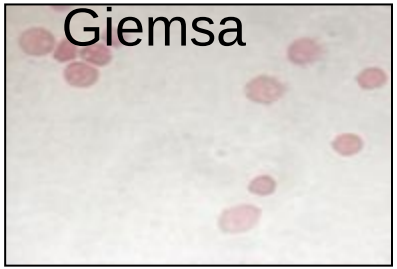


- more cells and more retics
- slower growing

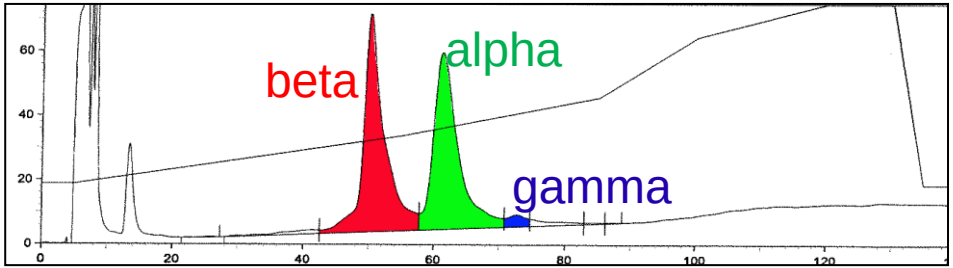
PRODUCTION OF CRBCS FROM ADULT CD34+ CELLS

40-70% enucleation; 95-99% after filtration



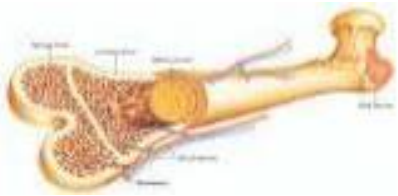


95-98% Hb A HPLC



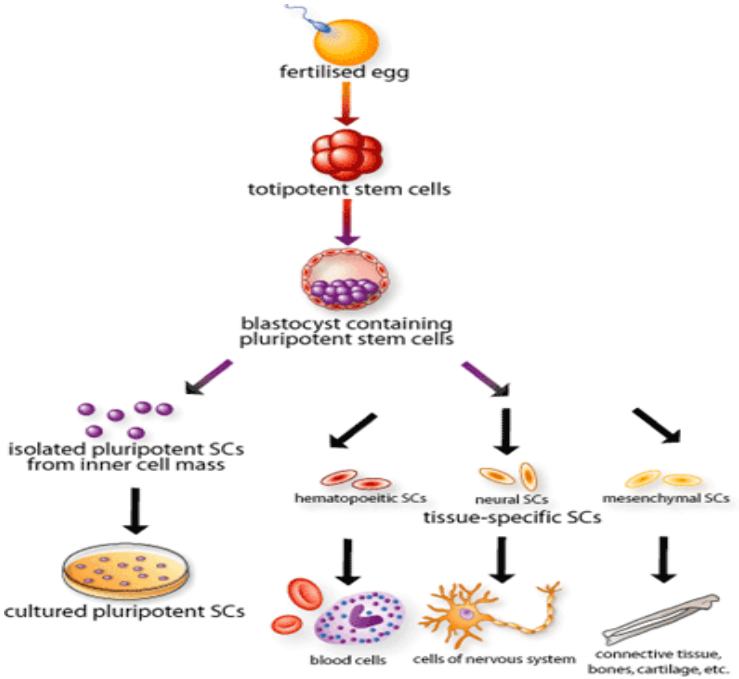
MAJOR CHALLENGES

- **Scale Up**
  - 5 x 10^9 RBCs **in each ml** of blood
    - **1 unit** = 2.4 X 10^{12} RBCs = **trillions**
  - 1 cord blood
    - 2-5 million CD34+ cells
    - ~ 2 units of blood MAXIMUM
- **Human – 10 billion RBCs every hour**
    - 2,777 RBCs per second
- **High cost**
  - Growth factors $$$$: erythropoietin, SCF, IL-3, transferrin
- **Source of cells**
  - cord blood
  - adult peripheral blood stem cells

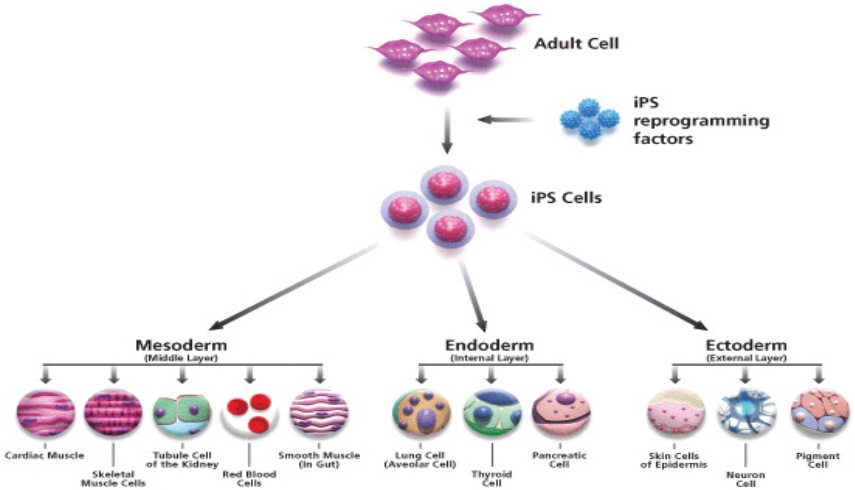


STEM CELL RESEARCH: REPROGRAM

YESTERDAY



TODAY



2006 - Reprograming of adult cells

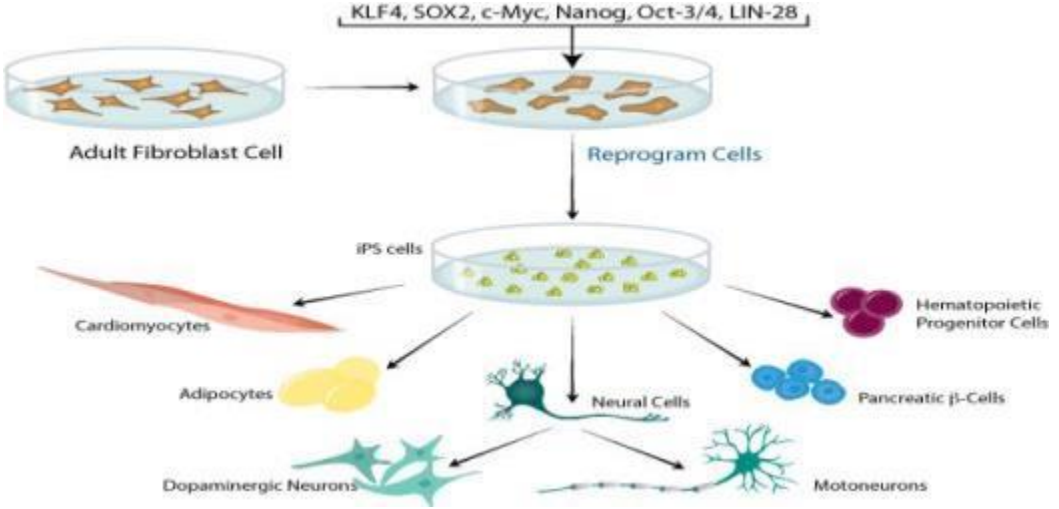
Yamanaka's lab

Kyoto, Japan

IPSC'S- INDUCED PLURIPOTENT STEM CELLS

- 4 transcription factor genes (Klf4, Sox2, c-Myc, Oct4)
- convert adult cells into "pluripotent" stem cells
  - generate any type of cells with appropriate growth factors
  - continuous supply
  - can do genetic modification
- patient's adult cells could provide immune-matched supply of cells

2018
human iPSCs
14,756 references



DIFFERENTIATION OF HUMAN IPSCS TO RBCS INDEPENDENT OF DONOR CELL TYPE OF ORIGIN

Haematologica 2015 Jan;100(1):32-41

- **human neural stem cells and human cord blood CD34+ stem cells equivalent potential for differentiation into mature red blood cells**
- **Problem is enucleation**

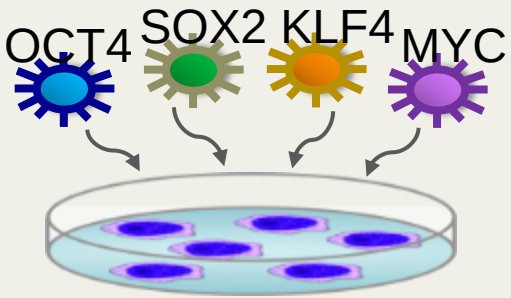
  iPSC-derived erythroid cells
  - low enucleation - suboptimal final maturation
  - for transfusion - need enucleated cells
- **Problem is expansion numbers**
  - adult transfusion needs require vast numbers of cells
    - approximately 2.4×10^{12} cells in each blood unit (trillions)
  - transfusion of a chronically transfused patient who receives 6 units per year would require about 1.5×10^{13} cells per year

HOW CAN GROWING RBCS IN CULTURE CURRENTLY BE USEFUL?

- **Biological insights - erythroid expression system**
  - terminal erythroid differentiation
  - hematologic diseases
  - structure and function of erythrocyte proteins and blood group antigens
- **Reagent red cells – for antibody identification**
  - 250,000 – 500,000 RBCs/assay
    - Rare donor RBCs
    - Rh null, Kell null, etc.
    - Genetically engineer combinations not found in natural populations
- **Studying molecules involved in parasite invasion**
  - identification of Babesia receptors on the RBC
  - Genetic engineer removal of specific proteins

INDUCED PLURIPOTENT STEM CELLS (IPSCS) AS REAGENTS

Reprogram cells from Rare Donors



1. Lacking high prevalence Rh antigens (RHCE, hrB-, hrS-)
2. Lacking combinations of antigens (D, U, Fya/b, Jkb, etc.)

- **Project**
  - Recruit rare donors
    - harvest buffy coat
    - reprogram as iPSCs
    - differentiate to RBCs in culture
    - test in Blood Bank assays
    - compare with original RBCs

Adapted from Redman Pract Ed 2016

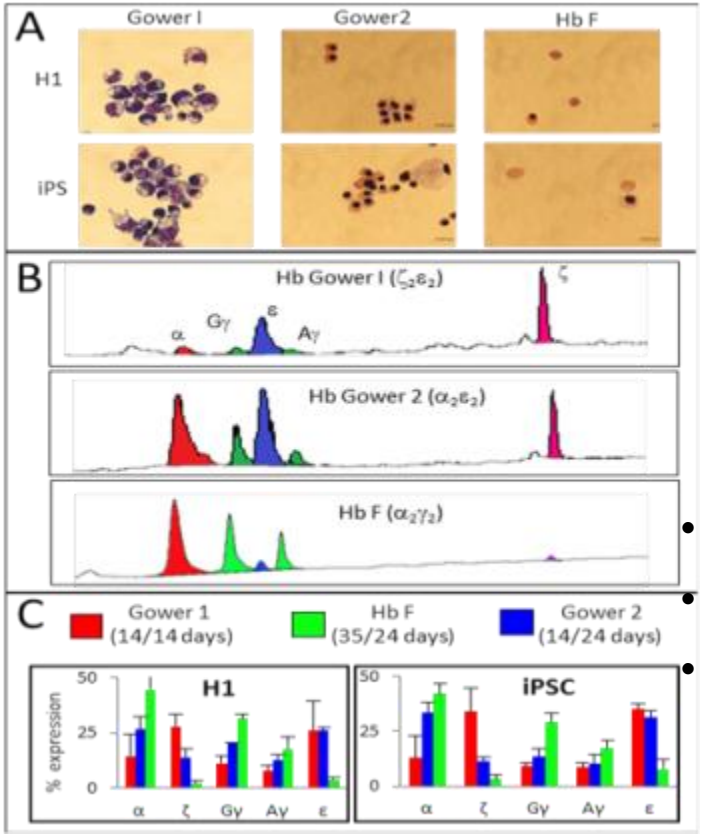
IPSCS CELL LINES FROM RARE DONORS

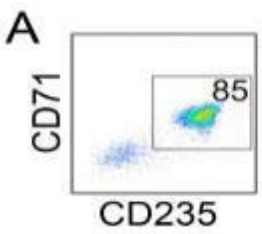
Donor	Blood Group Phenotype	Relevant Genotype	Comments
1	Group O, **D − −** lack RhCcEe	*RHD* *inactive RHCE*	useful for patients who have altered RHCE genes and make antibodies to all forms of RhCE
2	Group O−, **hrB−** E−,S−, Jk(b−), Fy(a−b−)	*RHD*DIIa(3-7)CE* *RHCE*ceS*	allows rapid distinction between antibodies directed to e antigen (often called Rh17)
3	Group O, **hrS−** E−,S− Jk(b−), Fy(a−b−)	*RHD*DAR* *RHCE*ceAR*	
4	Group O, E−,S− Jk(b−), Fy(a−b−), **hrS−and hrB−**	*RHD*DAUO* *RHCE*ceMO*	allows distinction between different hrS antibodies
5	Group O−, **Jk(b−),Fy(a−b−), S−s− U−**	*GYPB*01N*	Glycophorin B null; also negative for combinations of antigens often need for sensitized patients with SCD
6	Group O+, **e−**, **Jk(b−),Fy(a−b−), S−s− U−**	*GYPB*01N*	

- *antibody identification*
  - *2.5 x 10^5 cells/assay*
- *transfusion*

2.5 x 10^{12} cells/unit - not yet feasible

IPSC DERIVED CULTURED RBCS





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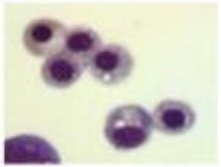
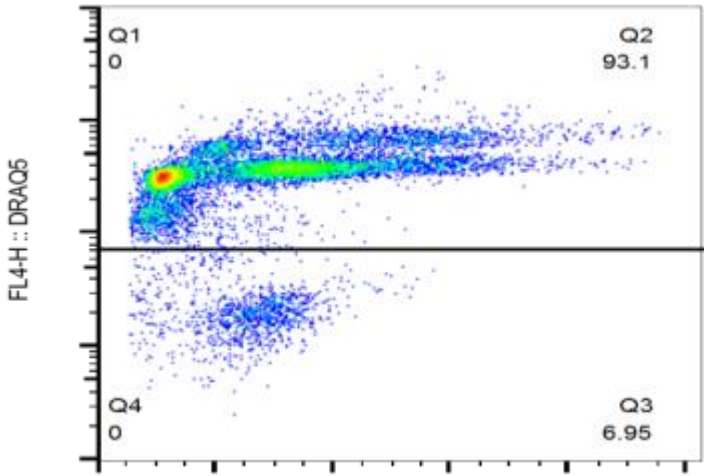
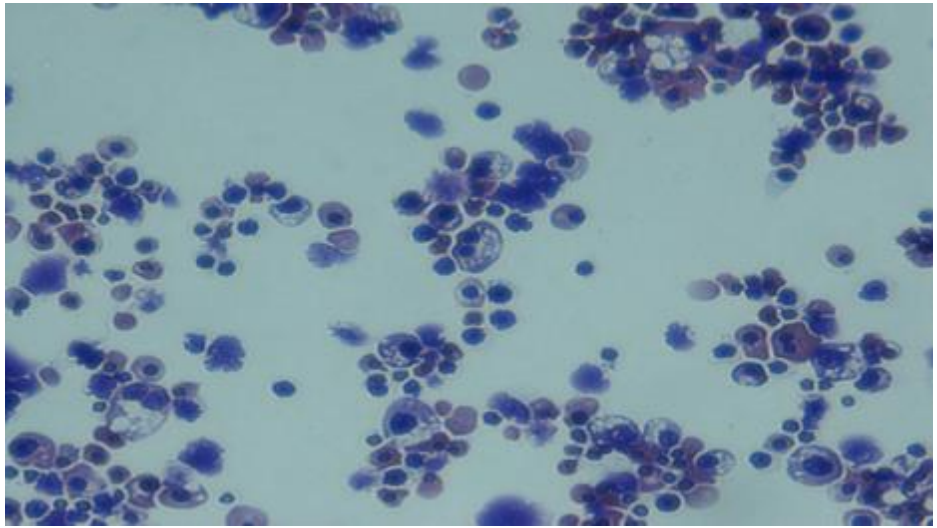


- **5-10% beta-globin**
- **90-95% gamma-globin**
- **traces of epsilon**

ENUCLEATION RATE IN CRBCS PRODUCED FROM IPSCS

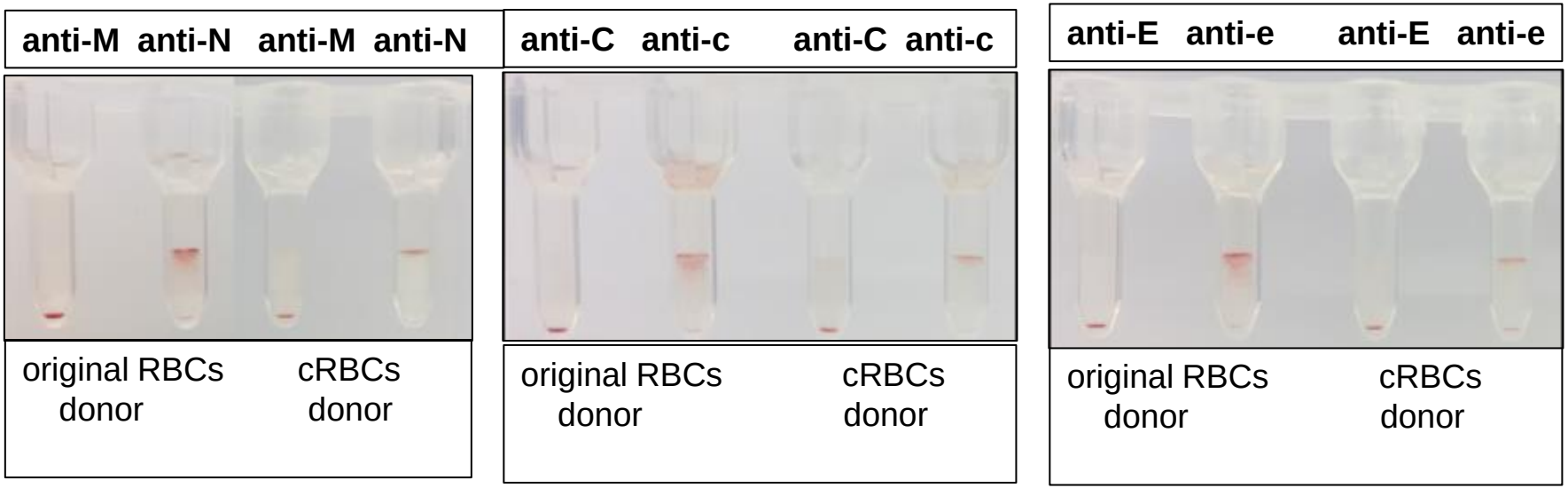




5-10%

TYPING FOR M/N, C/c and E/e

(donor is M-N+, C-c+, E-e+)



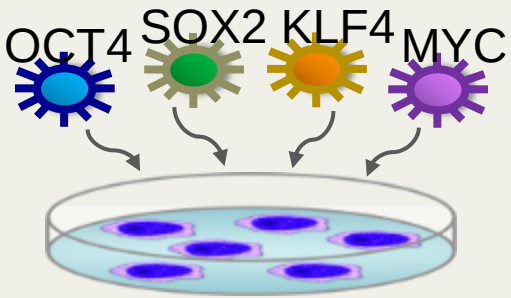
anti-M Bio Rad seraclone
anti-N Immucor gamma-clone

anti-C Immucor gamma-clone
anti-c Immucor Series1

anti-E Immucor gamma-clone
anti-e Immucor gamma-clone

INDUCED PLURIPOTENT STEM CELLS (IPSCS) AS REAGENTS

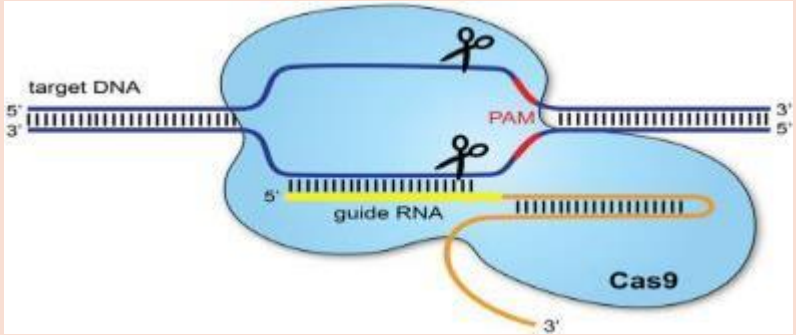
Reprogram rare donor cells



1. Lacking high prevalence Rh antigens (RHCE, hrB-, hrS-)
2. Lacking combinations of antigens (D, U, Fya/b, Ss, etc.)

Adapted from Redman Pract Ed 2016

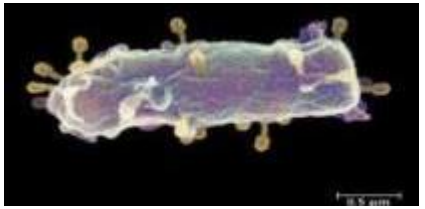
Gene edit iPSCs



1. Rh null
2. RhCE null (D--)
3. Variant Rh (e.g. DAK, Go(a))

GENE EDITING
CRISPR/CAS9 "TARGETED"

- **Precise-target** gene editing
- **Bacteria / archaea**
  - adaptive immunity to eliminate bacteriophage infection
    - discovered in 1980's
  - 2007 - bacteria acquire resistance against infection by integrating a genome fragment of the virus into its "CRISPR locus." Barrangou, R., et al. *Science,* 315, 1709–1712.
  - 2012 - Doudna and Charpentier realized potential to change or repair DNA at a *precise gene location*

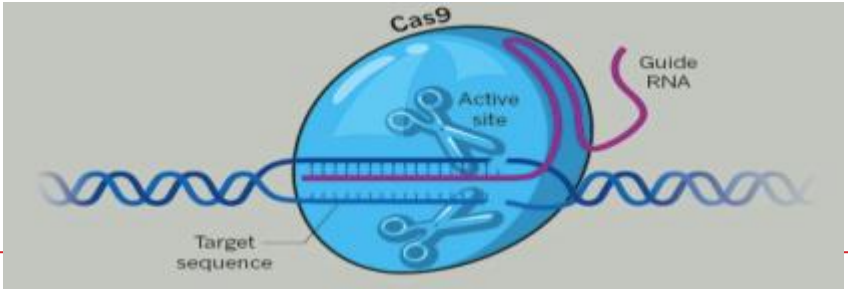


2015 "breakthrough of the year"

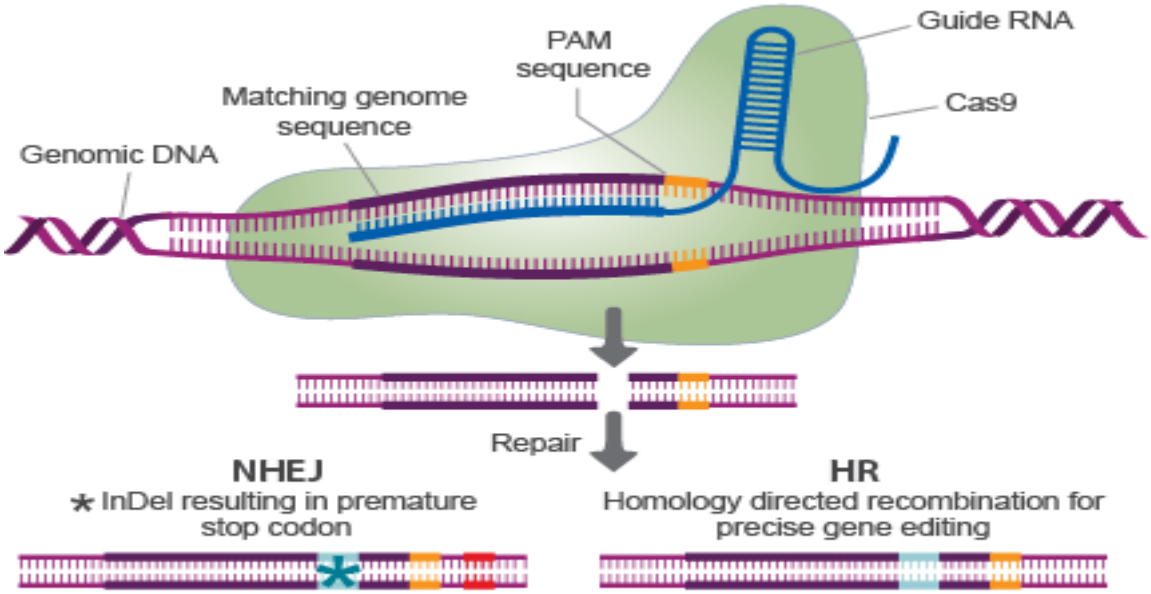
CRISPR/CAS9 GENOME EDITING

To introduce change into the DNA:

- **guide RNA** (gRNA) **-** design RNA sequence (~20 bases) **complementary to the target locus**
  - Guides Cas9 to the right part of the genome
- **enzyme Cas9** – 'molecular scissors' cuts the DNA at that specific location
- DNA is repaired – replaced with mutation OR with correct DNA



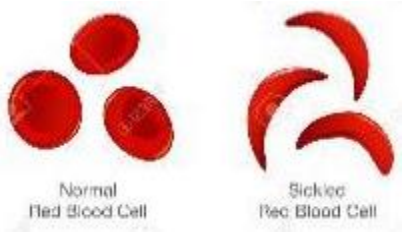
CRISPR/CAS9 GENOME EDITING



INACTIVATE GENE

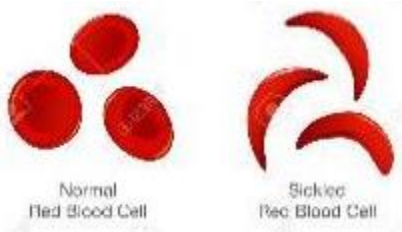
REPAIR OR REPLACE GENE

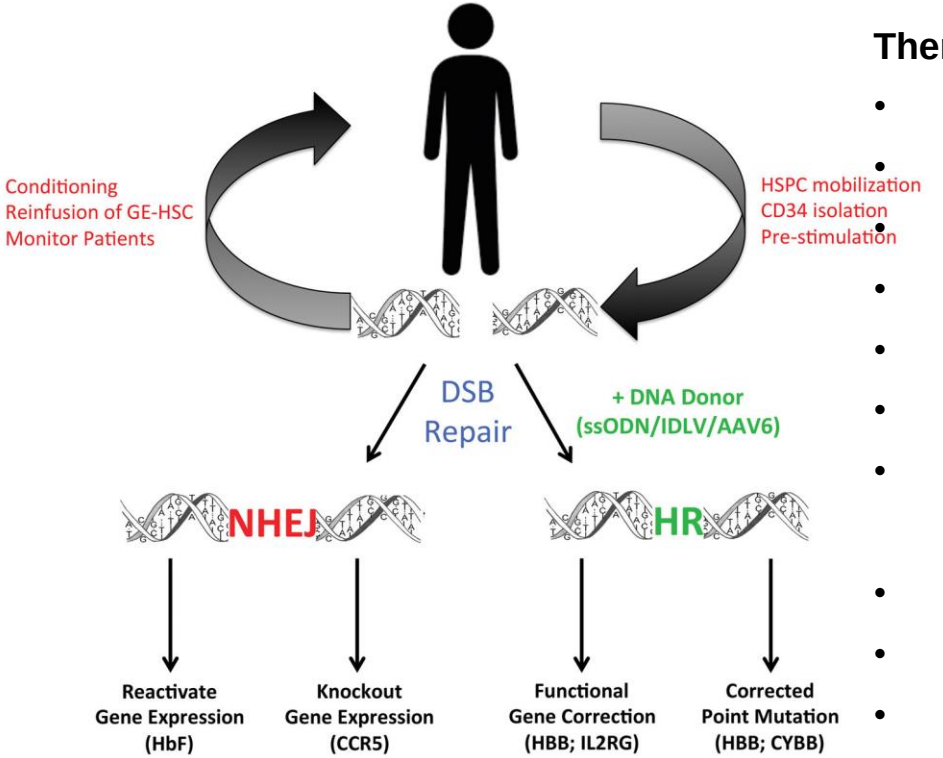
CURE OPTION FOR SCD ??



- CRISPR/Cas9 - revolutionized genome engineering but also brought the possibility of translating into a clinically meaningful reality
- Sickle cell disease (SCD)
- disease caused by a single gene mutation
  - A-T mutation: Adenine (A) to thymidine (T) transversion in the HBB gene
  - Single amino acid change from glutamic acid to a valine in hemoglobin molecule
- Genome editing as a curative option?
- To correct the mutation in patient hematopoietic stem/progenitor cells (HSPCs)
- Site-specific correction of the sickle mutation would allow for permanent production of normal red blood cells

CURE OPTION FOR SCD ??





Therapeutic Gene editing of autologous HSPCs

- HSPCs - harvested from bone marrow - GCSF or Plerixafo
- PBMCs enriched using the CD34 marker
- CD34+ cells are stimulated in stem cell cytokine media
- site-specific engineered double strand breaks (DSBs)
- repaired by HR homologous recombination
- to correct mutation for functional gene correction
- patient conditioned using myeloablative regiments to clear non-corrected resident bone-marrow
- Genetic engineered (GE) HSC are reinfused into patient
- Patient monitored for engraftment
- FDA requirement of a 15-year follow-up

GENE EDIT FROM EXISTING IPSCS

Stella Chou

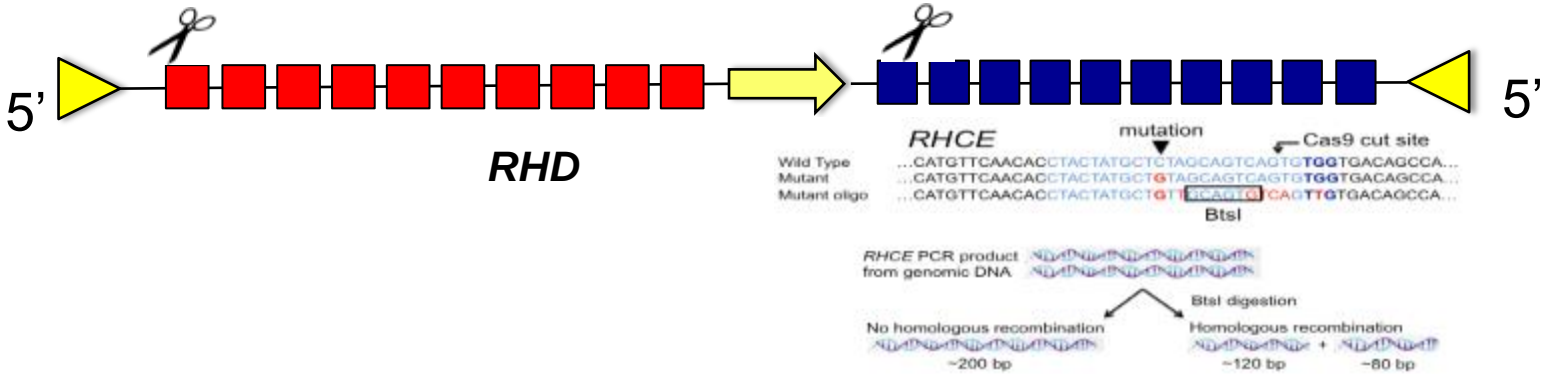
Children's Hospital of Philadelphia
iPSC CORE

- **Genotyped 12 "wild-type" iPSCs lines**
  - ABO, Rh, and extended antigen phenotypes
  - Identify Group O lines – 4 of 12

iPSC line	Method	Cell of origin	ABO genotype	RHD genotype	Predicted ABO/D type	Predicted extended antigen type by genotype
CHOPWT8	Lentivirus	**Peripheral blood**	***01/*01**	***RHD***	**Group O, RhD+**	C+ E- c+ e+ K- Jka+ Jkb+ Fya- Fyb+ S- s+ U+ Doa- Dob+
CHOPWT9	**Sendai**	**Peripheral blood**	***01/*01**	***RHD***	**Group O, RhD+**	C- E+ c+ e- K- Jka+ Jkb+ Fya- Fyb+ S- s+ U+ Doa+ Dob+
CHOPWT4	**Sendai**	**Fibroblast**	***01/*01**	**No RHD gene**	**Group O, RhD-**	C- E- c+ e+ K- Jka- Jkb+ Fya+ Fyb- S- s+ U+ Doa+ Dob+
CHOPWT10	**Sendai**	**Peripheral blood**	***01/*01**	**No RHD gene**	**Group O, RhD-**	C- E- c+ e+ K- Jka- Jkb+ Fya+ Fyb- S- s+ U+ Doa+ Dob+

RH NULL RBC'S

- **CRISPR/Cas9 – to target *RH* locus**



CRISPR/Cas9 gene editing to mutate RHCE in RHD negative cell line

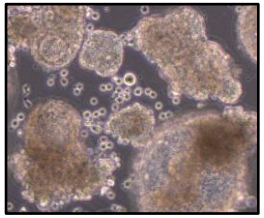
- Guide RNAs target a sequence close to the desired mutation site.
- If mutant oligonucleotide sequence is introduced, restriction enzyme digestion results in two fragments.

Abstract: IGT6-TU2-12 **Induced Pluripotent Stem Cell-Derived Red Cells for Use as Reagents to Resolve Rh Specificities**

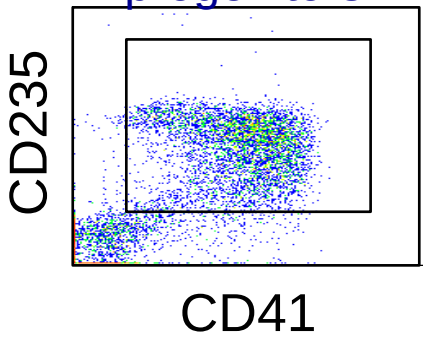
Children's Hospital of Philadelphia and New York Blood Center
Hyun H. An, J Aeschlimann, D.Posocco, JA Maguire, P Gadue, DL French, CM.Westhoff , ST. Chou

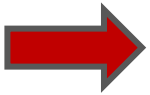
DIFFERENTIATION TO RED CELLS

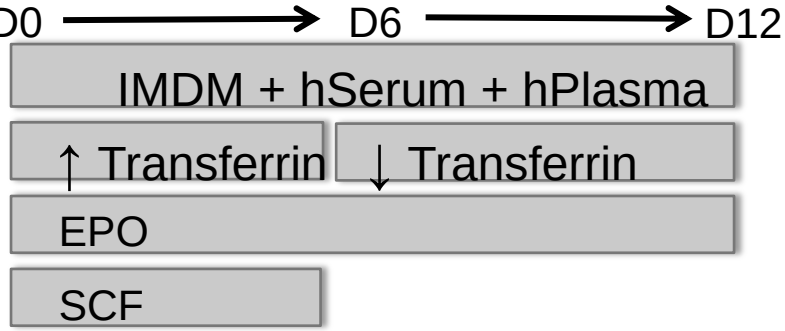
Embryoid body differentiation



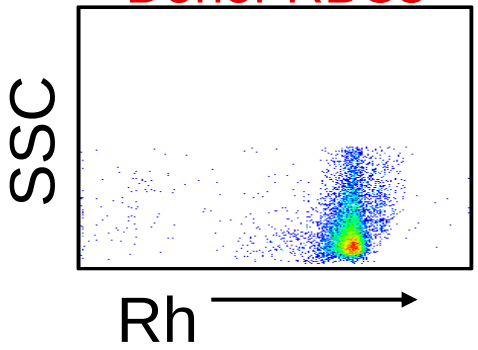
Hematopoietic progenitors



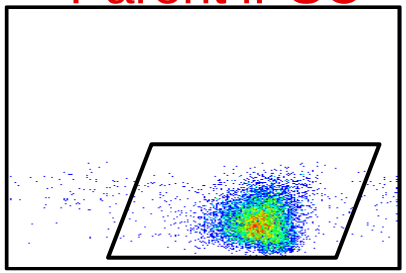




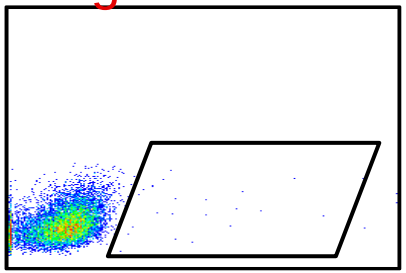
Donor RBCs



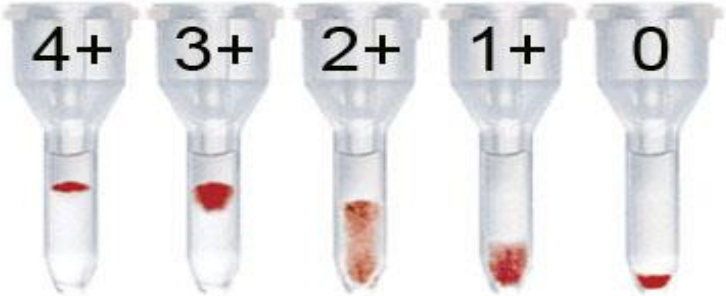
Parent iPSC



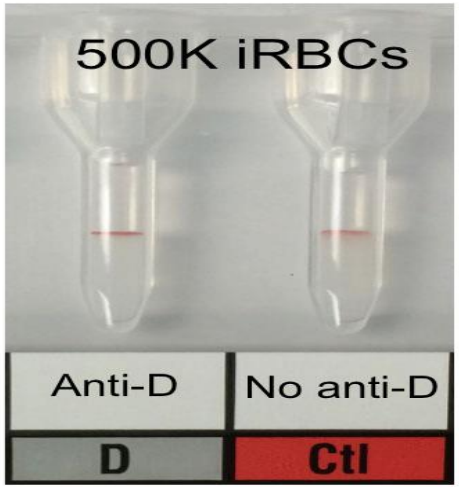
Targeted Rhnull



PERFORMANCE IN BLOOD BANK ASSAYS



Standard Gel Card Assay

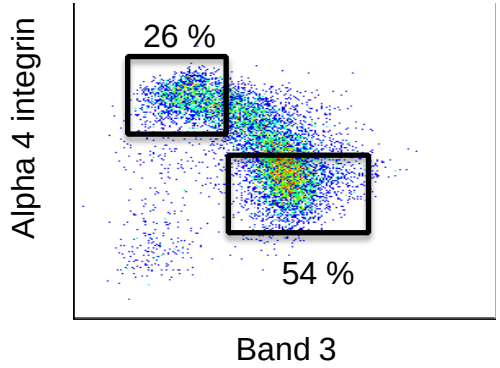


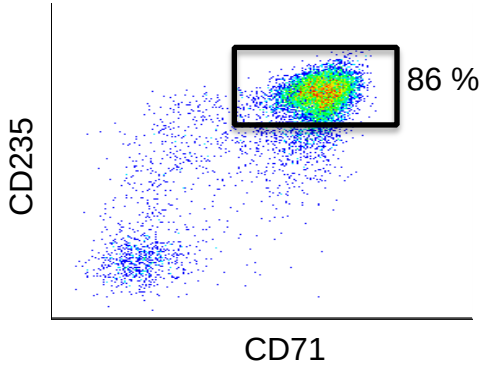
“Stuck” at top of gel matrix related to cell size

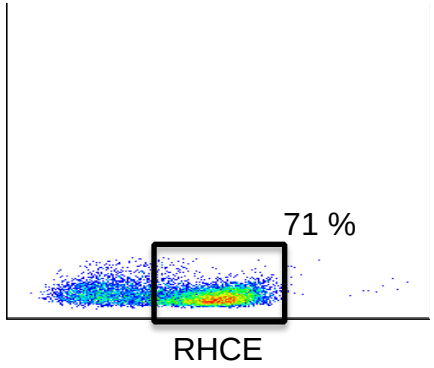
Day 6 iPSC-derived RBCs

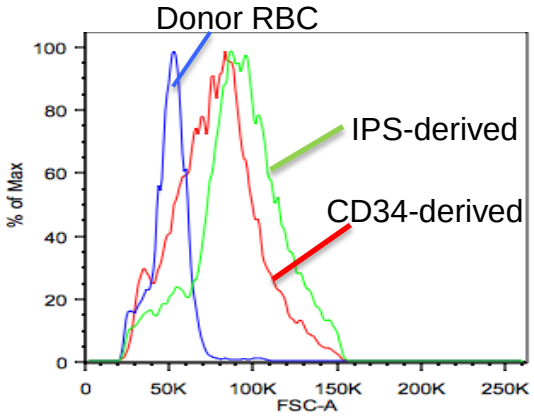
WT4.1 O, RhD-
D8 EB derived
progenitors:

EPO 2 U/ml
SCF 100 ng/ml
IGF1 25 ng/ml

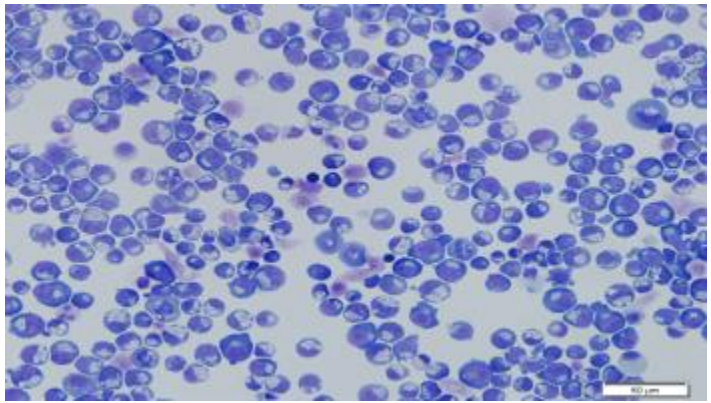








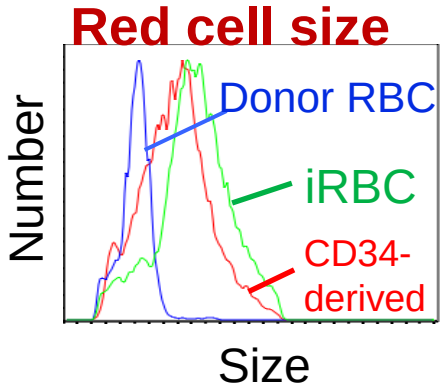
-Donor RBC
-CD34-Derived
-IPS-Derived

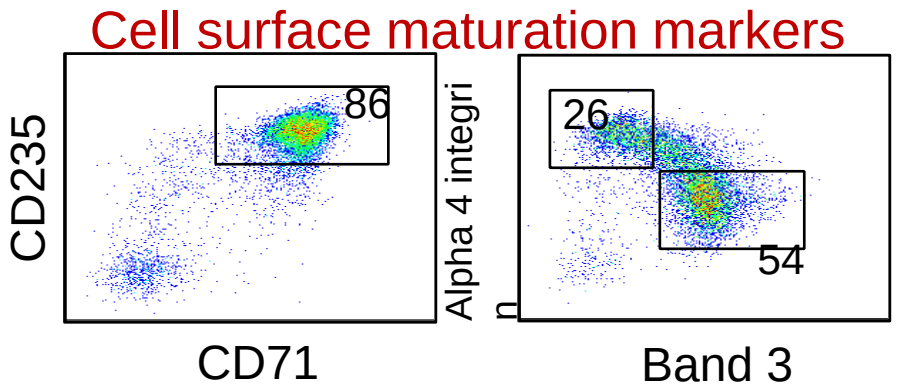


iPSC-derived RBC D6 liquid culture

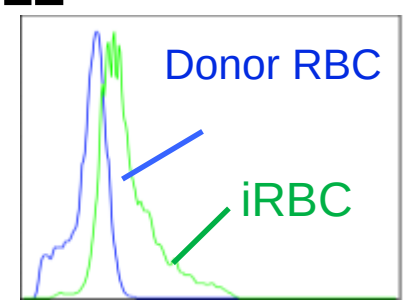
MATURATION AND SIZE OF IRBCS

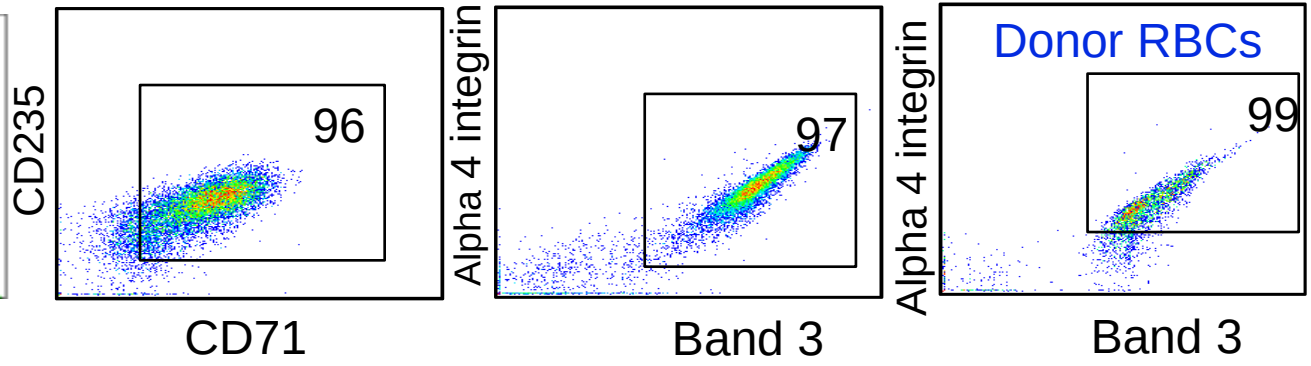
Day 6



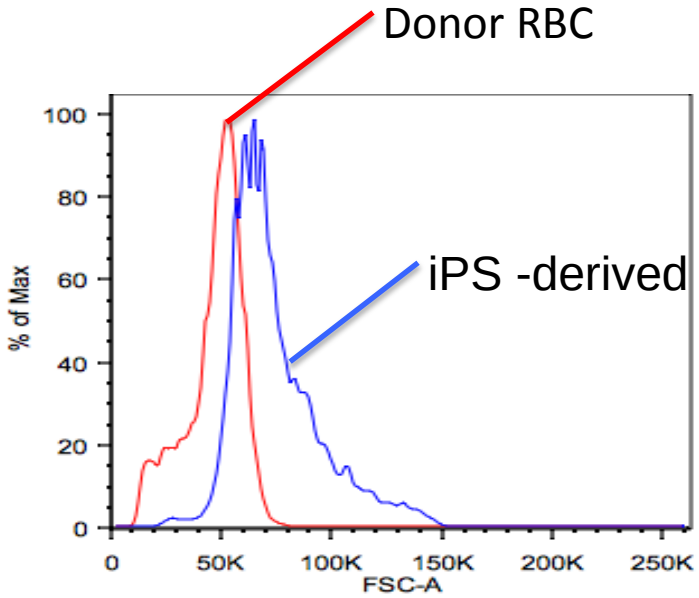


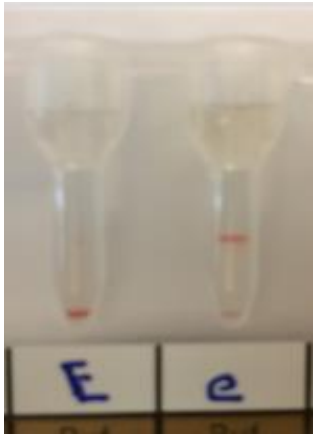
Day 12





iRBCs - Rh antigen typing gel card





WT 4.1 (D- E- e+)

D18 liquid cx

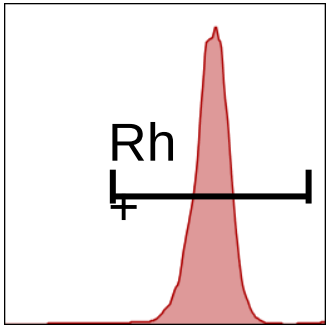
6d iPSC ery media +

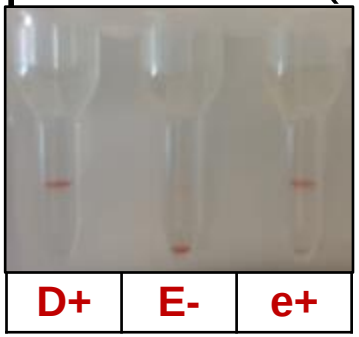
12d CD34 ery phase II media

RH TYPING OF IRBCS

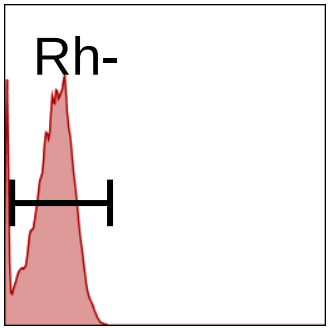
Gel card assay

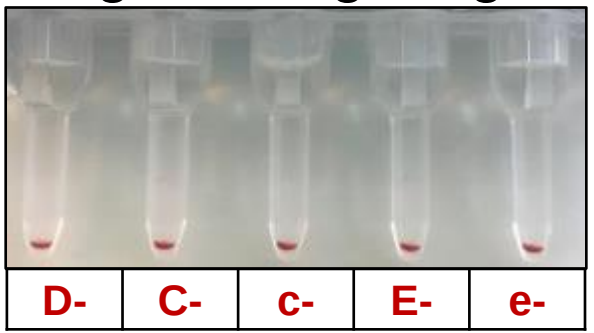
iRBCs from parent line (D+ E- e+)





iRBCs with RH gene targeting

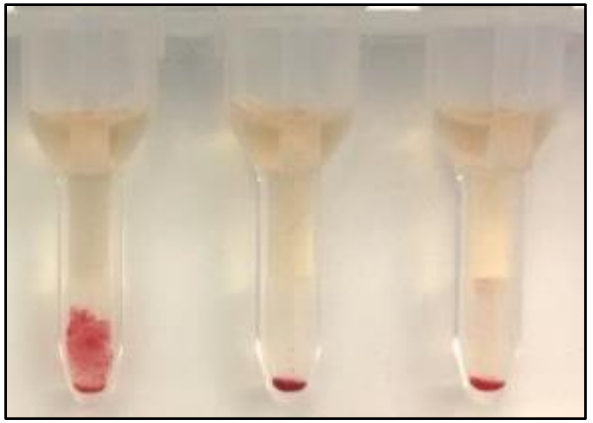




ANTIBODY FROM PATIENT WITH SCD IS NON-REACTIVE WITH RH NULL IRBCS

Patient: e+ with anti-e in plasma

RHCE genotype:**ce733G /ce733G*
partial e with allo anti-e



Donor e+	Donor e-	iRBC Rh null
2+	(-)	(-)

SUMMARY

iPSC technology and gene editing can be combined to generate customized red cell panels for blood banks

iRBCs with novel antigens or lacking any number of antigen combinations is possible

iRBCs can undergo sufficient maturation to be used in common blood bank assays

Potential to be one of the first clinical applications of iPSC-derived blood cells to impact patient care

iRBCs from rare donors will be important for transfusion when scale-up technology is available for in vitro RBC production

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