

Genome-edited peripheral blood stem cell-derived erythroid progenitor-4 cell line as the unique and beneficial panel cell for antibody identification

Irregular antibody screening and identification in pre-transfusion testing are indispensable to prevent hemolytic transfusion reactions. Serological testing, especially agglutination testing using the conventional tube test or gel column method, is a simple and quick way to confirm the existence of alloantibodies in the patient's plasma against antigens on red blood cells (RBC).^{1,2} Panels of cells, which consist of several types of red cells to detect almost all clinically relevant alloantibodies against major blood group antigens, have been widely used for serological testing. However, the existence of antibodies against high-frequency antigens or mixed alloantibodies sometimes makes antibody identification difficult and inadequate unless RBC which lack corresponding antigens are used. This study aimed to produce an erythroid cell line that expresses few blood group antigens by deleting the major blood group antigens (Rh, Duffy, P1PK, Kidd, JR, and MNS) from the original cell line using genome editing via CRISPR/Cas9^{3,4} in order to simplify the identification of irregular antibodies. The Diego blood group defined by BAND3 was excluded from the targets in this study because it was predicted that destroying the *BAND3* gene would make cells fragile.^{5,6} Corresponding antibodies against these blood group antigens are frequently identified as irregular antibodies in clinical settings in the Japanese population; therefore, antibody screening and identification are performed using panel cells including these antigens in Japanese blood centers. In this study, we used peripheral blood stem cell-derived erythroid progenitor (PBDEP)-4 cells established from an O-type donor.⁷ PBDEP-4 can differentiate into cells largely consisting of late erythroblasts with a small proportion being enucleated, and their duration of differentiation can be shortened to 10 days at most. Furthermore, PBDEP-4 cells are advantageous for serological testing with visual checks because they synthesize hemoglobin and are red-colored after differentiation. Here, we report the

outcomes and discuss their applicability to pre-transfusion testing. The research complied with relevant national regulations and institutional policies and was conducted in accordance with the Helsinki Declaration. This research was approved by the Ethics Review Committee of the Japanese Red Cross Society (approval numbers: 2018-006, 2019-018, 2022-014, and 2023-002).

Before genome editing, the PBDEP-4 genotype was confirmed by polymerase chain reaction with sequence-specific primers or Sanger sequencing. As a result, its major blood group antigen genotype other than ABO was typical in the Japanese population (Table 1 upper column). Then, flow cytometry using PBDEP-4 on day 7 of differentiation confirmed the expressions of antigens corresponding to the verified genotypes (Figure 1A, PB4). The antibodies used are listed in *Online Supplementary Table S1*. We subsequently attempted to delete blood group antigens, such as the Rh, Duffy, P1PK, Kidd, JR, and MNS, by genome editing via CRISPR/Cas9.

After these attempts, the eight target genes (*RHD*, *RHCE*, *ACKR1*, *A4GALT*, *SLC14A1*, *ABCG2*, *GPA*, and *GPB*) were knocked out, and we succeeded in generating PBDEP-Di^b cells that lack target antigens (Figure 1A, DIB and Table 1 lower column), as verified by flow cytometry. Sanger sequencing suggested that target genes other than *ACKR1* were modified with frameshift mutations, resulting in replacement with truncated protein (*Online Supplementary Table S2*). The modified *ACKR1* had an in-frame deletion, but flow cytometry showed that the cells lost reactivity with antibodies, suggesting that the Duffy antigen was knocked out. This result was consistent with a previous report by Hawksworth *et al.*, who knocked out the Duffy antigen using the same knockout vector.⁸ Remarkably, PBDEP-Di^b also demonstrated normal proliferation, as well as parent PBDEP-4 cells under the condition of maintenance and dif-

Table 1. Phenotypes of PBDEP-4 before and after genome editing.

Cell line	Rh					MNSs				P	Diego		Duffy		Kidd		JR
	D	C	c	E	e	M	N	S	s	P1	Di ^a	Di ^b	Fy ^a	Fy ^b	Jk ^a	Jk ^b	Jr ^a
PBDEP-4	+	+	0	0	+	+	+	0	+	+	0	+	+	0	+	+	+
PBDEP-Di ^b	KO	KO	KO	KO	KO	KO	KO	KO	KO	KO	0	+	KO	KO	KO	KO	KO

PBDEP-4: peripheral blood stem cell-derived erythroid progenitor-4; PBDEP-Di^b: genome edited cell line lacking target antigens; +: exists; 0: does not exist; KO: deleted antigen.

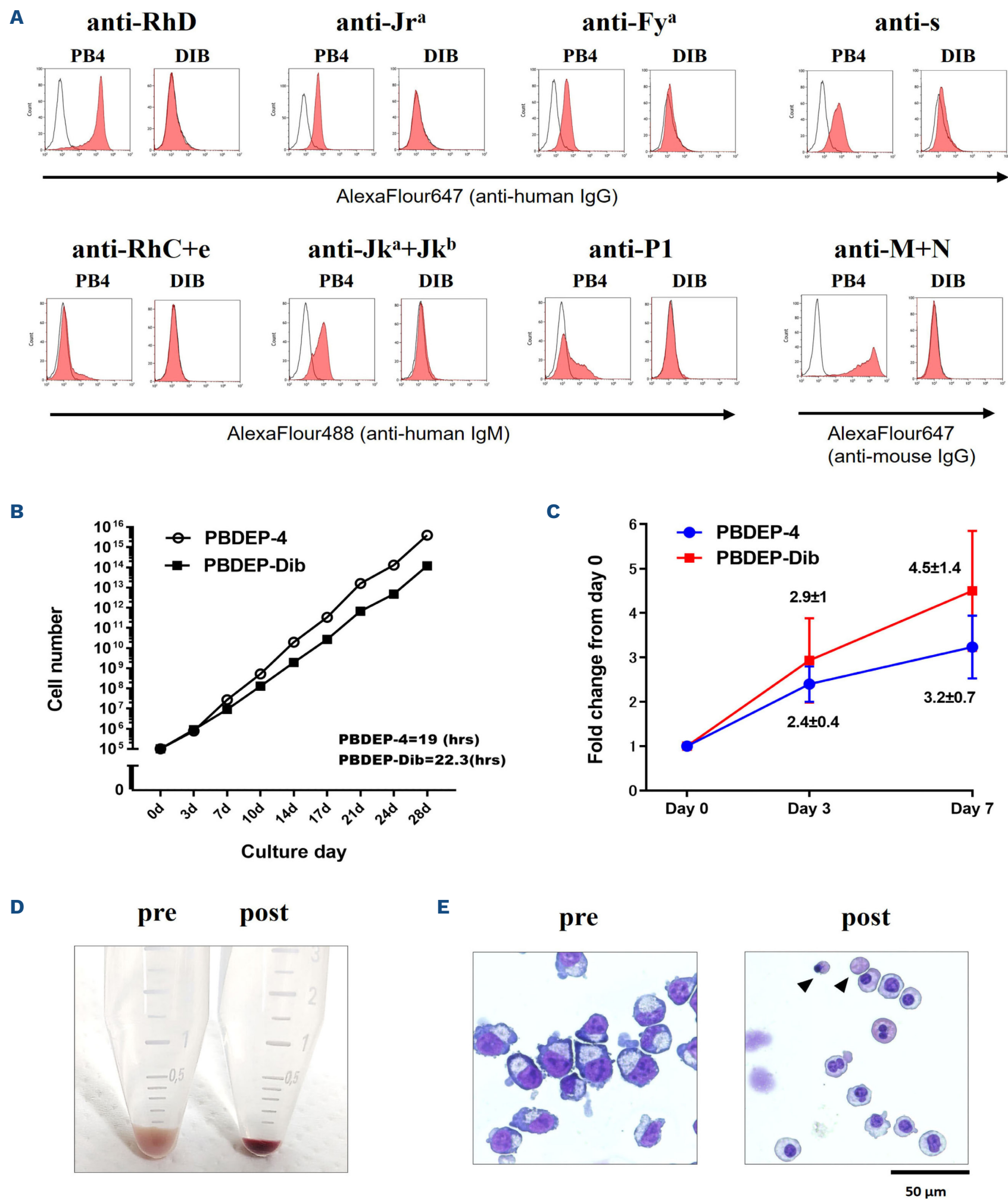


Figure 1. Reactivity with antibodies against major blood group antigens in peripheral blood stem cell-derived erythroid progenitor-4 (PBDEP-4) cells, and comparison of cell character between pre- and post-genome editing. (A) Flow cytometry analysis of differentiated PBDEP-4 and PBDEP-Di^b cells using antibodies against the corresponding target antigens except for Di^b. The histograms of PBDEP-4 (PB4) and PBDEP-Di^b cells (DIB) are shown side by side. The antibodies used are presented above. Each histogram overlay plot includes isotype control (black line without filling color) and antibody staining (plot with pale red filling color). (B) Growth curves of PBDEP-4 and PBDEP-Di^b cells for 28 days. (C) Fold changes in cell number during differentiation. The experiments were performed three times. The graph shows the mean number of fold changes, and the error bar shows mean $\pm$ standard deviation. (D) Appearance and (E) cell morphology of PBDEP-Di^b cells before (pre) and after (post) differentiation (7 days). Black arrowheads indicate enucleated erythroblasts. Scale bar, 50 μ m.

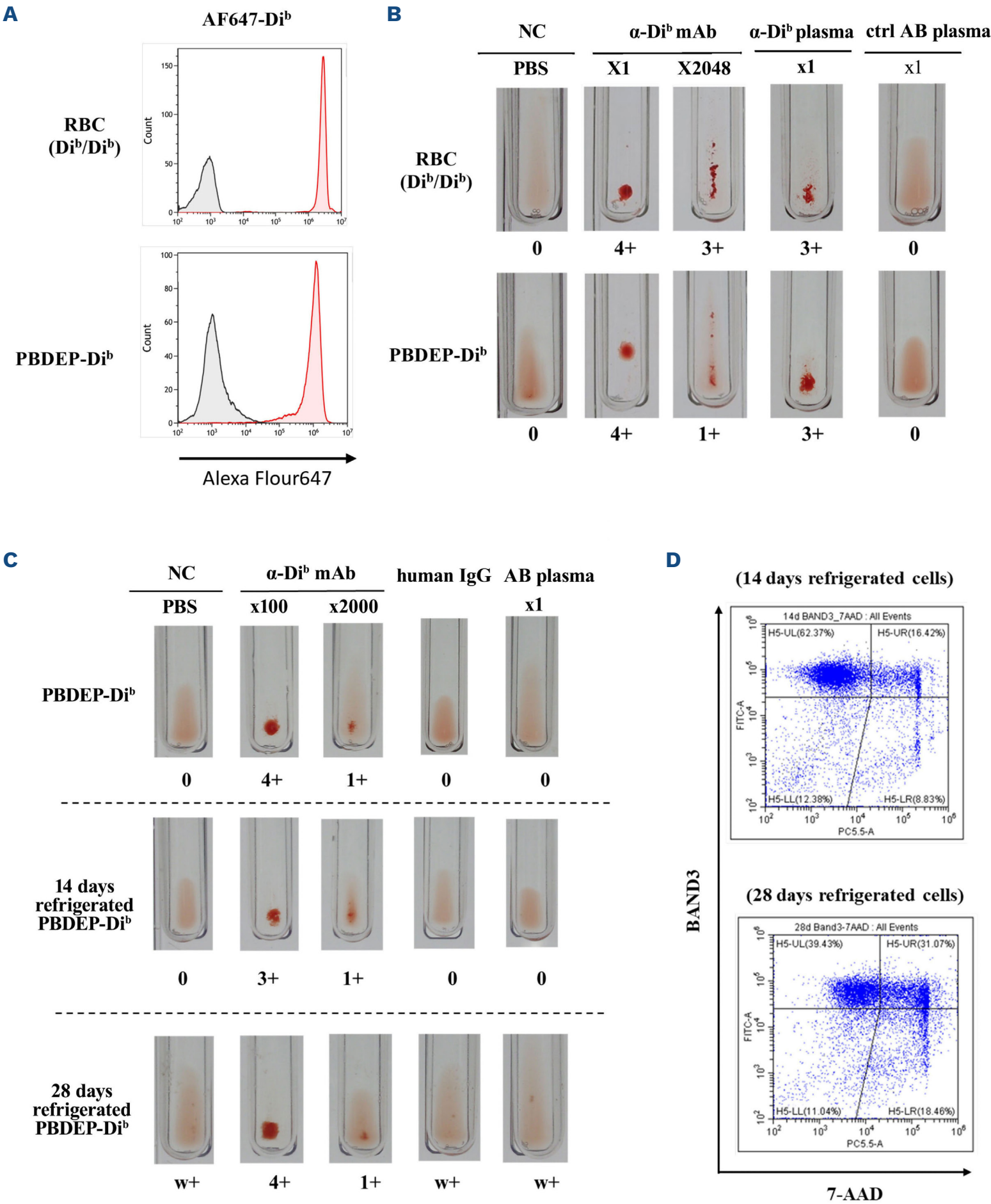


Figure 2. Reactivity with anti-Di^b antibody using differentiated PBDEP-Di^b cells. (A) Expression of the Di^b antigen in peripheral red blood cells (Di^b/Di^b) and differentiated PBDEP-Di^b cells was analyzed by flow cytometry using anti-Di^b monoclonal antibody. The histogram overlay plot includes isotype control (gray) and antibody staining (pale red). (B) Agglutination images of red blood cells (Di^b/Di^b) and differentiated PBDEP-Di^b cells by tube test using anti-Di^b monoclonal antibody, anti-Di^b plasma, and control human AB plasma. Dilution factors and agglutination scores are shown above and below the test tube images, respectively. (C) Reactivity of anti-Di^b monoclonal antibody with differentiated PBDEP-4 cells that were refrigerated for 0, 14, and 28 days using 10% fetal bovine serum-supplemented Iscove's modified Dulbecco's medium. Images of agglutination of PBDEP-Di^b cells detected by the tube test using anti-Di^b monoclonal antibody, human IgG as an isotype control, and control human AB plasma are shown. Dilution factors of anti-Di^b and agglutination scores are shown at the top. (D) Viability staining of differentiated PBDEP-4 cells that were refrigerated for 14 and 28 days using BRIC6 (anti-CD233) and 7-AAD. The cells were grouped into three groups: living cells (BAND3⁺/7-AAD⁻), dead cells (BAND3⁻/7-AAD⁺ plus BAND3⁺/7-AAD⁺), and cell debris (BAND3⁻/7-AAD⁻). The antibodies used are listed in *Online Supplementary Table S1*. RBC: red blood cells; NC: > negative control; mAb: monoclonal antibody; PBS: phosphate-buffered saline; ctrl: control; 7-AAD: 7-aminoactinomycin.

ferentiation (Figure 1B, C). Furthermore, the deletion of the blood group antigens hardly affected erythroid differentiation and cell morphology (Figure 1D, E) and cell death rarely occurred after differentiation (*data not shown*). Next, we confirmed the expression of Di^b antigen by flow cytometry and tube testing. Residual Di^b was detected at a high level of expression by flow cytometry (Figure 2A). Agglutination capability was confirmed by the tube test using an anti-Di^b monoclonal antibody and could be scored by the degree of agglutination; however, the sensitivity of detection was lower than that of RBC with Di^b/Di^b, at a high antibody dilution (x 2,048). Nevertheless, when PBDEP-Di^b was mixed with the actual donor plasma including anti-Di^b antibody, anti-Di^b could be detected with a sufficient agglutination score (3+) in a manner similar to RBC with Di^b/Di^b (Figure 2B). To assess the agglutination sensitivity after 14 and 28 days of cold storage, agglutination testing was performed using refrigerated PBDEP-Di^b. At 14 days the cells could be scored in a fashion similar to those in the common tube test (Figure 2C). However, in the tubes using the cells at 28 days, small cell clumps were formed in the negative control tubes using human IgG as an isotype control or control human AB plasma (Figure 2C). We subsequently performed viability staining to detect dead cells. After 14 days of storage, 62.4% of the cells were alive (BAND3⁺/7-AAD⁻) and 39.4% were alive after 28 days. The increased population of dead cells (BAND3⁻/7-AAD⁺ plus BAND3⁺/7-AAD⁺) may contribute to the formation of small cell clumps as found in the control tubes after 28 days (Figure 2D). These results suggest that a high proportion of cells die after long-term refrigeration, which would result in subsequent nonspecific aggregation and reduced sensitivity. We believe that refrigeration is appropriate for the storage of this cell line; cells refrigerated for up to 14 days can be used for serological testing. However, it is necessary to improve the storage condition in which the proportion of dead cells does not increase for a sufficient time.

PBDEP-Di^b is a distinctive cell line that possesses an extremely rare blood type with major blood group antigens being deleted. Although multiple mutations in blood group antigens affect membrane topology and morphology of mature RBC, apparent abnormality of nucleated erythroblasts caused by a mutation of blood group antigens has seldom been reported previously. For instance, Rh-null red cells in peripheral blood are known to have membrane fragility and morphological abnormalities, which make the cells vulnerable to hemolysis.^{9,10} Given the lack of specific descriptions regarding the morphological abnormality of cultured Rh-null cells in previous studies, deficiency of RhD and RhCE antigens in cultured erythroblasts may not cause severe membrane fragility, which results in immediate hemolysis.^{11,12} Moreover, even the cell line that lost five major blood group antigens presented normal morphology and underwent serological testing in a fashion similar to that of cultured RBC without editing of blood group antigens.⁸ In

contrast, BAND3, which was not included in these five targets, may contribute to membrane stability. As differentiated PBDEP-Di^b were scarcely enucleated, the nucleated state and cytoskeletal proteins around the nucleus may physically play a role in membrane stability after genome editing, to which the unedited BAND3 may also contribute.

In serological testing, the PBDEP-Di^b cell line is suitable for flow cytometry and tube testing. Our results suggest that PBDEP-4 offers considerable flexibility in modifying antigens, as exemplified by major blood group antigen-deleted cells (Figure 2B) and Fy^b-forced expressing cells (*Online Supplementary Figure S1*), which was established by the transfection of the *ACKR1* gene that was not possessed in parent PBDEP-4 and functioned almost similarly to RBC with Fy^b for the tube test, as mentioned in the legend to *Online Supplementary Figure S1*. To simplify antibody identification, we suggest that major blood group antigens and specific high-frequency antigens should be deleted first to create a near-null state, such as PBDEP-Di^b, and then multiple panel cells should be prepared by forced expression of the target antigens in question.

There are a few considerations that should be made for future studies. First, the cost of the maintenance of the cell line is extremely high because the maintenance medium is expensive. We have been working on improving the culture protocol, which has not yet been optimized. Three-dimensional culture is possible, but we have not started it in earnest due to cost constraints. These costs would decrease significantly if inexpensive media and efficient culture methods could be developed. Second, the applicability of these cells to the gel column method will have to be examined. Nucleated red cells do not pass through gel columns so far examined; however, An *et al.* previously detected antibodies using nucleated red cells in combination with certain gel columns that are not commercially available in Japan.¹² Hopefully, our PBDEP-4 cells may also become applicable to the gel column method using appropriately optimized columns.

In conclusion, the adoption of PBDEP-4 cells and the CRISPR/Cas9 system enables the artificial production of panel cells whose antigens are editable. Despite the lack of major blood group antigens, genome-edited PBDEP-4 cells demonstrated normal differentiation and were suitable for flow cytometry analysis and the conventional tube test. We expect that this method of using PBDEP-4 and genome editing can be applied to the manipulation of any RBC antigen to produce artificial panel cells for simple identification of alloantibodies in blood donors and recipients. In addition, it will greatly contribute to improving the accuracy of pre-transfusion testing and the basic study of blood group systems.

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References

1. White J. Pre-transfusion testing. ISBT Science Series. 2009;4(1):37-44.
2. British Committee for Standards in Haematology, Milkins C, Berryman J, et al. Guidelines for pre-transfusion compatibility procedures in blood transfusion laboratories. British Committee for Standards in Haematology published correction appears in Transfus Med. 2022;32(1):91. Transfus Med. 2013;23(1):3-35.
3. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823-826.
4. Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol. 2013;31(3):230-232.
5. Jarolim P, Murray JL, Rubin HL, et al. Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood. 1996;88(11):4366-4374.
6. Tanner MJ. The structure and function of band 3 (AE1): recent developments (review). Mol Membr Biol. 1997;14(4):155-165.
7. Ito S, Kaito S, Miyazaki T, et al. A new antigen SUMI carried on

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Disclosures

No conflicts of interest to disclose.

Contributions

KF and RK conceived and designed the study. KF, GK, KM and KI performed the experiments. KF wrote the manuscript. RK, TM, MO, SM, MS and YT read and edited the manuscript. RK supervised the study.

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Data-sharing statement

For original data, please contact r-kurita@jrc.or.jp.

glycophorin A encoded by the GYP A* M with c.91A>C (p.Thr31Pro) belongs to the MNS blood group system. Transfusion. 2020;60(6):1287-1293.

8. Hawksworth J, Satchwell TJ, Meinders M, et al. Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. EMBO Mol Med. 2018;10(6):e8454.
9. Sturgeon P. Hematological observations on the anemia associated with blood type Rhnull. Blood. 1970;36(3):310-320.
10. Ballas SK, Clark MR, Mohandas N, et al. Red cell membrane and cation deficiency in Rh null syndrome. Blood. 1984;63(5):1046-1055.
11. Cambot M, Mazurier C, Canoui-Poittrine F, et al. In vitro generated Rh(null) red cells recapitulate the in vivo deficiency: a model for rare blood group phenotypes and erythroid membrane disorders. Am J Hematol. 2013;88(5):343-349.
12. An HH, Gagne AL, Maguire JA, et al. The use of pluripotent stem cells to generate diagnostic tools for transfusion medicine. Blood. 2022;140(15):1723-1734.