SOX11 in Mantle Cell Lymphoma - Novel Tools for Diagnostic, Prognostic and Functional Investigations

Nordström, Lena

2014

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SOX11 in Mantle Cell Lymphoma

Novel Tools for Diagnostic, Prognostic and Functional Investigations

Lena Nordström

ACADEMIC THESIS which, by due permission of the Faculty of Engineering, Lund University, will be publicly defended in Stora hörsalen at Ingvar Kamprad Designcentrum (IKDC), Sölvegatan 26, Lund, Friday 23rd of May 2014 at 9.15 a.m

Faculty opponent is Prof. Helena Jernberg Wiklund, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden
SOX11 in Mantle Cell Lymphoma

Novel Tools for Diagnostic, Prognostic and Functional Investigations

Lena Nordström

2014
Faculty of Engineering
Department of Immunotechnology

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Cover: a schematic representation of the DNA binding by the HMG-box domain within the SOX protein family


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Original papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I-IV). The papers are appended in the end of the thesis.


Published papers are reproduced with permission from the publishers.
My contribution to the papers

Paper I  I took part in the design of the study and performed the majority of the experiments. I took part in writing the manuscript.

Paper II  I performed the statistical analysis and took an active part in writing the manuscript.

Paper III  I was responsible for the study design, analysis of the data and wrote the manuscript.

Paper IV  I was actively involved in the design of the study, performed parts of the experimental work and had the main responsibility for writing the manuscript.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>BTK</td>
<td>Burton’s tyrosine kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse large B cell lymphoma</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FL</td>
<td>Follicular lymphoma</td>
</tr>
<tr>
<td>FLIPI</td>
<td>Follicular lymphoma international prognostic index</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Histone H3 lysine 4 tri-methylation</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>Histone H3 lysine 9 di-methylation</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Histone H3 lysine 27 tri-methylation</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>IgV</td>
<td>Immunoglobulin variable</td>
</tr>
<tr>
<td>IPI</td>
<td>International prognostic index</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIPI</td>
<td>Mantle cell lymphoma international prognostic index</td>
</tr>
<tr>
<td>MZL</td>
<td>Marginal zone lymphoma</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PrC</td>
<td>Polycomb repressive complex</td>
</tr>
<tr>
<td>SLL/CLL</td>
<td>Small lymphocytic lymphoma/chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>SP</td>
<td>Side population</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TrxG</td>
<td>Trithorax group proteins</td>
</tr>
</tbody>
</table>
Biomarker discovery has dominated the cancer research field the last twenty years. The goal of biomarker research includes early detection, prediction of survival, response to treatment, and ultimately to develop personalized therapies based on the molecular profile of each patient. Although the clinical integration is slow, successful examples include the mutations in the epidermal growth factor receptor, predicting response to the tyrosine kinase inhibitor gefitinib. Additionally, development of targeted antibody therapies such as trastuzumab and rituximab, for treatment of HER2-positive breast cancer and CD20-positive B-cell lymphomas, has significantly improved patient survival. Critics argue that the cost of personalized diagnostics and medicine will be too high for the healthcare system to handle while the research community see and strive towards a more effective and quality driven healthcare. Personalized medicine has been the main driving force for the massive efforts to identify novel cancer biomarkers.

A biomarker could be a mutation of a gene, an RNA transcript, including mRNA and miRNA, a protein or a combination thereof. Depending on how the biomarker should be used in the clinic different criteria needs to be fulfilled; a biomarker for screening purpose should be easily accessible such as in blood or urine, while a biomarker for tumor stratification could also include tissue examinations.

This thesis focus on the transcription factor SOX11, a protein discovered in mantle cell lymphoma in 2008 (Ek et al. 2008) which shows great potential to fulfill the criteria of a clinically relevant biomarker, as stated by McKay et al., in the guidelines for mantle cell lymphoma (MCL) in the British Journal of Haematology (McKay et al. 2012):
"It is possible that SOX11 immunostaining may have a role in the characterization of MCL in the future when sensitive and specific antibodies become generally available"

The aim of this thesis is to extend the use of SOX11, by providing novel tools for research applications as well as for diagnosing and stratifying MCL patients. In Paper I we generated the first monoclonal antibody targeting SOX11 and demonstrated the ability to use it in immunohistochemistry and flow cytometry, two assays highly used for lymphoma diagnostics. In Paper II we further used this antibody to demonstrate the prognostic use of SOX11 in the homogenously treated Nordic MCL2/MCL3 cohort. Patients with a weak SOX11 expression were significantly correlated to a worse survival compared to patients with a strong SOX11 expression. Additionally, by combining molecular markers into a well-established prognostic index, MIPI, we further demonstrated a role of SOX11 and p53 to improve patient classifications.

The tumor specific expression of SOX11 makes it an attractive target in future cancer therapies. However, transcription factors are generally difficult to target with either antibody therapies or small molecular compounds. An alternative strategy could be to target SOX11 signaling pathways. The protein network surrounding SOX11 is highly unexplored and we and others have previously used gene expression studies to identify SOX11 signaling pathways, but no consensus has been determined. In paper III we further assess the specificities of SOX11 targeting antibodies in immunoprecipitation assays, with the aim to get further insight into SOX11 signaling pathways using chromatin immunoprecipitation or co-immunoprecipitation coupled to either next generation sequencing or mass spectrometry.

SOX11 is expressed in a few other malignancies including ovarian cancer, breast cancer and glioma. In Paper IV we study the epigenetic regulation of SOX11, including both DNA methylation and histone modifications, in both lymphomas and solid tumors. Non-expressing lymphomas show a heavy methylation of SOX11 while several solid tumors show a much more diverse degree of methylation. In breast cancer, we associate SOX11 methylation with the estrogen receptor (ER) positive subtype. We further show that epigenetic drugs, such as histone
deacetylase inhibitors enable re-expression of SOX11 in otherwise silenced cells.

In summary, this thesis presents novel tools, including the developed SOX11 antibody and the improved prognostic index for stratifying mantle cell lymphoma patients. Additionally, the developed SOX11 antibody has expanded our research applications to include flow cytometry and immunoprecipitation assays. In Chapter 2 and 3, my work will be put into the context of B-cell lymphomas with the specific focus on the molecular interactions in MCL. The importance of antibodies as a tool in biomarker discovery will be further discussed in Chapter 4. Chapter 5 presents an introduction to epigenetics, focusing on the aberrant epigenetic regulations in MCL and particular how SOX11 is regulated. Finally, in Chapter 6, the main findings of my work will be discussed and speculations on the future use of SOX11 in MCL presented.
Chapter 2

B-cell lymphomas

The immune system defends our body from pathogenic organisms. Several cell types and proteins constitute the immune system, including lymphocytes (B-cells, T-cells, and NK-cells), which are present in lymph nodes, bone marrow, spleen and blood. Lymphoproliferative disorders are characterized by abnormal proliferation of the lymphocytes and lymphomas are the term used to describe solid tumors of mature lymphocytes, which although related to each other, have very diverse clinical outcomes.

This chapter will focus on the biology and diagnosis of B-cell lymphomas and pin-point why we need to identify new biomarkers.

Subgroups of B-cell lymphomas

B-cell lymphomas are a heterogeneous group of tumors with either a very aggressive or indolent clinical course. The aggressive lymphomas, such as diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL) and Burkitt’s lymphoma (BL) consist of medium-to-large sized cells with high proliferation rate. Due to recent therapeutic improvements, including novel combinations of chemotherapy, rituximab and stem cell transplantation, subgroups of patients with DLBCL and BL are today cured in the majority of cases (Blum et al. 2004; Foon et al. 2011). MCL is still considered an incurable disease although novel treatment combinations have achieved long-term survival in younger patients (Geisler et al. 2012). The most common indolent lymphomas include small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL), follicular lymphoma (FL), and marginal zone lymphoma
(MZL) that consist of small-to-medium sized cells with a low proliferation rate (Lunning and Vose 2012). Although major therapeutic improvements have been achieved, increasing the overall survival time to more than 10 years, indolent lymphomas are still incurable in most cases (Brenner et al. 2008; Gribben 2010; Smith 2013). The incidences of mature B-cell lymphomas vary, as for many other tumors, among different geographic regions. The incidence rates per 100,000 per year in European countries are presented in Table 1.

**Table 1. Incidence rate per 100,000 for indolent and aggressive mature B-cell malignancies (Sant et al. 2010).**

<table>
<thead>
<tr>
<th>Indolent B-cell lymphoma</th>
<th>Incidence rate</th>
<th>Aggressive B-cell lymphoma</th>
<th>Incidence rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLL/CLL</td>
<td>4.92</td>
<td>DLBCL</td>
<td>3.81</td>
</tr>
<tr>
<td>FL</td>
<td>2.18</td>
<td>MCL</td>
<td>0.45</td>
</tr>
<tr>
<td>MZL</td>
<td>0.42</td>
<td>BL</td>
<td>0.22</td>
</tr>
</tbody>
</table>

B-cells are particular prone to malignant transformations due to the nature of the VDJ recombination and the germinal center reactions including somatic hyper mutation and class switching. B-cells undergoing clonal expansion with the breaking and joining of DNA could lead to chromosomal translocations. Indeed, most B-cell lymphomas are characterized by a chromosomal translocation most often involving the Ig heavy chain locus (14;32) and an oncogene such as BCL-2, CCND1 or C-MYC, leading to constitutive protein expression driving cell proliferation (Vega and Medeiros 2003). Nearly every case of FL, BL and MCL are characterized by a single translocation hit while multiple translocations have been associated with DLBCL (Table 2). In MZL, the (11;18)(q21;q21) translocation generate a fusion protein between API2 and MALT1. This fusion protein constitutively activates the NF-KB pathway. Most translocations are necessary but not sufficient to transform a B-cell and it has been shown that more mutations, chromosomal deletions or other alterations are necessary for tumor development. In fact, although unusual, healthy individuals could have small populations of clonal cells with chromosomal translocations such as t(11;14) and t(8;14) (Janz et al. 2003; Lecluse et al. 2009).
Table 2. Frequency of the most common chromosomal translocations in non-Hodgkin lymphoma (Vega and Medeiros 2003).

<table>
<thead>
<tr>
<th>Lymphoma</th>
<th>Translocation</th>
<th>Frequency</th>
<th>Oncogene</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLL/CLL</td>
<td>t(18;22)(q21;q11)</td>
<td>&lt; 5 %</td>
<td>BCL-2</td>
</tr>
<tr>
<td>FL</td>
<td>t(14;18)(q32;q21)</td>
<td>80-90%</td>
<td>BCL-2</td>
</tr>
<tr>
<td>MZL</td>
<td>t(11;18)(q21;q21)</td>
<td>10-40%(^1)</td>
<td>API2, MALT1</td>
</tr>
<tr>
<td>DLBCL</td>
<td>t(14;18)(q32;q21)</td>
<td>20-30%</td>
<td>BCL-2</td>
</tr>
<tr>
<td>MCL</td>
<td>t(11;14)(q13;q32)</td>
<td>90-100%</td>
<td>CCND1</td>
</tr>
<tr>
<td>BL</td>
<td>t(8;14)(q24;q32)</td>
<td>80-85%</td>
<td>C-MYC</td>
</tr>
</tbody>
</table>

\(^1\)The frequency varies with anatomic site (Staros 2010)

**Tumor origin and the existence of tumor stem cells**

Most malignant B-cells resemble a stage in the normal B-cell differentiation process in terms of genetic and molecular markers (gene expression, immunoglobulin variable (IgV) mutation status and immunophenotype). However, whether this normal counterpart is the actual cell of origin or merely a final differentiation state is uncertain. Although the naïve B-cells are by far more common than germinal center (GC) B-cells, most lymphomas are thought to have a GC or post GC origin (Kuppers 2005). For example, FL shares many features with normal GC B-cells including gene expression, surface markers, and a follicular growth pattern (Kridel et al. 2012). However, the origin of other lymphomas, such as MCL has been more difficult to determine. In MCL, the tumor cells have disrupted the normal lymph node structure and grow in a diffuse pattern. The immunophenotype of MCL (CD5+, CD10-, CD23-) resemble the naïve B cells in the follicular mantle zone, which historically has been considered as the cell of origin. However, 15-40 % of MCLs display hypermutated V\(_H\) genes, indicating a GC or post-GC cell origin (Thorselius et al. 2002; Walsh et al. 2003; Lai et al. 2006; Hadzidimitriou et al. 2011). Hypermutated MCLs have been associated to a better survival (Lai et al. 2006) and are more frequently observed in indolent MCL (Fernandez et al. 2010).
B-cell lymphomas are initially chemosensitive but most patients relapse with a refractory disease. It is therefore highly possible that subsets of the cancer cells were already primarily resistant to the therapy, subsequently causing the relapse. This ability of resistance and self-renewal is a general characteristic of stem cells. The concept of cancer stem cells have been demonstrated in a number of solid tumors including breast, prostate, pancreatic and glioma (Li et al. 2007; Kasper 2008; Park and Rich 2009; Pece et al. 2010). However, the existence of lymphoma stem cells has been more uncertain. Cells with stem cell-like features can be identified by flow cytometry, either by their expression of stem cell markers or as a result of their drug resistance. Side population (SP) analysis has lately been an important tool to identify cancer stem cells. Recently, SP cells have been identified in various lymphomas including MCL (Teshima et al. 2013), DLBCL (Koch et al. 2014), FL (Lee et al. 2012) and CLL (Foster et al. 2010). Additionally, transplantation of stem cell-like/SP cells into immunodeficient mice has demonstrated an increased ability to induce tumors compared to more limited effects observed for the bulk cells (Chen et al. 2010; Teshima et al. 2013).

In summary, a few studies have started to explore the presence and function of cancer stem cells in the pathogenesis of B-cell lymphomas. However, it is still unclear if the transformation of normal stem cells is the first step in the pathogenesis or if the lymphoma stem cell population constitutes a group of cells that have dedifferentiated and gained stem cell-like features. Irrespectively, it is important to understand the biology of lymphoma stem cells, find reliable biomarkers for identification and design novel therapeutic drugs targeting lymphoma stem cells for a future curative therapy.

Diagnostic classification of B-cell lymphomas

As described above, B-cell lymphomas constitute a heterogeneous group of tumors where each subclass has its own clinical behavior and need for treatment. Thus, it is extremely important to diagnose the different subtypes correctly. Today, the different subclasses are identified by a combination of clinical and biological features such as genetics,
morphology and immunophenotype (Campo et al. 2011). The diagnosis could be made on different types of specimens such as a lymph node biopsy or aspirate, bone marrow biopsy or aspirate, and/or peripheral blood. The golden standard of lymphoma diagnosis is an histopathological examination of the involved lymph node and immunophenotypic analysis with flow cytometry, although additional methods such as FISH and PCR sometimes aid in diagnosis (Dey et al. 2006).

The immunohistochemistry analysis begins with a morphologic and architectural investigation of the tissue, using hematoxylin and eosin staining. A lymphoma is most often easily identified by a disrupted lymph node structure while single tumor cells could often be recognized due to aberrant cell size, nuclei morphology and chromatin characteristics (Higgins et al. 2008). Besides analyzing tumor architecture, antibody panels are used for immunophenotyping. Different antibody panels are used depending on the suspected subclass, but general B-cell markers such as CD19, CD20, CD22 and CD79 are normally used. To further specify the lymphoma subtypes, more uniquely expressed markers are analyzed (Table 3). Additionally, other tumor related antigens such as BCL-2, BCL-6, or Cyclin D1 are also of importance to classify each subtype correctly (Swerdlow 2008).

**Table 3. Phenotypic features of mature B-cell malignancies (Swerdlow 2008)**

<table>
<thead>
<tr>
<th>Lymphoma</th>
<th>CD5</th>
<th>CD10</th>
<th>CD23</th>
<th>CD43</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLL/CLL</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FL</td>
<td>-</td>
<td>+/-</td>
<td>-/+</td>
<td>-</td>
</tr>
<tr>
<td>MZL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DLBCL</td>
<td>&quot;</td>
<td>-/+</td>
<td>NA</td>
<td>-/+</td>
</tr>
<tr>
<td>MCL</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BL</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

+ > 90 % of cases, +/- > 50 % of cases, -/+ < 50 % of cases, - < 10 % of cases, * expressed by some DLBCL, NA Not Available.

As a complement to immunohistochemistry, flow cytometry could be equally informative to determine clonality, tumor phenotype, monitoring response to treatment and minimal residual disease (Craig and Foon 2008; Barrena et al. 2011). Most large neoplastic B-cell populations can easily be distinguished from normal B-cells by a skewed kappa-lambda
ratio while identification of smaller tumor subsets or classification of B-cell lymphomas requires the analysis of aberrant antigen expression (Craig and Foon 2008). Additionally, flow cytometry easily quantifies signal intensities which could be used to evaluate targets for antibody therapy or for prognostic information, the latter recently demonstrated in B-cell acute lymphoblastic leukemia (Weng et al. 2013).

**Biomarkers in lymphoma**

Both indolent and aggressive lymphomas are a clinical challenge. Although the prognosis of non-Hodgkin lymphoma has improved dramatically over the last decade, with the inclusion of rituximab as the most important event, relapsed and/or refractory disease is still a major problem. The molecular understanding of lymphoma has increased dramatically through the introduction of gene expression arrays (1990s) and genome sequencing (2000s). Nevertheless, diagnosis still relies on general marker panels and very few tumor specific markers have been identified. With the aim of personalized medicine, novel biomarkers with a predictive, prognostic or disease monitoring abilities needs to be discovered. Additionally, several new companion biomarkers with the ability to prospectively predict response treatment are needed. A plethora of potential biomarkers have been presented in the literature, including gene mutations, protein expressions, miRNA expression and DNA methylation signatures (Greiner et al. 1996; Wang et al. 2008; Iqbal et al. 2012; Enjuanes et al. 2013). However, these markers are normally presented in a proof-of-concept study and must demonstrate its biomarker capability in large prospective studies to become approved. Lymphoma therapies are continuously changing, which could make old prognostic and predictive biomarkers less important. Routine and novel biomarkers need to be assessed for novel treatment protocols. Today, no molecular tool is available to aid clinicians in treatment selection. Instead, clinical data, most often incorporated into a prognostic index such as the international prognostic index (IPI) for DLBCL, follicular lymphoma IPI (FLIPI) and mantle cell lymphoma IPI (MIPI) are used to stratify patients into low, intermediate and high risk groups is used (Shipp 1993; Solal-Celigny et al. 2004; Hoster et al. 2008). However, at this point, risk
stratification does not affect treatment decisions. Instead, therapy decisions are limited by the patient’s tolerability to treatment, which in turn is dependent on other parameters such as age and performance status (Foon et al. 2011; Chao 2013; Smith 2013).
Chapter 3

A molecular dissection of mantle cell lymphoma

MCL is a rare but very aggressive B-cell lymphoma with a median age at diagnosis around 68 years (Caballero et al. 2013). The European incidence rate of MCL is approximately 0.45 cases per 100,000 per year with a clear male predominance (Sant et al. 2010). MCL has been considered an incurable disease although the survival has increased from a median survival of three years in the 1980s to around five years in the early 2000s (Herrmann et al. 2009). Recently, new treatment protocols combining high dose cytarabine with autologous stem cell transplantation and rituximab has achieved significant improvement in long term survival in younger patients. One such study, the Nordic MCL2 trial, has shown very encouraging results with a median survival of more than 10 years (Geisler et al. 2012).

This chapter focus on three key molecules in MCL; Cyclin D1, p53, and SOX11. The cellular context of these molecules will be described with a discussion on how they could be utilized in diagnostic and prognostic applications. Finally, novel treatment agents inhibiting pathways in which these molecules potentially act through will be highlighted and discussed.

Cyclin D1

The t(11;14) translocation leading to Cyclin D1 overexpression is a hallmark of MCL. Still, the importance of Cyclin D1 in the pathogenesis of MCL has been difficult to elucidate. The most described role of Cyclin D1 is in the cell cycle, controlling the transition of G1 to S phase by binding and activating cyclin-dependent kinase 4 (CDK4) and CDK6.
This activation results in phosphorylation of the retinoblastoma protein followed by release of the transcription factor E2F, which in turn initiates transcription of genes controlling DNA synthesis (Figure 1) (Stacey 2003).

Figure 1. The t(11;14)(q13;q32) translocation in mantle cell lymphoma. A) Schematic overview of translocation t(11;14) leading to Cyclin D1 over expression. B) Cyclin D1 and CDK4/6 complex phosphorylate retinoblastoma (Rb) which releases transcription factor E2F that up-regulate cell cycle driving genes.

The existence of Cyclin D1 negative MCL (7-15 %) has challenged the view of the importance of this protein (Yatabe et al. 2000; Rosenwald et al. 2003). Early reports suggested a correlation of Cyclin D1 negative cases to a more indolent form of MCL (Yatabe et al. 2000; Hashimoto et al. 2002) while later reports have shown similar clinicopathological and gene expression profiles as conventional Cyclin D1 positive MCL (Fu et al. 2005). Additionally, Fu et al. demonstrated that most Cyclin D1 negative MCL overexpress either Cyclin D2 or Cyclin D3 further
supporting a role for the D-type cyclins in the pathogenesis of MCL. Similarly, *in-vitro* knockdown of Cyclin D1 has been reported to only have a limited effect on cell proliferation and apoptosis, possibly explained by the up-regulation of Cyclin D2 (Klier et al. 2008). Although Cyclin D1 is overexpressed in MCL, it has been shown that a Cyclin D1 driven mouse model does not induce MCL development (Beltran et al. 2011). Similarly, healthy individuals with small fractions of B-cells carrying the t(11;14) translocations has been identified further supporting that more genetic hits are needed for a cell to transform into MCL (Lecluse et al. 2009). In addition, a part of our results in paper II demonstrate that the intensity of Cyclin D1 staining does not contain any prognostic information. By using Cox univariate analysis we show that no significant correlation to overall survival and event free survival was observed in the Nordic MCL2/MCL3 cohort, further demonstrating the poor correlation between Cyclin D1 and survival.

Recently, several CDK-independent functions of Cyclin D1 have been shown. Cyclin D1 has been discovered to interact with the pro-apoptotic protein BAX in the cytoplasm and inhibit its action in the apoptotic pathway (Beltran et al. 2011). Additionally, a recent study by Jirawatnotai et al. demonstrated a novel role in DNA repair by direct interactions to RAD51 (Jirawatnotai et al. 2011).

Oncogenes such as Cyclin D1 are attractive targets for specific therapy (Musgrove et al. 2011). However, proteins without enzymatic activity are generally difficult to inhibit with small molecular drugs due to lack of binding pockets (Arkin and Wells 2004). Instead, inhibitors to Cyclin D1 interaction partners, CDK4/CDK6, are under development. One such inhibitor, PD0332991, has been evaluated in a phase I clinical trial with 17 relapsed MCL patients. In the study, demonstrating a well-tolerated dose, 30 % (n=5) of the patients achieved progression free survival for more than 1 year, with one complete remission and two partial remissions (Leonard et al. 2012). In all, although Cyclin D1 does not correlate to survival, the Rb-E2F pathway is frequently deregulated in MCL and CDK inhibitors have shown effects in some relapsed MCL patients.
The protein p53, encoded by *TP53*, is a transcription factor known as the “master guardian of the genome” due to key regulatory functions of cellular growth, senescence and apoptosis in response to cellular stress and DNA damage (Lane 1992; Levine 1997). In normal cells, p53 is kept in low levels due to its negative regulator MDM-2, which ubiquitylates p53 for subsequent degradation by the proteasome (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997). In damaged cells, p53 is stabilized through phosphorylation by several different kinases including JNK, p38, ATM, Chk1 and Chk2 and others, thereby inhibiting its degradation and initiating transcription of target genes (Lavin and Gueven 2006) (Figure 2).

**Figure 2. p53 regulation in normal and damaged cells.** A) p53 is degraded by the proteasome in normal cells. B) p53 becomes phosphorylated in damaged cells and translocate to the nucleus where transcription of target genes is initiated.

The p53 pathway is frequently disrupted in mantle cell lymphoma. Mutation frequencies of the *TP53* gene itself have been reported in several studies. *TP53* mutations has been correlated to an unfavorable prognosis and reported to occur in 14-20% of MCL patients (Greiner et
Additionally, several other components of the p53 pathway have been reported to be deregulated in MCL including ATM mutations (Schaffner et al. 2000) and overexpression of MDM-2 (Hartmann et al. 2007). In paper II, we show that strong p53 staining (11% of our cohort) is significantly associated with a very poor survival. If these p53 strong cases, determined by immunohistochemistry, are correlated to *TP53* mutation was not investigated but such a correlation has previously been observed (Stefancikova et al. 2010). We further demonstrate the potential to add p53 staining to MIPI, improving patient classification compared to MIPI alone.

Activation of wild-type p53 is an attractive approach to target cancer cells. Nutlins is a class of drug compounds that interact with MDM-2 and inhibit its interaction with p53, thereby promoting p53 stabilization and activation (Vassilev et al. 2004). Nutlin-3 has been evaluated in preclinical studies for its effect in inducing cell death due to the relatively low frequency of *TP53* mutations and overexpression of MDM-2 in MCL (Drakos et al. 2009; Tabe et al. 2009; Jin et al. 2010). Nutlin-3 treatment of MCL cell-lines resulted in reduced cell proliferation, increased apoptosis and cell cycle arrest. Importantly, a combinatorial synergistic effect of Nutlin-3 and bortezomib (a proteasome inhibitor) has been observed, which minimize the risk of drug resistance (Jin et al. 2010). However, to the best of my knowledge, no MDM-2/p53 targeting drugs have entered clinical trials for treating MCL although other MDM-2 inhibitors such as RG7112 have successfully been evaluated for its efficacy in acute or chronic relapsing and refractory leukemia (www.clinicaltrials.gov). In summary, we and others have correlated p53 expression and deregulated pathways to clinical outcome in MCL, and thus novel p53 activating drugs have the potential to successfully treat MCL.

**SOX11**

The transcription factor SOX11 is highly expressed during cellular development, specifically in tissue remodeling and during both adult and
embryonic neurogenesis, regulating cell proliferation and differentiation (Hargrave et al. 1997; Dy et al. 2008; Wang et al. 2013b). The expression of SOX11 is very restricted in adult differentiated tissues (Penzo-Mendez 2010). Recently, up-regulation of this stem cell related protein has been demonstrated in MCL, glioma, medulloblastoma as well as in subgroups of BL, ovarian cancer, and breast cancer (Weigle et al. 2005; de Bont et al. 2008; Ek et al. 2008; Brennan et al. 2009; Dictor et al. 2009; Zvelebil et al. 2013). Expression of SOX11 has been observed in approximately 95 % of MCL (Ek et al. 2008; Wang et al. 2008; Mozos et al. 2009; Nygren et al. 2012), thus making it a disease defining marker.

The prognostic impact of SOX11 has been the topic of a few studies showing contradictory results. Increased levels of SOX11 have been correlated to an improved survival (Wang et al. 2008; Nygren et al. 2012). This was further supported by us in Paper II, where we investigated the prognostic power of SOX11 in the homogenously treated Nordic MCL2/MCL3 cohort. Our study is unique in relation to the homogenously treated material, which is, as discussed above, important due to the constantly changing treatment of MCL. However, up-regulation of SOX11 has also been associated with an inferior survival (Fernandez et al. 2010; Navarro et al. 2012; Meggendorfer et al. 2013). The reason for this controversy is that lack of SOX11 expression defines the rare indolent MCL from aggressive MCL. Thus, SOX11 is correlated to an inferior survival comparing indolent (SOX11 negative) MCL with aggressive (SOX11 positive) MCL while increased expression of SOX11 within the aggressive MCL subtype is correlated to improved survival.

The role of SOX11 in the pathogenesis of MCL remains largely unknown. In-vitro SOX11 knock-down experiments using siRNA showed that decreased levels of SOX11 results in increased cell proliferation. Similarly, forced over-expression of SOX11 resulted in a decreased proliferation rate (Gustavsson et al. 2010). To further address the biological function of SOX11 in MCL, several studies have used gene expression analysis to identify involved pathways. Gustavsson et al. demonstrated that the Cyclin D1 related pathway Rb-E2F (Figure 1B) mediates the reduced cell growth observed upon SOX11 overexpression (Gustavsson et al. 2010). Furthermore, Conrotto et al. found that SOX11 knockdown co-regulated several MCL associated genes, which further indicate that SOX11 directly or indirectly regulate these genes (Conrotto
et al. 2011). Chromatin immunoprecipitation is a method to assess transcription factor binding sites and two independent research groups have assessed the genome wide binding of SOX11, showing contradictory results. Vegliante et al. report an interaction to PAX5 leading to blocking of B-cell differentiation while Kuo et al. recently suggested several target genes involved in Wnt signaling and transforming growth factor beta (TGF-β) pathways (Vegliante et al. 2013; Kuo et al. 2014). Although a few genes and pathways are overlapping in these studies, no consensus on the molecular function of SOX11 has been determined. In paper III we assessed antibodies for immunoprecipitation assays, to gain deeper insight into the molecular interactions of SOX11, both at a protein level and at a transcriptional level. Immunoprecipitation assays are highly dependent on the specificity of the antibody, and we further demonstrated cross-reactivity to SOX4 of one of the most commonly used SOX11 antibodies. We show that antibodies developed against a C-terminal peptide of SOX11 show strong target specificity. Although none of our monoclonal antibodies were suitable for ChIP assays, we demonstrated proof of concept to analyze the SOX11 interacting network with mass spectrometry. Additionally, we identified elongation factor 1 alpha as a possible SOX11 interacting partner. Interestingly, elongation factor 1 alpha have been reported to interact with p53 (Blanch et al. 2013).

Novel targeted therapies in MCL

The survival of MCL has increased dramatically during the last decade. However, although novel treatment combinations have increased the overall survival rate, most patients will end up with relapsed and/or refractory disease. Moreover, fractions of patients, commonly the elderly, do not tolerate these high dose therapies. At the moment, several new targeted small molecules, targeting different molecules of the B-cell receptor (BCR) signaling pathways, are in different phases of clinical evaluation for treatment of relapsed non-Hodgkin’s lymphoma, here reviewed with the focus of MCL.
Signaling through the BCR is a requirement for cell survival during B-cell development. Similarly, it has been shown that BCR signaling promotes growth and survival in multiple B-cell malignancies, including DLBCL, FL, CLL and MCL (Young and Staudt 2013). The BCR downstream signaling cascade includes a complex network of kinases including spleen tyrosine kinases (SYK), Burton’s tyrosine kinase (BTK) and Phosphoinositide 3-kinase (PI3K). Finally, the signal is transferred to more distal networks such as AKT/mTOR, MAKP or NF-KB pathways driving cell proliferation and survival (Figure 3).

**Figure 3. Simplified overview of the B-cell receptor signaling pathways.** Inhibitors targeting spleen tyrosine kinase (SYK), Burton’s tyrosine kinase (BTK), Phosphoinositide 3-kinase (PI3K), AKT and mTOR are under development for treating malignant lymphomas.

Several proteins in the BCR signaling cascade have been shown to be active in MCL. Pighi et al. detected phosphorylated levels of both LYN
and SYK and Cinar et al. demonstrated that BTK is commonly overexpressed (Pighi et al. 2011; Cinar et al. 2013). Ibrutinib is an effective inhibitor of BTK and in a recent phase II study, ibrutinib showed a high response rate of 68 % and a complete response rate of 21 % in patients with relapsed or refractory MCL. The use of ibrutinib provides new possibilities of less intensive treatment in MCL and demonstrates a low toxicity profile (Wang et al. 2013a). Ibrutinib received US Food and Drug Administration (FDA) approval in November 2013 for treatment of relapsed MCL and in February 2014 as second line therapy in CLL (www.fda.gov).

Several other inhibitors of the BCR signaling cascade are under development. A phase I/II study involving the efficacy and safety of the SYK inhibitor fostamatinib has been evaluated in relapsed and refractory malignant lymphomas. The efficacy varied between different subtypes with the highest response rate in CLL (55 %), followed by DLBCL (22 %), MCL (11 %) and FL (10 %) (Friedberg et al. 2010). Additionally, several inhibitors of the PI3K/AKT/mTOR signaling pathway are in early clinical trials. Recently, Wagner-Johnsson et al. presented initial results on the use of idelalisib (GS-1101/CAL-101, a PI3K inhibitor) in 22 relapsed MCL patients (Data presented in the 2013 ASCO Annual Meeting). In this study, idelalisib was assessed in combination with everolimus (mTOR inhibitor), bortezomib (proteasome inhibitor) or bendamustine (chemotherapy) and rituximab. Although only four patients were included in the idelalisib/bendamustine/rituximab arm, an overall response rate of 100 % with a complete remission rate of 50 % indicate future clinical evaluations of this combination in larger cohorts. Other inhibitors of the PI3K/AKT/mTOR pathway includes AKT inhibitor perifosine and mTOR inhibitor everolimus and temsirolimus which have shown promising results in other non-Hodgkin lymphomas, however not in MCL and not further considered here (Smith et al. 2010; Witzig et al. 2011; Guidetti et al. 2012).
Antibodies are important tools in diagnostic and preclinical research, being able to detect, quantify and localize specific proteins. The conventional method for producing antibodies is by animal immunization, generating polyclonal antibodies. The development of hybridoma technology revolutionized the use of antibodies, to include large scale production of clonal antibodies for both research and therapeutic use.

This chapter gives a short introduction to antibody production and discusses important aspects between monoclonal and polyclonal antibodies for research use. Finally, the impact of antibodies as research tools will be discussed, with the specific focus of chromatin immunoprecipitation and co-immunoprecipitation assays.

**Antibody production**

Diagnostic and research antibodies are most often produced by immunization of animals, such as mouse, rabbits, goats and sheep (Lipman et al. 2005). A protein specific peptide is selected, and immunized together with an adjuvant to raise the antibody response. The immunized peptide present numerous of epitopes, thus activating multiple B-cell clones and yielding a polyclonal antibody sera. Monoclonal antibodies can be produced by hybridoma technology, developed more than three decades ago (Kohler and Milstein 1975). In this technology, single antibody producing B-cells are isolated after immunization and immortalized by cell fusion with myeloma cells. These fusion cells can be cultured and thus provide an unlimited source of clonal antibodies.
The choice of using monoclonal or polyclonal antibodies depends on several factors (Table 4). Monoclonal antibodies demonstrate single specificity and an essentially unlimited supply, generating low batch variations. However, the production time is long and more expensive compared to polyclonal antibodies (Lipman et al. 2005). Immunization of rabbits provides a rather inexpensive method to generate relatively large amounts of polyclonal antibodies, particularly useful in immunoprecipitation and western blot (Cooper and Paterson 2009). Additionally, polyclonal antibodies are generally less sensitive to conformational changes of the epitope, as a result of fixation agents or due to altered pH and salt concentrations (Lipman et al. 2005).

**Table 4. Comparison of polyclonal and monoclonal antibody characteristics**

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<th>Polyclonal antibodies</th>
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<td>+ Inexpensive to generate</td>
<td>- Expensive to generate</td>
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<tr>
<td>+ Shorter generation time</td>
<td>- Longer generation time</td>
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<td>- Batch variations</td>
<td>+ Consistency</td>
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<tr>
<td>- Lower concentration (µg/ml)</td>
<td>+ Higher concentrations (mg/ml)</td>
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<td>+/- recognize multiple epitopes</td>
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**Antibody validation**

The human genome consists of 20,000-25,000 protein coding genes while it is estimated that the number of proteins, including splice variants and post translational modifications exceed 1 million variants (Jensen 2004; Clamp et al. 2007). The antibody market has grown substantially over the years and is today estimated to contain more than 500,000 commercially available antibodies (Colwill et al. 2011). Most commercial antibodies are produced against proteins that are frequently studied, while there is still a huge lack of antibodies targeting the vast majority of proteome (Taussig et al. 2007). The human protein atlas is a Swedish initiative with the aim of mapping all human proteins in normal and cancer tissues by the generation of polyclonal antibodies. In their latest release (v.12) over 21,900 antibodies have been generated, mapping a total of 16,600 proteins with total genome coverage of 83 % (www.proteinatlas.org). An antibody search on Biocompare (www.biocompare.com) on “p53”...
resulted in over 4500 hits while a search on “SOX11” resulted in 103 hits. Although there are more than hundreds of different antibodies for some proteins, it is common to hear researchers complain over the quality of commercial antibodies. By studying the SOX11 literature published the last two years, it became clear that less than 10% of the commercial available antibodies have been used. This is just one example of the presence of poorly validated antibodies for commercial sale that waste researchers time, money and important patient material. However, also the research community has an important part of in the responsibility for unreproducible experiments, as most published papers generally lack description about antibody supplier, product number or protocol details (Helsby et al. 2013).

Most commercial antibodies are polyclonal, which by due to limited production volumes and batch variations generate a costly and never ending validation process. Although monoclonal antibodies are more expensive to develop in a short perspective, it is probably the solution for a sustainable production of well characterized antibodies. In paper I, we developed the first monoclonal antibody targeting SOX11. The antibody was well characterized and showed superior target specificity in ELISA, western blot, immunohistochemistry, immunofluorescence and flow cytometry. We further demonstrate the ability of using this antibody in clinical laboratories analyzing SOX11 expression in MCL samples with either immunohistochemistry or flow cytometry.

**Antibodies as a research tool – focusing on immunoprecipitation**

Antibodies have become a fundamental tool in preclinical research for identification, quantification and localization of specific molecules or cells. Antibodies are used in several assays such as ELISA, western blot, immunohistochemistry, flow cytometry as well as protein and chromatin immunoprecipitation and affinity based proteomics. Due to recent technical advances, immunoprecipitation assays have become key assays to identify protein network and transcription factor target sites (Weinmann and Farnham 2002; Free et al. 2009).
Fueled by the sequencing of the human genome (Lander et al. 2001; Venter 2001), next-generation sequencing technologies have had a rapid development during the last decade and are today offering high coverage and decreasing costs (Park 2009). Genome wide mapping of protein-DNA interactions, previously limited to microarray hybridization (ChIP-chip), are today commonly coupled to next-generation sequencing (ChIP-seq)(Ren et al. 2000; Barski et al. 2007). ChIP-chip and ChIP-seq have led to important understandings of transcriptional regulation and the epigenetic landscape during development and disease. Takahashi and Yamanaka presented one of the most impressive findings during recent years, the induction of pluripotency by four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka 2006). Multiple studies using ChIP-seq have now assessed the global transcriptional program of these key transcription factors (Goke et al. 2012; Lodato et al. 2013).

ChIP assays are highly dependent on antibody specificity and sensitivity. Cross-linking agents are most often used to preserve the DNA-protein interaction. However, this treatment can modify or mask several epitopes which have made polyclonal antibodies as the preferred choice over monoclonal antibodies. However, polyclonal antibodies show reactivity to several epitopes and the risk of cross-reactivity is higher compared to monoclonal antibodies. A cross-reactive antibody will generate false positive signals.

The molecular function of SOX11 is largely unknown (discussed in Chapter 3). In paper III, we generate and characterize four antibodies for their use in immunoprecipitation assays of SOX11. SOX proteins are commonly overexpressed in several human neoplasms, and SOX4 and SOX11 show up to 91% sequence homology within the HMG box (Dy et al. 2008). Shim et al. reported that they have validated 12 commercial available SOX4 antibodies and 10 commercial available SOX11 in western blot, immunohistochemistry and ChIP. However, only three of each antibody targeting SOX4 and SOX11 recognized a single band in western blot while none were suitable in immunohistochemistry or ChIP (Shim et al. 2012). Also at our lab, three commercial available SOX4 antibodies have been validated in immunohistochemistry and none turned out to be specific. Furthermore, in paper III we found that the most commonly used SOX11 antibody are cross-reactive to recombinant
SOX4 in ELISA, although this cross-reactivity remains to be demonstrated in other assays. Hence, it is not an understatement to notify researchers to be very restricted and selective in the choice of SOX antibodies in their experiments. Cross-reactivity between SOX4 and SOX11 antibodies is most probably not a major issue in MCL, where expression levels of SOX4 relatively low, but are of importance in other neoplasms, which possibly express both SOX11 and SOX4. In these cases, the use of several antibodies targeting different regions of SOX11 could be used to reduce false positive signals.

Immunoprecipitation could also be utilized to discover protein-protein interactions. Current improvements in the mass spectrometry field has resulted in higher resolution and increased sensitivity, which provides an ability to discover and identify novel interaction partners (Trinkle-Mulcahy 2012). As an example, Fang et al. used co-immuoprecipitation coupled to mass spectrometry to reveal the SOX2 interactome in glioblastoma (Fang et al. 2011). However, the interacting proteome surrounding SOX11 have not been well characterized. In paper III, we further demonstrated the concept of analyzing SOX11 and its potential interacting proteins with mass spectrometry. Although further optimizations are needed regarding buffer composition to preserve the protein complex, we demonstrated the ability to detect SOX11 in mass spectrometry and identified a potential interacting partner, elongation factor 1 alpha.
The term epigenetics refers to external changes that modify gene expression without changing the DNA sequence. Epigenetic changes are heritable during each cell division. Examples of such changes are DNA methylation, histone modifications and non-coding RNAs. This chapter covers an introduction to epigenetics, a discussion on the epigenetic regulation of SOX11 and a short review of current and upcoming epigenetic drugs. Here, the term epigenetic refers to DNA methylation and histone modifications, while the impact of non-coding RNAs is outside the scope of this thesis.

**Introducing epigenetics**

Human cells contain identical DNA sequences encoding our genes, packed into 46 chromosomes containing both DNA and proteins. The DNA encodes our genes while the proteins, mainly histones, are responsible for packaging and protection. The nucleosome consists of two copies of each four histones, H2A, H2B, H3 and H4, which have protruding tails that can undergo several post translational modifications (PTMs) including acetylation, phosphorylation, methylation, ubiquitination and sumoylation (Kouzarides 2007). Additionally, DNA can also undergo methylation at specific nucleotides. Together, these modifications work in a dynamic, complex interplay to control chromatin structure and gene expression.
DNA methylation

DNA methylation is a process performed by a group of DNA methyl transferases (DNMTs), which add a methyl group (-CH$_3$) to the 5’ position of the cytosine ring. DNA methylation occurs almost exclusively when a cytosine (C) follows next to a guanine (G) at so called CpG sites. About 80% of the human CpG sites are methylated (Bird 2002). However, the presence of local hypomethylated stretches with a high CpG content (CpG islands) are often associated with promoter regions. It has been estimated that about 60% of the human promoters contain CpG islands, and the majority of these regions remain unmethylated throughout development independent of gene expression (Lander et al. 2001; Venter 2001). A methylated promoter is a hallmark of silenced genes (Figure 4A), and methylation can inhibit transcription directly, by blocking the access of transcription factors, or indirectly, by recruiting methyl-CpG-binding domain proteins (Jones et al. 1998; Nan et al. 1998).

Histone modifications

Post translational modifications of the histone tails have a large impact on gene expression, either by altering the chromatin structure or by inhibiting and/or recruiting other regulatory proteins (Bannister and Kouzarides 2011). The post translational modifications most frequently studied are histone acetylation and methylation. Open chromatin, associated with transcriptional expression, is characterized by histone acetylation, catalyzed by histone acetyl transferases (HATs) and histone methylation of histone 3 on lysine 4 (H3K4me3), catalyzed by the Trithorax group proteins (TrxG) (Figure 4B). Histone acetylation neutralizes the positive charge of lysine and thus decreases the interaction with the negatively charged DNA. The mechanism underlying TrxG activation remains largely unexplored, but a recent report demonstrated a physical interaction between TrxG and HATs (Tie et al. 2014). To balance the effect of histone acetylation and TrxG methylation, histone deacetylases (HDACs) and polycomb repressive complex (PrC) repress gene expression by tightly packaging of the chromatin (Figure 4C). HDACs catalyze the removal of acetyl groups, thus restoring the positive charge of lysines and promote tight interactions between DNA and
The polycomb repressive complex catalyze trimethylation on lysine 27 on histone 3 (H3K27me3), a hallmark of silenced genes. During the latest years it has been shown that there is a significant crosstalk between DNA methylation and histone modifications to silence genes. For example, Vire et al. demonstrated that the polycomb group protein EZH2 physically interacts with DNMTs and control methylation (Vire et al. 2006).

Figure 4. Epigenetic regulation of gene expression. A) Repressed chromatin and inactive genes could be silenced by CpG methylation (by DNMTs). B) Active chromatin is characterized by acetylation (by HATs) and H3K4me3 (by TrxG). C) Repressed chromatin and inactive genes are also characterized by removal of acetyl groups (by HDACs) and H3K27me3 (by PrC).
Epigenetic modifications in MCL

Aberrant expression of enzymes regulating DNA methylation and histone modifications are known to contribute to tumor development. In terms of DNA methylation, cancer cells most often show a genome wide hypomethylation and a site specific hypermethylation. Loss of methylation, most often within repetitive regions, can lead to a general genomic instability and activation of oncogenes while site specific hypermethylation most often silence tumor suppressors (Ehrlich 2002). Additionally, several histone modifying enzymes are altered in cancer including overexpression or mutations of histone deacetylases and/or methyltransferases. Interestingly, several reports have shown that target genes of EZH2, a member of the polycomb complex, often become methylated in lymphomas (Velichutina et al. 2010). However, very few studies have demonstrated altered activity and expression of DNMTs in lymphomas. Interestingly, although the level of DNMT3A, partly responsible for de novo methylation, is elevated in MCL (unpublished data) twice as many hypomethylated promoters compared to hypermethylated promoters has been reported in MCL (Leshchenko et al. 2010), reflecting the difficulty in comparing expression levels with the amount of methylated target genes. An altered expression of histone modifying enzymes has also been described in MCL. Two important components of the PRC2 complex, SUZ12 and EZH2, have been reported to be over expressed in MCL (Martin-Perez et al. 2010; Kanduri et al. 2013). Additionally, another component of PRC1, BMI1, is frequently overexpressed in MCL (Bea et al. 2001) and was recently shown to be upregulated in MCL SP cells (Teshima et al. 2013). Other modifications described in lymphomas, although not yet MCL, are mutations in the trithorax group proteins MLL, MLL2 and MLL3 and truncated histone acetyltransferase p300 (Shaknovich and Melnick 2011).

Epigenetic regulation of SOX11 in normal and cancer cells

The expression of SOX11 in MCL is not a consequence of the genetic instability of the disease. Instead, SOX11 expression has recently been shown to be regulated by epigenetic events such as DNA methylation and
his histone modifications (Mikkelsen et al. 2007; Gustavsson et al. 2010; Vegliante et al. 2011).

SOX11 is silenced in almost all adult differentiated tissues (Penzo-Mendez 2010). Initial investigations by Vegliante et al. in peripheral B-cells have shown that SOX11 is regulated by epigenetic events with an unmethylated promoter and therefore only silenced by histone methylation of H3K9me2 and H3K27me3 (Vegliante et al. 2011). In Paper IV, we performed a detailed analysis of the epigenetic regulation of SOX11 in healthy cells and show that SOX11 is silenced by enrichment of H3K27me3 and displays a low degree of methylation. This is of importance to be able to assess and compare tumor related epigenetic changes causing SOX11 expression or specific silencing by DNA methylation.

The mechanism for SOX11 re-expression in MCL and other tumors is widely unknown. In paper IV, we further demonstrate that HDAC inhibitors such as vorinostat and trichostatin A can induce SOX11 expression in cell-lines with a low degree of methylation, indicating a role of histone acetylation in the control of SOX11. Vorinostat and trichostatin A strongly inhibit broad classes of HDACs including HDAC1–4, HDAC6–7 and HDAC9 (Witt et al. 2009) and any of these targets could be the cause for the altered expression of SOX11. To get deeper insight into which of these HDACs that regulate SOX11, individual knockdowns could be performed. Glohini et al. have studied the presence of different HDACs in lymphoma and found that most HDACs were homogenously expressed over all tested cell-lines, again demonstrating the complexity of linking individual epigenetic alterations to a general protein level (Gloghini et al. 2009).

In addition to the re-expression of SOX11 observed in several malignancies we and others have shown that SOX11 undergo specific methylation in some cancers. It has been shown that many genes associated with CpG islands and the silencing mark H3K27me3 undergoes de novo methylation in cancer (Schlesinger et al. 2007) and a few studies have reported a correlation between SOX11 methylation and cancer, as discussed below in this chapter. With the methylation status of SOX11 in normal cells reveals, we show that many tumors de novo methylate SOX11 during their pathogenesis. SOX11 methylation have
previously been reported by Gustavsson et al in non-expressing B-cell lymphomas (Gustavsson et al. 2010) and by Sernbo et al in subgroups of epithelial ovarian cancer (Sernbo et al. 2011). Furthermore, methylation of SOX11 has been demonstrated to positively correlate with lymph node metastasis in nasopharyngeal carcinoma (Zhang et al. 2013) and has been proposed in a signature of five genes to detect bladder cancer in urine sediments (Chung et al. 2011). In Paper IV, we further show that the promoter of SOX11 is heterogeneously methylated in many subsets of solid tumors (breast cancer, ovarian cancer, lung cancer and glioma). Interestingly, in breast cancer cells, we could correlate the methylated status of SOX11 to ER positivity with a negative correlation to gene expression. If the methylation status of the SOX11 promoter could be correlated to any known subtypes in the other tumors remains to be studied and are highly dependent on large clinical cohorts to be investigated. Most interesting would be to assess the relation between ER status and SOX11 expression in ovarian cancer, since this malignancy show several similarities with breast cancers. Interestingly, both high grade serous ovarian cancer and basal-like breast cancer are associated with a stem cell-like subtype and have been reported to express SOX11 (Sernbo et al. 2011; Schwede et al. 2013; Zvelebil et al. 2013).

Epigenetic biomarkers

Global maps on epigenetic changes have been created for most human malignancies which could be used in the clinic as diagnostic, prognostic or predictive biomarkers. Aberrant DNA methylation has shown most promise as an epigenetic biomarker for several reasons. First, DNA methylation has been shown to be an early event during tumor development and may precede classical transforming events such as mutations in suppressor or oncogenes (Baylin and Ohm 2006). Hence, detection of specific methylated CpG islands has potential in identifying early lesions. Secondly, an attractive feature of DNA methylation is that it is stably preserved in several body tissues and fluids and could be detected with PCR-based techniques (Rodriguez-Paredes and Esteller 2011). For example, hypermethylation of the glutathione S-transferase genes (GSTP1) has been detected in 100 % of prostate cancer tissues,
with the potential of also detecting the methylated promoter in urine, providing a non-invasive method (Lee et al. 1994; Cairns et al. 2001). SOX11 has also been suggested to enable early detection of bladder cancer in urine (Chung et al. 2011). In *paper IV* we further demonstrate the presence of SOX11 methylation in several malignancies. Although not subtype specific in non-Hodgkin lymphoma, a methylated SOX11 promoter could have a potential to be used in screening of high risk patients for early detection. Additionally, we demonstrate the presence of SOX11 methylation in subgroups of breast, ovarian, lung and glioma, which at least in breast cancer correlate to clinical subtype. The most important feature of a DNA methylation biomarkers in screening of early lesions are a low level of methylation in non-malignant cells, which needs to be confirmed for SOX11 in larger cohorts of patients.

**Epigenetic drug therapies**

During the last decade, a novel approach to target cancer cells has emerged in epigenetic drugs. Instead of killing cancer cells by DNA damaging agents or pathway inhibitors, epigenetic drugs attempts to reprogram the cancer cells by reactivation of tumor suppressor genes controlling cell growth and survival. Four epigenetic drugs, including inhibitors of DNA methylation and histone acetylation, are FDA approved for treatment of some hematologic malignancies while their use in solid tumors currently is evaluated in several clinical trials.

**DNA methyltransferase inhibitors**

DNA promoter methylation is a hallmark of silenced genes, and many tumor suppressor genes involved in the cell cycle (*p16^{INK4a}, p15^{INK4b}, p14^{ARF}*) apoptosis machinery (*DAPK, TMS1*) and DNA repair (*BRCA1, hMLH1, MGMT*) becomes *de novo* methylated in cancer (Esteller 2002). DNMTs, the enzymes responsible for the methylation, can be inhibited by two classes of compounds; nucleoside analogues and non-nucleoside inhibitors. Nucleoside analogs such as azacitidine (5-azacytidine) and decitabine (5-aza-2’-deoxycytidine) were the first epigenetic drugs to
become FDA approved in 2004 and 2006 respectively for treatment of myelodysplastic syndromes (Issa et al. 2005; Kantarjian et al. 2006). The drugs mainly target highly proliferating cancer cells by being phosphorylated and incorporated into the DNA strand during cell division. The incorporated nucleoside analog then trap DNMT by a covalent bond, and the complex becomes degraded by the proteasome (Gros et al. 2012). The use of azacitidine and decitabine as single drug treatments have been evaluated in several clinical trials targeting mostly leukemia and lymphoma but also solid tumors such as breast, bladder or prostate cancer (Nebbioso et al. 2012). However, both azacitidine and decitabine are chemically unstable and have unspecific toxic effects. Thus, novel DNMT inhibitors are during development and include more stable nucleoside analogs and small molecules blocking the catalytic site (Nebbioso et al. 2012). Promoter hypomethylation is not always enough to induce gene expression because a gene can be silenced by repressive histone marks as discussed above. Therefore, several studies are now evaluating the combination of demethylating agents with HDAC inhibitors.

**HDAC inhibitors**

The balance between HATs and HDACs play a key role in regulating transcription. HDAC inhibitors are a class of molecules which inhibit the catalytic site of HDACs, thus favoring acetylation over deacetylation. HDAC inhibitors also target several non-histone proteins, including transcription factors, DNA repair proteins and chromatin remodeling proteins (Dokmanovic et al. 2007). In fact, although HATs and HDACs are widely distributed over our genome, only 2-10 % of our genes are changed upon HDAC inhibitor treatments (Van Lint et al. 1996; Glaser et al. 2003; LaBonte et al. 2009) . Interestingly, these inhibitors are highly tumor specific and normal cells remain unaffected (Minucci and Pelicci 2006). Although this resistance of normal cells remains unclear, studies have shown that HDAC inhibitors induce DNA damage in both normal and cancer cells but only normal cells are able to repair their DNA (Lee et al. 2010). HDAC inhibitors have been reported to have cell specific effects including increased apoptosis and cell cycle arrest. Importantly, these inhibitors have been reported to target cancer stem cells by inducing
cell differentiation (Svechnikova et al. 2008; Culmes et al. 2012; Salvador et al. 2013). Today, HDAC inhibitors are investigated in hundreds of clinical trials, evaluating the effect as a single agent or in combinations with cytotoxic compounds (www.clinicaltrials.gov)

Vorinostat (SAHA) was the first HDAC inhibitor to be approved by FDA in 2006 for treating recurrent cutaneous T-cell lymphoma (Mann et al. 2007). Since then, the use of vorinostat has been evaluated in both lymphoid and solid tumors (www.clinicaltrials.gov). Last year, another HDAC inhibitor, entinostat in combination with exemestane, was presented as a FDA breakthrough therapy for treating recurrent or metastatic ER positive breast cancer (Yardley et al. 2013). Together, these examples demonstrate proof of concept for HDAC inhibitors and show promise for the release of several new compounds within the next coming years.

**Other epigenetic inhibitors**

Although most attention regarding epigenetic drugs are about HDAC inhibitors, several other approaches are under development including inhibitors of histone methyltransferases. The polycomb protein EZH2 is overexpressed in several tumors including MCL (Kleer et al. 2003; Karanikolas et al. 2009; Kanduri et al. 2013). 3-deazaneplanocin A (DZNep) originally developed as an inhibitor of S-adenosyl-L-homocysteine hydrolase (Tseng et al. 1989) has been shown to deplete EZH2 levels and inhibit H3K27me3 silencing of tumor suppressors in various cell lines, including MCL (Miranda et al. 2009; Fiskus et al. 2012). EPZ-6438, another EZH2 inhibitor is currently in a phase I trial for treatment of non-Hodgkin lymphoma patients harboring the EZH2 point mutation A682G (Knutson et al. 2014).
SOX11 was discovered as a diagnostic antigen in mantle cell lymphoma in 2008. In contrast to the vast majority of biomarkers published each year, SOX11 has proven its usefulness and is today widely accepted as a biomarker in lymphoma diagnosis.

At the start of my thesis, our research was significantly hampered by the general lack of SOX11 specific antibodies with low batch variations. Commercial SOX11 antibodies were polyclonal which are known to show batch variations, and thus making continued pathological investigations of SOX11 in MCL tissues difficult. Therefore, we decided to develop the first monoclonal antibody targeting SOX11. In Paper I we present the work of developing and evaluating this antibody, called SOX11-C1. This antibody specifically stains SOX11 in MCL cells in both immunohistochemistry and flow cytometry assays while all non-expressing lymphomas were not stained, and thus demonstrate its use in clinical settings. SOX11-C1 is today commercially available and contributes to the diagnosis of MCL patients as well as to the SOX11 research community.

The prognostic relevance of SOX11 in MCL patients has been a topic for discussion the last years. In Paper II, we correlate high levels of SOX11 with improved overall and event free survival in the homogenously treated Nordic MCL2/MCL3 cohort. Our view of SOX11 as a tumor suppressor is supported by in-vitro data as well as by other independent research groups. However, the absence of SOX11 in indolent MCL has resulted in papers correlating SOX11 expression to an inferior survival, comparing indolent and aggressive MCL. To my opinion, one could argue that indolent MCL is a clinically separate subtype and that lack of a protein, not normally expressed in healthy cells, doesn’t add a prognostic
value. However, lack of SOX11 is unquestionable useful for identifying indolent MCL in a diagnostic setting. We further explored the prognostic value of two other important molecules, Cyclin D1 and p53. A strong p53 staining was significantly correlated to an inferior survival while the intensity of Cyclin D1 staining showed no correlation to survival.

The prognostic relevance of SOX11, together with previously published in-vitro experiments suggests that SOX11 is a disease defining molecule. To further understand the function of SOX11, signaling pathways needs to be identified. In Paper III, we assessed the specificity of SOX11 targeting antibodies in immunoprecipitation assays with the aim to get further insight into SOX11 signaling pathways. Although the methods need further optimization, we identified elongation factor 1 alpha, a p53 interacting protein, as a possible SOX11 interacting protein.

The epigenetic modifications of SOX11, described and discussed in Chapter 5 and Paper IV, are important in relation to the growing interest of using epigenetic drugs such as HDAC inhibitors. In addition to the increased acetylation, HDAC inhibitors have several non-histone targets. For example, the three molecules discussed in Chapter 3, Cyclin D1, p53 and SOX11, are all targets of HDAC inhibitors. Both trichostatin A and vorinostat has been shown to rapidly decrease Cyclin D1 levels in MCL cell lines (Kawamata et al. 2007). Similarly, different HDAC inhibitors have been reported to either increase or decrease the stability of p53, probably depending on the specific inhibitor used, the cellular context as well as exposure times and concentrations. Finally, we show in Paper IV that SOX11 also are affected by HDAC inhibitors and could be re-expressed using either trichostatin A or vorinostat. SOX11 itself could potentially also be post-transcriptionally regulated by HATs and HDACs. Within the SOX protein family, it has been shown that both SOX2 and SRY could be acetylated by p300 and that HDAC3 can deacetylate SRY (Thevenet et al. 2004; Baltus et al. 2009)

As discussed in this thesis, protein levels of SOX11 could be used as a diagnostic and prognostic biomarker in MCL. Additionally, the methylated state of SOX11 promoter could potentially be used in screening high risk groups for early lesions, although more studies needs to validate our finding and prove the presence of SOX11 methylation as an early tumorigenic event.
Finally, I would like to speculate in the future use of SOX11 in a therapeutic approach. Several novel targeted therapies, exemplified with the BCR pathway and Ibrutinib, have shown great promise due to the low background in normal cells, and SOX11 targeting therapies have the potential to be very selective. All proteins inside a cell is continuously processed and presented by the major histocompatibility complex (MHC). The concept of tumor associated antigens presented on MHC molecules is the basis of T-cell based immunotherapies. This therapy is based on activation of cytotoxic CD8+ T-cells, capable of destroying tumor cells that express the antigen/MHC complex. Interestingly, Schmitz et al. have reported about an SOX11-derived peptide presented on a MHC molecule in glioma cells, capable of activating cytotoxic T-cells in-vitro (Schmitz et al. 2007). Additionally, Dao et al. recently demonstrated the concept of using monoclonal antibodies to target transcription factor WT1 by directing the antibody to the WT1 peptide-MHC complexes displayed at the surface of the tumor cells (Dao et al. 2013). Since a SOX11-MHC peptide already have been defined, both of these methods are likely to be explored to specifically target SOX11 expressing mantle cell lymphoma cells.

In conclusion, this thesis has extended the use of SOX11 as a marker in MCL. By providing new tools, including a monoclonal antibody and a combined molecular prognostic index I hope that the use of SOX11 in diagnostic and prognostic applications could be implemented in the clinic. Finally, the optimized protocols including flow cytometry and immunoprecipitation assays potentially enable a future discovery of SOX11 interacting pathways, which could contribute to the development of SOX11 targeting therapies.
Populärvetenskaplig sammanfattning


SOX11 är ett protein som enbart finns i celler under fostertiden, bl.a. under utvecklingen av det centrala nervsystemet. Att detta protein plötsligt återuttrycks i mantelcellslymfom är något man kan utnyttja för att ställa en säker diagnos. För att identifiera vilka patienter som har SOX11 (och därmed mantelcellslymfom) används antikroppar, ett protein som effektivt märker in det protein det var designat att binda, i detta fall...


För att kunna utveckla nya målinriktade behandlingar mot mantelcelllymfom krävs stora kunskaper om vad som gått fel i tumörcellerna och vad som driver den okontrollerade tillväxten och dess motståndskraft mot cellekött. Eftersom SOX11 enbart finns i tumörceller

Förutom i mantelcellslymfom, är SOX11 uttryckt i subgrupper av äggstockscancer, bröstcancer och i hjärntumörer. I Paper IV använde jag laboratoriemodeller, cell-linjer, av dessa tumörer för att studera reglering av SOX11. Jag visar att genen för SOX11 i normala celler binds av ”tystande” proteiner medan genen för SOX11 uttryckande tumörer binds av ”aktiverande” proteiner. Slutligen visar jag även att en ny typ av cancerbehandling, med så kallade epigenetiska läkemedel i vissa fall påverkar cancercellerna att återuttrycka SOX11.

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Chapter 9

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