An *in vivo* model for analysis of developmental erythropoiesis and globin gene regulation

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ABSTRACT **Expression of fetal γ-globin in adulthood ameliorates symptoms of β-hemoglobinopathies by compensating for the mutant β-globin. Reactivation of the silenced γ-globin gene is therefore of substantial clinical interest. To study the regulation of γ-globin expression, we created the GG mice, which carry an intact 183-kb human β-globin locus modified to express enhanced green fluorescent protein (eGFP) from the Gγ-globin promoter. GG embryos express eGFP first in the yolk sac blood islands and then in the aorta–gonad mesonephros and the fetal liver, the sites of normal embryonic hematopoiesis. eGFP expression in erythroid cells peaks at E9.5 and then is rapidly silenced (>95%) and maintained at low levels into adulthood, demonstrating appropriate developmental regulation of the human β-globin locus. *In vitro* knockdown of the epigenetic regulator DNA methyltransferase-1 in GG primary erythroid cells increases the proportion of eGFP$^+$ cells in culture from 41.9 to 74.1%. Furthermore, eGFP fluorescence is induced >3-fold after treatment of erythroid precursors with epigenetic drugs known to induce γ-globin expression, demonstrating the suitability of the Gγ-globin eGFP reporter for evaluation of γ-globin inducers. The GG mouse model is therefore a valuable model system for genetic and pharmacologic studies of the regulation of the β-globin locus and for discovery of novel therapies for the β-hemoglobinopathies.—McColl, B., Kao, B. R., Lourthai, P., Chan, K., Wardan, H., Roosjen, M., Delagneau, O., Gearing, L. J., Blewitt, M. E., Svasti, S., Fucharoen, S., Vadolas, J. An *in vivo* model for analysis of developmental erythropoiesis and globin gene regulation. *FASEB J.* 28, 2306–2317 (2014). www.fasebj.org**

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Hematopoiesis during mammalian development is characterized by the progressive appearance of distinct populations of cells at stage-specific sites within the embryo. In concert with the progression from primitive to definitive hematopoiesis, the developing erythroid system expresses stage-specific forms of hemoglobin (Hb) in a process known as Hb switching (1).

The Hb tetramer is composed of 2 α-like and 2 β-like proteins, encoded by the α- and β-globin gene loci. During early embryogenesis, clusters of cells in the yolk sac generate "primitive" nucleated red blood cells (RBCs) containing embryonic Hb (Hb Gower-1; $\zeta_2\varepsilon_2$). As development progresses, a second wave of hematopoiesis occurs within the aorta-gonad mesonephros (AGM) region. In the latter stages of embryogenesis, the fetal liver becomes the major site of definitive erythropoiesis, giving rise to RBCs containing fetal Hb (HbF; $\alpha_2\gamma_2$). Finally, the postnatal decline in the expression of fetal γ-globin and the concomitant increase

Abbreviations: AGM, aorta-gonad mesonephros; BAC, bacterial artificial chromosome; BCL11A, B-cell lymphoma/leukemia 11A; DNMT1, DNA methyltransferase 1; E, embryonic day *post coitum*; eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FISH, fluorescence *in situ* hybridization; Hb, hemoglobin; HbA, adult hemoglobin; HbF, fetal hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; LCR, locus control region; MFI, median fluorescence intensity; PCR, polymerase chain reaction; PFGE, pulse field gel electrophoresis; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RBC, red blood cell; RNAi, RNA interference; SCA, sickle cell anemia; WT, wild-type

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of β-globin synthesis results in the production of adult Hb (HbA; $\alpha_2\beta_2$).

This conceptually simple pattern of globin gene expression during development is the result of a complex series of regulatory events. Numerous epigenetic and transcriptional regulators are necessary for switching to occur, in what remains an incompletely understood process, despite several decades of research (2). The study of Hb switching has been stimulated by recognition of the therapeutic potential of globin switching in relation to the inherited β-hemoglobinopathies sickle cell anemia (SCA) and β-thalassemia. Hereditary persistence of fetal hemoglobin (HPFH) is a nonpathologic condition in which the postnatal silencing of the γ-globin gene is reduced or absent, resulting in the maintenance of $\alpha_2\gamma_2$ into adulthood. Coinheritance of HPFH with the SCA or β-thalassemia genotypes results in milder symptoms, due to complementation of the mutant β-globin by the persistent expression of γ-globin (3). Understanding the process whereby γ-globin is silenced is therefore of clinical significance, with the ultimate goal of identifying compounds capable of reactivating γ-globin expression in the adult. This goal has been the driving force behind the generation of several *in vitro* and *in vivo* model systems that replicate the human HbF to HbA switch.

Similar to humans, mice also display β-like globin gene switching through development. Hb switching in mice commences at embryonic day *post coitum* ~10 (~E10), when the predominantly expressed embryonic globins (βh1 and εγ) are replaced by those of adulthood (β_1 and β_2) (4). The first transgenic mice created to investigate human β-globin switching carried only part of the human locus control region (LCR; refs. 5, 6). Consequently, these animals failed to silence γ-globin, suggesting that additional sequences within the native locus are important for correct developmental gene switching. Later models incorporated the entire human β-globin locus, transferred as yeast artificial chromosomes (YACs) or bacterial artificial chromosomes (BACs), and demonstrated expression patterns of the human β-like globin transgenes that closely replicate normal globin switching (7–11).

In previous studies, we demonstrated that a 183-kb BAC clone containing the human β-globin locus can be used to model specific β-thalassemia mutations in transgenic mice (12–14). Subsequently, to facilitate the detection of human γ-globin expression, we modified the same BAC by replacing the ^{G}γ- and ^{A}γ-globin coding regions with that of the enhanced green fluorescent protein (eGFP) reporter. Here, we report the generation and characterization of a transgenic mouse model carrying the γ-eGFP-modified human β-globin locus (termed GG mice).

GG mice express eGFP specifically in the erythroid lineage and undergo progressive silencing of the reporter gene during embryogenesis. Silencing of eGFP is initiated at midgestation, and its expression declines in a manner similar to the pattern of γ-globin expression observed in transgenic mice carrying a native human β-globin locus. Adult mice exhibit a low level of eGFP fluorescence in the RBCs throughout life.

These observations led us to further explore the GG mice as a tool for examining human γ-globin gene silencing. Notably, depletion of DNA methyltransferase-1 (DNMT1) by RNA interference (RNAi) in GG erythroid cells produced a robust reactivation and induction of eGFP, demonstrating the utility of this mouse model for genetic analysis of γ-globin gene expression. Butyrate and decitabine are known inducers of γ-globin and have been used in clinical trials to treat patients with SCA or β-thalassemia (15–17). These agents increased eGFP expression in erythroid precursor cells, both individually and in combination treatments. Because the eGFP fluorescent reporter permits rapid and sensitive detection of γ-globin promoter activity, the GG mouse model is a valuable model system for investigation of γ-globin gene expression by unbiased genetic screens intended to identify components of the regulatory networks by RNAi knockdown and for the identification of novel pharmacologic activators of γ-globin gene expression by *in vitro* high-throughput drug screening.

MATERIALS AND METHODS

Generation of transgenic mice

All animal experiments were undertaken with the approval of the Murdoch Childrens Research Institute animal ethics committee. A BAC clone (pEBAC148β) containing the human β-globin locus was modified by recombineering, replacing the coding regions of the ^{G}γ- and ^{A}γ-globin genes with that of eGFP (pEBAC^{G}γ^{A}γeGFP), thereby placing the reporter under control of the ^{G}γ promoter (18). In brief, the start codon of the EGFP gene was targeted in frame with the start codon of the ^{G}γ-globin gene, whereas the stop codon of the Neo/Kan gene in the eGFP-Neo/Kan cassette was placed at the termination codon of the ^{A}γ-globin gene.

The eGFP-modified human β-globin locus was excised from pEBAC^{G}γ^{A}γeGFP by *Not*I digestion and separated by pulse field gel electrophoresis (PFGE). The genomic fragment was purified by using β-agarase I digestion and concentrated by microdialysis in microinjection buffer (10 mM Tris-HCl, pH 7.4; 0.2 mM EDTA; and 100 mM NaCl). DNA concentration was adjusted to 0.4 ng/μl before microinjection into C57BL/6 fertilized oocytes. Founder mice were identified by polymerase chain reaction (PCR) screening of tail DNA for the eGFP and human β-globin genes. One transmitting founder line was bred with C57BL/6 mice, to establish the independent GG colony.

Fluorescence *in situ* hybridization (FISH)

Approximately 3×10^6 fibroblasts were isolated from tail biopsies and treated with 100 ng/ml colcemide for 1 h. The cells were harvested by centrifugation, incubated in 0.56% KCl for 10 min, and fixed overnight with 3:1 methanol:acetic acid at −20°C before they were dropped onto slides. BAC DNA (pEBAC^{G}γ^{A}γeGFP) was labeled with Spectrum Green-dUTP (Abbott Molecular, Des Plaines, IL, USA) by nick translation. The labeled probe was ethanol precipitated and resuspended in hybridization buffer (50% formamide, 10%

dextran sulfate, and 2× SSC) at 30 ng/μl, then denatured at 72°C for 8 min. Metaphase spreads were denatured in 70% formamide and 2× SSC at 70°C for 2 min, dehydrated through an ethanol series, and hybridized to denatured probe beneath coverslips. Hybridizations were incubated overnight at 37°C and washed in 1× SSC at 70°C for 10 min. After a final PBS wash, the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 500 ng/μl 4,6-diamidino-2-phenylindole and examined with a BX60 fluorescence microscope (Olympus, Tokyo, Japan). Images were captured with Cytovision software (Applied Imaging, San Jose, CA, USA).

Southern blot analysis

Genomic DNA from GG and transgenic mice containing the normal human β-globin locus (as control) was digested with *Mss*I and separated by PFGE. DNA was transferred to nitrocellulose membrane by Southern blot, and the DNA fragments were cross-linked to the membrane by UV irradiation. The membrane was hybridized overnight at 65°C with an eGFP probe and ^{32}P-labeled by random priming, and the hybridizing species was visualized by autoradiography.

Hematologic studies

Blood samples were collected from wild-type (WT) and GG mice by retroorbital eye-bleeding into EDTA tubes. Full blood examinations were performed with an Advia automated hematology analyzer (Bayer, Pittsburgh, PA, USA). Blood smears were prepared and stained with May-Grunewald (Sigma-Aldrich, Castle Hill, NSW, Australia) and Giemsa (Sigma-Aldrich) stains before examination under a light microscope.

Embryo dissection and fluorescence-activated cell sorting (FACS) analysis

WT C57BL/6 females were mated with GG males and euthanized at the times indicated. Embryos were dissected and imaged with a M205FA fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). For antibody staining and FACS, dissected tissues were disaggregated in PBS and 1% fetal calf serum (FCS), washed twice, and counted. A total of 4×10^5 cells were labeled on ice for 20 min with 0.2 μg of APC-conjugated anti-mouse CD71 and 0.2 μg of PE-Cy7-conjugated anti-mouse TER119 (BD-PharMingen, San Diego, CA, USA). The samples were washed 3 times with PBS and 1% FCS and analyzed on an LSR II flow cytometer with FACS Diva software (BD Biosciences, Franklin Lakes, NJ, USA). For analysis of blood from GG mice, the samples were diluted 1:100 with PBS before FACS. For quantitative reverse transcription PCR (qRT-PCR), the tissue samples were prepared as above and sorted according to eGFP expression with a MoFlo cell sorter (Beckman Coulter, Gladesville, NSW, Australia).

qRT-PCR

RNA was extracted from cells with an RNeasy microkit (Qiagen, Chadstone Centre, VIC, Australia), and cDNA was prepared with SuperScript III (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with the primers listed in Supplemental Table S1 with SYBR Green qPCR Supermix (Invitrogen). Reactions were run on an Applied Biosystems 7300 real-time PCR machine (Applied Biosystems, Mulgrave, VIC, Australia). Relative expression was quantified by the Pfaffl method (19), and expression levels were normalized to β-actin.

Primary erythroid culture

Fetal livers from E14.5 GG pups were washed in PBS, disaggregated by gentle pipetting, counted, resuspended in expansion medium at 4×10^6 cells/ml, and maintained at that concentration by daily passaging (37°C, 5% CO_2). Cultures were maintained for 5 d minimum before drug assay or knockdown experiments. Differentiation cultures were established by washing the cells twice with PBS, resuspending them in differentiation medium at 4×10^6 cells/ml, and maintaining them with daily medium changes. Expansion medium consisted of Iscove's modified Dulbecco's medium containing 15% (v/v) FCS, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone, 20 ng/ml insulin-like growth factor, 180 ng/ml stem cell factor, and 2 U/ml erythropoietin. Differentiation medium consisted of Iscove's modified Dulbecco's medium containing 17% (v/v) FCS, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 160 μg/ml biotin, 8.3 ng/ml hypoxanthine, 10 U/ml erythropoietin, 4×10^{-4} IU/ml insulin, 1mg/ml holotransferrin, and 1 μM mifepristone.

Retroviral production and cellular transduction

Retroviruses were produced by calcium chloride cotransfection of HEK293T cells with LMP mir30-based shRNA vector and packaging constructs. Viral supernatants were collected 48 h after transfection and stored at −80°C. For transduction of primary cells, the culture plates were coated with retronectin (32 μg/ml) for 1 h at room temperature. After the plates were blocked and washed, viral supernatant was added, and the plates were spun for 1 h at 3200 *g*. After a wash with PBS, 1.5×10^6 primary erythroid progenitor cells were seeded in each well (MOI 10) in 2 ml expansion medium containing 4 μg/ml polybrene. The cells were incubated overnight and resuspended in fresh expansion medium the following day. Subsequent culture was as described above.

Drug treatment of primary cells

Primary GG erythroid precursor cells were seeded 1.5×10^5/well in 96 well U-bottomed plates, in a total volume of 100 μl of expansion medium/well containing compounds to be tested. Drug solutions were made fresh from powder immediately before the assays. After 24 h, the plates were centrifuged to pellet cells, and the media were removed and replaced with fresh differentiation medium lacking drugs. For flow cytometric analysis of eGFP fluorescence, 20-μl aliquots of the cultures were transferred to a fresh plate and measured with an LSR Fortessa cell analyzer equipped with a robotic high-throughput sampler (BD Biosciences).

RESULTS

Generation and characterization of GG mice

A BAC clone (pEBAC148β) containing the human β-globin locus was modified by recombineering, replacing the coding regions of the ^{G}γ- and ^{A}γ-globin genes with that of eGFP, thereby placing the reporter under control of the ^{G}γ promoter (pEBAC^{G}γ^{A}γeGFP; **Fig. 1** ***A***), as described elsewhere (18). After sequence confir-

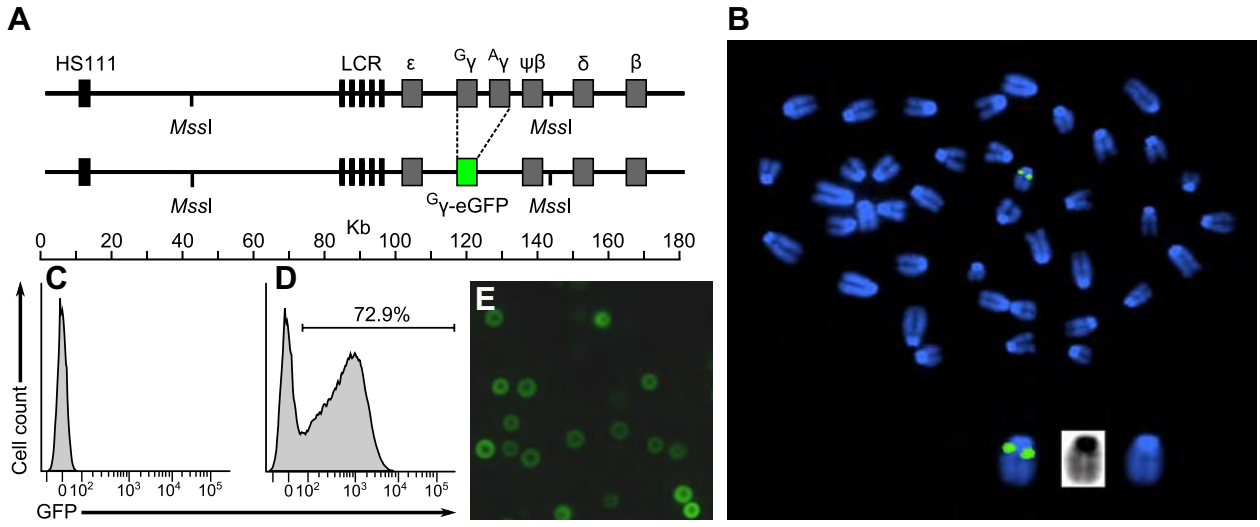

Figure 1. Construction of the GG mice. *A*) Diagram of the modified human β-globin locus. *B*) FISH analysis of metaphase spreads from fibroblasts of a GG mouse. Hybridization of the BAC probe to chromosome 17 is shown in green and in higher magnification (inset). *C*, *D*) Flow cytometric analysis of eGFP fluorescence in peripheral blood from WT control (*C*) and GG littermate (*D*). *E*) eGFP fluorescent cells in peripheral blood viewed under UV illumination.

mation, the purified eGFP-modified human β-globin fragment was microinjected into C57BL/6 fertilized oocytes to generate transgenic mice. Eight oocytes were transferred, yielding 45 pups, of which 4 (9%) were transgenic. Founder mice were identified by screening of tail DNA *via* PCR specific for eGFP and human β-globin. One transmitting founder was identified and bred with C57BL/6 mice, to establish the independent GG colony.

FISH analysis was used to detect the sites of transgene integration in the GG mice with a probe specific to pEBAC$^{G}\gamma^{A}\gamma$eGFP. A single signal was detected on each sister chromatid of metaphase chromosome 17, indicating a single site of integration (Fig. 1 *B*). The integrity of the transgene in GG mice was investigated by Southern blot. After *Mss*I digestion of genomic DNA from 2 GG mice, a single 106-kb species was detected with an eGFP-specific probe, and an identical-sized band was detected in digests of the purified pEBAC$^{G}\gamma^{A}\gamma$eGFP vector (Supplemental Fig. S1 *A*). This observation is in agreement with the predicted sequence of the pEBAC$^{G}\gamma^{A}\gamma$eGFP vector and indicates that most of the transgene sequence is present in GG mice, spanning the region from the LCR to the pseudo-β-globin gene. No hybridizing signal was detected in the lanes containing genomic DNA from transgenic mice containing unmodified pEBAC148β, demonstrating the specificity of detection.

Flow cytometric analysis of the peripheral blood of adult GG mice readily detected eGFP fluorescence at high frequency (65–80% of cells; Fig. 1 *C*, *D*). Also, eGFP fluorescence was visible in unfixed RBCs when viewed under UV illumination (Fig. 1 *E*). Maintenance of the colony was therefore performed by flow cytometric analysis of blood, in preference to PCR genotyping of tail DNA.

Hematologic studies

Blood samples were collected from WT and GG littermates, and full blood examinations were performed (Supplemental Fig. S1 *B*). Examination of Hb concentrations, RBC counts, hematocrit, and mean RBC volume showed no significant difference between WT and transgenic mice. Of all the parameters examined, only RBC distribution width (RDW) showed a statistically significant difference between the GG and WT littermates. The increased RDW indicated a degree of variation in the volume of GG RBCs (anisocytosis). Blood smears showed a small number of poikilocytes in samples from GG mice (data not shown).

These observations suggest a degree of dysmorphology in GG RBCs, presumably stemming from the expression of eGFP. However, it should be noted that extended (>12 mo) maintenance and observation of the GG mouse colony detected no evidence of impaired health or life span. GG mice are fertile, and pups are recovered at the expected mendelian ratios, indicating that the BAC transgene does not significantly impair embryonic development.

eGFP expression in embryogenesis

Low-magnification fluorescence microscopy of GG embryos was performed to examine the distribution of eGFP expression during development. At the earliest time point examined (E7.5–E8.0), dense eGFP fluorescence was present in the proximal region of the yolk sac, the location of the yolk sac blood islands responsible for primitive erythropoiesis in the developing embryo (**Fig. 2** ***A***). Punctate eGFP fluorescence was visible, extending into the distal areas of the yolk sac. Examination along the longitudinal axis at this time point demonstrated that eGFP fluorescence was localized to

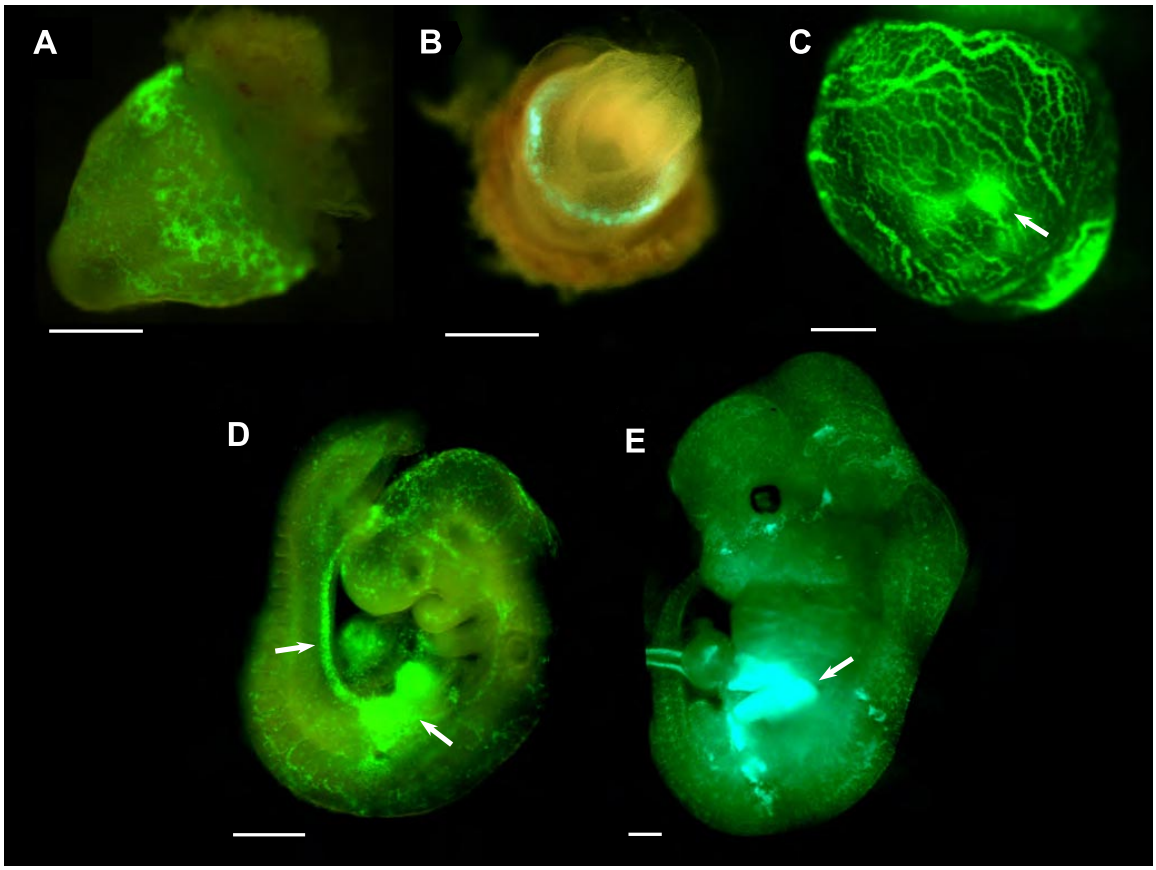

Figure 2. Developmental expression of eGFP in GG mice. *A*) E7.5 embryo. *B*) E7.5 embryo viewed longitudinally, showing the head fold and eGFP fluorescence in the yolk sac. *C*) E9.5 amnion showing eGFP fluorescence localized within the vasculature. Arrow indicates fetal liver. *D*) Dissected E9.5 embryo, showing eGFP fluorescence in the AGM and fetal liver (arrows). *E*) E12.5 embryo; note bright fetal liver fluorescence (arrow). Scale bars = 0.5 mm.

the yolk sac surrounding the embryo, rather than within the embryo proper (Fig. 2 *B*). This distribution of eGFP expression is notably similar to previously reported patterns of murine embryonic globin gene expression (εy and βH1; refs. 20, 21), suggesting that expression of the transgene is localized within the developing erythroid tissue.

During midgestation (E9.5–E10), intense eGFP fluorescence was observed within the AGM region and fetal liver (E9.5–E10, Fig. 2 *C*), corresponding to the sites of definitive erythropoiesis (1). As development progressed (E12.5), eGFP fluorescence became restricted to the fetal liver, demonstrating the migration of hematopoiesis away from the AGM (Fig. 2 *E*). Fluorescence was also clearly visible within the embryonic vasculature, due to high levels of eGFP protein within circulating erythrocytes (Fig. 2 *C*). High-power fluorescence imaging of unfixed blood clearly showed the eGFP fluorescence of individual RBCs (Fig. 1 *E*). Furthermore, eGFP fluorescence within the embryonic vasculature diminished with exsanguination during dissection (data not shown), indicating that fluorescence was localized within the circulating blood cells, rather than in the vascular endothelial cells.

These observations demonstrate that, by visual inspection, the human γ-globin promoter in the BAC transgene is active within the mouse embryo at the locations of normal hematopoiesis. Flow cytometric analysis of $eGFP^+$ cells was therefore performed to further investigate the regulation of the GG transgene.

Flow cytometric analysis of embryonic transgene expression

Flow cytometric analysis was used to examine eGFP fluorescence in embryonic hematopoietic cells, to determine whether the BAC transgene is regulated in a manner reflecting normal globin switching. Multiple parameters were assessed to examine the regulation of the transgene, including fluorescence intensity, the proportion of erythroid cells expressing eGFP, and the specificity of eGFP expression within the erythroid lineage.

Erythroid progenitor cells were identified by dual antibody staining for CD71 and TER119 (**Fig. 3 *A–E***). We observed a rapid and substantial decrease in the intensity of eGFP fluorescence in the erythroid population ($CD71^+/TER119^+$) during embryogenesis. Median fluorescence intensity (MFI) in the $CD71^+/TER119^+$ population decreased ~95% between E9.5 and E17.5 (Fig. 3 *F*), indicating substantial reduction in the activity of the γ-globin promoter during this period.

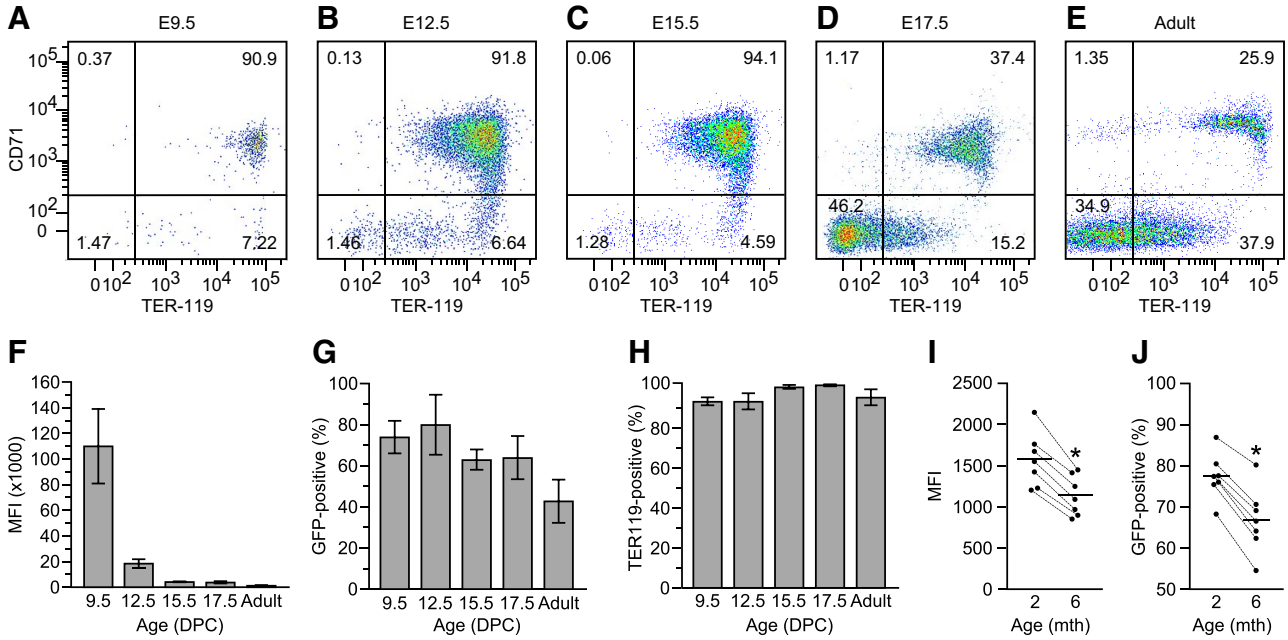

Figure 3. Flow cytometric analysis of developmental eGFP expression in GG mice. *A–E*) Flow cytometric analysis of CD71 and TER119 expression in GG mice at E9.5 (*A*), E12.5 (*B*), E15.5 (*C*), E17.5 (*D*), and adult (*E*) developmental time points. E9.5 samples were generated by disaggregating entire embryos, and fetal livers were dissected and stained for other embryonic time points. Adult samples are from dissected bone marrow. *F, G*) $CD71^+/Ter119^+$ cells were analyzed for eGFP median fluorescence intensity (MFI; *F*) and $eGFP^+$ proportion (*G*). *H*) Samples in panels *A–E* were gated to identify the proportion of $eGFP^+$ cells that are also $TER119^+$. Values are averages ± SD of 3 littermates. *I, J*) Blood of aging GG mice underwent a reduction in eGFP MFI (*I*) and proportion of $eGFP^+$ cells (*J*). * $P < 0.05$; Student's *t* test.

Interestingly, this decrease in eGFP expression was not accompanied by a similar reduction in the overall proportion of $eGFP^+$ erythroid cells. This proportion decreased throughout embryogenesis from >80% at E9.5 to >60% at E17.5; however, most of the erythroid cells maintained a detectable level of eGFP fluorescence (Fig. 3 *G*). This observation demonstrates that, although the activity of the γ-globin promoter is substantially reduced during embryogenesis, it is not completely silenced in most erythroid cells. Furthermore, ~50% of $CD71^+/TER119^+$ cells from GG adult bone marrow exhibited detectable eGFP fluorescence (Fig. 3 *F*), albeit at a greatly reduced intensity relative to that observed during embryogenesis.

The persistent expression of the transgene in a large proportion of erythroid cells when it might be anticipated to be silenced outright led us to examine more closely the specificity of eGFP expression. Flow cytometric data were therefore reanalyzed with a new gating protocol to address this question. By first gating intact cells according to eGFP status (positive or negative) and then analyzing TER119 expression in the $eGFP^+$ population, it is possible to determine the proportion of $eGFP^+$ cells that are of the erythroid lineage. This analysis revealed that expression of the transgene is specific for the erythroid lineage throughout development (Fig. 3 *H*). It therefore appears that the activity of the γ-globin promoter is restricted to the erythroid lineage, indicating that the transgene is regulated in a physiologically relevant fashion.

The activity of the transgene was also examined over time in adult mice, by comparing eGFP fluorescence in peripheral blood of individuals sampled at 2 mo of age and again at 6 mo. Results of this analysis show that the ^{G}γ-eGFP transgene undergoes a further reduction in activity with age. Both the intensity of eGFP fluorescence and the proportion of $eGFP^+$ cells in peripheral blood were significantly reduced with time (Fig. 3 *I, J*), suggesting that silencing of the γ-globin promoter continues into adulthood.

qRT-PCR analysis of globin gene expression during development

Given the erythroid-specific nature of transgene activity, it was possible to recover a largely pure population of erythroid cells by flow cytometric sorting of embryonic tissues on the basis of eGFP expression. Sorted cells were analyzed by qRT-PCR to examine expression of the eGFP transgene and human β-globin genes encoded by the BAC, as well as expression of the native mouse globins.

Results from qRT-PCR further demonstrated that regulation of the BAC transgene follows the physiological pattern of globin switching. Examining the mouse β-like globin genes revealed approximately equal proportions of ε*y* and β*H1* transcripts (**Fig. 4 *A***) at E9.5. By E12.5, ε*y* expression predominates in the liver, while adult β_1 and β_2 transcripts are also detected in that organ, marking the onset of definitive erythropoiesis. By E17.5, as switching continues, β_1 and β_2 are the dominant β-like globin species in both liver and blood,

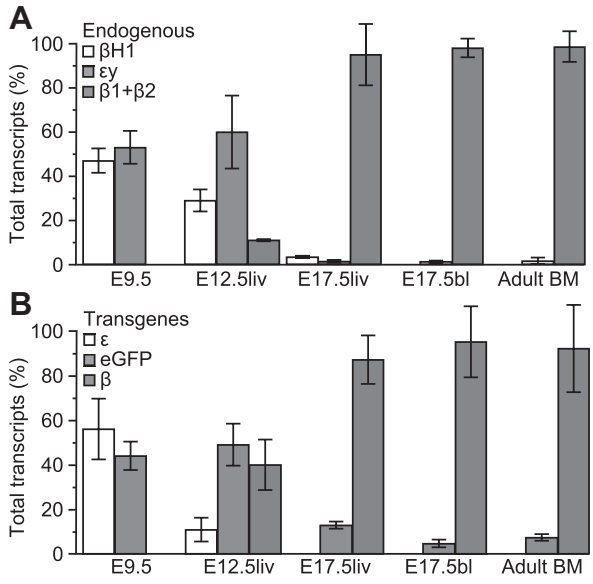

Figure 4. *In vivo* globin gene expression in eGFP$^+$ cells. qRT-PCR analysis of mouse endogenous β-like globin genes (*A*) and transgene expression from the β-globin BAC (*B*). Results are expressed as relative proportions of the 3 genes tested at each time point, averages ± SD of 3 littermates. Note that β_1 and β_2 were undetectable at E9.5, whereas βH1 was not detected in E17.5 blood and adult bone marrow. Human ε was not detected in E17.5 samples or adult bone marrow. bl, blood; liv, liver.

with ε*y* and β*H1* transcripts contributing only a minor proportion of the total (<5%). This pattern of globin switching replicates that previously described (7, 8, 21), validating our strategy of using eGFP expression as a marker of erythroid cells.

Examining levels of eGFP and human ε- and β-globin transcripts from the BAC transgene shows a pattern of expression consistent with normal globin switching. E9.5 samples display approximately equal levels of ε-globin and eGFP transcripts, whereas β-globin expression is undetectable at this time point, demonstrating transgene activity during primitive erythropoiesis. By E12.5, substantial β-globin expression is detected in the liver and is the dominant transcript detected through the remainder of embryogenesis and into adulthood. The expression of eGFP declines to ~10% of the total transcripts by late embryogenesis, consistent with the expected pattern of globin switching. It should be noted that the experimental approach used (sorting for eGFP$^+$ cells) ensures that eGFP$^-$ cells will not be analyzed. However, since eGFP expression is restricted to erythroid cells (Fig. 3 *H*), this strategy is likely to provide an accurate sampling of the erythroid lineage.

Switching of the γ-globin promoter in cultured GG primary erythroid cells

To undertake further analysis of globin switching in the GG mice, we cultured primary erythroid progenitor cells from fetal livers in an erythroid culture system, under conditions that stimulate ongoing expansion of erythroid cultures. After fetal liver harvest at E14.5, the cultures demonstrated a progressive reduction in both fluorescence intensity and the overall proportion of GFP$^+$ cells (**Fig. 5 *A*, *B***). Sampling of cultures for qRT-PCR analysis over the same period demonstrated a reduction of eGFP transcription and a progressive increase in expression of the human adult β-globin gene (Fig. 5 *C*, *D*). In concert with these observations, endogenous mouse globins underwent the expected pattern of switching, with adult β_1 and β_2 becoming predominant after 4 d of culture. Human ε-globin transcripts were undetected after 4 d in culture, in parallel with a reduction in expression of the mouse fetal ε*y* and β*H1* globin genes. It can therefore be concluded that erythroid cells from the GG mice undergo normal globin switching *in vitro*, and the process can be monitored by eGFP fluorescence.

Genetic examination of DNMT1 in globin switching

Our demonstration that cultured primary GG erythroid progenitors undergo the anticipated pattern of globin switching suggests that these cells are a suitable model system to interrogate the β-globin locus *in vitro*. shRNA knockdown of silencing regulators is a potential approach to dissecting the mechanisms underlying repression of the fetal globins. Furthermore, the rapid and sensitive detection of fetal globin promoter activity permitted by the eGFP reporter simplifies this strategy.

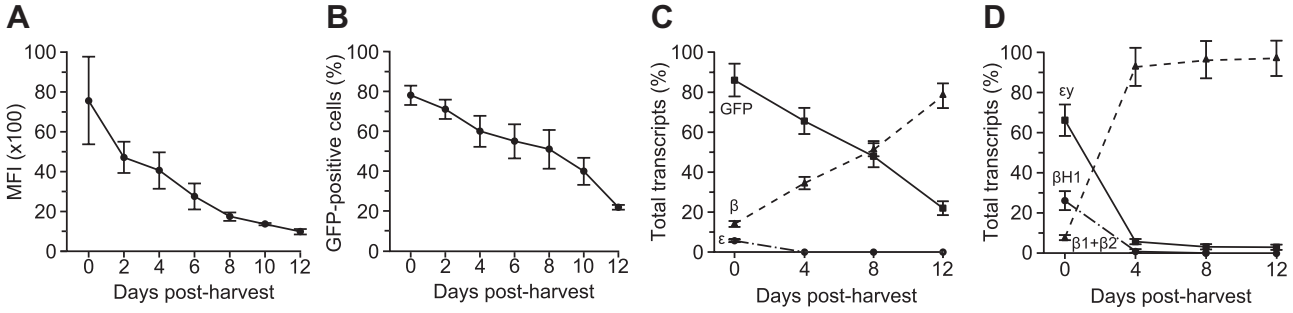

Figure 5. Analysis of cultured GG primary erythroid precursor cells. *A, B*) Flow cytometric analysis of eGFP MFI of cultured GG cells (*A*) and proportion of eGFP$^+$ cells in culture (*B*). *C*) qRT-PCR analysis of transgene expression from the GG BAC. *D*) qRT-PCR analysis of native mouse β-like globins. Results are expressed as relative proportions of the 3 genes tested at each time point. Values are averages ± SD of 3 parallel cultures.

DNA methylation is known to play a role in silencing of the embryonic globin genes (22, 23). Therefore, retrovirally delivered shRNA was used to knock down expression of the *Dnmt1* gene. Two shRNAs targeting DNMT1 were tested (shDnmt1.1 and shDnmt1.2), in comparison to a scramble negative control shRNA. After virus treatment, GG erythroid progenitor cells were cultured for 24 h in expansion medium, followed by transfer to differentiation medium. Transduced cells were identified by expression of the enhanced blue fluorescent protein (eBFP) reporter carried by the retroviral vector, and eGFP expression was measured daily.

No alteration in reporter fluorescence was observed in the 24-h expansion phase immediately following transduction with shDnmt1 retroviruses. However, a significant induction of eGFP fluorescence was observed after 48 h of culture in differentiation medium (**Fig. 6 *A–C***). Furthermore, a substantial increase in the proportion of $eGFP^+$ cells was observed after DNMT1 knockdown (Fig. 6 *A*, *B*, *D*). This observation suggests that the switching event is not irreversible, given that eGFP expression can be restored in cells that were previously $eGFP^-$.

Pharmacologic activation of the $^G\gamma$-eGFP transgene

Pharmacologic treatment of cultured primary GG erythroid progenitor cells was performed to test the potential to develop an *in vitro* screening assay for inducers of γ-globin expression based on detection of eGFP fluorescence. Drug treatments were performed with minimal cells (1.5×10^5/assay) in a 96-well format, to test the suitability of the assay for adaptation to a high-throughput format. Experiments were conducted using the 2-phase erythroid culture system, by treating cells with drugs for 24 h in expansion medium, then changing to differentiation medium free of drug for 72 h. Flow cytometric analysis of eGFP fluorescence was performed every 24 h. For all compounds tested in this study, the eGFP signal declined to that of untreated controls after 48 h in differentiation medium, demonstrating the transient nature of the induction. For clarity, values at 48 and 72 h differentiation are not shown.

Cells were treated with sodium butyrate and decitabine, both known inducers of γ-globin expression (15). The use of the 96-well format allows the simultaneous testing of multiple compounds and drug combinations and the conduct of sufficient replicates to achieve statistical significance. In this study, cells were treated with each drug at high and low concentrations (butyrate 1 mM and 330 μM; decitabine 100 and 33 nM) and with the 2 drugs in combination.

Treatment of cells with 1 mM sodium butyrate resulted in a substantial increase in median eGFP fluorescence, greater than double that of untreated controls after 24 h of drug treatment in expansion medium (**Fig. 7 *A***). After the change to differentiation medium, this increase was maintained after a further 24 h in culture. Median eGFP fluorescence declined at later time points, reaching the level of untreated controls after 48 h in differentiation medium (data not shown). Treatment with the lower of the concentrations of butyrate (330 μM) demonstrated the dose-responsive nature of the assay, giving an ~50% increase in median eGFP fluorescence relative to untreated controls. As with the 1 mM butyrate treatment, elevated eGFP fluorescence was maintained after 24 h under differentiation conditions and then declined.

Treatment of cells with decitabine also increased eGFP fluorescence, although the kinetics of induction were notably dissimilar to those of butyrate, presumably reflecting the differing modes of action of the two compounds. After 24 h in expansion medium containing 100 nM decitabine, no alteration in median eGFP fluorescence was observed. However, at the subsequent time point, after 24 h in differentiation medium lacking the drug, a significant (~60%) induction of fluorescence was observed. Given the mode of action of decitabine as an inhibitor of DNA methyltransferases (24), it is likely that the delayed response is due to the necessity for cells to undergo a cycle of cell division for DNA methylation marks to be lost.

Significant increases in eGFP fluorescence were also observed in response to the two drugs when presented

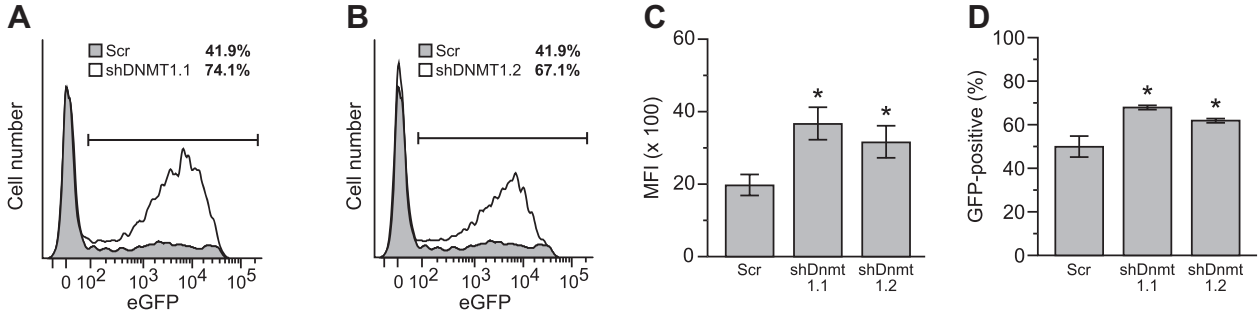

Figure 6. Depletion of DNMT1 reactivates γ-globin promoter in GG primary erythroid precursor cells. Cultured primary cells were treated with retroviruses expressing shRNA and successfully transduced cells identified by flow cytometry on the basis of blue fluorescent protein (BFP) expression. *A*, *B*) Overlay histogram displaying eGFP expression following knockdown with shDNMT1.1 (*A*) and shDNMT1.2 (*B*). *C*, *D*) eGFP MFI (*C*) and proportion of $eGFP^+$ cells following DNMT1 knockdown (*D*). Measurements are averages ± SD of 3 simultaneous experiments. * $P < 0.05$; Student's *t* test.

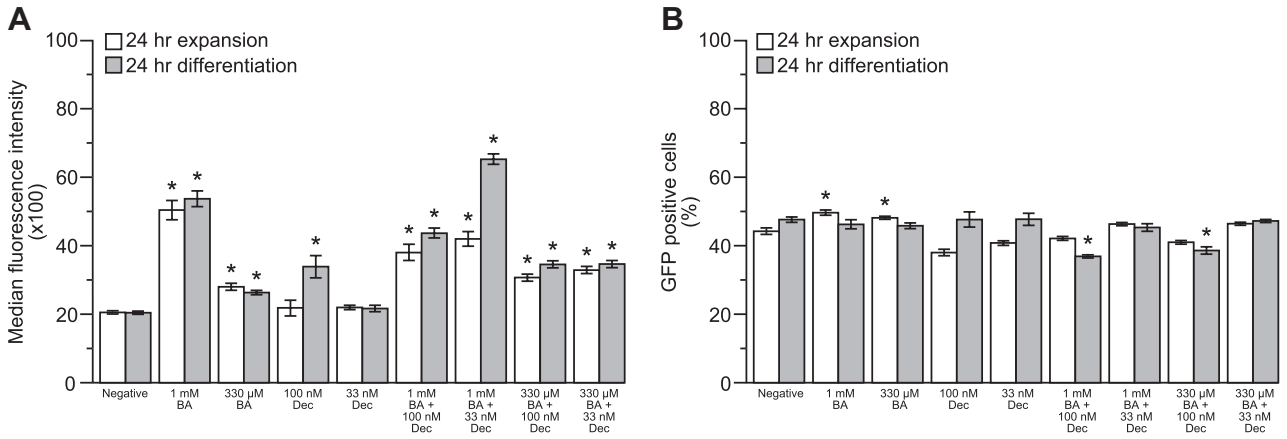

Figure 7. Pharmacologic reactivation of the γ-globin promoter in GG primary erythroid precursor cells. Cultured primary cells were exposed to drugs for 24 h in expansion medium, and then shifted to differentiation medium. Cultures were analyzed for eGFP MFI (*A*) and proportion of eGFP$^+$ cells every 24 h (*B*). BA, butyrate; Dec, decitabine. Values are averages ± SD of parallel triplicate drug treatments and are representative of repeated independent experiments. * $P < 0.05$; Student's *t* test.

in combination to GG erythroid precursors. Treating cells with 1 mM butyrate and 100 nM decitabine in combination resulted in a lower induction of eGFP fluorescence than 1 mM butyrate alone. This perhaps unexpected result is most likely due to toxicity; this combination also reduced the percentage of eGFP$^+$ cells in the culture (Fig. 7 *B*). However, the combination of 1 mM butyrate with 33 nM decitabine was the most effective of all tested, tripling the median eGFP fluorescence at the 24 h differentiation time point, without reducing the proportion of eGFP$^+$ cells in culture.

Combination treatments with 330 μM butyrate combined with high or low concentrations of decitabine resulted in moderate induction of eGFP fluorescence, greater than that of 330 μM butyrate alone. Of note was the effect of these combinations on the percentage of eGFP$^+$ cells in culture. The combination of 330 μM butyrate with 100 nM decitabine resulted in a diminished proportion of fluorescent cells, particularly when measured after the switch to differentiation medium. Reducing the decitabine concentration to 33 nM maintained the proportion of eGFP$^+$ cells relative to untreated controls and still induced equivalent expression of eGFP. This observation suggests that drug treatments can be optimized to maximize induction of γ-globin while minimizing toxic effects. Note also that shRNA knockdown of DNMT1 resulted in an increase in the proportion of eGFP$^+$ cells in culture (Fig. 6), suggesting that the decrease in eGFP$^+$ cells observed after 100 nM decitabine treatment is not due to the effect of the drug on DNMT1, but rather is the result of off-target toxic effects.

DISCUSSION

Treatments for the β-hemoglobinopathies based on reactivating γ-globin gene expression have been under consideration for several decades; however, therapeutics intended to induce synthesis of γ-globin have demonstrated only limited clinical success (15, 25). Recent advances in our understanding of γ-globin silencing have come from the study of individuals with HPFH (26–28). Genome-wide association studies of the genetic basis for HPFH identified single-nucleotide polymorphisms located within the *HBS1L-MYB* intergenic region, as well as within the gene encoding the transcription factor B-cell lymphoma/leukemia 11A (BCL11A) (26, 27, 29–31). Mutations in the gene encoding the erythroid transcription factor Krüppel-like factor 1 (KLF1) have also been identified in individuals with HPFH (28). In addition, molecular studies have identified several critical determinants of Hb switching, such as the erythroid transcription factors GATA1 (32), FOP1 (33), and SOX6 (34) and the TR2/TR4 orphan nuclear receptors (35).

In vivo studies of Hb switching have been made possible by the creation of several humanized mouse models carrying an intact β-globin locus (7–11). Notably, transgenic mice carrying the native human β-globin locus can replicate the regulation of the human β-like globin genes. It should be noted that transgenic mice undergo the γ- to β-globin switch before birth, whereas in humans it occurs after birth. Despite this, such mice have proven to be useful model systems in which to study human globin switching, a body of work that we have built on with the creation of the GG mice. By incorporating a ^{G}γ-eGFP reporter into an intact human β-globin locus, we created a mouse model that replicates the native pattern of γ-globin gene expression, while taking advantage of the readily quantifiable fluorescence generated by eGFP. Previous models have used fluorescent reporters within the hematopoietic system (36), demonstrating the feasibility of this approach. Analysis of the GG mice demonstrated activation of the ^{G}γ-eGFP reporter at the appropriate sites of developmental hematopoiesis. High eGFP expression is observed during early embryogenesis in the yolk sac, followed by expression at lower levels in the AGM and fetal liver at later stages of embryogenesis. Notably, characterization of eGFP expression patterns during

development demonstrated that the reporter is appropriately regulated, in agreement with previous observations of other mouse models containing the human β-globin locus (7–11).

The GG mice exhibit widespread (65–80%) expression of eGFP in adult blood. This observation appears anomalous when compared to the reported frequency of γ-globin-containing F cells in adult human blood (<5%; ref. 37) or in β-globin transgenic mice (7). It may be that a small number of transcripts from the γ-globin promoter are sufficient for detection by flow cytometry, and therefore the apparently pan-cellular expression of eGFP is simply due to minimal activity of the γ-globin promoter, rather than to the inappropriate regulation of the transgenic locus. Alternatively, there is evidence that sequences in the $^{G}\gamma$–$^{A}\gamma$ intergenic region function in silencing (6, 38, 39) and may harbor a BCL11A binding site (34). The absence of this region in the GG construct may therefore result in incomplete silencing of the $^{G}\gamma$-eGFP transgene. However, we have demonstrated by multiple methods that the $^{G}\gamma$-eGFP transgene undergoes substantial developmental repression and closely follows the patterns of expression reported for similar mouse models, indicating that the silencing process is functioning as anticipated in the GG mice.

Previous mouse models of globin switching have not provided the capacity to measure activity of the γ-globin promoter in a rapid and sensitive manner. A similar mouse model incorporating dual fluorescent reporters in the human β-globin locus has recently been described (40); however, in that model, expression from the reporters is not sufficiently high to allow unambiguous detection by flow cytometry. The GG mice are not subject to such limitations; indeed expression of the reporter gene is sufficiently high to allow visualization and quantitation of erythroid-specific eGFP expression at sites of hematopoiesis during embryogenesis.

Recent epigenetic profiling of the β-globin locus has provided insights into the nature of globin switching and has shown that modifiers of chromatin, such as lysine-specific demethylase (KDM1) and DNMT1, are involved in the process (22, 41, 42). Multimeric transcriptional regulators such as the direct-repeat erythroid-definitive (DRED) complex, CoREST, and nucleosome remodeling deacetylase (NuRD) have also been identified as being involved in γ-globin globin gene regulation (39, 42–44), revealing the complexity of the molecular mechanism responsible for γ-globin switching. Since the eGFP fluorescent reporter can be used for rapid and sensitive detection of γ-globin promoter activity, we explored the utility of the GG mice for investigating the mechanism governing γ-globin silencing. Repression of γ-globin during development correlates with the methylation of the regulatory regions of the gene (45, 46). The role of DNMT1 in this process was demonstrated by the observation that depletion of the protein in bone marrow cells from mice carrying the human β-globin locus reduces methylation of the ε- and γ-globin gene promoters, leading to elevated expression of both genes (47). Of note, recent proteomic screens for corepressors of γ-globin identified DNMT1 in complexes with two key determinants of Hb switching: BCL11A and TR2/TR4 (43, 48). These findings indicate a functional role for DNMT1 in the maintenance of γ-globin gene silencing in adult erythroid cells. This body of work is consistent with the observed reactivation of the γ-globin promoter in GG cells after DNMT1 depletion. Notably, *in vitro* treatment of primary GG erythroid cells with DNMT1-specific RNAi increased both the intensity of eGFP fluorescence and the overall proportion of $eGFP^{+}$ cells in culture, further demonstrating the significance of DNMT1 as an essential epigenetic component of γ-globin gene silencing. Furthermore, the demonstration that the eGFP reporter can be reactivated is encouraging, in that the ultimate goal of these studies is to identify compounds capable of reactivating silenced γ-globin *in vivo*.

With the growing recognition of the epigenetic nature of globin switching, efforts have been made to identify compounds that can reactivate γ-globin by chromatin modification, thereby providing novel therapies for the β-hemoglobinopathies. We tested the suitability of the GG mice to assess γ-globin gene reactivation following *in vitro* treatment of primary erythroid cells with known HbF inducers that target chromatin state. Butyrate is a member of the short-chain fatty acids (SCFAs), chemicals that increase gene expression by inhibiting histone deacetylase, thereby affecting nucleosome stability and chromatin accessibility (49). Decitabine is one of the cytidine nucleoside analogues that deplete DNMT1, leading to hypomethylation of DNA (24). In this study, *in vitro* treatment of primary erythroid cells from the GG mice with butyrate or decitabine increased eGFP expression. Notably, combination treatments with butyrate and decitabine further enhanced eGFP expression, and the effects of decitabine were readily detectable at concentrations below those considered to be myelotoxic (50). Cotreatment with butyrate and high levels of decitabine reduced the proportion of $eGFP^{+}$ cells in culture, possibly as a result of cytotoxic effects. This observation highlights the possible limitations of such combination therapy, but also demonstrates the utility of the GG model as a tool for drug screening.

Previous drug screening approaches have used hematopoietic cell lines transfected with reporters under control of minimal globin promoters. Dual reporter cell lines have been developed by our laboratory, in which the dsRed and eGFP coding regions are inserted into the human γ- and β-globin genes, respectively, thereby creating a reporter within an intact β-globin locus with all regulatory regions in place (51). The GG mice extend on this approach by providing the intact reporter locus in primary cells, thereby avoiding the potential for biologically irrelevant artifacts that accompany all cell line-based experiments. Furthermore, the high *in vitro* replicative capacity of fetal liver–derived erythroid precursors simplifies acquisition of the large number of cells required for any screening programs. It

is therefore envisaged that the GG mice will be a valuable model system for identification of pharmacologic activators of γ-globin gene expression or for unbiased genetic screens intended to identify components of γ-globin gene silencing by RNAi knockdown.

In addition to reactivation of γ-globin, the labeling of sites of hematopoiesis in the developing GG embryo and adult suggest that this model would also be useful for investigation of the basic biology of early blood cell development. Many questions of fundamental biology remain unanswered in the hematopoietic field, in particular relating to the origins of the hematopoietic precursors and the molecular details underlying the transition from the primitive to definitive lineage. Several characteristics of the GG mice indicate the potential of the model to investigate these questions. The erythroid-specific expression of the transgene permits purification of a largely pure population of cells by single-color FACS. Also, expression of the GG transgene is observed in both the primitive and definitive lineages, and is detectable in hematopoietic precursors. By taking advantage of these attributes, further work will be undertaken to examine the molecular details of hematopoiesis, possibly through expression array studies of eGFP-sorted cells. Such an approach has the potential to comprehensively examine the changing patterns of gene expression in the developing hematopoietic system.

Examining the processes now known to be part of globin switching also sheds light on the magnitude of the therapeutic challenge this system presents. In particular, epigenetic events play a major role in silencing γ-globin expression. The epigenetic field is one that is undergoing rapid advances; however, until recently, there has been only slow progress in understanding the relationship between gene expression and epigenetic modification of the genome. Consequently, while knowledge of the significance of chromatin modification in gene activity remains limited, progress in the comprehension of globin switching is also likely to be slow. The GG mouse model has the potential to accelerate the study of globin switching, by providing a platform suitable for high-throughput analysis of genetic and pharmacologic modifiers of γ-globin expression. Such advances will form the basis of the next generation of therapies for β-thalassemia and sickle cell anemia. FJ

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Dedication: This paper is dedicated to the memory of the authors' friend and colleague Maria Kastoras, who died on December 25, 2013, and is in tribute to her valuable work for Thalassaemia Australia.

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