Original Research

Generation of non-deletional hereditary persistence of fetal hemoglobin β-globin locus yeast artificial chromosome transgenic mouse models: -175 Black HPFH and -195 Brazilian HPFH

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Abstract

Fetal hemoglobin is a major genetic modifier of the phenotypic heterogeneity in patients with sickle cell disease and certain β-thalassemias. Normal levels of fetal hemoglobin postnatally are approximately 1% of total hemoglobin. Patients who have hereditary persistence of fetal hemoglobin, characterized by elevated synthesis of γ-globin in adulthood, show reduced disease pathophysiology. Hereditary persistence of fetal hemoglobin is caused by β-globin locus deletions (deletional hereditary persistence of fetal hemoglobin) or γ-globin gene promoter point mutations (non-deletional hereditary persistence of fetal hemoglobin). Current research has focused on elucidating the pathways involved in the maintenance/reactivation of γ-globin in adult life. To better understand these pathways, we generated new β-globin locus yeast artificial chromosome transgenic mice bearing the ^{A}γ-globin -175 T > C or -195 C > G hereditary persistence of fetal hemoglobin mutations to model naturally occurring hereditary persistence of fetal hemoglobin. Adult -175 and -195 mutant β-YAC mice displayed a hereditary persistence of fetal hemoglobin phenotype, as measured at the mRNA and protein levels. The molecular basis for these phenotypes was examined by chromatin immunoprecipitation of transcription factor/co-factor binding, including YY1, PAX1, TAL1, LMO2, and LDB1. In -175 HPFH versus wild-type samples, the occupancy of LMO2, TAL1 and LDB1 proteins was enriched in HPFH mice (5.8-fold, 5.2-fold and 2.7-fold, respectively), a result that concurs with a recent study in cell lines showing that these proteins form a complex with GATA-1 to mediate long-range interactions between the locus control region and the ^{A}γ-globin gene. Both hereditary persistence of fetal hemoglobin mutations result in a gain of ^{A}γ-globin activation, in contrast to other hereditary persistence of fetal hemoglobin mutations that result in a loss of repression. The mice provide additional tools to study γ-globin gene expression and may reveal new targets for selectively activating fetal hemoglobin.

Keywords: Globin gene, sickle cell disease, hemoglobinopathies, HPFH, fetal hemoglobin, transgenic mice

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Introduction

Fetal hemoglobin (HbF) is the major genetic modifier of phenotypic heterogeneity in patients with the β-globin chain disorders like sickle cell disease (SCD) and certain β-thalassemias. Continued expression of γ-globin chains results in increased HbF levels, which acts to replace missing normal adult hemoglobin (HbA) resulting from mutated or absent β-globin chains in these two β-hemoglobinopathies, respectively.[1] In newborns, the HbF level comprises 60 to 80% of total hemoglobin; however, the level gradually decreases to approximately 1% between 6 and 12 months of age. The β-like chain component of this hemoglobin switching is characterized by the progressive silencing of the *HBG1* (^{A}γ-globin) and *HBG2* (^{G}γ-globin) genes with concomitant activation of *HBB* (β-globin), and to a lesser extent *HBD* (δ-globin), gene expression in bone marrow.[2] Although the normal level of HbF postnatally is approximately 1% of total hemoglobin, some individuals have a condition known as hereditary persistence of fetal

hemoglobin (HPFH), marked by elevated synthesis of γ-globin chains in adulthood.[3,4] HPFH is classified as one of two types: (1) deletional HPFH or (2) non-deletional HPFH. Deletional HPFH is characterized by large deletions (13 to 106 kb), which may include partial or full deletion of the *HBD* and *HBB* genes. Increased *HBG* gene expression and thus, higher HbF levels, are associated with variable compensation for the partial or total lack of *HBD* and/or *HBB* gene expression. Non-deletional HPFH is a complex inherited trait caused by inheritance of one of the three major quantitative trait loci (QTL), the *Xmn*I site upstream of the *HBG2* gene (chromosome 11p15), BCL11A (chromosome 2p16), or the HBS1L-MYB intergenic polymorphism (HMIP, chromosome 6q23), or by a Mendelian inherited trait, caused by point mutations and minor deletions in the promoter regions of the *HBG* genes.[5]

Pharmacological agents such as butyrate, decitabine, and hydroxyurea are effective in inducing HbF *in vitro* and *in vivo*. To date, hydroxyurea is the only drug approved for clinical use in sickle cell patients, although the efficacy level is variable between patients and the long-term effects of this drug remain uncertain.[6,7] Therefore, current research has focused on elucidating the pathways involved in the maintenance/reactivation of γ-globin gene expression in adult life. Heterozygous individuals for the Mendelian non-deletional HPFHs have HbF levels varying from 5 to 40%, but the mechanism that leads to the increased HbF expression has not been totally unveiled. The most likely hypothesis is that the point mutations or minor deletions in the γ-globin gene promoter can alter the binding sites for transcription factors that regulate *HBG1* and *HBG2* gene expression. Many studies have demonstrated the role of ubiquitous or stage-specific transcription factors in β-like globin gene switching, indicating their potential as therapeutic targets in the treatment of β-hemoglobinopathies. The transcription factor B-cell chronic lymphocytic leukemia (CLL)/lymphoma 11A (BCL11A) was shown to function as a repressor of HbF expression, interacting with numerous co-repressors in the β-globin locus leading to reconfiguration of the chromatin in this region.[1,8] When Krüppel-like factor 1 (KLF1), an *HBB*-specific transcriptional activator, was knocked down in $CD34^+$ cells, γ-globin expression was induced.[9] Moreover, many mutations in the KLF1 gene were associated with increased levels of HbF.[10,11] Since KLF1 is also a direct activator of BCL11A, it may play a crucial role in the switch from *HBG* to *HBB* expression, by direct activation of *HBB* and indirect silencing of the γ-globin genes via activation of BCL11A.[9] Forced expression of orphan nuclear receptor proteins TR2/TR4 enhanced HbF expression in adult erythroid cells and ameliorated many of the pathophysiological characteristics of SCD in humanized SCD mice.[12] In addition, many HPFH mutations directly or indirectly affect the binding of transcription factors. For example, GATA-1 is a constituent of the repressor complex GATA-1-FOG-1-Mi2β that binds at the -567 ^{G}γ/-566 ^{A}γ-globin GATA motifs.[13–15] Point mutations (T > G transversions) at these sites alter the GATA-1 binding motifs, increasing HbF levels *in vitro* and *in vivo*. The -567 ^{G}γ-globin transversion was associated with HPFH in two members of an Iranian-American family.[15]

To better understand the molecular pathways involved in regulating γ-globin gene expression, we generated two new mutant β-globin locus yeast artificial chromosome (β-YAC) transgenic mouse models: (1) ^{A}γ-globin -175 T > C Black HPFH β-YAC mice and (2) ^{A}γ-globin -195 C > G Brazilian HPFH β-YAC mice. This study describes the phenotypic characterization of these lines, as well as some basic insight into the molecular mechanisms underlying these HPFHs. Inclusion of these models with previously characterized wild-type β-YAC mice and mutant ^{A}γ-globin -117 G > A Greek HPFH β-YAC mice as controls, both in the data presented here and in future studies, will more comprehensively demarcate the complex mechanics of γ-globin gene silencing and reveal potential targets for therapeutic manipulation of HbF expression, thus leading to effective treatments for SCD and certain β-thalassemias.

Material and methods

-175 and -195 ^{A}γ-globin HPFH β-YAC transgenic mice

β-YAC transgenic mice were generated essentially as described[14] using a 213 kb β-YAC containing the human β-globin locus. Briefly, yeast-integrating plasmid (YIP) pRS406 containing a 5.4 kb marked ^{A}γ-globin gene ($^{A}\gamma^{m}$) *Ssp*I fragment (GenBank file U01317 coordinates 38,683–44,077) was mutagenized using the Quick Change Site-Specific Mutagenesis Kit (Stratagene, La Jolla, CA, USA) to introduce the ^{A}γ-globin -175 T > C Black HPFH or the -195 C > G Brazilian HPFH mutations. The presence of the mutations was confirmed by DNA sequence analysis of a PCR-amplified fragment encompassing the mutated region. Transformation of wild-type β-YAC-bearing yeast, ''pop-in'', ''pop-out'' homologous recombination in yeast, screening of positive yeast clones, YAC purification, mouse transgenesis, and structural analysis/copy number determination of YAC transgenes was performed as described previously.[14]

Real-time quantitative RT-PCR (qRT-PCR)

β-like globin gene expression was measured as previously described.[14] In summary, total RNA was isolated from transgenic mouse adult blood using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Seven hundred nanograms of total RNA was used to generate cDNA using the iScript Reverse Transcription Supermix RT-qPCR kit (Bio-Rad, Hercules, CA, USA). PCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using a CFX96 real-time PCR detection system (Bio-Rad) to assess the expression of the murine α-globin genes, and human β- and γ-globin genes. Three sets of cDNA for duplicate samples were amplified, and the $\Delta\Delta C_T$ method was used to calculate the relative expression of the target genes. The expression of the human β- and γ-globin genes was normalized to expression of the murine α-globin genes. Results are shown as fold increase relative to wild-type β-YAC samples.

RNase protection assay (RPA)

RNase protection assay (RPA) reactions were carried out essentially as described.[16] Data include the means and standard deviations from three separate experiments, each of which included total RNA from the adult blood of two individual animals for each transgenic line.

Flow cytometry

Flow cytometry was carried out as previously described with some modifications.[14] Blood was collected from the tail vein of mice in phosphate-buffered saline/ethylenediaminetetraacetic acid (PBS/EDTA) and washed by adding 10 μL to 990 μL PBS and centrifuging at 200 × *g* for 5 min at 4°C in a swinging bucket rotor. Pellets were resuspended in 4% paraformaldehyde, incubated at room temperature for 30 min, followed by centrifugation. The pellet was resuspended in 1 mL ice-cold acetone/methanol (4:1), incubated on ice for 1 min, and centrifuged. Pellets were washed twice in PBS/0.1% BSA (PB) and blocked in 50 μL PBT (PB containing 0.1% triton X 100) containing 40 μg/mL normal goat immunoglobulin G (IgG) (sc-2028, Santa Cruz Biotechnology, Saint Louis, MO, USA) at room temperature for 30 min. Cells were washed twice in PB and resuspended in PBT containing 0.5 μg sheep anti-human hemoglobin F conjugated with fluorescein isothiocyanate (FITC) (A80-136A, Bethyl Laboratories, Montgomery, TX, USA) for each sample. A sample lacking primary antibody was included as a negative control. Cells were incubated 30 min at room temperature, washed twice in PB, resuspended in 400 μL PB, and analyzed using a BD LSRII Flow Cytometer (BD Biosciences, San Jose, CA, USA) with a 530/30 nm (FITC/GFP) emission filter. Data from 30,000 events were acquired for analysis using BD FACS Diva software (BD Biosciences).

Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

Mouse hemoglobin samples were prepared according to Masala and Manca[17] Briefly, each 30 μL sample was treated with anticoagulant (3 μL 50 mM EDTA, pH 8.0). Red blood cells (RBCs) were collected by centrifugation (250 × g, 10 min, 4°C). The pellet, comprising packed RBCs, was washed three times with 120 μL aliquots of 0.9% (w/v) NaCl before lysis by vortexing (10 s pulse) in 30 μL of deionized water and 12 μL of neat CCl_4. The hemoglobin-bearing aqueous phase was collected by centrifugation (20,000 × *g*, 10 min, 4°C) and analyzed by liquid chromatography-mass spectrometry (LC-MS) within 24 h of extraction. During the interval between extraction and analysis, hemoglobin samples were stored at 4°C. All samples were filtered (Millipore 25 mm, 0.45 μm Millex-LCR hydrophilic PTFE filter, EMD Millipore, Billerica, MA, USA) prior to analysis. Total protein concentration in the clarified cell lysates was determined using a Bio-Rad Quick Start Braford Protein assay kit according to manufacturer's instructions. These data were used to insure that the efficiency of hemoglobin extraction was not attenuated by variations in the ratio of reagent to sample.

High-pressure liquid chromatography (HPLC)-based analysis was carried out using an Acquity Classic UPLC System (Waters, Milford, MA, USA). Mouse hemoglobin samples and human hemoglobin instrument qualification standards (10 μl) were initially loaded in 95% solvent A (0.05% trifluoroacetic acid (TFA) in deionized water), 5% solvent B (0.05% TFA in 7:3 acetonitrile:isopropanol) onto a Sigma-Aldrich Supelco BioShell C4 (2.1 mm × 150 mm, 400 Å pores, 3.4 μm fused core particles) RP-HPLC column. The globin chains were resolved with a programmed gradient (500 μL/min) comprised of solvents A and B (95% A, 5% B from 0–4 min, 65% A, 35% B from 4–19 min, 50% A, 50% B from 19–19.5 min, 100% B from 19.5–22 min, 95% A, 5% B from 22–25 min). The effluent from the column was unevenly split (post-column) between a Waters Synapt HDMS hybrid time of flight mass spectrometer (150 μL/min), equipped with an electrospray source, and solvent waste (350 μL/min). The split was accomplished by back-pressure manipulation using tubing of varying length to control the flow through a high pressure (0.020 in) tee junction. The instrument was operated in sensitivity mode with the cone voltage ramped between 20 and 40 V. Positive ion spectra (1 s scans) were acquired over a mass range of 250–2500 m/z, while simultaneously collecting lock mass correction data (for postacquisition correction) using the Synapt HDMS auxiliary sprayer to introduce (1 s scans) leucine enkephalin (YGGFL) peptide every 30 s. The spectra of NaI cluster ions were collected under the same conditions and used to perform time to mass calibration.

Instrument function and data acquisition were controlled using Waters Mass Lynx 4.1 (SCN 872) software. The identity of the hemoglobin species residing within each peak was determined using the Mass Lynx MaxEnt 1 algorithm to deconvolute the multiply-charged ESI spectra associated with each peak-spectrum and to determine the zero-charge spectrum for the parent molecule that gave rise to the chromatographic peak. Quantification of each hemoglobin species was accomplished by manually integrating the area under the total ion current chromatographic peaks using Mass Lynx software.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described with some modifications.[14] Single-cell suspensions from postconception day E18 fetal livers from wild-type, -175 HPFH, and -195 HPFH β-YAC transgenic mice were prepared as described. Cells were incubated with 2 mM ethylene glycol bis[succinimidylsuccinate] (EGS) for 30 min. Cellular proteins and DNA were cross-linked by incubation in the presence of 1% fresh paraformaldehyde (10 min, room temperature) before the reaction was quenched by the addition of glycine to a final concentration of 125 mM. Chromatin was sonicated to a size range between 200 and 1000 bp and 20 μg of chromatin were used for each immunoprecipitation. Immunoprecipitations were carried out with specific antibodies against YY1 (sc-1703), PAX1 (sc-25407 X), GATA-1 (sc-265 X), TAL1 (sc-12984), LDB1 (sc-11198), from Santa

Cruz Biotechnology and LMO2 from R&D Systems (AF2726, Minneapolis, MN, USA) and control isotype-matched IgG antibodies (rabbit and goat from Santa Cruz Biotechnology) overnight at 4°C. The chromatin samples were incubated for 4 h at 4°C with 50 μL of Dynabeads Protein G (ThermoFisher Scientific, Grand Island, NY, USA). The immunoprecipitate was washed, the cross-links were reversed, and the genomic DNA was purified by the phenol:chloroform:isoamyl alcohol method. Recruitment of the transcription factors listed above was measured by real-time qPCR, using gene-specific primers for the γ-globin gene promoter region.[13]

Statistical analysis

Statistical analyses were performed with Prism version 5 software (GraphPad, La Jolla, CA, USA). Data presented are mean ± standard deviation (SD). Unpaired two-tailed *t*-test was used and P-values < 0.05 were considered significant.

Results

The -175 T > C and -195 C > G HPFH mutations elevate HbF levels in β-YAC transgenic mice

Three -175 HPFH and two -195 HPFH β-YAC transgenic mouse lines were produced with a 213 kb β-globin locus YAC, containing a 187 kb human chromosomal insert that encompassed the entire 82 kb β-globin locus from 5′HS5 of the locus control region (LCR) to 3′HS1, approximately 20 kb downstream from the β-globin gene (Figure 1). UPLC-MS analysis showed that -175 HPFH mice had the highest average level of γ-globin chains (mean of lines = 25.8% $\gamma/[\gamma+\beta]$), followed by -195 HPFH mice (mean of lines = 18.4%; Table 1). Wild-type β-YAC control mice did not express γ-globin, and -117 Greek HPFH β-YAC control mice displayed an average of 13.8%. Measurement of $^{A}\gamma$-globin mRNA by RPA(Figure 2) supported the HPLC data; $\gamma/(\gamma+\beta)$ was 34%, 12%, 14.1%, and 0% for -175 HPFH, -195 HPFH, -117 HPFH, and wild-type β-YAC animals, respectively (Table 1). Relative mRNA levels as determined by RT-qPCR were consistent with the RPA results (Table 1).

Flow cytometry was used to determine HbF distribution in adult RBCs (Figure 3). -175 and -195 HPFH mice exhibited heterocellular HbF distribution, whereas -117 HPFH control mice showed a pancellular distribution as previously documented.[18] These data are consistent with the phenotypes observed in human subjects, except for the –175 distribution, which is pancellular in patients.[3] The HbF levels also are consistent with those found in human patients, ranging between 36–41%, 4.5–16%, 12–16% and less than 1% in -175 T > C, -195 C > G and -117 G > A HPFH, and normal subjects, respectively.[3,19] Since our new HPFH models show γ-globin expression levels (transcript and chain) and HbF distribution patterns reflective of those found in the human population, they are useful tools for exploring fetal γ-globin gene regulatory mechanisms. Copy number-corrected human β- and γ-globin gene expression normalized to copy number-corrected mouse α-globin gene expression demonstrated that the resultant increase in γ-globin gene expression associated with the –175 and –195 HPFH mutations was accompanied by a concomitant β-globin decrease, supporting the competition model between γ- and β-globin for interaction with the LCR. The average values were 58.44% β, 21.01% γ for –175, 62.75% β, 15.53% γ for -195, and 74.00% β, 11.01% γ for –117, compared to 96.50% β, 0% γ for wild-type.

TAL1, LMO2 and LDB1 bind the -175 T > C gene promoter when HBG1 is activated in -175 HPFH β-YAC transgenic mice

Several studies have shown that regulation of β-like globin gene expression is mediated in part by a LDB1/LMO2/GATA-1/TAL1 complex, which promotes the interaction via looping between the gene promoters and the LCR.[20–25]

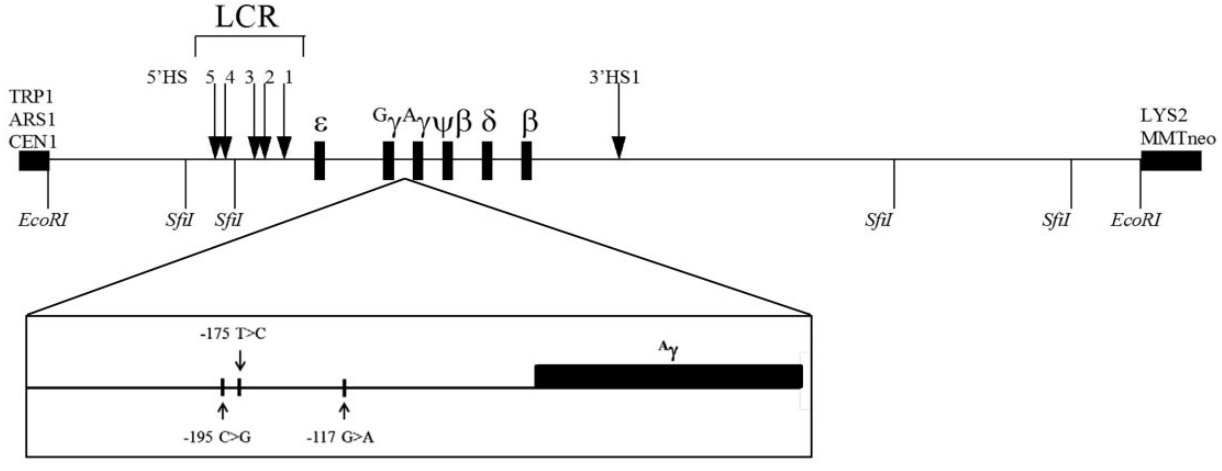

Figure 1 Human β-globin locus 213 kb yeast artificial chromosome (β-YAC). Upstream of the HBE gene (ε) is the locus control region (LCR), defined as a set of four erythroid specific and developmentally stable DNase I-hypersensitive sites (5' HS). Another developmentally stable HS, termed 3' HS1, is present ~20 kb 3' to the HBB gene (β). Also present in this construct are the yeast TRP1 gene, origin of replication, ARS1 (autonomously replicating sequence), a centromere, CEN1, the LYS2 gene, and a mammalian selectable marker cassette, MMTneo, encoding G418 resistance. Two restriction sites for *Eco*RI and four for *Sfi*I relevant to generation and structural characterization of this YAC are indicated below the line. In the expanded square below the YAC map, the position and nucleotide changes of the three HPFH mutations included in this study are shown

Table 1 Mean ± standard deviation for γ-globin chain (UPLC) and mRNA levels (RPA and RT-qPCR), in different lines of β-YAC mice, normalized to the total β-like globin chain or mRNA levels, respectively; $[\gamma/(\beta+\gamma)] \times 100$

		% γ/(γ + β)[a]		RPA		RT-qPCR
Mouse line	**Copy number**	**UPLC**	**RPA**	**Per copy γ-globin expression[b]**	**Per copy β-globin expression[b]**	**γ/α[c]**
−175 #4	2	26.39 ± 2.68	25.33 ± 2.52	13.23 ± 5.88	51.00 ± 19.18	448 ± 234
−175 #27	2	20.75 ± 0.88	34.67 ± 4.04	27.80 ± 7.33	79.15 ± 19.01	1323 ± 147
−175 #57	1	29.88 ± 2.22	42.00 ± 5.20	22.01 ± 7.59	45.18 ± 32.25	234 ± 20
−195 #9	3	15.91 ± 2.08	10.67 ± 1.82	10.19 ± 4.83	73.00 ± 25.71	245 ± 89
−195 #18	4	14.67 ± 0.22	13.38 ± 3.49	20.87 ± 10.07	52.50 ± 9.15	223 ± 32
−117 (control)	2	13.75 ± 0.85	14.05 ± 1.05	11.01 ± 3.08	74.00 ± 12.00	420 ± 76
Wild type β-YAC	3	0	0	0	96.50 ± 10.61	1 ± 0.11

Note: RT-qPCR values are normalized to the murine α-globin mRNA.
UPLC: ultra performance liquid chromatography, four experimental replicates; RPA: RNase protection assay, three experimental replicates; RT-qPCR: real time quantitative polymerase chain reaction, three experimental replicates.
[a]Mean ± standard deviation.
[b](Hu γ or β/β-YAC copy number)/(Mo α/4) × 100; mean ± standard deviation.
[c]Fold increase relative to wild-type β-YAC.

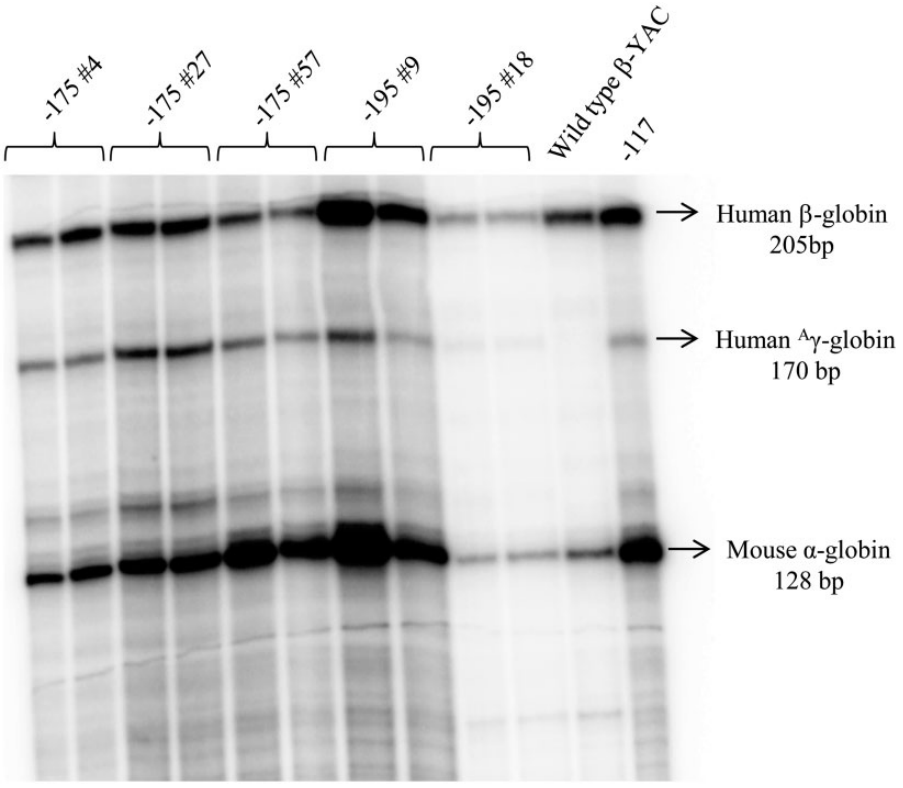

Figure 2 RPA results of the human β-globin and $^{A}\gamma$-globin and mouse α-globin mRNA in the -175 #4, -175 #27, -175 #57, -195 #9, and -195 #18 HPFH mouse lines compared to the wild-type β-YAC line (negative control) and the -117 line (positive control)

A recent study using cell lines demonstrated that the -175 HPFH mutation results in recruitment of part of this protein complex to the $^{A}\gamma$-globin gene promoter with concomitant gene activation.[26] The -175 HPFH T > C mutation results in the creation of an E-box motif, to which TAL1 binds. We performed ChIP using antibodies against these four transcription factors to verify that this complex is involved in γ-globin gene activation *in vivo* in -175 HPFH mutant mice. Postconception day E18 fetal liver is a developmental time point where γ-globin is normally silent; thus, it was chosen for our analysis. Our data indicate that the transcription factors TAL1, LMO2, and LDB1 showed a significant difference in occupancy ($P < 0.05$; Figure 4) of the *HBG1* gene promoter in the presence of the -175 C > T mutation compared to the control wild-type β-YAC mice and the IgG antibody control.

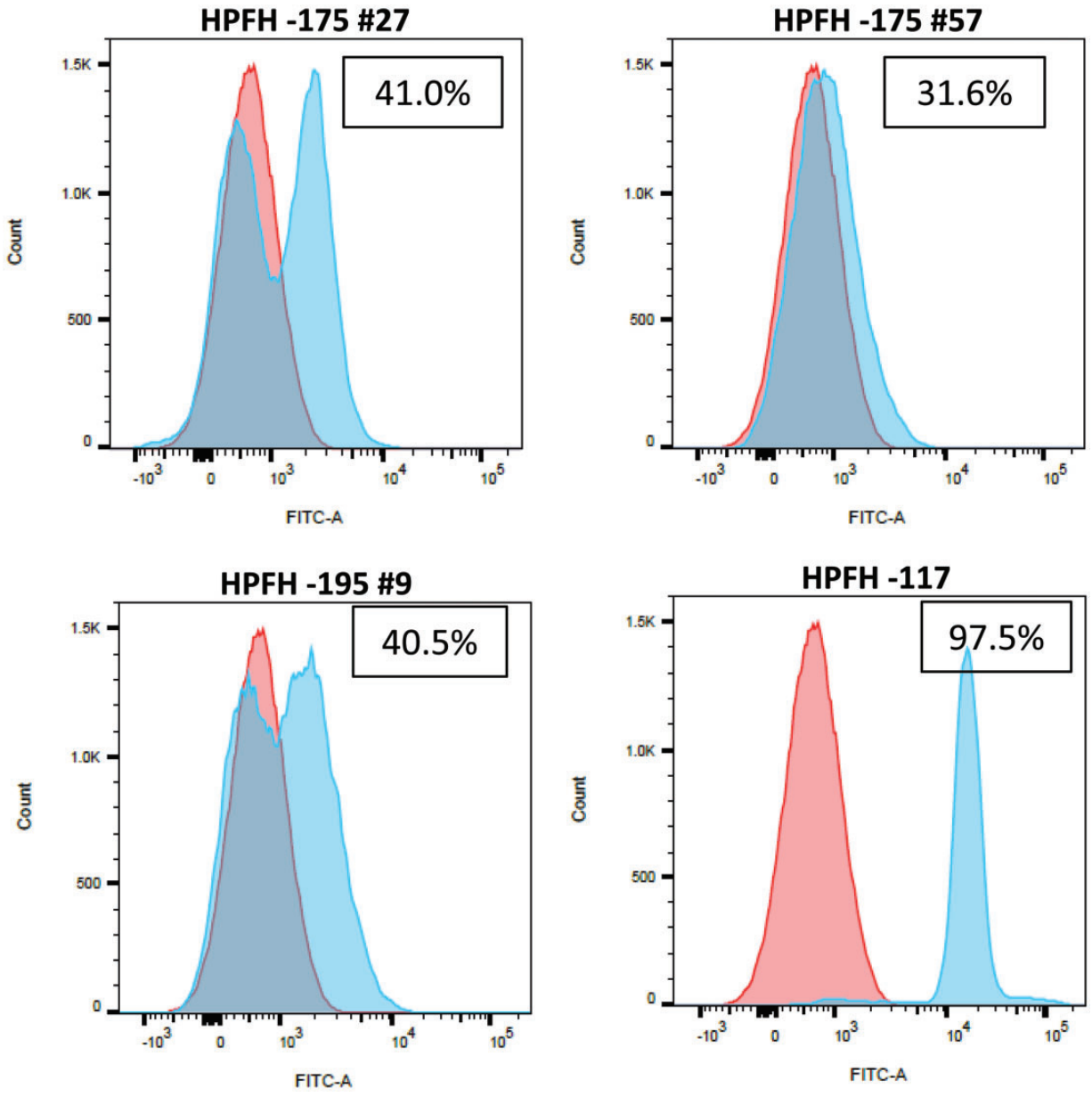

Figure 3 Flow cytometry analysis of cells isolated from adult mouse blood samples labeled with HbF antibody conjugated with FITC. Each panel shows a comparison between a HPFH line (in blue) and the non-transgenic control mice sample (in red). The percentage shown in each graphic is the HbF-expressing cell population after subtraction of the non-transgenic (non-HbF-expressing) background. Data representative of duplicate experiments are shown

GATA-1 often works in combination with TAL1 to activate erythroid gene expression[27,28] and the TAL1 binding we observe could be dependent upon GATA-1 occupancy.[22] However, our results did not show a statistically significant difference in the recruitment of GATA-1 between the wild-type and -175 β-YAC mice to the *HBG1* gene promoter (data not shown). Our results are consistent with those of Weinert et al.,[26] who demonstrated that in murine mouse erythroleukemia (MEL) and human K562 cells in the presence of the -175 T > C Black HPFH mutation, fetal globin expression was increased through *de novo* recruitment of the activator TAL1 which interacts with LDB1 and LMO2 to promote looping of the distal LCR to the modified $^{A}\gamma$-globin promoter.

The molecular mechanism involved in HBG1 gene activation in the presence of –195 C > G HPFH mutation remains uncertain

We did not find any statistically significant enrichment for any of the transcription factors tested for by ChIP in the presence of the -195 HPFH mutation compared to wild-type β-YAC transgenic mice. We included YY1 and PAX1 in our analysis since previous data by our research group using serial analysis of gene expression (SAGE), DNA-protein array, and ChIP with human erythroid progenitor cell culture implicated the involvement of these two transcription factors in *HBG1* gene regulation in the -195 promoter (data not shown).

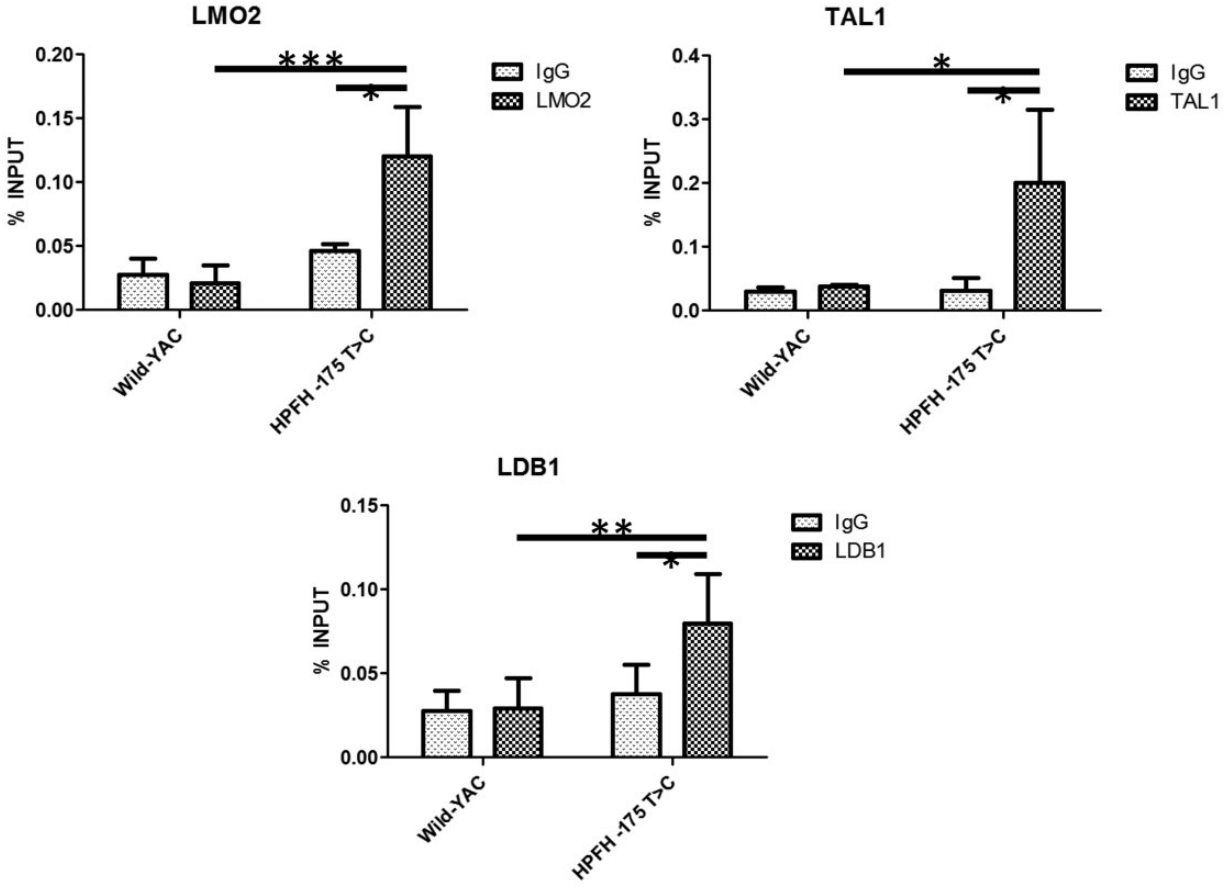

Figure 4 Chromatin Immunoprecipitation (ChIP) analysis using fetal liver samples of wild-type β-YAC transgenic mice (wild-YAC) and β-YAC transgenic mice carrying the HPFH -175 T > C mutation (HPFH -175 T > C), using anti-TAL1, anti-LMO2, and anti-LDB1 antibodies. For these three transcription factors, significant enrichment of occupancy ($P < 0.05$) in the HBG1 gene promoter region was verified only in the samples with the -175 T > C mutation. Data are shown as mean ± standard deviation for at least four experiments. * = P-value < 0.05; ** = P-value < 0.01; *** = P-value < 0.001

Discussion

The C > G point mutation identified at position -195 of the *HBG1* gene was described by Costa et al.[29] and is associated with moderate elevation of HbF levels in heterozygotes, ranging between 6.0 and 16.0%. Not much is known about the molecular mechanism regulating *HBG1* gene expression via the -195 promoter region. In three different cell lines (K562, HEL, and HEK293), the -195 C > G mutation increases promoter-linked luciferase gene expression in the presence of a LCR 5′ hypersensitive site 2 (5′HS2) fragment.[30,31] Although *HBG1* gene reactivation in the -198 T > C British HPFH mutation is mediated by the SP1 transcription factor, the same mechanism does not apply to the -195 HPFH mutation.[31] Our results using SAGE, DNA-protein array, and ChIP in human erythroid progenitor $CD34^+$cells isolated from six individuals (three normal donors and three individuals carrying the -195 C > G HPFH mutation) indicated the involvement of YY1 and PAX1 in *HBG1* gene regulation (unpublished data not shown). $^A\gamma$-globin reactivation appears to be characterized by a decrease in YY1 binding, since the -195 C > G substitution in the *HBG1* gene promoter abrogates YY1 binding with a concomitant increase of PAX1 binding in this DNA region. Although PAX1 has never been implicated in globin gene regulation, YY1 has been shown to be involved in embryonic ε-globin regulation. A YY1 binding site located near -269 of the *HBE* gene promoter overlaps two GATA-1 binding sites.[32] Mutation of this YY1 binding site leads to continued ε-globin expression in adulthood, indicating that YY1 functions as part of a *HBE* gene repressor.[33]

However, we did not find the same occupancy pattern of YY1 and PAX1 at the promoter region of *HBG1* gene. At first, we hypothesized that the observed difference between human erythroid progenitor $CD34^+$ cells and transgenic mice might be due to the inherent nature of the two experimental systems. In postconception day E18 fetal livers of wild-type β-YAC transgenic mice, the γ-globin to β-globin (HbF to HbA) switch already occurred and the γ-globin genes are silenced by this time. In contrast, in human erythroid progenitor cells, on day 13 of culture on which the experiments were carried out, expression of γ-globin was relatively high due to the addition of erythropoietin in the culture medium (erythropoietin was added to the cell culture medium at day 0 of culture). Thus, analysis of fetal livers at postconception day E12 may be more appropriate,

since the γ-globin to β-globin switch has not occurred and the γ-globin genes are still expressed. However, we did not find statistically significant difference in the occupancy of YY1 and PAX1 between the wild-type and -195 C > G *HBG1* gene promoter regions in post-conception day E12 fetal liver samples from β-YAC transgenic mice (data not shown). We will continue to perform experiments to ascertain the proteins that bind this region of the ^{A}γ-globin promoter, both at the wild-type sequence and the HPFH mutant sequence.

ChIP analysis of wild-type β-YAC and -175 HPFH β-YAC mice showed a significant difference in occupancy of the -175 C > T *HBG1* gene promoter sequence by the transcription factors TAL1, LMO2, and LDB1. TAL1 is a basic helix-loop-helix transcription factor required for the development of all hematopoietic lineages[34] that binds to different regulatory regions of the globin genes in the human β-globin locus.[22,23,25,26] LDB1 acts as a bridging factor to bring protein complexes bound at distant DNA binding sites in close proximity for interaction with one another via DNA looping.[35] LDB1 has a C-terminal LIM domain allowing the protein to interact other members of the complex by binding LMO2.[22] LMO2 predominantly interacts with TAL1 in hematopoietic cells. The interaction between TAL1 and LMO2 in turn stabilizes the LDB1 complex and modulates the activity of the complex.[36] Several studies indicate that GATA-1 binds in the -175 region of the *HBG1* gene[37,38] and interacts with TAL1 to activate erythroid gene expression.[27,28] In addition, TAL1 binding is dependent upon GATA-1 occupancy.[22] However, our results show that GATA-1 is not part of the activation complex in the presence of the -175 mutant *HBG1* gene promoter. Similarly, Wienert et al.[26] demonstrated that in the presence of the -175 mutation in mouse MEL cells or human K562 cells, fetal globin expression was increased through *de novo* recruitment of TAL1 which interacted with LDB1 and LMO2 to promote chromatin looping of the distal LCR to the modified ^{A}γ-globin promoter. Their data showed that GATA-1 recruitment was enhanced only 1.7-fold in the presence of the -175 mutation compared to the wild-type promoter sequence, indicating that GATA-1 does not play a key role in the activation of *HBG1* gene in the presence of this HPFH mutation.

Maintenance of *HBG1* expression in our adult HPFH mice parallels the phenotypes associated in human subjects carrying either of the HPFH mutations, demonstrating the validity of our -175 and -195 HPFH mouse models. Our preliminary ChIP data looking at the molecular mechanisms underlying these HPFHs indicate that these HPFH β-YAC transgenic lines are highly relevant for *in vivo* studies of β-like globin gene regulation, particularly mechanisms of γ-globin gene repression and activation.

Authors' contributions: CAB designed and performed experiments, interpreted data, and wrote the manuscript; FCC designed and performed experiments and interpreted data, HF performed experiments and interpreted data; RYN performed experiments and interpreted data; LVN performed experiments and interpreted data; MPP performed experiments and interpreted data; RDW performed experiments and interpreted data; KRP designed and performed experiments, interpreted data, and wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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