

# Gene therapy approaches to the treatment of hemoglobinopathies

## Review Article

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### Summary

**Hemoglobinopathies such as thalassemia and sickle cell anemia are potentially amenable to gene therapy. Applicable gene therapy strategies can be divided into four categories: those that replace the faulty gene with a complete transcriptional unit, those that activate transcription of fetal hemoglobin genes, those that modify the endogenous gene itself, and those that attempt to repair the defective globin RNA transcripts transcribed from the gene. Before becoming valuable in the treatment of human patients, each of these methodologies must overcome obstacles in efficiency of delivery, level of effectiveness, and length of time the treatment remains effective.**

## I. Introduction

Gene therapy techniques show increasing promise for use in the treatment of genetic disease. Gene therapy has been tested in treatment of adenosine deaminase deficiency (Tolstoshev, 1993, Fenjves, 1997), cystic fibrosis (Knowles, 1995), and other genetic disorders (Acsadi, 1991, Dunbar, 1996) both in animal models and in the clinic. The therapeutic effects are normally accomplished by replacing the defective gene with the correct one or by expressing a transgene whose product substitutes for its defective counterpart. Although, in principle, gene therapy should be applicable to any gene-based disorder, the difficulties with vectors suitable for efficient delivery of large transgenes or providing sustained expression of the transfected genes in a tissue-specific, properly regulated manner (Byun, 1996, Shi, 1997) limit its clinical applicability.

An alternative approach to gene replacement is correction of the defect in an existing gene or gene product. This method has been particularly useful in the treatment of hemoglobinopathies such as thalassemia and sickle cell anemia. Correct formation of the  $\alpha$ - and  $\beta$ -globin chains of hemoglobin is critical for the formation of hemoglobin in

normal red blood cells. In thalassemia, a decrease or absence of  $\alpha$ - or  $\beta$ -globin synthesis leads to low levels of hemoglobin, causing anemia. In sickle cell anemia a point mutation leads to production of a mutant  $\beta$ -globin ( $\beta^S$ ) that polymerizes and accumulates in erythrocytes, resulting in changes in membrane morphology and properties, leading to vaso-occlusion (Platt, 1993). To correct these disorders, researchers utilized oligonucleotides, small nuclear RNAs (snRNAs), ribozymes, and other strategies to restore the production of correct globin mRNA and protein.

## II. Gene therapy strategies

### A. Gene replacement

Replacement of defective  $\beta$ -globin genes with a functional transcription unit has been particularly difficult to accomplish (reviewed in Rivella, 1998). Although the  $\beta$ -globin gene is small, regulated expression from a transgene is difficult to achieve because it is controlled by a large locus control region (LCR) (Grosveld, 1998, Orkin, 1990). Since expression of  $\beta$ -globin is only useful if it occurs in erythroid precursor cells in concert with the  $\alpha$ -globin genes, tight regulation of the  $\beta$ -globin transgene is particularly important in treatment of sickle cell anemia

or thalassemia. Effective gene replacement will require efficient transfer of the  $\beta$ -globin gene into hematopoietic stem cells along with sustained expression at an appropriate, developmentally regulated level. Good candidates for vectors will stably integrate into the cell's chromosomal DNA or remain episomal. Possible viral vectors that could be useful include adeno-associated virus (AAV), adenovirus, and retroviral-, or simian virus 40 (SV40)-based vectors. Each of these vectors has advantages and disadvantages when factors such as insert size capacity, integration, and potential for long-term expression are considered. Recently, a construct, based on Epstein-Barr virus, which remains episomal in cell culture and is able to accommodate large DNA fragments containing the  $\beta$ -globin gene and complete regulatory region, has been developed (Westphal, 1998). However, proper expression of the  $\beta$ -globin gene in hematopoietic cells have not yet been tested. Additional approaches include the replication-deficient viral vectors or non-viral vectors as gene replacement carriers (Walsh, 1993, Gunzburg, 1996, Rivella, 1998). The latter are not limited by size, but more by difficulties in delivery into the nuclei of target cells and lack of chromosomal integration (Rivella, 1998).

To circumvent the problem of the large size of the required  $\beta$ -globin insert several truncated constructs have been tested (Zhou, 1996, Ellis, 1997).  $\beta$ -globin transcripts modified by removal of introns and/or reorganization of the LCR showed improvement in stable proviral transmission (Sadelain, 1995, Leboulch, 1994, Takekoshi, 1995). One of the constructs was used in a mouse transplant model and showed some evidence of long-term, high level expression of human  $\beta$ -globin (Raftopoulos, 1997). Although this study represents significant progress toward somatic gene therapy, it will be necessary to achieve more consistent and high-level expression of the replacement genes before this approach can be tested in patients. This may involve the development of more efficient transfection protocols or improvement in  $\beta$ -globin constructs. Such constructs might contain different LCR components, stem cell targeting components, nuclear localization signals, or different promoters and/or enhancers (Raftopoulos, 1997).

### B. Repair of defective splicing by oligonucleotides

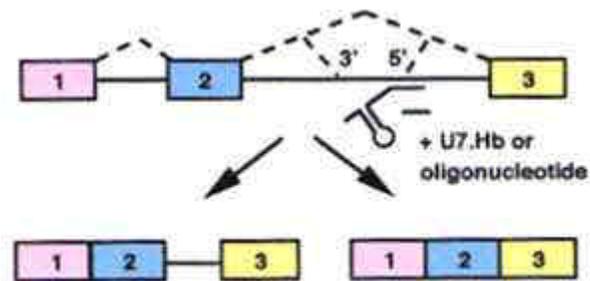
Work in this laboratory showed that antisense oligonucleotides may restore the production of normal  $\beta$ -globin in cells expressing thalassemic  $\beta$ -globin genes (**Figure 1**). Three thalassemic mutations in intron 2 of the  $\beta$ -globin gene: IVS2-654, -705, and -745 (Dominski, 1993, Sierakowska, 1996, 1997, unpublished data) were studied. The RNAs transcribed from these genes are aberrantly spliced due to point mutations that create

aberrant 5' splice sites and activate a common 3' splice site upstream. 18-mer 2'-O-methyl-oligoribonucleoside phosphorothioates targeted to the aberrant splice sites restored the correct splicing pattern in a sequence specific and dose dependent manner by causing the splicing machinery to use only the correct splice sites. The correction of splicing was accompanied by translation of the resultant  $\beta$ -globin mRNA into full-length  $\beta$ -globin protein.

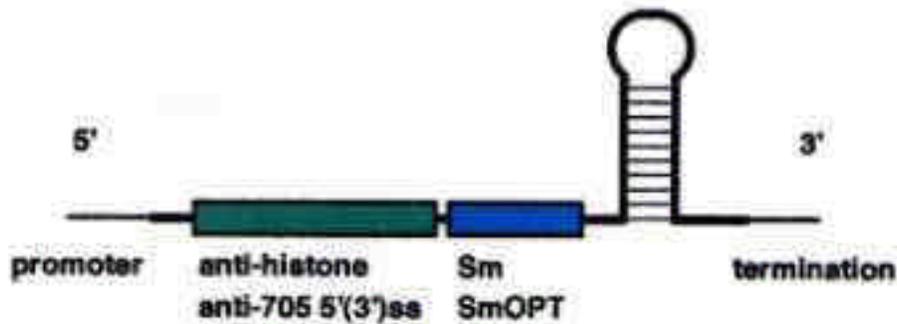
A promising feature of this approach is that in patients, the antisense oligonucleotides would restore the correct splicing of pre-mRNA, properly transcribed from the  $\beta$ -globin gene which remains in its natural chromosomal environment. This precludes the possibility of overexpression or inappropriate expression of  $\beta$ -globin mRNA and protein, an important consideration in treatment of hemoglobinopathies. Note, that the oligonucleotides do not remove the mutation and would therefore require periodic, life-long administrations. This approach is, thus, more akin to a pharmacological treatment than to a gene therapy one.

### C. Repair of defective splicing by small nuclear RNAs (SnRNAs)

SnRNAs are small, capped RNA molecules that are located in the nucleus and participate in splicing and other RNA processing reactions. Many of the snRNAs contain sequences antisense to the target RNAs and perform their functions upon binding to their target (Birnstiel, 1988). Thus, by analogy to the oligonucleotides discussed above, they can be used as antisense reagents in gene therapy protocols.



**Figure 1.** Correction of splicing of IVS2-705  $\beta$ -globin pre-mRNA by oligonucleotides or modified U7 snRNA. Boxes - exons, lines - introns. The dashed lines represent correct and aberrant splicing pathways. The oligonucleotides or modified U7 snRNA (U7.Hb) targeted to the IVS2-705 cryptic splice site (3') are depicted under the pre-mRNA (Sierakowska, 1996, Gorman, 1998).



**Figure 2.** Structure of U7 snRNA constructs. Wild-type U7 snRNA (heavy line) includes a stem-loop structure, the U7-specific Sm sequence (blue box) and a sequence antisense to the 3' end of histone pre-mRNA (green box). In anti-705 U7 snRNAs, the two sequences are replaced with the SmOPT sequence and with antisense sequences to the aberrant 3' or 5' splice sites in the  $\beta$ -globin gene, respectively. The promoter and 3' end forming (termination) regions are indicated (Gorman, 1998).

There are several advantages to using snRNAs in this approach. SnRNAs are capped and associate tightly with proteins which protect them from ribonucleases present in the cellular milieu and making them much more stable than naked RNA. SnRNA genes are driven by strong promoters leading to high level of expression, up to  $10^6$  copies of the snRNA per cell. Since splicing occurs in the nucleus, the nuclear localization of snRNAs makes them ideal for use in correction of splicing defects frequent in thalassemia (Birnstiel, 1988).

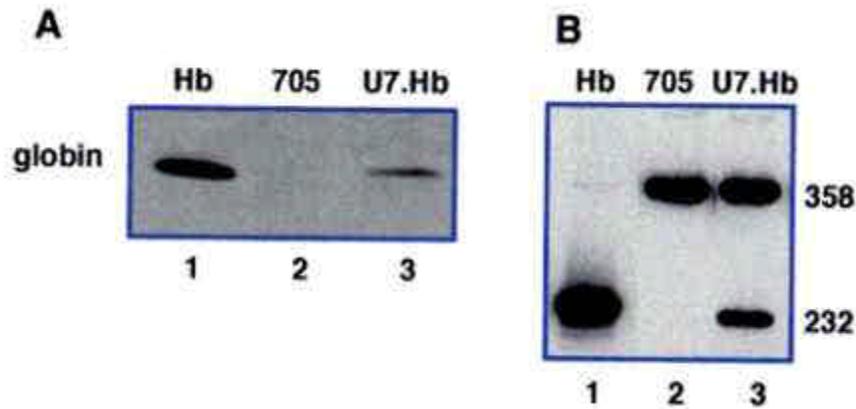
Utilization of an snRNA as a therapeutic agent involves replacement of the natural antisense sequence with that targeted to the desired RNA. The modified snRNA gene is incorporated into a plasmid and transfected into the cell. The relatively short insert carries the snRNA promoter and therefore exogenous promoters are not necessary. The transcribed snRNA migrates to the cytoplasm where it is complexed with specific proteins and subsequently returns to the nucleus where it can bind to the desired target. It is anticipated that snRNAs as antisense carriers will allow for long term, possibly permanent, expression of RNA antisense to its targets such as the aberrant thalassemic splice sites in  $\beta$ -globin pre-mRNA.

Anti- $\beta$ -globin sequences were incorporated into the gene for murine U7 snRNA (**Figure 2**) (Gorman, 1998). U7 snRNA complexes with at least two U7 specific proteins and eight common Sm proteins (Smith, 1991), forming a ribonucleoprotein particle (U7 snRNP). U7snRNP is involved in the processing of the 3' end of histone pre-mRNAs (Galli, 1983, Birchmeier, 1984, Birnstiel, 1988). The first 18 nucleotides of this 62 nucleotide-long RNA function as a natural antisense sequence by hybridizing with the so-called spacer element

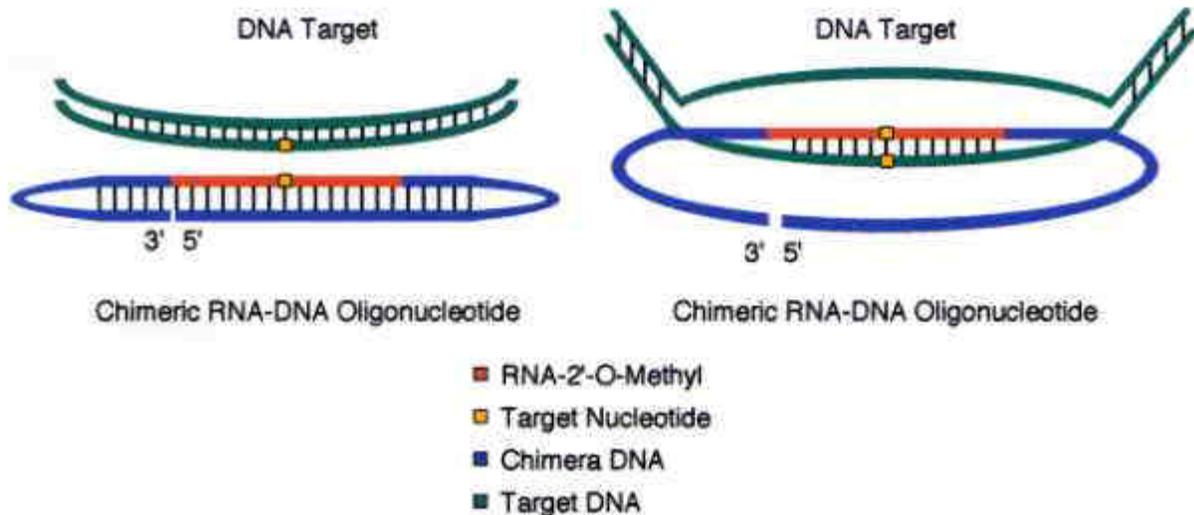
of histone pre-mRNA during its 3' processing (Bond, 1991, Spycher, 1994). It seemed possible that upon replacement of the anti-histone sequence with a sequence complementary to the  $\beta$ -globin aberrant splice sites, the resulting U7 snRNA molecule would bind equally well to the new target sequences and correct aberrant splicing in a manner similar to antisense oligonucleotides. Indeed, it was found that the insertion of appropriate antisense sequences into the U7 snRNA prevented its function in histone mRNA processing and allowed it to modify alternative splicing of  $\beta$ -globin pre-mRNA (**Figure 1**). Stable transfection of cells expressing thalassemic  $\beta$ -globin gene with vectors carrying a modified U7 snRNA gene led to a permanent correction of the splicing pattern of the  $\beta$ -globin pre-mRNA. Levels of correction reached 65% in transient expression and 55% in stable cell lines. The treatment also resulted in the accumulation of significant amounts of  $\beta$ -globin protein (**Figure 3**) (Gorman, 1998).

#### D. Removal of mutations by chimeric RNA-DNA oligonucleotides

It has recently been shown in model cell culture systems that double stranded chimeric RNA-DNA oligonucleotides may induce site specific removal from the human  $\beta$ -globin gene of the mutation responsible for sickle cell anemia (Cole-Strauss, 1996). The  $\beta^S$  allele is caused by an A to T mutation in the sixth codon of the  $\beta$ -globin gene which leads to replacement of valine by glutamic acid. This point mutation in a coding sequence represents a good candidate for using the chimeric oligonucleotides as a potential treatment.



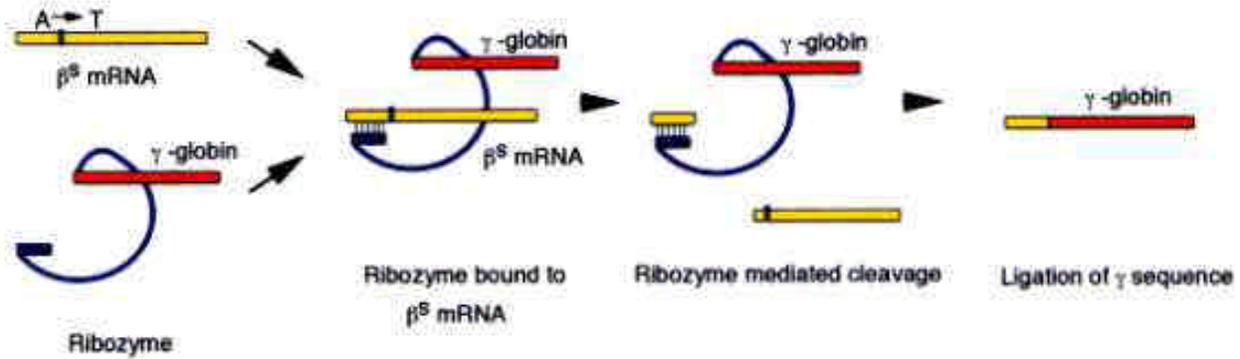
**Figure 3.** Correlation of protein levels (A) with mRNA (B) expression in a stable cell line expressing modified U7 snRNA (U7.Hb) (A) Western blot of cell line expressing full length  $\beta$ -globin protein (Lane 1), IVS2-705 cell line (Lane 2), and stable cell line expressing U7.Hb snRNA (Lane 3). (B) RT/PCR products from RNA from human blood (Lane 1), IVS2-705 cell line (Lane 2), and stable cell line expressing U7.Hb snRNA (Lane 3) (Gorman, 1998).



**Figure 4.** Diagram of basic structure of chimeric oligonucleotide. Blue lines represent chimera DNA residues, red lines represent 2'-O-methyl RNA residues, yellow box represents target nucleotide, green lines represent target DNA (Cole-Strauss, 1996).

The chimeric oligonucleotides are composed of a stretch of RNA and DNA residues in a duplex formation with double hairpin caps at the ends (**Figure 4**). The RNA residues were modified by 2' O-methylation of the ribose increasing the oligonucleotide resistance to nuclease degradation. The sequences of these oligonucleotides align with the target sequence except at the position of the

mutation. This single base mismatch is recognized by the endogenous cellular repair systems and either the oligonucleotide or the target is changed. Use of the chimeras resulted in almost equal amounts of  $\beta^S$  and normal  $\beta^A$  globin ( $\beta^A$ ) suggesting 50% correction of the mutation at the DNA level (Cole-Strauss, 1996).



**Figure 5.** Ribozyme mediated repair strategy. Scheme to convert  $\beta^S$  transcripts (yellow) to  $\gamma$ -globin (red) expressing transcripts (Lan, 1998).

### E. Ribozyme-mediated repair of mRNA

An alternative treatment for sickle cell anemia utilizes ribozymes to repair the defective  $\gamma$ -globin RNA transcripts (Lan, 1998). This work was based on the finding that the self-splicing intron from *Tetrahymena thermophila* mediates trans-splicing of RNA fragments in vitro (Inoue, 1986, Been, 1986). A shortened form of the ribozyme, L-21 (Zaug, 1988), was able to repair defective *lacZ* transcripts in *E. coli* and mammalian cells (Sullenger, 1994, Jones, 1996). To test whether ribozymes could be used in a therapeutic manner they were designed to convert  $\beta^S$  RNAs into  $\gamma$ -globin messages (Lan, 1998).  $\gamma$ -globin was selected since it was found that fetal,  $\gamma$ -globin containing, hemoglobin retards the polymerization of  $\beta^S$  hemoglobin (Sunshine, 1978, Behe, 1979). The trans-splicing group I ribozyme was modified to carry the 3' portion of the  $\gamma$ -globin mRNA. When the ribozyme base paired with the mutant  $\gamma$ -globin transcript upstream of the mutation, the transcript was cleaved, thereby releasing the portion containing the mutation, and subsequently the  $\gamma$ -globin 3' exon sequence was spliced in (Lan, 1998) (**Figure 5**). The ribozyme converted the  $\beta^S$  globin RNA to RNA that encoded  $\gamma$ -globin not only in model cell lines but also in red blood cell precursors from human cord blood (Lan, 1998).

### F. Activation of $\delta$ globin genes

Another alternative to gene replacement has been described by Donze et al. (Donze, 1996). In this approach the endogenous  $\delta$ -globin gene is activated with a modified erythroid-specific transcription factor, EKLF (Erythroid

Krupple-like factor). Normally, high levels of  $\delta$ -globin are expressed when, in concert with other proteins that bind to locus control region sequences, EKLF binds to the  $\delta$ -globin CACCC box. The  $\delta$ -globin gene promoter has a defective CACCC box that does not bind EKLF, which may be one reason for the low level of  $\delta$ -globin expression. Using a modified EKLF that binds to the defective  $\delta$ -globin gene promoter, increased levels of  $\delta$ -globin were produced. Such an approach would lead to increased HbA<sub>2</sub> ( $\alpha_2\delta_2$ ) production. Since HbA<sub>2</sub> has been shown to be an inhibitor of sickle cell HbS ( $\alpha_2\beta^S_2$ ) polymerization, transduction of erythroid stem cells with the modified EKLF gene, could be a useful treatment for sickle cell patients.

### G. $\alpha$ -globin reduction

The accumulation of excess  $\delta$ -globin in the red blood cell precursors of  $\delta$ -thalassemia patients results in premature cell death before the reticulocyte stage (Weatherall, 1972). Reducing the levels of  $\delta$ -globin can alleviate the imbalance between the  $\delta$ - and  $\beta$ -globin chains, leading to increased production of healthy reticulocytes. Ponnazhagen et al (1994) utilized recombinant adeno-associated virus 2-based antisense vectors to inhibit  $\delta$ -globin expression. Adeno-associated virus 2 (AAV) was selected since it is non-pathogenic and integrates in a site-specific manner into human chromosome 19. Different promoters (herpes virus thymidine kinase (TK) promoter, the SV40 early gene promoter, and the human  $\delta$ -globin gene promoter) were tested to achieve proper level of reduction of the  $\delta$ -globin

mRNA. The observed levels of  $\beta$ -globin inhibition were 0, 29, and 91%, respectively, at the transcriptional level. Thus it may be possible to adjust the level of the  $\beta$ -globin to achieve the desired 1:1 ratio of  $\alpha$ - and  $\beta$ -subunits. Complete inhibition of  $\beta$ -globin production would lead to a new imbalance, with excess  $\alpha$ -globin chains building up in the cells with detrimental consequences.

## H. Induced gene expression by drugs

Currently, patients with  $\beta$ -thalassemia are treated with periodic blood transfusions and iron chelation therapy. Blood transfusions can lead to iron overload, the leading cause of death in thalassaemic patients (Zurlo, 1989). Clinically relevant alteration of globin gene expression can be achieved by relatively simple pharmacological treatments. For example, hydroxyurea or butyric acid and its derivatives induce the expression of fetal hemoglobin ( $\beta_2$ ) which partially compensates for the lack of correct  $\beta$ -globin expression in sickle cell anemia or thalassemia (Charache, 1995, Charache, 1996). In clinical trials, patients receiving hydroxyurea exhibited a decrease in crisis rates within the first three months of treatment (Charache, 1995, Charache, 1996). The lower crisis rates were accompanied by lower neutrophil and platelet counts, as well as higher mean corpuscular volume and mean corpuscular hemoglobin concentrations (Charache, 1995, Charache, 1996). Use of hydroxyurea in patients with sickle cell anemia was so successful that the trial was stopped early and the drug moved to the clinic.

Sodium butyrate is used to treat urea-cycle disorder (Brusilow, 1991). It was found that one of the side effects from the drug was an increase in the patient's fetal hemoglobin levels, although the exact mechanism involved is unknown. These findings led to attempts to use this drug for the treatment of  $\beta$ -thalassemia. These treatments elicited varying levels of success in clinical trials (Perrine, 1989, Charache, 1996, Sher, 1995, Collins, 1995). In one study, 36% of all patients or 50% of non-transfused patients exhibited an increase in fetal hemoglobin after treatment with sodium phenylbutyrate (Collins, 1995). The increase in hemoglobin was concomitant with an increase in the number of red blood cells and mean corpuscular volume (Collins, 1995). Unfortunately, until it can be determined which subset of  $\beta$ -thalassaemic patients will respond to butyrate analogues, this therapy will remain of limited usefulness as a treatment.

## III. Conclusions

Many possible treatments for hemoglobinopathies are currently explored. Strategies range from simple drug treatments to a wide range of gene therapy techniques.

Although further testing and improvements are clearly needed before most of the approaches can be used on patients there is hope that some of them will lead to successful treatment for patients with thalassemia and sickle cell anemia. For any of the gene therapy techniques described here, the main hurdles appear to be delivery, achievement of long-term effects of the treatment and proper expression limited to the small population of the target cells, the erythroid precursors. The effects should be, preferably, accomplished by systemic treatment, although *ex vivo* bone marrow treatment and autologous reimplantation can also be considered. Even if the permanent curative effects of the treatment were difficult to achieve the temporary effects may be of clinical value. The increase in the production of correct  $\beta$ - or other globins in thalassemia and sickle cell anemia should improve survival of the erythroblasts and promote their maturation into red blood cells. Since erythrocytes have a life span of approximately 120 days (Eadie, 1955), the treated cells would remain in the bloodstream for an extended time period.

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