

polybed, sectioned, and stained with uranyl acetate and lead citrate. Ultra-thin sections were examined and photographed with the Hitachi-7000 transmission electron microscope.

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Transgenic Knockout Mice with Exclusively Human Sickle Hemoglobin and Sickle Cell Disease

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To create mice expressing exclusively human sickle hemoglobin (HbS), transgenic mice expressing human α -, γ -, and β^S -globin were generated and bred with knockout mice that had deletions of the murine α - and β -globin genes. These sickle cell mice have the major features (irreversibly sickled red cells, anemia, multiorgan pathology) found in humans with sickle cell disease and, as such, represent a useful in vivo system to accelerate the development of improved therapies for this common genetic disease.

A single base pair change in codon 6 of the β -globin gene causes sickle cell anemia in individuals who are homozygous for the mutation (1). Sickle hemoglobin [HbS ($\alpha_2\beta^S_2$)] undergoes polymerization upon deoxygenation, thereby distorting erythrocytes into a variety of sickled shapes, damaging the erythrocyte membrane, and ultimately causing anemia, ischemia, infarction, and progressive organ dysfunction. Despite the impressive body of knowledge that has accumulated (2), many aspects of sickle cell disease are still poorly understood and treatment options remain limited. Because of the inhibitory effects of mouse α - and β -globin on sickling, transgenic mice expressing various sickle hemoglobins (HbS, HbSAD, HbS-Antilles) develop almost none of the clinical manifestations of sickle cell disease (3). Some sickle cell disease pathology has been reported in transgenic mice bred to produce higher concentrations of the "supersickling" hemoglobins (HbSAD and HbS-Antilles) (4); however, these animals still lack important features that are commonly found in humans with sickle cell disease

(5). To overcome these limitations, we have created mice that no longer express mouse α - and β -globin; instead, they express exclusively human α - and β^S -globin.

Three fragments of human DNA were co-injected into fertilized mouse eggs to generate transgenic founders expressing human α - and β^S -globin (6). Because γ -globin has antisickling properties, we included the $G\gamma$ - and $A\gamma$ -globin genes to decrease the likelihood that erythrocytes would sickle during gestation and cause fetal death. In the particular transgenic

line that was generated [Tg(Hu-miniLCR $\alpha I^{G\gamma A\gamma\delta\beta^S}$)], $G\gamma$ - and $A\gamma$ -globin are expressed during the embryonic and fetal stages of development and not in adult mice (Fig. 1A) (7). Through successive rounds of breeding with knockout mice heterozygous for deletions of the murine α - and β -globin genes, $Hba^{o//+}Hbb^{o//+}$ (8, 9), mice homozygous for the α - and β -globin deletions and containing the sickle transgene were generated—Tg(Hu-miniLCR $\alpha I^{G\gamma A\gamma\delta\beta^S}$) $Hba^{o//Hba^o}Hbb^{o//Hbb^o}$, hereafter called sickle cell mice (10). Many of these mice turned purple and died a few hours after birth; their death was apparently a result of hypoxia brought about by respiratory distress. Because γ -globin concentrations are relatively low [range, 4 to 26% ($\gamma/\gamma+\beta^S$)] in newborn sickle cell mice (Fig. 1B) compared with newborn humans, it is likely that these deaths are caused by the sickling of erythrocytes during the critical period just after birth when the lungs must begin the task of supplying oxygen. Sickle cell mice that survived this early critical period were able to reach adulthood (many are now more than 7 months old) with normal appearance, activity, and fertility (11). Erythrocytes in adult sickle cell mice contain exclusively human α - and β^S -globin (Fig. 1B). There is an excess of α -globin chain synthesis (α/β^S , 1.26 ± 0.02 ;

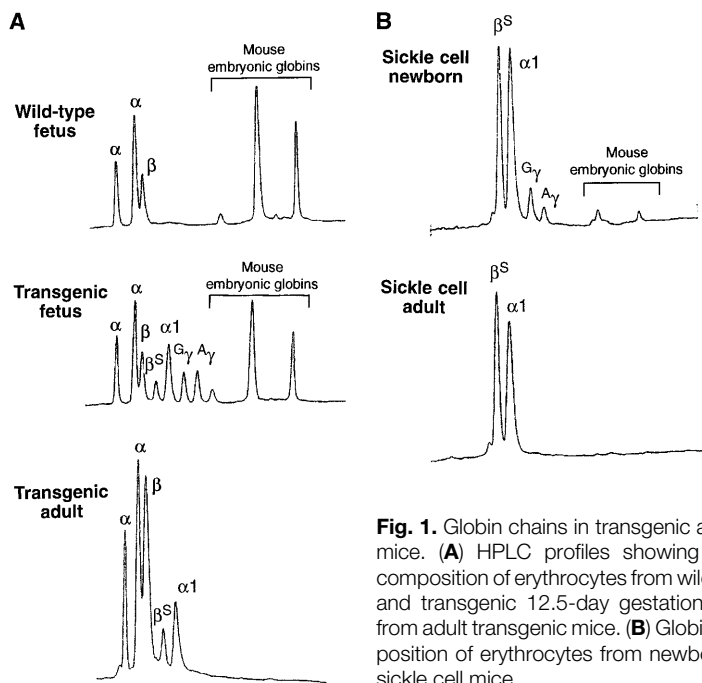


Fig. 1. Globin chains in transgenic and sickle cell mice. (A) HPLC profiles showing globin-chain composition of erythrocytes from wild-type (+//+) and transgenic 12.5-day gestation fetuses and from adult transgenic mice. (B) Globin-chain composition of erythrocytes from newborn and adult sickle cell mice.

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$n = 5$) (12), which indicates that these sickle cell mice are slightly β -thalassemic.

Common findings in humans with sickle cell anemia include irreversibly sickled cells (ISCs), anemia, and increased rigidity of erythrocytes. Sickle cell mice are anemic, with average hematocrits only 65% of normal, and have markedly elevated reticulocyte counts (Table 1). ISCs, indicative of repeated cycles of *in vivo* sickling and unsickling, were observed at a frequency of 5 to 10% in oxygenated sickle cell mouse blood (Fig. 2A). Upon deoxygenation *in vitro* (13), classically sickled cells formed at high frequency. Erythrocytes from sickle cell mice have significantly decreased osmotic fragility and increased dynamic rigidity (Fig. 2B) as measured by osmotic gradient ektacytometry (14). The various hematologic and erythrocytic perturbations that exist in these sickle cell mice closely parallel those observed in humans with sickle cell anemia (2).

The final and most serious manifestation of sickle cell disease in humans is damage to multiple organs. In sickle cell mice, kidney and heart weights increased 2-fold, and spleen weight increased 13-fold compared with wild-type controls (15). Long-term increases in cardiac output and splenic erythropoiesis, both in response to the chronic anemia that exists in these mice, are likely to be responsible for the observed increases in heart and spleen weights. Histologic analysis (16) revealed tissue damage in multiple organs (Fig. 3): kidney (fibrosis, atrophy, infarcts, cysts; Fig. 3B), lung (vascular congestion;

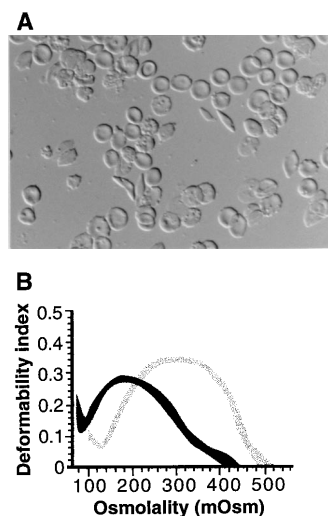


Fig. 2. Morphology and cellular characteristics of erythrocytes from adult sickle cell mice. (A) Oxygenated sickle cell mouse blood showing ISCs (elongated cells). (B) Osmotic deformability profiles of erythrocytes from wild-type (stippled curve) and sickle cell (solid curve) mice.

Fig. 3D), liver (multifocal ischemic infarcts; Fig. 3, E and F), and spleen (congested sinusoidal channels). Increased iron deposits were found in liver (Kupffer cells) and kidney (tubular epithelium). The extent and nature of the congestion, atrophy, fibrosis, and infarct found in organs of these sickle cell mice is very sim-

ilar to what has been reported in humans with sickle cell disease (17).

We have extensively reengineered the murine globin system to create mice that express exclusively human sickle hemoglobin and that faithfully recapitulate the major genetic, hematologic, and histopathologic features of humans with sick-

Table 1. Hematologic values for adult sickle cell and wild-type mice. Hct, hematocrit; MCH, mean corpuscular hemoglobin content; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; HDW, standard deviation of hemoglobin concentration histogram distribution width. MCH, MCV, MCHC, and HDW were determined with an automated hematology analyzer (H*3 System, Bayer Diagnostics, Tarrytown, New York). Animals were 3 to 7 months of age. Values are shown as mean \pm standard error of the mean ($n = 7$ in each group). Means for each parameter were significantly different for sickle cell and wild-type mice ($P < 0.006$, t test).

	Hct (%)	Reticulocytes (%)	MCH (pg)	MCV (fl)	MCHC (g/dl)	HDW (g/dl)
Wild type	43.6 \pm 1.2	3.4 \pm 0.5	13.2 \pm 0.3	40.3 \pm 0.2	33.8 \pm 0.7	4.2 \pm 0.2
Sickle cell	28.7 \pm 2.5	26.8 \pm 2.2	8.3 \pm 0.4	34.0 \pm 1.1	26.5 \pm 0.7	8.3 \pm 0.1

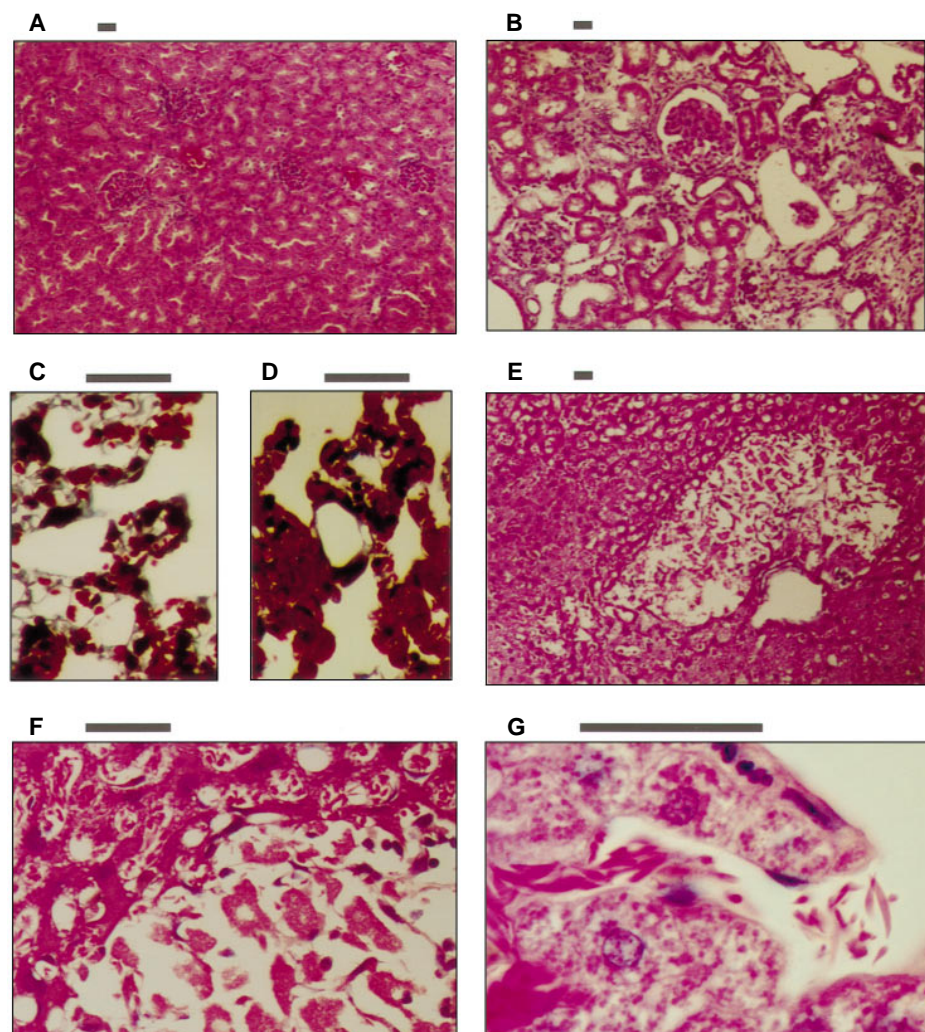


Fig. 3. Sickle cell mouse organ histopathology. (A) Wild-type kidney (cortex). (B) Sickle cell kidney (cortical microinfarct and cysts). (C) Wild-type lung parenchyma. (D) Sickle cell lung parenchyma (congested capillary bed). (E) Sickle cell liver (microinfarct surrounded by healthy tissue). (F) Higher magnification showing coagulative necrosis with loss of nuclear detail in hepatocytes. (G) Sickled erythrocytes in hepatic vascular channels. Scale bars = 15 μ m; stained with hematoxylin and eosin.

le cell anemia. In contrast to the limited studies that can be performed in humans, these animals provide an opportunity for rapidly exploring an expanded range of inquiry in an *in vivo* setting. As such, these sickle cell mice are likely to play an important role in furthering our understanding of the pathophysiology of sickle cell disease and in developing improved therapies for treating the more than 100,000 individuals born each year with this genetic disease.

Note added in proof: With a similar approach, we have also created mice that express exclusively normal human hemoglobin (HbA).

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6. Three DNA fragments were used to create transgenic mice: (i) 6.5-kb mini-LCR (Locus Control Region) [D. Talbot *et al.*, *Nature* **338**, 352 (1989)], (ii) 1.5-kb Pst I fragment containing the human $\alpha 1$ -globin gene, (iii) 39-kb Kpn I fragment containing the human fetal ($\zeta\gamma$, $\gamma\gamma$) and adult (δ , $\beta\delta$) globin genes in their normal genomic context [F. S. Collins, C. J. Stoeckert Jr., G. R. Serjeant, B. G. Forget, S. M. Weissman, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4894 (1984)].
7. Globin chain compositions were determined by high-performance liquid chromatography (HPLC) on a Vydac C₄ column as described (8).
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10. Genotyping was as follows. Polymerase chain reaction (PCR) conditions: 0.5 μ M each primer, 200 μ M deoxynucleotide triphosphate, 1 unit of Taq polymerase, 20- μ l reaction volume; 94°C (3 min); 35 cycles of 94°C (30 s), 58°C (30 s), 72°C (1 min). Sickle transgene, forward primer, $\delta 1$ (5'-GTATGGAGAGAGGCTCCAACCTC-3'), reverse primer, $\delta 2$ (5'-TCTGCCAAATCTTAGACAAAAC-3'). $\delta 1\delta 2$ PCR product, 480 base pairs (bp). Mouse α -globin genotypes (+/+ , *Hba*⁰¹/+, *Hba*⁰¹/*Hba*⁰²); *Hba*⁰² allele: forward primer, *Hba*⁰¹ (5'-ATAGATGGGTAGCCATTTAGATTCC-3'), reverse primer, *Hba*⁰² (5'-CCGGTTATAATTACCTCAGGTC-3'). *Hba*⁰¹/*Hba*⁰² PCR product, 461 bp. Also used previously published α -globin multiplex PCR (8). Mouse β -globin genotypes (+/+ , *Hbb*⁰¹/+, *Hbb*⁰¹/*Hbb*⁰²); *Hbb*⁰² allele: forward primer, *Hbb*⁰¹ (5'-AGATGTTTTTTTCACATCTTTGAGC-3'), reverse primer, *Hbb*⁰² (5'-AATGCCTGCTCTTACTGAAGG-3'). *Hbb*⁰¹/*Hbb*⁰² PCR product, 398 bp. Also used PCR for part of region deleted in the *Hbb*⁰² allele: forward primer, $\beta del1$ (5'-TTAGTGGTCTTAAAACCTTTGTGG-3'), reverse primer, $\beta del2$ (5'-ACTGGCACAGAGCATGTTATG-3'). $\beta del1$ / $\beta del2$ PCR was with Deep Vent (Exonuclease⁻) instead of Taq. Genotypes of sickle cell mice were confirmed by cellulose acetate gel electrophoresis of hemoglobins (Helena Laboratories, Beaumont, TX) [J. B. Whitney III, *Biochem. Genet.* **16**, 667 (1978)].
11. Sickle cell mice are fertile; however, females appear to have trouble feeding their pups and as a result many of the sickle cell mice we have produced thus far have come from sickle cell males bred with non-sickle cell females [Tg(Hu-miniLCR $\alpha 1^{\zeta\gamma}$ $\gamma\delta\beta\delta$) *Hba*⁰¹/*Hba*⁰¹/*Hbb*⁰¹/+]. Interestingly, there was a fivefold increase in sickle cell newborn survival when sickle cell males were bred with non-sickle cell females (number obtained/number expected = 32/122) compared with when non-sickle cell males were bred with non-sickle cell females (number obtained/number expected = 4/82). One possible explanation for this is the presence of segregating "survival" alleles in the mixed genetic background (FVB/N, 129, DBA/2, C57BL/6, Black Swiss) of the breeding population. These alleles would be inherited at a higher frequency from sickle cell males than from non-sickle cell males. Another possible explanation involves the presence of fewer healthy non-sickle cell newborns in litters from sickle

cell males. This might allow sickle cell newborns to compete more effectively for maternal attention and enable them to survive more readily. The following animal husbandry practices may influence the survival of sickle cell mice: Micro-Barrier Systems cages (17.8 × 27.9 cm) (Allentown Caging, Allentown, NJ), Bed-O-Cobs bedding (size, 0.32 cm) (Andersons Industrial Products, Maumee, OH), Nestlets nesting material (Ancare Corp., Bellmore, NY), and Purina 5008 food (PMI Inc.).

12. Globin chain synthesis was as described (8). Controls were C57BL/6 (1.0 ± 0.02; n = 3) and *Hbb*⁰¹/+ β -thalassemic mice (1.38 ± 0.05; n = 3).
13. Blood was mixed (1:1) with a 2% sodium bisulfite solution [G. A. Daland and W. B. Castle, *J. Lab. Clin. Med.* **33**, 1082 (1948)] and wet mounts were prepared immediately. After 15 min, 80% of cells were completely sickled (60% of cells had a single domain or a few parallel domains, 20% had multisplated holly leaf morphology).
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15. The increases (fold) in organ weights were calculated from organ weights expressed as a percentage of total body weight [sickle cell (n = 4) versus wild type (n = 5): kidneys, 2.5% versus 1.3%; heart, 1.0% versus 0.5%; spleen, 6.5% versus 0.5%].
16. Histological analysis was performed on four sickle cell and two wild-type mice, all 5 to 7 months of age.
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TECHNICAL COMMENTS

Government Funding of Research and Development

In a Policy Forum, Robert M. May (1) uses bibliometric data from an Australian benchmarking study (2) to show that the United Kingdom had the most cost-effective science base among G7 countries (3), as measured by citations attracted per million pounds (per £million) spent. In doing so, May, who is the Chief Scientist of the United Kingdom, has established a baseline that raises the profile of quantitative studies in science policy and against which investigators can measure future performance. Part of May's analysis rests on the assumption

that there is a relationship between financial investment in research and development (R&D) and scientific impact, as measured by citations to papers published in the peer reviewed serial literature. Although it is reasonable to assume that there is such a relationship, we have two main concerns with May's analysis. First, he does not allow for a time lag between expenditure on R&D and the evaluation of a country's scientific impact. By taking this into account, we demonstrate that United Kingdom (U.K.) science is not the most cost-

effective of the G7. Second, we argue that it is incorrect to relate the citation performance of the national science system only to government expenditure, because private and overseas funders have made an increasingly large contribution to public domain U.K. science in recent years.

May estimates (1) return on investment for a single year (1991) based on the yearly average number of citations over the period 1981-94 (4). However, expenditure in 1991 will have little effect on citations before about 1997 because there is commonly a 4-year lag before papers emerge from the funded research and at least a further 2-year period before the citation peak is reached. The preferred analysis would be to compare expenditure figures for each year with citations achieved (say, 4 to 6 years later) and to track this over time. Because May takes