Next generation research and therapy in red blood cell diseases

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Pathogenetic studies on red blood cell (RBC) diseases have always represented a powerful model for the study of medical genetics and for technology innovation in both diagnostics and research. This has mainly been due to the availability of these cells compared to others, such as neurons, myocytes, and so on, that are not so easily available. Indeed, the first and the best molecular characterization of genetic diseases was carried out in RBC disorders.

It is now over 50 years since the first pioneer studies on abnormal globin and glucose 6-phosphate dehydrogenase (G6PD) genes, the forerunners of the current research and molecular diagnosis of Mendelian disorders, and completion of the Human Genome Project was a crucial milestone in the diagnosis and research of genetic disorders. The assembly and refinement of the reference genome provide the mainstay for current knowledge in the field of human genetics. In recent years, scientists have spent much time and effort in identifying genes and mutations that are causative of several diseases, with great success. Although the identification of these genetic variants has improved our knowledge of disease etiology, there is still a considerable gap in our understanding of the genetic factors that modify disease severity. In this context, it is important to consider that there has been a substantial evolution in diagnostic and research technologies. The implementation of the new technologies is changing the approach to diagnosis and research. We started out using Sanger sequencing, and we are now embracing next generation sequencing (NGS), moving from a monogenic approach to an oligo/multigenic one. The application of next generation approaches will increase our knowledge of genetic and genomic differences among individuals, gradually leading to a shift in the clinical management and the therapeutic plan from a population-based approach to personalized therapy for the individual patient.

The 'next generation' era in the field of RBC physiopathology provided important insights into the molecular mechanisms of normal and diseased RBC homeostasis. These findings generated several novel therapeutic approaches that are now being examined in clinical trials.

In the last few years, several studies have supplied new concepts about the regulation of erythrocyte volume. In particular, *PIEZO1* has been discovered to be the causative gene of hereditary xerocytosis, also known as dehydrated hereditary stomatocytosis (DHS, OMIM 194380), an autosomal dominant hemolytic anemia characterized by primary erythrocyte dehydration.[1,2] Piezo proteins have recently been identified as ion channels mediating mechanosensory transduction in mammalian cells.[3] Mutations in PIEZO1 show a partial gain-of-function phenotype with delayed inactivation of the channel suggesting increased cation permeability that leads to erythrocyte dehydration.[1,2] In 2015, a second causative gene of DHS was identified, *KCNN4*, encoding a Gardos channel (a Ca2+ sensitive, intermediate conductance, potassium selective channel).[4-6] Similarly to gain-of-function genetic variants in *PIEZO1*, heterozygous dominantly inherited mutations in the *KCNN4* gene lead to greater activity of the channel when compared to the wild type.[4] The identification of *PIEZO1* and *KCNN4* variants in DHS patients strongly indicates that both genes play a critical role in normal erythrocyte deformation and in maintenance of erythrocyte volume homeostasis. Moreover, the identification of variants in these genes will open up new studies on their role in the improvement or worsening of RBC hydration in patients with primary (DHS) and secondary erythrocyte hydration disorders such as sickle cell disease (SCD). Thus, the routine introduction of NGS targeted panels would most likely facilitate, not only the diagnosis, but also the prognostic evaluation of these patients.

Among the disorders of secondary erythrocyte hydration, recent advances in the pathophysiology of SCD and β-thalassemia have elucidated new possible therapeutic approaches. A clinical trial on senicapoc (ICA-17043), a potent blocker of the Gardos channel, demonstrated that treatment of SCD patients resulted in increased hemoglobin and reduced markers of hemolysis, strongly suggesting that the survival of sickle red blood cells was improved.[7] Despite the lack of any reduction in the frequency of pain episodes, the increasing recognition that hemolysis contributes to the development of several SCD-related complications suggests that senicapoc may be beneficial in this disease by decreasing hemolysis.[7] Thus, blockers of Gardos and PIEZO1 channels could be used in future clinical practice for the treatment of primary and secondary disorders of erythrocyte hydration.

Likewise, another promising approach for the treatment of both β-thalassemia and SCD is gene replacement therapy. In this approach, samples of multipotent hematopoietic stem progenitor cells (HSPCs) are collected from the patient and subsequently modified to express a β-like globin gene in erythroid precursors; these cells are then re-infused.[8] The modified HSPCs will reconstitute the hematopoietic system, thus producing normal, gene-corrected RBCs. This approach still presents many challenges: i) to reduce the tendency of integrated viral vectors; ii) to activate nearby genes; and also iii) to further increase β-like globin expression. Early results of a clinical trial in β-thalassemia major patients treated with improved vectors are promising, and it is hoped that they will lead to advances in the treatment of thalassemic patients.[9]

The application of gene therapy to treat erythroid disorders regards not only β-thalassemia and hemoglobinopathies. For example, gene therapy has been investigated for Diamond Blackfan anemia (DBA) and other erythroid diseases, such as red cell enzyme disorders, including severe forms of G6PD and pyruvate kinase deficiency.[10,11]

For most of the anemias due to RBCs defects, blood transfusion therapy or treatment by erythropoiesis stimulating agents (ESAs), such as recombinant EPO, are the front-line therapies. However, neither of these treatment approaches is without

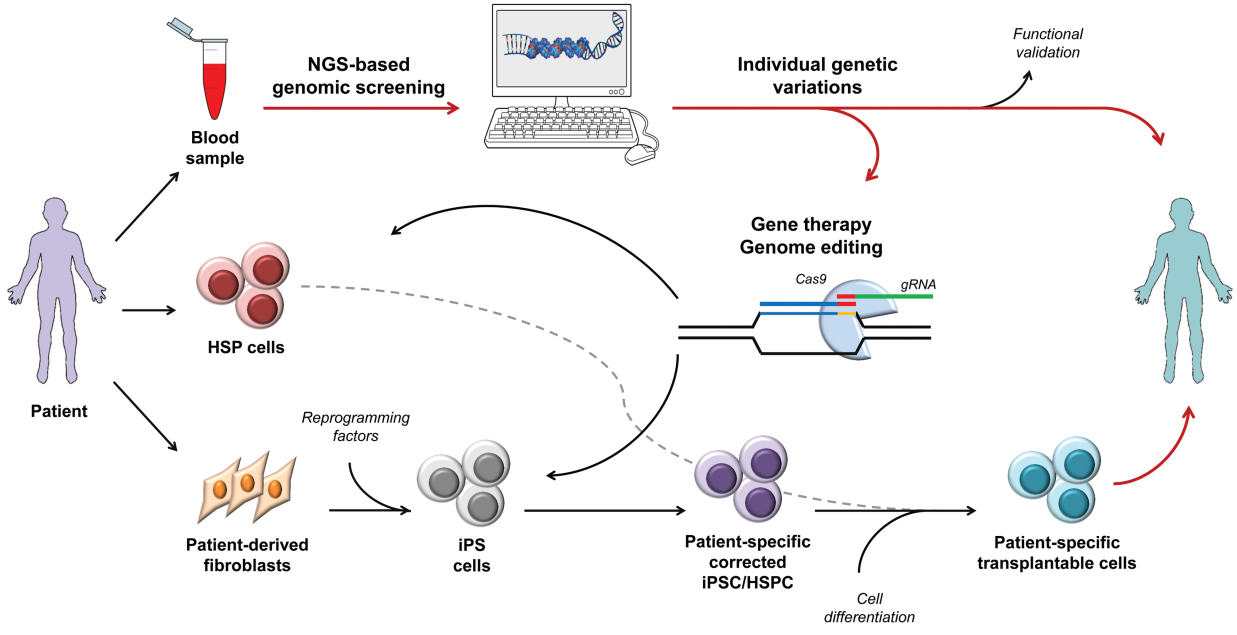

Figure 1. Integration between technological updates and clinical applications in diagnosis and therapy of red blood cell (RBC) diseases. Adult hematopoietic stem progenitor cells (HSPCs) or induced pluripotent stem cells (iPSCs) can be used for gene-therapy approaches. DNA extracted from a peripheral blood sample can be used to identify genetic variations by next generation sequencing (NGS). The causative role of these variations can be validated by *in vitro/in vivo* functional studies and then the commonly used CD34$^+$ HSPCs may be corrected directly by gene therapy or genome editing by CRISPR/CAS9 technology. Alternatively, somatic cells can be isolated by fibroblasts of the patient and reprogrammed to pluripotency, with the resulting iPSCs then being corrected by gene therapy or genome editing and differentiated through erythroid lineage.

risks and they are not effective in all cases. For example, patients with ineffective erythropoiesis do not respond to EPO. Thus, there is a clinical need for novel agents with a different mechanism of action from current ESAs.

Members of the transforming growth factor beta (TGF-β) superfamily, which include activins (A-B), growth differentiation factors (GDFs), and bone morphogenetic proteins (BMPs), have been studied as potential regulators of erythropoiesis, iron regulation and globin expression. Some recent studies have investigated the role played by two drugs, an activin receptor IIA (ActRIIA) ligand trap (ACE-011 or sotatercept) and a modified ActR type IIB (ActRIIB) ligand trap (ACE-536) in the regulation of late-stage erythropoiesis. It has been recently demonstrated that a mouse version of both drugs, termed RAP-011 and RAP-536, is able to induce differentiation of erythroid cells, improve ineffective erythropoiesis, correct anemia, and limit iron overload in a mouse model of β-thalassemia intermedia.[12,13] Both drugs act through inhibition of GDF11, a newly identified regulator of erythropoiesis that will contribute significantly to the understanding of the fine regulation of erythropoiesis and iron metabolism, and to the development of new drugs. So far, two phase II clinical trials have provided proof of the importance of ActR ligand trap molecules in the use of sotatercept in adults with β-thalassemia (*clinicaltrials.gov identifier: 01571635*) and in transfusion-dependent DBA patients (*clinicaltrials.gov identifier: 01464164*).

Finally, another no less interesting approach for future therapy in RBC diseases is represented by genome editing technologies. These have mainly been used to study gene function, in the discovery of therapeutic targets, and to develop disease models in several disorders. Considerable progress has been made in genome editing in the past decade *via* the use of either engineered nucleases systems, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or of the RNA-guided engineered nucleases based on CRISPR-Cas9 (clustered regularly inter-spaced short palindromic repeats/CRISPR-associated nuclease 9).[14-16] The most promising progress has been seen in the use of CRISPR/Cas9 technology for genome correction of specific DNA sequences including changes in either coding or non-coding regions of autologous cell genome.[17] This system has become a simple-to-design and cost-effective tool for various genome editing purposes, including gene therapy studies; indeed, it offers several advantages, the main one being its ability to edit multiple genes simultaneously.[18] Current challenges for genome editing of HSPCs include optimizing the delivery of gene-editing tools, improving the efficiency of introducing targeted modifications, and avoiding the creation of potentially harmful off-target mutations. β-thalassemia mutations have been corrected by gene editing in induced pluripotent stem cells (iPSCs) by converting β-thalassemic mutations from homozygous to heterozygous state, thus restoring *HBB* gene expression in erythrocytes differentiated from the corrected iPSCs.[19] This gene editing strategy will provide a crucial step to cure monogenic disease by genetic repair of patient-specific iPSCs. We can envision a future in which the functional integration between next generation technologies for genomic screening and genomic editing will allow us to achieve our goal of targeted diagnosis and therapy (Figure 1).

The importance and the advantages of next generation technologies are obvious. However, despite the widespread

use of these tools in clinical practice, some considerations on their limitations and/or disadvantages should be made. On the one hand, will the different stages of data processing represent a major limitation of NGS genome screening, or will the need to accurately profile and control the off-target effects of genome editing compromise its use in gene therapy? Unlike *ex vivo* cell therapies, genome-editing technologies can potentially affect the human germline, and international committees to study the ethical, legal, and social implications of human gene editing have already been appointed. For example, the Hinxton Group is working to guide decision-makers on the use of these technologies in humans (*http://www.hinxtongroup.org/*). Thus, the education and training of all professional figures involved in the clinical practice of molecular medicine still remains one of the main aims of the scientific community.

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