HUMANIZED β-THALASSEMA MOUSE MODEL CONTAINING THE COMMON IVSI-110 SPlicing MUTATION

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Running Title: Humanized IVS1-110 β-thalassemia mouse model

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In memory: Panayiotis A. Ioannou died in April 2005. This work was inspired and led by him, and his colleagues dedicate this research to his memory.

Splicing mutations are common causes of β-thalassemia. Some splicing mutations permit normal splicing as well as aberrant splicing, which can give a reduced level of normal β-globin synthesis causing mild disease (thalassemia intermedia). For other mutations, normal splicing is reduced to low levels, and patients are transfusion dependent when homozygous for the disease. Development of therapies for β-thalassemia will require suitable mouse models for preclinical studies. In this study, we report the generation of a humanized mouse model carrying the common IVSI-110 splicing mutation on a BAC including the human β-globin (huβ-globin) locus. We examined heterozygous murine β-globin knockout mice (mβth-3/+ ) carrying either the IVSI-110 or the normal huβ-globin locus. Our results show a 90% decrease in huβ-globin chain synthesis in the IVSI-110 mouse model compared to the mouse model carrying the normal huβ-globin locus. This notable difference is attributed to aberrant splicing. The humanized IVSI-110 mouse model recapitulates accurately the splicing defect found in comparable β-thalassemia patients. This mouse model is available as a platform for testing strategies for the restoration of normal splicing.

Thalassemia is one of the common inherited genetic disorders affecting hemoglobin synthesis. Approximately 300,000 patients with clinically relevant hemoglobinopathies are born each year (1). In the case of β-thalassemia, which is characterized by a reduction or absence of β-globin chain synthesis resulting in free or unpaired α-globin chains, which aggregate and precipitate within red cells, causing ineffective erythropoiesis and severe anemia.

Over two hundred different mutations have been found that cause β-thalassemia, with splicing mutations among the most common. Most of these mutations activate aberrant cryptic 5’ donor or 3’ acceptor splice sites without completely abolishing normal splicing. These mutations lead to the production of variable amounts of normal transcripts. Some mutations allow a significant level of normal splicing (such as IVSI-6), leading to thalassemia intermedia, while others reduce normal splicing to low levels (such as IVSI-110) or very low levels (such as IVSI-5 and IVSII-654), causing transfusion-dependent disease in homozygotes.

Because of the complex pathophysiology associated with the hemoglobinopathies, transgenic mouse models are an essential platform for delineating the pathological mechanisms, and, as in vivo model systems, for validating future therapeutic strategies. The humanized mouse model approach, where the transgenic mouse model contains large human genomic fragment(s), has provided valuable insight into the regulatory elements required for the developmental expression of human globin genes (2-6). To date, the YAC transgenic mouse for sickle cell anemia, exclusively expressing human βs-globin, is the only mouse thalassemic model expressing a disease gene from the intact huβ-globin locus (7). Other transgenic mice exclusively expressing human HbA, HbC, HbF and HbS have also been reported, but these mouse models were created by co-injecting large genomic DNA fragments encompassing truncated or hybrid versions of the β-globin locus containing the α1-globin gene (8-10). The
only β-thalassemia splicing mouse model reported to date is a knock-in model that contains the common IVSII-654 β-thalassemia splicing mutation in the context of the mouse β-globin (\textit{\textmu}\beta-globin) locus (11).

With the recent development of targeted modification techniques for BACs such as recE/recT-based homologous recombination (12-15), and the availability of site-specific BAC mutagenesis (16-19), the main obstacle to generating transgenic mouse models for disease-causing mutations has been overcome. We have recently reported the first humanized mouse model for a deletion mutation, causing β-thalassemia, at codons 41-42 (20). Unlike the situation with the normal \textit{\textmu}β-globin locus, these mice fail to show phenotypic complementation in heterozygous β-globin knockout (KO) background (\textit{\textmu}β<sup>m</sup>\beta<sup>th-3/4</sup>), or homozygous β-globin KO mice (\textit{\textmu}β<sup>m</sup>\beta<sup>th-3/3</sup>). This mouse is a suitable in vivo model system to investigate gene correction strategies in hematopoietic stem cells, and to identify HbF inducers.

We describe the development and characterisation of a humanized mouse model carrying the IVSI-110 splicing mutation in the context of the genomic \textit{\textmu}β-globin locus. This mutation was first described in 1981 and is one of the most common splicing mutations found in the Eastern Mediterranean region (21). Our results indicate that this humanized IVSI-110 mouse model recapitulates the splicing defect that is typical for IVSI-110 in β-thalassemia patients. This mouse will serve as a platform for testing novel strategies for the restoration of normal splicing.

**MATERIAL AND METHODS**

**Characterisation of the 183 kb genomic fragment containing the \textit{\textmu}β-globin locus -** The PAC clone 148O22 containing the \textit{\textmu}β-globin locus in a 183 kb genomic fragment was first isolated from the RPCI I PAC library (22) (http://www.chori.org/bacpac/) and shown to contain the β-globin locus (23). The 183 kb genomic fragment was retrofitted into the pEBAC140 cloning vector as a single Not I fragment to generate pEBAC/148β (Fig. 1A) (24). Sequencing of the 5’ and 3’ ends of the genomic insert (data not shown) and alignment with the human genome sequence (GenBank Accession number NT_028310.10) revealed that the genomic insert is 183,039 bp long, with 122,076 bp upstream of the start codon of the ε-globin gene and 17,672 bp downstream of the stop codon of the β-globin gene.

**Introduction of the IVSI-110 mutation into \textit{\textmu}β-globin locus -** Using GET recombination, an inducible homologous recombination system for Escherichia coli, the IVSI-110 mutation was introduced into the intact \textit{\textmu}β-globin locus contained in a BAC vector (14). The β-globin locus containing the IVSI-110 mutation was purified from DH10B cells using the Qiagen BAC purification procedure (Qiagen, Hilden, Germany). The 183 kb genomic insert was prepared for microinjection as previously reported (25).

**Genotyping of transgenic mice -** Genotyping was performed as previously described (25). Briefly, transgenic founder mice and F1 progeny were screened by PCR using the following primer pair: \textit{\textmu}β<sup>h</sup>F 5’-ACAAGACAGGTTAAGGACCA-3’ \textit{\textmu}β<sup>h</sup>R 5’- GTCTGTTCTCCATCTAAACTGTA 3’. These primers amplified a 447 bp product of the \textit{\textmu}β-globin gene. F1 progeny were bred with heterozygous knockout mice (\textit{\textmu}β<sup>h</sup>\beta<sup>th-3/4</sup>) to generate transgenic mice on a heterozygous and knockout background. The genotyping was performed by multiplex PCR using the following primer pairs: (i) \textit{\textmu}β<sup>H</sup>F/ \textit{\textmu}β<sup>H</sup>R (as above), (ii) HPRT-F 5’-GATGGGAGCCATCACATTGGTAG-3’ HPRT-R 5’-GGGACCTTGACCATCTTTTGA-3’ and (iii) \textit{\textmu}β<sup>h</sup>-F 5’-TGAGAAGGCTGTGTCCTTGTA3’ \textit{\textmu}β<sup>h</sup>-R 5’-CAGAGTAGGTCTCCAAAGCTA-3’. The conditions used for the multiplex PCR were: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min. After 30 cycles, the PCR products were resolved by 1.5% agarose gel-electrophoresis.

**Determination of transgene integrity -** The long-range integrity of the \textit{\textmu}β-globin locus in transgenic lines was examined by Southern blot analysis after digestion of genomic DNA with the Cfr9 I and Sfi I restriction endonucleases (Fermentas, Hanover, MD, USA). Cfr9 I digestion of the \textit{\textmu}β-globin locus yields a single 36 kb fragment that contains the γ-, δ- and β-globin genes, while Sfi I digestion cuts the genomic fragment twice in the middle of the fragment. (Fig. 1). To avoid shearing the genomic DNA, splenocytes from representative transgenic mice were suspended at a final concentration of 2x10<sup>7</sup> cells/ml in 0.5% InCert agarose (Cambrex Bio Science, Rockland,
USA). The solidified plugs were incubated overnight in buffered Proteinase K buffer (100 mM EDTA at pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 0.2 mg/ml of Proteinase K) at 50°C. After several washes in 20 mM Tris / 50 mM EDTA at pH 8.8, the plugs were digested overnight with Cfr9 I or Sfi I. The plugs were loaded into the wells of a 1% agarose gel and genomic DNA was separated in a CHEF-DRII pulsed-field gel electrophoresis system (BioRad, CA, USA). The DNA was alkali-blotted onto a Hybond membrane (Amersham BioScience, Buckinghamshire, UK) after partial depurination and hybridized with a PCR probe amplified from the huβ-globin locus using primers ββ-F / ββ-R.

**Determination of transgene copy number -** Transgene copy number in mice was performed by quantitative radioactive PCR as previously reported (25). In brief, PCR was performed using the primer pair HM-F 5’-GATGAAATTGTTGAGTGGACGCTCT-3’ /HM-R 5’-CCTGAAAGTCTCTCAGAGATCCA-3’ on mouse tail genomic DNA. These primers amplify the huβ-globin and muβ-globin sequences equally, yielding 381 bp and 367 bp products respectively. The human and mouse products can be readily distinguished since the murine product contains a unique NcoI site that yields two fragments, 242 bp and 125 bp. The PCR products were run on a 2.0 % 3:1 Nusieve gel (BMA, Rockland, USA). The gel was washed for 45 min, in 7% trichloroacetic acid and vacuum-dried at 65°C onto blotting paper. The dried radioactive gel was exposed to a Low Energy Storage Phosphor Screen (Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire, UK) and the Phosphor Screen (Molecular Dynamics, Buckinghamshire, UK) after partial depurination and hybridized with a PCR probe amplified from the huβ-globin locus using primers ββ-F / ββ-R.

**RT-PCR analysis -** Total RNA was prepared using the Tri-Reagent BD system (Molecular Research Center, Cincinnati, USA) as described by the manufacturer. cDNA was synthesized by random hexamers as primers. Primers used to amplify the sequence between exon 1 and exon 2 were: forward primer 5’-CGCCTCGAGGAAGTCTCCGTAC-3’ and reverse primer 5’-CGCGAATTCCCATACGACACAGGGAT -3’. The PCR was performed using the following conditions: denaturation at 94°C for 0:30 min, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. After 30 cycles, the PCR products resolved on a 2% Nusieve GTG agarose gel. The above primers amplified a 170 bp and a 189 bp product from the huβ-globin gene, which corresponds to the normal and aberrantly spliced mRNA respectively.

**Sequencing of RT-PCR products -** The PCR amplified products were digested with Xho I and EcoR I and cloned into the respective sites of pBluescript-KS+. Colonies were picked based on blue/white color selection and verified by sequencing using Big Dye Terminator Kit V3.1 (Perkin-Elmer, USA) according to manufacturer’s specifications, and using the RT-PCR forward primer as above. In brief sequencing PCR was performed using the following conditions: rapid thermal ramp to 96°C, hold at 96°C for 30 sec, rapid thermal ramp to 50°C, hold at 50°C for 15 sec, rapid thermal ramp to 60°C, hold at 60°C for 4 min. After 25 cycles, rapid thermal ramp to 4°C.

**Hematological analysis -** Blood was collected from transgenic mice ≥8 weeks of age by retro-orbital bleeding. Full blood examination (FBE)
was performed on whole blood collected into tubes containing EDTA. FBE was performed using an automated Roche Cobas Helios hemoglobin analyser at the Walter and Eliza Hall Institute, Melbourne, Australia. A minimum of two weeks was allowed between repeat samples, when such samples were necessary, to minimize the distortion of hematological parameters by frequent bleeding. Cellulose acetate gel electrophoresis was used to visualize hemoglobin tetramers. Samples containing approximately equal amounts of hemoglobin were run on cellulose acetate membranes (Helena Laboratories). Hemoglobin bands were visualized using Ponceau S stain.

**Measurement of globin chain synthesis** - The measurement of \( \mu \beta \)-globin and \( \nu \beta \)-globin chain synthesis was performed by high-pressure liquid chromatography (HPLC) as previously described (25). Red blood cells (20 \( \mu \)l) were lysed in 500 \( \mu \)l of lysis buffer (0.1 M 2-mercaptoethanol, 0.1M HCl), and then made up to 1 ml with 500 \( \mu \)l of 50 % aqueous acetonitrile. The lysate was centrifuged and the supernatant was analysed by HPLC. Globin chain separation was performed by loading 30 \( \mu \)l of lysed red cells onto a Vydac C4 column (Vydac, Hesperia, USA). The proteins eluting from the column were measured at 220 nm, 280 nm and 413 nm with an Agilent 1100 Diode Array Detector. Peak protein fractions were collected and analysed by tandem mass spectrometry in order to confirm globin chain type.

**RESULTS**

**Creation and characterisation of BAC transgenic mice** - In this study, our aim was to establish a humanized mouse model carrying the IVSI-110 splicing mutation and determine whether the \( \nu \beta \)-globin pre-mRNA produced in mice was aberrantly spliced as in humans. Founder mice were carrying either the normal or IVSI-110 genomic fragment following \( Cfr9 \) I digestion in both transgenic lines correlated with the \( \gamma \)-, \( \delta \)- and \( \beta \)-globin genomic fragment as being intact. The presence of a single \( Sfi \) I fragment approximately 160 kb in size in the transgenic line containing the normal \( \beta \)-globin locus, is consistent with the integration of two genomic fragments in a ‘tail’ to ‘tail’ orientation (Fig. 1C). The presence of two \( Sfi \) I fragments, 140 kb and 175 kb in the IVSI-110 transgenic line is consistent with the integration of two genomic fragments in a ‘head’ to ‘tail’ orientation (Fig. 1C).

**FISH analysis of transgenic mice** - Transgenic mice carrying the normal or the IVSI-110 \( \nu \beta \)-globin transgene were analysed by FISH. Metaphase spreads of fibroblast cells were prepared from F0 progeny. The transgenic founders were all found to contain a single integration site (Fig. 2A). The integrated BAC DNA was located using labelled insert DNA and chromosome identification made on the basis of the banding pattern obtained from the inverted DAPI metaphase image. In the founder containing the normal \( \nu \beta \)-globin locus, the transgene was proximally located on chromosome 14, while in the founder containing the IVSI-110 locus the transgene was medially located on chromosome 1 (Fig. 2A).

**Genotype determination** - To determine the degree of hematological complementation of the \( \nu \beta \)-globin locus in a thalassemic environment, transgenic mice were bred onto a \( \mu \beta \text{th}^{3+} \) and a \( \mu \beta \text{th}^{3-} \) background. The transgenic mice were genotyped by multiplex PCR using genomic DNA. Three genes were amplified by PCR (Fig. 2B). The bottom band represents the \( \mu \beta \)-globin gene (198 bp), the middle band represents the HPRT KO cassette (315 bp) and the top band corresponds to the \( \nu \beta \)-globin transgene (447 bp). Transgenic mice on a \( \mu \beta \text{th}^{3+} \) background were obtained for both transgenic lines, whereas...
viable transgenic mice on a \( \beta_{th-3/+} \) could only be generated with the normal \( \beta_{th-3} \) mouse, with the mice displaying normal hematological parameters as measured by FBE. We were unable to identify any IVSI-110 transgenic on a \( \beta_{th-3} \) background at birth indicating that the level of globin expression is insufficient to support fetal growth to birth. This is due to the low level of \( \beta_{th} \)-globin expression caused by the IVSI-110 mutation.

**Transgene copy number determination** - Two transgenic lines were analysed by quantitative radioactive PCR. Since the human and the mouse globin genes are very similar at the DNA sequence, the primers were designed to amplify both the \( \beta_{th} \)-globin and \( \beta_{mu} \)-globin genes. However, only the amplified mouse PCR product contains an internal \( \text{Necl} \) I restriction site, which was used to differentiate between the mouse and human-specific \( \beta \)-globin PCR product. The PCR products generated using the HM primers set were digested with \( \text{Necl} \) I and separated by agarose gel electrophoresis (Fig. 2C). The two lower bands represent the two mouse-specific products, 125 and 242 bp while the top band represents the undigested \( \beta_{mu} \)-globin gene, 381 bp. As expected, the wild type \( \text{Necl} \) I digested mouse control PCR produced two mouse-specific PCR products, while the human control PCR reaction produced one human-specific PCR product. The relative intensity of \( \beta_{mu} \)-globin-specific PCR product was compared to the \( \beta_{mu} \)-globin-specific PCR products on a wild type background (which contains four adult \( \beta \)-globin genes) and on a \( \beta_{mu} \)-thal specific PCR product (which contains two adult \( \beta \)-globin genes).

Using this approach, the transgene copy number was determined. Both transgenic lines contained two copies of the \( \beta_{mu} \)-globin locus (Fig. 2C).

**Expression of \( \beta_{mu} \)-globin in adult transgenic mice** - The transgenic lines containing the normal or IVSI-110 \( \beta_{mu} \)-globin locus were bred with \( \beta_{th-3/+} \) mice to generate mice hemizygous for the \( \beta_{mu} \)-globin locus on a \( \beta_{th-3/+} \) background. The expression level of \( \beta_{mu} \)-globin chain was 10% in wild type transgenic mice and 35% in \( \beta_{mu} \)-thal mice. Importantly, further breeding produced viable \( \beta_{mu} \)-th-3 mice expressing only the \( \beta_{mu} \)-globin locus (Fig. 3A), thus confirming that the 183 kb genomic fragment, containing the \( \beta_{mu} \)-globin locus, can fully complement \( \beta_{th-3/+} \) mice.

Measurement of globin chain synthesis in the humanized IVSI-110 mouse on wild type background was below measurable levels as seen by HPLC (data not shown), however \( \beta_{mu} \)-globin chain synthesis by the humanized IVSI-110 mouse on \( \beta_{th-3/+} \) background expressed \( \beta_{mu} \)-globin chain at ~3% relative to \( \alpha \)-globin chain.

It was noted that the level of \( \beta_{mu} \)-globin chain synthesis in the humanized IVSI-110 transgenic mouse was ~90% lower compared to \( \beta_{mu} \)-globin chain synthesis in the humanized normal \( \beta_{mu} \)-globin transgenic mouse on a similar background. These results suggest that this mouse model recapitulates accurately the \( \beta \)-globin gene defect that is typical for IVSI-110 in \( \beta \)-thalassemia patients.

In addition, there was a significant increase in the level of \( \beta_{mu} \)-globin chain in the \( \beta_{th-3/+} \) mice when compared to wild type transgenic mice as detected by HPLC globin chain separation and cellulose acetate gel electrophoresis (Fig. 3). This is thought to be due to post translational events, where reduced synthesis of \( \beta_{mu} \)-globin chain in the \( \beta_{th-3/+} \) mouse results in reduced competitive binding between the \( \beta_{mu} \)-globin chain for \( \alpha \)-globin resulting in an increase in the level of chimeric hemoglobin.

**Detection of aberrant splicing** - Splicing of \( \beta_{mu} \)-globin pre-mRNA in the humanized IVSI-110 mouse model was examined. RT-PCR was performed on RNA derived from whole blood, BM and spleen from the humanized IVSI-110 mouse model and compared with RNA derived from blood isolated from a homozygous IVSI-110 \( \beta \)-thalassemia patients (Fig. 4). PCR primers specific for the \( \beta_{mu} \)-globin gene were used to amplify the correctly spliced \( \beta_{mu} \)-globin mRNA spanning the 3’ end of exon 1 to the 5’ end of exon 2 regions. As a control we used transgenic mouse containing the normal \( \beta_{mu} \)-globin (Fig. 4).

As determined by RT-PCR transgenic mice that containing the IVSI-110 \( \beta \)-globin locus displayed one aberrantly spliced product. Detailed sequence analysis of cloned BM RT-PCR products, revealed that the +110 mutation was recognized as the expected 3’ splice site. This aberrant splice product was identified in the BM, spleen and blood of mice as well as in the blood of IVSI-110 \( \beta \)-thalassemia patients.
We noted quantitative differences in the amount of aberrant RT-PCR splice products amplified by PCR. In the BM and spleen, +110 splice product was present in greater amounts compared with correctly spliced β-globin mRNA, while in the blood of mice and IVSI-110 β-thalassemia patients the normal splice product was the predominate mRNA species. Presumably the difference in relative mRNA levels is due to mRNA instability.

We compared the strength of the authentic and aberrant splice sites using the Sapiro and Senapathy (S&S) matrix (26). The +110 3′ss scored 80.1, while the natural 3′ss scored 84.5 (Fig. 4). While the +110 3′ss scored lower than the natural 3′ss, it is not clear how the spliceosome can selectively discriminate against the authentic 3′ss in preference for the weaker aberrant splice site.

There are several studies, which report that RNA sequence elements bound by serine/arginine-rich (SR) family of proteins promotes the use of 5′ and 3′ splice sites through protein-protein and protein-RNA interactions. SR proteins can be either splicing activators or repressors, depending on where they bind to the pre-mRNA. Intrinsic binding of SR proteins has previously been reported to inhibit splicing (27). Since the IVSI-110 mutation promoted intrinsic aberrant splicing, the exonic splice enhancer algorithm ESEfinder was used to investigate whether the IVSI-110 mutation interfered with four putative SR splicing factors; SR protein SF2/ASF, SC35, SRp40 and SRp55 (28). Although other SR protein-binding sites have been reported the ESEfinder predicted that the IVSI-110 mutation disrupted an SRp40 binding motif. At this stage, we can only speculate that the IVSI-110 mutation may have disrupted the activity of an SR protein repressor.

Full blood examination - Full blood examination was performed on transgenic mice carrying the normal or IVSI-110 huβ-globin locus, including RBC count, total hemoglobin, hematocrit, erythrocyte indices, and reticulocyte counts. A summary of the results is shown in Table 1. The mβth-3/+ mice were found to be anemic, as reflected in the marked decrease in RBCs, hematocrit, and hemoglobin concentration. The reticulocyte counts in mβth-3/+ mice were significantly higher when compared with wild type mice, indicating active erythropoiesis. When the mβth-3/+ mice carry the normal huβ-globin locus, the levels of RBCs, hematocrit, and hemoglobin concentration increase to levels similar to wild type mice. We also noted that reticulocyte counts of mβth-3/+ mice carrying the huβ-globin locus decreased approaching wild type levels (Table 1). The low levels of huβ-globin chain synthesis by the humanized IVSI-110 transgenic mouse failed to complement the hematological abnormalities in full. However, the small amount of huβ-globin chain synthesis (~3%) by the humanized IVSI-110 mouse generated a small but measurable improvement in hematological values, which were statistically significant (Table 1).

DISCUSSION

Transgenic mice containing large genomic fragments are increasingly being developed, as in vivo model systems to facilitate our understanding of the biology and etiology of human diseases. With most of the human genome available as BAC clones, humanized BAC/YAC transgenic mice are increasingly the in vivo model systems of choice to map regulatory regions and to unravel the molecular mechanisms underlying developmental regulation and expression of functional genomic loci (2-6). With the growing demand for more sophisticated murine models to emulate human disease, BAC transgenics in combination with other gene knockout technologies are seen as important experimental tools for disease modelling.

In this study, we demonstrate that mice transgenic for the human β-globin locus, with the IVSI-110 G to A mutation produce the same aberrant spliced product found in IVSI-110 β-thalassemia patients. We noted quantitative differences in the level of aberrant +110 RT-PCR splice product between bone marrow and peripheral blood. While the bone marrow sample showed more aberrant +110 RT-PCR splice product relative to normal β-globin, the blood samples derived from IVSI-110 transgenic mice and IVSI-110 β-thalassemia patients exhibited the reverse. We attribute the difference in relative ratios of RT-PCR products to mRNA instability. The +110 aberrant splice product generates a premature stop codon, which is known to trigger the nonsense-mediated mRNA decay process (NMD). This pathway degrades mRNAs containing premature termination codons generated by point mutations and/or splicing alterations, whose
translation product may be detrimental to the cell (29).

Although large genomic transgenes are likely to contain all the necessary regulatory elements required for tissue-specific and position-independent expression, mouse models using such transgenes do not always mirror the expression of the endogenous gene in humans (25,30). The exact reason for this is not clear, but it may reflect the level of transcriptional compatibility between the two species.

We next investigated $\mu^\beta_{\text{th}-3/4}$ carrying two copies of the IVSI-110 $\beta$-globin locus ($\mu^\beta_{\text{th}-3/4}$, IVSI-110$^{+/0}$). Human $\beta$-globin chain synthesis was found to be ~3% relative to total $\beta$-globin chain synthesis. The low level of $\nu^\beta$-globin chain synthesis by the $\mu^\beta_{\text{th}-3/4}$, IVSI-110$^{+/0}$ mouse failed to complement the hematological abnormalities. However, the small amount of $\nu^\beta$-globin chain synthesis produced a small but measurable improvement in hematological values. When we compared the level of $\nu^\beta$-globin chain synthesis between the normal and IVSI-110 $\nu^\beta$-globin locus transgenic mice, the IVSI-110 mutation produced a 90% reduction in human $\beta$-globin synthesis. Interestingly, this 90% reduction of $\nu^\beta$-globin synthesis in mice is equivalent to the reduction of $\nu^\beta$-globin chain observed in IVSI-110 $\beta$-thalassemia patients.

Despite the many mutations causing alternative splicing identified to date, the reasons why mutations vary from mild to severe in their effects are poorly understood. Splicing relies on the correct recognition of cis-elements (the 5’ and 3’ splice sites and the branch point), but these are not sufficient to define exon-intron boundaries. Additional cis-acting elements such as exonic or intronic splicing enhancers and silencers significantly influence normal and alternative splice site selection. These elements are recognized by trans-acting factors, including the SR proteins and huRNPs (27). We can only speculate that the IVSI-110 mutation may have disrupted the binding of an intronic silencer, reducing exon definition and allowing the recruitment of the splicing machinery to upstream aberrant 3’ splice sites.

Kole and colleagues were the first to attempt therapeutic modulation of alternative splicing by targeting antisense oligonucleotides (AO) to the aberrant splice site to restore splicing specificity. This strategy prevents the selection of the aberrant splice site and thus favouring the recognition of the correct splice site by the splicing machinery. By using in vitro and cellular assays, they demonstrated inhibition of aberrant splicing in two common $\beta$-thalassemia mutations, IVSII-654 and IVSII-705 (31,32). In the case of the IVSII-110 mutation, efficient inhibition of aberrant splicing was achieved not by targeting the aberrant splice site, but the natural branch point sequence of the first intron (32). More recently, AO have been shown to correct aberrant splicing in vivo using a transgenic mouse model in which EGFP expression was interrupted by an intron containing IVSII-654 splicing mutation (33). Systemic delivery of AO restored EGFP expression in all tissues that took up AO, providing evidence that AO can be used in vivo to restore normal splicing. They found correction of aberrant splicing was variable for different tissues, but this may reflect the levels of transgene expression. Although this animal model represents an excellent in vivo model system for the investigation of delivery and activity of various AO chemistries, it is not clinically relevant with respect to anemia and extramedullary erythropoiesis.

An alternative approach to restore splicing specificity, lost through mutations, would be the use of bifunctional AO’s. By combining an antisense-targeting domain and peptide-recruiting domain it may be possible to recruit specific splicing factors that might influence splicing in a positive or negative manner. This approach has recently been shown to specifically restore wild type splicing when directed to defective BRACA1 or SMN2 pre-mRNA (34,35).

In this study, we show that this humanized IVSII-110 mouse model recapitulates the splicing defect that is typical for $\beta$-thalassemia patients carrying the IVSII-110 mutation. We show that the humanized transgenic $\mu^\beta_{\text{th}-3/4}$, carrying the IVSII-110 $\nu^\beta$-globin locus, represents a physiologically relevant in vivo model system, which can provide a unique opportunity for the development and evaluation of various therapeutic strategies such as AO and pharmacological-based therapies.
REFERENCES


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FIGURE LEGENDS

Fig. 1. The map of the ^5^μβ-globin locus contained in a 183 kb genomic fragment. (A) The 183 kb fragment contains seven olfactory receptor (OR) genes upstream of the LCR and the hypersensitive site 5'HS-111, located approximately 111 kb upstream of the e-promoter. Cfr9I restriction sites are indicated by “C” and the SfiI restriction sites are indicated by “S”. A 36 kb fragment is released after Cfr9I digestion, containing the ^5^γ-^7^γ-, ^δ^ and ^β^-globin genes. (B) Southern blot mapping of transgenic mice containing the normal ^5^β-globin locus (left panel) and the IVSI-110 ^5^β-globin locus transgenic mice. (C) Schematic diagram indicating transgene copy number and proposed orientation corresponding to the normal (top) and IVSI-110 (bottom) ^5^β-globin locus.

Fig. 2. Characterisation of transgenic lines containing the ^5^β-globin locus. (A) FISH analysis of a transgenic mouse carrying the normal ^5^β-globin locus (top panel): the probe (red) has hybridized to the inserted DNA proximally on chromosome 1; transgenic mouse carrying the IVSI-110 ^5^β-globin locus (bottom panel): the probe (red) has hybridized to the inserted DNA medially on chromosome 14. (B) Genotyping analyses of transgenic mice by multiplex PCR. Lane 1, molecular weight marker X (Roche); lane 2, wild type C57BL/6; lane 3, heterozygous β-globin knockout mouse (^5^β-th-3/+); lane 4, transgenic mouse.
containing the \( ^{hb} \beta \)-globin locus on a wild type background (\( ^{hb} \beta^{+/-}, ^{mub}\beta^{+/-} \)); lane 5, hemizygous transgenic mouse on a heterozygous knockout background (\( ^{hb} \beta^{+/-}, ^{mub}\beta^{+/0, th-3/+} \)); lane 6, transgenic mouse on a homozygous knockout background (\( ^{hb} \beta^{+/-}, ^{mub}\beta^{+/0, th-3/-} \)). (C) Transgene copy number determination by quantitative multiplex PCR. T, transgenic mice on a wild type background (\( ^{hb} \beta^{+/-}, ^{mub}\beta^{+/-} \); DH, hemizygous transgenic mice on a heterozygous knockout background (\( ^{hb} \beta^{+/-}, ^{mub}\beta^{+/0, th-3/+} \)). The transgene copy number was determined for each transgenic line as indicated.

**Fig. 3.** Analysis of \( ^{hb} \beta \)-globin gene expression in transgenic mice. (A) HPLC globin chains separation of (i) wild type mouse, (ii) wild type mouse carrying normal \( ^{hb} \beta \)-globin locus, (iii) \( ^{mub}\beta^{+/-} \) mice carrying the normal \( ^{hb} \beta \)-globin locus, (iv) \( ^{mub}\beta^{th-3/+} \) mice carrying the normal \( ^{hb} \beta \)-globin locus, (v) \( ^{mub}\beta^{+/-} \) mice carrying the IVSI-110 \( \beta \)-globin locus. The percentage of each globin chain expressed in transgenic mice is also indicated. (B) Cellulose acetate gel electrophoresis. Lane 1, wild type mouse Hb; lanes 2 and 3, \( ^{mub}\beta^{th-3/+} \) mice carrying the normal \( ^{hb} \beta \)-globin locus; lanes 4 and 5, \( ^{mub}\beta^{th-3/-} \) mice carrying the normal \( ^{hb} \beta \)-globin locus; lane 6 and lane 7, \( ^{mub}\beta^{th-3/+} \) mouse carrying the IVSI-110 \( \beta \)-globin locus.

**Fig. 4.** Comparison of normal and aberrant spliced \( \beta \)-globin mRNA by RT-PCR. Resolution of normal and aberrant \( ^{hb} \beta \)-globin RT-PCR products derived from mice and IVSI-110 \( \beta \)-thalassemia patients were performed on 2% Nusieve GTG agarose. Lane 1, Bone marrow from transgenic mouse carrying the normal \( ^{hb} \beta \)-globin locus; lane 2, bone marrow from the IVSI-110 transgenic mice; lane 3, blood sample from the IVSI-110 transgenic mice; lane 4, blood sample from a human IVSI-110 \( \beta \)-thalassemia patient. (B) Schematic representation of the \( ^{hb} \)-globin exonic and intronic regions. Capital letters represent exon sequence and lower case letters correspond to intron sequence. The strength of the 5' splice site and 3' splice site was determined using the Shapiro and Senapathy (S&S) consensus matrix. The S&S values of the natural donor and acceptor sites sites as well as the 3' IVSI-110 aberrant splice site are shown. (C) Sequence of cloned RT-PCR products derived from the BM of the IVSI-110 transgenic mice.

**Table 1:** Full blood examination

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hb (g/dL)</th>
<th>RBC (10^6/µl)</th>
<th>MCV (fl)</th>
<th>MCH (pg/rbc)</th>
<th>RDW</th>
<th>HCT (%)</th>
<th>RET (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (C57BL/6)</td>
<td>15.0±1.0</td>
<td>9.4±2.0</td>
<td>48.7±1.2</td>
<td>15.3±0.6</td>
<td>13.5±1.1</td>
<td>48.5±2.7</td>
<td>2.6±1.5</td>
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<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( ^{mub}\beta^{th-3/+} ) (n=10)</td>
<td>7.8±0.2</td>
<td>5.4±0.4</td>
<td>41.7±3.0</td>
<td>14.6±0.8</td>
<td>34.1±1.0</td>
<td>22.2±1.3</td>
<td>8.7±3.9</td>
</tr>
<tr>
<td>( ^{hb} \beta^{+/-}, ^{mub}\beta^{th-3/+} ) (n=8)</td>
<td>15.5±0.9</td>
<td>9.7±0.2</td>
<td>53.3±1.4</td>
<td>16.3±1.1</td>
<td>16.1±1.1</td>
<td>50.9±0.9</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>IVSI-110( ^{b}_{\beta}, ^{mub}\beta^{th-3/+} ), (n=8)</td>
<td>8.6±0.6</td>
<td>5.5±0.6</td>
<td>44.2±1.3</td>
<td>15.6±0.8</td>
<td>32.7±0.8</td>
<td>24.4±2.3</td>
<td>9.3±3.0</td>
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**Student’s t-Test**

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<tr>
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<th>( P=0.001 )</th>
<th>NS</th>
<th>( P=0.04 )</th>
<th>( P=0.04 )</th>
<th>( P=0.002 )</th>
<th>( P=0.02 )</th>
<th>NS</th>
</tr>
</thead>
</table>

Hemoglobin concentration (Hb), red blood cell counts (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), hematocrit (HCT), and reticulocytes (RET) are shown. The hematological values are expressed as means ± S.D. The differences between the \( ^{mub}\beta^{th-3/+} \) mice and the IVSI-110\( ^{b}_{\beta}, ^{mub}\beta^{th-3/+} \), are statistically significant as measured by Student’s t-test. NS corresponds to not statistically significant (\( P>0.05 \)).
Figure 1

A

Chromosome 11p15

5' HS-111

OR7 OR6 OR5 OR4 OR3 OR2 OR1 LTR

C C C S S C C C

γδ βψβ

5' HS5-HS1

ε γ λ β δ

Centromeric

Telomeric

20 40 60 80 100 120 140 160 180 (kb)

B

Cfr9 Sfr I

158 kb

36 kb

Normal locus

IFSI-110 locus

C

158 kb

tail to tail orientation

175 kb 140 kb

head to tail orientation

5' 3' 5' 3'

5' 3' 5' 3'
Figure 2

A

Normal locus

IVS1-110 locus

B

C

<table>
<thead>
<tr>
<th>Normal locus</th>
<th>IVS1-110 locus</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>huβ</td>
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<tr>
<td>muβmajor</td>
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</tr>
<tr>
<td>muβminor</td>
<td></td>
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</tr>
<tr>
<td>DH</td>
<td>DH</td>
<td>T</td>
</tr>
<tr>
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<tr>
<td>2</td>
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<td></td>
</tr>
</tbody>
</table>

Copy No.
Figure 3

A

(i) \( \text{mu}_\alpha \rightarrow \text{mu}_\beta \)

(ii) \( \text{hu}_\beta \) (10%)

(iii) \( \text{hu}_\beta \) (35%)

(iv) \( \text{hu}_\beta \) \& \( \text{hu}_\delta \)

B

1 2 3 4 5 6 7

mu_\alpha \text{hu}_\delta
mu_\alpha_2 \text{mu}_\beta
mu_\alpha_2 \text{mu}_\delta
mu_\alpha \text{hu} \beta_2

IVS1-110 locus

(v) \( \text{hu}_\beta \) (3%)
Figure 4

A

Transgenic
Normal
BL
Transgenic
IVS I-110
SP
BM
BL
Human
IVS I-110
BL

I VS1-110 189 nt (+19 nt)
Normal mRNA 170nt

B

Exon 1

+1
SS=80.1

Exon 2

+110
SS=80.1 (G to A)

Exon 3

+131
SS=84.5

C

Normal
GTGTTGTTGAGGCCCCTGGGCAG
VGEALS

Aberrant
GTGTTGTTGAGGCCCCTGGGCAG
VGEALS

IVS1-110 189 nt (+19 nt)
Normal mRNA 170nt

SS=80.1

110
SS=80.1 (G to A)

131
SS=84.5

GTGCTGG
VGEALS

gtt
tagtc

+1
SS=80.1

+110
SS=80.1 (G to A)

+131
SS=84.5

GTTGGTGAGGCCCTGGGCAG----------------------------------tctattttcccacccttagGCTGCTGG
VGEALS

GTTGGTGAGGCCCTGGGCAG----------------------------------tctattttcccacccttagGCTGCTGG
VGEALS

Normal
Aberrant

Normal

Aberrant

VGEALS

VGEALS

VGEALS

VGEALS

VGEALS

VGEALS

VGEALS

VGEALS
Humanized β-thalassemia mouse model containing the common IVSI-110 splicing mutation
Jim Vadolas, Mikhail Nefedov, Hady Wardan, Sima Mansooriderakshan, Lucille Vouillaire, Duangporn Jamsai, Robert Williamson and Panayiotis A. Ioannou

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