Genome Editing and Engineering

Course No: BT-637

LECTURE-29

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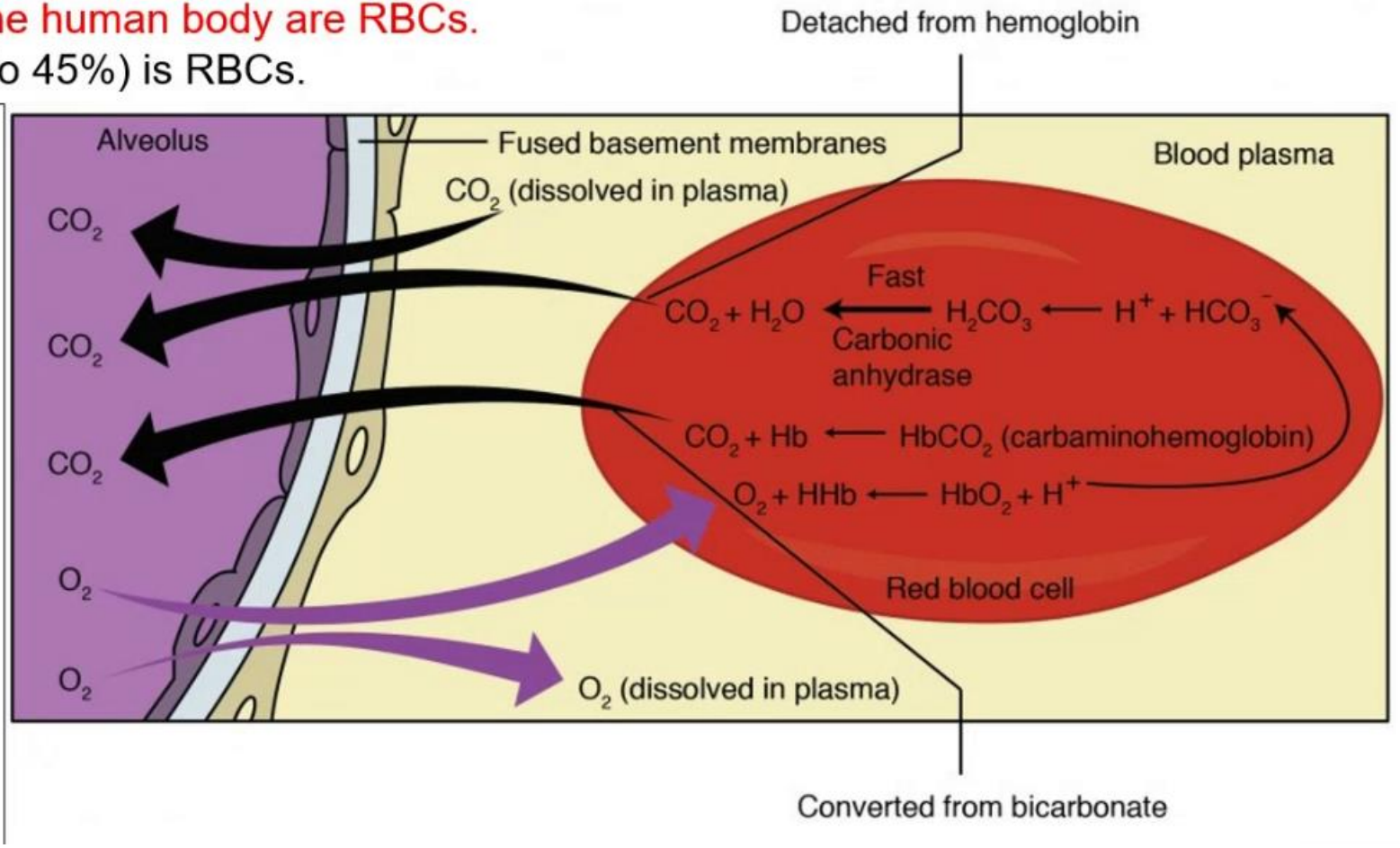
Red Blood Cells (RBCs)

- In humans, mature RBCs are flexible and oval biconcave disks.
- They lack a cell nucleus and most organelles, in order to accommodate maximum space for hemoglobin.
- They can be viewed as sacks of hemoglobin, with a plasma membrane as the sack.
- Approximately 2.4 million new RBCs are produced per second in human adults.
- The cells develop in the bone marrow and circulate for about 100–120 days in the body before their components are recycled by macrophages.
- Approximately a quarter of the cells in the human body are RBCs.
- Nearly half of the blood's volume (40% to 45%) is RBCs.

- A RBC consists of 250 million hemoglobin (Hb) molecules, a complex metalloprotein containing heme groups. The color of RBCs is due to the heme group of hemoglobin.
- The iron atoms of heme temporarily bind to oxygen molecules (O2) in the lungs and release them throughout the body.
- Oxygen can easily diffuse through the RBC's cell membrane.
- Hemoglobin in the RBCs also carries some of the waste product carbon dioxide back from the tissues; most waste carbon dioxide, however, is transported back to the pulmonary capillaries of the lungs as bicarbonate (HCO_3^-) dissolved in the blood plasma.



β-thalasemmia

- Red blood cells (erythrocytes) fulfil the unique role of supplying all tissues and organs with oxygen and are thus pivotal to human health.

- Hemoglobin, a tetrameric protein consisting of two α- and two β-like globin chains, is abundant in erythrocytes and is responsible for transporting oxygen from the lungs to the various tissues of the body.

- Not surprisingly, defects in hemoglobin proteins or in the expression of globin genes can result in severe diseases termed hemoglobinopathies.

- Hemoglobinopathies are amongst the most common genetic disorders world-wide, with an estimated 350,000 severely affected children born each year.

- Mutations in the globin genes may generate an abnormal form of hemoglobin, as in sickle cell disease (SCD) and hemoglobin C, D, and E disease, or result in reduced production of the a or b polypeptides and thus an imbalance of the globin chains in the cell. These latter conditions are termed a- or b-thalassemias, depending on which globin chain is impaired.

Wienert, B., Martyn, G. E., Funnell, A. P., Quinlan, K. G., & Crossley, M. (2018). Wake-up sleepy gene: Reactivating fetal globin for β-hemoglobinopathies. Trends in Genetics, 34(12), 927-940.

β-thalasemmia

- Beta thalassemia is a **inherited/genetic (monogenic) blood disorder** characterized by reduced **production of hemoglobin.**
- Hemoglobin is the **iron-containing protein in red blood cells** that carries oxygen to cells throughout the body.
- In people with beta thalassemia, low levels of hemoglobin lead to a lack of oxygen in many parts of the body.
- Often there is mild to severe anemia (low red blood cells or hemoglobin).
- It is caused due to mutations found in the β-globin gene (resultingin partial or complete lack of synthesis of β-globin chains), leading to anemia and requiring sporadic or chronic blood transfusions for survival.
- β-thalassemia is caused by more than 400 mutations, mostly associated with one or a limited number of nucleotides.
- The vast majority of β-thalassemias are caused by point mutations within the gene or its immediate flanking sequences and are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing and mRNA translation.
- Although defective β-globin gene expression and β-globin deficiency can be attributed to almost 400 thalassemic mutations, only ten mutations are responsible for the majority of cases worldwide. Of these, some of the most frequent cause aberrant splicing of intron 1 (IVS1–110, IVS1–6 and IVS1–5) or intron 2 (IVS2–654 and IVS2–745) of the human β-globin gene.

β-thalasemmia

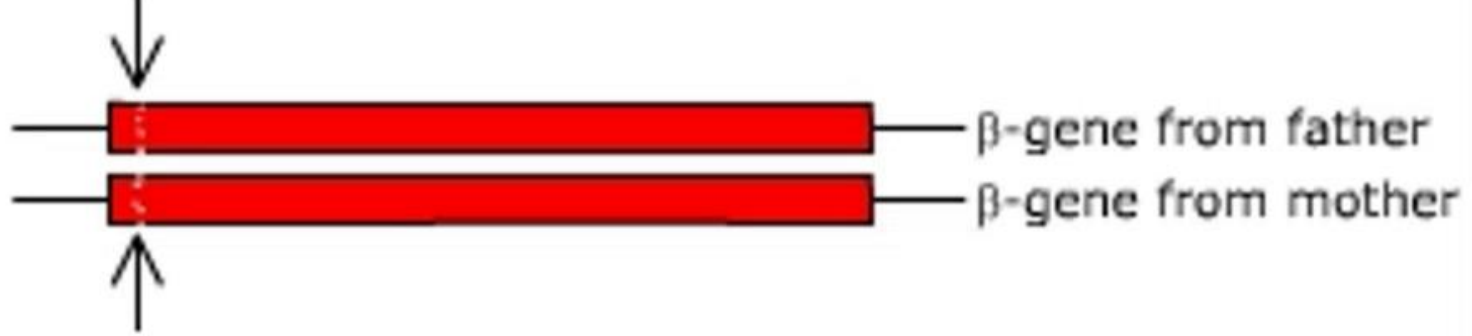
- The adult form of hemoglobin (HbA) is a heterotetramer consisting of two globin protein subunits, α- and β-globin, each encoded by separate genes and bound to a heme prosthetic group.

- Beta thalassemia is caused by mutations that inhibit the production of beta-globin polypeptides.

- Consequently, free alpha-globin molecules form insoluble aggregates that damage and destroy erythroid precursors in a process termed ineffective erythropoiesis.

- β-thalassemia is the result of single nucleotide substitutions, such as small insertions and deletions within the HBB gene or its flanking sequence. Rarely, β-thalassemia may occur due to large deletions. Only a fraction of β-thalassemia cases are the result of deletions in the HBB gene coding sequence. At present, 1,811 haemoglobin gene variants are known, of which, 404 mutations are associated with β-thalassemia. These mutations include causative mutations, mutations that modify disease presentation and neutral polymorphisms (IthaGenes database; ithanet.eu/db/ithagenes). It has been estimated that ~1.5% of the world's population are carriers of β thalassemia trait

With a mutation on one of the two ß-globin genes, a carrier is formed with lower protein production, but enough hemoglobin



Without a mutation enough Hemoglobin

No thalassemia carrier

With one mutation less Hemoglobin

β-thalassemia carrier without illness, but less hemoglobin (slight aneamia)

With two mutations no β-globin

β-thalassemia major patient with severe aneamia

Types of globin

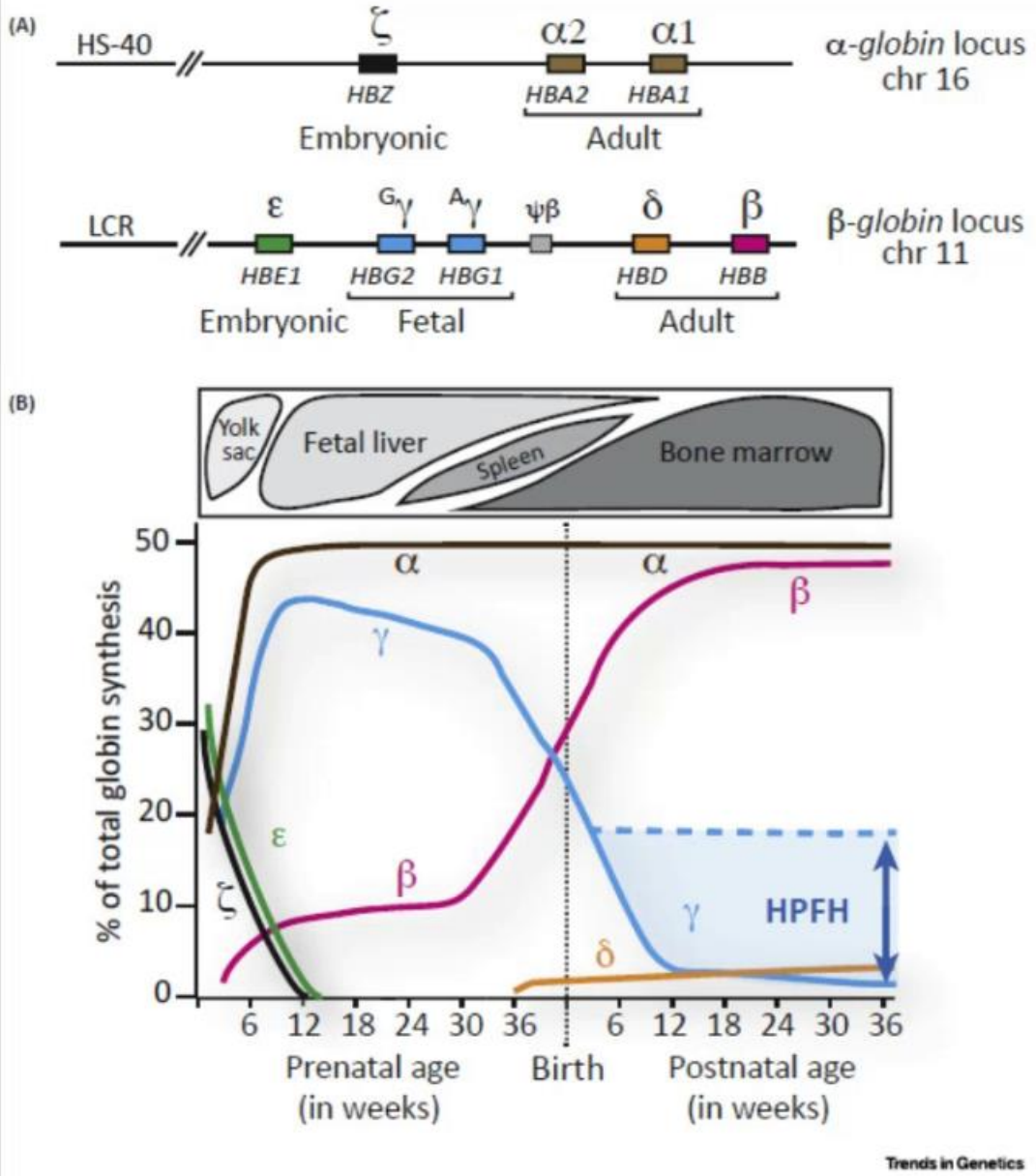


Figure 1. The Human *β-globin* Locus and its Developmental Regulation. (A) Schematic of the human *globin* loci. Shown are the *α-globin* locus on chromosome 16 and the *β-globin* locus on chromosome 11. Both loci are regulated by strong enhancer elements upstream of the *globin* genes, HS-40, and the locus control region (LCR). A pseudogene (Ψβ) is located between the fetal and adult *globin* genes on the *β-globin* locus. (B) Location of globin production (top) and developmental expression of *globin* chains in humans (bottom). People with hereditary persistence of fetal hemoglobin (HPFH) show abnormally high levels of *γ-globin* after birth.

Normal hemoglobin

There are 4 types of globin chain:
alpha (α), beta (β), delta (δ), gamma (γ)

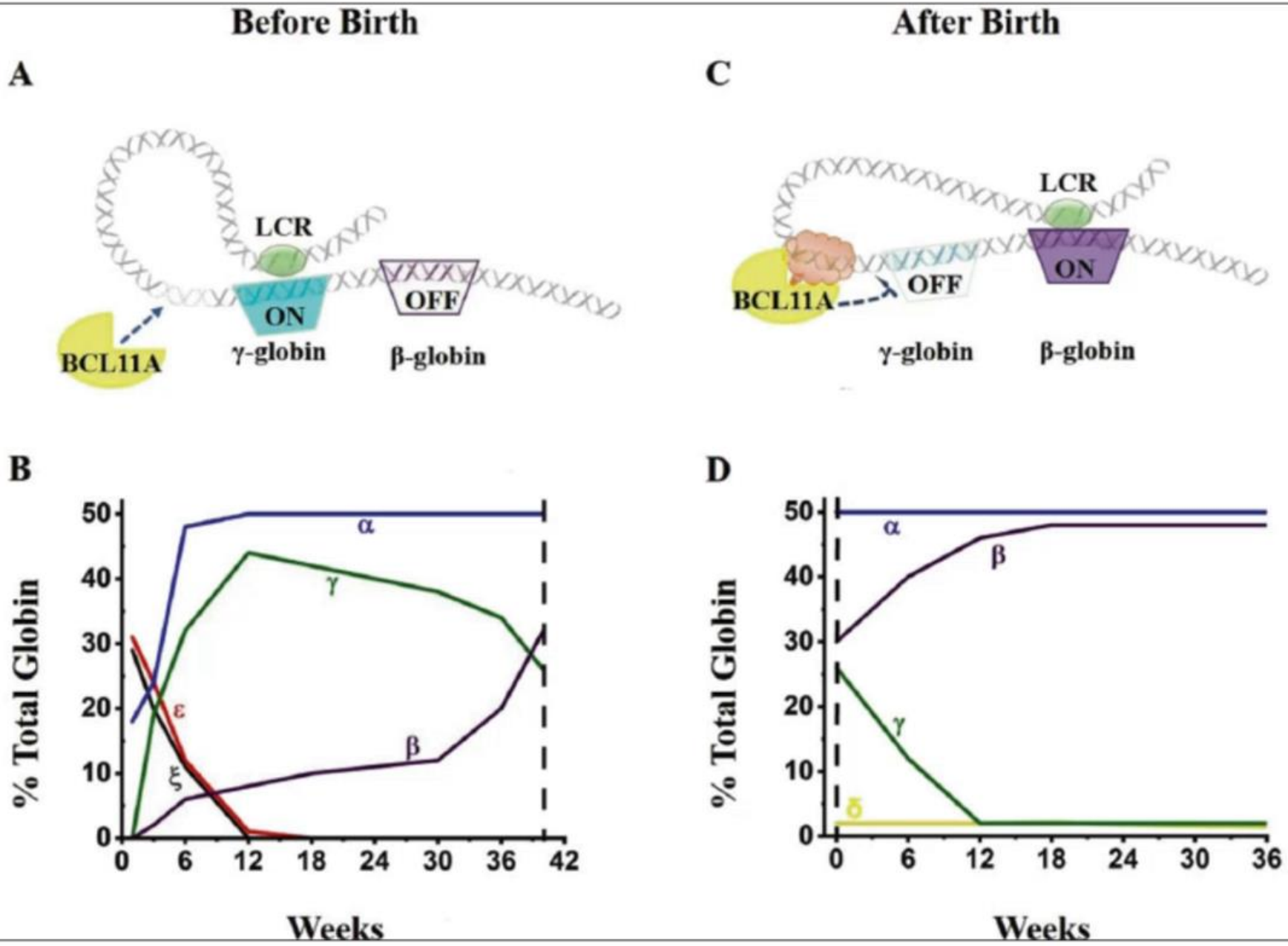
Hb type	Chains	Neonate	Adult
F	$\alpha_2\gamma_2$	75%	< 1%
A	$\alpha_2\beta_2$	25%	>97%
A2	$\alpha_2\delta_2$	<1%	< 3%

- During the first trimester of pregnancy, the ε-globin pairs up with ζ-globin to form functional human embryonic hemoglobin.
- There are two very important globin switches in human development: the first one takes place at the end of first trimester and the second one occurs around the time of birth. After first trimester, ε-globin gene is silenced and fetal γ-globin genes are upregulated. These γ-globin chains pair up with α-globin to form (Fetal hemoglobin) HbF.
- After birth, second switch is activated and γ-globin expression is virtually silenced. β-globin gene is upregulated and pairs with α-globin to form adult hemoglobin throughout adulthood.

Hereditary Persistence of Fetal Hemoglobin (HPFH) is an unusual condition in which red blood cells contain greater than normal amounts of hemoglobin F (fetal hemoglobin).

The fetal γ-globin genes are ordinarily silenced at birth and replaced by the adult β-globin genes. However, mutations that cause lifelong persistence of fetal γ-globin, ameliorate the debilitating effects of β-globin mutations. Therefore, therapeutically reactivating the fetal γ-globin gene is a prime focus of researchers.

Fig. 2 The Human Globin Locus and Its Developmental Regulation. **a**, **c** Shown are β-globin locus on chromosome 11 before and after birth. **b**, **d** shows the developmental shift of globin chains in humans Before and after Birth.



List of potential therapies to improve the management of β-thalassemia

Anemia

(is a condition in which you lack enough healthy red blood cells to carry adequate oxygen to your body's tissues)

- ❑ **Blood transfusion** (requiring sporadic or chronic blood transfusions to maintain their hemoglobin levels for survival)

- ❑ In β-thalassemia, red blood cell (RBC) transfusions are a life-saving treatment.
- ❑ However, every unit of transfused blood contains 200–250 mg of iron, and the human body has no mechanism to actively excrete excess iron.
- ❑ Therefore, cumulative **iron overload** is an inevitable consequence of chronic transfusion therapy.
- ❑ Therefore, management of tissue iron overload through iron chelation therapy (drugs) is a primary focus of β-thalassemia - to prevent iron accumulation in the patients organs and tissues.

List of potential therapies to improve the management of β-thalassemia

- **Allogenic bone marrow transplantation (BMT)** (advantage: potentially curative; issue: chronic graft-versus-host disease (GvHD) - White blood cells of the donor's immune system which remain within the donated tissue/cells (the graft) recognize the recipient (the host) as foreign (non-self). The white blood cells present within the transplanted tissue then attack the recipient's body's cells, which leads to GvHD. This should not be confused with a transplant rejection, which occurs when the immune system of the transplant recipient rejects the transplanted tissue; GvHD occurs when the donor's immune system's white blood cells reject the recipient). In addition, availability of allogeneic bone marrow is subject to finding a donor with an identical HLA.
- **Cord blood transplantation (CBT)**

Bone marrow transplantation – Hematopoietic stem cells (HSCs)

- The rationale of bone marrow transplantation in a β-thalassemic patient is to restore the tissue's capability of producing functional hemoglobin.

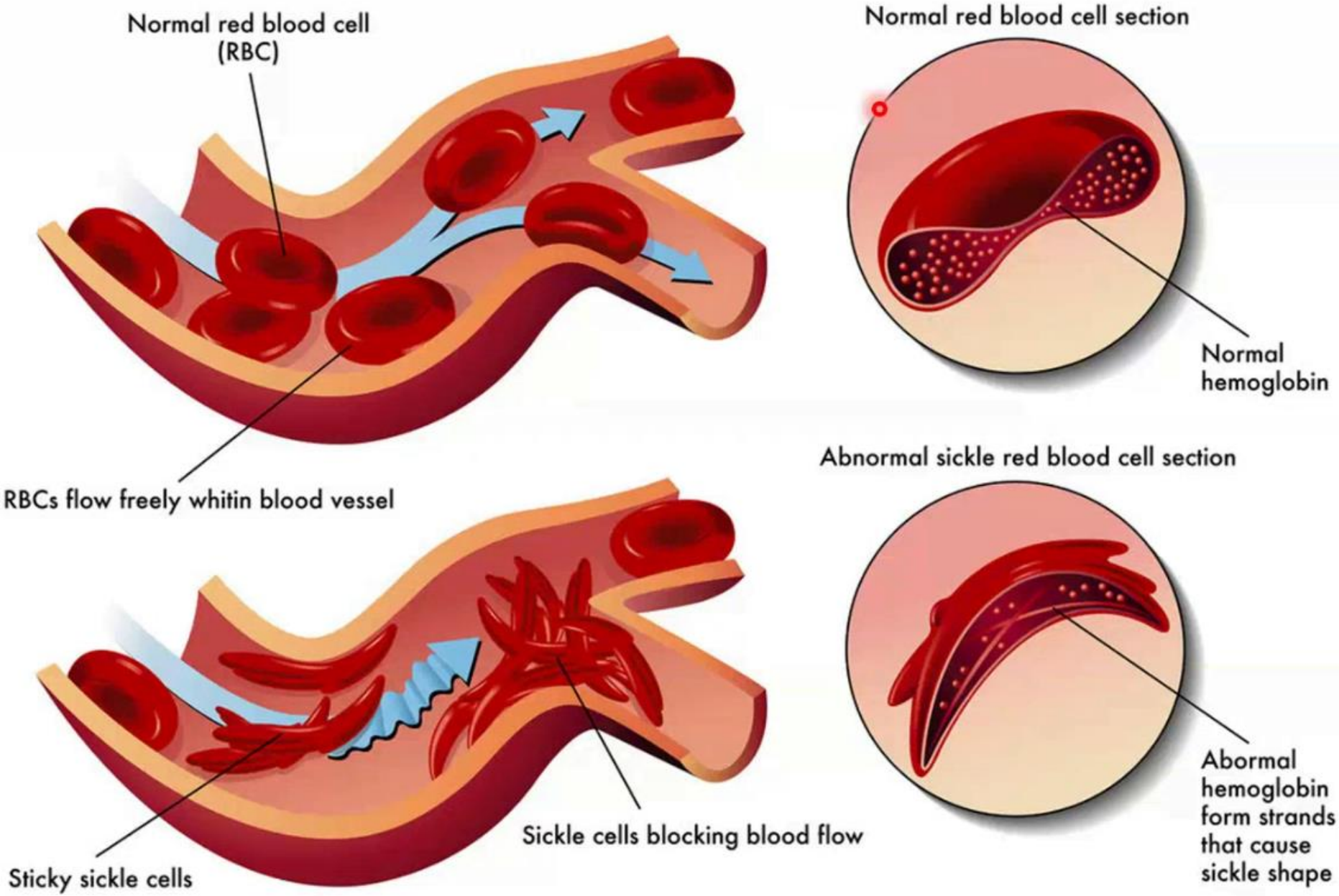
- Donald Thomas performed the first successful HSC transplantation in an 18 months old thalassemia major child using an HLA matched elder sibling as a donor 37 years ago.
- Bone marrow transplant stands on the following principles: i) Destroy defective stem cells to stop them from proliferating; ii) suppress the immune system of the host to ensure good engraftment; iii) infuse stem cells with normal genes; and iv) prevent graft vs. host disease (GVHD). This procedure requires progenitor stem cells to be administered in an individual.
- The procedure is sub-categorized on the basis of the source of progenitor cells (42) as follows: i) Progenitor stem cells from the recipient (autologous transplant); ii) stem cells from someone other than the recipient (allogeneic transplant); or iii) umbilical cord blood transplant.

List of potential therapies to improve the management of β-thalassemia

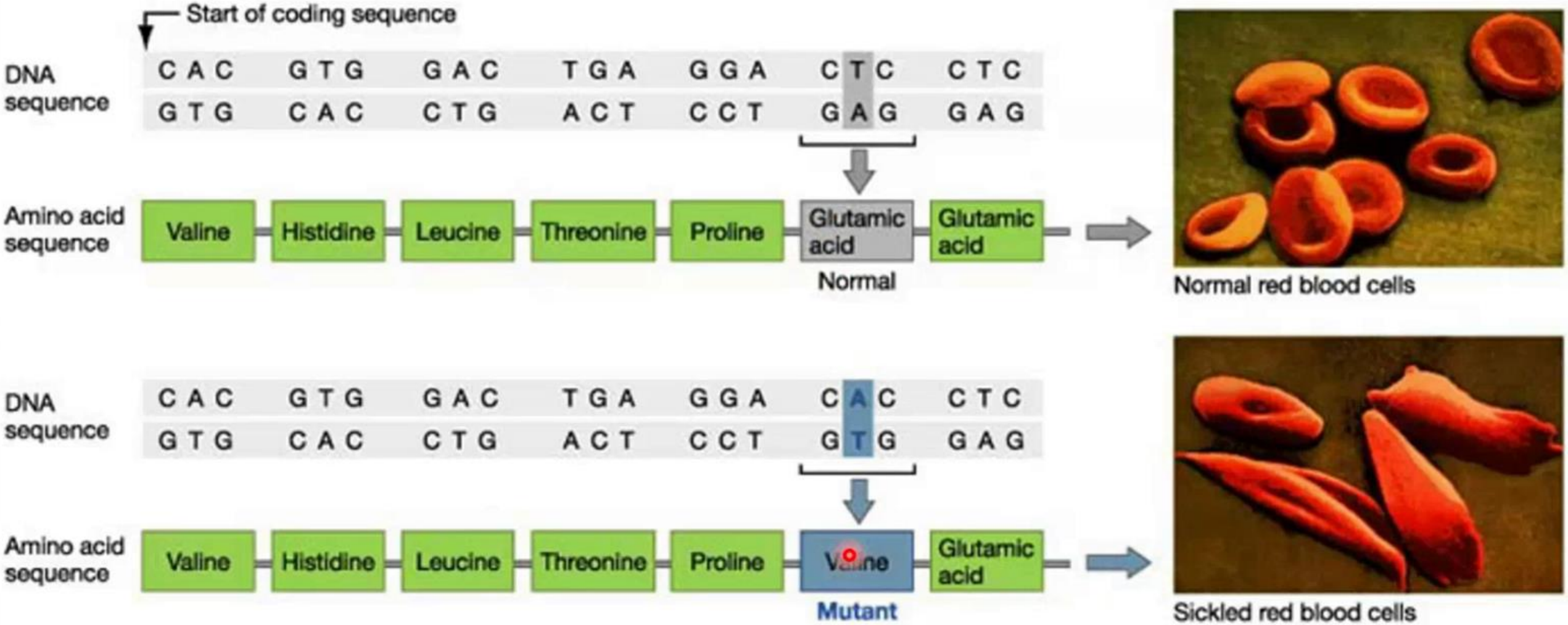
- **Reactivation of fetal hemoglobin (HbF) –** using drugs such as Hydroxyurea
- Fetal Hemoglobin: A Sleeping 'Back-up'

Rivella, S., & Rachmilewitz, E. (2009). Future alternative therapies for β-thalassemia. Expert review of hematology, 2(6), 685-697.

Sickle cell anemia



Sickle cell anemia



The change in amino acid sequence causes hemoglobin molecules to crystallize when oxygen levels in the blood are low. As a result, red blood cells sickle and get stuck in small blood vessels.

Hydroxyurea

- Certain drugs have been developed to enhance the expression of the γ-gene, which ultimately results in augmentation of fetal haemoglobin (HbF) levels and total haemoglobin levels in the body. However, its effectiveness is dependent on the genetic makeup of the individual patient.

- In the 1980s, a DNA demethylating compound, 5-azacytidine, was shown to boost HbF levels in baboons and humans. However, the high cytotoxicity and potential genotoxicity of 5-azacytidine stimulated the search for alternatives. Two years later **hydroxyurea** was identified as a potent HbF inducer.

- **Hydroxyurea (a cell cycle blocker) is a good and cost-effective drug** that normalizes the activity of several signalling pathways resulting in **augmented HbF production**, which ultimately results in reduction in the frequency of blood transfusions required.

- Hydroxyurea activates the γ-globin gene and enhances the production of HbF.
- Hydroxyurea is a cheap and cost effective drug that is effective in certain patients with thalassemia for reducing the frequency of blood transfusions required.
- Two α chains combine with the γ-globin chains and form HbF that functions in place of the defective haemoglobin. Hydroxyurea not only augments the HbF levels, but also increases the levels of total haemoglobin in the body.
- Its effectiveness is dependent on the genetic makeup of the patient, and it has proven to be a suitable treatment option for several thalassemia major patients.
- Despite this discovery in the early 1980s, its mode of action remains incompletely understood. While it has been shown to be effective in many patients, the magnitude of response varies from patient to patient for reasons that are still unknown. Thus, better understanding of the molecular processes of globin switching remained the primary focus of many researchers intent on developing more targeted therapies.

- The expression of individual globin genes is regulated by the interaction of an upstream enhancer cluster, the locus control region (LCR), with the gene promoters.
- Transcription factors at the promoters are thought to influence the recruitment of the LCR to turn the globin genes on and off at specific developmental stages.
- About 25 years ago, Krüppel-Like Factor 1 (KLF1), an erythroid specific transcriptional activator, was shown to bind to the b-globin promoter and to be critical for expression of adult β-globin. However, the factors required for fetal γ-globin silencing remained elusive.
- From 2007, a series of studies identified B Cell Lymphoma 11A (BCL11A) as a modulator of HbF levels , and follow-up studies established it as a major fetal globin repressor.
- A few years later, Zinc Finger And BTB Domain Containing 7A (ZBTB7A, also known as LRF) was established as the second major fetal globin repressor by a knockout study in HUDEP-2 cells and transgenic mice.
- Together, BCL11A and ZBTB7A account for the majority of γ-globin silencing.

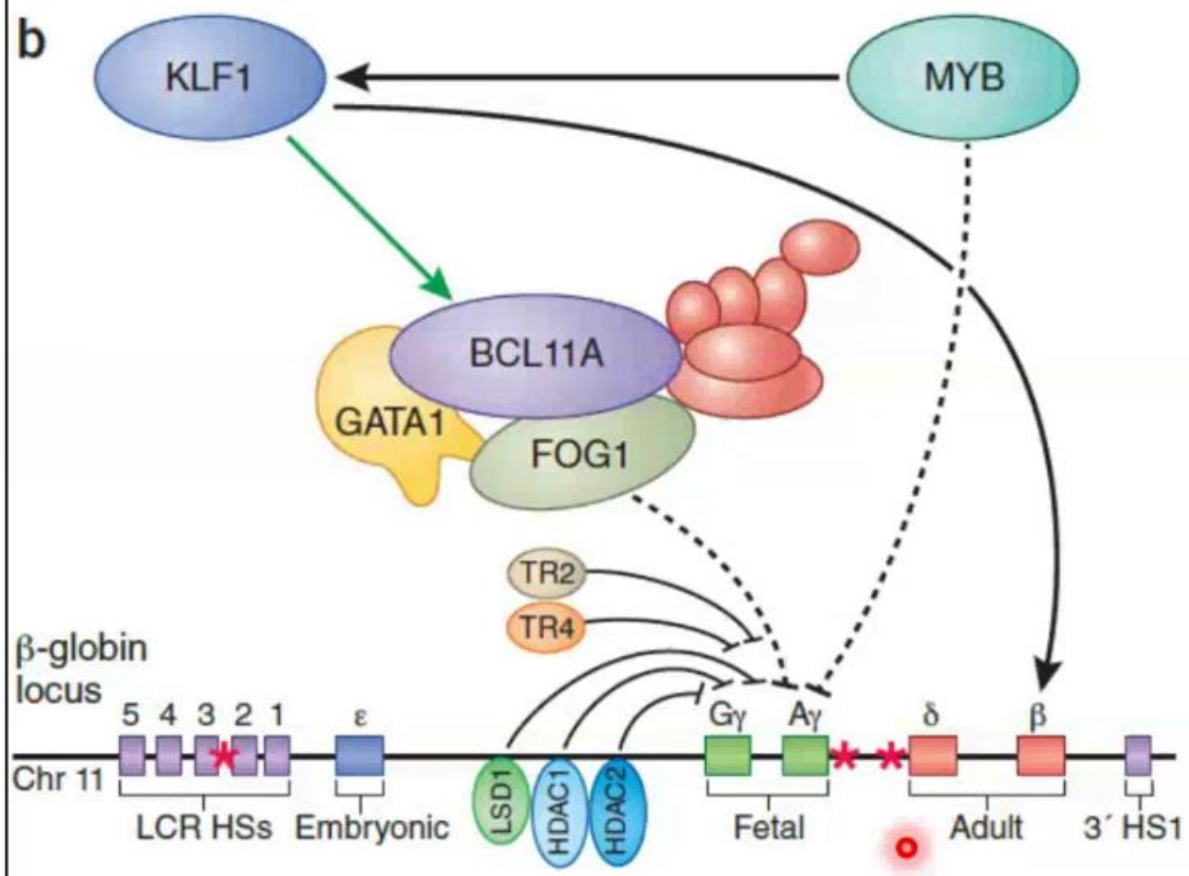
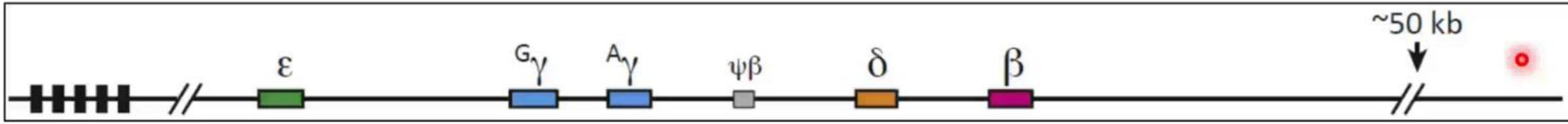


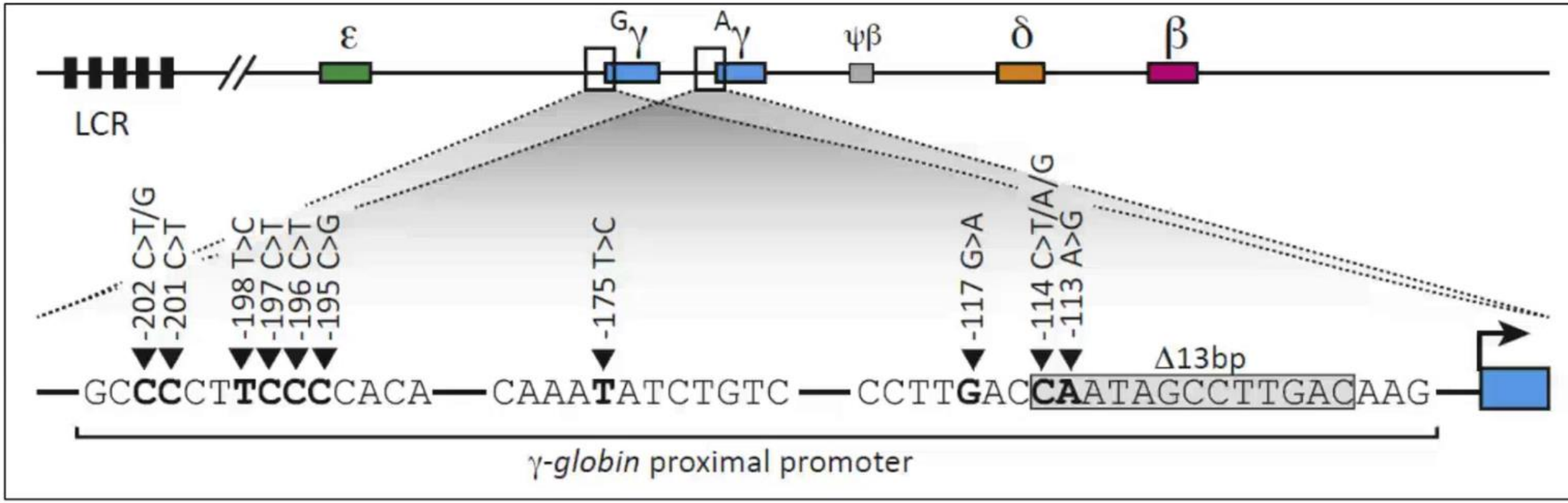
Figure 3 Regulation of HbF production. (**a**) Timing of β-like globin subunit switching during human ontogeny. The embryonic, fetal and adult stages are shown in blue, green and red, respectively. (**b**) Regulators of hemoglobin switching including BCL11A complexed with GATA1 and Fog1, KLF1, MYB, TR2, TR4, LSD1, HDAC1 and HDAC2 as well as their modes of proposed regulation are depicted here. Gene activation is depicted with an arrow, and gene repression with a blunt-ended arrow. Some factors, including the BCL11A complex, repress γ-globin through indirect mechanisms of action and are therefore shown with dashed lines. BCL11A binding sites are indicated with an asterisk (*). Co-repressor complexes that associated with BCL11A and other regulators are depicted in red. LCR, locus control region; HS, DNase I hypersensitivity site.

Sankaran, V. G., & Weiss, M. J. (2015). Anemia: progress in molecular mechanisms and therapies. Nature medicine, 21(3), 221-230.



LCR
(enhancer region)

A locus control region (LCR) is a long-range cis-regulatory element that enhances expression of linked genes at distal chromatin sites.



Wienert, B., Martyn, G. E., Funnell, A. P., Quinlan, K. G., & Crossley, M. (2018). Wake-up sleepy gene: Reactivating fetal globin for β-hemoglobinopathies. Trends in Genetics, 34(12), 927-940.

Genome editing

- Restore the normal β-globin function.
- It is hypothesized that the CRISPR/Cas9 system may be used to correct specifically harmful mutations of the β-globin gene, and this could be confirmed by normal erythrocyte differentiation and their normal expression.

- Reactivation of γ-globin that can replace the role of defective β-globin.

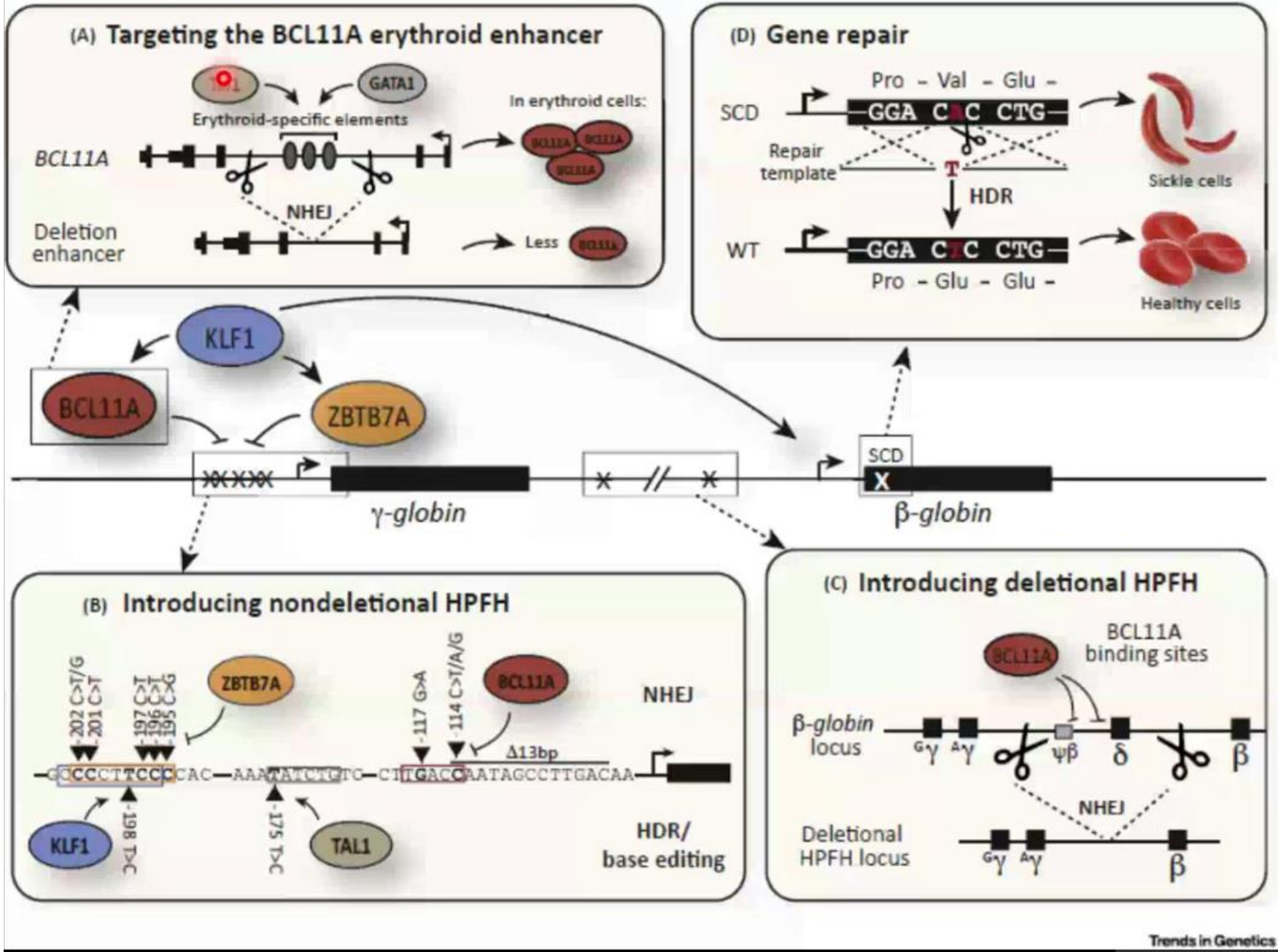
- BCL11A and ZBTB7A is a major γ-globin repressor. CRISPR/Cas9 is used to reactivate the genes associated with γ-globin by degrading either their repressor, BCL11A or ZBTB7A, or the binding of BCL11A to its binding site, thus increasing the production of γ-globin and minimizing the clinical severity of β-thalassemia.

- Additionally, BCL11A is an inhibitor of HbF. The low levels of BCL11A expression ultimately enhances HbF production.
- Pomalidomide, a pharmacological HbF inducer, has been found to induce HbF by lowering the expression of BCL11A.
- According to different molecular analyses, BCL11A knockdown is sufficient for increasing HbF expression.

- Various transcription factors are important in switching gene expression from γ-globin to β-globin.
- Shariati et al described one such transcription factor, SOX6. A mutation was introduced in the SOX6 binding gene region with the γ-globin gene promoter using CRISPR/Cas9 preventing its binding, and this resulted in reactivation of γ-globin gene expression. Increased levels of γ-globin mRNA expression was observed in K562 cells transfected with the CRISPR/Cas9 vectors. Thus, CRISPR/Cas9 can be used as a therapeutic approach for treating patients with β-thalassemia.
- The erythroid-specific transcription factor Krüppel-like factor 1 (KLF1) is a known positive regulator of BCL11A expression in both mouse models and in human cells.

- **Several clinical trials are investigating the safety and efficacy of gene addition and gene editing to restore Hb synthesis in β-thalassemia.**
- **Most of the products are gene addition-based technologies, while few are gene-editing strategies that aim to reactivate fetal Hb (HbF).**

Therapeutic Genome Editing Strategies to Treat β-Hemoglobinopathies



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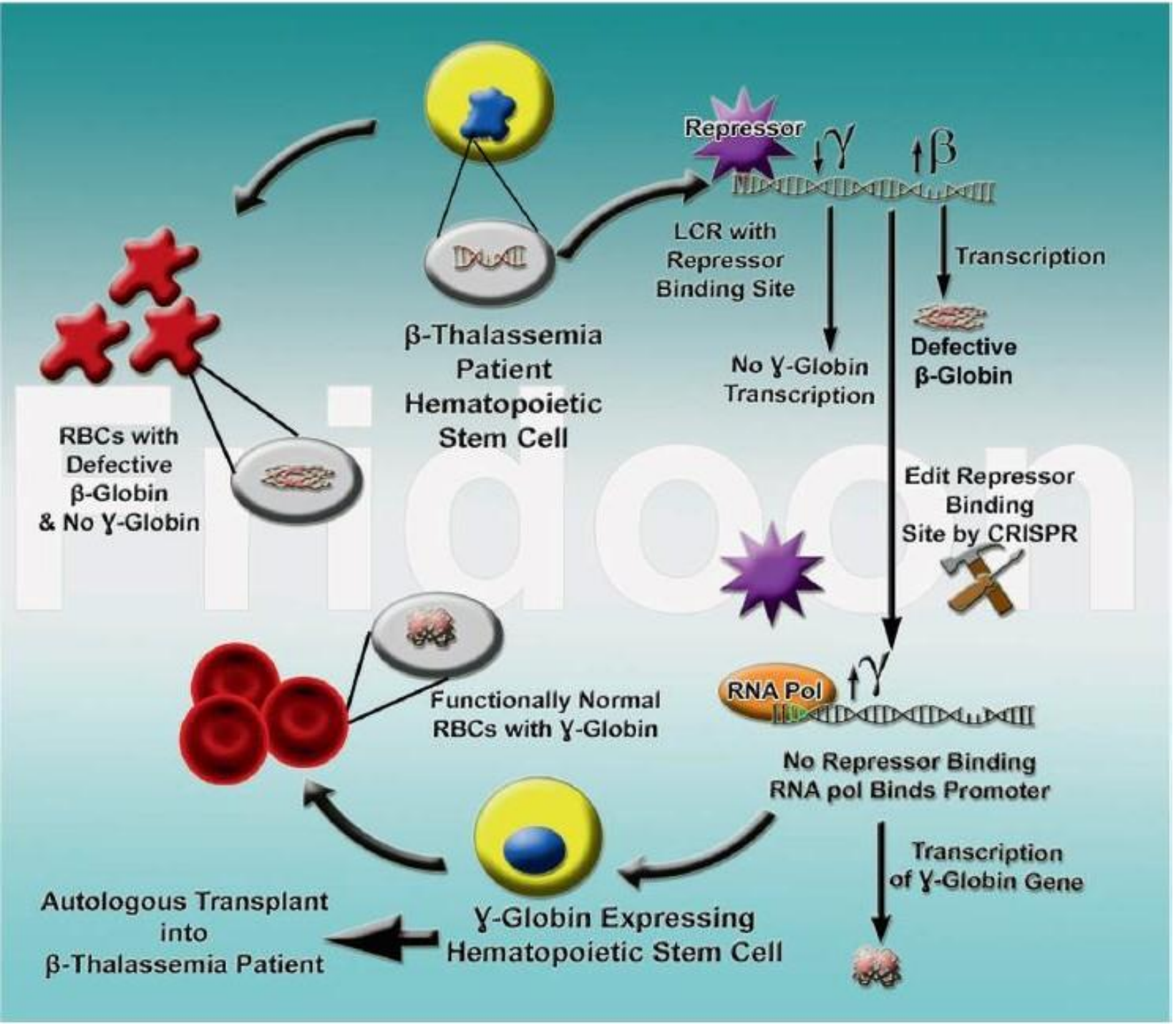


Gene Editing and β-thalassemia. Schematic representation of CRISPR/Cas9-based gene editing in autologous hematopoietic stem cells of thalassemia patients.

Figure 3. Summary of genome editing strategies and known molecular mechanisms within the *β-globin* locus. BCL11A and ZBTB7A repress *γ-globin* through direct binding to the proximal promoter and their expression is driven by the activator KLF1. KLF1 also drives the expression of adult *β-globin* by binding to the *β-globin* promoter. To elevate HbF levels one could (A) modify *BCL11A* expression by targeting erythroid-specific elements in the BCL11A enhancer, (B) target the binding sites of ZBTB7A or BCL11A within the promoter mimicking nondeletional HPFH or introduce known gain-of-function hereditary persistence of fetal hemoglobin (HPFH) mutations for activators TAL1 or KLF1, or (C) introduce deletional HPFH to delete BCL11A binding sites that are necessary for HbF silencing. (D) Lastly, repairing the sickle cell disease (SCD) point mutation in β-*globin* back to the wild-type (WT) sequence by homology-directed repair (HDR) or base editing may be feasible. NHEJ, Non-homologous end joining.

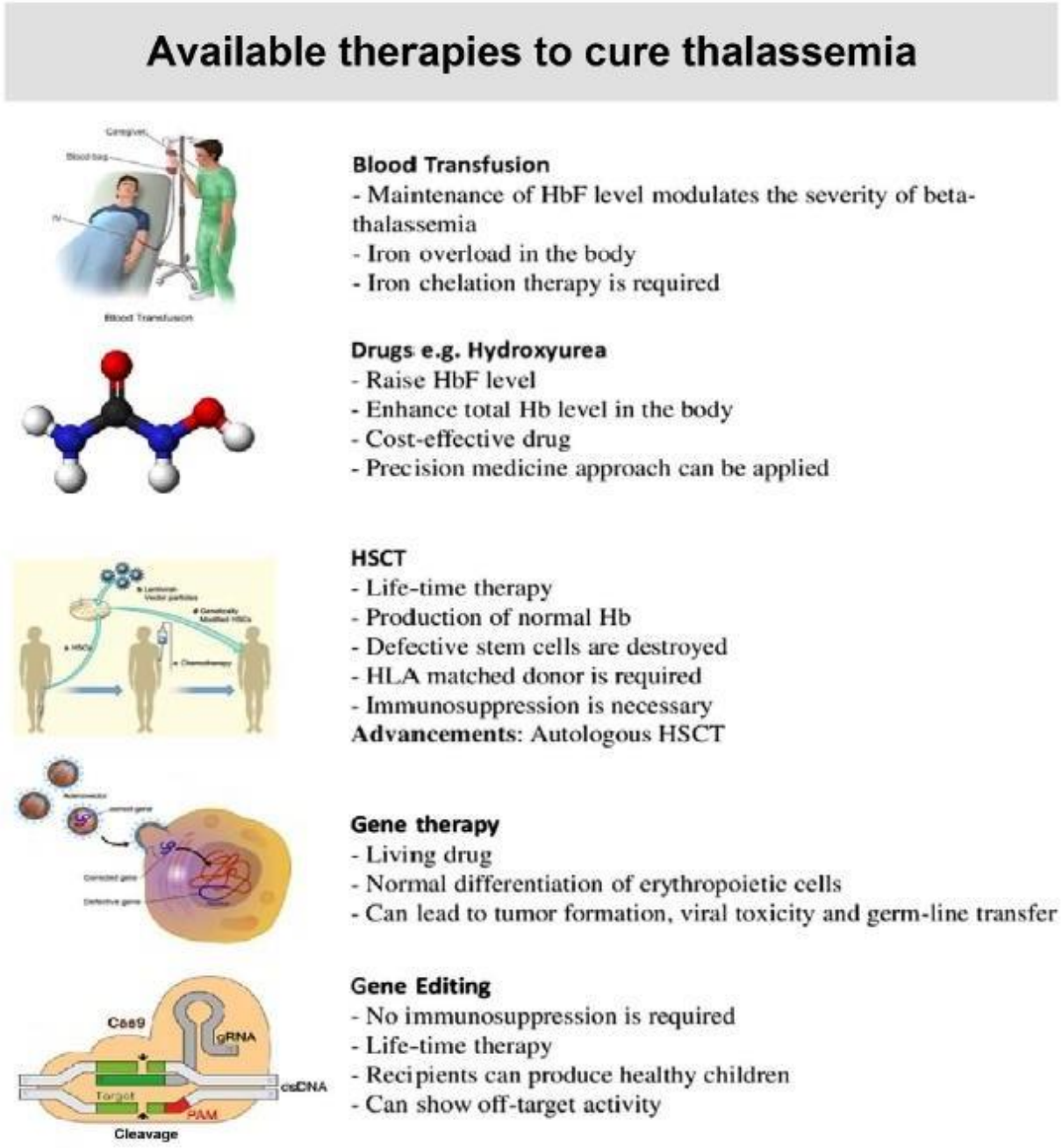


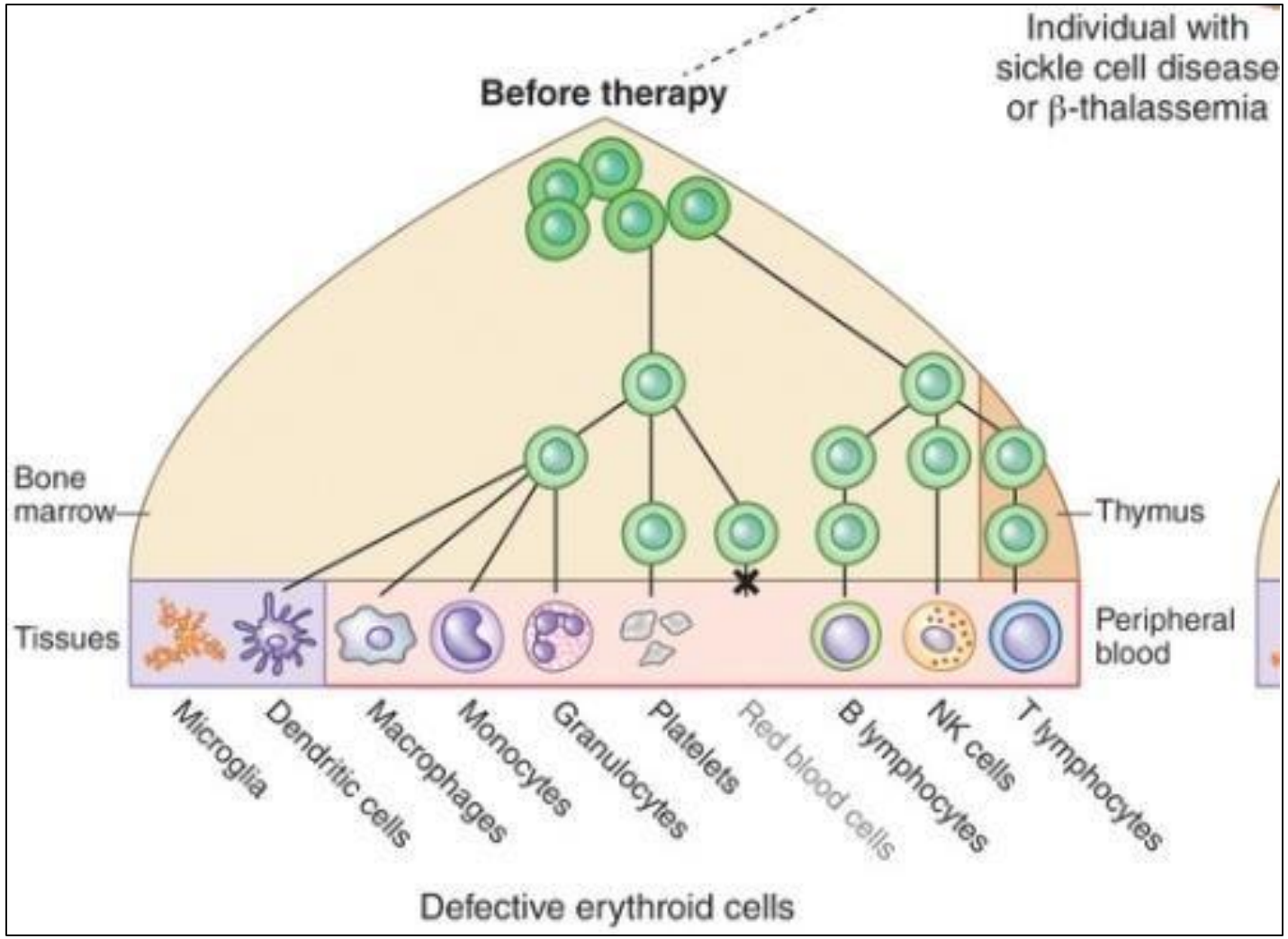
Figure 2. Available therapies for curing thalassemia. Modified and reproduced from Persons (136). Hb, haemoglobin; HbF, fetal Hb; HSCT, haematopoietic stem cell transplant; HLA, Human Leukocyte Antigen.

List of potential therapies to improve the management of β-thalassemia

❑ Generation of induced pluripotent stem cells and correction of the genetic defect(s)

- Patient derived induced pluripotent stem cells (iPSCs) have been corrected using the CRISPR/Cas9 system ex-vivo.
- The corrected iPSCs were then differentiated into fully developed red blood cell precursors which were used for transplantation.

Rivella, S., & Rachmilewitz, E. (2009). Future alternative therapies for β-thalassemia. Expert review of hematology, 2(6), 685-697.


Individual with sickle cell disease or β-thalassemia
Before therapy
Bone marrow
Thymus
Tissues
Peripheral blood
Microglia
Dendritic cells
Macrophages
Monocytes
Granulocytes
Platelets
Red blood cells
B lymphocytes
NK cells
T lymphocytes
Defective erythroid cells

Publications using CRISPR/Cas9 system-1

Genome Res. 2014 Sep;24(9):1526-33. doi: 10.1101/gr.173427.114. Epub 2014 Aug 5.

Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac.

Xie F[1], Ye L[1], Chang JC[1], Beyer AI[2], Wang J[3], Muench MO[4], Kan YW[5].

⊕ Author informatic

Abstract

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provide a rich source
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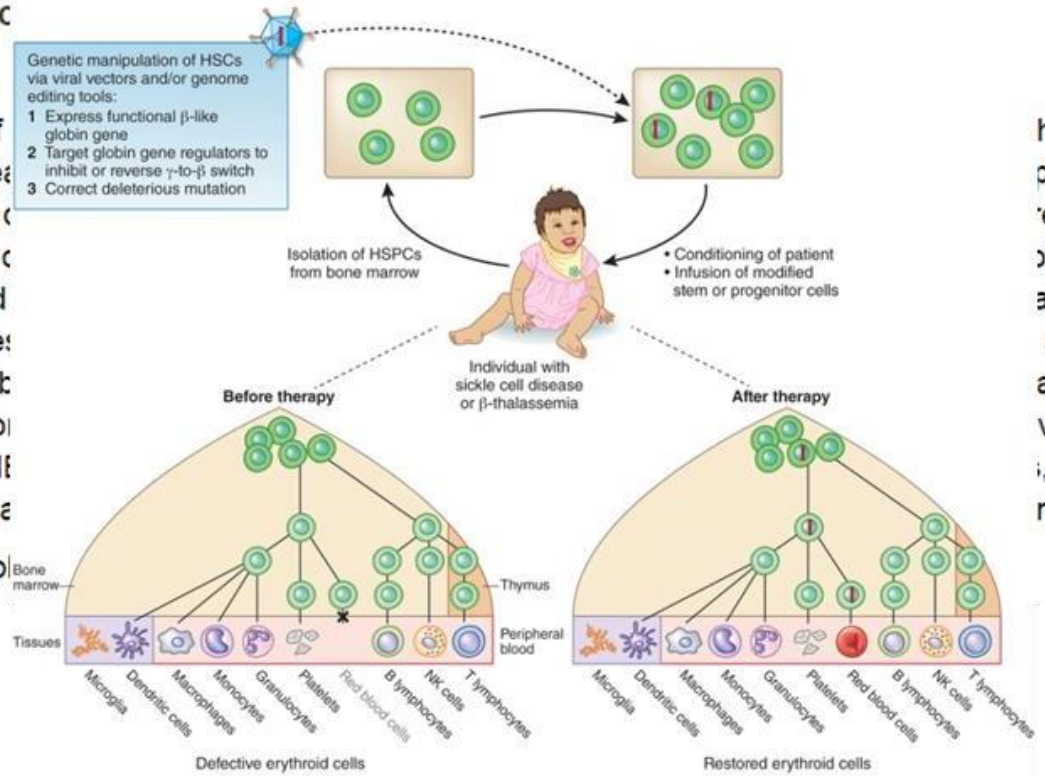

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patients could offer an
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Correction of the human hemoglobin beta (HBB) gene in induced pluripotent stem cells from β-thalassemia patients using CRISPR-Cas9 and the piggyback transposon

Picture is from Nature Medicine 21, 221–230 (2015)

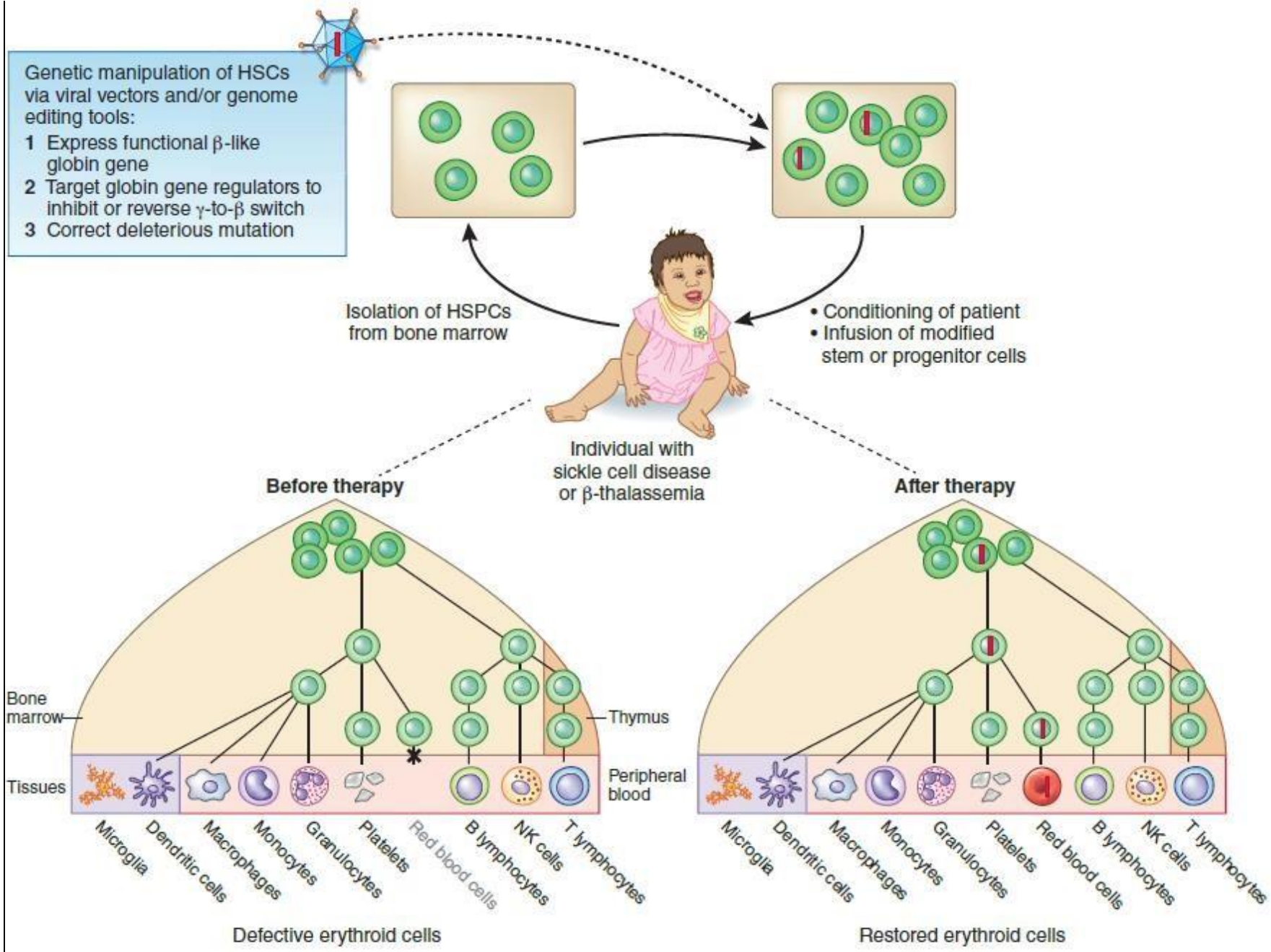


Figure 5 Gene therapy or editing to treat congenital forms of anemia. Potential corrective approaches for β-thalassemia or sickle cell disease are shown. These approaches can also be used to correct various other severe anemias caused by monogenic mutations. Adapted with permission from ref. 114, Nature Publishing Group.

Genome Res. 2014 Sep;24(9):1526-33. doi: 10.1101/gr.173427.114. Epub 2014 Aug 5.

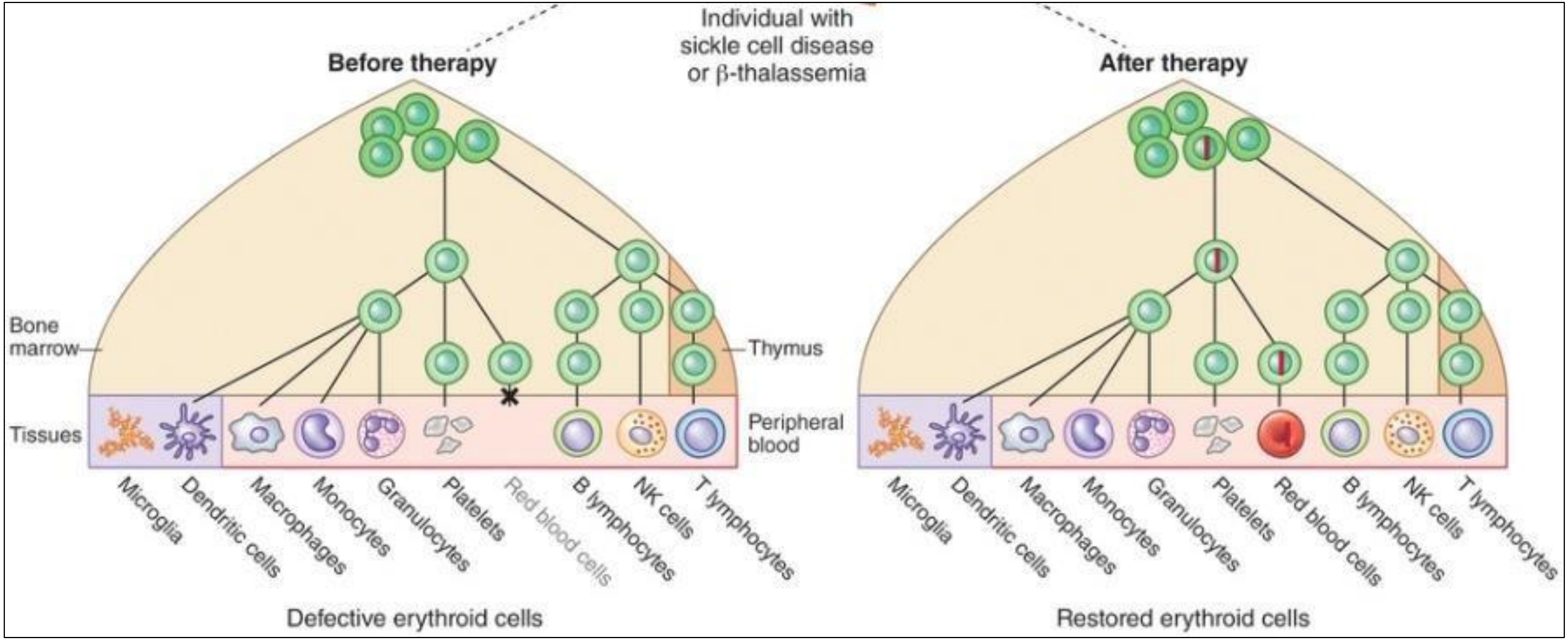
Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac.

Xie F[1], Ye L[1], Chang JC[1], Beyer AI[2], Wang J[3], Muench MO[4], Kan YW[5].

Author information

Abstract

β-thalassemia, one of the most common genetic diseases worldwide, is caused by mutations in the human hemoglobin beta (HBB) gene. Creation of human induced pluripotent stem cells (iPSCs) from β-thalassemia patients could offer an approach to cure this disease. Correction of the disease-causing mutations in iPSCs could restore normal function and provide a rich source of cells for transplantation. In this study, we used the latest gene-editing tool, CRISPR/Cas9 technology, combined with the piggyBac transposon to efficiently correct the HBB mutations in patient-derived iPSCs without leaving any residual footprint. No off-target effects were detected in the corrected iPSCs, and the cells retain full pluripotency and exhibit normal karyotypes. When differentiated into erythroblasts using a monolayer culture, gene-corrected iPSCs restored expression of HBB compared to the parental iPSCs line. Our study provides an effective approach to correct HBB mutations without leaving any genetic footprint in patient-derived iPSCs, thereby demonstrating a critical step toward the future application of stem cell-based gene therapy to monogenic diseases.


Individual with sickle cell disease or β-thalassemia
Before therapy
After therapy
Bone marrow
Thymus
Tissues
Peripheral blood
Microglia
Dendritic cells
Macrophages
Monocytes
Granulocytes
Platelets
Red blood cells
B lymphocytes
NK cells
T lymphocytes
Defective erythroid cells
Restored erythroid cells

Thank You!