In 2013, the 6th year of the Ludwig Boltzmann Cluster Oncology (LBC ONC) several projects were conducted and were in part finalized, and a number of interesting data have been collected. Individual projects were performed in 3 project-lines (PL) according to the master-plan of the LBC ONC. In these projects, several new stem cell markers and targets have been identified and characterized in myeloid, lymphatic and mast cell leukemias. In addition, stem cell-niche interactions and the effects of certain drugs on niche-related cells have been examined. Several new stem cell targets have been validated by using shRNA and/or by applying specific targeted drugs.

1. Overview of the LBC ONC

1.1. Introduction and Aims

In the past 15 years, numerous studies have shown that most if not all leukemias are composed of two different fractions of neoplastic cells, a bulk-population of cells with limited capacity to proliferate, and a smaller subset exhibiting the capacity of unlimited self-renewal, the so-called neoplastic stem cells (NSC), also referred to as leukemic stem cells (LSC) in the context of a leukemia. This hypothesis is now widely accepted and predicts that anti-cancer therapy is curative only when eliminating most or all LSC (NSC) in a given leukemia (neoplasm). During the past few years, more and more data have shown that NSC fractions represent heterogeneous populations of cells, reflecting plasticity and genetic instability in these populations. So far, however, little is known about the expression of relevant targets in LSC (Figure 1). From all these considerations it seems important to learn more about target expression profiles of LSC and about responses of these cells to targeted drugs, conventional drugs, and immunotherapy.

The aims of the LBC ONC are to identify and characterize LSC in various human leukemias, to define target expression profiles in these cells, to validate molecular targets, and to examine the effects of various targeted drugs on growth and survival of LSC. Drugs and markers were selected based on results obtained in previous years and the available literature. The long-term goal of the project is to develop curative therapies for human leukemias by applying drugs that recognize and eliminate LSC.
Expression of cell surface and cytoplasmic targets in leukemic cells
Many therapeutic targets have been identified in ‘bulk leukemic cells’. However, little is known about the expression of molecular targets in leukemic stem cells (LSC).

General Aims in LBC ONC Projects:

- Identification and phenotypic characterization of LSC in various leukemias
- Characterization of target expression profiles in LSC
- Delineation of stem cell plasticity and stem cell resistance: relationship to genetic instability and characterization of relevant LSC subclones
- Targeting of LSC using specific targeted drugs and drug combinations
- Evaluation of interactions between LSC and the so-called SC niche
- Evaluation of effects of endogenous immune-mediators (cytokines) and death regulators on growth and survival of neoplastic stem cells
1.2. Budget of the LBC ONC

The budget plan for 2013 was established in cooperation with the LBG (Ludwig Boltzmann Society). Budget was mainly used to employ personnel and to purchase consumables. Part of the budget was used to invite experts in the field or to send LBG-employed LBC ONC postdocs to conferences and stem cell meetings. The LBC ONC budget of 2013 amounted to 799,975.- €.

1.3. Partners and Internal Structure of the Cluster

Partners
As in the previous project-years of the LBC ONC, two academic partner institutes were involved in 2013, the Hanusch Hospital and the Medical University of Vienna (MUV).

Project Groups and Scientists
The LBC ONC is running 3 project lines (PL) that were also maintained in 2013: a) myeloid neoplasms, b) lymphoid neoplasms, and c) mast cell neoplasms including mast cell leukemia (MCL). Group ´a´ is also working on normal bone marrow stem cells and myelodysplastic syndromes (MDS). According to the Evaluation Report 2010 and the recommendations of the Scientific Advisory Board (SAB) all projects on solid tumors and melanomas had been finalized in 2011; papers from these studies are still being submitted and will be published. The following researchers were employed in 2013: Heidrun Karlic (MDS/AML), Gregor Eisenwort (AML, CML, MCL), Sabine Cerny-Reiterer (ALL, CML), Barbara Peter (MCL) and Emir Hadzijusufovic (MCL, CML). The group organized weekly staff-report meetings (1.5 hours), one week-start meeting (Monday, 10:00), and a Lecture Series (Friday 16:00). The group was also involved and participated actively in several workshops and other scientific meetings.

Administration
As in previous years, administration was coordinated by the administrative head of the LBC ONC, Prof. Dr. Thomas Grunt and was supported by our secretary, Sabine Sonnleitner, who handled all technical and minor administrative issues in the LBC ONC in 2013.

Core Facility Groups
All core facility (CF) groups that were established in cooperation with the MUV in the LBC ONC in previous years, were maintained, and supported LBC ONC projects in 2013: these CF-Platforms (PF) include a LSC-sort PF (employing a high speed sorter), a gene array PF (including proteomics and sequencing facilities), a NOD/SCID (NSG) mouse Xeno-TX-PF (in cooperation with the University of Veterinary Medicine Vienna), and a clinical PF (including a biobanking-system and disease-registries; coordinated by Prof. Dr. Wolfgang R. Sperr). These CF-PF were essential for the progress in our LBC ONC projects in 2013. In the next project year, the LBC ONC will establish an additional CF-PF dedicated to lentiviral-mediated gene delivery and the establishment of various stem cell lines.
1.4. Scientific Advisory Board (SAB)

In collaboration with the LBG, the LBC ONC has established an international SAB consisting of three experts in the field (Dominique Bonnet, Joos Jonkers and Charles Theillet). In 2013, the SAB members were also attending the evaluation meeting in Vienna (December 18+19, 2013). For the next project period, LBC ONC projects will be adjusted according to the evaluation report and the recommendations of the SAB.

1.5. Personnel and Career Development

The following scientists were employed via the LBG in the LBC ONC Cluster in 2013:

- Heidrun Karlic     Hanusch Hospital       01-12/2013
- Gregor Eisenwort   Medical University of Vienna   01-12/2013
- Sabine Cerny-Reiterer  Medical University of Vienna   01-12/2013
- Barbara Peter     Medical University of Vienna   01-12/2013
- Emir Hadzijusufovic  Medical University of Vienna   01-12/2013
- Alexandra Keller   Medical University of Vienna   03-12/2013

Career development steps: Harald Herrmann, previously employed in the LBC ONC via the LBG, started a position at the Radiation Department of the MUV in 2013. The other LBG-employed Postdocs work on their scientific career in the LBC ONC environment. Within the group of non-LBG-employed LBC ONC colleagues, one new Assistant Professorship (Karoline V. Gleixner) at the MUV should be mentioned.

1.6. Infrastructure

The infrastructure in 2013 included three labs (3x30 m²) dedicated to LSC research at the MUV, several other labs of participating scientists at the MUV, and a lab at the Hanusch Hospital. In addition, one office for our secretary (Sabine Sonnleitner) as well as several core facility rooms were made available and were used to run LBC ONC projects in 2013. All in all, the labs and infrastructure shared by partners as well as the scientific environment provided optimal conditions for our LBC ONC projects in 2013.

1.7. Scientific Highlights

Among several scientific achievements in 2013, the following highlights should be mentioned: 1. The identification of RAS as a driver of CD52 expression in myeloid stem and progenitor cells in MDS and AML, 2. The effects of various TKI on niche-related cells, i.e. endothelial cells, 3. The impressive effects of ponatinib on neoplastic mast cells, 4. the characterization of the target expression profiles of LSC in MCL, and 5. the presentation and validation of a new LSC concept that discriminates between pre-malignant neoplastic stem cells (NSC) and malignant (leukemic) NSC (=LSC).
1.8. Public Relation

The mission of the LBC ONC cluster is to increase awareness and to gain knowledge in the field of neoplastic (leukemic) stem cells in leukemias and other blood cell malignancies, to establish pathogenetic and targeting concepts in these neoplasms, and to establish LSC-eradicating treatment concepts, with the ultimate aim to improve anti-neoplastic therapy in patients with MDS, AML, CML, ALL, and MCL. As in previous years, the LBC ONC team transferred its mission-intention to the public in 2013 through publications and meetings organized by the LBG. In addition, members of the LBC ONC were involved in the establishment of a new special research program (SFB) on myeloid neoplasms at the Medical University of Vienna and in the organization of an international top-meeting on myeloid neoplasms in Vienna in October 2013.

2. Results obtained in the LBC ONC in 2013

2.1. Results obtained in Individual Projects

2.1.1. Aberrant expression of CD52 on LSC and role of the RAS Pathway

During the past few years the LBC ONC was able to show that LSC in patients with certain MDS- and AML variants express the Campath-1 antigen (CD52) in an aberrant manner. As assessed by flow cytometry, CD52 was found to be expressed in a majority of patients with MDS and isolated 5q−, and also in a few other patients with MDS. In addition, CD52 was found to be expressed in CD34+/CD38− LSC in a substantial subset (40%) of patients with AML, including patients with complex karyotype including 5q−, but also in other patients with AML, including cases with inv(3), trisomy 8 (+8) or monosomy 7 (-7). In all these patients, CD52 was found to be expressed on the surface of putative LSC, and we were also able to show that CD52 serves as a target of therapy in these patients. In particular, alemtuzumab, an antibody directed against CD52, produced complement-dependent lysis of LSC in vitro in these patients. Moreover, alemtuzumab induced hematologic responses, including remissions, in patients with MDS. Normal stem cells expressed only trace amounts or did not express CD52. In 2013, the LBC ONC also examined the regulation of expression of CD52 in leukemic cells. Since CD52 expression has been associated with EVI1- and CD300a expression, we first explored whether EVI1 and CD300a transcripts are detectable in LSC and correlate with CD52 expression. Indeed, in all MDS samples and AML samples tested, CD34+/CD38− LSC expressed EVI1- and CD300a mRNA. We also found a rough correlation between EVI1 mRNA expression and CD52 mRNA expression in CD34+/CD38− cells (R=0.66; p<0.05) (Figure 2). However, no correlation between CD300a and CD52 mRNA expression was demonstrable (p>0.05) (Figure 2). These data suggest that CD52 may be triggered by an EVI1-dependent pathway. In a next step, we screened for more specific molecular pathways contributing to the expression of CD52 on LSC in MDS and AML.
Figure 2
Correlation between expression of CD52- and EVI1 transcript levels in LSC expression of CD52 mRNA- and EVI1 mRNA levels (left panel) and CD52 mRNA- and CD300a mRNA levels (right panel) in highly enriched (sorted) LSC were compared. A rough correlation between CD52- and EVI1 mRNA expression levels was found. R values and p values are shown. RNA was isolated from sorted CD34+/CD38− cells in patients with MDS and AML. CD52 mRNA expression was quantified by qPCR. Human ABL served as a reference gene.

Since RAS activation has been associated with EVI1 expression in myeloid leukemias, we tested the RAS pathway that is known to be somatically altered and hyper-activated in several groups of patients with MDS and AML. Specifically, we asked whether activated RAS can trigger the expression of CD52 in AML cells. As visible in Figure 3, four different oncogenic RAS mutants (HRAS G12V, KRAS G12V, NRAS G12D, NRAS Q61K) induced the expression of CD52 in HL60 cells. Incubation of these CD52+ HL60 cells with alemtuzumab resulted in a dose-dependent cell lysis and in decreased cell numbers, whereas no drug effect was seen in empty-vector-transduced cells. These data suggest that CD52 expression on AML cells can be induced by RAS activation.

Figure 3
Effects of various RAS mutants on expression of CD52 in HL60 cells
HL60 cells were transfected with empty vector, HRAS G12V, KRAS G12V, NRAS G12D, and NRAS Q61K. Expression of CD52 was analyzed by flow cytometry. The black open histograms show the isotype-control and the red histograms represent CD52 expression.
We were also interested to learn more about the clinical consequences and prognostic impact of expression of CD52 on CD34+/CD38- NSC/LSC in MDS and AML. In MDS this was of particular interest as the 5q- anomaly is known to define a MDS subset with relatively good prognosis and only a subset of these patients progress to AML after a certain latency period. We found that in both groups of patients (MDS and AML), expression of CD52 correlates with survival (Figure 4). In AML the impact of CD52 expression in LSC on survival was found to be significant (p<0.05) whereas in patients with MDS, the difference was not significant, which may be explained by the relatively small numbers of patients or the relatively good overall prognosis of the 5q- population. The LBC ONC has launched a new prospective study in order to clarify this important point. In this new study, more patients with MDS and AML will be enrolled.

![Figure 4](image)

**Figure 4**
Influence of expression of CD52 in LSC on overall survival in MDS and AML
The probability of survival in patients with MDS (A, n=29) and AML (B, n=62) was determined for 2 subgroups of patients, namely those in whom a) CD34+/CD38− LSC expressed high levels of CD52 (high) and b) CD34+/CD38− LSC expressed either low levels or did not express CD52 (low). The probability of survival was calculated by the product limit method of Kaplan and Meier. The difference in overall survival in AML patients was found to be significant (p<0.05).

### 2.1.2. Expression of other Targets on LSC in Myeloid Neoplasms

During the past few years, the LBC ONC has screened for and has identified a number of cell surface receptors and target antigens in LSC in patients with MDS, AML and CML. Several cytokine receptors, such as the IL-2 receptor alpha chain (CD25), Siglec-3 (CD33), the stem cell invasion receptor CD44, the stem cell factor receptor KIT (CD117) and the IL-3R alpha chain (CD123) turned out to serve as potential targets of therapy. During the 6th project-year, these targets were further validated in functional assays and the effects of various targeted drugs have been examined. In CML, several of these projects have been performed in collaboration with a newly established research program (SFB) on myeloproliferative neoplasms and the Ludwig
Boltzmann Institute for Cancer Research (LBI-CR). CD25 was found to be expressed almost invariably on CML LSC in all patients, whereas in AML, LSC expressed CD25 in a subset of patients, and in MDS CD25 was not detected in LSC in most cases. During successful therapy with imatinib or novel (second generation) BCR/ABL-targeting TKI, the numbers of CD25+/CD26+ LSC decreased to minimal numbers and disappeared at the time of complete molecular response (CMR). In most of these patients, the remaining CD34+/CD38- (normal) stem cells were all CD25-negative and CD26-negative. However, in a few patients, the remaining CD26-negative stem cells were found to express CD25. Currently, the LBC ONC explores whether these remaining CD25+ stem cells at CMR are clonal cells, whether they express BCR/ABL and whether they represent an immature (premalignant) stage of LSC evolution. In the CD26-project, the LBC ONC has extended experiments to an in vivo mouse model to test the effects of the CD26-targeting drug vildagliptin, and to drug combinations consisting of nilotinib and vildagliptin. Whereas preincubation of CML cells with vildagliptin resulted in a reduced engraftment in NSG mice, continuous exposure to vildagliptin (in drinking water) as single agent was found to lead to an accumulation of engrafting CML cells in the bone marrow of these mice. In a next step, mice will be treated with a combination of vildagliptin and nilotinib. The hypothesis is that the drug combination will lead to a complete eradication of CML LSC which is supported by the observation that LTC-IC formation is affected in vitro by this drug combination. With regard to CD33 the LBC ONC will examine the effects of gemtuzumab-ozogamicin on engraftment of AML- and CML LSC in NSG mice in 2014. Based on the LSC-targeting effects of this drug in our in vitro experiments we will also propose a clinical trial in CML with the aim to eradicate LSC early by application of a drug combination consisting of gemtuzumab-ozogamicin and a BCR/ABL-targeting TKI.

2.1.3. New Targets identified in Acute Lymphoblastic Leukemia (ALL)

During the past few years, the LBC ONC has identified a number of novel markers and targets on CD34+/CD38- and CD34+/CD38+ progenitor cells in patients with ALL. These studies focused on both the Ph+ subset of ALL patients as well as patients with Ph- ALL. In 2013, the LBC ONC extended these analyses to more patient-samples. In these studies we confirmed that LSC in Ph+ ALL express CD25 and often also CD26. The expression of CD26 on LSC is restricted to those patients in whom neoplastic cells express the p210-form of BCR/ABL. The identity of these LSC was confirmed by demonstrating the expression of CD19 (B cell marker) by flow cytometry and by expression of BCR/ABL by qPCR. The target receptor CD20 was found to be expressed on LSC in about 50% of all patients independent of the presence of BCR/ABL. In all ALL patients examined, the LSC-enriched fractions also expressed the stem cell-homing receptor CD44, Campath-1 (CD52), AC133 (CD133), FLT3 (CD135), and CXCR4 (CD184). In a majority of the ALL patients tested (14/25) LSC expressed Siglec-3 (CD33). We also extended our drug validation studies in ALL. In these experiments, we were able to confirm that alemtuzumab induces rapid cell lysis in CD52+ LSC and that gemtuzumab-ozogamicin induces apoptosis in CD33+ ALL LSC. Currently, the effects of rituximab (anti-CD20-Ab) on growth and survival of CD20+ ALL LSC are examined.
We have also extended our studies on the effects of various signal transduction inhibitors and Bcl-2-targeting agents on ALL cells. Two drugs were examined in detail in 2013, namely the pan-Bcl-2 blocker obatoclax and the PI3 kinase/mTOR blocker BEZ235. Both drugs were found to inhibit the growth and survival of various Ph+ ALL cell lines, including Z-119, BV-173, TOM-1 and NALM-1 (Figure 5).

Figure 5
Dose-dependent effects of BEZ235 and obatoclax on growth of ALL cells
The Ph+ ALL cell lines TOM-1 (upper panels) and NALM-1 (lower panels) were incubated in control medium (Co) or in various concentrations of BEZ235 (left panels) or obatoclax (right panels) at 37°C for 48 hours. Then, \(^3\)H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments. Asterisk: p<0.05.

In a next step, we applied various drug combinations, using BEZ235, obatoclax, and diverse BCR/ABL TKI, including imatinib, nilotinib and ponatinib. In these experiments, we found that obatoclax and BEZ235 produce synergistic growth-inhibitory effects on Ph+ ALL cells when combined with BCR/ABL TKI (not shown). In addition, we found that the drug combination obatoclax+BEZ235 induces synergistic growth-inhibitory and apoptosis-inducing effects on ALL cells. Currently, the LBC ONC is examining the effects of obatoclax and BEZ235 (alone and in combination with TKI) on proliferation and survival (apoptosis) of ALL LSC obtained from patients with Ph+ and Ph- ALL.

In the 6\(^{th}\) project year, the LBC ONC was also able to finalize all studies on the expression and function of heme oxygenase-1 (HO-1) in ALL LSC. The data obtained show that HO-1 serves as a therapeutic target in ALL LSC, and that the HO-1 blockers employed (SMA-ZnPP and PEG-ZnPP) synergize with BCR/ABL TKI in producing growth inhibition and apoptosis in ALL cells (Cerny-Reiterer et al, Oncotarget 2014, in press).
2.1.4. Effects of TKI on vascular endothelial cells and angiogenesis

During the 6th project-year, the LBC ONC has started to investigate the effects of various targeted drugs on niche-related cells. Recent data suggest that the arteriolar cells in the bone marrow play a particular role in LSC evolution, LSC quiescence and LSC redistribution (Kunsika et al, Nature 2013;502:637-643). Other studies have shown that sinusoidal cells and post-capillary venules may play a role in stem cell biology. We have recently found that certain BCR/ABL TKI can promote atherosclerosis and provoke vascular occlusive events in patients with Ph+ CML. Therefore, we examined the effects of these TKI (nilotinib, ponatinib) on growth and survival of human vascular endothelial cells. We found that in patients with CML, the microvascular density (MVD) and thus angiogenesis decrease during treatment with nilotinib. Moreover, we found that nilotinib and ponatinib suppress the growth of vascular endothelial cells (HUVEC) in vitro whereas imatinib showed no effects on endothelial cell growth (Figure 6).

Figure 6
Effects of imatinib, nilotinib and ponatinib on growth of vascular endothelial cells
Cultured human umbilical vein-derived endothelial cells (HUVEC) were incubated in control medium or in various concentrations of imatinib, nilotinib or ponatinib at 37°C for 48 hours. After incubation, 3H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments. Asterisk: p<0.05.

In a next step, we examined potential targets of endothelial cells that may play a role in the inhibitory effects of the TKI tested. Among several potential targets recognized by nilotinib but not imatinib, several are known to play a key role in angiogenesis, such as KDR, Tie-2/TEK and ABL2. Currently, the LBC ONC is validating these targets in endothelial cells. The major question is whether these (or other) targets play a role in bone marrow angiogenesis and in niche-related functions mediating LSC survival and evolution. A related emerging hypothesis is that nilotinib and ponatinib exert superior effects on CML cells (compared to imatinib) not only because of their stronger effects on BCR/ABL but also because of their additional effects on the vascular stem cell niche and bone marrow angiogenesis. The notion that CML LSC expand and survive independent of BCR/ABL would favor this new hypothesis. The LBC ONC will test this exciting new hypothesis during the next 3 years. We have also confirmed anti-angiogenic effects of nilotinib in a migration assay, a vascular tube formation assay and in an in vivo model of hind-limb ischemia in mice. These data were obtained in collaboration with a research group at the Medical University of Innsbruck and were presented at the ASH meeting 2013 (Hadzijusufovic et al, Blood...
Moreover, the LBC ONC was able to show that nilotinib induces apoptosis in human endothelial cells.

### 2.1.5. Identification of Markers and Targets in putative LSC in MCL

During the past 3 years, members of the LBC ONC have been able to characterize the phenotype of neoplastic mast cells in patients with mast cell leukemia (MCL). In addition, we were able to define the phenotype of putative LSC in MCL. As assessed by *in vitro* testing and NSG xenotransplant experiments, LSC reside within a small CD34+ fraction of the MCL clone. This fraction was detected in patients with acute or subacute MCL, but was not detectable (is probably too small to be detected) in patients with chronic MCL. In the 6th year of our LBC ONC, we examined the putative MCL LSC in more detail.

In a first step, we extended our phenotypic analyses. In these studies, we were able to confirm that in all patients with advanced SM including MCL, putative LSC express CD33, CD52, CD117 (KIT) and CD123. By contrast, these cells did not express CD2, CD25, CD26 or CD30. Next, we performed gene array analyses in order to identify novel MCL-specific markers and targets in LSC. Gene arrays were performed using highly enriched CD34+/CD38- cells obtained from a MCL patient as well as CD34+/CD38- cells obtained from cord blood samples (n=3). When comparing these two cell types, a number of upregulated mRNAs were detected in CD34+/CD38- cells in MCL LSC compared to normal stem cells. Among these were CD9, CD88 (C5aR) and CD163 as well as several cytoplasmic targets, including the TEC family member ITK. A list of top-upregulated genes identified in neoplastic stem cells in MCL is shown in Table 1.

**New Molecular Lesions identified in neoplastic cells in advanced SM and MCL**

Deep sequencing studies were performed using MCL cells in freshly diagnosed and relapsed patients, in order to learn what additional molecular lesions (apart from mutated KIT) are responsible for disease initiation, disease progression and the development of drug resistance. Notably, MCL is characterized by multidrug resistance and most patients have refractory disease and a very poor prognosis with median survival times of less than 1 year. These studies are ongoing. First data revealed a number of molecular lesions (mutations in TET2, SRSF2, ASXL1, CBL, and RUNX1) in patients with SM who suffer from an associated hematologic non-mast cell-lineage disease (AHNMD). The prognosis was found to correlate with the number of additional molecular lesions in these patients. These data have been published in collaboration with The University of Mannheim in 2013 (Schwaab et al, Blood 2013). Moreover, we were able to identify several additional mutations in *KIT* in our sequencing studies. In particular, we identified one KIT D816H mutation as well as KIT M514L, a known SNP, in a patient with acute MCL, and an isolated KIT S476I mutation in another case of chronic MCL. In both MCL patients, deep sequencing studies are ongoing in order to identify additional lesions, and in both cases, all mutants and targets will undergo pharmacologic validation. In a first step, the D816H mutant was found to be resistant against imatinib but responsive against PKC412, whereas the S476I mutant was found to be responsive to both TKI. We were
also able to confirm that the sorted LSC fractions obtained from patients with MCL contained the KIT mutations identified by our sequencing studies.

Table 1

Upregulated markers and targets identified in MCL LSC by gene array analysis

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>MCL vs. CB fold-change</th>
<th>Target</th>
<th>MCL vs. CB fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD163</td>
<td>83.18</td>
<td>TPSAB1</td>
<td>52.32</td>
</tr>
<tr>
<td>CD88 (C5aR)</td>
<td>20.86</td>
<td>PDK4</td>
<td>33.26</td>
</tr>
<tr>
<td>LYVE1</td>
<td>16.01</td>
<td>HDC</td>
<td>26.98</td>
</tr>
<tr>
<td>CD87 (uPAR)</td>
<td>9.94</td>
<td>C3aR1</td>
<td>14.43</td>
</tr>
<tr>
<td>CD68 (LDLR)</td>
<td>9.38</td>
<td>CCL2</td>
<td>12.61</td>
</tr>
<tr>
<td>THBS1</td>
<td>9.11</td>
<td>CCNA1</td>
<td>11.3</td>
</tr>
<tr>
<td>CD327 (Siglec-6)</td>
<td>9.03</td>
<td>HO-1</td>
<td>10.73</td>
</tr>
<tr>
<td>TYROBP</td>
<td>8.73</td>
<td>ITK</td>
<td>9.27</td>
</tr>
<tr>
<td>CD86 (B7-2)</td>
<td>6.97</td>
<td>BCL2A1</td>
<td>5.5</td>
</tr>
<tr>
<td>CD9 (p24)</td>
<td>6.31</td>
<td>SGK1</td>
<td>5.15</td>
</tr>
</tbody>
</table>

Markers and targets that were overexpressed at least 5-fold in CD34+/CD38- MCL LSC compared to CD34+/CD38- cord blood (CB) stem cells, are depicted. Gene array analyses were performed by Affymetrix algorithms. Results are expressed as fold-change (MCL-SC vs. CB-SC). Abbreviations: LYVE1, lymphatic vessel endothel hyaluron receptor 1, uPAR, urokinase receptor; LDLR; low density lipoprotein receptor; THBS1, thrombospondin 1; Siglec-6, sialic acid-binding Ig-like lectin-6; TYROBP, TYRO protein tyrosine kinase binding protein; TPSAB1, tryptase alpha/beta 1; PDK4, pyruvate dehydrogenase kinase, isozyme 4; HDC, histidine decarboxylase; C3aR1, complement C3a receptor 1; CCL2, chemokine (C-C) ligand 2; CCNA1, cyclin A1; ITK, IL2-inducible T-cell kinase; SGK1, serum/glucocorticoid regulated kinase 1.

Identification of FES as a New Target in SM

During the past few years, the LBC ONC was able to show that several KIT-downstream kinase molecules may serve as potential targets of therapy. As assessed by chemical proteomic profiling and MS, several targets of PKC412 have been identified. Among these, FES appeared to be of major interest. Whereas PKC412 and its metabolite CGP62221 were found to block the activity of KIT D816V and FES, a second PKC412 metabolite, CGP52421, that accumulates in vivo during treatment, did not bind to and did not block KIT D816V and FES. To validate FES as a potential target in advanced SM, we performed experiments using a FES-specific shRNA and HMC-1.2 cells. We found that the shRNA-induced knock-down results in growth inhibition of HMC-1.2 cells (Figure 7).
HMC-1.2 cells were transfected with a FES-specific shRNA which resulted in an almost complete knock down of FES as determined by Western blotting (A). In “B” untransfected HMC-1.2 cells were mixed 1:1 with either control shRNA-transfected HMC-1.2 cells (■■) or with HMC-1.2 cells transfected with shRNA against FES (●●). Cell mixtures were cultured in complete medium with 10% FCS for 3 weeks. At various time points, the numbers (percentage) of GFP+ cells were determined by flow cytometry. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments.

Since FES has been implicated in survival pathways and growth regulation, we were also interested to learn whether the two metabolites differ in their potential to induce apoptosis in neoplastic mast cells in MCL. To address this point, HMC-1 cells were incubated with the two metabolites and with PKC412. We found that PKC412 as well as CGP62221 induce apoptosis and an increase in the numbers (percentage) of active caspase 3-positive cells and thus an increase in the numbers of apoptotic cells, whereas CGP52421 showed no comparable effects on HMC-1 cells (Figure 8).

Collectively, these data suggest that FES is a relevant target in neoplastic mast cells and that the effects of the TKI on FES may be important clinically. In addition, our data show that the PKC412 metabolite CGP52421 does not exert effects on mutant KIT and downstream FES which may explain its weak effects on survival and proliferation of neoplastic mast cells in MCL. Currently, the LBC ONC explores whether the PKC412 metabolites, including CGP52421, synergize with cladribine (2CdA) in producing growth-inhibitory effects in neoplastic mast cells obtained from patients with aggressive SM or MCL. This is of particular interest as drug combination studies are planned in patients with advanced SM and MCL. Initial data suggest that not only PKC412 but also CGP52421 synergize with 2CdA in inhibiting malignant cell growth in MCL cells.
Figure 8
Effects of PKC412, CGP52421 and CGP62221 on viability of HMC-1 cells
HMC-1.1 cells (left panel) and HMC-1.2 cells (right panel) were incubated in control medium or various concentrations of PKC412 or its metabolites (CGP52421, CGP62221) at 37°C for 24 hours. Then, cells were stained with an antibody against active caspase 3 and analyzed by flow cytometry. Results show the percentages of active caspase 3+ cells and represent the mean±S.D. of 3 independent experiments. Asterisk: p<0.05. Almost the same results were obtained when counting the numbers of apoptotic HMC-1 cells on Wright-Giemsa-stained slides by microscopy.

Effects of ponatinib on growth and survival of neoplastic mast cells
Ponatinib is a novel promising TKI that blocks a number of key kinase targets, including KIT, in various neoplastic cells. In our experiments, ponatinib was found to inhibit the kinase activity of KIT G560V and KIT D816V in HMC-1 cells. In addition, ponatinib was found to suppress Lyn- and STAT5 activity in these cells. Ponatinib was found to induce growth inhibition and apoptosis in HMC-1 cells as well as in primary neoplastic mast cells in all patients with advanced SM (including several MCL cases) tested. The effects of ponatinib were dose-dependent, with higher IC50 values obtained in HMC-1.2 cells harboring KIT D816V than HMC-1.1 cells lacking KIT D816V (Figure 9).

Figure 9
Ponatinib inhibits the proliferation of HMC-1 cells
HMC-1.1 cells (left panel) and HMC-1.2 cells (right panel) were incubated in control medium or in various concentrations of ponatinib (0.001-1 µM) at 37°C for 48 hours. After incubation, ³H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. from 3 independent experiments.
In drug combination experiments, ponatinib was found to synergize with midostaurin in producing growth inhibition and apoptosis in HMC-1 cells (Figure 10) and in primary neoplastic mast cells (not shown). The ponatinib+midostaurin combination induced substantial inhibition of KIT-, Lyn-, and STAT5 activity, but did not suppress Btk activation. We also applied a Btk siRNA and found that the Btk knock-down sensitizes HMC-1 cells against ponatinib. Finally, we were able to show that ponatinib synergizes with the Btk-targeting drug dasatinib to produce growth inhibition in HMC-1 cells. These data suggest that multiple signaling pathways are activated and play a functional role in neoplastic mast cells, and that broadly-acting drugs or drug-combinations are required to block all these pathways and to control neoplastic cell growth successfully.

**Figure 10**
Ponatinib synergizes with midostaurin in inducing growth inhibition in HMC-1 cells
HMC-1.1 cells (left) and HMC-1.2 cells (right) were incubated in control medium or in various concentrations of midostaurin (■■), ponatinib (●●) or a combination of both drugs (▲▲) (at fixed ratio of drug concentrations) for 48 hours. Then, ³H-thymidine incorporation was measured. Results represent the mean±S.D. of triplicates and are expressed in percent of medium control. Drug synergism was confirmed by calculating combination index (CI) values using the CalcuSyn program. A CI value below 1 (indicating synergism) is indicated by asterisk (*).

Further validation of BRD4 as a novel Drug Target in MCL
During the past few years, various cytoplasmic drug targets have been identified in neoplastic mast cells in LBC ONC projects, and several of these targets have been validated. One novel promising target is the epigenetic reader BRD4. The LBC ONC has identified this target in AML LSC and was also able to show that neoplastic mast cells in advanced mastocytosis, including MCL, express nuclear and cytoplasmic BRD4, whereas in indolent SM (ISM), BRD4 was found to be expressed primarily in the nuclei but not in the cytoplasm of neoplastic mast cells. We were also able to show that the BRD4-blocking drug JQ1 inhibits growth and survival in neoplastic mast cells. In 2013, the LBC ONC screened for drug partners in order to enhance JQ1-effects on neoplastic mast cells. Of several different targeted drugs examined, we were able to identify all-trans retinoic acid (ATRA) and PKC412 as most the potent drug partners that synergize with JQ1 in producing growth-inhibition and apoptosis in neoplastic mast cells (Figure 11).
Figure 11
Effects of JQ1 and PKC412 on survival (apoptosis) of HMC-1 cells
HMC-1 cells were incubated in control medium (Co), in various suboptimal concentrations of JQ1 and PKC412, and in the drug combinations JQ1+PKC412 at fixed ratio of drug concentrations (5:1) at 37°C for 48 hours. Thereafter, the numbers (percentage) of apoptotic cells were determined by staining for AnnexinV by flow cytometry. Results represent the mean±S.D. of triplicates.

2.1.6. Novel Human Mast Cell Lines

During the past few years the LBC ONC has established a number of novel human mast cell lines, including the MCPV series (MCPV1 through MCPV4) which represent an immature stage of mast cell development and thus can be employed as a model of MCL. Another cell line, ROSA, was established in collaboration with a French partner (Michel Arock) at the Ecole Normale Supérieure de Cachan (Paris, France). Both cell lines represent immature mast cell precursors and should therefore be helpful models to study the biology and target expression profiles of MCL. We have also established several subclones (MCPV and ROSA) expressing KIT D816V. In addition, both cell lines express a number of target antigens on their surface (e.g. CD30, CD33, CD44, CD52, CD117, CD123) and in their cytoplasm (e.g. BRD4, STAT5, RAS). Whereas ROSA cells express a functional IgE receptor, MCPV cells lack IgE-binding sites. Both mast cell lines will be employed in several LBC ONC projects in the next project period (2014-2017) and both may help to keep the numbers of mouse experiments to a minimum. Finally, the LBC ONC has generated one canine mastocytoma cell line, NI-1. Similar to ROSA cells, NI-1 cells express a functionally active IgE receptor. NI-1 cells will also be employed in various LBC ONC projects in the next project period.

2.1.8. Clinical Significance of the Subclone-Concept of LSC

During the past few years, the LBC ONC has established a new concept around neoplastic stem cells in hematopoietic and solid malignancies. In this novel concept, cancer stem cell evolution is a step-wise process that includes premalignant and malignant stages of stem cell development. The concept predicts that neoplastic stem cells develop in distinct and more and more independent subclones during NSC-CSC evolution. The resulting subclone-formation is associated with a time-dependent diversification of the cancer-genome, characterized by multiple pro-oncogenic lesions.
that accumulate in different patterns in neoplastic stem cells over time. Finally, the concept predicts that in an early phase of cancer stem cell evolution, neoplastic (stem) cells may be slowly-cycling cells, and these subclones remain small and cannot expand or replace or invade the normal organ within a short time period. By contrast the fully malignant CSC (LSC in leukemias) have an immediate capacity to expand and to generate a cancer or a leukemia (LSC). In other words, neoplastic stem cells (NSC) can be divided into premalignant NSC and fully malignant NSC, and only the malignant NSC are capable of (immediately) producing an overt malignancy. Over time, some or more of the premalignant NSC will acquire further lesions and hits and then will transform into fully malignant LSC (CSC). In an overt malignancy (leukemia) premalignant NSC as well as LSC (CSC) can be detected. In contrast to LSC, premalignant (preleukemic) NCS are often highly resistant against various anti-cancer agents, especially against cell-cycle-dependent drugs (but also targeted drugs). Therefore, late relapses often derive from (initially) premalignant rather than from malignant NSC. This concept has nicely been confirmed in the paradigmatic model of CML. In these patients, most BCR/ABL mutations are hardly detectable (only by nested PCR) at diagnosis because they all reside in small-sized subclones maintained by preleukemic NSC. However, at the time of relapse when these patients have developed imatinib resistance, one or more BCR/ABL mutations are often detected. These relapses may occur after several months or years. As assessed by quantitative PCR, the mutant-bearing subclones expand steadily over time before a relapse occurs in these patients, and these sub-clones all develop independently from each other in the same individual patients, supporting the concept of subclone-evolution of LSC. The LBC ONC has also validated the subclone- and multi-step concept of LSC evolution in other clinical models. In each model examined, the new hypothesis could be confirmed. The clinical implications of this new concept have been summarized in an overview article published in Cancer Res (Valent et al, Cancer Res 2013;73:1037-1045). In the next project-period the LBC ONC will further validate this exciting new LSC concept in various preclinical and clinical models in MDS, AML, CML and MCL.

Strategic Aims reached in the project year 2013:

The strategic aims defined for the Cluster for 2013 were all reached in this project year. One important aim was to establish more cell lines models in order to study rare diseases such as MCL. Indeed the LBC ONC was able to establish several new MCL-like cell lines and thereby augmented research in this important disease model. Another strategic aim was to intensify those projects that examine stem cell-niche interactions and the effects of various drugs on these interactions. This aim was also reached. In addition, the LBC ONC started to explore the effects of various targeted drugs on niche-related cells which may be a novel, hitherto underestimated, aspect of targeted drug therapies. Indeed, the LBC ONC found that certain BCR/ABL-targeting TKI exert major inhibitory effects on vascular endothelial cells which may have clinical implications for drug indications and for the anti-cancer effects of these agents. In the next project-period, the LBC ONC will further intensify studies on stem cell-niche interactions and will examine the effects of various targeted drugs on niche cells in the bone marrow of patients with CML and other leukemias. Finally, the LBC
ONC had a strong focus on a select group of (most promising) targets and attempted to explore the effects of certain drug combinations in various malignancies. In general, two types of target-structures were studied in the LBC ONC in 2013, namely i) druggable cell surface antigens that may be employed to eliminate even quiescent LSC, and ii) cytoplasmic signaling molecules and epigenetic targets relevant to growth and survival of LSC. For the next project year of the LBC ONC, an important strategic aim will be to further validate novel LSC markers and targets in human leukemias, and to characterize and validate new LSC-niche interactions in the leukemic bone marrow. Following the recommendations of the SAB and the evaluation report, the LBC ONC will focus on myeloid neoplasm and will complete most studies conducted in the field of ALL in 2014 and 2015.

2.2. Publications in 2013 – Overview

A number of original publications and review articles have been published in the project year 2013. Among these papers are several first- and/or senior authorships in Blood, Cancer Res, Haematologica, J Leukoc Biol, and other top journals. A novel stem cell concept has been published in Nat Rev Cancer in 2012, and the clinical implications of this new concept have been highlighted in a review article in Cancer Res in 2013. It should be pointed out that several of these studies were conducted in collaboration with groups in the LBI for Cancer Research (LBI-CR), St.Anna Children’s Hospital and CeMM working in a newly established special research program (SFB) on myeloproliferative neoplasms (MPN) in Vienna (coordinated by P. Valent). Finally, the LBC ONC cluster was again involved in several clinical trials in 2013.

2.3. Patents

No patents were filed during the period 2013 in our LBC ONC consortium. However, the cluster is actively scouting for potentially ‘patentable’ results.

2.4. Attending Conferences and Meetings

Members of our LBC ONC consortium attended several important national and international conferences in the fields of Hematology and LSC Research. In addition, members of LBC ONC were involved in the organization of national and international conferences and meetings dedicated to stem cell research in 2013 (see below).

2.5. Lectures and Presentations

Members of the LBC ONC consortium presented their data in a number of invited lectures and other presentations in national and international top conferences and workshops in 2013. The effects of Nilotinib on vascular endothelial cells in the context of CML were presented in an oral lecture by Emir Hadzijusufovic at the Annual Meeting of the American Society of Hematology (ASH) in New Orleans (December 2013).
3. Additional Information

3.1. Scientific Collaborations

Scientific Collaborations within the Cluster
During the past few years, several internal collaborations have been established in the scientific environment of the LBC ONC. These collaborations have also been exploited by members of the Cluster consortium in 2013 in order to strengthen LBC ONC projects. Collaborations between the Hanusch Hospital and the MUV were maintained and in part intensified in 2013. In particular, we have established a new collaboration on MCL involving both partner institutions (Hanusch and MUV). In addition, ongoing cooperations with the Departments of Laboratory Medicine (MCL models), Pathology (stem cell-niche interactions), Internal Medicine I (leukemias and myeloma) and Radiation Therapy (leukemia models) were maintained in 2013.

Scientific Collaborations with other Groups in Vienna
During the past few years, the LBC ONC has established several essential collaborations with a number of groups working in the field of LSC research and Translational Hematology in Vienna. These cooperations were maintained and extended in 2013. Highlighting examples of fruitful collaborations are projects conducted together with our colleagues at CeMM (Giulio Superti-Furga), the LBI for Cancer Research (LBI-CR, with Richard Moriggl), St. Anna Childrens’ Hospital (Thomas Lion) and the VetmedUni Vienna (Veronika Sexl, Michael Willmann & Thomas Rülicke). Most of these collaborations are embedded in a new Special Research Program (SFB) on Myeloproliferative Neoplasms in Vienna (Speaker and Coordinator: P. Valent).

3.2. Organization of Conferences and Meetings

As mentioned above, members of the LBC ONC consortium were actively involved in the organization of various national and international conferences and meetings, including a basic science seminar (topic: novel targets in hematopoietic malignancies) in the Annual Spring-Meeting of the Austrian Society for Hematology and Oncology (Linz, April 2013), a Consensus Workshop on Mastocytosis and MCL in Vienna (June 2013), the Annual ECNM Meeting in London (September 2013) and an international meeting on Myeloid Neoplasms in Vienna in October 2013. LBC ONC members participated and were actively involved in the organization of these meetings. Finally, the LBC ONC contributed to the Health Science Meeting of the LBG in December 2013.

3.3. Education and Ph.D. Program

As in previous years, members of the LBC ONC were actively involved in teaching master students, M.D. students and Ph.D. students in 2013. Our students exploited all teaching facilities of the LBC ONC and its partners on Campus and attended the main lecture series of the newly established SFB program dedicated to myeloid neoplasms. Our Ph.D. students were involved in the Ph.D. program of the Medical University of
Vienna that is coordinated successfully by Brigitte Marian, who is a valuable member and CF-PF coordinator (dedicated to student’s education) of our cluster.

4. Outlook and Aims for the Next Project-Year

Based on the master-plan of the LBC ONC and results obtained in 2013 and in previous project years, the LBC ONC will continue to focus on novel markers and targets in neoplastic stem cells in myeloid and mast cell leukemias. Following the evaluation report 2013 and the recommendation of the SAB, the LBC ONC will complete its projects on lymphoid neoplasms in 2014 and 2015. The strategic aims for 2014 are to establish more stem cell line models, to intensify research on stem cell-niche interactions and to focus even more on myeloid and mast cell neoplasms (MCL).

In addition, the LBC ONC will try to initiate and maintain collaborations with other clusters and institutes of the LBG, including the LBI-CR (leukemia models), LBI for Osteology (stem cell-niche interactions), Cluster for Cardiovascular Research (vascular stem cell niche) and the Cluster for Translational Oncology (CSC-niche interactions). With regard to targets, the LBC ONC will continue to explore major cell surface target antigens, such as CD25, CD26, CD33, CD44, CD52, CD117, and CD123. In several instances, preclinical concepts should be translated into clinical practice using the clinical trial platform of the LBC ONC. Among cytoplasmic targets, the epigenetic reader BRD4 as well as key signalling molecules, such as the PI3 kinase, RAS or STAT5 will be validated in various disease models in LBC ONC projects in 2014. Another major aim will be to define novel LSC markers and targets in AML, CML and MCL by gene array screening and deep sequencing approaches. Such sequencing studies have been initiated in MCL, CML and AML. Newly identified markers and lesions will undergo validation and testing in various preclinical (in vitro and in vivo) models examining LSC. In the next project years (2014-2017) markers and targets will be examined for expression in niche-related cells in the bone marrow of patients with MDS, AML, CML and MCL. Markers, targets, and somatic aberration profiles will also be compared in various phases of the disease and correlated with clinical parameters and outcomes. Finally, we will continue to study genomic diversification in LSC in our leukemia models in order to confirm our subclone-and-latency hypothesis of LSC evolution. The long-term goal in all LBC ONC projects remains to develop new improved (curative) therapies for leukemias by applying drug combinations that have the capacity to eliminate all premalignant NSC and LSC in these leukemias.

5. Publications

As mentioned above, the LBC ONC consortium published a series of publications in peer-review journals in 2013, including manuscripts in Blood, Cancer Research, Stem Cells Dev, Haematologica, PloS One, or J Leukoc Biol.


**Review Articles**


Other Publications: Letters, Editorials, Book-Chapters, Meeting Reports


Oral Presentations / Lectures


Valent P. Systemische Mastozytose. Annual Meeting of the German, Austrian and Swiss Society for Hematology and Oncology, Vienna, Austria, October 19, 2013.


Valent P. Die leukämische Stammzelle als Zielstruktur für therapeutische Effekte von BRD4-Inhibitoren und anderen chromatin-assoziierten Hemmstoffen. Annual Meeting of the German, Austrian and Swiss Society for Hematology and Oncology, Vienna, Austria, October 21, 2013.


Grunt TW. Targeting growth signaling and metabolic pathways in ovarian cancer cells. Annual Meeting of the German, Austrian and Swiss Society for Hematology and Oncology, Vienna, Austria, October 22, 2013.


Abstracts


