Int. J. Dev. Biol. 52: 873-886 (2008)
doi: 10.1387/ijdb.082597fm

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Developmentally regulated expression of hemoglobin subunits in avascular tissues

FIONA C. MANSERGH[1,2], SUSAN M. HUNTER[1], JENNY C. GEATRELL[3], MIGUEL JARRIN[3], KATE POWELL[3], MARTIN J. EVANS[1] AND MICHAEL A. WRIDE[3,4,*]

[1]*Cardiff School of Biosciences, Cardiff University, Wales, UK* [2]*Smurfit Institute of Genetics, Trinity College Dublin, Ireland* [3]*School of Optometry and Vision Sciences, Cardiff University, Wales, UK and* [4]*Ocular Development and Neurobiology Research Group, Department of Zoology, School of Natural Sciences, Trinity College Dublin, Ireland*

ABSTRACT We investigated the spatio-temporal profile of hemoglobin subunit expression in developing avascular tissues. Significant up-regulation of hemoglobin subunits was identified in microarray experiments comparing blastocyst inner cell masses with undifferentiated embryonic stem (ES) cells. Hemoglobin expression changes were confirmed using embryoid bodies (derived from *in vitro* differentiation of ES cells) to model very early development at pre-vascular stages of embryogenesis; i.e. prior to hematopoiesis. We also demonstrate, using RT-PCR, Western blotting and immunocytochemistry, expression of adult and fetal mouse hemoglobin subunits in the avascular ocular lens at various stages of development and maturation. Hemoglobin proteins were expressed in lens epithelial cells (cytoplasmic) and cortical lens fiber cells (nuclear and cell-surface-associated); however, a sensitive heme assay demonstrated negligible levels of heme in the developing lens postnatally. Hemoglobin expression was also observed in the developing eye in corneal endothelium and retinal ganglion cells. Gut sections showed, in addition to erythrocytes, hemoglobin protein staining in rare, individual villus epithelial cells. These results suggest a paradigm shift: hemoglobin subunits are expressed in the avascular lens and cornea and in pre-hematopoietic embryos. It is likely, therefore, that hemoglobin subunits have novel developmental roles; the absence of the heme group from the lens would indicate that at least some of these functions may be independent of oxygen metabolism. The pattern of expression of hemoglobin subunits in the perinuclear region during lens fiber cell differentiation, when denucleation is taking place, may indicate involvement in the apoptosis-like signaling processes occurring in differentiating lens fiber cells.

KEY WORDS: *hemoglobin, lens, differentiation, embryoid body, stem cell*

Introduction

Globins, small globular heme-binding proteins, have been identified in bacteria, plants, fungi and animals, highlighting their ancient evolutionary origins (Garrocho-Villegas *et al.*, 2007, Vinogradov *et al.*, 2007). Four globin families, differing in structure and tissue distribution (hemoglobin, myoglobin, neuroglobin and cytoglobin) have been identified in vertebrates (Pesce *et al.*, 2002, Hankeln *et al.*, 2005). The major functions of hemoglobin pertain to oxygen transport, especially in the hematopoietic system. Moreover, evidence is emerging for hemoglobin expression outside the erythroid lineage. The first such reports demonstrated neuronal expression and suggested that iron-catalyzed oxidation of neuronal hemoglobin could have a role in motor neuron degeneration (Ohyagi and Goto, 1994, Ohyagi *et al.*, 1994).

Abbreviations used in this paper: EB, embryoid body; Eraf, erythroid associate factor; ES, embryonic stem; GZ, lens germinative zone; ICM, inner cell mass; LEC, lens epithelial cell; LFC, lens fiber cell; LIF, leukemia inhibitory factor; mo, month; OD, optical density; OFZ, organelle free zone; p, postnatal; pc, post coitum; PCR, polymerase chain reaction; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; trHbs truncated hemoglobins.

***Address correspondence to:** Michael A. Wride. Head, Ocular Development and Neurobiology Research Group, Department of Zoology, School of Natural Sciences, Trinity College, Dublin, Dublin 2, Ireland. Tel: +353-1-896-1036. Fax: +353-1-677-8094. e-mail: wridem@tcd.ie
Web: http://tcdlocalportal.tcd.ie/pls/public/staff.detail?p_unit=zoology&p_name=wridem

Supplementary Material (six figures and a movie) for this paper is available online at: http://dx.doi.org/10.1387/ijdb.082597fm

Received: 29 January 2008; *Evaluated:* 28 February 2008; *Accepted:* 1 April 2008; *Published online:* 19 May 2008.

0214-6282/2008/$35.00

Hemoglobin was also shown to be expressed in macrophages and alternative roles for it in oxygen or nitric oxide sensing were proposed (Liu *et al.*, 1999). The results suggested that individual hemoglobin genes could be selectively expressed in various cell types (as opposed to blood, in which α- and β-hemoglobin subunit expression is coordinated stoichiometrically in order to form the heme-containing, 2α, 2β tetramers required for oxygen transport). Recently, expression of adult α- and β-hemoglobins has been noted in primate and rodent alveolar type II (ATII) cells (stem cells that differentiate into alveolar type I cells) and Clara cells (the primary producers of pulmonary surfactant), implying an important role for hemoglobin in lung physiology and possibly pathophysiology (Bhaskaran *et al.*, 2005, Newton *et al.*, 2006).

Suggested extra-hematopoietic roles for hemoglobins include detoxification of NO, as O_2-scavenging/sensing/consuming enzymes (Pesce *et al.*, 2002) and in apoptosis in the absence of the heme moiety (Brecht *et al.*, 2005a, Brecht *et al.*, 2005b). Small cathepsin-mediated proteolytic cleavage products of hemoglobin proteins (hemorphins) have cytotoxic and anti-proliferative effects on cells and act as "atypical" opioid peptides (Fruitier *et al.*, 1999, Blishchenko *et al.*, 2002, Ianzer *et al.*, 2006). Finally, truncated hemoglobins (trHbs) are small hemoproteins (20-40 residues shorter than full-length mammalian hemoglobin chains found in bacteria, higher plants, and unicellular eukaryotes) that define a distinct phylogenetic group within the globin super-family (Nardini *et al.*, 2007).

We became interested in avascular expression of hemoglobin subunits for two reasons. Firstly, microarrays were used to investigate gene expression differences between embryonic stem (ES) cells and the blastocyst inner cell masses (ICMs) from which ES cells are derived (Hunter *et al.*, 2008)(GEO: GSE8881). The genes encoding the adult α-hemoglobin and embryonic β-hemoglobin subunits (Hba-a1/2 and Hbb-Y) were significantly upregulated in ICMs at two different stages of development. As early embryos give insufficient material for protein studies (our array studies were carried out using mRNA derived from two rounds of amplification), here we used *in vitro* ES cell differentiation with embryoid bodies (EBs) to model early embryonic development and to investigate hemoglobin subunit expression prior to hematopoiesis. ES cells are pluripotent cells, which can contribute to all cell types of the embryo proper (Evans and Kaufman, 1981, Smith, 2001). EBs are floating aggregates of ES cells that, when grown without leukemia inhibitory factor (LIF), mimic the developmental potential of early-stage embryos, albeit in a disorganized way (Leahy *et al.*, 1999, O'Shea, 2004). Precursors, representative of all three germ layers, are produced and directed differentiation can be used to yield specific cell types (Keller *et al.*, 1993, Kennedy *et al.*, 1997, Bain *et al.*, 2000, Desbaillets *et al.*, 2000, O'Shea, 2004).

Secondly, our gene expression profiling study (Wride *et al.*, 2003) of wild type post-natal mouse lens maturation showed expression of embryonic and adult hemoglobin subunits (Hba-a1/2, Hba-X, Hbb-b1, Hbb-b2, and Hbb-Y) and a chaperone (erythroid associate factor; EraF/α-hemoglobin stabilizing protein; AHSP [α-hemoglobin stabilizing protein], which is involved in α-hemoglobin folding). We went on to demonstrate that these genes are significantly downregulated in Sparc knockout mouse lenses during cataract progression (Mansergh *et al.*, 2004). Other microarray studies revealed downregulation of the α-hemoglobin gene in the human lens epithelium and in the mimecan null mouse lens during cataract progression (Hawse *et al.*, 2003, Tasheva *et al.*, 2004).

The lens is an excellent model system for studies of development, maturation and aging (Lovicu and Robinson, 2004). It consists of an outer layer of lens epithelial cells (LECs), which contain a stem cell niche centrally and anteriorly (Zhou *et al.*, 2006), while peripheral LECs proliferate, migrate and differentiate into lens fiber cells (LFCs), a process that begins during embryogenesis and continues throughout life (Griep, 2006). Therefore, a cross section of a lens at any age reveals a spectrum of cells in various stages of quiescence, proliferation and differentiation. Lens transparency requires removal of LFC nuclei and organelles during differentiation, resulting in formation of an organelle free zone (OFZ). Components of apoptosis signaling and proteolytic pathways are utilized in this process (Bassnett, 2002, Wride, 2007); defects can cause lens opacity or cataract (Pendergrass *et*

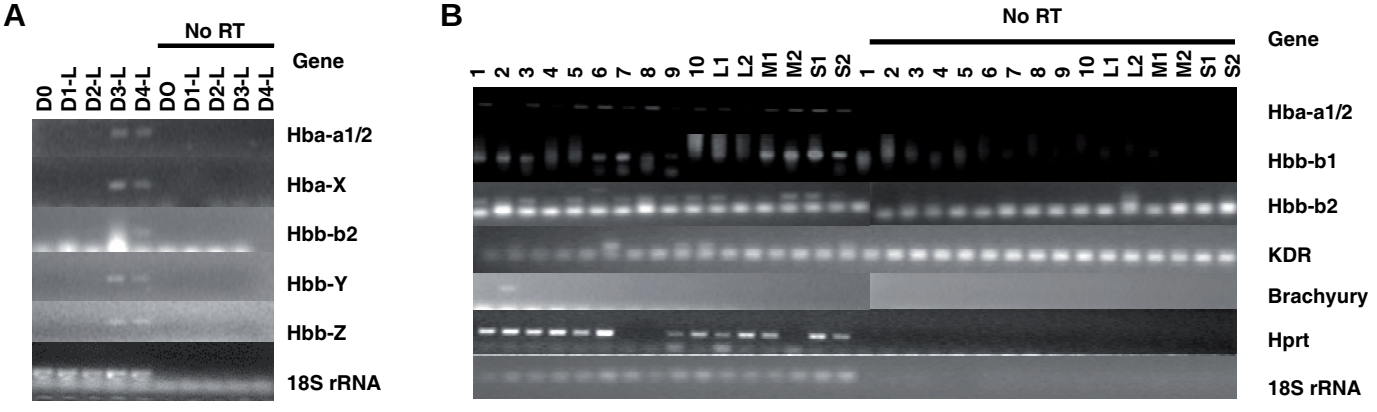


Fig. 1. Semi-quantitative RT-PCR of hemoglobins during early EB differentiation in cultures -LIF. (A) *Unamplified RNA from EB culture, 1000 cells/hanging drop, -LIF. D0 (Day 0), undifferentiated ES cells; D1 (Day 1) etc. PCR conditions used (gene: cycles, annealing temp): Hba-a1/2: 30, 58; Hba-X: 32, 61; Hbb-b2: 40, 61; Hbb-Y: 32, 61; Hbb-Z: 40, 58; 18sRNA: 22, 63).* **(B)** *Amplification of RNA from individual EBs, day 4 (D4)-LIF. L, large; M, medium; S, small EBs. KDR = early erythropoietic marker, Brachyury = early mesodermal marker, Hprt = alternative loading control giving variable results (as do actin and Gapdh; data not shown). PCR conditions used (gene: cycles, annealing temp): Hba-a1/2: 40, 58; Hbb-b1: 40, 61; KDR: 59, 45; Brachyury: 55, 40; Hprt: 58, 32; 18s RNA: 26, 62). 18s RNA = loading control. No-RT controls were run for each sample.*

al., 2005). LFCs, erythrocytes, blood platelets and keratinocytes are the only cell types that lose nuclei and organelles during terminal differentiation (Lockshin and Zakeri, 2004). Notably, the lens is entirely avascular and can be dissected free of hematopoietic contamination, potentially making it an ideal model system for the study of hemoglobin subunit function outside of the hematopoietic system (Wride *et al.*, 2003, Mansergh *et al.*, 2004).

Here, we present a thorough spatio-temporal analysis of embryonic and adult α- and β-hemoglobin subunit expression during early embryonic development, early ES cell differentiation and mouse lens development. The expression of hemoglobin protein subunits in pre-vascular embryos and in an avascular tissue (the lens), suggests a paradigm shift. In addition to the accepted function of hemoglobin in hematopoietic oxygen transport, we postulate developmental roles for hemoglobin in apoptosis signaling and/or early development and stem cell differentiation. These results pave the way for functional studies to determine the developmental roles of hemoglobin subunits outside the hematopoietic system.

Results

ES cell-embryo microarrays and EB RT-PCR

Microarray studies, comparing individually picked undifferentiated ES cell colonies with various microdissected ICM stages from which ES cells are derived, revealed differential expression of hemoglobin between blastocyst ICMs at 88 and 105 h post coitum (pc) and undifferentiated ES cells. Fold changes were as follows: Hbb-Y (mean of 3 different spots): +3.29 (P=0.0014) and Hba-a1/2 (mean of 2 different spots): +3.21 mean (p=0.00065). The entire MIAME compliant dataset has been submitted to and approved by GEO (http://www.ncbi.nlm.nih.gov/geo/; series accession number GSE8881). Given the paucity of starting material, two rounds of RNA amplification were required prior to arraying. Amplification can lead to bias in results; in order to minimize this, amplification and arraying methods were exhaustively optimized. These studies are described comprehensively elsewhere (Hunter *et al.*, 2008). Genes known to be involved in ES cell pluripotency and early development were also noted as differentially regulated in other results obtained from this array study (GSE8881), suggesting a high level of accuracy.

Nevertheless, we have attempted to confirm the reliability of these results using the reverse transcriptase-polymerase chain reaction (RT-PCR). Unamplified RNA from pooled ICMs can be used for RT-PCR if the genes in question are expressed at levels similar to β-actin, Gapdh or 18S rRNA (Hunter *et al.*, 2008); however, less abundantly expressed RNAs, such as the globin genes, are undetectable. We have detected adult hemoglobin subunits using one round of amplification with single EBs prior to RT-PCR with 40 cycles (see below). However, these EBs had an initial cell number of 1000 and could have contained up to 16,000 cells by day 4, as ES cells double roughly every 24 hours. ICMs 88 hours pc contain 48 cells; even 15-20 pooled ICMs would have given a total cell number of under 1000. PCR using double amplified RNA gives very erroneous results as the length of individual RNAs is reduced to 200-400bp on average and is very 3' biased. Moreover, sufficient material for protein studies cannot be obtained with ease. We therefore asked the question as to whether hemoglobins are expressed during undirected early *in vitro* differentiation of ES cells. EBs, with an initial cell number of 1000, were grown in suspension for four days after the initiation of differentiation via formation of hanging drops and withdrawal of LIF from the culture medium. RNA was extracted from ES cells and from day 1-4 -LIF EBs and subjected to RT -PCR. Given the sequence similarity between genes within the α- and β-hemoglobin gene clusters, expression of all hemoglobin genes was tested. No bands were identified at day 0 (undifferentiated ES cells), but expression of Hba-a1/2, Hba-X, Hbb-b2, Hbb-Y and Hbb-z appeared at day 3 of culture and was maintained on day 4 (Fig. 1A). Expression of Hbb-b1 was not detected; notably, other studies (see discussion) have referred to adult β-hemoglobin without differentiating between the two adult β-hemoglobin genes (Hbb-b1, Hbb-b2), which are identical in sequence at the protein, but not mRNA level.

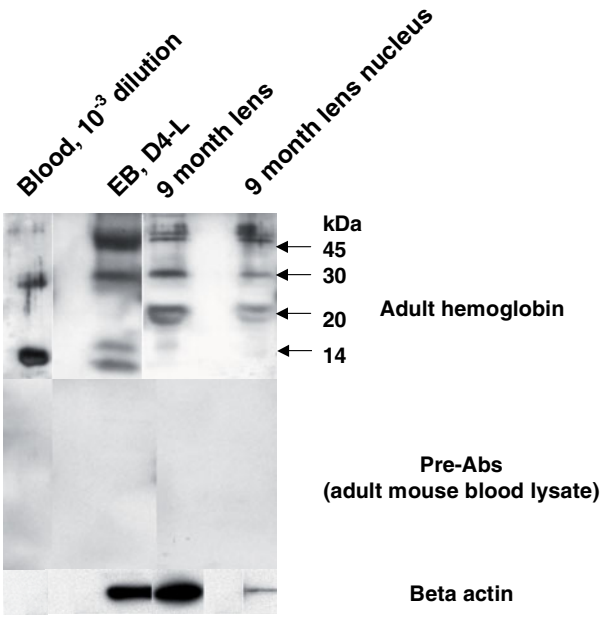


Fig. 2. Western blotting of hemoglobins during early EB differentiation day 4 EB-LIF compared to adult lens lysates. *Mouse blood lysate was used as positive control. β-actin was used as a loading control following stripping of blots. β-actin was not detectable in blood lysate as we used a 10^{-3} dilution of blood (approximately 0.024 μg) in order not to overload the gel. The blood blot gave a similar banding pattern as seen in the lens samples, with major bands at 14 kDa (corresponding to α- and β-globin monomers of 10 kDa and 16 kDa) and 30 kDa (corresponding to either alpha-globin trimers (30 kDa) and/or beta-globin dimers; see also Figure 5). In addition, there was a fainter band at approximately 45 kDa. The EB blot was similar to the blood blot except that there were two bands at around 14 kDa. These bands were much fainter in the 9 month lens samples (particularly the lens nucleus sample). The doublet band in both lens samples at approximately 20 kDa was not present in the day 4 EB (-LIF) samples. Blots were stripped and re-probed with anti-hemoglobin antibody pre-absorbed with either blood lysate or human haemoglobin powder (data not shown), which completely abolished staining.*

Given results suggesting expression of hemoglobin protein in each EB by immunofluorescence (see below), we tested whether the corresponding mRNAs were also expressed in each individual EB, as many genes upregulated in early development are only expressed in a minority of EBs at day 4 (e.g. Brachyury, Kdr, Afp,

Goosecoid; Fib 7B and FCM, SMH and MJE, unpublished results). To ensure that immunocytochemical staining of all EBs (see below) was not non-specific (even given the lack of staining in IgG controls), we used one round of amplification to amplify RNA from 16 individual EBs. Hba-a1/2 was consistently expressed in each EB, regardless of its size, and was more consistently expressed than any other gene tested (including the early hematopoietic marker KDR, the mesodermal marker Brachyury and the house-keeping gene Hprt (Fig. 1B) as well as actin and Gapdh; data not shown); only 18S rRNA showed more consistent expression across all samples at day 4-LIF (Fig. 1B). Expression of both Hbb-b1 and Hbb-b2 was more variable (each being expressed in 10 out of 16 individual EBs; 62.5%). Embryonic hemoglobin subunits (Hba-X, Hbb-Y and Hbb-Z) were not detectable in individual EBs using this method. This may simply be because they are expressed in a minority of EBs (i.e. none of those tested). However, this is more likely to be a result of the difficulties of PCR from low starting quantities of RNA; even one round of amplification shortens the length of most amplified mRNA (aRNA) fragments making PCR correspondingly more difficult, especially if primer sets are designed towards the 5' end of genes. We often have to increase PCRs by at least 6-8 cycles when using RNA generated from 1 round of amplification. Moreover, the number of cycles required does not always relate well to cycles used for unamplified RNA (FCM, SMH and MJE, unpublished results), which may explain also why Hbb-b1 appears to be expressed when the RNA is amplified, but not when unamplified RNA is tested.

Western blotting of hemoglobin proteins during early EB differentiation

Western blotting was used to investigate hemoglobin protein expression in differentiating EBs at day 4 -LIF using the rabbit anti-mouse antibody to pan-mouse hemoglobins (ICN/Cappel; Fig. 2), β-actin as a loading control and blood lysate as a positive control. Expression in EBs was compared with 9 mo lens and 9 mo lens nucleus. The blood blot gave a similar banding pattern as seen in the lens samples (see Fig. 5). In addition, there was a faint band at approximately 45kDa. The EB blot was similar to the blood blot with two bands at around 14kDa, which were faint in the 9 mo lens samples. The doublet band in lens samples at approximately 20kDa was not present in day 4 EBs -LIF. Blots were stripped and re-probed with anti-hemoglobin antibody pre-absorbed with either blood lysate or human hemoglobin powder (data not shown), which abolished staining. In the blood lysate blot, no β-actin band was obtained at the exposure times used, since we used blood lysate at a 10^{-3} dilution (approximately 0.024 μg) in order not to overload the gel; hemoglobin is readily detectable at these concentrations as it comprises 98% of RBC protein and is the largest protein constituent of blood; so, actin is below the level of detection.

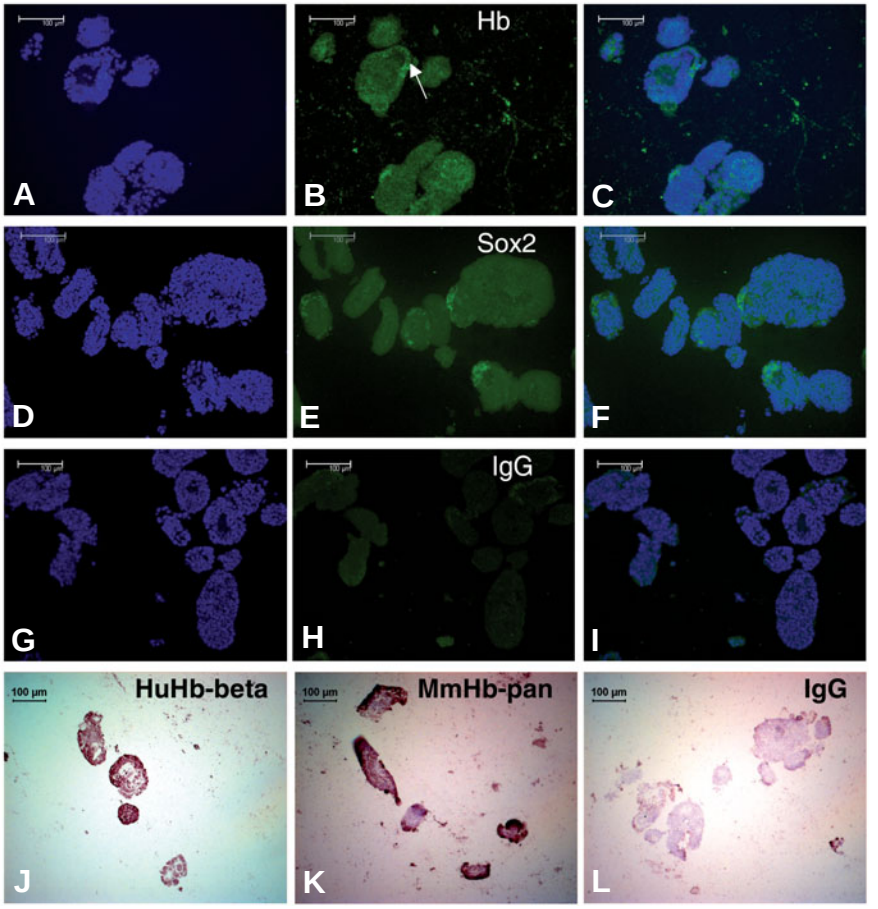


Fig. 3. Immunostaining of early differentiating EBs in culture. (A-I) *Immunofluorescence and* **(J-L)** *immunoperoxidase for hemoglobin* **(B,J,K)** *and a positive control, Sox2* **(E,F)**. *Anti-mouse pan hemoglobin was used in B, C and K, while anti-human β-hemoglobin was used in J. Hemoglobin staining was associated with the cytoplasm of nearly all cells in the EB, but not the nuclei (arrow in B).* **(H,I,L)** *Sections stained with rabbit IgG at the same concentration as the hemoglobin antibody are negative for staining. Bars, 100 μM.*

Immunofluorescence and immunoperoxidase for hemoglobin during early EB differentiation

Immunofluorescence was carried out on day 4 -LIF EBs using the polyclonal antibody to mouse hemoglobin and an antibody to human β-hemoglobin (Fig. 3). Using the anti-mouse antibody with immunofluorescence, expression was observed in the cytoplasm of the majority of cells in the EBs (Fig. 3 B, C). An antibody to Sox2 was used as a positive control; this was localized to distinct regions of the EBs (Fig. 3 E, F). Immunoperoxidase was also used to reveal staining for hemoglobin in early stage EB differentiation (Fig. 3 J-K); both the anti-mouse and anti-human antibodies revealed staining in EBs. The rabbit IgG controls were negative using both methods (Fig. 3 H, I).

Semi-quantitative RT-PCR of hemoglobins in the developing lens and cornea

Previous results demonstrated expression of 5 of 6 hemoglobin subunit mRNAs in newborn, p7, p14 and 9 mo adult mice (Wride *et al.*, 2003, Mansergh *et al.*, 2004). Here, we show expression of all 6 adult and embryonic hemoglobin subunit mRNAs in the embryonic eye (E12.5) and lens (E14.5, E16.5, NB, p7, p14, and 4 wk; Fig. 4A). Hba-a1/2 had a fairly constant profile of

expression, with a slight reduction in expression in the lens from newborn (NB) to 4 wks. The other hemoglobin chains and Ahsp/Eraf exhibited largely decreasing expression levels towards maturity (although Hba-x and Hbb-y both showed a slight peak of expression at day 7, after an initial postnatal drop). Hbb-Z is expressed in the embryonic eye and lens at E12.5 and E14.5, but not subsequently. PCR cycles used reflect high expression of these genes at embryonic stages; use of higher cycles reveals more substantial expression at postnatal stages (Wride *et al.*, 2003, Mansergh *et al.*, 2004). The corneas of 9 mo wild type and Sparc knockout mice showed expression of Hba-a1 and Hbb-b2 (adult hemoglobin subunits, Fig. 4B). Both Hba-a1 and Hbb-b2 were significantly upregulated in Sparc knockout mouse corneas (Fig. 4B).

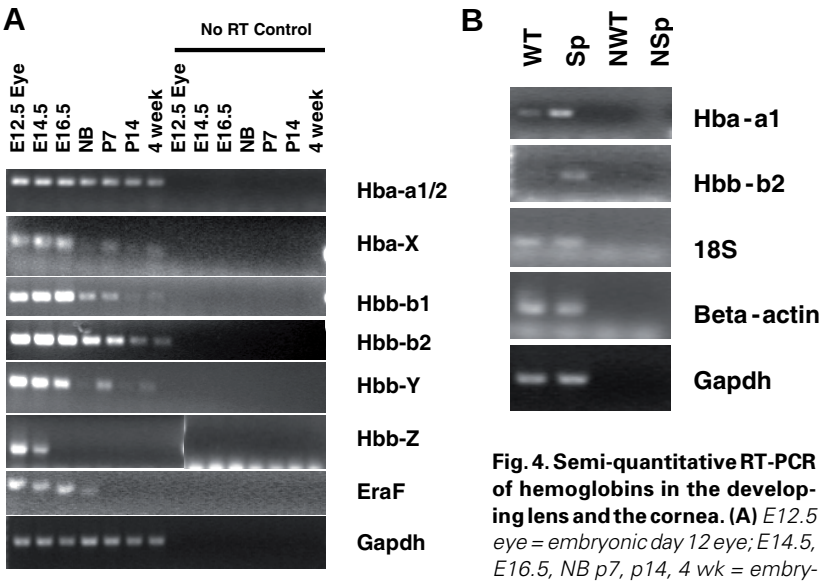


Fig. 4. Semi-quantitative RT-PCR of hemoglobins in the developing lens and the cornea. (A) *E12.5 eye = embryonic day 12 eye; E14.5, E16.5, NB p7, p14, 4 wk = embryonic and postnatal lens samples. Hemoglobin α, adult chains 1 and 2 were assessed together (Hba-a1/2) as Hba-a1 and Hba-a2 are identical in sequence. Hemoglobin, β adult major chain = Hbb-b1, hemoglobin, β adult minor chain = Hbb-b2. Hemoglobin X, α-like embryonic chain = Hba-X; hemoglobin Y, β-like embryonic chain = Hbb-Y and hemoglobin Z, β-like embryonic chain = Hbb-Z. Gapdh is used as a loading control.* **(B)** *Expression of globins in the mouse cornea; corneas from 9 mo old mice. WT, wild-type (129 Sv/Ev) mice, Sp, Sparc knockout mice (129 Sv/Ev). 18s rRNA, β-actin and Gapdh = loading controls. NWT, NSp = no RT controls. PCR conditions used in A and B (gene: cycles, annealing temp): Hba-a1/2: 32, 58; Hba-X: 32, 61; Hbb-b1: 32, 61; Hbb-b2: 32, 61; Hbb-Y: 32, 61; Hbb-Z: 45, 58; EraF: 32, 61; Gapdh: 22, 62.*

Western blotting of hemoglobins in post-natal and adult lens lysates

Western blotting revealed hemoglobin protein expression in the lens from newborn (NB) to 9 mo using a rabbit anti-mouse antibody to pan-mouse hemoglobin (ICN/Cappel). A monoclonal antibody to human fetal hemoglobin (AbCam cat# ab20517-250) was used to investigate embryonic and fetal hemoglobin expression in the 9 mo mouse lens (Fig. 5). We also attempted Western blotting with 3 anti-human antibodies, none of which worked (immunocytochemical staining with these antibodies was faint). In all Western blots, β-actin was used as a loading control, while blood lysate was used as a positive control

Two major bands were detected on the blood blot at 14 kDa and 30kDa (Fig. 5A). In the NB to p14 lens samples, bands were observed at 26 kDa and 30kDa. In the 4 wk lens, the 26kDa band disappeared. In the 9 mo whole lens, the 30kDa band predominated. Also in the 9 mo lens, bands were present at 20 kDa and 45kDa. The 45kDa band and the upper band of the doublet at 20kDa were fainter in the 9 mo lens nucleus sample. Blots were stripped and re-probed with anti-hemoglobin antibody pre-absorbed with blood lysate or human hemoglobin powder and most staining was abolished. Blots were stripped and re-probed with β-actin, confirming equal protein loading. β-actin was not detectable in blood lysate at 10^{-3} dilution (approx 0.024 μg) because blood comprises 98% hemoglobin; therefore, actin is below levels for detection. Using the antibody to fetal hemoglobin on individual 9 mo lens lysates, bands were observed at 20 kDa and 14kDa (Fig. 5B).

Immunofluorescence for hemoglobin during lens, cornea and retina development and maturation

Embryonic day 16.5 (ED16.5) and NB

Hemoglobin protein expression in the NB lens (Fig. 6 A-F) was detected in the LFCs in the cortex and was more intense in the anterior LFCs, compared to the posterior LFCs; it was faint in the cytoplasm of LECs. Staining was reduced towards the core of the lens, in which nuclei and other organelles are degenerating to form the OFZ. Intense staining was observed in erythrocytes associated with the hyaloid vasculature (Fig. 6 B, E, H; arrows). Staining was also associated with retinal ganglion cells (RGCs), the retinal pigment epithelium (RPE; Fig. 6 G-I) and the corneal endothelium (CE; Fig. 6E). The rabbit IgG control was negative (Fig. 6 J-L). These data were similar to the staining observed in the eye of the ED16.5 embryo (Supplementary Fig. 1).

P7 and p14

Hemoglobin immunofluorescence was very intense in lens cortex LFCs that have not yet lost their nuclei (as indicated by 4',6-diamidino-2-phenylindole (DAPI) staining) in both coronal (Fig. 7 A-F) and transverse (Fig. 7 G-O) sections, but was much less intense in the OFZ. Staining was also associated with the cytoplasm/cell surfaces of LECs, but not the nuclei (Fig. 7E and K). The RGC layer, other areas within the inner neuroblast layer (INBL), the corneal endothelium (CE) and the lens capsule (LC) were also positive. The rabbit IgG control was negative (Fig. 7 M-O). Staining patterns in the p14 lens were similar to those at p7 (Supplementary Figs. 2,3).

4 wks, 9 wks and 9 mo

In the 9 wk mouse lens, immunofluorescence was associated with cortical LFCs, particularly the border zone (BZ; Fig. 8 B-C), but, most-strikingly, it was associated with a peri-nuclear compartment and/or the nuclear membrane of cortical LFC nuclei (Fig. 8 E, H, F, and I). A laser scanning confocal microscope movie illustrates this (Supplementary Movie 1; http://dx.doi.org/10.1387/ijdb.082597fm). Nuclear-associated staining was significantly reduced in nuclei positioned further towards the anterior and core of the lens (arrowheads; Fig. 8C). A different plane of focus demon-

strated hemoglobin immunofluorescence in LECs (Fig. 8K and L). Staining was associated with the cytoplasm of LECs in the germinative zone (GZ), but became localized to nuclei at the lens equator (EQ) in which LECs migrate, stop proliferating and begin their terminal differentiating into LFCs. The rabbit IgG control was negative (Fig. 4 M-O). 4 wk (Supplementary Fig. 4) and 9 mo (Supplementary Fig. 5) lenses showed similar staining patterns. At 4 wks and 9 wks, hemoglobin immunofluorescence was associated with the surfaces of posterior cortical LFCs (Fgure 4 H and I; Supplementary Fig. 4 A-D). However, this pattern of immunofluorescence was different between 4 wks and 9 wks (compare Fig. 4B and 4D).

Diaminofluorene heme assay reveals presence of heme in EBs, but not in the lens

A diaminofluorene assay (Kaiho and Mizuno, 1985) was used to investigate whether heme was present in both lens and EB samples. A colorimetric heme assay using blood lysates at various dilutions (10^{-4}, 10^{-5} and 10^{-6}) as positive controls and water as a negative control, was used to test various lens and EB homogenates (Fig. 9; Table 1). Calculating the mean of all values at 30s and 45s for each condition revealed statistically significant differences for blood lysates at dilutions of 10^{-4} and 10^{-5} (but not 10^{-6}) compared to the water blank (see Table 1). The optical density (OD) was not significantly different to the water blank in any of the lens lysates. However, the level of heme in EBs was intermediate between the blood lysate samples diluted 10^{-4} and 10^{-5} (p=0.0016, Table 1).

We also used the diaminofluorene reaction as an histological stain for heme on tissue sections and on whole lenses (Supplementary Fig. 6). Positive staining (blue/green) was noted in the E16.5 embryo; there was positive staining for heme in the eye, including the lens and the retina (Supplementary Fig. 6 I-J). However, there was no detectable heme staining in sections of lenses from NB to p14, or in whole 9 mo lenses after 15 minutes in reaction mixture (Supplementary Fig. 6 E-F). However, in 9 mo corneas, a blue reaction color had developed afer 15 minutes owing to the presence of erythrocytes in the attached iris material (arrow in Supplementary Fig. 6H). EBs showed a low level of staining reflecting the levels of heme identified in EBs using the colorimetric heme assay (Fig. 9).

TABLE 1

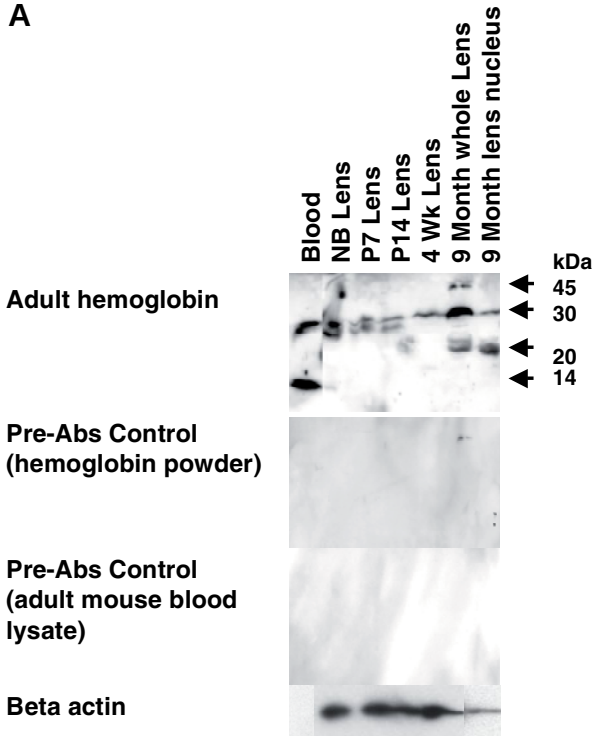
STATISTICAL ANALYSIS OF HEME ASSAY DATA PRESENTED IN FIG. 9

t-test	p value	n
blank vs blood x10-4	0.0000011*	n=6
blank vs blood x10-5	0.0000006*	n=6
blank vs blood x10-6	0,442809	n=6
blank vs 9m lens	0,414334	n=7
blank vs post natal lens	0,350203	n=4
blank vs EB	0.0015942*	n=3

Using the un-paired students' t-test, significant differences are revealed in the heme assay between the water blank and blood lysates at 10^{-4} and 10^{-5} dilutions and between the water blank and EBs, but not between the water blank and blood lysate at 10^{-6} and the water blank and postnatal or 9 mo mouse lens lysates. Mean of values compared at 1 min and 1 min 30 s.

Immunofluorescence for hemoglobin in adult mouse gut villus epithelium

The gut is richly supplied with blood and, as such, represents a "normal" vascularized tissue and a positive control for hemoglobin



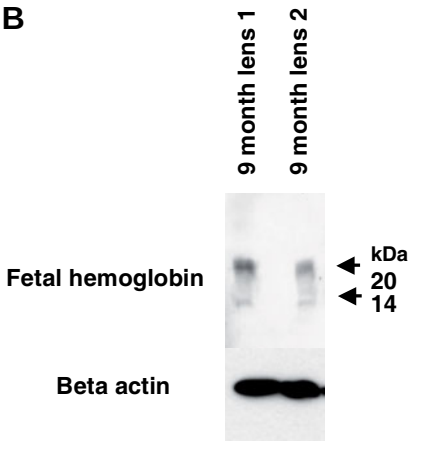


Fig. 5. Western blotting of hemoglobins in post-natal and adult lens lysates. (A) *Mouse blood lysate was used as a positive control. Two major bands were detected in blood, one at approximately 14 kDa (corresponding to α- and β-globin monomers of 10 kDa and 16 kDa) and 30 kDa (corresponding to either alpha-globin trimers and/or beta-globin dimers). In the NB to p14 lens samples, two bands were observed at approximately 26 kDa and 30 kDa (corresponding to α-globin-β-globin dimers (26 kDa), α-globin trimers (30 kDa) and/or β-globin dimers (32 kDa)). In the 4 wk lens, the lower band of the doublet at around 30 kDa disappeared. In the 9 mo whole lens, the upper of the two bands at approximately 30 kDa predominated, with the lower band very faint. In the 9 mo lens, a doublet band was also present at 20 kDa (corresponding to α-globin dimers). In addition, there was a doublet band at 45 kDa to 48 kDa, corresponding to β-globin trimers (48 kDa) and/or 3 x α-globin, 1 x β-globin tetramers (46 kDa). The 45 to 48 kDa band and the upper band of the doublet at 20 kDa were reduced in intensity compared to the 9 mo lens nucleus sample.* **(B)** *Fetal hemoglobin expression in two 9 mo old mouse lenses. The major band, in each case, was at 20 kDa (corresponding to fetal-globin dimers) with a fainter band at 14 kDa (corresponding to fetal-globin monomers). Blots were stripped and re-probed with anti-hemoglobin pre-absorbed (Pre-Abs) with either blood lysate or human hemoglobin powder and this abolished staining (one representative blot shown). Blots were also stripped and re-probed with β-actin antibody to confirm equal protein loading. β-actin was not detectable in blood lysate as we used a 10^{-3} dilution (approximately 0.024 μg) of our original blood lysate in order not to overload the gel.*

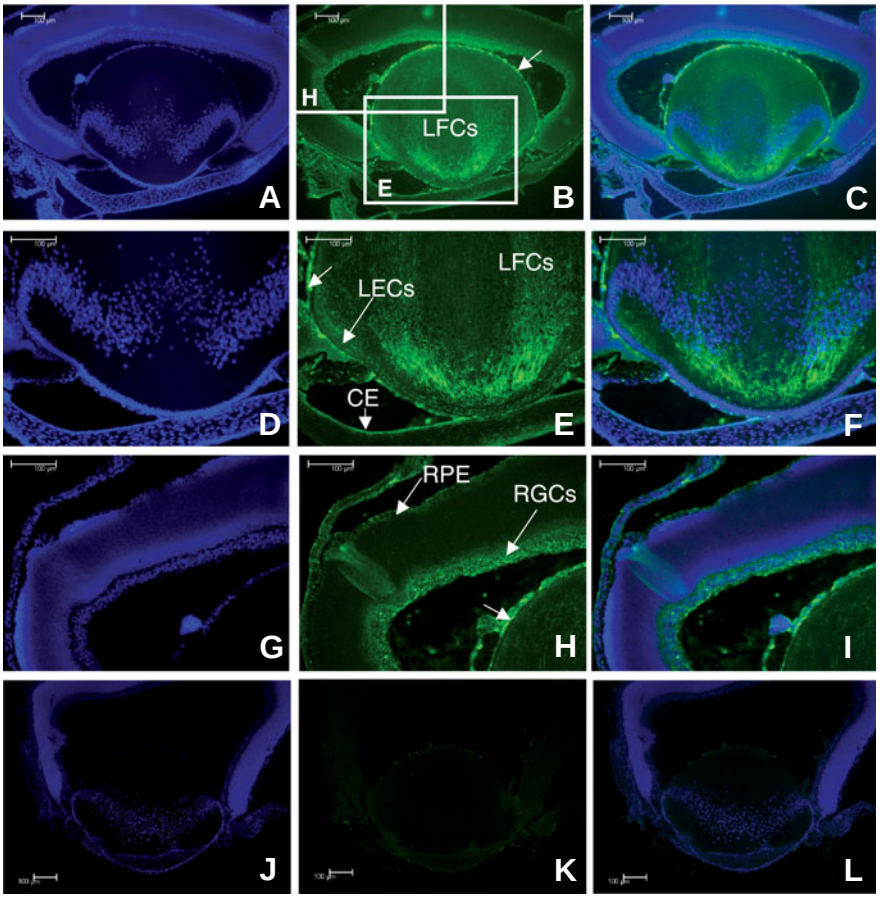


Fig. 6. Immunofluorescence for hemoglobin, newborn (NB) mouse eye. (A-J) *DAPI labelling.* **(B-K)** *Hemoglobin immunofluorescence.* ***(C-L)*** *Merged images. Regions defined by white rectangles in B are those in E and H. Intense immunofluorescence for the lens caspule and/or erythrocytes can be seen at the back of the lens (arrows in B, E, H). Staining is intense in the anterior region of the LFCs, decreasing in a gradient towards the posterior of the lens, whereas staning is faint in the cytopasm of LECs* **(E)**. *In the retina, staining is mainly associated with the developing RGC layer* **(H)**. *RPE cells are also positive for staining as is the corneal endothelium (CE)* **(H)**. **(J-L)** *Sections stained with rabbit IgG at the same concentration as the hemoglobin antibody are negative for staining. Bars, 100 μM.*

immunofluorescence. Furthermore, since our evidence above suggested expression of hemoglobin in differentiation of stem cells in both LECs and EBs, we sought to investigate whether hemoglobin immunofluorescence was associated with gut villi, which contain stem cells in their crypts that migrate within villi epithelia and subsequently undergo terminal differentiation (Yen and Wright, 2006, Barker *et al.*, 2007). In addition to erythrocytes (identified by positive staining for hemoglobin and lack of DAPI staining, since their nuclei have disappeared; arrowheads, Fig. 10 A-C), we identified a small, but significant number of hemoglobin positive cells at various positions within villus epithelia, excluding the crypts (Fig. 10 A-I, arrows).

Discussion

Hemoglobin subunit expression in early embryos and EBs prior to hematopoiesis

Our array results (GEO, GSE8881) demonstrated the expression of Hba-a1 and Hbb-y RNAs in the blastocyst ICM prior to implantation, a stage which can give rise to multipotent ES cells and is relatively undifferentiated. We have also shown hemoglobin subunit expression in EBs (a model of early differentiation) at stages several days prior to the accepted time of erythroid differentiation and blood island formation, in accordance with previously published work (Schmitt *et al.*, 1991, Keller *et al.*, 1993, Baird *et al.*, 2001). However, we have demonstrated expression of a larger number of hemoglobin subunits than previously examined and hemoglobin protein subunit expression was also shown using immunofluorescence and Western blotting.

EBs have long been used for hematopoietic differentiation *in vitro*. Many groups have noted early expression of hemoglobin RNAs in EBs during initiation of hematopoietic differentiation protocols. Despite large variations in the starting size of the EBs (1-1000 cells), tissue culture media and detection methods (RT-PCR, RNase protection assay, subtractive library sequencing, staining for α-hemoglobin protein), hemoglobin subunits are expressed consistently from day 3-5 of differentiation (Schmitt *et al.*, 1991, Wiles and Keller, 1991, Keller *et al.*, 1993, Baird *et al.*, 2001). Variations in onset of expression are most likely related to the varying sensitivities of the assays. Our study, using RT-PCR on single EBs (with RNA amplification), revealed expression of the adult α-hemoglobin gene in every single EB studied, suggesting an important role for it in early EB differentiation.

Hematopoietic stem cell differentiation studies tend to interpret the early expression of hemoglobin subunits as a sign of 'pre-conditioning' for the hematopoietic lineage. Further differentiation does indeed lead to hematopoietic specification and subsequent appearance of blood islands within the EBs, as well as primitive blast colonies that can be used to generate hematopoietic cells *in vitro*, and, to some extent, *in vivo* (Schmitt *et al.*, 1991, Wiles and Keller, 1991, Keller *et al.*, 1993, Kennedy *et al.*, 1997, Baird *et al.*, 2001). However, there are some differences between these prior studies and our results presented here. We show here that hemoglobins (in the previous studies associated with terminal erythropoiesis) are up-regulated simultaneously with Gata-1, a transcription factor involved in very early hematopoietic specification (Keller *et al.*, 1993) and Brachyury, a very early marker of mesodermal differentiation. Moreover, we show that both KDR, a marker of early hematopoiesis (Ziegler *et al.*, 1999) and Brachyury are expressed very inconsistently between individual EBs at day 4 -LIF (Fig. 1B), significantly more inconsistently than adult hemoglobin subunits (Hba1/2, Hbb-b1 and Hbb-b2). Hemoglobin subunit expression initiates 3-4 days prior to the obvious appearance of blood islands in the EBs (day 7 to 8 -LIF)(Keller *et al.*, 1993). Moreover, in the embryo, the first signs of yolk sac hematopoiesis also appear at embryonic day 8 (Lindenbaum and Grosveld, 1990). Therefore, the timing of obvious hematopoietic differentiation is remarkably

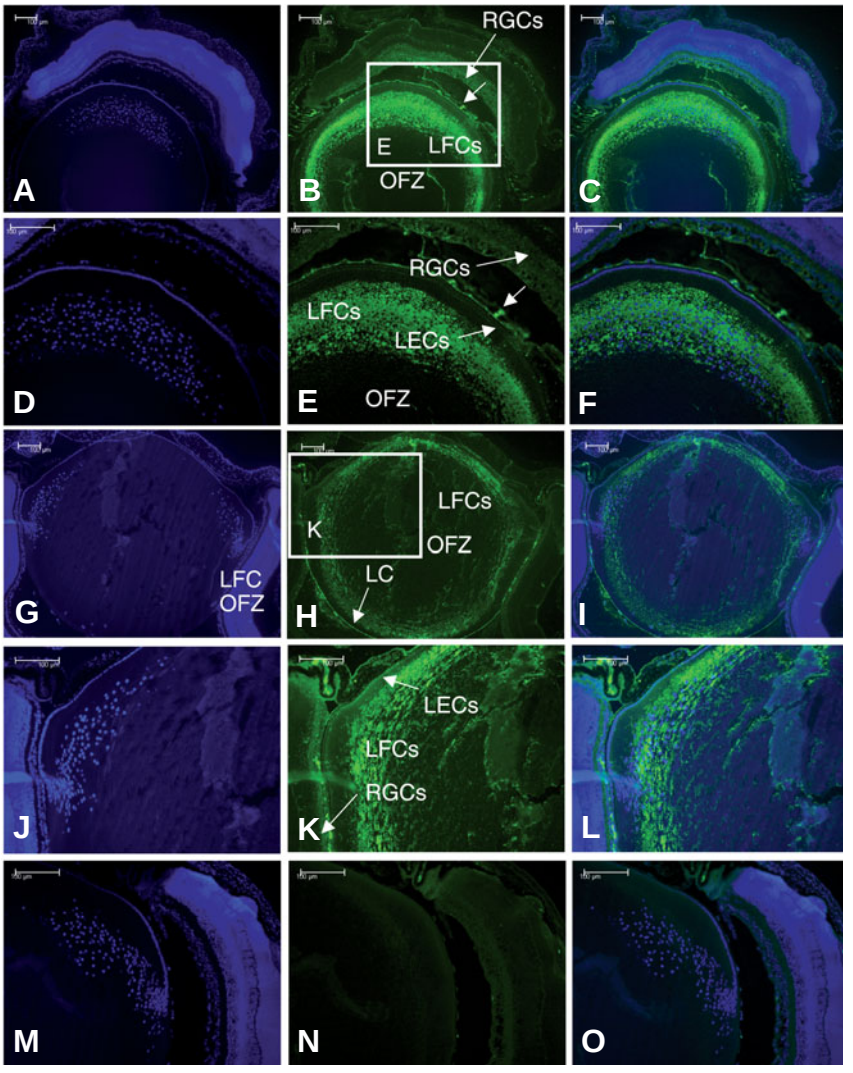


Fig. 7. Immunofluorescence for hemoglobin proteins, p7 mouse eye. (A,D,G,J,M) *DAPI labelling.* **(B,E,H,K,N)** *Hemoglobin immunofluorescence.* **(C,F,I,L,O)** *Merged images.* **(A-F)** *Coronal section through the eye.* **(G-O)** *Transverse section through the eye. Regions defined by white rectangles in (B) and (H) are those in (E) and (K). Intense immunofluorescence for erythrocytes and/or the lens capsule can be seen at the surface of the lens associated with the vitreous (arrows in B, E). Intense staining is observed in LFCs in the lens cortex in the layer in which lens fiber cells still retain their nuclei* **(B-K),** *but not significantly in LECs* **(K)** *or the OFZ* **(B-N)**. *Immunofluorescence is also associated with the lens capsule (LC)* **(H)**. *In the retina, staining is associated with the RGC layer* **(H)**. **(M-O)** *Sections stained with rabbit IgG at the same concentration as the hemoglobin antibody are negative for staining. Bars, 100 μM.*

similar in embryos and in EBs (day 8). In contrast, our arrays show up-regulation of Hba-a1 and Hbb-Y in the undifferentiated ICM at 88 h and 105 h pc (3.5-4.5 days). Furthermore, the expression of hemoglobin subunits appears at a stage when both embryos and EBs are showing very early signs of differentiation (day 3-4), rather than hematopoiesis *per se*. Notably, the ability of ES cell-derived hematopoietic precursors to reconstitute hematopoiesis *in vivo* is "surprisingly low" (Hole, 1999) or requires forced expression of the BCR/ABL oncogene (Perlingeiro *et al.*, 2001) or HoxB4 (Kyba *et al.*, 2002).

While it is possible that hemoglobin subunits may be specifying early hematopoietic 'pre-conditioning' (Baird *et al.*, 2001), we propose, given that we have found them to be expressed in avascular tissues during development: in the GZ of the lens epithelium and during differentiation of LFCs as well as in the cornea, that it is more likely that hemoglobin subunits have a more general role in the differentiation (or 'preconditioning') of numerous lineages during early embryonic development.

Hemoglobin subunit expression in the lens

Here, and in our previous studies, we have demonstrated hemoglobin subunit RNA and protein expression in prenatal and postnatal lenses and their down-regulation during cataract progression (Wride *et al.*, 2003, Mansergh *et al.*, 2004). During development, the lens is provided with oxygen and nutrients by the hyaloid vasculature, a structure removed by programmed cell death between p12 and p16 in the mouse (Ito and Yoshioka, 1999). The mature lens receives nutrients and removes waste products through the aqueous and vitreous humors without direct recourse to the vascular system. The expression of hemoglobin subunits in the lens is, therefore, suggestive of a new role for these proteins.

Notably, cataract and other ocular pathologies, including RPE degeneration, have been observed in association with sickle cell anemia and both α- and β-thalassemias (Bloomfield *et al.*, 1978, Lorenzen *et al.*, 1979, Gartaganis *et al.*, 1989, Sorcinelli *et al.*, 1990, Babalola *et al.*, 2000, Athanasiadis *et al.*, 2007). In the majority of these studies, the ocular anomalies observed have been attributed to iron metabolism disturbances. However, Babalola *et al.* (2000) described hereditary congenital cataracts associated with sickle cell anemia, while Lorenzen *et al.* (1979) found an association between sickle cell anemia and senile cataracts. It should also be pointed out that neither sickle cell anemia nor major thalassemias result in total loss of hemoglobin function. Therefore, it may be the case that expression of mutated β-hemoglobin (sickle cell disease), or 2 adult α- or β-hemoglobin subunit genes instead of 4 (thalassemia) is still sufficient to carry out any non-hematopoietic functions of the hemoglobin subunits and that pathology in these diseases is due entirely to hematopoietic insufficiency. However, given our results, it is possible that at least some of the ocular and/or systemic pathologies observed in sickle cell anemia and thalassemias may result from a diminution of non-hematopoietic functions of hemoglobin subunits and this possibility merits further analysis.

The immunofluorescence expression patterns we observe are consistent with roles for hemoglobin subunits in primary (ED16.5) and secondary LFC differentiation. Secondary lens fiber differentiation in the mouse peaks postnatally (p1-p7), correlating with increased hemoglobin subunit expression at p7. This process continues throughout life as new LFCs differentiate from LECs at the equator region of the lens. Furthermore, adult central LECs can

be considered to be stem cells that divide very infrequently in normal circumstances, while their progeny, more peripheral LECs, provide limited proliferative potential and move into the GZ towards the lens equator where they begin to cycle actively and to differentiate into LFCs (Zhou *et al.*, 2006). Hemoglobin proteins are expressed in the cytoplasm of LECs of the GZ (Fig. 8B, K (9 wks) and Supplementary Fig. 5; 9 mos). As these cells move towards the lens EQ, they acquire nuclear-associated expression of hemoglobin protein, which persists in cortical LFCs. There is prior evidence of nuclear cytoglobin expression (Geuens *et al.*, 2003). Moreover, nuclear expression of a non-symbiotic plant hemoglobin is increased during hypoxia (Seregelyes *et al.*, 2000). Notably, the nuclear association that we observe in the lens occurs spatially in shells of LFCs just outside those in which loss of LFC nuclei and organelles is occurring to form the OFZ during terminal LFC differentiation. The significance of immunofluorescence at the surface of LFCs (Fig. 8H and I; Supplementary Fig. 4) remains to be determined.

Since the heme moiety is undetectable in the lens from NB stages onwards, our prior hypotheses that hemoglobin subunits function as oxygen transporters, oxygen sinks or in iron homeostasis in the lens now seem unlikely (Wride *et al.*, 2003, Mansergh *et al.*, 2004). Therefore, given their expression patterns, the most credible hypothesis now is that hemoglobin subunits expressed in the lens are involved in aspects of LEC differentiation, specifically the denucleation/organelle loss undergone by maturing LFCs. This process appears to require components of apoptosis signaling pathways (Bassnett, 2002, Wride, 2007). Furthermore, Gata-2-mediated up-regulation of α-globin (i.e. α-hemoglobin protein without the association of the heme moiety) is pro-apoptotic in hematopoietic and non-hematopoietic cell lines (Brecht *et al.*, 2005a, Brecht *et al.*, 2005b), acting through suppression of bcl-2, activation of bax, release of cytochrome c and activation of caspases. Erythrocytes in most mammals undergo a loss of nuclei during erythroblast differentiation, albeit by enucleation (extrusion of the nucleus), rather than by denucleation (degradation of the nucleus). This process occurs concomitantly with hemoglobin up-regulation in erythroblasts (Koury *et al.*, 1987). Furthermore, a number of erythroid differentiation denucleation factors (EDDFs) have been identified (Xue *et al.*, 2006). It will be interesting to investigate whether EDDFs are also expressed in LFCs and have a role in LFC denucleation through their influence on hemoglobin expression.

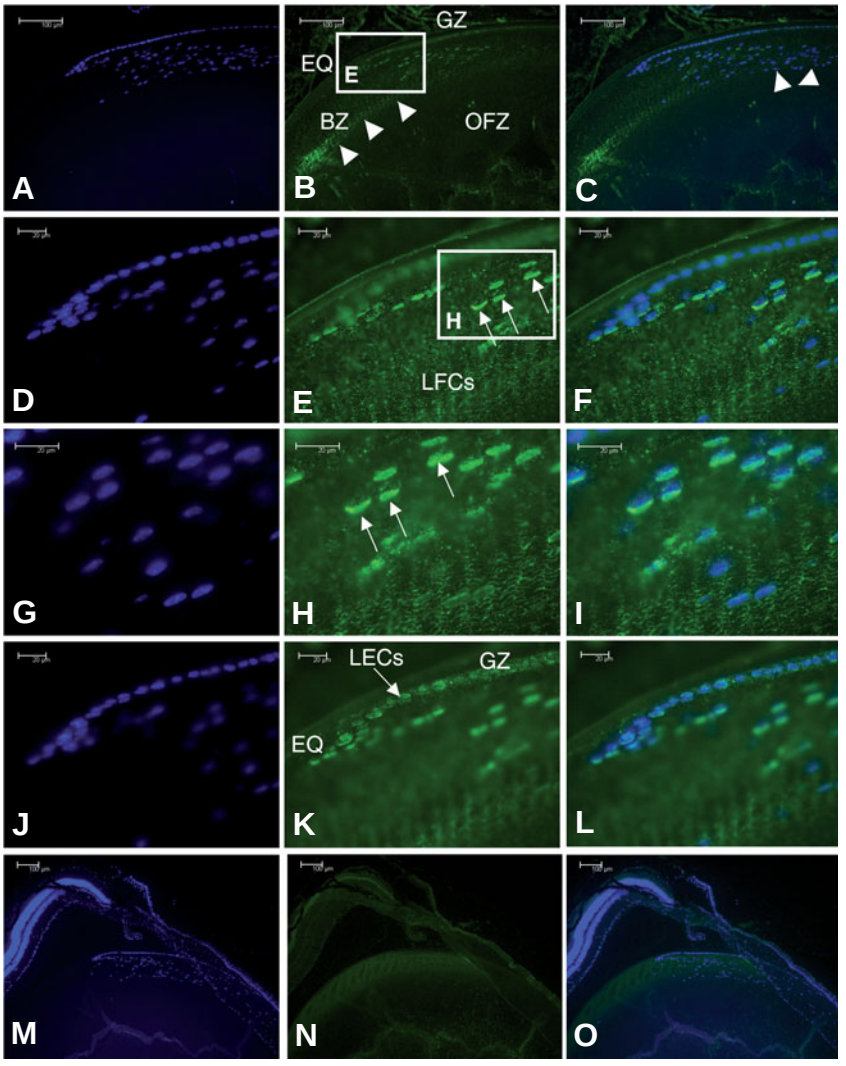


Fig. 8. Immunofluorescence for hemoglobin proteins, 9 wk mouse lens. (A,D,G,J,M) *DAPI labelling.* **(B,E,H,K,N)** *Hemoglobin immunofluorescence.* **(C,F,I,L,O)** *merged images. Regions defined by white rectangles in (B) and (E) are those in (E) and (H). Immunofluorescence is associated with LFCs at the boundary zone (BZ; arrowheads)* **(B,C)** *and with the surfaces of LFCs* **(E,F)**. *Immunoflourescence is associated with a nuclear-associated sub-compartment* **(E, F, H, I)** *in cortical LFCs. Immunofluorescence is also associated with the LECs* **(K, L)**. *LEC staining is associated with the cytoplasm of LECs in the GZ, but becomes progressively more associated with nuclei of LECs approaching the lens equator (EQ) at which point LECs are differentiating into LFCs.* **(M-O)** *Sections stained with rabbit IgG at the same concentration as the hemoglobin antibody are negative for staining. Mag bars 100 μM (A-C; M-O); 20 μM (D-L). A laser scanning confocal microscopy movie (available at http://dx.doi.org/10.1387/ijdb.082597fm) taken from the same region of the lens as presented here is provided in the Supplemantary Information accompanying this paper online.*

Hypoxia is known to trigger apoptosis and, intriguingly in the context of the current data, oxygen levels within the developing chick lens can influence LFC denucleation/organelle loss. As new fiber cells bury existing LFCs during development, they are subjected to increasing hypoxia; in hyperoxic lenses, the organelle loss was triggered at a greater depth than under normoxic conditions (McNulty *et al.*, 2004). We cannot totally exclude the possibility that heme is present in the lens at undetectable levels, which are still sufficient to sense oxygen when associated with hemoglobin subunits. In any case, we suggest that the differentiation of LFCs is an ideal system with which to complete functional studies, given the lack of hematopoietic contamination.

Expression of hemoglobin in villus epithelial cells

Gut sections were stained in order to check for normal hemoglobin expression in erythrocytes from a highly vascularized

tissue (as a positive control). Staining was noted, as expected, in anucleate erythrocytes, but also in rare, individual cells of the villus epithelium (Fig. 10). Stained epithelial cells were, as indicated by villus positioning, differentiating, migrating cells with nuclei (as indicated by DAPI staining). Stem cells are located in known positions in the crypts between villi, which give rise to daughter cells that mature, migrate, differentiate and are lost from the villus epithelium after approximately 7 days (Yen and Wright, 2006, Barker *et al.*, 2007). Recent work has revealed hemoglobin expression in ATII cells; stem cells that differentiate into alveolar type I cells and Clara cells in the lung (Bhaskaran *et al.*, 2005, Newton *et al.*, 2006), supporting a wider role for hemoglobins in stem cell differentiation in general, in a manner consistent with the results presented here in early embryos and stem cells.

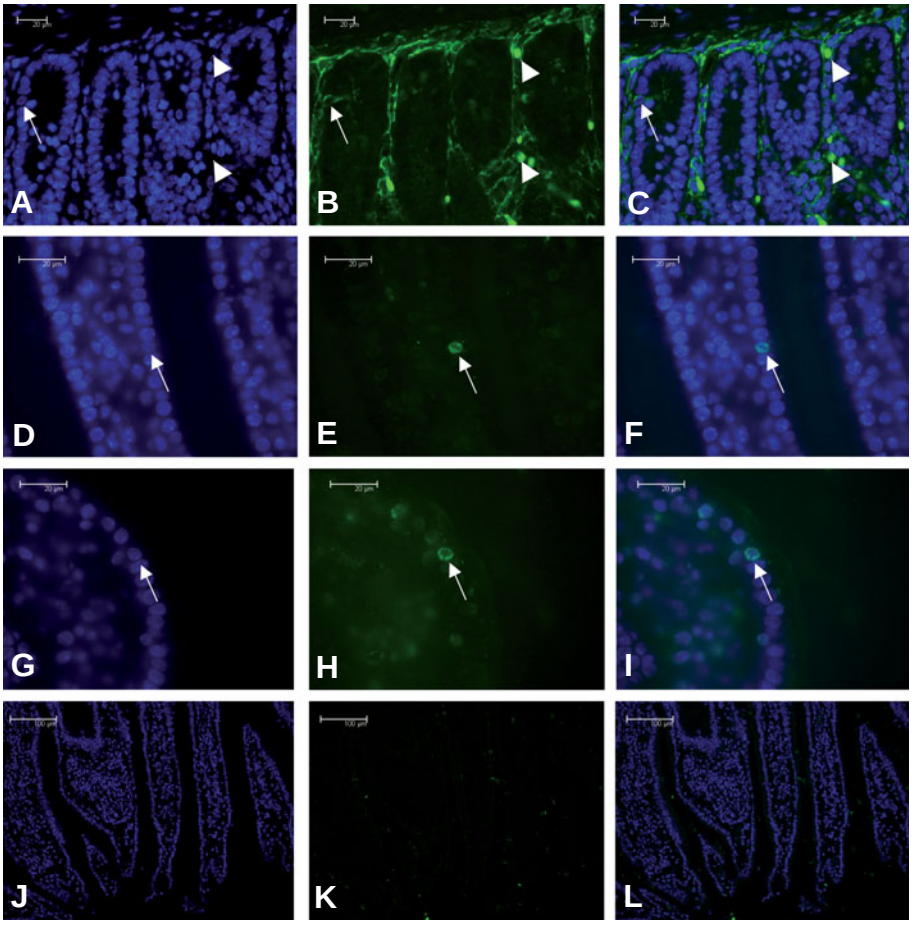


Fig. 10. Immunofluorescence for hemoglobin in adult mouse gut villus epithelium. (A,D,G,J) *DAPI labelling.* **(B,E,H,K)** *Immunofluorescence.* **(C,F,I,L)** *Merged images.* **(A-C), (D-F)** and **(G-I)** *are three different sections stained with anti-mouse hemoglobin. In* **(A-C)**, *hemoglobin immunofluorescence is associated with erythrocytes, identified by lack of DAPI nuclear staining in addition to the hemoglobin staining (arrowheads). Occasional individual nucleated cells of the gut villus epithelium are positive for hemoglobin immunoflourescence (arrows).* **(J-L)** *Sections stained with rabbit IgG at the same concentration as the hemoglobin antibody are negative for staining. Bars, 20 μM (A-I); 100 μM (J-L).*

RT-PCR and Western banding patterns and subunit stoichiometry

With the exception of Hbb-Z, the hemoglobin subunit expression patterns we observed in the lens using RT-PCR differed markedly from those in blood (where expression of the embryonic Hbb-Y and Hba-X is down-regulated postnatally and is largely replaced by expression of Hba-1/2 and Hbb-b1/2)(Lindenbaum and Grosveld, 1990). It is intriguing that "adult" hemoglobin subunits are expressed in the lens during embryonic development, while "embryonic" hemoglobin subunits continue to be expressed in the adult lens. The sizing of Western bands (Figs. 2 and 5) in the lens at different stages, blood and EBs were subtly different from each other (Figs. 2 and 5). It is possibile that monomers, dimers, trimers and even tetramers of hemoglobin exist in EBs and the lens. The results also confirmed fetal globin protein expression in adult lenses.

It is possible that different combinations of hemoglobin subunits have different functional consequences. Neither possible splice variants of hemoglobin genes or post-translational modifications of hemoglobins have been investigated to date. However, "mix and match" combinations of hemoglobin protein subunits, differential splicing and posttranslational modifications could all explain banding pattern variation. Moreoever, polyacrylamide gel electrophoresis (PAGE) reducing conditions may not completely disassociate subunits from each other because of the electrostatic mechanisms by which they associate (Royer *et al.*, 2001) and the different subunit interface strengths of embryonic, fetal, and adult hemoglobins (Manning *et al.*, 2007). Notably, the usual tetrameric hemoglobin assembly is unlikely, at least in the lens, given the apparent absence of heme, while loss of hemoglobin subunit stoichiometry is thought to have functional consequences (Liu *et al.*, 1999). Notably, Gata factors regulate hemoglobin

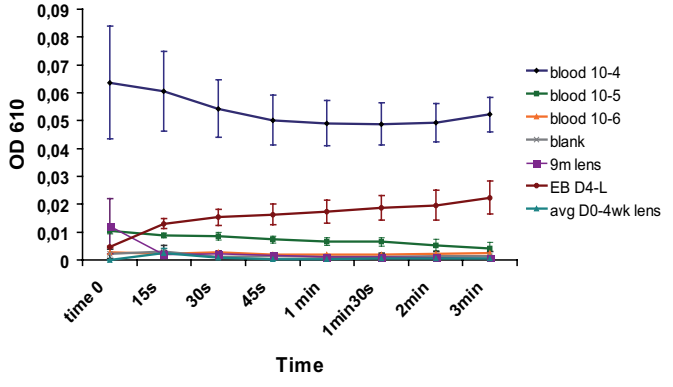


Fig. 9. Colorimetric difluoroacetate heme assay. *OD610 versus time of reaction of the assay. Lens lysates (average of day 0 to 4 wk lens lysate and 9 mo lens lysate) exhibit backround levels only (equivalent to the blank and blood lysate diluted at 10^{-6}). The heme assay with EB lysates reveals the presence of the heme moiety.*

expression in the erythroid lineage and we have identified GATA binding protein 1 in the lens in our previous array studies (http://watson-bios.grid.cf.ac.uk/array/), while Gata-3 is expressed in LFCs (Oosterwegel *et al.*, 1992). However, it is likely that the process of transcriptional initiation in the lens is substantially different from that in erythrocytes.

Eliminating hemoglobin contamination by erythrocytes

We have taken multiple steps to ensure lack of erythrocyte contamination in the experiments described here. Lenses were carefully dissected free of any contaminating iris material (methods and lens pictures described previously)(Wride *et al.*, 2003, Mansergh *et al.*, 2004). Furthermore, mature erythrocytes, which comprise the vast majority of cells in circulation outside the bone marrow, lack nuclei, and therefore also lack substantial quantities of nucleic acid. This makes it relatively easy to avoid contamination at the PCR level. We also found persistent expression of fetal hemoglobin subunits in adult lenses; these genes are not expressed in the adult hematopoietic system. Hemoglobin protein contamination is a more noteworthy problem, but we would point to the fact that we could not detect any heme in adult lenses as an indication that these samples were not contaminated in any way; blood controls had to be diluted by 10^{-6} before heme was undetectable (see Fig. 9). The persistence of hemoglobin subunit protein in the central adult lens nucleus (obtained by removal of cortical LFCs and LECs), which has lost all nucleic acid, is also noteworthy. The results are similar to those with whole lenses, confirming that the bands detected are highly unlikely to be a consequence of erythrocyte contamination, since removal of the outer lens compartments would also remove any contaminating red blood cells from the iris and other adherent tissue invisible under the dissecting microscope. Finally, with regard to immunofluorescence, erythrocytes are immediately visible if present due to intense staining. Furthermore, slides were counterstained with DAPI to label DNA and erythrocytes are visible, especially on the gut sections, as cells staining bright green, but lacking nuclei.

Concluding comments

Hemoglobin subunits are expressed in the process of early differentiation from the stem cell population in various tissues (lens epithelium GZ, gut villi and lung) and in early embryogenesis (the blastocyst ICM and a stem cell model thereof; EBs). The expression of hemoglobin protein subunits (in the absence of heme) has been associated with apoptosis when over-expressed in various cell lines (Brecht *et al.*, 2005a, Brecht *et al.*, 2005b), while globin expression in LFCs and in erythroid cells is spatiotemporally associated with loss of nuclear DNA and other organelles (although the mechanism of loss differs). Moreover, apoptosis is a process intimately associated with early development and differentiation; therefore, hemoglobin subunits may be carrying out similar functions in early embryos and in differentiating cells of the lens, cornea and lung. The common theme between the embryo and the more mature tissues may be the extensive “remodeling” that these cells undergo as they differentiate. We propose that hemoglobin subunits (possibly without heme) have roles in development in apoptosis, nuclear degeneration/organelle loss and/or stem cell differentiation, in addition to their long-accepted roles in oxygen transport.

Further studies are now required to investigate novel function(s) of hemoglobin subunits in the development and differentiation of avascular tissues. We suspect that lower levels of non-oxygen transport related hemoglobin subunit expression outside of the hematopoietic system have been overlooked, owing to the difficulties of separating most tissues from their blood supply; nevertheless, secondary functions of hemoglobin may be common to most self-renewing tissues, and hence very important. Our future work will be directed towards determining the nature of these functions. The early embryo, ES cell differentiation *in vitro* and the avascular lens and cornea represent particularly useful model systems.

Materials and Methods

Animal husbandry, tissue isolation

Mice (129SvEv) were maintained under Home Office, U.K. license on a 12 h light/12 h dark light cycle with food and water *ad libitum*. Blood samples were obtained via decapitation immediately after cervical dislocation. Approx 250 ml blood was dissolved in 750 ml 1 X RIPA buffer (Upstate), with 1 mini protease inhibitor tablet (Roche) per 10mls. Fetal blood samples were obtained from dissection of 16.5 embryos around the embryonic liver; a few microliters of blood were dissolved in RIPA buffer. Lenses were dissected from eyes by making a slit in the cornea, subsequent pressure on the sides of the eyeball resulted in extraction of the lens. Touching of the lens to clean tissue resulted in the removal of most excess adherent tissue, the remainder was dissected away using forceps under a dissecting microscope, in 1X PBS. Lens nuclei (cores) were obtained via the removal of the lens capsule and epithelium with forceps. Whole lenses and lens nuclei from 6 mice each were pooled and homogenized in mini-homogenizers (Fisher) in 1ml 1 X RIPA buffer + protease inhibitor. Samples were chilled for 30 minutes, then spun at 13000 rpm for 5 minutes at 4ºC (Heraeus Picofuge). Supernatant was frozen at -20ºC in 250 ml aliquots.

Protein concentration assay

Protein standards were prepared using serial BSA dilutions in 20 ml water, containing 2 ml of lysis buffer + protease inhibitors. Unknown samples were made up with 2 ml cell extract plus 18 ml water. The BioRad DC assay kit was used and OD750 readings taken (CamSpec) according to the manufacturers protocol.

ES cell culture and differentiation

Undifferentiated ES cells were maintained as previously described (Hunter *et al.*, 2008) in MEM-Alpha (GibcoTM, Invitrogen Ltd, Paisley, Renfrewshire, UK) supplemented with mercaptoethanol (Merck KGaA, 64293 Darmstadt, Germany), 2mM glutamineb10-4 M and 10-3 U/ml murine LIF (ESGRO™, Chemicon), 10% FBS and 10% NBS (selected batches, PAA Laboratories GmbH, Linz, A-4020 Austria). The cells were maintained at 37ºC in a humidified atmosphere with 5% CO2 on 0.1% gelatin (Stem Cell Technologies) coated tissue culture grade plastic (NUNCTM, Fisher Scientific, Loughborough, Leics, UK). EBs were obtained from cell suspensions of trypsinized (0.25% trypsin/EDTA, Invitrogen) undifferentiated ES cells. Hanging drops were assembled using standard protocols and 1000 cells per 10 ml hanging drop, omitting the LIF from the medium. After 1 day of differentiation, EBs were flooded with 20mls of media –LIF. Growth was continued for a total of 4 days, RNA samples were extracted at each stage. Random EB cultures were generated as above, but allowing EBs to aggregate from 100 ES cell/ml cell suspension, without hanging drops.

Arrays

Full methods for microarray analysis are available through GEO (GSE8881) and are also described elsewhere (Hunter *et al.*, 2008). In brief, individual undifferentiated ES colonies were picked and embryonic

sub-compartments were microdisected. 10-20 of each type were pooled, RNA was extracted using the Zymo Mini RNA isolation kit, amplified twice using the Arcturus RiboAmp kit (according to the manufacturer's protocols) and labeled using the Atlas™ PowerScript™ Fluorescent Labeling kit.

RNA extraction, RT-PCR procedures, EB amplification

RNA extraction, RT-PCR procedures and primers are as described previously (Wride *et al.*, 2003, Mansergh *et al.*, 2004, Hunter *et al.*, 2008). Individual EBs were picked from two separate day 4- LIF cultures. 10 EBs were derived from hanging drop cultures (standard size), whereas 6 were derived from random aggregation (2 small, two medium and two large EBs). RNA extraction and one round of amplification were carried out (as per the array protocol), followed by DNase treatment (Turbo DNAfree kit, Ambion) and RT-PCR as above, but using random hexamers instead of oligo-dT.

Tissue fixation and processing

Whole E16.5 mouse embryos or enucleated post-natal or adult mouse eyes were fixed in 4% fresh paraformaldehyde in PBS overnight to 24 h, washed in PBS and dehydrated through a graded series of ethanol before repeatedly soaking in 50:50 ethanol:xylene. After an overnight incubation, eyes were moved to xylene and washed twice, for 1 h, then placed in hot wax for 2x2 h (65ºC). Eyes or embryos were embedded in paraffin wax and sectioned using a microtome (HM 325, Microm) at 7μm. Sections were mounted on Histobond slides (Fisher Scientific).

Immunohistochemistry and immunofluorescence

Tissue sections were de-paraffinized in xylene and re-hydrated through a graded series of alcohols to distilled H_2O prior to treatment with antigen unmasking solution (Vector Laboratories) according to the manufacturer's protocol. Slides were washed 3 times in PBS-T for 5 minutes each time, then quenched in a solution composed of 2.5mls hydrogen peroxide, 2.5 mls methanol, and 20mls ddH2O. Further steps were carried out using the VectaStain Elite ABC kit (Vector Laboratories), according to the manufacturer's protocol, except that primary antibody incubations were carried out for 2 hours. Slides were incubated with one of 3 anti-hemoglobin primary antibodies as follows: Hemoglobin-b (H-76): sc21006 and Hemoglobin-a (H-80): sc21005 (both of which are anti-human and were used at a 1/50 dilution) as well as a rabbit anti-mouse hemoglobin antibody (ICN Biomedicals, cat no: 55447, diluted 1/400-1/800). Control rabbit IgG antibody (Sigma) was also used for each stage, concentrations were varied to match each of the antibodies tested. Slides were stained with the Vector Laboratories' VIP substrate kit for peroxidase. For immunofluorescence, the antigen retrieval and primary antibody incubation steps were carried out as above with antibodies at the same dilution, but an AlexaFluor-488 labeled secondary antibody (Invitrogen) was used instead. Slides were mounted in Vectashield Hardmount with DAPI (Vector Laboratories, UK) and photographed on a digital microscope (Leica DM4000/5000 B; Leica Microsystems Ltd, UK). We also carried out laser scanning confocal microscopy (TCS SP2 AOBS spectral confocal microscope system; Leica Microsystems Ltd, UK) and generated a movie through a section of the 9 wk lens cortex using Leica Confocal Software in order to highlight the nuclear-associated staining.

PAGE and Western blotting

Sodium dodecyl sulfate (SDS)-PAGE was carried out using the Mini-Protean® 3 cell system (Bio-Rad, UK) with 15% gels. 10 μg of protein/loading buffer mix was added to each well along with GE Healthcare Rainbow molecular weight markers. Proteins were transferred to a nitrocellulose membrane (Hybond™-P, Amersham Biosciences, UK). 5% milk/TBST was used for blocking; for some washes and antibody incubations. TBST washes alone were also used. A range of dilutions were used for optimization, the ICN/Cappel Hb antibody was used at 1/200-1/400 dilutions, the AbCam fetal antibody was used at 1/300, while the human antibodies failed to recognize mouse hemoglobin subunits (including that from blood samples) at any concentrations tested (1/200-1/50 gave no result, while background was very high at 1/50). Secondary antibody solution (1% milk and antibody at 1:2000) was added to the membrane for one hour at room temperature. Enzyme chemiluminescense (ECL) was used for detection (ECL Plus Western Blotting Detection Reagents, Amersham Biosciences, UK). Membranes were stripped and reprobed as necessary, a maximum of two times.

Heme assay

Heme detection was carried out using the diaminofluorene assay (Kaiho and Mizuno, 1985). Protocols for tissue, slide and lysate assay were derived directly from this paper. For histological staining of tissues sections and whole lenses for heme, 100mg diaminofluorene was dissolved in 10ml of 90% acetic acid (10mg/ml) and tissues to be stained were prepared. 1ml of diaminofluorene/acetic acid solution was mixed with 100 ml of 30% hydrogen peroxide and 10ml of 200mM Tris, pH7.0, containing 6M urea. This solution was added to whole lenses and corneas or deparaffinized sections for staining. Samples were incubated for 5 minutes at room temperature and photographed under a dissecting microscope as described previously (Mansergh *et al.*, 2004). Tissue sections on slides were prepared and re-hydrated as for immunohistochemistry (described above), exposed to the above stain, and then dehydrated prior to mounting. For examination of heme in cell lysates, spectrophotometry was used. Blood and lens homogenates, as prepared for Western blotting (described above), were resuspended in 1ml of deionized H_2O. 10mg/ml diaminofluorene solution was diluted to 5mg/ml using more acetic acid. 100ml of the above solution was added to 10mls 100mM Tris, pH7.0, containing 6M urea. 1ml of this solution was mixed with 0.5ml cell extract in a disposable cuvette. The reaction was initiated via addition of 5ul 30% hydrogen peroxide. Changes in absorbance (OD610) were monitored at room temperature for 3 minutes (see Fig. 9 for intervals). The spectrophotometer (CamSpec) was blanked using deionized water plus all other components described above in the same proportions, but without any homogenate.

Acknowledgments

This work was funded by grants to MJE and MW from the Biotechnology and Biosciences Research Council (BBSRC), The Royal Society, the National Eye Research Centre (NERC), and the Medical Research Council (MRC) as well as the Higher Education Funding Council for Wales (HEFCW). FCM is currently supported by RP Ireland, Fighting Blindness. We would like to thank Dr Alicia Battersby for the gift of anti-sox2 antibody, Dr Karen Reed and Prof. Alan Clarke, Cardiff School of Biosciences for providing mouse small intestine slides and all three for constructive discussion of results. We would also like to thank Heather Phillips for technical assistance with histology and Llinos Williams for help with immunofluorescence and immunohistochemistry.

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