RAPID COMMUNICATION

Reversal of Lethal α- and β-Thalassemias in Mice by Expression of Human Embryonic Globins

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Genetic mutations that block α- or β-globin gene expression in humans can result in severe and frequently lethal thalassemic phenotypes. Homozygous inactivation of the endogenous α- or β-globin genes in mice results in corresponding thalassemic syndromes that are uniformly fatal in utero. In the current study, we show that the viability of these mice can be rescued by expression of human embryonic ζ- and ϵ-globins, respectively. The capacity of embryonic globins to fully substitute for their adult globin homologues is further demonstrated by showing that ζ- and ϵ-globins reverse the hemolytic anemia and abnormal erythrocyte morphology of mice with nonlethal forms of α- and β-thalassemia. These results illustrate the potential therapeutic utility of embryonic globins as substitutes for deficient adult globins in thalassemic individuals. Moreover, the capacity of embryonic globins to functionally replace their adult homologues brings into question the physiologic basis for globin gene switching.

MOLECULAR DEFECTS that affect the level of α- or β-globin expression result in a collection of clinically heterogeneous disorders known as α- and β-thalassemias, respectively.[1-3] As a group, thalassemias comprise the most common genetic defects in humans, with a particularly high prevalence in certain Mediterranean, central African, and south Asian populations.[2-4] Clinically significant thalassemias are also increasingly recognized in members of many North American immigrant communities.[5] The phenotypes of specific thalassemic mutations are directly proportional to the quantitative deficits in globin gene expression and the resulting imbalance in α:β-chain synthesis. Severe forms of thalassemia are characterized by retarded growth and development resulting from a marked hemolytic anemia, with a compensatory expansion of hematopoietic tissues and a consequent hypermetabolic state.[1-3] Complete loss of α-globin gene expression results in midgestational fetal demise (α-thalassemia hydrops fetalis),[5] while complete loss of β-globin expression (β-thalassemia major) is lethal in untreated children.[2,3] Therapeutic options for severely affected individuals are limited to allogeneic bone marrow transplantation (BMT) or lifelong transfusions, neither of which is universally available and both of which are attended by significant cost and risk.[6-8]

Two α-like and two β-like globin chains assemble into functional hemoglobin (Hb) heterotetramers that reversibly and cooperatively bind four O_2 molecules under physiologic conditions. The expression profile of the constituent globin monomers is developmentally regulated. There are three functional α-like globin genes in both mouse and humans: ζ, α2, and α1. Expression of human ζ-globin is specific to the primitive, nucleated erythroblasts in the blood islands of the extraembryonic yolk sac.[9,10] As the site of active erythropoiesis migrates to the fetal liver, there is silencing of the ζ-globin gene and reciprocal induction of the two α-globin genes, which continue to express at high levels into adulthood. The human β-like globins are encoded by an embryonic ϵ-globin gene, expressed in yolk sac erythroblasts, two fetal γ-globin genes, and a β-globin gene that is maximally induced at birth and encodes almost 98% of the β-like globin chains in normal adult erythrocytes.[1,9,11] Coordinate switching of genes within the α- and β-globin clusters results in the sequential assembly of characteristic Hbs during embryonic ($\zeta_2\epsilon_2$), fetal ($\alpha_2\gamma_2$), and adult ($\alpha_2\beta_2$) developmental stages. The major evolutionary factor driving wide phylogenic conservation of this system is unknown, although developmental stage-specific Hbs may optimize O_2 delivery to embryonic, fetal, and adult tissues.

The presence of developmentally silenced, yet structurally intact, globin genes in the α- and β-globin clusters suggests a potential therapeutic approach to severe forms of thalassemia based on reactivation of these ''back-up'' loci. Hypothetically, globin chains expressed from these embryonic and fetal loci could substitute for their deficient adult globin homologues in individuals with severe thalassemias. This approach would be useful only if the Hbs assembling from existing adult and reactivated embryonic globin subunits (eg, Hbs $\zeta_2\beta_2$ and $\alpha_2\epsilon_2$) were functional in adult (definitive) erythrocytes. The feasibility of this approach is demonstrated by the phenotypic reversion in individuals with β-thalassemia who overexpress fetal γ-globin, assembling functional Hb $\alpha_2\gamma_2$.[2,12] The capacity of embryonic ϵ-globin to substitute for its adult β-globin homologue in definitive erythrocytes has not previously been explored. Likewise, the possibility that embryonic ζ-globin might substitute for adult α-globin, which has no fetal homologue, has never been investigated.

A major concern about stage-discordant globin substitution is the likelihood that Hbs incorporating embryonic subunits would be physiologically irrelevant in definitive erythrocytes. The widely held model in which developmental globin switching reflects some crucial difference between the functions of

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Submitted July 29, 1998; accepted August 20, 1998.

Supported in part by Grants No. HL-K11-02623 (J.E.R.) and HL-38632 (S.A.L.). J.E.R. is the recipient of a research fellowship from the Cooley's Anemia Foundation; S.A.L. is an Investigator of the Howard Hughes Medical Institute.

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0006-4971/98/9209-0070$3.00/0

embryonic and adult globins predicts that Hbs assembled from embryonic subunits should perform poorly in adult erythrocytes. Recent studies demonstrating that these Hbs exhibit substantially elevated O_2 affinities in vitro appear to support this model.[13,14] However, these predictions do not account for adaptive mechanisms that permit adults expressing mutant Hbs with a wide spectrum of O_2-binding affinities to grow and develop normally, and to sustain normal pregnancies.[15,16] For these reasons, in vivo assessments may prove to be more valuable predictors of embryonic globin function.[17] In the current work, we demonstrate the capacity of human embryonic ζ- and ϵ-globins to reverse the phenotypes of adult α- and β-thalassemic mice, by substituting for deficient α-and β-globin chains, respectively. Moreover, we show that ζ- and ϵ-globins maintain viability in mice with homozygous lethal inactivation of their endogenous adult globin genes, respectively. The data indicate that embryonic globins can function efficiently in vivo in definitive erythrocytes, providing a rational basis for the design of novel therapies aimed at their reactivation in individuals with clinically-severe thalassemias.

MATERIALS AND METHODS

Transgene construction. A 2.4-kb human (h) ζ transgene, comprising a 0.6-kb human α-globin promoter fragment linked to the 1.8-kb ζ-globin transcribed region and proximal 3′ flanking region, was constructed as previously described.[18] A 4.1-kb hϵ transgene was constructed using a two-step splice-overlap-extension/polymerase chain reaction synthesis,[19] in which the 1.5-kb hϵ transcribed region was inserted between 0.8-kb and 1.7-kb fragments containing the hβ promoter and enhancer elements, respectively.[20] The hζ and hϵ transgenes were inserted into polylinker *Eco*RI and *Cla* I/*Eco*RV sites of plasmid pSP72/βLCR, respectively, immediately adjacent to a 6.5-kb DNA fragment containing core elements of the human β-globin LCR DNase I hypersensitive sites 1-4.[18,21] All polymerase chain reaction (PCR)-amplified fragments were subsequently verified by dideoxy sequencing.[22] Linked 8.9-kb μβLCR/hζ and 10.6 kb μβLCR/hϵ fragments were released by *Sal* I/*Eco*RV or *Eco*RI digestion, respectively, and purified over an Elutip filtration column (Schleicher & Schuell, Keene, NH) before microinjection.[18]

Transgenic mice. Transgenic founders were generated according to a standard protocol by the University of Pennsylvania Transgenic and Chimeric Mouse Facility.[18] Founder mice identified by Southern transfer analysis of tail DNA were mated with CD-1 females or C57BL6 males to generate F1 progeny with germline transgene integration. Generation and characterization of mice with targeted deletions of the endogenous α- and β-globin genes are reported elsewhere.[23-26] The genotypes of progeny with combinations of human transgenes and gene deletions were determined by Southern analysis and/or PCR analysis of tail DNA, and/or deduced from their globin phenotypes (detailed below).

Southern analysis. Southern transfers were performed as previously described on 5 μg DNA purified from the tails of candidate mice.[18] [^{32}P]-random-primer labeled probes were generated from agarose gel-purified DNA templates. The hζ transgene was identified as a 1.2-kb *Pst* I fragment using a 610-bp *Nco* I template encompassing the hα-globin promoter region.[18] The hϵ transgene was identified as a 5.1-kb *Bam*HI DNA fragment using a 572-bp *Bam*HI/*Dra*III template encompassing the junction of the constituent β-globin promoter and ϵ-globin transcribed regions. When necessary, a 1.3-kb *Bam*HI fragment of intergenic DNA from the mouse (m) α-globin cluster (the 'X' region) was identified using itself as template.[18] Endogenous wild-type and α-globin knockout loci were identified as 13.0-kb and 9.0-kb *Hin*dIII DNA fragments, respectively, using a 1.3-kb *Bam*HI fragment originating 5′ to the tandem mα-globin genes.[23] hϵ transgene copy numbers were estimated as described,[18] based on two copies of ϵ-globin gene/human genome and two copies of 'X' region/mouse genome.

PCR amplification. Wild-type mβ-globin genes were identified as a 250-bp PCR product by amplification of mouse tail DNA using an oligomer pair specific to exons I and II of the mouse β^{Major}-, β^{Minor}-, and β^{Single}-globin genes (5′CAACCCCAGAAACAGACATC3′ and 5′CCAAGGGTAGACAACCAGC3′). The β-globin knockout allele (containing an HPRT mini-gene)[24] was identified as a 179-bp product with a sequence-specific oligomer pair (5′GACTGAACGTCTTGCTCGAG3′ and 5′AGCTCTTCAGTCTGATAAAATC3′). Primers (100 pmol each) were added to 0.25 μg DNA in a 50-μL reaction assembled according to the manufacturer's recommendations (Perkin Elmer Cetus, Norwalk, CT) with $MgCl_2$ adjusted to 2 mmol/L. Amplifications were performed for one cycle at 95°C × 3 minutes, 60°C × 15 seconds, and 72°C × 15 seconds, and for an additional 29 cycles using a modified (92°C × 1 minute) denaturation step. PCR products were analyzed on an EtBr-stained 5% polyacrylamide gel.

Globin analysis. Five to 10 μL of phosphate-buffered saline (PBS)-washed transgenic erythrocytes were lysed in 4 vol 1 mmol/L $MgCl_2$, and membranes pelleted at 4°C in a desktop centrifuge after addition of 1 vol 1.5 mol/L KCl. Globins were resolved by Triton-acid-urea gel electrophoresis in 5% acetic acid and visualized by Coomassie blue staining.[27]

Blood counts and peripheral blood smears. Complete blood counts were performed on EDTA-anticoagulated blood from duplicate age- and sex-matched mice using a Cell-Dyne 3500 analyzer (Abbott Laboratories, Chicago, IL). Manually prepared peripheral blood smears were stained with Wright-Giemsa reagent and photographed under oil at 100× magnification through an Optifoto microscope (Nikon, New York, NY) using Ektachrome 160T film (Eastman Kodak, Rochester, NY).

RESULTS

Generation of adult mice that express human embryonic globins. Transgenes capable of expressing embryonic globins in adult erythrocytes were constructed by linking the transcribed regions of the human ζ- and ϵ-globin genes to transcriptional control elements from the human adult α- and β-globin genes, respectively (Fig 1A and B). The ζ-globin transcribed region was linked to the α-globin promoter to generate the human ζ (hζ) transgene,[18] while the human ϵ (hϵ) transgene was constructed by inserting the ϵ-globin transcribed region between the β-globin promoter and 3′ enhancer elements.[20] Each chimeric gene was linked to core elements of hypersensitive sites 1-4 from the β-globin locus control region (μβLCR) to promote integration site-independent expression.[18,21] Multiple independent mouse lines containing the hζ and hϵ transgenes were generated using standard methods,[18] and the transgene copy number for each line was established in F1 mice.[18] The 5′ cap and 3′ poly(A) addition sites of the transcribed hϵ- and hζ-globin mRNAs were shown to be normally positioned using RNase protection analysis of RNA from adult reticulocytes (not shown). The expression of human embryonic globins was demonstrated by gel electrophoresis of clarified erythrocyte lysates (Fig 1C).[27,28] Based on their high-level expression of hζ- or hϵ-globin, one hζ-and one hϵ-globin line, with transgene copy numbers of 14 and 7, respectively, were identified for use in all subsequent studies.

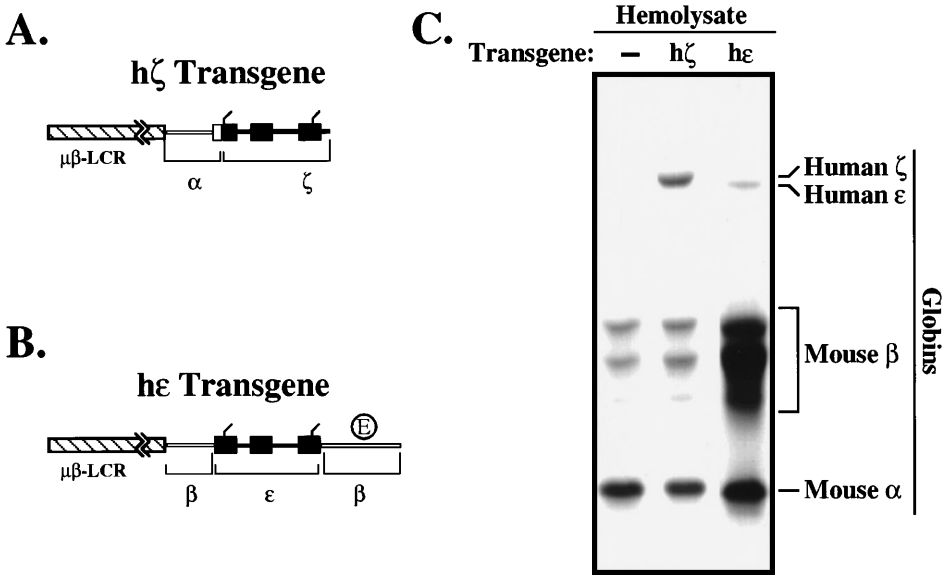

Fig 1. Generation of adult mice expressing human embryonic ζ- and ϵ-globins. (A) Construction of the hζ transgene. The full human ζ-globin transcribed region (black) was linked to the human α-globin gene promoter and 5′ untranslated region (light).[18] This chimeric hζ transgene was subsequently linked to a μβLCR cassette containing core elements of DNase I hypersensitive sites 1-4.[21] Exons are indicated as filled boxes, and translation initiation and termination codons by tick marks. (B) Construction of the hϵ transgene. The full-length ϵ-globin transcribed region (black) is bracketed by the β-globin promoter and 3′ flanking region (light), including the 3′ β-globin enhancer element (E). Exons and translation initiation and termination sites are indicated as in (A). (C) Expression of embryonic globins in definitive erythrocytes from mice carrying the hζ and hϵ transgenes. Clarified erythrocyte lysates from a wild type control (−), and hζ and hϵ adult transgenic mice were resolved by Triton-acid-urea gel electrophoresis and visualized by Coomassie blue staining.[27,28] Globin bands are identified to the right. The third lane (hϵ) was overloaded to demonstrate the hϵ-globin product.

Expression of embryonic hζ-globin reverses an α-thalassemic phenotype. Embryonic ζ-globin function in adult erythrocytes was initially assessed by determining its capacity to reverse an α-thalassemia phenotype. Transgenic mice expressing hζ-globin were mated with mice heterozygous for combined deletion of the closely linked mouse α2- and α1-globin genes ($m\alpha^{+/-}$; kind gift of J. Chang and Y.W. Kan, University of California, San Francisco).[23] Globin genotypes of the offspring were determined by Southern analysis of tail DNA (Fig 2A), and phenotypes of compound hemizygous $m\alpha^{+/-}$/hζ mice were subsequently compared with those of sex-matched sibling $m\alpha^{+/-}$ controls. The size and shape of $m\alpha^{+/-}$ erythrocytes varied widely (Fig 2C), modeling the changes typically observed in human α-thalassemic erythrocytes.[1-3] In contrast, erythrocytes from sex-matched $m\alpha^{+/-}$ littermates that coexpressed hζ-globin were morphologically normal. The hypochromic, microcytic anemia of $m\alpha^{+/-}$ mice (low Hb [Hb], elevated erythrocyte number [RBC], small erythrocyte size [mean corpuscular volume or MCV], and depressed intracellular Hb [mean corpuscular Hb or MCH]) reverted to normal in $m\alpha^{+/-}$ mice that coexpressed hζ-globin. (Fig 2E). The correction of the α-thalassemia phenotype in $m\alpha^{+/-}$ mice coexpressing hζ-globin suggests that the embryonic globin assembles into Hb $\zeta_2\beta_2$ heterotetramers that are functional in adult erythrocytes.

Expression of embryonic hϵ-globin reverses a β-thalassemic phenotype. To assess the function of embryonic ϵ-globin in adult erythrocytes, transgenic hϵ mice were mated with mice heterozygous for deletion of the two tandem endogenous adult β-globin genes ($m\beta^{+/-}$ mice, kind gift of O. Smithies, University of North Carolina, Chapel Hill).[24] The genotypes of offspring were determined by Southern and PCR analysis of tail DNA (Fig 2B). Both male and female $m\beta^{+/-}$ mice were growth retarded and displayed marked splenomegaly, characteristics shared by humans with severe forms of β-thalassemia.[1-3] Both of these phenotypic abnormalities resolved in $m\beta^{+/-}$ littermates coexpressing hϵ-globin (data not shown). Moreover, $m\beta^{+/-}$ mice displayed severe erythrocyte morphologic changes comprising marked variation in erythrocyte size and shape, while erythrocytes from sex-matched $m\beta^{+/-}$ littermates coexpressing embryonic hϵ-globin appeared normal (Fig 2D). Coexpression of the hϵ-globin transgene also normalized Hb levels, RBC, MCV, and MCH in erythrocytes from $m\beta^{+/-}$ littermates (Fig 2F). The underlying functional abnormalities in $m\beta^{+/-}$ erythrocytes that result in their abnormal morphologies are thus corrected by expression of hϵ-globin. These data imply that hϵ-globin acts as a β-like globin in adult erythrocytes by assembling into functional Hb $\alpha_2\epsilon_2$ heterotetramers.

Embryonic hζ-globin rescues the viability of mice with homozygous lethal α-globin gene deletion. The function of hζ-globin in adult erythrocytes was more rigorously tested by inbreeding $m\alpha^{+/-}$/hζ mice to generate embryos that were homozygous for the $m\alpha^{-}$ deletion ($m\alpha^{-/-}$). Although the expression of endogenous mζ-globin permits normal embryonic development, mice with the $m\alpha^{-/-}$ genotype die in utero at the time of the embryonic ζ- to adult α-globin switch.[25] Remarkably, however, these matings generated a number of $m\alpha^{-/-}$ mice whose viability was rescued by expression of embryonic hζ-globin. These $m\alpha^{-/-}$/hζ mice, which do not synthesize any mα-globin chains, appear identical to wild-type controls, are fertile, and bear normal litters (Fig 3A and C, and data not

shown). The rescue of mα$^{-/-}$ mice directly shows that embryonic hζ-globin is a fully sufficient substitute for adult α-globin in definitive erythrocytes.

Embryonic hϵ-globin rescues the viability of mice with homozygous lethal β*-globin gene deletion.* A similar strategy was used to determine whether human embryonic ϵ-globin could substitute for adult β-globin in definitive erythrocytes. mβ$^{+/-}$/hϵ mice were inbred, and genotypes of the weaned pups determined by analysis of genomic DNA (not shown). We failed to identify any mβ$^{-/-}$ pups, consistent with previous reports that mice with homozygous deletion of the endogenous β-globin genes (mβ$^{-/-}$) die in utero.[26] Remarkably, though, the matings generated liveborn mβ$^{-/-}$/hϵ mice that survived into adulthood, despite the complete absence of adult β-globin in their erythrocytes (Fig 3B). Although viable mβ$^{-/-}$/hϵ mice are smaller than nontransgenic controls (Fig 3D; see Discussion), their survival demonstrates the functional overlap between embryonic and adult β-like globins.

DISCUSSION

This report demonstrates that embryonic ζ- and ϵ-globins can functionally substitute for their adult α- and β-globin homologues in adult erythroid cells. These findings suggest that individuals with α- or β-thalassemia would likely benefit from reactivation of their endogenous embryonic ζ- or ϵ-globin genes, respectively. Moreover, the results bring into question the basis for the phylogenic conservation of globin gene switching.[15,16,29]

Transgenic mice are a valuable model system in which to study human embryonic globin function. The organization of the globin genes,[1,30-32] molecular controls of their expression,[1,33] and functions of their encoded globin proteins[25,26] are closely conserved between mice and humans. Although mice do not exhibit a clearly defined fetal stage of β-globin gene expression, they parallel human development by expressing different α-like and β-like globins during intrauterine (embryonic/fetal) and extrauterine (adult) stages. The physiologic characteristics of Hb function and O_2 transport in mice and humans are also quite similar. Previous reports have shown that deficiencies of α- or β-globin chains in the mouse result in disorders that closely model human α- or β-thalassemia, respectively (Fig 2), and that human α- and β-globins can fully substitute for their mouse counterparts.[25,26] These data indicate that the mouse model is an appropriate one in which to study human globin function.

To test the thesis that embryonic globins could sustain adult life, it was first necessary to overcome their tight developmental stage-specific regulation.[1,9-11] Silencing of both ζ- and ϵ-globin genes at the embryonic-to-fetal transition appears to be mediated primarily through their transcriptional regulation,[34-38] although recent evidence suggests that posttranscriptional events

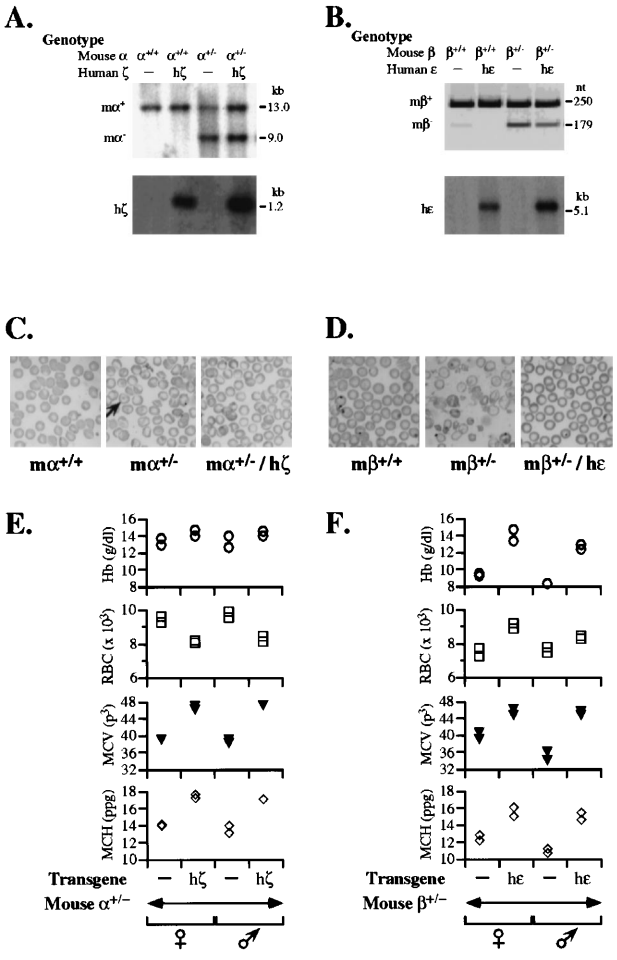

Fig 2. Function of human embryonic ζ- and ϵ-globins in adult murine erythrocytes. (A) Determination of mouse α-globin genotypes by Southern analysis. Duplicate Southern transfers of *Pst* I–digested DNA from wild-type mice (α$^{+/+}$), mice carrying the hζ transgene (α$^{+/+}$/hζ), mice heterozygous for deletion of the mα-globin genes (α$^{+/-}$), and mice heterozygous for deletion of the mα-globin genes that carried the hζ transgene (α$^{+/-}$/hζ). Blots were probed with a 1.3-kb fragment originating 5′ to the deleted mα-globin sequences (upper autoradiograph)[23] or the hα-globin promoter (lower autoradiograph).[18] The sizes and identities of the wild-type (mα^{+}) and deleted mα-globin loci (mα^{-}), and the hζ transgene (hζ) are indicated to the left and right of each autoradiograph, respectively. (B) Determination of mouse β-globin genotypes by combined Southern and PCR analyses. Tail DNA from mice was coamplified using paired oligomers recognizing wild-type mβ-globin genes (250-bp product) or a fragment of the HPRT cDNA comprising the knockout 'socket' (179-bp product).[24] Reaction products were resolved and visualized on an ethidium bromide–stained 5% polyacrylamide gel. Genotypes are indicated at top. Southern analysis of the same DNA (bottom) using the 572-bp hε probe indicates the presence/absence of the hϵ transgene. The sizes and identities of the wild-type (mβ^{+}) and deleted mβ-globin loci (mβ^{-}), and the hϵ transgene (hϵ) are indicated to the left and right of each autoradiograph, respectively. (C) Thalassemic erythrocyte morphology in mα$^{+/-}$ mice is corrected by coexpression of embryonic ζ-globin. Wright-Giemsa–stained peripheral blood smears from wild-type, mα$^{+/-}$, and mα$^{+/-}$/hζ mice, viewed under oil at 100 × magnification. A typical 'target cell' is indicated (arrow). (D) Thalassemic erythrocyte morphology in mβ$^{+/-}$ mice is corrected by coexpression of embryonic ϵ-globin. Wright-Giemsa–stained peripheral blood smears from wild-type, mβ$^{+/-}$, and mβ$^{+/-}$/hϵ mice viewed under oil at 100 × magnification. (E) Resolution of anemia and normalization of erythrocyte indices in mα$^{+/-}$ mice coexpressing embryonic ζ-globin. Erythrocyte analyses were performed on anticoagulated whole blood collected from duplicate sex-matched 13-week adult siblings. The Hb (○), RBC number (□), MCV (▼), and MCH (◇) are plotted for mice with the globin genotypes and sexes indicated at bottom. (F) Resolution of anemia and normalization of erythrocyte indices in mβ$^{+/-}$ mice expressing embryonic ϵ-globin. Analyses were performed on anticoagulated whole blood collected from duplicate sex-matched 9-week adult siblings as described in (E). The globin genotypes and sexes are indicated at bottom.

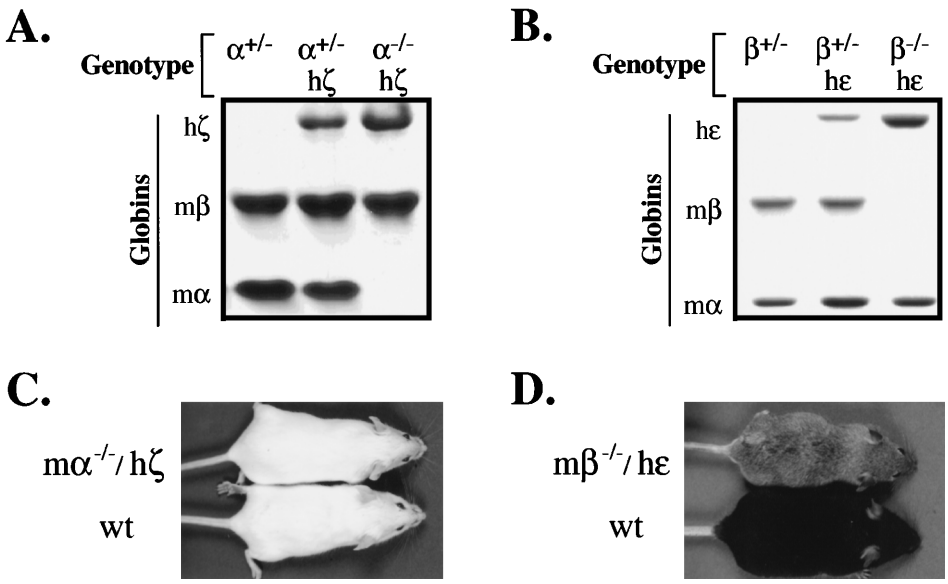

Fig 3. Rescue of viability in mice homozygous for deletion of adult α- and β-globin genes by expression of human embryonic ζ- and ε-globin. (A) Globin profile of mα$^{-/-}$/hζ mice. Clarified erythrocyte lysates prepared from mice with the indicated genotypes were resolved by Triton-acid-urea gel electrophoresis and visualized by Coomassie Blue staining. The identity of each globin band is indicated to the left of the gel. (B) Globin profile of mβ$^{-/-}$/hε mice. Clarified erythrocyte lysates were analyzed as described in (A). Genotypes are shown at the top, and globins identified to the left. (C) Physical appearance of mα$^{-/-}$/hζ mice. Female mα$^{-/-}$/hζ and control CD-1 mice are shown. (D) Physical appearance of mβ$^{-/-}$/hε mice. Male mβ$^{-/-}$/hε and control C57BL6 mice are shown.

contribute to this process.[18,39] Elements mapping to the 5′ flanking region regulate transcriptional silencing of the ε-globin gene,[34-36] while transcriptional silencing of the ζ-globin gene requires interaction of elements within both the 5′ and 3′ flanking regions.[18,37,38] To maximize ζ- and ε-globin expression in adults, these regulatory elements were replaced by promoter and enhancer elements from the corresponding human adult globin genes (Fig 1A and B). By using this approach, and by linking each construct to a μβLCR before microinjection,[18,21] it was possible to generate transgenes expressing high levels of embryonic globins in definitive mouse erythrocytes (Fig 1C).

The phenotypic severity of a thalassemic phenotype parallels the imbalance in α- and β-globin chain expression in terminally differentiating erythroid cells. The excess globin chains—particularly free α-globin chains—exhibit cytotoxic effects that manifest clinically as inefficient erythropoiesis and chronic hemolytic anemia.[1-3,5] Hence, the initial evidence that the embryonic hζ- and hε-globins could effectively substitute for deficient adult α- and β-globins, respectively, was inferred from their ability to reverse the thalassemic phenotypes of mα$^{+/-}$ and mβ$^{+/-}$ mice. As in humans who are heterozygous for loss of both α-globin loci (α-thalassemia trait), mα$^{+/-}$ mice grow normally despite a morphologically distinct hypochromic, microcytic anemia (Fig 2C and E).[25] Heterozygosity for loss of β-globin gene expression (mβ$^{+/-}$) in mice results in a slightly more severe phenotype, comprising growth retardation, splenomegaly, and marked erythrocyte abnormalities (Fig 2D and F, and data not shown),[26] as also occurs in humans.[1-3] Remarkably, the expression of embryonic hζ-and hε-globins reverses the growth retardation, anemia, and abnormal erythrocyte morphologies that characterize the thalassemic phenotypes in mα$^{+/-}$ and mβ$^{+/-}$ mice (Fig 2C through F, and data not shown). These results suggest that embryonic globins can substitute for their deficient adult counterparts, bringing the α:β ratio back into balance.

The functional properties of embryonic ζ- and ε-globins were further tested by asking whether they were fully sufficient to replace their corresponding adult α- and β-globins, respectively. As in humans,[5] the α$^{-/-}$ genotype in mice is lethal in utero.[25] Coexpression of embryonic hζ-globin in mα$^{-/-}$ mice results in viable adults which appear identical to age- and sex-matched wild-type controls (Fig 3A and C). The mβ$^{-/-}$ genotype, which is also lethal to mice in utero,[26] differs from its corresponding genetic disorder in humans, because human γ-globin supports fetal growth and development through birth.[1-3] Mice with homozygous lethal deletion of both β-globin loci (mβ$^{-/-}$) were rescued by expression of embryonic hε-globin (Fig 3B and D). Although these mβ$^{-/-}$/hε mice were viable and fertile, they appear to be small relative to wild-type controls. This modest growth retardation may reflect a residual deficit in hε-globin levels, although functional deficiencies cannot be excluded. Nonetheless, the data demonstrate that embryonic ζ- and ε-globins are remarkably effective substitutes for their adult α- and β-globin counterparts. This implies assembly of functional Hb $\zeta_2\beta_2$ and $\alpha_2\epsilon_2$ heterotetramers that bind and discharge O_2 in a manner that is physiologically valuable in adult erythrocytes.

The demonstration that expression of ζ- and ε-globins reverses the phenotypes in adult mice with deficient levels of α- and β-globins indicates that clinically significant α- and β-thalassemias in humans might be mitigated by reactivation of endogenous embryonic ζ- or ε-globin genes, respectively. The feasibility of this approach is demonstrated by the phenotypic reversion resulting from high-level γ-globin expression in β-thalassemics with concomitant HPFH mutations.[12] Embryonic globin genes are structurally and functionally unaffected by the vast majority of α- and β-thalassemic mutations, making them theoretically available for reactivation in most fetuses or adults with thalassemia. The current work shows that ζ-globin, encoded by the single extant nonadult α-like globin gene, has potential value to individuals with severe forms of α-thalassemia (Hb H disease), as well as to developing fetuses with otherwise lethal α-thalassemia hydrops fetalis.[5] The parallel recognition that ε-globin can substitute for β-globin in adults

provides an alternate to the γ-globin genes as a target for developing molecular therapies. The ϵ-globin gene may be particularly valuable in this regard, as its transcriptional control is gene-autonomous,[40] in contrast with the fetal γ-globin genes, whose expression may be affected by the structural integrity of the adjacent β-globin gene.[41-43] Theoretically, this functional linkage of γ- and β-globin gene expression might complicate attempts to fully reactivate γ-globin gene expression in individuals with thalassemias resulting from nondeletional mutations of the β-globin gene. In addition to its O_2-transporting capacity, ϵ-globin may possess other properties, such as antipolymerization activity, which might benefit individuals with sickle cell disease or related hemoglobinopathies. These functional characteristics of human embryonic globins remain to be explored.

The current data bring into question the widely held model that the highly conserved process of globin gene switching in higher vertebrates reflects some crucial difference between the functions of embryonic and adult globins. The viability of $m\alpha^{-/-}/h\zeta$ mice indicates that ζ-globin can subserve the spectrum of functions required of α-like globins in both embryonic and fetal/adult erythroid environments. The lack of a strong physiologic requirement for globin gene switching is further suggested by the recent demonstration that α-globin can also subserve both embryonic and adult/fetal functions in mice with homozygous inactivation of endogenous mζ-globin expression.[44] These observations would support previous claims that maintenance of a *trans*-placental O_2 gradient may not be the major force underlying the evolutionary conservation of the ζ-to-α-globin switch.[5,16] The phylogenic conservation of globin switching may instead reflect a marginal survival advantage due to mechanisms that have not yet been established. However, from the present data it is clear that reactivation of the embryonic genes in erythroid progenitors from α- or β-thalassemic adults would result in a striking survival advantage.

ACKNOWLEDGMENT

The authors thank Y.W. Kan and J. Chang, and O. Smithies for generously providing $m\alpha^{+/-}$ and $m\beta^{+/-}$ mice, respectively; K. Horiuchi for photomicroscopic assistance; A. Lee for technical assistance; and S. Surrey and N. Cooke for critical reading of the manuscript.

REFERENCES

1. Russell JE, Liebhaber SA: Molecular genetics of thalassemia, in Verma RS (ed): Advances in Genome Biology, vol 2. Greenwich, CT, JAI, 1993, p 283

2. Bunn HF, Forget BG: Hemoglobin: Molecular, Genetic, and Clinical Aspects. Philadelphia, PA, Saunders, 1986

3. Weatherall DJ, Clegg JB: The Thalassemia Syndromes. Oxford, UK, Blackwell Scientific, 1981

4. Modell B, Bulyzhenkov: Distribution and control of some genetic disorders. World Health Stat Quart 41:209, 1988

5. Chui DHK, Waye JS: Hydrops fetalis caused by alpha thalassemia: An emerging health care problem. Blood 91:2213, 1998

6. Giardini C, Galimberti M, Lucarelli G: Bone marrow transplantation in thalassemia. Annu Rev Med 46:319, 1995

7. Giardina PJ, Grady RW: Chelation therapy in beta thalassemia: The benefits and limitations of desferrioxamine. Semin Hematol 32:310, 1995

8. Feitelson M, Lega L, Guo J, Resti M, Rossi ME, Azzari C, Blubberg BS, Vierucci A: Pathogenesis of posttransfusion viral hepatitis in children with beta thalassemia. Hepatology 19:558, 1994

9. Peschle C, Mavilio F, Care F, Migliaccio G, Migliaccio AR, Salvo G, Samoggia P, Petti S, Guerriero R, Marinucci M, Lazzaro D, Russo G, Mastroberardino G: Haemoglobin switching in human embryos: asynchrony of zeta → alpha and epsilon → gamma globin switches in primitive and definitive erythropoietic lineage. Nature 313:235, 1985

10. Albitar M, Care A, Peschle C, Liebhaber SA: Developmental switching of messenger RNA expression from the human alpha-globin cluster: Fetal/adult pattern of theta globin gene expression. Blood 80:1586, 1992

11. Ley TJ, Maloney KA, Gordon JI, Schwartz AL: Globin gene expression in erythroid human fetal liver cells. J Clin Invest 83:1032, 1989

12. Wood WG: Increased Hb F in adult life, in Higgs DR, Weatherall DJ (eds): The Hemoglobinopathies. London, UK, Balliere Tindall, 1993

13. Mould RM, Hofmann OM, Brittain T: Production of human embryonic hemoglobin (Gower-II) in a yeast expression system. Biochem J 298:619, 1994

14. Hofmann OM, Mould R, Brittain T: Allosteric modulation of oxygen binding to the three human embryonic hemoglobins. Biochem J 306:367, 1995

15. Adamson JW, Parer JT, Stamatoyannopoulous G: Erythrocytosis associated with hemoglobin Ranier: Oxygen equilibria and marrow regulation. J Clin Invest 48:1376, 1969

16. Charache S, Catalano P, Burns S, Jones RT, Koler RD, Rutstein R, Williams RR: Pregnancy in carriers of high-affinity hemoglobins. Blood 65:713, 1985

17. Adachi K, Konitzer P, Lai CH, Kim J, Surrey S: Oxygen-binding properties and mechanical stability of human hemoglobin made in yeast. Protein Eng 5:807, 1992

18. Liebhaber SA, Wang Z, Cash F, Monks B, Russell JE: Developmental silencing of the embryonic zeta-globin gene: Concerted action of the promoter and the 3′ flanking region combined with stage-specific silencing of the transcribed region. Mol Cell Biol 16:2637, 1996

19. Russell JE, Liebhaber SA: The stability of human beta-globin mRNA is dependent on structural determinants positioned within its 3′ untranslated region. Blood 87:5314, 1996

20. Trudel M, Constantini F: A 3′ enhancer contributes to the stage-specific expression of the human beta-globin gene. Genes Dev 1:954, 1987

21. Talbot D, Collis P, Antoniou M, Vidal M, Grosveld F, Greaves DR: A dominant control region from human beta-globin locus conferring integration site-independent gene expression. Nature 33:352, 1989

22. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463, 1977

23. Chang J, Lu RH, Xu SM, Meneses J, Chan K, Pederson R, Kan YW: Inactivation of mouse alpha-globin gene by homologous recombination: Mouse model of hemoglobin H disease. Blood 88:1846, 1996

24. Detloff PJ, Lewis J, John SWM, Shehee WR, Langenbach R, Maeda N, Smithies O: Deletion and replacement of the mouse adult beta-globin genes by a ‘‘plug and socket’’ repeated targeting strategy. Mol Cell Biol 14:6936, 1994

25. Pastzy C, Mohandis N, Stevens ME, Loring JF, Liebhaber SA, Brion CM, Rubin, EM: Lethal alpha-thalassemia created by gene targeting in mice and its genetic rescue. Nat Genet 11:33, 1995

26. Ciavatta DJ, Ryan TM, Farmer SC, Townes TM: Mouse model of human beta(o) thalassemia: Targeted deletion of the mouse beta maj- and beta min-globin genes in embryonic stem cells. Proc Natl Acad Sci USA 92:9259, 1995

27. Rovera G, Magarian C, Borun TW: Resolution of hemoglobin subunits by electrophoresis in acid urea polyacrylamide gels containing Triton X-100. Anal Biochem 85:506, 1978

28. Alter BP: Gel electrophoretic separation of globin chains. Prog Clin Biol Res 60:157, 1981

29. Stryer L: Biochemistry. New York, NY, Freeman, 1993, p 157

30. Jimenez G, Gale KB, Enver T: The mouse beta-globin locus control region: hypersensitive sites 3 and 4. Nucleic Acids Res 20:5797, 1992

31. Hug B, Moon AM, Ley TJ: Structure and function of the murine beta-globin locus control region 5′ HS-3. Nucleic Acids Res 20:5771, 1992

32. Kielman MF, Smits R, Bernini LF: Localization and characterization of the mouse alpha-globin locus control region. Genomics 21:431, 1994

33. Enver T, Li Q, Gale KB, Hu M, May GE, Karlinsey JE, Jiminez G, Papayannopoulou T, Constantini F: Analysis of the developmental and transcriptional potentiation functions of 5′ HS-2 of the murine beta-globin locus control region in transgenic mice. Dev Biol 165:574, 1994

34. Raich N, Papayannopoulou T, Stamatoyannopoulous G, Enver T: Demonstration of a human epsilon-globin gene silencer with studies in transgenic mice. Blood 79:861, 1992

35. Trepicchio WL, Dyer MA, Baron MH: Developmental regulation of the human embryonic beta-like globin gene is mediated by synergistic interactions among multiple tissue- and stage-specific elements. Mol Cell Biol 13:7457, 1993

36. Peters B, Merezhinskaya N, Diffley JFX, Noguchi CT: Protein-DNA interactions in the epsilon-globin gene silencer. J Biol Chem 268:3430, 1993

37. Sabeth DE, Koehler KM, Yang WQ, Patton K, Stamatoyannopoulous G: Identification of a major positive regulatory element located 5′ to the human zeta-globin gene. Blood 82:2899, 1995

38. Sabath DE, Koehler KM, Yang WQ: Structure and function of the zeta-globin upstream regulatory element. Nucleic Acids Res 24: 4978, 1996

39. Russell JE, Morales J, Makeyev A, Liebhaber SA: Sequence divergence in the 3′UTRs of human zeta- and alpha-globin mRNA mediates a difference in their stabilities and contributes to efficient zeta-to-alpha gene developmental switching. Mol Cell Biol 18:2173, 1998

40. Raich N, Enver T, Nakamoto B, Josephson B, Papayannopoulou T, Stamatoyannopoulous G: Autonomous developmental control of human embryonic globin gene switching in transgenic mice. Science 23:1147, 1990

41. Behringer RR, Ryan TM, Palmiter RD, Brinster RL, Townes TM: Human gamma- to beta-globin gene switching in transgenic mice. Genes Develop 4:380, 1990

42. Enver T, Raich N, Ebens AJ, Papayannopoulou T, Constantini F, Stamatoyannopoulous G: Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. Nature 344:309, 1990

43. Shih DM, Wall RJ, Shapiro SG: Developmentally regulated and erythroid-specific expression of the human beta-globin gene in transgenic mice. Nucleic Acids Res 18:5465, 1991

44. Leder A, Daugherty C, Whitney B, Leder P: Mouse zeta- and alpha-globin genes: embryonic survival, alpha thalassemia, and genetic background effects. Blood 90:1275, 1997

1998 92: 3057-3063

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.