Freie Universität Berlin

Regulation of the proto-oncogene *ERG* by microRNAs and the role of microRNAs in the distinct ETP-ALL subgroup of T-ALL

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"Knowledge exists potentially in the human soul like the seed in the soil; by learning the potential becomes actual."

Abu Hamid al-Ghazali

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1 INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis describes the process of blood cell formation during both the embryonic and adult stages of an organism. The cells from the hematopoietic system are derived from stem cells known as pluripotent hematopoietic stem cells (HSCs)[1–3]. HSCs have the ability to self-renew and differentiate into various progenitor cells that subsequently commit to further maturation along specific cell lineages of hematopoietic system. Furthermore, recent studies revealed that HSCs could contribute to the regeneration of multiple tissue cell types, including brain, muscle, and hepatocytes[4–6]. In adults, most of the HSCs are in a proliferatively inactive state; approximately 95% of them are in the G0 phase of the cell cycle. Several models have been suggested regarding the differentiation and maturation of human hematopoietic cells[7]. According to the classical hematopoietic ontogeny the HSCs begin to differentiate towards fully mature blood cells, and lose the multilineage potential, and commit to a single hematopoietic lineage. HSCs can be subdivided into long-term HSCs (LT-HSCs), capable of self-renewal and differentiation, and short-term HSCs (ST-HSCs), which have limited self-renewal properties[2,8]. As the cells mature to multipotent progenitors (MPPs), they gradually lose their self-renewal ability and become more mitotically active, and give successively rise to two kinds of oligolineage-restricted progenitors: common lymphoid progenitors (CLPs), and common myeloid progenitors (CMPs) (**Figure 1**). These cells are highly proliferative and express receptors for specific growth and survival factors, so called the colony stimulating factors (CSF). The CLPs further differentiate into T-lymphocytes, B-lymphocytes, and natural killer cells[9], whereas CMPs after several differentiation stages give rise to mature monocytes/macrophages, granulocytes, erythrocytes, and megakaryocytes/platelets[10]. However, beside the blood

stem cells in the classical hematopoietic ontogeny, recent discoveries indicate that the HSC compartment consists of biased HSCs that give rise to skewed ratios of myeloid and lymphoid cells[11–13].

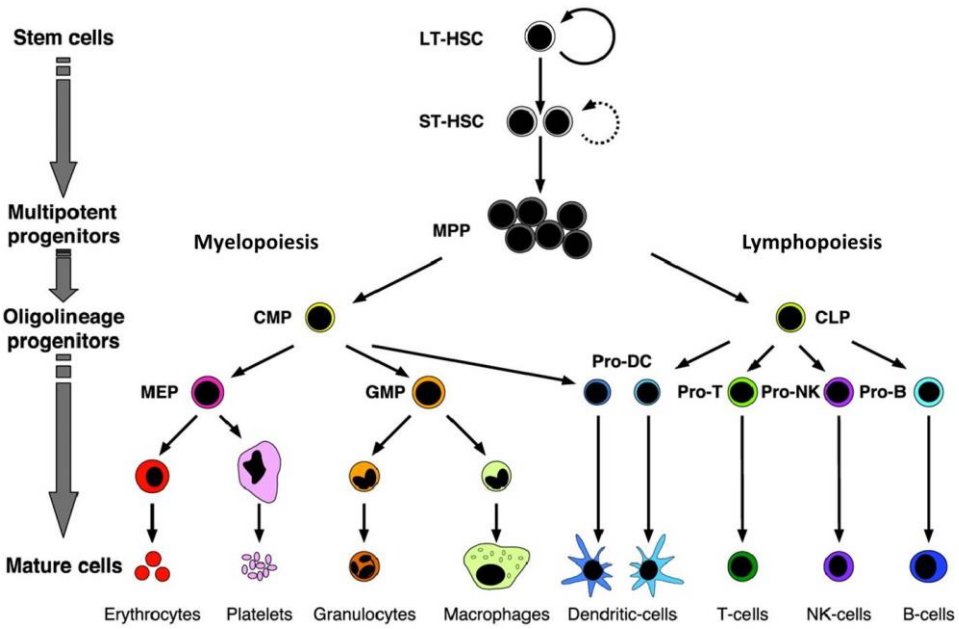


Figure 1. Hematopoietic differentiation. Schematic illustration of differentiation and maturation of human hematopoietic cells. The hematopoietic stem cells (HSC) differentiate into multipotent progentiors (MPP), which in turn give rise to either common myeloid progenitors (CMP), which are myeloid-cell and erythroid-cell lineage restricted, or common lymphoid progenitors (CLP), which are lymphoid-cell-lineage restricted progenitors.(Adapted from Passegue et al. 2003).

The human HSCs are identified by the specific surface markers ($CD34^+$, $CD59^+$, $Thy1/CD90^+$, $CD38^{-/low}$, lin^-) found on the stem cells at their undifferentiated state. As these cells begin to differentiate into mature cell lineages the cell surface markers are no longer identified. Cytokines [such as stem cell factor (SCF), thrombopoietin (TPO), and FLT3 (Fms-like tyrosine kinase 3) ligand] and transcription factors (TF, such as stem cell leukemia (SCL), GATA-2, LMO-2, and AML-1) have been found to be essential for the survival and proliferation of HSCs[14–17]. The lineage commitment and maturation of the HSCs is also determined by specific TFs (PU.1, GATA-1, CEBPA, IL-7, NOTCH1, and E2A), and cytokines (erythropoietin=EPO for erythroid maturation, TPO for megakaryopoiesis, and granulocyte- and granulocyte/macrophage colony-

stimulating factors=G-CSF and GM-CSF for granulopoiesis), which stimulate the differentiation and maturation of CMPs[18].

The maintenance and regulation of self-renewal and differentiation of HSCs depends on their specific microenvironment in bone marrow, which consists of stromal cells, including specialized fibroblasts, endothelial cells, osteoblasts, and adipocytes. These cells promote storage of HSCs, self-renewal and inhibition of differentiation. There is a continual molecular crosstalk between HSCs and these cells, which involves cadherins, integrins, chemokines, cytokines, signaling molecules and receptors that mediate adhesive cell-extracellular-matrix interactions and cell-cell interactions. Furthermore, ligand-receptor interactions activate intracellular signaling pathways after ligand binding to receptors that are expressed by HSCs. Important signaling molecules that mediate long-term maintenance and self-renewal of HSCs are among others NOTCH signaling, SCF-KIT pathway, and the angiopoietin-1 (ANG1)-tyrosine kinase receptor 2 (TIE2) signaling. NOTCH signaling was shown to be crucial for the self-renewal and clonal expansion of HSCs[19,20]. Furthermore, membrane bound SCF on osteoblasts was shown to stimulate the adhesion of HSCs to stromal cells by binding to and activating KIT, expressed by LT-HSCs[21].

Taken together, a continual signaling occurs between HSCs and the cells from the microenvironment. The stress signals from the periphery influence the properties of the HSCs, which response by differentiation and maturation into the effector cells (**Figure 1**).

1.2 T-cell development

The T-cells originate from CLPs in the bone marrow or fetal liver, and migrate to the thymus, in which the T-cell differentiation takes place. However, it has been shown that thymus alone is not sufficient for maintaining long-term T-cell development, and that replacement from the bone marrow is necessary[22]. The hematopoietic progenitors in blood contribute to the formation of thymic settling progenitors in thymus, which in

turn maintain the production of thymocytes[23,24]. The T-cell commitment includes loss of alternative lineage gene expression, loss of alternative lineage potential, expression of a T-cell specific gene program and initiation of T-cell specific gene rearrangements[25–27]. Normally, few precursor cells migrate into the thymus per day, and start to proliferate enormously upon arrival into the thymus. These cells receive proliferation-inducing signals by cytokines, such as SCF and later on IL-7. Development of T-cell precursors occur through distinct stages, which are identified by differences in cell surface markers, gene expression and developmental potential. The developmental stages occur successively in an ordered manner as follows: early T-cell precursors (ETP) which reside in the double negative (DN) 1 stage; DN2 and DN3 cells; CD4/CD8 double positive (DP) cells; and CD4 or CD8 single positive T-cells (**Figure 2**).

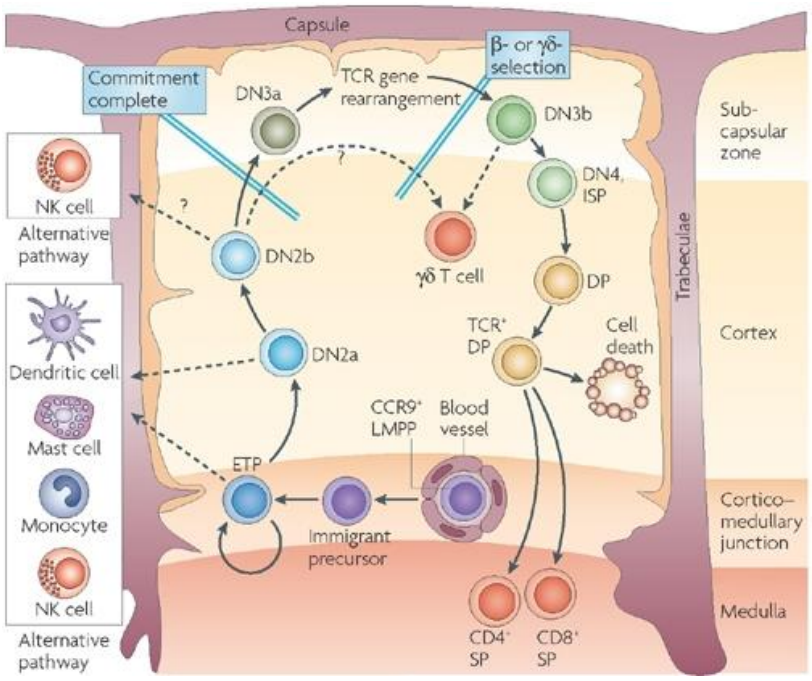


Figure 2. An overview of T-cell development. Immigrant precursors enter the thymus through blood vessels. Subsequently, the early T-cell precursors (ETP) migrate, and differentiate from double negative (DN) to double positive (DP) to single positive (SP) stages, through the distinct microenvironments of the thymus. (Rothenberg et. al. 2008).

The ETP cells possess the ability to differentiate into T-cells, myeloid cells, natural killer (NK) cells, and dendritic cells (DC)[28–30]. Thus, it is proposed that not all ETPs are derived from lymphoid restricted CLPs and that the thymus is seeded by various different types of progenitors with different lineage potential and different quantitative capacity to generate T-cells and commit to the T-cell lineage[31,32].

The ETP cells migrate through the thymic cortex, proliferating extensively and enter the DN2 stage by acquiring new surface markers (CD24 and CD25). In the DN2 stage the T-cell receptor (TCR) gene rearrangements take place by the V(D)J recombination, which gives rise to genes encoding TCRβ, TCRγ and/or TCRδ. After reaching the DN3 stage, the cells stop proliferating, and increase the TCR rearrangements to generate the first fully rearranged TCR loci. Only the T-cells which succeed in making in-frame TCR gene rearrangements survive and become activated by TCR-dependent selection (DN3b cells), which distinguishes them from DN3 cells that are not yet selected (DN3a cells). If the DN3 cells are successful at TCRβ rearrangement, then these cells turn on CD4 and CD8 expression and further proliferate and differentiate into DP cells, in a process called β-selection. Additionally, these cells further rearrange their TCRα genes to generate the conventional TCRαβ cells, which in turn undergo positive and negative selection for self/nonself discrimination for creation of CD4+ or CD8+ TCRαβ+ T-cells. Alternatively, the cells that succeed in expressing TCRγ and TCRδ proteins develop into mature TCRγδ cells.

The thymic epithelial cells provide specific receptor ligand and growth factors that trigger and support the pro-T-cell differentiation, proliferation and survival[33]. For instance, production of ligands, such as delta-like-ligand (DLL) 1 and DLL4, for the transmembrane protein NOTCH1 has been shown to be the most important of the environmental inputs for T-cell development. NOTCH signaling is essential for the very early steps of T-cell development and is sufficient to specify T-cell fate in bone marrow hematopoietic progenitors[34,35]. Expression of the Notch target genes *Deltex1* and *Hes1* was observed in ETP cells and development of ETP cells was inhibited when Notch signals were blocked in hematopoietic progenitor cells[36,37]. Additionally, loss of Notch1 expression in lymphoid progenitor cells resulted in B-lymphopoiesis in the thymus, at

the expense of T-cell development[38]. Thus, the NOTCH signaling is continuously required for promoting survival or proliferation throughout the DN stages. Furthermore the KIT expression, which promotes the cell proliferation, was shown to be essential in the earliest precursors, and gradually downregulated through the DN3 stage[39].

It has been shown that the expression of specific genes changes during the T-cell development and is turned on in a precise order. This includes T-cell identity genes, as well as TFs that regulate their expression. The T-cell identity genes include genes important for the TCR rearrangement (such as recombination activating gene (*RAG*) 1 and *RAG2*), TCR complex assembly (CD3 chains), and signaling components (kinases, phosphatases and adaptor proteins such as LCK,ZAP70 and LAT)[40]. Furthermore, various other TFs retain the plasticity in ETP cells (such as PU.1, SCL, GATA-2, and CEBPA) and cooperate with NOTCH1 in the T-cell specification process [such as GATA-3, MYB, RUNX1-CBFβ complexes E proteins (E2A and HEB), TCF1, GFI1, and Ikaros family members]. The TFs that contribute to the onset of T-cell lineage gene expression in the DN2 stage include the zinc-finger TF and tumor suppressor factor BCL-11b, HEBAlt and GLI2. Subsequently, the increased T-cell gene expression at the DN3 stage is directed by factors such as ETS-family and zinc-finger factors ETS1, ETS2, TCF1, LEF1, which are strongly upregulated at the DN2 to DN3 stage transition. Once the β- or γδ-selection is initiated multiple regulatory changes occur. At this stage the thymic microenvironment and the DN3-cell TFs have been shown to be of less importance in the maintenance of T-cell identity of the cells. NOTCH signal-dependent transcription was shown to be unessential, and a reduction of E-protein activity, and TFs that participated in the early T-cell precursor stages was observed[39,41].

1.3 Transcriptional regulation of hematopoiesis

The specification and differentiation of HSCs is one of the best characterized vertebrate developmental systems. The lineage-specific expression profiles in hematopoiesis are controlled mostly by distinct sets of TFs. The production of

transcriptional programs is controlled by the interaction of cellular context and extracellular signals. For instance, when an external stimulus activates a signal transduction pathway, the TFs get activated which in turn target gene promoters and enhancers. The expression levels and activities of these TFs selectively increase or repress gene expression to determine hematopoietic cell fate. The TFs are integrated into wider regulatory networks, which are characterized by a high degree of connectivity between TFs and transcriptional cofactors, which are part of multiprotein enzymatic complexes that facilitate or inhibit gene transcription by modification of chromatin. Several TFs are known to play critical roles in self-renewal, proliferation, and differentiation of HSCs (such as BCL2, MCL1, HOX family TFs, and GFI1). Furthermore, it has been shown that HSCs express TFs, which are responsible for the development of multiple lineages, such as PU.1, MPO, CEBPA, and MCSFR for myeloid differentiation, and GATA-1, EPOR, and MPL for erythroid/megakaryocytic differentiation, and preTα and PAX5 are TFs observed in cells at the CLP stage.

Apart from TFs the hematopoietic gene expression is regulated by epigenetic factors, including DNA methylation and covalent histone tail modifications, such as acetylation, methylation, phosphorylation, SUMOylation, and ubiquitylation. The epigenetic regulation of gene transcription implicates stable and heritable changes in gene expression by chromatin remodeling, which do not involve DNA sequence alterations. DNA methylation involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring, the CpG sites, which reduces the gene expression and can be inherited through cell division. The CpG methylation is catalyzed by DNA methyltransferase enzymes DNMT1, DNMT3A and DNMT3B. It has been shown that HSCs with deficient Dnmt1 expression showed impaired self-renewal and de-repressed expression of myeloerythroid regulators[42,43]. Additionally, absence of Dnmt3a progressively impaired the HSC differentiation and led to accumulation of HSC in the BM, implicating a critical role for Dnmt3a in HSC differentiation[44]. One of the best characterized repressive histone modifications in HSCs is exerted by Polycomb-group (PcG) complexes, which comprise a set of proteins than can assemble into multiple chromatin-associated complexes, such as Polycomb Repressive Complex (PCR) 1 and

2, whose deregulation leads to restriction of self-renewal and proliferation in HSCs and their progeny.

microRNAs (miRNAs) are another group of molecules regulating the expression of genes at a post-transcriptional level. It was shown that miRNAs play a very important role in normal hematopoiesis by regulating hematopoietic differentiation in almost every stage. Furthermore, aberrant expression of miRNAs has been associated with development of hematological malignancies. The role of miRNAs in normal hematopoiesis and in leukemogenesis will be described in detail in the coming chapters.

The hematopoietic process is tightly controlled by several signaling pathways and transcription factors. Deregulation of these regulatory networks is increasingly implicated in the development of hematological malignancies, including leukemogenesis.

1.4 Leukemogenesis

Leukemia is a heterogeneous family of blood diseases that originate from genetically and epigenetically altered hematopoietic progenitor cells. It is proposed that this malignant transformation occurs by a stepwise process in which increasing numbers of somatic mutations in a normal hematopoietic cell, possibly a HSC or a more restricted hematopoietic progenitor cell, convert these cells into a leukemic stem cell (LSC) characterized by a differentiation arrest, increased proliferation, enhanced self-renewal, decreased apoptosis and telomere maintenance. Furthermore, LSCs are defined by the ability to initiate, maintain and serially propagate leukemia *in vivo* while retaining the capacity to differentiate into committed progeny that lack these properties. LSCs reside at the apex of a hierarchy of malignant cells that is analogous to the hierarchy found in normal hematopoiesis and were found to reside within the normal bone marrow microenvironment, using the HSC machinery for survival and maintenance[45,46].

Identification and characterization of chromosomal translocations and other genetic/epigenetic alterations in leukemia revealed that chromosomal rearrangements

are associated with biologically distinct subtypes of leukemia, such as *PML-RARα* fusion gene associated with acute promyelocytic leukemia (APL), *CBFβ-SMMHC* fusion found in acute myelomonocytic leukemia, and translocations involving the *MLL* gene on chromosome 11q23, which is associated with poor prognosis in B-cell acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML)[47–50]. Although these genetic rearrangements have a critical role in the leukemogenesis, they require additional cooperative mutations to induce fully malignant diseases. Furthermore, molecular characterization has revealed other recurrent lesions, including mutations in the nucleocytoplasmic shuttling protein nucleophosmin (*NPM1*) in ~25% of AMLs, loss of function mutations and deletions in the TF *PAX5* in ~30% of B-ALL cases, and activating mutations in the *FLT3* receptor tyrosine kinase in both AML and acute lymphoblastic leukemia (ALL). Moreover, proto-oncogenes, that either suppress or promote programmed cell death, or apoptosis in normal hematopoiesis, have been shown to play critical roles in oncogenesis, as well[51,52].

1.4.1 Acute leukemia

Acute leukemias represent clonal expansion and arrest at a specific stage of normal myeloid or lymphoid hematopoietic cells that displace the normal bone marrow cells with higher numbers of immature white blood cells. Acute leukemia can be subdivided into two major subtypes, which are AML and ALL. ALL is the most common type of childhood leukemia, whereas AML occurs more commonly in adults. Acute undifferentiated leukemia, and acute mixed-lineage leukemia are other rare types of acute leukemia.

In acute leukemia, symptoms, which can be divided into primary and secondary, appear and get worse quickly. The primary symptoms caused by among others hypoplasia of normal cellular elements of bone marrow is infection from neutropenia, bleeding from thrombocytopenia and coagulopathies, and anemia. Secondary symptoms include fever, sore throat, weakness, fatigue, and weight loss.

In order to establish a diagnosis for acute leukemia, several diagnostic measures are undertaken. Application of the tests, such as complete blood count, bone marrow aspirate and biopsy, flow cytometry, cytogenetics, and polymerase chain reaction (PCR) are sufficient for categorizing the leukemia type[53]. The categorizing of acute leukemia is based on the World Health Organization (WHO) classification scheme, which divides the leukemia into several entities based on morphological and cytochemical criteria, which predominated in the previous French-American-British (FAB) classification, but also specifies the subtypes of leukemia that have distinct clinical, cytogenetic, molecular genetic, and immunophenotypic features[54]. According to this classification the blast percentage in the bone marrow must be at least 20% for a leukemia diagnosis.

1.4.2 Acute myeloid leukemia

AML is the most common acute leukemia in adults, accounting for about 80% of acute leukemia in adults. AML is a stem cell malignancy, resulting in accumulation of immature myeloid cells in the bone marrow, peripheral blood, and soft tissues, resulting in disruption of differentiation of hematopoietic progenitor cells. The median age at diagnosis is about 70 years of age, and the annual incidence of AML is 2.7 per 100,000 population.

Although the etiology for AML is unknown in most cases, several risk factors have been identified, including exposure to ionizing radiation, petroleum, benzene, and benzene-containing compounds. Furthermore, chromosomal abnormalities in autosomal recessive disorders, such as Bloom syndrome, fanconi anemia, and ataxia telangiectasia, as well as Down's syndrome have been associated with AML.

In order to define the 20% blast portion as AML, the cells must have surface antigens associated with myeloid differentiation, such as CD33 or CD13. Furthermore, more than 80% of monocytes in the blast population, establish acute monocytic leukemia diagnosis, whereas more than 50% of normoblasts or pronormoblasts defines the erythroleukemia.

Specific cytogenetic abnormalities have been used to help define distinct disease entities among myeloid disorders. The majority of the patients with AML display chromosome abnormality at the diagnosis and prognosis. The incidence of karyotypes with clonal chromosome aberrations is more frequent in children with *de novo* AML, with 70 to 85% of patients, compared to adult patients with 55 to 60%. The cytogenetic findings at diagnosis have been shown to be among the most important, independent prognostic factors in AML. In addition, cytogenetic risk systems have been suggested categorizing AML patients into one of three risk groups (favorable, intermediate, or adverse) based on cytogenetic findings at diagnosis.

The largest cytogenetic subset of AML constitutes the patients with a normal karyotype of marrow cells, which are classified in the intermediate prognostic category by major classification schemata. By using molecular genetic techniques, such as reverse transcription polymerase chain reaction (RT-PCR), global gene expression profiling, or direct sequencing, several recurrent genetic abnormalities, with prognostic significance have been detected in these cytogenetically normal AML (CN-AML) patients[55]. Mutations, such as the internal tandem duplication (ITD) of the *FLT3* gene (*FLT3*-ITD), the partial tandem duplication of the *MLL* gene, and mutations of the *CEBPA*, and *NPM1* genes have been discovered[56–59]. *NPM1* mutations are found in 48-64% of the patients, which makes it as the most common somatic gene mutation in AML. It has been shown that patients with *NPM1* mutations who lacked *FLT3*-ITD mutations had a favorable outcome[60].

Apart from genetic abnormalities such as translocations and mutations, aberrant expression of genes has also been shown to have prognostic significance in AML. For instance, overexpression of *BAALC* (brain and acute leukemia, cytoplasmic), ETS transcription factor *ERG* (v-ets erythroblastosis virus E26 oncogene homolog), and *MN1* (meningioma 1) genes have been associated with poor prognosis in CN-AML[61–63].

Treatment of AML is divided into induction and consolidation therapy. The induction therapy aims to reduce the leukemic cells and achieve a complete remission (CR), which is defined as having less than 5% blasts in the bone marrow, normal peripheral blood counts, and no extramedullary disease. The agents used in induction

therapy include cytarabine, and anthracycline, such as daunorubicin and doxorubicin. If residual leukemia exists, the patients are treated with a second course of induction therapy using an abbreviated schedule of the original induction. Consolidation is given to maintain the CR, and include intensive repeated cycles of chemotherapy, with high-dose cytarabine alone or a high-dose cytarabine-containing combination. It has been shown that patients without consolidation therapy relapse within 4-8 month after achieving CR[64].

1.4.3 Acute lymphoblastic leukemia

ALL is a highly heterogeneous disease, which develops from lymphoid cells that originates in a single B- or T-lymphocyte progenitor, blocked at a particular stage of development. According to the WHO classification ALL is classified as B-ALL, and T-cell lymphoblastic leukemia (T-ALL). B-ALL is further subdivided based on recurrent molecular and cytogenetic abnormalities[54].

The annual incidence of ALL is roughly 1.5 per 100,000 population and is age related, with 60% of cases observed in children. The exact pathogenetic events leading to development of ALL are unknown. Less than 5% of the cases are associated with inherited, predisposing genetic syndromes, such as Down's syndrome, or with ionizing radiation or exposure to specific chemotherapeutic drugs.

Cytogenetic and molecular aberrations occur in about 80% of children and 60-70% of adults, and serve as prognostic factors. Specific chromosomal translocations identify unique subtypes of ALL, and occur more frequently either in adult ALL [such as *BCR-ABL1* fusion gene encoded by the Philadelphia chromosome t(9;22)(q34;11)], or in pediatric ALL [such as *TEL-AML1*, generated by the t(12;21)(p13;q22) chromosomal translocation)][65].

The treatment for ALL is broadly categorized into induction phase, consolidation therapy, maintenance therapy, CNS prophylaxis, and allogeneic stem cell transplant. ALL induction regimes include chemotherapies, such as cyclophosphamide,

anthracycline, vincristine, asparaginase, and glucocorticoids. Furthermore, tyrosine kinase inhibitors such as imatinib, dasatinib and nilotinib, were shown to improve the CR and overall-survival (OS) rates in patients with *BCR/ABL* translocation[66,67]. CNS prophylaxis, such as intrathecal methotrexate, cytarabine, and corticosteorids are administered in order to avoid CNS relapse. The consolidation regimens, which include combinations of cytarabine, anthracycline, cyclophosphamide, methotrexate and 6-mercaptopurine, are implicated in order to improve OS and eradicate any possible minimal residual disease (MRD). Patients with poor prognosis are referred for allogeneic stem cell transplantation, which might improve long-term survival and cure rates in AML, as well as, ALL.

Hereafter, T-ALL, and a newly identified subgroup, early T-cell precursor ALL (ETP-ALL) will be described in more detail.

1.4.3.1 T-cell acute lymphoblastic leukemia

T-ALL is thought to result from malignant transformation of thymocytes that arise at defined stages of intrathymic T-cell differentiation, and represents about 15% and 25% of childhood and adult ALL, respectively. T-ALL is very heterogeneous with respect to immunophenotype, cytogenetics, molecular genetic abnormalities, and clinical features, including response to therapy, and prognosis. Patients with T-ALL indicate high white blood cell counts and present organomegaly, particularly mediastinal enlargement and central nervous system (CNS) involvement. Different systems have been used to classify T-ALL into subtypes. The European Group for the Immunological Characterization of Leukemias (EGIL) classification is the most commonly used one, and is based on the expression of immunophenotypic markers on T-lymphoblasts, reflecting the stage of thymic maturation and development. According to EGIL, T-ALL is defined by the presence of the expression of CD3, and is further subdivided into four subgroups: pro-T ($CD7^+$), pre-T ($CD2^{+/-}CD5^{+/-}CD8^{+/-}$, cortical (thymic, $CD1a^+$), and mature ($CD3^+CD1a^-$) T-ALL. Furthermore, T-ALL can be subdivided into two

subgroups, group a and group b, depending on mutually exclusive membrane expression of αβ or γδ TCR, respectively. The EGIL classification is very similar to the one of *German Multicenter Study Group for Adult ALL* (GMALL), from which the T-ALL patient samples were obtained for this study. According to GMALL the patients with early (pro- and pre-T according to EGIL), and mature T-ALL is classified as high-risk, with leukemia-free-survival (LFS) of 25% and 28%, respectively, whereas the thymic T-ALL patients are standard risk patients with LFS of 63%[68].

Cytogenetic abnormalities (structural changes) are observed in approximately 50% of T-ALL, which results in oncogene activation. The most common cytogenetic and molecular changes in T-ALL are included in Table 1. The chromosomal translocations that occur in T-ALL include juxtaposition of strong promoter and enhancer elements from the *TCR* genes (*TCRA/D* on 14q11 and *TCRB* on 7q32) with TF genes, such as *LYL1, TAL1, TAL2, BHLH1, LMO1, LMO2, HOXA* genes, and *HOX11*, which leads to aberrant expression of these genes or activation of oncogenes[69]. Additionally, formation of fusion genes (such as *SIL-TAL1*, *CALM-AF10*, and *NUP214-ABL1*), cryptic deletions (the most frequent is *INK4/ARF* locus, which leads to loss of G1 control of cell cycle) are other cytogenetic and molecular changes found in T-ALL. T-ALL with *MLL* fusion represents a distinct molecular subtype with a specific expression profile, which is characterized by differentiation arrest at an early stage of thymocyte development. Furthermore, it was shown that in the absence of chromosomal abnormalities, aberrant expression of T-cell oncogenes *HOX11, TAL1, LYL1, LMO1,* and *LMO2* were involved in leukemic transformation and associated with maturation arrest linked to defined stages of normal thymocyte development[70]

Table 1. Common cytogenetic abnormalities and mutations in T-ALL

Cytogenetic abnormality	Frequency	Involved oncogenes or fusion genes
Translocations involving TCR genes on chromosomes 7q34 (*TCRB* and *TCRG*) and 14q11 (*TCRA* and *TCRD*)	35%	*HOX11, HOX11L2, TAL1, TAL2, LYL1, BHLHB1, LMO1, LMO2, LCK, NOTCH1*, *cyclin D2*
Abnormal expression	9-30%	*SIL-TAL* fusion
Fusion gene formation	10%	*CALM-AF10* fusion
	4-8%	*MLL* fusions
	6%	*ABL1* fusions
	Rare	*NUP98* fusions
Activating gene mutations	50-60%	*NOTCH1, FLT3, NRAS*

Adapted from Aifantis *et. al*[71]

Activating mutations in T-ALL were observed in *NRAS, NOTCH1,* and *FLT3*, which will be described in more detail. Furthermore, mutations in *FBXW7*, a gene associated with *NOTCH1* mutations, have also been observed. *FBXW7* is a tumor suppressor gene, and has been found to suppress *NOTCH1* signaling[72]. Mutations in *FBXW7* have been found in primary T-ALL samples leading to abrogation of *NOTCH1* binding and thereby enhancing *NOTCH1* signaling.

Aberrant expression of genes was shown to be of prognostic significance in T-ALL as was shown for AML. Overexpression of genes such as *BAALC*, *ERG*, and *IGFBP7* was shown to be associated with a poor prognosis[73–75].

1.4.3.2 Early T-cell precursor acute lymphoblastic leukemia

Apart from the genetic subgroups of T-ALL described above, a new subgroup with developmental arrest at a very early stage of T-cell development was defined by a characteristic ETP signature in pediatric and adult T-ALL [76,77]. ETPs are early immigrants from bone marrow to the thymus as previously described (chapter 1.2). This T-ALL subgroup, termed as ETP-ALL, comprises up to 15% and 8% of pediatric and

adult T-ALL, respectively, and is described by an immature surface immunophenotype: absence of CD1a and CD8 expression, weak CD5 expression and expression of one or more myeloid-associated and/or stem cell-associated markers (such as CD117, CD34, (HLA)-DR, CD13, CD33, CD11b, or CD65). Additionally, an increased genomic instability, a high frequency of remission failures and hematologic relapse characterized this highly unfavorable T-ALL subgroup in pediatric patients. In adults, the outcome of ETP-ALL patients was comparably poor to the high-risk early T-ALL. Furthermore, molecular characterization revealed an association of ETP-ALL with high expression of stem cell associated genes, and genes of prognostic significance in AML, such as *BAALC, IGFBP7, MN1* and *WT1*[77]. Additionally, the mutational status of ETP-ALL revealed differences compared to the non-ETP T-ALL, with low frequency of *NOTCH1* mutations and high frequency of *FLT3* mutations. In a recent study, whole genome sequencing revealed that the mutational status of ETP-ALL was in part similar to myeloid leukemias[78]. Furthermore, comparison of gene expression profiles of ETP-ALL to purified normal and myeloid leukemic hematopoietic stem cell and progenitor cell populations showed similarities between ETP-ALL and normal and myeloid leukemic stem cells, indicating ETP-ALL to be poorly differentiated stem cell leukemia.

1.5 Relevant genetic aberrations involved in acute leukemia

Acute leukemia is a clinically heterogeneous disease characterized by a large number of chromosomal abnormalities, and gene mutations. These alterations disrupt normal differentiation by changing cellular functions like maintaining or enhancing the self-renewal capacity, blocking the control mechanism of normal proliferation, and apoptosis. The genetic aberrations, which are of relevance in this work, will herein be described in more detail.

1.5.1 ETS transcription factor ERG

ERG is a member of the ETS family TFs and plays a crucial role in hematopoiesis. Erg gene dosage was shown to be critical for the maintenance of HSC function, and was required to sustain definitive hematopoiesis in mice [79,80]. Homozygous mutant *ERG* mice was shown to die as a result of defects in definitive hematopoiesis[79,81]. Furthermore, by directly regulating the expression of Runx1 and Gata-2, Erg played an essential role in HSC self-renewal and for maintenance of definitive hematopoiesis during development. In hematopoietic differentiation, a potential role for ERG was conferred in T-cell development, as the expression of ERG was high in early thymocytes and diminished as the cells underwent T-cell commitment[82]. Additionally, ectopic expression of ERG was shown to induce megakaryocytic differentiation in human K562 cells and promoted expansion of megakaryocytes in hematopoietic progenitors[83,84]. In another study, forced expression of ERG was shown to promote the growth of hematopoietic cells and induced expansion of T, erythroid, and precursor B cells[85].

ERG has shown to act as a potent oncogene. Ectopic expression of *ERG* in hematopoietic cells induced the development of leukemia in mice[85,86]. Furthermore, trisomy of chromosome 21 leading to *ERG* overexpression has been strongly implicated in the development of hematological malignancy in Down syndrome[84].

In AML, *ERG* has been shown to be involved in the rare t(16;21)(p11;q22), which encodes the chimeric *FUS/ERG* gene fusion, and overexpression of *ERG* was found in the prognostically inferior subgroup of AML with a complex karyotype[87]. In addition, high levels of *ERG* in CN-AML was associated with an immature phenotype and was an independent risk factor predicting inferior outcome[62].

In T-ALL, high level expression of *ERG* constitutes an independent adverse prognostic factor and identifies T-ALL patients with a high-risk of relapse and inferior survival[73]. Additionally, deletions of *ERG* was observed in ALL, and amplification of the *ERG* locus was shown in a T-cell lymphoma mouse model[88,89].

1.5.2 NOTCH1

NOTCH1 is a heterodimeric transmembrane receptor consisting of extracellular (NEC) and transmembrane (NTM) subunits that are noncovalently associated by the heterodimerization domain (HD). Binding of the ligand to NEC results in activation of intracellular NOTCH1 (ICN, consisting of a C-terminal PEST domain, responsible for the short half-life of NOTCH1), which in turn translocates to the nucleus as part of a large transcription activator complex. In normal hematopoiesis, NOTCH signaling is essential for the generation of definitive embryonic HSCs and controls several steps in T-cell development[90,91]. The NOTCH1 gene was initially found to be involved in the rare translocation t(7;9)(q34;q34.3) in T-ALL[92]. The oncogenic activity of constitutively active NOTCH1 was demonstrated by the rapid development of T-ALL in mice transplanted with hematopoietic progenitors infected with retroviruses driving the expression of ICN[93]. Additionally, it was shown that transgenic mice expressing ICN1 in hematopoietic progenitor cells or in immature thymocytes developed T-cell tumors[93]. In further studies, mutations in the HD domain, which result in ligand independent ICN production, and PEST domain, which extends the half-life of ICN transcription activator complex, were observed in more than 50% of T-ALL from all molecular subtypes[94]. For the activation of NOTCH1 γ-secretase cleavage is required. Thus, implementation of γ-secretase inhibitors (GSIs) has revealed blockage of activated NOTCH1 protein, and transcriptional downregulation of NOTCH1 target genes in T-ALL cell lines harboring activating mutations in *NOTCH1*[94,95].

1.5.3 FLT3

The FLT3 receptor tyrosine kinase plays an important role in the development of hematopoietic stem cells. In normal bone marrow, *FLT3* expression is restricted to early progenitors, including CD34+ cells with high levels of C-KIT expression. Activation of the receptor by its FLT3 ligand induces oligomerization leading to phosphorylation and activation of downstream pathways. The FLT3 ligand is released as a soluble

homodimeric protein, and is expressed in cells of the hematopoietic bone marrow microenvironment, including fibroblasts, as well as in hematopoietic cell lines of myeloid, and B- and T-cell lineages. Both the membrane bound and souble forms activate the tyrosine kinase activity of the receptor and stimulate growth of progenitor cells in the marrow and blood, by synergizing with other hematopoietic growth factors and interleukins. The FLT3 ligand is a growth factor for immature myeloid cells and stem cells and can expand CD34+ cells *in vitro* and *in vivo*. In acute leukemia, approximately 40% of CN-AML patients harbor *FLT3*-ITD mutations in the juxtramembrane domain or activating point mutations in the activation loop of the tyrosine kinase domain (TKD), which promote autophosphorylation of FLT3. Constitutive activation of *FLT3*-ITDs activates the downstream targets, including STAT5 and RAS/MAPK pathways, which confers factor-independent growth of hematopoietic cells[96,97]. Both *FLT3*-ITD and *FLT3*-TKD have been associated with a poor outcome in AML[56,98]. These findings have led to the implementation of tyrosine kinase inhibitors for the treatment of *FLT3* mutated AML patients[99].

In T-ALL, mutations in the *FLT3* gene, including *FLT3*-ITD and *FLT3*-TKD, occur in a very low frequency, and is mainly described in only small sets of patients[100,101]. It has been shown that the newly identified subgroup ETP-ALL had high frequency of *FLT3* mutations[77].

The development of acute leukemia is thought to be the result of a series of genetic alterations conferring various types of growth and cellular survival advantages. In addition to the genetic abnormalities described above, more alterations might collaborate with these to contribute to the pathogenesis of leukemia. Recent studies have shown that miRNAs, which are small non-coding RNAs, play an important role in the regulation of normal hematopoiesis and in the pathogenesis of acute leukemia.

1.6 microRNAs

1.6.1 microRNA biogenesis

miRNAs are ~22 nt small non-coding RNAs that regulate gene expression post-transcriptionally by binding to specific mRNA targets and promoting their degradation and/or translational inhibition[102]. In mammals, miRNAs are predicted to control the activity of ~50% of all protein-coding genes. Many of the miRNA genes reside in intronic regions of neighboring genes, indicating that these miRNAs might be co-transcribed with their host genes[103]. In addition, there are other miRNAs which are intergenic and are likely transcribed as independent units[104,105]. The processing of the miRNAs initiates in the nucleus and proceeds in the cytoplasm (**Figure 3**). Transcription of the majority of the miRNAs is guided by the RNA polymerase II yielding a long, primary transcript (pri-miRNA)[106]. The miRNAs, like the mRNAs, bear the 7-methyl-guanylate cap at the 5'-end and poly (A) tail at the 3'-end[105,106]. The location of the transcription initiation sites to the miRNA coding region varies, with a distance from a few hundred bases up to 20 kb upstream[107]. The promoters of the miRNAs are similar to the promoters of protein coding genes (i.e. frequencies of CpG islands, TATA box, TFIIB recognition, initiator elements and histone modifications)[106,108]. Transcription of pri-miRNAs is regulated by different TFs (i.e., c-myc, p53, MEF2, PU.1, and REST)[109–112], and growth factors (platelet-derived growth factor (PDGF), transforming growth factor-ß (TGF-ß), and bone-derived neutrophic factor)[112–115]. Furthermore, miRNA transcription is also regulated by diverse epigenetic mechanisms[116–118]. This complex network of transcriptional regulation confers tissue- and cell-specific expression patterns.

Following transcription, the pri-miRNAs, which are generally several thousand nucleotides, fold into hairpin structures consisting of one or more inverted repeats, which act as substrates for two members of the RNase III family of enzymes, Drosha and Dicer. Both are double-stranded RNA (dsRNA) specific endonucleases that

generate 2-nucleotide-long 3' overhangs at the cleave site. Drosha executes its function within the nucleus, whereas Dicer is active in the cytoplasm.

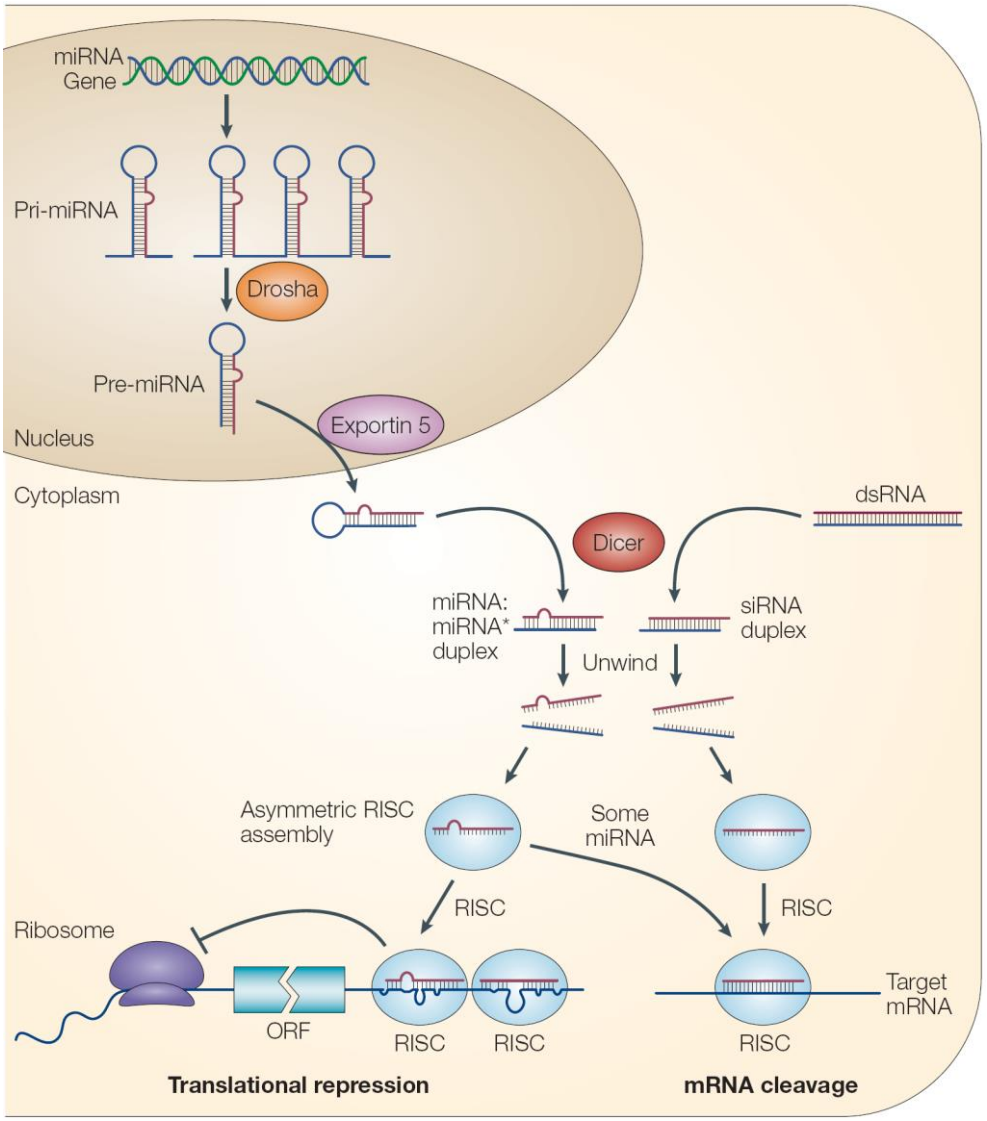


Figure 3. An overview of miRNA biogenesis. (He and Hannon, 2004)

Drosha cleaves the pri-miRNA transcripts into ~70bp pre-miRNAs regardless of the primary sequences and structures of the pri-miRNAs[119]. In order to cleave the pri-miRNAs Drosha associates with at least 20 distinct polypeptides and forms a large complex known as the "Drosha microprocessor complex"[120]. This complex minimally

includes the enzyme Drosha and its dsRNA binding partner DGCR8 (also known as Pasha in invertebrates). DGCR8 might recognize the junction between the single stranded RNA (ssRNA) and double stranded regions at the hairpin structures in order to direct Drosha cleavage one helical turn away from the ssRNA-dsRNA junction[121]. The regulation of Drosha/DGCR8 is tightly controlled by DEAD-box RNA helicases p68 and p72. pri-miR processing is further regulated by p68-interacting proteins, including the Smads, p53, and estrogen receptor α (ER-α). This regulatory control by different TFs enables a rapid regulation of miRNA expression in response to extracellular stimuli. An alternative pathway for the miRNA biogenesis includes miRNAs, which are embedded within short introns, so called "mirtrons". The mirtrons are directly spliced out of introns as pre-miRs, without getting processed by Drosha microprocessor complex[122].

Cleavage by Drosha leaves a ~2 nt 3' overhang, which is recognized by the export factor Exportin-5[123]. Exportin-5 cooperates with the small GTPase Ran to mediate the transport of pre-miRs into the cytoplasm[124]. In the cytoplasm, the cytoplasmic enzyme Dicer cleaves the pre-miRs at about two helical turns away from the base of the stem loop, and generates transient unstable ~22 nt dsRNA composed of the active guide strand (miRNA) and the passanger strand (miRNA*). One of these strands gets further incorporated into the RNA-induced silencing complex (RISC)[125,126]. In order to exert its function, Dicer associates with proteins such as TAR RNA-binding protein (TRBP), and protein kinase R-activating protein (PACT), which enhances Dicer stability and processing activity[127]. Knockdown of either of these cofactors destabilizes Dicer and results in a subsequent loss of miRNA biogenesis[128,129]. Along with these two cofactors, Dicer associates with Argonaute (Ago) proteins and forms the RISC-loading complex (RLC). The Ago proteins are the primary components of the RISC complex, and affect the miRNA-mediated repression. The human genome contains eight Ago-family proteins (Ago1-4 and Piwi1-4)[130]. Ago2 is the only one with cleavage activity and might therefore have a prominent impact on miRNA-mediated silencing. The RLC unwinds the double-stranded miRNA in a manner that facilitates the incorporation of the single stranded mature miRNA into miRNA-containing ribonucleoprotein (miRNP),

and the miRNA* strand gets degraded. Selection of the miRNA strand which gets incorporated into the RISC depends partly on the relative stability of the tow ends of the duplex, and the ratio of miRNA:miRNA* strand. The strand that enters the RISC is nearly always the one whose 5' end is less tightly paired[131,132]. Furthermore, deep sequencing of small RNAs revelad a ~100:1 ratio of miRNA:miRNA* strand, suggesting that miRNA* strand is actively getting degraded[133]. Following the loading of Ago with the single-stranded mature miRNA, Dicer dissociates and the miRNP complex capable of selecting miRNA targets is formed through the association of additional proteins[134,135].

1.6.2 microRNAs: Mechanisms of action

The gene silencing by the RISC complex occur either via translational inhibition or by mRNA degradation. The destiny of the target mRNA is in part decided by the sequence complementarity of miRNA to mRNA. The miRNA will direct the cleavage of the mRNA if the mRNA has sufficient complementarity to the miRNA, or just repress the translation if the mRNA is partly complementary to the miRNA[136–138]. In metazoans, the miRNAs pair imperfectly with their targets. The most important requirement for translational repression or cleavage is a contiguous and perfect base pairing of the miRNA 5' nucleotides 2-8, which represents the 'seed' region, to the 3' UTR of the target mRNA[139,140]. Furthermore, complementarity to the 3' miRNA stabilizes the interaction, and is important when matching in the seed region is suboptimal[141,142]. miRNAs mediate gene repression by interfering with translation of the target genes. Translation can generally be divided into initiation, elongation, and termination. miRNAs repress translation at initiation, either by targeting the cap recognition step or by inhibiting ribosome 80S complex assembly (**Figure 4)**.

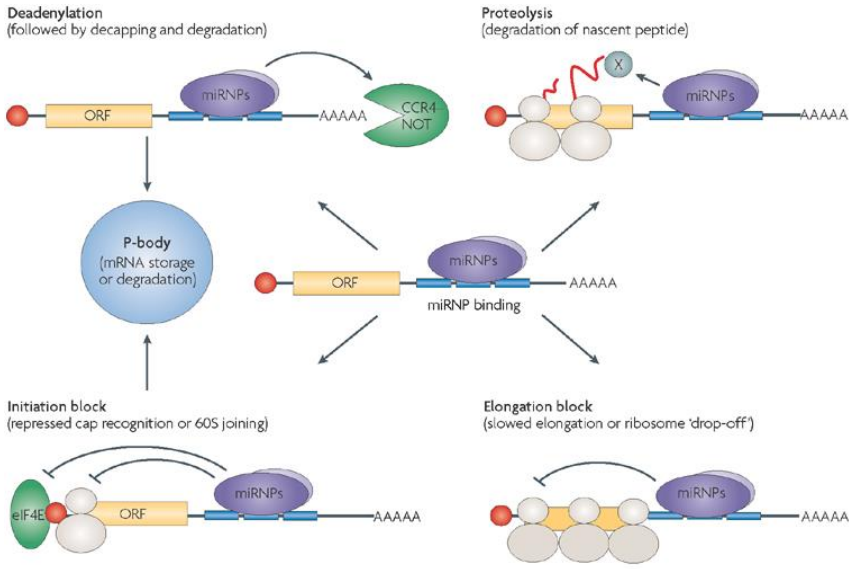


Figure 4. Possible mechanisms of the miRNA mediated post transcriptional gene repression in animal cells. Translational gene repression occurs either at the initiation step (upper left, and bottom left) or post-initiation step (upper right, and bottom right). (Filipowicz et. al. 2008).

At the initiation step miRNAs interfere with the eIF4F-cap recognition process. The AGO2 from the RISC complex compete with cap-binding protein eIF4E for m^7G cap binding and prevent translation of capped mRNAs[143,144]. Furthermore, it has been shown that miRNAs mediate deadenylation of the target mRNAs by interfering with the CCR4-NOT1 deadenylase complex[145]. Thus, miRNAs might cause translational block at the initiation stage by preventing the interaction between the 5'cap and 3'poly A tail, through targeting either the m^7G cap or the poly A tail. mRNAs repressed by these way are moved to cytoplasmic foci called P-bodies for either degradation or storage[143,146].

The inhibition of the 80S ribosome assembly occurs by association of the RISC with eIF6, which is involved in the biogenesis of 60S ribosomal subunit, and subsequently prevents the interaction between the 60S subunit with the 40S subunit[147].

Furthermore, repression at post-initiation steps has also been reported. Studies in *C.elegans* and in mammalian cells revealed that mRNAs, inhibited by miRNAs, remained associated with polysomes despite a reduction in their protein products[148–150]. Thus, a drop-off model was suggested, in which miRNAs are proposed to antagonize translation elongation by causing premature termination and subsequent ribosome drop-

off. Another study proposed that miRNA machinery recruited proteolytic enzymes to polysomes, which leads to the degradation of nascent polypeptides[151].

Recent findings indicate that under specific cellular conditions, miRNA mediated repression can be prevented or reversed[152–155]. The inhibition of *CAT1* mRNA by miR-122 was abrogated by the RNA-binding protein ELAVL1 in hepatoma cells subjected to different stress conditions[152]. It is possible that miRNA-mediated repression is regulated by RNA-binding proteins (RBPs), and other way around the RISC might act as translational activators by either displacing or modulating inhibitory RBPs bound at the 3'UTR[156,157].

1.6.3 microRNAs in normal hematopoiesis

Hematopoiesis is controlled by complex molecular events that regulate commitment, differentiation, proliferation, and apoptosis of HSCs. This process is mainly guided by a network of transcription factors, which initiates the commitment of HSCs to different lineages. The expression of the transcription factors during hematopoiesis is regulated by chromatin modifications including histone acetylation and DNA methylation[158]. However, recent studies implicate a crucial role for miRNAs in the regulation of hematopoietic differentiation by targeting the expression of transcription factors and genes involved in the regulation of cell cycling and proliferation. miRNAs might have an impact on almost every stage of hematopoiesis (**Figure 5**).

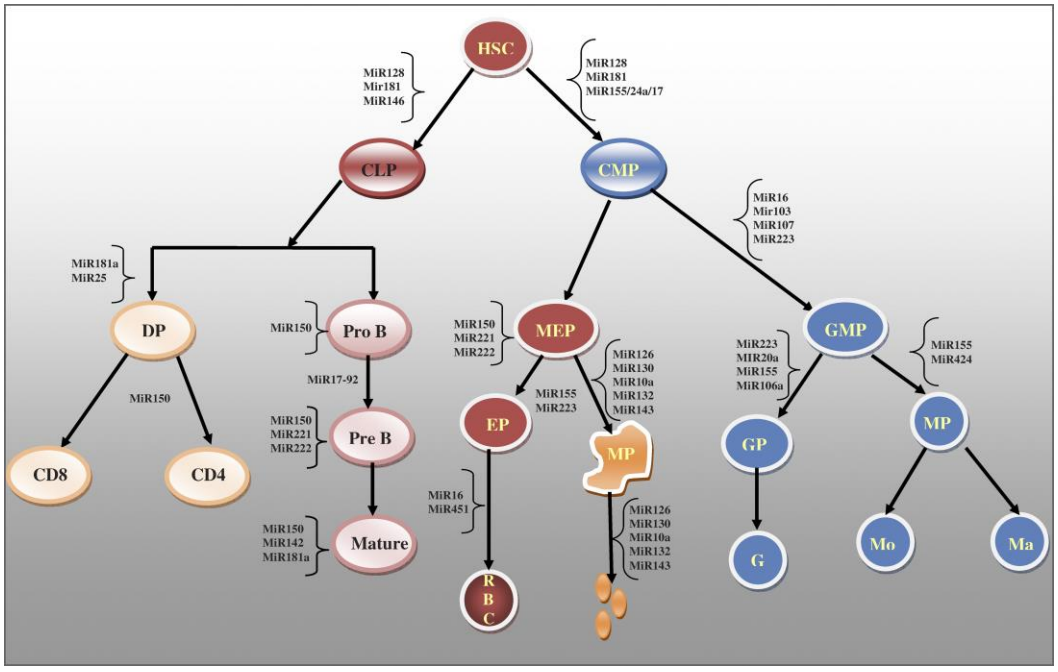


Figure 5. Role of different miRNAs in normal hematopoiesis. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DP, double positive; EP, erythroid progenitor; G, granulocyte; GMP, granulocyte macrophage progenitor; GP, granulocyte progenitor; HSC, hematopoietic stem cells; MEP, macrophage erythroid progenitor; MP, monocyte/macrophage progenitor. (Bhagavathi et al, 2010).

The first study demonstrated that miRNAs were differentially expressed in mice hematopoietic tissues[159]. miR-181 was found to be expressed at high levels in the murine thymus and differentiated B lymphocytes, whereas it was lower expressed in undifferentiated progenitor cells. Ectopic expression of miR-181 in hematopoietic progenitor cells resulted in B lymphocyte proliferation upon hematopoietic reconstitution. Furthermore, miR-223 was expressed at high levels in myeloid cells and miR-142 expression was highest in B-lymphoid tissues. The importance of miRNAs in hematopoiesis was further confirmed by experiments ablating Dicer in hematopoietic cells. Premature death of Dicer-deficient mice was observed with a lack of detectable multipotent stem cells[160]. Furthermore, deletion of Dicer resulted in developmental block at the pro- to pre-B-cell transition in early B-cell progenitors, and impaired T-cell development and aberrant T helper cell differentiation and cytokine production[161,162].

miRNAs might also have a major role in the earliest stages of hematopoiesis. From a large scale profiling of miRNAs using human CD34+ selected HSCs from normal human bone marrow and mobilized peripheral blood stem cell harvests 33 miRNAs were found to be expressed in CD34+ progenitor cells[163]. The most abundant miRNAs in human BM CD34+ cells were; miR-191, miR-181, miR-223, miR-25, miR-26, miR-221, and miR-222. In another study, miR-520h was found to promote HSC differentiation into committed progenitors by inhibiting *ABCG2* expression[164].

Several studies have highlighted the importance of miRNAs on lineage commitment in progenitor cells and mature effector cell function. In the erythropoiesis miR-221 and miR-222 were found to be downregulated during erythroid differentiation and maturation[165]. The downregulation of both miR-221 and miR-222 was necessary to promote early erythroid proliferation of umbilical cord blood (UCB). Downregulation of these miRNAs unblocked the tyrosine kinase receptor c-KIT, shown to be a direct target of miR-221 and miR-222, and which is critical for development of early erythroblasts[166]. Furthermore, examination of miRNA expression in erythrocyte precursors obtained from peripheral blood mononuclear cells, cultured in a three phase liquid system, revealed a progressive downregulation of miR-155, miR-221, and miR-222, upregulation of miR-16 and miR-451 at late stage of differentiation, and biphasic

expression pattern of miR-339 and miR-378[167]. The most consistent and upregulated miRNA after erythroid differentiation is miR-451, which is transcribed in cluster with miR-144 by the transcription factor GATA-1[168]. Overexpression of miR-451 was shown to promote erythroid differentiation and hemoglobin content[169]. Furthermore, miR-223 was downregulated in unilineage erythroid culture of UCB CD34+ progenitors. Ectopic expression of miR-223 targeted *LMO2*, playing critical role in hematopoietic differentiation, and thereby inhibited differentiation and maturation of erythroid cells[170].

miRNA expression profiling during *in vitro* megakaryocytic differentiation of BM CD34+ progenitor cells identified 19 miRNAs to be downregulated during the differentiation (including miR-10a, miR-10b, miR-106, miR-126, miR-130a, and miR-20)[171]. miR-130 was found to target *MAFB* , which is involved in the activation of the GPIIB promoter (a key protein for platelet physiology) and miR-10a was found to target *HOXA1* in megakaryopoiesis. Both of these genes are overexpressed during megakaryopoiesis and downregulation of the miRNAs might unblock their expression. Furthermore, the expression of miR-150 was increased in cells undergoing megakaryocytic differentiation, whereas it was downregulated in cells differentiating to erythrocytes[172]. Overexpression of miR-150 drove megakaryocyte/erythrocyte precursors toward megakaryocytes at the expense of erythroid cells by targeting the transcription factor c-Myb, a critical player in the cell fate decision between megakaryocyte/erythrocyte differentiations. Moreover, downregulation of two other miRNAs was observed during megakaryocytic differentiation, miR-146a and miR-155[173,174]. Ectopic expression of these miRNAs in CD34+ HSCs impaired the proliferation and differentiation of megakaryocytes, through direct targeting the pro-megakaryocyte transcription factor *ETS1* by miR-155, and the chemokine receptor 4 (CXCR4) by miR-146a.

The first miRNA identified to have a role in the myelopoiesis was miR-223. Myeloid differentiation in APL cells revealed that miR-223 upregulates mouse granulopoiesis in association with the transcription factors NFIA (negative nuclear factor IA) and CEBPA[175]. NFIA was found to downregulate, whereas CEBPA upregulated miR-223 expression. Two other miRNAs critical for granulocytic and monocytic differentiation

are miR-21 and miR-196b, which are regulated by the GFI-1 required for normal granulopiesis[176]. GFI-1 was found to repress the transcription of the two miRNAs by binding to their promoter region. Ectopic expression of miR-21 in Lin- murine BM cells resulted in a significant increase of monocytic colonies and miR-196b overexpression caused significant loss of granulocytic colonies. Thus, these two miRNAs might be critical players in the GFI-1 activity controlling the transcription between granulocytic-monocytic progenitors to granulocyte precursors. Furthermore, monocytopoiesis was found to be controlled by a circuitry involving miR-17-5p, miR-20, miR-106 (downregulated during monocytic differentiation), and AML-1 and M-CSF (upregulated during monocytic differentiation)[177].

miRNAs have also been implicated in lymphoid cell development and immune function. One of the most important miRNA involved in the modulation of this lineage is miR-150, which is preferentially expressed in mature, resting T and B cells but not in their progenitors. Ectopic expression of miR-150 in hematopoietic stem cell progenitors impairs the formation of mature B cells and inhibits the transition from pro-B to pre-B cell stage in part by increasing apoptosis, whereas it has little effects on T-cells[178,179]. It was shown that c-MYB, an essential transcription factor for early lymphoid development, was a direct target of miR-150, and the effects observed by miR-150 overexpression was in part caused by the interaction between miR-150 and c-MYB. Overexpression of another crucial miRNA, miR-181, caused in murine HSCs an increase in the percentage of CD19+ B cells and decrease of CD8+ T-cells without affecting other hematopoietic lineages in hematopoietic reconstitution assays *in vivo*[159]. Furthermore, miR-181a is upregulated in double positive (CD4+CD8+) thymocytes and targets *BCL-2*, *CD69*, and *TCRα*, genes involved in positive T-cell selection[180]. Moreover, miR-17-92 cluster was found to specifically act during the transition from pre-B to pro-B lymphocyte development, thus enhancing the survival of the B cells at this stage by targeting Bim[181].

Altogether, these data implicate a critical role for miRNAs in controlling hematopoietic system and dysregulation might, thus, disturb the tight control of

hematopoietic differentiation process and may contribute to hematological malignancies.

1.6.4 microRNAs in acute leukemia

miRNAs have been implicated in different types of leukemia. The first study connecting miRNAs and leukemia reported frequent downregulation of the miRNA cluster miR-15a/miR-16-1 in chronic lymphocytic leukemia (CLL)[182]. This cluster is localized at chromosome 13q14.3, which is deleted in about 65% of CLL patients. Additional work revealed that this cluster targets the anti-apoptotic *BCL-2*[183], which indirectly explains the overexpression of *BCL-2* observed in CLL.

Depending on the target gene, miRNAs act as either tumor suppressor miRNAs or oncogenic miRNAs (oncomiRs). Decrease in tumor suppressor miRNAs may cause increased expression of oncogenes that might contribute to increased cell proliferation, invasion, and a decrease in apoptosis, which in turn will give rise to solid tumor formation and blood-borne malignancies. Conversely, by targeting tumor suppressor genes the oncomiRs might also contribute to increased cell proliferation, invasion, and decreased cell death. Specific signatures of deregulated miRNAs which harbor diagnostic and prognostic implications have been described in both AML and ALL. Furthermore, miRNA genome-wide expression studies revealed altered miRNA expression pattern of acute leukemia patients compared to normal hematopoietic cells.

There are several suggested reasons for the dysregulation of miRNAs in acute leukemia. Genomic aberrations including amplifications and translocations may be one reason. Since miRNAs are encoded on such fragile sites, they are more prone for such alterations. For instance, high levels of the miR-17-92 gene cluster observed in *MLL*-rearranged patients, was due to the *MLL*- fusion proteins, and the oncogene c-myc[184,185]. In T-ALL, the overexpression of this cluster was caused by the newly identified translocation t(13;14)(q32;q11)[186]. Furthermore, genomic deletions and mutations may also be responsible for the dysregulation of the miRNAs, such as a 7Mb deletion of the chromosome 12, which reduced the expression of miR-203 in murine T-cell

leukemias/lymphomas[187]. Furthermore, mutation in the *miR-128* gene reduced the amount of mature miR-128b leading to prednisone resistance in *MLL*-rearranged leukemia[188,189]. Aberrant expression of neighboring protein coding genes may also affect the dysregulation of miRNAs. For instance, in *MLL*-rearranged ALL, T-ALL, and AML patients, the miR-196b is co-expressed with its neighboring genes *HOXA9*, and *HOXA10*[190,191]. Furthermore, modification of miRNAs in leukemia by epigenetics can be another regulatory level of miRNA expression. miR-203, controlling the expression of the oncogene ABL and BCR-ABL fusion products, was found to be silenced through CpG hypermethylation[187]. Moreover, in *MLL*-rearranged ALL, high miR-196b expression was associated with CpG island hypomethylation of the promoter region of miR-196b/HOXA locus[190]. Apart from the epigenetic regulation of miRNAs, it has been shown that miRNAs themselves can regulate the transcription of genes involved in the epigenetic machinery[192].

1.6.4.1 microRNAs in AML

Significantly and differentially expressed miRNAs have been identified for genetic subtypes of AML[193]. miRNA expression was shown to be closely associated with selected cytogenetic and molecular abnormalities, such as t(11q23) and *FLT3*-ITD mutations[194]. The expression of the miR-29 family member miR-29b was downregulated in primary AML cells compared to bone marrow CD34+ progenitors. Ectopic expression of miR-29b in AML cell lines and primary AML samples revealed a reduction in cell growth and induction of apoptosis by downregulating Mcl-1 expression in these cells, indicating that miR-29b acts as a tumor suppressor miRNA[195]. This notion was further supported by the dramatic reduction of tumors in a xenograft leukemia model in response to miR-29b overexpression. miR-29b was shown to target pathways involving the cell cycle, proliferation, and apoptosis[195]. Furthermore, in a study the miRNA expression was explored in cytogenetically normal AML (CN-AML) patients carrying *NPM1* and *FLT3*-ITD mutations[196]. Several upregulated miRNAs

(including miR-10a, miR-10b, and miRNAs from the let-7 and miR-29 family members) distinguished *NPM1* mutated from the *NPM1* unmutated cases. In addition, AML samples with FLT3–ITD were characterized by upregulation of miR-155, which was independent of FLT3 signaling.

In another study, the expression of two (miR-126/126*), three (miR-224, -368, and -382), and seven (miR-126, -126*, -224, -368, -382, -17-5p, and -20a) was able to distinguish Core Binding Factor (CBF), t(15;17), and *MLL* gene rearrangement AMLs, respectively, from one another[197]. Ectopic expression of miR-126/126* induced cell growth and inhibited apoptosis in AML cell lines, proposing a role for these miRNAs as oncomiRs in the leukemogenesis of AML.

miRNA expression was further associated with prognosis in CN-AML patients. miR-181a and miR-181b were part of miRNA expression signature associated with outcome and their expression levels were inversely associated with the risk of an event (failure to achieve complete remission, relapse, or death)[198,199]. These miRNAs contribute to an aggressive AML phenotype possibly through mechanisms associated with the activation of pathways of innate immunity mediated by toll-like receptors and interleukin-1β. Furthermore, patients with t(6;11), who showed increased levels of miR-21, which inhibits the tumor suppressor *PTEN*, was associated with a poor prognosis[199].

1.6.4.2 microRNAs in ALL

A large-scale genome wide miRNA expression profile assay identified 27 miRNAs to be differentially expressed between ALL and AML[200]. Among them, the expression of miR-128a and miR-128b was significantly upregulated, whereas the expression of let-7b and miR-223 were significantly downregulated in ALL compared to AML. The miR-128b expression was also higher in ALL compared to CD19+ cells, which in part was associated with promoter hypomethylation. Furthermore, it was found that five miRNAs (miR-128b, -204, -218, -331, and -181b-1), as well as the miRNAs belonging the miR-17-92 cluster, were the most highly expressed miRNAs in ALL compared to

CLL[201]. In pediatric ALL miRNA expression was linked to genetic subgroups and a specific miRNA expression profile was shown to be highly predictive of clinical outcome[202]. Another study identified differentially expressed miRNAs among different molecular ALL subclasses including B-ALL and T-ALL[203]. Additionally, specific miRNAs (miR-19b, miR-20a, miR-26a, miR-92 and miR-223) were found to promote T-ALL development in a mouse model and produced overlapping and cooperative effects on tumor suppressor genes implicated in the pathogenesis of T-ALL[204].

Taken together, the hematopoietic differentiation is tightly controlled by specific transcriptional programs, epigenetic mechanisms and miRNAs. Deregulation of this mechanisms leads to hematological malignancies, including acute leukemia. The aberrant expression of miRNAs was shown to be responsible for various genetic alterations observed in different kinds of leukemia. Deregulated miRNA expression was further associated with prognosis and was shown to contribute to the pathogenesis of acute leukemia.

2 AIM

Acute leukemias are complex heterogeneous group of disorders that differ with regard to biology, clinical course, and prognosis. Over the past years the knowledge of these hematological malignancies has increased, which has resulted in a classification based on morphology, immunophenotype, molecular and clinical features. These classifications attempt to define biologically and clinically relevant entities. However, in many cases the underlying pathomechanisms are unknown, and also the well-defined subgroups of leukemia exhibit extensive heterogeneity. Therefore, an improved understanding of the disturbed underlying molecular mechanisms would shed light on to the tumor biology of acute leukemias.

Aberrant expression of genes in acute leukemia, such as the ETS transcription factor *ERG,* involved in normal hematopoiesis, has been shown to be independent risk factor predicting inferior outcome in T-ALL and CN-AML. The underlying biology of the oncogenic properties of ERG and its expression regulation remain unknown. ERG was shown to be regulated partly by epigenetic mechanisms. Thus, in addition to the epigenetic regulation, the regulation of *ERG* may also be directed by other mechanisms, such as miRNAs.

Considerable progress has been made for the characterization of molecular genetics of T-ALL, allowing identification of new subgroups of T-ALL. The newly identified subgroup ETP-ALL is associated with stem cell and myeloid characteristics and poor prognosis in pediatric T-ALL, which makes this subgroup of biological and clinical relevance. Thus, the investigation of molecular genetics of adult ETP-ALL might unravel the pathomechanisms and potential therapeutic implications (targeted therapies), which might improve the outcome for these patients with poor prognosis.

Apart from the genetic abnormalities, miRNAs, which are post-transcriptional regulators of gene expression, have been shown to play crucial roles in normal

hematopoiesis and in the pathogenesis of acute leukemia. miRNAs fine-tune the hematopoietic system and manage the control of both lymphoid and myeloid lineages. Thus, deregulation of miRNAs might contribute to leukemogenesis by disturbing the tight control of normal hematopoietic processes.

In this context, the focus was laid on the following aspects:

1. Regulation of *ERG* by miRNAs

Regulation of *ERG* by miRNAs, by functional assays, and the potential role of the miRNAs in normal hematopoiesis and acute leukemia.

2. Molecular and clinical characterization of adult ETP-ALL

Investigation of the specific molecular alterations in ETP-ALL by performing a comprehensive molecular and clinical study on a large cohort of adult ETP-ALL. Furthermore, since high frequency of *FLT3* mutations were observed in ETP-ALL, the impact of tyrosine kinase inhibitors on T-ALL cell lines harboring *FLT3* mutations was also investigated.

3. Identification of aberrantly expressed miRNAs in ETP-ALL

Examination of the global miRNA expression in the high-risk subgroup of ETP-ALL and other subgroups of T-ALL, and the potential functional role of the miRNAs in acute leukemia. The expression pattern of miRNAs might shed new light on to the specific myeloid and stem cell like features of ETP-ALL.

3 PRESENTATION OF THE ARTICLES

This thesis is based on the following original articles:

1. Coskun E, von der Heide EK, Schlee C, Kühnl A, Gökbuget G, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. The role of microRNA-196a and microRNA-196b as *ERG* regulators in acute myeloid leukemia and acute T-lymphoblastic leukemia. ***Leukemia Research*** **2010**; 35(2):208-213

2. Neumann M, Coskun E, Fransecky L, Bartram I, Sartangi NF, Heesch S, Gökbuget N, Schwartz S, Brandts C, Schlee C, Haas R, Dührsen U, Griesshammer M, Döhner H, Ehninger G, Burmeister T, Blau O, Thiel E, Hoelzer D, Hofmann WK, Baldus CD. FLT3 mutations in early T-cell precursor ALL characterize a stem cell like leukemia and imply the clinical use of tyrosine kinase inhibitors. ***PloS One*** **2013**; 8(1):e53190

3. Coskun E, Neumann M, Schlee C, Liebertz F, Heesch S, Goekbuget N, Hoelzer D, Baldus CD. MicroRNA profiling reveals aberrant microRNA expression in adult ETP-ALL and functional studies implicate a role for miR-222 in acute leukemia. ***Leukemia Research*** **2013**; 37(6):647-656

3.1 Manuscript: "The role of microRNA-196a and microRNA-196b as *ERG* regulators in acute myeloid leukemia and acute T-lymphoblastic leukemia"

Deregulation of transcription factors, which promote normal differentiation of hematopoiesis, is critical for the development of leukemias. One such gene is ETS1 transcription factor *ERG* that plays an important physiological role in hematopoiesis and was of independent adverse prognostic significance in CN-AML, and T-ALL[62,73]. Little is known about the underlying regulatory mechanisms of *ERG*. For instance, *ERG* was found to be regulated partly by epigenetic mechanisms[205]. Thus in addition to epigenetic regulation, the regulation of *ERG* may also be directed by other mechanisms.

Herein, we addressed this issue by investigating the regulation of *ERG* by miRNAs, as they are post-transcriptional regulators of gene expression. By using four different databases (Targetscan http://www.targetscan.org, PicTar http://pictar.mdc-berlin.de, mirBase http://microrna.sanger.ac.uk, Human microRNA targets http://www.microrna.org) we identified the predicted potential miRNAs that regulate *ERG*. Eleven miRNAs (miR-9, miR-27a, miR-30b, miR-137, miR-142-3p, miR-145, miR-196a, miR-196b, miR-219, miR-361 and miR-544), common in at least two of the databases, were chosen for further analyses.

In order to examine the effect of miRNA overexpression on *ERG* mRNA downregulation, KG1a (AML) and MOLT-4 (T-ALL) cell lines were transfected with Pre-miR™ miRNA precursor molecules (Applied Biosystems/Ambion, Darmstadt, Germany), and scrambled RNA oligomer controls (Pre-miR negative control), using the Nucleofector systems (Lonza Cologne AG, Cologne, Germany) according to the manufacturer's recommendations. Subsequently, the cells were harvested 24 hours (hrs) and 48 hrs after transfection, for gene expression analysis. RNA was extracted using Trizol® Reagent (Invitrogen GmbH, Karlsruhe, Germany). Total RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany), and cDNA for miRNA analyses was converted using miRNA specific stem-loop primers (Applied Biosystems/Ambion,

Darmstadt, Germany). Quantitative real time RT-PCR (qRT-PCR) was performed on a region common to all *ERG* isoforms (pan-*ERG*), *ERG2* and *ERG3* isoforms using Glucose Phosphate Isomerase (GPI) as housekeeping gene. The relative levels of miRNAs were determined by stem-loop real time RT-PCR using miRNA specific primers according to the Taqman MicroRNA Assay protocol (Applied Biosystems/Ambion, Darmstadt, Germany), and RNU6B small nuclear RNA endogenous control was used for normalization. The quantification was performed on Real-Time Cycler Rotor-Gene RG3000 (Corbett Research, Wasserburg, Germany).

Of all miRNAs tested, only transfection of the miRNA precursor molecules of miR-196a (Pre-miR-19a) and miR-196b (Pre-miR-196b) induced a significant reduction of pan-*ERG*, *ERG2,* and *ERG3* mRNA expression levels in KG1a and MOLT-4 cell lines, respectively (**Figure 6 A-C**).

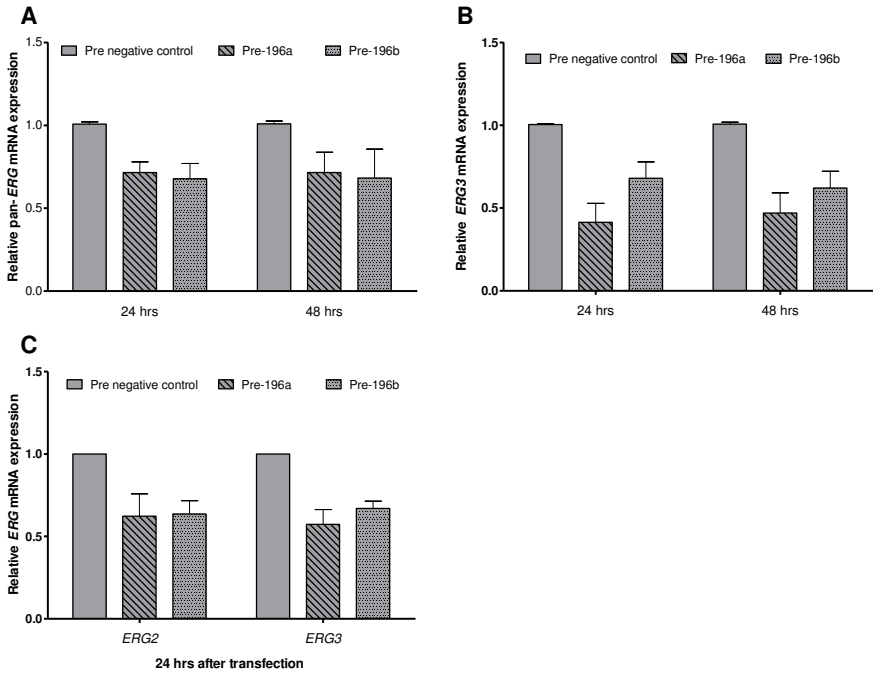


Figure 6. Transfections with precursor molecules Pre-miR-196a and Pre-miR-196b downregulate the expression of pan-*ERG* and *ERG3* mRNA in KG1a (Fig. 6A-B), as well as *ERG2* and *ERG3* mRNA expression in MOLT-4 (Fig. 6C) compared to cells transfected with unspecific Pre-miR negative control. The data depict the mean ± S.D. of three independent experiments.

Furthermore, the direct binding of miR-196a and miR-196b was confirmed by a luciferase reporter assay. Briefly, the predicted miR-196 recognition site of the *ERG* 3'UTR was cloned into a luciferase reporter vector (either as a wild-type or mutated sequence) and was subsequently cotransfected with PremiR-196a or PremiR-196b molecules into 293T cells. After 24 hrs, a 33% and 28% reduction of luciferase activity was observed in cells contransfected with the reporter vector containing the wild-type sequence and PremiR-196a or PremiR-196b molecules, respectively, compared to the control cells cotransfected with PremiR negative control. In contrast, transfection of the miRNA precursor molecules did not reduce the luciferase activity in the cells cotransfected with mutated *ERG* 3'UTR sequence.

Moreover, we assessed the expression of miR-196a and miR-196b during cell differentiation of normal CD34+ hematopoietic stem cells. The cells were isolated from human bone marrow of healthy donors and were kept in maintenance culture using the cytokines SCF and IL-3. Additionally, the lineage-specific differentiation was induced by the addition of EPO (for erythroid differentiation) and G/GM-CSF (for granulocytic differentiation). Flow cytometry analysis of the surface markers GlyA (expressed on erythrocytes) and CD15 (expressed on granulocytes) confirmed the lineage specific differentiation of the cells during the course of 9 days. The expression of miR-196a was nearly constant during differentiation, whereas the expression of miR-196b was high in undifferentiated CD34+ cells and decreased with early onset of differentiation stimulus (maintenance culture: 20-fold reduction, EPO: 20-fold reduction, G/GM-CSF: 14-fold reduction-day 0 vs. day 9).

To examine the role of miR-196a and miR-196b in acute leukemia, we measured the expression of these miRNAs in pretreatment bone marrow samples from T-ALL and AML patients. Both of the miRNAs were significantly higher expressed in AML compared to T-ALL (miR-196a, $P<0.01$, and miR-196b, $P<0.01$), and healthy controls (miR-196a, $P=0.05$, and miR-196b, $P=0.05$), whereas no significant changes were observed in the expression of the miRNAs in T-ALL compared to healthy controls. Furthermore, high expression of miR-196a and miR-196b was associated with expression of the early progenitor antigen CD34, and with aberrant expression of the myeloid antigen CD33. miR-196a was further significantly associated with a more immature T-ALL immunophenotype. Additionally, in AML,

association of the miRNAs with molecular features revealed that patients having *NPM1* mutations had higher expression levels of miR-196a and miR-196b compared to *NPM1* wild-type patients (P=0.01).

In conclusion, this study demonstrates miR-196a and miR-196b as regulators of *ERG,* and implicate a potential role for these miRNAs in acute leukemia.

The contributions in this work include design, preparation, and completion of the investigations. Immunophenotyping of the patient samples was performed in the GMALL reference laboratory at the Charité. Further works include evaluation of all presented data, illustrations and creation of the manuscript.

3.2 Manuscript: "*FLT3* mutations in early T-cell precursor ALL characterize a stem cell like leukemia and imply the clinical use of tyrosine kinase inhibitors"

ETP-ALL was identified in pediatric T-ALL as a high-risk subgroup, characterized by an ETP-like immature surface immunophenotype and a distinct gene expression profile[76]. Furthermore, in adult T-ALL, ETP-ALL was identified as a subgroup of the high-risk subgroup of early T-ALL[77]. In this small cohort of ETP-ALL, the expression levels of stem cell-associated genes, and genes known to be of prognostic significance in AML were examined, conferring ETP-ALL stem cell like and myeloid characteristics. To further unravel the molecular pathomechanisms for this distinct T-ALL subgroup, herein, a large cohort of adult ETP-ALL patients were examined for their molecular alterations and clinical outcome.

For this purpose 68 immunophenotypically identified ETP-ALL patients and 94 T-ALL patients from the GMALL trial 07/2003, used as reference group[73], were investigated. Pretreatment bone marrow samples were used for DNA and total RNA extraction using Trizol® Reagent (Invitrogen GmbH, Karlsruhe, Germany). Complementary DNA was synthesized of total RNA using avian myeloblastosis virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany). The samples were investigated by qRT-PCR for expression of eight genes (*BAALC, ERG, IGFBP7, WT1, MN1, GATA3, BCL11B,* and *MEF2C*). The *NOTCH1* mutation status was defined by direct sequencing of the N-terminal and the C-terminal region of the HD domain, the N-terminal and the C-terminal region of the PEST domain, and the TAD domain[206]. *WT1* mutations were analyzed in exons 7 and 9 by DNA sequencing of amplified PCR products[207]. Mutations were confirmed by cloning the specific PCR products and by sequencing independent clones. *FLT3* mutations, including internal tandem duplications (ITD) and tyrosine kinase domain (TKD), were analyzed using a commercially available FLT3 mutations assay (InVivoScribe Technologies, San Diego, USA). The TCR rearrangement status was assessed by the IdentiClone TM TCRG Gene Clonality Assay (InVivoScribe Technologies, San Diego, USA).

The examination of outcome with respect to the treatment of chemotherapy only or the allocation to allogenic stem cell transplantation (alloSCT) revealed that ETP-ALL patients receiving an alloSCT showed a favorable outcome (n=20; 3-year overall survival: 74%) compared to ETP-ALL patients that were treated with chemotherapy only (n=19; 3-year overall survival; 37%, *P*=0.006). Gene expression analysis revealed high expression of *BAALC* and *IGFBP7* in ETP-ALL compared to non-ETP T-ALL patients (*P*<0.001, and *P*=0.009, respectively). Furthermore, expression levels of *WT1*, *MN1*, and *MEF2C* were also higher expressed in ETP-ALL compared to non-ETP T-ALL. In contrast, *GATA3* and *BCL11B*, transcription factors playing crucial roles in the differentiation program of T-lymphopoiesis, were lower expressed in ETP-ALL compared to non-ETP T-ALL (*P*=0.005, and *P*<0.001, respectively). The TCR rearrangement status revealed that 59% of ETP-ALL patients lacked clonal TCR rearrangements, whereas only 22% of the non-ETP T-ALL patients showed the same status.

Furthermore, the mutational status of *NOTCH1* and *FLT3* between ETP-ALL and non-ETP T-ALL was examined. We found a low rate of *NOTCH1* mutations in the ETP-ALL subgroup (15%), whereas it was frequent (40%) in non-ETP T-ALL patients (*P*<0.001). On the other hand, the frequency of *FLT3* mutations in ETP-ALL was 35.3% compared to non-ETP-ALL displaying a frequency of only 1.2% (*P*<0.001).

Interestingly, within the ETP-ALL subgroup the patients having *FLT3* mutations (*FLT3*mut ETP-ALL) showed a specific immunophenotype and molecular characteristics compared to *FLT3* wild-type patients (*FLT3*wt ETP-ALL). *FLT3*mut ETP-ALL had a higher frequency of positivity for CD117 (83% vs. 28%), CD2 (88% vs. 30%), and CD13 (100% vs. 37%). In contrast, *FLT3*wt ETP-ALL was characterized by positivity for CD5 (54% vs. 4%), and CD33 (54% vs 4%). Differences in gene expression profile in *FTL3*mut ETP-ALL vs. *FLT3*wt ETP-ALL were as follows: higher expression levels of *WT1* (P=0.003) and lower expression of *IGFBP7* (P<0.001), and *GATA3* (P<0.001) were observed in *FLT3*mut ETP-ALL. TCR rearrangement analysis demonstrated that *FLT3*mut ETP-ALL patients predominantly lacked clonal TCR rearrangements (79%), while *FLT3*wt ETP-ALL patients showed more frequently TCR rearrangements (52%, *P*=0.01). Furthermore, *FLT3*mut ETP-ALL lacked *NOTCH1* mutations, while 23% *FLT3*wt ETP-ALL had *NOTCH1* mutations.

Since high rates of *FLT3* mutations were observed in ETP-ALL, the sensitivity of tyrosine kinase inhibitors (TKI), already studied in *FLT3* mutated AML[99], was assessed in a model of T-ALL with *FLT3*-ITD mutations. T-ALL cell lines Jurkat, BE13, and MOLT-4 were transfected with FLT3-ITD or FLT3-wt constructs and empty vector as control. Subsequently, the cells were treated with three different kinds of TKIs (sorafenib, PKC412, or TKI258) for 48 hrs and were tested for cell growth and apoptosis. The cell proliferation was measured with the WST-1 reagent (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Cell lines transfected with the FLT3 constructs revealed a growth advantage compared to empty vector transfected cells in untreated cells. However, treatment with all three TKIs resulted in a selective and significant inhibition of the proliferation of FLT3-ITD or FLT3wt transfected cells compared to empty vector transfected cells. This was observed in all three T-ALL cell lines treated with the TKIs. The apoptosis assay was performed by labeling the Jurkat cells, treated with TKIs, with Annexin V and 7-amino-actinomycin D (7-AAD), using Annexin V Apoptosis Detection Kit (BD Pharmingen, Heidelberg, Germany), and then analyzed by FACS Calibur (Becton-Dickinson) to determine the percentage of apoptotic cells. All TKIs induced enhanced apoptosis in cells transfected with FLT3 expressing constructs compared to empty vector controls.

Taken together, ETP-ALL patients represent a distinct molecular subgroup of adult T-ALL patients. In particular, the *FLT3*mut ETP-ALL constitutes a distinct subgroup within the ETP-ALL with unique immunophenotypical and molecular features pointing to a stem cell leukemia.

The contributions in this study include the experiments involving gene expression analyses, with subsequent data analysis, and contributing to the writing of the manuscript.

3.3 Manuscript: "MicroRNA profiling reveals aberrant microRNA expression in adult ETP-ALL and functional studies implicate a role for miR-222 in acute leukemia"

ETP-ALL is a newly identified subgroup of T-ALL characterized by a specific gene expression profile and poor prognosis. As described in the previous study (chapter 3.2), ETP-ALL was identified as a subgroup of high-risk early T-ALL in adults. Molecular characterization revealed an association of ETP-ALL with high expression of stem cell associated genes and genes of prognostic significance in AML. miRNAs have been implicated in normal hematopoiesis and in the pathogenesis of leukemia by modulating the expression of oncogenes and tumor suppressor genes. Thus, miRNAs may impact the specific gene expression signature observed in ETP-ALL.

To address this issue we examined global miRNA expression in ETP-ALL (n=8) compared to non-ETP T-ALL (n=6) and mature CD3+ cells from healthy controls (n=2), using Taqman Low Density Arrays (TLDA, Applied Biosystems/Ambion, Darmstadt, Germany) enabling quantification of 754 miRNAs. Briefly, the RNA was converted into cDNA using Megaplex™ Primer Pools and Taqman miRNA Reverse Transcription Kit (Applied Biosystems/ Ambion, Darmstadt, Germany). Quantitative real-time PCR was carried out using an Applied Biosystems 7900HT Fast real-time PCR sequence detection system according to manufacturer's protocol. Data were quantified and analyzed using Sequence Detection System (version 2.3, Applied Biosystems). miRNAs with threshold cycle (Ct) values of ≥37 were excluded from the analysis. The relative miRNA expression was normalized against RNU48 using the equation: $2^{-\Delta Ct}$, where ΔCt= (Ct miRNA- CtU48). Significant differences in miRNA expression were detected using the Mann Whitney U test, by comparing miRNA expression values in healthy controls and T-ALL subgroups. The miRNA expression data were assigned *P*-value, where a *P*-value ≤0.05 (two-sided) was considered to indicate a significant difference. Thirty percent of the miRNAs represented by the TLDA were expressed at detectable levels in at least two-thirds of the samples and the

controls. Of these, 55 miRNAs were differentially expressed in T-ALL vs. healthy controls: 49 miRNAs were upregulated and 6 miRNAs were downregulated. Furthermore, 8 miRNAs were found to be differentially expressed in ETP-ALL compared to non-ETP T-ALL (**Figure 7**): miR-221 and miR-222 were upregulated and 6 other miRNAs (miR-151-3p, miR-19a, miR-20b, miR-342-3p, miR-363, and miR-576-3p) were downregulated.

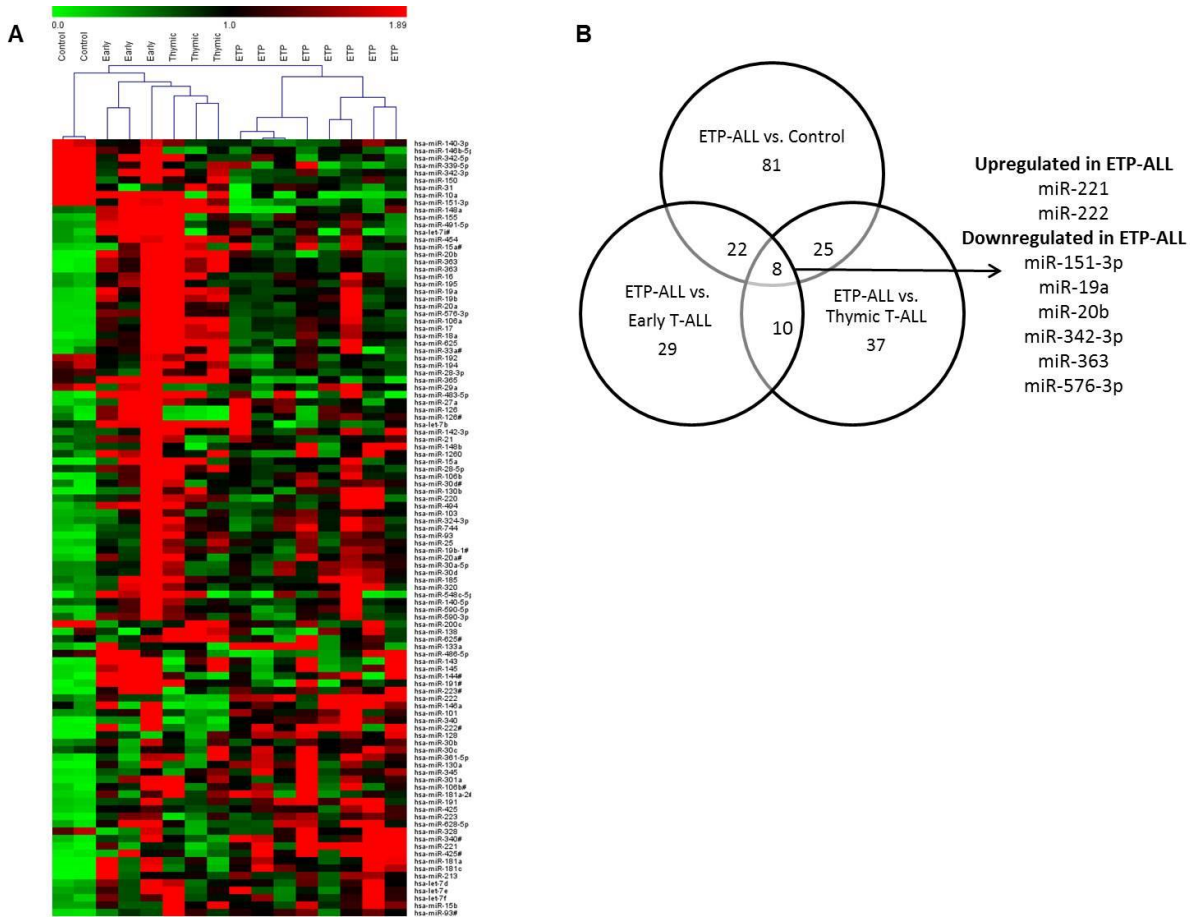
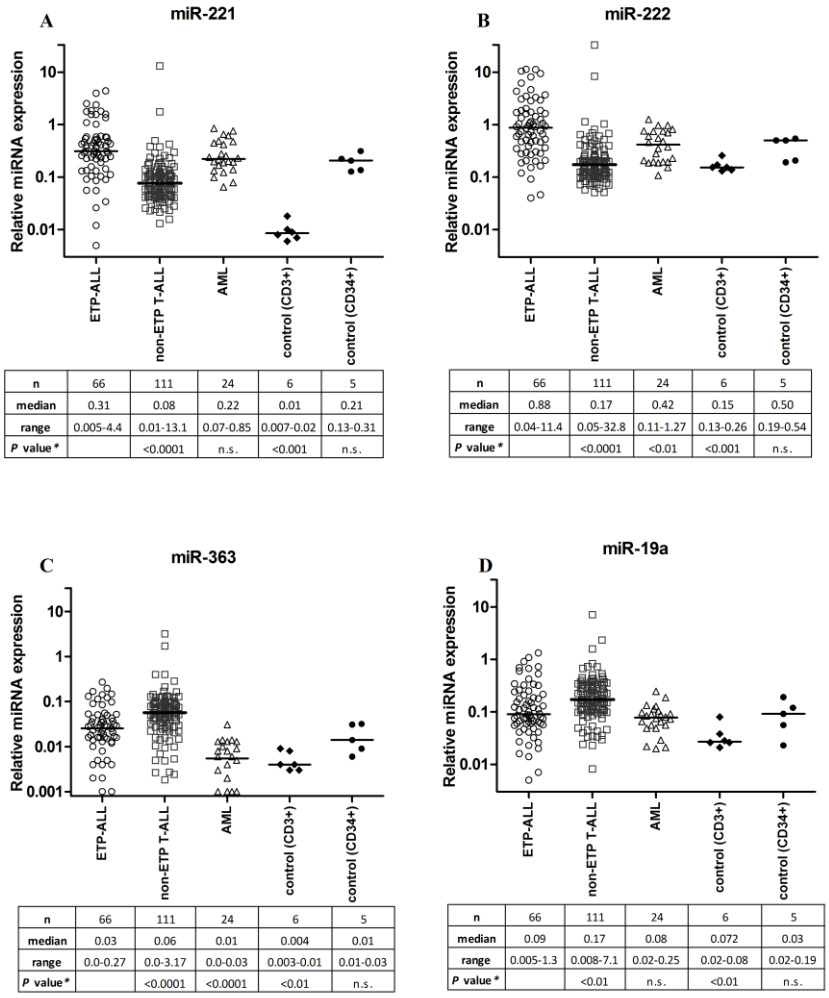


Figure 7. (A) Heatmap of differentially expressed miRNAs. Hierarchical clustering of healthy controls and T-ALL subgroups based on their miRNA expression levels. The scale bar indicates the relative expression of each miRNA with respect to the median expression level, correlated to an increase (red) or decrease (green) in the expression level. (B) Venn diagram displays differentially expressed miRNAs in ETP-ALL compared to control, early-, and thymic- T-ALL subgroups; miR-221 and miR-222 were upregulated, whereas miR-151-3p, miR-19a, miR-20b, miR-342-3p, miR-363, and miR-576-3p were downregulated in ETP-ALL.

For further analysis we validated the expression of miR-221, miR-222, miR-19a and miR-363 in a larger cohort of T-ALL patients (ETP-ALL, n=66, and non-ETP T-ALL, n=111). Additionally, we measured the miRNA expression in AML samples (n=24), mature CD3+ healthy controls (n=6), and CD34+ hematopoietic stem cells (n=5) (**Figure 8**).



n	66	111	24	6	5
median	0.31	0.08	0.22	0.01	0.21
range	0.005-4.4	0.01-13.1	0.07-0.85	0.007-0.02	0.13-0.31
P value *		<0.0001	n.s.	<0.001	n.s.

n	66	111	24	6	5
median	0.88	0.17	0.42	0.15	0.50
range	0.04-11.4	0.05-32.8	0.11-1.27	0.13-0.26	0.19-0.54
P value *		<0.0001	<0.01	<0.001	n.s.

n	66	111	24	6	5
median	0.03	0.06	0.01	0.004	0.01
range	0.0-0.27	0.0-3.17	0.0-0.03	0.003-0.01	0.01-0.03
P value *		<0.0001	<0.0001	<0.01	n.s.

n	66	111	24	6	5
median	0.09	0.17	0.08	0.072	0.03
range	0.005-1.3	0.008-7.1	0.02-0.25	0.02-0.08	0.02-0.19
P value *		<0.01	n.s.	<0.01	n.s.

Figure 8. Validation of miRNA expression in a larger cohort of T-ALL and AML. Quantitative real-time RT-PCR was performed in ETP-ALL (n=66), non-ETP T-ALL (n=111), AML (n=24), mature CD3+ cells (n=6), and CD34+ hematopoietic stem cells (n=5). For each miRNA (miR-221; Fig. 2A, miR-222; Fig. 2B, miR-363; Fig. 2C, and miR-19a; Fig. 2D) the median is shown by a horizontal bar. P-value*= Indicates statistical significance compared to ETP-ALL (Mann-Whitney U test).

miR-221 and miR-222 were upregulated in ETP-ALL compared to non-ETP-ALL and mature CD3+ healthy controls, whereas no significant changes were observed for miR-221 and miR-222 expression in ETP-ALL compared to CD34+ hematopoietic stem cells, and AML in case of miR-221. Furthermore, high expression of miR-221 and miR-222 was associated with expression of the early progenitor antigen CD34, and with a more immature T-ALL immunophenotype. These results imply miR-221 and miR-222 as stem cell associated miRNAs, and support the notion that ETP-ALL is an immature leukemic subgroup.

In contrast, the expression of miR-363 and miR-19a was downregulated in ETP-TALL and AML compared to non-ETP-TALL patients. Furthermore, low miR-363 expression was significantly associated with an immature immunophenotype of T-ALL, CD34 expression (P=0.05), and with aberrant expression of myeloid markers CD33 (P=0.03) and CD13 (P<0.01). Low miR-363 was further correlated with high expression of *MN1* (P<0.001). Similar results were obtained for miR-19a, however no significant correlations were observed between low and high miR-19a expression with regard to CD34 surface expression (P=0.63), and aberrant expression of CD33 (P=0.39), and CD13 (P=0.45). Low miR-19a expression was further correlated with high expression of *IGFBP7* (P<0.01), *MN1* (P<0.001), and *WT1* (P=0.05), suggesting these miRNAs to be associated with a more mature subtype of T-ALL.

Since miR-221 and miR-222 were the only upregulated miRNAs in ETP-ALL we searched for putative target genes found within the gene expression signature in ETP-ALL. Microarray-based gene expression profile (GEP) data were available for an independent set of ETP-ALL (n=18) and non-ETP T-ALL (n=68) patient samples[208]. First we identified genes differentially expressed (with an at least 2-fold change and with a significance of $P\leq0.05$) between ETP-ALL and non-ETP T-ALL cases (in total 223 probe sets) and subsequently searched for putative target genes of miR-221 and miR-222 using the Targetscan database (www.targetscan.org). Following genes were identified as putative miR-221 and miR-222 targets: *BCL2L11, ETS1*, and *E2F2*. Among them we verified the proto-oncogene *ETS1* as a direct target of miR-222.

miR-222 has previously been implicated in different kind of cancers. Depending on the cell type, miR-222 was shown to act as either tumor suppressor or oncogene[165,209]. In order to examine the role of miR-222 in acute leukemia we performed functional analyses in the leukemic cell lines. miR-222 was overexpressed in Jurkat (T-ALL) and KG1a (AML) cell lines. Subsequently, the total number of viable cells were detected by trypan blue exclusion using hemocytometer. Overexpression of miR-222 resulted in a significant reduction of cell growth in both cell lines compared to control cells. The inhibition of growth by miR-222 overexpression was further confirmed by WST-1 proliferation assay (Roche Diagnostics GmbH, Mannheim, Germany). miR-222 significantly reduced the proliferation in Jurkat and KG1a cells. Determination of the incorporated thymidine analog Bromodeoxyuridine (BrdU; Cell Proliferation ELISA; Roche Diagnostics GmbH, Mannheim, Deutschland) during the S-phase of the cell cycle revealed a 43% reduction of DNA replication in KG1a cells after overexpressing the miR-222. Furthermore, we examined if the growth inhibition observed in the leukemic cell lines was also caused by apoptosis and could show that overexpression of miR-222 induced apoptosis in both Jurkat (1.3-fold, P=0.02) and KG1a (3-fold, P<0.01).

In summary, ETP-ALL constitutes a specific subgroup of T-ALL with a specific expression pattern of miRNAs, which may to some extent explain the stem cell and myeloid character of ETP-ALL. Stem cell and AML related miRNAs miR-221 and miR-222 were significantly upregulated in ETP-ALL, and subsequent *in vitro* studies implicate a functional role for miR-222 in acute leukemia.

The contributions in this work include design, preparation, and completion of the investigations, with respect to miRNA expression profiling, gene expression analyses (miRNAs and *ETS1*), and functional assays. The evaluation of GEP data was performed in collaboration with Dr. Martin Neumann. Immunophenotyping of the patient samples was performed in the GMALL reference laboratory at the Charité. Further works include evaluation of all presented data, illustrations, and creation of the manuscript.

4 DISCUSSION

Although the prognosis in adult acute leukemia has improved in the past decades, long-term disease-free survival only reaches rates of 30-60% in T-ALL and 20-45% in AML depending on prognostic factors and subgroups. In acute leukemia the patients are assigned to specific risk groups at diagnosis for treatment optimization. Rapid diagnosis and classification is standardized as the basis for treatment stratification. Increasing understanding about the molecular aberrations in leukemogenesis allows treatment optimization for patients with leukemia. Prognostically relevant molecular markers can guide risk-adapted treatment strategies and will also be the basis for the development of new targeted therapies. In AML, specific cytogenetic or molecular aberrations, which have been identified by immunophenotypic analyses and by molecular genetic techniques are of prognostic significance. However, in adult T-ALL only a few molecular markers have been associated with clinical outcome. Thus, further improvement may be achieved by enhanced risk stratification and development of novel treatment approaches based on the implementation of targeted therapies.

4.1 The regulation of *ERG* by miR-196a and miR-196b

Aberrant expression of genes encoding TFs in acute leukemia might be partly due to the aberrant expression of regulatory miRNAs, displaying an active role in acute leukemias. One such transcription factor is the ETS transcription factor ERG, which has been implicated in normal hematopoiesis, and was found to be an adverse prognostic factor in a subset of adult patients with CN-AML and T-ALL[62,73]. Previously, it was shown that *ERG* was partly regulated by epigenetic mechanisms[205]. To further gain insight into the underlying regulatory mechanisms of *ERG,* herein, the regulation of *ERG* by miRNAs was examined. Among several miRNAs predicted to regulate *ERG*,

only miR-196a and miR-196b specifically modulated *ERG* expression at a post-transcriptional level. By overexpressing miR-196a and miR-196b in leukemic cell lines, downregulation of the mRNA levels of *ERG* as well as the isoforms *ERG2* and *ERG3* was observed, which further was confirmed by luciferase reporter assay. miR-196a and miR-196b have already been implicated in normal cell differentiation, proliferation, and in tumorgenesis of various cancer types[210–212]. miR-196a was found to inhibit proliferation and promote osteogenic differentiation in adipose tissue derived mesenchymal stem cells by targeting *HOXC8*. On the other hand, targeting of annexin A1 by miR-196a in esophageal cancer promoted cell proliferation, anchorage-independent growth and suppressed apoptosis[212], suggesting the oncogenic potential of miR-196a and implying its diverse functions in various cell types. Moreover, expression analysis of these miRNAs in CD34+ hematopoietic stem cells revealed that miR-196b was highly expressed in undifferentiated CD34+ hematopoietic stem cells and decreased with onset of differentiation, whereas the expression of miR-196a was nearly constant during the differentiation. These results suggest a role for miR-196b in early hematopoiesis, and are in line with previous studies showing high expression of miR-196b levels in human BM and spleen cells compared to other organs, and in short-term hematopoietic stem cells in mouse, which subsequently was downregulated in more differentiated hematopoietic cells[210,213]. Furthermore, the expression levels of miR-196a and miR-196b were measured in T-ALL and AML patients, which correlated with specific molecular characteristics. In particular, in T-ALL, high expression of both of the miRNAs was associated with an early immunophenotype of T-ALL, CD34-positivity and with aberrant expression of CD33. In AML, the expression of miR-196a and miR-196b was significantly higher in the patient samples compared to healthy donors. Additionally, both of the miRNAs were also higher expressed in the molecular subgroup of AML carrying *NPM1* mutations compared to *NPM1* wt. This is in line with the study from Jongen-Lavrencic et al, showing up-regulated miR-196a and miR-196b expression levels in AML carrying *NPM1* mutations[193]. In addition, this finding was supported by a study showing increased *ERG* expression to be associated with patients that have wild-type *NMP1*[214]. miR-196a and miR-196b are located within the genomic

clusters of *HOXB* and *HOXA* families that are overexpressed in AML with *NPM1* mutations[215]. Furthermore, miR-196a was found to regulate *HOXB8*[216], and the regulation of miR-196b was found to be similar to that of the surrounding *HOX* genes[210]. Thus, these observations implicate a role for miR-196a, miR-196b, and HOX genes in AML with *NPM1* mutations.

The expected inverse correlation between the expression of miRNAs and *ERG* mRNA that was not observed possibly reflects the heterogeneity in AML and T-ALL populations. Additionally, it must be kept in mind that the miRNA regulatory mechanism is a complex system involving regulation of a single gene by multiple miRNAs, which in turn can regulate multiple genes. Thus, the involvement of additional regulatory mechanisms of *ERG* needs to be further explored.

The alterations observed in *ERG* expression might affect many genes involved in hematopoiesis. In a mouse cell line, representing HSCs, Chip-seq analysis identified 36,000 ERG target regions, suggesting ERG to have a broad gene program. Furthermore, ERG was shown to be part of a complex consisting of several other TFs, which have been associated with normal hematopoietic development, such as SCL, LYL1, LMO2, GATA-2, RUNX1 and FL1[217], suggesting that aberrant regulation of ERG might directly interfere with other hematopoiesis associated TFs and as a result expression of their target genes. Certainly, in a recent study it was shown that the expression of the WNT11 gene was regulated by ERG, which implies that aberrant expression of ERG correlates with its target genes, and also that the WNT signaling pathway might be a primary target of ERG[218]. Furthermore, high expression of *ERG* mediated growth advantage in leukemic cells treated with pharmacological agent 6-bromoindirubin-3'-oxime (BIO). Recently, it was shown that ERG directly promoted the development of acute leukemia[86]. Transgenic expression of *ERG* caused T-ALL in mice and its knockdown reduced the proliferation of MOLT4 T-ALL cells. Thus, targeting of deregulated ERG as a therapeutic tool could be of potential value for treatment of acute leukemia. One opportunity could involve altering *ERG* expression through overexpression of miR-196a and miR-196 in leukemic cells, which in turn would

downregulate *ERG* and its target genes involved in uncontrolled cell proliferation (**Figure 9**).

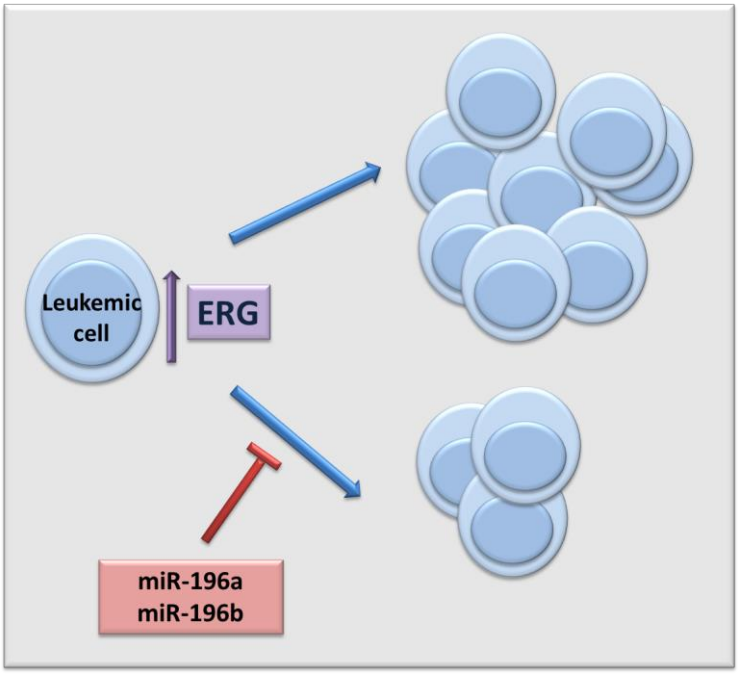


Figure 9. A hypothetic model of overexpression of miR-196a and miR-196b as therapeutic tool in leukemic cells with upregulated *ERG* expression. High expression of *ERG* promotes proliferation of leukemic cells. Thus, overexpression of miR-196a and miR-196b might downregulate *ERG* expression, which in turn would lead to inhibition of leukemic cell proliferation.

4.2 ETP-ALL defines a specific subgroup within T-ALL

Recently, the new subgroup of pediatric T-ALL, termed as ETP-ALL, was described by an immature surface immunophenotype, increased genomic instability, high frequency of remission failures and hematologic relapse[76]. On the basis of these results the treatment schedules for children with ETP-ALL was modified towards a more intensive regime including allogeneic stem cell transplantation. Furthermore, in adult T-ALL, ETP-ALL was identified as a subgroup of the high-risk group of early T-ALL[77]. The outcome of ETP-ALL patients was comparably poor to early T-ALL. Furthermore, molecular characterization revealed an association of ETP-ALL with high expression of stem cell associated genes and genes of prognostic significance in AML. To further

unravel the pathomechanisms of this distinct T-ALL subgroup, herein, a larger cohort of 68 adult ETP-ALL patients were examined for their molecular alterations and clinical outcome and were compared to 94 non-ETP T-ALL patients. Expression analyses of candidate genes revealed significant higher expression of stem cell associated genes and genes of prognostic significance in ETP-ALL versus non-ETP T-ALL. The expression of *BAALC* and *IGFBP7*, associated with an immature leukemic phenotype in adult T-ALL and AML[61,75], was upregulated in ETP-ALL, which underscores the immature nature of this subgroup. In addition, *MN1* identified to be associated with ETP-ALL[219], and *WT1*, a gene known to be of unfavorable prognosis in AML as well as in T-ALL in the presence of a mutation[207,220], were also overexpressed in this large cohort of ETP-ALL.

Furthermore, distinct differences were observed in the mutation profile of ETP-ALL, which showed less frequent *NOTCH1* mutations (15%) compared to non-ETP T-ALL (40%). Generally, T-ALL comprises high rate of *NOTCH1* mutations, which is involved in the pathogenesis of T-ALL. This makes the *NOTCH1* signaling pathway an interesting candidate for targeted therapies by the implementations of gamma secretase inhibitors[221,222]. However, the low frequency of *NOTCH1* mutations in ETP-ALL demonstrates a clear pathogenic difference to non-ETP T-ALL. Additionally, the high rate of *FLT3* mutations observed in ETP-ALL, affirms its distinct mutational profile compared to non-ETP T-ALL, and underscores the myeloid character of ETP-ALL, since *FLT3* mutations are the most frequent somatic alterations in AML[223], whereas *FLT3* is only rarely mutated in leukemic lymphoblasts[224]. These results indicated in this study implicate ETP-ALL to be a stem cell leukemia. In contrast, a recent study showed that the expression profile of ETP-ALL was similar to DP or SP T-cells in mouse, and suggested that some human ETP-ALLs may arise from more differentiated T-cells[225]. Thus, further studies are required to clarify the mechanisms of ETP-ALL origin, in order to find novel treatment options for this unfavorable subgroup of T-ALL. Another support for the immature character of ETP-ALL was revealed from the TCR rearrangement experiments. In normal human T-cell development, TCR rearrangements are rare in prothymocytes as these cells have not yet started with the TCR

rearrangement, but they are commonly found at the prethymocyte stage. The analysis of the TCR rearrangement status revealed a clear difference in clonal TCR rearrangements in ETP-ALL, with less common TCR rearrangements compared to non ETP T-ALL, indicating that ETP-ALL represents a separate leukemic subtype.

4.3 *FLT3* mutated ETP-ALL constitute a distinct subgroup within ETP-ALL

Interestingly, within the ETP-ALL subgroup *FLT3* mutated (*FLT3*mut) ETP-ALL defined a new molecular distinct entity, with a specific immunophenotype and molecular characteristics compared to *FLT3* wild-type (*FLT3*wt) ETP-ALL. Moreover, *FLT3*mut ETP-ALL showed specific molecular alterations indicating its stem cell like nature. *FLT3*mut ETP-ALL predominantly lacked clonal TCR-rearrangements pointing to a leukemic transformation before the prothymocyte stage of T-cell development. The absence of TCR rearrangements had already been linked to early treatment failure in children with T-ALL[226], providing an indirect support for the poor prognosis of ETP-ALL. The early developmental arrest of *FLT3*mut ETP-ALL is also emphasized by the low *GATA3* expression. In normal T-cell development, GATA3 plays a definite role in the early T-lineage specification as it is required for the transformation of the ETP/DN1 to the DN2a stage[227]. Thus the leukemic transformation in *FLT3*mut ETP-ALL lacking *GATA3* expression might occur at a stem cell pluripotent prothymic stage before *GATA3* expression is induced. These data in combination with the absence of activating *NOTCH1* mutations reflect an even more immature nature of the *FLT3*mut ETP-ALL within the ETP-ALL subgroup.

As ETP-ALL is defined as a subgroup of the high-risk subgroup of early T-ALL, an alloSCT is planned after the first complete remission for these patients. In this study, ETP-ALL patients receiving an alloSCT showed a remarkable favorable outcome in the studied cohort, whereas the outcome for ETP-ALL patients receiving chemotherapy only was relatively poor. The poor response to lymphoid cell-directed ALL

chemotherapy, might be due to the immature nature and myeloid characteristics of ETP-ALL. Thus, to further improve the outcome for these high-risk patients the implementation of targeted therapies should be considered. Due to the high frequency of *FLT3* mutations in ETP-ALL, tyrosine kinase inhibitors (TKIs) already studied in *FLT3* mutated AML would be an attractive treatment option. To evaluate the efficacy of TKIs in T-lymphoblasts, we assessed the sensitivity of T-ALL cell lines transfected with FLT3-ITD and FLT3-wt expression constructs and observed that FLT3 transfected T-ALL cells, despite of their enhanced proliferation, were particular sensitive to TKIs similar to results in AML. This further supports the rational for the clinical use of TKIs in FLT3mut ETP-ALL. However, it is noteworthy to mention that the T-ALL cell lines used in this study was not of ETP-ALL origin, due to lack of ETP-ALL like cell lines. Therefore, it is important to validate these experiments *in vitro* in primary ETP-ALL cells or *in vivo* in mouse with ETP-ALL.

4.4 The role of microRNAs in ETP-ALL

Apart from the genetic alterations of molecular markers, miRNAs, which are post-transcriptional regulators of genes, have been proposed to have crucial impact on the leukemogenesis of acute leukemia. Since recent studies have focused on the genetic alterations in ETP-ALL, by performing gene expression profiling and mutational analyses[77,78], this study aimed to investigate the expression pattern of miRNAs in ETP-ALL. miRNAs might be an attractive object for implementation of potential novel therapeutical targets for this distinct and high-risk subgroup of T-ALL. Using miRNA expression profiling differentially expressed miRNAs were identified in ETP-ALL compared to non-ETP T-ALL and mature CD3+ T-cells from healthy controls. Among the differentially expressed miRNAs miR-221 and miR-222 were found to be the only significantly higher expressed miRNAs in ETP-ALL compared to non-ETP T-ALL. Furthermore, both of the miRNAs were similarly high in ETP-ALL and CD34+ hematopoietic stem cells, which is in concordance with previous studies showing high

expression of miR-221 and miR-222 in CD34+ stem cells[165]. Consistently, high expression of miR-221 and miR-222 was associated with expression of the early progenitor antigen CD34, and with a more immature T-ALL immunophenotype, implying these miRNAs as stem cell associated miRNAs, and indirectly support the notion that ETP-ALL is an immature leukemic subgroup. Additionally, miR-221 and miR-222 have been implicated in acute leukemia, as being highly expressed in AML patient samples. As ETP-ALL has been associated with myeloid features, the expression of these miRNAs was further measured in AML patient samples, revealing high expression of miR-221 and miR-222 in ETP-ALL and AML compared to non-ETP T-ALL. In concordance, high expression of these miRNAs was also associated with expression of the myeloid antigens CD33 and CD13. Global gene expression profiling data of ETP-ALL pointed to putative targets for miR-221 and miR-222, as genes down regulated in ETP-ALL compared to non-ETP T-ALL. Among them the proto-oncogene *ETS1* was identified and verified as a direct target of miR-222. A previous study associated ETS1 with granulocytic differentiation [228]. Inhibition of ETS1 activity increased the capability of leukemic cells to undergo chemical-induced granulocytic differentiation. Thus, it is reasonable to propose that miR-222 might to some extent contribute to the myeloid character of ETP-ALL by down modulating *ETS1* expression. However, this hypothesis must further be validated with additional experiments. Furthermore, in previous studies, inactivation of ETS1 lead to increased apoptosis and defective activation and survival of T-cells [229,230]. In addition, ETS1 levels were found to be downregulated during apoptosis of T-cells [231], suggesting a potential role for *ETS1* in the functional studies showing increased apoptosis in leukemia cells by miR-222 overexpression. In previous studies, miR-222 was shown to either mediate growth advantage or inhibition of cell differentiation and proliferation, depending on the cancer type and target genes[165,232]. In this study, overexpression of miR-222 repressed growth in leukemic cells, and caused cell cycle arrest. Since ETP-ALL patients have a poor prognosis and fail to respond to standard intensive chemotherapy, miR-222 may possibly counteract the efficacy to conventional chemotherapy by causing cell cycle

arrest, since the drugs are affecting rapidly dividing cells. This notion emphasizes the importance of development of specific targeted therapies for ETP-ALL.

The expression profiling analysis revealed further six miRNAs (miR-151-3p, miR-19a, miR-20b, miR-342-3p, miR-363, miR-576-3p) downregulated in ETP-ALL compared to non-ETP T-ALL. The expression of miR-363, which is part of the oncogenic *miR-106-363* cluster and miR-19a, belonging to the oncogenic *miR-17-92* cluster, was validated in a larger cohort of T-ALL and AML samples. Both of these miRNAs were significantly downregulated in ETP-ALL and AML compared to non-ETP T-ALL, suggesting these miRNAs to be associated with a more mature subtype of T-ALL. In a previous study miR-19a was found to be upregulated in T-ALL compared to AML[233], showing consistency with the findings in this study. They further showed that overexpression of miR-19 in Jurkat cells induced growth advantage, contributing to the leukemogenesis in T-ALL. Moreover, miR-19 was sufficient to promote leukemogenesis in Notch1-induced T-ALL *in vivo*, by targeting crucial genes such as *Pten, Bim, Prkaa1*, and *Pp2a*[234], indicating miR-19 as an important oncogenic miRNA in T-ALL. However, since ETP-ALL showed low frequency of *NOTCH1* mutations[77], indicating its molecular difference to non-ETP T-ALL with 60% *NOTCH1* mutations, miR-19a might have less oncogenic importance in the pathogenesis of ETP-ALL.

Herein, the importance of miRNAs in acute leukemia was once more signified. To begin with, this study showed for the first time that a specific miRNA expression pattern exist in adult ETP-ALL patients, with miR-221 and miR-222 as the most overexpressed miRNAs, and secondly that miR-222 exert a functional role in acute leukemia by targeting the proto-oncogene *ETS1* which has an aberrant gene expression in profile of ETP-ALL.

Taken together, in this dissertation specific miRNAs have been identified with novel role in the pathogenesis of acute leukemia. Especially, miR-221 and miR-222 were found to be overexpressed in the newly identified subgroup ETP-ALL, which is characterized by a very immature phenotype and poor prognosis. As previously mentioned, the gene expression profile of ETP-ALL has been linked to the expression signature of ETPs. ETPs are intrathymic c-Kit^{hi} DN1 cells, which represent immature

progenitors that have recently immigrated from the bone marrow to the thymus and commit to the T-cell lineage, progressing through the DN1, DN2, DN3 and DN4 stages.

Specific miRNA expression pattern has been implicated in the different developmental stages of T-cell development[180]. Individual miRNAs were regulated during T-cell differentiation, with at least one miRNA or miRNA family overrepresented at each developmental stage. Interestingly, enrichment of the miRNAs was correlated with depletion of the transcript levels of the targets. For instance, miR-181a, enriched at the CD4+CD8+ stage of thymocyte development repressed the expression of Bcl-2, CD69, and the T-cell receptor; genes involved in positive selection. A significant enrichment of miR-221/222 expression was observed in the DN1 stage, which decreased with the differentiation into mature T-cells, indirectly supporting the notion that ETP-ALL is transformed from normal ETP cells. As previously mentioned, *ETS1*, downregulated in ETP-ALL, was shown to be a target of miR-222. During the T-cell development the *ETS1* expression was found to be low in ETPs and increased with differentiation into DN3 stage, where it plays a crucial role by collaborating with RUNX1 in TCR gene expression[235]. Thus, it can be hypothesized that the low expression of *ETS1* might partly be due to the high expression of miR-222 in ETPs. Consequently, by the transformation of ETPs into leukemic cells, miR-222 expression may remain high, leading to constant inhibition of *ETS1* expression post transcriptionally in ETP-ALL leukemic cells, and subsequently contributing to the immature nature of ETP-ALL (**Figure 10**).

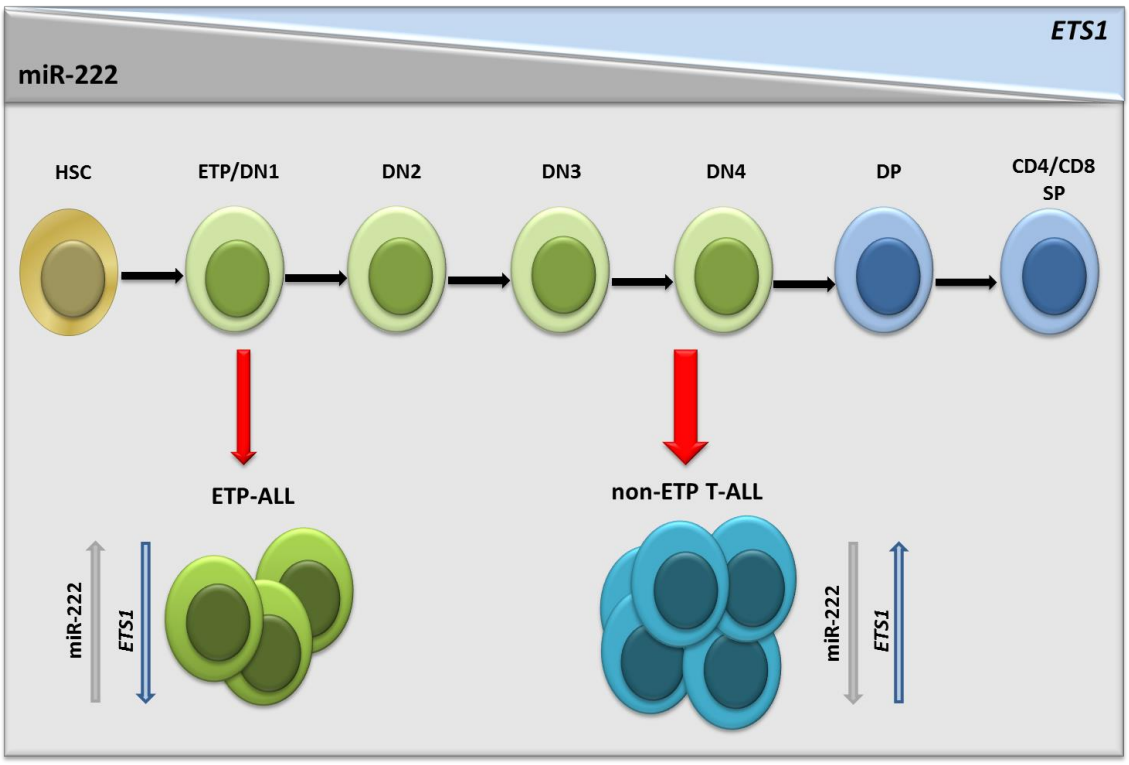


Figure 10. The hypothetic role of miR-222 in contribution to the transformation of ETP-ALL. The expression of miR-222 is high in normal ETP cells, and decreases with the differentiation of the cells into mature T-cells. In contrast the expression of *ETS1*, a target of miR-222 increases with the T-cell differentiation. Thus, by transformation of the normal ETPs into leukemic cells, the overexpression of miR-222 may inhibit the *ETS1* expression and contribute to the immature nature of ETP-ALL. The low miR-222 vs. high *ETS1* expression in non-ETP T-ALL cells may be an indirect support for the notion that these cells have been transformed from more differentiated cells in the T-cell development. HSC= hematopoietic stem cell, DN=double negative, DP= double positive, SP=single positive.

4.5 Implementation of microRNA-based therapy

As miRNAs have been shown to be powerful regulators of gene expression, the use of miRNAs-based therapy in acute leukemia should be considered. miRNAs have been implicated to have a tumor suppressive and/or oncogenic role in acute leukemia. The distinct expression pattern of miRNAs in different subgroups of acute leukemia suggest that miRNAs have the potential to classify patients and that specific subgroups may benefit from treatment strategies opposing the gain of oncogenic or loss of tumor suppressor function of miRNAs. Thus, attempts have been made to modulate the

miRNA expression in different cancer types by reintroducing miRNAs lost in cancer (precursor miRNAs), either via synthetic molecules or viral vectors. Furthermore, inhibition of oncogenic miRNAs has been achieved by using antagomirs and locked nucleic acid-anti-miR oligonucleotides that bind to miRNAs following the Watson-Crick complementarity principle. For instance, antagomir against miR-19 reactivated silenced tumor suppressors in leukemic cells, including *PTEN*, thereby opposing miR-19-driven leukemogenesis[186]. Inhibition of a single miRNA might provide significant therapeutic benefit, as treatment with a miR-196b specific antagomir was shown to abrogate the growth advantage of *MLL-AF9* transformed/miR-196b overexpressing bone marrow cells[210]. Furthermore, *in vivo* studies have also revealed promising effects of downregulation of miRNAs by antagomirs and locked nucleic acid-anti-miRs, which has prompted the investigator to explore, for instance, miR-122 as therapeutic tool for liver disease caused by hepatitis C virus (target of miR-122), as part of a worldwide clinical trial[236]. Thus, the effectivity of anti-miRNA molecules to treat acute leukemia must further be evaluated.

Apart from anti-miRNA molecules, which mediates direct downregulation of miRNAs, miR-masks (22 nt long oligonucleotides that compete with endogenous miRNA), and miRNA sponges (short oligonucleotide sequences that contain multiple miRNA binding sites to which the target miRNAs can bind) have been developed for therapeutical use.

The delivery of precursor miRNAs and miRNA sponges requires viral vectors with a tissue-specific promoter, in case of precursor miRNAs, or integration in the host genome, in case of miRNA sponges, which makes the therapeutic controlling and dosing in patients more difficult than in case of anti-miRNA molecules. Therefore, before applying miRNA-related drugs in acute leukemia, the issue concerning the delivery of these drugs to the bone marrow, must be elucidated. For instance, for delivery of synthetic miRNA mimics and anti-miRNA molecules, aptamers, which are RNA oligonucleotides fused to the miRNA molecules, and liposomes can be used, which can direct the attached miRNA molecule to specific cell surface receptors[237]. For instance, lipid coated synthetic miR-34 molecule was intravenously delivered in mice

with lung tumors and blocked growth of these tumors[238]. Furthermore, the systemical delivery of miR-34 molecule did not induce an immune system response, and had no effect on normal cells, as the affected pathways were already activated by the endogenous miR-34 in these cells[238]. The miRNAs identified in this dissertation, may also act as promising therapeutic option, as they were shown to target oncogenic transcription factors playing crucial roles in the pathogenesis of acute leukemia, as previously described for ERG and miR-196a/196b. However, further investigations are required in this field, since the regulation of miRNAs is a complex regulatory network, involving miRNAs that have the ability to target multiple molecules (such as *ETS1*, and *C-KIT* in case of miR-222). Hence, the cellular and molecular circuits where the miRNAs are involved must further be explored in order to use these molecules as new targets for a specific therapy.

In conclusion, the findings in this dissertation show that the oncogenic transcription factor *ERG* is regulated by miRNAs. Furthermore, the underlying pathomechanisms of the newly identified subgroup ETP-ALL is partly unraveled, by revealing molecular aberrations, including abnormal expression and mutations of genes of prognostic significance in acute leukemia. Implementation of myeloid directed therapy, by using TKIs in ETP-ALL would be an attractive treatment option. In addition, the importance of miRNAs in acute leukemia was anew confirmed by the findings showing distinct expression pattern of miRNAs in ETP-ALL, revealing high expression of stem cell and myeloid associated miRNAs and showing that the regulation of the oncogenic transcription factor *ETS1* is directed by miRNAs. These findings describe the function of miRNAs and regulatory influences of miRNAs in acute leukemia, providing the background and starting point for novel targeted concepts and strategies for more specific therapies.

5 SUMMARY

Acute leukemias are a complex heterogeneous group of disorders that differ with regard to biology, clinical course, and prognosis. Over the past years the knowledge of these hematological malignancies has increased, which has resulted in a classification based on morphology, immunophenotype, molecular and clinical features. Aberrant expression of genes in acute leukemia, such as the ETS transcription factor *ERG,* involved in normal hematopoiesis, has been shown to be independent risk factor predicting inferior outcome in T-ALL and CN-AML. The underlying biology of the oncogenic properties of ERG and its expression regulation remain unknown. Thus, we examined the regulation of *ERG* by miRNAs, and showed that miR-196a and miR-196b downregulated *ERG* expression on mRNA level. High expression of these miRNAs was associated with *NPM1* mutations in AML, and with an immature phenotype, and myeloid markers in T-ALL. Thus, these results implicate a potential role for miR-196a and miR-196b in acute leukemia.

Recently, a new subgroup of T-ALL, termed ETP-ALL, was identified and was shown to be associated with stem cell and myeloid characteristics and poor prognosis in pediatric T-ALL, which makes this subgroup of biological and clinical relevance. Therefore, we assessed molecular alterations as well as the clinical outcome in a large cohort of adult ETP-ALL in comparison to non-ETP T-ALL patients. High rates of *FLT3* mutations were observed in ETP-ALL. Interestingly, *FLT3* mutated ETP-ALL was identified as a molecular distinct leukemic subgroup characterized by a specific immunophenotype, a distinct gene expression pattern (aberrant expression of *IGFBP7, WT1, GATA3*) and mutational status (lack of *NOTCH1* mutations and low frequency of clonal TCR rearrangements). With respect to clinical course of these high-risk patients, ETP-ALL patients undergoing allogeneic stem cell transplantation showed an encouraging overall survival. To further explore and underscore the efficacy of targeted

therapies, we demonstrate that T-ALL cell lines transfected with FLT3-ITD expression constructs were particularly sensitive to tyrosine kinase inhibitors. In conclusion, ETP-ALL with *FLT3* mutations defines a molecular distinct stem cell like leukemic subtype. These data warrant the implementation of a specific treatment with FLT3 inhibitors in addition to early allogeneic stem cell transplantation for this high-risk subgroup.

Apart from the genetic alterations of molecular markers, miRNAs, which are post-transcriptional regulators of genes, have been proposed to have crucial impact on the pathogenesis of acute leukemia. In this study, we further investigated the global expression of miRNAs in ETP-ALL. The miRNA profiling revealed miR-221 and miR-222 as the most upregulated and six miRNAs (miR-151-3p, miR-19a, miR-20b, miR-342-3p, miR-363, miR-576-3p) as downregulated in ETP-ALL compared to non-ETP T-ALL. The expression of miR-221, miR-222, miR-19a, miR-363 was validated in a larger cohort of T-ALL patient samples. *ETS1*, downregulated in ETP-ALL, was identified as direct target of miR-222. Furthermore, in our *in vitro* studies miR-222 significantly inhibited proliferation, and caused cell cycle arrest and apoptosis in leukemic cells, implicating a role for miR-222 in the leukemogenesis by altering expression of the proto-oncogene *ETS1* in acute leukemia.

In conclusion, aberrantly expressed miRNAs, with a functional role in acute leukemia was identified. These findings may serve as useful resource for future studies and aid in development of novel therapeutic targets to improve the treatment of leukemia patients.

6 ZUSAMMENFASSUNG

Akute Leukämien stellen sehr heterogene Erkrankungen dar. In den letzten Jahren ist es möglich geworden akute Leukämien basierend auf morphologischen, immunologischen, molekularbiologische und zytogenetische Eigenschaften besser zu klassifizieren. Dies ermöglicht eine Therapieoptimierung von Leukämie Patienten. Es konnte gezeigt werden, dass die aberrante Expression von Genen als molekulare Prognosemarker dienen können und diese erklären teilweise auch die zugrundeliegende pathogenetischen Mechanismen von akuten Leukämien. Für den ETS Transkriptionsfaktor *ERG,* der an der Regulation der linienspezifischen Differenzierung von hämatopoetischen Progenitorzellen beteiligt ist, wurde gezeigt, dass er mit einer ungünstigen Prognose in CN-AML und T-ALL assoziiert ist und einen neuen molekularen Risikofaktor für Leukämie Patienten darstellt. Untersuchungen zur Expressionregulation von *ERG* konnten zeigen, dass *ERG* teilweise durch epigenetische Mechanismen reguliert ist. In der vorliegenden Arbeit ist die Regulation von *ERG* durch microRNAs untersucht worden. Die Überexpression von miR-196a und miR-196b führte zu einer Herunterregulation der *ERG* mRNA Expression in leukämischen Zellen. Eine erhöhte Expression der beiden miRNAs war mit *NPM1* Mutationen in der AML, mit einem unreifen Immunophänotyp und der Expression von myeloischen Oberflächenmarkers in T-ALL assoziiert. Zusammfassend kann somit festgestellt werden, dass miR-196a und miR-196b eine mögliche funktionelle Rolle in der Pathogenese der akuten Leukämie darstellt. Weiterhin war die molekulargenetische Charakterisierung einer ETP-ALL Subgruppe der T-ALL durchgeführt. Die ETP-ALL ist eine neue Subgruppe, die sich durch eine spezifische Genexpressionssignatur, einem unreifen Immunophänotyp und durch die Expression von myeloischen Antigenen und/oder Stamzellmarkern auszeichnet. Innerhalb der pädiatrischen T-ALL ist die ETP-ALL mit einer ungünstigen Prognose assoziiert. Deswegen ist die ETP-ALL biologisch

wie klinisch von besonderem Interesse. Hier konnte gezeigt werden, dass die ETP-ALL eine hohe Frequenz von FLT3 Mutationen besitzt. Interessanterweise konnte die FLT3 mutierte ETP-ALL als eine molekulargenetisch sehr distinkte ETP-ALL Subgruppe identifiziert werden, die sich durch eine spezifischen Imunophänotyp, Genexpressionsmuster (aberrante Expression von *IGFBP7, WT1, GATA3)* und Mutationsstatus (eine niedrige Frequenz von NOTCH1 Mutationen und TCR Rearrangement) auszeichnet. Darüber hinaus zeigten ETP-ALL Patienten einen Überlebensvorteil durch eine konsolidierende allogene Stammzelltransplantation im Vergleich zu Patienten die eine alleinige konsolidierende Chemotherapie erhalten haben. Weiterhin wurde die Wirksamkeit von Tyrosinkinase Inhibitioren in der T-ALL geprüft und es konnte gezeigt werden, dass FLT3-transformierten T-ALL Zelllinien eine hohe Sensitivität gegenüber Tyrosinkinase Inhibitoren besitzen. Diese Daten bestätigen die Einführung von spezifischen Behandlungsansätzen mit FLT3 Inhibitoren zusätzlich zur frühen allogenen Stammzelltransplantation für diese Risikogruppe. Darüber hinaus wurde die differentielle Expression von miRNAs in der ETP-ALL untersucht. Die miRNA Expressionsprofile zeigten eine erhöhte Expression von miR-221 und miR-222 und eine verminderte Expression von 6 weiteren miRNAs (miR-151-3p, miR-19a, miR-20b, miR-342-3p, miR-363, miR-576-3p) im Vergleich zur typischen T-ALL. Die Expression von miR-221, miR-222, miR-19a und miR-363 konnte in einer großen Kohorte von T-ALL Patienten validiert werden. Das Gen *ETS1*, welches in der ETP-ALL niedrig exprimiert ist, konnte als miR-222 Target identifiziert werden. Für die miR-222 konnte weiterhin eine Rolle in der Proliferationshemmung von leukämischen Zellen gezeigt werden; zum Einen durch die Inhibition der S-phase im Zellzyklus und zum Anderen durch die induktion von Apoptose. Zusammenfassend unterstreichen diese Daten, dass die Deregulation von miRNAs eine funktionelle Rolle in der akuten Leukämie spielt und können als Basis für die Entwicklung von miRNA-basierten Therapiestrategien dienen, um die Therapie von Leukämie Patienten zu verbessern.

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8 CURRICULUM VITAE

Due to data privacy the curriculum vitae is not available in the online version

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9 LIST OF PUBLICATIONS

9.1 Publications

Kirdis E, Jonsson IM, Kubica M, Potempa J, Josefsson E, Masalha M, Foster SJ, Tarkowski A. Ribonucleotide reductase class III, an essential enzyme for the anaerobic growth of Staphylococcus aureus, is a virulence determinant in septic arthritis. Microb Pathog. 2007; 43(5-6):179-188.

Coskun E, von der Heide EK, Schlee C, Kühnl A, Gökbuget N, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. The role of microRNA-196a and microRNA-196b as *ERG* regulators in acute myeloid leukemia and acute T-lymphoblastic leukemia. Leuk Res. 2011; 35(2):208-13.

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Coskun E, Neumann M, Schlee C, Liebertz F, Heesch S, Goekbuget N, Hoelzer D, Baldus CD. MicroRNA profiling reveals aberrant microRNA expression in adult ETP-ALL and functional studies implicate a role for miR-222 in acute leukemia. Leuk Res. 2013; 37(6):647-656.

9.2 Poster presentations

Coskun E, von der Heide EK, Schlee C, Goekbuget N, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. The Role of Microrna-196a-1 and Microrna-196b in Acute T-Lymphoblastic Leukemia and Acute Myeloid Leukemia. Blood (ASH Annual Meeting Abstracts), Nov 2009; 114: 1607.

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Coskun E, Neumann M, Schlee C, Goekbuget N, Hoelzer D, Thiel E, Baldus CD. MicroRNA Profiling Reveals Aberrant MicroRNA Expression in Adult ETP-ALL and Functional Studies Demonstrate a Role of Mir-222 in Acute Leukemia. Blood (ASH Annual Meeting Abstracts), Nov 2011; 118: 3458.

10 APPENDIX - PUBLICATIONS

Manuscript: "The role of microRNA-196a and microRNA-196b as *ERG* regulators in acute myeloid leukemia and acute T-lymphoblastic leukemia"

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Manuscript: "*FLT3* mutations in early T-cell precursor ALL characterize a stem cell like leukemia and imply the clinical use of tyrosine kinase inhibitors"

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Manuscript: "MicroRNA profiling reveals aberrant microRNA expression in adult ETP-ALL and functional studies implicate a role for miR-222 in acute leukemia"

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PLOS ONE

FLT3 Mutations in Early T-Cell Precursor ALL Characterize a Stem Cell Like Leukemia and Imply the Clinical Use of Tyrosine Kinase Inhibitors

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Abstract

Early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) has been identified as high-risk subgroup of acute T-lymphoblastic leukemia (T-ALL) with a high rate of *FLT3*-mutations in adults. To unravel the underlying pathomechanisms and the clinical course we assessed molecular alterations and clinical characteristics in a large cohort of ETP-ALL (n = 68) in comparison to non-ETP T-ALL adult patients. Interestingly, we found a high rate of *FLT3*-mutations in ETP-ALL samples (n = 24, 35%). Furthermore, *FLT3* mutated ETP-ALL was characterized by a specific immunophenotype (CD2+/CD5-/CD13+/CD33-), a distinct gene expression pattern (aberrant expression of *IGFBP7*, *WT1*, *GATA3*) and mutational status (absence of *NOTCH1* mutations and a low frequency, 21%, of clonal TCR rearrangements). The observed low *GATA3* expression and high *WT1* expression in combination with lack of *NOTCH1* mutations and a low rate of TCR rearrangements point to a leukemic transformation at the pluripotent prothymocyte stage in *FLT3* mutated ETP-ALL. The clinical outcome in ETP-ALL patients was poor, but encouraging in those patients with allogeneic stem cell transplantation (3-year OS: 74%). To further explore the efficacy of targeted therapies, we demonstrate that T-ALL cell lines transfected with FLT3 expression constructs were particularly sensitive to tyrosine kinase inhibitors. In conclusion, *FLT3* mutated ETP-ALL defines a molecular distinct stem cell like leukemic subtype. These data warrant clinical studies with the implementation of *FLT3* inhibitors in addition to early allogeneic stem cell transplantation for this high risk subgroup.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive leukemia accounting for 10–15% of childhood and 25% of adult ALL cases. Based on molecular studies, T-ALL can be divided into at least four molecular-cytogenetic subgroups, i.e. the *TAL/LMO*, the *TLX/HOX11*, the *TLX3/HOX11L2* and the *HOXA* subgroups [1–3]. Apart from these genetic subgroups, a fifth subgroup of T-ALL cases with developmental arrest at a very early stage of T-cell development was defined by a characteristic early T-cell precursor (ETP) signature in pediatric T-ALL [4]. This T-ALL subtype, termed as ETP-ALL, is described by an immature surface immunophenotype: absence of CD1a and CD8 expression, weak CD5 expression and expression of one or more myeloid-associated and/or stem cell-associated markers. In addition, an increased genomic instability, a high frequency of remission failures and hematologic relapse characterize this highly unfavorable T-ALL subgroup in pediatric patients [4].

Oncogenic alterations that lead to a differentiation arrest at specific stages of T-cell development are well known for specific subgroups of T-ALL. Of these, the overexpression of the orphan homeobox proteins *TLX1* and *TLX3* lead to a maturation block due to ETS1-mediated TLX recruitment to the Eα core [5]. Most recently, for the group of ETP-ALL a mutational spectrum similar to acute myeloid leukemia (AML) was observed, however no single genetic alterations could be tracked down [6]. For the majority of T-ALL, activation of *NOTCH1* signalling is a driving force in the pathogenesis [7]. Activating *NOTCH1* mutations have been found in more than 60% of T-lineage leukemias and result in a ligand-

independent release of the intracellular NOTCH1 domain, which subsequently translocates to the nucleus, where it acts as transcriptional co-activator [8–11]. Various groups have shown that activated *NOTCH1* signalling causes activation of downstream targets including *HES1, DTX1, PTCRA*, and *MYC* and clinical studies have explored gamma secretase inhibitors (GSI) as targeted therapeutic strategy in T-ALL [12–14].

In contrast to the high frequency of *NOTCH1* mutations, activating *FLT3* mutations (*FLT3*mut) occur only in a very low frequency of T-ALL cases (1–3%), but were evaluated in only limited patient series [15–17]. In contrast, mutations of the *FLT3* gene, including internal tandem duplications (ITD) and tyrosine kinase domain (TKD) mutations, are one of the most frequent somatic alterations in AML. About one third of AML patients harbor these alterations, which are associated with a poor prognosis in both, adult and pediatric AML [18,19]. These findings have promoted the use of tyrosine kinase inhibitors (TKI) in patients with *FLT3* mutated AML [20,21].

Recently, we have characterized ETP-ALL as a subgroup of early T-ALL in adults [22]. To unravel the underlying pathomechanisms of ETP-ALL and to extend our insights on *FLT3mut* ETP-ALL, we performed a comprehensive molecular and clinical study on a large cohort of adult ETP-ALL patients. We were able to demonstrate that *FLT3mut* ETP-ALL could be classified by its specific immunophenotype and distinctive stem cell like characteristics. Moreover, T-lymphoblastic cells transfected with FLT3-ITD constructs were particular sensitive to tyrosine kinase inhibition making this a new and potentially useful therapeutic option.

Materials and Methods

Patients and treatment

We screened 1241 peripheral blood and bone marrow samples of T-ALL patients that were sent to the central diagnostic reference laboratory of the German Acute Lymphoblastic Leukemia Multicenter Study Group (GMALL). Most cases were characterized with monoclonal antibodies to precursor cells (CD10, CD34, CD117, TdT and HLA-DR) and with a selection of lymphoid-associated antigens including surface and cytoplasmic (c) antigens (cCD79a, CD22, cIgM, CD19, CD20, CD24, CD3, TCR, CD2, CD5, CD4, CD8, CD7, CD1a) and myeloid-associated antigens including myeloperoxidase (MPO), CD13, CD33, CD65s, CD15, CD14, CD64. An antigen was considered positive, if they were expressed in ≥20% of leukemic cells (10% for cytoplasmic antigens). Classification of ETP-ALL was based on the immunophenotypic diagnostic criteria as originally described [4]: CD5 <75%; CD1a and CD8 <5%; CD117, CD34, HLA-DR, CD13, CD33, and CD65s >25%. CD11b was not determined (Suppl. Table S1). Of all immunophenotypically identified ETP-ALL patients (n = 142), sufficient material for further investigations was available in 68 cases. Sixteen of these 68 patients were already included in a previous work [22]. For 52 of these 68 patients clinical follow-up data were available. The median follow-up was 9.4 months (range: 0–124.6 months). Most patients were treated according to protocols of the GMALL study group (43/46, 93% by medical report, Table 1). In addition, 94 T-ALL patients from the GMALL trial 07/2003 were used as reference group, of which nine patients showed an ETP-ALL immunophenotype and were included in the cohort of 68 ETP-ALL patients [23,24]. Of the remaining 85 non-ETP T-ALL patients, 17 had an immunophenotype of early T-ALL, 15 of mature T-ALL, and 53 of thymic T-ALL. All patients gave written informed consent to participate in the study according to the Declaration of Helsinki. The studies were approved by the ethics board of the Johann Wolfgang Goethe-Universität Frankfurt/Main, Germany.

Table 1. Gene expression levels in ETP-ALL compared to non-ETP T-ALL.

Expression		ETP-ALL (N = 68)	non-ETP T-ALL (N = 85)	P-value
BAALC	median (range)	0.69 (0.0–27.1)	0.08 (0.0–160.3)	<.001
IGFBP7	median (range)	1.24 (0.01–4.2)	0.49 (0.0–16.2)	.009
WT1	median (range)	0.53 (0.0–4.2)	<0.01 (0.0–1.6)	<.001
ERG	median (range)	1.16 (0.0–18.6)	10.69 (0.5–136.7)	<.001
MN1	median (range)	4.59 (0.0–33.1)	0.66 (0.01–2.7)	<.001
BCL11B	median (range)	0.09 (0.0–1.4)	0.44 (0.0–9.9)	<.001
GATA3	median (range)	2.11 (0.0–27.3)	3.91 (0.3–32.4)	.005
MEF2C	median (range)	0.50 (0.0–5.1)	0.20 (0.0–1.7)	.001

P values were calculated by Mann-Whitney-U-test.
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Nucleic acid preparation and molecular characterization

Pretreatment bone marrow and peripheral blood samples from patients were used for DNA and total RNA extraction using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with minor modifications. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA and avian myeloblastosis virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany) in the presence of RNase inhibitor (RNasin; Roche, Mannheim, Germany).

The samples were investigated by comparative real-time PCR (RT-PCR) for expression of eight genes (*BAALC*, *ERG*, *IGFBP7*, *WT1*, *MN1*, *GATA3*, *BCL11B*, and *MEF2C*). The mRNA expression levels for *WT1* [25], *BAALC* [24], *IGFBP7* [26], and *ERG* [24] were determined by RT-PCR as previously described. *MN1*-primers were designed as reported [27]. Primer sequences for the expression analysis of *GATA3*, *BCL11B* and *MEF2C* are available on request.

The *NOTCH1* mutation status was defined by direct sequencing of the N-terminal and the C-terminal region of the HD domain, the N-terminal and the C-terminal region of the PEST domain, and the TAD domain [28,29].

WT1 mutations were analysed as recently reported [25]. *FLT3mut* (ITD/TKD) were analyzed using a commercially available FLT3 mutation assay (InVivoScribe Technologies, San Diego, USA). The TCR rearrangement status was assessed by the IdentiCloneTM TCRG Gene Clonality Assay (InVivoScribe Technologies, San Diego, USA).

Statistical analysis

Differences in the clinical characteristics were tested by the Pearson χ^2 test. For overall survival (OS) in the different subgroups, Kaplan-Meier curves were created and compared by the Log-rank test. OS was calculated from the time-point of study entry to the time-point of death or last follow-up (censored).

The statistical difference of gene expression between two independent groups was tested by the nonparametric Mann-Whitney-U-test. Differences in the mutation rates were analyzed by the Pearson χ^2 or the Fisher's exact test. For all tests a *P*-value<0.05 (two-sided) was considered to indicate a significant difference. All calculations were performed using the SPSS software version 19 (SPSS Inc., Chicago, IL, USA) and GraphPad

Prism® software version 5 (GraphPad Software Inc., LA Jolla, CA, USA).

Cell culture and chemicals

The human mature T-cell leukemia cell lines Jurkat, BE13 and MOLT-4 were obtained from the German Resource Center for Biological Material, DSMZ (Braunschweig, Germany) and previously characterized on a molecular level [30]. They were grown in RPMI media with 10% fetal bovine serum. All cell lines were cultured at 37°C in a 5% CO2 humidified chamber. TKI258 was a kind gift from Novartis (Basel, Switzerland). Tyrosine kinase inhibitors Sorafenib and PKC412 were purchased from Alexis/Enzo Life Sciences (BAY 43-9006; Loerrach, Germany) and LC Laboratories (Woburn, MA, USA) respectively. The chemotherapeutic agent cytarabine (AraC) was provided by Merck Chemicals (Darmstadt, Germany).

Plasmid constructs and transfection

For transduction of Jurkat, BE13 and MOLT-4, 2×10^6 cells were transfected with either FLT3-ITD, or FLT3-wt expression constructs and with the empty vector as a control, using the Nucleofector systems (Lonza Cologne AG, Cologne, Germany) according to the manufacturer's recommendations. The final concentration of the constructs and the empty vector control was 2 µg. FLT3-wt and FLT3-ITD expression constructs were previously described [31].

Cell proliferation assay

Cell proliferation was measured with the WST-1 reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Briefly, 48 hours (hrs) after transfection with FLT3 constructs and empty vector control, the cells were seeded in a 96-well plate with 2×10^5/well. Subsequently, the cells were cultured for 48 hrs with Sorafenib, PKC412, TKI258 and AraC as a chemotherapy agent or Dimethylsulfoxid (DMSO) as negative control. Absorbance was measured after 48 hrs by optical density absorption analyses at 450 nm using an ELISA multiplate reader.

The 50% growth inhibitory concentrations (IC_{50}) of Sorafenib, PKC412 and TKI258 were determined by plotting the logarithm of the drug concentrations (Sorafenib: 0–500 µM, PKC412: 0–18 nM, TKI258: 0–500 nM) and the growth rate of the cells treated with FLT3-ITD or FLT3-wt constructs and empty vector, using the WST-1 assay.

Apoptosis assay

The cellular apoptosis was measured transfected with FLT3-ITD or FLT3-wt constructs and with the empty vector. Briefly, after 48 hrs treatment with Sorafenib, PKC412, TKI258, and AraC cells were labelled with Annexin V and 7-amino-actinomycin D (7-AAD), using Annexin V Apoptosis Detection Kit (BD Pharmingen, Heidelberg, Germany) and then analyzed by FACS Calibur (Becton-Dickinson) to determine the percentage of apoptotic cells.

Results

Characteristics and clinical outcome of adult ETP-ALL patients

Based on the ETP-ALL specific immunophenotype, we identified pre-treatment samples of 68 newly diagnosed ETP-ALL patients. The median age was 38 years (range: 17–74 years). More patients were male (81%). Of all 68 ETP-ALL patients, follow-up data were available in 52 patients. Forty-five patients were treated according to a GMALL-like protocol, three patients to an AML-like protocol. Fifty-eight percent of patients achieved a complete remission (CR) after induction therapy (Supplementary Table S2). The cumulative 3-year OS was 60%. We further examined outcome with respect to the treatment of chemotherapy only or the allocation to allogenic stem cell transplantation (alloSCT). With the limitation of a potential selection bias for patients undergoing alloSCT, we observed for ETP-ALL patients receiving an alloSCT a favorable outcome (n = 20; 3-year OS: 74%) compared to ETP-ALL patients that were treated with chemotherapy only (n = 19; 3-year OS: 37%, P = 0.006; Figure 1). To address the potential selection bias we have also performed a landmark analysis with a time to transplant of two months. In this analyses patients undergoing alloSCT showed a favourable however not significant benefit compared to patients only receiving chemotherapy.

Aberrant gene expression and mutational analyses in ETP-ALL compared to non-ETP T-ALL

To further characterize ETP-ALL on the molecular level, we analyzed this large ETP-ALL cohort for the expression of selected genes involved in the pathogenesis and with prognostic implications in adult acute leukemia [22]. *BAALC* and *IGFBP7* were higher expressed in ETP-ALL compared to non-ETP T-ALL patients (*BAALC*, 8.6-fold, P<.001; *IGFBP7*, 2.5-fold, P = .009). Furthermore, expression levels of *WT1* and *MN1* were higher in ETP-ALL compared to non-ETP T-ALL (*WT1*, P<.001; *MN1*, 7-fold, P<.001). Additionally, expression of *MEF2C*, a gene associated with ETP-ALL [32], was significantly higher in ETP-ALL versus non-ETP T-ALL (2.6-fold, P = .001). As critical players in the differentiation program of T-lymphopoiesis, we explored the expression of the transcription factors *GATA3*, required for the development of normal ETPs [33], and *BCL11B*, necessary for the subsequent T-cell lineage commitment [34]. Both, *GATA3* and *BCL11B*, were lower expressed in ETP-ALL compared to non-ETP T-ALL (1.9-fold, P = .005; and 4.9-fold, P<.001; respectively). Similarly, ETS transcription factor *ERG* was also significantly downregulated in ETP-ALL vs. non-ETP T-ALL (9.2-fold, P<.001; Table 1) [35].

The analysis of the TCR rearrangement status revealed that 40 ETP-ALL patients (59%) lacked clonal TCR rearrangements, while 28 patients (41%) showed a monoclonal status. In contrast, 66 (78%) of non-ETP T-ALL patients showed clonal TCR rearrangements, whereas only 18 of these patients (22%) lacked monoclonal TCR rearrangements (Table 2). In addition, differences of the *NOTCH1* and *FLT3* mutation status between ETP-ALL and non-ETP T-ALL cases were explored. We found a low rate of *NOTCH1* mutations in the ETP-ALL subgroup (n = 10/68, 15%), whereas *NOTCH1* mutations were more frequent (40%) in non-ETP T-ALL patients (P<.001). In contrast, we found a high rate of *FLT3* mutations in ETP-ALL compared to non-ETP T-ALL patients: 24 of the 25 *FLT3* mutations were found in the ETP-ALL group, displaying a frequency of 35.3%, whereas non-ETP T-ALL showed a *FLT3* mutations frequency of only 1.2% (P<.001). Ten cases (6.5%) had TKD mutations, of which nine occurred in the ETP-ALL group (P = .003). ITD mutations were found in 15 cases, all belonging to the ETP-ALL group (P<.001, Table 2). In a multivariate analysis, *NOTCH1* mutation status, low expression of *BAALC*, *WT1*, *ERG*, *IGFBP7*, and TCR rearrangement had no additional prognostic impact in the subgroup of ETP-ALL.

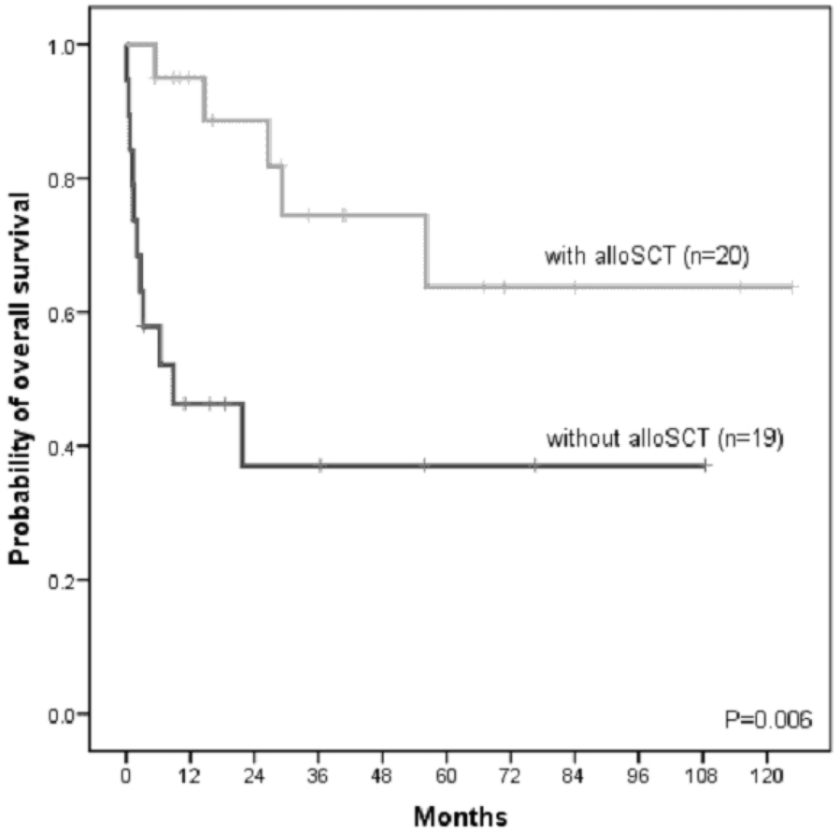

Figure 1. Kaplan Meier analysis of overall survival in adult ETP-ALL patients receiving chemotherapy only (without alloSCT) or undergoing alloSCT. P-value was calculated by the Log-Rank test. Abbreviations: alloSCT, allogeneic stem cell transplantation.
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Immunophenotype of *FLT3*mut ETP-ALL compared to *FLT3*wt ETP-ALL

In addition to the distinct immunophenotype, *FLT3*mut ETP-ALL showed specific immunophenotypic and molecular characteristics compared to *FLT3*wt ETP-ALL. In this study, 83% (20/24) of *FLT3*mut ETP-ALL patients were positive for CD117, compared to only 28% (13/44) of *FLT3*wt ETP-ALL cases (P<.001). Furthermore, *FLT3*mut ETP-ALL had a higher rate of positivity for CD2 (88% vs. 30%, P<.001) and CD13 (100% vs. 37%, P<.001) compared to *FLT3*wt ETP-ALL patients. *FLT3*wt ETP-ALL was characterized by expression of CD5 (54% vs. 4%, P<.001) and CD33 (54% vs. 4%, P<.001; Figure S1 in Supplementary Figures).

Table 2. Mutational events in ETP-ALL compared to non-ETP T-ALL.

Mutation Status		ETP-ALL	non-ETP T-ALL	P-value
TCR rearrangement	monoclonal	28 (41%)	66 (79%)	<.001
n = 153	polyclonal	40 (59%)	18 (21%)	
NOTCH1	mut	10 (15%)	35 (41%)	<.001
n = 151	wt	56 (85%)	50 (59%)	
***FLT3* total**	mut	24 (35%)	1 (1%)	<.001
n = 153	wt	44 (65%)	84 (99%)	
***FLT3* ITD**	mut	15 (22%)	0 (0%)	<.001
	wt	53 (78%)	85 (100%)	
***FLT3* D835**	mut	9 (13%)	1 (1%)	.003
	wt	59 (87%)	84 (99%)	

P values were calculated by Pearson's Chi-square test and Fisher's exact test, respectively.
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A recent study described the immunophenotype of TdT+/CD7+/CD13+/CD34+/CD117+ as highly specific for the prediction of *FLT3* mutations in an unselected cohort of T-ALL [17]. In our ETP-ALL cohort, 75% (18/24) of patients with *FLT3* mutations showed this immunophenotype, while only 7% (3/44) without *FLT3* mutations displayed this phenotype. Another *FLT3* mutation associated marker profile (sCD3-/CD117+/CD34+/CD62L+/CD56-/CD2+/CD7+/CD1a-/CD4-/CD5-/CD8-/CD13+ [36] can be adopted to 71% (17/24) of our *FLT3*mut ETP-ALL patients; this profile was highly specific for *FLT3* mutations without a false prediction. Here we established the combination of CD2+/CD5-/CD13+/CD33-, able to detect 21 of the 24 *FLT3*mut ETP-ALL patients, as highly sensitive (88%) and specific (95%) algorithm (Table 3).

Molecular characteristics of *FLT3*mut ETP-ALL in contrast to *FLT3*wt ETP-ALL

*FLT3*mut ETP-ALL showed a specific gene expression profile compared to *FLT3*wt ETP-ALL. Higher expression levels of *WT1* (2.2-fold, P = .003) and lower expression of *IGFBP7* (0.11-fold, P<.001) were characteristic for *FLT3*mut ETP-ALL. Remarkably,

Table 3. Combinations of antigens as a surrogate marker for *FLT3* mutations in ETP-ALL.

		FLT3mut (n = 24)	FLT3wt (n = 44)	P-value	Sensitivity	Specificity
CD117	pos	20	13	**<.001**	83%	70%
	neg	4	31			
TdT+/CD7+/CD13+/CD34+/CD117+[§]	pos	18	3	**<.001**	75%	93%
	neg	6	41			
CD117/CD34+/CD62L+/CD56/CD7+/CD2+/CD5-/CD13+[#]	pos	17	0	**<.001**	71%	100%
	neg	7	44			
CD2+/CD5-/CD13+/CD33-[&]	pos	21	2	**<.001**	88%	95%
	neg	3	42			

Abbreviations:
[§]combination of markers suggested by Hoehn *et al.* [17],
[#]combination of markers suggested by Paietta [36],
[&]combination of markers suggested in this paper. All combinations were adapted to the subgroup of ETP-ALL.
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*FLT3*mut ETP-ALL had a significantly lower expression of the T-cell transcription factor *GATA3* compared to *FLT3*wt ETP-ALL (P<.001). All except one *FLT3*mut ETP-ALL case had *GATA3* expression levels below the median. No significant differences were found for *BAALC*, *ERG*, *MN1*, *BCL11B*, and *MEF2C* (Table 3). As for other lymphoblastic leukemias described [37,38], *FLT3* itself is overexpressed in ETP-ALL. In this subgroup *FLT3*mut ETP-ALL showed a higher expression compared to *FLT3*wt ETP-ALL samples (P<.01, Figure S2 in Supplementary Figures).

TCR rearrangement analysis demonstrated that *FLT3*mut ETP-ALL patients predominantly lacked clonal TCR rearrangements. Only 21% of the *FLT3*mut ETP-ALL patients in contrast to 52% of the *FLT3*wt ETP-ALL patients showed a TCR rearrangement (P = .01). In addition, none of the *FLT3*mut ETP-ALL patients showed a *NOTCH1* mutation, while 23% (10/44) *FLT3*wt ETP-ALL had *NOTCH1* mutations (Table 4).

Table 4. Molecular characteristics of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients.

A Expression		FLT3mut (n = 24)	FLT3wt (n = 44)	P-value
WT1	median (range)	**0.78** (0.2–4.2)	**0.36** (0.0–3.4)	**.003**
IGFBP7	median (range)	**0.30** (0.02–4.0)	**2.52** (0.1–9.9)	**<.001**
GATA3	median (range)	**0.06** (0.0–5.7)	**5.82** (0.0–6.7)	**<.001**
BAALC	median (range)	0.44 (0.01–17.7)	0.95 (0.0–27.1)	.41
ERG	median (range)	0.94 (0.2–18.6)	1.56 (0.0–4.2)	.16
MN1	median (range)	6.35 (0.3–16.8)	7.42 (0.0–33.1)	.29
BCL11B	median (range)	0.20 (0.0–1.4)	0.05 (0.0–1.0)	.09
MEF2C	median (range)	0.71 (0.02–2.2)	0.46 (0.0–5.1)	.22
B Mutation Status				
NOTCH1	mut	0 (0%)	10 (23%)	**.01**
	wt	24 (100%)	34 (77%)	
TCR status	monoclonal	5 (21%)	23 (52%)	**.01**
	polyclonal	19 (79%)	21(48%)	

A: P-values were calculated by Mann-Whitney-U-test.
B: P-values were calculated by Pearson's Chi-square test and Fisher's exact test, respectively.
doi:10.1371/journal.pone.0053190.t004

Clinical characteristics of *FLT3* mutated ETP-ALL patients

With respect to clinical characteristics, no differences were observed between the *FLT3*mut ETP-ALL and the *FLT3*wt ETP-ALL patients regarding sex and age. The response to induction therapy was similar between both groups (CR: 13/21 vs. 13/24). Three of the 24 *FLT3*mut ETP-ALL patients were treated with an AML protocol, but none of the patients with *FLT3*wt ETP-ALL (Supplementary Table S3). The overall survival rate was similar between *FLT3*mut ETP-ALL and *FLT3*wt ETP-ALL patients (3-year survival: 58% versus 61%, P = 0.86; Figure S3 in Supplementary Figures).

Sensitivity of T-ALL cell lines transfected with *FLT3* expression constructs to TKI

In order to assess the sensitivity of TKI in a model of T-ALL with FLT3-ITD mutations, we transfected the T-ALL cell lines Jurkat, BE13 and MOLT-4 with FLT3-ITD or FLT3-wt constructs and an empty vector as control. Transfection of *FLT3* expression constructed did not alter the surface expression of myeloid (CD13, CD33) or stem cell (CD34, CD117) markers (data not shown). Cell lines transfected with FLT3-wt and FLT3-ITD constructs revealed a growth advantage compared to the empty vector transfected cells DMSO (first columns in Figure 2A–C). Treatment with TKI resulted in a selective and significant inhibition of the proliferation of FLT3-wt or FLT3-ITD transfected cells: Jurkat cells transfected with FLT3-ITD or FLT3-wt constructs showed a significant decrease in proliferation compared to empty vector transfected cells when treated with TKIs (including Sorafenib, PKC412, TKI258; Figure 2A). FLT3-ITD and FLT3-wt transfected cells were almost equally sensitive to PKC412 and TKI258, whereas empty vector transfected cells were relative insensitive. Similar results were observed for FLT3-ITD transfected MOLT-4 (Figure 2B) and BE13 cells (Figure 2C). FLT3-wt transfected cells behave different for MOLT-4 and BE13; MOLT-4 cells were more sensitive to sorafenib and BE13 cells were more resistant to PKC412 and TKI258. No differences in proliferation were observed with respect to the *FLT3* status for AraC treated cells (Figure 2A–C).

We further examined the TKI mediated apoptosis in Jurkat cells transfected with FLT3 expressing constructs. All TKIs induced enhanced apoptosis in cells transfected with FLT3 expressing constructs compared to empty vector controls (Figure S4 in Supplementary Figures). Cells treated with Sorafenib revealed a

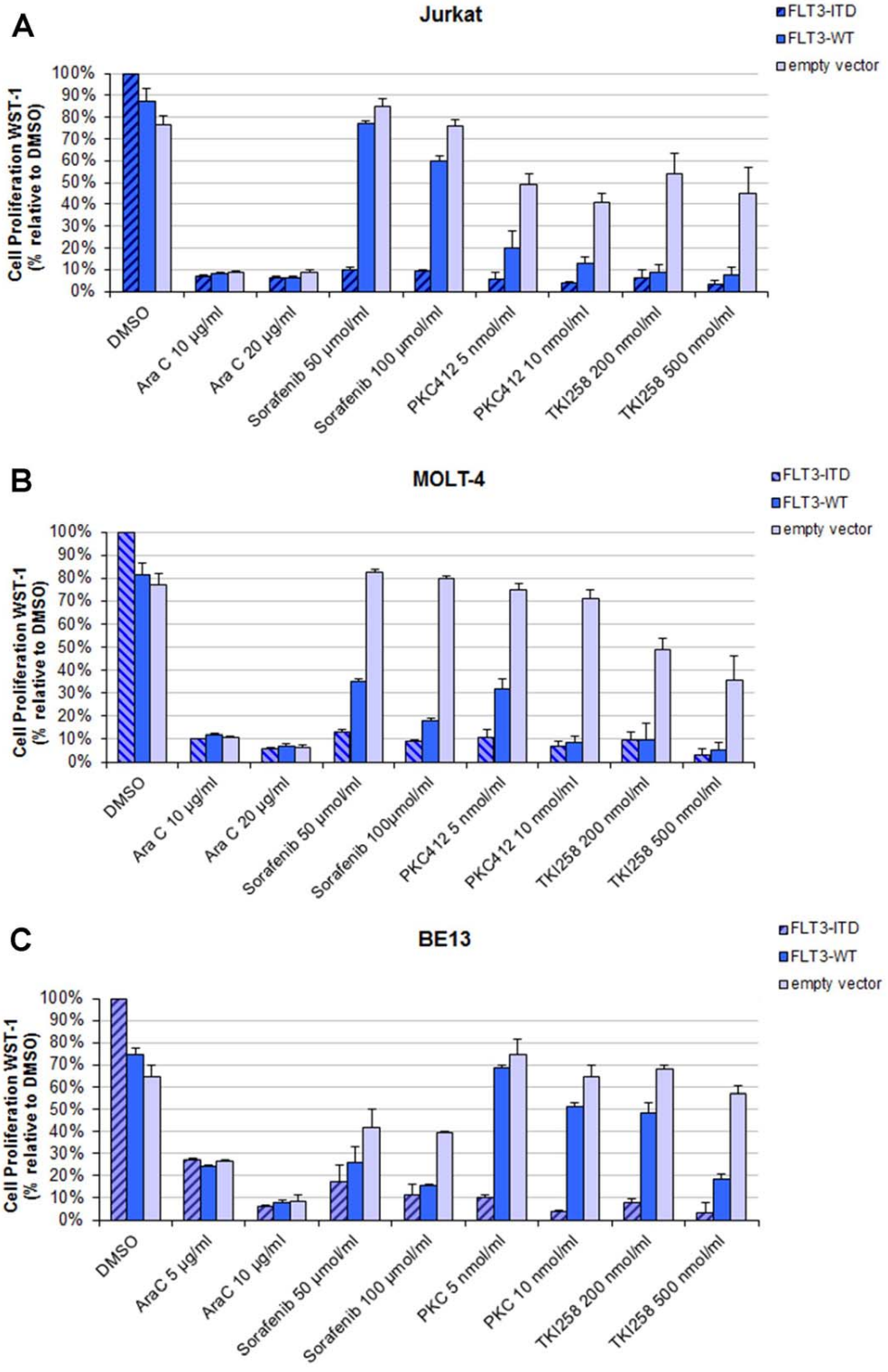

Figure 2. Effects of tyrosine kinase inhibitors on proliferation in T-ALL cell lines transfected with FLT3 expression constructs (A–C). Fourty-eight hours (hrs) after transfection, cells were seeded and cultured for additionally 48 hrs with tyrosine kinase inhibitors (PKC412, TKI258, and Sorafenib) and chemotherapy (AraC). Cell proliferation was measured using the WST-1 proliferation reagent. The mean optical density (OD) values corresponding to non-treated FLT3-ITD transfected cells were taken as 100%. The results were expressed in percentages of the OD of treated versus untreated control cells. Two experiments were performed in duplicates. For each drug two different doses were used. All results were expressed as means ±S.D. **A**: Jurkat cells. **B**: MOLT4 cells. **C**: BE13 cells.
doi:10.1371/journal.pone.0053190.g002

3-fold and 2-fold increase in apoptosis in FLT3-ITD and FLT3-wt transfected cells, respectively. Similar results were observed for PKC412 and TKI258 treated cells, whereas no significant changes in apoptosis were observed for AraC (Figure S4 in Supplementary Figures). Finally, we defined the concentration (IC50) of Sorafenib, PKC412, TKI258 and AraC that induced 50% growth inhibition of Jurkat cells (Figure 3A–D). The IC50 for Sorafenib was 25.7 μM in FLT3-ITD transfected cells, compared to to 305.5 μM and 486.5 μM in FLT3-wt, and empty vector transfected cells, respectively (Figure 3A). The IC50 for PKC412 was 2.8 nM in FLT3-ITD transfected cells, compared to 7.1 nM and 15.5 nM in FLT3-wt and empty vector transfected cells, respectively (Figure 3B). Similar growth inhibitory effects were observed for TKI258 (Figure 3C). No differences in the IC50 between the different transfected cells were seen for AraC (Figure 3D).

Discussion

In the past decades the molecular characterization of T-ALL broadly expanded and unraveled key events that drive malignant transformation. These genetic alterations may in future lead to the development and the implementation of targeted therapy. Coustan Smith et al. first identified ETP-ALL as a high risk subgroup of pediatric T-ALL characterized by a specific immature immunophenotype and a distinct gene expression profile [4]. Most recently, the genetic heterogeneity of pediatric ETP-ALL was further assessed by whole genome sequencing and next generation sequencing [6]. While various novel somatic mutations were identified, no single alteration could be detected pointing to the heterogeneous genetic background of ETP-ALL, despite the apparently common clinical and immunophenotype features. However, features shared with myeloid leukemias were present as well as mutations in genes of cytokine receptor and RAS signaling and genes involved in histone-modification were frequently observed [6]. In this work, we now focused on genes with already established prognostic and pathogenic value in AML and/or T-ALL.

We have previously characterized ETP-ALL as a high risk subgroup of early T-ALL in adults [22]. To further delineate the molecular pathomechanisms for this distinct T-ALL subgroup with stem cell like and myeloid features, we examined molecular alterations and clinical outcome in a large cohort of adult ETP-ALL patients (n = 68).

ETP-ALL is defined by a specific immunophenotype as described [4]. Recently, an additional score based on the immunophenotype was suggested to define ETP-ALL [39]. In our ETP-ALL cohort, 96% (65/68) of patients had a score greater than 6, which we used as the defining cut-off. In addition to this distinct phenotype, expression analyses of candidate genes revealed significant higher expression of stem cell associated genes and genes with adverse prognostic significance in ETP-ALL versus non-ETP T-ALL. High expression of *BAALC* and *IGFBP7*, associated with an immature high risk leukemic phenotype in adult T-ALL and AML [26,40], underscores the immature nature of ETP-ALL. Similarly, *IGFBP7*, like *MEF2C*, were also found to be significantly upregulated in pediatric ETP-ALL [4]. In addition, *MN1* identified to be associated with ETP-ALL [32], and *WT1*, a gene known to be of unfavorable prognosis in AML as well as in T-ALL in the presence of a mutation [25,41], were also overexpressed in the cohort of ETP-ALL.

We further observed distinct differences in the mutational profile: compared to non-ETP T-ALL, ETP-ALL patients showed less frequent *NOTCH1* mutations (15%). On the other hand, a

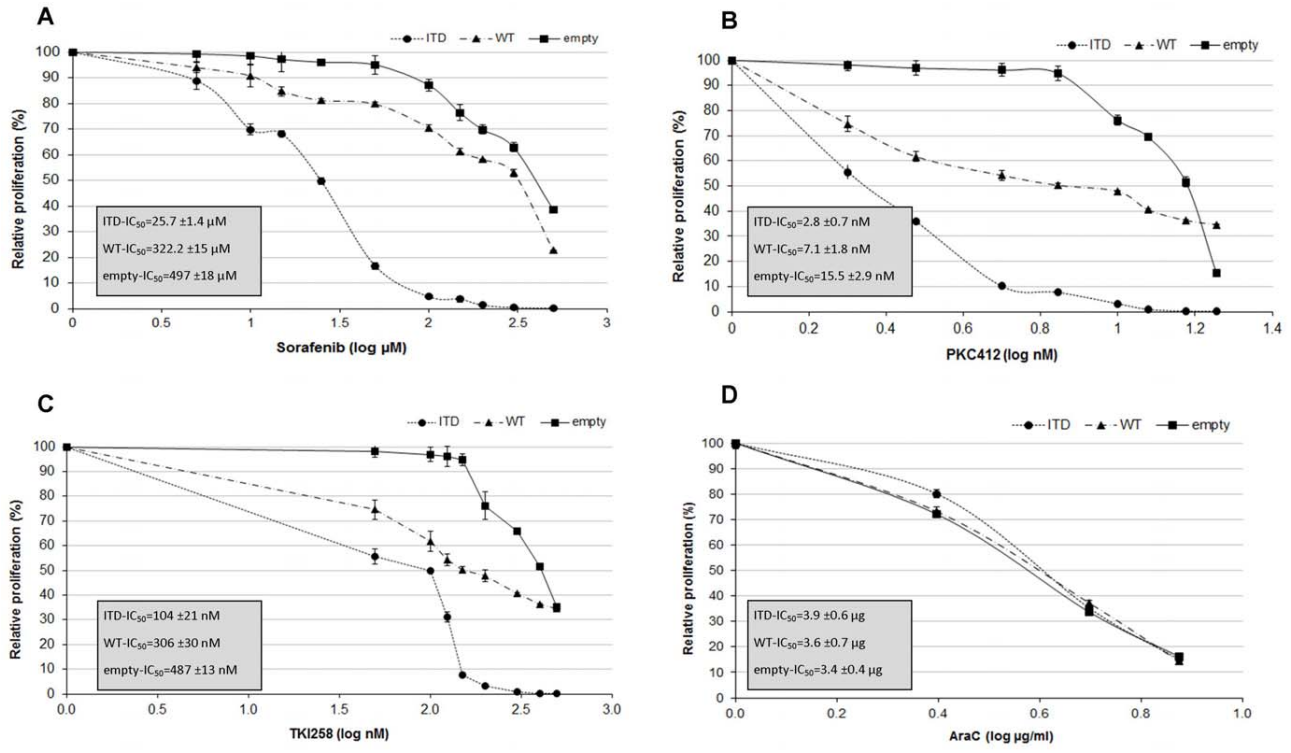

Figure 3. Growth inhibition of Jurkat cells transfected with FLT3 expression constructs (FLT3-ITD, FLT3-wt, and empty vector) and treated with Sorafenib, PKC412, TKI258 and AraC. IC_{50} was determined by WST-1 assay with different concentrations.
doi:10.1371/journal.pone.0053190.g003

high rate of *FLT3* mutations was observed in ETP-ALL (35%), contrasting the mutational profile of non-ETP T-ALL. *FLT3* mutations are one of the most frequent genetic alterations in AML [18], whereas *FLT3* is only infrequently mutated in leukemic lymphoblasts [42]. This underscores to some extent the association of ETP-ALL with early myeloid differentiation. In pediatric patients, a similar low rate of *NOTCH1* mutations (16%) was seen in ETP-ALL. *FLT3* mutations, although in a lower frequency (14%), occurred exclusively in ETP-ALL [6]. In addition, we analyzed the TCR rearrangement status in these ETP-ALL patients. In normal human T-cell development, TCR rearrangements are rare in prothymocytes, but are commonly found at the prethymocyte stage [43,44]. The frequent absence of TCR rearrangement in our cohort confirms the immaturity of ETP-ALL. Together, these data further indicate that ETP-ALL represents a distinct leukemic subtype.

Interestingly, within the ETP-ALL subgroup *FLT3*mut ETP-ALL define a new molecular stem cell entity as these cases show a specific immunophenotype and molecular characteristics compared to *FLT3*wt ETP-ALL. We observed a high expression of CD2, the myeloid antigen CD13, and CD117 in the *FLT3*mut ETP-ALL. CD117, encoded by the *c-KIT* protooncogene, is highly expressed at the early stages of hematopoietic development [45], and in acute leukemia the highest frequency of CD117 expression is found in AML [46–48]. Expression of CD117 has been associated with *FLT3* mutations in rare cases of T-ALL [15,16]. Here, in this yet largest cohort of *FLT3*mut T-ALL cases, only four *FLT3*mut patients lacked CD117 expression. Recently, combinations of surface markers were suggested as surrogate marker for *FLT3* mutations in T-ALL [17,36]. However, while these combinations yield a high sensitivity, none could detect all of the ETP-ALL cases with *FLT3*mut. In our study, a combination of CD2+/CD5-/CD13+/CD33- resulted in the highest sensitivity for the presence of *FLT3* mutations in ETP-ALL with a high specificity. For the routinely performed diagnostic flow cytometry, these combinations may help to identify ETP-ALL patients that should be tested for *FLT3* mutations.

We further observed that *FLT3*mut ETP-ALL predominantly lacked clonal TCR-rearrangements pointing to a leukemic transformation before the prothymocyte stage of T-cell development. The absence of TCR rearrangements had already been linked to early treatment failure in children with T-ALL [49], providing an indirect support for the poor prognosis of ETP-ALL. The early developmental arrest of *FLT3*mut ETP-ALL is also emphasized by the low *GATA3* expression. In normal T-cell development, GATA3 plays a definite role in the early T-lineage specification as it is required for the transformation of the ETP/DN1 to the DN2a stage [34]. Thus the leukemic transformation in *FLT3*mut ETP-ALL lacking *GATA3* expression might occur at a stem cell pluripotent prothymic stage before *GATA3* expression is induced. These data in combination with the absence of activating *NOTCH1* mutations reflect an even more immature nature of the *FLT3mut* ETP-ALL within the ETP-ALL subgroup.

ETP-ALL as a subgroup of early T-ALL reflects a high risk entity with an overall survival of approximately 50% in adults [22]. Based on the findings of the GMALL study group [50], an alloSCT should be planned in first complete remission for early T-ALL patients. Even though the selection for patients undergoing alloSCT is biased due to various confounding parameters, ETP-ALL patients receiving an alloSCT showed a remarkable favorable outcome in our cohort, whereas the outcome for ETP-ALL patients receiving chemotherapy was relatively poor. The poor response to lymphoid cell-directed ALL chemotherapy only, as already reported for pediatric ETP-ALL [4], might be due to the immature nature and myeloid characteristic of the ETP-ALL. Thus, to further improve outcome for these high risk patients, in addition to alloSCT the implementation of targeted therapies should be considered. Due to the high frequency of *FLT3* mutations in ETP-ALL, TKIs already studied in *FLT3* mutated AML [51,52] would be an attractive treatment option. We assessed the sensitivity of T-ALL cell lines transfected with FLT3-ITD and FLT3-wt expression constructs and observed that *FLT3* transfected T-ALL cells, despite of their enhanced proliferation, were particular sensitive to TKIs similar to results in AML [31]. Although the transfection of *FLT3* expression constructs in T-ALL cell lines remains an *in vitro* system, the distinct sensitivity to TKIs together with the positive experience in AML support the rational for the clinical use of TKIs in *FLT3*mut ETP-ALL. In this work, TKI side effects and the impact of TKI on the *FLT3* D835Y mutation were not evaluated. However, in analogy to AML it would be expected that the tested TKI are also able to target TKD mutations. Regarding side effects in the clinical use of TKI, the experience in AML have shown that chemotherapy backbone in combination TKIs have to be carefully chosen.

Herein, we describe that ETP-ALL patients represent a distinct molecular subgroup of adult T-ALL patients with a low frequency of *NOTCH1* mutations and a high rate of *FLT3* mutations. Moreover, we characterize *FLT3*mut ETP-ALL as a new subgroup of ETP-ALL with unique immunophenotypical and molecular features pointing to a stem cell leukemia. To further improve outcome of this high risk leukemia, targeted therapies with TKIs as well as the allocation to alloSCT should further explored.

Supporting Information

Figure S1 Expression of surface antigens comparing *FLT3*mut ETP-ALL patients and *FLT3*wt ETP-ALL patients. Median and quartiles of the percentage of positive cells in the flow cytometry are pictured. Abbreviations: * statistically significant; ns, not significant.
(DOC)

Figure S2 *FLT3* mRNA expression in 68 adult ETP-ALL samples measured by quantitative RT-PCR. The *FLT3* expression was significantly higher in *FLT3*mut ETP-ALL (n = 21) compared to *FLT3*wt ETP-ALL (n = 37) ($p<.01$).
(DOC)

Figure S3 Clinical outcome of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients. The plot shown is the Kaplan Meier analysis of overall survival. P-value was calculated by the Log-Rank test.
(DOC)

Figure S4 Effects of tyrosine kinase inhibitors on apoptosis in Jurkat cells transfected with FLT3 expression constructs. Fourty-eight hrs after transfection the cells were cultured with tyrosine kinase inhibitors (**A**: Sorafenib, **B**: PKC412, and **C**: TKI258) or **D**: AraC. Apoptosis assay was performed by Annexin V/7AAD labeling of the cells. The results are expressed in percentage of apoptotic cells. Experiments were performed in duplicates. All results were expressed as means ±S.D.
(DOC)

Table S1 Immunphenotype used for the classification of the 68 ETP-ALL patients.
(DOCX)

Table S2 Clinical characteristics of ETP-ALL patients.
(DOCX)

Table S3 Clinical characteristics of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients.
(DOCX)

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Conceived and designed the experiments: MN NG DH ET CDB. Performed the experiments: MN EC LF LM IB NFS SH SS CS OB. Analyzed the data: MN EC TB CDB. Contributed reagents/materials/analysis tools: CB RH UD MG HD GE. Wrote the paper: MN EC SS WKH CDB.

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