Erythrocytes and Erythropoietin

Guest Editors: Michael Föller, Lars Kaestner, Elisabetta Straface, and Johannes Vogel
Erythrocytes and Erythropoietin
Erythrocytes and Erythropoietin

Guest Editors: Michael Föller, Lars Kaestner, Elisabetta Straface, and Johannes Vogel
Editorial Board

Paul N. Adler, USA
Emad Alnemri, USA
Eyal Bengal, Israel
Avri Ben-Ze’ev, Israel
J. Chloé Bulinski, USA
Michael Bustin, USA
John Cooper, USA
Adrienne D. Cox, USA
J. R. Davie, Canada
Govindan Dayanithi, France
Arun M. Dharmarajan, Australia
Dara Dunican, Ireland
William Dunn, USA
Roland Foisner, Austria
Hans Herman Gerdes, Norway
Richard Gomer, USA
Hinrich Gronemeyer, France
Mehran Haidari, USA
Thomas Hays, USA
W. J. Hendriks, The Netherlands
Paul J. Higgins, USA
Michael Hortsch, USA
Pavel Hozak, Czech Republic
Jeremy Hyams, France
Anton M. Jetten, USA
Edward M. Johnson, USA
Daniel P. Kiehart, USA
Sharad Kumar, Australia
P. Marks, USA
Seamus J. Martin, Ireland
Manuela Martins-Green, USA
Helga M. Ögmundsdóttir, Iceland
Shoichiro Ono, USA
Howard Beverley Osborne, France
Markus Paulmichl, Austria
H. Benjamin Peng, Hong Kong
Craig Pikaard, USA
Liza Pon, USA
Jerome Rattner, Canada
Afshin Samali, Ireland
Michael Peter Sarras, USA
R. Seger, Israel
Barry D. Shur, USA
Arnoud Sonnenberg, The Netherlands
G. S. Stein, USA
Tung Tien Sun, USA
Ming Tan, USA
Guido Tarone, Italy
Richard Tucker, USA
Andre Van Wijnen, USA
Gerhard Wiche, Austria
Steve Winder, UK
Timothy J. Yen, USA
Contents

**Erythrocytes and Erythropoietin**, Michael Föller, Lars Kaestner, Elisabetta Straface, and Johannes Vogel
Volume 2011, Article ID 972536, 2 pages

**The Red Blood Cell as a Gender-Associated Biomarker in Metabolic Syndrome: A Pilot Study**, Elisabetta Straface, Lucrezia Gambardella, Antonella Mattatelli, Emanuele Canali, Francesca Boccalini, Luciano Agati, and Walter Malorni
Volume 2011, Article ID 204157, 7 pages

**Insights into the Function of the Unstructured N-Terminal Domain of Proteins 4.1R and 4.1G in Erythropoiesis**, Wataru Nunomura, Philippe Gascard, and Yuichi Takakuwa
Volume 2011, Article ID 943272, 13 pages

**Band 3 Missense Mutations and Stomatocytosis: Insight into the Molecular Mechanism Responsible for Monovalent Cation Leak**, Damien Barneaud-Rocca, Bernard Pellissier, Franck Borgese, and Hélène Guizouarn
Volume 2011, Article ID 136802, 8 pages

**Designing a Long Acting Erythropoietin by Fusing Three Carboxyl-Terminal Peptides of Human Chorionic Gonadotropin β Subunit to the N-Terminal and C-Terminal Coding Sequence**, Fuad Fares, Avri Havron, and Eyal Fima
Volume 2011, Article ID 275063, 7 pages

**Cellular Reprogramming toward the Erythroid Lineage**, Laura J. Norton, Alister P. W. Funnell, Richard C. M. Pearson, and Merlin Crossley
Volume 2011, Article ID 501464, 6 pages
Editorial

Erythrocytes and Erythropoietin

Michael Föller,1 Lars Kaestner,2 Elisabetta Straface,3 and Johannes Vogel4

1 Department of Physiology, University of Tübingen, Gmelinstraße 5, 72076 Tübingen, Germany
2 Institute for Molecular Cell Biology, School of Medicine, Saarland University, 66421 Homburg/Saar, Germany
3 Section of Cell Degeneration and Gender Medicine, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
4 Institute of Veterinary Physiology, Vetsuisse Faculty and Zürich Center for Integrative Human Physiology, University of Zürich, 8057 Zürich, Switzerland

Correspondence should be addressed to Michael Föller, michael.foeller@uni-tuebingen.de

Received 23 October 2011; Accepted 23 October 2011

Copyright © 2011 Michael Föller et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This special issue focuses on erythrocytes and erythropoietin including original research and review articles on the cellular physiology of erythrocytes and of erythrocyte-associated disorders. Moreover, the special issue includes papers on the action of erythropoietin and hypoxia-inducible factors on a cellular level. In the following, we present the original papers published in this special edition.

D. Barneaud-Rocca et al. deal with one of the most abundant proteins in red blood cells, the band 3 protein (Anion Exchanger 1). The paper reviews recent work hypothesizing the contribution of band 3 point mutations to a sodium and potassium leak. It is compared to alternative explanations suggesting that point mutations in band 3 regulate the cation leak through other transporters. The topic is driven by the fact that mutations in the band 3 protein have been associated with hereditary stomatocytosis. The molecular mechanisms discussed in the paper link the stomatocytosis and the sodium potassium leak of the mutated band 3 protein.

W. Nunami et al. review the function of unstructured N-terminal domain of protein 4.1R and 4.1 G and characterize the binding profiles of proteins 4.1R80, 4.1R35 and protein 4.1G in erythropoiesis. The regulation of the binding profiles of these proteins by the presence or absence of the N-terminal 209 amino acid sequence (headpiece region (HP)) and unstructured domain of the protein as well as of 4.1R35 (which contains the HP) by both Ca2+ and Ca2+/CaM is discussed. Knowing the different regulation and expression of the 4.1 protein isoforms will foster our understanding of erythropoiesis.

F. Fares et al. fused one Carboxyl-Terminal Peptide (CTP) of the human chorionic gonadotropin beta subunit to the N-terminal end and two CTPs to the C-terminal end of erythropoietin (Epo). This artificial erythropoiesis-stimulating agent had increased in vivo activity as well as half-life compared with recombinant human Epo and the hyperglycosylated Epo analogue darbepoetin alfa (Aranesp). As erythropoiesis-stimulating agents often need to be injected repeatedly over long time periods, for example, for anemia treatment in kidney diseases or cancer, more effective and longer lasting Epo derivates improve the patients’ quality of life.

E. Straface et al. present the results of a pilot study investigating new peripheral sex-associated markers in patients with metabolic syndrome and subclinical atherosclerosis. The metabolic syndrome, which is characteristic of hypertension, obesity, insulin resistance, hypertriglyceridemia, and hypercholesterolemia, is a major risk factor for cardiovascular mortality in the developed world. In their study, E. Straface et al. analyzed glycoporphin A, CD47, and phosphatidylserine exposition at the cell surface as hallmarks of erythrocyte damage. They report significant gender differences of those parameters in patients with metabolic syndrome.

L. J. Norton et al. review the recent advance in cellular reprogramming with the particular emphasis on its potentially beneficial use for the future treatment of hemoglobinopathies. Typical and frequent hemoglobinopathies are genetic disorders such as thalassaemia and sickle cell disease. To date, blood transfusion is the principal therapy of those
diseases. In their review article, L. J. Norton et al. outline the recent development in stem cell research such as classic cellular reprogramming and transdifferentiation and discuss their potential application for the treatment of anemia following hemoglobinopathies.

We hope that this special issue will alert researchers to some recent development in the field of erythrocytes and erythropoietin, particularly the correlation between protein alterations and clinical symptoms, and that a better understanding of this correlation can direct our efforts to the discovery of new therapeutic strategies for the treatment of anemia and metabolic disorders.

Michael Föller
Lars Kaestner
Elisabetta Straface
Johannes Vogel
Research Article
The Red Blood Cell as a Gender-Associated Biomarker in Metabolic Syndrome: A Pilot Study

Elisabetta Straface,1 Lucrezia Gambardella,1 Antonella Mattatelli,2 Emanuele Canali,2 Francesca Boccalini,2 Luciano Agati,2 and Walter Malorni1,3

1 Section of Cell Degeneration and Gender Medicine, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy
2 Department of Cardiology, “Sapienza” University, 00161, Rome, Italy
3 San Raffaele Institute Sulmona, 67039, L’Aquila, Italy

Correspondence should be addressed to Elisabetta Straface, elisabetta.straface@iss.it

Received 30 March 2011; Revised 28 June 2011; Accepted 15 July 2011

Academic Editor: Michael Föller

Copyright © 2011 Elisabetta Straface et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In the present pilot study (56 patients), some red blood cell parameters in samples from patients with metabolic syndrome and subclinical atherosclerosis, but without any sign of coronary artery disease, have been analyzed. The main goal of this work was to determine, in this preclinical state, new peripheral gender-associated bioindicators of possible diagnostic or prognostic value. In particular, three different “indicators” of red blood cell injury and aging have been evaluated: glycophorin A, CD47, and phosphatidylserine externalization. Interestingly, all these determinants appeared significantly modified and displayed gender differences. These findings could provide novel and useful hints in the research for gender-based real-time bioindicators in the progression of metabolic syndrome towards coronary artery disease. Further, more extensive studies are, however, necessary in order to validate these findings.

1. Introduction
Metabolic syndrome (MetS) is a cluster of risk factors for atherosclerosis, including insulin resistance, hypertension, glucose intolerance, hypertriglyceridemia, and low high-density lipoprotein-cholesterol (HDL-C) levels [1]. Affected patients have a significantly increased risk of developing atherosclerotic disease, diabetes, and cardiovascular disease (CVD). This is probably due to a blood hypercoagulability as well as to endothelial cell activation. It has been hypothesized that the hypercoagulability state could predispose patients to venous thromboembolism [2].

Several epidemiological studies, the Framingham, in particular [3], have investigated into the evolution of cardiovascular disease hypothesizing the presence of a gender difference in the pathogenetic and progression determinants detectable in men and women. For instance, women were found to outlive men and to experience fewer atherosclerotic cardiovascular events, with an incidence lagging behind that in men by 10 to 20 years [4]. This gap in incidence closes with advancing age, when CVD becomes the leading cause of death in women as well as in men [5, 6]. In consideration of the high incidence of morbidity and mortality, due to CVD, and of the paucity of well-established gender-associated markers, further studies focused at identifying novel bioindicators should be considered as mandatory.

On these bases, a pilot study has been conducted in a low number of patients with MetS of both sexes and subclinical atherosclerosis with the aim to identify innovative peripheral blood biomarker in this preclinical phase [7]. We focused our attention on the red blood cell (RBC) as a candidate possibly implicated in these pathologic conditions. RBCs are peculiar cells aimed at the delivery of oxygen and nitric oxide to the periphery and carbon dioxide to the lungs. In addition, they also exert, under physiological conditions, a scavenging activity towards reactive oxygen and nitrogen species often overproduced in morbidity states, for example, in inflamed tissues. Their deformability, essential for their circulation in
small blood vessels, is an important prerequisite for such vascular “antioxidant” functions. Conversely, when the redox state of RBCs is altered, erythrocytes can turn out to be a source of reactive species, and, consequently, its typical structural and functional features are lost [8, 9]. Importantly, the oxidatively modified erythrocyte increases its aggregability and adhesiveness to the endothelium and to other blood cells, thus contributing to vascular damage. In addition, CVD risk factors, namely, insulin resistance, obesity, and hypertension, all share a common abnormal ion profile in RBCs. This might help to explain their frequent clinical coexistence. Specifically, it has been hypothesized that RBC intracellular pH levels are lower and inversely linked to both body mass index (BMI) and fasting insulin concentrations either in normotensive or hypertensive individuals. Moreover, ionic imbalance, for example, of intracellular potassium, magnesium, and calcium, can decrease intracellular pH levels also resulting in a reduced GSH/GSSG ratio [10]. In this work, three different putative “indicators” of RBC injury and aging have been evaluated: glycophorin A (GA), CD47, and phosphatidylserine (PS). The first is a glycoprotein that is widely expressed at the surface of RBC and is downregulated during senescence [11]; the second, CD47, as for other cells, is an integrin-associated protein that acts as a “marker of self” [12]; the third is a phospholipid localized to the inner leaflet of the plasma membrane, which is externalized to the outer leaflet during cell remodeling leading to cell death, for example, by eryptosis [13, 14]. Notably, it has been reported that phosphatidylserine-exposing RBCs may adhere to the vascular walls [14] and may interfere with microcirculation as it has been proposed to occur in the metabolic syndrome [9]. Importantly, GA loss, PS externalization (evaluated in terms of positivity to its ligand: annexin V), and reduced expression of CD47, respectively, have been reported as critical events responsible for the removal of RBCs at the end of their lifespan [15–17].

2. Patients and Methods

2.1. Study Population. The study population consisted of 56 ambulatory subjects with MetS (31 men and 25 women, aging 50–70 years) and 40 age-matched healthy donors (HDs) (22 men and 18 women). All patients and HDs were Caucasian. All study subjects underwent a complete cardiovascular evaluation which has included: history and physical examination, heart rate, blood pressure, fasting serum glucose; fasting plasma lipids, Fibrinogen, CRP, comprehensive two-dimensional echocardiogram, carotid echocolor Doppler and exercise ECG testing. Healthy donors were identified on the basis of the absence of CVD risk factors and a completely normal CVD screening.

MetS was diagnosed according to the amended National Cholesterol Education Program’s Adult Treatment Panel III (ATP-III) Guidelines in individuals meeting three or more of the criteria reported elsewhere [1]. Healthy donors were identified on the basis of the absence of CVD risk factors and a completely normal CVD screening. We included in the study (i) patients with an increased (N1 mm) carotid intima-media thickness (IMT), but in the absence of known or suspected coronary artery disease (CAD), and (ii) only women in postmenopausal disease and without hormone replacement therapy. The patient characteristics have been reported in Table 1.

Patients with previous myocardial infarction, previous coronary artery by-pass graft, coronary angioplasty or positive exercise ECG testing, depression, inflammatory diseases, and ACEI treatment were excluded from the study. The nature and the purpose of the study were explained to all participants who gave their informed consent following the rules of good medical practice. This study was approved by the Institutional Review Board of “Sapienza” University of Rome (Italy). The investigation was conformed to the principles outlined in the Declaration of Helsinki.

2.2. Isolation of Erythrocytes. Human erythrocyte suspensions were prepared from fresh venous blood collected as previously reported [11].

2.3. Analysis of the Redox Balance in RBCs. For intracellular ROS production, RBCs (5 × 10^5 cells) were incubated in Hank’s balanced salt solution (HBSS, pH 7.4) containing dihydrorhodamine 123 (DHR 123, Molecular Probes, USA). Intracellular content of reduced thiols was explored by using 5-chloromethylfluoresceindiacetate (CMFDA, Molecular Probes). Samples were then analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif, USA). The median values of fluorescence intensity histograms were used to provide semiquantitative evaluation of reduced thiols content and reactive oxygen species (ROS) production.

2.4. Evaluation of RBC Injury. Quantitative evaluation of RBCs with phosphatidylserine externalization [11] was performed by flow cytometry after double staining using FITC-conjugated annexin V and 0.05% Trypan blue for 10 min at room temperature and analyzed by fluorescence-activated cell sorting (FACS) in the FL3 channel to determine the percentage of dead cells.

2.5. Quantitative and Qualitative RBC Protein Analyses. For glycophorin A detection, RBCs were stained with anti-glycophorin A (Saint Louis, Mo, USA) monoclonal antibodies and subsequently incubated with anti-mouse IgG-fluorescein-linked whole antibodies (Sigma). For CD47, RBCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Sigma Chemical Co, Mo, USA), stained with monoclonal anti-CD47 (Santa Cruz Biotechnology, CA, USA), and subsequently incubated with antimouse IgG-fluorescein-linked whole antibody (Sigma). Finally, all the samples were analyzed with a FACScan flow cytometer or observed with a Nikon Microphot fluorescence microscope.

2.6. Morphometric Analyses. Whole blood from MetS patients and healthy donors was stripped on the slide, dried at room temperature, and observed by light or differential interference contrast (DIC) microscopy. Altered erythrocyte shape was evaluated by counting at least 500 cells (50 RBCs
Table 1: Patients’ characteristics. Significant differences are in bold. Data are the mean ± SD of 56 MetS patients (31 male and 25 female) and 40 HD (22 male and 18 female).

| Variables                        | 56 MetS          | 40 HD           | P-values |
|----------------------------------|------------------|-----------------|----------|
| **Risk factors**                 |                  |                 |          |
| Body mass index (Kg/m²)          | 31.98 ± 4.84     | 21.05 ± 2.01    | 0.0001   |
| Waist circumference (cm)         | 112.7 ± 14.98    | 71.79 ± 6.22    | 0.0001   |
| Systolic blood pressure (mmHg)   | 134.57 ± 17.7    | 120.63 ± 8.2    | 0.03     |
| Diastolic blood pressure (mmHg)  | 84.13 ± 9.37     | 74.95 ± 5.68    | 0.001    |
| Glucose (mg/dl)                  | 122.71 ± 34.87   | 63.84 ± 9.38    | 0.001    |
| Total cholesterol (mg/dl)        | 197.62 ± 38.83   | 178.39 ± 20.23  | 0.15     |
| LDL-cholesterol (mg/dl)          | 125.4 ± 36.08    | 114.27 ± 28.51  | 0.31     |
| HDL-cholesterol (mg/dl)          | 44.11 ± 8.47     | 47.09 ± 8.8     | 0.78     |
| Triglyceride (mg/dl)             | 144.8 ± 72.28    | 118.94 ± 48.96  | 0.26     |
| Family history of CAD            | 12 (50%)         | 4 (21%)         | 0.60     |
| Family history of diabetes       | 13 (54%)         | 5 (26%)         | 0.80     |
| Currently smokers                | 5 (21%)          | 12 (63%)        | 0.67     |

**Echocardiography parameters**

| LV EF (%)                       | 52.46 ± 6.16     | 59.68 ± 2.82    | 0.001    |
| SIV (mm)                        | 11.58 ± 0.92     | 8.74 ± 1.28     | 0.0001   |
| PP (mm)                         | 11.13 ± 0.90     | 9.21 ± 1.22     | 0.001    |
| LVEDV (mL)                      | 126.78 ± 31.19   | 115.00 ± 18.93  | 0.05     |
| LVESV (mL)                      | 58.57 ± 17.20    | 40.05 ± 6.32    | 0.002    |
| LVM-I (g)                       | 114.25 ± 16.84   | 77.36 ± 31.24   | 0.0001   |

**Carotid echo-color Doppler parameters**

| CCA Sx (mm)                     | 1.25 ± 0.36      | 0.73 ± 0.22     | 0.0001   |
| ICA Sx (mm)                     | 1.68 ± 0.73      | 0.67 ± 0.26     | 0.001    |
| CCA Dx (mm)                     | 1.30 ± 0.39      | 0.56 ± 0.28     | 0.001    |
| ICA Dx (mm)                     | 1.96 ± 0.79      | 0.66 ± 0.27     | 0.0001   |

for each field at a magnification of 1500x) from MetS patients and healthy donors.

2.7. Statistical Analyses. Cytofluorimetric results were statistically analyzed by using the nonparametric Kolmogorov-Smirnov test using Cell Quest Software. At least 20,000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semiquantitative analysis. Statistical analyses of collected data were performed by using Student’s t-test.

3. Results

3.1. Redox Balance. Considering that changes in the redox state can contribute to the loss of RBC structure and function [8], two important parameters have been analyzed. We measured the reactive oxygen species (ROS) production and the total thiol content (essentially referred to as reduced glutathione). However, no significant differences were detected in the ROS and total thiol production in RBCs from patients with MetS in comparison with that from healthy donors (Figures 1(a) and 1(b)). Furthermore, no gender differences were observed.

3.2. Morphological Analyses. Changes of RBC viscosity, adhesiveness, and aggregability have been detected in many human pathologic conditions. In particular, changes of erythrocyte adhesiveness/aggregation and morphology have been proposed as useful markers to detect inflammatory conditions, plaque instability, and atheroma progression in patients with coronary artery disease [18–20]. In order to determine whether RBCs could be considered as biomarkers of diagnostic or prognostic value in metabolic syndrome, their morphology and adhesiveness/aggregation properties were studied in cells from both healthy donors and in patients with MetS. These analyses were carried out by means of light microscopy (two representative micrographs are shown in Figure 2(a)) and DIC microscopy evaluations (not shown). A significant increase of RBCs displaying morphological alterations has been detected in samples obtained from patients with MetS with respect to that from healthy donors (Figure 2(b); note that the percentage of altered RBCs, about 20%, was within the normal values) [21]. In particular, RBCs with numerous surface protrusions (acanthocytes) have been detected (arrows). Moreover, when patient gender was taken into consideration, a difference in terms of morphological alterations was detected. In particular, the percentage of altered RBCs was higher in men...
than in women with MetS. As expected, no gender difference was detected in RBCs from healthy donors (black histograms in Figure 2(b)).

3.3. RBC Senescence and Death. The analysis of GA and CD47 was carried out by both flow cytometry (Figures 3(a) and 4(a)) and immunofluorescence microscopy (Figures 3(b) and 4(b)). These analyses clearly demonstrated that the expression of GA and CD47 was substantially similar in RBCs from patients with MetS and in those from healthy donors (Figures 3(a) and 4(a)). However, when cells from males and females were analyzed separately, the expression of these molecules was found significantly \( P < 0.05 \) different: lower in RBCs from men with MetS in comparison with RBCs from women with MetS. Representative immunofluorescence micrographs displaying GA and CD47 positivity in RBCs from healthy donors and men with MetS are shown in Figures 3(b) and 4(b). Moreover, as concerns PS externalization, a significantly higher translocation of PS to the outer plasma membrane leaflet was detected in RBCs from patients with MetS in comparison with those from HD (Figure 5). Furthermore, a gender difference was also appreciable. In fact, in RBCs from male patients with MetS, the surface positivity for PS was twice that of controls, whereas no difference, in term of PS externalization, has been detected between RBCs from HD females and RBCs from females with MetS.

4. Discussion

In the present pilot study, we focused our attention on the red blood cell as candidate biomarker of metabolic syndrome. We also investigated about possible gender differences. We analyzed in detail three different “indicators” of RBC injury and aging: glycoporphin A, CD47, and phosphatidylserine. To this aim, diverse blood determinants were evaluated in a low
number (n = 56) of male and female patients that did not display any sign of CAD. To be included in the study, these patients should have at least 3 major criteria for MetS and a pathologically abnormal carotid IMT. Carotid IMT was in fact linked to many cardiovascular outcomes, including cerebral and coronary events, and it has been proposed as an index of subclinical atherosclerosis [22]. From a clinical point of view, despite a similar incidence of risk factors and IMT, men with MetS showed a significantly higher LV function and structure involvement in the absence of patent CAD symptoms.

It is a matter of fact that circulating erythrocytes could contribute to the pathogenesis of cardiovascular diseases [2, 8, 23]. The shape maintenance as well as mechanical deformability and elasticity of RBCs (7 μm) is essential prerequisites for their circulation, specifically in small blood vessels (about 5 μm). If the RBC is altered, its aggregability and adhesive properties change, thus contributing to vascular damage [23]. In our study, we detected differences, in terms of cell aging, cell adhesion, and/or aggregation, in RBCs from MetS patients with respect to those of healthy donors. In particular, we observed that the ability to pile was modified in erythrocytes either from women or from men with MetS. Moreover, they appeared to increase their adhesiveness to a substrate. This was probably associated with the increased PS externalization, a well-known marker of RBC aging and death, that has been associated with increased cell adhesion properties. In fact, studies have shown that RBC injury due to energy or antioxidant depletion causes breakdown of membrane phosphatidylserine asymmetry, with consequent exposure of phosphatidylserine at the erythrocyte surface (eryptosis) and binding to the phosphatidylserine receptors at macrophages and liver Kupffer cells, which then engulf and degrade the affected RBCs [14]. Locally, these phagocytic cells produce superoxide anion, which activates NF-κB and c-JN, inflammatory signalling pathways that regulate cellular transcriptional events, thereby leading to greater production of TNF-α, IL-6, and other proinflammatory mediators [9].

**Figure 3:** Glycophorin A analysis. (a) Histogram representing flow cytometry evaluations of glycophorin A. The numbers represent the mean ± SD of 40 HD (22 male and 18 female) and 56 MetS patients (31 male and 25 female). No changes, in terms of GA expression, were detectable in RBCs from MetS patients with respect to those from healthy donors. Conversely, a significant (°P < 0.05) decrease of this protein was evident in RBCs from men with MetS versus healthy male donors. (b) Representative immunofluorescence micrographs displaying different arrangement and positivity for GA of RBCs from a healthy man and a man with MetS.

**Figure 4:** CD47 analysis. (a) Histogram representing flow cytometry evaluations of CD47. The numbers represent the mean ± SD of 40 HD (22 male and 18 female) and 56 MetS patients (31 male and 25 female). No changes, in terms of CD47 expression, were detectable in RBCs from MetS patients with respect to those from healthy donors. Conversely, a significant (°P < 0.05) decrease of this protein was evident in RBCs from men with MetS versus healthy male donors. (b) Representative immunofluorescence micrographs displaying different arrangement and positivity for CD47 of RBCs from a healthy man and a man with MetS.
This could be of relevance in the light of recent works that describe the disparity of vascular cells from males and from females in terms of their “basal” redox state and their susceptibility to oxidative stress [24, 25] contributing to the pathogenesis of vascular diseases [26].

Interestingly, as concerns gender, we also found significant differences (morphological alterations, aging-associated molecules GA, and adhesion-associated molecules CD47 and PS). These results are in accord with several literature data [23] that suggest RBC as real-time biomarkers of disease progression and pathogenetic determinants in cardiovascular diseases. RBCs can in fact contribute to atherosclerotic plaque formation [19] and can behave as prooxidants, thus contributing to the pathogenetic mechanisms of vascular diseases [8]. For example, it has been demonstrated that oxidized erythrocytes can represent potential sources of systemic inflammation: the increase of exogenous or endogenous CO2 deoxygenates haemoglobin favouring the formation of methemoglobin [9].

Altogether the results of this pilot study are in line with the literature data indicating erythrocytes as possible biomarkers of vascular diseases [23]. We also hypothesize that gender could represent a key variable in this issue [3]. Further studies appear, however, as mandatory in order to assess if the gender-specific biomarkers analyzed here could be detected in a larger study population, thus providing useful insights for a gender-based management of MetS progression.

Acknowledgments

The authors thank Noemi Gabrielli, Romano Arcieri, and Alessio Metere for their precious help. This work has been supported by Grants from “Sapienza” University to L. Agati and from Ministero della Sanità to W. Malorni.

References

[1] S. M. Grundy, J. I. Cleeman, S. R. Daniels et al., “Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung, and Blood Institute scientific statement: expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. Executive summary,” Circulation, vol. 112, no. 17, pp. e285–e290, 2005.
[2] C. Ay, T. Tengler, R. Vormittag et al., “Venous thromboembolism—a manifestation of the metabolic syndrome,” Haematologica, vol. 92, no. 3, pp. 374–380, 2007.
[3] E. Ingelsson, M. J. Pencina, G. H. Tolfer et al., “Multimarker approach to evaluate the incidence of the metabolic syndrome and longitudinal changes in metabolic risk factors. The Framingham Offspring Study,” Circulation, vol. 116, no. 9, pp. 984–992, 2007.
[4] P. Mølstad, “Coronary heart disease in women: less extensive disease and improved long-term survival compared to men,” Scandinavian Cardiovascular Journal, vol. 43, no. 1, pp. 10–16, 2009.
[5] W. B. Kannel, “The Framingham Study: historical insight and longitudinal changes in metabolic risk factors. The Framingham Offspring Study,” Circulation, vol. 43, no. 1, pp. 27–37, 2002.
[6] M. J. Legato, A. Gelzer, R. Goland et al., “Gender-specific care of the patient with diabetes: review and recommendations,” Gender Medicine, vol. 3, no. 2, pp. 131–158, 2006.
[7] P. Prandoni, “Links between arterial and venous disease,” Journal of Internal Medicine, vol. 262, no. 3, pp. 341–350, 2007.
[8] M. Minetti, L. Agati, and W. Malorni, “The microenvironment can shift erythrocytes from a friendly to a harmful behavior: pathogenetic implications for vascular diseases,” Cardiovascular Research, vol. 75, no. 1, pp. 21–28, 2007.
[9] D. Zappulla, “Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO2 increases?” Journal of the Cardiometabolic Syndrome, vol. 3, no. 1, pp. 30–34, 2008.
[10] L. M. Resnick, “Ionic basis of hypertension, insulin resistance, vascular disease, and related disorders. The mechanism of “syndrome X”,” American Journal of Hypertension, vol. 6, no. 4, pp. 1235–1345, 1993.
[11] P. Matarrese, E. Straface, D. Pietraforte et al., “Peroxynitrite induces senescence and apoptosis of red blood cells through the activation of aspartyl and cysteinyl proteases,” FASEB Journal, vol. 19, no. 3, pp. 416–418, 2005.
[12] S. Khandelwal, N. Van Rooijen, and R. K. Saxena, “Reduced expression of CD47 during murine red blood cell (RBC) senescence and its role in RBC clearance from the circulation,” Transfusion, vol. 47, no. 9, pp. 1725–1732, 2007.
[13] D. Mandal, A. Mazumder, P. Das, M. Kundu, and J. Basu, “Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes,” Journal of Biological Chemistry, vol. 280, no. 47, pp. 39460–39467, 2005.
[14] F. Lang, E. Gulbins, P. A. Lang, D. Zappulla, and M. Föller, “Ceramide in suicidal death of erythrocytes,” Cellular Physiology and Biochemistry, vol. 26, no. 1, pp. 21–28, 2010.
[15] E. Daugas, C. Candé, and G. Kroemer, “Erythrocytes: death of a mummy,” Cell Death and Differentiation, vol. 8, no. 12, pp. 1131–1133, 2001.
[16] D. Pietraforte, P. Matarrese, E. Straface et al., “Two different pathways are involved in peroxynitrite-induced senescence.
and apoptosis of human erythrocytes," *Free Radical Biology and Medicine*, vol. 42, no. 2, pp. 202–214, 2007.

[17] P. A. Oldenborg, A. Zheleznyak, Y. F. Fang, C. F. Lagena, H. D. Gresham, and F. P. Lindberg, "Role of CD47 as a marker of self on red blood cells," *Science*, vol. 288, no. 5473, pp. 2051–2054, 2000.

[18] R. Matsuda, N. Kaneko, M. Kikuchi et al., "Clinical significance of measurement of plasma annexin V concentration of patients in the emergency room," *Resuscitation*, vol. 57, no. 2, pp. 171–177, 2003.

[19] D. Modun, I. Music, J. Vukovic et al., “The increase in human plasma antioxidant capacity after red wine consumption is due to both plasma urate and wine polyphenols,” *Atherosclerosis*, vol. 197, no. 1, pp. 250–256, 2008.

[20] P. A. Kyrle, G. Hron, S. Eichinger, and O. Wagner, "Circulating P-selectin and the risk of recurrent venous thromboembolism," *Thrombosis and Haemostasis*, vol. 97, no. 6, pp. 880–883, 2007.

[21] G. Lucantoni, D. Pietraforte, P. Matarrese et al., “The red blood cell as a biosensor for monitoring oxidative imbalance in chronic obstructive pulmonary disease: an ex vivo and in vitro study,” *Antioxidants and Redox Signaling*, vol. 8, no. 7-8, pp. 1171–1182, 2006.

[22] M. W. Lorenz, H. S. Markus, M. L. Bots, M. Rosvall, and M. Sitzer, "Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis," *Circulation*, vol. 115, no. 4, pp. 459–467, 2007.

[23] S. Berliner, O. Rogowski, S. Aharonov et al., “Erythrocyte adhesiveness/aggregation: a novel biomarker for the detection of low-grade internal inflammation in individuals with atherothrombotic risk factors and proven vascular disease,” *American Heart Journal*, vol. 149, no. 2, pp. 260–267, 2005.

[24] W. Malorni, E. Straface, P. Matarrese et al., “Redox state and gender differences in vascular smooth muscle cells,” *FEBS Letters*, vol. 582, no. 5, pp. 635–642, 2008.

[25] E. Straface, R. Vona, L. Gambardella et al., “Cell sex determines anoikis resistance in vascular smooth muscle cells,” *FEBS Letters*, vol. 583, no. 21, pp. 3448–3454, 2009.

[26] P. Matarrese, T. Colasanti, B. Ascione et al., “Gender disparity in susceptibility to oxidative stress and apoptosis induced by autoantibodies specific to RLIP76 in vascular cells,” *Antioxidants & Redox Signaling*. In press.
Review Article

Insights into the Function of the Unstructured N-Terminal Domain of Proteins 4.1R and 4.1G in Erythropoiesis

Wataru Nunomura,1 Philippe Gascard,2 and Yuichi Takakuwa1

1 Department of Biochemistry, Tokyo Women’s Medical University, Kawada 8-1, Shinjuku, Tokyo 162-8666, Japan
2 Department of Pathology, University of California, San Francisco (UCSF), San Francisco, CA 94143-0511, USA

Correspondence should be addressed to Yuichi Takakuwa, takakuwa@research.twmu.ac.jp

Received 20 April 2011; Accepted 20 June 2011

Academic Editor: Johannes Vogel

Copyright © 2011 Wataru Nunomura et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Membrane skeletal protein 4.1R is the prototypical member of a family of four highly paralogous proteins that include 4.1G, 4.1N, and 4.1B. Two isoforms of 4.1R (4.1R135 and 4.1R80), as well as 4.1G, are expressed in erythroblasts during terminal differentiation, but only 4.1R80 is present in mature erythrocytes. One goal in the field is to better understand the complex regulation of cell type and isoform-specific expression of 4.1 proteins. To start answering these questions, we are studying in depth the important functions of 4.1 proteins in the organization and function of the membrane skeleton in erythrocytes. We have previously reported that the binding profiles of 4.1R80 and 4.1R135 to membrane proteins and calmodulin are very different despite the similar structure of the membrane-binding domain of 4.1G and 4.1R135. We have accumulated evidence for those differences being caused by the N-terminal 209 amino acids headpiece region (HP). Interestingly, the HP region is an unstructured domain. Here we present an overview of the differences and similarities between 4.1 isoforms and paralogs. We also discuss the biological significance of unstructured domains.

1. 4.1R in the Erythrocyte Membrane Skeleton

The membrane skeleton, which underlies the erythrocyte plasma membrane, is made of a spectrin/actin lattice anchored to various transmembrane proteins via two specialized cytoskeletal proteins, 4.1R and red blood cell ankyrin, ankyrin-R [1]. 4.1R80 stabilizes horizontal interactions between spectrin heterodimers (α2-β2) and short actin (~14 molecules) filaments. Actin filaments interact with numerous accessory proteins, such as tropomyosin, myosin, tropomodulin, and adducin [1], which ensure reorganization of actin filaments. 4.1R80 interacts also with the transmembrane protein, glycophorin C (GPC) and with the membrane-associated guanylate kinase (MAGUK) protein p55, which also acts as an erythrocyte scaffolding protein (Figure 1).

1.1. GPC. GPC is 32 kDa single transmembrane protein expressed at ~50,000–100,000 molecules/erythrocyte. The cytoplasmic domain consists of 47 amino acids residues (ID: P04921). The R82HK sequence has been identified as the 4.1R binding sequence [2–4]. This RHK motif is highly conserved in the cytoplasmic domain of Neurexin IV, Paranodin, and TSLC1 (Tumor Suppressor Lung Cancer 1) [5]. Girault et al. have designated this RHK motif “GNP-motif,” after the single transmembrane 4.1R binding proteins, GPC, Neurexin IV, Paranodin [6]. GPC and other GNP-motif containing proteins possess a p55 binding motif, EYFI, in their C-terminal region (Figure 2).

1.2. p55. p55 is a 55 kDa erythrocyte scaffolding protein that belongs to the membrane-associated guanylate kinase homologues (MAGUK) family (ID: Q00013). This protein is characterized by the presence of a PDZ (Postsynaptic density protein-95, Dlg (Drosophila disc large tumor suppressor), ZO-1 (Zonula Occludens-1)) domain, an SH (src-homology) 3 domain, and a catalytic inactive guanylate kinase like (GUK) domain, all of which function as protein-protein interaction modules (Figure 2) [8]. The number of p55 copies in the human erythrocytes is ~80,000. p55 is also called Membrane Palmitoylated Protein 1 (MPP1) since...
Figure 1: Structure of human erythrocyte membrane. Spectrin dimers underlying the membrane interact with transmembrane proteins band 3 through ankyrin and glycophorin C (GPC) through an actin complex and protein 4.1R80(4.1R80). 4.1R80 also forms a ternary complex with p55 and GPC. CaM binds to 4.1R80 in a Ca\(^{2+}\)-independent manner.

Figure 2: Organization of the R30/GPC/p55 ternary complex. The NMR structure of GPC peptide and PDZ domain complex has been previously reported [7] (PDB accession no. 2ejy). The HOOK domain is the 4.1R binding site for p55 [4].
1.3. Band 3. Membrane stability is also controlled in part by band 3-ankyrin-spectrin interaction (as shown in Figure 1). Band 3 is a 102 kDa 14-transmembrane protein which mediates exchange of HCO$_3^-$ and Cl$^-$ and is therefore referred to as anion exchanger 1 (AE1) [1] (ID: P02730). It is expressed at 1,200,000 molecules/cell. It forms dimers that assemble into tetramers, each tetramer binding to one molecule of ankyrin. This is the base for the organization of the band 3-ankyrin-spectrin complex [13].

4.1R binds to the L$^{386}$RRRY and L$^{343}$RRRY sequences in band 3-cytoplasmic domain [14]. Although the crystal structure of the N-terminal cytoplasmic domain of band 3 has been reported, this structure is putative as the N-terminal 55 residues, including the L$^{343}$RRRY sequence, were missing in the crystal [15]. The results indicate that band 3 has four 4.1R binding sites. The stoichiometry of band 3 binding to 4.1R is still unknown. The importance of band 3 in membrane architecture results from its role in anchoring the spectrin network through interaction with the scaffold protein ankyrin. We have demonstrated that 4.1R$^{80}$ modulates band 3 interaction with ankyrin [16]. We have characterized a similar function for 4.1R$^{80}$ in modulating ankyrin interaction with CD44, a single transmembrane protein which acts as receptor for hyaluronic acid [17].

The absence of 4.1R, ankyrin, or spectrin or selected mutations in these proteins result in alterations in erythrocyte shape and mechanical properties (reviewed in [1, 12]). We have demonstrated that 4.1R interacts with membrane protein analogues in zebrafish (Danio rerio) using in vitro binding assays [18, 19] (ID: NP_778259). Salomao et al. have documented that protein 4.1R$^{80}$ can bind in vitro to additional erythrocyte transmembrane proteins, such as Kell, XK, Rh, and Duffy [20]. These interactions remain to be validated in vivo. The function of 4.1R has been inferred from the hematopoietic phenotype observed in human 4.1R-deficient patients, in transgenic 4.1R knock-out mice, and...
in zebrafish (Danio rerio) subjected to chemical mutagenesis [21]. 4.1R deficiency leads to hereditary elliptocytosis (HE), erythrocytes losing their typical biconcave disc shape to become elliptical. Thus, 4.1R acts in concert with other membrane proteins for maintaining normal erythrocyte shape [22].

2. PART I: 4.1R<sup>80</sup> and 4.1R<sup>135</sup> in Erythropoiesis

2.1. Overview of 4.1R Structure. 4.1R forms multimolecular complexes with transmembrane proteins and membrane-associated proteins, such as spectrin and actin [1]. Such complexes, which are critical for maintaining structural stability in red blood cells, could well be involved in other functions in nonerythroid cells, such as, for example, signal transduction at sites of cell-cell and/or cell-matrix contacts.

4.1R<sup>80</sup> (ID: P11171), present at approximately 200,000 copies per erythrocyte, can be extracted by high salt treatment of inside-out vesicles (IOVs), which correspond to erythrocytes membranes depleted of spectrin and actin. Based on its 622-amino-acid composition (reviewed in [1, 12]), the predicted molecular weight of 4.1R is only ~70 kDa, the discrepancy with the apparent molecular weight resulting in part from the unstructured domains of 4.1R. Limited α-chymotryptic digestion of 4.1R generates four polypeptides: a 30 kDa N-terminal membrane-binding domain, a 16 kDa domain, a 10 kDa spectrin-actin binding domain, and a 22/24 kDa C-terminal domain (reviewed in [1, 12]). A 4.1R isoform expressed in erythroblasts, but not in mature erythrocytes, contains an extra N-terminal 209 amino acids headpiece (HP) region. The apparent molecular weight of this 4.1R isoform in SDS-PAGE is ~135 kDa, and it is therefore referred to as 4.1R<sup>135</sup>. However, its theoretical molecular weight is ~100 kDa. This discrepancy results from the unstructured state of the HP region [23].

2.2. Unstructured N-Terminal and Structured 30 kDa FERM Domains of 4.1R<sup>135</sup>. We calculated the disorder probability of the N-terminal HP region and the FERM domain using the PrDOS software (http://prdos.hgc.jp/cgi-bin/top.cgi) [26]. A value greater than 0.5 reflects a disordered structure, with a probability of false prediction of 5% or less. Our analysis indicates a highly disordered structure for the HP region (amino acids 1–209) that contrasts with a highly ordered structure for the 30 kDa FERM domain (amino acids 210–507). Of particular note, while the overall 209aa HP region adopts a disordered structure, a short polypeptide (amino acids 70–80), corresponding to a previously identified Ca<sup>2+</sup>-dependent CaM-binding site [27, 28], does not (Figure 4).

We experimentally demonstrated that the HP is an unfolded region by SDS-PAGE, size exclusion chromatography (SEC), and dynamic light scattering (DLS). The theoretical molecular weight of 4.1R HP (RHP) is 23 kDa but we estimate its apparent molecular weight as 55 kDa by SDS-PAGE [29]. Furthermore, SEC analysis reveals that RHP is eluted between IgG (150 kDa) and albumin (68 kDa) on a Sephacryl S-300 column. While the theoretical molecular weights of the proteins corresponding to amino acids 1–507 of 4.1R<sup>135</sup> (RHP-R30) and to R30 (30 kDa FERM domain) are 56 kDa and 32 kDa, respectively, they migrate as polypeptides of >100 kDa and 35 kDa, respectively, on SDS-PAGE [29]. By DLS measurements, the hydrodynamic diameters of RHP, RHP-R30 and R30 are 7.6, 9.4, 5.6 nm, respectively (Nunomura, W., Shiba, K. and Takakuwa, Y., unpublished data). These hydrodynamic parameters enabled us to estimate the molecular weight of RHP, RHP-R30 and R30 to be 77, 127, and 40 kDa, respectively. The discrepancies between theoretical and apparent molecular weights for proteins containing RHP reflect the unfolded nature of this peptide.

In contrast, the consistency between theoretical and apparent molecular weights for R30 illustrates the folded nature of R30. Importantly, PrDOS-based analysis of full length 4.1R<sup>135</sup> predicted the 30 kDa domain to be the only region in the whole protein to adopt an ordered (folded) structure. The crystal structure of 4.1R 30 kDa domain is reminiscent of the shape of a cloverleaf or of a propeller, with three clearly distinct lobes (PDB: 1GG3) [25]. First, the N-lobe, corresponding to the first 78 amino acids and which includes the band 3 binding motif L<sup>37</sup>EEDY, consists of 4 double-stranded β-strands. Second, the α-lobe, corresponding to the following 90 amino acids and which includes the GPC binding site, consists of 4 α-helices. Third, the COOH-terminal lobe (C-lobe), which contains the p55 binding surface, is made of seven β-strands, and ends with an α-helix (Figure 4). Although many membrane skeletal proteins contain intrinsically disordered (unfolded) regions, there are very few reports describing the function(s) of these intrinsically disordered region [30–35]. Our findings will contribute not only to a better understanding of the structure of membrane skeletal proteins but also of the function of intrinsically disordered proteins.

2.3. Expression of 4.1R<sup>135</sup> and 4.1R<sup>80</sup>. In early stages of erythroblasts (CD34<sup>+</sup> cells), 4.1R<sup>135</sup> is the only isoform detected, 4.1R<sup>80</sup> being completely absent. After the middle stage, which is reached after approximately 7 days in culture, expression of 4.1R<sup>80</sup> increases dramatically. In mature erythrocytes, 4.1R<sup>80</sup> predominates, 4.1R<sup>135</sup> being hardly seen by immunocytochemical methods [29]. The complex mechanistic of 4.1R<sup>135</sup>-4.1R<sup>80</sup> gene switching has been recently described by Parra et al. [36, 37].

2.4. Binding Profiles of 4.1R<sup>135</sup> and 4.1R<sup>80</sup> to Membrane Proteins and CaM Differ. Previous studies have shown that, while 4.1R<sup>80</sup> binds to both band 3 and GPC in native inside-out vesicles (IOVs), it binds only to GPC in trypsinized IOVs [32]. Scatchard analysis indicates an apparent dissociation constant at equilibrium, K′, of 76 nM for 4.1R<sup>80</sup> binding to trypsinized IOVs (i.e., to GPC). In contrast, K′ for 4.1R<sup>80</sup> binding to native IOVs (i.e., to both GPC and band 3) reaches 340 nM. A similar analysis for 4.1R<sup>135</sup> revealed that 4.1R<sup>135</sup> binding to trypsinized IOVs (i.e., to GPC) is markedly weaker (K′ ~2 μM) than that of 4.1R<sup>80</sup>. In contrast, K′ for 4.1R<sup>135</sup> binding to native IOVs is 230 nM, similar to that
Figure 4: Primary structure of 4.1R isoforms and map of known binding partners for 4.1R. Translation of the prototypical red blood cell 80 kDa 4.1R isoform (4.1R80) is initiated at AUG-2, which is located in exon 4. Translation of the 135 kDa 4.1R isoform (4.1R135), an isoform expressed in early erythroblasts and other nucleated cells, is initiated at AUG-1, which is located in exon 2′ (ID: P11171). The 30 kDa membrane-binding domain is the so-called “FERM” domain. Disorder prediction for each domain has been established through the use of the PrDOS software package. An updated list of the binding partners identified for each domain of 4.1R is displayed. CPAP refers to a “centrosomal protein 4.1R-associated protein” reported by Hung et al. [24]. A 3D representation of the 30 kDa FERM domain of 4.1R, visualized with the MolFeat Ver. 4.6 software, is displayed (PDB accession no. 1GG3). The 30 kDa domain consists of three lobes (N-, α-, and C-lobe) and adopts a three-leaf clover shape [25].

Figure 5: Mapping of the CaM-binding sites in 4.1R. 4.1R135 has three CaM-binding sites: pep2 in the HP region, S185 being the key residue for Ca2+-sensitive site, and Ca2+-independent sequence; pep9 and pep11 in the FERM domain. Numbers in parenthesis indicate amino acid numbering for 4.1R80 (AUG2 form in Figure 4).
observed for 4.1R\(^{80}\). These findings imply that the presence or absence of HP in 4.1R isoforms modulates their binding affinity for GPC but not for band 3 [29].

In order to obtain independent confirmation of the binding affinities of 4.1R\(^{135}\) to band 3cyt and GPCcyt, we used the IAsys system based on the resonant mirror detection method [29]. In agreement with the binding data using IOVs described above, there was a dramatic difference in the binding affinity of 4.1R\(^{135}\) to band 3cyt and GPCcyt, the binding affinity being much higher for band 3cyt (23 ± 2 nM) than for GPCcyt (1327 ± 103 nM). In marked contrast, \(K_{(D)}\) values for binding of 4.1R\(^{80}\) to both band 3cyt and GPCcyt were very similar, in the submicromolar range. This confirmed an important role for HP in regulating 4.1R affinities for its two major transmembrane binding partners. In contrast to the marked differences in the binding affinities of 4.1R\(^{135}\) and 4.1R\(^{80}\) to band 3cyt and GPCcyt, the two isoforms bound to p55 with very similar affinities, in the submicromolar range.

As expected from the data obtained with 4.1R\(^{80}\) and 4.1R\(^{135}\) isoforms, the addition of RHP to R30 (RHP-R30) results in a profound change in the ability of R30 to bind to band 3cyt and GPCcyt. Thus, the binding affinity of RHP-R30 for band 3cyt is 35-fold higher than for GPCcyt. Together, these findings highlight an important role for RHP in modulating the interaction of R30 with its two membrane-binding partners.

2.5. Differences in CaM Binding to 4.1R Isoforms. We have previously documented that 4.1R\(^{80}\) binds to CaM with a \(K_{(D)}\) in the submicromolar range, both in the presence and absence of Ca\(^{2+}\) implying that this interaction is Ca\(^{2+}\)-independent [38]. We have also examined the nature of the interaction between 4.1R\(^{135}\) and CaM. Kinetic analysis of 4.1R\(^{135}\) interaction with CaM using the IAsys system identified a very strong interaction with a \(K_{(D)}\) of 51 ± 5 nM in the presence of Ca\(^{2+}\). In the absence of Ca\(^{2+}\), the binding affinity decreased by over 100-fold. Thus, in contrast to 4.1R\(^{80}\), the interaction of 4.1R\(^{135}\) with CaM is strongly Ca\(^{2+}\)-dependent. Probing of the HP region alone confirms a Ca\(^{2+}\)-dependent interaction with CaM, implying that this region harbors the CaM-binding site [27, 28]. Our observations are in accordance with Leclerc and Vetter’s study that identifies the S\(^{58}\)RGLSRFLSFSLKRPKS peptide as the Ca\(^{2+}\)-dependent binding site.
Figure 7: Effect of EGTA on the distribution of 4.1R135 in human erythroblasts (cultured for 7 days). Human erythroblasts were cultured in the presence or absence of 1 mM EGTA and immunostained with a rabbit antibody to RHP as previously described [29].

CaM binding sequence in RHP [27, 28] (Figure 5). The stoichiometry of 4.1R135 binding to CaM in the presence of Ca^{2+} is 1:1 as assessed by the quartz crystal microbalance (QCM) method. These results indicate that Ca^{2+}/CaM binds to the HP region but not to the 30 kDa domain [29].

2.6. Regulation of 4.1R135 Interactions with Membrane Proteins by Ca^{2+}/CaM. The binding affinity of 4.1R135 for band 3cyt is decreased by almost 2 orders of magnitude by Ca^{2+}/CaM. Moreover, Ca^{2+}/CaM completely abolishes the ability of 4.1R135 to bind to either GPCcyt or p55. Either 5 μM CaM or 100 μM Ca^{2+} alone has no effect on binding affinities. 4.1R135 binding to band 3cyt starts to decline at [Ca^{2+}]i greater than 10 nM (pCa < 8) with a maximal inhibition at 100 μM (pCa > 4). Half maximal binding is observed at a [Ca^{2+}]i of 3.2 μM (pCa = 5.5). In the case of 4.1R80, Ca^{2+}/CaM binding to the 30 kDa domain reduces about 10 times the binding affinity for band 3. Thus, we noted significant differences between 4.1R135 and 4.1R80 in the Ca^{2+}-dependence for the binding of these two isoforms to CaM. In contrast to the Ca^{2+}-independent binding of CaM to 4.1R80, CaM binding to 4.1R135 is strongly Ca^{2+} dependent. This difference is once again directly attributable to the HP region present in 4.1R135. Importantly, in contrast to band 3 and GPC that do not directly bind to the HP region, this region by itself binds to CaM in a Ca^{2+}-dependent manner. Thus, it must be inferred that the CaM-binding site in the HP region is the dominant binding site for CaM in 4.1R135 and that this site prevents the binding of CaM to the Ca^{2+}-independent binding site in 4.1R80. Furthermore, our finding that CaM dramatically decreases the binding of 4.1R135 to band 3 in a Ca^{2+}-dependent manner and abolishes its binding to GPC and p55 has implications for the function of this 4.1R isoform in early erythroblasts. Indeed, while low levels of Ca^{2+} in early erythroblasts will lead to membrane association through high-affinity interaction with band 3, increasing levels of Ca^{2+} during erythroid differentiation will lead to the displacement of the protein from the membrane and to a possible degradation and loss of this isoform from erythroblasts. Our findings that, in early erythroblasts, a fraction of 4.1R135 is actually associated with the membrane lends support to this hypothesis [29] (Figure 6). Strikingly, in human erythroblasts cultured for 7 days and treated with 1 mM EGTA, 4.1R135 is more clearly distributed at or near the plasma membrane than in nontreated cells (Figure 7). Precise quantitative measurements of Ca^{2+} levels in erythroblasts at different stages of maturation need to be performed to validate further this hypothesis.

3. PART II: 4.1R135 and 4.1G in Erythroblasts

4.1R135 and 4.1G are simultaneously expressed in erythroblasts and in nonerythroid cells, such as epithelial cells [42, 43]. The structure of the 30 kDa (FERM) domain of 4.1R and 4.1G is very similar. To date, there has not been any report about functional differences between 4.1R135 and 4.1G. We have shown for the first time differences in binding profiles of these two 4.1 proteins to membrane proteins.

3.1. Structural Similarity between 4.1R135 and 4.1G. The primary amino acid sequence of the 30 kDa domain of 4.1G is 71% identical to that of 4.1R [42] (ID: O43491). 4.1G is therefore predicted to bind to many of the previously identified 4.1R binding partners. In contrast to the high
conservation of the 30 kDa domain, the amino acid sequence identity of the HP region of 4.1G and 4.1R\textsuperscript{135} is quite low (35%). We therefore hypothesized that the HP region of 4.1R and 4.1G might regulate differently the binding properties of their respective 30 kDa domain.

Computer analysis of the 3D structure of the 30 kDa domain of 4.1G has demonstrated that its folded clover-like structure is very similar to that of 4.1R [41] (Figure 8). This observation validates the structural basis for 4.1G binding to previously defined 4.1R binding partners through its 30 kDa domain. As observed for the 30 kDa domain of 4.1R, 4.1G could also interact with CaM in a Ca\textsuperscript{2+}-independent manner.

Using a combination of computational calculations (aimed at calculating the disorder probability based on PrDOS software analysis), SDS-PAGE analysis and size exclusion chromatography, we established that, like the HP region of 4.1R, the HP region of 4.1G adopts an unstructured state [41]. As expected from their similar structure, R30 and G30 are both folded polypeptides, this 30 kDa region representing the only structured (folded) domain for both proteins [41].

3.2. Expression of 4.1G and 4.1R\textsuperscript{135} in Erythroblasts. In erythroblasts, both 4.1G and 4.1R are expressed whereas the two other 4.1 gene products, 4.1B and 4.1N, are not (personal communication, Narla Mohandas, New York Blood Center). 4.1G is expressed after 7–12 days of culture as a ~70 kDa isoform containing the HP region. This suggests the occurrence of alternative splicing events targeting domains downstream of the HP region (FERM domain, spectrin-actin binding domain and/or C-terminal domain) in 4.1G.

3.3. Differences in Binding Profiles of 4.1R\textsuperscript{135} and 4.1G to Membrane Proteins. 4.1G binds to IOVs prepared from erythrocyte membranes. The apparent $K'$ values for 4.1G FERM domain (G30) and full length 4.1G binding to IOVs are 169 ± 67 nM and 207 ± 49 nM, respectively, as assessed by Scatchard plot analysis. These values are similar to those obtained using resonant mirror detection [41]. These findings demonstrate that 4.1G can bind to transmembrane proteins of the erythrocyte membrane through its 30 kDa domain.

4.1G interacts \textit{in vitro} with band 3cyt and GPCcyt with $K_{D(D)}$s in the ~200 nM range. Importantly, the binding affinities of 4.1G for band 3cyt and GPCcyt are different from those of 4.1R\textsuperscript{135} despite the presence of an HP region in both proteins. Thus, 4.1G interacts with band 3cyt with a much lower affinity than 4.1R\textsuperscript{135}, the reverse being observed for GPCcyt. These differences result mainly from differences in the association rate constant $k_a$. In contrast, both 4.1G and 4.1R\textsuperscript{135} interact with p55 with similar affinities [44].

Binding affinities of full length 4.1G and of its 30 kDa domain (G30) for the membrane proteins described above are very similar, suggesting that 4.1G interacts with its binding partners primarily through G30, the headpiece GHP having a negligible effect on these interactions. This is in marked contrast to the interactions of the 30 kDa domain of 4.1R (R30) which are significantly affected by RHP [29].
Interestingly, recombinant chimera proteins consisting of either RHP and G30 (RHP-G30) or GHP and R30 (GHP-R30) showed similar binding affinities as G30 and R30. This implied significant differences in the structure and function of RHP and GHP. It should be noted that neither GHP nor RHP binds to any of these membrane proteins.

We showed an important role for the HP region in regulating 4.1R^{135} 30 kDa domain binding to membrane proteins. Thus, the HP region improves accessibility of the N-lobe to band 3, but impairs accessibility of the α-lobe to GPC whereas it does not have a significant effect on the C-lobe [29]. 4.1G HP does not appear to modulate the accessibility of the three lobes in G30 to their respective binding partners, the binding profile of 4.1G being similar to that of G30.

We demonstrated that 4.1G binds to various previously characterized 4.1R binding partners, including transmembrane proteins band 3, GPC, and p55, through its 30 kDa domain. The HP domain does not affect these interactions. However, Ca^{2+}-dependent CaM binding to the HP region has a profound effect on the interaction of 4.1G with its binding partners. The documented binding profiles for 4.1G are markedly different from those previously reported for 4.1R^{135} [29]. Since the primary structure of the 30 kDa domain of 4.1G and 4.1R is highly conserved (71% sequence similarity), the differences in binding profiles are likely to arise primarily from the nonconserved HP region.

3.4. Similarities and Differences of CaM Binding to HP and 30 kDa Domains of 4.1R and 4.1G. Both full length 4.1G and GHP bind to a CaM Sepharose 4B column in the presence of Ca^{2+} and can be eluted with 5 mM EGTA. The $K_D$ for CaM binding to 4.1G and GHP increases dramatically following chelation of Ca^{2+} with EGTA. These findings establish that CaM binds to 4.1G HP region in a Ca^{2+}-dependent manner. These data recapitulate previous observations made for CaM binding to RHP and 4.1R^{135} [29]. The binding affinity of 4.1G to Ca^{2+}/CaM is ~10 nM, and the stoichiometry is ~1:1 [41]. This observation strongly supports the importance of the HP region in mediating Ca^{2+}-dependent CaM binding to 4.1G.

However, although CaM binds to the HP region of 4.1G in a Ca^{2+}-dependent manner, it does not bind to 4.1G 30 kDa domain, as previously documented for 4.1R^{80} [38]. The HP region of 4.1G contains a sequence

---

**Figure 9:** Model proposed for Ca^{2+}/CaM-dependent regulation of 4.1G binding to membrane proteins. Erythroblast intracellular Ca^{2+} concentration is normally maintained at less than 0.1 μM ($10^{-7}$ M) as described in Figure 7 [39, 40]. 4.1G binds to band 3, GPC and p55 with a $K_D$ of $10^{-7}$ M. At higher Ca^{2+} concentrations, CaM binds to the HP region with a $K_D$ of $10^{-8}$ M. This results in a conformational and/or electric surface change which alters 4.1G binding sites, 4.1G interacting consequently with lower affinity with its binding partners GPC and no longer interacting with band 3 and p55. This model implies a Ca^{2+}/CaM-dependent regulation of 4.1G binding to transmembrane proteins.
In erythroblasts, we showed that, consistent with earlier structure and function during evolution and development (S76RGLSRLFSSFLKRPKS) [27, 28]. Although the Ca^{2+}-independent CaM-binding sequence previously identified in the 30 kDa domain of 4.1R^R{80} is conserved in 4.1G [41], our results indicate that CaM binds to the HP region but not to the 30 kDa domain of 4.1G. It should be emphasized that although the HP by itself does not affect binding of the 30 kDa domain of 4.1G to various membrane proteins, Ca^{2+}/CaM binding to the HP markedly inhibits the ability of the 30 kDa domain of 4.1G to interact with its various binding partners. These findings have enabled us to document similarities and differences in the structural and functional properties of 4.1G and 4.1R^{135}.

3.5. Ca^{2+}/CaM-Dependent Regulation of 4.1G Binding to Membrane Proteins. We have shown that Ca^{2+}/CaM binding to the headpiece of 4.1G results in a complete inhibition of 4.1G binding to band 3cyt and p55 and in a significant increase in the $K_{D}$ for 4.1G binding to GPCcyt. In light of the fact that CaM binds to the 30 kDa domain on 4.1R^R{80} in the absence of Ca^{2+} and that CaM binding decreases the $K_{D}$ of R30 for its binding partners in a Ca^{2+}-dependent manner [17, 44], we examined the effect of CaM binding to G30 on its binding properties to membrane proteins using a RHP-G30 chimera protein. Binding affinities of RHP-G30 for band 3cyt, GPCcyt, p55, and CD44cyt were measured in the presence or absence of Ca^{2+} and CaM. The $K_{D}$'s obtained for each binding partner in the absence of CaM were similar to those obtained with full-length 4.1G. In contrast, binding assays performed with RHP-G30 preincubated with Ca^{2+}/CaM showed a major decrease in binding affinity (7–10 fold in $K_{D}$) of RHP-G30 for band 3cyt, GPCcyt, and p55. These results indicate that although CaM can bind to G30 independently of Ca^{2+}, G30 interactions with membrane proteins can be regulated by CaM in a Ca^{2+}-dependent manner. These results also indicate that the regulation of 30 kDa domain binding properties by unfolded HP domain has unique features in the case of 4.1R^{135} and 4.1G.

The Ca^{2+} concentration dependence of the CaM-modulated interaction of 4.1G with band 3cyt and GPCcyt has been demonstrated [38, 44]. The half maximal binding of 4.1R^{135} and 4.1G to band 3cyt and GPCcyt occurs at Ca^{2+} concentrations in the submicromolar range [39, 40], supporting the potential biological relevance of our biochemical findings [29]. Ca^{2+}/CaM-dependent modulations of 4.1R^{135} and 4.1G binding to membrane proteins may be triggered upon signal transduction during erythroid development. Indeed, it has been documented that, at early stages of erythropoiesis, intracellular calcium levels increased from a basal level of 55 ± 5 nM to 259 ± 49 nM following binding of erythropoietin to its receptor [39]. Such an increase in intracellular calcium levels would be sufficient to modulate the interaction of 4.1R^{135} and 4.1G with its binding partners in erythroid cells. Our findings further suggest that 4.1G offers a unique opportunity to explore divergence of protein structure and function during evolution and development. In erythroblasts, we showed that, consistent with earlier reports [42, 43], 4.1G and 4.1R^{135} are both expressed during terminal erythroid differentiation and that both proteins can interact with common transmembrane proteins, such as band 3, GPC, and p55. Different binding affinities and Ca^{2+}/CaM-dependent modulation of interaction with band 3 and GPC suggest that these 4.1 proteins may play specific roles in membrane biogenesis during terminal erythroid differentiation (Figure 9).

Thus, the unstructured HP domains of 4.1R and 4.1G seem to play a unique role in regulating the membrane-binding properties of those proteins. Understanding the structural basis for differences and similarities in 4.1 binding properties will help us unveil novel biological functions for various 4.1 gene products. To that end, we are currently carrying out a structural analysis of the HP-Ca^{2+}/CaM complex using NMR and small-angle X-ray scattering (SAXS). These biophysical analyses should help us further understand the structural basis for the regulatory role of the unstructured HP domain.

4. Conclusion

During erythropoiesis, the HP domain acts as a regulator of 4.1R and 4.1G interaction with the plasma membrane. We hypothesize that these regulatory properties are in part the result of the unstructured conformation of the HP region. We also show that these regulatory properties depend on intracellular calcium concentrations, with these concentrations varying during erythropoiesis. Thus, the function of the HP domain may evolve depending on the structure of the 4.1 protein isoforms expressed at each stage of erythropoiesis.

5. Future Studies on 4.1R^{135} and 4.1G

This paper focuses on the structure and function of the N-terminal intrinsically disordered region (HP) and membrane-binding FERM domain of 4.1R^{135} and 4.1G and on the role of Ca^{2+} in regulating binding to membrane proteins through CaM. Our findings are based on in vitro binding assays. Direct evidence for these interactions and their regulations in living cells remains to be established. Although it is known that the RHP contains phosphorylation sites [28, 45], the relationship between Ca^{2+}/CaM regulation and phosphorylation remains to be investigated. 4.1G binds to spectrin/actin [46, 47] and receptors through its C-terminal region [48, 49]. Does Ca^{2+}/CaM binding to HP also regulate these interactions? Answering such mechanistic questions will help us define the biological significances of 4.1R^{135} and 4.1G in the late stage of erythropoiesis.

Appendix

Tables 1 and 2 summarize 4.1R^{135} and 4.1G binding kinetic parameters to the cytoplasmic tails of band 3 and GPC and to CaM in the presence or the absence of Ca^{2+}. Although both 4.1R^{135} and 4.1G bind to Ca^{2+}/CaM in the $10^{-8}$ M $K_{D}$ range, the $K_{D}$ of 4.1R^{135} binding to CaM is 5 times higher than 4.1G in the absence of Ca^{2+}. These results suggest that
the binding profiles of 4.1R and 4.1G may differ in respect to CaM.

**Abbreviations**

| Analyte | Ligand   | Condition | $K_a$ (M⁻¹ s⁻¹) | $K_d$ (s⁻¹) | $K_D$ (nM) |
|---------|----------|-----------|-----------------|-------------|------------|
| 4.1R₁₃₅ | band 3cyt| EGTA      | $3.1 \pm 0.2 \times 10^3$ | $7.1 \pm 0.2 \times 10^{-3}$ | 23 ± 2 |
| 4.1R₁₃₅ | GPCcyt   | Ca²⁺      | $8.0 \pm 0.2 \times 10^3$ | $1.1 \pm 0.1 \times 10^{-2}$ | 1327 ± 103 |
| 4.1G    | band 3cyt| EGTA      | $8.0 \pm 0.1 \times 10^4$ | $1.4 \pm 0.1 \times 10^{-2}$ | 185 ± 23 |
| 4.1G    | GPCcyt   | Ca²⁺      | $5.6 \pm 0.1 \times 10^4$ | $8.1 \pm 0.2 \times 10^{-3}$ | 144 ± 5 |

**Acknowledgment**

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education Culture, Sport, Science and Technology of Japan 15570123 for W. Nunomura.

**References**

[1] V. Bennett and A. J. Baines, “Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues,” *Physiological Reviews*, vol. 81, no. 3, pp. 1353–1392, 2001.

[2] N. J. Hemming, D. J. Anstee, W. J. Mawby, M. E. Reid, and M. J. A. Tanner, “Localization of the protein 4.1-binding site on human erythrocyte glycophorins C and D,” *Biochemical Journal*, vol. 299, no. 1, pp. 191–196, 1994.

[3] N. J. Hemming, D. J. Anstee, M. A. Staricoff, M. J. A. Tanner, and N. Mohandas, “Identification of the membrane attachment sites for protein 4.1 in the human erythrocyte,” *Journal of Biological Chemistry*, vol. 270, no. 10, pp. 5360–5366, 1995.

[4] S. M. Marfatia, R. A. Lue, D. Branton, and A. H. Chiashi, “Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the Drosophila disc-large tumor suppressor protein,” *Journal of Biological Chemistry*, vol. 270, no. 2, pp. 715–719, 1995.

[5] M. Yageta, M. Kuramochi, M. Masuda et al., “Direct association of TSLC1 and DAL-1, two distinct tumor suppressor proteins in lung cancer,” *Cancer Research*, vol. 62, no. 18, pp. 5129–5133, 2002.

[6] J. A. Girault, G. Labesse, J. P. Morrow, and I. Callebaut, “Janus kinases and focal adhesion kinases play in the 4.1 band: a superfamily of band 4.1 domains important for cell structure and signal transduction,” *Molecular Medicine*, vol. 4, no. 12, pp. 751–769, 1998.

[7] H. Kusunoki and T. Kohno, “Structural insight into the folding protein p55/MPP1 functions as an essential regulator of neutrophil polarity,” *Biochemical and Biophysical Research Communications*, vol. 359, no. 4, pp. 972–978, 2007.

[8] S. D. Dimitratos, D. F. Woods, D. G. Stathakis, and P. J. Bryant, “Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family,” *BioEssays*, vol. 21, no. 11, pp. 912–921, 1999.

[9] B. J. Quinn, E. J. Welch, A. C. Kim et al., “Erythrocyte scaffolding protein p55/MPP1 functions as an essential regulator of neutrophil polarity,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 47, pp. 19842–19847, 2009.

[10] P. Mburu, Y. Kikkawa, S. Townsend, R. Romero, H. Yonekawa, and S. D. M. Brown, “Whirlin complexes with p55 at the stereocilia tip during hair cell development,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 29, pp. 10973–10978, 2006.

[11] P. Mburu, M. R. Romero, H. Hilton et al., “Gelsolin plays a role in the actin polymerization complex of hair cell stereocilia,” *PLoS ONE*, vol. 5, no. 7, article e11627, 2010.
[12] W. Nunomura and Y. Takakuwa, “Regulation of protein 4.1R interactions with membrane proteins by Ca\(^{2+}\) and calmodulin,” *Frontiers in Bioscience*, vol. 11, no. 2, pp. 1522–1539, 2006.

[13] P. Michaela and V. Bennett, “The ANK repeats of erythrocyte ankyrin form two distinct but cooperative binding sites for the erythrocyte anion exchanger,” *Journal of Biological Chemistry*, vol. 270, no. 37, pp. 22050–22057, 1995.

[14] T. Jons and D. Drenchkahn, “Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte anion exchanger,” *EMBO Journal*, vol. 11, no. 8, pp. 2863–2867, 1992.

[15] D. Zhang, A. Kiyatkin, J. T. Bolin, and P. S. Low, “Crystalllographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3,” *Blood*, vol. 96, no. 9, pp. 2925–2933, 2000.

[16] L. An, Y. Takakuwa, W. Nunomura, S. Manno, and N. Mohandas, “Modulation of band 3-ankyrin interaction by protein 4.1: functional implications in regulation of erythrocyte membrane mechanical properties,” *Journal of Biological Chemistry*, vol. 271, no. 52, pp. 33187–33191, 1996.

[17] W. Nunomura, Y. Takakuwa, R. Tokimitsu, S. W. Krauss, M. Kawashima, and N. Mohandas, “Regulation of CD44-protein 4.1 interaction by Ca\(^{2+}\) and calmodulin. Implications for modulation of CD44-ankyrin interaction,” *Journal of Biological Chemistry*, vol. 272, no. 48, pp. 30322–30328, 1997.

[18] W. Nunomura, Y. Takakuwa, G. N. Cherr, and K. Murata, “Characterization of protein 4.1R in erythrocytes of zebrafish (Danio rerio): unique binding properties with transmembrane proteins and calmodulin,” *Comparative Biochemistry and Physiology B*, vol. 148, pp. 124–138, 2007.

[19] K. Murata, W. Nunomura, Y. Takakuwa, and G. N. Cherr, “Two different unique cardiac isoforms of protein 4.1R in zebrafish, Danio rerio, and insights into their cardiac functions as related to their unique structures,” *Development Growth and Differentiation*, vol. 52, no. 7, pp. 591–602, 2010.

[20] M. Salomao, X. Zhang, Y. Yang et al., “Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 23, pp. 8026–8031, 2008.

[21] E. Shafizadeh, B. H. Paw, H. Foot et al., “Characterization of zebrafish merlot/chablis as non-mammalian vertebrate models for severe congenital anemia due to protein 4.1 deficiency,” *Development*, vol. 129, no. 18, pp. 4359–4370, 2002.

[22] Y. Takakuwa, “Protein 4.1, a multifunctional protein of the erythrocyte membrane skeleton: structure and functions in erythrocytes and nonerythroid cells,” *International Journal of Hematology*, vol. 72, no. 3, pp. 298–309, 2000.

[23] W. Diakowski, M. Grzybek, and A. F. Sikorski, “Protein 4.1, a component of the erythrocyte membrane skeleton and its related homologue proteins forming the protein 4.1/FERM superfamily,” *Folia Histochemica et Cytobiologica*, vol. 44, no. 4, pp. 231–248, 2006.

[24] L. Y. Hung, C. J. C. Tang, and T. K. Tang, “Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the γ-tubulin complex,” *Molecular and Cellular Biology*, vol. 20, no. 20, pp. 7813–7825, 2000.

[25] B. G. Han, W. Nunomura, Y. Takakuwa, N. Mohandas, and B. K. Jap, “Protein 4.1R core domain structure and insights into regulation of cytoskeletal organization,” *Nature Structural Biology*, vol. 7, no. 10, pp. 871–875, 2000.

[26] T. Ishida and K. Kinoshita, “PrDOS: prediction of disordered protein regions from amino acid sequence,” *Nucleic Acids Research*, vol. 35, pp. W460–W464, 2007.

[27] G. M. Kelly, B. D. Zelus, and R. T. Moon, “Identification of a calcium-dependent calmodulin-binding domain in Xenopus membrane skeleton protein 4.1,” *Journal of Biological Chemistry*, vol. 266, no. 19, pp. 12469–12473, 1991.

[28] E. Leclerc and S. Vetter, “Characterization of a calcium-dependent calmodulin-binding domain in the 135-kD human protein 4.1 isoform,” *European Journal of Biochemistry*, vol. 258, no. 2, pp. 567–571, 1998.

[29] W. Nunomura, M. Parra, M. Hebiguchi, K. I. Sawada, N. Mohandas, and Y. Takakuwa, “Marked difference in membrane-protein-binding properties of the two isoforms of protein 4.1R expressed at early and late stages of erythrocyte differentiation,” *Biochemical Journal*, vol. 417, no. 1, pp. 141–148, 2009.

[30] D. Eliezer, “Biophysical characterization of intrinsically disordered proteins,” *Current Opinion in Structural Biology*, vol. 19, no. 1, pp. 23–30, 2009.

[31] L. M. Espinoza-Fonseca, “Reconciling binding mechanisms of intrinsically disordered proteins,” *Biochemical and Biophysical Research Communications*, vol. 382, no. 3, pp. 479–482, 2009.

[32] A. K. Dunker, M. S. Cortese, P. Romero, L. M. Iakoucheva, and V. N. Uversky, “Flexible nets: the roles of intrinsic disorder in protein interaction networks,” *FEBS Journal*, vol. 272, no. 20, pp. 5129–5148, 2005.

[33] Y. Minezaki, K. Homma, and K. Nishikawa, “Intrinsically disordered regions of human plasma membrane proteins preferentially occur in the cytoplasmic segment,” *Journal of Molecular Biology*, vol. 368, no. 3, pp. 902–913, 2007.

[34] A. B. Sigalov, “Membrane binding of intrinsically disordered proteins: critical importance of an appropriate membrane model,” *Self/Nonself—Immune Recognition and Signaling*, vol. 1, no. 2, pp. 129–132, 2010.

[35] A. Nörholm, R. Hendus-Altenburger, G. Bjerre, M. Kjaergaard, S. F. Pedersen, and B. B. Kragelund, “The intracellular distal tail of the Na\(^{+}/\text{H}\)\(^{+}\) exchanger NHE1 is intrinsically disordered: implications for NHE1 trafficking,” *Biochemistry*, vol. 50, pp. 3469–3480, 2011.

[36] M. K. Parra, S. Gee, N. Mohandas, and J. G. Conboy, “Efficient in vivo manipulation of alternative pre-mRNA splicing events using antisense morpholinos in mice,” *The Journal of Biological Chemistry*, vol. 286, pp. 6033–6039, 2011.

[37] M. K. Parra, J. S. Tan, N. Mohandas, and J. G. Conboy, “Intrapspin coordinates alternative first exons with alternative splicing in the protein 4.1R gene,” *EMBO Journal*, vol. 27, no. 1, pp. 122–131, 2008.

[38] W. Nunomura, Y. Takakuwa, M. Parra, J. G. Conboy, and N. Mohandas, “Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent calmodulin binding sites in erythrocyte protein 4.1. Implications for regulation of protein 4.1 interactions with transmembrane proteins,” *Journal of Biological Chemistry*, vol. 275, no. 9, pp. 6360–6367, 2000.

[39] R. V. Telamarty, B. A. Miller, R. C. Scaduto Jr., F. T. S. Yu, D. L. Tillotson, and J. Y. Cheung, “Three-dimensional intracellular gradients in single human breast-forming units-erythroblast-derived erythroblasts induced by erythropoietin,” *The Journal of Clinical Investigation*, vol. 85, pp. 1799–1809, 1990.

[40] B. A. Miller, R. C. Scaduto Jr., D. L. Tillotson, J. Botti, and J. Y. Cheung, “Erythropoietin stimulates a rise in intracellular free calcium concentration in single early human erythroid
precursors,” The Journal of Clinical Investigation, vol. 825, pp. 309–315, 1988.

[41] W. Nunomura, K. Kinoshita, M. Parra et al., “Similarities and differences in the structure and function of 4.1G and 4.1R\textsuperscript{135}, two protein 4.1 paralogues expressed in erythroid cells,” Biochemical Journal, vol. 432, no. 2, pp. 407–416, 2010.

[42] M. Parra, P. Gascard, L. D. Walensky, S. H. Snyder, N. Mohandas, and J. G. Conboy, “Cloning and characterization of 4.1G (EPB41L2), a new member of the skeletal protein 4.1 (EPB41) gene family,” Genomics, vol. 49, no. 2, pp. 298–306, 1998.

[43] P. Gascard, G. Lee, L. Coulombel et al., “Characterization of multiple isoforms of protein 4.1R expressed during erythroid terminal differentiation,” Blood, vol. 92, no. 11, pp. 4404–4414, 1998.

[44] W. Nunomura, Y. Takakuwa, M. Parra, J. Conboy, and N. Mohandas, “Regulation of protein 4.1R, p55, and Glycophorin C ternary complex in human erythrocyte membrane,” Journal of Biological Chemistry, vol. 275, no. 32, pp. 24540–24546, 2000.

[45] S. C. Huang, E. S. Liu, S. H. Chan et al., “Mitotic regulation of protein 4.1R involves phosphorylation by cdc2 kinase,” Molecular Biology of the Cell, vol. 16, no. 1, pp. 117–127, 2005.

[46] A. Kontrogianni-Konstantopoulos, C. S. Frye, E. J. Benz Jr., and S. C. Huang, “The prototypical 4.1R-10-kDa domain and the 4.1G-10-kDa paralog mediate fodrin-actin complex formation,” Journal of Biological Chemistry, vol. 276, no. 23, pp. 20679–20687, 2001.

[47] J. A. Gim, X. An, W. Nunomura, and N. Mohandas, “Functional characterization of spectrin-actin-binding domains in 4.1 family of proteins,” Biochemistry, vol. 41, no. 23, pp. 7275–7282, 2002.

[48] D. Lu, H. Yan, T. Othman, C. P. Turner, T. Woolf, and S. A. Rivkees, “Cytoskeletal protein 4.1G binds to the third intracellular loop of the A1 adenosine receptor and inhibits receptor action,” Biochemical Journal, vol. 377, no. 1, pp. 51–59, 2004.

[49] M. Saito, M. Sugai, Y. Katsushima, T. Yanagisawa, J. Sukegawa, and N. Nakahata, “Increase in cell-surface localization of parathyroid hormone receptor by cytoskeletal protein 4.1G,” Biochemical Journal, vol. 392, no. 1, pp. 75–81, 2005.
Band 3 Missense Mutations and Stomatocytosis: Insight into the Molecular Mechanism Responsible for Monovalent Cation Leak

Damien Barneaud-Rocca,1,2 Bernard Pellissier,1,2 Franck Borgese,1,2 and Hélène Guizouarn1,2

1 Institut de Biologie du Développement et Cancer, UMR6543, CNRS, 28 Avenue Valrose, 06108 Nice Cedex 2, France
2 Institut de Biologie du Développement et Cancer, Université de Nice, 06108 Nice Cedex 2, France

Correspondence should be addressed to Hélène Guizouarn, guizouar@unice.fr

Received 6 April 2011; Revised 27 May 2011; Accepted 29 May 2011

Academic Editor: Lars Kaestner

Copyright © 2011 Damien Barneaud-Rocca et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Missense mutations in the erythroid band 3 protein (Anion Exchanger 1) have been associated with hereditary stomatocytosis. Features of cation leaky red cells combined with functional expression of the mutated protein led to the conclusion that the AE1 point mutations were responsible for Na⁺ and K⁺ leak through a conductive mechanism. A molecular mechanism explaining mutated AE1-linked stomatocytosis involves changes in AE1 transport properties that become leaky to Na⁺ and K⁺. However, another explanation suggests that point-mutated AE1 could regulate a cation leak through other transporters. This short paper intends to discuss these two alternatives.

1. Introduction

Band 3 or anion exchanger 1 (AE1) is the major red cell membrane protein. It belongs to the Solute Carrier 4A family (SLC4A) grouping bicarbonate transporters [1–3]. This protein catalyzes electroneutral chloride-bicarbonate exchange and it is also expressed in kidney α-intercalated cells and in cardiomyocytes [4, 5]. In red cells it is involved in two main tasks: enhancement of carbon dioxide transport and structuration of cell shape. It is found in red cells from all vertebrates except lampreys which naturally do not express erythrocyte AE1 [6]. Besides this exception, its complete absence from mammalian red cells leads to red cell defects which consequences on health depends on the species. Dyserythropoiesis, severe haemolytic anaemia and often premature death have been reported in mouse [7] and human [8] whereas cow or zebra fish seems to better withstand red cell AE1 deficiency [9, 10].

In human, many different mutations in SLC4A1 gene coding for AE1 have been reported [11]. Some of them are asymptomatic whereas some others are associated with red cell pathologies characterized by alteration of red cell shape and rheological properties. As this protein is also expressed in kidney, a renal phenotype can be associated with SLC4A1 mutations. In this review we will focus on red cell AE1 and the reader interested in kidney AE1 is therefore addressed to very exhaustive recent reviews on this subject [12–15].

When a red cell phenotype is associated with SLC4A1 mutations the symptoms are hyper haemolysis and anaemia, icterus, splenomegaly. However, these symptoms may vary widely in intensity. It appears that the SLC4A1 mutations can be divided into two classes according to the way they impair AE1 function: (1) those that prevent correct folding of the protein so that it is not addressed to plasma membrane. This leads to a lower amount of AE1 in red cell membrane that impairs connection of skeleton and membrane, a feature of hereditary spherocytosis condition [16, 17].

(2) those that are associated with an increased cation permeability of red cell membrane. This latter condition is the hallmark of hereditary stomatocytosis [18, 19].

Since the initial discovery that 5 point mutations in SLC4A1 gene (responsible for: L687P, D705Y, S731P, H734R or R760Q substitutions in AE1) were associated with increased red cell Na⁺ and K⁺ leak [20], 4 other point mutations associated with similar red cell phenotype have been reported (G796R, E758K, S762R, R730C) [21–24].
been proposed that the molecular mechanism accounting for cation leaky red cells in these hereditary stomatocytoses was a change in AE1 transport properties induced by the point mutations. The exchanger itself mediates cation leak by a conductive mechanism [25]. However, this interpretation leads to dramatically change our way of thinking about band 3 transport mechanism. Moreover, the transport features of some of these AE1 mutants, lead to another interpretation, that is cation leak is due to the activation of endogenous Na+ and K+ transporters (or channels) in red cell membrane by mutated AE1 [23, 24, 26]. This short review intends to discuss the molecular mechanism of red cell cation leak associated with AE1 point mutations in hereditary stomatocytosis.

1.1. Position of Amino Acid Substitutions in AE1 Polypeptide. AE1 polypeptide can be divided in 3 functional domains: a cytoplasmic amino-terminal domain, about 400 amino acids, interacting in red cells with various enzymes, haemoglobin, ankyrin and band 4.2; a membrane spanning domain where anion exchange takes place and a short carboxy terminal end in the cytoplasm that associates with carbonic anhydrase II [27]. The protein forms part of a macromplex, combining membrane and cytoplasmic proteins that is thought to improve the efficiency of gas transport by red cells [28].

Figure 1 illustrates the position of each of the point mutations that have been identified in patients with cation leaky red cells. The substitutions concern highly conserved amino acids among known electroneutral anion exchangers (SLC4A1, A2 and A3) and they are all located in the membrane spanning domain.

1.2. Point Mutated AE1 and Permeability Features. The permeability of red cells from patients bearing heterozygous mutation on AE1 has been investigated and the transport features of the point mutated AE1 have been studied by expression in amphibian oocytes. Thus in most cases, it was possible to combine data from red cells to data from heterologous expression system. These data are presented here and will be discussed in the third part. Table 1 summarized the main features of cation leaky red cells and mutated AE1.

The red cell leaks Na+ and K+ by an ouabain and bumetanide resistant mechanism that is temperature dependent, it is increased by temperatures below 37°C. This has been extensively studied in red cells of patients heterozygous for L687P, D705Y, S731P, H734R, R760Q and S762R AE1 mutants [20, 21]. The diffusion of K+ and Na+ according to their electrochemical gradients leads to osmotic fragility of the red cells. At body temperature, the cation leak can be more or less compensated depending on the mutants. An artefactual rise in plasma K+ (pseudohyperkalaemia) can be observed after cooling blood to room temperature [30]. The shape of cation leak temperature dependence is not identical between the mutants [20] and the morphology of red cells also shows some differences between patients: blood smears exhibit stomatocytes or spherocytes. In red cells with H734R or G796R AE1 mutations, an increased activity of Na+/K+-2Cl− cotransporter, K+−Cl− cotransporter, Na+/H+ exchanger or K+/Na+/H+ exchanger has been reported [22, 26]. R730C mutant is also associated with increased activity of Na+/H+ exchanger and Na+/K+ -pump, whereas the Gardos channel fluxes are reduced [23]. Thus, the red cell permeability results from both, the monovalent cation leak induced by AE1 point mutations and the activity of solute carriers that have been stimulated by the initial Na+ and K+ movements. The regulation of these other carriers could differ between patients as well as how the body cope with cation leaky red cells. This could explain variations in patient's phenotypes (Table 1).

For all the studied patients, the abundance of AE1 in red cells is grossly normal. However, the anion permeability of these cells is decreased suggesting a loss of anion exchange...
Table 1: Features of cation leaky red cells and point mutated AE1.

| AE1 point mutation | Red cell shape | Abundance of AE1 (band 3) in red cell membrane | Red cell cation leak rate at 0°C (multiple of normal) | Anion exchange activity of mutated AE1 | Pharmacology of the red cell cation leak | Pharmacology of the cation leak in heterologous expression system (cation conductance or cation flux) |
|-------------------|---------------|-----------------------------------------------|------------------------------------------------------|--------------------------------------|---------------------------------------|----------------------------------------------------------------------------------|
| L687P             | Stomatocyte   | 82%                                           | 7-8                                                  | abolished                           | NS1652, SITS, dipyridamol             | Cation conductance: SITS, Zn²⁺, La³⁺ sensitive                                   |
| D705Y             | Spherocyte    | 77%                                           | 8                                                    | abolished                           | NS1652, SITS, dipyridamol             | Cation conductance: SITS, Zn²⁺, La³⁺ sensitive                                   |
| R730C             | Stomatocyte   | normal                                        | 6 (at 37°C)                                          | abolished                           | NT                                    | NT                                                                               |
| S731P             | Stomatocyte   | 79%                                           | 30–57-58–87                                          | abolished                           | NS1652, SITS, dipyridamol             | Cation conductance: SITS, Zn²⁺, La³⁺ sensitive                                   |
| H734R             | Stomatocyte   | 74–82%                                        | 87–94                                                | abolished                           | NS1652, SITS, dipyridamol             | Cation conductance: SITS, Zn²⁺, La³⁺ sensitive                                   |
| E758K             | Spherostomatocyte | Mild deficiency                              | NT                                                   | Normal with GPA coexpression        | NT                                    | Rb flux: DIDS, Zn²⁺, Gd³⁺ sensitive, Conductance: Zn²⁺, SITS and WW-781 sensitive |
| R760Q             | Spherocyte    | 85–92%                                        | 4–6                                                  | 74% of wt (with GPA)                | NS1652, SITS, dipyridamol             | NT                                                                               |
| S762R             | Stomatocyte   | NT                                            | 7                                                    | abolished                           | NT                                    | NT                                                                               |
| G796R             | Stomatocyte   | normal                                        | NT                                                   | abolished                           | NT                                    | Li uptake insensitive to SITS or H₂DIDS                                        |

This table summarized data collected from different publications. For L687P, D705Y, S731P, H734R: [20, 25]. For R730C: [23]. For E758K: [24]. For R760Q: [20, 31]. For S762R: [21]. For G796R: [22]. NT: not tested.

1 Each number refers to features of red cells from different patients carrying the same AE1 mutation.

2 The anion exchange was assessed in amphibian oocyte expressing the mutated AE1. The loss of anion exchange is confirmed in heterozygote red cells by ≈50% decreased anion flux.

function of the protein [20]. Indeed, functional characterization of L687P, D705Y, R730C, S731P, H734R, S762R and G796R mutants expressed in xenopus oocytes shows that they are no more able to exchange Cl⁻ and HCO₃⁻ [21, 25]. In contrast E758K and R760Q mutants keep an anion exchange activity [24, 31]. Another interesting difference for these two mutants is that their abundance in plasma membrane is highly dependent on glycoporphin A (GPA) co-expression. GPA is known to bind AE1 and to act as a chaperone. Moreover this interaction stimulates AE1 transport activity [32–34].

Very few studies are available about conductance of these stomatocytic red cells. Only conductance of red cells from two patients with R730C or H734R mutation on AE1 has been reported. Patch current recordings on 3 red cells from a patient with R730C AE1 mutation do not allow to detect increased cation conductance compared to normal red cells [23]. Similar conclusions have been drawn from conductance analyses on red cells with H734R AE1 mutation [26]. Expression of L687P, D705Y, S731P, H734R, R760Q, S762R or G796R AE1 mutants in xenopus oocytes induces a reversal in xenopus oocyte Na⁺ and K⁺ contents after 3 days in medium with ouabain and bumetanide. This cation leak is associated with increased ouabain and bumetanide-resistant Rb⁺ or Li⁺ uptake which is similar to the red cell cation leak [20–22]. The Na⁺ and K⁺ transport associated with AE1 missense mutations shows independent movement of Na⁺ and K⁺ with a 1 for 1 stoichiometry. Moreover, when assessed, a conductance has been associated with these cation movements. Thus the molecular mechanism responsible for the observed cation movement is a channel like transport mechanism [25].

Pharmacology of transport activities of the mutants have been assessed in red cells or in heterologous expression systems. DIDS (4,4′-disothiocyanostilbene-2,2′-disulfonate) has long been known to inhibit anion exchange at micromolar concentrations [35, 36]. Moreover, sulfonate radical of DIDS can link two different lysines (K539 and K851) in putative transmembrane helices (TM) 5 and 12 of AE1 membrane spanning domain [36]. It is thus possible to covalently bind DIDS on each or both of these two lysines. The DIDS derivative SITS (4-acetamido-4′-isothiocyanato-2,2′-stilbene disulfonate), flufenamic acid and niflumic acid are also potent inhibitors of AE1 activity. It is observed that the point mutations impair the protein sensitivity to classical anion exchanger inhibitors. For instance, S731P...
mutation prevents DIDS covalent binding to the exchanger [20]. For the two mutants keeping anion exchange activity, the DIDS sensitivity of the transport is also impaired. Figure 2 illustrates Cl⁻/HCO₃⁻ exchange as a function of DIDS concentrations for R760Q mutant compared to wt AE1. The R760Q mutation decreases AE1 DIDS sensitivity as shown by the right shift of the dose-response curve. The anion uptake mediated by E758K mutant is also less sensitive to DIDS than wtAE1 [24].

Pharmacology of the cation leak induced by AE1 point mutations has also been investigated. Inhibition of the Na⁺ and K⁺ leak induced by L687P, D705Y, S731P, H734R and R760Q mutations has been observed in red cells with SITS, dipyridamole and NS1652 also known to block anion exchange by DIDS. However, inhibition of the Na⁺ and K⁺ conductance induced by expression of mutated AE1 was observed with SITS and plurivalent cations such as Zn²⁺, La³⁺ and Gd³⁺ [24, 25]. Amidst AE1 point mutations associated with cation leaky red cells, two mutants (R730C and E758K) exhibit peculiar transport features in amphibian oocytes. R730C AE1 mutant induces only a weak ouabain and bumetanide resistant cation leak in xenopus oocytes. It is not possible to measure a significant increase in ouabain and bumetanide-resistant ⁸⁶Rb⁺ uptake and only a 2.5 fold increase in Li⁺ uptake is observed (to compare to ~8 fold increase in oocytes expressing S731P mutant for instance). Moreover expression of R730C mutant is associated with an increased activity of the Na⁺/K⁺ ATPase [23]. For E758K mutant permeability features depend on the expression system. It has been studied in two different amphibian oocytes, xenopus and ambystoma. In both species, its abundance in plasma membrane is dependent on the coexpression of GPA. The mutant keeps anion exchange activity and induces a ⁸⁶Rb⁺ uptake in both systems. However, it appears that this Rb⁺ permeability is not correlated to the expression level of the mutant when expressed in ambystoma: the higher number of transporters when coexpressed with GPA does not induce a higher ⁸⁶Rb⁺ uptake. The expression of E758K mutant also slightly increases xenopus oocyte conductance but this conductance does not account for the observed Rb⁺ permeability as deduced from differences in pharmacological pattern [24].

1.3. What Is the Pathway for Cations in Cells Expressing Point Mutated AE1? Point mutations in AE1 are associated with monovalent cation leak in red cells as in heterologous expression systems. This monovalent cation leak could be correlated to a non selective cation conductance and to elevated activity of endogenous transport systems. Two possibilities that are not exclusive could be envisioned: the missense mutations in AE1 polypeptide change the transport properties of the protein that becomes leaky to Na⁺ and K⁺, or the mutated AE1 stimulates native transporters for Na⁺ and K⁺ in red cells as in heterologous expression systems.

The work on trout AE1 has shown that this protein could interact with Na⁺-K⁺-2Cl⁻ cotransporter by its carboxy terminal domain, stimulating the activity of this transporter in xenopus oocytes [39]. Reports on E758K and R730C AE1 mutations suggest that the cation leak associated with these mutations likely involves activation of still undefined endogenous transporters. As AE1 forms part of a macrocomplex it functionally interacts with carbonic anhydrase [27, 40] and it is also involved in many molecular interactions in red cells, with ankyrin, glycophorin A (GPA), glycolytic enzymes or haemoglobin for instance [28, 41–43]. It is thus plausible that point mutated AE1 could interfere with different endogenous transporters (understood as pumps, channels or carriers) in red cells in heterologous expression systems. It could be proposed that point mutations by changing AE1 conformation enable molecular interactions regulating the activity of various endogenous transporters. An AE1 mutated conformation could be envisioned which would not dramatically change AE1 transport features but would activate endogenous monovalent cation permeabilities in red cells as in heterologous expression systems. That should happen with different point mutations in AE1 membrane spanning domain.

Since pioneer work of electrophysiologists on red cells in the 80s [44], numerous cation and anion conductances have been characterized. Anion conductances (maxi-anion channels [45, 46]) or cation conductances such as non-selective Ca²⁺ permeable cation channels (L-type Ca²⁺ channel, voltage-dependent) or non-selective voltage-independent cation channels (NSVCC) [47–51] and Ca²⁺ sensitive K⁺ channel (Gardos channel) [44] are well characterized in human red cells. It has been proposed that the TRPC6, member of
the Transient Receptor Potential family proteins, contributes to the non-selective voltage-independent cation current in red cells [52]. However, the molecular identity of channels responsible for most of the electrophysiologically described conductances is unknown. It could be proposed that the non selective Na⁺ and K⁺ leak induced by AE1 point mutations could be mediated by one of these conductances. However, the features of the cation leak associated with AE1 point mutations do not point out any of the red cell channels described so far. In particular, this cation leak is insensitive to amiloride known to block the NSVCC; it is insensitive to extracellular Cl⁻ concentration, known to stimulate red cell cation channels and it is insensitive to extracellular Ca²⁺ concentration [25]. Thus, in red cells as in heterologous expression system, the molecular identity of the transporters eventually activated by point mutated AE1 still remains unknown as their activating mechanism.

Whatever the origin of the cation leak induced by AE1 point mutations, the consecutive alteration of cation permeability will impair red cell homeostasis and modulate the activity of different transporters. The permeability features of red cells with H734R, G796R or R730C AE1 mutations show that the activity of different transporters could be stimulated: Na⁺-K⁺-2Cl⁻ cotransporter, Na⁺/K⁺ ATPase, Na⁺(K+)/H⁺ exchanger and K⁺-Cl⁻ cotransporter [22, 23, 26]. This could be due to functional interactions between the AE1 point mutation induced cation leak and the mentioned transporters. As a consequence, a same AE1 mutation could lead to diverse phenotypes in red cells depending on how the endogenous transporters react to the initial cation leak.

In the absence of identified native transporter mediating the cation leak associated with AE1 point mutations, the hypothesis of a cation leaky AE1 is challenging and the simplest to propose. Moreover, it provides an attractive approach to understand the transport mechanism of this protein. Whereas it is depicted as a typical electroneutral anion exchanger, the anion exchange rate through AE1 is extremely fast (10 000 per second) and Cl⁻ slippage occurs occasionally (1 for 10 000 exchange). Crystallographic structures are not available yet with enough resolution to help understand the transport mechanism [53]. This mechanism should allow very rapid conformational changes that could resemble alternately opened gates for instance. Previous work on trout AE1 has shown that this exchanger can behave as an anion conductance permeable to organic solutes (taurine, sorbitol) and to monovalent cations (Na⁺ and K⁺) [54, 55]. The work on a truncated human AE1 has also shown that this protein could behave like a conductance when it was deleted of transmembrane segments 6 and 7 [56]. Thus, it appears feasible to convert the electroneutral anion exchanger into a conductive pathway by different manoeuvres. Former studies in red cells, based on pharmacological evidence, have also suggested that a monovalent cation leak through AE1 could be induced by decreasing extracellular Cl⁻ concentration [57]. The speculations about a monovalent cation leak through AE1 polypeptide have to combine the following considerations.

The point mutations induce a conformational change of the protein as suggested by changes in pharmacology sensitivity (Figure 2), by impairment of anion exchange capacity or by requirement of GPA for correct addressing to plasma membrane. Moreover, diverse point mutations are likely to produce a similar AE1 conformation as deduced from similar permeability patterns. The transport features of cells expressing AE1 suggest at least three functional states for AE1: the wt state, a mutated state where no anion exchange occurs only cation leak exists and a B mutated state where anion exchange and cation leak coexist (Figure 3). Whether or not these functional states are linked to different structural states of AE1 has to be shown but it is likely.

It appears that apparent unrelated positions in AE1 membrane spanning domain are susceptible to impair anion exchange function in the same way: point mutations L687P, D705Y, R730C, S731P, H734R, S762R and G796R mutations. The R760Q mutations.

![Figure 3: Putative transport states of AE1](image)

**Figure 3:** Putative transport states of AE1. Whereas the wt AE1 does only exchange Cl⁻ and HCO₃⁻, a A mutated state only conducts monovalent cations and a B mutated state shows both transport activities, anion exchange and cation leak. The A state should be obtained with L687P, D705Y, R730C, S731P, H734R, S762R and G796R mutations. The B state should be obtained with E758K and R760Q mutations.

In wtAE1, the anion exchange site involves transmembrane helix 8 (TM 8) and an anion selectivity filter including a region at the top of TM 12 and 13 and amino acids in the loop connecting TM 7 and 8 [61, 62]. Our recent work on the cation leaky H734R mutant has shown that the same TM 8 was also involved in cation movement suggesting a common pathway for anions and cations through AE1 [63]. Moreover, it was shown that amino acids in the intracellular loop connecting TM 8 and TM 9 play an important role in AE1
transport features. For instance, substitution of the charged residues in this loop induces a cation leak and severely impairs anion exchange activity. The position of the point mutations 5731P, H734R, E758K, R760Q and S762R at both extremities of the next loop connecting TM 9 and 10, also suggests an important functional role for this central part of the membrane spanning domain. Amino acid substitutions in these two loops could change the orientation, rotation or movements of TM 8, 9 and 10 and impair AE1 transport site. The leak could be seen as a broken seal in the transport system that leaks Na\(^{+}\) and K\(^{+}\) for which a high driving force exists. This leads to consider that the transport site is susceptible to structural changes that could be induced by diverse but specific amino acid substitutions. This change unmarshes a conductance for monovalent cations that does not seem to interfere with the ability to exchange anions since some mutants exhibit both transport activities.

The possibility for a carrier to function as a channel seems conflicting. Indeed channels are seen as structures that could simultaneously connect intra and extracellular medium, what should never happen through a carrier. However, there are increasing examples of membrane proteins with ambiguous behaviour between channels and transporters. A historical example of transporter with channel activity is the glutamate transporter which is also a chloride channel [64–66]. Chloride channels, Na\(^{+}\)-K\(^{+}\) pump are other examples of ambiguous transport mechanisms between channels and transporters which strengthen our simple hypothesis of cation leaky AE1 [65–69]. In red cells, monovalent cation leak has also been associated with heterozygous mutations on RhAG (Rhesus Associated Glycoprotein) gene. Two different amino acid substitutions in RhAG could turn on a cation pore through this membrane protein proposed to be a NH\(_3\)/NH\(_4\)\(^{+}\) transporter in red cells [70].

2. Conclusion

Whereas specific AE1 mutations are undoubtedly linked to cation leaky red cells responsible for hereditary haemolytic anaemia, it is observed that all the 9 AE1 mutations presented here do not impair AE1 transport features in a similar manner. Moreover, membrane permeability of cells expressing point mutated AE1 shows some differences suggesting a complicated regulation of this permeability. The proposition of point mutations altering AE1 transport mechanism is an attractive hypothesis supported by experimental evidence. However, this does not exclude the possibility for some mutated AE1 to also regulate the activity of other transporters.

Resolution of the 3D structure of AE1 would greatly help to understand the peculiar transport properties of this surprising protein. It would be of particular interest to know how the studied AE1 point mutations alter AE1 structure, if these different point mutations have a common mechanism of action on the structure. A better understanding of the mechanistic of interactions between AE1 and its partners would also help to assign new regulatory function to AE1.

References

[1] S. L. Alper, “Molecular physiology of SLC4 anion exchangers,” Experimental Physiology, vol. 91, no. 1, pp. 153–161, 2006.
[2] A. Pushkin and I. Kurtz, “SLC4 base (HCO\(_3^{-}\), CO\(_2^{-}\)) transporters: classification, function, structure, genetic diseases, and knockout models,” American Journal of Physiology, vol. 290, no. 3, pp. F580–F599, 2006.
[3] M. F. Romero, “Molecular pathophysiology of SLCA4 bicarbonate transporters,” Current Opinion in Nephrology and Hypertension, vol. 14, no. 5, pp. 495–501, 2005.
[4] N. Hamasaki and K. Okubo, “Band 3 protein: physiology, function and structure,” Cellular and Molecular Biology (Noisy-le-Grand, France), vol. 42, no. 7, pp. 1025–1039, 1996.
[5] M. Pucet, I. Koricheva, R. Cassoly, and G. Vassort, “Identification of band 3 like proteins and Cl-/HCO3/- exchange in isolated cardiomycocytes,” The Journal of Biological Chemistry, vol. 270, no. 3, pp. 1315–1322, 1995.
[6] H. Hägerstrand, M. X. Daniellsit, M. X. Bobrowska-Hägerstrand et al., “Influence of band 3 protein absence and skeletal structures on amphiphile- and Ca\(^{2+}\)-induced shape alterations in erythrocytes: a study with lamprey (Lamperia fluviatilis), trout (Onchorhynchus mykiss) and human erythrocytes,” Biochimica et Biophysica Acta, vol. 1466, no. 1-2, pp. 125–138, 2000.
[7] C. D. Southgate, A. H. Chishiti, B. Mitchell, S. J. Yi, and J. Palek, “Targeted disruption of the murine erythroid band 3 gene results in spherocytosis and severe haemolytic anaemia despite a normal membrane skeleton,” Nature Genetics, vol. 14, no. 2, pp. 227–230, 1996.
[8] M. L. Ribeiro, N. Alloisio, H. Almeida et al., “Severe hereditary spherocytosis and distal renal tubular acidosis associated with the total absence of band 3,” Blood, vol. 96, no. 4, pp. 1602–1604, 2000.
[9] M. Inaba, A. Yawata, I. Koshino et al., “Defective anion transport and marked spherocytosis with membrane instability caused by hereditary total deficiency of red cell band 3 in cattle due to a nonsense mutation,” The Journal of Clinical Investigation, vol. 97, no. 8, pp. 1804–1817, 1996.
[10] B. H. Paw, A. J. Davidson, Y. Zhou et al., “Cell-specific mitotic defect and dyserythropoiesis associated with erythroid band 3 deficiency,” Nature Genetics, vol. 34, no. 1, pp. 59–64, 2003.
[11] P. Jarolím, “Disorders of band 3,” in Red Cell Membrane Transport in Health and Disease, J. C. Ellory and I. Bernhardt, Eds., pp. 603–619, Springer, Berlin, Germany, 2003.
[12] S. L. Alper, “Diseases of mutations in the SLC4A1/AE1 polypeptide,” in Membrane Transport Diseases, S. Broer and C. Wagner, Eds., pp. 39–63, Kluwer Academic, Boston, Mass, USA, 2003.
[13] S. L. Alper, “Familial renal tubular acidosis,” Journal of Nephrology, vol. 23, supplement 16, pp. S57–S76, 2010.
[14] L. J. Bruce and M. J. A. Tanner, “Erythroid band 3 variants and disease,” Bailliere’s Best Practice and Research in Clinical Haematology, vol. 12, no. 4, pp. 637–654, 1999.
[15] A. M. Toye, “Defective kidney anion-exchanger 1 (AE1, Band 3) trafficking in dominant distal renal tubular acidosis (dRTA),” Biochemical Society Symposium, vol. 72, pp. 47–63, 2005.
[16] A. Iolascon, E. M. Del Giudice, S. Ferrotta, N. Alloisio, L. Morlè, and J. Delaunay, “Hereditary spherocytosis: from clinical to molecular defects,” Haematologica, vol. 83, no. 3, pp. 240–257, 1998.
[17] P. Jarolim, J. L. Murray, H. L. Rubin et al., “Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency,” Blood, vol. 88, no. 11, pp. 4366–4374, 1996.
H. Guizouarn, S. Martial, N. Gabillat, and F. Borgese, “Point
A. Bogdanova, J. S. Goede, E. Weiss et al., “Cryohydrocytosis:
International Journal of Cell Biology 7
G. W. Stewart, “Hemolytic disease due to membrane ion
A. K. Stewart, P. S. Kedar, B. E. Shmukler et al., “Functional
A. Iolascon, L. De Falco, F. Borgese et al., “A novel erythroid
H. Guizouarn, F. Borgese, N. Gabillat et al., “South-east
L. J. Bruce, H. C. Robinson, H. Guizouarn et al., “Monovalent
L. J. Bruce, R. Beckmann, M. L. Ribeiro et al., “A band 3-based
J. C. Ellory, H. Guizouarn, F. Borgese, L. J. Bruce, R. J.
J. D. Groves and M. J. A. Tanner, “Glycophorin A facilitates
R. C. Williamson and A. M. Toye, “Glycophorin A; band 3 aid,”
Blood, Cells, Molecules, and Diseases, vol. 41, no. 1, pp. 35–43, 2008.
M. T. Young and M. J. A. Tanner, “Distinct regions of human
glycoporphin A enhance human red cell anion exchanger
(band 3; AE1) transport function and surface trafficking,” The
Journal of Biological Chemistry, vol. 278, no. 35, pp. 32954–32961, 2003.
M. J. Jennings and H. Passow, “Anion transport across the
erthrocyte membrane, in situ proteolysis of band 3 protein,
and cross-linking of proteolytic fragments by 4,4’-
diisothiocyanato dihydrostilbene-2,2'-disulfonate,” Biochimica et
Biophysica Acta, vol. 554, no. 2, pp. 498–519, 1979.
K. Okubo, D. Kang, N. Hamasaki, and M. L. Jennings, “Red
blood cell band 3. Lysine 539 and lysine 851 react with the same
H2DIDS (4,4’-diisothiocyanatohydrostilbene-2,2’-
disulfonic acid) molecule,” The Journal of Biological Chemistry,
vol. 269, no. 3, pp. 1918–1926, 1994.
A. Bielfeld-Ackermann, C. Range, and C. Korbmacher,
“Maitotoxin (MTX) activates a nonselective cation channel in
Xenopus laevis oocytes,” Pflugers Archiv European Journal of
Physiology, vol. 436, no. 3, pp. 329–337, 1998.
A. Diakov, J. P. Koch, O. Ducoudret, S. Müller-Berger, and E.
Frömter, “The disulfonic stilbene DIDS and the marine poison
maitotoxin activate the same two types of endogenous cation
conductance in the cell membrane of Xenopus laevis oocytes,”
Pflugers Archiv European Journal of Physiology, vol. 442, no. 5,
pp. 700–708, 2001.
H. Guizouarn, N. Gabillat, and F. Borgese, “Evidence for
Up-regulation of the Endogenous Na+-K+-2Cl– Copporter
by Molecular Interactions with the Anion Exchanger tAE1
Expressed in Xenopus Oocyte,” The Journal of Biological
Chemistry, vol. 279, no. 12, pp. 11513–11520, 2004.
D. Sterling, R. A. F. Reithmeier, and J. R. Casey, “A transport
metabolon: functional interaction of carbonic anhydrase II
and chloride/bicarbonate exchangers,” The Journal of Biological
Chemistry, vol. 276, no. 51, pp. 47886–47894, 2001.
M. E. Campanella, H. Chu, N. J. Vandersee et al., “Character-
zization of glycolytic enzyme interactions with murine
erythrocyte membranes in wild-type and membrane protein
knockout mice,” Blood, vol. 112, no. 9, pp. 3900–3906, 2008.
H. Chu and F. S. Low, “Mapping of glycolytic enzyme-binding
sites on human erythrocyte band 3,” Biochemical Journal,
vol. 400, no. 1, pp. 143–151, 2006.
G. Chétrie and R. Cassoly, “Affinity of hemoglobin for the
cytoplasmic fragment of human erythrocyte membrane band
3. Equilibrium measurements at physiological pH using
matrix-bound proteins: the effects of ionic strength, deoxy-
genation and of 2,3-diphosphoglycerate,” Journal of Molecular
Biology, vol. 185, no. 3, pp. 639–644, 1985.
R. Grygorczyk and W. Schwarz, “Properties of the Ca2+-
activated K+ conductance of human red cells as revealed by the
patch-clamp technique,” Cell Calcium, vol. 4, no. 5–6, pp.
499–510, 1983.
G. Decherf, G. Bouyer, S. Egèe, and S. L. Y. Thomas, “Chloride
channels in normal and cystic fibrosis human erythrocyte
membrane,” Blood Cells, Molecules, and Diseases, vol. 39, no.
1, pp. 24–34, 2007.
E. Głogowska, A. Dydra, A. Cufel et al., “Anion conductance of
the human red cell is carried by a maxi-anion channel,” Blood
Cells, Molecules, and Diseases, vol. 44, no. 4, pp. 243–251, 2010.
P. Bennekou, T. L. Barksman, B. I. Kristensen, L. R. Jensen,
and P. Christophersen, “Pharmacology of the human red cell
voltage-dependent cation channel. Part II: inactivation and
blocking,” Blood Cells, Molecules, and Diseases, vol. 33, no. 3,
pp. 356–361, 2004.
[48] P. Bennekou, T. L. Barksmann, L. R. Jensen, B. I. Kristensen, and P. Christophersen, “Voltage activation and hysteresis of the non-selective voltage-dependent channel in the intact human red cell,” Bioelectrochemistry, vol. 62, no. 2, pp. 181–185, 2004.

[49] P. Christophersen and P. Bennekou, “Evidence for a voltage-gated, non-selective cation channel in the human red cell membrane,” Biochimica et Biophysica Acta, vol. 1065, no. 1, pp. 103–106, 1991.

[50] L. Kaestner and I. Bernhardt, “Ion channels in the human red blood cell membrane: their further investigation and physiological relevance,” Bioelectrochemistry, vol. 55, no. 1-2, pp. 71–74, 2002.

[51] L. Kaestner, P. Christophersen, I. Bernhardt, and P. Bennekou, “The non-selective voltage-activated cation channel in the human red blood cell membrane: reconciliation between two conflicting reports and further characterisation,” Bioelectrochemistry, vol. 52, no. 2, pp. 117–125, 2000.

[52] M. Föller, R. S. Kasinathan, S. Koka et al., “TRPC6 contributes to the Ca2+ leak of human erythrocytes,” Cellular Physiology and Biochemistry, vol. 21, no. 1–3, pp. 183–192, 2008.

[53] T. Yamaguchi, T. Fujii, Y. Abe et al., “Helical image reconstruction of the outward-open human erythrocyte band 3 membrane domain in tubular crystals,” Journal of Structural Biology, vol. 169, no. 3, pp. 406–412, 2010.

[54] H. Guizouarn, N. Gabillat, R. Motais, and F. Borgese, “Multiple transport functions of a red blood cell anion exchanger, tAE1: its role in cell volume regulation,” Journal of Physiology, vol. 535, no. 2, pp. 497–506, 2001.

[55] S. Martial, H. Guizouarn, N. Gabillat, B. Pellissier, and F. Borgese, “Consequences of point mutations in trout anion exchanger 1 (tAE1) transmembrane domains: evidence that tAE1 can behave as a chloride channel,” Journal of Cellular Physiology, vol. 207, no. 3, pp. 829–835, 2006.

[56] M. D. Parker, M. T. Young, C. M. Daly, R. W. Meech, W. F. Boron, and M. J. A. Tanner, “A conductive pathway generated from fragments of the human red cell anion exchanger AE1,” Journal of Physiology, vol. 581, no. 1, pp. 33–50, 2007.

[57] G. S. Jones and P. A. Knauf, “Mechanism of the increase in cation permeability of human erythrocytes in low-chloride media,” Journal of General Physiology, vol. 86, no. 5, pp. 721–738, 1985.

[58] P. Jarolim, J. Palek, D. Amato et al., “Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis,” Proceedings of the National Academy of Sciences of the United States of America, vol. 88, no. 24, pp. 11022–11026, 1991.

[59] S. J. Allen, A. O’Donnell, N. D. E. Alexander et al., “Prevention of cerebral malaria in children in Papua New Guinea by Southeast Asian ovalocytosis band 3,” American Journal of Tropical Medicine and Hygiene, vol. 60, no. 6, pp. 1056–1060, 1999.

[60] B. Fiévet, N. Gabillat, F. Borgese, and R. Motais, “Expression of band 3 anion exchanger induces chloride current and taurine transport: structure-function analysis,” The EMBO Journal, vol. 14, no. 21, pp. 5158–5169, 1995.

[61] X. B. Tang, M. Kovacs, D. Sterling, and J. R. Casey, “Identification of residues lining the translocation pore of human AE1, plasma membrane anion exchange protein,” The Journal of Biological Chemistry, vol. 274, no. 6, pp. 3557–3564, 1999.

[62] Q. Zhu and J. R. Casey, “The substrate anion selectivity filter in the human erythrocyte Cl–/HCO3– exchange protein, AE1,” The Journal of Biological Chemistry, vol. 279, no. 22, pp. 23565–23573, 2004.

[63] D. Barneaud-Rocca, F. Borgese, and H. Guizouarn, “Dual transport properties of anion exchanger 1: the same transmembrane segment is involved in anion exchange and in a cation leak,” The Journal of Biological Chemistry, vol. 286, no. 11, pp. 8909–8916, 2011.

[64] H. P. Larsson, S. A. Picaut, F. S. Werblin, and H. Lecar, “Noise analysis of the glutamate-activated current in photoreceptors,” Biophysical Journal, vol. 70, no. 2, pp. 733–742, 1996.

[65] R. M. Ryan and R. J. Vandenberg, “A channel in a transporter,” Clinical and Experimental Pharmacology and Physiology, vol. 32, no. 1-2, pp. 1–6, 2005.

[66] R. J. Vandenberg, S. Huang, and R. M. Ryan, “Slips, leaks and channels in glutamate transporters,” Channels, vol. 2, no. 1, pp. 51–58, 2008.

[67] L. J. DeFelice and T. Goswami, “Transporters as channels,” Annual Review of Physiology, vol. 69, pp. 87–112, 2007.

[68] C. Miller, “ClC chloride channels viewed through a transporter lens,” Nature, vol. 440, no. 7083, pp. 484–489, 2006.

[69] A. Takeuchi, N. Reyes, P. Artigas, and D. C. Gadsby, “The ion pathway through the opened Na+K+-ATPase pump,” Nature, vol. 456, no. 7220, pp. 413–416, 2008.

[70] L. J. Bruce, H. Guizouarn, N. M. Burton et al., “The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein,” Blood, vol. 113, no. 6, pp. 1350–1357, 2009.
Research Article

Designing a Long Acting Erythropoietin by Fusing Three Carboxyl-Terminal Peptides of Human Chorionic Gonadotropin β Subunit to the N-Terminal and C-Terminal Coding Sequence

Fuad Fares,1 Avri Havron,2,3 and Eyal Fima2,3

1 Department of Human Biology, Faculty of Natural Sciences, University of Haifa and Department of Molecular Genetics, Carmel Medical Center, Mount Carmel, 31905 Haifa, Israel
2 ModigeneTech, Weizmann Science Park, 74140 Nes-Ziona, Israel
3 PROLOR Biotech, Weizmann Science Park, 74140 Nes-Ziona, Israel

Correspondence should be addressed to Fuad Fares, ffares@sci.haifa.ac.il

Received 26 February 2011; Accepted 26 June 2011

Academic Editor: Johannes Vogel

Copyright © 2011 Fuad Fares et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

A new analog of EPO was designed by fusing one and two CTPs to the N-terminal and C-terminal ends of EPO (EPO-(CTP)3), respectively. This analog was expressed and secreted efficiently in CHO cells. The in vitro test shows that the activity of EPO-(CTP)3 in TFI-1 cell proliferation assay is similar to that of EPO-WT and commercial rHEPO. However, in vivo studies indicated that treatment once a week with EPO-(CTP)3 (15 μg/kg) dramatically increased (~8 folds) haematocrit as it was compared to rHuEPO. Moreover, it was found that EPO-(CTP)3 is more effective than rHuEPO and Aranesp in increasing reticulocyte number in mice blood. The detected circulatory half-lives of rHuEPO, Aranesp, and EPO-(CTP)3 following IV injection of 20 IU were 4.4, 10.8, and 13.1 h, respectively. These data established the rational for using this chimera as a long-acting EPO analog in clinics. The therapeutic efficacy of EPO-CTP analog needs to be established in higher animals and in human clinical trials.

1. Introduction

Erythropoietin (EPO) is a 34-kDa glycoprotein hormone produced primarily by cells of the per tubular capillary endothelium of the kidney and regulates red blood cell production through stimulation of erythropoiesis [1, 2]. EPO synthesis in the kidney is increased following reduction in tissue oxygenation, it binds to specific receptors on red blood cell precursors in the bone marrow leading to proliferation, differentiation, and to an increase in haematocrit [3]. Biological responses associated with EPO include activation of intracellular signaling molecules such as transcription factors like signal transducer and activator of transcription (STAT) proteins leading to cellular growth and differentiation. EPO receptor belongs to a family of homodimerization receptors where dimerization of the receptor is required to trigger the biological responses associated with EPO [4–7]. Anemia in patients with chronic kidney disease is due to a number of factors, the most common of which is abnormally low erythropoietin levels. Anemia of EPO deficiency is recognized in advanced renal failure but not in early renal disease. Deficiency in EPO production results in anemia in humans and in animal models. EPO is heavily glycosylated with one O-linked and three N-linked oligosaccharide chains. It was found that O-linked oligosaccharide chain has no effect on secretion, receptor binding affinity, and in vitro or in vivo bioactivity. On the other hand, N-linked oligosaccharide chains have no role in in vitro activity, but it is critical for in vivo bioactivity [8].

The gene encoding human erythropoietin was cloned in 1985 leading to the production of recombinant human EPO (rHuEPO) [9, 10]. rHuEPO was used successfully in treating anemia associated with chronic kidney disease. It has also been approved for the treatment of anemia associated with cancer, HIV infection, and in the surgical setting in order to reduce blood transfusions [11–13]. One major issue regarding the clinical use of EPO is its relatively short half-life in vivo due to its rapid clearance (~5 hours) from the
circulation when it is injected intravenously [14]. Thus, the clinical therapeutic protocols of available stimulating agents used in the treatment of patients require frequent injections of EPO. The recommended therapy with rHuEPO is 2-3 times per week by subcutaneous or intravenous injections. Therefore, it can be anticipated that enhancing the in vivo half-life of EPO would reduce the number of injections per week. Previous studies indicated that there is a direct relationship between the sialic acid-containing carbohydrate content of the molecule and its serum half-life and in vivo bioactivity [15–17]. It was shown that fusing the carboxyl-terminal peptide (CTP) of hCGβ subunit that associated with four sites of O-linked oligosaccharide chains to the C-terminal of FSH, TSH, GH, and EPO cDNA did not affect secretion, receptor binding affinity, and in vitro bioactivity. On the other hand, the addition of O-linked oligosaccharides to the backbone of the protein significantly increased the half-life and longevity in vivo [18–22]. We hypothesis that the addition of 12 O-linked oligosaccharide chains to the backbone of EPO will dramatically increase the longevity of EPO. Therefore, in the present study, three carboxyl-terminal peptides (CTP) of hCGβ subunit that each contains four O-linked oligosaccharide recognition sites was fused to N-terminal (one) and to the C-terminal (two) of human EPO coding sequence, respectively. Our results indicate that ligation of three CTPs to the coding sequence of EPO dramatically increased both in vivo potency and half-life in the circulation.

2. Materials and Methods

2.1. Materials. Enzymes used in the construction of DNA vectors and constructs were purchased from New England BioLabs (Beverly, Mass, USA). Cell culture media and reagents were obtained from Biological Industries (Beit Haemek, Israel). Rabbit antisera against EPO were purchased from Fitzgerald (Concord, Mass, USA). Commercial human recombinant EPO (Eprex) was purchased from Janssen-Cilag (North Ryde, NSW, Australia).

2.2. Crystalllography. The interaction between EPO and its receptor was crystallized as described previously [23] by the Department of Structural Biology, Weizmann Institute of Science, Rehovot, Israel.

2.3. Construction of Chimeric Genes and Expression Vectors. A cassette gene containing the CTP of hCGβ was fused in tandem to the coding sequence of EPO at the N-terminal (one CTP) and to the C-terminal end (two CTPs) (Figure 1). DNA fragment containing sequences of hEPO-cDNA and coding sequence of CTP were synthesized by GeneArt (Regensburg, Germany). The DNA fragments contain the recognition sites of the restriction enzymes; Xba I (in the N-terminal) and Not I (in the C-terminal). Fragment containing hEPO and CTP sequences was completely sequenced to ensure that no errors were introduced during synthesis and ligated into the XbaI—Not I sites at the cloning site of the eukaryotic expression vector, pCI-DHFR. Similarly, cDNA of human EPO (EPO-WT) was constructed into pCI-DHFR vector.

2.4. Cell Culture and DNA Transfection. Chinese hamster ovary (CHO)-DG44 cells, which are DHFR negative, were used. Cells were cultured in MEM-α medium (Gibco BRL, USA) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), L-glutamine (2 mM), and 10% heat-inactivated fetal bovine serum at 37°C in humidified incubator containing 5% CO₂. These cells were transfected with 2 μg DNA of plasmid by using FuGENE6 (Roche, Mannheim, Germany) according to manufacturer protocol. Cells were selected for insertion of the plasmid DNA by growth in culture medium of CD DG44 without hypoxanthine and thymidine (HT) (Gibco BRL, USA) supplemented with 8 mM L-Glutamine (Biological Industries, Beit Haimic, Israel) and 18 mL/L of 10% Pluronic F-68 solution (Gibco BRL, USA).

2.5. Western Blotting. Samples of condition medium which were collected from stable clones were electrophorised on denaturing 15% SDS-polyacrylamide gels as described before [24]. Gels were allowed to equilibrate for 10 min in 25 mM Tris and 192 mM glycine in 20% (vol/vol) methanol. Proteins were transferred to a 0.2 μm pore size nitrocellulose membrane (Sigma, Saint Louis, Mo, USA) at 250 mA for 3 h using a Mini Trans-Blot electrophoresis cell (Biorad Laboratories, Richmond, CA) according to the method described in the manual accompanying the unit. The nitrocellulose membrane was incubated in 5% nonfat dry milk for 2 h at room temperature. The membrane was incubated with EPO antiserum (1:1000 titers) for overnight at 4°C followed by three consecutive washes in PBS containing 0.1% Tween (10 min/wash). Then, the membrane was incubated with secondary antibody conjugated to Horse Radish Peroxidase (HRP) (Zymed, San Francisco, CA) for 2 h at room temperature followed by three washes. Finally, the nitrocellulose paper was reacted with enhanced chemiluminescent substrate (ECL) (Pierce, Rockford, Ill, USA) for 5 min, dried with Whatman sheet and exposed to X-ray film.

2.6. In Vitro Bioactivity. Bioactivity of EPO variants was assayed by testing the proliferation dependence of the human erythroleukemic cell line TF-1 (Kitamura) (DSMZ) in the presence of EPO and EPO variants [25]. Cultures were routinely grown at 37°C, 5% CO₂ for 72 hrs in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes, 1 mM sodium pyruvate, 2.5 g/L glucose, 2 mM glutamine, and 2 μg/mL rhGM-CSF. Before transferring the cells to 96-well plates, the TF-1 cells were washed three times with cold PBS and suspended in the assay medium (1640 medium supplemented with 10% fetal bovine serum (FBS) but without addition of rhGM-CSF) at a density of 200,000 cells/mL. The assay was performed in 96-well plates containing 50 μL of cell suspension per well.
50 μL of assay medium containing 3 IU of EPO variants were then added to the wells of 96-well plates for 72 hrs. Cell viability was measured using MTT reagent kit (Cell Biolabs, San Diego, CA) according to manufacturer procedures.

2.7. Animals. Male ICR mice were obtained from Charles River Laboratories, Jerusalem, Israel, and housed in air-conditioned quarters with a 12 h light/dark schedule. Standard food and water were available ad libitum. Institute ethical committee approved the in vivo protocols. Animals were treated with EPO variants as specified.

2.8. In Vivo Bioassay. Groups of 7 male ICR mice (7-week-old males) were used. EPO-WT, EPO-(CTP)3, or commercial rHuEPO were injected to anesthetized animals as described in Table 1.

The animals were weighed, and each received an identical amount (5 or 15 μg/kg) of EPO variants by IV injections (0.2 mL/animal). The frequency of treatment was either thrice weekly (days 1, 3, and 5) or once weekly. The level of haematocrit was determined three times a week and the experiment was stopped after three weeks. Haematocrit was determined using blood samples obtained by filling two heparinized microhematocrit tubes from the inferior caval vein under anesthesia. In addition, reticulocyte counts were conducted since these cells are present in blood for ~48 hours before developing into mature red blood cells. Reticulocytes represent an appropriate evaluation for the acute experimental system employed. Blood was obtained from each pup by cardiac puncture and placed in EDTA coated tubes. This was then mixed with brilliant cresyl blue and incubated for 20 min at 37°C. The blood and stain were then smeared onto a glass slide and the number of reticulocytes assessed using a × 100 oil objective lens.

2.9. Metabolic Clearance Rate. The metabolic clearance of EPO-WT, Aranesp, and EPO-(CTP)3 was determined after IV injection of 20 IU/animal into male ICR mice. At selected intervals after injection, blood samples were collected and EPO immunoreactivity was determined by RIA.

2.10. Statistical Analysis. Data were expressed as the mean ± SEM. Statistical analysis of the data were performed using Student’s t-test and 1-way multivariate analysis of variance (ANOVA1) to calculate P value. P values < 0.05 were considered statistically significant.

3. Results and Discussion

Chrstallographic studies indicated that the N-terminal and C-terminal of EPO are not involved in the binding of the hormone to the receptor (Figure 2).

Therefore, we hypothesized that ligation of CTP to the N-terminal and to the C-terminal of EPO will not affect receptor binding affinity and thus bioactivity. Therefore, three CTPs (one in the N-terminal and two in the C-terminal) were ligated to the coding sequence of EPO. The cDNA of human EPO-WT and EPO-(CTP)3 was inserted into the eukaryotic expression vector, pCI-DHFR, and transfected into CHO cells. Stable clone expressing human EPO-WT or EPO-(CTP)3 was selected. Secretion of EPO was assessed by Western blot analysis under denaturing conditions using human EPO-specific antiserum. The EPO-WT migrated faster than EPO-(CTP)3 (Figure 3).
Table 1: Comparative 3 week induction of haematocrit by EPO-(CTP)₃ and rHuEPO.

| Group number | Mice/group | Compound          | Treatment       | Dose μg/kg | Regimen: IV |
|--------------|------------|-------------------|-----------------|-----------|-------------|
| 1            | n = 7      | Vehicle (control) | 0               |           |             |
| 2            | rHuEPO     | 15 μg/kg          | One dose per week |
| 3            | EPO-(CTP)₃ | 15 μg/kg          |                 |           |             |
| 4            | Commercial rHuEPO | 5           | 3 doses per week |

Figure 3: Expression of EPO-WT and EPO-(CTP)₃ from transfected CHO cells. Conditioned media from transfected cells were prepared for SDS/PAGE and proteins were detected by Western blot as described under “Materials and Methods.”

EPO-(CTP)₃ exhibited high molecular weight (∼57 kDa) comparing to EPO-WT (∼36 kDa) due to the addition of 84 amino acids and the O-linked oligosaccharides linked to CTP. These data may indicate that the O-linked glycosylation recognition site of the C-terminal region is preserved even though the sequence is fused to different proteins. Levels of EPO-WT and EPO-(CTP)₃ were quantities in condition medium by using a monoclonal antibody-based RIA.

The in vitro biological activity of EPO analogs was demonstrated by measuring their ability to stimulate the proliferation of TF-1 cells as described under “Materials and Methods.” The activity of EPO-(CTP)₃ in TF-1 cell proliferation assay was similar to that of EPO wild-type (prepared by Modigene Tech) and commercial rHuEPO (Figure 4).

For further pharmacological evaluation of EPO-(CTP)₃, comparative pharmacodynamic studies of EPO-(CTP)₃ and commercial rHuEPO were performed in male ICR mice (n = 7/group) using different frequencies and dose range as described in Table 1. The in vivo efficacy was obtained by measuring the mean values of haematocrit percentage in the blood. The results indicated that EPO-(CTP)₃ is significantly (P < 0.001) more efficient than rHuEPO when administered IV once a week with a dose of 15 μg/kg (Figure 5). EPO-(CTP)₃ can successfully increase the haematocrit when administered once a week with a dose of 15 μg/kg (Figure 5). Once weekly dosing with the same concentration of commercial rHuEPO or EPO-WT was significantly (P < 0.001) less efficient than once weekly dosing of EPO-(CTP)₃. An interesting observation from the present study was the ability of a single injection once a week of EPO-CTP (15 μg/kg) to increase dramatically (∼8 folds) the levels of haematocrit. Whereas administration of the same total dose of rHuEPO administered three times a week as 5 μg/kg per injection resulted in significantly (P < 0.001) lower effect (Figure 5).

Previously, we have shown that single injection over a week of EPO-CTP, an EPO that contains one CTP at the carboxyl-terminal end, (15 μg/kg) increased the level of haematocrit, whereas the same effect was achieved by administration of the same total dose of rHuEPO administered three times a week as 5 μg/kg per injection [21]. These results indicated the importance of sustained blood levels, rather than total dose of EPO. These findings are consistent with the hypothesis that the ability of a single injection of EPO-CTP to increase haematocrit results from its increased stability in the circulation.

Effect of EPO-WT, (EPO-CTP)₃, and Aranesp in reticulocyte counts is shown in Figure 6. The results indicated that a single IV injection of 15 μg/kg (EPO-CTP)₃ dramatically
administration of a single dose (20 μg) of EPO-WT, rHuEPO, or EPO-(CTP)3. In addition, mice were treated with 5 μg/kg of rHuEPO three times a week for 3 weeks. Control animals were injected IV with saline. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (%) ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 5: In vivo bioactivity of recombinant HuEPO derivatives. ICR mice (n = 7/group) received a single IV injection/week (15 mg/kg) for three weeks of EPO-WT, rHuEPO, or EPO-(CTP)3. In addition, mice were treated with 5 μg/kg of rHuEPO three times a week for 3 weeks. Control animals were injected IV with saline. Blood samples were collected after 72 h and reticulocytes were counted. Each point represents the group average of reticulocyte (%) ± SE. ***P < 0.001.

Table 2: Mean pharmacokinetic parameters following IV administration of a single dose (20 μg/kg) of rHuEPO, EPO-(CTP)3, and Aranesp in male ICR mice. Parameters were generated for individual rats and the mean data are presented here.

| Parameters | rHuEPO | EPO-(CTP)3 | Aranesp |
|------------|--------|------------|---------|
| AUC (hr×μg/L) | 31739 | 306072 | 178661 |
| Cmax (μg/L) | 10766 | 16466 | 13266 |
| Tmax (hr) | 0.25 | 0.25 | 0.25 |
| T1/2 (α) (hr) | 4.4 | 13.11 | 10.84 |

increased reticulocyte number compared to rHuEPO and to Aranesp. The increased biopotency of the chimera may reflect a change in their in vivo longevity. Therefore, the circulatory half-lives of the hormones were determined. EPO-WT, Aranesp, or EPO-(CTP)3 was injected IV into immature male mice and RIA monitored the plasma half-lives. The results indicated that EPO-(CTP)3 has the highest half-life in circulation (Figure 7).

The estimated half-lives of EPO-WT, Aranesp, and EPO-(CTP)3 are 4.4, 10.8, and 13.1 hours, respectively (Table 2). These data suggest that the mechanism of EPO clearance is affected by the presence of CTP. Estimation of area under the curve (AUC) and the maximal plasma concentration (Cmax) of EPO-(CTP)3 are higher than that of rHuEPO and Aranesp. However, the maximal concentration reached in plasma (Tmax) is similar (Table 2).

Previous studies indicated that the CTP sequence can be shuttled into different proteins and still be an acceptor for the O-linked oligosaccharides [18–20]. It was postulated that the O-linked oligosaccharides add flexibility, hydrophilicity, and stability to the protein [26]. This may explain the disinterference of CTP on the protein conformation and, thus, on receptor binding and bioactivity in vitro. On the other hand, it was suggested that the O-linked oligosaccharides play an important role in preventing plasma clearance and thus increasing the half-life of the protein in the circulation [18, 21, 22]. These roles have been postulated since the O-linked oligosaccharides are ended with sialic acid, which is negatively charged. It is known that negatively charged forms of the hormones are less cleared through the glomerular filtration [27]. Thus, addition of 12 O-linked oligosaccharide chains to the backbone of EPO significantly decreased renal clearance; the kidney is the main site of clearance for glycoprotein hormones and, thus, prolonged its half-life in the circulation.

Other studies described long acting hyperglycosylated EPO analog that prepared by addition of N-linked oligosaccharides to the backbone of the protein. In order to add N-linked oligosaccharide chains, the DNA sequence of the cloned human EPO gene was modified by site-directed mutagenesis [28]. This analog was 3-fold longer serum half-life and created in vivo potency comparing to human recombinant EPO-WT. However, its relative affinity for the EPO receptor was ~4-fold lower than that of rHuEPO. Moreover, changing 5 amino acids in the backbone of the protein may increase the immunogenicity of the new derivative.

Addition of CTP to the coding sequence of hormones FSH, TSH, and GH, do not affect secretion, receptor binding affinity, or bioactivity in vitro [18–22]. On the other hand, it was found that ligation of CTP to the coding sequence significantly increases the in vivo potency and half-life of the
with 20 IU of rHuEPO, Aranesp and EPO-(CTP) and blood
± were determined by RIA. Mean samples were drawn at the indicated times. Serum levels of EPO

Abbreviations

- EPO: Erythropoietin
- RHuEPO: Recombinant human erythropoietin
- WT: Wild-type
- Hcg: Human Chorionic Gonadotropin
- FSH: Follicl tropin
- TSH: Thyrotropin
- CTP: Carboxyl-terminal peptide
- CHO: Chinese hamster ovary cells
- PCR: Polymerase chain reaction
- WT: Wild-type.

Acknowledgment

The authors would like to thank the Israel Ministry of Industry and Trade for supporting this paper.

References

[1] W. Jelkmann, “Erythropoietin: structure, control of production, and function,” Physiological Reviews, vol. 72, no. 2, pp. 449–487, 1992.
[2] S. Schuster, J. H. Wilson, A. J. Erslev, and J. Caro, “Physiologic regulation and tissue localization of renal erythropoietin messenger RNA,” Blood, vol. 70, no. 1, pp. 316–318, 1987.
[3] D. A. Parry, E. Minasian, and S. J. Leach, “Conformational homologies among cytokines: interleukins and colony stimulating factors,” Journal of Molecular Recognition, vol. 1, no. 3, pp. 107–110, 1988.
[4] S. S. Watowich, A. Yoshimura, G. D. Longmore, D. J. Hilton, Y. Yoshimura, and H. F. Lodish, “Homodimerization and constitutive activation of the erythropoietin receptor,” Proceedings of the National Academy of Sciences of the United States of America, vol. 89, no. 6, pp. 2140–2144, 1992.
[5] H. Youssoufian, G. Longmore, D. Neumann, A. Yoshimura, and H. F. Lodish, “Structure, function, and activation of the erythropoietin receptor,” Blood, vol. 81, no. 9, pp. 2223–2236, 1993.
[6] J. N. Ihle, “Cytokine receptor signalling,” Nature, vol. 377, no. 6550, pp. 591–594, 1995.
[7] I. Remy, I. A. Wilson, and S. W. Michnick, “Erythropoietin receptor activation by a ligand-induced conformation change,” Science, vol. 283, no. 5404, pp. 990–993, 1999.
[8] L. C. Wasley, G. Timony, P. Murtha et al., “The importance of N- and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin,” Blood, vol. 77, no. 12, pp. 2624–2632, 1991.
[9] K. Jacobs, C. Shoemaker, R. Rudersdorf et al., “Isolation and characterization of genomic and cDNA clones of human erythropoietin,” Nature, vol. 313, no. 6005, pp. 806–810, 1985.
[10] F. K. Lin, S. Suggs, C. H. Lin et al., “Cloning and expression of the human erythropoietin gene,” Proceedings of the National Academy of Sciences of the United States of America, vol. 82, pp. 7580–7584, 1985.
[11] L. C. Platianias, C. B. Miller, R. Mick et al., “Treatment of chemotherapy-induced anemia with recombinant human erythropoietin in cancer patients,” Journal of Clinical Oncology, vol. 9, no. 11, pp. 2021–2026, 1991.
[12] D. H. Henry, “Experience with epoetin alfa and acquired immunodeficiency syndrome anemia,” Seminars in Oncology, vol. 25, no. 3, pp. 64–67, 1998.
[13] H. Ludwig, E. Sundal, M. Pecherstorfer et al., “Recombinant human erythropoietin for the correction of cancer associated anemia with and without concomitant cytotoxic chemotherapy,” Cancer, vol. 76, no. 11, pp. 2319–2329, 1995.
[14] I. C. Macdougall, “Optimizing the use of erythropoietic agents—pharmacokinetic and pharmacodynamic considerations,” Nephrology Dialysis Transplantation, vol. 17, supplement 5, pp. 66–70, 2002.
[15] M. M. Matzuk, A. J. W. Hsueh, P. Lapolt, A. Tsafiri, J. L. Keene, and I. Boime, “The biological role of the carboxyl-terminal extension of human choric gonadotropin β-subunit,” Endocrinology, vol. 126, no. 4, pp. 376–383, 1990.
[16] J. C. van den Hamer, A. G. Morell, I. H. Scheinberg, J. Hickman, and G. Ashwell, “Physical and chemical studies on ceruloplasmin. IX. The role of galactosyl residues in the clearance of ceruloplasmin from the circulation,” Journal of Biological Chemistry, vol. 245, no. 17, pp. 4397–4402, 1970.

[17] J. Pierce and T. F. Parsons, “Glycoprotein hormones: structure and function,” Annual Review of Biochemistry, vol. 50, pp. 465–495, 1981.

[18] F. A. Fares, N. Suganuma, K. Nishimori, P. S. LaPolt, A. J. W. Hsueh, and I. Boime, “Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the β chorionic gonadotropin subunit,” Proceedings of the National Academy of Sciences of the United States of America, vol. 89, no. 10, pp. 4304–4308, 1992.

[19] P. S. Lapolt, K. Nishimori, F. A. Fares, E. Perlas, I. Boime, and A. J. W. Hsueh, “Enhanced stimulation of follicle maturation and ovulatory potential by long acting follicle-stimulating hormone agonists with extended carboxyl-terminal peptides,” Endocrinology, vol. 131, no. 6, pp. 2514–2520, 1992.

[20] L. Joshi, Y. Murata, F. E. Wondisford, M. W. Szukulinski, R. Desai, and B. D. Weintraub, “Recombinant thyrotropin containing a β-subunit chimera with the human chorionic gonadotropin-β carboxy-terminus is biologically active, with a prolonged plasma half-life: role of carbohydrate in bioactivity and metabolic clearance,” Endocrinology, vol. 136, pp. 3839–3848, 1994.

[21] F. Fares, S. Ganem, T. Hajouj, and E. Agai, “Development of a long-acting erythropoietin by fusing the carboxyl-terminal peptide of human chorionic gonadotropin β-subunit to the coding sequence of human erythropoietin,” Endocrinology, vol. 148, no. 10, pp. 5081–5087, 2007.

[22] F. Fares, R. Guy, A. Bar-Ilan, Y. Felikman, and E. Fima, “Designing a long-acting human growth hormone (hGH) by fusing the carboxyl-terminal peptide of human chorionic gonadotropin β-subunit to the coding sequence of hGH,” Endocrinology, vol. 151, no. 9, pp. 4410–4417, 2010.

[23] M. L. Raves, M. Harel, Y. P. Pang, I. Silman, A. P. Kozikowski, and J. L. Sussman, “Structure of acetylcholinesterase complexed with the nootropic alkaloid, (-)-huperzine A,” Nature Structural Biology, vol. 4, no. 1, pp. 57–63, 1997.

[24] U. K. Laemmli, “Cleavage of structural proteins during the assembly of the head of bacteriophage T4,” Nature, vol. 227, no. 5259, pp. 680–685, 1970.

[25] T. Kitamura, T. Tange, T. Terasawa et al., “Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin,” Journal of Cellular Physiology, vol. 140, no. 2, pp. 323–334, 1989.

[26] N. Jentoft, “Why are proteins O-glycosylated?” Trends in Biochemical Sciences, vol. 14, no. 8, pp. 272–275, 1990.

[27] L. Wide, “The regulation of metabolic clearance rate of human FSH in mice by variation of the molecular structure of the hormone,” Acta Endocrinologica, vol. 112, no. 3, pp. 336–344, 1986.

[28] J. C. Egrie and J. K. Browne, “Development and characterization of novel erythropoiesis stimulating protein (NESP),” British Journal of Cancer, vol. 84, supplement 1, pp. 3–10, 2001.

[29] P. M. Bouloux, D. J. Handelsman, F. Jockenhövel et al., “First human exposure to FSH-CTP in hypogonadotrophic hypogonadal males,” Human Reproduction, vol. 16, no. 8, pp. 1592–1597, 2001.

[30] I. J. Duijkers, C. Klipping, P. J. Boerrigter, C. S. M. Machielsen, J. J. de Bie, and G. Voortman, “Single dose pharmacokinetics and effects on follicular growth and serum hormones of a long-acting recombinant FSH preparation (FSH-CTP) in healthy pituitary-suppressed females,” Human Reproduction, vol. 17, no. 8, pp. 1987–1993, 2002.

[31] P. Devroey, B. C. Fauser, P. Plateau, N. G. Beckers, M. Dhont, and B. M. Mannaeerts, “Induction of multiple follicular development by a single dose of long-acting recombinant follicle-Stimulating hormone (FSH-CTP, corifollitropin alfa) for controlled ovarian stimulation before in vitro fertilization,” Journal of Clinical Endocrinology and Metabolism, vol. 89, no. 5, pp. 2062–2070, 2004.

[32] J. W. Eschbach, J. C. Egrie, M. R. Downing, J. K. Browne, and J. W. Adamson, “Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined Phase I and II clinical trial,” New England Journal of Medicine, vol. 316, no. 2, pp. 73–78, 1987.

[33] R. W. Evans, B. Rader, and D. L. Manninen, “The quality of life of hemodialysis recipients treated with recombinant human erythropoietin. Cooperative Multicenter EPO Clinical Trial Group,” JAMA, vol. 263, no. 6, pp. 825–830, 1990.

[34] C. G. Winears, D. O. Oliver, M. J. Pippard, C. Reid, M. R. Downing, and P. M. Coles, “Effect of human erythropoietin derived from recombinant DNA on the anemia of patients maintained by chronic haemodialysis,” Lancet, vol. 2, no. 8517, pp. 1175–1178, 1986.

[35] L. C. Platanias, C. B. Miller, R. Mick et al., “Treatment of chemotherapy-induced anemia with recombinant human erythropoietin in cancer patients,” Journal of Clinical Oncology, vol. 9, no. 11, pp. 2021–2026, 1991.

[36] H. Ludwig, E. Sundal, M. Pecherstorfer et al., “Recombinant human erythropoietin for the correction of cancer associated anemia with and without concomitant cytotoxic chemotherapy,” Cancer, vol. 76, no. 11, pp. 2319–2329, 1995.
Review Article

Cellular Reprogramming toward the Erythroid Lineage

Laura J. Norton, Alister P. W. Funnell, Richard C. M. Pearson, and Merlin Crossley

School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Correspondence should be addressed to Merlin Crossley, m.crossley@unsw.edu.au

Received 30 March 2011; Accepted 8 May 2011

Academic Editor: Michael Föller

Copyright © 2011 Laura J. Norton et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Haemoglobinopathies such as thalassaemia and sickle cell disease present a major health burden. Currently, the main forms of treatment for these diseases are packed red blood cell transfusions and the administration of drugs which act to nonspecifically reactivate the production of foetal haemoglobin. These treatments are ongoing throughout the life of the patient and are associated with a number of risks, such as limitations in available blood for transfusion, infections, iron overload, immune rejection, and side effects associated with the drug treatments. The field of cellular reprogramming has advanced significantly in the last few years and has recently culminated in the successful production of erythrocytes in culture. This paper will discuss cellular reprogramming and its potential relevance to the treatment of haemoglobinopathies.

1. Introduction: Globin Gene Regulation and Haemoglobinopathies

The various blood cell lineages in mammals arise from a multipotent haematopoietic stem cell via particular differentiation pathways. One of these pathways, erythropoiesis, leads to the production of red blood cells (RBCs), which transport oxygen and carbon dioxide around the body by means of the intracellular metalloprotein haemoglobin (Hb). Hb is a tetramer, consisting of two α-globin and two β-globin subunits. In mammals, these globin chains are encoded by two gene loci: the α-globin locus and the β-globin locus. In humans, the α-globin locus consists of the embryonic γ- and adult α-globin genes, and the β-globin locus comprises the embryonic ε-, foetal γG- and γA-, and adult δ- and β-globin genes [1, 2]. The globin genes expressed from these loci differ from embryonic to adult erythropoiesis in order to meet varying oxygen demands and facilitate placental transfer of oxygen from mother to embryo [3].

There are a number of severe diseases caused by the disruption of adult globin genes, including thalassaemias and certain types of anaemia. According to the World Health Organisation, approximately 5% of the world’s population carry genes involved in Hb disorders, and as such, they present an enormous health burden. Thalassaemia is caused by a reduction or abolition of the expression of one or more globin genes, resulting in an imbalance of α- and β-globin chains in red blood cells and consequent anaemia [1, 4]. Sickle cell anaemia is another prevalent haemoglobinopathy and is caused by a mutation in the adult β-globin gene which generates a single glutamic acid to valine amino acid substitution. This mutation leads to the polymerisation of globins in venous circulation [5, 6], which can trigger a rigid and sickled cell phenotype [7, 8] and results in a number of acute conditions such as vaso-occlusion, splenic sequestration, and haemolytic anaemia [9].

There are currently a number of treatments available for patients suffering from thalassaemia and sickle cell anaemia. The most common is packed red blood cell transfusion, but this is associated with a number of problems, such as sufficiency of supply, bacterial and viral infection, biochemical and biomechanical changes during storage (red blood cell storage lesions), and the risk of immune rejection from the patient [10, 11]. Furthermore, blood transfusions are ongoing throughout a patient’s life and often lead to a potentially fatal buildup of iron and associated reduction in organ activity.

Another potential therapeutic option involves the reactivation of foetal γ-globin expression in adult patients. Residual production of foetal γ-globin persists naturally
throughout life, and levels vary between individuals [12, 13]. This persistent expression allows two γ-globin chains to combine with two adult α-globin chains to form what is known as foetal Hb (HbF). As only the adult β-globin gene is mutated in sickle cell anaemia, affected infants are protected from severe symptoms until they reach several months of age, due to the large amount of HbF still in circulation at birth [14]. Furthermore, patients who have inherited alleles associated with increased levels of HbF, known as hereditary persistence of foetal Hb (HPFH), are protected into adulthood [15]. Similarly, a more asymptomatic disease phenotype has also been shown in patients with β-thalassaemia who exhibit higher levels of HbF [16]. Together, these observations indicate that increased foetal γ-globin is able to compensate in part for the loss of adult β-globin function and thus ameliorates the symptoms of certain adult haemoglobinopathies. Accordingly, a number of drug treatments for β-thalassaemia and sickle cell disease, for instance, 5-azacytidine, hydroxyurea, and butyrate, all act by nonspecifically reactivating foetal γ-globin gene expression by various mechanisms. The effects of these drug treatments are transient and thus require ongoing administration. There is evidence that long-term administration of these drugs has chronic side effects, consistent with their lack of specificity [8, 17].

As the existing methods of treatment for these haemoglobinopathies remain inadequate, alternative forms of therapy are currently being sought, and stem cell therapies should be considered. This paper will discuss progress in utilising novel cellular reprogramming techniques to treat RBC diseases.

2. Cellular Reprogramming

Stem cells, both embryonic and adult, have the ability to differentiate into various cell types, making them a potentially attractive treatment option. Embryonic stem cells (ESCs) and adult stem cells (ASCs) each have their own strengths and disadvantages in these strategies. ESCs are more easily grown in culture and are pluripotent, meaning that they are able to differentiate into any cell of the body. The practicality of widespread ESC use for therapeutic purposes, however, has been questioned due to issues of supply and ethical and legal considerations. Moreover, these cells carry the risk of allogeneic immune rejection. ASCs, on the other hand, overcome some of these problems as they can be harvested from each individual patient. These cells, however, offer a different set of challenges. They are not abundant and are difficult to obtain, often being harboured in internal organs such as the gut and bone marrow. They have proven difficult to culture in vitro [18], and furthermore, they are widely believed to be multipotent rather than pluripotent and are thus only able to differentiate into certain cell types [19]. Cellular reprogramming potentially overcomes these issues and may offer new treatment methods for a range of diseases, including those of RBCs.

2.1. Classic Cellular Reprogramming. Takahashi and Yamanaka [20] were the first to show that it is possible to take differentiated somatic cells and transform them into cells with pluripotent potential. They began by identifying a pool of 24 transcription factors which are important in maintaining stem cell traits and used retroviral transduction to express these factors in murine embryonic and adult fibroblasts. They found that these cells then displayed characteristics and properties comparable to those of pluripotent ESCs. They were able to further refine the required transcription factors by setting up numerous combinations to determine which were essential to this process and identified four factors, Oct4, Sox2, Klf4, and c-Myc, needed to transform a somatic cell to an induced pluripotent stem cell (iPSC). Takahashi et al. [21] then applied these four factors to human cells and transformed neonatal and adult human fibroblasts into human iPSCs (hiPSCs). This was a valuable subsequent step as it showed that this process can be reproduced with human cells and could thus potentially be utilised for stem cell-based therapies of human disease. Several other transcription factor combinations have since been shown to be sufficient to reprogram somatic cells [22–24] and one particular combination, involving OCT4, SOX2, NANOG, and LIN28, is particularly efficient and is now widely utilised [25]. Figure 1(a) depicts the process of cellular reprogramming.

There are several potential issues associated with using iPSCs to treat disease. For example, a number of the transcription factors used to generate these cells, including c-Myc, Klf4, and Lin28, are oncogenes, and their misexpression can lead to cancer [26–28]. In order for iPSCs to be differentiated into a cell type of choice, the retrovirally transmitted genes need to be switched off or removed to reduce the possibility of their inducing tumours [22, 29]. Another potential issue is that the transplantation of any cells that have not been fully differentiated from the iPSC state could lead to the formation of cancerous teratomas. Any cells remaining which are still in a pluripotent state could multiply and, without the appropriate growth controls, would have the potential to result in tumours in transplant patients. To potentially avoid the issue of tumour formation and uncontrolled proliferation of iPSCs, an alternative methodology known as transdifferentiation is also being considered as a potential treatment strategy.

2.2. Transdifferentiation. Transdifferentiation is achieved by introducing various exogenous factors into a differentiated cell, such as a fibroblast, to directly convert it into another type of differentiated cell, thereby bypassing the pluripotent stage (Figure 1(b)). As early as 1990, Choi et al. [30] were able to convert various cells types, including dermal fibroblasts and chondroblasts, into mononucleated, striated myoblasts that were indistinguishable from normal myoblasts in vivo. This was achieved through the expression of the myogenic regulatory factor MyoD, a transcription factor known to be involved in the determination of muscle cells. Shortly after this, another group showed that it was possible to turn myeloid 416B cells into mast cells through the forced expression of GATA-2 and GATA-3, two transcription factors which play important roles in haematopoiesis [31]. Halder et al. [32] investigated the effects of ectopically expressing the gene eyeless (ey), an orthologue of the mammalian Pax6 gene,
in Drosophila and found that eye structures formed in places such as the wings and legs.

Since Takahashi and Yamanaka’s pioneering studies in cellular reprogramming, the field of transdifferentiation has advanced considerably. Zhou et al. [33] investigated the effects of expressing Ngn3, Pdx1, and Mafa, transcription factors involved in β-cell differentiation, on exocrine cells of the adult pancreas. They found that the coexpression of these factors was able to convert the exocrine cells into β-cells. The induced β-cells were identical to endogenous β-cells in morphology and showed similar expression of genes associated with β-cell function. These cells can also rescue the phenotype of hyperglycaemia, as they are able to secrete insulin and remodel surrounding vasculature. In other work, Vierbuchen et al. [34] utilised the neural-specific transcription factors, Ascl1, Brn2, and Myt1l, to rapidly convert both murine embryonic fibroblasts and adult fibroblasts directly into functional neurons. These induced neuronal cells express neuron-specific proteins, generate action potentials, and form functional synapses. A further advance has shown that it is also possible to transdifferentiate fibroblasts into functional neural progenitor cells by transient induction of Oct4, Sox2, Klf4, and c-Myc in cells cultured in a defined neural reprogramming medium [35]. This process bypassed the generation of iPSCs and gave rise to multipotent progenitors with the capacity to expand and differentiate into a number of neural lineages. All of these experiments indicate that it is possible to direct a differentiated cell to another cell fate through the application of extragenic factors.

3. Cellular Reprogramming as a Potential Treatment for Anaemia

Advances in cellular reprogramming have raised the attractive possibility that this technology could be utilised to generate a limitless source of immune-matched, pathogen-free erythrocytes for transfusion. Efforts were thus made to produce mature erythroid cells from hiPSCs in culture. An initial study by Feng et al. [28] revealed some practical difficulties. They found that hiPSCs are capable of generating haematopoietic cells with phenotypic and morphological characteristics similar to those derived from hESCs; however, these hiPSC-derived cells exhibited a dramatically reduced capacity (by greater than 1000-fold) to generate erythroid cells.

A subsequent study by Lapillone et al., however, showed that it was indeed possible to produce significant numbers of mature erythroid cells from hiPSCs in vitro [36]. This group employed the methods outlined by Thomson’s group [25] using OCT4, SOX2, NANOG, and LIN28 to convert fibroblasts to hiPSCs. They then cultured these cells in medium containing the cytokines SCF, TPO, FLT3 ligand, rhu BMP4, rhu VEGF-A165, IL-3, IL-6, and Erythropoietin (Epo). These culture conditions were optimised to obtain embryoid bodies that display early erythroid commitment. They analysed the expression profiles of these cells over 20 days of culture and found that pluripotent stem cell markers decreased whilst the erythroid markers CD36, CD235a, and CD71 increased. Cells at day 20 were found to have a high erythroid potential and were plated in sequential
cocktails of cytokines comprising SCF, IL-3, and/or Epo. Erythroid maturation was achieved after another 25 days and was confirmed by morphological examination and by flow cytometric analysis of erythroid markers (CD235a^hi, CD71^hi, and CD36^lo). Furthermore, these erythroid cells were able to enucleate, albeit with reduced capacity compared to hESCs, and were found to express functional Hb (predominantly HbF). These hiPSC-derived erythroid cells were compared to those differentiated from hESCs, and no significant differences were detected in terms of erythroid commitment, expression of erythroid markers, and type and functionality of Hb.

The study by Lapillone et al. revealed that while functional RBCs could be generated from hiPSCs, when compared to hESCs, hiPSCs were shown to have reduced (approximately 8-fold) amplification potential in producing erythroid cells, and were found to express functional Hb (predominantly HbF). These hiPSC-derived erythroid cells were compared to those differentiated from hESCs, and no significant differences were detected in terms of erythroid commitment, expression of erythroid markers, and type and functionality of Hb.

4. Cellular Reprogramming as a Potential Cure for Anaemia

In order to cure, rather than treat, RBC diseases, healthy progenitor cells must be transplanted into patients and subsequently repopulate the haematopoietic system. A promising study has already shown that it is possible to use reprogrammed cells to treat sickle cell anaemia in mice. Hana et al. [42] took cells from the tail tips of a humanized sickle cell anaemic mouse model, in which the mouse cDNA encoding the sickle cell anaemia mutation (HbS) was introduced into the genome of the mouse through homologous recombination. Following transplantation of these corrected haematopoietic progenitors, the treated mice had increased RBC numbers, Hb levels, and packed cell volume compared to untreated mice. These results demonstrate that it is possible to differentiate haematopoietic lineages from these cells although they have not explicitly shown mature erythrocyte differentiation. This possible treatment method circumvents many of the problems associated with current treatments, such as questionable quality, lack of supply, and immune-rejection. However, issues still remain, such as cost and the risk of iron overloading.
of these parameters were ameliorated in the treated mice. This study thus provides an important proof of principle that cellular reprogramming can be employed to correct erythroid disorders, albeit in this case, in conjunction with gene therapy.

5. Conclusions

Normal erythropoiesis is dependent upon the correct expression of globin genes. Where globin genes are incorrectly expressed, or are mutated, anaemia or thalassaemia results. Current therapy for these disorders involves packed red blood cell transfusions, which are limited by supply, risk of infection, expense, and patient rejection. Drug-based therapies involve the nonspecific reactivation of foetal globins and have long-term side effects. In seeking alternative strategies, recent advances have shown that cellular reprogramming can now generate large quantities of red blood cells in culture, potentially for use in transfusions. Furthermore, these strategies have been successfully combined with gene therapy to treat a sickle cell anaemia mouse model, suggesting that cellular reprogramming will provide a realistic future alternative to conventional treatment of haemoglobinopathies.

References

[1] A. C. Perkins, K. R. Peterson, G. Stamatoyannopoulos, H. E. Witkowska, and S. H. Orkin, “Fetal expression of a human Ay globin transgene rescues globin chain imbalance but not hemolysis in EKLF null mouse embryos,” Blood, vol. 95, no. 5, pp. 1827–1833, 2000.
[2] T. Trimborn, J. Gräbna, F. Grosveld, and P. Fraser, “Mechanisms of developmental control in the murine α and β-globin loci,” Genes and Development, vol. 13, no. 1, pp. 112–124, 1999.
[3] P. A. Oneal, N. M. Gant, J. D. Schwartz et al., “Fetal hemoglobin silencing in humans,” Blood, vol. 108, no. 6, pp. 2081–2086, 2006.
[4] M. H. Baron, “Transcriptional control of globin gene switching during vertebrate development,” Biochimica et Biophysica Acta—Gene Structure and Expression, vol. 1351, no. 1–2, pp. 51–72, 1997.
[5] O. Galkin and P. G. Vekilov, “Mechanisms of homogeneous nucleation of polymers of sickle cell anemia hemoglobin in deoxy state,” Journal of Molecular Biology, vol. 336, no. 1, pp. 43–59, 2004.
[6] L. Pauling, H. A. Itano, S. J. Singer, and I. C. Wells, “Sickle cell anemia, a molecular disease,” Science, vol. 110, no. 2865, pp. 543–548, 1949.
[7] P. S. Frenette and G. F. Atweh, “Sickle cell disease: old discoveries, new discoveries, and new concepts,” Journal of Clinical Investigation, vol. 117, no. 4, pp. 850–858, 2007.
[8] R. Mabaera, R. J. West, S. J. Conine et al., “A cell stress signaling model of fetal hemoglobin induction: what doesn’t kill red blood cells may make them stronger,” Experimental Hematology, vol. 36, no. 9, pp. 1057–1072, 2008.
[9] O. S. Platt, D. J. Brambilla, W. F. Rossie et al., “Mortality in sickle cell disease. Life expectancy and risk factors for early death,” New England Journal of Medicine, vol. 330, no. 23, pp. 1639–1644, 1994.
[10] J. Mountford, E. Olivier, and M. Turner, “Prospects for the manufacture of red cells for transfusion,” British Journal of Haematology, vol. 149, no. 1, pp. 22–34, 2010.
[11] M. H. Antonelou, A. G. Kriebardis, and I. S. Papassideri, “Aging and death signalling in mature red cells: from basic science to transfusion practice,” Blood Transfusion, vol. 8, supplement 3, pp. s39–s47, 2010.
[12] C. Garner, T. Tatu, J. E. Reitie et al., “Genetic influences on F cells and other hematologic variables: a twin heritability study,” Blood, vol. 95, no. 1, pp. 342–346, 2000.
[13] S. L. Thein and S. Menzel, “Discovering the genetics underlying foetal haemoglobin production in adults,” British Journal of Haematology, vol. 145, no. 4, pp. 453–467, 2009.
[14] J. Watson, “Sickling in Negro newborns—its possible relationship to fetal hemoglobin,” American Journal of Medicine, vol. 5, no. 1, pp. 159–160, 1948.
[15] S. Friedman and E. Schwartz, “Hereditary persistence of fetal hemoglobin with beta-chain synthesis in cis position (gammagamma-beta+-hpfh) in a Negro family,” Nature, vol. 259, no. 5539, pp. 138–140, 1976.
[16] D. J. Weatherall and J. B. Clegg, “Inherited haemoglobin disorders: an increasing global health problem,” Bulletin of the World Health Organization, vol. 79, no. 8, pp. 704–712, 2001.
[17] R. M. Böhmer, “Reactivation of fetal hemoglobin in adult stem cell erythropoiesis by transforming growth factor-β,” Journal of Hematology and Stem Cell Research, vol. 12, no. 5, pp. 499–504, 2003.
[18] J. Czyz, C. Wiese, A. Rolletschek, P. Błyszczuk, M. Cross, and A. M. Wobus, “Potential of embryonic and adult stem cells in vitro,” Journal of Biological Chemistry, vol. 384, no. 10-11, pp. 1391–1409, 2003.
[19] O. J. Borrie, “Embryonic and adult stem cells,” Acta Veterinaria Scandinavica, vol. 99, pp. 39–43, 2004.
[20] K. Takahashi and S. Yamanaka, “Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors,” Cell, vol. 126, no. 4, pp. 663–676, 2006.
[21] K. Takahashi, K. Tanabe, M. Ohnuki et al., “Induction of pluripotent stem cells from adult human fibroblasts by defined factors,” Cell, vol. 131, no. 5, pp. 861–872, 2007.
[22] S. Yamanaka, M. Nakagawa, M. Koyanagi et al., “Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts,” Nature Biotechnology, vol. 26, no. 1, pp. 101–106, 2008.
[23] H. H. Ng, J. C. D. Heng, B. Feng et al., “The Nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells,” Cell Stem Cell, vol. 6, no. 2, pp. 167–174, 2010.
[24] K. Plath, R. Ho, and C. Chronis, “Mechanistic insights into reprogramming to induced pluripotency,” Journal of Cellular Physiology, vol. 226, no. 4, pp. 868–878, 2011.
[25] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., “Induced pluripotent stem cell lines derived from human somatic cells,” Science, vol. 318, no. 5858, pp. 1917–1920, 2007.
[26] J. A. West, S. R. Viswanathan, A. Yabuuchi et al., “A role for Lin28 in primordial germ-cell development and germ-cell malignancy,” Nature, vol. 460, article U151, no. 7257, pp. 909–913, 2009.
[27] S. R. Viswanathan, J. T. Powers, W. Einhorn et al., “Lin28 promotes transformation and is associated with advanced human malignancies,” Nature Genetics, vol. 41, article U109, no. 7, pp. 843–848, 2009.
[28] Q. Feng, S. J. Lu, I. Klimanskaya et al., “Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence,” Stem Cells, vol. 28, no. 4, pp. 704–712, 2010.
[29] K. Okita, T. Ichisaka, and S. Yamanaka, “Generation of germine-competent induced pluripotent stem cells,” Nature, vol. 448, no. 7151, pp. 313–317, 2007.
[30] J. Choi, M. L. Costa, C. S. Mermelstein, C. Chagas, S. Holtzer, and H. Holtzer, “MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes,” Proceedings of the National Academy of Sciences of the United States of America, vol. 87, no. 20, pp. 7988–7992, 1990.

[31] J. Visvader and J. M. Adams, “Megakaryocytic differentiation induced in 416B myeloid cells by GATA-2 and GATA-3 transgenes or 5-azacytidine is tightly coupled to GATA-1 expression,” Blood, vol. 82, no. 5, pp. 1493–1501, 1993.

[32] G. Halder, P. Callaerts, and W. J. Gehring, “Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila,” Science, vol. 267, no. 5205, pp. 1788–1792, 1995.

[33] Q. Zhou, J. Brown, A. Kanarek, J. Rajagopal, and D. A. Melton, “In vivo reprogramming of adult pancreatic exocrine cells to [bgr]-cells,” Nature, vol. 455, no. 7213, pp. 627–632, 2008.

[34] T. Vierbuchen, A. Ostermeier, Z. P. Pang, Y. Kokubu, T. C. Südhof, and M. Wernig, “Direct conversion of fibroblasts to functional neurons by defined factors,” Nature, vol. 463, no. 7284, pp. 1035–1041, 2010.

[35] J. Kim, J. A. Efe, S. Zhu et al., “Direct reprogramming of mouse fibroblasts to neural progenitors,” Proceedings of the National Academy of Sciences of the United States of America, vol. 108, no. 19, pp. 7838–7843, 2011.

[36] H. Lapillonne, L. Kobari, C. Mazurier et al., “Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine,” Haematologica, vol. 95, no. 10, pp. 1651–1659, 2010.

[37] J. Dias et al., “Generation of red blood cells from human induced pluripotent stem cells,” Stem Cells and Development. In press.

[38] E. Szabo, S. Rampalli, R. M. Risueño et al., “Direct conversion of human fibroblasts to multilineage blood progenitors,” Nature, vol. 468, no. 7323, pp. 521–526, 2010.

[39] R. C. Perlingeiro, M. Kyba, and G. Q. Daley, “Clonal analysis of differentiating embryonic stem cells reveals a hematopoietic progenitor with primitive erythroid and adult lymphoid-myeloid potential,” Development, vol. 128, no. 22, pp. 4597–4604, 2001.

[40] K. H. Chang, A. M. Nelson, H. Cao et al., “Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin,” Blood, vol. 108, no. 5, pp. 1515–1523, 2006.

[41] A. Seifinejad, A. Taei, M. Totonchi et al., “Generation of human induced pluripotent stem cells from a Bombay individual: moving towards “universal-donor” red blood cells,” Biochemical and Biophysical Research Communications, vol. 391, no. 1, pp. 329–334, 2010.

[42] J. Hanna, M. Wernig, S. Markoulaki et al., “Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin,” Science, vol. 318, no. 5858, pp. 1920–1923, 2007.

[43] L. C. Wu, C. W. Sun, T. M. Ryan, K. M. Pawlik, J. Ren, and T. M. Townes, “Correction of sickle cell disease by homologous recombination in embryonic stem cells,” Blood, vol. 108, no. 4, pp. 1183–1188, 2006.