

## RNA Silencing: An Approach for the Treatment of $\beta$ -Thalassemia

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### Abstract

Thalassemia is an inherited disorder of blood, which is passed from one generation to another, represents the most common hemoglobinopathies caused. It occurs due to reduce or minute production of hemoglobin. Augmented levels of fetal hemoglobin (HbF) can revolutionize the severity of the  $\beta$ -hemoglobin disorders, like  $\beta$ -thalassemia. More recently, major advances have been made in the discovery of critical modifier genes, such as BCL11A (B cell lymphoma 11A), a master regulator of HbF (fetal hemoglobin) and hemoglobin switching. Down regulation of BCL11A expression or function by siRNA or small molecules may offer a new therapeutic approach to directed activation of HbF in adult erythroid cells of patients with  $\beta$ -thalassemia and other disorders of  $\beta$ -hemoglobin. RNA interference (RNAi) is a commonly used procedure for the analysis of regulation of gene expression in a variety of cells, by which target messenger RNA (mRNA) is cleaved by small interfering complementary RNA (siRNA). RNA silencing with direct delivery of siRNA can be used to suppress BCL11A gene expression. This review discusses the role of globin gene regulation in thalassemia, RNAi interference and related approaches available for the treatment of thalassemia major patients.

**Keywords:** BCL11A; Hemoglobin; Thalassemia; RNAi; siRNA

### Introduction

The thalassemias are worldwide genetic disorders that result in defective globin-chain production as a consequence of a large number of different genetic lesions [1]. The term thalassemia is derived from the Greek, thalassa (sea) and haima (blood). Thalassemia is an inherited disorder of blood which is passed from one generation to another. It occurs due to reduce or minute production of hemoglobin. Almost 25 million people in India are carriers of  $\beta$ -thalassemia gene. Over 9,000 thalassemic children are born every year in India [2]. It is not an infectious disease and cannot be passed from one individual to the other by personal or any other contact, or through blood transfusion, food or air. Thalassemias arise due to a quantitative defect in the globin chain production [3]. The thalassemias became the first diseases to be characterized at the molecular level, work that provided some indications of the repertoire of mutations that underlie human genetic disease.

### Types of Thalassemia

Thalassemia is characterized into two types i.e.  $\alpha$  (alpha) thalassemia and  $\beta$  (beta) thalassemia. Both of these disorders affect the production of normal hemoglobin, a key constituent of human red blood cells. In thalassemia there is impaired production of alpha or beta chains. If the production of alpha chains is impaired, the condition is called alpha thalassemia and if the production of beta chains is impaired the condition is called beta thalassemia [4].

### Alpha thalassemia

$\alpha$ -Thalassemia is a heterogeneous group of inherited disorders characterized by the reduced or absent synthesis of  $\alpha$ -globin chains [5]. It is probably the most common monogenic gene disorder in the world and is especially frequent in Mediterranean countries, South-East Asia, Africa, and in the Indian subcontinent [6]. Alpha-thalassaemia is more frequently caused by deletion than single point mutations or nucleotide insertions and deletions involving the canonical sequences controlling gene expression. In general the non-deletion  $\alpha$ -thalassaemia determinants may give rise to a more severe reduction in  $\alpha$ -chain synthesis than the  $\alpha$ -deletion type of chromosomes. Many mutations have been described affecting

mRNA processing, mRNA translation, and  $\alpha$ -globin stability. Alpha globin chain production is controlled by two genes on each chromosome 16 [7,8]. Deficient production is usually caused by a deletion of one or more of these genes.

### Beta thalassemia

$\beta$ -thalassemia, one of the most common single gene disorders, results from the decreased production of  $\beta$ -globin chains. More than 180 different mutations of the  $\beta$ -globin genes have been found in patients with  $\beta$ -thalassemia [9]. They may affect gene function at any level between transcription, processing of the primary messenger ribonucleic acid transcript, translation, or post-translational stability of the gene product. Beta globin synthesis is controlled by one gene on each chromosome 11. Estimates of newborns with homozygous  $\beta$ -thalassemia in India vary considerably from 6,000 to 7,500 per year and even more depending on the gene prevalence, population, and birth rate of the region [10]. The one gene defect,  $\beta$ -thalassemia trait (minor), is asymptomatic and results in microcytosis and mild anemia. If the synthesis of both genes is severely reduced or absent, the person has  $\beta$ -thalassemia major, also known as Cooley anemia. Persons with  $\beta$ -thalassemia major are almost never symptomatic at birth because of the presence of HbF, but symptoms begin to develop by six months of age. If the synthesis of beta chains is less severely reduced, the person has  $\beta$ -thalassemia intermedia. These persons experience symptoms that are less severe and do not require lifelong transfusions to survive past 20 years of age [11].

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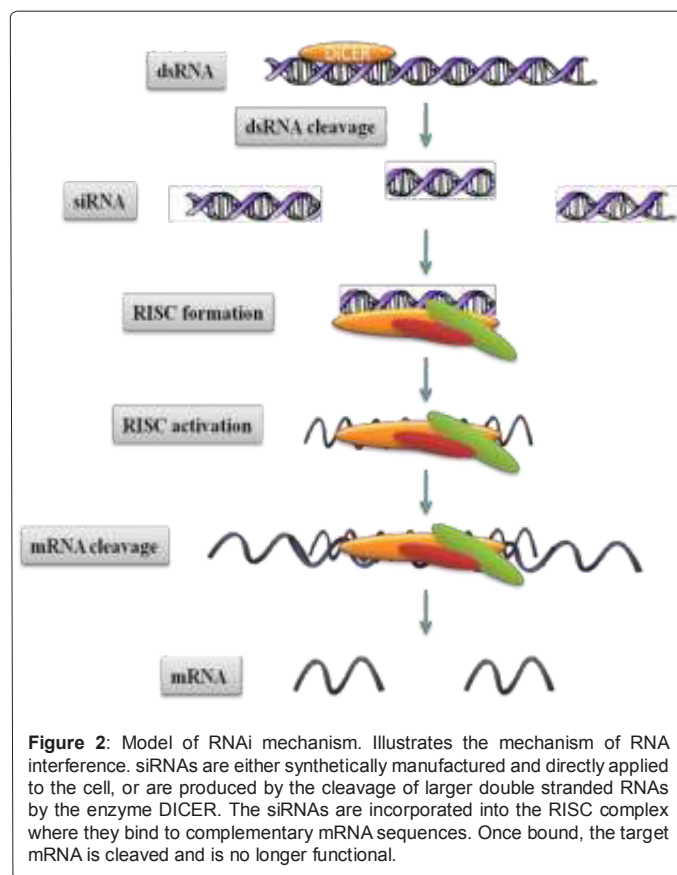
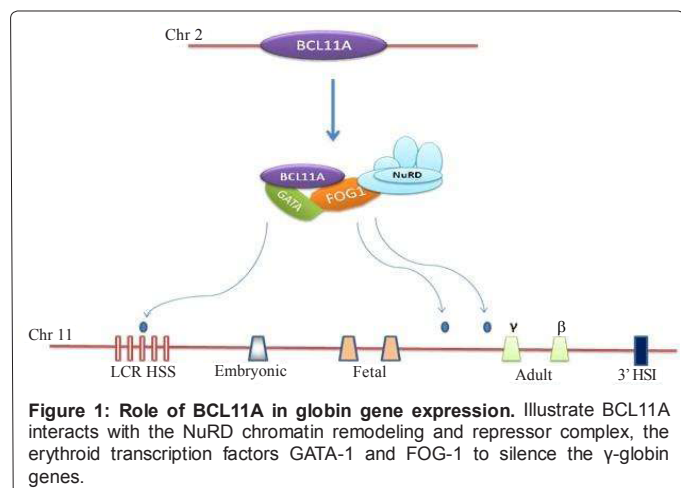
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### Role of BCL11A in globin gene regulation

BCL11A gene encodes a C2H2 type zinc-finger protein by its similarity to the mouse Bcl11a/Evi9 protein. The equivalent mouse gene is a general site of retroviral integration in myeloid leukemia, and may function as a leukemia disease gene, to a certain extent, through its interaction with BCL6. BCL11A acts as a transcriptional repressor in B lymphocytes. Figure 1 illustrates how a genetic variant within the BCL11A gene in humans that is associated with different levels of HbF is in turn associated with variation in expression of BCL11A (top of diagram). This variation in expression appears to have an effect on the function of BCL11A at the human  $\gamma$ -globin locus, where it is considered to act with the erythroid transcription factors GATA-1 and FOG-1, as well as the NuRD remodeling and repressor complex to silence the  $\gamma$ -globin genes [12]. BCL11A is expressed as short variant proteins in cells that robustly express  $\gamma$ -globin (primitive and fetal liver erythroid progenitors) and as full-length forms at the adult stage when the  $\gamma$ -globin genes are silenced. The developmental switch from human fetal ( $\gamma$ ) to adult ( $\beta$ ) hemoglobin represents a clinically important example of developmental gene regulation. Delay of the switch or reactivation of fetal hemoglobin (HbF) in the adult stage greatly ameliorates the clinical severity in the principal  $\beta$ -hemoglobin disorders sickle cell anemia and  $\beta$ -thalassemias. The transcription factor BCL11A is a central mediator of  $\gamma$ -globin silencing and hemoglobin switching. In earlier studies it was found that BCL11A maintains silencing of  $\gamma$ -globin expression in adult erythroid cells and functions as a direct transcriptional regulator of the fetal to adult hemoglobin switch in humans [13]. Moreover, BCL11A plays a central role in the evolutionarily different globin gene switches of mammals. As a factor critical for  $\gamma$ -globin gene silencing, BCL11A should be considered as a beneficial target to increase HbF in a directed manner in beta-thalassemia patients [14].

### BCL11A as a therapeutic target for reactivation of HbF

HbF is compiled of 2  $\alpha$  and 2  $\gamma$  chains that commonly disappear in the neonatal period. Throughout gestation, the replicated  $\gamma$ -globin genes represent the predominant genes transcribed in the  $\beta$ -globin cluster. After birth,  $\gamma$ -globin is replaced by adult  $\beta$ -globin, a process referred to as the “fetal switch.” In nonanemic persons, HbF composition is less than 1% of total hemoglobin. On the other hand, persons having sickle cell disease and  $\beta$ -thalassemia, higher levels of  $\gamma$ -globin expression partially compensates for defective or damage  $\beta$ -globin gene production, which upgrade the clinical brutality in these diseases. It was also shown that BCL11A directly interacts with



chromatin at the human  $\gamma$ -globin locus in primary erythroid cells and that it appeared to act as part of a complex with the transcription factor GATA-1 and the NuRD chromatin remodeling and repressor complex [14]. In the earlier genetic studies it was found that the gene BCL11A has the identified sequences that influence HbF levels. BCL11A protein levels emerged to associate with the developmental stage of expression, such that primordial and fetal liver erythroid cells that expressed high levels of  $\gamma$ -globin had low or absent expression of the full-length forms of BCL11A, whereas shorter variant forms of the protein were noted in these cells. This result suggested that this gene product may act as a repressor of the  $\gamma$ -globin genes [15].

### RNA interference

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) induces the post-transcriptional degradation of homologous transcripts, and has been observed in a variety of organisms including plants, fungi, insects, protozoans, and mammals [16]. RNA interference (RNAi), a process by which target messenger RNA (mRNA) is cleaved by small interfering complementary RNA (siRNA), is widely used for investigations of regulation of gene expression in various cells [17]. The delivery of siRNA (small interfering RNA) into cells *in vitro* has been shown to clearly inhibit gene expression. In the past few years RNA interference (RNAi) has become the most widely used technology for gene knockdown. RNAi is a natural powerful mechanism that is thought to have arisen for protection from viruses and transposons [18]. RNAi stands for a superior approach for tempering gene expression. In comparison with other mRNA targeting strategies, RNAi get the benefit of the physiological gene-silencing machinery, which may clarify the exceptional potency of RNAi.

## RNAi mechanism

RNA interference (RNAi) is the knockdown of gene expression by small double stranded RNA (dsRNAs) fragments in which one strand is complementary to a section of the gene's mRNA. Figure 2 gives an overview over the RNAi pathway. In an RNAi process dsRNAs get introduced into the cell. In the cytoplasm they are processed by an enzyme of the Dicer family into small interfering RNAs (siRNAs). RNAi can however be started by direct delivery of siRNA molecules. Next, siRNA is accumulated into an RNA-Induced Silencing Complex (RISC). Lastly, antisense siRNA strands direct the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA. This process leaves the genomic DNA intact but suppresses gene expression by RNA degradation.

## siRNA as a tool for *in vitro* screening

Recently, RNAi technology can be emerged as a powerful tool for studying the knockdown of genes *in vitro*. For that purpose, siRNA mediated gene silencing can be used for the down regulation of a gene phenotype *in vitro* with striking potency at relatively low compound costs. But the quantity of siRNAs must also be carefully regulated. Too many siRNAs can set off an undesirable immune response. In addition to these difficulties, studies have shown that RNAi can silence genes that have not been targeted. Although difficulties exist, RNAi does have some distinct advantages over traditional disease treatments. The main advantage is that RNAi is a natural cell pathway. The pathway's continued existence of the prokaryote eukaryote split to the present is evidence to its effectiveness. Another advantage of RNAi is that siRNAs can be easily mass produced. This speeds up experiments, and could mean that future treatments involving RNAi will be inexpensive. RNAi can be used against a multitude of targets. In earlier studies it was found that siRNA mediated knockdown has become a valuable tool for studying the genetic impact on human diseases. In order to achieve maximum mRNA knockdown with minimizing undesired effects the siRNA must be designed carefully. Effective design of synthetic siRNAs relied on a detailed investigation of the characteristics of naturally occurring siRNA molecules, to ensure effective uptake by RISC and specific silencing of the targeted gene. Although siRNA silencing appears to be extremely effective by selecting a single target in the mRNA, it may be desirable to design and employ two independent siRNA duplexes to control for the specificity of the silencing effect. Recently published data also indicate that the thermodynamic stability of siRNA duplexes can severely influence mRNA knockdown [19]. Numerous guidelines on siRNA design have been published and also various online siRNA design tools are available on the web from academic institutions or commercial siRNA suppliers.

## Overview of siRNAs as a therapeutics drug

The most commercially RNAi is used as a therapeutic agent. While there are many technical hurdles to be overcome before the technology would be of application to man, there has been much research in animals into the potential of RNAi as a therapeutic [20]. There are many powerful approaches that qualifying siRNA compounds for developing as therapeutic drugs. Due to its mRNA targeting strategies, it has many advantages over traditional therapeutic drugs. siRNAs are raising as new generation biodrugs. Several studies have supported the therapeutic potential of siRNA. The double stranded RNA based molecule, siRNA, has a high potential as biopharmaceutical therapeutics. As RNAi interferes with translation, and not with DNA transcription, siRNA may not interact with chromosomal DNA. This lack of DNA interaction greatly reduces concerns about possible

adverse gene alteration that might result from DNA-based gene therapy [21]. The interaction of siRNA with mRNA, not protein, also makes it possible to reduce the production of harmful proteins before synthesis. Another merit of siRNA as a therapeutic drug is that a wide range of target proteins can be utilized for gene silencing to treat diseases [22].

## Other approaches available for thalassemia treatment

**Stem cell transplantation:** Bone marrow transplantation was the original term used to describe the collection and transplantation of hematopoietic stem cells, but with the recent demonstration that the peripheral blood and umbilical cord blood are also useful sources of stem cells, hematopoietic cell transplantation has become the preferred generic term for this process. Hematopoietic Stem Cell Transplantation is a choice treatment of many malignant, nonmalignant, and genetic diseases.

**Bone marrow transplantation (BMT):** The correction of this hematopoietic disorder by bone marrow transplantation was first demonstrated by Thomas et al. [23] in a young patient who had not undergone transfusion. Patients with homozygous beta-thalassemia, who have a good prognosis during treatment with conventional therapy, appear to have an especially high probability of hematologic cure with bone marrow transplantation, although the morbidity and mortality associated with such treatment are not established [24]. It was concluded that for patients under 16 years of age, transplantation of bone marrow from an HLA identical donor offers a high probability of complication-free survival, particularly if they do not have hepatomegaly or portal fibrosis [25].

**Gene therapy:** The objective of GENE therapy is to insert a normal beta-globin gene into the patient's stem cells, thus allowing increased production of beta globin and healthy red blood cells. Gene transfer of a regulated  $\beta$ -globin gene in HSCs would reduce the imbalance between  $\alpha$ - and  $\beta$ -globin chains in erythroid cells. In recent studies, human immunodeficiency virus-based lentiviral (LV) vectors were shown to stably transmit the human beta-globin gene and a large LCR element, resulting in correction of beta-thalassemia intermedia in mice [26]. Gene transfer by using onco-retroviral vectors and lentiviral vectors are useful for the treatment of beta-thalassemia. Lentiviral vectors have an advantage over onco-retroviral vector due to integration of larger element and minimal sequence rearrangement.

**Hydroxyurea:** Hydroxyurea is a type of drug, thought to increase gamma globin by inducing stress erythropoiesis. In the womb, the foetus builds this type of protein instead of beta globin. It is not until after birth, when the foetus no longer produces gamma globin that the beta g lobin deficiency becomes apparent. Hydroxyurea has been shown to induce production of fetal hemoglobin. Fetal hemoglobin has a pair of gamma-globin molecules in place of the typical  $\beta$ -globins of adult hemoglobin. Higher-than-normal levels of fetal hemoglobin can ameliorate some of the symptoms of thalassemia. Effects in patients with thalassemia major are controversial. It was reported that a marked elevation of total Hb levels with HU that permitted regular transfusions to be stopped in some children with transfusion dependent thalassemia. Because of the associated risks and the limited experience with this drug in thalassemia intermedia patients, the use of hydroxyl urea should be restricted to patients with severe clinical problems such as extended extra medullary erythropoiesis or severe osteoporosis and those requiring transfusion therapy complicated by alloimmunization [27].

## Conclusion

Although lots of the trials controlling the action of the  $\beta$ -globin



locus are known, the facts of those regulating normal human hemoglobin switching and reactivation of HbF in adult hematopoietic cells remain to be illuminated. If the molecular trials in hemoglobin switching or  $\gamma$ -globin gene reactivation were better appreciated and HbF could be totally reactivated in adult cells, the approaching might show the way to cure for these disorders. One right away related approach would be to use gene therapeutic approaches to deliver siRNAs that target the molecules described above. The above review addresses the role of BCL11A in silencing the  $\gamma$ -globin genes and also a brief report on siRNA based strategy for the inhibition of BCL11A gene expression. This approach may also demonstrate the ability of RNAi (RNA Interference) to minimize viral reproduction *in vitro*. Such approaches that are achieved on a relatively small scale may help to disclose which therapeutic targets would be effective *in vivo* for more broadly applicable small molecule approaches. Also small molecules would be ideal as therapies in these globally widespread diseases. However, inhibition of BCL11A gene expression by RNAi approach can also be used as a convenient model for studying pathophysiology of thalassemic cells *in vitro*, and may possibly be applied to increase the fetal Hb levels in severe forms of  $\beta$ -thalassemia in a clinical setting.

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