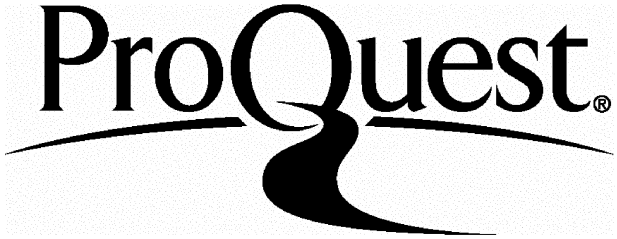
Analysis of the LYL-1 null mutant mouse- a possible role in haematopoietic progenitor cell mobilisation.

Emerson Randall King B.Sc. (Hons)

Department of Medicine, University College London, London University.

Submitted for a PhD degree in Molecular Genetics.



Abstract

The gene that encodes the Lymphoblastic Leukemia protein 1 (LYL-1) (Cleary, et al, 1988) was identified originally through the characterisation of a tumor specific t(7;19) chromosomal translocation involving the T-cell receptor (TCR) Cβ gene. The LYL-1 protein is a member of the class II basic helix-loop-helix (bHLH) family of transcriptional factors (Mellentin, et al, 1989; Massari and Murre, 2000). LYL-1 forms heterodimers, both in-vitro and in-vivo, with class I bHLH proteins E2A, and HEB (Miyamoto, et al, 1996), and has been shown to interact with motifs present in the NFκB1 precursor p105 (Ferrier, et al, 1999). LYL-1 mRNA is expressed primarily within the haematopoietic system (Kuo, et al, 1991) and displays a pattern of expression that overlaps considerably with stem cell leukemia protein (SCL) (Visvader, et al, 1991). Both SCL and LYL-1 mRNA are expressed in erythroid and myeloid cell lineages as well as in megakaryocytes, but not in normal T-cells (Mellentin, et al, 1989; Mouthon, et al, 1993). LYL-1, unlike SCL, is expressed in cell lines established from leukemias of B lymphocyte origin (Visvader, et al, 1991; Miyamoto, et al, 1996).

The amino acid sequence of the LYL-1 protein shares 78% overall identity with it's human counterpart (Visvader, et al, 1991; Kuo, et al, 1991) and possess an identical bHLH domain except for a conservative amino acid substitution in the loop region. Although the role of LYL-1 protein in the oncogenic transformation of T-cells has not been defined, as a consequence of the translocation it's de-regulated expression may alter the precise balance of transcriptional regulators, and thereby precipitate leukaemogenic events in T-cell. The almost identical E-box DNA-binding site for SCL and LYL-1 bHLH heterodimers (Miyamoto, et

al., 1996), in conjunction with the critical function SCL haematopoiesis (Begley and Green, 1999), suggests a role for LYL-1 within the haematopoietic cells. The function of LYL-1 in haematopoiesis was examined by disruption of the LYL-1 gene by homologous recombination in ES cells. A lacZ/ neomycin gene cassette was cloned in-frame into the fourth exon, replacing a 0.7kb HpaI fragment encoding the bHLH, as well as the entire 3'-end of the LYL-1 gene.

The absence of a functional LYL-1 protein was associated with increased numbers of c-Kit-positive cells, early (BFU-e) and late (CFU-e) erythroid progenitors (approximately 3-5 times) in the spleen. These progenitors generated haemoglobinised erythroblasts *in vitro*. More BFU-e in the LYL-$1^{-/-}$ spleen responded to Epo only, and in the presence of Epo and SCF some of these BFU-e produced colonies that comprised a large number of erythroid cells. *In vivo*, splenic erythroid progenitors and precursors expressed SCL mRNA, GATA-1, TER-119, and haemoglobin, and were distributed throughout the red pulp. LYL-$1^{-/-}$ spleen comprised 5 to 10-fold more erythroblasts than normal, which were arranged into clusters that often contained megakaryocytes. There was a 2-fold increase in the number of CFU-GEMM in the LYL-$1^{-/-}$ spleen, but no change in CFU-G. The bone marrow of LYL-$1^{-/-}$ mice contained 1.4-fold more CFU-e compared to LYL-$1^{+/+}$ controls, but there was no change in BFU-e number. The erythroblasts in the LYL-$1^{-/-}$ spleen were not apoptotic. The loss of a functional LYL-1 protein was further associated with a reduction in the number of erythrocytes, and the amount of stored iron, in the spleen, but no change in the number of erythrocytes in the peripheral circulation, or in the amount of stored iron in the bone marrow.

Abstract

The results presented suggest that LYL-1 has a role in events associated with mobilisation of haematopoietic progenitors from the bone marrow. Expression of a non-functional LYL-1 protein in LYL-$1^{-/-}$ mice induced mobilisation of erythroid progenitors, followed by their expansion in the spleen.

Acknowledgements.

I would like to acknowledge the efforts of Professor Fred Sablitzky. Without his financial support, the research presented in this thesis would not have been possible. His supervision during all stages of the research inspired confidence. Thank you Fred.

I would like to especially thank my wife Zeba. A perfect friend during times of difficulty and comfort.

Thank you Mum and Dad.

This is dedicated to The One in Whose Hand my soul lies.

Contents. Page

Title Page i

Abstract ii

Acknowledgements v

Dedication vi

Contents Page vii

Chapter 1 Introduction. 1

Section 1.1 *Haematopoiesis.* 1

Section 1.2 *Characteristics of the haematopoietic stem cell.* 1

Figure A. Fates of the HSC. 2

Figure B. Hierarchical model of haematopoiesis. 4

Section 1.3 *The location of haematopoietic stem cells.* 5

Section 1.4 *Development of haematopoietic stem cells during embryogenesis.* 6

Section 1.5 *The relationship between haematopoietic stem cells and the vasculature during development.* 8

Figure C. Location of HSCs. 9

Section 1.6 *Regulation of haematopoietic stem cell differentiation.* 10

Figure D. Haematopoiesis. 11

Section 1.7 *The role of transcription factors in haematopoiesis.* 12

Section 1.8 *Erythropoiesis.* 13

Section 1.9 *Development of erythroid cells.* 13

Figure E. Erythropoiesis. 14

Section 1.10 *Transcription factors critical for erythropoiesis.* 15

Section 1.11	*Growth factor receptors involved in haematopoiesis.*	18
Section 1.12	*Growth factors critical for erythropoiesis.*	19
Section 1.12.1	*Erythropoietin (Epo.)*	19
Section 1.12.2	*c-Kit.*	20
Section 1.13	*Interaction between Epo/ EpoR and SCF/ c-Kit signaling during erythropoiesis.*	22
Section 1.14	*Regulation of haematopoietic cell development, and migration, through adhesion receptors.*	23
	Figure F. Homing and mobilisation of HSC.	24
Section 1.15	*Class I bHLH proteins in haematopoiesis.*	26
Section 1.16	*Role of the class II bHLH protein SCL, in haematopoiesis.*	27
Section 1.2	*The Lymphoblastic Leukemia protein-1 (LYL-1).*	30
Section 1.2.1	*The LYL-1 gene.*	30
	Figure G. The LYL-1 gene and protein structure.	32
Section 1.2.2	*The LYL-1 protein.*	34
Section 1.2.3	*Expression of LYL-1.*	36
Section 1.2.4	*Function of LYL-1.*	38
Section 1.3	*Aim of the study.*	40
Chapter 2.	**Methods and Materials.**	42
Section 2.1	***Histology and Histochemistry.***	42
Section 2.1.1	*Prelude.*	42

Section 2.1.2	*Genotyping of the LYL-1 mice.*	42
Section 2.1.3	*Extraction and purification of genomic DNA.*	42
Section 2.1.4	*Preparation of high purity genomic DNA.*	43
	Figure 1. Genotyping of LYL-1 mice.	44
Section 2.1.5	*Fixation of tissues.*	46
Section 2.1.6	*Cytocentrifugation of bone marrow, spleen, and peripheral blood cells.*	47
Section 2.1.7	*Paraffin wax embedding of tissue.*	47
Section 2.1.8	*Cryoprotection and freezing of tissue.*	48
Section 2.1.9	*Preparation of tissue sections and cytocentrifuged cells for histological analysis.*	48
Section 2.1.10	*TUNEL assay for apoptotic cells.*	49
Section 2.1.11	*May-Grunwald and Giemsa stain of haematopoietic cells in sections of spleen.*	50
Section 2.1.12	*May-Grunwald and Giemsa stain of cytocentrifuged bone marrow and peripheral blood cells.*	50
Section 2.1.13	*Perl's Prussian blue stain for ferric iron in and cytocentrifuged bone marrow cells.*	51
Section 2.1.14	*β-galactosidase assay in cryosections of tissue.*	51
Section 2.1.15	*1,2-diaminofluorene stain of haemoglobin in cytocentrifuged bone marrow and spleen cells and sections of spleen.*	52
Section 2.2	***Materials used in histology and histochemistry.***	52

Section 2.2.1	*10X Phosphate buffered saline (PBS).*	52
Section 2.2.2	*4% paraformaldehyde solution in 1X PBS (pH7.5).*	52
Section 2.2.3	*Helly's fluid.*	53
Section 2.2.4	*Buffered 10% formalin (pH7).*	54
Section 2.2.5	*30% sucrose/ 1X PBS (pH 7.5) with 2mM $MgCl_2$.*	54
Section 2.2.6	*Clearing solution.*	55
Section 2.27	*Rinse buffer for X-GAL staining.*	55
Section 2.2.8	*X-GAL staining solution.*	56
Section 2.2.9	*May-Grunwald and Giemsa staining solutions.*	56
Section 2.2.10	*Fixative solution for cytocentrifuged cells.*	57
Section 2.2.11	*3% acetic acid in H_2O.*	57
Section 2.2.12	*Perl's Prussian blue stain for ferric iron.*	58
Section 2.2.13	*1,2 diaminofluorene (1,2-DAF) solution.*	58
Section 2.2.14	*1% Neutral red.*	59
Section 2.2.15	*Eosin Y solution.*	59
Section 2.2.16	*Buffered H_2O.*	59
Section 2.2.17	*Preparation of 90%, and 70% ethanol.*	59
Section 2.3	***Immunohistochemistry.***	60
Section 2.3.1	*Prelude.*	60
Section 2.3.2	*Immunohistochemical detection of target molecules in sections of spleen.*	60
Section 2.3.3	*3,3-diaminobenidine (DAB) colour development.*	61

Table 1. Concentration of antibodies used for immunohistochemistry. 62

Section 2.4 ***Materials used for immunohistochemistry.*** 63

Section 2.4.1 *Target retrieval solution.* 63

Section 2.4.2 *Periodic acid (0.01M).* 63

Section 2.4.3 *Sodium borohydride ($0.1 mgmL^{-1}$).* 64

Section 2.4.4 *Normal swine serum (1:20)/ 1X PBS.* 64

Section 2.4.5 *1.5%v/v normal swine serum/ 1X PBS.* 64

Section 2.4.6 *Anti-mouse biotinylated primary antibody and isotype control.* 65

Section 2.4.7 *Species-specific biotinylated secondary antibody.* 65

Section 2.4.8 *ExtrAvidin/ 1X PBS.* 65

Section 2.4.9 *3,3'-Diaminobenzidine (DAB) solution.* 66

Section 2.5 ***In situ Reverse Transcription (RT) Polymerase Chain Reaction (PCR) by direct incorporation of digoxigenin labelled dUTP.*** 67

Section 2.5.1 *Prelude.* 67

Section 2.5.2 *Digestion of spleen sections with Proteinase K.* 67

Section 2.5.3 *In situ Reverse Transcription.* 67

Section 2.5.4 *In situ Polymerase Chain Reaction.* 68

Section 2.5.5 *Signal development for in situ RT-PCR.* 68

Figure 2. Digestion of spleen sections with proteinase K. 70

Figure 3. SCL specific in situ RT-PCR protocol including DNAse and oligodT. 72

Figure 4. SCL specific in situ RT-PCR protocol including oligodT and without DNAse. 75

Figure 5. SCL specific in situ RT-PCR protocol in the presence of SCL specific primers, and without DNAse. 77

Section 2.6 ***Material used for in situ RT-PCR.*** 80

Section 2.6.1 *Diethylpropylcarbonate (DEPC)-treated H_2O.* 80

Section 2.6.2 *Proteinase K solution.* 80

Section 2.6.3 *Proteinase K buffer in 0.1M Tris/ 50mM Na_2EDTA.* 81

Section 2.6.4 *0.1M glycine in 1X PBS.* 81

Section 2.6.5 *10X DNAse buffer.* 81

Section 2.6.6 *Oligo-dT_{18} solution.* 82

Section 2.6.7 *In situ reverse transcriptase mixture.* 82

Section 2.6.8 *In situ polymerase chain reaction mixture.* 83

Section 2.6.9 *Taq DNA polymerase-Taq antibody for hot start of in-situ PCR.* 84

Section 2.6.10 *Buffer 1.* 85

Section 2.6.11 *Buffer 2.* 85

Section 2.6.12	*Buffer 3.*	86
Section 2.6.13	*Nitro blue tetrazolium chloride (NBT) and 5-bromo-4 -chloro-3-indolyl phosphate, toluidine salt (BCIP) substrate solution.*	86
Section 2.7	***Semi-quantitative RT-PCR.***	87
Section 2.7.1	*Prelude.*	87
Section 2.7.2	*Estimation of PCR cycles required to remain within the exponential range of amplification of cDNA.*	87
	Table 2. PCR mixture compositions.	88
	Table 3. Sense and Anti-sense primer sequences used in PCR.	89
	Table 4. Thermal profiles used for PCR.	89
	Figure 6. Determination of cycle number required to remain within the exponential range of amplification.	90
Section 2.7.3	*Experiments to determine the sensitivity of the semi-quantitative RT-PCR assay.*	92
	Figure 7. Exponential range of amplification for SCL and LYL-1 cDNA.	93
	Figure 8. Exposure time of hybridisation membranes.	95
	Figure 9. Signal intensity after different exposure times of hybridisation membranes.	97

Table 5. Signal intensity and cycle number for β-actin PCR. 99

Table 6. Signal intensity and cycle number for SCL and LYL-1 PCR. 99

Table 7. Signal intensity and exposure time for β-actin PCR. 99

Table 8. Signal intensity and exposure time for SCL and LYL-1 PCR. 100

Table 9. cDNA dilution and changes in signal intensity for β-actin PCR. 100

Table 10. cDNA dilution and changes in signal intensity for SCL and LYL-1 PCR. 100

Figure 10. Sensitivity of semi-quantitative RT-PCR assay. 102

Figure 11. Sensitivity of the *β-actin and SCL semi-quantitative RT-PCR assay.* 104

Section 2.8 ***Materials used for semi-quantitative RT-PCR.*** 106

Section 2.8.1 *Extraction and purification of total RNA from tissue.* 106

Section 2.8.2 *Reverse transcription mixture.* 106

Section 2.8.3 *Purification of cDNA.* 107

Section 2.8.4 *Polymerase chain reaction mixture.* 108

Section 2.8.5 *Southern hybridisation of PCR amplified cDNA.* 108

Section 2.8.6 *Radiolabeling of nucleic acid probes.* 109

Section 2.8.7	*Probe hybridisation.*	109
Section 2.8.8	*Church and Gilbert wash solution for Southern hybridisation.*	110
Section 2.8.9	*Church and Gilbert hybridisation solution for southern hybridisation.*	111
Section 2.8.10	*$Na_2HPO_4.2\ H_2O$ solution (1M).*	111
Section 2.9	***In vitro colony assays in methylcellulose media.***	112
Section 2.9.1	*Preparation of cells for in vitro colony assays in methylcellulose.*	112
Section 2.9.2	*In vitro colony assay of bone marrow and spleen cells in the presence of stem cell factor (SCF), erythropoietin (Epo), interleukin-3 (IL-3) and interleukin-6 (IL-6).*	112
Section 2.9.3	*In vitro colony assay of bone marrow and spleen cells in the presence of Epo.*	112
Section 2.10	***Materials used for in vitro colony assay.***	113
Section 2.10.1	*Colony assay in the presence of Epo only.*	113
Section 2.10.2	*Colony assay in the presence of SCF, Epo, IL-3, and IL-6.*	113

Section 2.11	***Other molecular biology techniques employed in this study.***	113
Section 2.11.1	*Northern hybridisation.*	114
Section 2.11.2	*Southern hybridisation of genomic DNA.*	114
Section 2.11.3	*Restriction endonuclease digestion of plasmid DNA.*	115
Section 2.11.4	*Electroporetic transformation of competent bacterial strain JM109 with plasmid DNA.*	115
Section 2.11.5	*Amplification of plasmid DNA.*	116
Section 2.11.6	*Extraction and Purification of plasmid DNA.*	116
Section 2.11.7	*Ligation of PCR products into plasmid vector pGemT.*	116
Section 2.12	***Materials used in molecular biology.***	117
Section 2.12.1	*10X 3-[N-Morpholino] propane sulphonic acid (MOPS).*	117
Section 2.12.2	*20X SSC.*	118
Section 2.12.3	*0.25M HCl.*	118
Section 2.12.4	*10X TBE.*	118
Section 2.12.5	*0.4M NaOH.*	119
Section 2.12.6	*1X TBE (pH8.3) electrophoresis buffer for agarose gel electrophoresis of DNA.*	119
Section 2.12.7	*40X Wash buffer for GlassMax cDNA Purification.*	120
Section 2.12.8	*Rinse solution for formaldehyde/ agarose gel.*	120
Section 2.12.9	*Lysis buffer for extraction of genomic DNA.*	121

Section 2.12.10	*Preparation of agarose gel for electrophoresis of DNA.*	122
Section 2.12.11	*Preparation of formaldehyde/ agarose gel for electrophoresis of RNA.*	122
Section 2.12.12	*Preparation of RNA electrophoresis buffer.*	123
Section 2.12.13	*Preparation of 10X DNA electrophoresis buffer.*	124
Section 2.13	*Statistical analysis.*	125
Chapter 3.	**Results.**	126
Section 3.1	*Relative expression of SCL and LYL-1 mRNA in mouse tissues.*	126
	Figure 12. Semi-quantitative RT-PCR in LYL-1 mouse tissues.	127
Section 3.2	*SCL mRNA expression is increased in the spleen of LYL-1$^{-/-}$ mice.*	129
	Figure 13. Increased expression of SCL mRNA in the LYL-1$^{-/-}$ spleen.	130
Section 3.3	*The spleen of LYL-1$^{-/-}$ mice contain more cells expressing SCL mRNA than the spleen of LYL-1$^{+/+}$ mice.*	132
	Figure 14. The LYL-1$^{-/-}$ spleen contain more cells that express SCL mRNA than LYL-1$^{+/+}$ spleen.	133
Section 3.4	*The spleen of LYL-1$^{-/-}$ mice contain more*	

erythroid cells than the spleen of LYL-1$^{+/+}$ mice. 136

Figure 15. Increase in erythroid cells in the LYL-1-/- spleen. 137

Figure 16. Increased number of GATA-1+ve cells in the LYL-1$^{-/-}$ spleen. 140

Section 3.5 *The majority of erythroid cells in the spleen of LYL-1$^{-/-}$ mice do not express the LYL-1^{lacZ} fusion protein.* 143

Figure 17. Cells in the LYL-1$^{-/-}$ spleen express the LYL-1^{lacZ} fusion protein. 144

Section 3.6 *The spleen of LYL-1$^{-/-}$ mice contain more erythroblasts but fewer erythrocytes than LYL-1$^{+/+}$ mice.* 146

Figure 18. LYL-1$^{-/-}$ spleen contain less erythrocytes than LYL-1$^{+/+}$ spleen. 147

Figure 19. LYL-1$^{-/-}$ spleen contain more erythroblasts than LYL-1$^{+/+}$ spleen. 149

Section 3.7 *LYL-1$^{-/-}$ spleen contain more cells that expressed c-Kit than LYL-1$^{+/+}$ spleen.* 152

Figure 20. Increased number of c-Kit $^{+ve}$ cells in the LYL-1$^{-/-}$ spleen. 153

Section 3.8 *LYL-1$^{-/-}$ spleen contain more CFU-e, BFU-e and CFU-GEMM than LYL-1$^{+/+}$ spleen.* 155

Figure 21. LYL-1$^{-/-}$ spleen contain more CFU-e, BFU-e and CFU-GEMM than

	LYL-1$^{+/+}$ spleen.	156
	Table 11. Number of CFU-e, BFU-e, and CFU-GEMM in the spleen.	159
	Table 12. Number of CFU-e, BFU-e, and CFU-GEMM in the bone marrow.	159
	Figure 22. BFU-e and CFU-GEMM colonies grown from LYL-1 spleen cells.	161
Section 3.9	*LYL-1$^{-/-}$ mice have similar numbers of peripheral erythrocytes to LYL-1$^{+/+}$ mice.*	164
	Figure 23. LYL-1$^{-/-}$ mice display normal haematological parameters.	165
Section 3.10	*LYL-1$^{-/-}$ mice have less haemosiderin in the spleen, and normal levels in the bone marrow compared to LYL-1$^{+/+}$ mice.*	167
	Figure 24. LYL-1$^{-/-}$ spleen contain less haemosiderin than LYL-1$^{+/+}$ spleen.	168
	Figure 25. The level of haemosiderin in LYL-1$^{-/-}$ bone marrow is normal.	170
Section 3.11	*Erythroblasts in the LYL-1$^{-/-}$ spleen do not undergo apoptosis.*	172
	Figure 26. LYL-1$^{-/-}$ spleen does not contain more apoptotic cells than LYL-1$^{+/+}$ spleen.	173
Section 3.12	*Summary.*	175

Chapter 4	**Discussion.**	176
Section 4.1	*SCL does not compensate for the loss of a functional LYL-1 protein in haematopoietic cells.*	176
Section 4.2	*LYL-1 was not expressed in erythroblasts, but may be expressed in activated splenic B cells.*	177
Section 4.3	*LYL-1 does not have a role in regulating proliferation of haematopoietic progenitor cells.*	178
Section 4.4	*LYL-1 does not negatively regulate SCL expression.*	179
Section 4.5	*The LYL-1 phenotype resulted from migration of haematopoietic progenitor cells.*	182
Section 4.6	*A possible role for LYL-1 in c-Kit mediated migration of haematopoietic progenitor cells from the bone marrow.*	182
Section 4.7	*A mechanism for LYL-1 control of c-Kit mediated haematopoietic progenitor cell migration.*	184
Section 4.8	*LYL-1 was not essential for the maintenance of normal haematological parameters.*	186
Section 4.9	*LYL-1 does not perform an anti-apoptotic function in haematopoietic progenitor cells.*	187
Section 4.10	*LYL-1 has a limited role in the regulation of haematopoietic progenitor cell migration to the spleen.*	188
Section 4.11	*LYL-1 regulates migration of distinct haematopoietic progenitor cell types.*	190
Section 4.12	*Conclusion.*	192

Section 4.13	*Future work.*	193
Section 4.14	*References.*	195

Chapter 1. Introduction

Section 1.1 Haematopoiesis.

Mammalian blood formation originates from a small common pool of pluripotent cells called haematopoietic stem cells (HSCs) (Figure D). Along with the bone marrow, HSCs can be found in umbilical cord blood and in peripheral blood, particularly after mobilisation treatments (To, et al., 1997). The stem cell concept originated from the work of Till and McCulloch (Till and McCulloch, 1961), which showed the formation of nodules of haematopoietic cells in the spleens of lethally irradiated mice after reconstitution with bone marrow from normal syngeneic donors (Till and McCulloch, 1961). HSCs are responsible for sustained production of sufficient numbers of blood cells of all lineages. This complex process is strictly regulated by intrinsic genetic programs involving the expression of cell-type and stage-specific regulators, and by the haematopoietic microenvironment, which comprises stromal cells, extracellular matrix molecules and soluble regulatory factors.

Section 1.2 Characteristics of the haematopoietic stem cell.

Several decades of research to elucidate the biology of HSCs have revealed three defining properties of these cells (Figure A). First, the HSC can generate more HSCs by a process of self-renewal (Godin, et al., 1999). Second, the HSC can differentiate into multipotential progenitor cells, which commit to further maturation and give rise to at least eight different cell types (Orkin, 1996) (Figure D). Third, HSCs have the capacity to provide long-term reconstitution of the haematopoietic system when injected intravenously into adult recipients (Krause, et al., 2001).

The mechanisms that govern the fate decisions of HSCs are under tight yet flexible control, and although a number of molecules have been shown to play a role at early stages their

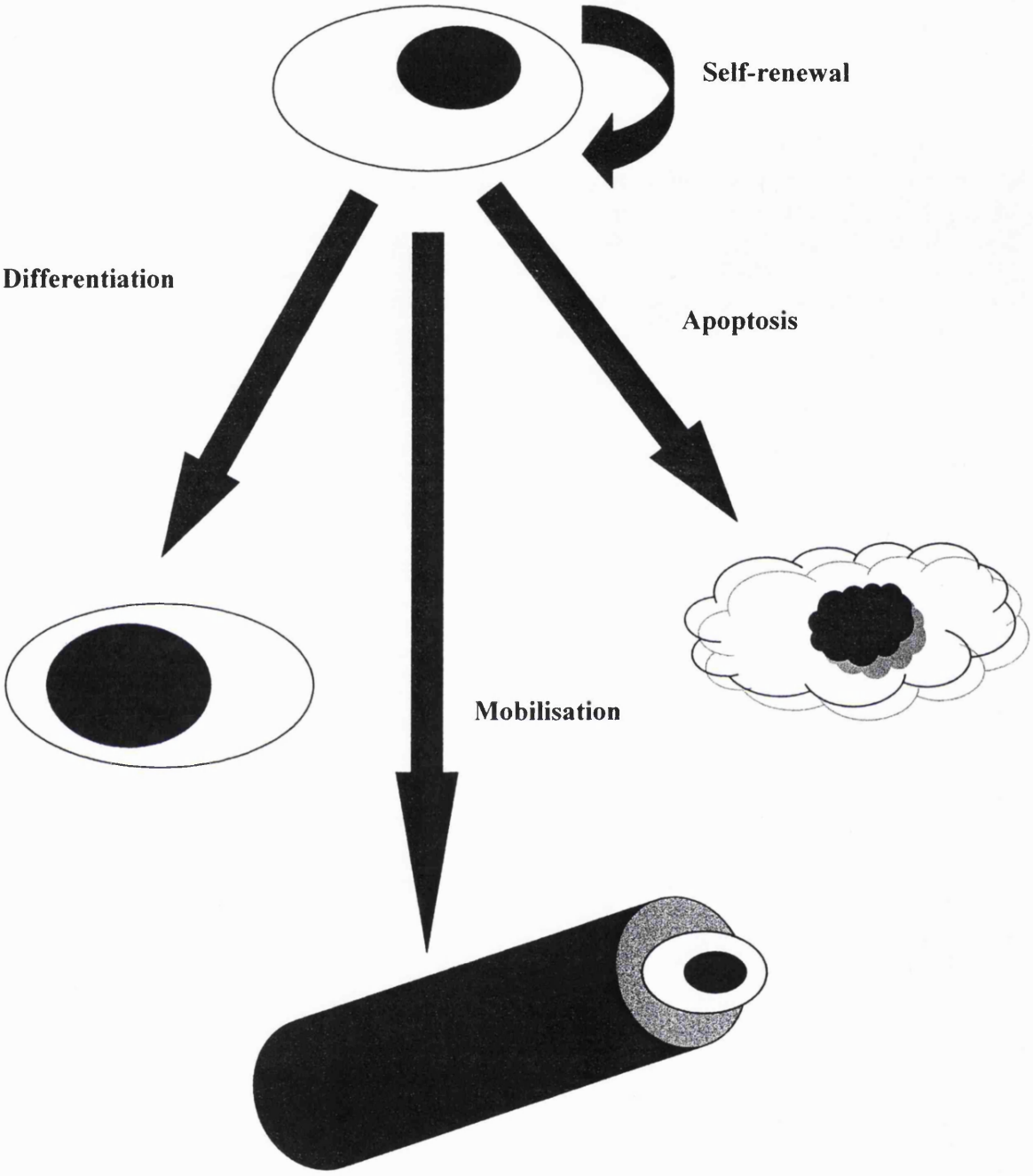


Figure A. The potential fates of HSCs. HSCs are capable of self-renewal; HSCs can differentiation into any of the haematopoietic lineages; HSCs can undergo apoptosis; HSCs can be mobilised to enter, or leave, the peripheral circulation.

development (Phillips, et al., 2000), the molecular nature of these mechanisms are only now starting to become clear. This is, in part, due to the elusiveness of the HSC (Scott and Gordon, 1995) present in the mouse bone marrow at a frequency of 1 in 10^5 cells (Harrison, et al., 1990), and the absence of specific markers for the HSC (Donnelly, et al., 1999) and appropriate in vitro assays for HSCs (Weilbaecher, et al., 1991; Choi, et al., 1998). Currently, the only reliable functional assay system for the most primitive HSC compartment is long-term in vivo transplantation. The majority of HSCs are normally quiescent, as shown by their resistance to treatment with 5-fluorouracil or 4-hydroperoxycyclophosphamide, which eliminated dividing cells without adversely affecting the long-term repopulating ability of bone marrow derived HSCs (Morrison, et al., 1995). Estimates of periodicity of mitosis vary widely (once a month to once in a few years), and direct examination of the cell cycle of long-term cells indicated that at any moment only 4% of them are in the S/ G2/ M phases (Morrison, et al., 1995). Separation of progenitor and precursor cell populations on the basis of surface marker expression and developmental potential in functional assays has established the hierarchical model of haematopoiesis (Figure B). In this model, HSCs give rise to progenitors, which develop into precursors that are committed to a specific cell fate (Orkin, 2000). Intrinsic genetic programs and microenvironmental factors influence the decision to self-renew or differentiate (Morrison, et al., 1995). Upon induction to cycling, HSCs may undergo symmetric divisions i.e. self-replication or differentiation, and/or asymmetric divisions i.e. generation of a self-replicated and a differentiated cell (Brecher, et al., 1993). HSCs differentiation has been interpreted in terms of stochastic (Till, et al, 1964), inductive (Curry and Trentin, 1967) or hybrid models (Just, et al., 1991). The stochastic hypothesis submits that random intrinsic molecular events are responsible for committment, while exogenous hematopoietic growth factors allow survival and proliferation of cells already programmed to a particular fate (Suda, et al., 1984). According to the inductive model,

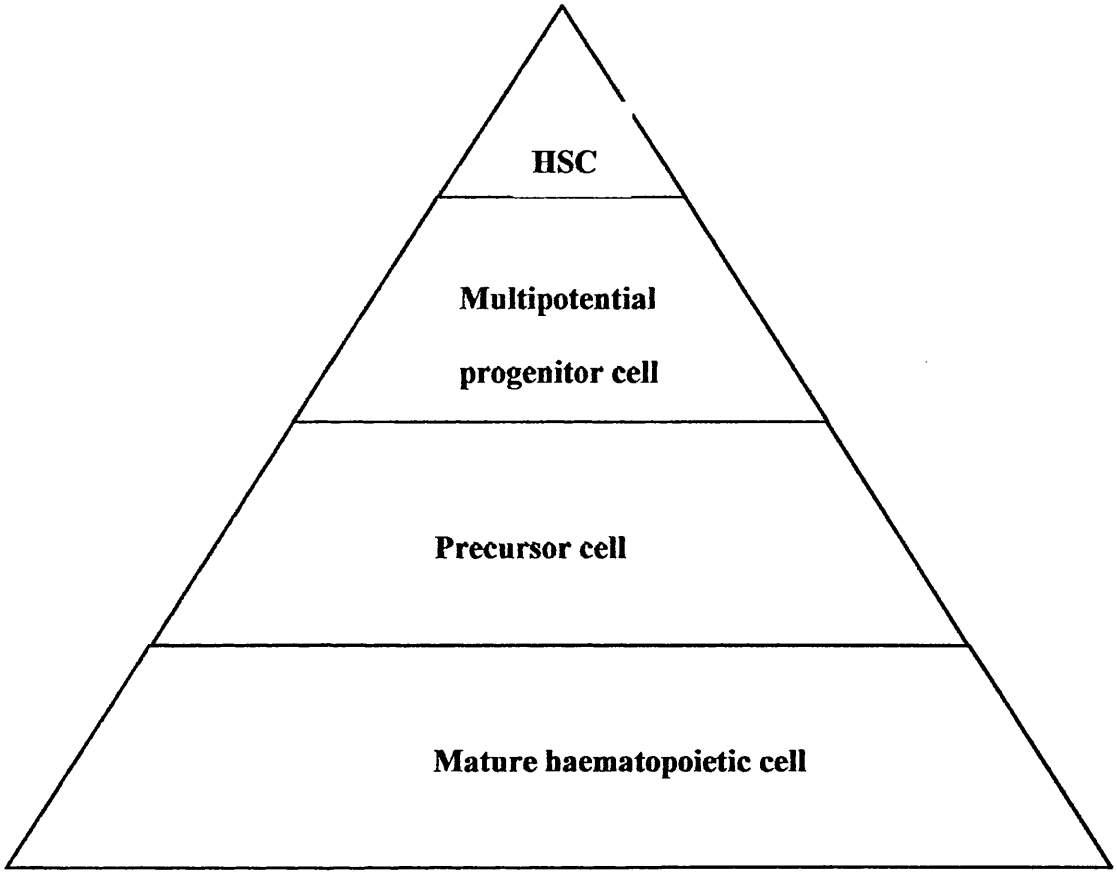


Figure B. The hierarchical model of haematopoiesis. HSCs give rise to progenitors, which develop into precursors that are committed to a specific cell fate.

growth factors and cell-mediated regulatory mechanisms trigger HSCs to differentiate along a particular lineage or lineages (Metcalf, 1991). Alternatively, the hybrid model proposes that stochastic events prevail at early developmental stages, and growth factor-mediated inductive events function later (Testa, et al., 1996). Regardless of which model is correct, it is accepted that lineage committment involves activation of specific differentiation programs through a network of factors that regulate these programs at the level of transcription (Orkin, 1995).

Section 1.3 The location of haematopoietic stem cells.

The origin of the HSC has been, and still is a controversial issue for debate (Moore and Metcalf, 1970; Dieterlen-Lievre, 1975; Lord and Dexter, 1995; Cumano, et al., 2000). Although HSCs proliferate and differentiate in haematopoietic organs such as fetal liver, bone marrow, thymus and spleen, HSCs do not originate in these organs. Rather, the HSC has an exogenous origin, arising through specification of mesodermal cells that, after several stages of development, become HSCs.

In the mouse extra- and intra-embryonic areas are well defined at 7.5 days post-coitum (dpc). The extra-embryonic territory comprises the yolk sac, which is derived from mesodermal and primitive endodermal cells, whereas the intra-embryonic area is demarcated by the embryo proper, which comprises the ectoderm, mesoderm and endoderm formed during gastrulation. The mesoderm of the embryo proper divides to form close associations with the ectoderm (somatopleura), and the endoderm (splanchnopleura). The vasculature of the paired dorsal aortas develops from the splanchnopleura at 8 dpc, and is linked to the yolk sac vasculature through the vitelline artery by 8.5dpc (Garcia-Porrero, et al., 199). Primoridiums of the mesonephros and gonads are apparent at 9dpc (Kaufmann, 1992) and develop into the metanephros (kidney), and the gonads by 12 dpc (Kaufmann, 1992). The region that

comprises the aorta-gonad-mesonephros (AGM region) extends from the forelimbs to the hindlimbs between 9.5dpc and 12.5dpc (Kaufmann, 1992). Each of the three developing organ systems has been associated with haematopoietic cells (de Bruijn, et al., 2000; Medvinsky, et al., 1996; Rich, et al., 1995).

Section 1.4 Development of haematopoietic stem cells during embryogenesis.

The first haematopoietic cells to form are the embryonic red cells located within structures known as blood islands in the yolk sac at about 7.5 dpc (Moore and Metcalf, 1970). These cells were large nucleated erythrocytes that expressed embryonic isoforms of haemoglobin βH1 and ε and did not require erythropoietin for late stages of differentiation (Palis, et al., 1999). Embryonic red cell production in the yolk sac, known as primitive erythropoiesis, signifies the first wave of blood cell development that supplies the growing embryos' increasing demand for oxygen. The second wave of blood cell development, known as definitive haematopoiesis, occurs in the fetal liver at about 14.5pdc, and continues in the bone marrow throughout adulthood. Erythrocytes generated by definitive haematopoiesis were small, express α1, α2, βmaj and βmin adult isoforms of haemoglobin, and required activation of erythropoietin-mediated differentiation pathways for complete development (Palis, et al., 1999).

The proposition that HSCs responsible for definitive haematopoiesis originated from an intraembryonic site rather than the yolk sac came from studies on avian embryos, which found that yolk sac derived stem cells did not contribute to definitive erythropoiesis, whereas cells isolated from the splanchnopleura-AGM provided long-term reconstitution of haematopoietic progenitors (Dieterlen-Lievre, 1975). HSCs derived from the yolk sac, unlike those from bone marrow, and fetal liver, are not capable of long-term reconstitution of

haematopoiesis when injected into an irradiated recipient mouse (Muller, et al., 1994; Medvinsky and Dzierzak, 2000; Orlic and Bodine, 1994). Furthermore, HSCs derived from the yolk sac prior to establishment of circulation do not generate haematopoietic multipotential progenitors *in vitro* (Nishikawa, et al., 1998). These observations indicated that yolk sac derived HSCs are different to those responsible for blood cell development in the adult. It has also been suggested that the microenvironment of the bone marrow may not provide the conditions for the yolk sac derived HSCs to develop into multipotential progenitors. This is because yolk sac derived cells isolated between 8 and 10.5 dpc provided long term reconstitution of the haematopoietic system when injected into the newborn liver or *in-utero* (Yoder, et al., 1997). Although the possibility that HSCs migration from intra-embryonic sites to the yolk sac once circulation was established could not be ruled out, this report suggested that the microenvironments of fetal liver and adult bone marrow regulated development of HSCs through distinct mechanisms.

There is now a considerable body of work that supports the concept that intra-embryonic sites such as the AGM generate HSCs independently, and that these cells are different to yolk sac derived HSCs in their capacity to reconstitute bone marrow (Medvinsky, et al., 1993; Godin, et al., 1993). Cells from the AGM with the capacity for long-term reconstitution of the haematopoietic system in mice comprised cells that generated colonies of haematopoietic cells in the spleen (CFU-S) as well as lymphocyte precursors (Muller, et al., 1994; Medvinsky and Dzierzak, 1996). *In vitro* studies in mice have shown that AGM derived HSCs generated haematopoietic progenitors prior to blood circulation, which confirmed a purely intraembryonic site for these HSCs (Nishikawa, et al., 1998).

Section 1.5 The relationship between haematopoietic stem cells and the vasculature during development.

The presence of independent intra-embryonic sites for HSCs has been elucidated further by studies using Xenopus embryos (Ciau-Utiz, et al., 2000). The Xenopus equivalent yolk sac and AGM were found to develop from different blastomeres (Ciau-Utiz, et al., 2000). Furthermore, clusters of haematopoietic cells associated with the aorta were derived from the same blastomere as the splanchnopleura-AGM region (Ciau-Utiz, et al., 2000). These results were consistent with studies in mice, which showed that of all components of the AGM, haematopoietic cells were most highly concentrated around the aorta between 9 and 12.5 dpc (Godin, et al., 1999). A close relationship between blood vessel and blood cell development was confirmed by studies that identified HSCs in the mesenchyme around the umbilical and vitelline arteries (De Bruijn, et al., 2000).

The apparent generalization of HSCs to blood vessels such as the aorta could be the result of a common progenitor for endothelium and haematopoietic cells, known as the haemangioblast (Pardanaud, Dieterlen-Lievre, 1999). The spatial and temporal association of embryonic red cell and vascular cell development within the yolk sac eluded to the existence of the haemangioblast, an elusive cell type that has be generated by in vitro differentiation of murine embryonic stem cells (Choi, et al., 1999). These cells established both haematopoiesis and vasculogenesis in embryos, and may be related to another putative bipotential progenitor cell in the AGM, the haemogenic endothelium (Jaffredo, et al., 1998). Studies have shown that cells, which expressed either of the endothelium-specific markers TIE2/ TEK (Hamaguchi, et al., 1999) or VE-cadherin (Nishikawa, et al., 1998) isolated from the AGM, as well as yolk sac, differentiated *in vitro* and generated endothelial and lymphohaematopoietic progeny. Although the molecular mechanisms responsible for

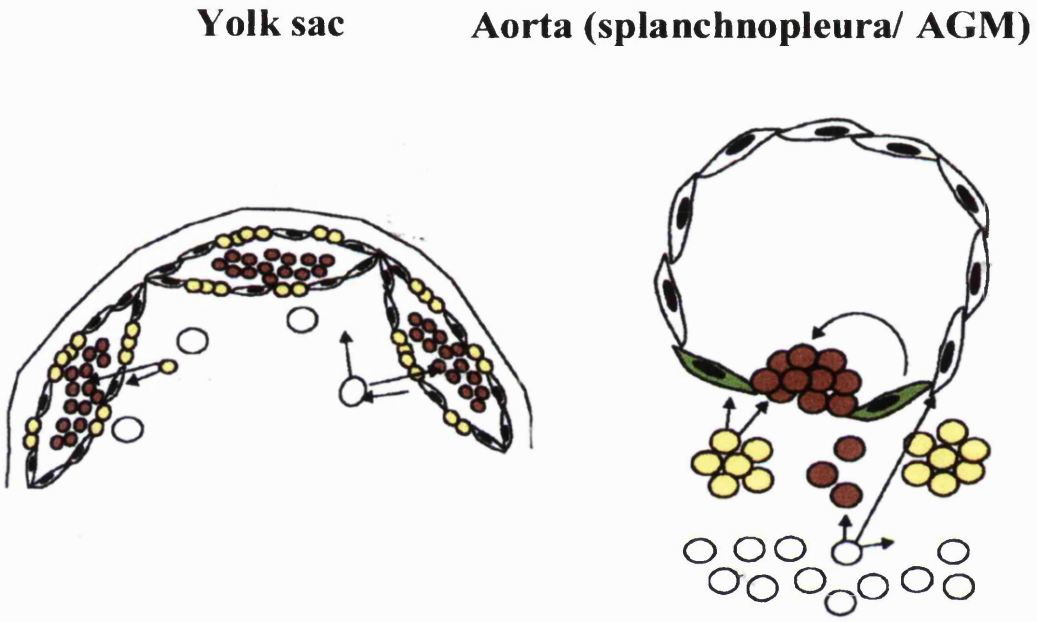


Figure C. Haematopoietic cells (red) may develop from several cell types (i) pluripotent mesodermal cells (white) or (ii) haemangioblasts (yellow) in the yolk sac and aorta, or (iii) haemogenic endothelium (green) in the aorta.
(Image taken from Cumano and Godin, 2001)

differentiation of the haemangioblast or haemogenic endothelium from the mesoderm into vasculogenic or haematopoietic cell types are not understood fully, these studies show that a close relationship exists between haematopoietic and vascular endothelial cell development at an early stage of embryogenesis (Figure C).

At about 14.5dpc the fetal liver is the site of definitive haematopoiesis (Orkin, 1996). At birth and throughout adult life, the bone marrow is responsible for the generation of blood cells, although during periods of stressed bone marrow haematopoiesis, the spleen can also become a site for blood cell production (Broudy, et al., 1996). The mechanisms responsible for these ontogenic shifts are not well known, but are likely to involve changes in the developing haematopoietic cells (Houssaint and Hallet, 1988), and in the haematopoietic microenvironment (Slaper-Cortenbach, et al, 1987; Friedriclh, et al, 1996). Experimental dissection of the mechanisms involved in decisions of self-renewal versus differentiation of the HSC has yielded valuable information on the contribution of intrinsic genetic programs, and extrinsic factors (Ogawa, 1993; Lansdorp, 1995) to the decision-making processes during HSCs development.

Section 1.6 Regulation of haematopoietic stem cell differentiation.

Blood cells generated by the haematopoietic system are classified into two main classes, lymphoid (B, T and natural killer lymphocytes) and myeloid (erythrocytes, megakaryocytes, granulocytes and monocytes) (Figure D). The life span of the fully differentiated mature blood cell varies considerably, ranging from several hours for granulocytes, several weeks for erythrocytes to several years for memory cells. Alterations in the balance between self-renewal and differentiation can lead to the emergence of cells that survive and grow in situations unfavorable to the growth of normal cells and hence to the establishment of

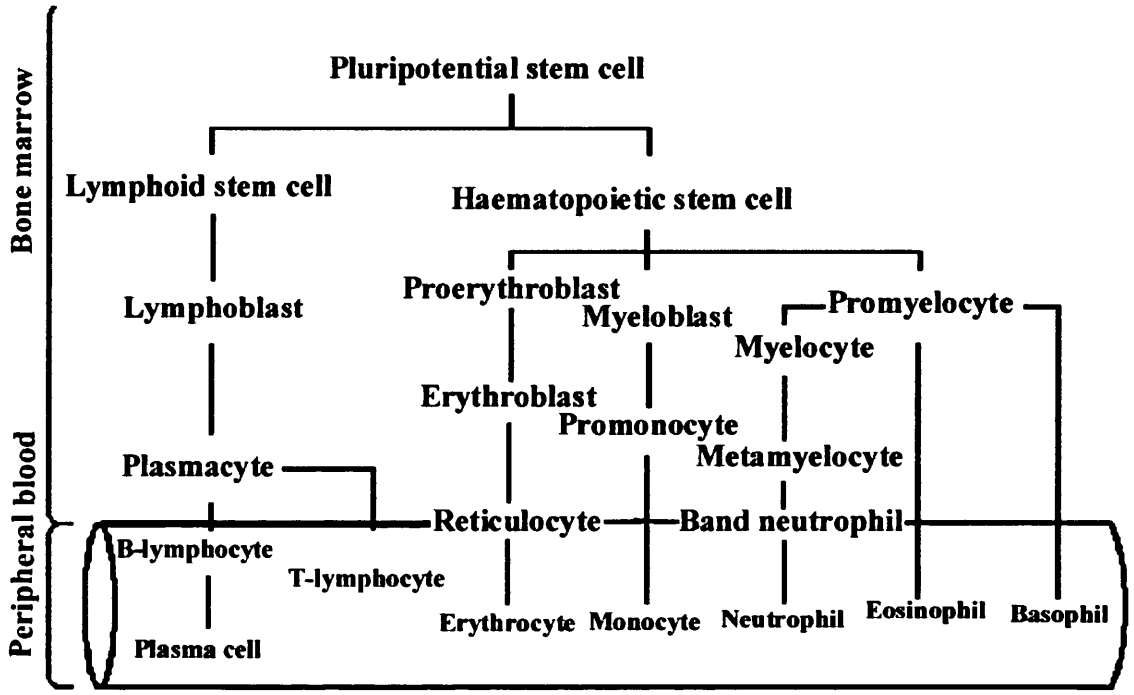


Figure D. The different haematopoietic lineage fates of a HSC.
Regulated diffrentiation of the HSC can generate eight different cell types.

leukemias. Haematopoiesis produces about 1 trillion cells each day (in a 70-kg man), including 200 billion erythrocytes and 70 billion neutrophilic leukocytes as well as maintaining a pool of undifferentiated stem cells for rapid response to situations such as acute stress (Ogawa, 1993). Stress situations can lead to production of a specific cell type such as haemolysis induced erythroid hyperplasia and granulocyte hyperplasia in response to bacterial infection.

The transition from HSC to mature haematopoietic cells occurs through several intermediate steps characterized by the progressive loss of the self-renewal capacity and the commitment to a specific cell lineage (Orkin, 1996; Paulson and Bernstein, 1996). The progenitor cell compartment, which can be identified by the expression of specific lineage markers, comprises cell types that are determined to differentiate into precursor cells. The developmental potential of progenitor cells is generally limited to only one or two of the haematopoietic lineages. Precursor cells progressively display the antigenic, biochemical, and morphological features characteristic of the mature cells of the appropriate lineages (Orkin, 2000). Proliferation of both progenitor and precursor cell compartments is tightly controlled, so that mature cells leaving the bone marrow possess little or no proliferative potential. Immunohistological analyses of the haematopoietic system, in conjunction with experimental assays such as the colony formation assay, have identified regulatory mechanisms that govern development of the HSCs, multipotential progenitor cells, and precursor cells.

Section 1.7 The role of transcription factors in haematopoiesis.

Discovery of transcription factors unique to haematopoietic cells and subsequent gene knockout experiments argue strongly for a predetermined differentiation program that is modulated by cytokines. For example, disruption of the gene encoding the haematopoietic

transcription factor SCL blocked haematopoiesis completely (Shivdasani, et al., 1995; Robb, et al., 1995a). Gene targeting of GATA-1 also severely affected erythropoiesis. (Penvy, et al., 1991). However, the ability of cytokines to initiate haematopoiesis both in vitro and in vivo suggests that the transcriptional regulators ultimately controlling differentiation are influenced by haematopoietic cytokines (Orkin, 1996).

Section 1.8 Erythropoiesis.

Erythropoiesis is a process that describes the production of erythrocytes. In vertebrates erythrocytes are generated early in embryogenesis (see section 1.4) reflecting commitment of mesoderm to a haematopoietic fate. As circulation is established, generation of primitive erythrocytes is vital for survival and subsequent development of the embryo. The production of definitive erythrocytes in the bone marrow during adult life maintains the supply of oxygen to respiring cells.

Section 1.9 Development of erythroid cells.

As mentioned above HSCs give rise to multipotential progenitors that generate precursors committed to differentiation along selected lineages (see section 1.6). Progenitor cells that generate erythroid precursors are not distinguishable form other progenitors morphologically, but can be detected by *in vitro* colony assay (Romeo, et al., 1990). Indeed, unlike lymphocytes the erythroid precursor cells cannot be distinguished by expression of stage specific surface markers and must be defined morphologically after histological staining with May-Grunwald and Giemsa stain. This stain produces a range of cytoplasmic and nuclear colour, and allows the erythroblast to be distinguished by its large nucleus and basophilic cytoplasm (Figure E). Erythroid burst-forming units (BFU-e), and colony-forming erythroid units (CFU-e) are early and late stage

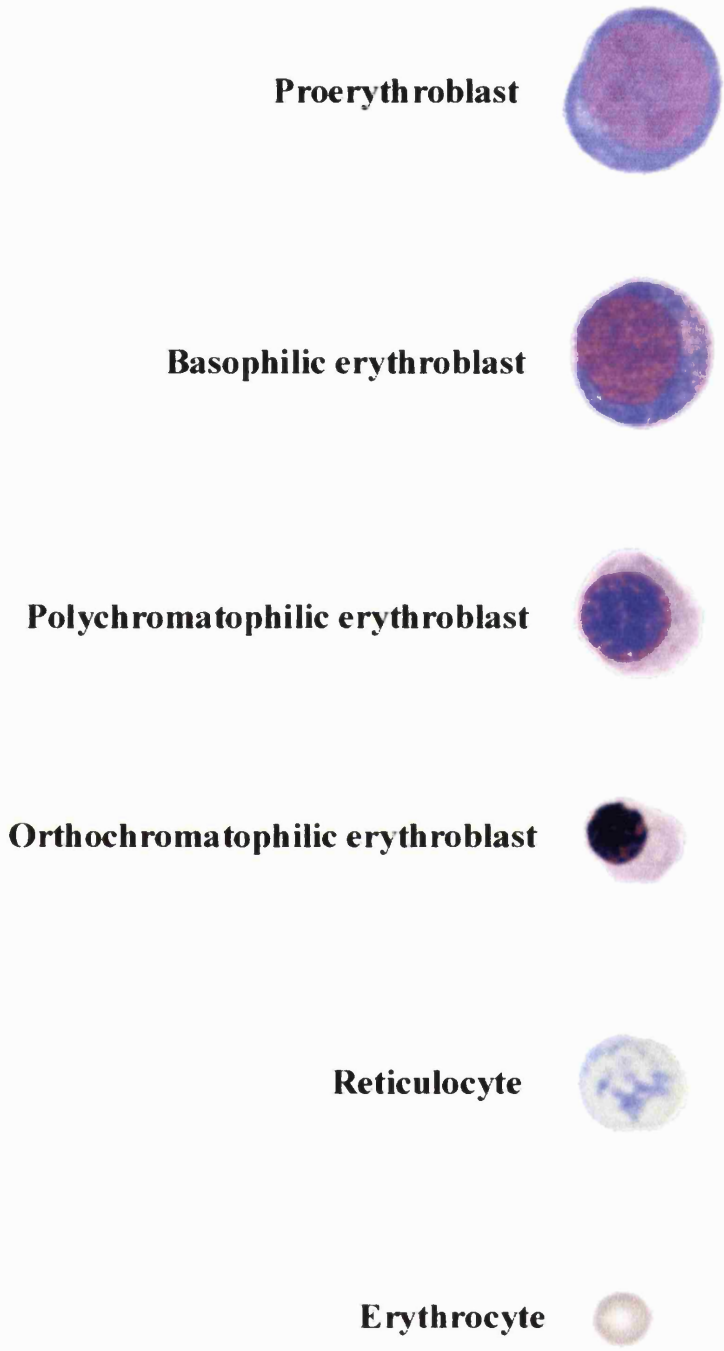


Figure E. May-Grunwald and Giemsa stained erythroid cells. Erythoid cells are characterised by their large nucleus and basophilic cytoplasm. As the cells develop the nucleus condenses and the haemoglobin content increases. The mature erythrocyte contains no nucleus (pyknosis).

erythroid progenitors that generate proerythroblasts. These proerythroblasts develop into basophilic, polychromatic, and orthochromatic erythroblasts. Each stage of development involves increased production of haemoglobin and further condensation of the nucleus (pyknosis) with eventual enucleation (Bernard, 1991) characteristic of the mature erythrocyte. A complex network of signaling pathways within cells regulates erythropoiesis.

Section 1.10 Transcription factors critical for erythropoiesis.

The role of cell restricted DNA binding proteins that mediate erythroid specific gene transcription has be dissected by two approaches. Genes discovered by analysis of chromosomal translocations and deletions associated with leukemia have been associated with essential proliferation and/ or differentiation decisions in haematopoietic cells (Rabbitts, 1994). Loss of function and gain of function experiments have identified a number of genes critical for erythropoiesis (Orkin and Zon, et al, 1997). Indeed, some of these genes, such as SCL, are involved at early stages in haematopoietic development (Yamaguchi, et al., 1993; Gering, et al, 1998). A preliminary hierarchy of transcriptional regulators with distinct and overlapping patterns of expression in erythroid progenitors and precursors has been fashioned from these studies (Orkin and Zon, 1997).

SCL, LMO2, FOG1, GATA-1 and GATA-2, and EKLF (as well as many others) have a role in either early and/ or late stages of erythropoiesis (Green et al., 1991; Aplan, et al., 1992; Penvy, et al., 1991; Simon, et al., 1992; Porcher, et al, 1996; Robb, et al, 1995a; Tsang, et al., 1998; Nuez, et al., 1995; Warren, et al., 1994; Tsai, et al., 1994). SCL was activated ectopically by chromosomal translocations in T-cell acute lymphoblastic leukemia (T-ALL) (Begley, et al, 1989; Chen, et al, 1990), and is essential for the development of all haematopoietic cell lineages (Porcher, et al, 1996). Disruption of the SCL gene in mice led to

early embryonic lethality and a complete absence of blood (Robb, et al, 1995a). Specifically, immature erythrocytes were lacking in the embryo, placenta, or yolk sac of mutant mice (Shivdasani, et al, 1995). SCL protein was found to be a positive regulator of normal erythroid differentiation (Aplan, et al, 1992) and growth (Green, et al, 1991) and may act with GATA-1 to form an erythroid specific multimeric DNA binding complex (see Section 1.16).

Many erythroid specific genes contain a GATA-1 motif in cis-regulatory sequences (Evans, et al., 1988). Extensive analysis of the role of this zinc finger protein, found exclusively in haematopoietic cells, with the exception of sertoli cells in the testis (Yamamoto, et al., 1997), has shown it to essential for maturation of erythroblasts (Penvy, et al., 1991; Weiss and Orkin, 1995; McDevitt, et al., 1997). GATA-$1^{-/-}$ embryos contained proerythroblasts in their yolk sac (Fujiwara, et al., 1996). However, erythroid precursors generated by *in vitro* differentiation of GATA-$1^{-/-}$ ES cells, arrested at the proerythroblasts stage and underwent apoptosis (Weiss, et al., 1994; Weiss and Orkin, 1995). GATA-1 appears to be expressed in erythroid progenitors (Silver and Palis, 1997), so their development into proerythroblasts in the absence of GATA-1 may be explained by an increase in GATA-2 expression (Weis, et al., 1994), although recent evidence suggests that during adult erythropoiesis GATA-1 and GATA-2 are not functionally equivalent (Takahashi, et al., 2000).

An interesting role for GATA-1 later in erythroid development has been elucidated recently. Studies suggest that erythroid cell signaling through the erythropoietin receptor (EpoR), and activation of death receptors may compete through GATA-1 to elicit differentiation, reversible maturation arrest or death of developing erythroid cells (Dai, et al., 1998; DeMaria, et al., 1999; Whyatt, et al., 2000). Normal GATA-1 function was therefore critical for

controlling red blood cell production (Orkin, and Weiss, 1999). The importance of GATA-1 in primitive and definitive erythropoiesis appears to involve independent functions of, as well as collaborative relationships between the two zinc fingers and N-terminal region of the protein (Shimizu, et al, 2001).

GATA-2 is not the functional equivalent of GATA-1, and targeted mutation of the GATA-2 gene reduces yolk sac and fetal liver erythropoiesis dramatically (Tsai, et al, 1994). GATA-2$^{-/-}$ ES cell contribution to adult haematopoiesis was marginal (Tsai, et al., 1994). As such, GATA-2 is believed to maintain survival and/ or cytokine responsiveness of early multipotential progenitors.

The function of erythroid-specific genes in later erythropoiesis is qualified by lacZ knock-in experiments targeted to the erythroid Kruppel-like factor (ELKF) gene (Nuez, et al., 1995). ELKF is a zinc finger protein that regulated adult β-globin expression (Kulozik, et al., 1991) and facilitated the switch from γ to β haemoglobin isoform expression (Perkins, et al., 1995). The yolk sac of EKLF$^{-/-}$ mouse embryos contained erythroid progenitors, but embryos died when haematopoiesis switched to the fetal liver (Perkin, et al., 1995; Nuez, et al., 1995). Interestingly, mature erythrocytes formed in the fetal liver contained reduced amounts of haemoglobin (Nuez, et al., 1995). ELKF was required for appropriate terminal erythroid maturation but is dispensable for erythroid progenitor commitment and expansion of precursors (Perkins, et al., 1995; Nuez, et al., 1995). GATA-1 has also been associated with steps in heme synthesis, since GATA-1 induced expression of a mitochondrial specific transporter during erythroid differentiation (Shirihai, et al., 2000).

Section 1.11 Growth factor receptors involved in haematopoiesis.

Cytokines produce multiple biological effects that are often dependent upon the stage of differentiation of the target cell (Wu, et al., 1997). The pleiotropic actions of cytokines can be explained by the array of signaling components and transcription factors present in a cell at the time of exposure to the cytokine (Smithgall, 1998). Furthermore, a given cytokine receptor can activate a variety of downstream signaling pathways, so many cytokines have overlapping effects in a cell. The molecular basis for this lies in the fact that the receptors for cytokines share common subunits that are important for signal transduction (Lowry, 1995).

Haematopoiesis is regulated in part through the differential expression and activation of lineage- and stage-specific hematopoietic growth factor receptors of the type I superfamily (Ihle, et a., 1998). For example, lymphocyte production depends on interleukin-7 (IL-7) receptor expression and signaling (von Freeden-Jeffry, et al., 1995), whereas EpoR expression and activation is required for the development of erythroid progenitor cells beyond the colony-forming unit-erythroid stage (Wu, et al., 1995). One key effect exerted by growth factors and their receptors is an inhibition of apoptosis. For example, T-cell development in IL-7 receptor-deficient mice can be rescued by transgenic expression of the survival factor Bcl-2 (Akashi, et al., 1997). However, certain growth factors affect more than the survival of targeted lineage-restricted progenitor cells. Neither Bcl-2 nor Bcl-xL expression in erythroid progenitor cells was able to rescue erythropoiesis in the absence of an Epo signal (Laronique, et l., 1997; Gregory, et al., 1998; Chida, et al., 1999). Efforts have focused on establishing the nature of Epo receptor-induced signals that promote erythroid progenitor cell survival, growth, and/or differentiation (Krantz, 1991).

Section 1.12 Growth factors critical to erythropoiesis.

Section 1.12.1 Erythropoietin (Epo).

The Epo is the principal regulator of erythropoiesis (Krantz and Goldwasser, 1984). Its cognate receptor, EpoR consists of a single polypeptide chain with an extracellular portion for high-affinity binding of Epo and a cytoplasmic portion essential for the transmission of signals (Wells and de Vos, 1996; Ihle, 1995). Distinct domains of the EpoR appear to control different functional responses via discrete downstream signaling pathways (Watowich, et al., 2000; Zochodne, et al., 2000). Epo bound to EpoR dimers on the surface of BFU-e and CFU-e and activated the tethered Janus family kinase (JAK) 2 (Remy, et al., 1999). JAK2 (Klingmuller, et al., 1995) can mediate the phosphorylation of eight cytoplasmic tyrosine sites within the Epo receptor, and via these sites, a complex set of Src homology 2 domain-encoding effectors (and associated cofactors) were engaged, including STAT 5A and B (Wojchowski, et al., 1999). Negative effectors are likely to be important in Epo-mediated events during erythropoiesis. These include Cis, which appeared to compete with STAT5 for binding at Tyr-343 (Quelle, et al., 1996), an amino acid site that was indispensable for EpoR function (Matsumoto, et al., 1997).

Epo stimulated erythroid cell proliferation and differentiation of erythroid progenitors capable of colony forming unit activity (CFU-e) (Krantz, 1991). CFU-e required Epo for their survival (Sawyer and Penta, 1996; Ruff-Jamison, et al., 1993), and Epo/ EpoR signaling induced an increase in β-globin mRNA production (Koury and Bondurant, 1990) consistent with erythroid differentiation. Studies indicated that Epo/ EpoR signals contributed to preventing committed erythroid progenitors from undergoing apoptosis (Kelley, et al., 1993). Embryonic lethality in $EpoR^{-/-}$ mutant mice was due to a block in differentiation of erythroid cells at the proerythroblasts stage in the fetal liver but had little effect on yolk sac

erythropoiesis (Lin, et al., 1995). Unlike erythroid precursors, which required Epo/ EpoR signaling for proliferation (Koury and Bondurant, 1990) and differentiation (Imada, et al., 1992), the generation of BFU-e and CFU-e did not require EpoR (Wu, et al., 1995). The capacity of Epo to promote proliferation, as well as differentiation of erythroid precursors may result from the differential effect of Epo concentration on cell cycle (Carroll, et al, 1995). Low concentrations of Epo induced a lag in cell growth and a prolongation of G1 that was associated with enhanced β-globin expression. Higher concentrations of Epo did not promote a lag in cell growth or a prolongation of G1 (Carroll, et al., 1995). Interestingly, Epo-induced differentiation was blocked by IL-3, which induced exit of cells from G1 and entry into S phase. These data indicated that Epo exerted its differentiation activity by regulating the cell cycle of responsive erythroid progenitors (Carroll, et al, 1995).

Interaction of cytokine signal responses was responsible for the SCF/ c-kit and Tpo/ c-mpl mediated rescue of $EpoR^{-/-}$ derived erythroid precursors *in vitro* (Kiernan, et al., 1996). This result showed that Epo/ EpoR and SCF/ c-Kit signaling shared similar transduction pathways during erythropoiesis.

Section 1.12.2 c-Kit

c-Kit is the cellular tyrosine kinase receptor for stem cell factor (SCF) and a member of the type III subfamily (Fleischman, 1993). Loss of function of c-Kit or SCF caused a lack of skin and hair pigmentation, lack of intestinal pacemaker activity, sterility, and anemia. These effects correlated with the normal expression of c-Kit on melanocytes, (Orr-Urtreger, et al., 1990; Funasaka, et al., 1992) the interstitial cells of Cajal in the gut (Huizinga, et al., 1995) germ cells (Orr-Urtreger, et al., 1990; Manova, et al., 1990) and many hematopoietic cell lineages, including stem cells and erythroid progenitors (Broudy, 1997; Broudy, et al., 1994;

Ashman, et al., 1991; Papayannopoulou, et al., 1991; Simmons, et al., 1994; Avraham, et al., 1992; Ogawa, et al., 1994) and demonstrated a requirement for c-Kit activity in the development of these cell lineages. Two naturally occurring mutations in mice led to the identification of the tyrosine kinase receptor c-Kit and its ligand SCF (Chabot, et al., 1988; Fleischman, 1993). These mutations, known as W and *Sl* respectively, both resulted in mice with haematopoietic defects. Mutations in the W gene (encoding c-Kit) generated abnormal HSCs, while mutations in *Sl* gene (encoding SCF) impaired normal stromal cell function. c-Kit-deficient mice exhibited a severe reduction of CFU-e in the fetal liver and died of anemia (Nocka, et al., 1989). Inhibition of c-Kit function with an anti-c-Kit monoclonal antibody blocked myelopoiesis but had no effect on lymphopoiesis (Ogawa, et al., 1994). c-Kit signaling promoted survival of early HSCs (Okada, et al., 1991) as well as erythroid progenitors (Wu, et al., 1997). The important function of c-Kit in haematopoietic cell development was further emphasized by studies that shoed transduction of c-Kit into selected progenitor cells enhanced proliferation and decreased apoptosis (Lu, et al., 1999). c-Kit mediated proliferation of haematopoietic progenitors, may, in part, be regulated by differential c-Kit expression, which selected progenitors for recruitment into a proliferative state (Lu, et al., 1999). In most haematopoietic lineages, particularly the erythroid lineage, c-kit is down modulated after early commitment (Katayama, et al., 1993; Lyman and Jacobsen, 1998). In the later stages of erythropoiesis, i.e. from CFU-e through to proerythroblast, c-kit expression declined (Gabbianelli, et al., 1995). c-Kit was also capable of synergistic interaction with the EpoR (Wu, et al, 1996), which induced differentiation of erythroid precursors (Wu, et al., 1997).

Section 1.13 Interaction between Epo/ EpoR and SCF/ c-Kit signaling during erythropoiesis.

c-Kit and EpoR co-operate during distinct stages of haematopoietic cell development (Kiernan, et al., 1996; Wu, et al., 1995). It has been proposed that interaction between Epo-R and c-Kit (Wu, et al., 1995) was required for erythroid colony formation at a stage prior to that at which EpoR-deficient precursors were arrested (Kiernan, et al, 1996). This was supported by studies that showed c-Kit was able to promote survival and proliferation of erythroid progenitors (Broudy, 1997) and interacted with the EpoR (Wu, et al, 1996) to induce differentiation of erythroid precursors (Wu, et al., 1997). Through colony-forming assays (Wu, et al., 1997; Miller, et al., 1999; Sui, et al., 1998) and in vivo analyses (de Haan, et al., 1995), SCF and Epo have been shown to act in synergy on co-targeted erythroid progenitor cells. The interaction seemed to involve the trans-phosphorylation of the EpoR by c-Kit (Wu, et al., 1996). GATA-1 has been shown to bind the EpoR promoter directly and induce its expression (Gregory, et al., 2000). GATA-1 induced the expression of Bcl-xL and co-operated with Epo to promote the survival of G1E-ER2 cells (Gregory, et al., 1999). Recently, utilising an embryonic stem cell-derived erythroid progenitor cell line from GATA-$1^{-/-}$ mice, restoration of GATA-1 function resulted in terminal erythroid differentiation (Kapur and Zhang, 2001), which was associated with down regulation of c-Kit and Bcl-2, up-regulation of Epo-R and Bcl-xL, and survival of differentiating erythroid progenitors.

These data confirmed that committed erythroid progenitors could not survive, proliferate, or differentiate unless both the c-Kit and the EpoR signal transduction pathways were functional. Although the nature of the synergy between c-Kit and EpoR is not known precisely, it is likely that EpoR activation occurs by distinct mechanisms that generate

different intracellular signals essential for proliferation and terminal differentiation of committed erythroid progenitors (Wu, et al., 1997).

Section 1.14 Regulation of haematopoietic cell development, and migration, through adhesion receptors.

Hematopoietic chimerism after liver transplantation suggests that extramedullary transplantable progenitor cells exist (Starzl, et al., 1993), and may contribute to the mobilizable pool of HSCs (Rao, et al., 1997; Wright, et al., 2001). The general consensus of opinion is that mobilized blood progenitor cells originate from the bone marrow. Modulation of the progenitor cell: bone marrow stroma interaction, followed by directed migration toward marrow sinuses and eventual egression through the basement membrane and the endothelial layer, represent several critical events during progenitor cell mobilization (Figure F):

The localization of haematopoiesis to the bone marrow involves developmentally regulated adhesive interactions between primitive hematopoietic cells and the stromal-cell-mediated hematopoietic microenvironment (Simmons, et al., 1992). These interactions, along with the broad range of agents that can induce transient increases in blood progenitor cells (To, et al., 1994) suggested that mobilization involved a perturbation of the adhesive interactions with stromal elements which, under steady-state conditions, are responsible for the physiologic retention of primitive hematopoietic progenitor cells in the bone marrow (Turner, et al., 1992). Many of the receptors expressed on the surface of HSCs and multipotential progenitor cells have adhesive properties (Watowich, et al., 1996; Long, 1999; Telen, 2000). Bone marrow stromal cells express ligands for these cell adhesion molecules (CAMs) (Long, et al., 1992).

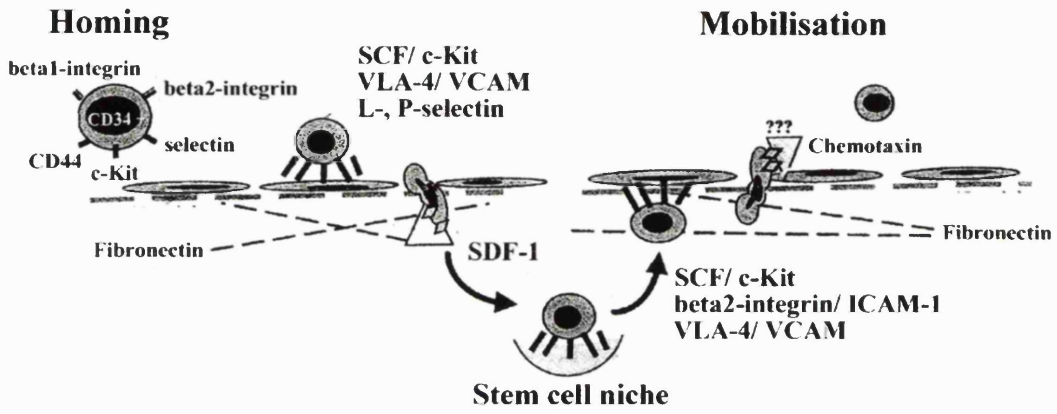


Figure F. Homing of HSCs and progenitors involves adhesion to the surface of endothelial cells, transmigration through the endothelium and anchorage to the stem cell niche in the bone marrow. Mobilisation involves release from the niche, and migration out through the endothelial layer, a process presumed to involve the reverse of processes involved in homing to the stem cell niche. (Image taken from Prosper, et al., 2001)

CAMs including members of the integrin, selectin, immunoglobulin superfamily and CD44 families of adhesion molecules are involved in regulating haematopoiesis (Prosper and Verfaillie, 2001) (Figure F). Adhesion of $CD34^{+ve}$ cells to bone marrow stromal cells through the very late antigen-4 (VLA-4), a member of the β_1-intergin family, as well as vascular cell adhesion molecule-1 (V-CAM 1), rescued them from apoptosis (Wang, et al., 1998). VLA-4 has been shown to be essential for normal differentiation and homing of progenitor cells (Arroyo, et al., 1999; Papayannopoulou, 1998). The very late antigen-5 (VLA-5), another member of the β_1-intergin family, also mediates adhesion of haematopoietic progenitors to the bone marrow stroma. Proliferation of committed progenitors (Verfaillie, 1992) and long-term culture initiating cells (Verfaillie and Cantazarro, 1996) was inhibited when $CD34^{+ve}$ cells were cultured in contact with stroma. G_1/S progression of progenitor cells was blocked through β_1-integrin (Hurley, et al., 1997), and activation of VLA-5/ fibronectin signalling pathways have been shown recently to inhibit progenitor cell proliferation (Jiang, et al., 2000). β_1-integrin mediated adhesion of $CD34^{+ve}$ cells was associated with up-regulation of $p27^{Kip}$ (Jiang, et al., 2000). Although mechanisms involved in adhesion-mediated inhibition of proliferation are not well understood, they are likely to have a critical role in physiological migration of HSCs and multipotential progenitors.

c-Kit was involved in the mobilization of BFU-e from the bone marrow to the spleen (To, et al., 1994), and could modulate haematopoietic progenitor cell adhesion/ mobilization by regulation of β1 integrins (Papayannopoulou, 1998). Blockage of c-Kit with an anti-c-Kit monoclonal antibody attenuated splenic erythropoiesis to a greater extent than bone marrow erythropoiesis in mice with phenylhydrazine-induced haemolytic anemia (Broudy et al., 1996). In this study the spleen was the primary site of erythropoietic expansion during stressed bone marrow erythropoiesis and suggested that splenic erythropoiesis was more

dependent on SCF/ c-Kit signaling than bone marrow erythropoiesis. Furthermore, homing of HSCs pre-treated with anti-c-Kit antibody from bone marrow to the spleen was severely perturbed (Broudy, et al., 1996).

Epo was shown to induce migration of BFU-e from the bone marrow to the spleen where they differentiated into CFU-e (Kato, et al, 1999). Epo-mediated migration of BFU-e from the bone marrow, after repeated administration of recombinant human Epo, was responsible for up-regulation of uptake clearance by the spleen (Kato, et al., 1998). Since W/W failed to up-regulate up take clearance (Kato, et al., 1998), BFU-e migration to the spleen was considered to be dependent on the presence of functional c-Kit.

An adhesion molecule expressed on HSCs and progenitor cells specifically has not been characterised, so the pathways that provide specificity to entering, or leaving the bone marrow are not known. It is likely that interaction between these cells and stromal elements are mediated by as yet unidentified receptors and ligands.

Section 1.15 Class I bHLH proteins in haematopoiesis.

Several bHLH transcription factors have been implicated in normal and malignant development of haematopoietic cells (Begley and Green, 1999; Bain, et al., 1997; Bain, et al., 1994; Engel and Murre, 1999). The E2A gene encodes for the class I bHLH proteins E12 and E47, which are capable of forming homodimers and heterodimers (Murre, et al., 1989b) that function as transcriptional activators (Henthorn, et al., 1990). Transactivation activity has been mapped to two N-terminal domains named AD1 and AD2 (Massari, et al., 1996). A motif termed LDFS within AD1 was responsible for interaction with chromatin modifying enzymes (Massari, et al., 1999). E2A mediated recruitment of molecules with HAT activity

(Qiu, et al., 1998) may be the mechanism by which E2A gene products regulate transcriptional activation in haematopoietic cells.

E2A homodimers can bind B cell specific E-box sites (Murre, et al., 1991; Shen and Kadesch, 1995; Bain, et al., 1993) and mediate B cell specific gene expression (Kee and Murre, 1998). Gene targeting experiments have shown that the E2A gene was essential for normal development of B-lymphocytes (Bain, et al., 1994; Zhuang, et al., 1994). However, E2A null mutant mice not only lacked mature B cells and had fewer B cell progenitors, they also suffered from defective development of αβ- and λδ- T lymphocytes (Bain, et al., 1997). Interestingly, E2A null mutant mice developed T cell lymphomas (Bain, et al., 1997; Yan, et al.,1997), a result that was consistent with their proposed role as tumor suppressors (Park, et al., 1999; Engel and Murre, 1999). It has been postulated that de-regulated expression of class II bHLH proteins such as SCL and LYL-1 may sequester E12/ E47, or conspire with other proteins to form aberrant complexes, that contribute to the block in development of T cells (Bain, et al., 1997; Park, et al., 1999).

Section 1.16 Role of the class II bHLH protein, SCL, in haematopoiesis.

SCL and tal-2 are activated ectopically by chromosomal translocations in T-ALL cells (Begley, et al., 1989; Chen, et al., 1990). Several groups of transcriptional regulators are associated with chromosomal breakpoints and deletions (Kennedy, et al., 1991; Cleary, 1991). This is of particular relevance with regard to SCL, since lesions within this gene were the most common molecular abnormality found in human T cell leukaemia (Robb and Begley, 1997). The SCL protein appears to modulate apoptosis in leukemic T-cells (Leroy-Viard, et al., 1995), and ectopic expression of SCL can protect human leukaemic T-cells from cytotoxin-induced apoptosis (Bernard, et al 1998). Ectopic expression of SCL in T-cells

disturbs normal transcriptional regulation and leads to development of tumors (Condorelli et al., 1996; Kelliher, et al., 1996). However, the leukemogenic potential of SCL in transgenic studies manifested in a proportion of mice only, and appeared context dependent (Robb, et al., 1995). Indeed, the incidence of tumor development was increased in mice that over-expressed both SCL and Lmo1 (Aplan, et al., 1997), or SCL and Lmo2 (Larson, et al., 1996). Lmo1 and Lmo2 are members of the LIM-only family of genes. Lmo2 has been shown to form part of an erythroid DNA binding complex with SCL, E47, Ldb1 and GATA-1 that bound a bipartite E-box-GATA-1 recognition site (Wadman, et al., 1997). A similar complex was found in T-cell lines derived from CD-2-Lmo2 transgenic mice, which bound to an E-box-E-box site (Grutz, et al., 1998) and may regulate T cell specific expression of genes different to those putative genes controlled by the SCL-Lmo multimeric complex in erythroid cells. It is possible that deregulated expression of SCL, and Lmo proteins, facilitate formation of an aberrant complex that influence the T cell developmental program. However, it is also conceivable that the leukaemogenic effect of de-regulated SCL expression may be to sequester individual proteins and inactivate normal patterns of gene expression.

Transgenic studies indicate that the relative concentration of Lmo proteins is related to development of tumors (Fisch, et al., 1992), and that SCL interacts, and interferes with E2A transcriptional activity (Hai-Ling, et al., 1994; Park and Sun, 1998). Furthermore, recent work by Herblot et al (Herblot, et al., 2000) demonstrated that ectopic expression of SCL and Lmo1 specifically targeted to the thymus both delayed and decreased expression of the pTα gene leading to decreased numbers of Tγδ+ thymocytes, a block in αβT cell differentiation at the double negative ($CD4^-CD8^-$) to double positive ($CD4^+CD8^+$) transition and marked atrophy in double positive cells. These phenotypic features were displayed by E2A and HEB homozygous null mutant mice (Bain, et al., 1997; Barndt, et al., 1999). The phenotype of

SCL-Lmo1 transgenic mice, was caused by a block in HEB-E2A mediated activation of pTα and TCRα genes, a process that was critical for normal T-cell development (Bain, et al., 1997). SCL-mediated conversion of E2A-HEB transcriptional activity from activation, to repression, may underlie the role of SCL in T-ALL. Unraveling the precise mechanism of action will require a better understanding of the role of class I and II bHLH proteins in thymopoiesis (Barndt, et al., 1999).

The SCL gene is expressed in early haematopoietic cells throughout ontogeny, in endothelial cells of the spleen, and in the developing brain (Baer, 1993; Mouthon, et al., 1993; Green, et al., 1992; Hwang, et al., 1993; Visvader, et al., 1991; Elefanty, et al., 1998; Sanchez, et al., 1999). SCL protein forms heterodimers with class I b HLH proteins E12 and E47 (Hsu, et al, 1991; Hsu, et al, 1994). Although a role for SCL in neurogenesis has not been described, Tal2 (a member of the TAL subfamily of bHLH genes) is required for normal development of the brain in mice (Bucher, et al., 2000). SCL may have a role in angiogenesis since the yolk sac capillary network in chimeric mice generated with $SCL^{-/-}$ null mutant ES cells tagged with an endothelial expressing lacZ gene did not undergo remodeling into vitelline vessels (Visvader, et al., 1998). Within the haematopoietic system SCL was expressed in myeloid progenitors, erythroid and myeloid cells as well as in megakaryocytes (Visvader, et al., 1991; Elefanty, et al., 1998) but not in mature T-cells (Visvader, et al., 1991). SCL has been shown to be essential for the development of all haematopoietic cell lineages (Porcher, et al., 1996); specification of haemangioblast development from the early mesoderm in zebrafish (Gering, et al, 1998), and was required for transition of mesodermal cells to a haematopoietic fate (Robertson, et al., 2000). Disruption of the SCL gene in mice caused early embryonic lethality and a complete absence of blood (Robb, et al., 1995a). Specifically, immature erythrocytes were absent in the embryo, placenta, or yolk sac of mutant mice (Shivdasani, et

al., 1995). These studies indicated a key role for SCL in primitive erythropoiesis, definitive haematopoiesis , and angiogenesis.

In adult haematopoiesis the SCL protein was a positive regulator of normal erythroid differentiation (Aplan, et al., 1992) and growth (Green, et al., 1991). Ectopic expression of SCL by retroviral transfer into erythroid, megakaryocytic, and granulomonocytic colony forming units augmented erythroid and megakaryocytic progenitor cell growth, but attenuated granulopoietic differentiation (Valtieri, et al., 1998). More recently SCL has been identified as a target for the acetyltransferases p300 and P/CAF in erythroid cells (Huang, et al., 2000), an effect that augmented SCL DNA binding and inhibited interaction with the co-repressor mSin3A. The results elucidated possible mechanisms for SCL-mediated transcriptional regulation. Interaction of SCL with specific co-activators, or co-repressors, may explain how SCL can act as a transcriptional activator or repressor (Huang and Brandt, 2000a; Huang, et al., 1999).

Based on their sequence and structural homologies and patterns of expression, LYL-1, Tal2 and SCL may constitute a subfamily of bHLH proteins, which possess leukemic properties mediated by recognition of a common group of target genes (Baer, 1993).

Section 1.2 The Lymphoblastic Leukaemia protein-1 (LYL-1).

Section 1.2.1 The LYL-1 gene.

A chromosomal 19p13 transcriptional unit named LYL-1 was identified provisionally by Cleary and co-workers (Cleary, et al, 1988) through the characterisation of a tumor specific t(7;19) chromosomal translocation involving the T-cell receptor (TCR) Cβ gene (Figure G). The structural effect of this breakpoint on the LYL-1 gene was assessed by limited nucleotide

sequence and restriction endonuclease analyses of the human SUP-T7 cell line, which was established from patients with T-cell acute lymphoblastic leukemia (T-ALL) and harbored the t(7;19) translocation. These studies showed that the breakpoint occurred in intron 1 of the LYL-1 gene and resulted in loss of exon 1 and juxtaposition of the LYL-1 gene with the TCR β chain gene in opposite transcriptional orientations (Figure G). The 5' end of the LYL-1 gene, along with it's promoter and start site for transcription are also lost as a consequence of the translocation, and truncated LYL-1 mRNA species could be detected in the SUP-T7 cell line by Northern blot analysis (Mellentin, et al, 1989). The transcription start site in the human LYL-1 gene is about 291bp upstream of a CTG site and lies in a GC- rich region containing four Sp1 binding sites but no TATA box homology sequence. Although no activators of LYL-1 protein expression have been identified, the presence of Sp1 binding sites within the transcription start site may suggest a role for Sp1 family proteins (Lin J-X. and Leonard W.J., 1997) or other transcription factors such as Egr-1 in regulating LYL-1 gene expression (Pagliuca, et al, 1998).

Isolation of the mouse LYL-1 gene was achieved through reduced stringency hybridisation of a mouse genomic DNA library with a human genomic DNA probe containing exons 2 and 3 (Kuo, et al, 1991). Restriction hybridisation and Southern blot analyses of two overlapping clones confirmed the presence of the complete LYL-1 gene on a 3.5kb BamHI/ EcoRI fragment (Kuo, et al, 1991). The LYL-1 gene was located on the central region of chromosome 8 and linked to Jund, Ucp, and Junb loci (Kuo, et al, 1991). Analysis of the nucleotide sequence of the LYL-1 gene revealed that, like its human counterpart, it consists of four exons (Figure G). Three ATG codons at nucleotides 1615, 1654 and 1672 (at the 5' end of exon 2), along with an in-frame CTG codon at nucleotide 894 (located at the 3' end of exon 1) provide alternative sites of translation initiation that are also shared by the human

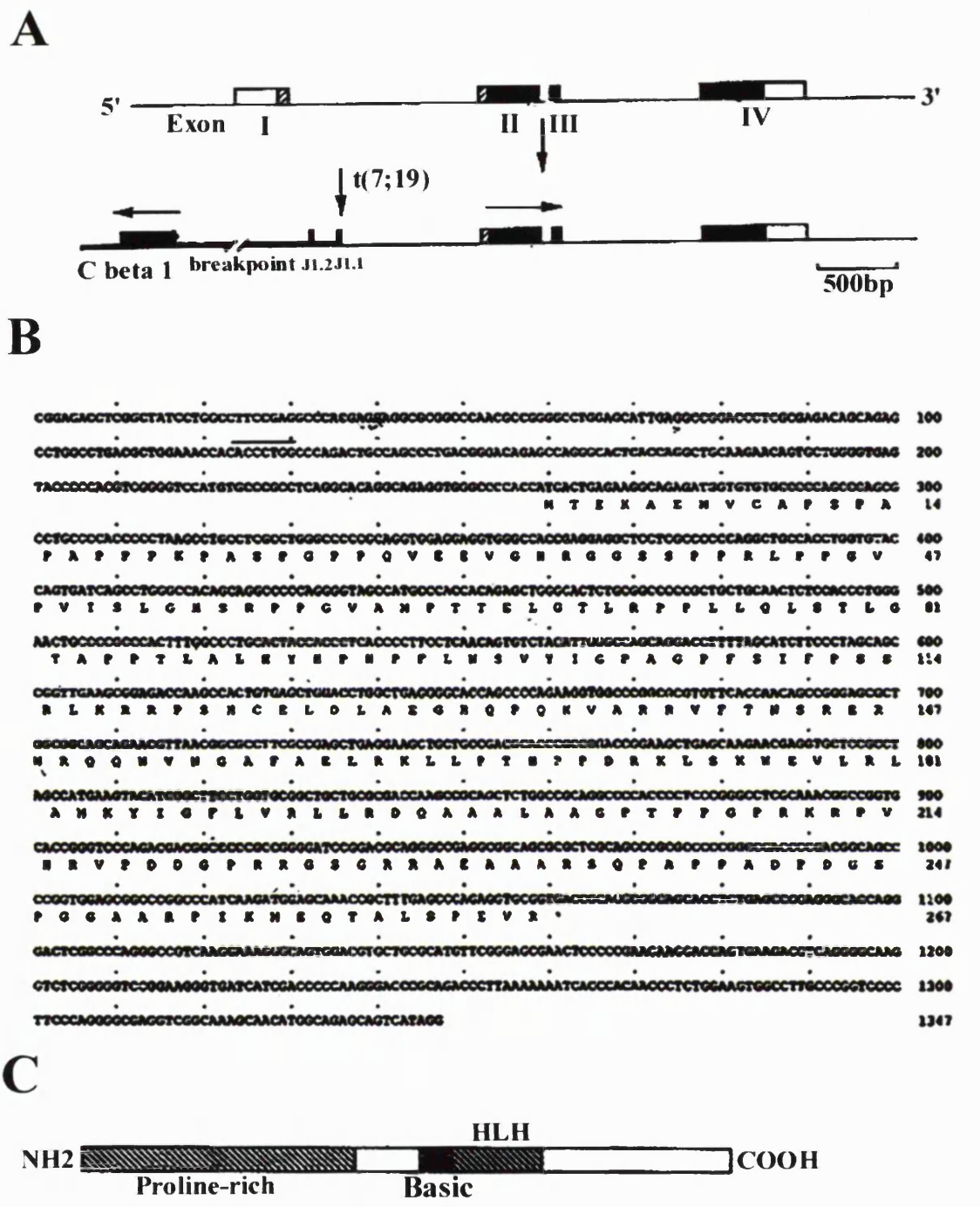


Figure G. (A) The LYL-1 gene comprises four exons (solid black regions are protein coding regions, and hatch regions are alternative translation initiation sequences from the 5' CTG codon). (B) The nucleotide sequence and predicted amino acid sequence; the overline shows the CTG codon favoured for translation initiation. (C) Schematic representation of LYL-1. The proline-rich,basic DNA-binding, and helix-loop-helix regions of the protein are indicated in boxes (Mellentin, et al., 1989).

LYL-1 gene, with the ATG at 1654 being the most favourable site (Kozak, 1986). Although the t(7;19) translocation harbored by the SUP-T7 cell line leads to loss of the LYL-1 promoter and transcription start site (Cleary, et al, 1988), cryptic promoter sites in intron 1 allow transcription of truncated mRNA molecules that do not hybridise with 5' exon probes as do the full-length 1.5kb mRNA transcripts synthesized from the intact human LYL-1 locus (Mellentin, et al, 1989).

In the mouse the expression of LYL-1 was complex (Visvader, et al, 1991). Screening of a mouse macrophage cDNA library with oligonucleotides specific for the HLH motif identified four clones with different 5' un-translated and 5' coding regions. LYL-1 mRNA expression was found to be regulated differentially in different haematopoietic cell types and lead to the generation of two size classes of transcript with distinct 5' un-translated regions. The smaller class size of between 1.5kb and 1.8kb was expressed in erythroid, macrophage and myeloid cell lines whereas the higher molecular weight transcript of between 2.0kb and 2.3kb was found in B-lymphoid and some myeloid cell lines. Furthermore, the difference in size was found to be due to variation at the 5' terminus of the LYL-1 transcripts with the 1.5kb to 1.8kb class consisting of a shorter 5' terminus. These differences in gene products reflects either alternative promoter usage or differential splicing of the LYL-1 gene, and that different haematopoietic cell types can use distinct 5' exons to process LYL-1 proteins with diverse structure. To this end it is interesting that the SUP-T7 cell line can express a 4kd amino terminal truncated 29kd human LYL-1 protein but not the predicted full-length 33kd protein (the 29kd protein comprises an intact, and presumably functional bHLH domain), and that the 1.5-1.8kb LYL-1 mRNA specie may encode a N-terminally truncated LYL-1 protein. LYL-1, like other bHLH proteins, possesses a transactivation domain at the N-terminal.

Section 1.2.2 The LYL-1 protein.

The LYL-1 protein is a member of the basic helix-loop-helix (bHLH) family of transcriptional regulators (Mellentin, et al, 1989; Massari and Murre, 2000). These proteins have been shown to be involved in important cellular activities such as differentiation and determination (Davis, et al, 1987; Murre, et al, 1989), gene regulation (Massari and Murre, 2000; Weintraub, 1993) and oncogenesis (Begley and Green, 1999; Bain, et al, 1997; Robb and Begley, 1997; Engel and Murre, 1999; Cleary, et al, 1988). bHLH proteins share a sequence motif comprising a region of hydrophilic (often basic) amino acid residues immediately adjacent to two amphipathic helices separated by an intervening loop (Murre, et al., 1989a). Dimerisation of LYL-1, as for all bHLH proteins, is mediated through association of the hydrophobic residues of the corresponding faces of the two helices (Murre, et al, 1989; Davis and Halazonetis, 1993; Murre, et al, 1994). bHLH protein dimers recognize the short palindromic sequence CANNTG, known as the E-box, such that each protein in the dimer contributes one half of the binding site. Therefore, DNA binding specificity resides in the combination of the two basic domains in the dimer (Massari and Murre, 2000).

LYL-1 is a class II bHLH protein that has been shown to form heterodimers, both in-vitro and in-vivo, with E2A proteins (Miyamoto, et al, 1996) that bind preferentially to the DNA sequence 5'-AACAGATGT(T/g)T-3'. The 3' ATGTT comprises the LYL-1 half site and displays a strong preference for A at position 6 of the overall consensus. The dimerisation, and DNA-binding features of LYL-1 was shared by another class II bHLH protein stem cell leukemia protein (SCL), which bound E47 and had a similar DNA-binding site to that of LYL-1 (Hsu, et al, 1994a; Hsu, et al., 1994b). Indeed, LYL-1 and SCL are unique amongst HLH proteins in preferring the AT to the CG base pair (Blackwell and Weintraub, 1990;

Blackwell, et al, 1993). LYL-1 was also reported to form heterodimers with HEB that bound the same site as LYL-1-E2A heterodimers (Miyamoto, et al, 1996).

LYL-1 has also been shown to interact with motifs present in the NFκB1 precursor p105 (Ferrier, et al, 1999). A yeast two-hybrid screen of a cDNA library derived from Jurkat T cells and transfection experiments showed that the HLH domain of LYL-1 interacts with the C-terminal ankyrin –like repeats of p105. A minority of the LYL-1 was bound to p105, which was postulated to be the result of transient interaction within the nuclear compartment (p105 being retained in the cytoplasm). Dysfunction of the NF-κB system is associated with T-cell neoplasia (Gilmore, et al, 1996), and over-expression of LYL-1, but not SCL, in Jurkat T-cells decreased NF-κB-dependent transcriptional activity (Ferrier, et al, 1999). A relationship between de-regulated expression of LYL-1 and dysfunctional NF-κB activity, although not proven experimentally, may provide a potential mechanism for oncogenic transformations in T-cells mediated by LYL-1. The N-terminus of the LYL-1 protein contains a proline rich region (Figure G), which in c-myc and ATF/ CRBP transcription factors has been shown to have a role in transactivation (Schwab, 1989; Mermod, et al, 1989). When fused to the heterologous DNA binding domain GAL4, LYL-1 did not display activator properties (Miyamoto, et al, 1996). However, the accuracy of this assay in determining the activity of this domain on promoters *in vivo* has not be examined experimentally and therefore its role in LYL-1 mediated transactivation remains to be elucidated.

The amino acid sequence of the LYL-1 protein shares 78% overall identity with it's human counterpart (Visvader, et al, 1991; Kuo, et al, 1991) and possess an identical bHLH domain except for a conservative amino acid substitution in the loop region. The LYL-1 protein also

lacks a single amino acid residue in the N- and C- termini leaving it two amino acids shorter than human LYL-1.

Although the role of LYL-1 protein in the oncogenic transformation of T-cells has not been defined, as a consequence of the translocation, it's de-regulated expression may alter the precise balance of transcriptional regulators in cells, and thereby mediate T-cell leukaemogenesis. The similarity of the SCL and LYL-1 bHLH domains, and almost identical DNA-binding sites, in conjunction with the known effect of other HLH proteins such as c-myc on cellular proliferation suggests a role for LYL-1 within the haematopoietic cells.

Section 1.2.3 Expression of LYL-1.

The association of LYL-1 with chromosomal abnormalities in T-ALL suggested a role for this bHLH protein in the regulation of haematopoiesis. Northern hybridisation analysis of mouse tissues indicated that LYL-1 was found in the spleen, but was un-detectable in thymus, liver, lung, brain and kidney (Kuo, et al, 1991). LYL-1 was not detected in T-lymphoma and non-leukaemic T-cell lines, or in stimulated splenic T-cells (Visvader, et al, 1991). However, a low level of LYL-1 mRNA was identified in the thymus (presumably due to resting T-cells in this organ) whereas high levels were found in splenic B-cells and mesenteric lymph nodes (Visvader, et al, 1991). These studies indicated a role for LYL-1 expression in population of cells in the spleen. Further northern blot analysis of a range of haematopoietic cell lines showed LYL-1 expression in non-lymphoid as well as in B-lymphoma cell lines (Visvader, et al, 1991). A range of myeloid, macrophage, mast and erythroid cell lines expressed LYL-1 mRNA along with high levels of expression in immature B-cell lines (Visvader, et al, 1991; Kuo, et al, 1991). Interestingly, the level of LYL-1 mRNA appeared to decrease in

plasmacytoma cells indicating that LYL-1expression was down regulated upon terminal B-cell differentiation.

Hybridisation of polyA PCR-amplified cDNAs from single normal explant marrow progenitor cells harvested from colony starts grown under growth factor permissive conditions showed a highly restricted pattern of LYL-1 expression compared to that of SCL, E12/ E47 and Id1 (Quesensberry, et al, 1996). One neutrophil progenitor and several neutrophil/ mast and erythroid/ megakaryocytic progenitors expressed LYL-1 mRNA whereas expression was absent in tri-lineage progenitors. This was in contrast to SCL expression in tri-, bi-, and uni-lineage progenitors and E12/E47 expression in tri-, bi-, and most uni-lineage progenitor cells (Quesensberry, et al, 1996). In the presence of cytokines LYL-1 expression decreased gradually in synchronised FDC-P1 cells during progression from G1 into S phase, while in the absence of cytokines LYL-1 mRNA expression fluctuated through the cell cycle and had decreased at 12hr post-feed, indicating that cytokines have little effect on LYL-1 mRNA expression.

The study of Quesensberry (Quesensberry, et al., 1996) suggested a role for LYL-1 in the development of lymphoid cells and committed haematopoietic progenitors. The restricted pattern, and complex regulation of LYL-1 expression is indicative of a role in the development of haematopoietic cells but does not elucidate the function of LYL-1 within these cells e.g. its role in regulating proliferation, differentiation and apoptosis. To this end, studies on the function of the LYL-1 protein have lagged behind its somewhat richer cousin SCL, so our understanding of LYL-1 function is limited comparatively. The functions of SCL may elucidate the function of LYL-1 in haematolymphoid cells. However, since LYL-1 cannot compensate for loss of SCL (Robb, et al, 1995a; Shivdasani, et al, 1995), LYL-1 and

SCL may possess unique, non-overlapping functions similar to those described for the myogenic-specific bHLH proteins (Weintraub, 1993).

Section 1.2.4 Function of LYL-1.

The LYL-1 gene was targeted by homologous recombination in ES cells (Rupping, E., Ph.D. thesis). The bHLH motif, and entire 3' non-coding region was replaced by a lacZ/neomycin reporter gene cassette (Figure H). This allowed the expression pattern of LYL-1 to be followed by the β-galactosidase function of the LYL-1^{lacZ} fusion protein (Rupping, E., Ph.D. thesis).

LYL-1^{lacZ} was expressed in the blood islands of the yolk sac, endothelial cells of blood vessels, and in mesenchyme of the hindbrain at 8.5dpc. At 10.5dpc, LYL-1^{lacZ} expression was high in the endothelial cells of the dorsal aorta and aortic arch arteries, and could be seen also in the fetal liver. Sections of 14.5dpc fetal liver stained with X-GAL showed LYL-1^{lacZ} expression in megakaryocytes and presumed pro-myelocytes. Both megakaryocytes and c-Kit^{+ve} cells from bone marrow were found to express LYL-1^{lacZ}. With the exception of a few LYL-1^{lacZ}-positive cells in the subcapsular region of the thymus, LYL-1^{lacZ} expression was not identified in any other cell type, including the spleen. Although this study did not show LYL-1^{lacZ} expression in spleen cells, LYL-1 mRNA expression in the spleen had been shown in previous studies (Kuo, et al, 1991;Visvader, et al, 1991). Further studies showed that the number of platelets; the development, and number of bone marrow and splenic T and B-lymphocytes, and the growth of bone marrow-derived CFU-GM and CFU-mixed colonies were normal in LYL-$1^{-/-}$ mice. X-GAL staining of LYL-$1^{+/-}$ and LYL-$1^{-/-}$ embryos showed that loss of LYL-1 had no obvious effect on development.

It was concluded that a functional LYL-1 protein was not required for normal development of the haematopoietic or endothelial system.

Section 1.3 Aim of the study.

The lack of an obvious phenotype in LYL-1$^{-/-}$ mice raises several issues regarding the role of LYL-1 in haematopoiesis. The loss of a functional LYL-1 protein in haematopoietic cells in the yolk sac, fetal liver, and bone marrow suggests that this protein has a redundant role in the formation of haematopoietic cells. However, it is evident that bHLH proteins act in a combinatorial manner (Murre, et al, 1989a; Hu, et al., 1992; Bockamp, et al., 1994), and can affect transcription in different ways depending on the choice of heterodimer (Ma, et al., 1999; Zhuang, et al., 1996), and the specific combinations of transcription factor binding sites (Bain, et al., 1993; Wadman, et al., 1997; Gruntz, et al., 1998). Furthermore, ubiquitous and cell-type, or cell-stage restricted forms of bHLH proteins possess both distinct and overlapping functions that determine cell-specific patterns of gene expression (Weintraub, 1993; Amati and Land, 1994; Bockamp, et al, 1994; Zhuang, et al, 1996). In the case of MyoD, a muscle-specific bHLH protein, the lack of effect on skeletal muscle cell development in MyoD$^{-/-}$ mutant mice was associated with upregulation of Myf5 (Rudnicki, et al., 1992). Gene targeting experiments have shown that MyoD, Myf5, and the related bHLH proteins myogenin and MRF4 have overlapping functions in skeletal muscle development (Rudnicki, et al, 1993; Braun, et al, 1992; Weintraub, 1993). A quantitative neurogenic phenotype was absent in neurogenin2 (Ngn2) single mutant mice due to compensation by Mash1, which was up-regulated in Ngn-2 mutant cortical progenitors (Fode, et al, 2000). Therefore, it is possible that the role of LYL-1 could be compensated via mechanisms similar to those operating for skeletal muscle-, and neuronal-specific bHLH proteins. Interestingly, up-regulation of GATA-2 in erythroid precursors generated by in-vitro differentiation of GATA-1$^{-/-}$ ES cells (Weiss, et al., 1994; Weiss and Orkin, 1995) may allow erythroid progenitors to develop into proerythroblasts in the yolk sac of GATA-1$^{-/-}$ mutant mice (Fujiwara, et al., 1996). This suggests that changes in the level of expression of related

transcriptional regulators may result in functional compensation of haematopoietic-specific transcription factors.

It may be that the effect of disrupting the LYL-1 gene was subtle. For example, disruption of the murine β-globin locus control region (LCR) 5' hypersensitive 3 (HS3) had a minimal effect in yolk-sac derived erythroid cells, and reduced β^{maj} and β^{min} expression by 30% in adult erythrocytes (Hug, et al., 1996). Loss of Ngn2 changed neuronal identity without affecting neuronal progenitor number, organization, or proliferative properties (Fode, et al., 2000). Small changes in haematopoietic cell development and/ or function may occur as a consequence of LYL-1 loss rather than overt defects

With these considerations in mind, we proposed the following hypothesis. The absence of an obvious phenotype in the LYL-$1^{-/-}$ mice was the result of functional compensation through altered expression of another gene. Since LYL-1 and SCL constitute a subfamily of bHLH proteins, which may regulate haematopoiesis by recognition of a common group of target genes (Baer, 1993), SCL was considered an appropriate candidate.

Chapter 2. Methods and Materials.

Section 2.1 Histology and histochemistry.

Section 2.1.1 Prelude.

Microscopic analysis was performed using an Axiophot 2 MOT microscope and Axiocam digital camera with supporting AxioVision imaging software (Carl Zeiss). Animals were handled in accordance with the terms of the Personal License and local guidelines.

Section 2.1.2 Genotyping of LYL-1 mice.

LYL-1 mice were genotyped by PCR amplification, and Southern hybridisation of EcoRV digested genomic DNA. Genomic DNA was extraction and purified from the tail of LYL-1+/+, LYL+/-, and LYL-/- mice.

Section 2.1.3 Extraction and purification of genomic DNA.

Approximately 0.3cm of mouse tail was incubated for at least 6 hours at 55^0C whilst shaking in 0.7mL of Lysis buffer (see Section 2.12.9) comprising 50mM Tris-HCl (pH 8), 100mM Na_2EDTA, 100mM NaCl, 0.2% SDS and 0.5mgmL^{-1} Proteinase K. After incubation the lysate was centrifuged at 14,000 rpm for 15 minutes to pellet un-digested tissue, and the supernatant removed to a clean microcentrifuge tube. 20µl of a 13µgmL^{-1} RNAse A solution was added to the sample followed by a 2 hour incubation at 37^0C. The samples were then mixed with an equal volume of isopropanol and centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed so not to disturb the genomic DNA pellet and replaced with 1mL of 70% ethanol followed by centrifugation at 14,000 rpm for 10 minutes. The 70% ethanol was removed by aspiration and the pellet allowed to air dry thoroughly. The pellet of genomic DNA was dissolved in 50-100µl of sterile H_2O overnight at 37^0C, and 1µl used to assess both concentration and integrity by ethidium bromide (0.5µgmL^{-1}) stained agarose gel

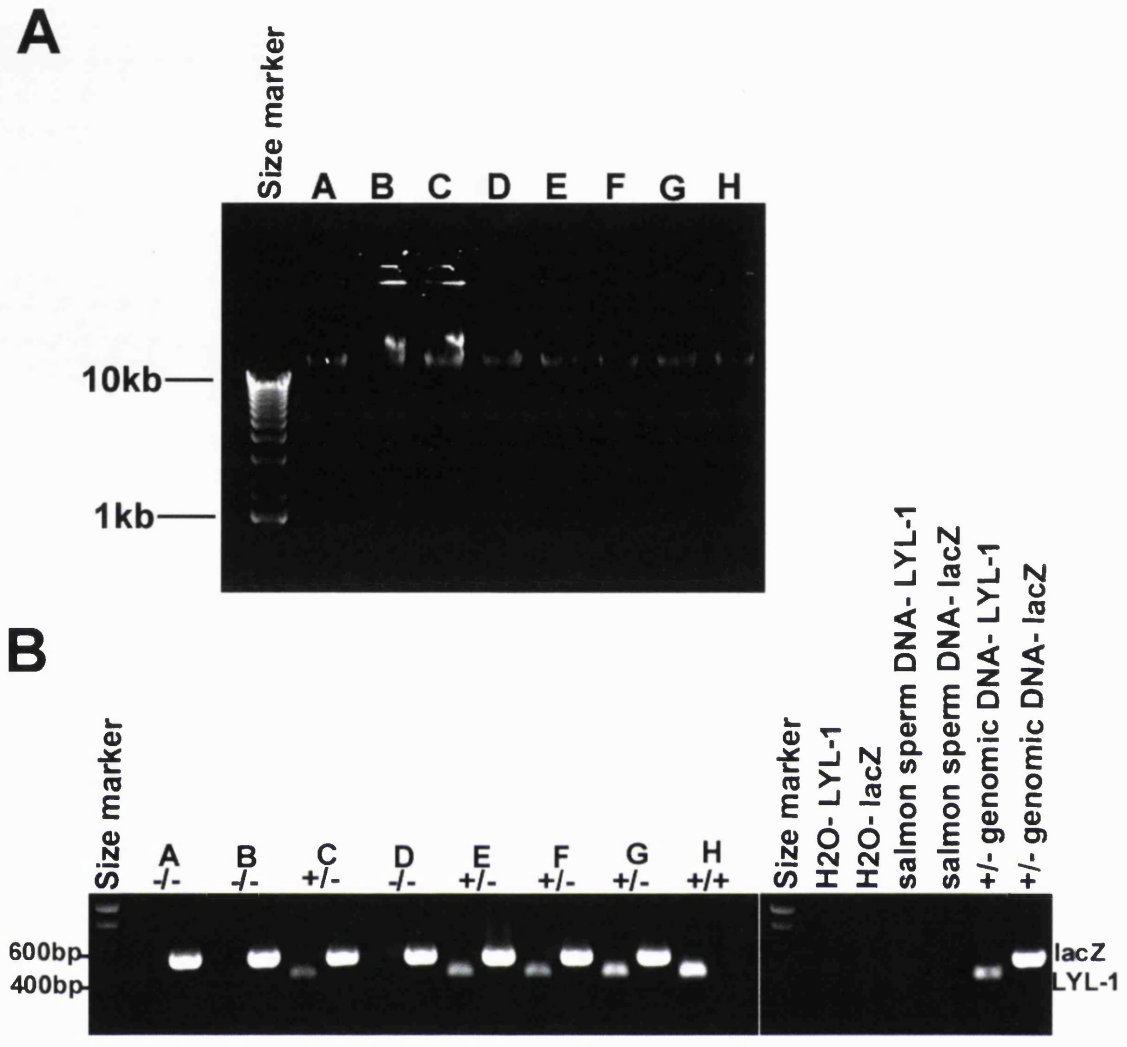
(0.8%/ 1X TBE (pH 8.3) electrophoresis (Figure 1A). The concentration of genomic DNA was also be determined by preparing a 1:50 dilution of the genomic DNA and measuring the OD at 260nm.

Approximately 500ng of genomic DNA was used in LYL-1, and lacZ-specific PCR reactions (see Tables 1, 2, and 3). Figure 1B shows that a LYL-1 cDNA fragment of 0.391kb, and a 0.5kb lacZ fragment was generated by PCR amplification of genomic DNA.

Section 2.1.4 Preparation of high purity genomic DNA.

The genomic DNA used in Southern hybridisation was extracted by mixing the tail lysate (after centrifugation to remove debris-see Section 2.1.3) with an equal volume of phenol (pH8) (Sigma, Cat No- P4557) for 60 minutes at room temperature by continual rotation. The sample was then centrifuged at 14,000rpm for 15 minutes to separate the phases, and the upper aqueous phase removed to a clean microcentrifuge tube containing an equal volume of phenol: chloroform (Sigma, Cat No- P2069). The sample was mixed by rotation for 45 minutes, centrifuged at 14,000rpm for 15 minutes, and the aqueous phase removed to a tube containing an equal volume of chloroform (BDH, Cat No- 100776B). The sample was mixed for a further 30 minutes and, following centrifugation at 14,000rpm for 15 minutes, the aqueous phase, containing the purified genomic DNA, was added to 0.6 volumes of isopropanol (BDH, Cat No- 102244J) and centrifuged at 14,000rpm for 15 minutes to precipitate the genomic DNA. The isopropanol was removed by aspiration and replaced with 1mL of 70% ethanol. The sample was vortexed for a few seconds and the sample centrifuged at 14,000rpm for 10 minutes. The ethanol was removed and the pellets allowed dried completely. The genomic DNA was incubated overnight at 37^0C in 25-50μL of sterile H_2O.

Figure 1. Genomic DNA (A) (see Section 2.1.3) was used in LYL-1-specific, and lacZ-specific PCR to routinely genotype LYL-1 mice (see Table 2). A 0.39kb LYL-1 fragment and, and a 0.5kb lacZ fragment (B) was generated with specific primers (Tables 3) and thermal profiles (Table 4). For establishment of LYL-1 mouse colonies, high purity genomic DNA (see Section 2.1.4) was digested with EcoRV, separated by ethidium bromide stained agarose gel electrophoresis, transferred to hybridisation membrane, and hybridised with an α^{32}P-dCTP labeled 2.6kb SalI-EcoR1 (C). Wildtype LYL-1 loci generate a 10kb fragment whereas targeted LYL-1 loci generated a 4.6kb fragment.


A
Size marker
A B C D E F G H
10kb
1kb
B
Size marker
A -/-
B -/-
C +/-
D -/-
E +/-
F +/-
G +/-
H +/+
Size marker
H2O- LYL-1
H2O- lacZ
salmon sperm DNA- LYL-1
salmon sperm DNA- lacZ
+/- genomic DNA- LYL-1
+/- genomic DNA- lacZ
600bp
400bp
lacZ
LYL-1
C
Targeting vector
EcoRV
Sal I EcoR I
EcoRV
LYL-1
lacZ
Neo
LYL-1 locus
EcoRV
Sal I EcoR I EcoRV
10kb
Targeted LYL-1 locus
EcoRV
EcoRV
EcoRV Sal I EcoR I
LYL-1
lacZ
Neo
Sal I-EcoR I
4.6kb
+/- -/- +/- +/- +/+
10kb
4.6kb

The concentration, and integrity of the genomic DNA was determined by agarose gel electrophoresis, and spectrometry (as described in section 2.1.3).

Approximately 500μg to 1mg of genomic DNA (20μl of a 25-50μgμl^{-1}) was incubated for 18-24 hours at 37^{0}C with an EcoRV digestion mixture comprising 3μl of a 10X BSA (fraction V), 3μl of 10X reaction buffer, 2μl of EcoRV (40U/μl) and 2μl of sterile H_2O to give a total volume of 30μl. After incubation the samples were separated by agarose (0.8% / 1X TBE) gel electrophoresis. After electrophoresis the gel was submerged in 0.25M HCl and shaken gently until the bromophenol blue has changed colour (approximately 15 minutes). The gel was then transferred to 0.4M NaOH and shaken gently until the bromophenol blue colour had returned. The gel was prepared for alkali capillary transfer to Hybond N+ nylon transfer membrane (Amersham) overnight at room temperature. The membrane was hybridised with a radiolabeled (see Sections 2.8.6 to 2.8.8) 2.6kb SalI-EcoRI probe and then exposed to X-ray film. Figure 1C shows the results of southern hybridisation of EcoRV digested genomic DNA from LYL-1$^{+/+}$, LYL$^{+/-}$, and LYL$^{-/-}$ mice. A 10kb fragment was obtained from a wildtype LYL-1 locus, whereas a targeted LYL-1 generated a fragment of 4.6kb (refer to Figure G).

Section 2.1.5 Fixation of tissues.

Tissues were dissected and rinsed immediately in ice cold 1X PBS (pH 7.5) and placed directly into 4% paraformaldehyde/ 1X PBS (pH 7.5) (see Sections 2.2.1 and 2.2.2) at 4^{0}C. Tissues were cut e.g. brain and liver, or perforated with a needled (271/2 gauge) syringe (1mL) e.g. spleen and thymus. Samples of spleen were also fixed in Helly's fluid (see Section 2.2.3) at room temperature. The volume of fixative exceeded the specimen volume by approximately 10 times, and incubation was for 12 to 24 hours. Samples of tissue fixed in 4%

paraformaldehyde/ 1X PBS (pH 7.5) were rinsed in 1X PBS (pH 7.5), and processed for histological analysis as either frozen or paraffin wax-embedded specimens. Samples of spleen fixed in Helly's fluid were washed in running H_2O for 12 to 24 hours and processed as paraffin wax-embedded specimens.

Section 2.1.6 Cytocentrifugation of bone marrow, spleen, and peripheral blood cells.

Peripheral blood cells were collected into 0.5mL microcentrifuge tubes containing 250µL of 1.5mgmL^{-1} K_2EDTA in 1X PBS (pH7.5). Spleen and bone marrow (flushed from the femur and tibia of both hind legs) cells were collected into 1X PBS (pH7.5). Spleen, bone marrow, and peripheral blood cells were diluted to 1:50 and 1:100 in 3% acetic acid in H_2O (see Section 2.2.11), and the number of nucleated cells counted on a haematocytometer (Neubauer). Cells were diluted to 1x10^6, 1x10^5, and 1x10^4 in 1X PBS (pH7.5) and centrifuged at 1500*g* for 1 minute onto poly-L-lysine treated glass microscope slides (Sigma, Cat No- P0425) in a Megafuge 1.0 (Heraeus Sepatech D37520). Excess solution was blotted onto filter paper, and the cytocentrifuged cells and allowed to air dry.

Section 2.1.7 Paraffin wax embedding of tissue.

The tissues were dehydrated by incubation in 70% ethanol for 5 hours, 90% ethanol for 12 hours and absolute ethanol for 1 and 2 hours with 1 change of absolute ethanol at room temperature. Following dehydration, specimens were incubated in Clearing solution (see Section 2.2.6) for 12 to 24 hours at room temperature. Specimens were removed from the Clearing solution and incubated in toluene (BDH, Cat No-30454 5Q) for 10 minutes. This was followed by a 2 hour incubation in liquid paraffin wax (Sigma, Cat No- A6630), followed by a further 2 hours in liquid paraffin wax under vacuum. Each specimen was

orientated appropriately in a mould filled with liquid paraffin wax, which was then solidified on a cold plate. 5μm serial sections were cut with a microtome (Leica DCS1), and placed on a drop of sterile H_2O on poly-lysine treated glass slides at 45^0C. The sections were dried overnight at 45^0C and stored in a dry, dust-free slide rack, protected from light, at room temperature until use.

Section 2.1.8 Cryoprotection and freezing of tissue.

Specimens were blotted to remove excess fixative and placed in a Falcon tube containing 50mL of 30% sucrose/ 1X PBS (pH 7.5) containing 2mM $MgCl_2$ (see Section 2.2.5) at 4^0C. The specimens were allowed to sink to the bottom of the Falcon tube. The cryo-protected specimens were placed into a mould filled with O.C.T. compound (BDH- Cat No- 361603E) and orientated appropriately. The mould was lowered into isopentane (BDH, Cat No-103616V) cooled in liquid nitrogen until the O.C.T. solidified. Specimens were stored at -80^0C. Alternatively, specimens were frozen by a crushed dry ice and, immediately prior to sectioning, mounted in O.C.T. and orientated appropriately. 10μm sections were cut on a cryostat (Leica, CM1850) at -20^0C, mounted onto poly-lysine treated microscope slides and stored at -20^0C until use.

Section 2.1.9 Preparation of tissue sections and cytocentrifuged cells for histological analysis.

5μm paraformaldehyde-fixed paraffin sections were de-waxed in xylene (BDH, Cat No-360716Y) for 5 minutes, and re-hydrated through absolute, 90%, 70% ethanol (see Section 2.2.17) and sterile H_2O for 5 minutes each.

Sections from spleen fixed in Helly's fluid were bleached in order to remove mercury pigment. Each section was incubated in approximately 1mL of Lugol's solution (Sigma, Cat No- L6146) for 2 minutes. The Lugol's solution was drained from each section, and replaced by 1mL of 5% sodium thiosulphate (Sigma, Cat No- S1648) for 5 minutes. The sections were washed in running H_2O for 5 minutes and then equilibrated in sterile H_2O or 1X PBS (pH 7.5). Cryosections were washed with 3 changes of sterile H_2O for 10 minutes each. Cytocentrifuged bone marrow, spleen and peripheral blood cells were fixed in methanol/ glacial acetic acid (see Section 2.2.10) for 1 minute and washed 3 times in sterile H_2O for 5 minutes each. After analysis, sections to be mounted in DPX (BDH, Cat No- 360294H) were dehydrated through 70%, 90% and absolute ethanol and cleared in xylene for 1 minute each, whereas sections to be processed in an aqueous mountant were washed in 3 changes of sterile H_2O for 1 minute each.

Section 2.1.10 TUNEL assay for apoptotic cells.

Apoptotic cells were detected using the principal of TUNEL (Terminal Deoxynucleotidyl Transferase (TdT)-mediated fluorescein-12-dUTP Nick End Labeling) with the Apoptosis Detection System, Fluorescein (Promega, Cat No- G3250). De-waxed and re-hydrated 5μm paraformaldehyde fixed paraffin sections (see Section 2.1.7) were equilibrated in 1X PBS (pH 7.5) for 5 minutes and then post-fixed in 4% paraformaldehyde/ 1X PBS (pH 7.5) for 10 minutes at room temperature, followed by incubation in 2 changes of sterile H_2O, and once in 1X PBS (pH 7.5), for 10 minutes each. 100μL of Proteinase K ($20\mu gmL^{-1}$) (see Sections 2.6.2 and 2.6.3) was added to each section for 10 minutes at room temperature followed by 3 changes of 1X PBS (pH 7.5), for 10 minutes each, and a further post-fixation in 4% paraformaldehyde/ 1X PBS (pH 7.5) for 5 minutes at room temperature.

The excess fixative was removed by immersing the sections in 1X PBS (pH 7.5) for 10 minutes. The sections were incubated in 100µL of Equilibration Buffer for 10 minutes, and then in 50µL of reaction mixture comprising 45µL Equilibration Buffer, 5µL Nucleotide mix, and 1µL TdT, for 1 hour at 37^0C. Following incubation in reaction mixture, the sections were washed in 2X SSC (se Section 2.12.2), and immersed in a solution of propidium iodide (PI)/ 1X PBS (pH7.5) for 15 minutes at room temperature. After nuclear staining in PI solution, the sections were washed in sterile H_2O 3 times for 5 minutes each. A drop of anti-fade solution (Molecular Probes, Cat No- S7461) was placed on each section, and covered with a glass coverslip (ESCO, Cat No- 92600). Apoptotic cells were identified by fluorescence microscopy using FITC, and rhodamine filter settings on an Axiophot 2 MOT microscope and Axiocam digital camera with supporting AxioVision imaging software (Carl Zeiss).

Section 2.1.11 May-Grunwald and Giemsa stain of haematopoietic cells in sections of spleen.

Paraformaldehyde and mercuric-fixed spleen sections (see Section 2.1.6) were placed directly into May-Grunwald solution (BDH, Cat No- 352065W) for 5 minutes. An equal volume of buffered H_2O (pH 6.8) (see Section 2.2.16) was added to the staining solution and the sections incubated for a further 5 minutes, after which the sections were transferred directly to Giemsa solution (BDH, Cat No- 350865P) and incubated for 15 minutes. Sections were then washed in sterile H_2O for 1 minute and mounted in DPX.

Section 2.1.12 May-Grunwald and Giemsa stain of cytocentrifuged bone marrow, and peripheral blood cells.

Cytocentrifuged bone marrow, spleen and peripheral blood cells were immersed in May-Grunwald solution (BDH, Cat No-352065W) for 3 to 5 minutes. An equal volume of buffered

H_2O (pH 7.3) (see Section 2.2.16) was added to the May-Grunwald solution and the slides incubated for a further 5 minutes. Slides were then transferred directly to Giemsa solution (BDH, Cat No- 350865P) and incubated for 15 minutes. Excess Giemsa solution was removed by a single wash in sterile H_2O for 1 minute and allowed to air dry.

Section 2.1.13 Perl's Prussian blue stain for ferric iron in sections of spleen and cytocentrifuged bone marrow cells.

Spleen sections, and cytocentrifuged bone marrow cells were immersed in a solution of 5% $K_4Fe(CN)_6$ and 5% HCl in equal volumes (preheated in a microwave at 750W for 30 seconds- see Section 2.2.12) for 5 minutes. The sections were then rinsed with 3 changes of sterile H_2O for 5 minutes each and counterstained in 1% neutral red for 2 to 3 minutes (see Section 2.2.14). Following the counter-stain, sections were washed in sterile H_2O for 1 minute, and mounted in DPX. Blue haemosiderin deposits, indicating, were examined microscopically.

Section 2.1.14 β-galactosidase assay in cryosections of tissue.

Cryosections (see Sections 2.1.5 and 2.1.6) were equilibrated in Rinse buffer (pH 7.6) (see Section 2.2.7) with no X-GAL at 37^0C for 1 hour. Cryosections were then incubated in X-GAL staining solution (see Section 2.2.8) containing X-GAL (Eurogentec, Cat No- OP-0020-10) at $1 mgmL^{-1}$ in dimethyformamide (DMF) (BDH, Cat No- 103224J), for between 2 and 18 hours at 37^0C. After incubation, the sections were washed in 3 changes of sterile H_2O and counterstained with either 1% neutral red or eosin Y (see Sections 2.2.14 and 2.2.15) solution for 2 to 3 minutes. Sections were then rinsed in sterile H_2O for 1 minute and mounted in DPX.

Section 2.1.15 1,2-diaminofluorene (DAF) stain of haemoglobin in cytocentrifuged bone marrow and spleen cells and sections of spleen.

A solution comprising 0.1% 1,2-DAF in 0.2M Tris (pH 7) and 0.3% H_2O_2 was prepared immediately prior to use (see Section 2.2.13). Cytocentrifuged bone marrow and spleen cells ($1x10^6$ cells) or 10μm cryosections of spleen were immersed in the DAF solution for between 2 and 4 minutes at room temperature. The DAF solution was drained from each slide and the samples dried completely. Cells that contained haemoglobin were blue in colour.

Section 2.2 Materials used in histology and histochemistry.

Section 2.2.1 10X Phosphate buffered saline (PBS).

COMPOSITION	AMOUNT
NaCl	80g
KCl	2g
Na_2HPO_4	14.4g
KH_2PO_4	2.4g
H_2O	1000mL

80g of NaCl (Fisher, Cat No- S/ 3160/ 63), 2g of KCl (Sigma, Cat No-), 14.4g of Na_2HPO_4 (Sigma, Cat No-S7907), and 2.4g KH_2PO_4 (Sigma, Cat No- P5655) were dissolved in 900mL of sterile H_2O and the pH adjusted to 7.5 with dilute HCl. The solution was autoclaved and used at a working concentration of 1X. This solution was stored at room temperature for up to 6 months.

Section 2.2.2 4% paraformaldehyde solution in 1X PBS (pH7.5).

COMPOSITION	AMOUNT
Paraformaldehyde	4g
10X PBS (pH7.5)	10mL
$MgCl_2$	0.2mL of 1M
Na_2EDTA	0.25mL of 0.5M
DEPC- treated H_2O	100mL

4g of paraformaldehyde powder (Sigma, Cat No-P6148) was added to 80mL of DEPC-treated H_2O (see Section 2.6.1) and heated to 65^0C until paraformaldehyde had dissolved. 1-2 drops of 10M NaOH (Fisher, Cat No- S/4880/53) facilitated solubilisation of the paraformaldehyde. Once cooled to room temperature, 10mL of 10X PBS (pH7.5) was added to the solution and the pH adjusted to 7.5 with dilute HCl. 0.2mL of 1M $MgCl_2$ and 0.25mL of 0.5M Na_2EDTA was added to the solution to give 2mM and 1.25mM final concentrations respectively. The volume was adjusted to 100mL with DEPC- treated H_2O and stored at 4^0C for up to 2 weeks.

Section 2.2.3 Helly's fluid.

COMPOSITION	AMOUNT
Mercuric chloride	5g
Potassium dichromate	2.5g
Sodium sulphate	1g
10% Formalin (pH7)	5mL
Sterile H_2O	100mL

5g mercuric chloride (Sigma, Cat No- M6529), 2.5g potassium dichromate (Sigma, Cat No- P6435), and 1g of sodium sulphate (Sigma, Cat No- 6547) were added to 100mL of sterile H_2O. Immediately prior to use, 10mL of buffered 10% Formalin (see Section 2.2.4) was added to the fixative. The solution (minus 10% Formalin) was stored at room temperature for up to 4 months.

Section 2.2.4 Buffered 10% formalin (pH7).

COMPOSITION	**AMOUNT**
37% paraformaldehyde solution	100mL
acidic $NaH_2PO_4.H_20$	4g
anhydrous Na_2HPO_4	6.5g
Sterile H_2O	900mL

4g of acidic sodium phosphate (Sigma, Cat No- S-7907) monohydrate was added to 900mL of sterile H_2O, followed by 6.5g of anhydrous disodium phosphate (BDH, Cat No- 102454R) and 100mL of 37% paraformaldehyde solution (Sigma, Cat No- F1635). This fixative solution was stored at 4^0C for up to 2 weeks.

Section 2.2.5 30% sucrose/ 1X PBS (pH 7.5) with 2mM $MgCl_2$.

COMPOSITION	AMOUNT
Sucrose	30g
$MgCl_2$	0.2mL of 1M stock solution

PBS (p H7.5)	10mL of 10X stock solution
Sterile H_2O	89.8mL

30g of sucrose (BDH, Cat No- 102744B) were dissolved in 89.8mL of sterile H_2O. 10mL of 10X PBS (pH7.5) was added to the sucrose solution followed by 0.2mL of 1M $MgCl_2$ to give a final concentration of 2mM $MgCl_2$, and 1X PBS (p H7.5). The sucrose solution was stored at 4^0C for 1 week.

Section 2.2.6 Clearing solution.

COMPOSITION	**AMOUNT**
Benzyl alcohol	50mL
Benzyl benzoate	100mL

Clearing solution was prepared by mixing 50mL of benzyl alcohol (Sigma, Cat No- B1042) and 100mL of benzyl benzoate (Sigma, Cat No- B6630). Approximately 10 times the tissue volume of clearing solution was used and the solution stored at room temperature for 6 months.

Section 2.2.7 Rinse buffer for X-GAL staining.

COMPOSITION	**AMOUNT**
Na_2HPO_4	50mL of 1M stock solution
$MgCl_2$	1mL of 1M stock solution
Sodium deoxycholate	0.05g

NP40	0.1mL
Sterile H_2O	up to 500mL

0.5g of sodium deoxycholate (Sigma, Cat No- D5670) was added to 400mL of sterile H_2O followed by 0.1mL NP40 (BDH, Cat No- 560092L), 50mL of 1M Na_2HPO_4, and 1mL of 1M $MgCl_2$ (Merck, Cat No- S832). The volume was adjusted to 500mL with sterile H_2O to give a final concentration of 100mM Na_2HPO_4, 2mM $MgCl_2$, 0.1% sodium deoxycholate, and 0.02% NP40. Rinse buffer was stored at room temperature for 4 months.

Section 2.2.8 X-GAL staining solution.

COMPOSITION	**AMOUNT**
$K_3Fe\ CN)_6$	0.5mL of 0.5M stock solution
$K_4Fe(CN)_6$	0.5mL of 0.5M stock solution
X-GAL	2mL of 25mgmL^{-1} stock solution in DMF
Rinse Buffer	47mL

50mL of X-GAL staining solution was prepared by adding 0.5mL of 0.5M $K_3Fe\ CN)_6$ (Sigma, Cat No- P3667) and 0.5mL of 0.5M $K_4Fe(CN)_6$ (Sigma, Cat No- P3289) to 47 mL of Rinse buffer (see Section 2.2.7). Immediately before use, 2mL of 25mgmL^{-1} X-GAL in DMF was added to the solution to give a final concentration of 1mgmL^{-1} X-GAL and 5mM of $K_3Fe\ CN)_6$ and $K_4Fe(CN)_6$. X-GAL/ DMF stock solutions were stored at -20^{0}C, and $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ solutions were kept protected from light at room temperature.

Section 2.2.9 May-Grunwald and Giemsa staining solutions.

May-Grunwald staining solution (BDH, Cat No-352065W) and Giemsa staining solution (BDH, Cat No- 350865P) were used to stain for haematopoietic cells in the spleen according to a protocol modified from Sheehan's Modified May-Grunwald technique (Sheehan D, Hrapchak B, Theory and practice of Histotechnology, 2nd Ed, 1980, pp217-218, Battelle Press, Ohio). Cytocentrifuged bone marrow, spleen and peripheral blood cells were stained with May-Grunwald and Giemsa staining solutions according to the Pappenheim's panoptic technique (Atlas of Clinical Hematology, Begemann, H., and Rastetter, J, 4th edition, 1989, Springer-Verlag Berlin Heidelberg) (see Sections 2.1.11 and 2.1.12).

Section 2.2.10 Fixative solution for cytocentrifuged cells.

COMPOSITION	**AMOUNT**
Glacial acetic acid	3mL
95% methanol	100mL

5mL of sterile H_2O was mixed with 95mL of methanol (Fisher, Cat No- M/ 4000/ 17), followed by 3mL of glacial acetic acid (Fisher, Cat No- A/ 0400/ PB17). The fixative was prepared immediately prior to use.

Section 2.2.11 3% acetic acid in H_2O.

COMPOSITION	**AMOUNT**
Glacial acetic acid	3mL
Sterile H_2O	100mL

3mL of glacial acetic acid (Fisher, Cat No- A/ 0400/ PB17) was mixed with with 100mL of sterile H_2O. Nucleated cells counted on a haematocytometer were diluted to 1:50, and 1:100. This solution was prepared just prior to use.

Section 2.2.12 Perl's Prussian blue stain for ferric iron.

COMPOSITION	AMOUNT
$K_4Fe(CN)_6$	100mL of 5% stock solution
HCl	100mL of 5% stock solution

5% $K_4Fe(CN)_6$ was prepared by dissolving 5g of $K_4Fe(CN)_6$ (Sigma, Cat No- P3289) in 100mL of sterile H_2O. 5% HCl was prepared by adding 5mL of concentrated HCl (Sigma, Cat No- 920-1) to 100mL of sterile H_2O. 100mL of 5% HCl was added to 100mL of 5% $K_4Fe(CN)_6$, and the solution heated in a microwave on high power (800W) for 30 seconds.

Section 2.2.13 1,2 diaminofluorene (1,2-DAF) solution.

COMPOSITION	AMOUNT
1,2-DAF	1g
Glacial acetic acid in sterile H_2O	10mL of 90%
Tris-HCl	10mL of 0.2M stock solution
H_2O_2	0.1mL of 30% stock solution

10mL of a 1% 1,2-DAF stock solution was prepared by dissolving 0.1g of 1,2-DAF (FLUKA, Cat No- 32980) in 10mL of 90% acetic acid (1mL of glacial acetic acid and 9mL

of sterile H_2O). This 1% 1,2-DAF stock solution was stored at 4^0C for up to 1 month. A working concentration of 0.1% 1,2-DAF solution was prepared by adding 1mL of 1% 1,2-DAF to 9mL of 0.2M Tris-HCl (pH7). Immediately prior to use, 0.1mL of 30% H_2O_2 (Sigma, Cat No- H-1009) was added to 10mL of the 0.1% 1,2-DAF solution.

Section 2.2.14 1% Neutral red.

COMPOSITION	**AMOUNT**
Neutral red	1g
Sterile H_2O	100mL

1g of Neutral red (Sigma, Cat No- N6634) was dissolved in 100mL of sterile H_2O. The staining solution was stored at room temperature for 6 months.

Section 2.2.15 Eosin Y solution.

Eosin Y solution (Sigma, Cat No- HT110-2-32) was used as a cytoplasmic counter-stain for paraffin and cryosections. Sections were stained for approximately 2 minutes in the Eosin Y solution and then processed as described in section 2.1.6.

Section 2.2.16 Buffered H_2O.

Buffer tablets dissolved in 10mL of sterile H_2O produced buffered H_2O solutions of pH6.8 (BDH, Cat No- BDH 362242D), and pH7.2 (BDH, Cat No- 362232B) that were stored at room temperature.

Section 2.2.17 Preparation of 90%, and 70% ethanol.

COMPOSITION	AMOUNT	%
Absolute ethanol	450mL	
Sterile H_2O	50mL	90%
Absolute ethanol	350mL	
Sterile H_2O	150mL	70%

500mL of 90% ethanol was prepared by mixing 450mL of 99.7% ethanol (BDDH, Cat No-10107) with 50mL of sterile H_2O. Similarly, 70% ethanol was prepared by mixing 350mL of 99.7% ethanol with 150mL of sterile H_2O.

Section 2.3 Immunohistochemistry.

Section 2.3.1 Prelude.

Microscopic analysis was performed using an Axiophot 2 MOT microscope and Axiocam digital camera with supporting AxioVision imaging software (Carl Zeiss). Gene frames (65µL) (Abgene, Cat No-HB-OS-SSEZ2E) were used routinely to prevent dehydration.

Section 2.3.2 Immunohistochemical detection of target molecules in sections of spleen.

Mouse antigens were detected in 10µm cryosections, and 5µm paraformaldehyde fixed paraffin sections by immunoperoxidase assay. Cryo-, and paraffin sections were prepared as described in section 2.1.6. The sections were then incubated in target retrieval solution (DAKO), pre-heated to 95-99^{0}C (pH 6) (see Section 2.4.1) in a water bath for 20 minutes.

The solution was allowed to cool for 20 minutes at room temperature, washed in sterile H_2O 3 times for 5 minutes each, and incubated in 0.01M periodic acid for 10 minutes (see Section 2.4.2). The sections were rinsed rapidly in sterile H_2O, and then incubated in $0.1 mgmL^{-1}$ sodium borohydride for 10 minutes (see Section 2.4.3). Incubation in periodic acid and sodium borohydride inhibited endogenous peroxidase activity in the spleen cells (see Chapter 3 Results Figure 15a). The sections were washed in sterile H_2O 3 times for 5 minutes each and incubated in normal swine serum (1:20)/ 1X PBS (pH7.5) (see Section 2.4.4) at room temperature for 1 hour. The sections were then washed in 1X PBS (pH7.5) 3 times for 5 minutes each, followed by incubation at 4^0C overnight, or at room temperature for 1 hour, in 1.5%^{v}/v normal swine serum/ 1X PBS (pH7.5) containing an anti-mouse primary antibody at optimal concentration (see Sections 2.4.5, and 2.4.6, see Table 1).

Sections incubated with a biotinylated anti-mouse primary antibody were washed in 1X PBS (pH7.5) 3 times for 5 minutes each (see Section 2.4.6 and Table 1), followed by incubation in $40\mu gmL^{-1}$ horseradish peroxidase (HRP)-conjugated ExtrAvidin (see Section 2.4.8) in 1X PBS (pH7.5) for 60 minutes at room temperature. Sections incubated with an un-conjugated anti-mouse primary antibody were incubated with a species-specific biotinylated secondary antibody (see Section 2.4.7) for 1 hour at room temperature immediately proceeding the wash in 1X PBS (pH7.5). This was followed by 3 washes in 1X PBS (pH7.5) for 5 minutes each and a further 1 hour incubation at room temperature with HRP-conjugated ExtrAvidin/ 1X PBS (pH7.5). After incubation, the sections were washed with 1X PBS (pH7.5) twice for 5 minutes each and once in sterile H_2O for 5 minutes before development of DAB colour.

Section 2.3.3 3,3-diaminobenzidie (DAB) colour development.

Table 1. Concentration antibodies used for immunohistochemistry.

Anti-mouse primary antibody.	**Dilution**
Biotinylated rat anti-mouse TER-119 (Clone Ly-76- IgG_{2A}; Pharmingen, Cat No- 09082D)	1:100
Rat anti-mouse GATA-1 (Clone N6- IgG_{2A}; Santa Cruz, Cat No- sc-265)	1:200
Biotinylated rat anti-mouse c-Kit (Clone 2B8- IgG_{2B}; Pharmingen, Cat No- 01902D)	1:50

Species-specific secondary antibody.	**Dilution**
Goat anti-rat biotinylated IgG (Santa Cruz, Cat No- sc-2041)	1:200

Isotype control	**Dilution**
Biotinylated rat IgG_{2A} (Clone KLH/ G2a-1-1; Insight Biotechnology, Cat No- 13-4321-81))	1:100/ 1:200
Biotinylated rat IgG_{2B} (Clone KLH/ G2b-1-2; Insight Biotechnology, Cat No- 13-4331-81))	1:50

One DAB tablet (Sigma, Cat No- D4418) was dissolved in 0.05M Tris-HCl (pH7.6) to give a final concentration of $1mgmL^{-1}$. 4µL of an 8% $NiCl_2$ solution (1.3mM final concentration) was added per 0.5mL of DAB solution. Sections were immersed in the solution and immediately followed by addition of 100µL of 30%^{v}/v H_2O_2 per 10mL of DAB solution, providing a final concentration of 0.3%^{v}/v H_2O_2 (see Section 2.4.9). The sections were incubated in this solution and the colour development monitored microscopically (typically 5 to 10 minutes). Sections were washed 3 times in sterile H_2O. The sections were processed and mounted in DPX (see Section 2.1.6).

Section 2.4 Materials used for immunohistochemistry.

Section 2.4.1 Target retrieval solution.

Target retrieval solution (pH6) was purchased from DAKO (Cat No- S1700). Coplin jars were filled with approximately 25mL of Target retrieval solution, placed in a water bath and heated to 95^{0}C-98^{0}C. De-waxed, re-hydrated spleen sections were immersed in the preheated Target Retrieval Solution for approximately 20 minutes. The coplin jar was removed from the water bath and allowed to cool for 20 minutes at room temperature. Sections were then washed with 3 changes of 1X PBS (pH7.5) for 5 minutes each.

Section 2.4.2 Periodic acid (0.01M).

COMPOSITION	**AMOUNT**
Periodic acid	2.279g
Sterile H_2O	100mL

2.279g of periodic acid (BDH, Cat No- 298422P) was dissolved in 1000mL of sterile H_2O to give a final concentration of 0.01M. The solution stored for 1 week at room temperature.

Section 2.4.3 Sodium borohydride ($0.1 mgmL^{-1}$)

COMPOSITION	AMOUNT
Na_2-borohydride	0.1g
Sterile H_2O	1000mL

100mg of sodium-borohydride (Sigma, Cat No- S9125) was dissolved in 1000mL of sterile H_2O. $0.1 mgmL^{-1}$ sodium-borohydride solution was stored at room temperature for up to 1 week.

Section 2.4.4 Normal swine serum (1:20)/ 1X PBS.

COMPOSITION	AMOUNT
Normal swine serum	0.1mL
1X PBS (pH7.5)	1.9mL

Normal swine serum was purchased from DAKO (Cat No- X0901). A 1 in 20 dilution of the normal swine serum in 1X PBS (pH7.4) was used to block non-specific binding of anti-mouse antibodies in spleen sections. Typically, 100µL of normal swine serum was mixed with 1.9mL of 1X PBS (pH7.4), and aliquots of 100µL stored at -20^0C until use.

Section 2.4.5 1.5%^{v}/v normal swine serum/ 1X PBS.

COMPOSITION	AMOUNT
Normal swine serum	15μL
1X PBS (pH7.5)	985μL

Antibodies were prepared in 1.5% normal swine serum in 1X PBS (pH7.5). 15μL of normal swine serum was mixed with 985μL of 1X PBS (pH7.5) and stored in 100μL at -20^{0}C. Antibodies were diluted to the optimal working concentration (see Table 1) freshly thawed 1.5% normal swine serum in 1X PBS (pH7.5) immediately prior to use.

Section 2.4.6 Anti-mouse biotinylated primary antibody and isotype control.

The working concentration of primary antibody used to detection of specific antigens in section of spleen was determined by titration experiments over a dilution range of 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800 in 1.5% normal swine serum/ 1X PBS (pH7.5). Dilutions were prepared from antibody stock solutions (see Table 1). Experiments were performed in the presence of isotype control rat biotinylated IgG (see Table 1).

Section 2.4.7 Species-specific biotinylated secondary antibody.

As in section 2.4.6, the working concentration of the species specific secondary antibody used detection the primary antibody was determined by titration experiments over a dilution range of 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800 in 1.5% normal swine serum/ 1X PBS (pH7.5). Dilutions were prepared from antibody stock solutions (see Table 1).

Section 2.4.8 ExtrAvidin/ 1X PBS.

ExtrAvidin (Sigma, Cat No- E2886) was diluted to a working concentration of 40μgmL^{-1} in 1X PBS (pH7.5). The ExtrAvidin/ 1X PBS (pH7.5) solution was prepared immediately prior to use.

Section 2.4.9 3,3'-Diaminobenzidine (DAB) solution.

COMPOSITION	AMOUNT
DAB	10mg
Tris-HCl (pH7.6)	9mL of a 0.5M stock solution
$NiCl_2$	4μL of an 8% stock solution
H_2O_2	100μL of a 30%^{v}/v stock solution

DAB was dissolved in 9mL of 0.05M Tris-HCl (pH7.6) to give a final concentration of 1mgmL^{-1}. 80μL of an 8% $NiCl_2$ solution (4μL per 0.5mL of DAB solution) was added to the DAB solution. Sections were immersed in the solution and immediately followed by addition of 100μL of 30%^{v}/v H_2O_2 per 10mL of DAB solution (comprising a final concentration of 0.3%^{v}/v H_2O_2). The sections were incubated in this solution and the colour development monitored microscopically (typically 5 to 10 minutes). Washing the sections in sterile H_2O 3 times for 5 minutes each stopped the reaction. Sections were mounted in DPX (see Section 2.1.6).

Section 2.5 In situ Reverse Transcription (RT)- Polymerase Chain Reaction (PCR) by direct incorporation of digoxigenin labeled dUTP.

Section 2.5.1 Prelude.

The *in situ* RT-PCR experiments were performed on 5μm paraformaldehyde fixed paraffin sections of spleen using a Primus 96 plus *in situ* thermal block (MWG Biotech). Gene frames (65μL) (Abgene, Cat No- HB-OS-SSEZ2E) were used routinely to prevent dehydration.

Section 2.5.2 Digestion of spleen sections with Proteinase K.

De-waxed and re-hydrated spleen sections (see Section 2.1.6) were equilibrated in 1X PBS (pH 7.5) for 5 minutes and then post-fixed in 4% paraformaldehyde/ 1X PBS (pH 7.5) (see Section 2.2.2).for 10 minutes at room temperature. Following incubation in 3 changes of sterile H_2O for 10 minutes each, sections were completely covered with 2, 4, 8, or 10μgmL^{-1} Proteinase K in 0.1M Tris-HCl (pH 7.4) containing 50mM Na_2EDTA (see Section 2.6.2, and 2.6.3) for 20 minutes at 37^{0}C. Sections were then immersed in 0.1M glycine/ 1X PBS (pH 7.5) (see Section 2.6.4) for 10 minutes, and incubated in 3 changes of sterile H_2O for 5 minutes each. To remove genomic DNA, sections were incubated in 1UμL^{-1} RNAse-free DNAse I (Roche Biochemicals, Cat No-776 785) in 1X DNAse I buffer (see Section 2.6.5) at 37^{0}C for at least 12 hours. After DNAse treatment, the sections were placed in 1mM Na_2EDTA solution (pH 8.0) for 15 minutes at 70^{0}C, and washed 3 times in 50mM Tris-HCl (pH 7.4) for 10 minutes each at room temperature.

Section 2.5.3 In situ Reverse Transcription.

The spleen sections were incubated in a solution containing 2.5μg Oligo-dT_{18} (see Section 2.6.6) at 70^{0}C for 10 minutes and then placed on ice immediately for 1 minute. Each section

was incubated in reverse transcription mixture containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 30mM DTT, 3mM $MgCl_2$, 1000U MMuLv SuperScript RT II (GIBCO BRL, Cat No-18064-014) and 1.5mM each of dATP, dCTP, dGTP, and dTTP (see Section 2.6.7). The sections were subjected to the following thermal profile: 10 minutes at 25^0C, 50 minutes at 42^0C, 15 minutes at 70^0C and then 1 minute on ice.

Section 2.5.4 In situ Polymerase Chain Reaction.

Excess reverse transcriptase mixture was blotted from each section and replaced with PCR mixture (see Sections 2.6.8, and 2.6.9). The thermal profile for SCL specific RT-PCR was optimized in soluble phase experiments. SCL cDNA was amplified *in situ* with the following thermal profile: 30 cycles of 95^0C for 30 seconds; 62^0C for 30 seconds and 72^0C for 30 seconds with a final extension for 5 minutes at 72^0C. After the PCR, the sections were washed twice with 0.1X SSC (pH 7) (see Section 2.12.2) at 60^0C for 15 minutes, and then processed for colour development.

Section 2.5.5 Signal development for in situ RT-PCR.

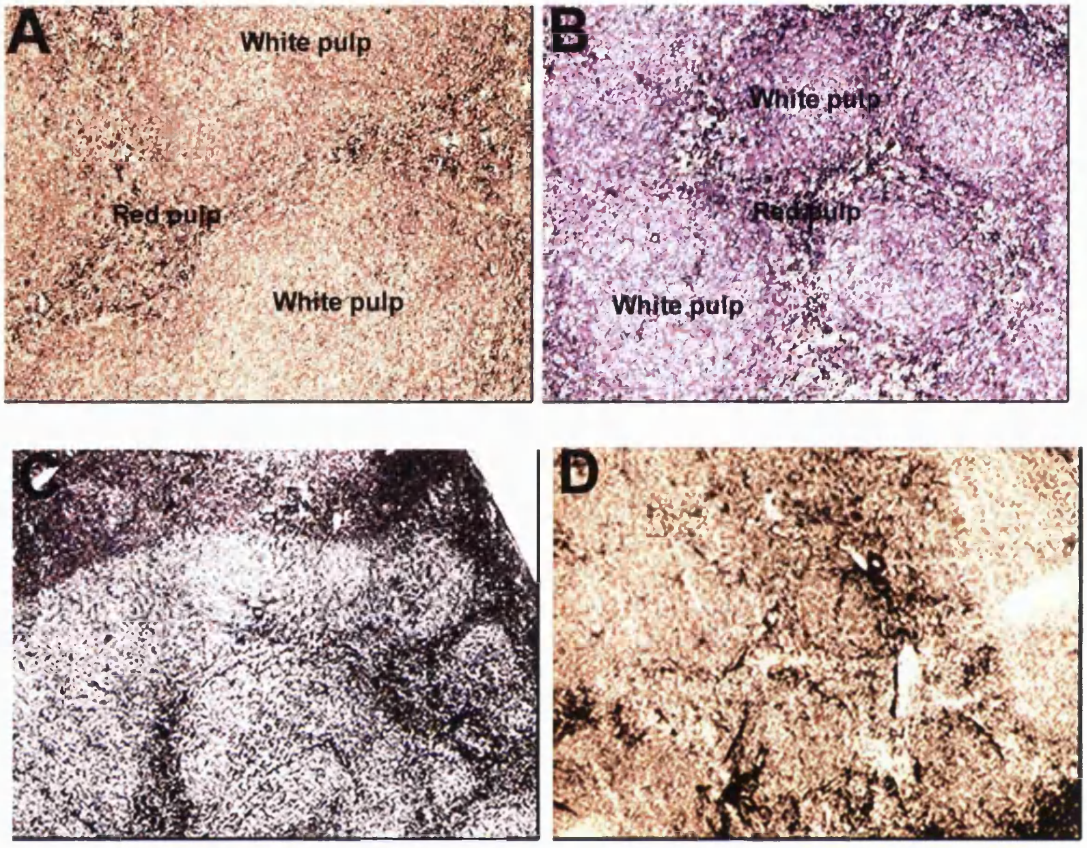
Sections were incubated in Buffer 1 (see Section 2.6.10) for 2 hours at room temperature. Excess Buffer 1 was blotted from the sections and replaced with fresh Buffer 1 containing alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (Roche, Cat No- 1 175 041) at a dilution of 1:500. Sections were incubated in the antibody solution for 1 hour at 37^0C, washed twice in Buffer 1 for 10 minutes each at room temperature, and then equilibrated Buffer 2 (see Section 2.6.11) for 10 minutes. The sections were transferred to fresh Buffer 2 containing 1X NBT/ BCIP (Roche, Cat No- 1 681 451) and incubated at 37^0C in the dark. Colour development was monitored microscopically and the reaction stopped by immersing the sections in Buffer 3 (see Section 2.6.12) when the desired colour intensity was reached.

Sections were mounted in a permanent aqueous mountant (Sigma, Cat No- 1000-4), covered with a coverslip and examined under appropriate magnification.

The initial step in designing an SCL cDNA-specific *in situ* RT-PCR protocol was to determine the length of time required to make the target accessible to the enzymes and substrates in the RT and PCR solutions without removal of the paraformaldehyde amplicon migration barrier (Nuovo, et al., 1993; Nuovo, 1996). Detection of the SCL cDNA PCR product by ethidium bromide stained gel electrophoresis and southern hybridisation with an α^{32}-dCTP labeled SCL specific probe (see Sections 2.8.4- 2.8.9) was taken as a measure of the extent of Proteinase K digestion on sections processed by *in situ* RT-PCR. In was clear that the integrity of the spleen sections was lost after incubation with 8 and 10μgmL^{-1} (Figure 2C and 2D respectively). The sections appeared to remain intact with 2 and 4μgmL^{-1} Proteinase K (Figure 2A and 2B respectively). However, hybridisation showed that SCL cDNA was released into the surrounding PCR solution from spleen sections incubated in 4μgmL^{-1} Proteinase K (Figure 2E and 2F), but not with 2μgmL^{-1} Proteinase K (Figure 2E and 2F). This result showed that digestion of 5μm paraformaldehyde-fixed paraffin sections of spleen with 2μgmL^{-1} for 20 minutes at 37^{O}C retained tissue integrity and the amplicon migration barrier. Figure 3 shows that SCL mRNA specific signals can be seen in cells in both $LYL^{+/+}$ (Figure 3A-C) and $LYL^{-/-}$ (Figure 3D-F) spleen. These SCL $cDNA^{+ve}$-cells were absence in experiments performed without reverse transcriptase (Figure 3G) and SCL specific primers (Figure 3H), indicating that the NBT/ BCIP-stained cells contained digoxigenin-labeled SCL cDNA. Although cells expressing SCL mRNA specifically can be seen, it was evident that the high background obscured clear discrimination of SCL $cDNA^{+ve}$ and SCL $cDNA^{-ve}$ cells. Since DNase treatment of the sections is known to generate non-specific signals (Martinez, et al, 1995), the DNase treatment was omitted. It was predicted

Figure 2. Proteinase K digestion of 5μm paraformaldehyde-fixed paraffin sections of LYL-1$^{-/-}$ spleen. Each section was incubated in 65μL of 2μgml^{-1} (A), 4μgml^{-1} (B), 8μgml^{-1} (C), and 10μgml^{-1} (D) Proteinase K for 20 minutes at 37^{0}C (see Section 2.5). Following the PCR step, the PCR solution was aspirated from each section, separated by ethidium bromide stained agarose gel electrophoresis (see Section 2.8), and transferred to a hybridisation membrane. The membranes were hybridised with an α^{32}P-d CTP labeled SCL probe and exposed to X-ray film for 48 hours at -80^{0}C (E, and F). SCL cDNA was detected in PCR solution after digestion with 4, 8 and 10μgml^{-1} proteinase K, whereas no SCL cDNA was present in the PCR solution after digestion with 2μgml^{-1} proteinase K. Images A-D are taken at 10x magnification. Images E and F are from separate sets of experiments.

Digeston of spleen sections with proteinase K



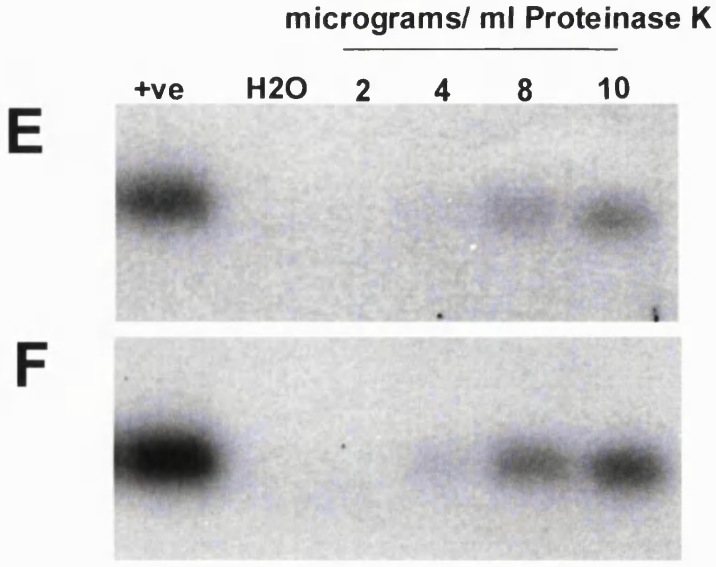
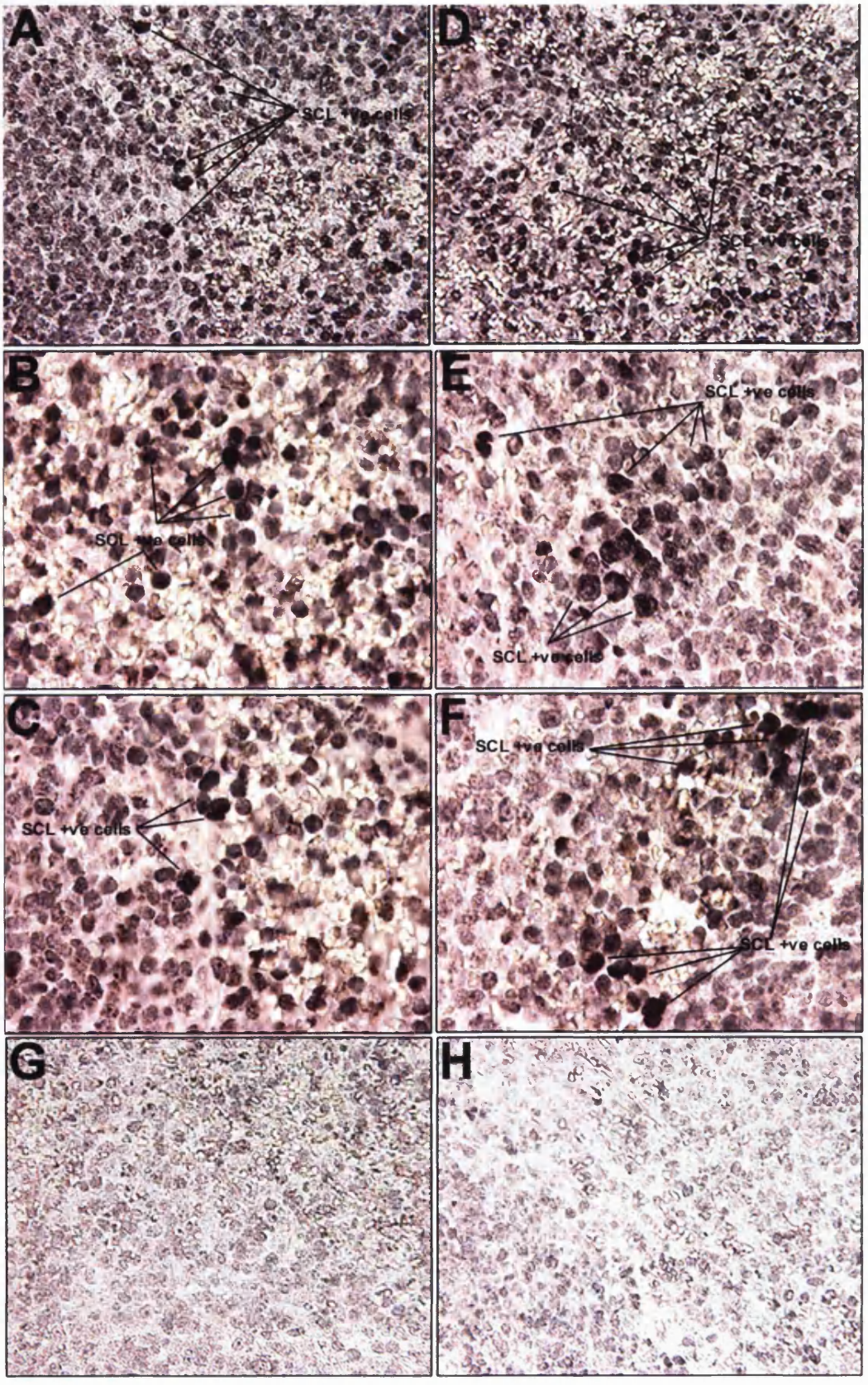


Figure 3. SCL specific *in situ* RT-PCR protocol included DNAse treatment and reverse transcription of oligo dT_{18}-primed mRNA *in situ* (see Section 2.8). SCL $mRNA^{+ve}$ cells were located in the red pulp of LYL-$1^{+/+}$ (A-C), and LYL-$1^{-/-}$ (D-F) 5μm paraformaldehyde-fixed paraffin sections of spleen. SCL $mRNA^{+ve}$ cells were arranged into clusters (E, F). SCL $mRNA^{+ve}$ cells were absent in spleen sections when either the reverse transcriptase (G), or the SCL-specific primers (H) were omitted from the *in situ* RT-PCR protocol. Images A,D,G, and H were taken at 10x magnification; images B, C, E, and F were taken at 20x magnification.

SCL specific in situ RT-PCR protocol including DNAse and oligodT.



that this would not increase staining due to amplification of SCL nucleic acid from a genomic template because the SCL primers were designed to amplify cDNA specifically.

Figure 4 shows that omitting the DNAse treatment reduced the non-specific background signal from spleen sections. SCL cDNA^{+ve}-cells could be seen clearly in LYL$^{+/+}$ (Figure 4A-C) and LYL$^{-/-}$ (Figure 4D-F) spleen, and were absent when reverse transcriptase (Figure 4G) and SCL-specific primers (Figure 4H) were omitted from the reaction solutions. There appeared to be an increase in the number of SCL mRNA expressing cells in the LYL$^{-/-}$ spleen (Figure 4D) compared to LYL$^{+/+}$ controls (Figure 4A). In general, cells in LYL$^{-/-}$ spleen were stained more intensely. To improve the signal to noise ratio further, the reverse transcriptase step was performed with an SCL-specific primer instead of oligodT_{18} (see Chapter 2 Section 2.6.8).

Figure 5 shows the result of *in situ* RT-PCR optimized for specific amplification of SCL mRNA in 5μm paraformaldehyde-fixed paraffin sections of spleen. The background staining was reduced considerably by using SCL-specific primers, rather than oligodT_{18}. SCL mRNA expressing cells were located in the subcapsular region of LYL$^{-/-}$ (Figure 5D) and LYL$^{+/+}$ spleen (Figure 5A). SCL mRNA expressing cells were also present in the red pulp of LYL$^{+/+}$ (Figure 5B, C, and G) and LYL$^{-/-}$ (Figure 5E, F, and H) spleen. The SCL cDNA^{+ve}-cells were not present in experiments performed without reverse transcriptase (Figure 5I) and SCL-specific primers (Figure 5J). Under high magnification, SCL cDNA^{+ve}-cells were found individually, and in clusters, in LYL$^{+/+}$ and LYL$^{-/-}$ spleen (see Results section 3.3).

Figure 4. SCL *in situ* RT-PCR protocol performed by reverse transcription of oligo dT_{18}-primed mRNA *in situ* (see Section 2.8) but without DNAse treatment, improved the signal to noise ratio from 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A-D) and LYL-$1^{-/-}$ (E-H) spleen. The SCL-specific primers were designed to amplify from a cDNA template only, and SCL $mRNA^{+ve}$ cells were distinct in both LYL-$1^{+/+}$ (A, B), and LYL-$1^{-/-}$ (E, F) spleen. There were more SCL $mRNA^{+ve}$ cells in LYL-$1^{-/-}$ spleen (G, H) compared to LYL-$1^{+/+}$ spleen (C, D), and spleen sections processed for *in situ* RT-PCR in the absence of reverse transcriptase (I), or SCL-specific primers (J) did not display any SCL $mRNA^{+ve}$ cells. Images A, E, I, and J were taken at 10x magnification; images B and F were taken at 15x magnification; images C, D, G, and H were taken at 40x magnification.

SCL specific in situ RT-PCR protocol including oligodT, and without DNAse.

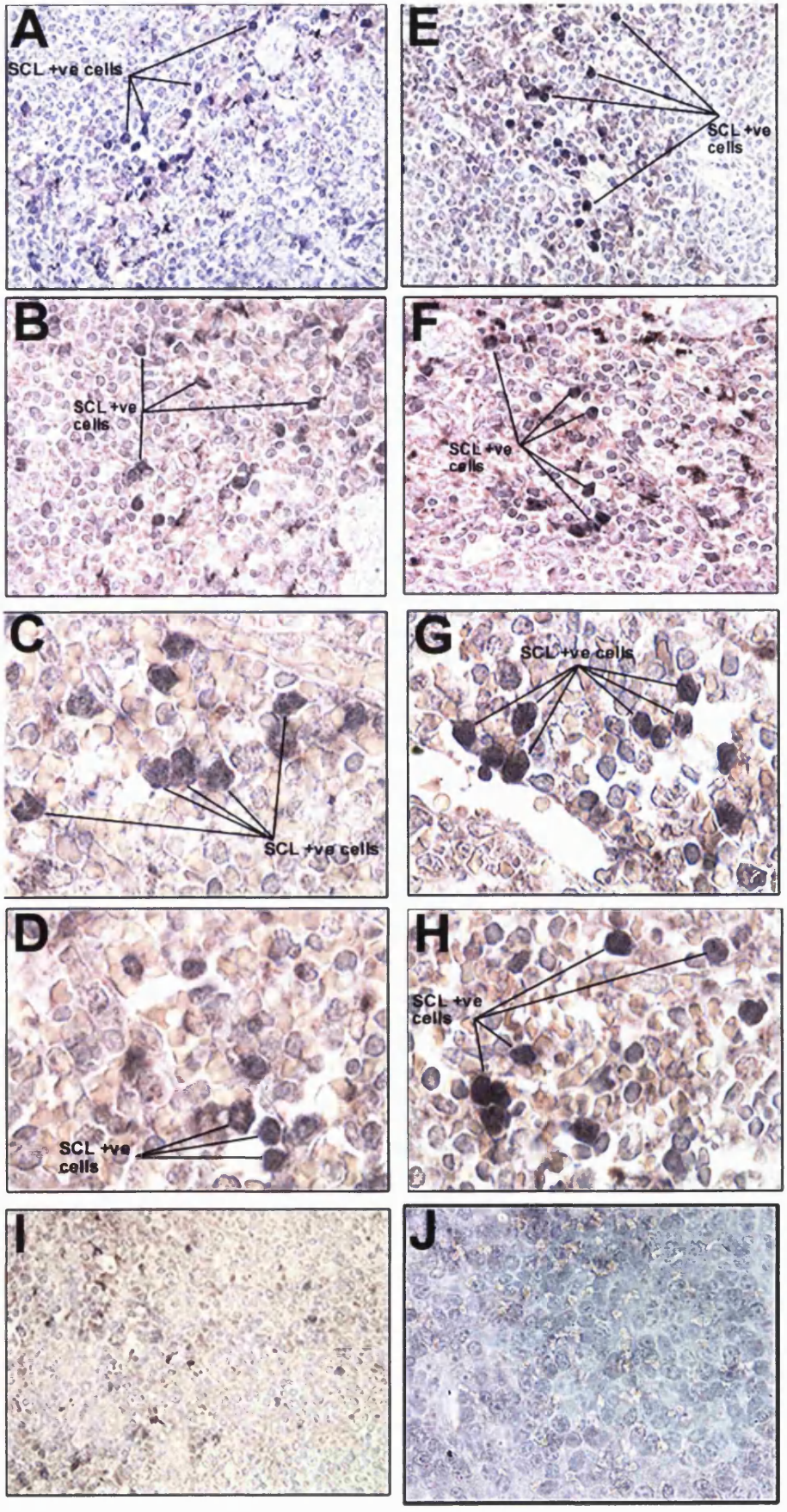
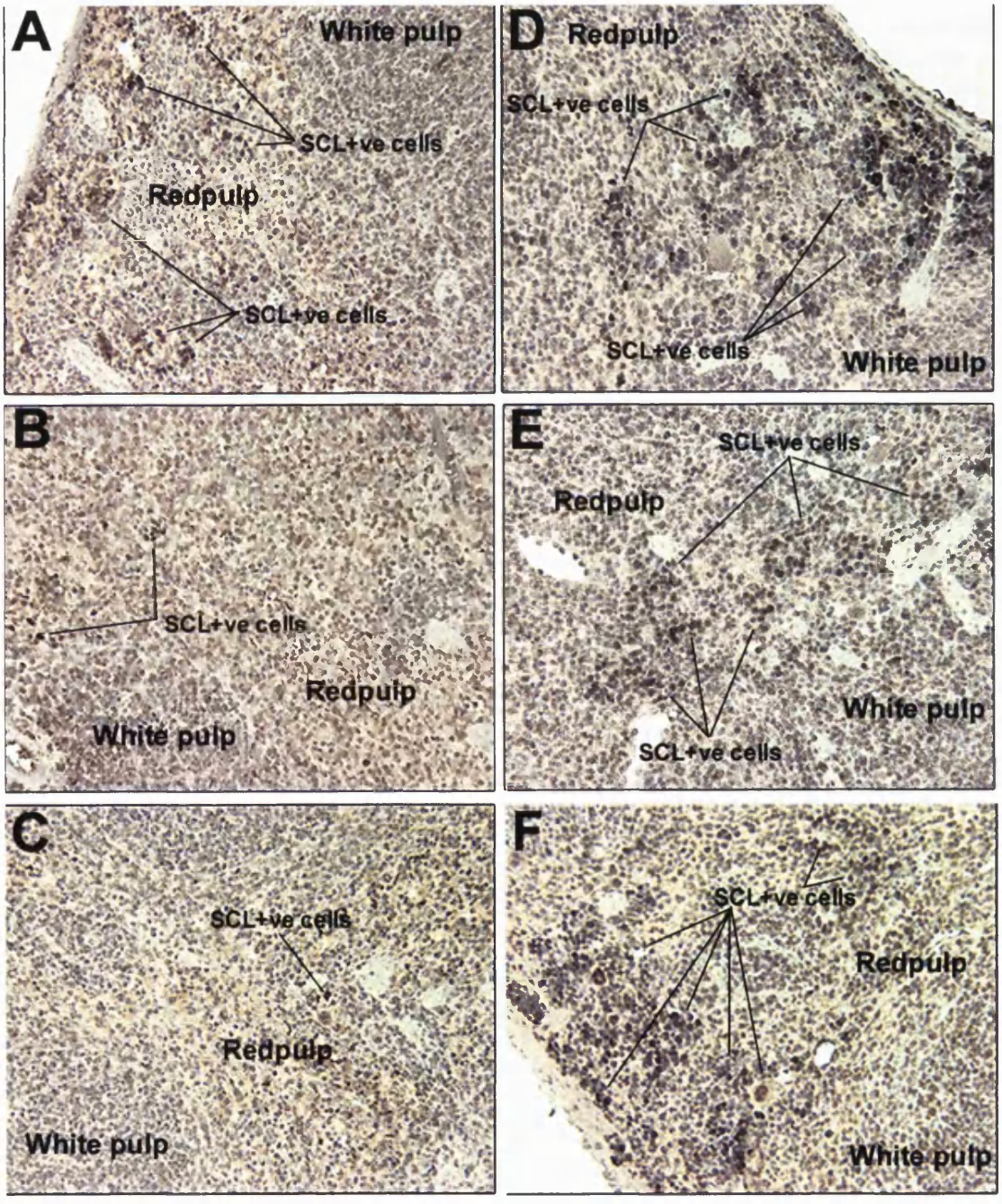
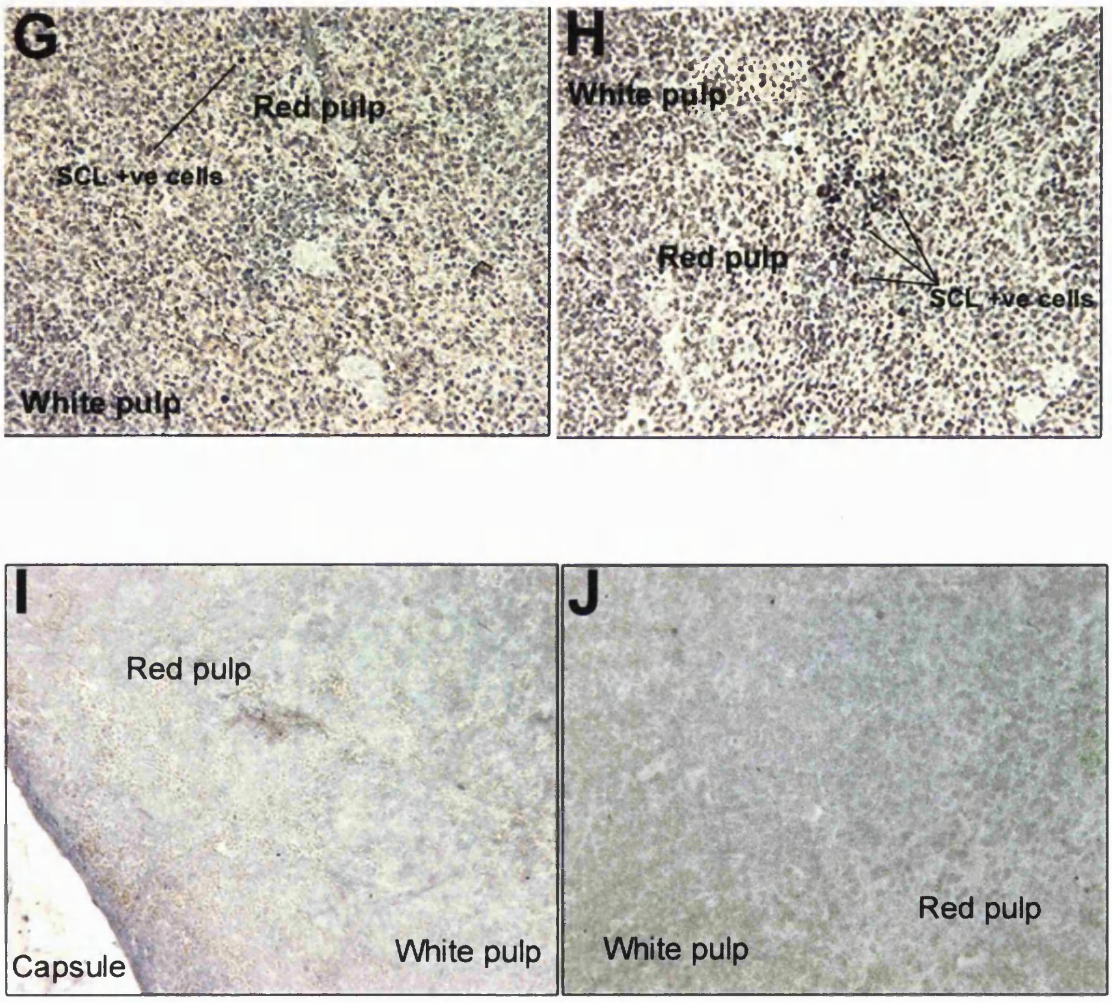


Figure 5. SCL mRNA specific *in-situ* RT-PCR performed on 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A, B, C, and G) and LYL-$1^{-/-}$ (D, E, F, and H) mice (see Section 2.5) with SCL specific primers and without DNAse treatment. SCL $mRNA^{+ve}$ cells were not present in the spleen sections processed for *in situ* RT-PCR in the absence of reverse transcriptase (I) and in the absence of SCL specific primers (J). SCL-specific reverse transcription improved the signal to noise ratio significantly. Cells expressing SCL mRNA were located in the subcapsular region of LYL-$1^{+/+}$ (A) and LYL-$1^{-/-}$ spleen (D, and F), as well as in the internal parenchyma of LYL-$1^{+/+}$ (B, C, and G) and LYL-$1^{-/-}$ (E, H) spleen. The LYL-$1^{-/-}$ spleen contained 5 to 10 times more cells expressing SCL mRNA than LYL-$1^{+/+}$ controls. All images were taken at 20x magnification.

SCL specific in situ RT-PCR protocol in the presence of SCL specific primers, and without DNAse.



SCL in situ RT-PCR protocol
in the presence of SCL specific primers and without DNAse.



Section 2.6 Materials used for in situ RT-PCR.

Section 2.6.1 Diethylpropylcarbonate (DEPC)-treated H_2O.

COMPOSITION	AMOUNT
DEPC	100μL
Sterile H_2O	0.9mL

100μL of DEPC (BDH, Cat No- 441703D) was added to 0.9mL of sterile H_2O to give a final concentration of 0.1%. The solution was incubated at 37^{0}C overnight and then autoclaved. DEPC-treated H_2O was used routinely during the preparation of solutions used for in situ RT-PCR.

Section 2.6.2 Proteinase K solution.

COMPOSITION	AMOUNT
Proteinase K	0.1g
0.1M Tris/ 50mM Na_2EDTA	1mL

0.1g of proteinase K (Sigma, Cat No) was added to 1mL of 0.1M Tris containing 50mM Na_2EDTA (pH8.0), to give a final concentration of 1mgmL^{-1}. The stock solution was incubated at 37^{0}C until the proteinase K was dissolved completely and 100μL aliquots stored at -20^{0}C. Serial dilutions of the proteinase K stock solution at 1, 2, 4, 8, and 10μgmL^{-1} were stored in 100μL aliquots at –20^{0}C for up to 2 months.

Section 2.6.3 Proteinase K buffer in 0.1M Tris/ 50mM Na_2EDTA.

COMPOSITION	AMOUNT
Tris	1mL
Na_2EDTA	1mL
DEPC-treated H_2O	98mL

100mL of 0.1M Tris/ 50mM Na_2EDTA solution was prepared by adding 1mL of 1M Tris (GIBCO BRL, Cat No- 15504-020), and 1mL of 0.5M Na_2EDTA (pH8) (BDH, Cat No- 100935V) to 98mL of DEPC-treated H_2O.

Section 2.6.4 0.1M glycine in 1X PBS.

COMPOSITION	AMOUNT
Glycine	1.875g
10X PBS (pH7.5)	25m L
DEPC-treated H_2O	225mL

1.875g of glycine (Sigma, Cat No- G7403) was dissolved in 225mL DEPC-treated H_2O. The volume was adjusted to 250mL by addition of 25mL 10X PBS (pH7.5) to give a working concentration of 0.1M glycine/ 1X PBS (p H7.5). The solution stored at 4^0C for up to one week.

Section 2.6.5 10X DNAse buffer.

COMPOSITION	AMOUNT
Tris-HCl (p H.5)	5mL of 1M Tris-HCl (p H7.5)
$MgCl_2$	100µL of 1M stock solution
BSA	1mL of $1mgmL^{-1}$ BSA stock solution
DEPC- treated H_2O	up to 10mL

10mL of 10X DNAse buffer comprising 500mM Tris-HCl (p H.7.5), 10mM $MgCl_2$ and $1mgmL^{-1}$ bovine serum albumin (BSA) fraction V (Sigma, Cat No- A-4503) was prepared by adding 5mL of 1M Tris-HCl (p H.5), 100µL of 1M $MgCl_2$, and 1mL of $1mgmL^{-1}$ BSA to 3.9mL of DEPC- treated H_2O. The buffer was stored in 100µL aliquots at -20^0C. The buffer was used at a working concentration of 1X.

Section 2.6.6 Oligo-dT_{18} solution.

COMPOSITION	AMOUNT
oligodT_{18}	5µL of a $0.5\mu g\mu L^{-1}$ stock solution
DEPC- treated H_2O	up to 65µL

OlligodT_{18} was purchased as part of the SuperScript™ Preamplification system (GIBCO BRL Cat No- 18089-011) at a concentration of $0.5\mu g\mu L^{-1}$. 5µL of oligodT_{18} solution was added to 60µL of DEPC- treated H_2O. 65µL aliquots of oligodT_{18} containing 2.5µg of oligodT_{18} were stored at -20^0C until use.

Section 2.6.7 In situ reverse transcriptase mixture.

COMPOSITION	AMOUNT
First strand buffer	13μL of 5X stock solution
dNTP mixture	9.75μL
Dithiotherol (DTT)	19.5μL
DEPC- treated H_2O	17.75μL

Reverse transcriptase purified from E. coli containing the *pol* gene of Moloney Murine Leukemia virus (MMuLV) (SuperScript RT II- GIBCO BRL Cat No- 18064-014) was used to synthesize cDNA from polyA$^+$ mRNA in situ. The reverse transcriptase mixture comprised 13μL of 5X First strand buffer (GIBCO BRL Cat No- 18064-014); 9.75μL of a dNTP mixture containing 10mM of each dATP, dCTP, dGTP, and dTTP; 19.5μL of 0.1M dithiotherol (DTT), and 17.75μL DEPC- treated H_2O. Each 65μL of mixture comprised 50mM Tris-HCl (pH 8.3), 75mM KCl, 30mM DTT, 3mM $MgCl_2$, 1000U MMuLV reverse transcriptase and 1.5mM each of dATP, dCTP, dGTP, and dTTP. 5μL of MMuLV reverse transcriptase ($20U\mu L^{-1}$) was added to freshly thawed 65μL aliquots of stock solution immediately prior to use. OligodT (2.5μg) or SCL primer (1μM) was added prior to use.

Section 2.6.8 In situ polymerase chain reaction mixture.

COMPOSITION	AMOUNT
PCR buffer	6.5μL of 10X stock solution
dNTP mixture	13μL
Digoxigenin-11-d UTP	6.5μL of 10μM stock solution
SCL sense primer	3.25μL of a 4μM stock solution

SCL anti-sense primer	3.25μL of 4μM stock solution
$MgCl_2$	5.2μL of 25mM
Q-solution	13μL of 5X solution
Taq DNA polymerase	3.6μL of a $0.42U\mu L^{-1}$ solution
DEPC- treated H_2O	10.7μL

Each 65μL of PCR mixture comprised 6.5μL of 10X PCR buffer (Helena Biosciences, Cat No- HP3002), 13μL of a dNTP mixture containing 1mM of each dATP, dCTP, dGTP, and dTTP, 6.5μL of 10μM digoxigenin-11-d UTP (Roche, Cat No- 1 093 088), 3.25μL of 4μM SCL sense and anti-sense primers, 5.2μL of 25mM $MgCl_2$, 13μL of 5X Q-solution (Qiagen, Cat No- 1-271-318), 3.6μL of a $0.42U\mu L^{-1}$ Taq DNA polymerase (Helena Biosciences, Cat No- HP3002) solution (see Section 2.6.9), and 10.7μL DEPC-treated H_2O. *In situ* PCR mixture comprised comprising 50mM Tris-HCl (pH9.1), 16mM NH_4SO_4, $150\mu gmL^{-1}$ BSA, 200μM of each dNTP, 10μM digoxigenin labeled dUTP; 0.2μM of each sense and anti-sense primer; 2mM $MgCl_2$, 1X Q-solution, and 1.5U Taq Polymerase was prepared prior to use from stock solutions stored at -20^0C.

Section 2.6.9 Taq DNA polymerase-Taq antibody for hot start of in-situ PCR.

COMPOSITION	**AMOUNT**
TaqStart antibody	4.4μL
Dilution buffer	17.6μL
Taq DNA polymerase	4.4μL of a $2.5U\mu L^{-1}$ solution

4.4μL (1.4μM) of TaqStart antibody (Clontech, Cat No- 5400-1) was mixed with 17.6μL of Dilution buffer (50mM KCl, 10mM Tris-HCl (pH 7.0)). 4.4μL of a 2.5UμL^{-1} Taq DNA polymerase solution (Helena Biosciences, Cat No- HP3002) was added to the TaqStart antibody mixture, which was incubated at room temperature for 15 minutes. 3.6μL of the Taq DNA polymerase-TaqStart antibody was used in each *in situ* PCR.

Section 2.6.10 ***Buffer 1.***

COMPOSITION	**AMOUNT**
Tris	12.11g
NaCl	5.84g
$MgCl_2$	0.4g
BSA	30g
Sterile H_2O	up to 1000mL

12.11g of Tris base, 5.84g of NaCl, 0.4g of $MgCl_2$, and 30g of BSA (fraction V) were dissolved in 900mL of sterile H_2O and the pH adjusted to 7.5 with dilute HCl. The volume was adjusted to 1000mL with sterile H_2O. Buffer 1 was stored at 4^0C for 2 weeks.

Section 2.6.11 ***Buffer 2.***

COMPOSITION	**AMOUNT**
Tris	12.11g
NaCl	5.84g
$MgCl_2$	10g

Sterile H_2O	up to 1000mL

12.11g of Tris base, 5.84g of NaCl, and 10g of $MgCl_2$ were dissolved in 900mL of sterile H_2O and the pH adjusted to 9.5 with dilute HCl. The volume was increased to 1000mL with sterile H_2O and stored at 4^0C for 3 months.

Section 2.6.12 ***Buffer 3***

COMPOSITION	**AMOUNT**
Tris	2.42g
Na_2EDTA	1.86g
Sterile H_2O	up to 1000mL

2.42g of Tris base and 1.86g of Na_2EDTA was dissolved in 900mL of sterile H_2O and the pH adjusted to 7.5 with dilute HCl. The volume was increased to 1000mL with sterile H_2O and stored at 4^0C for 3 months.

Section 2.6.13 ***Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) substrate solution.***

COMPOSITION	**AMOUNT**
NBT/ BCIP solution	1mL of 50X stock solution
Levamisole	50µL of 1M stock solution
Buffer 2	48.95mL

50mL of NBT/ BCIP substrate solution was prepared by adding 1mL of 50X NBT/ BCIP (Roche, Cat No- 1 681 451) and 50µL of 1M levamisole (Sigma, Cat No- L9756) to 48.95mL of Buffer 2 (see Section 2.6.10). The substrate solution was prepared immediately prior to use.

Section 2.7 Semi-quantitative RT-PCR.

Section 2.7.1 Prelude.

The PCR experiments were performed on 96- and 25-well Primus PCR thermal cyclers (MWG Biotech).

Section 2.7.2 Estimation of PCR cycles required to remain within the exponential range of amplification of cDNA.

In order to determine the number of cycles of PCR required to remain within the exponential range of amplification for each mRNA species, 10% (5µl) of total cDNA synthesized from 2.5µg of oligodT_{12-18} primed total RNA, isolated from LYL-$1^{+/+}$ 14.5dpc fetal liver, was used in individual reactions amplified over a range of cycles (see Sections 2.8.1- 2.8.4). β-actin is an abundant gene, so the signal generated after 10, 15, 20, 25 30 and 35 cycles was determined, whereas SCL and LYL-1 signals were determined after 20, 25, 30 and 35 cycles of amplification because they are both expressed at lower levels than β-actin (Figure 1).

Under these conditions (see Tables 2, 3, and 4), β-actin RT-PCR generated an amount of product, after 20, 25, 30, and 35 cycles of amplification, that was clearly visible on an ethidium bromide stained agarose gel following electrophoresis (Figure 6A, Table 5). The

Table 2: PCR mixture compositions.

β-actin, and lacZ specific PCR mixture composition.

COMPOSITION	AMOUNT	MOLARITY
PCR Buffer	2μL of a 10X stock solution	50mM Tris-HCl (pH9.1), 16mM NH_4-sulphate
dNTP mix	1μL of a 4mM stock solution	200μM each dNTP
BSA (fraction V)	1μL of a 2mM stock solution	0.1mgmL^{-1}
$MgCl_2$	1.2μL of a 25mM stock solution	1.5mM
Sense primer (5')	1μL of a 4μM stock solution	0.2μM
Anti-sense primer (3')	1μL of a 4μM stock solution	0.2μM
H_2O	3.3μL	
Total volume	10.5μL	

SCL, and LYL-1 specific PCR mixture composition.

COMPOSITION	AMOUNT	MOLARITY
PCR Buffer	2μL of a 10X stock solution	50mM Tris-HCl (pH9.1), 16mM NH_4-sulphate
dNTP mix	1μL of a 4mM stock solution	200μM each dNTP
BSA (fraction V)	1μL of a 2mM stock solution	0.1mgmL^{-1}
$MgCl_2$	1.2μL of a 25mM stock solution	1.5mM
Sense primer (5')	1μL of a 4μM stock solution	0.2μM
Anti-sense primer (3')	1μL of a 4μM stock solution	0.2μM
DMSO	1.8μl	9%
Tween-20	1μl of a 1% stock solution 0.01%	
H_2O	3.3μL	
Total volume	10.5μL	

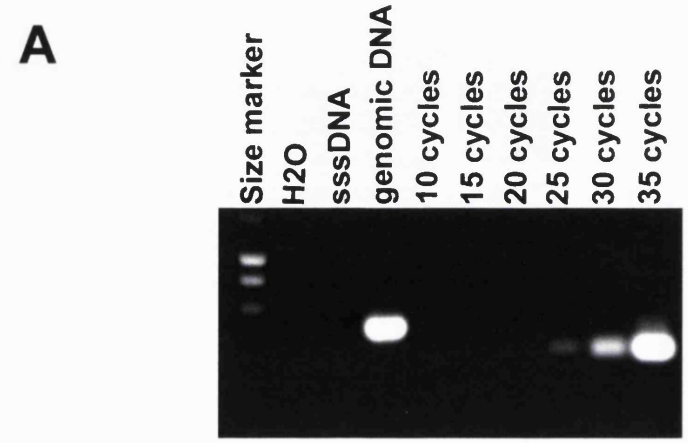
Table 3. Sense and Anti-sense primer sequences used in PCR.

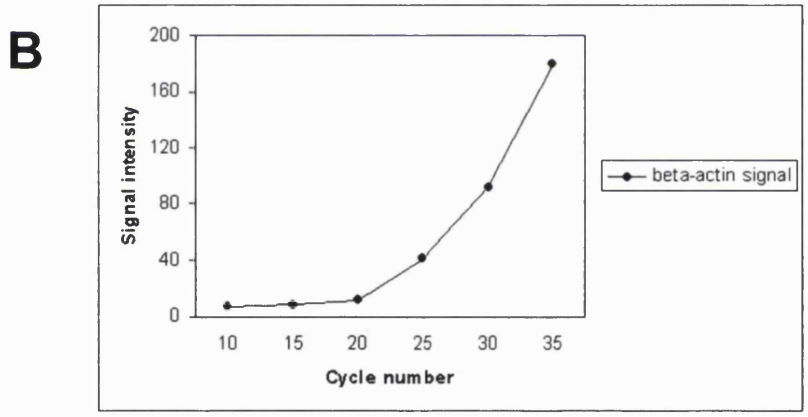
Product	Sense primer	Anti-sense primer
β-actin	5'-TAA AAC gCA gCT CAg TAA CAg TCC g-3'	5'-Tgg AAT CCT gTg gCA TCC ATg AAA C-3'
LYL-1	5'-CAA CCC Cag AAg gTg gCT Cg-3	5'-CTC Agg gAC gTC TgC TCC-3'
SCL	5'-ACT AgA ggg ACA ggA CgC gg-3'	5'-TTC CCC AAA gAA CCC ACT gCC-3'
GATA-1	5'-TCA gCA CTg gCC TAC TAC Ag-3'	5'-TAA gCA CTg CCg gTg ACA gg-3'
lacZ	5'-CgT CgT gAC Tgg gAA AAC CC-3'	5'-CgC gTA AAA ATg CgC TCA gg-3'

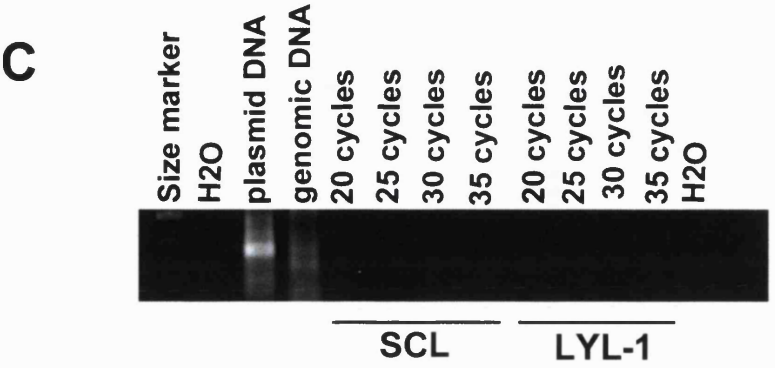
Table 4. Thermal profiles used for PCR.

Product	Initial Denaturation	Denaturation	Annelation	Extension	cDNA (kb)	Genomic DNA (kb)	Cycle n°
β-actin	95°C for 5min	95°C for 1min	69C for 1min	72°C for 1min	0.348	0.472	32
LYL-1	95°C for 5min	95°C for 1min	62°C for 30sec	72°C for 30sec	0.391	0.391	32
SCL	95°C for 5min	95°C for 1min	62°C for 30sec	72°C for 30sec	0.418	1.5	32
GATA-1	95°C for 5min	95°C for 1min	55°C for 30sec	72°C for 1min	0.27	0.7	32
lacZ	95°C for 5min	95°C for 1min	62°C for 1 min	72°C for 2min	0.498	0.498	32

Figure 6. Determination of cycle number required to remain within the exponential range of amplification for β-actin (A), SCL (C), and LYL-1 (C) cDNA. cDNA (10% of total preparation) was prepared from LYL-$1^{+/+}$14.5dpc fetal liver and amplified by PCR with specific primers (see Tables 1, 2, and 3). β-actin-specific reactions were performed with 10, 15, 20, 25, 30, and 35 cycles of amplification (A), whereas SCL-specific and LYL-1-specific reactions were amplified with 20, 25, 30, and 35 cycles of PCR (C). The reaction products were separated by ethidium bromide stained agarose gel electrophoresis (see Sections 2.8.5, 2.12.10, 2.12.13), visualized with UV illumination () controlled by AlphaImager software. The intensity of the signal from each β-actin PCR was measured with AlphaImager software and plotted against cycle number (B; see Table 4). SCL- and LYL-1-specific PCR after 20, 25, 30, and 35 cycles did not generate enough cDNA to be reliably measured by ethidium bromide stained agarose gel electrophoresis (C).


A
Size marker
H2O
sssDNA
genomic DNA
10 cycles
15 cycles
20 cycles
25 cycles
30 cycles
35 cycles


B
Signal intensity
200
160
120
80
40
0
10
15
20
25
30
35
Cycle number
beta-actin signal


C
Size marker
H2O
plasmid DNA
genomic DNA
20 cycles
25 cycles
30 cycles
35 cycles
20 cycles
25 cycles
30 cycles
35 cycles
H2O
SCL
LYL-1

signals could be measured from the gel by AlphaImager software, but 10 and 15 cycles of amplification did not generate a signal that could be measured reliably (Figure 6B- see Table 5). SCL and LYL-1 (Figure 6C) RT-PCR, using the same amount of cDNA as in the β-actin specific reactions, produced signals that were weak and could not be reliably measured from an ethidium bromide stained agarose gel. It can be seen in Figure 6C, 30 and 35 cycles of amplification are required to generate an SCL signal, while LYL-1 RT-PCR generates a signal that can be measured only after 35 cycles. To improve the sensitivity of the assay, SCL and LYL-1 reaction products were transferred to hybridisation membranes by capillary action under alkali conditions (see Section 2.8.5). Membranes were hybridised with α^{32}-dCTP labeled SCL and LYL-1 cDNA probes see Section 2.8.6). Pre-flashed X-ray film was exposed to the membranes, which generated SCL (Figure 7A) and LYL-1 (Figure 7B) RT-PCR signals that were measured by densitometry (Figure 7C- Table 6).

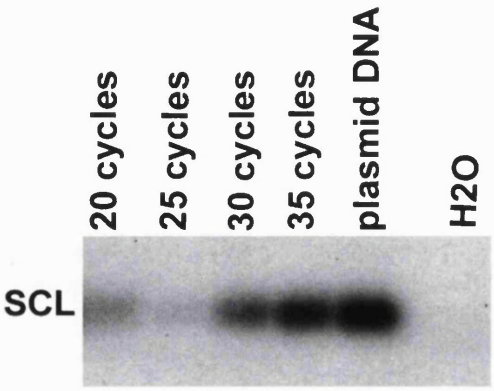
The data showed that 25 cycles was sufficient to remain in the exponential range of amplification for β-actin (Figure 6B, Table 5), while 30 cycles was required for both SCL and LYL-1 (Figure 7C, Table 6).

Section 2.7.3 Experiments to determine the sensitivity of the semi-quantitative RT-PCR assay.

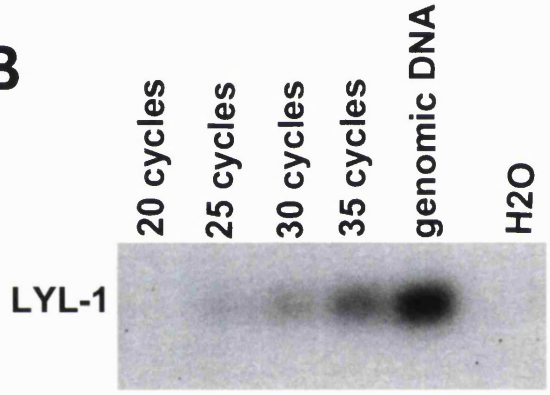
The intensity of the RT-PCR signals measured by densitometry was dependent on the length of time the radioactivity on the membrane was exposed to the X-ray film (Figure 8). Optimal exposure times determined for signals from 10% of LYL-$1^{+/+}$ 14.5dpc fetal liver cDNA amplified in β-actin specific reactions (Figure 8A) were linear between 10 and 40 minutes of exposure to the X-ray film (Figure 8A and 9A, Table 7). LYL-1 (Figure 8B and 9B, Table 8) and SCL (Figure 8C and 9C- Table 8) specific reactions generated linear signals between 60

Figure 7. The amount of SCL and LYL-1 specific PCR product generated by 20, 25, 30, and 35 cycles of amplification was determined by densitometry. PCR products separated by electrophoresis (see Section 2.7.2) were transferred to membranes and hybridised with α^{32}P-dCTP labeled SCL or LYL-1 cDNA probes (see Sections 2.8.5- 2.8.9). The membranes were exposed to X-ray film for 30 minutes prior to development, and the intensity of each signal measured with a Densitometer (). Signals generated from SCL (A) and LYL-1 (B) PCR were plotted against cycle number (C), and showed that the number of cycles of required to remain within the exponential range of amplification was 30 for both SCL and LYL-1 RT-PCR (see Table 5).

A


20 cycles
25 cycles
30 cycles
35 cycles
plasmid DNA
H2O
SCL

B


20 cycles
25 cycles
30 cycles
35 cycles
genomic DNA
H2O
LYL-1

C

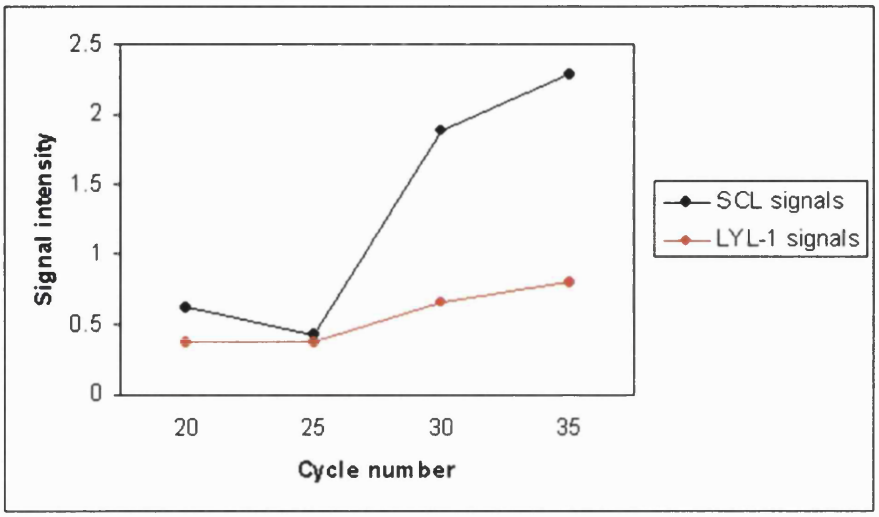

2.5
2
1.5
1
0.5
0
Signal intensity
20
25
30
35
Cycle number
SCL signals
LYL-1 signals

Figure 8. Estimation of exposure time required for a linear change in signal intensity. Membranes hybridised with a β-actin (A) labeled α^{32}P-dCTP probe was exposed to X-ray film for 5, 10, 20, 40, and 80 minutes, whilst those hybridised with LYL-1 (B) or SCL (C) labeled α^{32}P-dCTP probes were developed after 30, 60, 120, and 240 minutes exposure (see Tables 7 and 8).

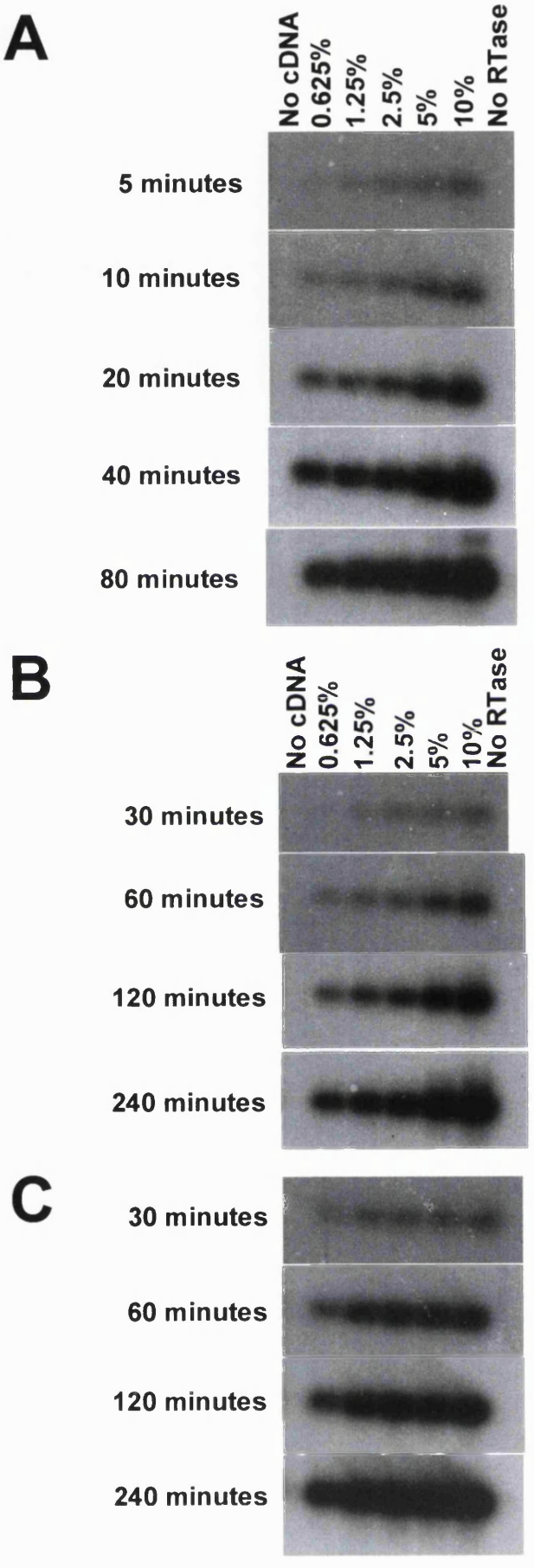
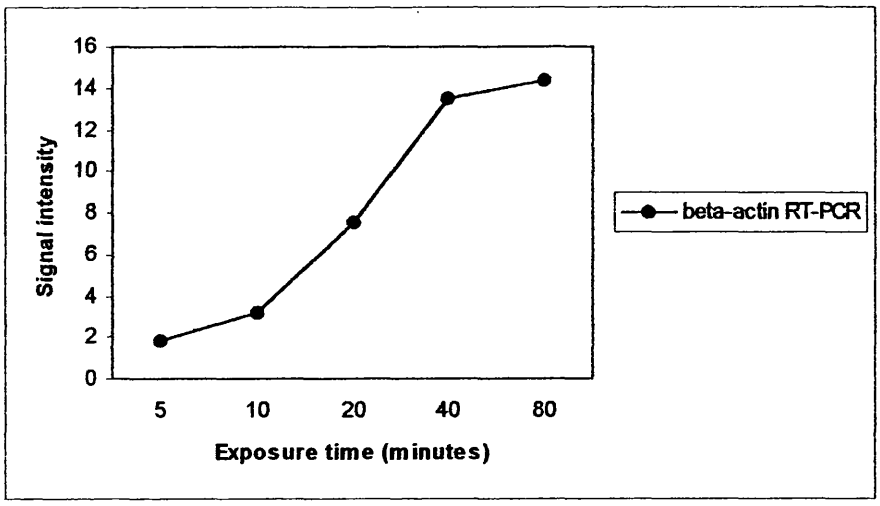
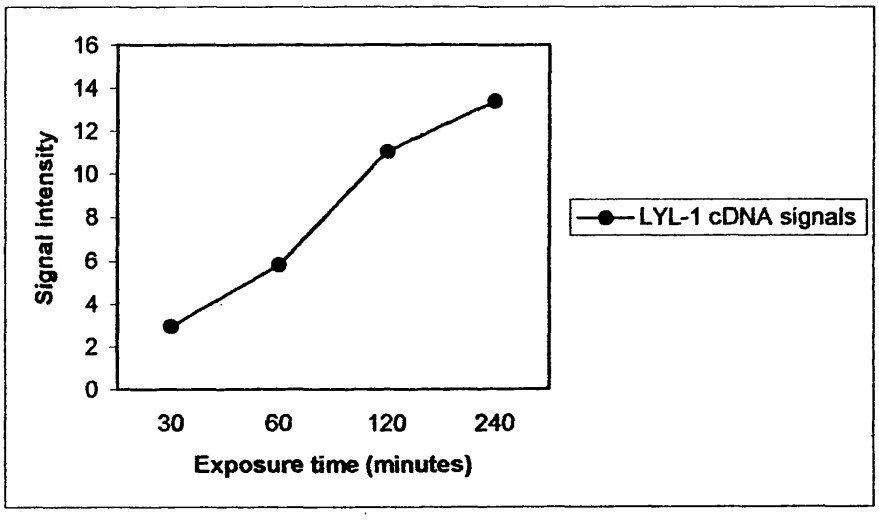

A
No cDNA
0.625%
1.25%
2.5%
5%
10%
No RTase
5 minutes
10 minutes
20 minutes
40 minutes
80 minutes
B
No cDNA
0.625%
1.25%
2.5%
5%
10%
No RTase
30 minutes
60 minutes
120 minutes
240 minutes
C
30 minutes
60 minutes
120 minutes
240 minutes

Figure 9. The signal intensity from 10% of the total cDNA synthesized from $LYL^{+/+}$ 14.5dpc fetal liver (Figure 3) was plotted against exposure time of the membrane to the X-ray film for β-actin (A), LYL-1 (B), and SCL (C). The optimal exposure time for membranes hybridised with a β-actin labeled α^{32}P-dCTP probe was 20 minutes (see Table 7), whereas 90 minutes of exposure was required for those hybridised with LYL-1 (B), or SCL (C) labeled α^{32}P-dCTP probes (see Table 8). Each data set followed a linear distribution according to y=mx+b.

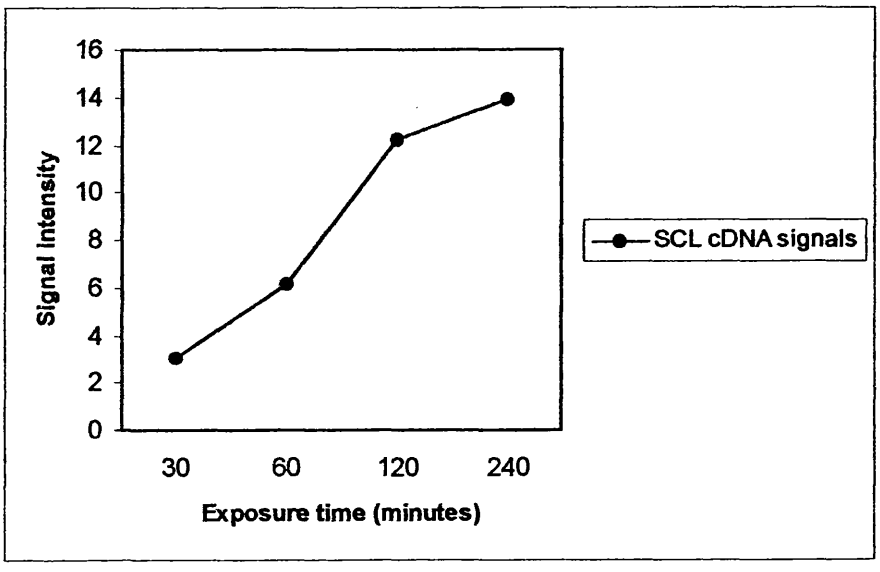
A


16
14
12
10
8
6
4
2
0
Signal intensity
5
10
20
40
80
Exposure time (minutes)
beta-actin RT-PCR

B


16
14
12
10
8
6
4
2
0
Signal intensity
30
60
120
240
Exposure time (minutes)
LYL-1 cDNA signals

C


16
14
12
10
8
6
4
2
0
Signal intensity
30
60
120
240
Exposure time (minutes)
SCL cDNA signals

No of cycles	β-actin signal
10	7.42
15	8.37
20	11.16
25	41.3
30	91.74
35	179.81

Table 5. The intensity of the signal obtained after β-actin specific PCR amplification of cDNA derived from $LYL^{+/+}$ 14.5dpc fetal liver (refer to Figures 1A and 1B- for details see Section 2.7).

No of cycles	SCL signal	LYL signal
20	0.62	0.37
25	0.42	0.38
30	1.88	0.66
35	2.28	0.8

Table 6. The intensity of the signal obtained after SCL and LYL-1 specific PCR amplification of cDNA derived from $LYL^{+/+}$ 14.5dpc fetal liver (refer to Figure 1C, and Figure 2- for details see Section 2.7).

Exposure time	β-actin signal
5 min	1.85
10 min	3.18
20 min	7.47
40 min	13.49
80 min	14.37

Table 7. The intensity of the signal obtained after different lengths of exposure time of the membrane to X-ray film was determined by densitometry (refer to Figure 3A). 10% of the total cDNA synthesized from $LYL^{+/+}$ 14.5dpc fetal liver total RNA was amplified by β-actin specific PCR (for details see Section 2.7).

Exposure time	LYL-1 signal	SCL/tal-1 signal
30 min	2.90	3.07
60 min	5.81	6.14
120 min	11.05	12.25
240 min	13.37	13.87

Table 8. The intensity of the signal obtained after different lengths of exposure time of the transfer membrane to X-ray film was determined by densitometry (refer to Figures 3B and 3C). 10% of the total cDNA synthesized from LYL$^{+/+}$ 14.5dpc fetal liver total RNA was amplified by SCL and LYL-1 specific PCR (for details see Section 2.7).

% Total cDNA	SCL signal	LYL-1 signal	β-actin signal
0.63%	1.17	0.71	0.64
1.25%	3.38	1.56	1.36
2.50%	5.21	3.77	4.48
5%	6.49	4.88	6.24
10%	7.99	5.14	8.82

Table 9. The intensity of the signal generated by β-actin, SCL, and LYL-1 specific PCR amplification of serial dilutions of 10% of the total cDNA (refer to Figure 4) synthesized from LYL$^{+/+}$ 14.5dpc fetal liver total RNA (for details see Section 2.7).

Total cDNA	β-actin signal	sem	SCL signal	sem
0.63%	1.21	0.30	1.53	0.18
1.25%	2.53	0.69	2.87	0.56
2.50%	4.97	0.91	3.92	0.64
5%	6.75	0.44	5.75	1.03
10%	8.44	0.21	6.89	0.62

Table 10. The signal generated by β-actin and SCL specific PCR amplification of cDNA was measured by densitometry (refer to Figure 5), and the average obtained from three independent experiments (for details see Section 2.7).

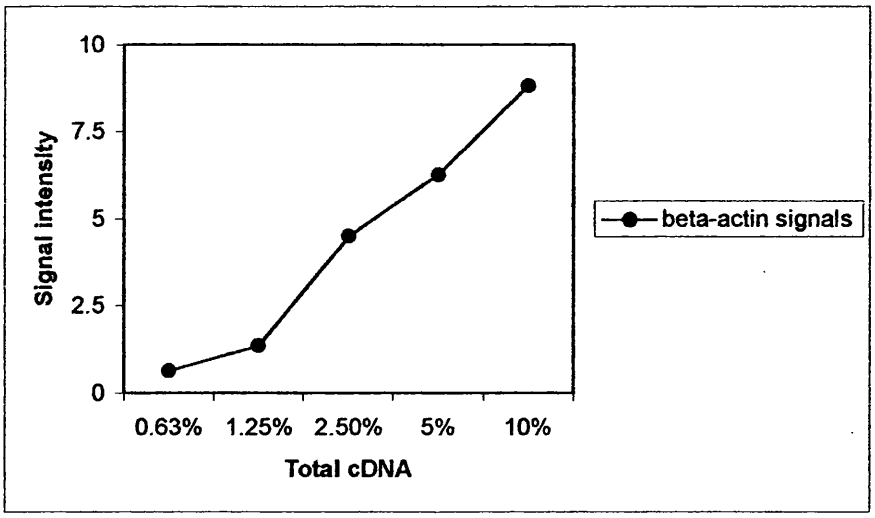
and 120 minutes of exposure. β-actin signals measured after 20 minutes of exposure, and SCL and LYL-1 specific signals after 90 minutes, were used to assess the relationship between signal intensity and concentration of cDNA (Figure 10). The signals obtained from serial dilutions of 10% of the total cDNA (5%, 25%, 1.25%, and 0.625%) fitted a linear distribution (y=mx+b) for β-actin, SCL and LYL-1 RT-PCR reactions (Figure 10- Table 9).

The β-actin signals from three independent RT-PCR reactions performed with LYL-$1^{+/+}$ 14.5dpc fetal liver cDNA (Figure1A) showed that the signals from 10%, 5%, 2.5%, 1.25%, and 0.625% of the total cDNA were significantly different, indicating that no less than a 2-fold difference in the relative expression level of β-actin mRNA can be measured accurately (Table 10). The SCL signal from 10% of the total cDNA was not significantly different to that obtained with 5% total cDNA (Figure 11B- Table 10). The difference between the signal from 10% total cDNA and 2.5% total cDNA was significant, indicating that no less than a 4-fold difference in the level of SCL mRNA expression can be measured accurately with the semi-quantitative RT-PCR assay.

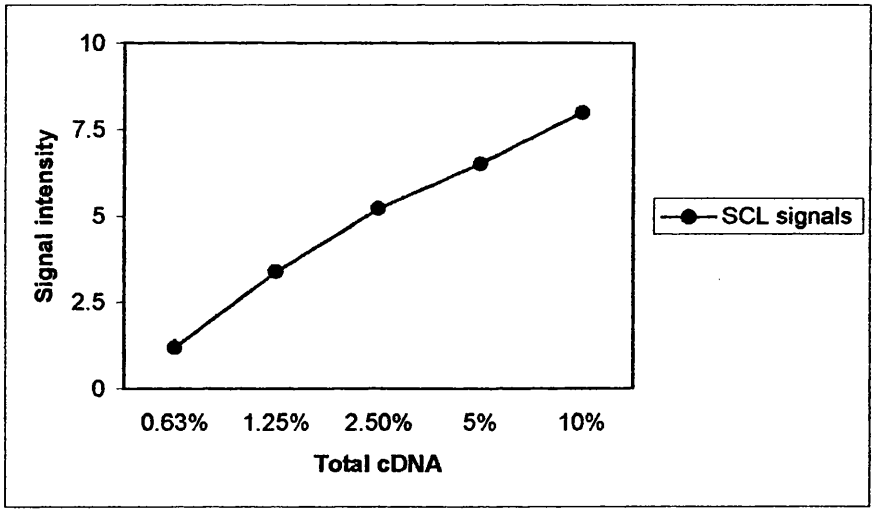
These data show that differences in the relative level of a transcript that has a high copy number, such as β-actin, can be measured more accurately than differences in the relative level of a low copy number transcript, such as SCL. With this assay differences of at least 2-fold in the expression level of β-actin mRNA, and at least 4-fold in the level of SCL mRNA can be measured accurately (Figure 11, Table 10). The signal intensity from the β-actin RT-PCR experiments performed with LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ cDNA samples were used to standardize the SCL and LYL-1 signals. This allowed for comparison of relative mRNA expression levels in LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice. The relative level of expression of SCL and LYL-1 mRNA was determined for brain, liver, thymus,

Figure 10. The change in signal intensity generated by 10%, 5%, 2.5%, 1.25%, and 0.625% of the total cDNA (input cDNA) in β-actin (A), SCL (B), and LYL-1 (C) specific PCR is linear. Serial dilutions (1:2 to 1:16- fold) were prepared from 10% of the total LYL-$1^{+/+}$ 14.5dpc fetal liver cDNA and each used in β-actin, SCL, and LYL-1 specific PCR. Signal intensity was measured by densitometry (see Table 8).

A


10
7.5
5
2.5
0
Signal intensity
0.63%
1.25%
2.50%
5%
10%
Total cDNA
beta-actin signals

B


10
7.5
5
2.5
0
Signal intensity
0.63%
1.25%
2.50%
5%
10%
Total cDNA
SCL signals

C

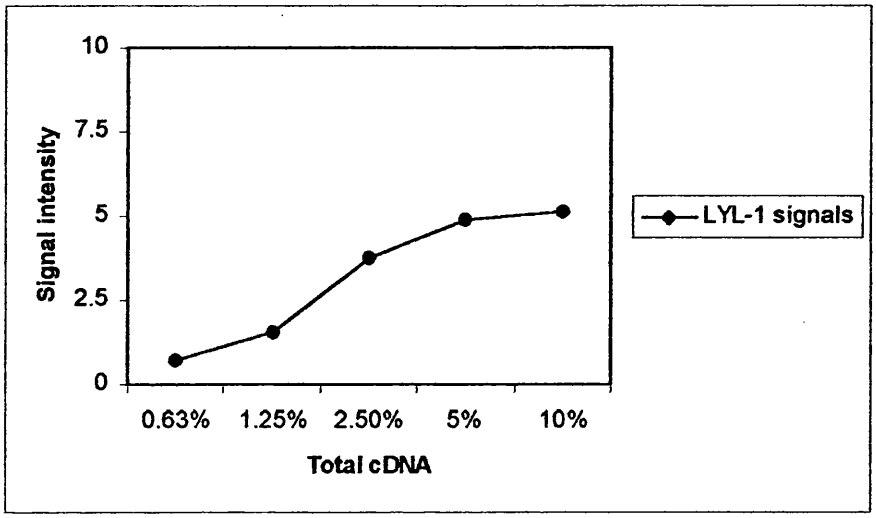
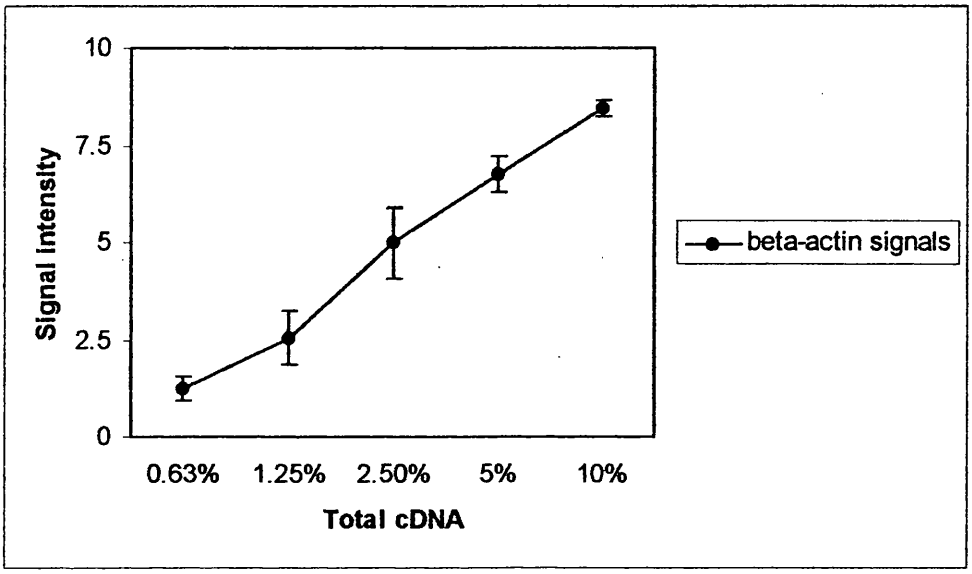
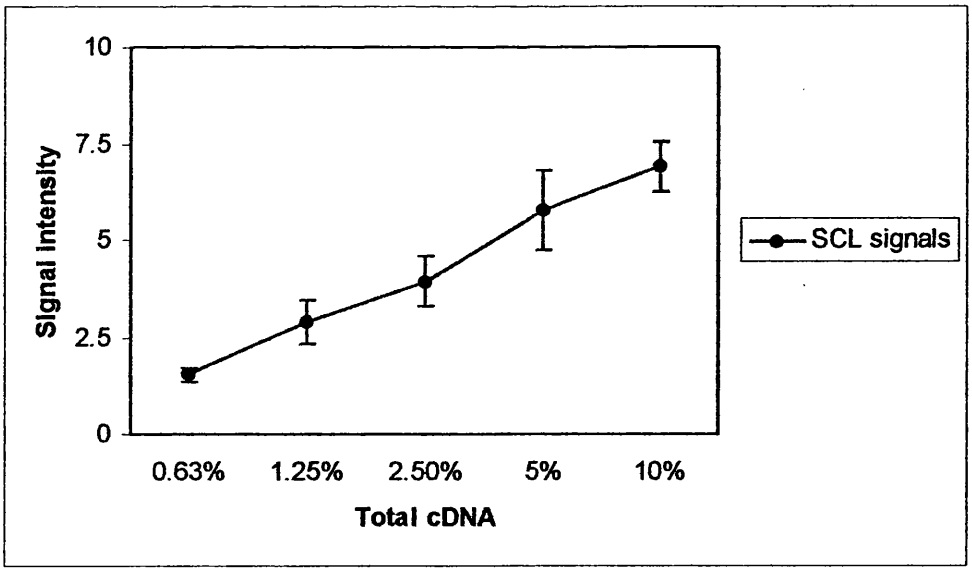

10
7.5
5
2.5
0
Signal intensity
0.63%
1.25%
2.50%
5%
10%
Total cDNA
LYL-1 signals

Figure 11. β-actin (A), and SCL (B), PCR using 10% to 0.625% of the total cDNA were repeated 3 times and the average signal intensity plotted against total input cDNA. A 2-fold change in input cDNA generated a β-actin specific RT-PCR signal was significantly different ($P<0.05$), whereas a 4-fold change in the input cDNA was required to generate a significantly different SCL-specific RT-PCR signal (see Table 9).

A


10
7.5
5
2.5
0
Signal Intensity
0.63%
1.25%
2.50%
5%
10%
Total cDNA
beta-actin signals

B


10
7.5
5
2.5
0
Signal Intensity
0.63%
1.25%
2.50%
5%
10%
Total cDNA
SCL signals

spleen, and bone marrow of adult LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice, as well as for 14.5dpc fetal liver (Figure 7).

Section 2.8 Materials used for semi-quantitative RT-PCR

Section 2.8.1 Extraction and purification of total RNA from tissue.

RNAzol^{TM}B (Biogenesis- Cat No- CS-105) was used to isolate and purify total RNA from tissues according to manufacturer protocols. The tissue sample was placed in a sterile 50mL Falcon tube with 2mL of RNAzolB per 100mg of tissue. The tissue was homogenized for at least 30 seconds with a homogeniser (Janke and Kunkel, IKA Labortechnik, Ultra Turrax T25) and then placed on ice. 0.2mL of chloroform was added to the homogenate and the sample shaken vigorously for 15 seconds. The sample was placed on ice for 5 minutes, and then centrifuged at 14,000 rpm for 15 minutes. After centrifugation, the upper aqueous phase containing the RNA was transferred to an RNAse-free microcentrifuge tube containing an equal volume of isopropanol. The sample was stored for 30 minutes at 4^0C, and then centrifuged at 14,000 rpm for 15 minutes at 4^0C. After centrifugation, the supernatant was removed and the pellet washed in 1mL of 70% ethanol by centrifugation at 14,000 rpm for 10 minutes at 4^0C. The ethanol was discarded and the pellet allowed to dry. The RNA was dissolved 10-20μL of RNAse-free H_2O. The integrity, and concentration of the total RNA was determined by measuring the 260nm:280nm ratio, and by formaldehyde agarose gel (1%) electrophoresis in 1X MOPS (pH 7) (see Sections 2.12.1, 2.12.11, and 2.12.12).

Section 2.8.2 Reverse transcription mixture.

COMPOSITION	**AMOUNT**

Total RNA	2μL of a 1.25μgmL^{-1} solution
oligodT_{18}	2μL of a 50μM stock solution
DEPC-treated H_2O	8μL
Reverse transcriptase mixture	7μl
SuperScript RT II	1μL (200UμL^{-1})

First-strand cDNA was synthesized from total RNA in accordance with the SuperScript II RT protocol (GIBCO BRL, Cat No- 18064-014). 2.5μg of total RNA was mixed with oligodT_{18} (5μM), and DEPC-treated sterile H_2O in a microcentrifuge tube to a final volume of 12μL, then heated at 70^{0}C for 10 minutes. The oligodT_{18}-primed total RNA was placed on ice immediately for 1 minute and then mixed with 7μL of reverse transcriptase reaction mixture comprising 25mM Tris-HCl (pH8.4), 37.5mM KCl, 1.5mM $MgCl_2$, 10mM DTT, and 500μM of each dNTP. The reverse transcriptase reaction was equilibrated at 42^{0}C for 1 minute and 1μL of SuperScript RT II (200UμL^{-1}) added to initiate cDNA synthesis, which was allowed to proceed for 50 minutes at 42^{0}C. The reaction was heated at 70^{0}C for 15 minutes to inactivate the reverse transcriptase, and then incubated with RNAse H (2U) (GIBCO BRL, Cat No- 18021-014) at 37^{0}C for 20 minutes to degrade the RNA templates.

Section 2.8.3 Purification of cDNA.

The cDNA was purified with the GlassMax DNA Isolation Spin Cartridge System (GIBCO BRL, Cat No- 15590-052) according to manufacturers instructions. 120μL of NaI-binding solution (6M) was mixed with the 20μL cDNA sample (section 2.8.2) at room temperature, and the mixture transferred to a GlassMax spin cartridge and centrifuged at 13,000x g for 20 seconds. The column was washed 4 times with ice cold 1X wash buffer containing 1mM

Na_2EDTA, 40mM Tris-HCl (pH7.4) and 400mM NaCl, followed by 2 washes with ice cold 70^0C ethanol by centrifugation at 13,000x g for 20 seconds each. The spin column was centrifuged at 13,000x g for 1 minute to remove residual 70% ethanol and the first-strand cDNA eluted from the column, into a clean microcentrifuge tube, with 50µL RNAse/ DNAse-free H_2O at 65^0C. The cDNA preparation was either placed on ice for immediate use, or stored at -20^0C.

Section 2.8.4 Polymerase chain reaction mixture.

cDNA, plasmid DNA or genomic DNA was mixed with PCR mixture comprising 50mM Tris-HCl (pH9.1), 16mM ammonium sulphate, 150µgmL^{-1} BSA, 200µM each dNTP, 0.2µM each sense and anti-sense primer (see Table 2 and 3) and $MgCl_2$. Taq Supreme (Helena Biosciences, Cat No- HP3002) (1.25U) was added to each 50µL PCR prior to initial denaturation or, in experiments that used cDNA, Taq Supreme was added by hot start. Each PCR reaction was subjected to a specific thermal profile (see Table 4) for amplification of target cDNA.

Section 2.8.5 Southern hybridisation of PCR amplified cDNA.

The PCR amplification products were separated on an ethidium bromide (0.5µgmL^{-1}) stained agarose (0.8% / 1X TBE) gel by electrophoresis (see Section 2.12.10). cDNA products were visualized by UV illumination recorded on a gel documentation system and analysed with AlphaImager software. Capillary transfer apparatus was constructed (see Section 2.12.6) and the cDNA products transferred from the gel to Hybond N+ hybridisation membrane (Amersham, Cat No- RPN 303B) with 0.4M NaOH transfer buffer (see Section 2.12.5). The transfer was allowed to proceed overnight at room temperature, after which the hybridisation membrane was air dried and stored at 4^0C until use.

Section 2.8.6 Radiolabeling of nucleic acid probes.

COMPOSITION	AMOUNT
cDNA	24μL
random oligonucleotide primers	10μL
dCTP buffer	10μL of a 5X solution
[α-^{32}P]-dCTP	5μl of a 10μCiμL^{-1} solution
Exo(-) Klenow fragment (5Uμl^{-1})	1μL

The Prime-It^{R} Random Primer Kit (Stratagene, Cat No-300385) was used to generate α^{32}P-dCTP labeled DNA probes according to manufactures protocol. 25 to 50ng of purified double stranded DNA in a volume of 24μL was mixed with 10μL of random oligonucleotide primers in a sterile microcentrifuge tube and heat to 100^{0}C for 5 minutes. The sample was allowed to cool to room temperature, then 10μL of 5X dCTP buffer comprising 0.1mM of each unlabeled deoxynucleotide was added to the sample followed by 5μL of [α-^{32}P]-dCTP (10μCiμL^{-1}) (Amersham, Cat No- AA0005) and 1μL of Exo(-) Klenow fragment (5Uμl^{-1}). The sample was mixed gently but thoroughly and incubated at 37^{0}C for 1 to 3 hours. After incubation 2μL of Na_2EDTA (0.5M) was added to stop the reaction. The radiolabeled probe was denatured by heating to 100^{0}C for 5 minutes, and then cooled on ice for 1 minute. The reaction was collected by centrifugation at 14, 000 rpm for 1 minute, and then added to the hybridisation solution.

Section 2.8.7 Probe hybridisation.

Nucleic acid transfer membranes comprising β-actin, SCL and LYL-1 cDNA products were pre-hybridised in Church and Gilbert hybridisation solution comprising 0.5M $Na_2HPO_4.2H_2O$, 1mM Na_2EDTA (pH 8), 7% SDS, 1%BSA (fraction V) and 10μgmL^{-1} denatured sonicated salmon sperm DNA (GIBCO BRL, Cat No- 15632-011) (see Section 2.8.9) for 2 to 3 hours at 65^{0}C. Then approximately 25ng of denatured α^{32}P-dCTP labeled probe (see Section 2.8.6) was added to the hybridisation solution and the hybridisation allowed to proceed for at least 14 hours at 65^{0}C. After hybridisation the membranes were washed in Church and Gilbert high stringency solution (see Section 2.8.8) comprising 40mM Na_2HPO_4 (pH7.2), 1mM Na_2EDTA and 1%SDS four times for 30 minutes each at 65^{0}C. The radioactive signal from the membranes was measured by autoradiography using pre-flashed X-ray Fuji Film and processed on an automated system (Kodak M35 X-OMAT).

Section 2.8.8 Church and Gilbert Wash solution for southern hybridisation.

COMPOSITION	**AMOUNT**
Na_2HPO_4	40mL of 1M stock solution
Na_2EDTA	2mL of 0.5M stock solution
SDS	50mL of 20%w/v stock solution
Sterile H_2O	up to 1000mL

40mL of 1M Na_2HPO_4, 2mL of 0.5M Na_2EDTA, and 50mL of 20%w/v sodium dodecyl sulphate (SDS- Fisher, Cat No- S/ P530/53) was added to 908mL of sterile H_2O at room temperature to give a final concentration of 40mM Na_2HPO_4, 1mM Na_2EDTA, and 1% SDS. The Wash solution was stored at room temperature for up to 3 months.

Section 2.8.9 *Church and Gilbert hybridisation solution for Southern hybridisation.*

COMPOSITION	AMOUNT
$Na_2HPO_4.2\ H_2O$	500mL of 1M stock solution (pH 7.2)
Na_2EDTA	2mL of 0.5M stock solution (pH 8)
SDS	350mL of 20%w/v stock solution
BSA (fraction V)	10g
Sterile H_2O	up to 1000mL

10g of BSA (fraction V- Sigma, Cat No- A-4503) was dissolved slowly in 500mL of sterile H_2O. After complete dissolution of the BSA, 500mL of 1M $Na_2HPO_4.2\ H_2O$, 2mL of 0.5M Na_2EDTA, and 350mL of 20%w/v SDS was added to the solution and the volume made up to 1000mL with sterile H_2O to give a final concentration of 0.5M $Na_2HPO_4.2\ H_2O$, 1m M Na_2EDTA, 7% SDS, and 1% BSA. The solution was then filtered through 0.45μm filters and stored in 50mL aliquots at -20^0C. Immediately prior to use, freshly denatured sonicated salmon sperm DNA (GIBCO BRL, Cat No- 15632-011) was added to the hybridisation solution to a final concentration of $10\mu gmL^{-1}$.

Section 2.8.10 *$Na_2HPO_4.2\ H_2O$ solution (1M).*

COMPOSITION	AMOUNT
$Na_2HPO_4.2\ H_2O$	71g
Sterile H_2O	up to 1000mL

71g of $Na_2HPO_4.2\ H_2O$ (BDH, Cat No- 103834G) was slowly added to 900mL of sterile H_2O. The pH was adjusted to 7.2 with concentrated phosphoric acid (Sigma, Cat No- P6560) and the volume made up to 1000mL with sterile H_2O to give a final concentration of 1M $Na_2HPO_4.2\ H_2O$. The solution was autoclaved and stored at room temperature for up to 3 months.

Section 2.9 In vitro colony assays in methylcellulose media.

Section 2.9.1 Preparation of cells for in vitro colony assays in methylcellulose.

Spleen and bone marrow cells (flushed from the femur and tibia of both hind legs) were collected in 200µl of Iscove's modified Dulbeccos medium (IMDM) containing 2% fetal bovine serum (FBS). The cells were diluted to 1:50 and 1:100 in 3% acetic acid in H_2O, and the number of nucleated cells counted on a haematocytometer.

Section 2.9.2 In vitro colony assay of bone marrow and spleen cells in the presence of stem cell factor (SCF), erythropoietin (Epo), interleukin-3 (IL-3) and interleukin-6 (IL-6).

Cells were diluted to $1.5x10^5\ mL^{-1}$ in IMDM. 0.3mL was mixed thoroughly with 3mL of Methocult™ GF (see Section 2.10.1). 1.1mL of the cell suspension was dispensed evenly into two 35x10mm culture dishes at a final concentration of $1.5x10^4$ cells per dish, and incubated at 37^0C in a 5% CO_2 humidified (>95%) incubator. Immature BFU-e and CFU-GM colonies were scored at day 7, and day 10 respectively. CFU-GEMM was scored at day 12.

Section 2.9.3 In vitro colony assay of bone marrow and spleen cells in the presence of Epo.

Cells assayed in the presence of Epo only were diluted to $2x10^6$ cells mL^{-1} in IMDM, and 0.3mL mixed thoroughly with 3mL of Methocult™ (see Section 2.10.1). 1.1mL of the cell suspension dispensed evenly into two 35x10mm culture dishes at a final concentration of $2x10^5$ cells per dish. CFU-e colonies were scored at day 2, and mature BFU-e scored at day 4.

Section 2.10 Materials used for in vitro colony assay.

Section 2.10.1 Colony assay in the presence of Epo only.

Colony assay of murine spleen and bone marrow cells was performed using methylcellulose-based medium. Cells assayed in the presence of Epo only were mixed with MethoCult (Stem Cell Technologies- Cat No M3434) comprising1% methylcellulose in Iscoves MDM, 15% FBS, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 1% BSA, 10μgmL^{-1} human transferrin, and 3UmL^{-1} Epo.

Section 2.10.2 Colony assay in the presence of SCF, Epo, IL-3, and IL-6.

Colony assay of murine spleen and bone marrow cells was performed using methylcellulose-based medium. Cells assayed in the presence of SCF, Epo, IL-3, and IL-6 were mixed with MethoCult (Stem Cell Technologies- Cat No M3334) comprising 1% methylcellulose in Iscoves MDM, 15% FBS, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 1% BSA, 10μgmL^{-1} bovine pancreatic insulin, 200μgmL^{-1} human transferrin, 3UmL^{-1}Epo, 10ngmL^{-1} IL-3, 10ngmL^{-1} IL-6 and 50ngmL^{-1}SCF.

Section 2.11 Other molecular biology techniques employed in this study.

Section 2.11.1 Northern hybridisation.

Approximately 30ng of total RNA (see Section 2.8.1) was mixed with 25µl of Electrophoresis Sample Buffer (see Section 2.12.16) and heated at 70^0C for 15 minutes. Samples were placed on ice for 1 minute, centrifuged at 14,000 rpm for 30 seconds and mixed with 1µL of 1mgmL^{-1} RNAse-free ethidium bromide. The total RNA samples were loaded on to a formaldehyde/ agarose gel immersed in 1X MOPS (pH 7) (see Section 2.12.1). Samples were separated by electrophoresis (typically 80V for 1 hour), examined by UV illumination and transferred to a Hybond^{TM}N nylon membrane (Amersham, Cat No- RPN 303N) by capillary transfer in 10X SSC transfer buffer (see Section 2.12.2). The membrane was then baked in an oven at 80^0C for 2 hours and stored in a dust-free environment at room temperature until used in hybridisation experiments (see Sections 2.8.8 and 2.8.9).

Section 2.11.2 Southern hybridisation of genomic DNA.

Southern hybridisation was used to determine the genotype of LYL-1 mutant mice. Approximately 500µg-1mg of genomic DNA (20µl of a 25-50µgµL^{-1}) extracted from tail (see Section 2.11.2) was incubated for 18-24 hours at 37^0C with an EcoRV digestion mixture comprising 3µL of a 10X BSA (fraction V) (Sigma, Cat No- 4503), 3µL of 10X Reaction Buffer (Boehringer Mannheim, Cat No- 85116922-34), 2µL of EcoRV (40U/µl) (Boehringer Mannheim, Cat No- 84787320-43) and 2µL of sterile H_2O to give a total volume of 30µL. After incubation the samples were separated by agarose (0.8% / 1X TBE) gel electrophoresis. The gel was submerged in 0.25M HCl (see Section 2.12.3) and shaken gently until the bromophenol blue has changed colour (approximately 15 minutes). The gel was then transferred to 0.4M NaOH and shaken gently until the bromophenol blue colour returned. Alkali capillary apparatus was set up to transfer the contents of the gel to Hybond N+ nylon transfer membrane (Amersham, Cat No- RPN 303B) overnight at room temperature. The

membrane was air dried and stored in a dust-free environment until used in hybridisation experiments (see Sections 2.8.7, 2.8.8, and 2.8.9).

Section 2.11.3 Restriction endonuclease digestion of plasmid DNA.

The method used for restriction digestion of plasmid DNA was modified from the protocols described in Sambrook, et al, 1989 (Molecular Cloning, A Laboratory Manual, 2nd edition 1989, Book 1, section 5.28). 0.2 to 1.0μg of plasmid DNA was used routinely in restriction endonuclease experiments. The plasmid DNA was mixed with 2μL of restriction enzyme digestion buffer (10X), 1 to 2U of restriction enzyme and sterile H_2O up to 20μL in a 0.2mL microcentrifuge tube. Digestion reactions were incubated at 37^0C for 1 to 2 hours then stopped by addition of 1μL of Na_2EDTA (0.1M) (BDH, Cat No-100935V). Reaction products were precipitated by addition of 2 volumes of absolute ethanol and one quarter volume ammonium acetate (7.5M) (BDH, Cat No-100134T), followed by incubation at -80^0C for 1 hour and centrifugation at 14,000 rpm for 15 minutes at 4^0C. The supernatant was removed and replaced by 0.5mL of 70% ethanol proceeded by centrifugation at 14,000 rpm for 10 minutes. The supernatant was removed by aspiration and the pellet air dried thoroughly before re-suspension in sterile H_2O. When double digestions were performed the re-suspended DNA was mixed with the appropriate buffer and restriction enzyme and incubated for a further 1 to 2 hours, after which the reactions products were precipitated as just described. The reaction products were examined by ethidium bromide ($0.5\mu gmL^{-1}$) stained agarose gel (0.8%) electrophoresis in 1X TBE electrophoresis buffer containing $0.5\mu gmL^{-1}$ ethidium bromide (see Section 2.12.10, and 2.12.13).

Section 2.11.4 Electroporetic transformation of competent bacterial strain JM109 with plasmid DNA.

1μL of plasmid DNA (approximately 250ng) was mixed with 50μL JM109 (strain of E. coli) in a sterile microcentrifuge tube. The mixture was transferred to an ice cold electroporation curvette and place in an electroporator (BioRad Gene Pulser II) pre-programmed to deliver an electric current of 2.5kV, at 25μF capacitance and 200 ohms resistance across the plasmid DNA/ cell mixture. Immediately after electroporation 0.5mL of Liquid Broth was added to the curvette and the sample mixed thoroughly before being transferred to a microcentrifuge tube and shaken for 40 minutes at 37^0C. 1μL, 10μL and 100μL of sample was smeared onto separate Luria agar Petri dishes containing 100mgmL^{-1} ampicillin. The plates incubated in an oven at 37^0C for 12 to 18 hours.

Section 2.11.5 Amplification of plasmid DNA.

10 individual colonies were isolated with the narrow end of a sterilised pipette tip and the tip placed in a 50mL Falcon tube containing 2mL of LBA. The culture was stirred vigorously in an oven at 37^0C for 12 to18 hours, and then between 100μL and 500μL of the 2mL culture was added to a sterilised 500mL Conical flask containing 100mL of LBA. The culture was stirred vigorously at 37^0C for 12 to18 hours.

Section 2.11.6 Extraction and Purification of plasmid DNA.

The Qiagen Mini- and Midi Plasmid Prep Kits (Qiagen, Cat No- 12243) were used for the routine extraction and purification of plasmid DNA in accordance with manufacturer protocols.

Section 2.11.7 Ligation of PCR products into pGemT plasmid vector.

Ligation of PCR products in to pGemT vector (Promega- Cat No- A3610) was performed according to manufacturers guidelines. 25ng of pGemT vector (50ngμL^{-1}) was placed in a

0.5mL centrifugation tube and mixed with 25ng of cDNA insert (1:1 molar ratio), 2X DNA Ligase buffer (10X), and 3U of T4 DNA ligase ($3U\mu L^{-1}$). The final volume was made up to 10μL with sterile H_2O. The reaction mixture was incubated at 4^0C overnight. The ligation products were precipitated by incubating the 10μL with 2.5 volumes of ice-cold absolute ethanol, and one-quarter-volume ammonium acetate (7.5M) for 1 hour at -80^0C, followed by centrifugation at 14,000 rpm for 30 minutes at 4^0C. The supernatant was removed and replaced with 70% ethanol, followed by a further centrifugation at 14,000 rpm for 15 minutes at room temperature. The ethanol was removed and the pellets air-dried completely before addition of 10μL of sterile H_2O. The pellet of ligation product was allowed to dissolve at room temperature for 1 hour, and then 2μL was mixed with 50μL JM109 competent cells and the sample prepared for transformation (see Section 2.11.4).

Section 2.12 Materials used in molecular biology.

Section 2.12.1 10X 3-[N-Morpholino] propane sulphonic acid (MOPS).

COMPOSITION	**AMOUNT**
MOPS	41.2g
Na_2-acetate	800mL of 100mM stock solution
Na_2EDTA	20mL of 0.5M stock solution
Sterile H_2O	up to 1000mL.

41.2g of MOPS (Sigma, Cat No-.M5162) was dissolved in 800mL of 100mM Na_2-acetate solution. The pH was adjusted to 7 with 2N NaOH. Then, 20mL of 0.5M Na_2EDTA (pH 8) was added and the volume adjusted to 1000mL with sterile H_2O to give a final concentration

of 0.2M MOPS, 0.5M Na_2.acetate, and 0.01M Na_2EDTA. The MOPS is used at a working concentration of 1X. MOPS buffer was stored at room temperature.

Section 2.12.2 20X SSC.

COMPOSITION	AMOUNT
NaCl	87.7g
Na_3- citrate	44.1g
Sterile H_2O	up to 1000mL

87.7g of NaCl (Fisher, Cat No- S/ 310/ 63) was dissolved in 900mL of sterile H_2O. 44.1g of tri-sodium citrate (Fisher, Cat No- S/ 3320/ 53) was added to the solution, and the pH adjusted to 7 with 0.25M HCl (see Section 2.12.3). SSC was autoclaved, and stored at room temperature. This stock solution was used to prepare working concentrations e.g. 2X.

Section 2.12.3 0.25M HCl.

COMPOSITION	AMOUNT
HCl	7.7mL
Sterile H_2O	up to 1000Ml

7.7mL of concentrated HCl (Sigma, Cat No- 920-1, 32M stock solution) was added to 992.3mL sterile H_2O. 0.25M HCl was stored at room temperature for 3 months.

Section 2.12.4 10X TBE.

COMPOSITION	AMOUNT
Tris	108g
Boric Acid	55g
Na_2EDTA (pH8)	40mL of a 0.5M stock solution
Sterile H_2O	up to 1000mL

108g of Tris (GIBCO BRL, Cat No- 15504-020) and 55g of boric acid (BDH, Cat No-274094Q) was dissolved in 900mL of sterile H_2O, followed by 40mL of 0.5M Na_2EDTA (pH8). The pH of 10X TBE was adjusted to 8.3 with 0.25M HCl. The final volume was made up to 1000mL with sterile H_2O. The TBE was autoclaved, and used at a working concentration of 1X (89mM Tris- borate, 2mM Na_2EDTA).

Section 2.12.5 0.4M NaOH.

COMPOSITION	AMOUNT
NaOH pellets	16g
Sterile H_2O	1000mL

16g of NaOH pellets were dissolved in 1000mL sterile H_2O. The solution was used as a buffer in alkali capillary transfer procedures (see Section 2.12.5).

Section 2.12.6 1X TBE (pH8.3) electrophoresis buffer for agarose gel electrophoresis of DNA.

COMPOSITION	AMOUNT
TBE	100mL of 10X TBE (pH8.3) stock solution
Ethidium bromide	50µL of a 10mgmL^{-1} stock solution
Sterile H_2O	up to 900mL

100mL of 10X TBE (p H.3) was added to 900mL of sterile H_2O, followed by 50µL of a 10mgmL^{-1} ethidium bromide stock solution to give a final concentration of 0.5µgmL^{-1} ethidium bromide and a working concentration of 1X TBE (89mMTris-borate, 2mM Na_2EDTA).

Section 2.12.7 40X Wash buffer for GlassMax cDNA Purification.

COMPOSITION	AMOUNT
Na_2EDTA	8mL of 0.5M stock solution
Tris-HCl (pH 7.5)	40mL of 1M stock solution
NaCl	23.37g
Sterile H_2O	up to 100mL

23.7g of NaCl was dissolved in 50mL of sterile H_2O. 8mL of 0.5M Na_2EDTA, and 40mL of 1M Tris-HCl (pH 7.5) was added to the solution and the volume made up to 100mL with sterile H_2O. The wash buffer was autoclaved and used at a working concentration of 1X to give a final concentration of 1mM Na_2EDTA, 10mM Tris-HCl (pH 7.5) and 0.1M NaCl.

Section 2.12.8 Rinse solution for formaldehyde/ agarose gel.

COMPOSITION	AMOUNT
0.4M NaOH	125mL
20X SSC	50mL
Sterile H_2O	up to 1000mL

125mL of 0.4M NaOH, and 50mL of 20X SSC was added to 825mL of sterile H_2O, to give final concentrations of 0.05M NaOH and 1X SSC. This solution was stored at room temperature for 6 months.

Section 2.12.9 Lysis buffer for extraction of genomic DNA.

COMPOSITION	AMOUNT
Tris-HCl (pH 8)	6g
Na_2EDTA	18.6g
NaCl	2.92g
SDS	5mL of a 20%w/v stock solution of SDS
Sterile H_2O	up to 500mL

6g of Tris (GIBCO BRL, Cat No-15504-020), 18.6g of Na_2EDTA, and 2.92g of NaCl were dissolved in 450mL of sterile H_2O. 4mL of a 20%w/v SDS (Fisher, Cat No- S/ P530/ 53) was then added to the solution and the volume made up to 500mL with sterile H_2O. Tail lysis buffer comprised 50m M Tris-HCl (pH 8), 100mM Na_2EDTA, 100mM NaCl, and 0.2% SDS. Immediately prior to use, 35µL of a $10mgmL^{-1}$ stock solution of Proteinase K was added to each 0.7mL of tail lysis buffer used to digest the tail. This gave a final concentration of $0.5mgmL^{-1}$ Proteinase K.

Section 2.12.10 Preparation of agarose gel for electrophoresis of DNA.

COMPOSITION	AMOUNT
Agarose	1.6g
TBE (pH8.3)	20mL of 10X stock solution
Ethidium bromide	10μL of a 10mgmL^{-1} stock solution
Sterile H_2O	up to 200mL

1.6g of agarose (Boehringer, Cat No- 85525620/20) was added to 150mL of sterile H_2O and heated until completely dissolved. 20mL of 10X TBE (pH8.3), and 10μL of a 10mgmL^{-1} ethidium bromide solution was added to the agarose solution, and the volume made up to 200mL with sterile H_2O to give a final concentration of 0.5μgmL^{-1} of ethidium bromide, 1X TBE and 0.8%^{w}/v agarose.

Section 2.12.11 Preparation of formaldehyde/ agarose gel for electrophoresis of RNA.

COMPOSITION	AMOUNT
Agarose	1.5g
MOPS (pH7)	10mL of 10X stock solution
Formalin (37% formaldehyde)	5.1mL
Sterile H_2O	87mL

1.5g of agarose (Boehringer Mannheim, Cat No-85525620/ 20) was added to 87mL of sterile H_2O. The solution was heated until the agarose completely dissolved. The agarose solution was cooled to 50^0C and then 10mL of 10X MOPS (pH7), followed by 5.1mL of Formalin (Sigma, Cat No- F1635) was added and the solution mixed thoroughly. The formaldehyde/ agarose gel solution was poured and allowed to set for at least 60 minutes. Once set, the gel was immersed in 1X MOPS (p H7) (Sigma, Cat No- M5162), and electrophoresed at 50V for 15 minutes, and the gel wells flushed to remove residual formaldehyde prior to loading RNA samples.

Section 2.12.12 Preparation of RNA electrophoresis buffer.

COMPOSITION	**AMOUNT**
Deionised formamide	0.75mL
MOPS (pH8)	0.15mL of a 10X stock solution
Formaldehyde	0.24mL of 37% stock solution
Glycerol	0.1mL of autoclaved stock solution
Bromophenol blue	0.08mL of a 10%w/v stock solution
H_2O	0.1mL

5μL of total RNA (10-30μg) was mixed with 25μL of RNA electrophoresis buffer (5 volumes). The sample was incubate at 70^0C for 15 minutes, chilled on ice for 1 minute and centrifuged for 30 seconds at 14,000 rpm to collect the sample. 1μL of a $1mgmL^{-1}$ ethidium bromide stock solution was added to the sample, which was loaded onto a formaldehyde/ agarose gel (see Section 2.12.15). The samples were separated by electrophoresis at 5 to 10V for 18 hours.

Section 2.12.13 Preparation of 10X DNA electrophoresis buffer.

COMPOSITION	AMOUNT
Xylene cyanole	0.04g
Glycerol	5mL
Bromophenol blue	0.04g
H_2O	up to 10mL

0.04g of bromophenol blue (BDH, Cat No-44305), and 0.4g of xylene cyanole (Sigma, Cat No- X4126) was dissolved in 5mL of sterile H_2O. 5mL of 50% glycerol was added to the mixture and the volume made up to 10mL with sterile H_2O to give a final concentration of 0.4% bromophenol blue, 0.4% xylene cyanole, and 50% glycerol. The 10X DNA electrophoresis buffer was stored in 100µL aliquots at -20^0C.

Section 2.13 Statistical analysis.

All values are reported as mean +SEM. Statistical significance for two unpaired groups was assessed by the Student's t test ($P<0.05$). ANOVA was used to assess variance between multiple groups.

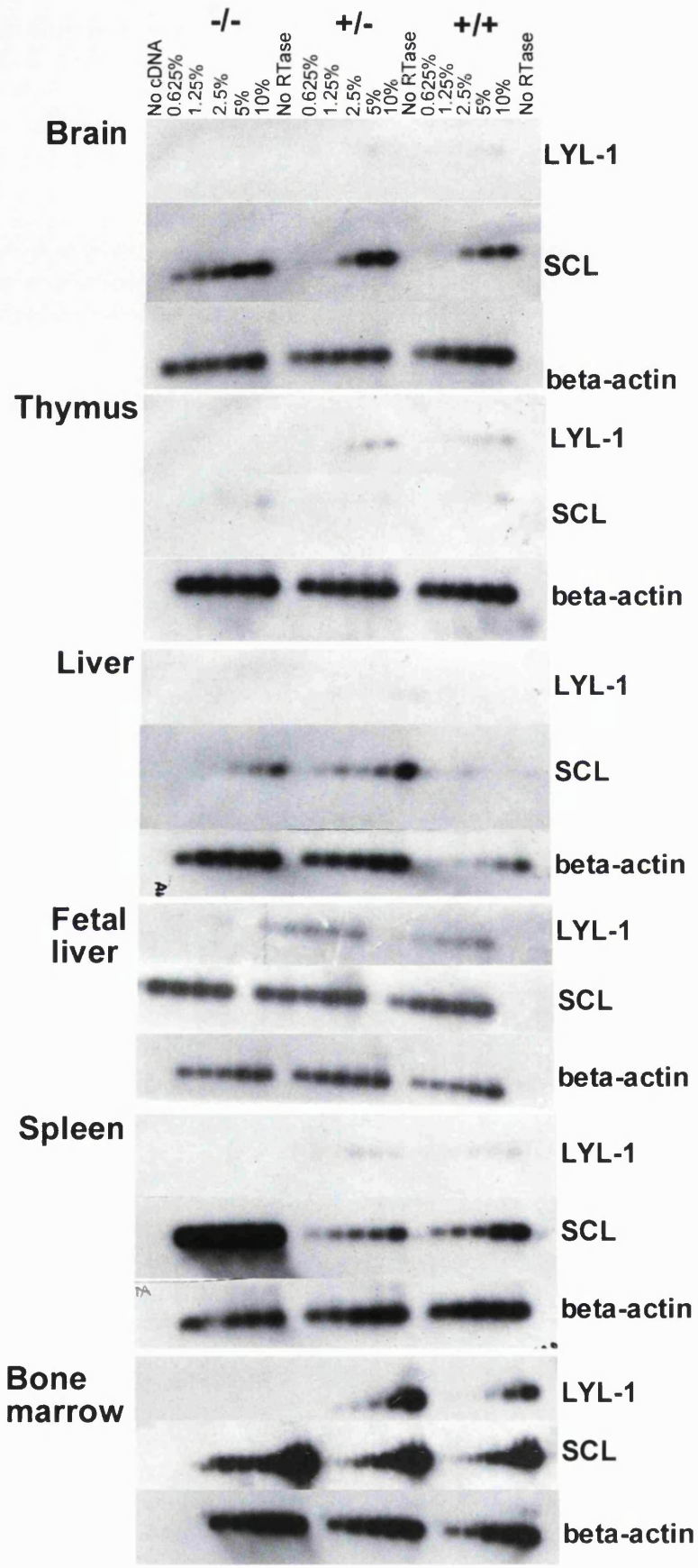
Chapter 3. Results.

Section 3.1 Relative expression of SCL and LYL-1 mRNA in mouse tissues.

Semi-quantitative RT-PCR was used to determine the relative expression of SCL mRNA between samples of cDNA derived from the brain, thymus, liver, 14.5dpc fetal liver, spleen and adult bone marrow of LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice (for details see Chapter 2 Methods and Materials section 2.7).

Figures 12 show the β-actin, SCL and LYL-1 signals for brain, liver, thymus, 14.5dpc fetal liver, bone marrow and spleen of LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice. Disruption of the LYL-1 locus was confirmed by the absence of LYL-1 mRNA in LYL-$1^{-/-}$ mice, and the presence of lacZ transcript in RT-PCR experiment specific for the E coli derived lacZ (data not shown). The intensity of the signals from the β-actin RT-PCR reactions were directly related to the amount of cDNA used in each reaction. Therefore, the relative amount of cDNA in each LYL$^{+/+}$, LYL$^{+/-}$, and LYL-$1^{-/-}$ β-actin RT-PCR could be estimated. For example, the amount of 14.5dpc fetal liver cDNA used in SCL and LYL-1 specific RT-PCR reactions was highest in LYL$^{+/-}$, followed by LYL-$1^{-/-}$, and LYL$^{+/+}$ (Figure 12). Therefore, the SCL and LYL-1 signals from the LYL-$1^{-/-}$, and LYL$^{+/+}$ 14.5dpc fetal liver could be corrected for the difference in the amount of cDNA relative to the LYL$^{+/-}$ β-actin signal. Using this analysis, level of LYL-1 mRNA expression was found to be highest in the bone marrow and 14.5dpc fetal liver (Figure 12). LYL-1 mRNA expression was 16-fold lower in the spleen and thymus, and at least 32-fold lower in the brain. The liver had an almost undetectable level of LYL-1 mRNA. These results confirm the presence of LYL-1 mRNA at highest levels in haematopoietic organs. However, LYL-1 mRNA may also expressed in cells present in non-haematopoietic organs, such as brain, in the adult mouse.

Figure 12. Semi-quantitative RT-PCR for SCL mRNA isolated from brain, thymus, liver, 14.5dpc fetal liver, spleen and bone marrow of LYL-$1^{+/+}$, LYL-$1^{+/-}$ and LYL-$1^{-/-}$ mice. 5µl (1:1) of each cDNA, and 5µl of 5%, 2.5%, 1.25%, and 0.625% of the total cDNA (see Section 2.7) were amplified in LYL-1 (top panel), SCL (middle panel), and β-actin (bottom panel) specific PCR reactions. Reactions with water replacing cDNA, and with water replacing the reverse transcriptase were performed as negative controls. Targeting of the LYL-1 locus was confirmed by the absence of LYL-1 mRNA in each tissue examined. There was no significant difference in the relative expression of SCL mRNA between LYL-$1^{-/-}$ (left), LYL-$1^{+/-}$ (middle), and LYL-$1^{+/+}$ (right) brain, thymus, liver, 14.5dpc fetal liver, and bone marrow. The spleen of LYL-$1^{-/-}$ mice expressed more SCL mRNA than LYL-$1^{+/-}$ and LYL-$1^{+/+}$ littermate controls.

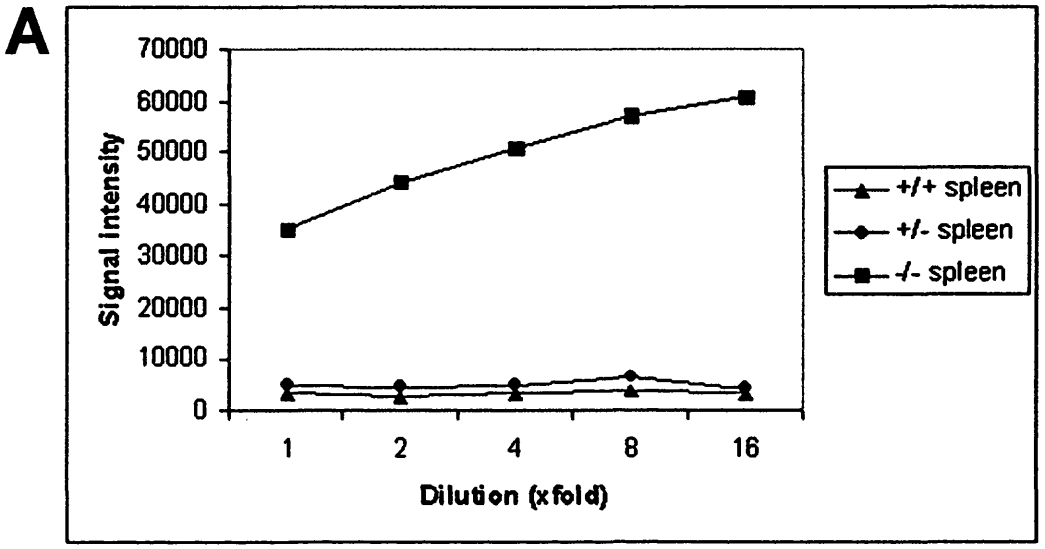

-/-
+/-
+/+
No cDNA
0.625%
1.25%
2.5%
5%
10%
No RTase
Brain
LYL-1
SCL
beta-actin
Thymus
Liver
Fetal liver
Spleen
Bone marrow

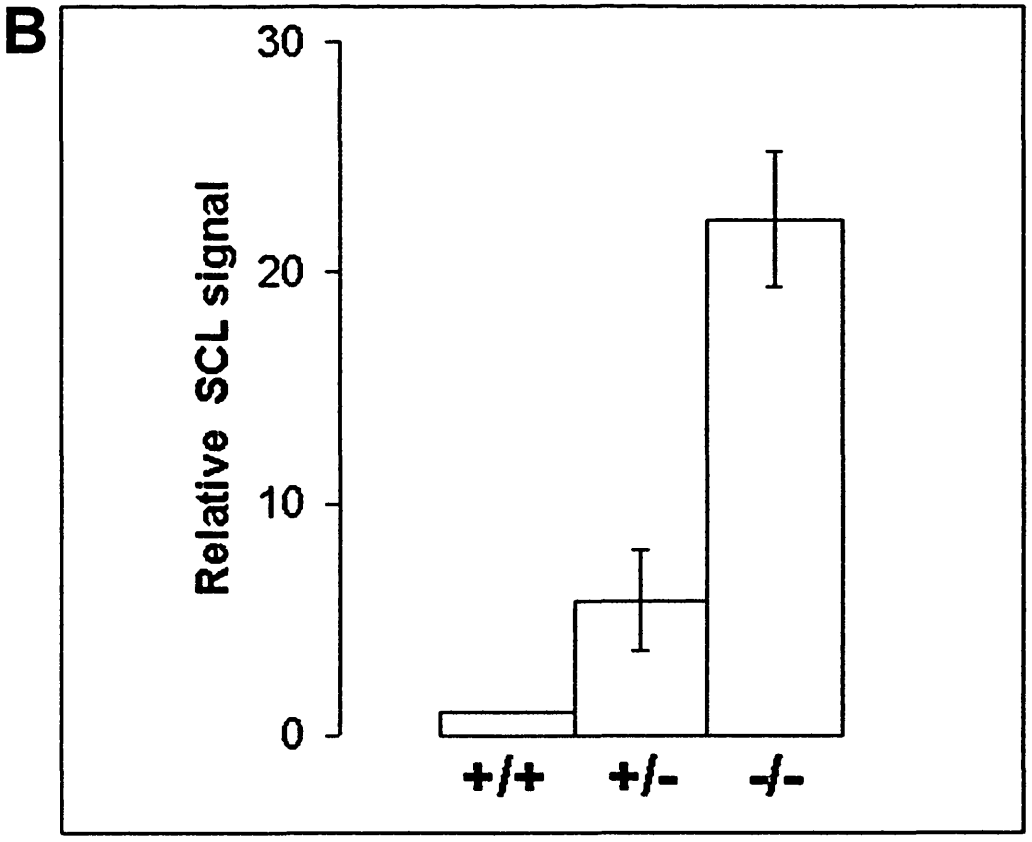
SCL mRNA expression mirrors that of LYL-1 with highest levels in the 14.5dpc fetal liver and bone marrow (Figure 12). Correction of the SCL mRNA signals for differences in cDNA input showed that these organs expressed approximately 8-fold more SCL mRNA than the spleen. The brain expressed 16-fold less SCL mRNA, and the liver expressed at least 32-fold less SCL mRNA than 14.5dpc fetal liver. The thymus expressed the lowest level of SCL mRNA (Figure 12). These data show that SCL mRNA expression is highest in haematopoietic organs such as bone marrow, fetal liver and spleen, but was also expressed at a somewhat lower level in the brain and liver. The rank order of expression of LYL-1 mRNA was 14.5dpc fetal liver/ bone marrow, thymus/ spleen, brain and liver; and for SCL the order of expression was 14.5dpc fetal liver/ bone marrow, spleen, brain, liver and thymus. As predicted, the level of LYL-1 mRNA in cDNA samples derived from LYL-$1^{+/-}$ tissues was approximately half the level in corresponding LYL-$1^{+/+}$ tissues (data not shown).

Section 3.2 SCL mRNA expression is increased in the spleen of LYL-$1^{-/-}$ mice.

The semi-quantitative RT-PCR assay employed in this study was able to identify changes of no less than 4-fold in the level of SCL mRNA expression in heterogeneous cell populations from the different tissues analysed (for details see Chapter 2 Methods and Materials section 2.1). With this degree of sensitivity, there was no difference in the level of SCL mRNA expression in the 14.5dpc fetal liver, brain, thymus and liver of LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice (Figure 12). There was no difference in level of SCL mRNA expression in LYL-$1^{-/-}$ bone marrow or fetal liver compared to controls, although a marginal increase in the bone marrow may have been at the very limit of the assay and therefore could not be determined accurately. However, the level of SCL mRNA in the LYL-$1^{-/-}$ spleen was markedly increased compared to controls (Figure 12). Results from five independent sets of data are represented in Figure 13. The LYL-$1^{-/-}$ spleen expressed 22.2 +2.9-fold more SCL

Figure 13. Enhanced expression of SCL mRNA in LYL-1$^{-/-}$ spleen. (A). SCL signals corrected for differences in the amount of cDNA in each reaction showed LYL-1$^{-/-}$ spleen to contain more SCL mRNA than controls. (B). The β-actin signals from the semi-quantitative RT-PCR reactions were used to standardize the SCL signals from LYL-1$^{+/+}$, LYL-1$^{+/-}$ and LYL-1$^{-/-}$ spleen. LYL-1$^{-/-}$ spleen expressed 22.2 + 2.9-fold more SCL mRNA than LYL-1$^{+/+}$ littermates (n=4) ($P<0.05$), and 5.8 + 2.2-fold more than LYL-1$^{+/-}$ littermates (n=4) ($P<0.05$). The level of SCL mRNA in LYL-1$^{+/-}$ mice (n=6) was not statistically different to SCL mRNA in the LYL-1$^{+/+}$ spleen (n=6) ($P<0.05$) but was significantly lower than SCL mRNA expression in the LYL-1$^{-/-}$ spleen.


A
70000
60000
50000
40000
30000
20000
10000
0
Signal intensity
1
2
4
8
16
Dilution (xfold)
+/+ spleen
+/- spleen
-/- spleen


B
30
20
10
0
Relative SCL signal
+/+
+/-
-/-

mRNA than the spleen of LYL-$1^{+/+}$ mice. Although there was a tendency towards increased SCL mRNA expression in the LYL-$1^{+/-}$ spleen compared to LYL-$1^{+/+}$ spleen, this increase was not significant (Figure 13). The increase in SCL mRNA expression in the LYL-$1^{-/-}$ spleen was not gender specific, and was not affected by age, since it was observed in the spleen from male and female mice, and in mice of eight weeks up to nine months of age (data not shown).

These data showed that in the absence of a functional LYL-1 protein, there is a dramatic, and specific increase in the level of SCL mRNA in the spleen.

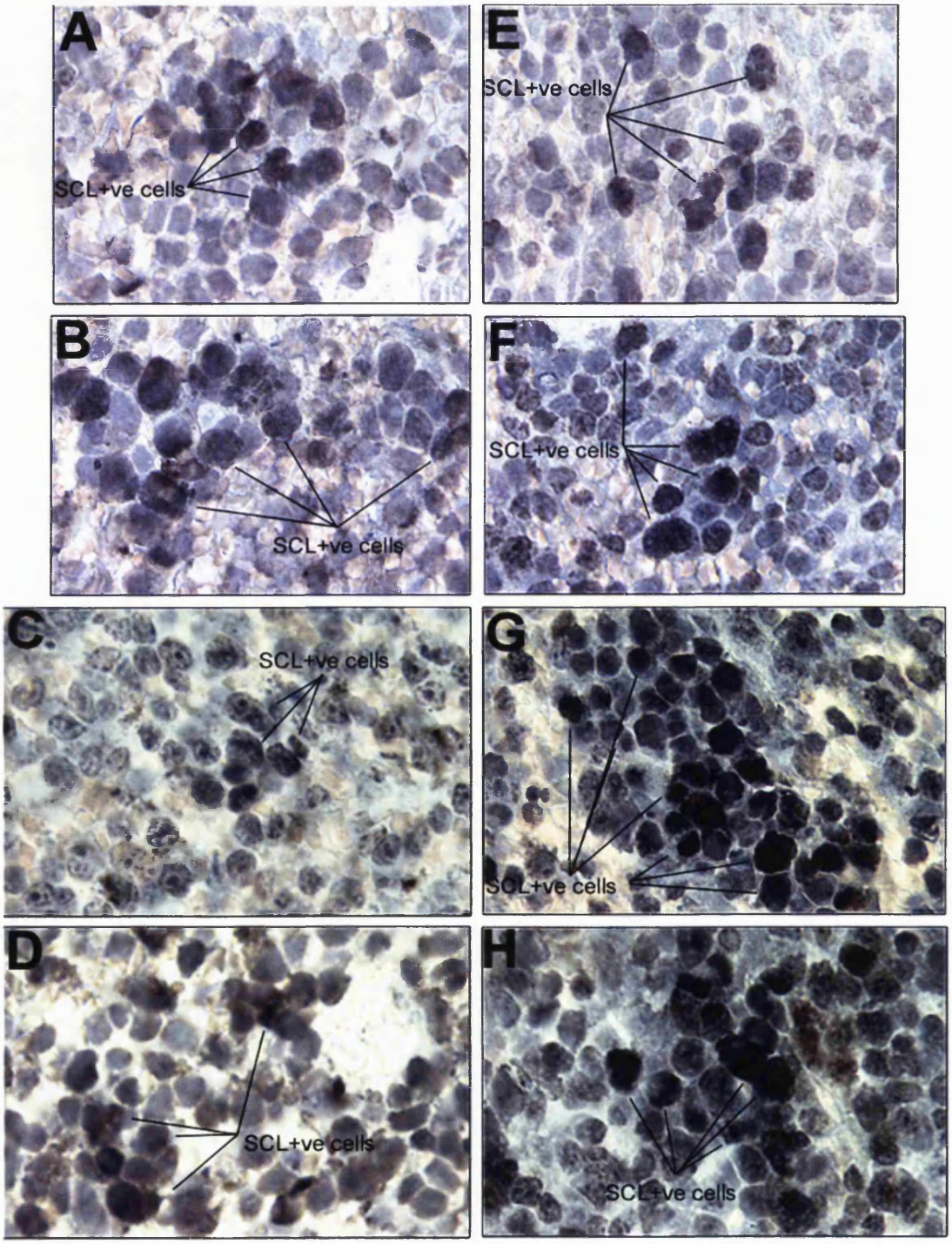
The increase in SCL mRNA in the spleen of LYL-$1^{-/-}$ mice could be due to an increase in the number of cells expressing SCL mRNA, or to an increase in the level of SCL mRNA expression in individual cells. The semi-quantitative RT-PCR data did not elucidate the contribution of these (or other) causes for the increase in SCL mRNA. In order to determine the expression of SCL mRNA at the cellular level, *in situ* RT-PCR specific for SCL mRNA was performed in 5μm paraformaldehyde-fixed paraffin sections of spleen from LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice.

Section 3.3 The spleen of LYL-$1^{-/-}$ mice comprised more cells expressing SCL mRNA than the spleen of LYL-$1^{+/+}$ mice.

Figures 5, and 14 show the result of *in situ* RT-PCR specific for SCL mRNA in the spleen of LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice (see Chapter 2 Methods and Materials section 2.5). Cells positive for SCL mRNA (SCL $mRNA^{+ve}$) were located in the red pulp of the spleen of both LYL-$1^{+/+}$ (Figures 5A-C, G) and LYL-$1^{-/-}$ mice (Figures 5D-F, H). The specificity of the signals was confirmed by negative control experiments performed in the absence of reverse

Figure 14. Many of the SCL mRNA^{+ve} cells are arranged into clusters in the LYL-1$^{+/+}$ (A-D) and LYL-1$^{-/-}$ (E-H) spleen. LYL-1$^{-/-}$ spleen contained more clusters that were larger (G, H) than those found in LYL-1$^{+/+}$ spleen (C, D). The level SCL mRNA expression in some cells in the LYL-1$^{-/-}$ spleen (E, F, and G) was higher than in the majority of cells in LYL-1$^{+/+}$ spleen (A, B, and C). All images were taken at 60x magnification.

LYL-1 -/- spleen comprise more cells that express SCL mRNA than LYL-1+/+ spleen.



transcriptase (Figure 5I) or in the absence of SCL specific primers (Figure 5J). SCL mRNA^{+ve} cells were found in the splenic parenchyme (Figures 5B, C, and G; 5E, F, H) and the subcapsular region (Figures 5A; 5D, and F). LYL-1$^{-/-}$ spleen contained more SCL mRNA^{+ve} cells (Figure 5D-E, H) than LYL-1+/+ spleen (Figure 5A-C, G). Many of the SCL mRNA^{+ve} cells were arranged in clusters in the spleen of LYL-1$^{+/+}$ (Figures 14A-D), and LYL-1$^{-/-}$ (Figures 14E-H) mice. Most clusters in the LYL-1$^{-/-}$ spleen were composed of more SCL mRNA^{+ve} cells (Figure 14E, G, H) than in LYL-1$^{+/+}$ spleen (Figures 14A-C). It was apparent that some of the cells in the LYL-1$^{-/-}$ spleen stained stronger (Figures 14G) than many of the SCL mRNA^{+ve} cells in the LYL-1$^{+/+}$ controls (Figure 14C). Although the number of SCL mRNA^{+ve} cells in the LYL-1$^{+/+}$ and LYL-1$^{-/-}$ spleen were not quantified accurately, the spleen of LYL-1$^{-/-}$ mice comprised approximately 5 to 10 times more SCL mRNA^{+ve} cells than the LYL-1$^{+/+}$ spleen.

These data showed that the increase in SCL mRNA in the LYL-1$^{-/-}$ spleen determined by semi-quantitative RT-PCR was primarily due to an increase in the number of cells expressing SCL mRNA. It also suggested that in the absence of a functional LYL-1 protein, the number of cells expressing SCL mRNA was increased in the spleen.

The spleen can serve as a site for erythroid expansion when bone marrow erythropoiesis is stressed (Broudy et al., 1996) and serves as a station for the scavenging of aged erythrocytes by macrophages (a process known as erythrophagocytosis) (Caceci, http://www.cvm.tamu.edu/). Development of the most primitive HSCs and progenitors in the spleen is documented in many studies (Broudy, et al., 1996; Hendrikx, et al., 1996; Hunt, 1998) and the spleen is the major site of regenerative re-population of haematopoietic progenitors in mice (Saitoh, et al., 1999). Furthermore, Epo induced splenic erythropoiesis

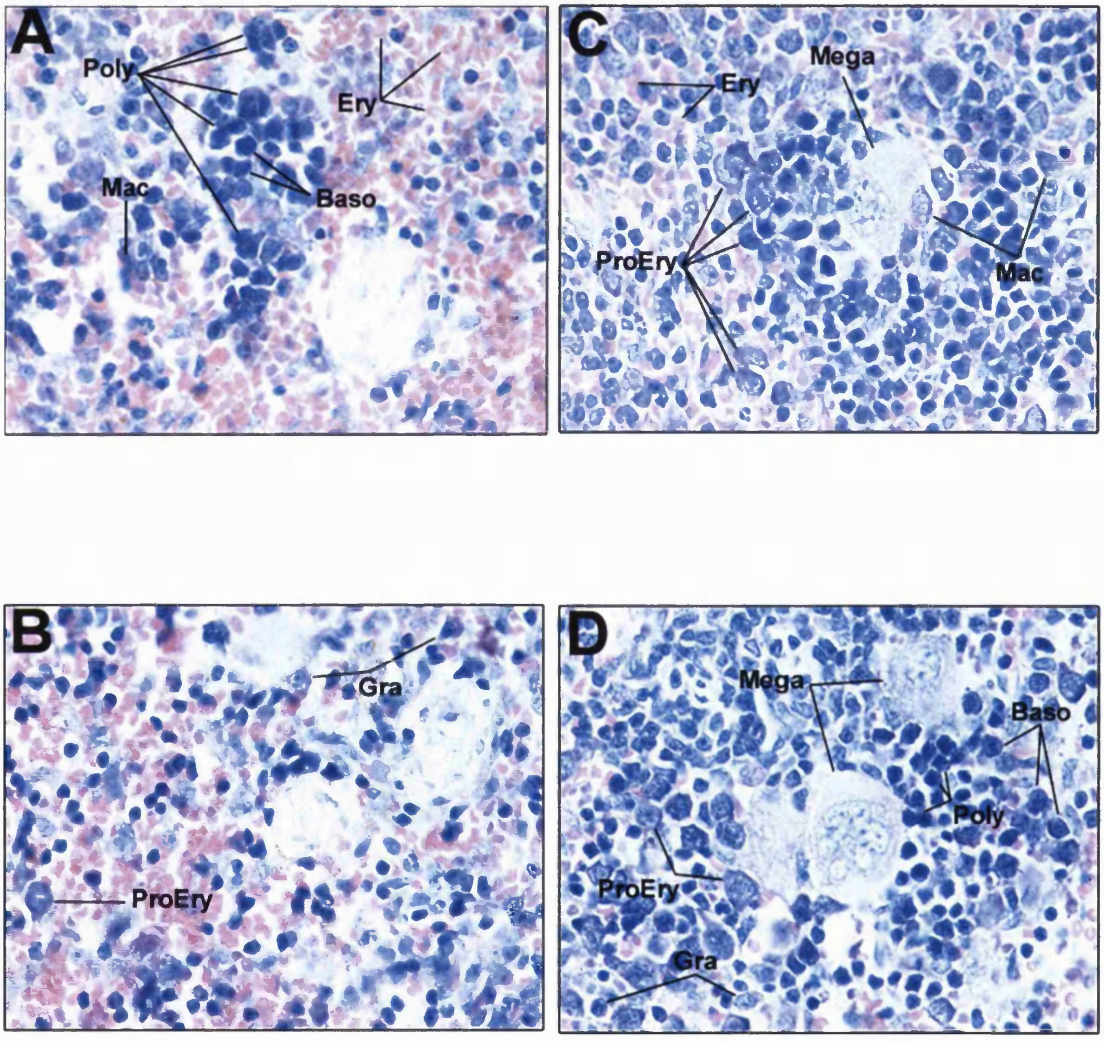
can be optimized by manipulation of the spleen microenvironment (Nijhof, et al., 1993). Since SCL is expressed in day 12 spleen colony forming units (CFU-S), in almost all erythroid colony forming cells (CFCs) (Elefanty, et al., 1998), and is up-regulated upon erythroid differentiation (Elefanty, et al., 1998; Aplan, et al., 1992) and growth (Green, et al., 1991), it was likely that many of the cells expressing SCL mRNA in the spleen of LYL-$1^{-/-}$ mice were erythroid. To examine this proposition, sections of spleen were subjected to specific histological and immunohistochemical analyses to establish the presence, and distribution of erythroid cells.

Section 3.4 The spleen of LYL-$1^{-/-}$ mice comprised more erythroid cells than the spleen of LYL-$1^{+/+}$ mice.

The May-Grunwald and Giemsa stain was used to identify haematopoietic cells in sections of spleen from LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice (see Chapter 2 Methods and Materials section 2.1.11). This stain allowed the different types of blood cell to be distinguished morphologically. Several different haematopoietic cell types were identified in the spleen of LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice including granulocytes (Gra), macrophages (Mac), erythrocytes (Ery) and erythroblasts (ProEry, Poly, Baso) (Figure 15). Spleens of both LYL-$1^{+/+}$ (Figure 15A, B) and LYL-$1^{-/-}$ (Figure 15C, D) LYL-1 mice contained clusters of erythroblasts. However, the clusters in the LYL-$1^{-/-}$ spleen (Figure 15C, D) were more frequent and comprised many more cells than in LYL-$1^{+/+}$ spleen (Figure 15A, B). The clusters in LYL-$1^{-/-}$ spleen were larger because they composed more proerythroblasts, polychromatic and basophilic erythroblasts (Figure 15C, D). It was apparent that the red pulp of LYL-$1^{+/+}$ spleen (Figure 15A, B) contained more erythrocytes than the LYL-$1^{-/-}$ spleen (Figure 14C, D). These results supported the proposition that the clusters of SCL mRNA^{+ve} cells, identified in the LYL-$1^{-/-}$ spleen by SCL-specific *in situ* RT-PCR, were erythroid.

Figure 15. May-Grunwald and Giemsa stain of haematopoietic cells in mercuric-fixed paraffin sections of LYL-$1^{+/+}$ (A, B) and LYL-$1^{-/-}$ (C, D) spleen (see Section 2.1.8). Several different haematopoietic cells can be identified including erythroblasts (Poly- polychromatic; Baso- basophilic; ProEry- proerythroblasts); erythrocytes (Ery), macrophages (Mac), granulocytes (Gra), and megakaryocytes (Mega) in spleen sections. Erythroid clusters were larger and more frequent in the LYL-$1^{-/-}$ spleen (C, D) compared to LYL-$1^{+/+}$ erythroid clusters (A, B). Erythrocytes (stained pink) were less frequent in the LYL-$1^{-/-}$ spleen sections (D) compared to LYL-$1^{+/+}$ controls (B). All images were taken at 20x magnification.

Increase in erythroid cells in the LYL-1-/- spleen.

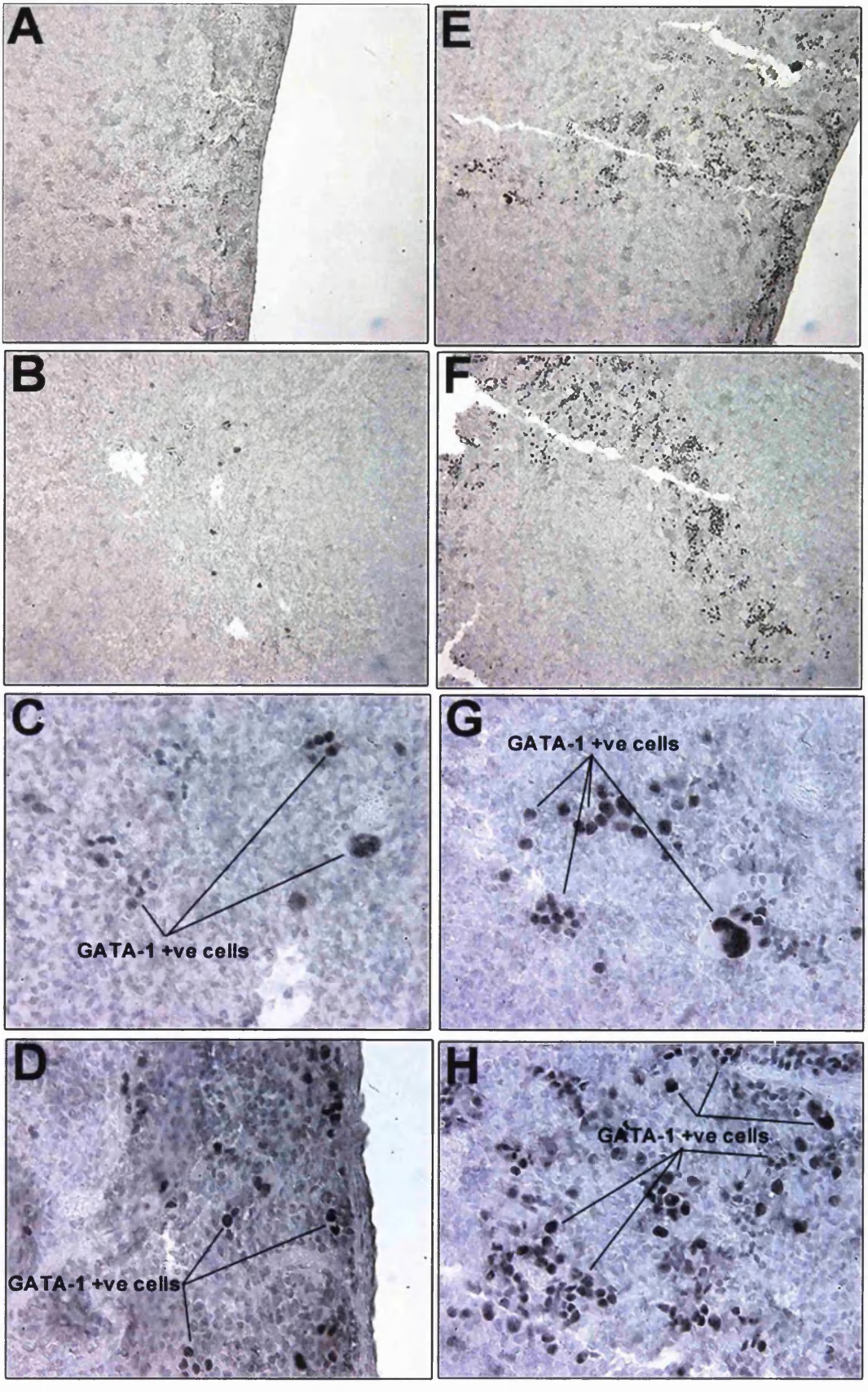


Sections of spleen were processed for immunoperoxidase staining of cells expressing GATA-1 (see Chapter 2 Methods and Materials section 2.3). GATA-1 is an erythroid lineage-restricted zinc finger transcription factor (Yamamoto, et al., 1997) that is critical for development of erythroid cells (Whyatt, et al., 2000; Penvy, et al., 1991; Simon, et al., 1992; Orkin, 1994) (see Chapter 1 Introduction section 1.10). 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{-/-}$ spleen stained for GATA-1 contained many more cells expressing GATA-1 (GATA-1^{+ve}) (Figure 16E-H) than found in LYL-$1^{+/+}$ spleen (Figure 16A-D). Although the immunohistochemical assay was not quantitative, there were approximately 5 to 10 times more GATA-1^{+ve} cells in LYL-$1^{-/-}$ spleen (Figure 16E, F) compared to LYL-$1^{+/+}$ controls (Figure 16A, B). These cells were located in the red pulp exclusively and were found in clusters (Figures 16C, D: G, H). The clusters of cells in the spleen of LYL-$1^{-/-}$ mice were larger and composed of more cells (Figure 16G, H) than those in LYL-$1^{+/+}$ controls (Figure 16C, D). The specificity of the assay was confirmed by the lack of stained cells after incubation with an irrelevant rat biotinylated IgG_{2a} isotype control (Figure 16I), without a secondary antibody (Figure 16J), and in the absence of ExtraAvidin (Figure 16K). Analysis of serial sections indicated that the GATA-1^{+ve} cells co-expressed SCL mRNA.

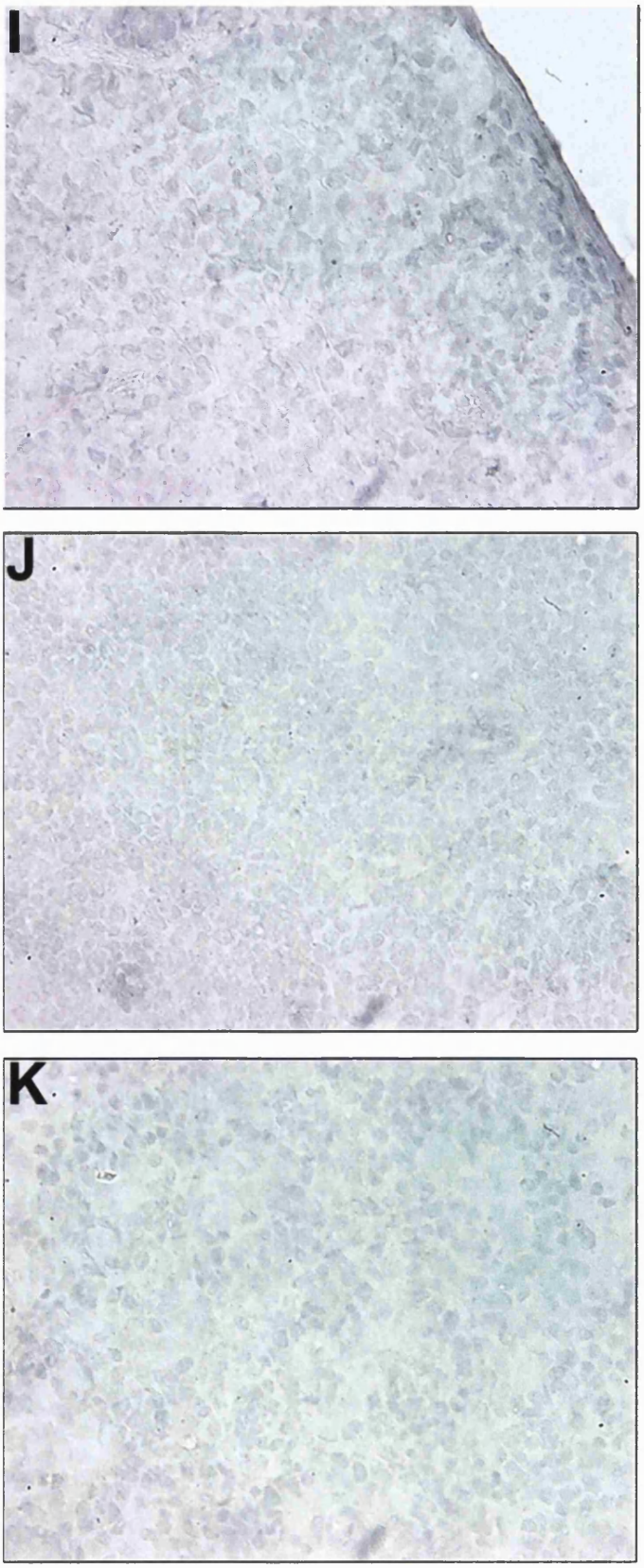
SCL and GATA-1 are co-expressed in erythroid cells (Aplan, et al., 1992) and contribute to formation of an erythroid DNA-binding complex in vivo (Wadman, et al., 1997). The increased number of SCL $mRNA^{+ve}$ and GATA-1^{+ve} cells in the LYL-$1^{-/-}$ spleen suggested that in the absence of a functional LYL-1 protein, there was an enhancement of splenic erythropoiesis. Although SCL and GATA-1 are co-expressed in erythroid cells, they are found in other cell types. Indeed, megakaryocytes were stained for GATA-1 in LYL-$1^{-/-}$ spleen (Figure 16G), which was consistent with previously published data (Visvader, et al., 1992). LYL-1^{lacZ}-positive cells (LYL-$1^{lacZ+ve}$) were found in fetal liver and bone marrow

Figure 16. 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A-D) and LYL-$1^{-/-}$ (E-H) spleen immunoperoxidase stained for GATA-1 (see Section 2.3). Cells expressing GATA-1 were found in the red pulp of LYL-$1^{+/+}$ (A, B) and LYL-$1^{-/-}$ (E, F) spleen exclusively. LYL-$1^{-/-}$ spleen contained many more GATA-1-expressing cells (E, F) than LYL-$1^{+/+}$ controls (A, B). Many of the GATA-1-expressing cells were in clusters in both LYL-$1^{+/+}$ (C, D) and LYL-$1^{-/-}$ (G, H) spleen. The clusters in LYL-$1^{-/-}$ spleen comprised more cells (H) than LYL-$1^{+/+}$ clusters (D). Specifically stained cells were not present in experiments performed in the presence of a biotinylated IgG_{2a} isotype control (I), rabbit anti-rat biotinylated immunoglobulins (J) and strepavidin/ avidin conjugated HRP (K) (see Table 1). Images A, B,E, and F were taken at 5x magnification; images C, D, G, and H were taken at 20x magnification; images I and J were taken at 40x magnification.

Increased numbers of GATA-1+ve cells in the LYL-1-/- spleen.



Negative controls for GATA-1 immunohistochemistry.



(Rupping, E. Ph. D. thesis), and LYL-1 mRNA was expressed in cells in the spleen as well as in erythroid cell lines (Visvader, et al, 1991; Kuo, et al, 1991). To determine whether LYL-1 was expressed in the erythroid cells identified in LYL-$1^{-/-}$ spleen, cryosections of LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ spleen were assayed for β -galactosidase activity.

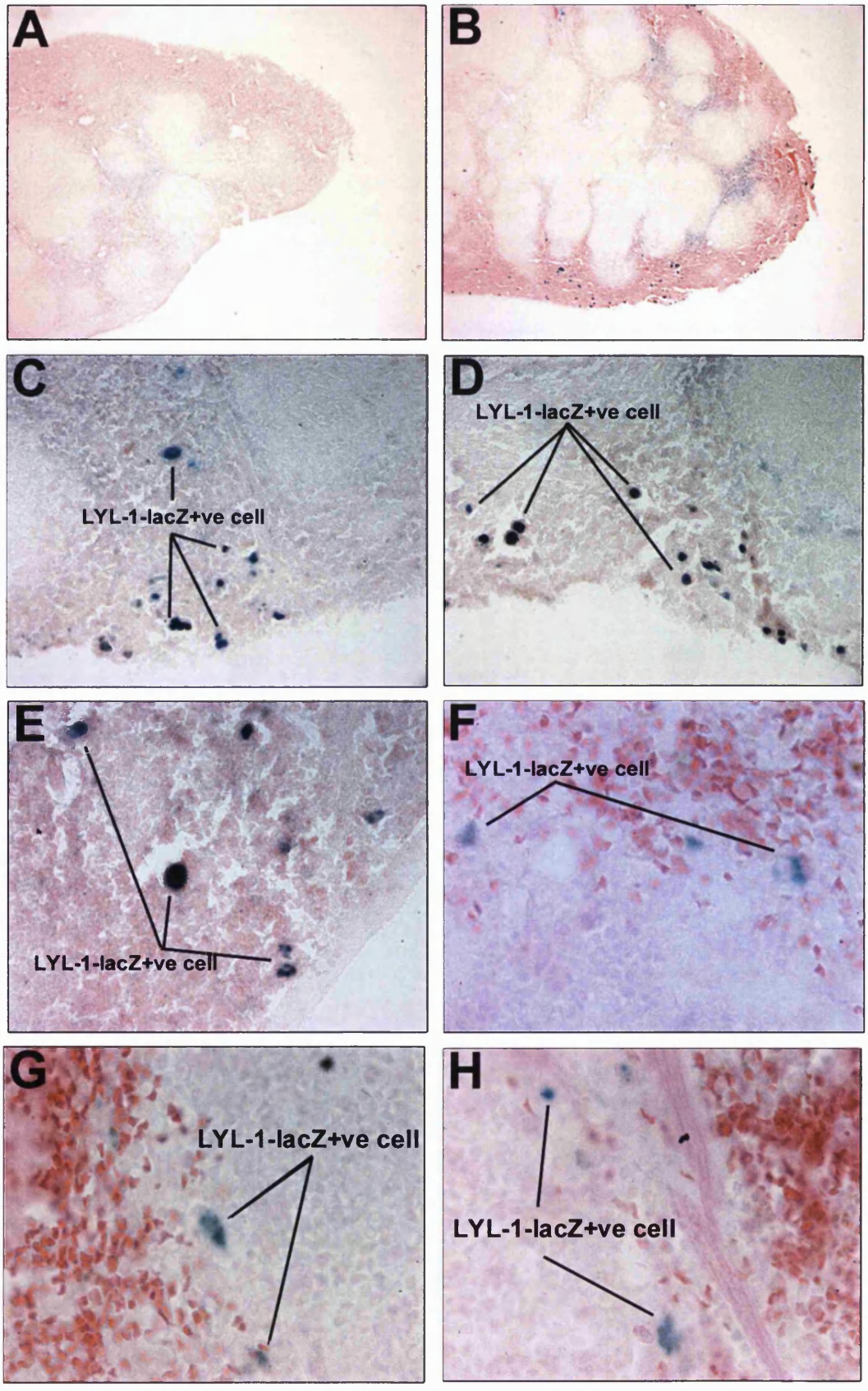
Section 3.5 The majority of erythroid cells in the spleen of LYL-$1^{-/-}$ mice do not express LYL-1^{lacZ} fusion protein.

The spatial pattern of LYL-1 expression in the spleen was examined by X-GAL staining 10μm paraformaldehyde-fixed cryosections (see Chapter 2 Methods and Materials section 2.1.14). Figure 17 shows LYL-1 expressing cells were located in the red pulp of the spleen (Figure 17B-E), particularly in the subcapsular region (Figure 17E), but were also located in the marginal zone (Figure 17F-H). The frequency of X-GAL stained cells was similar in the spleen of LYL-$1^{+/-}$ and LYL-$1^{-/-}$ (data not shown). The intensity of the blue staining of the cells ranges from weak (Figure17C) to strong LYL-1^{lacZ} expression (Figure 17D). Non-specific blue staining was found in the sections of LYL-$1^{+/+}$ (Figure 17A) and LYL-$1^{-/-}$ spleen (Figure 17B) but was clearly distinguishable from positively stained cells. The identity of the LYL-$1^{lacZ+ve}$ cells in the spleen could not be elucidated from the β -galactosidase assay. However, it was evident that LYL-$1^{lacZ+ve}$ population of cells was heterogeneous, since their size varied from small, blast-like cells (Figures 17C, and F) to larger cells (Figure 17D, and E).

X-GAL staining of spleen cryosections showed that most of the erythroid cells, which expressed SCL mRNA and GATA-1, did not co-express LYL-1. Co-expression of SCL mRNA, GATA-1 and LYL-1 in the same cell could not be excluded by the experiments described in sections 3.2- 3.5. However, it was evident from the spatial expression pattern of

Figure 17. X-GAL stained 10μm cryosections of LYL-$1^{+/+}$ (A) and LYL-$1^{-/-}$ (B-H) spleen allowed detection of LYL-$1^{lacZ+ve}$ cells (see Section 2.1.11). LYL-$1^{lacZ+ve}$ cells were located in the red pulp (C, D), the subcapsular region (E), and the marginal zone (F, G) of the spleen. Images A and B were taken at 4x magnification; images C and D wee taken at 20x magnification; images E, F, G, and H were taken at 60x magnification.

Cells in the LYL-1-/- spleen express the LYL-1-lacZ fusion protein.



LYL-1^{lacZ} that the population of cells, which expressed SCL mRNA and GATA-1 in LYL-$1^{-/-}$ spleen, did not express LYL-1. This suggested that LYL-1 was not required for development of erythroid cells in the spleen of LYL-$1^{-/-}$ mice. In order to assess the number, and distribution of erythroid cells specifically, expression of the erythroid-specific antigen TER-119 was determined by immunoperoxidase assay of spleen sections from LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice.

Section 3.6 The spleen of LYL-$1^{-/-}$ mice contained more erythroblasts but fewer erythrocytes than LYL-$1^{+/+}$ mice.

To examine the presence of erythroid precursors and their differentiated progeny including erythrocytes in the spleen, 5μm paraformaldehyde-fixed paraffin sections were immunoperoxidase-stained for the erythroid cell-specific membrane protein TER-119 (see Chapter 2 Methods and Materials section 2.3). TER-119 is expressed on early erythroblasts through to mature erythrocytes but not in erythroblasts with BFU-e or CFU-e activity (Ikuta, et al., 1990). The red pulp of LYL-$1^{+/+}$ spleen stained more strongly for TER-119 (Figure 18A, C and Figure 19A) than LYL-$1^{-/-}$ spleen (Figure 18B, D and Figure 19E). The TER-119 signal was absent in sections that were incubated with an irrelevant IgG_{2a} isotype control (Figures 18E) and HRP-conjugated streptaidin/avidin (Figure 18F).

Closer examination of the sections showed that the difference in TER-119 staining intensity was due to reduced numbers of erythrocytes in the red pulp of LYL-$1^{-/-}$ spleen (Figures 18B, D; 19E) compared to LYL-$1^{+/+}$ spleen (Figures 18A, C; 19A). Further examination of the red pulp revealed TER-119 expression on cells (TER-119^{+ve}) that displayed blast-like morphology (Figure 19B, F, and G). TER-119. These TER-119^{+ve} erythroblasts were found in clusters in both LYL-$1^{+/+}$ (Figure 19D) and LYL-$1^{-/-}$ spleen (Figure 19G). However, these

Figure 18. 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A, C) and LYL-$1^{-/-}$ (B, D) spleen immunoperoxidase stained for TER-119 (see Section 2.3 and Table 1). The red pulp of the LYL-$1^{-/-}$ spleen stained weaker than LYL-$1^{+/+}$ controls for TER-119. Assays performed in the presence of a biotinylated IgG_{2a} isotype control (E), or strepavidin/ avidin conjugated HRP (F) did not stain cells in the red pulp, and confirmed the specificity of the primary antibody stain in sections of spleen. All images were taken at 10x magnification.

LYL-1-/- spleen contains less erythrocytes than LYL-1+/+ spleen.

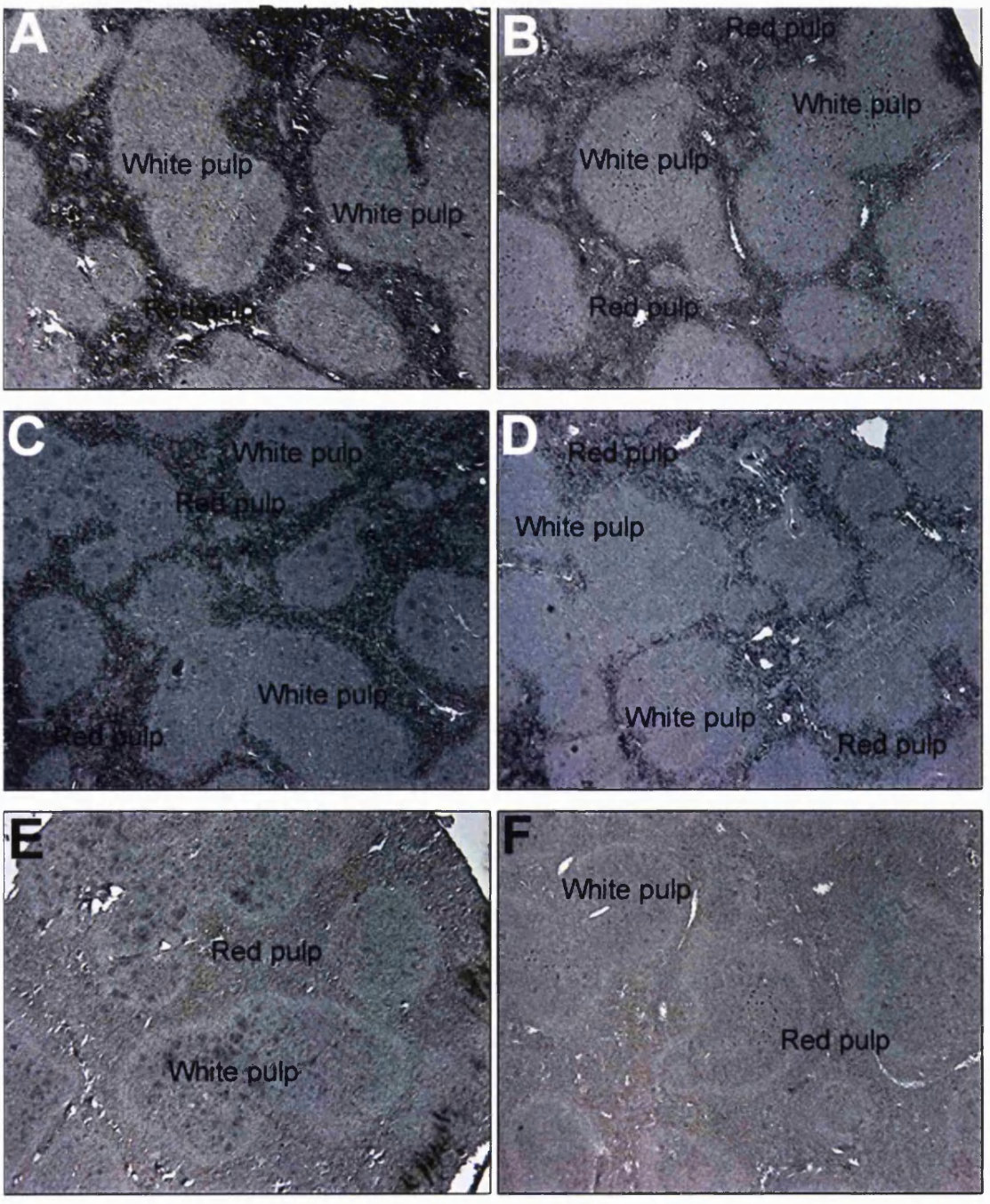
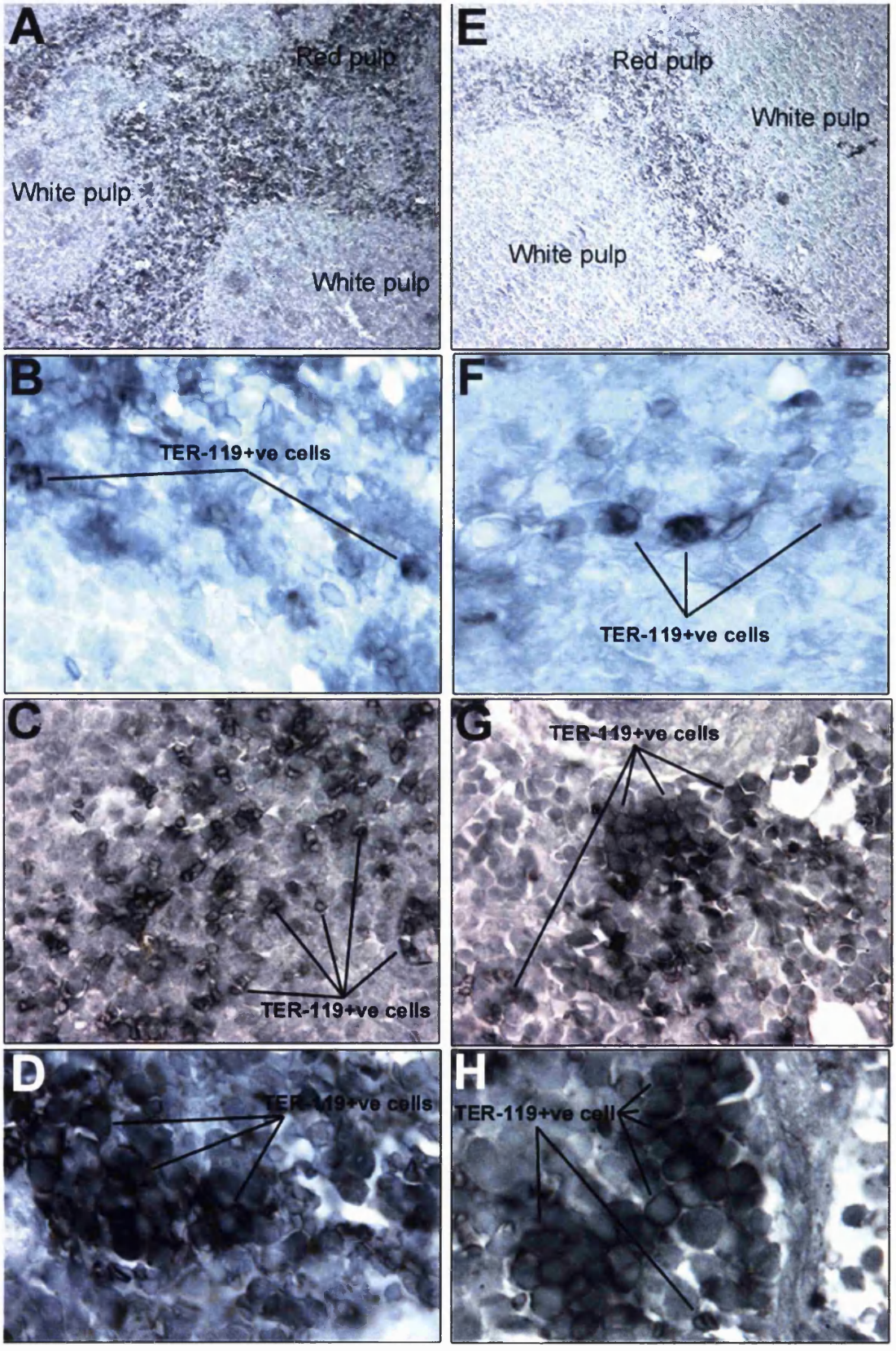


Figure 19. LYL-1$^{+/+}$ spleen sections stained for TER-119 contained more erythroid cells in the red pulp (A) than LYL-1$^{-/-}$ spleen (E) (see Section 2.3, and Table 1). Erythrocytes were reduced in number in LYL-1$^{-/-}$ spleen (G) compared to LYL-1$^{+/+}$ controls (C) (also see Figure 18), but LYL-1$^{-/-}$ sections contained more erythroblasts (F) than LYL-1$^{+/+}$ control spleen (B). Many of the erythroblasts in LYL-1$^{-/-}$ spleen were in clusters (G) that were more frequent and comprised more cells (G, H) than those in LYL-1$^{+/+}$ spleen (D). Images A, and E were taken at 20x magnification; C and G were taken at 40x magnification; images B, D, F, and H were taken at 60x magnification.

LYL-1 -/- spleen contains more erythroblasts than LYL-1+/+ spleen.



were more frequent, and larger in the LYL-1$^{-/-}$ spleen (Figure 19G, H). The LYL-1$^{-/-}$ spleen appeared to comprise at least 5 times more TER-119-positive erythroblasts than LYL-1$^{+/+}$ spleen. These data confirmed that the population of cells expressing SCL mRNA and GATA-1 in the LYL-1$^{-/-}$ spleen were erythroblasts, and showed the presence of increased numbers of erythroblasts specifically in the spleen of LYL-1$^{-/-}$ mice.

Based upon the results of the TER-119 specific immunohistochemical experiments, two conclusions were reached. First, in the absence of a functional LYL-1 protein, the spleen became a site for expansion of erythroid precursor cells. Erythroid precursor cells were defined morphologically by histological staining of haematopoietic cells, and by expression of GATA-1, SCL mRNA, and TER-119. Second, as indicated by the May-Grunwald and Giemsa and TER-119 staining, the absence of a functional LYL-1 protein was associated with reduced numbers of erythrocytes in spleen. The increased numbers of erythroid precursors could be the consequence of more progenitor cells in LYL-1$^{-/-}$ spleen. A rise in the number of haematopoietic progenitors in the LYL-1$^{-/-}$ spleen could result in more erythroid precursor cells. However, reduced numbers of erythrocytes in LYL-1$^{-/-}$ spleen suggested that these erythroid precursors did not differentiate into mature erythrocytes. Alternatively, the erythrocyte development in the LYL-1$^{-/-}$ spleen may be normal, but erythrocytes leave the spleen and enter the peripheral system more rapidly in LYL-1$^{-/-}$ mice.

In order to test the hypothesis that the LYL-1$^{-/-}$ spleen comprised more haematopoietic progenitor cells than LYL-1$^{+/+}$ spleen, sections of spleen were immuno-stained for the tyrosine receptor kinase c-Kit, an antigen that is expressed on haematopoietic progenitor cells.

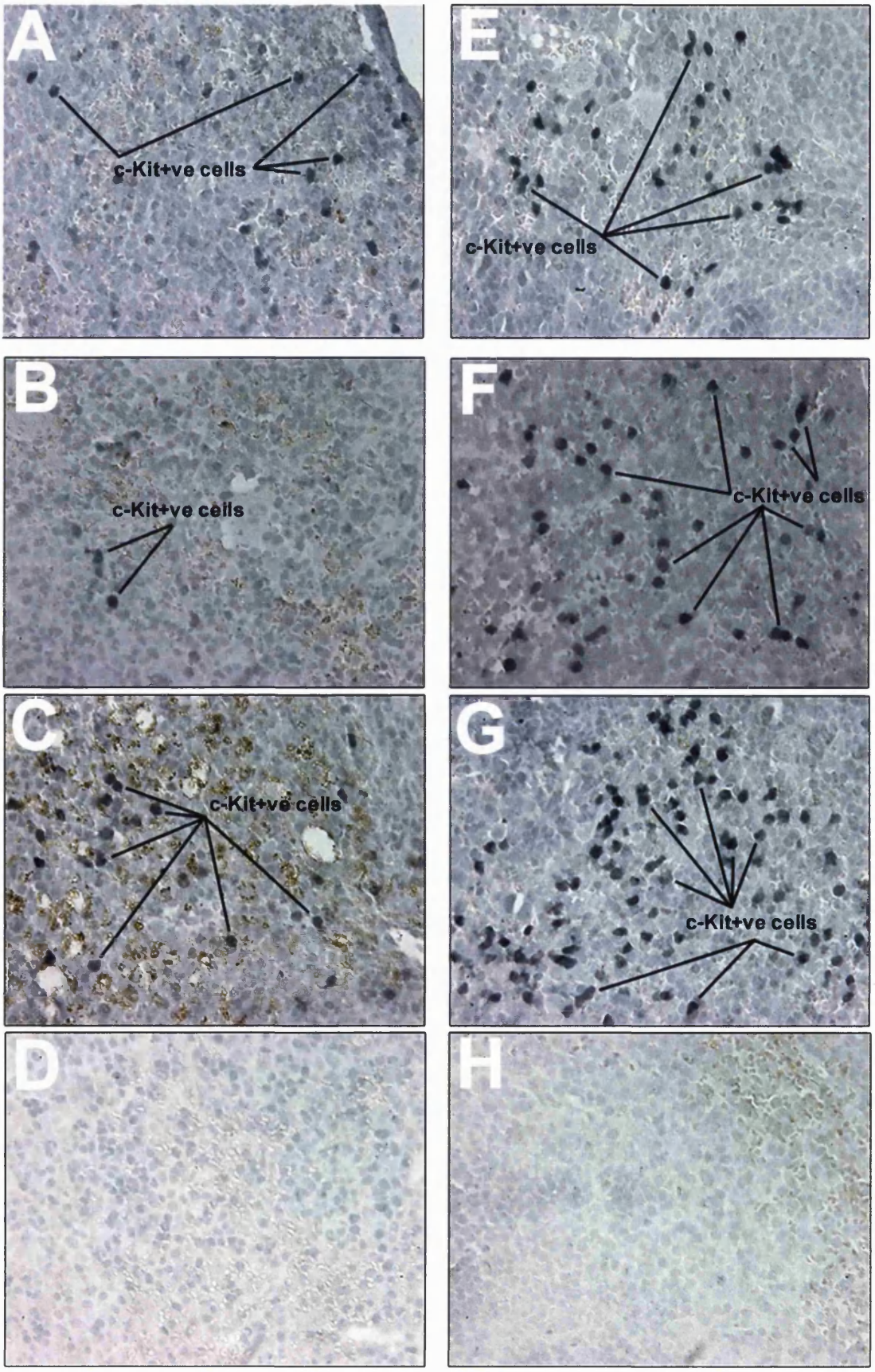
Section 3.7 LYL-1$^{-/-}$ spleen contained more cells that expressed c-Kit than LYL-1$^{+/+}$ spleen.

To establish whether LYL-1$^{-/-}$ spleen comprised more haematopoietic progenitors than LYL-1$^{+/+}$ spleen, 5μm paraformaldehyde-fixed paraffin sections were immunoperoxidase- stained for the tyrosine kinase receptor c-Kit (see Chapter 2 Methods and Materials section 2.3). c-Kit has been associated with proliferation, differentiation and mobilization of haematopoietic cells (Ogawa, et al, 1991; Ashman, 1999; Paulson and Bernstein, 1996), and is a marker for early multipotential haematopoietic progenitor cells (Ikuta and Weissman, 1992) capable of long term reconstitution of the haematopoietic system (Jong, et al., 1996; Lian, et al, 1997). c-Kit positive cells (c-Kit^{+ve}) were located almost exclusively in the red pulp in both LYL-1$^{+/+}$ and LYL-1$^{-/-}$ spleen (Figure 20). c-Kit^{+ve} cells displayed different staining intensities, in both LYL-1$^{+/+}$ and LYL-1$^{-/-}$ spleen. The LYL-1$^{-/-}$ spleen (Figure 20E- G) contained more c-Kit^{+ve} cells than LYL-1$^{+/+}$ controls (Figure 20A- C). When the sections were incubated with an irrelevant rat biotinylated IgG_{2b} isotype control (Figure 20G), or when the ExtrAvidin was omitted from the reaction (Figure 20H), the c-Kit specific signals were absent from spleen sections. LYL-1$^{-/-}$ spleen comprised approximately 4 times more c-Kit^{+ve} cells than LYL-1$^{+/+}$ controls. (Figures 20E, F, G and 20A, B, and C respectively).

Although the fate of an individual c-Kit^{+ve} cell could not be determined from this assay, these data suggested that the expansion of erythroid cells in the LYL-1$^{-/-}$ spleen was the result of increased numbers of c-Kit^{+ve} haematopoietic progenitor cells. Proliferation and differentiation of these cells in the spleen may account for the increased numbers of erythroid cells. Since c-Kit is expressed on cells that display erythroid burst- forming unit activity (BFU-e) (To, et al., 1994), it is possible that many of the cells in the LYL-1$^{-/-}$ spleen were committed erythroid progenitors, which migrated from the bone marrow. In order to

Figure 20. 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A-C) and LYL-$1^{-/-}$ (E-G) spleen immunoperoxidase stained for c-Kit (see Section 2.3, and Table 10). The LYL-$1^{-/-}$ spleen contained more c-Kit^{+ve} cells (G) than LYL-$1^{+/+}$ spleen (C) and these cells were almost exclusively in the red pulp of LYL-$1^{-/-}$ (E, F) and LYL-$1^{+/+}$ (A, B) spleen. Cells did not stain when spleen sections were immunoperoxidase stained in the presence of a rat biotinylated IgG_{2b} isotype control (D), or strepavidin/ avidin conjugated HRP (H). Images A to H were taken at 20x magnification.

Increased number of c-Kit+ve cells in the LYL-1-/- spleen.



investigate the developmental potential of the c-Kit^{+ve} cells in the LYL-1$^{-/-}$ mouse, cells were isolated from the spleen and bone marrow and grown *in vitro* in semi-solid methylcellulose based media containing fetal bovine serum (FBS) and defined cytokines.

Section 3.8 *LYL-1$^{-/-}$ spleen contained more CFU-e, BFU-e and CFU-GEMM than LYL-1$^{+/+}$ spleen.*

In vitro colony assays using methylcellulose media were employed to compare the growth and differentiation of haematopoietic progenitor cells isolated from the spleen and bone marrow of LYL-1$^{+/+}$ and LYL-1$^{-/-}$ mice (for details see Chapter 2 Methods and Materials section 2.9.1). Progenitor cells grown in the presence of Epo (3Uml^{-1}) generated CFU-e and mature BFU-e. After 2 days incubation in the presence of Epo (3Uml^{-1}), the LYL-1$^{-/-}$ spleen produced 7.2 +2 (n=4) CFU-e per 2.5x10^4 cells compared to 3.25+0.9 (n=4) from LYL-1$^{+/+}$ controls (Figure 21A), an increase of 2.2x in the number of CFU-e in the LYL-1$^{-/-}$ spleen. Bone marrow from LYL-1$^{-/-}$ mice produced 14+0.6 (n=3) CFU-e per 2.5x10^4 cells compared to 10+0.07 (n=3) CFU-e from LYL-1$^{+/+}$ bone marrow (Figure 21A), corresponding to an increase of 1.4x in CFU-e in LYL-1$^{-/-}$ bone marrow. After 4 days incubation under the same conditions, the LYL-1$^{-/-}$ spleen generated 2+0.5 (n=4) mature BFU-e per 2.5x10^4 cells compared to 0.4+0.07 (n=4) from LYL-1$^{+/+}$ controls, an increase of 5.5x in the number of mature BFU-e in the LYL-1$^{-/-}$ spleen (Figure 21B). This was in contrast to the bone marrow where no significant difference in mature BFU-e number was found between LYL-1$^{+/+}$ and LYL-1$^{-/-}$ mice (Figure 21B). The mature BFU-e colonies derived from in the LYL-1$^{-/-}$ spleen and bone marrow were red in colour, which indicated production of haemoglobin and mature erythrocytes.

Figure 21. Bar graphs represent the number of CFU-e and BFU-e colonies isolated from LYL-$1^{+/+}$ spleen and bone marrow, and LYL-$1^{-/-}$ spleen and bone marrow, grown in the presence of Epo only ($3Uml^{-1}$) (A and B), and in the presence of Epo ($3Uml^{-1}$), IL-3 ($10ngml^{-1}$), IL-6 ($10ngml^{-1}$) and SCF ($50ngml^{-1}$) (C) (see Section 2.5). After 2 days incubation in Epo only, LYL-$1^{-/-}$ spleen contained 7.16+2.17 (n=4) CFU-e per $2.5x10^4$ cells compared to 3.25+0.97 (n=4) from LYL-$1^{+/+}$ controls (A), an increase of 2.2-fold ($P<0.05$) (Table 11). Bone marrow from LYL-$1^{-/-}$ mice produced 13.75+0.6 CFU-e per $2.5x10^4$ cells compared to 10+0.07 CFU-e from LYL-$1^{+/+}$ bone marrow (A), an increase of 1.4-fold ($P<0.05$) (Tables 12).

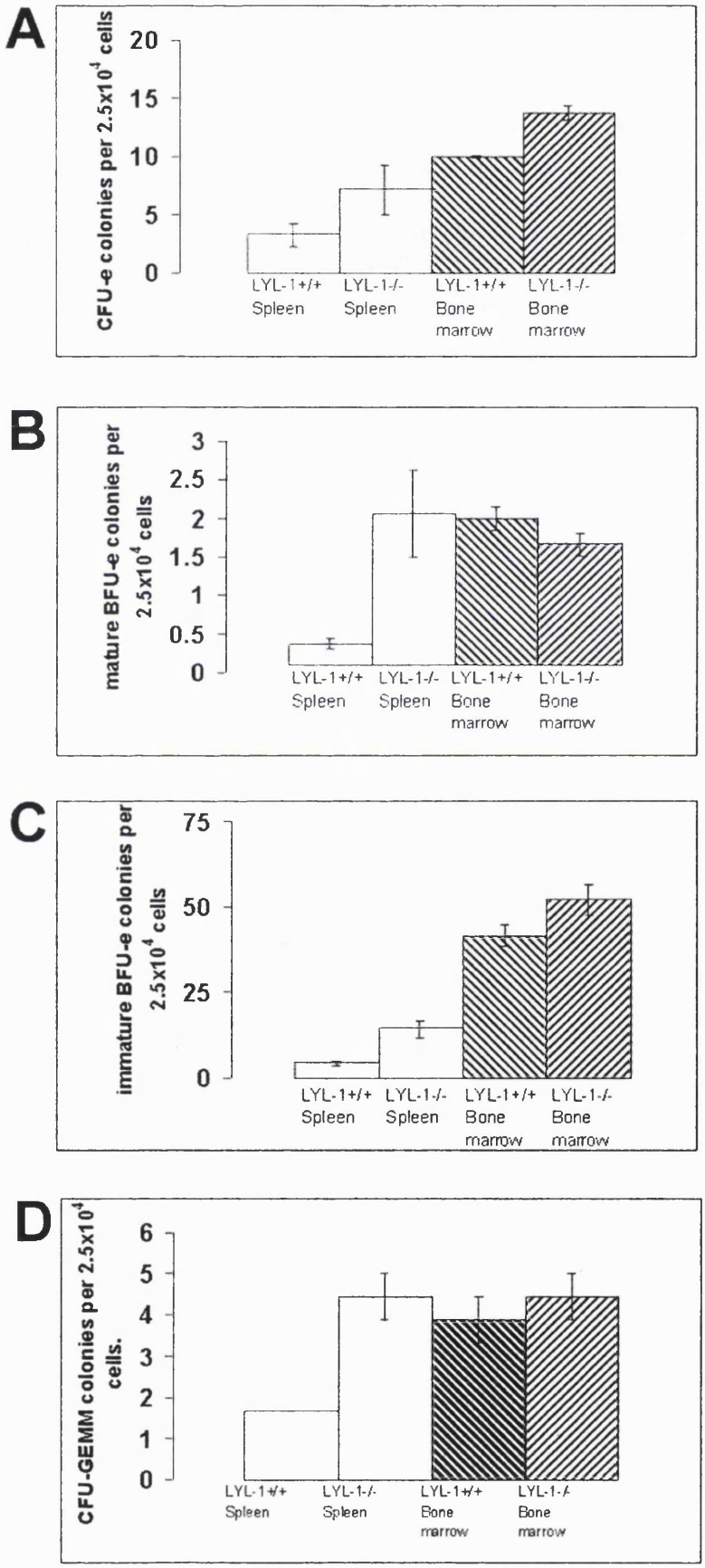
After 4 days of incubation in Epo only, the LYL-$1^{-/-}$ spleen produced 2+0.56 (n=4) mature BFU-e per $2.5x10^4$ cells compared to 0.37+0.07 (n=4) from LYL-$1^{+/+}$ controls (B), an increase of 5.5-fold ($P<0.05$) (Table 11). This is in contrast to the bone marrow where no significant difference in mature BFU-e number was found between LYL-$1^{-/-}$ (1.66 + 0.04, n=3) and LYL-$1^{+/+}$ (2 + 0.14, n=3) mice (B) (Tables 12).

After 7 days of incubation in Epo ($3Uml^{-1}$), IL-3 ($10ngml^{-1}$), IL-6 ($10ngml^{-1}$) and SCF ($50ngml^{-1}$), the number of immature BFU-e isolated from LYL-$1^{-/-}$ spleen was 14.4+2.42 (n=3) per $2.5x10^4$ cells compared to 4.44+0.55 (n=3) from LYL-$1^{+/+}$ controls, an increase of 3.2-fold ($P<0.05$) (Table 11). This was in contrast to the bone marrow where no significant difference in immature BFU-e number was found between LYL-$1^{-/-}$ (52.2+4.3, n=3) and LYL-$1^{+/+}$ (41.7, n=3) mice (Table 12).

After 10 days incubation in Epo ($3Uml^{-1}$), IL-3 ($10ngml^{-1}$), IL-6 ($10ngml^{-1}$) and SCF ($50ngml^{-1}$), the LYL-$1^{-/-}$ spleen generated 4.4 +0.55 (n=3) CFU-GEMM colonies per $2.5x10^4$

cells compared to 1.7 (n=3) colonies per $2.5x10^4$ cells from LYL-$1^{+/+}$ spleen (D), an increase of 2.6-fold ($P<0.05$) (Table 11). The number of CFU-GEMM in LYL-$1^{-/-}$ bone marrow was similar to LYL-$1^{+/+}$ bone marrow (Table 12).

LYL-1-/- spleen contains more CFU-e, mature and immature BFU-e, and CFU-GEMM than LYL-1+/+ spleen.



	Colony number (per $2.5x10^4$ cells)	
Spleen	**$LYL^{+/+}$**	**$LYL\text{-}1^{-/-}$**
CFU-e	3.25+0.97 (n=4)	7.16 +2.17 (n=4)
Mature BFU-e	0.37 +0.07(n=4)	2 +0.56 (n=4)
Immature BFU-e	4.44 +0.55 (n=3)	14.4 +2.42 (n=3)
CFU-GEMM	1.7 +0.15 (n=3)	4.4 +0.55 (n=3)

Table 11. The number of CFU-e (Day 2), and mature BFU-e (Day 4) in the spleen were scored in the presence of Epo only. Immature BFU-e (Day 7), and CFU-GEMM (Day 10) were scored in the presence of SCF, IL-3, IL-6, and Epo (for details see Section 2.9).

	Colony number (per $2.5X10^4$ cells)	
Bone marrow	**$LYL\text{-}1^{+/+}$**	**$LYL\text{-}1^{-/-}$**
CFU-e	10 +0.07 (n=4)	13.75 +0.6
Mature BFU-e	2 +0.14 (n=4)	1.66 +0.04 (n=4)
Immature BFU-e	41.7 +3.3 (n=3)	52.2 +4.3 (n=3)
CFU-GEMM	4.6 +0.51 (n=3)	3.9 +0.40 (n=3)

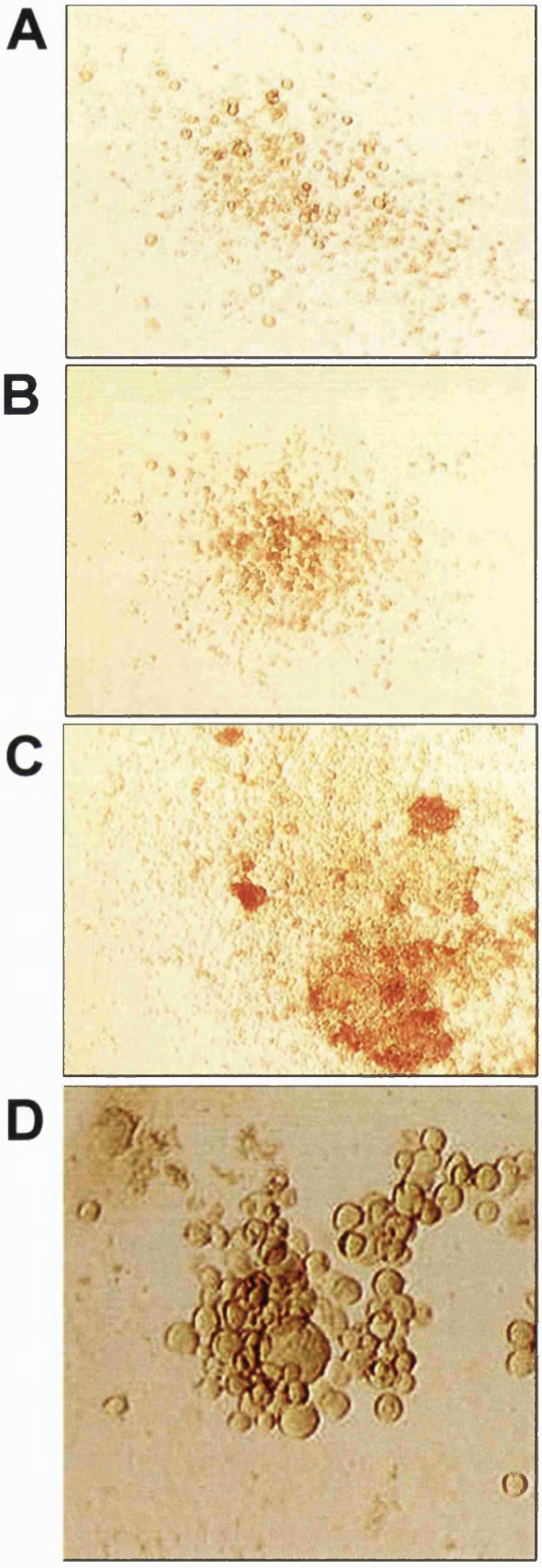
Table 12. The number of CFU-e (Day 2),and mature BFU-e (Day 4) in the bone marrow were scored in the presence of Epo only. Immature BFU-e (Day 7), and CFU-GEMM (Day 10) were scored in the presence of SCF, IL-3, IL-6, and Epo (for details see Section 2.9).

These data confirmed the presence of increased numbers of CFU-e and mature BFU-e in the spleen of LYL-1$^{-/-}$ mice (see Table 11). Under *in vitro* conditions, these cells formed colonies at the same rate, and of similar size to those grown from LYL-1$^{+/+}$ spleen. Therefore, mature BFU-e and CFU-e from LYL-1$^{-/-}$ spleen were capable of normal growth that was independent of the splenic microenvironment. It is known that the expression of c-Kit on immature BFU-e has declined at the CFU-e stage (Gabbianelli, et al., 1995). c-Kit^{+ve} in both LYL-1$^{+/-}$ and LYL$^{-/-}$ stained with different intensities, a result consistent with the presence of both early, and late stage erythroid cells. These data also showed the LYL-1$^{-/-}$ bone marrow contained 1.4x more CFU-e than LYL-1$^{+/+}$ bone marrow.

In vitro colony assays were also performed in the presence of defined cytokines SCF (50ngml^{-1}), IL-3 (10ngml^{-1}), IL-6 (10ngml^{-1}) and Epo (3Uml^{-1}) (for details see Section 2.9.2). Under these conditions, colonies were generated from immature BFU-e, and CFU-GM progenitors after 7 days, and CFU-GEMM progenitors after 12 days incubation (www.stemcell.com). LYL-1$^{-/-}$ spleen contained 14+2.4 (n=3) immature BFU-e per 2.5x10^4 cells compared to 4+0.6 (n=3) immature BFU-e from LYL-1$^{+/+}$ spleen (Figure 21C), an increase of 3.2x in the number of immature BFU-e. Bone marrow from LYL-1$^{-/-}$ mice did not have a significantly difference number of immature BFU-e (52.2+ 4.3, n=3) compared to LYL-1$^{+/+}$ mice (41.6+ 3.3, n=3) (Figure 21C, Table 12). A population of immature BFU-e from LYL-1$^{-/-}$ spleen generated very large colonies (Figure 22C) that contained about 10x more erythroid cells than BFU-e colonies generated from LYL-1$^{+/+}$ spleen (Figure 22A). Approximately 2 such colonies were present in each dish (1.5x10^4 cells), but they were not present in the LYL-1$^{+/+}$ spleen. Interestingly, the number of CFU-GEMM colonies increased in the LYL-1$^{-/-}$ spleen. 1.7 +0.15 (n=3) CFU-GEMM colonies per 2.5x10^4 cells were generated from LYL-1$^{+/+}$ spleen, compared to 4.4 +0.55 (n=3) colonies generated from

Figure 22. Progenitors isolated from LYL-1$^{-/-}$ spleen generated BFU-e colonies that were haemoglobinised (B), and some were very large (C) compared to the BFU-e colonies generated from progenitors isolated from LYL-1$^{+/+}$ spleen (A). CFU-GEMM colonies derived from LYL-1$^{-/-}$ spleen after 12 days of incubation (D) were 2.75 +0.25-fold (n=2) more frequent than in LYL-1$^{+/+}$ (data not shown). Images A and B were taken at 5x magnification; C was taken at 2x magnification; image D was taken at 10x magnification.

BFU-e and CFU-GEMM colonies grown in vitro from LYL-1 spleen cells.



LYL-1$^{-/-}$spleen (Figure 21D), an increase of 2.6-fold in the number of CFU-GEMM in the LYL-1$^{-/-}$ spleen (see Table 11). There was no significant difference in the number of CFU-GEMM in the LYL-1$^{-/-}$ bone marrow (4.6 +0.55, n=3), compared to LYL-1$^{+/+}$ controls (3.9 +0.55, n=3) (Figure 21D, Table 12). This suggested that a proportion of the c-Kit^{+ve} cells identified in LYL-1$^{-/-}$ spleen were multipotential progenitors capable of developing into colonies that comprised granulocytes, erythrocytes, and megakaryocytes. There was no difference in the number of CFU-GM scored at day 10 from LYL-1$^{+/+}$ and LYL-1$^{-/-}$ mice (data not shown), a result that was consistent with those of the previous study on LYL-1$^{-/-}$ mice (Rupping, E., Ph. D. thesis).

Synergy between the effects of Epo, IL-3, IL-6, and SCF on BFU-e in spleen and bone marrow elicited approximately 7-10x more BFU-e colonies in spleen, and 20-25x more BFU-e colonies in the bone marrow (Table 11 and 12). This was consistent with the effects of SCF and Epo on erythroid progenitor cells (Wu, et al., 1997; Miller, et al., 1999; Sui, et al., 1998). These data confirmed that the majority of BFU-e in the LYL-1$^{+/+}$ and LYL-1$^{-/-}$ spleen and bone marrow were immature, and that LYL-1$^{-/-}$ spleen contained more immature, as well as mature, BFU-e.

The results of the *in vitro* colony assays confirmed the presence of more BFU-e and CFU-e progenitor cells in the spleen of LYL-1$^{-/-}$ mice. These results were consistent with the increase in c-Kit^{+ve} cells identified by immunohistochemistry (see Section 3.7). It is proposed that these progenitors are responsible for the clusters of cells that expressed SCL mRNA, GATA-1, and TER-119 identified in LYL-1$^{-/-}$ spleen.

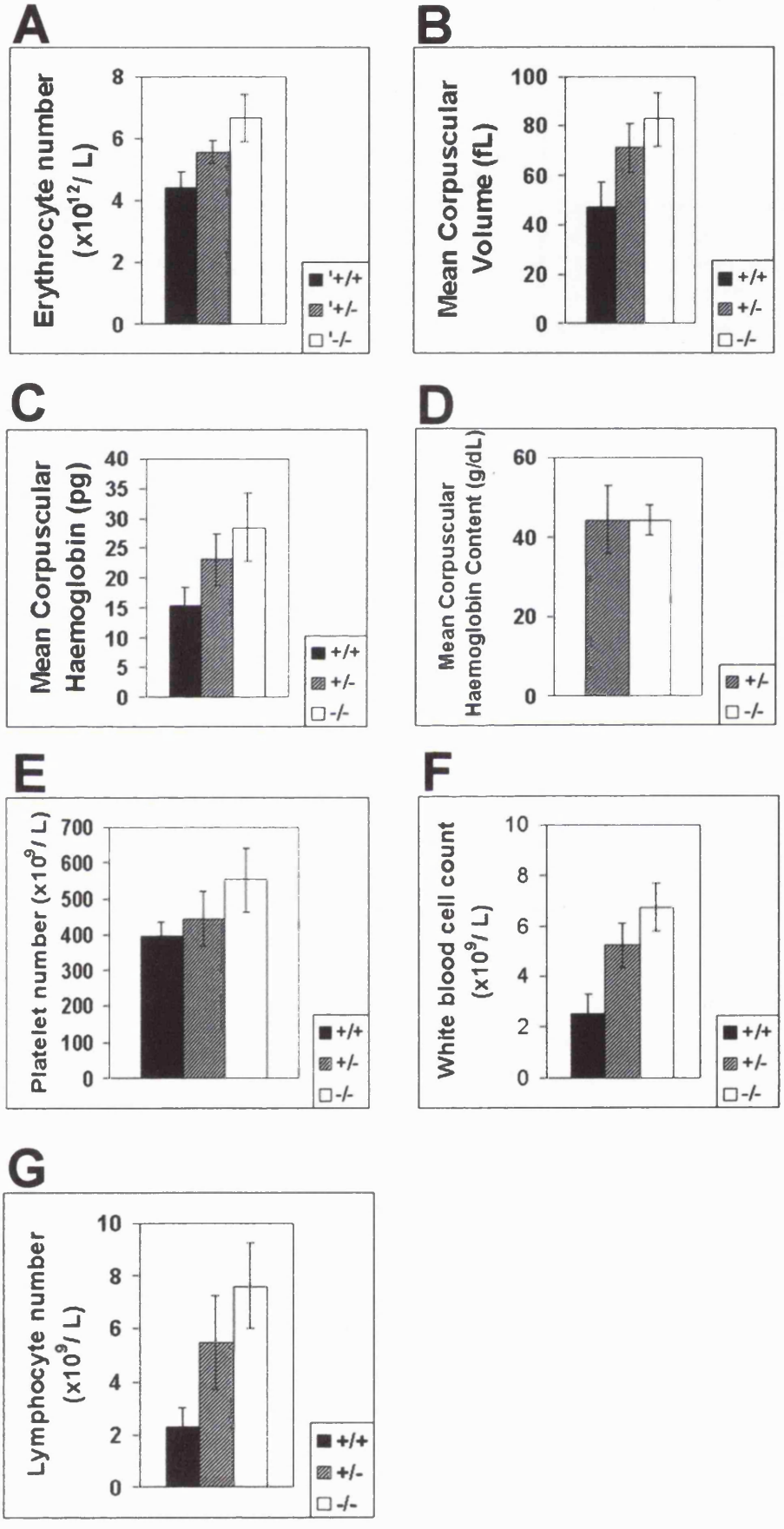
The CFU-e and BFU-e progenitors in the spleen of LYL-$1^{-/-}$ mice formed cells that developed normally *in vitro* i.e. synthesized haemoglobin. However, TER-119 staining of erythrocytes in LYL-$1^{-/-}$ spleen was less than in LYL-$1^{+/+}$ controls (see Chapter 3 Results section 3.6). One explanation for this difference could be that the microenvironment of the LYL-$1^{-/-}$ spleen did not provide the required signals necessary for terminal differentiation of the erythroblasts. Therefore, the development of the erythroblasts was blocked. A block in development would have occurred at a late stage in erythropoiesis, since polychromatic and basophilic erythroblasts were identified in the erythroid clusters in LYL-$1^{-/-}$ spleen. These clusters comprised cells that expressed GATA-1 (Chapter 3 Results section 3.4), which further indicated that BFU-e and CFU-e in LYL-$1^{-/-}$ spleen were able to develop into late stage erythroblasts *in vivo*, and the putative block occurred at point prior to terminal differentiation. Alternatively, the reduced number of erythrocytes in the LYL-$1^{-/-}$ spleen may be explained by an increase in the number of erythrocytes released into the peripheral circulation of LYL-$1^{-/-}$ mice. The peripheral blood of LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice was examined on a Coulter-like cell counter for differences in a range of haematological parameters.

Section 3.9 *LYL-$1^{-/-}$ mice have similar numbers of peripheral erythrocytes to LYL-$1^{+/+}$ mice.*

Analysis of peripheral blood collected from the tail vein revealed that LYL-$1^{-/-}$ mice had similar numbers of erythrocytes to LYL-$1^{+/-}$, and LYL-$1^{+/+}$ mice (Figure 23). The number of erythrocytes per µl of peripheral blood was highest for LYL-$1^{-/-}$ mice and lowest for LYL-$1^{+/+}$ mice (Figure 23A). However, this number varied greatly for LYL-$1^{-/-}$, LYL-$1^{+/-}$, and LYL-$1^{+/+}$ mice resulting in no significant difference between genotypes. Erythrocytes from LYL-$1^{-/-}$ mice displayed normal haematological parameters such as the mean corpuscular volume (MCV) (Figure 23B), mean corpuscular haemoglobin (MCH) (Figure 23C), platelet

Figure 23. Haematological parameters of LYL-1$^{-/-}$ peripheral blood were normal. Peripheral blood isolated by tail vein bleed, was collected into potassium-EDTA and analysis on a Coulter-like counter. There was no statistically significant difference in the number of erythrocytes (A), platelets (E), white blood cells (F), or lymphocytes (G) between groups. Mean corpuscular volume (B), mean corpuscular haemoglobin (C) and mean corpuscular haemoglobin content of erythrocytes did not vary significantly between genotypes.

LYL-1-/- display normal haematological parameters.



number (Figure 23E), white blood cell count (Figure 23F), and lymphocyte number (Figure 23G). The absence of a statistically significant difference in peripheral erythrocyte number between LYL-$1^{+/+}$, LYL-$1^{+/-}$, and LYL-$1^{-/-}$ mice suggested that the splenic erythropoiesis in the LYL-$1^{-/-}$ mice did not increase the number of erythrocytes in peripheral blood. To establish that the LYL-$1^{-/-}$ mice possessed normal levels of stored iron, bone marrow cytospins and sections of spleen were stained for haemosiderin.

Section 3.10 LYL-$1^{-/-}$ mice have less haemosiderin stores in the spleen, and normal stores in the bone marrow compared to LYL-$1^{+/+}$ mice.

Perl's stain showed that the red pulp of the LYL-$1^{-/-}$ spleen (Figures 24C, D) contained much less haemosiderin than LYL-$1^{+/+}$ controls (Figure 24A, B). Haemosiderin deposits were found throughout the red pulp and, to a lesser extent, in the marginal zone of the LYL-$1^{+/+}$ spleen (Figure 24A). The reduced amount of haemosiderin in the LYL-$1^{-/-}$ spleen may have been the result of increased utilization of iron by increased numbers of developing erythroblasts *in-situ*. Alternatively, defective recycling of scavenged iron through macrophage- mediated erythrophagocytosis, or insufficient total iron could explain these observations. If the level of iron was not sufficient to supply enhanced splenic erythropoiesis, the bone marrow iron stores may have been affected. The Perl's stain was performed on bone marrow cytospins comprising $1x10^6$ cells (see Section 2.1.13) and showed no qualitatively significant difference between the amount of haemosiderin in LYL-$1^{+/+}$ (Figure 25A-D) and LYL-$1^{-/-}$ (Figure 25E-H) bone marrow cells. Reticular cells, sideroblasts and sideromacrophages were present in both populations of cells, as well as erythroblasts that stained with a characteristic blue hallow. These results showed that by Perl's stain, LYL-$1^{-/-}$ spleen contained less haemosiderin than LYL-$1^{+/+}$ controls, and that bone marrow erythropoiesis in LYL-$1^{-/-}$ mice was not iron deficient.

Figure 24. Perl's stain of haemosiderin in 5μm paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ (A and B), and LYL-$1^{-/-}$ (C and D) spleen (see Section 2.1.10). LYL-$1^{+/+}$ spleen contained more haemosiderin (A) than LYL-$1^{-/-}$ spleen (C). Analysis at high magnification showed much of the haemosiderin to be associated with macrophages (B and D), those in LYL-$1^{+/+}$ spleen comprise heavy deposits of haemosiderin (B) compared to macrophages in the LYL-$1^{-/-}$ spleen (D). Images A and C were taken at 20x magnification and images B and D were taken at 60x magnification.

LYL-1-/- spleen contain less haemosiderin than LYL-1+/+ spleen.

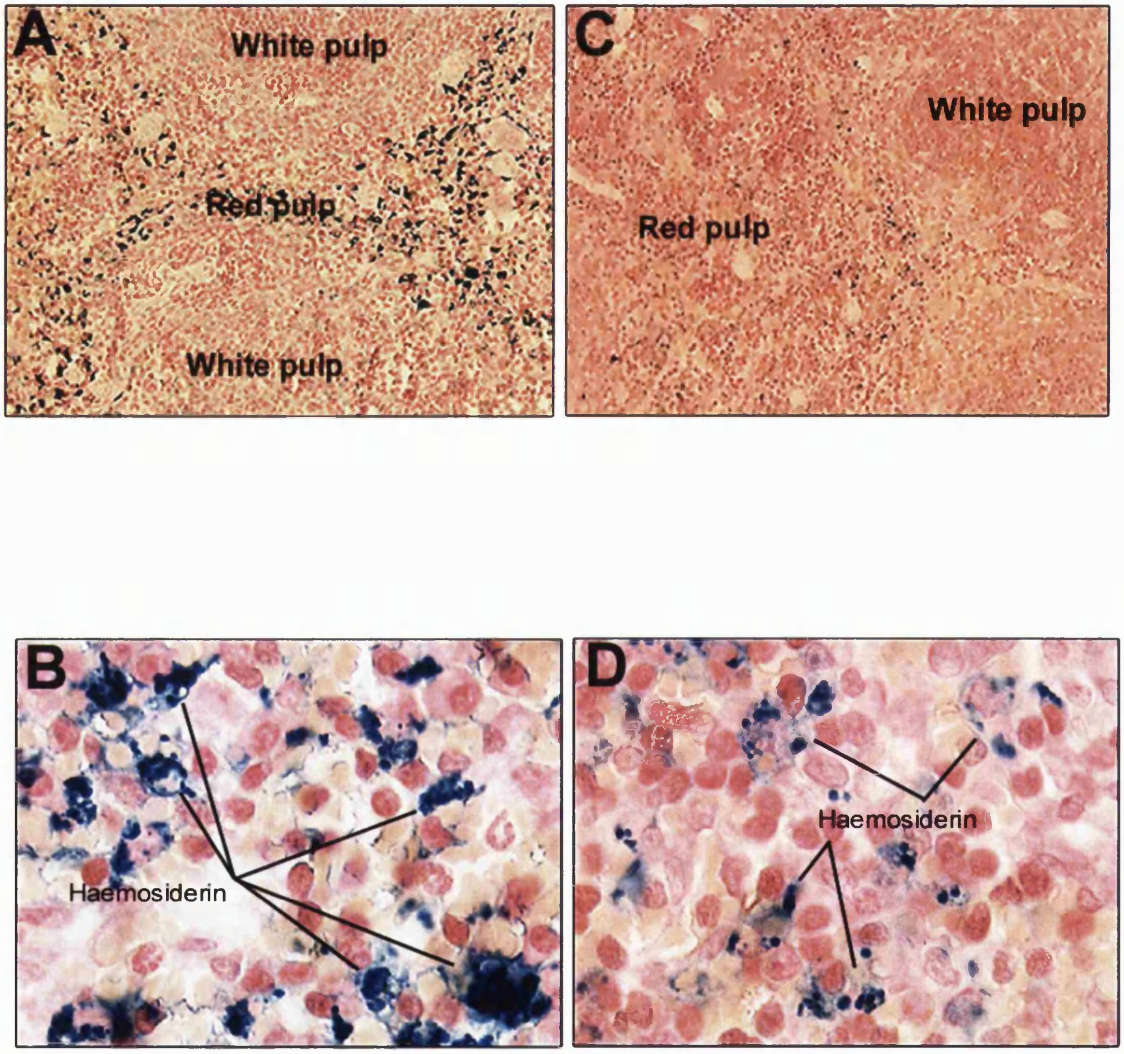
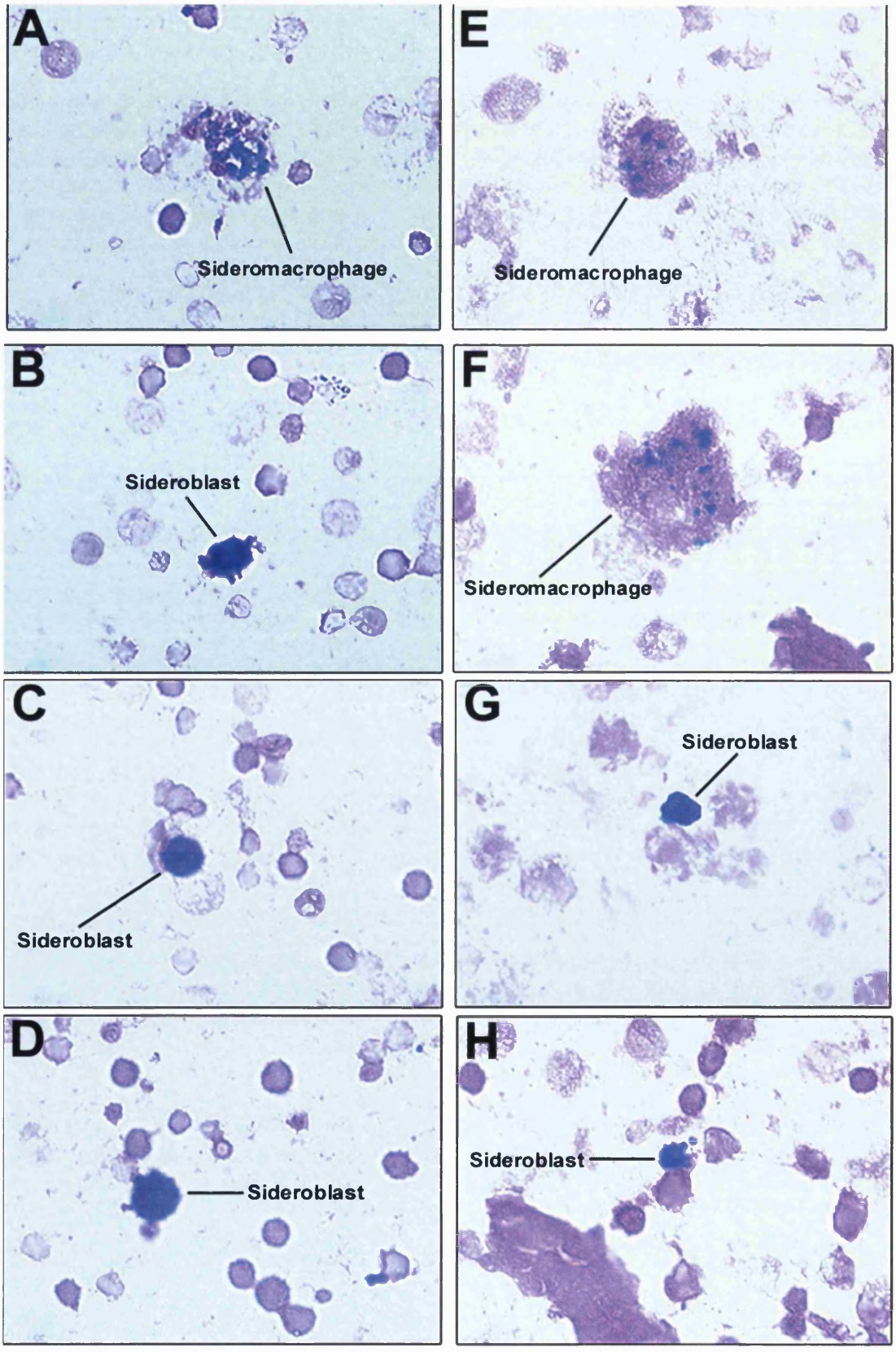


Figure 25. The amount of haemosiderin in cytocentrifuged LYL-$1^{-/-}$ (E-H) bone marrow cells (1x 10^6 cells) was similar to that of LYL-$1^{+/+}$ controls (A-D) (see Section 2.1.10). LYL-$1^{-/-}$ (G-H) and LYL-$1^{+/+}$ (B-D) bone marrow contained sideroblasts, and sideromacrophages (E-F, and A respectively). All images were taken at 60x magnification.

The amount of haemosiderin in the LYL-1-/- bone marrow is normal



As mentioned above, the reduced number of erythrocytes in the LYL-$1^{-/-}$ spleen may be the result of a block at the late stages of erythropoiesis, or apoptosis of erythroblasts prior to terminal differentiation in to erythrocytes. TUNEL assay was performed on sections of spleen to identify apoptotic cells.

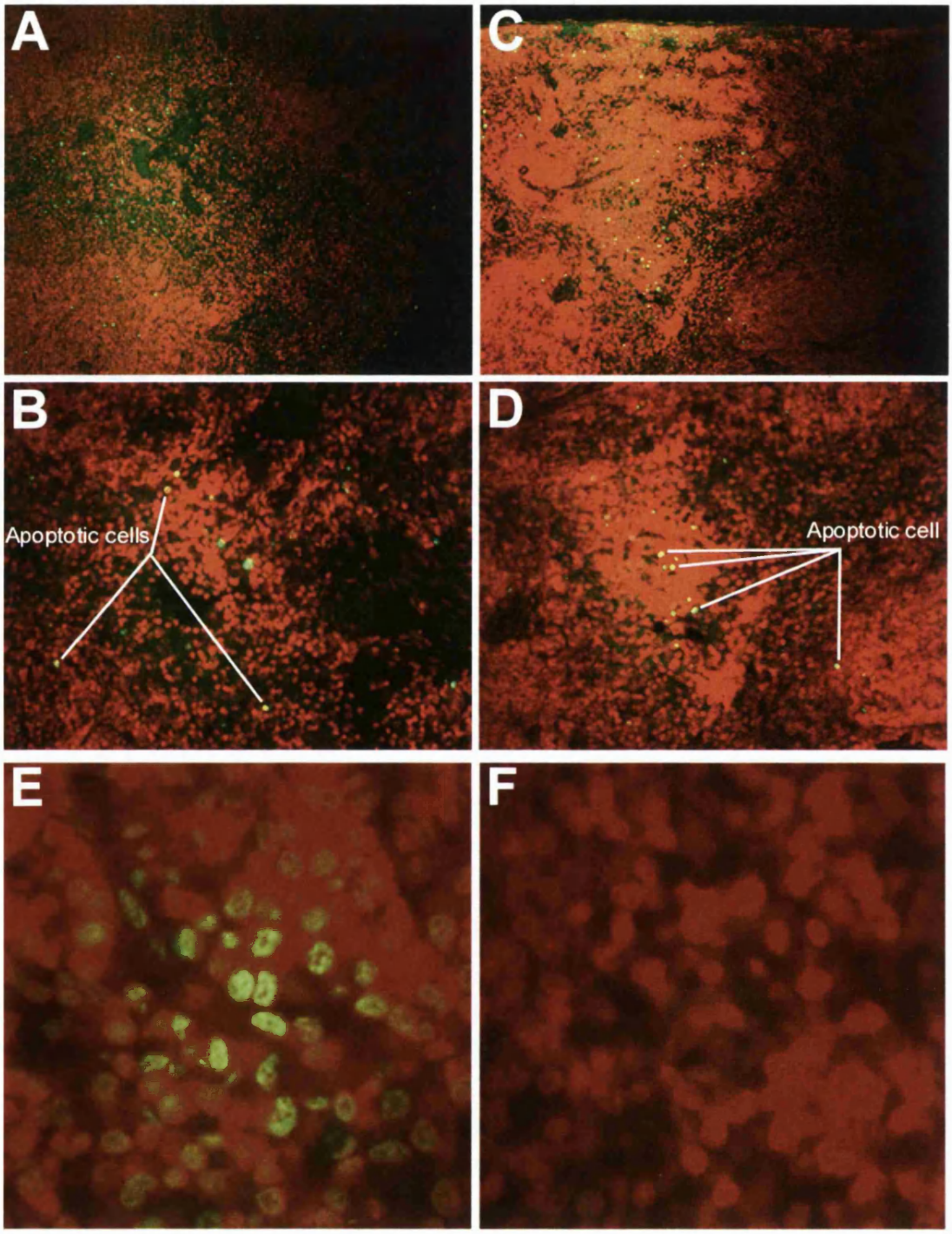
Section 3.11 Erythroblasts in the LYL-$1^{-/-}$ spleen do not undergo apoptosis.

Figure 26 shows the results of TUNEL assay performed on paraformaldehyde-fixed paraffin sections of LYL-$1^{+/+}$ and LYL-$1^{-/-}$ spleen (see Section 2.1.10). Lipofuscin deposits compromised the effectiveness of fluorescent methods of detection in the spleen (Figure 26A, C). Lipofuscin is the accumulation of lysosomes within cells from the oxidation of lipids and lipoproteins. Lipochrome, the wear and tear pigments found most commonly in the spleen, exhibits a strong orange autofluorescence in paraformaldehyde-fixed, unstained paraffin sections. Nonetheless, it was clear that the red pulp of LYL-$1^{-/-}$ spleen did not comprise more apoptotic cells than that of LYL-$1^{+/+}$ mice (Figure 26C-D and Figure 26A-B). This result suggested that the relatively normal number of peripheral erythrocytes in LYL-$1^{-/-}$ mice was not due to premature death of the erythroid progenitors or erythroblasts in the LYL-$1^{-/-}$ spleen.

An alternative mechanism may be responsible for the apparent block in erythroblast development in LYL-$1^{-/-}$ spleen, but it was not identified in this study. It is possible that the normal erythrocyte number, in the presence of increased splenic erythroid progenitors and erythroblasts, was the result of haemolysis. However, the data presented in this study does not address this possibility, and the reason(s) for reduced erythrocytes in the in LYL-$1^{-/-}$ spleen remains to be elucidated.

Figure 26. LYL-$1^{-/-}$ (C and D) and LYL-$1^{+/+}$ (A and B) spleen comprised similar numbers of apoptotic cells. Apoptosis was determined by TUNEL assay using the fluorescein detection kit (see Section 2.1.7). Image E represents a positive control (spleen sections exposed to DNAse I to promote DNA fragmentation) and F is a negative control showing propidium iodide staining alone (fluorescein 12 dUTP was omitted from the reaction). Images A and C were taken at 5x magnification; images B and D were taken at 10x magnification; images E and F were taken at 100x magnification.

LYL-1-/- spleen does not contain more apoptotic cells than LYL-1+/+ spleen.



Section 3.12 Summary.

Based upon the results described above, a phenotype was attributed to the LYL-1$^{-/-}$ mouse. The absence of a functional LYL-1 protein was associated with increased numbers of immature BFU-e (3.2-fold), mature BFU-e (5.5-fold), CFU-e (2.2-fold), and CFU-GEMM (2.6-fold) in the spleen specifically. Erythroid progenitors from the LYL-1$^{-/-}$ spleen generated haemoglobinised erythroblasts *in vitro*. The LYL-1$^{-/-}$ spleen also comprised BFU-e that produced very large colonies of erythroblasts in the presence of Epo and SCF. *In vivo*, splenic erythroid progenitors and precursors expressed SCL mRNA, GATA-1, and TER-119, and were distributed throughout the subcapsular region and red pulp. Erythroblasts in the LYL-1$^{-/-}$ spleen were 5 to 10 times more frequent than normal, and were arranged into clusters that were occasionally associated with megakaryocytes. The loss of a functional LYL-1 protein was further associated with a reduction in the number of erythrocytes, and amount of stored iron, in the spleen, but no change in the number of erythrocytes in the peripheral circulation, or levels of haemosiderin in the bone marrow.

Chapter 4. Discussion.

Section 4.1 SCL does not compensate for the loss of a functional LYL-1 protein in haematopoietic cells.

Disruption of the LYL-1 gene by homologous recombination in murine ES cells generated animals that displayed a phenotype consistent with a role for the LYL-1 protein in haematopoiesis. The results described in Chapter 3 do not support the hypothesis that SCL was able to compensate for the loss of LYL-1 in LYL-$1^{-/-}$ mice. Increased numbers of haematopoietic progenitors in the LYL-$1^{-/-}$ spleen, in the presence of a functional SCL protein, showed that SCL was not able to functionally compensate for the loss of LYL-1, and thereby maintain normal numbers of splenic haematopoietic progenitors.

Several lines of evidence support this conclusion. The distribution of cells expressing the LYL-1^{lacZ} fusion protein in LYL-$1^{+/-}$ and LYL-$1^{-/-}$ spleen sections (Figure 17) did not match that of erythroblasts expressing SCL mRNA (Figures 5 and 14), GATA-1 protein (Figure 16) and TER-119 protein (Figure 18 and 19). Therefore, LYL-1 expression did not overlap with that of SCL in erythroblasts in the LYL-$1^{-/-}$ spleen. The increase in SCL mRNA determined by semi-quantitative RT-PCR in LYL-$1^{-/-}$ spleen (Figure 13) was shown, by SCL-specific *in situ* RT-PCR, to be due to an increase in the numbers of erythroid cells expressing SCL mRNA, and not to an increase in the level of SCL mRNA expressed in individual erythroid cells (Figure 5, and 14). This showed that, unlike Myf-5 in myoblasts of the MyoD$^{-/-}$ mouse (Rudnicki, et al, 1993), Mash-1 in Ngn-$2^{-/-}$ mutant cortical progenitors (Fode, et al, 2000), and GATA-2 in erythroid precursors generated by in-vitro differentiation of GATA-$1^{-/-}$ ES cells (Weiss, et al., 1994; Fujiwara, et al., 1996), an increase in SCL mRNA expression in

individual murine erythroblasts was not able to functionally compensate for loss of LYL-1 protein.

Section 4.2 LYL-1 was not expressed in erythroblasts, but may be expressed in activated splenic B cells.

Absence of the LYL-1^{lacZ} fusion protein in the clusters of erythroblasts in the LYL-$1^{-/-}$ spleen indicated that LYL-1 was not involved in transcriptional regulation during erythropoiesis. Examination of May-Grunwald Giemsa/ X-GAL double stained bone marrow cytocentrifuged cells from LYL-$1^{+/-}$ mice confirmed the absence of LYL-1^{lacZ} fusion protein expression in morphologically distinct erythroblasts (data not shown). LYL-1 transcript was identified in several erythroid cells by northern hybridisation analysis (Visvader, et al, 1991). However, the expression of LYL-1 in erythroid cell lines may be different during normal development of erythroid cells, and its presence in these cell lines may not represent LYL-1 protein expression during normal erythropoiesis. This may not be the case for B lymphoid cell lines, which were shown to express the highest levels of LYL-1 mRNA (Kuo, et al., 1991). Along with LYL-1 mRNA expression in B-cell lines, LYL-1 mRNA was also identified in activated splenic B cells, mesenteric lymph nodes, and spleen (Visavder, et al., 1991: Kuo, et al., 1991). In the present study, few LYL-$1^{lacZ+ve}$ cells were identified within the white pulp of spleen from LYL-$1^{+/-}$ and LYL-$1^{-/-}$ mice (Figure 17). Indeed, these cells were almost exclusively located in the red pulp and marginal zone. The marginal zones in the spleen comprised activated B cells (Nolte, et al., 2000). It is interesting to speculate that the LYL-$1^{lacZ+ve}$ cells identified in the marginal zone of the LYL-$1^{+/-}$ and LYL-$1^{-/-}$ spleen were activated B cells. The LYL-$1^{lacZ+ve}$ cells found in the splenic red pulp were not identified morpholgically. However, their modest frequency within a heterogeneous population of mature haematopoietic cells, would suggest an immature cell type, possibly progenitor cell. It

can be concluded that LYL-1 may be expressed in a population of haematopoietic cells in the spleen including activated B-cells.

In the present study, LYL-1 was found in spleen, brain, thymus, and liver (Figure 12). RT-PCR is a more sensitive assay than northern hybridisation for detection of mRNA, and would reveal lower levels of LYL-1 mRNA expression. The presence of LYL-1 mRNA in the liver may result from haematopoietic cell contamination, rather than hepatocytes-specific LYL-1 expression. The presence of LYL-1 mRNA in brain was not due to blood cells, but X-GAL stained cryosections of brain were negative for the LYL-1^{lacZ} fusion protein (data not shown). SCL was identified in the developing brain (Green, et al., 1992) but not in the adult. The absence of a neuronal phenotype showed that the role of LYL-1 was redundant in murine brain cell development.

Section 4.3 LYL-1 does not have a role in regulating proliferation of haematopoietic progenitor cells.

Analysis of X-GAL stained bone marrow cytocentrifuged cells (Rupping, Ph.D. thesis) revealed that the majority of c-Kit^{+ve} cells expressed LYL-1^{lacZ} fusion protein. This association may allude to a relationship between LYL-1 and c-Kit expression, which has been confirmed for SCL (Vandenbark, et al., 1996; Vitelli, et al., 2000). However, in the present study, the distribution pattern, and number of LYL-$1^{lacZ+ve}$ cells in the spleen of LYL-$1^{+/-}$ and LYL-$1^{-/-}$ mice were similar, while the number of c-Kit^{+ve} cells increased by approximately 4-fold in LYL-$1^{-/-}$ spleen compared to $LYL^{+/+}$ (Figure 20). This was consistent with an increase in splenic CFU-e and BFU-e (Figure 21) determined by *in vitro* assay. The rate of development, and in the majority of cases (see below), the size of $LYL^{+/+}$ and LYL-$1^{-/-}$ splenic CFU-e and BFU-e colonies were similar (data not shown). $LYL^{+/+}$, and LYL-$1^{-/-}$

splenic BFU-e generated colonies comprising haemoglobinised cells (Figure 22). These data showed that LYL-1 was not expressed in the majority of c-Kit^{+ve} cells in the LYL-1$^{-/-}$ spleen and its absence in haematopoietic progenitor cells did not affect their normal development *in vitro*. LYL-1$^{-/-}$ bone marrow BFU-e and CFU-e colonies also developed normally *in vitro* and, with the exception of a marginal, but significant change in CFU-e, the numbers of haematopoietic progenitor cells remained normal in LYL-1$^{-/-}$ bone marrow. It can be concluded that LYL-1 does not have a role in the proliferation and/ or differentiation of c-Kit^{+ve} haematopoietic progenitors in mice.

Section 4.4 LYL-1 does not negatively regulate SCL expression.

The possibility that LYL-1 was expressed in a proportion of c-Kit^{+ve} cells in the spleen cannot be ruled out, since c-Kit^{+ve}, and LYL$^{lacZ+ve}$ cells were present within the red pulp of LYL$^{+/+}$ spleen (Figures 17 and 20). Co-localisation of c-Kit and LYL^{lacZ} to individual cells was not confirmed in this study, so a function for LYL-1 in these LYL$^{lacZ+ve}$c-Kit^{+ve} cells in the LYL$^{-/-}$ spleen cannot be elucidated. Assuming that LYL-1 was expressed in a subpopulation of c-Kit^{+ve} progenitors in the spleen, their development may indicate a role for LYL-1 in these cells. The *in situ* RT-PCR protocol revealed cell-to-cell changes in staining intensity in LYL-1$^{+/+}$ and LYL-1$^{-/-}$ sections of spleen (Figures 5 and 14). Some cells in the LYL-1$^{-/-}$ spleen appeared to express higher levels of SCL mRNA than seen in LYL-1$^{+/+}$ spleen. These apparent differences in the level of SCL expression may be the result of discrete differences in the integrity of the amplicon migration barrier. The proteinase K-mediated digestion of the paraformaldehyde-fixed spleen sections was sufficient to provide access of enzymes and substrates e.g. Taq DNA polymerase-Taq antibody, nucleotides (see Section 2.5.4) to the target, without disrupting the paraformaldehyde lattice to the extent that cDNA synthesized *in situ*, was able to migrate from its site of production, and into the

surrounding solution. However, the variables inherent to the protocol for *in situ* RT-PCR allow for discrimination between cells that express, and those that do not express SCL mRNA, but do not allow accurate estimation of SCL mRNA in cells that express different levels of the SCL transcript. It is tempting to speculate that within a subpopulation of c-Kit^{+ve} progenitors in the LYL-1$^{-/-}$ spleen, the absence of LYL-1 promoted up-regulation of SCL mRNA expression. SCL mRNA expression was induced upon erythroid differentiation (Aplan, et al., 1992), and patterns of SCL transcript expression differed in distinct erythroid subpopulations (Green, et al., 1991), consistent with the differential level of SCL mRNA expression in the clusters of erythroid cells in LYL-1$^{-/-}$ spleen (Figure 14). Increased SCL mRNA expression was correlated with proliferation of BFU-e, and SCL protein was required for self-renewal of erythroid progenitors (Green, et al., 1991). Enforced SCL protein expression in haematopoietic progenitor cells increased the number and size of BFU-e as well as the number of megakaryocytic colony-forming units (Valtieri, et al., 1998). Although the size of BFU-e colonies was normal in LYL-1$^{-/-}$ spleen, BFU-e as well as CFU-GEMM numbers increased significantly.

Clearly, negative regulation of SCL mRNA expression by LYL-1 does not occur in the erythroblastic clusters identified in the LYL-1$^{-/-}$ spleen. The effect, of a putative increase in SCL mRNA expression within a sub-population of c-Kit^{+ve}-LYL-1$^{lacZ+ve}$ haematopoietic progenitor cells in the LYL-1$^{-/-}$ spleen, on erythroid development would require assessment of SCL protein expression in this population of cells. SCL mRNA levels undergo biphasic modulation during HBMA-induced erythroid differentiation of MEL cells (Green et al., 1991). Differentiation produced a transient fall followed by a rise in SCL mRNA levels. The discordant regulation of SCL mRNA and protein during erythroid differentiation (Murrell, et al., 1995) suggested that additional levels of control regulated SCL expression. The

mechanisms involved in regulating SCL expression are poorly understood. Measurement of SCL mRNA expression in individual c-Kit^{+ve}-LYL-1$^{lacZ+ve}$- progenitors would confirm whether SCL mRNA expression was elevated in a sub-population of c-Kit^{+ve}-LYL-1$^{lacZ+ve}$ haematopoietic progenitors.

Several lines of evidence from the data presented in this study argue against a role for LYL-1 as a negative regulator of SCL mRNA expression in c-Kit^{+ve} haematopoietic progenitor cells. First, there was not a dramatic increase in the level of SCL mRNA expression in LYL-1$^{-/-}$ bone marrow (Figure 12), as seen in the spleen. This was consistent with the normal number of BFU-e in the LYL-1$^{-/-}$ bone marrow. The marginal increase in CFU-e in LYL-1$^{-/-}$ bone marrow (Figure 21A) may have been responsible for the small, but insignificant increase in SCL mRNA expression (Figure 12). However, this was not comparable to the increase in splenic BFU-e and CFU-e (Figure 21A, B, C), and SCL mRNA^{+ve} (Figure 13) in LYL-1$^{-/-}$ mice. If the absence of LYL-1, in a sub-population of c-Kit^{+ve} progenitors in LYL-1$^{-/-}$ spleen, facilitated erythroid proliferation and differentiation through elevated SCL mRNA expression, the number of BFU-e and CFU-e in LYL-1$^{-/-}$ bone marrow would have increased, as seen in the spleen. Second, the majority of c-Kit^{+ve} progenitors in the LYL-1$^{-/-}$ spleen do not express LYL-1^{lacZ}. As mentioned in Chapter 3, the expression of c-Kit on cells in the spleen does not indicate their fate. However, since the rise in c-Kit^{+ve} progenitors is consistent with elevated BFU-e, CFU-e and erythroblasts in the LYL-1$^{-/-}$ spleen, an increase in the number of LYL-1$^{lacZ+ve}$ cells in the spleen would be expected if LYL-1 were involved in the regulation of SCL mRNA expression in c-Kit^{+ve} progenitors. Third, semi-quantitative RT-PCR did not identify any change in SCL mRNA expression in 14.5dpc fetal liver (Figure 12). Fourth, there was no difference in the rate of growth or the number of cells in BFU-e and CFU-e colonies *in vitro*. It can be concluded that the phenotype of LYL-1$^{-/-}$ mice was not due

to elevated SCL mRNA expression in a sub-population of c-Kit^{+ve} haematopoietic progenitors. Therefore, LYL-1 is not a negative regulator of SCL mRNA expression in erythropoiesis.

Section 4.5 The LYL-1 phenotype resulted from migration of haematopoietic progenitor cells.

Over-expression of SCL mRNA in a sub-population of haematopoietic progenitors was not consistent with the experimental data, and therefore does not explain the increased numbers of erythroid progenitors and precursors in the spleen of LYL-1$^{-/-}$ mice. Since the proliferation of LYL-1$^{-/-}$ splenic BFU-e and CFU-e was normal (Figure 20), the most reasonable explanation for the LYL-1$^{-/-}$ phenotype was an increase in the migration of c-Kit^{+ve} haematopoietic progenitors from the bone marrow to the spleen.

Extramedullary haematopoietic progenitor cells have been identified (Starzl, et al., 1993), but progenitors are known to migrate from the bone marrow to the spleen via the peripheral blood (To, et al., 1994). Since LYL1 was expressed in the majority of c-Kit^{+ve} cells in the LYL$^{-/-}$ bone marrow (Rupping, Ph.D. thesis), the role of LYL-1 in these cells may elude to its function in their migration.

Section 4.6 A possible role for LYL-1 in c-Kit mediated migration of haematopoietic progenitor cells from the bone marrow.

Migration of haematopoietic progenitors involved perturbation of their adhesive interactions with stromal elements that were, under steady-state conditions, responsible for retention of the progenitor cells in the bone marrow (Turner, 1994). Haematopoietic progenitor cells are known to express a wide range of cell adhesion molecules (CAMs) including integrin,

selectin, immunoglobulin superfamily and the CD44 family of CAMs (Simmons, et al., 1994). The importance of the β1-integrin very late antigen-4 (VLA-4) to homing, lodgment and retention of primitive haematopoietic progenitor cells has been confirmed by studies that identified mobilized $CD34^+$ cells consistently demonstrated reduced expression of VLA-4 (Mohle, et al., 1993), and that perturbation of VLA-4 function, by administration of anti-VLA-4 antibodies, induced progenitor cell mobilization (Papayannopoulou, et al., 1993). Interestingly, the action of the anti- VLA-4 antibodies required functional c-Kit (Papayannopoulou, et al., 1998).

SCF and c-Kit interactions have been shown to play an important role in cell adhesion and migration (To, et al., 1997; Papayannopoulou, et al., 1998; Kinashi and Springer, 1994; Kodama, et al., 1994). SCF was a potent agent for mobilization of progenitors from the bone marrow to the peripheral blood (To, et al., 1994). A decrease in the density of c-Kit on the surface of $CD34^+$ cells in mobilized peripheral blood compared to levels on steady-state bone marrow and peripheral blood $CD34^+$ cells after four types of mobilisation protocol (To, et al., 1994) indicated that mobilisation events involved signaling pathways, which down-regulated c-Kit expression. Although the mechanism responsible for this reduction remains unknown, the change in c-Kit density occurred in the bone marrow before the egress of haematopoietic progenitors into the circulation (Simmons, et al., 1994). Further support for the role of c-Kit in haematopoietic progenitor cell mobilisation has come from studies that have used W/W mice. G-CSF mediated mobilization of haematopoietic progenitors was less efficient in these mice compared to wildtype control (Cynshi, et al., 1991). W/W mice were also unable to respond to Epo in the same way as normal mice (Kato, et al., 1998). These workers proposed that up-regulation of uptake clearance by the spleen, after repeated administration of recombinant human Epo, was due to Epo-mediated migration of BFU-e from the bone

marrow to the spleen (Kato, et al., 1999), and since W/W failed to up-regulate up take clearance, BFU-e migration to the spleen was dependent on the presence of functional c-Kit. These studies supported a role for c-Kit in regulating migration of different haematopoietic cell types, particularly the BFU-e, from the bone marrow to the spleen.

The absence of a functional LYL-1 protein in LYL-$1^{lacZ+ve}$ cells in the bone marrow of LYL-$1^{-/-}$ mice may have had an effect on c-Kit expression, which led to an increase in the migration of erythroid progenitor cells from the bone marrow to the spleen. As such, LYL-1 would function as a positive regulator of c-Kit expression, promoting adhesion of progenitor cells to the stromal cell matrix.

Section 4.7 A mechanism for LYL-1 control of c-Kit mediated haematopoietic progenitor cell migration.

Studies of the human c-kit promoter (Vandenbark, et al., 1996; Yamamoto, et al., 1993) revealed that c-kit expression was controlled at the transcriptional level, and that regulation of transcription involved several activators and repressors. cis-acting sequences in the promoter included putative binding sites for Sp1, AP-2, Ets-like proteins, GATA-1, and c-Myb. Myb and Ets proteins acted cooperatively as positive regulators of c-kit (Ratajczak , et al., 1998). In addition, selective Sp1 binding was critical for c-kit core promoter activity (Park, et al., 1998). The c-Kit promoter has also been shown to contain a consensus binding site for SCL (Vandenbark, et al., 1996). Anti-sense SCL inhibited c-Kit expression in several haematopoietic cell lines (Krosl, et al., 1998), which suggested that c-Kit was a downstream target for SCL in primitive haematopoietic progenitor cells. Recent studies have indicated that SCL forms part of a pentamer transcriptional complex with pRb, E2A, Lmo2, and Ldb1 in human adult proerythroblasts and erythroblasts (Vitelli, et al., 2000). However, this

complex negatively regulated c-Kit expression by binding to two inverted E box-2 type motifs in the c-kit promoter. These differences could be due to the effect of SCL on c-Kit expression in different haematopoietic cell types (Krosl, et al., 1998; Vitelli, et al., 2000). Sequence analysis of the c-kit promoter revealed two adjacent E boxes at 374 and 381 that are conserved between human and mouse (Tsujimura, et al., 1996). These E boxes may contribute to SCL-mediated c-Kit promoter activity (Vandenbark, et al., 1996; Vitelli, et al., 2000). Since LYL-1 and SCL have almost identical preferred E-box binding sequences, and share the same heterodimer partners (Miyamoto, et al., 1996), it was possible that LYL-1 might regulate c-Kit expression.

A putative role for LYL-1 in c-Kit-mediated migration would be to maintain the adhesive interactions between progenitor cells and stromal elements in the bone marrow. The absence of a functional LYL-1 protein would facilitate the release of erythroid progenitors from the bone marrow into the peripheral circulation. It is clear that the expression of c-Kit is not dependent on LYL-1 expression, and the release of progenitors from the LYL-$1^{-/-}$ bone marrow is not due to the absence of c-Kit on cells. However, as mentioned above, a reduction in c-Kit antigen density was inversely correlated with the presence of $CD34^{+}$ progenitors in the peripheral circulation (To, et al., 1994). Mobilised $CD34^{+}$ cells still expressed c-Kit (To, et al, 1997), as did cells in the $LYL^{+/-}$, and LYL-$1^{-/-}$ spleen. Therefore, one could speculate that the LYL-1 protein positively modulated c-Kit expression in progenitors, and thereby mediated retention of c-Kit^{+ve} cells in LYL-$1^{+/+}$ bone marrow. A non-functional LYL-1 protein could not form transcriptionally active elements that could either bind to sites in the c-Kit promoter directly, or elicit transcription of genes that induced c-Kit mediated-anchorage of progenitor cells. The putative mechanism of action proposed for LYL-1 function in c-Kit^{+ve} cells would explain the presence of increased numbers of haematopoietic, particularly

erythroid progenitors in the spleen in terms of increased cell migration from the bone marrow.

Section 4.8 LYL-1 was not essential for the maintenance of normal haematological parameters.

Since expression of a non-functional LYL-1 protein did not affect the proliferation, differentiation or survival of erythroid progenitors from LYL-$1^{-/-}$ spleen and bone marrow *in vitro*, other factors must be involved in maintaining apparently normal numbers of erythrocytes in LYL-$1^{-/-}$ mice (Figure 23). The absence of an increase in erythrocytes in the peripheral circulation, as well as haematological parameters such as the MCV, and MCHC suggested a block in the later stages of erythroid cell development in the LYL-$1^{-/-}$ spleen. It is possible that the tail vein method of blood retrieval may have affected the number of intact erythrocytes (and platelets) in the sample due to un-avoidable clotting of the severed blood vessel. Blood collected by cardiac puncture would not have been compromised in this way and may have provided less variable data. However, the results of the cell count showed a general increase in all the haematological parameters for LYL-$1^{-/-}$ blood (Figure 23) including the white blood cell count (Figure 23F). The haematological data suggested that increased numbers of haematopoietic progenitors, particularly erythroid progenitors in the LYL-$1^{-/-}$ spleen did not contribute cells to the peripheral blood system.

It was also conceivable that haemolysis at an unknown site could maintain an apparently normal number of erythrocytes in the peripheral circulation, even though production of erythrocytes in the spleen was enhanced. Haemolytic anemia induced by phenylhydrazine increased the erythropoietic demand on bone marrow (Broudy, et al., 1996). Under these conditions, bone marrow erythropoiesis became stressed, and promoted mobilisation of

erythroid progenitors to the spleen (Broudy, et al., 1996). Mobilisation was associated with enhanced splenic erythropoiesis that was responsible for expansion of the erythroid compartment during, and some time after re-establishment of normal erythrocyte numbers in the peripheral blood (Broudy, et al., 1996). The LYL-$1^{-/-}$ mice were not anemic, and had normal levels of iron stored in the bone marrow (Figure 25), which indicated that migration of increased numbers of erythroid progenitors from the bone marrow in LYL-$1^{-/-}$ mice occurred when bone marrow erythropoiesis was apparently capable of producing a sufficient number of erythrocytes. It can be concluded that bone marrow erythropoiesis was not defective in LYL-$1^{-/-}$ mice, and that migration of increased numbers of LYL-$1^{-/-}$ erythroid progenitor cells from the bone marrow to the spleen occurred when haematopoiesis was normal.

Section 4.9 LYL-1 does not perform an anti-apoptotic function in haematopoietic progenitor cells.

The spleen of LYL-$1^{+/+}$ and LYL-$1^{-/-}$ mice comprised similar numbers of apoptotic cells (Figure 26). Differentiation of erythroid precursors did not require a functional LYL-1 protein because, as mentioned above, LYL-1^{lacZ} was not expressed in erythroblasts (Figure 17). Furthermore, there was no indication that the progenitors from LYL-$1^{-/-}$ spleen died prematurely, or that colonies generated from LYL-$1^{+/-}$ spleen survived longer *in vitro*. The method for detection of apoptotic cells used in this study was not ideal for spleen sections due to significant autofluorescence in the tissue (Figure 26). Nevertheless, marked differences in apoptotic cell numbers between LYL-$1^{+/+}$ and LYL-$1^{-/-}$ spleen, and *in vitro* development of progenitors in methylcellulose medium comprising Epo, IL-3, IL-6, and SCF, were not apparent.

c-Kit mediated signals have been shown to protect serum-deprived erythroid cells from apoptosis (Endo, et al., 2001), and SCF, in conjunction with Epo, promoted development of BFU-e (Dai, et al., 2000; von Lindern, et al., 1999; Wessely, et al., 1999). SCL was able to secure the survival of primitive haematopoietic cells through upregulation of c-Kit (Krosl, et al., 1998) It would be interesting to speculate on whether the block in differentiation of erythroid progenitors at the late erythroblast stage was related to the loss of LYL-1, given the putative role of LYL-1 in c-Kit expression. Clearly, the absence of LYL-1 in erythroblasts in the LYL-$1^{-/-}$ spleen rules out a possible role in erythropoiesis. As discussed above, the majority of c-Kit^{+ve} cells in the LYL-$1^{-/-}$ spleen did not express LYL-1^{lacZ}, and grew normally in semi-solid methylcellulose media. The physiological conditions presented within the microenvironment of the spleen are altogether different to the *in vitro* assay medium, so an effect on the proliferation of LYL-$1^{lacZ+ve}$c-Kit^{+ve} progenitor cells in the absence of a functional LYL-1 protein, *in vivo*, cannot be ruled out completely. Examination of the growth of LYL-$1^{lacZ+ve}$c-Kit^{+ve} progenitors specifically, under different conditions, would be useful in this regard.

Section 4.10 LYL-1 has a limited role in the regulation of haematopoietic progenitor cell migration to the spleen.

The role of the spleen in haematopoiesis is well documented (Till and McCulloch, 1964; Moore and Metcalf, 1970; Krause, et al., 2001). The spleen served as a site for erythroid expansion when bone marrow erythropoiesis was stressed (Broudy et al., 1996) and was the major organ for regenerative re-population of haematopoietic progenitors in mice, in particular CFU-e progenitors (Saitoh, et al., 1999). As mentioned above, Epo is capable of stimulating migration of BFU-e from the bone marrow to the spleen, which was followed by differentiation into CFU-e (Kato, et al., 1999). Prolonged treatment of mice with recombinant

human Epo revealed differences in the development of BFU-e, CFU-e, and erythroid precursors in the bone marrow and spleen (Nijhof, et al., 1993). BFU-e produced CFU-e, which developed into erythroblasts with much higher efficiency in spleen compared to bone marrow (Hendrikx, et al., 1996). The distribution of ^{3}H-radiolabeled BFU-e into rats appeared to be selective, and indicated that unlike in the lung and liver, erythroid progenitors were capable of moving into the spleen specifically (Kato, et al., 1999). The increase in erythroid progenitor cell migration from the LYL-1$^{-/-}$ bone marrow to the spleen is modest in comparison to the expansion of splenic erythropoiesis associated with phenylhydrazine-induced haemolytic anemia (Broudy, et al., 1996). Indeed, the consequent increase in splenic erythropoiesis restored hematocrit and reticulocyte count (Boudy, et al., 1996), whereas no change in haematological parameters was found in LYL-1$^{-/-}$ peripheral blood compared to LYL-1$^{+/+}$ controls (Figure 23).

The BFU-e and CFU-e pool of erythroid progenitors did not re-distribute to the spleen, so the ability of LYL-1$^{-/-}$ haematopoietic progenitor cells to adhere to the bone marrow was not completely dependent on LYL-1 expression. It may be surmised that LYL-1 was able to regulate c-Kit-mediated anchorage in a population of c-Kit^{+ve} haematopoietic progenitors in the bone marrow, but was not required in all LYL-1$^{lacZ+ve}$ c-Kit^{+ve} progenitors. In this regard, the prominent increase in erythroid progenitors in the LYL-1$^{-/-}$ spleen may suggest that the proposed function of LYL-1 in the regulation of c-Kit-mediated anchorage of haematopoietic progenitors was specific for committed BFU-e. Alternatively, the absence of LYL-1 may have affected migration of all c-Kit^{+ve} cells in the bone marrow, but only a proportion of these cells lodged in the spleen. Interestingly, the ability of haematopoietic progenitors pre-treated with anti-c-kit antibody to migrate, or 'home', to the spleen was severely perturbed (Broudy, et al., 1996). Similarly, a defect in the ability of c-Kit^{+ve} progenitors to anchor in the

spleen of LYL-$1^{-/-}$ mice would be consistent with increased migration from the bone marrow. Furthermore, it may explain the 1.4-fold increase in LYL-$1^{-/-}$ bone marrow CFU-e as the result of elevated CFU-e generated in the spleen, circulating through the bone marrow. We can predict that, under steady-state conditions, more c-Kit^{+ve} progenitor cells are circulating in the peripheral blood of LYL-$1^{-/-}$ mice than normal. An assessment of the number of LYL-$1^{lacZ+ve}$ c-Kit^{+ve} cells in the LYL-$1^{-/-}$ peripheral blood would provide an estimate of how many of the LYL-$1^{lacZ+ve}$c-Kit^{+ve} progenitor cells in the bone marrow migrate into the blood, and then into the spleen.

Section 4.11 LYL-1 regulates migration of distinct haematopoietic progenitor cell types.

An interesting feature of the LYL-1 knockout phenotype was the presence of progenitor cells that generated very large BFU-e colonies *in vitro* (Figure 22C), and increased numbers of CFU-GEMM colonies, which comprised megakaryocytes *in vitro* (Figure 22D).

LYL-1 was expressed in megakaryocytes in the bone marrow (Rupping, Ph. D. thesis), but it's absence did not affect either megakaryocyte or platelet numbers. In this study, the spleen of LYL-$1^{-/-}$ mice comprised more progenitors that gave rise to CFU-GEMM (Figure 20, Tables 11 and 12) with no change in the number of CFU-GM from LYL-$1^{-/-}$ bone marrow (data not shown). Recently, a bipotent erythroid and megakaryocytic precursor was identified in the spleen of phenylhydrazine (PHZ)-treated mice (Vannucchi, et al., 2000). These authors reported high numbers of these precursor cells in PHZ-spleen, and suggested that normal BFU-e expressed megakaryocyte-specific genes, and gave rise to morphologically and biochemically distinct megakaryocytes when cultured in the presence of Tpo. The bipotential nature of BFU-e, and the erythroid-megakaryocytic precursor cells may arise from qualitative

and/ or quantitative changes in the equilibrium of the shared transcriptional regulators such as GATA-1 (Shivdasani, et al., 1997). Some of the LYL-1$^{-/-}$ splenic megakaryocytes expressed GATA-1 (Figure 16G, H), and may have developed from c-Kit^{+ve} erythroid-megakaryocytic bipotential progenitor cells that migrated from LYL-1$^{-/-}$ bone marrow to the spleen.

Progenitors that gave rise to large BFU-e may be a different cell type to those responsible for the majority of BFU-e in the LYL-1$^{-/-}$ spleen. Since these progenitors, as well as those that generated CFU-GEMM, probably expressed c-Kit, the putative role of LYL-1 in these progenitors as a regulator of c-Kit mediated migration is likely to be the same in these different types of haematopoietic progenitor cell. One may conclude that a lack of LYL-1 in c-Kit^{+ve} haematopoietic progenitors affected migration of cells with different developmental programs.

Section 4.12 Conclusion

A complex network of signaling pathways exist to regulate haematopoietic cell development and migration (Laronique, et al., 1997; Wright, et al., 2001). The precise role of LYL-1 in c-Kit-mediated migration of haematopoietic progenitor cells from the bone marrow to the spleen would require further research. The data presented does not define a function of LYL-1, but does describe a phenotype in the LYL-$1^{-/-}$ mice consistent with a role for the LYL-1 bHLH protein in regulating events associated with migration of haematopoietic progenitor cells in the adult mouse. It is proposed that LYL-1 may mediate these events by regulating the expression of c-Kit in haematopoietic progenitor cells.

Section 4.2 Future work.

The first set of experiments to investigate the role of LYL-1 in c-Kit expression would be to examine the level of c-Kit on LYL-$1^{-/-}$ haematopoietic progenitor cells. It is hypothesized that the level of c-Kit expression is reduced on these cells, and that progenitors egress the bone marrow due to dysfunctional c-Kit mediated anchorage. Flow cytometry could be used to analyse FDG- and c-Kit-stained haematopoietic progenitor cells from LYL-$1^{+/-}$ and LYL-$1^{-/-}$ bone marrow. It is predicted that c-Kit expression would be reduced on LYL-$1^{lacZ+ve}$c-Kit^{+ve} cells from LYL-$1^{-/-}$ spleen and bone marrow compared to LYL-$1^{+/-}$ controls.

Measurement of the number of c-Kit^{+ve} progenitors in the peripheral blood would indicate whether the number of circulating progenitors in the LYL-$1^{-/-}$ mice was higher than normal. It is predicted that the peripheral blood of LYL-$1^{-/-}$ mice contains more c-Kit^{+ve} haematopoietic progenitors than the blood of $LYL^{+/-}$ mice. Mobilisation assays, in conjunction with *in vitro* colony assays could also be employed to examine whether LYL-$1^{lacZ+ve}$c-Kit^{+ve} progenitors were able to mobilize to bone marrow and spleen. It is hypothesized that, due to deregulated expression of c-Kit in the absence of a functional LYL-1 protein, these progenitors would not locate to haematopoietic organs normally.

The proposed action of LYL-1 on c-Kit expression may be direct, or indirect. A direct action would be LYL-1 heterodimer DNA-binding to E-boxes within the c-Kit promoter. The hypothesis that LYL-1 acts directly on c-Kit promoter activity could be examined using gel shift, co-immunoprecipitation, and reporter assays. It is predicted that bHLH-dependent LYL-1 DNA-binding to the E-box binding sites within the c-Kit promoter would enhance expression of the c-Kit gene.

The discovery that the bHLH of SCL was dispensable for primitive erythropoiesis in ES cells, and not absolutely necessary for emergence of definitive haematopoietic cells (Porcher, et al., 1999) raises the possibility that the function of LYL-1 in haematopoietic progenitor cells was not entirely dependent on an intact bHLH domain. Although the proposed mechanism of LYL-$1^{-/-}$ function in haematopoietic progenitors must be mediated by DNA-binding of LYL-1 heterodimers such as LYL-1-E47, or indeed LYL-1-HEB (Miyamoto, et al., 1996), the role of other regions in regulation of c-Kit expression cannot be ruled out. Transfection assays using LYL-1 variants comprising different regions of the protein would identify whether, for example, the N-terminal proline rich region of LYL-1 was associated with c-Kit promoter activity *in vivo*.

The role of c-Kit in haematopoietic progenitor cell migration is closely associated with the function of β1- integrins. It is conceivable that the absence of a functional LYL-1 protein affected expression of these, and may be other genes encoding adhesion molecules such as P, and E-selectin, and CD44. Semi-quantitative RT-PCR would show whether expression of, for example VLA-4, or VLA-5, was altered in LYL-$1^{lacZ+ve}$ haematopoietic progenitor cells.

Examination of the growth of LYL-$1^{lacZ+ve}$ c-Kit^{+ve} erythroid progenitors isolated from LYL-$1^{-/-}$ spleen would reveal LYL-1 involvement in proliferation and differentiation of these cells *in vitro*. Experiments using *in vitro* colony assays could be designed to thoroughly investigate the effect of cytokines, such as Epo and SCF, on development of LYL-$1^{lacZ+ve}$ c-Kit^{+ve} erythroid progenitors from LYL-$1^{-/-}$ spleen, as well as bone marrow. In this context, comparison of bone marrow and spleen would elucidate differences in the role of haematopoietic organs on erythroid cell development in LYL-$1^{-/-}$ mice. A study of this type could also reveal if the development of erythroblast in the LYL-$1^{-/-}$ spleen was blocked.

Section 4.14 References.

Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R., Weissman, I.L. (1997). Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell, volume 89, p1033-1041.

Aplan, P.D., Jones, C.A., Chervinsky, D.S., Zhao, X.F., Ellsworth, M., Wu, C.Z., McGuire, E.A., and Gross, K.W. (1997). An scl gene product lacking the transactivation domain induces bony abnormalities and co-operates with LMO-1 to generate T-cell malignancies in transgenic mice. EBMO Journal, volume16, p2408-2419.

Aplan, P.D., Nakahara, K., Orkin, S. and Kirsch I.R. (1992). The SCL gene product: a positive regulator of erythroid differentiation. EMBO Journal. volume 11; p4073-4082.

Amati, B. and Land, H. (1994). Myc-Max-Mad: a transcription factor network controlling proliferation, differentiation and death. Current Opinions in Genetics and Development, volume 4, p102-108.

Arroyo, A. G.,Yang, J. T., Rayburn, H. and Hynes, R.O.(1999). Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo." Immunity, volume 11(5), p555-566.

Atchley, W., and Fitch W. (1997). A natural classification of the basic helix-loop-helix class of transcription factors. Proceedings of the National Academy of Sciences. USA. volume 94, p5172-5176.

Ashman, L.K., Cambareri, A.C., To, L.B., Levinsky, R.J. and Juttner, C.A.(1991). Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in normal human bone marrow. Blood, volume 78, p30-37.

Avraham, H., Vannier, E., Cowley, S., Jiang, S., Chi, S., Dinarello, C.A., Zsebo, K.M. and Groopman, J.E. (1992). Effects of the stem cell factor, c-kit ligand, on human megakaryocytic cells. Blood, volume 79 (number 2), p365-371.

Baer, R. (1993). TAL1, TAL2 and LYL1: a family of basic helix-loop-helix proteins implicated in T cell cute leukemia. seminars in Cancer Biology. volume 4; p341-p347.

Bain, G., Engel I., Maandag E. R., te Riele H., Voland J., Sharp L., Chun J., Huey B., Pinkel D., and Murre C.. (1997). E2A deficiency leads to abnormalities in T-cell development and to the rapid development of T-cell lymphomas. Molecular and Cellular Biology. volume 17, p4782-4791.

Bain, G., Gruenwald, S., and Murre, C. (1993). E2A and E2-2 are subunits of B-cell-specific E2-box DNA-binding proteins. Molecular and Cellular Biology, volume 13, p3522-3529.

Bain, G., Maandag E., Izon D., Amsen D., Kruisbeek A., Weintraub B., Krop I., Schlissel M., Feeney A., van Roon M., van der Valk M., te Riele H., Berns A., and Murre. C. (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell, volume 79, p885-892.

Barndt, R., Dai, M.F., and Zhuang, A. (1999). A novel role for HEB downstream or parallel to the pre-TCR signaling pathway during αβ thymopoiesis. Journal of Immunology, volume 163, p3331-3343.

Begley, C.G. and Green, A.R. (1999). The SCL gene: from case report to critical haematopoietic regulator. Blood. volume 93, p2760-2770.

Begley, C.G., Aplan P.D., Denning, S.M., Haynes, B.F., Waldmann, T.A. and Kirsch I.R. (1989). The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proceedings of the National Academy of Science. USA. volume 86; p10128-10132

Bernard, J. (1991). The erythroblastic island: past and future. Blood Cells, volume 17, p5-14.

Bernard, M., Delabesse, E., Novault, S., Hermine, O., and Macintyre, E.A. (1998). Antiapoptotic effect of ectopic TAL1/SCL expression in a human leukemic T-cell line. Cancer Research, volume 58, p2680-2687.

Blackwell, T.K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2a protein complexes revealed by binding site selection. Science, volume 250, p1104-1110.

Blackwell, T.K., Huang, J., Ma, A., Kretzner, L., Alt, W., Eisenmann, R.N, and Weintraub, H. (1993). Binding of Myc protein to canonical and noncanonical DNA sequences. Molecular and Cellular Biology, volume 13, p5216-5224.

Bockamp, E.O., McLaughlin, F., Murrell, A., and Green, A.R. (1994). Transcription factors and the regulation of hematopoiesis: Lessons for GATA and SCL proteins. BioEssays, volume 16 (number 7) July, p481-488.

Brecher G, Bookstein N, Redfearn W, Necas E, Pallavicini MG, Cronkite EP: Self-renewal of the long-term repopulation stem cell. (1993). Proceedings of the National Academy of Sciences, USA, volume 90, p6028-

Broudy, V. C. (1997). Stem cell factor and haematopoiesis. Blood, volume 90, p1345-1364.

Broudy, V.C., Kovach, N.L., Bennett, L.G., Lin, N., Jacobsen F.W. and Kidd, P.G. (1994). Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of c-kit receptor. Blood, volume 83, p2145-2152.

Broudy, V.C., Lin, N.L., Priestly, G.V., Nocka, K. and Wolf, N.S. (1996). Interaction of stem cell factor and its receptor c-kit mediates lodgment and acute expansion of hematopoietic cells in the murine spleen. Blood, volume 88 (number 1), p75-81.

Bucher, K., Sofroniew, M.V., Pannell, R., Impey, H., Smith, A.J.H., Torres, E.M., Dunnett, S.B., Jin, Y., Baer, R., and Rabbitts, T.H. (2000). The T cell oncogene Tal2 is necessary for normal development of the mouse brain. Developmental Biology, volume 227, p533-544

Carleton's Histological Technique, 4th edition, 1967, Oxford University Press

Cellular Pathology Technique. 4th. ed. C.F.A. Culling, R.T. Allison, W.T. Blair Butterworths, London, England.

Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the W locus. Nature, volume 335, p88-89.

Chen, Q., Cheng, J.-T., Tsai L.-H., Schneider, N., Buchanan, G., Carrol, A., Crist, W., Ozanne, B., Siciliano M.J. and Baer, R. (1990). The tal-1 gene undergoes chromosomal translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J. volume 9; p415-424.

Chida, D., Miura, O., Yoshimura, A., Miyajima, A. (1999). Role of cytokine signaling molecules in erythroid differentiation of mouse fetal liver hematopoietic cells: Functional analysis of signaling molecules by retrovirus-mediated expression. Blood, volume 93, p1567-1578.

Ciau-Utiz, A., Walmsley, M. and Patient, R. (2000). Distinct origins of adult and embryonic blood in Xenopus. Cell, volume 102, p787-796.

Cleary, L., Mellentin, J.D., Spies, J. and Smith, S.D. (1988). Chromosomal translocation involving the beta T-cell receptor gene in acute leukemia. Journal of Experimental Medicine, volume 167, p682-687.

Cleary, M.L. (1991). Oncogenic conversion of transcription factors by chromosomal trnaslocation. Cell, volume 66, p619-622.

Condorelli, G.L., Facchiano, F., Valtieri, M., Proietti, E., Vitelli, L., Lulli, V., Heubner, K., Peschle, C., and Croce, C.M. (1996). T-cell-directed TAL-1 expression induces T-cell malignancies in transgenic mice. Cance Research, volume 56, p5113-5119.

Crookham,J, Dapson,R, Hazardous Chemicals in the Histopathology Laboratory, 2nd ED, 1991, Anatech.

Cumano, A., Dieterlen-Lievre, F. and Godin, I. (2000). The splanchnopleura/AGM is the prime site for the generation of hemopoietic precursors in the mouse embryo. Vaccine, volume 18, p1621-1623.

Curry, J.L. and Trentin, J.J. (1967). Hemopoietic spleen colony studies I. Growth and Differentiation. Developmental Biology, volume 5 (May 15), p395-413.

Cynshi, O., Satoh, K., Shimonaka, Y., Hattori, K., Nomura, H., Imai, N., Hirashima, K (1991). Reduced response to granulocyte colony-stimulating factor in W/W^V and SL/SL^d mice. Leukaemia, volume 5, p75-77.

Dai, C.-H., Price, J.O., Brunner, T. and Krantz, S.B. (1998). Fas ligand is present in human erythroid colony-forming cells and interacts with Fas induced by interferon gamma to produce erythroid cell apoptosis. Blood, volume 91, p1235-1242.

Dao, M.A., Hashino, K. and Kato, I. And Nolta, J.A.(1998). Adhesion to fibronectin maintains regenerative capacity during *ex vivo* culture and transduction of hematopoietic stem and progenitor cells. Blood, volume 92, p4612-4621.

Damen, J. E., Wakao, H., Miyajima, A., Krosl, J., Humphries, R. K., Cutler, R. L., and Krystal, G. (1995) Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. EMBO J, volume 14, p5557-5568.

Davis L.J, and Halazoetis T.D. (1993). Both the helix-loop-helix and the leucine zipper motifs of the c-Myc contributes to its dimerisation specificity with Max. Oncogene, volume 8, p125-132.

Davis, R.L., and Weintraub, H., and Lasser, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell, volume 51, p987-1000.

Debili, N., Coulombel, L., Croisille, L., Katz, A.,Guichard, J., Breton-Gorius, J. and Vainchenker, W. (1996). Characterisation of a bipotent erythro-megakaryocytic progenitor in human bone marrow. Blood, volume 88, p1284-1296.

De Bruijn, M.F.T.R., Speck, N.A., Peeters, M.C.E. and Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. Journal EMBO, volume 19, p2465-2474.

De Haan, G., Dontje, B., Nijhof, W., and Loeffler, M. (1995) Stem Cells 13, 65-76

DeMaria, R., Testa, U., Luchetti, L., Zeuner, A., Stassi, G., Pelosi, E., Riccioni, R., Felli, N., Samoggia, P. and Peschle, C. (1999). Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood, volume 93, p796-803.

DeMaria, R., Zeuner, A., Eramo, A., Domenichelll, C., Boncl, D., Grignani, F., Srinivasula, S.M., Alnemri, E.S., Testa, U. and Peschle, C. (1999). Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. Nature, volume 401, p489-493.

De Silva, D. M., Askwith, C. C., Eide, D., and Kaplan, J. (1995). The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. Journal of Biological Chemistry, volume 270, P1098-1101.

Dieterlen-Lievre, F. (1975). On the origin of hematopoietic stem cells in the avian embryo: an experimental approach. Journal of Embryology and Experimental Morphology, volume 33, p607-619.

Donnelly, D.S., Zelterman, D., Sharkis, S., and Krause, D.S. (1999). Functional activity of murine CD34+ and CD34- haematopoietic stem cell populations. Experimental Hematology, volume 27, p788-796.

Elefanty, A.G., Begley, C.G., Metcalf. D., Barnett, L., Kontgen, F. and Robb, L. (1998). Characterisation of hematopoietic progenitor cells that express the transcription factor SCL, using a lacZ "knock-in" strategy. Proceeding of the National Academy of Sciences, volume 95, p11897-p11902.

Engel, I., and Murre, C. (1999). Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. Proceedings of the National Academy of Science, USA, volume 96: p996-1001.

Evans, T., Reitmann, M. and Felsenfeld, G. (1988). An erythroid specific DNA binding factor recognizes a regulatory sequence common to all chicken globin genes. Proceedings of the National Academy of Sciences, volume 85, p5976-5980.

Ferrier, R., Nougarede, R., Doucet, S., Kahn-Perles, B., Imbert, J. and Mathieu-Mahul, D. (1999). Physical interaction of the bHLH LYL1 protein and NF-κB1 p105. Oncogene, volume 18, p995-1005.

Fisch, P., Boehm, T., Lavenir, I., Larson, J., Arno, A., Forster, A., and Rabbitts, T.H. (1992). T-cell acute lymphoblastic lymphoma induced in transgenic mice by the RBTN1 and RBTN2 LIM-domain genes. Oncogene, volume 7, p2389-2397.

Fleischman, R.A. (1993). From white spots to stem cells: The role of the Kit receptor in mammalian development. Trends in Genetics, volume 9, p285-290

Fleming, M.D., Trenor 3rd, C.C., Su, M.A., Foernzler, D, Beier, D.R., Dietrich, W.F.and Andrews, N.C. (1997). Microcytic anemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nature Genetics, volume 16, p383-386.

Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J. and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes and Development, volume 14, p67-80.

Friedrich, C., Zausch, E., Sugrue, S.P. and Gutierrez-Ramos, J.C. (1996). Haematopoietic supportive functions of mouse bone marrow and fetal liver microenvironment: dissection of granulocyte, B-lymphocyte, and haematopoietic progenitor support at the stroma cell clone level. Blood, volume 87, p4595-4606.

Fujiwara, Y., Browne, C.P., Cunniff, K., Goff, S.C. and Orkin, S.H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. Proceedings of the National Academy of Sciences, volume 93, p12355-12358.

Funasaka, Y., Boulton, T., Cobb, M., Yarden, Y., Fan, B., Lyman, S.D., Williams, D.E., Anderson, D.M., Zakut, R., Mishima, Y., Halaban, R. (1992). c-Kit-kinase induces a cascade of protein tyrosine phosphorylation in normal human melanocytes in response to

mast cell growth factor and stimulates mitogen-activated protein kinase but is down-regulated in melanomas. Molecular and Cellular Biology, volume 3, p197-209.

Gabbianelli, M., E. Pelosi, E. Montesoro, M. Valtieri, L. Luchetti, P. Samoggia, L. Vitelli, T. Barberi, U. Testa, S. Lyman, et al. (1995). Multi-level effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood, volume 86, p1661-1670

Garcia-Porrero, J.A., Manaia, A., Jimeno, J., Lasky, L.L., Dieterlen-Lievre, F., and Godin, I.E. (1998). Antigenic profiles of endothelial and hematopoietic lineages in murine intraembryonic hemogenic sites. Developmental and Comparative Immunology, volume 22, p303-319.

Gering, M., Rodaway, A.R.F., Gottgens, B., Patient, R.K. and Green A.R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. EMBO Journal. volume 17(14); p4029-4045.

Gilmore, T.D., Koedood, M., Piffat, K.A., and White D.W. (1996). Rel/NF-kappaB/IkappaB proteins and cancer. Oncogene, volume 13, p1367-1378.

Gobert, S., Porteu, F., Pallu, S., Muller, O., Sabbah, M., Dusanter-Fourt, I., Courtois, G., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1995) Tyrosine phosphorylation of the erythropoietin receptor: role for differentiation and mitogenic signal transduction. Blood, volume 86, p598-606

Godin, I., Garcia-Porrero, J., Coutinho, A. Dieterlen-Lievre, F., Marcos, M.A.R. (1993). Para-aortic splanchnopleura from early mouse embryo contains B1a lymphocytes. Nature, volume 364, p67-70.

Godin, I., Garcia-Porrero, J.A., Dieterlin-Lievre, F., and Cumano, A. (1999). Stem cell emergence and hematopoietic activity are incompatible in mouse intraembryonic sites. Journal of Experimental Medicine, volume 190, p43-52.

Green, A. R., Visvader, J., Lints, T., Harvey, R., and Begley, C. G. (1992). SCL is co-expressed with GATA-1 in haemopoietic cells but is also expressed in developing brain. Oncogene, volume 7, p653–660.

Green, A.R., DeLuca, E. and Begley, C.G. (1991). Antisense SCL suppresses self-renewal and enhances spontaneous erythroid differentiation of the human leukemic cell line K562. EMBO Journal. volume 10 (13); p4153-4158.

Gregory, R.C., Jiang, N., Todokoro, K., Crouse, J., Pacifici, R.E. and Wojchowki, D.M. (1998). Erythropoietin receptor and STAT5-specific pathways promote SKT6 cell hemoglobinization. Blood, volume 92:1104-1108.

Grutz, G., Bucher, K., Lavenir, I., Larson, R., Larson, T. and Rabbitts, T.H. (1998). The oncogenic LIM- protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells. EMBO Journal, volume 17, p4594-4605.

Guillemot, F., Lo, L-C., Johnson, J., Auerbach, A., Anderson, D., and Joyner, A. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell, volume 73, p463-476.

Hai-Ling, H., Wadman, I., and Baer, R. (1994). Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proceedings of the National Academy of Sciences, volume 91, p3181-3185.

Hann, S.R., King, M.W., Bentley, D.L., Anderson, C.W., and Eisenman R.N. (1988). A non-AUG translation initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell, volume 52, p185-195.

Hansen, J. A., Lindberg, K., Hilton, D. J., Nielsen, J. H., and Billestrup, N. (1999). Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. Molecular Endocrinology, volume 13, p1832-1843.

Harrison, D.E., Stone, M, and Astle, C.M. (1990). Effects of transplantation on the primitive immunohematopoietic stem cell. Journal of Experimental Medicine, volume 172, p431-437.

Hendrikx, P.J., Martens, C.M., Hagenbeek, A., Keij, J.F. and Visser, J.W. (1996). Homing of fluorescently labeled murine hematopoietic stem cells. Experimental Hematology, volume 24 (number 2), p129-140.

Herblot, S., Steff, A-M., Hugo, P., Aplan, P.D., and Hoang, T. (2000). SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-Tα chain expression. Nature Immunology, volume 1(number 2), p138-144.

Houssaint , E. and Hallet, M-M. (1988). Inability of adult circulating haemopoietic stem cells to sustain haemopoiesis in mouse fetal liver microenvironment. Immunology, volume 64, p463-467.

Hsu, H.- L., Cheng, J.- T., Chen, Q. and Baer, R. (1991). Enhancer-binding of the tal-1 oncoprotein in association with the E47/ E12 helix-loop-helix proteins. Molecular and Cellular Biology, volume 11, p3037-3042.

Hsu, H.- L., Wadman, I. And Baer, R. (1994b). Formation of in vivo complexes between TAL1 and E2A polypeptides of leukemic T cells. Proceedings of the National Academy of Sciences, volume 91, p5947-5951.

Hsu, H.-L., Huang, L., Tsan, J.T., Funk, W., Wright, W.E., Hu, J.- S., Kingston, R.E. and Baer, R. (1994a). Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. Molecular and Cellular Biology, volume 14, p1256-1265.

Hu, J.S., Olson E.N., and Kingston R.E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA binding ability of myogenic regulatory factors. Molecular and Cellular Biology, volume 12; p1031-p1042.

Huang, S. and Brandt, S.J. (2000a). mSin3A regulates murine erythroleukaemia cell differentiation through association with the TAL1 (or SCL) transcription factor. Molecular and Cellular Biology, volume 20, p2248-2259.

Huang, S., Qiu, Y., Shi, Y., Xu, Z., and Brandt, S.J. (2000b). P/CAF-mediated acetylation regulates the function of the basic helix-loop-helix transcription factor TAL1/SCL. EMBO Journal, volume 19 (number 24), p6792-6803.

Huang, S., Qiu, Y., Stein, R.W., and Brandt, S.J. (1999). p300 functions as a transcriptional co-activator for the TAL-1/ SCL oncoprotein. Oncogene, volume 18, p4958-4967.

Huizinga, J.D., Thuneberg, L., Kluppel, M., Malysz, J., Mikkelsen, H.B., Bernstein, A. (1995). W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature, volume 373, p347-349.

Humphries, R.K., Eaves, A.C., Eaves, C.J. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. Proceedings of the National Academy of Sciences USA, volume 78, p3629-3633.

Hurley, R., McCarthy, J. and Verfaillie, C. (1997). Monoclonal antibody crosslinking of the alpha 4 or beta 1 integrin inhibits committed clonogenic hematopoietic progenitor proliferation. Experimental Hematology, volume 25, p21-328.

Hwang, L.-Y., Siegelman, M., Davis, L., Oppenheimer-Marks, N., S., and Baer, R. (1993). Expression of the TAL-1 proto-oncogene in cultured endothelial cells and blood vessels of the spleen. Oncogene, volume 8, p3043–3046.

Ihle, J.N., Thierfelder, W., Teglund, S., Stravapodis, D., Wang, D., Feng, J.and Parganas, E: (1998). Signaling by the cytokine receptor superfamily. Annual Academy of Science, New York, volume 865 (number1), p1-9.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y.-H., Weissman, I.L. and Katsura, Y. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of the hematopoietic stem cell. Cell, volume 62, p863-874.

Ikuta, K. and Weissman, I.L. (1992). Evdence that hematopoietic stem cells express c-kit but do not depend on steel factor for their generation. Proceedings of the National Academy of Sciences, USA, volume 89, p1502-1506.

Imada, K., Tsudo, M., Kodaka, T.-I., Itoh, K., Arima, N., Hattori, T., Okuma, M. and Uchiyama, T. (1992). Interleukin-2 (IL-2) induces erythroid differentiation and tyrosine phosphorylation in ELM-I-1 cells transfected with a human IL-2 receptor beta chain cDNA. Biochemica. Biophysica Research Communication, volume 188, p352-357.

Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lievre, F (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development, volume 125, p4575-4583.

Jiang, Y., Prosper, F. and Verfaillie, C. (2000). Opposing effects of engagement of integrins and stimulation of cytokine receptors on cell cycle progression of normal human hematopoietic progenitors. Blood, volume 95, p846-854.

Jong, M.O., Rozemuller, H., Kieboom, D., Visser, J.W., Wognum, A.W. and Wagemaker, G. (1996). Purification of repopulating hemopoietic stem cells based on binding of biotinylated Kit ligand. Leukemia, volume 10 (number 11), p1813-1822.

Just, U., Stocking, C., Spooncer, E., Dexter, T.M.and Ostertag W (1991). Expression of the GM-CSF gene after retroviral transfer in hematopoietic stem cell lines induces synchronous granulocyte-macrophage differentiation. Cell, volume 64, p1163-1173.

Kapur, R. and Zhang, L. (2001). A Novel Mechanism of Cooperation between c-Kit and Erythropoietin Receptor. Journal of Biological Chemistry, volume 276 (2), p1099-1106.

Katayama, N., Shih, J. P., Nishikawa, S., Kina, T., Clark, S.C.and Ogawa, M. (1993). Stage-specific expression of c-kit protein by murine hematopoietic progenitors. Blood, volume 82, p2353-2360.

Kato, M., Kato, Y. and Sugiyama, Y. (1999). Mechanism of the upregulation of erythropoietin-induced uptake clearance by the spleen. American Journal of Physiology, Endocrinology and Metabolism, volume 276 (number 5), E887-E895.

Kato, M., Miura, K., Kamiyama, H., Okazaki, A., Kumaki, K., Kato, Y. and Sugiyama, Y. (1998). Pharmacokinetics of erythropoietin in genetically anemic mice. Drug Metabolism and Disposal, volume 26, p126-131.

Kaufmann, M. (1992). The Atlas of Mouse Development, Academic Press, London, United Kingdom.

Kee, B., and Murre, C. (1998). Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. Journal of Experimental Medicine, volume 188, p699-713.

Keller G, Wall C, Fong AZ, Hawley TS & Hawley RG (1998). Overexpression of HOXI 1 leads to the immortalization of embryonic precursors with both primitive and definitive haematopoietic potential. Blood, volume 92, p877-887.

Kelley, L. L., Koury, M. J., Bondurant, M. C., Koury, S. T., Sawyer, S. T., and Wickrema, A. (1993). Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. Blood, volume 82 (number 8), p2340-2352.

Kelliher, M.A., Seldin, D.C., and Leder, P. (1996). Tal-1 induces T cell acute lymphoblastic leukemia accelerated by caesin kinase II α. EMBO Journal, volume 15, p5160-5166.

Kennedy, M.A., Gonzalez-Sarimento, R., Kees, U.R., Lampert, F., Bear, M., Boehm, T., and Rabbitts, T.H. (1991). HOX-11, a homeobox contaiing T-cell oncogene on human chromosome 10q24. Proceedings of the National Academy of Sciences, USA, volume 88, p8900-8904.

Kiernan, M.W., Perkins, A.C., Orkin, S.H.and Zon, L.I. (1996). Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proceedings of the National Academy of Sciences, USA, volume 93, p9126-9131.

Kinashi, T. and Springer, T.A. (1994). Steel factor and c-kit regulate cell-matrix adhesion. Blood, volume 83, p1033-1038.

Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995). Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell, volume 80, p729-738.

Kodama, H., Nose, M., Niida, S., Nishikawa, S. and Nishikawa, S. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cell to stromal cells. Experimental Hematology, volume 22, p979-984.

Koury, M.J. and Bondurant, M.C. (1990). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science, volume 248, p378-381.

Kozak , M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation at eukaryotic ribosomes. Cell, volume 44, p283-292.

Krantz, S.B. (1991). Erythropoietin. Blood, volume77, p419-443.

Krantz, S.B. and Goldwasser, E. (1984). Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus. Proceedings of the National Academy of Sciences, USA, volume 81, p7574-7578.

Krause, D.S., Thiese, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S.J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell, volume 105, p369-377.

Krosl, G., He, G., Lefrancois, M., Charron, F., Romeo, P.H., Jolicoeur, P., Kiirsch, I.R., Nemer, M. and Hoang, T. (1998). Transcription factor SCL is required for c-kit expression and c-Kit function in hemopoietic cells. Journal of Experimental Medicine, volume 188, p439-450.

Kulessa, H., Frampton, J., and Graf, T. (1995). GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts and erythroblasts. Genes and Development, volume 9, p1250-1262.

Kuo, S.S., Mellentin J.D., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and Cleary, M.L. (1991). Strucutre, chromosomal mapping, and expression of the mouse Lyl-1 gene. Oncogene. volume 6; p961-968.

Kurnit, D.M., Matthysse, S., Papayannopoulou, T., Stamatoyannopoulou, G. (1985). Stochastic branching model for hemopoietic progenitor cell differentiation. Journal of Cell Physiology, volume 123, p55-63.

Lacronique, V., Varlet, P., Mayeux, P., Porteu, A., Gisselbrecht, S., Kahn, A., Lacombe, C (1997). Bcl-2 targeted overexpression into the erythroid lineage of transgenic mice delays but does not prevent the apoptosis of erythropoietin-deprived erythroid progenitors. Blood, volume 90, p3050-3056.

Lansdorp, P.M. (1995). Developmental changes in the function of haematopoietic stem cells. Experimental Hematology, volume 23, p187-191.

Larson R.C., Lavenir, I., Larson, T.A., Baer, R., Warren, A.J., Wadman, I., Nottage, K., and Rabbitts, T.H. (1996). Protein dimerization between lmo2 (Rbtn2) and tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. Journal EMBO, volume 15, p1021-1027.

Lemischka, I.R. (1997). Microenvironmental regulation of hematopoietic stem cells. Stem Cells, volume 15 (Supplement 1), p63-68.

Leroy-Viard, K., Vinit, M.-A., Lecointe, N., Jouault, H., Hidner, U., Romeo, P.-H., and Mathieu-Mahul, D. (1995). Loss of TAL-1 protein activity induces premature apoptosis of Jurkat leukemic T cells upon medium depletion. Journal EMBO, volume 14, p2341–2349.

Lian, Z., Toki, J., Yu, C., Hayashi, H., Yasumizu, R., Sugiura, K., Jin, T., Inaba, M., Hisha, H., Li, Y., Yu, W., Fan, H. and Ikehara, S. (1997). Intrathymically injected hemopoietic stem cells can differentiate into all lineage cells in the thymus. Stem Cells, volume 15 (number 6), p430-436.

Lin, C.-S., Lim, S., Agati, V. and Constantini, F. (1995). Differential effects of an erythropoietin gene disruption on primitive and definitive erythropoiesis. Genes and Development, volume 10, p154-164.

Lin, C., Lin, J., Chen, S., Jiang, M. and Chiu, C. (1992) Comparison of hemoglobin and red blood cell distribution width in the differential diagnosis of microcytic anemia. Archives of Pathology and Laboratory Medicine 116: 1030-1032.

Lin, J-X and Leonard, W.J. (1997). The immediate-early gene product Egr-1 regulates the human Interleukin-2 receptor -chain promoter through a noncanonical Egr and Sp1 binding sites. Molecular and Cellular Biology, volume 17(no 7), p3714-3722.

Long, M.W. (1999). Blood cell cytoadhesion molecules. Experimental Hematology, volume 20, p288-301.

Lord BI & Dexter TM (1995). Which are the haematopoietic stem cells? (or: Don't debunk the history!). Experimental Hematology, volume 23, p1237-1241.

Lowry, P.A. (1995). Hematopoietic stem cell cytokine response. Journal of Cellular Biochemistry, volume 58, p410-415.

Luna L, Manual of Histologic Staining Methods of the AFIP, 3rd Ed, 1968, pp183, McGraw-Hill, NY.

Lyman, S. D., and S. E. Jacobsen. 1998. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood, volume 91, p1101-1134.

Ma, Q., Fode, C., Guillemot, F., and Anderson, D.J. (1999). Neurogenin1 and neurogenin 2 control two distinct waves of neurogenesis in dorsal root ganglia. Genes and Development, volume 13, p1717-1728.

Ma, Q., Kintner, C., and Anderson, D.J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene, Cell, volume 87, p43-52.

Mahajan, M.A., Park, S.T., and Sun, X.H. (1996). Association of a novel GTP binding protein, DRG, with TAL oncogenic proteins. Oncogene, volume12, p2343-2350.

Manova, K., Nocka, K., Besmer, P. and Bachvarova, R.F. (1990). Gonadal expression of c-kit encoded at the W locus of the mouse. Development, volume 110, p1057-1069.

Martinez, A., Miller, M.-J., Unsworth, E.J. and Cuttitta, F. (1995). Protocol for In-Situ RT-PCR. Taken from proceedings of the Third Analytical Morphology Meeting, Atlanta City, N.J.- June 5^{th}-9^{th}, 1995.

Maruyama K, Miyata K and Yoshimura A (1994) Proliferation and erythroid differentiation through the cytoplasmic domain of the erythropoietin receptor. Journal of Biological Chemistry, volume 269, p5976-5980.

Massari, M., Grant P., Pray-Grant M., Berger S., Workman J., and Murre, C. (1999). A conserved motif present in a class of helix-loop-helix proteins activates transcription by direct recruitment of the SAGA complex. Molecular and Cellular Biology, volume 4, p63-73.

Massari, M., Jennings, P., and Murre, C. (1996) The AD1 transactivation domain of E2A contains a highly conserved helix which is required for its activity in both Saccharomyces cerevisiae and mammalian cells. Molecular and Cellular Biology, volume 16, p121-129.

Massari, M.E. and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic cells. Molecular and Cellular Biology, volume 20, p429-440.

Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Ohtsubo, M., Misawa, H., Miyajima, A., and Yoshimura, A. (1997). CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood, volume 89, p3148-3154.

McDevitt, M.A., Shivdasani, R., Fujiwara, Y., Yang, H., and Orkin, S.H. (1997). A "knockdown" mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. Proceedings of the National Academy of Sciences, volume 94, p6781-6785.

McNiece, I.K., Langley, K.E., Zsebo, K.M. (1991). Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and EPO to stimulate human progenitor cells of the myeloid and erythroid linages. Experimental Hematology, volume 19, p226-231.

Medvinsky, A. and Dzierzak, E. (1996). Definitive haematopoiesis is autonomously initiated in the AGM region. Cell, volume 86, p897-906.

Medvinsky, A. and Dzierzak, E. (2000). Development of the hematopoietic stem cell: can we describe it? Blood, volume 94, p3613-3614.

Medvinsky, A., Samoylina, N., Muller, A., Dzierzak, E. (1993). An early pre-liver intra-embryonic source of CFU-S in the developing mouse. Nature, volume 64, p64-67.

Mellentin, J.D., Smith, S.D. and Cleary, M.L. (1989). lyl-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. Cell, volume 58, p77-83.

Mermod, N., O'Neill, E.A., Kelly, T.J., and Tjian, R. (1989). The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell, volume 58, p77-83.

Metcalf, D. (1991). Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: Influence of colony-stimulating factors. Proceedings of the National Academy Science, USA, volume 88, p11310-11314.

Miller, C. P., Liu, Z. Y., Noguchi, C. T., and Wojchowski, D. M. (1999) A Minimal Cytoplasmic Subdomain of the Erythropoietin Receptor Mediates Erythroid and Megakaryocytic Cell Development. Blood, volume 94, p3381-3387.

Miyamoto, A., Cui, X., Naumovski, L. and Cleary, M.L. (1996). Helix-loop-helix protein LYL-1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells. Molecular and Cellular Biology. volume 16 (5); p2394-2401.

Mohle, R., Haas, R. and Hunstein, W. (1993). Expression of adhesion molecules and c-Kit on CD34+ hematopoietic progenitor cells: Comparison of cytokine mobilized blood stem cells with normal bone marrow and peripheral blood. Journal of Hematotherapy, volume 2, p483-489.

Moore, M.S. and Metcalf, D (1970). Ontogeny of the haematopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. British Journal of Haematology, volume 18, p279-296.

Morrison, S.J., Uchida, N. and Weissman, I.L. (1995). The biology of haematopoietic stem cells. Annual Review of Cell Development Biology, volume 11, p35-71.

Mouthon, M-A., Bernard, O., Mitjavila, M-T., Romeo, P-H., Vainchenker, W. and Mathieu-Mahul, D. (1993). Expression of tal-1 and GATA-binding proteins during human hematopoiesis. Blood. volume 81; p647-655.

Mucenski, M. L.,McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T.A., Pietryga, D. W., Scott, W. J., Jr., Potter, S. (1991). A functional c-myb gene is required for normal fetal hepatic haematopoiesis. Cell, volume 65, p677-689.

Muller, A., Medvinsky, A., Strouboulis, J., Grosveld, F., Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity, volume 1, p291-301.

Murre, C., Bain, G., v. Dijk, M.A., Engel, I., Furnari, B.A., Massari, M.E., Matthews J.R., Quong, M.W., Rivera, R.R., and Stuiver M.H. (1994). Structure and function of the helix-loop-helix proteins. Biochimica Biophysica Acta, volume 1218, p129-135.

Murre, C., McCaw, P.S., and Baltimore D. (1989a). A new DNA binding and dimerisation motif in immunoglobulin enhancer bindng, daughterless, MyoD, and c-myc proteins. Cell, volume 56, p777-783.

Murre, C., McCaw P. S., Vaessin H., Caudy M., Jan L. Y., Jan Y. N., Cabrera C. V., Buskin J. N., Hauschka S. D., Lassar A. B., Weintraub H., and Baltimore, D. (1989b). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell, volume 58: p537-544.

Murre, C., Voronova A., and Baltimore, D. (1991). B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. Molecular and Cellular Biology, volume 11, p1156-1160.

Murrell, A. M., Bockamp, E. O., Gottgens, B., Chan, Y. S., Cross, M. A., Heyworth, C.M.and Green, A. R. (1995). Discordant regulation of SCL/TAL-1 mRNA and protein during erythroid differentiation. Oncogene, volume 11 (number 1), p131-139.

Nakahata, T., Gross, A.J., Ogawa, M. (1982). A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. Journal Cell Physiology, volume 113, p455-458.

Nathan, D. G., Piomelli, S., Gardner, F. H., and Lemire, V. B. (1961). The synthesis of heme and globin in the maturing human erythroid cell. Journal of Clinical Investigation, volume 40, p940-946.

Nijhof, W., Goris, H., Dontje, B., Dresz, J. and Loeffler. (1993). Optimal erythroid cell production during erythropoietin treatment of mice occurs by exploiting the splenic microenvironment. Experimental Hematology, volume 21, p496-501.

Nishikawa, S-I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H. and Katsura, Y. (1998). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embyos. Immunity, volume 8, p761-769.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. Genes and Development, volume 3, p816-826.

Nolte, M. A., Hoen, E. N., van Stijn, A., Kraal, G., Mebius, R. E. (2000). Isolation of the intact white pulp. Quantitative and qualitative analysis of the cellular composition of the splenic compartments. European Journal of Immunology, volume 30 (number 2), p626-634.

Nuez, B., Michalovich, D., Bygrave, A., Polmacher, R. and Groseveld, F. (1995). Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature, volume 375, p316-318.

Nuovo, G.J., Gallery, F., Hom, R., MacConnell, P. and Bloch, W. (1993). Importance of different variables for optimizing in-situ detection of PCR-amplified DNA. PCR Methods and Applications, volume 2, p305-312.

Nuovo, G.L. (1996). PCR in situ hybridization: Protocols and Applications, 3rd edition. Lippincott, Raven Press, New York, 538.

Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. Blood, volume 81, p2844-2853.

Ogawa, M., Matzusaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H. and Nishikawa, S. (1994). Expression and function of c-kit in hemopoietic progenitor cells. Journal of Experimental Medicine, volume 174, p63-71.

Okuda, T., Deursen, Jv, Hiebert, S.W., Grosveld, G. and Downing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver haematopoiesis. Cell, volume 84, p321-330.

Orkin, S.H. (1995). Transcription factors and hematopoietic development. Journal of Biological Chemistry, volume 270, p4955-4958.

Orkin, S.H. (1996). Development of the hematopoietic system. Current Opinions in Genetics and Development, volume 6, p597-602.

Orkin, S. (2000). Diversification of hematopoietic stem cells to specific lineages. Nature Reviews Genetics, volume 1, p57-p64.

Orkin, S.H. and Weiss, M.J. (1999). Cutting red cell production. Nature, volume 401, p433-436.

Orkin, S.H. and Zon, L.I. (1997). Genetics of erythropoiesis: Induced mutations in mice and zebrafish. Annual Review of Genetics, volume 31, p33-60.

Orlic, D. and Bodine, D.M. (1994). What defines a pluripotent haematopoietic stem cell (PHSC): will the real PHSC please stand up! Blood, volume 84, p3991-3994.

Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y. and Lonai, P. (1990). Developmental expression of c-kit, a protooncogene encoded by the W locus. Development, volume 109, p911-923.

Pagliuca, A., Cannada-Bartoli, P., and Lania, L. (1998). A role for Sp and helix-loop-helix transcription factors in the regulation of the human Id4 gene promoter activity. Journal of Biological Chemistry, volume273 (no 13), p7668-7674.

Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development, volume 126, p5073-5084.

Papayannopoulou, T.and Nakamoto, B.(1993). Peripheralisation of hematopoietic progenitors in primates treated with anti-VLA-4 integrin. Proceedings of the National Academy of Sciences, USA, volume 90, p9374-9378.

Papayannopoulou, T., Brice, M., Broudy, V.C. and Zsebo, K.M. (1991). Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood and fetal liver: functional properties and composite antigenic profile. Blood, volume 78, p1403-1412.

Papayannopoulou, T., Priestley, G.V. and Nakamoto, B. (1998). Anti-VLA-4/ VCAM-1-induced mobilization requires cooperative signaling through the kit/ mkit ligand pathway. Blood, volume 91, p2231-2239.

Pardanaud, L. and Dieterlen-Lievre, F. (1999). Manipulation of the angiogenic/ hemangiopoietic commitment in the avian embryo. Development, volume 126, p617-627.

Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G. and Ihle, J. N. (1998) Cell, volume 93, p385-395.

Park, S. T., Nolan G. P., and Sun X. H.. (1999). Growth inhibition and apoptosis due to restoration of E2A activity in T cell acute lymphoblastic leukemia cells. Journal of Experimental Medicine, volume 189, p501-508.

Park, G. H., H. K. Plummer III, and G. W. Krystal. (1998). Selective Sp1 binding is critical for maximal activity of the human c-kit promoter. Blood, volume 92, p4138-4149

Park, S.T. and Sun, X-H. (1998). The Tal1 oncoprotein inhibits E47-mediated transcription. The Journal of Biological Chemistry, volume 273 (number 12), p7030-7037.

Paulson, R.F., and Bernstein, A. (1996). Receptor tyrosine kinases and the regulation of hematopoiesis. Seminars in Immunology, volume 7, p267-277.

Penvy, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.F., D'Agati, V., Orkin, S.H. and Constantini, F. (1991). Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature, volume 349, p257-260.

Perkins, A.C., Sharpe, A.H. and Orkin, S.H. (1995). Lethal –thalassemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature, volume 375, p318-322.

Phillips, R.L., Ernst, R.E., Brunk, B., Ivanova, N., Mahan, M.A., Deanehan, J.K., Moore, K.A., Overton, G.C., and Lemischka, I.R. (2000). The genetic program of hematopoietic stem cells. Science, volume 288, p1635-1640.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. and Orkin, S. (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all haematopoietic lineages. Cell. volume 86: p47-57.

Prosper, F. and Verfaillie, C. (2001). Regulation of haematopoiesis thrugh adhesion receptors. Journal of Leukocyte Biology, volume 69, p307-316.

Qiu, Y., Sharma A., and Stein, R (1998). p300 mediates transcriptional stimulation by the basic helix-loop helix activators of the insulin gene. Molecular and Cellular Biology, volume.18, p2957-2964

Quelle, F. W., Wang, D., Nosaka, T., Thierfelder, W. E., Stravopodis, D., Weinstein, Y., and Ihle, J. N. (1996). Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. Molecular and Cellular Bioogy, volume 16, p1622-1631

Quesenberry, P.J., Iscove, N.N., Cooper, C., Brady, G., Newburger, P.E., Stein, G.S., Stein, J.S., Reddy, G.P.V., and Pearson-White, S. (1996). Expression of basic helix-loop-helix transcription factors in explant hematopoietic progenitors. Journal of Cellular Biochemistry, volume 61, p478-488.

Rao, S. S., Peters, S. O., Crittenden, R. B., Stewart, F. M., Ramshaw, H. S. and Quesenberry, P. J. (1997). Stem cell transplantation in the normal nonmyeloablated host: relationship between cell dose, schedule, and engraftment. Experimental Hematology, volume 25, p114-121.

Ratajczak, M. Z., D. Perrotti, P. Melotti, M. Powzaniuk, B. Calabretta, K. Onodera, D. A. Kregenow, B. Machalinski, and A. M. Gerwitz. (1998). Myb and ets proteins are candidate regulators of c-kit expression in human hematopoietic cells. Blood, volume 91, p1934-1946

Rabbitts, T.H. (1994). Chromosomal translocations in human cancer. Nature, volume 372, p143-149.

Remy, I., Wilson, I. A., and Michnick, S. W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. Science, volume 283, p990-993.

Rich, I.N. (1995). Primordial germ cells are capable of producing cells of the haematopoietic cell system in vitro. Blood, volume 86, p463-472.

Robb L. and Begley CG. (1997). The SCL/TAL1 gene: roles in normal and malignant haematopoiesis. Bioessays. July, volume19 (number 7), p607-613.

Robb, L., Lyons, I., Li, R., Hartley L., Kontgen, F., Harvey, R.P., Metcalf, D. and Begley, C.G. (1995a). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proceedings of the National Academy of Science, USA,. volume 92; p7075-7079.

Robb, L., Rasko, J.E.J., Bath, M.L., Strasser, A., and Begley, C.G. (1995b). scl, a gene frequently activated in human T cell leukemia, does not induce lymphomas in transgenic mice. Oncogene, volume 10, p205-209.

Robertson, S.M., Kennedy, M., Shannon, J.M. and Keller, G. (2000). A transition stage in the commitment of mesoderm to hemopoiesis requiring the transcription factor SCL/ tal-1. Development, volume 127 (number 11), p2447-2459.

Romeo, P. H., Prandini, M. H., Joulin, V., Mignotte, V., Prenant, M., Vainchenker, W., Marguerie, G. and Uzan, G. (1990). Megakaryocytic and erythrocytic lineages share specific transcription factors. Nature, volume 344, p477-479.

Rudnicki M.A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf5 and results in apparently normal muscle development. Cell, volume 71, p383-390.

Ruff-Jamison, S., Chen, K., and Cohen, S. (1993). Induction by EGF and interferon-gamma of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science, volume 261, p1733-1736.

Saitoh, T., Morimoto, K., Kumagai, T., Tsuboi, I., Aikawa, S. and Horie, T. (1999). Comparison of erythropoietic response to androgen in young and old senescence accelerated mice. Mechanisms in Ageing and Development, volume 109 (number 2), p125-139.

Sánchez, M.-J., Göttgens, B., Sinclair, A. M., Stanley, M., Begley, C. G., Hunter, S., and Green A. R. (1999). An SCL 3′ enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors. Development, volume 126 (number17), p3891-3904.

Sawyer, S. T., and Penta, K. (1996). Association of JAK2 and STAT5 with erythropoietin receptors. Role of receptor phosphorylation in erythropoietin signal transduction. Journal of Biological Chemistry, volume 271, p32430-32437.

Schwab, M. (1989). The myc-box oncogenes. In The Oncogene Handbook. E.P. Reddy, A.M. Skalka, and T. Curran, editors. Elsevier Science, Germany, p381-391.

Scott, M.A. and Gordon, M.Y. (1995). In search of the hemopoietic stem cell. British Journal of Haematology, volume 90, p738-743.

Sheehan D, Hrapchak B, Theory and practice of Histotechnology, 2nd Ed, 1980, pp217-218, Battelle Press, Ohio.

Shen, C. P., and Kadesch, T. (1995). B-cell-specific DNA binding by an E47 homodimer. Molecular and Cellular Biology, volume 15, p4518-4524.

Shimizu, R., Takahashi, S., Ohneda, K., Engel, J.D. and Yamamoto, M. (2001). In vivo requirements for GATA-1 functional domains during primitive and definitive erythropoiesis. EMBO Journal, volume 20 (number 1), p5250-5260.

Shirihai, O.S., Gregory, T., Yu, C., Orkin, S.H. and Weiss, m.j. (2000). ABC-me: a novel mitochondrial transporter induced by GATA-1 during erythroid differentiation. EMBO Journal, volume 19 (number 11), p2492-2502.

Shivdasani, R.A., Fujiwara, Y., McDevitt, M.A. (1997). A lineage selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO Journal, volume 1, p3965-3973.

Shivdasani, R., Mayer, E. and Orkin, S.. (1995a). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature. volume 373: p432-434.

Shivdasani, R. Mayer, E. and Orkin, S.H.(1995b). Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. Proceedings of the National Academy of Sciences, USA, volume 92, p8690-8694.

Shivdasani, R. A., Rosenblatt, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Saris, C. J., Orkin, S. H. (1995c). Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MDGF in megakaryocytic development. Cell, volume 81, p695-704.

Silver, L. and Palis, J. (1988). Initiation of murine embryonic erythropoiesis: a spatial analysis. Blood, volume 89, p1154-1164.

Simon, M.C., Penvy, L., Wiles, M.V., Keller, G., Constantini, F. and Orkin, S.H. (1992). Rescue of erythroid development in gene targeted GATA-1 mouse embryonic stem cells. Nature Genetics, volume 1, p92-98.

Simmons, P.J., Aylett, G.W., Niutta, S., To, L.B., Juttner, C.A. and Ashman, L.K. (1994). c-kit is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine-supplemented culture. Experimental Hematology, volume 22, p157-165.

Simmons, P.J., Masinovsky, B., Longenecker, B.M., Berenson, R., Torok-Storb, B. and Gallatin, W.M. (1992). Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitors. Blood, volume 80, p388-395.

Simmons, P.J., Zannettino, A., Gronthos, S.and Leavesley, D. (1994). Potential adhesion mechanisms for localization of hematopoietic progenitors to the bone marrow stroma. Leukemia and Lymphoma, volume 12, p353-363.

Slaper-Cortenbach, I., Ploemacher, R. and Lowenberg, B. (1987). Different stimulative effects of human bone marrow and fetal liver stromal cells on erythropoiesis in long-term culture. Blood, volume 69, p135-139.

Smithgall, T.E. (1998). Signal transduction pathways regulating hematopoietic differentiation. Pharmacological Reviews (Issue 1), volume 50, p1-20.

Socolovsky, M., Dusanter-Fourt, I., and Lodish, H. F. (1997). The prolactin receptor and severely truncated erythropoietin receptors support differentiation of erythroid progenitors. Journal of Biological Chemistry, volume 272, p14009-14012.

Starzl, T.E., Demetris, A.J., Tucco, M., Ricordi, C., Ildstad, S., Terasaki, P.I., Murase, N., Kendall, R.S., Kocova, M., Rudert, W.A., Zeevi, A. and Thiel, D.V. (1993). Chimerism after liver transplantation for type IV glycogen storage disease and type I Gaucher's disease. New England Journal of Medicine, volume 328, p745-749.

Suda, T, Suda J, Ogawa M. (1984). Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proceedings of the National Academy of Sciences, USA, volume 81, p2520-2524.

Sui, X., Krantz, S. B., You, M., and Zhao, Z. (1998). Synergistic activation of MAP kinase (ERK1/2) by erythropoietin and stem cell factor is essential for expanded erythropoiesis. Blood, volume 92, p1142-1149.

Takahashi, S., Shimizi, R., Suwabe, N., Kuroha, T., Yoh, K., Ohta, J., Nishimura, S., Lim, K.C., Engel, J.D. and Yamamoto, M. (2000). Blood, volume 96 (number 3), p910-916.

Telen, M.J. (2000). Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. Seminars in Hematology, volume 37, p130-142.

Testa, U., Fossati, C., Samoggia, P., Masciulli, R., Mariani, G., Hassan, H.J., Sposi, N.M., Guerriero, R., Rosato, V., Gabbianelli, M., Pelosi, E., Valtieri, M. and Peschle, C. (1996). Expression of growth factor receptors in unilineage differentiation culture of purified hematopoietic progenitors. Blood, volume 88, p3391-3406.

Testa, U., Pelosi, E., Gabbianelli, M., Fossati, C., Campisi, S., Isacchi, G., Peschle, C. (1993). Cascade transactivation of growth factor receptors in early human hematopoiesis. Blood, volume 81, p1442-1456.

Till, J.E. and McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Research, volume 14, p213-218.

Till, J.E., McCulloch, E.A. and Siminovitch, L. (1964). A stochastic model of stem cell proliferation based on the growth of spleen colony forming cells. Proc Natl Acad Sci USA, volume 51, p29

To, L.B., Haynock, D.N., Dowse, T., Simmons, P.J., Trimboli, S., Ashman, L.K. and Juttner, C.A. (1994). A comparative study of the phenotype and proliferative capacity of

peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and bone marrow CD34+ cells. Blood, volume 84, p2930-2939.

To, L.B., Haylock, D.N., Simmons, P.J. and Juttner, C.A. (1997). The biological and clinical uses of blood stem cells. Blood, volume 89, number 7, p2233-2258.

Tronik-Le, R.D., Roullot, V., Schweitzer, A., Berthier, R., Marguierie, G. (1995). Suppression of erythro-megakaryopoiesis and the induction of reversible thrombocytopenia in mice transgenic for the thymidine kinase gene targeted by the platelet glycopotein αIIb promoter. Journal of Experimental Medicine, volume 181, p2141-2151.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature, volume 371, p221-226.

Tsang, A.P., Fujiwara, Y., Horn, D.B. and Orkin, S.H. (1998). Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking GATA-1 transcriptional cofactor FOG. Genes and Development, volume 12, p1176-1188.

Tsang, A.P., Visvader, J.E., Turner, C.A. (1997). FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1, in erythroid and megakaryocytic differentiation. Cell, volume 90, p109-119.

Tsujimura, T., E. Morii, M. Nozaki, K. Hashimoto, Y. Moriyama, T. Takebayashi, T. Kondo, Y. Kanakura, and Y. Kitamura. (1996). Involvement of transcriptional factor encoded by the mi locus in the expression of c-kit receptor tyrosine kinase in cultured mast cells of mice. Blood, volume 88, p1225-1233

Turner, M.L. (1994). Regulation of hematopoietic progenitor cell migration, mobilization and homing. Stem Cells, volume 12, p227-229.

Valtieri, M., Tocci, A., Gabbianelli, M., Luchetti, L., Masella, B., Vitelli, L., Botta, R., Testa, U., Condorelli, G.L. and Peschle, C. (1998). Enforced TAL-1 expression stimulates primitive, erythroid, and megakaryocytic progenitors but blocks the granulopoietic differentiation program. Cancer Research, volume 58, p52-569.

Vandenbark, G.R., Chen, Y., Friday, E., Pavlik, K., Anthony, B., DeCastro, C. and Kaufman, R.E. (1996). Complex regulation of human c-kit transcription by promoter repressors, activators and specific myb elements. Cell Growth and Differentiation, volume 7, p1383-1392.

Vannucchi, A.M., Paoletti, F., Linari, S., Cellai, C., Caporale, R., Ferrini, P.R., Sanchez, M., Migliaccio, G., and Migliaccio, A.R. (2000). Blood, volume 95 (number 8), p2559-2568.

Verfaillie, C. (1992). Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. Blood, volume 79 (number 11), p2821-2826.

Verfaillie, C. and Catanzarro, P. (1996). Direct contact with stroma inhibits proliferation of long-term culture initiating cells. Leukemia, volume 10, p498-506.

Visvader, J., Begley, C.G. and Adams J.M. (1991). Differential expression of the LYL, SCL and E2A helix-loop-helix genes within the hematopoietic system. Oncogene. volume 6; p187-194.

Visvader, J.E., Elefanty, A.G., Strasser, A. and Adams, J.M. (1992). GATA-1 but not SCL induces megakaryocytic differentiation in an early myeloid line . EMBO Journal, volume 11, p4557-4564.

Visvader, J.E., Fujiwara, Y. and Orkin, S.H. (1998). Unsuspected role for the T-cell leukemia protein SCL/ tal-1 in vascular development. Genes and Development, volume 12 (number 4), p473-479.

Vitelli, L., Condorelli, G., Lulli, V., Hoang, T., Luchetti, L., Croce, C.M. and Peschle, c. (2000). A Pentamer Transcriptional Complex Including tal-1 and Retinoblastoma Protein Downmodulates c-kit Expression in Normal Erythroblasts. Molecular and Cellular Biology, volume 20 (July), p.5330-5342.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E. and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. Journal of Experimental Medicine, volume 181, p1519-1526.

Vuori, K., Hirai, H., Aizawa, S., Ruoslahti, E. (1996). Induction of p130as signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Molecular and Cellular Biology, volume 16, p2606-2613.

Wadman, I.A., Osada, H., Grutz, G.G., Agulnick, A.D., Westphal, H., Forster, A. and Rabbitts, T.H. (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-indng complex which includes TAL-1, E47, GATA-1 and Ldb1/ NLI proteins. EMBO Journal, volume 16 (number 11), p3145-3157.

Wang, M.W., Consoli, U., Lane, C.M. (1998). Rescue from apoptosis in early (CD34-selected) verses late (non-CD34-selected) human hematopoietic progenitor cells by very late antigen 4- and vascular cell adhesion molecule (VCAM) 1-dependent adhesion to bone marrow cells. Cell Growth and Differentiation, volume 9, p102-112.

Warren, A.J., Colledge, W.H., Carlton, M.B.L., Evans, M.J., Smith, A.J.H. and Rabbitts, T.H. (1994). The oncogenic cysteine-rich LIM domain protein Rbtn2 is essential for erythroid development. Cell, volume 78, p45-57.

Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., Lodish, H. F. (1996). Cytokine receptor signal transduction and the control of

hematopoietic cell development. Annual Reviews in Cellular and Developmental Biology, volume 12, p91-128.

Weilbaecher, K., Weissman, I., Blume, K., and Heimfeld, S. (1991). Culture of phenotypically defined haematopoietic stem cells and other progenitors at limiting dilution on Dexter monolayers. Blood, volume 78, p945-952.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell, volume 75, p1241-1244.

Weintraub, H., Dwarki, V., Verma, J., Davis R., Hollenberg, S., Sinder, L., Lasser, A., and Tapscott, S. (1991). Muscle-specific transcriptional activation by MyoD. Genes and Development. volume 5, p1377-1386.

Weiss, M.J., Keller, G. and Orkin, S.H. (1994). Novel insights into erythroid development revealed through in-vitro differentiation of GATA-1-/- embryonic stem cells. Genes and Development, volume 8, p1184-1197.

Weiss, M.J. and Orkin, S.H. (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proceedings of the National Academy of Sciences, USA, volume 92, p9623-9627.

Whyatt, D., Lindeboom, F., Karis, A., Ferreira, R., Milot, E., Hendriks, R., de Bruijn, M., Langeveld, A., Gribnau, J., Grosveld, F. and Philipsen, S. (2000). An intrinsic but cell-

nonautonomous defect in GATA-1-overexpressing mouse erythroid cells. Nature, volume 406, p 519-523.

Wojchowski, D. M., Gregory, R. C., Miller, C. P., Pandit, A. K., and Pircher, T. J. (1999). Signal transduction in the erythropoietin receptor system.Experimental Cell Research, volume 253, p143-156.

Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L. and Weissman, I.R. (2001). Physiological Migration of Hematopoietic Stem and Progenitor Cells. Science, volume 294, p1933-1936.

Wu, H., Liu, X., Jaenisch, R., and Lodish, H.F. (1995). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell, volume 83, p59-67.

Wu, H., Klingmuller, U., Acurio, A., Hsiao, J.G. and Lodish, H.F. (1997). Functional interaction of erythropoietin and stem cell factor receptors is essential for erythroid colony formation. Proceedings of the National Academy of Sciences, USA, volume 94, p1806-1820.

Wu, H., Klingmuller, U., Besmer, P. and Lodish, H.F. (1996). Interaction of erythropoietin and stem cell factor receptors. Nature, volume 377, p242-246.

Yamaguchi, T.P., Dumont, D.J., Conlon, R.A., Breitman, M.L. and Rossant, J. (1993). Flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development, volume 118, p489-498.

Yamamoto, K., A. Tojo, N. Aoki, and M. Shibuya. (1993). Characterization of the promoter region of the human c-kit proto-oncogene. Japanese Journal of Cancer Research, volume 84, p1136-1144

Yamamoto, M., Takahashi, S., Onodera, K., Muraosa, Y. and Engel, J.D. (1997). Upstream and downstream of the transcription factor GATA-1. Genes and Cells, volume 2, p107-115.

Yan, W., Young A. Z., Soares V. C., Kelley R., Benezra R. and Zhuang Y. (1997). High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. Molecular and Cellular Biology. volume 17, p7317-7327.

Yoder, M.C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D.M. and Orlic, D. (1997). Characterization of definitive lymphohematopoietic stem cells in the day nine murine yolk sac. Immunity, volume 7, p335-344.

Zhuang, Y., Cheng, P., and Weintraub, H. (1996). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. Molecular and Cellular Biology, volume 16, p2898-2905.

Zhuang, Y., Soriano P., and Weintraub H. (1994). The helix-loop-helix gene E2A is required for B cell formation. Cell, volume 79, p875-884.

Zochnodne, B., Truong, A.H., Stetler, K., Higgins, R.R., Howard, J., Dumont, D., Berger, S.A. and Ben-David, Y. (2000). Epo regulates erythroid proliferation and differentiation through distinct signaling pathways: implications for erythropoiesis and Friend virus-induced erythroleukemia. Oncogene, volume 19 (number 19), p2296-2304.

Analysis of the LYL-1 null mutant mouse- a possible role in haematopoietic progenitor cell mobilisation.

Emerson Randall King B.Sc. (Hons).

Department of Medicine, University College London, London University.

Submitted for a PhD degree in Molecular Genetics.

CORRECTIONS.

Section 1.2.4 Function of LYL-1.

The LYL-1 gene was targeted by homologous recombination in ES cells (Rupping, E., Ph.D. thesis). The bHLH motif, and entire 3' non-coding region was replaced by a lacZ/neomycin reporter gene cassette (Figure 1). This allowed the expression pattern of LYL-1 to be followed by the β-galactosidase function of the LYL-1^{lacZ} fusion protein (Rupping, E., Ph.D. thesis).

Section 1.2.5 Expression of LYL-1 mRNA in mice.

Determination of the normal expression pattern of the LYL-1 by RT-PCR indicated that LYL-1mRNA was up-regulated during embryogenesis and was highly expressed in fetal liver (Figure H1). In adult organs LYL-1 mRNA was abundant in bone marrow, and was detectable in spleen and brain, but not in testis, kidney or liver (Figure H1). LYL-1 mRNA was weakly expressed in an endothelial cell line (sEnd.1), but not in ES cells (D), or in carcinoma cells (F3) (Figure H1).

Section 1.2.6 Expression LYL-1^{lacZ} fusion protein of during embryogenesis.

To determine the temporal and spatial pattern of expression of the LYL-1^{lacZ} fusion gene during embryogenesis, whole embryos and/ or sections of them were stained with X-GAL. LYL-1^{lacZ} was expressed in the blood islands of the yolk sac, endothelial cells of blood vessels, and in mesenchyme of the hindbrain at 8.5dpc (Figure H2). At 11.5dpc, LYL-1^{lacZ} expression was absent in the brain, but high in endothelial cells of the dorsal aorta and aortic arch arteries, and could be seen in the fetal liver (Figure H2). At 14.5dpc a network of LYL-$1^{lacZ+ve}$ blood vessels were visible including in the umbilical cord (data not shown).

Sections of 14.5dpc fetal liver were stained with X-GAL and showed LYL-1^{lacZ} expression in large, polymorphonuclear cells that were positive for acetylcholinesterase in their cytoplasm (Figure H3). This indicated that LYL-1 was expressed in megakaryocytes. Furthermore, small blast-like cells expressed the LYL-1^{lacZ} fusion protein (Figure H4).

Section 1.2.7 Expression LYL-1^{lacZ} fusion protein in adult mice.

X-GAL staining of cytocentrifuged cells isolated from the bone marrow of adult LYL-1$^{-/-}$ mice showed that, as in the fetal liver, LYL-1^{lacZ} was expressed in megakaryocytes, as well as small blast like cells (Figure H4). To further characterise the latter cells, c-Kit^{+ve} cells were sorted from LYL-1$^{-/-}$ bone marrow by FACS, cytocentrifuged and stained with X-GAL. Almost all c-Kit^{+ve} cells were found to express the LYL-1^{lacZ} whereas c-Kit^{-ve} cells were unstained (data not shown). Fetal and adult peripheral blood cells, spleen cells and thymocytes did not stain with X-GAL. However, LYL-1^{lacZ}-positive cells were identified in the subcapsular region of the thymus (Figure H5). These cells were not characterised. Although this study did not show LYL-1^{lacZ} expression in spleen cells, LYL-1 mRNA expression in the spleen had been shown in previous studies (Kuo, et al, 1991;Visvader, et al, 1991). In this study, the absence of LYL-1$^{lacZ+ve}$ cells in the spleen may be due to the inability of the X-GAL staining solution to penetrate the intact spleen, or the limited sensitivity of the β-galactosidase assay.

Section 1.2.8 Development of LYL-1 null mutant mice.

The development of LYL-1$^{+/-}$ and LYL-1$^{-/-}$ mice during embryogenesis was examined in X-GAL stained whole mount embryos (Figure H6). No quantifiable differences were observed in development of LYL-1$^{-/-}$ embryos. Both the haematopoietic and vascular compartments (as well as brain) were unaffected by the absence of LYL-1 protein comprising an intact bHLH domain.

Furthermore, LYL-1$^{-/-}$ pups were born and grew normally, and displayed no obvious phenotype e.g. neonatal lethality, abnormal growth, premature death, and infertility (data not sown).

To further establish the developmental potential of haematopoietic progenitor cells in LYL-1$^{-/-}$ mice, bone marrow cells were isolated and the frequency of colony-forming units (CFUs) determined *in vitro*. The frequency of granulocyte, macrophage and mixed CFUs in LYL-1$^{-/-}$ bone marrow was similar to that of LYL-1$^{+/-}$ and LYL-1$^{+/+}$ bone marrow, indicating that LYL-1 was not required for development of these haematopoietic cell types (Figure H7). Indeed, the number of platelets was normal in LYL-1$^{-/-}$ mice, suggesting that although expressed strongly in megakaryocytes, LYL-1 was not necessary for apparently normal megakaryopoiesis and platelet production.

Furthermore, the lymphoid compartments in thymus, spleen and bone marrow were analysed by flow cytometry. B-cell development was assessed using CD45R/B220, CD43/ S7and IgM and was found to be normal in LYL-1$^{-/-}$ mice (Figure H8). The development of thymocytes and splenic T cells was also normal in these mutant mice.

It was concluded that a functional LYL-1 protein was not required for normal development of the haematopoietic or endothelial system.

Clarification of Aims.

Section 1.3 *Aim of the study.*

The lack of an obvious phenotype in LYL-1$^{-/-}$ mice raises several issues regarding the role of LYL-1 in haematopoiesis. The loss of a functional LYL-1 protein in haematopoietic cells in the yolk sac, fetal liver, and bone marrow suggests that this protein has a redundant role in the formation of haematopoietic cells. However, it is evident that bHLH proteins act in a combinatorial manner (Murre, et al, 1989a; Hu, et al., 1992; Bockamp, et al., 1994), and can affect transcription in different ways depending on the choice of heterodimer (Ma, et al., 1999; Zhuang, et al., 1996), and the specific combinations of transcription factor binding sites (Bain, et al., 1993; Wadman, et al., 1997; Gruntz, et al., 1998). Furthermore, ubiquitous and cell-type, or cell-stage restricted forms of bHLH proteins possess both distinct and overlapping functions that determine cell-specific patterns of gene expression (Weintraub, 1993; Amati and Land, 1994; Bockamp, et al, 1994; Zhuang, et al, 1996). In the case of MyoD, a muscle-specific bHLH protein, the lack of effect on skeletal muscle cell development in MyoD$^{-/-}$ mutant mice was associated with upregulation of Myf5 (Rudnicki, et al., 1992). Gene targeting experiments have shown that MyoD, Myf5, and the related bHLH proteins myogenin and MRF4 have overlapping functions in skeletal muscle development (Rudnicki, et al, 1993; Braun, et al, 1992; Weintraub, 1993). A quantitative neurogenic phenotype was absent in neurogenin2 (Ngn2) single mutant mice due to compensation by Mash1, which was up-regulated in Ngn-2 mutant cortical progenitors (Fode, et al, 2000). Therefore, it is possible that the role of LYL-1 could be compensated via mechanisms similar to those operating for skeletal muscle-, and neuronal-specific bHLH proteins. Interestingly, up-regulation of GATA-2 in erythroid precursors generated by in-vitro differentiation of GATA-1$^{-/-}$ ES cells (Weiss, et al., 1994; Weiss and Orkin, 1995) may allow erythroid progenitors to develop into proerythroblasts in the yolk sac of GATA-1$^{-/-}$ mutant mice (Fujiwara, et al., 1996). This suggests that changes in the level of expression of

related transcriptional regulators may result in functional compensation of haematopoietic-specific transcription factors.

It may be that the effect of disrupting the LYL-1 gene was subtle. For example, disruption of the murine β-globin locus control region (LCR) 5' hypersensitive 3 (HS3) had a minimal effect in yolk-sac derived erythroid cells, and reduced β^{maj} and β^{min} expression by 30% in adult erythrocytes (Hug, et al., 1996). Loss of Ngn2 changed neuronal identity without affecting neuronal progenitor number, organization, or proliferative properties (Fode, et al., 2000). Small changes in haematopoietic cell development and/ or function may occur as a consequence of LYL-1 loss rather than overt defects

With these considerations in mind, we proposed the following hypothesis. The absence of an obvious phenotype in the LYL-$1^{-/-}$ mice was the result of functional compensation through altered expression of another gene. Since LYL-1 and SCL constitute a subfamily of bHLH proteins, which may regulate haematopoiesis by recognition of a common group of target genes (Baer, 1993), SCL was considered an appropriate candidate. The aims of the study are as follows:

- To identify changes in the expression of SCL that may elucidate the apparent absence of a haematopoietic phenotype in LYL-$1^{-/-}$ mice. To achieve this, a detailed analysis of the relative level of SCL mRNA expression in lymphoid, as well as non-lymphoid, organs of LYL-$1^{-/-}$, LYL-$1^{+/-}$ and LYL-$1^{+/+}$ mice will be performed using semi-quantitative reverse transcription- polymerase chain reaction (RT-PCR).

- To develop a reproducible and sensitive semi-quantitative RT-PCR assay for the purpose of assessing relative SCL mRNA levels. This will be determined by autoradiography and densitometry. A difference in the amount of cDNA synthesized and amplified in each reaction will be corrected by comparing densitometric signals from β-actin RT-PCR experiments.

- To examine in more detail the expression of the LYL-1^{lacZ} fusion protein in the spleen. To achieve this, spleen from adult LYL-$1^{-/-}$, LYL-$1^{+/-}$ and LYL-$1^{+/+}$ mice will be processed for 10μm sectioning on a cryostat. Sections will then be mounted onto slides and stained with X-GAL to identify LYL-$1^{lacZ+ve}$ cells.

- To develop the results of the semi-quantitative RT-PCR using histochemical and immunohistochemical techniques.

LYL-1$^{+/-}$ spleen		LYL-1$^{-/-}$ spleen	
Cluster	Number of SCL mRNA^{+ve} cells in cluster	Cluster	Number of SCL mRNA^{+ve} cells in cluster
1	5	1	15
2	5	2	35
3	10	3	>40
		4	>40
		5	>50

Table 13. The number of individual clusters of SCL mRNA^{+ve} cells, and the number of SCL mRNA^{+ve} cells in each cluster in the spleen of LYL-1$^{+/+}$ and LYL-1$^{-/-}$ mice are tabulated above. LYL-1$^{-/-}$ spleen comprised more SCL mRNA^{+ve} clusters (about 4 to 5-fold) and each cluster contained more SCL mRNA^{+ve} cells (about 4 to 8-fold) than the spleen of LYL-1$^{+/+}$ littermates.

Colony description- CFU-GEMM.

Section 3.8 ***LYL-1$^{-/-}$ spleen contain more CFU-e, BFU-e and 'CFU-Mk/ CFU-GEMM-like' progenitors than LYL-1$^{+/+}$ spleen.***

In vitro colony assays using methylcellulose media were employed to compare the growth and differentiation of haematopoietic progenitor cells isolated from the spleen and bone marrow of LYL-1$^{+/+}$ and LYL-1$^{-/-}$ mice (for details see Chapter 2 Methods and Materials section 2.9.1). Interestingly, the number of colonies counted on Day 12 of incubation that comprised megakaryocytes increased in the LYL-1$^{-/-}$ spleen. 1.7 +0.15 (n=3) megakaryocyte-containing colonies per 2.5x10^4 cells were generated from LYL-1$^{+/+}$ spleen, compared to 4.4 +0.55 (n=3) colonies generated from LYL-1$^{-/-}$spleen (Figure 21D), an increase of 2.6-fold in the number of these progenitor cells in the LYL-1$^{-/-}$ spleen (see Table 11). Close examination of the colonies did not allow the progenitors to be defined as CFU-GEMM. The appearance of these colonies after 12 days of incubation is consistent with the development of CFU-GEMM under these assay conditions (www.stemcelltechnologies.com). However, the contribution of erythroid and granulocytic-type cells to the colonies derived from both LYL-1$^{+/+}$ and LYL-1$^{-/-}$spleen did not suggest that the progenitors were CFU-GEMM. Rather, these progenitors were more like a megakaryocyte colony-forming unit (CFU-Mk), and also generated cells that appeared red in colour and may have been erythroid cells (Figure 21D). There was no significant difference in the number of these CFU-Mk/ CFU-GEMM-like progenitors in the LYL-1$^{-/-}$ bone marrow (4.6 +0.55, n=3), compared to LYL-1$^{+/+}$ controls (3.9 +0.55, n=3) (Figure 21D, Table 12). This suggested that a proportion of the c-Kit^{+ve} cells identified in LYL-1$^{-/-}$ spleen were multipotential progenitors capable of developing into colonies that comprised megakaryocytes. May-Grunwald and Giemsa staining of the cells in these colonies would have identified the type(s) of cells that comprised them, and have provided a clearer definition of the type of progenitor (CFU-Mk/ CFU-GEMM-like) from which they were derived.

LYL-1^{lacZ} fusion protein expression- correlation to endogenous expression.

Section 3.5 ***The majority of erythroid cells in the spleen of LYL-1$^{-/-}$ mice do not express the LYL-1^{lacZ} fusion protein.***

The spatial pattern of LYL-1 expression in the spleen was examined by X-GAL staining of 10μm paraformaldehyde-fixed cryosections (see Chapter 2 Methods and Materials section 2.1.14). Figure 17 showed LYL-1 expressing cells were located in the red pulp of the LYL-1$^{+/-}$ spleen (Figure 17B-E), particularly in the subcapsular region (Figure 17E), but were also located in the marginal zone (Figure 17F-H). The frequency of X-GAL stained cells was similar in the LYL-1$^{+/-}$ spleen (data not shown). The intensity of the blue staining of the cells ranges from weak (Figure17C) to strong LYL-1^{lacZ} expression (Figure 17D). Non-specific blue staining was found in the sections of LYL-1$^{+/+}$ (Figure 17A) and LYL-1$^{-/-}$ spleen (Figure 17B) but was clearly distinguishable from positively stained cells. The identity of the LYL-1$^{lacZ+ve}$ cells in the spleen could not be elucidated from the β -galactosidase assay. However, it was evident that LYL-1$^{lacZ+ve}$ population of cells was heterogeneous, since their size varied from small, 'blast-like' cells (Figures 17C, and F) to larger 'megakaryocyte-like' cells (Figure 17D, and E).

X-GAL staining of spleen cryosections showed that most of the erythroid cells, which expressed SCL mRNA and GATA-1, appeared not to co-express the LYL-1^{lacZ} fusion protein. Co-expression of LYL-1 in the same cells that expressed SCL mRNA, GATA-1 could not be excluded by the experiments described in sections 2.1.14 and 3.5. The absence of visible blue stain in the erythroid cells could be due to the limited sensitivity of the β-galactosidase, so that although present, the activity of the LYL-1^{lacZ} fusion protein was not sufficient to generate a discernable chromagenic pigment. Therefore, the LYL-1^{lacZ} reporter gene may not "report" the endogenous LYL-1 gene expression. Indeed, LYL-1 mRNA was detected in brain and liver by

semi-quantitative RT-PCR (see section 2.7), whereas no X-GAL stained cells were found in either tissue. Furthermore, whole mount *in situ* hybridisation experiments (Neidhardt, L., et al. Mechanisms of Development, vol 98, p77-93, 2000) showed a more extensive pattern of LYL-1 mRNA expression than that of the LYL-1^{lacZ} fusion protein observed in X-GAL stained of LYL-1^{lacZ} mutant embryos (see section 1.26 and 1.28). It is possible that the level of LYL-1 expression varied in different haematopoietic cell types and could not be detected in erythroid cells in the LYL-$1^{-/-}$ spleen (and LYL-$1^{+/-}$ spleen) with the X-GAL staining method of the β-galactosidase assay employed in this study. The population of cells, which expressed SCL mRNA and GATA-1 in LYL-$1^{-/-}$ spleen, either did not express the LYL-1^{lacZ} fusion protein, or did so at a level that could not be detected by X-GAL staining of 10μm cryosections. Experiments to assess the expression of LYL-1^{lacZ} mRNA at the single cell level in spleen sections by lacZ- specific *in situ* RT-PCR were compromised by significant background signal (data not shown). Detection of β-galactosidase activity in living single cells with a fluorescent substrate such as fluorescein di-β-galactopyranoside (FDG) would allow a more sensitive analysis of LYL-1^{lacZ} expression in spleen cells by flow cytometry. Co-staining for erythroid-specific antigens, such as TER-119, would allow an accurate assessment of LYL-1 expression during splenic erythropoiesis.

DISCUSSION.- Interpretation of results.

The involvement of LYL-1 in the development of haematopoietic cells cannot be ruled out categorically. The phenotype described is consistent with a function for LYL-1 in regulating mobilisation of haematopoietic cells in the bone marrow. However, limitations in the sensitivity of the X-GAL staining procedure, which may have compromised the ability of the LYL-1^{lacZ} fusion protein to report endogenous LYL-1 mRNA expression, in conjunction with an increase in the number of erythrocytes, platelets, white blood cells and lymphocytes in the peripheral blood of LYL-$1^{-/-}$ compared to LYL-$1^{+/+}$ mice (Figure 23), allow alternative interpretations of the results presented in Chapter 3.

Alterations in c-Kit signaling may also interfere with proliferation and/ or differentiation programs in haematopoietic progenitor cells (Ogawa, et al., 1994). Since expression of LYL-1^{lacZ} fusion protein in the c-Kit^{+ve} progenitors, and the SCL $mRNA^{+ve}$, GATA-1^{+ve}, and TER-119^{+ve} erythroid precursors comprising the erythroid clusters cannot be ruled out, enhanced proliferation and differentiation of these cells specifically in the spleen due to loss of a functional LYL-1 protein may explain the results described in Chapter 3. As such, the phenotype of the LYL-$1^{-/-}$ mice suggests a role for this bHLH protein as a negative regulator of c-Kit mediated proliferation and/ or differentiation of splenic erythroid and megakaryocytic cells. The absence of an intact bHLH in the LYL-1 protein resulted in an increase c-Kit mediate proliferation and differentiation of erythroid cells in spleen. This is consistent with an increase in the number of c-Kit^{+ve} cells (Figure 20), as well as SCL $mRNA^{+ve}$ (Figure 14), GATA-1^{+ve} (Figure 17), and TER-119^{+ve} (Figure 19) erythroblasts in the LYL-$1^{-/-}$ spleen, as well as increased numbers of splenic BFU-e and CFU-e (Figure 21, Tables 11 and 12). Frequent association of the erythroid clusters in the LYL-$1^{-/-}$ spleen with megakaryocytes (Figure 15), and an increase in the number of

megakaryocyte-like colonies *in vitro* (Figure 22, Tables 11 and 12) suggests that LYL-1 protein may also regulate megakaryocyte development in the spleen. The bipotential nature of BFU-e, and the erythroid-megakaryocytic precursor cells may arise from qualitative and/ or quantitative changes in the equilibrium of the shared transcriptional regulators such as GATA-1 (Shivdasani, et al., 1997). Some of the LYL-$1^{-/-}$ splenic megakaryocytes expressed GATA-1 (Figure 16G, H), and may have developed from c-Kit^{+ve} erythroid-megakaryocytic bipotential progenitor cells in the spleen. However, with the exception of the cells responsible for the large erythroid colonies present only in LYL-$1^{-/-}$ spleen, the majority of the LYL-$1^{-/-}$ splenic erythroid progenitor cells did not generate colonies sooner, or colonies that remained longer, when grown in vitro (data not shown). Neither was there any clear difference in the number of apoptotic cells in the LYL-$1^{-/-}$ splenic erythroid clusters (Figure 26). Taken together, these data indicate no change in the intrinsic ability of most of the LYL-$1^{-/-}$ splenic erythroid progenitors to proliferate, differentiate, and survive, and that enhanced growth was targeted to a subpopulation of splenic progenitors in the absence of a functional LYL-1 protein, and these cells generated large numbers of erythroid cells, and possibly contributed to the increase in megakaryocytes. Alternatively, the semi-solid media used to culture the LYL-$1^{-/-}$ splenic progenitors may not have provided an appropriate environment for an intrinsic difference in their proliferation and/ or differentiation to manifest. As mentioned above, the spleen provides a complex microenvironment that was not recapitulated by the semi-solid methylcellulose medium. Therefore, without rejecting the possibility that the phenotype of the LYL-$1^{-/-}$ mice was the result of non-cell autonomous effects due to changes specifically in the splenic microenvironment of LYL-$1^{-/-}$ compared to control mice, enhanced proliferation of the majority of LYL-$1^{-/-}$ splenic erythroid progenitors may not be observed *in vitro*.

The absence of a significant change in the number of erythroid progenitors in LYL-1$^{-/-}$ bone marrow suggests that the LYL-1$^{-/-}$ phenotype is spleen specific, such that enhanced proliferation of, at least a subpopulation of erythroid cells, was challenged in LYL-1$^{-/-}$ bone marrow, and did not lead to the generation of more erythroid progenitors. Indeed, the development of BFU-e, CFU-e, and erythroid precursors in the spleen differed to bone marrow (Nijhof, et al., 1993). BFU-e produced CFU-e, which developed into erythroblasts with much higher efficiency in the splenic microenvironment compared to that of bone marrow (Hendrikx, et al., 1996), and erythroid expansion can be optimized by exploitation of the splenic microenvironment (Nijhof, et al, 1993). Once in the splenic microenvironment, conditions may be optimal for expression of the LYL-1$^{-/-}$ erythroid progenitor cell phenotype, which manifested as enhancement proliferation and differentiation of splenic erythroid progenitors and erythroblasts. c-Kit signaling is more closely associated with expansion of haematopoietic cells, particularly erythroid progenitors and precursors, in the spleen than the bone marrow following phenylhydrazine treatment (Broudy, et al, 1996). Enhanced proliferation of erythroid cells in the spleen was also observed after mice were treated with erythropoietin (Kato, et al, 1999) and was shown to be the result of migration of BFU-e from the bone marrow to spleen, followed by differentiation into CFU-e. The feature(s) peculiar to spleen that is responsible for expression of the erythroid and megakaryocytic cell phenotype in LYL-1$^{-/-}$ mice remain to be elucidated. The close association of c-Kit signaling and splenic erythropoiesis under normal conditions in the mouse, and when bone marrow haematopoiesis is stressed (Broudy, et al, 1996), in conjunction with expression of LYL-1^{lacZ} in c-Kit^{+ve} cells in the bone marrow (Rupping, E. PhD thesis- refer to section 1.2.7 for details) may, taken together with the data presented in this study, elude to a role for LYL-1 in regulating development of a population of c-Kit^{+ve} erythroid/ megakaryocytic progenitors in the spleen, such that in the absence of an intact bHLH in the LYL-1 protein, c-Kit mediated proliferation

and differentiation of these progenitor cells is augmented, leading to an expansion in the numbers of erythroblasts and megakaryocytes in the spleen.

The importance of c-Kit signaling in the proliferation, differentiation, and survival of haematopoietic progenitor/ precursor cells has been confirmed in many studies. c-Kit-deficient mice exhibited a severe reduction of CFU-e in the fetal liver and died of anemia (Nocka, et al., 1989). Inhibition of c-Kit function with an anti-c-Kit monoclonal antibody blocked myelopoiesis but had no effect on lymphopoiesis (Ogawa, et al., 1994). c-Kit signaling promoted survival of early HSCs (Okada, et al., 1991) as well as erythroid progenitors (Wu, et al., 1997). The important function of c-Kit in haematopoietic cell development was further emphasized by studies that showed transduction of c-Kit into selected progenitor cells enhanced proliferation and decreased apoptosis (Lu, et al., 1999). c-Kit mediated proliferation of haematopoietic progenitors, may, in part, be regulated by differential c-Kit expression, which selected progenitors for recruitment into a proliferative state (Lu, et al., 2000). Recently, matrix-metalloproteinase-9 (MMP-9) mediated release of soluble SCF was shown to enable translocation of bone marrow HSCs to a permissive vascular environment that favored differentiation into progenitors (Heissig, et al, 2002), and SCF-mediated chemotaxis involved SRC family kinase and PI3-kinase that required intact c-kit tyrosine residues 567 and 719 respectively (Ueda, et al, 2002). These studies indicate a strong association between signaling pathways involved in HSC/ MHPC mobilisation, and those responsible for proliferation and differentiation events. Clearly, c-Kit has important roles in both mobilisation and development of HSCs/ MHPCs (Ueda, et al, 2002; Heissig, et al, 2002). Therefore, as proposed in this thesis, a putative function for LYL-1 in regulating c-Kit mediated haematopoietic progenitor cell mobilisation may also necessitate changes in the regulation of proliferation and/ or differentiation events. This may also be considered in the

reverse, and a change in c-Kit mediated development may precipitate shifts in the mobilisation of progenitors. The very late antigen-5 (VLA-5), a member of the β_1-intergin family, also mediates adhesion of haematopoietic progenitors to the bone marrow stroma. Proliferation of committed progenitors (Verfaillie, 1992) and long-term culture initiating cells (Verfaillie and Cantazarro, 1996) was inhibited when $CD34^{+ve}$ cells were cultured in contact with stroma. G_1/S progression of progenitor cells was blocked through β_1-integrin (Hurley, et al., 1997), and activation of VLA-5/ fibronectin signalling pathways have been shown recently to inhibit progenitor cell proliferation (Jiang, et al., 2000). β_1-integrin mediated adhesion of $CD34^{+ve}$ cells was associated with up-regulation of $p27^{Kip}$, and could be overcome by addition of higher concentrations of cytokines (Jiang, et al., 2000). Although mechanisms involved in adhesion-mediated inhibition of proliferation are not well understood, they are likely to involve c-Kit. As proposed, a reduction in c-Kit mediated adhesion of a population of LYL-1- expressing haematopoietic progenitor cells to the bone marrow stroma, and consequent mobilisation into the peripheral blood in the $LYL\text{-}1^{-/-}$ mice may underlie their ability to expand in the splenic microenvironment. Once in the spleen, the $LYL\text{-}1^{-/-}$ erythroid and/ or megakaryocytic progenitors do not receive adhesion-mediated signals through c-Kit sufficient to inhibit proliferation. Under these conditions, the $LYL\text{-}1^{-/-}$ progenitors expand in number, giving rise to more BFU-e and megakaryocytic progenitors, which undergo differentiation and generate more CFU-e, erythroblasts and megakaryocytes. A detailed analysis of the immunophenotype of the erythroid/megakaryocytic and lymphoid compartments of $LYL\text{-}1^{-/-}$ spleen, in conjunction with assessment of c-Kit, β_1-intergin and $LYL\text{-}1^{lacZ}$ fusion protein expression would provide valuable information about the nature of the haematopoietic cells in the spleen of $LYL\text{-}1^{-/-}$ mice.

The increased number of erythrocytes in the LYL-1$^{-/-}$ peripheral blood may explain the reduction in erythrocyte number in the LYL-1$^{-/-}$ spleen. Since LYL-1$^{-/-}$ progenitors grew normally *in vitro* (Figures 21 and 22) and did not apoptose prematurely *in vivo* (Figure 26), it seems likely that more erythrocytes are being produced and expelled from the spleen into the periphery via the splenic vein. Peripheralisation of erythrocytes is regulated, in part, through a member of the Ig superfamily, CD147 (Coste, et al, 2001). Blockade of CD147 binding to an unidentified ligand on endothelial cells, with F(ab')$_2$ fragments of anti-CD147 monoclonal antibody disrupted circulation of erythrocytes and promoted their selective trapping in the spleen. Enhanced peripheralisation of erythrocytes from the LYL-1$^{-/-}$ spleen may suggest that the absence of a functional LYL-1 protein in erythroid progenitor cells causes the production of erythrocytes with increased CD147 expression and/ or function, which detach from the surrounding endothelial cells, and lead to a reduction in the number of splenic erythrocytes in LYL-1$^{-/-}$ mice. Alternatively, the reduced number of splenic erythrocytes in LYL-1$^{-/-}$ mice may result from an up-regulation in the release of erythrocytes from the spleen following expansion of splenic erythroid cells.

REFERENCES.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.S., Werb, Z. and Rafii, S. (2002). Recruitment of stem cells and progenitor cells from the bone marrow niche requires mmp-9 mediated release of kit-ligand. Cell, volume 109 (issue 5 May 31), p625-637.

Ueda, S., Mizuki, M., Ikeda, H., Tsujimura, T., Matsumura, I., Nakano, K., Daino, H., Honda, Z-I., Sonoyama, J., Shibayama, H., Sugahara, H., Machii, T. and Kanakura, Y. (2002). Critical roles for c-Kit tyrosine residues 567 and 719 in stem cell factor-induced

chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. Blood, volume 99 (number 9), p3342-3349.

Coste, I., Gauchat, J-F., Wilson, A., Izui, S., Jeannin, P., Delneste, Y., MacDonald, H.R., Bonnefoy J-Y. and Renno, T. (2001). Unavailability of CD147 leads to selective erythrocyte trapping in the spleen. Blood, volume 97 (number 12), p3984-3988.

Lu, L., Dai, M.S., Ge, Y., Wang, L.S., Braun, S.E., Wait, C.L., Griffith, D.J., Heinrich, M.C.and Broxmeyer, H.E. (2000). Co-transduction of cDNAs for c-kit and steel factor into single CD34+ cord blood cells further enhances the growth of erythroid and multipotential progenitors. Journal of Hematotherapy and Stem Cell Research, volume 9(number 6), p813-825.

Lu, L., Heinrich, M.C., Wang, L.S., Dai, M.S., Zigler, A.J., Chai, L., Broxmeyer, H.E. (1999). Retroviral-mediated gene transduction of c-kit into single hematopoietic progenitor cells from cord blood enhances erythroid colony formation and decreases sensitivity to inhibition by tumor necrosis factor-alpha and transforming growth factor-beta1. Blood, volume 94 (number 7), p2319-2332.