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Role miR-29 v mikroprostředí chronické lymfocytární leukémie

Diplomová práce

KAROLINA SEDLÁČKOVÁ

Vedoucí práce: Mgr. Miroslav Boudný, PhD.

Ústav experimentální biologie
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Autor:	Karolina Sedláčková Přírodovědecká fakulta Masarykova univerzita Ústav experimentální biologie
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Author:	Karolina Sedláčková Faculty of Science Masaryk University Department of Experimental Biology Department of Experimental Biology
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Abstrakt

Chronická lymfocytární leukémie (CLL) je nejčastější leukémií dospělých v západním světě. Na její vznik a vývoj má vedle dalších faktorů vliv také deregulace mikroRNA. Tyto krátké nekódující molekuly RNA regulují genovou expresi prostřednictvím degradace mRNA nebo inhibice translace. Deregulace mikroRNA bývá u CLL spojena s přítomností dalších prognostických faktorů, jako jsou mutace IgV_H nebo genomické aberace. Mezi tyto mikroRNA patří i rodina *miR-29*, jejíž nízké hladiny jsou časté u agresivní formy CLL. Tato diplomové práce se zabývá identifikací a validací nových cílových genů *miR-29* v kontextu CLL a jejího mikroprostředí.

Abstract

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world. Its onset and progression can be influenced by multiple factors including deregulation of microRNAs. These short non-coding RNA molecules regulate gene expression by mRNA degradation or translational inhibition. Moreover, microRNA deregulation is often linked with other prognostic factors of CLL, such as IgV_H mutations or genomic aberrations. This is also the case of the *miR-29* family which is often downregulated in the aggressive form of CLL. The aim of this thesis is identification and validation of novel *miR-29* target genes in the context of CLL and its microenvironment.

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MASARYKOVA UNIVERZITA
PŘÍRODOVĚDECKÁ FAKULTA
KOTLÁŘSKÁ 2, 611 37 BRNO
IČ: 00216224
DIČ: CZ00216224

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Oficiální zadání:
MikroRNA jsou krátké nekódující RNA regulující řadu různých mRNA prostřednictvím negativního ovlivnění jejich stability nebo translace. Je popsáno, že deregulace mikroRNA je spojena s malignitami B lymfocytů, zejména u chronické lymfocytární leukémie (CLL), nejčastějšího typu leukémie dospělých v Evropě a Severní Americe. Existuje přibližně 30 různých mikroRNA, o nichž je známo, že jsou u CLL různě exprimovány v závislosti na stavu IgHV nebo přítomnosti genomických aberací, včetně miR-29. Nízké hladiny miR-29 (a/b/c) jsou spojeny s agresivní formou CLL. MiR-29 u CLL reguluje i některé antiapoptické proteiny, konkrétně MCL1 a TCL1. Cílem této diplomové práce je zjistit, zda existují nějaké další cíle miR-29, které se potenciálně podílejí na interakcích v mikroprostředí. Tyto cíle plánujeme experimentálně ověřit a studovat jejich roli v patogenezi onemocnění.

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Konzultant:	Sonali Sharma, Ph.D.
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Čestné prohlášení

Prohlašuji, že jsem svoji diplomovou práci vypracovala samostatně pod vedením vedoucího práce s využitím informačních zdrojů, které jsou v práci citovány.

V Brně 20. dubna 2022

.....
Karolina Sedláčková

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List of abbreviations

Ago – Argonaute protein

AKAP5 – A-kinase anchor protein 5

Akt – protein kinase B

APRIL – a proliferation-inducing ligand

BAFF - B cell-activating factor

Bcl-2 – B-cell lymphoma 2

Bcl-XL – B-cell lymphoma-extra large

BCR – B cell receptor

BL – Burkitt lymphoma

BMSCs – bone marrow stromal cells

bp – base pair

BSA – bovine serum albumin

Btk – Bruton's tyrosine kinase

IGV_H – immunoglobulin heavy chain variable region

BM – bone marrow

BM MSCs – bone marrow mesenchymal stromal cells

C. elegans – Caenorhabditis elegans

CD40L – CD40 ligand

CLL – chronic lymphocytic leukemia

CM – conditioned medium

DCs – dendritic cells

DLBCL – diffuse large B cell lymphoma

DGCR8 – DiGeorge syndrome critical region gene 8

ECM – extracellular matrix

ERK 1/2 - extracellular signal-regulated kinase 1/2

EZH2 – enhancer of zeste homolog 2

FAK – focal adhesion kinase

FBS – fetal bovine serum

FDCs – follicular dendritic cells

FOXP3 – forkhead box protein P3

FRCs – fibroblastic reticular cells

GC – germinal centre

HDAC – histone deacetylase

ICAM-1 – intracellular adhesion molecule 1

ICOS – inducible T cell co-stimulator

ICOS-L – ligand of inducible T cell co-stimulator

IFN-γ – interferon gamma

IL – interleukin

ILK – integrin-linked kinase

ITGβ1 – integrin β1

KLF4 – Krüppel-like factor 4

LFA-1 – lymphocyte function-associated antigen 1

LN – lymph node

MAPK – mitogen-activated protein kinase

MCs – mononuclear cells

MCL – mantle cell lymphoma

Mcl-1 – induced myeloid leukemia cell differentiation protein

MIF – macrophage migration inhibitory factor

miRNA – microRNA

MMP-9 – gelatinase B/matrix metalloproteinase 9

MSCs – mesenchymal stromal cells

mTOR – mammalian target of rapamycin

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

NHL – non-Hodgkin lymphoma

NLCs – nurse-like cells

OS – overall survival

PB – peripheral blood

PBMCs – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PD-1 – programmed cell death protein 1

PI3K – phosphatidylinositol 3-kinase

PMA – phorbol-12-myristate-13-acetate

PRC2 – polycomb-repressing complex 2

pri-miRNA – primary miRNA

PVDF – polyvinylidene difluoride

RGD – arginine-glycine-aspartic acid

RISC – RNA-induced silencing complex

STAT3 – signal transducer and activator of transcription 3

SYK – spleen tyrosine kinase, also known as tyrosine-protein kinase

TAMs – tumour-associated microphages

TIM3 – T-cell immunoglobulin and mucin-domain containing-3

Tcl-1 – T-cell leukemia/lymphoma 1 oncoprotein

TFS – treatment-free survival

T_{fh} cells – follicular helper T cells

T_h cells – helper T cells

TRAF4 – tumour-necrosis factor receptor-associated factor 4

T_{reg} cells – regulatory T cells

TRBP – transactivating response RNA-binding protein

SCs – stromal cells

VCAM-1 – vascular cell adhesion protein 1

VLA-4 – very late antigen 4

3'-UTR – 3' untranslated region

ZAP70 – ζ-chain-associated protein kinase 70

1 Introduction

Chronic lymphocytic leukemia (CLL) represents, together with non-Hodgkin lymphomas (NHLs), a heterogenic group of mature B cell malignancies. The variability of these B cell malignancies is in their aggressivity, progression or resistance to treatment. Such parameters influence the outcome of the disease – while some patients do not require any treatment for years, for others the disease can be lethal within months. Despite recent significant progress in the development of various drugs and molecular inhibitors, B cell malignancies remain incurable.

The surrounding tumour microenvironment is one of the key players contributing to development or progression of cancer in general. However, signals coming from the microenvironment are essential for the bare survival of malignant B lymphocytes (Panayiotidis *et al.*, 1996).

Microenvironmental stimuli are recognised by various receptors on the cellular surface. When triggered with their ligand, these receptors thereupon activate respective signalling pathways, subsequently affecting cell survival, proliferation, differentiation, or adhesion. The most important receptor of B lymphocytes is the B cell receptor (BCR). While its signalling cascade involves multiple protein kinases and other regulatory molecules, the regulation of epigenetic modifiers by BCR signalling is equally important. MicroRNAs (miRNAs), short non-coding RNAs, represent one group of such modifiers. The *miR-29* family of miRNAs is one example of miRNAs with cellular levels in B cells influenced by microenvironmental signals (Sharma *et al.*, 2021). Binding of *miR-29* miRNAs to their target mRNAs then finetunes expression of multiple proteins, including those involved in cell survival, migration, or proliferation.

The aim of this thesis is the characterisation of new targets of *miR-29* in the context of CLL microenvironment. Selection of such candidates will be based on earlier gene expression analysis. Moreover, the role of selected proteins in microenvironmental interactions, cellular signalling or resistance to treatment will be also examined.

2 Chronic lymphocytic leukemia

CLL is the most common leukemia of adult population in the Western world. The median age of diagnosis is 70 years and male CLL patients are prevalent over female (Bosch and Dalla-Favera, 2019). This lymphoproliferative disorder is characterised by the accumulation of long-lived malignant $CD5^+$ $CD19^+$ B lymphocytes in bone marrow (BM), peripheral blood (PB) and secondary lymphoid organs (Caligaris-Cappio and Hamblin, 1999). Within these lymphoid organs, CLL cells gather in areas called proliferation centres or pseudo-follicles where they undergo proliferation (Figure 1). Such structures are typical for CLL histopathology (Stein *et al.*, 1980). In addition to the long lifespan of CLL cells, a vast majority of them is arrested in the G0/G1 phase of cell cycle (Delmer *et al.*, 1995).

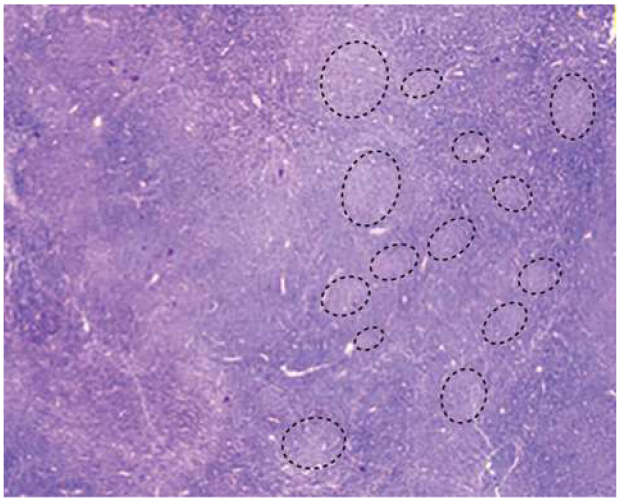


Figure 1: Lymph node biopsy of CLL patient stained with haemotoxylin and eosin. The circled areas represent pseudofollicles of CLL cells (Kipps *et al.*, 2017).

Remarkably, CLL cells are highly dependent on the lymphoid organ microenvironment – signalling molecules and ligands coming from the surrounding stroma or expressed on the membranes of other types of immune cells contribute to anti-immune protection and increased survival or proliferation of malignant B lymphocytes (Collins *et al.*, 1989). Recirculation of malignant cells between PB and secondary lymphoid organs is thus characteristic for the malignancy. PB-resident CLL cells migrate, influenced by chemokines produced by various microenvironmental cells, to lymph nodes (LNs), spleen or BM, to be eventually released back to PB. The main orchestrator of the homing of CLL cells to lymphoid organs is the CXCL12 chemokine which binds its receptor CXCR4 on the surface of malignant lymphocytes. Surface CXCR4 expression of PB-resident neoplastic cells is increased compared to CLL cells which have only recently left the lymphoid organ. CXCL12 is produced by different stromal cells.

Moreover, differential expression of CXCR4 between PB CLL cells and CLL cells localised within the lymphoid microenvironment allows the distinguishment of two subsets of CLL cells varying in their phenotype – $CD5^{dim}CXCR4^{bright}$ and $CD5^{bright}CXCR4^{dim}$ cells (Burger *et al.*, 1999; Calissano *et al.*, 2009). $CD5^{bright}CXCR4^{dim}$ cells represent a proliferative fraction of cells that have recently exited the microenvironment, while $CD5^{dim}CXCR4^{brigh}$ cells correspond to resting cells circulating in PB for an extended period of time (Figure 2).

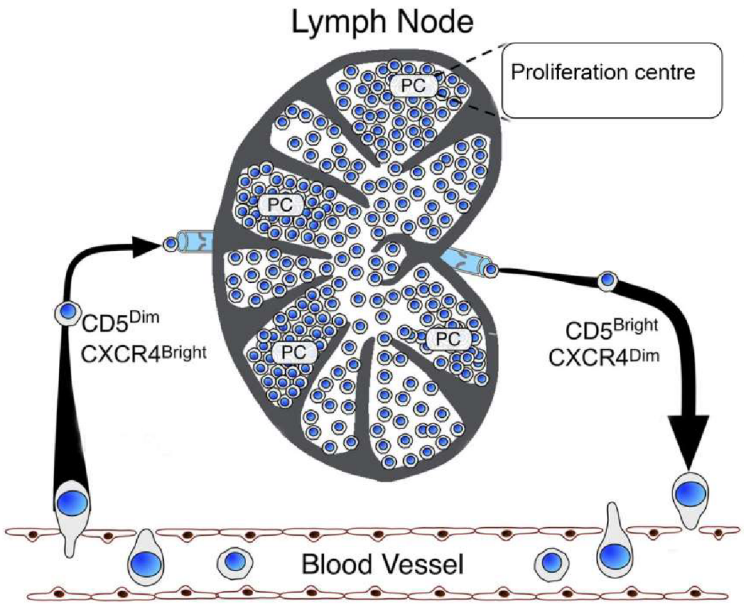


Figure 2: CLL cells resident in LN or PB can be distinguished according to levels of CD5 and CXCR4 molecules expressed on their surface (Cui *et al.*, 2014; edited).

Furthermore, interactions between CLL cells and the microenvironment also contribute to higher progression of the malignancy, and this is one of the reasons why, despite initially good responses to chemoimmunotherapy, CLL remains incurable. Similarly to other malignancies of mature B cells, CLL is a highly heterogenous disease, differing from cases not requiring any treatment to cases which eventually undergo so-called Richter's transformation and subsequently develop an aggressive lymphoma, e.g. diffuse large B cell lymphoma (DLBCL) or Hodgkin lymphoma. This transformation can also be partially induced by therapeutic pressure during CLL treatment (Rossi *et al.*, 2018).

Abnormal organisation of BCR is one of the main characteristics of CLL cells. In contrast to normal B lymphocytes, CLL cells express only a limited repertoire of immunoglobulin heavy chain variable (IGV_H) regions. Presence of somatic mutations in IGV_H region distinguishes more aggressive subtype of CLL while unmutated IGV_H is typical for CLL of more indolent phenotype (Coscia *et al.*, 2011; Hamblin *et al.*, 1999; Kröber *et al.*, 2002). Moreover, since the process of somatic mutations takes place in germinal centres (GC) of lymphoid organs,

it is possible to consider pre-GC naïve lymphocytes or GC-independent memory B cells as precursors of CLL cells with unmutated IGV_H. CLL with mutated IGV_H is on the other hand believed to develop from post-GC B lymphocytes (Seifert *et al.*, 2012). However, the cell of origin of CLL has still not been certainly identified.

Additionally, CLL cells with unmutated IGV_H manifest increased responsiveness to BCR stimulation. Contrarily, a phenomenon of BCR anergy – a state of lowered BCR responsiveness to antigen – is often a characteristic of CLL with mutated IGV_H (Mockridge *et al.*, 2007).

Besides IGV_H mutations, expression of ζ-chain-associated protein kinase 70 (ZAP70) is yet another prognostic factor in CLL. This kinase functions as a signal transducer involved in the BCR pathway in malignant as well as in normal activated B cells. Thus, $ZAP70^+$ cells are more responsive to BCR stimulation. Interestingly, cells with IGV_H mutations typically do not express ZAP70 while lack of mutations in IGV_H is linked with positivity for ZAP70 (Chen *et al.*, 2002; Rassenti *et al.*, 2004; Wiestner *et al.*, 2003).

The most frequent chromosomal alteration in CLL is (del)(13q14), with (del)(11q), (del)(17p) and trisomy of chromosome 12 being other common aberrations (Döhner *et al.*, 2000). The (del)(13q14) affects *miR-15a/16-1* locus, leading to the decreased level of these miRNAs typical for CLL cells. Moreover, genes for other two non-coding RNAs – *DLEU1* and *DLEU2* – are also located in this chromosomal area (Migliazza *et al.*, 2001; Calin *et al.*, 2002). The (del)(17p) is linked with disruption of the *TP53* gene while the (del)(11q) triggers ATM, a serine/threonine kinase controlling the presence of DNA damage. Interestingly, the (del)(13q14) is more often detected in CLL with mutated IGV_H, in contrast to the other frequent aberrations (Bosch and Dalla-Favera, 2019). Besides chromosomal mutations, single gene mutations also represent prognostic factors of CLL. For instance, mutations of *NOTCH1*, an oncogene and important transcription regulator, are linked with poor prognosis (Fabbri *et al.*, 2011). Some of these mentioned diagnostic and prognostic markers are shown in Figure 3.

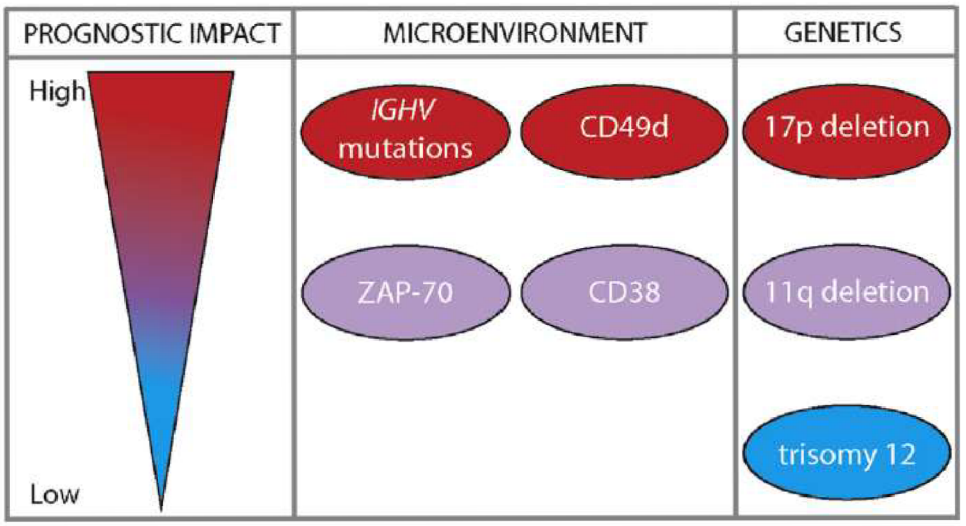


Figure 3: The most frequently detected prognostic factors of CLL and their level of prognostic impact (Dal Bo *et al*., 2014). While some of the factors were described earlier, role of CD49d and CD38 will be described later.

3 Lymphoid organs microenvironment

Besides B lymphocytes, other immune and non-immune cells – T cells, mononuclear cells or stromal cells – can be found inside of a lymphoid organ, contributing to the balance of its microenvironment (Figure 4). Signals involved in creation of such lymphoid milieu include both soluble cytokines released by present cells and signalling molecules expressed on the surface of the cells. These ligands then trigger various cellular pathways and events in B cells in order to maintain their survival and immunological functions. Moreover, stimuli from the microenvironment also affect other occurring cell types. The protective role of microenvironmental signals can be also utilised by malignant B cells, thus leading to their increased proliferation, survival, or drug resistance. Understanding the microenvironmental interactions therefore represents an important step in revealing the layers of complex biology of B cell malignancies, and it can also provide us with new possible therapeutic targets.

This chapter describes cellular composition of lymphoid microenvironment with relevance to CLL.

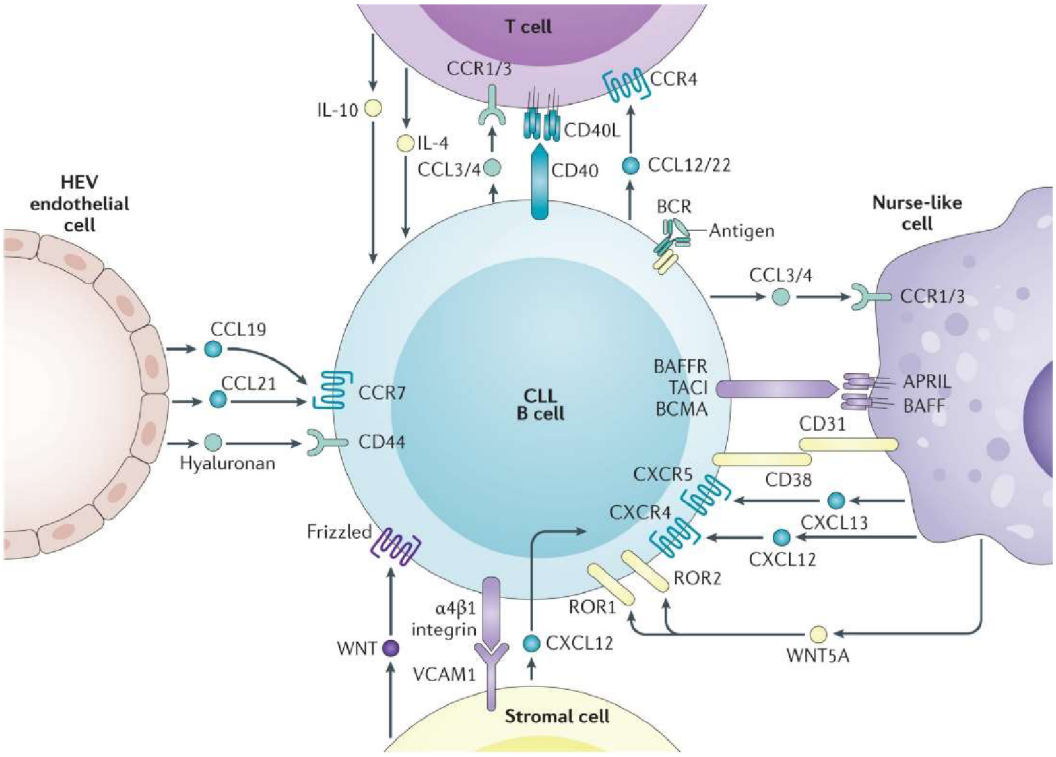


Figure 4: Scheme of CLL microenvironment (Kipps *et al.*, 2017).

3.1 T cells

The presence of all T cell subsets within the lymphoid microenvironment is crucial for the activation of normal B lymphocytes and antigen presentation. Besides their physical involvement in the immunological synapsis, T lymphocytes also produce various cytokines and interleukins (ILs) that support survival and proliferation of B cells. Some of these molecules are even key factors for the survival of CLL cells. Moreover, production of a specific type of IL together with expression of typical CD molecules are factors allowing the division of T cells into multiple subsets.

3.1.1 CD8+ T cells

$CD8^+$ T lymphocytes represent the key element of cellular immunity. These cells are also known as cytotoxic T cells due to their ability to kill cells infected with pathogens. Thus, $CD8^+$ T lymphocytes have, to some extent, the potential to recognise and destroy cancer cells.

However, the phenomenon of so-called $CD8^+$ T cell exhaustion frequently occurs in infections as well as in malignancies including CLL. Under such conditions, $CD8^+$ cells express changed spectrum of cytokines and surface receptors, leading to lowered responsiveness to antigenic stimulation, or to limited proliferation (Wherry *et al.*, 2003; Zajac *et al.*, 1998). This can be followed by metabolic changes of $CD8^+$ T lymphocytes or even by a decrease in their number. For instance, the hypo-responsive state of T cells in CLL is linked with increased expression of inhibitory receptors programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM3). Induction of these two molecules has been shown to be influenced by malignant B lymphocytes since increased levels of their ligands can be detected in the leukemic microenvironment (Siska *et al.*, 2016).

Furthermore, besides impaired proliferation, Taghiloo *et al.* (2017) reported defects in cytotoxic abilities of $CD8^+$ T cells in CLL, illustrated by lowered degranulation and production of pro-inflammatory cytokines. Additionally, the number of exhausted $CD8^+$ T lymphocytes was increased significantly in CLL when compared to non-malignant microenvironment. Such abundance of exhausted $CD8^+$ T cells corresponded with further disease progression.

3.1.2 $CD4^+$ T cells

The most important task of $CD4^+$ T cells, also known as helper T cells (T_h cells), is the regulation of immune response. This is provided by the release of various cytokines or expression of surface signalling molecules. Based on characteristically produced cytokines and on the impact various $CD4^+$ lymphocytes have on their surrounding cells, several subsets of $CD4^+$ T cells can be distinguished. Specifically, T_h1, T_h2 and T_h17 subgroups of T_h cells are recognised based on ILs they typically produce – interferon-gamma (IFN-γ), IL-4 and IL-17, respectively (Mosmann and Coffman, 1989; Park *et al.*, 2005) (Figure 5).

An enrichment of T_h cells is a frequent phenomenon in CLL LNs since various cytokines and other molecules produced by distinct subsets of $CD4^+$ T cells function as important anti-apoptotic and pro-proliferative signals for CLL cells. This has been confirmed repeatedly with IL-4 and CD40 ligand (CD40L), two of the most substantial cytokines produced by T_h cells in the context of CLL microenvironment. Stimulation of CLL lymphocytes with CD40L was linked with upregulation of the anti-apoptotic protein B-cell lymphoma-extra large (Bcl-X_L) levels. Likewise, CD40L and IL-4 stimulation of CLL cells causes an increase in the level of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), and similar trend was detected with the expression of other pro-survival proteins, e.g. induced myeloid leukemia cell differentiation protein (Mcl-1) (Dancescu *et al.*, 1992; Kitada *et al.*, 1999).

Moreover, Pascutti *et al.* (2013) discovered that exposition of CLL cells to CD40L combined with IL-21 promotes their antigen-independent expansion. This phenomenon is connected with changed gene expression similar to the one detected in CLL cells cocultured with autologous T cells. Combination of IL-4 and CD40L also supported proliferation, although to a lower extent.

Additionally, Ghia *et al.* (2002) noticed that one of the molecules increased upon CD40L stimulation of CLL cells is CCL22, an attractor of activated T_h1 and T_h2 cells. Since these $CD4^+$ T cells produce CD40L, this subsequently contributes to the existence of a positive feedback loop between CLL and T_h cells. On the other hand, no CCL22 induction was detected in media from unstimulated CLL cells.

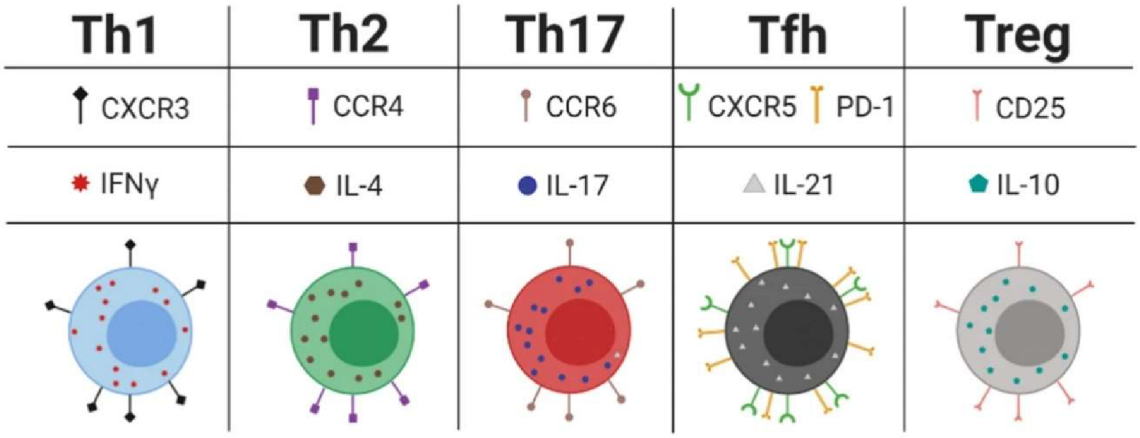


Figure 5: Subsets of $CD4^+$ T cells present within CLL microenvironment and their typical surface markers and produced cytokines (Roessner and Seiffert, 2020; edited).

3.1.3 Follicular helper T cells

The most abundant compartment of $CD4^+$ T cells in lymphoid organs consists of follicular helper T cells (T_{fh} cells). T_{fh} cells are usually located within lymphoid follicles and their presence has a positive impact on proliferation or activation of healthy B cells as well as on immunoglobulin production (Breitfeld *et al.*, 2000). Localisation of T_{fh} cells to follicles is mediated by expression of CXCR5 on their surface. CXCR5 is the receptor of CXCL13, a chemokine produced extensively by CLL cells, aside from other microenvironmental cells (Sivina *et al.*, 2021).

Besides IL-4 or CD40L, T_{fh} cells express IL-21, and combination of IL-4 and IL-21 was observed to even increase the T_{fh} cells-mediated proliferation (Ahearne *et al.*, 2013; Pascutti *et al.*, 2013). Contrarily, IL-21 alone can trigger apoptosis of CLL cells via activation of signalling pathway with caspases 3 and 8 as its final members. Such observation illustrates the importance of the balance between pro-survival and pro-apoptotic signals within the CLL microenvironment (De Totero *et al.*, 2006).

3.1.4 Regulatory T cells

While the most general definition is that regulatory T cells (T_{reg} cells) comprise a subset of $CD4^+$ lymphocytes, some $CD8^+$ lymphocytes can also be labelled as T_{reg} cells (Cosmi *et al.*, 2003). T_{reg} cells generally are of immunosuppressive character and they therefore contribute to maintenance of homeostasis. More specifically, T_{reg} cells play an important role in the context

of immunological self-tolerance (Zhao *et al.*, 2006). Limited functions of T_{reg} cells can therefore be of benefit for cancer cells within the malignant microenvironment.

The surface marker distinguishing T_{reg} cells is the IL-2 receptor CD25. Exposition to IL-2 is necessary for development and activation of T_{reg} cells (Sakaguchi *et al.*, 1995; Taams *et al.*, 2001). Furthermore, expression of forkhead box protein P3 (FOXP3) transcription factor is also typical for T_{reg} cells (Hori *et al.*, 2003).

Interestingly, multiple reports show an abundance of T_{reg} cells in CLL microenvironment when compared to healthy controls. This abundance corresponds with staging or progression of the disease (D'Arena *et al.*, 2011, Weiss *et al.*, 2011). Moreover, the amount of T_{reg} cells was shown to decrease following the treatment with the chemotherapeutic fludarabine (Beyer *et al.*, 2005).

Remarkably, Jadidi-Nigaragh *et al.* (2013) reported the capability of T_{reg} cells to inhibit proliferation of autologous B cells as well as malignant B lymphocytes or $CD4^+ CD25^-$ effector T cells in the context of CLL microenvironment. Under such conditions, malignant B cells thus probably develop some form of resistance to such T_{reg} cells-mediated inhibition.

3.2 Stromal cells

Stromal cells (SCs) mediate cellular contact inside of the lymphoid follicle as well as distribution of signalling molecules through the various types of extracellular matrix. They are therefore involved in the maintenance of supportive environment for both healthy and malignant B cells located in follicles.

The key chemokines produced by divergent subtypes of SCs in the context of lymphoid microenvironment are CXCL13 and CXCL12. There are specific receptors for CXCL12 and CXCL13 on the surface of B lymphocytes – CXCR5 and CXCR4, respectively. Interestingly, CLL cells circulating in PB have significantly increased CXCR4 expression compared to LN resident cells. This is possibly caused by CXCL12 dose-dependent endocytosis of CXCR4 which has been detected subsequently to CXCL12 binding (Burger *et al.*, 1999). Such observation implicates the significance of CXCL12 in the homing and migration of CLL cells. Similar results were obtained by López-Giral *et al.* (2004) who studied the expression of CXCR5 on CLL cells and their attraction to CXCL13. Furthermore, both cytokines support the survival of CLL cells – activated CXCR4 and CXCR5 can both subsequently trigger the anti-apoptotic extracellular signal-regulated kinase 1/2 (Erk 1/2) pathway (Burger *et al.*, 2005; Bürkle *et al.*, 2007).

In the context of microenvironmental interactions, fibroblastic reticular cells and follicular dendritic cells together with mesenchymal stromal cells represent the three most important subsets of SCs. Furthermore, due to BM infiltration of CLL cells, the protective role of bone marrow stromal cells (BMSCs) is also studied.

3.2.1 Fibroblastic reticular cells

Fibroblastic (also called follicular) reticular cells (FRCs) are located in secondary follicles, outside of the zone of proliferating B cells. Here, FRCs regulate the formation of the network of collagenous reticular extracellular fibres which, besides its mechanistic function, are also important for the transport of cytokines or other leukocytes (Sixt *et al.*, 2005). Accordingly, FRCs morphologically resemble fibroblasts from other tissues. The extracellular network created by FRCs contributes to the formation of immunological synapsis or antigen recognition by effector immune cells (Blonska *et al.*, 2015).

Additionally, signalling molecules produced by FRCs have the potential of triggering migration and homing of both healthy and malignant B lymphocytes – CCL19 and CCL21, activating the CCR7 receptor, represent just one such example (López-Giral *et al.*, 2004). CCR7 is usually considered to be the main orchestrator of B cell homing into LNs. Remarkably, higher expression of CCR7 correlates with ZAP70 positivity of CLL cells (Richardson *et al.*, 2006). Moreover, ZAP70 can even increase CCR7 expression, thus contributing to increased migration of CLL cells (Calpe *et al.*, 2011).

3.2.2 Follicular dendritic cells

Follicular dendritic cells (FDCs) can be found within primary lymphoid follicles as well as in GCs of secondary lymphoid organs. Here, FDCs represent the prevailing population of antigen-presenting cells. However, FDCs are also crucial for the development of GCs and their typical follicular structuralisation. This is linked with the production of CXCL13 provided by FDCs. Thus, CXCL13 signalling could in context of CLL contribute to formation of proliferation centres and pseudofollicles (Ansel *et al.*, 2000).

Furthermore, FDCs express the surface molecule vascular cell adhesion protein 1 (VCAM-1) which is recognised by very late antigen 4 (VLA-4) – a heterodimer consisting of α_4 and β_1 integrins. Other important surface adhesion molecule of FDCs is ICAM-1 which is

recognised by lymphocyte function-associated antigen 1 (LFA-1). This receptor consists of $\alpha_L\beta_2$ integrins (Koopman *et al.*, 1991).

Besides the previously mentioned CXCL13, IL-15 or B-cell activating factor (BAFF) are other two examples of cytokines generated by FDCs. While BAFF is a molecule with anti-apoptotic effect on B cells, IL-15 has a pro-proliferative character (Park *et al.*, 2004; Thompson *et al.*, 2000).

3.2.3 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are sometimes also referred to as mesenchymal stem cells – this is due to the pluripotency these cells maintain for example in the context of BM. Bone marrow mesenchymal stromal cells (BM MSCs) have the potential to differentiate into mesodermal cells such as fibroblastic cells, adipocytes or osteoblasts (Krebsbach *et al.*, 1999). Under the circumstances of B cell malignancies, MSCs support cancer cells not only via differentiation into other kinds of supportive cells – even non-differentiated MSCs contribute to the forming of favourable lymphoid microenvironment.

The pro-survival role of MSCs was also observed in CLL (Ding *et al.*, 2009). Co-culture with BM MSCs prevented both spontaneous and chemotherapy-induced death of leukemic cells. Such protection of CLL cells also correlated with increased levels of anti-apoptotic proteins Mcl-1 or XIAP. Moreover, those authors describe the reciprocal relationship of MSCs and malignant lymphocytes – activation of Erk 1/2 and protein kinase B (Akt) in MSCs was detected. Such effect of malignant lymphocytes was shown to be both cell contact-dependent and contact-independent.

3.3 Mononuclear cells

The myeloid lineage represents the second linage of cells differentiating from haematopoietic stem cells. Cells of this linage eventually develop into granulocytes or mononuclear cells (MCs).

MCs are further divided in two groups – monocytes (also referred to as peripheral blood mononuclear cells, PBMCs) and tissue macrophages. Macrophages are of importance for malignancies in general – in the context of tumour microenvironment they are also called tumour-associated macrophages (TAMs). Historically, two groups of TAMs have been established – M1 or M2. M1 macrophages show mostly anti-tumoral phenotype whereas the M2 subset

contributes to support of cancer cells. Presence of cells resembling M2 macrophages in CLL LNs is therefore not surprising. Moreover, cytokines and other soluble factors present in CLL serum have the potential to drive differentiation of monocytes into M2 macrophages (Van Attekum *et al.*, 2017).

The main function of MCs in general is phagocytosis. Besides this, MCs also regulate immune reactions as well as survival and growth of B cells. Production of soluble CD14 by MCs is one of such pro-survival signals and its generation can be increased by the presence of CLL cells. One possible mechanism behind CD14-mediated CLL cells protection from apoptosis is induced activity of the pro-survival nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Seiffert *et al.*, 2010).

In addition, Reinart *et al.* (2013) observed, in a mouse model of CLL, the anti-apoptotic effect of macrophage migration inhibitory factor (MIF). Levels of this chemokine are elevated in plasma of CLL patients and even malignant cells themselves have increased production of MIF. Moreover, production of MIF by CLL cells was found to reciprocally improve motility and quantity of macrophages. Pro-survival pathways Akt and MAPK were determined to be the main targets of MIF-induced signalling in CLL cells. Furthermore, when Galletti *et al.* (2016) eliminated MCs in CLL mice models, it led to up-regulation of pro-apoptotic proteins Bid, Bax, Fas or caspase 3. Eradication of macrophages thus caused higher rate of apoptosis of CLL cells.

3.4 Nurse-like cells

It has been revealed that under the influence of CLL cells, blood monocytes can evolve into M2 macrophages-resembling cells of a distinct phenotype – nurse-like cells (NLCs). They were given such name due to their functional analogy with thymic nurse cells. NLCs attract neoplastic cells and support their survival or proliferation in a contact-dependent way. Such mentioned features are analogical to functions of TAMs in solid tumours.

NLCs were first discovered by Burger *et al.* (2000). They demonstrated that separation of NCLs from a long-term culture of CLL PBMCs leads to declined viability of primary CLL cells. Furthermore, production of CXCL12 by NLCs was confirmed, and usage of a CXCL12 antibody reverted the anti-apoptotic protection of CLL cells provided by NLCs.

Bhattacharya *et* al. (2011) confirmed that NLCs are specific for CLL microenvironment. While $CD14^+$ PBMCs co-cultured with CLL B cells developed a character of NLCs, PBMCs co-cultured with non-malignant B cells did not resemble NLCs.

Despite originating from MC precursors, expression profile of proteins in NLCs differs from other MCs. For instance, NLCs manifest reduced expression of CD14 compared to other $CD14^+$ cells (Tsukada *et al*., 2002). Nishio *et al*. (2005) reported that, in addition to CXCL12, BAFF and a proliferation-inducing ligand (APRIL) can be considered typical products of NLCs. The pro-survival character of these molecules is in line with the supportive role of NLCs. Moreover, co-culture of CLL cells with NLCs lead to induced level of Mcl-1 in malignant cells, and this up-regulation was at least partially linked with increased BAFF and APRIL signalling.

4 Integrin β1

As mentioned earlier, stromal cells represent an important component of the lymphoid microenvironment. One of the signalling molecules expressed on their surface is VCAM-1 which is subsequently recognised by the $\alpha_4\beta_1$ integrin heterodimer present on B cells. This interaction leads to activation of intracellular signalling pathways influencing survival or adhesion. Similarly to other proteins, expression of these surface receptors can be influenced by miRNAs. Remarkably, integrin β_1 (ITGβ1) is a potential target gene of *miR-29* confirmed by the TargetScan database. ITGβ1 has shown the most significant and reproducible anticorrelation with *miR-29* in our experiments identifying novel *miR-29* targets. Thus, this chapter briefly describes the role of ITGβ1 in CLL and its impact on course of the malignancy.

ITGβ1 is a surface protein from the group of integrins. To form a functional surface receptor, it associates with various α integrins. There are 18 α and 8 β integrin subunits, together forming 24 variants of integrin heterodimer receptors (Figure 6). Each of these heterodimers has diverse affinity to distinct elements of the extracellular matrix (ECM) (Hynes, 2002). Out of the 24 possible subunits combinations, ITGβ1 is involved as the β chain in 12 of them. Integrin receptors represent one type of cell adhesion molecules – they are involved in cellular adhesion, connecting cytoskeleton with different components of ECM. Moreover, they also participate in cell-cell interactions.

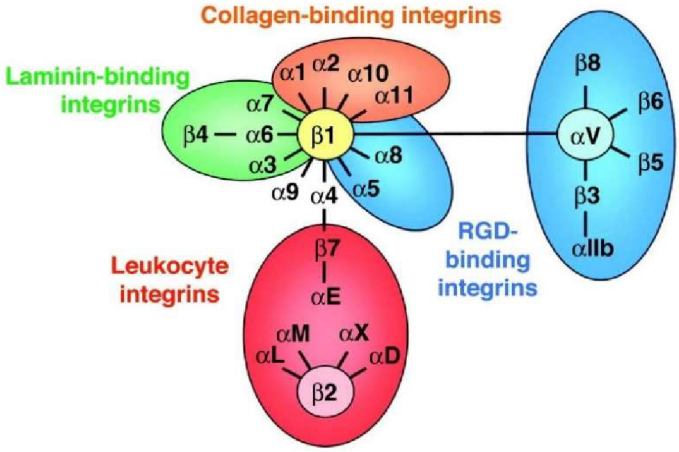


Figure 6: Combinations of α and β integrin subunits present in mammals. Typical ligands of each group of integrin heterodimers are also described (Margadant *et al.*, 2011; edited).

In general, every integrin chain consists of a large extracellular domain, a transmembrane segment and a short cytoplasmic signal-transducing domain. Subsequently to the interaction between integrins and ECM, the cytoplasmic domain triggers various signalling pathways regulating cell survival, proliferation or migration (Hynes, 1987). Such extracellular signals are

further transduced with contribution of intracellular proteins or kinases associated with integrins: while talin mediates the outside-in signals to components of cytoskeleton (in this case actin), focal adhesion kinase (FAK) or integrin-linked kinase (ILK) activate various intracellular signalling cascades (Hannigan *et* al., 1996; Mitra and Schlaepfer, 2006).

Besides this so-called outside-in signalling, integrins also participate in inside-out signalling (Figure 7). Inside-out signalling leads to modifications of integrin extracellular structure and interactions, subsequently modulating cell adhesion. These integrin alterations are consequences of various cellular signals cascade emerging in reaction to distinguished stimuli, e.g. grow factors (Hynes, 1992).

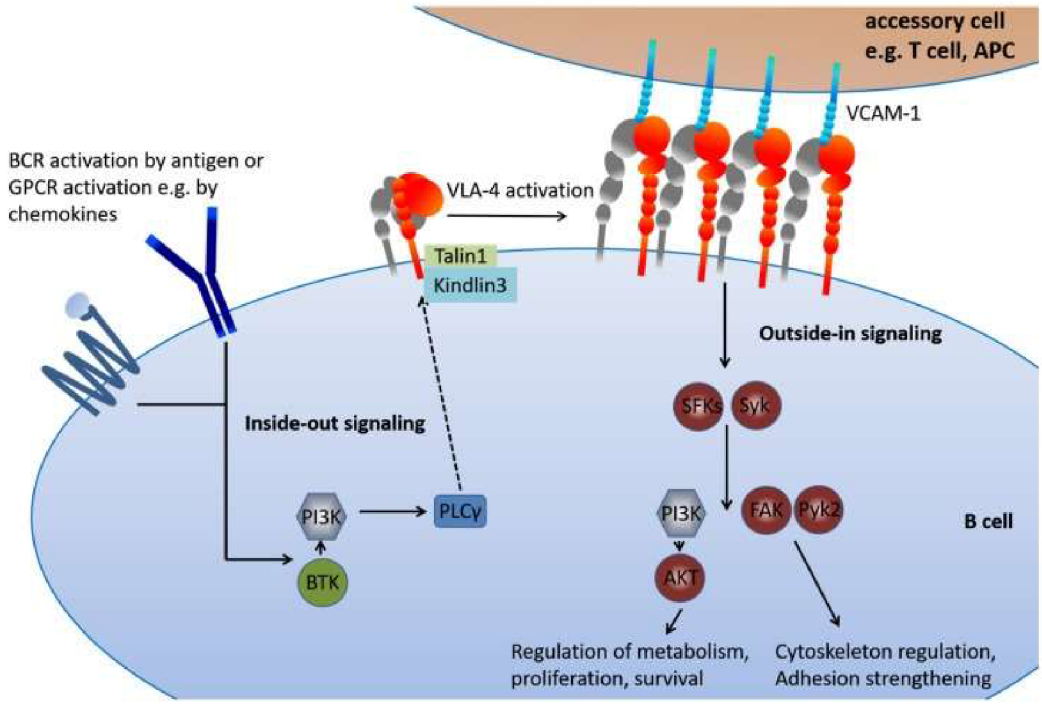


Figure 7: The two possible mechanisms of integrin signalling – outside-in and inside-out signalling. Some of the members of cellular pathways influenced by integrin triggering are showed as well (Härzschel *et al*., 2020).

Typical ligands of the ITGβ1 integrin heterodimers are components of the ECM which contain the arginine-glycine-aspartic acid motif (RGD) – fibronectin, laminin or collagen, as well as surface molecules of SCs (e.g. VCAM-1 expressed on FDCs).

Distinctive combinations of the α and β chains are characteristic for various tissues and organs where these receptors influence tissue integrity or cell motility, migration and adhesion, as well as the already mentioned intracellular cascades controlling cell proliferation or survival. Thus, deregulated expression of integrins is frequently observed in malignancies. Increased levels of integrins may contribute to heightened invasiveness (Klemke *et* al., 2007; Thibaudeau

et al., 2014), tumour angiogenesis, or resistance to apoptosis as well as to treatment (Berrazouane *et al.*, 2019; Guha *et al.*, 2019; Yang *et al.*, 2018).

4.1 ITGβ1 in CLL

In the context of CLL and NHLs, the heterodimer of $\alpha_4\beta_1$ integrins is the most important receptor containing ITGβ1. This structure is also known as VLA-4. In the CD nomenclature, the α_4 subunit is marked as CD49d while β_1 molecule is labelled as CD29. Interestingly, while CLL cells adhere to fibronectin, it is not the case of collagen or laminin which are ligands of other integrins (Vincent *et al.*, 1996). This illustrates the significance of $\alpha_4\beta_1$ integrin for CLL cells migration.

The fact that ITGβ1 contributes to survival of CLL cells has already been postulated by Lagneaux *et al.* in 1999. Those authors observed that inhibition of surface markers containing ITGβ1 as well as integrin β_2 chains leads to apoptosis despite the presence of SCs. Moreover, when de la Fuente *et al.* (1999) cultured primary CLL cells on fibronectin the percentage of apoptotic cells was significantly lower, even when compared to co-culture of the cells with pro-survival cytokines IL-4 and IL-6. Similar results were obtained after CLL cells were cultured on VCAM-1-coated wells. Next, levels of the anti-apoptotic protein Bcl-2 and pro-apoptotic Bax were determined in the cells cultured on fibronectin. Importantly, while levels of Bcl-2 decreased with time of culture without fibronectin, the decrease was only moderate – if any – in CLL cells on fibronectin. Expectedly, expression of Bax showed exactly opposite trend, with an increase in control cells and decrease in co-cultured cells. Thus, an elevated ratio of Bcl-2/Bax levels corresponded with higher survival of cells cultured on fibronectin (de la Fuente *et al.*, 1999).

Additionally, the involvement of $\alpha_4\beta_1$ integrin complex in the adhesion and motility of both non-malignant and CLL lymphocytes was tested by Lee *et al.* (2001). They further investigated the mechanism of CLL cells adhesiveness to SCs and later reported that blocking of α_4 and β_1 integrins with respective antibodies inhibited the adhesion. In contrast, antibodies against lymphocyte function-associated antigen 1 (LFA-1) consisting of α_L and β_2 integrins did not alter the adhesiveness to SCs. This study thus emphasised the significance of VLA-4 in CLL cells adhesion to stroma.

Moreover, the motility of CLL cells and their adhesion and migration through the endothelium, processes important for homing into tissues, are also dependent on the $\alpha_4\beta_1$ heterodimer. Furthermore, Maffei *et al.* (2012) observed the pro-survival character of CLL cells

adhesion to endothelial cells. Usage of CD49d or CD29 antibody reduced survival of CLL lymphocytes cultured in conditioned media from HUVEC endothelial cells. In line with this, presence of endothelial cells rescued CLL cells from fludarabine-induced apoptosis.

Furthermore, Bulian *et al.* (2014) pointed out that VLA-4 – or more specifically its CD49d subunit – has the potential to be the strongest flow cytometry-based predictor of overall survival (OS) as well as treatment-free survival (TFS) for CLL. Patients with CLL cells positive for CD49d generally had worse disease progression and positivity for CD49d correlated with other prognostic markers such as ZAP70 expression or unmutated IGH_V.

The prognostic value of VLA-4 is also of significance for the study done by Zucchetto *et al.* (2012). They demonstrated a constitutive association between VLA-4 and CD38. CD38 is a transmembrane molecule of enzymatic character. In the context of CLL CD38 can, besides $\alpha_4\beta_1$ integrin complex, couple with the 190-kDa isoform of CD44 (also known as CD44v) and gelatinase B/matrix metalloproteinase 9 (MMP-9) (Redondo-Muñoz *et al.*, 2008). Interestingly, the CD44v isoform is not present in non-CLL B cells. Moreover, these authors revealed that the VLA-4-CD44 complex binds the soluble form of MMP-9 – proMMP-9 – and that this interaction contributes to proMMP-9 activation. MMP-9 then participates, via its proteolytic activity, in the migration of CLL cells through endothelium or basal membranes. In addition, siRNA knockdown of ITGβ1 or CD44 reduced the amount of surface-bound proMMP-9 on CLL cells while the levels of secreted soluble proMMP-9 increased. Existence of this membrane-located macromolecular complex was further confirmed by Buggins *et al.* (2011).

The significance of the interaction between (pro)MMP-9 and the $\alpha_4\beta_1$-CD44v membrane complex for CLL cells is important not only for migration of malignant lymphocytes – a link between proMMP-9 binding to VLA-4 and cell viability has been reported by Redondo-Muñoz *et al.* (2010). Adhesion of primary CLL cells to plates coated with proMMP-9 resulted in considerable increase in viability, in contrast to cells treated only with bovine serum albumin (BSA). Usage of soluble proMMP-9 brought analogous results. Moreover, treatment of the cells with antibodies against $\alpha_4\beta_1$ integrins and CD44 interfered with adhesion to proMMP-9 and limited its anti-apoptotic effect. Importantly, upregulation of Mcl-1 was detected in samples cultured on proMMP-9. Furthermore, Redondo-Muñoz *et al.* discovered that blocking of Lyn kinase lead to decreased viability of CLL cells cultured on proMMP-9. Moreover, the proMMP-9-$\alpha_4\beta_1$ interaction causes phosphorylation of Lyn together with its dissociation from the β_1 integrin subunit. Thus, when Lyn silencing was performed, cellular adhesion was not affected, this intervention was however followed by induced apoptosis and Mcl-1 downregulation. To get the full picture, the authors have characterised signal transducer and activator of

transcription 3 (STAT3) as the signal transducer between Lyn activation and Mcl-1 upregulation.

Binding of VLA-4 to VCAM-1, another ligand of ITGβ1, also increases the viability of CLL cells. However, Redondo-Muñoz *et al.* (2010) revealed that the signalling pathways causing such increased survival are different from these related to (pro)MMP-9 ligation. Phosphorylation of Akt but not Lyn was detected following adhesion to VCAM-1. Subsequently, this intracellular cascade results in upregulation of the anti-apoptotic protein Bcl-X_L while levels of Mcl-1 were not affected. Thus, while binding of both (pro)MMP-9 and VCAM-1 to $\alpha_4\beta_1$ integrins causes increased survival, it is realised by distinct mechanisms (Redondo-Muñoz *et al.*, 2010).

Besides VCAM-1, the PI3K/Akt pathway is too activated by CLL cells adhesion to fibronectin. Next step of this signalling cascade is the activation of NF-κB pathway. In addition, blockage of PI3K/Akt pathway impaired the transendothelial migration of CLL cells, process regulated by MMP-9 as described earlier (Redondo-Muñoz *et al.*, 2006).

4.2 ITGβ1 in CLL resistance to treatment

As has been already mentioned, VLA-4 interactions with the tumour microenvironment contribute to resistance to various drugs. This is also the case of CLL, where the inhibitory effects of different therapeutics can lead to increased activation of the pro-survival pathways activated by VLA-4 signalling. CLL cells can thus acquire so-called microenvironment-mediated resistance.

Spaargaren *et al.* (2003) reported that BCR stimulation induces adhesion of avian B cells to VCAM-1 and fibronectin. Moreover, this adhesion was shown to be dependent on presence of functional Bruton's tyrosine kinase (Btk), a kinase critical for signalling downstream of BCR. These findings are of significance for the targeting of VLA-4 in CLL since inhibitors of Btk and other members of BCR pathway are extensively used in the treatment of CLL.

For instance, many studies on changes in adhesion of post-therapy CLL cells were performed using the Btk inhibitor ibrutinib. Herman *et al.* (2015) observed that CLL cells from patients treated with ibrutinib have impaired adhesion to fibronectin as well as to SCs. Moreover, ibrutinib contributed to mild detachment of malignant lymphocytes from fibronectin. This is connected with a modest decrease in expression of molecules mediating the adhesion – integrins $\alpha_4\beta_1$ and CD44. Blocking of VLA-4 with the ITGβ1 antagonist firategrast did not further reduce the adhesion decreased after ibrutinib. This illustrates that the mechanism behind

reduced adhesion is only partially mediated by the adhesive molecules, and that the BCR pathway is another important regulator of this process.

Furthermore, de Rooij *et al.* (2012) reported that the mentioned BCR stimulation-mediated increase in adhesion can be almost completely abolished by ibrutinib. On the other hand, stimulation of CLL cells with phorbol-12-myristate-13-acetate (PMA) to induce adhesion was also performed. Since this process bypasses Btk, adhesion of PMA-stimulated CLL cells was not influenced by ibrutinib.

Besides ibrutinib, other inhibitors of kinases are also being used in CLL therapy. Fiorcari *et al.* (2013) discovered that treatment with the PI3K inhibitor idelalisib interferes with adhesion of CLL cells to endothelial cells or VCAM-1. Moreover, while VLA-4 expression was colinear with adhesion to endothelial cells, this trend was disrupted by idelalisib. The drug was also shown to repress phosphorylation of Akt which otherwise follows VLA-4 interaction with endothelial cells.

5 MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules, 20-23 nucleotides in length. They represent one type of important post-transcriptional regulators and epigenetic modulators. MiRNAs regulate gene expression by binding to the 3‘ untranslated region (UTR) of a complementary mRNA (Wightman *et al.*, 1991). So-called seed sequence of the miRNA is responsible for this 3’ UTR binding. Seed sequence consists of nucleotides 2-7 or 2-8 at the 5’ end of miRNA (Brennecke *et al.*, 2005). Based on the level of complementarity between mRNA 3’ UTR and miRNA seed sequence, mRNA translation can be inhibited either by mRNA degradation or repression of translation (Bagga *et al.*, 2005; Lee *et al.*, 1993). One miRNA can target large number of mRNAs and similarly, one mRNA can be recognised by multiple miRNAs (Lim *et* al., 2005). In the human genome, there are currently more than 2500 miRNAs identified (Hammond, 2015).

The first miRNA, *lin-4*, was described by Lee *et al.* in 1993 as a posttranscriptional regulator of *Caenorhabditis elegans* (*C. elegans*) larval development. During the following years, presence of miRNAs has been described in majority of animal species as well as in plants. They regulate various cellular functions, including cell survival, proliferation, adhesion, or cellular communication.

Due to the initial discovery that *lin-4* action can influence development of *C. elegans,* some of the first studies on miRNAs were already focusing on the role of these molecules in the context of not only development but also various illnesses. Calin *et al.* (2002) pioneered in demonstrating a link between miRNA function and development of cancer. They observed that two miRNAs, *miR-15* and *miR-16*, are encoded by loci which are frequently deleted in CLL cells. Down-regulation of *miR-15* and *miR-16* is therefore typical for CLL cells, causing overexpression of their target genes which are of oncogenic character. Aberrant expression of miRNAs has been so far observed in almost every type of cancer. Besides reduced expression typical for tumour suppressors, miRNAs can also be of oncogenic character, causing downregulation of their target proteins.

5.1 MiRNA biogenesis

MiRNA biogenesis is a multistep process taking place in both the nucleus and cytoplasm (Figure 8). During the process, the initial miRNA precursor known as primary miRNA (pri-miRNA) is modified into mature miRNA which can subsequently regulate target mRNA

translation. The pri-miRNA is a result of miRNA gene transcription implemented by RNA polymerase II/III (Borchert *et al.*, 2006; Lee *et al.*, 2004). While one pri-miRNA can be a transcript of one gene, it can also be subsequently modified into multiple mature miRNAs (Winter *et al.*, 2009). Pri-miRNA has the typical hairpin structure with 5' and 3' overhangs (Lagos-Quintana *et al.*, 2001).

Co-transcriptionally, pri-miRNA cleavage is realised by the protein complex known as microprocessor. Microprocessor consists of the Drosha endonuclease and the double-stranded RNA-binding DiGeorge syndrome critical region gene 8 protein (DGCR8). DGCR8 navigates Drosha to one of the pri-miRNA ends where Drosha cleaves the structure 11 base pairs (bp) from the loop. Drosha cleaving results into new stem-loop structure – a pre-miRNA, 60-70 nucleotides in length (Lee *et al.*, 2003). Pre-miRNA is subsequently recognised by the complex of nuclear transport receptor Exportin-5 and Ran-GTP, and translocated into cytoplasm (Lund *et al.*, 2004).

In the cytoplasm, another cleavage of pre-miRNA occurs, mediated by the endonuclease Dicer. Dicer removes the loop, leaving a double-stranded, 20-23 bp miRNA duplex with 2 nucleotides overhangs at 3' ends. This duplex is then unwound into the guide strand, representing the mature miRNA, and passenger strand (miRNA*), which is usually degraded (Schwarz *et al.*, 2003).

The guide strand is subsequently loaded into RNA-induced silencing complex (RISC). RISC comprises Dicer together with protein kinase RNA activator (PACT) and transactivating response RNA-binding protein (TRBP). Argonaute protein 2 (Ago2), protein from the Ago family, is also involved in RISC. RISC with loaded miRNA is then navigated to the target mRNA and translation interference is realised. When the complementarity between 3' UTR and miRNA seed sequence is perfect, the target mRNA is cut, and this cleaving is catalysed by Ago2 (Zeng and Cullen, 2003; Liu *et al.*, 2004). In case of imperfect complementarity, inhibition of mRNA translation is based on interaction between RISC and translation initiation factors. Furthermore, presence of the 5' cap and more importantly 3' polyA is also important for miRNA-mediated inhibition of translation. It has been observed that mRNA deadenylation, followed by decapping and subsequent mRNA decay, is yet another mechanism of translational silencing (Behm-Ansmant *et al.*, 2006; Humphreys *et al.*, 2005).

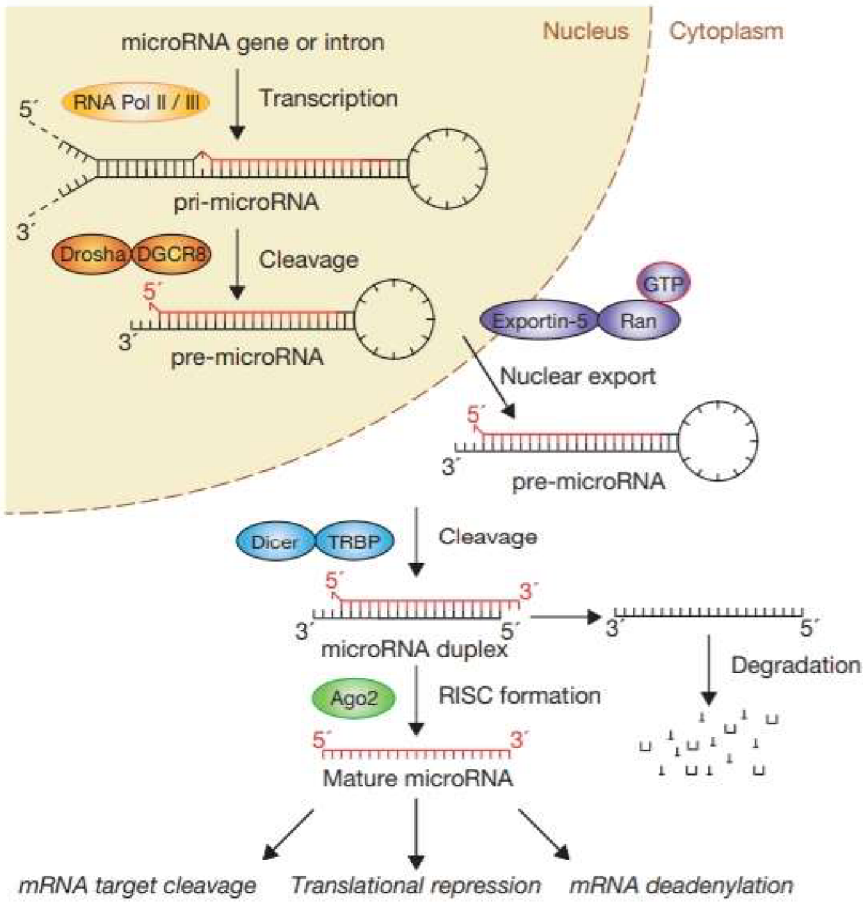


Figure 8: Scheme of miRNA biogenesis (Winter *et al*., 2009).

5.2 *miR-29* in B cell malignancies

miR-29 is a miRNA family consisting of three members – *miR-29a*, *miR-29b* and *miR-29c*. These miRNAs are encoded by gene clusters located on two chromosomes – while the *miR-29a*/*b1* can be found at 7q32.3, *miR-29c*/*b2* cluster is located at 1q32.2. Thus, mature *miR-29b* can develop from two pre-miRNAs (*pre-miR-29b1* and *pre-miR-29b2*) encoded by different loci. Nevertheless, all mature miRNAs of the family have similar stem structures and target the same sequences (Yang *et al*., 2015). In cancer in general, *miR-29* is usually considered a miRNA of tumour suppressing character – its downregulation is linked with increased levels of target proteins which are often anti-apoptotic or pro-proliferative. Altered levels of *miR-29* can be the result of chromosomal aberrations or mutations occurring in its loci. However, various cell signalling cascades can also influence *miR-29* expression.

In malignant B cells, the relationship between *miR-29* family and the BCR pathway is of relevance. Specifically, *miR-29* has been shown to be negatively regulated by the oncogene *Myc*, transcription factor and a regulator of cell proliferation and growth. *Myc* is often

deregulated in B cell malignancies. Importantly, activation of BCR signalling in CLL cells is followed by induction of Myc levels and subsequent *miR-29* downregulation (Sharma *et al*., 2021). Furthermore, these authors postulated that such decrease in *miR-29* levels results in up-regulation of tumour-necrosis factor receptor-associated factor 4 (TRAF4). TRAF4 has been shown to negatively regulate responsiveness to microenvironmental CD40L. Reduced CD40 signalling can then be linked with lowered activity of its downstream targets – components of the NF-κB pathway (Figure 9).

In addition, multiple Myc binding sites were detected in both *miR-29a*/*b1* and *miR-29c*/*b2* promoter regions in cells of the P493-6 lymphoblastoid cell line. Correspondingly, knockdown of *Myc* in lymphoma cell lines resulted in *miR-29* upregulation (Zhang *et al*., 2012).

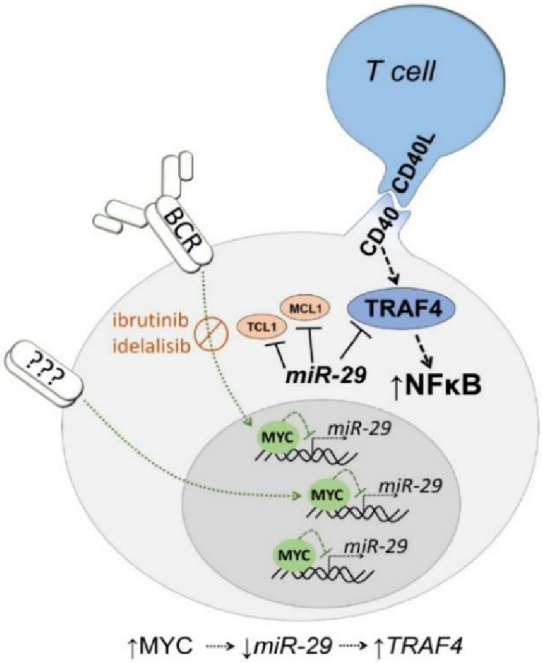


Figure 9: Schematic illustration of the BCR-Myc-*miR-29*-TRAF4-NFκB interaction in CLL cells (Sharma *et al*., 2021; edited).

Furthermore, the Myc-mediated *miR-29* downregulation can be realised only via Myc recruitment of histone deacetylase 3 (HDAC3) and polycomb repressing complex 2 (PRC2), two epigenetic modulators important for gene silencing (Figure 10). Knockdown of HDAC3 and enhancer of zeste homolog 2 (EZH2) – a member of PRC2 – averted the Myc-mediated *miR-29* silencing, further confirming the importance of the cooperation between Myc and the two regulators. The link between Myc and *miR-29* downregulation has been again shown by Mazzoccoli *et al*. (2018) in Burkitt lymphoma (BL) cells.

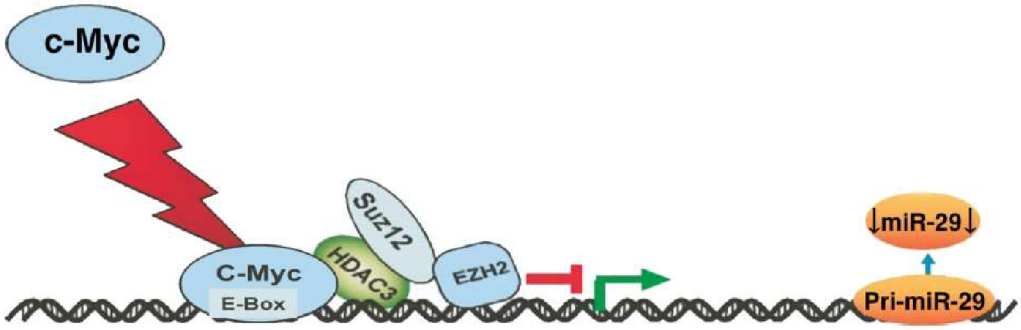


Figure 10: Indirect downregulation of miR-29 via Myc-mediated induction in PRC2 expression as proposed by Zhang *et al*. (2012). Edited.

Another mechanism of *miR-29* involvement in the BCR pathway is its regulation of Akt, kinase functioning downstream of BCR and involved in the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. Transfection of BL cell lines with *miR-29* mimic was followed by reduced levels of both total and phosphorylated Akt, confirming the relationship between the two molecules (Mazzoccoli *et al*., 2018). Furthermore, *miR-29* also affects the Akt pathway indirectly, by binding mRNA of the *Tcl-1* oncogene, a known co-activator of Akt (Laine *et al*., 2000).

Remarkably, in malignant B cells, *miR-29* has been shown to trigger mRNAs of multiple known oncogenes or genes linked with cell survival and proliferation. Additionally, some important epigenetic regulators are also influenced by *miR-29*. In CLL, the link between *miR-29* and Tcl-1 expression was confirmed by Pekarsky *et al*. (2006). Similarly, levels of Mcl-1 are also directly negatively influenced by *miR-29* expression (Mazzoccoli *et al*., 2018; Sharma *et al*., 2021).

6 Aims of the thesis

- Analysis of sequencing data from CXCR4/CD5 subpopulations and search for possible targets of *miR-29*.
- Study of selected *miR-29* targets in the context of CLL and its microenvironment, for better understanding of its overall role in the malignancy.
- Validation of the interaction between *miR-29* and selected genes in cell lines by various methods and approaches.
- Confirmation of *miR-29* targeting of the selected genes in primary CLL cells.

7 Material and methods

7.1 Cell lines

Overall, two CLL cell lines (HG-3 and MEC-1) and one NHL cell line (SU-DHL-4) were used for experimental work. Conditions of cell lines cultivation are described in Table 1. The IMDM and RPMI 1640 media (both Biosera, United Kingdom) used for cell cultivation were enriched to contain 10 % of fetal bovine serum (FBS; Biosera, United Kingdom) with penicillin/streptomycin antibiotics (Sigma-Aldrich, USA).

Cell line	Cell type	Medium	Passaging	Conditions of cultivation
MEC-1	chronic B cell leukemia	IMDM	every 2-3 days in 1:1 ratio (cell density 1-2×10^6 cells/ml)	37 °C, 95% humidity, 5% CO_2 atmosphere
HG-3	chronic B cell leukemia	RPMI 1640	every 2-3 days in 1:1 ratio (cell density 1-2×10^6 cells/ml)	37 °C, 95% humidity, 5% CO_2 atmosphere
SU-DHL-4	B cell lymphoma	RPMI 1640	every 2-3 days in 1:1 ratio (cell density 1-2×10^6 cells/ml)	37 °C, 95% humidity, 5% CO_2 atmosphere

Table 1: Used cell lines and conditions of cultivation.

7.2 Primary CLL cells

Primary CLL cells were isolated from peripheral blood obtained from patients diagnosed with chronic lymphocytic leukemia (C91.1). The patients have signed an informed consent form and the use of patient samples in the research has been approved by ethics committee of The University Hospital Brno. For isolation of CLL cells from peripheral blood the RosetteSep™ Human B Cell Enrichment/CD3 Depletion Cocktail kit (StemCell Technologies, Canada) was used, with subsequent separation by gradient centrifugation with the Ficoll-Paque™ medium (GE Healthcare Life Sciences, USA). Isolated CLL cells were then counted and transfected. After the transfection, cells were cultured for 48 or 72 hours in 20 % RPMI medium without antibiotics.

7.3 Transfections

Transfections of both CLL cell lines and primary cells were performed by electroporation with the Neon Transfection System Kit (Invitrogen, USA). The cells were transfected with

either MISSION® miRNA negative control, MISSION® miRNA hsa-miR-29c mimic (both Sigma-Aldrich, USA), miRCURY LNA miRNA inhibitor negative control or hsa-miR-29c miRCURY LNA miRNA inhibitor (both Qiagen, Germany). In case of cell lines, these reagents were of 500 nM and 1000 nM concentration per each condition. 5 million of cells was used for each reaction. In case of primary cells, 1000 nM concentration was used and 10 million of cells was transfected in one reaction. Additionally, monitoring of transfection efficiency was done by transfection with either 500 nM or 1000 nM siGLO Green Transfection Indicator (Horizon Discovery, USA) and subsequent detection using flow cytometry. The cells were harvested after 24 hours for siGLO and cell viability measurement (see below). For protein and RNA lysates, harvesting was typically performed 24, 48 and 72 hours after transfection.

7.4 Flow cytometry detection of cell viability, transfection efficiency and surface markers

For the determination of viability, the suspension of transfected cells in respective medium was diluted in 1:1 ratio with phosphate-buffered saline (PBS) containing 1,5 µl of 80 nM 3,3‘-Dihexyloxacarbocyanine Iodide (DIOC6; Invitrogen, USA) and 0,5 µl of 500 µg/l propidium iodide (Sigma-Aldrich, USA). The samples were incubated in dark at 37 °C/15 min. Next, the measuring was performed with the FACS Verse flow cytometer (BD Biosciences, USA).

Cells transfected with siGLO were spined down and resuspended in PBS multiple times. Afterwards, 1 µl of SYTOX™ Blue Dead Cell Stain (ThermoFisher Scientific, USA) was added to each sample and flow cytometry measurement was performed. Flow cytometry data were analysed in the FlowJo software.

Moreover, to determine the influence of *miR-29c* upregulation on selected surface markers, flow cytometry detection of these markers was performed. 250000 cells were spined several times and the supernatant was aspirated. Pellet was resuspended in 100 µl of FACS buffer (PBS with 2 % FBS and 10 mM EDTA) containing 2 µl of antibody (see Table 2). Samples were then stained for 20 minutes and 4 °C in dark. Subsequently, samples were washed with 0,5 ml of PBS and pellets were resuspended in 200 µl of FACS buffer and measured using the FACS Verse flow cytometer. Obtained results were analysed using the FlowJo software.

Name of the product	Catalogue number	Producer
APC anti-human CD29, clone TS2/16	2115035	Sony Biotechnologies, USA
BV510 anti-human CD49d, clone 9F10	2121590	Sony Biotechnologies, USA
FITC anti-human CD22, clone S-HCL-1	2417540	Sony Biotechnologies, USA
FITC anti-human CD5, clone UCHT2	2103030	Sony Biotechnologies, USA
FITC anti-human CD19, clone HIB19	2111030	Sony Biotechnologies, USA
APC anti-human CXCR4, clone 12G5	2132550	Sony Biotechnologies, USA
A-647 anti-human CD20, clone 2H7	2111590	Sony Biotechnologies, USA
A-647 anti-rabbit IgG (H+L), F(ab')2 Fragment	4414	Cell Signaling, USA

Table 2: List of used flow cytometry antibodies.

7.5 Intracellular staining

Intracellular staining is another flow cytometry method allowing visualisation of protein expression. For this method primary CLL cells were first fixed with methanol and stained for viability markers and CD5/CXCR4 expression. An aliquot of this solution containing 1 million of cells was then washed twice with cold 5 % FBS/PBS. Next, 50 µl of ITGβ1 primary antibody diluted 1:100 with 5 % FBS/PBS was added. The cells were stained for 1 hour. Afterwards, the cells were washed twice with cold 5 % FBS/PBS and stained with secondary antibody conjugated to the AlexaFluor® 647 fluorescent dye (A647, listed in Table 2). The antibody was diluted 1:500 with 5 % FBS/PBS and 100 µl of this solution was used. After 30 minutes of staining, samples were washed twice with 5 % FBS/PBS and the pellet was resuspended in 150 µl of FACS buffer. The samples were measured on the FACS Verse flow cytometer and the data were analysed using the FlowJo software.

7.6 Treatment with dactolisib

To confirm the link between the PI3K/Akt/mTOR pathway and ITGβ1 (see chapter 8.9), MEC-1 cells were treated with the dual PI3K-mTOR1/2 inhibitor dactolisib (also known as BEZ235; Selleckchem, USA). The cells were cultivated with multiple concentrations of the reagent (10 nM, 100 nM, 200 nM, 500 nM, 1000 nM) for either 5 or 24 hours. Cells treated with DMSO were used as negative control.

Moreover, to test the influence of microenvironmental molecules on the dactolisib-mediated modulation of ITGβ1 levels, the cells were cultivated with dactolisib and conditioned medium (CM) obtained from the fibroblast-like cell line HS-5 was used. Due to the adherence of HS-5 cell, the CM was obtained simply by removing medium from the cells after 2-3 days of cultivation. For this experiment, 200 nM and 1000 nM concentrations of dactolisib were used and the cell were cultivated for 24 hours. For each concentration, the cells were either cultivated with dactolisib alone, or CM was added to the cells after 4 hours of cultivation with dactolisib (Figure 11).

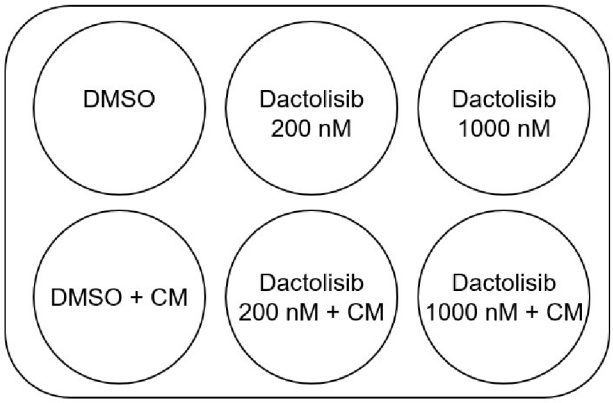


Figure 11: Scheme of the experiment with treatment of MEC-1 cells with dactolisib alone or with dactolisib and HS-5 CM.

7.7 Protein lysates preparation

Harvested cells were spined down (4°C/350 g/5 min) and resuspended in cold PBS, then spined down again (4°C/350 g/5 min). Following aspiration of PBS, cells were resuspended in lysis buffer (1% SDS, 10% glycerol, 100 mM TRIS-HCl, pH 7,4) containing inhibitors of phosphatases and proteases, both in 1:100 ratio (Sigma-Aldrich, USA). Samples were stored in -80 °C.

For the determination of protein concentration, lysates were sonicated (Hielscher, Germany) for 15 seconds and the concentration was then determined with usage of the DC Protein Assay (BIO-RAD Laboratories, USA). Subsequent measurement of absorbance was performed on the Spark® spectrophotometer (Tecan, Switzerland). Moreover, a calibration curve was prepared using standards with protein concentrations 8; 4; 2; 1; 0,5; 0,25; 0,125; 0 µg/ml. Protein concentration of samples was calculated according to the calibration curve equation. Next, loading buffer containing β-mercaptoethanol and bromphenol blue dye was added to the samples and denaturation was performed (98°C/5 min). Samples were stored in -80 °C.

7.8 Western blotting

Detection of protein levels was done by electrophoretic separation followed by immunodetection using specific antibodies. Protein electrophoresis was based on the SDS-PAGE method.

First, 10% polyacrylamide gels were prepared. The gel consists of two parts – separating gel (MiliQ H_2O, 30 % acrylamide (Serva, Germany), 1,5M TRIS (pH 8,8), 10% SDS, tetramethylenediamine (Sigma-Aldrich, USA), 10% ammonium persulfate (Sigma-Aldrich, USA) and stacking gel (all components the same as for separating gel, except of TRIS – 1M TRIS with pH 6,8 is used).

Denatured and dyed protein lysates were loaded into the wells of the gel. Electrophoretic chamber was filled with the TRIS-glycine electrophoretic buffer. The electrophoresis run for initial 15 min with voltage of 60 V, followed by 100 V applied for 90 min.

When the electrophoresis was finished, proteins from the gel were transferred to a polyvinylidene difluoride membrane (PVDF; Millopore, Germany). The wet-blot method was used for the transfer. PVDF membrane was first activated in methanol for 1 min. The transfer was done at 4 °C in TRIS-glycine blot buffer containing 20 % of methanol, and 100 V was applied for 90 min.

As the next step, membranes were blocked in 5% BSA (Biosera, United Kingdom) dissolved in wash buffer (150mM NaCl, 20mM TRIS (pH 7,4), 0,1% Tween 20) for 1 hour. Membranes were then incubated in primary antibodies (listed in Table 3) diluted with 5% BSA at 4 °C overnight. After washing with wash buffer (3-4×, 10 min for each washing), membranes were incubated in secondary antibody (listed in Table 4) diluted with 5% BSA at room temperature for 1 hour. Subsequently, membranes were washed again and then incubated for 5 min with a 1:1 mix of the enzyme and substrate contained in the Clarity Western ECL Substrate Kit

(BIO-RAD Laboratories, USA). Following the incubation, visualisation of membranes was performed using the UVITEC Alliance 4.7 machine (UVITEC Cambridge, United Kingdom).

Name of the protein	Molecular weight (kDa)	Animal of origin	Dilution with 5% BSA	Producer	Catalogue number
AKAP5	79	Rabbit	1:1000	Cell Signalling, USA	5671
p-Akt (S473)	60	Rabbit	1:2000	Cell Signalling, USA	4060
Akt	60	Mouse	1:2000	Cell Signalling, USA	2920
p-Btk (Y223)	77	Rabbit	1:2000	Cell Signalling, USA	5082
BTK	77	Rabbit	1:1000	Cell Signalling, USA	3533
CD20	33	Rabbit	1:1000	Abcam, USA	ab78237
DUSP2	32	Mouse	1:1000	Santa Cruz, USA	sc32776
p-Erk 1/2 (pT202/pY204)	44/42	Rabbit	1:2000	Cell Signalling, USA	4377
Erk 1/2	44/42	Mouse	1:2000	Cell Signalling, USA	4696
ITGB1	115, 135	Rabbit	1:1000	Cell Signalling, USA	4706
GAPDH	37	Mouse	1:1000	Cell Signalling, USA	97166
KLF4	62	Rabbit	1:1000	Cell Signalling, USA	4038
c-Myc	57-70	Rabbit	1:1000	Cell Signalling, USA	9402
PTEN	54	Rabbit	1:2000	Cell Signalling, USA	9554S
STAT3	79, 86	Mouse	1:2000	Cell Signalling, USA	9139
p-Syk (Y323)	72	Mouse	1:1000	Cell Signalling, USA	2715
Syk	72	Rabbit	1:1000	Cell Signalling, USA	12358
TRAF4	50	Rabbit	1:1000	Cell Signalling, USA	18527
Vinculin	124	Mouse	1:1000	Santa Cruz, USA	sc73614

Table 3: List of used primary antibodies.

Secondary antibody	Animal of origin	Dilution with 5% BSA	Producer	Catalogue number
anti-rabbit IgG	Goat	1:2000	Cell Signalling	7074
anti-mouse IgG	Horse	1:2000	Cell Signalling	7076

Table 4: List of used secondary antibodies.

7.9 RNA lysates preparation

The first step of RNA lysates preparation was cell lysis with the usage of TriReagent. Harvested cells were spined down (4°C/350 g/5 min) and resuspended in cold PBS, then spined down again (4°C/350 g/5 min). Following aspiration of PBS, pellets were resuspended in 1 ml of cold TriReagent (Molecular Research Centre, USA). Afterwards, 100 µl of 1-bromo-3-chloropropane (Sigma-Aldrich, USA) was added to each sample and samples were centrifuged (4 °C/12000 g/16 min). This centrifugation led to separation of the upper RNA-containing phase and bottom DNA-containing phase. On the interface of these two phases there was a ring containing proteins. RNA-containing phase was removed and translocated into new Eppendorf tube. Next, 500 µl of isopropanol and 1 µl of linear acrylamide were added to each tube and the samples were left at -20 °C overnight and then spined down (4 °C/12000 g/10 min). Aspiration of the supernatant followed, and pellet was washed with cold 75% ethanol. This step was

repeated twice and after the final aspiration the pellet was left in the box for 30 min to dry out completely. Dry pellet was then dissolved in 30 µl of nuclease-free water (NF water; Qiagen, Germany). After 15 min/4 °C isolated RNA was measured on NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA) to determine its purity and concentration.

7.10 Reverse transcription

Reverse transcription was performed to obtain cDNA of candidate genes as well as of *miR-29c*.

7.10.1 Reverse transcription of protein-coding genes mRNA

Reverse transcription of mRNA of protein-coding genes was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Reaction mix was prepared according to kit instructions – however, the final volume was reduced from 20 µl to 10 µl. RNA was first diluted with NF H_2O to 100 ng/µl and reverse transcription was performed with 250-400 ng of RNA (Table 5). Reaction was performed under conditions recommended by the producer of the kit (Table 6). When the reverse transcription was completed, 1 µl of NF H_2O per 10 ng cDNA was added to each obtained cDNA-containing mix.

Reagent	Volume (µl)
10× RT Buffer	1
10× RT Random Primers	0,4
25× dNTP Mix (100 mM)	1
MultiScribe™ Reverse Transcriptase	0,5
RNase Inhibitor	0,5
NF H_2O	1,6
Total per reaction	5

Table 5: Components of the 2× RT master mix and their respective volumes used for each reaction.

Condition	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 6: Conditions of the reverse transcription reaction according to kit instructions.

7.10.2 Reverse transcription of miRNAs

Reverse transcription of miRNAs contained in RNA isolates was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Reaction master mix was prepared to the total volume of 5 µl according to the instructions of the producer (Table 7). Isolated RNA was diluted to the concentration of 1,666 ng/ µl. 2,5 µl of the diluted RNA was combined with the master mix. Three specific sets of TaqMan primers were used for amplification of cDNAs of miR-29c and two endogenous controls – RNU 48b and RNU 38b. Thus, three types of master mix were prepared. The reverse transcription reaction was performed under the conditions recommended by the producer of the kit (Table 8).

Reagent	Volume (µl)
10× RT Buffer	0,75
5× TaqMan primer	1,5
dNTP Mix (100 mM)	0,075
MultiScribe™ Reverse Transcriptase	0,5
RNase Inhibitor	0,095
NF H_2O	2,08
Total per reaction	5

Table 7: Components of the miRNA RT master mix and their respective volumes used for each reaction.

Condition	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	16	42	85	4
Time (min)	30	30	5	∞

Table 8: Conditions of the reverse transcription reaction according to kit instructions.

7.11 Quantitative PCR

Quantitative PCR (qPCR) of the prepared cDNAs was performed with the aim to determine relative expression of *miR-29c* and mRNAs of studied genes. Each reaction was performed in total volume of 10 µl and qPCR was performed in triplicates for all the samples. Reaction mix was prepared combining Absolute qPCR ROX Mix (ThermoFisher Scientific, USA), NF H_2O and specific TaqMan probe (volumes of respective reagents can be found in Table 9). 1 µl of the diluted cDNA was then added to the mix. qPCR was performed in 384-well

plate. When the pipetting was finished, the plate was covered with adhesive foil and shortly centrifuged. The reaction was done in the ABI 7900 thermocycler (Applied Biosystems, USA), reaction conditions are listed in Table 10. Obtained results were later analysed using the QuantStudio™ 12K Flex software (Applied Biosystems, USA). For each of the triplicates, outlying Ct values were eliminated. For miRNA qPCR, *RNU 48b* and *RNU 38b* were used as endogenous controls. For protein-coding genes, *GAPDH* and *TBP* or *GAPDH* and *HPRT* genes were used as the endogenous controls.

Reagent	Volume (µl)
Absolute qPCR ROX Mix	5
NF H_2O	3,5
Specific TaqMan probe	0,5 (20× concentrated)
	0,1666667 (60× concentrated)
cDNA	1
Total per reaction	10

Table 9: Components of the qPCR reaction mix and their respective volumes. RNU 38b was 60× concentrated, the rest of the probes was 20× concentrated.

		50× repeated	
Condition	**Step 1**	**Step 2**	**Step 3**
Temperature (°C)	95	95	60
Time (min)	10	0,25	1

Table 10: Conditions of the qPCR reaction.

7.12 *miR-29c* stable overexpression

Besides transient overexpression achieved by transfection, stable overexpression of *miR-29c* was performed in HG-3 and SU-DHL-4 cell lines. For the cloning, pLKO.1 puro plasmid (Addgene, USA) was used.

The *miR-29c* stem loop sequence was inserted into the pLKO.1 puro vector using restriction enzymes AgeI and EcoRI. Restriction sites for these enzymes are located just after the U6 promoter which has been used. While the presence of resistance factor against ampicillin was used for selection of transformed bacteria, the transduced cell lines were selected utilising the puromycin resistance (Figure 12).

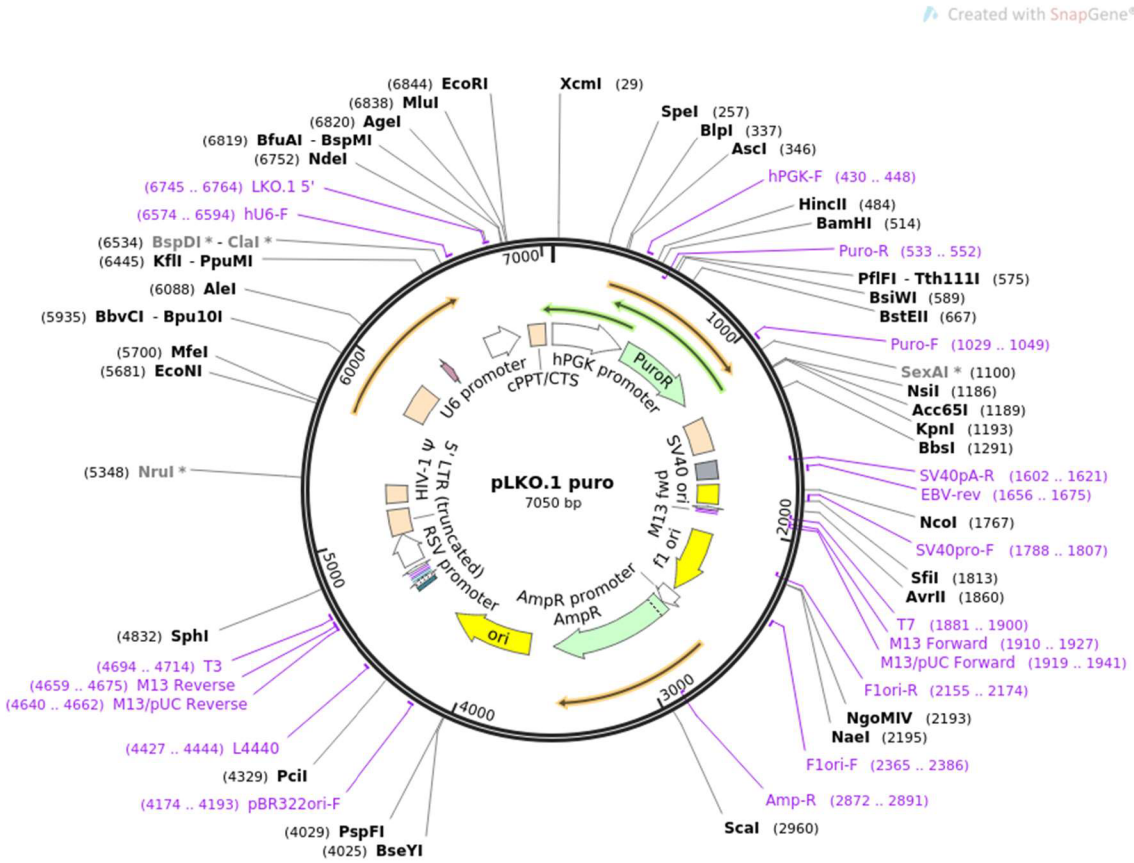


Figure 12: Map of the pLKO.1 puro plasmid backbone used for the *miR-29c* transduction.

7.12.1 Preparation of the vector plasmid for cloning

The pLKO.1 puro plasmid was prepared for cloning by digestion with AgeI and EcoRI (New England Biolabs, USA). The reagents used for the reaction are listed in Table 11. Total volume of the reaction was 60 µl. The reaction mix was incubated using thermoblock at 37° C/2 hours. The enzymes were inactivated by incubation at 80 °C/20 min. To avoid self-ligation of the digested constructs, 3 µl of shrimp alkaline phosphatase (New England Biolabs, USA) was subsequently added to dephosphorylate the 3' ends. The mix was incubated at 37 °C/40 min and afterwards the enzyme was deactivated at 65 °C/5 min. To obtain the digested plasmid, agarose gel electrophoresis was performed. 0,8% agarose gel was used and 60 V was applied for 1,5 hours. The digested plasmid fragment was then cut from the gel and extracted and purified according to the instructions of the kit QIAquick® Gel Extraction Kit (Qiagen, Germany).

Reagent	Quantity
CutSmart® Buffer (New England Biolabs, USA)	2 µl
AgeI	0,5 µl
EcoRI	0,5 µl
Vector plasmid	5 µg
NF H_2O	X µl to 20 µl of the total volume

Table 11: Components of the digestion reaction and the quantities used of each component.

7.12.2 Preparation of the insert for cloning and ligation

First, the oligonucleotides corresponding to *miR-29c* sequence (Figure 13) were annealed and phosphorylated. Components of the reaction mix are described in Table 12. The reaction mix was incubated in thermocycler – first at 37 °C/30 min, then at 95 °C/5 min and the temperature was gradually ramped down to 25 °C at 5°C/min. The annealed and phosphorylated oligonucleotides were subsequently 140× diluted with double-distilled water. Next, the ligation reaction was prepared, combining the annealed and phosphorylated oligonucleotide duplex, and the purified digested plasmid. The components of the ligation mix are listed in Table 13. The mix was incubated overnight at 16 °C and the reaction was subsequently stopped at 65 °C/10 min.

```
5´ - CCGGTGACCGATTTCTCCTGGTGTTCAGAGTCTGTTTTTGTCTAGCACCATTTGAAATCGGTTATTTTTG - 3´
5´ - AATTCAAAAATAACCGATTTCAAATGGTGCTAGACAAAAACAGACTCTGAACACCAGGAGAAATCGGTCA - 3´
```

Figure 13: Oligonucleotides containing *miR-29c* sequence and overhangs for AgeI (CCGG) and EcoRI (AATTC) restriction.

Reagent	Quantity
NF H_2O	6,5 µl
Oligo 1 (100 µM)	1 µl
Oligo 2 (100 µM)	1 µl
10× T4 Ligation Buffer (NEB, USA)	1 µl
T4 polynucleotide kinase (NEB, USA)	0,5 µl

Table 12: Components of the annealing and phosphorylation reaction. NEB stands for New England Biolabs.

Reagent	Quantity
Purified digested plasmid	100 ng
Diluted oligo duplex	1 µl
10× T4 Ligation Buffer (NEB, USA)	1 µl
T4 Ligase (NEB, USA)	1 µl
NF H2O to total volume of 10 µl	x µl

Table 13: Components of the ligation reaction. NEB stands for New England Biolabs.

7.12.3 Transformation of competent bacteria

The One Shot™ Stbl3 Chemically Competent E. coli strain (ThermoFisher Scientific, USA) was transformed with plasmid containing the inserted oligos. 5 µl of the ligation reaction mixture was added to the bacteria, followed by 30 min of incubation on ice. Subsequently heat shock was performed (42 °C/20 sec) and the cells were again incubated on ice for 3 min. Afterwards, 200-250 µl of pre-warmed 37 °C S.O.C medium (VWR, USA) was added and the cells were incubated in the thermoblock at 37°C/1 hour/225 rpm. Next, 25-100 µl of the bacterial suspension was spread into an agar plate pre-warmed to 37 °C. The plate contained ampicillin which was used for selection of transformants. The plate was incubated at 37 °C overnight. The colonies were then picked and grown overnight in 3-5 ml of LB medium with ampicillin. The cells were spined down at the maximal possible centrifugal force and plasmid isolation was performed using the Monarch® Plasmid Miniprep Kit (New England Biolabs, USA) following the kit instructions.

Next, due to laboratory workload of the virus lab, the production of lentiviral particles and transduction of HG-3 cell line was done by the supervisor. The *miR-29c*-overexpressing SU-DHL-4 cells were prepared by identical approach by Mgr. Daniel Filip.

7.13 Statistical analysis

Statistical analysis of obtained results was performed in GraphPad Prism v. 8.0.1 software using paired t-test. Results with p value <0,05 were considered statistically significant.

8 Results

8.1 Selection of candidate genes

Selection of candidate genes was based on sequencing data from the next generation sequencing (NGS) of transcriptome of primary CLL cells which has been previously performed in our lab. For this, paired samples from primary CLL cells were sorted to obtain the $CD5^{bright}CXCR4^{dim}$ subpopulation and the $CD5^{dim}CXCR4^{bright}$ subpopulation. Additionally, since one of the main attributes differing these two subpopulations is the activity of BCR pathway, another RNAseq analysis was performed with paired patient samples of primary CLL cells before and after therapy with ibrutinib. This small molecule blocks Btk activity and it is one of the most perspective BCR inhibitors. Upregulation of *miR-29* levels following exposure to ibrutinib has been observed before (Sharma *et al.*, 2021).

The $CD5^{bright}CXCR4^{dim}$ subpopulation represents CLL cells resident to LNs or those that have only recently relocated into PB. The other subgroup consists of $CD5^{dim}CXCR4^{bright}$ cells – CXCR4 positivity is one of the main attributes of PB-located CLL cells. MiRNA profiling was also performed on these two subpopulations, confirming *miR-29* downregulation in $CD5^{bright}CXCR4^{dim}$ cells (Figure 144). 7 pairs of samples were used for the miRNA profiling while mRNA profiling was performed on 10 pairs of samples.

For prediction of *miR-29* target genes the TargetScan database (Agarwal *et al.*, 2015) was searched for genes with conserved *miR-29* binding site. Our RNAseq data comparing CLL cell subpopulations were searched for *miR-29* target mRNAs (validated by TargetScan) which were anticorrelated with *miR-29* expression. These genes could be then considered potential *miR-29* targets in CLL cells.

This NGS datasets analysis brought a list of 53 *miR-29* target genes upregulated in the $CD5^{bright}CXCR4^{dim}$ subpopulation (fold change≥1,5; $p<0,05$). In addition, 413 genes showed significantly reduced expression after ibrutinib treatment (fold change≥1; $p<0,05$). Intersection of these two groups reduced the number of possible candidate genes to 11 – these genes and their brief annotations are listed in Table 14. Furthermore, although not significantly downregulated following ibrutinib treatment (fold change=1,1; $p=0,584$), *ITGβ1* has been added to the candidates group. Addition of *ITGβ1* has been done not only due to its downregulation in the $CD5^{bright}CXCR4^{dim}$ subpopulation but also for its important role in the context of CLL microenvironment. Moreover, reduced expression of *ITGβ1* after ibrutinib treatment has been previously demonstrated by others (Herman *et al.*, 2015; Zhao *et al.*, 2017). Additionally, we were

able to detect downregulation of *ITGβ1* levels in samples from MEC-1 cells treated with ibrutinib (Figure 16).

The strategy of the selection of possible candidates is summarised in Figure 15.

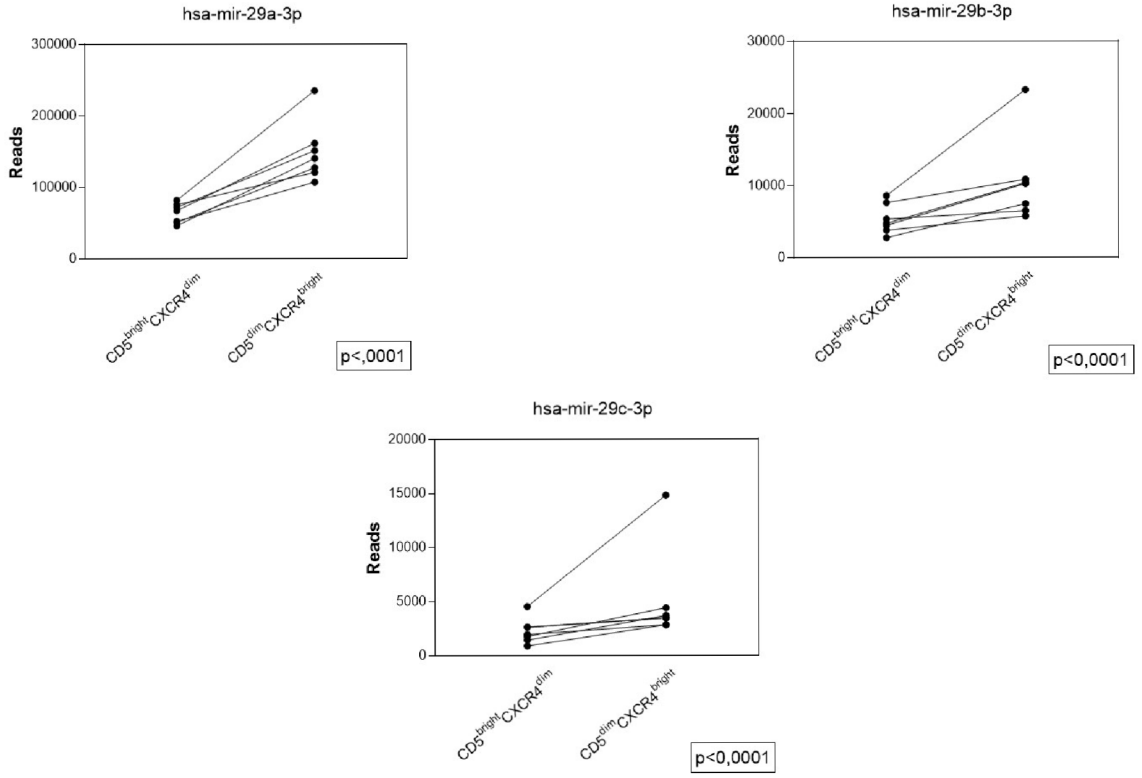


Figure 14: Differential expression of members of *miR-29* family between the $CD5^{bright}CXCR4^{dim}$ and $CD5^{dim}CXCR4^{bright}$ subpopulations sorted from CLL patient samples (7 pairs, N=14).

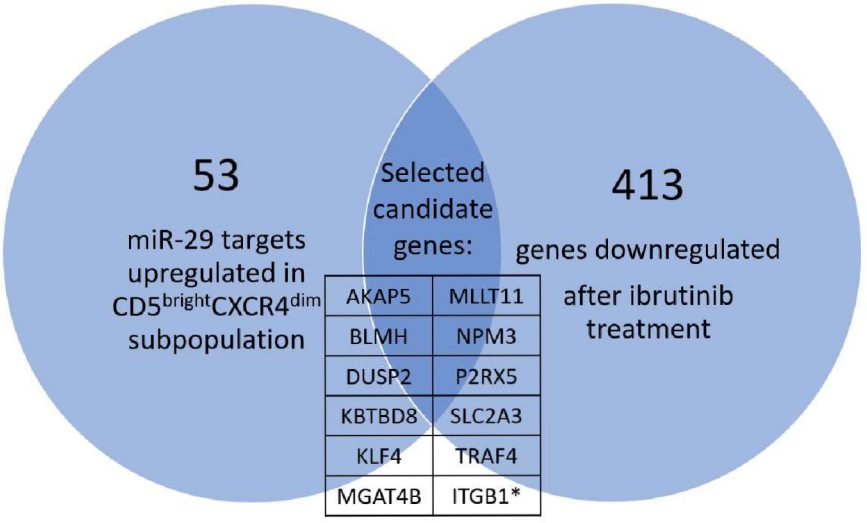


Figure 15: Strategy of the selection of possible candidate genes. *ITGβ1* is arrowed since it has not been significantly downregulated after ibrutinib treatment in our RNAseq data. The reasons why it was included in the candidates group are mentioned earlier.

Gene	Protein	Function
AKAP5	A-kinase anchor protein 5	scaffold protein involved in cAMP signalling due to its binding of PKA, PKC or calcineurin
BLMH	bleomycin hydrolase	cytoplasmic cysteine peptidase participating in bleomycin metabolism
DUSP2	dual specificity protein phosphatase 2	negative regulator of MAPK pathway
ITGB1	integrin beta 1	cell surface receptor forming an ECM-binding complex by pairing with various α integrins
KBTBD8	Kelch repeat and BTB domain-containing protein 8	ubiquitin ligase
KLF4	Krüppel-like factor 4	transcription factor regulating cell growth or proliferation
MGAT4B	alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase B	glycosyltransferase involved in sugar metabolism
MLLT11	AF1q	co-factor of the transcription factor TCF7, regulating the transition of HSCs into T cells
NPM3	nucleoplasmin-3	participating in various processes such as chromatin remodelling or rRNA biogenesis
P2RX5	P2X purinoceptor 5	ATP receptor with the character of ligand-gated cation channels
SLC2A3	solute carrier family 2, facilitated glucose transporter member 3	transmembrane tansporter of glucose and other monosaccharides
TRAF4	TNF receptor-associated factor 4	adapter protein with E3 ligase activity, interacts with Akt of NFκB pathways

Table 14: Brief annotation of genes filtered by the intersection of treated and untreated samples. The description of protein functions is based on annotations available in the UniProt database.

Since the downregulation after ibrutinib treatment was used as one of the main criteria of candidate genes selection, Western blot analysis of protein lysates from MEC-1 cells exposed to ibrutinib was performed (Figure 16). This analysis showed significant reduction in ITGβ1 levels (p=0,0079). This finding further validated our interest in *ITGβ1* as a valid candidate gene.

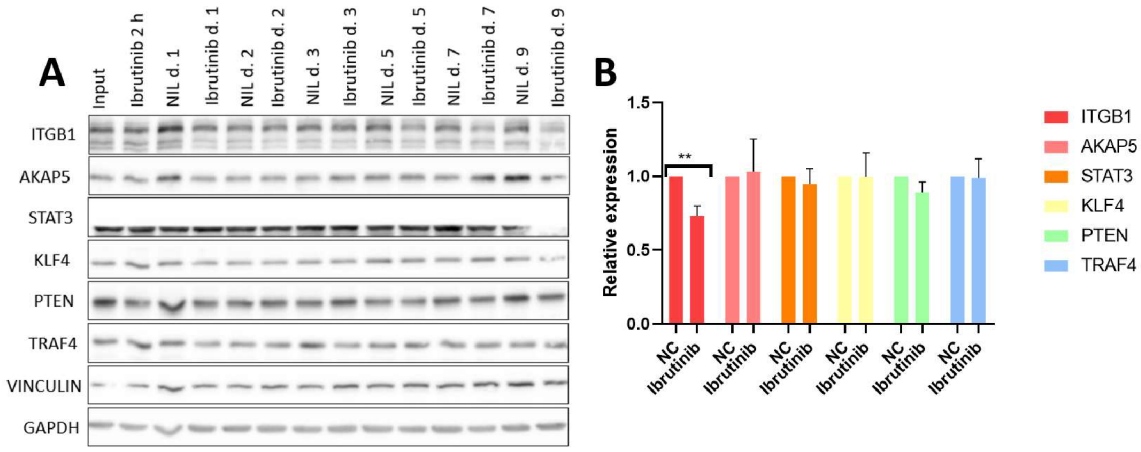


Figure 16: Levels of candidate genes in MEC-1 cells treated with ibrutinib. A. Western blot showing expression of candidate genes after multiple timepoints of exposure to ibrutinib (NIL = non-treated cells, d. 1 = day 1 after treatment). B. Normalised levels of selected candidate proteins in MEC-1 cells treated with ibrutinib.

Next part of the selection of the main candidates was literature research on the 11 enlisted genes. Possible relevance for B cell malignancies and their microenvironment has been considered. Moreover, some of the candidate genes were previously shown to be regulated by *miR-29 – DUSP2* (Li *et al*., 2009), *KLF4* (Cittelly *et al*., 2013) or *ITGβ1* (Sekiya *et al*., 2011).

This literature research resulted in a group of main candidate genes (Table 15, Figure 17). As the next step, it was possible to start with experimental validation of the relationship between these candidates and *miR-29*. Importantly, *DUSP2* has been excluded from experimental validation due to non-specificity of the antibody against the protein.

Gene name	UTR binding sites	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}
ITGB1	1 conserved	-0.36	-0.40	0.83
KLF4	1 conserved	-0.32	-0.32	0.86
AKAP5	1 conserved	-0.29	-0.30	0.93
DUSP2	1 conserved	-0.23	-0.30	0.88

Table 15: Ranking of the main candidate genes in the TargetScan database.

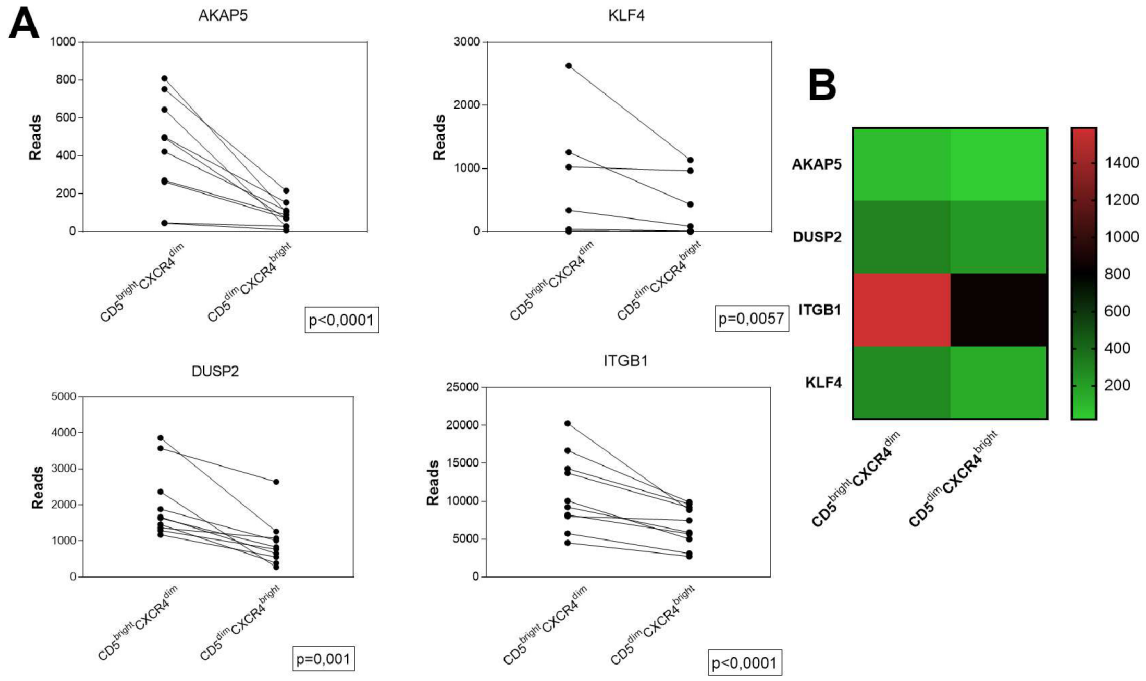


Figure 17: Changes in mRNA levels of the main candidate genes between the the $CD5^{bright}CXCR4^{dim}$ and $CD5^{dim}CXCR4^{bright}$ subpopulations. Sorted CLL patient samples were used for this analysis (10 pairs, N=20). A. Graphic depiction of RNAseq results for selected candidate genes. B. Heatmap showing changed expression of the main candidate genes between the two subpopulations. Colour gradient represents number of reads.

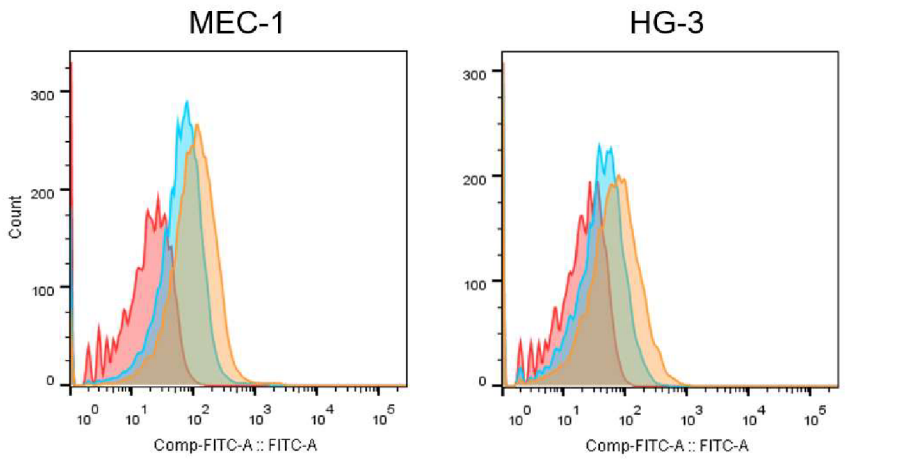
8.2 Regulation of candidate genes expression by *miR-29c* in CLL cell lines

Although regulation of some of the selected candidate genes by *miR-29* has been shown elsewhere, it has not been done in the context of CLL. To validate this interaction on protein (and subsequently also mRNA) level, transient overexpression of *miR-29c* by transfection was used as the first method. For this, various CLL cell lines were used.

The transfections were performed as mentioned earlier, using two concentrations of *miR-29c* mimic, 500 nM and 1000 nM. The cells were harvested 24, 48 and 72 hours after the transfection to obtain protein lysates for Western blot analysis. Results of Western blots were considered valid only when they matched the following criteria: consistent level of endogenous control (GAPDH/vinculin), TRAF4 downregulation after *miR-29c* overexpression, and efficiency of the transfection determined by flow cytometry siGLO detection. Transfections with efficiencies lower than 10 % for both siGLO concentrations were excluded. In general, transfection efficiencies were around 15 % for 500 nM concentration of siGLO and around 40 % for 1000 nM concentration of siGLO (Figure 18). TRAF4 has been used as the main positive control since it has been previously validated in our lab as a *miR-29* target in CLL cells (Sharma *et al.*, 2021).

Besides TRAF4, PTEN and STAT3 were also used as controls due to the fact that they have been previously confirmed as *miR-29* targets (Hines *et al.*, 2020; Li *et al.*, 2019).

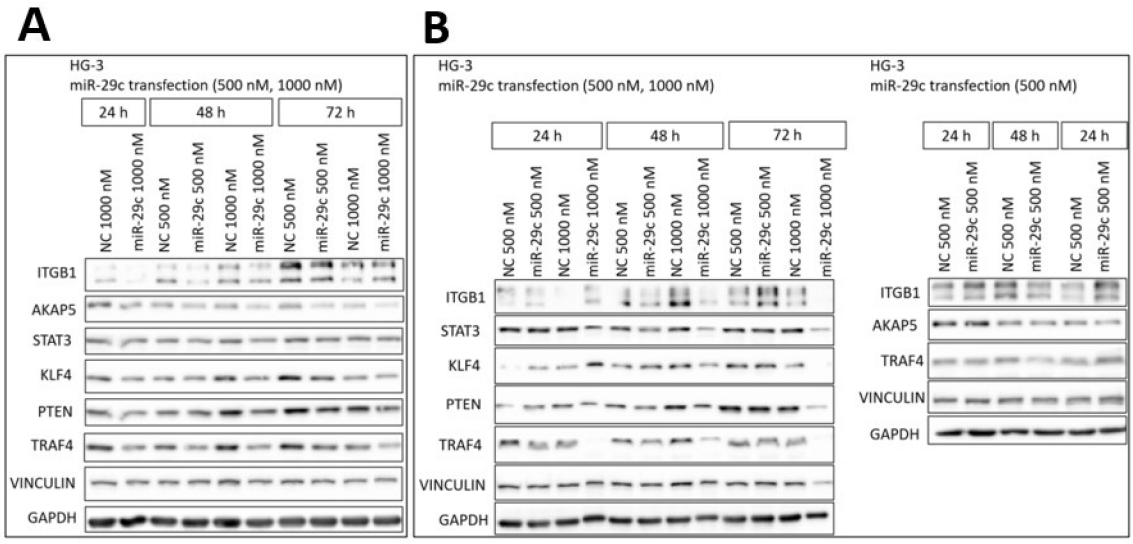
In the majority of performed Western blots, most constant results were obtained with the HG-3 cell line (Figure 19, Figure 21). Additionally, consistent downregulation of selected *miR-29c* targets was most prominent in samples harvested 48 hours after transfection. Blots from this timepoint were thus also selected for quantification and statistical analysis. When the 500 nM concentration of *miR-29c* mimic was used, statistically significant downregulation has been observed in case of *ITGβ1* (p=0,0074), *AKAP5* (p=0,0272) and *TRAF4* (p=0,0039). In case of the 1000 nM concentration, downregulation of *PTEN* (p=0,0108) and *TRAF4* (p=0,0209) was evaluated as statistically significant 48 hours after transfection (Figure 199 C).



siGLO concentration	% of positive cells
500 nM	41,2
1000 nM	65,7

siGLO concentration	% of positive cells
500 nM	16,1
1000 nM	32,6

Figure 18: Representative example of transfection efficiency determination by siGLO detection. The upper part shows fluorescence graphs obtained after data analysis in FlowJo software. The bottom part shows percentage of positive cells for both siGLO concentrations.



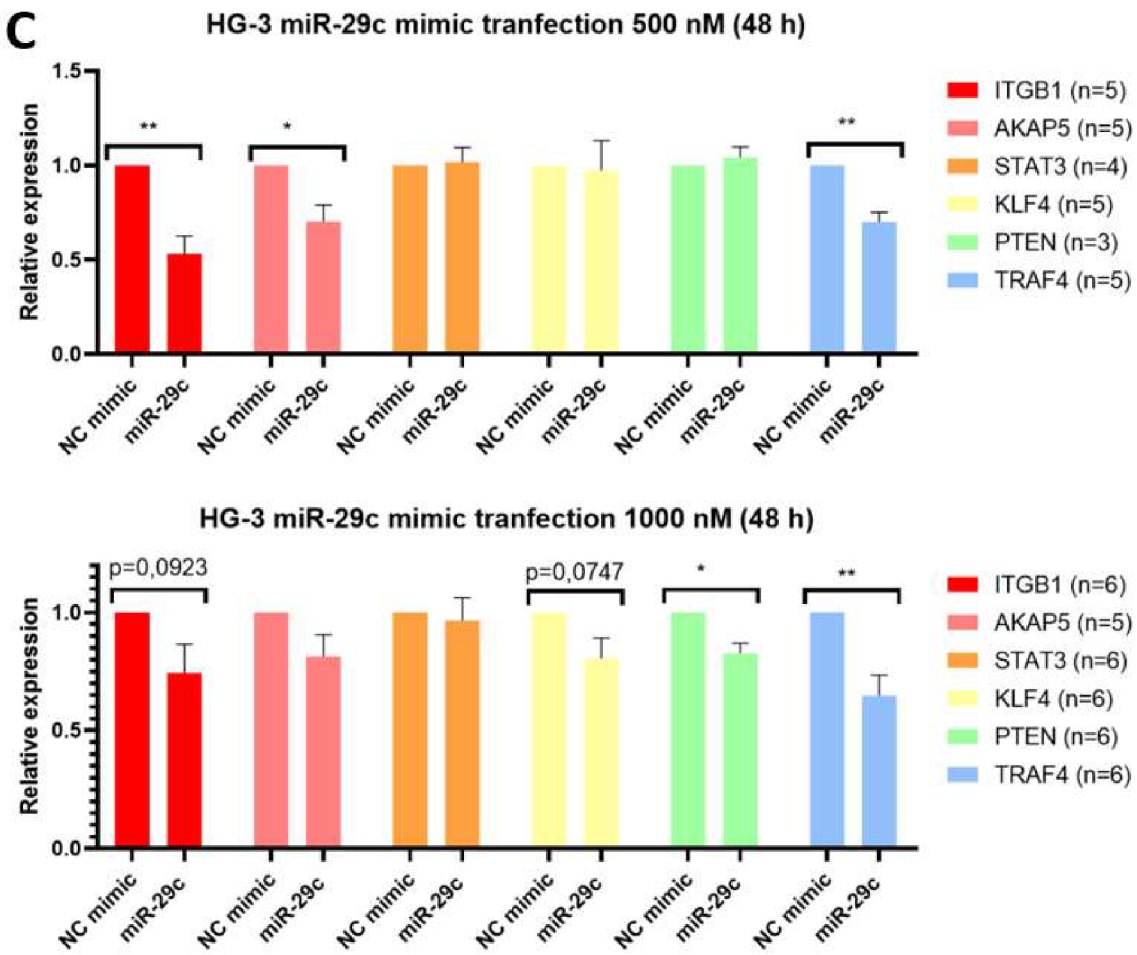


Figure 19: A, B. Scans of Western blots performed with lysates from HG-3 cells transfected with *miR-29c* mimic (500 nM and 1000 nM). The shown Western blots were selected based on the criteria mentioned earlier. C. Downregulation of candidate genes in the HG-3 cell line 48 hours after transfection with *miR-29c* mimic. Summarizing graphic depiction and statistical analysis of expression changes detected on Western blots shown in Figure 16 and Figure 21. 500 nM concentration in the upper part of the figure, 1000 nM concentration in the bottom part. NC stands for negative control.

In addition to HG-3 cells, *miR-29c* transfection of MEC-1 and SU-DHL-4 cells was also performed (Figure 20). The SU-DHL-4 cell line is of DLBCL origin. However, since CLL can progress into DLBCL during Richter's transformation, data obtained from SU-DHL-4 cells can be also valid for studies of CLL and better understanding of its progression. Results from the two cell lines further validated the expression trends observed in HG-3 cells. All the studied genes (*ITGβ1*, *STAT3*, *KLF4*, *PTEN* and *TRAF4*) were less expressed in SU-DHL-4 cells transfected with *miR-29c* mimic. In case of MEC-1 cells, downregulation of *ITGβ1*, *AKAP5*, *KLF4* and *TRAF4* was detected after *miR-29c* transfection.

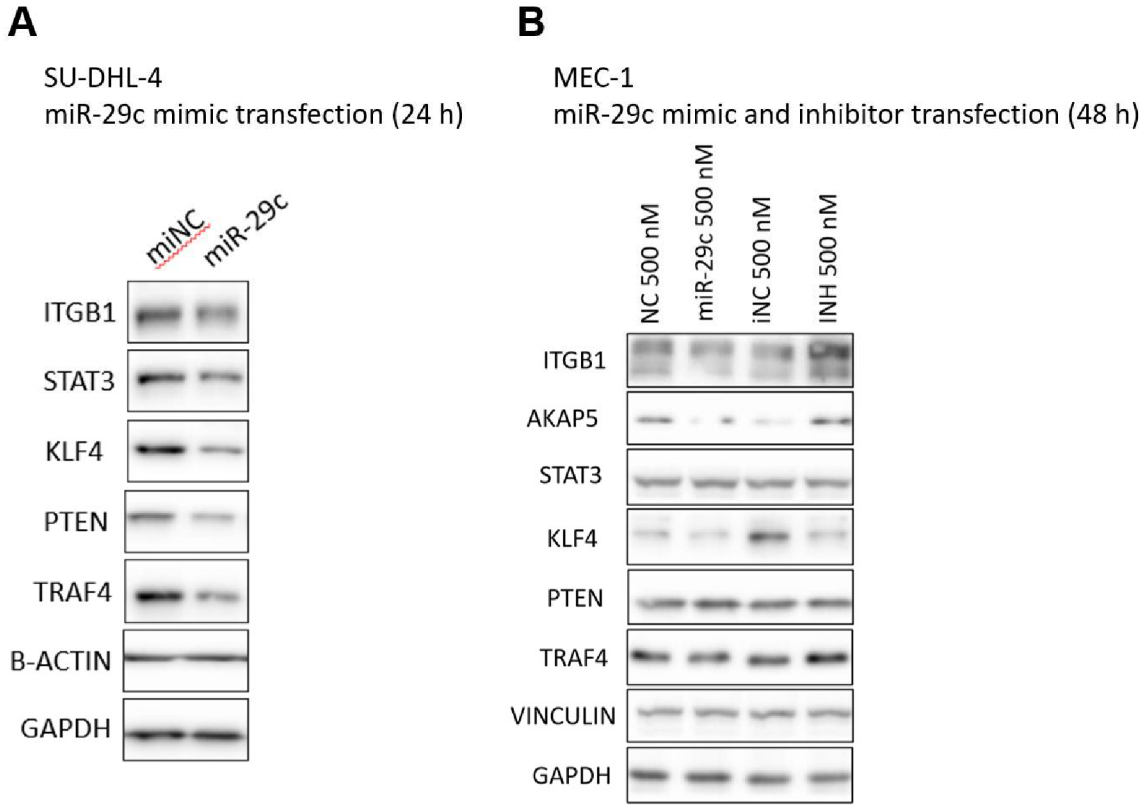


Figure 20: Expression of selected genes in SU-DHL-4 cell line and MEC-1 cell line transfected with *miR-29c* mimic or inhibitor. A. Western blot showing changed protein levels in transfected SU-DHL-4 cells. B. Western blot showing changed protein levels in transfected MEC-1 cells. iNC stands for inhibitor negative control, INH stands for inhibitor.

8.3 Inhibition of *miR-29c* leads to upregulation of candidate genes expression

Besides *miR-29c* mimic, the effect of *miR-29c* on candidate genes was analysed by transfection of CLL cell lines with *miR-29c* LNA inhibitor. This short double-stranded RNA molecule contains *miR-29c* binding site enriched with chemically modified nucleotides. Thus, mature *miR-29c* binds to *miR-29c* inhibitor molecule more strongly than to its target mRNA. Transfection of CLL cell line HG-3 with *miR-29c* inhibitor resulted in up-regulation of *ITGβ1* (p=0,3293), *AKAP5* (p=0,1704), *KLF4* (p=0,1925), *PTEN* (p= 0,3339) and *TRAF4* (p=0,1772) (Figure 21). The increase in protein level was not significant in case of any of the candidates probably due to higher standard deviation within samples. Nevertheless, since the trend is visible in the graph, a higher number of repetitions would probably increase statistical significance.

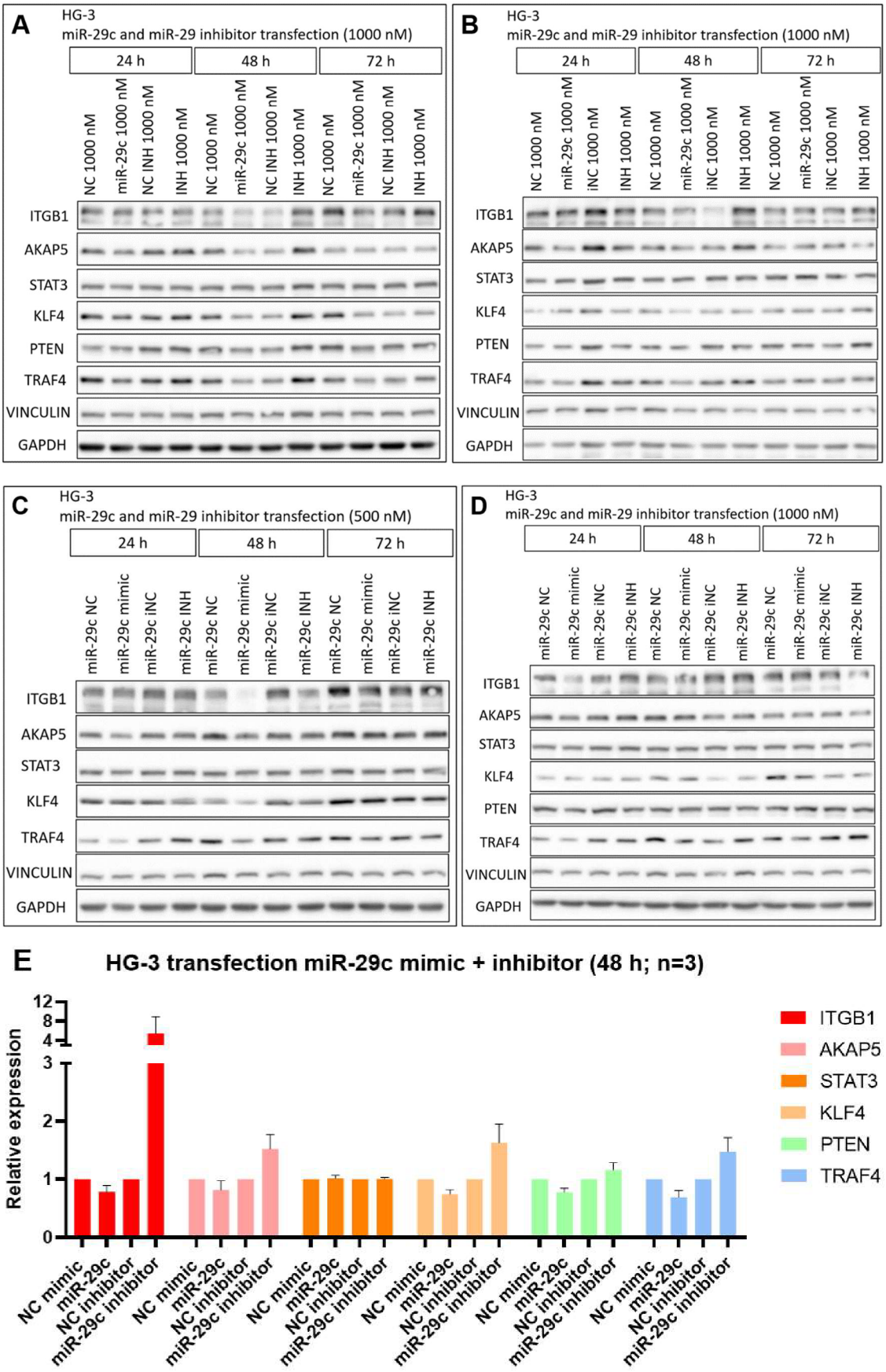


Figure 21: Inhibition of *miR-29c* leads to upregulation of selected genes. A, B, C, D. Scans of 48-hour timepoint from Western blots performed with samples from HG3 cells transfected with

500 nM or 1000 nM concentration of *miR-29c* mimic and inhibitor. E. Summarizing graphic depiction of Western blots from parts A, B and D (1000 nM *miR-29c* mimic).

8.4 qRT-PCR analysis of *miR-29c* mimic and inhibitor-transfected CLL cell lines

Expression of candidate genes following *miR-29c* mimic and inhibitor transfection of HG-3 cells was analysed not only by Western blot but also by qRT-PCR. First, changed levels of *miR-29c* in transfected cells were confirmed (Figure 22 A, B). Interestingly, while a significant increase in *miR-29c* levels was detected 24 hours after the transfection, the overexpression declined rather abruptly after 48 hours and 72 hours. The *miR-29c* inhibitor, on the other hand, did not have significant influence of *miR-29c* levels. Interestingly, similar results were observed with both 500 nM and 1000 nM concentration of mimic and inhibitor.

qRT-PCR analysis of *ITGβ1* and *TRAF4* mRNA levels post-transfection was performed as well. In general, these results were in line with expression trends observed on protein levels. When the 500 nM concentration of miR-29c mimic was used, downregulation in samples from the three timepoints was statistically significant for both *ITGβ1* (p=0,0197) and *TRAF4* (p=0,0302) (Figure 22 C, D).

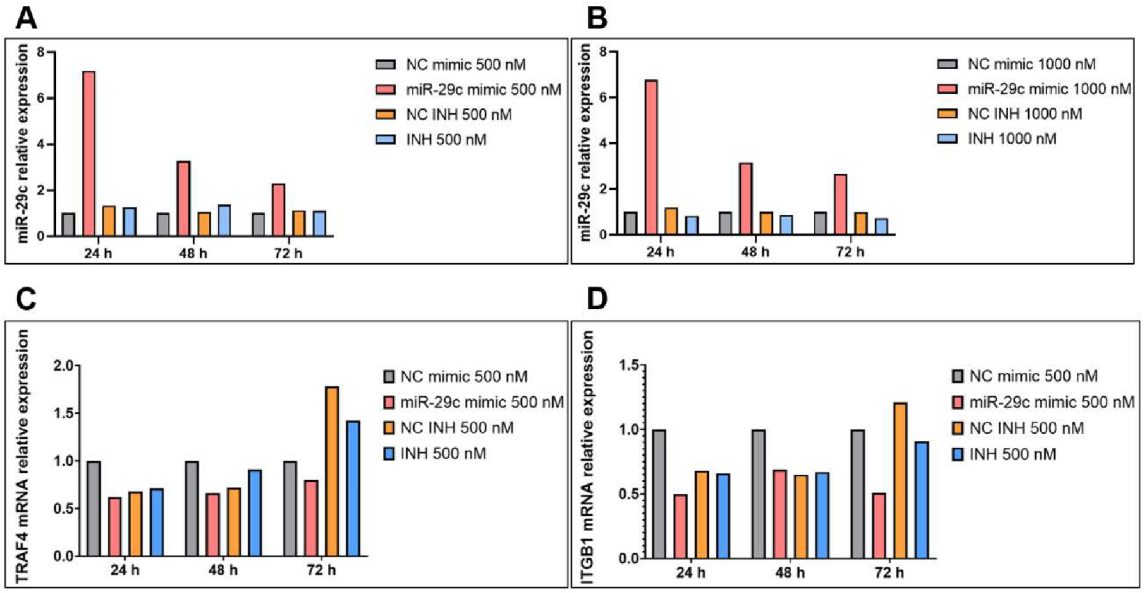


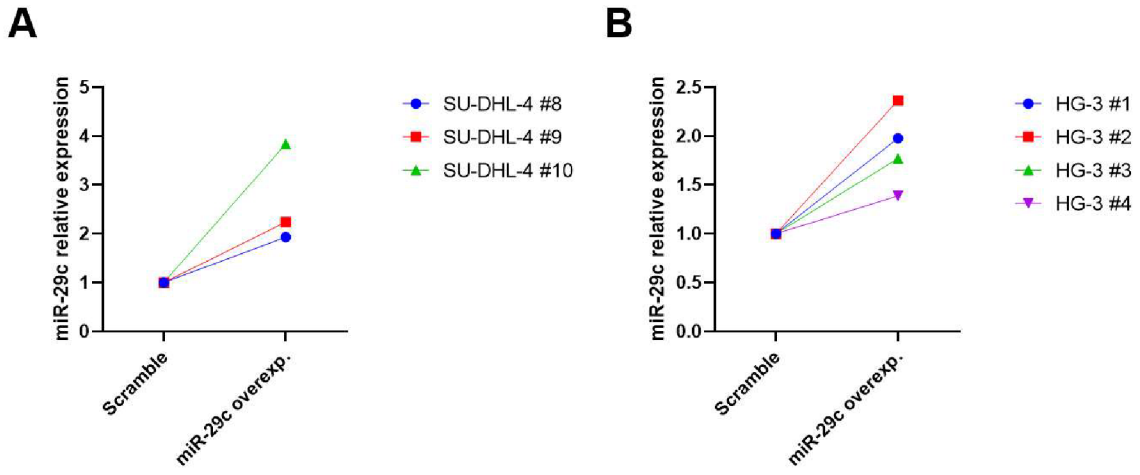
Figure 22: Changed levels of *miR-29c, ITGβ1* and *TRAF4* mRNAs following HG-3 cells transfection with *miR-29c* mimic and inhibitor. A, B. Graphic depiction of relative *miR-29c* expression in cell transfected with 500 nM concentration (A) or 1000 nM (B) concentration of *miR-29c* mimic and inhibitor. C. Graphic depiction of changed *TRAF4* mRNA levels following transfection with 500 nM concentration of *miR-29c* mimic and inhibitor. D. Graphic depiction

of changed *ITGβ1* mRNA levels following transfection with 500 nM concentration of *miR-29c* mimic and inhibitor.

8.5 Expression of candidate genes in cell lines with stable *miR-29c* overexpression

Because we were able to detect changed levels of candidate genes in cells transiently transfected with *miR-29c* mimic, we have decided to examine whether such results can be also observed in cells with stable overexpression of *miR-29c*. For such experiment, HG-3 and SU-DHL-4 cell lines were transduced with lentiviral vector containing either *miR-29c* or with scramble. To confirm level of the overexpression, harvesting of the cells for protein and RNA lysates has been done multiple times. Moreover, induction of *miR-29c* levels has been confirmed by qRT-PCR (Figure 23 A, B).

In case of SU-DHL-4 cells, statistically significant decrease was detected in levels of PTEN (p=0,0048) and TRAF4 (p<0,0001). Unfortunately, levels of AKAP5 in this cell line could not be studied due to low basal expression of the protein in SU-DHL-4 cells. Stable overexpression of *miR-29c* in HG-3 cells lead to statistically significant downregulation of ITGβ1 levels (p=0,0244). These results are further depicted in Figure 23 D-F.



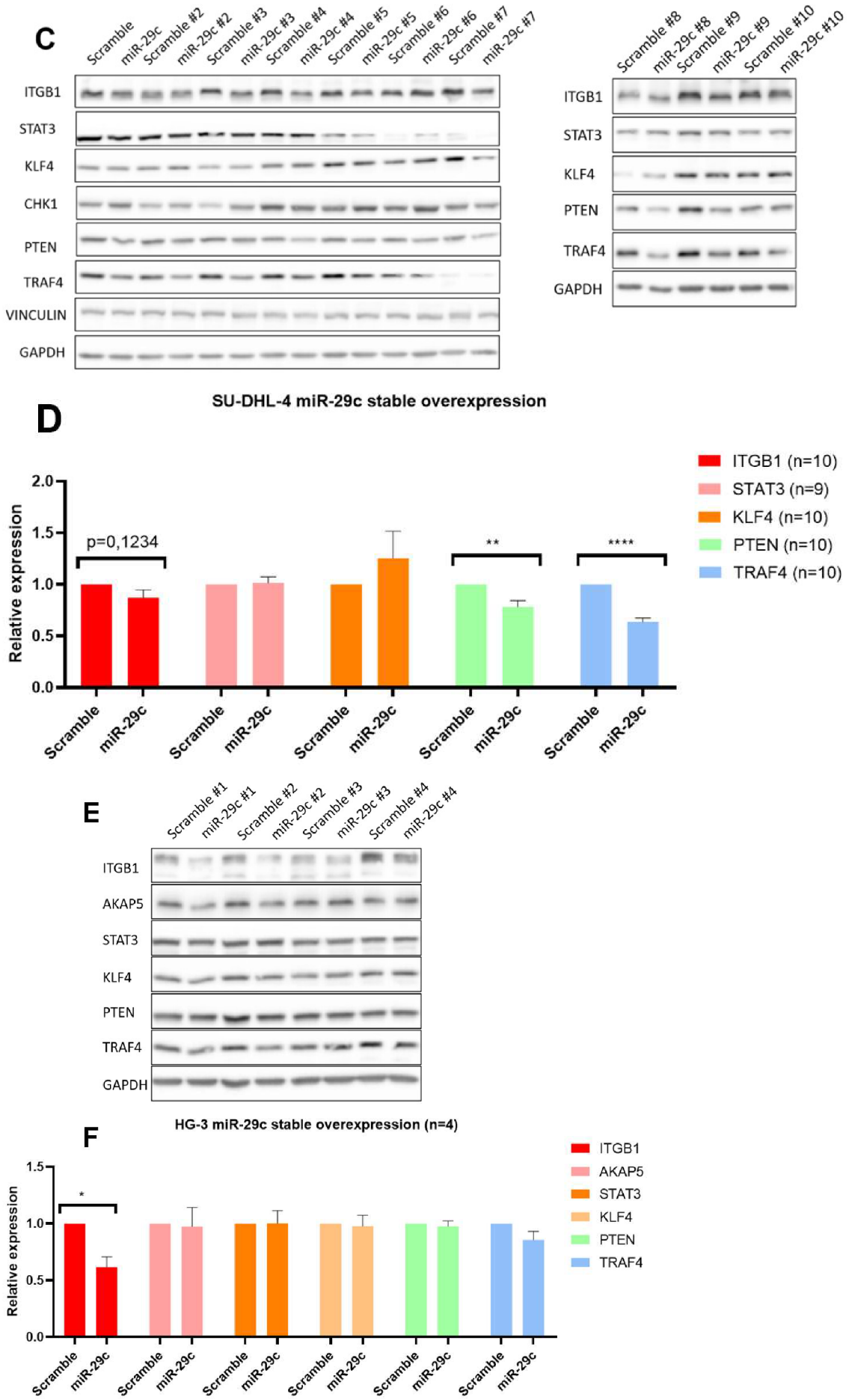

C
Scramble
miR-29c
Scramble #2
miR-29c #2
Scramble #3
miR-29c #3
Scramble #4
miR-29c #4
Scramble #5
miR-29c #5
Scramble #6
miR-29c #6
Scramble #7
miR-29c #7
ITGB1
STAT3
KLF4
CHK1
PTEN
TRAF4
VINCULIN
GAPDH
Scramble #8
miR-29c #8
Scramble #9
miR-29c #9
Scramble #10
miR-29c #10
ITGB1
STAT3
KLF4
PTEN
TRAF4
GAPDH
D
SU-DHL-4 miR-29c stable overexpression
Relative expression
2.0
1.5
1.0
0.5
0.0
p=0,1234
**
****
Scramble
miR-29c
ITGB1 (n=10)
STAT3 (n=9)
KLF4 (n=10)
PTEN (n=10)
TRAF4 (n=10)
E
Scramble #1
miR-29c #1
Scramble #2
miR-29c #2
Scramble #3
miR-29c #3
Scramble #4
miR-29c #4
ITGB1
AKAP5
STAT3
KLF4
PTEN
TRAF4
GAPDH
F
HG-3 miR-29c stable overexpression (n=4)
Relative expression
1.5
1.0
0.5
0.0
*
Scramble
miR-29c
ITGB1
AKAP5
STAT3
KLF4
PTEN
TRAF4

Figure 23: Levels of studied genes in CLL cell lines with stable *miR-29* overexpression. A. Graphic depiction of *miR-29c* overexpression detected in SU-DHL-4 lysates from three different timepoints. B. Graphic depiction of *miR-29c* overexpression detected in HG-3 lysates from four different timepoints. C. Western blot showing decreased levels of selected proteins in SU-DHL-4 cells with *miR-29c* overexpression harvested in multiple timepoints. D. Graphic summary of relative expression of selected proteins from the Western blot from part C. E. Western blot showing decreased levels of selected proteins in HG-3 cells with *miR-29c* overexpression harvested in multiple timepoints. F. Graphic summary of relative expression of selected proteins from the Western blot from part E.

Based on the results of transient transfections as well as on the results obtained from cells with stable *miR-29c* overexpression, we have decided to mainly focus on *ITGβ1* as the main candidate gene (Table 16). Another reason for further study of this gene was the already mentioned importance of ITGβ1 protein in the context of CLL and its microenvironment. Furthermore, these experiments validated *TRAF4* as *miR-29* target which can be used as a reliable positive control for detection of other genes regulated by this miRNA.

	Transient tranfection of HG-3 cells with miR-29c mimic		Stable overexpression of miR-29c in HG-3 cells		Stable overexpression of miR-29c in SU-DHL-4 cells	
Gene name	**Statistical significance**	**Reproducible downregulation**	**Statistical significance**	**Reproducible downregulation**	**Statistical significance**	**Reproducible downregulation**
ITGB1	p=0,0074 (500 nM); p=0,0923 (1000 nM)	Yes	p=0,0244	Yes	p=0,1234	Yes
AKAP5	p=0,0272 (500 nM); p=0,1151 (1000 nM)	Yes	p=0,8853	No	—	expression undetectable
KLF4	p=0,8808 (500 nM); p=0,0747 (1000 nM)	Yes	p=0,8355	No	p=0,3562	No
TRAF4	p=0,0039 (500 nM); p=0,0095 (1000 nM)	Yes	p=0,1521	Yes	p<0,0001	Yes
STAT3	p=0,8278 (500 nM); p=0,7430 (1000 nM)	No	p=0,9826	No	p=0,8296	No
PTEN	p=0,5315 (500 nM); p=0,0108 (1000 nM)	No	p=0,6687	No	p=0,0048	Yes

Table 16: Summary of changes of candidate proteins detected in transiently transfected cells and *miR-29c* overexpressing cells.

8.6 Phenotypic characterisation of *miR-29c*-overexpressing SU-DHL-4 cells

Besides detection of the mentioned proteins of interest, we have also decided to characterise phenotypic features of SU-DHL-4 cells with stable *miR-29c* overexpression. Thus, a Western blot was performed to determine levels of various kinases involved in BCR pathway – both phosphorylated and total forms of Btk, Syk, Akt and Erk, as well as Myc and CD20 were analysed. An increase in levels of phosphorylated Akt (p=0,0037) and Myc (p=0,1831) has

been observed in all samples with overexpressed *miR-29c*. However, no trend has been observed with the rest of the proteins (Figure 24).

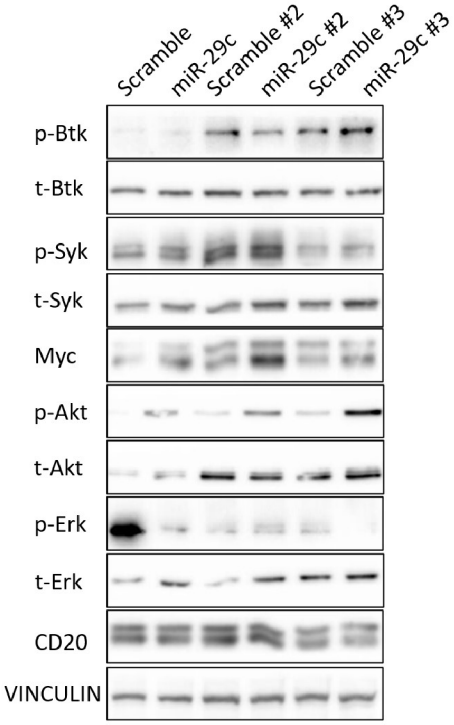


Figure 24: Levels of proteins involved in the BCR pathway in SU-DHL-4 cell with stable *miR-29c* overexpression.

Since CD29 (ITGβ1) is an important diagnostic and prognostic surface marker of CLL cells, we have decided to analyse its levels in transduced SU-DHL-4 cells not only by Western blotting but also by surface staining and subsequent flow cytometry detection. Moreover, various other surface markers were also measured to further characterise the phenotype of cells with stable *miR-29c* overexpression (Figure 25).

Interestingly, although downregulation of ITGβ1 levels after *miR-29c* induction has been confirmed as significant by Western blot analysis, surface expression of the protein did not show such trend. In contrast, a slight decrease was observed in levels of CD49d (integrin α_4), the other subunit of the CD49d-CD29 dimer. Cells with *miR-29c* overexpression also showed reduced expression of surface CXCR4 and CD20. Contrarily, an upregulation was detected in levels of CD22.

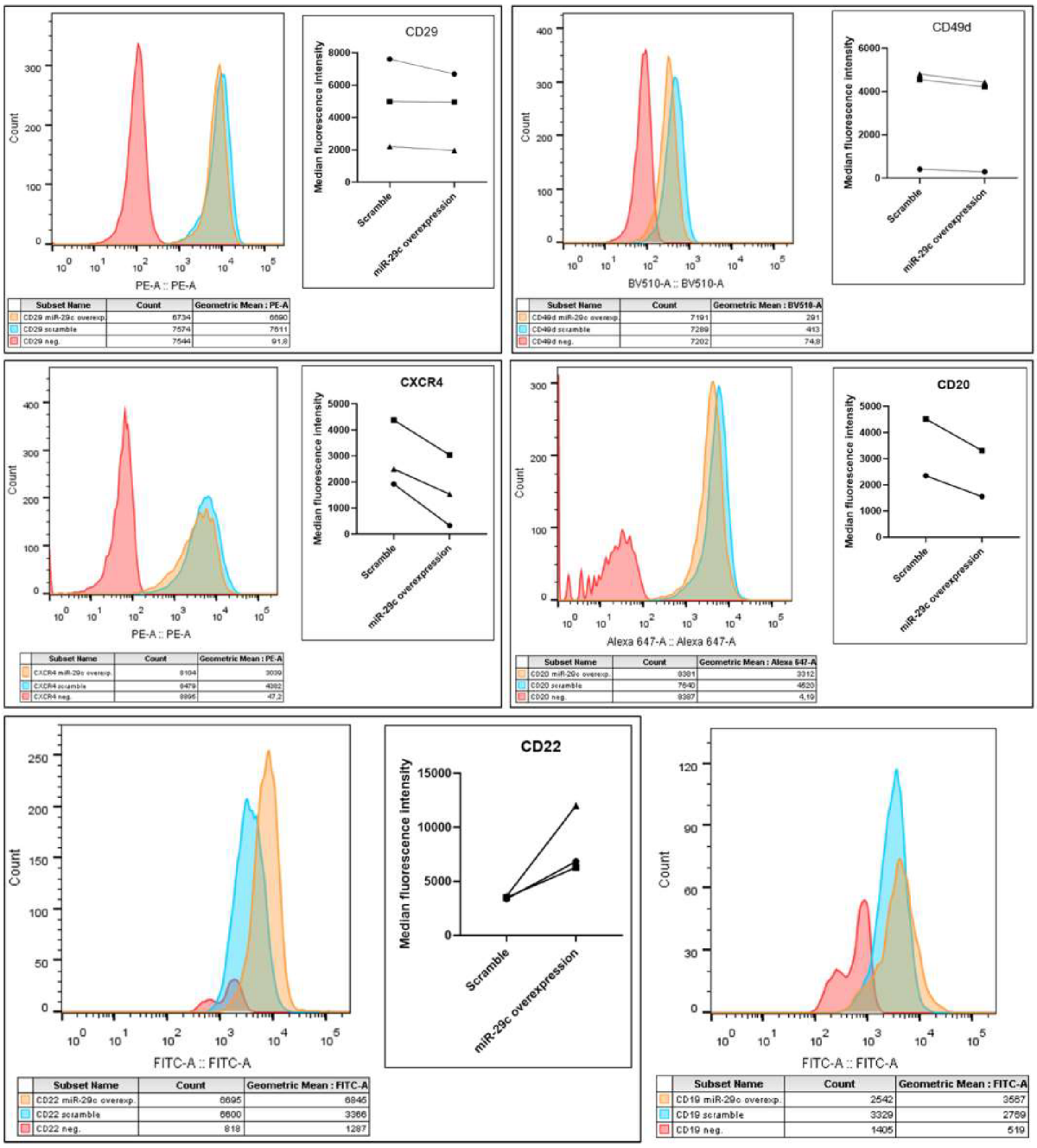
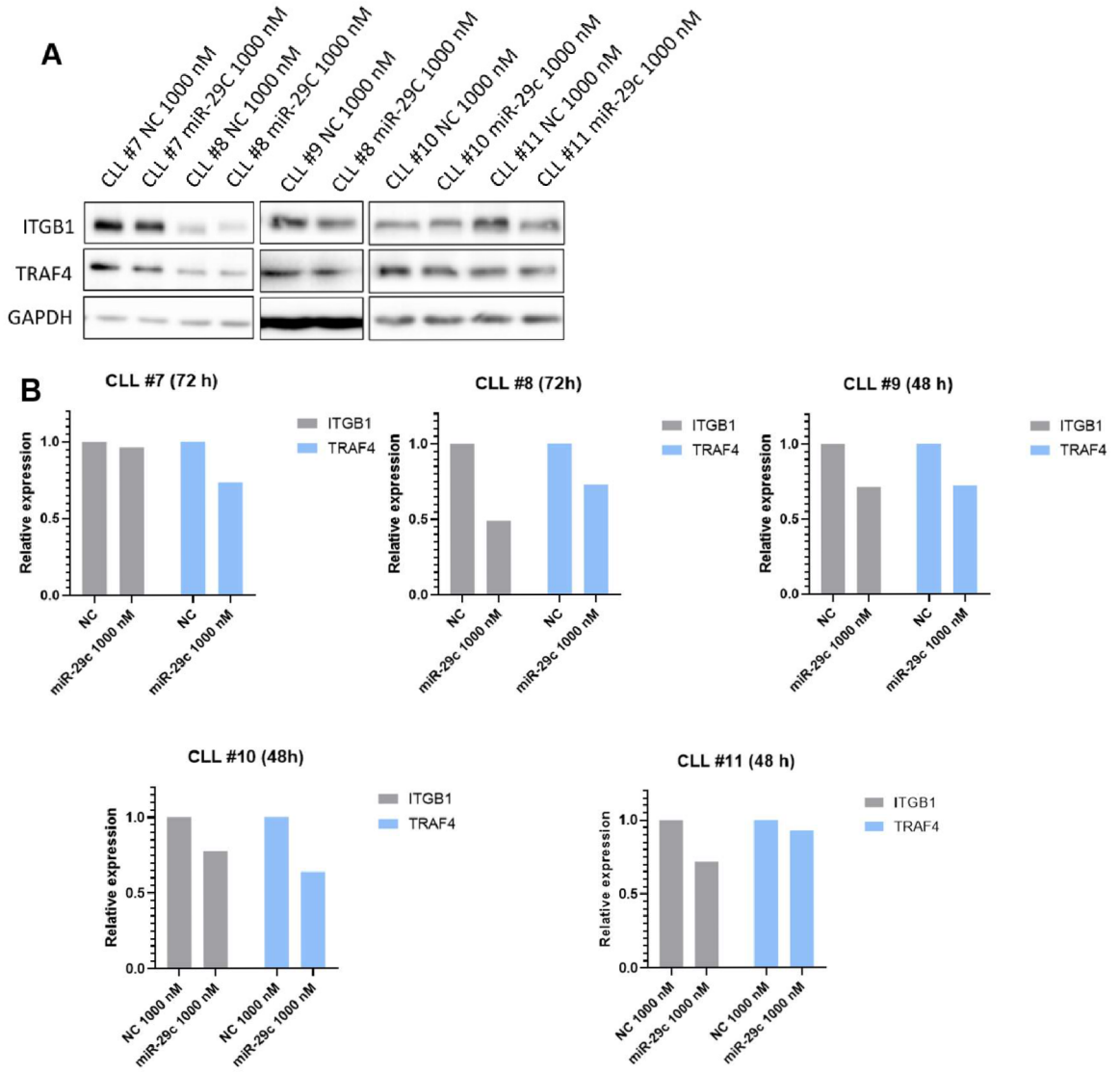


Figure 25: Graphs showing levels of various surface markers detected by flow cytometry on SU-DHL-4 cells with *miR-29c* overexpression. The histograms show representative results for each marker. Non-stained cells are coloured in red, cells transduced with scramble are shown in blue and cells overexpressing *miR-29c* are represented by orange curve. The additional graphs show changes in median fluorescence intensity (MFI) between cell transduced with scramble and *miR-29c* overexpressing cells from multiple repetitions (n=3, in case of CD20 n=2).

8.7 Regulation of ITGβ1 by *miR-29c* in primary CLL cells

To confirm the interaction between *miR-29c* and *ITGβ1* not only in CLL cell lines but also in primary CLL cells, transient transfections of primary cells from CLL patients (n=5) were performed (Figure 26). The cells were transfected with 1000 nM concentration of negative control or *miR-29c* mimic and subsequently harvested after 48 or 72 hours. Importantly, levels of ITGβ1 and TRAF4 were reduced following the transfection.



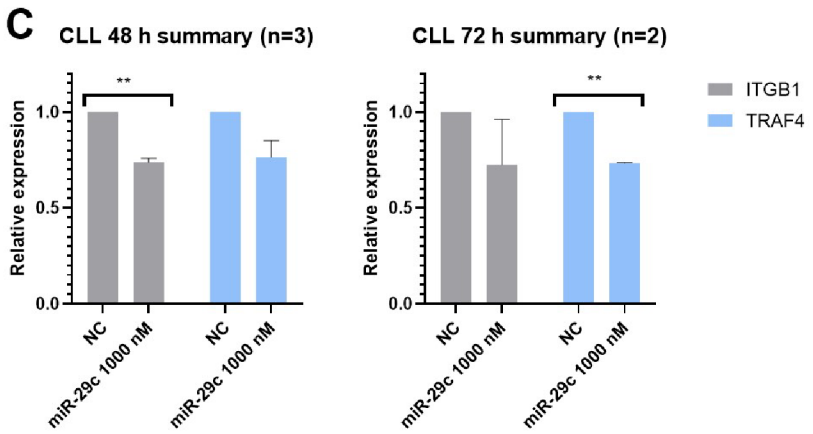


Figure 26: Expression of ITGβ1 and TRAF4 in primary CLL cells transfected with *miR-29c* mimic. A. Western blot showing levels of the two proteins in transfected primary CLL cells. B. Relative expression of ITGβ1 and TRAF4 from Western blots shown in part A. C. Graphic summary of relative expression of ITGβ1 and TRAF4 from Western blots shown in part A.

8.8 Levels of candidate genes differ between $CD5^{bright}CXCR4^{dim}$ and $CD5^{dim}CXCR4^{bright}$ subpopulations of primary CLL cells

While the differential expression of candidate genes in the two subpopulations of CLL cells has been shown by the previously mentioned RNAseq analysis, it has not been confirmed on protein level. After isolation from PB, primary cells from CLL patients (n=3) were sorted using flow cytometry. The sorting was based on expression of the surface markers CD5 and CXCR4. Besides $CD5^{bright}CXCR4^{dim}$ and $CD5^{dim}CXCR4^{bright}$ subpopulations, the $CD5^{int}CXCR4^{int}$ subpopulation was also obtained. Typically, this subgroup comprises the majority of CLL cells which express intermediate levels of CD5 and CXCR4 (Calissano *et al.*, 2009). Next, Western blot analysis was done with protein lysates from the sorted cells and ITGβ1 expression was studied. Since *miR-29* levels are downregulated in the context of lymphoid microenvironment (here represented by the $CD5^{bright}CXCR4^{dim}$ subpopulation), we expected ITGβ1 to be upregulated in this subpopulation. Indeed, decreased expression of ITGβ1 was detected in $CD5^{bright}CXCR4^{dim}$ subpopulation while the opposite trend was observed in $CD5^{dim}CXCR4^{bright}$ subpopulation which represents PB-resident CLL cells (Figure 27 A, B).

Besides the Western blot analysis, levels of ITGβ1 in the two CLL subpopulations were also analysed by intracellular staining. For this method, samples from three CLL patients were stained with primary ITGβ1 antibody and secondary antibody conjugated to the A647 fluorescent dye. The reversed expression of ITGβ1 between $CD5^{bright}CXCR4^{dim}$ and

CD5^{dim}CXCR4^{bright} CLL cells was again confirmed and it was evaluated as statistically significant (p=0,0206) (Figure 27 C).

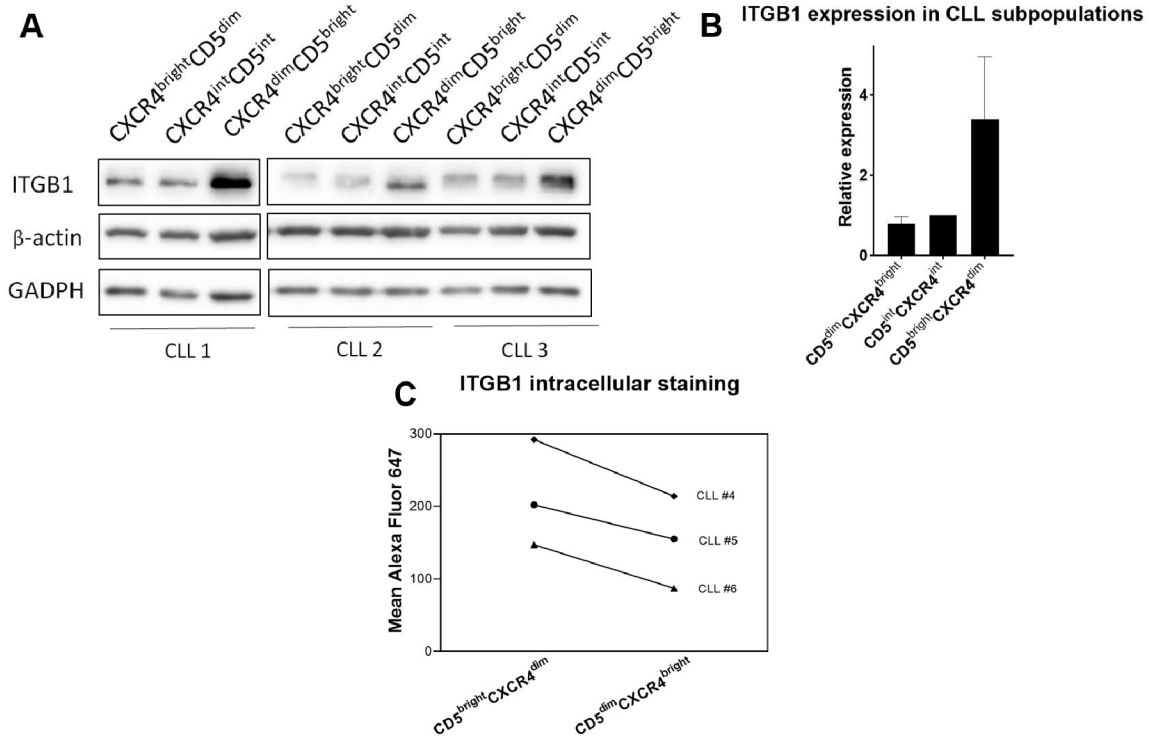


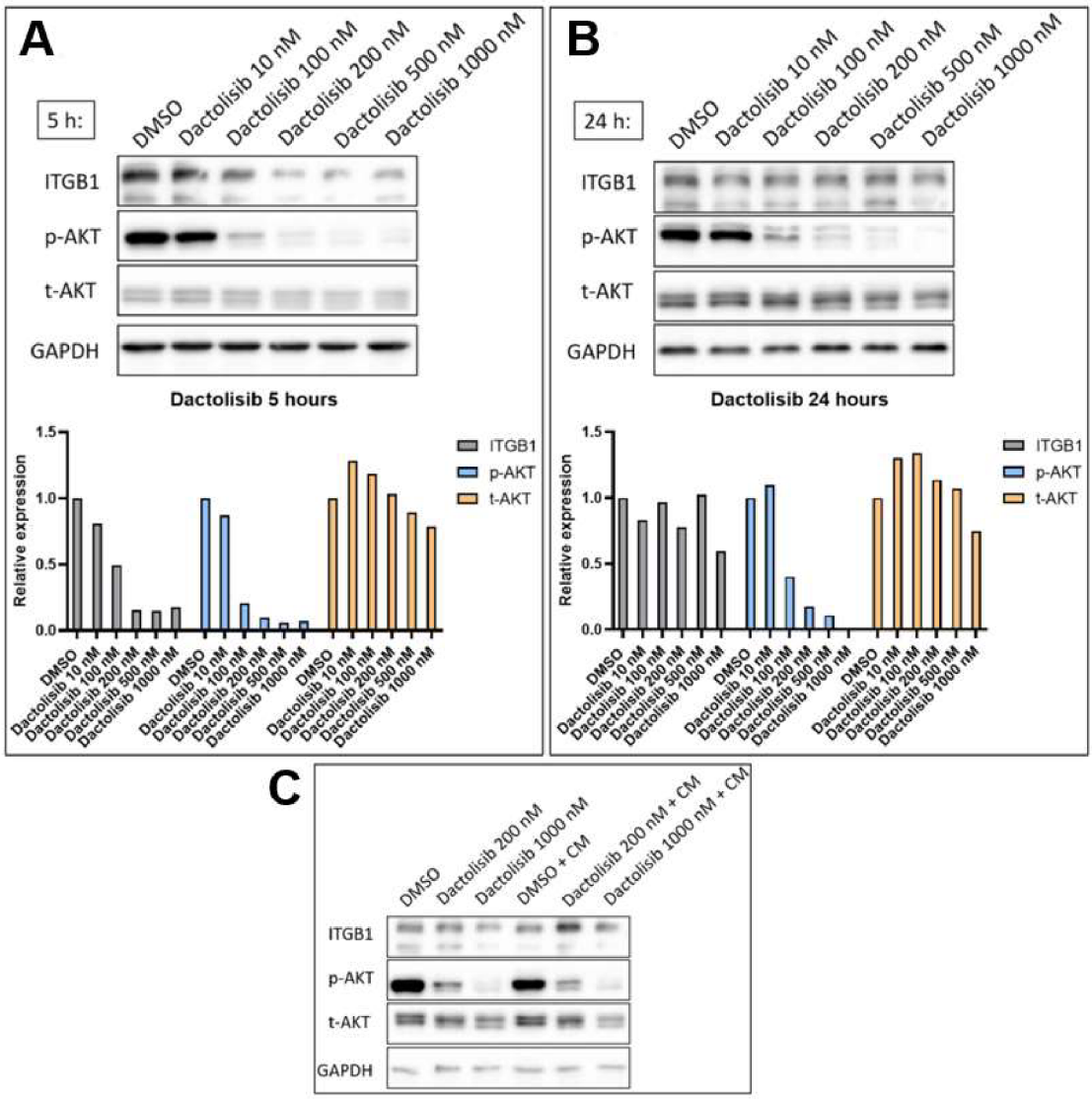
Figure 27: Expression of candidate *miR-29* targets varies between CD5^{bright}CXCR4^{dim} and CD5^{dim}CXCR4^{bright} subpopulations of primary CLL cells. A. A Western blot of sorted primary CLL samples (n=3). B. Relative protein levels of ITGβ1 in sorted primary CLL samples (n=3). C. Intracellular staining analysis of relative ITGβ1 expression in primary CLL cells (n=3).

8.9 Expression of ITGβ1 is influenced by blockage of PI3K/Akt/mTOR pathway in CLL cells

Finally, apart from its regulation by *miR-29*, we have decided to look on the influence of other cellular pathways on *ITGβ1* expression. Since *ITGβ1* induction by activity of the PI3K/Akt/mTOR pathway was observed in MCL cells (Zhao *et al.*, 2017), possible existence of this link in CLL lymphocytes was examined. MEC-1 cells were cultivated with increasing concentrations of the dual PI3K-mTOR1/2 inhibitor dactolisib for 5 and 24 hours (Figure 28 A, B). Cells treated with DMSO were used as negative control. Decrease of both phosphorylated and total Akt levels after dactolisib treatment confirmed functionality of the inhibitor. Importantly, exposition of MEC-1 cells to dactolisib lead to dose-dependent decrease of ITGβ1 levels. Linear reduction of both ITGβ1 and Akt expression was detected in cells treated for

5 hours. However, the trend was not so clear 24 hours after the treatment, probably due to high cytotoxicity of the inhibitor.

Next, the effect of lymphoid microenvironment on dactolisib-induced *ITGβ1* downregulation was studied. For this purpose, CM from cultivation of the fibroblast-like cell line HS-5 was obtained. Due to the character of the cells, the CM thus contains signalling molecules also present in the CLL microenvironment (e.g. produced by SCs). MEC-1 cells were first cultivated with dactolisib alone and after 4 hours the CM was added (Figure 28 C, D). The total time of cultivation was 24 hours. Remarkably, while ITGβ1 levels were reduced after dactolisib treatment, effect of the inhibitor was at least partially reverted following CM addition. However, such influence of CM was not observed with both forms of Akt. These results once again illustrate the complex regulation of *ITGβ1* expression by both cellular signalling cascades and microenvironmental signals.



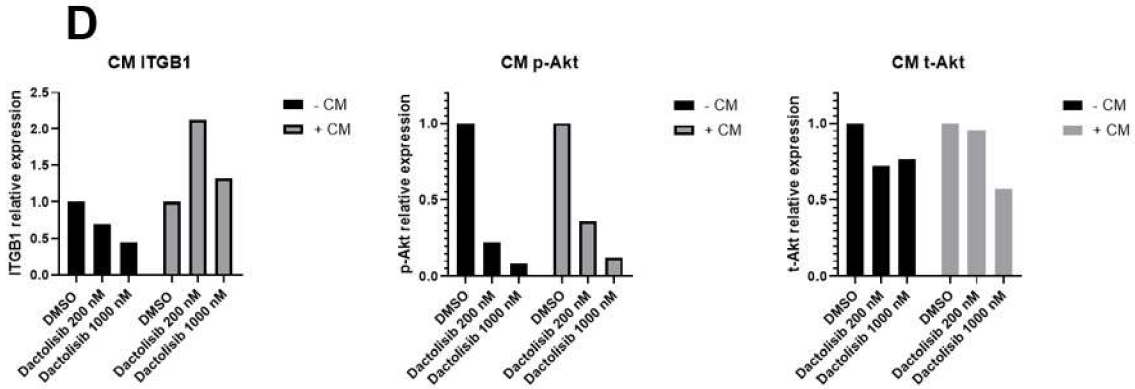


Figure 28: *ITGβ1* expression is regulated by the PI3K/Akt/mTOR pathway in CLL cells. A. Scan of Western blot performed with lysates from MEC-1 cells treated with various concentrations of dactolisib for 5 hours (up), graphic depiction of the Western blot (bottom). B. Scan of Western blot performed with lysates from MEC-1 cells treated either with various concentrations of dactolisib for 24 hours (up), graphic depiction of the Western blot (bottom). C. Scan of Western blot performed with lysates from MEC-1 cells treated with either dactolisib alone of with dactolisib and HS-5 CM added after 4 hours of the cultivation. D. Graphs summarizing results of the Western blot from part C.

9 Discussion

Chronic lymphocytic leukemia (CLL) is the most common leukemia of adult population in the Western world. The disease is characterised by accumulation of $CD5^+$ $CD19^+$ malignant mature B lymphocytes in PB, secondary lymphoid organs or BM. CLL cells typically circulate between PB and lymphoid organs. This phenomenon is linked with the necessity of contact with the lymphoid microenvironment. Cells present within the microenvironment provide pro-survival or pro-proliferative signals for malignant lymphocytes. Such signalling molecules, either soluble or surface-located, subsequently activate various cellular pathways important for well-being of both non-malignant and malignant B cells. Importantly, it has been shown repeatedly that miRNAs, short non-coding RNAs, have influential roles in these pathways.

MiRNAs represent important epigenetic regulators of gene expression. Similarly to oncoproteins or tumour suppressors, miRNAs can be of oncogenic or tumour-suppressive character in the context of CLL. Additionally, altered expression of various miRNA targets further contributes to aggressivity or progression of the malignancy. In line with these facts miRNAs thus have the potential of being used as biomarkers.

The *miR-29* family of miRNAs represents an example of tumour-suppressive, microenvironment-influenced miRNAs. This is linked with the fact that levels of *miR-29* are typically downregulated in aggressive CLL, resulting in increased expression of *miR-29* targets which often are of oncogenic character. For instance, lower *miR-29* expression was shown to be linked with positivity for ZAP70 and unmutated IGV_H (Li *et al.*, 2011). Although the members of the *miR-29* family (*miR-29a*, *miR-29b* and *miR-29c*) differ in their sequences even in their mature forms, the seed sequence complementary to targeted mRNAs is identical for all three mature *miR-29* molecules – *miR-29a*, *miR-29b* and *miR-29c*.

Importantly, levels of *miR-29* in CLL cells can be, besides other regulators, modulated by the activity of BCR signalling pathway. Thus, it is not surprising that *miR-29* expression is influenced by some inhibitors of molecules involved in the BCR pathway. Upregulation of *miR-29* after treatment with ibrutinib, a small molecular drug directly interfering with Btk, is one example of such influence. Ibrutinib is one of the most prominently used molecular inhibitors in CLL treatment. Moreover, another layer of *miR-29* regulation is represented by Myc binding to *miR-29* promoter regions. This interaction leads to decrease in *miR-29* levels. Stimuli from the microenvironment, such as CD40L, also contribute to *miR-29* downregulation (Sharma *et al.*, 2021).

In line with all these facts, it is rather predictable that *miR-29* targets in malignant B cells include some known oncoproteins, such as the *Bcl-2* family members *Tcl-1* (Pekarsky *et al.*, 2006) and *Mcl-1* (Mazzoccoli *et al.*, 2018). While contribution of other *miR-29* targets to CLL pathology might be less clear, their involvement in various signalling pathways or response to microenvironmental interactions is of importance as well, e.g. in the case of *TRAF4* which modulates the responsiveness to CD40 signalling as shown in our lab previously (Sharma *et al.*, 2021).

The aim of this thesis was to study and validate novel *miR-29* targets in the context of CLL microenvironment. Initial selection of potential candidate genes was based on the analysis of data from RNAseq performed in our lab previously. For this analysis, CLL cells were sorted into two subpopulations. The $CD5^{bright}CXCR4^{dim}$ subpopulation represents CLL cells which have only recently left the microenvironment of a lymphoid organ. In contrast, $CD5^{dim}CXCR4^{bright}$ cells are PB resident and thus in need of translocation into a lymphoid organ (Calissano *et al.*, 2011). Levels of *miR-29* were observed to vary between these subpopulations – expression of *miR-29* is lower in $CD5^{bright}CXCR4^{dim}$ cells when compared to $CD5^{dim}CXCR4^{bright}$ cells. Moreover, results of RNAseq from CLL cells treated with ibrutinib were also considered during the selection of candidate genes. Since *miR-29* has been previously shown to be downmodulated in ibrutinib-treated CLL cells (Sharma *et al.*, 2021), genes with increased expression in such cells thus were of potential interest.

After intersection of the list of genes inversely correlated with *miR-29* levels from CLL cell subpopulation data and the list of genes downmodulated following ibrutinib treatment of CLL cells, a group of 11 candidate genes was obtained. Moreover, these genes are listed in the TargetScan database as predicted *miR-29* targets. Importantly, although downregulation of ITGβ1 after ibrutinib treatment was not statistically significant in our data, it is a confirmed *miR-29c* target, and its downmodulation in ibrutinib-treated cells has been previously confirmed in lymphoma cells (Zhao *et al.*, 2017). Additionally, we were able to detect downmodulation of ITGβ1 protein levels after ibrutinib treatment in MEC-1 cells. To further narrow the list of candidate genes, literature research on these *miR-29* targets has been performed, leading to a group of main candidate genes which could be of relevance in the context of CLL and its microenvironment. This list contained *AKAP5*, *DUSP2*, *KLF4* and *ITGβ1*.

The hypothesized anti-correlation between expression of the selected genes and *miR-29* levels has been validated in CLL cell lines by various approaches. First, transient transfections with *miR-29c* mimic were performed. Following the initial transfections, *DUSP2* has been excluded from the candidate group due to non-specific binding of the used antibody. Western blot

analysis of lysates from transfected cells confirmed lower levels of all three remaining candidates after *miR-29c* induction. Interestingly, during multiple Western blot analyses no reproducible expressional trend has been observed in expression of *PTEN* and *STAT3*, other known *miR-29* targets. These two proteins were thus subsequently used as additional controls. In case of *STAT3*, absence of any detectable change after *miR-29c* transfection could be explained by its low ranking score when compared to other *miR-29* targets in the TargetScan database.

Besides the *miR-29c* mimic, we have also performed transfections with the *miR-29c* LNA inhibitor. This short double stranded RNA molecule interferes with *miR-29c* function due to presence of *miR-29c* binding site with chemically modified nucleotides in sequence of the inhibitor. Thus, endogenous *miR-29c* binds to this molecule with higher affinity compared to its target mRNA. The target mRNA can therefore be translated. Correspondingly, increased levels of all three candidates were observed after transfection with *miR-29c* inhibitor. However, these results were not statistically significant nor as reproducible as results observed after mimic transfections, probably because of lower number of repetitions and higher standard deviation within the samples. Adding more repetitions would probably increase the statistical significance of the results.

In addition to detection of changed protein expression in transiently transfected cells, regulation of *ITGβ1* and *TRAF4* by *miR-29c* in these cells has been validated on mRNA level. Interestingly, qPCR of *miR-29c* showed that while 24 hours post-transfection levels of *miR-29c* increased approximately sevenfold, this induction of *miR-29c* decreased rather prominently 48 and 72 hours after the transfection. Nevertheless, even 72 hours after transfection with *miR-29c* mimic, the detected levels of *miR-29c* were still more than twofold higher when compared to cell transfected with negative control. On the other hand, levels of *ITGβ1* and *TRAF4* mRNA showed statistically significant decrease in samples transfected with *miR-29c*. No consistent or statistically significant upregulation of the two mRNAs was detected after transfection with *miR-29c* inhibitor, confirming what we have previously detected on Western blot.

Besides transient transfections, we have decided to induce *miR-29c* levels in cell lines by yet another method. For this, sequence of *pre-miR-29c* was transduced, using a lentiviral vector, into two cell lines – HG-3 and SU-DHL-4. This manipulation led to creation of cell lines stably overexpressing *miR-29c*. Despite being of DLBCL origin, the SU-DHL-4 cell line was used due to the fact that CLL can progress into NHLs including DLBCL during the process of Richter's transformation. Thus, comparison of data obtained from this cell line could be of relevance for studies of this transformation.

Western blot results obtained from cells with stable *miR-29c* overexpression validated trends observed with transient transfection. Observed downregulation of potential *miR-29* targets was consistent throughout various timepoints of cells harvesting, thus confirming stability of the overexpression. Interestingly, although downregulation of candidate proteins was detected on protein level, we did not see any significant changes on mRNA level. One of the reasons behind this could be occurrence of translation inhibition rather than mRNA degradation after the *miR-29c*-target mRNA interaction. Another explanation could be that the change of mRNA level was relatively small – e.g. fold change 1,5 would correspond to Ct change of 0,58 which is close to the standard deviation of the measurement.

Besides concentrating on the candidate proteins, we have also decided to further characterise the phenotype of SU-DHL-4 cells with stable *miR-29c* overexpression to see the influence of the overexpression on important proteins and surface markers. Since the BCR pathway is one of the most frequently deregulated pathways in malignant B lymphocytes, Western blot detection of multiple proteins and kinases involved in BCR signalling was performed. Remarkably, induced levels of phosphorylated Akt as well as of Myc were present in samples from *miR-29c*-overexpressing cells. However, no other protein involved in BCR pathway showed reproducible expressional change in transduced cells.

Another part of the phenotypic characterisation of SU-DHL-4 cells was flow cytometric analysis of a panel of surface markers which are routinely detected in CLL cells. It is of importance to mention that while significant decrease in ITGβ1 protein levels after *miR-29c* overexpression was demonstrated by Western blot, flow cytometry showed only very mild decrease in surface levels of ITGβ1. Interestingly, integrin α_4, the binding partner of ITGβ1, showed slightly more noticeable downregulation on transduced cells.

Comparing the results of both stable and transient overexpression, we have decided to further concentrate on *ITGβ1* as the main candidate gene. This is also due to its significance for CLL cells. Together with integrin α_4, integrin β_1 forms a dimer on the cell surface. This molecular complex then binds components of extracellular matrix such as fibronectin. Another ligand of the $\alpha_4\beta_1$ integrin dimer is VCAM-1, molecule expressed on cells present within the lymphoid microenvironment. The $\alpha_4\beta_1$ integrin mediates adhesion and influences cell motility and migration. These events are linked with activation of various cellular pathways which are often of anti-apoptotic character (Redondo-Muñoz *et al.*, 2010). Correspondingly, $\alpha_4\beta_1$ integrin and the signalling cascades it triggers in the cells can contribute to CLL cells resistance to treatment (Spaargaren *et al.*, 2003). Furthermore, flow cytometric detection of CD49d and CD29 has the potential to help with better prognostication and characterisation of CLL (Bulian *et al.*, 2014).

To validate the involvement of ITGβ1 in various signalling pathways in the context of CLL cells and their microenvironment, we have decided to focus on the possible positive regulation of ITGβ1 by PI3K/Akt/mTOR pathway which has been observed by Zhao *et al.* (2017) in MCL cells. For this, MEC-1 cells were cultivated with increasing concentrations of dual PI3K-mTOR1/2 inhibitor dactolisib. Indeed, we were able to detect decreased levels of both phosphorylated and total Akt and ITGβ1. Remarkably, cultivation of MEC-1 cells with dactolisib and CM from HS-5 cells reverted the decrease in ITGβ1 levels observed after dactolisib treatment. On the other hand, levels of the two forms of Akt did not show such trend. These observations could thus indicate the strong influence microenvironmental signals have on the expression of *ITGβ1*.

Besides cell lines, the anti-correlation between *miR-29c* and *ITGβ1* together with *TRAF4* has been detected in primary CLL cells as well. Moreover, the varying expression of *ITGβ1* between $CD5^{bright}CXCR4^{dim}$ and $CD5^{dim}CXCR4^{bright}$ subpopulations was also verified by both Western blot analysis and intracellular staining. For these experiments, sorted primary CLL cells were used. Predictably, while $CD5^{bright}CXCR4^{dim}$ CLL cells showed increased ITGβ1 levels, opposite tendency was detected in $CD5^{dim}CXCR4^{bright}$ cells. These observations confirmed the previous RNAseq results.

In conclusion, considering results of experiments mentioned in the thesis we have evaluated a group of novel *miR-29* targets in the context of CLL. *PTEN* and *TRAF4* have been previously shown to be regulated by *miR-29* in the malignancy (Hines *et al.*, 2020; Sharma *et al.*, 2021) and they were thus used as positive controls. Downregulated protein levels after *miR-29c* transfection were repeatedly observed for all three candidate genes – *AKAP5*, *KLF4* and *ITGβ1*. However, only *ITGβ1* showed reproducible and statistically significant downregulation in both HG-3 and SU-DHL-4 cells with stable *miR-29c* overexpression. Moreover, involvement of ITGβ1 in cellular adhesion as well as in various signalling pathways is of high importance for the survival or motility of CLL cells. For all these reasons we have also evaluated the *miR-29-ITGβ1* relationship by other methods, further confirming the interaction. Additionally, ITGβ1 is one of the most frequently detected surface markers important for CLL diagnostics or staging. Identification of *miR-29* direct influence on *ITGβ1* thus emphasises the potential use of *miR-29* as a CLL biomarker.

However, due to ITGβ1 impact on CLL adhesion and motility in the future it would be interesting to examine whether some changes in migration of CLL cells occur after *miR-29* overexpression. Furthermore, closer study in levels of the integrin $\alpha_4\beta_1$ dimer and its respective subunits in cells with *miR-29c* upregulation could contribute to better understanding of possible

reciprocal expressional regulation of these two subunits. Treatment of *miR-29*-overexpressing CLL cells with various molecular inhibitors and subsequent detection of ITGβ1 levels could also better define the influence of various signalling pathways on the described *miR-29-ITGβ1* axis.

10 Souhrn

Chronická lymfocytární leukemie (CLL) je nejčastějším hematologickým nádorovým onemocněním dospělých v západním světě. Je charakterizována cirkulací a hromaděním $CD5^+$ $CD19^+$ maligních B lymfocytů v periferní krvi, sekundárních lymfatických orgánech a kostní dřeni. Tato cirkulace a kontakt s buňkami a extracelulární matrix v mikroprostředí lymfatického orgánu jsou pro CLL lymfocyty klíčové. Elementy mikroprostředí poskytují signální molekuly, na něž navazují dráhy receptorů CLL buněk, které v důsledku vedou až k aktivaci genů, jejichž produkty podporují přežití či proliferaci. V kontextu CLL je nejvíce studovanou signální drahou dráha B buněčného receptoru (BCR). Kromě proteinů a kináz, jež jsou do ní zapojeny, má tato signální kaskáda vliv také na molekuly mikroRNA (miRNA).

MiRNA jsou krátké nekódující molekuly RNA, jež představují významné posttranskripční regulátory genové exprese. Na základě komplementarity se vážou na mRNA a způsobují jejich degradaci či inhibici translace. MiRNA jsou v poslední době extenzivně studovány v souvislosti s onemocněními včetně nádorových – jejich cílové geny často mají povahu onkogenů či nádorových supresorů. Deregulace exprese miRNA tak může mít vliv na vznik či progresi malignit včetně CLL. Na hladiny miRNA u CLL má navíc vliv také mikroprostředí prostřednictvím signálních drah, které jsou okolními ligandy spouštěny. Toto je případ i miRNA z rodiny *miR-29*.

Cílem této diplomové práce bylo popsání nových cílových genů *miR-29* v kontextu CLL a jejího mikroprostředí. Na základě analýzy sekvenačních dat transkriptomu primárních CLL buněk byla získána skupina tří kandidátních genů – *AKAP5*, *KLF4* a *ITGβ1*. Interakce mezi *miR-29c*, jedním ze členů rodiny *miR-29*, a těmito geny byla potvrzena analýzou hladin jejich proteinů po transfekci *miR-29c* do buněk CLL linií i do primárních buněk CLL. Integrin β_1, produkt genu *ITGβ1*, je molekulou významnou pro buněčnou adhezi, čímž také např. zabraňuje buněčné smrti a podporuje proliferaci. *ITGβ1* vykazoval nejstabilnější a nejsignifikantnější snížení exprese po transfekci i po vložení plazmidu se stabilní expresí *miR-29c*. Inverzní korelace mezi *miR-29c* a *ITGβ1* byla proto potvrzena i dalšími metodami, např. pomocí kvantitativní PCR či průtokové cytometrie.

Získané výsledky zdůrazňují význam rodiny *miR-29* v kontextu buněk CLL a jejího mikroprostředí. Vzhledem k významným funkcím, jež cíle *miR-29* včetně zde potvrzeného integrinu β_1 zastávají, toto znovu podtrhuje potenciál využití *miR-29* jako prognostického markeru v diagnostice CLL.

11 Summary

Chronic lymphocytic leukemia (CLL) is the most common adult haematologic malignancy in the Western world. Accumulation of $CD5^+$ $CD19^+$ malignant B lymphocytes in peripheral blood (PB), secondary lymphoid organs and bone marrow is the main characteristic of this malignancy. Circulation between PB and lymphoid organs is essential for CLL cells survival. Within a lymphoid organ, CLL cells interact with components of the lymphoid microenvironment – various immune cells or elements of the extracellular matrix. Interactions with microenvironmental molecules lead to activation of pro-survival or pro-proliferative pathways in CLL cells. Out of these signalling cascades, the B cell receptor (BCR) pathway is the most studied in the context of CLL. Besides various proteins and kinases involved in its pathway, BCR also influences multiple microRNA (miRNA) molecules.

MiRNAs are short non-coding RNA molecules and important post-transcriptional regulators of gene expression due to their ability to block translation or trigger mRNA degradation. Genes regulated by miRNAs often are of oncogenic or tumour-suppressive character. Thus, deregulation of miRNA expression is commonly observed in malignancies including CLL. Moreover, some of these miRNAs can be influenced by signals coming from the microenvironment which is also the case of miRNAs belonging into the *miR-29* family.

The aim of this thesis was to identify and validate novel *miR-29* targets in the context of CLL and its microenvironment. After initial next generation sequencing analysis of transcriptome of primary CLL cells, a group of potential genes of interest was selected. The interaction between *miR-29c*, one of the members of the *miR-29* family, and the selected targets was studied in both CLL cell lines and in primary CLL cells. Downregulation of protein levels was observed for all three main candidate genes – *AKAP5*, *KLF4* and *ITGβ1*. Integrin β_1, product of the *ITGβ1* gene, is an important adhesive molecule of CLL cells which thus contributes to their survival or proliferation. Since the *ITGβ1* gene showed the most reproducible downregulation after *miR-29c* transfection or stable overexpression, its relationship with *miR-29c* has been confirmed by additional approaches such as qPCR or intracellular staining.

These results highlight the importance of *miR-29c* role in the context of CLL microenvironment. Moreover, they further confirm *miR-29c* potential as a reliable prognostic marker in CLL diagnostics.

12 Literature

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