**Mouse Bone Marrow and Peripheral Blood Erythroid Cell Counts are
Regulated by Different Autosomal Genetic Loci**

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Summary

Erythropoiesis is under fine control and genetic loci that effect it are likely to be important in a range of conditions. To assess the relative contributions of different genetic loci to parameters of erythropoiesis we have measured RBC counts in the peripheral circulation and committed erythroid cells (RBC and small normoblasts) in the bone marrow in a cohort of (CBA/H x C57BL/6) F2 mice to map quantitative trait loci (QTL). Candidate genes were assessed using bioinformatics and DNA sequencing. Different autosomal loci affect bone marrow (chromosomes 5, 11, and 19) and peripheral blood (chromosome 4) erythroid cell counts but there may be a common chromosome X locus. Spleen weight QTL were found on chromosomes 3, 15 and 17. Surprisingly, erythropoietin (*EPO*) is the best candidate quantitative trait gene (QTG) in the chromosome 5 locus that affects bone marrow but not peripheral blood erythroid cell counts. Epo gene expression is known to be genetically regulated in mice, but our data suggests a tissue-specific role for epo in mouse erythropoiesis that is also genetically determined. The identity of the other QTG will be important both to further knowledge of the control of erythropoiesis and as potential modifier genes for haematological disorders.

Keywords: Erythropoiesis erythropoietin QTL genetics marrow

Introduction

Erythropoiesis is necessarily under fine control, as the oxygen carrying capacity of erythrocytes in the peripheral blood has to be balanced by the increased viscosity of the blood with increasing cellularity. Thus, low peripheral red blood cell (pRBC) counts cause anaemia, whereas a high haematocrit increases the risk of cerebral infarction, cardiovascular disease and hypertension [1,2]. Maturation of erythrocytes is crucially dependent upon production of erythropoietin (epo) in response to tissue hypoxia. Epo protein binding to the erythropoietin receptor (epo-r) on erythroid progenitors activates the Jak2 tyrosine kinase and further signal transduction pathways, and mutations in the *EPO-R* and *JAK2* genes cause familial erythrocytosis and polycythemia vera respectively [3,4]. Erythrocyte stability is also a key determinant of pRBC counts as evidenced by the pathological consequences of haemolytic disorders such as sickle cell anaemia, the thalassemias, autoimmune haemolytic anaemia (AHA) and systemic lupus erythromatosus (SLE) [5,6].

Significant differences in normal and malignant haemopoiesis have been reported between inbred mouse strains, and these mouse strain-specific differences have enabled genetic studies to identify quantitative trait loci (QTL) that regulate these differences. For example, QTL that are linked to anti-red blood cell and antinuclear antibody production and splenomegaly have been mapped in mouse models of AHA and SLE [7-9]. Similarly, QTL have been identified that determine mouse stem, progenitor and mature white blood cell numbers [10-18]. However, despite the fact that many mouse haemopoietic

phenotypes have been extensively analysed for QTL, genetic analyses of mouse strain-specific differences in normal erythropoiesis and/or pRBC counts have yet to be reported.

There have not, up until now, been QTL studies reported using RBC counts as the phenotype, but Lin *et al* carried out a study using families from the Framingham Heart Study with haematocrit and haemoglobin levels as the phenotypes [1]. The study found evidence for a haematocrit QTL on chromosome 6q23-24 (mouse Chr10, 18-25Mb) and one effecting haematocrit and total haemoglobin on 9q34 (mouse Chr1, 65-70Mb). A rat study used haematocrit as the phenotype in a cross between spontaneously hypertensive and normotensive rats [19]. The results showed a QTL in the vicinity of the *ENO1* gene on rat chromosome 4 (mouse Chr6, 125Mb).

In adult humans, erythropoiesis is confined to the bone marrow, but in many other mammals including mice, it also occurs in the spleen. In the mouse, the peripheral blood erythrocyte count therefore represents the sum total of the contributions made by the two erythropoietic tissues. In ongoing studies of genetically determined differences in normal and malignant haemopoiesis in adult inbred CBA/H and C57BL/6 mice [20], we found that the peripheral blood RBC count in CBA/H mice was lower than in C57BL/6 mice ($p = 0.0009$) whereas, surprisingly, red blood cell counts in the bone marrow were higher in CBA/H mice (37% of total) than C57BL/6 mice (28%). Mouse strain-specific differences in peripheral blood erythrocyte counts could either reflect general differences in erythropoiesis that equally affect the two tissues, and/or reflect tissue-specific differences in the relative contributions that spleen and bone marrow make to peripheral

blood cellularity. To determine the genetic relationship between these two apparently contradictory phenotypes, F2 mice were scored for peripheral blood and bone marrow erythroid (RBC and normoblasts) cell counts, together with spleen weight, and QTL analyses carried out.

Materials and Methods:

Mice

All work in this report was carried out with ethical approval granted locally and nationally. CBA/H mice were from the Harwell colony, UK, and C57BL/6 mice from Harlan, UK. F2 intercross mice were produced using both (CBA/H x C57BL/6) and (C57BL/6 x CBA/H) breeding schemes, in Leicester, UK. Irradiated mice were exposed to 3 Gy X-rays (0.5 Gy/Min) and sacrificed 48 hours later.

Cell analysis:

2-5 month old mice were killed by cervical dislocation and blood taken by cardiac puncture; bone marrow, spleen and tail were collected. Blood and bone marrow was analysed using a Scharfe System Casey 1 Coulter counter (Reutlingen, Germany). The Casey analysis of bone marrow gave two distinct peaks based on cell size and shape. The 3-5 μM peak contained the mature red blood cells and small normoblasts. A second peak ($>6 \mu\text{M}$) contained larger white bone marrow cells.

Bone marrow cells were also analysed by Haematoxylin and Eosin (HE) staining of cytopins, which permits the quantitation of mature red cells, small densely staining normoblasts and white bone marrow cells. Biotinylated anti Ter119 antibody (Becton Dickinson-Pharmingen, Oxford, U.K.) and Streptavidin Ultra-load Magnetic Beads (Metachem Diagnostics, Northampton, UK) were used to immunodeplete Ter119 positive bone marrow cells.

Genetic Analysis

Microsatellite primer sequences were from the Whitehead Institute [www-genome.wi.mit.edu] and genetic positions from MGI or calculated from our own data. 133 informative polymorphic microsatellite markers across the 19 autosomes and the X chromosome were identified to give a genome wide screen at 15-20 cM intervals [20]. DNA was prepared from tails of the F2 mice.

QTL Mapping and Statistical Analysis

Trait data was normalised as appropriate (log transformation) and regressed onto age and sex, using residuals in the analysis. Windows QTL Cartographer Version 2.5 (WinCart) was used to implement composite interval mapping [21]. Significance levels were calculated by performing 10,000 permutations of the data and defined according to the guidelines of Lander & Kruglyak [22]. Boundaries of the QTL were estimated using one Lod support intervals. Direction of effect (i.e. which allele is the increaser allele) was inferred from the additive effect (a), which together with the dominance effect (d), were taken from the WinCart output. If the parameters are positive then BL6 is increaser or dominant allele, if negative then CBA.

Physical distances were taken from Ensembl based on Build 35 of the mouse genome.

Candidate gene analysis

Biological candidates were drawn from the published literature on erythropoiesis. Genes in the QTL intervals were checked for mouse haematological traits by performing a phenotype search at www.informatics.jax.org. Evidence of cis transcriptional regulation was determined using WebQTL analysis data from the BXD recombinant inbred mice with the haematopoietic stem cell data (if the gene was on the Affymetrix chip used). The

interval mapping function was carried out and presence of a suggestive QTL peak at the same genomic location of the gene was taken as evidence that genetic variation existed between BL6 and DBA (which is closely related to CBA) that effects the transcription of the gene. For example for the chromosome 5 peak stem cell data was used for: *EPO*; *SLC7A1*; *RAC1 AND MAFK*, but for: *RABGEF1*, *FZD9*, *HIP1*, *APS* and *CARD11* data was used from total brain. The remaining gene, *EIF2AK1*, is on neither chip. Probe set 99374 of the *EPO* cDNA shows a suggestive QTL on chromosome 5 ~10Mb distal of the *EPO* gene.

EPO and EPO-R sequence analysis

All exons and introns of the *EPO* and the *EPO-R* genes were PCR amplified from genomic DNA. Products were cloned using the Topo Cloning Kit (Amersham Life Technologies, UK) and sequenced. A 713bp fragment of the *EPO* promoter (-613 nucleotide to +100 nucleotide from ATG start site) was sequenced using primers 5'- TCG GTT TCC TGA CCA ATA GC -3' and 5'- GCC TCA AGA CAA GGA TGC TC -3'. A 736bp fragment of the *EPO-R* promoter was sequenced (-734 nucleotides to +2 nucleotides from ATG start site), using primers 5'- GAG CAC AAG GCA GGA GAG AG-3' and 5'- ATG CAG CCC TAG CTT CAG G-3'. Extragenic control regions were ascertained by interspecies homology using <http://ecrbrowser.dcode.org>.

Southern blot analysis used restriction digests of genomic DNA from the parental inbred strains probed with one of the two cDNAs and a GAPDH control probe for comparison.

Results

Discrimination of cell types

Mouse peripheral red blood cells (pRBC) from C57BL/6 and CBA have a mean corpuscular volume of 44-50 fL [24] which is smaller than the normal range for human pRBC of 78-98 fL. Using a Casey Coulter counter we measured the diameter of a pRBC as $<6\mu\text{m}$ (Figure 1A), suggesting that use of this method should be satisfactory for discriminating RBC from most WBC, given the greater size difference than in humans. A similar analysis of total mouse bone marrow (Figure 1B) reveals two discrete and well separated peaks, the smaller of which has the same apparent diameter as pRBC, evidence that the $<6\mu\text{m}$ diameter cells are predominantly small erythroid bone marrow cells, whereas as the larger and broader $>6\mu\text{m}$ peak contains most of the nucleated white bone marrow cells (WBMC). Cytospin analysis of HE stained bone marrow single cell suspensions confirmed that as a proportion of all bone marrow cells, CBA/H bone marrow had a higher erythrocyte count (37%; $n = 2$) than C57BL/6 (28%; $n = 2$) bone marrow. The number of nucleated bone marrow cells (Table 1) and small densely staining normoblasts was comparable in both mouse strains.

We further verified that we were correctly discriminating between the small erythroid and larger non-erythroid bone marrow cell types by two approaches. Firstly immunomagnetic removal of cells that are positive for the erythroid specific Ter119 cell surface marker using biotinylated anti-Ter119 antibody (Figure 1C) confirmed that 98-99% of the cells in the $<6\mu\text{m}$ peak were Ter119⁺, most of which will be terminally differentiated erythroid (erythrocytes and normoblasts) cells. Whilst there may be a small number of

nucleated $<6 \mu\text{M}$ diameter Ter119⁺ cells remaining, we estimate that this represents $<1\%$ of the total (Figure 1C). Secondly we compared the results with those 48 hours after the in vivo exposure of mice to 3 Gy X-rays, which is known to have no effect on pRBC counts, but kills 80-90% of nucleated WBMC and results in the concomitant increase $<6 \mu\text{M}$ peak (Figure 1D), further evidence the two distinct bone marrow peaks separated according to size (Figure 1B) predominantly represent erythroid and non-erythroid bone marrow cells.

Statistical description

Bone marrow and peripheral blood cellularity Casey analyses of inbred adult male CBA/H and C57BL/6 bone marrow revealed no significant difference in the recovery of non-erythroid cells ($p = 0.47$), whereas there was a statistically significant difference in the erythroid cell recovery ($p = 0.0002$), pRBC ($p = 0.0009$) and spleen weight (spleen; $p = 0.018$) (Table 1). To control for the day-to-day experimental and technical variation in the efficiency of bone marrow harvesting, bone marrow cellularity is henceforth expressed as the Erythroid:Non-erythroid (E/NE) ratio as this is independent of bone marrow harvesting efficiency, and the difference between the parental mouse strains is statistically significant ($p = 0.0001$). Importantly, the same approach using the cyto-spin cell counts revealed comparable E/NE ratios as the Casey analyses of the same bone marrow samples. As the normoblast counts were genotype-independent, this suggests that the genotype-dependent differences in erythroid bone marrow cell counts as assessed using the Casey cell counter was largely attributable to differences in mature red cell counts.

To map the QTL that determine the phenotypes, spleen weight, pRBC and E/NE bone marrow cell counts were analysed in adult (13.6 ± 3.3 weeks old) male and female (C57BL/6 x CBA/H)F2 mice. The average E/NE and pRBC values for the F1 and F2 mice are intermediate between those of the parental inbred mouse strains (Table 1). However, a statistical analysis of the data showed that the E/NE ratio was strongly influenced by sex (T-test $p = 4 \times 10^{-8}$) whereas the pRBC counts (T-test $p = 0.064$) and spleen weights (T-test $p=0.29$) were not. There was no significant correlation between age and the E/NE ratio or spleen weight but pRBC declined with age ($r = -0.41$, $p < 0.01$). There were no correlations between bone marrow E/NE ratio, peripheral blood RBC count phenotypes and spleen weight, suggesting that they are independent of each other.

	Non-erythroid ($\times 10^6/2$ femurs)	Erythroid ($\times 10^6/2$ femurs)	E/NE Ratio	pRBC ($\times 10^6/\mu\text{l}$)	Spleen weight ($\times \text{mg}$)
CBA/H (n = 37)	30.85 ± 0.36	24.5 ± 0.86	0.79 ± 0.03	6.63 ± 0.22	85.3 ± 2.2
C57BL/6 (n = 19)	32.29 ± 0.33	19.4 ± 0.65	0.61 ± 0.02	7.89 ± 0.23	94.2 ± 2.8
F1 (n = 22)	N/A	N/A	0.77 ± 0.03	6.72 ± 0.63	N/A
F2	N/A	N/A	0.74 ± 0.22 (n = 348)	7.31 ± 0.12 (n = 144)	100.3 ± 1.9 (n=113)
Female F2	N/A	N/A	0.82 ± 0.24 (n = 156)	7.53 ± 0.11 (n = 65)	102.4 ± 3.2 (n=56)
Male F2	N/A	N/A	0.69 ± 0.20 (n = 192)	7.13 ± 0.12 (n = 79)	98.2 ± 2.1 (n=57)

Table 1: Phenotype statistics for parental strains and F2 mice (mean $\pm$ s.d.). Sex was not recorded for 13 F2 mice.

QTL Analysis of the RBMC:WBMC ratio

F2 mice were genotyped at 133 microsatellite markers spaced across the genome

(approximately 10cM coverage). QTL analyses using the absolute NE or E counts as the

phenotype revealed no QTL and presumably reflects the day-to-day efficiency of bone marrow cell harvesting. In contrast, composite interval mapping of all F2 mice (M & F) revealed two QTL for the E/NE ratio trait with a Lod score higher than the threshold for significant linkage of 3.5 determined by permutation (Table 2). Using one LOD support intervals to define the QTL, a Lod score of 4.5 occurs on distal chromosome 5 (>75 cM), and a Lod of 6.3 on distal chromosome X (>65 cM) (Figure 2 and Table 2). The similarity of the Lod scores for the significant CBA/H and C57BL/6 increaser alleles (Table 2) is consistent with the intermediate phenotypes of the F2 mice compared to the parental inbred strains (Table 1). Three suggestive (threshold Lod > 2.7) QTL on chromosome 10 (14-43 cM), chromosome 15 (>47cM) and chromosome X (0-27cM) were also detected (data not shown).

The two chromosome X QTL identified in the analyses of all F2 mice (male and female; M & F) are consistent with the observation that the RBMC:WBMC ratio is strongly influenced by sex (Table 1), so this was investigated further by restricting the QTL analyses to either male (M) or female (F) F2 mice. In both cases, this uncovered QTL that were not observed in the original (M & F) analysis. The male mice showed a second locus on (proximal) chromosome 5 (Lod 3.5; Figure 3 and Table 2) and a suggestive QTL on chromosome 9 (Lod 2.7; 0-24 cM)(data not shown), whereas the female F2 mouse analysis uncovered one single significant QTL on chromosome 11 (Lod 4.4; Figure 4 and Table 2).

Table 2: QTL that determine the E/NE bone marrow cell ratio in F2 mice. Only those QTL that reached the threshold for significant loci are shown. Chromosome intervals were estimated using one Lod support intervals. The increaser allele is the strain allele that increases the phenotype i.e. it shows direction of effect.

	Chr.	Peak Lod	QTL peak in cM (1 Lod interval)	Physical interval: Mbp	Human synteny	Increaser allele	Dominant allele
M & F (n = 361)	5 X	4.5 6.3	78 (75-Tel) 69 (65-Tel)	136 (124 -150) 160 (147 –164)	7q22.1 Xp22.2	BL/6 CBA	No BL/6
M (n = 192)	5	3.5	57 (44-61)	100 (80-111)	4q21.2	BL/6	BL/6
F (n = 156)	11	4.4	30.0 (25.7-31.4)	56.5 (49-59)	5q33	CBA	CBA
M & F - Sex	5 19	4.5 4.3	78 (75-Tel) 42 (31-Tel)	136 (124-150) 43 (32-61)	7q22.1 10q24	BL/6 BL/6	No BL/6

To further investigate the sex effect we used sex as a covariate in the analysis (M & F, - sex) and compared to the original (M & F) analysis. As expected the chromosome X QTL were abolished, as were the suggestive QTL on chromosomes 10 and 15. However, the distal chromosome 5 QTL was present in both analyses, and a novel QTL on chromosome 19 uncovered (Table 2). Significantly, the telomeric chromosome 5 QTL interval contains the erythropoietin gene (*EPO*) at the centre of the peak (136.4Mb).

The significant QTL have been registered with the Mouse Genomic Nomenclature Committee as locus symbols: bmRBC1 (Chr 5 QTL); bmRBC2 (Chr 11), bmRBC3 (Chr 19) and bmRBC4 (Chr X).

QTL analysis of peripheral RBC count:

QTL analysis using pRBC counts as the phenotype revealed one significant QTL on chromosome 4 (Lod 4.0; 32.2-48.2 cM) and a suggestive QTL on distal chromosome X (Lod 3.3; >57 cM) (Figure 5 and Table 3). The CBA/H and C57BL/6 increaser alleles (Table 3) are again consistent with intermediate pRBC data in the F2 compared to parental inbred mice (Table 1). A comparison of the E/NE bone marrow cell ratio (Table 2) and pRBC (Table 3) QTL indicates that the distal chromosome X QTL for the two

traits overlap and therefore may represent a common sex-specific genetic effect.

However, all the other autosomal QTL are different (Table 2 and Table 3), strong evidence that the genetics underlying quantitative differences in the two phenotypes are largely independent.

Although the chromosome X (distal) QTL for the two phenotypes overlap, the increaser alleles are different for the two phenotypes (Table 2 and Table 3) and despite the suggestive chromosome X pRBC QTL, only a very weak and not statistically significant ($p = 0.064$) sex influence observed for the pRBC phenotype in the F2 mice (Table 1). Nevertheless, removing the influence of sex in the pRBC trait analysis by regression did abolish the distal X chromosome QTL without affecting the chromosome 4 QTL or uncovering any other QTL (data not shown), raising the possibility of sex- and phenotype-dependent epistatic effects of a single locus on distal chromosome X. The chromosome 4 QTL has been registered with the MGNC as pRBC1.

pRBC	No. of mice	Chr	Peak Lod	QTLpeak in cM (1 Lod interval)	Physical interval:Mb	Human synteny	Increaser Allele	Dominant Allele
CBA/H x C57BL/6	124 F2	4	4.0	41.9 (32.2-48.2)	84 (63-98)	9p22	CBA	BL/6
		X	3.3	69 (57-Tel)	160 (131-164)	Xp22	BL/6	BL/6
A/J x C57BL/6	29 RI lines (~8 mice/line)	4	7.5	40.0 (38.0-44.6)	82.3 (80.4-90.1)	9p22	BL/6	N/A
		1	4.9	58.5 (56-59.5)	92.8 (88.5-100.2)	2q37	A/J	N/A
		16	6.0	59.9 (57-66)	87.5 (84.9-92.3)	21q21	BL/6	N/A

Table 3 : Peripheral red blood cell QTL

To confirm this finding and refine the linkage interval we carried out a QTL analysis using genotype data on the AXB/BXA recombinant inbred strains from www.nervenet.org [23] and peripheral red blood cell counts (pRBC) from the Mouse

Phenome database [24,25]. The analysis finds two highly significant pRBC QTL on chromosome 16 (Lod 6.0, 57.0-66.0 cM) and chromosome 4 (Lod 7.5, 38.0 - 44.6 cM), and one significant QTL on chromosome 1 (Lod 4.9, 56-59.5cM). See Figure 6.

This data replicates the chromosome 4 QTL in a different cross and refines the chromosomal interval. The finding of additional QTL (chromosome 1 and 16 QTL) when using a different cross (A/J x C57BL/6 as apposed to CBA/H x C57BL/6) is a commonly observed phenomenon, dependent upon which strain-combinations segregate each QTL.

QTL analysis of spleen weight

Composite interval mapping using spleen weight as the phenotype found three suggestive QTL on chromosomes 3 (Lod = 3.0, interval 27-44cM), 15 (Lod = 3.1, interval 20-38cM) and 17 (Lod = 3.5, interval 15-34cM) (Figure 7). These do not overlap with any of the pRBC or E/NE bone marrow cell ratio QTL, although the chromosome 17 locus overlaps with a known spleen weight QTL Splq9 [26].

Analysis of candidate genes

We have used information from three sources to identify the most likely quantitative trait gene (QTG) from the inevitably large genomic intervals that are the result of QTL analysis. First we established a minimal list of 37 genes known to be directly involved in erythropoiesis. Secondly we identified genes in the QTL, alleles of which are known to have haematopoietic phenotypes e.g. gene knockouts (Mouse Genome Informatics). Thirdly we used the mRNA microarray studies using haematopoietic stem cells from

recombinant inbred mice [27], available at www.genenetwork.org, to identify locus genes that show evidence of genotype -dependent cis transcriptional regulation.

The telomeric chromosome 5 interval contains 325 known genes, with the erythropoietin gene (*EPO*) at the centre of the peak (136.4Mb). Ten genes in the region have alleles that cause haematopoietic phenotypes, none of which show cis regulation. There is however a suggestive QTL for *EPO* regulation within the locus, the most likely position for which is given by bootstrap analysis as being 145.5-146.5Mb. Biological candidates or those fulfilling the other two criteria are listed in Table 4.

Trait	Chr.	Lod	Interval (Mbp)	Candidates	Biol	Haem pheno	Cis reg
E/NE	5	4.5	124-150	<i>EPO</i>	Y	Y	N
E/NE	X	2.7	0-64	<i>GATA1</i>	Y	Y	N
E/NE (M & F, - sex)	19	4.3	32-61	<i>PTEN</i>	Y	N	N
E/NE (M)	9	2.7	0-42	<i>EPO-R</i> <i>FLII</i>	Y Y	Y Y	N Y
pRBC	1	4.9	88.5-100.2	<i>TWIST2</i>	N	Y	Y
pRBC	16	6.0	84.9-92.3	<i>IFNAR1</i>	N	Y	Y

Table 4: Candidate genes. Showing those that are biological candidates or suggested by having associated mouse haematological phenotypes and cis genetic regulation of transcription

Given that the phenotypes analysed involve erythroid cell counts in the peripheral blood and bone marrow, erythropoietin (*EPO*) and its receptor (*EPO-R*) are the most obvious candidate genes. The *EPO* gene maps to the centre of the peak (136.4Mb) of the significant (Lod 4.5) distal chromosome 5 QTL, whereas the *EPO-R* gene maps to the

comparatively weak (Lod 2.7) male-specific suggestive chromosome 9 QTL (Table 4). However, sequence analysis of CBA/H and C57BL/6 *EPO* and *EPO-R* cDNAs revealed no coding sequence polymorphisms. Similarly, no polymorphisms were detected in the introns (*EPO*), promoter sequences (*EPO* and *EPO-R*) or in three extragenic control regions (*EPO*) PCR amplified from genomic DNA. Southern blot analysis detected no signs of copy number variation between the two strains in either gene.

Discussion

Whilst variation in inbred mouse pRBC counts have previously been reported (Mouse Phenome database), we now also report that mouse bone marrow erythroid cell counts, as assessed using a surrogate E/NE ratio end-point, also vary in inbred mice. In the mice analysed, the pRBC and E/NE phenotypes are not obviously related, as there is no phenotypic correlation between the traits. Similarly, the E/NE ratio but not pRBC count was strongly influenced by sex. Mapping QTL for the two phenotypes in F2 mice showed that all the autosomal QTL were only detected by one of the phenotypes, further direct evidence that the two phenotypes are separate biological traits.

Our results are consistent with peripheral RBC counts being the net result of erythropoiesis, in both spleen and marrow, as well as erythrocyte clearance, whereas bone marrow RBC represent local erythropoiesis together with the contribution from circulating blood in the bone marrow.

We included spleen weight as a potential surrogate for spleen function, but there is no overlap in the spleen weight, pRBC or E/NE ratio QTL. These data show that this trait is under separate genetic control, which may indicate that spleen weight is not an appropriate surrogate measure of erythropoiesis. A more quantitative measure of erythropoiesis in the spleen is currently being investigated. The chromosome 17 locus overlaps with a known spleen weight QTL Splq9 [26].

In this study we found a significant QTL on chromosome 5 (bmRBC1) and a suggestive male-specific QTL on chromosome 9 that controls the bone marrow E/NE cell ratio. Erythropoietin and its receptor are obvious candidates although neither QTL appear to be involved in regulating pRBC counts. There is evidence that genetic variation near the *EPO* gene affects transcription levels (WebQTL), but no *EPO* or *EPO-R* gene coding sequence or promoter DNA sequence differences between the two parental CBA/H and C57BL/6 inbred mouse strains in this study were detected. However, the possibility of sequence variants in other upstream or downstream gene regulatory regions cannot be excluded.

No obvious candidate gene maps to the significant chromosome 4 QTL that controls pRBC counts and which has been replicated in two independent studies. However, other QTL that map to the 80-90 Mbp chromosome 4 interval include loci that control the immune response (*Ifa* and *Ifb*), splenomegaly (*Sbw2*, *Orgwg6* and *Spm1*), and anti-red blood cell IgG antibodies (*Arigg3*), which have been implicated in mouse models of autoimmune haemolytic anaemia and systemic lupus erythematosus [7-9]. Significantly, a mouse anti-erythrocyte autoantibody modifier locus (*Aem3*) and anti-nuclear autoantibody locus (*Bana2*) co-localise to the suggestive E/NE bone marrow cell ratio QTL on chromosome 10, and a human haematocrit level QTL has also been mapped to chromosome 6q23-24, syntenic to mouse chromosome 10 QTL [1].

One interpretation of our data is that natural variation in the *EPO* and *EPO-R* genes may affect erythrocyte production in the bone marrow but not peripheral blood. To our

knowledge this is the first time that natural genetic variation in these genes has been suggested to cause tissue-specific phenotypic variation. This work, if replicated, has a number of implications for clinical haematology, since variation in erythropoiesis impacts upon a number of conditions. Genetic loci that effect erythropoiesis are potential modifiers for diseases resulting in altered erythrocyte levels or functioning e.g. polycythaemia, anaemia and thalassaemia. Through the reported association between haematocrit and cerebral infarction and cardiovascular disease, the genetic loci could directly show association with these complex diseases.

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Figure 1: Casey coulter counter plots. (a) Peripheral RBC (b) Total bone marrow (c) Bone marrow depleted for erythroid cells by Ter119 (d) Bone marrow depleted for white cells by irradiation. Vertical dotted lines are the size cut-offs used to define cell populations

Figure 2: Linkage plot of bone marrow RBC:WBC ratio for chromosome 5. Top graph: X-axis distance in centimorgan, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.

Figure 3: Linkage plot Male-specific ratio QTL on chromosome 5. Top graph: X-axis distance in centimorgan, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.

Figure 4: Linkage plot of bone marrow RBMC:WBMC ratio on female mice for chromosome 11. Top graph: X-axis distance in centimorgan, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.

Figure 5: Linkage plot of peripheral RBC using CBA x BL6 F2 mice showing chromosome 4. Top graph: X-axis distance in centimorgan, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.

Figure 6: Linkage plot for chromosome 4 of pRBC using AXB/BXA mice. Top graph: X-axis distance in centimorgan, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.

Figure 7: Linkage plot for all chromosomes of spleen weight. Top graph: X-axis chromosome number, Y-axis Lod score plot. Bottom graph: Y-axis is additive effect, if >0 then C57BL/6 allele increases phenotype, <0 CBA is increaser allele.