

## RAPID COMMUNICATION

# Reversal of Lethal $\alpha$ - and $\beta$ -Thalassemias in Mice by Expression of Human Embryonic Globins

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Genetic mutations that block  $\alpha$ - or  $\beta$ -globin gene expression in humans can result in severe and frequently lethal thalassemic phenotypes. Homozygous inactivation of the endogenous  $\alpha$ - or  $\beta$ -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  $\zeta$ - and  $\epsilon$ -globins, respectively. The capacity of embryonic globins to fully substitute for their adult globin homologues is further demonstrated by showing that  $\zeta$ - and  $\epsilon$ -globins reverse the

hemolytic anemia and abnormal erythrocyte morphology of mice with nonlethal forms of  $\alpha$ - and  $\beta$ -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.

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**M**OLECULAR DEFECTS that affect the level of  $\alpha$ - or  $\beta$ -globin expression result in a collection of clinically heterogeneous disorders known as  $\alpha$ - and  $\beta$ -thalassemias, respectively.<sup>1-3</sup> 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.<sup>2-4</sup> Clinically significant thalassemias are also increasingly recognized in members of many North American immigrant communities.<sup>5</sup> The phenotypes of specific thalassemic mutations are directly proportional to the quantitative deficits in globin gene expression and the resulting imbalance in  $\alpha$ : $\beta$ -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.<sup>1-3</sup> Complete loss of  $\alpha$ -globin gene expression results in mid-gestational fetal demise ( $\alpha$ -thalassemia hydrops fetalis),<sup>5</sup> while complete loss of  $\beta$ -globin expression ( $\beta$ -thalassemia major) is lethal in untreated children.<sup>2,3</sup> 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.<sup>6-8</sup>

Two  $\alpha$ -like and two  $\beta$ -like globin chains assemble into functional hemoglobin (Hb) heterotetramers that reversibly and cooperatively bind four O<sub>2</sub> molecules under physiologic conditions. The expression profile of the constituent globin monomers is developmentally regulated. There are three functional  $\alpha$ -like globin genes in both mouse and humans:  $\zeta$ ,  $\alpha_2$ , and  $\alpha_1$ . Expression of human  $\zeta$ -globin is specific to the primitive, nucleated erythroblasts in the blood islands of the extraembryonic yolk sac.<sup>9,10</sup> As the site of active erythropoiesis migrates to the fetal liver, there is silencing of the  $\zeta$ -globin gene and reciprocal induction of the two  $\alpha$ -globin genes, which continue to express at high levels into adulthood. The human  $\beta$ -like globins are encoded by an embryonic  $\epsilon$ -globin gene, expressed in yolk sac erythroblasts, two fetal  $\gamma$ -globin genes, and a  $\beta$ -globin gene that is maximally induced at birth and encodes almost 98% of the  $\beta$ -like globin chains in normal adult erythrocytes.<sup>1,9,11</sup> Coordinate switching of genes within the  $\alpha$ - and  $\beta$ -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 phylogenetic conservation of this system is unknown, although developmental stage-specific Hbs may optimize O<sub>2</sub> delivery to embryonic, fetal, and adult tissues.

The presence of developmentally silenced, yet structurally intact, globin genes in the  $\alpha$ - and  $\beta$ -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  $\beta$ -thalassemia who overexpress fetal  $\gamma$ -globin, assembling functional Hb  $\alpha_2\gamma_2$ .<sup>2,12</sup> The capacity of embryonic  $\epsilon$ -globin to substitute for its adult  $\beta$ -globin homologue in definitive erythrocytes has not previously been explored. Likewise, the possibility that embryonic  $\zeta$ -globin might substitute for adult  $\alpha$ -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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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<sub>2</sub> affinities in vitro appear to support this model.<sup>13,14</sup> However, these predictions do not account for adaptive mechanisms that permit adults expressing mutant Hbs with a wide spectrum of O<sub>2</sub>-binding affinities to grow and develop normally, and to sustain normal pregnancies.<sup>15,16</sup> For these reasons, in vivo assessments may prove to be more valuable predictors of embryonic globin function.<sup>17</sup> In the current work, we demonstrate the capacity of human embryonic  $\zeta$ - and  $\epsilon$ -globins to reverse the phenotypes of adult  $\alpha$ - and  $\beta$ -thalassemic mice, by substituting for deficient  $\alpha$ - and  $\beta$ -globin chains, respectively. Moreover, we show that  $\zeta$ - and  $\epsilon$ -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)  $\zeta$  transgene, comprising a 0.6-kb human  $\alpha$ -globin promoter fragment linked to the 1.8-kb  $\zeta$ -globin transcribed region and proximal 3' flanking region, was constructed as previously described.<sup>18</sup> A 4.1-kb h $\epsilon$  transgene was constructed using a two-step splice-overlap-extension/polymerase chain reaction synthesis,<sup>19</sup> in which the 1.5-kb h $\epsilon$  transcribed region was inserted between 0.8-kb and 1.7-kb fragments containing the h $\beta$  promoter and enhancer elements, respectively.<sup>20</sup> The h $\zeta$  and h $\epsilon$  transgenes were inserted into polylinker *EcoRI* and *Clal*/*EcoRV* sites of plasmid pSP72/ $\beta$ LCR, respectively, immediately adjacent to a 6.5-kb DNA fragment containing core elements of the human  $\beta$ -globin LCR DNase I hypersensitive sites 1-4.<sup>18,21</sup> All polymerase chain reaction (PCR)-amplified fragments were subsequently verified by dideoxy sequencing.<sup>22</sup> Linked 8.9-kb  $\mu\beta$ LCR/h $\zeta$  and 10.6 kb  $\mu\beta$ LCR/h $\epsilon$  fragments were released by *Sal I*/*EcoRV* or *EcoRI* digestion, respectively, and purified over an Elutip filtration column (Schleicher & Schuell, Keene, NH) before microinjection.<sup>18</sup>

**Transgenic mice.** Transgenic founders were generated according to a standard protocol by the University of Pennsylvania Transgenic and Chimeric Mouse Facility.<sup>18</sup> Founder mice identified by Southern transfer analysis of tail DNA were mated with CD-1 females or C57BL/6 males to generate F1 progeny with germline transgene integration. Generation and characterization of mice with targeted deletions of the endogenous  $\alpha$ - and  $\beta$ -globin genes are reported elsewhere.<sup>23-26</sup> 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  $\mu$ g DNA purified from the tails of candidate mice.<sup>18</sup> [<sup>32</sup>P]-random-primer labeled probes were generated from agarose gel-purified DNA templates. The h $\zeta$  transgene was identified as a 1.2-kb *Pst I* fragment using a 610-bp *Nco I* template encompassing the h $\alpha$ -globin promoter region.<sup>18</sup> The h $\epsilon$  transgene was identified as a 5.1-kb *BamHI* DNA fragment using a 572-bp *BamHI*/*DraIII* template encompassing the junction of the constituent  $\beta$ -globin promoter and  $\epsilon$ -globin transcribed regions. When necessary, a 1.3-kb *BamHI* fragment of intergenic DNA from the mouse (m)  $\alpha$ -globin cluster (the 'X' region) was identified using itself as template.<sup>18</sup> Endogenous wild-type and  $\alpha$ -globin knockout loci were identified as 13.0-kb and 9.0-kb

*HindIII* DNA fragments, respectively, using a 1.3-kb *BamHI* fragment originating 5' to the tandem m $\alpha$ -globin genes.<sup>23</sup> h $\epsilon$  transgene copy numbers were estimated as described,<sup>18</sup> based on two copies of  $\epsilon$ -globin gene/human genome and two copies of 'X' region/mouse genome.

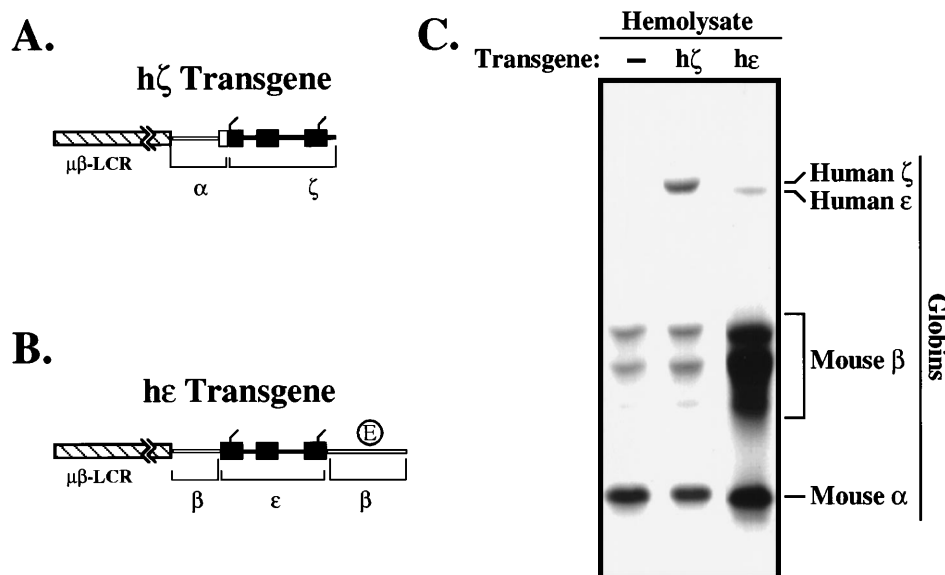
**PCR amplification.** Wild-type m $\beta$ -globin genes were identified as a 250-bp PCR product by amplification of mouse tail DNA using an oligomer pair specific to exons I and II of the mouse  $\beta^{\text{Major-}}$ ,  $\beta^{\text{Minor-}}$ , and  $\beta^{\text{Single-}}$  globin genes (5'CAACCCAGAAACAGACATC3' and 5'CCAAGGGTAGACAACCAGC3'). The  $\beta$ -globin knockout allele (containing an HPRT mini-gene)<sup>24</sup> was identified as a 179-bp product with a sequence-specific oligomer pair (5'GACTGAACGTCTTGCTC-GAG3' and 5'AGCTCTCAGTCTGATAAAATC3'). Primers (100 pmol each) were added to 0.25  $\mu$ g DNA in a 50- $\mu$ L reaction assembled according to the manufacturer's recommendations (Perkin Elmer Cetus, Norwalk, CT) with MgCl<sub>2</sub> adjusted to 2 mmol/L. Amplifications were performed for one cycle at 95°C  $\times$  3 minutes, 60°C  $\times$  15 seconds, and 72°C  $\times$  15 seconds, and for an additional 29 cycles using a modified (92°C  $\times$  1 minute) denaturation step. PCR products were analyzed on an EtBr-stained 5% polyacrylamide gel.

**Globin analysis.** Five to 10  $\mu$ L of phosphate-buffered saline (PBS)-washed transgenic erythrocytes were lysed in 4 vol 1 mmol/L MgCl<sub>2</sub>, 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.<sup>27</sup>

**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 $\times$  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  $\zeta$ - and  $\epsilon$ -globin genes to transcriptional control elements from the human adult  $\alpha$ - and  $\beta$ -globin genes, respectively (Fig 1A and B). The  $\zeta$ -globin transcribed region was linked to the  $\alpha$ -globin promoter to generate the human  $\zeta$  (h $\zeta$ ) transgene,<sup>18</sup> while the human  $\epsilon$  (h $\epsilon$ ) transgene was constructed by inserting the  $\epsilon$ -globin transcribed region between the  $\beta$ -globin promoter and 3' enhancer elements.<sup>20</sup> Each chimeric gene was linked to core elements of hypersensitive sites 1-4 from the  $\beta$ -globin locus control region ( $\mu\beta$ LCR) to promote integration site-independent expression.<sup>18,21</sup> Multiple independent mouse lines containing the h $\zeta$  and h $\epsilon$  transgenes were generated using standard methods,<sup>18</sup> and the transgene copy number for each line was established in F1 mice.<sup>18</sup> The 5' cap and 3' poly(A) addition sites of the transcribed h $\epsilon$ - and h $\zeta$ -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).<sup>27,28</sup> Based on their high-level expression of h $\zeta$ - or h $\epsilon$ -globin, one h $\zeta$ - and one h $\epsilon$ -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  $\zeta$ - and  $\epsilon$ -globins. (A) Construction of the  $h\zeta$  transgene. The full human  $\zeta$ -globin transcribed region (black) was linked to the human  $\alpha$ -globin gene promoter and 5' untranslated region (light).<sup>18</sup> This chimeric  $h\zeta$  transgene was subsequently linked to a  $\mu\beta$ -LCR cassette containing core elements of DNase I hypersensitive sites 1-4.<sup>21</sup> Exons are indicated as filled boxes, and translation initiation and termination codons by tick marks. (B) Construction of the  $h\epsilon$  transgene. The full-length  $\epsilon$ -globin transcribed region (black) is bracketed by the  $\beta$ -globin promoter and 3' flanking region (light), including the 3'  $\beta$ -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  $h\zeta$  and  $h\epsilon$  transgenes. Clarified erythrocyte lysates from a wild type control (-), and  $h\zeta$  and  $h\epsilon$  adult transgenic mice were resolved by Triton-acid-urea gel electrophoresis and visualized by Coomassie blue staining.<sup>27,28</sup> Globin bands are identified to the right. The third lane ( $h\epsilon$ ) was overloaded to demonstrate the  $h\epsilon$ -globin product.

*Expression of embryonic  $h\zeta$ -globin reverses an  $\alpha$ -thalassemic phenotype.* Embryonic  $\zeta$ -globin function in adult erythrocytes was initially assessed by determining its capacity to reverse an  $\alpha$ -thalassemia phenotype. Transgenic mice expressing  $h\zeta$ -globin were mated with mice heterozygous for combined deletion of the closely linked mouse  $\alpha 2$ - and  $\alpha 1$ -globin genes ( $m\alpha^{+/-}$ ; kind gift of J. Chang and Y.W. Kan, University of California, San Francisco).<sup>23</sup> Globin genotypes of the offspring were determined by Southern analysis of tail DNA (Fig 2A), and phenotypes of compound hemizygous  $m\alpha^{+/-}/h\zeta$  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  $\alpha$ -thalassemic erythrocytes.<sup>1-3</sup> In contrast, erythrocytes from sex-matched  $m\alpha^{+/-}$  littermates that coexpressed  $h\zeta$ -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  $h\zeta$ -globin. (Fig 2E). The correction of the  $\alpha$ -thalassemia phenotype in  $m\alpha^{+/-}$  mice coexpressing  $h\zeta$ -globin suggests that the embryonic globin assembles into Hb  $\zeta_2\beta_2$  heterotetramers that are functional in adult erythrocytes.

*Expression of embryonic  $h\epsilon$ -globin reverses a  $\beta$ -thalassemic phenotype.* To assess the function of embryonic  $\epsilon$ -globin in adult erythrocytes, transgenic  $h\epsilon$  mice were mated with mice heterozygous for deletion of the two tandem endogenous adult  $\beta$ -globin genes ( $m\beta^{+/-}$  mice, kind gift of O. Smithies, University of North Carolina, Chapel Hill).<sup>24</sup> 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  $\beta$ -thalassemia.<sup>1-3</sup> Both of these phenotypic abnormalities resolved in  $m\beta^{+/-}$  littermates coexpressing  $h\epsilon$ -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  $h\epsilon$ -globin appeared normal (Fig 2D). Coexpression of the  $h\epsilon$ -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  $h\epsilon$ -globin. These data imply that  $h\epsilon$ -globin acts as a  $\beta$ -like globin in adult erythrocytes by assembling into functional Hb  $\alpha_2\epsilon_2$  heterotetramers.

*Embryonic  $h\zeta$ -globin rescues the viability of mice with homozygous lethal  $\alpha$ -globin gene deletion.* The function of  $h\zeta$ -globin in adult erythrocytes was more rigorously tested by inbreeding  $m\alpha^{+/-}/h\zeta$  mice to generate embryos that were homozygous for the  $m\alpha^-$  deletion ( $m\alpha^{-/-}$ ). Although the expression of endogenous  $m\zeta$ -globin permits normal embryonic development, mice with the  $m\alpha^{-/-}$  genotype die in utero at the time of the embryonic  $\zeta$ - to adult  $\alpha$ -globin switch.<sup>25</sup> Remarkably, however, these matings generated a number of  $m\alpha^{-/-}$  mice whose viability was rescued by expression of embryonic  $h\zeta$ -globin. These  $m\alpha^{-/-}/h\zeta$  mice, which do not synthesize any  $m\alpha$ -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  $m\alpha^{-/-}$  mice directly shows that embryonic  $h\zeta$ -globin is a fully sufficient substitute for adult  $\alpha$ -globin in definitive erythrocytes.

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

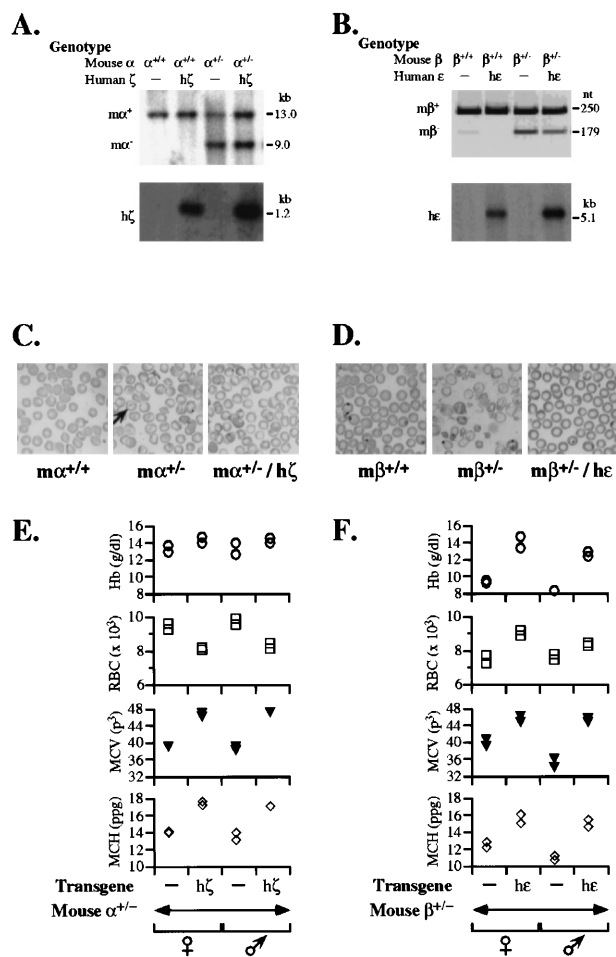
## DISCUSSION

This report demonstrates that embryonic  $\zeta$ - and  $\epsilon$ -globins can functionally substitute for their adult  $\alpha$ - and  $\beta$ -globin homologues in adult erythroid cells. These findings suggest that individuals with  $\alpha$ - or  $\beta$ -thalassemia would likely benefit from reactivation of their endogenous embryonic  $\zeta$ - or  $\epsilon$ -globin

genes, respectively. Moreover, the results bring into question the basis for the phylogenetic conservation of globin gene switching.<sup>15,16,29</sup>

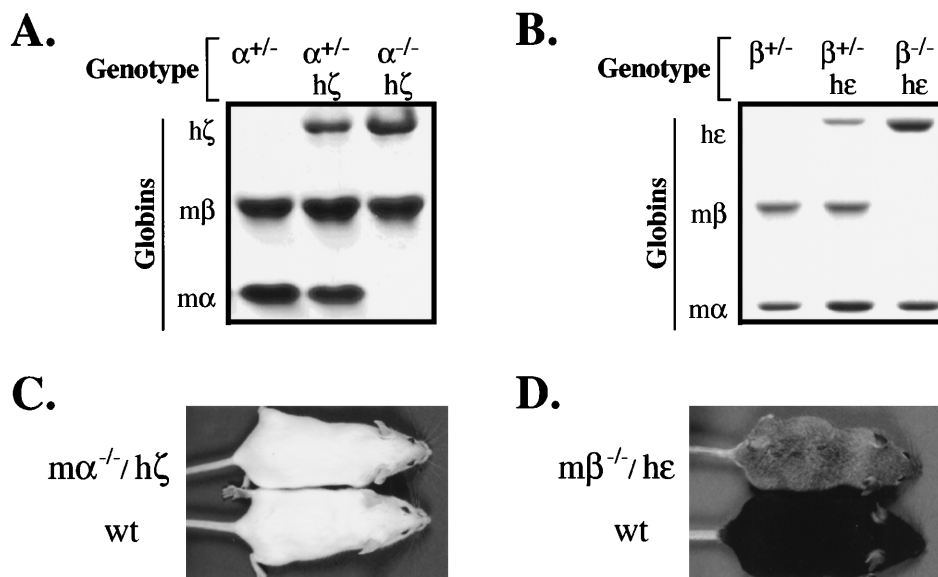
Transgenic mice are a valuable model system in which to study human embryonic globin function. The organization of the globin genes,<sup>1,30-32</sup> molecular controls of their expression,<sup>1,33</sup> and functions of their encoded globin proteins<sup>25,26</sup> are closely conserved between mice and humans. Although mice do not exhibit a clearly defined fetal stage of  $\beta$ -globin gene expression, they parallel human development by expressing different  $\alpha$ -like and  $\beta$ -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  $\alpha$ - or  $\beta$ -globin chains in the mouse result in disorders that closely model human  $\alpha$ - or  $\beta$ -thalassemia, respectively (Fig 2), and that human  $\alpha$ - and  $\beta$ -globins can fully substitute for their mouse counterparts.<sup>25,26</sup> 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.<sup>1,9-11</sup> Silencing of both  $\zeta$ - and  $\epsilon$ -globin genes at the embryonic-to-fetal transition appears to be mediated primarily through their transcriptional regulation,<sup>34-38</sup> although recent evidence suggests that posttranscriptional events



**Fig 2.** Function of human embryonic  $\zeta$ - and  $\epsilon$ -globins in adult murine erythrocytes. (A) Determination of mouse  $\alpha$ -globin genotypes by Southern analysis. Duplicate Southern transfers of *Pst* I-digested DNA from wild-type mice ( $\alpha^{+/+}$ ), mice carrying the  $h\zeta$  transgene ( $\alpha^{+/-}/h\zeta$ ), mice heterozygous for deletion of the  $m\alpha$ -globin genes ( $\alpha^{+/-}$ ), and mice heterozygous for deletion of the  $m\alpha$ -globin genes that carried the  $h\zeta$  transgene ( $\alpha^{+/-}/h\zeta$ ). Blots were probed with a 1.3-kb fragment originating 5' to the deleted  $m\alpha$ -globin sequences (upper autoradiograph)<sup>23</sup> or the  $h\alpha$ -globin promoter (lower autoradiograph).<sup>18</sup> The sizes and identities of the wild-type ( $m\alpha^+$ ) and deleted  $m\alpha$ -globin loci ( $m\alpha^-$ ), and the  $h\zeta$  transgene ( $h\zeta$ ) are indicated to the left and right of each autoradiograph, respectively. (B) Determination of mouse  $\beta$ -globin genotypes by combined Southern and PCR analyses. Tail DNA from mice was coamplified using paired oligomers recognizing wild-type  $m\beta$ -globin genes (250-bp product) or a fragment of the HPRT cDNA comprising the knockout 'socket' (179-bp product).<sup>24</sup> 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  $h\epsilon$  probe indicates the presence/absence of the  $h\epsilon$  transgene. The sizes and identities of the wild-type ( $m\beta^+$ ) and deleted  $m\beta$ -globin loci ( $m\beta^-$ ), and the  $h\epsilon$  transgene ( $h\epsilon$ ) are indicated to the left and right of each autoradiograph, respectively. (C) Thalassemic erythrocyte morphology in  $m\alpha^{+/-}$  mice is corrected by coexpression of embryonic  $\zeta$ -globin. Wright-Giemsa-stained peripheral blood smears from wild-type,  $m\alpha^{+/-}$ , and  $m\alpha^{+/-}/h\zeta$  mice, viewed under oil at 100 $\times$  magnification. A typical 'target cell' is indicated (arrow). (D) Thalassemic erythrocyte morphology in  $m\beta^{+/-}$  mice is corrected by coexpression of embryonic  $\epsilon$ -globin. Wright-Giemsa-stained peripheral blood smears from wild-type,  $m\beta^{+/-}$ , and  $m\beta^{+/-}/h\epsilon$  mice viewed under oil at 100 $\times$  magnification. (E) Resolution of anemia and normalization of erythrocyte indices in  $m\alpha^{+/-}$  mice coexpressing embryonic  $\zeta$ -globin. Erythrocyte analyses were performed on anticoagulated whole blood collected from duplicate sex-matched 13-week adult siblings. The Hb ( $\circ$ ), RBC number ( $\square$ ), MCV ( $\blacktriangledown$ ), and MCH ( $\diamond$ ) are plotted for mice with the globin genotypes and sexes indicated at bottom. (F) Resolution of anemia and normalization of erythrocyte indices in  $m\beta^{+/-}$  mice expressing embryonic  $\epsilon$ -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  $\alpha$ - and  $\beta$ -globin genes by expression of human embryonic  $\zeta$ - and  $\epsilon$ -globin.** (A) Globin profile of  $m\alpha^{-/-}/h\zeta$  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  $m\beta^{-/-}/h\epsilon$  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  $m\alpha^{-/-}/h\zeta$  mice. Female  $m\alpha^{-/-}/h\zeta$  and control CD-1 mice are shown. (D) Physical appearance of  $m\beta^{-/-}/h\epsilon$  mice. Male  $m\beta^{-/-}/h\epsilon$  and control C57BL6 mice are shown.



contribute to this process.<sup>18,39</sup> Elements mapping to the 5' flanking region regulate transcriptional silencing of the  $\epsilon$ -globin gene,<sup>34-36</sup> while transcriptional silencing of the  $\zeta$ -globin gene requires interaction of elements within both the 5' and 3' flanking regions.<sup>18,37,38</sup> To maximize  $\zeta$ - and  $\epsilon$ -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  $\mu$ LCR before microinjection,<sup>18,21</sup> 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  $\alpha$ - and  $\beta$ -globin chain expression in terminally differentiating erythroid cells. The excess globin chains—particularly free  $\alpha$ -globin chains—exhibit cytotoxic effects that manifest clinically as inefficient erythropoiesis and chronic hemolytic anemia.<sup>1-3,5</sup> Hence, the initial evidence that the embryonic  $h\zeta$ - and  $h\epsilon$ -globins could effectively substitute for deficient adult  $\alpha$ - and  $\beta$ -globins, respectively, was inferred from their ability to reverse the thalassemic phenotypes of  $m\alpha^{+/-}$  and  $m\beta^{+/-}$  mice. As in humans who are heterozygous for loss of both  $\alpha$ -globin loci ( $\alpha$ -thalassemia trait),  $m\alpha^{+/-}$  mice grow normally despite a morphologically distinct hypochromic, microcytic anemia (Fig 2C and E).<sup>25</sup> Heterozygosity for loss of  $\beta$ -globin gene expression ( $m\beta^{+/-}$ ) 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),<sup>26</sup> as also occurs in humans.<sup>1-3</sup> Remarkably, the expression of embryonic  $h\zeta$ - and  $h\epsilon$ -globins reverses the growth retardation, anemia, and abnormal erythrocyte morphologies that characterize the thalassemic phenotypes in  $m\alpha^{+/-}$  and  $m\beta^{+/-}$  mice (Fig 2C through F, and data not shown). These results suggest that embryonic globins can substitute for their deficient adult counterparts, bringing the  $\alpha$ : $\beta$  ratio back into balance.

The functional properties of embryonic  $\zeta$ - and  $\epsilon$ -globins were further tested by asking whether they were fully sufficient to

replace their corresponding adult  $\alpha$ - and  $\beta$ -globins, respectively. As in humans,<sup>5</sup> the  $\alpha^{-/-}$  genotype in mice is lethal in utero.<sup>25</sup> Coexpression of embryonic  $h\zeta$ -globin in  $m\alpha^{-/-}$  mice results in viable adults which appear identical to age- and sex-matched wild-type controls (Fig 3A and C). The  $m\beta^{-/-}$  genotype, which is also lethal to mice in utero,<sup>26</sup> differs from its corresponding genetic disorder in humans, because human  $\gamma$ -globin supports fetal growth and development through birth.<sup>1-3</sup> Mice with homozygous lethal deletion of both  $\beta$ -globin loci ( $m\beta^{-/-}$ ) were rescued by expression of embryonic  $h\epsilon$ -globin (Fig 3B and D). Although these  $m\beta^{-/-}/h\epsilon$  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  $h\epsilon$ -globin levels, although functional deficiencies cannot be excluded. Nonetheless, the data demonstrate that embryonic  $\zeta$ - and  $\epsilon$ -globins are remarkably effective substitutes for their adult  $\alpha$ - and  $\beta$ -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  $\zeta$ - and  $\epsilon$ -globins reverses the phenotypes in adult mice with deficient levels of  $\alpha$ - and  $\beta$ -globins indicates that clinically significant  $\alpha$ - and  $\beta$ -thalassemias in humans might be mitigated by reactivation of endogenous embryonic  $\zeta$ - or  $\epsilon$ -globin genes, respectively. The feasibility of this approach is demonstrated by the phenotypic reversion resulting from high-level  $\gamma$ -globin expression in  $\beta$ -thalassemics with concomitant HPFH mutations.<sup>12</sup> Embryonic globin genes are structurally and functionally unaffected by the vast majority of  $\alpha$ - and  $\beta$ -thalassemic mutations, making them theoretically available for reactivation in most fetuses or adults with thalassemia. The current work shows that  $\zeta$ -globin, encoded by the single extant nonadult  $\alpha$ -like globin gene, has potential value to individuals with severe forms of  $\alpha$ -thalassemia (Hb H disease), as well as to developing fetuses with otherwise lethal  $\alpha$ -thalassemia hydrops fetalis.<sup>5</sup> The parallel recognition that  $\epsilon$ -globin can substitute for  $\beta$ -globin in adults

provides an alternate to the  $\gamma$ -globin genes as a target for developing molecular therapies. The  $\epsilon$ -globin gene may be particularly valuable in this regard, as its transcriptional control is gene-autonomous,<sup>40</sup> in contrast with the fetal  $\gamma$ -globin genes, whose expression may be affected by the structural integrity of the adjacent  $\beta$ -globin gene.<sup>41-43</sup> Theoretically, this functional linkage of  $\gamma$ - and  $\beta$ -globin gene expression might complicate attempts to fully reactivate  $\gamma$ -globin gene expression in individuals with thalassemias resulting from nondeletional mutations of the  $\beta$ -globin gene. In addition to its O<sub>2</sub>-transporting capacity,  $\epsilon$ -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  $\alpha\zeta^{-/-}/h\zeta$  mice indicates that  $\zeta$ -globin can subserve the spectrum of functions required of  $\alpha$ -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  $\alpha$ -globin can also subserve both embryonic and adult/fetal functions in mice with homozygous inactivation of endogenous  $m\zeta$ -globin expression.<sup>44</sup> These observations would support previous claims that maintenance of a *trans*-placental O<sub>2</sub> gradient may not be the major force underlying the evolutionary conservation of the  $\zeta$ -to- $\alpha$ -globin switch.<sup>5,16</sup> The phylogenetic 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  $\alpha$ - or  $\beta$ -thalassemic adults would result in a striking survival advantage.

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