LUND UNIVERSITY

# Responses to Tumour initiating factors and Regulation of Normal and Malignant Haematopoiesis

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Doctoral Thesis
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# Responses to Tumour initiating factors and Regulation of Normal and Malignant Haematopoiesis

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LUND UNIVERSITY
Faculty of Medicine

**Academic Dissertation**

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Abstract

The haematopoietic stem cell (HSC) resides within a specific environment enabling it to retain its self-renewal capacity or quiescent state. It is proposed that the HSC niche is hypoxic, a milieu within which the HSC is protected from intrinsic and extrinsic stimuli. We have investigated the haematopoietic phenotype of an HSC in a mouse model where hypoxia-regulated *Vegfa* expression is abrogated. In *Vegfaδ/δ* mice, the HRE in the *Vegfa* promoter has been deleted at both alleles, thereby inhibiting HIF-binding and subsequent activation of Vegfa expression following hypoxia. We show that hypoxic regulation of *Vegfa* expression within the haematopoietic system affects haematopoietic differentiation and numbers of HSCs to a small extent. Interestingly, *Vegfa* expression was shown to be reduced in highly purified HSCs from bone marrow of *Vegfaδ/δ* mice but not in mature cells, suggesting that the niche of the HSC is hypoxic.

Acute lymphoblastic leukaemia (ALL) is the most common malignancy among children. Contemporary treatment protocols result in cure rates of 80-85% but 15-20% of children still experience relapse. A group of patients do therefore not benefit from conventional therapy underlining the urgent need to identify additional biomarkers at diagnosis. We have investigated the expression of VEGF-A, its receptors VEGFR-1 and VEGFR-2 as well as PTEN and SHP1 in childhood ALL using immunohistochemistry. We observed that the expression of VEGFR-1, PTEN and SHP1 in mononuclear cells of children with ALL were significantly different to the expression of mononuclear cells in children with no malignant disease. VEGFR-1, PTEN and SHP1 may be potential prognostic factors for childhood ALL.

Chromosomal translocations are reported in approximately 65% of all acute leukaemias. Reports have identified leukaemic translocations in human peripheral blood of healthy individuals supporting the hypothesis that leukaemic transformation is a multistep process. The t(10;11)(p13-14;q14-21) translocation is a reciprocal translocation and forms both an in-frame CALM·AF10 and AF10·CALM fusion. The long latency period prior to the onset of leukaemia in CALM·AF10 mice models suggests that the fusion protein alone does not cause leukaemic development. We hypothesise that AF10·CALM is required for the full leukaemic phenotype. In an in vitro model, we found that t(10;11)(p13-14;q14-21) reciprocal fusions have individual effects on cell biology and, when found in combination, have either a more pronounced or an inhibitory effect on leukaemogenesis. This highlights the importance of examining both fusion proteins in a two transcript reciprocal translocation as they on their own may have individual characteristics.

Key words:

Leukaemia, prognostic marker, ALL, HSC niche, reciprocal translocation, CALM·AF10, AF10·CALM

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*For my Parents*

*Utan tvivel är man inte klok*

Tage Danielsson

# List of Papers

This thesis is based on the following papers, referred to in the text by their respective roman numerals.

**I** Rehn M, Olsson A, Reckzeh K, **Diffner E**, Landberg G, Cammenga J. Hypoxia-regulated expression of vascular endothelial growth factor is not involved in maintenance of hematopoietic stem cells in steady state.
*Manuscript.*

**II** **Diffner E**, Gauffin F, Anagnostaki L, Nordgren A, Gustafsson B, Sander B, Gustafsson B, Persson JL. Expression of VEGF and VEGF receptors in childhood precursor B-cell acute lymphoblastic leukemia evaluated by immunohistochemistry.
*Journal of Pediatric Hematology/Oncology. 2009 Sep;31(9):696-701.*

**III** Gauffin F, **Diffner E**, Gustafsson B, Nordgren A, Wingren AG, Sander B, Persson JL, Gustafsson B. Expression of PTEN and SHP1, investigated from tissue microarrays in pediatric acute lymphoblastic leukemia.
*Pediatric Hematology and Oncology. 2009 Jan;26(1):48-56.*

**IV** **Diffner E**, Dempsey C, Alexander S, Landberg G, Saha V. Independent and combined effects of the reciprocal t(10;11) fusion proteins CALM·AF10 and AF10·CALM on leukaemogenesis through cloning and functional characterization.
*Manuscript.*

# Table of Contents

# Abbreviations

| | |
|---|---|
| AF10 | Myeloid/lymphoid MLL translocated to, 10 (or MLLT10) |
| AID | Activation Induced Deaminase |
| ALL | Acute Lymphoblastic (or Lymphoid) Leukaemia |
| AML1 | Acute Myeloid Leukaemia 1 protein |
| AML | Acute Myeloid Leukaemia |
| APL | Acute Promyelocytic Leukaemia |
| ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator |
| ATRA | All-Trans Retinoic Acid |
| B | B-cell/lymphocyte |
| BCR | Breakpoint Cluster Region |
| BM | Bone Marrow |
| BMPR1A | Bone Morphogenic Protein Receptor 1A |
| CALM | phosphatidylinositol binding Clathrin Assembly Protein (or PICALM) |
| CD | Cluster of Differentiation |
| CLL | Chronic Lymphoblastic (or Lymphoid) Leukaemia |
| CLP | Common Lymphoid Progenitor |
| CML | Chronic Myeloid Leukaemia |
| CMP | Common Myeloid Progenitor |
| DNA | Deoxyribonucleic Acid |
| DSB | Double Strand Break |
| E2A | DNA binding protein |
| EFS | Event-Free Survival |
| ES | Embryonic Stem |
| FAB | French-American-British |
| FACS | Fluorescence-Activated Cell Sorting |
| FISH | Fluorescence *In Situ* Hybridization |
| FLT-3 | FMS-Like Tyrosine kinase 3 |
| G-CSF | Granulocyte Colony-Stimulating Factor |
| GM-CSF | Granulocyte/ Macrophage Colony-Stimulating Factor |
| GMP | Granulocyte-Monocyte Progenitor |
| HIF | Hypoxia-Inducible Factor |
| HPC | Haematopoietic Progenitor Cell |
| HSC | Haematopoietic Stem Cell |
| HSPC | Haematopoietic Stem and Progenitor Cell |
| Hoe | Hoechst 33342 |
| HOX (A) | Hoemobox (A) cluster |
| Ig | Immunoglobulin |

| | |
|---|---|
| IL | Interleukin |
| ITD | Internal Tandem Duplications |
| LSC | Leukaemic Stem Cell |
| LT-HSC | Long-term HSC |
| M-CSF | Macrophage Colony-Stimulating Factor |
| MLL | Mixed Lineage Leukaemia |
| MPP | Multipotent Progenitor |
| MRD | Minimal Residual Disease |
| NOD | Non-Obese Diabetic |
| PCR | Polymerase Chain Reaction |
| PBX 1 | pre B-cell leukemia transcription factor 1 |
| Pre | Precursor |
| PLZF | Promyelocytic Leukaemia Zinc Finger |
| PML | Promyelocytic Leukaemia |
| PPR | Parathyroid hormone-related Peptide Receptor |
| R | Receptor |
| RAG | Recombinase-Activating Gene |
| RARα | Retinoic Acid Receptor alpha |
| RNAi | Ribonucleic Acid interference |
| ROS | Reactive Oxygen Species |
| RSS | Recombination Signal Sequences |
| qRT-PCR | quantitative Reverse Transcriptase PCR |
| SCF | Stem Cell Factor |
| SCID | Severe Combined ImmunoDeficient |
| SL-IC | SCID Leukaemia-Initiating Cell |
| SER | Slow Early Response |
| SKY | Spectral caryotype |
| SLAM | Signaling Lymphocyte Activation Molecule |
| ST-HSC | Short-Term HSC |
| T | T-cell/lymphocyte |
| TCR | T-Cell Receptor |
| TEL | Telomere elongation |
| TdT | Terminal deoxynucleotidyl Transferase |
| TPO | Thrombopoietin |
| VCAM-1 | Vascular Cell Adhesion Molecule-1 |
| VEGF | Vascular Endothelial Growth Factor |
| VEGFR | Vascular Endothelial Growth Factor Receptor |
| V(D)J | Variable (Diversity) Joining |
| WBC | White Blood Cell |
| WHO | World Health Organization |
| WT1 | Wilm's Tumour 1 |

# Haematopoiesis

Haematopoiesis is the continuous development of blood cells. The haematopoietic stem cell (HSC) has both the capacity to self-renew and the ability to produce the haematopoietic cells throughout life. The resultant mature cells of the haematopoietic hierarchy are important for the immune system (lymphocytes, macrophages, granulocytes etc.), tissue oxygenenation (erythrocytes) and thrombocytic involvement in haemostasis. The main sites of haematopoiesis within the adult human body are the ilium, ribs, cranium, sternum and vertebrae. This site can relocalise to the spleen and liver following haematopoietic stress[1]. A healthy adult human has a turnover of approximately $10^9$-$10^{12}$ haematopoietic cells per kg each day[2-4]. The haematopoietic system is a highly regulated entity and has a fundamental role in mammalian physiology.

## The Haematopoietic hierarchy

The haematopoietic system can be divided into two lineages – the lymphoid and the myeloid (Figure 1). Lymphocytes and natural killer cells are traditionally referred to as lymphoid cells whereas erythrocytes, macrophages and granulocytes may be referred to as myeloid cells. The common progenitor of these terminal cells is the HSC of which there are two subtypes - the long-term and short-term HSC (LT-HSC or ST-HSC). This distinction is based upon each HSC's capacity for self-renewal. The ST-HSC differentiate to the multipotential progenitor cells (MMPs) that can differentiate into either a lymphoid or myeloid progenitor cell forming either a common lymphoid or a common myeloid progenitor cell (CLP or CMP). The CMP can further differentiate into a megakaryocyte/ erythrocyte progenitor (MEP) or a granulocyte/ monocyte progenitor (GMP). Gene expression analysis has verified these findings, whilst also indicating that CMPs express myelo-erythroid but not lymphoid genes, whereas CLPs co-express B and T lymphoid but not myeloid genes[5, 6]. This classification is the 'original model' of the haematopoietic hierarchy proposed by Weissman and colleagues and was previously widely accepted[5,7]. During the past decade, Weissman's model has been challenged by several studies where CLPs and CMPs were found to possess macrophage or B lymphocyte potential, respectively[8-10].

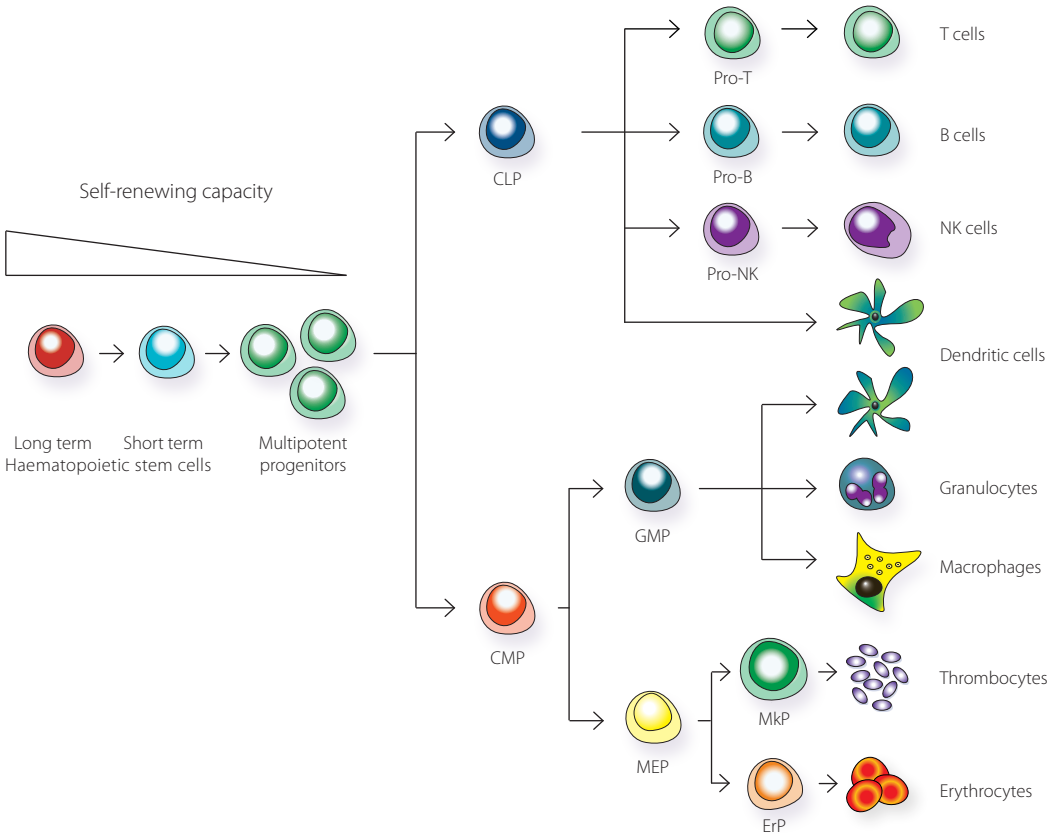


**Figure 1. Schematic illustration of the Haematopoietic hierarchy. The haematopoietic stem cell (HSC) can subdivide into a long-term or short-term HSC based upon its self-renewing capacity.** The HSC has the capacity to differentiate into all haematopoietic cells. MMP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/ macrophage progenitor; MEP, megakaryocyte/ erythrocyte progenitor; MkP, megacaryocyte progenitor; NK, natural killer.

Knowledge of the immunophenotype of haematopoietic stem and progenitor cells is important for clinical applications such as gene therapy, *ex vivo* expansion and transplantation. Aside from the clinical applications, a knowledge of surface markers of early-differentiated haematopoietic cells has been of great value in pre-clinical research regarding the biology and pathobiology of the haematopoietic compartment. The surface markers of human and murine haematopoietic stem and progenitor cells are summarized in Table 1 and are based on the 'original model'[11-13].

**Table 1. Immunophenotype of key players within the Haematopoietic hierarchy.** The cell types refer to a lineage negative (Lin-) population including the stated markers.

| | LT-HSC | MPP | CLP | CMP |
|---|---|---|---|---|
| Human | $CD34^+$/ $CD38^-$/ $CD90^+$/ $CD45RA^-$ | $CD34^+$/ $CD38^-$/ $CD90^-$/ $CD45RA^-$ | $CD34^+$/ $CD38^+$/ $CD10^+$/ $CD45RA^+$/ $Thy\text{-}1^-$ | $CD34^+$/ $CD38^+$/ $CD45RA^-$/ $IL\text{-}3R^{lo}$ |
| Mouse | $c\text{-}Kit^+$/ $Sca\text{-}1^+$/ $Thy\text{-}1^{lo}$/ $IL\text{-}7R\alpha^-$/ $Flk\text{-}2^-$/ $CD34^{-/lo}$ | $c\text{-}Kit^+$/ $Sca\text{-}1^+$/ $Thy\text{-}1^-$/ $IL\text{-}7R\alpha^-$/ $Flk\text{-}2^+$/ $Mac\text{-}1^{lo}$/ $CD34^+$ | $c\text{-}Kit^{lo}$/ $Sca\text{-}1^{lo}$/ $Thy\text{-}1^-$/ $IL\text{-}7R\alpha^+$/ $Flk\text{-}2^+$/ $CD24^{lo}$/ $CD27^+$/ $CD43^+$/ $TdT^+$/ $CD34^+$ | $c\text{-}Kit^+$/ $Sca\text{-}1^-$/ $Thy\text{-}1^-$/ $IL\text{-}7R\alpha$/ $Fc\gamma RII$/ $III^{lo}$/ $CD34^+$ |
| | SLAM: $CD150^+$/ $CD48^-$/ $CD244^-$ | SLAM: $CD150^-$/ $CD48^-$/ $CD244^+$ | | |

# The Haematopoietic stem cell niche

## *The osteoblastic and perivascular niche*

In the late 1970s Scholfield proposed that HSCs were located within a specific region (niche) in the bone marrow in order to maintain their multipotency and should HSCs be located elsewhere they would differentiate rather than retain the properties of a stem cell[14]. Since this original hypothesis was launched it has become clear that at least two haematopoietic stem cell niches exist - *the osteoblastic and the perivascular niche*.

The *osteoblastic niche* has been proposed due to a series of *in vito* and *in vivo* reports. Using osteobastic cells as feeder cells *in vitro*, it has been suggested that osteoblasts have an important role in the regulation of haematopoietic progenitor cells[15, 16]. It has been demonstrated that osteoblasts synthesise a variety of cytokines that are known to stimulate haematopoietic cells. These factors are for example: G-CSF, M-CSF, GM-CSF and IL-6[16-20]. In 2003 two independent studies reported that osteoblasts were critical regulators of haematopoiesis *in vivo*[21, 22]. In the first, Zhang *et al*. found that transgenic mice with conditional inactivation of bone morphogenic protein receptor 1A (BMPR1A) had approximately twice the number of LT-HSCs compared to wild-type mice. The increase in LT-HSCs was further examined and correlated to an increase of trabecular bone structures (or trabecular-bone-like-area), and a significantly increased number of osteoblasts[21]. It should be noted that other cell types may have been affected as the transgenic excision of BMPR1A was not specific for osteoblasts. The second study, by Calvi *et al*., supported the above findings using a transgenic model of which the constitutively activated parathyroid hormone-related peptide receptor (PPR) was controlled by an osteoblast-specific collagen promoter. PPR and parathyroid hormone are important regulators of calcium homeostasis, and therefore bone formation and resorption. The transgenic mice had a simultaneous increase of HSC and trabecular osteoblasts in the bone marrow[22]. A recent publication used an ossicle model to study the HSC within the osteoblastic niche. The model is based on transplanting mouse bone marrow stromal cells via a subcutaneous injection into the host mouse. The bone marrow stromal cells developed a region of cortical-like bone that was rich in trabecular structures – the ossicle. After a four week period the ossicle enriched with active haematopoiesis that was of endogenous origin. They found that host HSC homed to the ossicles via the peripheral circulation and that these HSC were also able to reconstitute lethally irradiated mice[23]. Another study demonstrated that HSCs homed to the endosteal bone surface and made direct contact with osteoblasts upon induced stress with 5-Fluorouracil treatment, a chemotherapeutic drug that induces apoptosis in proliferating cells[24]. In keeping with these studies, conditional ablation of osteoblasts results in a reversible decrease of bone marrow HSCs, indicating that osteoblasts are not only required for maintenance of bone marrow haematopoiesis, but are also an essential component of the niche[25].

The ability to rapidly mobilize HSCs, by the secretion of cytokines, to the vasculature has suggested that a subset of HSCs are in close proximity to the vascular system in the bone marrow[26, 27]. Knowledge of extramedullary haematopoiesis under stress conditions and foetal haematopoiesis which both lack the presence of osteoblasts or an osteoblastic niche indicate that other potential HSC niches are present. Murine haematopoietic stem and progenitor cells were imaged *in vivo*, and under a period of 70 days it was observed that these cells were localized to the microvasculature[28]. This proposed a functional *vascular endothelial niche* (or *perivascular niche*), however the population studied was not highly purified for HSCs. Another study the same year discovered that the family of SLAM antigens was highly enriched within the HSC population (Table 1). Using these markers the authors could identify that the majority of HSCs were in the perivascular region compared to the endosteal region where only 16% were located. With these data it is difficult to separate whether the accumulation of HSCs around the sinusoidal endothelial cells is due to the mobilization of HSCs to and from the circulation or rather due to a niche where the HSC would self-renew and maintain its multipotency. Several *in vitro* studies have supported the endothelial regulation of HSCs. In one study, HSCs increased in cell number, were induced to cell cycle and significantly increased the engraftment of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice when co-cultured with brain endothelial cells compared to isolated cells that had not been in co-culture[29]. Others have showed that endothelial cells from various organs support and promote HSCs to repopulate *ex vivo*[30, 31].

Taken together it seems as though both niches – the osteoblastic and the perivascular (and possibly several others) – collectively control HSC regulation of fate decisions. This was recently supported in a study from Scadden's group, where the endosteal cells were shown to be in close proximity to CD31 positive vascular cells. They therefore proposed that the osteoblastic niche is perivascular and that these niches overlap, yet they do not exclude that perivascular-only niches may be present in tissues absent of bone[32].

## *Hypoxic regulation of Haematopoietic stem and progenitor cells*

Except for the proposed cellular environmental niches (described above) it has also been suggested that haematopoietic stem and progenitor cells (HSPC) reside in hypoxic areas. It seems like the bone marrow has a lower oxygen level than peripheral blood of healthy donors[33] and that the area of hypoxia may be in the endosteal bone region as this area was stained positive for Pimonidazole (a chemical marker for hypoxia) at steady-state in mice[34]. In a study by Pamar and colleagues mice were intravenously injected with Hoechst 33342 (Hoe) dye. Based on the Hoe fluorescence intensity and thereby indirect oxygen gradient, they could demonstrate that cells on the lowest end of the scale had the highest HSC potential. Furthermore, by using Pimonidazole they demonstrated that HSCs had a higher staining of this compound than other bone marrow cells[35].

Several reports have demonstrated that hypoxic levels elevate the repopulating ability of both human and murine HSPC in *ex vivo* cultures[36-47]. One report also showed that more primitive haematopoietic cells were less sensitive to hypoxia[37]. By assuming that low levels of reactive oxygen species (ROS) are a measurement of hypoxia it has been proposed that a hypoxic niche provides the HSCs a long-term protection of oxidative stress and therefore self-renewal ability as haematopoietic populations with lower ROS have a higher HSC potential than populations with higher ROS levels[48].

At the molecular level more evidence is needed to fully understand the proposed hypoxic bone marrow niche. The transcription factor Hypoxia-Inducible Factor-1α (HIF-1α) is one of three hypoxia regulatory subunits that is stabilised at hypoxic levels. Although, it should be noted that there is evidence of a hypoxia-independent stabilisation of HIF-1α *in vitro* via various cytokines, SCF[49], G-CSF[50] and Tpo[51, 52]. Mouse models with direct or indirect effects on the HIF pathway exhibit extensive abnormalities within the haematopoietic development. HIF-1α deficient mice have an impaired proliferation of embryonic multi-lineage haematopoietic progenitors[53]. Furthermore, it has been demonstrated that HIF-2α deficient mice (HIF-2$\alpha^{-/-}$) exhibit pancytopenia due to defective haematopoietic microenvironment[54-56] and that the microenvironment of erythropoiesis is regulated by HIF-2α through VCAM-1 of endothelial cells[57]. Another important player in the HIF pathway is HIF-1β (or ARNT), which together with the HIF-α subunits are crucial in promoting hypoxic gene expression. ARNT deficient murine embryonic stem (ES) cells fail to differentiate to haematopoietic progenitor cells (HPC) under hypoxic conditions, however a decreased number of haematopoietic progenitors were rescued by exogenous VEGF (vascular endothelial growth factor)[58]. VEGF is the most well-known HIF-1α target gene and it is recognised as an essential mediator of vessel formation. Loss of one VEGF allele results in embryonic lethality due to absence of vascular structures however, interestingly these embryos also demonstrate a deficient HSC pool in the blood islands of the yolk sac[59, 60]. It has also been demonstrated that VEGF is required for maintenance of bone marrow activity and that VEGF mediates HSC survival and repopulation via an internal autocrine loop in mice[61].

# Leukaemia

Leukaemia is an umbrella term for all neoplasms that arise at any stage of haematopoiesis and predominantly involves the bone marrow. The exception is a neoplasm of a terminally differentiated B lymphocyte which is called myeloma instead of leukaemia. Leukaemias are termed chronic or acute based upon the speed of symptomatic onset and progression of the untreated disease. Chronic leukaemias show features of maturation while acute leukaemias on the other hand are characterized by a maturation arrest of progenitor or precursor cells accompanied by uncontrolled proliferation that results in bone marrow failure and is rapidly fatal without therapy. Myeloid leukaemia is broadly classified into chronic or acute myeloid leukaemia (CML or AML) compared to the lymphoid equivalent that is termed chronic or acute lymphoblastic (or lymphoid) leukaemia (CLL or ALL).

## Acute leukaemia

### *Risk factors*

Many risk factors have been proposed as a link to the development of leukaemia. The only factors that have been significantly associated as risk factors of both AML and ALL are environmental factors such as ionizing radiation and benzene exposure[62-64]. Retrospective studies following the atomic bomb explosions of Hiroshima and Nagasaki in 1945 demonstrated an elevated risk of leukaemic development in exposed personnel[65,66]. Benzene is a known carcinogen and exposure to this chemical has a strong increased risk of developing leukaemia[67-69]. The potential effects of environmental exposure on childhood leukaemia might occur either preconception, during pregnancy or during the postnatal period. Certain genetic or immunodeficiency syndromes (e.g. fanconi anemia[70], Down's syndrome[71], and ataxia telangiectasia[72]) place children at a higher risk of ALL. Also, a monozygotic twin has a higher risk of developing ALL than non-twin siblings[73].

### *Symptoms*

Acute leukaemia present with symptoms and signs of bone marrow failure with or without feature of extramedullary involvement, for example fatigue, fever, frequent infections, anemia, bleeding, musculoskeletal pain, as well as lymphadenopathy, hepathomegaly and splenomegaly (these latter signs are more common for patients with ALL than AML).

## *Classification and diagnosis*

Assigning patients to subtypes of AML and ALL has prognostic and in some cases such as acute promyelocytic leukaemia (APL), therapeutic significance. The diagnosis and classification of acute leukaemia are based upon tests of cells derived from the peripheral blood or aspirated from the bone marrow of the patient. The tests include examination of: morphologic, cytochemical, immunophenotypic and cytogenetic features, or by the combination of these.

Blood smears from the patient's peripheral blood are used to evaluate the morphological and cytochemical status, but also to assess the proportion of haematopoietic cells. The morphological evaluation is a first step to classify the lineage and both the French-American-British (FAB) classification system[74-78] and World Health Organization (WHO)[79] is used in the clinic today. Based on morphological and cytochemical examination categorises the FAB system AML into eight groups (M0-M7) or three groups for ALL (L1-L3). The ALL FAB groups have been found not to be of prognostic significance and the classification of ALL is rather based on the WHO system that combines morphology, immunophenotype and cytogenetics.

The immunophenotypic evaluation of peripheral blood or bone marrow aspirate has been extensively used in the classification of acute leukaemia following the introduction of flow cytometric analysis to the clinic. Using this method, AML and ALL may be further categorised into several sub-groups based upon the detection of different cellular antigens (mainly CD markers – Cluster of Differentiation). Using different panels of antibodies will the disease be classified to AML or ALL lineages (T or B-cell) as well as the status of B-cell maturation of the ALL clone i.e. pro-B, common-B or pre-B.

The cytogenetic status of the leukaemic cell offers important information as to the classification and diagnosis of the patient. Information of an abnormal cytogenetic profile may be identified using techniques such as FISH (fluorescence *in situ* hybridization),

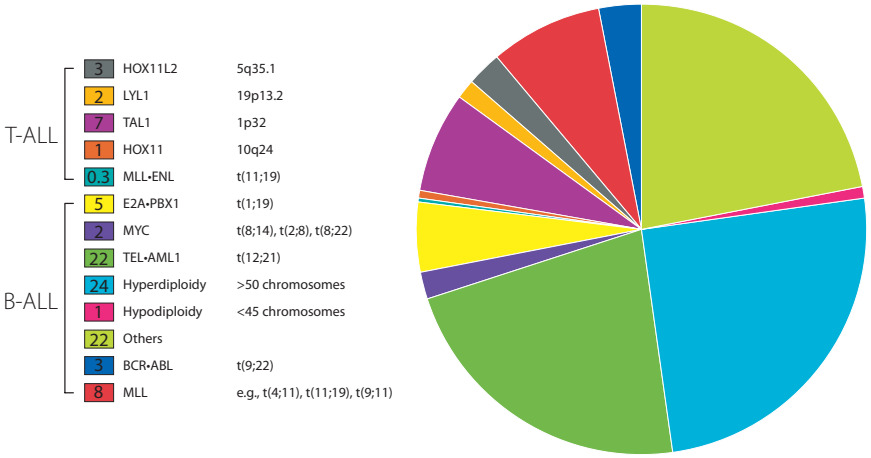


**Figure 2. Estimated frequency of specific genotypes of ALL in children.** Modified from Pui *et al.*[80]. Numbers in coloured squares represent the percentage value.

which can be performed on metaphase and interphase cells. Probes that may be used are: centromeric probes, specific DNA probes (e.g. sequences found at breakpoints in translocations or inversions), or SKY - a spectral caryotype, which detects sequences extending over the whole chromosome.

Based upon the above criteria a patient may be diagnosed with leukaemia and receive an allocation to a diagnostic sub-group.

# Childhood acute lymphoblastic leukaemia

## *Clinical aspects*

Acute lymphoblastic leukaemia is the most common malignancy among children (0-18 years). In Sweden approximately 60 children are diagnosed with ALL each year (Swedish Cancer Registry). ALL accounts for approximately a quarter of all childhood cancers and 80% of childhood leukaemias (Swedish Cancer Registry), with a peak in incidence between 2 and 5 years[81]. In Scandinavian countries (Sweden, Finland, Norway and Denmark) the incidence has remained stable at an approximate rate of 3.3 cases per 100,000 children from 1983 to 2002 through an increase in incidence between 1975 and 1983[82].

ALL can generally be divided into two categories: B-cell lymphoblastic leukaemia (B-ALL) and T-cell lymphoblastic leukaemia (T-ALL). Among the B-cell subtypes the pre-B ALL are the most common and accounts for approximately 70-80%.

ALL involves the bone marrow but the disease may be present at extramedullary sites (i.e. involving spleen, liver, mediastinum, lymph nodes, thymus, testes and CNS – central nervous system) at the time of diagnosis. The initial white blood cell (WBC) count can vary from being undetectable to greater than 100,000/ µl. Approximately 50% of all children have WBC counts of <10,000/ µl and 20% have a WBC count >50,000/ µl at the time of diagnosis (Normal WBC: 4-11,000/ µl).

## *Cytogenetics*

The most common genetic abnormality seen in leukaemic cells from children with ALL is hyperdiplody (>51 chromosomes) followed by the chromosomal translocation t(12;21)(p13;q22) that forms the TEL·AML1 (ETV6·RUNX1) fusion protein (Figure 2). These two abnormalities together comprise approximately half of all childhood ALL patients[80]. Children with hyperdiploid B-ALL usually possess extra copies of chromosomes 21, X, 14 and 4, with chromosomes 1, 2, and 3 being the least common[83]. Cytogenetic features play an important role in the risk-group stratification that will be described in more detail below.

## *Prognostic factors and risk-group stratification*

In the clinic today use haematologists clinical and laboratory features as the basis of treatment decisions for children diagnosed with ALL. Based on these features or 'prognostic factors' the children are then stratified into different risk-group criteria: standard-risk, intermediate-risk and high-risk (Table 2). Risk-tailored therapy is used to minimize over-treatment of low-risk patients and to ensure a more intense treatment to patients with a poor outcome and a higher risk of relapse and treatment failure. The main ALL prognostic factors used are:

- Age
- White blood cell (WBC) count
- Immunological subtype
- Cytogenetics and ploidy
- Response to therapy

Age and WBC count at the time of diagnosis are independent predictors of prognosis. Patients older than 10 years or infants (<12 months) have a less favourable outcome and are assigned to higher risk groups or to groups with special treatment protocols. A WBC count greater than 50,000/ µl is associated with a poor patient outcome, compared to a count of less than 50,000/ µl which is associated with a favourable prognosis[84, 85].

The immunological subtype T-ALL has historically been linked to a poorer prognosis. It is however not clear whether a patients immunogenic subtype is an independent predictor of prognosis or if it is due to the elevated initial WBC count and increasing age[85, 86].

Rearrangements of the mixed-lineage leukaemia gene (MLL) at chromosome 11q23, are detected in 5-8% of children with ALL but are occurring more frequently in infants in approximately 80% of all cases. Infants with MLL translocations have a very poor prognosis with a long-term event-free survival (EFS) rate of 10-30%[87,88]. The outcome of patients with MLL rearrangements seems to be age dependent, children (≥12

**Table 2. Risk group stratification.** Based on the current protocol in the United Kingdom (UKALL 2003).

| | |
|---|---|
| **Standard** | Children aged >1<10 years old and with a highest WBC count of <50,000/ µl. Cytogenetics: Do not have t(9;22), hypodiploidy (<44 chromosomes), or an MLL gene rearrangement |
| **Intermediate** | Children ≥10 years old and/or ≥50,000/ µl. Cytogenetics: Do not have t(9;22), hypodiploidy (<44 chromosomes), or an MLL gene rearrangement |
| **High** | All children independent of age (except infants) and WBC who are diagnosed with t(9;22) or have hypodiploidy (<44 chromosomes) or have a MLL gene rearrangement or have a slow early response to therapy. |

months) have a better prognosis than infants, but their outcome is poor compared to children the same age without a MLL translocation[88]. The t(9;22) translocation which creates the BCR·ABL fusion protein (also referred to the Philadelphia chromosome) is found in 3-5% of children diagnosed with ALL and is an unfavourable prognostic factor (5-year EFS 28%)[89]. The translocation is more common in older patients with pre-B ALL and patients with a high WBC count. Numerical chromosomal abnormalities (ploidy changes) are prognostic indicators in patients with childhood ALL. High hyperdiplody (50 or more chromosomes) is a favourable prognostic factor compared to hypodiplody (fewer than 45 chromosomes) and it is associated with a poor prognosis, with an 8-year EFS of 39%[90]. High hyperdiplody is observed in approximately 24% and hypodiplody in 5-6% of cases with childhood ALL.

One of the most important prognostic factors is initial response to therapy. Children with a slow early response (SER) to treatment have worse prognosis i.e. morphological detectable leukaemia in the bone marrow of patients at 7 or 14 days, or 7 to 10 days in peripheral blood following initiation of multi-agent chemotherapy[91-93]. SER is also linked to adverse cytogenetics as >50% of children with t(9;22) ALL have a slow response to therapy[94].

## *Therapy*

Survival rates for childhood leukaemia have improved dramatically since the 1980s, from a recurrent long-term cure rate of 50% to greater than 80%[95]. No new drugs have been introduced during this time period, for the effects seen are likely due to better risk-based classifications and standardised therapeutic strategies. The intensity of treatment is dependent on the clinical risk and involves often more than 10 drugs. A patients treatment regimen is typically divided into four phases: (1) remission

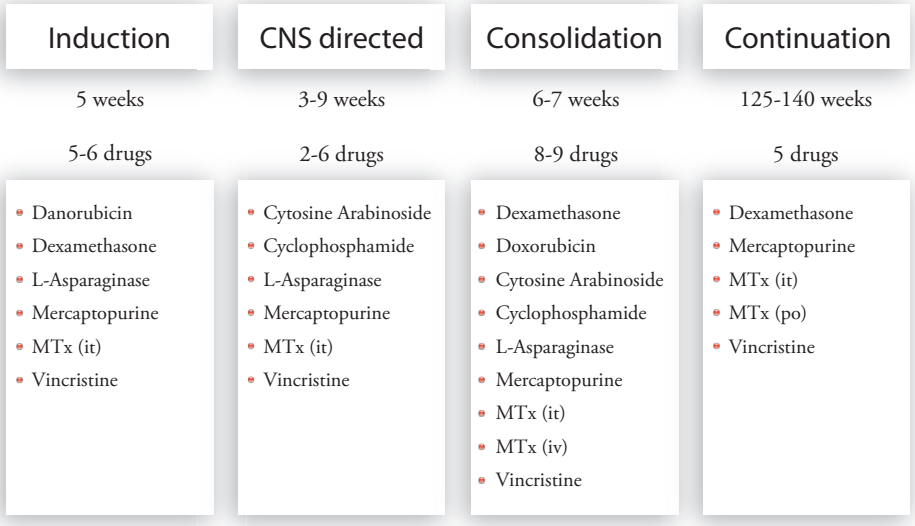


**Figure 3. Overview of chemotherapy given to children diagnosed with ALL.**
MTx= Methotrexate; it= intrathecal; iv= intravenous; po= peroral

induction, (2) CNS directed, (3) consolidation or intensification and (4) maintenance or continuation (Figure 3). In addition to this treatment protocol are in some cases CNS radiotherapy or bone marrow transplant required for cure. The duration of the treatment varies between the different prognostics risk-groups but spans over a minimum of 24 months. Therapy with imatinib holds very good promise for children diagnosed with t(9;22) ALL, as a recent trial showed data with more than twice improved 3-year EFS as historical controls[96].

## *Minimal residual disease and relapse*

Complete remission, after the treatment induction phase, is determined by morphological detectable leukaemia that has been set to a threshold value of <5% leukaemic cells (lymphoblasts) in the bone marrow (detected by light microscopy). Complete remission is achieved in more than 98% of children with ALL but with the current treatment protocol approximately 15-20% of children relapse and this is the most important adverse event in childhood ALL[97-99] with survival rates between 20-50%[100, 101]. Of the relapsed cases, approximately 30% have CNS involvement compared to approximately 3% of all ALL cases at diagnosis[94, 98, 102]. Relapse is thought to result from lymphoblasts that have survived the treatment and that are not detectable using conventional morphological assessments of 'complete' remission. The use of more sensitive techniques to detect minimal residual disease (MRD) is of increasing great clinical relevance. Several studies have demonstrated that MRD levels are independent predictors of outcome and that high levels are correlated to worse prognosis [103-106].

# Leukaemogenesis

The progression and development of leukaemia has been extensively studied over the past decade. Exploring whether every cell or a small subset of cells within leukaemia cell samples have leukaemia initiating properties has been the major research focus. This concept forms the basis of two different hypotheses termed the *Stochatic* and the *Leukaemic/Cancer stem cell model*, see Figure 4 (reviewed in[107]). The Stochatic model postulates that tumour heterogeneity is based on evolutionary abnormal genetic (intrinsic) or environmental (extrinsic) alterations, that each leukaemic cell will be equally sensitive to these alterations, that the cell of origin is a random cell which originally does not have the capacity to self-renew and that leukaemia-initiating activity cannot be enriched. Altertnatively, the leukaemic stem cell model is based upon a hierarchy where only a small subset of cells called leukaemic stem cells (LSCs) have unlimited self-renewal potential. These cells form the heterogenic leukaemic population with different self-renewal and proliferative properties and it is only the LSCs that can re-initiate malignant growth. The cell of origin is thought to be a progenitor of the HSC based on two differing theories. Firstly, HSCs already have functional self-renewal machinery compared to a differentiated cell, which would have to re-activate this characteristic. Secondly, HSCs have a long lifespan and may accumulate genetic abnormalities over time.

## The Leukaemic stem cell

The first cancer stem cell to be identified was the LSC from AML patients[108]. This population, termed the SCID leukaemia-initiating cell (SL-IC), was found to be $CD34^{+}/ CD38^{-}$ similar to the immunophenotype of normal HSCs. Interestingly, AML cells negative for CD34 or double positive for CD34 and CD38 were unable to engraft SCID mice. Another study showed that AML cells ($CD34^{+}/ CD38^{-}$) from patients of various FAB subgroups were able to engraft NOD/ SCID mice independent of their classification[109]. In serial transplants they found that the engrafting cells consisted only of a small subset of the initial AML population. Based on these findings Dick and Bonnet proposed a hierarchical organisation of the disease, with the LSC as the mother cell. This theory is termed the *Leukaemic stem cell model*. In a later study they even proposed that a hierarchy was found within the LSC population, similar

to the normal HSC compartment, with varying degrees of self-renewal[110]. Several studies have, since the original LSC immunophenotype was launched, revealed that the LSCs have different antigens expressed on the surface compared to the HSC. The immunophenotype of the AML LSC is: $CD34^+$, $CD38^-$, IL-$3R^+$ (CD123), $Thy1^-$ (CD90) and c-$kit^-$ (CD117)[111-113]. It is suggested that the difference seen between the normal and the malignant stem cell is part of the transformation event or that the transformation occurs in HSCs with unique properties. There are however contradictory reports describing that leukaemia-initiating cells are also found within the $CD34^-$ population, raising the possibility that $CD34^-$ cells may be target cells for transformation[114-116]. In addition, it has been shown that primary cord blood $CD34^-$ cells can repopulate NOD/ SCID mice, although with a low potential[117]. Another recent interesting finding in the AML LSC field was made by Bonnet and colleagues that anti-CD38 antibodies, used for FACS to sort populations for xenotransplantation, have inhibitory effects on the engraftment of both normal and leukaemic repopulating cells[118]. The immunodeficient NOD/SCID mouse model used eliminated cells positive for anti-CD38 antibody through immune clearance. The effect could be reversed when treating mice with immunosuppressive antibodies. When this effect was abrogated it was demonstrated that $CD34^+$/ $CD38^+$ AML cells could also initiate leukaemia. Again there is data challenging the original proposed immunophenotype of AML LSC.

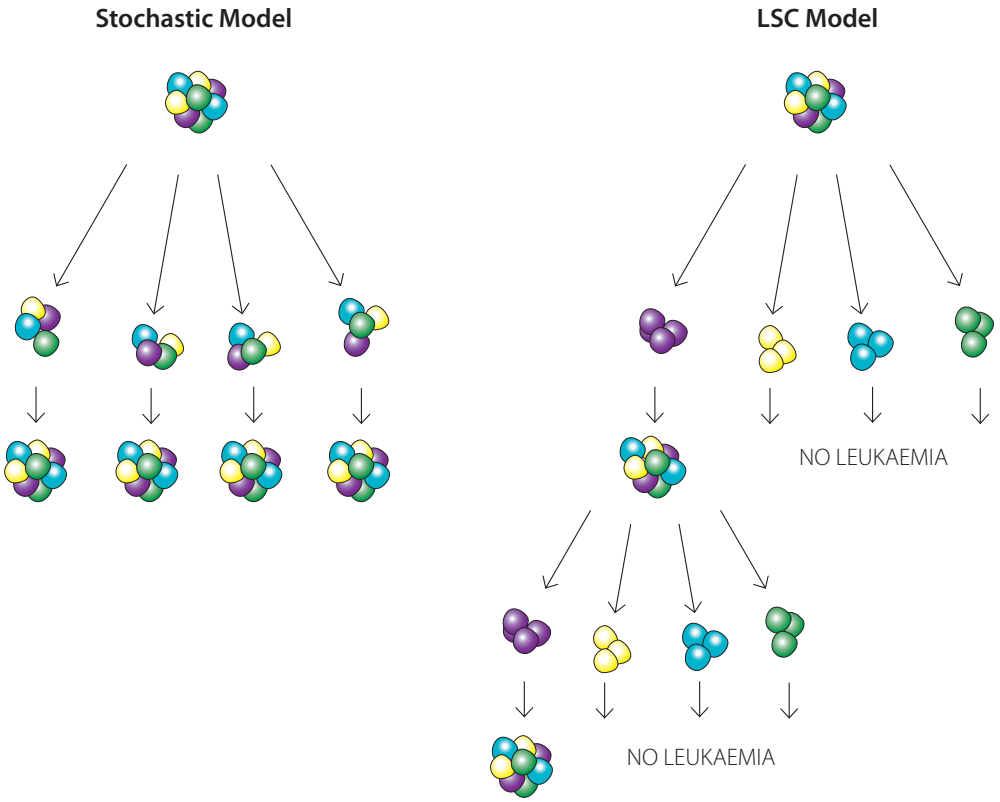


**Figure 4. Models of leukaemogenesis.** Leukaemia is composed of heterogeneous cells. According to the Stochastic model every leukaemic cell has leukaemia initiating properties. The LSC (Leukaemia Stem Cell) model postulates that only a small subset of cells, the LSCs, have leukaemia initiating properties.

The immunophenotype of ALL LSCs is a topic of debate within the ALL stem cell field[119]. Original reports described $CD34^+/ CD38^-$ or $CD34^+$ ALL cells lacking the expression of mature lymphoid markers (CD19 and CD10) as having leukaemia initiating properties[120, 121]. Also, a study from Greaves and colleagues showed that TEL·AML1 transduced cord blood formed an abnormal $CD34^+/ CD38^-/ CD19^+$ cell with a NOD/SCID engrafting potential[122]. Published data on the expression of lymphoid mature markers in ALL stem cells is conflicting. Based upon the above described data, it seems both an immature or a mature phenotype of lymphoid cells are able to engraft immunodeficient mice. In addition, a study showed that both ALL LSCs ($CD34^+/ CD19^-$) with or without CD38 expression could engraft mice in serial transplantations[123]. To further confuse the issue, a separate study showed that ALL cells at different stages of maturation ($CD34^+$ and $CD34^-$, $CD19^+$ and $CD19^-$ $CD20^+$ and $CD20^-$) possessed leukaemia initiating properties[124]. Whether or not the methodology used (antibody-mediated clearance, immunodeficient mouse model and a difference in risk classification of used patient samples) or the lack of stem like properties of ALL cells is the answer to the conflicting results is as yet unresolved.

## Hallmarks of Leukaemia

The hallmarks of leukaemia do not differ greatly from those of solid cancers. Leukaemia is a genetic disease caused by aberrant patterns of gene expression. The genes affected may be divided into two groups: *oncogenes* and *tumour suppressor genes*. Activation of an oncogene can arise from a specific point mutation within the sequence of a gene; from gene amplification or translocation of a gene to a more transcriptionally active site or from a gene fusion generating a new chimeric protein with enhanced biological activity. Tumour suppressor genes may be inactivated via deletion of that gene or following point mutation that disrupts proteomic function.

Leukaemic transformation is thought to be a multistep process involving activation of oncogenes and inactivation of tumour suppressor genes that together lead to impaired differentiation as well as increased proliferation, cell survival and self-renewal. As the most common genetic abnormality in leukaemia is chromosomal translocations and these have a major importance in leukaemogenesis, they will be described under a separate heading - Chromosomal translocations. The multistep process of leukaemic transformation will there also be described in more greater detail. Hear after will a few other common genetic abnormalities or impaired signalling pathways in acute leukaemia be described.

## *NOTCH-1*

NOTCH-1 mutations are present in over half of all T-ALL cases and are associated with a poor patient outcome[125, 126]. Notch-1 is a transmembrane protein that via cell-cell interactions results in proteolytic cleavages of both the extracellular and intracellular domains. The released intracellular domain thereafter translocates to the nucleus and associates with a transcriptional complex[127]. In normal haematopoiesis, Notch-1 signalling is an essential regulator of $\alpha\beta TCR^+$ versus $\gamma\delta TCR^+$ and $CD4^+$ versus $CD8^+$ T-cell lineage commitment[128]. It has also been postulated that NOTCH-1 has an important role in HSC maintenance (reviewed in[129]).

The mechanism by which NOTCH-1 functions as an oncogene in T-ALL is via a constitutively active signalling pathway - either via a ligand-independent activation or a stabilised intracellular Notch-1 protein. These observations have come from several *in vitro*, and *in vivo* studies where mice developed T-ALL like diseases due to aberrant Notch-1 signalling[130-132]. Over-expressed intracellular Notch-1 in bone marrow lineage negative cells led to accumulation of immature T-cells in the bone marrow, blood and lymphoid tissues of transplanted mice as early as 2 weeks post-transplantation. This demonstrates the potent oncogenic effect of Notch-1[132]. The aberrant Notch-1 signalling affect the malignant cell through enhanced cell survival, proliferation and cell metabolism[133]. Directed targeted therapy has mainly focused on γ-secretase inhibition of Notch-1 signalling, however *in vivo* studies[134, 135] have reported intestinal toxicity in mice which is thought to be due to active Notch-1 signalling in immature intestinal progenitors[136, 137]. Another study has demonstrated that this can be overcome by the concomitant use of glucocorticoids[138].

## *FLT-3*

FMS-like tyrosine kinase 3 (FLT-3) has been shown to play a crucial role in normal haematopoiesis[139, 140]. In haematopoietic malignancies FLT-3 is one of the most common mutated genes, accounting for approximately 15-35% of AML cases (ALL: 1-3%). In childhood AML, FLT-3 mutations (FLT-3 internal tandem duplications, ITD) have been shown to be independent predictors of poor clinical outcome. Studies in adult AML cohorts have not produced the same conclusions, but still an overall poor prognosis has been noted[141-144]. Gain-of-function FLT-3 mutations result in a constitutively activated tyrosine kinase that is a very potent oncogene. FLT-3 mutations disrupt normal proliferation, differentiation and apoptosis[145-149]. Early clinical trial data from studies involving inhibitory molecules that disrupt the gain-of-function of FLT-3 mutations have shown promising results [150-153].

## *WT1*

Wilm's Tumour 1 (WT1) is a zink-finger transcription factor that is highly expressed in haematopoietic progenitor cells[154]. An elevated expression and presence of mutations in acute leukaemia indicate an important role of the protein in the progression of leukaemia. WT1 mutations are generally rare in patients with ALL but have been found at the same frequency in both AML and childhood T-ALL. The frequency has been reported to vary between 10 to 15%[155-158]. The leukaemogenic function of WT1 is under debate. Whether the gene acts as an oncogene or a tumour suppressor gene is not fully understood. The fact that WT1 has an elevated expression in a majority of acute leukaemias and that knockdown of WT1 in leukaemic cell lines results in reduced growth and apoptosis suggests that WT1 is an oncogene[159, 160]. Some studies argue against WT1 oncogenic phenotype. $CD34^+$ cells and differentiation-competent leukaemia cell lines retrovirally transduced with WT1 induced growth arrest, quiescence and differentiation[161]. Also, loss of WT1 expression in leukaemic cell lines *in vivo* results in leukaemic development[162].

## *Angoigenic Factors*

VEGF-A and its tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR) are well-established key regulators in endothelial cell growth and in the process of tumour angiogenesis[61, 163]. VEGF and VEGFR are crucial in normal haematopoiesis and they have recently been shown to have an important role in haematological malignancy. They are not classified as oncogenes in leukaemia for they are not required for the leukaemic tranformation, but their involvement in maintaining the malignant phenotype is demonstrable. It was initially thought that angiogenesis was not important for haematological malignant progression due to the absence of tumour mass found in solid tumour equivalents. Intriguingly, several papers have reported augmented microvessel density within the bone marrow of patients with ALL[164, 165] and AML[164, 166-168]. The mechanism behind this is uncertain, however one report presented a positive correlation between increased VEGF expression and microvessel density in ALL and AML patients[166]. Higher VEGF protein expression has also been observed in both ALL and AML patients compared to control groups[164, 166], and elevated VEGF has prognostic significance in patients with AML[169]. Interestingly, VEGF expression in children have been found at lower levels relative to control groups. It is clear that an increase in microvessel density, similar to solid tumours, provides oxygen and nutrients to the malignant cells. Moreover, the increased endothelial cell mass may be important in the production of cytokines and growth factors that act on malignant cells in a paracrine fashion, promoting cell proliferation or survival.

Patterns of protein expression of VEGFR-1 in relation to VEGFR-2 have produced contradictory results in adults diagnosed with ALL and AML. In childhood ALL, however, VEGFR-1 is more commonly expressed than VEGFR-2[170, 171]. The receptors of VEGF have an important role in leukaemia as studies of small molecules inhibitors targeted against VEGF receptors has induced a decreased leukaemic phenotype both *in vitro* and *in vivo*[172, 173]. Also, studies have reported promising results using small molecule inhibitors in early clinical trials [174-177].

# Chromosomal translocations

Out of the main genetic chromosomal abnormalities found in leukaemia (deletions, inversions and translocations), chromosomal translocations are the most prevalent, as they occur in approximately 65% of all acute leukaemias[178]. Translocations can be associated with specific sub-types of leukaemias, for example the t(15;17) translocation fuses the transcription factor PML (promyelocytic leukaemia) to RARα (retinoic acid receptor alpha) and has only been reported in patients with acute promyelocytic leukaemia (AML-M3). Furthermore, approximately 98% of all CML patients harbour the BCR·ABL fusion and almost all cases with a CBFb·MYH11 fusion have an AML-M4Eo phenotype[179, 180]. This is not always the case as numerous translocations are found both in patients with ALL subtypes and in patients with different types of AML. One of these translocations, t(10;11)(p13-14;q14-21), will be discussed in greater detail below.

At a molecular level chromosomal translocations typify DNA breaks which occur in a part of the chromosome that subsequently join to another chromosome. The translocation can be *nonreciprocal* or *reciprocal* - defined by their net change of chromosomal material. The latter seem to be more common in haematological malignancies and often occur between genes with transcriptional properties, that are involved in normal haematological differentiation, self-renewal and proliferation[181]. In order to generate an expressed fusion protein that has oncogenic properties there are several requirements. Firstly, the genes must lie in close proximity[182], secondly the translocation has to occur within certain introns of specific genes in both chromosomes and thirdly the gene fusion has to be in frame and sufficient to encode a protein (fusion).

## The aetiology of chromosomal translocations

### *Molecular origin*

The mechanism by which chromosomal translocations occur has been intensively studied. It is hypothesised that normal recombination mechanisms become disorganised leading to junctions of two genes that may, together, form a hybrid protein with altered properties.

A high frequency of translocations (~35%) seen in patients with T-ALL involve the T-cell receptor (TCR) gene. Similarly, but less frequently, patients with B-ALL possess translocations involving the immunoglobulin (Ig) genes. From these observations, the V(D)J recombination and Ig class switch recombination of TCR or Ig genes have been suggested to be the necessary dysfunctional mechanisms required for programmed DNA damage. In the normal developing T or B lymphocyte the V(D)J recombination involves the recombinase-activating gene (RAG) 1/2 proteins that cut V(D)J gene fragments at specific recombination signal sequences (RSS). Together with several cooperating enzymes, including TdT (terminal deoxynucleotidyl transferase), the created double strand breaks (DSB) join together to form a new recombinant gene[183]. In a normal activated mature B lymphocyte, a process called Ig heavy chain class switch recombination can occur to improve Ig diversity as part of the humoral immune response. This normal process involves an enzyme called activation-induced deaminase (AID) that mutates cystedine residues to uracyl which mediate DSB formation within the Ig switch regions[184]. Two reports[185, 186] were published in 2008 proposing that not only are RAG and AID enzymes able to create Ig or TCR translocations but they also have the ability to create oncogene "off-target" DSBs[185,187]. Should DSBs of two "off-target" genes be created, this could generate a non-Ig, non-TCR translocation. Deregulated expression of AID in B lymphocytes has also demonstrated that every chromosome has the capacity to be affected by AID chromatid breaks and translocations[188].

Other proposed mechanisms that can cause DSBs followed by translocations are so called apoptogenic stimuli induced by chemotherapeutic drugs (such as topoisomerase-II inhibitors and etoposide)[189-192].

## *Prenatal origin – evidence of a multistep leukaemic transformation*

The founding of the 'in utero transformation' hypothesis came in 1962 when Wolman suggested that leukaemia can originate from one monozygotic twin in the uterus to be then transmitted to the other twin via the shared circulation[193]. Greaves and colleagues have provided molecular evidence that such suggestion is correct and that in some cases, leukaemogenesis occurs following at least two events similar to the Knudson-two-hit model of retinoblastoma[73, 194]. From data collected on twin siblings, it has been described that some translocations TEL·AML1[195-197], AML1·ETO[198] and some MLL rearrangements[199, 200] occur during embryogenesis. These translocations had identical breakpoints in both twin siblings indicating that the pre-leukaemic clone was 'infected' from one sibling to the other through a shared placenta[195-197]. More evidence came from the analysis of Guthrie cards (archived neonatal blood spots) where it was identified that oncogenic translocation was present at birth in children that were clinically healthy at the time of sampling. Clinically detectable disease occurred either within the first year of life or in some children after fourteen years, this suggests that a second postnatal 'hit' is required for a complete leukaemic transformation. The two-

hit hypothesis is supported further by reports detecting fusion genes within healthy individuals[190, 201-204]. Several mouse models have described that other genetic events together with a translocation are critical for a complete transformation and that, in some cases, the introduction of other genetic events shortens the latency period of the disease[205-207]. These data together suggest that some oncogenic fusions are capable of inducing leukaemia on their own whilst others require a second, or even several events to generate clinically detectable disease. It should be noted that a prenatal origin of chromosomal translocations in childhood leukaemia may not be unique, as childhood ALL cases with the E2A·PBX1 translocation only rarely have positive neonatal blood spots[208], and no cases of identical twins have been reported.

## Reciprocal translocations

Reciprocal translocations are one type of chromosomal translocations that are not affected by either the loss or gain of DNA, they are also referred to as balanced translocations (Figure 5). Reciprocal translocations are found in some solid malignancies, particularly childhood sarcomas, but have been mostly described and studied in haematological malignancies.

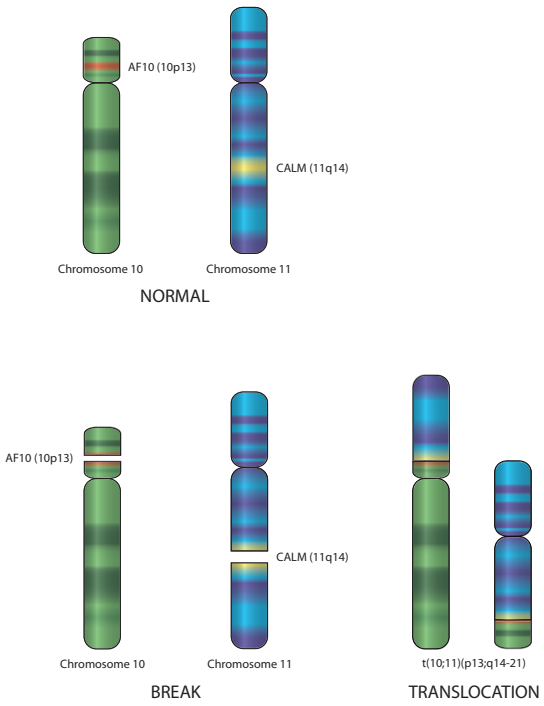


**Figure 5.** Reciprocal translocations are characterised by their inability to be affected by either loss or gain of DNA. t(10;11)(p13;q14-21) is a reciprocal translocation.

A reciprocal translocation will in theory form two chimeric genes that could subsequently form two functional chimeric proteins. However, in order to generate one chimeric protein (or fusion protein), the fusion gene must be in frame. In order to generate two fusion genes and therefore two expressed fusion proteins from one reciprocal translocation, both fusion genes must be in frame. In terms of oncogenic

potential, both fusion genes must involve relevant introns from relevant genes. It is possible that the second reciprocal translocation (which shall be referred to as the reciprocal translocation) is never expressed or that the reciprocal translocation is not detected in the majority of patients and is therefore thought to lack functionality.

Few studies describe the leukaemogenic involvement of reciprocal translocation in acute leukaemia, however some well characterised translocations - MLL·AF4, PML·RARα and PLZF·RARα - together with their reciprocal fusions play a key role in leukaemogenic transformation.

## *MLL·AF4*

MLL·AF4 t(4;11) is the most common translocation in infant, childhood and adult MLL gene-arranged leukaemias and is correlated to poor patient outcome. Both MLL·AF4 and AF4·MLL transcripts are expressed in 80% of cases[209]. Through adopting both an *in vitro* and *in vivo* model, Marschalek and colleagues demonstrated that the two reciprocal fusion proteins are involved in leukaemogenic transformation. The *in vitro* data[210] showed that cells expressing both MLL·AF4 and AF4·MLL have a more pronounced leukaemogenic potential compared to the single fusion expressing cells, however the AF4·MLL fusion was more dominant over the MLL·AF4 fusion and this was even more distinct *in vivo*[211]. In a transplantation mouse model it was shown that the dominant AF4·MLL fusion could induce ALL in mice independent of the MLL·AF4 fusion and that mice harbouring cells with both fusions had a similar outcome[211]. Mice transplanted with MLL·AF4 cells did not develop the malignant disease similar to previous studies where mice had a low penetrance and long latency[211-213].

## *PML·RARα and PLZF·RARα*

PML·RARα t(15;17) is a translocation that correlates with promyelocytic leukaemia (APL) and is unique because of its response to all-*trans* retinoic acid (ATRA) therapy. PML·RARα and RARα·PML transcripts are reported in 70% of cases. Mice models with the PML·RARα fusion develop leukaemia after a relatively long latency (6-12 months) and only 10-15% have a lethal form of leukaemia that resembles the human APL[214-216]. PLZF·RARα transgenic mice lack a complete APL leukaemic phenotype, for the mice had a phenotype similar to CML with mature myeloid cells rather than cells blocked at the promyelocytic stage of differentiation[217, 218]. These data suggest that PML·RARα and PLZF·RARα were crucial but not sufficient to cause APL leukaemogenesis. When examining the reciprocal RARα·PML and RARα·PLZF transgenic mice these did not have any sign of malignant disease. Interestingly, double transgenic PML·RARα/ RARα·PML mice increased the frequency of APL (~57%) and double transgenic PLZF·RARα/ RARα·PLZF mice now had an APL phenotype[217, 218]. However, for both double transgenic mouse models the disease had a long latency period that is not observed in human APL, suggesting that additional genetic events are required for a complete leukaemic phenotype.

# The t(10;11)(p13-14;q14-21) reciprocal translocation

## *Breakpoints*

The t(10;11)(p13-14;q14-21) translocation is a reciprocal translocation and fuses the gene CALM, which maps to chromosome 11q14, with AF10 (10p12) to form either a CALM·AF10 fusion or the reciprocal AF10·CALM fusion (Figure 4). The t(10;11)(p13;q14-21) translocations were first identified in U937 cells, a monocytic cell line established from a patient with histiocytic lymphoma. Dreyling *et al.* first identified the breakpoint between nucleotides 2090/2091 of CALM and 422/423 of AF10[219]. The following couple of years three independent reports described new evidence for the break point in U937 cells, now instead between nucleotides 2091/2092 of CALM and 423/424 of AF10[220, 221]. The fusion would thereby result in a 1595 amino acid sequence, where the final four amino acids of CALM were replaced by nearly the full length of the AF10 protein. To date, 12 different CALM·AF10 breakpoints and 9 AF10·CALM have been reported in patients with acute leukemia. In a number of studies the presence of the AF10·CALM translocation has not been analysed, however, studies evaluating both translocations indicate that the AF10·CALM translocation is found in approximately 60% of patients with a CALM·AF10 translocation (Table 3).

## *Clinical features*

CALM·AF10 is a rare translocation but has been reported in all the different FAB types of AML except M3 and M6. It is most commonly associated with T-ALL and the more undifferentiated AML subgroups (M0, M1)[220-232]. Most of these cases have clonal IgH, TCRG and/or TCRD, as well as both myeloid and lymphoid immunological phenotypes, suggesting that the leukaemic clone is a multipotent progenitor cell[233]. Asnafi *et al*. reported that of 144 patients diagnosed with T-ALL approximately 10% of paediatric and adult patients (ranged from 3-43 years, mean 20.7) expressed the CALM·AF10 transcript[223]. The T-ALL cases that were associated with the fusion were of either immature phenotype or TCRγ/δ. Despite the low patient number, the CALM·AF10 immature T-ALL patients identified a poor prognostic subgroup, as patients did not respond to treatment, relapsed or had a low survival.

## *In vivo models of CALM·AF10 (2092/424)*

At the present, there are three independent studies describing *in vivo* models of leukaemia that arise from the CALM·AF10 fusion transcript. In the first study, a vector-based RNAi approach was used to knock down the expression levels of CALM·AF10 in the U937 cell line. *In vitro*, fewer and smaller colonies were observed when the knockdown cells were cultured on methylcellulose. Cells were transplanted into mice and the control cells revealed extramedullary infiltration of spleen, kidney

**Table 3. Summary of reported cases with t(10;11)(p13;q14-21).** T-ALL patients from Asnafi *et. al.* are not included[223].

| | Sex/Age | Diagnosis | CALM·AF10 | AF10·CALM | Ref. |
|---|---|---|---|---|---|
| 1 | F/44 | AML-M0 | CALM 2091: AF10 424 | n/o | [222] |
| 2 | M/27 | AML-M0 | CALM 2091: AF10 424 | n/o | [227] |
| 3 | M/22 | AML-M0 | CALM 2091: AF10 883 | nd | [223] |
| 4 | F/12 | AML-M0 | CALM 2091: AF10 589 | AF10 588: CALM 2092 | [224] |
| 5 | M/47 | AML-M0 | CALM 1926: AF10 883 | nd | [221] |
| 6 | F/28 | AML-M1 | CALM 2091: AF10 883 | n/o | [222] |
| 7 | F/36 | AML-M1 | CALM 2091: AF10 978 | nd | [223] |
| 8 | M/19 | AML-M1 | CALM 2091: AF10 883<br>CALM 1926: AF10 883 | AF10 882: CALM 2092 | [221] |
| 9 | F/21 | AML-M1 | CALM 2091: AF10 589<br>CALM 1926: AF10 589 | AF10 588: CALM 2092 | [221] |
| 10 | F/12* | AML-M1 | CALM 2091: AF10 424 | AF10:CALM | [227] |
| 11 | F/12 | AML-M1 | CALM 2091: AF10 424 | nd | [223] |
| 12 | M/19* | AML-M1 | CALM 2091: AF10 424 | AF10:CALM | [227] |
| 13 | M/19 | AML-M1 | CALM 2091: AF10 424 | nd | [223] |
| 14 | M/47* | AML-M1 | CALM 2091: AF10 424 | AF10:CALM | [227] |
| 15 | M/47 | AML-M1 | CALM 2091: AF10 424 | nd | [223] |
| 16 | F/12 | AML-M1 | CALM 2091: AF10 424 (I-1)<br>CALM 2091: AF10 424 (I-2)<br>CALM 1986: AF10 424 (II)<br>CAML 1926: AF10 424 (III) | n/o | [218] |
| 17 | F/33 | AML-M2 | CALM 1926: AF10 883 | nd | [223] |
| 18 | M/36 | AML-M2 | CALM 1926: AF10 883 | n/o | [227] |
| 19 | F/23 | AML-M2 | CALM 2091: AF10 979 (IV) | AF10 978: CALM 2092 (IV') | [218] |
| 20 | M/16 | AML-M4 | CALM 1926: AF10 424 | AF10 423: CALM 1927<br>AF10 423: CALM 1987<br>AF10 423: CALM 2091 | [225] |
| 21 | M/41 | AML-M5 | CALM 2091: AF10 979 | n/o | [222] |
| 22 | M/2 | AML-M7 | CALM 2091: AF10 796 | nd | [219] |
| 23 | M/6 | AML-M7 | CALM: AF10 | | [228] |
| 24 | F/4 | AML-M7 | CALM 2091: AF10 423 | nd | [219] |
| 25 | F/19 | AML | CALM 2091: AF10 1048 | nd | [223] |
| 26 | M/39 | AUL | CALM 2091: AF10 424 | nd | [223] |
| 27 | F/10 | ALL-L1 | CALM 1926: AF10 424 (III) | n/o | [218] |
| 28 | M/22 | ALL-L1 | CALM 2091: AF10 883 | AF10 882: CALM 2092 | [224] |
| 29 | F/10 | ALL-L1 | CALM 1926: AF10 589 | AF10 588: CALM 1987 | [224] |
| 30 | M/7 | ALL-L2 | CALM 1926: AF10 883 (V) | AF10 882: CALM 1927 (V') | [218] |
| 31 | M/13 | T-ALL | CALM 2091: AF10 424 | AF10:CALM | [227] |
| 32 | M/26 | T-ALL | CALM 2091: AF10 979 | n/o | [222] |
| 33 | M/5 | Pre T-ALL | CALM 2091: AF10 979 | n/o | [221] |
| 34 | F/16 | Pre T-ALL | CALM 1926: AF10 883 | n/o | [221] |
| 35 | M/22 | T-ALL | CALM 2091: AF10 589 | n/o | [221] |
| 36 | F/38 | T-ALL | CALM 2091: AF10 589 | AF10:CALM | [227] |
| 37 | M/12 | T-ALL | CALM 1926: AF10 589 | AF10 588: CALM 1927<br>AF10 588: CALM 1987<br>AF10 588: CALM 2092 | [222] |
| 38 | F/14 | T-ALL | CALM 1926: AF10 424<br>CALM 1926: AF10 479 | AF10 423: CALM 1927<br>AF10 423: CALM 1987<br>AF10 423: CALM 2092<br>AF10 343: CALM 2092 | [217] |
| 39 | M/25 | LBL | CALM 1926: AF10 424 (III) | AF10 423: CALM 2092 (I')<br>AF10 423: CALM 1987 (II')<br>AF10 978: CALM 2092 (III') | [218] |
| 40 | M/25 | LBL → ALL | CALM 1926: AF10 980 | AF10 979: CALM 1987 | [224] |
| 41 | F/23 | NHL, Tcell LBL, AML | CALM:AF10 | nd | [222] |

n/o: not obtained, nd: not done. * Patient also included in ref. 223

and pancreas. In contrast, there were very few leukaemic cells in the organs of mice transplanted with the knockdown cells, and median survival was 27 versus 19.5 days. Using bone marrow transplantation assays, the authors demonstrated that CALM·AF10 is both necessary and sufficient for leukaemic transformation[234]. It should however be noted that the U937 cell line harbours both CALM·AF10 and AF10·CALM, why the genotype of the knockdown cells indirectly represents U937 cells with CALM·AF10 negative and AF10·CALM positive expression. In the second study, CALM·AF10 was retrovirally introduced into murine bone marrow cells and a bone marrow transplantation model was employed to generate mice with an AML phenotype. Interestingly, although the bulk of the leukaemic cells was myeloid, a rare population of cells expressing lymphoid markers and V(D)J rearrangements were able to propagate the leukaemia[235]. In a third model, transgenic mice expressing CALM·AF10 developed leukaemia at a median age of 12 months in at least 40 % of the first generation. Although the infiltrating cells were myeloid, staining for the lymphoid and myeloid markers and analysing the Ig and TCR status indicated that the target cell for transformation in some cases was multipotential[236].

## *Down stream targets of CALM·AF10*

Several reports have demonstrated that CALM·AF10 affects the HOXA family of genes. Dik *et al.* reported in a microarray-based comparison of CALM·AF10 positive and negative patients with T-ALL, that upregulation of HOXA5, HOXA9 and HOXA10 was found in CALM·AF10 positive cases[237]. In addition, all mice models exhibiting aberrant CALM·AF10 expression have demonstrated elevated levels of HOX genes, and it has been suggested that these genes are involved in the leukaemic transformation[234-236]. Both HOXA9 and HOXA10 have been observed to induce myeloid leukaemia and to interfere with both myeloid and lymphoid differentiation[238-240].

# The Present Investigation

## Aims

The general objectives of this thesis were to evaluate responses to different tumour initiating factors in leukaemia and examine regulation of normal and malignant haematopoiesis.

### *Specific aims*

- Examine the *in vivo* role of hypoxia-regulated expression of *Vegfa* in the haematopoietic compartment to evaluate the proposed hypoxic HSC niche.
- Evaluate the expression of VEGF-A and its two receptors (VEGFR-1, VEGFR-2) as well as SHP1 and PTEN in childhood ALL compared to non-malignant controls using immunohistochemistry.
- Indentify the independent and combined effects of the reciprocal t(10;11)(p13-14;q14-21) fusion proteins CALM·AF10 and AF10·CALM on leukaemogenesis through cloning and functional characterisation.

# Results and Discussion

### *Hypoxia-regulated expression of vascular endothelial growth factor is not involved in maintenance of murine haematopoietic stem cells in steady state. (Paper I)*

The haematopoietic stem cell (HSC) is thought to maintain its multipotency and stem cell properties within the HSC niche. If the HSC is located elsewhere it differentiates into specific haematopoietic terminal cells. In the bone marrow two niches have been proposed: the *perivascular niche* and the *osteoblastic niche*. Experimental data suggests that the HSC niche is hypoxic which is proposed to protect the HSC from DNA damage and reactive oxygen species. Vascular endothelial growth factor A (VEGF-A) is a well-known angiogenic factor that is induced by hypoxia and has interestingly been linked to play an important role in haematopoiesis, specifically in the survival and maintenance of HSCs. In this study we have evaluated whether the proposed hypoxic HSC niche is regulated by *Vegfa* expression in HSCs and thereby contributes to HSC maintenance. To test this hypothesis, we have investigated the haematopoietic phenotype in an *in vivo* model where hypoxia-regulated *Vegfa* expression is abrogated. In *Vegfa*δ/δ mice, the hypoxia response element (HRE) in the *Vegfa* promoter has been deleted at both alleles, thereby inhibiting binding of the transcription factor-Hypoxia-Inducible Factor (HIF) and subsequent activation of *Vegfa* expression following hypoxia.

Different bone marrow populations (total bone marrow cells (BM), c-Kit enriched cells (c-Kit$^+$), lineage-negative (Lin$^-$) and Lin$^-$/Sca-1$^+$/cKit$^+$/CD34$^-$ (LSK/ CD34$^-$) cells) from *Vegfa*δ/δ or wt mice were analysed for mRNA expression of *Vegfa* using qRT-PCR. Total bone marrow cells and c-Kit-enriched cells from wt and *Vegfa*δ/δ mice showed no differences in *Vegfa* expression levels whereas HSC with the LSK/ CD34$^-$ phenotype from the *Vegfa*δ/δ mice showed lower expression of *Vegfa* compared to wt cells. Furthermore, induction of *Vegfa* was tested in c-Kit enriched cells from wt or *Vegfa*δ/δ mice *in vitro* by incubating these cells either under normoxic or hypoxic culture conditions for 12 hours. While cells from the wt mice showed induction of *Vegfa* after 12 hours incubation in hypoxia, *Vegfa* levels in cells from *Vegfa*δ/δ mice remained low. These results show that hypoxia-regulated *Vegfa* expression occurs in hematopoietic cells from wt mice but is ablated in the *Vegfa*δ/δ mice. This data provides evidence that *Vegfa* is regulated by hypoxia/HIF in haematopoietic cells in wt mice and that HSCs (but not more mature haematopoietic cells) reside in a hypoxic microenvironment, which leads to the upregulation of *Vegfa* in normal steady-state conditions. Haematoxylin-Eosin stained histology sections and evaluation of microvessel density revealed that there were no major differences between *Vegfa*δ/δ and wt mice with regards to cellularity, differentiation and histology. Steady state haematopoiesis was investigated looking at peripheral blood cell counts, and lineage distribution within the blood, the spleen and the bone marrow in wt and *Vegfa*δ/δ

mice. Besides an increase in $CD3^+$ T-cells in the bone marrow of *Vegfa*δ/δ mice no statistically significant differences were observed in lineage distribution and cell number of differentiated cells in peripheral blood and bone marrow arguing against a prominent effect of the hypoxia/HIF-regulated VEGF-A regulation in steady-state haematopoiesis. *Vegfa* have previously been shown to be an important regulator of haematopoiesis and specifically for the HSCs. As the HSCs seem to reside in a hypoxic environment we wanted to evaluate if hypoxia/HIF-regulated *Vegfa* expression affects the cellularity of bone marrow and enumerate the hematopoietic stem/progenitor cells (HSPC). Total bone marrow cellularity was slightly decreased in *Vegfa*δ/δ mice but no differences in LSK percentage of lineage-negative cells between wt and *Vegfa*δ/δ mice were observed. No differences in percentage of LSK cells or LSK/ $CD34^-$ cells in the bone marrow were observed between *Vegfa*δ/δ mice and littermate wt control mice. To examine if the *in vitro* colony-forming ability of bone marrow cells were affected by the ablated hypoxia-regulated *Vegfa* expression in *Vegfa*δ/δ mice, bone marrow cells from wt and *Vegfa*δ/δ mice were plated in semi-solid medium in normoxic and hypoxic conditions. No significant differences in colony formation in normoxia or hypoxia were observed. This argues against a requirement for VEGF-A expression in HSC survival in steady state haematopoiesis. Finally, to investigate whether loss of hypoxia-regulated *Vegfa* expression has an effect on foetal liver haematopoietic stem and progenitor cells (HSPC) we analysed the total cellularity of foetal liver from dpc15.5. Similarly to the observation in bone marrow, foetal haematopoietic cellularity was decreased in *Vegfa*δ/δ mice. Strangely, against our hypothesis that the lack of hypoxia/HIF-regulated VEGF-A expression should lead to a decrease in HSC survival, a significant increase in foetal LSK cells was seen in the *Vegfa*δ/δ mice while the percentage of LSK/ $CD150^+$ cells was similar between wt and *Vegfa*δ/δ mice. The percentage of LSK cells of lineage-negative cells was increased in *Vegfa*δ/δ mice while percentage of LSK/ $CD150^+$ cells was identical.

### *Descriptive evaluation of potential biomarkers in childhood acute lymphoblastic leukaemia – VEGFR-1 and PTEN expression were significantly increased and SHP1 was decreased in the malignant group compared to non-malignant controls. (Paper II-III)*

Acute lymphoblastic leukaemia (ALL) is the most common malignancy amongst children with precursor B-cell ALL (pre-B ALL) being the major subtype. Complete remission is achieved in more than 98% of children with ALL but with the current treatment protocol approximately 15-20% of the children relapse and this is the most important adverse event in childhood ALL with survival rates between 20-50%. A group of patients do therefore not benefit or may even be over-treated by conventional therapy underlining the urgent need to identify additional biomarkers at diagnosis. This would allow for precise classification of risk groups and further individualized treatments to minimize disease recurrence. In these two studies we have evaluated the

protein expression of VEGF (VEGF-A) and its receptors (VEGFR-1 and VEGFR-2) as well as PTEN and SHP1 in childhood ALL cases compared to non-malignant controls using immunohistochemistry. All patients were treated at Karolinska University Hospital (Huddinge) or Linköping University Hospital between 1982 and 1999 according to standard criteria of the Nordic Society of Pediatric Haematology and Oncology (NOPHO). Patient follow-up was conducted over a 10-year period. Patients included in the non-malignant group were children who showed leukaemia associated symptoms, and underwent bone marrow aspiration as part of routine diagnostic procedures to exclude haematological malignancies. The stained specimens were assessed for the protein expression of VEGF and its receptors (Figure 6) as well as PTEN and SHP1, either by examining the staining intensity or both intensity and the percentage positive cells per at least 200 mononuclear cells, respectively.

We observed that the majority of children in the non-malignant group (n=15) and patients with pre-B ALL (n=32) have detectable VEGF protein expression. Even though the fraction of bone marrow samples with VEGF expression was slightly higher in the pre-B ALL group, we observed that the cellular expression intensity of VEGF was similar in bone marrow of both the groups examined. This data is contradictory to previous studies in which they found a decreased expression of secreted VEGF in plasma of childhood ALL patients compared with the control group. It is possible that the methodology used can explain this discrepancy. VEGFR-1 was significantly highly expressed in the pre-B ALL group compared to the non-malignant group. Expression of the receptors, VEGFR-1 and VEGFR-2 is not well described in bone marrow from patients with ALL, but two independent reports demonstrate a concomitant expression in both primary leukaemic cells and cell lines. We found that detectable protein levels of VEGFR-1 were more commonly found in mononuclear cells from pre-B ALL patients than VEGFR-2, similar to what has previously been described in both childhood and adult ALL. We did not observe any association between VEGF and its receptors or established prognostic factors such as risk-group, age, sex, and WBC count. It is however interesting to point out that a previous study did also show that VEGF levels are not significantly correlated with different clinical parameters in childhood ALL. As elevated VEGFR-1 expression has been associated with leukaemic cell invasion both *in vitro* and *in vivo* it would be interesting to follow-up our data with a larger study including clinical information regarding extramedullary disease.

Inactivation of the PTEN protein, either by gene deletion or gene mutations has been shown to hyperactivate the PI3K/Akt pathway, subsequently affecting the viability of the leukaemic cell. Surprisingly, when examining the protein expression of PTEN in children with ALL (n=31) in our study, we found that PTEN was significantly higher expressed in ALL patients compared to the non-malignant control group (n=13). However, the diagnostic ALL bone marrow samples were from children with a 5-year disease-free survival and when comparing these with bone marrow samples

from relapsed patients, the expression of PTEN was lower. This proposes that PTEN would be an interesting candidate as a prognostic marker. SHP1 is another tumour suppressor protein and loss of SHP1 in bone marrow biopsies has been observed in patients with myelodysplastic syndrome (MDS) who progressed more rapidly into acute leukaemia. Similarly to previous studies we observed that the expression of SHP1 was significantly lower in the ALL group compared to the non-malignant group.

In order to examine if VEGFR-1, PTEN and SHP1 may be potential prognostic markers of childhood ALL patients, several larger studies are necessary to determine if the level of protein expression are correlated to outcome and to examine if the expressed proteins are functionally active or not.

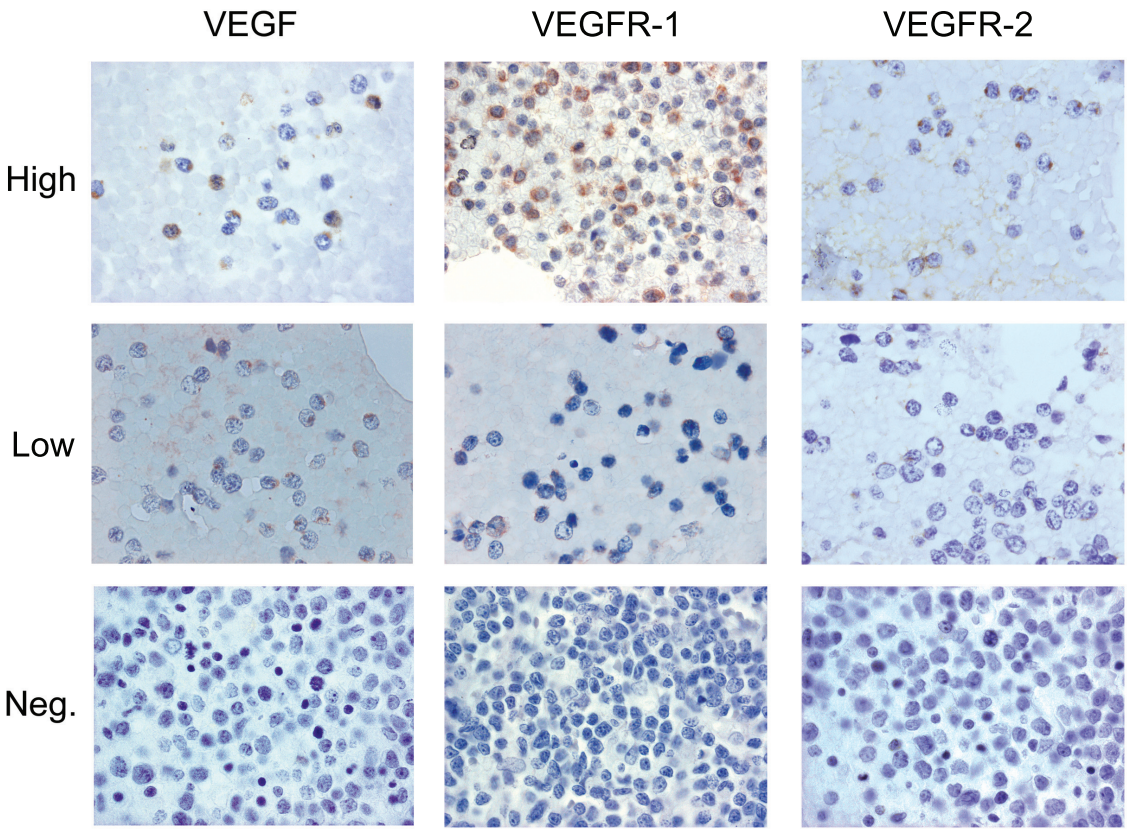


**Figure 6.** Staining for VEGF and its receptors, VEGFR-1 and VEGFR-2, in the bone marrow from childhood preB-cell ALL patients evaluated by immunohistochemical analysis. The images are presented as negative (neg), low, or high scores. Magnification 100. Image adapted from Paper II.

***The t(10;11)(p13-14;q14-21) reciprocal fusions have individual effects on cell biology and, when found in combination, have either a more pronounced or an inhibitory effect on leukaemogenesis. (Paper IV)***

Chromosomal translocations are reported in approximately 65% of all acute leukaemias. Knowledge of the chromosomal abnormality can both be of diagnostic and prognostic value subsequently affecting the patient outcome. Several reports have identified leukaemic translocations in human peripheral blood of healthy individuals supporting the hypothesis that leukaemic transformation is a multistep process. Recently well characterised reciprocal translocations such as MLL·AF4, BCR·ABL, PML·RARα and PLZF·RARα - together with their inverted partner, have been shown to have an important impact on leukaemic development. The t(10;11)(p13-14;q14-21) translocation is a reciprocal translocation and forms both an in-frame CALM·AF10 and AF10·CALM fusion. The t(10;11)(p13-14;q14-21) translocation is most commonly found in patients with T-ALL as is correlated to poor patient outcome. CALM·AF10 fusion has been examined in three independent mouse models to examine the leukaemogenic potential of the fusion. All three mouse models developed an AML-like disease with a relatively long latency. The long latency period prior to the onset of leukaemia and the consistent leukaemic phenotype suggests that the CALM·AF10 fusion protein alone does not cause the leukaemic development but rather that several genetic events are crucial. In this study have we examined if the reciprocal AF10·CALM fusion may be the other event and together with the CALM·AF10 fusion acts on the leukaemic transformation. To test this hypothesis we have used a lentiviral approach to stably transfect Jurkat T leukaemic cells and further analyse the independent or combined effect of the fusions on the potential of: proliferation, DNA synthesis, apoptosis, invasion and cytotoxic sensitivity (Figure 7).

We have observed that cells expressing the CALM·AF10 fusion alone had a proliferative advantage compared to control cells over time. These data are in line with previous studies that describe the CALM·AF10 fusion as having oncogenic potential. Further, cells expressing the AF10·CALM fusion showed a decreased proliferative ability and cells expressing both the CALM·AF10 and AF10·CALM fusion did not show any significant changes to control. The increase or decrease in cell number over time could not be explained by a change in cell cycle profile of the fusion expressing cells. Rather it seems like the decrease of cell growth is dependent on a pro-apoptotic function of the AF10·CALM fusion. To analyse how the fusion expressing cells behaved under induced stress, we treated the cells with Mitoxantrone. CALM·AF10 expressing cells showed a modest insensitivity to Mitoxantrone-induced apoptosis. Interestingly, cells expressing both fusions had a more pronounced insensitivity. AF10·CALM expression responded similarly to the treatment compared to control transduced cells. This suggests that although the AF10·CALM fusion may act in a pro-apoptotic fashion at a steady state level, the CALM·AF10 and AF10·CALM fusion proteins together may have an anti-apoptotic role upon treatment with Mitoxantrone. The t(10;11)

(p13-14;q14-21) fusions have, from previous published data, been associated with extramedullary diseases both in patients and in all three published CALM·AF10 transgenic mouse models. With this knowledge we aimed to analyse if the fusions are involved in invasion and whether the two fusions, independently or combined, have different properties in this feature as we found for apoptosis. Our data indicate that cells expressing the CALM·AF10 fusion have a significantly increased ability to invade an *in vitro* matrix compared to control. This observation is in line with the previous published *in vivo* data. What has not been described elsewhere is that cells expressing AF10·CALM or both fusions had a significantly decreased invasional potential compared to controls.

Our collective data suggests that the two fusions alone or in combination have different properties with regards to cellular regulation. The t(10;11)(p13-14;q14-21) reciprocal fusions have individual effects on cell biology and, when found in combination, have either a more pronounced or an inhibitory effect on leukaemogenesis. Our study highlights the importance of examining both fusion proteins in a two transcript reciprocal translocation as they on their own may have individual characteristics.

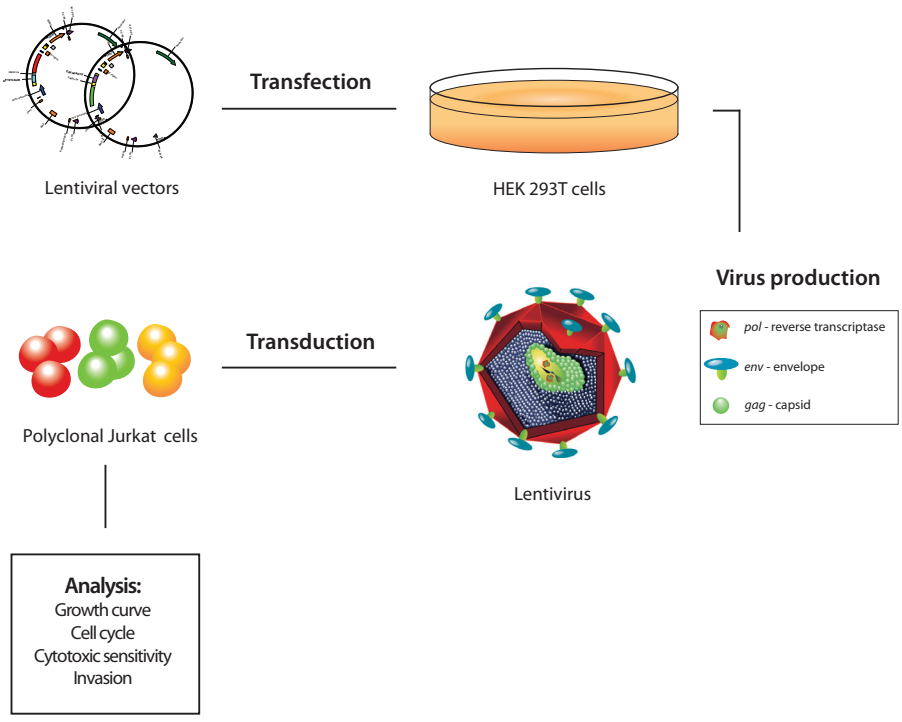


**Figure 7. Lentiviral transduction approach.** HEK 293T cells were transfected with Lentiviral vectors, created using the Gateway® system. Jurkat T-ALL cells were transduced with CALM·AF10 and/ or AF10·CALM (or control) lentiviral particles and sorted based on their reporter gene expression. The polyclonal Jurkat cells were then analysed for their leukaemogenic behaviour.

# Conclusions

In this thesis have we identified potential prognostic markers in childhood ALL, provided evidence that the HSC niche is hypoxic, as well as gained insight into that the inverted fusion in a two transcript reciprocal translocation may be a key regulator of leukaemic development.

We could conclude that:

- The HSC niche is hypoxic and hypoxia/HIF dependent *Vegfa* expression is not involved in maintenance of HSC at steady state.
- VEGFR-1, PTEN and SHP1 may be potential prognostic markers in childhood ALL.
- The two t(10;11)(p13-14;q14-21) fusion proteins have independent and combined characteristics on leukaemogenesis, why both fusions in a two transcript reciprocal translocation should be examined.

# Populärvetenskaplig sammanfattning

Vårt blod består av flera olika typer av celler så som leukocyter (vita blodkroppar) som ansvarar för vårt immunförsvar, erytrocyter (röda blodkroppar) som förser kroppen med syre, samt trombocyter (blodplättar) som har en viktig roll vid läkning av sår. Hematopoies är den process i benmärgen som bildar blodets alla celler och den hematopoietiska stamcellen (HSC) är urmodern till dessa celler. HSC utgör endast en liten fraktion av den totala benmärgen, men är mycket viktiga eftersom de bland annat har en speciell egenskap som gör att de kan bilda blodkoppar under hela vårt liv. Den här egenskapen innebär att de kan återskapa en identisk kopia av sig själv, så kallad självförnyelse. Om HSC inte skulle skapa en identisk kopia av sig själv skulle den differentiera till mer mogna celler. Differentieringsprocessen är irreversibel och mogna celler har en mycket kort livstid. Det finns flera studier som visar att HSC återfinns på en speciell plats i benmärgen, en plats där cellen är skyddad och där den kan bilda identiska kopior av sig själv (självförnyelse). Den här platsen kallas för den HSC nischen och det finns indikationer om att denna nisch är hypoxisk (syrefattig). Bland annat har man sett att om man låter HSC växa i en artificiell hypoxisk miljö så bibehålls deras förmåga att självförnya sig. Än så länge har man inte kunnat påvisa detta fullt ut *in vivo* – i en levande organism. VEGF (vascular endothelial growth factor) är en tillväxtfaktor som utsöndras från celler vid hypoxi och har fått sitt namn på grund av att denna faktor påverkar tillväxt av endotelceller (blodkärlens celler). Hypoxi i vävnad leder härmed till ökad kärlbildning, för att upprätthålla syrenivån. VEGF har även visats ha mycket stor påverkan på överlevnad samt självförnyelse av HSC.

I delarbete I har vi undersökt om HSC nischen i benmärg hos möss är hypoxisk genom att använda en musmodell som är defekt i VEGF genen (*Vegfa*δ/δ ). Defekten gör att mössen inte kan uttrycka VEGF vid hypoxi och vår hypotes var att mössens HSC påverkas av detta. Vi undersökte om *Vegfa*δ/δ -mössens blodbild samt benmärg var skild från normala möss, genom att använda olika metoder. Vi kunde dock inte se några påtagliga skillnader. När vi däremot direkt analyserade VEGF-nivåerna kunde vi se att det endast var HSC som påverkades och inte de mer differentierade cellerna. Detta indikerar att HSC återfinns i en hypoxisk miljö. Med detta som utgångspunkt, ville vi undersöka om HSC specifikt var påverkade. Vi undersökte om deras förmåga att differentiera samt om antalet HSC var förändrat i *Vegfa*δ/δ -möss jämfört med normala möss, men kunde inte se någon skillnad. Vi kunde däremot se en skillnad på det totala antalet celler i benmärgen, där *Vegfa*δ/δ -mössen hade ett minskat antal benmärgsceller. Detta kan antagligen förklaras med att mössen generellt är mindre i storlek. Slutligen undersökte vi levern hos musembryon (fetal lever). Levern är ett

viktigt säte för hematopoies innan födseln (även hos människor). Vi kunde se att den fetala levern från *Vegfa*δ/δ -möss hade ett totalt lägre antal celler än hos normala möss, med fler omogna celler men inte fler HSC. Sammanfattningsvis har vi kommit fram till att HSC nischen är hypoxisk men att hypoxireglerat VEGF-uttryck inte påverkar HSC i deras normala tillstånd.

Leukemi är en av blodets maligna sjukdomar och den karakteriseras av en ohämmad celldelning av omogna vita blodkroppar i benmärgen, och ofta även i andra organ som t.ex. perifert blod, mjälte och lever. Leukemi är ett samlingsnamn på flera olika subgrupper som alla har olika kännetecken. Akut leukemi skiljer sig från kronisk leukemi, främst genom att kronisk leukemi omfattar utmogna celler. Både akut och kronisk leukemi kan delas upp i myeloisk eller lymfoblastisk leukemi, denna uppdelning är beroende av vilka celler som leukemin härstammar ifrån. Om den leukemiska cellen har särdrag för att var en lymfoblastisk (eller lymfatisk) cell (dvs. en cell som skulle ha kunnat differentiera till lymfocyter) kallas sjukdomen för akut eller kronisk lymfoblastisk leukemi (ALL eller CLL). Har den leukemiska cellen särdrag för att vara myeloisk kallas leukemin istället för akut eller kronisk myeloisk leukemi (AML eller CML). ALL drabbar kvinnor och män, pojkar och flickor i alla åldrar, men det är vanligare att diagnostiseras med ALL som barn. Under de senaste tre decennierna har behandlingen för ALL lett till en dramatisk förändring, från att nästan vartannat barn dog av sjukdomen kan idag fler än 80 % av patienterna bli botade. Dessvärre får, trots den effektiva behandlingen, ungefär 15-20 % av barnen återfall vilket beror på att behandlingen inte är tillräckligt effektiv eller att fel typ av behandling givits. För att veta vilken typ av behandling patienterna behöver använder läkarna så kallade prognostiska faktorer. De vanligaste prognostiska faktorer som används i sjukvården idag är; mängden leukocyter i perifert blod, ålder, typ av leukemi, cytogenetisk information (leukemicellens arvsmassa), samt behandlingssvar. Genom att identifiera nya prognostiska faktorer skulle man på sikt kunna minska andelen patienter med återfall. I delarbete II och III har vi undersökt om vissa cancer-relaterade proteiner skiljer sig i uttryck mellan barn med ALL (n=31-32) och barn som inte har en malign sjukdom (n=13-15). Detta är ett första steg i att undersöka om dessa proteiner skulle kunna verka som prognostiska faktorer. De proteiner vi har undersökt är VEGF, och dess receptorer VEGFR-1 och VEGFR-2, samt PTEN och SHP1. Barnen behandlades på Karolinska Universitetssjukhus (Huddinge) eller Linköping Universitetssjukhus mellan 1982 and 1999 och benmärgsprover togs vid diagnos- eller återfallstillfället. Genom att använda en antikroppsbaserad teknik som kallas immunhistokemi har vi med mikroskopi detekterat proteinuttrycket i benmärgsbiopsierna. Vi kunde se att VEGFR-1, PTEN och SHP1 skiljde sig åt i proteinuttryck mellan barnen med ALL och barnen utan malign sjukdom. Proteinuttrycket var dock inte korrelerat med andra prognostiska faktorer. Våra data tyder på att VEGFR-1, PTEN och SHP1 skulle kunna vara intressanta faktorer ur ett prognostiskt perspektiv, men fler mer omfattande studier är nödvändiga innan dessa proteiner kan klassificeras som prognostiska faktorer i barn-ALL.

I delarbete IV har vi fokuserat på utvecklingen av leukemi. Den ursprungliga orsaken till leukemi är okänd, dvs. man kan inte förklara varför bara vissa personer drabbas, men det finns flera faktorer som kan bidra till den leukemiska utvecklingen (leukemogenes). Liksom de flesta cancerformer representeras leukemi av en ohämmad tillväxt, en blockering i differentiering, samt en ökad överlevnad och självförnyelse av leukemiceller. Leukemicellen har ett förändrat genuttryck jämfört med den normala leukocyten, dvs. arvsmassan i leukemicellen är förändrad. Vid ungefär 65 % av akut leukemi (AML eller ALL) uppvisar leukemicellen en så kallad kromosomal translokation. Detta innebär att två kromosomer utväxlar genetiskt material, vilket kan leda till ett onarturligt chimärt protein (fusionsprotein) med leukemogeniska egenskaper. I vissa fall har det visat sig att ett fusionsprotein inte är tillräckligt för att återskapa den leukemiska sjukdomen. Man vet att det i dessa fall krävs ytterligare en, eller ibland fler, genetiska förändringar för att sjukdomsförloppet ska kunna ske. Ofta används cellkultur eller möss för att undersöka fusionsproteinets leukemiska potential. Vid en så kallad reciprok translokation kan det i vissa fall bildas två fusionsproteiner. Studier har visat att det andra fusionsproteinet, det reciproka, kan vara den andra genetiska förändringen som gör att leukemogenesen blir fullständig. Den translokation som vi fokuserat på i delarbete IV är just en reciprok translokation, vilken kan ge upphov till två fusionsproteiner: CALM·AF10 och AF10·CALM. CALM·AF10 är det fusionsprotein som generellt har studerats, och patienter med denna fusion har mycket dålig prognos. Ofta får patienterna återfall och överlever inte sin sjukdom. Fusionen har återfunnits i patienter med både AML och ALL, men den vanligaste typen är T-ALL (T-lymfocyt ALL). CALM·AF10-fusionen har tidigare studerats i möss och den har visats ha leukemogenisk potential, men sjukdomsförloppet i möss är relativt långt. Vår hypotes är därför att den reciproka AF10·CALM-fusionen är den andra genetiska förändringen som leder till ett sjukdomsförlopp, och en sjukdom liknande det som återfinns i patienter. Vi har studerat detta genom att använda cellkulturer där en T-ALL cellinje (Jurkat celler) använts som modell. Vi använde fusionernas gener, tagna från en annan cellinje, och producerade virus. Viruspartiklarna användes sedan till att infektera Jurkat cellinjen. På så sätt framställde vi tre stycken olika cellinjer (plus tre olika kontrollcellinjer), en cellinje med CALM·AF10, en med AF10·CALM, samt en cellinje med båda fusionerna. På detta sätt kunde vi nu undersöka deras enskilda roll samt den gemensamma rollen i leukemi. Vi observerade att CALM·AF10-cellerna hade ett övertag i celltillväxt medan AF10·CALM cellerna förvånande nog visade en reducerad celltillväxt. Vi kunde vidare se att celler som uttryckte båda fusionerna, dvs. CALM·AF10/AF10·CALM-celler inte alls påverkades jämfört med kontroll-celler. När celler delar sig går de genom den så kallade cellcykeln, och genom att använda olika cellbiologiska metoder kan man undersöka cellernas förmåga att upprätthålla cellcykeln. Cellcykeln påverkades inte märkbart av de olika fusionsproteinerna, men däremot visade AF10·CALM-celler en högre nivå av celldöd, vilket kan förklara den minskade totala celltillväxten. För att efterlikna patienters behandling behandlade vi cellerna med en kemoterapeutisk drog och analyserade mängden celldöd. CALM·AF10-

cellerna påverkades till viss del av behandlingen, medan påvrkan på CALM·AF10/AF10·CALM-cellerna var betydligt mindre, vilket indikerar att båda fusionerna tillsammans skyddar leukemicellen från behandling. Vi ville också undersöka hur cellernas förmåga att röra på sig förändrades med fusionerna. Patienter med ALL har ofta leukemiceller i andra organ än i blod och benmärg, vilket tyder på att dessa leukemiceller har en ökad förmåga att infiltrera eller invadera ett organ. För att efterlikna detta fenomen använde vi en invasionsmetod, där cellerna får invadera en matris som påminner om vävnad. De celler som passerade matrisen räknades. Vi såg att CALM·AF10-celler hade en bättre förmåga att invadera än kontrollceller, och celler med båda fusionerna eller bara AF10·CALM hade en lägre förmåga. Sammanfattningsvis visar våra data att två fusionsproteiner i en reciprok translokation kan ha individuella egenskaper, och att fusionerna kan samverka och leda till ytterligare egenskaper i leukemicellen.

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