New blood in a dish: Towards Cost-Effective In-Vitro Production of Erythrocytes by Genetic Engineering

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Abstract

In vitro production of red blood cells (RBCs) has been an attractive alternative to minimize the gap between dwindling blood supply and growing clinical demand. However, available methods for in vitro production do not yet provide a sustainable supply at an effective cost, with the prohibitive cost being a major barrier. Recent advances in immortalized human erythroid cell lines have yielded promising results in producing RBCs, and have advantages relative to other sources of cells such as minimal culture and easy transduction. In this study, we propose three approaches to genetically engineer two immortalized human erythroid cell lines to reduce costs of raw materials in producing RBCs. The three approaches are expressing: a constitutively active EPO receptor (cEPOR); transferrin; divalent metal transporter (DMT1) and DMT1-associated genes respectively. Preliminary characterization has shown no differences in morphology, protein levels, deformability and oxygenation dynamics in the study as compared to mature RBCs produced by traditional in vitro methods. While the production process could be further optimized and scaled, this proof-of-concept study has demonstrated a feasible approach towards generating cost-effective RBCs in vitro.

1. Introduction

The worldwide demand for safe blood has been on the rise as growing life expectancy and diminishing blood donors continue to exacerbate the blood shortage problem in the world[1–3].

An adequate blood transfusion supply is essential for a functioning health-care system, but many low-income and middle-income countries still face significant challenges with access to safe and reliable blood transfusions. These challenges are particularly acute in regions where rates of blood donation are most inadequate, where the disparity between clinical demand and supply is often the highest[3]. Currently, available methods for generation of RBCs do not yet provide a reliable supply and the cost of producing RBCs remains a major barrier[4]. The cost is driven by raw materials[4,5], and the production process[6] which requires expensive biological inputs to satisfy extensive differentiation needs.

Major improvements in immortalized human erythroid cell lines make them one of the most promising sources to achieve cost-effective large-scale production of RBCs. Immortalized human erythroid cell lines are stable cell lines committed to the erythroid lineage, which provides a continuous supply of RBCs with few differentiation steps towards mature RBCs.[7] These cell lines such as Bristol Erythroid Line Adult (BEL-A) and Erythroid Line from Lund University (ELLU) demonstrate desirable properties such as efficient differentiation and easy isolation[1,8], which makes them an appealing system for genetic modifications in sustainable production of RBCs.

One of the major costs associated with in vitro RBC production is the cost of the proteins that are supplemented in the medium of culture for RBC maturation and iron uptake. Previous research has identified two major sources of cost: erythropoietin (EPO) and transferrin[4,5]. EPO

is a key hormone which binds EPO receptors to stimulate the production of RBCs in the body[9], the need of which may be bypassed by expressing a constitutively active EPO receptor (cEPOR). Transferrin is a protein produced by the liver that binds to iron ions and allows them to be transported in the blood to sites of erythropoiesis[10,11], which may be recombinantly expressed in the cell lines to lower the cost. Another way to enable dietary iron to be made available for RBCs is through the recombinant expression of an endogenous system allowing iron uptake by intestinal cells in RBCs. The expression of divalent metal transporter, (DMT1)[12], a membrane channel that allows iron uptake by intestinal cells, as well as the associated genes that allow the functioning of DMT1 (duodenal cytochrome B (DcytB) and sodium-hydrogen exchanger 3 (NHE3))[12,13] is proposed to reduce the cost of production by eliminating the need of supplemented transferrin.

In this study, we propose three unique methods to target EPO, transferrin, and DMT1 in reducing the cost of in vitro RBC production. The three approaches are inducible expression of: 1) cEPOR; 2) Transferrin; and 3) DMT1 and DMT1-associated genes in both BEL-A and ELLU. This proof-of-concept proposal could be scaled up to improve the efficiency and cost-effectiveness of in vitro RBC production, ultimately providing a sustainable solution to alleviate demands for safe and reliable blood transfusions in modern medicine.

2. Methods

2.1.1 Vector designs: Tet-ON inducible expression systems for constitutively active erythropoietin receptor (cEPOR), apotransferrin (ApoTF), and iron transport channel (DMT1, DCYTB, and NHE3)

The target genes are inserted into different pCMV6-AC-GFP-rtTA (Origene, PS100125) vectors between the TRE3G promoter and poly(A) to create expression plasmids, pTetON-cEPOR, pTetON-ApoTF, pTetON-SLC11A2, pTetON-CYBRD1, and pTetON-SLC9A3 (**Table 1**). The pCMV6-AC-GFP-rtTA vector (Origene, PS100125) contains Tet-ON or tetracycline-inducible expression system that is suitable for expression in mammalian cells. All the gene targets and the vectors are assembled using Gibson assembly.

2.1.2 Transient transfection of BEL-A and ELLU cell lines

After plasmid construction, plasmids are transfected into the BEL-A and ELLU cell lines by using TurboFectin Transfection Reagent (Origene, CAT#: TF81001) as described in the protocol[22]. The transfection method is optimized for nucleic acid delivery in eukaryotic cells. Briefly, cells are prepared by plating a density of 1-3 x 10^5 cells in complete growth medium in a 6-well plate and incubated overnight to get 50-70% confluence. Then, 1 µg of plasmid DNA is diluted in 250 µL of Opti-MEM I, a reduced serum medium, and gently vortexed. After that, 3 µL Turbofectin 8.0 is added to the diluted DNA and gently pipetted to complete the mixture. The mixture is incubated for 15 minutes at room temperature. The mixture is then introduced dropwise to cells, rocked vertically and horizontally, and incubated at 37°C for 24-48 hours.

Table 1. The plasmid designs in this study

Target genes	Plasmid backbone	Recombinant plasmid	Description
$EPOR_{R129C}$	pCMV6-AC-GFP-rtTA	pTetON-cEPOR	Tet-ON expression system for constitutively active EPO receptor (cEPOR), bearing single point mutation at nucleotide 484.
TF	pCMV6-AC-GFP-rtTA	pTetON-ApoTF	Tet-ON expression system for human apotransferrin (Gene ID: 7018)
SLC11A2	pCMV6-AC-GFP-rtTA	pTetON-SLC11A2	Tet-ON expression system for iron transport consisting of DMT1 (divalent metal transporter) encoded by *SLC11A2* (Gene ID: 4891), DCYTB (duodenal cytochrome B) encoded by *CYBRD1* (Gene ID: 79901) , and NHE3 (sodium-hydrogen exchanger 3) encoded by *SLC9A3* (Gene ID: 6550)
CYBRD1	pCMV6-AC-GFP-rtTA	pTetON-CYBRD1	
SLC9A3	pCMV6-AC-GFP-rtTA	pTetON-SLC9A3	

Table 2. Cell lines transfected with plasmids in the study

Cell lines		Description
BEL-A	B_cEPOR	BEL-A cell transfected with pTetON-cEPOR
	B_ApoTF	BEL-A cell transfected with pTetON-ApoTF
	B_DMT1-DCYTB-NHE3	BEL-A cell transfected with pTetON-SLC11A2, pTetON-CYBRD1, and pTetON-SLC9A3
ELLU	E_cEPOR	ELLU cell transfected with pTetON-cEPOR
	E_ApoTF	ELLU cell transfected with pTetON-ApoTF
	E_DMT1-DCYTB-NHE3	ELLU cell transfected with pTetON-SLC11A2, pTetON-CYBRD1, pTetON-SLC9A3

2.1.3 Testing for transferrin expression in BEL-A and ELLU cell lines

The cells are harvested and a Bradford assay is conducted to quantify the yield as described [ref]. The lysates are then subjected to a western blot as described[23]. Briefly, 20ug of protein in each lysate is prepared with the loading buffer, and denatured at 95°C for 5 minutes. The same amount of beta-actin protein is loaded as a control. Subsequently, the sample is loaded to a

precast 4-20% gradient polyacrylamide gel and electrophoresed. The gel is then transferred to a nitrocellulose membrane using the Trans-blot Turbo Transfer system for 7 minutes as described[24]. The membrane is then blocked with a blocking buffer (3% BSA in TBS-T) for an hour at room temperature to prevent non-specific binding of antibodies. The membrane is then incubated overnight at 4°C with a polyclonal transferrin antibody (Invitrogen). The blot is then washed 3 times for 5 minutes with TBS-T, incubated with a HRP-conjugated secondary antibody solution for 1 hr at room temperature. The blot is then rinsed by TBS-T and developed by adding a chemiluminescent substrate (ECL). The presence of transferrin expression is then validated by band intensity compared to the control in the X-ray image.

2.1.4 Testing cEPOR expression in BEL-A and ELLU cell lines

The expression of cEPOR is validated using the same protocol described in 2.1.3. The primary antibody used is EpoR monoclonal antibody 38409 (Invitrogen). The band intensity of the cEPOR protein is compared by loading a control protein GAPDH (glyceraldehyde-3-phosphate dehydrogenase) to validate the expression of cEPOR. Cells treated with different concentrations of doxycycline are used to determine the optimal doxycycline concentration for inducing cEPOR expression.

2.1.5 Testing expression of DMT1, DCYTB, and NHE3 in BEL-A and ELLU cell lines

The expression of DMT1, DCYTB, and NHE3 is validated with flow cytometry[25] as described as a rapid and non-invasive approach for the detection of cell surface proteins. The cells are washed twice with PBS to remove non-specific binding, and adjusted to 1 x 10^7 cells/ml with PBS on ice. 100uL of the cell suspension is allotted into three test tubes and incubated with each of anti-DMT1, anti-DCYTB, or anti-NHE3 respectively at 4 °C for a minimum of 30 minutes. The cells are then washed with cold PBS and centrifuged at 300-400 x g for 5 minutes to remove any unbound primary antibody. FITC conjugate is added to the mixture and incubated in the dark at 4 °C for an additional 30 minutes. The cells are then resuspended in 200 µl of PBS, and analyzed using flow cytometry to detect the presence of the relevant protein through the fluorescent signal emitted by the secondary reagent.

2.1.6 Preliminary Characterization of Cells

2.1.6.1 Morphological Examination and Quantification

A sample of cells is used to produce smears to examine their morphology under a light microscope. Cells resembling RBCs are observed (eRBCL cells; engineered RBC-like cells), and subsequent automated cell counting is performed to identify and quantify these cells based on cell size and granularity. Other RBC parameters such as hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are also determined. The expected range of these parameters are shown in **Table 3**.

Table 3. RBC-associated parameters - *Verbrugge and Huisman (2015)[12]

Parameter	Description*	Reference range
Red blood cells count (RBC)	Number of red blood cells per unit of volume	Male: 4.3-5.9 million/mm^3 Female: 3.5-5.5 million/mm^3
Hemoglobin concentration (HGB)	Hemoglobin concentration per unit of volume	Male: 13.5-17.5 g/dL Female: 12.0-16.0 g/dL
Mean corpuscular volume (MCV)	Average volume of red blood cells	80-100 μm^3
Mean corpuscular hemoglobin (MCH)	Amount of hemoglobin in a single red blood cell	25.4-34.6 pg/cell
Mean corpuscular hemoglobin concentration (MCHC)	Concentration of hemoglobin in a single red blood cell	31%-36% Hb/cell

2.1.6.2 Proteomic Analysis

The eRBCL cells are separated into two fractions, and the fraction containing RBC-like cells is lysed hypotonically as described in Crippen *et al.* (2001)[16]. The samples are then subjected to SDS-PAGE. The protein band of interest is excised, crushed, and processed by filter-aided sample preparation (FASP)[17]. They are then separated and analyzed by LC-MS, with spectra compared against a database of known protein sequences for identification.

2.1.6.3 Cell Membrane Shear Stress Test

The deformability of eRBCL cells is measured using a laser diffraction technique. Cell samples are first suspended and exposed to a range of shear stress values, and an osmotic gradient is then applied by adding varying concentrations of a hypertonic solution to the suspension. The laser diffraction patterns generated by suspensions are recorded. The RBC shape is observed to change from circular to elliptical as the shear stress increases. By varying defined values of shear stress and osmotic gradient, measurements by laser diffraction are used to produce a deformation index and plotted over time.

2.1.6.4 Assessment of Cell Oxygenation Dynamics

The oxygenation dynamics of cells are measured using a microfluidic analytical platform (MAP) as described[13]. Cell samples are washed, resuspended and flowed through the channel that isolates single RBCs in each microarray well. Deoxygenation is achieved by pumping N2 gas through the gas channels and oxygen concentration in single cells as measured by oxygen-sensitive probes is recorded at 100 frames/s.

3. Results

3.1 The expression plasmids: pTetON-cEPOR, pTetON-ApoTF, pTetON-SLC11A2, pCMV6-CYBRD1, and pCMV6-SLC9A3

The expression plasmids (**Figure 1**) are transfected into the ELLU and BELL-A cells in different conditions (**Table 2**). Here, we use the Tet-ON system to induce target gene expression in the

presence of doxycycline. The expression plasmids consist of two promoters, pCMV and pTRE3G. The human cytomegalovirus promoter (CMV) promoter is a constitutive promoter commonly used for recombinant protein expression in mammalian cells. Under the expression system, doxycycline or tetracycline binds the reverse tetracycline-controlled transactivator (rtTA) to generate a Dox-rtTA or Tet-rtTA complex, which induce the TRE3G promoter to express the target genes **(Figure 2).**

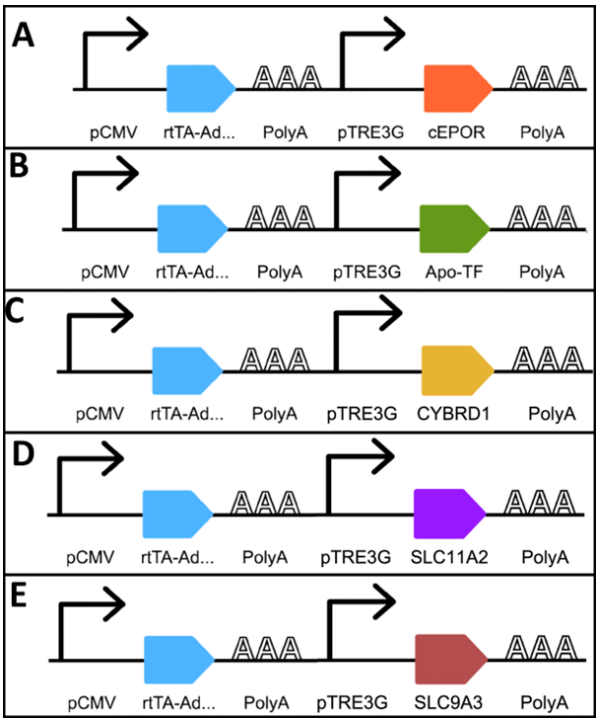

Figure 1. The design of transcription units for the expression of cEPOR, ApoTF, DMT1, DCYTB, and NHE3 proteins. For all expression plasmids, target gene expression is regulated by the Tet-ON system. This system consists of a reverse tetracycline-controlled transactivator (rtTA) gene regulated by human cytomegalovirus promoter (pCMV), and target genes regulated by the TRE3G promoter. Transcription units in (A) pTetON-cEPOR for cEPOR expression, (B) pTetON-ApoTF for transferrin (ApoTF) expressions, (C) pTetON-SLC11A2 for DMT1 expression, (D) pTetON-CYBRD1 for DCYTB expression, and (E) pTetON-SLC9A3 for NHE3 expression.

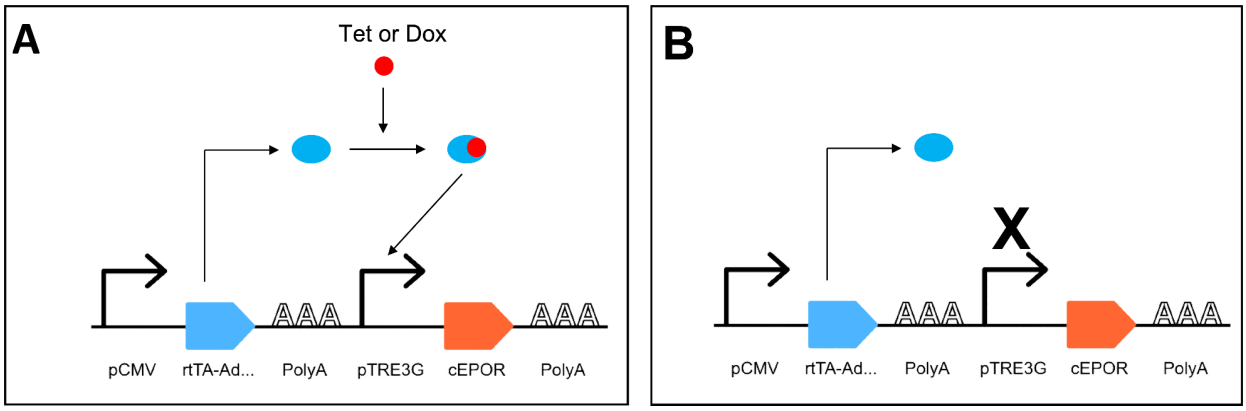

Figure 2. The Tet-ON expression system is used to regulate target gene expression. This system is regulated by the presence of tetracycline (Tet) or doxycycline (Dox). (A) In the presence of these substances, the reverse tetracycline-controlled transactivator (rtTA) binds to Dox or Tet to activate the TRE3G promoter and turn on the expression of cEPOR. (B) In contrast, the system will turn off or inactivate when Tet or Dox is absent from the system. The

other target genes (ApoTF, *SLC11A2, CYBRD1*, and *SLC9A3*) are also regulated by the same mechanism described here.

3.2 Dose-response curves of expressions of target genes

In the Tet-ON expression system, doxycycline is added to the medium to induce target gene expression for 72 hours. The expression of cEPOR, transferrin (ApoTF), DMT1, DCYTB, and NHE3 are measured in the presence of a range of doxycycline concentrations (0 to 1000 ng/mL) and the dose-response curves are shown in Figure 3. Protein expression is predicted to increase as doxycycline concentration increases that resembles a sigmoidal dose-response curve. A doxycycline concentration of 1000 ng/mL is expected to provide a maximal level of protein expression, as shown in a previous study(14).

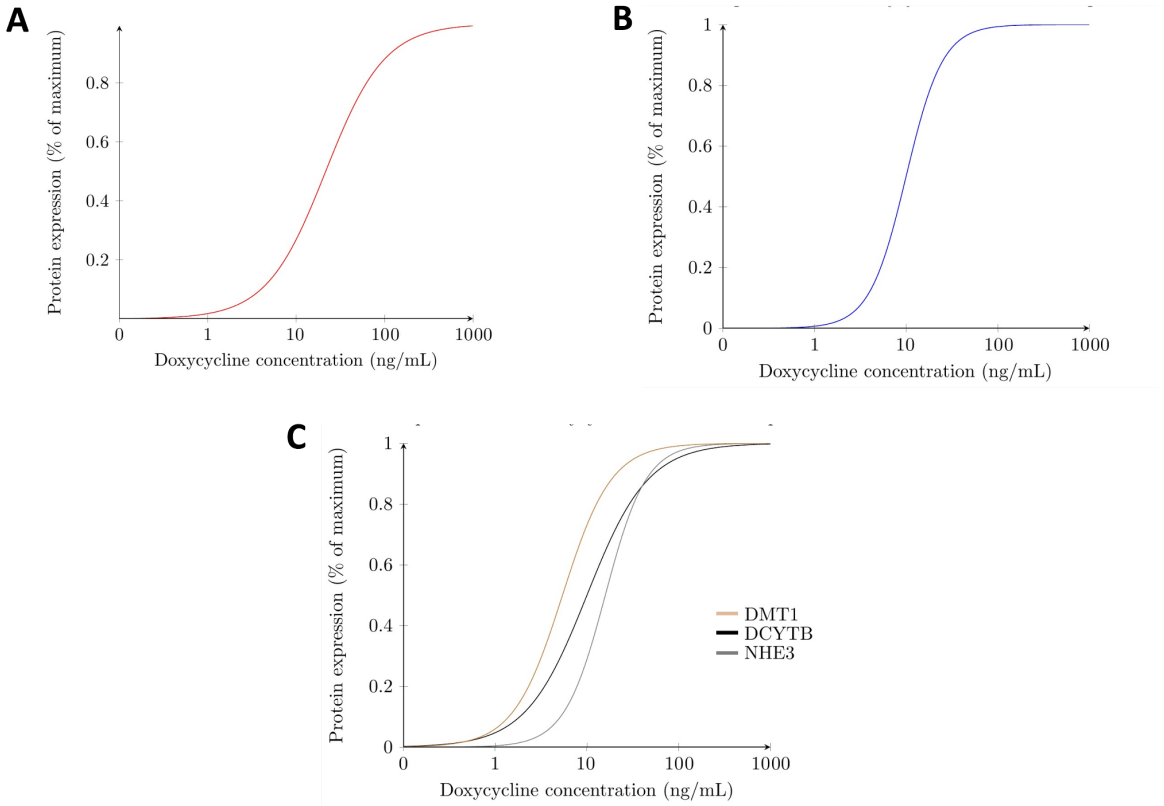

Figure 3. The dose-response curves - doxycycline concentration and target protein expression. The target genes are regulated by the Tet-ON system, which is induced by the presence of doxycycline in the medium. The **(A)** transferrin, **(B)** cEPOR and **(C)** DMT1, DCYTB, and NHE3 expressions are measured for different concentrations of doxycycline; from 0 to 1000 ng/mL. Target gene expression gradually increases as doxycycline concentration increases, with the optimum expression of all target genes expected at 1000 ng/mL[(14)].

3.3 Morphological characterization of RBCs

The eRBCL cells produced by the proposed method and the control method are characterized under a light microscope. The examination of the cells reveals that the eRBCL cells produced in the proposed method show a similar morphology compared to the control (**Figure 4)**, suggesting that there are no obvious alterations in cell morphology.

However, the automated cell count shows that the control consists of a higher proportion of eRBCL cells when compared to that of the proposed method. 80% of the cells in the control are observed to be eRBCL cells. 25% of cells produced in the proposed method are eRBCL cells, suggesting that it may be further optimized to improve the efficiency of RBC production. The 25% enucleation rate seen in our study is comparable to previous studies investigating means of in vitro production of RBCs from stem cells[15–17]. This is a common challenge among studies due to the demands of tight coordination and timely remodeling of cytoskeleton components in the enucleation process[18,19] that may not be met by current approaches.

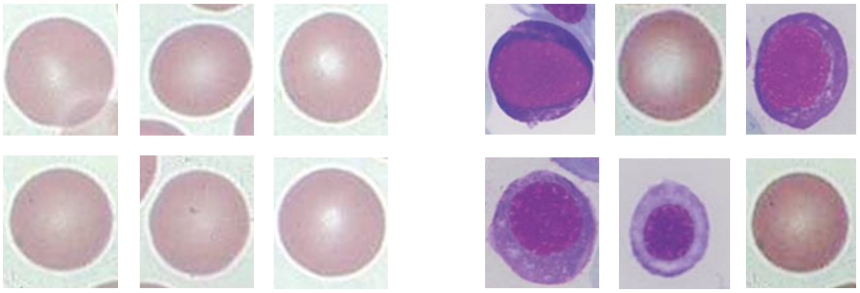

Figure 4. Morphology of the red blood cell product. The blood smears were taken from the conventional HSCs (left panel), and the proposed engineered HSCs (right panel).

3.4 Cell Proteomics

A proteomic analysis is carried out on eRBCL cells to understand potential factors preventing enucleation. It is revealed that the RBCs engineered are indistinguishable from the control. The LC-MS analysis showed that the deviation of proteins in RBCs engineered from the control is negligible, indicating that they have similar phenotypes. There is no aberrant protein expression in the eRBCL cells. While the proteomic analysis may not explain the low enucleation rates, the mechanism involved in the enucleation process is poorly understood with a variety of factors affecting the trajectory of the erythropoietic pathway[20]. Further efforts to examine the proteome and to understand underlying factors that cause a low enucleation rate are required.

3.5 Cell Membrane Resistance to Deformation

The resistance to deformation of eRBCL cells is compared to the control to understand the physical properties of the cells. The results from the shear stress test reveal that the deformability of the RBC cells produced from the proposed method is comparable to the control, indicating no significant differences in their mechanical properties. These results are in accordance with previous studies investigating membrane deformability of RBCs produced by in vitro methods[6,10]. Membrane deformability of RBCs in response to shear stress is an important characteristic for their ability to transit through capillaries to perform their oxygen delivery function[21]. Failure to reach similar tolerance to deformation may produce results similar to hemolytic anemia. The results obtained indicate eRBCL cells have similar mechanical properties to the control.

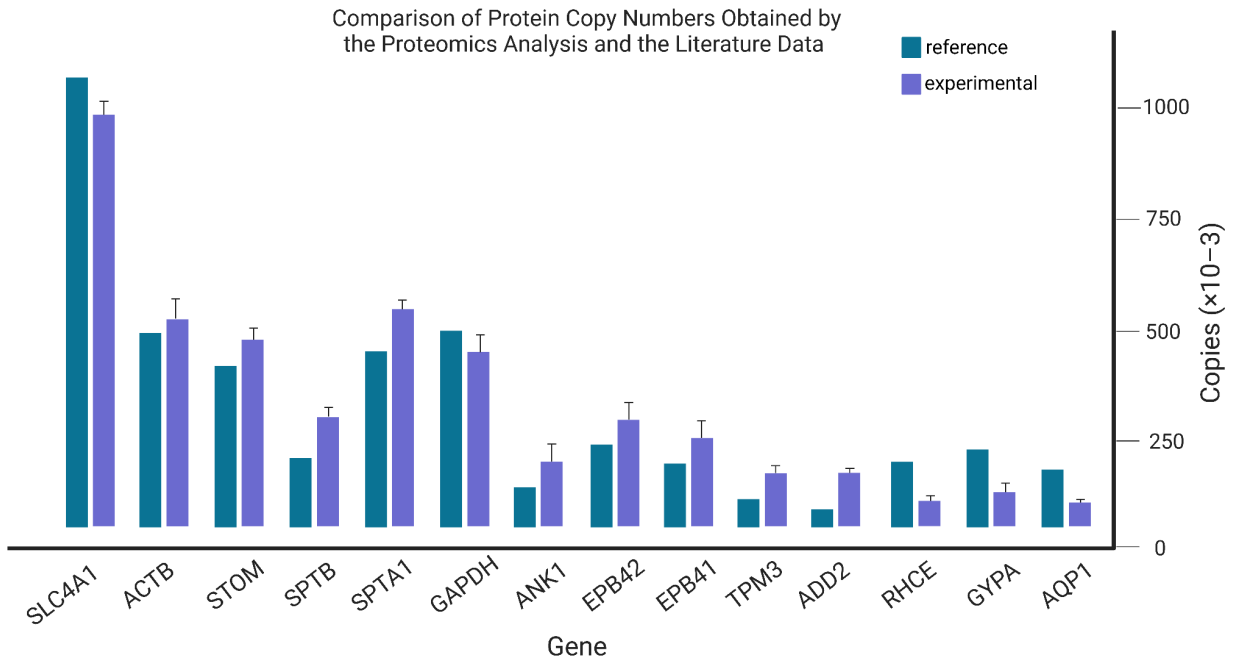

Figure 5. Representative selection of proteins from proteomic analysis. (control) RBCs, teal, and eRBCL cells (experimental), purple. Data in figure adapted from Table 1 https://pubs.acs.org/doi/10.1021/acs.jproteome.7b00025#.

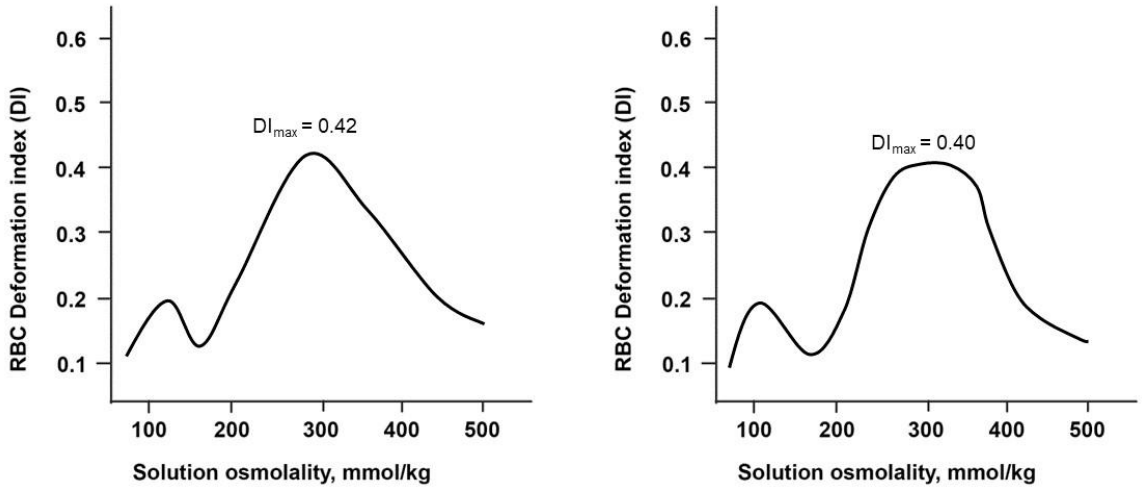

Figure 6. Deformability of RBC against different solution osmolality. Normal RBC control (left panel) and eRBCL cells (right panel). Figure adapted from figure 4 https://www.nature.com/articles/nbt1047.

3.6 Cell Oxygenation Dynamics

By trapping individual cells in a microarray, as part of a microfluidic analytical platform, the oxygenation dynamics of control RBCs and eRBCL cells are compared. There are no significant differences in the oxygenation dynamics between both populations of cells, indicating that the oxygen-carrying capacity of eRBCL cells is normal. Oxygen release is of particular concern because previous studies have shown older RBCs have higher oxygen release rates.[6].

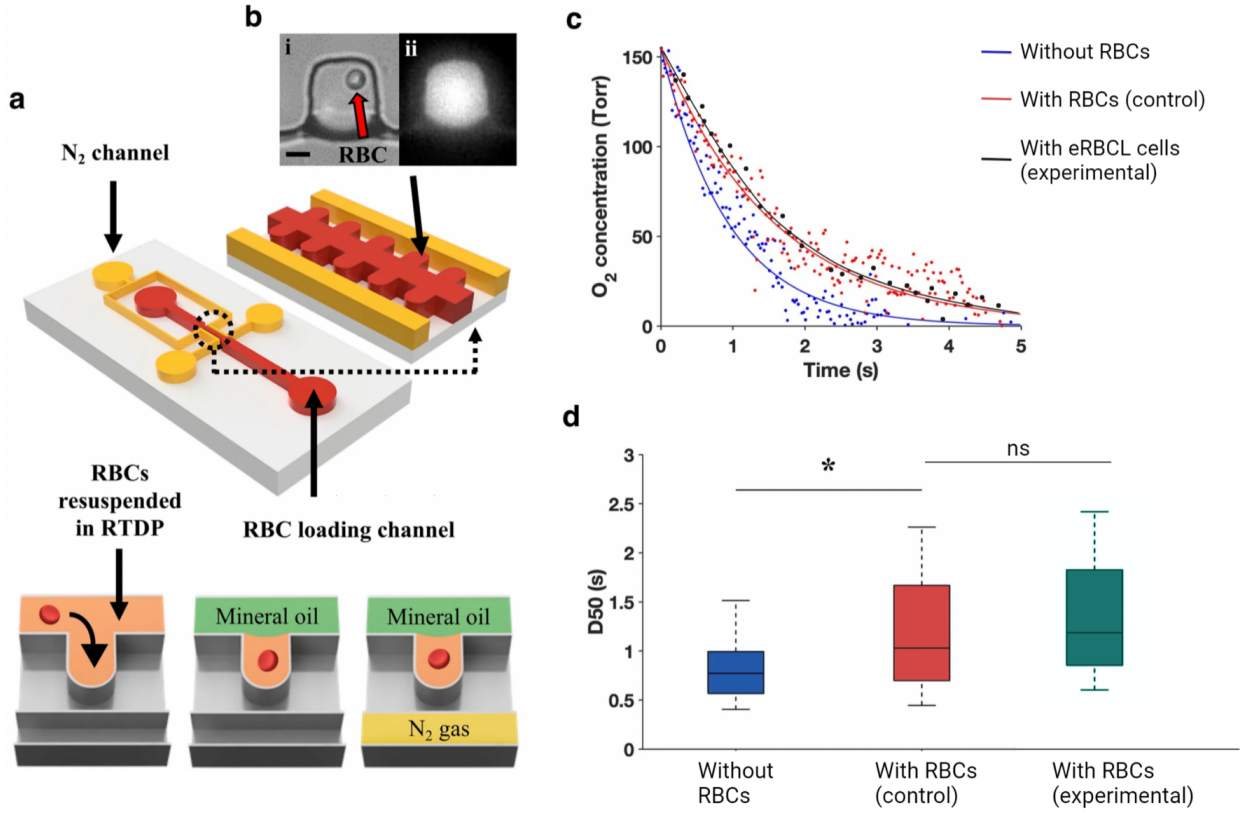

Figure 7. a) A figure depicting the microfluidic analytic platform (MAP). Red represents the RBC loading channel, and yellow represents N_2 flow. b) fluorescence microscopy of an RBC cell; i) trans-illumination, ii) epi-illumination. c) fluorescence intensity (over time) of RBCs (red) and eRBCL cells (black) compared to controls (blue). d) Comparison of oxygen partial pressure required to achieve 50% hemoglobin saturation. Adapted from fig 1 https://www.nature.com/articles/s42003-021-01793-z.

4. Conclusion

In conclusion, three novel approaches to genetically engineer two immortalized human erythroid cell lines are carried out to lower the cost of in vitro production of RBCs. Three constructs expressing a constitutively active EPO receptor (cEPOR), transferrin, and divalent metal transporter (DMT1) are expressed in two cell lines successfully. Preliminary characterization of the RBCs produced in the proposed method has shown no differences in morphology, protein levels, deformability, and oxygenation dynamics as compared to the controls. This study demonstrates a feasible approach to generate cost-effective RBCs in vitro with the potential to be further optimized and scaled.

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Supplementary Information

1. Project schedule plan

All tasks of the project proposals were planned in different sections. First stage consists of idea collection, literature review, and project selection that takes around 5 months. Then, the materials preparation and research conduction occur in the second stage. It might take 5-6 months. Finally, the cell testing and analysis will take place at the last stage. It uses about 3 months for cell evaluation and assessment which can parallel with research works. However, this plan can be changed or adapted during the real experiments and other unexpected results.

Task	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Idea collection												
Literature review												
Project selection												
Material preparation												
Research												
1. Cell passaging												
2. Plasmid construction												
3. Cell transfection and culturing												
Testing												
1. Gene expression												
2. Cell characterisation												

Table 1. Comparison of Protein Copy Numbers Obtained by the Proteomics Analysis and the Literature Data[26].

Protein	Gene	Copies (×10–3) literature	copies ± st. dev (×10–3) proteomics
AE1	SLC4A1	1200	1074 ± 114
b-Actin	ACTB	500	534± 65
Stomatin	STOM	430	467± 79
b-Spectrin	SPTB	242	369± 69
a-Spectrin	SPTA1	431	590 ± 67
Glyceraldehyde-P DH	GAPDH	500	475 ± 120
Ankyrin	ANK1	124	183 ± 32
Protein 4.2	EPB42	250	299± 20
Protein 4.1	EPB41	200	278± 35
Tropomyosin	TPM3	70	118± 66
b-Adducin	ADD2	30	101 ± 14
Rh polypeptide	RHCE, RHD	200 (D and CE)	63 ± 17
Glycophorin A	GYPA	251	103 ± 67 (52)
Aquaporin 1	AQP1	177	68 ± 10
Kell glycoprotein	KEL	3–18	50 ± 5.4
CD47	CD47	10–50	47 ± 5.4
RhAG	RHAG	100–200	53 ± 25
Nucleoside transporter	SLC29A1	10	26 ± 4.2
LW glycoprotein	ICAM4	3–5	21 ± 10
Urea transporter	SLC14A1	14	26 ± 8.2
AChE	ACHE	10	16 ± 2.9
Lutheran glycoprotein	BCAM	1.5–4	16 ± 5.7
CD99	CD99	1	10 ± 2.6

CD58 (LFA-3)	CD58	3–7	10 ± 0.9
KX	XK	1.5–4	8.1 ± 3.2
CD44	CD44	6–10	6.6 ± 2.2
DAF (CD55)	CD55	20	4.8 ± 2.6
Duffy	FY	6–13	5.0 ± 2.5
CR1	CR1	0.02–1.5	0.6 ± 0.3

Adapted from Bryk and Wisńiewski (2017)[26]