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Differential effects of young (growing) versus old (grown-up) bone marrow microenvironments on leukaemias by release of the cytokines CXCL13 and GDF11 from bone marrow stroma

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Für meine Familie und Freunde

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Abbreviations

5-FU	5-fluorouracil
(B-)ALL	(B-cell) acute lymphoid leukaemia
APC/ APC-Cy7	Allophycocyanin/ Allophycocyanin cyanine dye 7
BCR	breakpoint cluster region
BCR-ABL	B-Cell receptor-Abelson murine leukaemia viral oncogene homolog 1
BM	bone marrow
bp	base pair
CML	chronic myeloid leukaemia
dH2O	diethyldicarbonat-treated water
DMEM	Dulbecco's modified eagle medium
DNA	Desoxyribonucleic acid
dNTPs	Desoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
H_2Odd	H2O double-distilled
HBS	Hepes buffered saline
HSC	haematopoietic stem cell
kDa	kilodalton
L-Glu	L-Glutamine
LSC	leukaemic stem cell
Mab	monoclonal antibody
MIG	MSCV-IRES-GFP
MIR	MSCV-IRES-RFP
MSCV	Murine Stem Cell Virus (vector used for transduction)
NEAA	non-essential amino acids

P/S	penicillin/ streptomycin
p210	Philadelphia-Chromosom (210kDa)
PBS	phosphate buffered saline
PE	Phycoerythrin
RTqPCR	quantitative real-time PCR
RFP	Red Fluorescent Protein
shRNA	small hairpin RNA

1 Summary in English and German

1.1 Summary

While B-cell acute lymphoblastic leukaemia (B-ALL) can be described as the leukaemia of childhood, chronic myeloid leukaemia (CML) mostly develops in elderly individuals. Understanding and utilising mechanisms involved in the development and persistence of these leukaemias as possible targets for treatment strategies has received particular interest. Processes that happen in the vicinity of the cancerous cells themselves could influence cancer growth and behaviour and hence can serve as novel targets, leading to the development of two-pronged therapies that act both on leukaemic cells directly as well as their niche. The niche in the case of leukaemia is the bone marrow microenvironment (BMM) where these cells are not only generated but also instructed and protected. As the BMM is situated inside bones that undergo drastic changes and growth processes during the ageing process, the BMM itself is also being altered throughout life. These alterations and the very process of expansion itself may therefore also provide distinct regulatory influences on the cells (healthy or malignant) that are generated inside this niche, leading to the question: Does the age of the bone marrow microenvironment differentially influence the development of (“childhood”) B-ALL versus (“adult”) CML by the release of cytokines?

In previous studies by the host-laboratory the age distribution of B-ALL versus CML in a murine transduction/ transplantation model could be recapitulated; young mice which received the same number of leukaemia-initiating cells as their old counterparts died significantly earlier of B-ALL while showing a significantly delayed clinical course, when they were suffering from CML. The tumour load and other leukaemia-associated parameters also showed a clear disposition towards preferential induction of CML in elderly and B-ALL in younger mice.

In this project we could support the hypothesis that the age of the BMM differentially influences the proliferation of leukaemic cells and thereby the development and persistence of different types of leukaemias by utilising different *in vitro* culture experiments. Specifically, we could show that young (compared to old) bone marrow

stroma cells (BMSC) support the growth of (BCR-ABL1+) B-ALL cells both in a direct, cell on cell co-culture setting, as well as in young BMSC-derived conditioned medium. This supports the hypothesis that varying factors are differentially released from a young versus an old BMM and influence the growth of the leukaemia cells. The opposite might be true for CML cells (BCR-ABL1+ 32D cells); BMSC obtained from old animals showed a tendency to support their growth more profoundly than cells acquired from young animals.

Possible proteins responsible for the distinct regulation of myeloid versus lymphatic leukaemic cells by young versus old BMM have also been studied. We investigated C-X-C motif chemokine 13 (CXCL13) and growth differentiation factor 11 (GDF11) in their effect on leukaemia cells, as both proteins having previously been described to have tumour-modelling properties and age-dependent levels (see below).

We identified an increased secretion of CXCL13, a B-cell chemotactic factor, into conditioned medium from young versus old BMSC. In accordance with this we found migration of B-ALL cells towards BMSC from young compared to old mice to be improved, while adhesion of both B-ALL and CML cells to young versus old BMSC did not show any differences. By blocking CXCL13 the proliferation-supporting effect of young BMSC on B-ALL cells could be diminished. Similar effects could be demonstrated by blocking GDF11.

In the case of CML cells we could observe the opposite effect; blocking CXCL13 and GDF11 increased their proliferation in a co-culture with BMSC. This supported our hypothesis that both cytokines differentially regulate B-ALL and CML behaviour.

After the completion of this thesis, another member of the host-laboratory convincingly demonstrated the role of BMM age in the regulation of B-ALL via CXCL13 signalling (see discussion).

1.2 Zusammenfassung

Während B-Zell akute lymphatische Leukämie (B-ALL) als Leukämie der Kindheit beschrieben werden kann, manifestiert sich die chronische myeloische Leukämie (CML) hauptsächlich in älteren Patienten. Mechanismen, die wichtige Funktionen in

der Entstehung und der Persistenz dieser Leukämien spielen, müssen untersucht, verstanden und genutzt werden, um mögliche Zielstrukturen für neuartige Therapieoptionen aufzudecken. Insbesondere die Untersuchung von Prozessen, die außerhalb der Krebszelle selbst stattfinden, deren Wachstum und Verhalten jedoch maßgeblich beeinflusst, kann zur Entwicklung von Kombinationstherapien führen, die sowohl die leukämische Zelle selbst wie auch ihre Nische angreifen.

Im Fall von Leukämie ist diese Nische das Knochenmarksmikromilieu (KMM), wo kranke und gesunde Zellen nicht nur entstehen, sondern auch instruiert und beschützt werden. Da sich das KMM innerhalb von Knochen befindet, die drastischen Veränderungen und Wachstumsprozessen ausgesetzt sind, verändert sich auch das KMM während der gesamten Lebensspanne.

Diese Veränderungen und der Wachstumsprozess an sich könnten daher direkten regulatorischen Einfluss auf die gesunden und kranken Zellen nehmen, die sich im Inneren dieser Nische formen. Dies führt zu der Frage: Üben das Alter und Wachstum des Knochenmarksmikromilieus durch die Freisetzung von Zytokinen differenzielle Effekte auf (kindliche) B-ALL gegenüber (adulter) CML aus?

In vorherigen Untersuchungen durch andere Mitglieder der Laborgruppe konnte die altersabhängige Verteilung von Fällen der B-ALL und CML im murinen Transduktions-/ Transplantationsmodell reproduziert werden: Junge Mäuse, die die gleiche Anzahl Leukämie-initiierender Zellen wie Mäuse der alten Vergleichsgruppe erhielten, starben signifikant früher an B-ALL, während sich in jungen Mäusen die CML erst deutlich später manifestierte. Die Tumorlast und andere Leukämie-assoziierte Parameter zeigten ebenfalls einen klaren Trend in Richtung präferentieller Induktion von CML in alten versus B-ALL in jüngeren Mäusen.

In diesem Projekt konnten wir durch verschiedene *in vitro* Zellkultur-Experimente die Hypothese unterstützten, dass das Alter des KMM einen differenziellen Einfluss auf die Proliferation der leukämischen Zellen und damit auf die Entstehung und Persistenz der verschiedenen Leukämieformen ausübt. So konnten wir zeigen, dass junges (im Vergleich zu altem) KMM das Wachstum von (BCR-ABL1+) B-ALL Modellzellen sowohl in der direkten Zell-auf-Zell Kokultur als auch bei Kultur in Medium, das aus dem Überstand von jungen KMM-Zellen (bone marrow stroma

cells; BMSC) gewonnen wurde, unterstützt. Dies erhärtet die Vermutung, dass lösliche Faktoren, die von jungen und alten BMSC unterschiedlich freigesetzt werden, das Wachstum dieser Leukämiezellen beeinflussen.
Im Fall von CML könnte der gegensätzliche Effekt gelten; BMSC, die aus alten Mäusen gewonnen wurden zeigten die Tendenz, das Wachstum der CML-Modellzellen stärker als Zellen aus jungen Mäusen zu unterstützen.
Auch andere Charakteristika von Leukämiezellen in der Gegenwart von jungen gegenüber alten BMSC wurden von uns untersucht; die Migration von B-ALL Modellzellen in Richtung BMSC war signifikant erhöht, wenn BMSC aus jungen (gegenüber) alten Mäusen benutzt wurden. Die Adhäsion von B-ALL Modellzellen auf jungen gegenüber alten BMSC zeigte jedoch keinen Unterschied.
Des Weiteren identifizierten wir Proteine aus jungem gegenüber alten KMM, die möglicherweise einen differentiellen Einfluss auf die Regulation von myeloischen und lymphatischen Leukämien ausüben: Wir untersuchten die Zytokine C-X-C motif chemokine 13 (CXCL13) und growth differentiation factor 11 (GDF11) in ihrer Wirkung auf Leukämiezellen, da bei beiden Proteinen zuvor Tumor-modellierende Eigenschaften und altersabhängige Mengen beschrieben worden waren (s. unten). Durch antikörper-vermittelte Blockade von CXCL13 konnte der proliferationsunterstützende Effekt auf B-ALL Zellen durch junge BMSC verringert werden. Ähnliche Effekte konnten durch die Blockade von GDF11 nachgewiesen werden. Auch zeigten wir einen gegensätzlichen Effekt auf CML Modellzellen; die Blockade von CXCL13 und GDF11 verstärkte die Proliferation dieser Zellen in einer Kokultur mit BMSC.
Nach Abschluss dieser Arbeit konnte ein anderes Mitglied der Laborgruppe überzeugend die Rolle des Alters des KMM bei der Regulierung von B-ALL durch CXCL13 nachweisen (siehe Diskussion).

Unsere Hypothese lautete: Wachsendes gegenüber erwachsenem Knochenmarksmikromilieu übt durch die Freisetzung der Zytokine GDF11 und CXCL13 aus dem Knochenmarks-Stroma differenzielle Effekte auf Leukämien aus.

2. Introduction

2.1 Stem cells

Stem cells are undifferentiated cells with three major properties: they can divide indefinitely, they divide asymmetrically and they are not terminally differentiated[1]. These three features enable stem cells to fulfil their role as a source for all cells during development and for a wide range of cells throughout life. For example, damaged tissue is replaced by cells that originate from stem cells, new blood cells arise from haematopoietic stem cells and intestinal epithelium originates from intestinal stem cells every day.

Embryonic stem cells are totipotent and can differentiate into cells that make up all three different germ layers, thus they are able to form a complete organism[1]. Some of their daughter cells will become pluripotent stem cells that can still give rise to all the cells originating from the stem cell's respective germ layer. Even further differentiated stem cells will only replace a very specific class of cells, e.g., blood cells or intestinal epithelium. The other feature that was mentioned, asymmetrical division, is important to enable the cell to undergo these processes throughout the lifetime of the organism. It ensures that one of the emerging daughter cells will keep stem cell properties and remain a source for further cell supply, while the other will take on the role of a further differentiated cell.

2.2 Haematopoietic stem cells and haematopoietic system

Haematopoietic stem cells (HSCs) give rise to all circulating and non-circulating cells of the blood. According to the classical model, differentiation occurs in a hierarchical fashion with haematopoietic progenitor cells as intermediaries between HSCs and specific groups of blood cells[2] (Figure 1). The common myeloid progenitor originates from HSCs and gives rise only to myeloid cells like erythrocytes, thrombocytes and myeloblasts, which further differentiate into granulocytes and monocytes/ macrophages[3]. The common lymphoid progenitor also arises from an HSC but will produce cells of the lymphoid lineage, i.e. natural killer cells, B- and T-lymphocytes[4].

Mutations in haematopoietic stem or progenitor cells (HSPCs) can give rise to malignant alterations like leukaemias[5,6].

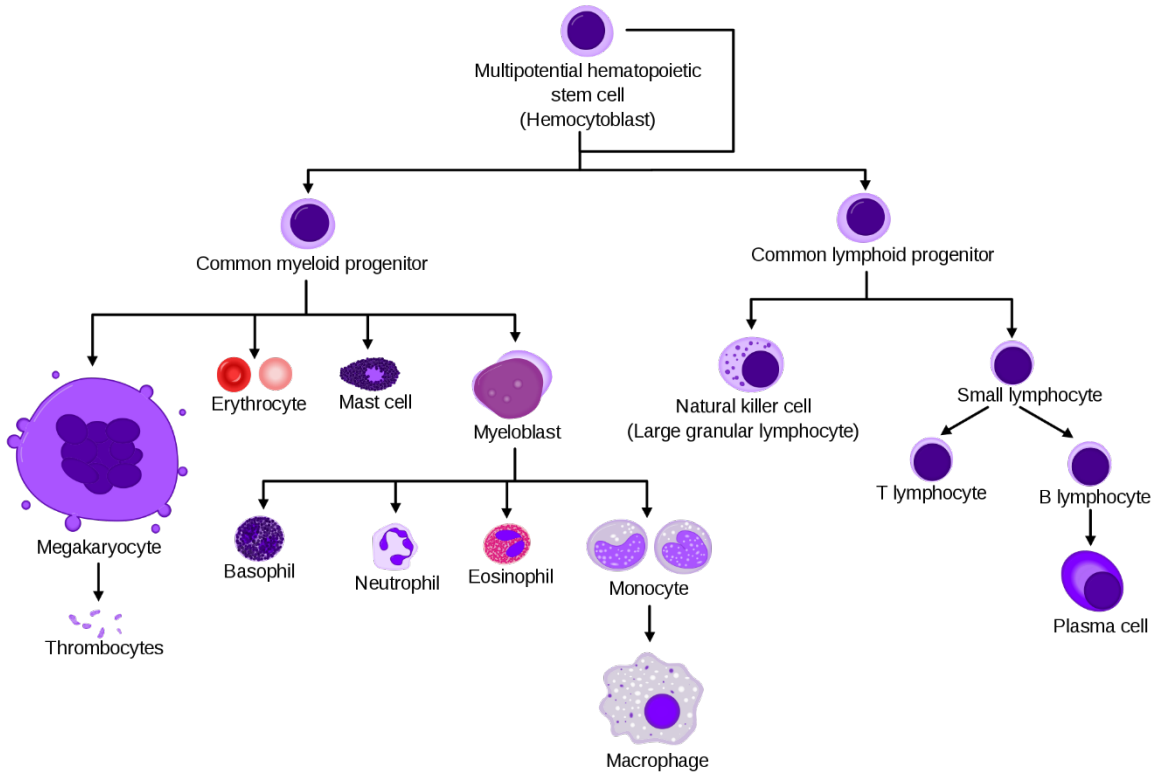

Figure 1: Haematopoiesis[7]

Multipotential hematopoietic stem cells differentiate into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) cells. CMP give rise to megakaryocytes, erythrocytes, mast cells and myeloblasts (and thus polymorphonuclear leukocytes), while CLP generate natural killer cells and lymphocytes.

Haematopoiesis occurs in the bone marrow and is tightly regulated by different circulating factors that stimulate the differentiation of specific types of blood cells. Some cells (like most myeloid cells) will terminally differentiate in the bone marrow cavity while most lymphatic cells will migrate towards primary and secondary lymphatic organs (thymus and lymph nodes) where they complete their development, maturation and activation[8].

In recent years it could be shown that the boundaries between different cell populations are not as clear-cut or distinct as previously stated. Haematopoiesis can rather be described as a continuous process that is undergone by overlapping cell populations[9,10].

2.3 Stem cell niche/ bone marrow microenvironment

As the bone marrow is the location for healthy and malignant haematopoiesis, the BMM plays a vital part in the development, persistence and resistance of different leukaemias to various therapies, such as chemotherapy, tyrosine kinase inhibitors etc.[11,12].

BMM is composed of a complex arrangement of different cell types such as bone marrow stroma cells, osteoblastic, osteoclastic, endothelial and mesenchymal stromal cells, neurons etc, along with different secreted cytokines and extracellular matrix components[13]. Physical properties like pH, ion or oxygen concentrations (**Figure 2**) are also reported to contribute to a functional BMM[14,15]. This niche supports HSCs and provides an environment in which these cells can undergo tightly regulated differentiation processes (i.e., haematopoiesis).

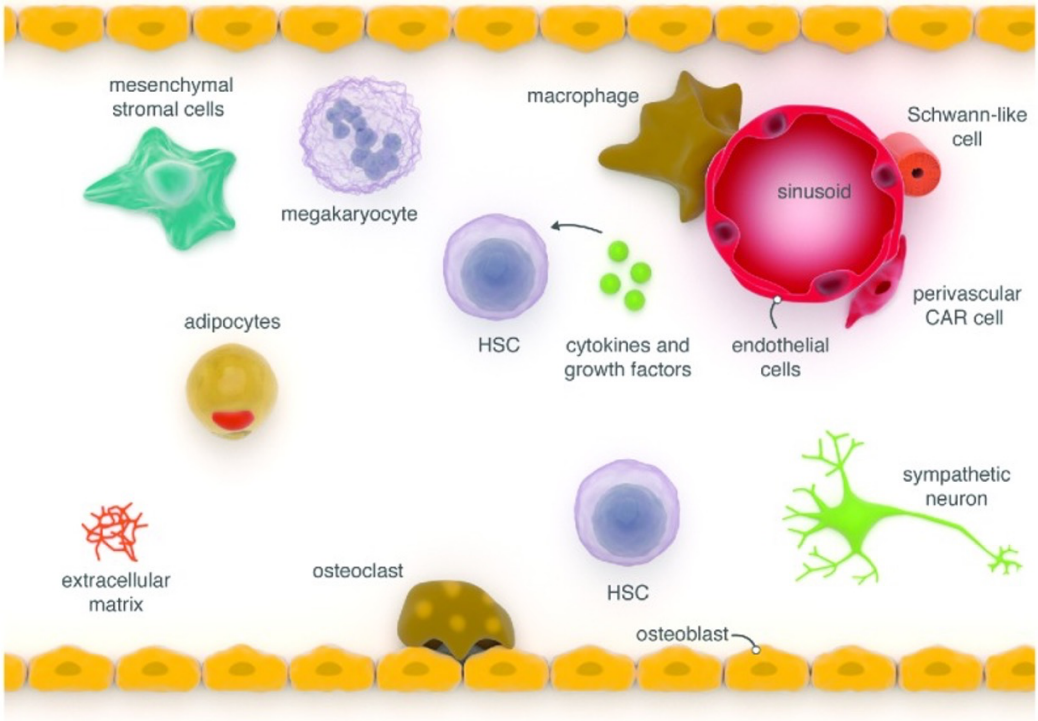

Figure 2: The healthy bone marrow (BM) niche[12]

The BM niche or microenvironment is composed of a variety of different cell types, among them mesenchymal stromal cells, osteoblasts and osteoclasts, macrophages, endothelial cells and neurons.

In the case of leukaemias the BMM protects LSCs from the influence of chemotherapeutic agents and tyrosine kinase inhibitors (TKI). BM-derived MSCs, for example, can increase adherence of human LSCs to the bone marrow niche and by that reduce the effect of TKIs[16] or up-regulate anti-apoptotic proteins in leukaemic cells[17].

By the modulation of the BMM in different types of leukaemias, it could be shown that the BMM can have important effects on LSCs depending on the type of leukaemia present.

Examples for these leukaemia-type-dependent interactions are the sensitivity of CML cells to BMM modulation by parathyroid hormone[11] and the protection of ALL cells from chemotherapy specifically by osteoblastic[18] and mesenchymal stromal cells[19,20]. These differences might, if further investigated, lead to the development of specific therapies that target the BMM directly instead of the LSCs.

These differential influences might also exist in the case of a paediatric BMM that sits within a bone that has not yet completed the process of growth when compared to that inside an adult and fully-grown bone.

2.4 Age-related development of bones and of haematopoiesis

Bone is a complex structure, which exists as long bone, short bone, flat bone and irregular bone. Bone consists of cortical or compact bone on the outside and spongy, trabecular bone tissue holding the bone marrow on the inside (Figure 3).

Bones undergo constant remodelling during life to adapt to changing biomechanical forces, to replace old, damaged bone with new and stronger bone and to grow during childhood and adolescence. Especially the ratio between the cortical bone tissue and the bone marrow cavity within changes during growth, with a relatively stronger expansion of the bone marrow tissue[21].

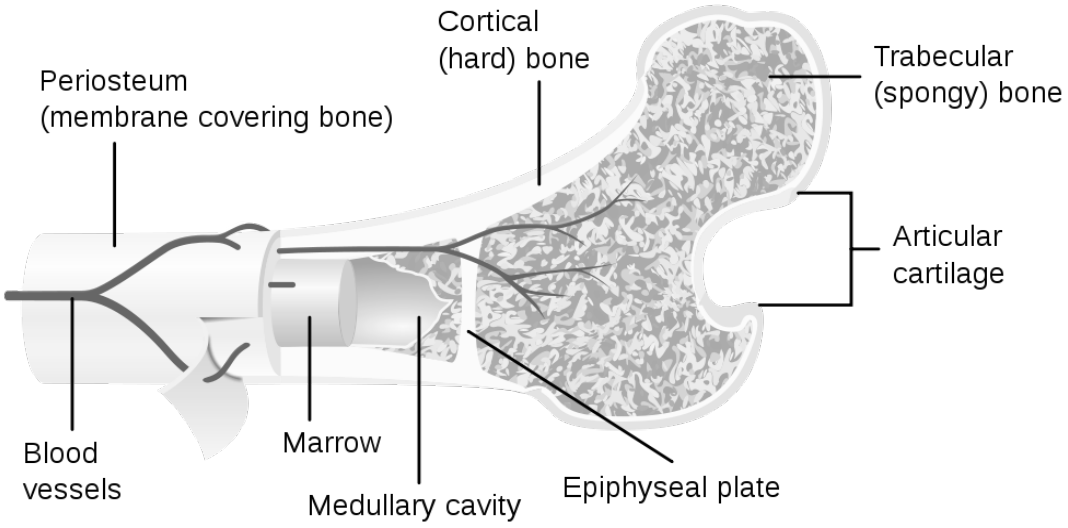

Figure 3: Bone anatomy[22]

Bones are highly perfused organs made of different tissues. The outer surface of the bone is called cortical (hard) bone, the interior is composed of trabecular (spongy) bone. A medullary cavity is formed in the shaft of long bones. In the medullary cavity and in the spaces between the spongiosa is the BM, where HSPC are found. The bone is surrounded by a connective tissue skin, the outer periosteum. On articular surfaces the bone is covered with cartilage.

Humans and rodents share the basic mechanism of bone growth. Longitudinal growth is achieved by enchondral bone formation at the growth plates while endocortical bone resorption and periostal bone formation lead to an increase of bone diameter. This process is regulated by sex hormones[23].

Cells that are involved in the growth process produce and secrete cytokines that further influence bone growth at other sites[24] but also influence other types of cells, such as cells responsible for angiogenesis[25] which is also critical for the functioning of the bone

Haematopoiesis is also influenced by aging. The rate of blood cell formation[26] and the potency of the immune system decline[27], myeloid-biased differentiation and the risk for myeloid malignancies increase[28], and anaemia frequently develops[29]. Aged long-term HSCs show an increased self-renewal and an increased expression of genes involved in myeloid lineage specification[30]. In recent large-scale studies, an increasing number of somatic mutations (associated with an increased risk of future haematologic cancer) in the cells of peripheral blood was detected with the highest incidence (18.4%) in individuals 90 to 108 years of age[31].

2.5 Leukaemia

Leukaemias are cancers of the haematopoietic system characterised by abnormalities of the leukocytes. They can be classified by the type of leukocytes affected (lymphoblastic/-cytic versus myeloid) and by the rate of the progression (chronic versus acute). All types of leukaemia show uncontrolled proliferation and accumulation of immature, cancerous leukocytes that lead to suppression of the formation of healthy and vitally needed blood cells. This again can lead to many symptoms related to the lack of healthy leukocytes (infections), thrombocytes (increased haemorrhaging) and erythrocytes (weakness and tiredness due to anaemia)[32]. In the UK leukaemia represents 3% of total cancer cases (in 2014) and has increased by 15% since the early 1990s[33]. The exact cause of leukaemia in most cases is unknown but some risk factors include “exposure to solvents, such as benzene and toluene, and unnecessary exposure to x-rays”[34] as well as viruses like the human T-lymphotropic virus[35].

2.6 B-ALL (B-cell Acute Lymphoblastic Leukaemia)

In B-ALL, B-cell lymphoblasts are overproduced in the bone marrow, interfering with healthy haematopoiesis. Leukaemic cells can impair physiological functions by infiltrating other organs and inhibiting the normal activity of the immune system leading to infections that make up most B-ALL-related deaths.

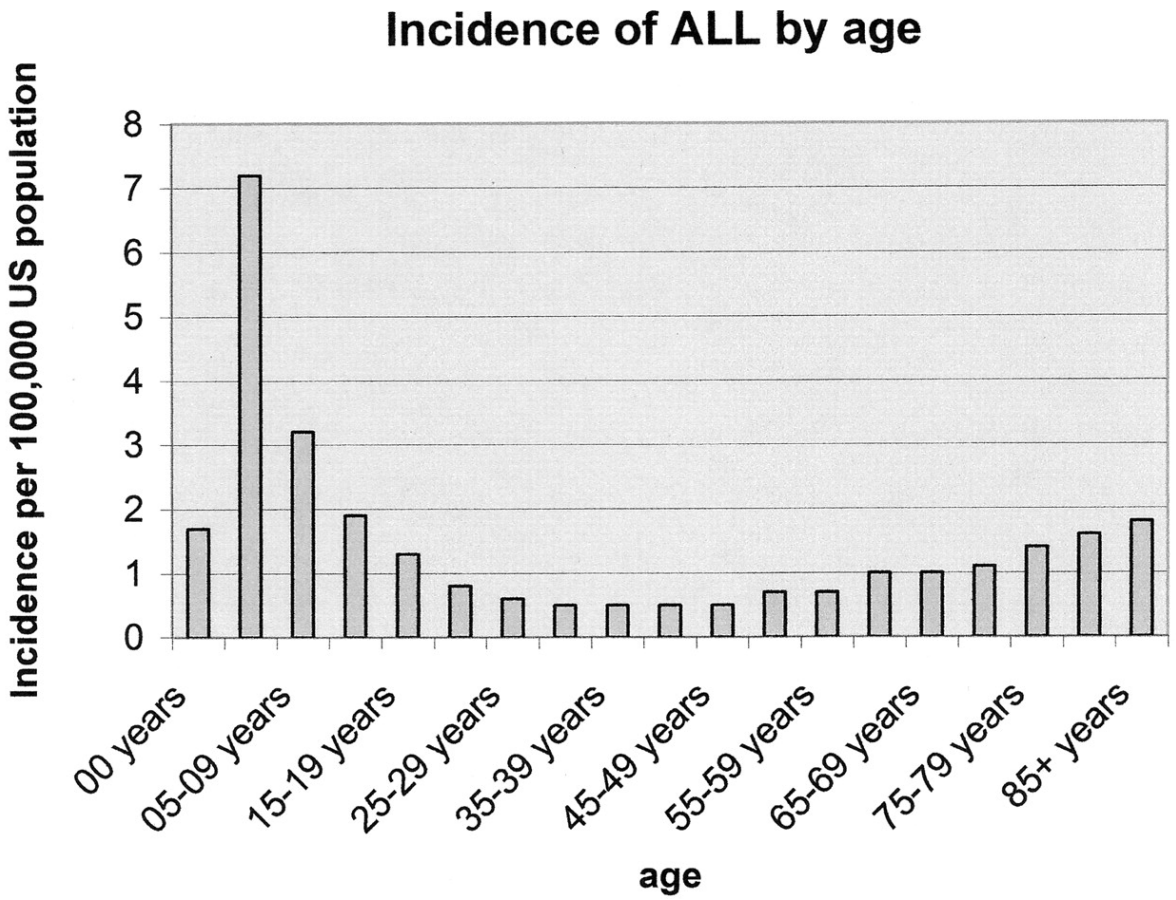

Figure 4: Incidence of acute lymphoblastic leukaemia (ALL) by age, SEER 1992–1999[36].

B-cell acute lymphoblastic leukaemia is the most common cancer in children[37] but relatively rare in adults. The incidence by age shows a peak between two and five years of age[38] (Figure 4). While some risk factors include high doses of radiation and exposure to some known carcinogenic substances, the majority of cases are most likely caused by a combination of genetic vulnerability, tumour oncogene activity (genes that can transform a cell into a cancer cell) and environmental factors[38]. One of these oncogenes is the result of a gene fusion, namely *BCR-ABL1*, which is due to a reciprocal translocation between chromosomes 9 and 22 and gives rise to the cytoplasmic, constitutively active tyrosine kinase BCR-ABL1. This kinase drives all cases of CML (see below) and 3% of paediatric B-ALL (and 25% of adult B-ALL)[39].

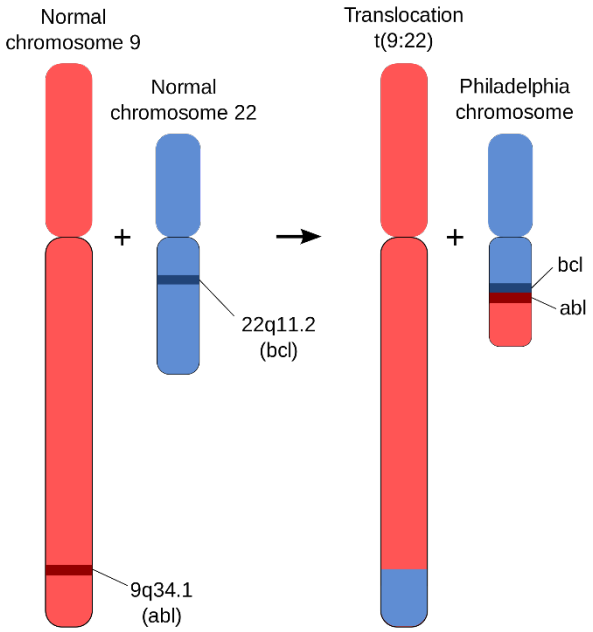

Figure 5. Translocation t(9:22)[1]

The introduction of the BCR-ABL1-targeting tyrosine kinase inhibitor (TKI) imatinib mesylate (IM) in 2001 has increased the 5-year survival rate of patients with CML to 90%[40], but responses in *bcr-abl*-positive B-ALL are much lower due to a higher frequency of mutations in the BCR-ABL1 kinase domain than in CML[41] and the non-eradication of leukaemic stem cells.

Using chemotherapy, and in the event of relapse stem cell transplantation, approximately 90% of cases of paediatric B-ALL can be cured[42] despite significant side effects. As approximately 10% of cases cannot be cured, novel strategies to overcome this disease need to be developed. For example, lower doses of chemotherapy could be combined with anti-cytokine therapy, or additional combination treatments can be used to help reduce systemic toxicity.

2.7 CML (Chronic Myeloid Leukaemia)

As the name suggests, cases of chronic myeloid leukaemia are characterised by the uncontrolled proliferation of myeloid cells of varying maturation stages. It progresses gradually ("chronic") and can be distinguished into three phases that are defined by the blast count and the intensity of symptoms[43]. Most patients present in the chronic phase (less than 5% blasts in peripheral blood) and show little to no symptoms. Without treatment, the disease will progress into the accelerated phase (less than 20% blasts) and further into blast crisis that shows features of acute leukaemias. Cases in chronic phase can often (more than 70% of cases)[44] be controlled by a TKI against BCR-ABL1 (see above) while in advanced stages or if TKI treatment is not effective, chemotherapy or stem cell transplantation may be utilised[43].

As opposed to B-ALL, CML is a disease of the elderly. Adults over the age of 50 represent 70% of all cases, and the average age at diagnosis of CML is around 64 years[45]. It is overall uncommon in children.

2.8 CXCL13 (C-X-C motif ligand 13)

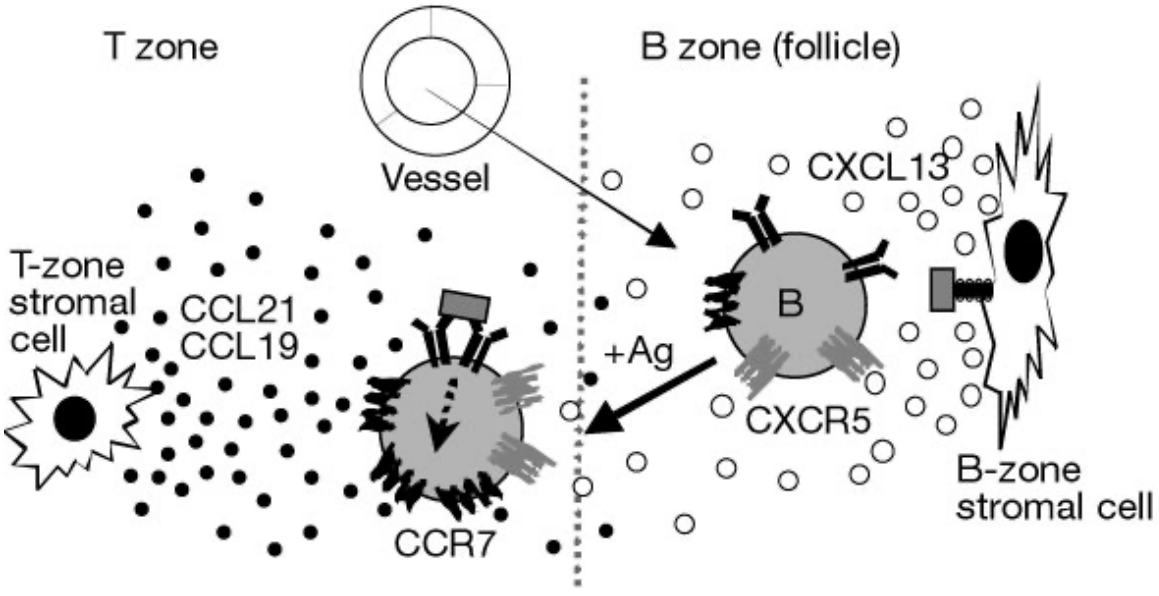

Figure 6: Schematic of B-cell movement into lymph node[46]

CXCL13 is a chemokine that induces cytotaxis in responsive cells. It has a strong attracting effect on B cells, organising their migrating into secondary lymphatic organs and the formation of germinal centres via the CXCR5 (spell out) receptor (see schematic)[47]. CXCR5 is also known as Burkitt lymphoma receptor 1 (BLR1) where it was originally identified and found to be highly expressed. The CXCL13/ CXCR5 axis also plays a role in systemic lupus erythematosus (SLE) by promoting the proliferation of mesangial cells[48] in the kidney??, in the pathogenesis of lupus nephritis [49] and in the early diagnosis of acute Lyme neuroborreliosis (LNB). In cases of LNB, CXCL13 can be found at elevated levels in the cerebrospinal fluid and is used as a marker for diagnosis[50].

Besides its chemoatractive properties, some studies could show that the "CXCL13-CXCR5 axis promotes the growth and invasion of colon cancer cells via PI3K/AKT pathway"[51] and that CXCL13 (and CXCl12) "significantly induce proliferation and collagen type I expression in osteoblasts from osteoarthritis patients"[52], thus indicating an influence on the growth and proliferation of various cell types other than B cells.

In chronic lymphocytic leukaemia (CLL), one group could show that the knockout of CXCR5 has a negative impact on the development of the disease in an Eμ-Tcl1 transgenic mouse line (an established model of murine CLL) and that CXCR5-negative Eμ-Tcl1 tumour cells have a gene expression prolife disadvantageous to cell proliferation[53]. On the one hand, this may be due to the localisation of CXCR5 positive cells within environments that have positive effects on the proliferation of the leukaemic cells. On the other hand, supplementing a steady co-culture of HK cells (a follicular dendritic cell line) and Eμ-Tcl1 leukaemia cell clones with CXCL13 could also improve proliferation significantly. Another paper on B-cell CLL could show that serum levels of CXCL13 were significantly elevated in CLL patients[54].

2.9 GDF11 (Growth Differentiation Factor 11)

GDF11 (Growth Differentiation Factor 11) is a cytokine of the TGF (Transforming Growth Factor)-β superfamily. It is homologous to myostatin (GDF8) and shares

some of its function and signalling pathways, using activin type II receptors that lead to the activation of the SMAD2/3 signalling components[55]. Myostatin is known to negatively control muscle tissue by enhancing protein degradation and limiting protein synthesis. Mutations in or loss of function of myostatin can lead to skeletal muscle hypertrophy[56]. GDF11 is important for regulating the anterior/posterior patterning of the axial skeleton during development[57] and plays a role in postnatal bone remodelling[58].

While some reports suggest a decline of circulating GDF11 with age[59], others have reported an increase in the systemic levels[60]. These different findings might be due to the non-sufficient specificity of the antibody against GDF11 used in some studies. This antibody is reported to also bind to GDF8, which is 500 times more abundant in the blood system[61].

It might still prove valuable to further investigate the role of GDF11 in processes related to aging, especially so in the haematological system.

2.10 TGF-β1 and leukaemia

Another member of the TGF-β superfamily, TGF-β1, has been shown to suppress leukaemic stem cells in chronic myeloid leukaemia[11]. In the experimental setup, parathyroid hormone was used to induce bone remodelling which released TGF-β1 from the extracellular matrix of the bone. This protein was shown to inhibit growth of CML cells *in vitro* and a knockdown of the receptor (TGFBR1) in BCR-ABL-expressing cells accelerated the development and increased the overall incidence of fatal CML-like MPN (spell out) in mice with increased bone turnover[11].

2.11 Preliminary data

The following data was obtained by other members of the host-laboratory prior to the project described in this thesis.

2.11.1 Induction of B-ALL and CML in young versus old mice

Using a retroviral transduction-transplantation-model of B-ALL and CML (manifested as a CML-like myeloproliferative neoplasia (MPN)) the survival of the transplanted animals and the behaviour of these leukaemias was analysed in young versus old mice.

CML (-like MPN) can be induced in recipient mice transplanted with donor bone marrow previously treated with 5-fluorouracil (5-FU) and transduced twice with BCR-ABL1 expressing retrovirus. In contrast, BCR-ABL1+ B-ALL can be reliably induced in recipient mice by transducing the donor BM of mice not previously treated with 5-FU with the same BCR-ABL1 expressing retrovirus[62] and transplanting this into recipient mice. Mice were considered young before completion of sexual maturation (around day 35[63]), matured once they had completed puberty after three months of age and old after 18 months. Consequently, cohorts of three to four-week-old mice were used as recipients and compared to groups of at least 1.5-year-old mice. All following experiments were designed according to these age-groups.

Resembling human disease incidences, young mice show a faster induction of B-ALL and show significantly shorter survival in this model (Figure 7), while in CML-like MPN their survival is significantly prolonged compared to old mice (Figure 8).

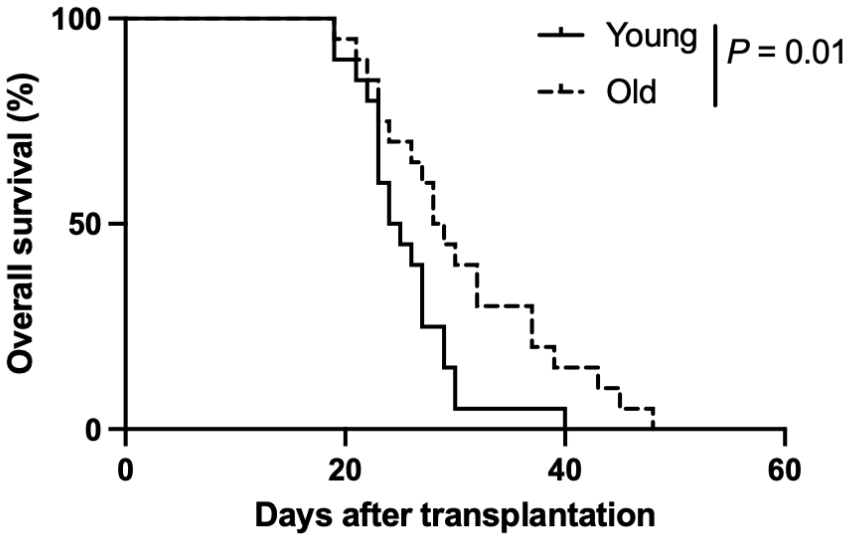

Figure 7: Survival of young versus old mice after induction of B-ALL

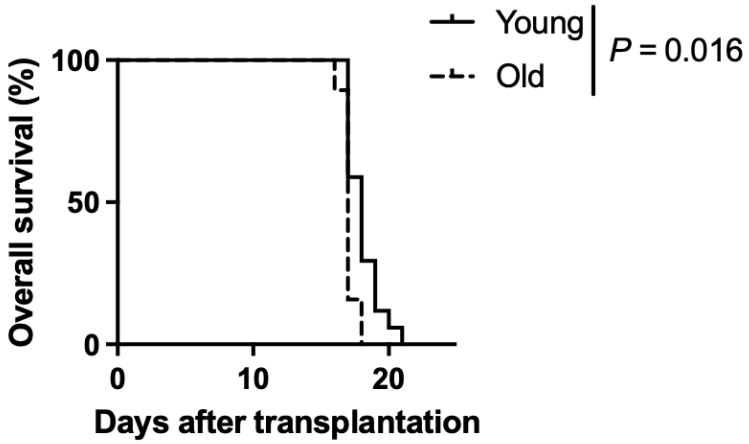

Figure 8: Survival of young versus old mice after induction of CML

The data suggest that the differences in survival must be explained by the recipients' age and specifically differences in their bone marrow microenvironment (Figure 8). Furthermore, examining the bone marrow cavity of young recipients of BCR-ABL1+ bone marrow in the CML model showed that it was not hypercellular with excess of myeloid cells as expected for CML (Figure 9). Instead, the bone marrow was hypocellular and infiltrated by an eosinophilic mass which has previously been associated with non-efficient induction of CML in this model[11].

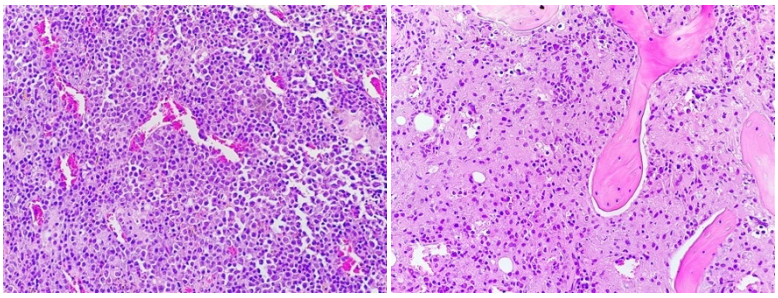

Figure 9: Haematoxylin & Eosin staining of a femur from representative old (left) and young (right) recipients of BCR-ABL1-transduced bone marrow in the retroviral transduction/transplantation model of CML.

Using Southern blotting to assess the number of virus-positive clones in the spleen, representing engrafted leukaemia-initiating cells[11], it was demonstrated that the number of clones was significantly decreased in young compared to old recipients of BCR-ABL1-transduced bone marrow in the CML model. This suggests that the microenvironment in old mice supports different disease-initiating clones while only few CML-clones can propagate in young mice.

Consistently, the number of myeloid colonies in methylcellulose containing only interleukin (IL)-3 (selectively promoting the growth of BCR-ABL1+ cells) was decreased when the bone marrow was derived from young compared to old mice with CML (data not shown). In case of cells derived from spleens, the number of total colonies was increased for young compared to old mice, while the number of myeloid colonies was unchanged. This suggests that myeloid progenitors are reduced selectively in the bone marrow of young mice with CML.

3. Hypothesis

Taking together the facts discussed above and the preliminary data, our hypothesis for this thesis was:
A young (growing) versus old (grown-up) bone marrow microenvironment differentially influences CML versus B-ALL development by release of the cytokines CXCL13 and GDF11 from the bone marrow stroma.

This medical doctorate project has focused on addressing questions about the influence of a young versus old bone marrow microenvironment on the development and the persistence of (paediatric and adult) B-cell lymphoblastic leukaemia (B-ALL) and chronic myeloid leukaemia (CML). Attention was given especially to the role of the two cytokines CXCL13 and GDF11 in the context of B-ALL and CML.
For this project, different *in vitro* cell culture techniques and quantifying assays (RTqPCR and ELISA) were used to investigate the behaviour of leukaemic model cell lines in the presence of young versus old bone marrow stroma cells and the expression levels of previously mentioned cytokines.

4. Experiments

4.1 Materials and chemicals

4.1.1 Equipment

Table 1: List of used equipment

Device	**Name/ Manufacturer**
Centrifuge	Hettich (Tuttlingen) Zentrifugen: Rotanta 460R VWR (Darmstadt) Mini Star
Pipette Controller	Integra Pipetboy 2
Pipettes	Gilson Pipettes 0,5-2 µL 1-10 µL 2-20 µL 50-200 µL 100-1000 µL
Heat block	Thermomixer Basic Cell Media (Elsteraue)
RTqPCR “Step One Plus”	Applied Biosystems (Darmstadt, GER)
Animal Blood Counter (Vet^{abc})	Scil (Viernheim, GER)
Flow cytometer “Calibur”	BD Biosciences (Heidelberg, GER)
Flow cytometer “Fortessa”	
Flow cytometer and –sorter “Aria”	
Flow cytometer and –sorter “Fusion”	
PCR T100TM Thermal Cycler	BioRad (Munich, GER)
Elisa Plate Reader	SpectraMax

4.1.2 Kits

Table 2: List of used kits

RNA Isolation kit (RNeasy Mini Kit)	Qiagen (Hilden, GER)
Invitrogen SuperScript III Platinum (RTqPCR)	Life technologies (Darmstadt, GER)
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems (Waltham, USA)
ProtoScript® First Strand cDNA Synthesis Kit	New England Biolabs (Ipswich, USA)
CXCL13 ELISA Kit	BioLegend (San Diego, USA)

4.1.3 Buffers and media

Table 3: List of used buffers and media

ACK lysis buffer	Life Technologies (Darmstadt, GER)
Phosphate buffered saline (PBS)	Life Technologies (Darmstadt, GER)
FACS buffer	500ml PBS 15ml FCS 2mM EDTA
HBS	8,0g NaCl 0,37g KCl 106,5mg Na_2HPO_4 1g dextrose 5g HEPES (1M) 450ml dH_2O
DMEM	Life Technologies (Darmstadt, GER)

RPMI	Life Technologies (Darmstadt, GER)
MEM α	Life Technologies (Darmstadt, GER)
293T (human embryonic kidney epithelial cells) medium	500ml DMEM 50ml FCS 10ml L-glutamine (xxxg/ml) 5ml NEAA 5ml P/S (100 U/mL penicillin; 100 µg/mL streptomycin)
3T3 (murine fibroblast cells) medium	500ml DMEM 50ml FCS 5ml L-glutamine (xxxg/ml) 5ml P/S (100 U/mL penicillin; 100 µg/mL streptomycin)
Ba/F3 (murine pro B-cells)/ 32D (murine bone marrow stroma cells) medium	500ml RPMI 50ml FCS 5ml L-glutamine (xxxg/ml) 5ml P/S (100 U/mL penicillin; 100 µg/mL streptomycin)
BMSC (MS-5) cell medium	500ml MEM α 100ml FCS 10ml L-glutamine (xxxg/ml) 5ml P/S (100 U/mL penicillin; 100 µg/mL streptomycin)

4.1.4 Antibodies

Table 4: List of used antibodies

	Application	**Antibody**	**Manufacturer**

	FACS	Mac1/CD11b-PE	BioLegend (Fell, GER)
lineage cocktail (depletion)	primary	Ter119-biotin	BD Biosciences (Heidelberg, GER)
		B220-biotin	
		Gr-1/Ly6G-biotin	
		CD5-biotin	
		F4/80-biotin	eBioscience (Vienna, AUS)
		BP1-PE	BD Biosciences (Heidelberg, GER)
		CXCR5-APC	BD Biosciences (Heidelberg, GER)
		TGFβR1-PE	
Neutralising		CXCL13 (MAB470)	R&D Systems (Minneapolis, USA)
		GDF11 (MAB19582)	
		Control: Mouse IgG1 (X0931)	Dako/ Agilent Technologies (Santa Clara, USA)

4.1.5 RTqPCR primers

Table 5: List of primers used for RTqPCR

Gene		**Sequence**	**Notes**
GAPDH	forward	-AGG TCG GTG TGA ACG GAT TTG-	murine, housekeeping gene
	reverse	-GGG GTC GTT GAT GGC AAC A-	
CXCL13	forward	-CTC CAG GCC ACG GTA TTC TG-	murine

	reverse	-CCA GGG GGC GTA ACT TGA AT-	
GDF11	forward	-GCA CCC CTA CCA GAT GTC C-	
	reverse	-CCA CAA CTT AGG AGC AGC CA-	

4.1.6 Chemicals

Table 6: List of used chemicals

Compound	**Manufacturer**	**Catalog Number**
ACK-Lysis Buffer	Gibco/ Thermo Fisher Scientific (Dreieich)	183644
CaCl2	Sigma Aldrich (Taufkirchen)	C1016
Chloroquine	Sigma Aldrich (Taufkirchen)	BCBK7067V
Dextrose	Sigma Aldrich (Taufkirchen)	SLBH3471V
DMEM	Life Technologies (Dreieich)	41966-052
Ethanol	Carl Roth (Karlsruhe)	166243011
HCl	Sigma Aldrich (Taufkirchen)	H1758
Isopropanol	Carl Roth (Karlsruhe)	236244487
KCl	Sigma Aldrich (Taufkirchen)	SLBH5524V
L-Glutamine	Gibco/ Thermo Fisher Scientific (Dreieich)	25030024
MEM alpha	Life Technologies (Dreieich)	22561-054

MEM Non-Essential Amino Acids Solution	Life Technologies (Dreieich)	11140-035
Mercaptoethanol	Sigma Aldrich (Taufkirchen)	M6250
Na_2EDTA	Sigma Aldrich (Taufkirchen)	
Na_2HPO_4	Sigma Aldrich (Taufkirchen)	BCBN2526V
NaCl	Sigma Aldrich (Taufkirchen)	SLBR1013V
NaOH	Applichem (Darmstadt)	R13454
Pen/ Strep	Gibco/ Thermo Fisher Scientific (Dreieich)	15149-122
Polybrene (Hexadimethrine Bromide)	Sigma Aldrich (Taufkirchen)	127H3691
Trypsin EDTA 0.05%	Gibco/ Thermo Fisher Scientific (Dreieich)	25300-054

4.1.7 Disposable laboratory supplies

Table 7: List of used disposable laboratory supplies

Product	**Manufacturer**
Cell culture dishes	Greiner Bio-One GmbH (Frickenhausen)
Cell scraper	Greiner Bio-One GmbH (Frickenhausen)

Cell strainer	Greiner Bio-One GmbH (Frickenhausen)
Conical tubes	Greiner Bio-One GmbH (Frickenhausen)
FACS tubes	Greiner Bio-One GmbH (Frickenhausen)
Multiple well plates	Corning (Corning, New York, USA)
Transwell® polycarbonate membrane cell culture inserts	Corning (Corning, New York, USA)

4.2 Methods

4.2.1 Extraction of RNA

RNA was used in quantitative real-time polymerase chain reactions (RTqPCR) to analyse (RNA-)expression levels of different proteins.

RNA from cell pellets was extracted following the manufacturer's protocol for "Qiagen RNeasy Mini Kit" or "Qiagen RNeasy Micro Kit" depending on the number of cells used.

4.2.2 Reverse transcriptase

RNA was extracted using Qiagen RNeasy Micro or Mini Kits and in case of low output reverse transcriptase was performed to increase the availability of cDNA in the following (two step) RTqPCR.

cDNA was created using "ProtoScript® First Strand cDNA Synthesis Kit" according to the manufacturer's recommendations.

4.2.3 RTqPCR

For RTqPCR analysis samples were prepared in three technical replicates and pipetted into 96-well plates.
When RNA was used, the following reaction was set up:

100 ng	RNA in 7,6µl dH_2O
0.4 µl	SuperScript® III Reverse Transcriptase
10 µl	2xSYBR green
1 µl	forward primer (2mM)
1 µl	reverse primer (2mM)

When cDNA was used, the following reaction was set up:

8 µl	cDNA in dH_2O
10 µl	2xSYBR green
1 µl	forward primer (2mM)
1 µl	reverse primer (2mM)

The RTqPCR was run on Applied Biosystems's "One Step Plus" and the results analysed using the $\Delta\Delta C_t$ method.

4.2.4 Extraction of plasmid DNA

Plasmid DNA is needed for transfection of the packaging cell line and subsequent virus production. Bacteria carrying either packaging plasmids or plasmids expressing a gene of interest were cultured and expanded in lysogeny broth overnight to which ampicillin (100 mg/mL; the plasmids transfer ampicillin resistance) had been added.

Plasmid DNA from bacteria was extracted using "QIAGEN Plasmid Maxi Kit" and the concentration of DNA measured on a "NanoDrop 1000".

4.2.5 Virus production by CaPO4 transfection of 293T cells

Retroviruses are used to deliver genes of interest into different cell types. They use their own reverse transcriptase to transcribe their RNA genome into DNA which is inserted into the host's genome by an integrase.

Retro- (MIG p210 containing BCR-ABL, empty vector) and lentiviruses (shRNA-expressing viruses) were produced by calcium-phosphate (CaPO4) transfection of 293T cells. The day prior to transfection $4*10^6$ 293T cells were plated on a 6 cm dish in 4 ml 293T-medium (see 4.1.3), achieving 80% confluence on the day of transfection.
Immediately before transfection, the medium was removed and replaced by 4 ml fresh 293T-medium to which the following mixture was added:

lentivirus

	7.5 µg	DNA with gene of interest (shRNA), including GFP
	1.5 µg	VSV plasmid
	3.75µg	Δ8.9 plasmid
438 µl		H_2O_{dd}
62µl		2M $CaCl_2$
500µl		HBS

retrovirus

	10 µg	DNA with gene of interest (MIG-p210 or empty vector including GFP)
	5 µg	EcoPak plasmid
438 µl		H_2O_{dd}
62µl		2M $CaCl_2$
500µl		HBS

The following day the medium was removed and replaced by 2.5 ml fresh 293T-medium, which was harvested 24 hours later, filtered through 0.2 µm strainers and frozen in 2ml aliquots.

4.2.6 Virus production by polyethylenimine (PEI) mediated transfection of 293T cells

Lentiviruses (shRNA-expressing viruses) were produced by polyethylenimine (PEI) transfection of 293T cells. The day prior to transfection $5*10^6$ 293T cells were plated on a 10 cm dish in 15 ml 293T-medium, achieving 80% confluence on the day of transfection.
Prior to tranfection, the old medium was removed and replaced by 6.5 ml fresh 293T-medium to which the following mixture was added:

60 µl	PEI-Max (1mg/ml)
1000 µl	DMEM (supplemented with 1% P/S, 2% L-Glu)
10 µg	DNA with gene of interest (shRNA), including GFP
6.5 µg	p8.91 plasmid
3.5 µg	pMD2.G plasmid

48 hours after transfection the supernatant was collected, filtered through 0.2 µm strainers and centrifuged at 51610 rcf at 4°C for 2:20 hours to concentrate the virus. The pellet was resuspended in an appropriate amount of medium and frozen to -80°C in 25 µl aliquots.

4.2.7 Titration of virus by transduction of 3T3 cells

In order to assess the transduction efficiency, i.e., the titre, of a given batch of virus-containing cell culture supernatant (or "virus"), different concentrations of this supernatant were used to transduce 3T3 cells. Because all virus constructs in our experiments contain genes that encode fluorescent proteins (either GFP or RFP

spell out), the proportion of fluorescent protein expressing cells can then be used to approximate the concentration of virus particles in the supernatant of the packaging cells (293T).

The day prior to transduction $2*10^5$ 3T3 cells were plated on a 6 cm dish in 4 ml 3T3-medium (see 4.1.3). The following day medium was removed, 1:3 and 1:30 dilutions of the virus (supplemented with 800 µg/ml polybrene to facilitate the uptake of the virus) were prepared and added to the plates. The medium was diluted after 5 hours of transduction and left in an incubator for 3 days. Transduction efficiency was measured by analysing the percentage of GFP- or RFP-positive cells by flow cytometry, using an uninfected sample of 3T3 cells as negative control. The concentration of transducing units was then calculated based on Poisson statistics. A titre of at least 35% GFP+ cells at a 1:30 dilution was considered usable.

4.2.8 Isolation of bone marrow stroma cells

Bone marrow stroma cells (BMSC) are used as a simple 2D-cell culture-model for the bone marrow microenvironment.

BMSC were isolated by crushing femur, tibia, pelvis and humerus of mice in PBS using mortar and pestle. PBS containing cells and bone material was then filtered through a 100 µm strainer, spun down, resuspended in BMSC culture medium (MEM alpha with supplements; see 4.1.3) and cultured in T75 tissue culture flasks. The medium was replaced every 2 to 3 days.
Conditioned medium was harvested after 48 hours and stored at -80°C.
When the cells reached confluence after 7 to 10 days, they were used for experiments. Later, the immunophenotype of the BMSC was shown to correspond predominantly to F4/80+/ CD169+ macrophages[64].

4.2.9 Knockdown of CXCL13 and GDF11 in bone marrow stroma cells by transduction with shRNA-expressing lentivirus

shRNA-expressing lentivirus is used to knock down specific proteins in the transduced cells. The shRNA interferes with the mRNA of the given protein and prevents its translation.

400,000 bone marrow stroma cells are plated per well in a 6-well plate. The following day, old medium was removed and replaced by the supernatant of transfected cells (containing the virus particles), diluted in BMSC culture medium supplemented with 800 µg/ml polybrene. Six hours later, the transduction medium was diluted six-fold and completely replaced by fresh culture medium on the following day. On the third day, cells were sorted by FACS for expression of GFP, a non-transduced sample serving as a GFP-negative control.
Cells were further cultured and expanded with a density of approximately 2500 cells/cm^2.

4.2.10 Sorting of Nestin+ mesenchymal stromal cells, Col2.3+ osteoblastic cells and Tie2+ endothelial cells

Different transgenic mice, so-called reporter mouse lines express a fluorescent protein under the control of a cell-type specific promotor, making it possible to sort for these cells by FACS. For expression analysis, transgenic mice with either GFP-labelled mesenchymal stromal cells, osteoblastic or endothelial cells were used because these cell types represent the most important sub-niches in the bone marrow microenvironment.

Transgenic Nestin-GFP+ (green fluorescent mesenchymal stromal cells), Col2.3-GFP+ (osteoblastic cells) or Tie2-GFP+ (endothelial cells) mice were sacrificed; femur, tibia, pelvis, spine and humerus removed, crushed in PBS (supplemented

with 2% FCS), filtered through 70 µm strainers and spun down. Red blood cells were lysed using ACK lysing buffer and the remaining cells resuspended in FACS buffer. Lineage positive cells were depleted with streptavidin-beads after staining with lineage cocktail (biotin-associated antibodies against lineage positive cells, i.e., Ter119, B220, Gr-1/ Ly6G, CD5 or F4/ 80 positive). Additional negative staining was performed with CD31- (not when sorting for endothelial cells)/ CD45- and Ter119-PE antibodies. The GFP+ PE- cells, representing MSCs, osteoblasts or endothelial cells, respectively, were sorted directly into *RLT plus buffer*,

4.2.11 Cell cycle staining

The cell cycle status of cells in each population can give further information on their proliferative behaviour.

The cell pellet was incubated for 15 minutes at room temperature with 50 µl of *BD Fixation/ Permeabilisation solution*, washed with 1 ml *BD Perm/Wash™ Buffer*, and the pelleted cells were stained with Ki-67 antibody at 4°C overnight. The following day, DAPI was added, achieving a final concentration of 5 µg/ml, and the cells were analysed using flow cytometry.
DAPI binds to DNA, its fluorescent signal is proportional to the amount of DNA in the cell. Cells within the G2- or M-phase of the cell cycle have replicated their complete genome and will show a fluorescent signal twice as strong as cells in G1- or G0-phase. S-phase cells show a signal somewhere between these two peaks. The additional staining with Ki-67 can identify cells that are actively dividing[65].

4.2.12 Apoptosis staining

During apoptosis, i.e., programmed cell death, cells show higher amounts of phosphatidylserine on their surface, which can be bound by a fluorescently tagged antibody to Annexin V. In combination with analysing DNA fragmentation by DAPI, the percentage of apoptotic and necrotic cells can be calculated after flow cytometry.

Increased numbers of apoptotic and necrotic cells suggest presence of cytotoxic stimuli or insufficient availability of cell supporting factors.

The cell pellet was incubated for 30 minutes on ice with 50 µl of Annexin V antibody, diluted in Annexin V Binding buffer and washed with Annexin V Binding buffer. DAPI was added at a dilution of 1:2500. The cells were analysed using flow cytometry.

4.2.13 Infection of BA/F3 and 32D cells with MIG-p210-, MIR-p210- or empty vector-virus

BA/F3 is a murine pro-B, and 32D a murine myeloblast-like cell line. Transduced with BCR-ABL-expressing retrovirus they can be used to model murine BCR-ABL1+ B-ALL or CML, respectively.

$5*10^6$ cells were resuspended in the transduction mixture, containing

2 ml	BA/F3-/ 32D-medium
2 ml	virus solution (supernatant from transfected cells) with at least 20% transduction efficiency in 3T3
40 µl	HEPES
10 µl	Polybrene (2 µg/ml)

Then the cells were plated in 6-well plates and “spinfected” in a centrifuge at 2000 RPM at 32°C for 90 minutes. After that they were resuspended and incubated for 2 hours at 37°C. Cells were then pelleted down and resuspended in BA/F3-/ 32D-medium. Because of a lower transduction efficiency in the case of 32D cells, this procedure was repeated daily for 4 consecutive days. After 4 days in culture, cells were sorted for either GFP- or RFP-positivity and further expanded in BA/F3-/ 32D-medium.

4.2.15 FACS staining

Cells for FACS analysis were pelleted and resuspended in cold FACS buffer containing the antibody (or antibodies) of interest in the appropriate dilution and stained in the dark at 4°C for 30 minutes. The cells were then washed with 2 ml of cold PBS, pelleted again and resuspended in the appropriate amount (usually around 300 µl) of cold FACS buffer.

4.2.16 Migration assay

The differential migratory behaviour of cells through a very fine mesh (in this case with 5µm pore-size) towards a monolayer of either young or old bone marrow stroma cells can indicate the presence or absence of cell-attracting factors or cytokines.

In order to perform the migration essay, 50,000 young versus old bone marrow stroma cells in stroma cell medium were plated into 24-well plates and allowed to grow until subconfluence. Corning® Transwell® polycarbonate membrane cell culture inserts were put into the wells, which were then filled with 100 µl low-FCS medium (i.e., 2% FCS). This medium contained 100,000 BCR-ABL1+ BA/F3 or BA/F3 empty vector cells as control in triplicates. Migration was analysed after 4, 8, 12 and 24 hours by carefully re-suspending the non-adherent cells in the lower compartment, taking photos at three random locations per well and counting the migrated BCR-ABL1+ or empty vector BA/F3 cells. This procedure was chosen, because especially at the first time-points the cell-density was not high enough for counting with a haemocytometer.

4.2.17 Adhesion assay

Adhesion between cells is mediated by different cell-surface and extracellular proteins and occurs among cells of the same type or between different cell types. It plays in important role in the bone marrow, where HSCs and LSCs adhere to the

surrounding niche that protects and stimulates them (see introduction). Differences in adhesion might therefore also play a role in the differential protection of distinct leukaemias by young versus old bone marrow stroma.

In order to perform the adhesion assay, 30,000 young and old BMSC were plated in 48-well plates and allowed to grow to subconfluence. 100,000 BCR-ABL1+ BA/F3, empty vector BA/F3, BCR-ABL1+ 32D or empty vector 32D cells were added per well, gently spun down onto the BMSC by centrifugation for 5 minutes at 1000 RPM and left in an incubator for 6 hours. Non-adherent cells were then removed, three photographs were taken at random positions in each well and the number of adherent cells was counted.

4.2.18 Expansion analysis by co-culture of bone marrow stroma cells with BA/F3 and 32D cells

Co-culturing bone marrow stroma cells and leukaemic cell lines is a basic in vitro *tool to mimic the interaction of leukaemic cells and the surrounding cells of the bone marrow microenvironment. The expansion or proliferation of leukaemic cells in the presence of young versus old bone marrow stroma cells can be analysed by counting the non-adherent cells.*
The co-culture can further be manipulated by adding cytokine-neutralising antibodies that prevent the supportive or non-supportive effect of cytokines, giving insight into the function or importance of these factors.
Another modification is the lentiviral, shRNA-mediated knockdown of these cytokines in the bone marrow stroma cells prior to co-culturing them with the leukaemic cells.

50,000 young versus old bone marrow stroma cells in BMSC culture medium (see above) were plated into 24-well plates and allowed to grow until subconfluence. 30,000 BCR-ABL1+ BA/F3, empty vector BA/F3, BCR-ABL1+ 32D and empty vector 32D cells in BMSC culture medium were added in triplicates. The following days 10

µl of carefully resuspended supernatant containing the non-adherent cells was taken, stained with trypan blue and counted in a haemocytometer.

When neutralising antibodies were used, these were added to a final concentration of 2.5 µg/ml.

5. Results

5.1 BCR-ABL1+ BA/F3 cells proliferate more on young bone marrow stroma cells

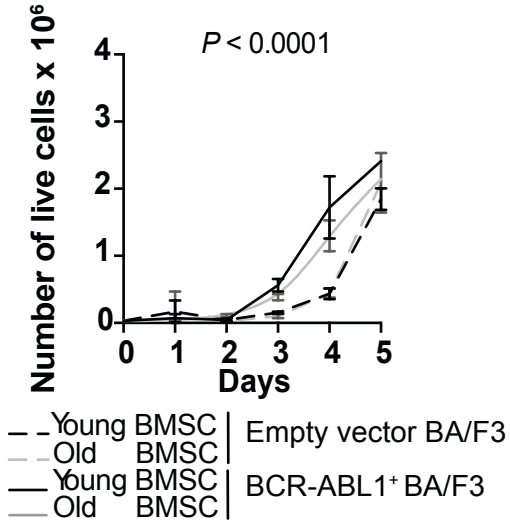

Figure 10: BCR-ABL+ BA/F3 cells proliferate more on young compared to old bone marrow stroma cells (BMSC).

Number of empty vector (dashed line)- or BCR-ABL1 (solid line)-transduced BA/F3 cells plated on bone marrow stroma cells from young (black) versus old (grey) mice (n = 3). P-*value as indicated (two-way ANOVA).*

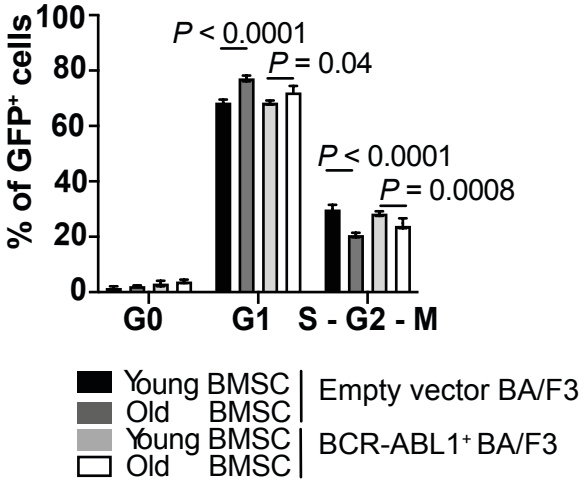

Figure 11: An increased proportion of empty vector and BCR-ABL+ BA/F3 cells can be found in the (S)-G2-M fraction of the cell cycle when plated on young bone marrow stroma cells (BMSC).

Cell cycle analysis by Ki67 and DAPI staining of empty vector- or BCR-ABL1-transduced BA/F3 cells plated on bone marrow stroma cells from young versus old mice (n = 3). Cell cycle analysis was performed on day 5. P-*values are as indicated.*

To test our hypothesis that the age of the BMM influences the growth of leukaemic cells, bone marrow stroma cells (BMSC) isolated from young and old mice were co-cultured with leukaemic and non-leukaemic murine pro-B cells, i.e., BCR-ABL1+ BA/F3 (retrovirally transduced with BCR-ABL1-expressing virus) and empty vector transduced BA/F3 cells.

It could be shown that the BCR-ABL1+ pro-B cells (BA/F3) proliferate significantly more when plated on young BMSC compared to cells cultured on old BMSC (Figure 10).

Additionally, analysis of the cell cycle showed that a significantly higher proportion of BA/F3 cells plated on young BMSC are within the proliferative phases of the cell cycle (S-G2-M) compared to cells cultured on old BMSC (Figure 11) suggesting a faster proliferation and complementing our proliferation assay (as mentioned above).

5.2 Old bone marrow stroma cells might support the proliferation of BCR-ABL1+ 32D cells

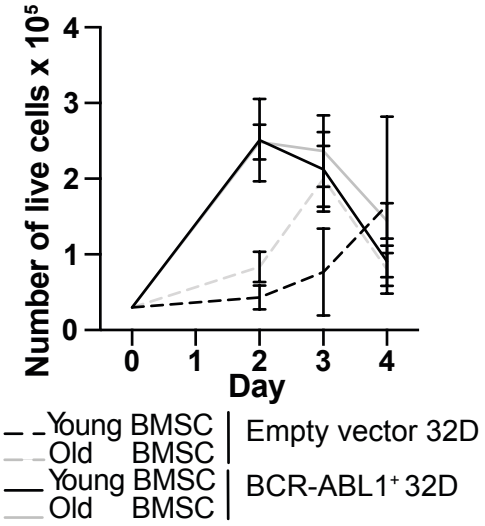

Figure 12: The number of BCR-ABL1+ 32D cells might be higher when cultured on old bone marrow stroma cells (BMSC).

Number of empty vector (dashed line)- or BCR-ABL1 (solid line)-transduced 32D cells plated on BMSC from young (black) versus old (grey) mice (n=3). 3 x 10^4 32D cells had been plated.

Since we also hypothesised that the occurrence of CML mainly in the elderly population could party be explained by differences in the interaction with an aged BMM, we cultured the (myeloblast-like) CML cell line, BCR-ABL1+ 32D cells, on young versus old BMSC, and the number of BCR-ABL1+ 32D cells was assessed over time.

We observed a trend towards BCR-ABL1+ 32D cells being found in higher quantity when cultured on old compared to young BMSC (Figure 12).

5.3 Conditioned medium from young bone marrow stroma cells increases the proliferation of BCR-ABL1+ BA/F3 cells

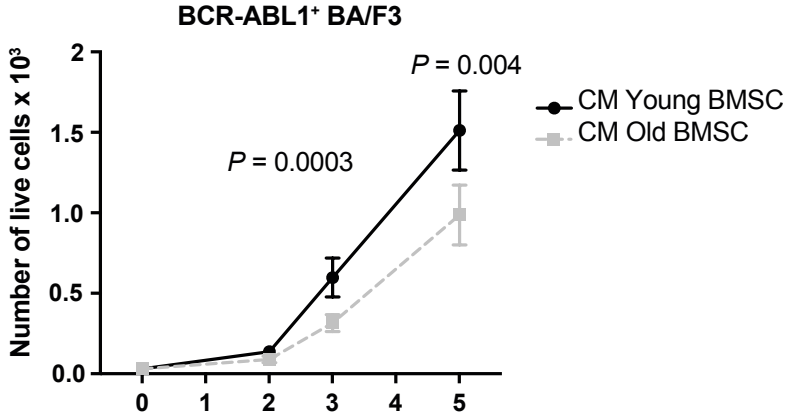

Figure 13: Leukaemic cells proliferate more when cultured in conditioned medium derived from young bone marrow stroma cells (BMSC).

Number of BCR-ABL1-transduced BA/F3 cells grown in the conditioned medium (CM) of BMSC from young (black) versus old (grey) mice (after 4 days, n = 3). P*-values as indicated.*

Considering the proliferation advantage of BCR-ABL1+ BA/F3 cells on young BMSC we hypothesised that soluble factors released differentially by young versus old BMSC could be responsible for the observed phenotype in Figures 10 and 11.

Therefore, cell culture supernatant (conditioned medium) from young versus old BMSC was collected. BCR-ABL1+ BA/F3 cells where exposed to the conditioned

medium and cell number and viability were measured by trypan blue staining in a haemocytometer under a bright field microscope.

Similar to the observations in co-culture experiments (Figures 10 and 11), BCR-ABL1+ BA/F3 cells proliferated more in conditioned medium collected from young compared to old BMSC (Figure 13).

5.4 The age of the bone marrow stroma cells does not influence the adhesion of BCR-ABL1+ BA/F3 cells

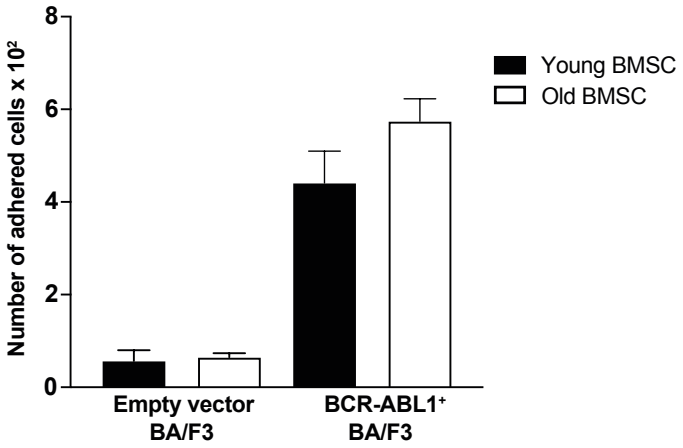

Figure 14: The adhesion of BCR-ABL1+ BA/F3 cells is not influenced by the age of the bone marrow stroma cells (BMSC).

Number of empty vector- or BCR-ABL1-transduced BA/F3 cells adhering to BMSC from young (black) versus old (white) mice (n = 3). 1 x 10^5 cells had been plated and were allowed to adhere for 6 hours.

Not only soluble factors but also cell-to-cell interactions and adhesion may play a role in the regulation of leukaemia cell proliferation by the BMM, since it was shown that adhesion might play an important role in haematopoiesis and leukemogenesis[66]. Therefore, we performed an adhesion assay to assess whether leukaemia cells differentially adhere on young or old BMSC. No differences in the adhesion of BCR-ABL1+ or empty vector BA/F3 cells were observed (Figure 14) to either young or old BMSC.

5.5 The migration of BCR-ABL1+ BA/F3 cells is enhanced towards young bone marrow stroma cells

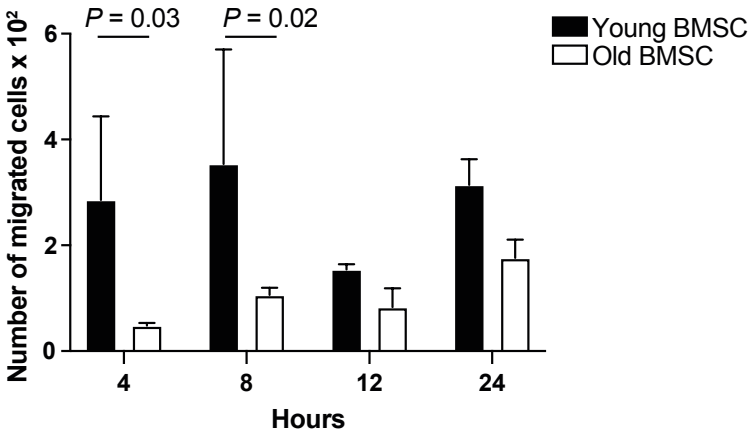

Figure 15: BCR-ABL1+ BA/F3 cells migrate faster towards young versus old bone marrow stroma cells (BMSC).

Number of BCR-ABL1-transduced BA/F3 cells that migrated towards young (black) versus old (white) BMSC after 4, 8, 12 and 24 hours (n = 3). P-*values are as indicated.*

Figure 13 suggests an involvement of factors released by BMSC on the proliferative and possibly migratory behaviour of the B-ALL cells. Therefore, we performed a trans-well migration assay. After BCR-ABL1+ BA/F3 cells were plated in the top chamber of the transwell, the cells that were able to migrate towards the bottom chamber through a porous membrane were counted at different time points. Young versus old BMSC had been plated in the bottom chamber.

When counting migrated cells at two of the four different time intervals (after four and eight hours), we observed a significant advantage of BCR-ABL1+ BA/F3 cells migrating towards young compared to old BMSC (Figure 15). This could provide additional insight into how factors released from young BMSC differentially influence the behavior of leukaemia cells.

5.6 The expression of *Cxcl13* and *GDF11* on a transcriptional level does not differ between young versus old bone marrow stroma cells

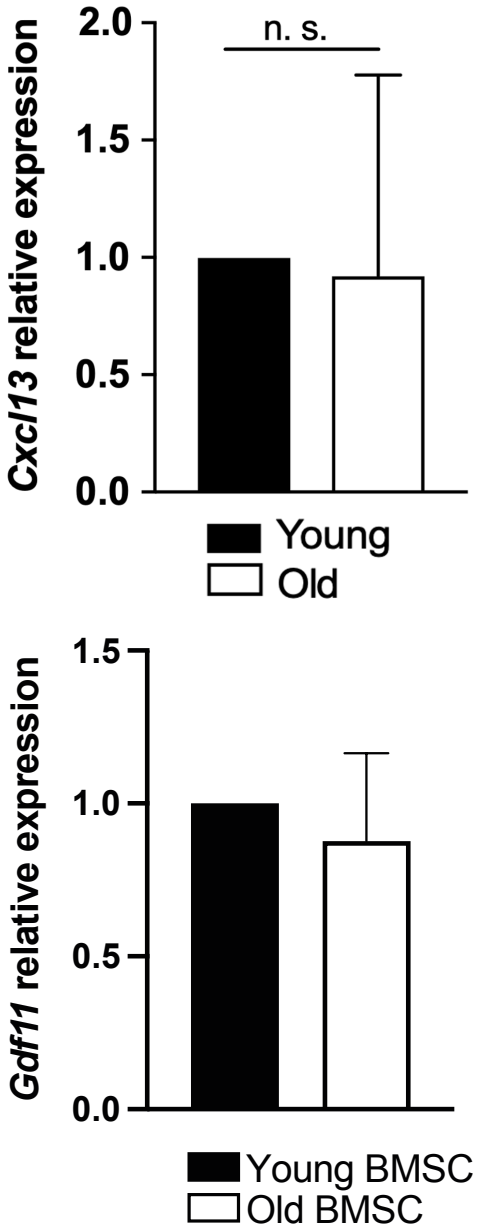

Figure 16: No differences of* Cxcl13 and *Gdf11 expression in young versus old bone marrow stroma cells (BMSC) were found on a transcriptional level.

Relative expression of Cxcl13 (top) and Gdf11 (bottom) in BMSC from young (black) versus old (white) mice (n=5).

As already mentioned in chapters 2.8 and 2.9 we identified potential targets that might be differentially expressed and hence be modulating the effect of young versus old BMSC on proliferation and migration of the leukaemic cell lines. We tested the expression of *Cxcl13* and *Gdf11* in BMSC from young and old mice via RTqPCR but no difference in the expression of both targets could be observed.

5.7 The level of *Cxcl13* expression does not differ between different bone marrow cell populations, while *Gdf11* is more expressed in Nestin+ cells

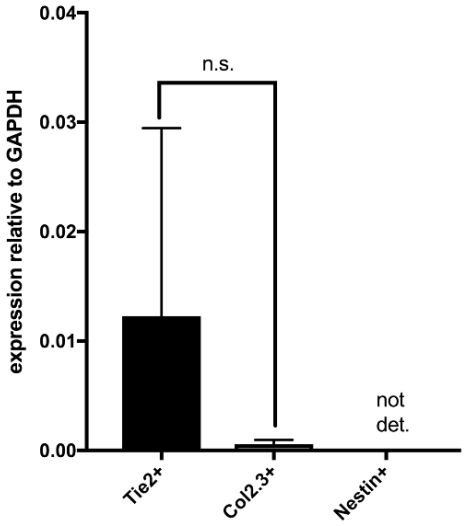

Figure 17: The transcriptional expression of Cxcl13 in Tie2+, Col2.3+ and Nestin+ cells does not differ.

Relative expression of Cxcl13 in sorted Tie2+, Col2.3+ and Nestin+ cells (n=5).

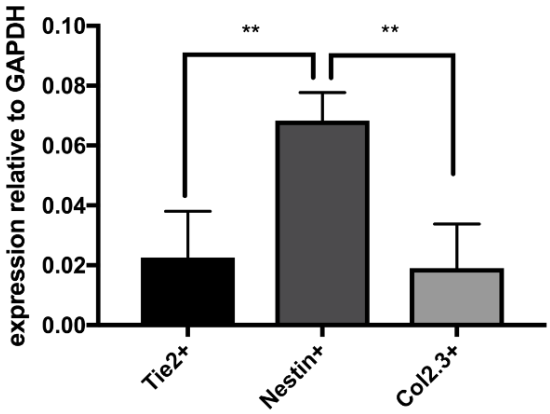

Figure 18: Gdf11 has a higher expression in Nestin+ cells.

Relative expression of Gdf11 in sorted Tie2+, Col2.3+ and Nestin+ cells (n=5).

To investigate the source of CXCL13 and GDF11 within different cell populations of the bone marrow microenvironment, RTqPCR was performed on RNA from endothelial, osteogenic and mesenchymal stromal cells. We used three different reporter mouse models with GFP under the promoters of *Tie2* (expressed in endothelial cells), *Col2.3* (expressed in osteogenic cells) and *Nestin* (expressed in mesenchymal stromal cells) respectively.

After analysis of the RTqPCR data using the $\Delta\Delta C_t$ method, it could be shown that endothelial cells tend to have a higher expression of *Cxcl13* when compared to osteoblastic cells (Figure 17).

The expression level of *Gdf11* was found to be higher in MSC compared to endothelial (P = 0.0039) and osteoblastic (P = 0.0022) cells (Figure 18).

5.8 Young bone marrow stroma cells produce higher levels of CXCL13

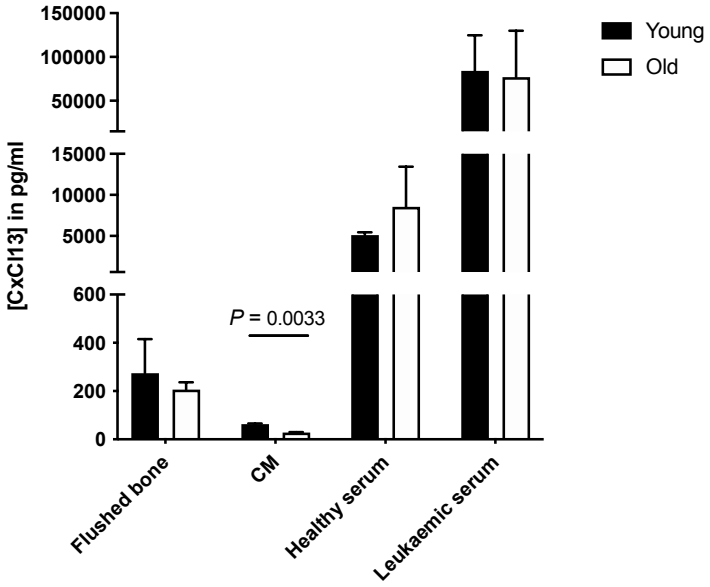

Figure 19: CXCL13 is released more from young compared to old bone marrow stroma cells (BMSC).

Concentration of CXCL13 in pg/ml in flushed bone, conditioned medium (CM) from BMSC and in the serum of peripheral blood from healthy and leukaemic young (black) versus aged (white) mice (n = 3). CM was harvested after 7 days. P*-value as indicated.*

Since we did not observe differences in the transcriptional level of *Cxcl13* (Figure 16), we tested for a potential alteration of the protein level upon ageing of CXCL13 by ELISA in conditioned medium of BMSC, flushed bone marrow, leukaemic and healthy serum from young and old mice respectively.

Conditioned medium from young BMSC shows a higher concentration of CXCL13 when compared to old, while no significant differences could be found in young versus old flushed bone marrow, leukaemic and healthy serum (Figure 19).

5.9 Blocking GDF11 and CXCL13 leads to a reduction of the proliferation of BCR-ABL1+ BA/F3 cells in the presence of young bone marrow stroma cells

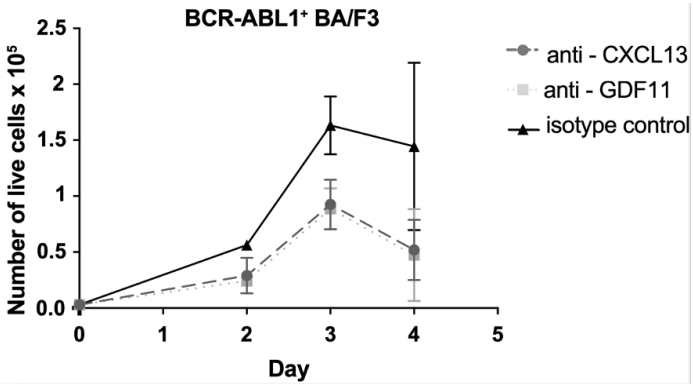

Figure 20: The proliferation of BCR-ABL1+ BA/F3 cells on young bone marrow stroma cells (BMSC) is reduced when CXCL13 and GDF11 are blocked by neutralising antibodies.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from young mice in the presence of isotype control antibodies (solid line), antibodies against CXCL13 (dashed line) or antibodies against GDF11 (dotted line). P*-values are* P = *0.03 and 0.002 on days 3 and 4, respectively, for control versus anti-CXCL13 and* P = *0.02 and 0.001 on days 3 and 4, respectively, for control versus anti-GDF11 (Two-way ANOVA).*

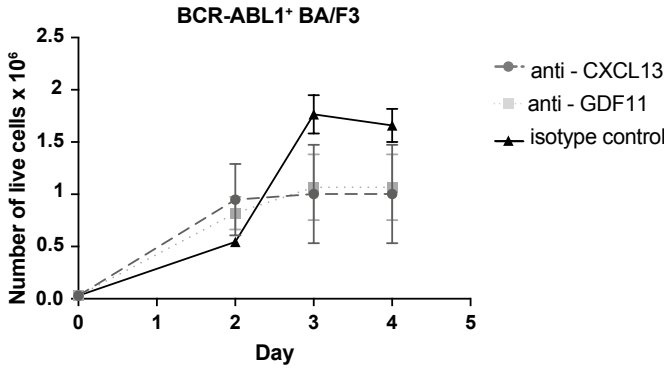

Figure 21: The proliferation of BCR-ABL1+ BA/F3 cells on old bone marrow stroma cells (BMSC) is not significantly reduced when CXCL13 and GDF11 are blocked by neutralising antibodies.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from young mice in the presence of isotype control antibodies (solid line), antibodies against CXCL13 (dashed line) or antibodies against GDF11 (dotted line).

To investigate the influence of GDF11 and CXCL13 on the proliferation of leukaemia cells, we used neutralising antibodies against those proteins in a co-culture setting of empty vector- and BCR-ABL1-transduced BA/F3 cells with young and old BMSC. The blockade of CXCL13 and GDF11 in a co-culture of young BMSC with BCR-ABL1+ BA/F3 cells reduced the proliferation of these cells compared to a culture with isotype-control antibodies on day 3 and 4 (Figure 20). No differences were observed when CXCL13 and GDF11 were blocked in co-culture with old BMSC (Figure 21).

5.10 Blocking GDF11 and CXCL13 increases the proliferation of 32D cells

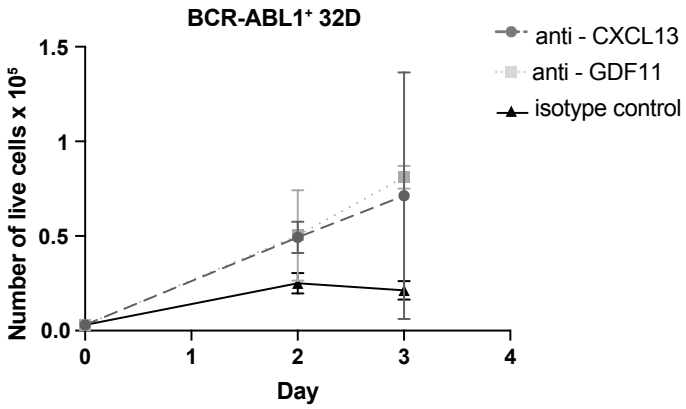

Figure 22: The proliferation of BCR-ABL1+ 32D cells on young bone marrow stroma cells (BMSC) is increased when CXCL13 and GDF11 are blocked by neutralising antibodies.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from young mice in the presence of isotype control antibodies (solid line), antibodies against CXCL13 (dashed line) or antibodies against GDF11 (dotted line). P-*values are* P = *0.001 and < 0.0001 for control versus anti-CXCL13 and anti-GDF11 respectively, on day 3 (Two-way ANOVA).*

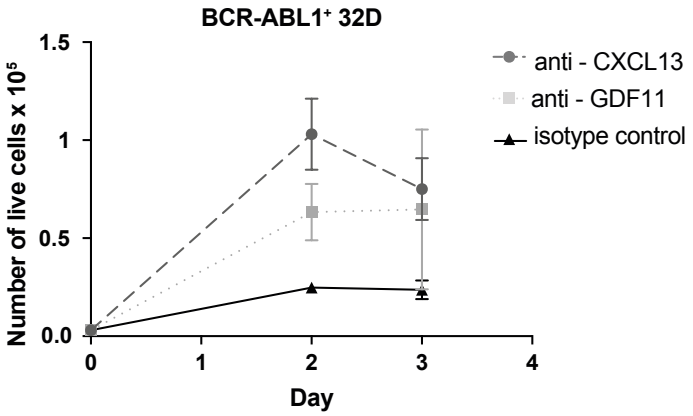

Figure 23: The proliferation of BCR-ABL1+ 32D cells on old bone marrow stroma cells (BMSC) is increased when CXCL13 and GDF11 are blocked by neutralising antibodies.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from old mice in the presence of isotype control antibodies (solid line), antibodies against CXCL13 (dashed line) or antibodies against GDF11 (dotted line). P-*values are* P < *0.0001 and P = 0.0009 on days 2 and 3 respectively, for control versus anti-CXCL13 and* P = *0.02 and 0.01 on days 2 and 3 respectively, for control versus anti-GDF11 (Two-way ANOVA).*

To test the hypothesis that GDF11 has a suppressive effect on CML progression, we used neutralizing antibodies against GDF11 in co-culture experiments of the CML cell line BCR-ABL1+ 32D with young and old BMSC.

Blocking GDF11 increases the proliferation of these cells significantly compared to the isotype control (Figure 22, Figure 23).

5.11 Efficient knockdown of *Cxcl13* and *Gdf11* in young and old bone marrow stroma cells

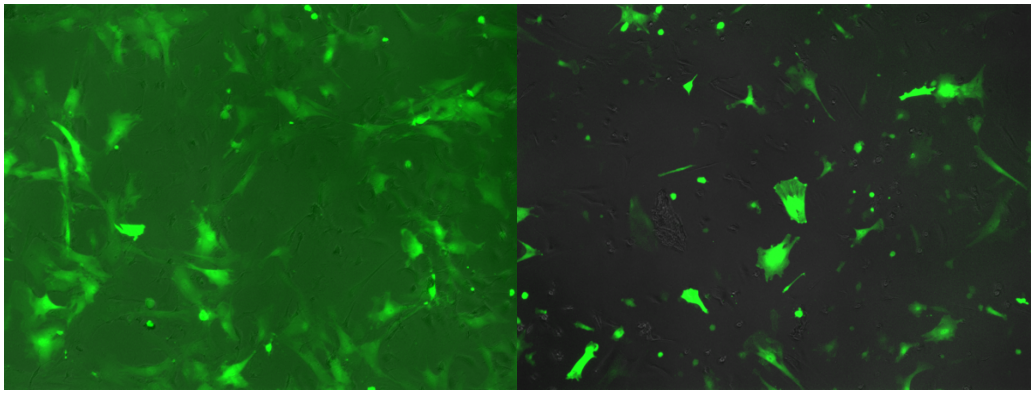

Figure 24: Representative images of bone marrow stroma cells (BMSC) after transduction with sh-Cxcl13 (left) or sh-Gdf11 (right) expressing lentivirus.

Representative images of BMSC transduced with sh-Cxcl13 (left) or sh-Gdf11 (right) and green fluorescent protein (GFP) expressing lentivirus.

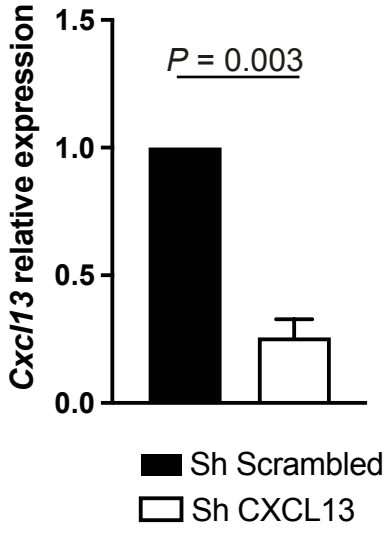

Figure 25: The expression of Cxcl13 is reduced after knockdown on a transcriptional level in bone marrow stroma cells (BMSC).

*Relative expression of Cxcl13 after knockdown in young BMSC transduced with sh-*scrambled*- or sh-Cxcl13-expressing lentivirus. P-value as indicated.*

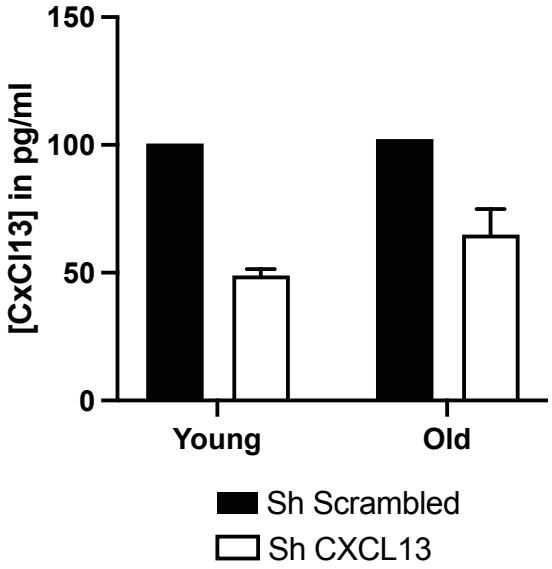

Figure 26: The levels of CXCL13 might be reduced after knockdown in bone marrow stroma cells (BMSC)

*The protein level of CXCL13 in pg/ml in the conditioned medium from young and old BMSC transduced with sh-*scrambled *or sh-Cxcl13-expressing lentivirus by ELISA (n = 2).*

In order to further investigate the role of CXCL13 and GDF11 from BMSC we transduced BMSC with sh-*Cxl13* and sh-*Gdf11* expressing lentivirus. Efficient transduction was assessed by imaging as shown in Figure 24. To confirm the knockdown, the RNA from BMSC after transduction was isolated and RTqPCR was performed. A significant reduction of *Cxcl13* was detected (Figure 25). The conditioned medium from the transduced BMSC was collected and the level of CXCL13 was assessed by ELISA (Figure 26). No significant differences were observed, although a trend towards a reduction may be present.

5.12 The knockdown of *Cxcl13* and *Gdf11* might increase the proliferation of BCR-ABL1+ BA/F3 cells

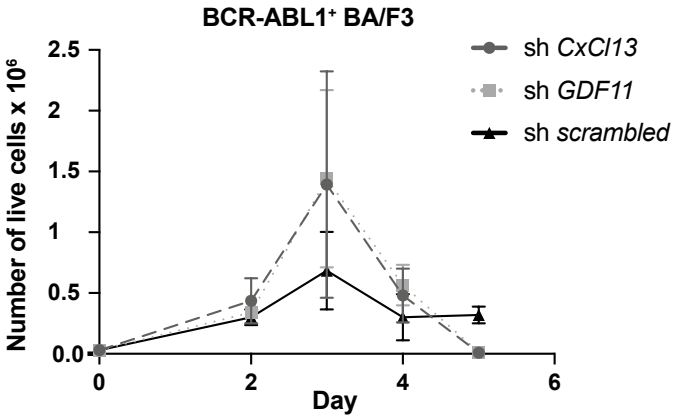

Figure 27: Knocking down Cxcl13-/ Gdf11 in young bone marrow stroma cells (BMSC) might increase the proliferation of BCR-ABL1+ BA/F3 cells.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from young mice after transduction with scrambled RNA- (solid line), sh-Cxcl13- (dashed line) or sh-Gdf11-expressing virus (dotted line). P-*values are* P *= 0.01 and 0.005 on day 3 for control versus sh-Cxcl13 and sh-Gdf11 respectively (Two-way ANOVA).*

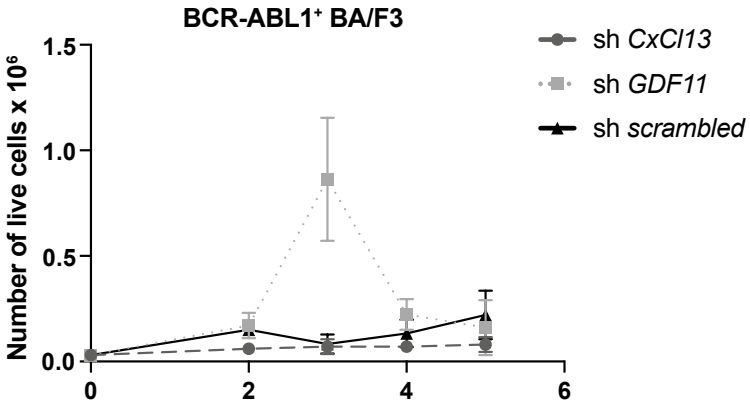

Figure 28: Knocking down Gdf11 in old bone marrow stroma cells (BMSC) might increase the proliferation of BCR-ABL1+ BA/F3 cells.

Number of BCR-ABL1-transduced BA/F3 cells plated on BMSC from old mice after transduction with scrambled RNA- (solid line), sh-Cxcl13- (dashed line) or sh-Gdf11-expressing virus (dotted line). P-*values are* P *= 0.003 on day 3 for control versus sh-Gdf11 (two-way ANOVA).*

Once we tested the transduction efficiency and knockdown of *Cxcl13* and *Gdf11* in BMSC, we performed a co-culture experiment with BCR-ABL1+ BA/F3 cells. In Figure 27 we observed that the knockdown of *Cxcl13* and *Gdf11* led to a proliferative advantage of the BCR-ABL1+ BA/F3 cells on day 3 in young BMSC. The same setting in old BMSC showed that the knockdown of *Gdf11* led to an increase of BCR-ABL1+ BA/F3 cell proliferation on day 3 (Figure 28).

6. Discussion

The role of the age of the bone marrow microenvironment and more specifically the influence of CXCL13 and GDF11 on leukaemia cell behaviour was investigated in this project. We could show that the age of bone marrow-derived stroma cells (BMSC) differentially regulates the growth of B-ALL and CML cell lines (BCR-ABL1+ BA/F3 and BCR-ABL1+ 32D respectively), that the CXCL13 concentration was higher in conditioned medium of young compared to old BMSC and that disrupting CXCL13- and GDF11-signalling might influence the growth of leukaemia cells.

6.1 The age of the bone marrow stroma influences the growth and migration of leukaemia cells

Similar to the situation in humans, where B-ALL is more frequent in children[67], while CML occurs predominantly in older adults[68], our preliminary data and further investigations by other members of the laboratory [64] showed a more aggressive phenotype of the B-ALL model in young mice, while CML showed a more aggressive disease course in old mice. Different mechanisms, that are mostly leukaemia cell-intrinsic, have been reported to be involved in this phenomenon, e.g. clonal expansion[69], an age-related bias towards myeloid differentiation[70] or impaired DNA repair[71]. But another promising field of research, the tumour microenvironment, might also be involved in this phenotype. The microenvironment could be shown to play a role in the development of a variety of different cancers[72,73] including leukaemias[12].

In the context of this thesis, we assessed the influence of BMSC as a simple model for the bone marrow microenvironment (BMM) on B-ALL and CML cells. Later, the immunophenotype of the BMSC was shown to correspond predominantly to F4/80+/ CD169+ macrophages[64]. We could show that young BMSC support the growth of the B-ALL cell line BCR-ABL1+ BA/F3 in direct co-culture as well as in BMSC-derived conditioned medium, while, reversely, old BMSC showed a tendency to support the CML cell line BCR-ABL1+ 32D in a cell-on-cell co-culture. Considering

previous reports that showed the importance of adhesion[66] and migration on the function of different blood cells, we performed adhesion and migration assays to test whether ageing alters these functions. While we could not find a difference in the adhesion of the B-ALL cells to young versus old BMSC, those cells migrated faster towards BMSC from young compared to old mice.

The growth controlling effect of BMSC seemed to be mediated either by direct cell-cell interactions or by the release of cytokines into the cell culture medium, but results in conditioned medium experiments suggest an effect of secreted cytokines that act on the leukaemia cells, even after the BMSC had been removed. Also considering the increased migration of BCR-ABL1+ BA/F3 cells towards young BMSC, we hypothesised that a B-cell supporting and cytoattractive cytokine might play a role in controlling proliferation and migration of the B-ALL cell line.

Since different BMM cell populations have been described to play a role in supporting both hematopoietic stem cells (HSC) and leukaemia initiating cells (LIC)[11-13,74], our results are in overall alignment with previous findings. However, a differential effect of BMSC ageing on B-ALL and CML is a novel concept.

6.2 CXCL13 levels in the BMM are age-dependent

We identified CXCL13 and GDF11 as potential cytokines that could explain the influence of the BMSC on leukaemia cell proliferation and migration. The cytoattractive properties on B-cells[47] and the role in the regulation of different tumours[75-77] of CXCL13 have been described, while GDF11 has been discussed to be involved in ageing and the development of various cancers[78]. GDF11 also plays a role in bone development and remodelling[79]. As mentioned above (2.9), some groups have previously reported declining levels of GDF11 with age[59]. We hypothesised that higher amounts of this protein might protect the young bone marrow niche from the development and/ or expansion of CML, just like TGF-β1 (another member from the same protein superfamily) was shown to suppress CML-like MPN in mice[11]. No changes on the transcriptional level of *Gdf11* were found in young versus old BMSC. Thus, we investigated whether different components of the

BMM might have differential expression of *Gdf11*. Our data suggest that mesenchymal stroma cells are the main source of *Gdf11* within the BMM. This is in line with studies that report MSC to produce and release TGF-β1[80], especially in a tumour setting. In the context of this project, no ELISA assay for GDF11 was available. Therefore, the level of the released protein could not be measured. Further investigations into the age dependency of GDF11 availability should be performed, e.g., by immunosorbent assays or Western blot.

In the case of CXCL13, while we also did not find age-dependent differences on the transcriptional level, we could find higher levels of the protein in young (compared to old) BMSC-derived conditioned medium. This might explain the increased B-ALL cell proliferation in both the co-culture and conditioned medium experiments. Zanetti et al. later demonstrated that higher protein levels of CXCL13 can also be found in bone marrow and peripheral blood plasma samples from young compared to old mice[64]. As mentioned before, CXCL13 is a chemoattractive cytokine for B-cells in different stages of maturation, while also supporting the organisation of secondary lymphatic organs (see 2.8). Decreased or altered activity of the immune system in ageing organisms may explain differences in the availability of cytokines which govern its development and maintenance[81]. During “immunosenescence” B clonotypic immune response to new extracellular pathogens decreases[82]. In the aged, circulating B-cells with less CD40, no IgD, and no CD27 are more frequent, indicating that these cells are no longer capable of acting as antigen-presenting cells or interacting with T lymphocytes[27]. These changes in the B-cell compartment during ageing might extend or be related to the interactions with secondary lymphatic organs and the BMM, possibly through decreased availability of CXCL13. This may reduce the susceptibility of an aged BMM to B-ALL.

6.2 Disrupting CXCL13- and GDF11-signaling might influence leukaemia cell proliferation

Taking together our results from proliferation, migration and CXCL13-/ GDF11-quantifying assays, we investigated the functional role of these proteins on leukaemia cell function.

Published data shows that neutralising antibodies against CXCL13 and GDF11 inhibit their binding to and signalling via receptors on the surface of the leukaemia cell lines[83,84]. In our assays these antibodies changed the growth of BCR-ABL1+ BA/F3 and 32D cells and by this might provide some insight into the role of these cytokines in the behaviour of leukaemia cells. Blocking the effect of CXCL13 and GDF11 on BCR-ABL1+ BA/F3 cells co-cultured with young (but not old) BMSC decreased their proliferation significantly, suggesting a beneficial effect of these cytokines on the growth of this leukaemia cell line.

A supporting role of CXCL13 on the growth of BCR-ABL1+ BA/F3 cells is plausible, since not only does CXCL13 attract B-cells to areas that will support the proliferation of these cells. It has also been reported to act on other malignant lymphoid, Eμ-Tcl1+ CLL cells in a co-culture with follicular dendritic cells via its cognate receptor CXCR5[53]. The proliferation-supporting effects could directly be controlled either by CXCR5-mediated signalling or by an indirect modification of the communication between the leukaemic cells and other cells in the BMM[85]. In light of this, it will be important to characterise the CXCR5-CXCL13-axis upon ageing in BMSC and its effect on leukaemia, e.g., by using CXCR5-deficient B-ALL cells in a co-culture setting.

The blockade of CXCL13 in co-cultures with old BMSC did not change the growth of the BCR-ABL1+ BA/F3 cells, further supporting our hypothesis that the influence of CXCL13 predominantly is in the young BMM.

GDF11 has not yet been described to influence the behaviour of B-cells. One study showed that GDF11 is involved in the crosstalk between human intestinal lymphatic endothelial cells (HILEC) and colorectal cancer and correlates with tumour stage[86].

Additional studies should look further into its effects on lymphatic and lymphoblastic cells.

In case of the CML- cell line, blocking CXCL13 and GDF11 by neutralising antibodies had the opposing effect compared to the experiments with the B-ALL cell line. Blocking CXCL13 and GDF11 led BCR-ABL1+ 32D cells to proliferate more, indicating an anti-proliferative effect of these factors. For GDF11 this observation falls in line with previous discoveries about another TGF-β family protein, TGF-β1, that strongly reduces the number of CML cells in leukaemic mice[11]. CXCL13 on the other hand has not yet been shown to have a strong impact on myeloid cells, although one group reports a role in the recruitment of myeloid-derived suppressor cells to gastric cancer via the CXCL13-CXCR5-axis[87]. Further experiments, possibly involving *Gdf11*- and *Cxcl13*-knockout mice, could shed more light on these proteins' role on myeloid cells in general and their influence on myeloid leukaemias. The inexact matching of naturally existing CML cells is one drawback of the 32D cells. In patients, CML originates from the *BCR-ABL1* fusion gene occurring in haematopoietic or myeloid stem cells while 32D cells are murine myeloblast-like cells into which we introduced *BCR-ABL1*. In future experiments, K562 cells from a human immortalised myeloid leukaemia cell line or primary cells from the CML mouse model could be used as an alternative.

Additional experiments by Zanetti et al. based on the methods described in this thesis could validate the observations made regarding the B-ALL model: neutralising antibodies against CXCL13 not only reduced the proliferation of BCR-ABL1+ BA/F3 cells but also of primary BCR-ABL1+ BP1+ (beta protein 1; a surface marker found on immature B-cells[62]) cells from a B-ALL mouse model cultured on young bone marrow macrophages[64].

The role of CXCL13 and GDF11 on leukaemia cells was also assessed via a knockdown of both genes in young and old BMSC and subsequent co-culture experiments with leukaemia cells in order to assess proliferation of the leukaemia cells. Technical complications didn't allow for a coherent interpretation of the data obtained. ELISA and RTqPCR results indicated the successful transduction and knockdown in BMSC in case of *Cxcl13*. For *Gdf11*, data collected from transduced

3T3 cells indicated a similar success in the knockdown of this gene. Transduction of BMSC with sh*Gdf11*-expressing lentivirus, however, was repeated several times, but in most cases, cells sorted after transduction did not expand adequately in cell culture. And in all cases, cells expressing shRNA against *Gdf11* and collected for RTqPCR analysis did not yield sufficient or sufficiently pure RNA. This assay should be repeated, ideally also with an ELISA setup for the detection of GDF11 protein levels in the conditioned medium of these cells. Observations in experiments that include sh*Gdf11*-transduced cells must therefore be critically evaluated. Our results do not imply a definite effect of this protein but might highlight the importance of and provide a path towards more research on this cytokine axis and its effects in stroma-HSC/ LSC interactions.

Knockdown *Cxcl13* and *Gdf11* in BMSC in a co-culture showed effects contradicting our results from neutralising antibody experiments. BCR-ABL1+ BA/F3 cells expanded more when *Cxcl13* or *Gdf11* had been knocked down in young BMSC while those co-cultured in medium supplemented with the neutralising antibodies grew more slowly (as mentioned above). The transduction and flow cytometry-assisted sorting process is strenuous for BMSC and might affect these cells in a way that changes their BA/F3 cell supporting characteristics, which is why empty vector-transduced and sorted cells were used as a control. These empty vector-transduced cells however expanded more stably and showed a morphology resembling un-transduced rather than shRNA-transduced cells. This might suggest an unintended effect of the knockdown on the overall performance of the BMSC, possibly altering their supporting role in ways beyond the release of the target cytokines, CXCL13 and GDF11. Culture conditions for transduced BMSC could possibly be improved by adding MSC-supporting factors and by limiting differentiation of the cells that stay in culture for the period necessary for this assay.

Selecting transduced cells by puromycin (the virus vector contains a puromycin N-acetyltransferase) as an alternative to flow cytometry-assisted cell sorting was tested and rejected because of apparent adverse effects on cell morphology and growth behaviour and the effect of residual antibiotic on other cells in later co-cultures, even

if puromycin was added at different concentrations to find out the best and most tolerable concentration for the cells (data not shown).

Zanetti et al. were able to improve the knockdown-model and the quality of the transduced bone marrow macrophages. Subsequently they showed that the knockdown of *Cxcl13* impaired the proliferation of BCR-ABL1+ BA/F3 cells on young and old macrophages[64], complementing our findings in the assays involving neutralising antibodies. These improvements should also be applied to *Gdf11*-knockdown BMSC so that the experiments can be repeated with higher quantities of adequately expanding cells.

In conclusion, we showed that the age of the BMM differentially impacts B-ALL and CML cell lines. In particular we demonstrated that young BMSC support the proliferation and migration of BCR-ABL1+ BA/F3 cells and that CXCL13 and GDF11 released from BMSC influence the growth of leukaemia cells.

Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

> Differential effects of young (growing) versus old (grown-up) bone marrow microenvironments on leukaemias by release of the cytokines CXCL13 and GDF11 from bone marrow stroma

im Institut für Tumorbiologie und experimentelle Therapie Georg-Speyer-Haus unter Betreuung und Anleitung von **Prof. Dr. Daniela Krause** mit Unterstützung durch **Costanza Zanetti** ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.
Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.
Vorliegende Ergebnisse der Arbeit (gezeigt in Figures 10 und 11) wurden in folgendem Publikationsorgan veröffentlicht:

1) Zanetti C, Kumar R, Ender J, Godavarthy PS, Hartmann M, Hey J, Breuer K, Weissenberger ES, Minciacchi VR, Karantanou C, Gu Z, Roberts KG, Metzler M, Stock W, Mullighan CG, Bloomfield CD, Filmann N, Bankov K, Hartmann S, Hasserjian RP, Cousins AF, Halsey C, Plass C, Lipka DB, Krause DS. The age of the bone marrow microenvironment influences B-cell acute lymphoblastic leukemia progression via CXCR5-CXCL13. Blood. 2021 Nov 11;138(19):1870-1884. doi: 10.1182/blood.2021011557. PMID: 34424946.

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