Molecular approach to the hemoglobinopathies

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- Hemoglobin disorders account for almost 5% of deaths in children under the age of five years.
- For a minority of patients, mostly in high-income countries, current therapies include lifelong monthly supportive red blood cell transfusions together with iron chelation or curative allogeneic HSC transplantation.

Thalassemia combined with hereditary cell membrane defect

Characteristic	Patient	Father	Mother	Older sister
RBC (x1012/l)	3.16	4.96	5.46	4.8
Hb (g/dl)	7.16	15.5	14.3	12.6
MCV (fl)	70.6	93.6	79.6	80.03
MCH (pg)	22.66	31.3	26.2	26.1
MCHC (g/dl)	32.09	33.4	32.9	326.5
MCV (fl)	72.76	93.3	83.1	83.2
PK	N	N	N	N
G6PD	N	N	N	N
Coombs test	N	N	N	N
Haemoglobin electrophoresis	N	N	N	N

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* Mother $-\alpha^{3.7}/\alpha\alpha$
* DNA sequencing showed SLC4A1 gene had two mutation sites: Exon 3 c.113A>C and intron 7 c.609+86G>A in the proband.
* In exon 3 c.113 A>C of SLC4A1, the GAC codon was mutated to GCC (Asp 38 Ala) missense mutation.
* Mutations were not detected in ANK1, SPTA1 (*AD/AR*), SPTB or EPB42.
* His father and older sister had the same mutations

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* β-thalassaemia combined with hereditary spherocytosis

Erythrocyte Indexes in Patients with Hereditary Spherocytosis (HS)

	Control	HS	HS+ beta-thal
Age (year)	21.64±12.74	18.70 ± 12.37	32.46 ± 10.90
RBC	4.82 ± 0.82	3.70 ± 0.90	5.27 ± 0.75
Hb (g/dL)	13.75 ± 1.11	10.63 ± 2.90	10.49 ± 1.18
Hct(%)	41.84 ± 2.76	31.5 ± 8.76	34.73 ± 4.62
MCV (fL)	87.20 ± 4.38	84.84 ± 10.16	66.13 ± 7.40
MCH (pg)	28.68 ± 1.80	28.70 ± 3.47	20.90 ± 3.32
MCHC (g/dL)	33.01 ± 1.06	33.89 ± 2.35	30.36 ± 1.16
RDW (%)	12.28 ± 0.72	17.16 ± 5.33	18.70 ± 5.17

δ -globin gene (HbA2) Variant- (HbB2)

Sample	HbA2	HbX	HUGO nomenclature	α-genotype
1	1.7-2.1	1.1-1.5	Cd16 GGC>CGC c.49G>C	αα/αα
2	1.1-1.5	1.0-1.4	Cd16 GGC>CGC c.49G>C	–α/αα
3	1.3-1.5	0.8-1.2	Cd16 GGC>CGC c.49G>C	–α/–α

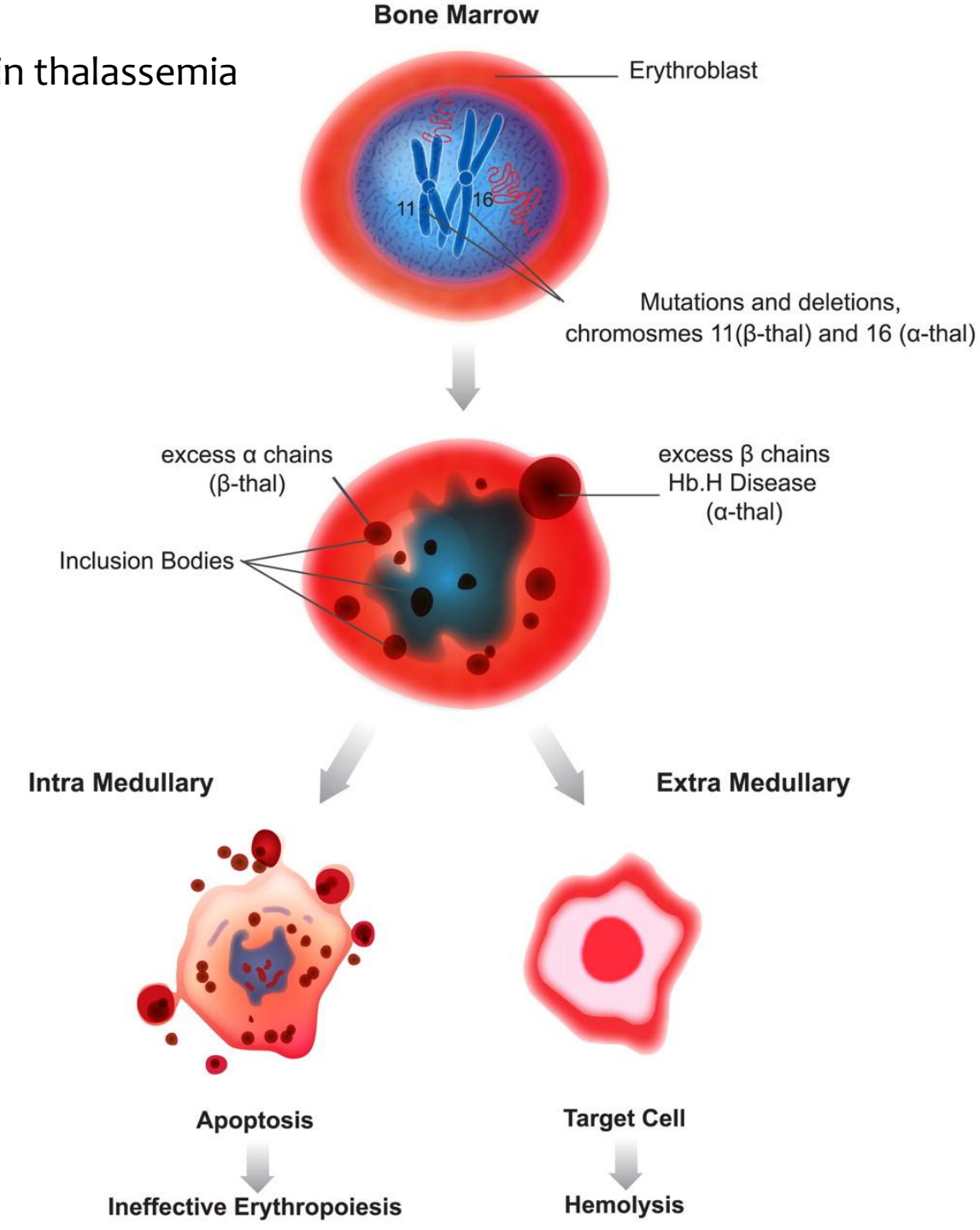
δ -globin gene -HbA2 Variant

Sample	HbA2	HbX	HUGO nomenclature	α-genotype
1	1.3		Cd116 CGC>CAC c.350G>A	–α/αα
2	1.6		Cd116 CGC>CAC c.350G>A	αα/αα
3	1.4		Cd116 CGC>CAC c.350G>A	–α/αα
4	1.6		Cd27 GCC>TCC c.82G>T	–α/αα
5	1.7		Cd27 GCC>TCC c.82G>T	–α/–α
6	0.6		Cd27 GCC>TCC/ IVS-I-128 G>C c.82G>T/c.93-1 G>C	–α/αα

Transcription Factor mutation & HbF

* A missense mutation in the KLF1 gene, p.Ser102Pro (c.304T>C), which was detectable in 10 of 23 cases with *elevated HbF* level.
* A transcription regulator of erythrocyte development that probably serves as a general switch factor during erythropoiesis.
* It is a dual regulator of fetal-to-adult globin switching. Binds to the CACCC box in the beta-globin gene promoter and acts as a preferential activator of this gene.
* It binds to the BCL11A promoter and activates expression of BCL11A, which in turn inhibits the HBG1 and HBG2 genes.
* *BCL11A deletions result in fetal hemoglobin persistence and neurodevelopmental alterations.*

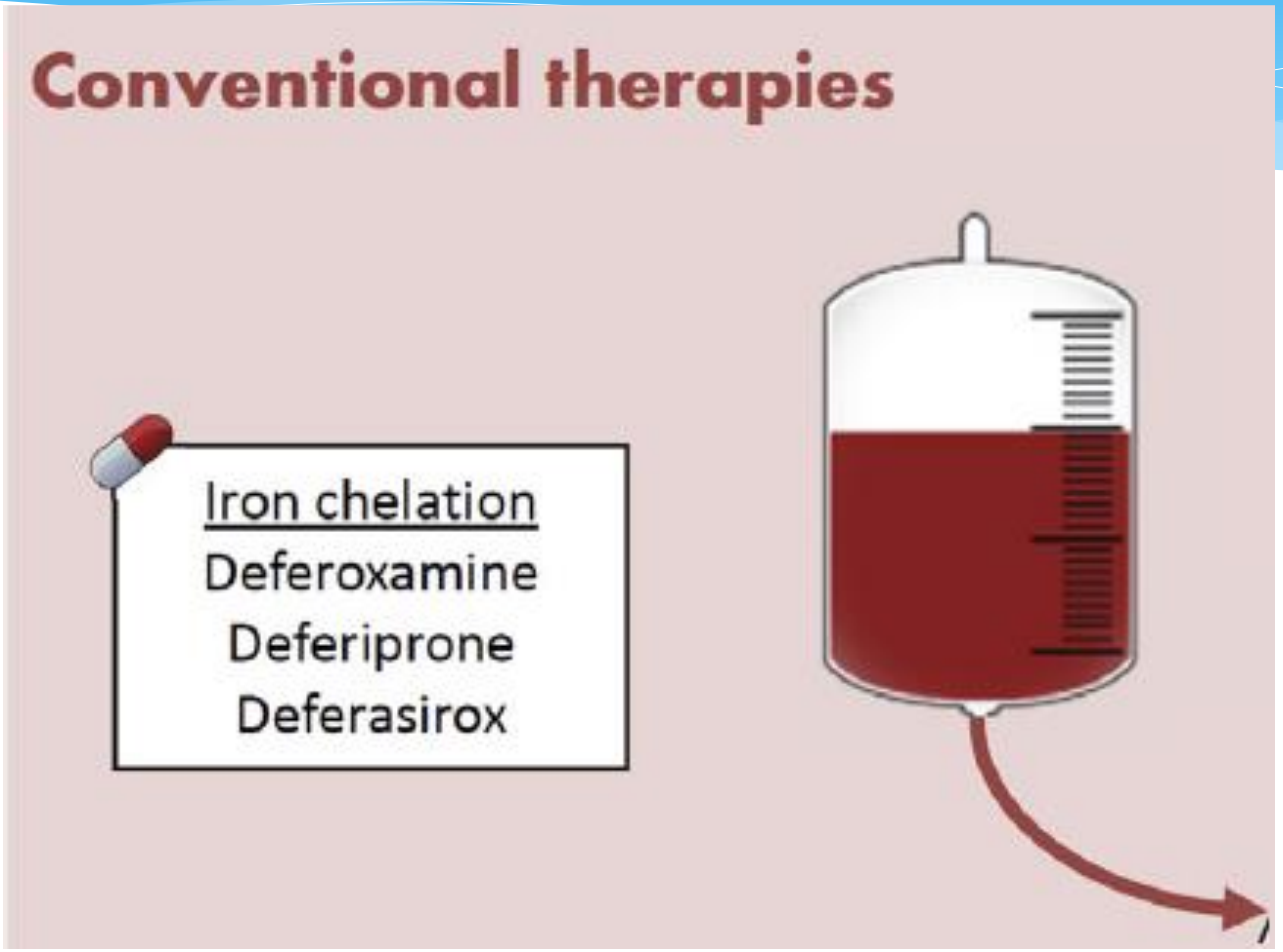
Mechanism of IE and hemolysis in thalassemia



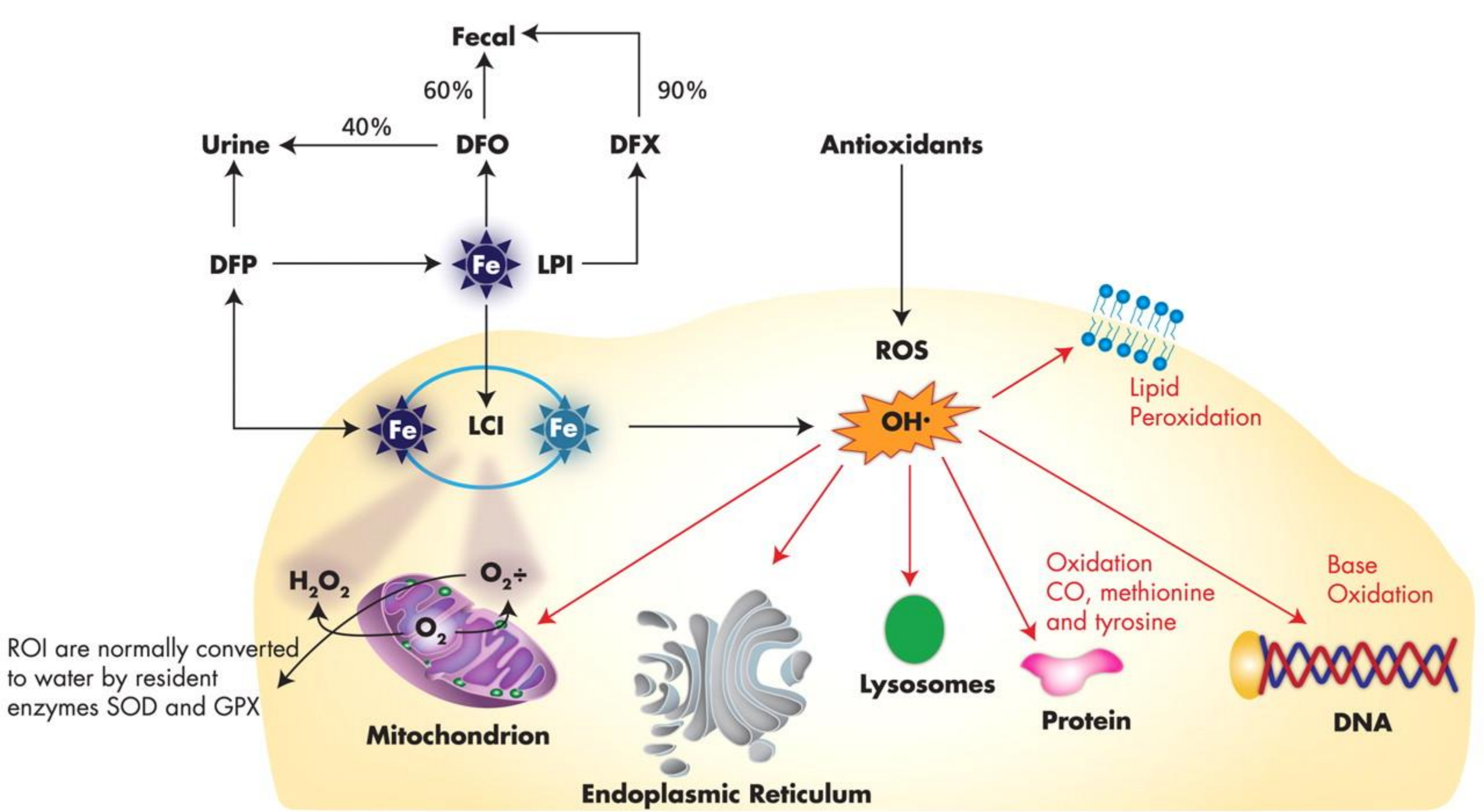
Current disease management of β-thalassemia

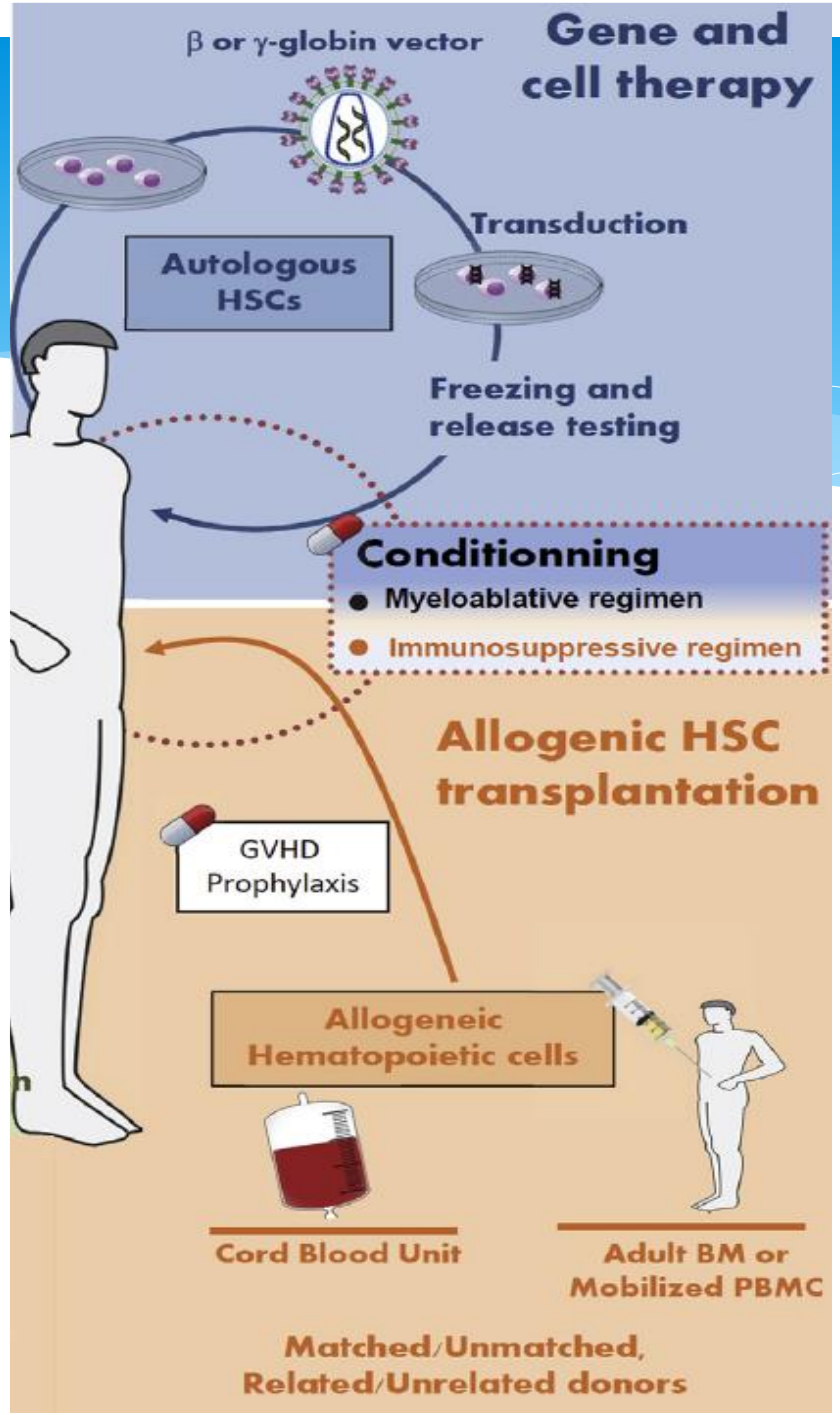
* Current disease management of β-thalassemia consists of:
  - Prenatal diagnosis
  - Transfusion therapy
  - Allogeneic bone marrow transplantation (BMT).

Current therapies for beta-thalassemia major



- Amelioration of free iron species (LPI and LCI) by iron chelators and antioxidants.
- Labile plasma iron (LPI) is penetrating through the cell membrane with a consequent accumulation of labile cell iron (LCI). Both LPI and LCI react with reactive oxygen intermediate (ROI) producing noxious reactive oxygen species (ROS), for example, OH' radicals, which are highly reactive and oxidize DNA, proteins and lipid components of the cell.
- Deferiprone (DFP) chelates LCI alone or in combination with LPI by Deferiozamine (DFO).
- Deferasirox (DFX) mainly removes LPI.


Fecal
60%
90%
40%
Urine
DFO
DFX
Antioxidants
DFP
Fe
LPI
ROS
Fe
LCI
Fe
OH·
Lipid
Peroxidation
H_2O_2
O_2÷
O_2
ROI are normally converted
to water by resident
enzymes SOD and GPX
Mitochondrion
Endoplasmic Reticulum
Lysosomes
Oxidation
CO, methionine
and tyrosine
Protein
Base
Oxidation
DNA


β or γ-globin vector
Gene and cell therapy
Transduction
Autologous HSCs
Freezing and release testing
Conditionning
Myeloablative regimen
Immunosuppressive regimen
Allogenic HSC transplantation
GVHD Prophylaxis
Allogeneic Hematopoietic cells
Cord Blood Unit
Adult BM or Mobilized PBMC
Matched/Unmatched, Related/Unrelated donors

The first curative allogeneic SCT to a thalassemia patient from an human leukocyte antigen (HLA) identical sibling donor was reported in 1982.

The probability of overall event-free survival has been recently reported as high as 89%-97% for patients with no advanced disease and of 80%-87% for patients with advanced disease.

Cord blood transplantation

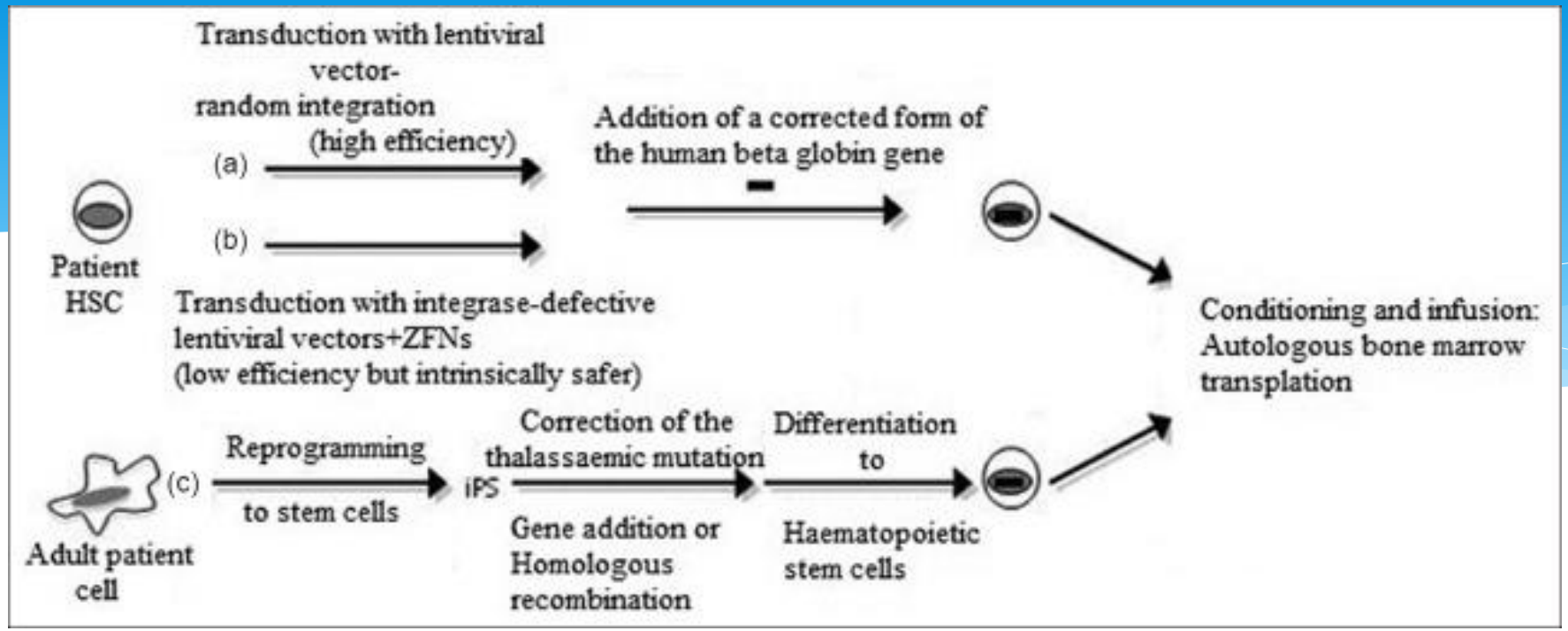
- The potential benefits of umbilical cord blood (UCB) treatment are the low risk of viral contamination from a graft, the decreased incidence of acute and chronic GVHD, and easier accessibility. The small size or small number of stem cells in the UBC collection relative to the number required for engraftment are probably the main causes of failure of UCB transplantation; therefore, this procedure is being used mainly in pediatric patients.
- Some patients have received UCB transplantation in combination with bone marrow or peripheral progenitor cells.

Genetic approaches

Gene transfer using onco-retroviral vectors

Gene addition mediated by retroviral vectors is an attractive approach for monogenic disorder. However, when applied to hemoglobinopathies (Thalassemia), this strategy raises major challenges in terms of controlling transgene expression, which should be:

1. Erythroid-specific;
2. Elevated;
3. Position-independent
4. Sustained over time.

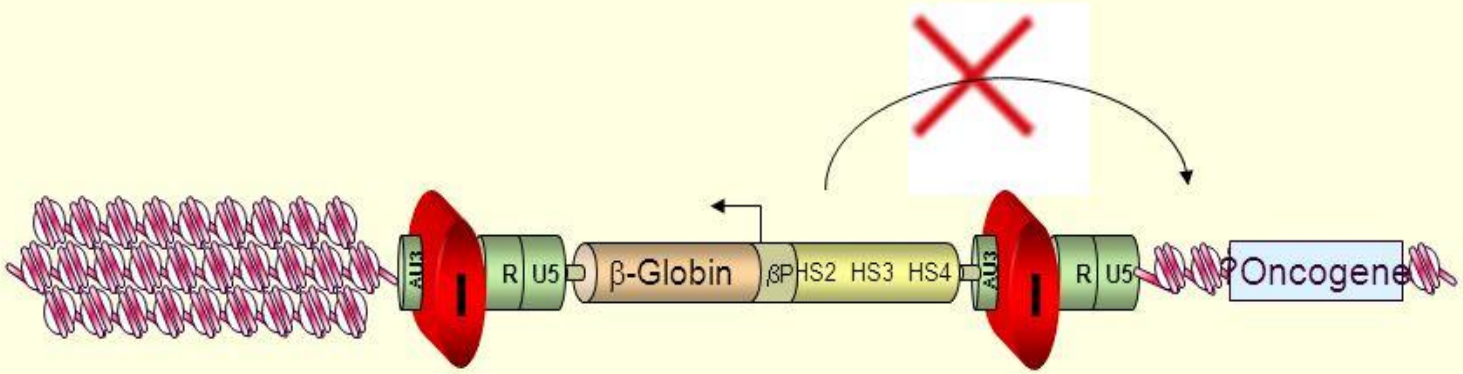

Transduction with lentiviral vector-random integration (high efficiency)
(a)
Addition of a corrected form of the human beta globin gene
(b)
Patient HSC
Transduction with integrase-defective lentiviral vectors+ZFNs (low efficiency but intrinsically safer)
Conditioning and infusion: Autologous bone marrow transplation
Reprogramming to stem cells
(c)
Adult patient cell
iPS
Correction of the thalassaemic mutation
Gene addition or Homologous recombination
Differentiation to
Haematopoietic stem cells

Limitations

* One of the most pressing problems is elimination of the transcription factors when they are no longer needed.

* Second, it is necessary to reestablish the correct re-programming so that the iPS cells do not develop into tumors.

Use of "Insulator" in a β-globin lentiviral vector for Gene Therapy of β-Thalassemia



Insertion of insulator sequences in a Lentiviral Vector to increase the safety of the vector, blocking the activity of the enhancer towards surrounding genes.

Future therapies-Fetal Hb inducers

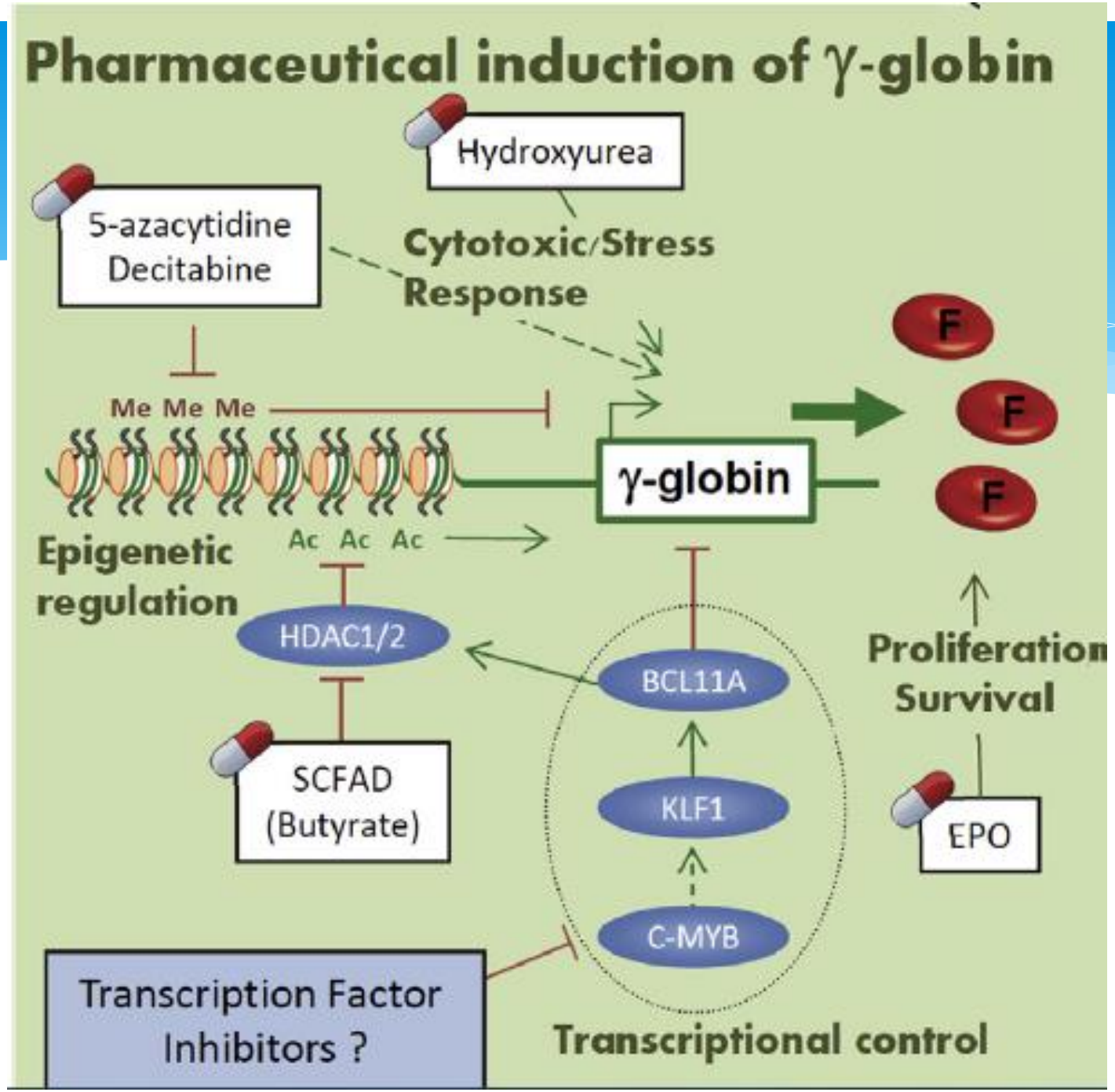
* Gamma-globin chain inducers aim to reduce the need for red blood cell transfusions.
* A number of drugs have been tested, including cytotoxic compounds and epigenetic regulators.
* The first drug shown to increase g-globin expression was the demethylating agent 5-azacitidine.
* Small-chain fatty acid derivatives, including arginine butyrate, have also been shown to increase g-globin expression most likely by inhibiting histone deacetylation.
* Hydroxyurea is the only drug currently approved for g-globin induction. It acts through multiple mechanisms. Its cytotoxic activity is thought to accelerate the differentiation process and to stimulate cellular stress response pathways, leading to an overall increase in the number of F cells.

Future therapies-Fetal Hb inducers

* Erythropoietin (EPO) has proliferative and anti-apoptotic properties. The combined administration of recombinant EPO together with cytotoxic drugs can be beneficial for patients with low baseline EPO levels.

Future therapies-Fetal Hb inducers

* Future treatments may target the transcription factors involved in g-globin repression, such as BCL11a and KLF1.
* BCL11a is an essential transcription factor involved in g-globin downregulation.
* It binds to intergenic regions of the HBB locus, promoting long-range interactions with the LCR that favors b-globin expression. It recruits histone deacetylase to repress g-globin. KLF1 is a strong inducer of b-globin expression that also activates BCL11A transcription when produced in large amounts in adult cells.
* KLF1 may itself be stimulated by c-Myb.


Pharmaceutical induction of γ-globin
Hydroxyurea
5-azacytidine
Decitabine
Cytotoxic/Stress
Response
Me Me Me
γ-globin
F
F
F
Epigenetic
regulation
Ac Ac Ac
HDAC1/2
BCL11A
Proliferation
Survival
SCFAD
(Butyrate)
KLF1
EPO
C-MYB
Transcription Factor
Inhibitors ?
Transcriptional control

Hematological parameters of patient with delta-beta thalassemia

Parameters	Patient	Father	Mother
Hemoglobin (g/dl)	9.8	15.0	12.2
RBC count (million/mm^3)	4.61	5.84	5.27
MCV (fl)	76.4	85.0	74.2
MCHC (pg)	27.7	30.1	31.2
RDW-CV (%)	17.3	15.4	17.0
G6PD screening test	NEGATIVE	-	-
HPLC	HbF 100% HbA 0% HbA_2 0%	F 19.2% A_2 2.7%	F 16.0% A_2 2.9%
SGOT (IU/L) SGPT (IU/L)	35 48	-	-
Alkaline phosphatase (IU/L)	144	-	-

Induction of fetal hemoglobin synthesis by CRISPR/Cas9

* large deletions in the β-globin locus result in hereditary persistence of fetal hemoglobin
* CRISPR/Cas9 strategy to disrupt a 13.6-kb genomic region encompassing the δ- and β-globin genes and a putative γ-δ intergenic fetal hemoglobin (HbF) silencer.

* Data analyses emphasized that the miRNAs at the imprinted 14q32 locus in fetal erythroblasts and the let-7 miRNA family in adult erythroblasts as key regulators of stage-specific erythroid transcriptional programs.
* It indicates that the mature let-7 miRNAs may be regulated in a post-transcriptional manner.
* miRNA was discovered in 1993.