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INTRINSIC AND EXTRINSIC MECHANISMS FOR B-CELL LYMPHOMA DEVELOPMENT AND PROGRESSION STUDIED BY GLOBAL GENE EXPRESSION PROFILING

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Intrinsic and extrinsic mechanisms for B-cell lymphoma development and progression studied by global gene expression profiling
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Mantle cell lymphoma (MCL) is a rare hematopoietic malignancy characterized by frequent relapses and poor survival, partly due to minimal residual disease, whereby a subset of malignant cells, harbored in protective niches, survive treatment. In vitro and ex vivo experiments have shown that MCL cells can be rescued from apoptosis through interactions with non-malignant cells such as stromal cells. The present thesis investigates the effect that extrinsic microenvironmental interactions may exert on MCL cells and discuss the presumptive role of these as well as intrinsic mechanisms in the development and progression of lymphomas.

MCL cells commonly grow in suspension but when co-cultured with stromal cells a fraction of the MCL cells strongly attached to the stromal cell monolayer. Analysis of transcript levels by next generation sequencing and species-specific read separation we identified sets of genes with altered transcript levels between the adherent MCL cells and those that remained in suspension. These genes could broadly be divided into four functional themes of which three exhibited increased transcript levels in the adherent fraction: B-cell signaling, anti-apoptosis and cell adhesion/migration, and one, early mitosis for which the associated genes exhibited lower transcript levels in the adherent MCL fraction. Additionally, we observed a significant overlap between the changed genes in the present co-culture model system and genes that change at the mRNA level between lymph node and cells in circulation in material from MCL and chronic lymphocytic leukemia patients. Suggesting that the model system faithfully represent aspects of microenvironment-promoted changes to the lymphoma cells also observed in vivo. As different MCL cell lines were subject to similar co-culture conditions we observed differences in engagement of and dependency on cell surface molecules, including the B cell receptor, for adhesion to stromal cells. Different responses to microenvironmental interactions for different MCL cells may therefore affect how different patients or different subsets of MCL cells within a patient will respond to different therapy regimens.

The transcription factor SOX11 is a key diagnostic marker in MCL. Commonly not expressed in hematological malignancies it is expressed in 90% of MCL cases and most importantly identifies cyclin D1 negative MCL. Its role in MCL development and progression is however debated. Here we show that in the context of the non-malignant pre-B-cell-like cell line Ba/F3 SOX11 does not exhibit oncogenic properties.

MYC is a transcription factor that is capable of regulating the expression of a vast and diverse set of genes and has a role in lymphoma development, progression and disease prognosis for many lymphoma types, including MCL. Here we show that progressively increasing MYC levels in B-cells lead to the gradual change in expression in a large set of genes and importantly the gradual increase of two lymphoma-associated MYC mutants significantly altered transcript levels differently from wild type MYC. Gene set enrichment analysis identified functions previously associated with regulation by MYC such as ribosome biogenesis and purine metabolism, while other, novel functions such as those related to B-cell identity and chemotaxis were observed. The MYC regulated genes overlapped with previous gene signatures observed in the Eμ-Myc mouse model and also with recently identified direct MYC targets, collectively indicating the utility of the present model system with inducible MYC for the identification of MYC dependent functions in lymphomagenesis. Two studied lymphoma-associated MYC mutants differentially affect largely distinct subsets on Myc-regulated genes via different mechanisms.

LIST OF SCIENTIFIC PAPERS

Scientific papers included in the thesis:

I. Gustav Arvidsson, Johan Henriksson, Birgitta Sander, and Anthony P. Wright **Mixed-species RNAseq analysis of human lymphoma cells adhering to mouse stromal cells identifies a core gene set that is also differentially expressed in the lymph node microenvironment of mantle cell lymphoma and chronic lymphocytic leukemia patients.** *Haematologica. 2018 103(4):666-678*

II. Laia Sadeghi, Gustav Arvidsson, Magali Merrien, Agata M. Wasik, André Görgens C.I. Edvard Smith, Birgitta Sander and Anthony P. Wright **Differential B-cell receptor signaling requirement for adhesion of mantle cell lymphoma cells to stromal cells.** *Unpublished manuscript*

III. Martin Lord, Gustav Arvidsson, Agata M. Wasik, Birger Christensson, Anthony P. Wright, Alf Grandien, and Birgitta Sander **Impact of Sox11 over-expression in Ba/F3 cells**. *Haematologica. 2018 103(12):e594-e597*

IV. Amir Mahani, Gustav Arvidsson, Laia Sadeghi, Alf Grandien and Anthony P. Wright **Differential transcriptional reprogramming by wild type and lymphoma-associated mutant MYC proteins as B-cells convert to lymphoma-like cells.** *Unpublished manuscript*

Related scientific papers not included in the thesis:

Gustav Arvidsson and Anthony P. Wright **A protein intrinsic disorder approach for characterising differentially expressed genes in transcriptome data: Analysis of cell-adhesion regulated gene expression in lymphoma cells.** *Int J Mol Sci. 2018 10;19(10):3101*

CONTENTS

LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
ASCT	autologous stem cell transplant
BCR	B-cell receptor
bHLH-LZ	basic helix-ollo -helix leucine zipper
BL	Burkitt's lymphoma
BM	bone marrow
B_{reg}	regulatory B-cell
CAF	cancer associated fibroblast
CAM-DR	cell adhesion mediated drug resistance
CAR?	chimeric antigen receptor
CFSE	carboxyfluorescein succinimidyl ester
CLL	chronic lymphocytic leukemia
DLBCL	diffuse large B-cell lymphoma
DSMZ	Deutsche sammlung von mikroorganismen und zellkulturen
FBS	fetal bovine serum
FDR	false discovery rate
FL	follicular lymphoma
FPKM	reads per kilobase per million of reads
GC	germinal center
GO	gene ontology
GSEA	gene set enrichment analysis
IG	immunoglobuline
KEGG	Kyoto encyclopedia of genes and genomes
LN	lymph node
MALT	mucosa-associated lymphoid tissue
MBI-IV	myc box I-IV

MCL	mantle cell lymphoma
MIPI	mantle cell lymphoma international prognostic index
MSigDb	molecular signure database
NGS	next generation sequencing
NHL	non-Hodgkins lymphoma
NIBS	national bioinformatics infrastructure Sweden
R-BAC	bendamustine and cytarabine in combination with rituximab
R-CHOP	cyclophosphamide, doxorubicin, vincristine, prednisone with rituximab
R-DHAP	dexamethasone, high-dose cytarabine, cisplatin with rituximab
RPKM	reads per kilobase per million of reads
SNIC	Swedish National Infrastructure for Computing
TAD	trans activation domain
TME	tumor microenvironment
TPM	transcript per million
T_{Reg}	regulatory T-cell
UCSC	University of California Santa Cruz
UPPMAX	Uppsala multidisciplinary center for advanced computational science
UPPNEX	UPPmax next generation sequencing cluster & storage

1 INTRODUCTION

1.1 B-CELL LYMPHOMAS

Normal B-cell differentiation and maturation depend on intrinsic and extrinsic signaling mechanisms where survival, proliferation, genomic rearrangements and mutations are supported at one or several instances during development. The process of B-cell development is inherently prone to malignant transformation given that the processes involved in antibody diversification may lead to unwanted chromosomal aberrations and oncogenic mutations. B-cell lymphomas are a heterogeneous group of malignancies, with different manifestations and clinical outcomes that acquire the first oncogenic event at some stage during B-cell development.[1,2] This first event may give survival advantages during further differentiation steps. The ultimate transformative event occurs later and can occur at different stages of the B-cell differentiation process. Consequently, the transformed B-cells will have a phenotypical resemblance to a normal B-cell counterpart at a particular developmental stage which may be reflected in terms of V(D)J rearrangements, *IGHV* mutation status as well as in distinct gene expression profiles and cell morphology. For example, the disease-defining oncogenic event in mantle cell lymphoma (MCL) occurs during the VDJ rearrangement of the immunoglobulin genes at the pre-B-cell stage, a translocational event juxtaposes cyclin D1—which is normally required for the G_1/S cell cycle transition—to the immunoglobulin heavy chain promoter, leading to deregulated expression and an aberrant cell cycle. This genetic event is however not sufficient to drive malignant transformation, as circulating B-cells carrying the MCL-associated translocation above have been observed in healthy individuals,[3] and secondary oncogenic events are required to promote malignant transformation.

The normal cellular counterpart for the majority of MCL cases and some cases of chronic lymphocytic leukemia (CLL) is the naïve, pre-germinal center B-cell and these cases are typically associated with an aggressive disease phenotype and a poor clinical outcome. For some MCL and CLL cases however the cell of origin is an antigen-experienced cell that has acquired somatic mutations when passing through the germinal center. Germinal center B-cell-like diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and Burkitt's lymphoma (BL) have their normal counterpart in the germinal center B-cell while activated B-cell-like DLBCL and mucosa associated lymphoid tissue lymphoma (MALT) resemble post-germinal center B-cells. The most common B-cell lymphomas are FL and DLBCL, comprising between 50-60% of B-cell lymphoma cases worldwide. In some B cell lymphomas such as FL, BL and MCL, key translocational events—where the oncogenes

BCL2, *MYC* and *CCND1*, respectively, are erroneously joined to the heavy chain locus on chromosome 14, leading to their constant activation—are present in the vast majority of cases. As these genomic rearrangements alone are not sufficient to induce malignant transformation, secondary genomic aberrations altering key pathways are necessary for cancer progression. While key translocational events related to the immunoglobulin heavy chain locus are considered hallmarks of these three B-cell lymphomas, most cases retain one allele of the immunoglobulin heavy chain locus and have the ability to express a functional B-cell receptor and can take advantage of its downstream signaling for increased survival and proliferative responses.

1.2 MANTLE CELL LYMPHOMA

1.2.1 Etiology and epidemiology of MCL

Mantle cell lymphoma (MCL) is an aggressive and in most cases incurable B-cell neoplasm that was recognized as a distinct non-Hodgkin's lymphoma (NHL) subtype in the early 1990s. MCL comprises about 7% of the NHL cases and it is characterized by a generally high relapse rate and mortality.[1] As in most NHL subtypes there is a strong male predominance among MCL cases (2.3–2.5 male to female ratio) and the median age at diagnosis is 67 years and 70 years for men and women respectivly.[4,5] Patients commonly present with advanced stage disease at diagnosis and more than 90% of the cases have extra-nodal manifestations with bone marrow (> 60% of cases) and gastrointestinal involvement.[6,7] The most recent classification of MCL makes a distinction between two main groups, the first one being classic MCL, which is typically *IGHV* unmutated, $SOX11^+$, genomically unstable with lymph node or extra-nodal involvement, and the second group being leukemic, non-nodal MCL which is typically negative for SOX11, genomically stable, carrying mutated *IGHV*.[2] Four main cytological variants of MCL are recognized: small cell, classic, pleomorphic and blastoid, ordered according to progressively worse disease outcome. The small cell type has often been found in MCL patients with tumors that follow an indolent clinical course.

While the median overall survival time has doubled over the past years—from 3–4 years to 5–7 years—the prognosis for MCL patients is usually poor and fewer than 15% of patients are long-term survivors.[8–10] There is however a small subgroup of patients (10–15%) with indolent disease, that may benefit from being left untreated under close supervision as the postponed therapy of these cases will not affect the clinical outcome.[11,12] A subset of the indolent cases are characterized by a non-nodal, leukemic disease presentation but also certain cases with nodal involvement have a slow disease course and a long survival without treatment.[13] There is a high risk of over-treatment due to the difficulty of diagnosing the

indolent disease type. These indolent cases have a higher number of somatic mutations in the *IGHV* locus and while the majority of cases are positive for the MCL hallmark translocation t(11;14)(q13;q32) they carry few other genetic aberrations.[14] When gene expression profiles were compared between indolent forms of MCL, conventional MCL and several other B-cell neoplasms the indolent MCLs showed a greater similarity to conventional MCL than to any other cell type in the study.[15] Thus this study showed that the indolent cases were indeed true MCL and not a subtype of other less aggressive B cell lymphomas such as CLL. Indolent MCL may progress into a more aggressive form at a later stage, often through the loss of a functional TP53 pathway.

In MCL, the Ki67 proliferative index is considered the most reliable prognostic factor for predicting long-term patient outcome.[16,17] A gene expression profiling study found a strong correlation between high expression of a proliferative gene signature consisting of 20 genes and poor survival.[18] Patients with the highest expression of proliferation index genes had a median survival of 0.8 years while those in the study with lowest expression had a median survival of 6.7 years.[18] In addition, deletions of genomic regions containing *CDKN2A* and *TP53* have been correlated with poor survival.[19]

1.2.2 MCL Phenotype

MCL was thought to derive from naïve, pre-germinal B-cells. Recent findings however suggest that a fraction of MCL cases (15–40%) show evidence of antigen experience manifested in the observed somatic mutations within the immunoglobulin genes, this patient subset has been associated with an indolent course and a more favorable clinical outcome.[20–22] A similar division has been observed in CLL where Ig-unmutated cases have been correlated with an inferior prognosis. Additionally, while stereotypically different from those observed in CLL, a bias in regards to the BCR repertoire has been reported in MCL, hinting of a role for antigen-driven clonal selection in subsets of MCL. While indolent MCL often have a higher number of somatic mutations in *IGHV*, a prognostic role for the *IGHV* mutation status in MCL is yet to be described. The immunophenotype of MCL is reminiscent of a mature naïve B-cell ($CD19^+$, $CD20^+$, $CD45^+$, $CD24^+$) with a medium to strong IgM expression along with $CD5^+$, $CD10^-$ and $CD23^-$ (In contrast, CLL has weak expression of CD20 and surface Ig and it is positive for both CD5 and CD23).[12]

1.2.3 Cyclin D1

As mentioned above, the defining feature of MCL is the t(11;14)(q13;q32) translocation which brings the cyclin D1 gene (*CCND1*) under the control of the immunoglobulin heavy

chain enhancer, leading to constitutive expression.[23,24] Binding of cyclin D1 to CDK4 or CDK6—which are both commonly overexpressed in MCL—activates E2F through phosphorylation of its inhibitor, the tumor suppressor RB1, which leads to a deregulation of the cell cycle promoting cells to undergo the G_1/S transition. The cyclin D1/CDK4 complex can in addition enable cyclin E/CDK2 activation by binding its repressor $p27^{Kip1}$ (is encoded by the *CDKN1B* gene), which in turn promotes further phosphorylation of RB1 and promotes entry into the S phase. Furthermore, additional mechanisms resulting in CCND1 overexpression have been observed in MCL. *CCND1* contains 5 exons that can be alternately spliced into two isoforms; cyclin D1a and D1b. While the latter is not involved in MCL pathogenesis, cyclin D1a is detected in virtually all CCND1 overexpressing MCL cases and harbors a destabilizing 3' UTR that, when truncated by chromosomal rearrangements or mutations, stabilizes the transcript leading to increased CCND1 levels. Truncated CCND1 transcripts have been correlated with poor survival.[25,26] In rare cases CCND1 levels can be further elevated by amplification events affecting the t(11;14)(q13;q32) region.[27] Interestingly, apart from the involvement of CCND1 in cell cycle progression, it has also been shown to bind RAD51 to promote DNA repair and homologous DNA recombination[28] and to impair the function of the proapoptotic protein BAX through direct interaction.[29]

The t(11;14)(q13;q32) translocation alone does not explain the aggressiveness of MCL nor is the deregulation of cyclin D1 sufficient to initiate disease progression.[30] The t(11;14)(q13;q32) translocation has been detected in 8% of peripheral blood samples from a population of healthy individuals.[3] Secondary genomic alterations are therefore necessary for malignant transformation. While the t(11;14)(q13;q32) translocation is considered a hallmark of MCL there is a small subset of cases with no cyclin D1 overexpression (~ 10%) that still present with a conventional MCL morphology and phenotype. These tumors have a gene expression profile almost identical to cyclin D1-positive MCL and about 50% of these cases express high levels of either cyclin D2 or D3 caused by chromosomal rearrangements fusing *CCND2* or *CCND3* to the *IG* loci.[31–33] As the cyclin D1 translocation is not present in all cases of MCL and as immunohistochemical detection is problematic for cyclin D2 and cyclin D3 since these cyclins, in contrast to cyclin D1, are expressed in normal B cells and other lymphomas, there has been an interest in identifying other diagnostic markers for MCL. SOX11 has proven a valuable diagnostic tool as it is expressed in almost all MCL cases, and importantly it is expressed in MCL cases lacking the CCND1 translocation.[34]

1.2.4 SOX11

The *SOX11* gene belongs to the Sry-related high-mobility group (HMG) box transcription factor family group C, together with *SOX4* and *SOX12*.[35–37] These genes are crucial for organogenesis.[38,39] Postnatally, SOX4 expression is restricted to B and T cells and gonad cells while the expression of SOX11 is primarily confined to neural tissue.[40,41] The role of SOX12 is less well described but expression has been observed across different cell types.[38] Members of the SOXC group preferentially bind to $C^{A}/_{T}TTGTT$ genomic motifs (at surprisingly low affinity levels compared to other transcription factors)[37] via the much-conserved N-terminally located HMG box, for which the SOXC group members share 84% identity.[38] In lymphopoiesis, SOX4 is essential for pro-B cell survival[42] while SOX11 has no known role.

While SOX11 is silenced in nearly all mature B-cells[43,44] it is expressed in over 90% of MCL cases and more importantly it is strongly expressed in cyclin D1-negative MCL while not expressed at all or at very low levels in other lymphomas.[34,43] In studies focusing on distinct non-hematopoietic malignancies such as ovarian cancer and gliomas, it has been suggested to act as a tumor suppressor,[45,46] while in aggressive breast cancer and head and neck cancer, SOX11 has been suggested to have oncogenic properties.[47,48] Some studies of MCL have argued that SOX11 has oncogenic properties, promoting proliferation and hampering terminal B-cell differentiation by down-regulation of BCL6 and PAX5.[49–51] The repression of BCL6 by SOX11 may impair MCL cell trafficking to and within germinal centers (GC). In a recently developed murine model with B-cell specific SOX11 overexpression it was shown to promote BCR signaling and proliferation.[52] This is however not without controversy as other studies investigating the oncogenic properties of SOX11 in MCL have rendered contradictory results indicative of a reduced proliferative response upon increased SOX11 expression.[53–55] Recent studies have additionally linked SOX11 to the regulation of functions related to tumor-supportive microenvironmental interactions, such as the migration of MCL cells below bone marrow derived mesenchymal cells or in promoting angiogenesis, suggesting that the role for SOX11 in lymphomagenesis may be context dependent.[56,57] SOX11 expression has proven a reliable tool for diagnosis of cyclin D1-negative MCL[34] and in addition studies have begun to evaluate a presumptive prognostic role for SOX11, as indolent MCL cases are often negative for SOX11. This is however a controversial subject as several independent groups have reported that SOX11 has no independent prognostic role.[13,15,55,58]

1.2.5 Secondary chromosomal aberrations in MCL

MCL commonly has a complex genetic landscape with a relatively high frequency of genomic abnormalities. Several chromosomal deletions and amplifications have been reported, and the majority of candidate genes related to the chromosomal aberrations are related to DNA damage response pathways, cell cycle regulation and cell survival.[59] Homozygous deletion at 9p21 is frequently observed in MCL[60] affecting the *CDKN2A* locus that encodes for both the CDK inhibitor INK4a as well as the tumor suppressor ARF, which protects TP53 from MDM2-regulated proteasome degradation. Mono-allelic deletions are frequent observed at 17p and 11q, containing *TP53* and *ATM* respectively. Amplifications have been reported for a number of chromosomal regions targeting *MDM2* and *CDK4* at 12q13 as well as the anti-apoptotic *BCL2* (18q21). Albeit rare, overexpression of the oncogene MYC either by amplification events at 8q24 or by other mechanisms such as the Burkitt translocation t(8;14)(q24;q32) has been observed in blastoid MCL cases where it has been correlated with a poor prognosis.[61,62]

1.2.6 Somatic mutations in MCL

The frequency of somatic mutations in MCL (1.2 per Mb)—while higher than for CLL and acute myeloid leukemia (AML)—is lower than for most hematopoietic and solid tumors. The most commonly mutated genes are *ATM*, *TP53* and *CCND1*.[63] *ATM,* which encodes for a protein important in cellular responses to DNA damage, is often subject to truncating or missense mutations in *IGHV*-non-mutated MCL and its inactivation has been correlated with increased genomic instability.[64] *CCND1* is frequently mutated in the indolent MCL subtype while *TP53* mutations are equally distributed between *IGHV* mutated and un-mutated cases irrespective of SOX11 expression.[63] Other notable reoccurring somatic mutations have been reported in the anti-apoptotic gene *BIRC3*, the cell surface receptor *TLR2* and in the three chromatin modifiers *WHSC1*, *MLL2* and *MEF2B*.[63] NOTCH1 and NOTCH2 are among the less frequently mutated genes in MCL, albeit with possible prognostic importance given the association between mutational status and inferior outcome.[63]

1.2.7 Constitutively activated pathways in MCL

Accumulated genomic gains and losses together with somatic mutational events give rise to constitutive activation of several pathways in MCL. The underlying mechanism for their activation and the implications thereof remains to be fully understood. The cell survival-promoting NF-κB pathway is frequently deregulated in MCL leading to an overexpression of several anti-apoptotic genes such as: *BCL2, BCL2L1*, *XIAP, MCL1* and *cFLIP*.[65] The mechanism for activation remains to be elucidated but signaling via the B-cell receptor

(BCR) pathway or via TNF-family receptors reportedly can lead to NF-κB activation.[65] The cell-surface BCR is connected to a network of kinases and phosphatases, which have roles with regards to survival, differentiation and proliferation during normal B-cell maturation. Chronic and tonic activation of signaling via the BCR is common in B-cell malignancies. Several kinases downstream of the BCR such as BTK, SYK and LYN are reportedly phosphorylated in MCL, indicative of an activated BCR signaling pathway.[66] Apart from observations of activated BCR signaling in MCL, emerging evidence suggests a role for BCR signaling in response to external stimuli in the proximal microenvironment, where, for instance, MCL and CLL cells residing in LN tissue have increased transcript levels of BCR signature genes.[67,68] It has recently become more evident that anti-apoptotic signaling via the tumor microenvironment may play a crucial role for the onset and progression of MCL and other B-cell malignancies than previously anticipated. In many cases the anti-apoptotic signaling may derive from pathways connected to the BCR. In addition, activated, phosphorylated key components of the PI3K/AKT/mTOR pathway have been found in MCL and several differentially expressed pathway components have been observed in MCL by global gene expression profiling, which can promote tumor proliferation and surviva[69,70] Gene expression profiling in primary MCL samples found several components of the Wnt pathway, known to be deregulated in several malignancies, to be up-regulated.[69] Another study found a transcriptionally activating form of beta-catenin present in the nucleus in MCL cell lines and primary samples, leading to an activated transcription of its targets, including cyclin D1 and MYC.[71] It is interesting to note the tremendous and maybe disproportionate effort that has been made to describe genetic abnormalities and dysregulated pathways in MCL and other hematological malignancies in relation to the research that has been conducted in regards to the cellular context. Recent efforts have however begun to dissect the different effects that the microenvironmental context has on the malignant cells in studies comparing malignant cells of different origin (peripheral blood, lymph node and bone marrow) in CLL and MCL.[67,68,72]

1.2.8 Treatment

MCL is notoriously difficult to treat, virtually incurable and no therapy is considered standard. For disease stratification the established Mantle Cell Lymphoma International Prognostic Index (MIPI), which makes use of the age of the patient, the Eastern Cooperative Oncology group performance index, lactate dehydrogenase level and leukocyte count, readily discriminates between indolent and aggressive cases while the resolution for intermediate cases makes interpretation difficult.[73] Incorporation of the Ki67 proliferation index[74] has

further improved stratification and studies have suggested a role for the additive prognostic value of *TP53* and *SOX11* status.[58] The rediscovered heterogeneity of the disease and the introduction of high-dose cytarabine chemotherapy combined with autologous stem cell transplantation (ASCT) and anti-CD20 treatment (Rituximab, a chimeric monoclonal antibody) have produced promising results. This and other improvements have led to a prolonged overall survival as well as a few MCL cases where the patients do experience long-term disease-free survival. Several regimens are currently under clinical investigation for management of different MCL patient groups. The choice of first line treatment depends on the age of the patient as well as fitness status. Young, fit patients may benefit from a high dose, R-CHOP/R-DHAP (R-CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone in combination with Rituximab; R-DHAP: dexamethasone, high-dose cytarabine and cisplatin in combination with Rituximab) treatment combined with an ASCT while unfit younger patients or fit elderly patients may be treated with conventional regimens such as standard dose R-CHOP or R-BAC (bendamustine and cytarabine in combination with Rituximab) followed by Rituximab maintenance.[75] Unfit and frail elderly patients require a less toxic immunochemotherapy treatment combined with Rituximab, followed by Rituximab maintenance. For patients presenting with an indolent disease manifestation; watch and wait until the disease advances is recommended, followed by therapy as conventional MCL upon disease progression. Relapsing MCL patient's response rates are generally lower regardless of choice of regimen. While some younger patients can respond well to second-line therapy the only treatment associated with long-term remission is the ASCT. Treatment of elderly or unfit patients that can no longer withstand chemotherapy and much less an ASCT calls for a palliative approach.

Recent findings have increased the hope that individualized medicine and small molecules targeting specific pathways will improve survival further. Two small molecules are currently being tested and integrated into conventional regimens for treatment of MCL and CLL, the proteasome inhibitor Bortezomib and the Bruton's tyrosine kinase (BTK) inhibitor Ibrutinib.[76,77] Bortezomib reversibly inhibits the proteolytic activity of the ubiquitin-proteasome system and Ibrutinib is an orally administrated small molecule that covalently binds to and inhibits BTK activity. Interestingly, a phase 1 study found an increase in $CD19^{+}$ $CD3^{-}$ $CD5^{+}$ cells in the peripheral blood of MCL patients following ibrutinib treatment,[78] indicative of a function for BCR signaling in relation to microenvironmental interaction and retention that could possibly be exploited to expel cells from tumor-harboring niches to the peripheral blood where they can more readily be targeted by other targeted therapy strategies or even by conventional chemotherapy regimens. Ibrutinib has also been shown to inhibit so

called pseudoemperipoesis (migration of cells below a stromal cell monolayer), in a CXCR4 dependent manner, using an in vitro co-culture system with a MCL cell line and mesenchymal stromal cell line.[78] Recently, the second generation BTK inhibitor Acalabrutinib was approved for treatment of refractory MCL, reportedly more specific to BTK than Ibrutinib, reducing off target effects, and with more potent in vivo activity.[79–81]

Other promising drugs in early phase clinical trials for treatment of MCL include the second-generation BCL2 inhibitor Ventoclax[82], a CDK4/6 inhibitor,[83] inhibitors of histone deacetylases as well as an anti-CD79 conjugated monoclonal antibody.[84] Additionally, the immunomodulatory small molecule lenalidomide has recently been approved for treatment of relapsed MCL by the FDA based on positive overall response rates in multiple phase II studies.[85,86] While targeting the anti-apoptotic BCL2 in MCL may pose a viable option, concerns arise as other BCL2 family members, such as MCL1 and BCL2L1 (encoding for Bcl-xL), have been shown to be up-regulated in response to signaling from proximal cells *in vitro*,[87,88] and increased mRNA levels of these two anti-apoptotic proteins have been observed in LN material as compared to peripheral blood in MCL.[87]

1.3 MYC IN LYMPHOMAS

The transcription factor MYC (encoded by *MYC* and sometimes referred to as c-Myc) is a basic helix-loop-helix protein that together with the paralogues MYCN, and MYCL constitutes the MYC family of transcription factors, all with similar structural and functional properties albeit with different expression across different tissues that have been implicated in the development and progression of a substantial fraction of human tumors. For the present discussion the focus will be on MYC. MYC has its name from its viral oncogenic homolog v-myc of the avian myelocytomatosis virus (MC29).[89–92] Its expression is essential during embryogenesis and a gene knock-out is embryonically lethal in mice.[93] Expression of Myc is important for the survival of B-cell precursors and their subsequent differentiation into mature B-cells and Myc expression can be detected at both pro- and pre-B-cell stages in mouse.[94,95] Mature, resting murine B-cells express MYC at very low levels.[95] MYC is expressed in a subset of GC B-cells and it is required for GC formation and maintenance.[96] In the GC increased MYC levels upon repression of its repressor BCL6 in the light zone allows for entry into the dark zone and somatic hypermutation reactions. Later, upon exiting the GC, MYC transcription in the B-cell is repressed by BLIMP1, allowing for differentiation into plasma cells.[97] *MYC* was early found to be translocated to the immunoglobulin heavy chain locus (t8;14)(q24;q32) in Burkitt's lymphoma cells, an event that leads to deregulated expression.[98,99] In 10-15% of Burkitt's lymphoma cases *MYC* is instead juxtaposed to either

the *Igκ* (2p12) or *Igλ* (22q11) immunoglobulin genes, which similarly leads to aberrant expression and a Burkitt's lymphoma phenotype.[100,101] A deregulated MYC expression is however not sufficient for malignant transformation and secondary mutational events are necessary to overcome responses that would otherwise lead to apoptosis or senescence.[102–104] More recently, and relevant for the present thesis, a study found that oncogenic transformation of murine B-cells was only possible if both intrinsic apoptosis and a TP53 dependent response were circumvented by the overexpression of BCL2L1 and BMI1 in conjunction with MYC.[105] Chromosomal rearrangements leading to the juxtaposition of MYC to the heavy chain locus have also been reported in 5-10% of DLBCL cases and the rearrangements have been associated with a poor prognosis.[106–108] In rare cases, the translocational event leading to aberrant MYC expression has also been observed in MCL where it, as in DLBCL, has been associated with an adverse prognosis.[109] Across aggressive B-cell lymphomas however, there are cases with elevated MYC levels irrespective of the chromosomal aberrations mentioned above, indicative of other mechanisms leading to increased MYC levels and cell transformation.[110] In MCL for example, increased MYC levels resulting from amplification events have been associated with a poor prognosis.[111] Across a variety of human cancers, the most frequent amplification events are those affecting 8q24 where the *MYC* gene is located.[112] Additionally, albeit rare, increased MYC levels due to translocation in CLL are associated with a poor prognosis.[113]

1.3.1 MYC structure, protein stability and mutational landscape

The MYC protein consists of a C-terminally located basic helix-loop-helix leucin zipper (bHLH-LZ) domain used for heterodimer formation with other bHLH-LZ transcription factors such as MAX and ZBTB17 (gene product also known as Miz-1) via the basic region located N-terminal to the HLH-LZ to bind DNA. The N-terminally located trans-activation domain (TAD) contains stretches of conserved regions entitled Myc box 0–IV (MB0–IV) where the MBI contains a proline-rich stretch of residues important for MYC activity and stability, where mutations frequently are found in lymphoma patients.[114–116]

Sequential phosphorylation of MYC at serine 62 and threonine 58 by ERK/CDK2 and GSK3β respectively, first activates MYC, and subsequently primes it for proteasomal degradation, summarized by Amy S. Farrell and Rosalie C. Sears.[117] The most frequent mutation found in lymphoma is T58I while the somewhat less frequent T58A is associated with protein stabilization and higher transforming capacity.[118] The half-lives for WT MYC and T58I are around 30 min while the T58A half-life is double that,[118] which is intriguing given removing the phosphorylation site at T58 is not the sole contributor to increased protein

stability but also properties of the residue such the effect it has in regards to phosphorylation at S62, which is impaired in T58I.[119] The T58A substitution has additionally been shown to promote a more rapid tumor development in mice as compared to WT MYC.[116,120] Gene regulation differences for MYC variants differing at T58 have been reported using fibroblast model systems with hundreds of genes reportedly differentially regulated between MYC WT and MYC T58I expressing cells.[118] How the mutational status affect gene expression in a more relevant, lymphoma-like system remains to be investigated and is addressed by the present thesis.

1.3.2 Genome recognition and gene regulation by MYC

MYC has a central role for transcriptional regulation of genes involved in many fundamental cellular processes such as growth, cell division and apoptosis. It can form heterodimers with MAX to bind DNA canonical E-box motifs (CACGTG) or variants thereof to, directly or indirectly, regulate the expression of a vast number of genes. While the MYC/MAX heterodimer has the highest affinity for the canonical E-box,[121,122] which, incidentally, is one of the most frequent motifs in the human genome.[123] MYC/MAX has additionally been shown to bind non-canonical E-boxes as well as genomic sequences without recognizable E-box motifs.[122,124] The progressive affinity for different genomic motifs allows for specific, MYC-level dependent regulation of sets of genes where those with high affinity canonical E-box motifs are occupied preferentially, sequentially followed by motifs with lower and lower affinity, with the capacity to regulate the associated genes.[125] The MYC has also been shown to repress the expression of sets of genes through interactions with ZBTB17 (also known as MIZ1),[126,127] later studies have questioned this as mutated MYC, unable to interact with ZBTB17, retained repressive capabilities and an additional role for ZBTB17 where it may affect MYC-promoted RNA polymerase II recruitment.[128] This simplified description of the regulatory involvement of MYC is probably misrepresentative as the role of MYC is pluralistic, given its multiple interaction partners and seemingly diverse involvement in functions like proliferation, growth and apoptosis. Additionally, MYC was one of four genes, alongside Sox2, Klf4 and Oct4 that could reprogram and de-differentiate fibroblasts into pluripotent stem cells and it is crucial for hematopoietic stem cell maintenance and survival.[129,130] There is currently little evidence for transcriptional regulation of specific genes being the only, or even the primary function, of MYC for propagating oncogenic responses, as pointed out in a review by Wolf et al.[131] MYC has for instance been shown to promote transcription by both RNA polymerase I[132] and III,[133] to promote the interaction between the rDNA and the nucleolar matrix via the intergenic spacer regions,[134] and has been

shown to release RNA polymerase II from transcriptional pausing.[135,136] Interestingly, MYC has been shown to bind to the promoter region of genes to elevate the transcription of all accessible genes, which led to the hypothesis that MYC is a global transcriptional amplifier.[137,138] This is however not a likely sole explanation model given the specific and context dependent gene regulation by MYC described more recently[127,128,139] and given the gradual specificity the MYC/MAX heterodimer shows for canonical E-box motifs and similar variants.[122,125] Additionally, direct expressional regulation of specific genes by MYC has recently been shown in a model system where the de novo transcription of a particular set of genes was shown to decrease upon rapid MYC degradation, several of these genes were directly involved in functions such as ribosome biogenesis and purine synthesis.[140] Interestingly, the identified signature of genes directly regulated by MYC was collectively increased at the mRNA level in data sets derived from a diverse set of human cancers in relation to the measured levels of MYC. Based on these recent findings a more plausible unified explanation model has been put forth where MYC specifically regulates subsets of genes based on promoter affinities while promoting transcription of accessible genes, the net effect being a significant increase in transcription of genes where MYC has the highest promoter affinity making it a non-linear amplifier of expression. This should however be context dependent and in cases where MYC levels are high, and high-affinity promoters saturated, the net effect could be increased expression of genes with lower promoter affinity for MYC.

1.4 THE TUMOR MICROENVIRONMENT

1.4.1 General implications of the tumor microenvironment

While tumors arise due to somatic mutations and genetic aberrations promoting clonal expansion and a growth advantage for the transformed cells, the events do not occur in isolation but within a complex tissue context, often involving multiple cell types. Over the past decades it has become increasingly evident that the microenvironment plays a vital role for tumor expansion, vascularization, metastasis and drug resistance. When Hanahan and Weinberg revised their “hallmarks of cancer“[141] in 2011 the effect that the tumor stroma has on the malignant cells was given substantial space and microenvironmental implications were discussed for every hallmark.[142] It is now widely appreciated that solid and hematopoietic tumor cells do not act in isolation nor as a homogenous cell-cluster but rather engage in a dynamic crosstalk with proximal and distal cellular components within the organism. A malignant lesion may therefore be seen more as an organ where both tumor and normal cell composition constitute important components for cancer initiation, growth, progression and,

as will be discussed further on, resistance to therapy. Several components of the tumor stroma, in the form of secreted molecules, adhesion molecules or surface receptors and their interactions with the malignant cells have been identified both as presumptive therapeutic targets as well prognostic markers for disease stratification.

Different cell-types frequently infiltrate and interact with malignant cells in the tumor tissue. These recruited or invading cells may be implicated in a diverse repertoire of pro tumorigenic mechanisms and can for instance: act to promote proliferation, confer resistance to cell death, evade destruction by the immune system or act to promote metastasis. Endothelial cells may infiltrate tumor tissue in response to vascular growth factors such as VEGF and FGF and see to the vascularization of the lesion together with pericytes. Cancer associated fibroblasts (CAFs) have been extensively studied in solid tumors over the past decade and are known to produce growth factors like TGF-β, EGF and HGF, mitogenic factors such as SFRP1 and IGF1 as well as chemokines like CXCL12 and CXCL14.[143] The CAF is thought to be a locally recruited fibroblast educated by the tumor environment to produce growth-promoting signals. Cytokine gradients of CXCL12, CXCL14 and others serve to recruit immune cells such as macrophages and B-cells to the lesion, reshaping the microenvironmental composition. Together with other microenvironmental signals they can polarize the recruitment of immune cells favoring an immune-dampening response where M2 macrophages and Th2 cells are attracted to the tumor tissue.

Infiltration of pro-tumorogenic immune cells into the tumor tissue is frequently observed in both solid and hematopoietic malignancies. Regulatory T and B cells (T_{Reg}, B_{reg}) can provide mitogenic stimuli to tumor cells while locally reducing immune responses by, for instance, suppressing cytotoxic T cells whose presence in tumors have been correlated with a favorable disease outcome in several malignancies.[144]

1.4.2 The tumor microenvironment in B-cell malignancies

Lymphoma cells often occupy natural B-cell niches such as the bone marrow or the lymph node, but different lymphomas have different relations to their immediate microenvironment, both in terms of microenvironmental dependencies and the capacity to recruit or re-educate proximal cells. Hence, the composition of the tumor microenvironment varies between B-cell malignancies.[145] In BL the lesions consist almost exclusively of autonomous, malignant cells with few, if any dependencies in regards to the microenvironment while, in contrast, the structure of the tumor tissue in FL is more reminiscent of normal lymphoid structures where the malignant cells take advantage of niches normally important for the survival and proliferation of non-malignant B-cells. In classical Hodgkin's lymphoma the infrequent

malignant cells are supported by a large stromal component, distinct from normal tissues, where supportive cells have been recruited to shape the microenvironment. Other lymphomas have microenvironmental dependencies that fall somewhere in between these more extreme cases. In FL and DLBCL, different compositions of non-malignant cells within the tumor stroma, such as infiltrating non-malignant immune cells have been correlated with survival, which further underlines the importance of the tumor microenvironment in lymphoma progression.[146,147] In MCL, infiltrating T-cells have been ascribed prognostic properties.[148]

Normal B-cells are harbored by and dependent on niche-specific signaling in different microenvironmental settings during their differentiation and maturation process. These signaling mechanisms are not random but precisely regulated, intrinsically through regulation by stage-specific transcription factors and in response to external stimuli. As the immediate environment is of great importance for B-cell homing and differentiation the notion and composition of the tumor microenvironment B-cell neoplasms is therefore inherently different from the microenvironment in most solid tumors. Malignant B-cells can make use of signaling mechanisms intended for their non-malignant counterparts as survival-promoting pathways are often functional in the malignant cells.[149] Lymphoma cells can also make use of surface molecules for accessing niches through migration towards normally occurring gradients of chemokines. This has for example been illustrated by knock-out studies of the CCR7 surface receptor in the Eμ-Myc mouse model where *CCR7*-deficient mice were tumor free significantly longer than wild type and as B-cells from the two genotypes were subsequently transplanted into congenic mice the *CCR7* deficient B-cells infiltrated LN, Spleen and bone marrow (BM) tissue slower and to a significantly lower extent.[150] Similar homing functions allow for interactions with mesenchymal stromal cells, lymphoma associated macrophages and regulatory T-cells among others and are implicated in immune evasion, proliferation and anti-apoptotic responses. Evasion of the immune system can be mediated by several mechanisms such as down regulation of MHC class II molecules, simultaneous down regulation of MHC I and CD58, which obliterates the effects of both $CD8^+$ T-cells and neutrophils, by increased expression of CD95L which induces apoptosis in $CD8^+$ T cells or via recruitment of cells with an immune dampening effect such as T_{Reg} or B_{Reg} cells. Interestingly, recent studies in CLL have shown that the malignant cells themselves can act in a regulatory fashion given right external stimuli.[151]

There is a growing appreciation for the BM as a survival-promoting niche for malignant B-cells in hematological cancers such as CLL and MCL, these cellular interactions may ultimately be responsible for the development of the phenomena referred to as minimal

residual disease. Even though the bulk of the malignant cells can often be efficiently eradicated by ever evolving and constantly refined chemotherapy regimens a minority of the malignant cells can still be protected by, for instance, niches of the BM. These surviving cells subsequently have the capacity to expand and repopulate the patient upon treatment termination. A better understanding of the overlap and differences between hematopoietic stem cell-homing and a presumptive cancer stem cell niche will therefore be of use when therapeutically targeting the tumor microenvironment.

Primary MCL and CLL can only be kept in culture for a few days before they undergo spontaneous apoptosis.[152,153] If co-cultured with mesenchymal stromal cells on the other hand, the in vitro cultures can be sustained for months.[153,154] The anti-apoptotic effect has been associated with an adhesion-mediated increased signaling via the canonical and non-canonical NF-κB pathway in the malignant cells leading to increased levels of the anti-apoptotic proteins BCL2L1 and MCL1.[87,88,153,155]

At diagnosis MCL patients frequently present with a disseminated disease often involving multiple extranodal tissues. Consequently, in MCL patients, several distinct microenvironmental niches exist that supposedly have different ways of supporting the lymphoma cells. An explanation for the frequent relapses in MCL may be the homing of a subpopulation of cells in survival-promoting, protective microenvironments where interactions between MCL cells and the stroma allow for treatment evasion and pave the way for minimal residual disease. While recent studies have brought forth questions related to the microenvironment in MCL much remains to be elucidated in regards to the effect that the different genomic abnormalities in MCL have on the cells capacity to interact with and shape their immediate microenvironment. In a recent review by Puente et al.[156] aiming to discuss genetics of MCL and CLL in relation to each other and to the microenvironment partially fails due to the strong focus on the genetic component, almost entirely omitting to discuss the cellular context.

The G-protein coupled surface receptor CXCR4 is important for homing and migration of B-cells to the bone marrow or to/within secondary lymphoid structures,[157–159] it is expressed at multiple instances during development.[160] Surface expression of CXCR4 allows for migration towards CXCL12 gradients, which is secreted by cells in the microenvironment such as stromal cells. Knocking out either the CXCR4 receptor or its ligand, the chemokine CXCL12, are both lethal in mice.[161162] MCL and CLL cells reportedly express high levels of the CXCR4 surface receptor which can be utilized to access supportive microenvironmental niches.[163,164] Additional surface receptors for chemoattractants important for B-cell homing

and migration are CXCR5, which is the receptor for CXCL13, as well as CCR7 which has been shown interact with CCL19 and CCL21 and more recently also with CXCL12.[165] CXCL12, in addition to promoting migration to protective niches, also has a direct role in promoting cell survival in CLL by reducing apoptotic responses.[166] Small molecules such as the agonist Plerixafor have been developed, which upon binding to CXCR4 leads to receptor internalization without triggering downstream signaling.[167] Plerixafor has been shown to expel different hematopoietic cells of both human and murine origin from tissues into the peripheral blood.[168,169] In MCL, inhibition of CXCR4 by plerixafor has been shown to impair migration of MCL cells beneath stromal cells.[163] Additionally, reduced CXCR4 surface levels have been reported in LN residing cells in MCL and CLL as compared to those in circulation.[67,78]

In the GC, interactions between B-cells and adjacent cells via ICAM1 or VLA-4, have been shown to decrease the number of apoptotic cells.[170] MCL cells frequently express high levels of VLA-4.[163] Signaling via VLA-4 can promote cell survival of NHL cells[171] and has been implicated in cell adhesion mediated drug resistance and the protective effect provided to the MCL cells by proximal stromal cells can be overcome by directly targeting the VLA-4 surface molecule by antibodies.[172] ICAM1 is commonly expressed on B-cells and facilitates their exit from circulation by enabling adhesion to the vascular wall.[173] High ICAM1 levels have been observed in a subset of MCL patients[174] and in CLL, ICAM1 surface expression and plasma levels have been corelated with clinical stages of CLL.[175]

MCL cells express the surface receptor CD40, permitting proliferation and survival support by $CD154^+$ T cells. In vitro experiments have shown a proliferative response in MCL upon stimulation with the CD40 ligand CD154 and a study of several B-cell malignancies that included a few MCL cases, found that infiltration of T_{Reg} cells could provide similar stimuli via the CD40-CD154 axis.[176–178] Infiltration of macrophages into the tumor tissue has been observed in aggressive MCL cases with increased mitotic activity, their role is yet to be investigated but may be similar to lymphoma associated macrophages which have been described and correlated with an inferior prognosis in FL.[179–181]

1.4.3 The B-cell receptor and the microenvironment in MCL

A constitutively active signaling via the BCR has been reported in MCL cell lines and patient samples[66] and interestingly, studies using primary material from CLL and MCL patients have identified the increased expression of BCR genes in cells in the LN when compared to those in the peripheral blood.[67,68] Consistently, the observed exodus of cells from lymphoid tissues in MCL patients treated with the BTK inhibitor Ibrutinib further supports the importance of

BCR signaling for the homing of lymphoma cells.[78] Interestingly, *in vitro* studies using MCL cell lines have shown that BTK inhibition by Ibrutinib has been shown to reduce migration towards both CXCL12 and CXCL13 gradients.[78] While interacting antigens and the underlying mechanism has not been determined, constitutive activation of Syk, PKCβ-II and PI3K, all downstream of the BCR, have been reported in MCL.[182–184] The activated BCR signaling leads to downstream activation of the NF-κB and MAP kinase pathways among others, promoting survival, for instance resulting from increased levels of the anti-apoptotic proteins BCL2L1 and MCL1, as well as cell division. While the microenvironmental mechanisms promoting activation of BRC signaling have yet to be elucidated the therapeutic potential remains high given the promising responses in clinical trials targeting central actors downstream of the BCR with small molecules.

1.4.4 Environment mediated drug resistance

So far, the discussion here has focused on how neoplastic B-cells take advantage of homing signals from the tumor microenvironment for direct growth support, survival and immune evasion. Accumulating evidence also suggests that intercellular interactions in the tumor microenvironment can alter the tumor cells sensitivity to chemotherapeutic agents. The special case of environment mediated drug resistance called cell adhesion mediated drug resistance (CAM-DR) was first described in myeloma cell lines.[185] Ex-vivo studies of MCL cells have shown that interactions with stromal cells can make the malignant cells less susceptible to cytotoxic treatment, partly through induction of the NF-κB pathway.[153] Notably, cells that did not adhere to the stromal cell monolayer were still sensitive to drug-induced apoptosis while soluble factors, such as the B-cell activating factor (BAFF)—produced by stromal cells upon contact with lymphoma cells—lowered the apoptotic effect.[153] Reports of similar effects have been observed in CLL, where a number of different stromal cell lines have been shown to protect CLL cells.[186] Additionally, direct interactions between lymphoma cells and stromal cells have been shown to induce cell cycle arrest in MCL and DLBCL, making the malignant cells less susceptible to cytotoxic agents aimed at proliferating cells.[187] The mechanisms and underlying microenvironmental cross talk giving rise to CAM-DR, survival of the malignant cells and subsequent relapse in patients remain to be fully characterized.

1.4.5 Therapeutic targets in the MCL microenvironment

Several approaches have been undertaken to target tumor-promoting microenvironmental interactions in MCL and here follows a brief description of four options that are or may develop into options for treatment of MCL. CXCR4 inhibitors such as Plerixafor have been

shown to disrupt homing signals and mobilize tumor cells into peripheral blood in both hematological and solid malignancies.[188] When deprived of protective signals from the microenvironment these cells can more efficiently be targeted by conventional chemotherapy.[189] As hematopoietic stem cells make use of the very same signaling for homing a combinatory treatment with molecules specifically targeting the malignant cells is desirable. In general, the issue with overlapping signaling mechanisms between cancer stem cell niches and hematopoietic stem cell niches will have to be addressed to efficiently target the tumor microenvironment while keeping toxicity low. The need for a better characterization of the different supportive signaling mechanisms cannot be emphasized enough and recent studies of global gene expression differences in MCL and CLL cells residing in different microenvironmental contexts have begun to identify presumptive targets that are important for intercellular communication and physical interactions.

Therapeutic targets in the tumor microenvironment include adhesion molecules such as VLA-4, which is frequently expressed in MCL and can be targeted by the humanized monoclonal antibody Nataluzimab[163]

Blocking signaling through the BCR via the BTK inhibitor Ibrutinib has produced promising results, mobilizing MCL cells from lymphoid tissue into the peripheral blood where they can be targeted by conventional therapy.[78] Ibrutinib has in addition been shown to impair migration and survival in CLL cells as well as their ability to secrete BCR dependent chemokines such as CCL3 and CCL4, both thought to promote interactions with T cells.[190] This underlines a central role for the BCR when it comes to migration and homing. The second generation BTK inhibitor Acalabrutinib reportedly have a higher specificity for its target and reports following a phase-2 trial in patients with relapsed or refractory MCL have shown promising results.[80,81] Targeting other central components of the BCR signaling pathway like Syk may have potential and the small-molecule Syk-inhibitor R406 has shown promising results in both pre-clinical studies and in a first clinical trial in CLL.[191]

2 AIMS

The overall aim of the thesis was to establish and study relevant model systems for investigating the implications of intrinsic and extrinsic stimuli of cells in lymphomagenesis. Specifically, the studies aimed to do the following:

2.1 PAPER I

Given that stromal cells of different origin can protect MCL and CLL cells from drug-induced apoptosis the aim of Paper I was to develop and characterize a model system for studying cellular changes arising in lymphoma cells upon interaction with and/or attachment to stromal cells.

2.2 PAPER II

Based on findings from Paper I the aim of Paper II was to evaluate the findings from Paper I in a similar albeit different model system and investigate to investigate the extent of common and divergent effects in response to MCL cell adhesion to stromal cells.

2.3 PAPER III

The aim of paper III was to investigate the effect of SOX11 overexpression in the non-malignant pre-B-cell Ba/F3, in order to determine whether it showed oncogenic activity.

2.4 PAPER IV

In Paper IV the aim was to develop a model system for testing the effect of progressively increasing MYC levels in B-cell lymphomagenesis and characterize the differences between wild type MYC and two lymphoma-associated MYC mutants under these circumstances, in order to determine the role of wild type and mutant MYC in the conversion of normal B-cells to lymphoma cells.

3 METHODOLOGICAL CONSIDERATIONS

Here follows a brief discussion of technical and methodological considerations and choices. Full descriptions of the materials and methods are available in the respective publications.

3.1 CELL SOURCES

For studies of microenvironmental interactions in MCL, two well-characterized MCL cell lines that grow in single-cells suspensions were chosen: JeKo-1 was established in 1998 from mononuclear cells of the peripheral blood from a 78 year old female patient diagnosed with MCL.[192] The rendered cell line had a B-cell phenotype expressing cell surface markers: IgM^+, IgD^+, $CD3^-$, $CD5^+$, $CD10^-$, $CD19^+$, $CD20^+$ and $CD23^-$ and carried the MCL-associated cyclin D1 translocation t(11;14)(q13;q32). The JeKo-1 cells used in the present studies were purchased from the DSMZ. Rec-1 was established in 1988 from lymph node material from a 61 year old male patient with malignant large-cell B-lymphoma positive for the t(11;14)(q13;q32) translocation, which had the following cell-surface characteristics: IgM^+, IgD^+, $CD3^-$, $CD5^-$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$, $CD23^-$ and $CD37^+$.[193,194] The Rec-1 cells used here were a kind gift from Dr. Christian Bastard, Ronan, France.

Recent studies have shown that an array of different stromal cell lines, of both human and murine origin, can protect lymphoma cells from undergoing both spontaneous and drug-induced apoptosis. [153,163,172,186,195] MS-5 is a bone marrow derived murine stromal cell line established in 1988 that has the capacity to support the growth of hematopoietic stem cells.[196,197] It was chosen for its documented capacity to protect lymphoma cells from spontaneous and drug-induced apoptosis[153,171,195] as well as for its murine origin, which would later allow for transcript discrimination between the lymphoma and stromal components in the adherent co-culture fraction by next generation sequencing and species-based read separation. The MS-5 cells used in the present study were purchased from the DSMZ.

For investing a presumptive role for the transcription factor SOX11 in non-malignant B-cells in terms on the effect it has on global gene expression, proliferation and presumptive oncogenic transformation we transduced the murine, bone marrow derived, pro-B cell line Ba/F3 with a Tet-On construct that allowed for doxycycline-regulated overexpression of SOX11.[198,199] Ba/F3 has previously been used to investigate oncogenic properties of proteins.[200–203]

For investigating the role of MYC in its WT form or in variants carrying lymphoma-associated missense mutations leading to the exchange of threonine 58 to either alanine

(T58A) or isoleucine (T58I), murine B-cells carrying Tet-On MYC constructs (WT, T58A and T58I) were generated through retroviral transduction of splenic B-cells with constructs for regulable expression of MYC and constitutive expression of BMI1 and BCL2L1 as described previously.[204] The expression of WT MYC or mutated MYC could be regulated from low levels to being overtly over-expressed by adding doxycycline (in the range of 0-1000 ng/ml) to the transformed B-cells.

3.2 CO-CULTURE CONDITIONS AND CHARACTERISATION

MCL cells were maintained in RPMI1640 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin. The MS-5 stromal cells were cultured in αMEM supplemented with 10% heat inactivated FBS, 2mM sodium pyruvate and 1% penicillin/streptomycin. The co-cultures were cultivated as the MS-5 stromal cells. The stromal cells were seeded in advance followed by the addition of MCL cells 24 hours later at a 10:1 MCL to stromal cell ratio. For quantitation of bound MCL cells the cells were labelled prior to co-cultivation using the Carboxyfluorescein succinimidyl ester (CFSE, CellTracer life technologies) in conjunction with previously labeled far red stromal cells. The ratio of bound MCL cells to stromal cells was determined by flow cytometry using a MACSQuant Analyzer 10 instrument and the data was analyzed using FlowJo (v10).

3.3 NGS LIBRARY PREPARATION AND SEQUENCING

For all RNA-seq experiments in the thesis, total RNA was extracted using the RNeasy kit (Qiagen), quality was assessed by TapeStation and subsequent cDNA libraries were created using the TrueSeq 2.0 library preparation kit from Illumina including a poly-A enrichment step by poly-T attached magnetic beads. The libraries were sequenced using different Illumina sequencing platforms depending on the experimental prerequisites in terms of read length, read count etc., e.g. the MiSeq platform was used for initial pilot experiments for species-based read separation for its cost-effectiveness and for the subsequent co-culture experiments a HiSeq2000/2500 instrument was used generating 2x101 base pair (bp) paired-end reads. The same platform was used for global gene expression assessment in MYC over-expressing cells, but in that study a paired-end approach was deemed unnecessary and the libraries were sequenced to generate 50 bp single-end reads.

3.4 NGS ANALYSIS

Most processor- and memory-intensive computational processes such as species-based read separation, alignment to reference genomes and per feature read quantification were conducted using the UPPNEX computer cluster infrastructure which is a member of the NIBS

infrastructure for bioinformatics and is a part of the UPPMAX and it is partially funded through the SNIC computational infrastructure for Swedish universities. Hence, all data processing, from quality assessment through alignment to the reference genomes and subsequent per-feature read-quantification was conducted in a Unix/Linux environment using dedicated, open-source software packages for reproducibility purposes.

3.4.1 Read quality and pre-processing

Quality assessment of the raw fastq data files was conducted using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter trimming was in most cases automatically performed immediately following sequencing by means of Illumina proprietary software. A subsequent quality-based trimming of read-ends using phred scores was conducted using Trimmomatic.[205]

3.4.2 Reference genomes

Reference genomes for alignment-based read quantification or species-specific read separation were initially acquired via the UCSC (unmasked hg19 and mm10 including scaffolds for human and mouse respectively). Following the release of the updated human reference genome in December 2013 by the Genome Reference Consortium new versions of unmasked genomes for both human and mouse, including scaffolds, were downloaded from the ENSEMBL ftp server and used for subsequent analysis (GRCh38.87 and GRCm38.87 for human and mouse respectively).

3.4.3 Species-based unmixing of short reads

Given the dual cell components in the co-cultures (human MCL cells co-cultured with murine stromal cells) and due to the fact that lengthy physical cell separation procedures have been shown to introduce artifacts affecting the expression levels of genes due to sample preparation procedures,[206–209] a decision was made to circumnavigate this by extracting total RNA from a mixed population of human and mouse cells followed by library preparation, sequencing and a subsequent *in silico* de-convolution of the short reads based on species origin.

Initially, a method for linear unmixing of reads based on their species origin was developed where a per-gene bleed over coefficient was calculated, using mono-cultured human and murine cells, which was subsequently used for per gene unmixing. The method had several drawbacks. First, it assumes that the global blead over between organisms is small. This was not an issue in the case of the present study as the long, paired-end reads readily differentiated

between the two species fractions for the majority of genes. Secondly, estimation of the constants for the linear unmixing model had to be determined empirically, something that was feasible in the case of the present study but that would be more difficult in other model system settings such as xenografts should the method be developed further and distributed for broader use. Lastly, the unmixing is done at the count level using aligned and quantified read count data. A method capable of classifying a read based on its inherent sequence information prior to counting would be preferable. The species-based and publicly available classifying tool for short read sequencing data named Xenome met all of the criteria mentioned above.[210] Initially developed for the deconvolution of NGS reads of xenograft origin Xenome uses a k-mer based approach for a mere classification of reads from mixed libraries of up to two species. During the indexing step a k-mer hash array based on the reference genomes fasta files is created which is thereafter used to classify each read as being derived from one or the other, neither or both of the different species. As indicated in the paper by Conway et al.[211] the drawback of an alignment-based method using a short read aligner such as Tophat with read processing software like SAMtools[212] is not so much the miss classification of reads but that a significant proportion of the reads cannot be assigned to either of the species exclusively which inflates the "both" category. Another approach would have been to use a k-mer based count approach omitting alignment such as the one employed by the program Sailfish.[213] During the course of the project other approaches for species-based read separation with marginally better performance than Xenome have been put forward.[214] Based on empirical benchmarking against the linear-unmixing approach (Figure 1) and the ease of use, the Xenome approach was employed in Paper I and Paper II using the default k-mer length of 25.

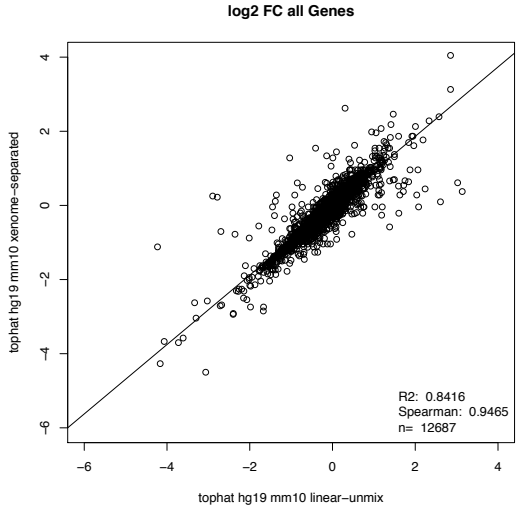

Figure 1. Comparison of *in silico*, species-based deconvolution of short read libraries by linear unmixing (x-axis) or Xenome (y-axis).

3.4.4 Alignment of short reads to the reference genome

Reads separated based on species origin were aligned to the respective reference genomes using splice-aware short read aligners. For paper I we made use of Tophat2,[215] which is a splice-aware aligner based on Bowtie2 that in turn employs a suffix-array-like so-called FM-index which is based on the burrows wheeler transformation[216] and therefore leaves a small memory footprint making it suitable for local desktop machines.[217] It is however processor intensive and the alignment is time consuming despite the possibility of multi-threading. While the precision of Tophat2 is only slightly inferior to more recently developed short read aligners,[218] the slow alignment process made us switch to the more recent, faster and more precise, splice-aware short-read aligner STAR[219] for the subsequent projects. STAR, like Tophat2, employs a suffix-array-based approach but in contrast to Tophat2 it loads the entire uncompressed suffix array into the RAM memory making it memory intensive but extremely fast while retaining mapping precision.[218] For comparison a typical fastq file that Tophat2 would align in 4 h using < 16 GB memory STAR would align in 15 min using ~ 80 GB of memory.

3.4.5 Read quantification and differential expression

In Paper I, Reads per feature (concatenated exons for one gene) were counted using the Bioconductor package GenomicAlignments (1.0.6) with the counting mode set to "Union" with gene annotations from the Bioconductor packages TxDb.Hsapiens.UCSC.hg19.knownGene (v.2.14.0) and TxDb.Mmusculus.UCSC.mm10.knownGene (v.2.14.0). For the subsequent studies reads per feature were derived using the function featurecounts from the subread package in conjunction with gtf gene annotation files downloaded from the ENSEMBL ftp server (GRCh38.87 for human and GRCm38.87 for mouse). The TxDb databases contain information for 23459 and 24421 genes for human and mouse respectively while the ENSEMBL database for the GRC38 genomes contain information about 58051 human genes and 49671 for mouse.

Count table objects were imported into the R environment and tests for differential transcript levels between groups were conducted using the well-established Biocounductor packages DESeq[220] and edgeR.[221] For Paper I sequential pair-wise comparisons using DESeq sufficed while the more complex experimental setups in Paper II and Paper IV required a more complex modeling approach which was implemented using the glmQLF[222] workflow from the edgeR package. Briefly about the normalization of read counts to library sizes and to feature (gene) length, Paper I present expression values as transcript per million (TPM) while

the subsequent Paper II and IV presents the expression as reads per kilobase per million of reads (RPKM). While the two are related they differ slightly in regards to how the data is normalized. RPKM, (or the equivalent fragments per kilobase per million of reads (FPKM), which is often used for paired end data) is calculated based on the relationship between the number of counted fragments for each feature in relation to the total number of mapped fragments in the cDNA library and to the length of the feature in base pairs.[223] TPM in addition takes both feature length and library size into consideration and also adjusts for the length of all expressed transcripts.[224]

3.4.6 Gene set overrepresentation tests and GSEA

To ascribe functional categories to sets of genes with transcript level differences between conditions gene set databases such as the KEGG pathway database[225,226], the GO biological processes[227,228] or the Hallmark[229] gene sets from the MSigDb[230] have been used in conjunction with methods to test for enrichment. Basic enrichment tests for significant overlaps between differentially expressed and/or clustered genes and gene lists from the databases above or gene signature lists acquired elsewhere were conducted using Fisher's exact tests, adjusting for multiple testing by the false discovery rate (FDR) when multiple sequential tests were conducted. Gene set enrichment analysis (GSEA) produces a similar result but the approach is slightly more powerful as it takes expression levels from the global gene expression data into account and thereby gives more weight to the most significant genes with the highest fold change.[231] All genes for which expression data has been acquired are ordered so that one end of the list is associated with the most interesting genes for one condition and the other end contain the most interesting genes for the other condition, for a pair-wise comparison one might use $\log_2$ transformed fold change values multiplied by the negative $\log_{10}$ transformed adjusted *P*-values. The GSEA can for instance be conducted either by submitting gene expression data to the software provided by Broad Institute or by feeding a pre-ranked list to the Bioconductor package fGSEA.[232] This produces a list of gene sets presented with the level of enrichment (expressed as a normalized enrichment score NES) and significance level (FDR adjusted *P*-value).

3.4.7 Genome-wide identification of E-box motifs

The canonical E-box motif CACGTG, which the MYC/MAX heterodimer has the highest affinity for, was aligned to the mouse reference genome GRCm38.87 using the short read aligner Bowtie (v1.2.0)[233] rendering 271113 unique alignment locations. The output bam file containing read alignment coordinates was converted to a bed file using BEDTools (v2.27.1) and distances between the aligned E-boxes to the closest transcription start site (TSS) for

known features in the ENSEMBL database were calculated using the script annotatePeaks.pl from the Homer package (http://homer.ucsd.edu/homer). To tests whether the clustered genes in Paper IV had E-box motifs closer to the TSS or whether they contained a higher number of E-box motifs within a certain distance from the TSS a Z-score was calculated based on 1000 resampling permutations within the set of non-regulated genes.

4 RESULTS AND DISCUSSION

4.1 PAPER I

As stromal cells can protect malignant B cells from undergoing both spontaneous and drug induced apoptosis[153,186] we sought to investigate the implications of microenvironmental signaling by stromal cells on MCL cells, both in relation to function and gene expression. For this purpose, a co-culture model system was developed using the murine stromal cell line MS-5 and the MCL cell line JeKo-1. A subset of the MCL cells, which normally grow in suspension, adhered strongly to the stromal cell monolayer. This has previously been observed in similar systems.[163,234] The intercellular interaction was stable as a subsequent addition of fresh MCL cells in 10-fold excess, after the first set had been allowed to adhere to the stromal cells for 24 hours and the suspension fraction had been poured off, could not displace the already bound MCL cells by competing for the same binding sites and the first bound cells remained bound during the following 24 hours. The stable adhesion of a subset of the MCL cells to the stromal cell mono-layer prompted us to investigate differences in global gene expression between the adherent fraction and the cells remaining in suspension by RNA sequencing.

Using a murine stromal cell line made possible immediate RNA isolation from the mixed fraction of adherent cells followed by library preparation and short read generation by massive parallel sequencing by means of the Illumina platform. The mixed libraries were subsequently deconvoluted in silico using species-based read separation. Isolated RNA from suspension cells in co-culture were subject to the same workflow as the mono-cultured MCL and stromal cells that were included as control. 1050 genes were found to have significantly changed transcript levels between adherent and suspension MCL cells in co-culture, including cytokines such as e.g. IL10, CSF1, CCL22, CCL3 and CCL4 as well as NF-κB pathway components like: NFKB1, NFKB2, NFKBID and NFKBIE and surface receptors such as e.g. TNF and CD40, anti-apoptotic BCL2L1 and the adhesion molecule ICAM1, all with higher expression in the adherent MCL cells. A subsequent functional classification of the differentially regulated genes between adherent and suspension cells in co-culture led to the identification of four functional themes, three for genes with higher mRNA levels in the adherent cells: B-cell receptor activation and immune cell signaling, apoptosis and anti-apoptosis and cell adhesion and migration, as well as early mitosis which was associated with genes with lower transcript levels in the adherent cells.

100 genes had significantly altered transcript levels between the mono- and co-cultured MS-5 stromal cells. While there were interesting genes among the regulated, such as an increase

in the immunomodulatory cytokines Ccl2 and Ccl7, the experimental setup was not designed for detecting gene expression changes in the stromal cell fraction as only a minority of these cells were physically interacting with an MCL cell, which diluted the signal for gene expression changes resulting from a physical cell-cell interaction.

When the 1050 differentially expressed genes between adherent and suspension MCL cells from the present study were interrogated for overlaps with previously published data sets that describe gene expression differences between lymphoma/leukemia cells residing in the lymph nodes and those in circulation for MCL and CLL patients, significant overlaps in regulated genes were found (Intersect: 348 genes (Fisher's exact test $P = 8.2x10^{-38}$) and 228 genes (Fisher's exact test $P = 2.7x10^{-38}$), respectively). 116 of these genes were present in all three data sets and out of these 65 displayed a conserved direction of regulation between the studies and out of these we identified a core set of thirteen genes that were all changed more than two-fold in the present study (*CCL3, CCL4, DUSP4, ETV5, ICAM1, IL15RA, IL21R, IL4I1, MFSD2A, NFKB1, NFKBIE, SEMA7A, TMEM2*). Future studies of these thirteen genes investigating their roles in adhesion, intercellular communication as well as dissecting possible prognostic properties would be of interest.

The observed increased levels of CCL3 and CCL4 are consistent with previous observations in co-cultures of MCL or CLL cells and for lymphoma cells stimulated with anti-IgM,[78,235] which may have implications for an immunomodulatory response, attracting regulatory T-cells.[236] Increased CCL3 and CCL4 serum levels have also been associated with an inferior prognosis in DLBCL[237] and in CLL[238] where they decrease rapidly upon Ibrutinib treatment initiation.[239] Increased secretion of the chemokine CCL22 by FL cells has been shown to actively recruit T_{Reg} cells to the tumor tissue.[178] Interestingly, and which was not granted space in the published manuscript, there was a strong up-regulation of IL-10 in the adhered MCL co-culture fraction along with the up-regulation of several genes related to B_{Reg} cells such as PDCD11[240] (2-fold increase in the adhered MCL fraction). This led us to investigate the intersect between genes with significantly altered transcript levels between suspension and adherent MCL co-culture fractions and an experimentally determined B_{Reg} gene signature (27 genes) where we found a significant enrichment for genes with a stronger mRNA expression in the adhered MCL cell fraction (intersect: 7 genes, Fisher's exact test *P*-value = 0.0009, Figure 2).[241] Among the differentially expressed genes overlapping with the B_{Reg} cell expression profile apart from IL-10 were the transcription factor IRF4, important during B cell development, HES1 implicated in notch signaling, the early growth response gene EGR3, the TNF superfamily member LTA, SRM which is a mediator of growth and differentiation

as well as the surface molecule SLAMF1. Although, most of the B_{Reg} signature genes did not exhibit differential gene expression between the adhesion–suspension MCL cell fractions, the notion of a malignant cell capable of acting immunosuppressive upon contact with stromal cells, a trait normally reserved for infiltrating regulatory T and B cells, is intriguing. Interestingly, it has been reported that CLL cells themselves can adopt regulatory characteristics in response to appropriate external stimuli.[151] The over-lapping genes identified here might represent a module of genes that can elicit a subset of B_{Reg} behavior.

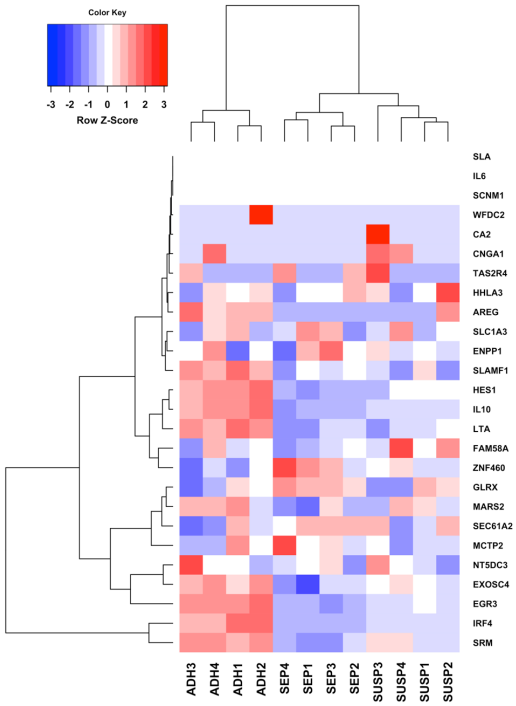

Figure 2. Relative transcript levels for 27 regulatory B-cell signature genes. ADH (adherent MCL cells in co-culture), SEP (mono cultured MCL cells), SUSP (suspension MCL cells in co-culture).

We concluded that, while there are differences between the results from the present *in vitro* study and those based on patient material, the co-culture model system reproduces a significant part of the gene regulation observed *in vivo* and that this model system, or future incarnations thereof, can be utilized for studying aspects of the microenvironmental interactions also observed in MCL and CLL patients.

4.2 PAPER II

Building and expanding on the results from Paper I a second co-culture model system was developed, using MS-5 stromal cells and the MCL cell line REC-1. Similar to JeKo-1, REC-1 adhered strongly to the stromal cell monolayer but significantly fewer cells attached and in contrast to JeKo-1, where an increase in cumulative adhesion over time could be observed, the number of adhered cells did not significantly increase following 4 hours of co-culture. Additionally, REC-1 cells migrated to a lower extent towards conditioned media from

stromal cells than JeKo-1 cells. Global gene expression data similar to that in Paper 1 was generated for the REC-1 co-culture conditions and for consistency the JeKo-1 data was reanalyzed in parallel using an updated workflow including: the most recent reference genome versions, quicker and more accurate alignment using the short-read aligner STAR combined with an analysis workflow based on generalized linear models by the edgeR Bioconductor package, which allow for more complex experimental designs. Given that every step of the analysis workflow was exchanged the net result for the reanalyzed JeKo-1 data set was relatively consistent (Figure 3).

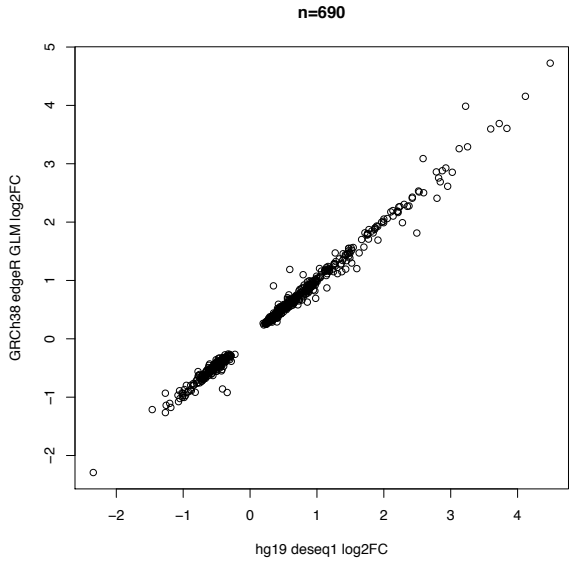

Figure 3 Comparison of gene expression changes (log2 fold change, genes with significantly altered transcript levels) between adherent and suspension JeKo-1 cells using the workflow presented in Paper I (deseq1, on x-axis) compared to the updated workflow implemented in Paper II (edgeR, y-axis).

Significant differences in transcript levels between adherent and suspension cells in co-culture were observed for 549 and 291 genes for JeKo-1 and REC- cells respectively (FDR q-value < 0.05, absolute fold change > 1.5). Surprisingly, merely 34 genes were significantly changed at the transcript level in both cell lines in response to adhesion to the stromal cell mono-layer. Several of the concordantly down-regulated genes were of mitochondrial origin with functions in the respiratory chain. The small overlap prompted an analysis of the differences in response to adhesion between JeKo-1 and REC-1 which rendered a list of 590 genes that were significantly differentially regulated between JeKo-1 and REC-1 as the cells adhered to the stromal cell mono-layer. Among these genes were several that had been identified belonging to a core signature of thirteen genes in Paper I such as *CCL3, CCL4, ICAM1, NFKB1* and *NFKBIE* that were also differentially regulated in MCL and CLL patient samples. A subsequent GSEA using a ranked list of genes based on differences in response to adhesion between JeKo-1 and REC-1 cells rendered a somewhat weak but significant

enrichment of nine gene sets from the KEGG pathway database, including B-cell receptor signaling and Apoptosis. Consistently, there was a significant overlap of genes with differential responses to adhesion between JeKo-1 and REC-1 and published signatures for BCR and NF-κB signaling. However, stimulating the cells with anti-IgM antibodies led to the phosphorylation of BTK and PLCγ in both cell lines and increased levels of the cytokines CCL3 and CCL4—which are known to be upregulated upon BCR stimulation[235]—in the conditioned media. Both JeKo-1 and REC-1 thus appeared to express a functional BCR but upon adhesion there was a significant difference in BCR signaling engagement between the two cell lines. Similarly, knocking down BTK, which functions downstream of the BCR, or inhibiting its function by the covalently bound small-molecule Ibrutinib led to a reduction in stromal cell-bound JeKo-1 cells but had no measurable effect on the number of adhered REC-1 cells.

The surface receptor CXCR4 was among the genes with significant differences in differential regulation upon adhesion. CXCR4 mRNA and cell surface levels were decreased in JeKo-1 and REC-1 cells upon adhesion and the negative regulation was significantly higher in JeKo-1. The reduced CXCR4 levels in the adherent fraction is consistent with previous studies where lower CXCR4 surface levels have been found for cells of lymph node origin when compared to cells in circulation.[67,72] Attenuation of CXCR4 signaling by addition of the agonist plerixafor (a small molecule also known as AMD3100) significantly decreased migration towards CXCL12 and reduced adhesion to the stromal cell mono-layer for JeKo-1cells, while no significant effect was observed for REC-1.

Reduced cell surface levels of ICAM1 by siRNA knock-down led to a reduction in the number of adhered MCL cells while knocking down sphingosine-1-phosphate receptor 1 (S1PR1) increased the number of adhered JeKo-1 and REC-1 cells. S1PR1 has been suggested to have diagnostic properties in MCL,[242] and missense mutations affecting S1PR1 have been shown to be more frequent among relapsing MCL patients.[243] Consistent with the present study, increased S1PR1 levels upon BTK inhibition by Ibrutinib[244] as well as a reduction in S1PR1 levels upon anti-IgM or CXCL12 stimulation or in co-cultures with nurse like cells[245] has been shown in CLL.

The importance of the BCR for lymphoma development and progression has been described previously as has the possibility of targeting BCR signaling for treatment of B-cell lymphomas, reviewed by R. Young and L. Staudt.[246] BCR signaling important for the survival of MCL cells can be conferred by the TME [66,68,247] To target intrinsic aberrant BCR signaling or TME-mediated BCR signaling, small molecules targeting the BCR-axis by inhibition of

e.g. the spleen tyrosine kinase (SYK),[191,248] Phosphatidylinositol 3-kinase δ (PI3Kδ)[249,250] or BTK[251] have been developed for treatment of MCL and CLL patients. The irreversibly-binding BTK inhibitor Ibrutinib, has proven successful for treatment of MCL[77,252] and CLL[76,253] and has been shown to promote a rapid migration of MCL cells into circulation following treatment initiation.[78] Resistance to Ibrutinib has however been reported,[254] with plausible mechanistic explanations being missense-mutations affecting cysteine 451 in the region where Ibrutinib binds to BTK, leading to a reversible binding, or through mutations to PLCγ, leading to constitutive downstream BCR signaling.[255] Another suggested, possible culprit for Ibrutinib resistance in MCL could be supportive signaling mechanisms provided by the TME.[256] A second generation BTK inhibitor, Acalabrutinib, allegedly with improved specificity for BTK, has recently been approved for the treatment of refractory MCL.[81] Blocking BCR signaling through BTK inhibition by Ibrutinib has been shown to reduce serum levels of the cytokines CCL3 and CCL4 in MCL and CLL,[78,239] which is consistent with *in vitro* observations where the levels of CCL3 and CCL4 increase in the conditioned media of MCL and CLL cells upon co-culture or when stimulated with anti-IgM.[235] High serum levels of CCL3 and CCL4 have additionally been associated with a poor prognosis in CLL and DLBCL.[237,238] The present study shows that different MCL cells have different dependencies on BCR signaling in relation to their interaction with their immediate microenvironment, which could lead to a differential response to treatments where aspects of BCR signaling are being targeted.

In conclusion, the two MCL cell lines JeKo-1 and REC-1 have different dependencies for adhesion to and intercellular communication with stromal cells. BCR signaling was important for the adhesion of JeKo-1 to stromal cells but not for REC-1. Similarly, the chemokine receptor CXCR4 was implicated in adhesion and migration of JeKo-1 cells but not for REC-1. Lowered surface levels of the adhesion molecule ICAM1 reduced the adhesion of both cells to stromal cells while reducing the levels of S1PR1 led to an increase in the number of attached cells. This prompts for future studies validating the findings from the present study and subsequently investigating the possibility of targeting proteins for which a common dependency was observed in relation to adhesion. The in vitro model system described in the present study may thus be used to identify pathways and mechanisms that in turn can be used to identify patients that will respond to different forms of treatment.

4.3 PAPER III

The study made use of an inducible system to investigate the impact of SOX11 over-expression in the Ba/F3 pro-B cell line. Constitutive over-expression of SOX11 for 72 hours

led to a gradual aggregation of cells. Following 72 hours of SOX11 over-expression the Ba/F3 cells formed large aggregates, had lower metabolic activity as measured by XTT and a lower 3H Thymidine incorporation rate. A global gene expression analysis by microarray identified 534 significantly changed genes with higher and 338 with lower transcript levels in the SOX11 over-expressing (SOX11-on) cells as compared to control, including higher transcript levels for Mmp8 and S110A8/9 as well as the protocatherin-β genes Pcdhb16 and Pcdhb17. In addition, the cells appeared to retain their B-cell identity as the pro-B-cell restricted genes Id1 and Tal were increased at the transcript level while no significant change was detected for other genes typically associated with specific B-cell developmental stages.[257] A gene set enrichment analysis of the global gene expression data identified several cell cycle related gene sets that were enriched for among the genes with increased transcript levels in the SOX11-on cells, and importantly they contained several checkpoint genes like Chek1 and Chek2 as well as DNA damage response genes like Trp53, Brca1 and Brca2. The net result was reduced proliferation. Among the enriched gene sets for genes with lower transcript levels were those associated with leukocyte function and immunomodulatory pathways. We concluded that in the context of the Ba/F3 cell system, SOX11 did not act as an oncogene. Interestingly, the oncogenic properties of SOX11 in MCL have been debated and the results have been divergent and collectively inconclusive.[50,52,54,55] If anything, the importance of the cellular context for the net response upon SOX11 induction/depletion has been underlined. It is therefore interesting to note that in this particular cellular system SOX11 did not elicit explicit oncogenic activity which of course does not exclude the possibility of other outcomes at other B-cell differentiation stages, in other cellular backgrounds, or in other microenvironmental contexts.

4.4 PAPER IV

A model system was developed for doxycycline-regulated control of wild type MYC levels or for MYC carrying either of the lymphoma-associated missense mutations T58A or T58I in murine splenic B-cells. The transformed cells were generated through simultaneous lentiviral transduction of BCL2L1 and BMI1, to inhibit apoptosis and DNA damage response, alongside a vector carrying Tet-on constructs allowing for progressive regulation of MYC levels. MYC protein levels, when measured by western blot, indicated consistently higher levels for T58A for any doxycycline dose when compared to WT or T58I, possibly due to increased protein stability as has previously been reported.[118] As the level of MYC increased so did cellular proliferation rate. A gradual increase in MYC levels also led a lower portion of cells in G_0/G_1 and a higher portion in the S phase of the cell cycle as the cells

stained with propidium were assessed by flow cytometry. Interestingly, consistently for all functional readouts, the response is more sensitive and subsequently of higher magnitude for T58A as compared to WT which has a more sensitive and higher response when compared to T58I, consistent with the order of previous observations in regards of transforming capacity.[119] The phenotypic differences together with previous observations of differences in gene regulation between WT and T58I[118] led us to investigate differences in gene regulation between the three B-cell types expressing different MYC genotypes by next generation sequencing.

mRNA was extracted for cells expressing seven different levels of MYC for WT, T58A and T58I and cDNA libraries were prepared and sequenced using the Illumina platform generating on average 29 million 50 bp single-end reads per sample. Reads were subject to quality control and trimmed based on sequence quality, and subsequently aligned to the latest mouse reference genome GRCh38.87 concatenated with the sequences for MYC, BCL2L1, BMI1 and EGFP, which were all over-expressed in the transduced cells. Aligned reads per gene (concatenated exons) were assessed by the featurecounts function of the SubRead package and, using the glmQLF workflow of the edgeR package, following threshold-based removal of genes with consistently low read counts, the per gene expression was modeled using generalized linear models that were then used to test for significant gene profiles. While the three MYC genotypes were different in terms of global gene expression level their response to progressively increasing MYC levels were consistent, as shown by the principal component analysis.

7263 genes were significantly altered at the transcript level as the levels of WT MYC increased, in addition 347 genes were regulated significantly differently in response to increasing MYC levels between WT and T58A while 683 were differentially regulated between WT and T58I. The union of lists with differentially regulated genes was thereafter subject to division into 12 clusters by agglomerative hierarchical clustering based on $\log_2$ transformed RPKM values. For two clusters a significant enrichment of non-coding genes was found. Consistent with previous observations about MYC and E-box motifs, most clusters containing a majority of genes with increasing transcript levels following an increase in MYC levels contained more genes with E-boxes within 1000 bp of the TSS.

The genes in each cluster were subsequently functionally classified through hypergeometric tests for overrepresentation in gene sets from the gene ontology database for biological processes and in gene sets from the KEGG pathway database. Some clusters contained genes related to processes that have previously been described to change in response to increased

MYC levels (e.g. de novo pyrimidine and purine synthesis[258], cell cycle progression as well as ribosome biogenesis[259]). Other clusters contained genes in gene sets which have not been described in relation to increased MYC levels before (e.g. functions related to B-cell identity and Chemotaxis, which were enriched for in clusters containing genes that were down-regulated as the level of MYC increased).

When interrogating the overlap between genes that were differentially expressed upon an increase in WT MYC levels in the present study and genes that were found to be differentially expressed in the Eμ-Myc mouse model between cells at three different stages of lymphomagenesis, with progressively increasing MYC levels, a significant intersect of commonly regulated genes was found (intersect 3991 genes, Fisher's exact test *P*-value = $1.7x10^{-8}$).[127] All previously defined clusters were represented in the intersect but notably we observed a significant enrichment of genes in the three clusters that contained genes involved in ribosome biogenesis, purine metabolism and pyrimidine metabolism. Interestingly, a significant overlap was also found between genes significantly changed upon progressively increasing WT MYC levels and a gene signature containing 100 direct MYC targets (intersect 71 genes, Fisher's exact test *P*-value = $1.6x10^{-28}$) and more importantly the genes in the intersect were significantly enriched for among the most significant genes in the present study.[140] These two observations strengthened the notion of a model system that represent aspects of gene expression regulation by MYC independently observed elsewhere.

Consistent with previous studies, MYC carrying lymphoma-associated mutations at threonine 58 regulate the expression of genes differently from WT,[118] and interestingly, while T58A and T58I commonly can regulate a set of genes differently from WT they do also regulate the expression of genes differently between the two. The mutant phenotype is not merely due to the inability to phosphorylate threonine 58 but seems also to reflect different conformational properties of the proteins containing the T58A and T58I substitutions. We concluded that the model system can be used to further study functional changes in response to different MYC levels and that it can also be used for a more elaborate characterization of how the lymphoma-associated MYC mutants affect cancer progression in B-cells.

5 CONCLUDING REMARKS

It is interesting to note the importance of the cellular background and the context in which the cell resides when assessing gene expression changes inflicted by external or internal events. With this in mind, the key for finding ways to manage MCL and other malignancies that are difficult to cure possibly does not lie in their genetic makeup and probably not in the microenvironmental context either but in a combination of the two. Developing and characterizing model systems such as the ones presented here may prove useful for designing and testing treatment approaches for targeting microenvironmental interactions that are specific to lymphoma subsets. The controlled nature of the in vitro models is of course compelling but their lack of complexity may cause issues as they may exclude important components, illustrated by the discrepancy between the efficacy of certain compounds in 2D monocultures as compared to their *in vivo* effect. Regardless of the level of refinement the relevance of these systems will have to be evaluated in relation to in vivo situations and for that the evolving single cell omics techniques, such as for instance: spatial transcriptomics, single cell ATAC-seq and single cell bisulfite sequencing will likely prove important.

Microenvironment-related findings from the present thesis such as the importance of surface molecules like for instance ICAM1, CXCR4, S1PR1 and the BCR will have to be evaluated in other in vitro and in vivo systems to investigate their universality in propagating survival or adhesion related mechanisms to the malignant cell. Including different model systems can have a confirmatory role but also a role in finding different genes that potentially could be used for disease stratification or for to be targeted in different patient subgroups. Future experiments could for instance involve different in vitro cell systems, possibly through the use of 3D cultures and inclusion of more than one supportive cell type. Similar systems could be developed for ex vivo propagation of lymphoma cells.

Interestingly, given the documented role of MYC in cell trafficking and homing, as illustrated by its role in the GC reaction, it would be of interest to subject the B-cells presented here, with regulable MYC levels, to the adhesion assay presented in Paper I and II. Given the omnipresent and pluralistic role of MYC in human malignancies it would be unsurprising if it also had a role in survival-promoting microenvironmental interactions.

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