

**Identification of genes transcriptionally
regulated by the E2A-HLF leukaemia-
associated fusion protein**

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A thesis submitted for the Degree of Doctor of Philosophy

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2005

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Abstract

The E2A-HLF chimaeric transcription factor arises from the t(17;19) translocation in some childhood pro-B acute lymphoblastic leukaemias (ALL). The t(17;19) translocation in childhood ALL is associated with a very poor outcome. Disseminated intravascular coagulopathy and hypercalcaemia at presentation are also associated with the translocation.

In vitro experiments have shown that E2A-HLF acts as an anti-apoptotic factor in murine IL-3-dependent cell lines, Baf-3 and FL5.12. A tetracycline-inducible system to express E2A-HLF in Baf-3 cells was established and which confirmed an anti-apoptotic role of E2A-HLF in these cells following IL-3 withdrawal. DNA microarrays were used to identify candidate targets of E2A-HLF that may contribute to its anti-apoptotic and leukaemogenic action. Several candidate target genes were identified and their induction by E2A-HLF was confirmed by Northern blotting analysis and promoter-reporter gene assays. Independent experiments demonstrated that E4BP4, a basic leucine zipper transcription factor related to E2A-HLF, was also a target of E2A-HLF.

Experiments assessing the role of E2A-HLF and its target genes on immortalisation and differentiation of primary murine haematopoietic progenitor cells revealed that E2A-HLF functions as a potent oncogene. Expression of E2A-HLF in a retrovirus and infection of bone marrow and foetal liver haematopoietic progenitors cells lead to the immortalisation of myeloid and lymphoid progenitors as shown by colony forming assays and liquid culture. Other workers have demonstrated that the co-expression of both E2A-HLF and BCL-2 was necessary to immortalise lymphoid progenitors. Data presented here shows for the first time that E2A-HLF alone is sufficient to immortalise primary lymphoid progenitors and that co-expression of BCL-2 has a synergistic effect on the immortalisation properties of E2A-HLF.

Acknowledgements

I would like to thank my supervisor, Dr Hugh Brady, for all his help and encouragement over the last few years.

I would also like to say thanks to my friends and colleagues in the Molecular Haematology and Cancer Biology Unit at for being a great bunch of people to work with and for being source of information, advice and protocols!

Special thanks to Elaine O'Sullivan (the first member of E2A-HLF/E4BP4 posse) for being my mentor over the last few years. Thanks for all your support and guidance.

I would also like to thank Lesley Smyth, Helena Kempinski and Mike Hubank for taking the time to read through my thesis chapters and give helpful comments.

I am also very grateful to Nipurna Jina and Danielle Fletcher for their patience and help with the microarray work.

Finally, I would like to thank my family for their love, support and for having faith in me.

*This thesis is dedicated to Mum, Dad, Karen, Anna, Amy, Simon, Aunty Ng Kiu,
Aunty Yuen Tai and the rest of the family.*

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Abbreviations

2-ME	2-mercaptoethanol
5-FU	5-fluorouracil
AD	activation domain
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APL	acute promyelocytic leukaemia
Apaf-1	Apoptotic protease activating factor-1
BCR	B cell receptor
bHLH	basic helix-loop-helix
Bio	biotin
BM	bone marrow
BMT	bone marrow transplant
bp	base pairs
bZIP	basic leucine zipper
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
cDNA	complementary DNA
CMP	common myeloid progenitor
CMV	cytomegalovirus
cRNA	complementary RNA
dd H ₂ O	double distilled water
DEPC	diethyl pyrocarbonate
DIC	disseminated intravascular coagulopathy
DNA	deoxyribonucleic acid
dNTP	dioxynucleotide triphosphate
Dox	doxycycline
DTT	dithiothreitol
E7	embryonic day 7
E13	embryonic day 13
E12	embryonic day 12
ECL	enhanced chemiluminescence
EDTA	ethylamine diamine tetra acetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FDR	false discovery rate
FISH	fluorescence <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
Flt-3L	<i>fms</i> -like tyrosine kinase-3 ligand
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSFR	granulocyte-colony stimulating factor receptor
GEMM-CFU	granulocyte, erythroid, macrophage, megakaryocyte colony forming unit

GM-CSF	granulocyte macrophage colony stimulating factor
GMP	granulocyte-macrophage progenitor
GST	glutathione-S-transferase
h	hour(s)
HLF	Hepatic Leukaemia Factor
HPC	haematopoietic progenitor cell
HRP	horse radish peroxidase
HSC	haematopoietic stem cell
HSV	<i>Herpes simplex</i> virus
Ig	Immunoglobulin
IL	Interleukin
IL-7R α	IL-7 receptor α subunit
IMDM	Iscoves modified Dulbecco medium
IRES	internal ribosomal entry site
kb	kilobases
kDa	kilo Daltons
LTR	long terminal repeat
M-CSR	macrophage-colony stimulating factor receptor
MEP	Megakaryocyte-erythroid progenitor
min	minute(s)
MOPS	3-[N-morpholino]-2-hydroxypropanesulfonic acid
mRNA	messenger RNA
MSCV	Murine stem cell virus
NK	natural killer
NLS	nuclear localisation signal
NP40	Nonidet [®] P 40
NSM	neurosecretory motor neurons
PAR	proline and acidic amino acid rich
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PE	phycoerythrin
PGK	phosphoglycerate kinase promoter
PI	propidium iodide
PI-3	phosphatidylinositol-3 (kinase)
PVDF	polyvinylidene fluoride
RDA	representational difference analysis
Rag	recombinase activating gene
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
rtTA	reverse tetracycline transactivator
SCF	stem cell factor
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second(s)
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween

TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	tetracycline
TetR	tet repressor protein
TRE	tetracycline response element
U	unit(s)
UV	ultraviolet
VP16	virion protein 16

CHAPTER 1

INTRODUCTION

1.1. Overview of haematopoiesis

Mature blood cells have a limited life span and need to be continuously replaced.

New cells are generated from the proliferation and differentiation a small number of haematopoietic stem cells (HSCs). The haematopoietic system in the mouse has been extensively studied. Haematopoiesis occurs at different organs during murine development and occurs in two phases: primitive and definitive haematopoiesis.

Primitive haematopoiesis occurs in extraembryonic yolk sac at embryonic day 7 (E7) in mice (Lensch and Daley, 2004; Kondo *et al.*, 2003). Primitive haematopoiesis is a transient phase of blood cell development that is characterised by the production of large, nucleated erythroid cells. Definitive haematopoiesis generates cells of the myeloid and lymphoid lineages and occurs within the foetal liver from embryonic day 12 (E12) (Lensch and Daley, 2004). The bone marrow subsequently replaces the foetal liver as the primary site of haematopoiesis after birth.

HSCs in the bone marrow are rare and represent less than 0.05% of bone marrow cells in mice (Spangrude *et al.*, 1988). Haematopoiesis is a progressive process whereby HSCs exist at the top of a haematopoietic hierarchy and divide to produce progeny that are more restricted in their multipotentiality, but possess an increased ability to differentiate and proliferate to generate lymphocytes, myeloid, erythroid and megakaryocytic cells (**Figure 1.1**).

1.1.1. B cell development in mice

Clonogenic common lymphoid progenitors (CLPs) have been isolated in murine bone marrow and foetal liver (Kondo *et al.*, 1997; Mebius *et al.*, 2001). The model of haematopoiesis depicted in **Figure 1.1** suggests that CLPs are restricted to production of T cells, B cells and natural killer (NK) cells. However, bipotential

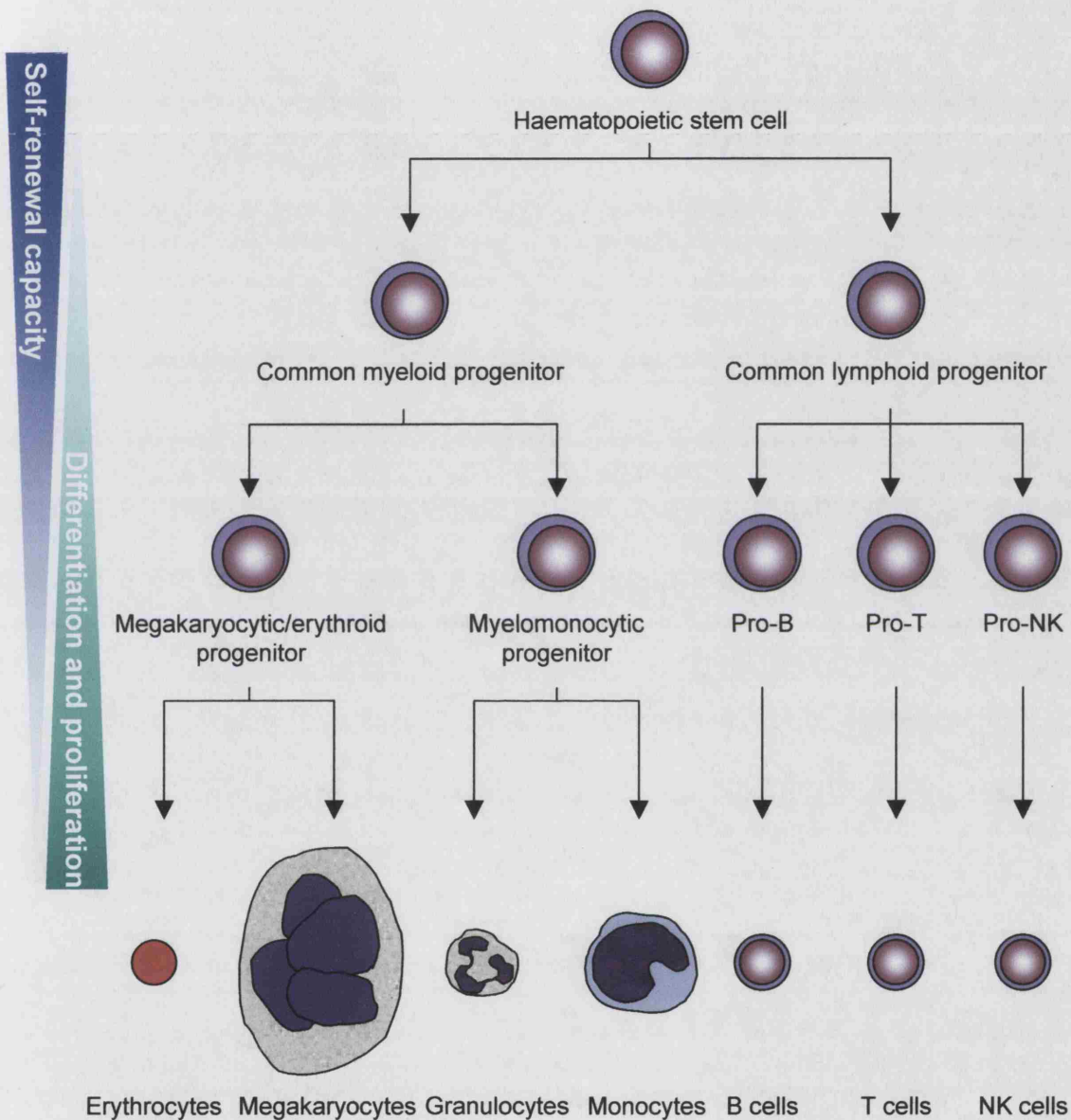


Figure 1.1. Overview of normal haematopoiesis.

Haematopoietic stem cells (HSCs) can generate all the cells of the haematopoietic system. HSCs can produce progeny which can undergo self-renewal or become more committed along a haematopoietic lineage which produce more differentiated cells with greater proliferative potential. Adapted from Janeway and Travers, 1997.

progenitors have been identified in both murine foetal liver and murine bone marrow which generated B cells and macrophages (Cumano *et al.*, 1992; Montecino-Rodriguez *et al.*, 2001).

The successive stages of mouse B cell development are illustrated in **Figure 1.2**.

Different stages of murine B cell development are characterised by the rearrangement and expression of immunoglobulin heavy chain (*IgH*) and immunoglobulin light chain (*IgL*) genes and by the expression of intracellular and cell surface molecules, e.g. terminal deoxynucleotidyl transferase (TdT), recombinase-activating genes (Rag-1/Rag-2), CD19, B220 (CD45RA isoform), CD43 and the Interleukin 7 receptor (IL-7R α) (Osmond *et al.*, 1998; Hardy and Hayakawa, 2001).

Critical stages in B cell development involve the rearrangement of immunoglobulin gene segments. Survival of developing B cells is dependent on successful rearrangement of the *IgH* and *IgL* chains which require the activities of Rag-1/Rag-2 and TdT (Hardy and Hayakawa, 2001). Generation of productive immunoglobulin heavy chains leads to the assembly and expression of the pre-B cell receptor (pre-BCR) composed of the heavy chain, the surrogate light chains ($\lambda 5$ and VpreB) and the $Ig\alpha$ and $Ig\beta$ signalling complex. Signalling through the pre-BCR allows survival and progression to the next stage of B cell differentiation which involves rearrangement of the κ and λ *IgL* loci. Productive rearrangement of the *IgL* gene segments allows the light chain to assemble with the heavy chain to generate the BCR on the cell surface of the subsequent immature B cell. The generation of non-productive *IgH* and *IgL* chains leads to death of the cells by apoptosis (Marsden and Strasser, 2003).

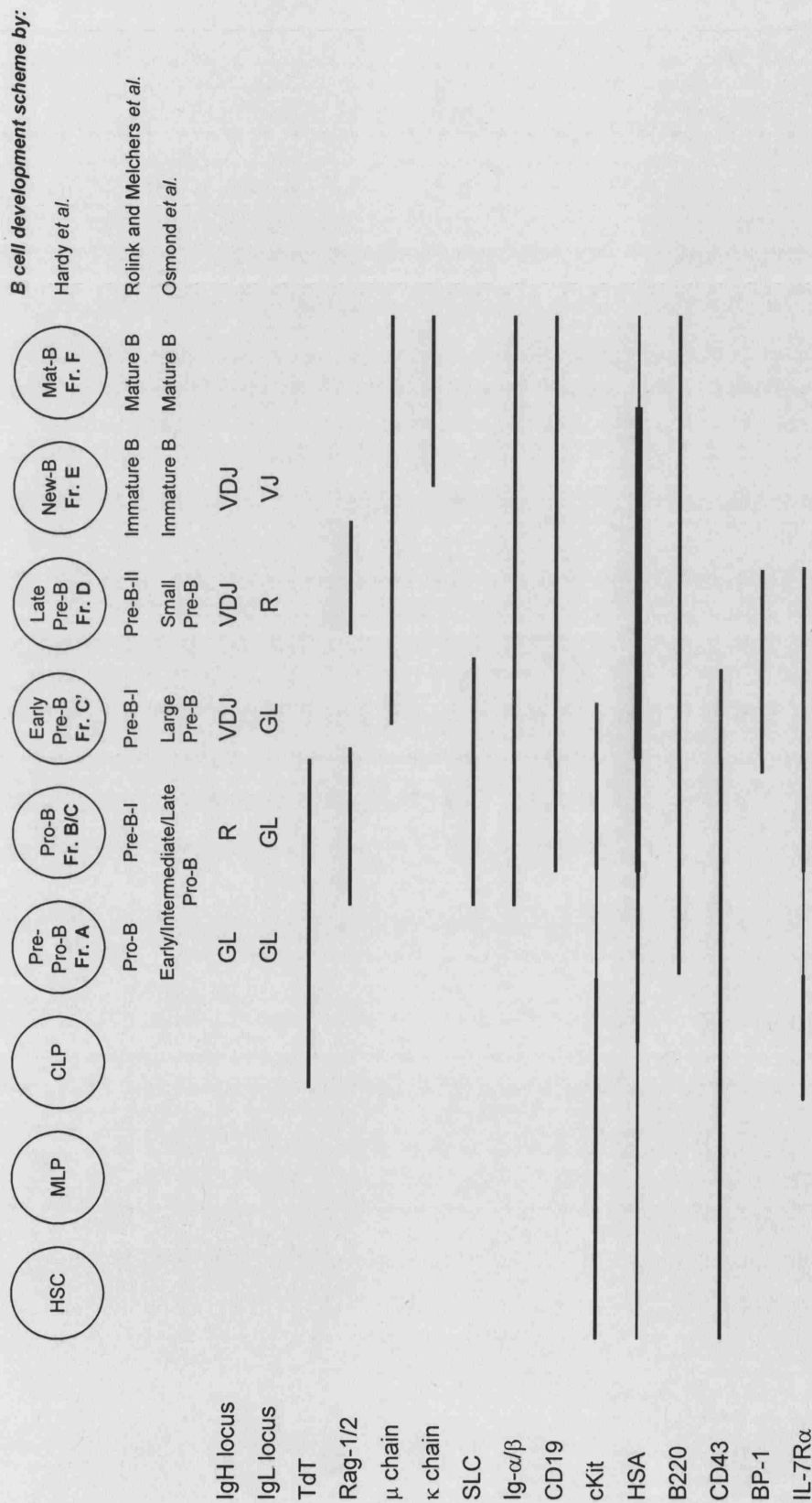


Figure 1.2. B cell development in mouse bone marrow.

Different stages of B cell development can be distinguished in the bone marrow by the expression of different cell markers and by the status of immunoglobulin gene locus rearrangement. Line thickness indicates the relative level of expression. Fr., Fraction. SLC, surrogate light chain. GL, germline. R, rearranging. MLP, multilineage progenitor. Adapted from Hardy and Hayakawa, 2001.

The phenotypic changes and rearrangement of the immunoglobulin genes that occur as B cells develop are tightly regulated. One of the regulatory mechanisms controlling B cell development involves ubiquitous and specific transcription factors which control the expression of the different markers of B cell development (Bartholdy and Matthias, 2004). Gene targeting studies have identified a number of genes required for B cell commitment and differentiation. Genes such as *E2A*, *EBF* and *Pax-5* have been shown to be critical for normal B cell differentiation (Bain *et al.*, 1994; Zhuang *et al.*, 1994; Lin and Grosschedl, 1995; Nutt *et al.*, 1997). Mice deficient for *E2A* or *EBF* exhibit a block in the earliest stage of B cell development before the initiation of IgH rearrangement. The expression of many B cell specific genes, such as *CD19*, *λ5* and *VpreB*, are affected by the loss of E2A and EBF expression. The role of E2A in B cell development will be examined in more detail later in section 1.4.

1.1.2. Myeloid cell development in mice

Common myeloid progenitors (CMPs) are the clonogenic precursors of the granulocyte-monocyte restricted progenitors (GMPs) and the megakaryocyte-erythrocyte restricted progenitors (MEPs) that generate granulocytes and monocytes or platelets and erythrocytes respectively (Akashi *et al.*, 2000; Traver *et al.*, 2001). Foetal liver CMPs have been shown to have some B differentiation potential (Traver *et al.*, 2001). As with B cell development, several transcription factors (e.g. PU.1, GATA-1, C/EBPs) play a vital role in the regulation of myeloid development (Zhu and Emerson, 2002; Friedman, 2002).

Neutrophils are the most abundant of the granulocytes which also include eosinophils and basophils. Several stages of neutrophil development can be

identified. Myeloblasts are the earliest recognisable stage of neutrophil differentiation, followed by promyelocytes, and then myelocytes and finally mature neutrophils. Specific markers are found at different stages. Prolmyelocytes acquire primary granules containing enzymes such as myeloperoxidase, neutrophil elastase and myeloblastin. Myelocytes acquire secondary granules which contain lactoferrin, neutrophil gelatinase and express the Gr-1 cell surface marker. Other markers of granulopoiesis include granulocyte colony stimulating factor receptor (G-CSFR), CD33 and CD13 (Friedman, 2002).

Development of monocytes proceeds from the monoblast which develop into promonocytes which then develop into monocytes. Monocytes are able to mature into tissue macrophages. Markers for monocyte development include Mac-1, macrophage-colony stimulating factor receptor (M-CSFR), Fcγ receptor (II/III), CD14 and CD18 (Friedman, 2002). Mac-1 and Gr-1 can be used to identify neutrophils and monocytes in mouse by their relative levels of expression by flow cytometric analysis (Legasse and Weissman, 1996).

1.2. Chromosome translocations in human leukaemias

Regulation of haematopoiesis is complex and involves the integrated effects of intrinsic transcription factors, signalling pathways initiated by regulatory cytokines and by extrinsic factors such as interaction with the bone marrow stroma.

Chromosome translocations or other genetic aberrations can occur in HSCs or very early progenitor cells and disrupt the normal regulation of haematopoiesis and subsequently contribute to the development of leukaemia.

Acute lymphoblastic leukaemia (ALL) is the most common type of childhood leukaemia. Chromosome translocations are detected in the majority of ALLs.

Distinct chromosome translocations are associated with specific subtypes of leukaemia (Rabbitts, 1994; Look, 1997). Many chromosome translocations have prognostic significance which may determine the treatment modality undertaken.

The consequences of chromosome translocations include activation of a proto-oncogene, e.g. *c-Myc* or *BCL-2*, by juxtaposition with a highly constitutively active regulatory element, e.g. *Ig* or T cell receptor (*TCR*) genes, leading to aberrant expression of the proto-oncogene (Rabbitts, 1994; Rubnitz and Look, 1999). More commonly, a chimaeric protein is generated which has unique properties. Chimaeric proteins may be kinases, e.g. BCR-ABL and TEL-JAK2, but are more commonly transcription factors, e.g. E2A-HLF, PML-Rar α and TEL-AML1 (Rabbitts, 1994; Lacronique *et al.*, 1997). These chimaeric transcription factors could affect the normal cellular transcriptional networks and lead to leukaemic transformation by interfering with the regulation of genes that control cell proliferation, differentiation and cell death.

1.3. Evolutionarily conserved mechanisms of apoptosis

Apoptosis, or programmed cell death, is a normal and physiologically important process in multicellular organisms which enables the removal of excess, defective, or damaged cells during development and during tissue homeostasis. Deregulation of apoptosis has been implicated in developmental abnormalities and diseases including cancer and autoimmune diseases. Apoptosis is important during B cell development at several stages particularly in deletion of cells which generate non-functional immunoglobulin rearrangements, do not respond to IL-7, or generate autoreactive B cells (reviewed by Marsden and Strasser, 2003).

Apoptosis is regulated by genes that are highly conserved between nematodes and mammals (Meier *et al.*, 2000). Four genes, *egl-1*, *ced-4*, *ced-3*, and *ced-9*, are involved in regulating the apoptotic death of 131 of 1090 somatic cells during *Caenorhabditis elegans* development. These genes have homologues in mammalian apoptosis pathways. The four *C. elegans* genes act in a simple genetic pathway whereby CED-9 (a homologue of anti-apoptotic members of the BCL-2 family) inhibits apoptosis by binding to the adaptor protein CED-4 (an APAF-1-like molecule), and prevents the activation of the CED-3 caspase (**Figure 1.3**).

Displacement of CED-9 from CED-4 by EGL-1 (a pro-apoptotic BH3-only member of the BCL-2 family) activates the proteolytic action of CED-3 and causes apoptosis. In *C. elegans*, CES-2 and CES-1 regulate the apoptotic death of the serotonergic neurosecretory neurons (NSM). Loss-of-function mutations in CES-2 and gain-of-function mutations in CES-1 block apoptosis in these neurons (Ellis and Horvitz, 1991).

CES-2 is a member of the basic leucine zipper (bZIP) family of transcription factors. Another member of this group of transcription factors is E4BP4. Both CES-2 and E4BP4 share homology with the proline-and-acidic-amino-acid rich (PAR) bZIP subfamily within the bZIP domain. These transcription factors are able to bind to similar or identical consensus sequences (Cowell, 2002; Zhang *et al.*, 1995). PAR bZIP members include D-box binding protein (DBP), thyrotroph embryonic factor (TEF) and hepatic leukaemia factor (HLF). Overexpression of E4BP4 or E2A-HLF, a product of the t(17;19) translocation in childhood ALL, have been shown to delay apoptosis in pro-B cells (Inaba *et al.*, 1996; Ikushima *et al.*, 1997; Inukai *et al.*, 1998; Kuribara *et al.*, 1999; Dang *et al.*, 2001).

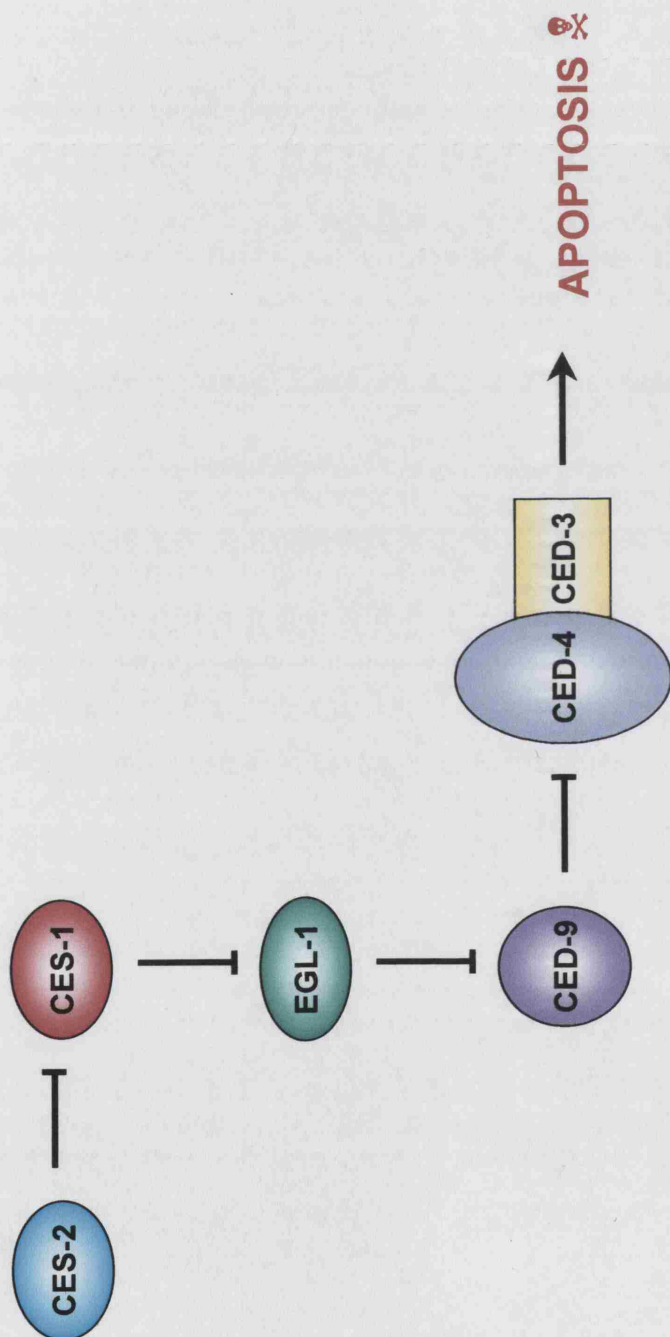


Figure 1.3. The apoptotic system in *C. elegans*.

CED-9 inhibits apoptosis by binding to the adaptor protein CED-4 and prevents the activation of a caspase. Displacement of CED-9 from CED-4 by EGL-1 activates the proteolytic action of CED-3 and causes apoptosis. BCL-2 is the mammalian functional homologue of CED-9. EGL-1 is a member of the pro-apoptotic BH3-only members of the BCL-2 family and CED-4 is a APAF-1-like molecule. In serotonergic neurosecretory neurons (NSM), the transcription factors, CES-2 and CES-1, act to regulate apoptosis. Gain-of-function mutations in CES-1 and loss-of-function in CES-2 block apoptosis in these cells. CES-1 is a member of the Snail/Slug family of family of zinc finger transcription factors. CES-2 shares homology with the bZIP protein E4BP4 and with the PAR bZIP subfamily.

1.4. E2A function and regulation

The *E2A* gene encodes two transcription factors, E12 and E47, due to differential splicing of exons which encode for the basic helix-loop-helix (HLH) domain (Murre *et al.*, 1989). E2A proteins are Class I HLH proteins and are ubiquitously expressed and can homodimerise or heterodimerise with tissue-restricted Class II proteins, e.g. MyoD, myogenin (Murre *et al.*, 1994; Massari and Murre, 2000). Class V bHLH proteins, also known as Id proteins, can antagonise the activity Class I HLH proteins by heterodimerisation as they lack a functional basic DNA binding region. E2A proteins, and other Class I HLH proteins such as E2-2 and HeLa E-box binding protein (HEB), recognise the canonical 'E-box' sequences (CANNTG). E-box elements are found in a number of different genes and enhancer elements. The IgH gene enhancer and Ig kappa enhancer contain E-boxes as well as muscle-, neuron- and pancreatic-specific genes. The role of the E2A proteins has been extensively studied in B and T cells.

1.4.1. E2A in B cell development

E2A proteins are involved in numerous stages during B cell development (Kee *et al.*, 2000). The importance of E2A in B cell development was determined by the generation of E2A deficient mice by the disruption of the bHLH-encoding exons. This showed that E2A is necessary for the earliest stages of B cell development (Bain *et al.*, 1994; Zhuang *et al.*, 1994). Two different strategies were used to generate the E2A deficient mice. One strategy involved replacement of the E12 bHLH domain encoding exon with a neomycin cassette (Bain *et al.*, 1994). Deletion of the E12 exon also abrogated the expression of the E47 protein product. The second strategy involved replacement of both the E12 and E47 encoding exons with

a neomycin cassette (Zhuang *et al.*, 1994). A summary of the phenotype of E2A knockout mice is given in **Table 1.1**. E2A knockout mice have a high rate of neonatal mortality and post-natal growth retardation. E2A knockout mice lack pre-B and mature B cells as well as a significantly reduced numbers of B cell progenitors (B220⁺/CD43⁺). An incomplete developmental block also occurred at a similar stage in T cell development. E2A knockout mice were highly susceptible to the development of T cell tumours (Bain *et al.*, 1997; Yan *et al.*, 1997). The expression of B-lineage specific genes such as *mb-1*, *VpreB*, *λ5*, *Pax-5* and *Rag-2* were severely impaired.

In vitro studies have shown that overexpression of E2A can activate expression of B-lineage specific genes in non-B cells such as a macrophage cell line and fibroblasts (Choi *et al.*, 1996; Kee and Murre, 1998). E2A also induced TCR γδ V(D)J rearrangements in a human embryonic kidney cell line, BOSC 23, in the presence of Rag-1 and Rag-2 (Ghosh *et al.*, 2001). E2A also has a role in tumour suppression as demonstrated by the ectopic expression of the E2A proteins in T cell lymphomas derived from E2A-deficient mice. The thymic lymphomas were transduced with retroviral constructs which expressed E12 or E47 which resulted in slowed cell growth rate and death (Engel and Murre, 1999).

Table 1.1. Phenotype of phenotype of E2A knockout mice

	Bain <i>et al.</i> , 1994; Bain <i>et al.</i> , 1997.	Zhuang <i>et al.</i> , 1994; Yan <i>et al.</i> , 1997.
Strategy	Replacement of exon encoding E12 bHLH domain with neomycin cassette by homologous recombination.	Replacement of exons encoding E12 and E47 domains with neomycin cassette by homologous recombination.
Mouse strain	129/Ola.	129/Sv.
Life expectancy	High rate of neonatal death mainly between post-natal day 1 and day 4.	High rate of neonatal death mainly within the first week. Nearly all die before weaning.
Growth	Some post-natal growth retardation.	Post-natal growth retardation.
Cell numbers	Reduced numbers of nucleated cells in peripheral blood. Reduced numbers of thymic and splenic lymphoid cells. Heterozygous mice have modest reduction of B220 ⁺ /IgM ⁺ cells in spleen. Erythrocyte and myeloid numbers not significantly changed.	Normal numbers of granulocytes and monocytes in bone marrow. Heterozygotes have reduced numbers of pro-B and mature B cells in E18.5 foetal liver, but corrected during first month of life.
Spleen	Reduced numbers of splenic T cells.	Degenerated quickly within first 3 weeks of life, but showed normal tissue architecture.
T cells	Reduced numbers of thymocytes. CD4 ⁺ and CD8 ⁺ cells present in spleen and thymus which express normal levels of TCR. Decreased percentage of CD4/CD8 DN and CD4/CD8 DP cells. Increased percentage of CD4 and CD8 SP cells.	Mature T cells found in peripheral organs. Normal subsets of CD4/CD8 DP and SP cells in thymus of 1-week-old mice. CD4/CD8 DP from thymus gradually reduced in 2- to 3-week-old mice.
B cells	Reduced B220 ⁺ /CD43 ⁺ progenitors. No B220 ⁺ /IgM ⁺ cells detected. No DJ or V(D)J rearrangements in foetal liver or bone marrow.	Little or no B220 ⁺ B cells in BM or spleen. No IgM ⁺ cells detected. No DJ rearrangements detected.
Expression of other genes	Reduced levels of CD19, immunoglobulin germ-line transcripts (<i>I_H</i>), <i>Rag-1</i> , <i>λ5</i> , <i>mb-1</i> and <i>Pax-5</i> transcripts in E15.5 foetal liver.	
Malignancies	Approx. 50% of mice at 4 months developed T cell lymphomas. Tumours were CD4 ⁺ /CD8 ⁺ . Most tumours express increased levels of <i>c-myc</i> transcripts.	6 out of 13 adult mice develop acute T cell tumours between 3 to 10 months. Tumours were CD4 ⁺ /CD8 ⁺ or CD4 ^{low} /CD8 ⁺ or CD4 ⁺ /CD8 ⁺ and little or no TCR expression. 1 heterozygous mouse developed T cell leukaemia at 2 years.

DP, double positive; SP, single positive; DN, double negative; TCR, T cell receptor.

1.5. HLF function and regulation

HLF was cloned by nature of its fusion to E2A and was found to be expressed in human brain, liver and kidney (Hunger *et al.*, 1992; Inaba *et al.*, 1992). Similar tissue expression was also determined in rats and mice (Falvey *et al.*, 1995; Hitzler *et al.*, 1999). HLF was not initially believed to be expressed in haematopoietic and

lymphoid cells, however a recent report showed that HLF was expressed in erythroid cells (Hunger *et al.*, 1992; Inaba *et al.*, 1992; Crable and Anderson, 2003).

Analysis of rat HLF expression revealed that the rat *HLF* gene encoded two transcriptional activators which differ in their tissue distribution and were expressed according to circadian rhythms (Falvey *et al.*, 1995). HLF stimulates transcription of reporter constructs containing the cholesterol 7 α hydroxylase promoter, albumin promoter, Factor VIII and Factor IX promoters and may therefore normally regulate the expression of liver-specific genes in the liver (Falvey *et al.*, 1995; Begbie *et al.*, 1999; Mitsui *et al.*, 2001; Newcombe *et al.*, 1998). HLF has also been shown to be able to transactivate the LMO2 promoter in reporter assays in an erythroid cell line (Crable and Anderson, 2003). Due to the similarity between HLF and CES-2, the role of HLF in programmed cell death was investigated in developing mice. No correlation was observed between the expression of HLF and tissues undergoing extensive apoptosis during development (Hitzler *et al.*, 1999).

The circadian pattern of the expression of HLF and the other PAR bZIP proteins has been investigated in the nervous system and liver. The PAR bZIP proteins and E4BP4 have been shown to be involved in the transcriptional regulation of the circadian clock mechanism in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The *Drosophila* protein, PAR domain protein 1 (Pdp1) was recently identified and has been shown to be an essential clock component in flies (Cyran *et al.*, 2003). The role of PAR bZIP proteins and E4BP4 in the regulation of the circadian rhythm will be discussed later in section 1.7.

1.6. E4BP4 function and regulation

E4BP4 (also known as NFIL3) was initially identified as an adenovirus E4 promoter-binding protein and as a transcriptional activator of the human interleukin-3 (IL-3) promoter in T-cells (Cowell *et al.*, 1992; Zhang *et al.*, 1995). E4BP4 is expressed in many different tissues and has been implicated in several cellular pathways including regulation of the circadian clock mechanism and the response to hormones and IL-3 (Lai and Ting, 1999; Cowell, 2002).

E4BP4 contains a repression domain (**Figure 1.4**) and has been characterised mainly as a repressor of transcription using reporter gene assays in various cell types (Cowell *et al.*, 1992; Chen *et al.*, 1995; Doi *et al.*, 2001). E4BP4 expression was shown to be modulated by calcium signalling pathways in rat smooth muscle cells and induced by parathyroid hormone in osteoclasts (Nishimura and Tanaka, 2001; Ozkurt and Tetradis, 2003). E4BP4 expression was also induced by the glucocorticoid, dexamethasone, in ID13 mouse fibroblasts (Wallace *et al.*, 1997). This study suggested that E4BP4 might play a role in the glucocorticoid-induced negative regulation of genes such as cyclooxygenase-2 (*Cox-2*), inducible nitric oxide synthase (*iNOS*) and cytoplasmic phospholipase A2 (*cPLA2*). These genes were found to have E4BP4-like binding sites within their promoters. The *Cox-2* promoter was shown to contain four E4BP4-binding sites. A luciferase reporter construct containing 1 kb of the *Cox-2* promoter was repressed by the overexpression of E4BP4 (Ozkurt and Tetradis, 2003).

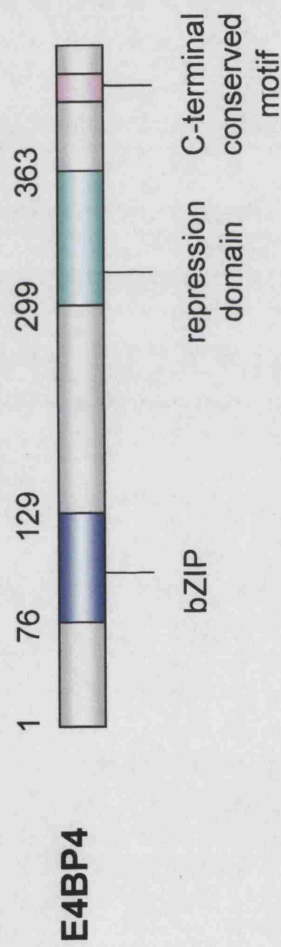


Figure 1.4. The structure of E4BP4.

E4BP4 is a 462 amino acid protein (~50 kD) that is related to the PAR ZIP family of transcription factors, but does not contain a PAR region. Mouse, rat and chicken E4BP4 share 90% sequence homology. E4BP4 is also similar within the bZIP to *C. elegans* CES-2 and *Drosophila* vrille. E4BP4 contains a repression domain and a C-terminal conserved motif that is also found in chicken and *Xenopus* E4BP4 homologues (Cowell, 2002).

E4BP4 expression requires the presence of IL-3 in murine IL-3-dependent pro-B cell lines, FL5.12 and Baf-3 (Ikushima *et al.*, 1997). Baf-3 and FL5.12 cells normally die by apoptosis in the absence of IL-3. Overexpression of E4BP4 in FL5.12 and Baf-3 cells was reported to confer enhanced survival following the withdrawal of IL-3 (Ikushima *et al.*, 1997; Kuribara *et al.*, 1999). However, overexpression of E4BP4 failed to rescue myeloid cells from p53-dependent apoptosis (Altura *et al.*, 1998). A recent study has shown that E4BP4 is involved in the regulation of motoneuron growth and survival (Junghans *et al.*, 2004). Overexpression of E4BP4 in motor neurons protects them against death induced by the removal of neurotrophic factors or activation of death receptors.

The Raf-mitogen-activated protein kinase and phosphatidylinositol-3 (PI-3) kinase pathways have also been proposed to regulate E4BP4 expression in Baf-3 cells expressing oncogenic Ras mutants and the human βc shared cytokine receptor chain (Kuribara *et al.*, 1999). The GATA-1 and GATA-2 transcription factors have been shown to be involved in the regulation of E4BP4 expression in response to IL-3 (Yu *et al.*, 2002). It has also been implied that the BCR-ABL fusion product can induce the expression of E4BP4 in Baf-3 cells (Kuribara *et al.*, 1999).

1.7. PAR bZIP proteins and E4BP4 in the circadian rhythm

E4BP4 and the PAR bZIP proteins have also been shown to be involved in the regulation of the circadian rhythm. Endogenous clocks drive the daily oscillations of physiological and behavioural processes. These circadian rhythms are regulated by the master circadian clock which resides in the SCN (Reppert and Weaver, 2001). The molecular basis of the regulation of the core clock mechanism involves interacting positive and negative transcriptional and translational feedback loops.

The rhythmic transcription of the three *Period* (*mPER*) genes and the two *Cryptochrome* (*mCRY*) genes and clock control genes (*CCGs*) in mice is driven by CLOCK and BMAL1 heterodimers which enhance transcription via E-box elements (Reppert and Weaver, 2001). *mPER* and *mCRY* proteins form complexes that translocate to the nucleus whereby they can inhibit the transcriptional activities of CLOCK/BMAL1 (**Figure 1.5**). *mPER2* also activates the transcription of *BMAL1* which is likely to promote the formation of CLOCK/BMAL1 heterodimers and restart the transcriptional cycles of *mPer* and *mCry*.

E4BP4 expression in the SCN, liver and pineal gland shows rhythmic patterns and oscillate opposite to that of DBP, TEF and HLF, i.e. when levels of E4BP4 expression are high, levels of DBP, TEF and HLF are lower and vice versa (Mitsui *et al.*, 2001; Doi *et al.*, 2001; Gachon *et al.*, 2004). E4BP4 is very closely related to the *Drosophila* gene, *vrille*, which is involved the regulation of the fly circadian rhythm. E4BP4 and the PAR bZIP proteins were shown to have antagonising effects on reporter constructs containing the *mPer2* promoter.

A proposed model of E4BP4 and PAR bZIP proteins in the transcriptional regulation of the circadian rhythm is illustrated in **Figure 1.5**. E4BP4 in the chicken pineal gland repressed chicken *Per2* expression and it has been proposed that E4BP4 is involved in synchronizing or adjusting the intrinsic circadian clock to the natural 24 hour cycle of light and dark (Doi *et al.*, 2001). E4BP4 antagonises the action of DBP, TEF and HLF in the circadian oscillatory mechanism in mice and they can therefore act as molecular switches which activate or repress clock control genes (Mitsui *et al.*, 2001). The deletion of all three PAR bZIP proteins does not affect the oscillatory expression of *BMAL1*, *Cry1* and *Per1* or any of the other core clock genes

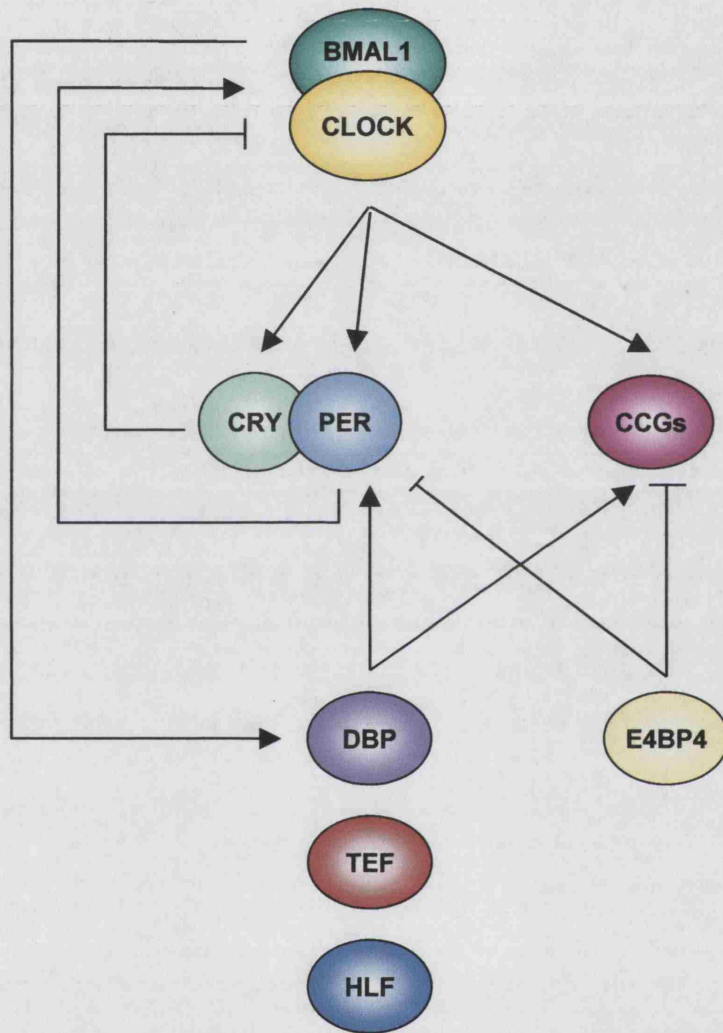


Figure 1.5. Transcriptional control of the mammalian circadian clock and the role of PAR bZIP proteins and E4BP4.

CLOCK/BMAL1 heterodimers regulate the rhythmic transcription of the *Per*, *Cry* and clock control genes (*CCGs*). PER and CRY proteins form complexes and translocate into the nucleus. CRY downregulates CLOCK/BMAL1 activity whilst PER can transactivate *BMAL1*. The PAR bZIP proteins and E4BP4 have opposite activities on the mouse *Per1* gene and *CCGs*. Adapted from Cowell, 2002.

which indicates that the PAR bZIP proteins are not required for the generation of circadian rhythm and therefore are involved in regulating the outputs of the mammalian timing system (Gachon *et al.*, 2004).

The *mPer2* gene has been implicated in tumour suppression. Homozygous *mPer2* mutant mice were prone to the development neoplastic growths, e.g. salivary gland hyperplasia, teratoma in male mice and lymphoma, with or without treatment with ionising radiation (Fu *et al.*, 2002).

1.8. E2A-HLF in childhood ALL

A study was carried out to identify new reoccurring chromosomal abnormalities in paediatric B-lineage ALL (Raimondi *et al.*, 1991). This study identified the t(17;19)(q22;p13) as a non-random translocation and estimated that approximately 1% of B-lineage ALLs carried this translocation. The t(17;19)(q22;p13) translocation results in the formation and expression of the E2A-HLF fusion protein (Hunger *et al.*, 1992; Inaba *et al.*, 1992). E2A-HLF retains the transactivation domains of E2A, but the bHLH dimerisation and DNA-binding domain of E2A is replaced by the bZIP domain of HLF (Figure 1.6).

1.8.1. Clinical aspects of children with t(17;19)⁺ ALL

Several ALL patients were found to have the t(17;19) translocation (Ohyashiki *et al.*, 1991; Yamada *et al.*, 1991; Raimondi *et al.*, 1991; Inaba *et al.*, 1992; Hunger *et al.*, 1992; Hunger *et al.*, 1994; Devaraj *et al.*, 1994; Takahashi *et al.*, 2001; Daheron *et al.*, 2002; Matsunaga *et al.*, 2004). The t(17;19) translocation is associated with disseminated intravascular coagulopathy (DIC), hypercalcaemia and bone lesions and is associated with a poor prognosis. DIC is an unusual finding in ALL and is

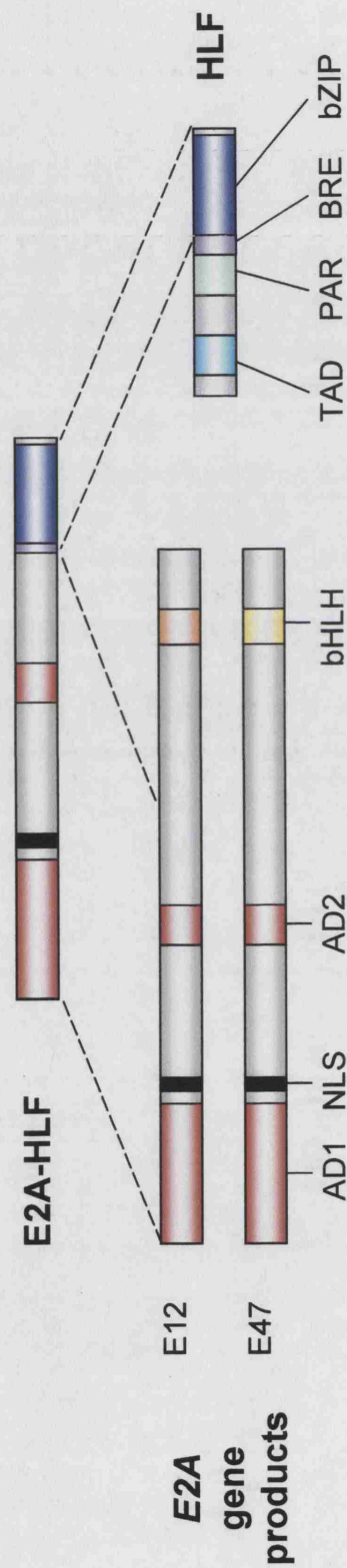


Figure 1.6. The structure of the E2A-HLF fusion.

The E2A-HLF chimaeric transcription factor is a protein of about 570 amino acids (~62 kD) and arises due to the t(17;19)(q22;p13) translocation in a percentage of childhood pro-B ALL. The E12 and E47 products of the E2A gene arise through differential splicing of the two adjacent HLF encoding exons. The t(17;19) translocation results in the replacement of the E2A bHLH domain with the bZIP domain from HLF. AD1, activation domain 1. AD2, activation domain 2. NLS, nuclear localisation signal. BRE, basic extended region.

more commonly associated with acute promyelocytic leukaemia (APL) which carries the t(15;17) translocation that generates the PML-RAR α fusion protein.

A summary of the published and unpublished cases of t(17;19) translocation in childhood ALL is shown in **Table 1.2**. The ages of the patients ranged between 2 to 17 years and almost all have died as a result of their leukaemia. Many but not all patients with the t(17;19) translocation have the E2A-HLF fusion product. These translocations may involve genes other than *E2A* and *HLF*. A subset of patients with t(17;19) translocation presented with DIC at the time of diagnosis and some with hypercalcaemia. The cause of DIC in t(17;19)-associated ALL is not known, but E2A-HLF was shown to transactivate reporter constructs containing the Factor VIII and IX coagulation factor promoters in the E2A-HLF-expressing UOC-B1 leukaemic cell line (Begbie *et al.*, 1999). The overexpression of Annexin II has also been suggested to contribute to the hypercalcaemia associated with t(17;19) translocation in childhood ALL (Matsunaga *et al.*, 2004).

The t(17;19)⁺ patients also differ on whether they have additional genetic alterations. Chromosomal analysis of leukaemic cells from some patients show the presence of only the t(17;19) translocation, whilst other patients have additional chromosomal changes. Other genetic changes have been documented in patients with the t(17;19) translocation. Using a *p16* exon 2 probe, two out of four t(17;19)⁺ leukaemic cell lines were found to have homozygous deletions of both the *p16* and *p15* genes (Maloney *et al.*, 1998). Hypermethylation of the *p15* promoter was found in one of the remaining two patients that did not have homozygous deletions of *p16* and *p15*. The deletion of exon 2 of the *p16* gene results in the inactivation of both the p16 and p19^{ARF} proteins and may therefore result in alterations to the

Table 1.2. Summary of childhood ALL patients with t(17;19) translocation.

	Age (y)	Sex	E2A- HLF type	Clinical information and Notes	Cell line generated	References
1	17	F	I	Complete remission not achieved. Died 10 weeks after admission. Homozygous deletion of <i>p15</i> and <i>p16</i> detected.	HAL-01	Ohyashiki <i>et al.</i> , 1991; Hunger <i>et al.</i> , 1992; Maloney <i>et al.</i> , 1998.
2	16.9	M	I	DIC on presentation. Died in clinical remission.		Raimondi <i>et al.</i> , 1991; Inaba <i>et al.</i> , 1992.
3	15.5	F	I	DIC on presentation. Clinical remission achieved. Homozygous deletion of <i>p15</i> and <i>p16</i> detected.	UOC-B1	Raimondi <i>et al.</i> , 1991; Inaba <i>et al.</i> , 1992; Maloney <i>et al.</i> , 1998.
4	2.2	M	N/A	Complete clinical remission and off therapy. Translocation possibly does not involve <i>E2A</i> or <i>HLF</i> .		Raimondi <i>et al.</i> , 1991.
5	11.1	M	N/A	Died in induction. ALL undetectable by immunophenotype.		Raimondi <i>et al.</i> , 1991.
6	-	-	II	Hypermethylation of <i>p15</i> promoter.		Hunger <i>et al.</i> , 1994; Maloney <i>et al.</i> , 1998.
7	-	-	II	No additional information		Hunger <i>et al.</i> , 1994.
8	17	F	II	Achieved first complete remission. Received BMT 13 months after diagnosis. Suffered two bone marrow relapses. Died 56 months from diagnosis.		Yamada <i>et al.</i> , 1991; Devaraj <i>et al.</i> , 1994.
9	11	M	II	Hypercalcaemia and bone lesions at presentation. Recurrence of bone pain and hypercalcaemia at 5 months post-induction therapy. Died 8.5 months after diagnosis.		Devaraj <i>et al.</i> , 1994.
10	13	M	I and II	Relapsed during maintenance treatment. Received BMT. Relapsed 4 months post-BMT. Died 3 weeks later.		Devaraj <i>et al.</i> , 1994.
11	4	M	I and II	Complete remission achieved. Relapsed 5 months later. Hypercalcaemia at diagnosis.	YCUB-2	Takahishi <i>et al.</i> , 2000.
12	12	F	I	DIC on presentation. Achieved clinical remission. Relapsed after 5 months. Died at 8 months after diagnosis.		Dahéron <i>et al.</i> , 2002.
13	14	F	II	Hypercalcaemia present. Died after 8 months.	Endo-kun	Matsunaga <i>et al.</i> , 2004.
14	12	F	II	Hypercalcaemia present. Died after 9 months.		Matsunaga <i>et al.</i> , 2004.
15	5.5	F	II	Died during relapse at 18 months after diagnosis. DIC during relapse.		This study.
16	8	F	II	Died during relapse.		This study.

-, not available. y, years. M, male. F, female. N/A, not applicable. DIC, disseminated intravascular coagulopathy; BMT, bone marrow transplant.

p53/MDM2/ARF, and pRB/cyclin D/p16 pathways which are altered in numerous tumours (Weinberg, 1995; Pomerantz *et al.*, 1998; Zhang *et al.*, 1998).

1.8.2. Different rearrangements generate the E2A-HLF fusion

The E2A-HLF fusion can arise from two different genomic rearrangements (Hunger *et al.*, 1994). Type I rearrangements involve splicing of the *E2A* exon 13 and *E2A* intronic sequences to exon 4 of *HLF* (Figure 1.7). Non-template nucleotides, i.e. nucleotides that are not encoded by chromosomal DNA but are added *de novo*, are inserted, possibly by TdT, to maintain the translational reading frame (Hunger *et al.*, 1994). Type II rearrangements involve direct splicing of exon 12 of *E2A* to exon 4 of *HLF*. *E2A* exon 12 and *HLF* exon 4 are in the same translational reading frame. In summary, these two rearrangements generate fusion proteins containing the same portion of *HLF*, but differ on whether or not *E2A* exon 13 and a cryptic exon are included. Despite these structural differences, the fusion proteins generated by the two types of rearrangements result in identical DNA-binding and transcriptional regulatory activity (Hunger *et al.*, 1994).

1.8.3. Other E2A fusion partners

The *E2A* gene is more commonly involved in the t(1;19) translocation with the *PBX1* gene. E2A-PBX1 is found predominantly in childhood pre-B cell ALL and contains the same portion of *E2A* (Kamps *et al.*, 1990; Nourse *et al.*, 1990). E2A-PBX1 is found in approximately 5% of pre-B cell ALL. A novel fusion partner of *E2A*, *FBI*, has been documented in four cases of pre-B ALL (Brambillasca *et al.*, 1999).

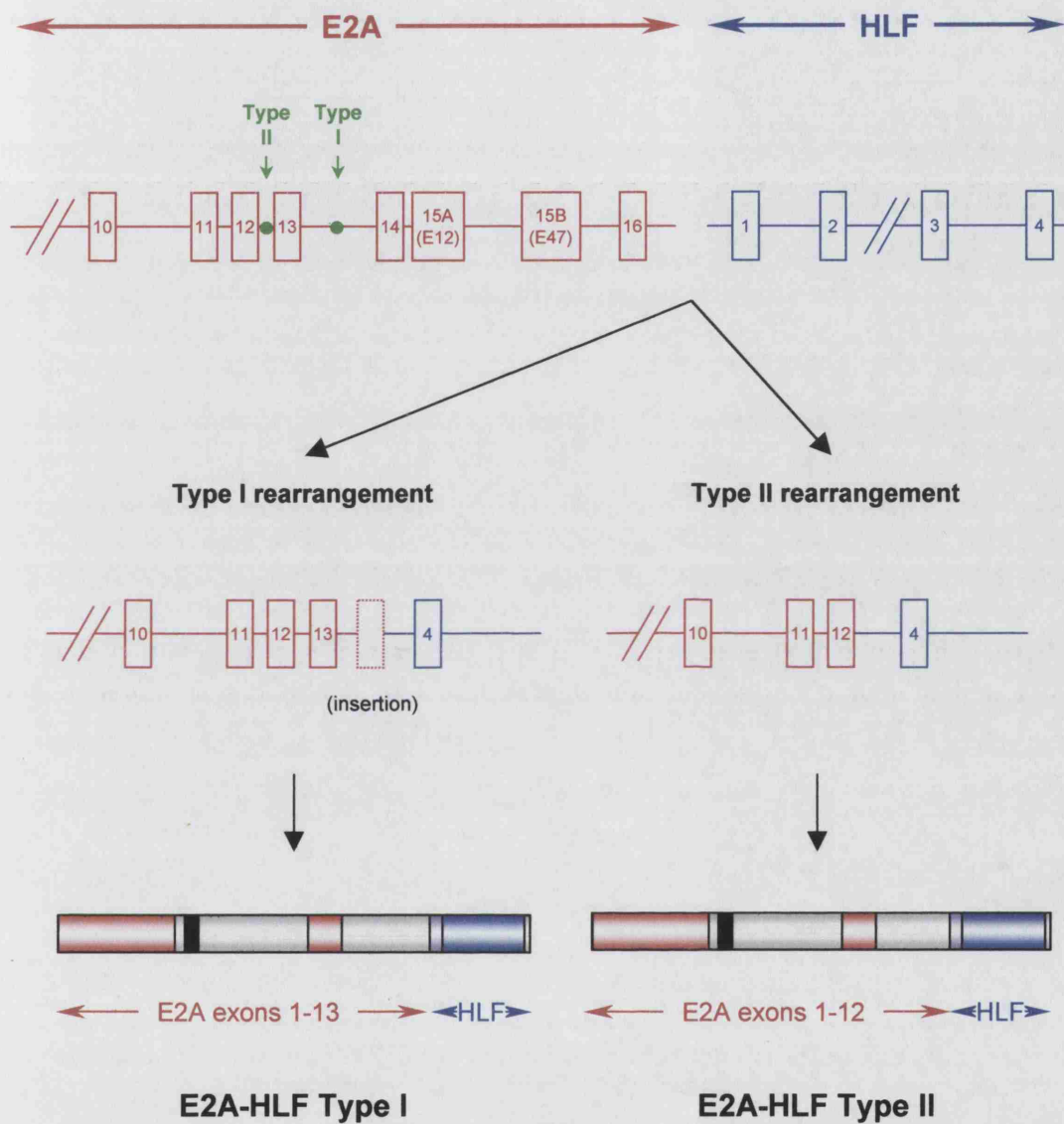


Figure 1.7. Two types of genomic rearrangement generate E2A-HLF.

A schematic representation of the genomic structure of the E2A and HLF genes is shown. Open boxes indicate exons. Green circles indicate positions of the breakpoints within exon 12 or intron 13 of the E2A gene which generate the two different types of rearrangement when fused to exon 4 of HLF. E12 and E47 bHLH domains are encoded by exons 15A and 15B respectively. Adapted from Hunger, 1996.

1.9. Properties of E2A-HLF fusion protein

The functions of E2A-HLF have been reviewed recently (Seidel and Look, 2001). E2A-HLF has been shown to be a transcriptional activator and transforms NIH 3T3 embryonic mouse fibroblasts in soft agar which develop into tumours in nude mice (Hunger *et al.*, 1994; Inaba *et al.*, 1994; Yoshihara *et al.*, 1995; Inukai *et al.*, 1997; Inukai *et al.*, 1998; Begbie *et al.*, 1999). Expression of this chimaeric transcription factor delays apoptosis following IL-3 deprivation of Baf-3 and FL5.12 cells and confers a greater protection against IL-3 withdrawal-induced apoptosis than that achieved by overexpression of E4BP4 (Inaba *et al.*, 1996; Ikushima *et al.*, 1997; Inukai *et al.*, 1998; Dang *et al.*, 2001).

As E2A-HLF and E4BP4 can recognise the same consensus sites, a model was proposed to explain how E2A-HLF could act as an anti-apoptotic factor in human ALL (**Figure 1.8**) (Ikushima *et al.*, 1997). IL-3 tightly controls the regulation of E4BP4 expression. E4BP4 transactivates its target genes via the consensus site and its target genes are involved in transducing critical survival signals. In ALL cells that carry the t(17;19) translocation, E2A-HLF is constitutively expressed and replaces the function of E4BP4 by the ability of E2A-HLF to recognise the same consensus sites and activate the same target genes.

E2A-HLF has been used in retroviral transduction studies and has been shown to immortalise myeloid progenitor cells (Ayton and Cleary, 2003; Smith *et al.*, 2003). In these studies, E2A-HLF was unable to immortalise lymphoid cells by itself. Co-expression of BCL-2 and E2A-HLF was required to immortalise lymphoid cells which were leukaemogenic *in vivo* after acquiring further mutations which resulted in stroma- and IL-7-independent growth (Smith *et al.*, 2002).

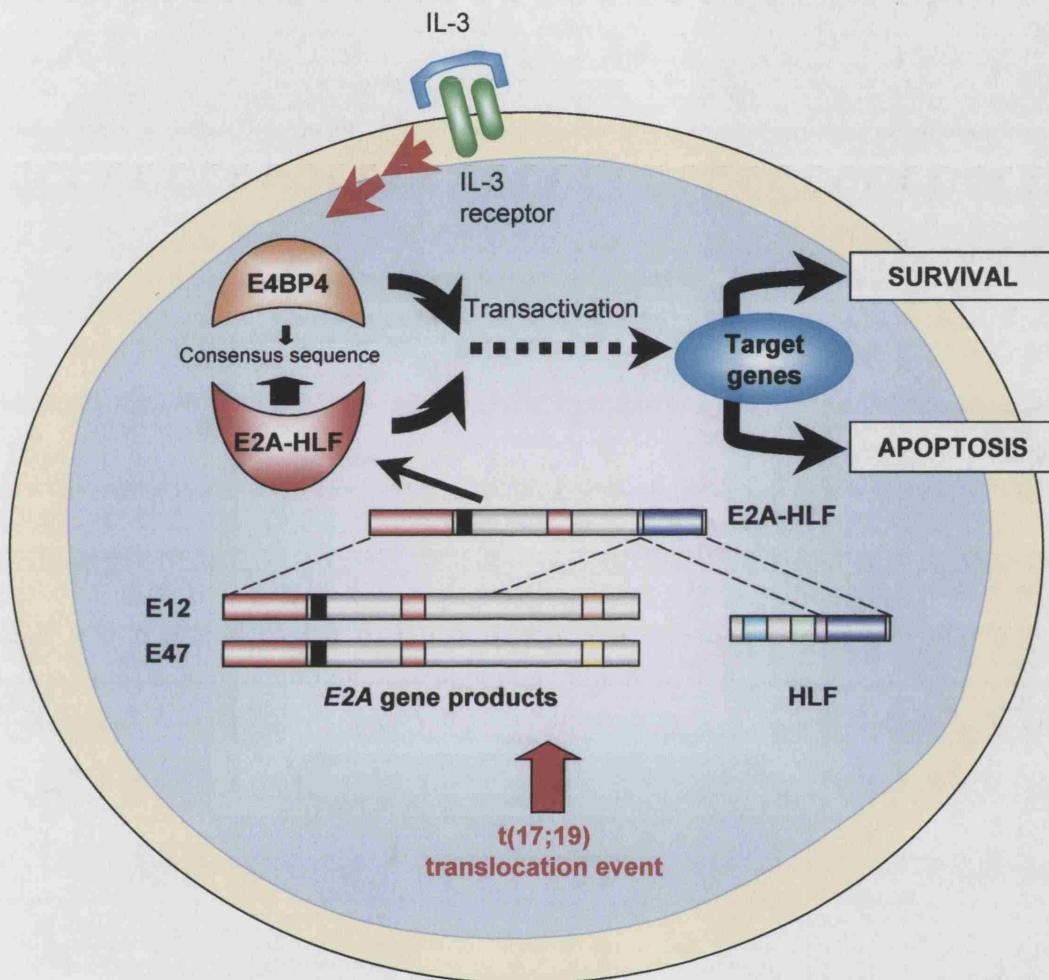


Figure 1.8. Model of E4BP4 function in cell survival pathways and a proposed mechanism of action of the E2A-HLF oncoprotein.

Ikushima *et al* (1997) showed that overexpression of E4BP4 delayed apoptosis in FL5.12 cells after IL-3 withdrawal. A model was proposed whereby E4BP4 and E2A-HLF recognised the same consensus site to modulate survival genes that are normally regulated by IL-3. The constitutive expression of E2A-HLF in ALL can replace the function of E4BP4 and mediate cell survival independently of IL-3 status.

The t(17;19) translocation in ALL also results in the loss of a functional allele of E2A. Homozygous *E2A* knockout mice are highly susceptible to the development of T-cell lymphomas (Bain *et al.*, 1997; Yan *et al.*, 1997). One of the heterozygous mice also developed T cell leukaemia. These data suggest that loss of a functional *E2A* allele in cells that harbour the t(17;19) translocation may contribute to leukaemogenesis.

E2A-HLF transgenic mice have been generated to determine its role *in vivo*. E2A-HLF cDNA derived from two patients were driven by the E μ enhancer and SV40 early promoter or the Ig enhancer and promoter (Smith *et al.*, 1999; Honda *et al.*, 1999). The phenotype of the transgenic E2A-HLF mice are summarised in **Table 1.3**. Transgenic mice which expressed E2A-HLF driven by the Ig promoter developed thymic hypoplasia, had increased levels of T-cell apoptosis, showed a block in B-cell maturation and developed T-cell ALL at 3 to 6 months of age (Honda *et al.*, 1999). Transgenic mice that expressed E2A-HLF driven by the E μ enhancer developed thymic hypoplasia, T-cell maturation arrest and increased T-cell apoptosis and developed T lineage, or more rarely, B lineage lymphomas after 10 months (Smith *et al.*, 1999).

Table 1.3. Phenotype of transgenic E2A-HLF mice.

	Smith <i>et al.</i> , 1999.	Honda <i>et al.</i> , 1999.
Transgene	E2A-HLF (HAL-01) driven by E μ SV40 promoter.	E2A-HLF (UOC-B1) driven by IgH enhancer/promoter.
Mouse strain	FVB.	C57BL/6 \times DBA/2.
E2A-HLF expression	High in thymus. Low in spleen. Low in bone marrow. Low levels in B cells.	High in thymus. Absent in spleen. Very low levels in B cells.
Thymus	Smaller thymuses than controls.	Smaller thymuses than controls.
Spleen	Normal B cells numbers. Reduced T cell numbers.	Enlarged spleens. Decreased lymphocyte numbers in spleen.
B cells	B cell subsets similar to controls.	Reduced numbers of CD43 ⁺ /B220 ⁺ early progenitors. Reduced numbers of CD43 ⁺ /B220 ⁺ late progenitors.
T cells	Normal CD4/CD8 populations at 4 weeks. Progressive loss of CD4 ⁺ /CD8 ⁺ DP and CD4 ⁺ SP, CD8 ⁺ SP cells after 10 weeks. Expansion of T cells with a progenitor-like CD3 ⁺ /CD4 ^{lo} /CD8 ⁺ phenotype expressing high E2A-HLF levels. Mature SP splenic T cells do not express E2A-HLF.	Normal CD4/CD8 subsets.
Other features		Increased susceptibility to infections due to reduced numbers of lymphocytes.
Malignancies	22/36 mice develop malignancies. Mean latency = 10 months. 19 mice T-lineage lymphoma. 3 mice B-lineage lymphoma.	5/26 mice develop malignancies. Mean latency = 6 months. Acute T cell leukaemia.

SP, single positive; DP, double positive.

1.10. Previously identified targets of E2A-HLF

To elucidate the role of E2A-HLF in leukaemogenesis, studies have been carried out to identify its downstream targets. Several downstream targets of E2A-HLF have been identified using cDNA representational difference analysis (RDA) and include *Annexin VIII*, *SRPUL*, *Slug*, *Groucho*-related genes and *Runx1/AML-1* and *Annexin II* (Kurosawa *et al.*, 1999; Inukai *et al.*, 1999; Dang *et al.*, 2001; Matsunaga *et al.*, 2004). *Annexin VIII*, *SRPUL* and *Slug* were identified as E2A-HLF targets in the E2A-HLF-expressing UOC-B1 leukaemic cell line induced to express a dominant

negative form of E2A-HLF that lacks transactivational activity. Of the previously identified targets of E2A-HLF, only overexpression of Slug, a mammalian homologue of CES-1, confers some resistance to IL-3 withdrawal-induced apoptosis in Baf-3 cells (Kurosawa *et al.*, 1999). However it is not expressed in all leukaemia cell lines that express E2A-HLF (Inukai *et al.*, 1999). The combined effects of the loss of one functional allele of *E2A* and the abnormal expression of genes in addition to other genetic alterations in B cell progenitors are likely to contribute to the development of the leukaemia (Yan *et al.*, 1997; Seidel and Look, 2001).

1.11. Transcriptional profiling of childhood leukaemias

Chromosomal aberrations and other genetic events that are associated with subtypes of leukaemia will result in changes in the expression of many different genes. The changes in the global expression of genes has been determined by the use of microarray technology. Several large scale gene expression profiling studies have been carried using samples from patients with childhood leukaemia (Armstrong *et al.*, 2002; Ferrando *et al.*, 2002; Yeoh *et al.*, 2002). The aim of these studies was to correlate gene expression profiles of leukaemias with the presence of a particular genetic lesion.

These studies showed that the different chromosome translocations and imbalances generated distinct genetic signatures involving large numbers of genes. Infant leukaemias which had the mixed lineage leukaemia (*MLL*) gene rearrangements had distinct expression profiles (Armstrong *et al.*, 2002; Ferrando *et al.*, 2002).

Transcriptional profiling of cells from childhood leukaemias with rearranged *MLL* show deregulation of *Hox* gene expression, suggesting that deregulation of *Hox*

genes may have a role in the leukaemic transformation induced by *MLL* oncogenes (Ferrando *et al.*, 2002).

The expression profiles of leukaemias can be used to classify, determine subtypes and predict the outcome of the leukaemia and also help determine the molecular mechanism by which the genetic lesion causes leukaemia. Data from large scale gene expression profiling can aid the accurate diagnosis of patients and help identify those who may be more at risk from relapse, those who require less aggressive therapy and those who require more aggressive therapy (Armstrong *et al.*, 2002; Yeoh *et al.*, 2002). The characteristic gene expression profiles for the different chromosomal abnormalities can provide information regarding the underlying biology of the leukaemia and identification of molecular targets for therapeutic intervention.

No gene expression profiling studies have been carried out using ALL patient samples bearing the t(17;19) translocation due to the rarity of this translocation and lack of available patient material. The benefit of using an *in vitro* model for the purpose of determining the gene expression profile caused by the expression of a specific genetic lesion, e.g. E2A-HLF, overcomes the problems associated with lack of sample material and will also allow the identification of genes involved in the very early stages when the oncoprotein is first expressed before the acquisition of other genetic lesions.

1.12. Strategy for the identification of E2A-HLF target genes

An *in vitro* inducible system can be used to model the *in vivo* events that follow the t(17;19) translocation and subsequent expression of the E2A-HLF oncoprotein. The

primary events that follow the expression of the oncoprotein can be determined as the level and temporal expression of the gene of interest can be tightly controlled.

Establishment of the Tet-On inducible system in Baf-3 cells would allow the identification of genes that are regulated by E2A-HLF and also determine whether the expression of these genes changes during the inhibition of apoptosis by E2A-HLF after IL-3 withdrawal. Baf-3 cells have the same immunophenotype as the leukaemic cells found in human ALL which carry the t(17;19) translocation. The differential expression of thousands of genes caused by E2A-HLF expression can then be determined using microarray technology.

1.13. Project Aims

The generation of *in vitro* inducible system to express E2A-HLF combined with the use of DNA microarrays will allow the identification of genes that are important in initial stages of E2A-HLF-induced leukaemia in humans and may identify novel targets for novel therapies.

CHAPTER 2

MATERIALS AND METHODS

Chemicals were purchased from VWR, Poole, Dorset, UK unless otherwise stated.

All tissue culture reagents were from Invitrogen, Paisley, UK unless otherwise

stated. Restriction enzymes were purchased from Promega, Madison, Wisconsin,

USA. Oligonucleotides were purchased from Sigma Genosys, Haverhill, UK. All

recombinant cytokines were purchased from Peprotech EC, London, UK.

2.1. Buffers and Solutions

Table 2.1. Buffers and solutions

Buffer or solution	Final concentration of components
FACS staining buffer	Phosphate buffered saline (PBS) (Invitrogen) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide.
Hypotonic buffer	0.1 % sodium citrate, 0.1 % Triton® X-100 (Sigma).
Luria-Bertani (LB)-agar	LB broth medium plus 15 g/l agar.
LB broth	1.0% tryptone, 0.5% yeast extract, 1.0% NaCl (pH 7.0).
NP40 lysis buffer	150 mM NaCl, 1.0 % Nonidet® P 40 (NP40), 50 mM Tris-HCl (pH 8.0). One Complete Mini protease inhibitor cocktail tablet (Roche, Lewes, UK) was dissolved per 10 ml lysis buffer.
Protein transfer buffer	12 mM Tris-HCl (pH 8.3), 96 mM glycine, 20% methanol.
Psi broth	2% tryptone, 0.5% yeast extract, 0.4% MgSO ₄ , 10 mM KCl, adjust pH to 7.6 with 1 M KOH.
Red cell lysis buffer	17 mM Tris-HCl (pH 7.2), 0.144 M NH ₄ Cl.
5× Sample Buffer	250 mM Tris-HCl (pH 6.8), 10% SDS, 2.5% bromophenol blue, 50% glycerol (Sigma). Dithiothreitol (DTT) was added to protein samples to final concentration of 100 mM.
SDS-PAGE electrophoresis buffer	25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS.
TBS	20 mM Tris-HCl (pH 7.5), 150 mM NaCl.
TBS-T	20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (Sigma).
TFBI	100 mM RbCl ₂ , 50 mM MnCl ₂ , 30 mM KOAc, 10 mM CaCl ₂ , 15% glycerol. Adjusted to pH 5.8 with 0.2 M AcOH.
TFBII	10 mM 3-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPS), 10 mM RbCl ₂ , 75 mM CaCl ₂ , 15% glycerol. Adjusted to pH 7.0 with NaOH.
TAE buffer	40 mM Tris-acetate and 1 mM EDTA at pH 8.3.

2.2. Cell Culture

Cells were maintained at 37 °C with 5% CO₂ in a humidified atmosphere.

2.2.1. *TonBaF.1*

TonBaF.1 cells, derived from the stable transfection of Baf-3 cells with the reverse tetracycline transactivator (rtTA) were kindly provided by Dr George Daley (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts).

TonBaF.1 cells were maintained in RPMI 1640 containing 10% foetal calf serum (FCS) (Globepharm, Surrey, UK), 10 % WEHI-3B conditioned medium (as a source of IL-3), 0.5 mg/ml geneticin, 100 units/ml (U/ml) penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.2.2. *WEHI-3B*

WEHI-3B cells were maintained in DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. WEHI-3B cells grow as a mixture of suspension and adherent cells. To split confluent cells, suspension cells were harvested and adherent cells were trypsinised with 1× trypsin-EDTA solution (Sigma). Suspension and adherent cells were pooled and used to seed fresh medium.

Conditioned medium from WEHI-3B cells was used as a source of IL-3. WEHI-3B cells were cultured until confluent (approximately 3 to 4 days). Cells were removed by centrifugation and the conditioned medium was sterile filtered using 0.22 µm filter (Millipore, Watford, UK) and stored at -20 °C.

2.2.3. *HeLa*

HeLa cells were cultured in DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine.

2.2.4. *CTLL-2*

CTLL-2 cells were cultured in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol (2-ME) and conditioned medium from cells stably transfected with an Interleukin 2 (IL-2)-expressing construct (a kind gift from Dr Owen Williams, Molecular Haematology and Cancer Biology Unit, Institute of Child Health, London Institute of Child Health, London).

2.2.5. *LinXE*

LinXE cells, an ecotropic retrovirus packaging cell line, were cultured in DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 75 µg/ml of hygromycin B (Calbiochem, Nottingham, UK).

2.2.6. *Jurkat*

Jurkat cells were maintained in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.2.7. *HAL-01*

HAL-01 cells were maintained in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.3. General molecular biology techniques

Standard protocols, buffers and solutions were from Sambrook *et al.*, 1989.

2.3.1. Small-scale plasmid preparation (Miniprep)

5 ml of LB broth with appropriate antibiotics was inoculated with a bacterial colony and cultured overnight at 37 °C with vigorous shaking. Plasmid DNA was extracted from 1.5 ml of bacterial culture using QIAprep Spin Miniprep kit (Qiagen, Crawley, UK) according to manufacturer's instructions. DNA was eluted in 50 µl of sterile double distilled water (dd H₂O) and stored at -20 °C.

2.3.2. Large-scale plasmid preparation (Maxiprep)

5 ml of LB broth with appropriate antibiotics was inoculated with a bacterial colony and cultured for 6 hours (h). 200 ml of LB broth with appropriate antibiotic was inoculated with 200 µl of the starter culture and cultured overnight at 37 °C with vigorous shaking. Plasmid DNA was extracted from 200 ml of bacterial culture using a Plasmid Maxi kit (Qiagen) according to manufacturer's instructions. DNA was eluted in 500 µl of dd H₂O and stored at -20 °C.

2.3.3. Preparation of chemically competent *Escherichia coli*

5 ml of Psi broth (**Table 2.1**) was inoculated with an *E. coli* colony and cultured overnight at 37 °C with shaking. 100 ml of pre-warmed (to 37 °C) Psi broth was inoculated with 5 ml of starter culture and cultured at 37 °C for approximately 1 h with vigorous shaking until an absorbance reading of approximately 0.48 at 550 nm was reached. Cells were transferred to pre-chilled 50 ml tubes and incubated on ice for 10 minutes (min). Cells were then centrifuged at 2500 rpm for 10 min at 4 °C. Cells were resuspended in 30 ml of cold TFBI buffer (**Table 2.1**) and incubated on

ice for 20 min. Cells were then centrifuged at 2500 rpm for 10 min at 4 °C. Cells were then resuspended in 4 ml of ice-cold TBFII buffer (**Table 2.1**). 200 µl aliquots were dispensed into 1.5 ml tubes in a dry ice/ethanol bath and stored at -80 °C.

2.3.4. Transformation of chemically competent *E. coli*

10 ng of plasmid DNA or 2 µl of a ligation reaction was gently mixed with 100 µl competent *E. coli* and incubated on ice for 30 min. Cells were heat shocked at 42 °C for 60 sec and placed immediately on ice for 2 min. 400 µl room temperature LB broth was added to the cells and incubated at 37 °C for 1 h with vigorous shaking. 100 µl to 200 µl of bacterial culture was plated on to LB-agar plates (**Table 2.1**) with appropriate antibiotics and incubated at 37 °C overnight.

2.3.5. Isolation of total RNA

Total RNA was extracted using TRIzol[®] Reagent (Invitrogen) following the recommended manufacturer's procedure. Briefly, pelleted cells were lysed in 1 ml TRIzol[®] per 10⁷ cells and left at room temperature for 10 min. 0.2 ml of chloroform (per 1 ml of TRIzol[®] used) was added to the sample, mixed and then centrifuged at 12,000 ×g for 15 min at 4 °C. The upper layer was transferred to a fresh tube and 0.5 ml of isopropanol (per 1 ml of TRIzol[®] used) and 1 µl of glycogen (Roche) added. RNA was precipitated at room temperature for 10 min. RNA was pelleted at 12,000 ×g for 10 min at 4 °C. RNA pellets were washed in 1 ml 75% ethanol and centrifuged at 7,500 ×g for 5 min at 4 °C. RNA pellets were air-dried before being dissolved in diethyl pyrocarbonate (DEPC)-treated dd H₂O and stored at -80 °C until used.

2.3.6. Transfection of cells with Lipofectamine™

Transfections were carried out using Lipofectamine™ (Invitrogen) according to manufacturer's instructions. Optimum amount of DNA, volume of Lipofectamine™ and cell density for transfections were established for each cell line in preliminary experiments.

For example, cells were seeded at the optimum density for transfection a day prior to transfection in 10 cm² dishes. The optimum amount of DNA was resuspended in 800 µl of OptiMEM™ medium (Invitrogen) and the optimum volume of Lipofectamine™ was made up to 800 µl with OptiMEM™. Lipofectamine™ solution was added drop-wise to the DNA solution and gently mixed. The DNA/Lipofectamine™ mix was then incubated at room temperature for 45 min. Cells for transfection were washed with 10 ml of OptiMEM™. 7.4 ml of OptiMEM™ was added to the DNA/Lipofectamine™ mix and transferred on to the cells. Cells were incubated at 37 °C/5% CO₂ for 5 h before a further 10 ml of complete medium containing 20% FCS was gently added. Medium was changed after 24 h.

2.3.7. Polymerase Chain Reaction (PCR)

PCR was used to incorporate a 5' epitope tag or specific flanking restriction enzyme sites to the cDNAs of interest prior to cloning into appropriate vectors. PCR was carried out in a 50 µl reaction volume containing 10 ng template DNA, 1× Pfx Amplification buffer (Invitrogen), 0.3 mM deoxynucleotide triphosphate (dNTP) mix (Promega), 1 mM MgSO₄, 0.3 µM of each primer and 2.5 U Platinum® Pfx DNA polymerase (Invitrogen). Typical cycling conditions were 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 2 min followed by final extension at 68 °C for 5 min.

For PCR using 5' epitope tagging primers, two step cycling conditions were used which consisted of 96 °C for 2 min, 10 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 68 °C for 2 min followed by 20 cycles of 94 °C for 30 sec, 65 °C for 30 sec, 68 °C for 2 min and a final extension at 68 °C for 5 min.

PCR products were electrophoresed in 1% agarose gel in TAE (Tris-acetate EDTA) buffer (**Table 2.1**) containing 0.5 µg/ml ethidium bromide and visualised using an ultraviolet (UV) transilluminator.

2.3.8. DNA sequencing

DNA sequencing was carried out by the DNA Sequencing Service, Wolfson Institute of Biomedical Research, University College London, UK.

2.4. Detection of E2A-HLF and actin transcripts in patient and normal cells by reverse transcriptase-PCR (RT-PCR)

100 ng of total RNA was reverse transcribed using an oligo-dT primer and SuperScript II™ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Control tubes contained all of the reagents for cDNA synthesis except RT. 2 µl of cDNA was used in 50 µl PCR reactions containing 1× Reaction buffer (Promega), 0.2 mM dNTP mix, 1.5 mM MgCl₂, 1 µM of each primer and 1.25 U *Taq* DNA polymerase (Promega).

E2A-HLF was amplified using the forward primer 19F (5'-TCGCCCAGCTACGACGGGGGTCTC-3') and reverse primer 17B (5'-GAGGCCCGGATGGCGATCTG-3') (Devaraj *et al.*, 1994). Cycling conditions for were 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 66 °C for 1 min, 72 °C for 2 min and final extension step of 72 °C for 5 min (Devaraj *et al.*, 1994).

PCR amplification of actin was used as a control for RNA integrity. Actin was amplified using the forward primer 5'-AACCGACTGCTGTACCTTCAC-3' and reverse primer 5'-GGCATCCACGAACTACCTTCAAC-3'. Cycling conditions were 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 30 sec and a final extension step of 72 °C for 5 min.

RT-PCR products were electrophoresed in 2% agarose gel in TAE buffer containing 0.5 µg/ml ethidium bromide and visualised using a UV transilluminator. RT-PCR products were excised from the gel and recovered using a QIAquick gel extraction kit (Qiagen). E2A-HLF RT-PCR products were cloned into the pGEM[®]-T-Easy vector (Promega) and subsequently sequenced.

2.5. Generation of tetracycline (tet)-inducible Baf-3 cell lines

2.5.1. Tet-inducible plasmid constructs

E2A-HLF cDNA (kindly provided by Dr Thomas Look, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts) was digested with *Bam*HI and *Xba*I restriction enzymes and ligated into the *Bam*HI and *Nhe*I sites of the pTRE2hyg vector (BD Clontech, Oxford, UK) to give pTRE2hyg-E2A-HLF.

Human E4BP4 cDNA (kindly donated by Dr Helen Hurst, Cancer Research UK, Hammersmith Hospital, London, UK) was digested with *Bam*HI and *Sal*II restriction enzymes and ligated into the *Bam*HI and *Xho*I sites of the pTRE2hyg vector to generate pTRE2hyg-E4BP4.

5×10^6 TonBaF.1 cells were electroporated in 250 µl of RPMI 1640 containing either 20 µg of pTRE2hyg, or pTRE2hyg-E2A-HLF, or pTRE2hyg-E4BP4 at 260 V/950 µF using the Gene Pulser[®] II electroporation system (BioRad, Hemel

Hempstead, UK). Cells were allowed to recover for 24 h at 37 °C/5% CO₂ prior to selection in medium containing 0.8 mg/ml hygromycin B for two weeks. Single cell clones were isolated, expanded and maintained in medium containing 0.4 mg/ml hygromycin B. Western blotting was used to screen for inducible protein expression after cells were cultured presence or absence of 2 µg/ml doxycycline for 24 h.

2.5.2. Cloning of tet-inducible cells by fluorescence activated cell sorting (FACS)

Single cell clones were generated from of tet-inducible mixed populations after FACS sorting. Mixed populations of inducible cells were centrifuged and resuspended at 0.5×10^6 /ml in medium containing 2% FCS (RPMI 1640 containing 10% WEHI-3B conditioned medium). The Autoclone[®] sorting option on the EPICS[®] ALTRA[™] FACS sorter (Beckman Coulter, High Wycombe, UK) was used to dispense a single live cell into each well of a 96-well flat-bottomed plate containing 100 µl complete medium. Cells were subsequently propagated and expanded.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cells were washed twice in cold PBS and lysed in NP40 lysis buffer (Table 2.1) containing protease inhibitors (Roche). Cell lysates containing 10 µg protein were resolved by electrophoresis in 10% SDS-PAGE gels. SDS-PAGE was carried out using Mini Protean III apparatus (BioRad) according to manufacturer's instructions. 10% resolving gels contained 10% acrylamide (National Diagnostics, Atlanta, Georgia, USA), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate (Sigma) and 0.4% N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma). Stacking gels contained 5% acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1%

SDS, 0.1% ammonium persulphate and 0.1% TEMED. Gels were electrophoresed in SDS-PAGE electrophoresis buffer (**Table 2.1**) at 80 V.

2.7. Protein transfer to polyvinylidene fluoride membrane (PVDF) membrane

Proteins in SDS-PAGE gels were transferred on to PVDF membrane (Millipore) in protein transfer buffer (**Table 2.1**) using a BioRad Mini Trans-Blot Electrophoretic Transfer system according to the manufacturer's instructions. Gels were transferred for 1 h 100 V.

2.8. Western blotting analysis

All antibodies were diluted in 5% milk powder in TBS-T (**Table 2.1**). Working dilutions of antibodies used for Western blotting analysis are given in **Table 2.2**. Membranes were incubated for 1 h at room temperature with gentle agitation in 5% milk powder in TBS-T. Membranes were subsequently incubated with primary antibody for 1 h at room temperature with gentle agitation. Membranes were washed in TBS-T for three 10 min washes. Membranes were subsequently incubated for 1 h with the relevant secondary antibody. Membranes were washed as previously described followed by a final 5 min wash with TBS buffer (**Table 2.1**). Proteins were visualised by ECL™ reagent (Amersham Biosciences, Little Chalfont, UK) and quantified using BioRad GS-800 densitometer and software.

Table 2.2. Antibodies used for Western blotting analysis.

Antibody	Manufacturer	Isotype	Working dilution
Mouse anti-E2A (Yae)	Santa Cruz Biotechnology, California, USA	IgG ₁	1:200
Goat anti-E4BP4 (v-19)	Santa Cruz Biotechnology	IgG	1:400
Rabbit anti-HLF (Ab-2)	Oncogene, Nottingham, UK	IgG	1:400
Rabbit anti-E4BP4	A gift from Dr Toshiya Inaba, Hiroshima University, Japan	IgG	1:500
Rat anti- α -tubulin (YL1/2)	Serotec, Oxford, UK	IgG _{2a}	1:2000
Donkey anti-mouse-HRP conjugate	Santa Cruz Biotechnology	IgG	1:2000
Goat anti-rat-HRP conjugate	Santa Cruz Biotechnology	IgG	1:2000
Donkey anti-rabbit-HRP conjugate	Santa Cruz Biotechnology	IgG	1:2000

2.9. Northern blotting analysis

10 μ g of total RNA or 2 μ g of poly A⁺ RNA, prepared using Oligotex mRNA Midi kit (Qiagen) following manufacturer's instructions, was electrophoresed in a 1.2 % agarose/formaldehyde gel. RNA was transferred on to nitrocellulose membranes by capillary action (Sambrook *et al.*, 1989). Membranes were probed with ³²P-labelled murine E4BP4, LMO2, IL-15 or BCL-2 probes and subsequently reprobbed with human β -actin or murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Bands were quantified using Typhoon 8600 variable mode imager and software (Amersham Biosciences).

2.10. IL-3 withdrawal experiments

TonBaF.1 cells were seeded at a density of 2×10^5 cells/ml in fresh medium at 12 h prior to IL-3 withdrawal experiments. Cells were washed three times with PBS and cultured in IL-3-free medium (RPMI 1640 containing 10 % FCS, 0.5 mg/ml

geneticin, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). Inducible E4BP4 and E2A-HLF Baf-3 clones were resuspended at a density of 2×10^5 cells/ml in fresh medium at 12 h prior to culturing with or without 2 µg/ml doxycycline for 12 h. Cells were washed three times with PBS and resuspended in the same volume of IL-3-free medium (RPMI containing 10 % FCS, 0.5 mg/ml geneticin, 0.4 mg/ml hygromycin B, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) with or without 2 µg/ml doxycycline. Cells were stained with 50 µg/ml propidium iodide (PI) in hypotonic buffer and then analysed by flow cytometry using EPICS[®] XL[™] flow cytometer and EXPO[™]32 software (Beckman Coulter) (Nicoletti *et al.*, 1991).

2.11. Luciferase reporter constructs and assays

Oligonucleotides containing HLF consensus sites (HLF-CS) (5'-CGCGTGATTACGTAACCGATCTGATTACGTAACCA-3') or E4BP4 consensus sites (E4BP4-CS) (5'-CGCGTTATGTAACGACCTGTTAATGTAACGATCCACTTATGTAACA-3') were annealed and ligated into the *Mlu*I and *Bgl*II sites of the pGL3-promoter luciferase reporter (Promega). The consensus sites are highlighted in bold. The presence of a single copy of each double-stranded oligonucleotide was confirmed by sequencing.

The -3190LMOLUC luciferase reporter construct (kindly donated by Dr Kathleen Anderson, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Ohio) contains 3.2 kb of the human LMO2 promoter cloned into the pGL3 basic vector (Promega). The mL15/Luc luciferase reporter construct (kindly provided by Dr N. Azimi, National Institute of Allergy and

Infectious Disease, National Institutes of Health, Bethesda) contains 1.2 kb of the murine IL-15 promoter cloned into the pGL3-basic vector.

1×10^7 of inducible Baf-3 cells were electroporated in 500 μ l RPMI 1640 containing 36 μ g of luciferase reporter construct and 4 μ g pRL-CMV (Promega) at 260 V/950 μ F. Cells were allowed to recover for 24 h prior to culturing with or without 2 μ g/ml doxycycline for a further 48 h. Luciferase assays were performed using the Dual Luciferase Assay System (Promega) according to the manufacturer's recommended procedures. To assess transfection efficiency, parallel electroporations were carried out using the pEGFP-N1 (BD Clontech) construct which expresses enhanced green fluorescent protein (EGFP). Transfection efficiency was measured by flow cytometry at 24 h after electroporations.

2.12. Chloramphenicol acetyltransferase (CAT) reporter gene assays.

2.12.1. E2A-HLF and E4BP4 expression constructs

E2A-HLF and E4BP4 were amplified by PCR to incorporate flanking *Bam*HI restriction enzyme sites. pTRE2hyg-E2A-HLF and pTRE2hyg-E4BP4 constructs were used as template DNA in PCR reactions. E2A-HLF was amplified using forward primer 5'- AAAGGATCCAGAATGAACCAGCCGCAGAAG-3' and reverse primer 5'- AAAGGATCCAGGGGCCCCGTGCCTGG-3'. E4BP4 was amplified using forward primer 5'- AAAGGATCCATGCAGCTGAGAAAAATGCAG-3' and reverse primer 5'- AAAGGATCCCCAGAGTCTGAAGCAGAGATTG-3'. PCR products were digested with *Bam*HI and ligated into the *Bam*HI site of pcDNA3.1/myc-HIS[®](-)(Invitrogen) to generate pcDNA3.1-E2A-HLF and pcDNA3.1-E4BP4 respectively.

2.12.2. Transfection of HeLa cells and CAT enzyme-linked immunosorbent assay (ELISA)

HeLa cells were seeded at 3×10^5 cells per well of a 6-well plate and transfected at 50% confluency using a total of 2 μ g of DNA and 8 μ l of Lipofectamine™ Reagent as described in section 2.3.6. pcDNA3.1-E4BP4 or pcDNA3.1-E2A-HLF or empty pcDNA3.1 were co-transfected with 0.75 μ g of p π S12(34)CAT reporter construct (kindly donated by Dr Ian Cowell, The Roslin Institute, Edinburgh, UK) and 0.25 μ g of pRL-CMV (Promega).

Cells were harvested at 48 h after transfection. Cells were washed with PBS and lysed using lysis buffer from a CAT ELISA kit (Roche). Protein concentrations were determined for each sample and adjusted so protein concentrations were equivalent. Levels of CAT were determined using CAT ELISA (Roche). The luciferase activity of each sample was determined by luciferase assay using a luciferase assay kit (Sigma) and used to normalise the CAT levels. Experiments were carried out in triplicate and normalised to the CAT levels obtained using empty pcDNA3.1.

2.13. Microarray Analysis

Three E2A-HLF clones were seeded at 2×10^5 cells/ml in fresh medium for 12 h prior to culturing with or without doxycycline for 12 h. Experiments were carried out in triplicate. Western blotting analysis was used to confirm the expression of E2A-HLF. Total RNA was extracted and the quality and quantity determined using an Agilent 2100 Bioanalyzer. Biotin-labelled cRNA (generated from 15 μ g total RNA) was fragmented and hybridised to Affymetrix MG-U74Av2 microarrays following protocols outlined in the Affymetrix Gene Expression Technical Manual.

For each replicate experiment, one probe array was used for cells grown in absence of doxycycline (the control) and one for cells grown in presence of doxycycline.

Affymetrix Microarray Suite 5.0 software was used to calculate gene expression values for each probe array. Data was analysed using Genespring 5.1 software. A normalisation step was applied to each array to allow comparison of gene signals across multiple arrays. Gene expression of the induced sample was normalised to the expression level of the uninduced control sample for each experiment. Data was subsequently filtered to identify genes reliably detected in both the induced and uninduced samples. These genes were further filtered by Welch t-test to identify genes which were statistically significantly changed by greater than 1.3-fold by the expression of E2A-HLF. Multiple testing correction on this set of genes was performed using Benjamini-Hochberg false discovery rate procedure.

2.14. IL-15 bioassay

CTLL-2 cells were used two days after the last medium changed to increase the sensitivity of the bioassay (Paxton, 1996). Cells were washed twice in 50 ml of medium without IL-2 and resuspended at 2×10^6 cells/ml. 50 μ l of cells (equivalent to 1×10^5 cells) was dispensed into each well of a 96-well plate. An equal volume of conditioned medium from inducible E2A-HLF Baf-3 cells induced to express E2A-HLF for 48 h was dispensed into each well. Serial dilutions of recombinant murine IL-15 were used to generate a standard curve. Cells were cultured for 48 h at 37 °C/5% CO₂ and cell viability was assessed using a MTS assay. 20 μ l of MTS assay reagent (Promega) was added to each well. Cells were incubated at 37 °C/ 5% for up to 4 h. Absorbance was measured at 490 nm and reference filter was set at 655 nm to account for background.

2.15. Transduction of primary haematopoietic progenitor cells.

2.15.1. Retroviral constructs

The pMSCV-IRES-hCD2t vector was derived from pMSCVneo (BD Clontech). The phosphoglycerate kinase promoter (PGK) and neomycin resistance gene was replaced with an internal ribosome entry site (IRES)-hCD2tailless cassette derived from the pMI-IRES-hCD2t construct, a kind gift from Dr M. J. Bevan, Department of Immunology, University of Washington, Seattle, USA.

5' Myc-epitope tagged E2A-HLF was generated by PCR using forward primer 5'-ATATGAATTCCACCATGGAACAAAACTTATTTCTGAAGAAGATCTGATGAACCAGCCGCAGAGG-3' and reverse primer (5'-ATATGAATTCTACAGGGGCCCCGTGCCT-3'). The PCR product was digested with *EcoRI* and ligated into the *EcoRI* site of pMSCV-based vectors.

3' Myc-tagged murine BCL-2 was generated by RT-PCR from murine thymocyte RNA and cloned into the *EcoRI* site of pMSCVpuro (a gift from Martin Woodward, Molecular Haematology and Cancer Biology Unit, Institute of Child Health, London).

2.15.2. Transfection of LinXE retrovirus packaging cell line and concentration of retroviruses

LinXE cells were transfected at 60% to 80% confluency with 8 µg of DNA and 40 µl of Lipofectamine™ as described in section 2.3.6. Retroviral supernatant was harvested after 48 h and cleared of cellular debris by centrifugation at 1800 rpm for 5 min. Viral particles were concentrated 10-fold by centrifugation at 16,000 ×g at 4 °C for 1 h and resuspended in DMEM medium containing 10% FCS, 2 mM, L-

glutamine, 100 U/ml penicillin 100 µg/ml streptomycin, 50 µM 2-ME and appropriate cytokines (see section 2.15.3).

2.15.3. Infection of haematopoietic progenitor cells

Bone marrow enriched for HPCs was harvested from four-week-old C57BL/6 mice previously treated for 5 days with 5'-fluorouracil (5-FU) at a dose of 150 mg/kg.

Bone marrow was harvested from the tibia and femur bones. Erythrocytes were lysed with red cell lysis buffer (**Table 2.1.**) at room temperature for 10 min. 1×10^6 /ml HPCs were cultured in wells of a 12-well plate in medium containing recombinant murine cytokines for 24 h prior to infection.

cKit⁺ HPCs were purified from the foetal liver of E12 of C57BL/6 or E13 of C57BL/10 mouse embryos by magnetic cell sorting (MACS) (Miltenyi Biotec, Bisley, Surrey, UK) using anti-cKit (clone 2B8) (BD Pharmingen). 0.5×10^6 cells/ml cKit⁺ HPCs were cultured in wells of a 12-well plate in medium containing recombinant murine cytokines for 34 h prior to infection. Foetal liver HPCs were typically 90% cKit⁺ after enrichment as determined by flow cytometry.

For B cell assays, HPCs were cultured in DMEM medium containing 10% FCS, 2 mM, L-glutamine, 100 U/ml penicillin 100 µg/ml streptomycin, 50 µM 2-ME with 100 ng/ml stem cell factor (SCF), 200 ng/ml IL-7 and 10 ng/ml *fms*-like tyrosine kinase-3 ligand (Flt-3-L). For myeloid assays, HPCs were cultured in DMEM medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6.

Infections were performed in 96-well plates (1×10^4 cells/100 µl/well) with concentrated retrovirus resuspended in DMEM medium containing 5 µg/ml

polybrene (Sigma), 20% FCS, L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME and appropriate cytokines. HPCs were infected by centrifugation at 700 ×g at 25 °C for 45 minutes. Fresh medium containing appropriate cytokines was added to infected cells 24 h after infections.

2.16. Methylcellulose assays

HPCs transduced with retroviral constructs were cultured in duplicate 1.1 ml methylcellulose cultures in 35 mm² plates (Stem Cell Technologies, London, UK) 48 h after infection.

For B cell assays, cells were cultured in Methocult M3231 methylcellulose medium (Stem Cell Technologies) supplemented with SCF, IL-7 and Flt-3L. Colonies containing more than 50 cells were scored, pooled and viable cells counted with trypan blue after 7 to 10 days in culture. Secondary and subsequent rounds were performed by replating 1×10^4 cells/ml under identical conditions.

For myeloid assays, cells were cultured in Methocult M3434 methylcellulose medium (Stem Cell Technologies) containing SCF, IL-3, IL-6 and erythropoietin (EPO) and supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). Colonies containing more than 50 cells were scored, pooled and viable cells counted with trypan blue after 5 to 7 days in culture. Secondary and subsequent rounds were performed by replating 1×10^4 cells/ml under identical conditions.

HPCs infected with retroviral constructs containing neomycin and/or puromycin resistance genes were selected during the first round of replating only with 1 mg/ml geneticin and/or 1 µg/ml puromycin (BD Clontech).

2.16.1. Generation of cell lines from immortalised cells in methylcellulose culture

Cells were pooled at the end of various rounds of replating and cultured at 1×10^5 /ml. Lymphoid cells were cultured in Iscoves modified Dulbecco medium (IMDM) (Sigma) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 50 µM 2-ME, SCF, IL-7 and Flt-3L. Myeloid cells were cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, SCF, IL-3 and IL-6.

2.16.2. Flow cytometric analysis of methylcellulose colonies and derived cell lines

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or biotin (Bio)-conjugated monoclonal antibodies specific for cell surface markers (see **Table 2.2**). Cells were pre-incubated with unlabelled anti-Fcγ III/II receptor mAb^{2.4G2} diluted in FACS staining buffer (see section 2.1.). Cells were subsequently incubated with 100 µl primary antibody diluted in FACS staining buffer. Biotin-conjugated monoclonal antibodies were visualised with Streptavidin-Tri-Color diluted in FACS staining buffer. Flow cytometry was performed using an EPICS[®] XL[™] flow cytometer and data was analysed using accompanying software.

Table 2.3. Antibodies used for flow cytometry.

Antibody	Manufacturer	Clone no.	Isotype	Working dilution
Anti-B220-PE	BD Pharmingen	RA3-6B2	Rat IgG _{2a} , κ	1:200
Anti-BP-1-Bio	BD Pharmingen	6C3	IgG _{2a} , κ	1:200
Anti-CD19-PE	BD Pharmingen	1D3	Rat IgG _{2a} , κ	1:200
Anti-cKIT-Bio	E Bioscience	2B8	Rat IgG _{2b} , κ	1:100
Anti-Gr-1-PE	E-Bioscience	RB6-8C5	Rat IgG _{2a} , κ	1:200
Anti-human CD2-FITC	E-Bioscience	RPA-2.10	IgG1, κ	1:100
Anti-Mac-1-FITC	BD Pharmingen	M1/70	Rat IgG _{2a} , κ	1:100
IgG _{2a} , κ -PE isotype control	BD Pharmingen	N/A	Rat IgG _{2a} , κ	1:100
IgG _{2b} -Bio isotype control	Caltag	N/A	Rat IgG _{2b}	1:100
Streptavidin-Tri-Color	Caltag	N/A	N/A	1:100

2.16.3. Cell proliferation assays for assessment of cytokine requirements of immortalised cell lines

Cells were washed with RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME. 5×10^3 myeloid cells or 1×10^5 lymphoid cells in a total volume of 50 μ l were dispensed in triplicate into wells of a 96-well plate. An equal volume of medium containing 2 \times cytokine concentration was added to each well. Cell viability was measured using MTS assay after 48 h at 37 °C/5% CO₂ as described in section 2.14. Absorbance was measured at 490 nm and reference filter was set at 655 nm to account for background.

CHAPTER 3

RESULTS (1)

3.1. Background

3.1.1. Identification of t(17;19) translocation and E2A-HLF in ALL patients

The two types of rearrangements which generate the E2A-HLF fusion are associated with different clinical features (Raimondi *et al.*, 1991; Hunger *et al.*, 1992; Inaba *et al.*, 1992; Hunger *et al.*, 1994; Devaraj *et al.*, 1994). Type I rearrangements are associated with DIC whereas Type II is associated with hypercalcaemia and bone lesions. DIC cannot be ruled out in all patients as complete coagulation studies were not carried out in all cases and therefore low grade DIC might be present in additional cases. All of the previously studied childhood ALL patients who have been found to have E2A-HLF expressed in their leukaemic cells have died from their leukaemia. Identification of patients who have t(17;19) translocation may help in the selection of treatment and also anticipate clinical problems.

Different methods have been used to identify the t(17;19) translocation in patients with ALL. Standard karyotyping methods have been used to identify translocations which involve chromosomes 17 and 19 (Raimondi *et al.*, 1991; Ohyashiki *et al.*, 1991). A case of ALL which had an apparently normal karyotype, as determined by R-banding, was found to have the t(17;19) translocation using fluorescence *in situ* hybridisation (FISH) using whole chromosome paints for chromosomes 17 and 19 (Daheron *et al.*, 2002). FISH using an *E2A* specific probe had also been used to identify translocations involving the *E2A* gene (Boomer *et al.*, 2001). Southern blotting has also been used to detect rearrangements of the *E2A* locus in patients in addition to karyotyping and FISH analysis (Hunger *et al.*, 1992; Devaraj *et al.*, 1994). These methods identify the t(17;19) translocation but do not determine whether the E2A-HLF is expressed. Other methods could have been used to

establish whether E2A-HLF is expressed. Western blotting analysis could be used to detect expression of the fusion protein whereas RT-PCR could be used to detect the expression of E2A-HLF transcripts.

3.1.2. Detection of type I and Type II E2A-HLF rearrangements

The two types of genomic rearrangement that generate the E2A-HLF fusion differ by whether or not they have a cryptic exon consisting of sequences from *E2A* exon 13 and intronic sequence (**Figure 1.7**) (Hunger *et al.*, 1994).

RT-PCR has been used in previous studies to characterise the nature of the E2A-HLF rearrangement in patient leukaemic cells. The advantage of using RT-PCR is that primers can be designed to distinguish between type I and type II rearrangements.

The expected RT-PCR products from type I and type II rearrangements is shown in **Figure 3.1** using a forward primer within *E2A* exon 11 (primer 19F) and a reverse primer within *HLF* exon 4 (primer 17B) that have been used to characterise E2A-HLF rearrangements in previous studies (Hunger *et al.*, 1994; Devaraj *et al.*, 1994).

The complete nucleotide sequence of type II E2A-HLF has not been published, therefore the type II sequence given in **Figure 3.1** is hypothetical and is based on the published type I E2A-HLF nucleotide sequence. The presence of a cryptic exon in the type I rearrangement would generate a larger RT-PCR product (approximately 476 bp) than the product obtained from a type II translocation which lacks the insertion (293 bp).

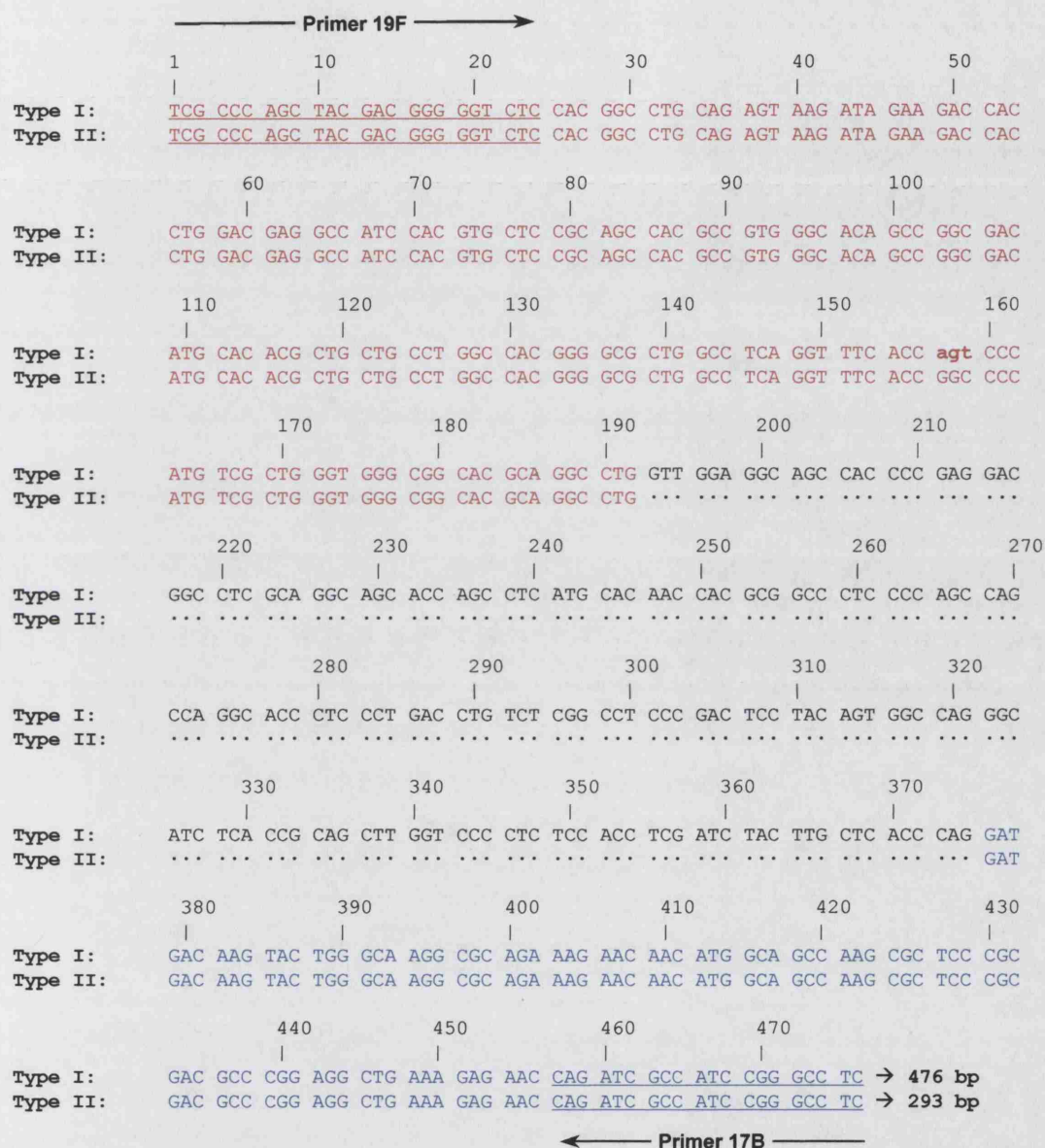


Figure 3.1. Type I and type II E2A-HLF RT-PCR products.

Type I E2A-HLF sequence is the published sequence of E2A-HLF from the UOC-B1 cell line (Inaba *et al*, 1992). The sequence for type II E2A-HLF is a hypothetical as it has not been published. Red letters indicate *E2A* sequences. Black letters indicate cryptic exon containing *E2A* exon 13 and intronic sequence and *HLF* intronic sequence. Blue letters indicate *HLF* exon 4. Lower case letters show the position of the glycine (wild type) residue which is substituted for serine in some patients. Underlined sequences indicate the positions of the RT-PCR primers.

3.2. Results

3.2.1. Two patients with *t(17;19)* translocation express *E2A-HLF*

A small study was done using patient material from two ALL patients who were found to have a *t(17;19)* translocation by routine karyotyping or by FISH. One patient was identified at Great Ormond Street Hospital, London (Patient 1) and one from Our Lady's Hospital, Dublin (Patient 2).

Patient 1 was a female patient who presented with ALL at the age of 5½ years. The patient was treated, but suffered a fatal relapse at 18 months from time of diagnosis. DIC was evident at the time of relapse. Chromosomal analysis of leukaemic cells revealed a *t(17;19)* translocation at the time of diagnosis and relapse.

FISH analysis was carried out by the Cytogenetics Unit at Great Ormond Street Hospital, London, using whole chromosome paints for chromosomes 17 and 19 on metaphase spreads from a diagnostic sample from Patient 1 (**Figure A.1** in Appendix). FISH analysis confirmed the presence of a translocation involving chromosomes 17 and 19. The derivative chromosomes 19 and 17 were evident. However, the signal from the reciprocal fusion on the derivative chromosome 17 was either very weak or not present (**Figure A.1**).

RT-PCR was performed on RNA samples extracted from bone marrow cells taken at the time of diagnosis and time of relapse. No PCR product corresponding to *E2A-HLF* was amplified in the negative controls (-RT) or in Jurkat cells, a T-ALL cell line which does not express *E2A-HLF* (**Figure 3.2**). A PCR product of approximately 300 bp was amplified in the presence of RT and was consistent with a type II rearrangement. As expected, the HAL-01 cell line, which has been

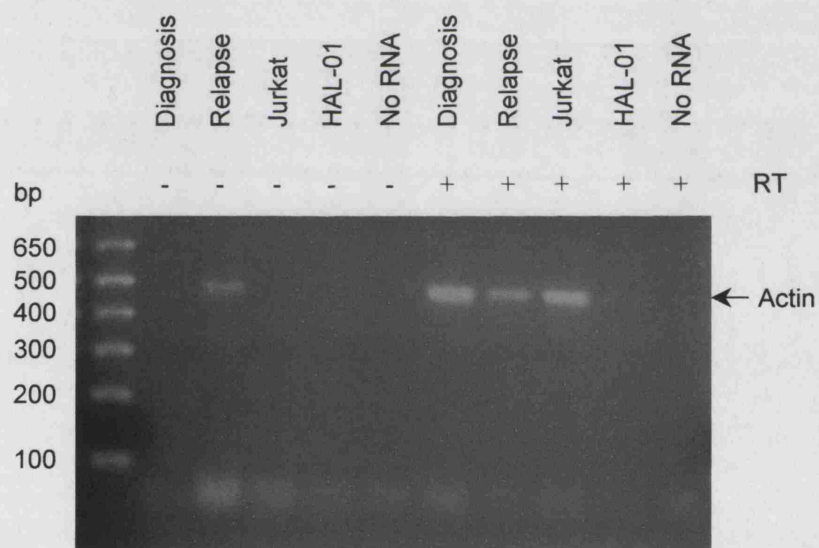
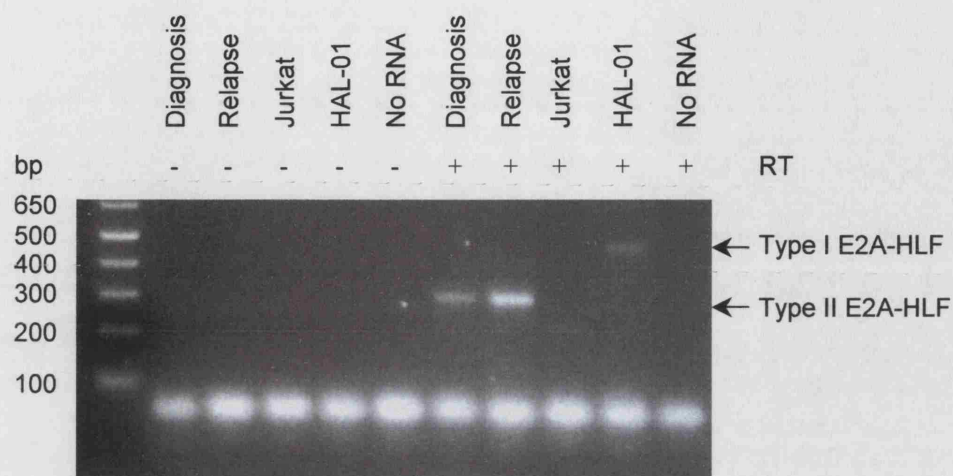


Figure 3.2. E2A-HLF transcript is expressed in the bone marrow of Patient 1. RT-PCR was performed using primers to amplify E2A-HLF and actin with cDNA derived from bone marrow from Patient 1 taken from the time of diagnosis and relapse.

determined to have a type I rearrangement, generated a larger PCR product of approximately 450 bp (Hunger *et al.*, 1994). RT-PCR for actin showed that some DNA contamination was found in the relapse sample as shown by the presence of a band in the sample amplified without the addition of RT. A very faint band for the actin RT-PCR product for the HAL-01 cells in the presence of RT was detected.

Patient 2 was an 8-year-old child with ALL that was found to have a t(17;19) translocation by karyotyping (**Figure A.2** in Appendix). RT-PCR was also carried using RNA derived from the bone marrow of this second patient taken at the time of relapse. RT-PCR amplified a product of approximately 300 bp (**Figure 3.3**). This result also suggested that this patient also had a type II E2A-HLF rearrangement.

3.2.2. Normal haematopoietic cells and leukaemic cells lacking t(17;19) translocation do not generate an E2A-HLF RT-PCR product

To further confirm the specificity of the RT-PCR primers for E2A-HLF, additional RT-PCR was carried out on using RNA derived from normal cells or primary leukaemia cells which do not have t(17;19) translocation. RNA was obtained from peripheral blood lymphocytes (PBL) from a normal adult donor, normal bone marrow (BM) from a paediatric patient and bone marrow from a patient with B cell ALL which had another translocation (MLL-AF9) unrelated to E2A-HLF. RNA from HAL-01 cells was used as a positive control to confirm that the PCR reaction had worked.

No E2A-HLF RT-PCR amplification product was detected in the PBL, BM or in the cells from patient with MLL-AF9 leukaemia (**Figure 3.4**). A specific amplification product was only found with the positive control using cDNA from HAL-01 cells in the presence of RT. This confirmed the specificity of the RT-PCR for E2A-HLF.

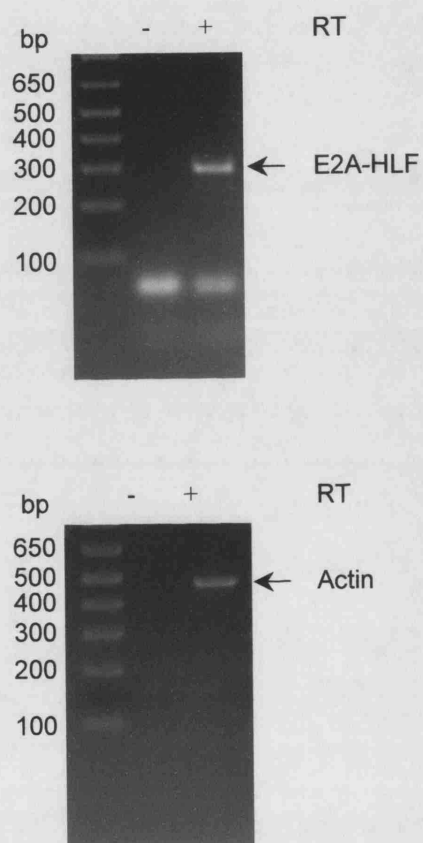


Figure 3.3. E2A-HLF transcript is expressed in the bone marrow of Patient 2. RT-PCR was performed using primers to amplify E2A-HLF and actin with cDNA derived from bone marrow from Patient 2 taken at the time of relapse.

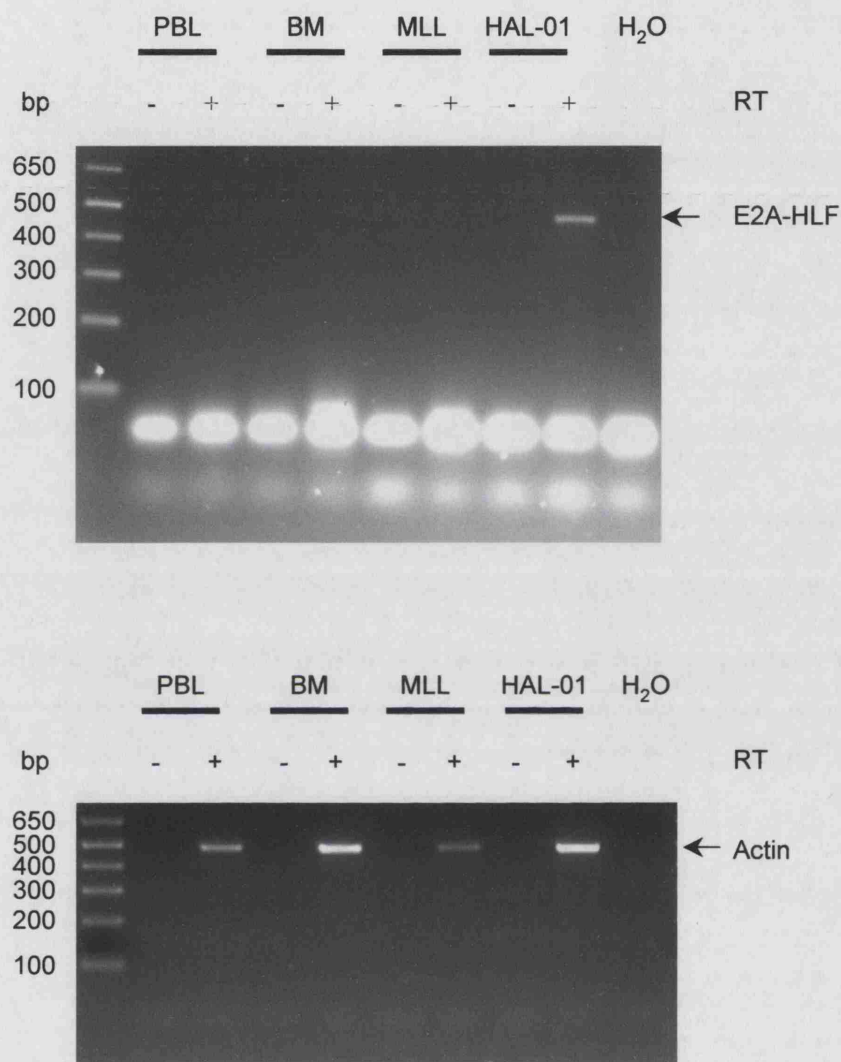


Figure 3.4. E2A-HLF is not expressed by normal haematopoietic cells or by leukaemic cells without the t(17;19) translocation.

RT-PCR was performed using primers to amplify E2A-HLF or actin with cDNA derived from normal adult peripheral blood lymphocytes (PBL), normal bone marrow (BM), bone marrow from a patient with MLL-AF9 translocation (MLL). HAL-01 cDNA was used as a positive control and water (no cDNA) was as a negative control.

RT-PCR using primers specific for actin demonstrated the absence of an E2A-HLF product in the negative control samples was not due to degradation of the RNA.

3.2.3. Further confirmation of Type II rearrangements in Patients 1 and 2

The sizes of the RT-PCR products from the samples from Patients 1 and Patient 2 indicated that E2A-HLF was expressed and that it was a type II rearrangement. The RT-PCR products amplified from the diagnostic and relapse samples from Patient 1 were subsequently sequenced as well as the RT-PCR product from the relapse sample from Patient 2. Sequence analysis confirmed that the RT-PCR products were indeed due to the presence of a type II rearrangement (**Figure 3.5**).

Patients 1 and 2 have a wild type glycine residue encoded by nucleotides 157-159 (**Figure 3.5**) which correspond to *E2A* residue number 425 (Hunger *et al.*, 1994). HAL-01 and UOC-B1 cell lines, which have type I rearrangements, have a serine residue at this position that may be a somatic mutation or an uncommon polymorphism (Hunger *et al.*, 1994). Patients 1 and 2 also have silent nucleotide substitutions at nucleotide positions 168 and 174 that do not result in a change in amino acid (**Figure 3.5**). These nucleotide substitutions were also found in two other patients (Devaraj *et al.*, 1994; Takahashi *et al.*, 2001). The sequence analysis also confirmed that the 3' end of *E2A* exon 12 was directly fused in frame to the 5' end of *HLF* exon 4 in Patients 1 and 2. As these exons are in the same translational reading frame, a functional E2A-HLF protein would be expected to be expressed.

	1	10	20	30	40										
Patient 1 (Diagnosis):	TCG	CCC	AGC	TAC	GAC	GGC	GGT	CTC	CAC	GGC	CTG	CAG	AGT	AAG	ATA
Patient 1 (Relapse):	TCG	CCC	AGC	TAC	GAC	GAG	GGT	CTC	CAC	GGC	CTG	CAG	AGT	AAG	ATA
Patient 2 (Relapse):	TCG	CCC	AGC	TAC	GAC	GGG	GGT	CTC	CAC	GGC	CTG	CAG	AGT	AAG	ATA
Amino Acid sequence:	S	P	S	Y	D	G	G	L	H	G	L	Q	S	K	I
	50	60	70	80	90										
Patient 1 (Diagnosis):	GAA	GAC	CAC	CTG	GAC	GAG	GCC	ATC	CAC	GTG	CTC	CGC	AGC	CAC	GCC
Patient 1 (Relapse):	GAA	GAC	CAC	CTG	GAC	GAG	GCC	ATC	CAC	GTG	CTC	CGC	AGC	CAC	GCC
Patient 2 (Relapse):	GAA	GAC	CAC	CTG	GAC	GAG	GCC	ATC	CAC	GTG	CTC	CGC	AGC	CAC	GCC
Amino Acid sequence:	E	D	H	L	D	E	A	I	H	V	L	R	S	H	A
	100	110	120	130											
Patient 1 (Diagnosis):	GTG	GGC	ACA	GCC	GGC	GAC	ATG	CAC	ACG	CTG	CTG	CCT	GGC	CAC	GGG
Patient 1 (Relapse):	GTG	GGC	ACA	GCC	GGC	GAC	ATG	CAC	ACG	CTG	CTG	CCT	GGC	CAC	GGG
Patient 2 (Relapse):	GTG	GGC	ACA	GCC	GGC	GAC	ATG	CAC	ACG	CTG	CTG	CCT	GGC	CAC	GGG
Amino Acid sequence:	V	G	T	A	G	D	M	H	T	L	L	P	G	H	G
	140	150	160	170	180										
Patient 1 (Diagnosis):	GCG	CTG	GCC	TCA	GGT	TTC	ACC	GGC	CCC	ATG	TCa	CTG	Ggc	GGG	CGG
Patient 1 (Relapse):	GCG	CTG	GCC	TCA	GGT	TTC	ACC	GGC	CCC	ATG	TCa	CTG	Ggc	GGG	CGG
Patient 2 (Relapse):	GCG	CTG	GCC	TCA	GGT	TTC	ACC	GGC	CCC	ATG	TCa	CTG	Ggc	GGG	CGG
Amino Acid sequence:	A	L	A	S	G	F	T	G	P	M	S	L	G	G	R
	190	200	210	220											
Patient 1 (Diagnosis):	CAC	GCA	GGC	CTG	GAT	GAC	AAG	TAC	TGG	GCA	AGG	CGC	AGA	AAG	AAC
Patient 1 (Relapse):	CAC	GCA	GGC	CTG	GAT	GAC	AAG	TAC	TGG	GCA	AGG	CGC	AGA	AAG	AAC
Patient 2 (Relapse):	CAC	GCA	GGC	CTG	GAT	GAC	AAG	TAC	TGG	GCA	AGG	CGC	AGA	AAG	AAC
Amino Acid sequence:	H	A	G	L	D	D	K	Y	W	A	R	R	R	K	N
	230	240	250	260	270										
Patient 1 (Diagnosis):	AAC	ATG	GCA	GCC	AAG	CGC	TCC	CGC	GAC	GCC	CGG	AGG	CTG	AAA	GAG
Patient 1 (Relapse):	AAC	ATG	GCA	GCC	AAG	CGC	TCC	CGC	GAC	GCC	CGG	AGG	CTG	AAA	GAG
Patient 2 (Relapse):	AAC	ATG	GCA	GCC	AAG	CGC	TCC	CGC	GAC	GCC	CGG	AGG	CTG	AAA	GAG
Amino Acid sequence:	N	M	A	A	K	R	S	R	D	A	R	R	L	K	E
	280	290													
Patient 1 (Diagnosis):	AAC	CAG	ATC	GCC	ATC	CGG	GCC	TC							
Patient 1 (Relapse):	AAC	CAG	ATC	GCC	ATC	CGG	GCC	TC							
Patient 2 (Relapse):	AAC	CAG	ATC	GCC	ATC	CGG	GCC	TC							
Amino Acid sequence:	N	Q	I	A	I	R	A								

Figure 3.5. Nucleotide and amino acid sequences of type II rearrangement RT-PCR products amplified from Patients 1 and 2.

Nucleotide and predicted amino acid sequences of the 293 bp RT-PCR products from Patient 1 and 2. Red letters indicate *E2A* sequences. Blue letters indicate *HLF* exon 4 sequences. Lower case letters indicate a nucleotide substitution which is different from the wild type *E2A* sequence but does not result in an amino acid change. Underlined letters indicate primer sequences.

3.3. Discussion

3.3.1. Type II rearrangements in Patients 1 and 2

In this study, RT-PCR and sequence analysis of the amplified E2A-HLF product from RNA samples obtained from two patients with ALL showed that both patients had type II rearrangements. Type II rearrangements appear to be more common than type I rearrangements (**Table 1.2**). No evidence of DIC or hypercalcaemia was evident at the time of diagnosis in Patient 1. However, DIC was present in this patient at the time of relapse. As DIC is more commonly associated with type I rearrangements and is usually detected at the time of diagnosis, the type II E2A-HLF rearrangement in Patient 1 may have had a role in DIC at the time of relapse.

Alternatively, this may be a coincidental finding and may have been the result of therapy or due to advanced stage of disease or other causes e.g. sepsis, spontaneous lysis of leukaemic cells.

Two patients who express both type I and type II E2A-HLF transcripts in their leukaemic cells have previously been identified (Devaraj *et al.*, 1994; Takahashi *et al.*, 2001). The presence of both types of rearrangements in the same patient has been proposed to be due to alternative splicing (Devaraj *et al.*, 1994; Takahashi *et al.*, 2001). The existence of two populations of leukaemic cells which either have the type I or type II rearrangement was also proposed as an alternative explanation for the presence of both types of rearrangement as both transcripts were detected in colonies derived from single cells in soft agar culture (Takahashi *et al.*, 2001).

Leukaemias are generally considered to be clonal in nature and it is likely that one genomic (type I) rearrangement has occurred in a cell which subsequently generates type I and type II E2A-HLF transcripts by alternative splicing. Both patients with

leukaemic cells which expressed the two rearrangements were found to have hypercalcaemia and no evidence of DIC. The type I rearrangements found in these two cases were slightly different to the normal type I rearrangements found in patients with a single E2A-HLF rearrangement. The type I transcript from one patient lacked the first 8 nucleotides of *HLF* exon 4 whereas the second case involved a truncated *E2A* exon 13 (Devaraj *et al.*, 1994; Takahashi *et al.*, 2001).

Type I rearrangements are unique for each patient due to the insertion of non-templated nucleotides in the joining region between the *E2A* derived sequences and the *HLF* derived sequences (Hunger *et al.*, 1994). Non-templated nucleotides are not encoded by chromosomal DNA, but are inserted *de novo* by the activity of TdT.

Although type I and type II E2A-HLF fusions are slightly different structurally and have associations with different clinical features, they have identical DNA-binding and transcriptional regulatory properties (Hunger *et al.*, 1994).

3.3.2. Patients with molecularly characterised E2A-HLF and clinical outcome

The patients presented here are aged 5½ and 8 years. Published cases of t(17;19)⁺ ALL show that patients are typically aged 11 years-old or older, but younger patients were found to have the translocation and express E2A-HLF (**Table 1.2**). No obvious correlation between age at presentation or type of rearrangement to clinical outcome was evident. There were more female patients with the E2A-HLF translocation than male patients, but this could be due to the small numbers of patients that have been studied.

3.3.3. Further work and conclusions

Sequence analysis of the RT-PCR products from Patients 1 and 2 indicated that an in-frame, and therefore functional, E2A-HLF protein could be expressed. The E2A-

HLF protein could be detected by Western blotting analysis or other techniques.

This is further discussed in Chapter 7.

One case of ALL with DIC was identified which had an apparent normal karyotype (Daheron *et al.*, 2002). However, subsequent RT-PCR and FISH analysis demonstrated the expression of a type I E2A-HLF transcript in the leukaemic cells of this patient. This suggests that the frequency of the t(17;19) translocation may be underestimated by using conventional banding techniques.

RT-PCR is a sensitive method which can be used to detect the E2A-HLF fusion in patient cells. The sensitivity of this method would allow it to be used in detecting minimal residual disease and therefore be used to determine effectiveness of treatment (Devaraj *et al.*, 1994).

CHAPTER 4

RESULTS (2)

4.1. Background

4.1.1. Establishment of a tetracycline inducible system to express E2A-HLF and E4BP4

An *in vitro* inducible system could be used to model the *in vivo* events that follow the t(17;19) translocation and subsequent expression of the E2A-HLF oncoprotein. This system has several advantages. Firstly, the primary events that follow the expression of the induced oncoprotein of interest can be determined as the level and temporal expression of the gene can be tightly controlled. Secondly, an inducible gene expression system overcomes problems associated with stable cell lines. The constitutive expression of a gene of interest may cause phenotypic changes in cells in long-term selection, particularly if the gene of interest has deleterious effects or is toxic to the cells.

Overexpression of human E4BP4 in FL5.12 and Baf-3 cells has been reported to delay apoptosis induced by IL-3 withdrawal (Ikushima *et al.*, 1997; Kuribara *et al.*, 1999). The generation of cell lines which either inducibly express E2A-HLF or human E4BP4 can be used to compare and contrast the effects of these two proteins.

Baf-3 cells are a suitable cell line to use to establish an inducible system to express E2A-HLF and E4BP4. Baf-3 cells and patients with t(17;19)⁺ ALL have a pro-B cell immunophenotype. In addition, as Baf-3 cells are dependent on IL-3 for survival and undergo apoptosis in absence of IL-3, therefore the effects of E2A-HLF and E4BP4 on apoptosis can be easily examined.

4.1.2. Tet-On inducible system

The Tet-On system is based on control components derived from the tetracycline (tet) resistance operon encoded by the *Tn10* transposon in *E. coli*. The first component of the Tet-On system is the reverse tetracycline transactivator (rtTA). This regulatory protein was generated by four amino acid changes in tetracycline repressor protein (TetR) which binds to the tet operator sequences (*tetO*) in presence of tetracycline or its derivatives, e.g. doxycycline. Fusion to virion protein 16 (VP16) activation domain of the *Herpes simplex* virus (HSV) converted TetR into a transactivator (rtTA) (Gossen *et al.*, 1995). The second component of the Tet-On system is the response plasmid which expresses the gene of interest under the control of the tetracycline response element (TRE). The TRE consists of 7 direct repeats of a 42 bp sequence containing the *tetO* located upstream of the minimal cytomegalovirus (CMV) promoter. This minimal CMV promoter lacks the strong enhancer sequences associated with the immediate early promoter and minimises background expression of the gene of interest. A schematic overview of the Tet-On system is depicted in **Figure 4.1**. Stable cell lines that express rtTA and the response element will express the gene of interest in response to tetracycline or doxycycline.

4.2. Results

4.2.1. E4BP4 protein is regulated by IL-3 in murine pro-B cells

TonBaF.1 cells are a clone derived from the stable transfection of Baf-3 cells with a rtTA-expressing construct which confers geneticin resistance (Klucher *et al.*, 1998). E4BP4 protein expression is regulated by IL-3 and it has been described as a ‘delayed-early’ IL-3-responsive gene in Baf-3 and FL5.12 cells (Ikushima *et al.*, 1997). Therefore, the endogenous levels of E4BP4 protein were examined in

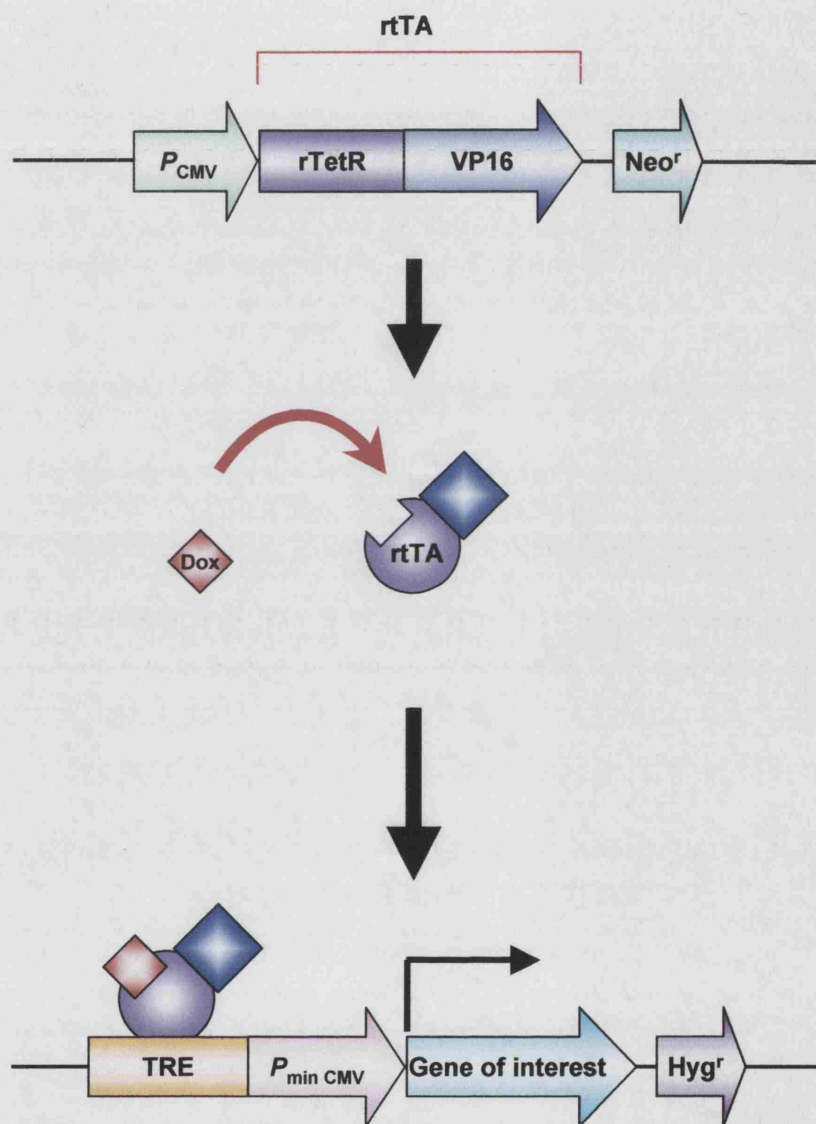


Figure 4.1. Gene regulation in the Tet-On system.

The reverse tetracycline transactivator (rtTA) fusion protein consists of the reverse Tet repressor (rTetR) fused to the VP16 activation domain. In the presence of the inducing agent, tetracycline or doxycycline (Dox), rtTA binds to the tetracycline response element (TRE) activates the transcription of the gene of interest. Neo^r, Neomycin resistance gene. Hyg^r, Hygromycin resistance gene.

TonBaF.1 cells to establish whether the cells retained IL-3-dependent expression of E4BP4.

Western blotting analysis determined that E4BP4 protein was expressed by the TonBaF.1 cells and that the levels changed depending on the presence or absence of IL-3. The level of E4BP4 protein (~50 kD) decreased by 2.1-fold within 2 h of culturing cells in the absence of IL-3 (**Figure 4.2A**). Protein levels continued to decrease to more than 28-fold by 6 h following IL-3 withdrawal. When cells were subsequently cultured in IL-3-containing medium, a 7.7-fold increase in E4BP4 protein was evident within 2 h.

Northern blotting analysis performed on RNA samples from the same time points showed that the E4BP4 mRNA levels decreased by 2.1-fold within 2 h of IL-3 withdrawal (**Figure 4.2B**). In contrast to the protein levels of E4BP4, the level of E4BP4 RNA showed a maximum decrease of 2.6-fold after IL-3 withdrawal. The E4BP4 mRNA levels subsequently increased by 1.5-fold within 2 h after the reintroduction of IL-3.

4.2.2. Generation of tet-inducible Baf-3 cells which inducibly express E2A-HLF or E4BP4

To generate the Baf-3 cells that inducibly express E2A-HLF or human E4BP4, the E2A-HLF and human E4BP4 cDNAs were cloned into the pTRE2hyg tetracycline responsive expression construct which also confers resistance to hygromycin B. TonBaF.1 cells were electroporated with pTRE2hyg-E2A-HLF or pTRE2hyg-E4BP4 constructs and immediately divided into ten different pools and cultured independently. After two weeks of selection with hygromycin B, the mixed

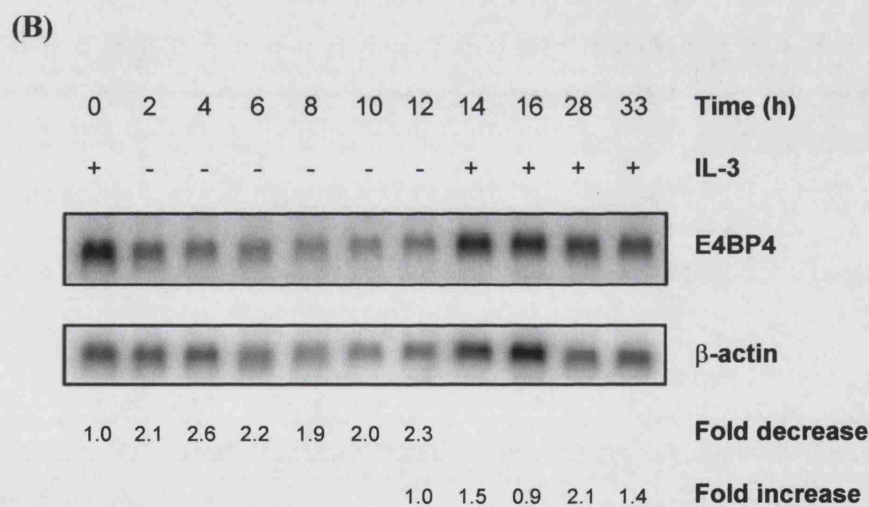
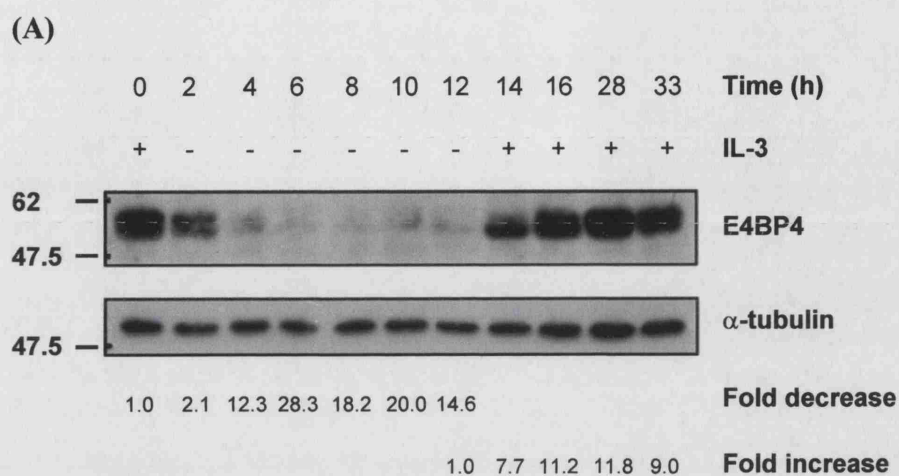


Figure 4.2. E4BP4 protein expression is regulated by IL-3 in Baf-3 cells.

(A) Western blotting detection of the levels of E4BP4 protein in TonBaF.1 cells when cultured in presence or absence of IL-3. Cells were cultured in IL-3-free medium for 12 h and subsequently seeded in IL-3-containing medium for an additional 21 h. E4BP4 levels were normalised to α -tubulin levels and the fold changes determined. **(B)** Northern blotting analysis was also performed using 2 μ g poly A⁺ RNA from the cells in **(A)**.

population of cells were analysed for their inducible expression of E2A-HLF or E4BP4 by doxycycline.

Inducible expression of both E2A-HLF and E4BP4 was confirmed by Western blotting analysis of representative mixed populations (**Figure 4.3**). Induced protein expression was achieved within 4 h after the addition of doxycycline to the culture medium. Maximum inducible expression of E2A-HLF or E4BP4 was achieved after 24 h or 48 h, respectively, in presence of doxycycline. Levels of E2A-HLF protein expression are reduced by 1.8-fold within 24 h after the removal of doxycycline from the culture medium and returned to below background levels by 48 h (**Figure 4.3A**). E4BP4 protein levels reached basal levels within 24 h after the removal of doxycycline from the culture medium (**Figure 4.3B**).

After confirmation of the expression of E4BP4 and E2A-HLF in the mixed population of inducible cells, single cell clones were established by FACS sorting single cells into each well of a 96-well microtitre plate. These clones were cultured and expanded and analysed for their inducible expression of E2A-HLF or E4BP4.

Approximately 80 clones were screened for inducible expression of E2A-HLF by Western blotting. A selection of analysed clones are shown in **Figure 4.4A** and **4.4B**. Different clones showed different levels of induction and background levels of E2A-HLF in absence of doxycycline. Analysis of a selection of clones induced with doxycycline over a period of 24 h demonstrated that maximum expression of E2A-HLF was achieved after culturing cells in presence of doxycycline for 24 h (**Figure 4.4C**).

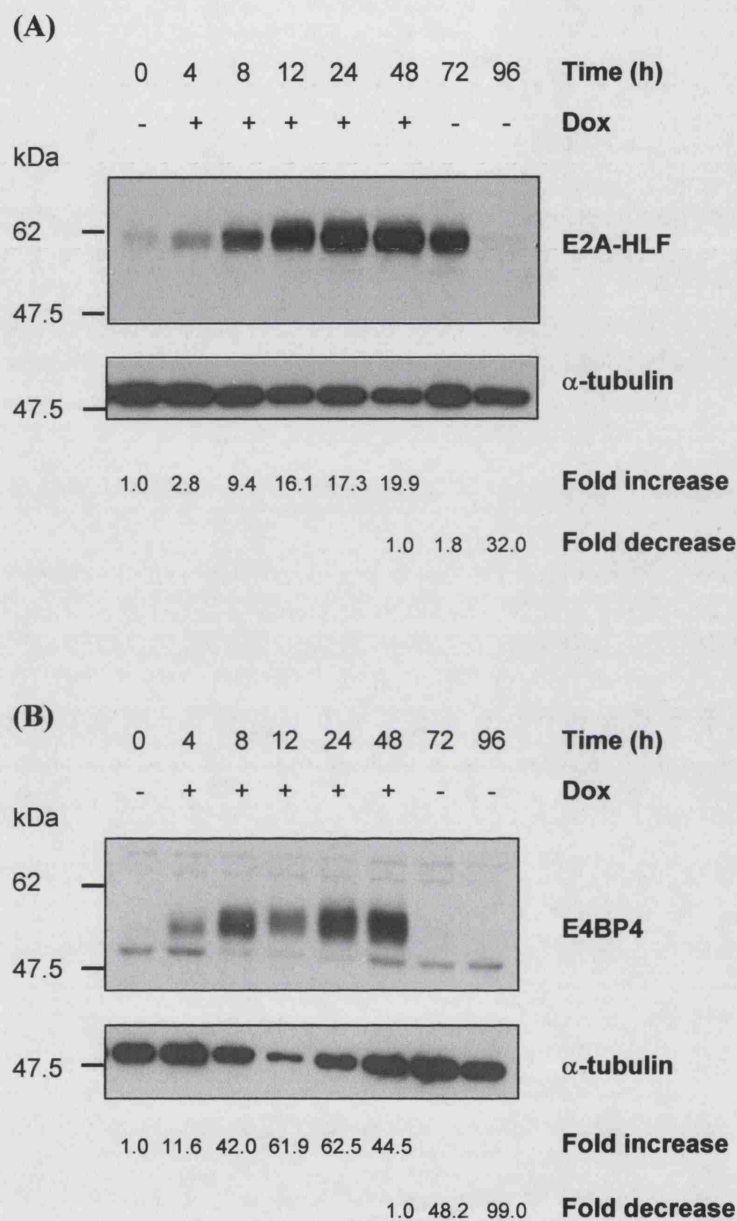


Figure 4.3. Inducible expression of E2A-HLF and E4BP4 in mixed populations of stable TonBaF.1.

(A) Western blotting analysis of inducible expression of E2A-HLF in a mixed population of stably transfected TonBaF.1 cells. **(B)** Western blotting analysis of inducible expression of E4BP4 in a mixed population of stably transfected TonBaF.1 cells. E2A-HLF and E4BP4 levels were normalised to α -tubulin levels and the fold induction relative to 0 h levels determined.

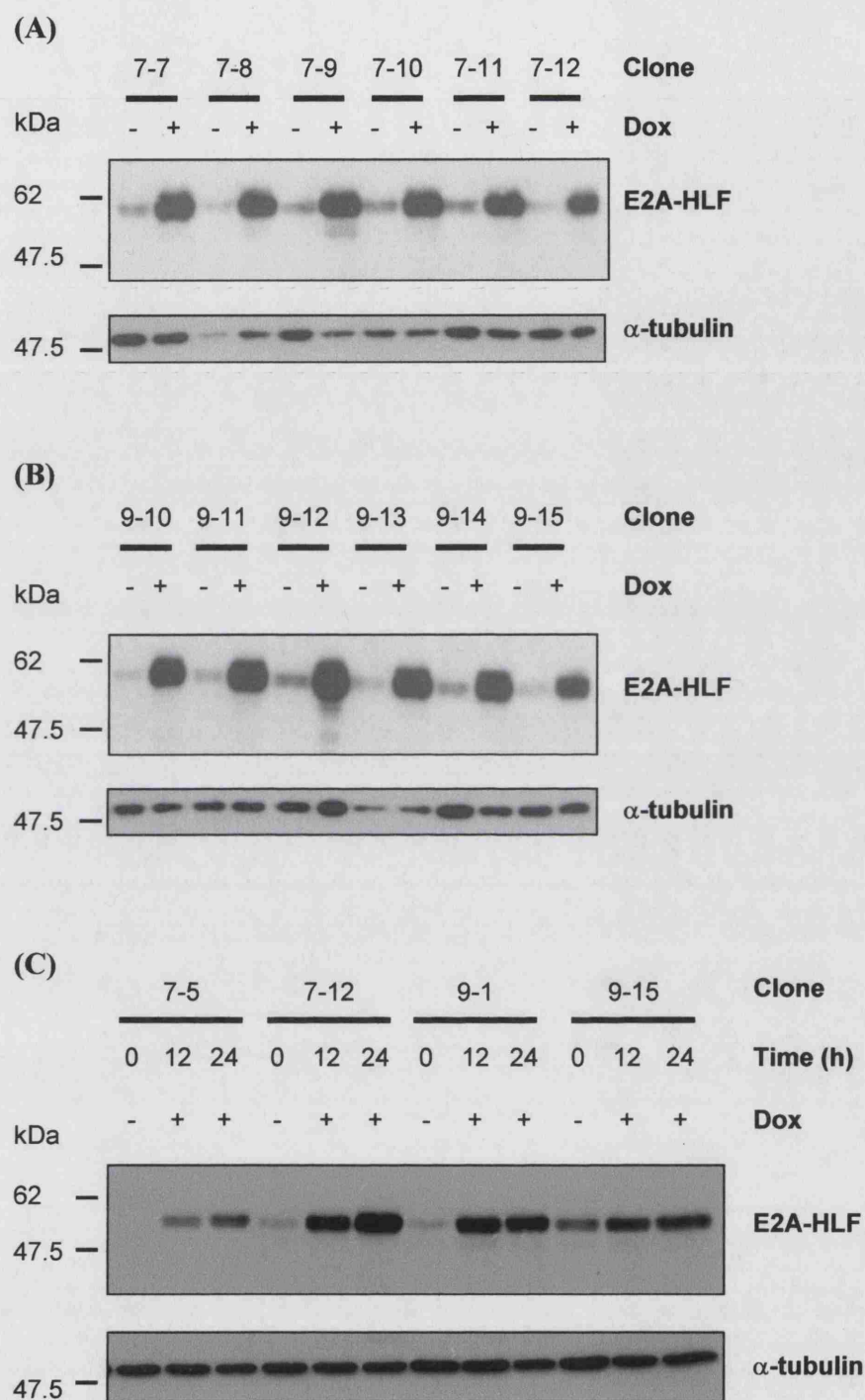


Figure 4.4. Inducible clones expressing E2A-HLF.

(A) and (B) Western blotting analysis of inducible expression of E2A-HLF in clones of stably transfected TonBaF.1 cells using anti-E2A after 24 h culture in presence of doxycycline for 24 h. (C) Level of expression of E2A-HLF protein over the course of 24 h of induction by doxycycline. E2A-HLF levels were normalised to α -tubulin levels and the fold induction relative to 0 h levels determined.

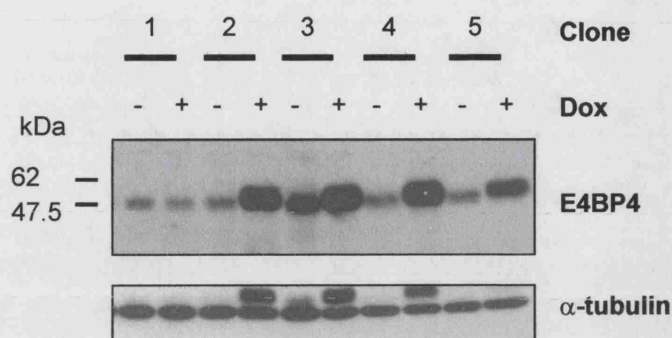
Approximately 20 clones were screened for the inducible expression of human E4BP4 by Western blotting using a commercially available anti-E4BP4 antibody. A selection of analysed clones are shown (**Figure 4.5A and 4.5B**). Different levels of inducible E4BP4 protein were expressed by different clones after induction with doxycycline for 24 h. Western blotting analysis of two inducible E4BP4 clones over the course of 24 h showed that a 1.3- to 1.6-fold increase above the endogenous level of E4BP4 was achieved within 4 h of induction, reaching maximum expression at 24 h (**Figure 4.5C**).

4.2.3. Expression of human E2A-HLF but not E4BP4 delays apoptosis induced by IL-3 withdrawal in murine pro-B cells

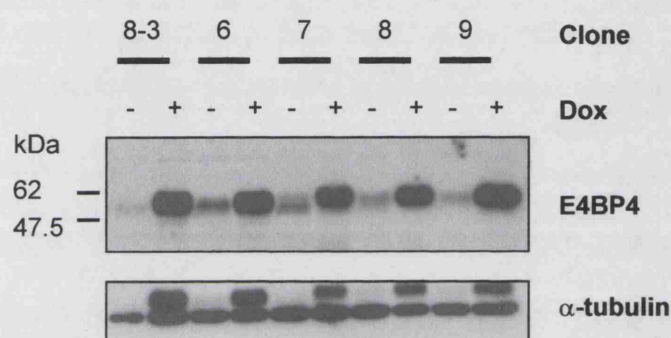
Expression of E2A-HLF in murine pro-B cells has been shown to delay apoptosis induced by IL-3 withdrawal (Dang *et al.*, 2001; Inaba *et al.*, 1996; Inukai *et al.*, 1998). To assess the anti-apoptotic effects of E2A-HLF, clones generated in the previous section which showed the highest inducibility with doxycycline after 24 h and expressed the lowest background in absence of doxycycline were further analysed in IL-3-withdrawal experiments. The degree of apoptosis was measured by quantification of hypodiploid ($\leq 2N$) DNA after PI staining followed by flow cytometry. The percentage of live cells was determined to be cells which were in G0/G1, S and G2/M phases of the cell cycle (2N to 4N DNA).

TonBaF.1 cells were initially analysed for the response of these cells to IL-3 withdrawal after culture in presence or absence of doxycycline (**Figure 4.6**). The presence or absence of doxycycline did not change the response of the cells to IL-3 withdrawal. Several inducible E2A-HLF clones were cultured in presence or absence of doxycycline for 12 h prior to IL-3 deprivation. Culture of the clones in

(A)



(B)



(C)

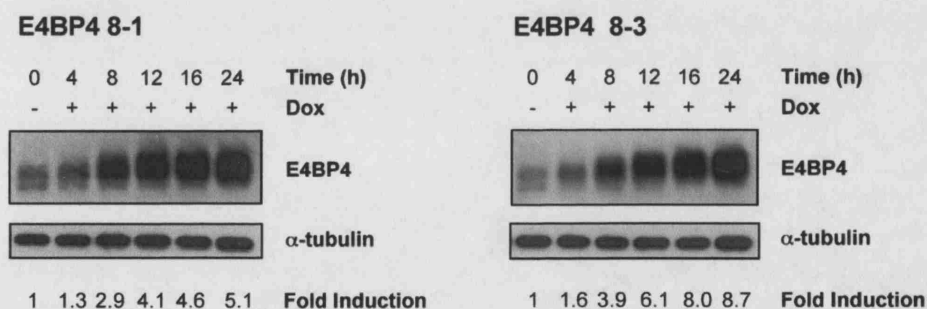


Figure 4.5. Inducible clones expressing E4BP4.

(A) and (B) Western blotting analysis of inducible expression of E4BP4 in clones of stably transfected TonBaF.1 cells after 24 h in presence of doxycycline. The upper band in the tubulin blots is E4BP4. (C) Level of expression of E4BP4 over the course of 24 h of induction by doxycycline.

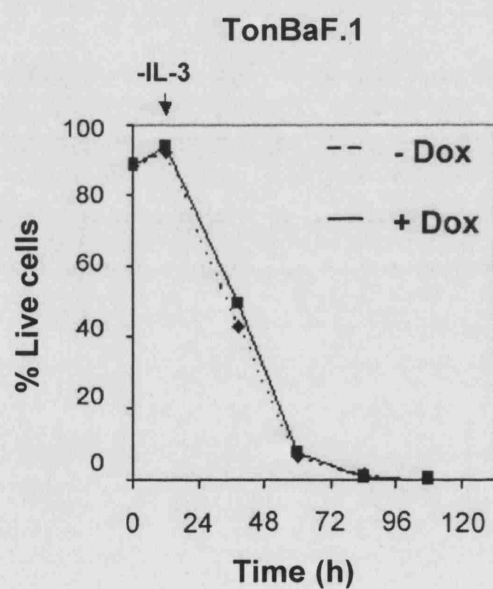


Figure 4.6. TonBaF.1 cells undergo apoptosis after IL-3 withdrawal.

TonBaF.1 cultured with or without doxycycline for 12 h and subsequently cultured in absence of IL-3 from the indicated time point (-IL-3). Percentage of live cells was determined by PI staining followed by flow cytometric analysis.

presence of doxycycline substantially delayed apoptosis induced by IL-3 deprivation (**Figure 4.7A, 4.7B and 4.7C**). Western blotting analysis of these three clones showed that the expression of E2A-HLF was dependent on the presence of doxycycline and that there was no or minimum background expression of E2A-HLF in absence of doxycycline (**Figure 4.7D, 4.7E and 4.7F**). The levels of α -tubulin were affected by the withdrawal of IL-3 in uninduced cells. E2A-HLF expression was maintained when cells were cultured in the presence of doxycycline over several days without IL-3.

The level of induced E2A-HLF expression as determined by Western blotting correlated with the level of protection conferred by E2A-HLF when clones were cultured in absence of IL-3. Clones E2A-HLF 7-12 and E2A-HLF 9-1 showed greater protection against IL-3 withdrawal induced apoptosis and higher levels of E2A-HLF protein expression in presence of doxycycline compared to clone E2A-HLF 7-5.

IL-3-withdrawal experiments were also performed using the inducible E4BP4 clones to assess the ability of human E4BP4 to promote survival of Baf-3 cells. In contrast to the inducible E2A-HLF clones, induction of E4BP4 in two clones for 12 h prior to culturing cells in the absence of IL-3 conferred no survival advantage compared with uninduced cells (**Figure 4.8A and 4.8B**). Western blotting analysis showed that human E4BP4 was induced and maintained by doxycycline throughout the different time points (**Figure 4.8C and 4.8D**). These results were similar for additional inducible E4BP4 clones in several other experiments.

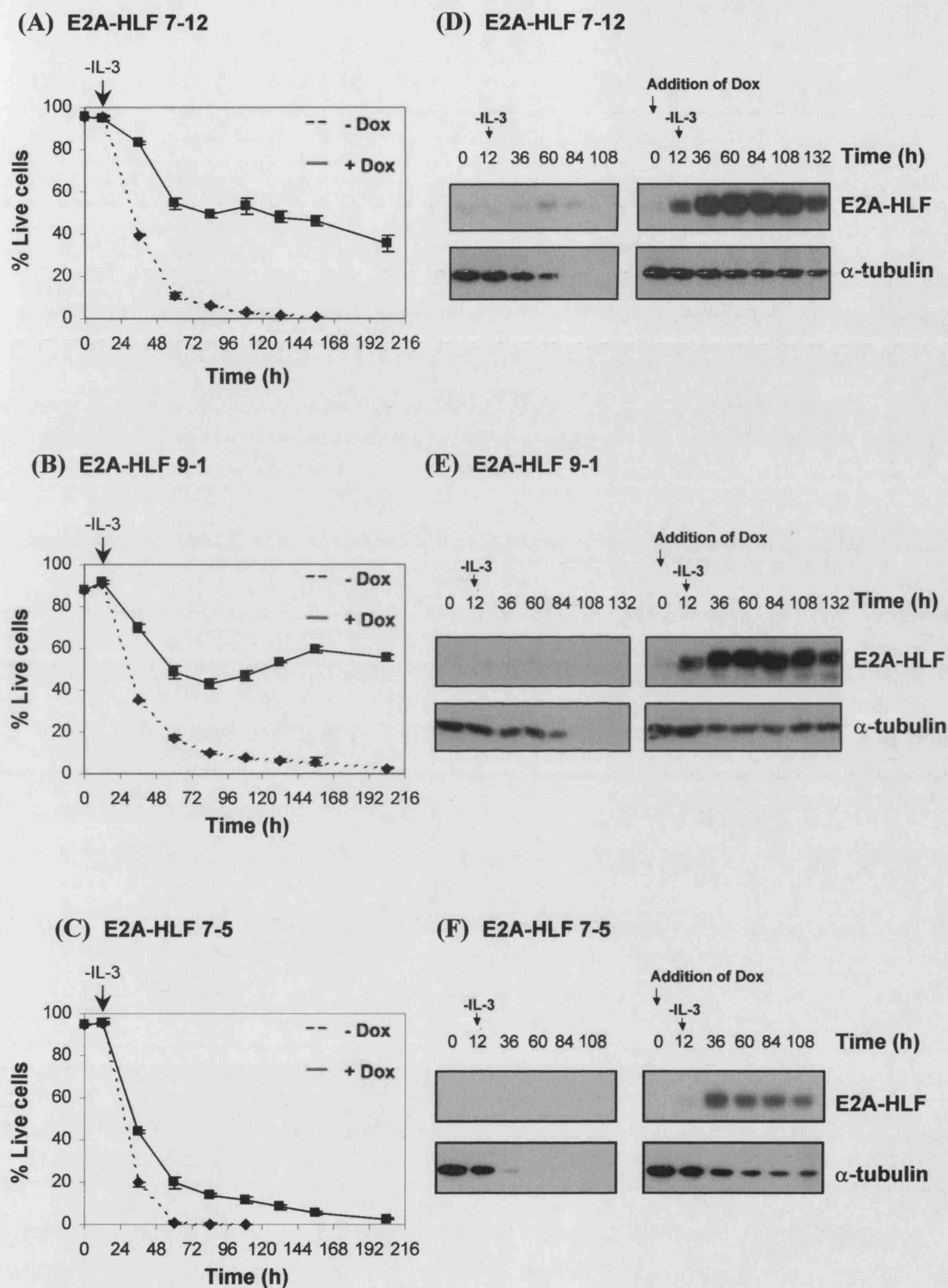
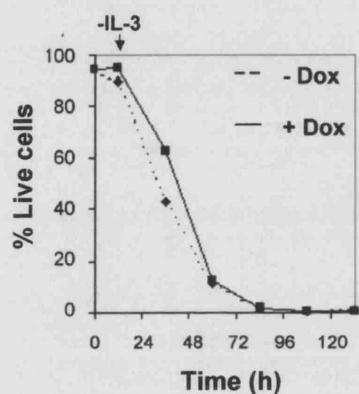


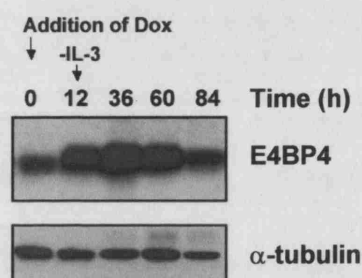
Figure 4.7. E2A-HLF protects against IL-3-withdrawal induced apoptosis in Baf-3 clones.

(A) (B) and (C) Three inducible E2A-HLF clones were cultured with or without doxycycline for 12 h and subsequently cultured in absence of IL-3 from the indicated time point (-IL-3). Percentage of live cells was determined by PI staining followed by flow cytometric analysis. Experiments were carried out in triplicate and bars represent standard error. (C) (E) and (F) Western blotting analysis of protein samples taken from the same clones at the indicate time points.

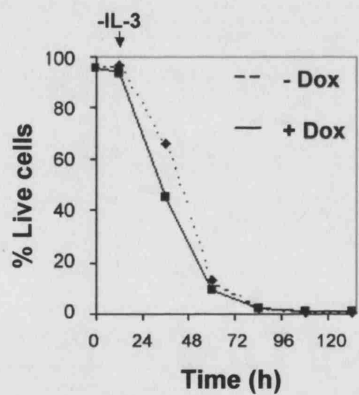
(A) E4BP4 8-1



(C) E4BP4 8-1



(B) E4BP4 8-3



(D) E4BP4 8-3

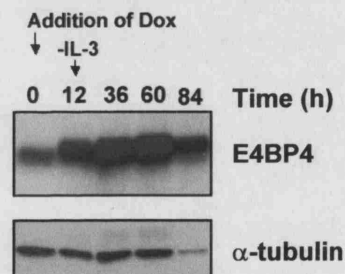


Figure 4.8. Overexpression of E4BP4 does not protect against IL-3-withdrawal induced apoptosis in Baf-3 clones.

(A) and (B) Two inducible E4BP4 clones were cultured with or without doxycycline for 12 h and subsequently cultured in absence of IL-3 from the indicated time point (-IL-3). (C) and (D) Western blotting analysis of protein samples taken from the same clones at the indicate time points.

4.2.4. E4BP4 and E2A-HLF recognise the same DNA sequences but have different transcriptional activities

To determine whether the absence of protection against IL-3 withdrawal induced apoptosis by the overexpressed human E4BP4 was due to expression of a non-functional protein, the function of the induced E4BP4 was further assessed using reporter assays to confirm that it functioned as a transcriptional repressor. The inducible E4BP4 clones were compared to the inducible E2A-HLF clones in reporter assays. Three inducible E4BP4 clones and three inducible E2A-HLF clones were electroporated with three different luciferase reporter constructs and cultured with or without doxycycline. The reporter constructs used were the pGL3-promoter (negative control), a pGL3-promoter containing either two HLF consensus sites (HLF-CS) or a pGL3-promoter containing three E4BP4 consensus sites (E4BP4-CS).

All the luciferase reporter constructs were transactivated by approximately 3-fold in the Empty Vector clone in the presence of doxycycline (**Figure 4.9**). This clone was derived from TonBaF.1 cells stably transfected with the pTRE2hyg construct. It was noted that doxycycline alone had an effect on the pGL3-promoter reporter construct in all the inducible clones (**Figure 4.9**). The presence of the HLF or E4BP4 recognition sequences overrode the non-specific transcriptional effects of doxycycline. The inducible E4BP4 clones showed repression of the HLF-CS reporter and the E4BP4-CS reporter constructs by the equivalent of 0.7- and 0.6-fold respectively when compared to the pGL3-promoter luciferase levels.

The transcriptional activity of E2A-HLF was assessed using the same reporter constructs. The inducible clones showed transactivation of the HLF-CS and E4BP4-

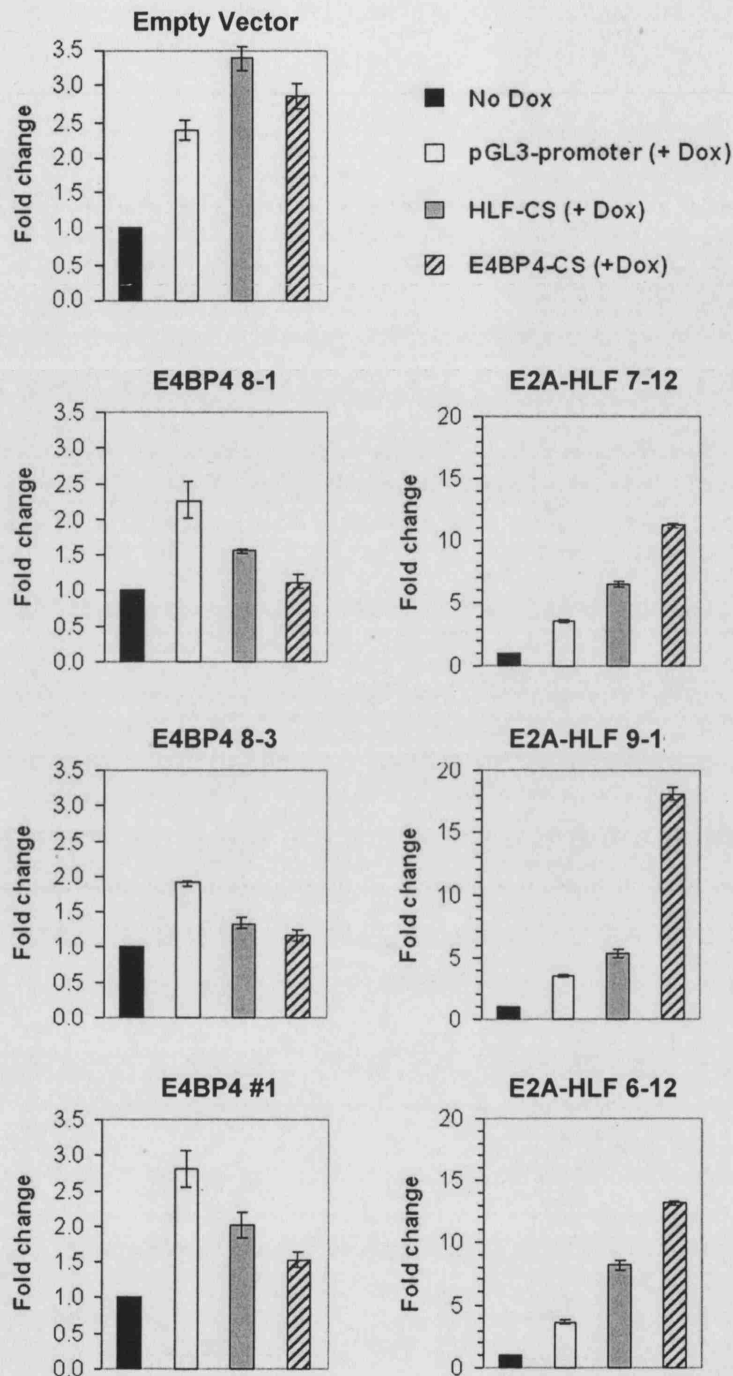


Figure 4.9. E4BP4 and E2A-HLF recognise the same DNA sequences but have different transcriptional activities in Baf-3 cells.

The transcriptional activities of E4BP4 and E2A-HLF were assessed by luciferase reporter gene assays using reporter constructs containing E4BP4 DNA consensus sites (E4BP4-CS) or HLF DNA consensus sites (HLF-CS) or neither (pGL3-promoter). An Empty Vector clone and inducible E4BP4 and E2A-HLF clones were electroporated with the reporter constructs and cultured with or without doxycycline for 48 h. Means of triplicate experiments after doxycycline induction are expressed relative to the luciferase activity obtained with uninduced cells. Bars indicate standard error.

CS reporter constructs after the cells were induced to express E2A-HLF by doxycycline. The increase in transactivation of HLF-CS and E4BP4-CS reporter constructs was 5- to 8-fold and 11- to 18-fold respectively. The level of transactivation of these reporter constructs was also greater than that obtained from the pGL3-promoter reporter construct.

The transcriptional repression activity of E4BP4 and transactivational activity of E2A-HLF was also confirmed in HeLa cells. Co-transfection experiments were carried out with the p π S12(34)CAT reporter construct which contains three E4BP4 binding sites upstream of a human glutathione-S-transferase (GST) π promoter (Cowell *et al.*, 1992). A dose-dependent transactivation of this reporter construct by E2A-HLF was demonstrated (**Figure 4.10A**). A 2-fold activation of the reporter construct was achieved with 0.01 μ g of the E2A-HLF expression construct.

Maximum activation of approximately 12-fold was achieved using 0.2 μ g of the E2A-HLF expression vector. A dose-dependent repression by E4BP4 was also demonstrated (**Figure 4.10B**). Approximately 0.8-fold change was evident with 0.01 μ g of the E4BP4 expression construct. Maximum repression of approximately 0.15-fold change was achieved with 0.5 μ g of E4BP4 expression construct.

These data indicate that induced human E4BP4 is functional in the E4BP4 clones, but does not confer a survival advantage in absence of IL-3. Furthermore, E4BP4 in Baf-3 and HeLa cells functions as a transcriptional repressor as previously described (Chen *et al.*, 1995; Cowell *et al.*, 1992; Doi *et al.*, 2001), whereas E2A-HLF functions as a transactivator (Hunger *et al.*, 1994; Begbie *et al.*, 1999).

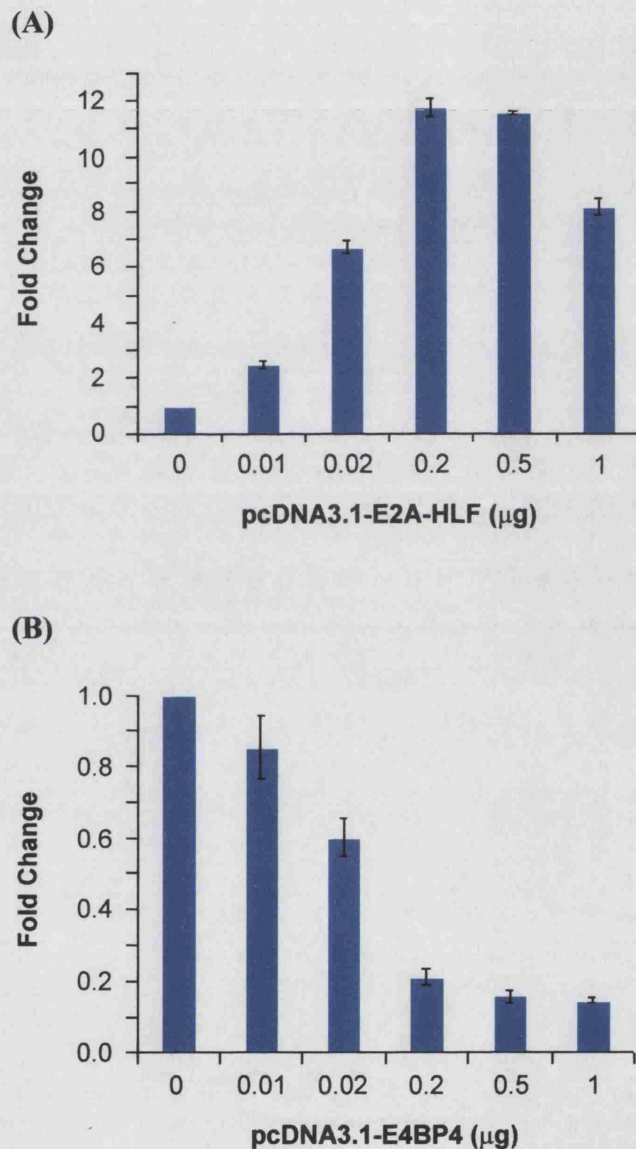


Figure 4.10. E2A-HLF and E4BP4 can recognise the same DNA sequences but have different transactivational activities in HeLa cells.

(A) E2A-HLF recognised the same DNA consensus sequence in the p π S12(34)CAT reporter construct but demonstrates a dose-response transactivation of CAT expression. Error bars represent standard error. (B) E4BP4 recognised the DNA consensus sequence in the p34CAT reporter construct and demonstrates a dose-response repression of CAT expression.

4.2.5. *E2A-HLF induces the expression of E4BP4*

The possibility that endogenous E4BP4 expression could be regulated by E2A-HLF was also investigated. E4BP4 mRNA and protein levels were analysed from cells that had been induced to express E2A-HLF for 12 h and then cultured in absence of IL-3 over a 24 h period. The E4BP4 protein levels initially decreased in all the clones after IL-3 withdrawal (**Figure 4.11A**). Whilst the E4BP4 protein levels in the Empty Vector clone continued to decrease over 24 h after IL-3 withdrawal, the E4BP4 protein levels increased by 24 h after IL-3 removal in all the clones induced to express E2A-HLF. A 1.7- to 2.5- fold increase in E4BP4 proteins levels was found at 24 h after IL-3 withdrawal compared to the levels at found at 12 h after IL-3 withdrawal. The E4BP4 levels induced by E2A-HLF are maintained for at least 72 h after IL-3 withdrawal (data not shown).

Northern blotting analysis also confirmed that expression of E2A-HLF induced E4BP4 RNA levels by 24 h after IL-3 removal. At this time point, E4BP4 mRNA levels were similar to those in the presence of IL-3. The E4BP4 RNA levels at this time point were approximately 1.6- fold to 2.9-fold greater compared to the E4BP4 mRNA levels at 12 h after IL-3 withdrawal (**Figure 4.11B**). Using the 28S and 18S ribosomal bands as an alternative loading control to the GAPDH RNA levels (which decreased after 12h of IL-3 starvation), these results indicate that E4BP4 is either a direct or indirect transcriptional target of the E2A-HLF oncoprotein in pro-B cells.

4.3. Discussion

4.3.1. *Regulation of E4BP4 by IL-3*

In order to understand the role of E4BP4 and E2A-HLF in the survival of pro-B cells, a model system was established to inducibly express E2A-HLF or inducibly

(A)



(B)



Figure 4.11. E2A-HLF can regulate E4BP4 expression.

(A) Western blotting was carried out on inducible E2A-HLF and Empty Vector clones to determine the levels of E2A-HLF and E4BP4 protein levels after induction with 2 μ M doxycycline either in the presence and absence of IL-3 for the indicated times. (B) Northern blotting analysis was also performed using 10 μ g total RNA from the cells in (A). Membranes were probed for E4BP4 and reprobed for GAPDH. The same amount of RNA used for the Northern blot was loaded separately and then stained with ethidium bromide for use as an alternative loading control.

overexpress E4BP4. E4BP4 and E2A-HLF clones were shown to rapidly express human E4BP4 or E2A-HLF on addition of doxycycline to the cell culture medium. The IL-3 regulated expression of E4BP4 protein and mRNA was also confirmed by Western blotting and by Northern blotting analysis. The levels of mRNA do not decrease as much as the protein levels. This could be due to other pathways that may be involved in regulating the levels of E4BP4 that are independent of the presence of IL-3. These pathways may involve Raf mitogen-activated protein kinase and PI-3 kinase signalling pathways which have been proposed to increase E4BP4 activity via Ras (Kuribara *et al.*, 1999).

4.3.2. Background expression of E2A-HLF and E4BP4 in the inducible Baf-3 cells

Although E2A-HLF and human E4BP4 were induced by doxycycline in the inducible Baf-3 clones that were generated, some background expression, of E2A-HLF in particular, was evident in absence of doxycycline. The background level of E2A-HLF in absence of doxycycline was variable between different clones and also between different experiments. This background expression was possibly due to small amounts of tetracycline present in the foetal calf serum used for these experiments. A combination of non-optimum cell density and non-optimum IL-3 levels in the medium may have influenced the levels of background expression particularly if the expression of E2A-HLF has a pro-survival function. Minimal transcription from the promoter in the absence of doxycycline may be due to the site of integration of the rtTA expression construct. The site of integration into euchromatin or heterochromatin can influence the level of expression of a transgene (Alberts *et al.*, 1989).

Background levels of inducible human E4BP4 in the absence of doxycycline was difficult to determine due to the presence of the endogenous murine E4BP4 protein. The endogenous levels of murine E4BP4 are probably determined by the levels of IL-3 in the medium which would change over time in a culture, and between cultures, as cells utilise the cytokine. This may account for the variable levels of endogenous E4BP4 expressed in the absence of doxycycline between clones (**Figure 4.5**). The level of endogenous E4BP4 detected may also have been due to the antibody used. A gift of a limited amount of a rabbit anti-E4BP4 antiserum (from Dr Toshiya Inaba, Hiroshima University, Japan) was initially used for Western blotting analysis (**Figure 4.3B**) and appeared to detect human E4BP4 better than murine E4BP4. A commercial goat anti-E4BP4 antibody became available and was used to detect E4BP4 in all other Western blotting analysis. This antibody was able to detect both murine and human E4BP4 to a similar extent. Use of an epitope-tagged human E4BP4 construct would have overcome the problems of differentiating between endogenous and leaky induced E4BP4 protein.

4.3.3. The function of induced expression of E2A-HLF and E4BP4

The ability of E2A-HLF and E4BP4 to function as survival factors in pro-B cells was assessed by inducing E2A-HLF or E4BP4 expression for 12 h prior to the removal of IL-3. E2A-HLF was confirmed to act as an anti-apoptotic factor. In contrast to previously published results (Ikushima *et al*, 1997; Kuribara *et al*, 1999), data presented here showed that overexpression of E4BP4 in Baf-3 cells does not confer any resistance to apoptosis induced by IL-3 deprivation. This suggests that E4BP4 by itself is unable to function as a survival factor in Baf-3 cells. Therefore, the data presented here opposes previous reports that have hypothesised that the anti-apoptotic function of E2A-HLF is due to the replacement of the function of E4BP4

in pro-B cells in which the t(17;19) translocation has occurred (Ikushima *et al.*, 1997).

4.3.4. Transactivational activities of E2A-HLF and E4BP4

The role of E4BP4 in regulating transcription was assessed in Baf-3 cells using luciferase assay reporter constructs containing three E4BP4 DNA-binding consensus sequences (E4BP4-CS) or two HLF DNA-binding consensus sequences (HLF-CS). Overexpression of E4BP4 repressed the expression of both E4BP4-CS and HLF-CS luciferase reporter constructs, whereas E2A-HLF transactivated the same reporters. These data demonstrate that E4BP4 and E2A-HLF have different effects on the same promoter. As E2A-HLF has anti-apoptotic properties, if anything, E4BP4 might therefore be expected to have pro-apoptotic properties. However, in the system used here, E4BP4 has no effect on the survival of Baf-3 cells following IL-3 withdrawal. E2A-HLF has been shown to block p53-mediated cell death in murine myeloid leukaemia cells which constitutively express a temperature-sensitive *p53* mutant gene (Altura *et al.*, 1998). The same study also showed that E4BP4 had opposite effects to those of E2A-HLF by potentiating p53-induced apoptosis.

E4BP4 has been mainly characterised as a transcriptional repressor (Cowell *et al.*, 1992; Chen *et al.*, 1995; Doi *et al.*, 2001). However, E4BP4 has also been shown to activate transcription of a reporter gene downstream of an IL-3 promoter in T cells (Zhang *et al.*, 1995). This suggests that the overexpression of E4BP4 could induce the expression of IL-3 in pro-B cells which could in turn promote survival via an autocrine mechanism in the absence of exogenous IL-3. However, IL-3 mRNA was not detected in FL5.12 cells overexpressing E4BP4 and conditioned medium from these cells did not promote survival or growth of wild-type cells (Ikushima *et al.*,

1997). The generation of E4BP4-null mice should definitively determine whether E4BP4 has any role in cell survival. The downstream targets of E4BP4 in pro-B cells have yet to be identified.

4.3.5. E4BP4 as a downstream target of E2A-HLF

E4BP4 was identified as a new target of E2A-HLF. E4BP4 may participate in downstream leukaemogenic process such as immortalisation or proliferation, either alone or in synergy with other targets of E2A-HLF in pro-B cells. Oncogenic Ras mutants have been shown to induce the expression of E4BP4 and it has also been implied that BCR-ABL expression in Baf-3 cells results in induction of E4BP4 (Kuribara *et al*, 1999).

A recent study that involved analysis of genes that were differentially expressed in the brains of mice which are deficient for all three PAR bZIP proteins (TEF^{-/-}/DBP^{-/-}/HLF^{-/-}) or which only expressed TEF (TEF^{+/+}/DBP^{-/-}/HLF^{-/-}) showed that E4BP4 was expressed at higher levels in the TEF^{+/+}/DBP^{-/-}/HLF^{-/-} mice compared to the triple knockout mice. This suggests that TEF upregulates the expression of E4BP4 (Gachon *et al.*, 2004). The ability of TEF to upregulate the expression of E4BP4 suggests that other PAR bZIP proteins, e.g. E2A-HLF, might also be able to upregulate E4BP4 and therefore supports the results presented in this chapter.

4.3.6. Conclusions

Data in this chapter confirmed that E2A-HLF has anti-apoptotic activities, is a transactivator in Baf-3 cells and one of its transcriptional targets is E4BP4. E2A-HLF may cause ALL by altering the transcriptional profile of pro-B cells so that the target genes which are activated contribute to the leukaemic phenotype. Previous reports have suggested that E2A-HLF might replace the function of E4BP4 in cells

in which the t(17;19) translocation has occurred and thus account for the anti-apoptotic function of E2A-HLF (Ikushima *et al*, 1997). Data presented in this chapter provides evidence opposing this hypothesis as overexpression of E4BP4 does not delay apoptosis induced by IL-3 withdrawal in Baf-3 cells whereas E2A-HLF delays apoptosis in IL-3-deprived Baf-3 cells.

CHAPTER 5

RESULTS (3)

5.1. Background

5.1.1. Microarray technology

Microarray technology enables thousands of genes to be analysed simultaneously to delineate the changes in the gene expression in cells that have been subjected to a particular treatment or express a specific protein. The Affymetrix GeneChip[®] probe arrays contain oligonucleotide probes located in specific areas (Affymetrix, 2004). Eleven to sixteen probes on the array represent each gene transcript. For each probe, a mismatch probe is generated which differs by a single central nucleotide. Mismatch probes act as controls for background and non-specific cross-hybridisation and help indicate the relative abundance of the specific RNA. Information regarding the oligonucleotide sequence, gene ontology, gene symbol, description and GenBank accession numbers for each probe are deposited in databases that are regularly updated. The Murine Genome U74v2 Set is comprised of three arrays and represents approximately 36,000 full-length genes and expressed sequence tag (EST) clusters. The sequences represented are derived from sequence clusters in Build 74 of the UniGene Database.

5.1.2. Sample preparation for Affymetrix GeneChip[®] arrays

A schematic overview of the steps involved in order to obtain gene expression data using Affymetrix GeneChip[®] arrays is illustrated in **Figure 5.1**. Total RNA derived from an experimental sample is initially reverse transcribed to cDNA. *In vitro* transcription is then used to generate biotin-labelled complementary RNA (cRNA). The cRNA is used in a hybridisation cocktail for hybridisation to a GeneChip[®] probe array. The probe array is stained with a streptavidin-phycoerythrin conjugate which binds to the biotin-labelled cRNA. The probe array is scanned and the intensity of

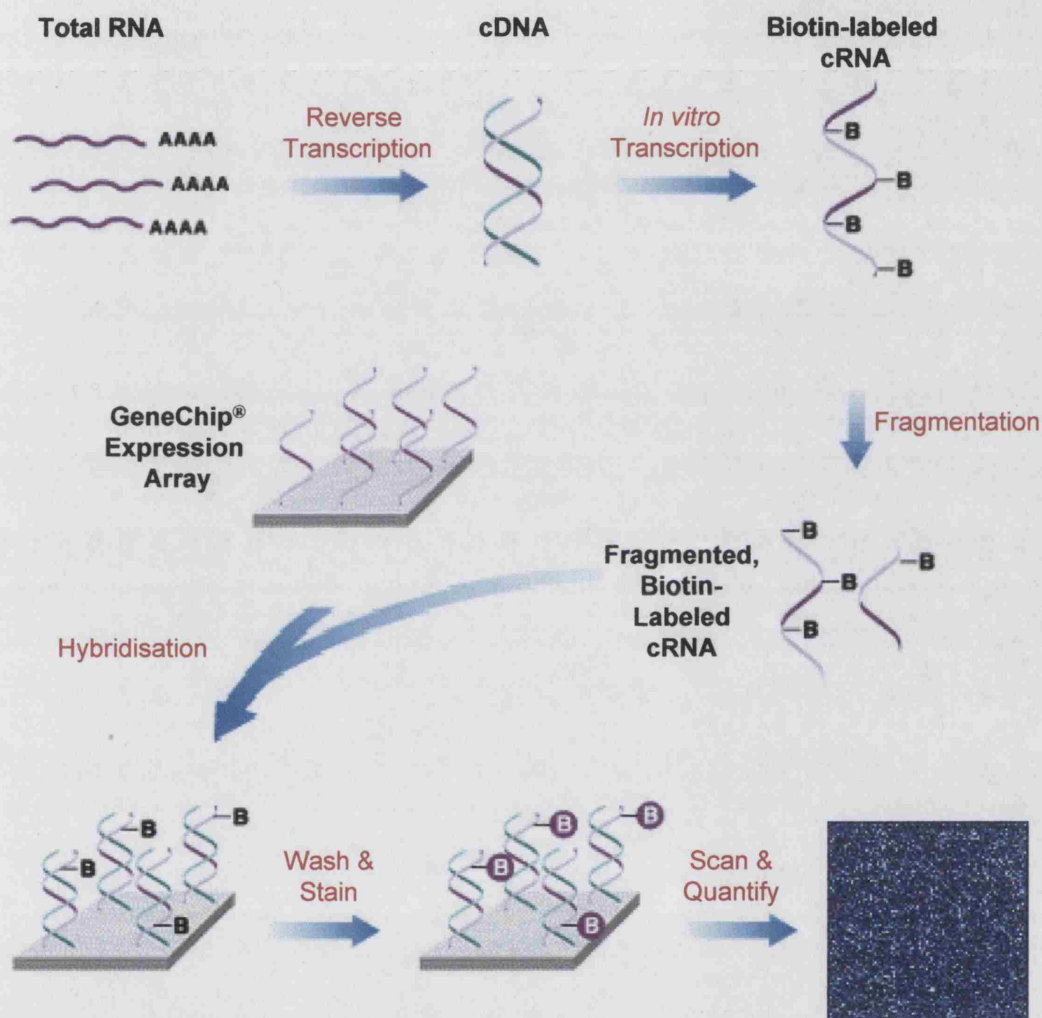


Figure 5.1. Overview of Affymetrix GeneChip[®] technology.

Labeled cRNA targets derived from the mRNA of an experimental sample are hybridised to nucleic acid probes attached to the solid support. The abundance of each mRNA species can be inferred by monitoring the amount of label associated with each DNA location. Adapted from an image from the Affymetrix website.

fluorescence emitted at 570 nm is proportional to the bound target at each location on the probe array. The relative expression levels of each gene on the probe array can be determined and subjected to statistical analyses to identify genes that are significantly changed compared to a control array.

5.2. Results

5.2.1. Differential gene expression in pro-B cells expressing E2A-HLF

The chimaeric nature of the E2A-HLF transcription factor suggested that the genes that it regulates are likely to be different to those normally regulated by either E2A or HLF. Microarray studies were carried out to identify genes that were regulated by E2A-HLF. Induced expression of E2A-HLF for 12 h in Baf-3 cells was sufficient to delay apoptosis after withdrawal of IL-3 (see Chapter 4). Three independent replicate inducible E2A-HLF Baf-3 clones were chosen and cultured in absence or presence of doxycycline for 12 h to induce expression of E2A-HLF. Three independent experiments were carried out using the three clones. Total RNA was extracted from cells, processed and hybridised to Affymetrix MG-U74Av2 microarrays.

Affymetrix Microarray Suite 5.0 software was used to calculate gene expression values for each probe array. Data was analysed using Genespring 5.1 software. A normalisation step was applied to each array to allow comparison of gene signals across multiple arrays. A scale factor was also given for each array as a measure of the array image intensity. The scale factors of the arrays used for further analysis varied between 0.52 to 1.50, with one array having a scale factor of 6.4. Data from one pair of arrays of a replicate experiment, using clone E2A-HLF 7-12, was excluded from further analysis as one of this pair of arrays had a scale factor of 18.

Data from this array chip was considered to be unreliable. Non-biological factors such as amount and quality of the cRNA, amount of stain, or other experimental variation can contribute to the overall variability in hybridisation intensities between different arrays (Affymetrix, 2004).

The gene expression level of the induced sample was normalised to the expression level of the uninduced control sample for each experiment. This allowed of the expression levels of genes between the induced and uninduced samples to be compared. Data was subsequently filtered to identify 6418 genes that were determined to be reliably detected in both the induced and uninduced samples. These genes were further filtered by Welch t-test to identify 46 genes which were statistically significantly changed by greater than 1.3-fold by the E2A-HLF expression. Multiple testing correction on this set of genes was performed using Benjamini-Hochberg false discovery rate procedure (FDR). Fifteen genes passed this restriction.

5.2.2. Genes upregulated by E2A-HLF

Of the 46 genes that were significantly changed by greater than 1.3-fold, twenty-six genes were upregulated by the expression of E2A-HLF (**Table 5.1**). The genes that were upregulated by E2A-HLF were changed by a maximum of 2.6-fold. Twelve of these genes passed Benjamini-Hochberg FDR procedure. The target genes upregulated by E2A-HLF were categorised according to their biological role. The products of genes upregulated by E2A-HLF were categorised as: (1) regulators of transcription; (2) signal transducers; (3) regulators of apoptosis; (4) involved in DNA repair; (5) cytokines; (6) enzymes or transporters and (7) receptors (**Table 5.3**). The

Table 5.1. Genes upregulated by E2A-HLF.

Probe set ID	Genbank ID	P-value	Fold Change	Gene symbol	Notes
1 94174_at	AF006071	0.0499	2.633	<i>Catn1</i>	<i>Catenin alpha-like 1</i> . Homologous to alpha-catenin. Downregulated in human pancreatic cell line AsPC-1 after NaB treatment. NaB induces growth inhibition and apoptosis AsPC-1 cells.
2 101649_at	AF048695	0.0278	2.552	<i>Pip5k1a</i>	Phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha. Synthesises phosphatidylinositol 4,5-bisphosphate which is hydrolysed by PKC to generate second messenger molecules. Implicated in G protein regulation. Wide tissue expression. Overexpression can delay apoptosis.
3 160895_at	L08266	0.0024	2.129	<i>Facc</i>	Fanconi Anaemia complementation group C. DNA repair enzyme involved in maintenance of normal chromosome stability. Required for optimal activation of STAT1 in response to cytokine and growth factors and for suppressing cytokine-induced apoptosis by modulating the activity of double-stranded RNA-dependent protein kinase.
4 104750_at	M63630	0.0016	1.880	<i>IRG-47</i>	Interferon gamma inducible protein 47. Contains motifs characteristic of guanine nucleotide-binding proteins. Is rapidly and transiently induced by interferon- γ in pre-B, B cell, stromal cells and fibroblast cell lines.
5 102963_at	L21973	0.0387	1.799	<i>E2f1</i>	Transcription Factor. Required for G1/S-phase transition during the mammalian cell cycle. Activates transcription of genes required for DNA replication. E2F activity is regulated by pRb. Myc induces transcription of the E2F1. Overexpression can induce apoptosis under certain conditions.
6 93856_at	M55512	0.0337	1.766	<i>Wt1</i>	Wilms' tumour homologue. Transcription Factor. Expressed in the kidney and a subset of haematopoietic cells. Overexpressed in most cases of adult and paediatric AML, ALL, CML and MDS. Suppresses transcription of haematopoietic-related proteins including M-CSF, TGF β , and RAR- α .
7 97957_at	AF072759	0.0493	1.712	<i>Slc27a4</i>	Solute carrier family 27 (fatty acid transporter), member 4/fatty acid transport protein 4 (<i>FATP4</i>).
8 96810_at	A1154017	0.0037	1.600	<i>Lmo2</i>	Lim only 2. Involved in the formation of multimeric DNA-binding complexes for the generation of haematopoietic lineages and angiogenesis (in mice). Is overexpressed in T acute lymphoblastic leukaemia with the t(11;14) translocation.
9 161037_at	U14332	0.0001	1.595	<i>IL15</i>	Interleukin 15. T-cell growth factor which affects T-cell activation and proliferation similarly to IL-2. IL-15 transgenic mice have early expansions in NK and CD8+ T lymphocytes and later develop fatal lymphocytic leukaemia with a T-NK phenotype.
10 102313_at	L09737	0.0134	1.546	<i>Gch1</i>	GTP cyclohydrolase 1. Involved in tetrahydrobiopterin biosynthesis. Defects cause an atypical severe form of phenylketonuria.
11 96640_at	A1644158	0.0005	1.500	<i>EST</i>	Weakly similar to human Drr1/TU3A. Is downregulated in renal cell carcinoma. Expressed in brain, heart, placenta, lung liver, skeletal muscle, kidney and pancreas. Unknown function.

Genes highlighted in bold indicates those that have passed the Benjamini-Hochberg false discovery rate procedure.

Table 5.1. Genes upregulated by E2A-HLF (continued).

Probe set ID	Genbank ID	P-value	Fold Change	Gene symbol	Notes
12 93627_at	AI852287	0.0001	1.483	Ankrd28	Ankyrin repeat domain 28
13 95426_at	AW048512	0.0016	1.469	Esch1	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial. Involved in fatty acid metabolism.
14 103699_i_at	AI646638	0.0285	1.455	Frat2	Frequently rearranged in advanced T-cell lymphomas 2. Binds GSK-3 and prevents GSK-3-dependent phosphorylation. Involved in Wnt signalling. Highest expression in pancreas, heart, spleen, placenta, skeletal muscle, liver, peripheral blood leukocytes and foetal kidney. Expression is higher in gastric cancer, cervical cancer and chronic myelogenous leukaemia cell lines than in other cancer cell lines.
15 95637_at	AI838592	0.0003	1.429	Flnb	<i>Filamin β</i> . Filamins are actin-binding proteins that stabilise actin webs and links them to cellular membranes. Also interact with numerous cellular proteins including Gp1b, FcR1, Rho, Rac, cdc42, Sek1.
16 97375_at	U70209	0.0324	1.417	Pdk1	Polycystic kidney disease 1 protein/polycystin. Encodes a membrane protein involved in cell-to-cell or cell-matrix interactions. Found in a complex containing E-cadherin and α -, β -, and γ -catenin. May be an ion-channel regulator. Induces resistance to apoptosis and regulates differentiation in MDCK cells.
17 103048_at	M12731	0.0004	1.383	Nmyc	Transcription factor. Functions similarly to c-myc. Amplified in neuroblastoma, retinoblastoma and small cell lung carcinoma. Activated by Pax-5 in pre-B cells.
18 98869_g_at 98868_at	L31532	0.0004 0.0007	1.376 1.301	Bcl2	Anti-apoptotic protein. Inhibits apoptosis in response to many cytotoxic insults. Is juxtaposed to the IgH locus by t(14; 18) chromosomal translocation of human follicular B-cell which results in inappropriately elevated levels of Bcl-2 RNA and protein.
19 102337_s_at	M31312	0.0282	1.374	FcgRII	Fc receptor, IgG, low affinity IIb isoform/CD32. Overexpressed in follicular lymphoma carrying the t(1;22) and may play a role in tumour progression.
20 160911_at	Z11574	0.0064	1.340	Sos1	Mouse son of sevenless 1 homologue. Interacts with Grb2. Guanine nucleotide exchange factor. Involved in IL-3 mediated signalling pathway via Ras.
21 160491_at	AW125589	0.0054	1.323	EST	NEDD8-conjugating enzyme/ubiquitin-conjugating enzyme.
22 92392_at	AI850484	0.0031	1.319	EST	Similar to rat K ⁺ channel forming (RCK) proteins
23 94046_at	U05247	0.0360	1.316	Csk	C-src tyrosine kinase. Phosphorylates Src family tyrosine kinases and downregulates their activities <i>in vitro</i> and <i>in vivo</i> .
24 101997_at	AI851598	0.0019	1.315	Apg12l	Autophagy 12-like (<i>S. cerevisiae</i>). Involved in a ubiquitin-like protein conjugation system in autophagy.

Genes highlighted in bold indicates those that have passed the Benjamini-Hochberg false discovery rate procedure.

Table 5.1. Genes upregulated by E2A-HLF (continued).

Probe set ID	Genbank ID	P-value	Fold Change	Gene symbol	Notes
25 95379_at	AA245183	0.0229	1.310	<i>Mab21/2</i>	<i>Mab-21-like 2</i> . Homologue of a <i>C. elegans</i> cell-fate specification gene, <i>Mab21</i> . May be downstream of TGFβ1 signalling.
26 102745_at	M18858	0.0001	1.304	<i>Tcrg-V4</i>	T-cell receptor gamma, variable 4

Genes highlighted in bold indicates those that have passed the Benjamini-Hochberg false discovery rate procedure.

Table 5.2. Genes downregulated by E2A-HLF.

	Probe set ID	Genbank ID	P-value	Fold Change	Gene symbol	Notes
1	98891_at	AW011716	0.0432	0.585	<i>EST</i>	Unknown
2	103813_at	D85926	0.0292	0.611	<i>Sh3yl1</i>	Sh3 domain YSC-like 1. Expressed in anagen hair follicle. Unknown function.
3	161228_f_at	AV260677	0.0026	0.638	<i>Mena</i>	Mammalian <i>enabled</i> homologue (<i>Drosophila</i>). Involved in actin filament organisation. Binds to ABL and Src SH3 domains. Is a target of Abl kinase.
4	97759_at	U09383	0.0286	0.657	<i>Kcnma1</i>	Calcium-activated potassium channel.
5	103690_at	AW125574	0.0350	0.660	<i>Whscr5</i>	Williams-Beuren syndrome chromosome region 5 homologue (human). Includes LIM kinase 1, a serine/threonine protein kinase which phosphorylates and inactivates the actin binding/depolymerising factor cofilin and induces actin cytoskeletal changes. LIMK1 is increased in invasive breast and prostate cancer cell lines in comparison with less invasive cells.
6	95284_at	AI662509	0.0155	0.663	<i>EST</i>	FcRX/Fc receptor homologue expressed in B cells.
7	93728_at	X62940	0.0001	0.668	<i>Tgrb1i4</i>	<i>TGF-β1</i> induced transcript 4/TSC-22. Decreased TSC-22 mRNA levels in human brain and salivary gland tumours. Overexpression of TSC-22-GFP fusion protein increases the sensitivity of the cells to anticancer drugs. Translocates to nucleus concomitant with radiation-induced apoptosis.
8	92472_f_at	AF099973	0.0064	0.670	<i>Slfn2</i>	Schlafen 2. Schlafen genes are differentially regulated during thymocyte maturation and are preferentially expressed in the lymphoid tissues.
9	97560_at	AF037437	0.0332	0.679	<i>Psap</i>	Prosaposin. Precursor glycoprotein for saposins A, B, C, and D which activate several lysosomal hydrolases involved in sphingolipid metabolism. Saposins accumulate in tissues of patients with lysosomal storage diseases. Prosaposin gene mutations causes sphingolipidosis.
10	162403_at	AV282092	0.0288	0.685	<i>EST</i>	Similar to hypothetical yeast DNA-binding domain containing protein. Unknown function.
11	160821_r_at	AW124336	0.0108	0.695	<i>Ppp6c</i>	Protein phosphatase 6 catalytic subunit. Human Ppp6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1 which are involved in regulating cell cycle progression.
12	93833_s_at	X05862	0.0001	0.701	<i>Hist1h2bc</i>	Histone 1, H2bc. Involved in chromosome organisation.
13	93836_at	AF041054	0.0447	0.709	<i>BNIP3</i>	E1B 19K/Bcl-2-binding protein 3. Pro-apoptotic mitochondrial protein that binds to adenovirus E1B 19 kDa protein or to Bcl-2. Can overcome Bcl-2 suppression. Interacts with Bcl-2, Bcl-X(L), and CED-9.

Genes highlighted in bold indicates those that have passed the Benjamini-Hochberg false discovery rate procedure.

Table 5.2. Genes downregulated by E2A-HLF (continued).

Probe set ID	Genbank ID	P-value	Fold Change	Gene symbol	Notes
14 98478_at	U95826	0.0316	0.718	<i>Cong2</i>	Cyclin G2. Expressed at high levels in cerebellum, thymus, spleen, and prostate and at low levels in skeletal muscle. Cyclin G1 induction is p53-dependent, but activation of cyclin G2 expression is occurs in absence of p53. Is upregulated as cells undergo cell cycle arrest or apoptosis in response to inhibitory stimuli independent of p53.
15 162272_r_at	AV365041	0.0269	0.720	<i>Stxbp2</i>	Syntaxin-binding protein 2. Involved in vesicle transport.
16 161241_at	AV272626	0.0273	0.722	<i>EST</i>	Similar to human hypothetical 58.5 KDa protein. Unknown function.
17 93853_at	AA763918	0.0208	0.724	<i>Dnajb4</i>	DNA subfamily B member 4 homologue/HP40. Chaperone protein. Involved in the folding of newly synthesised proteins, in protein assembly and disassembly and in the translocation of proteins into organelles under non-stress conditions.
18 97802_at	AI850195	0.0073	0.733	<i>EST</i>	RNA processing factor 1 homologue. Involved in ribosome biogenesis.
19 102972_s_at	Y08380	0.0423	0.735	<i>Dab1</i>	Disabled homologue 1 (<i>Drosophila</i>). Adapter molecule functioning in neural development. Phosphorylated upon reelin induction in embryonic neurons leading to activation of PI3K, Akt and the inhibition of GSK3 β .
20 162264_s_at	AV359014	0.0472	0.739	<i>BUB1</i>	Budding uninhibited by benzimidazoles 1 homologue (<i>S. cerevisiae</i>). Protein kinase required for mitosis checkpoint control.

Genes highlighted in bold indicates those that have passed the Benjamini-Hochberg false discovery rate procedure.

Table 5.3. Classification of genes upregulated by E2A-HLF^a.

Regulation of transcription	DNA repair
<i>E2f1</i>	<i>Facc</i>
<i>Lmo2</i>	
<i>Nmyc</i>	Receptors
<i>Wt1</i>	<i>FcrgRll</i>
	<i>Tcrg-V4</i>
Signal transduction	
<i>Catnal1</i>	Cytokines
<i>Csk</i>	<i>IL-15</i>
<i>Frat2</i>	
<i>IRG-47</i>	Regulation of apoptosis
<i>Pdk1</i>	<i>Bcl2</i>
<i>Pip5k1a</i>	
<i>Sos1</i>	Other
	<i>Apg12l</i>
Enzymes or transporters	<i>Flnb</i>
<i>Esch1</i>	<i>Mab21l2</i>
<i>Gch1</i>	
<i>Slc27a4</i>	

Table 5.4. Classification of genes downregulated by E2A-HLF^a.

Regulation of transcription	Enzymes or transporters
<i>Tgfb1i4</i>	<i>Kcnma1</i>
Regulation of cell cycle	Chromosome organization
<i>Bub1</i>	<i>Hist1h2bc</i>
<i>Ccng2</i>	
<i>Ppp6c</i>	Other
	<i>Dnajb4</i>
Signal transduction	<i>Mena</i>
<i>Dab1</i>	<i>Psap</i>
	<i>Slfn2</i>
Regulation of apoptosis	<i>Stxbp2</i>
<i>Bnip3</i>	

^aOnly genes with known functions are listed.

two largest categories consisted of four genes involved in the regulation of transcription and seven genes involved in signal transduction.

5.2.3. Genes downregulated by E2A-HLF

Twenty genes were downregulated by the expression of E2A-HLF by more than 1.3-fold (i.e. less than 0.585 fold change) (**Table 5.2**). Genes that were downregulated by E2A-HLF were changed by a maximum of 1.7-fold. Three genes passed the Benjamini-Hochberg FDR procedure. The target genes downregulated by E2A-HLF were also categorised according to their biological role. These categories were: (1) regulator of transcription; (2) regulators of cell cycle; (3) signal transducers; (4) enzymes; (5) regulators of apoptosis and (6) involved in chromosome organisation (**Table 5.4**). The largest group consisted of three genes involved in the regulation of the cell cycle.

5.2.4. Expression of previously identified as targets of E2A-HLF

Annexin VIII, *SRPUL*, *Slug*, *Groucho*-related genes, *Runx1* and *Annexin II* have all been previously identified as targets of E2A-HLF (Kurosawa *et al.*, 1999; Inukai *et al.*, 1999; Dang *et al.*, 2001; Matsunaga *et al.*, 2004). The expression of these genes was therefore assessed using the microarray data from this current study. *Slug* was the only previously identified target of E2A-HLF that was shown to have anti-apoptotic activities in pro-B cells (Inukai *et al.*, 1999). However, *Slug* was not expressed or overexpressed by the inducible E2A-HLF clones used in this current study. *Runx1*, which was previously shown to be downregulated by E2A-HLF (Dang *et al.*, 2001), was expressed, but was not changed by the expression of E2A-HLF in this current study. Similarly, *Annexin II* which was shown to be upregulated by E2A-HLF (Matsunaga *et al.*, 2004), was expressed, but not changed by the

expression of E2A-HLF in this study. These data suggest that Slug, Runx1 and Annexin II are not essential for the leukaemogenic activities of E2A-HLF.

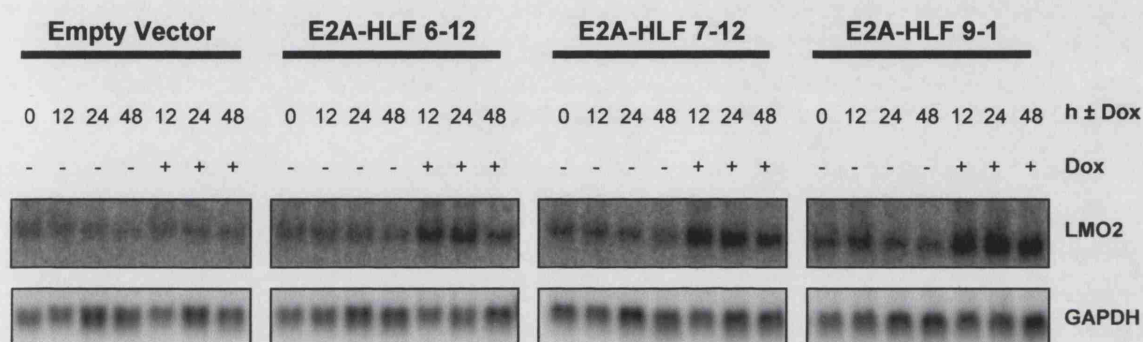
Probes for *SRPUL*, *Annexin VIII* and *Groucho*-related genes were not present on the arrays, therefore any changes in the expression of these genes could not be assessed. Genes for the coagulation factors, Factor VIII and Factor IX, which were transactivated in reporter studies by E2A-HLF (Begbie *et al.*, 1999) were also not expressed.

5.2.5. Validation of microarray data by Northern blotting

To confirm that genes identified by microarray analysis were true targets of E2A-HLF, Northern blotting analysis was performed using RNA extracted from Empty Vector and inducible E2A-HLF Baf-3 clones cultured with or without doxycycline for up to 48 h. *LMO2* and *IL-15* were both upregulated and maintained for up to 48 h in the inducible E2A-HLF Baf-3 clones, but not in the Empty Vector clone which does not express E2A-HLF (**Figure 5.2A and 5.2B**). The absence of *LMO2* and *IL-15* induction in the Empty Vector clone confirmed that their induction was due to E2A-HLF expression and not due to doxycycline alone.

Normalisation of the *LMO2* levels to the GAPDH levels showed that *LMO2* was induced by 2-fold (relative to the levels at 0 h) in each of the inducible E2A-HLF clones after 12 h of induction by doxycycline. Normalisation of the *IL-15* levels to the GAPDH levels showed that *IL-15* was induced by 2.4-fold in clone E2A-HLF 6-12, 1.9-fold in clone E2A-HLF 7-12 and 3.3-fold in clone E2A-HLF 9-1 when compared to the levels at 0 h.

(A)



(B)

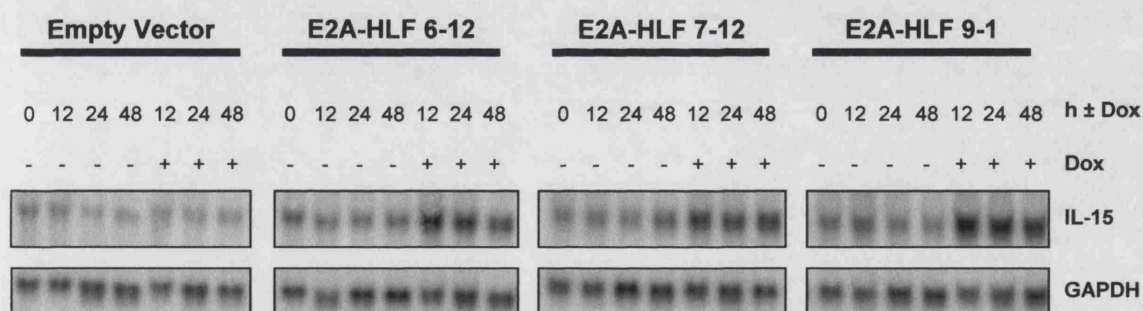


Figure 5.2. LMO2 and IL-15 are upregulated by the expression of E2A-HLF. Northern blotting analysis was performed using 10 μ g of total RNA from inducible clones cultured with or without doxycycline for up to 48 h using cDNA probes for either LMO2 (A) or IL-15 (B). Membranes were subsequently reprobbed for GAPDH.

The levels of BCL-2 expression was also analysed by Northern blotting (**Figure 5.3**). The levels of BCL-2 induced after 12 h and 24 h of induction by doxycycline, compared to the levels at 0 h, was modest compared to the induction of LMO2 or IL-15 by E2A-HLF. BCL-2 induced in clones E2A-HLF 6-12 and 7-12 was higher than that induced in clone E2A-HLF 9-1.

5.2.6. Validation of microarray data by luciferase reporter assays

Luciferase reporter constructs containing the promoter regions of human LMO2 (-3190LMOLUC) and mouse IL-15 (mIL-15/Luc) were used to further confirm that LMO2 and IL-15 were targets of E2A-HLF. Doxycycline had a non-specific effect on both luciferase reporter constructs in all clones (**Figure 5.4**). However, the expression of E2A-HLF overcame the non-specific effect of doxycycline. The mouse IL-15 promoter was transactivated by an average of 3.5-fold by E2A-HLF. E2A-HLF transactivated the LMO2 promoter by an average of 2-fold. This was similar to the level of induction achieved by HLF on the same reporter construct in erythroid cells (Crale and Anderson, 2003).

5.2.7. Determination of the expression of IL-15

To determine whether the inducible E2A-HLF Baf-3 cells expressed IL-15, a bioassay using CTLL-2 cells was performed. CTLL-2 cells are an IL-2-dependent T cell line which also proliferate in response to IL-4 and IL-7 and IL-15 (Paxton, 1996). The responsiveness of the CTLL-2 cells to recombinant murine IL-15 was initially assessed (**Figure 5.5A**). Increased levels of recombinant IL-15 corresponded to increased proliferation of CTLL-2 as determined by the increase in relative absorbance in a MTS assay from which a standard curved was generated. In parallel to the CTLL-2 cells grown in recombinant cytokine, CTLL-2 cells were also

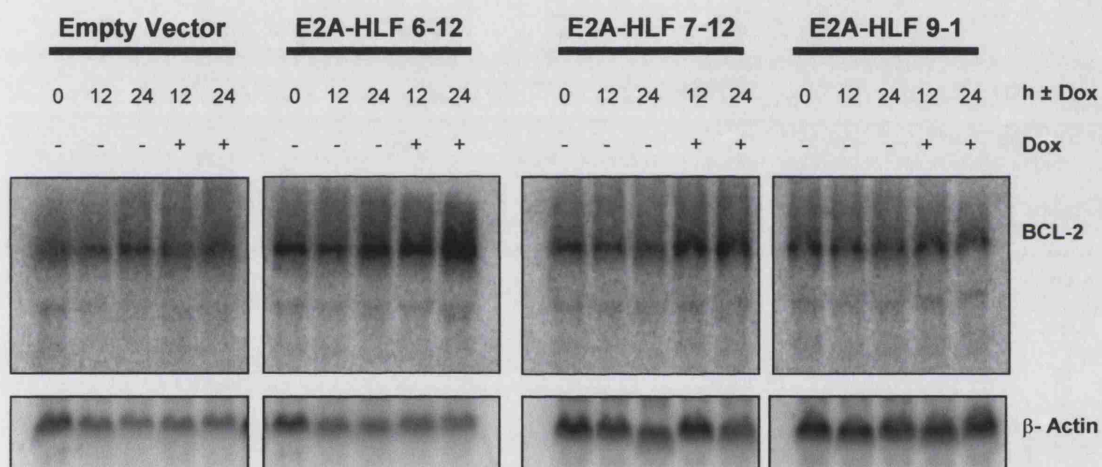


Figure 5.3. BCL-2 is upregulated by the expression of E2A-HLF.

Northern blotting analysis was performed using 10 μ g of total RNA from inducible clones cultured with or without doxycycline for up to 24 h using a cDNA probe for BCL-2. Membranes were subsequently reprobed for GAPDH.

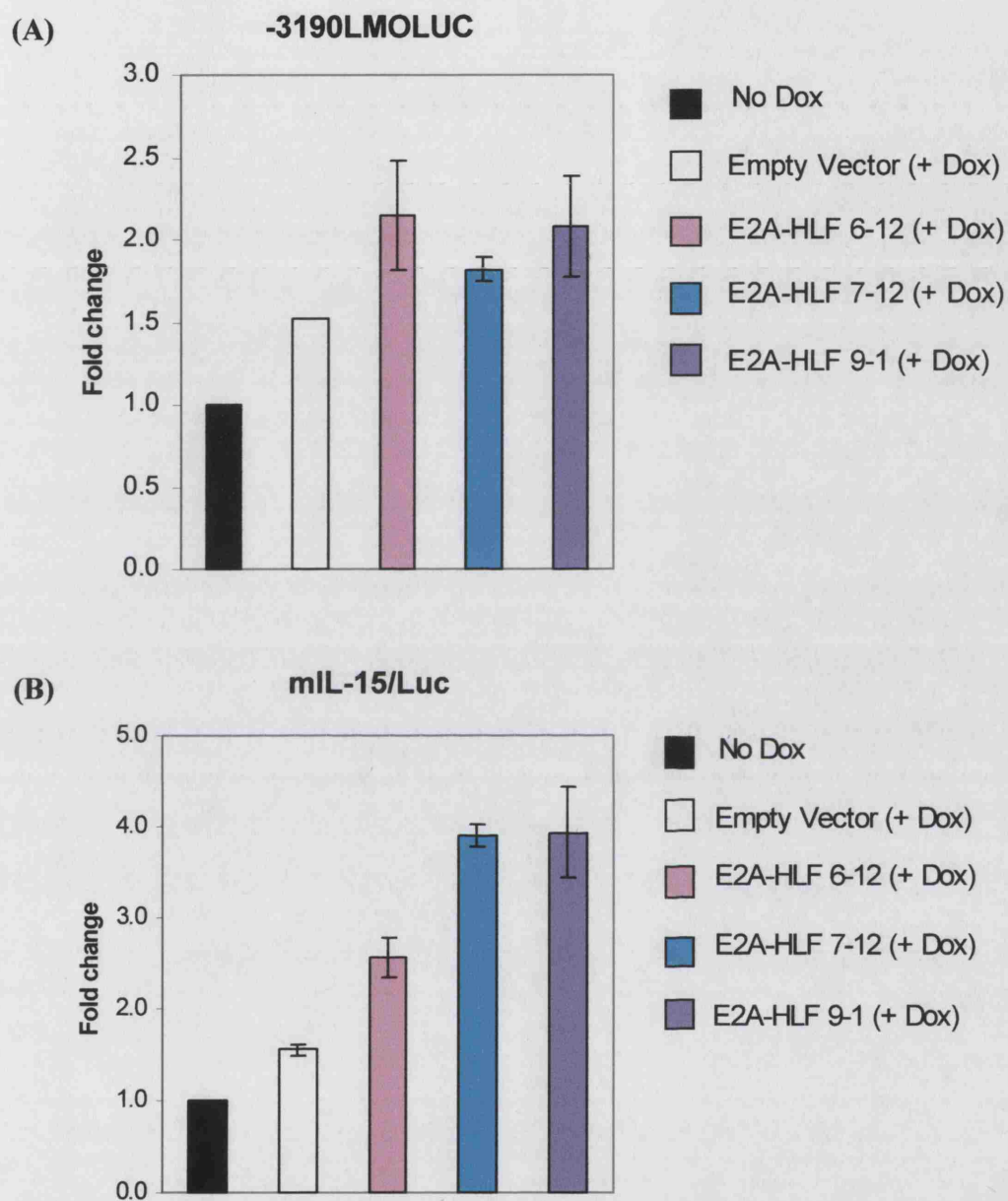
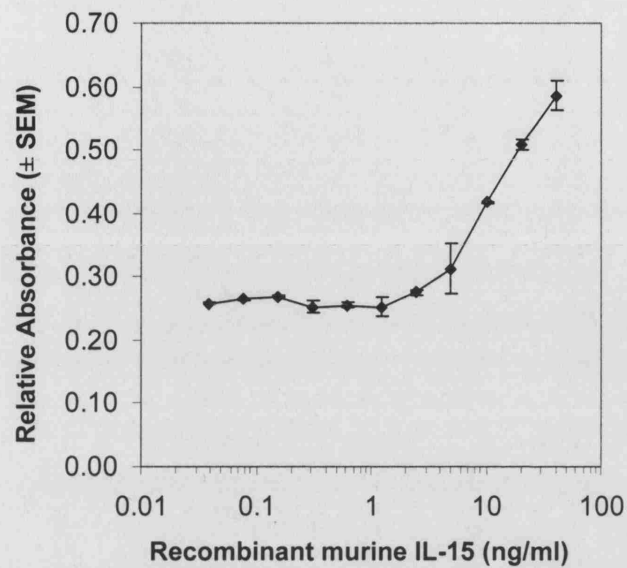


Figure 5.4. E2A-HLF transactivates the human LMO2 promoter and murine IL-15 promoter.

The transcriptional activity of E2A-HLF on **(A)** the human LMO2 promoter (-3190LMOLUC) and **(B)** murine IL-15 promoter (mIL-15/Luc) was assessed by luciferase reporter gene assays in an Empty Vector clone and three E2A-HLF clones. Clones were electroporated with the luciferase reporter constructs and cultured with or without doxycycline for 48 h. The mean fold changes after induction by doxycycline are expressed relative to the luciferase activity obtained when cells were not induced with doxycycline. Bars indicate standard error.

(A)



(B)

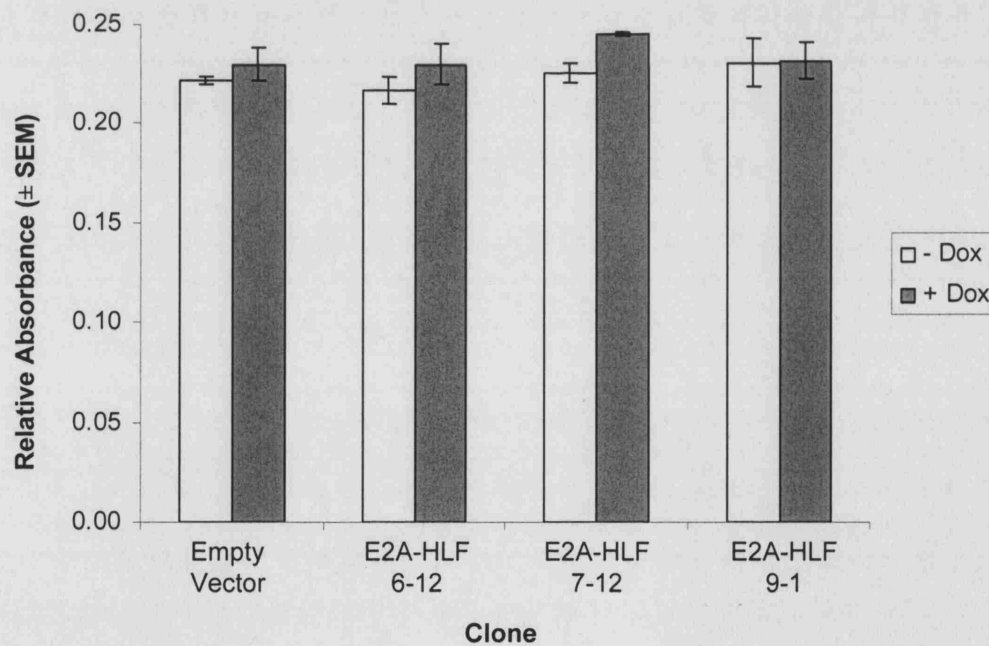


Figure 5.5. Insensitivity of CTLL-2 bioassay for the detection of secreted IL-15 using MTS reagent.

(A) Growth of CTLL-2 cells for 24 hours in medium containing increasing concentrations of recombinant murine IL-15. (B) Growth of CTLL-2 cells cultured in medium obtained from four inducible clones that had been cultured in presence or absence of doxycycline for 48 hours.

cultured in medium obtained from inducible E2A-HLF clones that had been culture with or without doxycycline for 48 h. No differences between the relative absorbance levels was found for any of the clones in the presence or absence of doxycycline (**Figure 5.5B**). Comparison of these relative absorbance readings with those from the standard curve showed that the relative absorbance levels were at or below the readings given by the lowest concentration of recombinant IL-15. This indicated that the CTLL-2 cells had not proliferated in medium from the inducible E2A-HLF Baf-3 cells. This MTS-based IL-15 bioassay was therefore not sensitive enough to detect low levels of proliferation which could occur in response to very low concentrations of IL-15.

5.3. Discussion

5.3.1. Identification of new targets of E2A-HLF

Expression of E2A-HLF has been proposed to alter the transcriptional profile of pro-B cells that carry the t(17;19) translocation. *Annexin VIII*, *SRPUL*, *Slug*, *Groucho*-related genes and *Runx1* have been identified as targets of E2A-HLF (Kurosawa *et al.*, 1999; Inukai *et al.*, 1999; Dang *et al.*, 2001; Matsunaga *et al.*, 2004). Only *Slug* delayed apoptosis in pro-B cells induced by IL-3 withdrawal. Microarrays were used to identify genes which may contribute to the leukaemic phenotype of ALLs expressing E2A-HLF and which may have a greater role in inhibiting apoptosis or other leukaemogenic processes.

5.3.2. Potential role of target genes in leukaemogenesis

E2A-HLF upregulated genes which encode transcription factors such as WT1, E2F1, N-Myc and LMO2. These genes are already known to be oncogenes and are deregulated in a variety of different human malignancies. WT1 is a zinc-finger

DNA-binding protein that is deleted in patients with Wilm's tumour (Rosenfeld *et al.*, 2003). WT1 is overexpressed in most cases of adult and paediatric acute myeloid leukaemia, acute lymphoblastic leukaemia, chronic myeloid leukaemia and myelodysplasia (Rosenfeld *et al.*, 2003). *N-myc* is overexpressed in neuroblastoma and other solid tumours. Transgenic mice which express *N-myc* under the control of the IgH E μ enhancer develop lymphoid malignancies (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Sheppard *et al.*, 1998).

LMO2 is involved T-cell ALL that have chromosome rearrangements involving 11q13 which result in the overexpression of LMO2 (Royer-Pokora *et al.*, 1991; Boehm *et al.*, 1991). Transgenic mice which ectopically expressed LMO2 in thymocytes developed T cell leukaemias (Neale *et al.*, 1995; Larson *et al.*, 1995; Larson *et al.*, 1994). LMO2 was previously found to be part of a multimeric DNA-binding complex with TAL1, E47, GATA-1 and Ldb1/NLI proteins in erythroid cells and was shown to be necessary for early stages of haematopoiesis and for normal haematopoietic development in adult mice (Warren *et al.*, 1994; Wadman *et al.*, 1997; Yamada *et al.*, 1998).

Several large scale microarray studies have previously been carried determine the gene expression profiles of different leukaemias in children (Armstrong *et al.*, 2002; Ferrando *et al.*, 2002; Yeoh *et al.*, 2002). LMO2 has been found to be upregulated in patients with *MLL* rearrangements. Furthermore, LMO2 was upregulated in B precursor ALL with a *MLL* translocation and in *MLL*-ENL-ERtm-transformed cells (Armstrong *et al.*, 2002; Zeisig *et al.*, 2004). The upregulation of LMO2 expression in *MLL* leukaemias and by E2A-HLF may suggest that LMO2 may be involved in a common leukaemogenic pathway.

E2A-HLF upregulated the expression of IL-15, a T-cell growth factor. The anti-apoptotic properties of E2A-HLF could therefore be mediated via IL-15, possibly by an autocrine mechanism. IL-15 has been shown to have an anti-apoptotic function in different cell types (Bulfone-Paus *et al.*, 1997; Hjorth-Hansen *et al.*, 1999; Lin *et al.*, 2001; Masuda *et al.*, 2001; Hoontrakoon *et al.*, 2002; Berard *et al.*, 2003). IL-15 transgenic mice showed early expansions in NK and CD8⁺ T lymphocytes and later developed fatal lymphocytic leukaemia (Fehniger *et al.*, 2001). The upregulation of *LMO2*, *IL-15* and other T-cell genes, e.g. *TCRG-V4*, by E2A-HLF may explain the susceptibility of E2A-HLF transgenic mice to develop mainly T-cell malignancies (Smith *et al.*, 1999; Honda *et al.*, 1999).

To detect expression of secreted IL-15 which may have been produced by the induced E2A-HLF clones, a MTS-based IL-15 bioassay using CTLL-2 cells was used. Unfortunately, this MTS-based bioassay was not sensitive enough to detect IL-15 or the cells did not secrete IL-15. CTLL-2 bioassays using tritiated thymidine are more commonly used and may be more sensitive in detecting very low levels of IL-15. As CTLL-2 cells are also responsive to IL-2 and IL-4, use of a neutralising IL-15 antibody would increase the specificity of the bioassay. A neutralising antibody would compete with the IL-15 receptors on the CTLL-2 cells for binding to IL-15. This would result in a decrease in the proliferation rate of the CTLL-2 cells compared to CTLL-2 cells cultured without the neutralising antibody.

E2A-HLF may also directly affect regulators of apoptosis machinery. The anti-apoptotic *BCL-2* was upregulated, whereas the pro-apoptotic *BNIP3* was downregulated by E2A-HLF. This data is interesting as a study by Smith *et al.*

(2002) showed that immortalisation of B-cell progenitor cells *in vitro* by E2A-HLF required the co-expression of BCL-2 (Smith *et al.*, 2002).

As previously discussed in Chapter 4, microarray studies using mice deficient for some or all of the PAR bZIP proteins (TEF, HLF and DBP) showed that TEF, and perhaps the other PAR bZIP proteins, upregulated the expression of E4BP4 (Gachon *et al.*, 2004). In addition, prosaposin was also shown to be a downregulated target of TEF (Gachon *et al.*, 2004). Prosaposin was a downregulated target of E2A-HLF in the inducible E2A-HLF Baf-3 clones in studied in this chapter.

5.3.3. Further work

Further validation of the targets of E2A-HLF identified by the microarray studies needs to be carried out. Changes in mRNA levels can be determined by Northern blotting, RT-PCR or by real-time PCR. Further analyses of mRNA levels have to be carried out to confirm that target genes identified by microarray analysis are indeed upregulated by E2A-HLF and not by doxycycline alone. Changes in protein expression of E2A-HLF target genes can be determined by Western blotting using mouse specific antibodies. Chromatin immunoprecipitation or electrophoretic mobility shift assays (EMSA) would be useful to determine whether E2A-HLF directly bound to the promoters of candidate target genes.

The targets that have been identified using microarrays could also be determined in available patient samples. A number of experiments are required to fully elucidate the role of E2A-HLF and its target genes in leukaemogenesis and inhibition of apoptosis.

CHAPTER 6

RESULTS (4)

6.1. Background

The ability of oncogenes to immortalise and transform myeloid and lymphoid progenitor cells has been assessed using colony-forming cell assays in methylcellulose (Lavau *et al.*, 1997). The clonogenic and self-renewal properties of HPCs can be determined by the numbers of colonies formed during successive rounds of replating. Both the self-renewal and the proliferative capacity of the HPCs can be determined by the numbers of cells generated by these colonies.

Normal HPCs generate colonies for a limited number of serial replatings in methylcellulose due to exhaustion of self-renewal capacity. However, expression of an immortalising oncogene in HPCs allows the cells to serially replated and generate colonies for significantly more rounds than the control HPCs. Culture of HPCs in the presence of specific cocktails of exogenous cytokines in methylcellulose can promote differentiation of the HPCs down either the myeloid or B cell lineages. This *in vitro* system may also be used to assess the contribution of the candidate target genes of E2A-HLF to immortalisation or transformation of primary HPCs. Furthermore, this system would allow analysis of the synergistic effects of co-expression of both E2A-HLF and its target genes, e.g. *LMO2* and *IL-15*.

The role of E2A-HLF in leukaemogenesis can be assessed *in vitro* using primary murine HPCs. Previous studies have shown that an E2A-HLF-expressing retrovirus was able to transduce and immortalise HPCs (Ayton and Cleary, 2003; Smith *et al.*, 2003; Smith *et al.*, 2002). These previous studies showed that myeloid progenitors were easily immortalised by E2A-HLF, however E2A-HLF was only able to immortalise lymphoid cells when co-expressed with BCL-2 (Ayton and Cleary, 2003; Smith *et al.*, 2003; Smith *et al.*, 2002).

To further assess the role of E2A-HLF in leukaemogenesis, E2A-HLF could be expressed in a retrovirus with or without an antibiotic selectable marker and used to transduce HPCs derived from either murine bone marrow or murine foetal liver.

The cDNAs for E2A-HLF and murine BCL-2 were epitope tagged with a Myc-epitope by PCR and cloned into pMSCV-based retroviral vectors (**Figure 6.1**).

E2A-HLF was cloned into the pMSCV-IRES-hCD2t retroviral vector which expresses the human CD2 gene lacking the cytoplasmic signalling domain under the control of an IRES. This retroviral construct allows the infection efficiency of the virus to be determined using an anti-human CD2 antibody. This retrovirus does not contain an antibiotic resistance gene to allow positive selection of infected cells by antibiotic selection. Any positive selection that occurs would be due to the expression of the oncogene expressed by the retrovirus.

The other retroviral vectors used in this study contain either a neomycin or puromycin resistance gene which allows for selection of infected cells by selection with geneticin or puromycin, respectively. After an initial round of antibiotic selection, all surviving cells should express the cDNA of interest.

An overview of the strategies used to transduce HPCs is illustrated in **Figure 6.2**.

Sources of HPCs from mice are the bone marrow and foetal liver. Unsorted bone marrow cells from 5-fluorouracil (5-FU) treated mice are enriched for HPCs and murine foetal livers contain large numbers of HPCs. The same infection conditions are used for HPCs derived from the different sources.

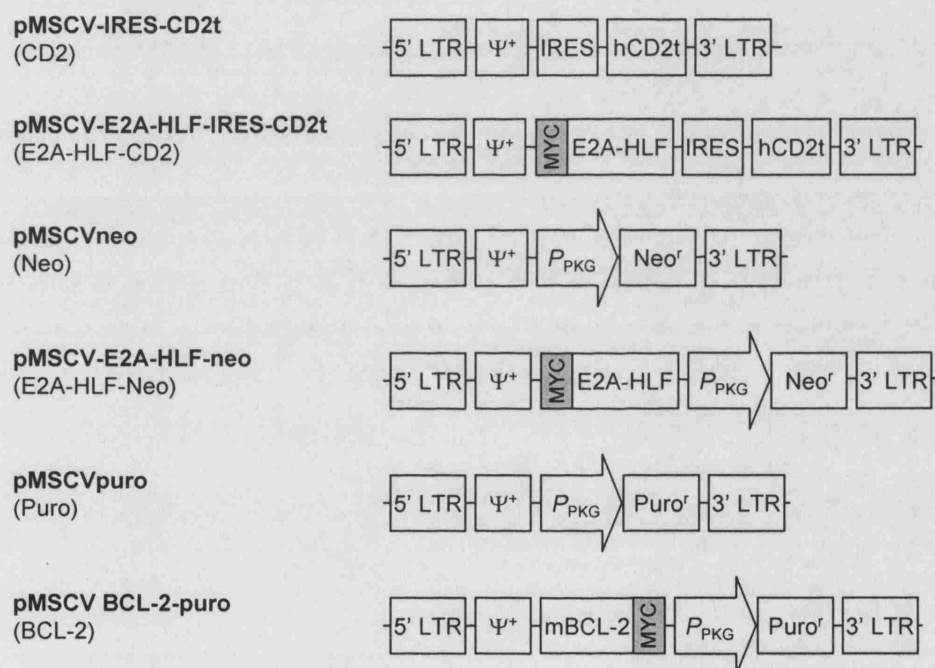


Figure 6.1. pMSCV constructs used to transduce primary HPCs

E2A-HLF and murine BCL-2 were cloned into pMSCV retroviral-based vectors. LTR, long terminal repeat. Ψ^+ , viral packaging signal. P_{PKG} , murine phosphoglycerate kinase promoter. Neo^r, neomycin resistance gene. Puro^r, puromycin resistance gene. hCD2t, tailless human CD2. IRES, internal ribosomal entry site. MYC, MYC epitope.

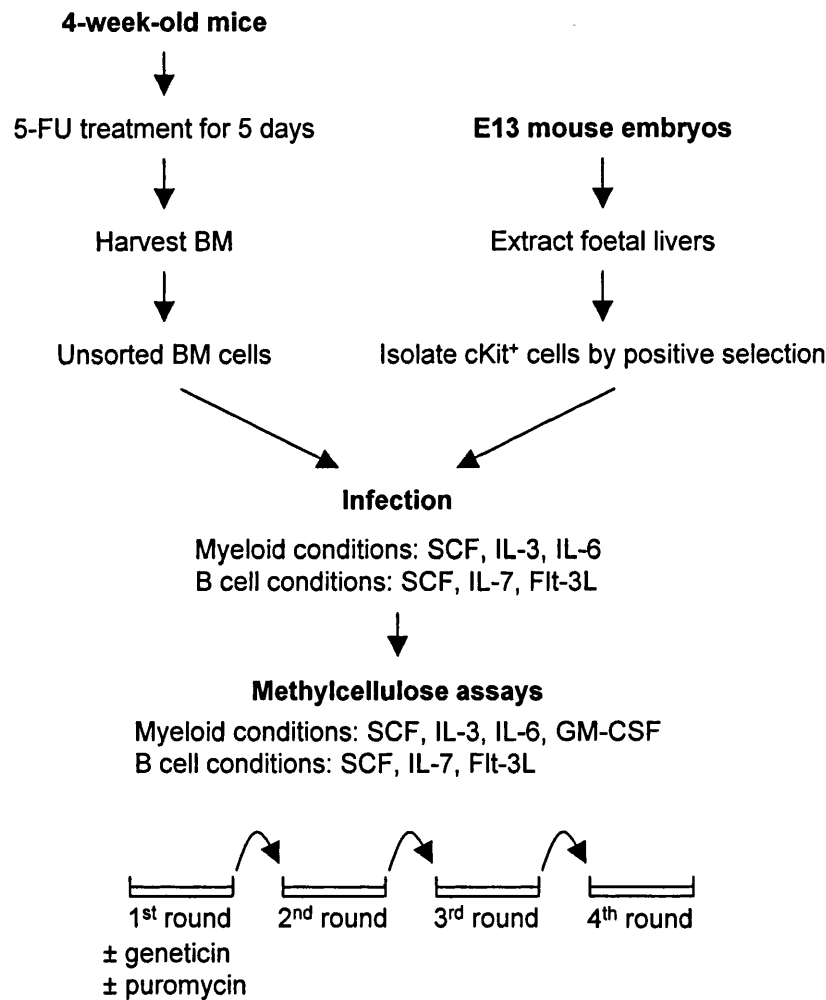


Figure 6.2. Experimental strategy used to transduce primary murine bone marrow or foetal liver HPCs.

HPCs obtained from bone marrow or foetal livers are cultured with cultured in cytokines which promote myeloid or lymphoid differentiation. HPCs are infected with retroviruses for 48 h prior to culture in methylcellulose. 5-FU, 5-fluorouracil.

6.2. Results

6.2.1. Murine bone marrow myeloid progenitors are immortalised by the E2A-HLF-CD2 retrovirus

To determine if E2A-HLF could initiate leukaemic transformation of primitive HSCs or HPCs and generate immortalised B cells, E2A-HLF expression was targeted to bone marrow HPCs by retroviral infection. As E2A-HLF was previously shown to immortalise bone marrow myeloid progenitors (Ayton and Cleary, 2003; Smith *et al.*, 2003), E2A-HLF-expressing retroviruses were used to infect bone marrow HPCs which were subsequently cultured in myeloid conditions in preliminary experiments to optimise infection conditions.

Unsorted bone marrow cells from C57BL/6 donor mice treated for 5 days with 5-FU were infected with the empty pMSCV-IRES-hCD2t (designated CD2) or the pMSCV-E2A-HLF-IRES-CD2t (designated E2A-HLF-CD2) retroviruses and cultured in presence of exogenous SCF, IL-3, IL-6 and GM-CSF which promote differentiation along the myeloid lineage.

To assess the efficiency of infection by the retroviruses, the level of CD2 expression was determined by flow cytometry at 48 h post-infection (prior to plating into methylcellulose medium). Similar levels of infection were achieved for both infections, whereby 50.1% of HPCs were transduced with empty CD2 retrovirus and 48.4% of HPCs were transduced with E2A-HLF-CD2 (**Figure 6.3A**). The expression of E2A-HLF protein by the E2A-HLF-CD2 retrovirus infected cells was confirmed by Western blotting (**Figure 6.3D**). No significant change in the levels of CD2 expressed by cells transduced by the empty CD2 retrovirus was observed in

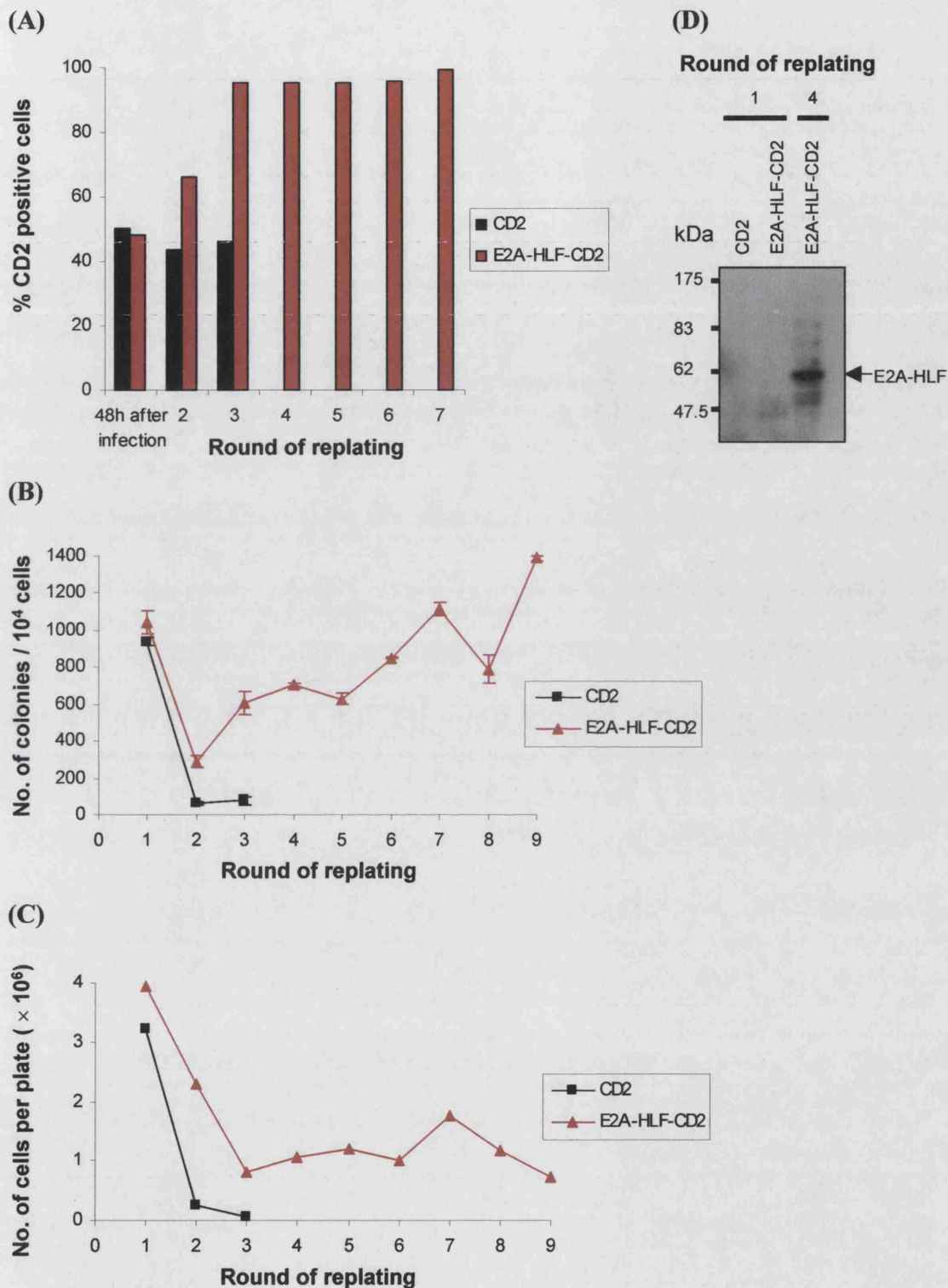


Figure 6.3. E2A-HLF-CD2 retrovirus readily immortalised bone marrow progenitor HPCs cultured in SCF, IL-3, IL-6 and GM-CSF.

(A) CD2 expression by transduced bone marrow cells determined 48 h after infection and after subsequent rounds of replating. (B) Mean number of colonies generated per 10^4 cells (\pm SEM) after 5-7 days in culture in methylcellulose in presence of SCF, IL-3, IL-6 and GM-CSF. (C) Total number of viable cells isolated from each plate. (D) Expression of E2A-HLF protein in cells transduced with E2A-HLF-CD2 retrovirus.

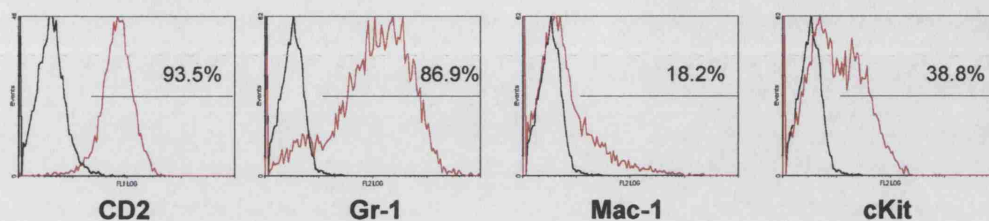
subsequent rounds of replating. In contrast, the cells transduced by the E2A-HLF-CD2 retrovirus showed enrichment of CD2 expression in subsequent rounds of replating. The percentage of CD2-expressing cells increased to 65% at the end of the second round, rose to 95.5% by the end of the third round and reached 99.4% by the seventh round (**Figure 6.3A**).

Cells which were transduced with the empty CD2 or E2A-HLF-CD2 retroviruses initially generated similar numbers of colonies at the end of the first round of replating (**Figure 6.3B**). As expected, the empty CD2 retrovirus-transduced cells generated significantly fewer colonies and viable cells than the E2A-HLF-CD2-transduced HPCs and failed to replate beyond the third round (**Figure 6.3B** and **6.3C**). In contrast, the E2A-HLF-CD2-transduced cells could be serially replated at least until the ninth round and generated large numbers of colonies and significantly more viable cells at the end of each round of replating compared to cells transduced with the empty CD2 retrovirus.

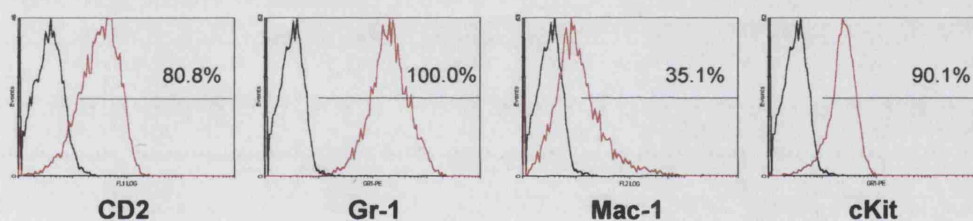
Cells immortalised by E2A-HLF-CD2 were analysed for the expression of different markers of myeloid differentiation (Mac-1 and Gr-1) and a marker of progenitor cells (cKit). Almost all the cells in the culture expressed the infection marker, CD2. Analysis of the CD2-positive cells taken from methylcellulose culture at the end of the fifth round of replating showed that 87% of the cells expressed high levels of the granulocyte marker Gr-1, low expression of Mac-1 and cKit (**Figure 6.4A**).

Analysis of a cell line that was established from methylcellulose colonies by continuous passage and expansion in liquid medium showed a similar expression of cell surface markers (**Figure 6.4B**). However, the cell line expressed higher levels of Gr-1 and cKit (**Figure 6.4B**). HPCs which were transduced with the empty CD2

(A) E2A-HLF-CD2 colonies



(B) E2A-HLF-CD2 cell line



(C) CD2 cell line

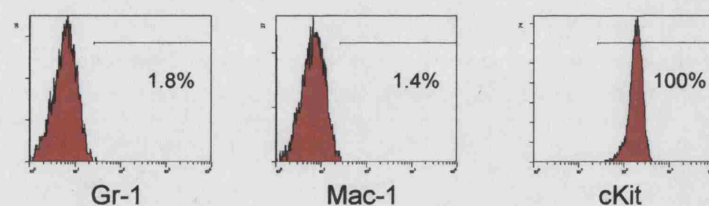


Figure 6.4. Bone marrow HPCs immortalised by E2A-HLF-CD2 express myeloid cell surface markers.

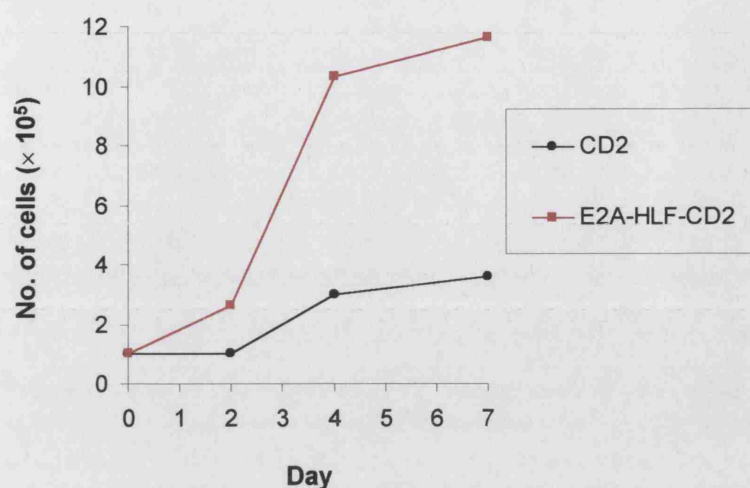
(A) Flow cytometric analysis of cells at the end of the 5th round of replating in methylcellulose. **(B)** Flow cytometric analysis a cell line derived from the 5th round of replating that had adapted to growth in liquid culture. **(C)** Flow cytometric analysis of a cell line derived from the 3rd round of replating of cells transduced with empty CD2 adapted to growth in liquid culture. Black lines indicate unstained cells.

retrovirus which were adapted to growth in liquid culture did not express Gr-1 or Mac-1, but expressed high levels of cKit (**Figure 6.4C**).

To study further the growth properties of cells immortalised by the E2A-HLF-CD2 retrovirus, the growth rate of the cell lines was determined. The E2A-HLF-CD2 cell line showed approximately 12-fold increase in cell numbers by seven days when cultured in presence of SCF, IL-3 and IL-6 (**Figure 6.5A**). However, over the same period, the empty CD2 cell line showed a 3-fold increase when cultured in the same cytokines. The growth of the empty CD2 cell line eventually stopped whilst cells that were transduced with E2A-HLF-CD2 proliferated well for at least 2 months after adapting from growth in methylcellulose medium before cells were frozen down and stored in liquid nitrogen.

As E2A-HLF confers resistance to apoptosis induced by IL-3 withdrawal from IL-3-dependent pro-B cells, the cytokine requirements of the cell lines derived from HPCs transduced by empty CD2 or E2A-HLF-CD2 retroviruses were assessed using a MTS cell viability assay. Both cell lines proliferated maximally in the presence of SCF, IL-3 and IL-6 and also in the presence of SCF and IL-3 only (**Figure 6.5B**). The E2A-HLF-CD2-transduced cell line showed a higher absorbance reading compared to the empty CD2 vector cell line in the presence of all three cytokines which is consistent with the increased growth rate of the E2A-HLF-CD2 cell line (**Figure 6.5A**). Both cell lines showed little viability in absence of all three cytokines. Differences in cell viability was evident when cells were cultured in SCF alone or in IL-3 alone. Compared to control cells cultured in the presence of all three cytokines, the viability of the CD2 cell line decreased to 62% and the E2A-HLF-CD2 cell line viability to 16% when cultured in SCF alone. In presence of IL-3

(A)



(B)

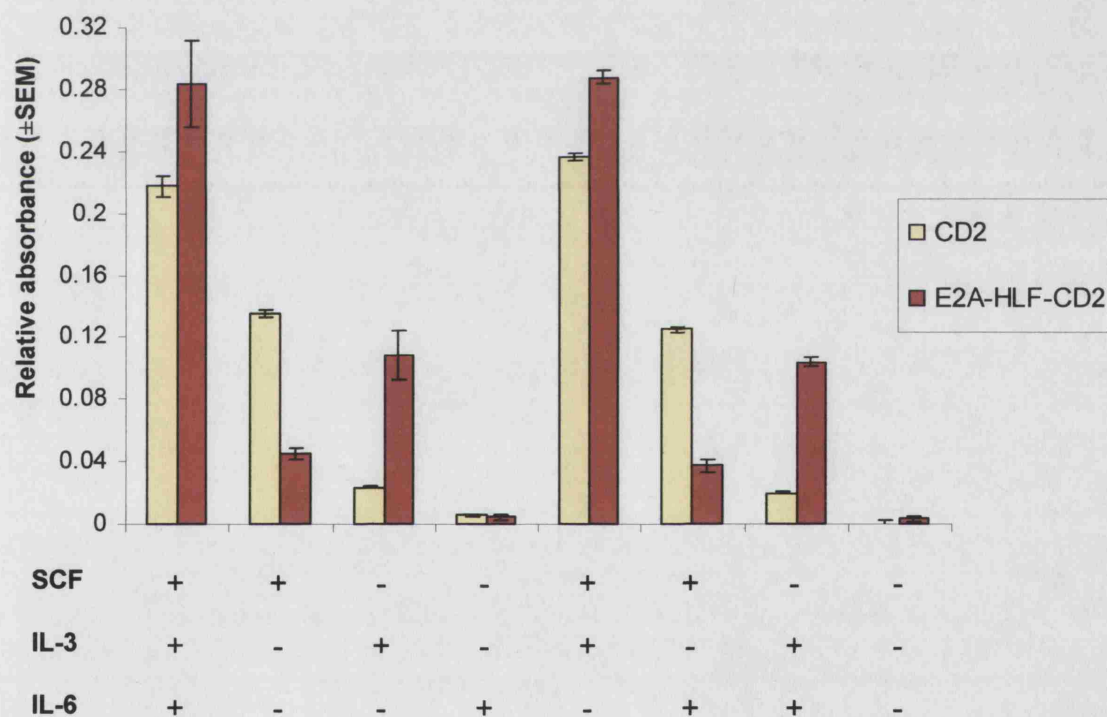


Figure 6.5. Bone marrow myeloid cells immortalised by E2A-HLF-CD2 are SCF and IL-3 dependent.

(A) The rate of growth of an E2A-HLF-CD2 immortalised myeloid cell line and a cell line derived from cells transduced with empty CD2 vector in liquid medium containing SCF, IL-3 and IL-6. (B) Assessment of the cytokine requirements of a E2A-HLF-CD2 cell line using a MTS assay.

alone, the CD2 cell line viability decreased to 11% whilst the E2A-HLF-CD2 cell line viability decreased to 38% compared to controls. IL-6 did not synergise with the other cytokines or promote the growth of either cell line alone.

6.2.2. Murine bone marrow myeloid progenitors are immortalised by the E2A-HLF-Neo retrovirus

Conditions were also established for the infection of bone marrow HPCs using the retroviral constructs pMSCVneo or pMSCVpuro which contain the neomycin or puromycin resistance genes, respectively. After an initial round of antibiotic selection, surviving HPCs and their colonies, should express the gene of interest. In contrast, transduction of HPCs using the E2A-HLF-CD2 retrovirus, as was shown in section 6.2.1, several rounds of replating are required to enrich for CD2-positive and therefore E2A-HLF-expressing cells.

Results obtained using the empty pMSCVneo (abbreviated to Neo) and pMSCV-E2A-HLF-Neo (abbreviated to E2A-HLF-Neo) were similar to those obtained using the E2A-HLF-CD2 (**Figure 6.6A and 6.6B**). E2A-HLF-Neo also immortalised cells cultured in presence of SCF, IL-3, IL-6 and GM-CSF. Due to the antibiotic selection during the first round of replating, the numbers of colonies and cells generated at the end of the first round were much lower than those obtained using the empty CD2 or E2A-HLF-CD2 retroviruses.

HPCs transduced with the empty Neo retrovirus generated colonies that did not replat beyond the third round (**Figure 6.6A**). In contrast, the HPCs transduced with E2A-HLF-Neo generated significantly more colonies and cells and were able to be replated for at least eight rounds (**Figure 6.6A and 6.6B**). Colony morphology in methylcellulose was significantly different between the Neo-transduced cells and the

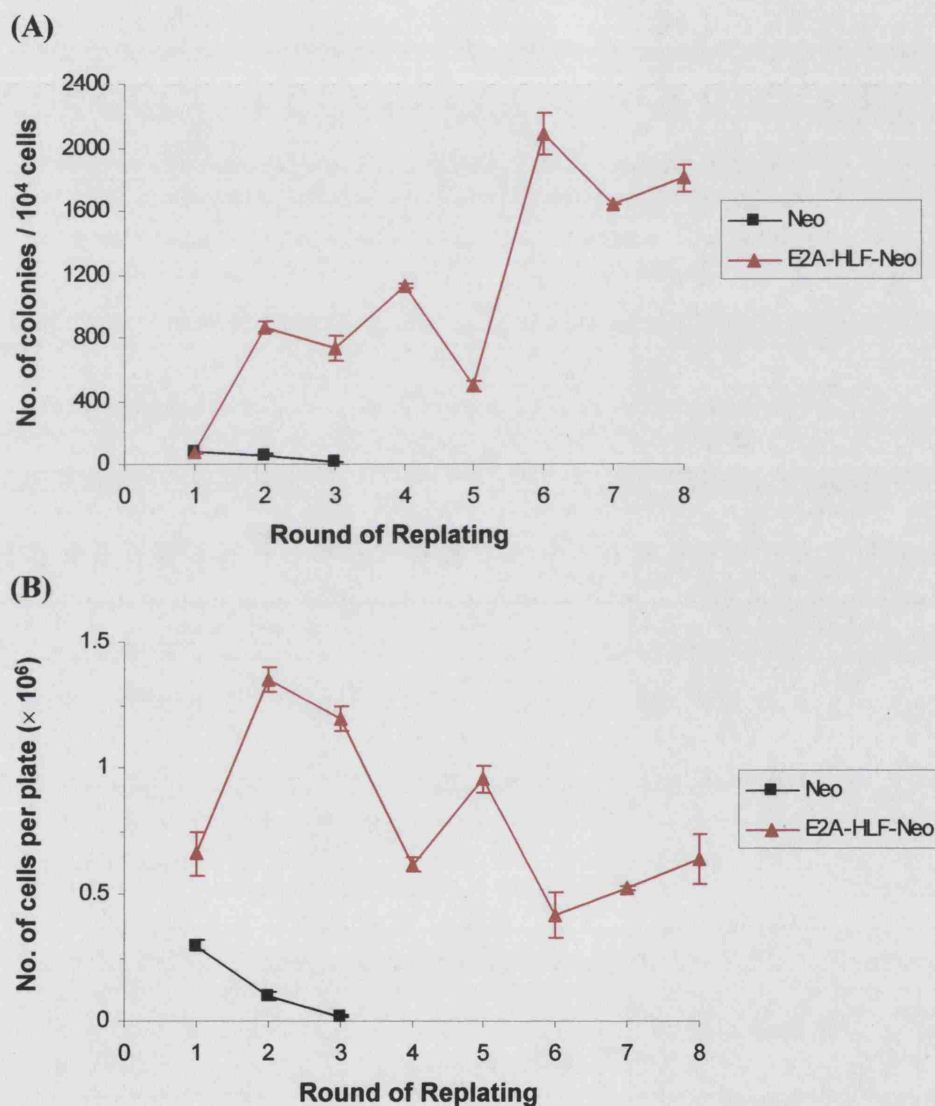


Figure 6.6. E2A-HLF-Neo retrovirus also immortalised bone marrow myeloid progenitor cells.

(A) Mean number of colonies generated per 10^4 cells (\pm SEM) after 5-7 days in culture in methylcellulose with SCF, IL-3, IL-6 and GM-CSF. Less than 10^4 cells were obtained at 48 h after infection therefore all available cells were used to seed the 1st round **(B)** Total number of viable cells isolated from each plate (\pm SEM).

E2A-HLF-Neo-transduced cells. Neo colonies were relatively small and represented granulocyte, erythroid, macrophage, megakaryocyte colony forming unit (GEMM-CFU) colonies (**Figure 6.7A**). However, E2A-HLF-Neo cells generated larger colonies consisting of densely packed cells (**Figure 6.7B**).

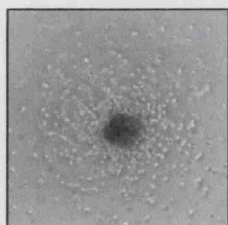
Flow cytometric analysis of cell surface markers indicated that the cells immortalised by E2A-HLF-Neo had a different phenotype to cells immortalised by the E2A-HLF-CD2 retrovirus (section 2.3.1). The cells immortalised by E2A-HLF-Neo expressed high levels of both Mac-1 and Gr-1 (**Figure 6.7C**). In contrast to cells immortalised by E2A-HLF-CD2 expressed low levels of Mac-1 and high levels of Gr-1 (**Figure 6.4A**). The cells immortalised by E2A-HLF-Neo cells expressed similar levels of cKit to the E2A-HLF-CD2 immortalised cells.

E2A-HLF-Neo transduced cells could be adapted to grow in liquid culture. However, this cell line did not proliferate as well as the cell line established using the E2A-HLF-CD2 retrovirus. The cells in liquid culture became adherent and eventually failed to proliferate.

6.2.3. Transduction of bone marrow HPCs in lymphoid conditions with E2A-HLF-CD2

Bone marrow HPCs were transduced with the empty CD2 and E2A-HLF-CD2 retroviral constructs and grown in the presence of the cytokines, SCF, IL-7 and Flt-3, which promote the differentiation of progenitor cells to B cells. E2A-HLF was shown to require BCL-2 co-expression in order to immortalise lymphoid cells in transduction experiments on unsorted bone marrow cells cultured on a bone marrow stromal cell line (Smith *et al.*, 2002). Therefore murine BCL-2 was cloned into a puromycin selectable pMSCVpuro retroviral vector (designated BCL-2) and used to

(A) Neo



(B) E2A-HLF-Neo



(C) E2A-HLF-Neo

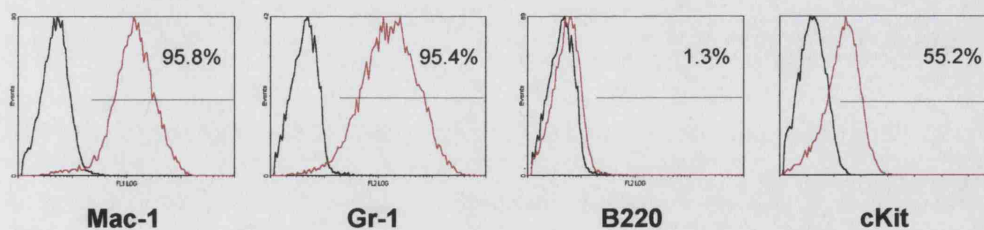


Figure 6.7. Myeloid cells immortalised by E2A-HLF-Neo retrovirus express high levels of Mac-1 and Gr-1.

(A) Typical morphology of Neo colonies obtained at the end of the 2nd round of replating ($\times 10$ magnification). **(B)** Typical morphology of E2A-HLF-Neo colonies obtained at the end of the 2nd round of replating ($\times 10$ magnification). **(C)** Flow cytometric analysis of cells immortalised by E2A-HLF-Neo retrovirus. Black lines indicate isotype control.

transduce bone marrow HPCs. Analysis of the infected bone marrow HPCs at 48 h post-infection, showed comparable levels of CD2 expression between each infection condition with an average expression of 53% (**Figure 6.8A**). The level of CD2 expression of the cells transduced with the empty CD2 retrovirus at the end of the second and third rounds of plating was greater than 39%. However, the levels of CD2 expressed by these cells decreased to 6% at the end of the third round of replating. CD2 expression by cells transduced with E2A-HLF-CD2 alone or with BCL-2 decreased to 15% and 9% respectively at the end of the second round and by the end of the third round, CD2 expression had decreased further to less than 10%.

Similar numbers of colonies were generated by cells infected with the different retrovirus combinations which ranged between 200 and 300 colonies per 10^4 cells plated at the end of the first round of replating (**Figure 6.8B**). Similar numbers of colonies were generated by the different infection conditions at the end of the next two rounds of replating. By the end of the fourth round, the cells that were transduced with E2A-HLF-CD2 alone generated significantly greater numbers than cells that had been transduced using the other retroviruses. However this difference in colony numbers did not persist in later rounds of replating (**Figure 6.8B**).

E2A-HLF-CD2-transduced cells generated greater numbers of viable cells than cells that had been transduced with the empty CD2 retrovirus at the end of the first and second round of replating (**Figure 6.8C**). However, co-infection of E2A-HLF-CD2 and BCL-2 generated greater numbers of viable cells than the other two infection conditions. On subsequent rounds of replating, the number of viable cells continued

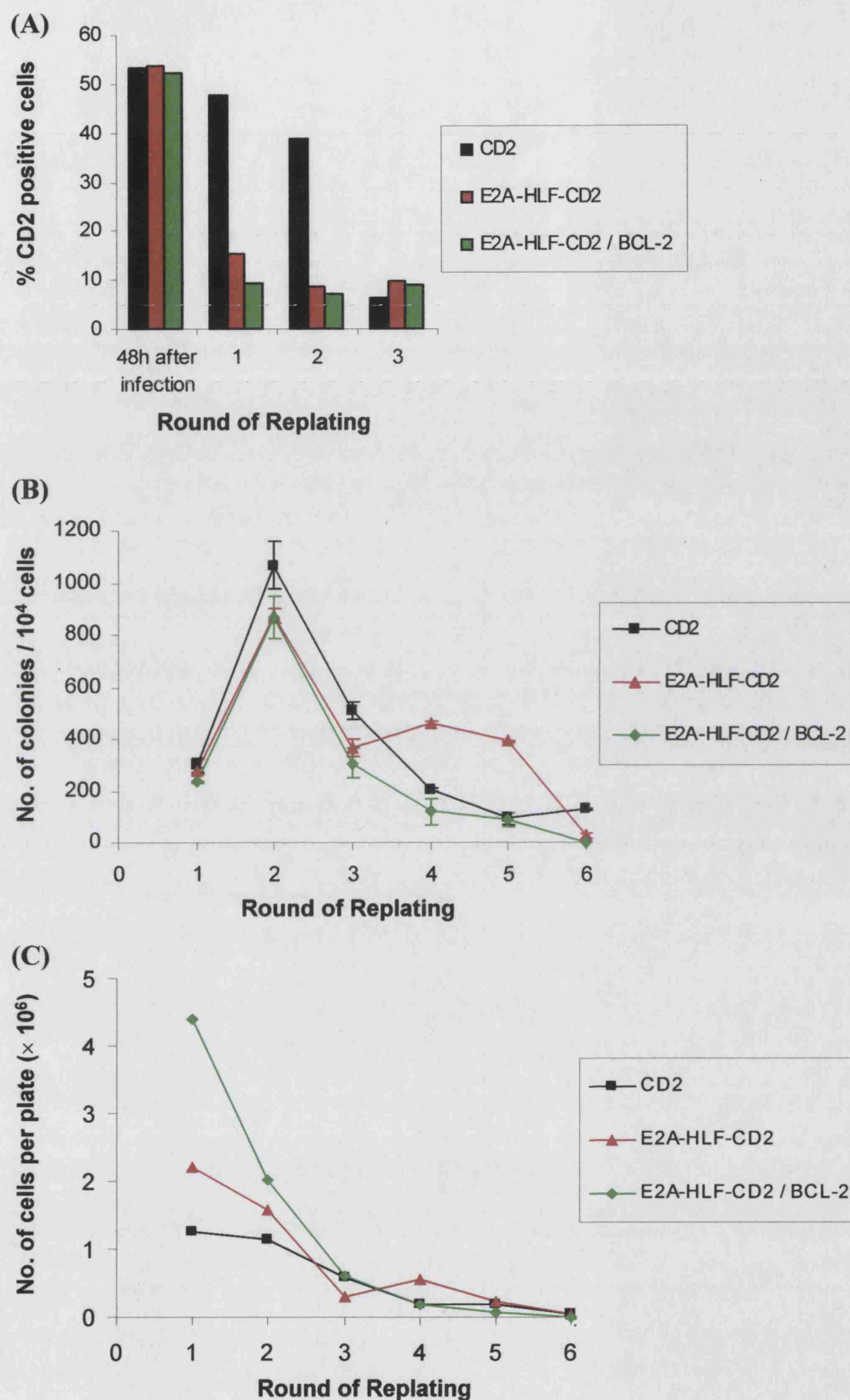


Figure 6.8. Bone marrow HPCs cultured with SCF, IL-7 and Flt-3L were not immortalised by the E2A-HLF-CD2 retrovirus.

(A) CD2 expression in bone marrow HPCs at 48 h after infection and after different rounds of replating. (B) Mean number of colonies generated per 10^4 cells after 7-10 days in culture in methylcellulose with SCF, IL-7 and Flt-3L. (C) Total number of viable cells isolated from each plate.

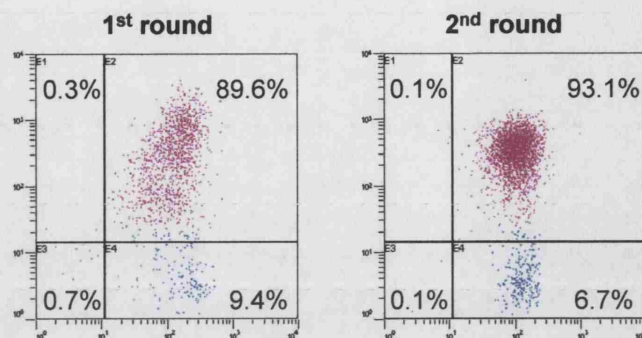
to decrease for each infection condition and any differences between each infection condition was no longer evident (**Figure 6.8**).

Analysis of the surface markers of the cells at the end of different rounds of replating by flow cytometry confirmed that the transduced HPCs were differentiating along the B cell lineage in the presence of SCF, IL-7 and Flt-3L. Transduced HPCs expressed B220, a B lineage marker. Most cells co-expressed BP1 indicating that the transduced cells were able to differentiate to the pre-B cell stage (**Figure 6.9**). However, E2A-HLF-CD2 alone and co-infection of E2A-HLF-CD2 and BCL-2 generated higher numbers of pro-B cells (B220⁺/BP1⁻ cells) compared to cells transduced by the empty CD2 retrovirus. However, as flow cytometric analysis was carried out on the total cell population, irrespective of CD2 expression, the increase in the percentage of pro-B cells could not be correlated to E2A-HLF-CD2 or BCL-2 expression. Western blotting analysis of the cells taken after the first round of replating, when the numbers of viable cells generated was highest, failed to demonstrate the expression of E2A-HLF either by using an anti-Myc antibody or an anti-E2A antibody (data not shown).

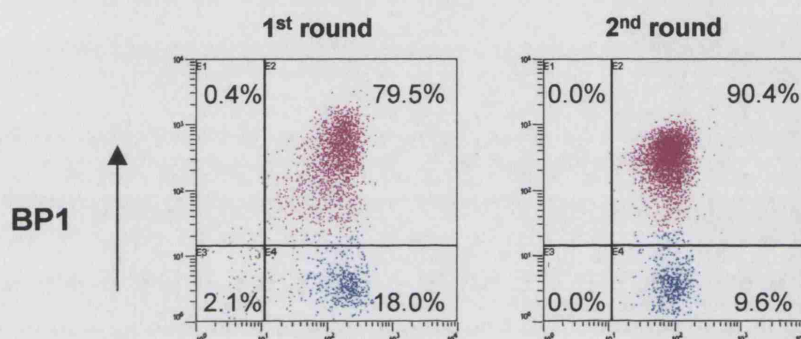
6.2.4. Foetal liver myeloid and B cell progenitors are immortalised by the E2A-HLF-Neo retrovirus

Bone marrow HPCs infected with E2A-HLF-expressing retroviruses that then were cultured in lymphoid conditions were not immortalised. This was not unexpected as previous retroviral transduction studies had shown that E2A-HLF was not able to immortalise bone marrow HPCs without co-expression of BCL-2 (Smith *et al.*, 2002). However, in this current study, co-infection of bone marrow HPCs with E2A-HLF-expressing and BCL-2-expressing retroviruses also failed to immortalise

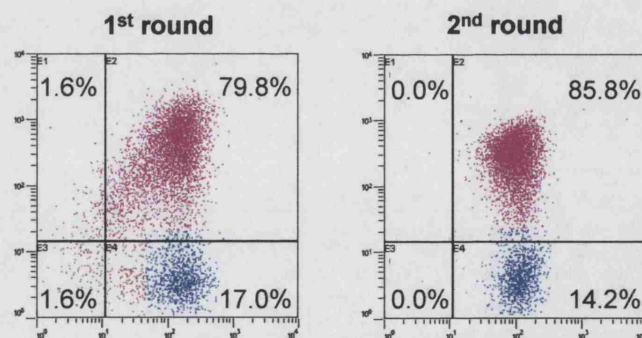
(A) CD2 Only



(B) E2A-HLF-CD2 Only



(C) E2A-HLF-CD2 / BCL-2



→
B220

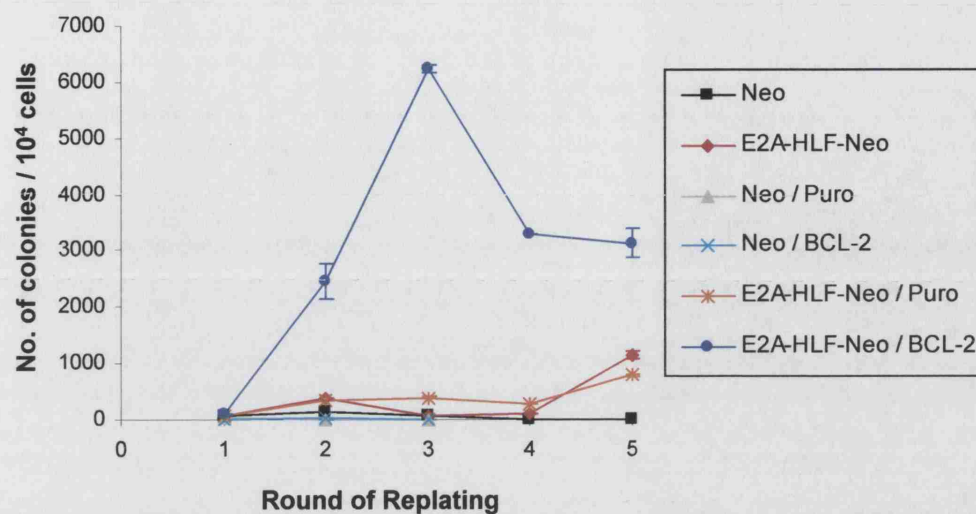
Figure 6.9. Bone marrow HPCs differentiate to B cells in presence of SCF, IL-7 and Flt-3L.

Flow cytometric analysis of pooled colonies from different rounds of methylcellulose replating with SCF, IL-7 and Flt-3L. B220 and BP1 expression was determined for total live cells.

bone marrow HPCs cultured in B cell lineage promoting cytokines. An alternative source of HPCs was therefore used. Foetal liver is the primary site of haematopoiesis during foetal development and is therefore another source of HPCs for *in vitro* experiments. It is not known whether the E2A-HLF fusion arises *in utero*. In order to determine whether E2A-HLF can immortalise HPCs derived from foetal liver, the E2A-HLF-Neo retrovirus was also used to transduce murine foetal liver HPCs. cKit-expressing HPCs from embryonic day 13 (E13) foetal liver of C57BL/10 mice were purified and transduced with E2A-HLF-Neo with or without BCL-2. Cells were subsequently cultured in the presence of SCF, IL-7 and Flt-3L. Single or double antibiotic selection was used where appropriate during the first round of replating only.

At the end of the first round of replating, 7 to 88 colonies were generated by the HPCs transduced with the different combinations of retroviruses (**Figure 6.10A**). The lowest number of colonies were generated by the cells transduced with Neo/BCL-2 retrovirus combination (**Figure 6.10A**). The other retrovirus combinations generated colony numbers ranging from 30 to 88. However, on subsequent rounds of replating, cells that had been transduced with E2A-HLF-Neo alone or E2A-HLF-Neo/Puro generated an average of 360 colonies per 10^4 cells plated and always generated more colonies than the cells transduced with Neo/Puro or Neo/BCL-2. E2A-HLF-Neo alone or E2A-HLF-Neo/Puro transduced cells could be serially replated until at least the fifth round whereas cells transduced with the empty retroviruses and the Neo/BCL-2 combinations did not replate beyond the third round. E2A-HLF-Neo/BCL-2 showed a synergistic action and generated significantly greater numbers of colonies and viable cells than all the other retroviral combinations with nearly 2500 colonies generated at the end of the second round of

(A)



(B)

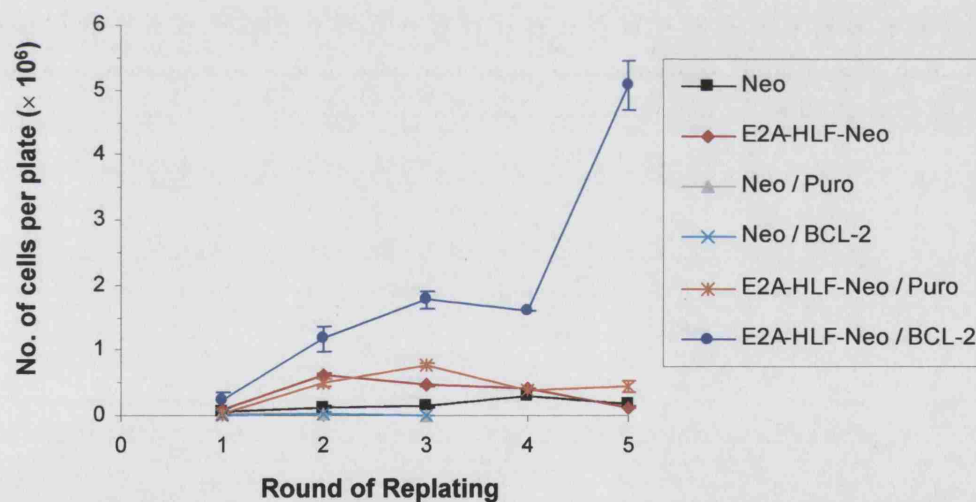


Figure 6.10. E2A-HLF-Neo immortalised foetal liver HPCs in B cell conditions and synergised with BCL-2.

(A) Mean number of colonies generated per 10^4 cells after 7-10 days in culture in methylcellulose with SCF, IL-7 and Flt-3L (\pm SEM). Fewer than 10^4 cells were obtained at 48 h after infection therefore all viable cells were plated for the 1st round. (B) Total number of viable cells isolated from each plate (\pm SEM).

replating (**Figure 6.10A and 6.10B**). This synergistic effect between E2A-HLF and BCL-2 on colony numbers and cell numbers was consistent throughout all rounds of replating.

Cells that were transduced with E2A-HLF-Neo/Puro were cultured in conditions which promote B cell differentiation. However, analysis of the cell surface markers of the immortalised cells showed enrichment of cells expressing the myeloid marker, Gr-1, and loss of cells that expressed the B cell marker, B220 (**Figure 6.11A**). Cells that had been transduced by E2A-HLF-Neo alone showed the same enrichment of Gr-1-expressing cells (data not shown). However, E2A-HLF-Neo/BCL-2 immortalised B cells as determined by the enrichment of B220-expressing cells under this condition (**Figure 6.11B**). Cell lines were established from the myeloid cells immortalised by E2A-HLF-Neo alone and by E2A-HLF-Neo/Puro as well as the B cells immortalised by E2A-HLF/BCL-2 in liquid medium containing SCF, IL-7 and Flt-3L. These cell lines proliferated in response to SCF, IL-7 and Flt-3L for at least 2 months. Flow cytometric analysis of these cell lines showed that the E2A-HLF-Neo alone or E2A-HLF-Neo/Puro cell lines expressed low levels of Mac-1, higher levels of Gr-1 but no expression of B220 as expected (**Figure 6.12A and 6.12B**). This phenotype was similar to the phenotype of the myeloid cell line generated by E2A-HLF-CD2 (**Figure 6.4**). The E2A-HLF-Neo/BCL-2 cell line expressed B220, but not Mac-1 or Gr-1 (**Figure 6.12C**).

Infection of foetal liver HPCs were repeated to confirm the synergistic effect between E2A-HLF and BCL-2 and to determine whether B cells could be immortalised at all by E2A-HLF-Neo without co-expression of BCL-2. E2A-HLF-Neo/BCL-2 was again able to synergise and generate greater numbers of colonies

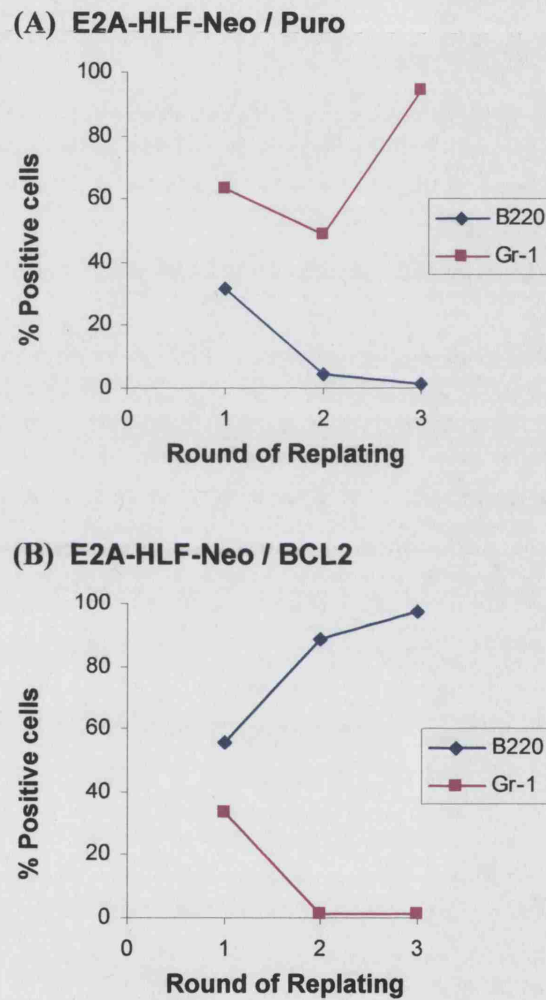
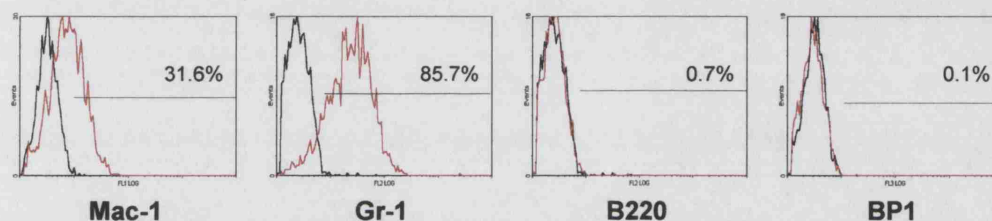


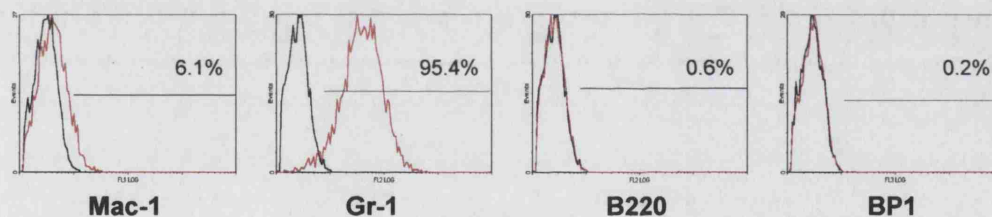
Figure 6.11. Enrichment of Gr-1-expressing cells by E2A-HLF-Neo/Puro and enrichment of B220-expressing cells by E2A-HLF-Neo/BCL-2.

Pooled cells from different rounds of relating in presence of SCF, IL-7 and Flt-3L were analysed by flow cytometry for expression of B220 and Gr-1 cell surface markers.

(A) E2A-HLF-Neo cell line



(B) E2A-HLF-Neo / Puro cell line



(C) E2A-HLF-Neo / BCL-2 cell line

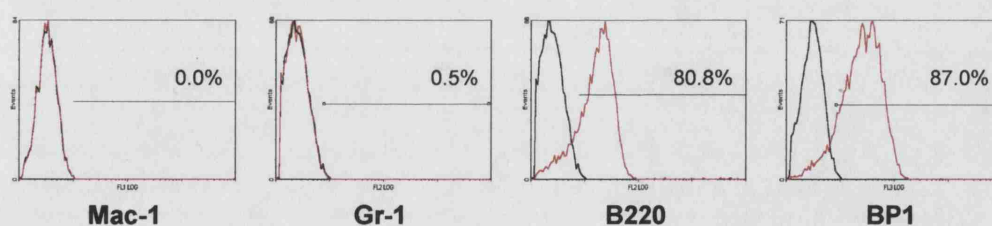


Figure 6.12. E2A-HLF-Neo immortalises foetal liver myeloid progenitors cultured in B cell conditions.

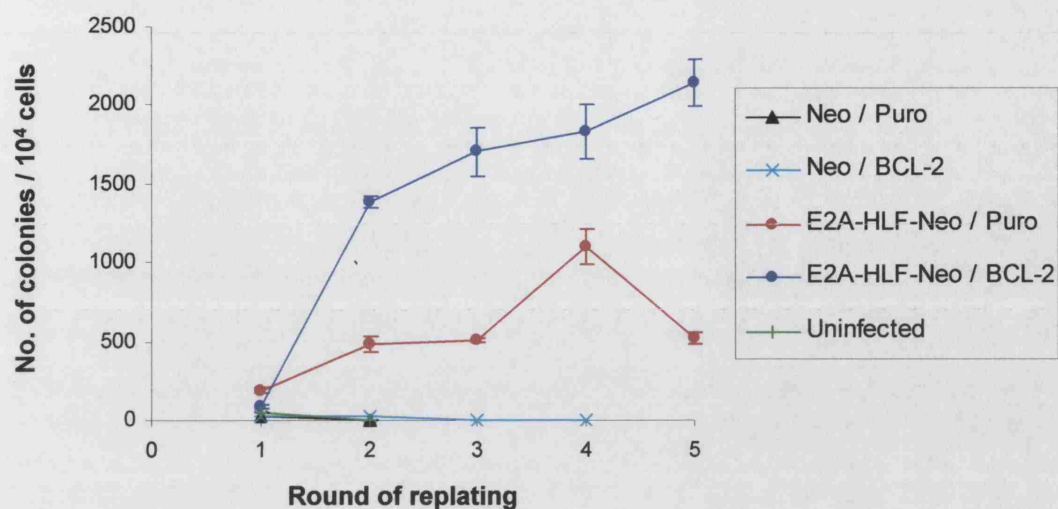
Cell lines established from pooled cells from the fifth round of replating were analysed for the expression of the myeloid markers or B cell markers by flow cytometry. Black lines indicate isotype control.

and viable cells than uninfected cells or cells transduced with Neo/Puro, or Neo/BCL-2, or E2A-HLF-Neo/Puro (**Figure 6.13A and 6.13B**). Uninfected cells, Neo/Puro and Neo/BCL-2 infected cells generated significantly fewer cells than E2A-HLF/Puro or E2A-HLF/BCL-2 and failed to replate beyond the third round.

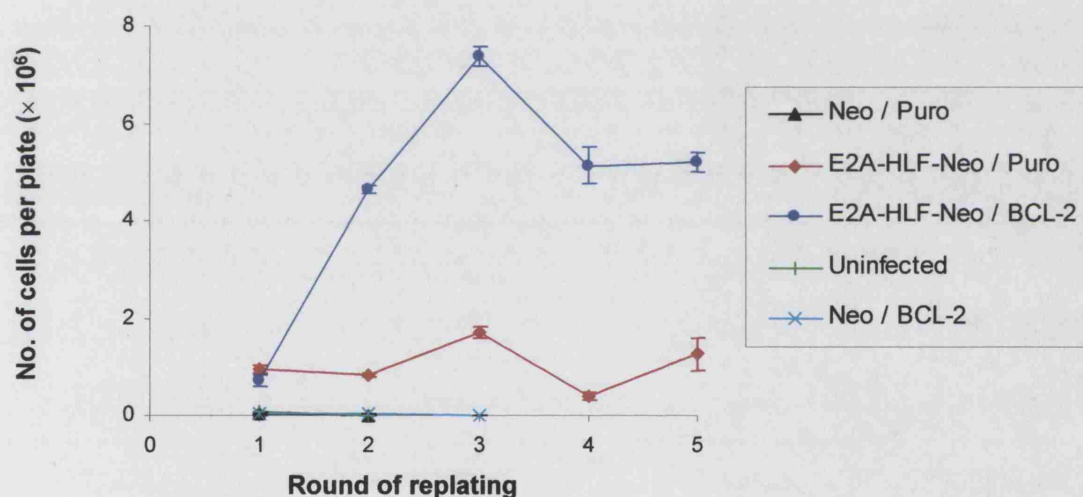
Colony morphology in methylcellulose shows that Neo/BCL-2 generated relatively small colonies which were consistent with the appearance of normal B cell colonies (**Figure 6.13C**) whereas E2A-HLF-Neo/Puro colonies were much larger and consisted of densely-packed cells. E2A-HLF-Neo/BCL-2 colonies were similar, but were smaller in size (**Figure 6.13D**). However, at the time when the images of the colonies were taken (the second round of replating), E2A-HLF-Neo/BCL-2 had generated 3-fold more colonies than E2A-HLF-Neo/Puro.

In contrast to the previous results using foetal liver HPCs, E2A-HLF-Neo/Puro was able to immortalise B cells as determined by the enrichment of B220-expressing cells in methylcellulose cultures (**Figure 6.14A**). Cells transduced with E2A-HLF-Neo/BCL-2 also showed enrichment of B220-expressing cells. However, initial rounds of replating also showed high levels of Gr-1-expressing cells indicating that some cells co-expressed both B cell and myeloid markers (**Figure 6.14**). Further flow cytometric analysis of cells derived from the fourth round of replating showed that E2A-HLF-Neo immortalised cells expressed B220, BP1 and CD19 with very little or no Mac-1 or Gr-1 and some cKit expression (**Figure 6.15A**). Analysis of E2A-HLF-Neo/BCL-2 immortalised cells also confirmed that the cells generated were B cells, however, under these conditions, the cells expressed lower levels of CD19 and higher levels of cKit than the E2A-HLF-Neo/Puro cells (**Figure 6.15B**). Cell lines could be established in liquid culture from cells transduced with E2A-

(A)



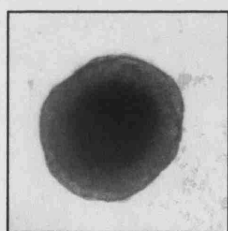
(B)



(C) Neo / BCL-2



(D) E2A-HLF-Neo / Puro



(E) E2A-HLF-Neo / BCL-2

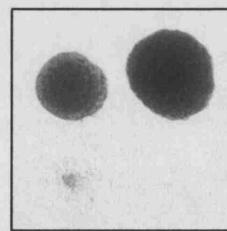
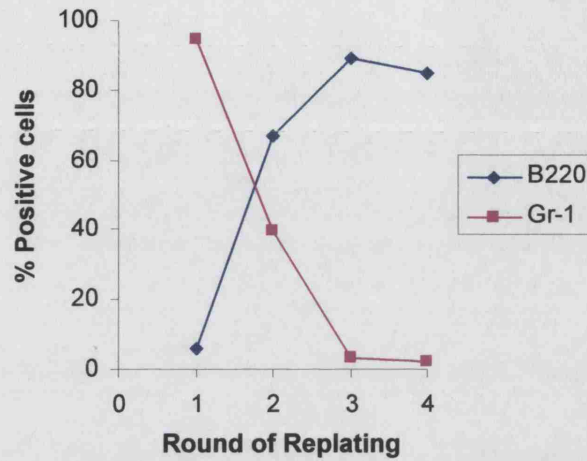


Figure 6.13. E2A-HLF-Neo immortalises foetal liver HPCs in B cell conditions and synergises with BCL-2.

(A) Mean number of colonies generated per 10^4 cells after 7-10 days in culture in methylcellulose with SCF, IL-7 and Flt-3L (\pm SEM). (B) Total number of viable cells isolated from plate (\pm SEM). (C), (D) and (E) Typical morphology of colonies in methylcellulose at the end of the second round of replating ($\times 10$ magnification).

(A) E2A-HLF-Neo / Puro



(B) E2A-HLF-Neo / BCL-2

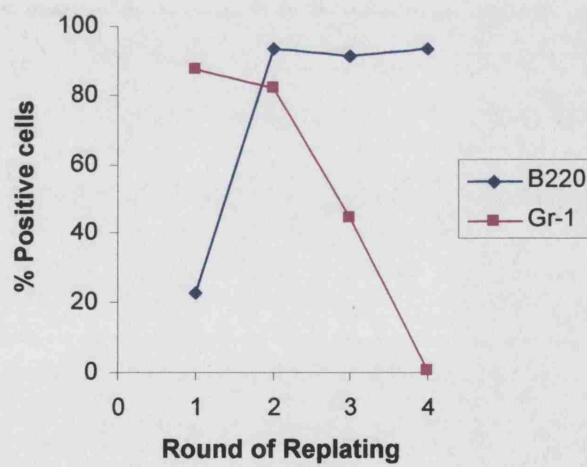
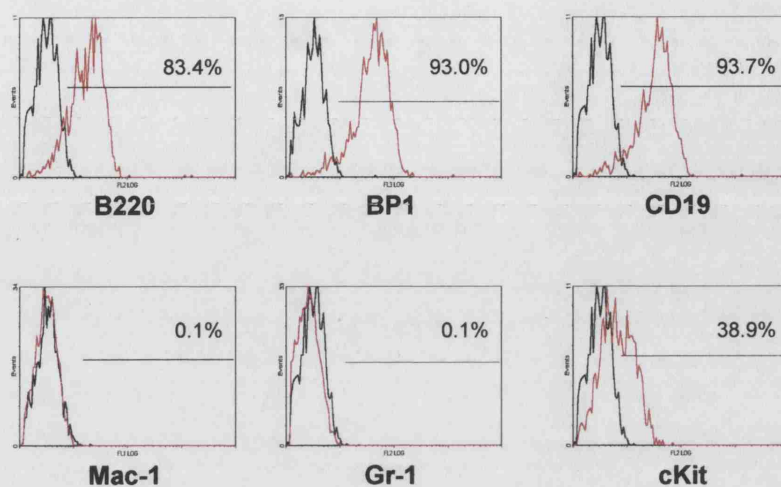


Figure 6.14. Enrichment of B220-expressing cells by E2A-HLF-Neo with or without BCL-2.

Pooled cells from different rounds of relating in presence of SCF, IL-7 and Flt-3L were assessed for expression of B220 and Gr-1 cell surface markers by flow cytometry.

(A) E2A-HLF-Neo / Puro



(B) E2AHLF-Neo / BCL-2

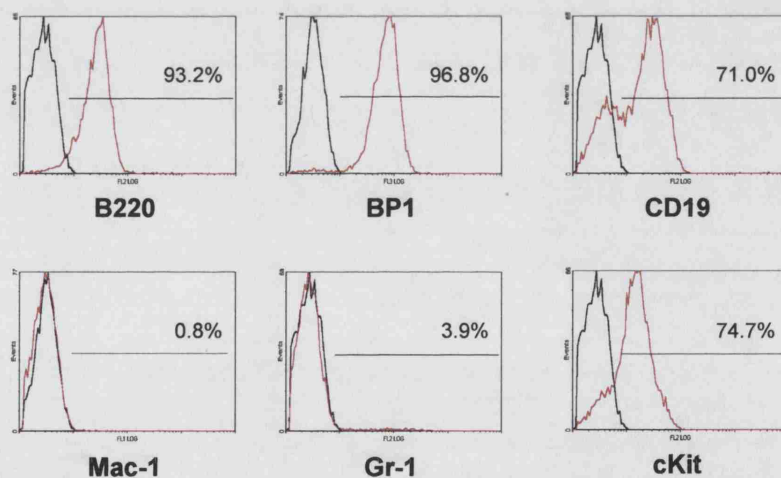


Figure 6.15. E2A-HLF-Neo with or without co-expression of BCL-2 immortalises B cells derived from foetal liver HPCs.

Flow cytometric analysis of pooled colonies from the fourth round of methylcellulose replating in presence of SCF, IL-7 and Flt-3L.

HLF-Neo/Puro and E2A-HLF-Neo/BCL-2 which proliferated well in liquid medium containing SCF, IL-7 and FLt-3L cytokines.

In an independent transduction experiment, immortalised B cells that had adapted to growth in liquid culture, were generated from foetal liver HPCs transduced with E2A-HLF-Neo alone, E2A-HLF-Neo/Puro and E2A-HLF-Neo/BCL-2. These cell lines were assessed for their cytokine requirements. Maximum viability was observed with all three cell lines when cultured in presence of SCF, IL-7 and Flt-3L or in presence of SCF and IL-7 (**Figure 6.16**). The E2A-HLF-Neo alone and E2A-HLF-Neo/Puro cell lines showed similar characteristics in the different cytokine combinations. These two cell lines showed a decreased viability (to 12%) when cultured in SCF alone compared to the control cells grown in presence of all three cytokines. However, the viability of the E2A-HLF-Neo/BCL-2 cell line decreased to 20% when cultured in SCF alone compared to control. In the presence of IL-7 alone, the E2A-HLF-Neo/BCL-2 cell line show approximately 14-fold greater viability than the other two cell lines. In absence of all three cytokines, the E2A-HLF-Neo/BCL-2 cell line was still viable whereas the other two cell lines were not. Flt-3L did not synergise with the other cytokines or promote the growth of any of the cell lines alone.

6.3. Discussion

Haematopoietic progenitor and stem cells have a limited self-renewal capacity. Self-renewal capacity is exhausted in normal haematopoietic cells and will fail to replate after a few rounds in methylcellulose. However, data presented in this chapter show that transduction of haematopoietic progenitor cells by E2A-HLF appears to increase

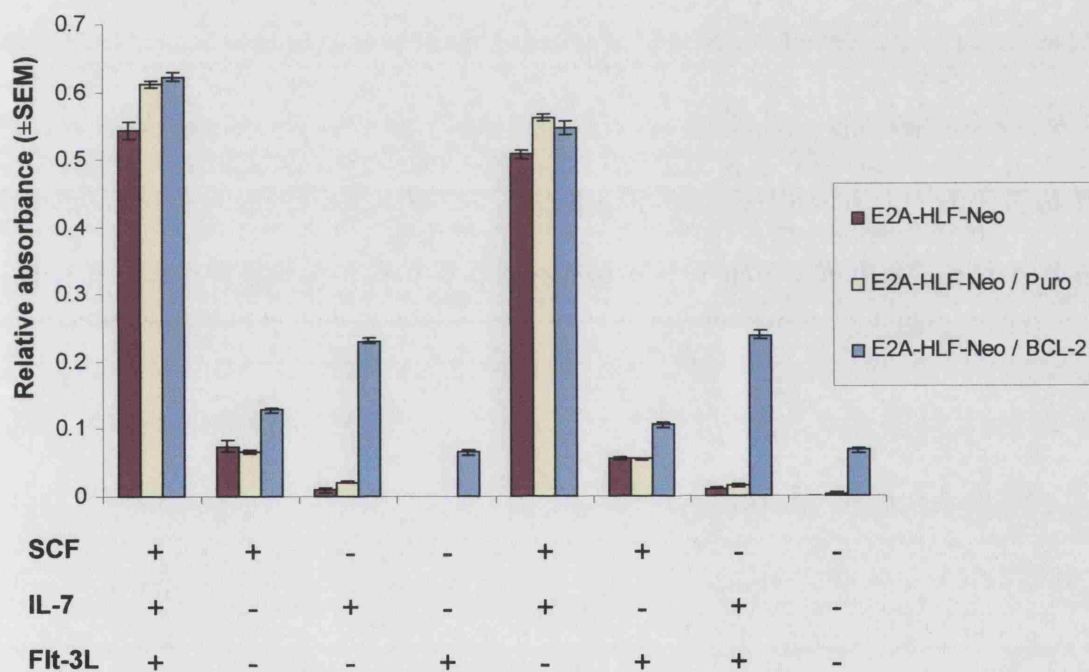


Figure 6.16. A B cell line established from foetal liver HPCs transduced with E2A-HLF-Neo/BCL-2 is viable in absence of SCF, IL-7 and Flt-3L.

Assessment of the cytokine requirements of B cell lines generated from transduced E12 foetal liver HPCs was culture of cells in different cytokine combinations for 48 hours. Viability was determined by a MTS assay.

the self-renewal capacity of the progenitor cells that are grown in myeloid and B cell conditions, thereby confirming the oncogenic potential of E2A-HLF.

6.3.1. *Immortalisation of bone marrow cells by E2A-HLF*

Data in this chapter confirmed previous studies that showed E2A-HLF readily immortalised HPCs grown in the presence of myeloid cytokines (Ayton and Cleary, 2003; Smith *et al.*, 2003). In addition, E2A-HLF also readily immortalised myeloid progenitors derived from both murine bone marrow and murine foetal liver. E2A-HLF-CD2 immortalised myeloid progenitors without any antibiotic selection. This indicated that the expression of E2A-HLF provided a survival or proliferative advantage over cells which do not express E2A-HLF.

The myeloid cell lines established from bone marrow HPCs transduced with E2A-HLF-CD2 or E2A-HLF-Neo showed different cell surface marker expression. The myeloid E2A-HLF-CD2 cell line derived from bone marrow HPCs expressed high levels of Gr-1 and low expression of Mac-1. Immortalised myeloid cell lines from E2A-HLF-Neo- and E2A-HLF-Neo/Puro-transduced foetal liver HPCs also expressed low levels of Mac-1 and high levels of Gr-1. However, the myeloid cell line derived from bone marrow HPCs transduced with the E2A-HLF-Neo retrovirus expressed high levels of both Mac-1 and Gr-1. This difference may be due to E2A-HLF having more than one myeloid progenitor target in the bone marrow. The differences in the expression of cell surface markers suggested that the immortalised myeloid cell lines were slightly different cell types and may explain why cells from the E2A-HLF-Neo-transduced bone marrow cell line were able to differentiate and become adherent and were unable to undergo long-term culture.

The ability of E2A-HLF-Neo to immortalise and generate a myeloid cell line from foetal liver HPCs when cultured only in the presence of the cytokines SCF, IL-7 and Flt-3L which promote the differentiation B cells was unexpected. This could be explained by a number of observations. Many myeloid colonies were generated during the first round of replating of uninfected and infected foetal liver HPCs in presence of SCF, IL-7 and Flt-3L. The myeloid progenitors present in these cultures are targets for E2A-HLF immortalisation. The cytokine requirements of myeloid bone marrow cells immortalised by E2A-HLF-CD2 showed that immortalised myeloid cells could grow in SCF alone. Therefore, the growth of the myeloid foetal liver E2A-HLF-Neo cell line could be also be supported by SCF alone and that differentiation of these cells along the myeloid lineage could be achieved without any requirement for exogenous IL-3, IL-6 or GM-CSF. Therefore, E2A-HLF may regulate genes which would promote the myeloid differentiation of HPCs or compensate for the lack of signalling induced by IL-3, IL-6 and GM-CSF.

E2A-HLF functioned as a potent oncogene in this retroviral system. Other workers have shown that unsorted bone marrow cells from 3.5-week old Balb/c mice transduced with E2A-HLF and grown on AC-6.21 bone marrow stromal cells were unable to generate long-lived lymphoid cells unless the cells co-expressed BCL-2 (Smith *et al.*, 2002). No myeloid progenitors were immortalised using this system. As unsorted bone marrow was used and no exogenous cytokines added, myeloid precursors could have been targeted and immortalised by E2A-HLF. AC-6.21 bone marrow stromal cells may not support propagation and growth of myeloid progenitors which were targeted by E2A-HLF.

E2A-HLF has not been documented to be associated with myeloid leukaemias in humans or cause myeloid malignancies in E2A-HLF transgenic mice. The translocation which generates the E2A-HLF fusion is hypothesised to occur during immunoglobulin rearrangement during B cell development (Inaba *et al.*, 1992; Hunger *et al.*, 1992). If this is the mechanism by which the translocation which generates E2A-HLF arises, then E2A-HLF will not be expressed in myeloid cells. The *E2A* promoter may not be active in myeloid cells and therefore any E2A-HLF product generated by gene rearrangement in myeloid cells may not be expressed. It is important to note that the E2A-PBX1 fusion protein also immortalises myeloid progenitors, but not B cell progenitors, and E2A-PBX1 transgenic mice develop acute myeloid leukaemias (Kamps and Baltimore, 1993; Thorsteinsdottir *et al.*, 1999; Sykes and Kamps, 2004). It is interesting that granulocytes in the homozygous E2A knock-out mice were less mature than in the wild type littermates as assessed by morphology and size scatter by flow cytometry (Zhuang *et al.*, 1994).

6.3.2. Inability to immortalise bone marrow HPCs with E2A-HLF

The E2A-HLF-CD2 retrovirus did not immortalise lymphoid HPCs derived from bone marrow whether or not BCL-2 was co-expressed. The level of CD2 expression, which was used as a marker of infection, decreased on serial rounds of replating, suggesting that the percentage of cells expressing E2A-HLF also decreased. This observation suggests that the expression of E2A-HLF, or high levels of E2A-HLF, in bone marrow B cells may be deleterious which might help explain why transgenic E2A-HLF mice show low level expression of E2A-HLF in B cells (Honda *et al.*, 1999; Smith *et al.*, 1999). Increased levels of apoptosis in bone marrow cells transduced with E2A-HLF and grown on stromal cells has previously been reported (Smith *et al.*, 2002). To overcome the loss of CD2, and therefore

E2A-HLF expression, as detailed in results section 6.2.3, bone marrow HPCs could have been transduced with the E2A-HLF-Neo retroviral construct and subsequently selected with geneticin to enforce E2A-HLF expression. Methylcellulose assays could be repeated to determine whether E2A-HLF can immortalise bone marrow HPCs cultured in B cell promoting cytokines.

6.3.3. Immortalisation of foetal liver HPCs by E2A-HLF

In contrast, E2A-HLF was able to immortalise B cells derived from foetal liver HPCs and co-infection of E2A-HLF-Neo and BCL-2 generated greater numbers viable cells than the other two infection conditions. This data indicates that the expression of BCL-2 synergised with E2A-HLF to further increase the number of viable cells. However, E2A-HLF-Neo/Puro colonies were significantly larger than E2A-HLF-Neo/BCL-2. This was most likely to be due to the significantly higher number of colonies generated by E2A-HLF-Neo/BCL-2 which limits colony sizes due to increased rates of nutrient depletion from the methylcellulose medium.

The cytokine requirements of the B cell line immortalised by E2A-HLF-Neo/BCL-2 showed the cells were able to survive in absence of exogenous cytokines over a 48 h period and therefore demonstrate some cytokine independence. It has yet not been determined whether these cells could survive in absence of exogenous cytokines over an extended time period. The most striking difference between the cytokine requirements of the B cell lines occurred when the cells were cultured in IL-7 only. Cells co-expression E2A-HLF and BCL-2 proliferated significantly more than cells that expressed E2A-HLF only. This suggests that the mechanism of E2A-HLF immortalisation is different to that achieved by E2A-HLF with BCL-2.

In humans, E2A-HLF is found in pro-B acute lymphoblastic leukaemias. It has not yet been established which are the human target cells for immortalisation by E2A-HLF. The target population may be a committed progenitor or a stem cell. The predominant phenotype of B cells immortalised by E2A-HLF with or without BCL-2 in the experiments detailed in this chapter were B220⁺/BP1⁺. This indicates that the cells were able to differentiate to the pre-B cell stage, a stage later than the pro-B stage (see **Figure 1.2**). This may be due to differences between human and murine lymphopoiesis or due to the differences between the *in vivo* and *in vitro* effects of E2A-HLF.

6.3.4. Conclusions

Data in this chapter demonstrated that E2A-HLF was capable of immortalising HPCs. For the first time, E2A-HLF has been shown to immortalise B lymphoid cell derived from foetal liver. However, *in vivo* experiments are needed to determine whether the immortalised cell lines are leukaemogenic. If the cell lines are leukaemogenic in mice, then this would generate a murine model of B lineage ALL leukaemia. Such a model would allow the investigation the effectiveness of chemotherapeutic drugs and studying the leukaemic progression and invasion.

It is interesting that E2A-HLF and BCL-2 synergise in these assays as BCL-2 is a candidate target gene of E2A-HLF. Other E2A-HLF target genes may also co-operate with E2A-HLF and contribute to the E2A-HLF-mediated leukaemogenesis. LMO2 and IL-15 were identified as targets of E2A-HLF in Chapter 5. The synergistic or cooperative effects of LMO2 and IL-15 should therefore be investigated in HPCs. These E2A-HLF target genes may be suitable targets for

inhibition or activation by chemotherapeutic agents which may improve treatment and clinical outcome.

CHAPTER 7

FINAL DISCUSSION AND CONCLUSIONS

7.1. Achievement of project aims

The original project aims of this project were to generate an *in vitro* inducible system to express E2A-HLF and identify novel target genes in order to elucidate the mechanisms by which E2A-HLF functions and causes leukaemia (section 1.13). In addition to this, an *in vitro* system involving retroviral transfer of target genes into primary HPCs was to be established in order to assess the function of the E2A-HLF target genes. A small-scale clinical study was also to be carried in order to characterise the product of the t(17;19) translocation in two cases of ALL. Data and material from these three areas of work can be used to examine the leukaemogenic properties of E2A-HLF.

7.2. Conclusions

Tet-inducible Baf-3 cells which inducibly expressed E2A-HLF or E4BP4 were generated (Chapter 4). These inducible Baf-3 cells were used to determine whether overexpression of E2A-HLF or E4BP4 conferred a survival advantage to Baf-3 cells induced to undergo apoptosis after IL-3 withdrawal. E2A-HLF was confirmed to have anti-apoptotic properties function as a transcriptional activator. E2A-HLF transactivated reporter constructs containing HLF/E4BP4 consensus sites in reporter assays. By contrast, E4BP4 did not confer protection from apoptosis induced by IL-3 withdrawal. The transcriptional repression functions of E4BP4 was also confirmed using reporter constructs containing HLF/E4BP4 consensus sites in reporter assays.

E4BP4 was found to be a target of E2A-HLF in Chapter 4. Other E2A-HLF targets in the inducible Baf-3 cells were identified using microarrays (Chapter 5).

Microarray studies identified several genes that were upregulated by E2A-HLF e.g. *LMO2*, *IL-15* and *BCL-2* and some were downregulated e.g. *BNIP3*. Northern

blotting analysis and reporter assays using reporter constructs containing the promoter region of putative E2A-HLF target genes validated the findings from the microarray studies.

Retroviral transduction of primary HPCs showed that E2A-HLF immortalised myeloid and lymphoid HPCs which were used to generate cell lines (Chapter 6). E2A-HLF and its candidate target, BCL-2, demonstrated synergistic actions in the immortalisation of B cells. These experiments enabled the conditions for immortalisation of B cells to be established. These conditions will allow the study of the effects of the overexpression of other candidate E2A-HLF target genes identified by microarray experiments.

Two ALL patients were identified and were shown to have the t(17;19) translocation in leukaemic cells. Leukaemic cells from these two patients were shown to express E2A-HLF (Chapter 3). This provided further information regarding the nature of the t(17;19) translocation in ALL. Material from these two patients could be analysed and combined with the data from the other results chapters to help elucidate the role of E2A-HLF in leukaemia.

A model by which E2A-HLF functions as an oncogene can be generated using data from Chapters 4, 5 and 6. Expression of E2A-HLF in patient cells may lead to the aberrant expression of E2A-HLF target genes. E2A-HLF and its target genes may function via a number of pathways which may synergise to cause leukaemia (**Figure 7.1**). E2A-HLF regulates the expression of genes that are involved in conferring the anti-apoptotic functions of E2A-HLF (e.g. BCL-2 and BNIP3). This may lead to the survival of cells which would otherwise be deleted by apoptosis. E2A-HLF and its

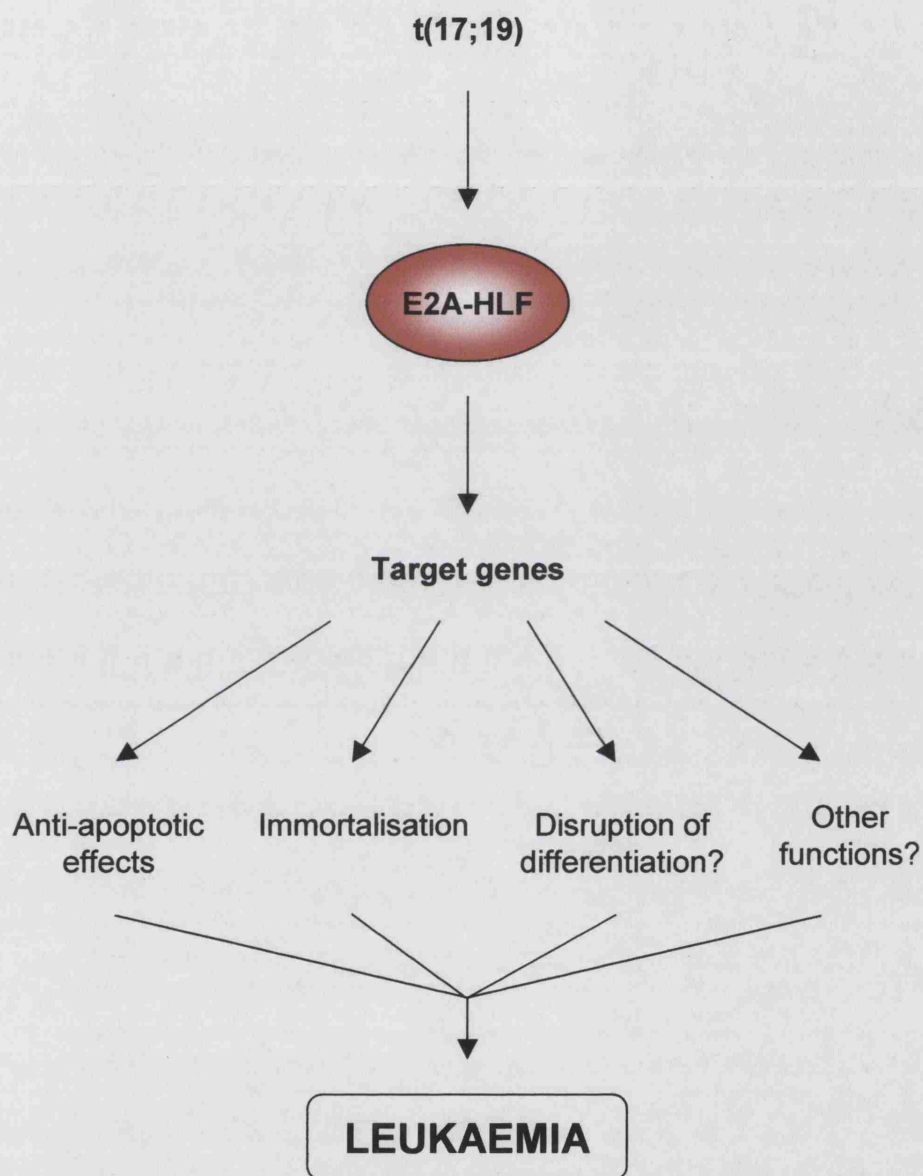


Figure 7.1. A model of leukaemogenic pathways mediated by E2A-HLF.

Expression of E2A-HLF due to the presence of a t(17;19) translocation in the leukaemic cells of patients with B lineage ALL leads to a change in the cellular gene expression. Upregulation or downregulation of E2A-HLF target genes confer the anti-apoptotic and immortalisation properties of E2A-HLF. Other possible leukaemogenic pathways initiated by E2A-HLF expression may be involved which are yet to be determined, e.g. disruption of differentiation. Genes involved in these different pathways are likely to function synergistically to cause leukaemia.

target genes may also have a role in immortalisation, disrupting or blocking differentiation.

7.3. Future work

7.3.1. E2A-HLF rearrangements in patient samples

Sequence analysis of the RT-PCR products from two patients, Patients 1 and 2, indicated that a type II E2A-HLF rearrangement had occurred in the leukaemic cells (Chapter 3). This rearrangement may result in the expression of a functional protein in the leukaemic cells. Western blotting needs to be carried out to determine whether the E2A-HLF protein was expressed. The fusion could have been detected using an anti-E2A antibody, but the antibody would also detect the normal E2A products. An anti-HLF antibody is therefore required to detect the same protein band in order to confirm the presence of a fusion. Unfortunately, the commercial anti-HLF antibody is no longer available, making this analysis difficult. An alternative method to detect the fusion protein is an EMSA. An EMSA using the HLF consensus sequence would be bound by the HLF part of the fusion protein. An anti-E2A antibody could be used to supershift the complex to ensure that the protein bound the probe is the E2A-HLF fusion and not HLF protein.

Several studies could be carried out using the available patient material. Southern blotting could be used to determine whether there are any homozygous deletions of the *p16* or hypermethylation of the *p15* genes to determine whether these additional genetic alterations are a common feature in ALL with the E2A-HLF fusion. RNA samples from patients may be used to determine whether the expression of previously identified E2A-HLF target genes and genes identified by microarray analysis of Baf-3 cells induced to express E2A-HLF. Changes in the expression of

these genes could also be investigated using RT-PCR on the limited number of available patient samples.

E2A-HLF was able to transform myeloid and lymphoid progenitors derived from murine foetal liver (Chapter 6). The origin of the translocation which generates E2A-HLF has not been determined. The translocation could potentially arise *in utero*. Guthrie cards which contain blood spots from neonates have been used to determine whether a translocation that is found in childhood and infant leukaemia arose pre- or post-natally (Gale *et al.*, 1997). The t(1;19) E2A-PBX1 fusion has been proposed to be of post-natal origin as normal V(D)J junctions formed during foetal development usually lack, or contain very few, *N*-nucleotides whereas fusions in children and adults usually contain these *N*-nucleotides (Wiemels *et al.*, 2002). Type I rearrangements contain *N*-nucleotides, however, type II rearrangements do not. It would be interesting to determine whether the t(17;19) translocation in ALL is of pre- or post-natal origin. It may be possible to obtain the Guthrie cards from patients which have ALL with the t(17;19) translocation and determine whether the translocation is present in neonatal blood spots.

As type II rearrangements do not have any inserted sequences between exon 12 of *E2A* and exon 4 of *HLF*, type II rearrangements would be expected to be the same or very similar for all patients that have this rearrangement. This would make type II rearrangements more amenable to the development for specific targeted therapy than a type I fusion. Although type I and type II E2A-HLF fusions are slightly different structurally and have associations with different clinical features, they have identical DNA-binding and transcriptional regulatory properties (Hunger *et al.*, 1994).

These future studies will help determine the molecular pathogenesis and origins of the E2A-HLF-induced leukaemias.

7.3.2. LMO2 and IL-15

The common γ chain subunit is utilised by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 cytokines. Deficiency of the common γ chain causes an X-linked severe immunodeficiency syndrome (SCID-X1) which has been treated using gene therapy involving retrovirus-mediated transfer of the common γ chain into autologous CD34 bone marrow cells (Cavazzana-Calvo *et al.*, 2000). Recently, a lymphoproliferative disorder developed in two patients in a gene therapy trial due to LMO2 activation via integration of the retroviral vector (Hacein-Bey-Abina *et al.*, 2003). LMO2 is a known oncogene in T-ALL and is also upregulated in B-lineage ALLs with MLL translocations (Armstrong *et al.*, 2002; Ferrando *et al.*, 2002).

A recent report has demonstrated that IL-15 can provide a proliferative signal to T-ALL cells (Barata *et al.*, 2004). IL-15 could potentially act in an autocrine manner and stimulate the survival or proliferation of leukaemic cells which express E2A-HLF. IL-15 and LMO2 could therefore potentially cooperate and elicit some of the leukaemogenic functions of E2A-HLF in pro-B cells. No studies have been carried out to determine whether LMO2 and the common γ chain cooperate *in vitro*.

Cooperation between LMO2 and IL-15 and also IL-15 and common γ chain may be assessed by retroviral transfer of these genes into HPCs and culture in methylcellulose.

7.3.3. Determination of the importance of E2A-HLF in HPCs

Several different studies can be carried using the immortalised cell lines established in Chapter 6. The expression of genes identified in Chapter 5 could be determined in the lymphoid and myeloid HPCs immortalised by E2A-HLF. RNA interference (RNAi) technology may be used to reduce the expression of E2A-HLF or its target genes to determine whether there are any changes in cell phenotype, proliferative capacity or the ability for maintenance as a cell line of HPCs immortalised by E2A-HLF. Changes in cell phenotype could be assessed using cell surface markers, e.g. B220, cKit, CD19, Mac-1 and Gr-1. RNAi could also be used on the inducible E2A-HLF clones generated in Chapter 4 in order to determine the effect of downregulation of expression of one of the target genes on the anti-apoptotic function of E2A-HLF following IL-3 withdrawal.

An alternative method of downregulating the expression of E2A-HLF after the oncogene has immortalised primary HPCs involves utilization of the Tet-Off system. The expression of E2A-HLF would be maintained in absence of doxycycline. Once cells were immortalised, doxycycline would be added to the cell culture medium to switch off the expression of E2A-HLF. The changes in gene expression can be determined by the use of microarrays.

Foetal liver derived myeloid and lymphoid HPCs were immortalised by E2A-HLF. Gene expression profiling could also be carried out to determine whether E2A-HLF regulates common genes to immortalise myeloid and lymphoid HPCs. A comparison could be made between normal, non-immortalised myeloid or lymphoid HPCs which have been derived from foetal liver HPCs and cultured and differentiated in methylcellulose. The expression of potential E2A-HLF target genes

identified in the different microarray studies could be checked in the patient samples by real-time PCR.

7.4. Final conclusions

E2A-HLF may contribute to the development of leukaemia in several different ways. Firstly, the t(17;19) translocation results in the loss of one allele of the E2A tumour suppressor. Secondly, the E2A-HLF chimaeric transcription factor regulates a unique set of genes in pro-B cells which are not regulated by their normal counterparts, E2A and HLF. These genes may have an anti-apoptotic effect or may be involved in other processes such as immortalisation and differentiation. The identification of these downstream genes may aid the development of treatments which may be more effective in treating E2A-HLF patients who tend to have an extremely unfavourable prognosis. In addition, any improved treatments for t(17;19)⁺ leukaemias may very well have applications in other cancers.

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APPENDIX

Patient 1

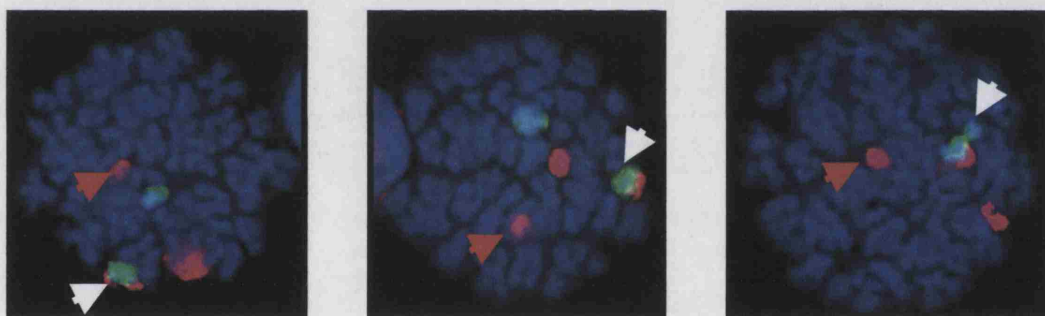


Figure A.1. Detection of t(17;19) translocation in Patient 1 by FISH.

FISH analysis of cells derived from Patient 1 using whole chromosome paint for chromosome 17 (red) and 19 (green). The derivative chromosome 19 is indicated by a white arrow. The derivative chromosome 17 is indicated by a red arrow. Three metaphases are shown. Images courtesy of Helena Kempski, Great Ormond Street Hospital, London.

Patient 2

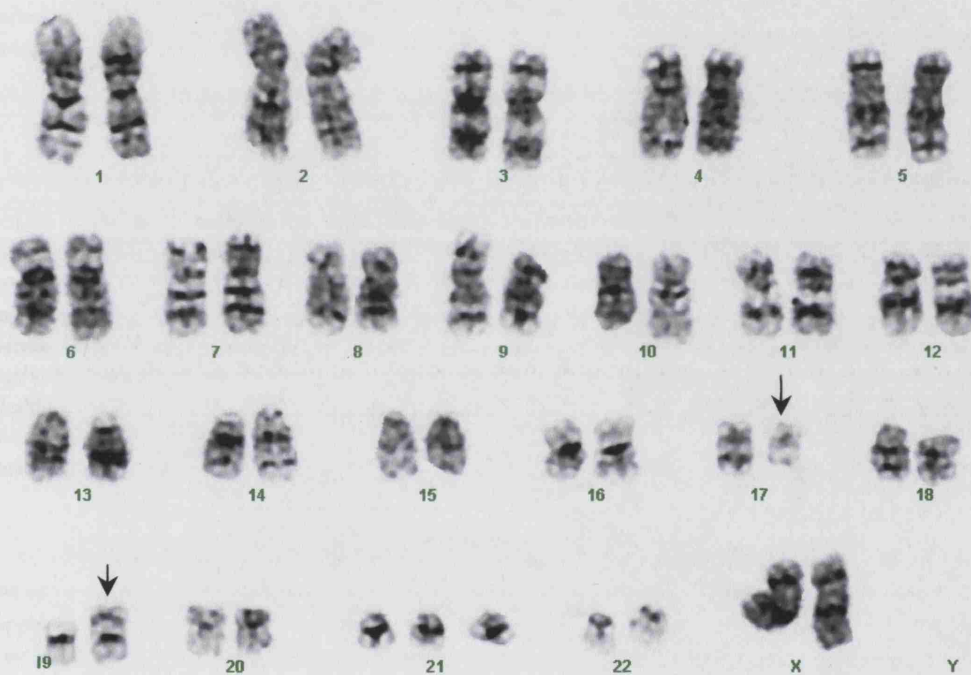


Figure A.2. Detection of t(17;19) translocation in Patient 2 by karyotyping. Karyogram demonstrating the presence of an rearrangement between chromosomes 17 and 19. The derivative chromosomes 17 and 19 are indicated by the arrows. Image courtesy of Professor Owen Smith, Our Lady's Hospital, Dublin.

