Leukemia-associated genetic aberrations in mesenchymal stem cells of children with acute lymphoblastic leukemia

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Not everything that can be counted counts,
and not everything that counts can be counted.

Nicht alles, was gezählt werden kann, zählt
und nicht alles, was zählt, kann gezählt werden.

Albert Einstein

CONTENT

SUMMARY

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. The disease is caused by malignant immature lymphocytes or their progenitors. Therapy for children with initial ALL has proven to be quite successful (85% long-term survival), but the treatment outcome for relapsed ALL remains unsatisfactory (approximately 40% long-term survival). ALL subtypes can be classified according to their immunophenotype, morphology and specific genetic alterations using cytogenetic analysis. These genetic alterations include structural or numerical chromosome changes such as translocations or hyperdiploidy, deletions and amplifications. Analysis of the chromosomal translocations and the resulting fusion genes has improved the understanding of the biology of leukemia cells (LC). However, as previously suggested, the understanding of leukemia pathogenesis also requires an extended knowledge of not only the specific genetic mutations implicated, but also of the cellular framework, in which they arise, develop and are maintained. The bone marrow (BM) microenvironment promotes survival and differentiation of hematopoietic stem cells (HSC) and lymphocytes. The BM contains at least two different types of progenitor cells, HSC and mesenchymal stem cells (MSC). MSC are able to differentiate into multiple mesenchymal lineages, including bone, cartilage, muscle, fat tissue and BM stroma cell. MSC facilitate engraftment of HSC and seem to persist in the BM after chemotherapy. The plasticity of MSC to differentiate into cells of the hematopoietic lineage has been demonstrated in mice. Furthermore, recent studies have demonstrated the reprogramming of B-cells and other somatic cell types like fibroblasts into pluripotent stem cells by ectopic expression of defined transcription factors, indicating the high plasticity of virtually any cell. Therefore, we analyzed MSC from B cell precursor ALL patients for the presence of leukemia-associated chromosomal translocations and immunoglobulin (*IG*) gene rearrangements. Leukemia-specific *IG* / T cell receptor (*TCR*) gene rearrangements and junctional regions are widely used as clonal leukemic markers for the detection of minimal residual disease (MRD). Leukemia cells from 10 of 49 patients showed one of the three translocations namely *TEL-AML1*, *E2A-PBX1* or *MLL* rearrangement. Leukemia-associated aberrations were detected in MSC of these 10 ALL patients independent of the sampling time points. Leukemia-specific *IG* gene rearrangements were detected in MSC from 3 of the 8 analyzed patients. These results suggest a clonal relationship between MSC and leukemia cells.

1 INTRODUCTION

1.1 Childhood acute lymphoblastic leukemia

1.1.1 Features of the ALL disease

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. The peak incidence occurs between 2 and 5 years of age. Children with certain genetic (e.g. Down syndrom) or immunodeficiency syndromes are at increased risk. The disease is caused by uncontrolled proliferation of malignant immature lymphocytes. Bone marrow (BM) and peripheral blood are involved in most of cases, with frequent extramedullary involvement, primarily of the central nervous system (CNS), lymph nodes, spleen, thymus, liver and testis. Therapy for children with initial ALL has proven to be quite successful. Long-term survival for newly diagnosed children with ALL approaches 85% (1-3). However, even with risk-stratified and more intensive frontline therapy, 15-20% of children with ALL still relapse. The treatment outcome for relapsed ALL remains unsatisfactory. Approximately only 40% of children with relapsed ALL show a long-term survival (4-6). Relapsed ALL remains more common than new diagnoses of many common pediatric malignancies. More children still die from ALL than are diagnosed with osteosarcoma, Ewing sarcoma or retinoblastoma. The yearly number of children with ALL and treatment failure is similar to the number of children with newly diagnosed acute myeloblastic leukemia, Hodgkin disease or rhabdomyosarcoma. At the present time, the origin of the relapse remains unclear (4-6). Childhood ALL is a heterogeneous disease. ALL subtypes can be classified according to immunophenotype, morphology and specific genetic alterations.

1.1.2 Characterization of ALL

1.1.2.1 Immunophenotype

Leukemia cells in ALL are classified according to immunophenotype using an extensive panel of monoclonal antibodies for cell surface markers (CD markers). The European group for the immunological classification of acute leukemias (EGIL) established criteria for the characterization of acute leukemia based on marker expression (7, 8) (Table 1 and 2). Approximately 70-80% of childhood ALL are of B-precursor lineage (i.e. precursor B-cell leukemia or early pre-B cell ALL), and 15-17% are of T-precursor lineage. In B-cell ALL, although the immunophenotype varies, most are $cCD79a^+$, $CD19^+$, $CD10^+$, $CD34^+$, and TdT^+, but do not express surface immunoglobulin (sIg). Cytoplasmic mu (μ) heavy chains are found in the pre-B cell

subtype of leukemia. Based on the immunophenotype, four subtypes of B-cell ALL can be identified (Table 1). Pro-B-ALL patients have a poor prognosis. Common-ALL represents 2/3 of all childhood ALL and is a good prognostic subgroup.

Table 1. B-cell ALL subtypes.

Immunophenotyp markers	Pro-B ALL	Common ALL	Pre-B ALL	B-ALL
CD34	+	+	+	+
CD19	+	+	+	+
CD10	-	+	-	±
TdT	+	+	+	+
cCD79	+	+	+	+
μ_H	-	-	+	+
slg	-	-	-	+

In T-cell ALL the immunophenotype also varies, but most leukemia cells express cytoplasmic $CD3^+$ ($cCD3^+$), $CD7^+$, TdT^+, and $CD34^+$. The expression of other T cell markers such as CD3, CD2, CD1a (cortical thymocyte marker), CD4, and CD8 is more variable. Some have a double negative ($CD4^-$, $CD8^-$) phenotype, and some a double positive ($CD4^+$, $CD8^+$) one. Three subtypes of T-cell ALL are identified on immunophenotype basis (Table 2).

Table 2. T-cell ALL subtypes.

Immunophenotype markers	Early T-ALL	Cortical T-ALL	Mature T-ALL
cCD3	+	+	+
CD1a	-	+	-
CD2	±	+	-
CD34	+	+	+
CD5	±	-	-
CD7	+	+	+
sCD3	-	±	+
TdT	+	+	+

The expression of specific maturation markers in B and T cells does not strictly correspond with those in leukemia cells. Some B-precursor ALL cells express myeloid markers (e.g. CD33) in addition to B-lymphocyte markers.

1.1.2.2 Morphology

Morphologically, ALL can be classified using the French-American-British (FAB) system (9) as L1, L2, or L3 (Figure 1). Although these system has been rarely used in recent years. The morphology of BM derived blasts on the aspirate smears stained with Wright-Giemsa shows a high nuclear-cytoplasmic ratio (L1) and the presence of nucleoli (L2). L3 ALL (Burkitt lymphoma) represents a lymphoid population of more mature B-cell lineage and usually shows a distinct morphology with a deep basophilic and vacuolated cytoplasm. 85 to 89 % of children with ALL are classified as having FAB L1.

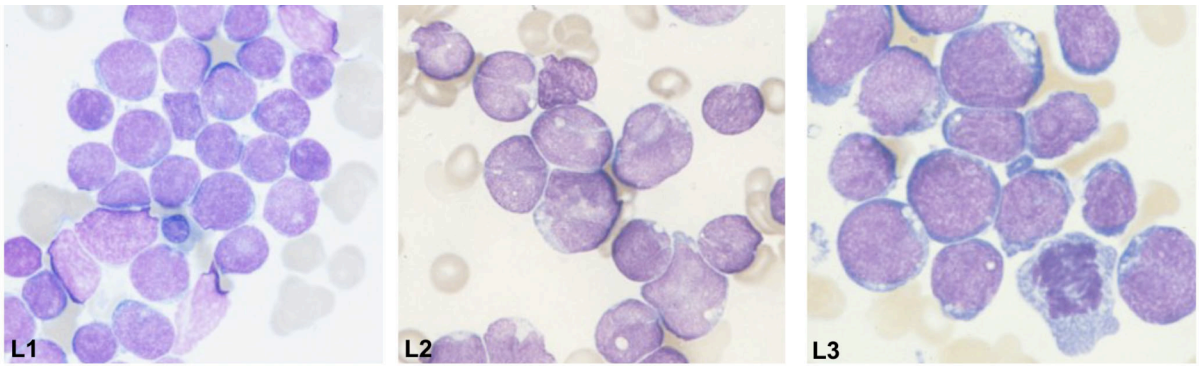


Figure 1. L1: BM aspirate from a patient with ALL. Blasts are small cells with homogenous nuclear chromatin and regular nuclear shape. The blast cells show a high nuclear to cytoplasmic ratio. **L2:** BM aspirate from a patient with ALL. Pleiomorphic blasts with variable amounts of cytoplasm, irregular nuclei and multiple indistinct nucleoli. **L3:** BM aspirate from a patient with Burkitt lymphoma at leukemic phase. Deeply basophilic blasts with dense nuclear chromatin and multiple cytoplasmic vacuoles (10).

1.1.2.3 Genetic aberrations

The main subtypes of ALL involve a large number of specific genetic alterations. Over the past century, it has become clear that genetic changes play a central role in cancer development. In 1914, Theodor Boveri postulated that the atypical chromosomes often seen in cancer cells are the basis of the aberrant behavior in these cells (11). In 1960, Nowell and Hungerford identified a small marker chromosome present in leukemia cells of patients with chronic myeloid leukemia, called Philadelphia chromosome (12, 13). The nature of the abnormality was unclear, but it was assumed that there was loss of chromosomal material and that this loss was associated with the development of leukemia disease. In 1973, Janet Rowley identified the first chromosomal translocation that involved a reciprocal rearrangement of chromosomes 8 and 21 in patients with acute myelogenous leukemia (AML). She also discovered that the Philadelphia chromosome is a

translocation involving chromosomes 9 and 22 (14). The products of chromosomal translocations can be fusion genes, generating either a dysregulated partner gene or a chimeric fusion protein with a new function. In the early 1980s, Heisterkamp et al. reported one of the first descriptions for fusion genes as the results of translocations. These studies showed, that the translocation t(9;22) caused the expression of the chimeric fusion protein BCR-ABL1, revealing a fusion of the *BCR* and *ABL1* genes. Several mechanisms have been proposed to explain the development of chromosomal translocations in leukemia cells. These mechanisms include: (1) illegitimate V(D)J or switch recombination; (2) homologous recombination (HR) mediated by repetitive sequences, such as Alu or long interspersed nuclear elements; (3) DNA topoisomerase II subunit exchange; and (4) error-prone non-homologous end-joining (NHEJ) following DNA double-strand breaks (DSBs) (15). Furthermore, it has been suggested that the aberrant karyotypes of tumors are caused by an extended genetic instability. For this reason the genetic instability has been postulated to be one of the hallmarks of cancer (16). Genetic alterations in ALL (Table 3) include structural or numerical chromosome changes such as translocations or hyperdiploidy (17), deletions and amplifications (15, 18).

Cell types involved	Chromosome abnormality	Molecular change	Frequency (%)	Functional product
Precursor B-cell	t(12;21)(p13;q22)	TEL-AML1 fusion	20-24	Chimeric transcription factors
	t(1;19)(q23;p13)	E2A-PBX1 fusion	5	Chimeric transcription factors
	11q23 translocations	MLL-AF4, MLL-ENL and other fusions	8	Modified transcription factor
	hyperdiploidy	Increased gene dosage	21-30	Unknown
	t(9;22)(q34;q11)	BCR-ABL1 fusion	3	Activated kinase
Precursor T-cell	1q deletion; t(1;14)(p32;q11)	SIL-SCL fusion	25	Dysregulated transcription factor (SCL/TAL1)
	Translocation involving TCR genes: 7q34 (TCRB and TCRG) 14q11 (TCRA and TCRD)	HOX, TAL, NOTCH,	35	Transcription factors
	t(10;11)(q21;p15)	CALM-AF10 fusion	10	Chimeric transcription factors

Table 3. Chromosomal aberrations in childhood ALL. The frequency of translocation is shown for specific ALL subtypes (B or T ALL).

It is thought that these genetic alterations contribute to the leukemia transformation by changing cellular functions like maintaining or enhancing the self-renewal capacity, blocking the control mechanism of normal proliferation, differentiation and apoptosis. Some genetic alterations affect only one of these pathways, whereas others act on more than one (2) (17, 18) . However, the detection of chromosomal translocations in healthy donors and experiments in transgenic mice indicate that a single chromosomal translocation is not enough to induce leukemia, depending on the translocation investigated. Therefore, Greaves postulated a two-step model for childhood leukemia with chromosomal translocations as initiating hit (17). Since the central question in this work is the presence of the fusion genes in MSC from precursor B-ALL patients, the contribution of the BCP-ALL fusion genes to leukemia transformation will be discussed in more detail.

Translocation-generated chimeric transcription factors

The transcription factors Runx-1 (AML1), MLL, Tel (ETV6) and E2A are required for HSC formation and function. They target the HOX-gene-mediated transcription cascade. The homeobox gene (HOX) superfamily encodes transcription factors that act as master regulators of development. They have the ability to activate or repress a diverse range of downstream target genes that regulate self-renewal, proliferation and differentiation. The AML1-CBFβ transcription factor complex (AML1) regulates either directly or indirectly the transcription of specific members of the HOX gene family. The MLL protein is required to maintain this transcription. HOX proteins collaborate with cofactors, including the PBX1 protein to induce the transcription of downstream target genes. The chimeric transcription factors, MLL fusion proteins, TEL-AML1 and E2A-PBX1, target also the HOX-gene-mediated transcription cascade (Figure 2). These three fusion genes modify the normal pattern of expression of members of the HOX gene family (18, 2).

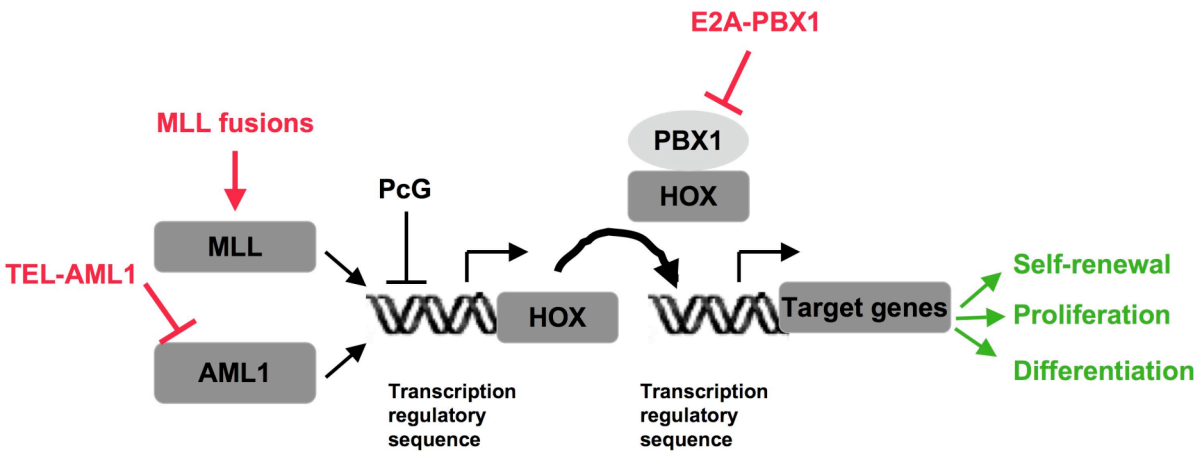


Figure 2: **The chimeric transcription factors TEL-AML1, E2A-PBX1 and MLL fusions**. MLL fusion proteins, TEL-AML1, and E2A-PBX1 target the HOX-mediated transcription cascade. The AML1 regulates the transcription of specific members of the *HOX* gene family. The MLL protein is required to maintain this transcription. HOX proteins cooperate with cofactors like PBX1 protein to induce the transcription of downstream target genes, which regulate self-renewal, proliferation, and differentiation of HSCs and their committed progenitors. The red arrows indicate the sites of action of three chimeric transcription factors. Partially adapted from reference 2.

TEL-AML1 fusion gene

The t(12;21) translocation that results in the *TEL-AML1* fusion gene is the most common genetic aberration in childhood ALL. The chimeric protein contains an HLH domain of TEL fused to nearly all parts of AML1 gene, including both the transactivation domain and the DNA- and protein-binding Runt homology domain. Transgenic TEL-AML1 expression did not induce leukemia either in vitro using human HSC, or in vivo using transgenic mice and zebrafish models, but increased the self–renewal of B-cell progenitors and the number of HSC (19-23). Twin studies and analysis of cord blood samples demonstrates that the childhood leukemia development is initiated in utero (24, 25). *TEL-AML1* is detectable in cord blood samples at a frequency approximately 100-fold in excess of the risk of leukemia, indicating that additional molecular hits are required for malignant transformation. Most of TEL-AML1^{+} leukemia cells show secondary events like *TEL* deletion, *AML1* amplification and/or abnormal numbers of chromosomes, supporting the observation that *TEL-AML1* fusion gene alone is not sufficient to induce leukemia.

E2A-PBX1 fusion gene

The *E2A-PBX1* fusion gene results from the t(1;19) translocation. The *E2A* gene on chromosome 19, fuses to the homeobox gene *PBX1*, on chromosome 1, leading to

the expression of different variants of hybrid E2A-PBX1 oncoproteins (26). Expression of the E2A-PBX1 fusion gene in the BM of mice results either in AML (27), or induces T-cell lymphomas in transgenic mice (28), demonstrating its oncogenic potential.

MLL 11q 23 rearrangements

The mixed-lineage leukemia (*MLL*) gene, which encodes a histone methyltransferase, can rearrange with more than 50 different fusion partners. MLL rearrangements are found in multiple-lineage leukemias, i.e. acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute biphenotypic leukemia (ABL). The leukemia-associated translocations involving the *MLL* gene on chromosome 11 band q23 result in chimeric proteins consisting of the N-terminal portion of *MLL* fused to the C-terminal portion of the partner gene. For example, the t(11;10), t(11;19) fuse *MLL* to *AF-9* and *ENL*, respectively. These two partners are small serine/proline-rich proteins with nuclear localization signals, suggesting that they may function as transcriptional transactivators (18). The MLL protein is a nuclear protein that maintains the expression of particular members of the HOX family. The MLL fusion proteins disrupt the normal pattern of expression of HOX genes, causing change in the self-renewal and growth of HSC and committed progenitors. Murine models of MLL-induced leukemia confirmed the oncogenic role of MLL fusion proteins (29, 30). ALL patients with *MLL* rearrangement have a poor prognosis.

BCR-ABL1 fusion gene

The reciprocal translocation t(9;22) gives rise to the *BCR-ABL1* fusion gene resulting from joining of 3´ sequence of the tyrosine kinase *ABL* proto-oncogene on chromosome 9 to the 5´ sequence of the *BCR* gene of chromosome 22. The BCR-ABL fusion proteins exhibit increased tyrosine kinase activity that alters signaling pathways that control the proliferation, survival and self-renewal capacity. The BCR-ABL1 fusion gene generates a benign or chronic myeloproliferative clonal expansion in both transgenic mice and adult patients (17).

1.1.3 Treatment of childhood ALL

Children with newly diagnosed and relapsed ALL are systematically included in clinical trial studies. ALL are diagnosed according to their specific characteristics, as mentioned above. In Germany virtually all children with ALL are treated using either

the ALL-BFM (Berlin-Frankfurt-Münster) /Co-ALL-07-03 study protocols for initial ALL (31, 32) or ALL-REZ-BFM study protocol for relapsed ALL (33-35). Based on biological and clinical risk factors, different stratification risk groups are defined (high S3/4, intermediary S2 and low S1). The type and intensity of therapy is dependent on these stratification risk groups. Treatment of ALL includes administration of several chemotherapeutic drug-combinations (polychemotherapy). These drug-combinations vary depending on the ALL study groups and treatment phases. ALL treatment consists of a remission-induction phase, an intensification (or consolidation) phase, and continuation therapy to eliminate residual disease (36, 3). The aim of remission-induction treatment is to induce complete remission and to restore normal hematopoiesis. The intensification treatment is used to eradicate drug-resistant residual leukemia cells. For reasons that currently remain unclear, ALL-patients require continuation treatment to prevent relapse. Allogeneic stem cell transplantation is usually indicated for patients who do not have respond to therapy or who are in high-risk stratification groups (36, 3). At diagnosis ALL patients usually have a total tumor burden of approximately 10^{12} to 10^{13} leukemia cells. The majority of patients show complete remission (CR) after about 4 weeks of chemotherapy. CR is defined as a status in which less than 5% of the cells in BM samples are morphologically blasts. At this time, up to 10^{10} malignant cells can still remain in the patient. These remaining cells are defined as the minimal residual disease (MRD) (37, 38). Several sensitive techniques are used to detect MRD, which allows a longer follow-up of the tumor burden during chemotherapy and subsequently more accurate stratification of therapy. The two principal methods for MRD detection in childhood ALL are the molecular analysis of leukemia-specific B- and T-cell receptor gene rearrangements or breakpoint fusion regions of chromosomal rearrangements using quantitative real-time PCR and the flow cytometric analysis of aberrant immunophenotypes (38). As an example, an overview of ALL-REZ-BFM-2002 treatment protocol is given in Figure 3.

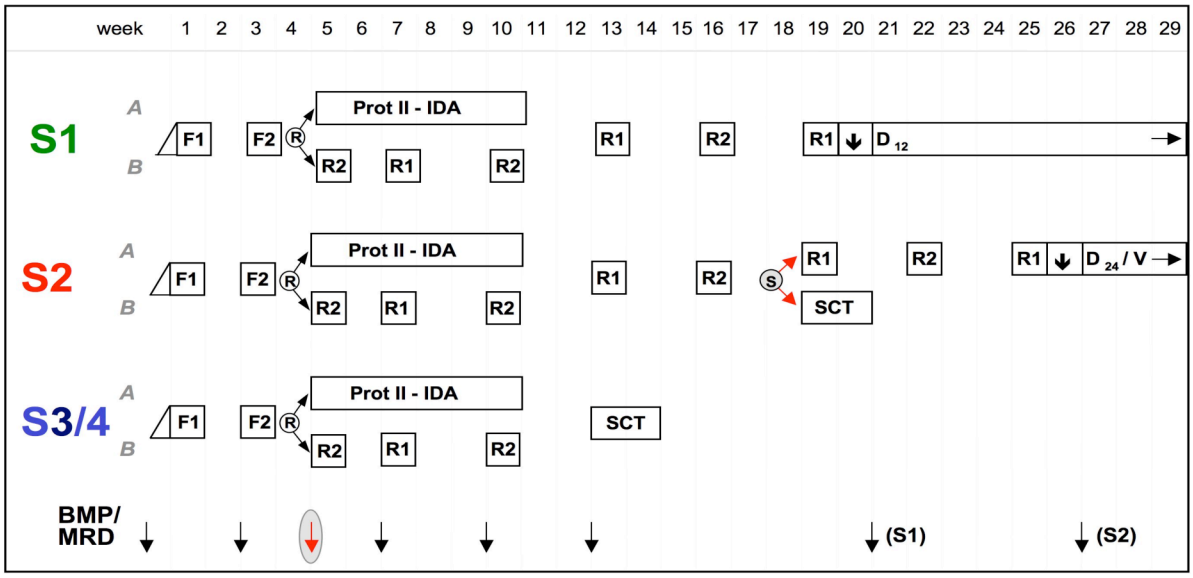


Figure 3.Therapy schedule of ALL-relapse study BFM 2002. D12/D24, 12/24 months maintenance therapy; ®, randomisation; (S), stratification; V, VP16-reinduction pulses;↓, local radiation; (↓), BMP-time-point for postremission-stratification in S2; SCT, stem-cell transplantation; BMP, bone marrow puncture; MRD, minimal residual disease; chemotherapy courses: F1, F2, R2, R1, protocol II-IDA. Kindly provided from Prof. Henze.

The factors, which are predictive for survival after relapse include immunophenotype, site of relapse, length of the first complete remission (CR1) and the response to induction therapy measured as MRD. Survival after BM-relapse is generally worse than after extramedullary relapse (CNS or testicular). In addition, prognosis after early relapse is much worse than after later relapse (4-6).

1.1.4 *IG gene* rearrangement

Lymphocytes can initiate specific immune responses against antigens by generating a nearly infinite diversity of antigen receptors. This is achieved in large part by a somatic recombination process known as variable, diversity, and joining V(D)J recombination. Through V(D)J recombination, the variable region of antigen receptor genes is assembled from component germline V, D, and J gene segments. There are seven different loci that are rearranged to generate the antigen receptors of T and B lymphocytes. These include the immunoglobulin (*Ig*) heavy chain locus (*IgH*) and the *Ig* light chain (*IgL*) loci (Igκ and Igλ), which encode the antigen receptor and secreted antibodies of B cells, and the T cell receptor (TCR) α, β, δ and γ loci. The rearrangement of these loci is tightly controlled in a lineage-, stage-, and allele-specific manner.

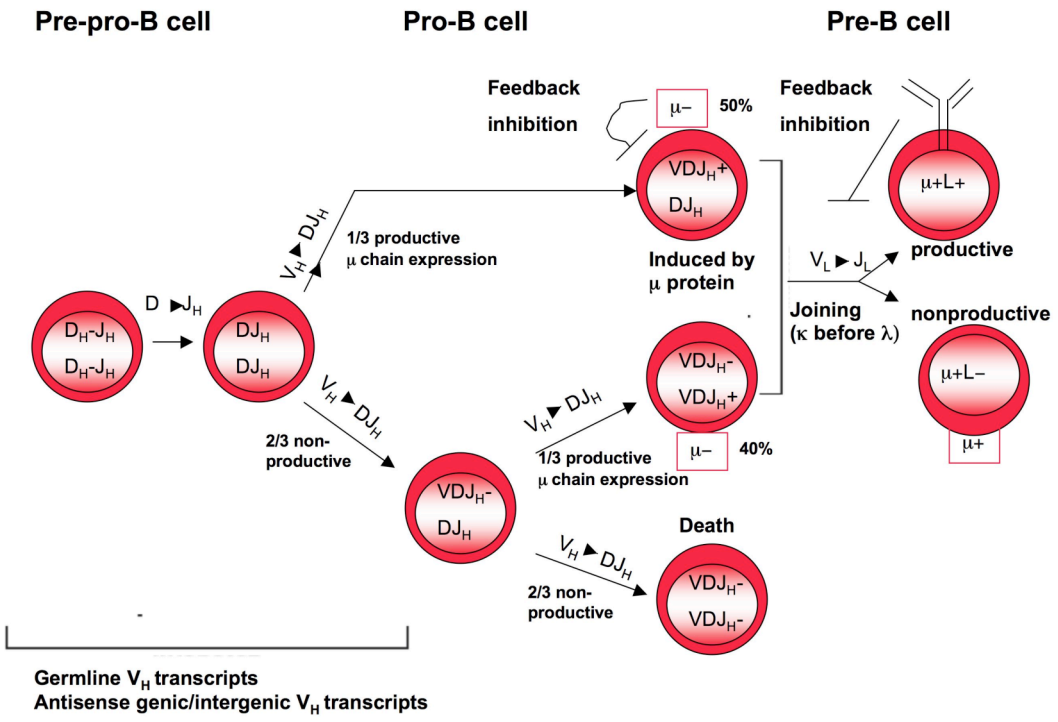


Figure 4. The regulated model of allelic exclusion. During B-cell development early B-cells similarly rearrange both of their IgH alleles before proceeding to rearrange their IgL genes. Ordered Ig gene rearrangement also extends to the order in which the gene segments of the IgH variable region are assembled. DH to JH joining occurs on both IgH alleles before VH to DJH joining on one allele. Furthermore, only one VHDJH rearrangement in each cell produced a functional μ chain. V(D)J recombination in bone marrow pro-B cells first assembles IgH V(D)J exons leading to μ chain expression. Subsequently, IgL VJ exons are assembled in pre-B cells, generating immature B cells that express μ plus IgL chains as surface IgM. The two types of IgL proteins (Igκ and Igλ) are encoded in distinct loci (Igκ and Igλ), and primary Igκ V(D)J recombination usually precedes that of Igλ. Individual B cells express either Igκ or Igλ. Partially adapted from reference 31.

During B cell maturation, sequential intrinsic genetic DNA sequences are rearranged in the heavy and light chain immunoglobulin loci (Figure 4) (39). Cells at the pro-B stage of development initiate immunoglobulin rearrangements, a process involving the assembly of V, D and J gene segments. Recombination activating gene 1/2 (RAG1/2) endonucleases initiate V(D)J recombination by cleaving V, D and J segments, which are joined exclusively by non-homologous end-joining NHEJ to form V(D)J exons. Assembly of the heavy chain locus (IgH) precedes that of the light chains loci (IgL) (39). In addition, the rearrangements of the IgH locus are sequential with D_H to J_H joining occurring on both alleles prior to V_H to D_HJ_H segment rearrangement (39). The productive assembly of the V_H-D_HJ_H variable gene regions indirectly induces differentiation to the next stage. In this next stage, IgL chains are assembled with Igκ rearrangements generally preceding those of Igλ (39), generating

immature B cells that express μ plus IgL chains as surface IgM. Surface IgM^+ B cells migrate to peripheral lymphoid tissues (for example, spleen) where they participate in antigen-dependent responses including class switch recombination. The random insertion and deletion of nucleotides at the junction sites of V, (D,) and J gene segments create fingerprint-like sequences of the junctional regions of *IG* and *TCR* genes. These sequences are specific for each lymphocyte and are therefore also specific for each malignant lymphoid clone. The junctional regions are actually used as leukemia-specific targets for analysis of MRD.

Furthermore, *TCRB* gene rearrangement can be detected in approximately 30-35% of precursor-B-ALL patients (Table 4), and seems to be dependent on immunophenotype, age and the presence of TEL-AML1 transcripts (40, 41). The types and/or characteristics of antigen receptor gene rearrangements in BCP-ALL vary with the patients' age, as well as with the genotype of the leukemia (42-46, 38, 47, 41, 48).

Gene	Type of rearrangement	Precursor-B-ALL (%)	T-ALL (%)
IGH	V_H -J_H	80-85	~5
	D_H -J_H	~20	~20
	Total IGH	>95	20–25
IGK	Vκ -Kde	45	0
	Intron-Kde	15-25	0
	Vκ -Jκ	30	-
	Total IGK-Kde	60-75	0
IGL	Vλ -Jλ	15-20	-
TCRB	V β -J β	25-30	80
	D β -J β	15	55
	Total TCRB	35	90
TCRG	Vγ -Jγ	50-60	95
TCRD	Vδ2-Dδ3 or Dδ2-Dδ3	40	5-10
	Vδ -Jδ1 or Dδ2-Jδ1	<1	50
	Total TCRD	40	55
TCRD/A	V δ 2-J α	40-45	-

Table 4. Frequency of *IG/TCR* gene rearrangements in childhood ALL. Abbreviations: *IGH*, immunoglobulin heavy chain; *IGK*; immunoglobulin kappa chain; *IGL:* immunoglobulin light chain; *TCRB*, T cell receptor beta chain; *TCRG*, T cell receptor gamma chain; *TCRD*, T cell receptor delta chain; *TCRD/A*, T cell receptor alpha/delta chain; V, variable; D, diversity; J, joining; H, heavy chain; κ, kappa chain; γ, gamma chain; α, alpha chain; β, beta chain; δ, delta chain; λ, lambda chain. Partially adapted from reference 41.

1.2 Hematopoiesis

1.2.1 Stem cells / hematopoietic stem cells

Stem cells are classified according to their developmental potential. The term “stem cell” describes cells with self-renewal and differentiation capacity to one or more functional lineages. The term *totipotent* is used to describe the ability of cells to form all lineages of the organism including extraembryonic tissues. In mammals only the zygote and early blastomeres are *totipotent*. Embryonic stem cells are *pluripotent* cells that can self-renew and differentiate into cells representing the three major germ layers: endoderm, mesoderm or ectoderm as well as any of the cell types of the body. Adult stem cells like hematopoietic stem cells are *multipotent* with the ability to differentiate into multiple cell types of one lineage. *Unipotent* stem cells like spermatogonial stem cells can form only one cell type (sperm). The traditional stem cell model is based on hierarchy of stem cell subtypes. According to this point of view, stem cells undergo a series of commitment steps that irreversibly generate in sequence pluripotent, multipotent, unipotent, and differentiated progeny. Furthermore, in the adult rare tissue-resident stem cells can generate only the mature cell types corresponding to their tissue of origin (49-51). In 1961, Till and MacCullough (52) discovered a cell population of clonogenic BM cells with capacity to generate myelo-erythroid colonies in the spleen of lethally-irradiated hosts. These cells were proposed to be hematopoietic stem cells (HSC), i.e. progenitor cells with the characteristics of self-renewal and differentiation capacity for all types of blood cells. HSC reside as rare cells in the BM of adult mammals at the top of the hematopoietic hierarchy and have the ability to differentiate to several progenitors (Figure 5). These progenitors generate blood precursors with the capacity to unilineage differentiation and production of mature blood cells, including erythrocytes, megakaryocytes, myeloid cell (monocyte/macrophage and neutrophil), and lymphocytes (53-55). Vertebrate hematopoiesis is thought to occur in two successive steps, primitive and definitive, those differ in cell types produced and anatomic location. In mammals, hematopoiesis (primitive) occurs first in the yolk sac (YS) blood islands, later at the aorta-gonad mesonephros (AGM) region, in which a hemangioblast differentiates to red blood cells and endothelial cells and/or hemogenic endothelial cells differentiate to HSC and endothelial cells. Subsequently definitive hematopoiesis takes place in fetal liver, thymus and ultimately BM. Hematopoiesis in each location favors the production of a specific blood lineage (56).

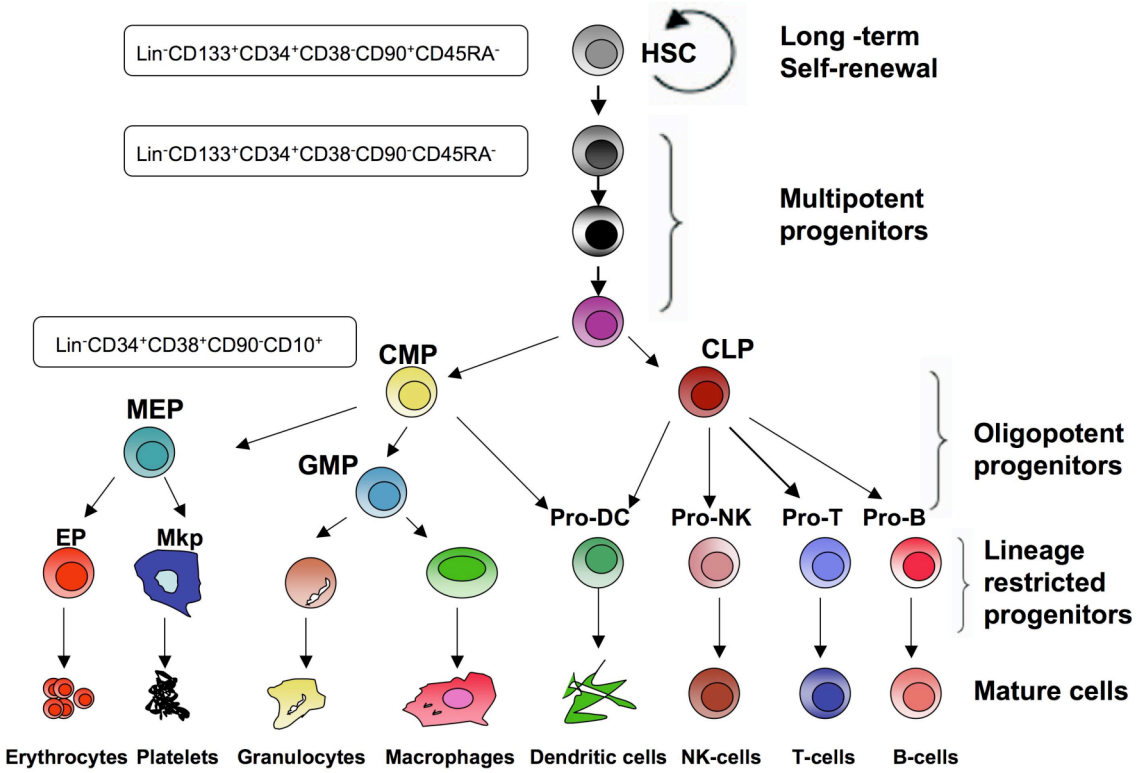


Figure 5. Scheme of hematopoietic development showing intermediates in the hierarchy of hematopoietic differentiation. Surface markers used for isolation are indicated at left for each stem and progenitor cell. HSC indicates long-term reconstituting, self-renewing cells; MPP, multipotent progenitors with limited self-renewal leading to transient but multilineage reconstitution; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; pro-B, B lymphocyte protenitor; Pro-T, T-cell progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythroid progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor. Partially adapted from reference 6.

As intrinsic determination of cellular phenotype, different transcription factors regulate how HSC develop during embryogenesis and how lineage-restricted differentiation is programmed. The transcription factors mostly required for HSC formation and function are Runx-1 (AML1), ML*L*, TEL (ETV6), SCL/tal-1 LMO-2 and GATA-2. The transcription factors mostly required for lineage-restricted differentiation especially for lymphocytes are Ikaros, PU.1, Notch, E2A and PAX-5 (56).

1.2.2 Bone marrow HSC niches

Regulation of HSC self-renewal and differentiation is thought to depend on their microenvironment, also called bone marrow stem-cell niche (57). The concept of a stem cell ´niche´ was first proposed in 1978 (58). Normal hematopoiesis requires a complex and reciprocal interaction between the BM microenvironment and HSC. The niche keeps the stem cells in a dynamic balance between self-renewal and

differentiation. Furthermore, stem cells are dependent on their microenvironment to maintain their ability of asymmetric or symmetric cell division. The BM stem cell niche is composed of specialized cells like osteoblasts, responsible for osteogenesis (bone growth) and controlling HSC numbers and stroma cells including sinusoidal endothelial cells, supporting proliferation, differentiation, and transendothelial migration of HSC. B-cells are generated from HSC and develop in BM (Figure 6). Afterwards mature B-cells migrate into the blood to reach peripheral lymphoid tissues. The development of B-cell precursors through various stages (Figure 4) requires a coordinated interaction between BM stroma cells producing different factors in BM niches, as well as the expression of specific transcription factors, such as Ikaros, E2A, and PAX5. In 1982, Whitlock and Witte described the growth of B-cell precursor on BM-derived stromal cells *in vitro*. For B-cell development several microenvironmental components have been identified, such as CXC-chemokine ligand 12 (CXCL12), FLT3 ligand, interleukin 7 (IL-7) and stem-cell factor (SCF). Different subtypes of BM stroma cells produce these factors (59, 60).

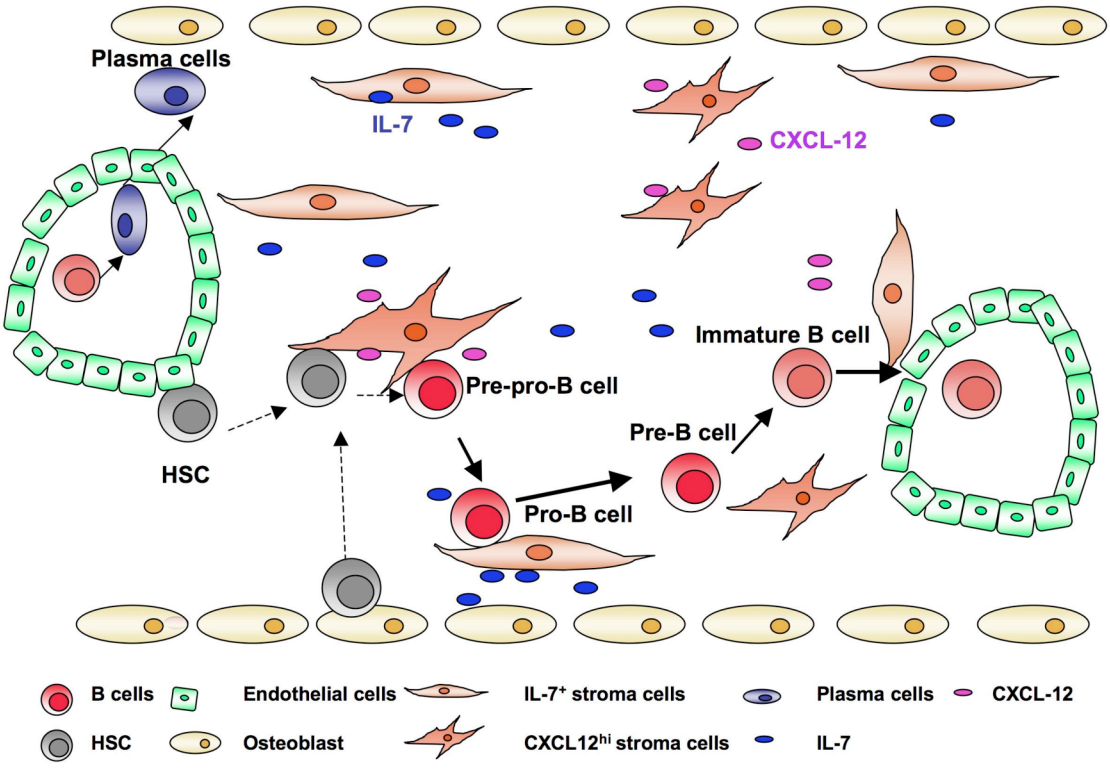


Figure 6. A model of cellular niches for B-cell development and movement in the bone marrow
In this model the intermediate precursor cells between HSC — which are located near the osteoblasts, endothelial cells or CXC-chemokine ligand 12^{hi} (CXCL12^{hi}) reticular cells — and pre-pro-B cells would move towards CXCL12^{hi} reticular cells. Pre-pro-B cells associate with CXCL12^{hi} reticular cells, whereas pro-B cells move to IL-7-expressing cells like MSC and stroma cells. Subsequently, pre-B cells leave IL-7-expressing cells. B cells expressing cell-surface IgM exit the bone marrow and

enter the blood to reach the spleen, where they mature into peripheral mature B cells. End-stage B cells (plasma cells) again locate to CXCL12^{hi} reticular cells in the bone marrow. This Figure is modified from reference 51.

1.2.3 MSC as origin of the HSC BM-niche

1.2.3.1 Characterization of MSC

The BM contains not only hematopoietic stem cells but also a second type of progenitor cells, mesenchymal stem cells (MSC). In 1974, Friedenstein described the isolation of spindle-shaped, clonogenic cells in monolayer culture. These fibroblastoid cells formed colonies when seeded at low density demonstrating their self-renewal capacity (colony-forming unit fibroblast). These CFU-F derived cells can be used as feeder layer for the culture of HSC (61). BM-fibroblastoid cells are able to differentiate into multiple mesenchymal lineages, including bone, cartilage, muscle, fat tissue and BM stroma cell (62, 63). Because of their self-renewal and differentiation capacity, they are called mesenchymal stem cells (MSC). MSC are a heterogeneous population of cells that have fibroblastoid morphology, form CFU-F in vitro, proliferate as plastic-adherent cells and show differentiation capacity. MSC do not express the hematopoietic markers CD45, CD34, whereas they express variable levels of CD105 (endoglin), CD73 (ecto-5´-nucleotidase), CD44, CD90, CD29 (β1 integrin) and STRO-1 (stromal antigen 1). As depicted in Figure 7, MSC have the ability to differentiate into cells of the mesodernal lineage, like bone, fat and cartilage cells but they also have endodermic (64) and neurodermic differentiation potential (65, 62). The plasticity of MSC to differentiate into cells of the hematopoietic lineage has been demonstrated in mice (66). It is postulated that this differentiation capacity is due to the developmental origin of mesenchymal tissues, which includes the mesoderm, and, in a lower proportion, the cranial neural crest. Adult MSC are commonly considered to be of mesodermal origin (67). The exact embryonic origin of MSC still remains unknown. However, it has recently been shown, that embryonic MSC derived from the neuroepithelium and neural crest (68). It has been suggested that in post-natal life the relative importance of MSC derived from other developmental lineages decreases due to the increasing importance of mesodermal MSC (69). Because of the differentiation capacity, it has been proposed that MSC could be used for the regeneration of different tissues.

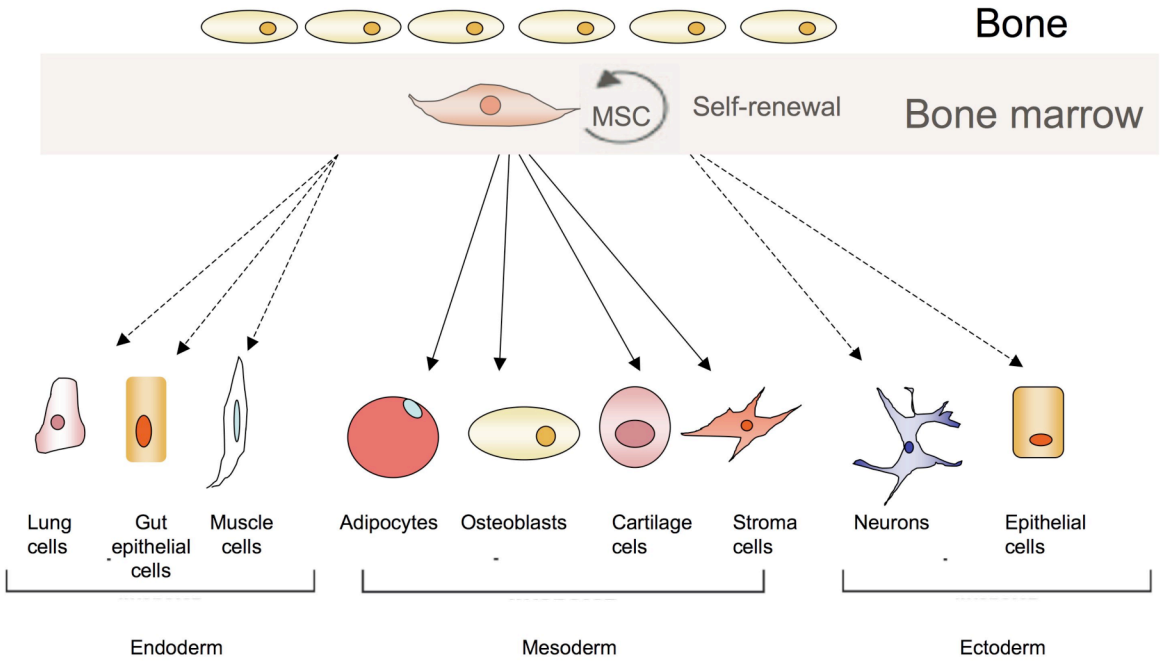


Figure 7. The multipotentiality of MSC. Mesenchymal stem cells (MSC) have the ability to self-renew (curved arrow) and to differentiate (straight, solid arrows) towards the mesodermal lineage to adipocyte, osteoblasts, cartilage cells and stroma cells in the bone-marrow. The reported ability to transdifferentiate into cells of other lineages (ectoderm and endoderm) is shown by dashed arrows, as transdifferentiation is controversial *in vivo*. This Figure is modified from reference 60.

1.2.3.2 Role of MSC in the physiology of BM-cells

MSC have been shown to differentiate into pericytes, myofibroblasts, BM stromal cells, osteocytes, osteoblasts and endothelial cells, after transplantation into the BM of non-obese diabetic-severe combined immunodefiency (NOD-SCID) mice (70, 69). All these cells form the functional components of the HSC niche that support hematopoiesis. Furthermore, stromal-cell progenitors and progenies can retain the HSC pool in BM by maintaining HSC in a resting state (71, 72). It has also been reported, that MSC facilitated the engraftment of transplanted HSC (73). Therefore, it is likely that MSC have the ability to form HSC niches in the bone marrow.

1.2.3.3 Interaction of MSC and immune cells

Immune responses can be subdivided into innate and adaptive responses. The innate response is immediate and non-antigen-specific. In contrast, the adaptive response is slower but antigen-specific. Immunomodulatory roles of MSC have previously been suggested (74, 75). Concerning the modulation of innate immunity, MSC have been shown to inhibit the maturation of monocytes, $CD34^+$ and

hematopoieic progenitor cells into dendritic cells (DC) in vitro (76-79). Mature DC incubated with MSC, have decreased cell-surface expression of MHC class II, CD11c and CD83, reducing the antigen-presenting function of the DCs (76, 78, 79). For adaptive immunity, it has been observed, that BM-derived MSC suppresse T-cell proliferation (80-87). After TCR engagement, T-cells proliferate and acquire several effector functions, including cytokine expression and cytotoxity in the case of $CD8^+$ T cells. It has been shown that MSC inhibit T-cell proliferation, but they do not promote T-cell apoptosis. Furthermore, the MSC-mediated anti-proliferative effect on T-cells is associated with the survival of T cells in a resting state (80, 81, 86).

1.2.3.4 Clinical applications of MSC

In several clinical trials, MSC have been used for regeneration of damaged tissue and for treatment of inflammation resulting from cardiovascular disease and myocardial infarction, brain and spinal cord injury, Crohn´s Disease, and graft-versus-host disease (GVHD) during BM transplantation (88). MSC have demonstrated significant potential for clinical use due to their suitable isolation, their lack of relevant immunogenicity allowing allogenic transplantation without immunosuppressive drugs, and their potential to differentiate into several tissue-specific cell types, to promote vascularization, and to show immunosuppressive effects (89).

1.3 Aberrant hematopoiesis

1.3.1 Leukemia stem cells theory

At the end of 19^{th} century, experimental pathologists (Virchow, Maximow) recognized that many tumors (solid or liquid) exhibit morphologic heterogeneity. Rudolf Virchow and Julius Cohnheim postulated that cancer results from resting embryonic tissue. Furthermore, in 1937, Furth and Kahn showed that a single leukemia cell could initiate a cancer graft (90). During the next several decades, different transplantation assays were done, showing a wide variation in the proportions of cells that initiate tumor growth. More recently, Pierce et al. showed that malignant teratocarcinoma cells could differentiate into mature benign cells (91). In 1961, Pierce and Speers suggested that tumors are altered pictures of normal development (91). Later, Park et al. showed that although the tumors arise from single cells, the tumor building cells are heterogeneous. Further studies demonstrated that most leukemia cells are unable to proliferate extensively in vivo using xenograft models and only a small

subset of cells (~1/10^6) was clonogenic (92, 93). This observation suggests that cancer stem cells or cancer initiating cells are distinct from the bulk of tumor and are able to propagate and maintain tumor growth (94). Furthermore, a previous study has shown that leukemia cells at different maturational stages were able to reconstitute and reestablish the complete leukemia phenotype in a xenograft model (95).

There are at least two different main theories about cancer stem cells. The first theory proposes that a small population of immature cancer stem cells maintains the malignancies. In the second one it has been postulated that the majority of malignant cells have stem cell characteristics like self-renewal and differentiation capacity.

On the other hand, because the tumor surrounding microenvironment and the immune system play a significant role in cancer progression, it was suggested, that the subpopulation of leukemia cells that appeared non-tumorigenic in xenograft model might actually be tumorigenic in the presence of the appropriate tumor stroma and immune system. Strasser and colleagues demonstrated that a very high frequency (~1 in 10) of lymphoma and leukemia cell of mouse origin induce tumor growth when transplanted into histocompatible mice (96). Recently, a study demonstrated the increased numbers of tumorigenic human melanoma cells (~1 in 4) using more immunocompromised NOD/SCID interleukin-2 receptor gamma chain deficient mice (97), indicating that the frequency of tumorigenic cells or cancer stem cells vary depending on tumor type and the specific experimental system used.

1.3.2 Origin of leukemia cells

For leukemia the initial cell type in which the transforming mutations develop still remains unknown. Many studies have tried to answer the question at which stage leukemia originates within the hematopoietic hierarchy. These studies have focused on the detection of leukemia-associated genetic alterations in different cell types. Leukemia-associated genetic alterations have been detected in hematopoietic stem cells (HSC). For example, the chromosomal translocations t(9;22) and t(4;11) were detected in $CD34^+CD19^-$ cells (98). It has been reported that different clones from the same patient show the *TEL-AML1* fusion gene but have independent B-cell receptor rearrangements, demonstrating that the initial hit may be propagated in cells not yet committed to the B-cell lineage (99, 100). Furthermore, *TEL-AML1* has been detected in $CD133^+$ cells, showing a primitive stem cell population as leukemia-initiating cells (101). In a recently reported TEL-AML1 mouse model it has been demonstrated that mice were predisposed to hematologic malignancies after

treatment with chemical carcinogens. This was only the case when TEL-AML1 was expressed in HSC but not when it was expressed in early lymphoid progenitors (22). These results suggest that TEL-AML1 is required to be expressed in HSC for promoting the development of malignancy. Because normal stem cells and leukemia stem cells (LSC) share the ability to self-renewal, it has been postulated that LSC are HSC that have become malignant as the result of accumulated genetic aberrations. Interestingly, “HSC transcription factors” like *MLL*, *TEL, AML1*, *SCL/tal1* and *LMO-2* are the genes which are mainly involved in leukemia-associated translocations (56). Moreover, it has been stated by Hong et al. that the understanding of leukemia pathogenesis requires an extended knowledge not only of the specific genetic mutations implicated, but also of the cellular framework, in which they arise and develop (25).

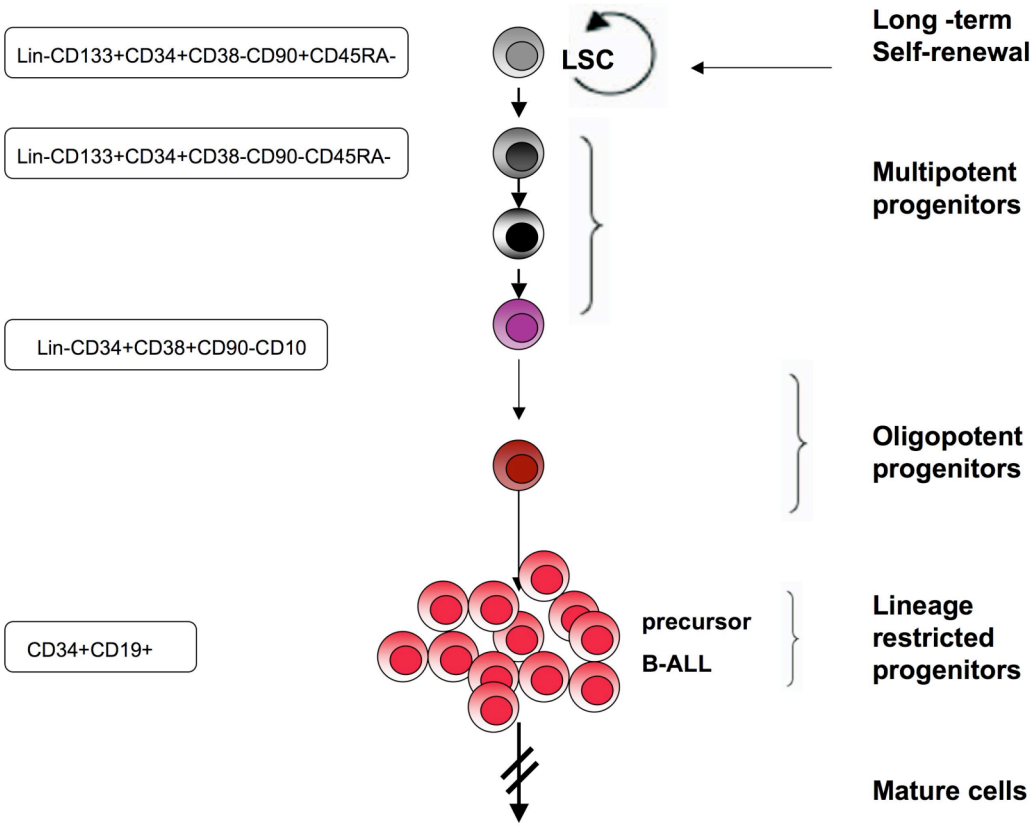


Figure 8. Origin of leukemia cells. Leukemia can be considered as a newly formed abnormal hematopoietic tissue initiated by a few LSC that undergo an aberrant and regulated process of organogenesis analogously to that of normal HSC. LSC can either be HSC, which have become leukemic as the result of accumulated genetic mutations, or more restricted progenitors, which have reacquired the stem cell abilities like self-renewal. This Figure is modified from reference 60.

1.3.3 Leukemia cells and MSC interaction

1.3.3.1 Tumor and MSC / stroma interaction

In the last two decades it has become clear that the interaction of tumor and surrounding stroma play a significant role in tumor development and progression, even though the “seed and soil” hypothesis from Page was already postulated in 1889. In this hypothesis he compared tumor cells with the seed of plants, in that they are both “carried in all directions; but they can only live and grow if they fall on appropriated soil”. Various studies have demonstrated that cancer cells modify their stroma to form a supportive environment for their development and progression by producing a wide range of different factors. Previous studies have shown that tumor-associated fibroblasts, as well as MSC play a significant role in the growth of epithelial tumors like breast cancer (102). The origin of tumor-associated fibroblasts is still controversially discussed. Furthermore, BM transplantation experiments in a gastric cancer mouse model indicated that the subset of gastric fibroblasts were from BM origin. Recent reports proposed that the BM-derived MSC have the ability to migrate to the stroma of developing tumors (103, 102, 104, 105). MSC have been shown to promote tumor growth by reciprocal interaction with tumors (102) and by their immunosuppressive character (106).

1.3.3. Leukemia cell niche

There is diverse evidence supporting the importance of the BM microenvironment in leukemia cell growth and survival, via reciprocal interactions between leukemia cells and cells of the bone marrow microenvironment such as stroma, osteoblasts and endothelium (107-109). Leukemia cells grow and accumulate in close contact to MSC. Direct cell-to-cell contact with BM-MSC is essential for the long-term survival and expansion of leukemia cells in vitro (110-116). Furthermore, BM-MSC and/or BM-stroma cells regulate survival and response of ALL cells during chemotherapy (117-121). Previous studies demonstrated that leukemia cells interact with the vascular endothelium in a similar manner as their benign lymphocyte counterparts. Disruption of the interaction between chemokine stromal-cell-derived factor 1 (SDF-1) and its receptor CXCR4 inhibits the homing of a variety of ALL cell lines (109). The growth and survival of B-cell precursor leukemia cells seems to require the presence of the same factors in BM niches as immature normal B cells. Furthermore, recent data suggest that leukemia cells are able to create abnormal bone marrow niches

and that this tumor microenvironment causes HSC dysfunction (Figure 9) (122, 123).

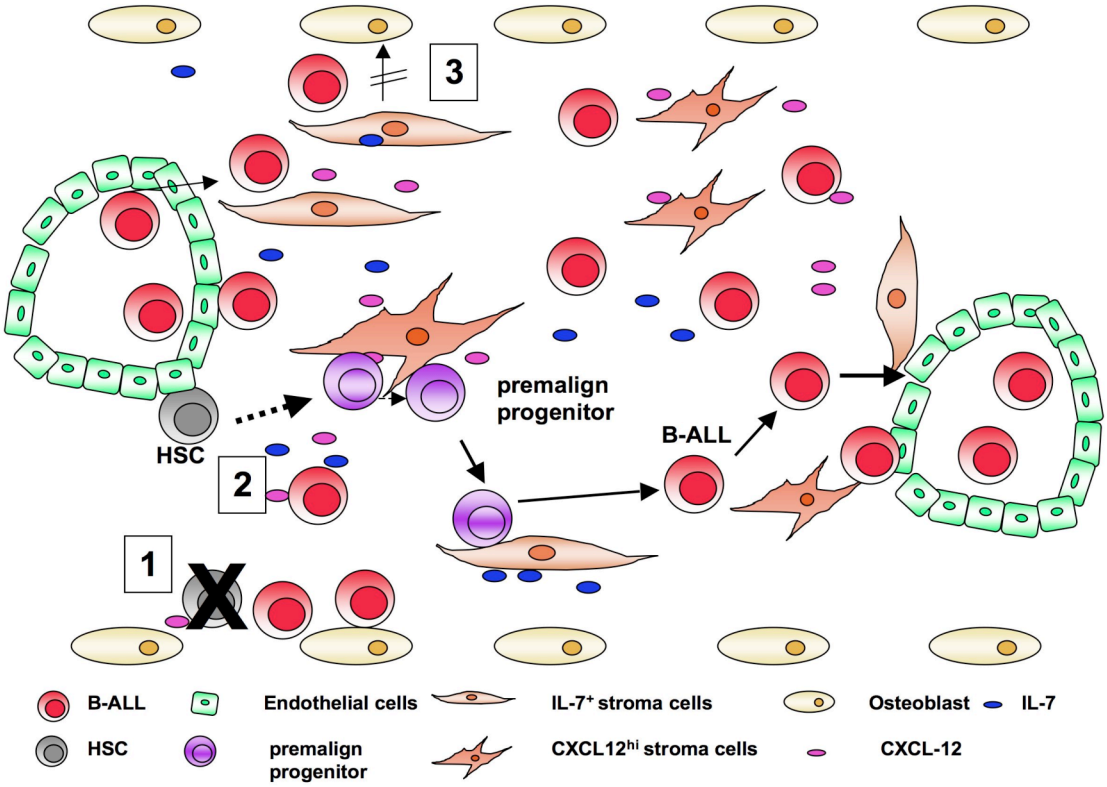


Figure 9. A model of cellular niches for B-ALL development and movement in the bone marrow. In this model, B-ALL cells change the BM microenvironment 1) by competition with HSC for space, displacing the normal hematopoiesis, 2) by competition with HSC for growth factors produced by the BM-niche building cells, like osteoblasts, endothelial cells or stroma cells, and 3) by regulation of the MSC proliferation and differentiation. The inhibition of MSC differentiation through Dkk-1 produced by myeloma cells is associated with the development of osteolytic lesions.

A further study has shown the importance of the microenvironment by injection of MLL-AF9 transduced $CD34^+$ cord blood cells into three types of non-obese immunodeficient mice a) NOD/SCID, b) NOD/SCOD/β2 microglobin null ($\beta2^{-/-}$), and c) NOD/SCID mice that overexpressed the human cytokines SCF, GM-SCF and IL-3 (SGM3). When cells were injected into NOD/SCID or NOD/SCOD/$\beta2^{-/-}$ mice, a B-ALL and/or a biphenotype leukemia resulted, but these cells caused uniformly AML when injected into NOD/SCID/SGM3 mice, showing that the lineage fate is probably determined by the host microenvironment (124). Homing to microenvironment also appears crucial in leukemia cell survival, since up-regulation of the adhesion molecule α4β1 integrin (VLA-4) ligand for VCAM-1 on stroma cells support leukemia cells survival and growth (125). Recently, a randomized clinical trial study has confirmed that cotransplantation of donor origin MSC and HSC results in both

reduced incidence of GVHD and an increased relapse rate, probably due to the immunosuppressive effect (less GVL) or as a consequence of their “niche function” (126).

1.3.3.3 Genetic alterations in tumor-associated stroma cells

As Mishra et al. have recently suggested, although the importance of cross-talk between tumor cells and stroma cells has been increasingly recognized, the question whether the stroma cells themselves harbor tumor-associated or tumor-promoting alterations is just beginning to be addressed (127). Furthermore, recent studies support the capability of tumor microenvironment to be the exclusive mechanism for cancer development. They demonstrated that alterations in the HSC niches lead to the development of myeloproliferative disease (128, 129). Mutations in TP53 tumor suppressor gene have also been described in stromal fibroblast of breast and prostate cancer in humans and animals (130-132). These somatic TP53 mutations are reported to be associated with regional lymph-node metastases in sporadic breast cancer (133). Furthermore, Leukemia-associated genetic aberrations have been reported for endothelial cells in patients with B-cell lymphoma (134) or chronic myelogenous leukemia (CML) (135). Nevertheless, the presence of tumor-associated alterations in stroma cells is still controversially discussed.

1.4 Plasticity of stem cells

In the concept of stem cell plasticity, it is suggested that under certain conditions various tissue-specific stem cells may have the ability to transdifferentiate to a cell of a completely distinct lineage. This suggested that the developmental specification of a stem cell is dependent on the general potential of the cell (pre-determined stem cell fate) as well as on microenvironmental effect, such as stimuli from growth factors (stem cell niche). Currently, different methods have been reported to induce pluripotenty in murine and human adult somatic cells. In mouse models, dedifferentiation of somatic cells was induced using nuclear transfer, fusion and ectopic expression of defined factors. In 2006, Takahashi and Yamanaka demonstrated that ectopic expression of transcription factors *Oct4*, *Klf4*, *Sox2* and *c-Myc* could reprogram murine somatic cells into induced pluripotent stem cells (iPS) (136). Furthermore, iPS cells were generated from pro- and pre-B cells by transduction with the reprogramming factors Oct4, Sox2, c-Myc and Klf4 and from mature B-cells by the additional specific knockdown of the Pax5 transcription factor

(137, 138). It has also been reported that long-term culture of BM cells can generate chimera-competent pluripotent stem cells. It is yet not clear how prolonged in vitro culture can induce transdifferentiation or pluripotency. The in vivo transdifferentiation of cells derived from BM, brain, or skin to cells of different lineage is still controversial (49).

1.5 Aim and outline of the thesis

Childhood acute lymphoblastic leukemia (ALL) is caused by malignant immature lymphocytes. Even though childhood ALL can be cured in a large number of patients, around 20% of the patients suffer a relapse after chemotherapy. The origin of the relapse is unclear at the present time. As described in the introduction, for leukemia the initial cell type in which the transforming mutations develop still remains unknown. Many studies have tried to answer the question at which stage leukemia originates, most of them by focusing on cells from the hematopoietic lineage. Moreover, while there are many studies, which have focused on stroma cells from solid tumors, the role of mesenchymal stroma cells in hematological malignancies is less well understood. Given the high plasticity of many cell types, we asked whether the tumor cells or tumor stem cells are pluripotent as well. Therefore, we searched for leukemia-associated genetic aberrations and *IG* gene rearrangements in MSC from childhood B cell precursor (BCP) ALL patients.

1. Therefore, MSC were isolated from bone marrow aspirates of 49 childhood ALL patients at different time points (diagnosis, during/after treatment and after stem cell transplantation) and from 3 healthy donors.
2. In the framework of the ALL relapse study group, data about cytogenetic analyses of leukemia cells for the presence of leukemia-associated chromosomal translocations and the sequence of leukemia-specific *IG* gene rearrangements were available. Leukemia cells from 10 of 49 ALL patients showed one of the three translocations, namely *TEL-AML1*, *E2A-PBX1* or *MLL* rearrangements. MSC from these 10 ALL patients were analyzed for the presence of leukemia-associated genetic aberrations using fluorescence in situ hybridization (FISH) in interphase and metaphase, genomic DNA sequencing, quantitative real-time PCR and Western blot analysis.
3. MSC were further analyzed for the presence of leukemia-specific *IG* gene rearrangements.
4. To exclude positive signals from contaminating leukemia cells in the MSC cultures, MSC from 3 ALL patients were transduced with the Simian Virus 40 Large T-antigen (SV40-Tag) and further characterized.

2 Materials and Methods

2.1 Patients

MSC cultures were established from bone marrow aspirates in 49 out of 60 cases. In 11 cases, either we did not observe adherent stroma cells, or the cells died after a short culture period. Leukemia cells (LC) from 10 of these 49 patients showed one of the three translocations (*TEL-AML1*, *E2A-PBX1* and *MLL* rearrangement) and were included in this study. 9 patients were enrolled in the relapse trial ALL-REZ of the BFM (Berlin-Frankfurt-Muenster) study group, and 2 with initial ALL in the frontline trial ALL-BFM (one patient was included in both trials). The clinical characteristics of the 10 patients with childhood ALL are shown in Table 5 and the pretreatment and time points when samples were taken are shown in Appendix Table 1. Informed consent was obtained from all patients and/or their guardians. This study was approved by the institutional ethics committees of the participating institutions.

2.2 Isolation and characterization of MSC

In order to isolate MSC, 0.5 to 2 ml bone marrow (BM) aspirates were cultured in complete culture medium (CCM) consisting of Iscove basal medium, 10% fetal bovine serum (FBS), 10% horse serum (Biochrom, Berlin, Germany), and 100 µg/ml primocin (Amaxa, Cologne, Germany). After 24 hours incubation and Ficoll gradient cell purification the cells were resuspended in CCM with 10^{-6} µM hydrocortisone at cell concentrations of 2-5x10^5 cells/ml and maintained at 32° C for 1-3 weeks and at 37°C for the following 1-12 weeks. Non-adherent cells were removed by weekly medium change and washing with phosphate buffered saline (PBS). When the cells had grown to 70-90% confluency, they were harvested with accutase solution (PAA Laboratories GmbH, Pasching, Austria). From all BM samples analyzed, MSC were harvested and characterized after the 2nd and 3rd passage (2-6 weeks). The cells were characterized by flow-cytometry with monoclonal antibodies (mAb) against CD3, CD4, CD8, CD11b, CD19, CD20, CD22 CD29, CD33, CD34, CD44, CD45, CD73 (SH-3), CD90, CD105, CD166, and IgG isotype controls using a FACScalibur cytometer and CellQuest software (Becton Dickinson, San Diego, CA, USA). After cytospin centrifugation or after growing cells in chamber slides, the cells were also stained with mAb against human CD19, prolyl-4-hydroxylase beta (PH), Stro-1, E2A-PBX1, SSEA-4, CD105 and polyclonal Abs against fibronectin and SSX1-9. For detection of primary antibodies, Alexa488, Alexa594 (Invitrogen) and Amca labeled secondary antibodies were used, prior to fluorescence microscope analysis (Axiovert

200, Zeiss, Jena, Germany). In control stainings the primary antibody was omitted or isotype Abs were used. MSC were further characterized by low-density fibroblast colony-forming unit assay (CFU-F). The multipotency of MSC was assessed by their capacity to differentiate into adipocytes and osteoblasts as previously described (139). Only cultured fibroblastoid cells that were negative for CD3, CD45, CD19, CD34, CD31 and CD11b (> 98%) and positive for CD29, CD166, CD73, CD90, CD105, SSEA-4, Stro-1, Fibronectin, Prolyl-4 hydroxylase, as well as positive in the CFU-F assay, and showing differentiation to adipocytes and osteoblasts, were classified as MSC. All chemicals used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Calbiochem Merck (Darmstadt, Germany). Antibodies either for Western blot, FACS, immunocytochemistry or FICTION were purchased from Becton Dickinson (San Diego, CA, USA), Acris (Hiddenhausen, Germany) or Invitrogen (Karlsruhe, Germany).

2.3 CFU-F assays

MSC were plated at 15 cells per cm^2 in tissue culture dishes. After incubation for 10-14 days at 37°C in 5 % CO_2 the cells were washed with PBS and stained with 0.5 % Crystal Violet (pH 4.5) in methanol for 5-10 min at room temperature. Cells were washed with PBS twice and colonies were counted.

2.4 Differentiation of MSC

For osteogenic differentiation MSC were incubated in MSC-medium supplemented with 10^{-8} M dexamethasone, 0.5 µM ascorbic acid, and 20 mM betaglycerol-phosphate. Deposition of mineral was assessed after fixation using cold 70% ethanol solution followed by Alizarin red staining, incubation with 40 mM Alizarin red (pH 4.1) for 10 min (Alfa Aesar GmbH, Karlsruhe, Germany). For adipogenic differentiation MSCs were incubated in MSC-medium supplemented with 5 µg/ml insulin, 10^{-6} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 60 mM indomethacin. Cells were fixed, using 10% fomaldehyde and stained with fresh oil-red solution (Amresco; Solon, OH, USA).

2.5 Immunostaining

MSC (chamber slides/cytospins) were fixed in cold acetone/methanol for 3 minutes and washed with PBS. To block unspecific antibody binding, the slides were first incubated with protein blocking agent (Immunotech, Marseille, France) for 15 min

and then with 1 % of normal donkey or goat serum in PBS for 30 min. For the staining of intracellular proteins, sections were incubated with primary antibodies in PBS/0.2% gelatine for 60 min at RT or overnight at 4°C. For the staining of cell surface molecules, sections were incubated with primary antibodies in PBS/1% BSA for 60 min at RT. After washing with PBS, secondary antibodies were added for 60 min at RT. As negative controls, samples were incubated with isotype-matched control antibodies or secondary antibodies only. After staining with DAPI, sections were covered with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and examined using an Axiovert 200 microscope with AxioVision Release 4.5 software (Zeiss, Jena, Germany).

2.6 Metaphase analysis

Cells in the logarithmic phase of growth were exposed to 0.1 µM colcemid (Roche Diagnostics; Mannheim, Germany) for 2 h at 37°C, and harvested. Subsequently, the cell pellets were treated dropwise with 0.4% KCl, and incubated for 20-30 min at 37°C. After hypotonic treatment, cells were fixed in methanol/acetic acid (3:1), by dropwise adding the fixative. This step was repeated 3 times. After final centrifugation, the cell pellet was resuspended in 0.1-0.2 ml of final fixative. 10-20 µl of cell suspension were dropped on to ice-cold slides and the slides were then air-dried.

2.7 Fluorescence in situ hybridization

MSC were analyzed using fluorescence in situ hybridizations (FISH) with LSI dual-color translocation gene probes (*TEL-AML1*; Vysis, Downers Grove, IL, USA) or dual-color break-apart rearrangement probes [*MLL*, *ETV6* (*TEL*); Vysis, *E2A* (*TCF3*), and DakoCytomation, Glostrup, Denmark] and using the whole chromosome paint (WCP) technique with specific gene probes for chromosome X, Y, 1, 11, 19, 21 (MetaSystems) or with chromosome enumeration probes (CEP) for chromosome X, Y, 10, 11, 12 (Vysis) and chromosome 21 (LSI probe, Vysis) according to the instructions of the provider. FISH analyses for the fusion genes were performed on cytospin or chamber slides. Ice-cold methanol/acetic acid (3:1 v/v) was used to fix the cells and the slides were air-dried. The hybridizations included different steps, as follows. Briefly, after a 2 min pepsin treatment (0.005% in 0.01M hydrochloric acid [pH 2.3], 37° C), slides were washed once in 2 x SSC (standard saline citrate: 20 x SSC, 3.0 M NaCl, 0.3 M sodium-citrate; pH 7.0) for 10 min, and PBS for 5 min,

respectively. After fixation in 3% formaldehyde for 5 min and two further washes with PBS, slides were dehydrated in ethanol series including 70%–100% ethanol steps. Air-dried slides and DNA probes were co-denatured for 10 min at 75°C and further hybridized overnight at 37°C (E2A-beak apart probes: at 45°C) using the HYBRrite denaturation/ hybridization system for FISH (Vysis). For post-hybridization washes, slides were incubated in 0.4 x SSC/ 0.3% NP-40 for 15 min and after 2 further washing steps with 2 x SSC/ 0.1% NP-40 were stained with DAPI. Afterwards, the slides were mounted in anti-fade solution (Vectashield) and further evaluated by using a fluorescence microscope (Axiovert 200). From each ALL sample, a total of 100-300 nuclei in interphase were analyzed. For patient 10 and each transduced clone, 8-20 metaphases were analyzed. The cut-off for the FISH results was determined with MSC from healthy donors (*MLL-rearrangements*: 4%; *TEL-AML*: 5-6%; *E2A-PBX1*: 5%). Hybridizations were also performed with MSC from 2 ALL patients without known fusion gene in the blasts, whose samples were taken during chemotherapy. Additionally, "criss cross" controls were performed for which MSC positive for one fusion gene were hybridized with FISH probes for an unrelated fusion gene. In both cases, false positive signals were in the same range as observed for MSC from healthy donors (never more than 5-6 % positive signals).

2.8 Multi-color FICTION

Further analyses were performed using FICTION procedure (combined FISH and immunocytochemistry) (140). After immunostainings as described above, slides were fixed in paraformaldehyde solution (1%) and Carnoy's fixative solution (methanol / acetic acid, 3:1), respectively. Thereafter, slides were dehydrated and air-dried. 10 µl FISH-probe were added to the slides, which were further covered with coverslips. Both probe and target DNA were incubated at 75°C for 10 minutes and hybridized overnight at 37°C. After hybridization, slides were washed first in 0.1 x SSC buffer at 60°C and then in 2xSSC buffer at RT. Slides were mounted in anti-fade solution (Vectashield) and evaluated by using a fluorescence microscope (Axiovert 200) with AxioVision Release 4.5 software (Zeiss, Jena, Germany).

2.9 Cell lines

The human B-cell precursor (BCP)-ALL cell lines REH (*TEL-AML1* positive) and 697 (*E2A-PBX1* positive) were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The BCP-ALL cell line Z-181 (*BCR-*

ABL1) (141) and the stromal cell line L87/4 (142) were kindly provided by Z. Estrov (Anderson Cancer Center, Houston, TX, USA) and K. Thalmeier (University of Munich, Germany), respectively.

2.10 Transduction of MSC with the SV40 Large T-antigen

The MSC were transduced with the SV40 large T-antigen (SV40-Tag), which has been shown to extend the life span of the transduced cells. Retroviral supernatants were generated by transient calcium phosphate cotransfection with the retroviral vector plasmid LoxP-HyTK-LT together with the expression plasmids encoding the Moloney MLV gag/pol genes (pcDNA3.1MLVg/p) and the MLV-10A1 env gene (pALF-10A1) in 293T cells. Two days after transfection, virus supernatants were harvested, filtrated (0.45 µm pore size) and MSC were transduced using standard methods (143). Transduced cells were selected with 10-20 µg/ml hygromycin-B (Calbiochem, Merck; Darmstadt, Germany) and diluted at 1 cell per well in 96-well plates. In one case one clone was subcloned again at 0.3-0.5 cells per well.

2.11 Immunoglobulin gene rearrangements

DNA was extracted using the Puregene DNA-Isolation kit (Biozym Diagnostic GmbH; Oldendorf, Germany). In DNA from bone marrow samples of ALL patients taken at initial or relapse diagnosis immunoglobulin heavy and light chains (*IgGH/IGK*) gene rearrangements were identified using screening PCR reactions (144-147). Clonality was assessed by homo- and hetero-duplex analysis (148). The PCR products, for which monoclonality had been confirmed, were subsequently sequenced using Big Dye Terminator reaction mix and capillary electrophoresis (Applied Biosystems). Quantification of patient specific junctional sequences in MSC samples was performed using real time quantitative PCR with combinations of a germline TaqMan-probe, a germline primer and an allele specific primer on a real time PCR machine (Light Cycler, Roche Diagnostics) (149, 150). DNA samples from 100,000 cells (approx. 660 ng DNA) were analyzed in triplicate by PCR. The DNA isolated from MNC in initial diagnosis or relapse sample was diluted in a mixture of DNA isolated from MNC of 10 healthy volunteers (in 10-fold steps from 100% to 0.001%), and used for quantification of the proportion of cells with leukemia-specific *IG* gene rearrangements. The undiluted DNA of 10 healthy volunteers was used as negative control in quintuplicate PCR reactions. When the proportion of positive cells in the tested MSC cultures was lower than 3%, the corresponding cultures were considered

as negative in this analysis. In the analyzed MSC of some patients, 300 bp regions of the corresponding *IG* gene rearrangements were further sequenced, in order to confirm the sequences previously identified in the corresponding leukemia blasts.

2.12 Sequencing of the *MLL-ENL* breakpoint in MSC

The genomic breakpoint sequence of the fusion gene MLL-ENL was identified in the blasts from patient 8 in order to design specific PCR-primers and a TaqMan gene probe (forward primer: *tacacatttacctgtagatacacatgt*, reverse primer: *tagctgagcatggtggtatgagand* and TaqMan probe: *tttgagaacaagttgcagacaaaaaaaaga*) for the corresponding breakpoint sequence (151). Positive MSC were detected by quantitative real-time PCR and confirmed by DNA-sequence analysis of genomic DNA from blasts and MSC.

2.13 Western blot analysis

MSC were lysed using 1% IGEPAL in NP-40 lysis-buffer (Sigma-Aldrich). Protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad, Hercules, CA USA). Equal protein amounts were separated by 8% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Buckinghamshire, United Kingdom). After blocking with 5% nonfat dry milk in PBS, the membranes were treated with monoclonal antibody mouse anti-human E2A-PBX1 (Becton Dickinson), followed by horseradish peroxidase (HRP)–linked secondary antibodies (Cell Signaling Technology, Danvers, USA). Blots were subsequently treated with anti–beta-actin Abs (Sigma-Aldrich) as control for equal loading. The bands were visualized by chemiluminescence phototope-HRP kit (Amersham) according to the manufacturer's instructions.

3 RESULTS

In order to study the involvement of mesenchymal stem cells (MSC) in childhood B cell precursor (BCP) ALL disease, the presence of leukemia-associated genetic aberrations and immunoglobulin (*IG*) gene rearrangement were analyzed in MSC from BCP-ALL patients.

3.1 Characterization of MSC from childhood ALL patients

Knowledge about MSC is mostly based on the characterization of cultured cells, because of the difficulty in defining MSC other than by the *in-vitro* self-renewal and the differentiation potential (62, 68). Since no specific marker for sorting of MSC is known at the present time, it was necessary to isolate MSC in vitro through prolonged cell culture. As shown previously, prolonged culture of human BM MSC does not induce genetic alterations (152). MSC cultures were established from bone marrow aspirates in 49 out of 60 children with ALL. In 11 cases, either adherent stroma cells were not observed, or the cells died after a short culture periods. Isolated MSC were phenotypically analyzed during a culture period of 4-16 weeks, as described previously (62, 63). The resultant fibroblastoid adherent cells were multipotent, as shown by the low density CFU-F assay (Figure 10A) and their capacity to differentiate to adipocytes and osteoblasts (Figure 10B).

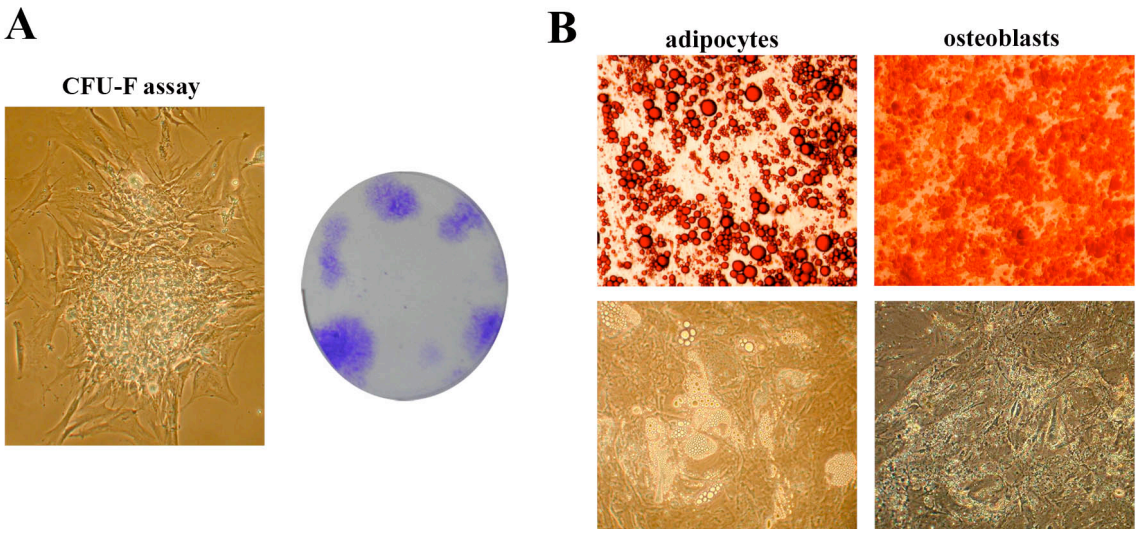


Figure 10. Self-renewal and differentiation capacity of MSC. (A) MSC from patient 5 after CFU-F assays without staining (left) and stained with Crystal Violet (right). **(B)** MSC from patient 5 after differentiation to adipocytes (top: oil-red staining; bottom: without staining) and to osteoblasts (top: alizarin-red; bottom: without staining).

The fibroblastoid cells were negative for hematopoietic- (e.g. CD34, CD45), lymphoid- (e.g. CD19, CD3), myeloid markers (e.g. CD11b) and positive for stroma lineages markers (e.g. CD166, CD73 and CD90) in flow-cytometric analysis (Figure11).

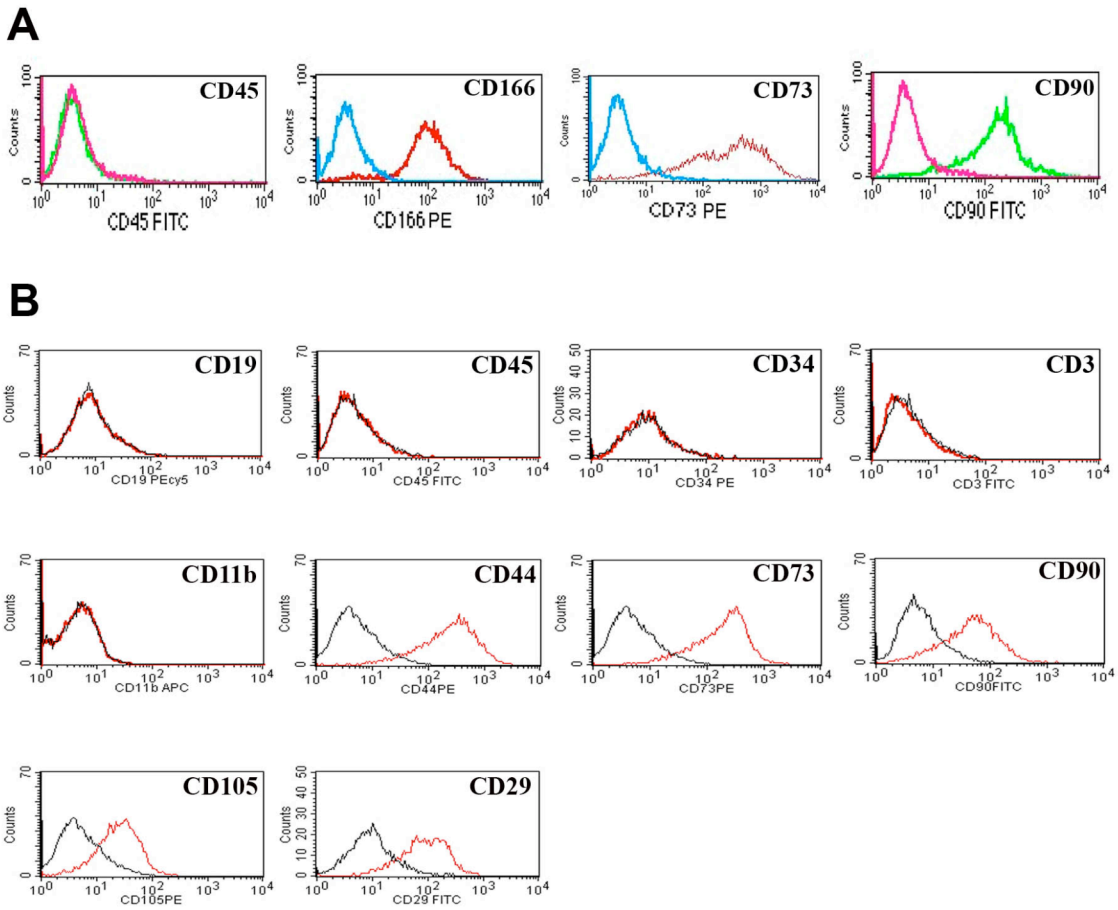


Figure 11. Immunophenotype analysis of MSC using FACS. (A) Flow cytometric analysis of MSC from patient 7, with mAb against CD45-FITC (green), CD166-PE (red), CD73-PE (red), CD90-FITC (green) and isotype controls as indicated. **(B)** FACS-analysis of MSC isolated from patient 10 stained with mAbs against CD19, CD45, CD34, CD3, CD11b, CD44, CD73, CD90, CD105, CD29 (red) and isotype controls (black). **(A-B)** All stainings were performed in parallel; therefore isotype controls for the same mAbs isotypes are shown repeatedly.

In immunocytochemistry, the fibroblastoid cells specifically stained with a mAb against SSEA-4 (stage-specific embryonic antigen 4) (Figure 12), but human primary fibroblast, the stroma cell line L87/4 and the leukemia cell line Z181 did not show SSEA-4 staining.

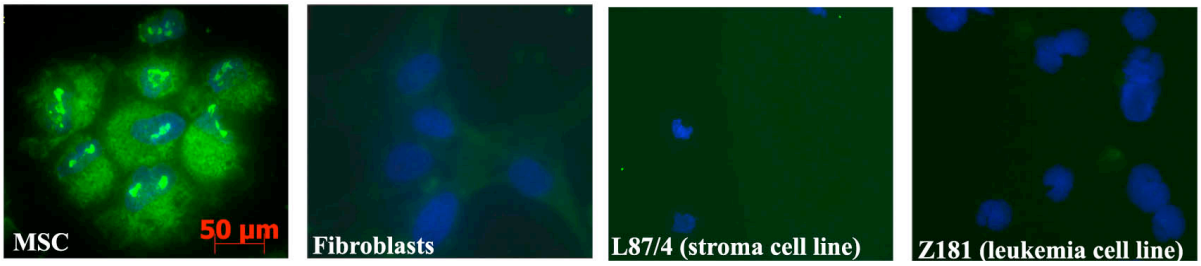


Figure 12. Immunocytochemical analysis of MSC. Immunocytochemical analysis with mAb against SSEA-4 (green) and nuclear staining with DAPI (blue). Results obtained with MSC from patient 7 (left), with control fibroblasts (middle left), with the stroma cell line L87/4 (middle right), and with the leukemia cell line Z181 (right).

The isolated fibroblastoid cells were positive for prolyl-4-hydroxylase (PH), whereas the leukemia cell line 697 showed no staining. Control stainings were performed with isotype control, revealing no background staining (Figure 13).

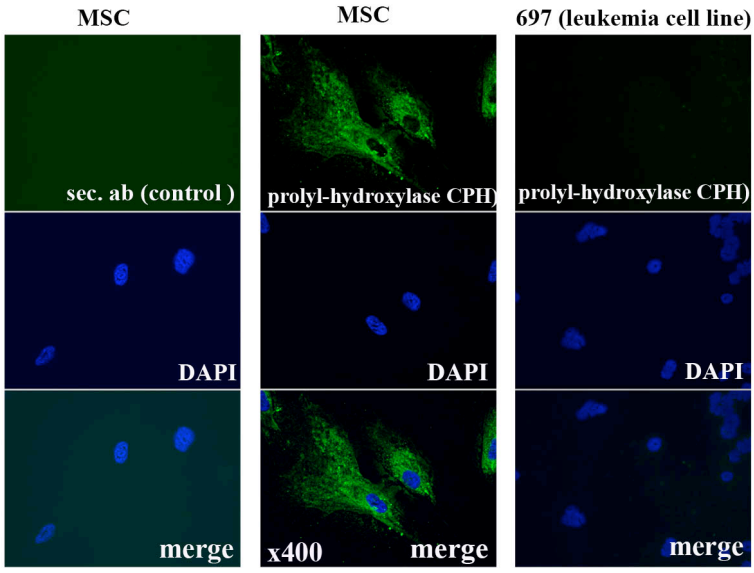


Figure 13. Immunocytochemical staining of MSC and leukemia cell line with prolyl-hydrxylase. MSC from patient 10 after immunocytochemical staining with mAbs against prolyl-hydroxylase (middle) and control staining without primary antibodies (left) both counterstained with DAPI (blue) at a 400x magnification. Leukemia cell line 697 (right) after immunocytochemical staining with mAbs against prolyl-hydroxylase, showed no staining.

Furthermore, the isolated fibroblastoid cells were positive for the markers STRO-1, PH, CD105, fibronectin, and SSX 1-9 but not for CD34 (Figure 14).

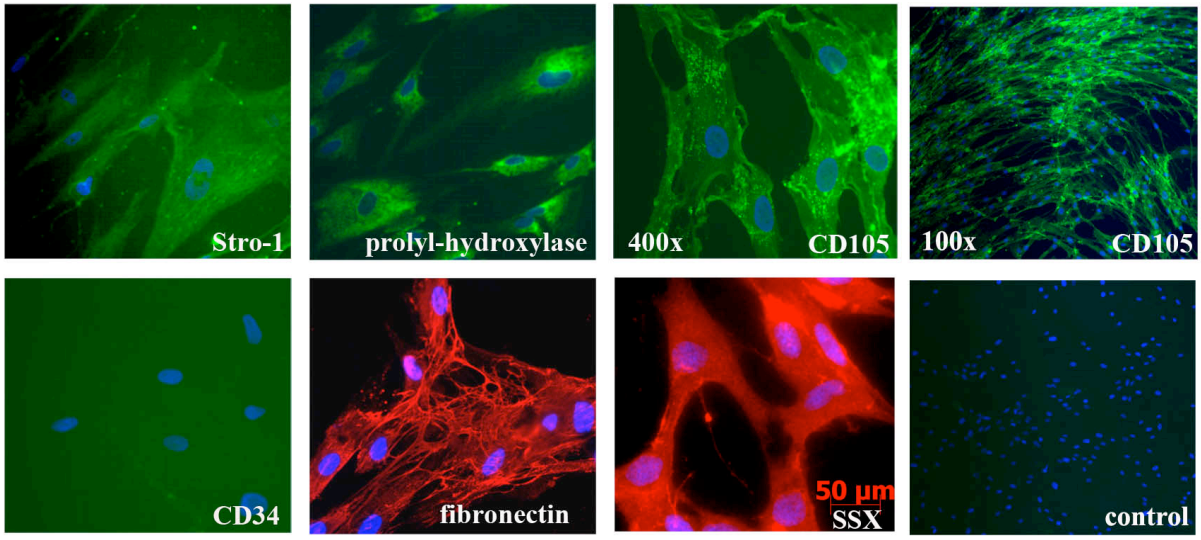


Figure 14. Immunocytochemical analysis of MSC. (A) MSC from patient 7 after immunocytochemical staining with mAbs against STRO-1, prolyl-hydroxylase (PH), CD105, CD34 and with antibodies against fibronectin and SSX 1-9, MSC were counterstained with DAPI (blue, magnification 400x). **(B)** MSC from patient 10 after immunocytochemical staining with mAbs against CD105 (top, magnification 100x), and control staining without primary antibodies (bottom).

Only cultured fibroblastoid cells that were negative for hematopoietic and myeloid lineage markers and positive for MSC and stroma markers, as well as positive in the CFU-F assay, and showing differentiation capacity were classified as MSC. MSC were isolated from BM samples at diagnosis, relapse and in most cases from remission time points, all of them showing purity higher than 99%, analyzed by FACS. MSC from BM aspirates of 3 healthy donors were used as controls, being phenotypically similar to MSC from the considered ALL patients.

3.2 Leukemia-associated genetic alterations in MSC

The most common chromosomal translocations in childhood ALL are *TEL-AML1* [t(12;21)], *E2A-PBX1* [t(1;19)] and 11q23 rearrangements involving the *MLL* gene (153). Therefore, we investigated their presence in MSC isolated from ALL patients. Leukemia cells (LC) from 10 of these 49 patients showed one of the three translocations, namely *TEL-AML1*, *E2A-PBX1* or *MLL* rearrangement, as detected in the current ALL-BMF study, by using FISH and real time-PCR. These 10 ALL-patients were included in this study. 9 patients were enrolled in the relapse trial ALL-REZ-BFM study group, and 2 with initial ALL in the frontline trial ALL-BFM (one patient was included in both trials). Detailed information concerning the clinical

characteristics of the patients and the remission are shown in Table 5 and in Appendix Table 1, respectively.

#	Disease stage at 1st analysis of MSC	Immuno-phenotype	Age at diagnosis [years, month]	Sex	Fusion gene[b]	Clinical status
1	1st relapse	c-ALL	11.6	m	*TEL-AML1*	CCR
2	1st relapse	preB-ALL	8.2	m	*TEL-AML1*	CCR
3	1st relapse	preB-ALL	3.8	f	*TEL-AML1*	loss of follow-up
4	Initial	preB-ALL	3.5	f	*TEL-AML1*	secondary AML[c] t(10;11)/ death
5	Initial 1st relapse	c-ALL c-ALL	5.0 10.1	f	*TEL-AML1* *TEL-AML1*	1st relapse CCR
6	1st relapse	c-ALL	10.2	f	*E2A-PBX1*	CCR
7	1st relapse	preB-ALL	3.6	m	*E2A-PBX1*	non-response/ death
8	5th relapse	proB-ALL	21.2	m	*MLL-ENL*	death
9	2nd relapse	proB-ALL	1.6	m	*MLL-ENL*	subsequent relapse/ death
10	1st relapse	c-ALL	10.3	m	*MLL-21.q22*	2nd relapse after SCT/ death
11*	initial	c-ALL	15.5	m	-	death
12*	1st relapse	c-ALL	10.2	m	-	SCT/CCR

Table 5: Clinical characteristics in ALL patients. Remarks: [a]age at diagnosis when patient was included in the study; [b]Fusion genes detected in a standard screening procedure of ALL at diagnosis: *TEL-AML1*, *E2A-PBX1*, *MLL*-rearrangements; [c] secondary AML diagnosed with t(10;11) translocation in LC. Abbreviations: #, patient number. ALL, acute lymphoblastic leukemia. AML, acute myeloid leukemia. m, male. f, female. CCR, continuous complete remission. SCT, stem cell transplantation, * control group without fusion gene.

3.3 *TEL-AML1* fusion gene

3.3.1 Frequency of *TEL-AML1* fusion gene in MSC of children with ALL

Five patients with *TEL-AML1* [t(12;21)] were analyzed, and the data from one of them (patient 5) are presented as an example in Figure 15. In this patient, the fusion gene *TEL-AML1* was detected in leukemia cells (89-91%, Table 6, Figure 15) as well as in MSC (11-33%, Table 6).

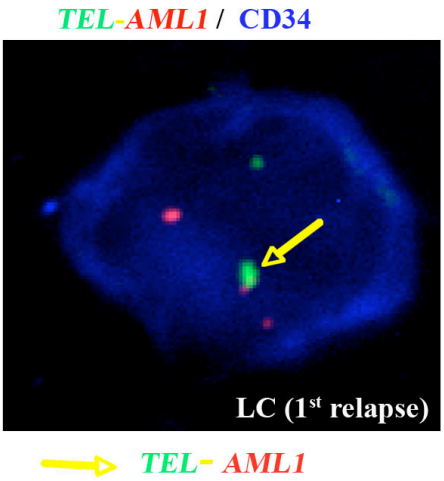


Figure 15. FICTION analysis of BM LC from patient 5 with *TEL-AML1* at the relapse diagnosis. LC were hybridized with gene probes specific for *TEL* (green) and for *AML1* (orange), and stained with mAb against CD34 (blue). The LC shows a yellow (green/red) signal indicating *TEL-AML1* fusion gene (yellow arrow), one green signal for *TEL*, and two orange signals for native *AML1* and residual *AML1*.

The detection of the *TEL-AML1* fusion gene in MSC was independent of the sample time points, e.g. initial diagnosis (Figure 16A), relapse (Figure 16B) or control time points taken during chemotherapy or remission (Table 6).

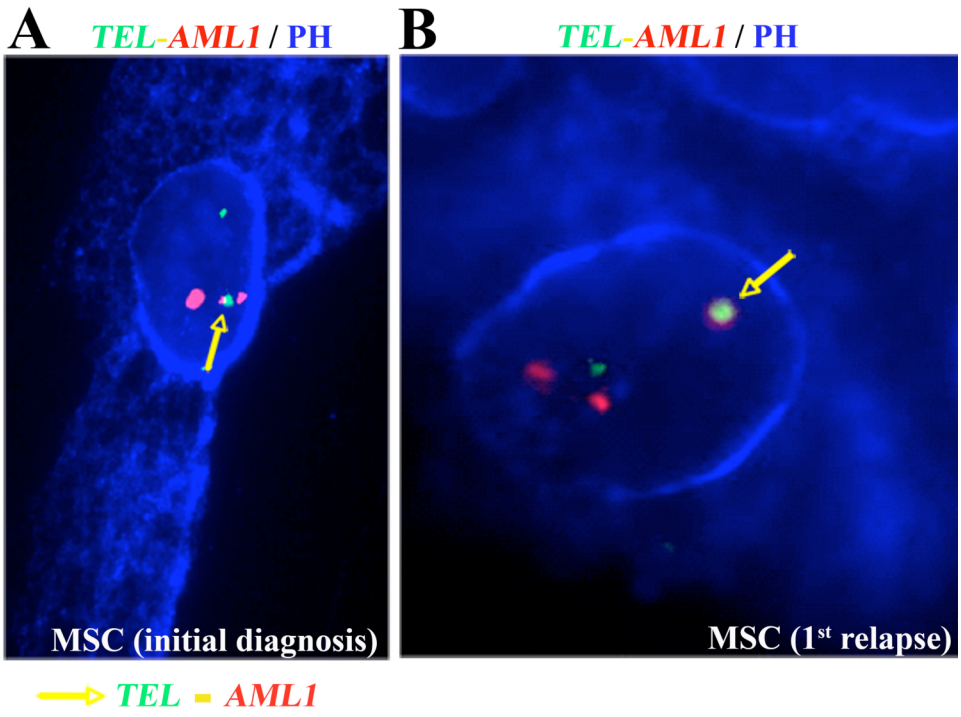


Figure 16. FICTION analysis of MSC from patient 5 with *TEL-AML1* fusion gene. (A) FICTION analysis of MSC (chamber slide) from patient 5 at initial diagnosis. MSC were hybridized with gene probes specific for *TEL* (green) and for *AML1* (orange), and stained with mAb against PH (blue). The MSC shows a yellow (green/orange) signal indicating *TEL-AML1* fusion gene (yellow arrow), one green signal for *TEL*, and two orange signals for native *AML1* and residual *AML1*, revealing a similar pattern as found in the LC (Figure 15). (B) FICTION analysis of MSC (cytospin) from 1st relapse with the *TEL-AML1* gene probes described for (A), combined with mAb against PH (blue), revealing a similar pattern of TEL-AML fusion gene as found in the LC and MSC from initial diagnosis (Figure 15).

FISH analysis combined with immunostaining against PH revealed the fibroblastoid character of *TEL-AML1* positive cells (Figure 16). Control hybridizations were performed with MSC from 2 healthy donors, as well as with MSC from 2 ALL patients without *TEL-AML1* fusion gene in LC, whose samples were taken during chemotherapy. The rates of false positive results were lower than 5% for healthy donors and also for the ALL patients without the fusion gene.

Similar to patient 5, in patients 1-4, the fusion gene *TEL-AML1* was detected in LC (82-90%), and in MSC (11-33%), independently of the corresponding sample time points (Table 6).

Patient	Time point of sampling		LC content in BM [%]	Fusion gene frequency [%]	
	Sample	Weeks after start of treatment		MNC	MSC
TEL-AML1					
1	1st rel	-	83	90	33
	rem 1	10	0		27
	rem 2	13	0		18
2	1st rel	-	95	90	20[a]
	rem 1	17	0		32
	rem 2	27	0		26
3	1st rel	-	98	92	21
	rem 1	2	0		14
	rem 2	17	0		25
4	diag	-	98	82	12
	sec AML	-	99	5[#]	15
5	diag	-	91	90	11[b]
	rem	5	0		20[b]
	1st rel [c]	-	89	90	33[b]
	rem	3	0		12[b]

Table 6. **Frequency of the *TEL-AML1* fusion gene in MSC of children with ALL.** [#] The cut-off for the FISH results (6%) was determined with MSC from healthy donors and 2 ALL patients without known fusion gene in the LC (*TEL-AML*: 5-6%). Remarks: [a] Only 50-60 cells were available for counting, [b] abnormal numbers of chromosome was detected in a range between 27-35% in all samples (see Table 7), [c] SV40-Tag clones were established (see Table 9). Abbreviations: BM, bone marrow; MNC, mononuclear cells from BM; MSC, mesenchymal stem cells from BM; LC, leukemia cells; SCT, stem cell transplantation; diag, initial diagnosis; rel; relapse; rem, sample taken during treatment and/or in remission (see Appendix Table 1).

3.3.2 Detection of additional aberrations in MSC from patient 5

Additional aberrations were observed in MSC from patient 5, even when the cells were negative for the *TEL-AML1* fusion gene. MSC at 1st relapse analyzed with

FISH showed abnormal numbers of signals for *TEL* and *AML1* genes with no detectable *TEL-AML1* fusion gene signal (Figure 17).

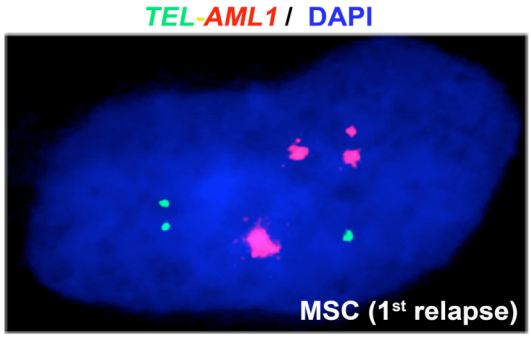


Figure 17. MSC from patient 5 at 1st relapse analyzed by FISH as described in Figure 15. MSC showed abnormal signals of *TEL* and *AML1* genes with no detectable *TEL-AML1* fusion gene signals (4 orange *AML1* signals and 3 green *TEL* signals, counterstained with DAPI).

In order to analyze whether the number of FISH signals described above corresponds with the number of involved chromosomes, MSC were hybridized with centromeric gene probes for chromosome 10, 11, 12, X and a probe for chromosome 21 (Table 7). In contrast to MSC from healthy donors (Figure 18C), abnormal numbers of chromosome were detected in MSC at initial diagnosis (Figure 18A) and in MSC at 1st relapse (Figure 18B).

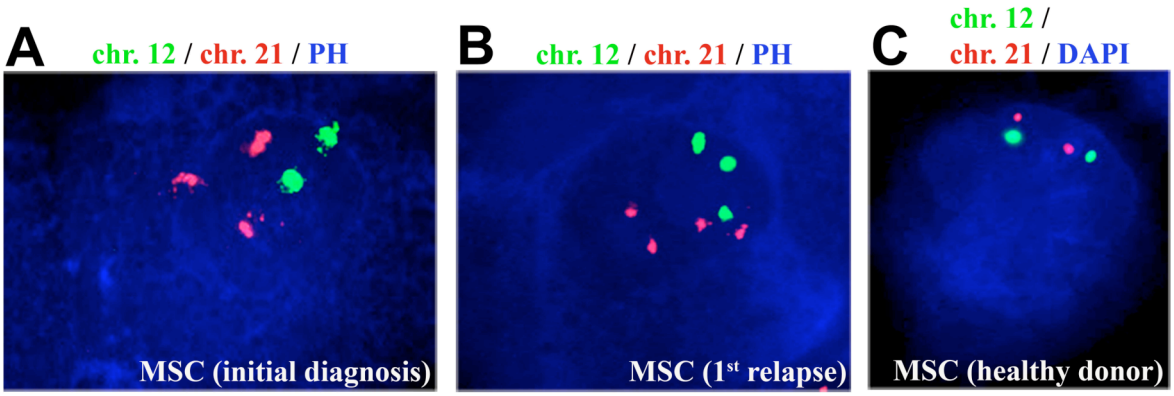


Figure 18. FISH analysis of MSC from patient 5 hybridized with specific enumerations gene probes for chromosomes 12 and 21. (A) MSC at initial diagnosis were analyzed after hybridization with gene probes for the chromosomes 12 (green) and 21 (orange), showing 3 signals for chromosome 21 and 2 signals for chromosome 12, combined with mAb against PH (blue). **(B)** MSC at 1st relapse analyzed with FICTION as described in (D), showing 4 signals for chromosome 21 and 3 signals for chromosome 12. **(C)** MSC from a healthy donor analyzed by FICTION with gene probes for the chromosomes 12 (green) and 21 (orange), showing 2 signals for chromosome 12 and 2 signals for chromosomes 21.

Abnormal numbers of chromosome were detected in 35% of MSC at initial diagnosis and in 27% of MSC at 1st relapse from patient 5 (Table 7). In healthy donor, abnormal

numbers of chromosome were not detected. Furthermore, the percentage of tetraploid cells was less than 3%.

Time point of sampling	Chromosome #	Frequency of abnormal numbers of chromosome (Based on 150 nuclei)			
		Copy number of chromosome / cell			
		1	2	3	4
diagnosis	10	9	128	13	-
	11	2	135	12	1
	12	7	134	8	1
	21	3	113	29	5
	X	4	117	26	3
1st relapse	10	10	132	5	3
	11	4	135	9	2
	12	9	125	15	1
	21	7	133	7	3
	X	7	132	8	3

Table 7. Frequency of copy number of chromosomes 10, 11, 12, 21 and X in primary MSC from patient 5. MSC in interphase were hybridized with centromeric gene probes for the chromosome 10, 11, 12, X and a LSI probe for chromosome 21. 100-200 nuclei were counted. The cut-off for FISH results was determined with MSC from a healthy donor (1%). Abnormal numbers of chromosome were detected in 35% of MSC at initial diagnosis (Figure 1D) and in 27% of MSC at 1st relapse (Figure 1E).

3.3.3 Frequency of leukemia-specific *IG* gene rearrangements in MSC

The MSC were investigated for the presence of leukemia-specific *IG* gene rearrangements by using quantitative real-time PCR and sequencing. In 2 of 5 patients (#4 and #5), leukemia-specific *IG* gene rearrangements were detected in MSC (Table 8).

#	Time point of sampling	*IG* gene rearrangement	
		Identified in MNC	Percentage in MSC
1	1st rel	*VH3JH4*	0
2	rem 1	*VH3DH1JH5*	0
	rem 2	*VkI-kde*	0
3	rem 2	*VkIII-kde*	0
		VkIV-kde	0
4	diag	*VH3DH3JH4*	99.9
		VH3JH4	77
5	1st rel	*VH3DH6JH6*	28

Table 8. Frequency of leukemia-specific *IG* gene rearrangements in MSC of children with ALL. DNA isolated from MSC was quantified for specific IG gene rearrangements identified in leukemic cells at diagnosis. Values of quantitative real-time PCR are given in percentages. Abbreviations: V, variable region; D, diversity region; J, joining region; H, heavy chain. k, kappa; kde, kappa deleting; *IG*, Immunoglobulin; negative (0), < 0.1.

The proportion of positive MSC from patient 5 was 99.9% for the specific *VH3DH3JH4* gene rearrangement. In patient 5, PCR analysis of leukemia cells and MSC using *VH3* family primer and *JH* consensus primer for *IG* heavy chain showed a PCR product for leukemia cells at diagnosis presenting a clonal *VH3JH* rearrangement in the main LC population and a clear, but weaker product of MSC of the same patient (Figure 19).

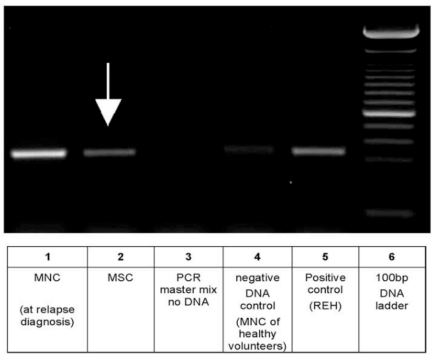
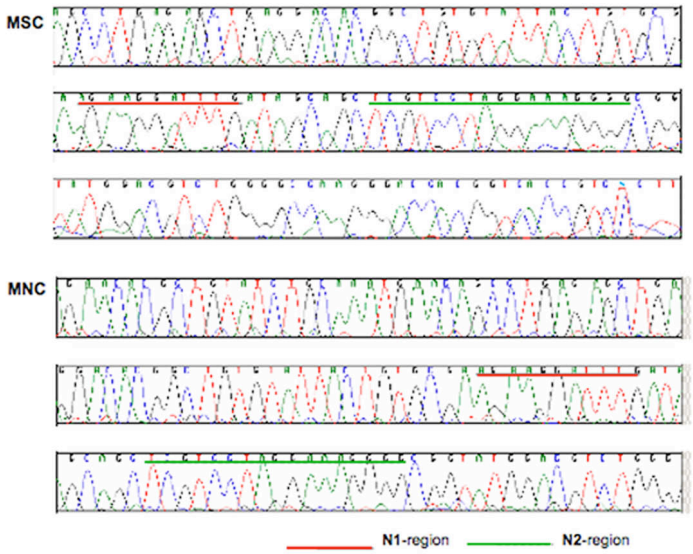


Figure 19. Analysis of *VH3JH* rearrangement in LC and MSC from patient 5. PCR analysis of LC and MSC from patient 5 using *VH3* family primer and *JH* consensus primer for *IG* heavy chain showed a PCR product for LC at diagnosis (lane 1) presenting a clonal *VH3JH* rearrangement in the main LC population and a clear, but weaker product of MSC of the same patient (lane 2). Lane 3 is a negative control (PCR mastermix), lane 4 shows a smear of products from a mononuclear cells pool of healthy donors, lane 5 is the positive control cell line REH having a clonal *VH3JH* rearrangement, and lane 6 is the 100 bp DNA marker.

This rearrangement (*VH3DH6JH6)* was further confirmed by genomic DNA sequencing and showed identical results for both leukemia cells and MSC (Figure 20).

A



B

Pt. 5		VH sequence (3')*		N1	DH sequence		N2	JH sequence (5')*
MSC	name	VH3-30*01			DH6-13*01			JH6*02
	deletions(-) insertions(+)		-4	+11	-4	-9	+17	-16
	sequence	GGCTGTGTATTACTGTGCGA		AGAAGGATTTG	ATAGCAGC		TCGTCCTAGGAAAGGGG	CGGTATGGACGTCTGGGGCC
MNC	name	VH3-30*01			DH6-13*01			JH6*02
	deletions(-) insertions(+)		-4	+11	-4	-9	+17	-16
	sequence	GGCTGTGTATTACTGTGCGA		AGAAGGATTTG	ATAGCAGC		TCGTCCTAGGAAAGGGG	CGGTATGGACGTCTGGGGCC

Figure 20. Analysis of *VH3JH* rearrangement in LC and MSC from patient 5. **(A)** DNA sequence chromatograms for VH3-30*1/DH6-13*01/JH6*02 rearrangements from MNC and MSC of patient 5. N1 region is underlined (red line) and N2 regions are marked with a green line. **(B)** Junctional sequences of the *VH3DH6JH6* rearrangement identified in MNC and in MSC of patient 5. In both MNC and MSC the same *VH3DH6JH6* rearrangement was detected between VH3-30*1 and DH6-13*01 segments (N1-region) and between DH6-13*01 and JH6*02 segments (N2-region). * 3' (VH) or 5' (JH) joining sites are shown. IgH: immunoglobulin heavy chain gene locus, V: variable, D: diversity, J: joining. Specific Primer (designed according to junctional region sequence): underlined sequence: 5'-*ACTGTGCGAAGAAGGATTTGATAG*- 3'

The proportion of *VH3DH6JH6* rearrangement-positive MSC was further determined by quantitative real-time PCR using a specific primer (underlined in Figure 20A) and a common *JH6* primer and a *JH6* hydrolysation (TaqMan) probe for the *VH3DH6JH6* rearrangement. The amount of *VH3DH6JH6* rearrangement-positive MSC was 28% (Figure 21).

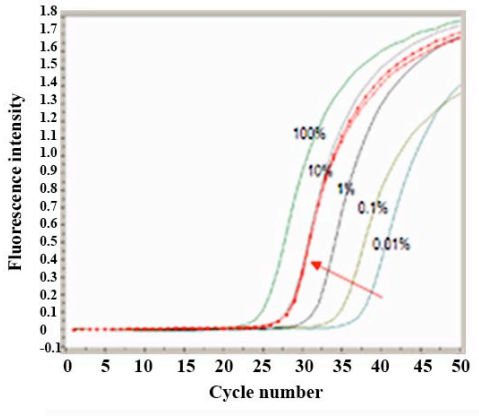


Figure 21. Quantitative real-time PCR analysis of MSC for the *VH3DH6JH6* rearrangement. Quantitative Real-time PCR using a specific primer (underlined in A) and a common *JH6* primer and a *JH6* hydrolysation (TaqMan) probe for the *VH3DH6JH6* rearrangement identified in MNC. Rearrangements in MSC were quantified using log-step serial dilutions from 100% to 0.01%. After correction with a single copy reference gene (β-globin) the amount of positive MSC were calculated to be 28% (red arrow).

3.3.4 SV40-Tag MSC from a TEL-AML1^{+} patient and a healthy donor

To exclude positive signals from contaminating leukemia cells in the MSC cultures and to immortalize the cells, MSC (passage 2-3) from patient 5 and a healthy donor were transduced with a Simian Virus 40 Large T antigen (SV40-Tag) encoding retrovirus, selected for Hygromycin and cloned. After 1 cell / well limiting dilution, 12 clones from patient 5 and three from one healthy donor were analyzed.

3.3.4.1 Characterization of SV40-Tag MSC

All characteristics described for primary MSC were also observed in SV40-Tag MSC. The SV40-Tag MSC were multipotent as shown by their capacity to differentiate to adipocytes and osteoblasts (Figure 22).

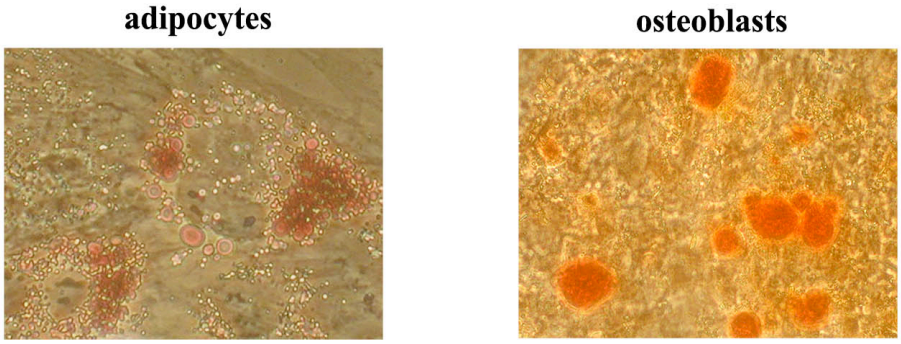


Figure 22. Differentiation capacity of SV40-Tag MSC. SV40-Tag MSC from patient 5 after differentiation to adipocytes (left: oil-red staining) and osteoblasts (right: alizarin-red).

The SV40-Tag MSC from Patient 5 and a healthy donor were negative for hematopoietic- (e.g. CD34, CD45), lymphoid- (e.g. CD19, CD33), myeloid markers (e.g. CD11b) and positive for stroma lineages markers (e.g. CD166, CD73 and CD90, CD29, CD44, Cd105) in flow-cytometric analysis (Figure 23).

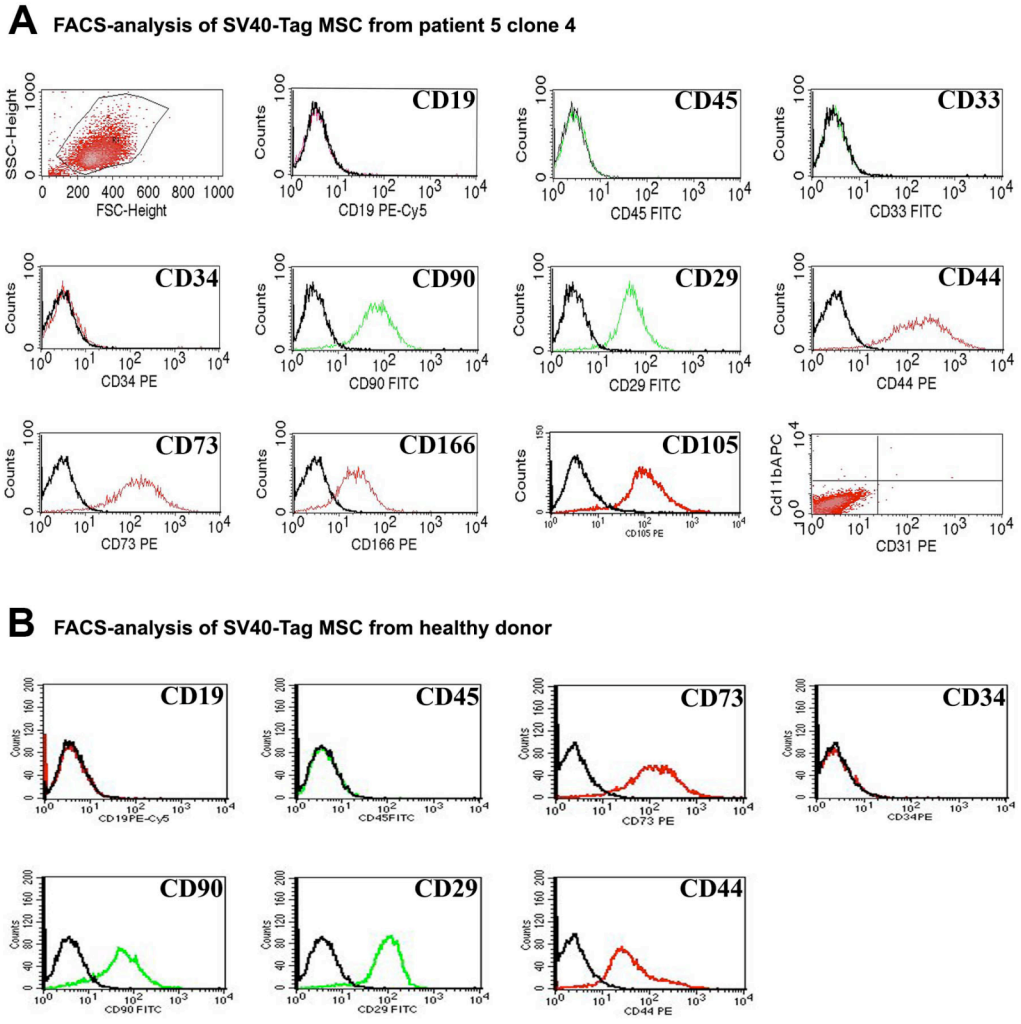


Figure 23. Immunophenotype analysis of SV40-Tag MSC using FACS. (A) Flow cytometry-analysis of SV40-Tag MSC from patient 5, with mAbs against CD19, CD45, CD33, CD34, CD90, CD29, CD44, CD73, CD166, CD105, CD31/CD11b. **(B)** FACS-analysis of representative SV40-Tag MSC from a healthy donor with mAbs against CD19, CD45, CD73, CD34, CD90, CD29, CD44.

3.3.4.2 Frequency of *TEL-AML1* and additional aberrations in SV40-Tag MSC

FISH / FICTION analysis of SV40-Tag MSC from patient 5 revealed the presence of the *TEL-AML1* fusion gene. The proportion of translocation-positive SV40-Tag MSC varied between 11% and 70% in different clones three weeks after cloning and between 11 and 23% two to five months after cloning (Figure 24). In SV40-Tag MSC from patient 5, four different types of clones were observed. The first and fourth type

showed absence of *TEL-AML1* fusion gene, while the second exhibited 11% of *TEL-AML1* positive cells (Table 9). In the third type, the transduced MSC clones showed 17-23% *TEL-AML1* positive cells in the interphase (Figure 24).

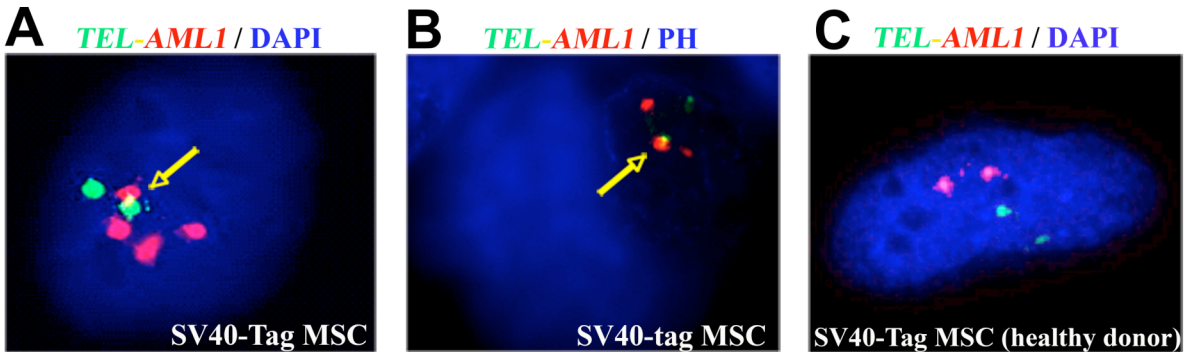


Figure 24. FISH/FICTION analysis of SV40-Tag MSC with *TEL-AML1* gene probes. (A) SV40-Tag MCS in interphase (patient 5) analyzed by FISH for *TEL-AML1* and counterstained with DAPI (blue). The SV40-Tag MSC showed one yellow signal (*TEL-AML1*, yellow arrow), one green signal (*TEL*) and 3 orange signals (native *AML1*, residual *AML1*, *AML1* amplification). **(B)** FICTION analysis of SV40-Tag MSC (cytospin) with the *TEL-AML1* gene probes combined with mAb against PH (blue) revealing the same pattern as in LC and primary MSC. **(C)** SV40-Tag MSC from a healthy donor analyzed with the *TEL/AML1* gene probes and counterstained with DAPI (blue), showing no translocation and 2 signals of *AML1* (orange) and *TEL* (green).

One of the clones from type 3 was further subcloned (0.3 cells / well dilution) and 6 clones were analyzed. One of them showed 70% *TEL-AML1* fusion gene. After one month culturing the proportion of *TEL-AML1* fusion gene changed, and was 10-15% (Figure 25).

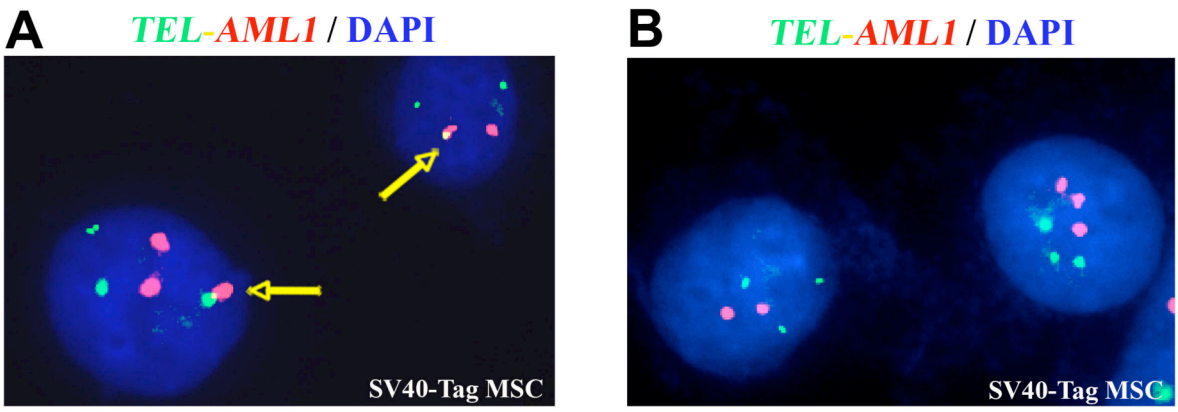


Figure 25. FISH/FICTION analysis of SV40-Tag MSC after subcloning. (A) SV40-Tag MCS after subcloning (0.3cells/well) in interphase analyzed by FISH for *TEL-AML1* and counterstained with DAPI (blue). The SV40-Tag MSC showed one yellow signal (*TEL-AML1*, yellow arrow), 2 green signals

(*TEL*) and 2 orange signals. **(B)** FICTION analysis of SV40-Tag MSC (the same clone, after one month culture) with the *TEL-AML1* gene probes and counterstained with DAPI (blue). The SV40-Tag MSC showed 3 green signals (*TEL*), 2 orange signals (left) and 3 orange signals (right).

The reason for the heterogeneous presence of *TEL-AML1* in cloned cells is unknown, but could be due to genetic instability (154). Detailed information concerning the proportions of leukemia-associated aberrations in SV40-Tag MSC is shown in Table 9. In addition to the *TEL-AML* fusion gene, SV40-Tag MSC from patient 5 showed abnormal numbers of chromosomes 12 and 21 in some cases (Figure 26). The first two types showed a standard number of chromosomes (44-46). Type 3 (Figure 26A) and type 4 showed both an aberrant number of chromosomes (65-81).

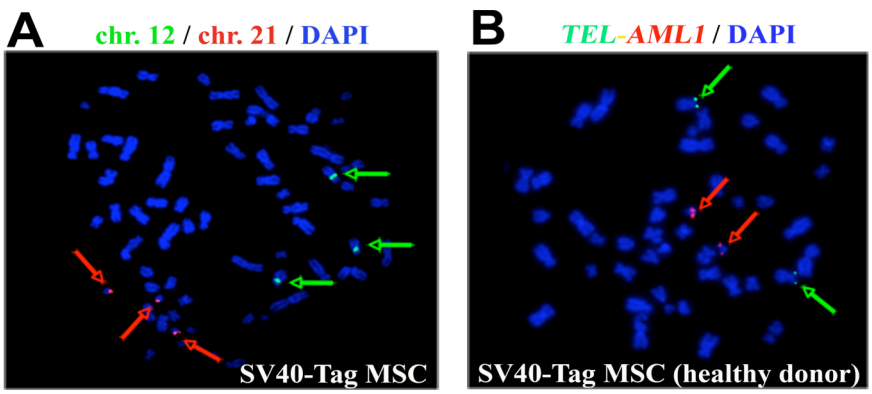


Figure 26. FISH analysis of SV40-Tag MSC in metaphase. (A) SV40-Tag MSC of one clone from patient 5 analyzed in metaphase with gene probes for the chromosomes 12 (green) and 21 (orange), showing 65 chromosomes with 3 dosages of chromosomes 12 and 21, counterstained with DAPI (blue). **(B)** SV40-Tag MSC from a healthy donor analyzed with *TEL/AML1* gene probes in metaphase, counterstained with DAPI (blue), showing 46 chromosomes with 2 signals of *AML1* (orange) and 2 signals of *TEL* (green).

The different types of SV40-Tag MSC reflected the heterogeneity found in primary MSC. Further hybridizations were performed with SV40-Tag MSC from one healthy donor, cultured for the same period of time, revealing a normal karyotype, and normal *TEL* and *AML1* copy numbers (Figure 24C and 26B), thus indicating that the leukemia-associated aberrations were not caused by SV40-Tag.

Pat. #	Status / Time of sampling	Type of clones	Number of clones	FISH interphase *TEL-AML1* [%]	Metaphase Total numbers of chromosome
SV40-Tag MSC					
5	1st relapse	5.1	3	0	46
		5.2	1	11	46
		5.3	4	17-23	69-81
		5.4	4	4	69-81
HD	-	HD.1	3	5	46

Table 9. Frequency of leukemia-associated aberrations in SV40-Tag MSC of children with ALL. Primary MSC (passage 2-3) from patient 5 and a healthy donor were transduced with a SV40-Tag encoding retrovirus, selected for Hygromycin and cloned. In SV40-Tag MSC from patient 5, four different types of clones were observed**.** The cut-off for the FISH results (6%) was determined with SV40-Tag MSC from healthy donors as described in Methods (*TEL-AML*: 5-6% in interphase, and normal numbers of chromosomes in metaphase). Abbreviations: MSC, mesenchymal stem cells from BM; HD, healthy donor.

3.4 *E2A-PBX1* fusion gene

3.4.1 Frequency of *E2A-PBX1* fusion gene in MSC of children with ALL

The fusion gene *E2A-PBX1* [t(1;19)] was identified in the LC from patients 6 and patient 7. *E2A* rearrangement was detected by FICTION in about 50% of the MSC at the time of 1st relapse and remission time points (Table 10, Figure 27).

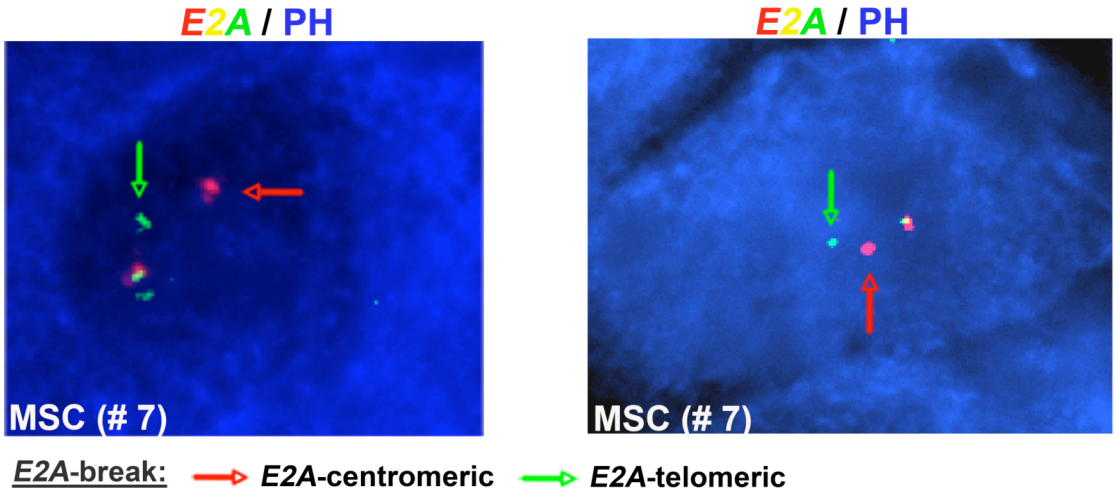


Figure 27. FICTION analysis of MSC isolated from patient 7 with *E2A-PBX1* fusion gene. MSC hybridized with split-signal FISH gene probes for *E2A* rearrangement (a red centromeric *E2A* gene probe and a green telomeric *E2A* gene probe) combined with mAb against PH (blue). The MSC show one yellow signal (green/red, no-translocation), one green signal and one red signal indicating *E2A* rearrangement.

Control hybridizations with MSC from a healthy donor and an ALL patient without *E2A-PBX1* fusion gene whose sample was taken during chemotherapy, showed false positive results lower than 5%.

Pat. #	Time point of sampling		LC content In BM [%]	Fusion gene frequency [%]	
	Sample	Weeks after start of treatment		MNC	MSC
E2A-PBX1					
6	1st rel [c]	-	62	50	n.d.
	rem 1[c]	13	0		46[a]
	rem 2	17	0		54
7	1st rel[c]	-	19	33	47

Table 10. Frequency of *E2A* rearrangements in MSC of children with ALL. The cut-off for the FISH results (6%) was determined with MSC from healthy donors and 2 ALL patients without known fusion gene in the LC as described in Methods (*E2A-PBX1*: 5%). [c] SV40-Tag clones were established (see Table 11). Abbreviations: BM, bone marrow; MNC, mononuclear cells from BM; MSC, mesenchymal stem cells from BM; LC, leukemia cells; SCT, stem cell transplantation; diag, initial diagnosis; rel; relapse; rem, sample taken during treatment and/or in remission (see Table S2); n.d., not done.

3.4.2 Frequency of *E2A-PBX1* fusion gene in SV40-Tag MSC

Primary MSC (passage 2-3) from patient 6, 7 and a healthy donor were transduced with a SV40-Tag encoding retrovirus, selected for hygromycin and cloned. After 1 cell / well limiting dilution, six clones from patient 6, five from patient 7 and three from one healthy donor were analyzed. The E2A translocation was detected by FISH analysis in some but not all SV40-Tag MSC clones (Figure 28). The proportions of *E2A*-rearrangements in different SV40-Tag MSC-clones from patient 7 were 4% (like healthy donor, Figure 28, left) to 55% (Figure 28, right).

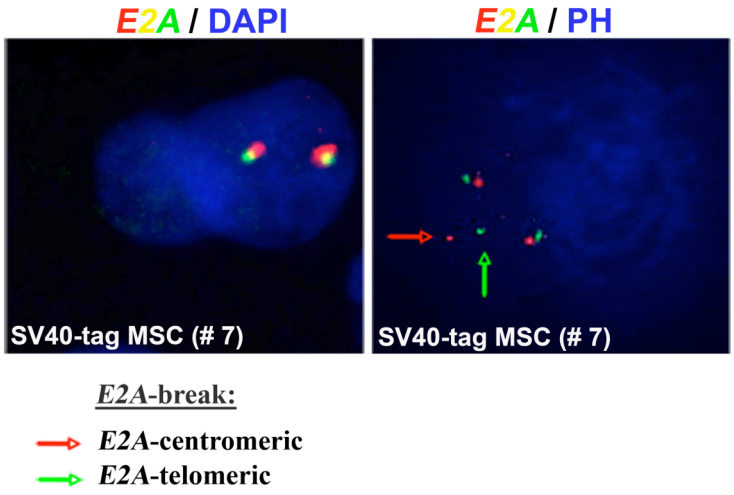


Figure 28. FISH/FICTION analysis of SV40-Tag MSC with *E2A* gene probes. SV40-Tag MSC from patient 7 analyzed by FISH for the *E2A* gene, as described in A, and counterstained with DAPI. Left: shows a cell from one clone (type 4) with two yellow signals indicating no translocation. Right: shows a cell of another clone (type1) with *E2A* translocation (one single green signal, one single red signal, and 2 green/red (yellow signals).

In *E2A* rearrangement positive clones, the proportion of positive MSC ranged between 18-55% (Table 12).

Pat. #	Status / Time of sampling	Type of clones	Number of clones	FISH interphase *E2A-PBX1* [%]	Metaphase Total numbers of chromosome
SV40-Tag MSC					
6	1st relapse	6.1	1	45	65-79
		6.2	1	50	65-79
	remision	6.3	3	30	65-79
7	1st relapse	7.1	2	55[a]	65-79
		7.2	1	18[a]	65-79
		7.3	1	28[a]	44-46
		7.4	1	4[a]	44-46
HD	-	HD.1	3	4	46

Table 12. Frequency of *E2A* rearrangements in SV40-Tag MSC of children with ALL. Remarks: [a]*E2A-PBX1* was detected by Western-Blot analysis. Abbreviations: remission, sample taken during treatment and/or remission. Primary MSC (passage 2-3) from patient 6, 7 and a healthy donor were transduced with a SV40-Tag encoding retrovirus, selected for Hygromycin and cloned. After 1 cell / well limiting dilution, six clones from patient 6, five from patient 7 and three from one healthy donor were analyzed. The proportions of *E2A*-rearrangements in different SV40-Tag MSC-clones from patient 6 were 30-50%. The SV40-Tag MSC-clones from patient 6 showed an aberrant number of chromosomes (65-81). The cut-off for FISH results was determined with SV40-Tag MSC from a

healthy donor (*E2A*-rearrangement: 4% in interphase and normal numbers of chromosomes in metaphase).

The fusion protein E2A-PBX1 was detected by Western blot analysis in the SV40-Tag MSC (Figure 29), showing a band of 77 kD, that has been reported to represent the E2A-PBX1 fusion protein, in addition to 85-90 kD bands detected in a control leukemia cell line (697) (26). Additionally, 2 bands of approximately 40 kD were detected, corresponding to PBX1a and PBX1b (155).

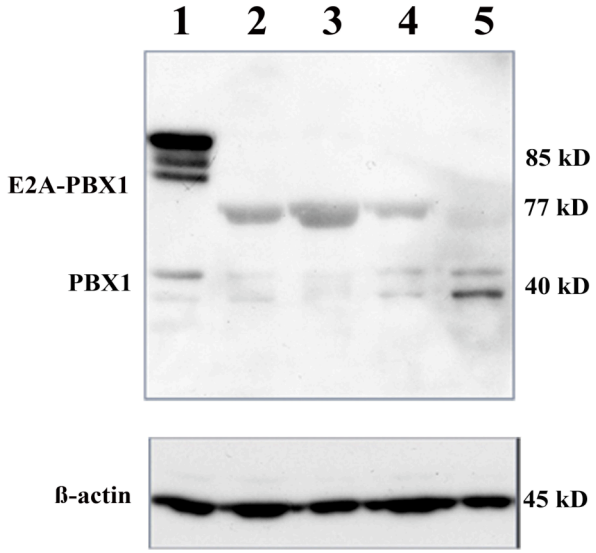


Figure 29. Western-blot analysis of SV40-Tag MSC from patient 7 using mAbs against the E2A-PBX1 fusion protein. The E2A-PBX1 positive cell line 697 was used as positive control (lane 1; 85-90 kD). The cell line L87/4 (lane 5) was used as negative control. Different clones of SV40-Tag MSC were included (lanes 2-4, approx. 77 kD). All lanes showed bands of around 40 kD, which corresponded to described PBX1 bands. Anti-beta actin mAbs were used as control for equal protein loading.

Some clones from SV40-Tag MSC showed additional aberrations like abnormal numbers of chromosome (Table 12). Three of five SV40-Tag MSC-clones from patient 7 showed an aberrant number of chromosomes (65-81). The results of different representative clones are presented in Table 12**.** Using whole chromosome paint (WCP) FISH probes, partial deletions in the chromosomes 1 and 19 and translocations between chromosomes 1 and 19 were detected in SV40-Tag MSC from patient 6 and 7 (Figure 30).

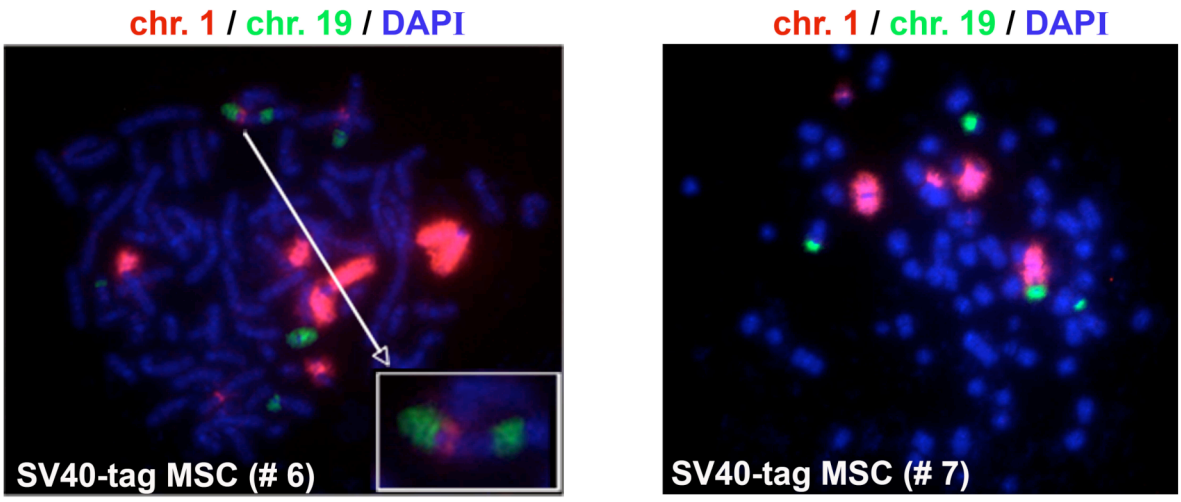


Figure 30. SV40-Tag MSC hybridized with whole chromosome gene probes in metaphase. SV40-Tag MSC from patient 6 (left) and patient 7 (right) were analyzed with WCP gene probes for chromosome 1 (red) and chromosome 19 (green) in metaphase, and counterstained with DAPI (blue). Abnormalities, such as translocations (1;19), deletions and variable numbers of both chromosomes were detected.

Control hybridizations with the SV40-Tag MSC from a healthy donor cultured in parallel for more than 2-3 months showed no translocation between chromosomes 1 and 19 (Figure 31).

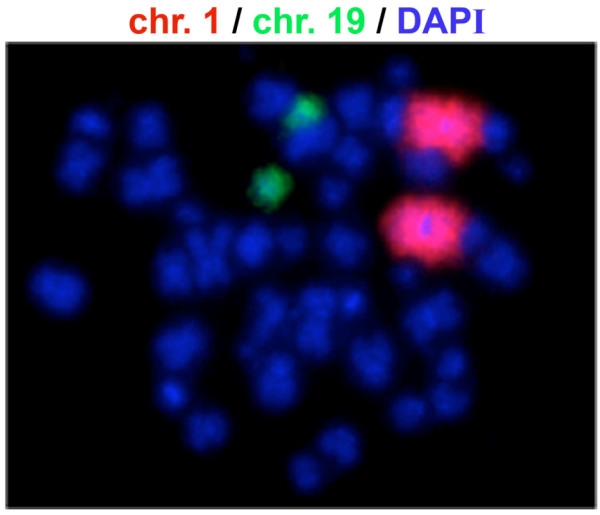


Figure 31. SV40-Tag MSC from a healthy donor hybridized with whole chromosome gene probes in metaphase. SV40-Tag MSC from a healthy donor, analyzed with WCP gene probes for chromosome 1 (red) and chromosome 19 (green) in metaphase and counterstained with DAPI (blue), showing 2 dosages of both chromosomes 1 and 19.

3.5 *MLL* (11q23) rearrangements

3.5.1 Frequency of *MLL-ENL* fusion gene in MSC of children with ALL

LC and MSC from patients 8 and 9 showed the fusion gene *MLL-ENL* resulting from a translocation [t(11;19)]. The male patient 8 experienced 5 relapses of ALL. In LC at the 5th relapse, the *MLL* gene rearrangement was detected in combination with partial loss of the *MLL* gene (Figure 32A). In addition, the LC showed duplication of the X chromosome and loss of the Y chromosome (Figure 32B).

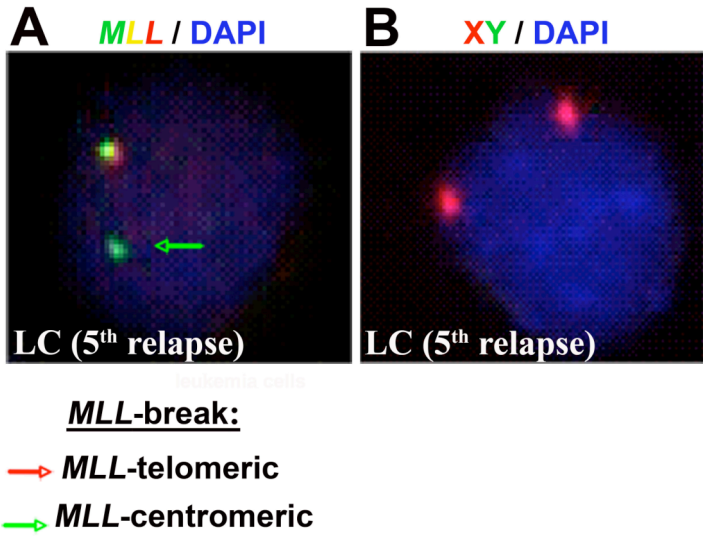


Figure 32. FISH analysis of LC from patient 8. (A) LC from patient 8 at 5th relapse hybridized with split-signal FISH gene probes for *MLL* rearrangement (an orange telomeric *MLL* gene probe and a green centromeric *MLL* gene probe) and counterstained with DAPI. The LC with *MLL* translocation shows one green/orange (yellow) signal for *MLL* gene, one single green signal indicating *MLL* rearrangement and lack of the second orange signal indicating loss of 3´ *MLL* gene. **(B)** LC from patient 8 (male) hybridized with probes for the X chromosome (orange), the Y chromosome (green) and counterstained with DAPI, showing loss of the Y chromosome and 2 signals for X chromosome.

In 18% of the MSC isolated from patient 8, the *MLL* rearrangement was detected using FISH, showing two different types of MSC with *MLL* rearrangements. Type I were MSC (9% of the 18% positive MSC) showing a *MLL* rearrangement in combination with partial loss of the *MLL* gene and loss of the Y chromosome (Figure 33A). Type I MSC corresponded to the genotype of the LC found at the 5th relapse. Type II MSC (91% of the 18% positive MSC) were characterized by *MLL* rearrangement and presence of the Y chromosome (Figure 33B). Type II MSC reflected the genotype of the LC found at the 1st relapse and at initial diagnosis more than 6 years before.

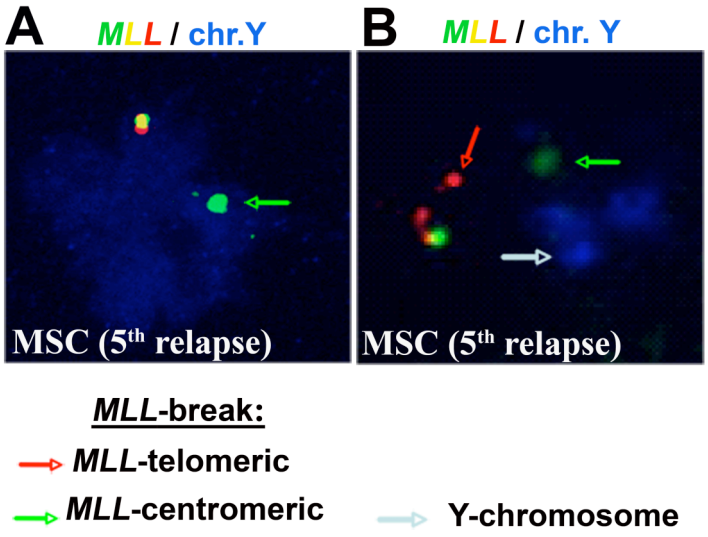


Figure 33. FISH analysis of MSC from patient 8. MSC at 5th relapse hybridized with the *MLL* gene probes described for Figure 30 and with a Y chromosome gene probe (blue). **(A)** The MSC shows one green/orange (yellow) signal and one green signal indicating *MLL* rearrangement (green arrow) combined with 3´ loss of *MLL* gene and loss of Y chromosome, as described above for LC from 5th relapse. (**B**) The MSC shows *MLL* rearrangement (one yellow, one green and one orange signal) in combination with presence of chromosome Y (blue signal), showing similar genetic pattern as previously found in initial diagnosis and 1st relapse.

The *MLL-ENL* fusion gene sequence present in LC from initial diagnosis to the 5th relapse (156) was also identified in MSC from the 5th relapse, indicating a clonal relationship between MSC and LC (Figure 34).

1. *MLL-ENL* sequence of LC from patient 8 at the 3rd relapse:
TTTTGTATTTTCTTTTTTTTGTCTGCAACTTGTTCTCAAAT
2. LC from 5th relapse:
TTTTGTATTTTCTTTTTTTTGTCTGCAACTTGTTCTCAAAT
3. MSC from 5th relapse:
TTTTGTNTTTTCTTTTTTTTGTCTGCAACTTGTTCTCAAAT

blue: *MLLT1* (*ENL*, Intron 1) red: inserted nucleotide black: *MLL* (Intron 11)

Figure 34. *MLL*-ENL sequence of LC and MSC from patient 8. The DNA sequences from LC at different time points (1. 3rd relapse, 2. 5th relapse) and MSC from 5th relapse (3) showed identical breakpoints.

Furthermore, a leukemia-specific *IG* rearrangement was detected in MSC from patient 8. The proportion of positive MSC was 7% for the specific *VH3DH3JH4* rearrangement (Appendix Table 2).

In MSC from patient 9 at 2nd relapse, the *MLL* rearrangement was detected by FICTION at diagnosis (22%), and at the control time point (18%) during chemotherapy, when no LC were detected (Table 13).

Patient	Time point of sampling		LC content in BM [%]	Fusion gene frequency [%]	
	Sample	Weeks after start of treatment		MNC	MSC
MLL rearrangement					
8	5th rel	-	73	87[c]	18 [d]
9	2nd rel	-	92	90	22
	rem	4	0		18
10	1st rel 1	-	90	96	n.d.
	1st rel 2	14	50	n.d.	11
	SCT	-	-	-	-
	rem 1[e]	11	0	n.d.	12
	rem 2[e]	27	0	2[#]	22
	rem 3[e]	33	0	0	14
	rem 4[e]	34	0	n.d.	18
	rem 5[e]	36	0	3[#]	19.8
	rem 6[e]	44	0	n.d.	20
	2nd rel 1	58	14	n.d.	23
	2nd rel 2	65	82	69	10

Table 13. Frequency of *MLL* rearrangement in MSC of children with ALL. [#] The cut-off for the FISH results (6%) was determined with MSC from healthy donors and 2 ALL patients without known fusion gene in the LC as described in Methods (*MLL*-rearrangements: 4%) Remarks: [d] *MLL-ENL* genomic breakpoint analyzed by real-time PCR (LC: 100%, MSC: 46%), [e] In the control samples 1-6, MNC showed always female donor genotype and MSC male host genotype. Abbreviations: BM, bone marrow; MNC, mononuclear cells from BM; MSC, mesenchymal stem cells from BM; LC, leukemia cells; SCT, stem cell transplantation; diag, initial diagnosis; rel; relapse; rem, sample taken during treatment and/or in remission (see Table); n.d., not done.

3.5.2 Disease time course of the male ALL patient 10 with the t(11;21) translocation and analysis of MNC and MSC

Finally, patient 10 gave us the opportunity to analyze aberrant MSC for a longer period of time. This patient had received two subsequent stem cell transplantations (SCT) from his sister. Therefore, it was possible to distinguish between donor and recipient by the sex chromosomes. Furthermore, we obtained enough BM aspirate to analyze primary MSC in metaphase. The leukemia cells from patient 10 showed a *MLL* rearrangement, involving chromosomes 11 and 21 [t(11;21)]. The course of

disease from this patient is depicted in Figure 34 and Table 13. After a first SCT from his sister, the patient suffered from a relapse, where the *MLL* translocation was found in 96% of the LC (Figure 35).

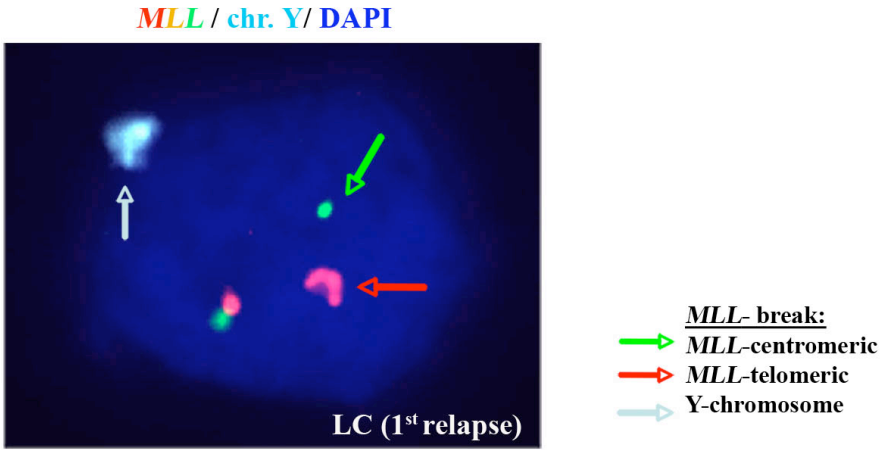


Figure 35. LC at 1st relapse hybridized with the *MLL* gene probes and with a Y chromosome gene probe. The leukemia cell shows *MLL* rearrangement (one yellow, one green and one orange signal) in combination with presence of Y chromosome (light blue).

The *MLL* rearrangement was found in 11% of the MSC isolated from the 1st relapse control time point. After a second SCT, again with his sister as donor, chimerism analysis of BM samples revealed that 100% of $CD34^+$, $CD19^+$ and $CD3^+$ cells were of donor origin, while 82-95% of MSC derived from the recipient. This was supported by FISH-analysis with X/Y probes, showing 99% BM mononuclear cells (MNC) with female donor genotype (Figure 36A) and more than 90% MSC with male recipient genotype (Figure 36B). In the following 12 months, 8 successive BM samples were analyzed for X and Y chromosomes and *MLL* rearrangement in MNC and MSC, confirming the donor origin in MNC in control samples 1-6 (Figure 36C) and always the host origin in MSC. Interestingly, in all 8 BM samples the *MLL* rearrangement was detected in MSC in a range between 10 and 23% by analyzing the cells in interphase (Figure 36D-E, Table 13). The analysis of MSC in metaphase confirmed the presence of *MLL* rearrangements (Figure 36F) and the translocation between chromosomes 11 and 21 (Figure 36G). Unfortunately, the patient experienced a further relapse, where Y chromosome and *MLL* rearrangement were found in 82% BM leukemia cells (Figure 36H).

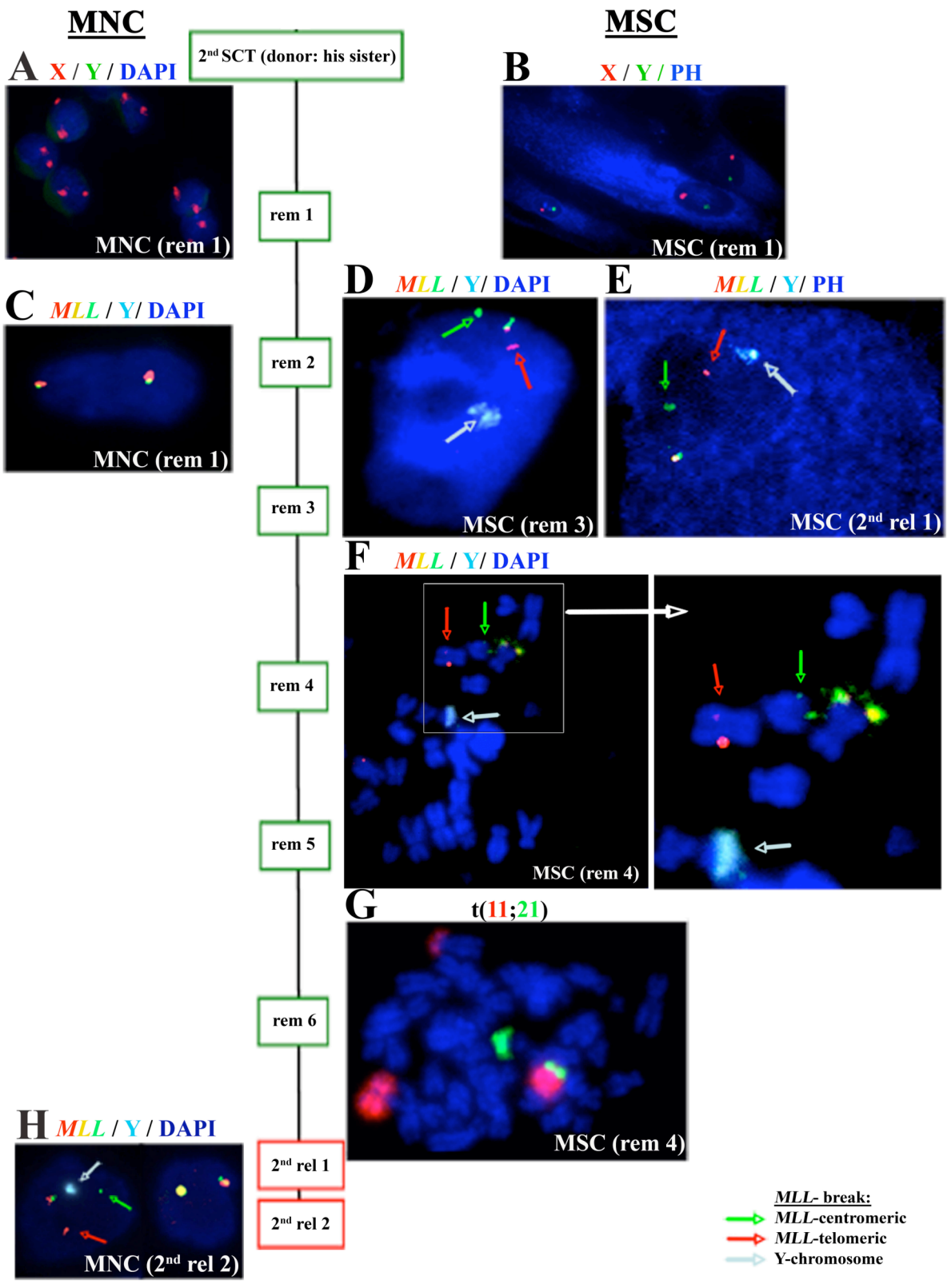


Figure 36: Disease time course of the male ALL patient (#10) and analysis of MNC and MSC during follow up. The disease course is depicted on a time axis beginning on the top with the second SCT from his sister as donor. Following 6 successive remission BM samples that were taken over a period of 65 weeks until the patient relapsed again. Shown are either MNC or MSC from BM aspirates at different time points after SCT. **(A)** MNC at control time point 1 (rem 1), hybridized with gene probes

for the X chromosome (orange) and the Y chromosome (green), and counterstained with DAPI, showing a female karyotype (2 orange signals). **(B)** MSC (chamber slide) at rem 1, hybridized with probes for X chromosome (orange) and Y chromosome (green), showing predominantly a male karyotype (one green and one orange signal). PH (blue) confirms a fibroblastoid phenotype. **(C)** MNC at rem 1 hybridized with the *MLL* and Y chromosome gene probes. The MNC shows 2 yellows signals, indicating no *MLL* rearrangement, and no Y chromosome signal (light blue). **(D-E)** MSC isolated from different remission samples hybridized with the *MLL* gene probes and with a Y whole chromosome gene probe (light blue). MSC at (D) rem 3 and (E) rem 7 show *MLL* rearrangement (one yellow, one green and one orange signal) in combination with presence of Y chromosome (light blue). (D) Counterstained with DAPI and (E) combined with mAb against PH (blue). **(F)** MSC (rem 4) analyzed in metaphase with *MLL* gene probes and counterstained with DAPI. Left: shows MLL rearrangement (one yellow, one green and one orange signal) in combination with presence of Y chromosome (light blue). Right: shows an enlargement of the labeled area. **(G)** MSC (rem 4) analyzed with WCP gene probes for chromosome 11 (red) and chromosome 21 (green) in metaphase, and counterstained with DAPI (blue), showing a translocation between chromosome 11 and 21. **(H)** MNC at 2nd relapse (65 weeks after SCT), hybridized with the *MLL* gene probes and with a Y chromosome gene probe (light blue) showing one cell (left) with *MLL* rearrangement (one yellow, one green and one orange signal) in combination with presence of Y chromosome (light blue), and one cell (right) with 2 yellows signals indicating no *MLL* rearrangement and no Y chromosome signal (light blue), counterstained with DAPI (blue), showing host and donor karyotypes, respectively.

All together, MSC from all 10 analyzed ALL-patients showed the chromosomal translocations that had been detected in leukemia cells (*TEL-AML1*, *E2A-PBX1* or *MLL* rearrangement). The proportions of translocation-positive MSC varied between 10 and 54% depending on the patients and the time point of analysis. Leukemia-specific *IG* gene rearrangements were detected in the MSC from 3 ALL patients.

4 DISCUSSION

We demonstrated that MSC from children with ALL carried leukemia-associated genetic aberrations. All patients included suffered from first or subsequent relapse, except one at initial diagnosis who developed a secondary leukemia (AML). Leukemia-associated translocations were present in MSC independent of the time point of sampling; they could be detected at diagnosis, at relapse or even in BM samples that were obtained in remission after chemotherapy or SCT.

4.1 Isolation and characterization of MSC

MSC were isolated and characterized from BM aspirates of 49 of 60 patients. As ALL-REZ BFM study group, the major focus was patients with relapsed ALL. Approximately only 40% of children with relapsed ALL show a long-term survival. It was not possible to directly isolate MSC from patients and analyze them for the presence of fusion genes, because MSC can currently only clearly be characterized by in vitro analysis and by their self-renewal and differentiation potential, and because there are no MSC specific markers available. Therefore, the only option was to isolate MSC via standard culture protocols and prove that they are indeed MSC by phenotype and function and that the cultured cells consist of a pure population. Any other alternative method would suffer from the risk of contaminating hematopoietic cells, blasts or undefined other cells. Initially, the aim of our study was also to compare two groups of patients, namely good and bad responders. The first problem encountered was, that isolation of MSC from most of frozen BM aspirates was not possible (no fibroblastoid cell were detectable), and accordingly, a retrospective study was not possible. Therefore, MSC were isolated from freshly isolated BM aspirates.

4.2 Leukemia-associated genetic aberrations in MSC

Multiple independent lines of evidence were obtained for the presence of chromosomal translocations in MSC. The presence of leukemia-associated genetic aberrations was first analyzed by FISH and afterward by several additional analyses. Additional analyses were performed in order to confirm the obtained results, showing that these data were caused neither by false positive hybridizations nor by contaminating LC. To exclude false positive hybridizations, which are a concern for FISH analysis, first, different probes were used (split signal, fusion signal and whole chromosome FISH probes), which gave compatible results. Second, the genomic

breakpoint of the fusion gene was sequenced in MSC and in LC of one patient, revealing the identical sequence. Third, the presence of the fusion protein for E2A-PBX1 was confirmed by Western blot analysis in one patient. Fourth, to exclude the possibility that the rate of false positive signals was higher in MSC derived from a marrow that had been exposed to chemotherapy, hybridizations were performed with MSC from ALL patients without a fusion gene in LC, whose BM aspirates were taken during chemotherapy. In these cases, the rates of false positive results were comparable to those from healthy donors. Fifth, the chromosomal translocations were detected in MSC in interphase and metaphase from a patient after SCT, when the BM mononuclear cells were of donor origin and no LC were detectable.

Furthermore, two different lines of evidence excluded the possibility of contaminating leukemia cells. First, FISH in combination with immunocytochemistry (FICTION) confirmed the presence of fusion genes in fibroblastoid cells. Second, analysis of SV40-Tag transduced MSC confirmed the results obtained with primary MSC. Taken together, multiple independent techniques demonstrated the presence of leukemia-associated aberrations in MSC from ALL-patients.

Leukemia-associated genetic aberrations have been reported for endothelial cells in patients with B-cell lymphoma (134) or chronic myelogenous leukemia (CML) (135). The *BCR-ABL* fusion gene was detected in fibroblastoid cells from one child with CML (157). However, leukemia-associated genetic aberrations were not detected in fibroblastoid cells or MSC of adult leukemia patients (158, 159). A possible explanation for the presence of fusion genes in MSC from children with ALL but not adult leukemia-patients could be a prenatal origin of the childhood B-precursor-ALL disease observed in some patients (160).

4.3 Clonal relationship between MSC and leukemia cells

Leukemia-specific *IG* gene rearrangements were detected in MSC from 3 of 8 analyzed patients. Furthermore, the sequence based analysis of leukemia-specific *IG* gene rearrangements and of the MLL-ENL fusion gene in MSC and LC revealed the identical sequence including the inserted nucleotides. As previously described, the sequence of VDJ and the junctional regions are specific for each leukemia clone, and are used in clinical trials for MRD detection. Therefore, these results suggest a clonal relationship between aberrant MSC and LC.

4.4 Models explaining the presence of leukemia-associated aberrations in MSC

The presence of leukemia-associated rearrangements in MSC can be explained by different mechanisms, which are not distinguishable from each other at the present time. A cell fusion model, a common progenitor cell model and a de/transdifferentiation model have been recently discussed to explain the plasticity of adult stem cells (51). It is unlikely that cell fusions were responsible for the described findings. In these cases tetraploid cells or abnormal numbers of analyzed genes and/or chromosomes would be expected and observed by using FISH (161). As shown in Figures 16A-B (patient 5), Figure 27 (patient 7), Figure 33 (patient 8) and Figures 36D-E (interphase) and Figure 36G (metaphase) for patient 10 no additional genes have been detected. This is clearly seen in metaphase analysis (Figure 36G), which shows a cell with no additional chromosomes (especially for chromosomes 11 and 21) that would not be expected after cell fusion. Moreover, the frequencies of fusion genes in MSC established from diagnosis samples (with 90% leukemia cells in culture) were comparable to those from remission samples (0% leukemia cells in culture). As indicated in Table 6, Patient #5 showed at diagnosis 11% fusion gene-positive MSC with 91% leukemia cells in the BM sample and in the remission sample 20% fusion gene-positive MSC were detected when no leukemia cells (0%) were present in the BM sample (similar results were obtained for patients #2, #3, #9 and #10). These results indicate that the frequencies of fusion genes found in MSC are neither due to the presence, nor to the proportion of leukemia cells in culture. Even more importantantly MSC from patient 10 were isolated after SCT when no blasts were detectable neither with flow cytometric analysis nor with more sensitive methods like a PCR for MRD. In some MSC cultures from patient 10, only 80-85% showed male karyotype (host), we never detected fusion genes in the remaining MSC with female karyotype (donor). Therefore, using *MLL* FISH probes in combination with Y-chromosome FISH probes in several independent samples of patient 10 allows us to exclude the possibility of cell fusion during cell culture. Furthermore, 7 of these 10 patients showed no leukemia-specific *IG* gene rearrangements in MSC but revealed fusion genes, arguing against the cell fusion of LC and MSC in these cases. Taken together, we can firmly exclude that cell fusion in vitro is responsible for the detection of fusion genes in the MSC.

However, it has been previously reported that tumor DNA can be horizontally transferred to the tumor stroma and endothelial cells through the uptake of apoptotic bodies in a human xenograft mouse model. These studies showed the presence of

tumor DNA in fibroblastoid cells with a frequency of 2-5% either after three weeks co-culturing with apoptotic bodies or in vivo (162, 163). Furthermore, replication of the tumor DNA was only possible when the p53-p21 pathway was inactivated either by an oncogene or by p53 mutations. As mentioned in the last paragraph, the detection of leukemia-associated genetic aberrations in MSC was neither due to the presence nor to the proportion of leukemia cells in culture, indicating that the horizontal transfer of leukemia cells DNA to MSC *in vitro* cannot explain the obtained results. Nevertheless, we cannot formally exclude the contribution of the transfer of genetic material from leukemia cells to MSC *in vivo*.

The alternative explanations "common progenitor cell model" and "de/transdifferentiation model" have to be considered. First, a common progenitor stem cell with leukemia-associated aberrations and/or leukemia-specific *IG* gene rearrangements could differentiate to MSC and leukemia cells (Figure 37). The ability to differentiate into both cell types, MSC and HSC, has been reported for $CD133^+$ stem cells (164). Interestingly, in $CD133^+CD19^-$ cells from ALL-patients, leukemia-associated fusion genes and *IG* gene rearrangement were detected (101). It is also possible that overexpression of oncogenes like c-Myc (18) in malignant cells would result in pluripotency of leukemia stem cells and would increase the differentiation capacity of these cells. Moreover, early recombinase activating gene expression in progenitor cells, which may simultaneously induce *IG/TCR* gene rearrangement and translocation, has been recently discussed (165-167).

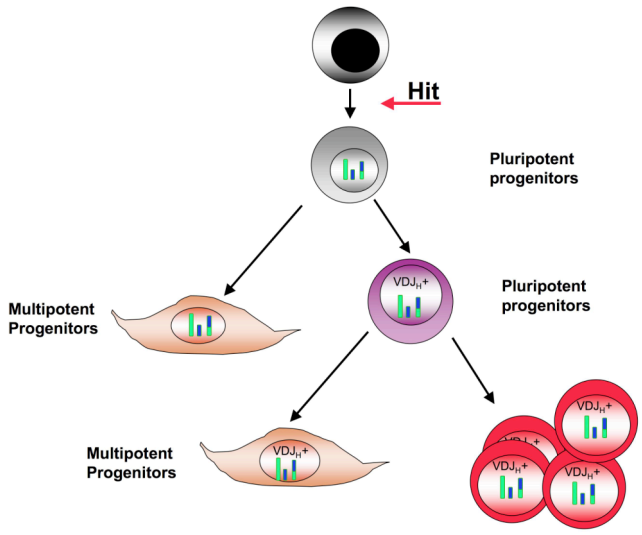


Figure 37. A common progenitor model. In this model either a progenitor cell with translocation (gray) or with translocation and IG gene rearrangements have the ability to differentiate into both cell types, LC (red) and MSC (beige), ***in vitro* and *in vivo*.**

The second model include that leukemia cells or leukemia stem cells could de- or transdifferentiate to MSC (Figure 38). It has been reported that pluripotent stem cells can be induced from adult fibroblast cultures and non-terminally differentiated B cells (138, 168). Furthermore, conditional *PAX5* deletion in mice allowed mature B cells to dedifferentiate into early uncommitted progenitors (137). A genome-wide analysis in childhood BCP-ALL has revealed that the *PAX5* gene was the most frequent target of somatic mutation being altered in 31.7% of patients (169). Assuming a high plasticity of various cell types, it is tempting to speculate that also aberrant MSC can de/transdifferentiate to leukemia cells.

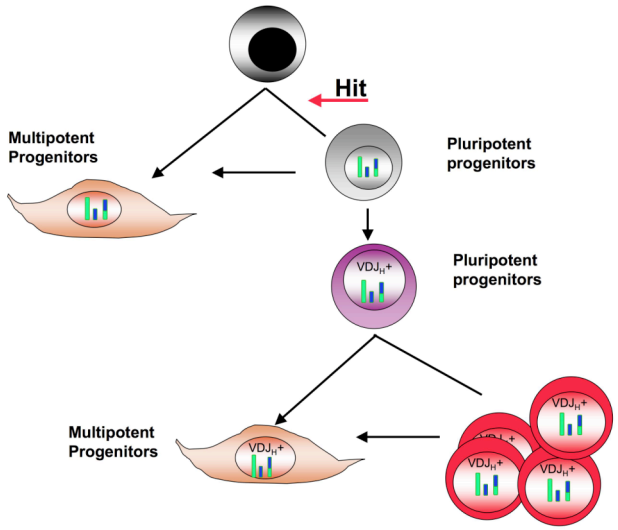


Figure 38. A de/transdifferentiation model. In this model either a progenitor cell with translocation (gray) or leukemia cells (red) with both translocation and *IG* gene rearrangement have the ability to de/transdifferentiate into MSC (beige), ***in vitro* and *in vivo*.**

By considering both models together, the fact that leukemia-associated translocations were detected in all MSC samples and that leukemia-specific *IG* gene rearrangement in only 3 of them, support the model of a common progenitor. On the other hand, the detection of leukemia-specific *IG* gene rearrangement in the 3 patients mentioned above would support the model of de/transdifferentiation. It would also be possible that common premalign cells containing fusion genes and *IG* gene rearrangements would differentiate to both cells types, but it is unlikely since it is assumed that HSC would reduce their multipotency character depending on their differentiation status. The considered models have been found to be dependent on the analyzed patients and the accumulation of genetic aberrations. The presented results cannot completely be explained just by one of these models so indicating that more than one model may be implicated.

4.5 Cell type specific role of fusion genes

The leukemia-associated genetic aberrations were less frequent in MSC than reported for HSC and leukemia cells from ALL patients (170). While the translocations probably cause a selective advantage for the HSC and leukemia cells, the translocations did not seem to confer a growth advantage to primary MSC, because the frequency of fusion genes appeared to remain constant in MSC isolated at different time points during the observation period. Furthermore, the cells with fusion genes did not outgrow those without fusion genes in vitro. Indeed, in the case of SV40-Tag MSC, the translocations seem to confer a growth disadvantage, because the proportions of SV40-Tag MSC with fusion genes decreased during culture. One of the reasons could be that the fusion genes may have different functions in MSC, leukemia cells and HSC (171).

4.6 Presence of additional genetic aberrations in MSC

The observation of genetic heterogeneity in primary MSC from some patients was confirmed by the fact that relapse samples display increased genetic heterogeneity as previously reported (172). The presence of abnormal numbers of chromosomes in MSC of some patients suggests genetic instability. For various cancers (173-175) as well as some cases of leukemia, genetic instability has been suggested to be the initial event for transformation. It has been postulated that different genetic alterations may occur in a common progenitor cell that segregates into a heterogeneous pool of stem cells of different lineages, including some pre-malignant clones which may further differentiate into malignant cells (165, 124). The resulting LC may be more homogeneous due to a selection process but heterogeneity would remain in the pre-malignant progenitor pool. The heterogeneity observed in the MSC described here could have been generated in a similar manner.

In other studies abnormal numbers of chromosomes have also been detected in MSC from patients with multiple myeloma (176) and myelodysplastic syndrom (177). Furthermore, the detection of abnormal numbers of chromosomes would have profound implications for understanding secondary bone tumors in the children that survive their leukemia disease. In a study including 1,376 ALL-patients, it has been reported that 19% of secondary malignancies were osteo-, Ewing-, and fibroblastic sarcomas (178). Since MSC are supposed to be the progenitor cells of Ewing sarcomas (179), osteoblasts and fibroblasts, the possibility exists that MSC may have contributed to the secondary malignancy in these patients.

4.7 Final remarks

Regardless of how leukemia-associated aberrations were generated in MSC of children with ALL, these results have shown for the first time that LC and MSC have genetic aberrations in common, and that they are clonally related. The presence of leukemia-associated aberrations and *IG* gene rearrangements in MSC indicates that different cell types are involved in the pathogenesis and pathophysiology of leukemia. Recently, different studies have reported that overlapping mechanisms may regulate the development of iPS-cells and cancer-cells, like in the case of the p53 pathway (180-185). If cancer cells arise through reprogramming-like processes, it would suggest that also cancer stem cells might have a pluripotent character. Furthermore, the presence of alterations in MSC also suggests that the BM niche seems to be part of the disease. Further studies have to be carried out, whether these alterations change the BM niche function. Additional experiments have to be done in order to properly answer the question about, whether alterations in stroma cells are required for leukemia development or not. Recently, a mouse model demonstrated that the expression of TEL-AML1 in HSC was not sufficient to induce leukemia (22). It would be of great interest to analyze whether the coexpression of TEL-AML1 in HSC and MSC would lead either to increase the incidence of leukemia, or to worsen the course of disease in this mouse model. Because the aberrant MSC appear to be long-lived and therapy-resistant, it is tempting to speculate about the involvement of MSC in the generation of relapse. Thus, a systematic monitoring and characterization of MSC and other involved cells during therapy and after SCT may be relevant for a better understanding and subsequent treatment of ALL patients.

5 ZUSAMMENFASSUNG

Die akute lymphoblastische Leukämie (ALL) ist die häufigste maligne Erkrankung im Kindesalter. Die Erkrankung wird durch entartete unreife Lymphozyten oder ihre Vorläufer verursacht. Die derzeit zur Verfügung stehenden Therapien erreichen bei Kindern mit Ersterkrankung eine Langzeit-Überlebenssrate von 85%, aber die Heilungsaussichten nach einem Rezidiv liegen bei nur etwa 40%. ALL-Subtypen werden anhand ihres Immunphänotyps, ihrer Morphologie und ihrer speziellen genetischen Mutationen klassifiziert. Diese genetischen Aberrationen beinhalten strukturelle oder numerische Veränderungen der Chromosomen, wie Translokationen oder Hyperdiploidie, Deletionen und Amplifikationen. Die Analysen der chromosomalen Translokationen und der daraus resultierenden Fusionsgenen haben zum besseren Verständnis der Biologie von Leukämiezellen beigetragen. Das bessere Verständnis der Pathogenese von Leukämien erfordert nicht nur das erweiterte Wissen über die speziellen Mutationen, die sie beinhalten, sondern auch über das zelluläre Netzwerk, in denen diese Mutationen entstehen und sich entwickeln. Das Mikromilieu des Knochenmarks (KM) fördert Überleben und Differenzierung von hämatopoetischen Stammzellen (HSZ) und Lymohozyten. Im KM befinden sich zwei verschiedene Typen von Vorläuferzellen, HSZ und mesenchymale Stammzellen (MSZ). MSZ haben die Fähigkeit zu verschiedenen Zelltypen aus der mesenchymalen Linie zu differenzieren, wie etwa zu knochenbildenden Zellen, Muskel- und Fettzellen sowie KM-Stromazellen. MSZ erleichtern das Anwachsen von transplantierten HSZ und scheinen auch chemoresistent zu sein. In Mäusen wurde die Fähigkeit von MSZ auch zu Zellen der hematopoietischen Linie zu differenzieren demonstriert. Darüber hinaus konnten neuere Studien zeigen, dass reife B-Zellen und andere somatische Zelltypen wie Fibroblasten zu pluripotenten Stammzellen reprogrammiert werden können, indem genau definierte Transkriptionsfaktoren in diesen Zellen exprimiert werden. Folglich zeigen diese Daten, dass fast alle Zellen eine hohe Plastizität besitzen. Aus diesen Gründen untersuchten wir MSZ aus Patienten mit B-Zell-Vorläufer-ALL auf das Vorhandensein von leukämiezellen-assoziierten chromosomalen Aberrationen und Immunglobulin-Genumlagerungen. Die Produkte der *TCR*/*IG*-Genumlagerungen stellen leukämieklon-spezifische Marker dar, mit denen minimale Resterkrankung (MRD) während der Behandlung nachgewiesen werden können. Leukämiezellen aus 10 von 49 Patienten zeigten eine von drei Translokationen, entweder *TEL-AML1*, *E2A-PX1,* oder *MLL*-Rearrangement. Leukämie-assoziierte Aberrationen wurden in

MSZ aus all diesen 10 ALL-Patienten nachgewiesen, unabhängig vom analysierten Zeitpunkt. Des Weiteren konnten in 3 von 8 Patienten leukämie-assozierte *IG*-Genumlagerungen detektiert werden. Diese Ergebnisse weisen auf eine klonale Verwandtschaft zwischen MSZ und Leukämiezellen hin.

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7 APPENDIX

#	Disease stage at 1st analysis of MSC [diagnosis sample]	Treatment protocol (before analysis of study sample)	Time between diagnosis and relapse [months]	Treatment protocol at analysis of sample	Irradiation (before analysis of diagnosis sample)	SCT
1	1st relapse	ALL-BFM 2000 (MR)	59	ALL-REZ BFM 2002 (S2)	-	-
2	1st relapse	COALL 06-97	69	ALL-REZ BFM 2002 (S2)	-	-
3	1st relapse	ALL-BFM 95 (MR)	10	ALL-REZ BFM 2002 (S2)	-	-
4	initial	n.a.	n.a.	ALL-BFM 2000 (MR)	-	-
	secondary AML	ALL-BFM 2000 (MR)	14	AML-BFM 2004	-	-
5	initial	n.a.	n.a.	ALL BFM 2000 (MR)	-	-
	1st relapse	ALL-BFM 2000 (MR)	65	ALL-REZ BFM 2002 (S2)	-	-
6	1st relapse	COALL 06-97	34	ALL-REZ BFM 2002 (S2)	-	-
7	1st relapse	COALL 06-97	11	ALL-REZ BFM 2002 (S4)	12 Gy	-
8	5th relapse	Initial: ALL VII/81, 1st relapse: ALL-REZ BFM P95, 2nd relapse: ALL-REZ BFM 96, 3rd relapse: ALL-REZ BFM02, 4th relapse: ALL-REZ BFM02	14[a]	ALL-REZ BFM 2002	36 Gy	2
9	2nd relapse	Initial: ALL-BFM 2000 1st relapse: ALL-REZ BFM 2002	3[a]	ALL-REZ BFM 2002	-	1
10	1st relapse[b]	ALL-BFM/ALL-REZ BFM 2002 + Rituximab	34	individual treatment	12 Gy	2
11*	initial	n.a.	n.a.	ALL BFM 2000 (MR)	-	-
12*	1st relapse	ALL BFM 2000 (MR)	36	ALL-REZ BFM 2002 (S2)	-	-

Table 1: Previous and current treatments of patients, time between disease stages and time points when MSC samples were taken. Remarks: ALL-REZ BFM P95/96/2002, standard protocols for treatment of children with relapsed ALL in Germany; ALL-BFM 95/2000 and COALL 06-97, standard protocols for treatment of children with newly diagnosed ALL in Germany. Abbreviation: MR, intermediate initial ALL risk group; S2, intermediate relapsed risk group; S4, high relapsed risk group; n.a. , not applicable. [a] time between the previous relapse and current relapse. [b] remission sample after SCT.

Pat. #	Time point of sampling	*IG* gene rearrangement	
		Identified in MNC	**Percentage in MSC**
6	rem 1	*VH1DH6JH5*	0
7			n.d.
8	5^{th} rel	*VH6DH3JH6*	7
		VH3JH4	6
9			n.d.
10	rem1, rem 2, rem 3, rem 4, rem 5, rem 6	*VkI-kde*	0
		VH4DH3JH4	0
		VH3DH2JH6	0

Table 2: Frequency of leukemia-specific *IG* gene rearrangements in MSC of children with ALL. DNA isolated from MSC was quantified for specific IG gene rearrangements identified in leukemic cells at diagnosis. Values of quantitative RT-PCR are given in percentages. Abbreviations: V, variable region; D, diversity region; J, joining region; H, heavy chain. k, kappa; kde, kappa deleting; *IG*, Immunoglobulin; ALL, acute lymphoblastic leukaemia; SCT, stem cell transplantation; n.d.: not done; negative (0), < 0.1.

8 ABBREVIATIONS

ABL	acute biphenotypic leukemia
ABL1	Abelson murine leukemia vial oncogene homolog 1
ALL	acute lymphoblastic leukemia
ALL-BFM	the frontline trial ALL Berlin-Frankfurt-Muenster study group
ALL-REZ-BFM	the relapse trial ALL Berlin-Frankfurt-Muenster study group
AML	acute myelogenous leukemia
AML1	transcription factors Runx-1
BCP-ALL	B cell precursor ALL
BCR	Breakpoint cluster region
BM	bone marrow
CFU-F	low-density fibroblast colony-forming unit assay
CML	chronic myeloid leukemia
CNS	central nervous system
CR	complete remission
DC	dendritic cells
E2A	Transcription factor 3 (TCF3)
FACS	Flow-cytometry
FBS	fetal bovine serum
FISH	Flourescence In situ hybridization
FICTION	combined FISH and immunocytochemistry
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia fetal calf serum
HOX	homeobox gene
HSC	hematopoietic stem cells
IG	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
iPS	induced pluripotent stem cells
kD	kilo dalton
LC	leukemia cells
LSC	leukemia stem cells
mAB	monoclonal antibodies
MLL	mixed-lineage leukemia gene
MNC	mononuclear cells

MRD	minimal residual disease
MSC	mesenchymal stem cells
NOD-SCID	non-obese diabetic-severe combined immunodefiency
PBS	phosphate buffered saline
PBX1	pre-B- cell leukemia transcription factor 1
PH	prolyl-4-hydroxylase beta
RT	room temperature
SCT	stem cell transplantation
SSC	standard saline citrate
SV40-Tag	Simian Virus 40 Large T-antigen
TCR	T cell receptor
TEL	ETS variant gene 6 (ETV6)

9 PUBLICATIONS

SHALAPOUR, S., ECKERT, C., SEEGER, K., PFAU, M., PRADA, J., HENZ, G., BLANKENSTEIN, T., and KAMMERTOENS, T. (2010) Leukemia-associated genetic aberrations in mesenchymal stem cells of children with acute lymphoblastic leukemia. *J Mol Med. In press.*

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Eidesstattliche Erklärung

Hiermit versichere ich, die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt zu haben.

Bei der Verfassung der Dissertation wurden keine anderen als die im Text aufgeführten Hilfsmittel verwendet.

Ein Promotionsverfahren zu einem früheren Zeitpunkt an einer anderen Hochschule oder bei einem anderen Fachbereich wurde nicht beantragt.

Berlin, 04.09.09

Shabnam Shalapour