Towards the genetic treatment of β-thalassemia: new disease models, new vectors, new cells

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β-thalassemia major is a severe congenital anemia for which there is currently no curative therapy other than allogeneic hematopoietic stem cell transplantation. This therapeutic option, however, is only available to less than a quarter of thalassemia patients who have an HLA-matched bone marrow donor. The transfer of a regulated globin gene in autologous hematopoietic stem cells is an attractive alternative approach since, in principle, it is applicable to all thalassemic subjects. This strategy, though simple in theory, creates a major challenge in terms of controlling transgene expression, which ideally should be erythroid-specific, differentiation stage-restricted, elevated, position independent, and sustained over time. It has been difficult to satisfy all these requirements with the classic murine γ-retroviral vectors. Nearly a decade ago, May *et al.* demonstrated that an optimized combination of proximal and distal β-globin transcriptional control elements borne by a recombinant lentiviral vector permits lineage-specific and elevated β-globin expression in vivo, resulting in therapeutic hemoglobin production and correction of anemia in β-thalassemic mice. Several groups have extended these findings to various models of β-thalassemia and sickle cell disease. New mouse models have since emerged which provide additional tools for testing and comparing globin vectors. The very recent discovery that adult differentiated cells can be reprogrammed to become pluripotent stem (iPS) cells from which hematopoietic cells can be derived, provides yet another opportunity for the further development of stem cell engineering for the cure of the severe hemoglobinopathies.

Introduction

Thalassemias are congenital, quantitative defects in hemoglobin synthesis that are mostly found in the temperate regions of the world. In the β-thalassemias, the defective globin gene encodes the β-chain of adult hemoglobin (HbA, α2:β2). The β-globin chain deficit leads to chain inbalance with intracellular precipitation of excess α-globin chains and ineffective erythropoiesis.[1-4] In the most severe forms, found in homozygotes or compound heterozygotes, the anemia is lethal within the first years of life in the absence of any treatment.[5] The majority of β-thalassemia patients require lifelong transfusion therapy to correct their anemia and suppress the massive erythropoiesis with the associated skeletal modifications.[1-4] Transfusion therapy, however, exacerbates the burden of iron overload, which must be aggressively treated to avoid severe and often fatal complications. Prevention and treatment of iron overload are the major goals of current patient management.[6] The only means to definitively cure the disease is through allogeneic bone marrow transplantation (BMT),[7-9] which is the treatment of choice when a related matched donor is available. However, severe complications may still arise even after a matched related donor bone marrow transplantation. In fact, mortality and morbility rates are in the range of 10-20% even in the best transplantation units. Globin gene therapy would, in principle, be applicable to every patient and, with a reduced myeloablative regimen, could even become preferable to bone marrow transplantation. Although highly promising, globin gene transfer raises a number of challenging biological questions. These concern the isolation and transduction of HSCs, the design of vectors that provide therapeutic levels of transgene expression with a minimal risk of insertional oncogenesis, and the implementation of non-toxic transplant conditions that permit host repopulation with reduced intensity conditioning. Many of these issues are common to all stem cell-based gene therapies. Globin gene transfer, however, is particular in its stringent transcriptional requirements: transgene expression has to be erythroid specific, differentiation stage specific and elevated. Achieving therapeutic β-globin expression had represented a tremendous obstacle for almost two decades,[10,11] but a body of research accumulated over the past seven years has demonstrated that therapeutic levels of hemoglobin synthesis can be achieved in the progeny of genetically modified murine HSCs. This commentary briefly reviews recent advances in animal modeling, globin vector design and stem cell isolation.

Mouse models of β-thalassemia

Progress in the gene therapy of β-thalassemia would not have been possible without the development of suitable animal models of the disease. Murine models are the most useful and are generally adopted as first line *in vivo* studies. The best known murine models are named in their order of discovery: Hbb^{th1}, Hbb^{th2}, Hbb^{th3} and Hbb^{th4}. The Hbb^{th1} model is a natural occurring deletion of the β-major gene.[12] The Hbb^{th2} and Hbb^{th3} models were generated by targeted gene disruption in the laboratory of Oliver Smithies, who obtained the Nobel prize for Medicine or Physiology in 2007. The Hbb^{th2} model[13] has an artificial disruption of the β-major globin gene introduced by homologous recombination, which leaves in place a *neo* gene and results in a more severe phenotype than Hbb^{th1}. The Hbb^{th3}model[14] has an artificial deletion of both mouse β-globin genes causing a β°-thalassemia and a homozygous phenotype more severe than the corresponding human disease. Hbb^{th4} is a deriv-

ative of Hbb^{th3} that has a single β°-thalassemic globin gene (*b-IVS2 nt 654*) substituting for the two deleted murine genes.[15] Since the added gene is a β°-thalassemic gene, the phenotype is comparable to Hbb^{th3}, but it is a better model for experiments aiming at gene correction of that specific mutation. A schematic representation of these models is provided in Figure 1.

With the exception of Hbb$^{th1/th1}$, these models are in utero lethal in the homozygous state and are, therefore, mainly used in the heterozygous state which recapitulates the phenotype of human thalassemia intermedia. Rivella *et al*. overcame this limitation and developed a murine model of β-thalassemia major by engrafting fetal liver cells from Hbb$^{th3/th3}$ mice into lethally irradiated, adult syngeneic mice.[16]

Three other murine models of β-thalassemia were derived from the Hbb^{th3} genotype by transgenesis with BAC carrying common thalassemia mutations in the context of a full 183 Kb human β-globin locus.[17-20] As in the homozygous state, all these murine models produce only human β-like globins and contain all known globin regulatory sequences. They are referred to as humanized murine models of β-thalassemia. In the first of these models, the mutant BAC contains the most common South-East Asia β°-thalassemia mutation, a 4 bp deletion of codons 41-42 (-TTCT),[17] in the second the IVS-110 splice mutation which is found worldwide,[19] and in the third the HbE point mutation at codon 26 of the β-globin gene.[18] The small amount of normal β-chain produced by the HbE mutant is sufficient to rescue the lethal phenotype of the homozygous Hbb$^{th3/th3}$ β-globin null model. All these humanized murine models should serve as valuable tools for gene correction in hemopoietic stem cells and for studying the effects of HbF inducers *in vivo*.

Engraftment of immune-deficient mice[21] with hematopoietic cells collected from β-thalassemia CD34$^+$ subjects provides an additional model for investigating the efficacy of gene therapies for human β-thalassemia.[22] While providing a useful means to measure globin transgene expression in human cells in an *in vivo* context, this model is limited in its ability to test true HSC engraftment and further supports minimal human erythropoiesis.[22]

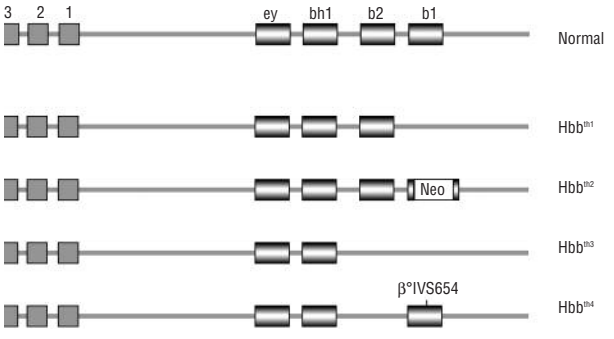

Figure 1. The structure of the globin gene cluster in the most common mouse models of thalassemia letal in Bbb^{th2}, Hbb^{th3} and Hbb^{th4}. The homozygous state is shown on the right.

Globin gene transfer vectors

Early gene therapy experiments were generally carried out with murine leukemia virus (MLV)-derived vectors, referred to as oncoretroviral or gamma-retroviral vectors. The vectors built in the 1980s encoded the β-globin gene, including its promoter and two proximal enhancers. With those constructs, β-globin expression was tissue-specific, but low and variable, usually varying between 0 and 2% of endogenous mouse β-major RNA levels.[23-27] The discovery of the locus control region (LCR), a powerful enhancer spreading over 20kb and located 60kb upstream of the human β-globin gene on chromosome 11, did not result in a quick resolution of globin vectors. Initial efforts to incorporate LCR subfragments into oncoretroviral vectors yielded low titers,[28] low expression[29] or unstable vectors prone to sequence rearrangements.[30] Incorporation of 1 kb long LCR made of core elements HS2, HS3, and HS4 significantly increased expression levels in murine erythroleukemia cells,[10,11] but failed to abolish positional variability of expression.[11] This suggested that a minimal enhancer comprising juxtaposed core elements was equivalent to a good but still ineffective erythroid-specific LCR.[31-39] These findings argued against the effectiveness of isolated core elements and were consistent with contemporary studies in transgenic mice establishing the limited effect of single copy core elements.[40] However, the incorporation of larger LCR into oncoretroviral vectors proved to be problematic because of vector instability and frequent genomic rearrangements. Investigators were forced to either seek novel transcriptional control elements or explore alternative vector systems. Limited success was achieved by swapping the α-locus HS-40 regulatory region for the β-LCR,[41-43] and alternative erythroid specific promoters, such as the ankyrin[44,45] and mutant HPFH γ-globin promoters,[46,47] in place of the β-globin promoter.

In the mid 1990s, May *et al*. hypothesized that lentiviral vectors might overcome the genomic instability of complex γ-retroviral vectors owing to the ability of lentiviruses to better regulate their RNA stability. In 2000, they reported[48] that a lentiviral vector harboring an optimized combination of proximal and distal β-globin transcription control elements, including large segments of the human β-globin LCR, could treat β-thalassemic mice of their anemia. This finding prepared the way for expressing therapeutic levels of hemoglobin in thalassemic mice and became an example of efficacy for subsequent β- or α-globin vector development. The TNS9 vector[48] encodes the human β-globin gene, deleted of a cryptic polyadenylation site within intron 2,[11] flanked by an extended promoter sequence and the β-globin 3' proximal enhancer, as well as large LCR ele-

ments (3.2 kb) spanning HS2, 3 and 4.[39] Several vectors similar in structure to TNS9 but encoding the γ-globin gene or mutated β-globin genes have been reported.[49-52]

The first severe hemoglobinopathy to be treated in mice was the $Hbb^{th3/+}$ model[48] (Table 1). In mice harboring on average 0.5-1.0 vector copies per cell in peripheral blood cells, the TNS9 vector expressed elevated and persistent levels of human β-globin appropriately restricted to the erythroid tissues. Peripheral blood cells showed hemoglobin levels of 11-13 g/dL compared with 8.0-8.5 g/dL in age-matched controls, a hematocrit of 39-45% compared with 29-32% in controls, and decreased reticulocyte counts, from 19-23% in control mice to 5-10% in the TNS9-treated cohort.[48] In contrast to TNS9, a control vector encoding core elements of the LCR silenced over time.[48] TNS9 maintained stable levels of human β-globin gene over a 40-week period in long-term primary transplant recipients and remained stable in secondary transplant recipients of TNS9-transduced bone marrow for up to 40 weeks after transplant with no indication of loss of expression. This level of β-globin gene expression was sufficient to achieve a durable improvement in anemia, correct extra-medullary hematopoiesis and markedly reduce hepatic iron accumulation.[53] To summarize, TNS9 vector provided good evidence that viral-mediated globin gene transfer could achieve major therapeutic benefit in a severe hemoglobinopathy.

Persons *et al.*[51] investigated a lentiviral vector with a slightly shorter LCR made of HS2-3-4, but encoding the human γ-globin gene, in normal C57Bl/6J mice transplanted with transduced thalassemic ($Hbb^{th3/+}$) bone marrow cells. Fifteen weeks after transplantation, the engrafted mice showed 7-90% HbF-containing red blood cells by fluorescent activated cell sorting (FACS) analysis. Expression was related to vector copy number and was therapeutic only in mice with a vector copy number (VCN) of 2.4 ±0.7 as evaluated by an improvement in the anemia, with hemoglobin levels of 11.6±0.3 g/dL and reticulocyte count of 12.6±2.8%.

To better evaluate the therapeutic efficiency of TNS9 and its potential clinical applicability to the most severe hemoglobinopathies, Rivella *et al.*[16] investigated its efficacy in the context of a fatal anemia. They developed a new model of β-thalassemia major by engrafting adult mice with $Hbb^{th3/th3}$ fetal liver cells (FLCs). Engrafted mice died of severe anemia usually within 60 days of FLC transplantation, but survived when engrafted with TNS9-transduced $Hbb^{th3/th3}$ FLCs. In chimeras with less than 5% murine endogenous hemoglobin (produced by residual host hematopoiesis) and surviving more than eight months after transplantation, hemoglobin levels averaged 6.5±2.9 g/dL for a mean vector copy number of 1.6±0.6. This indicates that TNS9 could generate 4 g/dL hemoglobin per vector copy in this *in vivo* setting, approximately half of hemizygous hemoglobin production (8.1±0.3 g/dL in $Hbb^{th3/+}$ chimeras).

Using the Hbb $^{th1/th1}$ model of thalassemia, Imren *et al.*[54] reported the pancellular permanent correction of the thalassemic phenotype with essentially the same cassette used in the successful gene therapy of a mouse model of sickle cell disease (SCD), except for the substitution of a normal in place of an antisickling β-globin gene.[54] Their vector boosted the hemoglobin production of 4.4 g/dL for an average of 3 vector copies per cell, an increase of 1.6 g/dL per VCN.

In an effort to further increase the expression of a lentiviral-encoded human β-globin cassette, Lisowski *et al.*[55] assessed different promoter lengths and the inclusion of HS1 or modifications of the HS4 fragment contained in their reference TNS9 vector. They found that HS4 could not be modified without significantly reducing the β-globin expression, whereas the addition of HS1, absent from alla available globin vectors, increased the human β-globin expression in mice by 50%. Their best vectors were able to correct the Hbb $^{th3/+}$ β-thalassemia phenotype with remarkably low values of vector copy number achieving an output of up to 9.5 grams per VCN.

A different approach to the treatment of β-thalassemia was taken by Li *et al.* who in this issue report the correction of the Hbb^{th4} model of mouse β-thalassemia by the embryonic injection of a lentiviral β-globin vector. In this model of β-thalassemia, the lentiviral vector determined a hemoglobin increase ranging from 1 to 1.6 g/dL in the different mouse generations. The transgene VCN deter-

Table 1. Hemoglobin production per vector copy number (VCN) achieved in thalassemic bone marrow chimeras with the globin lentiviral cassettes used in different studies.

Author	*Mouse donor model*	*Vector (globin gene)*	*VCN*	*Hb/VCN*	*Donor Hb g/dL*	*Recipient Hb g/dL*	*Hbb^{hu} %*	*LCR–HS*
May	$Hbb^{th3/+}$	TNS9 (β)	0.80	3.7	8.9	11.8±1.3	21±2.9	2-3-4
Rivella	$Hbb^{th3/th3}$	TNS9 (β)	1.60	4.0	0	6.5 ±2.9	25±8	2-3-4
Lisowsk	$Hbb^{th3/+}$	T10 (β)	0.42	9.5	8.9	12.9±0.9	41±9	1-2-3-4
Lisowski	$Hbb^{th3/+}$	S10 (β)	0.42	8.8	8.9	12.7±1.1	41±9	1-2-3-4
Lisowski	$Hbb^{th3/+}$	T9 (β)	0.71	6.4	8.9	11.9±0.8	27±6	2-3-4
Lisowski	$Hbb^{th3/+}$	S9 (β)	0.71	4.2	8.9	11.7±0.6	27±6	2-3-4
Imren	$Hbb^{th1/th1}$	(β)	3	1.6	8.0	12.4±0.7	32±4	2-3-4
Li	$Hbb^{th4/+}$	LBG (β)	1?	1.76	8.9	10.6±1.5		None
Person	$Hbb^{th3/+}$	(β/γ)	2.40	1.0	9.1	11.6±0.3	15	2-3-4

mined by FISH was estimated to be 1-2 per cell. Therefore, the output from the vector was 0.5-1.6 g/dl in the different generations, which is surprisingly high given the absence of LCR elements in their vector. This is an interesting and attractive experimental system. Since the transgene is present in all cells, and not only in the bone marrow progenitors, it makes it easier to evaluate the absolute erythroid specificity of the globin cassette. Furthermore, integration of only 1-2 vectors into a single embryonic cell reduces the risk of insertional mutagenesis when compared with the millions of events occurring in the infection of the bone marrow. It is not clear from this study whether the globin vector expresses adequately from only a subset of integration sites. However, these findings do suggest that introducing globin lentiviral vectors in ES cells, at least in the mouse, may occasionally escape silencing.

Puthenveetil *et al.*[22] tested a β-globin regulated lentiviral vector insulated by flanking cHS4 in a xenogeneic model in which transduced CD34$^+$ cells collected from subjects with thalassemia major were transplanted into NOD-SCID mice. These authors found a proportion of HbA+ cells and an average amount of HbA similar to those obtained with normal CD34 cells. The model does not allow the increase in hemoglobin to be determined and it is not possible, therefore, to evaluate the β-globin output.

Han *et al.*[56] used a variant of the TNS9 vector encoding the human α-globin gene to treat a lethal murine model of α-thalassemia developed in their laboratory by in utero gene transfer. Human α-globin gene expression persisted for a few months, but, in contrast to results obtained with the β-globin gene in bone marrow chimeras, human α-globin was expressed at low level and transiently. This may have been the consequence of limited, if any, transduction of HSCs.

New cell sources: embryonic stem cells and induced pluripotent stem cells

While HSCs are the natural vehicle for restoring long-term hematopoiesis, their use has some important limitations. The first is their relative scarcity, which can eventually preclude autologous HSC therapy when the harvested cellular product is too small. The second is the difficulty to perform biosafety testing such as integration site analysis and consequently to select cells with chosen integration sites, because adult HSCs cannot be replicated *in vitro*. The third limitation is that homologous recombination using current technologies is practically impossible thus compromising the advent of gene correction. All of these limitations are ultimately due to the fact that adult HSCs cannot be expanded *in vitro* without losing their stem cell potency. These limitations explain the critical importance of viral vectors such as gamma-retroviral and lentiviral vectors, which are remarkably quick and efficient in achieving stable gene transfer. This is essential when dealing with HSCs that are only available in limited quantities.

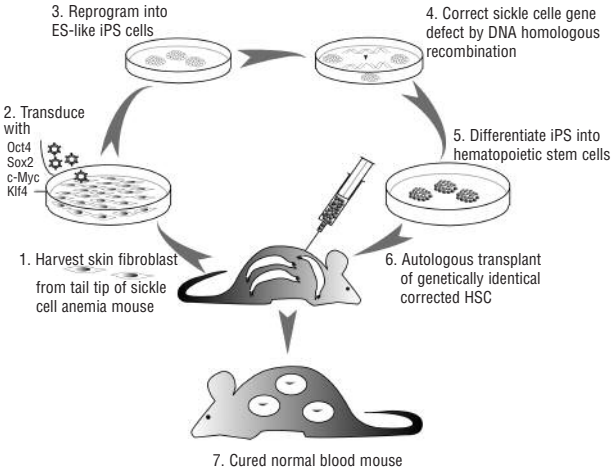

Figure 2. The steps involved in the process of gene correction of the mouse model of sickle cell anemia using induced pluripotent stem (iPS) cells.

The only cells that are amenable to gene targeting and correction, which requires unlimited *in vitro* cell division without losing multipotency, are the embryonic stem (ES) cells. Chang *et al.*[57] recently provided proof of principle of the feasibility of the homologous recombination approach in mice with sickle cell anemia. The current restrictions on human ES cell research, however, make the investigation of this intriguing strategy in humans problematic. Furthermore, the autologous ES cells to be used for stem cell engineering purposes would have to be generated from somatic cell nuclear transfer (SCNT) and therapeutic cloning, processes that are extremely inefficient and that require large supplies of human donor eggs as the recipient cells.

However, over the last year Takahashi *et al.*[58] have reported the successful reprogramming of fibroblasts to an embryonic stem-like state. Cells obtained by this reverse-differentiation process, called induced pluripotent stem (iPS) cells, were produced by exposing embryonic or young adult bulk fibroblast cultures to gamma-retroviral vectors encoding 4 transcription factors, which are physiologically active in the embryonic stem cells, but generally turned off when differentiation progresses. The cultured cells formed colonies similar to ES cell colonies. These findings have since been confirmed and extended by others to both mouse and human fibroblasts.[58-66] Recently, Rudolf Jaenisch and co-workers achieved a successful gene therapy in a mouse model of sickle cell disease, using homologous recombination in ES-like iPS cells.[67] The process, summarized in Figure 2, is applied to fibroblast harvested from a skin biopsy, which are then induced to become iPS by transduction with retroviral vectors that encode four stem cell transcription factors. iPS are amenable to the correction of the SC mutation by standard homologous recombination techniques and can then be differentiated *in vitro* into unlimited amounts of hematopoietic stem cells. The whole process ends with the autologous transplantation of the corrected HSC into

the original mouse donor, which will now be cured of its SC disease. For the moment, the technique has the disadvantage of requiring potentially oncogenic vectors and oncogenic transcription factors to induce the iPS state. Indeed, mice subjected to the procedure were reported to develop tumors more frequently than controls.[60] At least one of these factors, c-myc, can be eliminated from the reprogramming process.[59]

This technique is not only useful for homologous recombination, but may also enhance lentiviral-mediated globin gene transfer for the treatment of β-thalassemia by providing a means to perform detailed integration site analysis and adequate *in vitro* cell expansion before infusing cells into the recipient. However, many aspects of this exciting approach must still be clarified. It is especially important to note that it is still not known whether the genomic integrity of the reprogrammed cells and reprogramming itself are consistent with safe, long-term hematopoietic reconstitution. This approach is still a very long way from any clinical investigation.

Conclusions

Almost forty years have passed since the cloning of the β-globin gene gave birth to the hope of developing a gene therapy for thalassemia and hemoglobinopathies. It has taken since then to discover the multiple facets of the complex globin regulation and assemble most of the sequence relevant to globin gene expression. Over the last decade, a wider understanding of globin regulation together with advances in the development of more efficient and safer viral vectors has have led to the successful treatment of various models of murine thalassemia. While newer vectors with higher expression and titer are still being developed, current research is now focusing on safety issues and the initiation of upcoming clinical trials (*http://www4.od.nih.gov/oba/sacghs/meetings/July%202007/SACGHSJul2007meeting.htm*). Finally, over the last year, breakthrough discoveries in stem cell biology have led to the production of iPS cells, opening new horizons for a possible treatment for thalassemic patients from whom HSCs cannot be harvested in sufficient amounts. Major developments in somatic cell reprogramming without the use of oncogenes are still needed before such therapeutic cloning and either globin gene addition followed by integration site selection or homologous recombination can come closer to clinical reality. Whatever the stem cell source, achieving high-level and permanent hematopoietic reconstitution with genetically modified autologous cells and minimal toxicity remains an essential goal for these future stem cell therapies.

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