In vitro models of TEL/AML1^{+} acute lymphoblastic leukaemia

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Abstract

Acute lymphoblastic leukaemia (ALL) is the most common cancer in children, and is characterised by the proliferation of immature lymphoid cells in the bone marrow. The fusion gene TEL/AML1, generated by the chromosome translocation t(12;21), is the most common single genetic aberration in B-lineage ALL, and is formed from the transcription factors TEL and AML1 (coded for by the genes *ETV6* and *RUNX1*, respectively). However, it is still not clear how the presence of TEL/AML1 causes the development of leukaemia. In addition, the importance of a common secondary mutation in TEL/AML1$^+$ leukaemia, the loss of the untranslocated *ETV6* allele, is still subject to debate.

The role of TEL/AML1 in malignant haematopoiesis has been previously studied in a variety of models, include *in vivo* mouse models, *in vitro* TEL/AML1$^+$ cell lines, and *ex vivo* patient samples. Each of these models has contributed great amounts to our knowledge about TEL/AML1$^+$ leukaemia; however, each type of model has its disadvantages. Here, a new model is presented which aims to complement these other models, based upon human embryonic stem cells (hESCs) expressing a TEL/AML1 transgene. These transgenic hESCs were shown to be capable of multipotent haematopoietic development, including towards B lymphocytes.

The consequence of the loss of TEL in TEL/AML1$^+$ leukaemia is not fully known. Here I provide two insights into this frequent secondary mutation. Firstly, a gene expression microarray revealed the transcriptional role of TEL and illuminated how its loss might contribute to progression of TEL/AML1$^+$ leukaemia. Secondly, the functional role of TEL in proliferation and apoptosis was further investigated, which provided further insight about the clonal evolution of TEL/AML1$^+$ leukaemia. In addition, new discoveries were made of potential partners for heterodimeric transcription factor complexes and targets for TEL repression, which provide new avenues for future studies.

Taken together, the data presented in this thesis provides the basis for renewed study into TEL/AML1$^+$ leukaemia which could address remaining questions about the disease.

Index

Table of Contents

List of Figures

List of Tables

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Author's declaration

All data presented in this thesis is original. With the exception of cloning of pCAG-TEL/AML1 carried out by Jared Cartwright (Figure 3.2), confocal imaging of transgenic cell lines by Matt Lakins (Figure 3.5), karyotyping of transgenic hESC lines by Duncan Baker (Figure 3.7), cell sorting by Karen Hodgkinson (Figure 3.10, Figure 3.11, and Figure 4.1), and gene expression microarray carried out by Celina Whalley and Peter Ashton (Figure 4.1 and Figure 4.2), all the work presented here was carried out by Richard Berks.

Chapter 1: Introduction

1.1 General features of leukaemia

1.1.1 Disease & Classification

The leukaemias are a group of cancers of the bone marrow involving the proliferation of immature leukocytes called 'blasts', and make up about 2.5% of cancers in the United Kingdom, with an incidence of about 1 in 10,000 (Office of National Statistics, Cancer Registrations, 2008). Leukaemia can be classified as either acute or chronic; acute leukaemias are fast progressing and are the most common leukaemias in children, whereas chronic leukaemias can take months or years to progress, involving more mature blasts, and commonly occur in adults. These can be further subdivided into the haematopoietic lineage of the leukaemic blasts, either lymphoid or myeloid. Combining these classifications gives the four major groups of leukaemias (making up over 80% of all leukaemias), which are Chronic Myelogenous Leukaemia (CML), Chronic Lymphocytic Leukaemia (CLL), Acute Myelogenous Leukaemia (AML), and Acute Lymphoblastic Leukaemia (ALL).

1.1.2 Acute Lymphoblastic Leukaemia

Acute Lymphoblastic Leukaemia (ALL) is a disease typified by the proliferation of immature lymphocytes (termed lymphoblasts) in the bone marrow. The symptoms of the disease probably result from the 'crowding-out' of normal haematopoietic cells by these malignant lymphoblasts, and include anaemia, fatigue, frequent infections and fever, and bruising caused by the loss of platelets. In addition, leukaemia blasts infiltrate other organs, including the central nervous system (CNS), lymph nodes, spleen, and liver. According to the Haematological Malignancy Research Network, the incidence of ALL is roughly 1 in 100,000 in the United Kingdom, and peaks in 2-10 year olds, where it accounts for 80% of all leukaemias in this age group. Lymphoid leukaemias can be either of B-cell, T-cell, or Natural Killer (NK)-cell lineage, with B-cell lineage leukaemias making up approximately 88% of all lymphoid leukaemias, and T-cell ALL accounting for 15 to 25% of ALLs (Hoffbrand *et al* 2006; Knight 2010).

1.1.3 Subtypes of B-lineage ALL

The most common single genetic cause of childhood B-lineage ALL is the chromosome translocation t(12;21)(p13;q22), generating a fusion between two transcription factors: the gene *ETV6* (coding for the protein TEL) and *RUNX1* (coding for AML1), often referred to as TEL/AML1. Affecting roughly 25% of patients (1 in 10,000 children under the age of 15), the prognosis of TEL/AML1$^+$ leukaemia is good, with around 90% of children cured (Moorman 2012). The frequency of TEL/AML1 fusions in adult ALL is low, representing about 3% of adult ALL cases (Kwong & Wong 1997; Lee *et al* 2005).

The fusion protein BCR-ABL1, resulting from the t(9;22)(q34;q11.2) translocation (otherwise known as the 'Philadelphia chromosome'), is more commonly associated with adult ALL than childhood ALL, representing 30% and 3% of cases, respectively. BCR-ABL1 is associated with a poor prognosis in ALL, despite being associated with a good prognosis in CML (Knight 2010).

Other chromosome abnormalities associated with B-ALL include t(1;19)(q23;p13)/*E2A-PBX1*, t(v;11q23)/rearranged *MLL*, and t(5;14)(q31;q32)/*IL3-IGH*. Hyperdiploidy (chromosome numbers in excess of 50) and hypodiploidy (fewer than 45 chromosomes) are also frequently observed in B-ALL. In contrast, T-cell leukaemias do not have any defining cytogenetic abnormalities for the purposes of classification (WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue, 2008).

Down's Syndrome (DS, or Trisomy 21) is associated with a higher incidence of both ALL and AML (Maloney 2011) with most of the standard chromosome translocations represented in DS patients. The overall mortality rate in DS-associate leukaemia is higher; this is likely due to the different frequencies of favourable and unfavourable chromosome translocations in DS patients. For example, TEL/AML1$^+$ leukaemia is less frequently observed in DS-associated ALL (Forestier *et al* 2008; Maloney *et al* 2010).

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The first part of this chapter starts with an introduction to haematopoiesis and B cell development and function, followed by an overview of two cell processes critical in cancer: proliferation and apoptosis.

1.2 Haematopoiesis

Haematopoiesis, the generation of all blood cells from haematopoietic stem cells (HSCs), is one of the most studied tissue development systems in biology. Indeed, HSCs are the archetypal stem cell; Till, McCulloch, and colleagues were the first to prove that cells existed in the bone marrow that could give rise to multiple differentiated cell types (Becker *et al* 1963) and that these cells were capable of self-renewal (Siminovitch *et al* 1963), two defining characteristics of all stem cells. This started a new field of stem cell research, to determine how the decisions were made between self-renewal and differentiation (Iscove 2011).

Haematopoiesis is a vast subject, and it is neither possible nor necessary to summarise the whole body of research in this introduction. Presented here is an overview of the important features of haematopoiesis that are necessary background for the chapters to follow.

Differentiated blood cell types are not generated directly from HSCs, instead they arise via a series of intermediate progenitors. Figure 1.1 shows a current understanding of the hierarchy of haematopoietic development, adapted from Reya *et al* 2001. This model divides HSCs into three categories, based upon their ability to self-renew; long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSC), and multipotent progenitors with no self-renewal capacity. These categories were determined by the speed at which transplanted cells could restore haematopoiesis in mice subjected to lethal X-ray irradiation, and the duration for which this could be sustained (Morrison & Weissman 1994; Morrison *et al* 1997; Reya *et al* 2001).

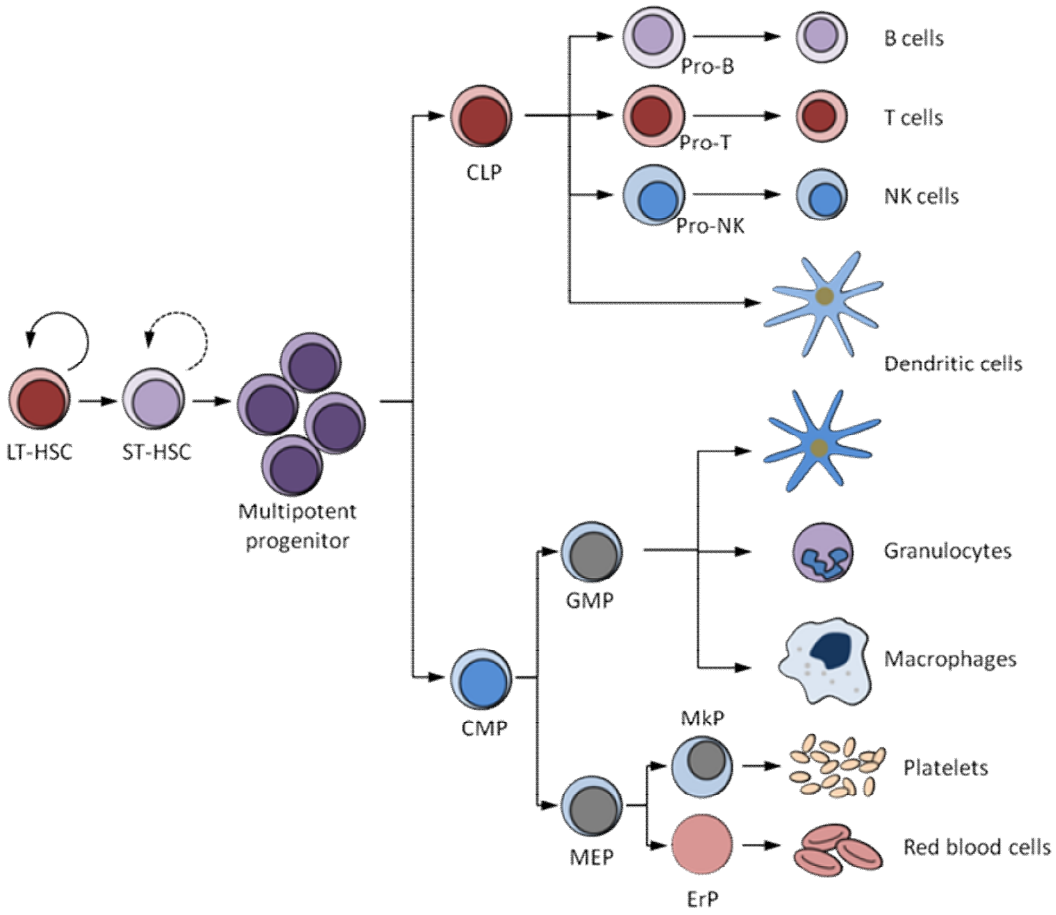

Figure 1.1 – Overview of haematopoietic development.
HSCs are divided into long-term and short-term HSCs (LT-HSC and ST-HSC, respectively), capable of self renewal (indicated by circular arrow), and multipotent progenitors, not capable of self-renewal. These give rise to Common Lymphoid Progenitors (CLP) and Common Myeloid Progenitors (CMP). CLPs give rise to B, T, and NK cells. CMPs give rise to granulocyte/macrophage precursors (GMPs) and Megakaryocyte/Erythrocyte Precursor (MEPs), which generate platelets and red blood cells through Megakaryocyte precursors (MkP) and Erythrocyte precursors (ErP), respectively. Dendritic cells are believed to arise from both CLPs and GMPs. Adapted from Reya *et al* 2001.

1.2.1 Stages of haematopoietic development

Haematopoiesis in the mammalian embryo originates in the extra-embryonic yolk sac (Dzierzak & Speck 2008). The emergence of the first haematopoietic cells, primitive erythrocytes, coincides with the development of vasculature at embryonic day 7.5 (E7.5). This lead to the discovery of what is believed to be a common precursor to haematopoietic and endothelial tissues, called the haemangioblast (Choi *et al* 1998; Fehling *et al* 2003), although there is still debate around the identity and differentiation potential of the haemangioblast (Hirschi 2012).

Definitive haematopoietic stem cells (HSCs), capable of reconstituting irradiated adult mice when transplanted, do not appear in the developing embryo until E10.5 in the aorta-gonad-mesonephros (AGM) region (Muller *et al* 1994; Medvinsky & Dzierzak 1996; de Bruijn *et al* 2000). HSCs emerge from the luminal surface of the dorsal aorta (de Bruijn *et al* 2002; North *et al* 2002; Taoudi & Medvinsky 2007), from endothelial cells which are collectively known as the 'haemogenic endothelium' (Zovein *et al* 2008). The foetal liver is a major source of HSCs in the developing embryo, but does not itself generate HSC *de novo* (Johnson & Moore 1975; Houssaint 1981); rather it is colonised by HSCs from the AGM and other tissues to which the circulation has transported HSCs, e.g. the placenta and yolk sac (Kumaravelu *et al* 2002; Gekas *et al* 2005).

Between the first haematopoietic cells in the yolk sac at E7.5 and the emergence of the first definitive HSCs at E10.5, a series of multipotent haematopoietic cells are also generated. After primitive erythropoiesis begins in the yolk sac and before blood circulation commences at E8.5, multipotent myeloid progenitors are generated in the yolk sac (Palis *et al* 1999). Similarly, before the onset of circulation, lymphoid-myeloid precursors are generated in the para-aortic splanchnopleura, the region which will become the AGM (Cumano *et al* 1996). Finally, a HSC population capable of repopulating neonatal mice, but not adult mice, is generated concomitantly in the yolk sac and the AGM region at E9 (Yoder *et al* 1997).

HSCs generated from the AGM region migrate to colonise the liver, and then the spleen and thymus are colonised either directly from the AGM or from the liver

(Bertrand *et al* 2006; Yokota *et al* 2006). However, it is likely that HSCs that populate the bone marrow in adult mice are derived not from the HSCs emerging from the embryonic AGM region, but from those in the yolk sac (Gothert *et al* 2005; Samokhvalov *et al* 2007; Tanaka *et al* 2012). The bone marrow becomes the main site of haematopoiesis as extra-medullary haematopoiesis in the liver, spleen, and thymus subsides and ceases.

1.2.2 Cell surface markers of early haematopoietic development

The elucidation of early haematopoietic development has been facilitated by cell surface markers that can be used to identify populations of cells by immunostaining.

The classical marker for endothelium is CD31, the cell-adhesion molecule PECAM-1 (Platelet/Endothelial Cell Adhesion Molecule-1; Newman 1994), which was used to identify that haematopoietic and endothelial cells arise from a common precursor (Choi *et al* 1998; Fehling *et al* 2003; Zovein *et al* 2008).

The clear identification of human HSCs capable of long-term engraftment (so called 'LT-HSC'; Kondo *et al* 2003) has been more complicated than the identification of the mouse equivalent, due to the assays being used to functionally identify such populations (Kondo *et al* 2003). HSCs are known to express CD34, but the majority of $CD34^+$ haematopoietic cells are committed progenitors (Hogge *et al* 1996). $CD34^+$ HSCs have been further characterised by the lack of expression of CD45RA (Mayani *et al* 1993) and CD38 (Hao *et al* 1995; Bhatia *et al* 1997), amongst others (Kondo *et al* 2003). A recent study has characterised human LT-HSCs further by $CD34^+ CD38^- CD45RA^- Thy1^+ CD49f^+$ (Notta *et al* 2011a). In addition, CD43 has been identified as the earliest exclusive marker of haematopoietic cells emerging from differentiation of human embryonic stem cells, or hESCs (Vodyanik *et al* 2006).

1.2.3 Haematopoietic transcription factors

SCL (Stem Cell Leukaemia), also known as TAL1 or TCL5, is a basic helix-loop-helix (bHLH) transcription factor (Massari & Murre 2000), and one of the earliest-acting regulators of haematopoiesis (Lecuyer & Hoang 2004). SCL is

essential for haematopoiesis *in vivo* (Shivdasani *et al* 1995), as *SCL*$^{-/-}$ ES cells do not contribute to any haematopoietic lineage in chimeric mice (Porcher *et al* 1996; Robb *et al* 1996). SCL appears to act at several stages of haematopoietic development: SCL is essential for the production of primitive haematopoietic cells from hemangioblasts in the yolk sac (Robb *et al* 1995), as well as definitive haematopoiesis (Porcher *et al* 1996). In adult haematopoiesis, SCL expression is highest in HSCs and progressively decreases in more committed cells in the haematopoietic hierarchy (Elefanty *et al* 1998), but still remains high in cells differentiating towards erythrocyte, megakaryocyte, and mast cell lineages (Lecuyer & Hoang 2004).

EKLF, also known as KLF1, is a member of the Krüppel-Like Factor family of transcriptional regulators (Siatecka & Bieker 2011). EKLF is essential for definitive erythropoiesis in the foetal liver, but dispensable for primitive erythropoiesis in the yolk sac (Nuez *et al* 1995), despite its expression throughout primitive haematopoiesis (Southwood *et al* 1996). EKLF is not expressed in early haematopoietic progenitors, but is expressed in common myeloid progenitors and their megakaryocytic-erythroid progenitor progeny, and increases as cells commit to erythrocyte lineage, whilst simultaneously inhibiting megakaryocytic differentiation. (Frontelo *et al* 2007).

PU.1, also known as SPI-1, is a member of the *ets* family of transcription factors, and is an important transcriptional regulator during definitive haematopoiesis (Kastner & Chan 2008). PU.1 knockout mice die late in gestation and have defects in multiple definitive haematopoietic lineages, including B- and T-lymphocytes, monocytes, and granulocytes (Scott *et al* 1994). PU.1 is essential for the maintenance of adult and foetal liver HSCs, and also for the differentiation of committed myeloid progenitors (DeKoter *et al* 1998; Iwasaki *et al* 2005). PU.1 has an important role in B cell development (Medina *et al* 2004), as B cell differentiation and maturation is inhibited, but not blocked, in PU.1 deficient mice (Iwasaki *et al* 2005).

Two of the GATA family of transcription factors, GATA1 and GATA2, have major roles in the regulation of haematopoiesis. GATA1 is an important regulator of

various myeloid lineages (Crispino 2005): it is essential for erythropoiesis, as GATA1 null mice embryos die at around E10.5 from anaemia, and are found to have defective primitive red blood cell development (Fujiwara *et al* 1996). Definitive erythroid development from GATA1-null ESCs is also blocked (Weiss *et al* 1994). In erythropoiesis, GATA1 is known to interact with multiple other transcription factors including PU.1 and EKLF (Cantor & Orkin 2002). Aside from its importance in erythoid development, GATA1 has a role in megakaryocyte growth and platelet genesis (Shivdasani *et al* 1997), eosinophil development (Crispino 2005), and mast cell differentiation (Migliaccio *et al* 2003).

GATA2 also has an important role in haematopoiesis (Vicente *et al* 2012). GATA2 is required for the production, proliferation, and survival of definitive HSCs right from their earliest emergences in the AGM region (Tsai *et al* 1994; Tsai & Orkin 1997; Ling *et al* 2004). In this role, GATA2 is known to interact with other haematopoietic transcription factors FLI1 and SCL (Pimanda *et al* 2007). GATA2 also has an overlapping role with GATA1 in the regulation of erythroid and megakaryocytic development (Ikonomi *et al* 2000; Grass *et al* 2003). Both GATA1 and GATA2 also exert effects on myeloid differentiation through the regulation of expression of PU.1 (Chou *et al* 2009).

1.2.4 Assays used to investigate haematopoiesis

Research in haematopoiesis, including the origins of haematopoietic precursors, has required a variety of standardised assays to test how haematopoietic development is affected. Here an overview is given of a selection of these methods, what function they perform, and examples of how they have been used to investigate haematopoietic development.

Perhaps the ‘gold standard’ test for determining the haematopoietic potential of a suspected stem cell is to transplant cells into an irradiated recipient mouse. Definitive HSCs are defined by their ability to reconstitute full long-term haematopoiesis in an irradiated recipient mouse and, by serial transplantation, that the same population can be transplanted from the recipient mouse into another irradiated mouse and reconstitute haematopoiesis once more, and so on. This can be

used to determine, for example, the origin of definitive HSCs in the developing embryo (e.g. Muller *et al* 1994), or the influence of certain genes on HSC populations (e.g. Hock *et al* 2004). However, with adaptation it can be used for other purposes. For example, it is assumed that only one HSC is necessarily to reconstitute haematopoiesis in an irradiated recipient mouse, and thus by performing a serial dilution of a suspected stem cell population before transplantation, one can determine how many definitive HSCs exist in the starting population by calculating backwards from the biggest dilution that allows full haematopoietic reconstitution. This limiting dilution assay has been used to determine, for example, the effects of Sonic Hedgehog (Shh) signalling on the numbers of HSCs in the bone marrow (Bhardwaj *et al* 2001). Another adaptation is xenograft into immunodeficient mice, such as the NOD-SCID mouse, to perform the same assays for human HSCs, either derived from primary bone marrow aspirates or cord blood (e.g. Larochelle *et al* 1996; Broxmeyer *et al* 2011), or from hESCs (e.g. Wang *et al* 2005; Ledran *et al* 2008).

The colony forming unit (CFU) assay was first described by Till and McCulloch (1961); irradiated mice that received injections of bone marrow were found to develop nodules in their spleen. The number of these nodules varied linearly with the numbers of cell injected, and these nodules contained a number of different haematopoietic cell types. These colonies were later found to be clonal in origin, i.e. derived from a single cell (Becker *et al* 1963). *In vitro* CFU assays today involve growing suspected haematopoietic progenitors in a semi-solid media (most commonly methylcellulose) with haematopoietic cytokines to promote differentiation. The resulting colonies of haematopoietic cells can be enumerated and different cell types identified to determine the frequency and haematopoietic potential of the cells in the starting population. This technique has been used, for example, to study how cells from different haematopoietic niches influence the differentiation of hESCs (Ledran *et al* 2008).

1.3 B cell development

To understand the progression of B cell ALL, it is important to give a brief overview of the function of B cells, and human B cell development, both normal and leukaemic.

1.3.1 Normal function of B cells

The primary role of B cells is to produce antibodies with affinity to foreign antigens. These antibodies bind to foreign pathogens, marking them for destruction by different cell types of the immune system, in a mechanism called the 'humoral immune response' (Murphy *et al* 2008). Antibodies can be secreted by B cells as a soluble protein, but can also be bound to the plasma membrane, where they form the main component of a signalling complex called the B-cell receptor (BCR). Two other essential components of the BCR are the invariable immunoglobulins Igα (otherwise known as mb-1 or CD79A) and Igβ (CD79B), which provide most of the cytoplasmic domains of the BCR (Borst *et al* 1996; Murphy *et al* 2008).
BCR signalling is enhanced by a co-receptor complex made up of the transmembrane proteins CD19, CD21, and CD81 (Murphy *et al* 2008). CD19 is expressed from early B cell development and is frequently used as a marker for B lineage commitment as a result.

Diversity in antibody binding is generated by a variety of mechanisms, and is an integral part of the development of B cells. The basic structure of an antibody consists of two heavy chains and two light chains. Both heavy and light chains have a "variable region" which come together to form the antigen binding surface, and an invariable "constant region", which forms the remaining antibody structure. The variable regions are produced by the recombination of multiple gene segments at the light and heavy chain loci. For heavy chains, this consists of roughly 65 'Variable' (V) segments, 27 'Diversity' (D) segments, and 6 'Joining' (J) segments. Light chains only have V and J segments; for the two light chain loci, called *kappa* and *lambda*, there are 40 V and 5 J segments for kappa, and 30 V and 4 J segments for lambda. (The numbers of segments are not the same for all people, and there are multiple non-coding 'pseudogenes' in each loci which are not included in this

number; Murphy *et al* 2008). Recombination of the segments is controlled by two Recombination Activating Genes, RAG1 and RAG2 (Schatz & Ji 2011).

Additional diversity is conferred by the removal and insertion of nucleotides at the joins between the V and J segments in light chains (and also partially in the sequence of the D region in heavy chains). Nucleotides called N-nucleotides are added by the gene Terminal deoxynucleotidyl Transferase (TdT; Komori *et al* 1993). Further diversity in mature activated B cells can be gained through a process called 'somatic hypermutation', which generates point mutations in the V gene segments. This allows further editing to generate enhanced binding to antigen (Di Noia & Neuberger 2007).

1.3.2 The humoral immune response

Upon infection by a pathogen, B cells expressing a BCR specific for a particular antigen will engulf the pathogen by phagocytosis. Once internalised, the pathogen is degraded, and peptides from the pathogen are presented back onto on the surface of the B cell by a MHC class II molecule. These peptides are recognised by helper T cells (T_H2 cells) specific for that peptide, which have been 'armed' by professional antigen presenting cells such as dendritic cells and macrophages. This causes the production of various cytokines by the helper T cell, which in turn causes 'B cell activation': the differentiation and proliferation of that B cell into a 'plasma cell', producing antibodies specific for the antigen it recognised on the pathogen (Parker 1993). How these antibodies are distributed throughout the body, and what happens when these antibodies bind to the antigen, is defined by the isotype of the antibody, which is controlled by the cocktail of cytokines produced by the helper T cell (Murphy *et al* 2008).

When bound to pathogens, isotypes such as IgM and IgG can activate the immune process of 'complement' and stimulate removal of the pathogen by phagocytes, such as neutrophils and macrophages. Phagocytes can also be stimulated directly via receptors which recognise the "constant regions" on the antibodies known as Fc receptors (Ravetch & Clynes 1998). Similarly, small particles such as bacterial toxins and cellular debris can be neutralised by most antibody isotypes, particularly

IgG and IgA. These antigen-antibody immune complexes are carried on erythrocytes in the blood by the activation of complement, and transported to the spleen or the liver where they are cleared by macrophages (Murphy *et al* 2008).

IgG antibodies binding to Fc receptors on natural killer (NK) cells stimulate these cells to kill the antibody-bound pathogen by a process called antibody-dependent cell-mediated cytotoxicity (ADCC; Lanier *et al* 1988); NK cells release granules containing perforin and granzymes, which kills the pathogen by creating pores in the membrane, and activating internal apoptosis pathways, respectively. Finally, antibodies of the IgE isotype bind to Fc recptors on mast cells, basophils, and activated eosinophils, which release chemicals such as histamine, increasing blood flow and making blood vessels more permeable, as a means to recruit more immune cells to the site of infection (Sutton & Gould 1993).

1.3.3 Early B cell development in humans

B-lineage cells develop in multiple locations during embryonic development, including the foetal liver, but from mid-gestation onwards, all B cells originate in the bone marrow (LeBien 2000). From the haematopoietic stem cell (HSC) in the bone marrow, B cell development goes through a Common Lymphoid Progenitor (CLP) stage, which is capable of differentiating towards B cells or T cells, Natural Killer Cells (NK), or Dendritic Cells (DCs; Galy *et al* 1995). CLPs then give rise to a cell termed ‘Early-B’, which is the first B-lineage committed cell, before progressing through ‘pro-B’, ‘pre-B’, and ‘immature B’ cell stages to make a functional naïve B cell (LeBien 2000). In this regard, human B cell development is largely similar to murine B cell development, though there are some differences and gaps in understanding of human B lymphopoiesis (LeBien 2000; Hardy *et al* 2007; LeBien & Tedder 2008).

CLPs are defined as $CD34^{+}CD45RA^{+}CD10^{+}CD19^{-}$ cells, which give rise to B, T, NK cells, and DCs, but lack myeloid and erythroid potential (Galy *et al* 1995). The next stage of B cell development, ‘Early-B’ cells, is defined by the same cell surface markers as CLPs, but has characteristics of B cell lineage rather than T, NK, or DC lineage. As such, Early-B cells are negative for the pan-B marker CD19, but express

RAG1 and RAG2 (Ryan *et al* 1997) and undergo DJ-recombination of the heavy chain immunoglobulin (Bertrand *et al* 1997; Davi *et al* 1997). Early-B cells also express Pax5 (Ryan *et al* 1997), as well as cytoplasmic Igα (Dworzak *et al* 1998) and VpreB (Wang *et al* 1998b), components of the pre-B Cell Receptor (pre-BCR) complex.

Pro-B cells are defined as expressing the cell surface markers CD34 and CD19, and high levels of CD10 (Loken *et al* 1987). These cells also express cytoplasmic TdT (Loken *et al* 1987), continue to express cytoplasmic VpreB (Wang *et al* 1998b), and the majority have undergone VDJ recombination (Bertrand *et al* 1997; Davi *et al* 1997).

The next stage of development, the pre-B cell, can be further subdivided into two stages. Pre-BI cells are large proliferating cells, and have lost some expression of TdT and all expression of the RAG genes. Pre-BI cells also express on their cell surface the *mu*-heavy chain (μHC), which had undergone VDJ recombination during the preceding stages (Loken *et al* 1987; Ghia *et al* 1996) and will later form the heavy chain of IgM. In addition, VpreB and the other components of the pre-BCR, λ5, Igα and Igβ, are also expressed on the surface of pre-BI cells, forming the pre-BCR complex (Benschop & Cambier 1999). The combination of VpreB and λ5 are known as the 'surrogate light chain' or ψLC, which, as the name suggests, take the role of the light chain immunoglobulin before rearrangements of the VJ gene segments take place. Pre-BI cells progress to form smaller, non-proliferating cells referred to as Pre-BII cells. These cells cease to express extracellular pre-BCR, but express once more the RAG genes and TdT, as VJ recombination of the light chain immunoglobulins takes place primarily at the *kappa*-light chain (κLC) locus (Ghia *et al* 1996). Therefore, the formation of the pre-BCR can be seen as an important checkpoint in the development of B cells, as it marks the transition from pro-B to pre-B cell, and allows the cell to progress to recombination of the light chain genes.

Once rearrangements of the VJ gene segments has ceased, the cell expresses complete IgM on the surface and the cell is designated an immature B cell. In the bone marrow, immature B cells are tested for autoreactivity; immature B cells that react to auto-antigens are eliminated in the bone marrow, those that do not are

allowed to mature and leave the bone marrow. Alternative splicing generates *delta*-heavy chains (δHC) and so the mature B cells express both IgM and IgD on their surface (Murphy *et al* 2008).

1.3.4 Control of B cell development

B cell development is controlled by a variety of mechanisms. Cell intrinsic mechanisms consist of the checkpoints of V(D)J recombination as described above, and the actions of networks of transcription factors, which stimulate the expression of the others sequentially. These intrinsic mechanisms are also controlled by extrinsic mechanisms of cytokines produced by the bone marrow stroma cells, on which haematopoietic development takes place. In addition to the production of certain chemokines necessary for B cell development, stroma cells also express cell adhesion molecules which developing B cells can attach to, presumably to enhance and enable the effects of soluble and membrane bound ligands described below.

In murine lymphoid development, the expression of the transcription factors PU.1 and Ikaros in multipotent haematopoietic progenitors promotes the expression of FLT3, the receptor for a ligand expressed on bone marrow stromal cells called FLT3-ligand. In CLPs, signalling through FLT3 acts together with PU.1 to induce the expression of the IL-7 receptor (IL-7R) signalling, which induces the expression of E2A. The production of the transcription factor Early B-cell Factor (EBF) is stimulated by PU.1, E2A, and IL-7R, which commits the lymphoid cell to B-lineage differentiation. Finally EBF promotes the expression of the pan-B marker Pax5, which directs the expression of key components of the BCR signalling complex, such as CD19, Igα, and BLNK (Singh *et al* 2005; Murphy *et al* 2008). Human B cell development is similar, however, the role of IL-7 signalling is not clear, as it is known that IL-7 is not essential for human B cell development (LeBien 2000).

1.3.5 B cell activation in secondary lymphoid tissues

As described above in section 1.3.2, B cells presenting peptides from a pathogen are activated by the co-stimulation of a helper T cell. However, it is clear that the chances of a B cell presenting peptides from a pathogen encountering a T cell that recognises those peptides in the blood stream are very slim. Instead, the chances of

such an encounter are increased by bringing B cells and T cells together in lymphoid organs such as the lymph nodes. The binding of antigen to B cells, and the recognition of peptides by T cells and subsequent differentiation to helper T cells, activates the expression of specific cell adhesion molecules. This 'traps' both primed helper T cells and antigen-bearing B cells within particular regions of the lymph node, thus greatly increasing the chances of a B cell encountering the appropriate helper T cell (Garside *et al* 1998).

Once a B cell is activated, it proceeds to differentiate into antibody-producing plasma cells. Some B cells form a 'primary focus', where B cell differentiation proceeds quickly; such primary foci form in the red pulp of the spleen or in the medullary cords where lymph drains from the lymph node. Alternatively, some B cells continue to proliferate and form a 'germinal centre', which could be described as an 'island' of dividing B cells in a 'sea' of resting B cells (Murphy *et al* 2008). In germinal centres, B cells undergo somatic hypermutation to further edit the variable regions of their antibodies, the aim being to improve the affinity of the antibody for the antigen should the pathogen not be cleared from the body promptly. Germinal centre (GC) B cells will differentiate to form plasma cells, as well as memory B cells which confer long-term protection against future infections by the pathogen (MacLennan 1994).

1.3.6 B cell development in ALL

The lymphoblasts that are present in the bone marrow of patients with TEL/AML1$^+$ leukaemia frequently exhibit a pro-B ($CD34^+CD19^+CD10^+$) phenotype with aberrant expression of some myeloid antigens (Golub *et al* 1995; Borowitz *et al* 1998; De Zen *et al* 2000), and the gene expression profile of TEL/AML1$^+$ lymphoblasts is similar to those seen in normal pro-B cells (Andersson *et al* 2010). This indicates an impairment in B cell differentiation at the transition from pro-B to pre-B cells, which is frequently (Tsuzuki *et al* 2004; Fischer *et al* 2005), but not always (Andreasson *et al* 2001; Morrow *et al* 2004; Schindler *et al* 2009) recapitulated in transgenic mouse models.

Mouse models involving xenografts of human cells, either from leukaemia patients themselves or from normal haematopoietic progenitors exogenously expressing TEL/AML1, present an opportunity to study characteristics of B cell development in the context of TEL/AML1$^+$ leukaemia. These have indicated that TEL/AML1 confers increased self-renewal properties on a CD34$^+$CD38$^-$CD19$^+$ population, which is not observed in normal bone marrow, but whose closest normal equivalent is pro-B cells (Castor *et al* 2005; Hong *et al* 2008).

Some of the symptoms of leukaemia are associated with a decrease in myeloid haematopoiesis, though it is not clear from investigating patient bone marrow alone whether this abnormal haematopoiesis is due to effects of TEL/AML1 on HSCs, or whether it is a result of the 'crowding out' of normal cells in the bone marrow by leukaemic blasts. Transgenic mouse models give indications of the effects of TEL/AML1 in mammalian haematopoiesis. Bone marrow transduced with TEL/AML1 has normal myeloid development (Morrow *et al* 2004; Tsuzuki *et al* 2004; Fischer *et al* 2005), even increasing myeloid progenitor self-renewal (Tsuzuki *et al* 2004; Fischer *et al* 2005). However, it has also been shown that TEL/AML1 increases self-renewal in HSCs, and possibly decreases differentiation towards lineage-specific progenitors (Fischer *et al* 2005; Schindler *et al* 2009).

A summary of the main gene expression changes during selected stages of B cell development is shown in Figure 1.2 below:

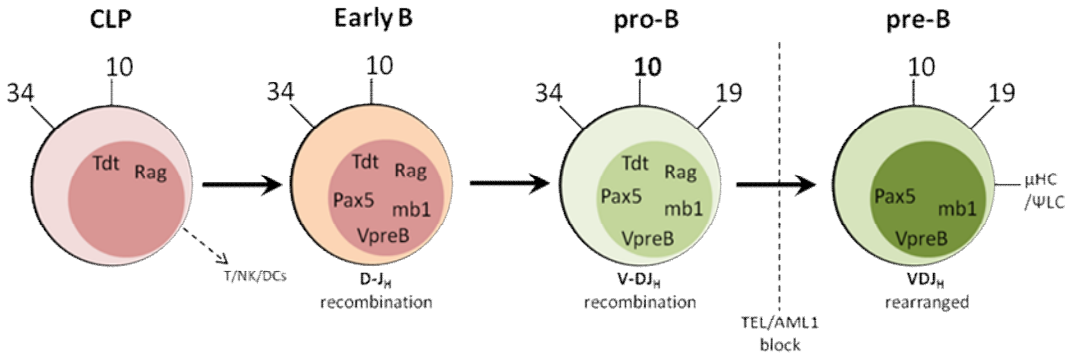

Figure 1.2 – Diagram of B cell development.
Outline of differentiation from common lymphoid progenitor (CLP) to pro-B and pre-B cells, via the Early B cell. Expression of CD markers (34 = CD34, etc.) and pre-B cell receptor components μHC and ψLC are shown on the outside of each cell, and expression of transcript for Rag1/2, Tdt, mb1, Pax5, and VpreB are shown on the inside of each cell. Below the B-lineage cells, the recombination status of the heavy chain VDJ genes is shown. Dotted line indicates the stage at which B cell differentiation appears to be blocked in TEL/AML1$^+$ leukaemia patients and mouse models.

1.3.7 B cell development and other B cell malignancies

As with the resemblance of B-cell ALL to normal pro-B cells, many of the various B cell malignancies also appear to be the counterparts of normal B cell developmental stages. This can be manifested in their V(D)J rearrangements, morphology, surface phenotype, and tumour site (Shaffer *et al* 2002; Murphy *et al* 2008). For example, Follicular Lymphomas tend to resemble germinal centre B cells: located in lymph nodes, expressing the GC B cell marker CD10, and are frequently infiltrated by follicular dendritic cells and T cells. A malignancy called Diffuse Large B Cell Lymphoma has a heterogeneous phenotype, but can be divided into two forms which resemble GC B cells (with somatic hypermutation) or activated peripheral B cells (without somatic hypermutation; Alizadeh *et al* 2000). A similar situation is found in Chronic Lymphocytic Leukaemia (CLL) where both gene expression profiling (Klein *et al* 2001; Rosenwald *et al* 2001) and mutation status of V gene segments (Hamblin *et al* 1999; Gurrieri *et al* 2002) have distinguished two subtypes of the disease, resembling normal memory B cells and activated peripheral B cells (Shaffer *et al* 2002).

1.4 Proliferation

Cancer is defined by the uncontrolled proliferation of cells, both by means of self-sufficiency in growth-promoting signals, and insensitivity to growth-inhibitory signals (Hanahan & Weinberg 2000). To describe how proliferation is affected in leukaemia, a brief introduction to the cell cycle and how it is controlled is given below.

1.4.1 The cell cycle

As a means to ensure that the processes of cell division (including DNA replication, chromosome condensation, and separation of chromosomes into daughter cells) are carried in the correct sequence and in a controlled fashion, these processes are divided into phases of the cell cycle. DNA Synthesis, which takes place during S-phase, and Mitosis, occurring during M-phase, are separated by two ‘Gap’ or G-phases, so that the cell cycle proceeds sequentially through G_1, S, G_2, and M-phase. G_1-phase can be seen as an opportunity for a cell to pause to assess

whether conditions (both external and internal) are favourable before committing to the effort of cell division. In the case that conditions are not favourable, cells can enter an optional resting phase known as 'G_0', in which the cell can remain for extended periods of time before restarting proliferation. Some differentiated cell types, for example neurones and skeletal muscle cells, remain in G_0 permanently and will not divide again before they die (Alberts *et al* 2002).

Progression through the cell cycle, and thus the proliferation of cells, is controlled at several checkpoints. As mentioned above, G_1-phase is where the cell checks that the environment is favourable for the cell to divide. Favourable external conditions for cell division will include a ready supply of nutrients. In addition, transition through the G_1-checkpoint into S-phase can be stimulated by growth factors and mitogens. Factors which might inhibit cell division include damage to DNA; the consequence of DNA damage is for the cell to attempt to repair the damage, or to commit suicide by apoptosis. The protein p53 is an important component in controlling this process. The transition from G_2-phase into M-phase, also known as the G_2-checkpoint, occurs in a similar fashion, by sensing DNA damage in the newly replicated DNA, and either repairing the damage or triggering apoptosis in the cell. Finally, the metaphase checkpoint can only be passed once, after the condensed chromosomes have aligned in the cell and microtubule spindles have attached to each chromosome. This allows the equal separation of sister chromatids, preventing an uneven distribution of chromosomes in the daughter cells (Alberts *et al* 2002).

1.4.2 Internal control of the cell cycle

The molecular control of the progression of the cell cycle has several layers, the foundation of which is the complexes formed by the cyclins and the cyclin-dependent kinases (Cdks). The activity of these cyclin-Cdk complexes increases and decreases cyclically, and the periods of activity for each complex, and thus their names, roughly correspond to one phase of the cell cycle; the G_1-Cdk complex starts the progression of the cell cycle, the G_1/S-Cdk complex commits the cell to DNA replication, the S-Cdk complex promotes DNA replication, and the M-Cdk complex promotes the events of mitosis (Morgan 1997; Alberts *et al* 2002).

As suggested by the name, cyclin-dependent kinases are not active until bound by cyclins. The full activation of the Cdk-cyclin complexes then requires phosphorylation by protein complexes known as Cdk-activating kinases (CAKs); in humans, this is performed by the kinase Cdk7 and its partners cyclin H and Mat1 (Morgan 1997). Further phosphorylation can also inhibit the activity of Cdk-cyclin complexes, a process particularly important in controlling M-Cdk complex at the onset of mitosis; phosphorylation by proteins related to the yeast kinase Wee1 inhibits Cdk-complexes, which can be reversed by phosphatases of the Cdc25 family. The formation and activity of Cdk-cyclin complexes can also be inhibited by the binding of proteins called Cdk-inhibitor proteins (CKIs), which is a significant regulatory mechanism during G_1 and S-phase. In humans, well characterised examples of such CKIs are p21-Cip1 (also known as CDKN1A), p27-Kip1 (CDKN1B), p16-Ink4A (CDKN2A), p15-Ink4B (CDKN2B), and p18-Ink4C (CDKN2C; Morgan 1997; Pavletich 1999). Many of these genes that inhibit progression of the cell cycle are known secondary mutations in leukaemias including TEL/AML1$^+$ acute lymphoblastic leukaemia, as described in section 1.9.2 below.

Destruction of cyclins during critical phases of the cell cycle is triggered by the attachment of ubiquitin molecules to specific residues on the protein. Two protein complexes that catalyse this reaction, known as ubiquitin-ligases, are important in this control mechanism. The SCF complex targets cyclins and CKIs that control S-phase initiation. The Anaphase-Promoting Complex (APC) ubiquitinates cyclins involved in the regulation of mitosis. These ubiquitin ligases are controlled by different mechanisms; ubiquitination by SCF is controlled by the phosphorylation state of the target cyclins, whereas APC is controlled by the addition of subunits to the complex, notably Cdc20 (Alberts *et al* 2002).

1.4.3 External control of the cell cycle

Cells can also receive cues from the environment to stimulate growth. This is particularly important in complex multi-cellular organisms, where cell numbers need to be controlled for the proper development of organs, limbs, and tissues, and when cells need to be replaced after other cells die. As cell volume is intrinsically linked to cell division, external factors can either promote proliferation by growth factors,

which promote an increase in cell volume, or mitogens, which promote progression in the cell cycle. Many factors can act as both growth factors and mitogens.

One well-known example of a mitogen signalling pathway that controls the cell cycle is the MAP kinase pathway. This signalling cascade is triggered by the binding of a growth factor (for example, EGF) to its receptor (e.g. EGFR), causing the exchange of GDP with GTP in the GTPase protein Ras. This starts a cascade of protein phosphorylation (made possible by GTPase), eventually leading to the phosphorylation of MAPK, which in turn leads to the activation of a protein called Myc. Myc is a transcription factor which stimulates the production of several cell cycle genes, such as cyclin D, subunits of SCF, and a protein called E2F which promotes transition into S-phase.

An example of growth factor signalling pathways which increase cell volume is the activation of a PI3-kinase (PI3K), which phosphorylates inositol phospholipids in the plasma membrane. These then act as docking sites for kinases with Pleckstrin Homology (PH) domains which are then activated by phosphorylation, triggering a phosphorylation cascade. An example of a protein phosphorylated in this pathway is S6 kinase, which phosphorylates the ribosomal protein S6, eventually leading to an increased production of ribosomes and thus an increase in protein synthesis (Stocker & Hafen 2000; Alberts *et al* 2002).

Other signalling molecules inhibit growth; for example, the TGF-β family of signalling proteins, which signal through cell surface receptors to alter the activity of transcription factors of the SMAD family, can in certain situations be inhibitors of cell cycle progression. TGF-β promotes the expression of p15-Ink4B and p21-Cip1, CKIs which block the activity of Cdk-cyclin complexes and thus prevent the phosphorylation (and inactivation) of the protein Retinoblastoma (Rb), an inhibitor of G_1 to S-phase transition (Datto *et al* 1997; Alberts *et al* 2002).

1.4.4 Dysregulation of proliferation in ALL

As described later in section 1.9.2, many genes frequently mutated in acute lymphoblastic leukaemia are cell-intrinsic regulators of the cell cycle (for example, *CDKN2A*; Takeuchi *et al* 1995). In addition to genes whose products dampen cell

cycle progression, many mutations in ALL act to alter the response to external factors – increasing sensitivity to growth factors and mitogens, and decreasing sensitivity to growth inhibitory signals – and leukaemic cells become dependent on these signalling pathways. For example, TEL/AML1 is known to reduce the growth inhibitory effects of TGF-β signalling (Ford *et al* 2009). Mutations that constitutively activate Ras signalling, and thus promote survival and proliferation through the MAP kinase pathway, are found in many human cancers and at a rate of about 15% in ALL (Perentesis *et al* 2004). Finally, PI3K signalling is required for proliferation and survival in TEL/AML1^{+} leukaemia cells (Fuka *et al* 2011a) and upregulation of the erythropoietin receptor, which acts via PI3K signalling, is frequent (Ross *et al* 2003; Fine *et al* 2004). Taken together, the examples above suggest that leukaemic cells become insensitive to both intrinsic and extrinsic growth inhibitory signals, and more sensitive to proliferation promoting signals, resulting in an increased cell growth.

1.5 Apoptosis

Apoptosis, the process of 'programmed cell death', is a critical mechanism throughout embryo development and tissue homeostasis, and is not simply the opposing force to proliferation. For example, during development, apoptosis is critical for the shaping of the limbs and for the correct innervation of muscles, and in normal immune development apoptosis eliminates self-reactive lymphocytes that would otherwise cause autoimmune disorders. To describe the role of apoptosis in leukaemia, a concise introduction to apoptosis is presented below.

1.5.1 The mechanisms of apoptosis

The mechanism of cell death is a controlled cascade of proteases called caspases, which have a cysteine in their catalytic site and cleave other proteins at specific aspartic acid residues. They are formed from an inactive procaspase, which is cleaved by other caspases. To start the cascade off, specific procaspases called 'initiator procaspases' are brought together by adaptor proteins, resulting in a complex of procaspases that mutually cleave each other. Once the initiator caspases are active, they cleave other caspase molecules, resulting in an amplifying chain reaction of caspase activation. Different caspases cleave cellular components, such

as nuclear lamins, resulting in the disassembly of the nucleus, and DNAse precursors which, when activated by cleavage, destroy the DNA in the cell. The cell dismantles itself and is quickly taken up and digested by phagocytes such as macrophages (Alberts *et al* 2002).

There are two routes by which the initiator caspases are brought together by adaptor proteins: extrinsic and intrinsic routes. The extrinsic route involves the activation of cell surface proteins called death receptors, an example of which is the protein Fas. Binding of Fas ligand to Fas results in the trimerisation of Fas, which recruits adaptor proteins such as FADD (Fas-Associated protein with Death Domain). FADD then promotes the self-cleavage of procaspase-8, which then activates caspases leading to apoptosis (Alberts *et al* 2002). Fas is a member of the Tumour Necrosis Factor Receptor (TNFR) superfamily of death receptors (Wallach *et al* 1999). Extrinsic death signalling is a common route for cytotoxic T lymphocytes to clear infected cells and control lymphocyte numbers (Murphy *et al* 2008).

Cells can also activate apoptosis via the intrinsic pathway. This is understood to be triggered by the release of cytochrome c from the mitochondria, which binds to the adaptor protein APAF1 (Apoptotic protease activating factor 1), leading to the activation of procaspase-9 (Green & Reed 1998). The intrinsic apoptosis activation pathway is controlled by the various members of the Bcl-2 family of mitochondrial proteins (Chao & Korsmeyer 1998). Bcl-2 and Bcl-X_L both inhibit the release of cytochrome c, whereas family members like Bax and Bak promote its release from mitochondria. In addition, these core Bcl-2 family members are regulated by other family members; Bad binds to inhibit the anti-apoptosis family members, and pro-apoptotic members Bax and Bak are activated by Bid amongst others. It is believed that the balance between Bcl-2 and Bax activity is a key determinant of the sensitivity of cells to apoptotic signals (Chao & Korsmeyer 1998; Alberts *et al* 2002). Another apoptosis regulator family is the IAP (Inhibitor of Apoptosis) proteins, which act primarily by binding to procaspases, preventing their cleavage and subsequent activation (Deveraux & Reed 1999; Alberts *et al* 2002; Salvesen & Duckett 2002).

The activity of the Bcl-2 family members is controlled in part by the cell cycle regulator p53, either transcriptionally by inducing the expression of pro-apoptotic Bax, or by directly binding and inhibiting anti-apoptotic Bcl-2 family members (Hemann & Lowe 2006). This therefore links the control of the cell cycle to apoptosis, and providing one mechanism by which, for example, DNA damage that cannot be repaired triggers cell death (Alberts *et al* 2002). The anti-apoptotic effects of IAP proteins can be induced by growth factor or mitogen signalling, for example, NFκB signalling (Salvesen & Duckett 2002) and PI3K signalling (Datta *et al* 1999).

1.5.2 Importance of apoptosis in lymphocyte development

Apoptosis is a major regulator of B cell development (see section 1.3.3), which in turn is regulated in part by binding of auto-antigens to the pre-BCR and binding of foreign antigens to the BCR (Niiro & Clark 2002). Bcl-2 expression can rescue immature B cells at various stages of early development when they would otherwise be eliminated, such as if a non-functional pre-BCR is produced (Marsden & Strasser 2003). In addition, whilst deletion of autoreactive B cells does not require Fas signalling it can be inhibited by Bcl-2 or Bcl-X_L; in transgenic mice, autoreactive B cells that overexpress Bcl-2 are not eliminated (Cory 1995; Marsden & Strasser 2003). Fas signalling might instead mediate the control of B cell numbers by cytotoxic T cells (Watanabe-Fukunaga *et al* 1992; Takahashi *et al* 1994; Marsden & Strasser 2003). In this regard, activation of B cells by BCR signalling confers resistance to Fas-mediated cell death induced by T cells (Rothstein *et al* 1995). Bcl-2 and Bcl-X_L are essential for maintaining a pool of B cells in adults; mice deficient in Bcl-X_L show a significant reduction in mature B cells, whereas Bcl-2 deficient mice, after initially showing normal post-natal B cell differentiation, quickly lose adult B cells to apoptosis (Chao & Korsmeyer 1998).

1.5.3 Methods for studying apoptosis

DNA fragmentation during the late stages of apoptosis can be visualised by DNA staining dyes such as DAPI, Propidium Iodide (PI), or 7-Aminoactinomycin D (7-AAD). More accurate identification of nuclei undergoing DNA fragmentation is permitted by an assay called a TUNEL (TdT dUTP nick end labelling) assay, to identify the ‘nicks’ in the DNA made in this process by the addition of labelled

terminal nucleotides by the enzyme Terminal deoxynucleotidyl transferases (TdT). Additionally, DNA dyes can be used to label cells that have increased permeability during the late stages of apoptosis, since live cells are not normally labelled by DNA dyes without fixation; this can be exploited in fluorescent labelling for flow cytometry.

Changes in the cell membrane can be used to indicate the earliest stages of apoptosis. During apoptosis, the membrane phospholipid phosphatidylserine is translocated from the cytosolic face to the exterior face of the plasma membrane, by an unknown mechanism. Annexin-V is a protein that has high affinity for phosphatidylserine, and so fluorescently labelled Annexin-V can be applied to identify cells undergoing the early stages of apoptosis using, for example, flow cytometry. Other assays which can also be used to measure apoptosis include, for example, the direct assessment of activated caspase activity in cell lysates by cleaving recombinant proteins which release fluorescent molecules.

1.5.4 Evasion of apoptosis in ALL

The PI3K signalling pathway is important in the survival of lymphoblasts in ALL (Levy *et al* 2009). TEL/AML1 promotes survival of leukaemia cells via the PI3K signalling pathway (Fuka *et al* 2011a), possibly through the upregulation of the erythropoietin receptor EPOR (Inthal *et al* 2008; Torrano *et al* 2011). In addition, TEL/AML1 is known to induce the upregulation of pro-survival factors HSP90 (which stabilises PI3K and proteins in its signalling pathway) and survivin (a member of the IAP family; Diakos *et al* 2007). Activation of anti-apoptotic Bcl-2 does not appear to be sufficient for ALL, however; less than 15% of mice that overexpress Bcl-2 have lymphoid malignancies, mostly lymphomas and mature B cell leukaemias (Cory 1995; Marsden & Strasser 2003). Conversely, mutations in the pro-apoptotic Bcl-2 family member Bax are frequently found in ALL cell lines (Meijerink *et al* 1998). The above evidence suggests that lymphoblast survival in ALL is mediated by increased insensitivity to pro-apoptotic signals.

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The second part of this chapter looks specifically at TEL/AML1 leukaemia, starting with an introduction to TEL and AML1 individually, and finishing with the experimental study of acute lymphoblastic leukaemia.

1.6 TEL (ETV6)

Information about TEL, its role in haematopoiesis, and its involvement in leukaemias and other cancers, is presented in depth in the introduction to Chapter 4. The *TEL* gene, located at 12p13 and also known as *ETV6*, was discovered in the context of Chronic Myelomonocytic Leukaemia (CMML; Golub *et al* 1994). TEL is a member of the *ets* family of transcription factors, which are identified by the DNA-binding region *ets* (Wasylyk *et al* 1993), and also shares with many of its family members a helix-loop-helix polymerisation domain known as *pointed* or sterile alpha motif (SAM) domain, which allows TEL to form homo- and heterodimers (Kim *et al* 2001). TEL is a transcriptional repressor, acting via two main mechanisms; recruiting histone deacetylases (HDACs) via the binding of corepressors N-CoR (Guidez *et al* 2000), SMRT and mSin3A (Chakrabarti & Nucifora 1999), or via an HDAC-independent mechanism of chromatin condensation involving the binding of L3MBTL via the *pointed* domain (Boccuni *et al* 2003). TEL itself is regulated by phosphorylation via the MAPK/ERK pathway (Arai *et al* 2002; Maki *et al* 2004). Little is known about the targets of transcriptional regulation by TEL.

TEL is ubiquitously expressed in the mouse embryo (Wang *et al* 1997; Wang *et al* 1998a), and *ETV6*$^{-/-}$ mice are embryonic lethal from defects in yolk sac angiogenesis (Wang *et al* 1997). TEL is not intrinsically required for embryonic or adult haematopoiesis, but rather acts as a selective regulator of haematopoietic stem cell (HSC) survival by controlling the colonisation of the bone marrow in the adult mouse (Wang *et al* 1998a; Hock *et al* 2004).

1.7 AML1 (RUNX1)

1.7.1 Discovery of AML1

The *AML1* gene, located at 21q22.3, was first identified in the context of Acute Myeloid Leukaemia. Breakpoints for the chromosome translocation t(8;21)(q22;q22)

were found to cluster within a specific region on a *NotI*-digested clone from chromosome 21. The surrounding cDNA clones were sequenced to identify a unique gene with no homology to any other gene at the time, and was named *AML1* after the association of the t(8;12) translocation with acute myeloid leukaemia (Miyoshi *et al* 1991). Soon after, it was discovered that the *AML1* gene had homology to a region in the Drosophila gene *runt* (Daga *et al* 1992) which had DNA binding properties (Ogawa *et al* 1993). *AML1* and its two related genes *AML2* and *AML3* (Levanon *et al* 1994) were later renamed *RUNX1*, *RUNX2*, and *RUNX3*, short for '*runt*-related transcription factor' (van Wijnen *et al* 2004). The proteins from these genes are also called Core Binding Factor alpha (CBFα) genes, as they form heterodimeric complexes with CBFβ (with the *AML1/RUNX1* gene coding for CBFα2 protein) to regulate gene expression (Speck *et al* 1999).

1.7.2 AML1 structure

The DNA binding activity of AML1 is conferred by a domain homologous to the Drosophila gene *runt* (Daga *et al* 1992), and has consequently been named '*runt* homology domain' (RHD), shared by all of the *AML/RUNX* gene family (Levanon *et al* 1994). The second major domain of AML1, not shared by its family members, is the 'transactivation domain' (Kanno *et al* 1998). This is required for binding to variety of cofactors for both activation and repressors functions (Ito 1999).

AML1 is known to bind to a consensus sequence originally found in the enhancer region of the gene SL3-3 (Amtoft *et al* 1997), the core of which is PyGPyGGTPy (where 'Py' is a pyrimidine nucleotide, i.e. C or T), for example TTTGCGGTTA/T (Thornell *et al* 1991). DNA binding by AML1 is greatly enhanced by the binding of the CBFβ subunit, coded for by the *CBFB* gene (Kanno *et al* 1998). CBFβ does not bind to DNA, but does bind to AML1 via the *runt* homology domain (Meyers *et al* 1993), increasing the activity of AML1-mediated transcription (Ogawa *et al* 1993; Zhang *et al* 1996).

The AML1 protein has a series of self-inhibitory regions which regulate its DNA binding (Ito 1999). A region N-terminal to RHD has a modest inhibitory effect on DNA binding (Gu *et al* 2000), but the main source of auto-inhibition comes from the

regions C-terminal to RHD (Kanno *et al* 1998), by masking the DNA-binding surface of AML1 i.e. the RHD. This auto-inhibition must be removed before heterodimerisation of AML1 and CBFβ can occur; one protein believed to be able to induce unmasking of the DNA binding surface and subsequent promote heterodimerisation is the *ets* family member ETS-1 (Ito 1999). Other proteins that can fulfil this function are yet to be determined, but must exist since CBF acts in tissues that do not express ETS-1.

1.7.3 AML1 activity

The CBF complex can act as both an activator and a repressor of gene expression, through its association with either histone acetyltransferases (HATs) or deacetylases (HDACs; Zelent *et al* 2004). AML1 recruits the adapter proteins p300 and CREB-Binding Protein (CBP) via the transactivation domain (Kitabayashi *et al* 1998), which have intrinsic HAT activity (Ogryzko *et al* 1996) and recruit the HAT protein P/CAF (Yang *et al* 1996). The decondensation of chromatin permits gene expression; genes known to be regulated by AML1 / CBF activity include the T-cell receptors *TCRα* and *TCRβ* (Ito 1999), *TCRδ* (Hernandez-Munain & Krangel 1994), *IL-3* (Uchida *et al* 1997) and *PU.1* (Okada *et al* 1998).

There are two potential routes by which AML1 recruits HDACs to repress gene expression. Firstly, via the C-terminal VWRPY motif, AML1 may recruit Groucho-family corepressors (Aronson *et al* 1997; Levanon *et al* 1998), which may act through HDACs (Chen *et al* 1999). Secondly, AML1 interacts with mSin3A (Fenrick *et al* 1999), an interaction which is regulated by phosphorylation (Imai *et al* 2004).

AML1 and CBFβ, when dimerised, are known to interact with other transcription factors, including c-Myb (Hernandez-Munain & Krangel 1995), C/EBP (Zhang *et al* 1996), MITF (Ogihara *et al* 1999), and SMAD proteins (Hanai *et al* 1999). It is believed that the binding of Core Binding Factors to other proteins creates gene expression complexes which enable specificity of expression in tissues and during developmental stages; one such example is the expression of *TCRα* (Ito 1999).

1.7.4 AML1 function

The role of AML1 and Core Binding Factor in haematopoiesis has been reviewed extensively, e.g. de Bruijn & Speck 2004. Provided here is an overview of the main functions of AML1 in embryonic and adult haematopoiesis.

AML1$^{-/-}$ mice are embryonic lethal at around E12.5 due to extensive haemorrhaging in the CNS (Okuda *et al* 1996; Wang *et al* 1996b). Primitive yolk sac erythropoiesis is unaffected in these mutants, but mice fail to initiate definitive haematopoiesis from any site, and *AML1*$^{-/-}$ ES cells fail to contribute to haematopoiesis in chimeric mice (Okuda *et al* 1996; Wang *et al* 1996b). The expression of only a specific set of haematopoiesis-related genes is absent in *AML1*$^{-/-}$, which include PU.1, Vav, flk-2/flt-3, M-CSF receptor, G-CSF receptor and c-Myb (Okada *et al* 1998).

However, despite the critical role that AML1 plays in foetal haematopoiesis, the loss of AML1 appears to have a subtler effect in adult haematopoiesis, as shown by conditional mouse mutants in which *Runx1* could be excised under the control of Cre-*loxP* system. Normal haematopoietic development was maintained in adult mice despite loss of *Runx1*, but a reduction in all white blood cells (particularly B- and T-lymphocytes) and platelets was observed (Ichikawa *et al* 2004; Growney *et al* 2005). The Common Lymphoid Progenitor (CLP) population was unaffected with loss of *Runx1*, indicating that it is the commitment to B- and T-lineage that is affected in these mice (Ichikawa *et al* 2004). The HSC compartment in these mice was increased but was otherwise functional. Interestingly, loss of *Runx1* in adult mice leads to mild myeloid expansion in the bone marrow and spleen, mirroring the myeloproliferative disorders in patients with homozygous loss of *RUNX1* (Growney *et al* 2005).

1.7.5 AML1 fusion proteins in leukaemias

The importance of AML1 in haematopoiesis and leukaemia can be recognised by its frequent involvement in fusion proteins resulting from chromosome translocations. The t(8;21)(q22;q22) translocation, giving rise to the AML1-ETO fusion protein, appears in about 18% of AML patients (Rowley 1973; Perry *et al* 2002). The rare t(3;21) translocation fuses AML1 to any of a number of genes in the 3q26 region,

including *EVI1*, *MDS1*, and *EAP*, and is often observed in AML, myelodysplasia syndrome, and in the 'blast crisis' phase of CML (Nucifora *et al* 1993; Nucifora & Rowley 1995; Perry *et al* 2002). Finally, the t(12;21) translocation was identified as a recurrent genetic abnormality in ALL (Romana *et al* 1994) and was soon discovered to fuse most of the AML1 protein with the N-terminus of the newly discovered TEL protein (Romana *et al* 1995a). These fusions act to inhibit the transcriptional activation activities of AML1 (Perry *et al* 2002).

1.8 TEL/AML1 and ALL

1.8.1 Discovery of TEL/AML1

The t(12;21) translocation was identified as a frequent feature of ALL by Romana and colleagues (1994), where three out of eight B-cell ALL patients were shown by Fluorescence *In Situ* Hybridisation (FISH) to harbour the t(12;21) translocation in cells from their bone marrow. Shortly after the identification of TEL as the partner of PDGFRβ in the CMML-associated translocation t(5;12)(q33;p13) (Golub *et al* 1994), it was discovered that in the t(12;21)(p13;q22) translocation, TEL was fused to AML1 (Golub *et al* 1995; Romana *et al* 1995a). The TEL/AML1 fusion protein was found to be frequently associated with childhood ALL (Romana *et al* 1995b), a surprising discovery considering these genes had independently been identified in the context of myeloid leukaemias (Miyoshi *et al* 1991; Golub *et al* 1994).

1.8.2 Structure of TEL/AML1

The fusion of TEL and AML1 commonly occurs between exons 5 and 6 of TEL and exons 1 and 2 of AML1; i.e. in intron 5 of TEL and intron 1 of AML1 (Wiemels & Greaves 1999). The TEL moiety of TEL/AML1 conserves the *pointed* domain, required for hetero- and homo-dimerisation (Kim *et al* 2001) and the central repression domain, to which HDACs are recruited (Wang & Hiebert 2001), but loses the DNA-binding *ets* domain (Golub *et al* 1994). Almost the entire of the AML1 is conserved, including both the DNA-binding *runt* homology domain (RHD; Levanon

et al 1994) and the transactivation domain which can recruit both HATs and HDACS (Kanno *et al* 1998).

1.8.3 Molecular biology of TEL/AML1

Whereas AML1 acts as both an activator and repressor of gene expression, TEL/AML1 constitutively represses AML1 targets, as discovered using reporter gene constructs of AML1 targets (Hiebert *et al* 1996; Fears *et al* 1997). One mechanism of this action is the by the recruitment of HDACs by the interaction of a complex containing N-CoR, SMRT, and mSin3A with TEL/AML1 (Fenrick *et al* 1999; Guidez *et al* 2000), which are recruited by domains present in both the TEL and AML1 moieties (Chakrabarti & Nucifora 1999; Fenrick *et al* 1999; Guidez *et al* 2000). CBFβ is required for the binding of AML1 to its targets via RHD (Zhang *et al* 1996), and similarly it is required for functional TEL/AML1 activity (Roudaia *et al* 2009).

The *pointed* dimerisation domain of TEL appears to be important in the gene repression activity of TEL/AML1, as its deletion removes most (but not all) of the inhibition of reporter gene activation (Hiebert *et al* 1996; Fears *et al* 1997). This is likely to be due to the stabilisation of interactions between TEL/AML1 dimers and their corepressors, rather than any intrinsic properties of the *pointed* domain itself, as the replacement of the *pointed* domain with a different oligomerisation domain preserves the gene repression activities (Lopez *et al* 1999).

TEL and TEL/AML1 can form heterodimers *in vitro* (McLean *et al* 1996) through their *pointed* domains. TEL does not appear to have any effect on the gene repression activity of TEL/AML1 on AML1 targets (Hiebert *et al* 1996), but TEL/AML1 can have a dominant-negative effect over TEL, inhibiting both its gene repression activity and its ability to repress transformed-NIH3T3 cell growth (Gunji *et al* 2004). Since the loss of TEL is a common secondary mutation in TEL/AML1^{+} leukaemia (Cave *et al* 1995), this dominant-negative effect of TEL/AML1 over TEL is significant as it indicates a possible mechanism of leukaemogenesis through the loss of heterozygosity of TEL. The role of TEL deletions in leukaemia will be discussed in Chapter 4 (section 4.1.7).

In addition to a negative effect on basal levels of expression of AML1 targets, TEL/AML1 has a dominant-negative effect over AML1-mediated transcriptional activation (Hiebert *et al* 1996) and the AML1-induced proliferation of NIH3T3 cells (Gunji *et al* 2004). *In vivo*, TEL/AML1 dominant-negative effects on both TEL and AML1 does not appear to copy the phenotype of knockouts of either of these genes (Schindler *et al* 2009), indicating that the negative effects of TEL/AML1 on its wild-type components is subtle, a contrast to observations in *AML1/ETO*-expressing mice, for example (Yergeau *et al* 1997).

1.8.4 Prenatal origins of the TEL/AML1 fusion

It is known that the TEL/AML1 fusion occurs prenatally; the breakpoint of TEL/AML1 in newly-diagnosed patients can be retrospectively matched by PCR with DNA samples from their dried blood spot cards taken at birth (Wiemels *et al* 1999a) which are normally used to diagnose phenylketonuria. In addition, such breakpoints are found to be identical in monochorionic twins (those that share a placenta) diagnosed with leukaemia (Wiemels *et al* 1999b); the only feasible explanation for this being the migration of a TEL/AML1$^+$ cell from the bone marrow of one twin, through the shared placenta, to the other twin (Greaves *et al* 2003).

It has been previously reported that TEL/AML1 has been found in 1% of cord blood samples (Mori *et al* 2002), which is 100-fold more frequent than the incidence of TEL/AML1$^+$ leukaemia, implying that only 1 in 100 newborns positive for TEL/AML1 will go on to develop ALL. However, the findings of Mori and colleagues has been disputed by a recent paper (Lausten-Thomsen *et al* 2011), which claims the frequency of the TEL/AML1 translocation event is closer to 0.01%, thus implying that most if not all of TEL/AML1$^+$ newborns will go on to develop ALL.

After the publication of the paper from Lausten-Thomsen and colleagues, another group revealed in correspondence evidence apparently confirming the original report by Mori and colleagues, that the TEL/AML1 transcript could be detected in approximately 1% of neonatal cord-blood samples (Zuna *et al* 2011). An examination of the methods used by the papers from Mori and Lausten-Thomsen and

possible explanations for their discrepancy followed (Greaves *et al* 2011; Schmiegelow *et al* 2011).

All three papers performed PCR for TEL/AML1 transcript on cDNA prepared from cord blood samples and found 1% of cord-blood samples from healthy neonates were TEL/AML1$^+$. However, the frequency of TEL/AML1$^+$ cells in the cord blood samples (as measured by the relative expression of TEL/AML1 in the samples) differed, with Mori estimating between 10^{-3} and 10^{-4} cells, and Lausten-Thomsen estimating less than 10^{-5}.

After this initial PCR screen, the three papers attempted to confirm the results using different methods. Lausten-Thomsen and colleagues, having used fresh cord blood samples for the first screen, re-performed the PCR on their TEL/AML1$^+$ samples by thawing out frozen samples and flow sorting for CD19$^+$ cells. However, all of the repeated samples were found to be negative for TEL/AML1; since they expected to see an increase in the level of TEL/AML1 transcript in the CD19$^+$ sorted populations, they concluded that these samples were indeed negative. They attributed the identification of TEL/AML1$^+$ samples in their first screen as low level contamination (Lausten-Thomsen *et al* 2011).

Mori and Zuna and their respective colleagues confirmed their TEL/AML1$^+$ samples by FISH after cell sorting for B cell markers CD19 and CD10 for the presence of TEL/AML1 fusions. Mori and colleagues positively confirmed the presence of TEL/AML1 fusions in the two TEL/AML1$^+$ samples they investigated by FISH (Mori *et al* 2002), and Zuna and colleagues confirmed TEL/AML1 in the one sample they analysed (Zuna *et al* 2011).

Ultimately, the discrepancy between the results of Mori's and Lausten-Thomsen's experiments has not yet been resolved, and it is likely there is a technical explanation, rather than biological explanation (such as the nationalities of the populations analysed). Both groups called for further, large scale investigations from other scientists (Greaves *et al* 2011; Schmiegelow *et al* 2011).

1.8.5 Progression of TEL/AML1$^+$ leukaemia

It is clear that two very different models of the progression TEL/AML1$^+$ leukaemia emerge if the frequency of the translocation that generates the fusion gene is 1% or 0.01%, given that the incidence of TEL/AML1$^+$ leukaemia in children is 1 in 10,000 (0.01%). If the latter is true, then the presence of TEL/AML1 is sufficient to cause leukaemia; if the former is true, then TEL/AML1 is not sufficient for leukaemia, and 'secondary hits' are required to progress to overt leukaemia.

Beyond the translocation frequencies presented by Mori and colleagues, there are several lines of evidence that support the 'two-hit' hypothesis, one of which is the study of leukaemia in twins. If a TEL/AML1$^+$ cell was all that was needed to cause leukaemia, and if both twins of identical genetic makeup had the same transformed clone (as it is known is possible via trans-placental transfer; Wiemels *et al* 1999b), then one would expect that both twins develop leukaemia, and at roughly the same time. However, from the few (albeit small) studies that have been performed on the subject, it is known that this is not the case; despite shared TEL/AML1$^+$ clones, concordance of childhood leukaemia in twins is low, and there is often a delay from the prenatal translocation and the presentation of overt leukaemia in both individual twins and singletons (Greaves *et al* 2003).

Commonly mutated, deleted, and abnormally regulated genes in TEL/AML1$^+$ patients have been identified through the analysis of patient material, which has recently entered a new era with the use of high-throughput and large scale screens. As such, screens for gene expression changes (Golub *et al* 1999; Fine *et al* 2004; Andersson *et al* 2005a; Andersson *et al* 2005b; Gandemer *et al* 2007; Andersson *et al* 2010), single nucleotide polymorphisms (SNPs) and copy number alterations (CNAs; Mullighan *et al* 2007; Kawamata *et al* 2008; Bateman *et al* 2010; Kuster *et al* 2011; Notta *et al* 2011b), chromosomal structural changes (Attarbaschi *et al* 2004; Mullighan *et al* 2007), and epigenetically regulated genes (Starkova *et al* 2007) in TEL/AML1$^+$ leukaemia patient samples have identified genes and pathways commonly disrupted in TEL/AML1$^+$ leukaemia, and thus suggest a 'two-hit' model of leukaemogenesis.

Mutations likely to drive leukaemia progression are always discordant in twins (Bateman *et al* 2010), and thus probably occur post-natally. One such common mutation, deletions in the short arm of chromosome 12 where *ETV6* is located, could not be 'backtracked' to birth when comparing samples from patients and their matched neonatal blood spot cards (Wiemels *et al* 2008).

A 'two-hit' model of disease progression in leukaemia has also been suggested in animal models. Most mouse models expressing TEL/AML1 on its own have failed to recapitulate overt B lineage ALL. Endogenous expression of TEL/AML1 under the control of immunoglobulin heavy chain enhancer/promoter, EμV_HP failed to cause haematological malignancies (Andreasson *et al* 2001; Ford *et al* 2009). Exogenous expression of TEL/AML1, by the viral transduction of haematopoietic precursors from bone marrow or foetal liver, failed to induce leukaemia (Morrow *et al* 2004; Tsuzuki *et al* 2004; Fischer *et al* 2005), but instead caused abnormalities in B cell development, either causing increased self-renewal of B cell precursors (Morrow *et al* 2004; Morrow *et al* 2007) or impairment of transition from pro-B to pre-B cells (Tsuzuki *et al* 2004; Fischer *et al* 2005). The result is the accumulation of persistent early B cell progenitors, which is believed to represent a 'pre-leukaemic state' that predisposes individuals for further transformation and ultimately overt leukaemia.

1.8.6 Infection in the aetiology of ALL

An interesting area of study has been the role that early childhood infections might play in the progression of ALL (Greaves 2006). This theory was originally speculated on the basis of three observations: firstly, the peak incidence of ALL, in children aged two to five, coincided strongly with that of common childhood infections such as measles; secondly, the presentation of ALL was usually associated with previous history or coincidence of respiratory infections; and thirdly, that that certain haematological malignancies could be caused by specific viral infections (such as adult T-cell leukemia/lymphoma by Human T-cell Lymphotropic Virus-1 [HTLV1] and Burkitt's lymphoma by Epstein-Barr Virus [EBV]; Greaves 2006).

Epidemiological studies lead to the proposal of two possible mechanisms of an immunological aspect of ALL. Firstly, the 'population mixing' hypothesis (Kinlen

1988) arose from studies of clusters of ALL cases in remote towns (including those of British towns with nuclear power plants such as Sellafield), and proposed that a large influx of migrants exposes the endogenous community to previously unencountered infections, such as a (symptomatically mild) virus. Secondly, the 'delayed exposure' hypothesis (Greaves 1988) arose from the observations of increased incidence in more affluent (and therefore more 'hygienic') societies and countries, and proposed that a delay in the exposure to common childhood infections might inadvertently lead to an inappropriate proliferative response to such an infection.

Kinlen's 'population mixing' hypothesis has been supported by a series of events in which populations mixed in a specific place at a specific time, the majority of which results in a transient increase in the incidence of childhood leukaemia (McNally & Eden 2004). There is an array of evidence to support Greaves' 'delayed exposure' hypothesis; for example children who attend day-care or a nursery in their first years of life (an indicator of increased exposure to infections) have a reduced risk of leukaemia (Ma *et al* 2002; Gilham *et al* 2005). Similar hypotheses to Greaves' have been put forward to explain the increased incidence of immunological diseases such as allergies (Wills-Karp *et al* 2001) and Type-I diabetes (McKinney *et al* 2000) in affluent societies; indeed the distributions of these diseases correlate with that of ALL (Stene & Nafstad 2001; Feltbower *et al* 2004).

It is worth noting that both of the theories are consistent with a 'two-hit' model of leukaemogenesis, and that such an infectious influence isn't a necessary component of the natural history of ALL, but might contribute to its observed characteristics. In this respect, Greaves (2006) proposes something akin to a 'three-hit' model; a pre-natal fusion event, an expansion of the transformed clone as a result of an infection, followed by subsequent genetic mutations in the transformed clone pool.

1.8.7 Clonal evolution

Clonal evolution of cancer is a concept parallel to that of Darwinian evolution of species, and is the result of the study of multi-step carcinogenesis (Greaves & Maley 2012). As a concept it incorporates many features typical of cancers, such how

mutations accumulated over time (i.e. during the lifetime of an individual) can lead to cancerous growth, the genetic diversity found in tumours, and maintain the potential for relapse after treatment.

Many random genetic changes in cells might be harmful for the cell, in which case the cell will die. However, genetic changes that confer an advantage to a cell are passed onto its daughter cells, and thus provide the opportunity for further mutations. Here, 'advantages' for the cell include the abilities to proliferate and survive, and thus resulting in populations of cells that rapidly proliferate and are resistant to apoptosis, i.e. cancer. Mutations occur concurrently in different cells, and so there may be several clones at any one point which have the ability to form a cancer, thus representing a tree of divergence of clones, rather than a step-wise linear progression. Typically however, only a minority of these will come to form the main bulk of the tumour, as they dominate over their competitor clones.

The concept of clonal evolution has been applied to acute lymphoblastic leukaemia, including the TEL/AML1^{+} subtype. In the case of monochorionic twins, the concordance rate for childhood ALL is typically 10-15% (Greaves *et al* 2003). There are published examples of such twins, discordant for TEL/AML1^{+} ALL, where the 'healthy' twin also harbours the pre-leukaemic clone (for example, Hong *et al* 2008). Mutations present in the twin with leukaemia were not present in the pre-leukaemic clone in the 'healthy' twin (Hong *et al* 2008). In addition, there are examples of identical monochorionic twins concordant for TEL/AML1^{+} (e.g. Bateman *et al* 2010) which are *discordant* for the mutations responsible for their leukaemia. These examples not only indicate a requirement for further mutations beyond the t(12;21) translocation event, but also the diversity of mutations that give rise to leukaemia, even in the genetically identical backgrounds found in the twins with a shared pre-leukaemic clone, support a branching, divergent model of leukaemia progression.

The importance of this divergence has been illustrated in relapsed leukaemia (Mullighan *et al* 2008). Access to samples of bone marrow from both the initial diagnosis and relapse of leukaemia allowed the comparison of the genetic differences between the clones present. By analysing the copy number alterations

(CNAs) present in the dominant clones, Mullighan and colleagues were able to map out the origins of the relapse clone with respect to the clone from the initial diagnosis of ALL. In 6% of cases, there were no common CNAs in the diagnostic or relapse clone, indicating the relapse might be a secondary, unrelated leukaemia. In 42% of cases, CNAs present in the initial diagnosis clone were all present in the relapse clone, indicating that these cases of relapsed leukaemia represented clonal evolution from the original leukaemia. However, in the majority of the cases (52%), the relapsed clone shared only a few of the CNAs present in the initial diagnosis clone, indicating that the relapse clones were derived from ancestral clones to the initial diagnosis clone, which remained in the bone marrow whilst the initial diagnosis clone dominated during the overt primary leukaemia.

The 'clonal architecture' of TEL/AML1^{+} (Anderson *et al* 2011) and BCR-ABL1^{+} (Notta *et al* 2011b) ALL has also been plotted out using similar techniques to track different clones within one bone marrow sample. An important finding of both of these studies that the interaction and competition between the different clones is dynamic; once bulk bone marrow is transplanted into immunodeficient mice, individual clones would continue to evolve, leading to previously minor clones that over time become the dominant clone. These findings are significant when considering the treatment of relapse; relapsed leukaemias are likely to be divergent from the primary leukaemias, and so therapies targeted at the initial diagnosis clone may not be effective for the treatment of the relapse. Indeed, in one study analysing CNAs in matched relapse and diagnosis samples from TEL/AML1^{+} patients, many of the CNAs unique to relapse samples were in genes involved in glucocorticoid signalling and so provide a potential mechanisms for acquired drug resistance at relapse (Kuster *et al* 2011).

Study of clonal evolution in leukaemias has also allowed the identification genes mutated at a higher frequency than expected, which are presumed to confer a selective advantage to clones, and so are called 'driver' mutations. Other genes which have a neutral effect on leukaemic clones are known as 'passenger' mutations, since they will frequently be passed along with 'driver' mutations, but are not as frequent nor believed to confer any advantage (Haber & Settleman 2007). In a study of CNAs present in TEL/AML1^{+} leukaemia in identical twins (Bateman *et al* 2010),

it was estimated that 5.1 CNAs were present in each case, 3.2 'driver' mutations (range 2-7) and 1.9 'passenger' mutations (range 1-4). Ultimately the distinction between 'driver' and 'passenger' mutations requires functional studies (Bateman *et al* 2010).

1.9 Common secondary mutations in acute lymphoblastic leukaemia

The identification of genetic alterations in ALL has allowed for the elucidation of common pathways of leukaemogenesis, as well as the investigation of mutations specific to certain leukaemia subtypes and patient groups. Karyotyping allows for the gross genetic changes such as translocations, deletions and amplifications, and aneuploidy to be identified quickly and cheaply, and is still the method by which many chromosome translocations that define subtypes of leukaemia are diagnosed. Fluorescence *In Situ* Hybridisation (FISH) can be used to confirm certain fusion genes resulting from translocations, and also to identify deleted alleles of specific genes. However, karyotype analysis is subjective and can only identify large changes in chromosome structure, far bigger than any single gene. FISH is labour intensive, and by definition requires a probe for a specific gene with a known sequence, precluding it from genome-wide studies.

Microarray platforms provide the opportunity for unbiased and detailed screens for genetic alterations (Mullighan & Downing 2009). Comparative genomic hybridisation (CGH) array screens use large fragments (up to ~200kb) of the human DNA, cloned into bacterial artificial chromosome (BAC) vectors, allowing coverage of most of the entire genome. Single Nucleotide Polymorphism (SNP) arrays use much shorter (20-100 bp), and were originally designed to identify known single nucleotide changes in the genome that are associated with susceptibility to certain diseases. However, by virtue of the probes being able to identify both maternal and paternal alleles, they can also be used to identify the copy number of genes, and as such deletions or amplifications of regions of chromosomes, known as copy number alterations, or CNAs. This ability of SNP arrays to detect CNAs added with the high resolution and the small amounts of DNA required have resulted in their widespread use to identify genetic alterations in acute lymphoblastic leukaemia.

1.9.1 ETV6

Deletions in 12p, and specifically deletions at *ETV6* locus, have been identified as a common feature of ALL (Irving *et al* 2005; Kuiper *et al* 2007; Mullighan *et al* 2007; Strefford *et al* 2007; Kawamata *et al* 2008; Bungaro *et al* 2009), in particular TEL/AML1^{+} leukaemia (Raimondi *et al* 1986; Raynaud *et al* 1996; Cave *et al* 1997; Attarbaschi *et al* 2004; Kawamata *et al* 2008). The role of *ETV6* deletion in the context of TEL/AML1^{+} leukaemia will be discussed in Chapter 4 (section 4.1.7).

1.9.2 Cell Cycle control genes

Recurrent mutations in a variety of proteins involved in cell cycle control have been identified in the context of ALL, for example *CDKN1B*, *CDKN2A*, *CDKN2B*, and *RB1* (Irving *et al* 2005; Kuiper *et al* 2007; Mullighan *et al* 2007; Strefford *et al* 2007; Kawamata *et al* 2008; Bungaro *et al* 2009; Bateman *et al* 2010; Notta *et al* 2011b). *CDKN2A*, found on chromosome 9p21 and also known as *P16INK4*, is a cyclin-dependent kinase inhibitor, an important regulator of cell cycle progression, as discussed above in section 1.4.2. It is also a known tumour suppressor gene, frequently mutated in various cancers (Smith-Sorensen & Hovig 1996). Deletions of *CDKN2A* are frequent in both B- and T-lineage ALL (Takeuchi *et al* 1995), though less frequent in TEL/AML1^{+} leukaemia (Sulong *et al* 2009), and genetic alterations within 9p are associated with relapse and poor prognosis in ALL (Heerema *et al* 1999; Hann *et al* 2001; Calero Moreno *et al* 2002; Mullighan *et al* 2008). The deletion of the *CDKN2A* locus in mice, in combination with exogenous expression of TEL/AML1, gives rise to frequent leukaemias (Bernardin *et al* 2002), and the deletion of *CDKN2A* in *RAG* knock-out mice induces B-ALL (Hauer *et al* 2011).

1.9.3 B cell development genes

Another group of genes frequently mutated or deleted in B-ALL are genes involved in B cell development (Kuiper *et al* 2007; Mullighan *et al* 2007). *PAX5* is a transcription factor which induces B cell specification in lymphoid progenitors by repressing alternative lineage pathways (Nutt *et al* 1999). Loss of functional PAX5 is observed through a variety of mechanisms, including mono-allelic deletions, exon deletions, small translocations, point mutations, and mutations in the *PAX5* promoter

(Mullighan *et al* 2007). Genes that are regulated by PAX5 are enriched in gene expression patterns in leukaemic cells from B-ALL patients without a common cytogenetic abnormality, indicating that the loss of PAX5 is of significant functional consequence in these leukaemic cells (Mullighan *et al* 2007). Mutations in two proteins that directly regulate *PAX5* expression, EBF and E2A, are also often identified in ALL (Kuiper *et al* 2007; Mullighan *et al* 2007).

1.9.4 Other genes associated with TEL/AML1$^+$ leukaemia

Throughout this thesis, a selection of the many genes involved in leukaemia was studied, in addition to some of those listed above. These genes, namely PIK3C3, TNFRSF7, SMAD1, ANGPTL4, EPOR, and IKZF2, are described below, in terms of their function and their relationship to TEL/AML1$^+$ leukaemia.

PIK3C3, also known as VPS34, belongs to a family of Phosphoinositide 3-kinases; PIK3C3 phosphorylates membrane-bound phosphotidylinositol which activates a signalling pathway (introduced in section 1.4.3) that regulates a variety of membrane transport functions such as endocytosis and biosynthetic trafficking (Simonsen *et al* 2001). PIK3C3 has been identified by three separate gene expression microarrays as a gene upregulated in TEL/AML1$^+$ leukaemia (Yeoh *et al* 2002; Andersson *et al* 2005b; Gandemer *et al* 2007). Upregulation of PIK3C3 was also observed in CML patients (Li *et al* 2002), indicating that PIK3C3 upregulation may represent a common pathway of haematological malignancies. PIK3C3 promotes survival of T cell; a lack of PIK3C3 in T lymphocytes results in increased cell death and reduced IL-7Rα (McLeod *et al* 2011). Mutations in another PI3K family member, PIK3CA (p110α), which lead to its increased activity, have been frequently observed in a variety of cancers (Karakas *et al* 2006).

TNFRSF7, also known as CD27, is a marker of memory B cells in humans (Agematsu *et al* 2000). TNFRSF7 and its ligand, CD70, is found on both T and B lymphocytes, and their interaction is believed to be important in the activation of both lymphocytes in the germinal centre (Borst *et al* 2005). High expression of TNFRSF7 has been associated previously with TEL/AML1$^+$ leukaemia (Yeoh *et al* 2002; Fine *et al* 2004; Gandemer *et al* 2007). Similarly, high levels of TNFRSF7

have been detected by flow cytometry on leukaemic B lymphoblasts (Nilsson *et al* 2005; Troeger *et al* 2008). In addition, TNFRSF7 in both membrane-bound and soluble forms has been associated with Chronic Lymphoid Leukaemia and Non-Hodgkin's Lymphoma (van Oers *et al* 1993; Ranheim *et al* 1995). The function of TNFRSF7 is not clear; however, it is believed to transduce pro-apoptotic signals through Siva-1 (Prasad *et al* 1997; Yoon *et al* 1999; Xue *et al* 2002), but can also activate both canonical and non-canonical NF-κB pathways (Ramakrishnan *et al* 2004), which are known to activate anti-apoptotic pathways (Borst *et al* 2005).

SMAD1 is a member of a family of signal transducers that act downstream from the TGF-β family of ligands, and have a variety of roles in proliferation and differentiation in many organs (Blank & Karlsson 2011). In haematopoiesis, the role of SMAD1 and its family members is believed to be highly context specific, and so the results from *in vitro* and *in vivo* data can sometimes be conflicting. In mice, a pulse of expression of SMAD1 during early development increases the numbers of haemangioblasts (Zafonte *et al* 2007), but SMAD1 appears to be redundant in adult haematopoiesis (Singbrant *et al* 2010). In zebrafish, SMAD1 deficiency leads to enhanced erythropoiesis but a lack of mature macrophages (McReynolds *et al* 2007). In human B cell lymphoma, SMAD1 overexpression and activation decreases proliferation (Munoz *et al* 2004). However, the role of SMAD1 in acute lymphoblastic leukaemia is not known.

Angiopoietin-like 4 (*ANGPTL4*), along with its family member ANGPTL2, was associated with leukaemia with TEL/AML1$^+$ leukaemia compared to normal haematopoietic populations (Andersson *et al* 2005b). In addition to their role of influencing angiogenesis, angiopoietin-like proteins are known to promote expansion of HSCs (Zhang *et al* 2006), and have been implicated in acute myeloid leukaemia.

The *EPOR* gene codes for the receptor for erythropoietin (EPO), a cytokine which promotes the production of erythrocytes (red blood cells). In two independent gene expression microarray studies, EPOR has been found to be consistently highly expressed in TEL/AML1$^+$ ALL compared to other subtypes (Ross *et al* 2003; Fine *et al* 2004). In addition, a mutation in the promoter of EPOR which leads to its upregulation has been identified as a mutation that contributes to leukaemogenesis in

a recently published mouse model of TEL/AML1$^+$ leukaemia (van der Weyden *et al* 2011), and two reports have shown that EPO signalling through EPOR promotes proliferation and survival in TEL/AML1$^+$ leukaemia (Inthal *et al* 2008; Torrano *et al* 2011).

IKAROS, encoded by the gene *IKZF1*, acts as a transcription factor via chromatin remodelling, and is involved in lymphoid lineage commitment, B cell development in particular (Georgopoulos 2002). A null mutation of *Ikaros* leads to the complete absence of both foetal and adult B cells and foetal T cells (Wang *et al* 1996a) and a reduction in the HSC pool (Nichogiannopoulou *et al* 1999). Deletions and dysregulation of *IKZF1* are commonly observed in B- and T-lineage leukaemia, and are associated with poor prognosis and increased risk of relapse (Mullighan *et al* 2008; Mullighan *et al* 2009; Payne & Dovat 2011). Since abnormal B cell development is a hallmark of TEL/AML1$^+$ leukaemia, the frequent deletion of genes associated with B lymphopoiesis provides a potential mechanism by which B cell progenitors can persist, allowing for further mutations to be acquired for progression towards overt leukaemia. In Chapter 4, the Ikaros family member IKZF2 (also known as 'Helios') is studied in relation the impact of gene expression of the loss of TEL. Deletion of IKZF2 has been identified as a recurrent mutation in relapsed B- and T- lineage ALL (Mullighan *et al* 2008), and overexpression of certain isoforms of IKZF2 has been associated with T-cell ALL (Nakase *et al* 2002) and adult T cell leukaemia/lymphoma (Fujii *et al* 2003).

1.10 Experimental study of leukaemia

As more is revealed about the nature of TEL/AML1$^+$ leukaemia, researchers will continue to use model systems as tools to address questions about fundamental aspects of the disease. Such model systems can be divided into three categories: *in vivo* models using transgenic animals, models using patient-derived material either *ex vivo* or in a xenograft animal model, and studies using cell lines *in vitro*. I will discuss the use of model systems in the study of TEL/AML1$^+$ leukaemia in more detail in the introduction to Chapter 3, but here I will give an overview of each of these.

1.10.1 *In vivo* models

Firstly, *in vivo* mouse models, either by studying transgenic animals (Schindler *et al* 2009) or recipients of TEL/AML1-transduced bone marrow (Tsuzuki *et al* 2004), which have been used to investigate the development and clonal evolution of TEL/AML1$^+$ HSCs. Using these models, it has been found that TEL/AML1 increases quiescence in HSCs (Bernardin *et al* 2002; Schindler *et al* 2009), but also self-renewal in downstream multipotent precursors and B lymphoid precursors (Morrow *et al* 2004; Fischer *et al* 2005), and that this activity requires domains present in both the TEL and AML1 moieties of the fusion protein (Morrow *et al* 2007). In addition, these transgenic mice have demonstrated the necessity of secondary mutations to provoke overt leukaemia (Schindler *et al* 2009; van der Weyden *et al* 2011), a finding also confirmed in a zebrafish model of TEL/AML1 (Sabaawy *et al* 2006).

1.10.2 Xenograft models

Secondly, models using leukaemia patient-derived material (le Viseur *et al* 2008) or TEL/AML1-transduced cord-blood samples (Hong *et al* 2008), either as a xenograft or *ex vivo*, have been described. These have further revealed the self-renewal capacity of committed progenitors (le Viseur *et al* 2008), and identified a serially transplantable pluripotent cell population, akin to a 'leukaemic stem cell' (Hong *et al* 2008) which responds abnormally to TGF-β (Ford *et al* 2009).

1.10.3 Cell lines

Thirdly, patient-derived cell lines and *in vitro* studies have been used to investigate molecular and cellular aspects of leukaemia. For example, using cell lines, TEL/AML1 was found to interfere with the activities of both TEL and AML1 (Gunji *et al* 2004), and bone marrow stromal cells were found to regulate chemotherapy resistance via TGF-β signalling (Dosen-Dahl *et al* 2008). RNAi silencing of TEL/AML1 in cell lines has revealed it is not necessary for survival of these cells (Zaliova *et al* 2011), but maintains a malignant gene expression program that differs to normal B cell precursors (Fuka *et al* 2011b).

One of the most widely used cell lines for studying TEL/AML1$^+$ leukaemia is the REH cell line. The REH cell line was derived from the peripheral blood of a 15-year-old in acute lymphoblastic leukaemia relapse (Rosenfeld *et al* 1977), making it the first cell line representative of a B cell precursor leukaemia (Matsuo & Drexler 1998). It was later discovered to harbour TEL/AML1 (Uphoff *et al* 1997), shortly after the discovery of the fusion gene in 1995 (Golub *et al* ; Romana *et al*). It was also shown that the REH cell line lacked the remaining untranslocated TEL allele; in this case by a second translocation t(5;12)(q31;p13) resulting in the deletion of TEL (Uphoff *et al* 1997). The use of the REH cell line in the study of TEL/AML1$^+$ leukaemia is further discussed in section 4.1.10.

1.11 Gene delivery methods

The experimental study of the role of a particular gene, including the work presented in the examples above and the current work presented in this thesis, often requires the transfer of genes into cells by gene vectors. Summarised below are the two main types of vectors used for gene delivery into mammalian cells; plasmid vectors and viral vectors. In the introduction to Chapter 3 (section 3.1.9), the methods of transgene expression in hESCs are further explored.

1.11.1 Plasmid vectors

DNA plasmid transfection is commonly used to transiently express proteins in cells as it is relatively simple to construct a DNA plasmid, and there is not the same limit on the size of the genetic material that can be delivered, unlike viral vectors (see below). The stable expression of the transgene requires the integration of the plasmid in the host genome, as plasmids are not commonly transferred to daughter cells when cells divide, and so are ‘diluted out’ over time. However, both the transfection efficiency and the efficiency of stable integration of plasmids is low, and certainly much lower than that of viral vectors such as lentiviruses (Washbourne & McAllister 2002).

The delivery of DNA plasmids across the plasma membrane into cells can be performed by a variety of methods, loosely defined in two categories: chemical and non-chemical methods. Chemical methods tend to induce endocytosis of DNA by enhancing the ability of DNA to bind to the plasma membrane, a process that generally does not occur normally because of the charged nature of DNA. For example, calcium phosphate precipitation (Graham & van der Eb 1973) allows for the binding of DNA to the plasma membrane. Alternatively, DNA plasmids can be delivered into the cell in liposomes, small lipid-membrane vesicles that bind to the plasma membrane and are taken into the cell by endocytosis. Non-chemical methods involve the 'forced entry' of DNA into cell. In the case of plant cells, DNA can be fired into the cell attached to tungsten particles in a method known as biolistics, but the most commonly used method in animal cells is electroporation. The precise mechanism of action for electroporation is unknown, but it is believed that firing a pulse of electricity through the cell causes large pores to open in the plasma membrane, through which charged particles like DNA can pass (Neumann *et al* 1982).

1.11.2 Viral vectors

The use of viral vectors for the expression of exogenous genes appears to be an obvious choice – viruses have evolved to deliver genetic material into cells and express it at high levels. As such, the advantage of using viral vector for gene delivery is the high transduction efficiency of many different types of tissues. Viral vectors used for laboratory research are frequently engineered for safety and thus lack genes required for replication. However, the advantages of viral vectors are also balanced by several disadvantages, namely the production of the high titres of viruses required is time consuming and labour intensive, and the size of the gene insert used is limited by the capacity of the virus.

Adenoviruses are DNA viruses that have a double-stranded DNA genome. They are non-integrating viruses, and so are often only used for transient transduction of cell lines. The cloning capacity of the virus is about 5-14kb, but the production of adenoviruses is time-consuming at about four to six weeks (Ross & Parks 2009). As such, adenoviruses are not often used in laboratory research, but closely related

viruses, so-called adeno-associated viruses (AAVs), are showing promise as gene delivery vectors for gene therapy, as unlike adenoviruses, AAVs do not elicit a significant immune response in humans (Flotte & Carter 1995).

Retroviruses are a family of RNA viruses which integrate their genes into host genomes using the enzyme reverse transcriptase to convert their genome into single-stranded DNA. The cloning capacity of retroviruses is about 10kb (Trono 2000), but retroviruses are mostly only able to infect dividing cells, and tend to insert randomly into the genome, which can lead to unintended consequences in host gene expression. Lentiviruses are a genus of the retrovirus family, but have the advantage over most retroviruses in that they are able to transduce both dividing and non-dividing cells. The integration of lentiviruses into the genome occurs at less random locations (Cattoglio *et al* 2007), and coupled with their high transduction efficiency and long-term stable expression, lentiviruses are a commonly used viral vector in laboratory gene expression experiments.

1.12 Conclusion and Aims

Acute Lymphoblastic Leukaemia (ALL) is the rapid proliferation of immature lymphoblasts in the bone marrow, and is the most common cancer in children. The t(12;21) translocation, generating the TEL/AML1 fusion protein, is the most frequently identified initiating genetic lesion in childhood ALL, and is most likely to occur prenatally, but further postnatal mutations are necessary for the progression to overt leukaemia.

The transcription factors TEL and AML1 (coded for by the genes *ETV6* and *RUNX1*, respectively) are key players in normal and malignant haematopoiesis. TEL/AML1 appears to interfere with the normal activities of both wild-type TEL and AML1, but little is known about the genes which are regulated by either, and how their aberrant regulation leads to the ‘pre-leukaemic state’ of a persistent clone that is predisposed to further mutations. In addition, it has not been directly proved in which haematopoietic progenitor cell the translocation needs to occur to generate this

leukaemia initiating cell, although it is assumed to be early multipotent cells such a HSCs (Schindler *et al* 2009).

A diagram showing a model of initiation and progression of TEL/AML1$^+$ leukaemia is shown in Figure 1.3. As depicted in Figure 1.3A, HSCs present in the developing embryo are subject to the chromosome translocation t(12;21), which generates HSCs of increase self-renewal (as described in Schindler *et al* 2009). These multipotent HSCs are capable of differentiation along any lineage, but in the event of commitment to B cell development, they can generate the a 'pre-leukaemic clone' (Hong *et al* 2008) with similar phenotype to pro-B cells, that have self-renewal properties and a reduced capacity to differentiate into pre-B cells and further along the B lymphocyte lineage. The main feature of this model is that TEL/AML1 on its own is not sufficient for leukaemia. In the first chapter of this thesis, the development of a model of TEL/AML1$^+$ leukaemia is described, which provides additional evidence to support this model.

From the TEL/AML1$^+$ pre-leukaemic clone, overt leukaemia develops resulting in lymphoblast population with TEL deficiency (Figure 1.3B), commonly manifested either by the genetic deletion of the remaining TEL allele or a down-regulation of TEL, as is observed in approximately 75% and 25% of TEL/AML1$^+$ patients, respectively (Cave *et al* 1997; Patel *et al* 2003). A third hypothetical possibility is the loss of TEL function through the loss of downstream mediators of TEL activity, or activation of opposing mechanisms, through either mutation or dysregulation.

What role this TEL deficiency plays in progression from pre-leukaemia to overt TEL/AML1$^+$ leukaemia is depicted as three possible models in Figure 1.3B. The loss of TEL could be sufficient for progression into overt leukaemia (1), though this does not take into other frequent mutations which may be significant (as described in section 1.9) or the influence of infection (described in section 1.8.6). Another possibility (2) is that TEL deficiency is a necessary requirement of the pre-leukaemic clone, but is not sufficient for progression to overt leukaemia, which requires other mutations or environmental factors (shown as a question mark). Finally, it is possible that overt leukaemia can be triggered without the loss of TEL (3), but instead that TEL deficiency confers an advantage to a leukaemic clone, such that it quickly

becomes the dominant clone in the population. In the second chapter of this thesis, these three possibilities for the role of TEL in the context of TEL/AML1^{+} will be examined by over-expressing TEL in the REH leukaemia cell line. This aims to illuminate the role TEL plays, both on a transcriptional and functional level, and thus what is lost during TEL/AML1^{+} leukaemia progression.

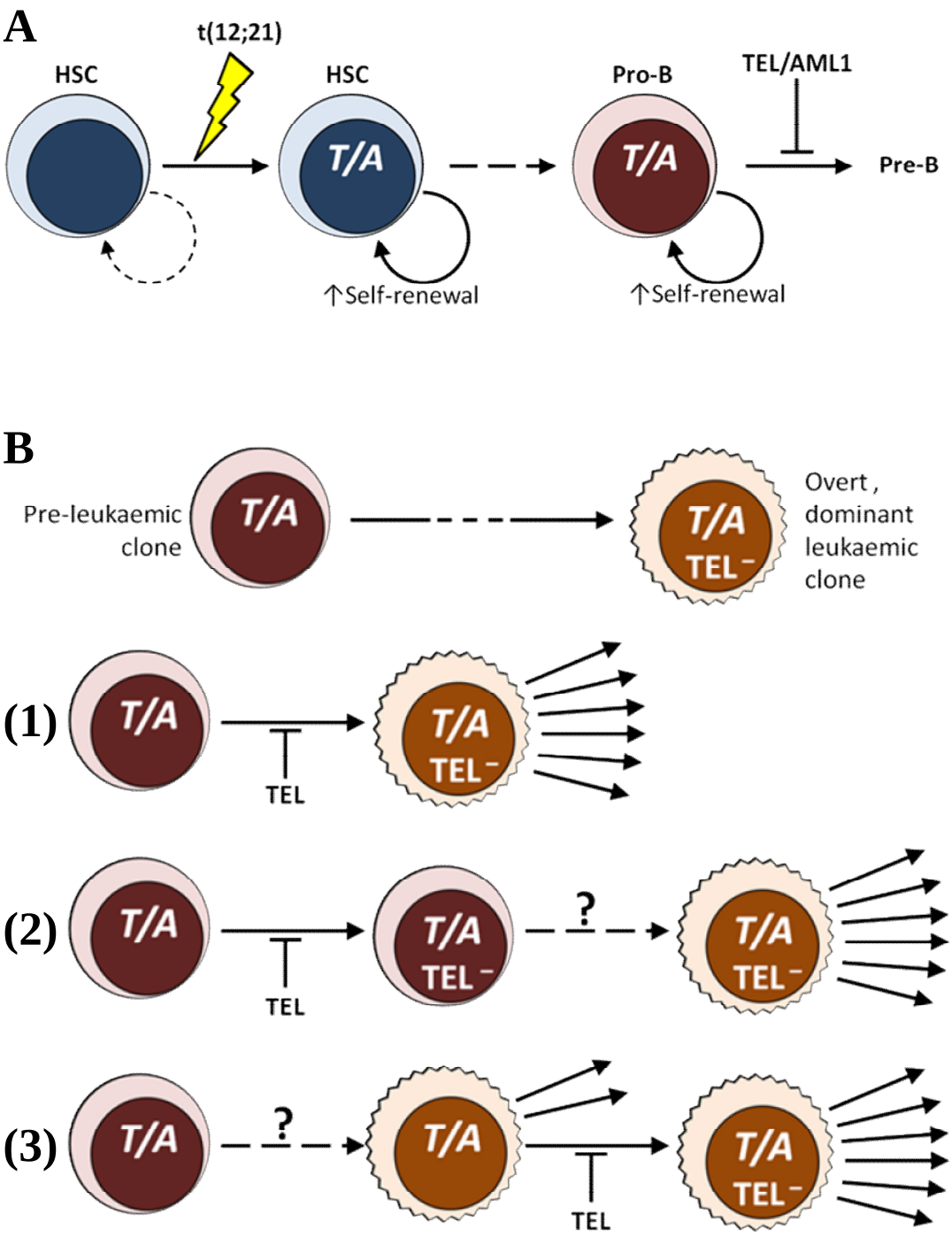


Figure 1.3 – Models for the progression of TEL/AML1+ leukaemia.
(A) TEL/AML1 increases self-renewal in transformed HSCs and can generate a persistent self-renewing 'pre-leukaemic clone' akin to a pro-B cell lineage, which has reduced ability to differentiate further along the B lymphocyte lineage. (B) The progression of this TEL/AML1$^+$ pre-leukaemic clone ends with TEL deficiency, either through a genetic loss or a functional loss. This suggests one of three possible models for progression of TEL/AML1$^+$ leukaemia: (1) TEL deficiency is both necessary and sufficient for progression; (2) TEL deficiency is necessary but not sufficient; (3) TEL deficiency is not necessary for progression to leukaemia, but confers a selective advantage to a leukaemic clone such that it eventually becomes the dominant clone. See text for further discussion of these models.

Chapter 2: Materials and Methods

2.1 hESC culture

2.1.1 Culture of human embryonic stem cells (hESCs)

The human embryonic stem cell (hESC) line H9 was obtained from Peter Andrews (Centre for Stem Cell Biology, University of Sheffield) with permission from WiCell Research Institute (University of Wisconsin). hESCs were cultured on irradiated mouse embryonic fibroblasts (iMEFs; MEFs obtained from UK Stem Cell Bank) seeded onto gelatinised 6-well plates. hESC culture media was made using Knockout Dublecco's Modified Eagle Medium (KO-DMEM, Gibco), supplement with 20% Knockout Serum Replacement (KO-SR), 2mM L-glutamine, 100µM Non-Essential Amino Acids (NEAA, Gibco), 100µM β-mercaptoethanol (Gibco), 4ng/ml human basic fibroblast growth factor (hbFGF), and 1× Penicillin-Streptomycin (PenStrep, Invitrogen). Media was replaced every day, 2ml per well.

Stably transfected hES cell lines ('T/A D1', 'T/A D2', 'T/A F1', 'T/A F2', 'GFP1', 'GFP2', 'ΔRHD5', and 'ΔRHD6') were maintained as above, except for the use of drug-resistant DR4 iMEFs (Tucker *et al* 1997; DR4 MEFs kindly provided by Peter Andrews at University of Sheffield), and the media was supplemented with 1µg/ml puromycin.

2.1.2 Preparation of mouse embryonic fibroblasts (MEFs)

To prepare frozen aliquots of low passage MEFs, E13.5 day mouse embryos were homogenised using a scalpel and incubated with Trypsin-EDTA at 37ºC for 10-20 mins in a centrifuge tube. MEF Media (DMEM supplemented with 10% Foetal Bovine Serum [FBS], 2mM L-glutamine, and 1× PenStrep) was added to the tube, large chunks left to settle and then the supernatant transferred to one T75 flask per embryo used, which was incubated at 37ºC until confluent. Cells were then detached using Trypsin-EDTA, then frozen in one aliquot per flask, in freezing media (FBS with 20% DMSO).

2.1.3 Preparation of frozen aliquots of inactivated MEFs (iMEF)

To prepare frozen aliquots of iMEFs, a frozen aliquot of low passage MEFs (as prepared above) was thawed and cultured in a T75 until confluent. These cells were then passaged into several T175 flasks (commonly from one T75 to eight T175 flasks), and cultured until confluent. The cells were then harvested using Trypsin-EDTA, and then resuspended in a small volume (~10ml) of MEF media in a 50ml centrifuge tube. MEFs were inactivated by X-ray irradiation (40Gy), and then counted. These iMEFs were centrifuged and resuspended in freezing media at a concentration of 2 million cells per ml. Aliquots of 2 million cells are then frozen.

2.1.4 Preparation of iMEF plates for hESC culture

To prepare plates of iMEFs on which to culture hESCs, 6-well plates (Corning) were gelatinised by diluting bovine Gelatin solution (Sigma-Aldrich) 1:20 in pre-warmed Phosphate Buffered Saline (PBS; PAA), dispensing 2 ml per well, and incubating at room temperature for at least 20 minutes. Frozen aliquots of 2 million iMEFs (as prepared above) were thawed and resuspended in 10ml of MEF media. These were centrifuged and resuspended in 12ml media, before dispensing into the gelatinised 6-well plate (after removing the gelatine solution) and incubating at 37ºC overnight.

2.1.5 Passaging hESCs

To split, cells were washed with PBS, and incubated with 1mg/ml Collagenase IV (Gibco) in KO-DMEM for 20-30mins. Cells were harvested from the plate by pipetting, centrifuged for 5 minutes at 200g, washed once with 2mls hESC media and centrifuged, and seeded into a freshly prepared gelatinised iMEF plate at a split ratio of between 1:6 and 1:10.

2.2 Transfection of hESC

Transfection of hESCs was performed using the NEON electroporation system (Invitrogen). H9 hESCs were dissociated into single cells using 0.05% Trypsin-EDTA, and resuspended at a concentration of 1×10^7 cells/ml, in Resuspension buffer R. 10µl of cells (i.e. 1×10^5 cells) was mixed with 0.5µg DNA, and was electroporated using the NEON transfection system, with 2 pulses of 1050V

with a pulse width of 30ms. Cells were then dispensed into gelatinised 6-well plates seeded with DR4 iMEFs, containing pre-warmed hESC media supplemented with 10μM Y-27632, a selective inhibitor of ROCK (Watanabe *et al* 2007). Media was supplemented with 1μg/ml puromycin from two days after transfection, Y-27632 was supplemented to media until colonies with more than ten cells were visible.

2.3 Western Blot

Cell extracts were prepared using radioimmunoprecipitation assay buffer (RIPA buffer, Sigma Aldrich) following manufacturer's instructions. Protein samples from hESC lines to detect TEL/AML1 were resolved on a 12.5% SDS-polyacrylamide gel. Protein was transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore), and blocked in PBS with 5% (w/v) milk powder for one hour. Primary antibodies were diluted in PBS with 5% milk and 0.1% Triton; 1 in 2000 for anti-HA tag, and incubated overnight at 4ºC (more information and manufacturers for antibodies used can be found in Table 2.1). Membranes were washed three times with PBS-Triton for five minutes, and then incubated with a Horseradish Peroxidase-conjugated secondary antibody at a dilution of 1 in 5000, in PBS with 5% milk. Membranes were washed as before and proteins detected using the chemiluminescent reagent ECL (GE Healthcare) and photographic film (Amersham Hyperfilm, GE Healthcare).

2.4 Imaging of hESCs using immunocytochemistry

hESC lines were grown on chamber slides onto which DR4 iMEFs had previously been seeded. After three or four days of culture, cells were fixed using 4% Paraformaldehyde (PFA) for 15 minutes, washed with PBS for 5 minutes, and permeabilised using PBS supplemented with 0.5% Triton X-100 and 3% goat serum (hereafter referred as PBS-Triton-Serum) for 30 minutes. The anti-HA tag primary antibody was diluted 1 in 500 in PBS-Triton-Serum and incubated for 1 hour. Cells were washed three times with PBS-Triton for 5 minutes, and fluorescently-conjugated primary and secondary antibodies applied (Goat anti-rabbit Alexa647 and anti-GFP-Alexa488, diluted 1 in 500 in PBS-Triton-Serum) for 45 minutes. The cells were washed three times for 5 minutes with PBS-Triton, with the first wash containing DAPI at a dilution of 1 in 2000. Cells were post-fixed with 4% PFA for 5

minutes, before being washed three times again with PBS-Triton. Finally, PBS-Triton was removed and replaced with PBS to prevent cells from drying out. Fluorescently labelled cells were imaged using a Multiphoton Zeiss LSM 780 NLO, and images processed using Volocity software (PerkinElmer).

2.5 Preparation of samples for karyotyping

hESCs in exponential growth were incubated with colcemid solution (KaryoMAX, Gibco), diluted to 2µg/ml in PBS, for four hours at 37ºC. Cells were detached from the culture dish by Trypsin-EDTA and aspirated to form a single cell suspension, which was transferred to a 15ml centrifuge tube, to which 6mls of prewarmed hypotonic KCl solution (37.5mM in dH_2O) was added, and incubated for 5 minutes at 37ºC. The tubes were centrifuged at 100g for 8 minutes to pellet the cells, before being fixed using a fixative (3:1 methanol:glacial acetic acid); the pellet was resuspended in the fixative and centrifuged three times. After the final centrifuge step, the samples ready for karyotyping were resuspended in the fixative and stored at -20ºC. Karyotyping was kindly performed by Duncan Baker (Sheffield Children's Hospital). All cell lines were found to be karyotypically normal female (46, XX).

2.6 Haematopoietic differentiation of hESC

2.6.1 Differentiation culture

hESCs were differentiated towards haematopoietic lineage following the OP9 co-culture method as described in Vodyanik & Slukvin 2007. To summarise, on day 0, hESCs were seeded onto overgrown OP9 cultures in 10cm dishes, in small aggregates at a concentration equivalent to 10^6 cells per plate. Media used for differentiation was Minimum Essential Medium Alpha-modification (α-MEM, Gibco), supplemented with 10% Foetal Bovine Serum (FBS, HyClone), 100µM monothioglycerol (MTG, Sigma-Aldrich), 50µM Ascorbic Acid (Sigma-Aldrich) and 1× PenStrep. On day 1, medium was replaced with 20ml differentiation medium, and on days 4 and 6, medium was half-changed, with 10ml fresh medium.

2.6.2 Harvesting of differentiated hESCs

On day 8, the cells from the differentiation cultures were harvested by 20 minutes incubation with Collagenase IV solution followed by 30 minutes in Trypsin-EDTA solution. Cells were washed three times with 'Washing Buffer': Phosphate Buffered Saline (PBS) without calcium or magnesium (PAA Laboratories), supplemented with 5% FBS and 2mM EDTA. On the third wash, cells were passed through a 70-µm Cell Strainer (BD Biosciences) to remove any clumps, and then cells were counted by trypan blue exclusion, either manually by microscope or automatically using the Vi-CELL XR cell counter (Beckman Coulter).

2.6.3 Analysis of differentiation by flow cytometry

For flow cytometric analysis, the cell concentration was adjusted to 5×10^6 cells /ml and 100µl of cells (i.e. 5×10^5 cells) were stained in 100µl staining volume for at least 30 minutes using combinations of the following monoclonal antibodies: CD31-PE, CD31-Alexa488, CD43-FITC, CD43-PE, and TRA-1-85-APC (more information and manufacturers for antibodies used can be found in Table 2.1). For dead cell discrimination, cells were stained with 7-Amino-actinomycin D (7-AAD). Cells were analysed using the CyAn-ADP flow cytometer (Beckman Coulter), and subsequent analysis was carried out using the Summit (Beckman Coulter) and FlowJo (TreeStar) software programs.

2.6.4 Sorting of $CD43^+$ cells by MACS and FACS

For MACS enrichment prior to sorting by FACS, harvested cells were subjected to two MACS enrichments: dead cell depletion and enrichment on CD43.

First, for the dead cell depletion, cells were washed once with PBS (with added magnesium, in the form of hydrous magnesium chloride at a final concentration of 0.1g/L) and then MACS Binding Buffer (Miltenyi Biotec). Cells were resuspended in 100µl Dead Cell Removal Microbeads per 10^7 cells (Miltenyi Biotec) and incubated at room temperature for 15 minutes. The mixture of cells and beads was then passed through a LS positive selection column in a MACS separator, which was

then washed four times with Binding Buffer. All the flow through was collected as the live cell fraction.

Second, for the enrichment for $CD43^+$ cells, the protocol described by Vodyanik & Slukvin was adapted. Briefly, cells were centrifuged, and the pellet was resuspended in an equal volume (i.e. if the pellet volume was 0.2ml, the pellet was resuspended in 0.2ml) – this is called the staining volume. The anti-CD43-PE antibody was added at a 10^{th} of this total volume (i.e. 40µl, in the above case), and the mixture was incubated at 4ºC for 20 minutes. Cells were washed using Washing Buffer (PBS, 5% FBS, and 2mM EDTA), resuspended in the same staining volume as before, and Anti-PE Microbeads added at a 10^{th} of the volume. This mixture was incubated for 20 minutes at 4ºC. Cells were washed using Washing Buffer, and the cells were passed through a LS positive selection column in a MACS separator. The column was washed three times with Washing Buffer, removed from the MACS separator, and then the cells adhering to the column were eluted in Washing Buffer.

For sorting of $CD43^+$ cells directly by FACS, harvested cells were stained with TRA-1-85-APC, CD43-PE, and 7-AAD, and cells were sorted using the MoFlo cell sorter (Beckman Coulter) on the basis of lack of 7-AAD staining, expression of TRA-1-85, and expression of CD43.

2.7 Haematopoietic Precursor Colony Forming Assay

To assess the haematopoietic capacity of differentiated hESCs, sorted $CD43^+$ were resuspended in 0.3ml differentiation media (αMEM, 10% FBS, 100µM MTG, 50µM Ascorbic Acid, and 1× PenStrep), and added to a pre-aliquotted tube containing 3ml of methylcellulose-based medium (MethoCult H4435, STEMCELL Technologies). The tube was vortexed to mix and incubated in a 37ºC water bath for 10 minutes to allow bubbles to dissipate. Using a blunt-end needle (STEMCELL Technologies) and a 1ml syringe (BD Biosciences), cells in MethoCult were dispensed into three 35mm low adherence dishes (BD Biosciences), which were placed inside a square petridish (Nalgene Nunc) with an open 35mm filled dish with dH_2O. After two weeks, the dishes were checked for colonies containing differentiated cells.

2.8 Differentiation of hESC-derived haematopoietic precursors towards B lymphoid lineage

Sorted $CD43^+$ haematopoietic precursors from hESC differentiation were differentiated towards B lymphoid lineage as described in Vodyanik *et al* 2006. To summarise, sorted $CD43^+$ cells were seeded onto Mitomycin-C-inactivated MS-5 stromal cell monolayers, at a density of 10^3 cells per well of 6-well plates (MS-5 cells kindly provided by Claudine Schiff, Centre d'Immunologie de Marseille Luminy, Université de la Méditerranée). Differentiation media consisted of αMEM supplemented with 10% FBS, 100µM MTG, 50ng/ml Stem Cell Factor (SCF), 50ng/ml Fms-related tyrosine kinase 3 ligand (Flt-3L), 20ng/ml Interleukin-7 (IL-7), and 5ng/ml Interleukin-3 (IL-3; all cytokines from Peprotech). Media was half-changed every 4 days, with media without IL-3. All cells were harvested on the fourth week of culture (both adherent and non-adherent), and analysed by qPCR.

2.9 REH cell culture

The REH cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 2mM L-glutamine. Cells were passaged by centrifugation at 300g for 5 minutes and were commonly split at a 1:10 ratio. Stably transfected REH cells (REH:TEL and REH:pCIneo) were maintain in media supplemented with 600µg/ml G418 and 1× PenStrep.

2.10 Transfection of REH cells with DNA plasmids

Transfection of REH cells was performed using the NEON electroporation system (Invitrogen). REH cells were resuspended at a concentration of 1×10^7 cells/ml, in Resuspension buffer R. 10µl of cells (i.e. 1×10^5 cells) was mixed with 0.5µg DNA, and was electroporated using the NEON transfection system, with 2 pulses of 1050V with a pulse width of 30ms. Cells were then dispensed into 6-well plates containing pre-warmed REH media. Two days after transfection, the media was replaced, supplemented with 600µg/ml G418.

2.11 Transduction of REH cells with viral vectors

Transduction of REH cells was performed by spinoculation; 10^6 REH cells were resuspended in 500µl of media without FBS, and mixed with 500µl of viral supernatant (either MSCV-TEL-IRES-eGFP or MSCV-eGFP, cited in Morrow *et al* 2007 and kindly provided by Owen Williams, Institute of Child Health, University College London) and Polybrene (Hexadimethrine bromide, at a final concentration of 5µg/ml, Sigma-Aldrich), and centrifuge at 800g for 45 minutes at room temperature. Cells were resuspended in the supernatant, and plated out into a 6-well plate. 1ml of media without FBS was added, and incubated at 37ºC. 24 hours later, media was replaced with media containing FBS. Cells expressing GFP were sorted by FACS 48 hours after the initial transduction.

2.12 Gene expression profiling by qPCR

RNA extract, cDNA synthesis, and qPCR for all cells followed the same protocol. Cells were lysed by resuspending in 350µl RLT buffer (Qiagen), and RNA was extracted using the RNAeasy Spin Column Kit (Qiagen), following manufacturer's instructions. RNA concentration was assessed using NANOdrop (Thermo Scientific). cDNA was synthesised using the EZ-First Strand Kit (Biological Industries), using between 0.5µg and 1µg of RNA, following manufacturer's instructions. cDNA was either used immediately or stored at -20ºC for future use.

Real-time quantitative PCR was performed with the SYBR Green PCR kit using the ABI Prism 7300 system (Applied Biosystems) following manufacturer's instructions. Expression of target genes was normalised to GAPDH and expressed as a fold change in expression using the change in cycle threshold (ΔΔcT) method. Primer sequences for target genes are listed in Table 2.2.

2.13 Microarray

The integrity of the RNA samples was assessed using Agilent 2100 Bioanalyzer, and were all deemed to be of good quality. RNA was subjected to a further RNA quality control test; Figure 4.1C shows the fragments of amplified (complimentary) RNA for loading onto the chip are of similar sizes between samples and within the

necessary range (200-1000 nucleotides). Raw data from the microarray was normalised so that median values and spread of data for both replicates in each group were the same (Figure 4.1D). The microarray platform used was Agilent Human Gene Expression 4×44k Microarray. The same RNA as used in the microarray was used again to validate the expression of a selection of genes (Table 4.3) by qPCR (Figure 4.3, Figure 4.8, and Figure 4.9).

2.14 EdU uptake assay

Proliferation of REH cells was assessed by directly measuring DNA synthesis using the 'Click-iT EdU Flow Cytometry Assay Kit' (Invitrogen). Briefly, the nucleoside thymidine analog EdU was added to cells in media at a final concentration of 10µM. After incubating for one hour, cells were washed, fixed with 4% PFA, and permeabilised with PBS-Triton (0.15% v/v), before reacting the EdU with a fluorescently-labelled azide (either azide-Alexa488 or azide-Alexa647, depending on the application) using the reagents included in the kit. EdU uptake was assessed by flow cytometry.

2.15 Cell cycle analysis

REH cells transduced with either GFP or TEL-GFP oncoretrovirus were seeded into culture for 96 hours. At 24 hour time points, samples were taken, and washed in PBS by centrifugation. Cells were then fixed with 4% PFA, before being permeabilised with PBS-Triton. The cells were finally stained with DAPI (1 in 2000 dilution in PBS-Triton), washed in PBS, and analysed by flow cytometry.

2.16 Apoptosis assay

REH cells transduced with either GFP or TEL-GFP oncoretrovirus were seeded into culture for 72 hours with or without staurosporine (STS; 1µM). At 24 hour time points, samples were taken, washed once in PBS, and washed again in "Annexin-V binding buffer" (10mM HEPES, 140mM NaCl, and 2.5mM $CaCl_2$ in water), by centrifugation. The cells were resuspended in 100µl binding buffer, to which 5µl Alexa647-conjugated anti-Annexin-V antibody and 5µl of pre-diluted DAPI (1% v/v

in binding buffer) was added. Cells were incubated at room temperature in the dark for 15 minutes, before 400µl binding buffer was added, and the cells were analysed by flow cytometry.

2.17 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. To perform statistical analysis on the data from the differentiation of hESC (Figure 3.8), I first assessed whether the data fitted a normal distribution using the in-built D'Agostino-Pearson omnibus normality test (D'Agostino & Pearson 1973). None of the data sets passed the test for normality, due to the small sample sizes used.
I therefore analysed data using non-parametric statistical tests, specifically the Mann-Whitney test for comparing two groups (the non-parametric counterpart to Student's *t*-test), and Kruskal-Wallis test for comparing three or more groups (the non-parametric counterpart to One-way ANOVA). Statistic significance was set to *p* values less than 0.05.

2.18 Antibody Table

Specificity	Label	Clone	Isotype	Manufacturer	Host	Use
Annexin-V	Alexa647	n/a	n/a	Molecular Probes	Goat	FC
Anti-CD31	PE	WM59	IgG1, κ	BD Pharmingen	Mouse	FC
Anti-CD31	Alexa488	M89D3	IgG_{2a}, κ	BD Pharmingen	Mouse	FC
Anti-CD43	PE/FITC	1G10	IgG1, κ	BD Pharmingen	Mouse	FC
Anti-GFP	Alexa488	-	IgG	Molecular Probes	Rabbit	ICC
Anti-HA tag	Purified	n/a (polyclonal)	IgG	AbCam	Rabbit	WB, ICC
Anti-SSEA-1	APC	MC-480	IgM	R&D Systems	Mouse	FC
Anti-SSEA-4	PE	MC-813-70	IgG_3	Millipore	Mouse	FC
Anti-TRA-1-60	FITC	TRA-1-60	IgM	Millipore	Mouse	FC
Anti-TRA-1-85	APC	TRA-1-85	IgG1	R&D Systems	Mouse	FC
Anti-Rabbit IgG secondary	HRP	n/a (polyclonal)	IgG	AbCam	Goat	WB

Table 2.1 – List of antibodies used in this thesis
Abbreviations- PE: R-Phycoerythrin; FITC: Fluorescein isothiocyanate; APC: allophycocyanin; HRP: Horseradish Peroxidase; FC: Flow Cytometry; ICC: Immunocytochemistry; WB: Western Blot.

2.19 Primer list

Gene Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
ANGPTL4	GGCCAAGCCTGCCCGAAGAA	CGGTGCAGGCGGCTGACATT
BCL-2	GTACCTGAACCGGCACCTGCAC	AGCCTCCCATTGCCCCAGGAG
CD10	TGCACAGGTGTGGTGTGGAACC	TGCGGCAGTGAAAGGCTTCTGA
CD19	GCCGGTCGCCAGGACAATGG	AGGGAGGTTGCTTCCCGGCT
CD34*	TGGACCGCGCTTTGCT	CCCTGGGTAGGTAACTCTGGG
CD43	GCCCCAGTGCTGCGTCCTTA	GCTGTTGTGCTCCCCAGAGCG
CDKN1B	AAGAGGCGAGCCAGCGCAAG	GGCCGCGGGGGTCTGTAGTA
CDKN2A	AGACCCAACCTGGGGCGACT	CGAGTGCTCGGAGGAGGTGC
CLIC5	GGCGACAGCTAACGGGGACG	CGCTGAGAGAAAGGACAGTTGCCG
EKLF*	CGGACACACAGGATGACTTC	GGCTGGTCCTCAGACTTCAC
EPOR	TGGACCCCCTCATCCTGACGC	AACTCGCTCTCTGGGCTCGGG
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
GATA1*	GGGATCACACTGAGCTTGC	ACCCCTGATTCTGGTGTGG
GATA2*	GCGTCTCCAGCCTCATCTT	GGAAGAGTCCGCTGCTGTAG
HPRT §	GTTGGATACAGGCCAGACTTTGTTG	GTTGGATACAGGCCAGACTTTGTTG
IKZF2	GCACAAACACACGTTAGGCACAG	ACCCCTCAAAGAGGAGGTGACAA
MB1	CCGCACAATAGCAGCAACAA	CCACGTGTAGTTGCCATGGA
NANOG	GCCCTGATTCTTCCACCAGTCCC	TGACCGGGACCTTGTCTTCCTT
NETO	ATCCCCCTGACCGGGAATGC	TTTGCACTCCCAAGACGGTTCAA
OCT4	GCCACATCGCCCAGCAGCTT	GCCCTTCTGGCGCCGGTTAC
p53 §	GGGACAGCCAAGTCTGTTATGTGC	CTGTCTTCCAGATACTCGGGATAC
PAX5	CGTTTAGTTTCAGCTCAGTGATCAG	CGCTCACAGGTCGGAATAATTC
PIK3C3†	GCCTTGGAACTTCTGGGAAAA	CAACAGCATAACGCCTCACAG
PU.1*	CACAGCGAGTTCGAGAGCTT	GATGGGTACTGGAGGCACAT
RAG1	TTTGCCGGGTCTGCATTC	TCAGTAGGGAAGCATGGATATCG
SCL*	ATGAGATGGAGATTACTGATG	GCCCCGTTCACATTCTGCT
SMAD1	GGCGGAGAAAGGAGAGGCCGAG	GTCCGTGTGGGAGCACGAGTGG
TDT	TTGCCCTGTTGGGATGGA	CATAGCGCCGGAGGTCTCT
TTBK	CGGGCGAGTGGAGCTACCTG	ACTGGCCTGTCGGTCAGCGT
TEL‡	TCCCAACGGACTGGCTCGACT	TAGTGGCGCAGGGCTCTGGA
TEL/AML1‡	CATCATGGTCTCTGTCTCCCCGCC	TGGCATCGTGGACGTCTCTAGAAGG
TNFRSF7†	AGGGACAAGGAGTGCACCG	AAGGTAAGTGGGTGGGCTGAG
VpreB	ACTGAAGACAGCAGAGGCAC	GCTCCACCCTCTTCCATGAC

Table 2.2 – List of primer s used in this thesis
* Primers from Zambidis *et al* 2005
† Primers from Gandemer *et al* 2007
§ specific for mouse gene, all others specific for human genes
‡ Primers for TEL amplify across exon 7-8 of TEL, TEL/AML1 is across the breakpoint of TEL/AML1.

Chapter 3: The effects of TEL/AML1 on haematopoietic development of human embryonic stem cells

3.1 Introduction

There are critical aspects of TEL/AML1$^+$ acute lymphoblastic leukaemia which are yet to be elucidated. For example, it is commonly assumed that the original translocation event occurs in the haematopoietic stem cell (HSCs), though that has not been proven (le Viseur *et al* 2008). In addition, the frequency of the translocation generating TEL/AML1 in the normal, non-leukaemic, population is subject to debate (Mori *et al* 2002; Brown 2011; Lausten-Thomsen *et al* 2011). Also, the interaction between different leukaemic sub-clones in a single patient, and their 'evolution' of over the course of the disease (Anderson *et al* 2011; Notta *et al* 2011b), complicates the interpretation of any study using whole bone marrow, and therapeutic approaches.

As more is revealed about the nature of TEL/AML1$^+$ leukaemia, researchers will continue to use model systems as tools to address questions about fundamental aspects of the disease. Such model systems can be divided into three categories: *in vivo* models using transgenic animals, xenograft models using *ex vivo* patient-derived material, and studies using cell lines *in vitro*.

3.1.1 *In vivo models*

Firstly, *in vivo* mouse models, either studying transgenic animals (Schindler *et al* 2009) or recipients of TEL/AML1-transduced bone marrow (Tsuzuki *et al* 2004), have been used to investigate the development and clonal evolution of TEL/AML1$^+$ HSCs. A common feature is that TEL/AML1 on its own is not sufficient to cause leukaemia in most of the mouse models, consistent with predictions made from the analysis of leukaemia in monozygotic twins (Wiemels *et al* 1999b). Neither endogenous expression (Andreasson *et al* 2001; Ford *et al* 2009) nor exogenous expression of TEL/AML1 (Morrow *et al* 2004; Tsuzuki *et al* 2004; Fischer *et al* 2005; Lyons *et al* 2010) caused overt leukaemia.

Expression of TEL/AML1 *in vivo* increased quiescence in HSCs (Schindler *et al* 2009), and caused abnormalities in B cell development, either causing increased self-renewal of B cell precursors (Morrow *et al* 2004; Morrow *et al* 2007; Lyons *et al* 2010) or impairment of transition from pro-B to pre-B cells (Tsuzuki *et al* 2004; Fischer *et al* 2005). The result is the accumulation of persistent early B cell progenitors, which is believed to represent a 'pre-leukaemic state' that predisposes individuals for further transformation and ultimately overt leukaemia.

Other groups have focussed on the cooperation between TEL/AML1 and common secondary mutations. Bernardin and colleagues (2002) transduced bone marrow with TEL/AML1 and transplanted into syngeneic mice, and only 2 of 9 mice developed leukaemia (one T-cell ALL, one B-cell ALL). Bone marrow from mice lacking p16-INK4a and p19-ARF genes (a common mutation in childhood ALL; Takeuchi *et al* 1995) transduced with TEL/AML1 gave rise to leukaemia in 6 of 8 recipient mice, but none could be diagnosed as B-lineage. TEL/AML1^{+} transgenic mice described by Schindler and co-workers (2009) have a predisposition for haematological malignancies when exposed to chemical mutagens (though mostly T cell lymphomas and leukaemias). Finally, a mouse model developed by van der Weyden and colleagues (2011) expressed TEL/AML1 under the control of the endogenous *Etv6* locus. Using this in combination with a 'Sleeping Beauty' transposase, they performed an insertional mutagenesis screen to identify genes that when mutated contribute towards leukaemia. 20% of the mice developed B lineage leukaemia, and the screen gave an insight into cooperating mutations, both known and novel.

3.1.2 Xenograft models

Secondly, models using xenografts of patient-derived cells or cord blood haematopoietic progenitors transduced with TEL/AML1 have been used to imitate human disease features. There is an increased morbidity in SCID mice receiving material from patients who suffered relapse shortly after discontinuing treatment than those whose leukaemia relapsed later or did not relapse at all (Kamel-Reid *et al* 1991), a feature that could even be used to predict which patients would relapse (Uckun *et al* 1995). Leukaemic cell invasion in NOD/SCID mice was commonly

observed in the spleen, but only rarely into other organs, mimicking the human disease (Nijmeijer *et al* 2001).

Subsequently, and following work from John Dick and contemporaries working in Acute Myeloid Leukaemia (Lapidot *et al* 1994; Bonnet & Dick 1997) the most common use of xenografts has been focussed on the identification of the cell which gives rise to the leukaemia, referred to as the 'leukaemic stem cell', or LSC. Cobaleda and colleagues identified from patients with the 'Philadelphia Chromosome' subtype of ALL ('Ph^+ ALL') a population of $CD34^+CD38^-$ cells which propagated leukaemia in SCID mice, also shown to be the engrafting population from AML (Bonnet & Dick 1997) as well as normal bone marrow (Cobaleda *et al* 2000). In TEL/$AML1^+$ ALL it was found that $CD34^+CD38^-CD19^+$ cells, a B lineage population not seen in normal bone marrow, were able to self-renew *in vitro* and *in vivo* giving rise to TEL/$AML1^+$ cells, whereas their $CD19^-$ counterparts gave rise to TEL/$AML1^-$ cells of various lineages (Castor *et al* 2005). This population could be observed in the peripheral blood of the healthy twin of a TEL/$AML1^+$ leukaemia patient (but not in that of an unrelated normal age-matched control), and that, by VDJ rearrangement sequencing, they are likely to derive from the same monoclonal population in both twins, indicating a pre-natal transfer from one twin to the other (Hong *et al* 2008). In addition, the transduction of cord-blood with TEL/AML1 generated the $CD34^+CD38^-CD19^+$ population, which could transfer a 'pre-leukaemic' state in serially transplanted mice. The concept of LSCs has clinical relevance; it was found that bone marrow from 'poor outcome' ALL patients lead to high engraftment in NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice, indicating high numbers of LSCs (Morisot *et al* 2010).

However, there has been some ambiguity about the identity of the LSC. Kong and colleagues (2008) showed that both $CD34^+CD38^-CD19^+$ and $CD34^+CD38^+CD19^+$ cells could initiate ALL in newborn NSG mice, whereas le Viseur and co-workers (2008) showed that cells comprising of either $CD34^+CD19^-$, $CD34^+CD19^+$, or $CD34^-CD19^+$ were able to re-establish all leukaemic cell phenotypes when transplanted into NOD/SCID mice. Various technical explanations have been put forward for these discrepancies, including sub-100% purity of flow-sorted populations, the genetic backgrounds and ages of mice used for xenografts (Shultz *et*

al 2007), and low levels of clearance of antibody-stained cells (Taussig *et al* 2008). However, it has been suggested that the immunophenotypically defined populations are more similar functionally than previously thought and that they fluctuate between different states (le Viseur *et al* 2008).

3.1.3 Models using cell lines

Thirdly, patient-derived cell lines and *in vitro* studies have been used to investigate molecular and cellular aspects of leukaemia. One of the caveats of this approach is that cell lines may acquire genetic changes to adapt to growth factor-independent proliferation *ex vivo*. Nevertheless, gene expression analysis of leukaemia cell lines and primary leukaemia samples has shown that cell lines harbouring similar genetic lesions cluster together, as well as with patients who also carry that lesion (Andersson *et al* 2005a), indicating that the transcriptional profile in primary leukaemia samples remains intact in cell lines despite extended *ex* vivo culture, and so are a valuable tool for studying leukaemia.

Using leukaemia patient-derived cell lines, common chromosomal changes (Uphoff *et al* 1997), other genomic changes (Tsuzuki *et al* 2007), and how TEL/AML1 is processed in the cell (Chakrabarti *et al* 2000) have been identified and confirmed in primary patient samples. In particular, the molecular biology of TEL/AML1 and its transcriptional effects have been studied using cell lines. It was discovered that the fusion of TEL to AML1 and its isoforms represses AML1 targets (Hiebert *et al* 1996), that TEL/AML1 can only have its suppressive effects on AML1 targets when dimerised (Fears *et al* 1997), and that it also has an effect on wildtype TEL (Gunji *et al* 2004).The role of the domains of TEL and AML1 in the DNA binding of the fused TEL/AML1 has also been studied using TEL/AML1 mutants transduced into cell lines (Morrow *et al* 2007).

The use of cell lines has also helped identify pathways that contribute to the survival and pathogenesis of leukaemic cells. For example, the role of TCL-1 has been identified and investigated using cell lines (Andreasson *et al* 2001; Fears *et al* 2002), as well as the circumvention of the mitotic checkpoint (Krapf *et al* 2010) and the anti-apoptosis networks that leukaemia cells exploit (Accordi *et al* 2007; Diakos *et al*

2007; Fuka *et al* 2011a). The protection from chemotherapeutic drugs that bone marrow stroma confers has been elucidated in leukaemia cell lines (Dosen-Dahl *et al* 2008). Cell lines have also been used to assess the effects of common mutations, for example, by the overexpression of TEL (Tsuzuki *et al* 2007) or the RNAi-mediated silencing of TEL/AML1 itself (Fuka *et al* 2011b; Zaliova *et al* 2011). The role of microRNA (miRNAs) in leukaemia, a relatively new direction in cancer research, has also been investigated using cell lines (Gefen *et al* 2009; Diakos *et al* 2010).

3.1.4 The prospect for a hESC-based model

Whilst important discoveries have been made with these models, each of these approaches has its disadvantages. Haematopoietic development in animal models does share many characteristics with that in humans, but there are subtle differences that limit the application of results from transgenic animal models to human disease (for a review of mouse models of leukaemia, see Bernardi *et al* 2002). For similar reasons, the xenograft of human haematopoietic precursors into mice is dependent on the mouse strain being used, some of which may skew haematopoietic development toward certain lineages (Shultz *et al* 2007; McDermott *et al* 2010). However, primary human material, whether in the form of bone marrow samples or cord blood samples, is difficult to obtain, and the inherent genetic variability between donors may conceal subtle but significant differences. Cell lines avoid this problem, but there are still concerns about the adaptations made by these cells to survive in growth factor free conditions (Matsuo & Drexler 1998), either as a result of their malignant transformation, or by exogenous factors such as Epstein-Barr virus (Gerber *et al* 1969). In addition, cell lines at best only provide a ‘snapshot’ of a particular human cell type and cannot provide information about its developmental origins.

The use of human embryonic stem cells (hESCs) as a source of haematopoietic precursors for a model of TEL/AML1^{+} leukaemia would complement the above models, but could also address some of their shortfalls. Working with a human system enables a more direct comparison to the disease than might be possible with mouse models, allowing, for example, gene expression changes to be compared with array data from patients. The use of hESCs – pluripotent cell lines that can be efficiently and genetically manipulated, expanded, and differentiated *in vitro* –

provides a potentially limitless source of syngeneic human haematopoietic precursors, capable of differentiating to mature blood cell types, and avoids the difficulties of culturing human HSCs *ex vivo*.

3.1.5 Use of ES cells in models of leukaemia and other diseases

There have been examples of the use of mouse ESCs (mESCs) to study leukaemias. The effects of BCR-ABL fusion gene (associated with Chronic Myeloid Leukaemia, or CML) upon the differentiation capacity of mESCs has been investigated, and has been shown to inhibit haematopoietic differentiation via activation of STAT3 (Coppo *et al* 2003), increasing the numbers of multipotent and myeloid-committed haematopoietic progenitors whilst decreasing erythroid progenitors (Era & Witte 2000), consistent with observations in patients in the chronic phase of CML. Haematopoietic progenitors derived from BCR-ABL-transduced mESCs cause leukaemia upon injection into mice which recapitulated specific aspects of CML, such as IL-3 production (Peters *et al* 2001).

Recently a hESC model of MLL/AF4^{+} leukaemia has been described (Bueno *et al* 2012). In infant pro-B ALL, MLL/AF4 (generated by the t(4;11) translocation) is associated with a short latency and a poor prognosis. hESCs expressing MLL/AF4 (transduced with lentiviral vector) show an increased ability to generate haemato-endothelial precursors, but that differentiation from these cells is skewed towards endothelium and away from haematopoiesis. The significance of these results is not known, although bone marrow mesenchymal stem cells carrying MLL/AF4 have been reported in infant MLL/AF4^{+} B-ALL, suggesting a pre-haematopoietic cell of origin for this leukaemia (Menendez *et al* 2009).

In addition, there have been several examples of the use of hESCs for modelling other diseases. Pre-implantation genetic diagnosis of blastocysts has allowed the identification of embryos carrying mutations for congenital diseases, which has permitted the derivation of hESCs (Mateizel *et al* 2006) and their use to model diseases such as Fragile-X syndrome (Eiges *et al* 2007), Turner's syndrome (Urbach & Benvenisty 2009), and Huntington's disease (Niclis *et al* 2009). Homologous recombination in hESCs has also been used to model disorders such as Lesch-Nyhan

disease (Urbach *et al* 2004), Paroxysmal nocturnal haemoglobinuria (Zou *et al* 2009), and Ataxia-telangiectasia (Song *et al* 2010).

These studies into the use of mESCs and hESCs to study leukaemia and other diseases demonstrate the potential of a hESC-based model which can be utilised at multiple levels of leukaemia research.

3.1.6 Embryonic stem cells

Embryonic stem cells (ESCs) were first described in 1981 by two groups attempting to culture the pluripotent cells in mouse pre-implantation blastocysts (Evans & Kaufman 1981; Martin 1981). These cells are able to form teratocarcinomas containing all three germ layers when injected into mice, a property previously described in cells from germ cell tumours, termed embryonic carcinoma cells, or ECCs (Kleinsmith & Pierce 1964). mESCs are also able to contribute to the embryo when injected into mouse blastocysts.

The derivation of embryonic stem cells from human blastocysts (hESCs) was demonstrated in 1998 by Thomson and colleagues, and shared the same characteristics as mESCs, namely derived from the pre-implantation blastocysts, indefinite pluripotent culture in vitro, and potential to form cells from all three germ layers (Thomson *et al* 1998). As of December 2011 there were over 1,000 described hESC lines, but three of the cell lines described in Thomson's paper, H1, H7, and H9, are still the most widely used today (Levine 2011).

3.1.7 Definition of hESCs by pluripotency markers

Embryonic stem cells are defined by two main characteristics – their capability for indefinite self-renewal, and their pluripotency, usually demonstrated by their ability to form teratomas containing tissues of all three germ layers when implanted into immunodeficient mice (Thomson *et al* 1998). The expression of certain genes and cell surface molecules has also been used as markers to define hESCs.

The transcription factor OCT4, coded for by the gene *POU5F1*, is a critical regulator of pluripotency. OCT4 protein is present in the mammalian oocyte, and is expressed

at low levels from the 4-cell stage of the fertilised zygote, before being upregulated from the 8-cell stage (Pesce *et al* 1998). In the blastocyst, whilst the expression of OCT4 remains high in the inner cell mass, its expression is lost upon differentiation to form the trophoblast, which go to form the extra-embryonic tissues (Nichols *et al* 1998). Consistent with this, *Oct4*$^{-/-}$ embryos develop up to blastocysts stage, but fail to form an embryo as the inner cell mass loses its pluripotency and differentiates towards the trophoblast lineages (Nichols *et al* 1998).

NANOG is another transcription factor required for the maintenance of pluripotency in hESCs. The loss of Nanog in mESCs caused a loss of pluripotency and differentiation to trophoblast lineages (Mitsui *et al* 2003), similar to the effects of the loss of OCT4. It has since been discovered that pluripotency is maintained by the cooperation of OCT4, NANOG, and another transcription factor SOX2, by inhibiting the expression of transcription factors involved in lineage specification, and stimulating the expression of other transcription factors involved in pluripotency (Boyer *et al* 2005). In addition, these transcription factors co-operate to stimulate their own expression, and in so doing form a self-regulatory 'feed-forward' loop (Boyer *et al* 2005).

The cell surface antigens Stage-Specific Embryonic Antigen-4 (SSEA-4) and SSEA-1 are glycolipids used to distinguish between pluripotent and differentiating cells. Production of SSEA-4 is high in undifferentiated pluripotent cells, such as the embryonic carcinoma cell lines 2102Ep, but as pluripotent cells differentiate, production of SSEA-4 ceases and SSEA-1 production increases (Kannagi *et al* 1983). TRA-1-60 is another antigen expressed on the cell surface of pluripotent cells (Draper *et al* 2002), and has been identified as a glycosaminoglycans (Badcock *et al* 1999), which might be attached to the proteoglycan podocalyxin (Schopperle & DeWolf 2007). The functions of these cell surface antigens are not known.

3.1.8 Haematopoietic differentiation of hESCs

Haematopoietic differentiation from hESCs was first described by Kaufman and colleagues (Kaufman *et al* 2001), using a co-culture methods with murine bone marrow stromal cells. Haematopoiesis from hESCs goes through waves of primitive

and definitive stages (Zambidis *et al* 2005), mimicking the development of blood lineages during embryonic development. To date, hESC have been differentiated to functional cells comprising of almost the full range of adult haematopoietic cell types, including megakaryocytes and platelets (Lu *et al* 2011), enucleated red blood cells (Lu *et al* 2008), mast cells (Kovarova *et al* 2010), neutrophils (Yokoyama *et al* 2009), eosinophils (Choi *et al* 2009), monocytes and macrophages (Klimchenko *et al* 2011), dendritic cells (Slukvin *et al* 2006), T cells (Timmermans *et al* 2009), and NK cells (Woll *et al* 2005, Vodyanik *et al* 2006). Differentiation towards B cells has been demonstrated up to pre-B cell stage with multiple genomic D-J_H rearrangements (Vodyanik *et al* 2006; Carpenter *et al* 2011), but as of yet no V_H-D_H rearrangements, and no secretion of antibodies typical of mature plasma cells. Basophilic granulocytes have been produced (e.g. Ma *et al* 2007), but no functional assays have been performed on such cells.

There are two main methods of haematopoietic differentiation of ES cells: embryoid bodies and stromal co-cultures. Differentiation of ES cells by embryoid bodies, small spheroid aggregates of ES cells, follows closely early embryonic development (Keller 1995; Leahy *et al* 1999). The resultant spontaneous lineage specification in human embryoid body cultures can be directed towards haematopoietic differentiation using a cocktail of haematopoietic cytokines, for example Stem Cell Factor (SCF), Flt-3 Ligand (Flt-3L), Interleukins 3 and 6 (IL-3 and IL-6), Granulocyte Colony-Stimulating Factor (G-CSF), and Bone Morphogenic Protein-4 (BMP-4; Chadwick *et al* 2003).

Haematopoietic differentiation by co-culture is dependent upon the contact between ES cells and stromal cell lines derived from sites of haematopoiesis (Wilson & Trumpp 2006). The OP9 cell line, derived from the bone marrow of *op/op* mice deficient in Macrophage Colony-Stimulating Factor (M-CSF) was first used to differentiate mESCs (Nakano 1995), and later for hESCs (Vodyanik *et al* 2005). S17 cells, generated from a long-term bone marrow cultured to enrich for myeloid cells (Collins & Dorshkind 1987), are another popular cell line used to differentiated hESCs (Kaufman *et al* 2001; Qiu *et al* 2005). Cell lines derived from the first site of definitive haematopoiesis in the mouse embryo, the aorta-gonad-mesonephros (AGM) region, have also been used to induce haematopoietic differentiation in

hESCs (Ledran *et al* 2008). One advantage of the co-culture systems is that they do not require supplementary cytokines.

3.1.9 Methods of transgene expression in hESCs

The recent derivation of iPSCs from cord blood (Giorgetti *et al* 2009; Haase *et al* 2009; Ye *et al* 2009) and peripheral blood (Chou *et al* 2011) will undoubtedly lead to models of haematological malignancies using iPSCs derived from leukaemia patients in the future (see section 3.1.10). In the meantime, hESCs could fulfil this role by the exogenous expression of TEL/AML1 (Lensch & Daley 2006; Bueno *et al* 2007). The methods by which this could be achieved fall in to three broad categories: constitutive exogenous promoters, inducible promoter systems, and the use of endogenous promoters.

Constitutive and stable expression of GFP has been achieved using the *phosphoglycerate kinase 1* (PGK) promoter, cytomegalovirus (CMV) promoter, human *elongation factor 1 alpha* (hEF1α) promoter, and chicken *β-actin* promoter (Eiges *et al* 2001; Kim *et al* 2005). A chimeric promoter pCAG, containing elements of human CMV, chicken *β-actin*, rabbit *β-globin*, and the virus polyoma mutant enhancer sequence PyF101, was found to sustain high levels of expression in hESCs over extended culture periods (Liew *et al* 2007). Other promoters have been quantitatively compared using lentiviral vectors and show varying levels of expression stability both in undifferentiated culture and upon differentiation (Norrman *et al* 2010).

There is concern that the overexpression of any transgene can aberrantly affect cells (including hESCs), presumably by the overloading of protein metabolism pathways, in a phenomenon called ‘transgene toxicity’ (Vallier *et al* 2007; Zafarana *et al* 2009). One solution to this problem is to express the gene under an inducible system. One of the first instances of inducible expression in hESCs used a variant of a Tetracycline-inducible system (Gossen *et al* 1995) to express GFP and phosphatidylinositol glycan A (PIG-A; Zhou *et al* 2007). Later work showed that inducible GFP expression could be maintained even upon directed differentiation *in vitro* and in teratomas formed *in vivo* (Xia *et al* 2008).

In this chapter, the inducible system I first used to express TEL/AML1 in hESCs is called Tet-ON, and is based upon the tetracycline system (Gossen *et al* 1995), further developed by Clontech. The expression of the gene-of-interest is controlled by the binding of a constitutively-expressed recombinant tetracycline-dependent transactivator (rtTA) to the tetracycline response element (TRE), which lies upstream of a minimum CMV promoter. This binding only occurs in the presence of doxycycline (a tetracycline derivative), so the expression of the gene-of-interest can be "switched on" by administering doxycycline. The advantages of using an inducible expression system, in particular a chemically inducible system like the Tet-ON system, are three-fold: (1) the timing of expression can be controlled; (2) the level of expression can be controlled by varying the concentration of doxycycline; and (3) expression is controlled by a non-cytotoxic drug which would not have an effect on non-transgenic cell lines, such as those used for co-culture differentiation methods.

The use of endogenous promoters to appropriate the cell's own gene expression machinery has been demonstrated in hESCs. This work has mostly been described in the context of the creation of 'reporter genes', where genes for fluorescent proteins are expressed concurrently with a gene of interest, either indirectly under the control of a promoter for the gene, or directly, by the disruption of one allele of this gene with the fluorescent protein gene. For example, the expression of a GFP driven by the *Rex1* promoter in a extra-genomic plasmid vector (Eiges *et al* 2001) and the disruption of the *POU5F1* (*Oct4*) locus by homologous recombination (Zwaka & Thomson 2003; Hockemeyer *et al* 2009) have been described to act as 'pluripotency reporter genes', whereas the homologous recombination of a fluorescent protein gene into the loci of *Mixl1* (Davis *et al* 2008), *Pitx3* (Hockemeyer *et al* 2009), *Fezf2* (Ruby & Zheng 2009), and *Olig2* (Ruby & Zheng 2009), all markers of differentiation towards specific lineages, have also been demonstrated. In addition, homologous recombination of transgenes has been achieved into loci such as the human equivalent of the mouse *ROSA26* gene (Irion *et al* 2007) and *AAVS1* (Hockemeyer *et al* 2009), both of which contain genes ubiquitously and strongly expressed in most cell types, and have been previously useful in the generation of transgenic mice. Finally, homologous recombination has been used to disrupt genes by insertional mutagenesis to demonstrate the use of hESCs to model diseases; for

example, the X-chromosome *HPRT* gene to model Lesch-Nyhan disease (Zwaka & Thomson 2003; Urbach *et al* 2004), X-chromosome *PIG-A* gene to model paroxysmal nocturnal hemoglobinuria (Zou *et al* 2009), and bialleic disruptions of *p53* and *ATM*, recapitulating various cancers and Ataxia-telangiectasia respectively (Song *et al* 2010).

3.1.10 Modelling human diseases with induced pluripotent stem cells

Differentiated somatic cells can be reprogrammed by transduction of transcription factors active in embryonic stem cells (ESCs) to form cells resembling ESCs, called 'induced pluripotent stem cells', or iPSCs. This was demonstrated first in fibroblasts taken from mice, where it was shown that the minimal set of genes required for reprogramming, called 'reprogramming factors', comprised of *Klf4*, *Sox2*, *c-Myc*, and *Oct4* (Takahashi & Yamanaka 2006). These iPSCs would form teratomas when injected subcutaneously into mice, and when injected into blastocysts would contribute to embryonic development, two key tests of pluripotency. Soon after it was shown that it was possible to generate iPSCs from human fibroblasts using the same four factors (Takahashi *et al* 2007).

The impact of the technology to generate iPSCs goes beyond the study of pluripotency or an alternative source of hESCs-like cells. The potential to use human iPSCs for disease modelling and even drug screening was instantly recognised (Zaehres & Scholer 2007). In theory, one could take fibroblasts from a diseased patient to generate iPSCs, which could then be used to provide an unlimited source of differentiated cells of any lineage to study their disease. This may be particularly useful for diseases which do not have a suitable animal model or appropriate cell line to study the disease (Saha & Jaenisch 2009). In addition, with respect to disease modelling, iPSCs have an advantage over the alternative of genetically-modified hESCs in that any disease-causing mutations do not necessarily have to be fully identified before they can be modelled by iPSCs; all that is required is a patient which to take cells from to be reprogrammed. However, there are challenges with modelling human diseases with iPSCs, which fall roughly into three groups; firstly, the reprogramming of human cells; secondly, the culture and differentiation of

iPSCs; and thirdly, how amenably a specific disease is to being modelled in this way.

The main concern with the method originally used to deliver the 'reprogramming factors' into differentiated cell types, retroviral or lentiviral transduction, is that the vectors will insert randomly into the genome and the genes will remain highly expressed after reprogramming, which could affect the differentiation towards the desired cell types, and/or any disease phenotypes from arising (Saha & Jaenisch 2009; Stadtfeld & Hochedlinger 2010; Grskovic *et al* 2011). To combat this, a number of methods to generate 'reprogramming factor-free' iPSCs have been developed. Integrating vectors can be removed by using either transposon vectors such as piggyBac (Kaji *et al* 2009) or Cre-recombinase mediated excision (Soldner *et al* 2009), but in the former the excision is inefficient and laborious, and the latter still leaves behind viral promoters (Saha & Jaenisch 2009). Non-integrating vectors, such as episomal vectors (Yu *et al* 2009), adenoviral vectors (Stadtfeld *et al* 2008), or transient plasmid transfection (Okita *et al* 2008) have been used, though the efficiency of transduction using these methods, and the possibility of fragments of vector integrating, have been a concern. 'DNA-free' methods, such as the use of the reprogramming factor proteins (Kim *et al* 2009), synthetic mRNA (Warren *et al* 2010), or miRNAs (Miyoshi *et al* 2011) have also been demonstrated, but reprogramming with these methods remains low efficiency.

The theoretical possibility of deriving any cell types by the differentiation of human ESCs or iPSCs *in vitro* is, in practise, much more difficult. Although various cell lineages have been derived from these human pluripotent stem cells, assessed by morphology and expression genetic and phenotypic markers, there have been only a few examples of functional studies in differentiated cell types from hiPSCs; for example with neural crest cell types (Lee *et al* 2009) and haematopoietic progenitors (Raya *et al* 2009). There are additional problems if one wanted to use an iPSC- or hESC-based disease model for drug screening, in that the large scale culture of iPSCs in bioreactors is still a significantly more complicated process than normal cell lines [ref]. In addition, the differentiation protocols required to differentiate hESCs and iPSCs can take weeks, going through intermediate stages and producing

a variety of different lineage which may not be the desired end product (Saha & Jaenisch 2009; Grskovic *et al* 2011).

Ultimately, although the modelling of any human disease using iPSCs is an attractive prospect, in reality some diseases will be less amenable to modelling in this way than others. Diseases most likely to be modelled successfully will be cell-autonomous (so that the phenotype of the disease will appear in the cells with little influence from other cell types or the environment) and should manifest the phenotype rapidly with high penetrance, so that the phenotype becomes apparent in a reasonable time-frame for cells in culture (Grskovic *et al* 2011). Unfortunately, many diseases do not have these traits; they are diseases that impact on multiple interacting cell types as well as environmental factors (such as diabetes), or diseases which have a long latency where symptoms manifest themselves over many years (such as Alzheimer's disease). It may be possible to replace some environmental influences and interacting cell types, and accelerate disease progression with factors such as oxidative stress, but the relevance of the model may then be called into question (Saha & Jaenisch 2009).

Researchers have already begun modelling a wide variety of diseases using iPSCs; a list of these models can be found in Grskovic *et al* 2011. As a couple of illustrative examples, I will give an overview of the iPSC models of Hutchinson-Gilford progeria syndrome and familial Parkinson's disease.

Hutchinson-Gilford progeria syndrome (HGPS) is a disease caused by a mutation in the Lamin A (*LMNA*) gene, which is characterised by early onset of ageing, with patients often dying in their teens from heart attack or stroke. Researchers studying the disease derived iPSCs from HGPS patients (Zhang *et al* 2011) and differentiated the cells towards the variety of mesenchymal cell lineages that are affected in the disease, including vascular smooth muscle cells (VSMCs). The cells affected all showed signs of DNA damage and nuclear abnormalities. The VSMCs from these patients responded poorly to both hypoxic stress and electrical stimulation attempting to mimic the pulse of blood circulation, compared to controls. This work was confirmed by other researchers studying iPSCs derived from HGPS patients

(Liu *et al* 2011); smooth muscle cells showed nuclear abnormalities and an accelerated senescence, an indicator of vascular ageing.

Models of familial Parkinson's disease using iPSCs have also been described, and represent a rarer group of iPSC-based models of diseases which commonly manifest themselves in adults. One example used iPSCs from a patient with a mutation in the gene encoding leucine-rich repeat kinase-2 (*LRRK2*), the most common mutation associated with Parkinson's disease (Nguyen *et al* 2011). Dopaminergic neurones derived from these iPSCs showed increased susceptibility to oxidative stress and inducers of apoptosis, which is consistent with current understanding of the early development of Parkinson's disease. Since familial Parkinson's disease has much in common with the sporadic disease, it is hoped that this iPSC model and others like it could be used to study the underlying biology of both.

So what are the prospects for acute lymphoblastic leukaemia models using iPSCs? ALL meets some of the preferred criteria for modelling with iPSCs; it is a relatively early onset disease (in children) that manifests itself rapidly (rapid progression leading to death if left untreated) with a clear phenotype in a particular cell type (in the form of lymphoblasts). Researchers have a choice of starting material to generate iPSCs for haematological disease modelling, since efficient reprogramming has been demonstrated from differentiated blood cells (e.g. Loh *et al* 2010) stem cells from mobilised peripheral blood (Loh *et al* 2009), cord blood (Haase *et al* 2009), bone marrow from both healthy donors and chronic myeloid leukaemia patients (Hu *et al* 2011), and even lymphoid cell lines (Choi *et al* 2011). In addition, methods for the differentiation of both hESCs (Vodyanik *et al* 2006) and iPSCs (Carpenter *et al* 2011) toward B-lymphoid lineages (as well as T cells e.g. Timmermans *et al* 2009) have already been established.

However, one potential obstacle to the modelling of ALL using a iPSC model are that leukaemia as a haematological malignancy is not solely cell autonomous, as there is evidence that leukaemia is as dependent on the interaction of the bone microenvironment as normal haematopoiesis (e.g. Colmone *et al* 2008; Doan & Chute 2012). In addition, it may be difficult to model the effects of infection in the development of leukaemia as suggested by Greaves and contemporaries (described

in the Introduction in section 1.8.6). Nevertheless, the opportunity to study leukaemias using iPSCs derived from leukaemic blasts is attractive, and one that I believe will be exploited in the near future. Such iPSCs could be used, for example, to study the effects of mutations present in the blasts on their survival and proliferation by the 'correction' of mutations using non-mutated transgenes.

3.1.11 Aims

The work presented in this chapter describes the development of a model of TEL/AML1$^+$ leukaemia, where transgenic hESCs expressing TEL/AML1 were differentiated to generate haematopoietic progenitors. As controls for the effects of TEL/AML1, I used hESCs expressing either GFP or a non-DNA binding mutant TEL/AML1, which has a 39 amino acid deletion in the runt homology domain (RHD) of the AML1 moiety of TEL/AML1 (the TEL/AML1-ΔRHD mutant ; Morrow *et al* 2007). These transgenic hESCs were then differentiated towards haematopoietic lineage and assessed for the expression of haematopoietic transcription factors. The haematopoietic precursors produced during differentiation were further directed B lymphocyte lineage to reproduce a leukaemic phenotype *in vitro*. I assessed this by the expression of markers of early B cell differentiation, and also tested for a selection of genes associated with TEL/AML1$^+$ leukaemia.

3.2 Results

3.2.1 Inducible expression system

To express TEL/AML1 in hESCs, I initially intended to make use of the tetracycline-inducible expression system 'Tet-ON' (Gossen *et al* 1995; developed by Clontech). A summary of the inducible expression mechanism was described in section 3.1.9, and is shown in Figure 3.1A.

Using the TRE-TEL/AML1 and pCMV-rtTA plasmid constructs (provided by Owen Williams, Institute of Child Health, UCL), I first tested the inducible expression system by transfecting the TRE-TEL/AML1 plasmid into a MEF cell line which contained the Tet-ON machinery to express a mutated form of p53 (Jiang *et al* 2001; provided by Jo Milner, University of York); when doxycycline is administered, the activated rtTA would bind not only to the TRE driving the expression of the mutant p53 transcript, but also that driving TEL/AML1 expression. TEL/AML1 transcript could then be detected by RNA extraction and cDNA synthesis, followed by PCR. When doxycycline was administered to the transfected 'Tet-ON MEFs', a small increase in the quantity of the PCR product for mutant p53 transcript was observed, but no change in that for TEL/AML1 (Figure 3.1B). I subsequently discovered that small quantities of the transfected TRE-TEL/AML1 plasmid remained in the RNA extract despite performing an on-column DNA digest (Figure 3.1C). Any carryover of the transfected plasmid DNA would end up acting as a template for the PCR.

To avoid any problems with significant TRE-TEL/AML1 plasmid carrying over into the PCR reaction, it was decided to test the system 'in reverse' instead; that is, to generate a stable cell line containing the TRE-TEL/AML1 (which has a neomycin resistance gene as a selection marker), and transfect the pCMV-rtTA into the cell line before administering doxycycline. However, despite several attempts, no expression of TEL/AML1 could be detected in the transgenic HT1080 cells transfected with the rtTA plasmid and treated with doxycycline (Figure 3.1D). It was decided that a constitutive expression system would be developed instead.

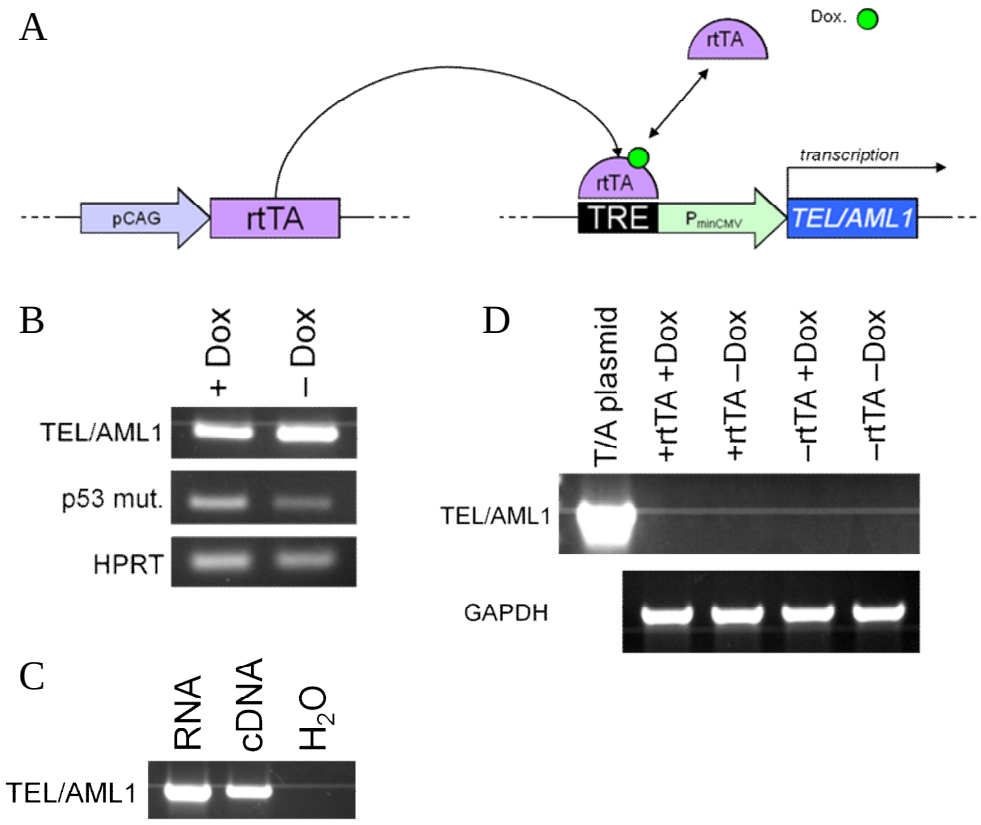


Figure 3.1 – Inducible expression system.
(A) Diagram showing mechanism of the Tet-ON inducible expression system. (B) RT-PCR showing no change in quantity of PCR product for TEL/AML1 in the presence or absence of doxycycline, in the p53 mutant TET cell line (C) RT-PCR showing presence of TRE-TEL/AML1 plasmid DNA in RNA extract, even after DNase treatment. (D) Lack of inducible expression of TEL/AML1 in the presence of both rtTA and Doxycycline in the 'reverse model' (see text for details).

3.2.2 Cloning of pCAG-TEL/AML1 plasmid

Constitutive expression of TEL/AML1 was controlled by the CAG promoter, a CMV based promoter which does not lose expression in hESCs (Liew *et al* 2007). I cloned the TEL/AML1 sequence into the pCAG-eGFP plasmid (provided by Peter Andrews) after removing the eGFP sequence (Figure 3.2A). After several unsuccessful attempts to make the plasmid using standard 'restriction digest-ligation' methods (not shown), the pCAG-TEL/AML1 construct was made by using the InFusion cloning system (Clontech), outlined in Figure 3.2B. Primers were designed to amplify the insert sequence that also contain a 15bp homology to the linearised vector sequence either side of the insertion site (Figure 3.2C), and PCR conditions optimised to give a single clean band by gel electrophoresis (Figure 3.2D). The vector was then linearised using two restriction enzymes (*AgeI* and *NotI*, Promega), excising GFP in the process (Figure 3.2E). Finally, the linearised vector and insert are joined together using the InFusion cloning reagents, giving rise to plasmid clones, from which clones comprising of both the vector and insert were selected (Figure 3.2F).

3.2.3 Cloning of pCAG-TEL/AML1-ΔRHD plasmid

As a control for studying the effects of TEL/AML1 expression on haematopoiesis, I used a non-DNA binding mutant TEL/AML1, which has a 39 amino acid deletion in the runt homology domain (RHD) of the AML1 moiety of TEL/AML1 (Figure 3.3A, reproduced from Morrow *et al* 2007; construct provided by Owen Williams). This TEL/AML1-ΔRHD sequence was inserted into the pCAG vector using a PCR-based cloning method, as shown in Figure 3.3B. To summarise, primers were designed to amplify the TEL/AML1-ΔRHD sequence and add restriction sites for *AgeI* and *NotI* at the 5' and 3' ends, respectively, with an appropriate length 'clamp sequence' (Figure 3.3C). The PCR reaction was optimised to produce a single clean band corresponding to the size of the TEL/AML1-ΔRHD sequence, and this product and the pCAG-eGFP vector were digested using *AgeI* and *NotI* restriction enzymes (Figure 3.3D). Finally the linearised pCAG vector and the digested TEL/AML1-ΔRHD PCR product were joined together by T4 Ligase (Promega), from which clones comprising of both the vector and insert were selected (Figure 3.3E).

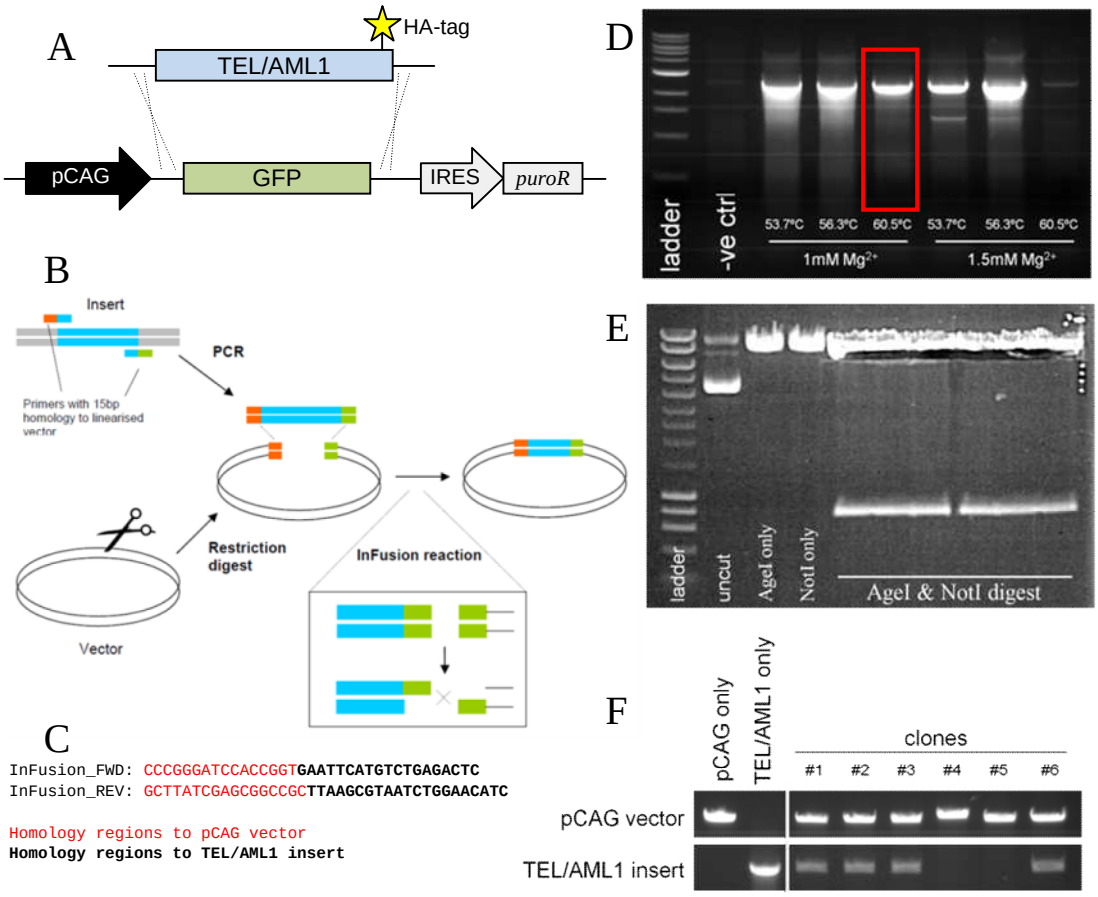


Figure 3.2 – cloning of pCAG-TEL/AML1.
(A) Cloning strategy to construct the pCAG-TEL/AML1 plasmid. (B) Overview of the InFusion reaction. (C) PCR primers used to amplify the TEL/AML1 insert for InFusion cloning, showing homology to pCAG vector and TEL/AML1. (D) Optimisation of TEL/AML1 PCR amplification using the InFusion primers, to give single clean band, indicated by the red box. (E) Digest of pCAG-eGFP, showing single digest with AgeI and NotI enzymes, and double digest; note that the band representing double-digested vector has been cut out of the gel to minimise mutagenesis by exposure to UV during imaging. (F) Gel showing the plasmid clones obtained after InFusion ligation and transformation of bacteria. Clones #4 and #5 do not contain the TEL/AML1 insert, whereas clones #1, #2, #3, and #6 do.

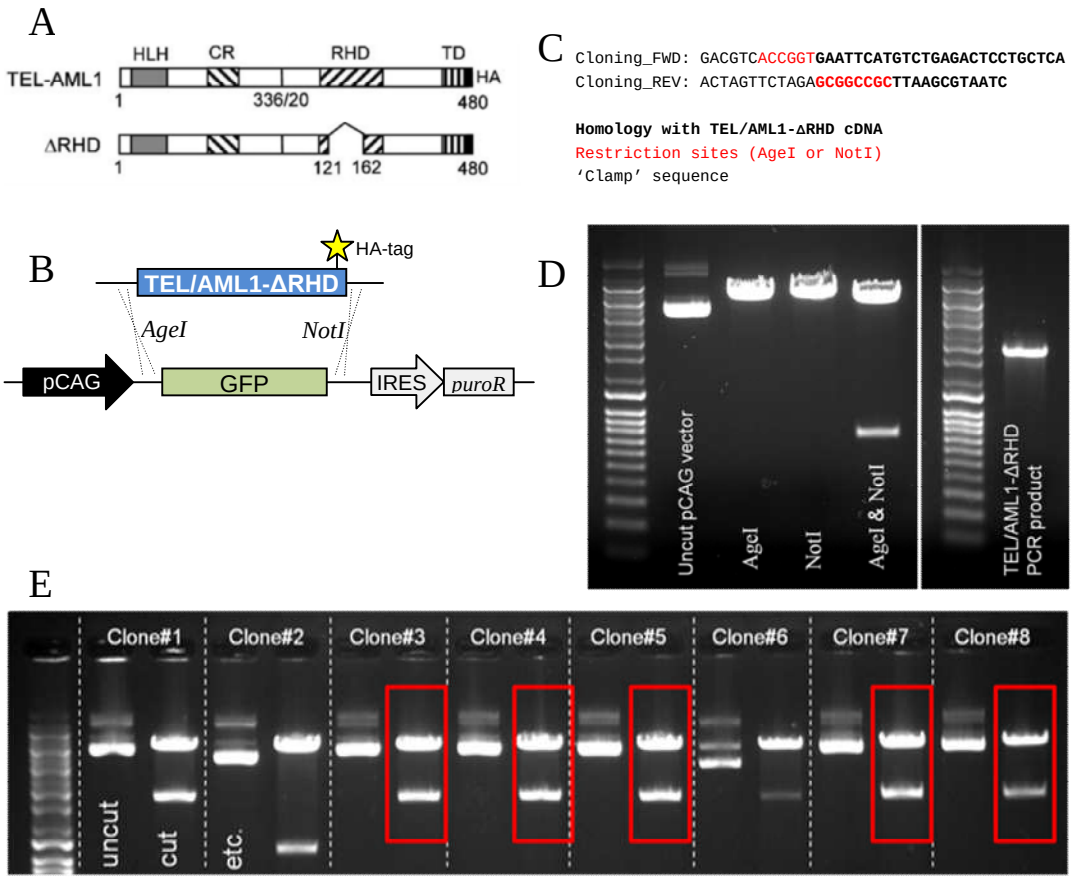


Figure 3.3 – Cloning of pCAG-TEL/AML1-ΔRHD.
(A) Diagrams showing deleted regions in Runt Homology Domain (ΔRHD) mutant; reproduced from Morrow *et al* 2007. (B) Cloning strategy for pCAG-TEL/AML1-ΔRHD (C) PCR primers used to amplify the TEL/AML1-ΔRHD sequence for cloning, with restriction sites and 'clamp' regions indicated. (D) left, digest of pCAG-GFP vector; right, PCR product of TEL/AML1-ΔRHD. (E) Gel showing plasmids (uncut and cut with *AgeI* and *NotI*) from eight bacteria colonies after ligation and transformation, red boxes indicating lanes containing fragments of the correct size.

3.2.4 Characterisation of TEL/AML1 expression in transgenic hESC lines

The human embryonic stem cell (hESC) line H9 was transfected with pCAG-TEL/AML1, pCAG-TEL/AML-ΔRHD, or pCAG-GFP, as described in Chapter 2. Eight monoclonal transgenic cell lines were generated; four expressing TEL/AML1 (referred to hereafter as T/AD1, T/AD2, T/AF1, and T/AF2), two expressing GFP (GFP1 and GFP2), and two expressing TEL/AML1-ΔRHD (ΔRHD5 and ΔRHD6). Expression levels of TEL/AML1 and TEL/AML1-ΔRHD were assessed by qPCR (using primers amplifying across the breakpoint of TEL/AML1, which is unaffected in the ΔRHD mutant; Figure 3.4A), and are shown in Figure 3.4B, comparing with TEL/AML1 expression in REH cells, a TEL/AML1$^+$ leukaemia patient-derived cell line (Rosenfeld *et al* 1977). Expression levels of TEL/AML1 and the ΔRHD mutant were high (from 3-fold to 43-fold that of TEL/AML1 expression in REH cells), with no TEL/AML1 expression in the GFP$^+$ cell lines. TEL/AML1 and TEL/AML1-ΔRHD protein levels were assessed by western blot against the HA-tag found at the 3' end of both the TEL/AML1 and ΔRHD mutant sequences. Protein levels are compared with the H9 hESC line, and were consistent with transcript levels shown by qPCR (Figure 3.4B).

To identify the location of the TEL/AML1 protein in the cell, immunocytochemistry was performed on cultures in glass chamber slides (as described in Materials and Methods), and images were acquired using confocal microscopy. TEL/AML1 was present in TEL/AML1-expressing hESC colonies and not in the 'feeder layer' of iMEFs (Figure 3.5A; T/AD1 cell line), and with higher magnification TEL/AML1 can be seen localising to the nucleus (Figure 3.5B; colocalisation with DAPI, staining double-stranded DNA). TEL/AML1 was not observed in GFP-expressing hESC lines (Figure 3.5C), which were confirmed as positive for GFP (Figure 3.5D).

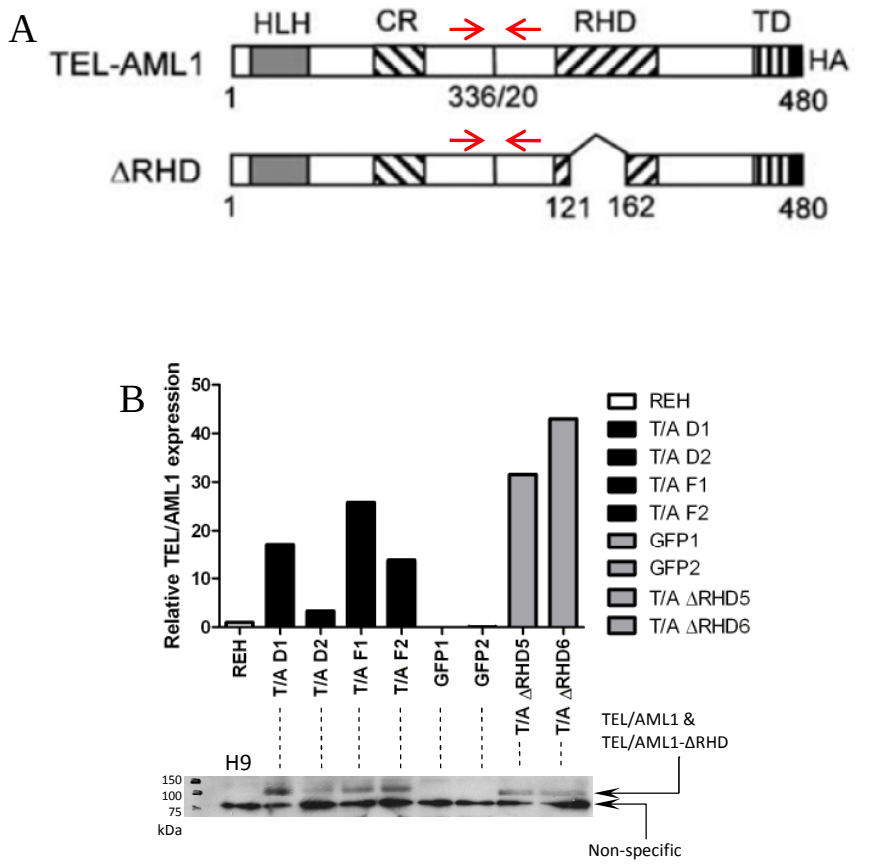


Figure 3.4 – Characterisation of TEL/AML1 expression in transgenic hESC lines.
(A) Diagram indicating features the cDNA sequences of TEL/AML1 and TEL/AML1-ΔRHD, running 5' to 3', left to right. Oligonucleotide primers to test for expression of TEL/AML1 or TEL/AML1-ΔRHD are shown in red, across the breakpoint of the TEL and AML1 moieties of these fusion genes. The HA tag, against which antibodies are used for Western blot and immunocytochemistry (see Figure 3.5) is shown at the 3' end as a solid black bar (B) Expression of TEL/AML1 or TEL/AML1-ΔRHD by qPCR, compared to REH cells, and by Western Blot against HA-tag, compared to H9 hESCs (mean of three technical replicates)**.**

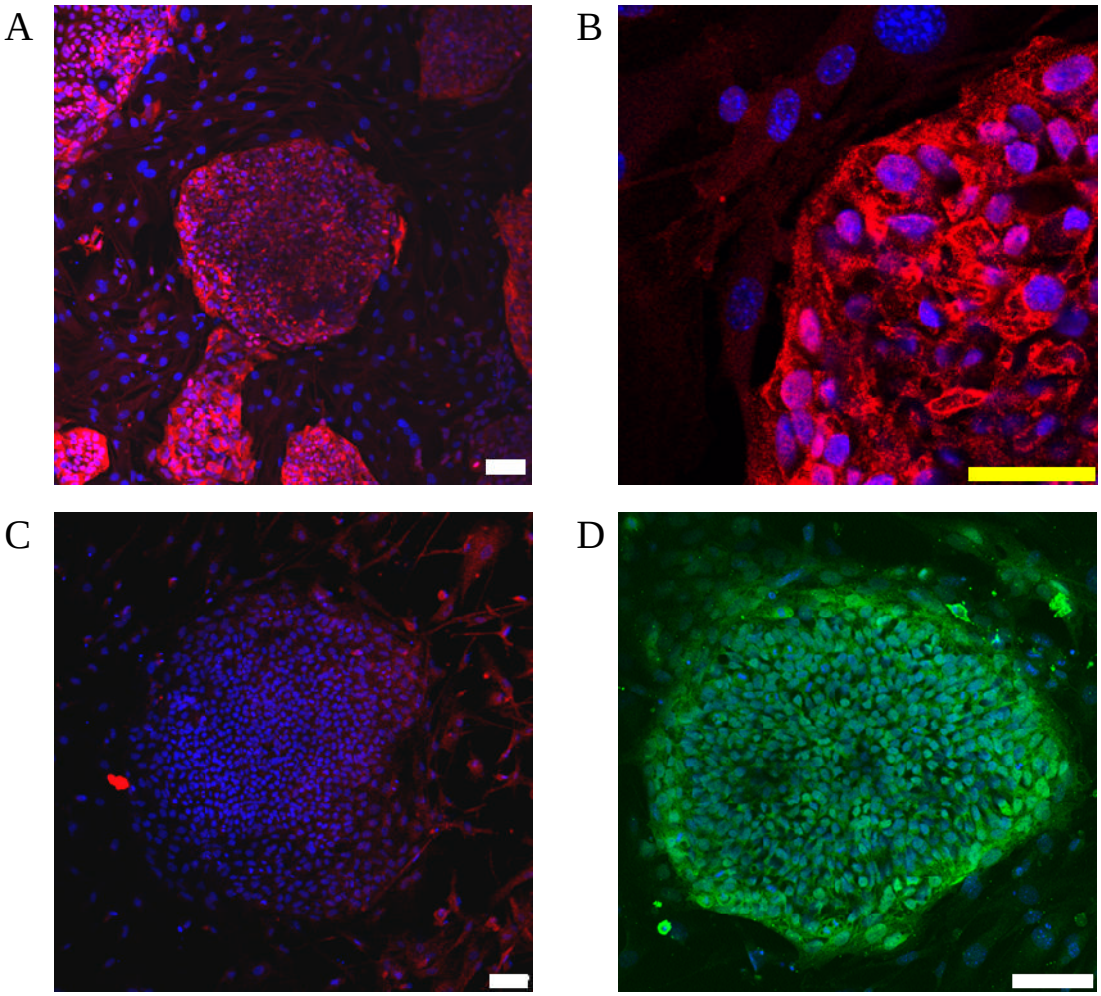


Figure 3.5 – Confocal images of transgenic hESCs.
Red = HA-tag. Blue = DAPI, Green = GFP; white scale bar = 100µm, yellow scale bar = 50µm. (A) T/AD1, showing several colonies, on DR4 iMEFs. (B) Close-up view of A, showing co-localisation of TEL/AML1 and DAPI in the nucleus. (C) GFP1, showing absence of TEL/AML1 expression. (D) GFP1, showing GFP expression.

3.2.5 Characterisation of pluripotency markers in transgenic hESC lines

To show that the expression of transgenes does not affect the pluripotency of hESCs, the expression of pluripotency markers Nanog (Figure 3.6A) and Oct4 (Figure 3.6B) was assessed by qPCR. All transgenic hESC lines showed no decrease in expression of either pluripotency marker, though the expression levels are higher than H9 in seven of the eight transgenic cell lines, the significance of which is unknown. However, the expression levels of *Oct4* and *Nanog* appeared to correlate in each cell line. This is consistent with Oct4 and Nanog (along with Sox2), forming parts of a self-regulatory 'circuitry' for pluripotency, consisting of 'feed-forward' loops to maintain high levels of all pluripotency genes (Boyer *et al* 2005).

Next, the expression of cell surface markers of pluripotency SSEA4 (Figure 3.6C) and TRA-1-60 (Figure 3.6D), and marker of differentiation SSEA1 (Figure 3.6E) were assessed by flow cytometry. All transgenic cell lines were shown to be positive for SSEA4 and TRA-1-60, and negative for SSEA1. Finally, all hESC lines were karyotyped to identify any karyotypic changes (Figure 3.7), but all were found to be of normal female karyotype (46,XX).

3.2.6 Differentiation of transgenic hESC lines

Differentiation of hESC lines was performed using an established differentiation protocol was used (Vodyanik & Slukvin 2007), outlined in Materials and Methods. Figure 3.8A shows the differentiation of hESCs, starting with a discrete hESC colony on OP9 cells on day 1, to a less distinct mesodermal colony on day 4, expanding on day 6, and generating small round cells of 'cobblestone' appearance by day 8, which represent haematopoietic precursors. Cells from these OP9 co-cultures were harvested and analysed by flow cytometry for cell surface markers of haemato-endothelial differentiation (Figure 3.8B). The endothelial marker CD31 was regularly present in about 5-15% of hESC-derived (TRA-1-85$^+$) cells, and CD31$^+$CD43$^+$ cells made up to 1% of hESC-derived cells. Since haematopoietic differentiation progresses through an endothelial (CD31$^+$CD43$^-$) precursor before gaining the early haematopoietic marker CD43, it was decided that CD31 would be useful as a surrogate marker of haematopoietic differentiation.

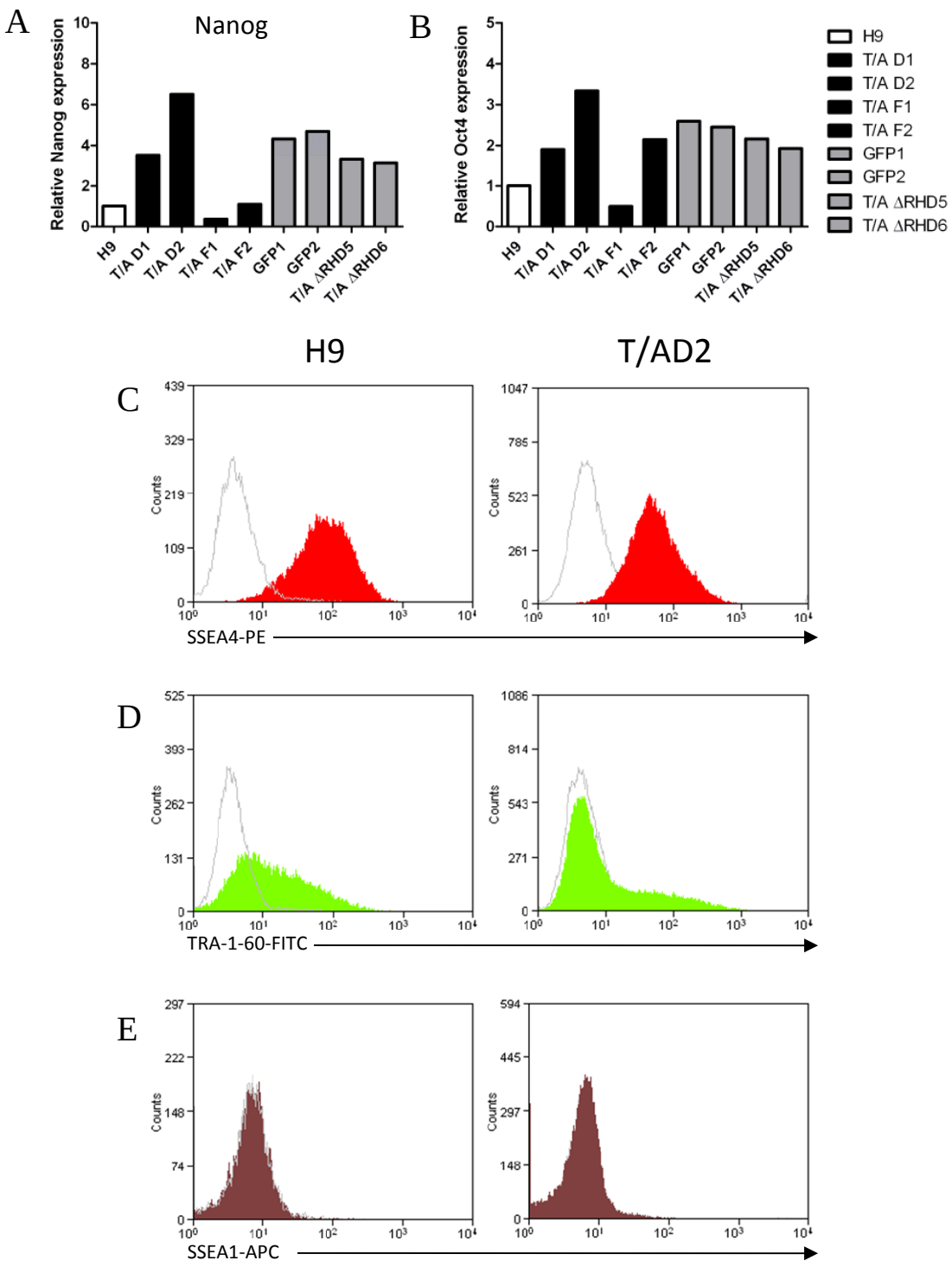


Figure 3.6 – Characterisation of pluripotency markers in transgenic hESC markers.
(A-B) Expression of pluripotency genes Nanog (A) and Oct4 (B) in the transgenic hESC lines by qPCR, compared to H9 hESCs. (C-E) Flow cytometry histograms showing expression of pluripotency markers SSEA-4 (C) and TRA-1-60 (D), and differentiation marker SSEA-1 (E), in the T/AD2 cell line, compared to H9 hESCs. Histograms are representative of all transgenic hESC lines

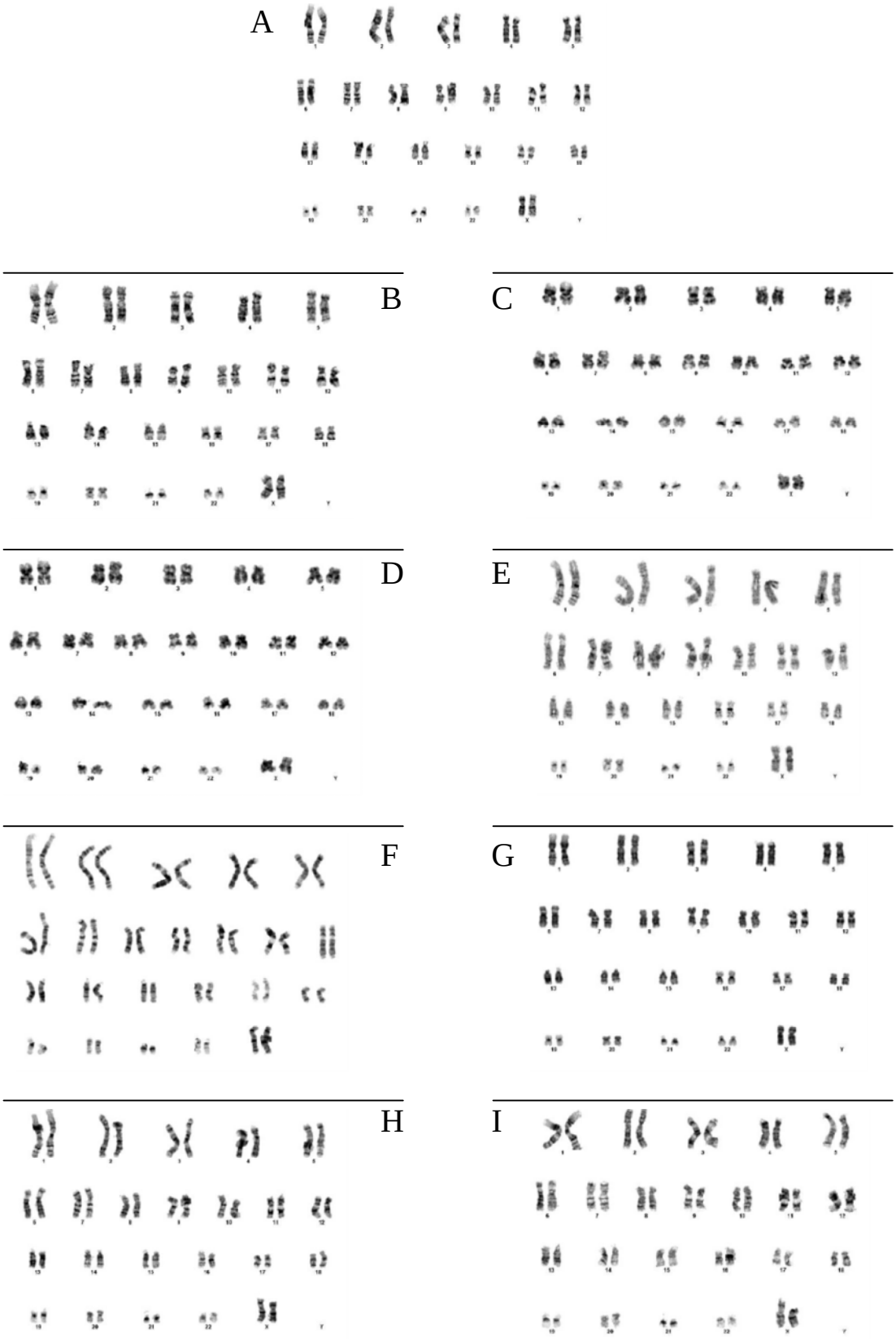


Figure 3.7 – Karyotypes of transgenic cell lines.
All transgenic hESC lines were found to be karyotypically normal (46, XX). Shown are the karyotypes for (A) H9, (B) T/AD1, (C) T/AD2, (D) T/AF1, (E) T/AF2, (F) GFP1, (G) GFP2, (H) ΔRHD5, and (I) ΔRHD6.

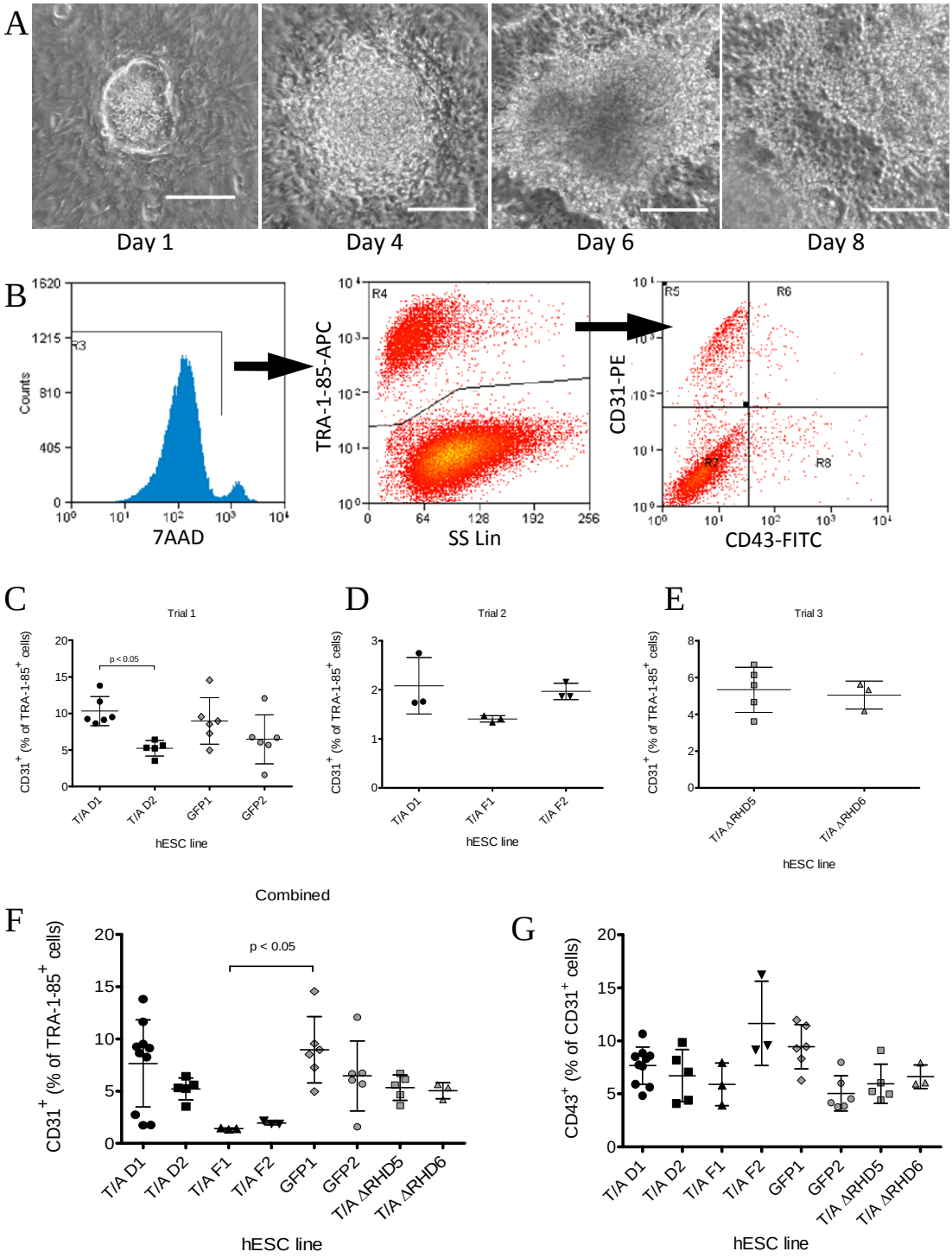


Figure 3.8 – Differentiation of transgenic hESC lines towards haematopoietic lineages.
(A) Sequential bright-field images showing the differentiation of hESCs, starting from day 1 (after seeding) ending at day 8 (when cells were harvested); Scale bar = 400µm. (B) Flow cytometry gating strategy for assessment of differentiation. Following the arrows from left to right, Live-Dead stain (7-AAD), human-mouse discrimination (human TRA-1-85 antibody), and CD31 vs. CD43. (C-F) Plot showing percentage $CD31^+$ cells derived from simultaneous differentiation trials with (C) T/AD1, T/AD2, GFP1, and GFP2, (D) T/AD1, T/AF1, and T/AF2, (E) ΔRHD5 and ΔRHD6, and (F) all transgenic lines combined, with lines showing mean and standard deviation. (G) plot showing percentage $CD43^+$ cells in $CD31^+$ to show consistent levels of haematopoietic development from endothelium. Statistical analysis was performed using the non-parametric Mann-Whitney and Kruskal-Wallis tests, as described in Material and Methods (section 2.17)

Differentiation experiments took place in a series of 'trials', using different combinations of transgenic cell lines each time. 'Trial 1' (Figure 3.8C), differentiating the cell lines T/AD1, T/AD2, GFP1, and GFP2, showed a significant difference in the differentiation capacity of T/AD1 and T/AD2, but no significant difference between any of the TEL/AML1-expressing and GFP-expressing cell lines. 'Trial 2' (Figure 3.8D), differentiating T/AD1, T/AF1, and T/AF2, showed low levels of expression for all cell lines, but no significant differences. Finally, 'Trial 3' (Figure 3.8E) showed no significant differences in the differentiation of the cell lines ΔRHD5 and ΔRHD6.

Compiling the differentiation data together (Figure 3.8F), there is no general trend to suggest that the overexpression of TEL/AML1 has a significant effect on the haemato-endothelial differentiation of hESCs. There is a significant difference in the level of differentiation between T/AF1 and GFP1, but since differentiation of T/AD1 during Trial 2 was low (which in Trial 1 gave rise to $CD31^+$ cells at a rate of 10% on average), it is possible that this significant difference is down to poor induction of differentiation in Trial 2, as opposed to an intrinsic block of differentiation in T/AF1 and T/AF2.

The percentage $CD43^+$ cells within the $CD31^+$ population did not differ significantly for the cell lines (Figure 3.8G), showing there was no block in differentiation from endothelial ($CD31^+CD43^-$) to haemato-endothelial ($CD31^+CD43^+$) cells, apart from in the T/AF2 hESC line. However, differentiation of T/AF2 was particularly low (Figure 3.8D), so even small numbers of $CD31^+CD43^+$ cells would make up a large percentage of the total $CD31^+$ population.

3.2.7 Analysis of hESC-derived $CD43^+$ cells

To further analyse haematopoietic precursors derived from the transgenic cell lines, $CD43^+$ cells were sorted from the bulk harvest by swapping the fluorescent colours conjugated to the CD31 and CD43 antibodies (Figure 3.9A), using CD31-Alexa488 and CD43-PE instead of CD31-PE and CD43-FITC. This allowed for a much better separation of the $CD31^+CD43^+$ population from the $CD31^+CD43^-$ population, and

also allowed for enrichment of the $CD43^+$ population from the bulk harvested cells by MACS, using anti-PE microbeads (Miltenyi Biotec).

Sorted $CD43^+$ cells derived from the T/AD1 hESC were able to generate differentiated haematopoietic colonies when incubated in semi-solid methylcellulose medium. By the size and density of the colonies, it was concluded that they consisted of monocytes and granulocytes (Figure 3.9B).

I investigated the expression of haematopoietic transcription factors in the co-cultures, namely SCL, EKLF, PU.1, GATA1, and GATA2 (Zambidis *et al* 2005). Genes were not found to be expressed in OP9 cells (Figure 3.9C) nor in hESCs, apart from low levels of GATA2 (Figure 3.9D). Expression of these genes in the differentiated transgenic hESCs was assessed using two approaches. Firstly, cDNA was prepared from the 'bulk harvest' (i.e. all the cells in the dish, including OP9s and undifferentiated hESCs) of differentiated T/AD1 and ΔRHD5 hESC lines (Figure 3.9E). Secondly, cDNA was prepared from sorted $CD43^+$ cells from day 8 and day 11 of co-culture of T/AD1 (Figure 3.9F). qPCR was performed on the cDNA samples and Ct values were normalised to CD43 to take into account different levels of haematopoietic differentiation in the co-cultures.

Comparing the T/AD1 and ΔRHD5 bulk cultures, no significant difference was observed in the expression of any of the transcription factors (Figure 3.9E). In the sorted $CD43^+$ cells, expression of all transcription factors was reduced in cells taken from day 11 of co-culture compared to day 8, apart from PU.1, which was highly upregulated. PU.1 is a transcription factor associated with differentiation towards B lymphocyte and macrophage lineages (DeKoter & Singh 2000), so this might indicate a shift of the $CD43^+$ cells from a 'stem cell' or 'precursor' identity towards a more differentiated phenotype.

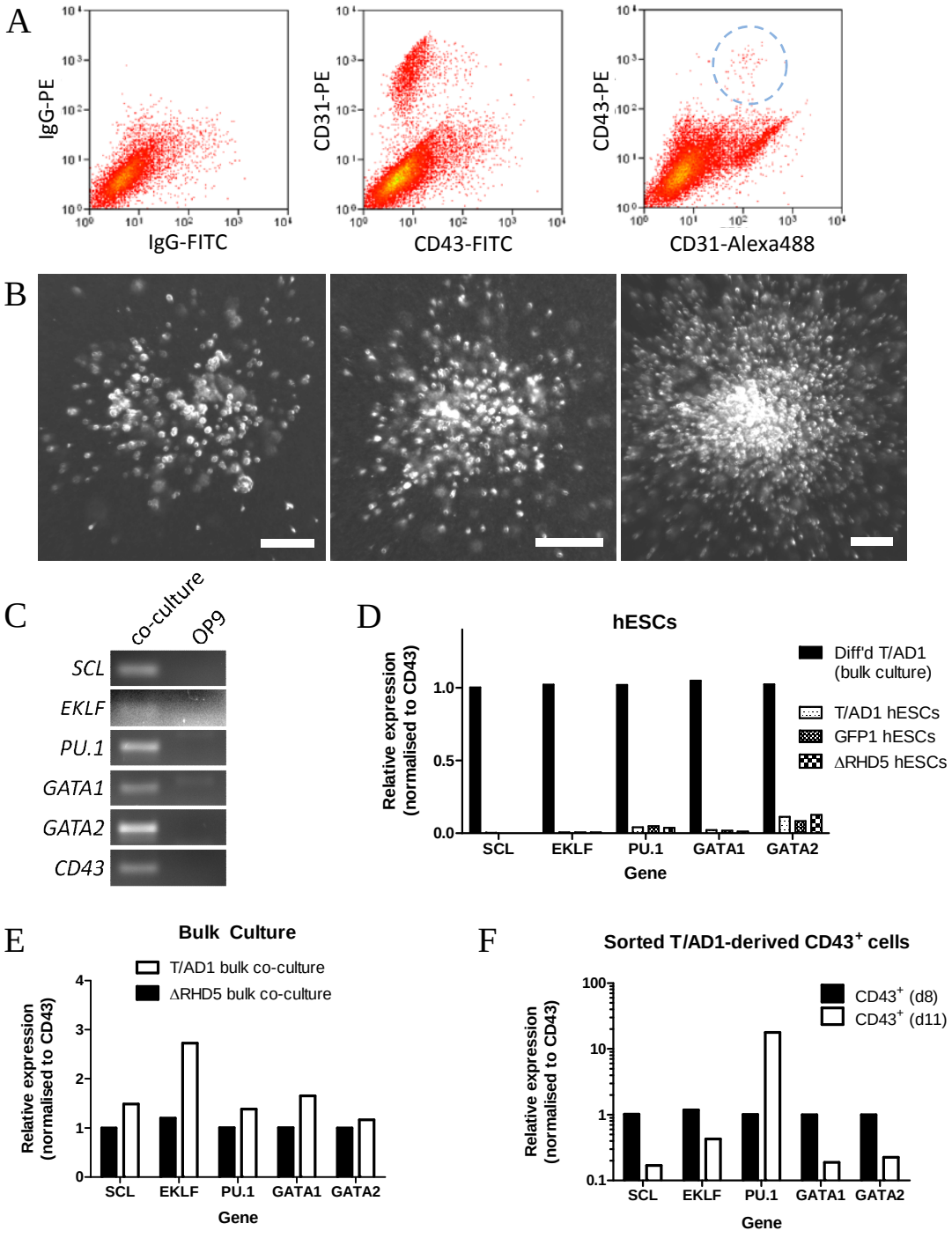


Figure 3.9 – Analysis of differentiated transgenic hESC lines.
(A) Representative plot showing how the swapping of fluorophores on the antibodies enable clear discrimination of $CD31^{+}CD43^{+}$ population (encircled in blue), which is the basis of MACS enrichment and FACS sorting. (B) Representative images showing differentiated colonies derived from sorted $CD43^{+}$ cells derived from T/AD1 hESCs cultured in methylcellulose based media for two weeks; Scale bar = 200µm. Colonies were made up of granulocyte and monocyte lineage cells. (C) PCR products using early haematopoietic genes (SCL, EKLF, PU.1, GATA1, GATA2) and CD43, showing no expression of these genes in OP9 cells (D-F) graphs showing the transcript expression level by qPCR of early haematopoietic genes normalised to CD43 expression from (D) undifferentiated hESC lines, (E) all cells harvested from co-cultures of T/AD1 or ΔRHD5 transgenic hESC lines ('Bulk Culture'), and (F) sorted $CD43^{+}$ cells from day 8 or day 11 of co-cultures of T/AD1 (each bar represents the mean of three technical replicates to measure one biological replicate per graph).

3.2.8 B lymphopoiesis

I next examined the capacity of the transgenic cell lines to differentiate towards B lymphocytes, by taking the sorted $CD43^+$ cells from the OP9 co-cultures of T/AD1, GFP1, and ΔRHD5, and co-culturing these with the bone marrow stromal cell line MS-5, for four weeks, with additional cytokines (see Materials & Methods and Vodyanik *et al* 2006). Figure 3.10A shows a basic schematic of the expression of markers of B cell development (TdT, RAG1, PAX5, mb1, VpreB, CD19, CD10, and CD34) from the haematopoietic stem cell (HSC), via the common lymphoid progenitor (CLP), and through pro-B and pre-B cells (LeBien 2000; Sanz *et al* 2003; Hystad *et al* 2007; Sanz *et al* 2010). The protein VpreB forms part of the ψ-light chain (ψLC), which is a component of the pre-B cell receptor, expressed on the cell surface of pre-B cells (Wang *et al* 1998b), and is also shown. The dotted line indicates at which stage the impairment in B cell differentiation commonly occurs in patients (Golub *et al* 1995), which is frequently (Tsuzuki *et al* 2004; Fischer *et al* 2005), but not always (Andreasson *et al* 2001; Morrow *et al* 2004; Schindler *et al* 2009) recapitulated in mouse models.

The numbers of $CD43^+$ cells used in the MS-5 co-cultures were small (1,000 cells per well of a six-well plate), and so it would have been difficult to sort these cells by FACS. Therefore, I assessed the B lymphocyte differentiation in MS-5 co-cultures by preparing cDNA samples from the entire culture (similar to the 'bulk culture' method used to analyses haematopoietic differentiation in Figure 3.9E) and analysing gene expression by qPCR. B cell markers expressed in REH cells were not expressed in MS-5 cells (Figure 3.10B), whereas the MS-5 co-cultures showed expression of CD10 and CD19 transcript (Figure 3.10C), indicating that differentiation towards B cell lineage in all co-cultures progressed at least as far as pro-B cells. MS5 co-cultures from differentiated T/AD1 hESCs showed a significantly increased level of CD34 compared to those from differentiated ΔRHD5 hESCs, possibly indicating a higher pro-B to pre-B ratio from TEL/AML1-expressing hESCs. In addition, the expression of CD10 was significantly decreased in MS5 co-cultures from differentiated GFP1 hESCs compared to those from ΔRHD5 hESC, but the implication of this is not clear. Comparing B cell marker expression within the MS-5 co-cultures of T/AD1-, GFP1-, and ΔRHD5-derived

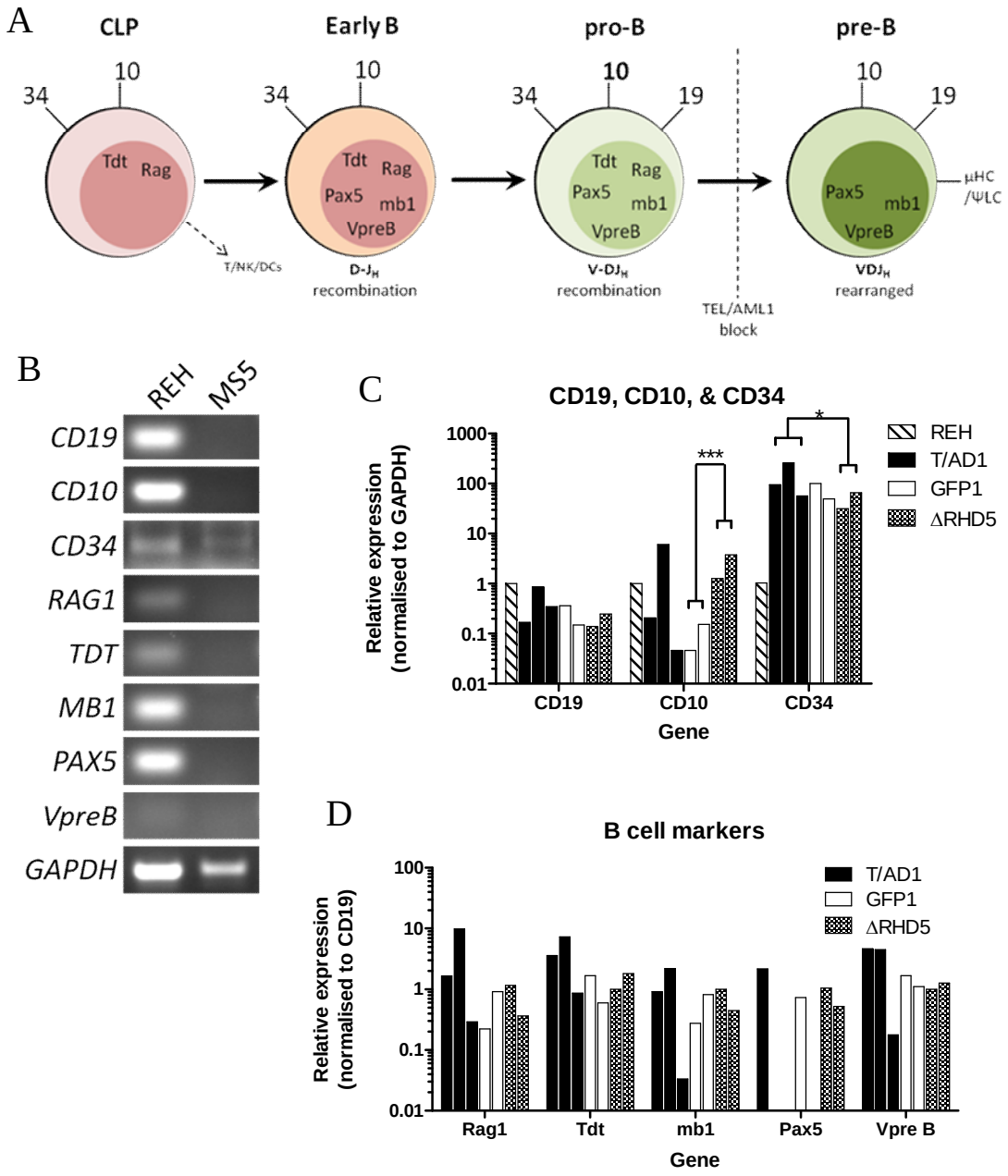


Figure 3.10 – B lymphopoiesis from transgenic hESCs.
(A) Outline of differentiation from common lymphoid progenitor (CLP) to pro-B and pre-B cells, via Early B cell. Expression of CD markers (34 = CD34, etc.) and pre-B cell receptor components μHC and ψLC are shown on the outside of each 'cell', and expression of transcript for Rag1/2, Tdt, mb1, Pax5, and VpreB are shown on the inside of each 'cell'. Below the B-lineage cells, the recombination status of the heavy chain VDJ genes is shown. Dotted line indicates the stage at which B cell differentiation appears to be blocked in TEL/AML1$^+$ leukaemia patients and mouse models. (B) Gel electrophoresis of qPCR products showing no expression of the indicated genes in MS-5 cells. (C-D) Expression by qPCR of (C) CD19 and CD10 or (D) B cell markers in MS-5 co-cultures of T/AD1-, GFP1-, and ΔRHD5-derived CD43$^+$ cells, bars representing individual experiments. Relative expression values are normalised against (C) REH and (D) one of the MS-5 co-cultures of ΔRHD5-derived cells (each bar represents the mean of three technical replicates, p-values as calculated using Mann-Whitney U test represented by asterixes: * $p<0.05$, *** $p<0.01$).

$CD43^+$ cells, there are no significant differences in expression between the different cell lines, though there are slightly higher levels of expression of Pax5 and VpreB in the T/AD1 MS-5 co-cultures than in those with GFP1 and ΔRHD5 (Figure 3.10D).

3.2.9 Leukaemia Associated genes

I also examined the expression of genes commonly associated with $TEL/AML1^+$ acute lymphoblastic leukaemia that have been identified in gene expression microarray screens of samples from ALL patients (Andersson *et al* 2005b; Gandemer *et al* 2007). I hypothesised that any differences in the expression of these genes would be best observed after B cell differentiation, since $TEL/AML1^+$ leukaemia is exclusively a B lineage leukaemia (Uphoff *et al* 1997).

Andersson and colleagues generated a gene expression profile that distinguished $TEL/AML1^+$ leukaemia from other types of ALL. They then compared this profile to the gene expression profile of normal haematopoietic cells, and identified expression patterns for 67 genes that were unique to leukaemia cells (Andersson *et al* 2005b). I compared this list to the $TEL/AML1^+$ gene expression profile created by Gandemer and colleagues (2007), and picked four genes (PIK3C3, TNFRSF7, SMAD1, and ANGPTL4) for further analysis. PIK3C3 was identified by both microarray screens, whilst family members of TNFRSF7 (identified by Gandemer's study) were also observed in Andersson's study. SMAD1 was chosen for its involvement in the BMP signalling pathway, known to regulate haematopoiesis and leukaemia (Bhatia *et al* 1999). Finally, ANGPTL4 was chosen as several of its related family members were identified in Andersson's study, which have been shown to support expansion of HSCs (Zhang *et al* 2006) and implicated in myeloid leukaemia (Loges *et al* 2005).

Out of these genes, two – *PIK3C3* and *ANGPTL4* – were expressed in the transgenic hESCs normally (Figure 3.11A), but not in MS-5 cells (Figure 3.11B). In the MS-5 co-cultures of $CD43^+$ cells derived from the transgenic hESCs, *TNFRSF7* and *SMAD1* were both significantly upregulated in the T/AD1 co-cultures compared to the ΔRHD5 co-cultures. *ANGPTL4* was significantly downregulated in co-cultures using differentiated GFP1 hESCs compared to those from ΔRHD5 hESCs (Figure 3.11C).

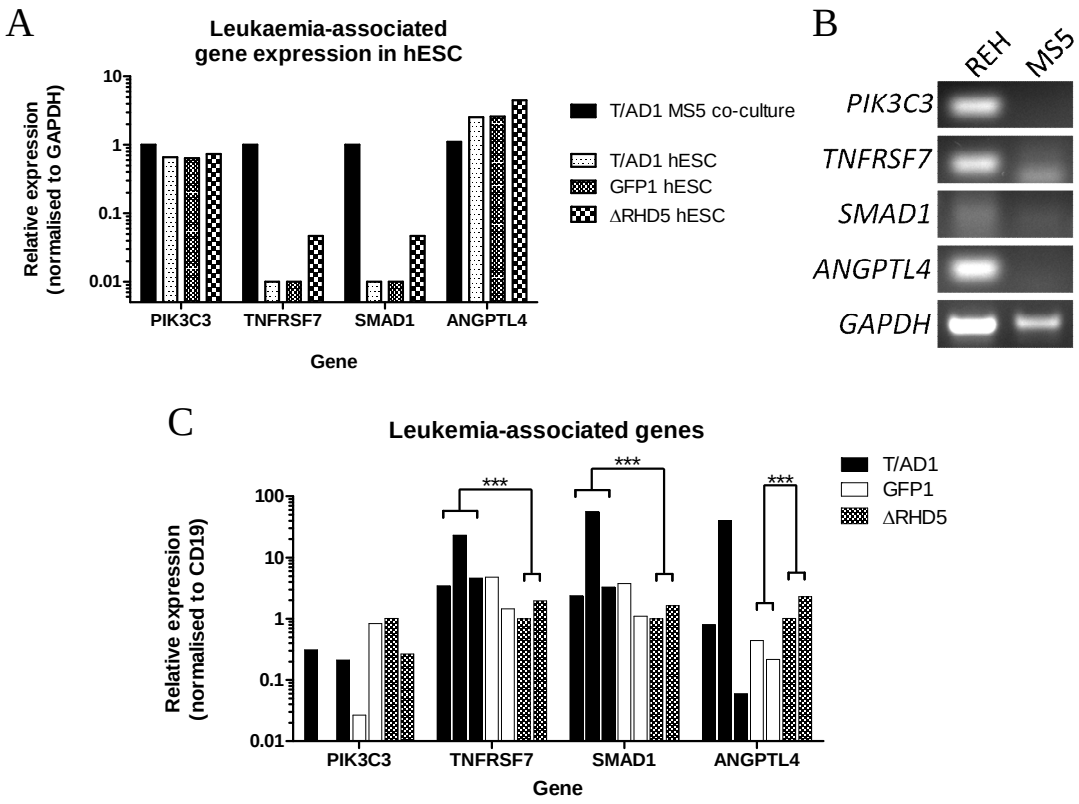


Figure 3.11 – Expression of genes associated with TEL/AML1$^+$ leukaemia in transgenic hESCs.
(A) Expression by qPCR of leukaemia-associated genes in undifferentiated transgenic hESCs. (B) Gel electrophoresis of qPCR products showing no expression of the indicated genes in MS-5 cells. (C) Expression by qPCR of leukaemia-associated genes in MS-5 co-cultures of T/AD1-, GFP1-, and ΔRHD5-derived CD43$^+$ cells, bars representing individual experiments. Ct values are normalised to CD19, and relative expression values are normalised one of the MS-5 co-cultures of ΔRHD5-derived cells. (each bar represents the mean of three technical replicates, p-values as calculated using Mann-Whitney U test represented by asterixes: *** $p<0.01$).

3.3 Discussion

The data presented in this chapter describes the creation of a model of TEL/AML1$^+$ acute lymphoblastic leukaemia using hESCs as a source of human haematopoietic progenitors. The stable expression of TEL/AML1 was not found to affect markers of pluripotency in hESCs nor cause any karyotypic changes. Differentiation towards haematopoiesis, performed using a co-culture method with OP9 cells, was not affected by the presence of TEL/AML1. CD43$^+$ hESC-derived haematopoietic precursors expressed markers of haematopoiesis and were capable of differentiating to mature blood cells. Directed differentiation of the haematopoietic precursors towards B lineage lymphocytes, using a co-culture method with MS-5 cells, was able to generate cells expressing markers of B cell development. The expression of TEL/AML1 resulted in a slight increase in CD34 expression, and in the expression of two of four genes investigated that have previously been associated with TEL/AML1$^+$ leukaemia.

3.3.1 Generating a model of leukaemia using hESCs

The hESC model of ALL presented here aims to complement previous models, including mouse *in vivo* models (Tsuzuki *et al* 2004; Schindler *et al* 2009), xenograft models (Hong *et al* 2008; le Viseur *et al* 2008), and models using cell lines (Diakos *et al* 2007; Gefen *et al* 2009; Zaliova *et al* 2011). Whilst expanding HSCs from cord blood, bone marrow, or mobilised peripheral blood, is still technically challenging (Dahlberg *et al* 2011), hESCs can provide a theoretically limitless supply of human haematopoietic precursors with which to study ALL.

The H9 hESC line was one of the original lines (along with H1 and H7) generated by James Thomson in the first description of hESC (Thomson *et al* 1998). Previously, I had used hESC lines developed at the University of Sheffield (SHEF1, SHEF3, and SHEF4), but found in our lab that the efficiency of haematopoietic differentiation from these cells was low; this was not necessarily surprising, since the capacity of different hESC lines to differentiate towards various lineages is known to vary significantly (Osafune *et al* 2008). I was granted access to H1, H7, and H9, and found H1 and H9 to be the easiest of the three hESC lines to grow in cell culture. These cell lines could be considered ‘gold standards’ with regards to haematopoietic

differentiation, with the use of H1 in particular widely reported in the literature. I chose the H9 cell line as the basis of our model as it was available to use first, and was reported to have equivalent differentiation capacity as H1 (Vodyanik *et al* 2005).

I initially aimed to inducibly express TEL/AML1 for several reasons. Firstly, it would allow the timing of TEL/AML1 expression to be controlled. This would avoid any potential problems with inhibition of differentiation, previously observed whilst expressing the acute myeloid leukaemia (AML)-associated fusion gene MLL/ENL in hESCs (Mark Coles, unpublished observations), but also allow an investigation of at which level of the haematopoietic developmental hierarchy TEL/AML1 has its influence, which is still under debate (Schindler *et al* 2009). Secondly, it would allow the control of the expression level of TEL/AML1, which would enable the study of the effects of physiological levels of TEL/AML1, as opposed to superfluous levels which may have unexpected effects. Unfortunately, I was not able to achieve inducible expression of TEL/AML1 when testing in cell lines (Figure 3.1). This may have been due to poor expression of or a mutation in the recombinant tetracycline-responsive transactivator (rtTA), or similarly a mutation in the tetracycline response element (TRE) to which rtTA binds.

It was decided to proceed instead with a constitutive expression system; however, there were two main concerns with this method that needed to be addressed. Firstly, a promoter needed to be selected that would consistently express TEL/AML1 without silencing, a problem often encountered with hESCs (Kim *et al* 2005). I opted to use the pCAG vector, which has been demonstrated to drive stable expression in hESCs, compared with other CMV derivatives (Liew *et al* 2007). Secondly, I wanted to ensure that expression of a transgene, in this case a mutant transcription factor associated with malignant haematopoiesis, would not cause the hESCs to spontaneously differentiate, but would allow differentiation when induced. After generating the pCAG-TEL/AML1 (Figure 3.2) and pCAG-TEL/AML1-ΔRHD (Figure 3.3) plasmid constructs and transfecting these (along with pCAG-eGFP) into H9 hESCs, I found the transgenic cell lines maintained their hESC morphology and pluripotency markers (Figure 3.6). hESCs are prone to acquiring karyotypic changes (Amps *et al* 2011) that are typically found in embryonic carcinoma cells, commonly

affecting chromosomes 17 and 12, the latter the location of the *ETV6* gene. To confirm that the transgenic hESC lines had not undergone similar structural changes, the cell lines were karyotyped, and were found to be normal (46, XX; Figure 3.7). I was thus convinced that the overexpression of TEL/AML1 in hESCs was a valid approach.

In absence of an inducible system which allowed the level of TEL/AML1 expression to be controlled, several cell lines were generated to form a panel with varying transgene expression. The resulting TEL/AML1 lines gave mRNA transcript levels ranging between 3- and 43-fold more than that in the TEL/AML1$^+$ cell line REH (Rosenfeld *et al* 1977).

In summary, I believe that the expression of TEL/AML1 did not aberrantly affect hESCs by itself, and that in the panel of TEL/AML1-expressing hESC lines I had enough range of expression to minimise any potential affects resulting from the act of overexpressing proteins, called 'transgene toxicity' (Zafarana *et al* 2009). In not generating the system to inducibly expression TEL/AML1 I was not able to fully control expression, so denying the opportunity to study, for example, the timing of when TEL/AML1 might have an influence on hESC differentiation. Despite this, the hESC lines generated provides the basis of a model for TEL/AML1$^+$ leukaemia.

3.3.2 Haematopoietic differentiation of transgenic hESCs

Differentiation of hESC lines was performed using the OP9 co-culture method, first set out by Nakano and colleagues (Nakano *et al* 1994), and adapted for hESC differentiation by James Thomson's group (Vodyanik *et al* 2005). I used an established protocol for the 'OP9 co-culture' method (Vodyanik & Slukvin 2007), and found this method to give consistent results over several weeks when a staggered regime was implemented for the splitting of hESCs and OP9 cells, and the seeding, feeding, and harvesting of co-cultures. I did attempt differentiation by the 'embryoid body' method (Chadwick *et al* 2003) but found it to be inefficient (data not shown) and more technically challenging, as well as the cost of supplemental cytokines to be expensive.

Differentiation of the various cell lines took place in a series of three 'trials', with each trial differentiating a number of cell lines simultaneously (Figure 3.8C-E). The TEL/AML1$^+$ cell line T/AD1 from trial one was also used trial two to act as a reference; whilst the level of differentiation for each cell line did vary, the levels across the cell lines did appear to follow each other, so using T/AD1 in the second trial gave an indication of the efficiency in that trial. It is not clear why the level of differentiation in trial two was much lower compared to either of the other, but since a fresh vial of OP9 cells was thawed for each trial, it is possible that Trial 2 made use of an OP9 batch less able to induce hESC differentiation.

Overall there was little difference in the level of differentiation towards CD31$^+$ haemato-endothelial precursors amongst the various cell lines (Figure 3.8F), and no significant difference in the percentage of CD43$^+$ cells in the CD31$^+$ population (Figure 3.8G). It had been previously reported that the level of differentiation depended on the proliferation rates of the cell lines (Vodyanik & Slukvin 2007). In normal maintenance culture, no differences in growth rates between the transgenic cell lines were observed. However, in co-culture with OP9 cells, the proliferation rates of the cell lines did vary; since the same number of cells for each cell line was seeded onto each OP9 plate, the percentage of TRA-1-85$^+$ (i.e. human) cells in the total harvested cell suspension could act as a surrogate marker for proliferation. This value varied from 11% to 82%, but no significant correlation was observed between the percentage TRA-1-85$^+$ cell count and the level of differentiation, neither for individual cell lines nor for all cell lines combined (data not shown).

The lack of a significant effect of TEL/AML1 on the differentiation is consistent with previous observations of the role of TEL and AML1 in embryonic stem cells and early embryonic development, if it is assumed the influence would be TEL/AML1 having a dominant negative effect on TEL and/or AML1 (Zelent *et al* 2004). Whilst *ETV6*$^{-/-}$ mice are embryonic lethal (due to angiogenic defects in the yolk sac), *ETV6*$^{-/-}$ chimeric mice show no defects in haematopoiesis, instead showing problems associated with the post-natal establishment of haematopoiesis in the bone marrow (Wang *et al* 1998a). Similarly, whilst *RUNX1*$^{-/-}$ mouse embryos and embryonic stem cells cannot transfer from primitive to definitive haematopoiesis (Lacaud *et al* 2002), haploinsufficent ESCs are still capable of definitive

haematopoiesis, showing accelerated haematopoietic commitment (Lacaud *et al* 2004). As such, any effects that TEL/AML1 expression might have on the expression or activity of either TEL or AML1 (Zelent *et al* 2004) are unlikely to have a significant effect during haematopoietic differentiation of hESCs.

I intended to use the $CD31^+CD43^+$ haematopoietic progenitors for three main purposes: (1) to study the general haematopoietic capacity of these progenitors by Colony Forming Unit (CFU) assays; (2) to investigate gene expression changes in these progenitors by qPCR; and (3) to differentiate these cells towards B lymphopoiesis by MS-5 co-culture for further analysis. The total number of cells harvested each time was regularly ~10^8 cells, of which 40% on average were human ($TRA\text{-}1\text{-}85^+$), and of these up to 1% were $CD31^+CD43^+$, meaning roughly 400,000 $CD31^+CD43^+$ cells were available. However, it was found that the number of cells that could be harvested from co-cultures (by FACS, sometimes in combination with MACS) was much lower than should be present in the culture; the actual number of cells harvested was rarely above 5,000. The likely reason was due to the 'clumpy' nature of endothelial cells, which prevents attaining single cell suspensions without harsh physical detachment, leading to cell death. This meant that there commonly were only enough cells to use for one of the three purposes above. I decided, given the small $CD31^+CD43^+$ yield, to focus the use of the cells for the MS-5 co-culture, as it was in B cell differentiation where I expected to be most likely to see significant differences between the cell lines, given the known impact of TEL/AML1 on B cell development in a variety of models (Tsuzuki *et al* 2004; Sabaawy *et al* 2006; Hong *et al* 2008), and B lineage induction only required 1,000 cells per well of a six-well plate (Vodyanik *et al* 2006). This unfortunately prevented meaningful numbers of replicates for the CFU assays and gene expression analysis from being carried out.

One limitation of this study was that the different transgenic hESC lines were differentiated in three separate trials. The differentiation capacity of T/AD1varied significantly between the two trials it was used in, indicating that the conditions of the three trials were not identical, and so limiting the strength of the conclusions that can be drawn by direct comparison between trials. The differentiation of the transgenic lines was divided in this way primarily because not all the hESC lines were ready at the same time. To more reliably compare the differentiation capacity

of these lines, the eight hESC lines would have to be differentiated at the same time using the same batch of OP9 cells, but it would then become a very difficult task in terms of cell culture. If these differentiation experiments were to be repeated and remain divided into trials, it would be important to ensure uniformity in the OP9 batches used for differentiation, and to use one or more cell lines in all trials, to allow for a more reliable comparison between the hESC lines.

3.3.3 B cell development from differentiated transgenic hESCs

Since there were not enough cells for analysis of B cell development in the MS-5 co-cultures by flow cytometry, I assessed the level of B lymphopoiesis by qPCR as it has the advantage of being very sensitive to small amounts of transcript. For the same reason, it is also more susceptible to contamination, but I found no expression in MS5 cells of any of the B cell development markers (Figure 3.10B) or the leukaemia-associated genes (Figure 3.11B). The genes analysed (Vodyanik *et al* 2006) are used to discriminate between different stages of B cell development (Ghia *et al* 1996; LeBien 2000; Hystad *et al* 2007; Sanz *et al* 2010).

In the expression of genes for cell surface 'CD' markers, there is an upregulation of CD34 in T/AD1 MS5 co-cultures compared to ΔRHD5 co-cultures (Figure 3.10C), which might indicate an increase ratio of pro-B to pre-B cells (since it is during this transition that CD34 expression is lost; Ghia *et al* 1996) which is consistent with the pro-B to pre-B blockade observed in a number of mouse models investigating the effects of TEL/AML1 on B cell development (Tsuzuki *et al* 2004; Fischer *et al* 2005). There was no significant difference between the expression of other B cell markers in T/AD1 compared to GFP1 or ΔRHD5 (Figure 3.10D). Comparison of these gene expression changes with mouse models of leukaemia is complicated since most papers analyse B cell development using flow cytometry (as opposed to gene expression; e.g. Tsuzuki *et al* 2004, Schindler *et al* 2009), and the stages of B cell development in a mouse do not have direct equivalents in humans (Hardy *et al* 1991; LeBien 2000). However, if the block in B cell development between the pro-B and pre-B stages commonly observed in patients (Golub *et al* 1995) were to be observed in this model, one might see much higher levels of *TdT* expression, higher levels of

VpreB and *Rag1*, and lower levels of *Pax5* as the proportion of pro-B cells compared to pre-B cells increases (Hystad *et al* 2007).

Using the same method, I also investigated the expression levels of genes commonly upregulated with TEL/AML1$^+$ leukaemia. These genes (PIK3C3, TNFRSF7, SMAD1, and ANGPTL4) were selected from studies of RNA samples from acute lymphoblastic leukaemia patients of various subtypes analysed by gene expression microarray (Andersson *et al* 2005b; Gandemer *et al* 2007). An increase in expression of TNFRSF7 and SMAD1 in T/AD1 MS5 co-cultures compared to the ΔRHD5 co-cultures was observed (Figure 3.11C). Amongst these four genes originally identified by Andersson and colleagues, only the genes also identified in the microarray by Gandemer (namely PIK3C3 and TNFRSF7) were validated by qPCR (Gandemer *et al* 2007). However, Andersson and colleagues found the genes in their TEL/AML1$^+$ profile to correlate significantly with those identified in a separate gene expression profiling study of acute lymphoblastic leukaemia subtypes (Ross *et al* 2003).

Of course, the influence of TEL/AML1 has not been fully revealed by the analysis of only four genes. Beyond testing the expression of other genes previously associated with TEL/AML1$^+$ leukaemia, one approach would be to conduct a gene expression microarray to identify the differentially expressed genes in differentiated transgenic hESCs. This would have the advantage of a direct list of genes affected by, and reveal potential functional consequences of, the expression of TEL/AML1. Such an approach would enable a more directed study of how TEL/AML1 affects haematopoietic development and early B cells.

Nevertheless, the lack of a strong 'leukaemia' phenotype, in terms of a block in B cell development and expression of genes commonly associated with leukaemia, is consistent with previous studies in mouse models. Mice receiving bone marrow transduced with TEL/AML1 do not show symptoms of leukaemia (Andreasson *et al* 2001; Tsuzuki *et al* 2004; Fischer *et al* 2005), but TEL/AML1 in combination with secondary mutations does give rise to B cell ALL (Bernardin *et al* 2002; Schindler *et al* 2009; van der Weyden *et al* 2011).

3.3.4 Conclusions

The results presented in this chapter have not shown that TEL/AML1 alone has a significant effect on the haematopoietic differentiation of hESCs. According to previous studies of TEL/AML1$^+$ leukaemia, the properties acquired by TEL/AML1 expression alone have been referred to as a 'pre-leukaemic state' (Greaves *et al* 2003), exemplified by high numbers of quiescent HSCs (Schindler *et al* 2009) and/or an impairment – but not a block – of B cell differentiation (Fischer *et al* 2005). In this model, I was not able to provide a strong demonstration of either of these two hallmarks, due to the limited numbers of haematopoietic cells that could be recovered from the OP9 and MS-5 co-cultures. However, if these technical difficulties can be overcome, there is no reason why this model could not be used to demonstrate these effects. Indeed, it is possible that these effects are an artefact of mouse models, and that one might not observe a similar effect in a human model such as the hESC model presented in this chapter. Nevertheless, our results are consistent with previous studies in mice that conclude TEL/AML1 is not sufficient for leukaemogenesis, and thus likely represents a 'first-hit' that gives transformed cells properties which allow it to acquire further mutations leading to leukaemia.

The strength of the hESC model of leukaemia is its ability to recapitulate haematopoietic differentiation at various stages. However, the technical challenges involved in this have impaired the current study, although I would anticipate that some of these challenges could be overcome with enough time to optimise methods (for example, the isolation of CD31$^+$CD43$^+$ haematopoietic precursors). Further technical challenges may be met, such as the culture of B cell precursors once derived from haematopoietic precursors, although it has been shown that it is possible to culture B cell progenitors from CD34$^+$ cord blood cells for more than 12 weeks *ex vivo*, without significant changes to their cell surface markers (Rawlings *et al* 1995), and B cells from peripheral blood for extended periods of culture (Wiesner *et al* 2008). Whether such results could be gained from hESC-derived B cell precursors remains to be seen.

In summary, this chapter describes the development of the first model of TEL/AML1$^+$ leukaemia using hESCs. Combining this study with the work of others

in haematopoietic differentiation of hESCs, I have shown that TEL/AML1-expressing hESCs are capable of haematopoiesis and differentiation towards B lymphocytes. Overcoming the technical challenges of haematopoietic differentiation of the transgenic hESCs will allow renewed study into TEL/AML1^{+} leukaemia using a human multipotent system.

Chapter 4: The role of TEL in TEL/AML1$^+$ leukaemia

4.1 Introduction

One of the most common secondary mutations in TEL/AML1$^+$ leukaemia is the loss of the untranslocated TEL allele (Raynaud *et al* 1996; Cave *et al* 1997). This frequent bialleic loss of TEL indicates its importance in the context of leukaemia, and yet there is relatively little known about the role of TEL in normal and malignant haematopoiesis, particularly its regulatory targets. This knowledge has implications for deepening the understanding of how leukaemia arises and for the discovery of drugs that act against dysregulated pathways.

4.1.1 Discovery of TEL

The *TEL* gene, located at 12p13, was first discovered in the context of Chronic Myelomonocytic Leukemia (CMML) by Golub and colleagues (1994). In this disease, the gene coding for platelet-derived growth factor receptor beta (PDGFRβ) was found to be frequently fused to an unidentified gene as a result of the t(5;12)(q33;p13) chromosome translocation. It was found that this gene shared the same DNA binding domain found in members of the *ets* family, and so it was named *TEL*, after 'Translocation, Ets, Leukaemia' (Golub *et al* 1994). The *TEL* gene was later renamed *ETV6* (after 'ETs Variant 6') to avoid confusion with the abbreviation for 'telomere'; however, both names are still used interchangeably for both the gene and the protein, though TEL is more commonly used when describing fusion proteins.

4.1.2 TEL structure

The *ets* group of transcription factors are defined by an 80 amino acid DNA binding domain called *ets* (Wasylyk *et al* 1993). The other domain that TEL shares with many of its family members is the *pointed* domain, also known as a sterile alpha motif (SAM), which forms a helix-loop-helix structure to form homo- and heterodimers (Kim *et al* 2001). Finally, TEL has a central repression domain, which is involved in the recruitment of co-repressors (Wang & Hiebert 2001). TEL is unique amongst its family members as it always acts as a repressor, whereas the rest

of the *ets* family can be activators or repressors of gene expression dependent on context (Mavrothalassitis & Ghysdael 2000; Oikawa & Yamada 2003).

4.1.3 TEL activity

TEL represses the expression of its targets by chromatin condensation, via two routes. The recruitment of histone deacetylases (HDACs) is mediated by corepressors N-CoR (Guidez *et al* 2000), SMRT and mSin3A (Chakrabarti & Nucifora 1999), which bind to the central region of TEL. Alternatively, L3MBTL, which causes chromatin condensation independent of HDACs, has a domain similar to the SAM domain and thus binds to the helix-loop-helix *pointed* domain (Boccuni *et al* 2003). The homodimerisation of TEL, via the *pointed* domain, increases its DNA binding affinity (Green *et al* 2010) and is necessary for its repression activities, which is likely due to the stabilisation of the repressor-corepressor interactions (Lopez *et al* 1999), rather than any intrinsic property of the dimerised *pointed* domain.

Despite its apparent importance in the regulation of haematopoiesis and its involvement in leukaemia and other cancers, little is known about the targets of TEL. The DNA-binding specificity of the *ets* family members centres on a GGAA/T consensus sequence (Karim *et al* 1990; Nye *et al* 1992), but appears *in vivo* to depend largely on availability of binding sites (Wei *et al* 2010). TEL has been demonstrated to bind to and inhibit the promoters for *stromelysin-1* (Fenrick *et al* 2000; Wang & Hiebert 2001) and *Bcl-X_L* (Irvin *et al* 2003). TEL has also been shown to repress the expression of *TCL1*, a gene associated with CML (Fears *et al* 2002).

TEL is known to bind with other transcription factors to repress their gene expression. TEL binds with the *ets* family member FLI-1 to inhibit the megakaryocytic-specific *GPIbα* and *GPIX* promoters, which are normally activated by FLI-1 (Kwiatkowski *et al* 1998). TEL also binds to STAT3 and represses its growth-inhibiting activities (Schick *et al* 2004). TEL represses the activation of the *MCSFR* promoter by AML1 and C/EBPα, and that the HLH *pointed* domain is required for full repression (Fears *et al* 1997).

Regulation of TEL activity is believed to occur through the Mitogen-Activated Protein Kinases (MAPK) pathway, after the discovery of MAPK phosphorylation sites in TEL (Poirel *et al* 1997). Two phosphorylation sites in particular have been linked to MAPK-induced regulation, serine-213 and serine-257, which both lie in the central region between the *pointed* and *ets* domains. Serine-257 is phosphorylated in response to stress by p38-MAPKs (Arai *et al* 2002), whereas both serine-213 and serine-257 are phosphorylated by Extracellular signal-Related Kinase (ERK), a family of MAPKs (Maki *et al* 2004). The phosphorylation of TEL by p38 or ERK MAPKs inhibits the gene repression activities of TEL by preventing DNA binding (Arai *et al* 2002; Maki *et al* 2004).

4.1.4 TEL function

ETV6 is expressed in mouse embryos from embryonic day 7 (E7). Expression increases through E8.5 and E9.5, where it can be observed throughout the embryo and in the yolk sac. At E12.5, *ETV6* is expressed at higher levels in lung, kidney and liver, as well as neural tissues, before peaking at E17. *ETV6* is expressed in many tissues in the adult mouse, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, testes, and haematopoietic tissues (Wang *et al* 1997; Wang *et al* 1998a).

ETV6$^{-/-}$ mice are embryonic lethal, owing to faulty blood vessel formation in the yolk sac at around E9.5 (Wang *et al* 1997). In addition, *ETV6* knockout demonstrate apoptosis in embryonic mesenchymal and neural tissues (particularly cranial nerve ganglia), indicating that *ETV6* expression is required for their survival. To observe the role of TEL in the adult mouse, chimeric embryos with *ETV6*$^{-/-}$ ES cells were generated (Wang *et al* 1998a). TEL was found not to be required for haematopoietic commitment nor maturation in these embryos, but rather it is required for the colonisation of bone marrow and maintenance of haematopoiesis in the adult. It was later confirmed using conditional *ETV6* mutant mice that TEL selectively regulates adult HSC survival but is unnecessary for lineage-specification (Hock *et al* 2004).

4.1.5 TEL fusion proteins in leukaemias

The high frequency of chromosome abnormalities involving the short arm of chromosome 12 (i.e. 12p) had been noted several years before TEL was identified

(Raimondi *et al* 1986). After the *ETV6* gene had been identified, it was found to be the gene frequently involved in fusion proteins in leukaemias and other haematological malignancies (reviewed in Bohlander 2005). The fusion partners of *TEL* can be roughly divided into two groups: protein tyrosine kinases, and transcription factors.

Genes for Protein Tyrosine Kinases (PTKs) that have been described as fusion genes with TEL include the aforementioned *PDGFRβ* (Golub *et al* 1994), *ABL1* (Papadopoulos *et al* 1995), *ABL2* (Cazzaniga *et al* 1999), and *JAK2* (Lacronique *et al* 1997). The characteristic feature of all these TEL-PTK fusion proteins is that they conserve the helix-loop-helix domain of TEL (known as *pointed*, and required for dimerisation of TEL; Kim *et al* 2001) and the kinase domain of the PTK partner. This result in a mechanism of action common to TEL-PTKs fusions; the protein dimerises via the *pointed* domains of TEL, which constitutively activates the auto-phosphorylation activity of the PTK partner, and leads to the phosphorylation and activation of downstream targets required for cell survival and proliferation.

The most common transcription factor (TF) that has been found in fusions with TEL is *RUNX1*, coding for AML1 (Golub *et al* 1995). Other TF fusion partners are much rarer; they include *MN1* (Buijs *et al* 1995), *MDS1* (Peeters *et al* 1997), *BTL* (Cools *et al* 1999), and *PAX5* (Cazzaniga *et al* 2001). The TEL/AML1 fusion protein conserves the *pointed* domain of TEL; the mechanism of action of TEL/AML1 is discussed in the Introduction (section 1.8.3). However, the pointed domain of TEL is not present in the other TEL-TF fusion proteins, and so there is little known about their mechanism of action.

4.1.6 TEL fusion proteins in other cancers

The above fusion genes are all involved in haematological malignancies, but TEL fusions have also been described in other cancers. The fusion of *ETV6* to the gene coding for neurotrophic tyrosine kinase, receptor type 3 (NTRK3) has been observed in mesoblastic nephroma (Knezevich *et al* 1998a), congenital fibrosarcoma (Knezevich *et al* 1998b), and secretory breast carcinoma (Tognon *et al* 2002).

Ets family members related to ETV6 also contribute towards gene fusions in prostate cancer (Narod *et al* 2008; Clark & Cooper 2009).

4.1.7 Role of TEL in TEL/AML1$^+$ leukaemia

The most common secondary mutation in TEL/AML1$^+$ leukaemia is the deletion of the second untranslocated *ETV6* allele (Raynaud *et al* 1996; Cave *et al* 1997). This loss of heterozygosity has been detected in about 75% of TEL/AML1$^+$ leukaemia patients (Cave *et al* 1997; Patel *et al* 2003). *ETV6* deletions do occur in TEL/AML1$^-$ leukaemia, but at a much lower frequency (Attarbaschi *et al* 2004). Two separate studies have shown that the gene expression profiles of TEL/AML1$^-$ leukaemia with a *ETV6* deletion are similar to the profiles from TEL/AML1$^+$ leukaemia (Yeoh *et al* 2002; Attarbaschi *et al* 2004), indicating that the common biology is a result of the impairment of the TEL gene, in either one or both alleles.

The frequent loss of heterozygosity of TEL in leukaemia (Cave *et al* 1995) – as well as ovarian cancer (Hatta *et al* 1997) – suggests a tumour suppressor role for TEL. Indeed, the expression of TEL in Ras-transformed NIH-3T3 cells inhibits their growth and inhibits their tumour invasion *in vivo* (Fenrick *et al* 2000), whilst predisposing them to apoptosis via the repression of *Bcl-X$_L$* (Irvin *et al* 2003). It has been shown that TEL/AML1 has a dominant negative effect on the growth suppressive activity of wild-type TEL, by heterodimerisation via the *pointed* domain (Gunji *et al* 2004), so providing a further mechanism how the loss of TEL activity contributes to leukaemia progression. Tsuzuki and colleagues have also shown that the forced expression of TEL in the TEL/AML1$^+$ cell lines REH slows down proliferation and cell division (Tsuzuki *et al* 2007).

However, few of the target genes of TEL are known, particularly in the context of TEL/AML1$^+$ leukaemia. Using two cell lines derived from the same TEL/AML1$^+$ ALL patient, one showing complete loss of TEL amongst other chromosomal changes, Fears and colleagues showed T Cell Leukaemia/Lymphoma Protein 1 (TCL1) was expressed at a higher level in the TEL-negative cell line, and that it could be downregulated by overexpression of TEL in these cells (Fears *et al* 2002).

4.1.8 Identifying targets of TEL repression

In addition to a core binding sequence (GGAA/T) which defines the DNA binding activity of the *ets* domain (Karim *et al* 1990), recent work has elucidated the preferred DNA sequences surrounding this motif to which the members of the *ets* family binds, including TEL (Wei *et al* 2010). Using a high-throughput DNA binding specificity assay, Wei and colleagues defined a series of high affinity binding sequences for human *ets* family members, which they then grouped into four clusters depending on their sequence preference. Representative members of each group were assessed for *in vivo* binding specificity using chromatin immunoprecipitation (ChIP); in the case of the group ETV6 belonged to, ELF1 was used. They found that whilst the ChIP-determined binding sites were highly enriched for the binding sequences defined *in vitro*, only a small fraction of the possible binding sites for *ets* family members were occupied by those proteins. This suggests that the binding of *ets* family members, including TEL, can be determined by accessibility to the DNA, regulated by chromatin packaging (Wei *et al* 2010).

Other researchers have also shown that the targets of TEL are determined by factors other than sequence specificity. Green and colleagues (2010) showed that the binding affinity of TEL to sequences is dependent on polymerisation. Monomeric TEL (in which two point mutations disrupt the activity of the *pointed* domain) has a lower affinity than expected for a TEL-specific binding sequence. They explained this by a C-terminal inhibitory domain (CID) to the DNA-binding *ets* domain, reducing its binding to DNA. Conversely, dimers of TEL have a higher affinity to DNA than monomeric TEL, and that DNA binding stabilises this dimer, evading the autoinhibition of the CID. Thus, Green and colleagues argued that TEL acts more readily at promoters with multiple binding sites (Green *et al* 2010), examples of which have already been defined, in the promoters of known TEL targets *Bcl-X_L* (Irvin *et al* 2003) and *stromelysin-1* (Wang & Hiebert 2001).

The examples above demonstrate that, to determine the role activity of TEL in leukaemia, it is not enough to know to which DNA sequences TEL binds, and that the activity of TEL, like that of any transcription factor, is context specific – dependent on factors such as the availability of binding sites through chromatin

packaging, and polymerisation. In addition, TEL is also known to regulate gene expression through the heterodimerisation with other transcription factors, including *ets* family members (Fears *et al* 1997; Kwiatkowski *et al* 1998; Schick *et al* 2004). Therefore, to determine the consequences of a loss of heterozygosity of TEL, I decided to pursue a more direct approach: analysing the changes in global gene expression caused by TEL using a gene expression microarray.

4.1.9 Use of microarrays to determine gene expression patterns

Gene expression microarrays provide the opportunity to determine on a large scale what genes are being expressed in a biological sample. Traditionally, mRNA extracted from cells is converted into cDNA and labelled with a fluorescent dye (e.g. Cy3); today, RNA synthesised from the extracted mRNA can be used instead of mRNA, though this does depend on a high quality of RNA. The labelled cDNAs or mRNAs are then hybridised with unique DNA probes printed onto a chip in spots; each probe unique to one gene. The levels of expression of genes represented on the chip can then be determined by measuring the fluorescence intensity of each probe spot, which is proportional to the amount of hybridised labelled cDNA.

These raw intensity values need to be corrected to counteract factors such as the concentration of the starting material, the intensity of the dye, the background fluorescence of the chip, and other factors that might influence the measurement and interpretation of fluorescence intensity values (Quackenbush 2002). Part of this normalisation process can involve adjusting the range of intensity values so that the average and spread for replicates within each group (e.g. condition or sample type) is the same. The gene expression values from the different samples, derived from the normalised intensity data, can be compared to give fold change in gene expression.

Following this, the data can be analysed depending on the question being addressed; for example, to identify a population of cells, mathematical clustering can be used to compare the gene expression profile to profiles from known cell profiles. For our purposes, it was useful to know what kinds of genes were differentially regulated; these could be identified by linking the gene expression changes to publicly available

databases for the function of genes (gene ontology), which could identify genes belonging to common pathways, such as proliferation, or apoptosis.

4.1.10 Studying leukaemia using cell lines

As discussed in the introduction to Chapter 3 (section 3.1.3), many molecular and cellular aspects of leukaemia have been investigated using cell lines, with the REH cell line in particular being frequently used to study TEL/AML1$^+$ leukaemia. Leukaemia cell lines that share specific genetic lesions with acute lymphoblastic leukaemia patient samples also share gene expression profiles, and this holds true for REH cells and lymphoblasts from TEL/AML1$^+$ leukaemia patients (Fine *et al* 2004; Andersson *et al* 2005a), endorsing its use as a model.

The REH cell line was used to demonstrate the molecular interactions of TEL/AML1 with its transcriptional corepressors Sin3A (Fenrick *et al* 1999) and N-CoR (Guidez *et al* 2000). The transcriptional profile defined by TEL/AML1 was investigated using REH cells and patient samples treated with HDAC inhibitors to remove the effect of gene repression by TEL/AML1 (Starkova *et al* 2007).

REH cells have been useful in identifying interactions between lymphoblasts and the stromal microenvironment, including how CXCR4 promotes chemotaxis (Burger *et al* 1999; Burger *et al* 2001), and how bone marrow stroma confers resistance to chemotherapy via TGFβ and BMP-6 induction of *TIEG1* expression (Dosen-Dahl *et al* 2008) and compensate for lost asparagines as a result of asparaginase chemotherapy (Iwamoto *et al* 2007). In addition, REH cells have been used to investigate the role of erythropoietin (EPO) signalling (Inthal *et al* 2008; Torrano *et al* 2011), survivin (Diakos *et al* 2010; Tyner *et al* 2011), and miRNAs (Gefen *et al* 2009; Diakos *et al* 2010) in TEL/AML1$^+$ leukaemia.

Finally, RNA interference (RNAi) has allowed researchers to knock down the expression of genes, and such approaches have been used frequently to ablate the expression of TEL/AML1 to investigate if and how it maintains the leukaemia phenotype of REH cells (Diakos *et al* 2007; Zaliova *et al* 2010; Fuka *et al* 2011a; Fuka *et al* 2011b; Zaliova *et al* 2011); results from these studies have indicated that

TEL/AML1 is not necessary for the survival of REH cells, suggesting that it becomes redundant after the initiation of leukaemia.

4.1.11 Aims

Given the loss of the untranslocated *ETV6* allele is a frequent feature of TEL/AML1$^+$ leukaemia, it is assumed to be the case that the loss of TEL confers some selective advantage to pre-leukaemic cells. To investigate this concept further, it was decided to overexpress TEL in the REH cell line so in effect reversing the progression of TEL/AML1$^+$ leukaemia. This would give an indication of what role TEL has in normal and TEL/AML1$^+$ 'pre-leukaemic' cells, and how its loss, or the dysregulation of the genes its controls, might represent a step towards overt leukaemia.

Using viral transduction, the gene expression profile of TEL-expressing REH cells was revealed using a gene expression microarray, and differentially expressed genes relevant to ALL were validated. I further investigated the effects of TEL on proliferation, cell cycle, and apoptosis.

4.2 Results

4.2.1 Gene expression microarray

As described in the introduction, despite the importance of TEL in both normal and malignant haematopoietic development, only a few of the targets of its transcriptional repression activity are known. I wished to address the question of why the loss of TEL is important in TEL/AML1$^+$ leukaemia. To do this, I decided to overexpress TEL in the REH cell line, which both carries the TEL/AML1 fusion gene and also lacks an endogenous TEL allele. By analysing the expression profile of TEL-overexpressing cells using a cDNA microarray, it could be determined what function TEL fulfils in these cells, and thus give an indication of the reason why the loss of TEL in the context of TEL/AML1$^+$ leukaemia is important for the progression of leukaemia.

To express TEL in REH cells, a Murine Stem Cell Virus (MSCV) oncoretroviral vector was used (kindly provided by Owen Williams). An Internal Ribosome Entry Site (IRES) allowed cells expressing TEL to be identified by simultaneous expression of GFP, with an oncoretroviral vector coding for GFP alone used as a control. The efficient of viral transduction was high; 48 hours after transduction, around 99% of cells transduced with either GFP-oncoretrovirus or TEL-GFP-oncoretrovirus were GFP-positive (Figure 4.1A).

REH cells transduced with either TEL-GFP or GFP oncoretrovirus were sorted by flow cytometry, with a post-sort purity of 99.7%. (Figure 4.1B). RNA was extracted from the sorted GFP$^+$ cells, and subjected to a RNA quality control test; Figure 4.1C shows the fragments of amplified (complimentary) RNA for loading onto the chip are of similar sizes between samples and within the necessary range (200-1000 nucleotides). Raw data from the microarray was normalised so that median values and spread of data for both replicates in each group were the same (Figure 4.1D).

The gene expression data from the microarray shows that, upon overexpression of TEL, there were more genes down-regulated than upregulated (Figure 4.2A). This might be expected, since TEL is a transcriptional repressor; however, not all the genes downregulated in the microarray will be direct targets of TEL. Figure 4.2B

shows the numbers of differentially expressed genes in graphical format, which illustrates more clearly the difference between the numbers of up- and downregulated genes and the levels of their expression. A heat map shows the differentially expressed genes in clusters of red (upregulated) and blue (downregulated) transcripts (Figure 4.2C)

A list of the top ten upregulated and downregulated genes in response to TEL overexpression is shown in Table 4.1 and Table 4.2, respectively. A set of genes were selected because of prior evidence of involvement in cancer or because of strong differential regulation indicated by the microarray, and are shown in Table 4.3. For reference, the analysis of microarray data gave TEL a fold-change value of 60.6.

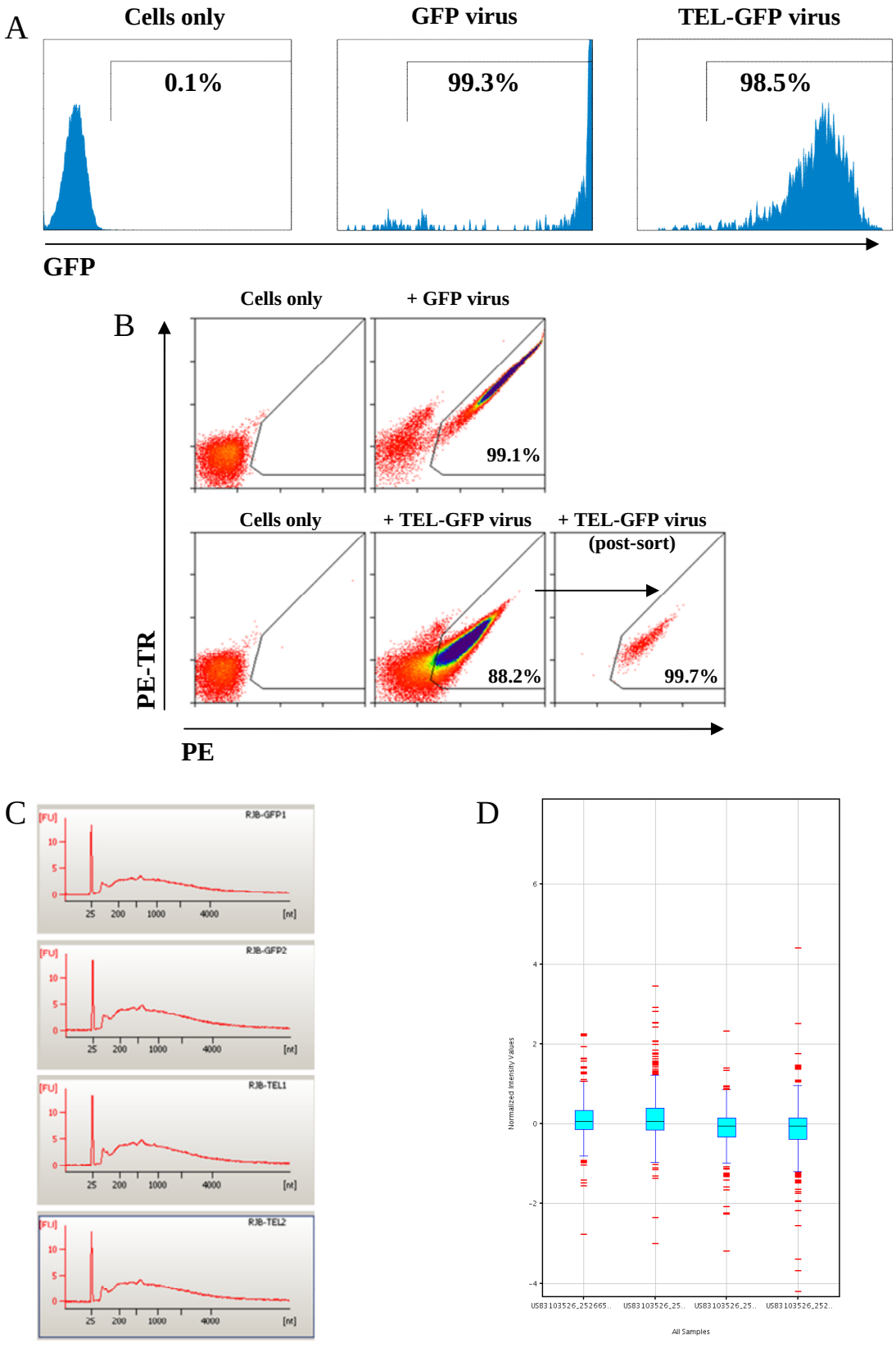
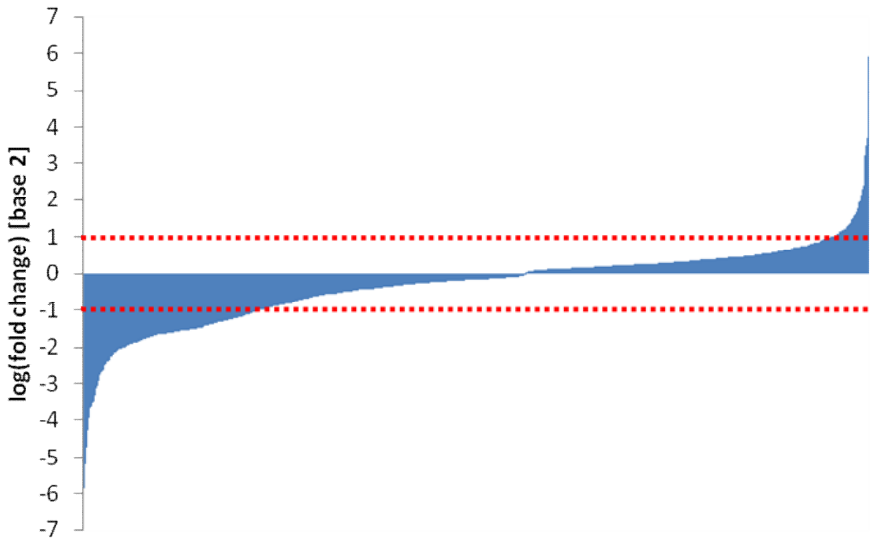


Figure 4.1 – Quality control for gene expression analysis
(A) Histograms showing the expression of GFP in cells transduced with oncoretrovirus coding for GFP (middle panel) or with oncoretrovirus coding for TEL and GFP (right panel); also shown is the percentage of cells in the gate displayed, designated GFP-positive. (B) Flow cytometry plots of cells transduced with oncoretroviruses for GFP (top row) or TEL-GFP (bottom row). (C) Electrophoretic plots to show RNA quality, from top to bottom, GFP1, GFP2, TEL1, TEL2. (D) Boxplot showing log intensity values from the microarray, normalised within each condition (GFP or TEL-GFP virus).

A

	up-regulated	down-regulated
Differentially expressed ($p \leq 0.05$)	710	915
>2-fold	84	361

B



C

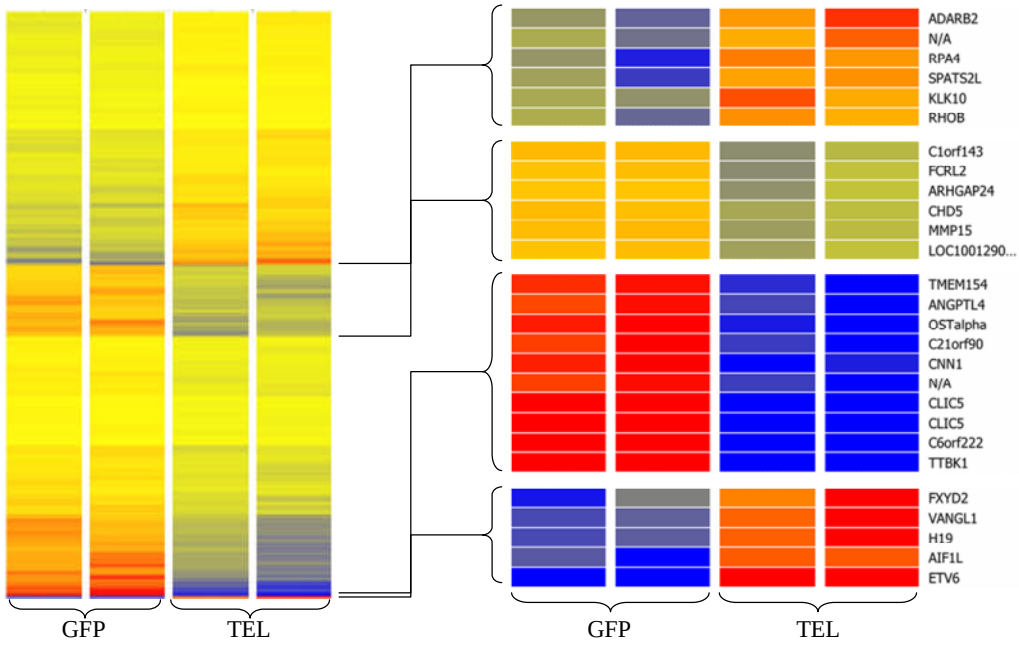


Figure 4.2 – Gene expression analysis by microarray.
(A) summary of the number of differentially up- and down-regulated genes on TEL overexpression, including those differentially expressed more than two-fold (B) graph showing the differentially expressed genes along the x-axis against their fold-change on the y-axis; red dotted line indicates two-fold difference (C) 'heat-map' showing the differentially expressed genes; red colouring indicates upregulated genes, blue indicates downregulated genes, and yellow indicates genes unchanged. Also shown are close-up views of selected regions of the heat map.

Gene code	RefSeq	Description	Fold-change
H19	NR_002196	H19, imprinted maternally expressed transcript (non-protein coding), non-coding RNA	13.02
AIF1L	NM_031426	allograft inflammatory factor 1-like, transcript variant 1, mRNA	12.39
VANGL1	NM_138959	vang-like 1 (van gogh, Drosophila), mRNA	10.44
FXYD2	NM_021603	FXYD domain containing ion transport regulator 2, transcript variant b, mRNA	9.39
ADARB2	NM_018702	adenosine deaminase, RNA-specific, B2 (RED2 homolog rat), mRNA	5.40
RPA4	NM_013347	replication protein A4, 34kDa, mRNA	5.33
MPO	NM_000250	myeloperoxidase, nuclear gene encoding mitochondrial protein, mRNA	5.12
IKZF2	NM_001079526	IKAROS family zinc finger 2 (Helios), transcript variant 2, mRNA	5.02
CTGF	NM_001901	connective tissue growth factor, mRNA	4.94
EPAS1	NM_001430	endothelial PAS domain protein , mRNA	4.77

Table 4.1 – Top ten genes up-regulated on TEL overexpression
(excluding TEL, which had a fold change value of 60.6)

Gene code	RefSeq	Description	Fold-change
TTBK1	NM_032538	tau tubulin kinase 1, mRNA	-56.64
C6orf222	NM_001010903	chromosome 6 open reading frame 222, mRNA	-49.98
CLIC5	NM_016929	chloride intracellular channel 5, transcript variant 2, mRNA	-46.16
CNN1	NM_001299	calponin 1, basic, smooth muscle, mRNA	-27.70
C21orf90	NR_026547	chromosome 21 open reading frame 90, transcript variant 1, non-coding RNA	-26.48
OSTalpha	NM_152672	organic solute transporter alpha, mRNA	-22.25
FAM69C	NM_001044369	family with sequence similarity 69, member C, mRNA	-19.35
TMEM154	NM_152680	transmembrane protein 154, mRNA	-18.11
FLJ32810	NM_152432	Rho-type GTPase-activating protein FLJ32810, mRNA	-15.15
ANGPTL4	NM_139314	angiopoietin-like 4, transcript variant 1, mRNA	-14.87

Table 4.2 – Top ten genes down-regulated on TEL overexpression

Gene code	RefSeq	Description	Fold-change
CDKN1B	NM_004064	cyclin-dependent kinase inhibitor 1B (p27, Kip1), mRNA	1.66
BCL-2	NM_000633	B-cell CLL/lymphoma 2, nuclear gene encoding mitochondrial protein, transcript variant alpha, mRNA	-1.83
EPOR	NM_000121	erythropoietin receptor, mRNA	-1.80
ANGPTL4	NM_139314	angiopoietin-like 4, transcript variant 1, mRNA	-14.87
IKZF2	NM_001079526	IKAROS family zinc finger 2 (Helios), transcript variant 2, mRNA	5.02
CLIC5	NM_016929	chloride intracellular channel 5, transcript variant 2, mRNA	-46.16
TTBK1	NM_032538	tau tubulin kinase 1, mRNA	-56.64
NETO1	NM_138999	neuropilin (NRP) and tolloid (TLL)-like 1, transcript variant 1, mRNA	-11.75

Table 4.3 – List of genes from microarray whose expression are validated in this chapter

4.2.2 Ingenuity Pathway Analysis

To identify the cellular functions affected by the gene expression changes as a result of TEL overexpression, the data was further analysed by Ingenuity Pathway Analysis (IPA) software. Functional analysis in the IPA depends upon knowledge about which genes are involved in a particular function (for example, 'differentiation of B lymphocytes'), and whether these genes positively or negatively affect this function. By comparing these genes set with the differentially expressed genes in our microarray dataset, it can be predicted what functions are being affected and in which direction (i.e. whether in our microarray the genes changed suggest an activation or an inhibition of a particular function).

Table 4.4 lists all the functions predicted to be affected by gene expression changes observed in the microarray. Each function is accompanied with the following information:

- Activation state – whether the gene expression changes in our dataset suggest either a decrease or an increase the function
- z-score – how strong directionally the association is, based upon how many genes have expression direction consistent with the activation state, and how

strongly each gene affects the function; negative values indicate the function is predicted to be decreased, positive values indicate the function is predicated to be increased (z-scores with absolute values larger than 2 are considered significantly affected; only these are shown in Table 4.4)

- p-value – the statistical measure of the overlap between the genes associated with the function and the differentially expressed genes in the microarray (p-values less than 0.05 are considered statistically significant)
- # genes – how many genes identified in our microarray are known to be involved in the function, whether they decrease or increase the function, or have an unknown or ambiguous effect

Functions	Predicted Activation State	z-score	p-value	# genes
proliferation of immune cells	Decreased	-3.372	5.98E-05	75
proliferation of lymphocytes	Decreased	-3.364	7.84E-05	71
proliferation of blood cells	Decreased	-3.155	1.46E-04	77
proliferation of T lymphocytes	Decreased	-3.118	3.66E-03	53
differentiation of leukocytes	Decreased	-3.105	2.97E-03	59
development of blood cells	Decreased	-2.918	4.04E-03	59
proliferation of B lymphocytes	Decreased	-2.795	4.46E-05	34
quantity of osteoclasts	Decreased	-2.750	8.69E-03	12
protein kinase cascade	Decreased	-2.667	7.03E-03	33
differentiation of mononuclear leukocytes	Decreased	-2.650	5.05E-03	49
differentiation of T lymphocytes	Decreased	-2.649	3.29E-03	35
activation of antigen presenting cells	Decreased	-2.580	3.15E-03	24
cell movement of endothelial cells	Decreased	-2.552	8.23E-03	24
differentiation of blood cells	Decreased	-2.510	3.85E-04	71
differentiation of lymphocytes	Decreased	-2.509	4.02E-03	46
proliferation of cells	Decreased	-2.368	1.72E-03	216
haematopoiesis	Decreased	-2.354	9.03E-05	89
cellular homeostasis	Decreased	-2.314	9.93E-04	114
differentiation of cells	Decreased	-2.311	5.70E-04	157
differentiation	Decreased	-2.233	2.46E-04	169
activation of macrophages	Decreased	-2.181	2.40E-03	18
function of blood cells	Decreased	-2.055	5.05E-03	53
proliferation of hepatocytes	Decreased	-2.015	2.21E-03	16
uptake of anion	Increased	2.018	7.05E-03	6
infection by bacteria	Increased	2.516	1.25E-03	40
infection by virus	Increased	2.749	8.33E-03	105

Table 4.4 – Functions predicted to be affected by TEL overexpression, based upon gene expression changes from the microarray
p-values are formatted as follows: 5.98E-05 = 5.98×10^{-5} = 0.0000598.

Table 4.4 indicates that functions predicted to be decreased include the proliferation of a variety of immune cell types, including T and B lymphocytes. This finding is significant in our model, since it suggests that in the context of leukaemia, the loss of TEL might result in an increase in proliferation of lymphoblasts. Several other functions predicted to be decreased include differentiation of blood cells, including lymphocytes. However, as leukaemic lymphoblasts are immature cells, it might be expected that if the loss of TEL contributes to the progression of the disease, that the overexpression of TEL might increase differentiation, rather than decrease. TEL appears to reduce the number of osteoclasts, a bone-resorbing haematopoietic cell derived from the monocyte/macrophage lineage (Boyle *et al* 2003). In addition, there are functions that are affected that do not appear to have any direct relevance to leukaemia; e.g. proliferation of hepatocytes, uptake of anion, infection by bacteria, infection by virus. These may be indications of other functions of TEL in non-haematopoietic tissues.

Since TEL is known to affect the function of other transcription factors by heterodimerisation (Fears *et al* 1997; Kwiatkowski *et al* 1998; Schick *et al* 2004), the gene expression data was subjected to an analysis for transcription factor activity. IPA compares the gene expression data with the known targets of a database of transcriptional regulators, in an attempt to identify which gene expression changes might be associated with the inhibition or activation of a transcriptional regulator. This produces a list of transcription factor proteins whose activities might be affected; note that this list is not dependent on any gene expression changes of these transcription factors, simply their respective targets. The output is a z-score for the direction in which the transcription factor is affected (i.e. decreased or increased), and a p-value to indicate the statistical significance of the overlap between the known targets of the transcription factor and the differentially expressed genes in the microarray dataset.

Table 4.5 shows the list of transcription factors predicted to be affected based upon the gene expression changes observed in our microarray. Transcription factors known to be regulated by heterodimerisation with TEL, for example STAT3 (Schick *et al* 2004) and FLI1 (Kwiatkowski *et al* 1998), are not seen in this list. However, the list of transcription factors might represent new partners of TEL. Of particular note is

the transcription factor SPI1 (also known as PU.1), which is another member of the *ets* family along with TEL and FLI1. PU.1 is known to be involved in both normal haematopoiesis and leukaemia (Gupta *et al* 2006; Kastner & Chan 2008; Gupta *et al* 2009).

Transcription Regulator	Predicted Activation State	z-score	p-value
WT1	**Inhibited**	**-2.671**	**2.70E-02**
RELA	Inhibited	-2.618	7.12E-02
EP300	Inhibited	-2.513	1.27E-01
Nfat family	**Inhibited**	**-2.238**	**5.75E-03**
HMGB1	Inhibited	-2.216	9.47E-02
SPI1	Inhibited	-2.175	1.07E-01
MYCN	Inhibited	-2.128	5.02E-01
HOXA9	**Inhibited**	**-2.072**	**2.49E-02**
FOXC2	Inhibited	-2.069	9.23E-02
NfkB-RelA	**Inhibited**	**-2.012**	**1.39E-02**
ESR1	Activated	2.064	7.81E-02
SMAD2	Activated	2.076	3.41E-01
SMARCA4	Activated	2.096	7.52E-02
PPARD	Activated	2.619	2.58E-01

Table 4.5 – Transcription factors predicted to be affected by TEL overexpression, based upon gene expression changes from microarray
In **bold** are predictions deemed statistically significant ($p < 0.05$)

In light of the importance of proliferation indicated by IPA I decided to further investigate the effect on proliferation of overexpression of TEL in REH cells.

4.2.3 Effects of TEL transduction on proliferation

Previous work on the role of TEL in leukaemia has indicated that a loss of TEL results in an increase in the proliferation (Fenrick *et al* 2000; Tsuzuki *et al* 2007). Analysis of the gene expression data indicated that a gene involved in proliferation and cell cycle control, namely p27-KIP1 (CDKN1B), was differentially expressed (Table 4.3). p16-INK4A (CDKN2A), another gene involved in cell cycle control, has been previously shown to be a common secondary mutation in acute lymphoblastic leukaemia, as described in the Introduction chapter (section 1.9.2). However, when the expression of these genes was analysed by qPCR, using the same RNA as used in the microarray, I found no evidence of differential expression of either of these genes (Figure 4.3A). Nevertheless, I decided to continue to investigate further the role of TEL on the proliferation of REH cells.

The effects of oncoretroviral transduction of TEL on proliferation were studied by analysing DNA content by the double stranded DNA stain DAPI and analysed by flow cytometry. DNA content histograms were deconvoluted by using FlowJo software, giving an estimate of the numbers of cells in the stages of cell cycle as shown in Figure 4.3B. Cells in a sub-G1 position on the DNA histogram represent apoptotic cells undergoing DNA fragmentation.

Frequencies calculated using the FlowJo software were compiled from GFP-transduced or TEL-GFP-transduced REH cells at 24 hour intervals over 96 hours. The expression of TEL in REH resulted in only subtle effects on cell cycle progression, with a small increase in the numbers of cells in G1 phase (Figure 4.3C), and small decreases in the numbers of cells in S (Figure 4.3D) and G2 phases (Figure 4.3E). Also, there was a small increase in the numbers of sub-G1 cells (Figure 4.3F) cells. Further replicates would have to be performed to show whether this is reproducible and statistically significant, but they indicate the expression of TEL results in only a slightly reduced proliferation in REH cells, and increased apoptosis.

I investigated the effect of TEL overexpression on proliferation further using a different expression method, that of DNA plasmid transfection. This would allow me to rule out any possibility that the gene delivery method was affecting the results.

4.2.4 Cloning of pCI-TEL-neo^{r}

To generate a plasmid to over-express TEL in REH cells, TEL cDNA was inserted into the multiple cloning site (MCS) of the pCIneo expression plasmid (Promega; Figure 4.4A), by standard restriction-ligation method using *SalI* and *NotI* at the 5’ and 3’ end of TEL, respectively. Digests of both the library plasmid in which the TEL cDNA was contained and pCIneo was performed (Figure 4.4B), and the fragments ligated with T4 Ligase, giving rise to plasmid clone, several of which were assessed to contain both the pCIneo vector and the TEL insert by restriction digest (Figure 4.4C).

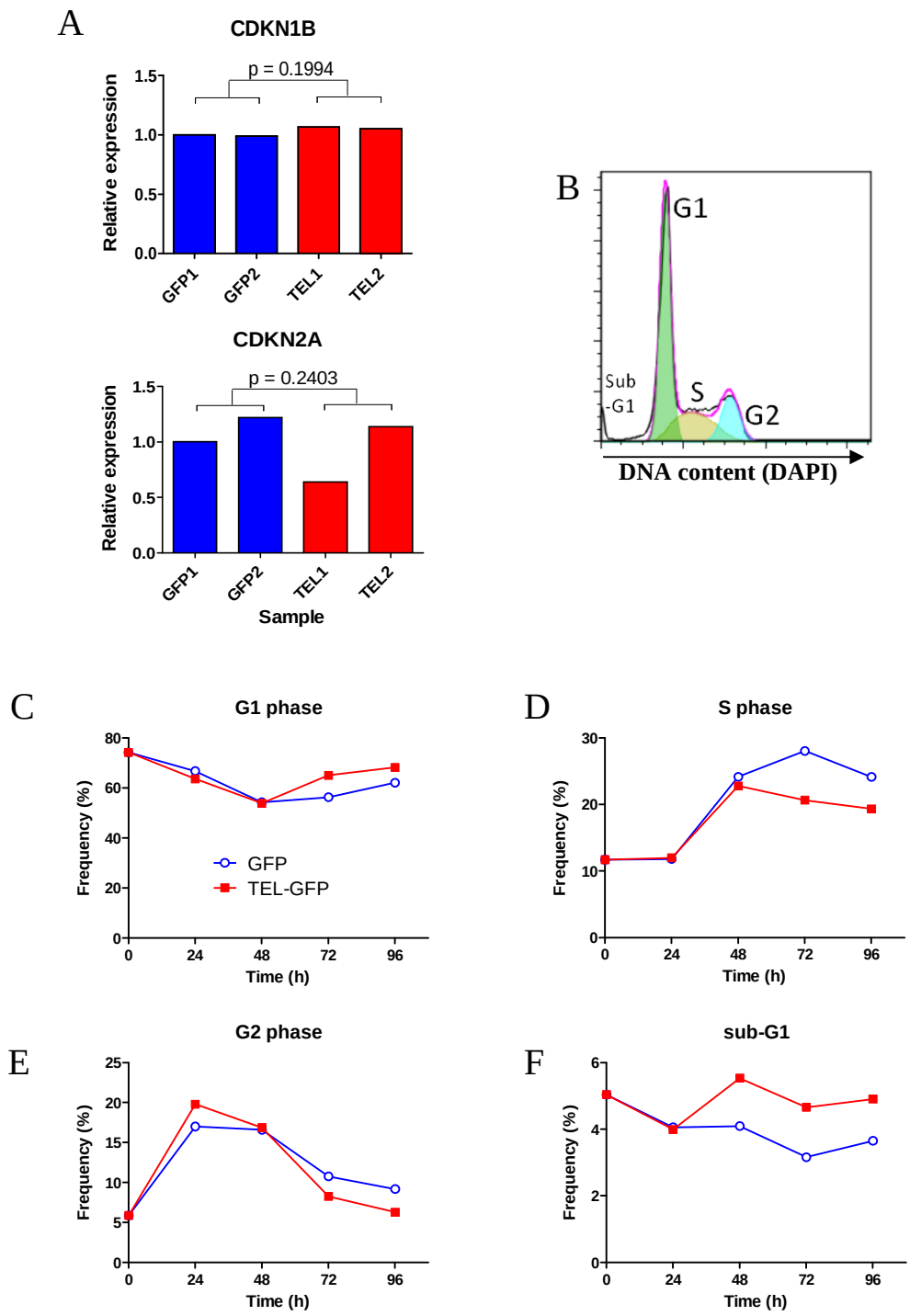


Figure 4.3 – Effects of oncoretroviral transduction of TEL on cell cycle.
(A) Expression of genes associated with cell cycle control (CDKN1B, upper, and CDKN2A, lower), as analysed by qPCR. (B) Representative histogram showing DNA content of cells, stained with DAPI after permeabilisation. Histograms were analysed on FlowJo software and cell cycle populations were fitted to the histogram as shown. (C-F) Percentage of cells transduced with GFP oncoretrovirus (open blue circles) or TEL-GFP oncoretrovirus (closed red squares) populating G1 phase (C), S phase (D), G2 phase (E), or sub-G1 (F) against time after transduction (n=1).

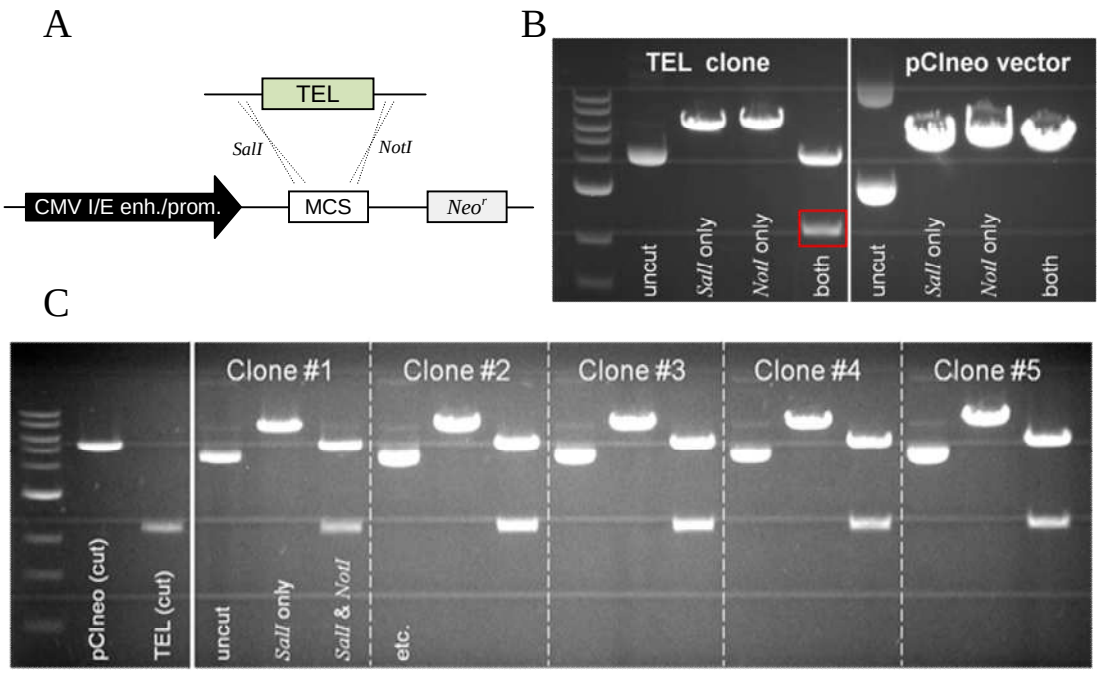


Figure 4.4 – Cloning of pCI-TEL-neo^{r}.
(A) Cloning strategy used to construct the pCI-TEL-neo^{r} plasmid. (B) Digest of TEL clone and pCIneo vector with *SalI* and *NotI* restriction enzymes. (C) Plasmid clones after ligation of digested TEL and pCIneo fragments. Plasmid clones are digested to show presence of both pCIneo and TEL.

4.2.5 Short term effect of TEL on growth

With the TEL plasmid, I first intended to investigate the short term effect of the expression of TEL in REH cells. However, without a fluorescently labelled TEL protein or a plasmid able to express a fluorescent label at the same time, and knowing the transfection efficiency of REH was less than 100%, it would not be obvious which cells were expressing TEL and which were not. I decided to circumvent this problem by co-transfecting TEL with a separate expression plasmid for a fluorescent protein.

Figure 4.5A demonstrates the phenomena of co-transfection using two fluorescent plasmids, one for the fluorescent protein Citrine (a Yellow Fluorescent Protein derivative; Griesbeck *et al* 2001) and one for Tomato (a DsRed derivative; Shaner *et al* 2004). Transfection of REH cells with both plasmids fluorescent plasmids results in cells that express both fluorescent proteins, and that the expression levels of Tomato and Citrine increases linearly; i.e. cells expressing high levels of Tomato express high levels of Citrine, and *vice versa*. There is also a subtle increase in the transfection efficiency of both plasmids in co-transfection, compared to when each was transfected individually (20% to 29% for Tomato, 40% to 43% for Citrine).

These phenomena were exploited to investigate the effect of TEL on the proliferation of REH cells by co-transfecting REH with both Citrine and TEL plasmids. Based upon Figure 4.5A, it could be assumed that most Citrine$^+$ cells will also express TEL, and that levels of Citrine expression could be used as a surrogate marker for levels of TEL expression.

I assessed the proliferation of REH cells by measurement of DNA synthesis using labelled nucleotide incorporation. In the current study, EdU taken up by the cell was labelled with Azide-Alexa647. The intensity of Alexa647 fluorescence in each cell is proportional to the amount of EdU incorporated into DNA by the cell (and thus the amount of DNA synthesis) during the incubation period. Thus, mean fluorescence intensity (MFI) of the fluorophore is a useful statistic to compare overall DNA synthesis rates; cells that are proliferating at a faster rate have more cells

synthesising DNA into which EdU is incorporated, resulting in a higher MFI than slowly proliferating cells.

Figure 4.5B shows the effect that transfection of Citrine and the co-transfection of Citrine and TEL (with co-transfection of Citrine & empty vector pCIneo as a control) has on EdU uptake. Cells that were cotransfected with Citrine and either the TEL plasmid or the empty vector frequently had a Citrine^{-} fraction, the overall transfection rate of Citrine being roughly 20%. It was found that the MFI of the fluorescently labelled EdU in the Citrine^{-} fraction varied between samples depending on what they had been transfected with, even though it was assumed that such cells would not be carrying any exogenous plasmids. To control for this variation, I used the ratio between the MFI of the labelled EdU from the Citrine^{+} and Citrine^{-} fractions, assuming that the effect that was giving rise to this variation would affect both fractions equally.

Figure 4.5C shows the MFI ratio was the same between cells transfected with Citrine and cells co-transfected with Citrine and empty vector pCIneo, indicating that the presence of the second plasmids had no intrinsic effect on the proliferation rate. However, the MFI ratio was slightly lower in cells co-transfected with Citrine and TEL, owing to a low MFI in the Citrine^{+} population, which I assumed (based upon Figure 4.5A) has high levels of TEL expression.

One explanation for this reduction in the proliferation rate is that it is caused by the 'burden' of overexpressing *any* protein, regardless of what role it has. However, I did not believe this to be the case in this instance, since the vector transfected as the empty vector contains the gene for the protein to confer neomycin resistance (Neomycin Phosphotransferase), which would also inflict such a 'burden' upon the cells, but there was no change between cells transfected with Citrine alone and those transfected with Citrine and the empty vector (Figure 4.5C). Therefore, I concluded that the immediate effect of TEL after transfection (<48hrs) is a subtle reduction in the proliferation in REH cells.

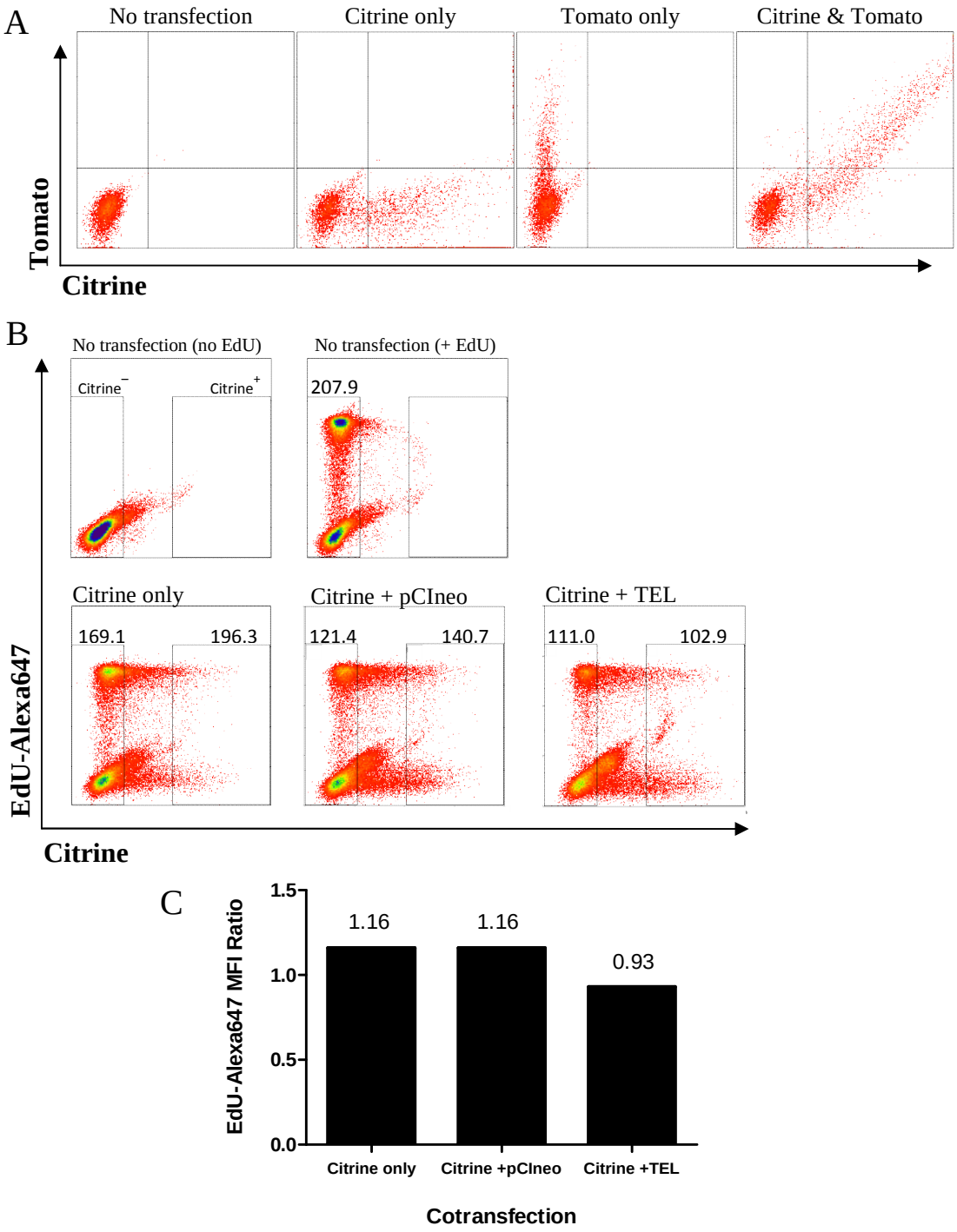


Figure 4.5 – Effects of TEL plasmid transfection on DNA synthesis
(A) Cotransfection of REH with two fluorescent protein plasmids (Citrine and Tomato), showing uptake of both plasmids and expression of both Citrine and Tomato at the same intensity. (B) Flow plots showing EdU uptake (visualised by azide-Alexa647 staining) and Citrine expression, when REH is transfected with Citrine alone, Citrine and pCIneo, or Citrine and pCI-TEL-neo. Numbers above gates indicate mean fluorescence intensity of Alexa647 of the events in the gate. (C) Bar graph showing the ratio between the mean fluorescence intensity (MFI) of EdU-Alexa647 staining of Citrine-positive cells and that of Citrine-negative cells (shown by gates in (B)); n=1.

4.2.6 Long term loss of TEL

REH cells were transfected with TEL or pCIneo, and the cultures were kept under selection (G418, 600µg/ml) for up to 22 weeks. However, after eight weeks (by which time all non-transfected REH cells would have been killed), the rates of EdU incorporation between TEL and pCIneo selected populations was similar (Figure 4.6A), as were the growth rates in culture (Figure 4.6B), and rates of cell death in response to Staurosporine (Figure 4.6C), a protein kinase inhibitor and apoptosis inducer (Bertrand *et al* 1994).

It was discovered by qPCR that the expression of TEL mRNA in the transfected REH cells decreased dramatically in the population between weeks 4 and 9, and remained low (Figure 4.6D), despite constant antibiotic selection in culture. This is consistent with the idea that TEL expression puts REH cells at a selective disadvantage in culture; since cell culture conditions favour the fastest growing cells, there is a strong selective pressure for any cells expressing genes which slow down cell growth (as has been shown for TEL in Figure 4.5) to silence the expression of these genes, and any cells doing so, by definition, will increase their numbers in the total population.

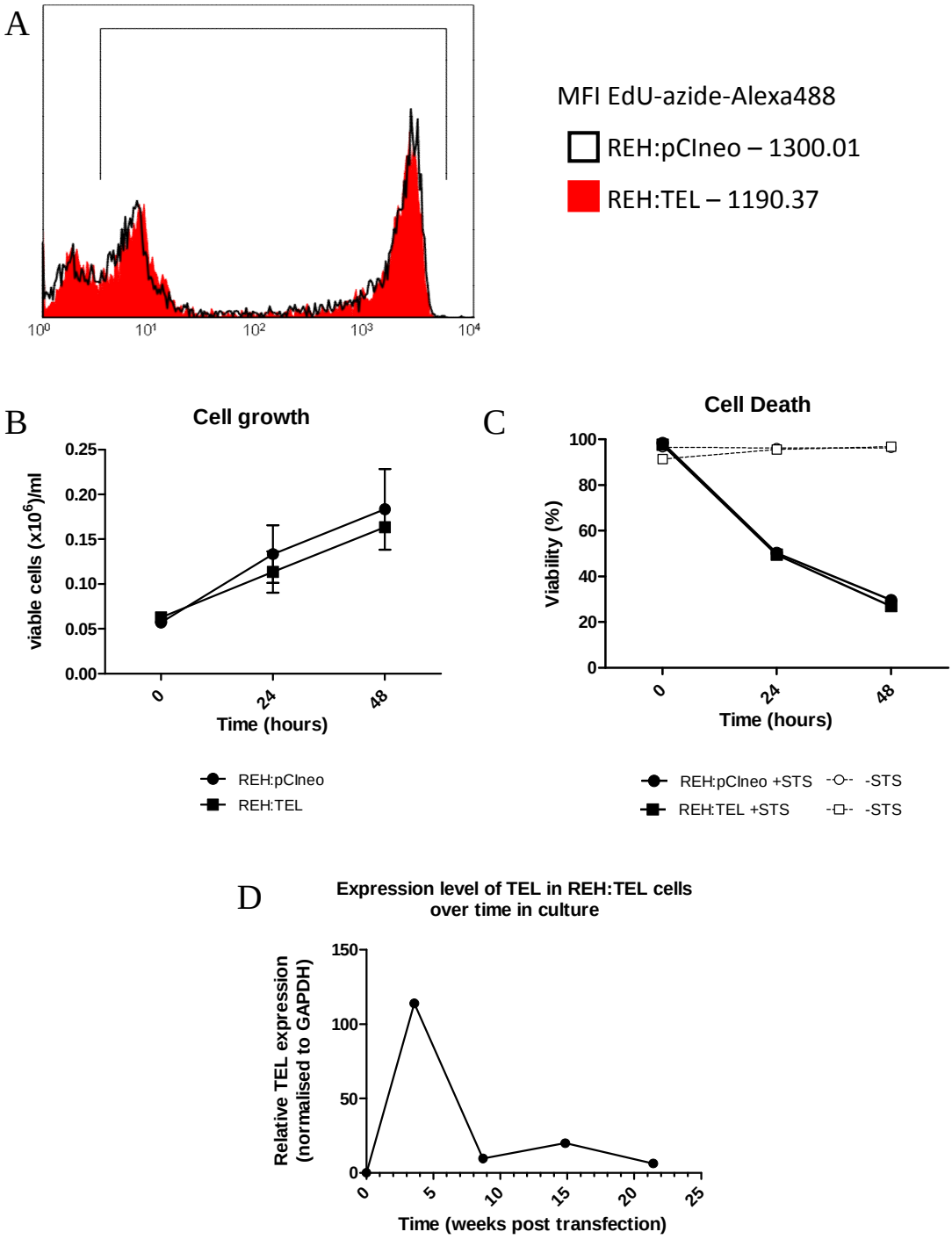


Figure 4.6 – Long term loss of TEL in culture.
(A) Comparing EdU uptake by flow cytometry in REH:pCIneo and REH:TEL cells after 8 weeks post-transfection in culture under G418 selection. (B) Graph showing growth of REH:pCIneo and REH:TEL cells seeded in triplicate, 12 weeks post-transfection in culture under G418 selection. (C) Graph showing death of REH:pCIneo and REH:TEL cells caused by 1µM Staurosporine (STS) after 14 weeks in culture under G418 selection. (D) Graph showing the relative expression level by qPCR of TEL in the bulk culture of REH:TEL over time (n=1).

4.2.7 Monoclonal REH:TEL cell lines

In an attempt to avoid the problems with 'bulk cultures' observed in Figure 4.6, I generated monoclonal cell lines to investigate proliferation. Fresh REH cells recently transfected with TEL or pCIneo were seeded into 96-well U-bottom plates in limiting dilution to ensure no more than one cell per well. Nine clones were generated; from TEL-transfected REH cells: T1C8, T1E10, T1G8, T2C5, and T2D10, and from pCIneo-transfected REH cells: p1C6, p1E9, p2B2, and p2D5.

A growth curve experiment was used to identify the slowest growing TEL-overexpressing clones (Figure 4.7A), and an estimated doubling time for each of the clones was calculated from these curves (Figure 4.7B). The doubling times from the curves vary from 27 hours to 49 hours, 27 hours being the approximate doubling time for untransfected REH cells (data not shown). However, unexpectedly, the two fastest growing clones were TEL-overexpressing (T1G8 and T1C5), and the slowest growing clone was a pCIneo-transfected clone (p2D5), and there appeared to be no consistent pattern in terms of the doubling times for the other TEL- or pCIneo-transfected clones.

To resolve this discrepancy, I assessed the transcript expression of TEL in the clones to see if the difference in growth rates could be attributed to the expression levels of TEL in the clones. TEL mRNA expression levels were all high in the clones from TEL-transfected REH (apart from clone T2D10) and low in the clones from pCIneo-transfected REH cells (Figure 4.7C). The expression levels of TEL did not appear to correlate with the growth rates of the clones.

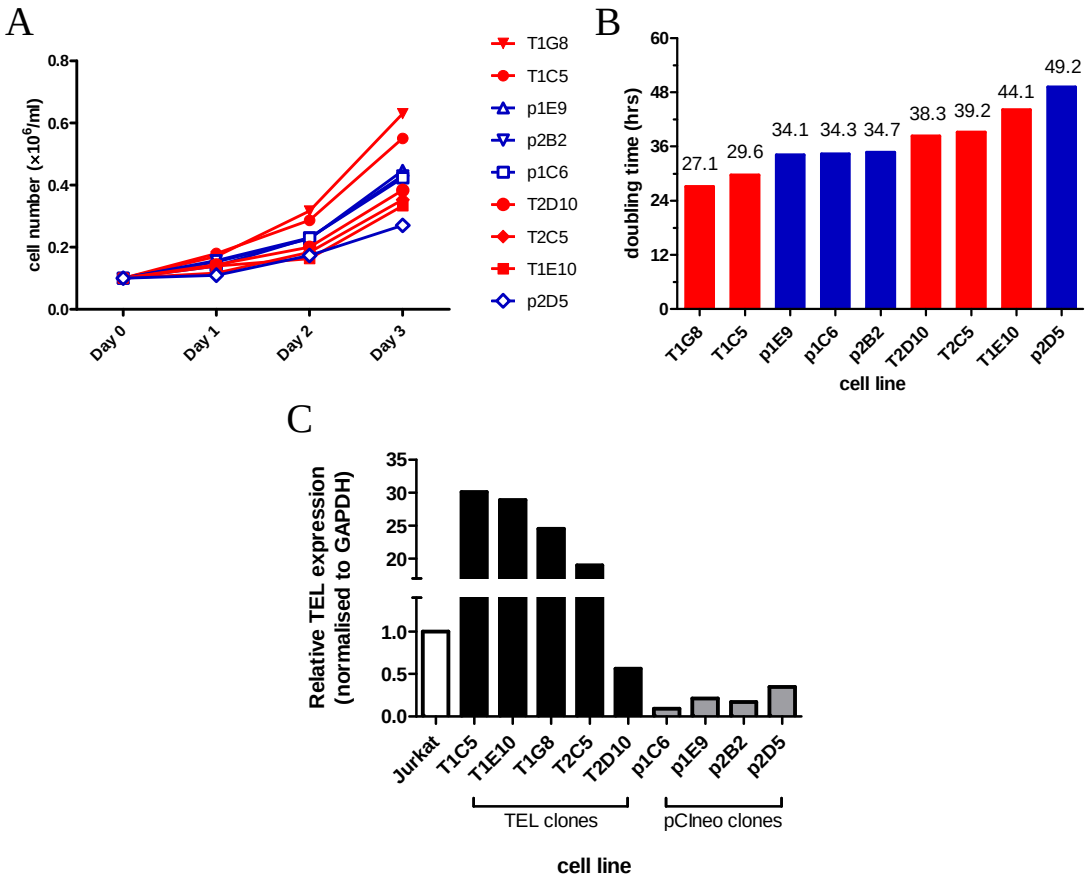


Figure 4.7 – Growth of monoclonal REH:TEL cell lines.
(A) Growth curves over three days calculated by automated cell counts starting with 10^5 cells. Legend lists cell lines in order of final cell count on day three, highest to lowest. Mean calculated from three technical replicates, n=1. (B) Doubling time of cell lines calculated from growth curves (C) Expression of TEL transcript in the monoclonal cell lines derived from REH transfected with TEL or pCIneo. Transcript expression level is normalised to GAPDH and compared to Jurkat cells.

4.2.8 Effects of TEL transduction on apoptosis

The microarray identified BCL-2, a gene associated with the control of apoptosis, as down-regulated 1.8-fold in TEL-transduced REH cells compared to GFP-transduced cells (Table 4.3). Analysis by qPCR, using the same RNA as was used in the microarray, indicated that BCL-2 was down-regulated in TEL-transduced REH cells compared to GFP-transduced cells (Figure 4.8A), but only by virtue of a high level of BCL-2 expression in one of the GFP-transduced cells. To further investigate the effect of TEL on apoptosis, cells were transduced with either GFP or TEL-GFP oncoretrovirus, and treated with or without Staurosporine (STS), an inhibitor of protein kinases, and a potent inducer of apoptosis (Jacobson *et al* 1993). The cells were stained with an antibody for Annexin-V and DAPI and analysed by flow cytometry to distinguish between live cells, and cells in the early or late stages of apoptosis (Figure 4.8B). The experimental schedule is shown in Figure 4.8C; briefly, REH cells were transduced with either GFP- or TEL-GFP-oncoretrovirus, and 24 hours later incubated with or without staurosporine, with samples taken at 24 hour intervals from before the transduction to two days after staurosporine treatment.

The frequencies of the populations in each condition are shown in Figure 4.8C. In the absence of staurosporine, the transduction of TEL did not increase either of the apoptotic cell populations compared to GFP-transduced cells. Staurosporine induced apoptosis in both GFP- and TEL-transduced cells, indicated by the dramatic decrease in live cells and the increase in the early and late apoptotic populations; however, there appeared to be no difference in the numbers of cells present in the apoptotic cell fractions between the GFP- or TEL-transduced cells, indicating that TEL did not have a significant effect on apoptosis in REH cells.

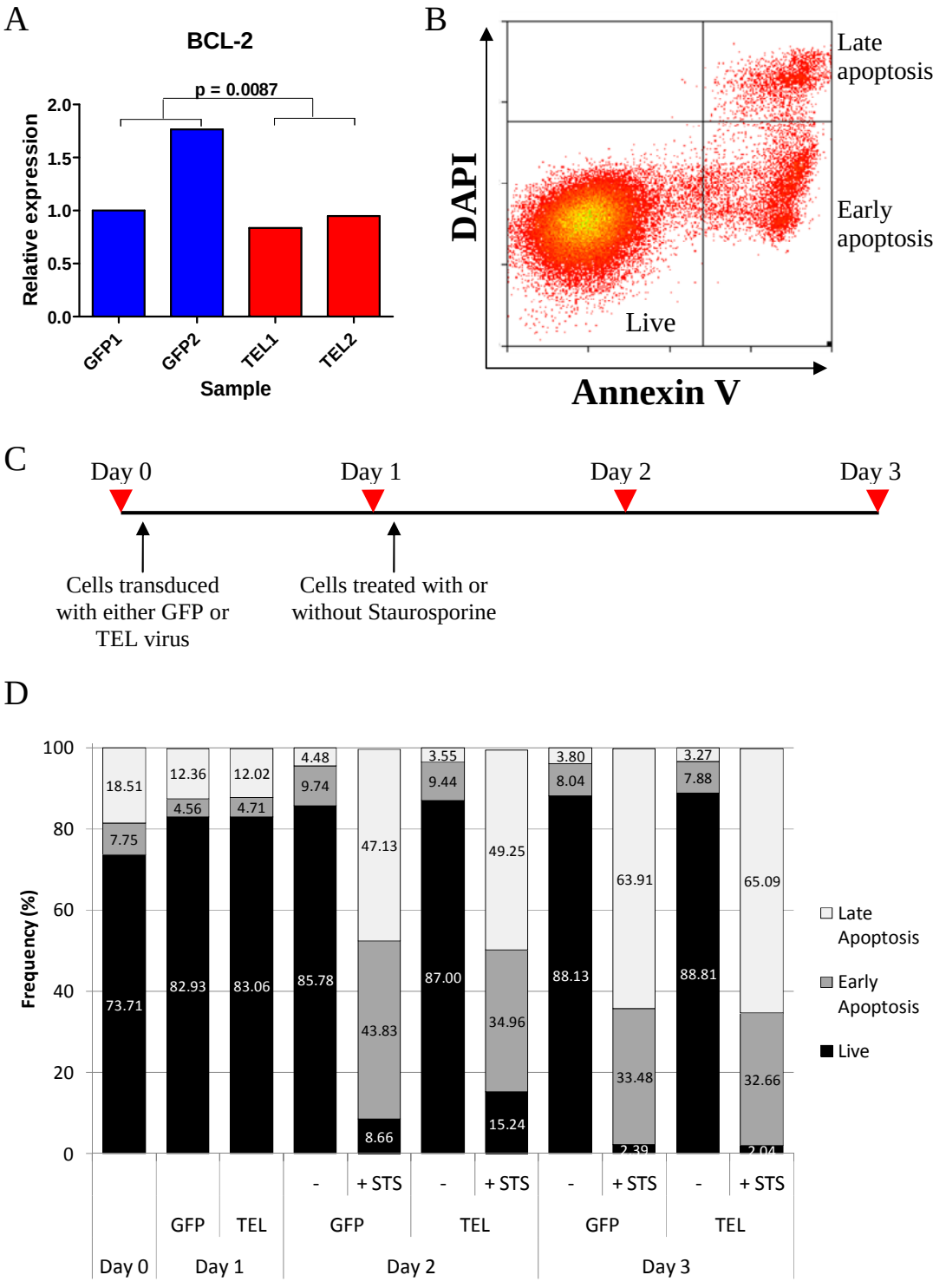


Figure 4.8 – Effects of oncoretroviral transduction of TEL on apoptosis.
(A) Expression of BCL-2, a gene associated with cell cycle control, as analysed by qPCR. (B) Flow cytometry histogram showing cells stained with Annexin-V antibody and DAPI to distinguish between live cells, cells in early apoptosis, and cells in late apoptosis. (C) Experimental set up; red arrow heads indicate when samples were taken for analysis, and black arrows indicate when cells were either transduced with GFP or TEL oncoretrovirus, or treated with staurosporine (STS; 1µM). (D) Frequencies of live cells, cells in early apoptosis, and cells in late apoptosis, as defined in (B) 'GFP' and 'TEL' indicate cells transduced with GFP- or TEL-oncoretrovirus, respectively, and '-' and '+ STS' indicated cells cultured in the absence or presence of 1µM staurosporine, respectively; n=1.

4.2.9 Further investigation of genes identified in microarray

In addition to identifying genes associated with proliferation and apoptosis, the gene expression microarray indicated other differentially expressed genes that have previously been connected to leukaemia, namely EPOR (erythropoietin receptor), ANGPTL4 (Angiopoietin-like 4), and IKZF2 (Helios; Table 4.3). Using the same RNA samples as were used in the microarray, the differential expression of these genes was further investigated by qPCR in Figure 4.9A. Despite being identified in the microarray as genes with differential expression, by qPCR no difference was observed in the expression of the genes in the GFP-transduced versus the TEL-transduced cells (Figure 4.9A).

Many of the genes identified in the microarray have not been previously linked to leukaemia, namely CLIC5, TTBK1, and NETO1. Each of these genes were strongly differentially expressed (Table 4.3), and so warranted further validation by qPCR.

CLIC5 (chloride intracellular channel protein 5) is a chloride ion channel found on internal membranes, that was originally identified in the microvilli of the placenta and found to associate with the actin cytoskeleton (Berryman & Bretscher 2000). CLIC5 has since been found in the cilia of hair cells of the inner ear, and is essential for the functions of the cochlea for hearing and the vestibular system for balance (Gagnon *et al* 2006). It is also highly expressed in skeletal muscle, and has a role in proliferation and differentiation of myoblasts (Li *et al* 2010). High expression of CLIC5 has been shown to be associated with TEL/AML1$^+$ leukaemia compared to other subtypes of ALL (Ross *et al* 2003), and also presented on murine follicular dendritic cells, which help form germinal centres, sites of rapid B cell proliferation (Huber *et al* 2005). Recently it has been identified as part of a multigene predictor of triple-negative breast cancer patients who are at low risk of metastasis (Yau *et al* 2010).

TTBK1 (tau tubulin kinase 1) is a brain-derived kinase that phosphorylates tau, β-tubulin, MAP2 and α-casein (Takahashi *et al* 1995). Variants of TTBK1 are associated with Alzheimer's disease (Vazquez-Higuera *et al* 2011; Yu *et al* 2011),

and transgenic mice expressing human TTBK1 show impaired memory (Sato *et al* 2008).

NETO1 (neuropilin and tolloid-like 1) is a transmembrane receptor that interacts with the NMDA receptor at neuron synapses, and is involved in memory and learning (Ng *et al* 2009). Deletion of the chromosome arm 18q where the NETO1 locus is situated has been associated with autism (O'Donnell *et al* 2010). NETO1 itself is a receptor for Semaphorin-3F (Sema3F; Gay *et al* 2011). Semaphorins are a class of signalling proteins involved in axonal guidance during neural development. SEMA3F has no known connection to leukaemia, but semaphorin family members have been linked to leukaemia in the past; for example, SEMA4D (also known as CD100) has been shown to help promote proliferation of B cell Chronic Lymphoid Leukaemia cells (Deaglio *et al* 2005). NETO1 has been shown to be upregulated in a human B cell line in response to hypoxic conditions (Kim *et al* 2006), purported to confer survival to leukaemia cells (Benito *et al* 2011).

Out of these three genes, two (CLIC5 and NETO1) were indicated by qPCR as being downregulated in the TEL-transduced REH cells compared to the GFP-transduced cells (Figure 4.9B). The expression of TTBK1 was not shown to be different between TEL-transduced and GFP-transduced cells.

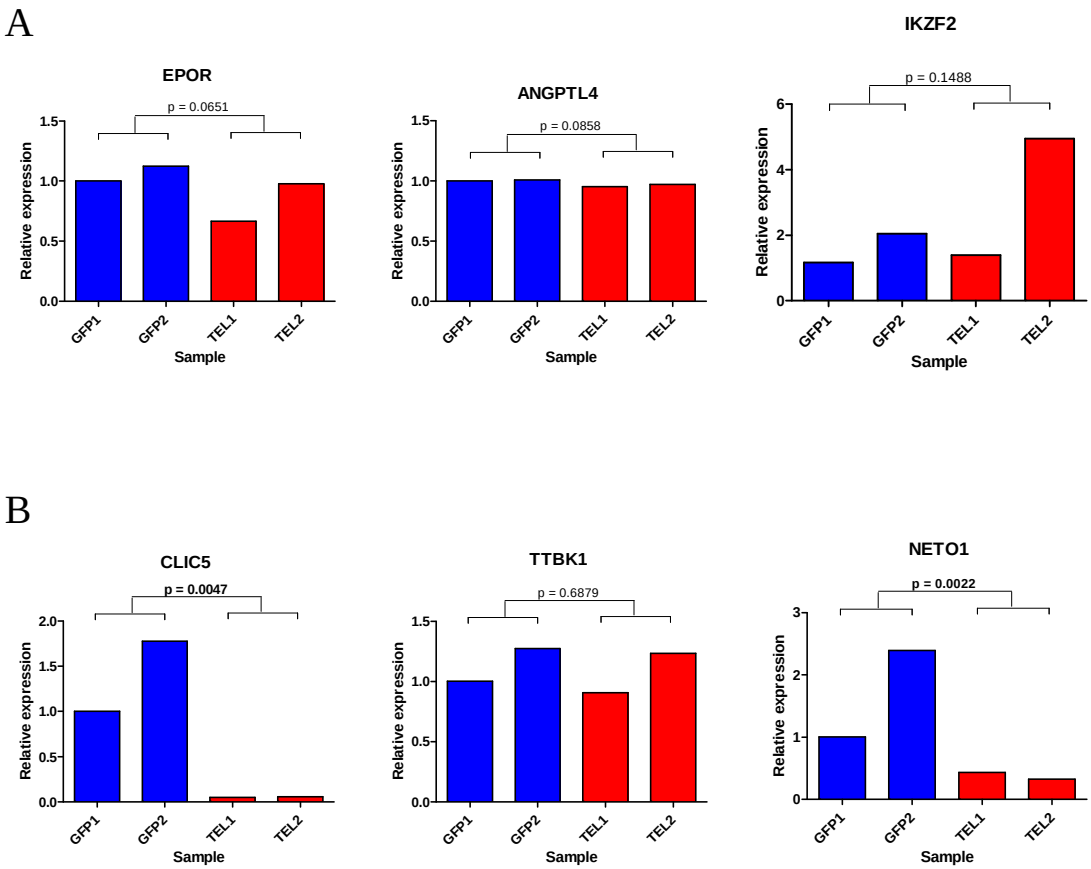


Figure 4.9 – qPCR data from other genes.
(A) Expression of EPOR, ANGPTL4, and IKZF2, by qPCR. (B) Expression of CLIC5, TTBK1, and NETO1, by qPCR.

4.3 Discussion

The data presented in this chapter describes the investigation of the role of TEL overexpression in the REH cell line, a model cell line of TEL/AML1$^+$ leukaemia. RNA extracted from REH cells after TEL or GFP transduction was run on a gene expression microarray. Of the differentially expressed genes, there were several genes involved in proliferation and apoptosis, two important factors in the development and progression of cancer.

I first investigated the effects of TEL on proliferation. REH cells transduced with oncoretroviruses expressing TEL and GFP only showed subtle changes in the progression of the cell cycle, as demonstrated by a slight decrease of cells in S and G_2 phases, compared to those transduced with GFP alone. I decided to further pursue the study of proliferation in REH by plasmid transfection. The early effects of the transfection of a TEL expression plasmid were to slow down proliferation compared to transfection with the empty vector, as demonstrated by reducing incorporation of EdU, a nucleotide analogue. However, this effect was lost over time in the bulk culture of TEL-transfected cells, concurrent with a loss of TEL expression, indicating a strong selective pressure of TEL-transfected REH cells to silence their TEL expression. The generation of monoclones gave conflicting results with regards to TEL mRNA and protein levels, as well as growth rates.

I next investigated the role of TEL in apoptosis. Despite a slight increase in sub-G_1 cells by cell cycle analysis, TEL transduction had no effect on cell death in response to staurosporine, as shown by Annexin-V staining. Finally I confirmed the differential expression of two sets of other genes from the microarray, one set of three genes that have previously been linked to leukaemia, and another set that have not had any connection to leukaemia before.

4.3.1 Gene expression microarray

There have been many examples of the use of gene expression microarrays to study acute lymphoblastic leukaemia. These have been used to identify the molecular subclasses of leukaemia and discover new subclasses (Fine *et al* 2004; De Pitta *et al* 2005; Den Boer *et al* 2009), and to generate a set of genes that can discriminate

between specific groups of ALL patients, for example TEL/AML1$^+$ patients (Gandemer *et al* 2007). The findings from such studies are useful in discovering what makes the subtypes different, which could be used in the future either for a more detailed diagnosis or for 'personalised treatment' for a specific subtype.

However, if one hopes to answer a fundamental question of leukaemia – 'what makes leukaemia different to normal haematopoiesis?' – then it would be more appropriate to look at the differences between leukaemic and normal blood cells. Analysing gene expression data to discriminate between different subtypes of ALL will, by definition, ignore gene expression patterns that are common to many of these subtypes. There are a few examples of studies that aim to identify the gene expression differences between leukaemic and normal haematopoietic cells (Andersson *et al* 2005b; Andersson *et al* 2010). Combining the results of these studies with those looking at specific subtypes will shed light on how specific causes can lead to common outcomes of malignant haematopoiesis. Similarly, the intention of this study was to investigate how the loss of TEL fits into the bigger picture molecular pathogenesis of leukaemia.

The differentially expressed genes were analysed by Ingenuity Pathway Analysis (IPA), which is designed to link genes with their known functions and interactions. Analysis of predicted perturbed functions in response to TEL overexpression identified decreases in several functions such as 'proliferation of immune cells', and 'proliferation of lymphocytes' (Table 4.4). This indicates that TEL has a role of suppression of proliferation in haematopoietic cells including B lymphocytes, which fits with the hypothesis that the loss of TEL is an important step in the progression of leukaemia, allowing lymphoblasts to proliferate unhindered. Genes whose differential expression identifies such perturbed functions would be interesting candidates for further analysis. In addition, other functions affected in this model, such as 'activation of antigen presenting cells' and 'activation of macrophages' may represent new functions for TEL. Other functions affected by TEL overexpression include decreases in 'quantity of osteoclasts' and 'cell movement of endothelial cells', and increases in 'uptake of anion', 'infection by viruses, and 'infection by bacteria' (Table 4.4). These may represent functions of TEL in other cells, but could

also be a result of 'false positives' in the differentially expressed genes from the microarray, and so not represent a physiological function of TEL.

Transcription factor analysis identified transcriptional regulators whose targets were differentially expressed in the microarray, and therefore suggests transcription factors whose activity may have been affected as a result of the overexpression of TEL (Table 4.5). Of particular note is the prediction of a decreased activity in the *ets* family member PU.1 (coded by the gene SPI1), which is involved in both normal and malignant haematopoiesis (Gupta *et al* 2006; Kastner & Chan 2008; Gupta *et al* 2009). The transcription factors predicted to be inhibited may represent new targets of TEL, and could be further investigated by protein binding studies.

I did not identify in the microarray genes previously shown to be regulated by TEL, such as *stromelysin-1* (Fenrick *et al* 2000; Wang & Hiebert 2001), *Bcl-X_L* (Irvin *et al* 2003), and *TCL1* (Fears *et al* 2002). It is possible that any differential expression of these genes did not meet the threshold of statistical significance, or are not expressed in REH cells. The expression of these genes could be analysed by qPCR.

The microarray identified some differentially expressed genes that have already been linked to TEL/AML1$^+$ leukaemia: for example, EPOR (Ross *et al* 2003; Fine *et al* 2004) and ANGPTL4 (Andersson *et al* 2005b). This was promising as it showed not only that our model was relevant to leukaemia, but it also suggests that the loss of functional TEL is a 'driver' mutation in TEL/AML1$^+$ leukaemia progression; that genes significantly associated with TEL/AML1$^+$ leukaemia are in fact the result of the loss of TEL. To identify further changes specific to TEL/AML1$^+$ leukaemia that could be caused by TEL, these results with other gene expression microarrays could be compared using a computational biology approach.

However, many of the highest deregulated genes have not been previously associated with leukaemia. I analysed three of the genes, CLIC5, NETO1, and TTBK1, all of which have had some involvement in neurons, neuronal development, or memory. TEL has been implicated in neuronal development and survival; TEL is expressed in the embryonic brain and *TEL$^{-/-}$* mouse embryos have defects of the neural tube and ganglia (Wang *et al* 1997). Both CLIC5 and NETO1 were confirmed as being

downregulated on overexpression of TEL (Figure 4.9B), which might suggest they are targets of TEL, or that they could be downregulated by an indirect mechanism. Whether either of these genes has any involvement in leukaemia is unconfirmed, but could be further investigated. For example, NETO1 is a receptor for the signalling protein Semaphorin-3F (Gay *et al* 2011), whose family members have been reported to be involved in proliferation of B-CLL cells (Deaglio *et al* 2005). To determine any effect of the loss of NETO1 in TEL/AML1$^+$ leukaemia and in particular in the role of the loss of TEL, using recombinant semaphorin-3F and anti-NETO1 blocking antibodies, the proliferation of TEL-transduced REH cells in the presence of semaphorin-3F could be assessed.

Nevertheless, out of the probes that were identified as differentially expressed in the microarray, it would not be expected that all of these are indeed differentially expressed *in vivo*. When comparing two groups for differentially expressed probes, a statistical significance threshold of $p = 0.05$ allows for 5% of the probes to be different purely by chance, or 1 in 20. For the 44,000 probe microarray, this equates to 2,200 probes that would be appear to be 'differentially expressed' by chance. Our microarray results show that roughly 1,600 probes are differentially expressed, meaning I cannot be confident that the genes that represent these probes are genuinely differentially expressed genes. A correction to adjust the p-values to take this into account was not successful due to the small sample size in each group (Peter Ashton, personal communication).

It was therefore decided to validate the differential expression of some of the genes directly by qPCR. Out of the eight genes I chose to validated that were identified as differentially expressed in the microarray, only three (BCL-2, CLIC5, and NETO1) were found to be differentially expressed in the qPCR. Therefore, I cannot be confident that the differentially expression of the probes identified in the microarray, and the subsequent analysis, is reliable. The small sample size is a limitation of this study; more replicate samples for each group would increase the statistical power of the microarray analysis and subsequent validation by qPCR.

4.3.2 The role of TEL in proliferation

As proliferation was predicted to be one of the main functions affected by the overexpression of TEL, and since uncontrolled growth is the defining feature of leukaemia and cancer, I decided to study the effects of TEL on proliferation of REH cells.

There have been previous studies into the influence of TEL on cell growth in the past. Fenrick and colleagues (2000) studied the effects of TEL on Ras-transformed 3T3 (fibroblast) cells. These transformed cells normally display growth in semi-solid media (agar), but the expression of TEL slowed down this growth considerably. In adherent cell cultures, TEL expressed in non-transformed 3T3 cells had no effect on proliferation, but the proliferation of Ras-transformed cells was slowed down by TEL expression. (Fenrick *et al* 2000). The growth inhibitory effect of TEL on 3T3 cells was also shown by Gunji and co-workers (2004).

Fears and colleagues (2002) derived two cell lines from samples taken from the same TEL/AML1$^+$ leukaemia patient, one at initial diagnosis (AT-1), which was positive for TEL, and one at relapse (AT-2), which was negative for TEL. They showed that the AT-2 (TEL-negative) cell line grew at a faster rate, although it was not discussed whether this was due to the loss of TEL or caused by other genetic abnormalities that arose in the AT-2 cell line, which includes a translocation involving chromosomes 1 and 17 (Fears *et al* 2002).

Tsuzuki and co-workers (2007) transduced REH cells with retroviruses containing either GFP alone or TEL and GFP. By tracking the changes in the GFP$^+$ population amongst all cells transduced, they showed that TEL-GFP-transduced cells lost their GFP expression quicker than cells transduced with GFP alone, and that cell division occurs at a slower rate (Tsuzuki *et al* 2007).

However, I found that the expression of TEL on the proliferation of REH cells had only a subtle effect of slowing down proliferation. I used a variety of different methods of measurement, including doubling time, growth curves, DNA synthesis analysed by EdU incorporation, and cell cycle analysis by DNA content. I also could

not confirm differential expression of the cell cycle genes *CDKN1B* and *CDKN2A* by qPCR (Figure 4.3A).

By oncoretroviral transduction, I measured the effects of TEL on the cell cycle, and only found a slight reduction in the numbers of cells in S phase of 5-7 percentage points (Figure 4.3D), and a similar reduction of cells in G_2 phase of 2-3 percentage points (Figure 4.3E). By plasmid transfection, I observed a modest decrease in EdU uptake (Figure 4.5C), but inconclusive results using clones derived from stably transfected cells when growth rate was assessed with growth curves and doubling times (Figure 4.7A-B).

This conflicts with previous work on the effects of TEL on cell growth, but might be explained by the differences between these studies and the work presented in this chapter. The differences in cell growth of the AT-1 and AT-2 cell lines (Fears *et al* 2002) may not be solely due to the absence of TEL in the AT-2 cell line; detailed spectral karyotyping identified a t(1;17)(q36;p13) translocation in the AT-2 cell line not present in the slower growing AT-1 cell line. Whilst TEL inhibited the growth of 3T3 fibroblast cells (Fenrick *et al* 2000; Gunji *et al* 2004), it cannot be ruled out that this effect is specific to 3T3 or fibroblast cells, and so it cannot be assumed TEL has the same effect on REH cells. Finally, Tsuzuki and co-workers did show that the transduction of REH cells with TEL slows down the proliferation of cells using a competition assay, but there was no further analysis with regards to cell cycle analysis or EdU uptake (Tsuzuki *et al* 2007).

I did observe some conflicting data in our results, particularly in the study of the transduced REH monoclones (Figure 4.7). A growth curve was generated by counting cells seeded into a cell culture plated over time (Figure 4.7A), and from these curves, a doubling time for each clone was calculated (Figure 4.7B). Unexpectedly, the fastest growing clones overall were not the empty vector clones, but the TEL-transfected T1C5 and T1G8 clones. In addition, the slowest growing clone, below the rest of the TEL clones, was the empty vector clone p2D5. Despite the best efforts to ensure a consistent numbers of cells (100,000) at the start of the experiment, if there were differences between the numbers of cells seeded due to counting errors, this could explain the discrepancy between the growth curves and

the doubling times of the clones. These experiments would have to be repeated for this to be investigated. By qPCR, the TEL-transfected clones expressed high levels of TEL, with the exception of T2D10 (Figure 4.7C). Low levels of qPCR amplification could be detected in all of the empty vector clones, which could be due to contamination, the expression of mutant transcripts in REH cells or none specific binding of the primers. The expression of TEL in these clones did not appear to correlate with their growth rate.

In summary, without additional replicates of the experiment, I cannot conclude that TEL has more than a subtle inhibitory effect on the proliferation of REH cells. One factor that was not examined was if TEL has an effect on the response of REH cells to external stimuli that either induce or inhibit proliferation. For example, the proliferation of REH cells has been shown to be stimulated by IL-3 (Accordi *et al* 2007) or seratonin (Prada *et al* 2011), and inhibited by IL-7 (Pandrau-Garcia *et al* 1994); an experiment using these in combination with TEL expression would reveal how REH cells respond to such stimuli, and possibly identify a role for TEL in signal transduction.

4.3.3 The role of TEL in apoptosis

In addition to proliferation, the evasion of apoptosis is an important feature of cancer and leukaemia. The microarray also identified several genes involved in apoptosis, so I decided to investigate further what role the expression of TEL has on the death of REH cells.

There have been previous studies of the influence of TEL on apoptosis. Irvin and colleagues (2003) found that TEL-transduced 3T3 fibroblast cells were much more sensitive to apoptosis induced by serum-starvation than control 3T3 cells, and that this correlated with a repression of the anti-apoptotic gene *Bcl-X_L* by TEL. Yamagata and co-workers (2006) discovered that the expression of TEL in the murine myeloid cell line 32Dcl3 increase the sensitivity of these cells to apoptosis induced by G-CSF through the upregulation of p53.

However, in REH cells overexpressing TEL, (Figure 4.8) I did not observe a significant effect on cell death. When analysing the cell cycle profile TEL

oncoretrovirus-transduced cells, I only saw a small increase in the number of the 'sub-G_1' (apoptotic) cells of 1-2 percentage points (Figure 4.3F). However, when I analysed TEL plasmid-transfected cells for Annexin-V staining (a marker of apoptosis), I saw no difference in the apoptosis response to the apoptosis inducer staurosporine (Figure 4.8D).

One explanation for the discrepancy between our results and that of previous work (Irvin *et al* 2003; Yamagata *et al* 2006) could be that, similar to the work in studying the effects of TEL in 3T3, the increase in sensitivity to apoptosis is due to cell-specific effects. However, since apoptosis can be induced in different ways, it cannot be ruled out that TEL does not have the influence on staurosporine-mediated apoptosis that it might on other means of stimulating cell death.

As described in Introduction chapter (section 1.5.1), apoptosis triggered by intrinsic signals is dependent on the balance between anti-apoptotic and pro-apoptotic signals of the *bcl-2* family of mitochondrial proteins (Tsujimoto 2003; Yamagata *et al* 2006). Although the apparent downregulation of BCL-2 in TEL-expressing cells might only be due to a higher level of TEL expression in one of the GFP-transduced clones (Figure 4.8A), a downregulation of BCL-2 might be expected to lead to increased levels of apoptosis. However, the microarray indicated the downregulation of a pro-apoptotic bcl-2 family member, *BAK1*, on TEL overexpression (data not shown). The level of differential expression was low (1.16-fold down regulation), which I did not confirm by qPCR, but the downregulation of *BAK1* might have been enough to balance out any real downregulation of *BCL-2*, and so lead to no net effect on apoptosis of TEL overexpression.

As described in the Introduction chapter (section 1.5.2), the elimination of self-reactive B cells is a critical stage in B cell development. Apoptosis of immature self-reactive B lymphocytes is believed to occur via the B-cell receptor (Niiro & Clark 2002), and acts via both caspase-dependent and -independent pathways (Berard *et al* 1999). The mechanism of staurosporine-induced apoptosis is unclear, but it is believed to act via both caspase-dependent and caspase-independent pathways (Belmokhtar *et al* 2001; Zhang *et al* 2004), the latter which could act independently of the *bcl-2* family proteins (Zhang *et al* 2004). To fully investigate

the effects of TEL on apoptosis, a variety of methods to induce apoptosis could be used, including those relevant to B lymphocytes; for example, activation of apoptosis via the B-cell receptor by anti-IgM antibody, and apoptosis via Fas by anti-Fas antibody (Graves *et al* 1998).

4.3.4 Conclusion

The data presented in this chapter has not demonstrated more than subtle effect of TEL on proliferation or apoptosis of the TEL/AML1$^+$ cell line REH. This is in conflict with previous work which has shown that forced TEL expression decreases proliferation of cells and increases apoptosis.

One possible reason for our observations, as outlined above, could be that I did not consider the use of methods to induce or inhibit proliferation, nor a variety of methods to induce apoptosis, which could have revealed an influence of TEL that I did not observe in this study. Another limitation of this study is the low number of replicates used for the experiments. If the effects of TEL on proliferation and apoptosis truly are as subtle as our results suggest, more replicates would increase the statistical power of the data.

It may be that the loss of TEL is not in fact a 'driver' mutation of TEL/AML1$^+$ leukaemia, or is only partially involved. It is possible that the loss of TEL is only one mutation in a multi-step process to leukaemia, all of which are necessary but not sufficient to bring about overt leukaemia. To this end, it would be necessary to investigate how TEL along with other common mutations (outlined in the Introduction, section 1.9) influence leukaemia.

However, given the frequency of the loss of the untranslocated *ETV6* allele observed in TEL/AML1$^+$ leukaemia patients (Cave *et al* 1997; Patel *et al* 2003), it seems unlikely that TEL only has a limited effect influence. To support this, when the gene expression changes were analysed by the Ingenuity Pathway Analysis, most of the functions predicted to be affected by TEL overexpression were proliferation of various subtypes of haematopoietic cells (Table 4.4). It is more likely that the loss of TEL results in changes in lymphoblasts that have not been studied in this chapter. Cancer is a disease of many features, with increased proliferation and evasion of

apoptosis just two such features, which can each be affected in many ways; for example, a hypothetical cancer promoting mutation might not affect the baseline rate of proliferation of a cell, but might change its sensitivity to a proliferation-inducing signal, which in turn means that the cell proliferates faster in response. More sophisticated experiments than the ones performed in this chapter would have to be performed to reveal what role TEL has on proliferation and apoptosis. A more directed approach would be to start with investigating the functions predicted by IPA to be affected by TEL overexpression (Table 4.4).

Ultimately, some of the roles of TEL may not be revealed by models using cell lines, and indeed, the limitation all model systems when studying any gene is that they cannot individually reveal all aspects of what that gene does in an organism. As a demonstration, mouse models were used to show that TEL is essential for adult haematopoietic engraftment but dispensable for adult haematopoiesis itself (Wang *et al* 1998a; Hock *et al* 2004), which would not have been possible to show using cell lines alone. Similarly, whilst important aspects of the role of the loss of TEL in TEL/AML1$^+$ leukaemia can be revealed using cell lines, such a model would not be appropriate for the study of, for example, how TEL influences B lymphocyte development, since cell lines rarely represent more than a ‘snapshot’ of a particular stage in development.

Chapter 5: Discussion

The fusion gene TEL/AML1, generated by the t(12;21) chromosome translocation, is the most common single genetic aberration in childhood leukaemia. However, despite extensive research using a variety of mouse and human models, it is still not clear how the activity of TEL/AML1 leads to leukaemia. In addition, the significance of one of the most frequent secondary mutations, the loss of the remaining untranslocated TEL allele, is still not known. The aims of this thesis were two-fold: to generate a new model based on human embryonic stem cells in which the aetiology of TEL/AML1$^+$ leukaemia might be further investigated (Chapter 3), and provide an insight into the role of the loss of TEL in the context of TEL/AML1$^+$ leukaemia using a human leukaemia cell line REH (Chapter 4).

A model of leukaemia using hESCs would combine the advantages of *in vivo* and *in vitro* models; it would demonstrate human multipotent haematopoietic development, and would be amenable to genetic manipulation whilst maintaining a reproducible genetic background. In Chapter 3 all of these characteristics were demonstrated in the hES cell line H9 expressing the TEL/AML1 transgene. Transgenic hESCs could generate haematopoietic progenitors, capable of differentiation towards a variety of haematopoietic lineages, whilst also maintaining genetic stability and pluripotency markers when cultured in an undifferentiated state. To my knowledge there are no published examples of models of TEL/AML1$^+$ leukaemia using hESCs, and thus the work presented in this thesis provides the basis of first model of its kind.

Differentiation of transgenic TEL/AML1$^+$ hESCs showed that there was no significant effect of TEL/AML1 on the numbers of haematopoietic precursors (CD43$^+$CD31$^+$ hESC-derived cells; Figure 3.8). This is consistent with previous studies concerning the haematopoietic development in *TEL* and *AML1* deficient mice (Wang *et al* 1998a; Lacaud *et al* 2004), demonstrating that any dominant negative effects that TEL/AML1 might have over TEL or AML1 (Zelent *et al* 2004) will not have a significant effect on the differentiation of TEL/AML1$^+$ hESCs. TEL/AML1-transgenic mice show that the expression of TEL/AML1 increases the self-renewal capacity of HSCs and an increase in myeloid progenitors with a

reciprocal decrease in lymphoid progenitors (Schindler *et al* 2009). Unfortunately, inefficient recovery of $CD43^{+}CD31^{+}$ haematopoietic precursors precluded a thorough analysis by CFU assays of the influence of TEL/AML1 in the haematopoietic capacity from the transgenic hESCs (Figure 3.9). However, it would theoretically be possible to conduct a more in depth study of the self-renewal and differentiation properties of $TEL/AML1^{+}$ hESC-derived haematopoietic progenitors, using both an *in vivo* approach by xenograft repopulation, with additional limiting dilution and competitive repopulation assays, and *in vitro* approach with serial transplantations in methylcellulose-based CFU assays (Schindler *et al* 2009).

Further differentiation of haematopoietic progenitors towards B cell lineage was demonstrated in Figure 3.10. A slight increase in CD34 transcript expression was observed in $TEL/AML1^{+}$ differentiation cultures, which suggests an inhibition of differentiation between pro-B and pre-B expression, consistent with murine models of $TEL/AML1^{+}$ leukaemia (Tsuzuki *et al* 2004; Fischer *et al* 2005). No study of the proliferative or self-renewal capacity of these B lineage cells was performed, which would have shed light on whether these cells contained a population of 'pre-leukaemic' blasts typical of $TEL/AML1^{+}$ leukaemia (Morrow *et al* 2004; Morrow *et al* 2007). Nevertheless, such studies could be performed by xenograft as detailed above or in an *in vitro* culture system (Rawlings *et al* 1995). In addition, harvesting of B lineage cells could enable further phenotypic studies of, for example, surface expression of B cell markers and immunoglobulin gene rearrangements (Carpenter *et al* 2011).

I demonstrated the upregulation of two genes, TNFRSF7 and SMAD1, associated with $TEL/AML1^{+}$ leukaemia in the B lineage differentiation cultures (Figure 3.11), indicating that the association of these genes with TEL/AML1 might be directly regulated by TEL/AML1 itself. However, if functional effects of TEL/AML1 in haematopoietic and B-lineage progenitors could be demonstrated using the experiments suggested above, it could be interesting to conduct a gene expression microarray to observe the changes involved. This might shed light on the pathways involved in maintaining the 'pre-leukaemic' state observed in TEL/AML1, and could provide therapeutic targets with which to proactively reduce the risk of progression to overt leukaemia. In any case it would be interesting to pursue the importance of

SMAD1 and TNFRSF7 in TEL/AML1$^+$ leukaemia, particularly the latter as previous studies have demonstrated it has a clear link to leukaemia, as described in the Introduction chapter (section 1.9.4).

A panel of hESC lines was generated that constitutively expressed TEL/AML1. Although no adverse effects of the overexpression of TEL/AML1 were observed, concerns about 'transgene toxicity' could be addressed by using homologous recombination in hESCs, which could insert *RUNX1* cDNA in the appropriate intron of the *ETV6* locus, similar to the systems used in some murine models (Schindler *et al* 2009; van der Weyden *et al* 2011). This would enable the expression of TEL/AML1 from the endogenous *ETV6* locus, and so generate a model more closely replicating the disease.

During the development of this model, I had attempted to set up a tetracycline-inducible expression system for TEL/AML1. However, in practise no expression could be induced when tested, and it was decided to switch to a constitutive expression system. The inducible system would allow the investigation of at what stage TEL/AML1 can contribute to leukaemogenesis, and so provide evidence as to the 'cell of origin' in leukaemia. Studies in mice with inducible expression of TEL/AML1 have shown that the presence of TEL/AML1 in HSCs can change the self-renewal properties of multipotent and lymphoid cells (Schindler *et al* 2009), but that TEL/AML1 expression restricted to lymphoid lineages has no effect (Andreasson *et al* 2001; Schindler *et al* 2009).

To elucidate the importance of the loss of TEL in the progression of TEL/AML1$^+$ leukaemia, TEL was overexpressed in the TEL/AML1$^+$ cell line REH (Chapter 4). A gene expression microarray showed that the presence of TEL had an effect on the transcription of genes involved in functions of significance to leukaemia; for example, TEL expression decreased genes involved in the proliferation of lymphocytes (Table 4.4). In addition, differentially expressed genes included some genes strongly associated with TEL/AML1$^+$ leukaemia from previous studies (Table 4.3). These two results vindicate the approach taken as they indicate that this model has some relevance to the clinical TEL/AML1$^+$ disease. Supporting this, it has been shown that despite concerns that culture adaptation of cell lines can result in cells

that no longer represent real diseases, leukaemia cells lines have similar transcriptional profiles to the leukaemia subtypes from which they were derived (Andersson *et al* 2005a).

Regarding the genes differentially regulated by TEL overexpression, some genes have been previously linked to leukaemia, and it might be interesting to use a computational approach to further compare the data set generated in this thesis with those of studies of ALL and/or TEL/AML1^{+} leukaemia. Analysis of gene expression changes also identified gene expression patterns that were consistent with the inhibition of various transcription factors (Table 4.5). Since TEL is known to form heterodimers with other transcription factors and interfere with their transcriptional profiles (Fears *et al* 1997; Kwiatkowski *et al* 1998; Schick *et al* 2004), this list of transcription factors could be an interesting starting point for further studies of functional heterodimer partners of TEL. Finally, through strong differential expression of genes in the presence of TEL (Table 4.1 and Table 4.2), new potential targets of TEL were identified, two of which, CLIC5 and NETO1, have been validated by qPCR (Figure 4.9); it would be interesting to pursue these genes with further studies as they have not been linked to leukaemia previously. It is our intention to make this gene expression data set publicly available so that it could be used by other groups for their own research, and thus would represent the first gene expression data set looking at the transcriptional consequences of TEL in the context of TEL/AML1^{+} leukaemia.

As an example of the further research that could be conducted on genes identified in the TEL microarray, one could investigate the role of CLIC5 in leukaemia. As discussed in Chapter 4 (section 4.2.9), CLIC5 is an intracellular chloride channel that has already been associated with TEL/AML1^{+} leukaemia (Ross *et al* 2003) that is associated with (Berryman & Bretscher 2000) and regulated by (Singh *et al* 2007) the actin cytoskeleton. Interestingly, intracellular chloride ion concentration is known to control, and be controlled by, progression through the cell cycle (Blackiston *et al* 2009), evidence of which is observed in various cell types (Valenzuela *et al* 2000; Miyazaki *et al* 2008), including lymphocytes (Bubien *et al* 1990). Connecting the actin cytoskeleton and proliferation is cell volume (Lang *et al* 1998), which has also been shown to be connected chloride concentration (Chen *et al*

2002). From the data presented in Chapter 4, and from previous gene expression studies of TEL/AML1 (e.g.Ross *et al* 2003), it is feasible that the upregulation of CLIC5 in TEL/AML1^{+} leukaemia, with the subsequent loss of heterozygosity of TEL, affects the intracellular chloride concentration, which could lead to a dysregulation of the cell cycle, possibly mediate by changes in cell volume. Experiments which could be performed to investigate this hypothesis could include the overexpression and knockdown of CLIC5, in coordination with the use of chloride-sensitive dyes, such as MQAE, to measure intracellular chloride concentration. The intracellular chloride concentration can be altered through the use of cell medium deficient in chloride (replaced instead with NO_3^- , e.g. Miyazaki *et al* 2008). The effect of such alterations on cell volume can be measured using a variety of techniques (Davis *et al* 2004), and on cell proliferation and cell cycle progression through techniques including those demonstrated in this thesis.

Following the study of the transcriptional consequences of TEL overexpression, the functional effects, namely proliferation and apoptosis, were investigated. It was found that TEL had no consistent effect on proliferation. Whilst this is conflicting with other studies of the role of TEL in proliferation (Fenrick *et al* 2000; Fears *et al* 2002; Gunji *et al* 2004; Tsuzuki *et al* 2007), there are several explanations for the discrepancies between previous studies and the work presented in this thesis (see section 4.3.2). In addition, the lack of a functional effect on proliferation may seem surprising given the transcriptional effect on genes involved with proliferation (Table 4.4), but it is possible that the proliferative effects of TEL could not be demonstrated in the experiments performed; one aspect that was not investigated was how TEL affected the proliferation of cells in response to stimulants of growth and/or cell division in REH cells, and so this would make a good starting point for further studies. Similarly, no influence of TEL on the induction of apoptosis was observed (Figure 4.8). Whilst I used an inducer of apoptosis in the form of staurosporine, it is not clear through which mechanism this chemical causes apoptosis, and so cell-specific differences in how apoptosis is controlled could account for discrepancies between this work and previous studies into the role of TEL in apoptosis (Irvin *et al* 2003; Yamagata *et al* 2006). It would be interesting to investigate if TEL regulates apoptosis by stimulating cell death through a better defined pathway, for example, by the polymerisation and activation of Fas receptor using antibodies.

In the introduction chapter, three possible models of the role of the loss of TEL in TEL/AML1$^+$ leukaemia were presented (Figure 1.3). The first showed that the loss of TEL is both necessary and sufficient for progression of a TEL/AML1$^+$ clone to overt leukaemia. The second showed that TEL deficiency is necessary for this, but other events (for example, further mutations) are needed for progression. Finally, the third showed that, after an event that causes proliferation of the leukaemic clone, loss of TEL is one of many mutations that could occur during this expansion, but that TEL deficiency confers a significant proliferative advantage to a clone so that it becomes the dominant clone in the leukaemia.

The first scenario seems unlikely to be true, based upon previous studies; the number of genes that are frequently mutated in TEL/AML1$^+$ leukaemia would indicate that TEL is not the only important factor in progression; similarly, the lack of TEL as a gene commonly mutated in other leukaemias not only suggest that it is the loss of both alleles of TEL that is important but also that TEL loss by itself is not sufficient for increased proliferation and survival. The work presented in this thesis also supports this view, suggesting that TEL has no more than a subtle effect on proliferation and survival. This also suggests that the third model is incorrect, in that TEL deficiency allows enough expansion of a leukaemic clone to 'out-compete' other clones, which is not supported by the results.

In light of this, the second scenario appears to be the most likely; that TEL deficiency is an essential feature of TEL/AML1$^+$ leukaemia, that somehow predisposes the pre-leukaemic clone to progress to overt leukaemia, but the 'switch' to progression appears to be in the form of another event, either another series of mutations, or possibly an immunological mechanism as put forward by Greaves (2006). The transcriptional changes observed as a result of the overexpression of TEL, presented in Chapter 4, provide a starting point for further studies as to how TEL deficiency poises pre-leukaemic clones for progression to overt leukaemia. Thus the work presented as a whole in this thesis confirm the view that

Further study of the role of TEL deficiency could be performed in the TEL/AML1 transgenic hESC model. In the TEL/AML1$^+$ background, the knockdown of TEL by RNA-interference (RNAi) directed at exons 6 to 8 of TEL (so avoiding any

knockdown of the TEL/AML1 fusion) could replicate TEL deficiency (Figure 5.1). This would replicate TEL deficiency in the hESC model and thus allow the study of TEL in a multipotent system, a valid approach given that TEL overexpression identified gene expression changes consistent with an inhibition of haematopoietic differentiation in various lineages (Table 4.4). This approach of changing the expression of genes in a TEL/AML1$^+$ background could be used, in theory, to investigate the function of any gene in the context of TEL/AML1$^+$ leukaemia. In particular, such an approach could provide further evidence for the importance of genes known to affect differentiation (e.g. Ikaros / IKZF1; Mullighan *et al* 2008), or indeed investigate genes identified in the TEL overexpression model that have previously not been linked to leukaemia (e.g. CLIC5 and NETO1). Recently, an unbiased screen exploiting random insertion mutagenesis in a TEL/AML1-transgenic mouse revealed a large number of genes that contribute to leukaemia, some of which were known common secondary mutations in humans, and others which were novel (van der Weyden *et al* 2011). Using standard overexpression or knockdown methods, it would be possible in our hESC model to conduct preliminary studies into what effects these novel mutations have on haematopoiesis and lymphopoiesis in a human system, reducing dependence on expensive and time-consuming mouse models.

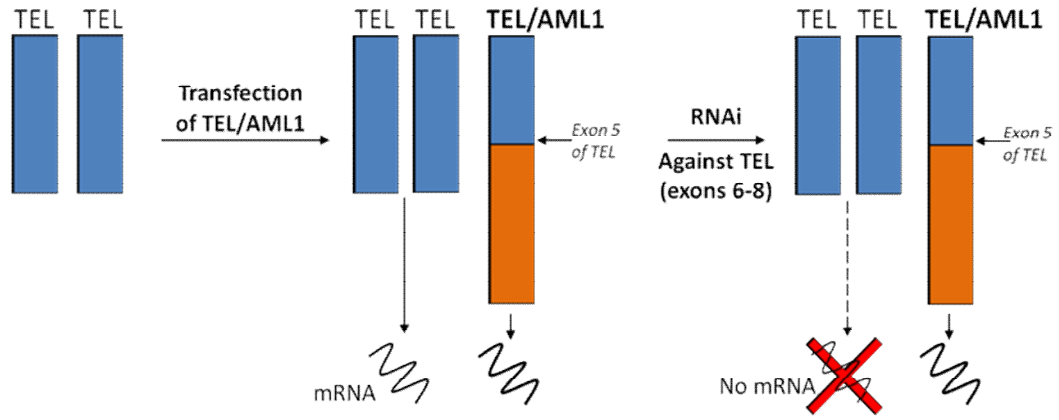


Figure 5.1 – TEL knockdown in the TEL/AML1$^+$ hESC model
Upon transfection of TEL/AML1, mRNA from both the TEL locus and TEL/AML1 cDNA is transcribed. RNAi against exons of TEL not present in TEL/AML1 (i.e. exons 6 to 8) would knockdown the expression of TEL but leave the expression of TEL/AML1 unaffected, and thus replicate TEL deficiency frequently observed in TEL/AML1$^+$ leukaemia.

The work presented in this thesis makes two novel contributions to the field of TEL/AML1^{+} leukaemia; firstly, a gene expression microarray data set showing the role of TEL in this leukaemia subtype; and secondly, a model based on hESCs that could be used to further investigate the roles of TEL and these differentially regulated genes in haematopoietic development. Both the gene expression microarray data set and the influence of TEL/AML1 in haematopoietic development are consistent with previous studies in the field, and thus are relevant to the study of ALL. This work has the potential to make further contributions to our understanding of leukaemia, as well as potential therapeutic targets for the treatment of leukaemia, and thus warrants further exploitation.

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