The β-thalassemias are characterized by a very heterogeneous group of inherited mutations causing abnormal expression of globin genes, leading to total absence or quantitative reduction of synthesis of β-globin chains. The reduction of β-globin chains is associated with a corresponding excess of the complementary α-globin chains in erythroid cells, causing premature hemolysis of red blood cells and destruction of erythroid precursors in the bone marrow and extramedullary sites (ineffective erythropoiesis). Several molecules appear to be not necessary or even harmful to the erythroid cell, i.e. abnormal β-globin mRNA molecules (for instance in the case of aberration of splicing) and α-globin mRNA molecules, present in large excess. In agreement, inhibition of the expression of abnormal β- or α-globin mRNAs could be beneficial. Gene silencing could be of interest also in experimental therapy employing activation of the expression of human γ-globin genes by interfering with transcriptional repressors. The conclusion of the experiments described in the present review is that experimental therapy of β-thalassemia is commonly dedicated to induce the forced expression (by gene therapy and gene corrections of the mutations) of the adult not functional β-globin gene. On the other hand induction of fetal γ-globin genes can be achieved by inhibiting putative transcription repressors. Finally, in addition to this primary issue, the inhibition of the excess of α-globin mRNA might turn to be an approach able to ameliorating the clinical parameters of β-thalassemia.

Key words: Gene silencing - Oligonucleotides - Globins - Fetal hemoglobin - Beta-thalassemia.

The β-thalassemias are characterized by a very heterogeneous group of inherited mutations causing abnormal expression of globin genes, leading to total absence or quantitative reduction of synthesis of β-globin chains. This disease is frequent in the Mediterranean area, in Middle-East, in Africa and Asia. The reduction of β-globin chains is associated with a corresponding excess of the complementary α-globin chain in erythroid cells, that causes premature hemolysis of red blood cells and destruction of erythroid precursors in the bone marrow and extramedullary sites (ineffective erythropoiesis) (Figure 1). More than 200 different mutations have been identified in β-thalassemia patients, including deletions of the β or δβ gene region, stop codons leading to premature termination of a non functional β-globin chain, mutations suppressing correct maturation of the β-globin RNA precursor.
In conclusion, as reviewed by Bank, several molecules appear to be not necessary or even harmful to the erythroid cells, i.e. abnormal β-globin mRNA molecules (for instance in the case of aberration of the splicing process) and α-globin mRNA molecules, present in large excess. The accumulation of unbalanced amounts of α-globin causes the presence of excessive free α-globin chains, which precipitate to the erythrocyte membrane, resulting in hemolytic anemia. In agreement with these considerations, inhibition of the expression of abnormal mRNAs could be beneficial. This research takes great benefits from the availability of several in vivo mice model system mimicking several features of β-thalassemia. For instance, Voon et al. recently demonstrated that coinheritance of α- and β-thalassaemia in mice improves the thalassaemic phenotype. In their experiments, heterozygous murine β-globin knockout (KO) mice (β<sup>+</sup>−) which display severe anaemia were mated with heterozygous α-globin KO mice (α<sup>+</sup>−). The resulting progeny were compared with wild-type WT (α<sup>+</sup>+/+, β<sup>+</sup>+/+), heterozygous α-KO (α<sup>+</sup>−/−; β<sup>+</sup>−), heterozygous β-KO (α<sup>+</sup>+/−; β<sup>+</sup>−) or double heterozygous (DH) α-KO/β-KO (α<sup>+</sup>−/−; β<sup>+</sup>−) with respect to blood parameters and spleen dimension.

Heterozygous β-KO mice (β<sup>+</sup>−) showed spleen enlargement, marked reductions in hemoglobin and hematocrit levels and significant increases in reticulocyte counts compared to WT mice. In contrast, α-KO/β-KO mice showed near normal dimension of the spleen and of the red blood cell indices.
These results indicate that reduction of \(\alpha\)-globin expression leads to correction of the globin chain imbalance in \(\beta\)-thalassaemic mice and therefore an improved phenotype. Similar situation is found in humans.\(^{1-3}\) In this respect, it is well established that \(\alpha\)-thalassemia patients can exhibit a milder phenotype.\(^{1-3}\) In agreement with the interplay between accumulation of \(\alpha\)-globin and severity of \(\beta\)-thalassemia are recent observations demonstrating that \(\alpha\)-haemoglobin stabilising protein is a quantitative trait gene that modifies the phenotype of \(\beta\)-thalassaemia.\(^4\) If this gene is down-regulated, the \(\beta\)-thalassemia phenotype is clinically mild.

It should also be underlined that post-transcriptional gene silencing is operated in nature; an example is the well described nonsense-mediated mRNA decay (NMD)\(^{12-15}\) of the \(\beta^\gamma\)-globin mRNA present in the most frequent type of \(\beta\)-thalassemia in Italy. In this case the CAG (Gln) codon of the in \(\beta\)-globin mRNA is mutated to the UAG stop codon,\(^12,\ 13\) leading to premature translation termination and to mRNA destabilization. Another example is constituted by a novel thallasemia mutation (insertion of a single A nucleotide at codon 18 of the exon 1 of the \(\beta\)-globin gene) associated with a \(13.4\) kb \(\delta\)-globin gene deletion in a hereditary persistence of fetal hemoglobin (HPFH) patient. The novel mutation causes a frame shift with the generation of a UGA stop codon. The levels of \(\beta\)-globin mRNA found by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis were found to be much lower than those expected, suggesting that the mutated \(\beta\)-globin mRNA was not stable.\(^4\) These data were confirmed by inhibiting transcription with ethidium bromide (Figure 2).

On the other hand, gene silencing could be relevant, rather than for eliminating or lowering the amount of aberrant globin mRNA molecules, for inducing the expression of \(\gamma\)-globin genes, with the aim of stimulate the production of fetal hemoglobin (HbF). In this respect it is firmly established that HPFH in \(\beta\)-thalassemia is associated with benign clinical parameters. Interestingly, coexistence of HPFH with homozgyous \(\beta\)-thalassemia often results in complete phenotypic complementation of the disease.\(^{16,\ 17}\) Therefore, there has been considerable interest in recent years in finding ways of increasing production of HbF.\(^7\) Accordingly, an alternative therapeutic approach for \(\beta\)-thalassemia is to devise strategies to reactivate the \(\gamma\)-globin genes.\(^8\) In addition, HbF induction in vivo (for instance with hydroxyurea) renders the patients not dependent from blood transfusions.\(^8\) As representative example, Dixit et al. reported a study on thirty-seven patients with \(\beta\)-thalassemia intermediate to assess response to HU therapy.\(^\text{18}\) Major response was defined as transfusion independence or Hb rise of more than 20 g/l and minor response as rise in Hb of 10-20 g/L or reduction in transfusion frequency by 50%. Twenty-six patients (70.2%) showed response to hydroxyurea (HU) therapy. Seventeen patients (45.9%) were major responders, and nine patients (24.3%) showed minor response. Mean Hb levels rose on HU therapy.\(^\text{18}\)

In conclusion down-regulation or silencing of the expression of specific gene could represent a strategy to ameliorate the biochemical parameters of erythroid cells from \(\beta\)-thalassemia patients.\(^\text{19}\) Accordingly, several research groups designed silencing strategies in this applied field of molecular medicine.

### Approaches for gene silencing

Several approaches are available for gene silencing, including post-transcriptional targeting of mRNA employing lentiviral vectors or short hairpin RNA (shRNA) and the use of decoy oligonucleotides targeting transcription factors. With respect of the use of the RNA interference approach, exogenous target gene-complementary short hairpin RNAs (shRNAs) are capable of down-regulating target gene expression through sequence-specific pre-mRNA degradation\(^{20-\text{22}}\) via a process known as RNA interference (RNAi).\(^\text{23}\) The transcription factor decoy approach (TFD), on the other hand, has as target molecules transcription factors.\(^{24-\text{26}}\) TFD has been proposed to modulate gene expression in vitro. This approach is based on the intracellular delivery of double stranded oligodeoxynucleotides mimicking binding sequences of transcription factors and causing inhibition of the binding of TF-related proteins to the specific consensus sequences in the promoter of TF-target genes. This treatment leads to inhibition of transcription if the target TF is an activator, and to transcriptional activation, if the target gene is a repressor.\(^\text{27, 28}\)

### Restoration of the balanced \(\alpha/\beta\)-globin gene expression in \(\beta\)-thalassemia mice using RNAi approach

Xie et al.\(^\text{27}\) explored post-transcriptional strategies aiming at the reduction of \(\alpha\)-globin chains on
β(654)[Hbb(th-4)/Hbb(+)] mouse, carrying a human splicing-deficient β-globin allele [Hbb(th-4)] (Figure 3). This mouse model system carries a normal mouse β-globin allele, and a defective human pIVS-2-654 allele associated with aberrant splicing due to C>T substitution at nt654 of intron 2. Therefore, the β654 mice produce half of the normal mouse β-globin chains but no functional human β-globin, manifesting typical signs of a moderate form of β-thalassemia, including anemia, splenomegaly, abnormal hematologic indices. Xie et al. 27 have explored combined employment of post transcriptional approaches for gene therapy of β-thalassemia, one aiming at correcting the pIVS-2-654 globin mRNA, the other aiming at "silencing" the α-globin mRNA, in order to achieve a reduction of the excess of α-globin chains. Through lentiviral vectors, three types of β654 transgenic mice have been produced, namely α_i-Hbbth-4/Hbb(+), β_i- Hbbth-4/Hbb(+) and α_β_i-Hbbth-4/Hbb(+), integrat-

Increase of γ-globin mRNA and HbF production with decoy oligonucleotides

Within their laboratory, the authors attempted to apply a transcription factors decoy strategy for subtracting putative repressor factors. One of the designed decoy oligonucleotides, mimicking a DNA region of the human γ-globin gene was able to 1) stably interact with nuclear proteins isolated from human leukemic K562 cells; 2) induce erythroid differentiation of K562 cells and increased γ-globin mRNA expression and HbF production in erythroid precursor cells from normal donors. 28 This demonstrates that down modulation of regulatory genes associated with the repression of target genes target can induce increase of the expression of target genes.

Conclusions

Experimental therapy of β-thalassemia is commonly dedicated to induce the forced expression (by gene therapy and gene corrections of the mutations) of the adult, not functional, β-globin gene. In addition to these "gain-of-function" strategies, "loss-of-function" approaches can be employed. The first strategy focuses on the inhibition of α-globin gene expression with the aim to reduce the unbalanced α-globin/β-globin ration in the erythroid cells. It has been indeed demonstrated that reduction of the excess of α-globin chains might turn to be an approach able to ameliorating the clinical parameters of β-thalassemia. The aim of the second strategy is to induce the expression of silent genes (such as the fetal γ-globin gene) by interfering with putative repressors. This might induce an increase of γ-globin chains, production of functional HbF and, consequently, also reduction of the excess of α-globin.
References