The role of MN1-TEL, MN1 and TEL2 in Leukemia

Cintia Carella

The Role of MN1-TEL, MN1 and TEL2 in Leukemia

De rol van MN1-TEL, MN1 en TEL2 in leukemie

Thesis

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"Cheshire Puss, ……Would you tell me, please,

which way I ought to go from here?"

"That depends a good deal on where you want to get to," said the Cat.

"I don't much care where—"
said Alice.

"Then it doesn't matter which way you go," said the Cat.

"--so long as I get *somewhere*," Alice added as an explanation.

"Oh, you're sure to do that," said the Cat,

"if you only walk long enough."

(from Alice's Adventure in Wonderland, by Lewis Carroll)

a Tommaso, Sara e……

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Scope of the thesis

Hematopoiesis is a complex process carried out by a small pool of totipotent self-renewing hematopoietic stem cells, able to generate every cell type of the blood throughout life. Blood cell differentiation is achieved by progressive loss of totipotency and self-renewal capacity, which gradually identifies the different blood cell lineages. Understanding the detailed mechanisms and the key players of this complex phenomenon is challenging, due to the intricate net of interactions upon which it relies. It is largely accepted however, that understanding of the mechanisms underlying the functioning of hematopoietic stem cells is necessary to comprehend the molecular alterations that lead to leukemia and ultimately to identify the possible treatments for such malignancies.

The scope of this thesis is to define the role of TEL2, MN1-TEL and MN1 in hematopoiesis and leukemogenesis. The basis of all the projects presented is to analyze the reconstitution capability and the leukemic potential of bone marrow cells forced to express the above-mentioned proteins through retroviral transduction.

Chapter 1 of this thesis provides an overview of the current knowledge regarding the development of the hematopoietic system, maintenance of homeostasis and its deviation towards malignancy. It also summarizes some of the most recent findings regarding the function of the proteins that are the subject of this thesis.

In chapter 2 we demonstrate that the ETS transcription factor TEL2 alone, a close homologue of TEL, is not able to trigger leukemia in a mouse model with reconstituted retrovirally-transduced bone marrow. However, it also shows that forced expression of TEL2 generates aberrations that are reminiscent of human myeloproliferative disease.

The possibility that TEL2 functions as an oncogene when associated with other mutations is the subject of Chapter 3. We show that TEL2 expression in $p19^{Arf}$-deficient bone marrow leads to development of B cell lymphoma due in part to significant upregulation of mTOR. We also present evidence that mTOR is a direct transcriptional target of TEL2.

In chapter 4 we explore the role of the fusion protein MN1-TEL in myeloid leukemogenesis and show that forced expression of the fusion protein in mouse bone marrow generates factor dependent cell lines and together with unknown secondary mutations causes AML in mice.

In chapter 5 we not only establish that expression of MN1 alone strongly stimulates the proliferation of myeloid progenitors causing the development of MPD in mice, but also that its overexpression is an important secondary event in the development of human AML characterized by the inv(16) chromosomal aberration.

In Chapter 6 we summarize and discuss the results of this thesis in relation to the current knowledge regarding hematopoietic malignancies.

Chapter 1

Introduction

1.1 Hematopoiesis

Among the tissues of the body blood is one of the most fascinating both for the complexity of its cellular composition and for its unique structure; thanks to these features, the blood can immunologically protect and support the diverse cell types present in every area of the body (Orkin, 1996; Orkin, 2000).

All cells of the hematopoietic system derive from a selected group of precursor cells of mesenchymal origin called *Hematopoietic Stem Cells* (HSCs) (Baum et al., 1992; Morrison and Weissman, 1994; Osawa et al., 1996; Spangrude et al., 1988). In the adult the majority of precursor cells are localized in the bone marrow, whereas only a small portion of them is also present in the bloodstream. It is estimated that only one every 10,000 to 15,000 bone marrow cells is a HSC, with this value dropping to 1 in 100,000 blood cells in the bloodstream (Abkowitz et al., 1996; Metcalf, 1999); therefore, the isolation of a candidate stem cell population has been a challenge. For a long time it was believed that the majority of HSCs in adult marrow neither divided nor differentiated and they were considered to be in a state of *prolonged intermitotic interval* (Becker et al., 1965; Hodgson and Bradley, 1979; Lerner and Harrison, 1990). However, there is now consolidated evidence that HSCs regularly divide throughout adult life (Bradford et al., 1997; Morrison et al., 1996; Ponchio et al., 1995; Rufer et al., 1999), albeit at a slow rate.

The introduction of the *Fluorescence-Activated Cell Sorter* (FACS) constituted a milestone for the isolation of cell populations highly enriched in HSCs (Morrison et al., 1997, Morrison, 1994), by virtue of their unique cell surface marker composition. HSCs express significant levels of c-Kit (c-Kit^{+}), and Sca-1 (Sca-1^{+})(Uchida and Weissman, 1992) but do not or hardly express any of lineage-specific markers (Lin$^{-/lo}$). Only a small fraction of the HSC population is able to self-renew indefinitely maintaining the numerical integrity of their pool (Passegue et al., 2003). When transplanted into irradiated mice, these cells reconstitute the hematopoietic compartment and sustain hematopoiesis throughout life. Because of these properties HSCs are defined as *Long Term Hematopoietic Stem Cells* (LT-HSCs), and are identified by the cell surface markers Thy1^{lo}, Lin^{-}, Sca-1^{+} and c-Kit^{+} (Smith et al., 1991; Wagers et al., 2002). In the mouse the LT-HSCs are CD34$^{-/lo}$ and therefore differ from human LT-HSC, which are CD34^{+} (Osawa et al., 1996). A restricted fraction of the LT-HSCs gives rise to the *Short-Term Hematopoietic Stem Cells* (ST-HSCs). These cells have a finite self-renewal capacity but generate a set of oligo-lineage restricted progenitors, called *Multipotent Progenitor Population* (MPP). The ST-HSCs show almost the same combination of surface markers as the LT-HSCs with the exception that they also express the receptor tyrosine kinase Flk-2 (Lin^{-}, c-Kit^{+}, Sca-1^{+}, Thy1.1^{lo}, Flk-2^{+}). ST-HSCs generate MPPs that lost the capacity to self-renew, maintain Flk-2 expression,

but loose Thy1 expression (Lin$^-$, c-Kit$^+$, Sca-1$^+$, Thy1$^-$ and Flk-2$^+$) (Christensen and Weissman, 2001) (Figure 1). It is generally accepted that passage of cells from one differentiation stage into the next is an irreversible process, linked to reduction in self-renewal capacity. The lineage-committed state is then irreversible. The cellular signals that influence the choice between self-renewal and differentiation are still not completely known.

The first decisive step in hematopoietic differentiation involves the commitment of the MPP to either the *myeloid or lymphoid* differentiation pathway. This event generates a progeny of cells with a more restricted differentiation potential, defined as the *Common Myeloid Progenitor* (CMP) and *Common Lymphoid Progenitors* (CLP), respectively. The progeny of cells committed to either pathway becomes obligated to specific sublines of differentiation and is referred as *committed progenitor cells*.

The CMP progeny is again divided in two different branches able to generate the entire myeloid compartment: the *Megakaryocytes-Erythrocytes Progenitors* (MEPs)

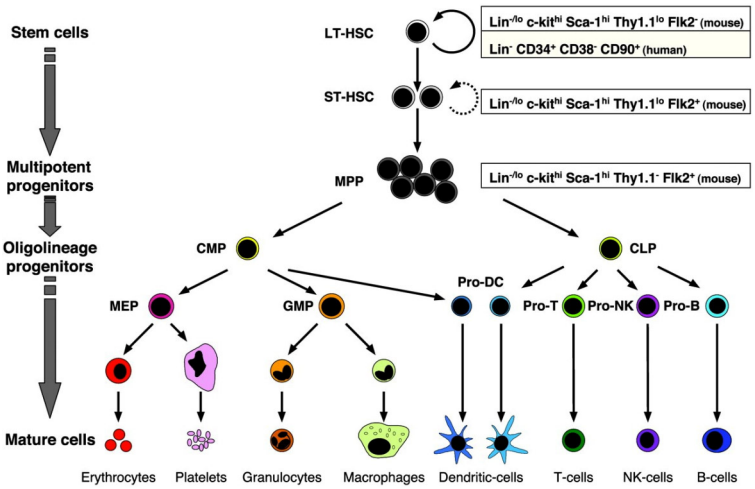

Figure 1: Hematopoietic and progenitor cell lineages.

HSCs can be divided into LT-HSCs, highly self-renewing cells that reconstitute an animal for its entire life span, or ST-HSCs, which reconstitute the animal for a limited period. ST-HSCs differentiate into MPPs, which do not or briefly self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny through functionally irreversible maturation steps. The CLPs give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells. The CMPs give rise to GMPs and MEPs. The GMPs then differentiate into monocytes/macrophages and granulocytes. The MEPs produce megakaryocytes/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. All of these stem and progenitor populations are separable as pure populations by using their specific cell surface markers combination.

Adapted from: Passegue' *et al.* (2003). *PNAS*, 100; pp.11842–11849

and the *Granulocytes-Monocytes Progenitors* (GMPs). The CLPs, on the other hand, are responsible for the production of the lymphoid compartment. Their downstream progeny is constituted of B- and T-lymphocytes and *Natural Killer* (NK) cells. Finally, these latter populations are able to complete the differentiation process and give rise to the broad spectrum of mature cells that circulate in the peripheral blood (Passegue et al., 2003) (Figure 1).

This elegant and dynamic process, which generally is restricted to the bone marrow and is responsible for blood cell production throughout life, is referred as *Hematopoiesis.*

During mammalian development this process occurs in distinct anatomical locations. More then 40 years ago, Moore and Owen demonstrated that the first site of hematopoiesis (*primitive hematopoiesis*) in the mouse was the embryonic yolk sac (Moore and Owen, 1965). Now we know that in the mouse both embryonic blood and endothelial progenitors first emerge in the extra-embryonic yolk sack blood islands at Embryonic day (E) 7.5 and that *definitive hematopoiesis* derives from the *Aorta/Gonad/Mesonephros* (AGM) region at E10 (Haar and Ackerman, 1971; Kondo et al., 2003; Orkin and Zon, 2002).

Already at E12 the mouse fetal liver becomes the principal hematopoietic organ from which HSCs migrate to the spleen, the thymus and finally, by day E16-17 to the bone marrow which will support hematopoiesis for the rest of the animal's life (Ikuta K, 1993; Jordan et al., 1995; Morrison et al., 1995; Zanjani et al., 1993). The common origin of the earliest blood cells and vascular endothelial cells suggests that both cell types are derived form a common progenitor, the *Hemangioblast* (Pardanaud and Dieterlen-Lievre, 1999a; Pardanaud et al., 1989). Particularly indicative in this regard are findings obtained with a knock-in mouse expressing the histochemical marker *LacZ* under the control of the *Runx1* locus (AML1) (North et al., 1999), a gene essential for definitive but not primitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996a). In this mouse *LacZ* staining is present in both the endothelial cells in the ventral wall of the dorsal aorta and in the associated hematopoietic clusters(de Bruijn et al., 2000). Based on this evidence, investigators proposed the existence of specialized endothelial cells, defined as "Hemogenic endothelium", present in the dorsal aorta and the vitelline and umbilical arteries, that give rise to HSCs (Caprioli et al., 1998; Cumano et al., 2000; Jaffredo et al., 1998; Pardanaud and Dieterlen-Lievre, 1999b; Pardanaud et al., 1989; Wood et al., 1997).

1.2 Regulation of hematopoiesis

The mechanisms underlying the stem cell decision to either self-renew or differentiate are still largely unknown. However, many of the important players have

been identified that specify whether a HSC differentiates down the myeloid, lymphoid or erythromegakaryocytic lineage. Decades of experimental work have shown that lineage commitment is strongly influenced by cytokines (Arai et al., 1990), constitutively produced or induced by the bone marrow stroma cells. *Interleukin* 3 (IL-3) stimulates proliferation of multipotent progenitors as well as various lineage-committed progenitors. *Granulocyte-Macrophage Colony Stimulatory Factor* (GM-CSF) also stimulates early progenitors, plus macrophages and granulocytes. On the other hand, IL-7, *Erythropoietin* (EPO), *Granulocyte-Colony Stimulatory Factor* (G-CSF), *Monocyte-Colony Stimulatory Factor* (M-CSF), and IL-5 predominantly stimulate their cognate progenitors defined as lymphoid progenitors, erythroblast, granulocytes, monocytes/macrophages and eosinophils, respectively. A unique feature of cytokines is the combination of their sometimes overlapping function and their distinct biological activity on specific cell types. A possible explanation lays in the structure of the cytokine receptors and the variety of intracellular signaling pathways they can activate. There are several structurally distinct families of cytokine receptors: the tyrosine kinase receptor family, the *Tumor Necrosis Factor* (TNF) receptor family, the *Transforming Growth Factor* (TGF)-β receptor family, and the chemokine receptor family. The binding of a cytokine to its receptor usually leads to dimerization of the receptor and activation of the receptor–associated kinases. The *Janus tyrosine kinases* (JAKs) are a key signaling element for cytokine function (Darnell et al., 1994). JAKs are cytoplasmic protein tyrosine kinases whose activation and subsequent phosphorylation create the proper binding sites for signaling molecules possessing a SH2 phospho-tyrosine binding domain. The SH2 proteins recruited to the phosphorylated receptor include tyrosine phosphatases, the p85 subunit of *phosphatidylinositol 3* (PI3) kinase, *Signal Transducers and Activators of Transcription* (STATs), and adaptor molecules like Shc and Grb (Ihle and Kerr, 1995). Currently four members of the JAK family are known: JAK1,2,3 and TYK2, whereas seven members of the STAT family have been reported. Each cytokine is able to activate a specific set of JAK and STATs. For example, IL-12 and IL-4 seem to activate specifically STAT4 and STAT6 (Kaplan et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996). In contrast, many cytokines, including IL-3 (Azam et al., 1995), GM-CSF (Gouilleux et al., 1995), IL-5, IL-2, EPO (Wakao et al., 1995), and *Thrombopoietin* (TPO) (Nagata et al., 1995; Nagata and Todokoro, 1995), mainly activate STAT5 (Mui et al., 1995). Although a variety of growth factors play a fundamental role in hematopoiesis, not every single one of them is considered to be ‘instructive’ for the chosen pathway of differentiation, but rather ‘permissive’ for cell viability and proliferation (Socolovsky et al., 1998; Stoffel et al., 1999). In this context it is important to mention the fundamental role of the interaction between blood progenitors and bone marrow stroma. Activation of these signaling pathways results in activation of a pivotal third element in the regulation of hematopoiesis: the specific expression of *Transcription Factors* (TFs). The differentiation process of progenitors is orchestrated by silencing genes that maintain stem cell characteristics and by

activating genes that define the identity of a specific lineage. A particularly instructive experimental system to study the influence of transcription factor expression on differentiation has been developed by Graf and colleagues, in which selected multipotent hematopoietic progenitors of chickens are transformed with a retrovirus expressing the *myb* and *ets* oncogenes. In this system, the transformation event occurs in two types of cells: multipotent progenitors called *Myb-Ets progenitors* (MEPs), and myeloblast (McNagny, 1997). Upon overexpression of GATA-1 in this system, it was possible to reprogram these progenitors to differentiate into erythroid-, eosinophil- and thromboblast (equivalent to megakaryocytes) lineages (Kulessa et al., 1995). Similarly, forced expression of GATA-1 in the murine myeloid 416B cell line converted them into a megakaryocytic phenotype (Visvader and Adams, 1993; Visvader et al., 1995; Visvader et al., 1992). In contrast, forced expression of the ETS-like factor PU.1 drove MEPs towards myeloid differentiation (Nerlov et al., 1998), while forced expression of PU.1 together with GATA-1 and C/EBPα resulted in production of eosinophils only (Nerlov et al., 1998). This raises the question which combination of TFs is responsible for the commitment to myeloblast and which for the commitment to eosinophils? To answer these questions the expression pattern of different TFs has been analyzed. GATA-1 expression was high in MEPs, absent in myeloblast, and moderate in eosinophils (Kulessa et al., 1995). The ETS family member PU.1 (Spi.1) is expressed in myeloblast but not in MEPs and eosinophils (McNagny, 1997). Finally, the bZip family CAAT enhancer binding proteins C/EBPα and are expressed in both myeloblast and eosinophils, but not in MEPs (Muller et al., 1995; Nerlov et al., 1998). Moreover the expression of PU.1 and C/EBPs in the myelomonocytic compartment of the MEP system confirmed their role in the regulation of myeloid-specific genes in normal cells.

GATA-1 and PU.1 are dominant factors for erythroid/megakaryocytic/eosinophilic and myeloid differentiation, respectively. A number of recent observations together with their non-overlapping expression pattern suggest that PU.1 and GATA-1 act as antagonists, in part through direct interaction (Rekhtman et al., 1999; Zhang et al., 1999). On the other hand PU.1 and C/EBPs synergize in the regulation of myeloid-specific cytokine receptor genes (Tenen et al., 1997). Besides the mere presence or absence of a TF, its concentration may influence lineage choice and differentiation. Eosinophilic differentiation requires low levels of GATA-1 whereas high levels are necessary for erythroid and megakaryocytic differentiation (Kulessa et al., 1995). High levels of PU.1 favor macrophages development, whereas low levels generate B cells (DeKoter and Singh, 2000) (Figure 2).

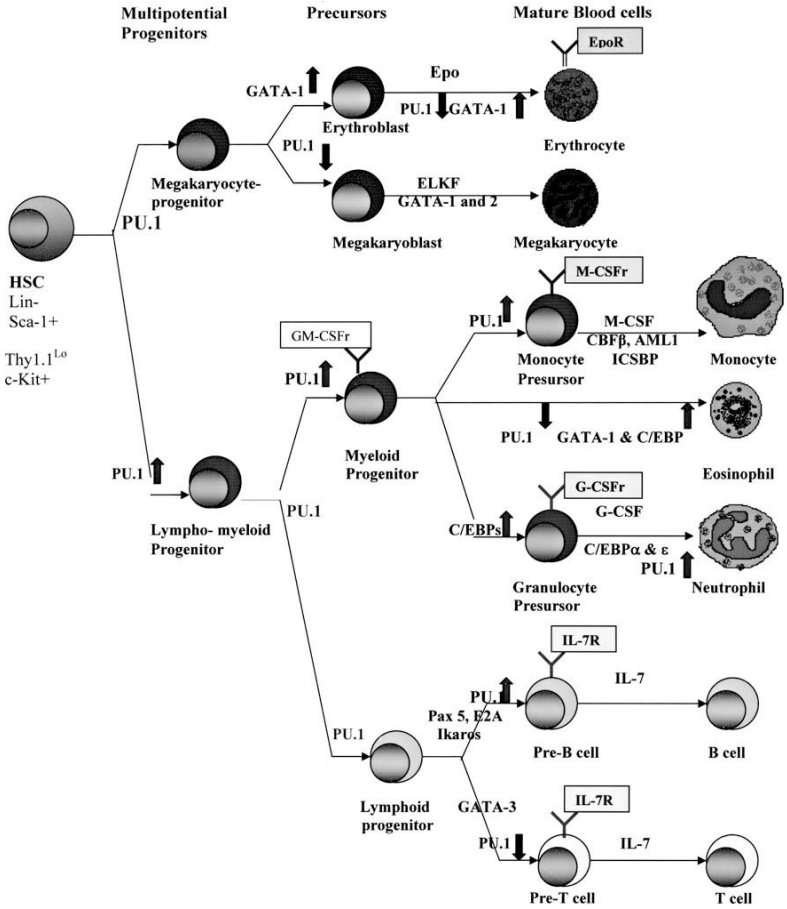

Figure 2: PU.1 involvement in the different stages of hematopoiesis.

PU.1 is expressed at low levels in HSCs and multipotent progenitors and is upregulated specifically at the beginning of the lympho-myeloid compartment differentiation. PU.1 concentration increases during macrophage differentiation, whereas its levels remain low during the B-lymphocyte differentiation. PU.1 is required for monocytic precursors as well as mature macrophage formation, but is downregulated by GATA-1 and C/EBPα during differentiation of eosinophils and functionally mature neutrophils. PU.1 downregulation is instead a necessary step during erythrocyte and T cell differentiation.

Adapted from: Gupta *et al.* (2006). Stem Cells and Development, 4; pp.609-617.

1.3 Hematopoiesis and Leukemia

The mammalian hematopoietic system is characterized by a hierarchy of cell lineages, in which the self-renewal, the proliferative and the differentiative potentials are regulated by combinations of intercellular interactions and cytokines.

HSCs are the best described stem cells and distinguish themselves from their downstream progeny by their higher self-renewal potential. Self-renewal of the HSCs pool is achieved either by symmetrical division that generates two identical daughter cells or by an asymmetrical division, which yields one HSC and a downstream progenitor with a reduced self-renewal potential but an increased capacity to differentiate. This secondary type of progenitor is able to cycle much faster and generate a clonally expanded population of cells (Warner et al., 2004). Further divisions of these cells is accompanied by an increase in their lineage commitment, which means an higher capacity to proliferate and differentiate at the cost of a loss in self renewal ability (Huntly and Gilliland, 2005). Understanding the tight regulation that guides this process is not only one of the major targets of modern cell biology but is also of great clinical significance given the proven close relationship between normal stem cells and cancer stem cells. Cancer stem cells are considered to be a small population of cells within the tumor with infinite proliferation potential, responsible for the formation and maintenance of the tumor itself (Reya et al., 2001). As HSCs are able to regenerate the entire hematopoietic system in a lethally irradiated mouse (Dick, 1996), cancer stem cells possess the capacity to regenerate the same tumor in a permissive recipient (Huntly and Gilliland, 2005). For many types of cancer the target cell that gives rise to the neoplastic event is unknown, but for some leukemias there is ample evidence that the initiating cell is a HSC that has accumulated a certain number of mutations. More then 30 years ago, several studies indicated that only a small subset of leukemic cells was capable of extensive proliferation *in vivo* and *in vitro* (Park et al., 1971). Since then, more evidence has been obtained that most leukemic cells are unable to proliferate extensively and that only a restricted pool was truly clonogenic (Blair et al., 1997; Bonnet and Dick, 1997); these cells have been defined as the *Leukemic Stem Cells* (LSCs) and, like their normal counterparts, are responsible for the perpetuation of their downstream neoplastic population through self-renewal and possibly, partial differentiation, analogous to the normal HSCs (Figure 3). Using various AML patient samples this cellular subset has been identified as $Thy1^-$, $CD34^+$, $CD38^-$, comprising not more than 1% of the entire leukemic population, which are solely responsible for the recreation of the same disease in NOD/SCID mice (Blair et al., 1997; Bonnet and Dick, 1997). Recently many investigators have demonstrated the similarity between the HSC and LSCs revealing that pathways, which have been classically associated with cancer, may also regulate normal stem cells development and *vice versa*. For example, over-expression of the *BCL-2* oncogene prevents apoptosis thereby increasing the number of HSCs *in vivo*, an observation that underscores the role played by programmed cell death on HSC homeostasis (Domen et al., 2000; Domen and Weissman, 2000). Also the Wnt signaling pathways appear to be relevant for both oncogenic and normal hematopoiesis (Taipale and Beachy, 2001). Wnt proteins are signaling molecules (Nusse and Varmus, 1982) that take part in the regulation of developmental processes in many different organisms (Cadigan and

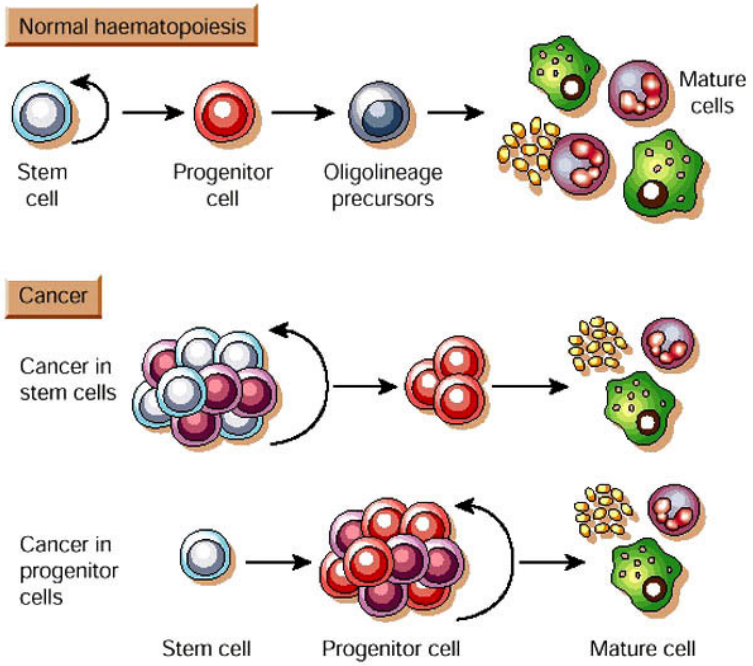

Figure 3: Progenitors cells as target of transformation.

Comparison of the self-renewal processes occurring during physiological hematopoiesis and during leukemic transformation. Due to their high level of self-renewal capacity, the stem cells are optimal targets for the leukemic transformation.

Differently from the normal hematopoiesis (top lane), in the case of stem cell transformation, the signaling pathways regulating the self-renewal processes, otherwise tightly regulated, appear to be uncontrolled (middle lane). Moreover, if the transformation event occurs at the level of one of the progenitors, this leads to the generation of a progenitor cell empowered of self-renewal capacity (bottom lane).

Adapted from: Reya *et al.* (2001). *Nature*, 414; pp.105-111.

Nusse, 1997). Wnt signaling is altered in many cancer types and its presence in the bone marrow (Reya et al., 2000) suggests a possible role in HSC homeostasis. To support this hypothesis, surrogate activation of Wnt signaling by overexpression of activated β-catenin drives the expansion of transplantable HSCs (Thy1.1^{lo}/Lin$^{-/lo}$/ Sca1^{+}/c-Kit^{+}) in long-term culture. Its inhibition instead leads to a reduction of HSCs proliferation, increased cell death *in vitro* and reduced reconstitution potential *in vivo* (Reya et al., 2003). Overexpression of some Hox genes, such as *HoxB4* and *HoxA9* (Antonchuk et al., 2002; Thorsteinsdottir et al., 2002) or activation of the Notch signaling pathway (Karanu et al., 2000; Varnum-Finney et al., 2000), all result in the

selective expansion of the HSCs pool *in vitro* and *in vivo.* Therefore, these pathways are also involved in HSC homeostasis, and their deregulation is involved in oncogenesis. Despite the supportive evidence, the postulation that LSCs are HSCs remains controversial, because it cannot be excluded that LSCs are a more restricted progenitor or even a terminally differentiated mature cell that, as a result of accumulated mutations, reacquired the stem cell capability of self-renewal.

1.4 Chromosomal Translocation in leukemia

In leukemogenesis normal regulation that leads to the lymphoid or myeloid cell development is often altered by genetic lesions. This can cause a differentiation arrest, a deregulation of proliferation, an aberrant survival potential that drives the clonal expansion of the affected cells, or a combination of these effects. Early on, cytogenetic analysis revealed that blast cells in patients with leukemia or lymphoma harbored clonal chromosomal abnormalities (Look, 1997; Rabbitts, 1994). These karyotypic alterations mostly involve chromosomal translocations or inversions, which alter expression of the genes located at the chromosomal breakpoints, and, together with point mutations and gene deletions, occur in 65% of human leukemias (Look, 1997; Pui et al., 1990; Raimondi, 1993; Raimondi, 1999; Rowley, 1990; Solomon et al., 1991). These aberrations are generally restricted to cells of a specific lineage that become arrested in their development, which suggests that the affected gene(s) is involved in the regulation of specific hematopoietic progenitors. Chromosomal translocations are caused by a double-strand DNA breaks and subsequent non-homologous end-joining recombination combined with a lack of fidelity in DNA-repair mechanisms (Greaves and Wiemels, 2003) within a specific genomic region. The latter are known as *Break-point Cluster Regions* (BCRs) and can measure from a few base pairs (Wiemels, 2003) to hundreds of kilobases (Reichel et al., 1998; Wiemels and Greaves, 1999; Xiao et al., 2001). Breakpoints occur scattered throughout the BCRs suggesting two possibilities: either these regions are particularly vulnerable to breaks or breaks occur randomly throughout the gene and only the ones that confer a clonal growth advantage lead to disease (Greaves and Wiemels, 2003). In leukemias, as well as in other types of cancer, stem cells are the suspected target for this kind of genetic alterations (Greaves, 2000; McCulloch, 1983; Reya et al., 2001). This is not because these cells are more prone to DNA damage when compared to differentiated cell types; on the contrary, the limited cycling of stem cells would protect them from DNA damage to their genome. Rather, it is the stem cells' long lifespan, together with their unique self-renewal capacity, that allows them to acquire the secondary genetic alterations necessary for uncontrolled clonal expansion. It is a well-known fact that expression of the product of a single chromosome translocation in transgenic mice is in most cases insufficient to cause leukemic growth (Hanahan and Weinberg, 2000; Higuchi et al., 2002; Yuan et al., 2001). Many examples support this hypothesis such as the fact that

the *BCR-ABL* fusion gene alone is able to initiate a benign or a chronic myeloproliferative clonal expansion in both patients and mice (Daley et al., 1990; Era and Witte, 2000), but the progression of CML to an acute phase leukemia (blast crisis) with differentiation arrest is due to additional mutations (Deininger et al., 2000). Moreover, the *BCR-ABL* fusion mRNA can be detected in the blood of many normal adults (Bose et al., 1998), but most of the same individuals are negative for the fusion gene in a second test. The possibility that the fusion gene had been generated in a cell with limited proliferative capacity would explain why the presence of the fusion gene is transient and why this event does not result in leukemia. Also AML1-ETO and TEL-AML1 fusion proteins represent a leukemic event but they are never present as a single hit. A well-documented second hit in TEL-AML1 cases is the deletion of the normal *TEL* allele (Raynaud et al., 1996), which may provide a selective growth advantage compared to cells that retain *TEL* (Lopez et al., 1999; Rompaey et al., 2000).

Chromosome translocations are somatic lesions that can directly activate proto-oncogenes, either by inappropriate expression, as happens in T- or B-lymphoid malignancies in which the proto-oncogene comes under the control of the highly expressed B- or T-cell receptor genes, or by fusion to another gene giving rise to expression of a leukemia-specific chimeric oncoprotein (Look, 1997; Rabbitts, 1991; Rabbitts, 1994). One example of an overexpressed oncogene is *MYC*, often

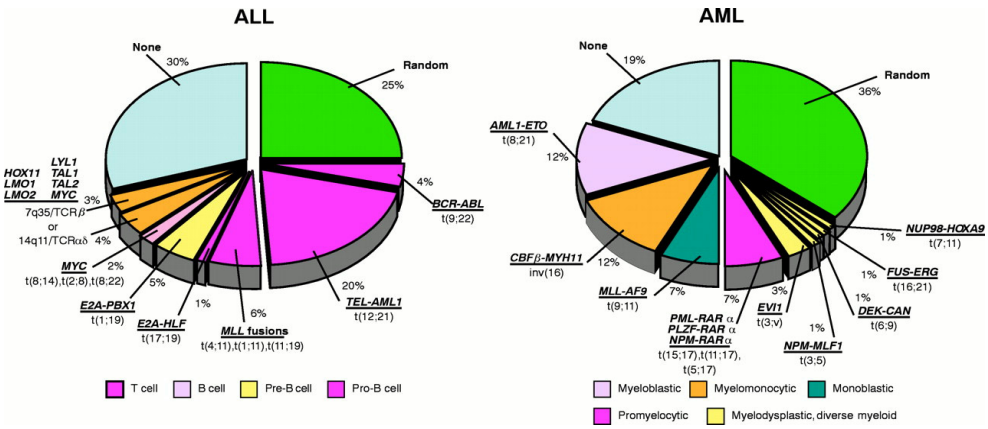

Figure 4: Distribution of translocation-generated fusion genes among the different type of leukemias.

The charts illustrate the distribution of translocation-generated fusion genes commonly found in the different immunological subtype of ALL (left) and AML (right). The “Random” label indicates sporadic rearrangements observed only in leukemic cells from single cases. The “None” label refers to leukemias that lack identifiable gene abnormalities.

Adapted from T. Look (1997). *Science,* 278; pp.1059-1064

translocated into the *Immunoglobulin Heavy chain* (IgH) in B-lymphoid malignancies or into the *T-Cell Receptor* (TCR) gene loci in T-lymphoid malignancies, both resulting in deregulated MYC expression (Rabbitts, 1994).

However, in the majority of the cases, chromosomal translocations result in the expression of fusion genes involving *Transcription Factors (*TFs) genes critical for the regulation of hematopoiesis (Shivdasani and Orkin, 1996). Alteration of transcription is a commonly observed mechanism to block normal differentiation (Pandolfi, 2001; Tenen, 2003).

In *Acute Lymphoid Leukemias* (ALLs) the type of gene alteration seems to correlate with a specific differentiation state of the leukemic cells, reflecting the immunologic subtype of the disease (Figure 4). The list of the genes that are found translocated into the TCR locus in many T-cell leukemias include those encoding the orphan homeobox protein HOX11, the cysteine-rich LIM domain-containing LMO1 and LMO2 proteins, the *basic Helix-Loop-Helix* (bHLH) proteins LYL1, TAL1, TAL2, and MYC. Moreover, as previously mentioned, the *MYC* gene is also found translocated into the proximity of one of the *Immunoglobulin* (Ig) loci in B-cell leukemia and Burkitt's lymphoma. *BCL-2* is another oncogene identified because of its translocation into the IGH locus by the t(14;18) in human B cell follicular cell lymphoma (Bakhshi et al., 1985; Cleary and Sklar, 1985; Cleary et al., 1986; Tsujimoto et al., 1985).

Instead, in pre- and pro-B ALL many chromosomal translocations involve the fusion of two different TFs, such as *E2A* and *PBX1* in pre-B lymphoblastic leukemia and *E2A*, *HLF*, *MLL*, *TEL* and *AML1* in pro-B cells. *MLL, TEL* and *AML1* are found translocated with more than 15 alternative partner genes and, in the case of *TEL* and *AML1,* even with each other (Lengauer, 2001; Romana et al., 1995a; Romana et al., 1995b). These translocations are often able to modify the normal expression pattern of HOX genes (Buske and Humphries, 2000; Ferrando and Look, 2003). *TEL-AML1*, resulting from the t(12;21), is one of the most common fusion genes, found in 20% of ALL patients (Shurtleff et al., 1995). In this translocation the 5' portion of TEL is fused to almost the entire coding region of AML1, encoding the α subunit of the *Core Binding Factor* (CBF) (Loh and Rubnitz, 2002). The resulting chimeric transcription factor maintains the protein-protein interaction domain of TEL and the DNA-binding domain of AML1. A consequence of this fusion is the inhibition of the transcriptional activity normally initiated when AML1 binds to its cognate DNA binding site within the core enhancer sequence of its target genes (Hiebert et al., 1996) (Figure 5). AML1 recruits other TFs and co-activators, including histone acetylases, generating a complex that drives the transcription of target genes. On the contrary, when TEL-AML1 binds to the same AML1 binding sites it recruits histone deacetylases, which induce compaction of the chromatin, causing transcriptional inhibition (Pui et al., 2004). As mentioned above, many products of chromosomal translocations are able to interfere with the normal expression of HOX genes (Buske and Humphries, 2000; Ferrando and Look, 2003). A

second player in the regulation of *HOX* gene expression is the *Mixed-Lineage Leukemia protein* (MLL) (Ayton and Cleary, 2001; Ernst et al., 2002). In this case the N-terminal portion of MLL, also known as the SET domain (Rozovskaia et al., 2000), can be fused with the C-terminal portion of more than 40 partner genes, and this type of alterations occur in more than 80% of infants with ALL (Pui et al., 2004).

AML1-ETO (Peterson and Zhang, 2004), resulting from the t(8;21), and *CBFβ-*

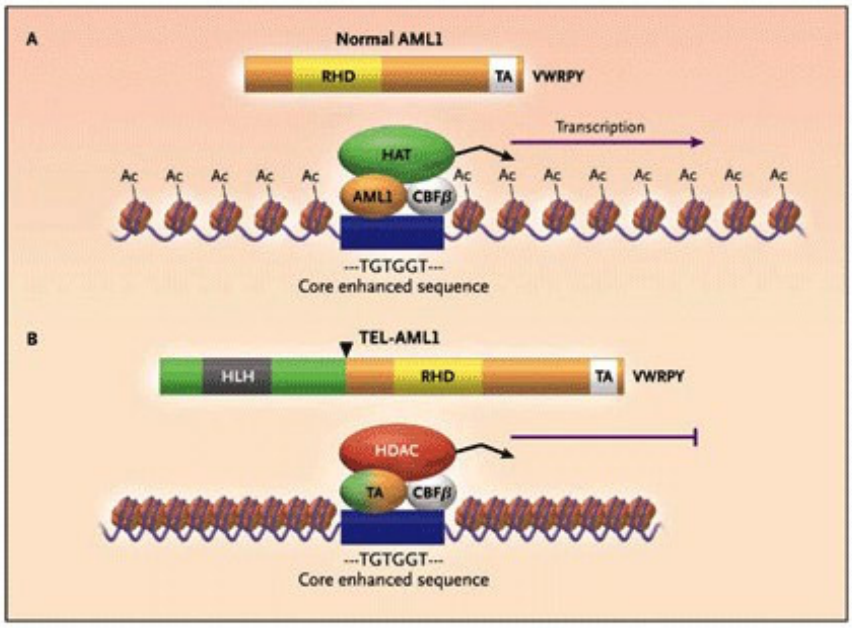

Figure 5: Transcriptional repression mechanism of TEL-AML1.

Panel A shows the structure of AML1, consisting of a central *runt homology domain* (RHD), responsible for DNA binding and hetero-dimerization with CBFβ, a transcription activation domain (TA) and the C-terminal amino acid sequence (VWRPY) which mediates the binding to the Groucho co-repressor.

AML1, together with CBFβ, recruits a transcriptional activation complex that includes *histone acetyl transferase* (HAT) that through lysine residues acetylation, opens the chromatin structure leading to the transcriptional activation (purple arrow).

Panel B shows the structure of the TEL-AML1 fusion protein, in which the TEL HLH domain is fused to the almost complete AML1 protein. Although it still maintains the ability to hetero-dimerize with CBFβ, TEL-AML1, instead of recruiting a transcriptional activation complex, recruits a transcriptional co-repressor complex that includes *histone deacetylases* (HDAC). In this case the removal of acetyl groups from histones results in the compacting of the chromatin and transcriptional repression.

Adapted from: Pui *et al.* (2004). *N Engl J Med*, 350; pp.1535-1548.

MYH11, resulting from the inversion on chromosome 16 (inv16) or t(16;16), account for 25% of all cases of human *Acute Myeloid Leukemia* (AML) (Langabeer et al., 1997a; Langabeer et al., 1997b) (Figure 4). Both events affect the function of CBF. This is a transcription factor composed of two subunits: AML1 (also known as RUNX1 or *CBF*α), discussed in the previous paragraph and also targeted by t(3;21) and t(16;21), and CBFβ (Liu et al., 1993). Normal function of both genes is critical for the hematopoietic development and lack of either one causes embryonic lethality due to a failure to switch to definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996a; Wang et al., 1996b). The expression of *AML1-ETO* increases the percentage of self-renewing stem cells without pathogenic consequences (Guzman et al., 2002; Mulloy et al., 2002). As mentioned before, a second mutation is necessary to provide the molecular context in which *AML1-ETO* causes leukemia. One possible event is the upregulation of HoxA9, commonly observed in AML (Golub et al., 1999; Lawrence et al., 1999). The other common chromosomal rearrangement, inv16, associated with 8-10% of the AML cases is specifically present in the FAB subtype M4E0 (Speck and Gilliland, 2002). In this case the *CBF*β gene is fused to *MYH11* gene, which encodes for a *Smooth Muscle Myosin Heavy Chain* (SMMHC) (Liu et al., 1993), generating a CBFβ-SMMHC fusion protein. Similarly to AML1-ETO, CBFβ-SMMHC is a dominant-negative inhibitor of CBF transcriptional activity (Liu et al., 1996; Lutterbach et al., 1999) and is unable to cause AML alone. In chapter 5 of this thesis we show that upregulation of the MN1 gene is an important secondary mutation in the development of inv(16) AML. In addition to the dominant negative mutations, loss of function mutations in AML1 have also been found in 20% of cases of the most immature subtype (M0) of AML (Osato et al., 1999; Preudhomme et al., 2000).

Translocations involving the RARα gene are invariably associated with *Acute Promyelocytic Leukemia* (APL). The t(15;17) generates the PML/RARα fusion protein and, in a significant portion of patients, also the reciprocal RARα/ PML protein (Alcalay et al., 2001). Almost the entire RARα protein is fused to almost the entire PML protein. PML is part of the “nuclear body”, complex of macromolecular structures within the nucleus (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994; Zhong et al., 2000b). Expression of PML/ RARα causes not only delocalization of normal PML protein, but also of other nuclear body components (Zhong et al., 2000a). PML/ RARα, like RARα, is able to bind *retinoic acid responsive elements* (RAREs) (Lin and Evans, 2000), *Retinoic Acid* (RA), and RXRα (He et al., 1999; Melnick and Licht, 1999). Like RARα, PML/RARα binds co-repressor molecules and, in absence of RA, represses transcription of RA-responsive genes. However, unlike RARα, physiological concentrations of RA are not sufficient to release the recruited co-repressors from PML/ RARα, and the repression state of the RA-responsive genes is maintained. Fortunately, pharmacologic concentrations of RA, achieved with the administration of *all-trans-retinoic acid* (ATRA), can reverse this repressive state (Grignani et al., 1998; He et al., 1998; Lin et al., 1998) and restores the normal nuclear localization of PML

(Dyck et al., 1994; Faretta et al., 2001; Koken et al., 1994; Weis et al., 1994). This explains why APL is uniquely sensitive to ATRA treatment (Soignet et al., 1997; Warrell, 1996).

Phosphorylation of tyrosine residues is a well-known mechanism conserved throughout evolution to transmit activating signals from the cell surface or specific intracellular structures to proteins in the cytoplasm and cell nucleus. *Receptor tyrosine kinases* (RTKs) mediate the cellular response of different extracellular signals involved in the regulation of cell proliferation, migration, differentiation and survival. The first tyrosine kinase oncogene*, BCR-ABL*, associated with human hematological disease was discovered more then 20 years ago as result of the t(9;22), also known as the Philadelphia (Ph) chromosome. Since then, ample evidence accumulated that shows involvement of multiple activated tyrosine kinases in different kinds of leukemias. In every case the constitutive tyrosine kinase activity causes enhanced proliferation and prolonged viability, but rarely blocks differentiation (Scheijen and Griffin, 2002).

In the case of BCR-ABL the chimeric protein is generated by the fusion of a variable portion of N-terminal BCR protein to the ABL tyrosine kinase domain (Shtivelman et al., 1985), resulting in a p185, a p210 or a p230. In *Chronic Myeloid Leukemia* (CML) the predominant form is $p210^{BCR\text{-}ABL}$, while in Ph^+ ALL it is $p185^{BCR\text{-}ABL}$. The $p230^{BCR\text{-}ABL}$ has been associated with chronic neutrophilic leukemias (Scheijen and Griffin, 2002). ABL can also be fused to the N-terminal portion of TEL, resulting in a TEL-ABL protein (Andreasson et al., 1997; Golub et al., 1996; Papadopoulos et al., 1995). All ABL fusion proteins show an elevated tyrosine-kinase activity compared to normal c-ABL, but of the three, $p185^{BCR\text{-}ABL}$ is the most potent (Scheijen and Griffin, 2002). The transforming properties of BCR-ABL proteins are evident in hematopoietic cells, and require the presence of a functional protein kinase domain. Activation of RAS, RAF, PI3K, and JNK/SAPK signaling pathways (Dickens et al., 1997; Sawyers et al., 1995; Skorski et al., 1997; Skorski et al., 1995a; Skorski et al., 1995b), as well as transcriptional activation of NF-$_\kappa$B, c-JUN and c-MYC are required for BCR-ABL-induced transformation (Raitano et al., 1995; Reuther et al., 1998; Sawyers et al., 1992). There is a wealth of evidence that cooperation between multiple signaling pathways, including RAS and PI3K, is required for the full oncogenic activity of BCR-ABL (Sonoyama et al., 2002). Moreover, STAT5 is found constitutively activated by tyrosine phosphorylation in BCR-ABL-transformed cells (Carlesso et al., 1996; Ilaria and Van Etten, 1996). BCR-ABL inhibits apoptosis in cells exposed to DNA damage, cytokine deprivation and FAS activation, blocking the mitochondrial release of cytocrome c and procaspase-3 (Amarante-Mendes et al., 1998b; Dubrez et al., 1998). This is accomplished via BAD phosphorylation (Neshat et al., 2000), and induction of increased levels of BCL2 and BCL-x_L (Amarante-Mendes et al., 1998a) (Sanchez-Garcia and Grutz, 1995). Furthermore BCR-ABL-mediated down-regulation of p27Kip1 may contribute to enhancing the survival signaling in hematopoietic cells (Gesbert et

al., 2000; Jonuleit et al., 2000; Parada et al., 2001). The understanding of the mechanisms underlying the BCR-ABL activity were fundamental for the development of an ABL-specific tyrosine kinase inhibitor, STI-571 (Druker et al., 2001), the poster child of target-specific therapeutic interference which is currently used to treat patients with Ph^+ leukemia.

1.5 ETS transcription factors in Leukemia

The *E26-transformation specific* (ETS) family of eukaryotic TFs, which is currently composed of more then 30 members, was discovered more than two decades ago. These genes are unique to the Metazoan Lineage, and are present in organisms as different as sponges and humans (Degnan et al., 1993; Laudet et al., 1993). Ets-1 was the first member of this family (Leprince et al., 1983; Nunn et al., 1983), identified as part of the *gag-myb-ets* oncogene, encoded by the transforming retrovirus E26. This virus induces both erythoblastic and myeloblastic leukemia in chickens (Karim et al., 1990; Leprince et al., 1983).

Some of the ETS members are ubiquitously expressed while others show a restricted tissue distribution (Graves and Petersen, 1998; Oikawa and Yamada, 2003). The better-characterized members are: ETS-1, ETS-2, PU.1 (SPI-1), SPI-B, FLI-1 (ERGB), ELF-1, ERG, EIL, and TEL.

The unifying feature of this family of TFs is the presence of the ETS DNA-binding domain, a Winged Helix-Turn-Helix motif (Graves and Petersen, 1998; Liang et al., 1994a; Liang et al., 1994b). This domain binds to the unique sequence GGAA/T named *ETS Binding Site* (EBS), which is present in the promoter/enhancer regions of more than 200 ETS target genes necessary for the proper regulation of fundamental cellular processes. A second highly conserved domain found in the N-terminal regions of a significant fraction of ETS proteins is the "*Pointed*" (PNT) domain, a *Helix-Loop-Helix* (HLH) structure important for protein-protein interactions (Kim et al., 2001). In TEL the PNT domain also functions as a self-association domain (Carroll et al., 1996; Golub et al., 1996; McLean et al., 1996). Many of the ETS genes are preferentially expressed in the hematopoietic system where they regulate both expansion and differentiation of hematopoietic progenitors. ETS-1, FLI-1 and ERG are initially expressed in the blood island where hemangioblasts are present (Maroulakou and Bowe, 2000). TEL is essential for angiogenesis in the yolk sack of the mouse as well as hematopoiesis in the adult animal (Wang et al., 1997; Wang et al., 1998). Expression of PU.1 is observed in $CD34^+$ hematopoietic progenitors, where its levels appear to steer differentiation toward myeloid or lymphoid lineages. High PU.1 levels promote macrophage differentiation and relatively low levels induce B-cell differentiation (DeKoter and Singh, 2000). $PU.1^{-/-}$ hematopoietic progenitors failed to express IL-7Rα because PU.1 directly regulates the IL7R-α promoter (DeKoter et al.,

2002). The absence of PU.1 causes defects in the lymphoid compartment, especially in B-cells, but the knockout mice also fail to develop mature macrophages and neutrophils (McKercher et al., 1996; Scott et al., 1994).

Several ETS proteins have been implicated in malignant and genetic disorders. For example, FLI-1, TEL and ERG, are located at chromosome translocation breakpoints in different leukemias and solid tumors (Dittmer and Nordheim, 1998; Mavrothalassitis and Ghysdael, 2000; Truong and Ben-David, 2000). *PU.1* (Moreau-Gachelin, 1994) and *Fli-1* (Ben-David et al., 1991) are found activated by the Friend retrovirus in mouse erythroleukemia, whereas transduction of PU.1 retrovirus into hematopoietic progenitors induces the proliferation of immortalized proerythroblast-like cells that nonetheless remain dependent on EPO for survival (Schuetze et al., 1993). Furthermore, PU.1 transgenic mice develop erythroleukemia soon after birth (Moreau-Gachelin et al., 1996), while mutations in PU.1 have recently been reported in approximately 7% of AML patients, suggesting that disruption of this gene could contribute to the differentiation arrest found in these patients (Mueller et al., 2002). Another example of an ETS family gene involved in leukemia is *TEL/ETV6* gene. This gene is a frequent target of chromosome translocations producing fusions with 3 types of partners:

1. *protein tyrosine kinases* (PTKs)
2. TFs
3. other genes, whereby the resulting fusion protein is not active (Figure 6).

The first PTK fusion partner discovered was the *Platelet-derived Growth Factor Receptor beta* (PDGFRβ) (Golub et al., 1994). *TEL-PDGFRβ* is a critical player in *Chronic Myelo-Monocytic Leukemia* (CMML) and exhibits transformation activity when expressed in cell lines. The N-terminal portion of TEL, including the PNT domain in fused to the C-terminal portion of PDGFRβ containing the kinase domain. This general structure typifies the TEL-PTK fusions in which the tyrosine kinase domain can be derived from ABL1, ABL2, JAK2, NTRK3, FGFR3, and SYK. The PNT domain of TEL provides a dimerization interphase, which constitutively activates the associated PTK (Bohlander, 2005).

TEL-PTKs are present in a wide range of hematological and non- hematological malignancies.

When expressed in murine bone marrow, TEL-JAK2 leads to fatal myeloid and lymphoproliferative disease (Schwaller et al., 1998), TEL-ABL1 to myeloproliferative syndrome with a long latency period (Million et al., 2002), and TEL-PDGFRβ to a rapid fatal myeloproliferative syndrome (Tomasson et al., 2000).

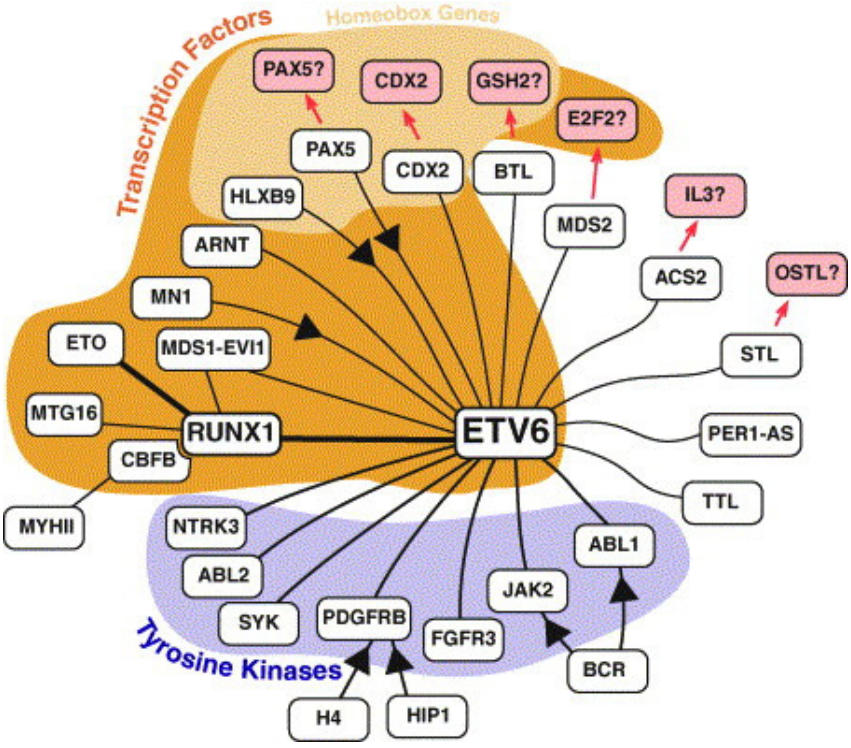

Figure 6: Representation of the fusion gene network of TEL/ETV6.

The darker line connects fusion genes associated to leukemia. The red arrow indicates transcriptional regulation. The question mark indicates that the upregulation or the relevance thereof has not been proved.

Adapted from: Bohlander S. (2005). *Sem Cancer Biol,* 15; pp.162-174.

Among the TF fusion partners of TEL, RUNX1 (AML1), was one of the earlier identified, and occurs in childhood acute B-cell lymphoblastic leukemia (Knezevich et al., 1998a). The t(12;21) is present in 25% of the childhood B-ALLs (Shurtleff et al., 1995) and, in some cases, has been shown to originate *in utero* (Ford et al., 1998). While the TEL-RUNX1 fusions are common in childhood ALL, all the other TEL fusions with TFs are much rarer. The t(12;22) translocation fuses TEL to MN1, in some AML (M1, M4, and M7) or in some myelodysplastic syndrome patients (Buijs et al., 1995). Two variants of the MN1-TEL fusion have been reported. The Type I fusion occurs within *TEL* intron 2 and incorporates an intact but inactive PNT domain as well as the ETS domain into the chimeric protein, whereas the Type II fusion occurs within TEL intron 3 disrupting part of the TEL PNT domain (Buijs et al., 1995; Golub, 1997). Initial experiments showed that MN1-TEL has weak transforming activity in NIH3T3 murine

fibroblasts (Buijs et al., 2000). In chapter 4 of this thesis we show that MN1-TEL functions as an effective oncogene in the mouse hematopoietic system.

1.6 Major players in this thesis

1.6.1 TEL2/ETV7

TEL2 is another member of the ETS family of transcription factors, closely related to TEL. Like TEL, TEL2 contains an N-terminal PNT domain that shows 62% homology with the PNT domain of TEL, while the C-terminal ETS domain shows 85% homology with that of TEL (Potter et al., 2000). Both proteins act as transcriptional repressors, and TEL2 inhibits expression of *RAR*α and *BMP-6* upon transfection into the osteosarcoma cell line MG-63 (Gu et al., 2001). Unlike TEL, that is expressed ubiquitously, TEL2 shows a more restricted expression pattern; it is predominantly present in human bone marrow and spleen, which suggests a possible role in the hematopoietic system (Gu et al., 2001; Potter et al., 2000). Moreover, TEL2 is expressed in a variety of human tumors, including leukemias (Cardone et al., 2005; Gu et al., 2001; Poirel et al., 2000). In transiently transfected Hela cells TEL2 localizes to the nucleus, excluding the nucleoli and can bind the same *Consensus DNA-Binding Sequence* (CDBS) as TEL. Via the PNT domain TEL2 can self-associate or form heterodimers with TEL (Potter et al., 2000). Although highly homologous to TEL, the 13 amino acid substitutions in the 80 amino acid-long ETS domain of TEL2 might enable it to bind to a unique set of target genes. This possibility is supported by the observation that TEL2 overexpression in the human myeloid leukemia cell line U937, blocks its monocytic differentiation, while similar overexpression of TEL fails to recapitulate this phenotype (Kawagoe et al., 2004). This effect of TEL2 depends on the presence of both an intact PNT and ETS domain (Kawagoe et al., 2004). Forced expression of TEL2 in *E*μ*-Myc* transgenic mice accelerates Myc-induced B-cell lymphomagenesis in these mice (Cardone et al., 2005).

1.6.2 MN1-TEL

A consistent group of human leukemia is associated with tumor-specific fusion proteins involving TEL, an ETS TF containing a PNT domain in its N-terminal portion (Golub et al., 1994; Sharrocks et al., 1997) that mediates homo/oligomerization (Jousset et al., 1997), association with FLI1 (Kwiatkowski et al., 1998) and TEL2 (Potter et al., 2000) and recruits the transcriptional co-repressor N-Cor (Lopez et al., 1999). TEL also recruits the Sin3a and SMRT co-repressors (Chakrabarti and Nucifora, 1999; Fenrick et al., 1999) enabling TEL to exert its function as transcriptional repressor. At its C-terminal portion TEL contains the EBS (Buijs et al., 1995; Szymczyna and Arrowsmith, 2000). As mentioned earlier, *TEL* translocations

most commonly generate chimeric proteins containing the PNT domain fused to phosphotyrosine kinase domains, such as that of the PDGF-β receptor (Golub et al., 1994), ABL (Golub et al., 1996; Papadopoulos et al., 1995), JAK2 (Lacronique et al., 1997; Peeters et al., 1997), ARG (Cazzaniga et al., 1999) and NTRK3 (Knezevich et al., 1998a; Knezevich et al., 1998b). In these fusions the TEL PNT domain mediates the activation of its partner's phosphotyrosine kinase domains, responsible of the transforming activity (Carron et al., 2000; Golub et al., 1994; Liu et al., 2000; Sjoblom et al., 1999; Tomasson et al., 1999). The TEL PNT domain is also found fused with several TFs such as AML1 (Romana et al., 1995a), MDS1 (EVI1) (Nucifora, 1997) and CDX2 (Chase et al., 1999). Among these TEL fusions that retain the ETS DNA-binding moiety are rare (Beverloo et al., 2001; Buijs et al., 2000; Cazzaniga et al., 2001). The first of such *TEL* fusions to be identified was the one with *MN1,* mediated by t(12;22)(p13;q11) associated with myeloid malignancies in humans (Buijs et al., 1995). This translocation leads to expression of an MN1-TEL fusion oncoprotein. Like AML1-ETO and other myeloid oncogenes, MN1-TEL has been detected only in myeloid leukemia but it is still unclear whether MN1-TEL alone is responsible for the myeloid phenotype. One reason for this could be that in patients MN1-TEL expression is confined to only GMPs thereby forcing myeloid expansion, a possibility supported by the results presented in chapter 5, which show specific expression of MN1 in the GMP. Recent work by Kawagoe and Grosveld has shown that expression of MN1-TEL in murine MPPs, under the control *of the AML1* regulating region, alters both myeloid and lymphoid development and causes T-lymphoid tumors (Kawagoe and Grosveld, 2005b). MN1-TEL expression increases the repopulation ability of myeloid progenitors *in vitro* and also partially inhibits their differentiation *in vivo*. Moreover, in these mice the proliferation of thymocytes is promoted while their differentiation is arrested at the early stage of $CD4^-/CD8^-$. This condition leads to the onset of T-lymphoid tumors in 30% of the mice (Kawagoe and Grosveld, 2005b). However, in the same animal model, the combined expression of MN1-TEL with HOXA9 causes AML in 90% of the mice while only 10% develop T-lymphoid leukemia (Kawagoe and Grosveld, 2005a). These combined data suggest that MN1-TEL has the potential to increase the proliferation of both myeloid and lymphoid progenitors but the lack of *MN1* expression in the CLP is the most likely reason that MN1-TEL is strictly associated with myeloid disease.

In this thesis we demonstrate that murine early progenitors, transduced with MN1-TEL, show an increased self-renewal capacity *in vitro*, and are able to originate immortalized myeloid cell lines that maintain a primitive morphology and still depend on IL3/SCF for growth and survival (Carella et al., 2006). The primitive nature of these cell lines, as well as their preserved ability to differentiate, was confirmed, *in vivo*, by their capability to repopulate the entire hematopoietic system of lethally irradiated mice. Three months post-transplantation all recipients died of promonocytic leukemia. Also freshly MN1-TEL-transduced progenitors reconstitute the hematopoietic system of

lethally irradiated mice and cause AML three months after transplantation (Carella et al., 2006).

1.6.3 MN1

MN1 is a gene localized on chromosome 22 and was discovered as the target of a reciprocal t(4;22) in a meningioma patient. The gene measures 70 Kb and comprises 2 large exons of 4.7 and 2.8 Kb, respectively, encoding a protein of 1319 amino acids (aa) (Lekanne Deprez et al., 1995). As result of the t(4;22) the 5'-exon was disrupted and the gene inactivated. No MN1 expression was observed in this patient, despite the fact that the other allele was intact. MN1 is an evolutionary conserved gene and is expressed ubiquitously at low levels (Lekanne Deprez et al., 1995). The *MN1* gene was also found fused to *TEL* as a result of the t(12;22) present in a subset of AML and myelodysplastic syndrome patients (Buijs et al., 1995). More recently, MN1 has been identified as a transcriptional co-activator that enhances the transcription activity of the Moloney sarcoma virus LTR (Buijs et al., 2000; van Wely et al., 2003). MN1's co-activation activity is not restricted to the RAR-RXR nuclear receptor, given that MN1 expression inhibits proliferation of an osteoblast cell line via co-activation of the vitamin D receptor (Sutton et al., 2005).

In chapter 4 of this thesis we show that MN1-TEL is a bona fide myeloid oncogene when expressed under the control of a retroviral vector in mouse bone marrow. Mapping of sequences essential for the transforming activity of MN1-TEL showed that deletion of the N-terminal 500 amino acids of MN1 but not inactivation of the ETS DNA binding domain abrogated transformation. This was in stark contrast with previous observations in NIH3T3 fibroblasts showing that a functional ETS domain was essential for the transformation by MN1-TEL. A possible explanation for this difference came from the observation that also retrovirally expressed MN1 was capable of transforming mouse myeloid cells. These observations in combination with reports that MN1 was consistently overexpressed in samples of inv(16) AML patients (Ross et al., 2004) prompted us to test whether MN1 overexpression cooperated with CBFβ-MYH11 in a mouse model for inv(16) AML. Our results support the notion that MN1 overexpression is an obligatory secondary event in this leukemia subtype.

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Chapter 2

The ETS factor TEL2 is a hematopoietic oncoprotein

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NEOPLASIA

The ETS factor TEL2 is a hematopoietic oncoprotein

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TEL2/ETV7 is highly homologous to the ETS transcription factor TEL/ETV6, a frequent target of chromosome translocation in human leukemia. Although both proteins are transcriptional inhibitors binding similar DNA recognition sequences, they have opposite biologic effects: TEL inhibits proliferation while TEL2 promotes it. In addition, forced expression of TEL2 but not TEL blocks vitamin D3–induced differentiation of U937 and HL60 myeloid cells. TEL2 is expressed in the hematopoietic system, and its expression is up-regulated in bone marrow samples of some patients with leukemia, suggesting a role in oncogenesis. Recently we also showed that TEL2 cooperates with Myc in B lymphomagenesis in mice. Here we show that forced expression of TEL2 alone in mouse bone marrow causes a myeloproliferative disease with a long latency period but with high penetrance. This suggested that secondary mutations are necessary for disease development. Treating mice receiving transplants with TEL2-expressing bone marrow with the chemical carcinogen *N*-ethyl-*N*-nitrosourea (ENU) resulted in significantly accelerated disease onset. Although the mice developed a GFP-positive myeloid disease with 30% of the mice showing elevated white blood counts, they all died of T-cell lymphoma, which was GFP negative. Together our data identify TEL2 as a bona fide oncogene, but leukemic transformation is dependent on secondary mutations. (Blood. 2006;107: 1124-1132)

Introduction

The ETS (E26 transformation specific) proteins belong to a large family of eukaryotic transcription factors (TFs) unique to the metazoan lineage and highly conserved throughout evolution.[1,2] Some ETS proteins are expressed ubiquitously while others show a restricted tissue distribution.[3,4] All ETS proteins possess a highly conserved 85–amino acid (aa) ETS domain that binds a purine-rich GGAA/T core motif present in promoters and enhancers of different target genes.[4,5] A subgroup of ETS TFs, including the TEL proteins, also contains a conserved pointed (PNT) protein-protein interaction domain, which mediates the formation of homodimers/oligomers[6,7] and heterodimers/oligomers.[8,9] In TEL this domain is involved in transcriptional repression.[10]

Vertebrate ETS TFs are implicated in many aspects of normal development and differentiation, including that of the hematopoietic system.[11] For example, Ets-1, Fli-1, and Erg are expressed early during mouse development in the blood islands of the yolk sac where hemangioblasts are present.[12] A high level of Pu-1 expression in $CD34^+$ hematopoietic progenitors directs their differentiation toward the macrophage lineage, while low Pu-1 expression induces B-cell differentiation.[13] The expression of IL-7Rα, a receptor essential for pro-B-cell development, is directly regulated by Pu-1 in lymphoid progenitors.[3,14] The ubiquitously expressed TEL/ETV6 is essential for normal embryonic yolk sac angiogenesis and is important for the maintenance of the adult bone marrow microenvironment.[15]

Several ETS genes, including *ETS1, ETS2, PU1, FLI1, TEL/ETV6,* and *ERG*, possess oncogenic properties.[4] Human *TEL/ETV6* is a frequent target of chromosome translocations[16] both in hematopoietic malignancies as well as in some solid tumors.[17] *TEL/ETV6* translocations mostly encode oncogenic fusion proteins[16,18-21] with some exceptions.[22-24] In addition, *TEL* might also have tumor suppressor activity because the gene is often deleted during later stages of t(12;21) childhood pre–B-cell acute lymphoblastic leukemia (pre–B-ALL).[25] This hypothesis is supported by the observation that TEL expression inhibits Ras-induced transformation of NIH3T3 fibroblasts.[26,27]

Recently, a novel *ETS* gene highly related to *TEL1* was isolated and coined *TEL2*.[9,19,28] *TEL2* is expressed predominantly in human hematopoietic tissues,[9] contains a PNT domain and an ETS DNA binding domain, and localizes to the nucleus. TEL2 self-associates via its PNT domain but can also form heterodimers/oligomers with TEL1, suggesting that these proteins might affect each other's function in vivo.[9,29] Although both proteins function as transcriptional repressors in transient transfection assays,[19,29] their biologic effects appear distinct. For example, TEL1 inhibits colony formation of Ras-transformed NIH3T3 fibroblasts, while TEL2 slightly stimulates colony formation.[29] During vitamin D3–induced differentiation of the promonocytic cell line U937, TEL2 but not TEL1 expression is down-regulated, and forced expression of TEL2 blocks differentiation. In contrast, overexpression of TEL2 mutants

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containing an impaired ETS or PNT domain had no effect on vitamin D3–induced differentiation of U937 cells.[29] Also TEL2, but not its PNT or ETS mutant versions, cooperates with Myc in B-lymphomagenesis in mice,[30] and its expression is up-regulated in some adult human leukemia samples[29] and in more than 30% of pediatric ALL patients.[30] Moreover, TEL2 is expressed in many human tumor cell lines.[31] Together these data suggest a role for TEL2 in hematopoietic malignancy. Herein we show that expression of TEL2 alone in the mouse hematopoietic system causes a nontransplantable myeloproliferative disease (MPD) after a long latency period. This suggests that secondary mutations are necessary for disease development. Indeed, treatment with the DNA-damaging agent *N*-ethyl-*N*-nitrosourea (ENU) in mice receiving transplants greatly accelerated tumorigenesis.

Materials and methods

Plasmid constructs, retroviral production, and viral titer determination

TEL2 cDNA was cloned upstream of the IRES element into the unique *Eco*RI site of the murine stem cell virus (MSCV) IRES-GFP vector.[29,32-35] Retroviral constructs were used to generate replication-incompetent retroviral stocks by transient transfection of the Ecotropic Phoenix 293T packaging cells line[36] using Fugene 6 (Roche, Indianapolis, IN). Viral titers were determined by infection of NIH3T3 cells in the presence of Polybrene (Sigma Chemical, St. Louis, MO) (8 μg/mL) and varied between 4×10^5 to 1×10^6 CFU/mL.

Bone marrow extraction, Lin$^-$ isolation, and retroviral transduction

Bone marrow (BM) cells were harvested by flushing the femurs and tibiae of FVB-J male donor mice 3 days after intraperitoneal injection of 150 μg/g body weight 5-fluorouracil (5FU) (Sigma Chemical). Lineage-negative (Lin$^-$) cells were purified by immunodepletion of cells presenting myeloid, erythroid, and lymphoid differentiation markers using biotinylated mouse antibodies Ly-6G (Pharmingen, San Diego, CA), Cd11b (Pharmingen), Cd45R/B220 (Pharmingen), CD5 (Pharmingen) and TER-119 (Pharmingen) and streptavidin-coated beads (Dynabeads M-280 streptavidin; Brown Deer, WI). The resulting progenitor-enriched population was prestimulated for 48 hours in Iscove medium (Gibco, Carlsbad, CA), 20% fetal bovine serum (Hyclone, South Logan, UT), supplemented with interleukin-3 (IL-3; 20 ng/mL) (Preprotech, London, United Kingdom), interleukin-6 (IL-6; 30 ng/mL) (Peprotech), interleukin-7 (IL-7; 10 ng/mL) (Peprotech), and stem cell factor (SCF; 50 ng/mL) (R&D Systems, Minneapolis, MN) in nontissue culture grade plastic Petri dishes. After prestimulation the cells were plated onto Retronectin (Takara, Otsu, Japan)–coated plates and incubated with the retroviral supernatants twice daily for 2 days in the presence of cytokines.

Colony-forming unit assays

Transduced and normal Lin$^-$ cells were plated in semisolid methylcellulose medium (MC1) plus cytokines (10 μg/mL insulin, 200 ng/mL human transferrin, 50 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6, 3 U/mL erythropoietin [Epo], StemCell Technologies 03434, Victoria, BC, Canada) at a concentration of 1×10^3 cells per dish Erythroid colony-forming unit (CFU-E), granulocyte erythrocyte monocyte macrophage colony-forming unit (CFU-GEMM), and granulocyte macrophage colony-forming unit (CFU-GM) colonies were scored 10 to 14 days later, pooled, and reseeded at a density of 1×10^3 cells per dish into secondary methylcellulose (MC2) cultures. Part of the cells were used to determine the fraction of GFP-positive cells using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). We continued MC replating until the cultures were exhausted (usually 3 to 4 consecutive rounds of plating) due to terminal differentiation of the cells.

Long-term culture-initiating cell (LTC-IC) assays

Transduced and normal BM cells (5×10^3 cells per dish) were plated onto irradiated M2-10B4 stromal layers[37] in Myelocult media (StemCell Technologies M5300) to which freshly prepared 0.5 μg/mL hydrocortisone (hydrocortisone 21-hemisuccinate; Sigma Chemical) was added. The cells were cultured for 4 weeks, while replacing half the medium every 7 days. At the end of this period the cells were harvested, analyzed for GFP expression by fluorescence-activated cell sorting (FACS), and plated in methylcellulose media. We scored the number and type of colonies 2 weeks later. Serial MC replating and concomitant GFP analyses were performed for 6 to 8 weeks.

Bone marrow transplantation (BMT)

Female 6- to 12-week-old FVB mice were lethally irradiated (single dose of 950 cGy) and 24 hours later received transplants by tail vein injection with 300 μL PBS plus heparin (30 U/mL) containing 3×10^5 to 5×10^5 Lin$^-$ cells transduced with retrovirus or not. After BMT, the animals were evaluated daily for possible signs of disease. Peripheral blood was obtained monthly by retro-orbital phlebotomy and was analyzed by FACS to determine the percentage of white blood cells (WBCs), red blood cells (RBCs), and platelets expressing GFP. Complete blood counts (CBCs) were performed with a Hemavet 3700 (Drew Scientific, Cumbria, United Kingdom), and Giemsa-stained blood smears were analyzed to verify the presence of abnormal cells.

ENU mutagenesis

TEL2 BMT was performed exactly as described in "Bone marrow transplantation (BMT)," but 5 weeks after transplantation the recipients received intraperitoneal injections twice, at a 48-hour interval, with 100 mg/kg ENU. The health status of the animals was followed as described in "Bone marrow transplantation (BMT)."

Analysis of diseased mice and tissue preparation

All the following animal procedures were conducted in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Peripheral blood of moribund animals was isolated for GFP analysis, blood smears, and CBCs. The animals were killed using CO_2 asphyxiation, and the weight of the spleen and liver was recorded. All organs were recovered, fixed in 10% neutral-buffered formalin, processed and embedded in paraffin, sectioned at 4 to 5 μm, and stained with hematoxylin and eosin (H&E) for routine histologic examination. Sternum and hind limbs were subjected to an additional decalcification step before tissue processing. Select tissues were also processed for immunohistochemical analysis with antibodies to the hematopoietic markers CD3 (Dako, Carpinteria, CA), CD45R/B220 (Pharmingen, San Diego CA), terminal deoxynucleotidyl transferase (TdT; Supertechs, Bethesda, MD), myeloperoxidase (MPO; Dako), TER-119 (Pharmingen, San Diego, CA), GATA1 (Santa Cruz Biotechnology, Santa Cruz, CA), and green fluorescent protein (GFP; Clontech, Palo Alto, CA).

Single-cell suspensions were prepared from bone marrow, spleen, and liver for analysis of GFP expression by FACS and for the preparation of cytospin slides (5×10^4 cells per slide) for morphologic examination after May-Grünwald-Giemsa (MGG) staining. After lysis of the red cells, the leukocytes were analyzed immediately for surface marker expression or frozen in fetal calf serum (FCS)/10% DMSO for later analysis. Images of tissue sections and cytospins were obtained using a BX51 microscope equipped with a Uplan FL 40 ×/0.75 numeric aperture (NA) or a 100 ×/1.30 NA objective (Olympus, Tokyo, Japan). Images were acquired using a SPOT camera and SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI). Original magnification for tissue sections and cytospins was 400 × and 1000 ×, respectively.

Flow cytometric analysis

Single-cell suspensions of BM, spleen, and liver were washed and incubated for 30 minutes on ice in staining medium (SM: DMEM supplemented with 10% fetal bovine serum) containing human γ-globulin (100 mg/mL; Sigma Chemical) to block Fc receptors. After washing, cells were incubated with monoclonal antibodies (CD3c, CD4, CD8, CD11b/Mac1, CD19, CD34, B220, TER-119, Gr1, Sca1, c-Kit, Flt3, all from Pharmingen, San Jose, CA; anti–mouse IgM from Southern Biotechnology Associates, Birmingham, AL) on ice for 30 minutes. After a final washing step, cells were resuspended in SM and analyzed using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), selecting single cells by gating on forward versus side light scatter. Wild-type and TEL2-expressing bone marrow cells were cultured for 2 weeks, and apoptotic cells were identified by FACS after annexin V–FITC staining. Propidium iodine staining was used to exclude dead cells.

Western blotting

For Western blotting, protein extracts were prepared from spleen samples of diseased TEL2 and healthy control mice, killed at the same time, using TRI Reagent (Sigma Chemical), following the manufacturer's instructions. After quantification using the BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL), 30 μg total protein was separated on 10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels under reducing conditions and transferred onto a polyvinylidine difluoride membrane (Millipore, Billerica, MA). The membranes were incubated with the antibodies specific for Bcl-x (Transduction Laboratories, Lexington, KY), recognizing both Bcl-xl and Bcl-xs, Bcl2 antibody (554218; Pharmingen), and TEL2 antibody.[30]

Secondary transplantations

Lethally irradiated (9.5 Gy [950 rad]) recipient mice received injections in the tail vein with 1×10^6 freshly isolated bone marrow or spleen cells from moribund primary recipients after red cell lysis. Mice receiving transplants were followed and killed as described for the primary recipients.

Affymetrix GeneChip analysis

BM from 5FU-treated mice was either mock transduced (Un-BM) or transduced with MSCV-IRES-GFP (GFP-BM) or MSCV-TEL2-IRES-GFP (TEL2-BM) retrovirus as described in "Bone marrow transplantation (BMT)." After transduction, cells were allowed to recover in culture for 48 hours and were sorted for GFP expression by FACS. RNA of the GFP-positive cells and mock-transduced cells was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations.

RNA quality was confirmed by UV spectrophotometry and by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). Ten micrograms of total RNA was processed in the St Jude Hartwell Center Core Facility according to the standard Affymetrix (Santa Clara, CA) protocol[38] and analyzed on the Affymetrix MOE-430A GeneChip array. Signal values, detection calls, and pairwise expression analyses were performed using the default parameters within the statistical algorithm of the Affymetrix GCOS software version 1.2. Signal values were scaled to a 2% trimmed mean target value of 500. Stringent selection criteria were applied to identify differential expression associated with TEL2. The following pairwise comparisons were performed: TEL2-BM versus GFP-BM, TEL2-BM versus Un-BM, and GFP-BM versus Un-BM. Initial selection was based on differential expression between TEL2-BM and GFP-BM. Probe sets with a $\log_2$ ratio greater than one (more than 2-fold change) plus a "change call" ($P < .006$, Wilcoxon signed rank test) were selected for further evaluation. To best identify TEL2-associated changes, the initial selection was filtered to exclude probe sets with less than 2-fold change in TEL2-BM versus Un-BM and those with more than 2-fold change in GFP-BM versus Un-BM. Probe set annotations were obtained from the Affymetrix website.[39] Functional classification of genes was performed using the Affymetrix Gene Ontology browser. Biologic processes significantly enriched within the TEL2-associated gene list were identified using the χ^2 test ($P < .001$).

Results

TEL2 expression affects the colony-formation potential of primitive myeloid progenitors in vitro

To test the effect of forced TEL2 expression in mouse hematopoietic cells, Lin$^-$ cells of 5-FU treated donor mice were transduced with MSCV-IRES-GFP or MSCV-TEL2-IRES-GFP retrovirus (Figure 1A). FACS analysis 4 days later showed that 10% to 50% of the

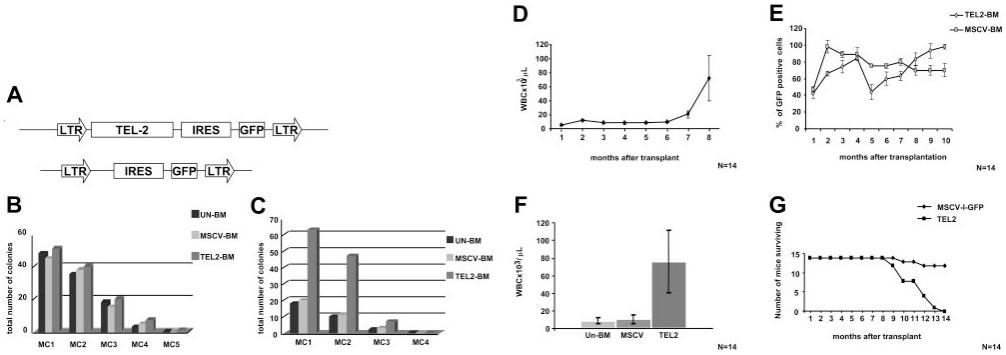

Figure 1. BM-expressing *TEL2* causes a myeloid proliferative disease in mice. (A) Schematic representation of the MSCV-IRES-GFP and MSCV-TEL2-IRES-GFP retroviral vectors used for the transduction of the Lin$^-$ BM cells. The *TEL2* cDNA was cloned in the single *Eco*RI site and is followed by an *IRES-GFP* marker gene. LTR, long terminal repeat. (B) Myeloid clonogenic activity of BM cells transduced with TEL2 retrovirus (TEL2-BM) compared with BM cells transduced with vector (MSCV-BM) or untransduced BM cells (Un-BM). Bars indicate the number of colonies counted at each round of serial replating in MC1 to MC5. CFU (1000 cells per dish plated) counts show no difference among the 3 samples with regard to their successive colony-forming capacity. This graph depicts the result of 1 of 4 experiments that gave almost identical results. (C) Successive MC assays of TEL2-BM, MSCV-BM, and Un-BM cells after 4 weeks of LTC-IC culture on an M2-10B4 stromal layer showing an increased colony-forming capacity of the TEL2-BM cells during the first 2 rounds of MC culture. This graph depicts the result of 1 of 4 experiments that gave almost identical results. (D) Average monthly peripheral blood leukocyte counts of all mice receiving transplants with TEL2-BM, showing an increase of the WBCs starting at 6 months after transplantation. Error bars show the standard deviation of each data point. (E) Monthly percentage of GFP-positive cells in the peripheral blood of mice receiving transplants with MSCV-BM or TEL2-BM, showing a steady increase in GFP-positive cells in mice receiving transplants with TEL2-BM starting at 5 months after transplantation. Error bars show the standard deviation of each data point. (F) Comparison of the average leukocyte count—in peripheral blood of mice receiving transplants with UN-BM, MSCV-BM and TEL2-BM—at the moment of death of the TEL2-BM mice. (G) Combined Kaplan-Meier survival plot of 14 (2×7) lethally irradiated mice receiving transplants with MSCV-BM or TEL2-BM ($n = 2$).

cells expressed GFP (not shown), and were used for in vitro colony-forming assays and in vivo bone marrow reconstitution of lethally irradiated recipient mice.

To assess whether TEL2 expression conferred a proliferative advantage to hematopoietic cells in vitro, we compared the growth characteristics of Lin^- cells transduced with empty retrovirus (MSCV-BM) or a TEL2 retrovirus (TEL2-BM) with that of uninfected Lin^- cells (Un-BM). To assess the colony-forming capacity of committed progenitors we plated the cells into MC1 and 2 weeks later scored the cultures for the number and type of colony-forming units (CFU-E, CFU-GEMM, CFU-GM). After counting, 10^3 cells from the MC1 were replated into fresh MC and CFUs were scored, and this process was repeated until the CFU activity was exhausted (3 to 4 rounds). However, we observed no differences in the type, size, or number of the Un-BM, MSCV-BM, and TEL2-BM colonies (Figure 1B). We next tested whether TEL2 expression affected the growth of primitive progenitors and followed the clonogenic potential of TEL2-BM and MSCV-BM progenitors in myeloid MCs after long-term culture-initiating cell (LTC-IC) cultures. This experiment was repeated twice with both types of bone marrow containing 10% GFP-positive cells. After 4 weeks of LTC-ICs, cells were collected and plated into serial MC assays. While the percentage of GFP-positive cells in MSCV-BM was similar before and after LTC-ICs, the percentage of GFP-positive cells in the MC1 of TEL2-BM LTC-ICs had increased to 60% and the number of colonies in the MC1 was 3-fold higher than that in the MC1 of MSCV-BM cells. Upon serial replating, the elevated percentage of GFP-positive cells in the TEL2 MCs remained, but the number of colonies rapidly dropped to a level similar to that of MSCV-BM cells (Figure 1C). Thus, TEL2-expressing primitive progenitors appeared to have a growth advantage in LTC-IC assays only. When transduced Lin^- bone marrow cells used for the LTC-ICs were directly plated onto tissue culture plastic, they formed their own stromal layer. Under these conditions TEL2-BM cells grew faster than control MSCV-BM cells (not shown), and the percentage of GFP-positive cells again increased from 10% to 60% during 3 weeks of culture. This suggested that interaction with stromal cells, whether in LTC-IC cultures or in stroma-forming BM cultures, provided a growth advantage to TEL2-BM cells.

Bone marrow–expressing TEL2 induces a myeloproliferative disease in FVB recipient mice

We simultaneously tested whether TEL2 expression in reconstituted BM[30] would affect normal hematopoiesis in vivo and transplanted Un-BM, MSCV-BM, and TEL2-BM cells into 7 lethally irradiated FVB recipients and assessed their peripheral blood parameters and the percentage of GFP-positive cells monthly. The same experiment was repeated 3 months later, and the combined results of these experiments are presented in Figure 1D-G. All animals receiving transplants engrafted, and the peripheral blood counts (PBCs) remained stable for 6 months, followed by an increasing leukocytosis (Figure 1D) until the mice became moribund 2 to 3 months later. At the time the animals were killed, they showed an average peripheral WBC count of 75×10^9/L ($75 \times 10^3/\mu L$) (Figure 1E), with all cells expressing GFP (Figure 1F). All TEL2-BM mice died within 8 to 14 months after transplantation (Figure 1G). Instead, mice receiving transplants with Un-BM or MSCV-BM cells showed normal blood parameters and remained healthy, although 2 MSCV-BM animals died of causes not related to hematopoietic disease.

Average CBCs of mice (Un-BM, MSCV-BM, and TEL2-BM) were determined at the time of death of the TEL2-BM mice (Table 1).

Assessment of cell morphology of peripheral blood smears (Figure 2A) showed anemia, large numbers of mature and immature neutrophils, and the presence of some blastlike cells. BM cytospins (Figure 2B) of affected mice revealed the presence of mostly myeloid cells with a similar abundance of mature and immature neutrophils, but also blastlike cells, monocytes, and promyelocytes. All TEL2-BM animals showed splenomegaly and hepatomegaly, due to extensive infiltration of a population of cells with a high mitotic index, which expressed GFP (Figure 2C-D). The spleen had completely lost its normal architecture (not shown), and the cell population in the red pulp consisted of erythroid precursors, megakaryocytes, and a vast excess of mature granulocytes and immature myeloid cells. Unlike the spleen, the liver infiltrate consisted of mature granulocytes and immature myeloid cells. The immature cells were the dominant constituent in both tissues, and most of them expressed MPO consistent with a granulocytic lineage (Figure 2E). Western blot analysis confirmed expression of TEL2 in the spleen of the diseased mice (Figure 2F). Given that the antiapoptotic effect of TEL2 on B-cell progenitors is associated with increased expression of Bcl2 but not Bcl-xl,[27] we also tested the expression of these 2 antiapoptotic proteins in the same spleen samples. Compared with MSCV-BM spleen cells, 2 TEL2-BM tumor samples expressed an increased amount of Bcl-xl and Bcl-xs, whereas Bcl2 expression was increased in 4 of 6 tumor samples. Two tumors samples showed no up-regulation of Bcl2 or Bcl-xl, indicating that the antiapoptotic proteins were not consistently up-regulated in all tumors. FACS analysis of BM (Figure 3) showed that the malignant cells were $Mac1^+$ and $Gr1^+$. BM also showed an increase in $Sca1^+$, c-Kit^+, and $Thy1.1^+$ progenitors. Together these features are consistent with a diagnosis of a chronic myelomonocytic leukemia (CMML)–like disease,[40] suggesting that TEL2 enlarges an early myeloid progenitor population.

Transplantation of the diseased bone marrow into 12 sublethally irradiated secondary recipients failed to reproduce disease. The percentage of GFP-positive cells in the peripheral blood of the secondary recipients ranged between 5% and 35% during the first 3 months after transplantation but diminished to almost undetectable levels thereafter. Only 1 of 12 secondary recipients showed a distinct myeloproliferation (93×10^9/L [$93 \times 10^3/\mu L$]) associated with organ infiltration, but the percentage of GFP-positive cells in peripheral blood was 12% and the population of cells infiltrating the different organs was GFP negative.

Table 1. Average complete blood count (CBC) in peripheral blood of mice receiving transplants with Un-BM, MSCV-BM, and TEL2-BM, at the moment of death of the TEL2-BM mice

	UN-BM	MSCV-BM	TEL2-BM*
Complete WBC count, $\times 10^9$/L	8.6 ± 3.6	10.3 ± 4.9	76 ± 35.32
Neutrophil count, $\times 10^9$/L	1.48 ± 0.52	2.36 ± 0.87	14.7 ± 13.94
Lymphocyte count, $\times 10^9$/L	4.99 ± 1.68	4.64 ± 1.6	15.3 ± 14.05†
Monocyte count, $\times 10^9$/L	0.55 ± 0.28	0.57 ± 0.39	3.2 ± 2.8
Eosinophil count, $\times 10^9$/L	0.019 ± 0.008	0.013 ± 0.005	1.14 ± 0.96
Basophil count, $\times 10^9$/L	0	0	0.23 ± 0.19
MCH, pg	13.42 ± 1.45	15.19 ± 1.31	17.9 ± 4.4
RBC count, $\times 10^{12}$/L	7.46 ± 0.71	7.6 ± 0.811	4.66 ± 1.65
MCV, fL	44.84 ± 3.14	49.23 ± 3.32	62.68 ± 14.29
Platelet count, $\times 10^9$/L	1176 ± 68.63	1406 ± 204	1094 ± 303
Hemoglobin level, g/L	127 ± 17	130 ± 17	84 ± 14

The results are expressed as average ± SD. The 2-tail unpaired Student *t* test was used to compare the data. $P \leq .05$ was considered significant.

MCH indicates mean corpuscular hemoglobin; MCV, mean corpuscular volume.

*P values calculated between the TEL2-BM and the MSCV-BM values were $< .05$.

†The high number of lymphocytes in the Hemavet counts of the TEL2-BM mouse blood samples is a gross overestimate because the blast cells and partially differentiated myeloid cells have the same size as lymphocytes and are therefore scored as "lymphocytes." This was confirmed by morphologic analysis of the blood smears (Figure 2A).

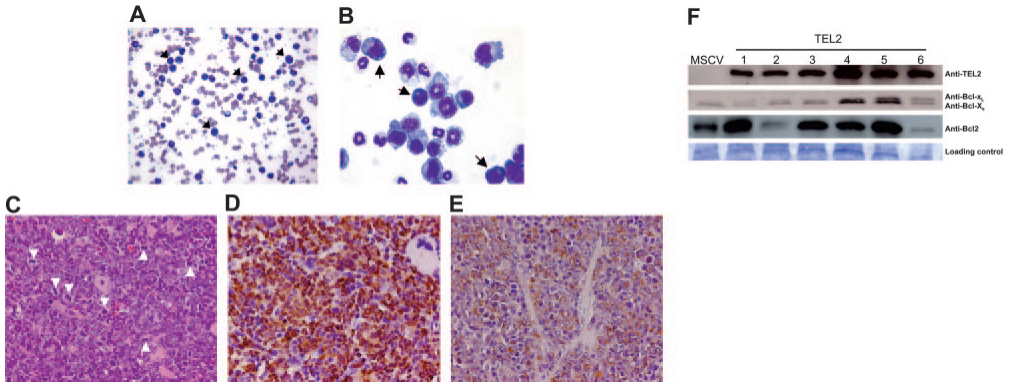

Figure 2. Mice receiving transplants with TEL2-BM develop a chronic myelomonocytic-like disease. (A) Peripheral blood smear stained with May-Grünwald-Giemsa (MGG) of a diseased mouse that underwent TEL2-BM transplantation. Arrows indicate blastlike cells. (B) Cytospin preparation after MGG staining of bone marrow of a diseased mouse that underwent TEL2-BM transplantation, showing an excess of myeloid cells in early stages of differentiation. Arrows indicate blast cells. (C) Spleen—of a diseased mouse that underwent TEL2-BM transplantation—with an extensive red pulp infiltrate of myeloid cells with a high mitotic index (H&E, magnification × 40). Mitotic figures are indicated by white arrowheads. (D) The myeloid cells expressed GFP (brown stain), confirming their transplant derivation (anti-GFP, magnification × 40). (E) The myeloid population was primarily composed of immature cells that expressed MPO (brown stain), consistent with their granulocytic lineage (anti-MPO with cytoplasm localization, magnification × 40). (F) Top row shows a Western blot analysis of TEL2 expression in spleen samples of 1 MSCV-BM (MSCV, 10 months after transplantation) and 6 diseased TEL2-BM mice at the moment of death. The second row shows Bcl-xl and Bcl-xs expression in the same samples. Bcl-xl and Bcl-xs expression in leukemic spleen samples is not altered compared with that in the control spleen sample. The third row shows that 4 of 6 leukemic TEL2 spleens show increased Bcl2 expression (samples 1, 3, 4, and 5). The bottom row shows protein loading of the blot after staining with Coomassie blue.

We concluded that this unique case was caused by a genetic event not related to overexpression of TEL2.

Annexin V staining of FVB Lin⁻ BM cells

Because TEL2 expression in pre-B cells inhibits their rate of apoptosis,[30] we assessed whether a similar effect of TEL2 in myeloid cells might provide a proliferative advantage in the mice receiving transplants. We analyzed the percentage of apoptotic cells by annexin V staining in Lin⁻ Un-BM cells or in GFP-positive MSCV-BM and GFP-positive TEL2-BM cells after culture for 7 days in liquid media promoting myeloid proliferation. Compared with both Un-BM and GFP-positive MSCV-BM cells, GFP-positive TEL2-BM cells showed a consistent 50% decrease in the number of apoptotic cells (Figure 4). This suggested that the myeloproliferative disease caused by TEL2-BM cells in mice might in part be attributed to a reduced level of apoptosis of myeloid progenitors in vivo.

To obtain information about TEL2-induced changes in gene expression, we transduced FVB Lin⁻ cells with MSCV-IRES-GFP (MSCV-Lin⁻ cells) or MSCV-TEL2-IRES-GFP retrovirus (TEL2-Lin⁻ cells) and sorted GFP-positive cells 72 hours later by FACS. Relative RNA abundance in these 2 GFP-positive cell populations and in untransduced Lin⁻ cells was determined by Affymetrix microarray analysis (MOE-430A). We then compared gene expression differences between Un-Lin⁻ and MSCV-Lin⁻ and MSCV-Lin⁻ and TEL2-Lin⁻ cells (Figure 5). This 3-way comparison allowed elimination of genes whose expression was changed due to

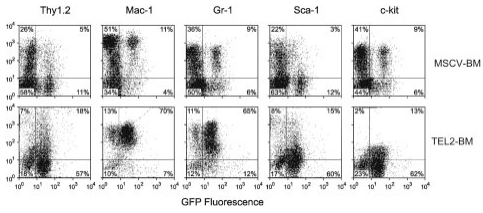

Figure 3. Flow cytometric cell surface marker analysis of BM cells of a diseased mouse that received TEL2-BM transplants and a healthy control mouse that received MSCV-BM transplants. (A) Top row shows expression of the indicated cell surface markers (y-axis) versus GFP expression (x-axis) in MSCV-BM cells. The bottom row shows expression of these markers and GFP in TEL2-BM cells. TEL2-BM cells show increased percentages of cells expressing all of the markers, but most cells are positive for Mac1 and Gr1. The malignant cells in the spleen, liver, and peripheral blood expressed the same complement of cell surface markers (not shown).

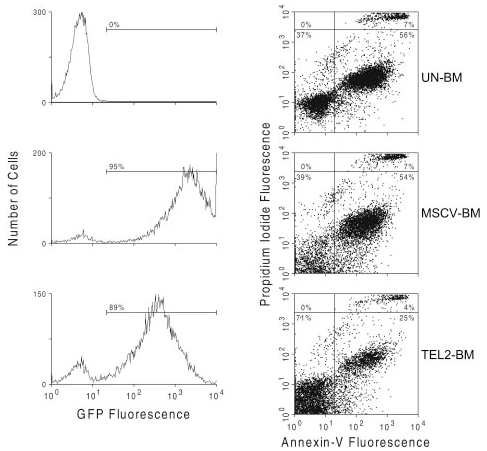

Figure 4. TEL2 expression reduces the rate of apoptosis of BM cells cultured in vitro. UN-BM cells (top 2 panels) and retrovirus-transduced MSCV-BM cells (middle 2 panels) and TEL2-BM cells (bottom 2 panels) were inoculated in liquid culture in presence of myeloid growth factors. After 4 days of culture and staining with propidium iodide, cells were analyzed for GFP expression (left panels) and for annexin V expression (right panels) by FACS. The number of apoptotic cells in the TEL2-BM sample (bottom right quadrant of the dot plots) was half of that in the UN-BM and MSCV-BM samples. The same was true for the percentage of dead cells (top right quadrant of the dot plots).

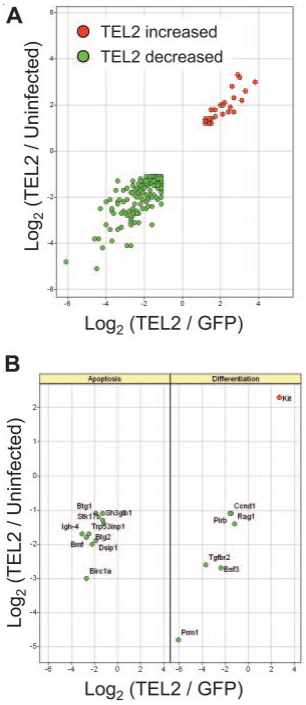

Figure 5. Microarray analysis of gene expression changes associated with TEL2 expression in mouse bone marrow. RNA from mouse bone marrow cells, either mock transduced (uninfected) or transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (GFP), were analyzed using the MOE-430A Affymetrix GeneChip microarrays. Log_2 ratio values from pairwise comparisons are plotted. (A) Relative expression of 195 probe sets identified as differentially expressed (see "Materials and methods") in both TEL2 versus GFP (x-axis) and TEL2 versus uninfected (y-axis) bone marrow. (B) Relative expression of differentially expressed probe sets found enriched ($P < .001$) in the Gene Ontology categories of apoptosis (left) or differentiation (right). Gene symbols are indicated in the panels.

GFP virus infection rather than TEL2 expression. A total of 195 genes was at least 2-fold up- or down-regulated (for the full list, see Table S1, available on the *Blood* website; see the Supplemental Table link at the top of the online article). Gene ontology analysis of this data set identified changes in expression of genes involved in apoptosis and differentiation/proliferation. This revealed changes in expression of numerous genes involved in apoptosis, including the proapoptotic genes *Trp53inp1, Stk17b* (DRAK2),[41] *Dapk2,*[42] and *Bmf.*[43] We also noticed down-regulation of antiproliferative genes including *Btg1,*[44] *Btg2,*[45,46] *Cdkn1B (p27Kip1),*[47,48] and *Rb1cc1* [49-51] and up-regulation of *cKit*, whose activation is associated with acute myelogenous leukemia (AML).[52] There was also a general down-regulation of differentiation-associated genes, including *cFes,*[53,54] *Btg1,* and *Btg2* and of the tumor suppressor gene *Tgfbr2*[55] (Figure 4; Table S1). Notably, there was no up-regulation of Bcl2 or Bcl-x mRNA levels in TEL2-Lin$^-$ cells.

ENU treatment of mice receiving transplants with TEL2 induces myeloid disease and T-cell lymphoblastic leukemia

The long latency of disease suggested that enforced TEL2 expression alone was insufficient to cause the myeloproliferative phenotype and that disease development depended at least on one or possibly more secondary mutations. To test this scenario we performed random mutagenesis by injecting 5 mice receiving transplants with vector-transduced BM and 10 mice receiving transplants with TEL2-transduced BM (30% GFP positive) with ENU 5 weeks after transplantation. The TEL2-BM-ENU animals started to die 2 months earlier than the MSCV-BM-ENU control group, and the average survival time was 2 months shorter (5 months) than that of MSCV-BM-ENU animals (7 months) (Figure 6A). Moribund mice had a large spleen and liver with cells staining positive for CD3 (Figure 6B) and TdT (Figure 6C), which were GFP negative (Figure 6D). Thus, the mice suffered from T-cell lymphoma not expressing TEL2. In addition, 3 of the mice had a significantly elevated WBC count (30×10^9/L to 50×10^9/L [$30 \times 10^3/\mu$L to $50 \times 10^3/\mu$L]) in the peripheral blood. These mice had an abundance of poorly differentiated dysplastic GFP-positive (50% to 70%) cells in the peripheral blood (Figure 6E), which were not T cells because only a few of them stained positive for CD3 (Figure 6F). The BM of these mice as well as of mice that had normal peripheral WBCs did show a high percentage of Mac1$^+$ (31% to 72%), Sca1$^+$ (25% to 30%), and GFP-positive (50% to 70%) cells, but no cells were positive for CD3, CD4, or CD8 T-cell markers. Therefore, the mice also suffered from a myeloid disease that in 30% of the mice gave elevated WBCs. Two of the 5 MSCV-BM-ENU control mice died of solid tumors, whereas the remaining 3 died of unknown causes. However, no controls died of a hematopoietic malignancy because their peripheral blood counts remained normal and the percentage of GFP-positive cells remained constant.

Discussion

TEL2, a new member of the ETS TFs, was identified by several groups a few years ago.[9,19,28] The protein was coined TEL2 because of its high homology with TEL (38.2% identity). However, despite this homology, its function appears to be quite distinct from that of TEL. TEL has an antiproliferative effect[26,27,56] whereas TEL2 has a mostly proliferative effect,[27,29] although a direct assessment of the tumorigenic activity of TEL2 alone has not been determined. Our in vitro LTC-IC studies strongly suggested that TEL2 stimulated the growth of primitive progenitors, provided they are in contact with a feeder layer, a condition that mimics the bone marrow environment. This effect on myeloid cells is different from TEL2's effect on B-cell progenitors, whose growth is stimulated in the absence of feeder cells.[27]

TEL2 is specifically expressed in the human hematopoietic system, and its expression is up-regulated in some adult human leukemias[29] and more than 30% of pediatric B-ALL.[30] TEL2 also accelerated B lymphomagenesis in Eμ-Myc mice, a model for Burkitt lymphoma, and possibly cooperates with N-MYC/C-MYC in pediatric B-cell lymphoma.[30] Therefore, we tested whether enforced TEL2 expression alone in mouse bone marrow predisposed the animals to hematopoietic malignancy. Our experiments indeed verified the oncogenicity of TEL2 in this setting, but the long latency of disease indicated that secondary mutations are necessary for disease development.

The disease was not transplantable in secondary recipients, although the proliferation of the myeloid cells appeared strictly cell autonomous given the tight correlation between GFP expression and the number of leukemic cells (Figure 1E). This indicated that

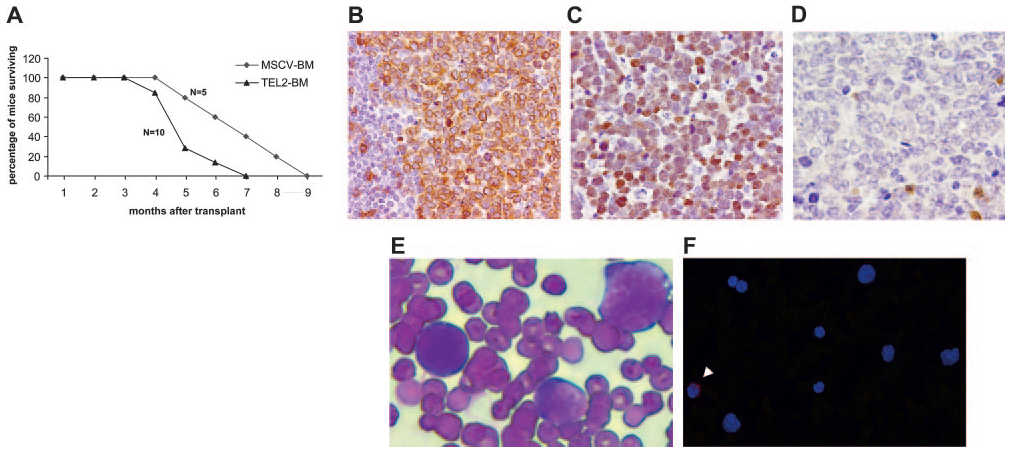

Figure 6. ENU treatment of mice receiving TEL2-BM transplants accelerates hematopoietic disease and causes T-cell lymphoma. (A) Kaplan-Meier survival curve of mice receiving transplants with bone marrow transduced with MSCV-IRES-GFP (MSCV-BM) or MSCV-TEL2-IRES-GFP (TEL2-BM) and treated with ENU 5 weeks after transplantation The mice receiving TEL2-BM transplants died 1.5 to 2 months earlier than the MSCV-BM mice, suggesting cooperation between TEL2 expression and ENU mutagenesis. (B) Spleen of a mouse with T-lymphoblastic lymphoma that underwent ENU treatment and TEL2-BM transplantation. The malignant lymphocytes in the red pulp express CD3 (brown color) while most of the lymphocytes associated with the follicular marginal zone do not express CD3 (anti-CD3 with cytoplasm/membrane localization, magnification × 40, marginal zone is on the left and the red pulp cells with CD3 expression are on the right). (C) The CD3$^+$ malignant lymphocytes also expressed TdT (brown color), which is consistent with a lymphoblastic lymphoma (anti-TdT with nuclear localization, magnification × 40). (D) The T lymphocytes in the spleen do not stain with a GFP antibody; the only cells that are positive for GFP (bottom right corner) are B lymphocytes, which also stain positive for B220 (not shown). (E) Peripheral blood smear of a TEL2-BM/ENU mouse with increased WBCs (30×10^9/L [$30 \times 10^3/\mu$L]) showing numerous dysplastic white cells (magnification × 100). (F) Blood smear of the same mouse stained with a CD3 antibody. Only a small percentage of cells (white arrowhead, red color) expressed this T-cell marker, indicating that the dysplastic cells are not of T-cell origin. The nucleated cells were counterstained with DAPI (blue color).

progenitor cells expressing TEL2 were not immortalized and that the conditions in the reconstituted bone marrow were unable to support reconstitution by the MPD. Similar observations have been reported for MPD caused by NUP98-Hox A9.[57] Annexin V analysis of TEL2-expressing bone marrow after a short period (1 week) of in vitro culture showed that TEL2 suppressed the apoptotic index of these cells as it does inhibit apoptosis in Myc-overexpressing or wild-type B-cell progenitors.[30] Given the large excess of myeloid progenitors in the BM and the outcome of the Affimetrix array analysis of Lin$^-$ cells 72 hours after transduction with TEL2 or vector retrovirus suggested that TEL2 also inhibits apoptosis of myeloid progenitors. Several proapoptotic genes were repressed, such as *Trp53inp1, Stk17b, Dapk2,* and *Bmf,* which might mediate this effect. In addition, 4 of 6 mice analyzed for Bcl2 or Bcl-xl expression in myeloid cells, which invaded the spleen, showed up-regulation of Bcl2, Bcl-xl and Bcl-xs. Similar to the effect of TEL2 in B cells,[30] up-regulation of Bcl2 and Bcl-x is probably not at the transcriptional level, because *Bcl2* and *Bclx* mRNA was not increased in the Affimetrix arrays of TEL2-transduced bone marrow. We believe that Bcl2 and Bcl-x overexpression contributes to expansion of the myeloid cells, but currently we do not know which events substitute for Bcl2 and Bcl-x overexpression in the 2 tumor samples that did not overexpress these proteins. However, changes in expression of genes inhibiting *(Btg1, Btg2, Cdkn1B, Rb1cc1)* or stimulating *(cKit)* proliferation could have an additive effect on the enlargement of the myeloid progenitor pool. It remains to be determined whether any of these genes are direct transcriptional targets of TEL2, an issue that we will address in future studies.

It is reasonable to speculate that myeloid progenitors in the bone marrow of mice receiving transplants have a reduced apoptotic rate, but this does not lead to increased numbers of myeloid cells in the peripheral blood during the first 6 months after transplantation, a feature also apparent in mice expressing MLL fusion oncogenes and AML1-ETO.[58,59] It is possible that compensatory mechanisms control the number of myeloid cells entering the periphery and that additional genetic changes, possibly those that affect cell proliferation, may override this check, resulting in accumulation of differentiating myeloid cells in the peripheral blood. In addition, expansion of the myeloid compartment during the first 6 months after transplantation would increase the frequency of secondary mutations and thereby promote development of disease. The extensive differentiation of the cells is the likely cause of the chronic character of the disease, which after initial detection took 2 to 3 months to kill the animal. The differentiation of the malignant cells in affected animals seems in conflict with the observation that TEL2 inhibits differentiation of HL60 and U937 myeloid cells.[29] The reason for this is unknown but, unlike bone marrow, cell lines harbor many mutations, which might account for this difference in behavior.

Given the development of myeloproliferative disease in mice receiving transplants with TEL2, we expected that ENU mutagenesis would induce myeloid leukemia. This occurs in mice expressing RUNX1-ETO or CBFβ-MYH11,[59,60] indicating that these fusion transcription factors specifically affect myeloid cells despite the fact that conditional RUNX1-ETO knock-in mice[59] also express the fusion protein in their T cells.[61] Our experiments indicate that in combination with ENU all TEL2-BM mice developed distinct myeloid aberrations in the bone marrow within 3 to 6 months after ENU treatment, which is much more rapid than TEL2-BM mice only (Figure 1G). However, only 3 mice showed elevated numbers of dysplastic cells in the peripheral blood, indicating a possible development of myeloid leukemia. Surprisingly, all mice also developed GFP-negative T-cell lymphoma. We must conclude that changes caused by

TEL2 expression promoted T-cell lymphomagenesis, because none of the control ENU-treated mice developed T-cell lymphoma. It is possible that T-cell proliferation is stimulated indirectly by TEL2-expressing hematopoietic cells, which could contribute to increased genetic instability, making these cells more susceptible to ENU-mediated leukemic transformation. The lethal lymphoid disease might have prevented the myeloid preleukemic state of these mice to develop into AML.

Together our results suggest that TEL2 is a hematopoietic oncogene in the mouse, and its overexpression in some adult and 30% of pediatric ALL samples[29,30] also argues for a causative role of TEL2 in human leukemogenesis.

Acknowledgments

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Chapter 3

The ETS factor TEL2 activates mTOR in mouse B lymphoid cells and causes B cell lymphoma when expressed in Arf-null bone marrow

The ETS factor TEL2 activates mTor in mouse B lymphoid cells and causes B cell lymphoma when expressed in Arf-null bone marrow.

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Running title: TEL2 activates mTOR in B cell leukemia.

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Summary

TEL2, a member of the ETS family of transcription factors, is mainly expressed in the human hematopoietic system and can interact with its relative TEL, a frequent target of recurrent chromosomal translocation associated with hematopoietic malignancies. Previously we showed that TEL2 has oncogenic activity. Its overexpression in mouse hematopoietic cells accelerated cell cycle traverse and reduced apoptosis causing a myeloproliferative disease when these cells were transplanted into lethally irradiated mice. TEL2 overexpression also cooperated with c-Myc in a mouse model of Burkitt's lymphoma. Indeed, combined overexpression of *TEL2* and *MYC* was found in over 30% of samples of pediatric patients suffering from acute lymphocytic leukemia (ALL).

Here we investigated the oncogenic cooperation between TEL2 and the Arf/Mdm2/p53 tumor suppressor pathway. Forced expression of TEL2 in $Arf^{-/-}$ mouse bone marrow caused expansion of a B cell population predisposing mice to ALL bearing features of human B-ALL. We show for the first time that this disease is in part caused by TEL2's ability to directly upregulate transcription of the *mTor* gene (target of rapamycin) via a canonical TEL binding site in its promoter. mTor encodes a central regulator of cell growth and apoptosis. However, treatment of $Arf^{-/-}$/TEL2 cells with the mTor inhibitor rapamycin only partially attenuated their growth in vitro, suggesting that other proliferative effects of TEL2 also contribute to growth of these cells. These results suggest that ALL patients overexpressing TEL2 might identify a sub-group of patients that might respond less favorably to treatment with rapamycin or its analogues.

Introduction

The ETS (E26- Transformation Specific) family of transcription factors (TFs) is involved in the regulation of a large number of genes, some of which are expressed ubiquitously while others are expressed in a tissue-specific fashion (Graves and Petersen, 1998; Oikawa and Yamada, 2003). All of these proteins share a highly conserved ETS DNA binding domain of approximately 85 amino-acid (aa) that binds the purine-rich GGAA/T core motif (EBS; ETS Binding Sequence). This sequence is present in the regulatory regions of more than 200 target genes involved in fundamental cellular process (Graves and Petersen, 1998; Janknecht and Nordheim, 1993). In addition, a subset of ETS TFs share a conserved Pointed (PNT) protein-protein interaction domain, which confers the ability to form homodimers/oligomers (Lacronique et al., 1997) (Kim et al., 2001), and heterodimers (Baker et al., 2001), and might abolish activation of gene transcription (Fenrick et al., 1999). A number of ETS proteins are essential for proper development of the hematopoietic system itself (Bassuk and Leiden, 1997), some of which exert their function in a lineage-specific manner. For example: PU1 plays an important role in lineage development and is essential for the generation of myeloid cells and B cells (McKercher et al., 1996) (Scott et al., 1994). Absence of PU1 causes a block in differentiation of the hematopoietic stem cell into the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) (Akashi et al., 2000; Iwasaki et al., 2005). Also, Fli1 is involved in megakaryopoiesis and loss of this gene in mouse embryos results in absence of megakaryocytes (Hart et al., 2000; Kawada et al., 2001). TEL/ETV6 is widely expressed in embryonic and adult tissues and is necessary for normal embryonic angiogenesis and maintenance of the adult bone marrow microenviroment (Scott et al., 1994; Wang et al., 1997; Yamamoto et al., 1998). TEL is a nuclear phosphoprotein, which acts as a sequence-specific transcriptional repressor in transient transcription experiments (Lopez et al., 1999) (Fenrick et al., 1999), and the gene is a frequent target of chromosome translocations in human hematopoietic malignancies (Golub et al., 1997) and in some solid tumors (Knezevich et al., 1998; Tognon et al., 2002). The resulting chimeric genes encode fusion proteins often containing the amino-terminal PNT domain of TEL fused to different fusion partners such as the protein tyrosine kinases ABL, JAK2, or the PDGFβ-R (Golub, 1997; Poirel et al., 1998; Rubnitz et al., 1999; Tosi et al., 1998), or to transcription factors such as RUNX1 and EVI1 (Golub, 1997; Nucifora, 1997). In addition, TEL might have a tumor suppressor function because in t(12;21) childhood pre B-ALL one allele of TEL is involved in a translocation with AML1, while the second allele is often deleted during the course of the disease (Fenrick et al., 2000; Golub et al., 1997; Rompaey et al., 2000).

We and others have identified a novel ETS gene highly related to TEL-1, named TEL-2/ETV7 (Gu et al., 2001; Poirel et al., 2000; Potter et al., 2000). A TEL-2 transcript of 1,55 Kb is predominantly present in human hematopoietic tissues both during

development and adult life (Potter et al., 2000). TEL2 contains a PNT domain and an ETS DNA binding domain and localizes to the nucleus, excluding the nucleoli. Interestingly, TEL2 is able to self-associate but can also form hetero-dimers with TEL1, suggesting that these proteins might affect each other's function *in vivo* (Kawagoe et al., 2004; Potter et al., 2000). In spite of their similarity in sequence and structure, TEL1 and TEL2 show major differences in biological activity. For example TEL suppresses Ras-induced transformation of NIH3T3 fibroblasts *in vitro* (Van Rompaey et al., 1999), while TEL2 promotes transformation (Kawagoe et al., 2004). Forced expression of TEL2 but not TEL1 inhibits vitamin-D3-induced differentiation of U937 cells and endogenous TEL2 but not TEL1 is downregulated during differentiation of U937 cells (Kawagoe et al., 2004). TEL2 might have oncogenic potential because it is overexpressed in some adult leukemia patient samples (Kawagoe et al., 2004) and in more than 30% of pediatric ALL patients (Cardone et al., 2005). TEL2 inhibits apoptosis in murine bone marrow and pre-B cells cultured *in vitro* and cooperates with Myc in murine B-lymphomagenesis (Cardone et al., 2005). Finally, forced TEL2 expression in normal murine bone marrow leads to a myeloproliferative disease, which is characterized by a long latency period (Carella et al., 2005). Our previous work suggested that secondary mutations accelerate the onset of disease. To further test this hypothesis we expressed TEL2 in bone marrow isolated from p19Arf$^{-/-}$ mice (Kamijo et al., 1997), which mostly develop sarcomas and T-cell lymphomas (Kamijo et al., 1997). Here we show cooperation of TEL2 with inactivation of the p53 pathway. After transplantation into congenic recipient animals TEL2/Arf-/- BM cells cause B-cell lymphomas in 100% of cases, with all tumors expressing increased levels of c-Myc.

We provide evidence that resistance to apoptosis and increased cell cycle traverse of pre-B cells overexpressing TEL2 is in part caused by direct upregulation of the master regulator of cell metabolism, *mTOR*, through binding of TEL2 to the *mTOR* promoter region.

Results

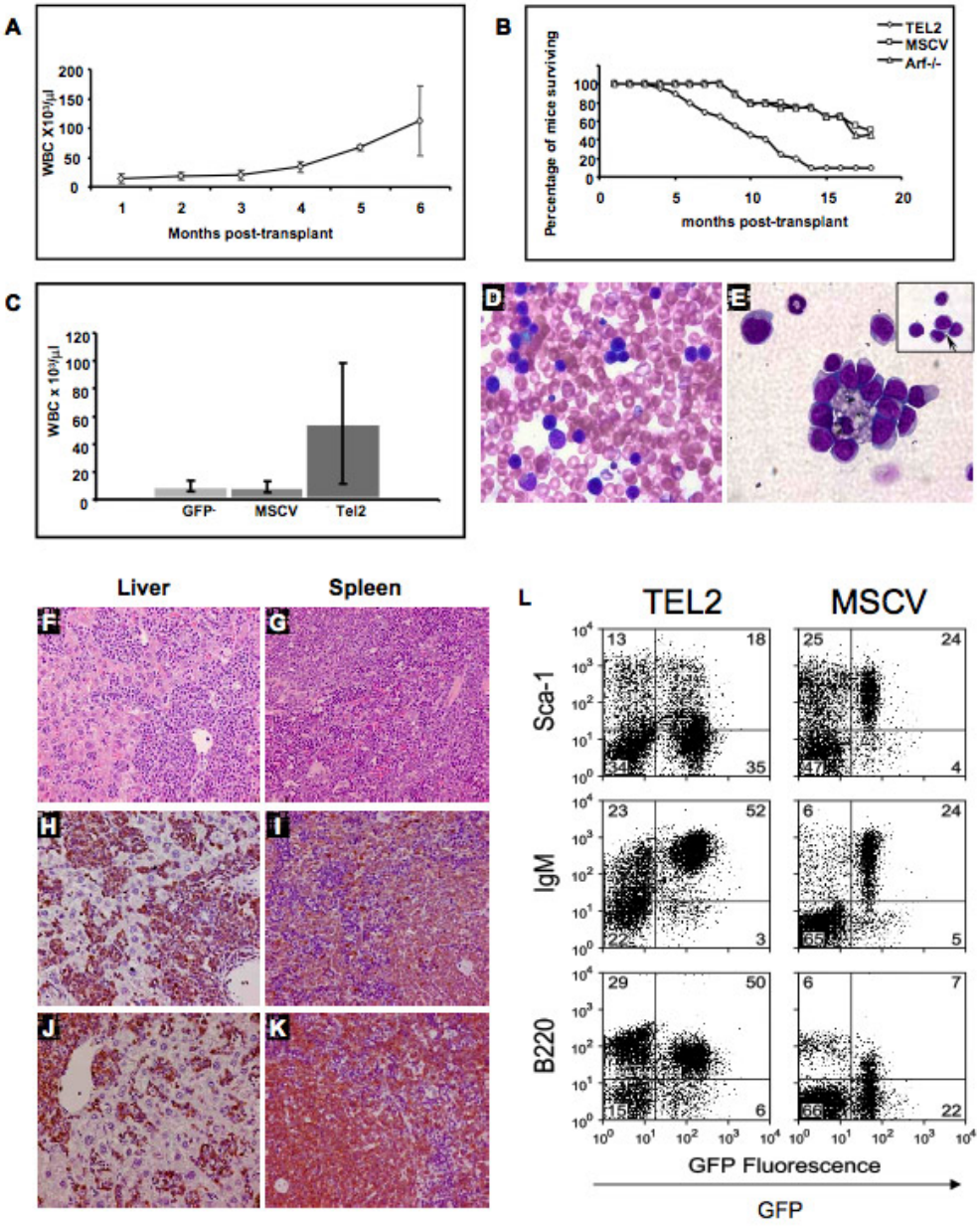
Expression of TEL2 in Arf$^{-/-}$ bone marrow causes B-cell leukemia in transplanted mice.

Overexpression of TEL2 in the mouse hematopoietic compartment leads to a proliferative advantage of selected cell populations due to reduced apoptosis and accelerated cell cycle traverse (Cardone et al., 2005; Carella et al., 2005). Because TEL2 cooperates with c-Myc in lymphomagenesis and c-Myc cooperates with inactivation of the tumor suppressor Arf (Kamijo et al., 1997), we tested whether TEL2 also cooperated with inactivation of Arf. Therefore we transplanted 2 cohorts of lethally irradiated recipients (n=18) with Arf$^{-/-}$ BM or with Arf$^{-/-}$ BM transduced with MSCV-IRES-GFP or MSCV-TEL2-IRES-GFP retrovirus. The CBCs, bloodsmears, and analysis of the percentage of GFP^{+} cells in the peripheral blood (PB) of transplanted mice were determined monthly and 2-3 months later we observed a consistent increase in the WBC counts (Fig 1A), due to increasing numbers of B lymphocytes (not shown). In 16 out of 18 primary recipients lethal disease developed within a period of 4-14 months post-transplant (medium survival: 9 months, Fig 1B). The remaining 2

Figure 1: TEL2 expression in p19$^{Arf-/-}$ BM causes B-cell lymphoma in mice.

A) Average monthly WBC counts in PB of mice (n=18) transplanted with Arf$^{-/-}$/TEL2 BM showing a steady increase during the first 6 months post-transplantation. Error bars show the variation in WBCs between individual mice at each data point. **B**) Combined Kaplan-Mayer survival plots of mice (n=18) transplanted with Arf$^{-/-}$ BM (GFP^{-}), or Arf$^{-/-}$ BM transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP retrovirus (MSCV). The 2 mice transplanted with MSCV-TEL2-IRES-GFP BM that were healthy at 14 months after transplantation represent 2 animals in which the transplant did not take and their BM and peripheral blood did not express GFP. **C**) WBC counts performed at the time of death of mice transplanted with Arf$^{-/-}$ BM (GFP^{-}), or Arf$^{-/-}$ BM transduced with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP. Error bars show the the variation in cell counts between individual mice receiving the same transplant. **D-E**) May-Grunwald-Giemsa (MGG) staining of a PB smear (D, 40X) and a BM cytospin (E, 60X) of a moribund mouse transplanted with Arf-/-/TEL2 BM, showing the presence of lymphoid blast cells. **F**), **H**), **J**), Liver sections of a diseased mouse transplanted with Arf$^{-/-}$/TEL2 BM, stained with H&E (F), anti-GFP(H) and anti-B220 (J) antibody (20x), respectively, revealing GFP+ (brown stain, H), CD45R/B220 (brown stain, J) leukemic cells infiltrating the portal tracts and sinusoids of the liver. **G**), **I**), **K**), Spleen sections showing the splenic red pulp and follicles. The H&E staining (G) shows the massive infiltration of dark staining cells that express GFP (brown stain in I) and the B lymphoid marker CD45R/B220 (brown stain, K). **L**) Flow cytometric analysis of cell surface markers of bone marrow cells of a diseased Arf$^{-/-}$/TEL2-BM transplanted mouse (TEL2) compared with BM of a mouse simultaneously transplanted with Arf$^{-/-}$/MSCV (MSCV) BM, confirming the B-cells lineage derivation of the malignant cells in the diseased mouse. The Y-axes indicate expression of different cell surface marker plotted against the expression of GFP on the X-axis. Most of the neoplastic cells expressed the pan-B cell marker B220 and IgM, indicating a more mature population of B-lymphocytes.

animals survived without developing disease but their PB did not express GFP, indicating that transplantation of the $Arf^{-/-}$/TEL2 bone marrow had failed. Compared to mice transplanted with $Arf^{-/-}$ BM or $Arf^{-/-}$/vector BM, $Arf^{-/-}$/TEL2 BM-transplanted mice showed an accelerated onset of disease. When moribund, these mice presented with peripheral blood leukocyte counts between 2-10.2x10^7/ml (Fig1C) with blast cells in the



PB and BM (Fig 1D-E). All mice showed massive hepatomegaly (Fig1F), splenomegaly (Fig1G), bone marrow invasion, and frequent lymphoadenopathy. Histopatology confirmed large numbers of GFP^+ leukemic cells in the bone marrow, liver (Fig 1H), spleen (Fig 1I), kidney, and to a lesser extent also in the lymphnodes, lungs, and meninges (data not shown). Immunohistochemical staining showed that these cells in the different tissues stained negative for TdT, MPO and CD3, but positive for CD45, IgM (data not shown), and B220 (Fig J-K). Additional FACS cell surface marker analysis of the neoplastic cells in bone marrow, spleen and liver showed that the predominant GFP^+ population was $Sca\text{-}1^+$, $B220^+$ and IgM^+ (Fig 1L), with some of the sick animals also containing a minor population of GFP^+ myeloid cells expressing c-Kit, Thy1.2, Gr-1 and Mac-1 (data not shown). We concluded that the mice suffered form disseminating B-cell lymphoma and that forced expression of TEL2 in $Arf^{-/-}$ bone marrow cells not only accelerated disease onset but also changed the type of disease from mostly T cell lymphoma in $Arf^{-/-}$ mice to B cell lymphoma in mice transplanted with $Arf^{-/-}$/TEL2 bone marrow.

TEL2/$Arf^{-/-}$ B-cell leukemia is transplantable

We next tested whether the B cell lympho-leukemia was transplantable and injected $1x10^6$ bone marrow cells (80-90% GFP+) of 4 diseased primary recipients each into 4 sub-lethally irradiated secondary recipients (16 mice in total). All mice died within 4-7 weeks post-transplant (Fig 2A) of fulminant disease. The leukocyte count in the PB reached values $>15x10^7$/ml (Fig 2B), with all cells expressing high levels of GFP (data not shown). All animals showed massive numbers of neoplastic cells in the spleen, liver, kidney, lungs, heart, lymphnodes, thyroid, and brain (data not shown). Surface marker analysis of cells in the bone marrow, spleen, and liver showed a uniform population of B lymphocytes positive for Sca-1, B220, IgM, CD43, but negative for BP1 (data not shown).

We also analyzed the identity of the antibody light chain within the GFP^+ population and found that some cells expressed □-chain whereas others expressed κ-chain, indicating an at least oligoclonal origin of the transformed cells (data not shown).

$Arf^{-/-}$/TEL2 tumor cells show chromosomal aberrations, amplification of *c-Myc, and increased c-Myc expression.*

We analyzed the karyotypic abnormalities present in malignant $Arf^{-/-}$/TEL2 cells in the bone marrow, spleen, and liver of 4 primary, and 2 secondary recipients. All samples showed structural chromosomal abnormalities, but numerical aberrations were more frequent. Interestingly, between 5%-60% of cells in all samples (average 30%) showed triplication of chromosome 15 (Fig 2C), a predominant feature present in spontaneous and carcinogen-induced murine B-cells lymphomas (Wiener et al., 1981)

that also display a Thy1^{+}/B220^{+} phenotype (Vasmel et al., 1989). Because many genes involved in B-cell proliferation (such as the *IL-7R*, the *Ly6* family of genes, and *c-Myc*)

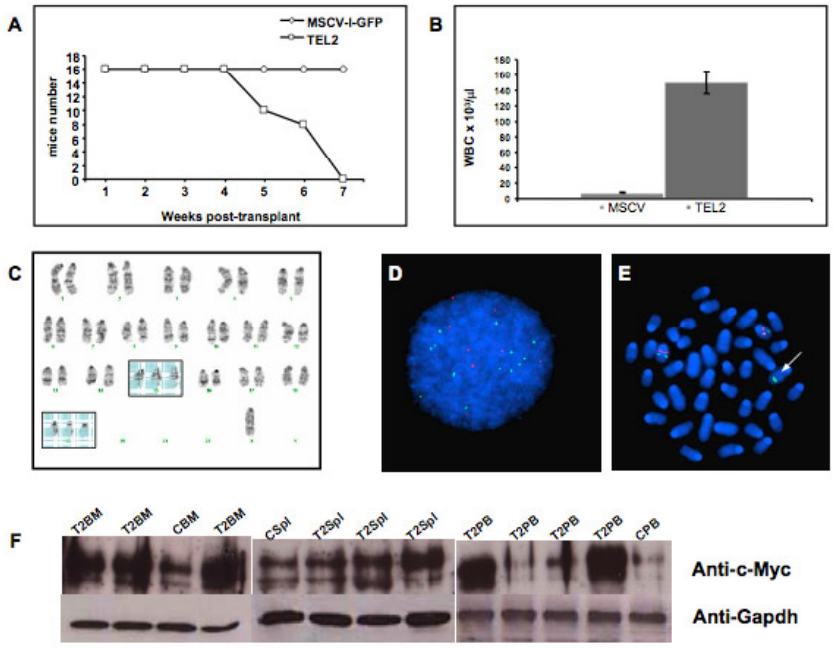


Figure 2: Arf-/-/TEL2-induced leukemia is transplantable and overexpresses c-Myc.

A) Combined Kaplan-Mayer survival plots of secondary recipients transplanted with leukemic Arf-/-/TEL2 BM (TEL2) or healthy Arf-/-/MSCV BM (MSCV-I-GFP) (n=16). Compared with the development of the primary lympho-leukemia, secondary recipients of leukemic BM showed a highly accelerated mortality rate and died between 4 and 7 weeks after transplantation. Recipients of control BM did not develop disease. **B**) WBC counts of moribund secondary recipients after receiving leukemic Arf-/- /TEL2 BM (TEL2) or of healthy recipients after receiving Arf-/- /MSCV BM killed at the same time (MSCV). Error bars show the variation in WBC counts between different mice within each group. **C**) Karyotype analysis of cells isolated from a diseased mouse, which received a transplant of leukemic Arf-/-/TEL2 BM. All such mice displayed chromosomal instability. In this particular case there is a triplication of chromosome 15, a common feature of these mice, but also a triplication of chromosome 19 and loss of the Y chromosome. **D**) Fluorescent In Situ Hybridization (FISH) of leukemic BM cells in interphase using a c-Myc BAC DNA (green signal) and a chromosome 15 specific BAC DNA (red signal) as probes. The abundance of green signals indicates amplification of the c-Myc gene in the leukemic cells. The chromosomes were counterstained with DAPI (blue). **E**) FISH analysis of BM cells of one of the diseased mice in which a triplication of the c-Myc gene (green signals) was present in 30% of the cells with one of the signals not associated with chromosome 15 (red signals). In this mouse the third c-Myc copy is present on the chromosome 12. A t(12;15) is a common event in mouse B-cell leukemia. **F**) Western blots of total protein extracts prepared from BM, spleen (spl) and PB WBC (PB) of Arf-/- mice transplanted with MSCV-TEL2-IRES-GFP- (T2) or MSCV-IRES-GFP-transduced BM , showing over-expression of c-Myc protein in almost all leukemic samples. As a loading control the detection of GAPDH is shown.

are located on chromosome 15, triplication of this chromosome might directly contribute to the tumor phenotype. We performed fluorescent in situ hybridization (FISH) of malignant interphase and metaphase cells of bone marrow, spleen, and liver, using a mouse c-Myc BAC probe and a chromosome 15-specific BAC probe, which localizes to band A2. This allowed us to assess whether tumor cells contained *c-Myc* translocations/amplifications and whether the *c-Myc* copy number coincided with the chromosome 15 ploidy. The results confirmed the initial karyotype analysis with *c-Myc* signals present on each of the three chromosomes 15 in 30-85% of the cells, while in 4 out of 6 samples 2-30% of the cells also showed other abnormalities involving *c-Myc*. Some cells showed 3 signals for *c-Myc* but only 2 signals for the chromosome 15 probe (Fig. 2D), while the remainder of the cells contained an aneuploid number of signals for both probes with the number of *c-Myc* signals always higher than those of chromosome 15 (Fig. 2E). This indicated an increased *c-Myc* copy number due to triplication of chromosome 15 and additional translocation events. Western blot analysis of protein extracts of bone marrow, spleen, or peripheral blood of a number of diseased animals showed overexpression of c-Myc as compared to MSCV-transplanted controls (Fig. 2F).

Other frequent chromosomal abnormalities in these tumor cells included trisomy chromosome 7, and the presence of a 1q+ chromosome, while many other, less common, abnormalities were also present (not shown).

Forced TEL2 expression accelerates cell cycle traverse and reduces the apoptotic rate of B-lymphocytes cultured *in vitro*.

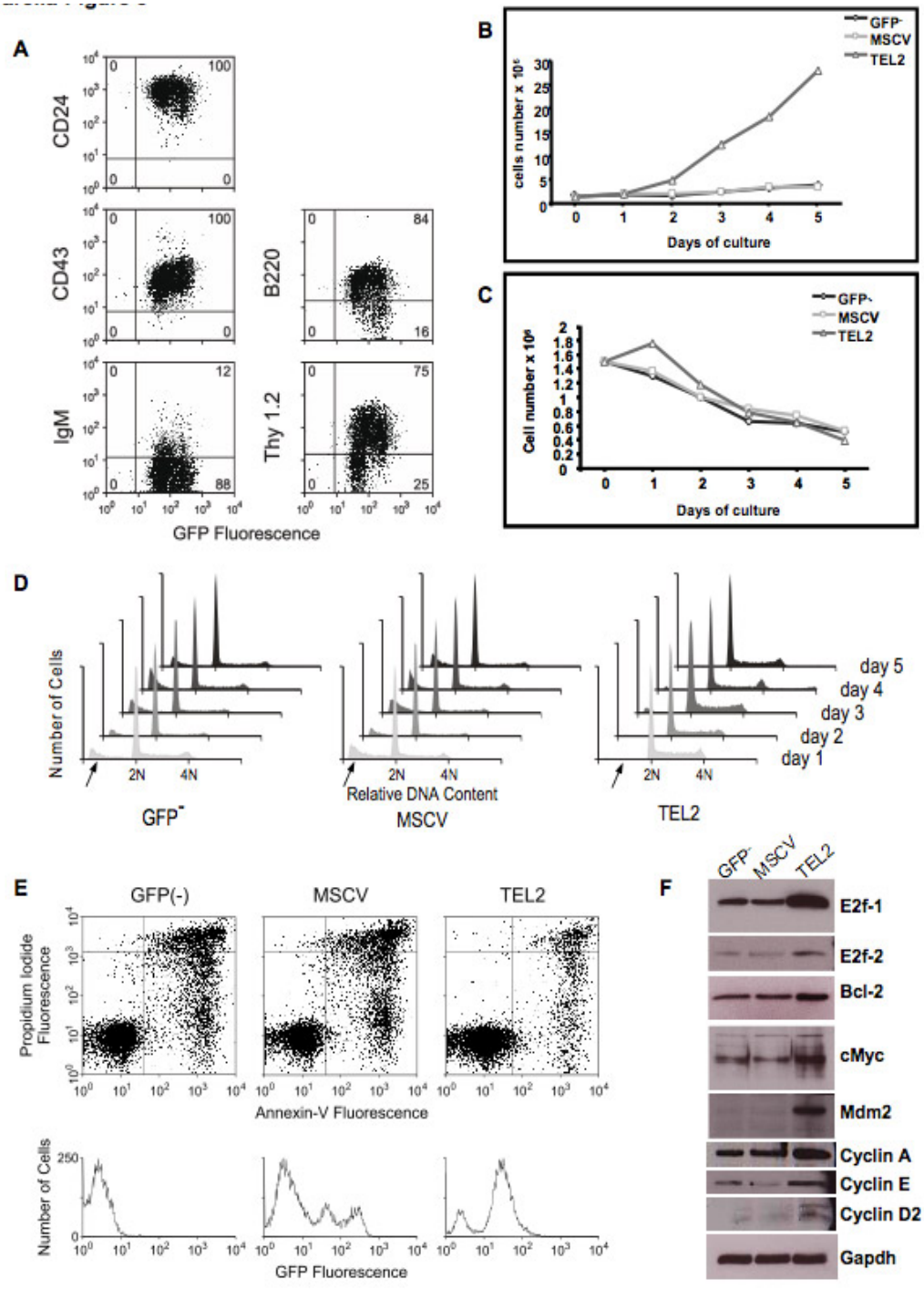
The long latency period necessary for TEL2 to induce disease in an p19Arf$^{-/-}$ background or an Eμ-Myc transgenic background (Cardone et al., 2005) suggested that yet additional events must occur for disease to emerge. To investigate which changes in growth characteristics TEL2 might impinge upon Arf$^{-/-}$ B220^{+} cells, we studied their altered growth characteristics *in vitro*. FACS cell surface marker analysis of retrovirus transduced cells cultured for 5 days on S-17 stroma cells in presence of IL-7 (Henderson and Dorshkind, 1990; Henderson et al., 1990) confirmed that >90% of cells expressed the B-lymphoid markers B-220, CD43, and CD24, but were mostly negative for IgM (Fig3A). In addition, the cells were Thy1.2^{+} but did not express CD3, CD4, and CD8, identifying these cells as pre-pro-B and early pro-B lymphocytes (Hathcock et al., 1992) ; Li et al. 1993 ; (Li et al., 1996) (Allman et al., 1999; Hardy et al., 1991; Li et al., 1993; Wells et al., 1994). In some of the diseased mice high Thy1.2 expression of was also a feature of malignant Arf$^{-/-}$/TEL2 B lymphocytes. After sorting, the GFP^{+} population was cultured on plastic or on S-17 stromal layers in the presence of IL-7. Irrespective of the presence of the S-17 feeders the TEL2-expressing cultures contained up to 10-fold more cells than MSCV-I-GFP vector transduced or non-transduced Arf$^{-/-}$ pre-B cell cultures within 5 days of culture (Fig 3B). However, the Arf$^{-/-}$

/TEL2 cells remained strictly IL-7 dependent, as withdrawal of this growth factor led to demise of the culture (Fig 3C). These data further confirmed that $Arf^{-/-}$/TEL2 B cells need additional mutations to become fully transformed.

To determine what caused the increased proliferation of $Arf^{-/-}$/TEL2 cells we determined their cell cycle profile and their rate of apoptosis using flow cytometry. As shown in Fig 3D, 5 consecutive days of cell cycle analysis showed that $Arf^{-/-}$/TEL2 cultures contained twice as many cells in S- (45-55%) and G2/M phase (5-10%) of the cell cycle than control cells (15-25% S-phase; 3-5% G2/M phase), indicating an accelerated cell cycle traverse. In addition, the amount of fragmented DNA, derived from apoptotic cells in $Arf^{-/-}$/TEL2 cultures was much less abundant than in cultures of control cells. This difference in apoptotic rate between the 3 cultures was confirmed by Annexin V staining showing that the number of dying and dead cells in the $Arf^{-/-}$/TEL2 pro B cell cultures (Fig. 3E, right panel) was much lower than in the control pro-B cell cultures (Fig. 3E, left and middle panel). Thus, TEL2 targets both cell cycle and apoptotic regulators causing the increased growth rate of $Arf^{-/-}$/TEL2 pro B cells.

Protein expression pattern in TEL2 expressing B-lymphocytes

To canvass proteins involved in these responses we performed Western blot analysis of cell lysates of pro-B cells 5 days after $Arf^{-/-}$ bone marrow had been transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (MSCV). Flow cytometry confirmed that the cells expressed B cell markers and lysates of sorted GFP^{+} cells were compared with that of non-transduced cells grown under the same conditions. Compared to $Arf^{-/-}$ or $Arf^{-/-}$/MSCV control pro-B cells, $Arf^{-/-}$/TEL2 cells expressed increased amounts of the cell cycle regulators cyclin D2, cyclin A, cyclin E, E2F1, E2F2 and cMyc (Fig. 3F). These cells also expressed more of the apoptotic regulator Bcl2 (Fig 3F), but not Bcl-X_L (not shown). Myc potently inhibits expression of Bcl2 and Bcl-X_L an effect apparently counteracted by TEL2, which might contribute to B lymphomagenesis (Eischen et al., 2001). Thus, the increased expression of these proteins might be directly responsible for the increased growth rate of $Arf^{-/-}$/TEL2 cells. Surprisingly, Mdm2 expression was also upregulated (Fig 3F), an effect not seen in wild type TEL2 expressing pro-B cells (Cardone et al., 2005). Other critical cell cycle regulators, such as p53, Rb, p21 and p27 did not show altered levels of expression in $Arf^{-/-}$/TEL2 pro-B cells compared to that in control cells (data not shown).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MSCV
TEL2
E2f-1
E2f-2
Bcl-2
cMyc
Mdm2
Cyclin A
Cyclin E
Cyclin D2
Gapdh

Figure 3: Forced TEL2 expression stimulates growth and survival of Arf$^{-/-}$ B cells in vitro.
A) Flow cytometric analysis of cell surface markers present on GFP^{+} Arf$^{-/-}$/TEL2 primary leukemia B cells. After transduction with MSCV-TEL2-IRES-GFP or MSCV-IRES-GFP retrovirus, BM was cultured on S17 feeder cells in the presence of IL-7. After 5 days the population was positive for the pan B cell marker CD45R/B220 and the pro- and pre-B cell marker CD24, The cells did not express surface IgM confirming the immature status of these cells. The Arf$^{-/-}$/TEL2 B cells consitently expressed Thy1.2. **B**) Growth curves comparing the proliferation rate of Arf$^{-/-}$ pro-B cells (GFP^{-}) with that of the same cells transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (MSCV) retrovirus cultured in presence of IL-7 over a period of 5 days. The results shown are representative of three independent experiments. **C)** Comparison of the survival rate of Arf$^{-/-}$ pro-B cells (GFP^{-}) with that of the same cells transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (MSCV) retrovirus after withdawal of IL-7. Although Arf$^{-/-}$/TEL2 cells appear less affected during the first 24 hours of IL-7 withdrawal, they remain strictly growth factor dependent as shown during days 2-5 of culture. **D**) Flow cytometric analysis of the DNA content of Arf$^{-/-}$ pro-B cells (GFP^{-}) and the same cells transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (MSCV) retrovirus during cell cycle progression at 5 consecutive days of culture in presence of IL-7. Arf$^{-/-}$/TEL2 pro-B cells showed a consistent increase of the percentage of cells in the S and G_2/M phases of the cell cycle. The percentage of cells within the G_1, S and G_2/M phases of the cell cycle were determined by analysis of the single-parameter DNA histogram using the computer program ModFit. Black arrows indicate fragmented DNA derived from apoptotic cells present in the control samples, which is absent in the Arf$^{-/-}$/TEL2 sample. **E**) TEL2 expression decreases the rate of apoptosis of Arf-null pro-B cells. The top 3 panels show the fraction of apoptotic cells (upper and lower quadrant of each panel) in Arf$^{-/-}$ pro-B cell cultures (GFP^{-}) and in cultures of the same cells transduced with MSCV-TEL2-IRES-GFP (TEL2) or MSCV-IRES-GFP (MSCV) retrovirus in the presence of IL-7. The rate of apoptosis was measured by the ApoAlert method. Cells were stained with an annexin-V-FITC antibody (X-axis) and propidium iodide (Y-axis) and analyzed by FACS. Bottom panels show the percentage of GFP^{+} cells in each sample. The results are representative of three independent experiments. **F**) Western blot analysis showing overexpression of E2f-1, E2f-2, Bcl2, c-Myc, Mdm2, Cyclin-A, -E, and -D2 in Arf$^{-/-}$/TEL2 pro-B cells (right lanes), compared to expression of these proteins in Arf$^{-/-}$ (left lanes) and Arf$^{-/-}$/MSCV (central lanes) pro-B cells.

TEL2 directly regulates *mTOR* expression

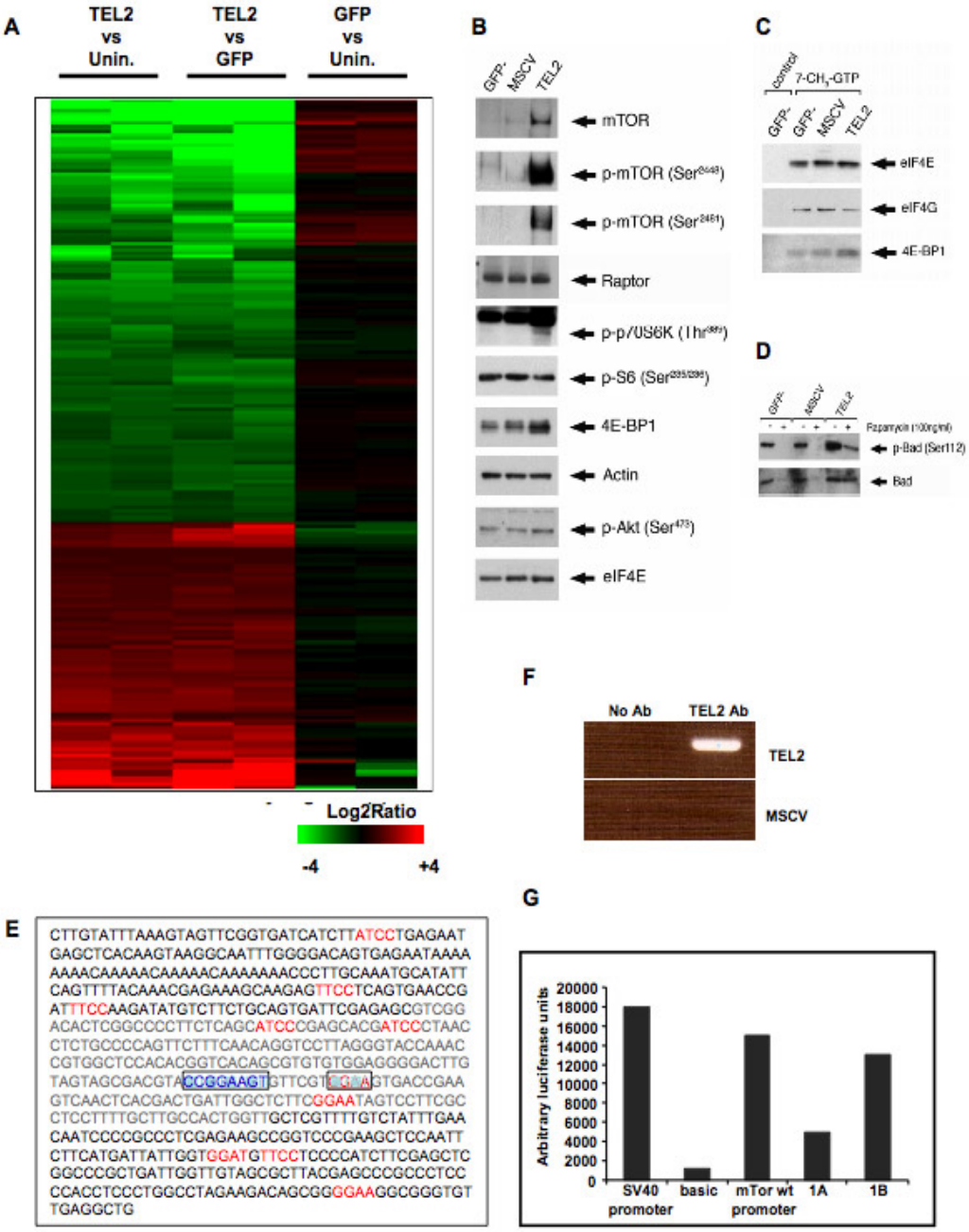
To identify other changes in gene expression that might contribute to the altered growth characteristics of Arf$^{-/-}$/TEL2 pro-B cells, we also performed Affymetrix microarray analysis, using the MOE 430A chip, of the same cells grown on S17 feeders for 1 week after Lin^{-} cell transduction. We adopted a three-way comparison considering only the subset of genes that showed a different expression level in TEL2-transduced versus non-transduced and TEL2-transduced versus MSCV-transduced pro-B cells, but that did not show changes in MSCV versus non-transduced pro-B cells (Fig 4A). This allowed elimination of genes whose expression might be altered due to retroviral transduction rather than to TEL2 expression. In total, we selected 217 genes that showed at least a 2-fold up-, or down-regulation in the TEL2 expressing pro-B cells compared to MSCV transduced cells (see complementary data, Table 1). One of the most intriguing upregulated genes was *Frap1/mTor*, the target of rapamycin

serine/theonine kinase, a member of the phosphoinositide-kinase-related kinases (PIKK) (Bjornsti and Houghton, 2004b). mTor senses mitogenic stimuli, nutrient conditions (Hara et al., 2002; Hardwick et al., 1999; Kim et al., 2002) and ATP (Dennis et al., 2001) and is a central regulator of cell size and proliferation (Dennis et al., 2001), differentiation (Coolican et al., 1997; Erbay and Chen, 2001; Shu et al., 2002), migration (Poon et al., 1996; Sun et al., 2001) and survival (Hosoi et al., 1999; Huang

Figure 4: TEL2 regulates mTOR expression in Arf$^{-/-}$/TEL2 pro-B cells.

A) Microarray analysis showing changes in expression of 217 probesets associated with TEL2 overexpression in Arf$^{-/-}$ pro-B cells. The heat-map compares the relative levels of transcripts present in Arf$^{-/-}$/TEL2 pro-B cells with that in Arf$^{-/-}$ pro-B cells (TEL2 versus Unin.) or Arf$^{-/-}$/MSCV pro-B cells (TEL2 versus MSCV). The differences in expression levels of these 217 probesets have been corrected for differences in their expression in Arf$^{-/-}$/MSCV and Arf$^{-/-}$ pro-B cells (GFP versus Unin.). Log_2Ratio values from the pair-wise comparisons are plotted in the heat-map. **B**) Western blot analyses of total protein extracts of Arf$^{-/-}$ pro-B cells (GFP$^-$), Arf$^{-/-}$/TEL2 pro-B cells (TEL2) and Arf$^{-/-}$/MSCV pro-B cells (MSCV), cultured for 1 week in presence of IL-7, showing the levels of expression in these 3 samples of mTor, its Ser^{2448}- and Ser^{2481}-phosphorylated forms (p-m-TOR), Thr^{389}-phopsphorylated p70S6K (p-p70S6K), 4E-BP1, Raptor, phosphorylated S6 (p-S6, Ser235/236), phosphorylated Akt(p-Akt, Ser473) and eIF4E. **C**) Western blot analysis of active translation complexes containing eIF4E and eIF4G recovered from lysates of Arf$^{-/-}$ pro-B cells (GFP$^-$), Arf$^{-/-}$/TEL2 pro-B cells (TEL2) and Arf$^{-/-}$/MSCV pro-B cells (MSCV) by binding to 7-methyl-GTP-Sepharose beads, Te control shows translation complexes recovered form Arf$^{-/-}$ pro-B cells (GFP$^-$) on standard Sepharose beads. Compared to control samples (GFP$^-$, MSCV) the amount of active translation complexes in Arf$^{-/-}$ /TEL2 pro-B cells is not increased. **D**) Western blot analyses of total protein extracts of Arf$^{-/-}$ pro-B cells (GFP$^-$), Arf$^{-/-}$/TEL2 pro-B cells (TEL2) and Arf$^{-/-}$/MSCV pro-B cells (MSCV) cultured in the presence of IL-7 with or without the mTor inhibitor rapamycin. Compared to controls, TEL2 overexpressing Arf$^{-/-}$ pro-B cells contain increased levels of Bad and p-Bad(Ser112), both in the presence and absence of rapamycin. **E**) Sequence of a 600 bp mTor promoter fragment (Ensembl ID ENSMUSG00000028991) 5' of the cap site. The 10 predicted ETS core binding sites are indicated in red, the canonical TEL binding site CCGGAAGT in blue. We generated 2 mutant promoter fragments; one in which the canonical TEL2 binding site was mutated (1A, first boxed sequence) the second carrying a mutation in the downstream GTGGAAGT sequence (1B, second boxed sequence). **F**) Chromatin Immunoprecipitation Assay (ChIP) of fragmented chromatin of GFP$^+$ Arf$^{-/-}$ pro-B cells transduced with MSCV-IRES-GFP or MSCV-TEL2-IRES-GFP retrovirus, using a TEL2 antibody (TEL2 Ab), followed by PCR amplification of mTOR promoter sequences. The agarose gel shows presence of the mTOR promoter fragment in the TEL2 immunoprecipitate (TEL2 Ab) of cells transduced with MSCV-TEL2-IRES-GFP retrovirus but not in cells transduced with MSCV-IRES-GFP retrovirus. No mTor promoter fragment was detected in a ChIP assay without added TEL2 antibody (NoAb). **G**) Primary Arf$^{-/-}$ /TEL2 pro-B-cells were transfected with luciferase reporters driven by the SV40-promoter (SV40 promoter), no promoter (basic), the wild type 600bp mTOR promoter (WT), the 1A (1A) or the 1B (1B) mutant mTor promoters. Luciferase activity was normalized via co-transfection of an active *Renilla* luciferase reporter. Mutation of the canonical TEL2 binding site in the 1A mutant promoter resulted in significant loss of promoter activity.

et al., 2001; Huang et al., 2003) by controlling cellular functions that include translation initiation, transcription and protein stability (Inoki et al., 2005; Jacinto and Hall, 2003).



The best-studied role of mTOR is its control of translation. First, we verified that upregulated transcription of *mTor* also resulted in increased amounts of mTor protein on western blots. As shown in Fig 4B, $Arf^{-/-}$/TEL2 pro-B cells expressed considerably more mTor than $Arf^{-/-}$ control cells. In addition, using phospho-specific antibodies mTOR also appeared to be activated as it was phosphorylated at both Ser^{2448} (Abraham, 2002; Jacinto and Hall, 2003) and the autophosphorylation site Ser^{2481} (Peterson et al., 2000). However, Raptor, a constituent of the mTor complex (Hara et al., 2002; Kim et al., 2002), and Akt, an upstream effector of mTor phosphorylation (Brazil and Hemmings, 2001; Kandel and Hay, 1999) (Scheid and Woodgett, 2001a; Scheid and Woodgett, 2001b), showed a comparable level of expression in all samples (Fig 4B). We next tested whether 2 important signaling targets of mTor, ribosomal p70S6K1 and 4E-BP1 (Fumagalli and Thomas, 2000) were altered. Ribosomal p70S6K1 controls the synthesis of ribosomal proteins and 4E-BP1 (eIF4E-binding protein 1) controls cap-dependent translation of mRNAs (Bjornsti and Houghton, 2004a; Bjornsti and Houghton, 2004b). P70S6K1 was phosphorylated at Thr^{389}, in $Arf^{-/-}$/TEL2 pro-B cells, indicating that it was active. The amount of 4E-BP1 on the other hand was increased but its phosphorylation level was similar to that in the control $Arf^{-/-}$ pro-B cells (Fig 4B). Surprisingly, phosphorylation of S6 at $Ser^{235/236}$, the downstream target of p70S6K1, was not increased in TEL2 cells, indicating that it was not activated. Also the amount of eIF4E, which is released upon 4E-BP1 phosphorylation, was not altered in $Arf^{-/-}$/TEL2 pro-B cells compared to control cells (Fig. 4B and C). Moreover, binding to 7-methyl-GTP-Sepharose beads of translation complexes containing activated eIF4E (Gingras et al., 1998), followed by Western blot analysis, showed that compared to $Arf^{-/-}$ control cells $Arf^{-/-}$/TEL2 cells neither contained more activated eIF4E, nor of the eIF4E-complexed scaffold protein eIF4G (Fig. 4C). Together these data indicated that these 2 pathways downstream of mTor were not more active in $Arf^{-/-}$/TEL2 pro-B cells than in $Arf^{-/-}$ control pro-B cells. Because p70S6 K1 was activated in $Arf^{-/-}$/TEL2 cells, but not its target S6, we checked the fate of other known p70S6 targets, such as the pro-apoptotic protein Bad (Harada et al., 2001). Bad is inhibited by p70S6K1 through phosphorylation at Ser^{112}. Indeed, blotting with both a Bad and a phospho-specific Bad antibody confirmed that $Arf^{-/-}$/TEL2 pro-B cells contained more Ser^{112}-phosphorylated Bad- than control cells (Fig 4D). This inactivation of Bad might also contribute to the reduced apoptotic rate of $Arf^{-/-}$/TEL2 pro-B cells.

mTor is a transcriptional target of TEL2 in $Arf^{-/-}$ pro-B cells.

Because Akt activity was not increased in $Arf^{-/-}$/TEL2 pro-B cells (Fig. 4B), it can not be responsible for the observed upregulation of mTor. Therefore, we investigated if TEL2 directly regulates mTOR transcription. Sequence analysis of a 600 bp mTor promoter fragment, directly upstream the Cap site revealed the presence of 10 ETS core binding sites (Wasylyk et al., 1993), one of which, CCGGAAGT, represents a canonical TEL binding site (Fig 4E) (Buijs et al., 2000). Given that this site can also

bind TEL2 (Potter et al., 2000) we determined whether TEL2 is bound to this site in Arf$^{-/-}$/TEL2 pro-B cells, using ChIP (chromatin immunoprecipitation) analysis. As shown in Fig. 4F, the central part of the 600 bp promoter fragment (indicated in grey in Fig 4E), which contains the CCGGAAGT site, was enriched in the chromatin immunoprecipitated with a TEL2 antibody. This suggested that TEL2 is associated with the mTOR promoter in Arf$^{-/-}$/TEL2 pro-B cells. We next assessed in transient transcription experiments whether a luciferase reporter gene under the control of the 600bp mTor promoter fragment was responsive to TEL2 expression. We used this mTOR promoter-luciferase reporter, a SV40 promoter-luciferase positive control reporter, and promoter-less negative control reporter to transiently transfect Arf$^{-/-}$/TEL2 pro B-cells. Fig. 4G shows that cells transfected with the wild type mTor promoter-driven *luciferase* reporter showed almost the same luciferase activity as cells transfected with the SV40 promoter driven reporter, whereas the promoter-less reporter generated no luciferase activity. To determine if TEL2 responsiveness was conferred via the CCGGAAGT ETS binding site or possibly also via the adjacent GTGGAAGT ETS site (designated 1A and 1B in Fig 4E, respectively), we also generated reporter plasmids with promoter fragments in which the 1A or the 1B ETS binding sites were mutated. Compared to the wild type promoter fragment, cells transfected with the 1B mutant promoter showed only a slight reduction in luciferase activity, while the 1A mutant promoter showed a 3-fold reduction in luciferase activity (Fig. 4G). Together these data suggested that TEL2 can directly upregulate the mTor promoter, in large part via binding to the 1A TEL site.

Rapamycin attenuates but does not stop proliferation of Arf$^{-/-}$/TEL2 pro-B cells.

To verify whether mTor upregulation contributed to the increased proliferation of Arf$^{-/-}$/TEL2 pro-B cells, we treated these cells, Arf$^{-/-}$, and Arf$^{-/-}$/MSCV control pro-B cells for 3 days with the specific mTor inhibitor rapamycin (Gingras et al., 2001). This treatment completely halted the proliferation of control cells and considerably slowed the growth of Arf$^{-/-}$/TEL2 pro-B cells, as shown by the 3-day growth curve (Fig. 5A) and FACS cell cycle analysis (Fig. 5B). This not only suggested that mTOR upregulation greatly contributed to the proliferation of Arf$^{-/-}$/TEL2 pro-B cells but also that other growth-stimulating effects emanating from TEL2 contribute to their accelerated cell cycle traverse. The cell cycle profiles also indicated that rapamycin induced much less cell death in the Arf$^{-/-}$/TEL2 pro-B cells than in control cells (fragmented DNA peaks in the flow charts, Fig. 5B). This notion was supported by FACS analysis of the three different types of cells stained with Annexin-V at day 0 and day 2 of rapamycin treatment. This showed that Annexin-V^{+} cells in the Arf$^{-/-}$/TEL2 pro-B cells is half of that in control cells both at day 0 and 2 of rapamycin treatment (Fig. 5C).

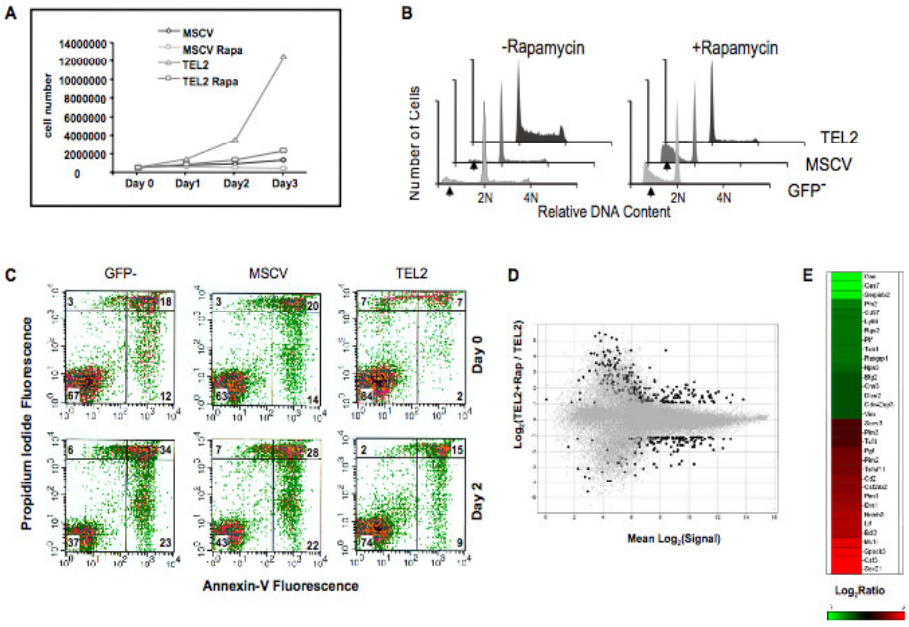


Figure 5:The growth promoting activity of TEL2 in Arf$^{-/-}$ pro-B cells is in part dependent on mTor activation.

A) Growth curves comparing the proliferation rate of Arf$^{-/-}$/TEL2 pro-B cells (TEL2) and Arf$^{-/-}$/MSCV pro-B cells (MSCV) in presence (Rapa) or absence of Rapamycin. Rapamycin greatly reduced but did not stop the proliferation of Arf$^{-/-}$/TEL2 pro-B cells. **B**) Rapamycin treatment induced cell cycle arrest of Arf$^{-/-}$ pro-B cells (GFP^{-}) and Arf$^{-/-}$/MSCV pro-B cells (MSCV) but not of Arf$^{-/-}$/TEL2 pro-B cells (TEL2). Arf$^{-/-}$/TEL2 pro-B cells proliferated slower in the presence of rapamycin as shown by the reduced fraction of cells in the S and G2/M phases of the cell cycle (compare +rapamycin with –rapamycin). Whereas addition of rapamycin induced apoptosis in Arf$^{-/-}$ pro-B cells and Arf$^{-/-}$/MSCV pro-B cells as indicated by the appearance of fragmented DNA (black arrowheads) the drug did not induce noticeable DNA fragmentation in Arf$^{-/-}$/TEL2 pro-B cells. **C**) FACS analysis of cultures of Arf$^{-/-}$ pro-B cells (GFP^{-}), Arf$^{-/-}$/MSCV pro-B cells (MSCV) and Arf$^{-/-}$/TEL2 pro-B cells (TEL2) after staining with an annexin-V-FITC antibody shows a significant increase in dead (upper quadrants) and apoptotic cells (lower right quadrant) in the control cultures (upper and lower left and middle panels) 48 hours after addition of rapamycin, while there is only a marginal increase in dead and apoptotic cells in Arf$^{-/-}$/TEL2 pro-B cell cultures (upper and lower right panels) upon rapamycin treatment. **D**) Microarray analysis showing changes in gene expression in Arf$^{-/-}$/TEL2 pro-B cells treated for 48 hours or not with rapamycin. All differentially expressed genes in this dataset are indicated by filled circles; GCOS analysis >2-fold plus “Change Call” (377 Probesets). **E**) List of differentially expressed genes implicated in cell growth and/or apoptosis in Arf$^{-/-}$/TEL2 pro-B cells in the presence or absense of rapamycin.

Rapamycin-induced differences in gene expression in Arf$^{-/-}$/TEL2 pro-B cells.

To characterize genes responsible for the proliferation of Arf$^{-/-}$/TEL2 pro-B cells in the presence of rapamycin, we performed Affymetrix micro array analysis of the cells grown in the presence or absence of rapamycin (Fig. 5D and E). Rapamycin-treated cells showed down-regulation of anti-proliferative genes like Btg2 (Duriez et al., 2004; Rouault et al., 1996), a member of the B-cell translocation gene family (Btg1,2,3 and Tob1) (Kuo et al., 2003). Among the up-regulated genes there are several that might contribute to the increased proliferation such as the anti-apoptotic gene Bcl2 (Letai et al., 2004; Sanchez-Beato et al., 2003) (Oltersdorf et al., 2005), and the anti-apoptotic and proliferation stimulating genes Pim 1,2 and 3 (Mikkers et al., 2004), the growth factor gene Lif (Estrov et al., 1992) and the growth stimulating gene Notch3 (Jonsson et al., 2001). Currently, we do not know how these genes are upregulated in the presence of rapamycin or whether indeed they are direct transcriptional targets of TEL2.

Discussion.

In previous work we have shown that forced expression of TEL2 in the mouse hematopoietic system causes a myeloproliferative disease (Carella et al., 2005). When expressed together with cMyc in an Eμ-Myc model of B lymphomagenesis, TEL2 shortens disease latency (Cardone et al., 2005). In those studies we found that TEL2 inhibited apoptosis of wild type myeloid-, as well as B-lymphoid of Eμ-Myc hematopoietic cells and we speculated that the reduced apoptotic rate in both models would enlarge a critical cell pool that, due to its increased cell number, would be more vulnerable to additional mutation, hence causing disease or shortening disease latency (Cardone et al., 2005; Carella et al., 2005). This scenario seems particularly plausible in the Eμ-Myc lymphomagenesis model, in which all Eμ-Myc/TEL2 B cell lymphomas indeed acquired additional mutations inactivating the p53 pathway (Cardone et al., 2005), eliminating the high apoptotic rate of Myc overexpressing B cells (Eischen et al., 1999). In addition, pro-B cells expressing TEL2 *in vitro* displayed accelerated cell cycle traverse, which coincided with the appropriate changes in expression of cell cycle regulators (Cardone et al., 2005). Because both c-Myc and TEL2 overexpression stimulate growth of B cells (Cardone et al., 2005), we tested whether TEL2 could cooperate with inactivation of the p53 pathway by expressing TEL2 in *Arf*-null bone marrow. Given that TEL2 alone produced myeloid disease (Carella et al., 2005), it was surprising that mice transplanted with $Arf^{-/-}$/TEL2 bone marrow developed B cell lymphoma rather than acute myeloid leukemia. However, C57Bl/6/129sv $Arf^{-/-}$ mice develop T-cell lymphoma (Kamijo et al., 1997) while expression of TEL2 changes the type of malignancy from T to B cell lymphoma. This might be caused by a distinct cell specificity of TEL2, favoring outgrowth of B lymphoid, rather than T lymphoid progenitors. Although we have not analyzed the effects of TEL2 on T cells, downstream effects of TEL2 do show cell specificity, given that forced expression of the protein in primary mouse embryo fibroblasts does not stimulate their growth (C. Carella and G. Grosveld, unpublished results). As shown in Fig. 3E,TEL2 expression suppressed the apoptotic rate of $Arf^{-/-}$ pro-B cells, possibly due to increased Bcl2 expression (Fig. 3F) and inactivation of Bad (Fig 4B). Thus, the combined effects of TEL2, faster cycling and reduced apoptosis, would enlarge the pool of pro-B cells, which is reflected in pre-leukemic mice by the steady increase of B lymphocytes in the peripheral blood (Fig. 1A). Although cultured pro-B lymphocytes expressing TEL2 contain increased amounts of Myc (Fig 3 F), in $Arf^{-/-}$/TEL2 B cell lymphomas there is an apparent selection for cells that amplified the *Myc* copy number, either by triplication of chromosome 15 or by a combination of triplication and additional translocations. These findings are relevant given that these features are consistently found in both murine B cell lymphomas (Vasmel et al., 1989) and human Burkitt's lymphomas (Boxer and Dang, 2001). Therefore, the end result in $Arf^{-/-}$/TEL2 B cell lymphomas is overexpression of Myc in combination with a compromised p53 pathway, which is

identical to B cell lymphomas in TEL2/Eμ-Myc mice, which express high levels of Myc and eliminate their p53 pathway (Cardone et al., 2005). In addition, the level of Mdm2 protein is highly increased in $Arf^{-/-}$/TEL2 B cell lymphomas, and it is significant that this protein, which silences the pro-apoptotic function of p53(Eischen et al., 2004; Eischen et al., 1999), can also exert mitogenic effects through a p53-independent activation of E2f1, whose protein level is also increased in our malignant cells (Martin et al., 1995). We believe that in both scenarios TEL2 functions as a mediator, which enlarges the cell pool by suppressing apoptosis in TEL2/Eμ-Myc B cells and by suppressing apoptosis and accelerating cell cycle traverse in $Arf^{-/-}$/TEL2 B cells. Enlargement of these cell pools increases the chance of the suitable cooperating mutations to occur.

It is noteworthy that increased protein levels of E2f-1, E2f-2, Bcl2, cMyc, and Cyclins A, E and D2 , common to many types of B cell lymphomas, in $Arf^{-/-}$/TEL2 cells must be due to increased protein synthesis or reduced turnover, or both, because in our Affymetrix analysis none of encoding mRNAs were upregulated. Therefore, increased amounts of mTor mRNA in our Affymetrix arrays, encoding a central regulator of translation, was a possible key event, directly responsible for the increased amount of the above proteins (Bjornsti and Houghton, 2004b).

First we investigated whether mTor was a transcriptional target of TEL2 in pro-B cells. Based on ChIP analysis with a TEL2 antibody in $Arf^{-/-}$/TEL2 pro-B cells and luciferase assays testing the activity of the wild type and mutant 600 bp *mTor* promoter fragment in these cells, we believe that *mTor* is a direct transcriptional target of TEL2. Respective mutation of the two putative TEL binding sites showed that TEL2 responsiveness was mainly mediated via the 1A (CCGGAAGT) site (Fig. 4E, F). Two conclusions can be drawn from this result: 1) TEL2 can act as a transcriptional activator, while it functions as a strong transcriptional repressor in transient transcription assays on an artificial promoter in NIH3T3 fibroblasts (Kawagoe et al., 2004; Potter et al., 2000). This suggests that the transcriptional readout of TEL2 is cell-specific and will be dictated by the transcription complexes with which it associates. 2) Given the hematopoietic-specific expression of TEL2 (Potter et al., 2000), transcriptional regulation of the *mTor* gene by TEL2 must be mainly restricted to hematopoietic cells.

Not only transcription of *mTor* was upregulated, but also the amount of mTor protein and its kinase activity (Fig. 4B). Therefore, it was puzzling that the known downstream pathways of mTor were only partially activated. Its phosphorylation target ribosomal p70S6K1 was activated, but S6, the target of p70S6K1 kinase was not. Another target of p70S6K1, Bad (Harada et al., 2001) did show rapamycin sensitive increased phosphorylation at Ser^{112} (Fig. 4B), suggesting that a mTor-to-p70S6K1-to-Bad phosphorylation cascade was operational. In addition, this suggested that inactivation of Bad via this phosphorylation step might contribute to the reduced apoptosis of $Arf^{-/-}$/TEL2 Pro-B cells. Also the amount of 4EBP1 in $Arf^{-/-}$/TEL2 Pro-B

cells was increased but, despite the presence of the vastly increased amount of activated mTOR, the overall amount of phosphorylated 4EBP1 was marginally increased (middle band in the 4EBP1 blot in Fig. 4B), which would indicate only marginal additional activation of translation initiation factor eIF4E. Indeed, binding of active translation complexes containing eIF4E and eIF4G to 7-methyl-GTP-Sepharose beads was not increased in $Arf^{-/-}$/TEL2 pro-B cells. Notwithstanding these puzzling results, the growth characteristics of $Arf^{-/-}$/TEL2 Pro-B cells was in large part dependent on activated mTor, as suggested by the reduced growth rate of the cells in the presence of rapamycin. Given that control cells did not proliferate and died in the presence of rapamycin it is clear that other growth promoting and anti apoptotic effects emanate from TEL2, not involving mTor. These include upregulation of growth stimulating (*Notch3, Lif*) and downregulation of anti-proliferation genes (*Btg2*) as well as upregulation of anti-apoptotic genes (Bcl2, Pim1, 2, and 3). In particular the upregulation of Bcl2 and Pim proteins are interesting. Pim proteins have been shown to be involved in oncogenic events (Allen et al., 1997; Konietzko et al., 1999; Moroy et al., 1991; Verbeek et al., 1991) promoting lymphomagenesis (Amson et al., 1989; Neill and Kelsell, 2001; Yoshida et al., 1999) and Pim2 can phosphorylate and inactivate Bad independent of mTor function (Fox et al., 2003). Therefore, we can speculate that both activation of Bcl2 and inactivation of Bad through Pim proteins provide $Arf^{-/-}$/TEL2 leukemic cells with an alternative pathway to achieve increased cell-survival (Hammerman et al., 2005)(Fig. 6). It is presently unknown whether any of these genes are direct transcriptional targets of TEL2, and we have insufficient insight in the mTor regulatory network to explain the changes in expression of these genes in $Arf^{-/-}$/TEL2 pro-B cells in the presence of rapamycin. Importantly, there are already clinical trials using rapamycin in B-CLL patients (Decker et al., 2003) (Hipp et al., 2005; Ringshausen et al., 2005) and we believe that understanding the molecular events that allow leukemic cells to overcome the rapamycin block, could be relevant to identify subgroups of leukemic patients non-responsive to rapamycin. Patients overexpressing TEL2 might constitute such a subgroup.

A recent report may also provide an explanation for the increased protein expression of E2F-1, E2F-2, Bcl2, cMyc, and Cyclins A, E and D2 in $Arf^{-/-}$/TEL2 pro-B cells in spite of only partial activation of the mTor pathway. It was shown that activated S6K1 phosphorylates Pdcd4, an inhibitor of eIF4A (Dorrello et al., 2006). Phosphorylated Pdcd4 is a substrate of the ubiquitin ligase SCF and becomes rapidly degraded by the proteasome, resulting in increased translation and cell growth. It will be important to determine whether Pdcd4 in $Arf^{-/-}$/TEL2 pro-B cells is indeed phosphorylated in an mTor-dependent manner.

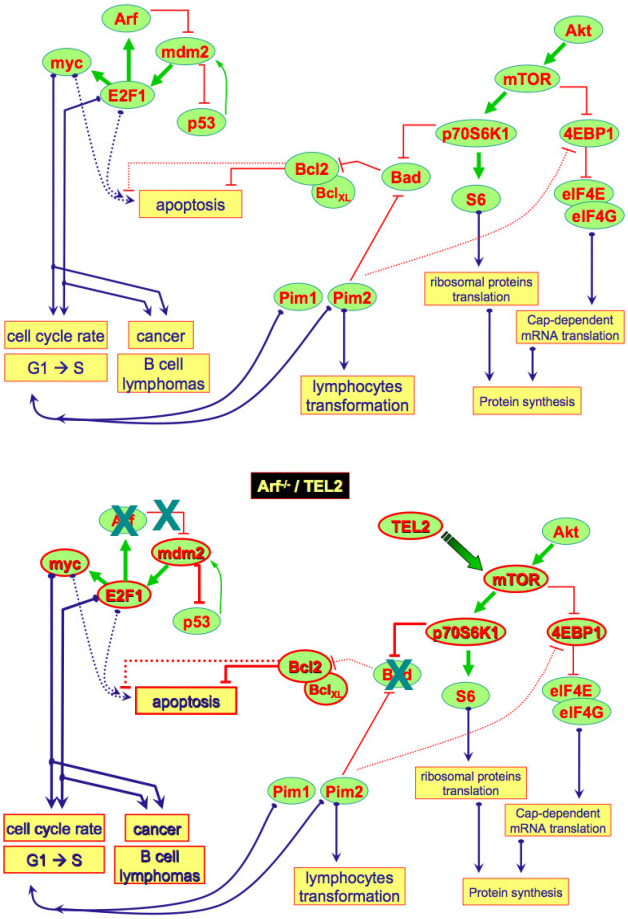


Figure 6: Diagram showing the hypothesized mechanisms of cooperation between TEL2 forced expression and p19^{Arf} deficiency .

Materials and methods.

Plasmid constructs, retrovirus production, determination of viral titers, mouse bone marrow transduction and transplantation.

MSCV-IRES-GFP and MSCV-IRES-TEL2 retroviral vectors have been described elsewhere (Kawagoe et al., 2004). Generation of retroviral stocks, determination of viral titers, isolation and transduction of Lin^- bone marrow cells, and bone marrow transplantation of mice were performed as described (Cardone et al., 2005). The retroviral titers ranged between $4x10^5$ - 10^6 cfu/ml.

Luciferase constructs for *m-Tor* promoter transient expression assays were prepared using the pGL3-basic vector (Promega, Madison, WI). The 600 bp *m-Tor* promoter fragments were generated by PCR using genomic mouse DNA as a template and primers containing a synthetic BglII restriction site at the 5' end and a HindIII restriction site at the 3' end to create a fragment compatible with cloning into the pGL-3 vector. To generate promoter fragments containing mutated TEL2 binding sites we used site directed mutagenesis employing PCR primers that changed the CCGGAAGT sequence (1A in Fig. 4C) into CCAGCTGT, creating a PvuII restriction site. For generation of the 1B mutant (1B in Fig. 4C) we followed the same strategy but changing the GTGGAAGT sequence into GTACTAGT, creating a SpeI restriction site. Primer sequences are available upon request.

Analysis of diseased mice and tissue preparation

Moribund transplanted animals were analyzed as described (Carella et al., 2005). For immunohistohemical analysis the following primary antibodies were used: CD3 and myeloperoxidase (MPO) (Dako, Carpinteria, CA); CD34, CD45R/B220, and IgM (PharMingen; San Diego, CA); terminal deoxynucleotidyl transferase (TdT; Super Techs, Bethesda, MD) and green fluorescent protein (GFP; Molecular Probes, Eugene, OR).

Flow Cytometric Analysis

Single cell suspensions of bone marrow, spleen and liver cells were washed, and incubated for 30 minutes on ice in staining medium (SM: DMEM supplemented with 10% fetal bovine serum) containing human gamma-globulin (100mg/ml, Sigma, St Loius, Mo) to block Fc receptors. After a second wash, cells were incubated with a titered excess of monoclonal antibody (CD3c, CD4, CD8, CD11b/Mac1, CD19, CD34, CD45R/B220, TER119, Gr-1, Sca-1, c-kit, Flt3, all from Pharmingen; anti-m-IgM from Southern Biotechnology Associates) on ice for 30 minutes. After a final wash, cells were resuspended in SM and analyzed using a BD Biosciences FACS Calibur flow cytometer (BD Biosciences, San Jose, CA), selecting single cells by gating on forward versus side light scatter.

Cell cycle analysis and Annexin V staining

P19$^{Arf-/-}$ pro-B-cells, GFP^{+} FACS sorted Arf$^{-/-}$ pro-B cells transduced with MSCV-IRES-GFP or MSCV-TEL2-IRES-GFP were seeded at equal density (5x10^5 cells/ml) in presence of IL-7, and cultured for 5 days. The cell number, their apoptotic rate and their cell cycle status were analyzed daily. The experiment was repeated twice in triplicate. The percentage of cells in the G_1, S, and G_2/M phases of the cell cycle were determined by analysis of the single-parameter DNA histogram using the computer program ModFit. The Annexin-V-isothiocyanate staining and the cell cycle analysis were performed as described previously (Cardone et al., 2005).

Secondary Recipients

Secondary transplants were performed by tail vein injection of 1x10^6 freshly isolated bone marrow or spleen cells of moribund primary recipients into sub lethally irradiated secondary recipients (950 Rad). Diseased mice were analyzed as described (Carella et al., 2005).

Karyotype analysis of malignant cells.

Single cell suspensions of bone marrow, spleen and liver were cultured as described (Carella et al., 2005) and treated with colcemid (50 ng/ml) for approximately 2 hours to arrest cells in metaphase. Cytospin slides were air dried and banded with trypsin-Wright's stain.

Fluorescent in situ hybridization (FISH).

Purified cMyc BAC DNA (218117) or a mouse chromosome 15 specific BAC (43G16) were used as probes to determine the copy number of the *cMyc* gene and the ploidy of chromosome 15 in B lymphoma cells. The chromosome 15 BAC DNA was labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN) and the *cMyc* BAC DNA was labeled with biotin-16-dUTP (Roche Molecular Biochemicals) by nick translation. The labeled probes were combined with sheared mouse DNA, heat denatured, and hybridized to metaphase and interphase nuclei in a solution containing 50% formamide, 10% dextran sulfate and 2xSSC. Probe detection was accomplished by incubating the hybridized slides in fluorescein labeled anti-digoxigenin (Roche Molecular Biochemicals) and Texas-red avidin (Vector Laboratories Inc., Burlingame, Ca). The nuclei were then stained with 4,6-diamidino-2-phenylindole (DAPI) and their chromosome content analyzed.

In vitro *culture, transduction, and transfection of Arf$^{-/-}$ pro-B cells.*

Bone marrow cells were extracted from C57Bl/6/129SVJ p19$^{Arf-/-}$ donor mice after 5FU treatment. The Lin^{-} selection and retrovirus transduction were performed as described (Cardone et al., 2005). After transduction, cells were plated on an S17 stromal layer (Saffran and Witte, 92) in RPMI 1640 medium supplemented with 10%

fetal calf serum (HyClone), 55 μM 2-mercaptoethanol, 2mM glutamine, penicillin (100IU/ml), streptomycin (100 μg/ml) and IL-7 (50 U/ml), to allow outgrowth of the pro-B cell population. After 1 week we confirmed their pro-B cell immunophenotype by flow cytometry and sorted the GFP^+ cells by FACS.

$Arf^{-/-}$ pro-B cells, transduced with retrovirus or not, were cultured for 3 days in the presence of Rapamycin (10g/ml dissolved in DMSO) and a reduced concentration of IL-7 (10 U/ml), to avoid possible inhibitory effects of rapamycin treatment (Brown Grupp PNas 03). Cell counts, Annexin-V staining and cell cycle analysis were performed daily.

$Arf^{-/-}$/TEL2 pro-B-cells were transfected with luciferase reporter plasmids using the Cell Line Optimization Nucleofector™ Kit (Amaxa Biosystem Inc, Gaithersburg, MD). Cells were harvested 24h after nucleofection and Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer's protocol.

Western Blots analysis

Protein extracts of mouse tissues or $p19^{Arf-/-}$ B-cells, transduced with retroviruses or not, were obtained using TRI Reagent (Sigma) following the manufacturer's instructions. The total protein concentration was determined using the BCA™ Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Protein (30 μg) was separated on 10% SDS-PAGE gels under reducing conditions and transferred onto a poly-vinylidene difluoride (PVDF) membrane (Immobilon PVDF, Millipore, Bedford, MA). The membranes were probed with any of the following antibodies: Bcl-X (556361; BD PharMingen), recognizing both Bcl-Xl and Bcl-Xs, Bcl2 (554218; BD PharMingen), E2f-1(32-1400; Zymed Laboratories Inc., South San Francisco, CA), E2f-2 (sc-633), E2f-3 (sc-878), c-Myc (sc-764), Mdm2 (sc-812), Cyclin A (sc-751), Cyclin E (sc-481), Cyclin D2 (sc-181), Cyclin D1 (sc-450), Cyclin D3 (sc-182;.), Gapdh (MAB374; Chemicon Int.,Temecula, CA), p53 (Ab-7; Calbiochem, La Jolla, CA.), Rb (sc-102; Santa Cruz Biotechnology Inc.), p21 (554085; BD PharMingen), Kip1/p27 (57; Transduction Laboratories, Lexingtion, Ky), mTor (26E3) mouse monoclonal hybridoma (produced in Peter Houghton's laboratory), p-mTor(Ser2448) rabbit polyclonal, p-mTor(Ser2481) rabbit polyclonal, Raptor rabbit polyclonal, p-p70S6K(Thr389) rabbit polyclonal, p-ribosomal S6(Ser235/236) rabbit polyclonal (all Cell Signaling Technology, Beverly, MA), 4E-BP1 rabbit polyclonal (Zymed Laboratories), Actin mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA), p-Akt(Ser473) rabbit polyclonal (Cell Signaling Technology), eIF4E mouse monoclonal (BD Transduction Laboratories), p-Bad(Ser112), Bad, p-eIF4G(Ser1108) (all from Cell Signaling Technology). Western blots were incubated with secondary IgG conjugated horseradish peroxidase antibodies (Pierce, Rockford,IL). Immunoreactive bands were visualized using Pierce SuperSignal chemiluminescence substrate (Pierce, Rockford, IL) and exposure of the blots to Kodak BiomaxTM MR film (Eastman Kodak Company, Rochester, NY).

Binding of active translation complexes containing eIF4E and eIF4G on 7-methyl-GTP-Sepharose beads.

Binding of active translation complexes containing eIF4E and eIF4G to 7-methyl-GTP-Sepharose beads was performed as described (Gingras et al., 1998).

Chromatin Immunoprecipitation (ChIP) Assay

Once obtained, the pro-B populations were sorted for GFP^+ cells, as described above, chromatin was prepared and immunoprecipitated with a TEL2 antibody (Cardone et al., 2005) using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate Cell Signaling Solutions) following the manufacturer's instructions.

Luciferase reporter assays

The transfection of the primary $Arf^{-/-}$/TEL2 pro-B-cell with the pGL3 vector, or the same vector containing the wild type, the 1A, or 1B mutant mTor promoter fragments was performed using the Cell Line Optimization Nucleofector™ Kit (Amaxa biosystem GmbH, Cologne, Germany), following the manufacturer's instructions. The cells were harvested 24h after nucleofection and Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer's instructions.

Affymetrix GeneChip analysis

Duplicate experiments were performed to identify changes in gene expression associated with TEL2 overexpression. Bone marrow of 5 FU-treated $p19^{Arf-/-}$ mice was either mock-transduced (Un-BM) or transduced with MSCV-IRES-GFP (GFP-BM) or MSCV-TEL2-IRES-GFP (TEL2-BM) retrovirus as described above. After transduction, cells were allowed to recover in culture for 48 hours and were then plated onto S17 stromal layers and cultured in presence of IL-7 for 1 week. GFP^+ B-lymphoid cells of all 3 cultures were sorted by FACS and RNA was isolated using Trizol (Invitrogen) following the manufacturer's recommendations.

RNA quality was confirmed by UV spectrophotometry and by analysis on an Agilent 2100 Bioanalyzer. Total RNA (10 μg) was processed in the St. Jude Hartwell Center Core Facility, following the standard Affymetrix protocol (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).
Expression analysis was performed using the Affymetrix MOE-430A GeneChip array. Signal values, detection calls and pair-wise GeneChip analyses were performed using the default parameters within the statistical algorithm of the Affymetrix GCOS software version 1.1. Signal values were scaled to a 2% trimmed mean target value of 500. Probeset annotations (March 14, 2005) were obtained from the Affymetrix website (http://www.affymetrix.com/analysis/index.affx).

In each experiment, three pair-wise comparisons were performed: TEL2-BM vs. GFP-BM, TEL2-BM vs. Un-BM, and GFP-BM vs. Un-BM. Stringent selection criteria were applied to detect differential expression. Only those probesets with a Log_2Ratio>1 (>2-fold change) and called “Increased/Decreased” ($p<0.0045$, Wilcoxon signed rank test) were retained for subsequent filtering. TEL2-associated changes were identified as probesets that displayed differential expression across each of the four comparisons of TEL2 vs. GFP and TEL2 vs. Un-BM.

A third experiment was set up to identify rapamycin-sensitive changes in the context of TEL2 expressing B-cells. The $Arf^{-/-}$/TEL2 B-cells were grown in presence or absence of Rapamycin (10ηg/ml) for 48h and RNA was extracted. As described above, the Affymetrix GCOS software was used to perform a pair-wise comparison between the two samples. Probesets with a Log_2Ratio>1 and with a “change call” ($p<0.006$, Wilcoxon signed rank test) were retained for further evaluation.

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Supplementary data

The Ets factor TEL2 activates mTor in mouse B lymphoid cells and causes B cell lymphoma when expressed in Arf-null bone marrow.

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Table1 description:

Differential expression in Arf-null B-cells associated with Tel2 expression. A total of 217 probesets had greater than 2-fold change in expression in both Tel2 vs. vector and TEL vs. GFP^- cells. Signal and fold-change values represent the average of duplicate experiments. Full details of the selection criteria are described in materials and methods. Gene annotations are from the Affymetrix update on 12/19/05.

Table 1

Probe Set ID	*TEL2 / vector Fold Change*	*TEL2 / GFP- Fold Change*	*Gene Symbol*	*Gene Title*	*Transcript ID*	*Entrez Gene*
1451181_at	22.6	6.5	2410008J05Rik	RIKEN cDNA 2410008J05 gene	AF488729	69195
1424470_a_at	21.9	2.6	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	BC020532	223864
1424471_at	16.6	17.8	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	BC020532	223864
1430700_a_at	16.6	12.1	Pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	AK005158	27226
1417439_at	13.9	6.3	Cd248	CD248 antigen, endosialin	NM_054042	70445
1422069_at	13.9	10.2	Mc1r	melanocortin 1 receptor	NM_008559	17199
1425675_s_at	13.5	5.7	Ceacam1	CEA-related cell adhesion molecule 1	M77196	26365
1419769_at	13.5	14.4	Cd22	CD22 antigen	AF102134	12483
1449005_at	9.8	7.5	Slc16a3	solute carrier family 16 (monocarboxylic acid transporters), member 3	NM_030696	80879
1451386_at	8.0	5.5	Blvrb	biliverdin reductase B (flavin reductase (NADPH))	BC027279	233016
1451721_a_at	8.0	2.9	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	M15848	14961
1423135_at	7.5	2.8	Thy1	thymus cell antigen 1, theta	AV028402	21838
1417249_at	6.5	2.9	Polm	polymerase (DNA directed), mu	NM_017401	54125
1450387_s_at	6.3	8.0	Ak3l1	adenylate kinase 3 alpha-like 1	NM_009647	11639
1418770_at	6.3	3.0	Cd2	CD2 antigen	NM_013486	12481
1450648_s_at	6.1	8.3	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	NM_010379	14961
1456306_a_at	5.5	2.7	Umod	uromodulin	BB747266	22242
1433604_x_at	5.5	5.9	Aldoa	aldolase 1, A isoform	BG065457	11674
1448477_at	5.3	4.1	Chst12	carbohydrate sulfotransferase 12	NM_021528	59031
1417592_at	5.3	3.5	Frap1	FK506 binding protein 12-rapamycin associated protein 1	NM_020009	56717
1434799_x_at	5.3	5.9	Aldoa	aldolase 1, A isoform	BG793658	11674
1419768_at	5.3	5.5	Cd22	CD22 antigen	AF102134	12483
1449937_at	5.1	5.5	Pp11r	placental protein 11 related	AI528824	19011
1460678_at	5.1	4.1	Klhdc2	kelch domain containing 2	BC005581	69554
1449938_at	5.1	4.8	Pp11r	placental protein 11 related	AI528824	19011
1421714_at	4.6	3.4	Tmprss3	transmembrane protease, serine 3	NM_080727	140765
1439148_a_at	4.6	4.8	Pfkl	phosphofructokinase, liver, B-type	BE914497	18641
1455898_x_at	4.3	6.1	Slc2a3	solute carrier family 2 (facilitated glucose transporter), member 3	BB414515	20527
1425086_a_at	4.1	2.2	Slamf6	SLAM family member 6	AF248636	30925

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP- Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1416921_x_at	4.1	4.6	Aldoa	aldolase 1, A isoform	NM_007438	11674
1415918_a_at	4.0	4.6	Tpi1	triosephosphate isomerase 1	NM_009415	21991
1435086_s_at	4.0	4.0	Klhdc2	kelch domain containing 2	AV298107	69554
1439375_x_at	3.9	4.3	Aldoa	Aldolase 1, A isoform (Aldoa), mRNA	AV030922	11674
1422580_at	3.9	2.5	Myl4	myosin, light polypeptide 4	NM_010858	17896
1437012_x_at	3.7	2.3	Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	BB226235	223864
1452927_x_at	3.7	4.3	Tpi1	triosephosphate isomerase 1	AW537828	21991
1451385_at	3.7	3.9	2310056P07Rik	RIKEN cDNA 2310056P07 gene	BC010826	70186
1418709_at	3.6	4.0	Cox7a1	cytochrome c oxidase, subunit VIIa 1	AF037370	12865
1419737_a_at	3.6	3.4	Ldh1	lactate dehydrogenase 1, A chain	NM_010699	16828
1417177_at	3.6	3.2	Galk1	galactokinase 1	NM_016905	14635
1426599_a_at	3.6	3.7	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	BM209618	20525
1435659_a_at	3.6	4.4	Tpi1	triosephosphate isomerase 1	AA153477	21991
1426554_a_at	3.5	3.2	Pgam1	phosphoglycerate mutase 1	BI407347	18648
1415978_at	3.5	3.6	Tubb3	tubulin, beta 3	NM_023279	22152
1416022_at	3.5	2.6	Fabp5	fatty acid binding protein 5, epidermal	BC002008	16592
1417308_at	3.2	3.1	Pkm2	pyruvate kinase, muscle	NM_011099	18746
1421173_at	3.1	3.0	Irf4	interferon regulatory factor 4	U34307	16364
1425802_a_at	3.1	2.5	Fcrlm1	Fc receptor-like mucin-like 1	AF329487	98752
1448525_a_at	3.1	3.2	Bnip3l	BCL2/adenovirus E1B 19kDa-interacting protein 3-like	AK018668	12177
1448752_at	3.1	2.7	Car2	carbonic anhydrase 2	NM_009801	12349
1418402_at	3.0	4.4	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta)	NM_009616	11492
1418649_at	3.0	2.7	Egln3	EGL nine homolog 3 (C. elegans)	BB284358	112407
1448890_at	3.0	4.6	Klf2	Kruppel-like factor 2 (lung)	NM_008452	16598
1417864_at	3.0	3.2	Pgk1	phosphoglycerate kinase 1	NM_008828	
1450269_a_at	3.0	2.8	Pfkl	phosphofructokinase, liver, B-type	NM_008826	18641
1434773_a_at	3.0	3.0	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	BM207588	20525
1424965_at	2.9	2.5	Lpxn	leupaxin	BC026563	107321
1419023_x_at	2.9	2.6	Eno1	enolase 1, alpha non-neuron	NM_023119	13806
1416923_a_at	2.9	3.1	Bnip3l	BCL2/adenovirus E1B 19kDa-interacting protein 3-like	AK018668	12177

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP- Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1451755_a_at	2.8	2.7	Apobec1	apolipoprotein B editing complex 1	BC003792	11810
1460682_s_at	2.8	2.5	Ceacam2	CEA-related cell adhesion molecule 2	BC024320	26367
1422470_at	2.8	3.5	Bnip3	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	NM_009760	12176
1428572_at	2.7	3.0	Basp1	brain abundant, membrane attached signal protein 1	AK011545	70350
1450081_x_at	2.7	2.5	Gpi1	glucose phosphate isomerase 1	NM_008155	14751
1426600_at	2.6	2.5	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	BM209618	20525
1419022_a_at	2.6	2.5	Eno1	enolase 1, alpha non-neuron	NM_023119	
1416069_at	2.6	2.5	Pfkp	phosphofructokinase, platelet	NM_019703	56421
1423748_at	2.5	2.4	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	BC027196	228026
1423967_at	2.5	2.4	Palm	paralemmin	BC015297	18483
1415964_at	2.5	2.1	Scd1	stearoyl-Coenzyme A desaturase 1	NM_009127	20249
1416922_a_at	2.5	2.8	Bnip3l	BCL2/adenovirus E1B 19kDa-interacting protein 3-like	AK018668	12177
1427404_x_at	2.5	2.5	LOC433182	similar to enolase 1, alpha non-neuron	BC004017	433182
1420997_a_at	2.5	2.5	Gpi1	glucose phosphate isomerase 1	NM_008155	14751
1438640_x_at	2.3	2.5	Pgk1	phosphoglycerate kinase 1	AV305101	
1422476_at	2.3	2.2	Ifi30	interferon gamma inducible protein 30	NM_023065	65972
1426510_at	2.2	3.0	Sccpdh	saccharopine dehydrogenase (putative)	AW537824	109232
1423418_at	2.2	2.1	Fdps	farnesyl diphosphate synthetase	BI247584	110196
1449991_at	2.2	2.1	Cd244	CD244 natural killer cell receptor 2B4	NM_018729	18106
1439435_x_at	2.2	2.5	Pgk1	phosphoglycerate kinase 1	BB411302	
1460188_at	2.2	2.3	Ptpn6	protein tyrosine phosphatase, non-receptor type 6	NM_013545	15170
1423466_at	2.1	2.1	Ccr7	chemokine (C-C motif) receptor 7	BB204380	12775
1434814_x_at	2.0	2.0	Gpi1	glucose phosphate isomerase 1	BE992059	14751
1427094_at	2.0	2.0	Pole2	polymerase (DNA directed), epsilon 2 (p59 subunit)	AF036898	18974
1427820_at	2.0	2.7	---	Mus musculus, clone IMAGE:3983821	BC021831	
1422662_at	-2.1	-2.1	Lgals8	lectin, galactose binding, soluble 8	AI987967	56048
1448775_at	-2.1	-2.2	LOC547362	similar to interferon-inducible protein 203	NM_008328	547362
1416930_at	-2.1	-2.9	Ly6d	lymphocyte antigen 6 complex, locus D	NM_010742	17068
1426716_at	-2.2	-2.2	Tdrd7	tudor domain containing 7	BC025099	100121
1427451_a_at	-2.2	-3.1	BC018473	cDNA sequence BC018473	AF118272	193217
1415936_at	-2.2	-2.5	Bcar3	breast cancer anti-estrogen resistance 3	NM_013867	29815
1434920_a_at	-2.2	-2.4	Evl	Ena-vasodilator stimulated phosphoprotein	AW553781	14026
1415673_at	-2.2	-2.1	Psph	phosphoserine phosphatase	NM_133900	100678

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP- Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1438993_a_at	-2.2	-2.1	Atp6v1d	ATPase, H+ transporting, V1 subunit D	AV105788	73834
1416008_at	-2.3	-2.3	Satb1	special AT-rich sequence binding protein 1	AV172776	20230
1417943_at	-2.4	-2.5	Gng4	guanine nucleotide binding protein (G protein), gamma 4 subunit	NM_010317	14706
1423626_at	-2.4	-3.0	Dst	dystonin	BB150886	13518
1452016_at	-2.4	-2.3	Alox5ap	arachidonate 5-lipoxygenase activating protein	BC026209	11690
1451564_at	-2.4	-2.6	Parp14	poly (ADP-ribose) polymerase family, member 14	BC021340	432411
1424784_at	-2.5	-3.1	1700029I01Rik	RIKEN cDNA 1700029I01 gene	AV047635	
1450033_a_at	-2.5	-2.5	Stat1	signal transducer and activator of transcription 1	AW214029	20846
1417141_at	-2.5	-2.3	Igtp	interferon gamma induced GTPase	NM_018738	16145
1427891_at	-2.5	-2.3	Gimap6	GTPase, IMAP family member 6	BB667753	231931
1427262_at	-2.5	-3.0	Xist	inactive X specific transcripts	L04961	213742
1450545_a_at	-2.5	-2.5	Dntt	deoxynucleotidyltransferase, terminal	AF316014	21673
1418826_at	-2.5	-2.8	Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	NM_027209	69774
1437110_at	-2.5	-2.4	2810474O19Rik	RIKEN cDNA 2810474O19 gene	BM232998	67246
1452231_x_at	-2.5	-2.4	LOC545386	similar to Interferon-activatable protein 205 (IFI-205) (D3 protein)	M74124	545386
1448233_at	-2.5	-2.1	Prnp	prion protein	BE630020	19122
1434150_a_at	-2.6	-3.0	3300001H21Rik	RIKEN cDNA 3300001H21 gene, UbiE-YGHL1 fusion protein	AV171622	
1425814_a_at	-2.6	-2.3	Calcrl	calcitonin receptor-like	AF209905	54598
1449360_at	-2.6	-3.1	Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	NM_007781	12984
1422468_at	-2.6	-2.2	Ppt1	palmitoyl-protein thioesterase 1	AF326558	19063
1433674_a_at	-2.6	-2.3	Rnu22	RNA, U22 small nucleolar	BQ177137	83673
1449038_at	-2.7	-2.7	Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	NM_008288	15483
1425737_at	-2.7	-2.5	2510016G02Rik	RIKEN cDNA 2510016G02 gene	BC002195	72438
1450143_at	-2.7	-4.0	Rasgrp1	RAS guanyl releasing protein 1	BB354696	19419
1456567_x_at	-2.7	-2.3	Grn	granulin	BB000455	14824
1426851_a_at	-2.7	-2.2	Nov	nephroblastoma overexpressed gene	X96585	18133
1438629_x_at	-2.7	-2.2	Grn	granulin	AV166504	14824

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP-Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1436570_at	-2.7	-2.8	---	Transcribed locus	BG143461	
1449757_x_at	-2.8	-2.5	Dntt	deoxynucleotidyltransferase, terminal	BB160593	21673
1425156_at	-2.8	-3.4	9830147J24Rik	RIKEN cDNA 9830147J24 gene	BC010229	229900
1434380_at	-2.8	-2.9	9830147J24Rik	RIKEN cDNA 9830147J24 gene (9830147J24Rik), mRNA	BM241271	229900
1450165_at	-2.9	-3.6	Slfn2	schlafen 2	NM_011408	20556
1437719_x_at	-2.9	-2.8	A230046K03Rik	RIKEN cDNA A230046K03 gene	BB387783	319277
1437503_a_at	-2.9	-2.7	MGI:1915044	scotin gene	BB533076	66940
1456028_x_at	-2.9	-2.1	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	BB454540	17118
1439399_a_at	-2.9	-2.2	Rnu22	RNA, U22 small nucleolar	BB493265	83673
1424775_at	-3.0	-2.7	Oas1a	2'-5' oligoadenylate synthetase 1A	BC018470	246730
1426065_a_at	-3.0	-2.7	Trib3	tribbles homolog 3 (Drosophila)	BC012955	228775
1418240_at	-3.0	-4.0	Gbp2	guanylate nucleotide binding protein 2	NM_010260	14469
1452348_s_at	-3.0	-2.3	Ifi204	interferon activated gene 204	AI481797	
1435331_at	-3.0	-3.1	AI447904	expressed sequence AI447904	BM241008	236312
1415971_at	-3.0	-2.4	Marcks	myristoylated alanine rich protein kinase C substrate	AW546141	17118
1418004_a_at	-3.0	-2.2	1810009M01Rik	RIKEN cDNA 1810009M01 gene	NM_023056	65963
1419043_a_at	-3.1	-2.7	Iigp1	interferon inducible GTPase 1	BM239828	60440
1433675_at	-3.1	-2.5	Rnu22	RNA, U22 small nucleolar	BQ177137	83673
1415973_at	-3.1	-2.5	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	AW546141	17118
1426906_at	-3.1	-2.5	LOC545386	similar to Interferon-activatable protein 205 (IFI-205) (D3 protein)	M74124	545386
1426852_x_at	-3.4	-3.1	Nov	nephroblastoma overexpressed gene	X96585	18133
1415972_at	-3.4	-2.6	Marcks	myristoylated alanine rich protein kinase C substrate	AW546141	17118
1420166_at	-3.4	-2.5	Dntt	Terminal deoxynucleotidyl transferase long isoform (Tdt) mRNA	BB160593	21673
1424923_at	-3.5	-2.8	Serpina3g	serine (or cysteine) peptidase inhibitor, clade A, member 3G	BC002065	20715
1450932_s_at	-3.5	-3.6	Dock9	dedicator of cytokinesis 9	BB795072	105445
1426243_at	-3.5	-3.2	Cth	cystathionase (cystathionine gamma-lyase)	BC019483	107869

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP- Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1419091_a_at	-3.6	-2.1	Anxa2	annexin A2	NM_007585	12306
1449591_at	-3.6	-3.5	Casp11	caspase 11, apoptosis-related cysteine peptidase	NM_007609	12363
1435906_x_at	-3.6	-5.3	Gbp2	guanylate nucleotide binding protein 2	BE197524	14469
1424254_at	-3.6	-3.6	Ifitm1	interferon induced transmembrane protein 1	BC027285	68713
1451474_a_at	-3.7	-4.0	Parp8	poly (ADP-ribose) polymerase family, member 8	BC022679	52552
1456700_x_at	-3.9	-2.8	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	BB100920	17118
1416498_at	-4.0	-3.7	Ppic	peptidylprolyl isomerase C	NM_008908	19038
1427263_at	-4.0	-6.1	Xist	inactive X specific transcripts	L04961	213742
1417185_at	-4.0	-2.8	Ly6a	lymphocyte antigen 6 complex, locus A	BC002070	110454
1451780_at	-4.0	-4.3	Blnk	B-cell linker	AF068182	17060
1419666_x_at	-4.1	-2.8	Nupr1	nuclear protein 1	NM_019738	56312
1417516_at	-4.1	-3.5	Ddit3	DNA-damage inducible transcript 3	NM_007837	13198
1452592_at	-4.1	-4.4	Mgst2	microsomal glutathione S-transferase 2	AV066880	211666
1456225_x_at	-4.3	-3.2	Trib3	tribbles homolog 3 (Drosophila)	BB508622	228775
1423555_a_at	-4.4	-3.9	Ifi44	interferon-induced protein 44	BB329808	99899
1417292_at	-4.6	-4.3	Ifi47	interferon gamma inducible protein 47	NM_008330	15953
1416612_at	-4.8	-2.1	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	BI251808	13078
1422567_at	-4.8	-2.6	Niban	niban protein	NM_022018	63913
1424631_a_at	-4.8	-4.8	Ighg	Immunoglobulin heavy chain (gamma polypeptide)	BC025447	380794
1444028_s_at	-5.1	-3.7	Dock9	dedicator of cytokinesis 9	AA410148	105445
1450680_at	-5.1	-4.9	Rag1	recombination activating gene 1	NM_009019	19373
1426174_s_at	-5.1	-4.9	Ighg	Immunoglobulin heavy chain (gamma polypeptide)	S69212	380794
1429947_a_at	-5.3	-7.0	Zbp1	Z-DNA binding protein 1	AK008179	58203
1449905_at	-5.3	-4.4	Clec4f	C-type lectin domain family 4, member f	NM_016751	51811
1418065_at	-5.5	-4.1	Rag2	recombination activating gene 2	NM_009020	19374
1450291_s_at	-5.7	-6.3	Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C	NM_022429	64380
1419665_a_at	-5.7	-3.7	Nupr1	nuclear protein 1	NM_019738	56312
1430523_s_at	-6.1	-5.7	Igl-V1	immunoglobulin lambda chain, variable 1	AK008145	16142
1460214_at	-6.1	-5.7	Pcp4	Purkinje cell protein 4	NM_008791	18546
1421276_a_at	-6.3	-4.6	Dst	dystonin	NM_134448	13518
1416953_at	-6.5	-4.4	Ctgf	connective tissue growth factor	NM_010217	14219
1419089_at	-6.7	-4.3	Timp3	tissue inhibitor of metalloproteinase 3	BI111620	21859
1419135_at	-7.0	-6.5	Ltb	lymphotoxin B	NM_008518	16994

Probe Set ID	TEL2 / vector Fold Change	TEL2 / GFP- Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1426808_at	-7.0	-2.7	Lgals3	lectin, galactose binding, soluble 3	X16834	16854
1422437_at	-7.0	-3.2	Col5a2	procollagen, type V, alpha 2	AV229424	12832
1416405_at	-7.2	-3.7	Bgn	biglycan	BC019502	12111
1423754_at	-7.2	-5.7	Ifitm3	interferon induced transmembrane protein 3	BC010291	66141
1449254_at	-7.2	-2.5	Spp1	secreted phosphoprotein 1	NM_009263	20750
1418162_at	-7.5	-5.3	Tlr4	toll-like receptor 4	AF185285	21898
1438676_at	-7.5	-6.1	Mpa2l	macrophage activation 2 like	BM241485	100702
1425917_at	-8.3	-10.2	H28	histocompatibility 28	BC024930	15061
1449009_at	-8.6	-7.0	Tgtp	T-cell specific GTPase	NM_011579	21822
1422571_at	-8.6	-3.0	Thbs2	thrombospondin 2	NM_011581	21826
1450663_at	-8.9	-3.9	Thbs2	thrombospondin 2	NM_011581	21826
1452141_a_at	-9.2	-9.2	Sepp1	selenoprotein P, plasma, 1	BC001991	20363
1449347_a_at	-9.5	-7.2	Xlr4b /// Xlr4a	X-linked lymphocyte-regulated 4B /// X-linked lymphocyte-regulated 4A	NM_021365	
1451310_a_at	-9.5	-3.7	Ctsl	cathepsin L	J02583	13039
1439065_x_at	-9.8	-5.1	---	CDNA clone MGC:76795 IMAGE:30111586	C77501	
1436172_at	-11.3	-5.9	9530028C05	hypothetical protein 9530028C05	BQ175154	330256
1426642_at	-11.7	-3.9	Fn1	fibronectin 1	BC004724	14268
1437165_a_at	-12.6	-5.9	Pcolce	procollagen C-endopeptidase enhancer protein	BB250811	18542
1415855_at	-13.0	-6.3	Kitl	kit ligand	BB815530	17311
1437889_x_at	-14.9	-5.3	Bgn	biglycan	AI931862	12111
1438650_x_at	-14.9	-6.5	Gja1	gap junction membrane channel protein alpha 1	AV330726	14609
1415800_at	-14.9	-5.1	Gja1	gap junction membrane channel protein alpha 1	M63801	14609
1448823_at	-16.0	-4.9	Cxcl12	chemokine (C-X-C motif) ligand 12	BC006640	20315
1424542_at	-16.6	-9.8	S100a4	S100 calcium binding protein A4	D00208	20198
1448392_at	-17.1	-12.6	Sparc	secreted acidic cysteine rich glycoprotein	NM_009242	20692
1420725_at	-19.7	-9.5	Tmlhe	trimethyllysine hydroxylase, epsilon	AY033513	192289
1421811_at	-19.7	-9.5	Thbs1	thrombospondin 1	AI385532	21825
1421375_a_at	-19.7	-8.0	S100a6	S100 calcium binding protein A6 (calcyclin)	NM_011313	20200
1448303_at	-20.4	-5.5	Gpnmb	glycoprotein (transmembrane) nmb	NM_053110	93695
1418672_at	-21.9	-16.6	Akr1c13	aldo-keto reductase family 1, member C13	NM_013778	27384
1437992_x_at	-22.6	-8.6	Gja1	gap junction membrane channel protein alpha 1	BB039269	14609
1415904_at	-23.4	-10.9	Lpl	lipoprotein lipase	BC003305	16956
1417327_at	-24.3	-13.9	Cav2	caveolin 2	NM_016900	12390

Probe Set ID	*TEL2 / vector Fold Change*	*TEL2 / GFP- Fold Change*	*Gene Symbol*	*Gene Title*	*Transcript ID*	*Entrez Gene*
1437687_x_at	-26.0	-8.3	Fkbp9	FK506 binding protein 9	BB026630	27055
1418580_at	-29.9	-24.3	5830458K16Rik	RIKEN cDNA 5830458K16 gene	BC024872	67775
1448323_a_at	-34.3	-16.0	Bgn	biglycan	BC019502	12111
1424769_s_at	-38.1	-17.1	Cald1	caldesmon 1	BI248947	109624
1449106_at	-46.9	-16.0	Gpx3	glutathione peroxidase 3	NM_008161	14778
1438945_x_at	-57.7	-28.8	Gja1	gap junction membrane channel protein alpha 1	BB142324	14609

Table2 description:

Differential expression in Tel2/Arf-null B-cells following rapamycin treatment. The selection has been performed comparing the Tel2 transduced Arf-null B-cells in culture for 48 hours in presence or not of Rapamycin. A total of 259 probesets had greater than 2-fold change in expression using the selection criteria described in materials and methods. Probesets with “Absent” detection calls in both conditions were excluded. Gene annotations are from the Affymetrix update on 12/19/05.

Table 2

ProbeSet ID	*Rapamycin/Control Fold Change*	*Gene Symbol*	*Gene Title*	*Transcript ID*	*Entrez Gene*
1417262_at	-14.9	Ptgs2	prostaglandin-endoperoxide synthase 2	M94967	19225
1425315_at	-13.0	Dock7	dedicator of cytokinesis 7	BC006868	67299
1429891_at	-11.3	Capsl	calcyphosine-like	AK006467	75568
1449682_s_at	-8.0	2410129E14Rik	RIKEN cDNA 2410129E14 gene	C79445	73710
1442019_at	-7.5	---	16d neonate cerebellum cDNA, RIKEN full-length enrich. library, clone:9630033M11	BB627097	
1417859_at	-7.0	Gas7	growth arrest specific 7	NM_008088	14457
1418870_at	-7.0	4930579J09Rik	RIKEN cDNA 4930579J09 gene	NM_133689	67752
1456312_x_at	-7.0	Gsn	gelsolin	AV224521	227753
1415812_at	-7.0	Gsn	gelsolin	NM_010354	227753
1437171_x_at	-7.0	Gsn	gelsolin	AV025667	227753
1419082_at	-6.5	Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2	NM_011111	18788
1419908_at	-6.5	Uqcrb	Fc receptor-like mucin-like 1 (Fcrlm1), mRNA	BB219290	67530
1429714_at	-6.5	---	---	BB822050	
1436991_x_at	-6.1	Gsn	gelsolin	AV025559	227753
1417473_a_at	-4.9	Ppcs	phosphopantothenoylcysteine synthetase	NM_026494	106564
1452679_at	-4.6	2410129E14Rik	RIKEN cDNA 2410129E14 gene	AA986082	73710
1440107_at	-4.3	9430023P16Rik	RIKEN cDNA 9430023P16 gene, mRNA (cDNA clone MGC:113724 IMAGE:6851591)	BB077622	226517
1456873_at	-4.3	Clic5	chloride intracellular channel 5	BB236747	224796
1453009_at	-4.0	Cpm	carboxypeptidase M	AK004327	70574
1457270_at	-4.0	B230343A10Rik	RIKEN cDNA B230343A10 gene	AI506234	320013
1417936_at	-4.0	Ccl9	chemokine (C-C motif) ligand 9	AF128196	20308
1447757_x_at	-3.7	Inpp5f	inositol polyphosphate-5-phosphatase F	AV033355	101490
1447302_at	-3.7	---	Transcribed locus	BE956835	
1415823_at	-3.7	Scd2	stearoyl-Coenzyme A desaturase 2	BG060909	20250
1415972_at	-3.7	Marcks	myristoylated alanine rich protein kinase C substrate	AW546141	17118
1449519_at	-3.5	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	NM_007836	13197
1415822_at	-3.5	Scd2	stearoyl-Coenzyme A desaturase 2	BG060909	20250
1415824_at	-3.2	Scd2	stearoyl-Coenzyme A desaturase 2	BG060909	20250
1415971_at	-3.2	Marcks	myristoylated alanine rich protein kinase C substrate	AW546141	17118
1429413_at	-3.2	Cpm	carboxypeptidase M	AK017670	70574
1418210_at	-3.0	Pfn2	profilin 2	NM_019410	18645
1419248_at	-3.0	Rgs2	regulator of G-protein signaling 2	AF215668	19735

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1420731_a_at	-3.0	Csrp2	cysteine and glycine-rich protein 2	NM_007792	13008
1428306_at	-3.0	Ddit4	DNA-damage-inducible transcript 4	AK017926	74747
1448147_at	-3.0	Tnfrsf19	tumor necrosis factor receptor superfamily, member 19	NM_013869	29820
1449383_at	-3.0	Adssl1	adenylosuccinate synthetase like 1	NM_007421	11565
1456700_x_at	-3.0	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	BB100920	17118
1459840_s_at	-3.0	Ccdc28b	coiled coil domain containing 28B	AV365721	66264
1426850_a_at	-2.8	Map2k6	mitogen activated protein kinase kinase 6	BB261602	26399
1428769_at	-2.8	Tatdn3	TatD DNase domain containing 3	AK005193	68972
1447820_x_at	-2.8	Cpt2	carnitine palmitoyltransferase 2	AV236319	12896
1448898_at	-2.8	Ccl9	chemokine (C-C motif) ligand 9	AF128196	20308
1419821_s_at	-2.8	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	AI788952	15926
1418394_a_at	-2.8	Cd97	CD97 antigen	NM_011925	26364
1417021_a_at	-2.6	Spo11	sporulation protein, meiosis-specific, SPO11 homolog (S. cerevisiae)	NM_012046	26972
1427600_at	-2.6	Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19, mRNA	BC025871	29820
1450430_at	-2.6	Mrc1	mannose receptor, C type 1	NM_008625	17533
1439505_at	-2.6	Clic5	Chloride intracellular channel 5, mRNA (cDNA clone MGC:73528 IMAGE:1227040)	AA210377	224796
1452944_at	-2.6	Syngr2	synaptogyrin 2	AK004030	20973
1418209_a_at	-2.6	Pfn2	profilin 2	NM_019410	18645
1419247_at	-2.6	Rgs2	regulator of G-protein signaling 2	AF215668	19735
1423176_at	-2.6	Tob1	transducer of ErbB-2.1	BQ266486	22057
1426243_at	-2.6	Cth	cystathionase (cystathionine gamma-lyase)	BC019483	107869
1427760_s_at	-2.6	Plf, Plf2, Mrpplf3	Proliferin, proliferin 2, mitogen regulated protein, proliferin 3	X75557	
1434295_at	-2.6	Rasgrp1	RAS guanyl releasing protein 1	BE691356	19419
1439247_at	-2.6	Dock10	dedicator of cytokinesis 10	BB763030	210293
1454200_at	-2.6	Zfhx1b	zinc finger homeobox 1b	AK012377	24136
1426510_at	-2.5	Sccpdh	saccharopine dehydrogenase (putative)	AW537824	109232
1415973_at	-2.5	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	AW546141	17118
1416318_at	-2.5	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a	AF426024	66222
1417394_at	-2.5	Klf4	Kruppel-like factor 4 (gut)	BG069413	16600
1425802_a_at	-2.5	Fcrlm1	Fc receptor-like mucin-like 1	AF329487	98752
1448883_at	-2.5	Lgmn	legumain	NM_011175	19141
1457218_at	-2.5	6430510M02Rik	RIKEN cDNA 6430510M02 gene	BB296225	319517
1417395_at	-2.3	Klf4	Kruppel-like factor 4 (gut)	BG069413	16600
1418402_at	-2.3	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta)	NM_009616	11492
1425093_at	-2.3	P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3	BC023089	228139

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1427347_s_at	-2.3	Tubb2	tubulin, beta 2	BC003475	
1449916_at	-2.3	Pbx4	pre-B-cell leukemia transcription factor 4	NM_030555	80720
1437213_at	-2.3	Nudt21	nudix (nucleoside diphosphate linked moiety X)-type motif 21	BG070110	68219
1441887_x_at	-2.3	Sh3bgrl	SH3-binding domain glutamic acid-rich protein like, mRNA	BB548587	56726
1446309_at	-2.3	5830483C08Rik	RIKEN cDNA 5830483C08 gene, mRNA	BB466955	209334
1447706_at	-2.3	Ppp1r2	Protein phosphatase 1, regulatory (inhibitor) subunit 2 (Ppp1r2), mRNA	BB535847	66849
1428897_at	-2.3	2610029I01Rik	RIKEN cDNA 2610029I01 gene	AK011611	77032
1417777_at	-2.3	Ltb4dh	leukotriene B4 12-hydroxydehydrogenase	BC014865	67103
1418403_at	-2.3	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta)	NM_009616	11492
1419907_s_at	-2.3	Fcrlm1	Fc receptor-like mucin-like 1	BB219290	98752
1422580_at	-2.3	Myl4	myosin, light polypeptide 4	NM_010858	17896
1426218_at	-2.3	Glcci1	glucocorticoid induced transcript 1	AA152997	170772
1426734_at	-2.3	BC022623	cDNA sequence BC022623	BB008324	224093
1428789_at	-2.3	Ralgps2	Ral GEF with PH domain and SH3 binding motif 2	AK008856	78255
1438322_x_at	-2.3	Fdft1	farnesyl diphosphate farnesyl transferase 1	BB028312	14137
1449374_at	-2.3	Pipox	pipecolic acid oxidase	BC013525	19193
1453472_a_at	-2.3	Slamf7	SLAM family member 7	AK016183	75345
1455439_a_at	-2.3	Lgals1	lectin, galactose binding, soluble 1	AI642438	16852
1456292_a_at	-2.3	Vim	vimentin	AV147875	22352
1442393_at	-2.3	Zfhx1b	Zinc finger homeobox 1b (Zfhx1b), mRNA	BB488200	24136
1445518_at	-2.3	Zfhx1b	Zinc finger homeobox 1b (Zfhx1b), mRNA	BG061923	24136
1447830_s_at	-2.3	Rgs2	regulator of G-protein signaling 2	BB034265	19735
1447903_x_at	-2.3	Ap1s2	adaptor-related protein complex 1, sigma 2 subunit	BB185861	108012
1454729_at	-2.3	B130017P16Rik	RIKEN cDNA B130017P16 gene	BB293313	81907
1429778_at	-2.1	Optn	optineurin	AK015354	71648
1421670_a_at	-2.1	Irak4	interleukin-1 receptor-associated kinase 4	NM_029926	266632
1422631_at	-2.1	Ahr	aryl-hydrocarbon receptor	BE989096	11622
1424967_x_at	-2.1	Tnnt2	troponin T2, cardiac	L47552	21956
1449757_x_at	-2.1	Dntt	deoxynucleotidyltransferase, terminal	BB160593	21673
1430311_at	-2.1	Marcks	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA	AV304251	17118
1433815_at	-2.1	MGI:1923321	gamma-aminobutyric acid (GABA-B) receptor binding protein	AV290082	76071
1435640_x_at	-2.1	A130040M12Rik	RIKEN cDNA A130040M12 gene	BE634869	319269
1446548_at	-2.1	---	---	AV242959	
1452963_at	-2.1	9530077C05Rik	RIKEN cDNA 9530077C05 gene	AK003384	68283
1425680_a_at	-2.1	Btrc	beta-transducin repeat containing protein	AF110396	12234

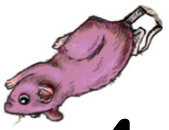
ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1416250_at	-2.1	Btg2	B-cell translocation gene 2, anti-proliferative	NM_007570	12227
1417104_at	-2.1	Emp3	epithelial membrane protein 3	BC001999	13732
1418189_s_at	-2.1	Malat1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	AF146523	72289
1424567_at	-2.1	Tspan2	tetraspan 2	BC007185	70747
1425344_at	-2.1	4430402O11Rik	RIKEN cDNA 4430402O11 gene	BI452475	67608
1427410_at	-2.1	Dleu2	deleted in lymphocytic leukemia, 2	BB812902	328425
1433443_a_at	-2.1	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	BB705380	208715
1433444_at	-2.1	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	BB705380	208715
1434378_a_at	-2.1	Mxd4	Max dimerization protein 4, mRNA (cDNA clone MGC:19425 IMAGE:3490469)	BG868949	17122
1435331_at	-2.1	AI447904	expressed sequence AI447904	BM241008	236312
1436213_a_at	-2.1	C430010P07Rik	RIKEN cDNA C430010P07 gene	AV023018	227227
1436759_x_at	-2.1	Cnn3	calponin 3, acidic	AV172168	71994
1436836_x_at	-2.1	Cnn3	calponin 3, acidic	BB724741	71994
1438118_x_at	-2.1	Vim	vimentin	AV147875	22352
1449087_at	-2.1	Rnf141	ring finger protein 141	AF353167	67150
1450700_at	-2.1	Cdc42ep3	CDC42 effector protein (Rho GTPase binding) 3	BB012489	260409
1450988_at	-2.1	Lgr5	leucine rich repeat containing G protein coupled receptor 5	BB751088	14160
1451972_at	-2.1	Glcci1	glucocorticoid induced transcript 1	AA152997	170772
1455570_x_at	-2.1	Cnn3	calponin 3, acidic	BB833102	71994
1428236_at	-2.1	Acbd5	acyl-Coenzyme A binding domain containing 5	AK005001	74159
1430379_at	-2.1	5830411K21Rik	RIKEN cDNA 5830411K21 gene	BM220939	78822
1433571_at	-2.1	Serinc5	serine incorporator 5	BQ175260	218442
1440283_at	-2.1	---	neonate thymus cDNA, RIKEN full-length enriched library, clone:A430088H08	BB131118	
1453365_at	-2.1	Rabgap1l	RAB GTPase activating protein 1-like	AK018430	29809
1454671_at	-2.1	Insig1	insulin induced gene 1	BB005488	231070
1454691_at	-2.1	Nrxn1	neurexin I	BB336165	18189
1420524_a_at	2.1	Masp2	mannan-binding lectin serine peptidase 2	NM_010767	
1432298_at	2.1	4921508M14Rik	RIKEN cDNA 4921508M14 gene	AK014845	70844
1441619_at	2.1	4732429I09Rik	RIKEN cDNA 4732429I09 gene	BB027977	243906
1416236_a_at	2.1	Eva1	epithelial V-like antigen 1	BC015076	14012
1417404_at	2.1	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	NM_130450	170439
1421992_a_at	2.1	Igfbp4	insulin-like growth factor binding protein 4	NM_010517	16010
1423000_a_at	2.1	Dgke	diacylglycerol kinase, epsilon	NM_019505	56077

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1423868_at	2.1	Txnrd3	thioredoxin reductase 3	AF349659	232223
1434380_at	2.1	9830147J24Rik	RIKEN cDNA 9830147J24 gene (9830147J24Rik), mRNA	BM241271	229900
1437100_x_at	2.1	Pim3	proviral integration site 3	BB206220	223775
1437614_x_at	2.1	Zdhhc14	zinc finger, DHHC domain containing 14	AV223474	224454
1448366_at	2.1	Stx1a	syntaxin 1A (brain)	NM_016801	20907
1452207_at	2.1	Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	Y15163	17684
1455447_at	2.1	D430019H16Rik	RIKEN cDNA D430019H16 gene	BM116882	268595
1455899_x_at	2.1	Socs3	suppressor of cytokine signaling 3	BB241535	12702
1434530_at	2.1	Odz4	odd Oz/ten-m homolog 4 (Drosophila)	BQ175876	23966
1434766_at	2.1	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit (Prkaa2), mRNA	BQ175911	108079
1434797_at	2.1	---	---	BB054275	
1436115_at	2.1	---	---	BB829749	
1436758_at	2.1	Hdac4	histone deacetylase 4	AI661423	208727
1439799_at	2.1	---	Transcribed locus	BE953350	
1440992_at	2.1	3110052M02Rik	RIKEN cDNA 3110052M02 gene	AV276161	73229
1454693_at	2.1	LOC545348	hypothetical protein LOC545348	BQ176116	545348
1458342_at	2.1	---	Adult male corpora quadrigemina cDNA, RIKEN f.l. enr. library, clone:B230339N19	BB313069	238321
1422320_x_at	2.3	Phxr5	per-hexamer repeat gene 5	NM_008836	18690
1450475_at	2.3	Dlx3	distal-less homeobox 3	U79738	13393
1435213_at	2.3	Nhlrc1	NHL repeat containing 1	BB309133	105193
1435396_at	2.3	C85317	expressed sequence C85317	BM218509	97823
1454304_at	2.3	Epn2	epsin 2	BB626346	13855
1451888_a_at	2.3	Odz4	odd Oz/ten-m homolog 4 (Drosophila)	AB025413	23966
1415978_at	2.3	Tubb3	tubulin, beta 3	NM_023279	22152
1417065_at	2.3	Egr1	early growth response 1	NM_007913	13653
1438511_a_at	2.3	1190002H23Rik	RIKEN cDNA 1190002H23 gene	BB408123	66214
1448263_a_at	2.3	Cndp2	CNDP dipeptidase 2 (metallopeptidase M20 family)	NM_023149	66054
1449009_at	2.3	Tgtp	T-cell specific GTPase	NM_011579	21822
1460177_at	2.3	Cndp2	CNDP dipeptidase 2 (metallopeptidase M20 family)	NM_023149	66054
1438355_at	2.3	---	Adult male corpora quadrigemina cDNA, RIKEN f.l. enr. library, clone:B230339N19	AI414870	238321
1438783_at	2.3	Tmepai	Transmembrane, prostate androgen induced RNA, mRNA	BB325257	65112
1445641_at	2.3	---	---	BB727879	

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1446293_at	2.3	Bcl11a	B-cell CLL/lymphoma 11A (zinc finger protein), mRNA	BB471990	14025
1456816_at	2.3	Rai17	Retinoic acid induced 17, mRNA (cDNA clone MGC:86031 IMAGE:6856060)	BG076071	328365
1457164_at	2.5	Trpa1	transient receptor potential cation channel, subfamily A, member 1	BB309395	277328
1452547_s_at	2.5	H2-D1, H2-T18	histocompatibility 2, D region locus 1, histocompatibility 2, T region locus 18	M16810	
1431235_at	2.5	1110061A14Rik	RIKEN cDNA 1110061A14 gene	BI654939	68827
1438436_at	2.5	6330407J23Rik	RIKEN cDNA 6330407J23 gene	BE979968	67412
1441822_at	2.5	1700008F19Rik	RIKEN cDNA 1700008F19 gene	AV209890	75631
1422188_s_at	2.5	Tcrg	T-cell receptor gamma chain /// T cell receptor gamma chain	NM_011558	
1448078_at	2.5	C76533	expressed sequence C76533	BG078968	98134
1424964_at	2.6	Rp1h	retinitis pigmentosa 1 homolog (human)	AF155141	19888
1435872_at	2.6	---	Transcribed locus	BE631223	
1440682_at	2.6	Odz4	Odd Oz/ten-m homolog 4 (Drosophila) (Odz4), mRNA	BB180429	23966
1447701_x_at	2.6	Idh3a	isocitrate dehydrogenase 3 (NAD+) alpha	BB468271	67834
1459937_at	2.6	Ass1	Argininosuccinate synthetase 1, mRNA (cDNA clone MGC:6218 IMAGE:3491910)	BM213298	11898
1418471_at	2.6	Pgf	placental growth factor	NM_008827	18654
1423756_s_at	2.6	Igfbp4	insulin-like growth factor binding protein 4	BC019836	16010
1423757_x_at	2.6	Igfbp4	insulin-like growth factor binding protein 4	BC019836	16010
1437223_s_at	2.6	Xbp1	X-box binding protein 1	AV051768	22433
1437405_a_at	2.6	Igfbp4	insulin-like growth factor binding protein 4	BB787243	16010
1448265_x_at	2.6	Eva1	epithelial V-like antigen 1	BC015076	14012
1433551_at	2.6	AI427515	expressed sequence AI427515	AV173683	270097
1457800_at	2.6	2900019G14Rik	RIKEN cDNA 2900019G14 gene, mRNA (cDNA clone IMAGE:5709418)	AW122190	72932
1460318_at	2.8	Csrp3	cysteine and glycine-rich protein 3	NM_013808	13009
1452383_at	2.8	Rps6ka3	ribosomal protein S6 kinase polypeptide 3	BE376079	110651
1444031_at	2.8	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	BB218576	108058
1418003_at	2.8	1190002H23Rik	RIKEN cDNA 1190002H23 gene	NM_025427	66214
1419515_at	2.8	Fgd2	FYVE, RhoGEF and PH domain containing 2	NM_013710	26382
1422189_x_at	2.8	Tcrg	T-cell receptor gamma chain	NM_011558	110067
1437406_x_at	2.8	Igfbp4	insulin-like growth factor binding protein 4	BB787243	16010
1460732_a_at	3.0	Ppl	periplakin	AF126834	19041
1439529_at	3.0	A430110N23	hypothetical protein A430110N23	AI845825	269855
1446181_at	3.0	C85699	expressed sequence C85699	BG067253	97104

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1453987_at	3.0	Btbd5	BTB (POZ) domain containing 5	AK013278	66689
1417216_at	3.0	Pim2	proviral integration site 2 /// similar to Pim2 protein	NM_138606	
1417943_at	3.0	Gng4	guanine nucleotide binding protein (G protein), gamma 4 subunit	NM_010317	14706
1420886_a_at	3.0	Xbp1	X-box binding protein 1	NM_013842	22433
1422938_at	3.0	Bcl2	B-cell leukemia/lymphoma 2	NM_009741	12043
1423006_at	3.0	Pim1	proviral integration site 1	NM_008842	18712
1451944_a_at	3.0	Tnfsf11	tumor necrosis factor (ligand) superfamily, member 11	AB032771	21943
1426288_at	3.2	Lrp4	low density lipoprotein receptor-related protein 4	AF247637	228357
1449360_at	3.2	Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	NM_007781	12984
1459903_at	3.2	Sema7a	sema domain, Ig domain, and GPI membrane anchor, (semaphorin) 7A	AA144045	20361
1439704_at	3.2	Hdac2	histone deacetylase 2	BB461532	15182
1435465_at	3.2	Kbtbd11	kelch repeat and BTB (POZ) domain containing 11	AW049906	74901
1416049_at	3.2	Gldc	glycine decarboxylase	NM_138595	104174
1418770_at	3.2	Cd2	CD2 antigen	NM_013486	12481
1425519_a_at	3.5	Ii	Ia-associated invariant chain	BC003476	16149
1435458_at	3.5	Pim1	proviral integration site 1	AI323550	18712
1450521_a_at	3.5	Tcrg	T-cell receptor gamma chain	NM_011558	110067
1436907_at	3.5	Nav1	neuron navigator 1	BE980206	215690
1457936_at	3.7	Mapk8	mitogen activated protein kinase 8	BB184171	26419
1416237_at	3.7	Eva1	epithelial V-like antigen 1	BC015076	14012
1417439_at	3.7	Cd248	CD248 antigen, endosialin	NM_054042	70445
1442659_at	3.7	Pcdh9	protocadherin 9	BB244656	211712
1447669_s_at	3.7	Gng4	guanine nucleotide binding protein (G protein), gamma 4 subunit	AV347903	14706
1421965_s_at	4.0	Notch3	Notch gene homolog 3 (Drosophila)	NM_008716	18131
1427371_at	4.0	Abca8a	ATP-binding cassette, sub-family A (ABC1), member 8a	BC026496	217258
1428718_at	4.0	Scrn1	secernin 1	AW490544	69938
1444403_at	4.0	Cbfa2t2h	Core-binding factor, runt domain, alpha subunit 2, translocated to, 2 homolog (human)	BB296343	12396
1457077_at	4.0	---	similar to hypothetical protein FLJ34960 [Mus musculus], mRNA sequence	BB306048	245683
1457973_at	4.0	---	Transcribed locus	BB750203	
1457687_at	4.0	Bcl2	B-cell leukemia/lymphoma 2	BI664467	12043
1421207_at	4.3	Lif	leukemia inhibitory factor	AF065917	16878
1430520_at	4.3	Cpne8	copine VIII	AW548480	66871

ProbeSet ID	Rapamycin/Control Fold Change	Gene Symbol	Gene Title	Transcript ID	Entrez Gene
1432391_at	4.3	Ccdc21	coiled-coil domain containing 21	AK010621	70012
1436825_a_at	4.3	Gm191	gene model 191, (NCBI)	BB807335	
1443837_x_at	4.3	Bcl2	B-cell leukemia/lymphoma 2	BB030997	12043
1435393_at	4.6	Mc1r	Melanocortin 1 receptor (Mc1r), mRNA	BB765942	17199
1437122_at	4.6	Bcl2	B-cell leukemia/lymphoma 2	BM119782	12043
1440147_at	5.3	Lgi2	leucine-rich repeat LGI family, member 2	BM118120	246316
1448080_at	5.3	---	---	AI256288	
1448724_at	5.3	Cish	cytokine inducible SH2-containing protein	NM_009895	12700
1422069_at	5.7	Mc1r	melanocortin 1 receptor	NM_008559	17199
1440157_at	5.7	Scml4	MFLJ00197 protein	BB235437	268297
1420515_a_at	6.5	Pglyrp2	peptidoglycan recognition protein 2	NM_021319	57757
1438201_at	6.5	---	Transcribed locus	AV290622	
1453087_at	7.0	6330403L08Rik	RIKEN cDNA 6330403L08 gene	AK018107	70744
1451186_at	7.0	Isg20l1	interferon stimulated exonuclease gene 20-like 1	BI440638	68048
1451840_at	7.5	Kcnip4	Kv channel interacting protein 4	BG261945	80334
1437059_at	9.8	Sox21	SRY-box containing gene 21	BB046776	223227
1454364_at	9.8	9530025L08Rik	RIKEN cDNA 9530025L08 gene	BB110590	77439
1459102_at	11.3	Myo1d	Myosin ID (Myo1d), mRNA	BG068811	338367
1439322_at	11.3	---	15d embryo head cDNA, RIKEN full-length enriched library, clone:D930042L20	BB080017	
1459076_at	11.3	---	---	BG069034	
1442913_at	12.1	---	12d embryo spinal ganglion cDNA, RIKEN f.l. enr. library, clone:D130048N24	BB456875	
1452861_at	12.1	2010300C02Rik	RIKEN cDNA 2010300C02 gene	AK008485	72097
1441702_at	13.9	Rfx3	Regulatory factor X, 3 (influences HLA class II expression), mRNA	BB363174	19726
1450555_at	18.4	Tex13	testis expressed gene 13	AF285576	83555
1427819_at	18.4	Bcl2	B-cell leukemia/lymphoma 2 (Bcl2), transcript variant 1, mRNA	BC027249	12043
1420353_at	21.1	Lta	lymphotoxin A	NM_010735	16992
1430739_at	32.0	4930471M09Rik	RIKEN cDNA 4930471M09 gene	AV261947	75787
1445061_at	42.2	---	15d embryo head cDNA, RIKEN full-length enriched library, clone:D930042L20	BM941356	



Chapter 4

MN1-TEL, the product of the t(12;22) in human myeloid leukemia, immortalizes murine myeloid cells and causes myeloid malignancy in mice.

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ORIGINAL ARTICLE

MN1-TEL, the product of the t(12;22) in human myeloid leukemia, immortalizes murine myeloid cells and causes myeloid malignancy in mice

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MN1-TEL is the product of the recurrent t(12;22)(p12;q11) associated with human myeloid malignancies. MN1-TEL functions as an activated transcription factor, exhibiting weak transforming activity in NIH3T3 fibroblasts that depends on the presence of a functional TEL DNA-binding domain, the N-terminal transactivating sequences of MN1 and C-terminal sequences of MN1. We determined the transforming activity of MN1-TEL in mouse bone marrow (BM) by using retroviral transfer. MN1-TEL-transduced BM showed increased self-renewal capacity of primitive progenitors *in vitro*, and prolonged *in vitro* culture of MN1-TEL-expressing BM produced immortalized myeloid, interleukin (IL)-3/stem cell factor-dependent cell lines with a primitive morphology. Transplantation of such cell lines into lethally irradiated mice rescued them from irradiation-induced death and resulted in the contribution of MN1-TEL-expressing cells to all hematopoietic lineages, underscoring the primitive nature of these cells and their capacity to differentiate *in vivo*. Three months after transplantation, all mice succumbed to promonocytic leukemia. Transplantation of freshly MN1-TEL-transduced BM into lethally irradiated mice also caused acute myeloid leukemia within 3 months of transplantation. We infer that MN1-TEL is a hematopoietic oncogene that stimulates the growth of hematopoietic cells, but depends on secondary mutations to cause leukemia in mice.
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Introduction

Recurrent chromosome translocations in human leukemia frequently produce tumor-specific fusion proteins involving TEL (ETV6), a member of the E26 transforming sequence (ETS) family of transcription factors.[1,2] TEL's N terminus contains a pointed (PNT) domain, which interacts with other proteins and defines a subclass of ETS proteins.[3,4] TEL's PNT domain mediates homo-oligomerization,[5] but also associates with the PNT domains of FLI1[6] and TEL2[7] and recruits the transcriptional corepressor N-Cor.[8] The domain between the PNT and DNA-binding domains recruits the corepressors Sin3a and SMRT.[9,10] These interactions are responsible for TEL's transcriptional repressor function. TEL's C-terminal ETS domain binds to DNA at sites (e.g., 5′-GGAA-3′) typical of those bound by ETS proteins.[11,12]

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Translocations affecting TEL, mostly results in fusion of the PNT domain with various partners,[1,13] including the phosphotyrosine kinases (PTKs) platelet-derived growth factor-β receptor, ABL,[14] janus kinase 2[15] and ARG[16] in hematopoietic diseases, and NTRK3 in some solid tumors.[17,18] Oligomerization via the PNT domain constitutively activates the PTK activity of the fused partner protein,[15,19,20] thereby tumorigenically transforming hematopoietic cells.[19,21–24] In other PNT-domain fusions, such as TEL-acute myeloid leukemia1 (AML1), recruitment of mSin3A via the PNT domain creates a dominant-negative transcription factor.[10,25–27] The PNT domain may similarly affect other transcription factor fusion partners, including TEL-MDS1 (TEL-EVI1)[28] and TEL-CDX2.[29]

TEL fusion proteins that retain the ETS DNA-binding moiety are rare.[11,30–32] The first identified, MN1-TEL1, is the product of the t(12;22)(q12;q11) associated with human myeloid malignancy. MN1-TEL activates transcription of reporter genes via TEL-binding sites.[11] *MN1* was discovered through its involvement in a t(4;22)(p16;q11) in meningioma.[33] MN1, a nuclear protein, is a transcriptional coactivator[11] recruited by the retinoic acid coactivators (RAC)3 and p300 in retinoic acid receptor α (RARα) retinoid X receptor-(RXR)-mediated transcription.[34] In NIH3T3 fibroblasts, MN1-TEL exhibits transforming activity that depends on DNA binding via TEL and on N-terminal transactivating sequences and C-terminal sequences in MN1. We showed that mice expressing MN1-TEL under the control of *Aml1* regulatory sequences develop T-cell lymphoma after a long latency.[35] Nonetheless, these mice can also develop AML provided they obtain the appropriate secondary mutation. AML results when MN1-TEL is combined with overexpression of HOXA9, a combination also found in patients with the t(12;22).[35,36] Here we assessed the *in vitro* and *in vivo* transforming activity of MN1-TEL in mouse BM, using a retroviral transduction/BM transplantation approach and identified MN1-TEL sequences necessary for transformation of myeloid cells.

Materials and methods

Plasmids and retrovirus production

MN1-TEL cDNA was cloned into the unique *Eco*R1 cloning site of murine stem-cell virus-internal ribosome entry site-green fluorescent protein (MSCV-IRES-GFP) plasmid, and high-titer virus (5×10^5 – 1.5×10^6 cfu/ml) was obtained as described previously.[37] As a control, we used empty MSCV-IRES-GFP vector.

BM extraction, isolation of Lin BM cells and retroviral transduction, and BM transplantation

BM was harvested from the femurs and tibiae of male, 8- to 12-week old, FVB-J, C57BL/6J, or C57BL/6/129svJ mice treated

with 5-fluorouracil (5FU) (Sigma, St Louis, MO, USA). Isolation of Lin$^-$ cells, viral transduction and BM transplantation into female, 8- to 12-week-old C57BL/6J or C57BL/6/129svJ recipients was performed as described previously.[37]

Hematopoietic colony-forming assays and LTC-IC assays

These assays were performed as described previously.[38]

Cell cycle analysis by FACS

Cell cycle analysis of MMT3 and vector control cells was performed as described previously.[37]

In vitro *differentiation of MMT3 cells*

MMT3 cells in liquid culture were induced to differentiate by adding dimethyl sulfoxide (DMSO) (1 and 2%), vitamin D3 (1×10^{-8} mol/l), trichostatin A (10 μg/ml), retinoic acid (1 mM) or one of the following growth factors: granulocyte colony-stimulating factor (G-CSF), 5 ng/ml; granulocyte–macrophage colony-stimulating factor (GM-CSF), 30 ng/ml; macrophage colony-stimulating factor (M-CSF), 5 ng/ml; IL-3, 50 ng/ml; erythropoietin (EPO), 2 U/ml; and IL-6, 50 ng/ml (Preprotech, Rocky Hill, NC, USA). Cell-surface markers (Sca1, cKit, Mac1, Gr1, Thy1, B220, CD3, CD4, CD8), identified by fluorescence-activated cell sorting (FACS) at 0, 3 and 7 days of culture, were compared with those on MMT3 cells cultured with IL-3, IL-6 and stem cell factor (SCF) (50 ng/ml).

Secondary BM transplantation

Mice (C57BL/6/129svJ) were given a single sublethal dose (8 Gy) of radiation and the next day were injected in the tail vein with $5–8 \times 10^5$ primary leukemic BM or spleen cells. Mice were inspected daily for signs of hematopoietic disease.

Analysis of diseased mice and tissue preparation

All animal procedures were conducted in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Retro-orbital cavity blood was collected monthly and analyzed by FACS to determine the percentage of GFP-expressing white blood cells (WBCs), erythrocytes and platelets. Blood counts were obtained with a Hemavet 3700 instrument (Drew Scientific, Cumbria, UK), and Giemsa-stained blood smears were examined for abnormal cells. Moribund animals were killed by CO_2 asphyxiation after methoxyflurane inhalation and analyzed as described previously.[37]

FACS of Hoechst 33342-stained side population cells

Exponentially growing MMT3 cells were harvested in phosphate-buferred saline (1×10^6 cells/ml) containing 10 μg/ml Hoechst 33342 (Sigma) and incubated for 90 min at 37°C. To positively identify the SP cells, the ATP binding cassette transporter inhibitor reserpine (10 μM; Sigma) was added to a small aliquot of cells. Cells were counterstained with propidium iodide to mark dead cells and sorted in a BD Biosciences FACS Vantage SE/DiVa instrument (BD Biosciences, San Jose, CA, USA) using laser excitation at 488 and 357 nm. Hoechst-33342 fluorescence was collected at 424 ± 22 nm (blue) and >640 nm (red). A bivariate display of red versus blue fluorescence was used to identify SP cells, which were absent from the aliquot incubated with reserpine. Viable GFP$^+$ cells displaying diminished Hoechst fluorescence were collected as the SP cells; all other cells were collected as the non-SP cells.[39]

Results

MN1-TEL increases the self-renewal capacity of primitive hematopoietic progenitors

Lin$^-$ BM from 5FU-treated FVB mice was transduced with MSCV carrying an IRES sequence linked to the *GFP* gene, without (control) or with *MN1-TEL* cDNA. FACS 2 days later revealed GFP expression in 30–40% of MN1-TEL-transduced and 50–60% of control BM cells (Figure 1a). After 2 weeks in methylcellulose (MC), MN1-TEL-expressing cells showed a proliferation advantage over vector-transduced cells, either owing to a shorter cell cycle traverse, reduced apoptosis or both. Serial replating increased this advantage: the percentage of GFP$^+$ MN1-TEL-expressing cells steadily increased to 100%, whereas that of GFP$^+$ control cells remained constant (around 60%; Figure 1a). Although the colony-forming activity of control cells was exhausted at MC3 or MC4, MN1-TEL-transduced cells produced colonies for two additional cycles (Figure 1b). Colonies produced by MN1-TEL-expressing cells were GFP$^+$, larger and more densely packed than those of control cells, especially at later rounds of MC assays (Figure 1c). After 4, 5 or 6 weeks of long-term-culture-initiating cell culture (LTC-IC),[40] the effect of MN1-TEL on colony formation was more pronounced: MN1-TEL cultures in initial MC assay produced, on average, 35 times as many colonies as did control cultures (Figure 1d). The Sca1$^+$/cKit$^+$/Lin$^-$ progenitor phenotype of MN1-TEL cells (determined by cell-surface marker analysis; not shown) suggested expansion of a primitive hematopoietic progenitor. However, MN1-TEL did not immortalize cells: no colonies were produced beyond MC 6 following LTC-IC. The same results were obtained with C57BL/6 BM.

When after 5 weeks of LTC-IC, cells were put in liquid culture in the presence of any of the following cytokines: IL-6, IL-3, SCF, GM-CSF, G-CSF, M-CSF, EPO or IL-3 + SCF + IL6, they proliferated in the presence of IL-3 + SCF + IL6 (Figure 1d), IL-3 and somewhat with SCF (not shown). Removal of cytokines completely inhibited proliferation (Figure 1e).

MN1-TEL sequences necessary for increased proliferation in LTC-IC assays

MN1-TEL's ability to transform NIH3T3 fibroblasts depends on TEL-mediated DNA binding and functions provided by N-terminal and C-terminal MN1 sequences. We assessed these sequences' involvement in growth promotion of BM by examining the effects of MN1-TEL mutants described previously:[11] MN1-TEL-DBDM (does not bind TEL-binding sites); MN1-TELΔ229–1223 (retains N-terminal part of MN1 sequences interacting with p300 and Rac3);[34] the complementary mutant MN1-TEL12–228 (retains C-terminal part f MN1 sequences interacting with p300 and Rac3); MN1-TELΔ692–1123 (retains all sequences interacting with p300 and Rac3); and MN1-TELΔ18–1123 (missing almost all MN1 sequences). We transduced Lin$^-$ BM cells with MSCV-IRES-GFP virus-expressing mutant MN1-TEL, wild-type (WT) MN1-TEL or neither. Western blotting of sorted GFP$^+$ cells with a C-terminal TEL antibody[11] showed the expression of all MN1-TEL proteins, although the MN1-TEL, MN1-TEL-DBDM and MN1-TELΔ692–1123 were expressed at a lower level than MN1-TEL12–228 and MN1-TELΔ229–1223 and at much lower level than MN1-TELΔ18–1123 (Figure 1d). Given that

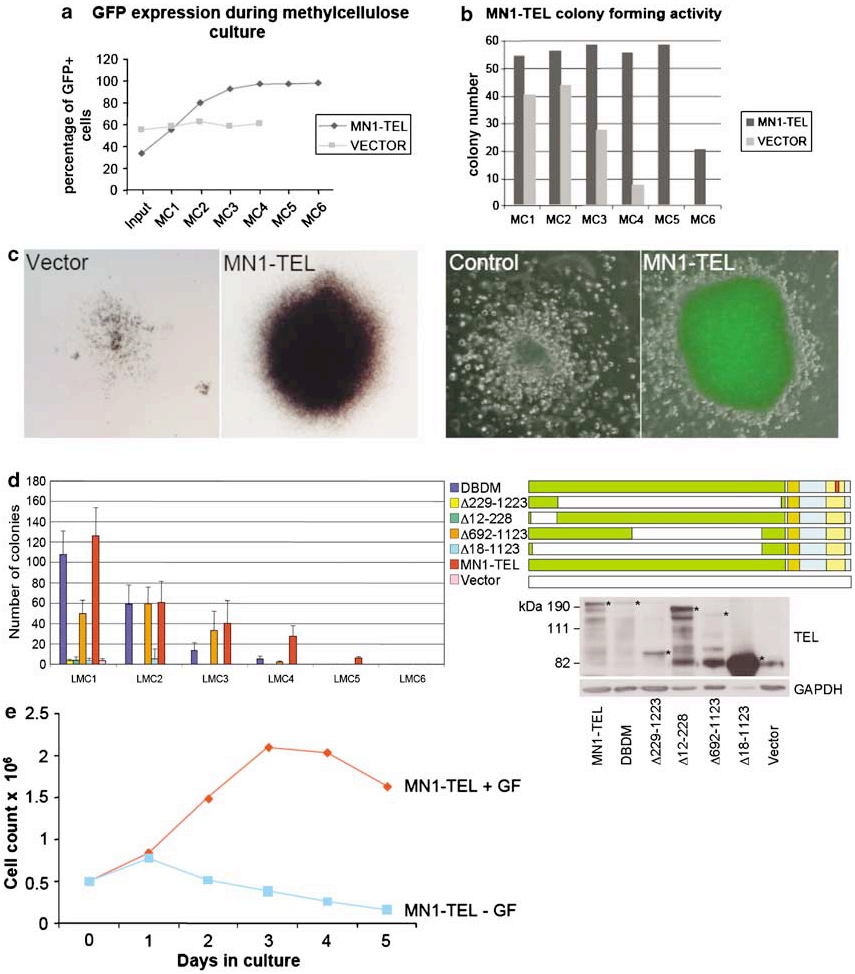

Figure 1 MN1-TEL promotes growth of mouse myeloid progenitors. (**a**) Mouse BM was transduced with MSCV-MN1-TEL-IRES-GFP or MSCV-IRES-GFP and serially plated into MC1–6. The percentage of GFP-expressing cells at each round of plating is shown. (**b**) The numbers of MN1-TEL or vector-transduced colonies at each round of MC culture are shown. (**c**) Left two panels compare the size of an MC3 colony of vector cells (left) and MN1-TEL$^+$ cells (right). Right two panels show immunofluorescence micrographs of a non-transduced GFP$^-$ colony (left) and a GFP$^+$/MN1-TEL$^+$ colony (right) in the same MC2 culture. (**d**) Colony-forming capacity of BM cells transduced with retrovirus encoding MN1-TEL, MN1-TEL mutants or GFP alone, in serial MC assays (LMC1–6) after 4, 5 or 6 weeks of LTC-IC (LMC1–6). A visual representation of the MN1-TEL mutants is shown at the right of the bar graph. Green represents MN1 sequences and interrupting white boxes represent deleted sequences. Gray represents TEL sequences with the PNT domain in orange and the ETS DNA-binding domain in yellow. The small red box indicates the mutation in the ETS domain of the DBDM mutant. The Western blot underneath shows expression of the different MN1-TEL mutants in transduced GFP$^+$ BM cells (indicated by asterisks at the right of the bands) detected with a C-terminal peptide TEL antibody. The glyceraldehyde-3-phosphate dehydrogenase loading control is shown underneath. (**e**) Mouse BM transduced with MSCV-MN1-TEL-IRES-GFP was cultured in LTC-IC for 5 weeks, followed by liquid culture with IL-3, SCF and IL-6 (4 weeks). Growth curves were determined in the presence of IL-3/SCF/IL-6 (MN1-TEL + GF) or without added growth factors (MN1-TEL-GF).

vector-transduced and non-transduced BM produce few MC colonies after LTC-IC, this analysis allowed us to identify MN1-TEL mutants that retained the capacity to stimulate BM self-renewal activity.

Combining data from two LTC-IC experiments after 4, 5 and 6 weeks of LTC-IC, we determined average numbers of colonies in serial MC assays (Figure 1d). All cells producing colonies in serial MC assays were GFP$^+$. Only MN1-TEL, MN1-TELDBDM and, to a lesser extent, MN1-TELΔ692–1123 cells showed increased self-renewal activity. Cells expressing Δ229–1223, Δ12–228 and Δ18–1123 MN1-TEL produced few colonies (Figure 1d). We concluded that N-terminal MN1 sequences but, surprisingly, not DNA binding by the ETS domain are important for MN1-TEL's growth-promoting activity.

BM cells expressing MN1-TEL can be immortalized in culture

Confirming our previous finding that BM from MN1-TEL knock-in mice can be immortalized,[35] BM cells transduced with MN1-TEL retrovirus became immortalized upon 3–5 weeks of liquid culture with IL-3, IL-6 and SCF using cells recovered from the MC 1 (2 weeks) after 4 weeks of LTC-IC. May–Grunwald–Giemsa staining revealed small, blast-like cells and large cells with basophilic granules all expressing GFP and MN1-TEL (Figure 2a, c and e). Immunophenotyping by FACS showed they were cKit$^+$, Sca$^+$, partly Thy1$^+$ and Mac1^{low} (Figure 2c). We obtained numerous morphologically and immunophenotypically similar MN1-TEL cell lines from independently transduced BM isolates and examined one line, MMT3, for its cell cycle characteristics and cytokine dependence. FACS cell cycle analysis after 2 months of liquid culture of MMT3 cells and vector-transduced cells showed that 44.5% of the former and only 3% of the latter were in the S-phase (Figure 2b), indicating that MN1-TEL cells cycled much faster than vector control cells. We next cultured MMT3 cells with IL-3; SCF; IL-6; M-CSF; G-CSF; GM-CSF; EPO; IL-3, IL-6 or SCF (Figure 2d). Cells proliferated well with IL-3, to some extent with SCF, vigorously with IL-3 + SCF + IL-6, and slowly with EPO. Non-proliferating cells were remarkably resistant to apoptosis induced by cytokine withdrawal and died slowly (half the population in 5 days). We compared the level of antiapoptotic BclX_L and Bcl2 proteins expressed in MMT3 and control cells cultured for 2 months (Figure 2e). MMT3 cells expressed a moderately increased amount of BclX_L and a substantially increased amount of Bcl2.

Transplantation of MMT3 cells into lethally irradiated mice

Attempts to induce differentiation of MMT3 cells *in vitro* by adding vitamin D3, retinoic acid, DMSO, trichostatin A or various cytokines (IL-6, G-CSF, GM-CSF, M-CSF, IL-7 and IL-2) failed and the cells remained immunophenotypically and morphologically unchanged (not shown). We next tested whether leukemia developed in lethally irradiated mice given MMT3 cell transplants. Surprisingly, transplantation rescued all ($n = 10$) recipients from radiation-induced BM failure; two mock-transplanted irradiated control mice died at 12 and 14 days after irradiation, respectively. One month after transplantation, the WBC counts of recipients were low ($0.26–1.04 \times 10^3/\mu l$) but reached normal values ($2.3–7.5 \times 10^3/\mu l$) after 2 months; 50–70% of cells were GFP$^+$ at each time point (Figure 3a and

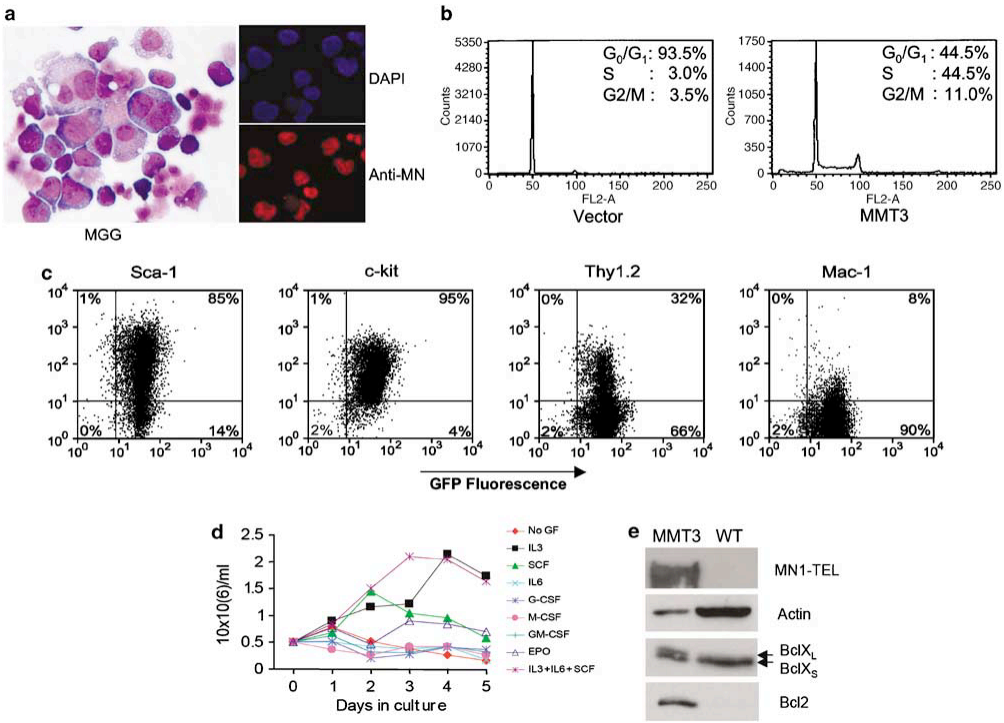

Figure 2 MN1-TEL-expressing BM cells are immortalized during liquid culture. (**a**) FVB BM was transduced with MSCV-MN1-TEL-IRES-GFP, seeded into LTC-IC, harvested 4 weeks later and plated in MC. Two weeks later, MC colonies were seeded into liquid cultures containing IL-3/SCF/IL-6 and 5 weeks later cell lines containing morphologically variable cells emerged (left panel). Immunolabeling of these cells with the 2F2 monoclonal MN1 antibody revealed nuclear localization of MN1-TEL (lower right panel). Nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI) is shown (upper right panel). (**b**) FACS cell cycle analysis of MMT3 cells and vector-transduced cells, both in culture for 2 months, showed that MMT3 cells cycled faster. (**c**) Cell-surface marker analysis of the MN1-TEL cell line MMT3. (**d**) MMT3 cells are growth-factor dependent, as shown by proliferation assays with different cytokines. (**e**) MMT3 cells and WT FVB BM cells grown in liquid culture with IL-3/SCF/IL-6 for 8 weeks were lysed and the proteins immunoblotted with anti-MN1, anti-actin, anti-BclX and anti-Bcl2 antibodies.

b). Gating and immunophenotyping by FACS of the peripheral blood of one transplant recipient killed at 2 months determined the proportions of erythrocytes (3%), B cells (53%), T cells (7%), granulocytes (68%), macrophages (83%) and platelets (35%) expressing GFP (Figure 3c). This analysis also identified GFP$^{+bright}$ and GFP^{+dim} populations (Figure 3c, right). The

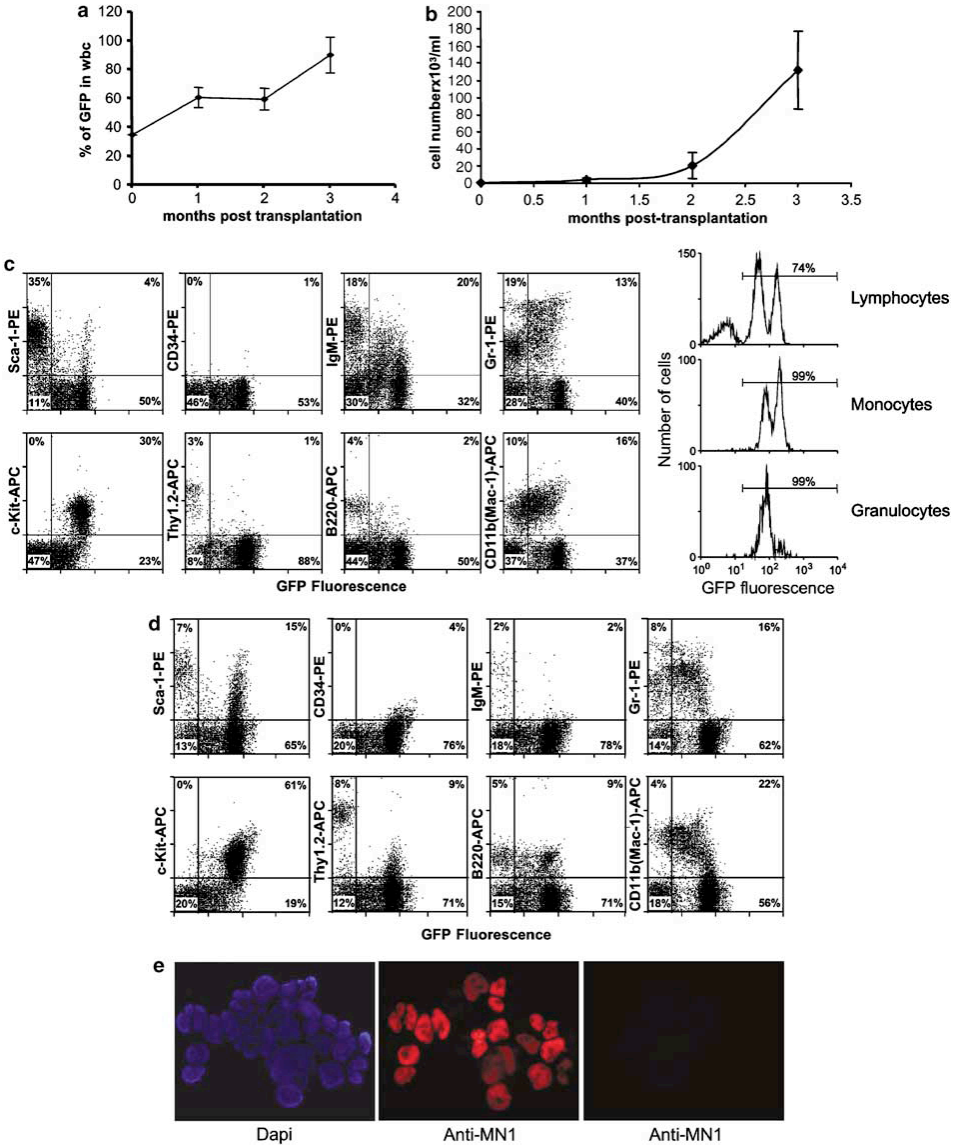

Figure 3 Hematopoiesis in lethally irradiated mice is temporarily restored by transplantation with MMT3 cells. (**a**) Average percentage of GFP$^+$ WBCs in the peripheral blood of 10 MMT3-transplanted mice 1, 2 and 3 months after transplantation. (**b**) Average WBC counts of MMT3-transplant recipients 1, 2 and 3 months after transplantation. Error bars indicate variation among mice. (**c**) Cell-surface marker analysis of peripheral blood from a mouse 2 months after transplantation with MMT3 cells (left eight panels). At the right is the GFP fluorescence in the peripheral blood of MMT3-transplant recipients, showing separate peaks representing GFP^{+dim} and GFP$^{+bright}$ lymphocyte and monocyte populations and GFP^{+dim} granulocytes. (**d**) Cell-surface marker analysis of BM from the same mouse 2 months after transplantation with MMT3 cells, also showing GFP^{+dim} and GFP$^{+bright}$ populations. (**e**) MN1-TEL immunofluorescence in BM from a mouse 2 months after transplantation with MMT3 cells, using an MN1 antibody. Left, DAPI staining; middle, MN1-TEL detected with an MN1 antibody; right, vector-transduced BM stained with an MN1 antibody.

former mainly consisting of cKit$^+$ cells that differentiated partially into immunoglobulin (Ig)M$^+$ B cells and more poorly into Mac1$^+$ monocytes/macrophages and Gr1$^+$ granulocytes. The GFP$^{+\,dim}$ population (cKit/Sca1) differentiated better into Mac1$^+$, Gr1$^+$ and B220$^+$ WBCs. The BM of this mouse (15% Sca1$^+$, 61% cKit$^+$, 4% Cd34$^+$, 9% Thy1$^+$, 9% B220$^+$, 2% IgM$^+$, 22% Mac1$^+$ and16% Gr1$^+$) showed a GFP$^{+\,bright}$ population consisting of cKit$^+$ and partly Sca1$^+$/Thy1$^+$ cells and the GFP$^{+\,dim}$ population consisting of Mac1$^+$/Gr1$^+$ cells IgM$^-$. (Figure 3d). Some cells in each population were B220$^+$ but IgM$^-$. Immunolabeling of cytospin preparations of this BM revealed 60% of cells expressing MN1-TEL (Figure 3e). FACS of eye blood from the nine remaining MMT3-transplant recipients also showed the two GFP$^+$ populations (Figure 3c, right). To avoid killing more transplant recipients, we deduced the percentage of GFP$^+$ cells in their peripheral blood from the FACS scatter plots. On average, erythrocytes were 3% GFP$^+$ and platelets, 50%. The aberrant size distribution of the MMT3-derived cells precluded the determination of GFP$^+$ contributions to the different WBC lineages.

Development of myeloid leukemia after transplantation

Myeloid leukemia (Sca1$^+$, Mac1$^+$, cKit^{dim}) developed in all mice 10–13 weeks after transplantation (Figure 4a and b). Peripheral WBC counts were high (100–180 $\times$ 10^3/μl) and GFP$^+$ cells had invaded most organs, including liver, spleen, kidney, lung, heart and caused brain hemorrhages (Figure 4c). The invading blast cells had distorted or effaced the architecture of the spleen, liver and femoral BM in association with dysplastic megakaryocytes. Immunohistochemical staining of paraffin sections showed that blasts in the BM and the invaded organs were positive for cKit, CD34, with some also positive for GATA1 (not shown). The blasts were negative for CD3, CD45, TdT, Mac2, lysozyme, myeloperoxidase and Factor 8. These features would be consistent with a myeloid leukemia with megakaryocyte maturation.

To determine which cells of the MMT3 cell line rescued the lethally irradiated mice, we incubated cells with Hoechst 33342 and sorted the unstained, GFP$^+$ side population (SP) by FACS (Figure 5a). SP cells variably constituted 5–25% of all GFP$^+$ cells. In normal BM, the SP is highly enriched in hematopoietic

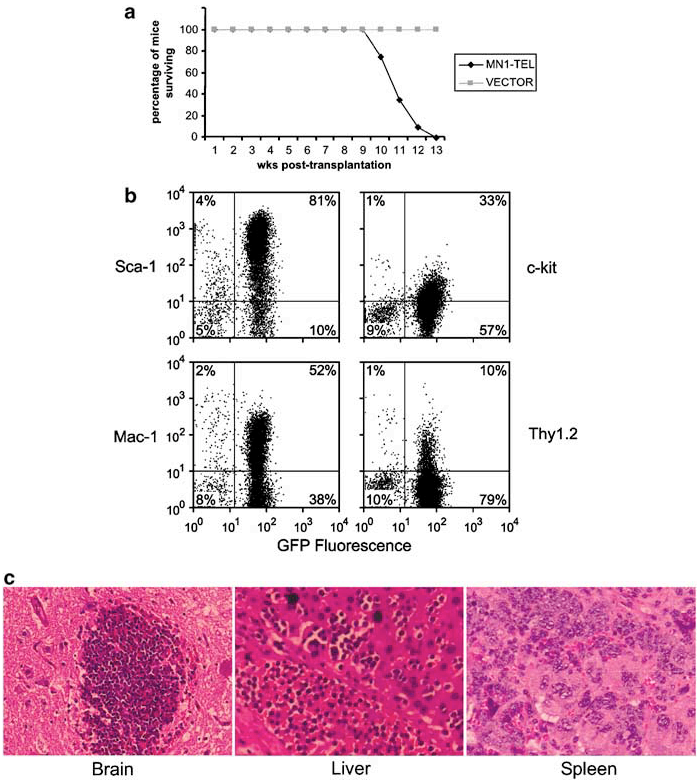

Figure 4 Transplantation with MMT3 cells causes myeloid leukemia in mice. (**a**) Kaplan–Meier survival curve for nine lethally irradiated mice given transplants of MMT3 cells (MN1-TEL) and five lethally irradiated mice given transplants of MSCV-IRES-GFP-transduced BM (Vector). (**b**) Representative cell-surface marker analysis of BM from one of nine mice in which leukemia developed 3 months after transplantation with MMT3 cells. (**c**) Tissue sections (hematoxylin–eosin stain) from a mouse in which leukemia developed 3 months after transplantation with MMT3 cells. Left, hemorrhage in the brain ($\times$ 100 magnification), a common feature in these mice; middle, liver with invading tumor cells ($\times$ 200); right, spleen with leukemic cells ($\times$ 200).

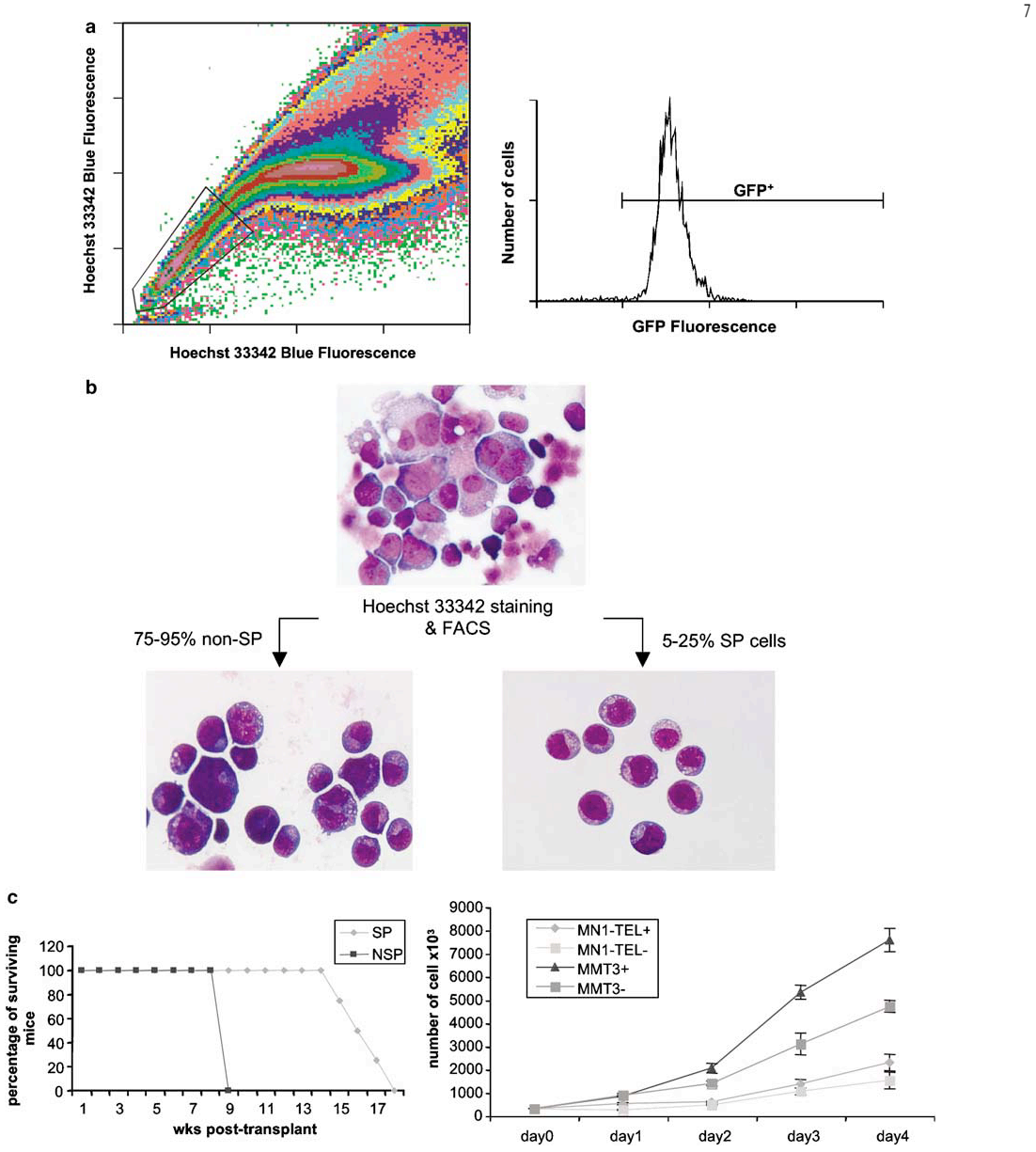

Figure 5 Lethally irradiated mice receiving transplants of MMT3 SP cells are rescued from radiation death but develop leukemia. (**a**) Left, a culture of fast-growing MMT3 cells was stained with Hoechst 33342 dye and sorted by two-color FACS (red and blue fluorescence) to separate stained (non-SP) and unstained (SP, boxed area in the left panel) cells, all expressing GFP (right). (**b**) Cytospin preparations of the MMT3 non-SP and SP fractions obtained in (**a**). (**c**, left) Kaplan–Meier survival curve for lethally irradiated mice given transplants of non-SP ($n=2$) and SP ($n=4$) MMT3 cells, rescuing them from hematopoietic failure. Non-SP recipients died of leukocytopenia 8 and 9 weeks later, whereas SP recipients died of myeloid leukemia between 15 and 18 weeks later. (Right) Leukemic cells were isolated from a moribund MMT3-transplant recipient and liquid cultured with IL-3/SCF/IL-6 (MMT3+) or without growth factors (MMT3−), and leukemic cells isolated from a recipient of MSCV-MN1-TEL-IRES-GFP BM (see Figure 6) were liquid cultured with IL-3/SCF/IL-6 (MN1-TEL+) or without growth factors (MN1-TEL−).

stem cells.[39] The MMT3 SP consisted of mainly small cells with scant cytoplasm; non-SP cells were larger, with more abundant cytoplasm (Figure 5b). Transplantation of SP-MMT3 cells into lethally irradiated recipients ($n=4$) recapitulated the results obtained with unsorted MMT3 cells; mice were rescued from radiation-induced death but succumbed to myeloid leukemia ($cKit^+/Sca1^+/Mac1^{+dim}$) 3.5–4 months after transplantation (Figure 5c, left). Non-SP MMT3 cell transplants also prolonged the lives of lethally irradiated recipients, but these mice ($n=2$) died of leukopenia 2 months after transplantation (Figure 5c, left panel). Clearly, the SP-MMT3 cells included primitive cells with repopulating and leukemogenic activity, whereas the non-SP cells included non-leukemic, short-term repopulating cells.

Next, we addressed whether individual cells in the MMT3 cell line are multipotent or whether multiple sub-populations, each with limited differentiation capacity, together conferred multipotentiality. We sorted six single SP-MMT3 cells, and expanded each to a total of 3×10^6 cells. These were then transplanted into 6 lethally irradiated mice (5×10^5 cells/mouse), but all 36 recipients died 11–14 days later. This result might indicate that the repopulating cells represent a minor subset (<17% of cells) of the SP-MMT3 cells or that the cells were no longer multipotent owing to expansion in culture.

Leukemic BM from MMT3-transplant recipients grew in culture without added cytokines, indicating that a secondary mutation(s) had occurred that rendered these cells IL3/SCF-independent (Figure 5c, right panel). The MMT3 leukemia was fully transplantable into secondary sublethally irradiated mice and caused disease ($Sca1^+$, $Mac1^+$, $cKit^{dim}$ $Thy1^{dim}$) within 3–4 weeks after transplantation.

Mice receiving freshly transduced MN1-TEL BM also develop myeloid leukemia

We also tested whether C57BL/6/129svJ mice receiving transplants of C57BL/6 BM freshly transduced with MSCV-MN1-TEL-IRES-GFP (MN1-TEL-BM, 10 mice) or MSCV-IRES-GFP (vector-BM, 10 mice) developed leukemia. MN1-TEL-BM recipients died of myeloid leukemia 8–14 weeks after transplantation. No disease developed in vector-BM recipients, but two mice died of graft failure (Figure 6a). MN1-TEL-BM recipients had high peripheral WBC counts ($1.5–2.0\times10^5/\mu l$), and most had brain hemorrhages. The peripheral blood contained blasts and poorly differentiated neutrophils; the BM consisted of monomorphic myeloid cells (Figure 6b). Leukemic cells invaded the spleen, liver, lungs, kidney, intestines and lymph nodes, showing mitotic figures in all organs. Leukemic BM of moribund animals was phenotypically variable: two samples contained GFP^{+dim} and $GFP^{+bright}$ cell populations (Figure 6c, lower panels); the remainder contained a single GFP^{+dim} population (Figure 6c, upper panels). In mice with two GFP^+ populations, GFP^{+dim} cells were mainly $cKit^{+dim}$, whereas the $GFP^{+bright}$ population was mainly $Mac1^+/Gr1^+/Thy1^+$. The leukemic cells in the BM of the other eight mice were $cKit^+/Mac1^+/Thy1^+$ with 10% of the cells also $Gr1^+$. Transplantation of the leukemic BM into sublethally irradiated secondary recipients generated fulminant myeloid leukemia 19–27 days after transplantation. Leukemic BM from these mice proliferated without added growth factors (Figure 5c), although not as vigorously as MMT3-derived leukemic cells.

Discussion

Previously, we established that MN1-TEL has weak transforming activity in NIH3T3 fibroblasts[11] and causes hematopoietic disease in mice when expressed from the endogenous *Aml1* locus.[35,36] Here we have shown that retrovirally expressed MN1-TEL has a more potent transforming activity than MN1-TEL expressed by *Aml1*. Retrovirally expressed MN1-TEL increased the self-renewal activity of myeloid progenitors, but serial MC cultures were always finite. Other oncogenic fusion transcription factors such as MLL-AF9 and AML1-ETO show similar activities.[41,42] Immortalized, cytokine-dependent cell lines arose only when MN1-TEL BM cells were expanded in liquid culture. Because the number of cells in liquid culture vastly exceeds that in MC assays, they are more likely to undergo secondary mutations causing immortalization. We have not yet attempted to identify these mutations.

Although cells of the MN1-TEL cell line MMT3 were impervious to differentiation stimuli *in vitro*, they displayed a remarkable capacity to differentiate along various lineages after transplantation into lethally irradiated mice. Although we analyzed the BM of only one mouse 2 months post-transplantation (i.e., 1 month before leukemia developed; Figure 3c), we obtained similar results from the peripheral blood of the nine remaining mice. However, results from recipients of MMT3-SP cells suggest that leukemic cells are not inherent to the cell line but are generated after transplantation. Given that SP cells (5×10^5 cells/mouse) were transplanted directly after FACS and, in this experiment, composed 25% of the MMT3 population, these mice received 2.5 times as many primitive cells as did mice receiving non-sorted MMT3 cells (8×10^5 cells/mouse). If the MMT3 line had contained leukemic cells, one would expect leukemia to have developed more rapidly in SP-MMT3-transplant recipients. However, there was no significant difference in disease onset (14–18 weeks versus 10–13 weeks; Figures 4a and 5c), consistent with the notion that MMT3 cells underwent additional leukemic events after transplantation. This suggestion was further supported by the observation that MMT3 cells recovered from the leukemic mice had become IL3-independent (Figure 5c), which could only have happened by additional mutation.

We noticed that during a year in culture, the surface markers of the MMT3 cells changed from 100% $cKit^+/Sca1^+$ to 60% also $Mac1^+$, a phenotype similar to that of the MMT3 leukemia cells in diseased mice. Leukemic cells in these mice might therefore have arisen from this sub-population of cells in the MMT3 line.

We addressed whether individual cells of the MMT3 line were multipotent or whether multiple sub-populations have limited differentiation capacity. The finding that none of the six single-cell-derived MMT3-SP cell batches rescued transplant recipients from radiation-induced death suggested either that only a small sub-population (<17%) of SP-MMT3 cells have repopulating activity, or that the cells' multipotency was lost during expansion to a population of 3×10^6 in culture. In either case, the outcome prevented us from determining whether individual MMT3-SP cells are multipotent.

Because MN1-TEL stimulates proliferation and slightly impairs differentiation of BM cells,[36] a second genetic event is needed to immortalize them *in vitro*. Full leukemic transformation required at least one additional genetic event to confer cytokine independence. Therefore, full transformation requires two or more events in addition to MN1-TEL expression.

We mapped MN1-TEL sequences essential for stimulation of myeloid cell proliferation and found one substantial difference from the findings of a similar analysis in NIH3T3 fibroblasts:[11] growth of myeloid progenitors in LTC-IC was independent of a functional TEL DNA-binding domain. We believe that MN1 is responsible for this finding, because it alone also promotes

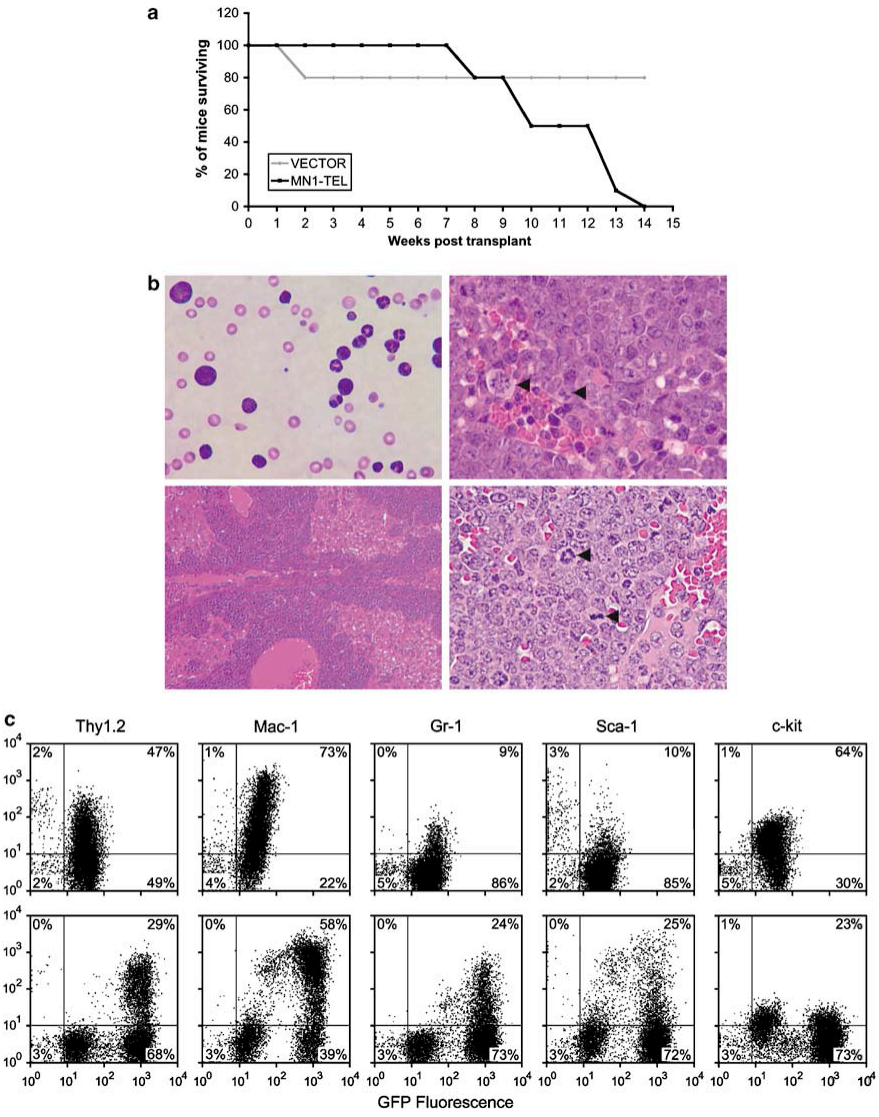

Figure 6 Myeloid leukemia develops in mice given transplants of MN1-TEL-expressing BM. (**a**) Kaplan–Meier survival curve of 10 lethally irradiated C57BL/6/129svJ mice that received C57BL/6 BM transduced with MSCV-MN1-TEL-IRES-GFP (MN1-TEL) and 10 mice that received MSCV-IRES-GFP-transduced BM (Vector). (**b**) The peripheral blood of moribund MN1-TEL leukemic mice contained blast cells and partially differentiated neutrophils (upper left panel). Leukemic cells filled the BM (upper right panel) and invaded most organs such as spleen (lower right panel) and the liver (lower left panel). Note the presence of numerous mitotic figures in the BM and spleen (black arrowheads). (**c**) Cell-surface marker analysis of BM from mice with MN1-TEL leukemia. Upper row, a single GFP^{+dim} population; lower row, GFP^{+dim} and $GFP^{+bright}$ cell populations.

growth of myeloid cells of a slightly different phenotype in LTC-IC (J Bonten, C Carella and G Grosveld, unpublished observation) but not of fibroblasts.[11] Therefore, the growth-stimulating effect of MN1-TEL-DBDM may be mediated entirely by its MN1 sequences. Further analysis is needed to resolve this issue.

We therefore concentrated on the contribution of the different MN1 (rather than TEL) sequences to the oncogenicity of the fusion protein. The growth-stimulating activity of MN1-TEL depended largely on the N-terminal 228 amino acids of MN1, a domain also important for interaction with p300 and Rac3 in

HepB3 cells.[34] Consistent with p300 and Rac3 interactions within the first 520 N-terminal amino acids of MN1,[34] MN1-TELΔ692–1123 retained some transforming ability. However, whether loss of transforming activity of MN1-TELΔ12–228 is owing to the loss of interaction with these two coactivators remains to be determined. Experiments with additional MN1-TEL mutants and co-immunoprecipitation studies will resolve this issue.

MN1's contribution to oncogenicity also depends on sequences between amino acids 693 and 1123. Interestingly, this region contains an arginine-methyltransferase target sequence (amino acids 939–947).[43] Arginine methylation is important for transcriptional activation of nuclear receptor transcription complexes,[44] and given MN1's interaction with RARα-RXR dimers,[34] this methylation sequence might contribute to MN1's function, a possibility we are investigating.

Finally, our finding that myeloid leukemia develops in mice given transplants of MN1-TEL-transduced BM (reported here) but not in mice carrying an MN1-TEL knockin construct[35] may be explained by the much higher level of expression of MN1-TEL mediated by the retroviral vector than by the *Aml1* knockin allele.

In summary, we have further established that MN1-TEL is a *bona fide* hematopoietic oncogene and that its function strongly depends on sequences in the N-terminal domain of MN1. We are determining which proteins interact with this domain and which downstream target genes mediate the growth-promoting effects of MN1-TEL in hematopoietic cells.

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Chapter 5

MN1 overexpression is a prerequisite for the development of inv(16) AML

MN1 overexpression is a prerequisite for the development of inv(16) AML.

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Summary

The gene encoding the transcriptional coactivator MN1 is target of the reciprocal chromosome translocation (12;22)(p13;q12) in some patients with acute myeloid leukemia (AML). In addition, expression array analysis showed that *MN1* was overexpressed in AML specified by inv(16), in some AML overexpressing EVI1, and in some AML without karyotypic abnormalities. Here we describe that mice receiving transplants of bone marrow overexpressing *MN1* rapidly developed myeloproliferative disease. This bone marrow also generated myeloid cell lines in culture. By mimicking the situation in human inv(16) AML, forced coexpression of MN1 and Cbfβ-SMMHC rapidly caused AML in mice. These findings identify *MN1* as a highly effective hematopoietic oncogene and suggest that MN1 overexpression is an obligatory and defining cooperative event in human inv(16) AML.

Significance

The *MN1* gene is the target of a rare t(12;22) in patients with myeloid leukemia and encodes a transcriptional coactivator. The gene is also overexpressed in 12% of AML patients characterized by the chromosome 16 inversion, which encodes the CBFβ-SMMHC fusion oncoprotein. Here we show that mice overexpressing MN1 in the bone marrow rapidly develop myeloproliferative disease, whereas mice overexpressing MN1 in conjunction with Cbfβ-SMMHC rapidly develop AML. Overexpression of MN1 is also found in other types of AML. Our results indicate that MN1 overexpression is an obligatory step in the development of inv(16) AML and future pharmacological interference with MN1 function might provide a novel therapeutic approach for the treatment of AML in which this protein is overexpressed.

Introduction

The *MN1* gene was identified as the target of a unique balanced t(4;22) in a patient with meningioma and was thought to be a prime candidate for the meningioma tumor suppressor gene on chromosome 22 (Meningioma 1; Lekanne Deprez, 1991)(Lekanne Deprez et al., 1991), but its relation to meningioma remains unresolved. *MN1* encodes a nuclear protein of 150kD, highly conserved among vertebrates and no homology to other proteins. Its amino acid (aa) sequence suggested a role in transcription(Lekanne Deprez et al., 1995), which was confirmed by the observation that MN1 activated transcription of the Moloney Sarcoma Virus long terminal repeat (MSV-LTR) in transient transcription assays(Buijs et al., 2000). MN1 appeared to activate transcription of the LTR via direct repeat sequences (DR5) that bind RAR-RXR nuclear receptor dimers. MN1 interacts with RAR-RXR most probably via the protein intermediates p300 and RAC3 (also known as nuclear receptor coactivator 3, NCOA3) (van Wely et al., 2003). RAC3 and MN1 are transcription coactivators(Chen et al., 1997; Leo and Chen, 2000), and coexpression of MN1 with p300 or RAC3 synergistically activated the transcriptional activity of RAR-RXR dimers in the presence of retinoic acid(van Wely et al., 2003). MN1's co-activation activity is not restricted to the RAR-RXR nuclear receptor, as MN1 expression inhibits proliferation of an osteoblast cell line via coactivation of the vitamin D receptor(Sutton et al., 2005).

MN1 is also the target of a balanced t(12;22) in myeloid leukemia(Buijs et al., 2000), in which its first exon is fused to *TEL,* a member of the family of ETS transcription factors(Golub et al., 1994). Although TEL generally functions as a site-specific transcriptional repressor(Chakrabarti and Nucifora, 1999; Fenrick et al., 1999; Lopez et al., 1999), MN1-TEL moderately activates transcription of TEL-responsive reporters in transient transfection experiments and the fusion protein has transforming activity in both NIH3T3 fibroblasts(Buijs et al., 2000) and mouse bone marrow (BM) (Carella et al., 2006). The transforming activity in NIH3T3 cells critically depends on DNA binding via the ETS domain of TEL and on the presence of the N-terminal 500 amino acids (aa) of MN1(Buijs et al., 2000). In BM cells the transforming activity also depends on the MN1 N-terminal 500 aa but it is independent of DNA binding(Carella et al., 2006).

Conditional *MN1-TEL* knock-in mice that expressed the gene under the control of *Aml1* regulatory sequences developed lymphoid or myeloid malignancies depending on the nature of the cooperating mutations(Kawagoe and Grosveld, 2005a; Kawagoe and Grosveld, 2005b). This confirmed MN1-TEL's role as a *bona fide* hematopoietic oncogene.

Interestingly, the association of *MN1* with myeloid malignancy might go beyond *MN1*'s involvement in the t(12;22), as the gene was found to be overexpressed in inv(16)(p13;q22) AML(Ross et al., 2004; Valk et al., 2004), in some AMLs overexpressing the transcription factor EVI1(Valk et al., 2004) and in some adult AMLs without karyotypic abnormalities(Heuser et al., 2006). In the latter case overexpression of *MN1* was associated with a worse prognosis and a shorter survival rate(Heuser et al., 2006).

Inv(16) is the chromosomal hallmark of one of two core binding factor (CBF) leukemias and encodes the CBFβ-SMMHC fusion protein(Liu et al., 1995). CBF consists of a CBFβ/RUNX1 heterodimer that regulates genes associated with lymphoid and myeloid differentiation(Otto et al., 2003). The RUNX1 subunit is the target of the recurrent t(8;21) in AML, giving rise to a RUNX1-ETO fusion protein. Both CBFβ-SMMHC and RUNX1-ETO have a dominant-negative effect on CBF function(Castilla et al., 1996; Okuda et al., 1996). This was concluded from the observation that *Runx1* and *Cbfb* knockout mouse embryos and heterozygous *Runx1-ETO* or *Cbfb-SMMHC* knock-in embryos all die at midgestation due to the inability to switch to definitive hematopoiesis(Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996). Mice chimeric for *Runx1-ETO* or *Cbfb-SMMHC* show alterations in multilineage differentiation of hematopoietic cells in the BM but do not spontaneously develop myeloid leukemia(Castilla et al., 1999; Higuchi et al., 2002). Consistent with the notion that leukemogenesis is a multi-step process(Look, 1997), such mice only developed myeloid disease after treatment with the chemical carcinogen N-ethyl-N-nitrosourea (ENU)(Castilla et al., 1999; Higuchi et al., 2002). Retroviral mutagenesis of *Cbfb-SMMHC* chimeric knock-in mice identified the cooperating zinc finger genes *Plag1* and *PlagL2(Castilla et al., 2004)*, which are also overexpressed in 20% of human AML samples with *PLAGL2* preferentially increased in inv(16) leukemia samples(Landrette et al., 2005).

Here we report that mice receiving transplants with BM overexpressing MN1 rapidly developed a fatal myeloproliferative disease, while mice receiving transplants with *Cbfb-SMMHC* chimeric BM overexpressing MN1 developed acute myeloid leukemia. In addition, Q-RT-PCR analysis of AML samples confirmed *MN1* overexpression in inv(16) patient samples but elevated expression was also found in other pediatric AML samples. Our data suggest that MN1 overexpression is an important secondary mutation in inv(16) AML but its upregulation may also contribute to the development of other AML subtypes.

Results

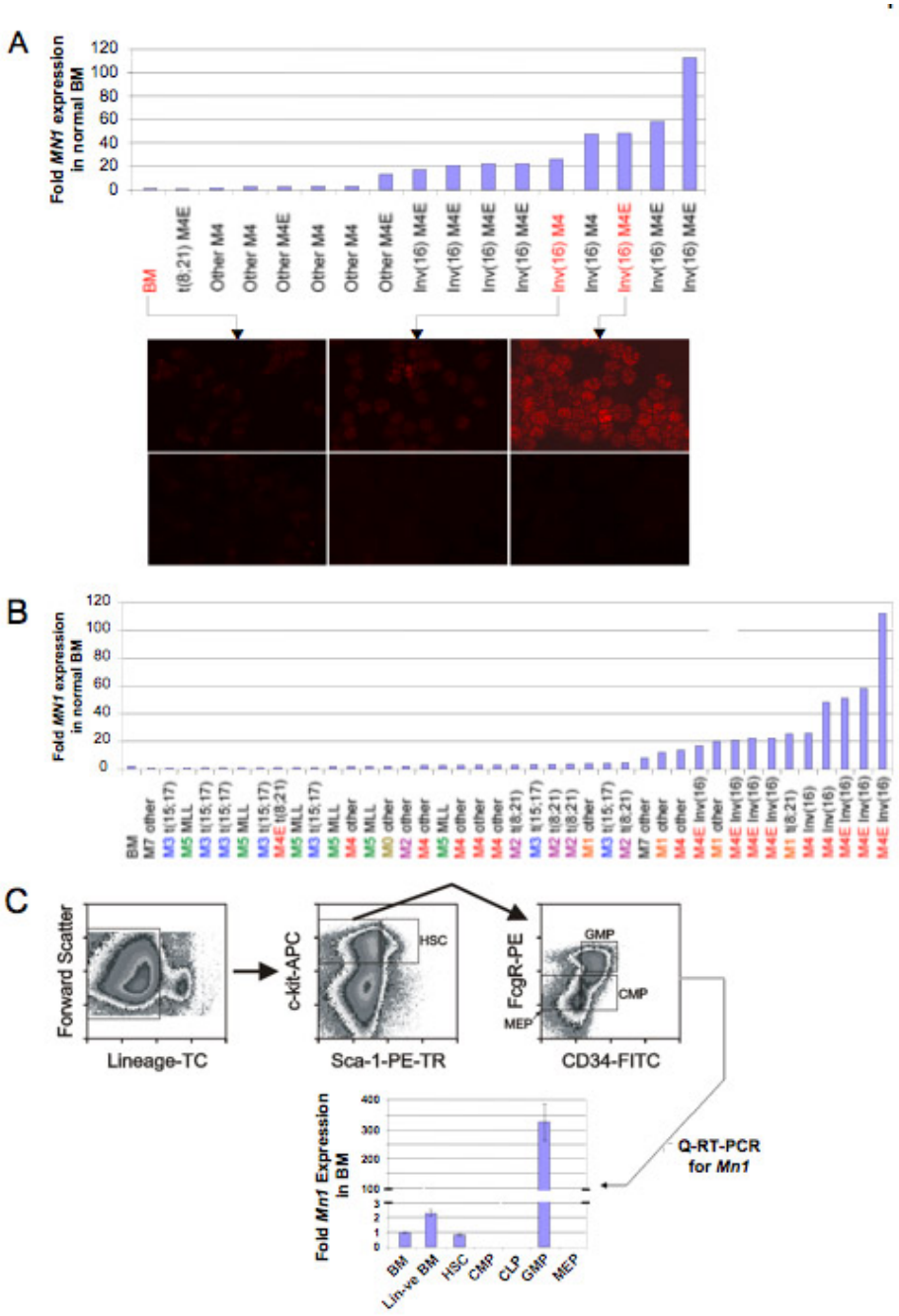
Inv(16) leukemia cells overexpress MN1.

Expression profiling of pediatric and adult patients with AML (Ross et al., 2004; Valk et al., 2004) has shown that BM from patients with inv(16) AML, who express the CBFβ-SMMHC fusion protein, expressed up to 10 times more MN1 mRNA than normal BM. Inv(16) AML is often of the French-American-British classification(Bennett et al., 1991) (FAB)-M4 subtype with eosinophilia. Also AML patients overexpressing EVI1 showed upregulated expression of *MN1*(Valk et al., 2004). We therefore assessed the level of *MN1* expression in 41 pediatric AML BM samples in comparison with expression in normal BM using Q-RT-PCR. This group included: 1 acute undifferentiated leukemia (FAB-M0), 4 acute myeloblastic leukemias [FAB-M1; 1 with t(8;21)], 5 acute myeloblastic leukemias with maturation [FAB-M2; 4 with t(8;21), 1 other], 7 acute promyelocytic leukemias [FAB-M3; all t(15;17)], 16 acute myelomonocytic leukemias [FAB-M4; 7 inv(16) with eosinophilia, 2 inv(16) without eosinophilia, 1 t(8;21) with eosinophilia, 6 other M4)], 6 acute monocytic leukemias (FAB-M5, all MLL translocations), and 2 megakaryoblastic leukemias (FAB-M7). All 9 inv(16) samples, with or without eosinophilia, showed between 17 to112-fold higher *MN1* expression than normal BM, whereas only 1 non-inv(16) FAB-M4 sample showed

Figure 1: *MN1* expression in AML patient samples and mouse bone marrow.

A) Upper panel; BM RNA of pediatric AML FAB-M4 patients [16 in total, of which 10 with eosinophilia (E), including 9 samples with inv(16), 1 with t(8;21) and 6 with other karyotypic abnormalities (other)] were analyzed for expression of MN1 by Q-RT-PCR. Expression levels are depicted as fold expression of *MN1* in normal human BM (BM). The upper row of the 6 fluorescence micrographs shows indirect immunofluorescence detection of MN1, using BM cytospin preparations stained with an MN1 monoclonal antibody of the 2 inv(16) patients (indicated by red labels in the upper panel) and a healthy individual. The lower row shows control staining of the same cytospin preparations using secondary antibody only. **B**) BM RNA of 41 pediatric patients with AML [1 FAB-M0 (olive green), 4 FAB-M1 (orange), 5 FAB-M2 (purple), 7 FAB-M3 (blue), 16 FAB-M4 (red), 6 FAB-M5 (green), and 2 FAB-M7 (black)] with the indicated chromosomal translocations or other karyotypic abnormalities (other) were analyzed for expression of *MN1* by Q-RT-PCR. Expression levels are depicted as fold expression of *MN1* in normal human BM. "MLL" signifies samples with different chromosomal translocations involving the *MLL* gene. "E" indicates eosinophilia. **C**) Mouse BM (FVB) was FACS sorted into fractions representing the hematopoietic stem cells (HSC), the common myeloid progenitors (CMP), the common lymphoid progenitors (CLP), the granulocyte/macrophage progenitors (GMP), and the megakaryocyte/eryrthrocyte progenitors (MEP) using the forward scatter of differentiation lineage-negative cells, expressing or not of the cell surface markers c-Kit, Sca-1, Fc□-receptor and CD34. RNA of each fraction was extracted and subjected to Q-RT-PCR to determine the level of *Mn1* expression in relation to *Hprt*. Expression levels are depicted as fold expression of *Mn1* in unfractionated mouse BM (BM). Lin-ve BM represents a sample containing all progenitors depleted of cells expression lineage markers.

medium to high *MN1* expression (Fig 1A, B and supplementary data Fig 1A). The 6 other FAB-M4 samples showed no (1 t(8;21)M4E) or only moderate upregulation (1.7-2.9 fold; Fig 1A, B). Also the 4 FAB-M1 leukemia samples showed significant *MN1* upregulation (4.1-25.3 fold) (Fig.1B and supplementary data Fig.1B). Of the remaining



21 AML samples 9 showed moderately increased expression (2.2-8 fold) of *MN1*, including all M2 samples, 4 of which carried the t(8;21), whereas 12 samples showed a lower than 2-fold increase in *MN1* expression (Fig. 1B). The latter group contained 5 of 6 FAB-M5 samples harboring MLL translocations, 5 of 7 t(15;17) FAB-M3 samples, 1 of 2 FAB-M7 samples, and 1 FAB-M0 sample.

These results not only confirmed overexpression of *MN1* in inv(16) FAB-M4 but also indicated that the level of *MN1* overexpression substantially exceeded that determined by expression profiling.

Because *Plag1* and *PlagL2* overexpression was shown to cooperate with *Cbfb-SMMHC* in a mouse model of inv(16) leukemia(Castilla et al., 2004) and *PLAGL2* expression was preferentially upregulated in BM samples of inv(16) leukemia patients(Landrette et al., 2005), we tested whether there was a direct correlation between the levels of *MN1* and *PLAGL2* expression in our 9 inv(16) patient samples. Q-RT-PCR for *PLAGL2* mRNA showed that only 1 FAB-M4E sample showed a 4.4-fold higher expression of *PLAGL2* than control BM, whereas the other 8 samples showed equal or lower *PLAGL2* expression than control BM (supplementary data Fig. 1A). This result suggested that upregulation of *MN1* and *PLAGL2* are not functionally linked and appear to be independent genetic events in inv(16) leukemia.

Because Valk and coworkers(Valk et al., 2004) also reported overexpression of MN1 in leukemia samples with upregulated EVI1 expression, we repeated the Q-RT-PCR analysis of the same 41 AML samples for *EVI1* expression. Only 5 samples showed increased expression of this gene (supplementary data, Fig. 1C), and 2 of those (1 FAB-M7 and 1 FAB-M1) also showed upregulated expression of MN1. These data suggest that upregulation of MN1 does occur in EVI1 leukemia but is not an obligatory step.

Using an MN1 monoclonal antibody(Buijs et al., 2000), immunofluorescence detection of MN1 in the BM cells of 2 inv(16) patients and a healthy subject (fold overexpression of *MN1* mRNA patient1: patient2: normal BM = 50: 25: 1; Fig. 1A) indeed confirmed increased speckled staining in the nucleus of tumor cells of patient1 and slightly increased staining in cells of patient2 (Fig. 1A). The same cells incubated with secondary antibody alone showed no staining (Fig. 1A).

To determine whether MN1 upregulation might be the result of gene amplification, we employed Q-PCR of DNA of the same 2 inv(16) patient samples that we used for MN1 immunofluorescence to determine whether their *MN1* copy number was higher than that in normal BM DNA However, no amplification could be detected (not shown).

Expression of mouse *Mn1* in selected mouse BM progenitor populations.

Because MN1 is upregulated in inv(16) leukemia, we wished to address which hematopoietic progenitor cells normally express this gene. To answer this question we used mouse BM and followed the method of Akashi and coworkers(Akashi et al., 2000) to sort populations representing the hematopoietic stem cell (HSC), the common myeloid progenitor (CMP), the common lymphoid progenitor (CLP), the myeloid/erythroid progenitor (MEP) and the granulocyte/monocyte progenitor (GMP) (Fig. 1C). We then determined the amount of *Mn1* mRNA present in these fractionated cell populations by using quantitative (Q)-RT-PCR with whole mouse BM RNA as a control. This showed that *Mn1* mRNA was present at a similar level in whole BM and in the HCS fraction, while Lin^- BM cells were 2-fold enriched in Mn1 expressing progenitors. Analysis of the other fractions showed that this signal was derived from the GMP fraction in which *Mn1* expression is 300-fold higher that in whole BM, whereas there is no *Mn1* expression in the CMP, CLP or MEP fractions. This result opens the possibility that *Mn1* expression in the GMP might be involved in the expansion of the progeny of this fraction and that forced overexpression of *MN1* causes this progenitor population to proliferate abnormally.

Overexpression of MN1 in mouse BM stimulates outgrowth of myeloid cells and produces immortalized cell lines.

We reported that MN1-TEL transforms NIH3T3 fibroblasts, an activity dependent on both the presence of MN1 N-terminal sequences and a functional TEL DNA binding domain (Buijs et al., 2000). Paradoxically, the self-renewal activity of mouse BM cells was equally stimulated by MN1-TEL as by an MN1-TEL mutant with a non-functional ETS DNA binding domain, as measured by colony forming assays in semisolid medium(Carella et al., 2006). To determine if MN1 alone also possessed this capacity, BM transduced with murine stem cell virus (MSCV) expressing MN1-IRES-GFP (30% GFP^+)(Fig 2A) was plated in methylcellulose and its colony forming activity compared with that of BM transduced with MSCV-IRES-GFP. In the first methylcellulose assay (MC1), MSCV-MN1-IRES-GFP-transduced cells gave twice as many colonies as vector-transduced BM (Fig. 2A). Upon serial replating (MC2, MC3, MC3), the number of $MN1^+$ BM and vector BM colonies dropped to equal numbers in the MC2 but the number of $MN1^+$ colonies increased drastically in the MC3 and MC4 whereas vector-transduced cells produced no colonies. Fluorescence-activated cell sorting (FACS) analysis of cells recovered from the MC3 culture revealed that >95% of cells were GFP^+ (not shown). This together with the observation that MN1-overexpressing cells recovered from the MC1 grew rapidly in liquid culture, and were 98% GFP^+ within 4 weeks of culture, strongly suggested that MN1 overexpressing cells acquired a distinct growth advantage over that of non-transduced cells. Immunofluorescence analysis with

an MN1 antibody showed mostly nuclear, punctate MN1 signals in all cells (Fig. 2B), whereas vector transduced cells showed only faint GFP fluorescence encoded by the retroviral vector.

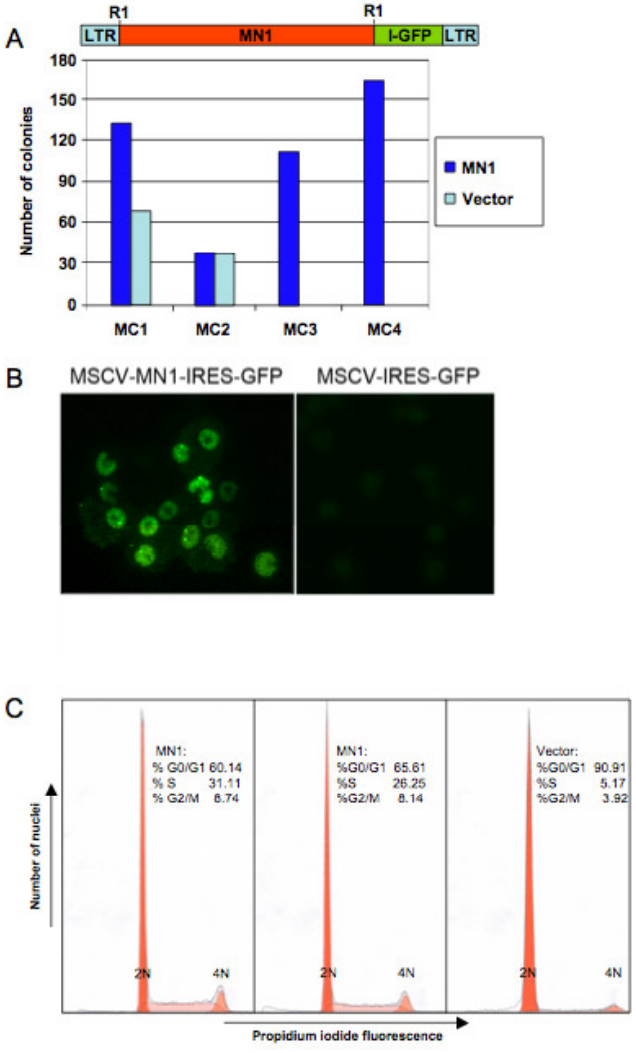


Figure 2: Mouse BM transduced with MN1 retrovirus shows increased proliferation and generates cytokine-dependent cell lines.

A) The map at the top depicts the structure of the MSCV-MN1-IRES-GFP retroviral vector used to transduce mouse BM. The MN1 open reading frame (MN1) is fused to IRES-GFP (I-GFP), flanked by MSCV long terminal repeat (LTR) sequences. As control we used the same vector not containing MN1 sequences. R1 indicates *EcoRI* sites flanking the insert. BM form C57Bl/6/129svJ mice, transduced with MSCV-MN1-IRES-GFP (MN1) or MSCV-IRES-GFP retrovirus (vector), were serially plated in methylcellulose semi-solid medium (MC1, MC2, MC3, MC4) and at each step colonies were counted after 2 weeks of culture. At each serial replating 10^3 cells from one MC were plated into the next MC and colonies were scored 2 weeks later. MN1 expressing BM replated much better than vector-transduced and produced more colonies BM. **B**) Fluorescence micrograph of an MSCV-MN1-IRES-GFP-transduced cell line and MSCV-IRES-GFP-transduced BM stained with an MN1 antibody. **C**) Flow cytomeric analysis by FACS of the DNA content of propidium iodide stained nuclei from 2 independent MSCV-MN1-IRES-GFP-transduced cell lines (MN1) and MSCV-IRES-GFP-transduced BM (Vector), cultured for 2 months after transduction of the BM. MN1-overexpressing cells proliferate much faster than vector-transduced BM cells.

Cell surface marker analysis showed that the cells were cKit+/Sca1+/Mac1+ with 10% of the cells also expressing Gr1 (not shown). Cells cultured this way were immortalized but their growth and survival was strictly dependent upon addition of the cytokines IL3 and SCF to the culture (not shown). Cell cycle analysis using flow cytometry of 2 independent *MN1* cell lines and vector-transduced BM, all cultured for 2 months after transduction, showed that the fraction of MN1-transduced cells in the S- and G2/M-phase of the cell cycle was drastically increased compared to that of vector-transduced cells (Fig. 2C). This showed that MN1 overexpression strongly stimulates cell cycle traverse.

Mice receiving transplants of BM transduced with *MN1* retrovirus rapidly develop myeloproliferative disease (MPD).

We next tested the effect of MN1 overexpression on mouse BM in vivo. Lethally irradiated C57Bl/6/129svJ mice (n=14) died of fulminant hematopoietic disease 5-8 weeks after receiving transplants of 3-5 x 10^5 MSCV-*MN1-IRES-GFP*-transduced (Fig 3A) C57Bl/6 Lin- BM cells (60% GFP^+), whereas mice receiving transplants with the same number of MSCV-*IRES-GFP* C57Bl/6 Lin- BM cells (60% GFP^+) (n=5) remained healthy (Fig. 3A). The average white blood cell count (WBC) of diseased mice was 1.5 x 10^7/ml, and their peripheral blood contained large numbers of neutrophils, neutrophil precursors and some blast-like cells (Fig. 3B) all expressing GFP (Fig. 3E). Southernblotting of the BM DNA of 3 of the diseased mice probed with IRES-GFP showed multiple integrations of the MSCV-MN1-IRES-GFP retrovirus (Fig. 3C), suggesting that the disease was oligoclonal. Malignant cells invaded the spleen, liver, and brain (Fig. 3D), and most of the mice died of brain hemorrhage that was probably caused by the high WBC. FACS analysis showed that BM and spleen were each composed of a single population of $Mac1^+/Gr1^+$ cells (Fig. 3E). Together, these features suggested that the mice suffered from a myeloproliferative disease (MPD).

Transplantation of pooled 5 x 10^5 BM cells from 2 of the diseased MN1 mice into 5 sublethally irradiated secondary recipients recreated the same fulminant MPD 18-23 days later (not shown), suggesting the disease was cell-autonomous. We conclude that *MN1* is a highly efficient oncogene that strongly promotes the growth of myelomonocytic cells but does not substantially inhibit their differentiation.

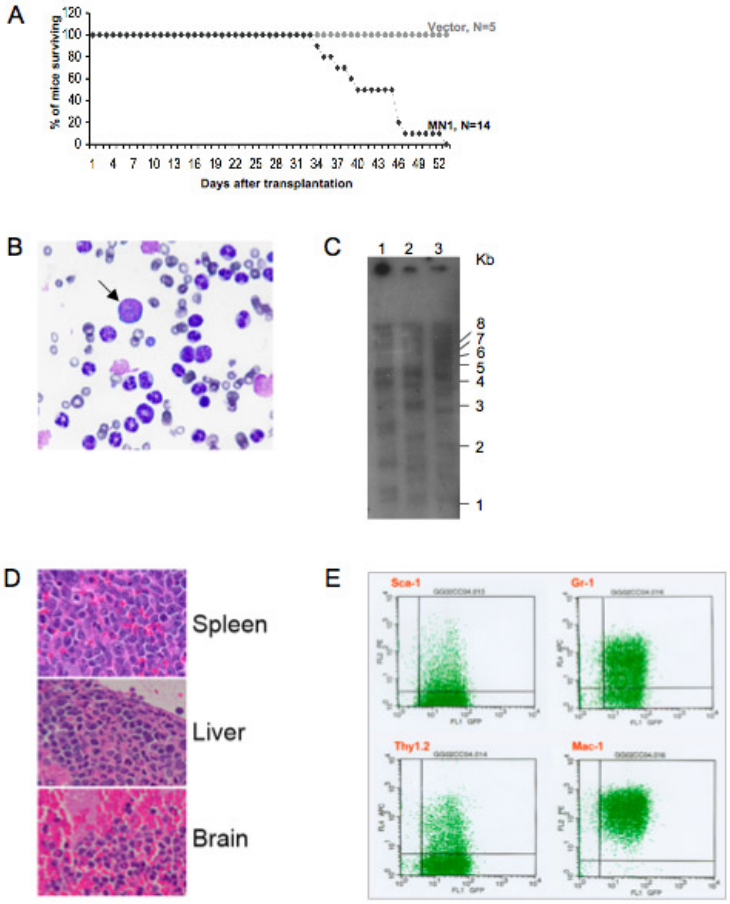


Figure 3: Mice receiving transplants of MSCV-MN1-IRES-GFP-transduced BM rapidly develop myeloproliferative disease (MPD).

A) Survival curve of lethally irradiated C57Bl/6 mice receiving transplants of syngeneic BM transduced with MSCV-MN1-IRES-GFP (MN1, n=14) or MSCV-IRES-GFP (Vector, n=5) retrovirus. Mice receiving MN1-overexpressing BM died of hematopoietic disease between 34-52 days after transplantation, whereas vector-transplanted mice remained healthy. **B**) Peripheral blood smear of a representative moribund MN1-BM-transplanted mouse (average WBC is 150 x 10^7/ml) showing large numbers of partially and fully differentiated neutrophils and some blast-like cells (arrow), showing the mice suffered of a myeloproliferative disease (MPD). **C**) Southernblot of BM DNA of 3 MPD mice overexpressing MN1, digested with EcoRI and hybridized with a GFP probe. All three samples contain multiple retroviral integrations, suggesting that the disease is oligoclonal. **D**) Malignant cells invaded the spleen and liver. All diseased mice showed brain hemorrhages, often causing sudden death. Secondary recipients receiving transplants of diseased BM rapidly (18-23 days) developed the same disease (not shown). **E**) FACS analysis of cell surface markers of MPD BM showing that cells were GFP+/Gr-1+/Mac-1+ and some also expressed Sca-1 and/or Thy-1. Cells were negative for c-Kit.

Coexpression of Cbfβ-SMMHC and MN1 causes AML in mice.

Because MN1 is consistently overexpressed in inv(16) AML, we tested whether its overexpression in BM of mice expressing Cbfβ-SMMHC cooperates to cause AML. Chimeric mice generated with *Cbfb-SMMHC* knock-in ES cells do not spontaneously develop hematopoietic disease and hematopoietic cells expressing the Cbfβ-SMMHC fusion protein fail to differentiate and remain in the BM(Castilla et al., 1999). We used *Cbfb-SMMHC* ES cells to generate 10 highly chimeric mice. Lin- BM cells from these mice were transduced with *MN1-IRES-GFP* retrovirus (30% GFP^+) and transplanted into 12 lethally irradiated C56Bl/6 recipients. As a control, we transplanted chimeric BM transduced with control *IRES-GFP* retrovirus (45% GFP^+) into 6 irradiated recipients. Given that all our human inv(16) samples overexpressed *MN1*, it was possible that transcription of *MN1* is regulated by Cbfβ-SMMHC. Therefore, we determined by Q-RT-PCR whether the level of endogenous *Mn1* mRNA in MSCV-IRES-GFP-transduced chimeric mouse BM was more abundant than in similarly transduced wild type BM. As shown in Fig. 4A *Mn1* expression in both types of BM was similar, opposing the possibility that Cbfβ-SMMHC directly or indirectly upregulated *Mn1* expression in mouse BM.

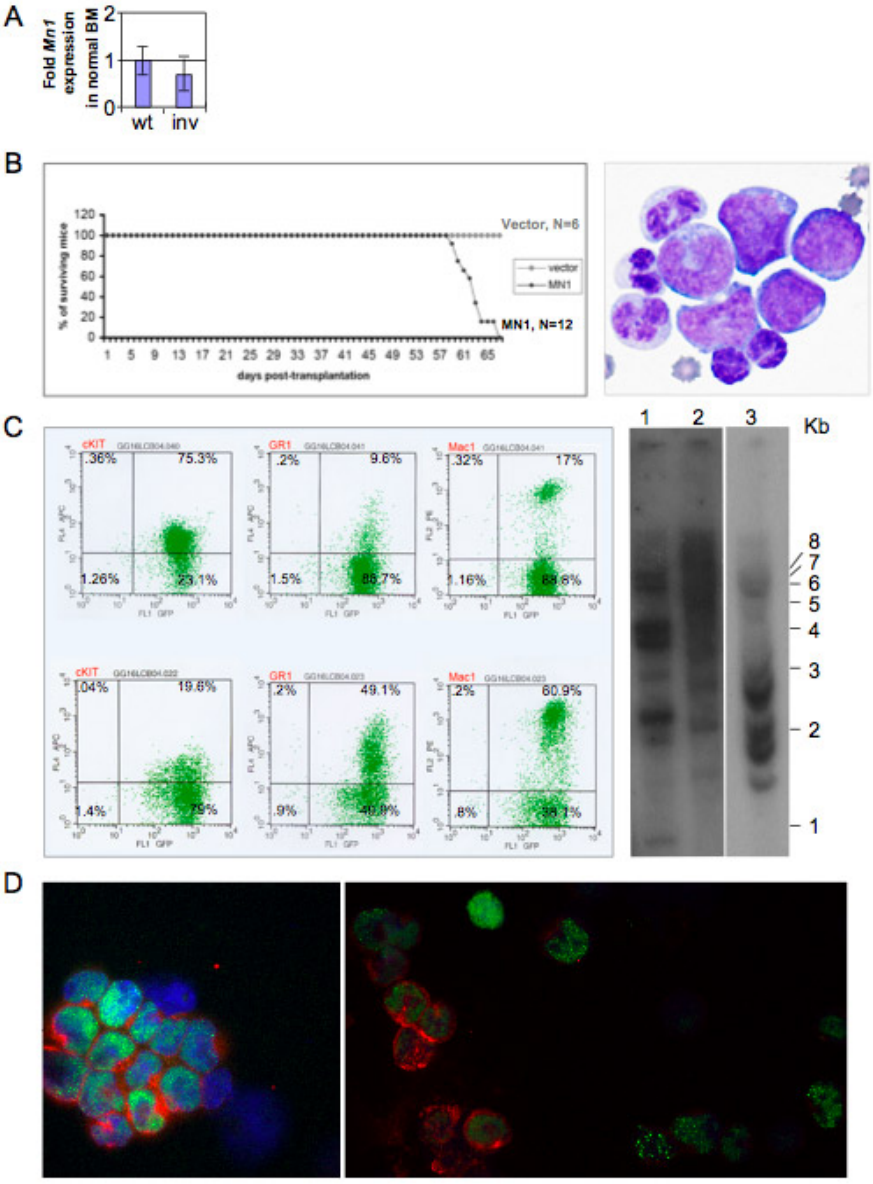
All 12 mice receiving inv(16)/MN1 transplants died of hematopoietic disease 58-68 days after transplantation, whereas the 6 mice given vector-transduced inv(16) transplants remained well (Fig. 4B). Leukemic cells infiltrated the spleen and lymph nodes and effaced their normal architecture (not shown). In addition myeloid cells infiltrated the liver, brain, uterus, lungs, stomach and heart (not shown). Southern blot analysis of BM DNA of 3 of these mice with a GFP probe showed multiple integrations (Fig. 4C), suggesting the disease was oligoclonal (see discussion). The diseased mice could be divided into those whose peripheral blood contained a predominance of blast cells (Fig. 4B) and those whose peripheral blood contained partly blast cells and partly more differentiated myeloid cells (neutrophils and neutrophil precursors). Cell surface marker analysis by FACS (Fig. 4C) showed that BM cells of the former type mostly expressed no markers other than cKit, with a small percentage of cells expressing Mac1 and Gr1, whereas the BM of the latter mice contained fewer $cKit^+$ cells and more $Mac1^+$ and $Gr1^+$ cells. The expression of cKit in the MN1/Cbfβ–SMMHC leukemic cells was in sharp contrast with the malignant cells of mice that received *MN1*-transduced BM, which expressed Mac1 and Gr1 but not cKit (Fig. 3E). This result is similar to *Cbfb-SMMHC* chimeric mice treated with ENU which also developed a cKit+ AML(Castilla et al., 1999). GFP signal was present in 99% of all cells (Fig. 4C). Immunofluorescense using an MN1 antibody and an antibody to the CBFβ-SMMHC fusion breakpoint(Wijmenga et al., 1996) to double label cytospin preparations of BM from both types of mice showed that in mice with mostly blasts in the peripheral blood, most BM cells expressed both MN1 and CBFβ-SMMHC, whereas mice with fewer blasts and more differentiated cells in the PB also contained cells in the BM expressing

MN1-only (Fig. 4D). This suggested that mice whose peripheral blood contains more differentiated cells have a mixed AML/MPD in which the AML arose from inv(16) cells overexpressing MN1 and the MPD from wild-type cells overexpressing MN1, whereas mice with mostly blast cells in the peripheral blood have inv(16)/MN1 AML.

We next assessed whether the level of *MN1* expression in the BM of our MN1/Cbfβ-SMMHC transplanted mice would be comparable with that in inv(16) patient BM. Using Q-RT-PCR with human *MN1* primers (our mouse *Mn1* primers do not amplify human *MN1* cDNA, not shown), we determined the amount of *MN1* mRNA in the leukemic BM of 4 Cbfb-SMMHC/MN1 transplanted mice, in BM of 3 inv(16) patients and in the inv(16) M4E cell line ME-1(Yanagisawa et al., 1991). This showed that *MN1* expression in these 4 Cbfb-SMMHC/MN1 BM samples was 0.7-3.9 fold as abundant as in the patient sample with the highest *MN1* expression (Figs. 5 and 1A). Thus, *MN1* expression in the BM of 3 of the mice was considerably higher than in inv(16) patients but in one mouse comparable expression also appeared sufficient to provoke disease.

Figure 4: Mice receiving transplants of chimeric *Cbfb-SMMHC* BM transduced with MSCV-MN1-IRES-GFP develop AML.

A) RNA from normal mouse BM (wt) and chimeric inv(16) BM (inv), both transduced with MSCV-IRES-GFP virus was analyzed by Q-RT-PCR for the expression of endogenous *Mn1*. The values are the average of 2 independent experiments. *Mn1* expression is not upregulated in inv(16) chimeric BM. **B**) Survival curve showing that lethally irradiated C57Bl/6 mice receiving transplants of chimeric *Cbfb-SMMHC* BM transduced with MSCV-MN1-IRES-GFP (n=12) developed hematopoietic disease 58-68 days after transplantation, while those receiving transplants of the same BM transduced with MSCV-IRES-GFP (n=6) retrovirus remained healthy. The blood smear to the right shows the peripheral blood of a mouse with AML with a preponderance of blast cells, but also showing neutrophil progenitors. **C**) Left panel: FACS cell surface marker analysis of the BM of a mouse with a preponderance of blast cells in the peripheral blood, with 75.3% of cells expressing c-Kit, 9.6% of cells expressing Gr-1, and 17% Mac-1 (upper 3 plots) and of the BM of a mouse with a lower number of blast cells in the peripheral blood showing 19.6% of cells expressing c-Kit, and 49.1% of cells expressing Gr-1 and 60.9% of cells expressing Mac-1 (lower 3 plots). More than 98% of cells express GFP. Right panel: Southern blot of BM DNA of 4 leukemic MN1/Cbfβ-SMMHC-transplanted mice (1-4), digested with EcoRI and hybridized with a GFP probe. All 4 samples contain multiple retroviral integrations. Due to DNA overloading of sample 4 a shorter exposure of this lane is shown. **D**) Fluorescence micrographs of BM cytospin preparations of a diseased mouse containing mainly c-Kit+ BM cells (left panel) and a mouse containing partial c-Kit+ BM cells (right panel), double stained with antibodies specific for the CBFβ-SMMHC fusion peptide (red), and MN1 (green). Most cells in the left panel are positive for both CBFβ-SMMHC (cytoplasm) and MN1 signals (nucleus), whereas only part of the cells in the right panel are positive for both signals with the remainder of the cells only positive for the MN1 signal. The nuclei of the cells in the left panel were counter stained with DAPI (blue).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Fold Mn1 expression in normal BM
2
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% of surviving mice
120
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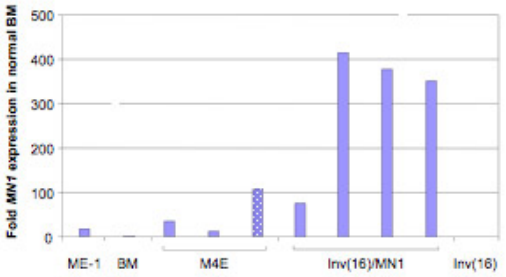

Figure 5: Comparison of *MN1* expression in BM of inv(16) patients and diseased mice transplanted with Cbfβ-SMMHC/MN1-expressing BM.

Q-RT-PCR with primers specific for human *MN1* was used to determine the level of *MN1* RNA expression in the human inv(16) cell line ME-1 (ME-1), in pooled BM of healthy individuals (BM), in BM of 3 inv(16) patients (M4E) and in BM of 4 diseased Cbfβ-SMMHC/MN1-transplanted mice. As a negative control we used BM of a mouse transplanted with vector-transduced Cbfβ-SMMHC chimeric BM [inv(16)]. Expression levels are depicted as fold expression of *MN1* in normal human BM. *MN1* expression in BM of Cbfβ-SMMHC/MN1-transplanted mice was between 0.7-3.9 fold higher than in BM of the inv(16) patient with the highest level of *MN1* expression (speckled box M4E).

Supplementary data

MN1 overexpression is a prerequisite for the development of inv(16) AML.

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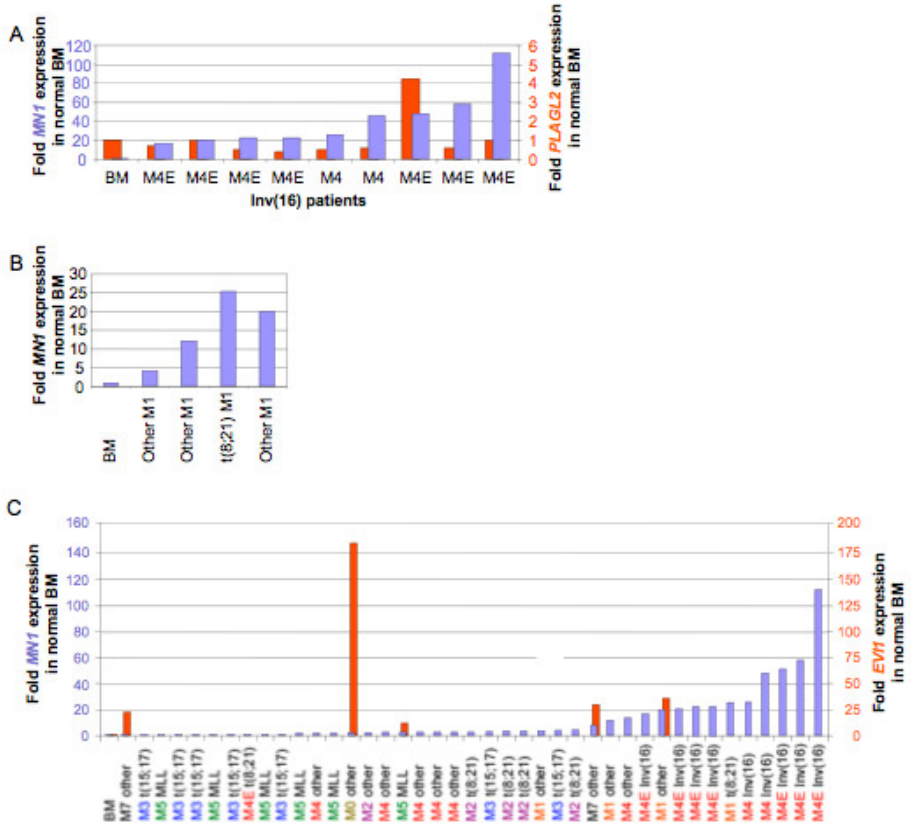

Supplementary data Figure 1. *MN1* and *PLAGL2* expression in pediatric inv(16) FAB-M4 patients, *MN1* expression in FAB-M1 patient samples, and *MN1* and *EVI1* expression in 41 pediatric AML samples.

A) BM RNA of pediatric inv(16) FAB-M4 patients with (M4E) or without eosinophilia (M4) and of a pool of 8 normal donors was analyzed for expression of *MN1* and *PLAGL2* by Q-RT-PCR. The histogram in purple shows the expression level of *MN1* in patient samples, depicted as fold expression of *MN1* in normal human BM (BM), the histogram in orange shows the expression level of *PLAG2* in the same patient samples, depicted as fold expression of *PLAGL2* in normal human BM (BM). All values were normalized for GAPDH expression. Note that the scales for *MN1* (left, purple) and *PLAGL2* (right, orange) expression are different. All inv(16) BM samples overexpress *MN1*, but only one sample overexpresses both *MN1* and *PLAGL2.* **B**) BM RNA of pediatric FAB-M1 patients or of a pool of 8 normal individuals was analyzed for expression of *MN1* by Q-RT-PCR. All 4 FAB-M1 patients overexpress *MN1*. Values were normalized for GAPDH expression **C**) BM RNA of pediatric AML patients of different FAB-classification (M0-M7) or of a pool of 8 normal donors was analyzed for expression of *MN1* and *EVI1* by Q-RT-PCR. The histogram in purple shows the expression level of *MN1* in the different samples, depicted as fold expression of *MN1* in normal human BM (BM), the histogram in orange shows the expression level of *EVI1* in the same patient samples, depicted as fold expression of *EVI1* in normal human BM (BM). All values were normalized for GAPDH expression. Only 2 patient samples coincidently overexpressed *MN1* and *EVI1* (one M7 and one M1).

Discussion

Myeloid leukemogenesis is a multi-step process(Look, 1997) and it has been well documented that in AML, specified by recurrent chromosome translocations, cooperating mutations are essential for disease development(Ayton and Cleary, 2003; Castilla et al., 1999; Castilla et al., 2004; He et al., 2000; Kawagoe and Grosveld, 2005a; Largaespada, 2000). In expression profiling experiments, the level of *MN1* mRNA was specifically elevated in BM samples of pediatric and adult patients with inv(16) AML(Ross et al., 2004; Valk et al., 2004). *MN1* upregulation was also found in a subtype of AML associated with very poor prognosis(Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2005), which is defined by overexpression of the immortalizing(Du et al., 2005) transcription factor EVI1(Valk et al., 2004). Given that MN1 sequences confer oncogenic properties to the MN1-TEL fusion protein(Buijs et al., 2000; Carella et al., 2006), the product of the t(12;22) in AML, it opened the possibility that overexpression of *MN1* in these AML subtypes actively contributed to the leukemic process. This suggestion was supported by the observation that retrovirus-mediated overexpression of MN1 in mouse BM boosted proliferation of myeloid cells, allowed the establishment of myeloid cell lines in vitro, and caused rapid development of MPD after transplantation into lethally irradiated mice. Moreover, the MN1 effects were cell-intrinsic as secondary recipients receiving transplants of BM of diseased primary recipients rapidly developed the same disease. Despite the rapid development of the primary MPD Southern blot analysis of the malignant cells showed that the disease was oligoclonal rather than polyclonal, which implied that additional genetic changes must have occurred for the disease to develop. Currently we do not know the nature of these additional genetic changes.

Expression of endogenous *Mn1* is present in the sorted HSC but not in the CMP CLP, MEP progenitor compartments. Given the profound proliferative effect of human MN1 expression on GMP-derived cells in mouse BM, we speculate that expansion of this compartment might be dependent on endogenous upregulation of *Mn1*. Therefore, forced expression using the *MN1* retroviral vector would lead to over-expansion. Why MN1 overexpression specifically affects the proliferation of GMP-derived but not MEP-derived cells is currently unknown. *Mn1* knockout mice have defects in the development of membranous bones of the cranial skeleton(Meester-Smoor et al., 2005) but whether these mice also harbor hematopoietic defects is a question we are currently addressing.

We also do not know via which mechanism MN1 stimulates growth of myelomonocytic cells. We reported that in Hep3B cells the protein is recruited to RAR/RXR dimers via the coactivators p300/CBP and RAC3(van Wely et al., 2003) and stimulates the transcription activity of RAR/RXR in the presence of retinoic acid. It is

possible that in myeloid cells MN1 participates in a similar protein complex or in protein complexes with other transcription factors that recruit p300/CBP, such as MYB, RUNX1, GATA1, 2, and 3, C/EBP, PU.1, and MLL(Blobel, 2000). Growth stimulation of myeloid cells by MN1 is opposite to its reported effects in an osteoblast cell line(Sutton et al., 2005), in which MN1-mediated coactivation of the vitamin D receptor inhibited proliferation. Growth inhibition of several types of epithelial cells by TGF-β, an inhibitor of epithelial cell proliferation, is also associated with induction of MN1 expression(Chen et al., 2001; Kang et al., 2003). This profound difference in response to MN1 upregulation can only be explained if MN1 can be recruited into different transcription factor complexes whose effects are cell type specific.

Previously we reported that BM of MN1-TEL knock-in mice showed increased self-renewal activity of myeloid progenitors(Kawagoe and Grosveld, 2005a) and produced myeloid cell lines in vitro. The same effects were observed with BM transduced with MN1-TEL retrovirus. In both scenarios MN1-TEL cell lines displayed a more primitive phenotype (cKit+/Sca1+) (Carella et al., 2006; Kawagoe and Grosveld, 2005a) than the MN1 cell lines (cKit+/Sca1+/Mac1+) reported here. We speculate that this difference is caused by the presence of TEL sequences in the fusion protein, which might recruit MN1-TEL to TEL recognition sites at promoter/enhancer areas of genes that inhibit differentiation.

As determined by Q-RT-PCR, *MN1* mRNA levels in inv(16) FAB-M4 samples were much greater than that determined by expression profiling. Q-RT-PCR analysis showed that *MN1* expression in our 9 inv(16) patient samples was on average 43.7 fold higher than in normal BM, while estimations using array analysis suggested an average 4.6-(Ross et al., 2004) or 9.2-fold(Valk et al., 2004) increase in *MN1* mRNA. This discrepancy leads to a distinct underestimation of MN1 expression using expression arrays. Indeed, inclusion of other AML subtypes in our Q-RT-PCR analysis showed substantial upregulation of MN1 in FAB-M1 samples (14.1-fold average) and moderate (2 to 8-fold) upregulation in all M2-, 1 M7-, 2 M3- and 1 M5 sample(s), not reported in the study by Ross et al., 2004 (Ross et al., 2004), which included the very same patient samples. *MN1* expression was also moderately increased in all but 2 non-inv(16) FAB-M4 samples. Q-RT-PCR of a much larger group of AML samples will have to determine whether it will support our initial results. Given the small numbers of patients analyzed it was impossible to obtain a statistically significant correlation between levels of MN1 expression and the survival rates of inv(16) and FAB-M1 patients. Nonetheless, it was reported that *MN1* overexpression in adult patient samples with a normal karyotype correlated with a worse prognosis and a shorter survival rate(Heuser et al., 2006).

Immunofluorescence analysis with an MN1 monoclonal antibody of 2 of our inv(16) BM samples, expressing 50 and 25-fold more *MN1* mRNA than normal BM, showed that the MN1 signal was elevated in leukemic cells but the difference was

much more modest than expected from the increase in *MN1* mRNA levels. Possible explanations could be that not all *MN1* mRNA is translated in these AML cells, or alternatively that MN1 over expression increases its protein turnover. To date all inv(16) patient samples analyzed showed upregulated *MN1* expression(Ross et al., 2004; Valk et al., 2004; this manuscript), strongly suggesting that overexpression of *MN1* is an obligatory step in the development of this disease.

An important question that remains to be answered is via which molecular mechanism *MN1* overexpression is obtained. Based on the observation that expression of the endogenous mouse *Mn1* gene was similar in Cbfb-SMMHC chimeric and normal BM (Fig 4A) we do not think that the gene is a direct transcriptional target of the CBF transcription factor. Given that the promoter regions of the mouse and human genes show extensive sequence conservation, their transcriptional regulation is likely to be similar. Also, *MN1* upregulation was reported in AML patients with a normal karyotype(Heuser et al., 2006), further indicating that other genetic changes than expression of CBF fusion proteins are responsible for *MN1* overexpression. Using PCR amplification of *MN1* first exon sequences in the genomic DNA of the two patient samples that overexpress MN1 protein (Fig1 A, indicated in red) and of normal BM sample revealed a diploid copy number in all 3 samples (not shown). Therefore the increased MN1 expression in these 2 patients was not the result of *MN1* gene amplification.

Cbfβ-SMMHC chimeric knock-in mice do not develop myeloid leukemia(Castilla et al., 1999; Landrette et al., 2005), whereas mice carrying a conditional *Cbfβ-SMMHC* knock-in gene do develop disease within 3-6 months after induction. This difference is most likely caused by the increased size of the Cbfβ-SMMHC^{+} preleukemic progenitor pool in the BM of the conditional knock-in mice(Kuo et al., 2006). Thus, the increased proliferative capacity of MN1 overexpressing cells may enlarge the pool of inv(16) cells enough to promote additional mutations allowing the leukemia to emerge. We favor this possibility because Southern blotting suggested that mouse inv(16)/MN1 leukemia is oligoclonal rather than polyclonal, although our analysis cannot exclude that the oligoclonality derives from the concomitant MN1-induced MPD in these transplanted mice. Irrespective of whether the MN1/inv(16) disease is monoclonal or oligoclonal the finding strongly suggests that additional mutations must occur during AML development in the transplanted mice. Known candidate genes are *Plag1* and *PlagL2*, which were identified as *Cbfb-SMMHC* cooperating genes in mouse inv(16) AML(Landrette et al., 2005), and were found to be upregulated in human inv(16) AML(Valk et al., 2004). We do not think *Plag1* or *PlagL2* are transcriptional targets of MN1 because the mRNA levels of these genes were not increased in Q-RT-PCR analysis of MN1 overexpressing mouse cell lines, MN1 MPD BM, or inv(16)/MN1 BM. The same holds true for *PLAGL2* in inv(16) leukemia as we did not find any correlation between the levels of *PLAGL2* and *MN1* expression in our patient samples

(supplementary Fig. 1C), of which only one showed increased expression of *PLAGL2*. We also investigated the mutation status of the *Npm* gene in BM of the 3 MN1 MPD mice shown in Fig. 3C and the 4 inv(16)/MN1 AML mice shown in Fig. 4C. Mutation in codons 288 or 290 of NPM causes relocalization of the protein from the nucleolus to the cytoplasm in 35% of AML patients (Grisendi and Pandolfi, 2005), which affects the p53 tumor suppressor pathway activity (den Besten et al., 2005). Sequencing of PCR-amplified *Npm* cDNA of these 8 mice showed that the gene was not mutated (not shown).

Given the cooperation between MN1 overexpression and CBFβ-SMMHC in inv(16) AML, interference with MN1 function might provide a novel therapeutic approach for this CBF leukemia. In addition, it will be interesting to determine whether MN1 overexpression similarly promotes leukemia development in a mouse model for t(8;21) AML, which also targets the Cbf transcription factor.

Together our experiments suggest that the differentiation-inhibiting protein Cbfβ-SMMHC and the proliferation-stimulating protein MN1 cooperate in the development of AML in this mouse model. Given that *MN1* expression is upregulated in all inv(16) patients investigated (Ross et al., 2004; Valk et al., 2004; this study), it is reasonable to speculate that these 2 proteins play a similar cooperative role in human inv(16) AML.

Experimental procedures:

Patient materials

All patients and patient materials used in this paper have been described previously(Ross et al., 2004). Informed consent for the use of the leukemic cells for research was obtained from parents, guardians, or patients (as age-appropriate) in accordance with the Declaration of Helsinki, and study approval was obtained from the SJCRH institutional review board (IRB).

Plasmids and retrovirus production

MN1 cDNA (Buijs et al., 2000) was cloned into the EcoRI site of MSCV-IRES-GFP and high-titer ecotropic virus ($5x10^5$ - $1x10^6$cfu/ml) was obtained as described(Cardone et al., 2005). As a control we used MSCV-IRES-GFP virus without cDNA insert.

Bone marrow transplantation.

For transplantation of MSCV-MN1-IRES-GFP transduced wild type BM into C57Bl/6 females, BM was harvested from femurs and tibiae of 8 week-old male C57BL/6 or C57BL/6/129svJ mice treated with 5-fluorouracil (5FU). 5FU injection, isolation of Lin- cells, viral transduction and BM transplantation into lethally irradiated female recipients was performed as described previously(Cardone et al., 2005). For transplantation of inv(16) chimeric BM, heterozygous *Cbfb-SMMHC* knock-in ES cells(Castilla et al., 1996) were injected into C57BL/6 blastocysts and 10 highly chimeric male mice were generated. After treatment with 5-FU, BM of these mice was isolated, transduced with MSCV-MN1-IRES-GFP or MSCV-IRES-GFP retrovirus, yielding cell populations that were 30% and 60% GFP^+, respectively. The MSCV-MN1-IRES-GFP-transduced cells were transplanted into 12 lethally irradiated female C57BL/6 recipients and the MSCV-IRES-GFP control cells into 6 such recipients.

Immunofluorescence

Cytospin preparations of BM of 2 pediatric inv(16) patients from the St Jude Children's Research Hospital tumor bank and BM of a healthy donor were fixed with 4% paraformaldehyde for 4 minutes. Cells were permeabilized with 0.2% Triton-X100 and incubated with MN1 monoclonal antibody 2F2(Buijs et al., 2000), followed by incubation with a CY3-labeled goat-anti mouse secondary antibody (1/400 dilution). MN1 staining of cytospin preparations of *MN1*-transduced BM-derived cell lines was performed following the same procedure. Fluorescent images of cytospin preparations were obtained using a BX-50 microscope (equipped with a UPlanFI 40X/0.75 or 100X/1.30 numeric aperture objectives, Olympus, Tokyo, Japan) with a SPOT camera and SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI).

Cell cycle analysis by FACS.

Cell cycle analysis of *MN1*-transduced BM cell lines and vector-transduced control BM cells was performed as described previously(Cardone et al., 2005).

Secondary bone marrow transplantation

Secondary BM transplantation of leukemic BM was performed as described previously(Carella et al., 2006).

Hematopoietic progenitor assays.

Sequential methylcellulose-based cultures (MC1-4) of BM cells was performed using MethoCult GFM3434 (StemCell Technologies, Vancouver, BC), containing mouse SCF (50 ng/ml), mouse IL-3 (10 ng/ml), human IL-6 (10 ng/ml), and human erythropoietin (3 units/ml) and were performed as described(Carella et al., 2006).

Analysis of diseased mice and tissue preparation

All animal procedures were conducted in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Collection of blood, GFP analysis of peripheral blood (PB), BM, spleen, and liver cell suspensions, euthanasia of diseased animals, tissue collection, fixation, paraffin embedding, sectioning and histological staining and May-Grunwald-Giemsa staining of PB were performed as described(Carella et al., 2006). Select tissues were also processed for immunohistochemical analysis with antibodies to hematopoietic phenotype markers: CD3 (Dako, Carpinteria, CA), CD45R/B220 (PharMingen, San Diego CA), terminal deoxynucleotidyl transferase (TdT, Supertechs, Bethesda, MA), myeloperoxidase (MPO, Dako, Carpinteria, CA), TER 119 (PharMingen, San Diego, CA), GATA. (Santa Cruz, Santa Cruz, CA) and green fluorescent protein (GFP, Clontech, Palo Alto, CA).

Cell surface markers

Marker analysis by FACS of single-cell suspensions of BM and spleen was performed as described(Carella et al., 2006). Cells were incubated with monoclonal antibodies (CD3c, CD4, CD8, CD11b/Mac1, CD19, CD34, B220, TER-119, Gr1, Sca1, c-Kit, Flt3, all from Pharmingen, San Jose, CA; anti–mouse IgM from Southern Biotechnology Associates, Birmingham, AL) on ice for 30 minutes. Stained cells were analyzed using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Sorting of mouse BM for stem cell and progenitor fractions

Following the methods of Akashi et al. (Akashi et al., 2000) different mouse BM progenitor populations representing the hematopoietic stem cell (HSC), the myeloid/erythroid progenitor (MEP), the common myeloid progenitor (CMP), the granulocyte/monocyte progenitor (GMP) and the common lymphoid progenitor (CLP)

were isolated and their identity verified by RT-PCR analysis for the expression of signature genes(Akashi et al., 2000).

Q-RT-PCR

The reverse trancriptase reactions of patient BM RNA were done with 50ng total RNA in 20ul total volume using TaqMan Reverse Transcription Reagent (Applied Biosystems) following the manufacturer's recommendations.

Primers and probes for genes were chosen with the assistance of the computer program Primer Express (PE Applied Biosystems, version 2.0.0). We confirmed the gene specificity of the nucleotide sequences chosen for the primers using BLASTN searches. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. The primers and probes are shown in Table 1. Each probe was synthesized with the 5' end reporter dye (FAM: 6-carboxyfluorescein) and 3' end BHQ1 dark quencher dye at St Jude's Hartwell Center for Bioinformatics and Biotechnology.

Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). All PCR reaction mixtures contained 2ul cDNA (corresponding to 5 ng reverse transcribed total RNA), 1X TaqMan Universal PCR Master Mix (PE Applied Biosystems), 1X Eukaryotic 18S rRNA endogenous control, 300nM of each primer and 200nM probe in 30ul reaction volume in 96 well plates. The thermal cycling conditions were as follows: after incubation at 50°C for 2 minutes and an initial denaturation step at 95°C for 10 min, 40 cycles were performed at 95°C for 15 seconds and 1 minute at 60°C. Standard curves were obtained using cDNA generated with human or mouse total bone marrow RNA (pooled human normal bone marrow from 8 male/female from BD Bioscience Clontech and pooled mouse BM from 3 male C57Bl/6/129svj mixed-background mice). Each PCR run included the 6 points of the standard curve (5-fold serially diluted human or mouse bone marrow cDNA), a non-template control with water, a calibrator cDNA, and the unknown cDNA samples. Baseline and threshold Ct value were analyzed manually with the ABI Prism SDS2.1 software. Values for each PCR product were normalized against 18S rRNA to compare expression in patient bone marrow samples with that in human bone marrow total RNA (BD Bioscience Clontech). Quantitative PCR assays were conducted in duplicate for each sample and a mean value was used to calculate mRNA levels.

Q-PCR reactions to compare the copy number of the *MN1* gene in inv(16) patient BM DNA samples with that in normal BM DNA was performed using the TaqMan Reverse Transcription Reagent following the manufacturer's protocols. A dilution series of 1, 0.5, 0.25, 0.125, 0.025, 0.005, 0.001, 0.0002 □g genomic BM DNA of 2 different inv(16) patients (indicated in red in Fig. 1) and a normal individual were subjected to one-step PCR with *MN1* first exon 1 and *GAPDH* exon 8 primers (Table

1), using 40 cycles of amplification (10 seconds 95°C, 1 minute 60°C) after incubation of the samples for 10 minutes at 25°C, 30 minutes at 48°C and 10 minutes at 95°C. The *GAPDH* 5' end reporter dye was tetrachlorofluorescein (TET) instead of FAM. Baseline and threshold Ct value were analyzed manually with the ABI Prism SDS2.1 software. Values for the *MN1* PCR product were normalized against that of the *GAPDH* product in inv(16) and normal BM DNA samples.

Analysis of *Mn1* expression in mouse hematopoietic stem cells and bone marrow progenitor populations was carried out using a two-step real-time RT-PCR protocol. FACS sorted hematopoietic stem cells and bone marrow progenitor fractions were pelleted by centrifugation and subsequently lysed in 800 µL of TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification Kit (Invitrogen Corporation) following the manufacturers protocol however, with addition of 10 µg of RNase-free glycogen prior to binding to the column. Genomic DNA was degraded by addition of 1 unit of DNase I for 15 minutes at room temperature, followed by inactivation of DNase I by addition of 1 µL of 25 mM EDTA and heating at 65°C for 10 minutes. First-strand cDNA synthesis was carried out on the total RNA isolate using SuperScript III First-Stand Synthesis SuperMix (Invitrogen Corporation), following the manufacturers instructions. Real time analysis was carried out using a modified version of the multiplexed tandem PCR approach(Stanley and Szewczuk, 2005). For first round multiplexed amplification, 5 µL of the first strand cDNA reaction was added to MN1 or HPRT outer primers (300nM final of each), 200nM dNTPs (Promega, Madison WI, USA), 1.5mM $MgCl_2$, GoTaq buffer (Promega), and 0.5U GoTaq (Promega) in a total volume of 20 µL. PCR was carried out on a PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) for 15 cycles using the following conditions: 95°C, 10 min for 1 cycle followed by 20 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec. The resulting products were mixed and first-round primers, dNTPs and Taq removed using the QIAGEN mini elute PCR reaction clean-up kit (QIAGEN Inc, Valencia, CA, USA). The real-time PCR reaction consisted of 5 µL of an appropriate dilution of the first-round amplified cDNA mixed with 15 µL of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM each of the inner *Mn1* or *Hprt* forward and reverse primers and 200 nM probe in a final reaction volume of 30 µL. Real time PCR was carried out in a 96-well plate on Bio-Rad iQ5 Multicolor Real-Time PCR Detection System using the following cycling conditions: one cycle each of 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For all samples, the expression level of *Mn1* was normalized to *Hprt* and expressed relative to the level in total bone marrow.

Table 1.

Human *PLAGL2* cDNA primers:
Forward: CACTGTGGCAAGGCTTTTGC
Reverse: GATGGTCCTTGCGGTGAAACAT
Probe: ATACAAGCTGTATAGGCACATGGCCACCC

Human *EVI1* cDNA primers:
Forward: AATGTGAAAACTGTGCCAAGGTT
Reverse: CCGACATGCTGAGAGCGAAT
Probe: TCACGGACCCTAGCAACCTTCAGCGGCA

Human *MN1* cDNA primers:
Forward: GAAGGCCAAACCCCAGAAC
Reverse: GATGCTGAGGCCTTGTTTGC
Probe: CCAACAGCAAAGAAGCCCACGACC

Human *MN1* exon 1 primers:
Forward: ATTGACCTGGACTCGCTGATG
Reverse: TGTCCACCAGGGCCTTGT
Probe: CAGCGCTGCCTGGTACATGCCC

Human *GAPDH* exon 8 primers:
Forward: ACCACAGTCCATGCCATCACT
Reverse: CCATCACGCCACAGTTTCC
Probe: CCCAGAAGACTGTGGATGGCCCC

Mouse *Mn1* cDNA primers:
Forward outer/inner: TGGTGGAGATGAGGACAAGA
Reverse outer: CTTGGGGTCACCATCTGTG
Reverse inner: GTGGCTGAGGCCTTGTTGG
Probe: CCCAACAACAAAGAAGCCCATGACC

Mouse *Hprt* cDNA primers:
Forward outer/inner: TTATCAGACTGAAGAGCTACT
Reverse outer: CTTAACCATTTTGGGGCTGT
Reverse inner: TTACCAGTGTCAATTATATCTTCAACAATC
Probe: TGAGAGATCATCTCCACCAATAACTTTTATGTCC

Retroviral integration sites in leukemia samples.

To determine the number of MN1 retroviral integration sites, Southern blots containing tumor DNA digested with EcoRI were hybridized with a GFP probe. This detects hybrid DNA fragments containing MSCV sequences downstream of the viral 3' EcoRI site (Fig. 1A) to the first downstream EcoRI site in mouse genomic DNA.

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Chapter 6

Discussion

The hematopoietic system is one of the most fascinating and intricate developmental systems in the vertebrate. Its complexity lies in its capability of producing a broad spectrum of cells, diverse not only in their differentiation stage, like in most of the other tissue compartments, but also in their nature and consequently, their function. One additional characteristic that distinguishes the hematopoietic system from many other organs is that most mature blood cells have a short life and therefore hematopoiesis has to be a continuous process throughout life. With the term hematopoiesis we define the totality of cellular processes that drive succession of lineage-commitment steps coupled with a restriction of differentiation potential and the establishment of lineage-specific expression profiles, starting from a restricted number of HSCs. Experimental work in the last 30 years has provided much information regarding the hematopoietic subpopulations and their overall regulation. Many regulatory factors involved in the control of the proliferation and differentiation of these populations have been identified, cloned and characterized. What makes these factors of special interest is that there is a continuously growing amount of evidence that many of them are not only responsible for physiological hematopoietic processes but are also involved in the pathologic mechanisms leading to hematological malignancies. Among the multitude of these factors we are particularly interested in the ETS transcription factors, a large family of winged helix-loop-helix DNA-binding proteins (Graves and Petersen, 1998; Janknecht and Nordheim, 1993; Oikawa and Yamada, 2003). Many of the ETS proteins are expressed in the hematopoietic system and are thought to be involved in cell differentiation and function (Maroulakou and Bowe, 2000). In the 1990s, many of these ETS factors have been studied using gene targeting experiments in murine embryonic stem cells. This approach elucidated their function and extensive biochemical and molecular studies contributed in the clarification of their role in hematopoiesis.

Our laboratory had focused its interest on the ETS protein TEL because of its involvement in the recurrent t(12;22)(p12;q12) in human myeloid malignancies. While studying TEL we became interested in the function of its close homologue TEL2, whose expression is mainly restricted to the hematopoietic system (Potter et al., 2000).

Several observations indicated a possible association of TEL2 with tumorigenesis: 1) TEL2 dimerizes with TEL (Kawagoe et al., 2004) a protein believed to have a tumor suppressor function, 2) TEL2 expression was found to be up-regulated in some B-ALL and AML patients (Kawagoe et al., 2004) and had been found in (non-hematopoietic) cDNA libraries used by the National Cancer Institute Cancer Genome Anatomy Project (NCI-CGAP), 3) TEL2 blocked the inhibitory effect of TEL1 on Ras-induced cellular transformation of NIH3T3 cells (Kawagoe et al., 2004), demonstrating that, in spite of their high similarity, TEL1 and TEL2 have divergent activities, 4) forced expression of TEL2 in U937 cells inhibited their vitamin D3-induced differentiation.

These observations prompted us to investigate the *in vivo* effects of TEL2 overexpression in murine bone marrow.

In Chapter 2 we showed that TEL2 behaves as an oncogene when overexpressed in murine bone marrow, causing a myeloproliferative disease. TEL2 inhibits apoptosis of myeloid progenitors, which might be causally involved in disease development. Affimetrix analysis showed that TEL2 downregulates several pro-apoptotic genes, such as p53, the executioner caspase7, Scythe, and the NfκB inhibitors *Bcl3* and *Faf1*, which could explain TEL2's inhibitory effect on apoptosis (Carella et al., 2005). However, it remains to be determined whether downregulation of these genes is a direct or indirect effect of TEL2, because it is unknown whether any of these genes are direct transcriptional targets. Because the latency of disease onset is long, we inferred that development of TEL2-induced myeloid disease depended on secondary genetic events. To confirm this, we treated TEL2-transplanted mice with the chemical mutagen ENU to determine cooperation between TEL2 expression and chemical mutagenesis. Although ENU treatment significantly shortened the latency of disease development the outcome was different than expected. Part of the mice developed significantly elevated WBC in the peripheral blood of poorly differentiated TEL2-expressing cells and/or showed increased numbers of myeloid cells in the BM relatively early after transplantation. Nevertheless, all mice died of T cell lymphoma not expressing TEL2 (Carella et al., 2005). This remains a puzzling result, which can only be explained by the assumption that TEL2 overexpression in the hematopoietic system indirectly promoted the development of lymphoid disease. Because we did not analyze the cells in the thymus prior to overt disease we do not know whether there was abnormal proliferation of T cells, possibly as a result of aberrant cytokine expression.

To further explore the possible secondary mutations cooperating with TEL2 in the leukemic process we decided to overexpress TEL2 in bone marrow already predisposed to develop neoplasia, such as bone marrow cells of Eμ-Myc transgenic mice or of Arf-null mice

Transgenic mice expressing the c-Myc oncogene driven by the immunoglobulin heavy chain enhancer (Eμ) develop a fatal lymphoma within a period of 6 months (Adams et al., 1985). Tumor development depends on secondary inactivation of the p53 pathway which eliminates c-Myc-induced apoptosis (Eischen et al., 1999). We found that TEL2 overexpression augments the proliferation and survival of normal murine B cells and accelerated B-lymphomagenesis in Eμ-Myc transgenic mice (Cardone et al., 2005). Despite a TEL2-induced 50% reduction in the apoptotic rate of Eμ-Myc pre-B cells, all TEL2/Eμ-Myc B-cell lymphomas underwent inactivation of the p53 pathway. The most likely explanation for the cooperative effect in this tumorigenesis model is that the TEL2-induced reduction in the apoptotic rate of the Eμ-Myc pre-B cells promoted further expansion of this compartment in transplanted mice. This increased the chance of additional mutations, but TEL2's effect alone was

insufficient to cause disease. A similar scenario might also be involved in human lymphomagenesis as suggested by the analysis of pediatric *B-cell acute lymphocytic leukemia* (B-ALL) samples, which showed increased co-expression of TEL2, MYC-C and/or MYC-N in over one-third of cases (Cardone et al., 2005).

In Chapter 3 we also performed the complementary experiment by expressing TEL2 in bone marrow of Arf-null mice. These mice with a compromised Arf-Mdm2-p53 pathway (Kamijo et al., 1997) are prone to spontaneous tumor development, and develop undifferentiated sarcomas (43%), lymphomas (29%) of predominantly T-cells origin, carcinomas (17%) and tumors of the nervous system (11%) (Kamijo et al., 1999). All mice transplanted with TEL2-overexpressing Arf-null bone marrow developed B-cell lympho-leukemia 4-6 months after transplantation. Thus, compared with TEL2-transduced wild type bone marrow (Chapter 2), the absence of Arf not only accelerated the onset of disease, but also changed the disease phenotype from myelodysplasia to B-cell lymphoma. Interestingly, TEL2/Arf-null lymphomas all showed *c-Myc* amplification resulting in overexpression of c-Myc. Thus, whether one combines TEL2 overexpression with Myc overexpression or with an impaired p53 pathway, the disease outcome is identical; a B cell lympho-leukemia that lost the p53 pathway overexpressing both TEL2 and c-Myc.

In vitro culture of $Arf^{-/-}$ $B220^{+}$ pro-B cells with or without TEL2 overexpression showed that TEL2 shortens cell cycle traverse and reduces the rate of apoptosis, thereby stimulating the proliferation of these cells. This phenotype was in agreement with increased expression of proteins affecting cell cycle and apoptosis such as c-Myc, Bcl2, E2f1, E2f2 and the cyclins A, E and D2. Affimetrix analysis of these cells did not show increased levels of RNA encoding for any of these proteins, suggesting that the upregulation of their protein levels was a post-transcriptional event. However, the combination of loss of the p53 pathway and overexpression of TEL2 was insufficient for tumorigenic transformation of these cells and they remained strictly dependent on IL-7 for survival. We believe that the oncogenic role of TEL2 overexpression in $Arf^{-/-}$ bone marrow in mice consists of increasing the pool of pre-leukemic pre-B cells through increased proliferation, thereby promoting the chance of additional mutations such as amplification and overexpression of *c-Myc*. Clearly the growth promoting effect of TEL2 alone is insufficient for tumorigenic transformation of these cells resulting in selection for overexpression of c-Myc. The combination of c-Myc overexpression on a mutant p53 pathway background is an essential and well-recognized combination for full transformation of B lymphoid cells (Eischen et al., 1999). Comparison of the transcriptome of *in vitro* cultured $Arf^{-/-}$ pro B cells expressing TEL2 with that of $Arf^{-/-}$ pro B cells also revealed upregulation of Frap1 (*FK506 binding protein 12-rapamycin associated protein 1* or FKBP12), also coined *mammalian Target of Rapamycin* (mTor), a member of the *phosphatidylinositol 3-kinase* (PI3K) family of proteins. mTor is a central player in the regulation of a wide collection of cellular functions, including

translation, transcription, mRNA turnover, protein stability, actin cytoskeleton organization and autophagy (Inoki et al., 2005; Jacinto and Hall, 2003). The best-characterized mTor function in mammalian cells is its regulation of translation (Bjornsti and Houghton, 2004), which would provide an explanation for the increased expression of part of the cell cycle and anti apoptotic proteins in $Arf^{-/-}$/TEL2 pro B cells. In Chapter 3 we also provide preliminary evidence that the protein level of activated mTor is upregulated in these cells, results that are currently being verified by others in the laboratory. Moreover, we found evidence that *mTor* is a direct transcriptional target of TEL2 via the CCGGAAGT binding site present at -289 to -281 in its promoter region. Despite the fact that proliferation of the $Arf^{-/-}$/TEL2 pro-B cells was inhibited by the addition of the mTor inhibitor rapamycin, we detected only partial upregulation of the 2 known downstream pathways of mTor. This seems in direct conflict with the notion that the increase in cell cycle proteins and anti-apoptotic proteins was a direct result of increased translation initiation due to mTor activation. However, recently it was reported that activated S6K1 phosphorylates Pdcd4, an inhibitor of eIF4A, resulting in its increased degradation via the ubiquitin ligase SCF (Dorrello et al., 2006) thereby stimulating translation and cell growth. This novel finding may well explain our results (S6K1 activity is upregulated in our cells) and calls for a comparison of the amount of Pdcd4 protein in $Arf^{-/-}$ pro-B cells with that in $Arf^{-/-}$/TEL2 pro-B cells.

Our findings point to the possibility that molecular identification of TEL2-overexpressing leukemia/lymphoma samples might identify patients that will be partly resistant to adjuvant treatment with inhibitors of the mTor pathway, such as rapamycin or rapamycin analogues (Yee et al., 2006; Zanesi et al., 2006) and will therefore have limited benefit from such treatment.

Our results in wild type, Eμ-Myc transgenic, and Arf-null mice combined with the fact that TEL2 expression is upregulated in some adult leukemias and over 30% of pediatric ALLs, leads us to propose that TEL2 is a bona fide oncogene involved in human leukemia.

The second focus of this thesis was to establish the oncogenic activity of MN1-TEL in the mouse hematopoietic system. MN1-TEL is the product of the chromosomal translocation t(12;22)(p12;q12) associated with human myeloid malignancies. We argued that confirming the oncogenic activity of MN1-TEL in the mouse hematopoietic system would make a strong case for a causative role of MN1-TEL in human leukemia. When we started this work It had been shown that MN1-TEL possessed weak oncogenic activity in NIH3T3 fibroblasts (Buijs et al., 1995; Buijs et al., 2000).

In Chapter 4 of this thesis we demonstrated that forced expression of MN1-TEL in murine bone marrow increased the self-renewal capacity of primitive hematopoietic progenitors, which upon in vitro culture produced immortalized IL-3/SCF-dependent myeloid cell lines. Generation of these cell lines requires at least one additional genetic or epigenetic event, given that serial replating of MN1-TEL expressing bone marrow

cells in methylcellulose was always finite. We believe that the much larger number of cells in liquid cultures increases the chance of secondary mutations resulting in immortalization of the cells. As yet we have not invested effort in identifying the nature of this event but its characterization might be important for understanding the process of myeloid leukemogenesis in general. Given the role of the p53 pathway in cell immortalization we speculate that candidate genes would directly or indirectly affect this pathway. Mutations affecting the proper function of this pathway have already been described in AML. For instance 35% of AML patients express a mutant NPM protein (Cazzaniga et al., 2005; Falini et al., 2005), which causes the protein to relocalize form the nucleolus to the cytoplasm. This perturbs both p53-dependent and independent functions of Arf (den Besten et al., 2005).

It was remarkable that lethally irradiated mice receiving transplants of MN1-TEL cell lines were rescued from radiation-induced bone marrow insufficiency. This indicated the presence of primitive progenitors that maintained the capacity to differentiate into all hematopoietic cell lineages. Notwithstanding, all transplanted mice developed acute myeloid leukemia within 3 months after transplantation. Leukemic cells recovered form these mice proliferated in culture without addition of growth factors, strongly suggesting that the cell line had undergone additional genetic alterations rendering it fully transformed.

The fraction of normal bone marrow cells that do not stain with the fluorescent dye Hoechst 33342, defined as *side population* (SP) are highly enriched in hematopoietic stem cells (Goodell et al., 2005). Using SP sorting we showed that the repopulation and leukemogenic activity of the MN1-TEL cell line resided within this fraction. Thus, only a small number of cells within the cell line maintains its self-renewal potential. We believe that cells within this fraction underwent additional mutation and generated the leukemic stem cell that eventually killed the mice. Given that this is a long living cell, we think that it would be a better target to undergo mutation than a short living, differentiating cell that would have to regain its capacity to self-renew.

In addition to generating myeloid cell lines, we also showed that bone marrow freshly transduced with MN1-TEL retrovirus caused AML in transplanted mice within 3 months after transplantation. Together these experiments led to 2 important conclusions: 1) MN1-TEL is an efficient hematopoietic oncogene but alone is insufficient to cause disease and 2) Methylcellulose cultures provided a reliable assay system to measure MN1-TEL's oncogenic activity in myeloid cells.

We next employed these methylcellulose assays to determine which sequences of MN1-TEL were essential for its transforming activity. These experiments led to the exciting but puzzling observation that MN1-TEL's transforming activity was not dependent on a functional ETS DNA binding domain, while such dependence was well established in fibroblasts (Buijs et al., 2000). It was even more surprising that bone

marrow cells transduced with a retroviral vector expressing a protein from which all TEL sequences had been deleted also showed increased self-renewal activity and generated myeloid cell lines during liquid cultures. The same was true for bone marrow transduced with a virus encoding full length MN1. Moreover, mice transplanted with such bone marrow samples rapidly developed myeloproliferative disease. Although the disease features resembled those of MN1-TEL-expressing bone marrow, there were important differences: MN1-TEL cell lines had a more primitive phenotype than MN1 cell lines and MN1-TEL expressing bone marrow caused AML rather than myeloproliferative disease upon transplantation into lethally irradiated mice. This suggested that presence of the TEL DNA binding domain in the fusion protein made it a more potent inhibitor of differentiation than MN1. Because MN1 interacts with transcription factors via its N-terminal domain (van Wely et al., 2003), one can envision that additional DNA binding via the ETS domain might target the fusion protein to the promoters/enhancers of a subset of genes that inhibit myeloid differentiation. Conformation of this possibility would need testing of additional MN1-TEL mutants combined with extensive transcriptional profiling and promoter studies, experiments that are well within our current technical reach.

Despite the rapidly developing myeloproliferative disease in mice transplanted with MN1-overexpressing BM, the disease was not polyclonal suggesting that there was selection of cells that underwent additional mutation. Whether these were de novo mutations or gene expression changes caused by integration of the MN1 provirus remains to be established. It would be worthwhile to isolate the MSCV-MN1-IRES-GFP integration sites form the malignant cells of different mice to establish whether there are common integration sites. If so, it would pinpoint the gene or genes whose altered expression cooperate(s) with MN1 in the development of the myeloproliferative disease. Together our results showed that MN1 strongly stimulates the growth of myeloid progenitors.

These findings combined with the observation that MN1 is consistently overexpressed in samples of patients suffering from inv(16) AML (Ross et al., 2004; Valk et al., 2004) prompted us to perform the experiments described in Chapter 5 of this thesis. Inv(16) AML is characterized by expression of a CBFB-MYH11 fusion protein (Liu et al., 1996). When expressed in mouse bone marrow CBFB-MYH11's dominant negative effect on the CBF transcription factor (Castilla et al., 1996; Okuda et al., 1996) enables it to cause alterations in multilineage differentiation of hematopoietic cells (Castilla et al., 1999), but expression of this fusion protein alone does not cause leukemia. Therefore, our goal was to assess whether MN1 overexpression is a cooperating event in inv(16) AML.

First we addressed which progenitors in the bone marrow express MN1. Using FACS we sorted bone marrow fractions representing the HSCs, CMPs, GMPs and the common MEPs. Quantitative-RT-PCR revealed that MN1 is expressed at a low level in

the HSC and is strongly upregulated in the GMP, while there is no expression in the CMP, CLP and MEP fractions. Cells causing the MN1 myeloproliferative disease are GMP-derived and expression of the retrovirus-encoded MN1 will become even higher. It is possible that the elevated levels of MN1 specifically affect the proliferation rate of these cells but not their differentiation, giving rise to a massive increase in partially and fully differentiated neutrophils. To prove this point one could transduce sorted GMP cells with MN1 retrovirus and compare their growth rate with that of vector-transduced GMP cells using methylcellulose assays and liquid cultures.

Second, we verified that *MN1* overexpression indeed occurs in inv(16) AML patients and confirmed by Q-RT-PCR that 9 out of 9 of our inv(16) AML samples contained increased amounts of *MN1 mRNA*. Moreover, in the 2 inv(16) samples available for analysis we also detected increased amounts of MN1 protein.

It was therefore exciting that mice transplanted with CBFB-MYH11 knock-in chimeric bone marrow transduced with MN1 retrovirus developed a c-Kit$^+$ AML 2 months after transplantation, in which cells expressed both MN1 and CBFB-MYH11. This result was distinctly different from what happens in CBFB-MYH11 chimeric mice. These animals only develop leukemia upon treatment with the chemical carcinogen *N-ethyl-N-nitrosourea* (ENU) (Castilla et al., 1999), a c-Kit$^+$ AML very similar to that in our MN1/CBFB-MYH11-transplanted mice. These results convinced us that MN1 overexpression truly cooperates with CBFB-MYH11 in the development of this AML and suggests a similar cooperation in human inv(16) leukemia. Whether this combination is sufficient to fully transform myeloid progenitors is however doubtful. The emerging leukemia was not polyclonal, again suggesting selection for leukemic subclones due to additional mutation. Also, using retroviral tagging in mice Castilla and coworkers (Castilla et al., 2004) reported that overexpression of the transcription factors Plag1 and PlagL2 cooperated with CBFB-MYH11 in the development of AML. Indeed, 20% of human AML samples show overexpression of PLAG genes with *PLAGL2* preferentially increased in inv(16) leukemia samples (Landrette et al., 2005). We determined that *PLAG* genes were not overexpressed in our diseased mice and that only 1 of our 9 human inv(16) samples showed increased expression of *PLAGL2.* This established that PLAG genes are not transcriptional targets of MN1 and their upregulation in some inv(16) AMLs is likely to be caused by a separate genetic event. It is therefore likely that in inv(16) patients without *PLAGL2* overexpression other mutations may substitute for its effects, supporting the notion that MN1 overexpression and CBFB-MYH11 alone are insufficient to cause disease.

We also established that MN1 overexpression is not restricted to inv(16) AML but occurs in poorly differentiated FAB-M1 AML. This is in agreement with a paper reporting overexpression of MN1 in AML samples without karyotypic abnormalities (Heuser et al., 2006). In these patients MN1 overexpression is associated with a worse

prognosis and a shorter survival rate. Given MN1's cooperative role in inv(16) AML, we infer that its role in M1 AML is likely to be similar.

Many aspects of MN1's role in AML remain unanswered. For instance it is currently unknown how expression of the gene is upregulated. In 2 inv(16) patient samples we excluded the possibility of gene amplification and *MN1* expression in mouse cells is not upregulated by CBFB-MYH11, a result in agreement with *MN1* overexpression in leukemia samples that do not carry CBF transcription factor mutations. This suggests that other as yet unknown genetic events are responsible for MN1 overexpression. Possibly, structure-function analysis of the MN1 promoter in the inv(16) cell line ME-1 might pinpoint sequences responsible for its upregulation. Once such sequences have been delineated it should be possible to identify the transcription factors that bind to these sequences. Also the mechanism by which MN1 stimulates cell growth remains to be defined. The sequences involved in MN1's growth stimulatory effect (at least in MN1-TEL) reside in the N-terminal domain of the protein (Chapter 4), which was shown to interact the transcriptional coactivators P300/CBP and RAC3 (van Wely et al., 2003), defining MN1 as a constituent of coactivator complexes. To understand how MN1 and MN1-TEL control growth of myeloid progenitors it will be crucial to determine via which transcription factor(s) MN1 exceeds its function. Identification of MN1 and MN1-TEL transcription factor complexes present in MN1 and MN1-TEL-overexpressing cells might provide insight into this question. In addition, it will be equally important to define the critical downstream targets of MN1, a problem that might be tackled by careful and extensive expression profiling. Together these approaches may provide us with a detailed insight into the molecular mechanisms with which MN1 and MN1-TEL affect the proliferation of myeloid progenitor cells.

Our work with MN1-TEL produced results that implicates MN1 in a much wider range of AMLs and in a much larger group of patients than those defined by the t(12;22)(p12;q12). Therefore interference with MN1's function might be of therapeutic relevance for this group of patients. Once the critical MN1 transcription factor complexes have been defined, we hope that it will be possible to develop small molecule inhibitors that interfere with association of MN1 with its cooperating factors. Given that MN1-TEL's function is dependent on the MN1 N-terminal domain we expect that treatment with such inhibitors will be beneficial both for patients overexpressing MN1 as well as for patients expressing MN1-TEL.

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Summary

The generation of all blood cells in the body relies on the ability of a restricted pool of totipotent hematopoietic stem cells, the long-term repopulating cells, to simultaneously differentiate and self-renew. This process guarantees that the pool of totipotent stem cell is maintained, and produces an array of pluripotent stem cells that gradually loose their self-renewing potential as they differentiate to produce mature blood cells.

Every cell type in the hematopoietic system can generate a cancer. This type of cancer is called leukemia. In the past decades particular emphasis has been put on identifying and understanding the molecular alterations that cause these diseases and that determine their pathological outcomes.

The continuous acquisition of molecular research tools enabled us to better understand the relationships among different cell types of the hematopoietic system and to formulate and study models of leukemogenesis more in detail.

The role played by ETS transcription factors during development of the hematopoietic system and their involvement in pediatric and adult leukemias, has been studied for years. Our group has developed an interest in this family of transcription factors, focusing in particular on the role of *TEL* in the chromosomal translocation t(12;22)(p12;q12) involved in human myeloid malignancies and on the possible role of its homologue *TEL2* in leukemia.

TEL2 is expressed in the hematopoietic system and increased expression of this gene has been found in some leukemias in adults and in over 30% of pediatric B-cell lymphoma patients. These observations, coupled to our previous *in vitro* results identified *TEL2* as a potential oncogene, which led us to directly test the hypothesis that *TEL2* can play an active role in leukemogenesis in a mouse model.

Our data, discussed in Chapter 2, clearly show that TEL2 behaves as a bona fide oncogene *in vivo*. Lethally irradiated wild type mice whose bone marrow had been reconstituted with TEL2 overexpressing bone marrow, developed a myeloproliferative disease (MPD) after a long latency period. This was a significant finding that identified *TEL2* as a gene that can cause alterations of the hematopoietic system. However, our data clearly suggested that TEL2 overexpression alone was not sufficient for the development of leukemia but required cooperation of secondary mutations.

This latter hypothesis was investigated in Chapter 3 by directing forced expression of TEL2 in mouse bone marrow cells harboring a compromised p53 pathway resulting from deletion of the tumor suppressor p19Arf (Chapter 3). This not only accelerated the onset of disease but also changed the phenotype from MPD to B-cell lymphoma. Most importantly, we uncovered a direct involvement of TEL2 in the regulation of Frap1, also known as mTor (Target of Rapamycin) in Arf-/- pro-B cells.

This finding led us to propose a hypothetical model, in which the upregulation of mTor by TEL2 is in part responsible for the increased proliferation and survival rates of malignant cells. Although additional studies are needed to detail the exact mechanisms by which these phenomena occur, our data possibly carry significant therapeutic implications. If subgroups of adult and/or pediatric leukemia patients can be identified that show increased expression of TEL2 and mTOR, such patients might only partially benefit from treatment with the mTOR inhibitor rapamycin, a drug which is being tested for treatment of other types of cancer. Our data suggest that in addition to mTOR activation additional growth promoting signals emanate from TEL2, which would call for a treatment of these patients with rapamycin in combination with other chemotherapeutic agents.

A second aim of this thesis was to elucidate the oncogenic potential of the fusion protein MN1-TEL in the mouse hematopoietic system. The data described in Chapter 4 further established the role of MN1-TEL as a hematopoietic oncogene and identified the N-terminal domain of MN1 to be essential for the oncogenic activity of the fusion protein. Next we will have to identify the proteins interacting with this domain and to identify the downstream target genes that mediate the growth-promoting activity of MN1-TEL in the hematopoietic system.

Our studies also paved the way to further explore the leukemogenic role of MN1 itself. In Chapter 5 we show that MN1 alone is also able to stimulate the growth of mouse myeloid progenitors, to cause MPD in mice, and to cooperate with Cbfβ-SMMHC, the product of the human chromosomal rearrangement *inv(16)* in the development of acute myeloid leukemia (AML) in mice. This findings combined with the observation that MN1 is consistently overexpressed in *inv(16)* AML patients underscore the importance to further elucidate the molecular mechanisms with which MN1 exerts its growth promoting activity in myeloid cells. This knowledge may eventually lead to the development of specific small molecule inhibitors of MN1 that might provide a more targeted mode of treatment of patients with myeloid leukemia overexpressing MN1.

Samenvatting

De produktie van alle bloedcellen in het lichaam is afhankelijk van een gelimiteerd aantal totipotente hematopoietische stamcellen, de lange termijn repopulerende cellen, die tegelijkertijd differentieëren en zichzelf vernieuwen. Dit proces garandeert dat de voorraad van totipotente cellen gehandhaafd blijft en dat er een verzameling van pluripotente stamcellen geproduceerd wordt. Deze pluripotente cellen verliezen, tijdens de differentiatie tot volwassen bloedcel, geleidelijk de capacitiet zichzelf te regenereren.

Van elke cel soort in het hematopietische systeem kan een kanker ontstaan. Dit type kanker noemt men leukemia. In voorgaande jaren is er veel tijd geinvesteerd in het vinden en begrijpen van de molekulaire veranderingen die deze ziektes veroorzaken en die de pathalogische eigenschappen van deze ziektes bepalen.

Het zich steeds uitbreidende arsenaal van onderzoeks methoden heeft ons in staat gesteld om de relatie tussen de verschillende soorten hematopoietische cellen te begrijpen en meer gedetailleerde leukemie modellen te genereren en te bestuderen.

De rol van ETS transcriptie faktoren tijdens de ontwikkeling van het hematopoieties systeem en de betrokkenheid van deze faktoren bij de ontwikkeling van leukemie in zowel kinderen als volwassenen wordt reeds vele jaren bestudeerd. Onze onderzoeksgroep raakte geinteresseerd in deze groep van transcriptie faktoren door de betrokkenheid van TEL bij de chromosomale translokatie t(12;22)(p12;q12) bij humane myeloïde leukemie en ook door de mogelijke rol van het homologe *TEL2* gen bij leukemie.

TEL2 komt tot expressie in het hematopoietisch systeem en over-expressie van het gen werd vastgesteld bij een aantal leukemieën bij volwassenen en bij meer dan 30% van de B cel lymfomen bij kinderen. Samen met eerdere *in vitro* resultaten bevestigden deze bevindingen *TEL2* als een potentieel oncogen, wat ons er toe bracht om direct de hypothese te testen dat TEL2 in een muismodel een aktieve rol kan spelen tijdens leukemogenese.

Onze resultaten, besproken in hoofdstuk 2, tonen duidelijk aan dat TEL2 zich *in vivo* gedraagt als een *bona fide* oncogen. Letaal bestraalde muizen die getransplanteerd werden met TEL2 over-expresserend beenmerg ontwikkelden een myeloproliferatieve ziekte (MPD) met een lange latentie tijd. Dit was een belangrijke vinding omdat het aantoonde dat TEL2 veranderingen in het hematopoietiese systeem teweeg kan brengen. Echter, onze resultaten suggereerden duidelijk dat de over-expressie van TEL2 alleen onvoldoende was om leukemie te veroorzaken, maar afhing van de samenwerking met secondaire mutaties.

Deze hypothese werd getest in hoofdstuk 3. Door TEL2 te over-expresseren in beenmerg waarin het p53 netwerk geinaktiveerd was door deletie van de tumor

suppressor p19Arf. Dit versnelde niet alleen het ontstaan van de ziekte maar veranderde ook het fenotype van MPD naar B cel lymfoma. De belangrijkste ontdekking was dat TEL2 in Arf-/- pro-B cellen direkt betrokken is bij de regulatie van Frap1, ook wel mTor genoemd (Target of Rapamycin). Deze vondst leidde tot een hypothetisch model waarbij de toegenomen expressie van mTor door TEL2 gedeeltelijk verantwoordelijk is voor de toename in groei en overleving van de maligne cellen. Hoewel aanvullende studies nodig zijn om het preciese mechanisme waarmee dit plaatsvindt te ontrafelen, hebben onze resultaten wellicht belangrijke therapeutische implicaties. Als er subgroepen volwassen en/of pediatriese leukemie patienten geindentificeerd worden die een verhoogde expressie van TEL2 en mTOR vertonen, zouden zulke patienten slechts gedeeltelijk voordeel hebben van een behandeling met de mTOR remmer rapamycine, een middel dat reeds gebruikt wordt voor de behandeling van andere soorten kanker. Onze data suggereren dat naast mTOR over-expressie TEL2 ook andere groei-stimulerende signalen teweeg brengt, wat zou betekenen dat zulke patienten behandeld zouden moeten worden met rapamycine in combinatie met ander chemotherapeutica.

Een tweede doel van dit proefschrift was om de oncogene capaciteit van het MN1-TEL fusie eiwit in het hematopoietisch systeem van de muis te bepalen. De data beschreven in hoofdstuk 4 bevestigen verder de rol van MN1-TEL als hematopoietisch oncogen en laten zien dat het N-terminale domein van MN1 essentieel is voor de oncogene aktiviteit van het fusie-eiwit. In navolging hierop zullen we zowel de eiwitten moeten identificeren die binden aan MN1 als wel de ‘target’ genen moeten bepalen die de groei-stimulerende aktiviteit van MN1-TEL in het hematopoietische systeem bewerkstellen.

Onze studies leidden er ook toe om de leukemogene rol van MN1 zelf te onderzoeken. In hoofdstuk 5 laten beschrijven we dat MN1 in staat is de groei van hematopoietische voorloper cellen te stimuleren, MPD te veroorzaken in muizen, en samen te werken met Cbfβ-SMMHC bij het veroorzaken van acute myeloide leukemie (AML) in muizen. Cbfβ-SMMHC is het produkt van de humane chromosomale verandering inv(16). Deze bevindingen gecombineerd met het feit dat *MN1* consistent over-geëxpresseerd wordt in inv(16) AML patienten onderstrepen het belang om het moleculaire mechanisme te ontrafelen waamee MN1 de groei van myeloïde cellen stimuleert. Deze kennis zou uiteindelijk kunnen leiden tot het ontwikkelen van specifieke klein-moleculaire remmers van MN1 die wellicht een meer gerichte behandeling kunnen verschaffen voor patienten met leukemieën die MN1 over-expresseren.

List of Abbreviations

AGM .....Aorta/Gonad/Mesonephros
ALL .....Acute Lymphoid Leukemia
AML .....Acute Myeloid Leukemia
APL .....Acute Promyelocytic Leukemia
ATRA .....all-trans-retinoic acid
B-ALL .....B-cell acute lymphocytic leukemia
BCR.....Break-point Cluster Region
bHLH .....basic Helix-Loop-Helix
CBC.....Complete Blood Count
CBF .....Core Binding Factor
CDBS .....Consensus DNA-Binding Sequence
CLP .....Common Lymphoid Progenitors
CML.....Chronic Myeloid Leukemia
CMML .....Chronic Myelo-Monocytic Leukemia
CMP .....Common Myeloid Progenitor
E .....Embryonic day
EBS .....ETS Binding Site
ENU.....N-ethyl-N-nitrosourea
EPO.....erythropoietin
ETS .....E26-transformation specific
FACS .....Fluorescence-Activated Cell Sorter
FISH .....Fluorescent *In situ* Hybridization
G-CSF .....Granulocyte-Colony Stimulatory Factor
GFP.....Green Fluorescent Protein
GM-CSF.....Granulocyte-Macrophage Colony Stimulatory Factor
GMP .....Granulocytes-Monocytes Progenitor
HAT .....Histone Acetyl Transferase
HDAC .....Histone De-Acetylase
HLH .....Helix-Loop-Helix
HSC.....Hematopoietic Stem Cell
Ig .....Immunoglobulin
IgH.....Immunoglobulin Heavy chain
IL-3 .....Interleukin 3
IRES.....Internal Ribosome Entry Site
JAK.....Janus tyrosine kinase
LSC .....Leukemic Stem Cell
LT-HSC .....Long Term Hematopoietic Stem Cell
M-CSF.....Monocyte-Colony Stimulatory Factor
MEP .....Megakaryocytes-Erythrocytes Progenitor
MEP .....Myb-Ets progenitor
MLL .....Mixed-Lineage Leukemia protein
MPP .....Multipotent Progenitor Population
MSCV.....Murine Stem Cell Virus
mTor.....mammalian Target of Rapamycin
NK .....Natural Killer
PB .....Peripheral Blood
PDGFRβ .....Platelet-derived Growth Factor Receptor beta
Ph.....Philadelphia
PI3.....phosphatidylinositol 3
PI3K .....phosphatidylinositol 3-kinase

PNT .....Pointed domain
PTK .....Protein Tyrosine Kinase
RA .....retinoic acid
RARE .....retinoic acid responsive element
RTK.....Receptor tyrosine kinase
SMMHC .....Smooth Muscle Myosin Heavy Chain
SP .....side population
STAT.....Signal Transducers and Activators of Transcription
ST-HSC .....Short-Term Hematopoietic Stem Cell
TCR.....T-Cell Receptor
TF .....Transcription Factor
TGF-β.....Transforming Growth Factor-β
TNF .....Tumor Necrosis Factor
TPO.....Thrombopoietin
WBC.....White Blood Cells

Curriculum Vitae

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1997-1998: ***Post-Graduate Fellow*** under the supervision of Dr. C. Luparello at the Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy" of the University of Palermo.

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that has been an example for me. Ivan and Adelì, you have been for us our Memphian family with whom to share holidays and birthdays. I miss all the four of you but I know we will see each other again (I still need to visit Perù).

Massimo dear, our friendship goes far beyond the scientific experience. Still I terribly miss your contagious enthusiasm for science, even in the difficult moments, and the long afternoons that we spent at the microscope when you were trying really hard to make me understand the difference between a blast and a big lymphocyte. I hope we will get the chance to work together again one day.

Maria and Eddie we have been sharing so many things together and I always felt that the friendship between us goes far beyond the distances that now separate us. But just to be sure that the ocean does not get too big I am starting to plan my puertorican vacation…….. Angela e Colin I just thank you for being always there for me and my family. After so long I am simply looking forward to share with you new great experiences. Sabrina e Roberto, we have met in the final steps of my Memphian journey and still we soon became good friends. AnnMarie, Dr Ashmun and all the FACS people, I am really grateful for your huge help and patience and now that I am doing FACS analysis by myself I miss all of you even more. Dr Hougthon I will always think about you as the perfect mix between kindness and a great knowledge. It is a big privilege for me to have met you. Dr Campana, beside the scientific aspect of our relationship that helped me to learn a lot, you are for me a valuable and wise advisor to whom I will always refer. I still want to thank Chill and Marike that have been my two guardian angels helping me in every way with their great competence and patience.

Thanks to Dr. Claus Nerlov for his patience in letting me finish this.

I am really grateful to Prof. Nadia Rosenthal for her help and precious suggestions. A breath of fresh air that gave me the energy to go through the final steps.

A special thanks to ST. Jude patients and families. I have never been able to help them, I am not brave enough, but they have been helping me, with their strength and courage in the everyday fight, to truly realize how good my life is in every aspect.

Many people say that you make so many good friends when you are so far from your home, family, friends and everything you are used to, just because you need to. In the attempt to restore what it is familiar to you, at least in part, you invest on the people you have around, just to feel warm and protected again in a comfortable environment. Many say that probably in other situations or in

different scenarios the kind of relationships with the same people would not be the same. I disagree.

Ringrazio I miei genitori e tutti i miei familiari (sono tanti e uno più prezioso dell'altro) che sono stati sempre con me anche quando l'oceano ci divideva. Mamma, tu sei la mia ispirazione.

I feel really fortunate to have all of you.