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Effects of human γ -globin in murine β -thalassaemia

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Summary

Murine models of β -thalassaemia have been used to test therapeutic globin gene vectors. However, the level of γ -globin expression necessary to achieve full phenotypic correction in these models is unclear. In order to address this issue, we carried out breeding and transplantation studies in murine models of β -thalassaemia intermedia ($Hbb^{th-3/+}$) and severe β -thalassaemia major (Hbb^{th-3}/Hbb^{th-3}) using transgenic lines expressing various levels of human γ -globin. Expression of γ -globin RNA at a modest 7–14% of total α -globin RNA resulted in the selective survival of HbF(+) erythrocytes, a fivefold increase in total HbF, and a phenotypic improvement in the β -thalassaemia intermedia model. Full normalisation of erythrocyte indices in this model required γ -globin RNA expression at 27% of α -globin, resulting in an average 40% (6.8 g/dl) HbF. Studies using the homozygous Hbb^{th-3} model of lethal β -thalassaemia major demonstrated that even this high level of γ -globin expression, for reasons related to the function of the hybrid globin tetramers, could only prolong, but not fully support, survival. Taken together, these results indicate that only the heterozygous Hbb^{th-3} model of β -thalassaemia intermedia can be reliably used for the pre-clinical assessment of γ -globin gene therapy vectors, as well as other means of γ -globin gene induction.

Keywords

haemoglobin; thalassaemia; molecular; animal model

Murine models of β -thalassaemia and sickle cell disease developed over the past decade have been used extensively to study the patho-physiology of, and potential therapies for, these diseases (Sadelain *et al*, 2004). In particular, such models have been used to assess the functional properties of recombinant virus vectors for variants of human β -globin and γ -globin designed to treat the β -chain haemoglobinopathies. Most of these studies have focused on one of three murine models of relatively mild β -thalassaemia intermedia or sickle cell disease (May *et al*, 2000, 2002; Pawliuk *et al*, 2001; Imren *et al*, 2002; Person *et al*, 2003; Hanawa *et al*, 2004), while only one study has focused on a murine model of severe β -thalassaemia major (Rivella *et al*, 2003). Likewise, most of these studies have also focused on the use of recombinant virus vectors for wild type (WT) or variant forms of human β -globin (May *et al*, 2000, 2002; Pawliuk *et al*, 2001; Imren *et al*, 2002), while relatively few have focused on the use of vectors for human γ -globin (Person *et al*, 2003; Hanawa *et al*, 2004). These and other studies (Person *et al*, 2001) have provided some information correlating the level of β -globin or γ -globin gene expression with the level of phenotypic improvement in these models. However, several critical questions remain regarding the level of human globin gene expression, and especially γ -globin gene expression that is necessary to improve or normalise the phenotypic sequela in these thalassaemia models. For example, it is unclear whether expression of γ -globin confers the same degree of selective RBC survival in murine models of

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β -thalassaemia as that observed in patients with equivalent disease. The amount of γ -globin expression that is necessary to fully normalise the wide spectrum of erythrocytic indices in murine models of β -thalassaemia has also remained unresolved. Finally, no studies have been reported to date on the ability of human γ -globin to rescue the murine model of lethal β -thalassaemia major characterised by a complete absence of β -chains.

In the studies described here, we sought to address these questions in order to better characterise and justify the use of these models as a means of validating candidate gene transfer vectors for human γ -globin prior to their use in clinical trials. For these studies, we focused on the *th-3* murine model of β -thalassaemia (Yang *et al*, 1995). In this model, both the *Hbb-b1* and *Hbb-b2* adult β -globin genes are deleted in *cis*. Homozygous *Hbb^{th-3}/Hbb^{th-3}* mice die perinatally, while heterozygous *Hbb^{th-3}/+* mice backcrossed to C57BL/6J exhibit a phenotype of β -thalassaemia intermedia with decreased haematocrit and haemoglobin (Hb) content, increased reticulocytes, and abnormal red blood cell (RBC) morphology.

We carried out a series of studies crossing *Hbb^{th-3}/+* mice to transgenic lines expressing various levels of human γ -globin. This included lines expressing a wider range of γ -globin and a more comprehensive analysis of RBC indices than previously reported (Person *et al*, 2001). We also included a comparison with non-thalassaemic controls in order to accurately assess the degree of selective RBC survival/expansion afforded by γ -globin expression in this thalassaemic environment. In a separate series of studies, we carried out breeding and fetal liver cell (FLC) transplantation studies so as to assess the ability of the human γ -globin transgenes to rescue the lethal phenotype in the *Hbb^{th-3}/Hbb^{th-3}* model of β -thalassaemia major. The results of these studies are compared to similar studies in murine models of β -thalassaemia with human β -globin and γ -globin, as well as to clinical observations of patients with varying degrees of β -thalassaemia and elevated levels of HbF.

Materials and methods

Mouse strains

Wild-type C57BL/6J mice and thalassaemic *Hbb^{th-3}/+* mice (Yang *et al*, 1995) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Thalassaemic *Hbb^{th-3}/+* mice are heterozygous for a deletion encompassing both the *Hbb-b1* and *Hbb-b2* adult β -globin genes in *cis*, and thus fail to express all β -like globins from this allele. The transgenic lines 1279 and 135/4-2 have been previously described (Enver *et al*, 1989; Constantoulakis *et al*, 1991; Stamatoyannopoulos *et al*, 1993). As shown in the top panel of Fig 1, line 1279 (herein labelled γ^{lo}) contains three copies in *cis* of a transgene consisting of a 2.5-kb composite of the human β -globin locus control region (termed a μ LCR) linked to a coding cassette for human γ -globin extending -1348 bp 5' from the transcriptional start site. This includes a putative stage-specific silencer that results in minimal transgene expression in adults. The bottom panel of Fig 1 shows that line 135/4-2 (herein labelled γ^{hi}) contains nine copies in *cis* of a transgene similar to that in line 1279, except that the γ -globin gene promoter is truncated to -382, effectively deleting the stage-specific silencer. The *Hbb^{th-3}/+* line and the two transgenic lines were backcrossed to C57BL/6J for more than 10 generations. Combinations of β -thalassaemia and γ -globin transgenic lines were then generated by appropriate crosses. Offspring were typed for the *Hbb^{th-3}* allele by polymerase chain reaction (PCR) analysis for the residual *hprt* gene from the knock-out cassette using the following primers: 5'-GGCAAAGGATGTGATACGTGGAAG-3' and 5'-CCAGTTTACTAATGACACAAACATG-3'. These results were confirmed by quantitative real-time PCR analysis for murine β -globin using the following primers: β -globin, 5'-GTCATCACCGAAGCCTGATT-3' and 5'-TGTCTGTTTCTGGGGTTGTG-3'; *Aire* (internal control), 5'-CACACAGGCAATTTGTCCAC-3' and 5'-AGAGGAAGCGGAAAGTTGT-3'. This latter approach was also used to confirm the

genotype of homozygous *Hbb^{th-3}/Hbb^{th-3}* fetuses. Offspring were typed for the γ -globin transgenes by immunofluorescent analysis (described below) to identify transgene-positive individuals, and real-time PCR to differentiate between heterozygotes and homozygotes using the following primers: γ -globin, 5'-TGGCTAAA CTCCACCCATGGGTTG-3' and 5'-CCAGAAGCGAGTGTGTGGAAGTCT-3'; *Aire* (internal control) described above. All animal studies were carried out in accordance with accepted standards and were approved by the University of Washington Institutional Animal Care and Use Committee.

Transgene expression analysis

Total RNA prepared from peripheral blood was analysed by RNase protection as previously described (Stamatoyannopoulos *et al*, 1993), using the following probes: pT7M α (128) linearised with HindIII to give a 128 bp protected fragment within exon 1 of the mouse α -globin gene; and pT7 γ (170) linearised with BstEII to give a 170 bp protected fragment within exon 2 of the human γ -globin gene. A total of 50 ng RNA was hybridised overnight at 48°C with 10⁶ cpm of each radiolabelled probe. A pilot experiment confirmed that the probe was in excess under these conditions. After digestion with RNase A and T1, the protected fragments were separated on 6% polyacrylamide 8 mol/l urea gels, and autoradiography was performed without intensifying screens. Signal intensities were quantified by Phosphorimager, and expression levels of the human γ -globin transgenes were calculated as a percentage of total mouse α -globin. For immunofluorescent analysis, 3 μ l of peripheral blood was fixed in 4% formaldehyde for 30 min at room temperature, permeabilised by sequential washes in cold acetone, stained with a phycoerythrin-conjugated monoclonal antibody for HbF (BD Bioscience, San Jose, CA, USA), and scanned on a FACScan flow cytometer (BD Bioscience) using cellquest software as previously described (Thorpe *et al*, 1994; Emery *et al*, 2002). Protein analysis was performed on an automated high-performance liquid chromatography (HPLC) analyser (Variant II β -thalassaemia short program; Bio-Rad Laboratories, Hercules, CA, USA).

Haematological analysis

For reticulocyte analysis, 1 μ l of peripheral blood was incubated in Hank's balanced salt solution with 0.01 μ g/ml of thiazole orange (Sigma-Aldrich, St Louis, MO, USA), 2 mmol/l of EDTA and 0.02% sodium azide for 1–2 h at room temperature as previously described (Davis *et al*, 1995). The stained cells were analysed on a FACScan flow cytometer using cellquest software. All other blood cell analyses were performed on a Cell-DYN 3500 hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA).

FLC transplantation

Donor fetal livers were harvested from embryonic day 15.5 embryos obtained by intercrossing either *Hbb^{th-3}/+* mice or *Hbb^{th-3}/+* mice homozygous for the γ^{lo} or γ^{hi} transgene alleles. Fetuses were diagnosed by colour phenotype and PCR so as to identify *Hbb^{th-3}/Hbb^{th-3}* homozygotes. These fetal liver preparations were mixed in various ratios and transplanted at a dose of 3×10^6 total cells per recipient into congenic C57BL/6J mice previously treated with 1100 cGy myeloablating total body irradiation. Transplant recipients were monitored routinely for signs of anaemia and were killed when moribund or profoundly anaemic with haematocrits $\leq 15\%$.

Results

Generation and expression analysis of β -thalassaemic/ γ -globin transgenic lines

The β -globin knockout mouse line *Hbb^{th-3}* (Yang *et al*, 1995), and two γ -globin transgenic mouse lines, line 1279 (γ^{lo}), which expresses low levels of γ -globin (Enver *et al*, 1989; Constantoulakis *et al*, 1991), and line 135/4-2 (γ^{hi}), which expresses high levels of γ -globin

(Stamatoyannopoulos *et al*, 1993), were backcrossed to C57BL/6J and then crossed to generate a series of β -thalassaemia/ γ -transgene hybrids. As shown in Fig 1 and discussed more fully in the *Materials and methods* section, these two transgenic lines express different levels of human γ -globin as a result of differences in 5'-promoter regions and transgene copy numbers. For further analysis, we chose to focus on three γ -globin transgene allele combinations: heterozygous γ^{lo} ($\gamma^{lo/-}$), homozygous γ^{lo} (γ^{lo}/γ^{lo}), and homozygous γ^{hi} (γ^{hi}/γ^{hi}). The amount of γ -globin expressed from these three transgene allele combinations against both a WT C57BL/6J background and on the heterozygous $Hbb^{th-3/+}$ - β -thalassaemia background were determined at the level of RNA (by RNase protection) and at the level of protein (by HPLC).

In the WT background, γ -globin RNA expression levels ranged from $7.0 \pm 0.8\%$ (*versus* total mouse α -globin) for the $\gamma^{lo/-}$ transgene allele, $13.9 \pm 1.3\%$ for the γ^{lo}/γ^{lo} transgene combination, and $27.2 \pm 5.9\%$ for the γ^{hi}/γ^{hi} transgene combination (Fig 2, left panel). These levels were consistent with previously reported expression levels for these transgenic lines (Enver *et al*, 1989; Constantoulakis *et al*, 1991; Stamatoyannopoulos *et al*, 1993) and were significantly different from one another ($P \leq 0.001$). In these same lines, protein expression levels ranged from $2.7 \pm 0.4\%$ (HbF *versus* total Hb) for the $\gamma^{lo/-}$ transgene allele, $5.2 \pm 0.7\%$ for the γ^{lo}/γ^{lo} transgene combination, and $12.0 \pm 2.7\%$ for the γ^{hi}/γ^{hi} transgene combination. These levels were also significantly different from one another ($P \ll 0.001$), but represented an average 60% reduction compared with RNA levels. This discrepancy between RNA and protein levels may reflect a modest competitive advantage of mouse β -globin over human γ -globin in associating with mouse α -globin or possibly a modest discrepancy in the relative efficiency of translation.

Evidence for selective advantage of murine thalassaemic red cells containing fetal Hb

RNA levels remained essentially unchanged when these same transgene allele combinations were crossed onto the $Hbb^{th-3/+}$ - β -thalassaemia background, ranging from $6.8 \pm 1.5\%$ for the $\gamma^{lo/-}$ transgene allele, $15.8 \pm 1.6\%$ for the γ^{lo}/γ^{lo} transgene combination, and $27.1 \pm 4.2\%$ for the γ^{hi}/γ^{hi} transgene combination (Fig 2, left). This suggests that: (i) γ -globin transgene expression fails to convey a selective survival advantage at the early stages of erythrocyte development when RNA is still present; and (ii) transcription of the γ -globin transgene is not effected when there is deficient β -globin transcription in *trans*. However, protein analysis provided strong evidence of a selective survival advantage for mature RBC expressing the γ -globin transgene, as shown by an increase in protein expression levels ranging from $13.7 \pm 1.2\%$ for the $\gamma^{lo/-}$ transgene allele, $25.4 \pm 3.5\%$ for the γ^{lo}/γ^{lo} transgene combination, and $40.0 \pm 4.7\%$ for the γ^{hi}/γ^{hi} transgene combination. This represented a highly significant increase in the levels of γ -globin protein compared to the levels in WT mice, with an average increase of 5.1-fold for the $\gamma^{lo/-}$ combination, 4.9-fold for the γ^{lo}/γ^{lo} transgene combination, and 3.3-fold for the γ^{hi}/γ^{hi} transgene combination.

To investigate further this apparent selective survival advantage for RBC expressing the γ -globin transgene, immunofluorescent staining and flow cytometry was used to assess the $\gamma^{lo/-}$ transgene expression pattern in both WT and β -thalassaemia backgrounds. As seen in the example presented in Fig 3A and summarised in Fig 3B, this transgene allele generated a clearly heterocellular pattern of expression in the WT background, with an average $64 \pm 7\%$ of RBC expressing HbF. This was consistent with the initial characterisation of this line, where the fraction of F cells was estimated at 25% by a less sensitive staining method (Constantoulakis *et al*, 1991). In contrast, this same allele generated a nearly pancellular expression pattern in the β -thalassaemia background, with an average $93 \pm 3\%$ of RBC expressing HbF. As seen in Fig 3A and summarised in Fig 3C, this shift was also accompanied by an increase in the absolute level of γ -globin expression within the population of HbF(+) RBC. In the WT background, HbF(+) RBC stained with an average intensity of 53 ± 10 fluorescent units, while in the β -

thalassaemia background the average intensity was 195 ± 70 units. Combining this 3.7-fold increase in the level of expression within HbF(+) cells with the 1.4-fold increase in the overall frequency of HbF(+) cells fully accounts for the 5.1-fold increase in total HbF expression determined by HPLC analysis. Taken together, these results provide clear evidence that human γ -globin can compensate for the diminished levels of endogenous β -globin in this murine model of β -thalassaemia intermedia, and that this compensation results in the selective survival and expansion of HbF(+) RBC. This in turn results in a post-transcriptional increase in both the fraction of RBC expressing HbF, as well as the overall level of HbF expressed within these RBC.

Human γ -globin production fully corrects the phenotype of murine β -thalassaemia intermedia

We assessed multiple haematological parameters for the three $Hbb^{th-3/+} \times \gamma$ -globin transgenic hybrids described above, as well as non-transgenic $Hbb^{th-3/+}$ and WT controls. As seen in the series of panels to the left of Fig 4, non-transgenic $Hbb^{th-3/+}$ mice exhibited a phenotype of β -thalassaemia intermedia, with a decrease in RBC ($10.4 \pm 0.4 \times 10^{12}/l$ vs. $11.7 \pm 0.6 \times 10^{12}/l$ for WT), total Hb (12.8 ± 0.7 g/dl vs. 17.5 ± 1.1 g/dl for WT), and haematocrit ($45.2 \pm 1.9\%$ vs. $57.8 \pm 2.6\%$ for WT), and a notable increase in reticulocytes ($22.1 \pm 4.0\%$ vs. $5.5 \pm 2.0\%$ for WT). Addition of a single γ -globin transgene allele ($\gamma^{lo/-}$) increased RBC counts to levels that were indistinguishable from WT ($11.6 \pm 0.8 \times 10^{12}/l$), and generated a modest improvement in Hb (15.2 ± 1.2 g/dl), haematocrit ($50.5 \pm 3.4\%$), and reticulocytes ($10.1 \pm 3.7\%$). Addition of two copies of this γ -globin transgenic allele (γ^{lo}/γ^{lo}) lead to the full normalisation of all four of these parameters, including RBC counts ($11.9 \pm 0.8 \times 10^{12}/l$), Hb (17.6 ± 0.9 g/dl), haematocrit ($58.9 \pm 2.7\%$), and reticulocytes ($5.5 \pm 1.0\%$). This was also the case for two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}), including RBC counts ($11.6 \pm 1.1 \times 10^{12}/l$), Hb (17.1 ± 0.9 g/dl), haematocrit ($57.2 \pm 1.1\%$), and reticulocytes ($3.8 \pm 1.9\%$). Taken together, these results demonstrate that the deficits in overall Hb and RBC production in this model of murine β -thalassaemia intermedia can be fully normalised by expression of hybrid mouse/human HbF at about 25% (4.5 g/dl) HbF, and that this can be achieved by expression of γ -globin RNA at about 15% of total mouse α -globin.

The higher levels of γ -globin expression were necessary to fully normalise multiple indices used to characterise mature RBC (Fig 4). RBC from $Hbb^{th-3/+}$ mice exhibited a decrease in mean corpuscular volume (MCV; 41.5 ± 1.7 fl vs. 50.3 ± 1.2 fl for WT) and mean corpuscular Hb (MCH; 12.3 ± 0.5 pg vs. 15.5 ± 0.4 pg for WT), and a notable increase in red cell distribution width (RDW; $35.6 \pm 2.2\%$ vs. $15.8 \pm 1.6\%$ for WT). Addition of either one or two copies of the γ^{lo} transgene allele ($\gamma^{lo/-}$ or γ^{lo}/γ^{lo} respectively) improved all of these indices relative to the $Hbb^{th-3/+}$ controls, but failed to fully normalise them relative to the WT controls. This included an increase in MCV (43.5 ± 1.4 and 47.3 ± 0.6 fl respectively) and MCH (13.1 ± 0.5 and 14.1 ± 0.3 pg), and a decrease in RDW ($26.4 \pm 2.8\%$ and $20.6.8 \pm 1.7\%$). It was not until two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}) were included that all these indices normalised, including MCV (48.6 ± 0.4 fl), MCH (14.8 ± 0.8) and RDW ($17.6 \pm 1.1\%$). As indicated in Fig 4, there were no significant differences in mean corpuscular Hb concentration between the various lines tested. These differences in RBC morphology were confirmed by histological analysis (Fig 5). In this case, $Hbb^{th-3/+}$ (Thal) RBC showed a significant level of dismorphic features, including hypochromic microcytosis, target cells, basophilic stippling, and poikilocytes, as well as acanthocytes, spherocytes, burr cells, and schistocytes. There was also evidence of Howell–Jolly bodies, polychromatophilia, and a low level of nucleated RBCs indicative of a modest level of RBC regeneration. These features were progressively diminished in $\gamma^{lo/+}$ and γ^{lo}/γ^{lo} RBC, and were completely normalised in γ^{hi}/γ^{hi} RBC compared with RBC from a normal C57BL/6J (WT) control.

Taken together, these results demonstrate that the deficits in multiple RBC indices observed in this murine model of β -thalassaemia intermedia can be fully normalised by hybrid mouse/human HbF, but that this requires expression at about 40% (6.8 g/dl) HbF, which in turn can be achieved by expression of γ -globin RNA at about 27% of total mouse α -globin.

Hybrid mouse α /human γ -Hb is unable to rescue from lethality the murine model of β -thalassaemia major characterised by complete β -chain deficiency

We next sought to determine the degree to which human γ -globin expression could mitigate the lethal phenotype in the homozygous Hbb^{th-3}/Hbb^{th-3} model of murine β -thalassaemia major. Crosses were produced in which the mice were homozygous for a complete β -globin chain deficiency and in which the only non- α -globin chains produced were human γ -globin. These mice were expected to have a very large excess of α -globin chains and the question was whether, and to what level, the synthesis of human γ -globin chains could rescue these animals from embryonic lethality. As detailed in Table I, crosses between $Hbb^{th-3}/+$ individuals containing no γ -globin transgenes (γ^-/γ^-) and two copies of the line γ^{lo} transgene allele (γ^{lo}/γ^{lo}), as well as intercrosses between $Hbb^{th-3}/+$ individuals containing two copies of the γ^{lo} allele (γ^{lo}/γ^{lo}), failed to generate viable offspring homozygous for the Hbb^{th-3} knockout (0 of 35 for the $\gamma^{lo}/-$ offspring and 0 of 68 for the γ^{lo}/γ^{lo} offspring, with an expectation rate of 1 in 4). This is consistent with the results of Person *et al* (2001), who, by similar crosses, found 0 of 49 viable β^0 -thalassaemia homozygotes containing a transgene expressing human γ -globin at 13%, with an expectation rate of 1 in 16.

We also performed intercrosses between $Hbb^{th-3}/+$ individuals containing two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}), which was shown to produce γ -globin protein at *c.* 40% in the thalassaemia intermedia mice described above. Surprisingly, these crosses produced only one offspring of 29 live births that was homozygous for the Hbb^{th-3} knockout allele, while the expected number was 7.5 (1/4). This animal had a total Hb level of 11.5 g/dl and haematocrit of 37%. This level of anaemia is compatible with survival in mice, and is equivalent to the haematological phenotype of W/W^v mice (Antonchuck *et al*, 2004). However, even this animal failed to survive past 2 months of age. These results indicate that the failure of even high levels of human γ -globin to rescue the Hbb^{th-3}/Hbb^{th-3} mice resides in the functional properties of the mouse/human hybrid Hb. This in turn suggests that the murine model of homozygous β -thalassaemia is not an appropriate animal model for testing the therapeutic potential of elevated levels of fetal Hb.

Studies in a murine β -thalassaemia major stem cell transplantation model

We next turned to the transplantation model for murine β -thalassaemia major, first described by Rivella *et al* (2003). This involved collecting liver cells from day 15.5 Hbb^{th-3}/Hbb^{th-3} fetuses that were also homozygous for the γ^{lo} or γ^{hi} transgene alleles, mixing these in different ratios with Hbb^{th-3}/Hbb^{th-3} FLCs containing no γ -globin transgenes, and transplanting the mixtures of cells into myeloablated congenic C57BL/6J recipients. Recipients of unmodified Hbb^{th-3}/Hbb^{th-3} FLCs were expected to die around 50 d post-transplant due to profound anaemia that was coincidental with the loss of normal recipient RBCs. If γ -globin gene expression could compensate for the complete β -globin chain deficiency and hybrid mouse α -globin/human γ -globin tetramers could functionally replace native mouse HbA, we would expect the transplant recipients to be corrected haematologically and to survive.

As seen in Fig 6, in the absence of γ -globin transgenes, transplant recipients in our studies survived a median of 48 d, eventually succumbing to profound anaemia coincident with the eventual loss of normal recipient RBC. Animals transplanted with FLCs from WT C57BL/6 donors or congenic $Hbb^{th-3}/+$ donors typically survived long-term (data not shown). The use of γ^{lo}/γ^{lo} FLCs in ratios of 10%, 25%, 50% and even 100% failed to significantly increase

survival in this model, with a median survival of 63 d at the highest ratio (Fig 6A). Likewise, the use of $\gamma^{\text{hi}}/\gamma^{\text{hi}}$ FLCs at a ratio of 10% failed to significantly increase survival, with a median survival of 52 d. In contrast, the use of $\gamma^{\text{hi}}/\gamma^{\text{hi}}$ FLCs at 25%, 50% and 100% did significantly increase survival, to a median of 77, 75 and 84 d respectively. Analysis at near 40 d post-transplant (latest time tested) indicated a clear dose–response in multiple haematological indices. This included an overall increase in haematocrit, ranging from $14 \pm 9\%$ for the 0% controls, $24 \pm 3\%$ for 100% $\gamma^{\text{lo}}/\gamma^{\text{lo}}$, and $31 \pm 9\%$ for 100% $\gamma^{\text{hi}}/\gamma^{\text{hi}}$, as well as in total Hb, ranging from 4.8 ± 3.8 g/dl for the 0% controls, 8.6 ± 1.1 g/dl for 100% $\gamma^{\text{lo}}/\gamma^{\text{lo}}$, and 9.4 ± 1.1 g/dl for 100% $\gamma^{\text{hi}}/\gamma^{\text{hi}}$. Taking into account the fraction of RBC expressing HbF ($40 \pm 14\%$) and the fraction of total Hb arising from HbF ($37 \pm 15\%$) in the 100% $\gamma^{\text{hi}}/\gamma^{\text{hi}}$ recipients at this time point, it was estimated that the $\gamma^{\text{hi}}/\gamma^{\text{hi}}$ transgenes resulted in the production of HbF at 7.7 ± 1.9 g/dl. This is slightly higher than the level observed in *Hbb^{th-3}/+* recipients containing this same transgene combination (6.8 g/dl), but well below the 11.5 g/dl observed in the one live-born individual with this same genetic constitution from the breeding studies described above. Immunofluorescent analysis at 83 d post-transplant of surviving $\gamma^{\text{hi}}/\gamma^{\text{hi}}$ transplant recipients demonstrated that essentially 100% of peripheral RBC consisted of HbF(+) donor cells.

This level of γ -globin gene expression was, however, clearly insufficient to support the long-term survival in this transplantation model of murine homozygous β -thalassaemia. This is in contrast to the findings of Rivella *et al* (2003), who reported that expression of a recombinant virus vector for human β -globin at levels resulting in production of mouse/human hybrid HbA at an average 6.5 ± 2.9 g/dl could support long-term survival in this same model. This discrepancy shows that human γ -globin may provide an even poorer substitute for mouse β -globin than does human β -globin in this and other murine models of β -thalassaemia, and implicates the function of the hybrid mouse α /human γ -Hb, rather than the amount of this hybrid Hb, in the failure of γ -globin to rescue the lethal phenotype in this model.

Discussion

The gene therapy approaches that are currently being pursued for the treatment of the β -chain haemoglobinopathies rely on the use of recombinant virus vectors to stably transfer expression cassettes for either human β -globin or γ -globin into reconstituting haematopoietic stem cells of thalassaemia patients. Vectors for human γ -globin have been pursued due to the potent anti-sickling properties of γ -globin for the treatment of sickle cell disease, as well as the therapeutic potential of γ -globin for the treatment of the β -thalassaemic patients. Several lines of evidence suggest that γ -globin gene transfer should prove curative for the β -thalassaemic patients. For example, compound heterozygotes for β -thalassaemia and deletional *HPFH* exhibit a very mild phenotype (Weatherall, 2001), compound heterozygotes for β -thalassaemia and a non-deletional *HPFH* allele exhibit a β -thalassaemia intermedia phenotype (Fessas & Stamatoyannopoulos, 1964; Sofroniadou *et al*, 1975; Tate *et al*, 1986), and compound $\delta\beta$ -thalassaemia/ β -thalassaemia heterozygotes exhibit a β -thalassaemia intermedia to mild phenotypes (Stamatoyannopoulos *et al*, 1969). Homozygous β^0 -thalassaemia individuals who have also coinherited an unlinked gene for heterocellular *HPFH* have higher levels of HbF and mild clinical course (Prchal & Stamatoyannopoulos, 1981). Homozygotes for $\delta\beta$ -thalassaemia have only fetal Hb in their blood and a phenotype of intermediate thalassaemia (Weatherall, 2001), while homozygotes for deletional *HPFH* have only fetal Hb in their blood and are clinically normal, although they have elevated haematocrits and dismorphic RBC indices, presumably secondary to the increased oxygen affinity of fetal Hb (Wheeler & Krevans, 1961; Charache *et al*, 1976). Collectively, the experience of a very large number of clinical studies over 40–50 years strongly indicated that a gene therapy approach based on γ -globin should prove effective for the treatment of β -thalassaemia.

As a prerequisite to gene therapy clinical trials, it is essential that the efficacy and safety of candidate globin gene transfer vectors be tested in animal models of β -thalassaemia. Several studies to date have used murine models of relatively mild β -thalassaemia intermedia (May *et al*, 2000, 2002; Imren *et al*, 2002; Person *et al*, 2003; Hanawa *et al*, 2004), and have shown that β -globin gene transfer with lentivirus-based vectors can fully correct the thalassaemic phenotype in this animal model. The studies reported here demonstrated that γ -globin gene transfer can fully correct the phenotype of murine β -thalassaemia intermedia, indicating that $Hbb^{th-3/+}$ mice represents a good model for testing candidate vectors for human γ -globin. In particular, we documented a clear dose-dependent improvement in multiple haematopoietic indices, culminating in the complete normalisation of the β -thalassaemia phenotype at the highest levels of γ -globin expression tested. We also documented the selective survival of RBC expressing threshold levels of γ -globin, a property predicted to be critical for the therapeutic success of globin gene therapy. These studies also establish the level of γ -globin expression necessary to fully normalise the phenotypic sequelae in this model ($27.1 \pm 4.2\%$ of mouse α -globin RNA, correlating to expression of hybrid HbF at $40.0 \pm 4.7\%$ or about 6.8 g/dl).

Given these results, it was a surprise to find that expression of human γ -globin at even higher levels (giving rise to total Hb levels in the 7.7–11.5 g/dl range) failed to support long-term survival of Hbb^{th-3}/Hbb^{th-3} homozygous mice that manifest a phenotype of embryonic lethal β -thalassaemia major. This was especially true in light of studies with virus vectors for human β -globin in this model where long-term survival was achieved at total Hb levels of 6.5 g/dl or less (Rivella *et al*, 2003). Likewise, clinical studies indicate that humans can survive, albeit with serious disease, with all or nearly all Hb consisting of HbF at levels of 6 g/dl or even lower (Weatherall & Clegg, 1981). Thus, it would appear that, in contrast to the heterozygous $Hbb^{th-3/+}$ mice with β -thalassaemia intermedia, the homozygous Hbb^{th-3}/Hbb^{th-3} mice constitute an inappropriate model for testing the efficacy of γ -globin gene transfer vectors.

We can propose two possible explanations for the failure of γ -globin to rescue the lethal phenotype in Hbb^{th-3}/Hbb^{th-3} mice. First, previous studies have demonstrated that γ -globin has a strong propensity for forming Hb Bart's (γ_4 homotetramers) in transgenic mice (Arcasoy *et al*, 1997). Although the underlying mechanisms for this propensity are unclear, it suggests a basic inefficiency of hybrid mouse α -globin/human γ -globin heterotetramer formation. The formation of Hb Bart's would further decrease the already limited pool of binding partners for excess α -globin chains, resulting in increased α -chain precipitation and proerythrocyte destruction. Secondly, previous studies have also demonstrated that hybrid mouse/human HbF, as well as Hb Bart's, have decreased oxygen affinity (Arcasoy *et al*, 1997; Shear *et al*, 1998), which, in combination, would diminish the ability of surviving RBC in these mice to efficiently deliver oxygen.

In summary, human γ -globin can fully correct the heterozygous $Hbb^{th-3/+}$ mouse model of β -thalassaemia intermedia in a dose-dependent manner and in association with an increased survival of RBC-containing hybrid mouse/human HbF. As such, $Hbb^{th-3/+}$ mice constitute a good model for the pre-clinical assessment of gene transfer vectors for human γ -globin. However, even high levels of human γ -globin cannot rescue the lethal phenotype of the homozygous Hbb^{th-3}/Hbb^{th-3} mouse model of severe β -thalassaemia major, and as such this model is not appropriate for testing therapeutic interventions based on human γ -globin gene expression.

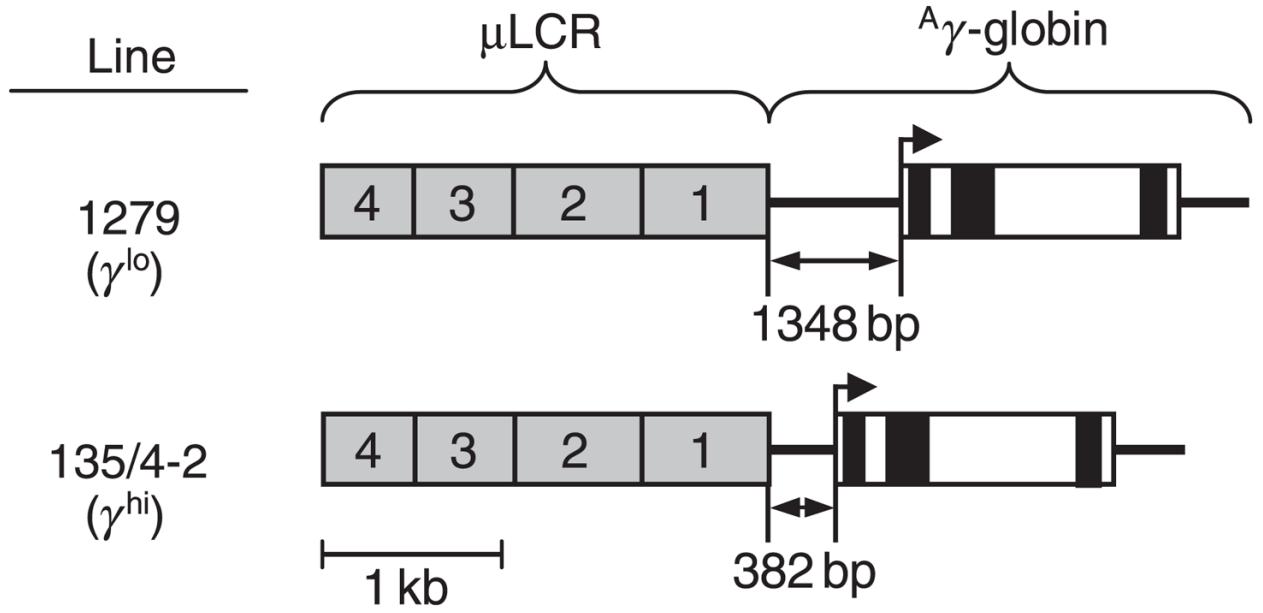
Acknowledgments

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**Fig 1.**

γ -Globin transgenic lines. The γ -globin expression cassettes in the transgenic mouse lines 1279 (Enver *et al*, 1989; Constantoulakis *et al*, 1991), and 135/4-2 (Stamatoyannopoulos *et al*, 1993), include a 2.5 kb locus control region (μ LCR) containing DNase hypersensitive sites 1–4 from the human β -globin locus linked to a coding cassette for human $\Lambda\gamma$ -globin that includes either a –1348 bp 5'-promoter region (line 1279) or a –382 bp 5'-promoter region (line 135/4-2). The line 1279 allele (denoted γ^{lo}) contains three transgene copies in *cis*, while the line 135/4-2 allele (denoted γ^{hi}) contains nine copies in *cis*. Grey boxes, μ LCR; filled and open boxes, $\Lambda\gamma$ -globin exons and introns respectively; heavy line: 5'- and 3'-regions flanking the $\Lambda\gamma$ -globin coding region.

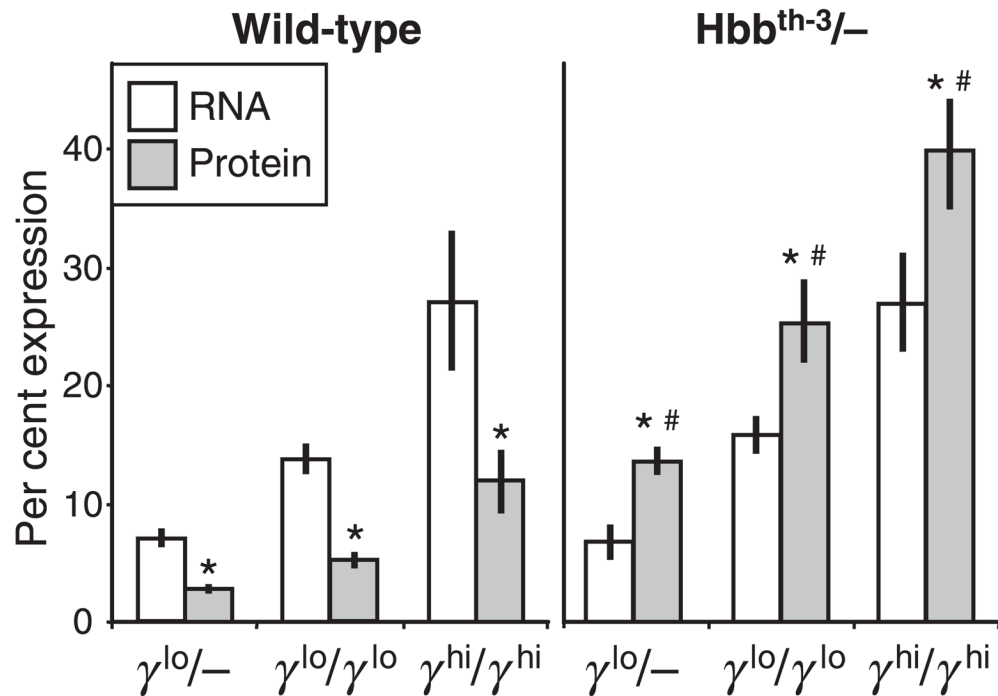


Fig 2.

γ -Globin expression in wild type and β -thalassaemia intermedia mice. Peripheral blood was collected at 10–20 weeks of age from wild type and *Hbb*^{th-3/+} individuals containing one or two copies of the γ^{lo} transgene alleles ($\gamma^{lo}/-$ and γ^{lo}/γ^{lo} respectively) or two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}). Samples were analysed for the amount of γ -globin RNA (as a percentage of total mouse α -globin, open bars) by RNase protection, and for the amount of γ -globin protein (in the form of hybrid mouse/human HbF as a percentage of total Hb, grey bars) by high-performance liquid chromatography. Results are presented as mean \pm SD for a total of five to 12 individuals for each group for the RNA analysis and seven to 29 individuals for each group for the protein analysis. * $P < 0.001$ vs. RNA; # $P < 0.001$ vs. wild type.

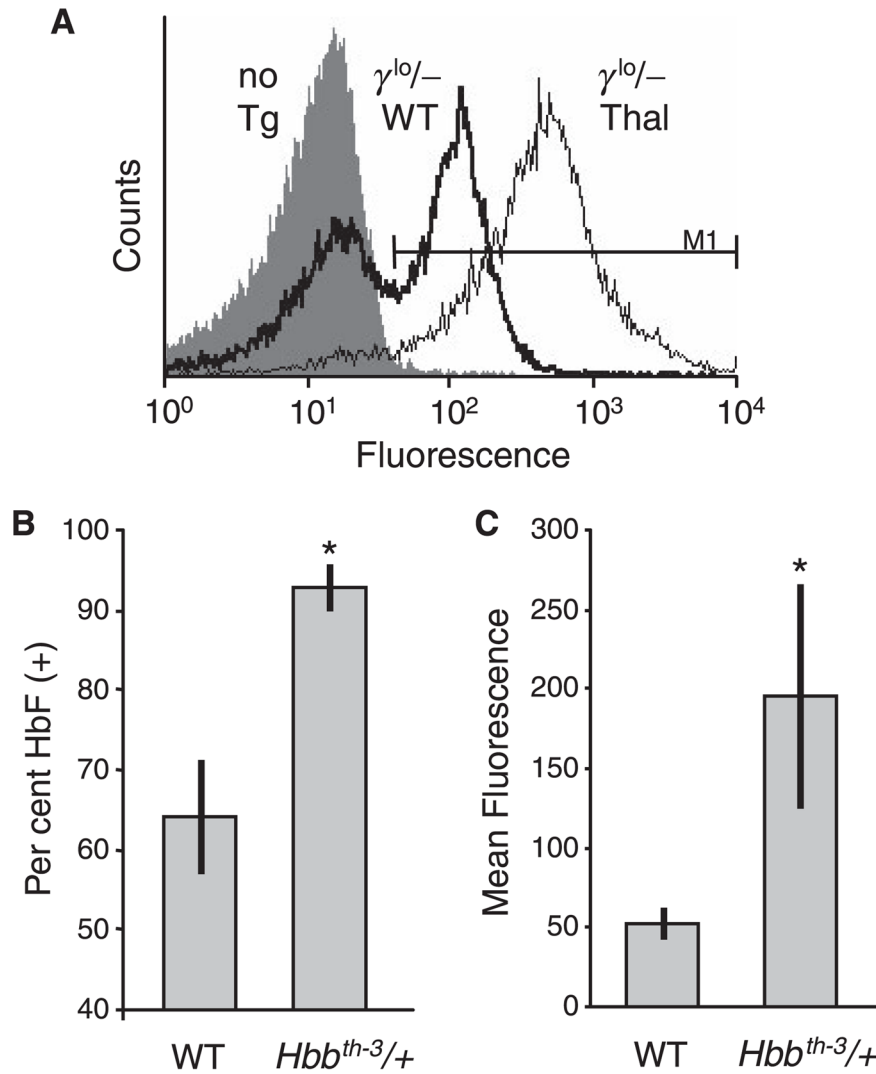
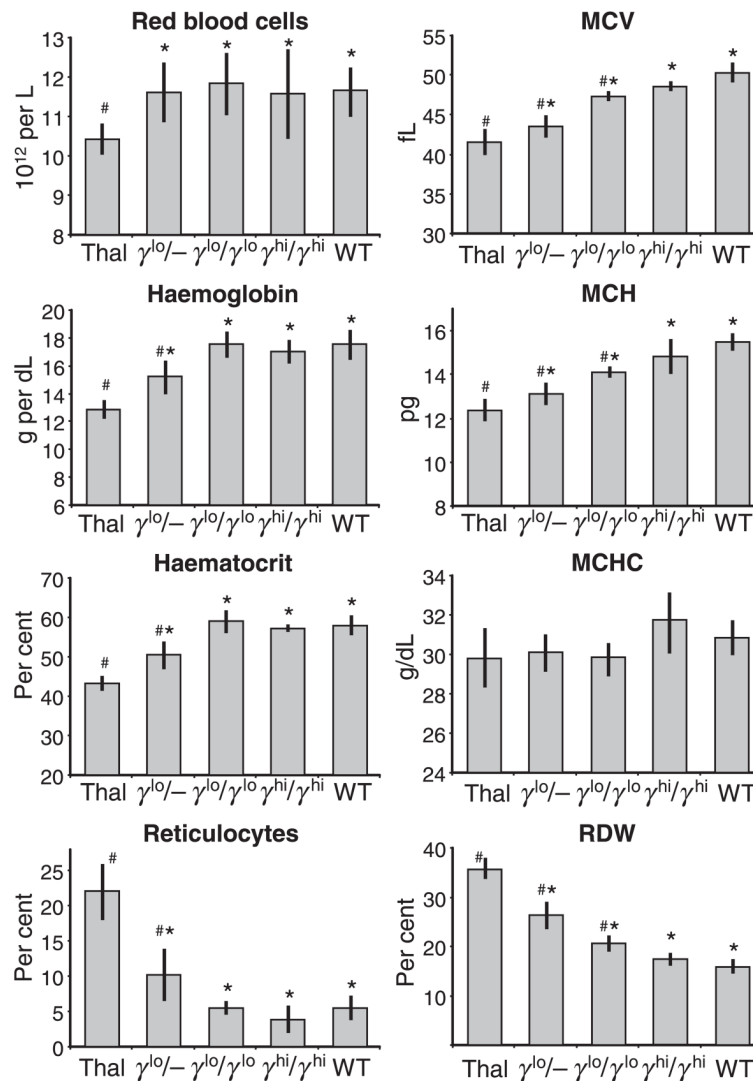


Fig 3. *In vivo* selection of red blood cell (RBC) expression γ -globin. Peripheral blood was collected from non-transgenic wild-type (WT) mice, WT mice containing a single copy of the γ^{10} transgene alleles ($\gamma^{10/-}$), and $Hbb^{th-3/+}$ thalassaemia mice containing a single copy of the γ^{10} transgene allele, fixed and stained with a phycoerythrin-conjugated monoclonal antibody to HbF, and analysed on a flow cytometer. (A) Typical analysis showing the lack of staining in a non-transgenic control (no Tg; filled histogram), as well as the shift from a heterocellular pattern of γ -globin expression in a transgenic WT mouse ($\gamma^{10/-}$ WT; heavy line) to a pancellular and significantly elevated pattern of expression in a $Hbb^{th-3/+}$ mouse ($\gamma^{10/-}$ Thal; light line). The marker used to define HbF(+) is indicated. (B) Percentage of HbF(+) RBC in WT versus $Hbb^{th-3/+}$ mice containing a single copy of the γ^{10} transgene allele showing a selective expansion of these cells in the thalassaemia background. Data represents mean \pm SD for at least six individuals. (C) Mean fluorescence of HbF(+) RBC from panel (B), showing a concomitant increase in the intensity of staining in the thalassaemia background. * $P < 0.01$ vs. WT.

**Fig 4.**

Effects of γ -globin expression on haemopoietic and red blood cell (RBC) indices. Peripheral blood was collected at 10–20 weeks of age from none-transgenic *Hbb^{th-3/+}* (Thal) and wild-type (WT) individuals, as well as from *Hbb^{th-3/+}* individuals containing one or two copies of the γ^{lo} transgene alleles ($\gamma^{lo/-}$ and γ^{lo}/γ^{lo} respectively) or two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}). Samples were analysed for total RBC counts, total haemoglobin, haematocrit, the percentage of reticulocytes, as well as mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and red cell distribution width. Results represent mean \pm SD for three to 11 individuals for each group. * $P < 0.05$ vs. the Thal controls; $P < 0.05$ vs. the WT control.

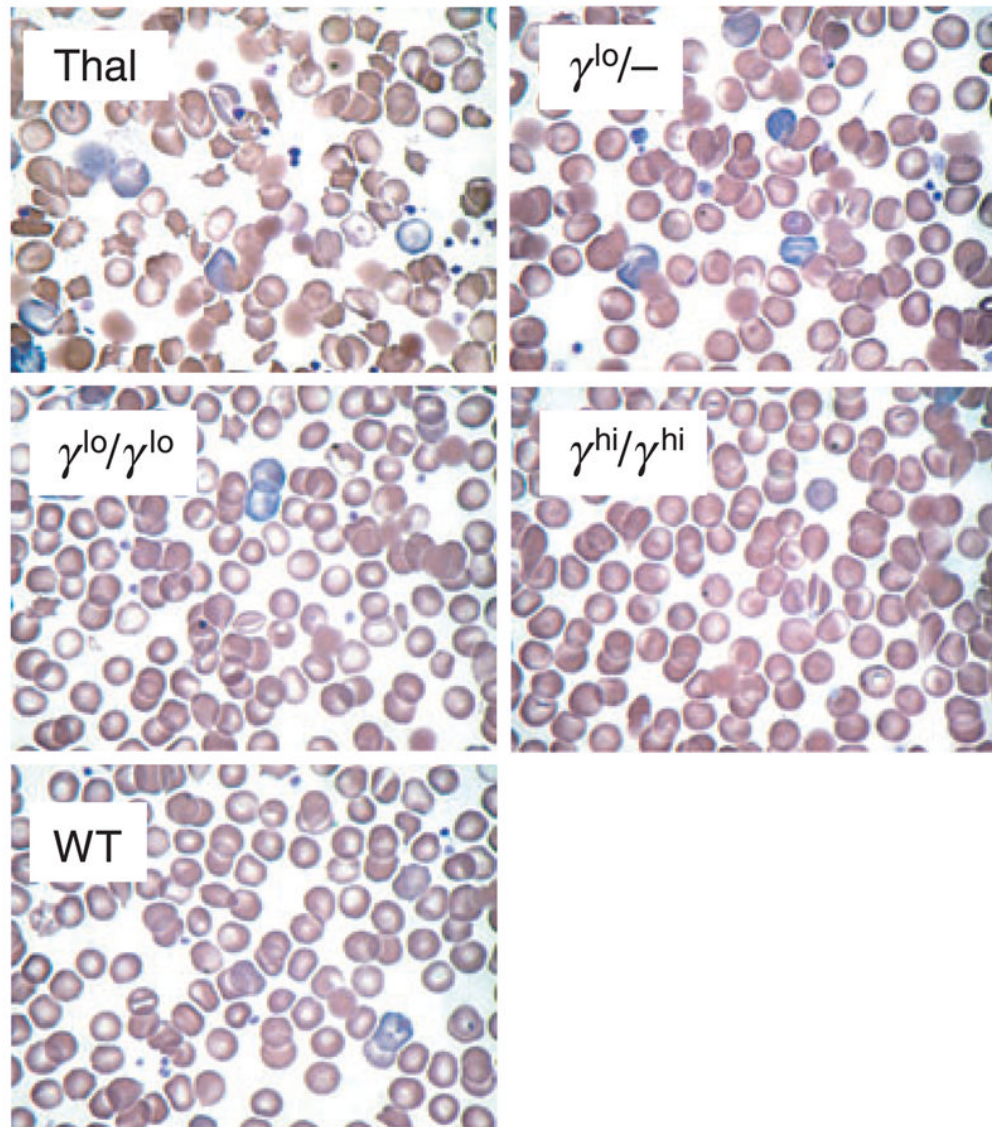
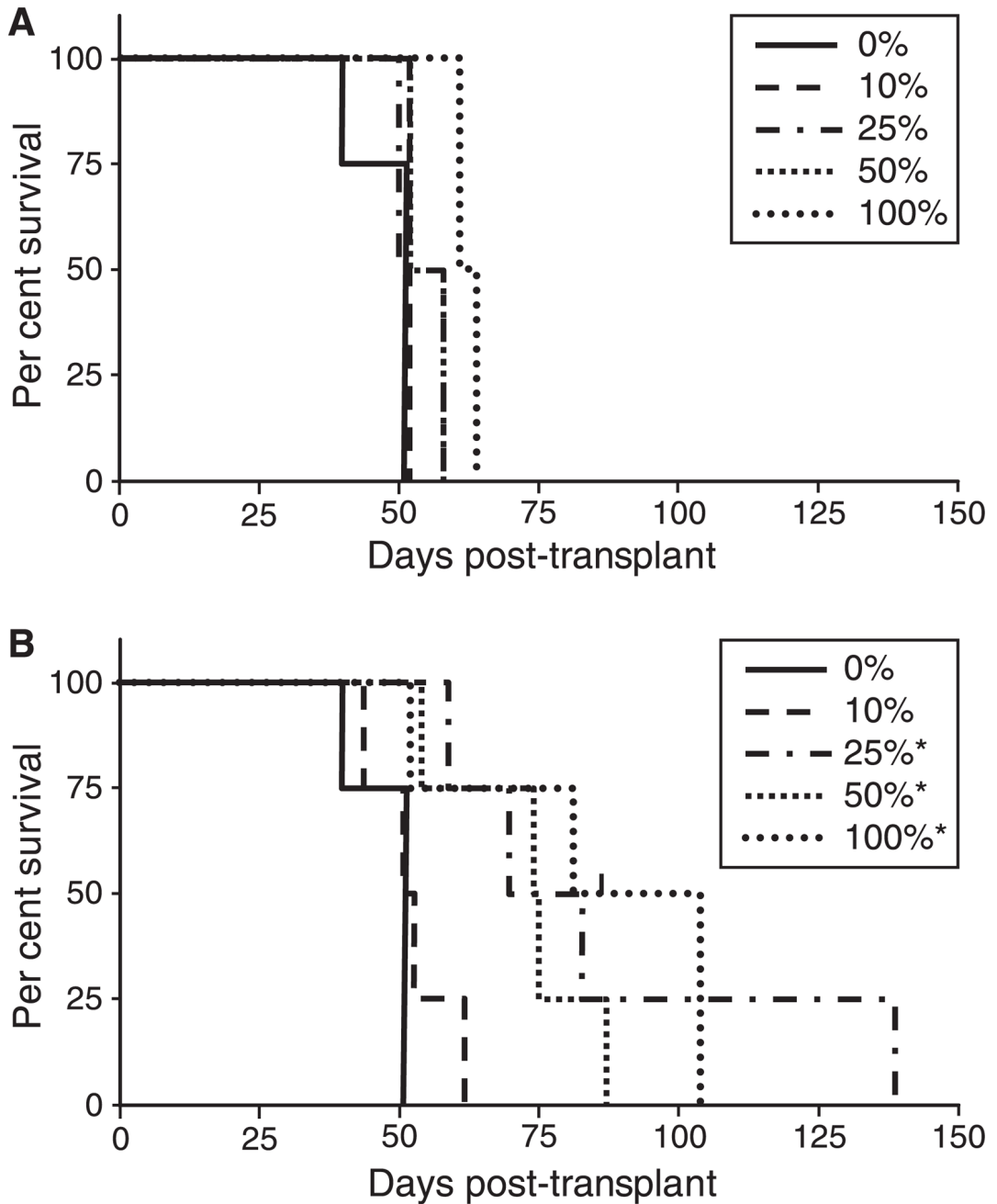


Fig 5. Effects of γ -globin expression on red blood cell histology. Wright–Giemsa stained peripheral blood smears from individual animals are presented, including wild-type C57BL/6J, non-transgenic β -thalassaemia intermedia $Hbb^{th-3/+}$ (Thal) or $Hbb^{th-3/+}$ individuals containing one copy of the γ^{lo} transgene allele ($\gamma^{lo}/-$), two copies of the γ^{lo} transgene allele (γ^{lo}/γ^{lo}) or two copies of the γ^{hi} transgene allele (γ^{hi}/γ^{hi}) (original magnification $\times 60$).

**Fig 6.**

Effects of γ -globin expression on survival of mice transplanted with Hbb^{th-3}/Hbb^{th-3} fetal liver cells (FLCs). FLCs were collected from day 15.5 homozygous Hbb^{th-3}/Hbb^{th-3} fetuses containing either no transgenes or two copies of either the γ^{lo} or γ^{hi} transgene alleles (γ^{lo}/γ^{lo} or γ^{hi}/γ^{hi} respectively). These cells were mixed in different ratios (shown as the percentage of transgenic FLCs), and transplanted into myeloablated congenic C57BL/6J recipients.

Transplant recipients were monitored closely and killed when moribund or severely anemic with hematocrits $<15\%$. (A) Survival of recipients transplanted with mixtures of γ^{lo}/γ^{lo} FLCs. (B) Survival of recipients transplanted with mixtures of γ^{hi}/γ^{hi} FLCs. * $P \leq 0.01$ vs. 0% controls.

Table IBreeding for β -null mice.

Parents	Target genotype	Target frequency of offspring (1/4 expected)
$Hbb^{th-3/+}, \gamma^{lo}/\gamma^{lo} \times Hbb^{th-3/+}, \gamma^{-}/\gamma^{-}$	$Hbb^{th-3}/Hbb^{th-3}, \gamma^{lo}/-$	0 of 35
$Hbb^{th-3/+}, \gamma^{lo}/\gamma^{lo} \times Hbb^{th-3/+}, \gamma^{lo}/\gamma^{lo}$	$Hbb^{th-3}/Hbb^{th-3}, \gamma^{lo}/\gamma^{lo}$	0 of 68
$Hbb^{th-3/+}, \gamma^{hi}/\gamma^{hi} \times Hbb^{th-3/+}, \gamma^{hi}/\gamma^{hi}$	$Hbb^{th-3}/Hbb^{th-3}, \gamma^{hi}/\gamma^{hi}$	1 of 29*

* Died at 2 months of age.