CDK5 Involvement in Cancer and in the Tumor Microenvironment

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CDK5 involvement in cancer and in the tumor microenvironment.

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

Floriana Farina

The Open University  JUNE 2020
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Figure 5. 1 p35 expression in THP-1 polarization. Representative immunoblot and quantification of THP1-derived macrophages differentiated 72h with PMA, then polarized toward pro-inflammatory macrophages (M1, 20ng/ml LPS and 100ng/ml IFNγ treated cells) and alternatively-activated macrophages (M2, 20ng/ml IL-4 treated cells) for 4h and 24h (A). p35 mRNA expression was measured after p35 overexpression in THP-1 cells (B). Representative immunoblot and quantification of THP1-derived macrophages overexpressing p35 (C). Results are representative of three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used in A, Student’s t-test was used in B and C. Significant differences are indicated by: ***P<0.001, **P<0.01.

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**Figure 7.2** M2-cytokines production in shCDK5 BMDM. Change in classical M2 cytokines mRNA expression after M2 polarization, in CDK5-lacking BMDM. BMDM cells were treated with 20ng/ml IL-4 and 20ng/ml IL-13 for 6h and 24h. Classical M2 cytokines expression was measured (IL-10 and TNF-α). Results are representative of three independent experiments. The data are presented as the means±SD. To compare means Student’s t-test was used. Significant differences are indicated by: **P<0.01.
Table 1. Schematic classification of DLBCL, according to Ann Arbor system. This is the landmark lymphoma staging classification system for both Hodgkin lymphoma and non-Hodgkin lymphoma. It is named after the town of Ann Arbor in the US state of Michigan where the Committee on Hodgkin's Disease Staging Classification met in 1971 to agree on it.

Table 2. Schematic representation of DLBCL patient features and outcome, in relationship with their classification.

Table 3. Schematic classification of Breast cancer classification, according to TNM staging.
ABC  Activated B-Cell Like
APC  Allophycocyanin
ARG1 Arginase I
ASCT Autologous Stem Cell Transplantation
ATCC American Type Culture Collection
B-L  B-Lymphocytes
BC  Breast Cancer
BCA Bicinchoninic Acid
BCR B-Cell Receptor
BRDU 5-Bromo-2'-Deoxyuridine
BTZ Bortezomib
C  Centroblasts
CDK5 Cyclin-Dependent Kinase 5
CDK5R1/P35 Cyclin-Dependent Kinase 5 Activator 1
CDS Coding Sequence
CGB-L Germinal Centre B-Lymphocytes
CHOP Cyclophosphamide, Doxorubicin, Vincristine, And Prednisone
DCS Dendritic Cells
DLBCL Diffuse Large B-Cell Lymphoma
DMSO Dimethyl Sulfoxide
EDTA Ethylenediaminetetraacetic Acid
FDC Follicular Dendritic Cells
FFPE Formalin-Fixed, Paraffin-Embedded
FPKM Fragments Per Kilobase Million
GC Germinal Centre
GCB Germinal Centre B-Cell Like
HC Healthy Controls
HD High Dose
HEK293T Human Embryonic Kidney 293t
HER2 Human Epidermal Growth Factor Receptor 2
HSC Hematopoietic Stem Cell
IF Immunofluorescence
IFN- Γ Interferon Gamma
IG Immunoglobulin
IHC Immunohistochemistry
IL Interleukin
ILC Innate Lymphoid Cells
INOS Inducible Nitric Oxide Synthase
LPS Lipopolysaccharide
MB-L Memory B-Cells
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<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression Free Survival</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone-Receptor</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>R</td>
<td>Rituximab</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
</tr>
<tr>
<td>SCFC</td>
<td>Small Cleaved Follicle Center B-Cell</td>
</tr>
<tr>
<td>SCR</td>
<td>Scramble</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic Hypermutations</td>
</tr>
<tr>
<td>SHRNA</td>
<td>Short Hairpin RNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer And Activator Of Transcription</td>
</tr>
<tr>
<td>T</td>
<td>Tyr</td>
</tr>
<tr>
<td>TAMS</td>
<td>Tumor Associated Macrophages</td>
</tr>
<tr>
<td>TDT</td>
<td>Deoxynucleotidyl Transferase</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor Microenvironment</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor Node Metastasis</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Uridine Nick-End Labeling</td>
</tr>
<tr>
<td>VSMCS</td>
<td>Vascular Smooth Muscle Cells</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
The work described in this dissertation was performed in IRCCS Istituto Clinico Humanitas (ICH). The work reported here was carried out entirely by the author, unless otherwise indicated. The first part of the results here discussed was accepted for publication in Cell Death and Disease (Farina F.M.*, Inguscio A.*, Kunderfranco P., Cortesi A., Elia L., Quintavalle M. MicroRNA-26a/cyclin-dependent kinase 5 axis controls proliferation, apoptosis and in vivo tumor growth of diffuse large B-cell lymphoma cell lines; 2017, Cell Death and Disease).
Part 1
Cyclin-dependent kinase 5 (CDK5) is a serine/threonine kinase belonging to the CDK family, firstly discovered as a major player of neuronal development, but several groups revealed additional roles of CDK5 especially in cancer. Indeed, CDK5 is reported to act as a tumor promoter in various types of solid tumors, but no reports indicated CDK5 involvement in hematopoietic malignancies development and progression.

Diffuse large B-cell lymphoma (DLBCL) is the most frequent type of non-Hodgkin lymphoma. Despite a favourable therapeutic response to first-line chemo-immunotherapy, still 30–40% of patients are refractory, or relapse after this treatment. Thus, alternative strategies must be sought.

We demonstrated that CDK5 and its activator, cyclin-dependent kinase 5 activator 1 (CDK5R1 or p35), are overexpressed in DLBCL. Their role is mediated through the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and regulate proliferation and survival of DLBCL cells.

MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate gene expression and are involved in cancer initiation and progression. We identify miR-26a as direct regulator of p35 expression and CDK5 activity. In addition, we show that miR-26a ectopic expression leads to a drastic reduction of DLBCL tumor progression both in vitro and in vivo.

In conclusion, these results demonstrate an important role for miR-26a and CDK5 together in the survival and growth of DLBCL cells, suggesting the existence of potential novel therapeutic targets for the treatment of DLBCL.

**Graphical abstract Part 1.** Schematic representation of CDK5 involvement in DLBCL progression.
Part 2
Breast cancer is one of the most common cancers and the leading causes of death in women worldwide. It is complex tissues composed of neoplastic cells, surrounded by several stromal cell types that comprise the tumor microenvironment. Within the microenvironment, immune cells and especially Tumor-Associated Macrophages (TAMs) have been reported to regulate cancer progression and metastasis formation, and that a paracrine interaction between cancer cells and macrophages is required for invasion and intravasation in vivo. Until now, no previous work described CDK5 involvement in breast cancer-associated Macrophages.

Firstly, we revealed that CDK5 is not only expressed in the tumor mass of human breast cancer specimens, but it is also detectable in the tumor microenvironment. In particular, its expression was found in TAMs.

To dissect CDK5 involvement in TAMs, we measured its expression through in silico analysis and in vitro assays. We found that CDK5 is expressed in differentiated macrophages and it is downregulated after inflammatory stimuli. Through gain and loss-of-function experiments, we found that CDK5 inhibition is necessary for M1 polarization and M1 cytokines production. In addition, CDK5 silencing in vitro, induced a reduction in podosome formation.

To conclude, these data suggest p35/CDK5 involvement in cancer invasion and in the regulation of immune escape mechanisms. In addition, CDK5 expression in TAMs might be used as prognostic marker for the outcome evaluation of cancer patients. We aim to demonstrate that CDK5 inhibition might be a viable strategy to treat invasive breast cancer.

**Graphical abstract Part 2.** Schematic representation of CDK5 involvement in TAMs.
Publication related to this thesis:

Publications not related to the PhD project:
- Bianchessi V., Vinci M.C., Nigro P., Rizzi V., Farina F., Capogrossi M.C., Pompilio G., Gualdi V., Lauri A. Methylation profiling by bisulfite sequencing analysis of the mtDNA Non-Coding Region in replicative and senescent Endothelial Cells; 2016, Mitochondrion.

Reviews

- Stratton M.S., Farina F.M., Elia L. Epigenetics and vascular diseases; 2019, Journal of Molecular and Cellular Cardiology
INTRODUCTION
1. **CANCER**

Cancer comprises a broad spectrum of diseases, characterized by abnormal and uncontrolled cellular growth. It represents the second leading cause of death globally, accounting for an estimated 9.6 million deaths, in 2018 (Figure 1). According to the estimation from World Health Organization (WHO), there will be 29.5 million people diagnosed as cancer and 16.3 million people died because of cancer by 2040.

![Figure 1](image_url) 
*Figure 1* Estimated age-standardized mortality rates of cancer per 100,000 (GLOBOCAN 2018, IARC).

Despite recent advances in early detection and targeted therapy, cancer remains a big health challenge with the highest priority for investigation [1].
2. IMMUNE SYSTEM

Immune system represents our host defence system, able to recognize foreign antigens and invading organisms (viruses, bacteria, fungi, protozoa). Immune cells are generated in the bone marrow from a multipotent precursor, known as hematopoietic stem cell (HSC) [2], through a process called haematopoiesis. This process can lead to two different commitment, categorized as lymphoid or myeloid lineage [3]. The myeloid progenitors develop into granulocytes (basophils, eosinophils and neutrophils) and monocytes, differentiating into macrophages and dendritic cells (DCs). On the other side, the lymphoid lineage gives rise to B cells, T cells, Natural Killer (NK) cells and innate lymphoid cells (ILC) [3]. These different types of immune cells are specifically enrolled in an effector response.

Classically, immune response is divided into two main sub-systems, known as innate and adaptive immunity (Figure 2). The innate or inborn immunity represents the first line of defence and consists of NK, mast cells, eosinophils, basophils, neutrophils, DC, and macrophages (Mφ), together with the “not-specific” complement system. On the other side, the adaptive immune system is able to respond specifically and selectively to different antigens, due to the ability of B and T lymphocytes. Innate immunity is characterized by the ability to support a rapid effector response, when encountering a pathogen for the first time. It takes place within minutes or hours of infection and therefore, it is responsible for the “first line” defence of host. The innate immune response consists of physical (e.g. skin and mucosal membranes), chemical (e.g. low pH and antimicrobial peptides), and cellular defences (e.g. phagocytic cells, the complement system, and inflammation) against pathogens and tissue damage [4].

The second line of defence, or adaptive immunity is referred to an “acquired” immunity that is specifically reactive to the pathogen presented. One of the peculiar characteristics of adaptive immunity is the ability to proliferate of effector cells, with the aim of efficiently counteract the infection. This process is achieved through cellular selection, differentiation, and clonal expansion. Clonal expansion of lymphocytes is the hallmark of the adaptive immune system, able to sustain a reproducible and balanced immune response with T or B lymphocyte sharing the same antigen receptor repertoire.
Figure 2. Cellular components of the mammalian immune system, consisting in “innate” and “adaptive” immunity. Basophils, eosinophils, neutrophils, macrophages, mast cells, natural killer cells, and dendritic cells mediate the innate immunity. The adaptive immune system includes B cell-mediated humoral immunity and T cell-mediated cellular immunity, both of which are directed towards the specific antigens.

In summary, while the innate immune response is an immediate process (minutes to hours) associated with the response against general pathogens or foreign particles, the adaptive immune response is highly specific by B cells activity and sustained through the activation of memory T cells (Figure 2).
3. **B LYMPHOCYTES**

B lymphocytes are small (6-10 µm) cells that represent the principal cellular niche of the humoral response, due to their ability to produce antibodies [5]. B cells are generated from HSC within the bone marrow in a multistep differentiation process [6], and they continue to differentiate until a functional B cell receptor is expressed.

B lymphocyte development consists of two phases: an antigen-independent phase that develops in the primary lymphoid tissues, followed by an antigen-dependent one that takes place in secondary lymphoid tissues (Figure 3).

*Figure 3. Schematic representation of the two compartments of B cell differentiation. Mature B cells develop from hematopoietic stem cells in the bone marrow. Then cells migrate into peripheral lymphoid tissues, where these cells can react with antigen.*

In the bone marrow, a primary B-cell repertoire is produced through several rearrangements that involve light and heavy chain immunoglobulin (Ig) rearrangement of the variable (V), diversity (D) and joining (J) gene segments that led to the generation of an unique antigen B-cell receptor (BCR). BCR expression is fundamental for B cell precursors maturation. These cells have not been exposed to external antigens and are known as “naïve B cells”.

Mature B cells, migrating from the bone marrow to the secondary lymphoid organs (spleen, lymph nodes and mucosa-associated lymphoid tissues), can co-express IgM and IgG before they start their clonal expansion. In these organs, the encountering of an antigen is required for the final stages of B cell development. Antigen-activated B cells may enter the germinal centre (GC), where they start proliferating and differentiating. This process, also known as clonal expansion, leads to the production of specific clonal lineages that will react against one specific antigen. Th-cell mediated stimulation of B cells induces the secretion of soluble form of IgM and class switch [7]. In particular, in the GC zone, a naïve B-
cell is activated by antigen receptor stimulation from a T helper cell and differentiates into a centroblast that continue proliferating in the “dark zone” of the GC. In this region, B cells undergo somatic hypermutation (SHM), where a single base-pair changes are randomly introduced \(1 \times 10^3\) bases per generation into the IgV regions), in order to select for immunoglobulin with higher affinity for a specific antigen [8]. At the end of the clonal expansion and SHM processes happening in the dark zone, B-cells move to the “light zone” and they morphologically became centrocytes. At this differentiation stage, B cell fate depends on other GC resident cells like T-cells and follicular dendritic cells [9, 10]. Indeed, B cells are selected in the light zone further depending on their ability to bind antigen presented to them by fDC and T cells. This process is a negative selection, where most B cells acquire deleterious mutations in the IgV regions and then undergo apoptosis. Further, cells with the highest affinity rate are selected for differentiation. Remaining B cells can also undergo Ig class switches (from IgM and IgD to other classes), by isotype switch recombination. This process facilitates the production of several antibodies with different effector functions due to replacing Ig heavy-chain (IgH) constant C region by a downstream C region gene (e.g. Cγ, Cα, Cε), which allows the expression of IgG, IgA, and IgE maintaining the specificity [11].

IgH recombination changes occur, while SHM induce mutations in the IgV region, able to produce a population of B cells with an increase (or decrease) affinity for a particular antigen. These genetic modifications are essential for normal immune response but also a source of DNA damage that can become pathological with lymphoma [12].

Due to the high plasticity and the profound genomic rearrangements occurring in this fine process, with the combination of other factors, including microenvironment and epigenetics, it is possible the arousal of some mutations that led to lymphomagenesis [13]. In addition, abnormal B cell are able to proliferate much more than normal cells, and they stop responding to inhibitory signals that usually limit the cellular growth.
4. **DIFFUSE LARGE B CELL LYMPHOMA (DLBCL)**

Diffuse large B-Cell Lymphoma (DLBCL) is the most common type of Non-Hodgkin Lymphoma (NHL) among adults, accounting for 30% of adult lymphomas [14, 15]. According to the updated WHO classification, DLBCL is a heterogeneous disease, which comprises several subgroups of mature B-cell neoplasms [12]. Its aetiology is largely unknown, but it is thought to be associated with immunosuppression, genetic susceptibility, autoimmune disease, infective agents as well as other environmental factors [16]. Several classification systems have been proposed, basically based on the common morphology, the immunophenotype, genetic alterations, and clinical outcomes in order to stratify patients, to dissect and to discover possible pathogenetic mechanisms, and to design an effective therapy. Most DLBCLs arise from germinial B lymphocytes at different stages of differentiation and recurrent genetic alterations mostly contribute to the molecular pathogenesis of the disease. Gene expression profiling technique allowed identifying at least two molecular subtypes of DLBCL with different prognoses: the germinal centre B-cell like DLBCL (GCB DLBCL) and the activated B-cell like DLBCL (ABC DLBCL), that originate from post-germininal center B cells that are interrupted during plasmocytic differentiation. (Figure 4). The GCB and the ABC groups represent lymphomas arising from different stages of B cell differentiation. Due to this classification, it has shown that the ABC subtype is characterised by poor prognosis and inferior outcome following standard chemotherapy regimens (three-years progression free survival of 40% as compared to 75%, p<0,001).

![Figure 4. Schematic representation of DLBCL development. GCB-DLBCL originate from light zone B cells. ABC-DLBCL shows characteristics of late post-GC B cells.](image-url)
Specific markers, including CD10, LM02, and BCL6 are expressed in GCB patients that displays a better response to traditional chemotherapy, on the contrary ABC patients express lower levels of BCL6 and are often refractory to standard chemotherapy regimen (R-CHOP) [17, 18]. This response to treatment can be linked to the molecular pattern of ABC patients, since they showed constitutive activation of NF-κB which may be related to the presence of mutations of multiple genes regulating this pathway [19, 20]. In addition, constitutively activated signal transducer and activator of transcription 3 (STAT3) is correlated with a more advanced clinical stage and overall poor survival in DLBCL [21, 22]. STAT3 is a member of the STAT family of transcription factors, able to dimerize and translocate into the nucleus after its activation, mediated by phosphorylation of a tyrosine residue Tyr705 (T705). STAT3 transcriptional activity and DNA binding can be further modified by the phosphorylation of Ser727 (S727). Activated STAT3 is fundamental in the regulation of tumor growth, invasion, cellular proliferation, angiogenesis, immune response, and survival [23, 24].

The identification of DLBCL molecular subtypes has improved their classification, but it can’t be translated in profound changes in the clinical practice (Table 1).

<table>
<thead>
<tr>
<th>ANN ARBOR CLASSIFICATION SYSTEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
</tr>
</tbody>
</table>
| I | Single lymph node  
| IE | Single extralymphatic organ or site  
| Stage II |  
| II | Two or more lymph nodes  
| IIE | Extralymphatic organ or site and one or more lymph node regions on the same side of the diaphragm  
| Stage III |  
| III | Lymph node regions on both sides of the diaphragm  
| IIS | Spleen involved  
| IIE | Extralymphatic site involved  
| Stage IV |  
| Diffuse or disseminated involvement of one or more extralymphatic organs or tissues, with or without associated lymph node involvement  

Table 1. Schematic classification of DLBCL, according to Ann Arbor system. This is the landmark lymphoma staging classification system for both Hodgkin lymphoma and non-Hodgkin lymphoma. It is named after the town of Ann Arbor in the US state of Michigan where the Committee on Hodgkin's Disease Staging Classification met in 1971 to agree on it.
Immuno-chemotherapy regimen represents the standard treatment for DLBCL patients. Rituximab (R), a monoclonal antibody against CD20, is administered in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) [25]. This approach (R-CHOP) is effective for the vast majority of cases, even in late stages [26]. However, still 30-40% of patients are refractory, or relapse after this treatment. The clinical approach to those patients includes high dose therapy and autologous stem cell transplantation (HD-ASCT). However, only around 50% of relapsed/refractory DLBCL patients are eligible for this approach. Further, of eligible patients, only <10% of patients with primary refractory disease are cured (Table 2).

Due to the very low response rates, seen in secondary therapies, and the high proportion of patients, ineligible for those treatments, alternative strategies and novel agents are urgently required.

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>GCB</th>
<th>ABC</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell of Origin</td>
<td>Germinal centre B-cell</td>
<td>Post-germinal centre B-cell</td>
<td>Unknown</td>
</tr>
<tr>
<td>Frequency</td>
<td>~45%</td>
<td>~35%</td>
<td>&lt;20%</td>
</tr>
<tr>
<td>Median age(years)</td>
<td>61</td>
<td>66</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Survival at 5 years</td>
<td>~60%</td>
<td>~40%</td>
<td>Low</td>
</tr>
<tr>
<td>Curable</td>
<td>~50%</td>
<td>~30%</td>
<td>Low</td>
</tr>
</tbody>
</table>

Table 2. Schematic representation of DLBCL patient features and outcome, in relationship with their classification.
5. BREAST CANCER

Breast cancer is the second most common cause of cancer-related death among women (WHO), accounting 2.1 million women each year. The incidence of new cases is increasing every year and the probability for a woman to get a breast cancer is very high (1:8) compared to other tumors (www.cancer.org) []. In 2018, it was reported to cause 15% of female death worldwide. Family history [27], genetic mutations (i.e. BRCA1, BRCA2) [28], hormonal factors [29], and unhealthy lifestyle habits like smoking, alcohol and unhealthy diet [30] are considered to be the main risk factors for breast cancer development. Breast cancer can be classified into non-invasive (carcinoma in situ) and invasive carcinomas, depending on its growth (Figure 5) [31].

![Figure 5. Schematic representation of breast cancer classification related to tumor growth.](image)

Invasive carcinomas are characterized by cellular spread to the surrounding connective tissues and the metastasization to distant organs of the body. Other less common histological groups are identified as inflammatory, medullary, apocrine, mucinous and tubular carcinomas.

The characterization of breast cancer depends on the Tumor Node Metastasis (TNM) classification, which considers tumor size, lymph node involvement and distant metastasis.

Breast cancer classification is of utmost importance and it is necessary to define the precise prognosis and plan an effective therapy. The most established classifications in breast cancer includes molecular subtypes [32], TNM staging system [33] and grade [34] (Table 3).
## TNM Staging

<table>
<thead>
<tr>
<th>T</th>
<th>Size or extent of the primary tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Tumor can't be defined</td>
</tr>
<tr>
<td>TIS</td>
<td>Ductal carcinoma in situ (DCIS)</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of tumor</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor mass is &lt;2cm</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor mass is 2-5cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor mass is &gt;5cm</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour has spread into the chest wall/skin</td>
</tr>
<tr>
<td>N</td>
<td>Spread to regional lymph nodes</td>
</tr>
<tr>
<td>NX</td>
<td>Lymph node spread can't be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Regional lymph node metastasis present</td>
</tr>
<tr>
<td>N2</td>
<td>Tumor spread to regional lymph nodes stucking to surrounding tissues</td>
</tr>
<tr>
<td>N3</td>
<td>Tumor spread to lymph nodes related to breastbone/collarbone</td>
</tr>
<tr>
<td>M</td>
<td>Presence of distant metastasis</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Metastasis to distant organs</td>
</tr>
</tbody>
</table>

*Table 3.* Schematic classification of Breast cancer classification, according to TNM staging.

Breast tumors can be further classified into four distinct different molecular subtypes taking advantage of four well-known biomarkers, including estrogen-receptor (ERα), progesterone-receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 [32]. This molecular classification is often a key element to define patient prognosis and to the correct choice of therapeutic strategy [35]. Taking advantage of this classification, it is possible to define mainly four different molecular subtypes of breast cancer that can be summarized as following:

1. **Luminal A** breast cancer is hormone-receptor positive (ER and/or PR positive), HER2 negative, and has low levels of the protein Ki-67, which helps control how fast cancer cells grow. Luminal A cancers are low-grade, tend to grow slowly and also has the best outcome with hormonal therapy [36].

2. **Luminal B** breast cancer is hormone-receptor positive (ER and/or PR positive), and either HER2 positive or HER2 negative with high levels of Ki-67. Luminal B cancers generally grow slightly faster and are more aggressive than luminal A cancers [37]. Nevertheless, patients with luminal B subtype can benefit from hormonal therapy in combination with treatment with anti-HER2 antibody Trastuzumab (Herceptin), depending on HER2 expression [38].
3. HER2-enriched breast cancer is hormone-receptor negative (ER and PR negative) and HER2 positive. This kind of tumors has a very fast-growing rate and a worse prognosis, but anti-HER2 regimen (fam-trastuzumab deruxtecan, trastuzumab, pertuzumab, lapatinib, and neratinib) is very often successful.

4. Triple negative breast cancer (TNBC) is hormone-receptor negative (ER and PR negative) and HER2 negative. This type of cancer is more common in women with BRCA1 gene mutations and it is characterized by poor prognosis, due to lack of specific drug targets [37].

The treatment of breast cancer includes surgery, chemotherapy, radiation, and hormone therapy [39]. Although the overall survival and relapse-free survival rates increased in the past few decades due to novel therapies, the treatment of breast cancer is not yet satisfying [40]. Therefore, the identification of new potential targets for breast cancer recognition and therapy is becoming of pivotal importance in the clinical practice, to deal with patients and to establish a targeted therapy.
6. **CDK5, AN ATYPICAL MEMBER OF CYCLIN-DEPENDENT KINASES**

Cyclin-dependent kinase 5 (CDK5), also known as neuronal Cdc2-Like Kinase (NCLK), is a small 33 KDa serine/threonine kinase belonging to the CDK family [41]. It was originally identified based on the structural similarity to Cdk1 (Cdc2) and Cdk2, key regulators of eukaryotic cell cycle progression. The proteins of this family are kinases that specifically phosphorylate threonine or serine residues. In particular, CDK superfamily is composed of nine small serine/threonine kinases (30–35kDa), numbered from Cdk1 to Cdk20, according to their discovery [42].

CDKs are mainly involved in biological functions related to cell cycle progression like mitosis, regulation of cellular processes [43], but also cellular senescence, differentiation and apoptosis [44-46], through gene transcription modification [47].

In proliferating cells, dysregulation of CDKs leads to cancer development, whereas their inhibition in neuronal precursors leads to terminal differentiation [48].

Generally, CDKs require the binding of an activator protein, named cyclins, to be activated and the phosphorylation in a loop of activation. Although specific CDKs are associated with different cell cycle phases, their activities can sometimes overlap, depending on the binding with different cyclins.

Unlike other members of the CDK family, CDK5 is not activated by cyclins, but rather by non-cyclin proteins, termed p35 and p39. When CDK5 is bound by its activators, it adopts a structural conformation comparable to those of a canonical CDKs [49]. The abundance of p39 and p35 in the nervous system cause CDK5 importance in its development and function [50-52].

In the last decade, CDK5 has been demonstrated to have a role in lots of other cellular types, like pancreatic β cells, monocytes, corneal epithelial cells, germ cells [53, 54].

Recently, CDK5 involvement in many other different biological mechanisms, such as activation of immune system [55], cell migration [56], angiogenesis [57], and regulation of gene expression [58, 59] in different cell types has been raised (all summarized in Figure 6).

Cyclin-dependent kinase 5 (CDK5), also known as neuronal Cdc2-Like Kinase (NCLK), is a small 33 KDa serine/threonine kinase belonging to the CDK family.
Moreover, several studies revealed a new role of CDK5 in the development and progression of cancer [60]. In particular, CDK5 increased expression is often associated with poor prognosis in numerous malignancies like head and neck squamous cell carcinoma [61], hepatocellular carcinoma [62], as well as glioblastoma [63], lung [64], prostate [65], breast [66], colorectal [67], thyroid [68], ovarian [69], pancreatic cancer [70] (Figure 7).
Figure 7. The emerging role of CDK5 in cancer [60]. CDK5 is emerged as one activator of cancer progression; through its phosphorylation activity is able to activate different substrates involving aberrant proliferation of tumor cells.
7. **miRNA**

microRNAs are small RNA molecules as short as 20-22 nucleotides that exert negative regulatory effects on mRNA translation [71, 72] (Figure 8). microRNA originate from longer precursors transcribed in the nucleus into primary transcript microRNA (pri-miRNA). Pri-miRNA are characterised by hairpin structures that undergo further processing finally resulting in mature miRNA. Hairpins are recognised by a type II endonuclease complex composed of DGCR8 and Drosha subunits that binds to a characteristic mismatch at the basis of the stem and excide it, leaving two overhanging nucleotides at 3’ end. Excided stem-loops are precursors of microRNA (pre-miRNA) and are exported to the cytoplasm by exportin V that recognise the overhanging nucleotides left by Drosha [73]. In the cytoplasm pre-miRNA are further cleaved by another endonuclease, the Dicer enzyme, which cuts the loop between 5p and 3p arms, leaving an imperfect miR-5p/miR-3p duplex [74]. The duplex denatures and one of the arms is loaded in the RISC complex. Although for most miRNAs only one of the arms is generally recruited in the RISC complex while the other undergoes degradation, for some miRNAs both strands may be loaded. The selected miRNA strand directs the riboproteic complex to its molecular targets and mediates translational silencing through different mechanisms [75]. This can be done by inhibiting translation, degrading the mRNA transcript, or silencing transcription [76]. Perfect complementarity between miRNA and target mRNA results in target degradation [77], while incomplete binding leads to mRNA silencing either by competition with ribosome binding or by sequestering the ribonucleic complex to Processing Bodies (P-bodies) [78]. The critical region for miRNA-mRNA interaction is named seed sequence [79]. It consists of a region of perfect match generally spanning 6 or 7 nucleotides from position 2 at the 5’ end of RISC-loaded mature miRNA. Since complementarity is restricted to the short sequence of the seed region every miRNA is allowed to bind a wide array of different targets. This feature designates miRNAs as regulatory molecules able to finely tune complex cellular processes.
Figure 8. miRNA processing and target recognition. The pri-miRNA is processed by the Drosha enzyme to a stem-loop-structured miRNA precursor molecule (pre-miRNA). The pre-miRNA is then transported in the cytoplasm where the Dicer enzyme cleaves off the double stranded portion of the hairpin and generates the mature miRNA, which is incorporated into miRNA-protein complexes. The mature miRNA binds to partially complementary recognition sequences (seed sequences) on 3’-UTRs of mRNAs and targets them for decay or translational repression [72].
8. miR-26a

The miR-26 family comprises three different members, residing on different chromosome and named miR-26a-1, miR-26a-2, and miR-26b. Several groups described miR-26 family as a key player of fundamental physiological processes related to cellular growth, development, and activation [80]. In particular, it is reported to be a fundamental key player in myogenesis, during myotubes differentiation [81], in angiogenesis [82], in Vascular smooth muscle cells (VSMCs) differentiation [83], and in fibrosis [84].

In addition, miR-26a anti-tumorigenic attitude was firstly described in high Myc inducible expressing tumors, both in vitro and in vivo. Moreover, an in vivo lymphoma selection model suggested a possible role of miR-26 as a tumor suppressor in Myc driven B-cell Lymphoma [85]. Further studies demonstrated that low miR-26 expression correlates with development and progression of several cancer types [86], including breast [87], prostate [88], and bladder [89] cancer.

On the contrary, in other tumor types, miR-26 is reported to exert a pro-tumoral role, as described in lung cancer [90], in T-cell lymphoblastic leukemia [91], and in glioma [92].

This Janus-faced attitude needs further investigation to better understand miR-26 involvement in cancer, and in particular in DLBCL.
9. TUMOR ASSOCIATED MACROPHAGES (TAMs)

The tumor microenvironment represents a potential therapeutic target that is still to be explored. The immune system interacts with tumor cells, acting as a key modulator of tumor biology: it can interfere with tumor growth and responsiveness to therapy, thus representing a major cause of cancer development and progression [93]. In this context, one of the major players in this context is represented by Tumor-Associated Macrophages (TAMs) [94]. TAMs are closely involved in multiple stages of carcinogenesis [95]: they contribute to cancer initiation [96], growth [97], invasion [98], and metastasis [99], through the production of a various array of cytokines, growth factors, pro-angiogenic factors, and matrix metalloproteinases [100, 101]. In the context of cancer, TAMs are particularly abundant within the tumor where are able to stimulate cancer cell proliferation, immunosuppression and promote neo-angiogenesis to sustain tumor growth and metastasis formation [102]. Macrophages, derived from monocyte precursors, undergo specific differentiation depending on local environmental influence. The various macrophage functions are due to receptor interaction on the macrophage surface and, crucially, on the cytokine milieu in the microenvironment.

TAMs classically exhibit two states of activation known as “M1-like” and “M2-like” polarization. Macrophages can go through polarization in response to Th1 cytokines, like LPS and IFNy into an M1 state [103, 104]. This signal activation induces macrophages to produce huge amounts of pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-23, and chemokines like CCL2, CCL15, CXCL9 and CXCL10 [103-106]. M1 macrophages are also able to upregulate Nos2 production, thus inducing inducible nitric oxide synthase (iNOS), which stimulates the synthesis of nitric oxide (NO) from L-arginine [107]. Moreover, pro-inflammatory macrophages spread their production of reactive oxygen species (ROS) [108]. In general, classically activated macrophages cytokine profile amplifies the Th1 immune response. Furthermore, IFNy and TNF increase the expression of MHC class II and costimulatory molecules (e.g. CD86) on M1 macrophages. These features augment the capacity of antigen presentation by macrophages and further support T cells responses [109].

On the other hand, in response to Th2 cytokines, like IL-4 and IL-13, macrophages polarized toward an M2 state [103-105]. These cytokines are produced by basophils, mast cells and other granulocytes and important drivers of Th2 immune responses. On the other side, M2 macrophages are involved in type 2 inflammation, tissue remodelling, angiogenesis, and are believed to promote tumor growth [103-105, 110]. M2 macrophages produce high levels of interleukin-10 (IL-10), an immunosuppressive cytokine and arginase I (Arg1), important for wound healing, and express numerous scavenger receptors. The classical M1-M2 paradigm has been recently revealed to represent two ends of a spectrum that includes a variety of activation states. It has been shown in
multiple studies that macrophages can show mixed states of activation and there are multiple sub-populations of TAMs [111]. High TAM numbers have been shown to be an independent prognostic factor in many forms of cancer [112].

Figure 8. Representative schematization of Tumor Associated Macrophages involvement within the tumor.
10. TAM-targeted immunotherapy

Cancer immunotherapy is a recent approach, aimed at enhancing immune response and restoring its reactivity in order to neutralize or eliminate cancer. TAMs represent a promising cellular target to specifically modulate cancer progression. Several approaches in the last years propose TAM targeting and some of them are now under clinical trials. One of the most relevant consideration is that TAMs can constitute as much as 50% of the cell mass in breast cancer [113] and in addition, several groups have shown a correlation between breast cancer prognosis and infiltrating macrophages in the tumor mass [112, 114, 115].

![Diagram of TAM-targeted immunotherapy](image)

Figure 9. TAM-targeted immunotherapy. Pro-tumoural TAM activities, like angiogenesis, immune escape, and dissemination have been targeted in either preclinical models or therapeutic trials in humans [116].

Among cancer immunotherapy it is possible to identify two possible approaches. The first one, also known as active immunotherapy is based on the activation on long lasting anti-tumor immune responses, through the administration of tumor-associated proteins and peptides used as immunogens [117]. The second approach, known as passive immunotherapy, is aimed at triggering T cells to directly target cancer cells or activate the host adaptive immune system through the administration of monoclonal antibodies [117].
Part 1

Diffuse large B-Cell Lymphoma (DLBCL) accounts for 30% of adult lymphomas. Despite standard immuno-chemotherapy regimen efficacy, still 30-40% of patients are refractory after treatment, or relapse after autologous transplantation. Therefore, novel targets aimed at the improvement of innovative therapeutical strategies are needed.

We observed that CDK5 and p35 are overexpressed in several DLBCL cell lines, compared to B lymphocytes from healthy donors.

We hypothesize that CDK5 could play a key role in the regulation of tumor growth by promoting cell proliferation and by inducing resistance to apoptotic stimuli in neoplastic cells, and as a consequence CDK5 targeting could be an effective and innovative therapeutic strategy in patients’ management.

Part 2

TAMs can promote cancer progression and metastasis, and a paracrine interaction between tumor cells and macrophages is required for invasion and extravasation.

Several works highlighted CDK5 role in cancer progression and invasiveness both in solid tumors and in hematopoietic malignancies. Until now, no previous work described CDK5 role in the TME and in TAMs.

Therefore, the study of CDK5 involvement within the TME could provide new insights in the regulation of tumor inflammation in breast cancers.

In addition, we hypothesize that CDK5 activity could mediate macrophage infiltration within the tumor by the regulation of podosome formation, thus affecting cancer promotion and metastatisation processes.

In light of this, CDK5 targeting in the TME could be a promising therapeutic strategy that could overcome chemotherapy resistance and provide new immunotherapeutic approaches.
MATERIALS AND METHODS
1. **Cells.**

Cell lines were cultured in RPMI-1640 (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA), 10000 U/ml penicillin, 10000 mg/ml streptomycin (Pen/Strep, Lonza Walkersville, MD), 20 mM L-Glutamine (Ultraglutamine, Lonza Walkersville, MD), and periodically tested for mycoplasma contamination.

**DLBCL cell lines.** In this study different types of DLBCL cell lines were used, including Germinal Centre B-cell (GCB) cell lines (SU-DHL-4, SU-DHL-6, SU-DHL-16) and Activated B-cell (ABC) cell lines (SU-DHL-2, SU-DHL-8, and RCK-8). All of them were kindly provided by L. Pasqualucci, M.D. (Columbia University, New York, USA).

**B-cells.** Normal B lymphocytes were negatively selected by an immunomagnetic technique (Human B Cell Enrichment Kit, Stem Cell Technologies, Vancouver, British Columbia, Canada) from the peripheral blood of consenting healthy donors.

**THP-1 cells.** Human monocytic leukaemia THP-1 cells were purchased from American Type Culture Collection (ATCC) consortium. Differentiated macrophages were obtained after 1,6 µM PMA treatment for 72h.

**BMDM.** Bone Marrow Derived Macrophages were collected from murine femurs and tibias. Bones were cleaned of remaining tissue and bone marrow cells were flushed from bones using a 0.5x16mm needle and 5ml syringe. Cells were counted using a haemocytometer and resuspended in complete at a concentration of 5x10⁵ cells/ml. Mesenchymal cells were removed after 24h of adherence on plastic. Further, macrophages were differentiated with 20ng/ml mM-CSF (Merk Millipore).

THP-1 and BMDM cells were treated with LPS from Escherichia coli serotype 055: B5 (Sigma-Aldrich). Human and murine IFNγ and IL-4, were purchased from R&D Systems. Dexamethasone was from Sigma-Aldrich. Prior to stimulation, exhausted media were removed and and cells were washed with PBS. Stimuli were prepared at the desired dilutions in serum-free culture media.

2. **Generation of loss- and gain-of-function experiments on DLBCL cell lines.**

Lentiviral CDK5 and p35 short hairpin RNA (shRNA) (shCdk5#1 and #2, shp35#1 and #2) were obtained from the RNAi Consortium (http://www.broadinstitute.org/rnai/trc). A scramble shRNA was used as a control. For p35-expressing lentiviral particles, a p35-expressing vector containing the human CDS p35 complementary DNA (NM_003885.2) was cloned in lentiviral plasmids. Lentiviral particles were generated using a three-plasmid system, in HEK293T cells. Supernatants were collected and lentiviral
particles were concentrated with PEG purification system. Cells were further transduced with lentiviral particles and polybrene at 8 µg/mL (Sigma-Aldrich, St. Louis, MO), followed by puromycin selection at 48 hours after the transduction. Efficiency of knockdown or over-expression was validated by immunoblotting and/or qRT-PCR.

3. **Proliferation assay.**

For growth curves, all cell lines were plated and grown under normal conditions for 1 to 4 days. Every day, cells were harvested, diluted in Trypan blue to assess viability, and counted in the Burker’s chamber. Cell proliferation was further assessed by MTT assay according to the manufacturer’s recommendations (Roche Diagnostics GmbH, Mannheim, Germany): 40,000 cells were seeded in triplicates in a 96-wells; 5 mg/mL MTT per well was added both 24 and 48 hours after seeding. Cells were then incubated for 4 hours and DMSO was used to dissolve crystals. 5-Bromo-2’-Deoxyuridine (BrdU) incorporation was also used to confirm cellular proliferation. Cells were plated and grown under normal conditions for 24 hours. Cells were labelled with 3 µg/ml BrdU for 4 hours, washed twice with PBS, fixed in 1% formaldehyde and then spotted on slides. Immunofluorescence was performed using anti-BrdU antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according manufacturer’s protocol.

4. **Apoptosis assays.**

Apoptotic cell death was detected by Annexin-V–Allophycocyanin (APC)/Propidium Iodide (PI) double staining (Immunostep, Salamanca, SP, EU), according to the manufacturer’s instructions. This staining allows quantification of early (annexin-V+/PI-) and late (annexin-V+/PI+) apoptotic cells, as well as necrotic cells (annexin-V-/PI+). In order to detect cell death, cells were recovered after 24h, double stained with Annexin-V and propidium iodide and analysed on a FACSCalibur flow cytometry system (Becton-Dickinson, San Jose, CA, USA) using BD CellQuest software version 3.3 (BD). Data were analysed using FlowJo 7.3.5 software for Windows (Tree Star, Inc. Ashland, OR).

5. **Cell cycle analysis.**

Cells (1 x 106) were washed twice with PBS and fixed in 70% ethanol and kept at 4°C prior to overnight DNA staining with 2.5 µg/ml PI (Calbiochem, Darmstadt, Germany) in the presence of 12.5 µg/ml RNAse (Sigma-tau, Rome, Italy). The number of cells at each stage of the cell cycle was measured using a
FACSCalibur flow cytometry system (BD). The histograms were analysed using the FlowJo 7.3.5 software version for Windows.

6. **Western blot analysis.**

Cell samples were homogenized in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 µg/mL aprotinin, 10 µg/mL leupeptin). Protein concentrations were determined by using bicinchoninic acid (BCA) Protein Assay. Cell lysates were resolved by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Immunocomplexes were visualized using an enhanced chemiluminescence western blotting detection system (Amersham Biosciences, Milano, Italy, EU). Blotting analysis was performed, according to the manufacturer’s protocols, using the following antibodies: CDK5 (1:1000), p35 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:1000) (Cell signaling, Danvers, MA, USA), Actin (1:1000) (Sigma-tau, Rome, Italy), STAT3 (1:1000) and pSTAT3 (Ser727) (1:1000) (Cell signaling, Danvers, MA, USA). HRP- conjugated secondary antibodies (1:5000) (Pierce) against mouse or rabbit were used to detect each protein line.

7. **RNA extraction and real-time PCR.**

Total RNA was extracted using NucleoZOL reagent (Machery-Nagel) following the manufacturer's instructions. 1000ng of total RNA was used for cDNA synthesis with Oligo d(T) primer. Real-time PCR was carried out on an Applied Biosystems ViiA7 Fast Real-Time PCR. PCR reactions were run in duplicate in, at least, three independent experiments. The mRNA levels were normalized to GAPDH as a housekeeping gene and analysed using the ΔΔCt method. All the results are reported as fold change, relative to “CTR”.

8. **Luciferase Reporter assay.**

For the 3′ UTR reporter assay, experiments were performed in 3T3 cells. 3′ UTR segments were sub-cloned by standard procedures into the psiCHECK-2 (Promega, Madison, WI, USA) immediately downstream of the stop codon of the Renilla gene using the primers p35–5′ → (5′-GAGGCTGCTCGATGGAGGA-3′); p35–3′ → (5′-TAAGATTTAACATCATCATATT-3′).

Seed sequence mutagenesis was performed as described by the manufacturer (Agilent Technologies). Cells were transfected with 10 ng of the reporter plasmid and 20 nM of the miRNA or CTR, using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells were lysed and assayed for luciferase
activity at 48 h after transfection, and the luciferase gene coexpressed in the same plasmid was used as internal control. All luciferase assays were performed with Dual Luciferase kit (Promega).

9. **Chromatin Immunoprecipitation assay.**

Cells were cross-linked for 15 min at RT using 1% formaldehyde. Cross-linking was quenched by adding glycine to a final concentration of 0.125 M. The cells were then resuspended in lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40 and protease inhibitors) and incubated on ice for 15 min. Chromatin was sheared to generate 200–400 bp fragments. The efficiency of sonication was assessed with agarose gel electrophoresis. Chromatin samples were pre-cleared for 1 h with protein-G beads and then immunoprecipitated overnight at 4 °C with anti-STAT3. Immunocomplexes were washed with low-salt wash buffer (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH 8, 1% Triton X-100, 150 mM NaCl and protease inhibitors), high-salt wash buffer (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH 8, 1% Triton X-100, 500 mM NaCl and protease inhibitors) and TE buffer. Immunocomplexes were then eluted in elution buffer (1% SDS and 100 mM NaHCO₃) and cross-linking reverted overnight at 65 °C. Samples were extracted with phenol/chloroform and precipitated with ethanol. Enriched regions relative to input DNA were detected by Real-Time PCR and analysed using the ΔΔCt method.

10. **In vivo tumor growth of shCDK5 SU-DHL-8 cells in NOD/SCID mice.**

Six- to eight-week-old NOD/SCID mice (Charles River Laboratories, Milano, Italy, EU) with body weights of 20 to 25g were used to generate xenografts of SU-DHL-8 cells. Experiments were performed according to Italian laws (D.L. 116/92 and following additions) and approved by the institutional Ethical Committee for Animal Experimentation. Engineered cells (10 × 10⁶ cells/mouse) were injected subcutaneously into the left flank of each mouse. The endpoint of the experiment was tumor weight. Tumors were measured with calipers, and their weights were calculated using the formula: \((a \times b^2)/2\), where \(a\) and \(b\) represented the longest and shortest diameters, respectively. Mice were monitored twice weekly and were sacrificed by CO₂ inhalation when they showed signs of terminal illness, including hind leg paralysis, inability to eat or drink, and/or moribund. Each experiment was performed on at least two separate occasions, using five mice per experiment.
11. **Apoptosis assay in vivo.**

Sections (2 μm) from formalin-fixed, paraffin-embedded tumor nodules were processed with DeadEnd™ Fluorometric TUNEL (Promega, Madison WI, USA) according to the manufacturer’s instructions. The sections were examined using a light microscope (BX53; Olympus, Tokyo, Japan). Image analysis was performed using open source ImageJ software (http://rsb.info.nih.gov/ij/).

12. **Podosome function and migration assays**

Quantification of podosomes was performed on merged F-actin stained samples on at least 50 randomly chosen fields representing ~100 total cells per experimental point. Cells containing at least one complete rosette of podosomes were scored as positive. Total cell numbers were calculated on the total number of cells, counting the nuclei (DAP I) on the same field.

13. **Immunohistochemical staining**

*In vivo tumor challenge*

Tumor necrosis/ apoptosis analysis were performed on 2μm sections from formalin-fixed, paraffin-embedded (FFPE) tumor nodules. Tumor necrosis was detected by terminal deoxynucleotidyl transferase of 2′-Deoxyuridine, 5′-Triphosphate (dUTP) nick end labeling staining with the DeadEnd™ Fluorometric TUNEL (Promega, Madison WI, USA), according to the manufacturer’s instructions.

**Breast cancer specimens characterization**

Expression of CDK5 and CD68 was analyzed by IHC in consecutive 2μm FFPE tumor sections, using rabbit polyclonal anti-CDK5 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD68 (1:1000) (Dako; KP-1 clone), and anti-CD163 (1:200) (Leica Biosystems; 10D6 clone). After deparaffinization and rehydration, antigen retrieval was performed by heat treatment using 10 mM citrate buffer (CDK5, Dako; pH 6.0) or EDTA buffer (CD68 and CD163, Dako; 0.25 mM, pH 8) in water bath at 98°C for 20 min. Endogenous peroxidases were blocked by incubation with Peroxidase-Blocking Solution for 15 min at room temperature, followed by incubation for 20 min with Background Sniper (Biocare Medical) to block nonspecific binding. The sections were then incubated with primary antibodies for 1 h at room temperature, followed by incubation with the 3,3′-diaminobenzidine (DAB; brown signal) (Dako). Then sections were counterstained with hematoxylin/eosin and tissue slides were digitized using a computer-aided slide scanner (Olympus VS120 DotSlide).

To identify the potential miR-26a targets, the algorithms miRanda (http://www.microrna.org), TargetScan (http://www.targetscan.org), and PicTar (http://pictar.mdc-berlin.de/) were used. The DLBCL gene expression datasets with confirmed COO subtypes were collected from a public data repository, the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The gene expression datasets retrieved from three GEO series (Compagno_GSE12195; Basso_GSE2350, and Brune_GSE12453) were mainly conducted on two different Affymetrix oligonucleotide microarray platforms, including the GeneChip Human Genome U133A Array (GSE12195, GSE12453) and the Affymetrix Human Genome U95 Version 2 Array (GSE2350). Among all the genes in the arrays we extract CDK5, CDK5R1, EME1, and CCND1 expression. Analysed data values were plotted with Prism (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA). Detailed descriptions of the specimen characteristics and clinical features are provided in the original studies.

The KM-Plotter database contains data regarding the survival of 3,955 patients with breast cancer (RFS data). The association between CDK5 mRNA expression levels and RFS was analyzed using an online KM-Plotter database using the gene expression data and the survival information of patients with breast cancer (https://kmplot.com/analysis/index.php?p=service). Cohorts of patients were split by median expression values through auto select best cut-off. A collection of clinical data, including estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2) status, lymph node status, tumor pathological grade, intrinsic subtype and TP53 status were collected.

15. Statistical analysis.

Statistical analyses were performed using Prism 7.2.5 (GraphPad Software, Inc., La Jolla, CA, USA). To test the probability of significant differences between untreated and treated samples, a two-way analysis of variance was employed, and individual group comparisons were evaluated using the Bonferroni post-hoc test. Terminal deoxynucleotidyl transferase dUTP nick end labelling and immunohistochemistry data were analysed using one-way analysis of variance and individual group comparisons were evaluated using the Bonferroni post-hoc test. A two-tailed value of P<0.05 was considered significant. Data are represented as mean ± SD unless otherwise stated.
Part one

1. **CDK5 is over-expressed and over-activated in lymphoma cell lines.**

Several groups identified CDK5 as a potential marker of cancer progression [60]. In particular, previous reports have shown that CDK5 is highly over-expressed during tumor development. Considerable efforts have been dedicated to the comprehension of CDK5 role in solid neoplasms, while its involvement in hematopoietic malignancies is completely an unexplored field. In light of this, we decided to better investigate its role in these tumors. In particular, we evaluated CDK5 and its activator p35 expression in lymphoma cell lines, including DLBCL, Hodgkin lymphoma, Burkitt lymphoma, and chronic lymphocytic leukemia cell lines compared to B lymphocytes. As shown in Figure 1.1, we observed that both CDK5 and p35 expression are highly increased in cancer cell lines we analysed, compared to B lymphocytes obtained from healthy donors (Figure 1.1A-C).

![Figure 1.1](image)

**Figure 1.1.** CDK5 and p35 are overexpressed in cancer cell lines, compared to B Lymphocytes. Representative quantification CDK5 (A) and p35 (B) protein expression, and relative immunoblot (C) in primary B lymphocytes (B CELLS), B-cell chronic lymphocytic leukemia (MEC-1), Hodgkin lymphoma (L-1236, L-428, L-540, SUPDH1, and KM-H2) and Burkitt’s lymphoma (RAMOS) cell lines. Relative expression is shown as normalized to the housekeeping and graphed as a fold change relative to B-cells. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA with Tukey’s multiple
comparisons test was used. Significant differences are indicated by: **P<0.01, and *P<0.05

Then, we focused our attention on DLBCL cell lines. In particular, we analysed several DLBCL cells to better clarify CDK5 and p35 role in this context. Coherently with previous results, we noticed an increased CDK5 expression at both mRNA and protein level in DLBCL cell lines, compared to B lymphocytes (Figure 1.2A-C).

CDK5 can phosphorylate numerous types of protein substrates, and in particular it is able to regulate the phosphorylation of Ser727 on STAT3. This has been demonstrated to have a role in the regulation of cancer cell survival and it is fundamental in the nuclear translocation of STAT3 [118]. We demonstrated that CDK5 activity in DLBCL cells specifically correlates with STAT3 phosphorylation rate at S727 (Figure 1.2C, D). On the other hand, while p35 expression is highly increased at mRNA level, we didn’t observe a concomitant increase in its levels of expression at the protein level, indicating that p35 translation is differently regulated in cancer cells in comparison with healthy ones (Figure 1.1C, E, F).
CDK5 and p35 are overexpressed DLBCL cell lines, compared to B lymphocytes. Relative CDK5 expression at mRNA (A) protein (B) level with relative immunoblot (C) in Germinal Centre B-Cell like (GCB; SU-DHL-4, SU-DHL-6, SU-DHL-16, RCK8), and Activated B-Cell like (ABC; SU-DHL-2, SU-DHL-8) DLBCL cell lines. CDK5 activity was measured via STAT3 phosphorylation rate (C-D). CDK5 expression was also related to its activator, p35. Its relative mRNA (E) and protein expression (F), were measured. Relative expression is shown as normalized to the housekeeping and graphed as a fold change relative to B-cells. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA with Tukey’s multiple comparisons test was used. Significant differences are indicated by: *** P<0.001, **P<0.01, and *P<0.05.

To further corroborate our in vitro results, we decided to measure both CDK5 and p35 expression levels in DLBCL patients and healthy individuals exploiting a published gene expression data set (Compagno_GSE12195; Basso_GSE2350...
CDK5 is increased in DLBCL patients, compared to HC in public datasets. Gene expression analysis of CDK5 (A) and p35 (CDK5R1, B) in primary DLBCL samples, compared to normal B cells (Germinal Centre B-Lymphocytes, CGB-L; Memory B-cells, MB-L; Naïve Pre Centroblastic B-cell, NPCB-L; B-Lymphocytes, B-L; Centroblasts, C; Naïve Pre Centroblast, NPC; Small Cleaved Follicle Center B-cell, SCFC; Plasma Cell, PC) (Compagno_GSE12195; Basso_GSE2350 Brune_GSE12453). As expected, in agreement with the results we obtained in vitro, there was no difference in p35 mRNA (Figure 1.3B). Overall, these results indicate that CDK5 is overexpressed in DLBCL.

Figure 1.3. CDK5 is increased in DLBCL patients, compared to HC in public datasets. Gene expression analysis of CDK5 (A) and p35 (CDK5R1, B) in primary DLBCL samples, compared to normal B cells (Germinal Centre B-Lymphocytes, CGB-L; Memory B-cells, MB-L; Naïve Pre Centroblastic B-cell, NPCB-L; B-Lymphocytes, B-L; Centroblasts, C; Naïve Pre Centroblast, NPC; Small Cleaved Follicle Center B-cell, SCFC; Plasma Cell, PC) (Compagno_GSE12195; Basso_GSE2350 Brune_GSE12453). Significant differences are indicated by the specific p Value.
2. CDK5 silencing impairs DLBCL cell lines proliferation in vitro.

To better characterize CDK5 involvement in DLBCL biology, we decided to perform loss-of-function experiments. In particular, we designed two different shCDK5-specific shRNAs to inhibit its expression. We used two different cell lines: SU-DHL-4 (Figure 2.1A) and SU-DHL-6 (Figure 2.1B) as a Germinal Centre B-Cell like (GCB) DLBCL cell lines, and SU-DHL-8 (Figure 2.1C, D), as Activated B-Cell like (ABC) one, since both of them were highly overexpressing CDK5 and p35. As shown in Figure 2.1, the silencing efficiency was evaluated measuring CDK5 expression at both mRNA and protein level. Since SU-DHL-8 cells line showed the best silencing efficiency, both at mRNA and protein level, we decided to focus our attention mainly on this cell line.

![Figure 2.1](image)

**Figure 2.1.** CDK5 loss-of-function in DLBCL cell lines. CDK5 mRNA expression was measured in Germinal Centre B-Cell like (GCB) SU-DHL-4 (A), and SU-DHL-6 (B); and Activated B-Cell like (ABC) SU-DHL-8 (C) DLBCL cell lines after lentiviral transduction with two different shRNAs. Further CDK5 expression was measured in SU-DHL-8 (D). Results are representative of minimum three independent experiments. Data are shown as fold change relative to shSCR. The data are presented as the means±SD. To compare means, Student’s t-test was used. Significant differences are indicated by: ***P<0.001, **P<0.01, and *P<0.05.
In addition, we further demonstrated that CDK5 silencing impaired STAT3 phosphorylation (S727), confirming that even in our context it is mediated by CDK5 activity (Figure 2.2A).

Figure 2.2. CDK5 inhibition induces pSTAT3 (S727) impairment in SU-DHL-8 cells. Densitometric analysis of normalized pSTAT3 to total STAT3 and relative immunoblot of pSTAT3 S727 phosphorylation level in SU-DHL-8 expressing CDK5-specific shRNAs. Results are representative of three independent experiments. The data are presented as the means±SD. To compare means, Student’s t-test was used. Significant differences are indicated by: **P<0.01, and *P<0.05.

Following, to better characterize CDK5 role in these cell lines, we performed cell proliferation assay to define its role in cell cycle involvement. Therefore, we measured cells proliferation rate, by MTT assay, BrdU incorporation capacity, and we analysed the cell cycle of all these cells in SU-DHL-8 cells. As shown in Figure 2.3A-D, CDK5 silencing is able to directly impair the proliferative capacity of cancer cells in vitro.

Figure 2.3. CDK5 loss inhibits cell proliferation in SU-DHL-8 cells. Specific CDK5 down-regulation with two different shRNAs significantly inhibited cell proliferation of SU-DHL-8 cells, measured by growth curve (A), BrdU incorporation assay (B), and MTT assay (C). In addition, CDK5 loss induced an impairment of the cell cycle of SU-DHL-8 cells (D). Results are representative of minimum three independent experiments. The data are
presented as the means±SD. To compare means, two-way ANOVA was used in A, D, and one-way ANOVA was used in B, C. Significant differences are indicated by: *** P<0.001, **P<0.01, and *P<0.05.

As a step further, we measured proliferative capacity of shCDK5 SU-DHL-4 and SU-DHL-6 we generated. Coherently, also in these cellular models we observed a reduction in proliferation rate, MTT assay and BrdU incorporation for both SU-DHL-4 (Figure 2.4A-C) and SU-DHL-6 (Figure 2.4D-F).

Figure 2.4. CDK5 role in proliferation is confirmed in SU-DHL-4 and SU-DHL-6 cells. Specific CDK5 down-regulation with two different shRNAs significantly inhibited cell proliferation of SU-DHL-4 cells, measured by growth curve (A), BrdU incorporation assay (B), and MTT assay (C), and of SU-DHL-6 cells, measured by growth curve (D), BrdU incorporation assay (E), and MTT assay (F). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, two-way ANOVA was used in A, D, and one-way ANOVA was used in B, C, E, F. Significant differences are indicated by: *** P<0.001, **P<0.01, and *P<0.05.
3. **p35 modulation regulates DLBCL cell lines proliferation in vitro.**

Since CDK5 activity is finely regulated by p35 expression, we wondered whether the proliferative capacity of lymphoma cells could be related to p35 modulation. Therefore, we designed two p35-specific shRNAs to evaluate proliferation capacity in SU-DHL-8 (ABC) cell lines. Silencing efficacy was measured by RT-qPCR and WB (Figure 3.1A, B).

![Figure 3.1](image-url)

**Figure 3.1.** p35 loss-of-function in SU-DHL-8 cells. Specific p35 down-regulation with two different shRNAs significantly inhibited p35 mRNA expression (A) and protein level (B). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, Student’s t-test was used. Significant differences are indicated by: *** P<0.001 and **P<0.01.

As expected, p35 impairment in DLBCL cell lines could affect the proliferation rate of these cells, to the same extent obtained by CDK5 inhibition. Indeed, as shown in Figure 3.2A-C, both growth rate, MTT assay, and BrdU incorporation were significantly reduced in p35-silenced SU-DHL-8 cells.

![Figure 3.2](image-url)

**Figure 3.2.** p35 impairment reduces cell proliferation in SU-DHL-8 cells. Specific p35 down-regulation with two different shRNAs significantly inhibited cell proliferation of SU-DHL-8 cells, measured by growth curve (A), BrdU incorporation assay (B), and MTT
assay (C), Results are representative of minimum three independent experiments. To compare means, two-way ANOVA was used in A, and one-way ANOVA was used in B, C. Significant differences are indicated by: ***P<0.001, **P<0.01, and *P<0.05.

In addition, to further strengthen these results, we performed gain-of-function experiments, taking advantage of SU-DHL-16 cell line, in which we observed the lowest CDK5 and p35 expression, compared to the other cell lines examined (Figure 1.1A-C). In particular, we stably over-expressed CDK5’s activator p35, measuring ectopic expression both at mRNA and protein level (Figure 3.3A, B). Consistently with previous results, stable expression of recombinant p35 in this cell line induced an increased proliferative capacity, compared to control cells (Figure 3.3C).

**Figure 3.3.** p35 ectopic over-expression improves cell proliferation in SU-DHL-8 cells. Specific p35 overexpression significantly increases p35 mRNA expression (A) and protein level (B). In addition, p35 ectopic increase induces an increased cell proliferation of SU-DHL-8 cells, measured by growth curve (C). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, Student’s t-test was used in A, B, and two-way ANOVA was used in C. Significant differences are indicated by: ***P<0.001 and **P<0.01.
4. **CDK5 and p35 expression are necessary to protect DLBCL cell lines from apoptosis.**

Previous report stated that CDK5 is able to enhance cell survival in murine neurons through c-Jun negative regulation [119], in post-mitotic murine cells by regulating Bcl-2 and Bcl-XL [120], and in human podocytes derived from diabetic patients via promoting MEKK1 phosphorylation [121]. In addition, our experiments demonstrated that CDK5 activity could affect proliferative capacity in DLBCL cell lines, thus we speculated that its modulation could even affect cell survival in our model of hematopoietic malignancies. Therefore, we performed Annexin V/PI staining by FACS in order to investigate if CDK5 could affect apoptosis rate in DLBCL cell lines. As shown in **Figure 4.1A**, we observed an increased cell mortality in CDK5-silenced SU-DHL-8. The same results were obtained analysing shp35 SU-DHL-8 cells (**Figure 4.1B**). Furthermore, we decided to chemically inhibit CDK5 with two different compounds (CGP74514A and Purvalanol) and the obtained results confirmed that CDK5 activity is important to protect cells from apoptosis (**Figure 4.1C**).

![Figure 4.1](image)

**Figure 4.1.** p35/CDK5 axis inhibition is crucial to induce apoptosis in SU-DHL-8 cells. Specific down-regulation via lentiviral downregulation of both CDK5 and p35 in SU-DHL-8 significantly increased apoptotic cell number, assessed by Annexin-V/PI staining, respectively (A, B). In addition, apoptosis rate was increased after the treatment with two different CDK5 inhibitors (pan-CDKs inhibitors, CGP74514A and Purvalanol) on SU-DHL-8 cell lines. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: ***P<0.001, **P<0.01, and *p < 0.05.

In addition, we investigated if CDK5 silencing could enhance DLBCL sensitivity to pro-apoptotic agent, such as TRAIL, since the combination of CDKs inhibition with chemotherapy can be very helpful to inhibit cancer progression in patients [122]. Intriguingly, we observed that the administration of a pro-apoptotic reagent
in combination to CDK5/p35 silencing increased apoptosis rate, dramatically stopping cellular proliferation in SU-DHL-8 cells (Figure 4.2A, B).

Figure 4.2. p35 overexpression or CDK5 inhibition entailed apoptosis rate in SU-DHL-8 cells. Specific down-regulation via lentiviral downregulation of both CDK5 (A) and p35 (B) in SU-DHL-8 significantly increased apoptotic cell number, assessed by Annexin-V/PI staining, in the presence of 5 µM Killer sTRAIL. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: *** P<0.001, **P<0.01, and *p < 0.05.

We also observed that CDK5-silenced SU-DHL-4 showed an increased apoptotic rate only in one shRNA (data not shown). This effect could be due to the reduced level of silencing achieved in this cell line (less than 50%; Figure 2.1A). Thereby, we wondered whether the combination of the pro-apoptotic agent TRAIL in shCDK5-SU-DHL-4 cells could affect apoptosis rate, and we observed that SU-DHL-4 cells showed an increased mortality rate in shCDK5 after the administration of low doses of TRAIL (Figure 4.3A).

Moreover, to test the hypothesis that CDK5 inhibition might be used in combination with cytotoxic agents to increase the cellular apoptotic rate, we inhibited CDK5 activity in SU-DHL-8 cells using CDK’s PAN-inhibitors in combination with the pro-apoptotic agent TRAIL. As hypothesized, we observed that CDK5 impairment induced an increased mortality rate in TRAIL-treated SU-DHL-8 (Figure 4.3B, C).
Figure 4.3. CDK5 role in apoptosis induction. Specific down-regulation via lentiviral downregulation of CDK5 significantly increased apoptotic rate in SU-DHL-4 cell lines (A). Further the combination of two different doses of TRAIL (2.5 µM and 5 µM) increased the apoptotic rate of SU-DHL-8 cells treated with two pan-CDK inhibitors as Purvalanol (B) and GGP74514A (C), assessed by Annexin-V/PI staining. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: ***P<0.001 and **P<0.01.

To further elucidate CDK5 anti-apoptotic activity, we measured the apoptosis level in SU-DHL-16 cells overexpressing p35 triggered with bortezomib (BTZ), since SU-DHL-16 were not sensitive to TRAIL in our hands. Consistent with previous results, we detected a substantial decrease in the BTZ–induced apoptosis after p35 over-expression (Figure 4.4). These results strongly suggest that CDK5 activity is able to prevent apoptosis in DLBCL cells.

Figure 4.4. p35 overexpression prevent apoptosis induction in SU-DHL-16 cells. p35 overexpression in SU-DHL-16 cells significantly reduced apoptotic rate, assessed by Annexin-V/PI staining, after the treatment with a pro-apoptotic agent to which those cells are insensible (Bortezomib, BTZ). Results are representative of minimum three
independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: *** $P<0.001$. 
5. **CDK5 activity regulates the transcription of STAT3 specific targets.**

Since STAT3 phosphorylation was strongly reduced in CDK5-silenced SU-DHL-8 cells (Figure 2A), we decided to measure the expression of STAT3-specific targets.

Intriguingly, the expression of two out of six STAT3-dependent genes resulted down regulated by the lack of CDK5: Essential meiotic endonuclease 1 homolog 1 (EME1) and Cyclin D1 (CCND1) (Figure 5.1A, B).

**Figure 5.1.** CDK5 silencing induces STAT3 downstream target inhibition. Relative expression of specific STAT3 downstream target in SU-DHL-8 cell lines lacking CDK5 with two specific shRNAs was measured via qPCR, compared to shSCR cells (A). Among all the target only Essential meiotic endonuclease 1 homolog 1 (EME1) and Cyclin D1 (CCND1) were significantly downregulated with both shRNAs (B). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: ***P<0.001, **P<0.01, and *p < 0.05.

Notably, these STAT3 targets are significantly upregulated in the DLBCL patients. In particular EME1 (Figure 5.2A) and CCND1 (Figure 5.2B) resulted significantly overexpressed, suggesting that CDK5 activity could mediate the transcription of STAT3 downstream targets.
**Figure 5.2.** STAT3 downstream targets are increased in DLBCL patients. Gene expression analysis of EME1 (A) and CCND1 (B) in primary DLBCL samples, compared to normal B cells (Germinal Centre B-Lymphocytes, CGB-L; Memory B-cells, MB-L; Naïve Pre Centroblastic B-cell, NPCB-L). Straight bars represent the median. The signal intensity is increased in human DLBCL tumor tissues as compared with non-tumor control tissues (data from a published dataset Compagno_GSE12195 NCBI GEO profiling dataset). Significant differences are indicated by the specific p Value.

To determine whether STAT3 could bind the promoter of these two genes in SU-DHL-8 cells, chromatin immunoprecipitation assay (ChIP) was performed. As shown in Figure 5.2, PCR yielded EME1 (Figure 5.2A) and CCND1 (Figure 5.2B) promoters DNA immunoprecipitated with an anti-STAT3 antibody in the region of the putative STAT3-binding sites. We observed a reduction in EME1 enrichment, but no significant differences regarding CCND1. These data provide evidence that STAT3 can directly bind EME1 promoter in SU-DHL-8 cells.

**Figure 5.3.** Validation of STAT3 binding to the EME1 promoter. ChIP assay was performed with untreated SU-DHL-8 cell lines with antibody against pSTAT3 (S727) and control IgG. Non immunoprecipitated chromatin was used as total input control. PCR primers were designed to include putative STAT3-binding sites in the promoter of selected genes: EME1 (A) and CCND1 (B). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: **P<0.01.
6. **CDK5 modulates DLBCL tumor growth in vivo.**

To test CDK5 role in vivo, CDK5-silenced SU-DHL-8 were injected subcutaneously into NOD/SCID mice. Palpable tumors were measured three times per week and mice were sacrificed at day 35. CDK5-silenced tumors showed very low replication rate, since they were not detectable for almost the entire study, while control group (shSCR) presented fast growing and prominent tumors (**Figure 6.1A, B**).

![Figure 6.1](image_url)

**Figure 6.1** CDK5 inhibition inhibit tumor growth in vivo. Specific CDK5-silenced SU-DHL-8 were subcutaneously injected in NOD/SCID mice. Tumors were monitored until 5 weeks after the challenge and tumor volume during time was calculated via the repetitive measurement of the tumor masses with the caliper (A). At sacrifice, the relative tumor weights were measured (B) Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To compare means, two-way ANOVA was used in A and one-way ANOVA was used. Significant differences are indicated by: **P<0.01.

To assess tumor proliferation, we performed immunohistochemical analysis for Ki67 on the tumor xenografts, but we could not detect any significant difference (data not shown). In addition, the amount of apoptosis among the tumor samples was assessed by TUNEL assay. The number of apoptotic cells per field was significantly higher in tumors with reduces CDK5 expression (**Figure 6.2**). These results clearly demonstrate that CDK5 regulates in vivo tumor growth and apoptosis rate of DLBCL cells.
Figure 6.2. CDK5 impairment induces an increased apoptosis rate in DLBCL cell in vivo. Apoptotic rate of DLBCL tumors were visualized and quantified using the TUNEL assay through Immunohistochemistry. Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To compare means, two-way ANOVA was used in A and one-way ANOVA was used. Significant differences are indicated by: **P<0.01.
7. **miR-26 is a direct p35 target in vitro.**

Since we noticed that p35 mRNA relative expression and protein level in DLBCL cell lines are inversely correlated (Figure 1.2E, F), we hypothesized that cancer cells could induce p35 post-translation modification. We hypothesized that it could be due to epigenetic modulation of p35 translation directly mediated by one miRNA. Thus, we performed a bioinformatics analysis to assess if some miRNAs could directly target p35 3'UTR. We noticed that miR-26a has one putative binding site in the p35 3'-UTR with a seed sequence located at positions 2061-2067 bp (Figure 7.1).

$$5'...\text{AAGGAACAACUGAUACUAGAG}...3' \quad \text{WT} \quad p35 \quad 3'\text{UTR}$$

$$3' \quad \text{UCGGAUAGGACCUAAGAACU} \quad 5' \quad \text{mature miR-26a (nt 2061-2067)}$$

$$5'...\text{AAGGAACAACUGAUACUC\text{CTCG}...3'} \quad \text{MUT} \quad p35 \quad 3'\text{UTR}$$

$$3' \quad \text{UCGGAUAGGACCUAAGAACU} \quad 5' \quad \text{mature miR-26a (nt 2061-2067)}$$

*Figure 7.1. miR-26a seed sequence compared to p35 WT 3'UTR and the MUT sequence. miR-26a and its putative binding sequence in the 3'-UTR of p35. In bold, the mutant seed sequence (WT, wild type; MUT, mutant type).*

To test the possible p35 regulation by miR-26a, its precursor (hsa-pre-miR-26a) was stably overexpressed into SU-DHL-8 cells (Figure 7.2).

*Figure 7.2. miR-26a over-expression in SU-DHL-8 cells. miR-26a relative expression was measured via qPCR in SU-DHL-8 overexpressing miR-26a through lentiviral approach. Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To compare means, Student's \(t\) test was used. Significant differences are indicated by: **\(P<0.01\).*

To assess that miR-26a could directly target p35 3'UTR, Western blotting analysis was performed. miR-26a overexpressing SU-DHL-8 showed a decrease of the
native p35 protein (Figure 7.3) compared to cells transduced with empty vector or over-expressing a control miRNA.

**Figure 7.3.** p35 expression is modulated by miR-26a. Representative immunoblot for p35 in SU-DHL-8 overexpressing miR-26a and two control miRNAs (miR-15/16). miR-26a significantly reduced p35 protein levels. Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: *P<0.05.

To further confirm that miR-26a can directly target p35 3’ UTR, we performed luciferase assay. We observed that miR-26a significantly repressed luciferase activity compared to a non-targeting control. In addition, mutagenesis of the seed sequence led to a recovery of the luciferase activity (Figure 7.4). Taken together, these results indicate that p35 is a direct target of miR-26a in DLBCL cells.

**Figure 7.4.** Luciferase assay confirm that miR-26a directly target p35 3’UTR. miR-26a significantly suppressed luciferase activity of plasmid carrying the WT but not the MUT 3’UTR of p35. Values are presented as fold change relative to E.V., which is indicated as 100%. Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To
compare means, one-way ANOVA was used. Significant differences are indicated by: **P<0.01.
8. miR-26a overexpression impairs DLBCL cell lines proliferation in vitro, targeting p35.

First, in order to demonstrate whether miR-26a might have a role on lymphoma biology, the expression level of miR-26a was measured in DLBCL cell lines. miR-26a was strongly down-regulated in DLBCL cell lines compared to B lymphocytes (Figure 8.1A). Following, we examined the effects of the hsa-miR-26a overexpression on the proliferation rate of SU-DHL-8 cells. Compared to controls, as shown in Figure 8.1B, hsa-miR-26a over-expression induced a reduction in cell proliferation. Moreover, hsa-miR-26a expression increased apoptosis rate in TRAIL treated SU-DHL-8 cells, even if we did not observe statistically significant differences in untreated cells (Figure 8.1C).

Figure 8. 1. miR-26a can modulate DLBCL cell growth in vitro. Relative miR-26a expression in Germinal Centre B-Cell like (GCB; SU-DHL-4, SU-DHL-6, SU-DHL-16, RCK8), and Activated B-Cell like (ABC; SU-DHL-2, SU-DHL-8) DLBCL cell lines (A). Relative expression is shown as normalized to the housekeeping and graphed as a fold change relative to B-cells. miR-26a upregulation significantly inhibited cell proliferation of SU-DHL-8 cells, measured by growth curve (B), and increased apoptotic rate, assessed by Annexin-V/PI staining, in the presence of 5 μM Killer sTRAIL (C). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA with Tukey’s multiple comparisons test was used in A and C; two-way ANOVA was used in B. Significant differences are indicated by: **P<0.01, and *P<0.05

Then, in order to demonstrate that the effect of miR-26a on proliferation and apoptosis of DLBCL was mediated by p35, we decided to test proliferation capacity and apoptosis rate in SU-DHL-8 overexpressing both miR-26a and p35 CDS sequence (miR-26/p35). The overexpression efficiency was measured by WB (Figure 8.2A).

Further, miR-26/p35 cells showed a markedly increase in cell proliferation and a decrease in apoptosis, compared to miR-26a/E.V (Figure 8.2B, C). In particular,
we observed that the protective effect of miR-26a was completely reverted after p35 overexpression. Further, in order to explore whether the miR-26a/CDK5/p35 axis exerts its functions through the CDK5/STAT3 pathway, we examined the expression level of the different STAT3 target gene that was significantly down-regulated upon CDK5 silencing, EME1. Expression levels of EME1 was decreased in SU-DHL-8 cells expressing miR-26/E.V., but not in SU-DHL-8 cells expressing miR-26/p35, compared to E.V. control cells (Figure 8.2D). These data indicate that miR-26a inhibits CDK5/STAT3 signalling in DLBCL cells.

**Figure 8.2.** miR-26 effects on SU-DHL-8 cell growth and apoptosis are rescued after p35 over-expression. Relative quantification of p35 protein level was measured in miR-26a overexpressing DLBCL and in cells in which miR-26a effect was rescued with a p35 CDS, lacking the 3'UTR (A). p35 overexpression rescued miR-26a effect on DLBCL as demonstrated by the increased proliferation rate measured with growth curve (B) and the reduction in the apoptotic rate, measured via AnnexinV/PI staining (C). EME1 mRNA expression was measured by qPCR in DLBCL overexpressing miR-26a and the in miR-26a overexpressing cells with the p35 mutated form (D). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA with Tukey’s multiple comparisons test was used in A, C and D; two-way ANOVA was used in B. Significant differences are indicated by: **P<0.01, and *P<0.05
9. miR-26 modulates DLBCL tumor growth in vivo, through p35 regulation.

SU-DHL-8 cells stably expressing miR-26a (miR-26) and cells overexpressing both miR-26a/p35 were injected subcutaneously into NOD/SCID mice. The tumors of the SU-DHL-8 miR-26 group were significantly smaller than control (E.V.) and the rescue group (miR-26/p35) for almost the entire study (Figure 9.1A, B).

Figure 9. 1. miR-26a/p35 modulation affect tumor growth in vivo. miR-26a and miR-26a/p35 overexpressing SU-DHL-8 were subcutaneously injected in NOD/SCID mice. Tumors were monitored until 5 weeks after the challenge and tumor volume during time was calculated via the repetitive measurement of the tumor masses with the caliper (A). At sacrifice, the relative tumor weights were measured (B) Results are representative of minimum three independent experiments in which eight animals per group were used. The data are presented as the means±SD. To compare means, two-way ANOVA was used in A and one-way ANOVA was used. Significant differences are indicated by: **P<0.01.
Part two

1. **CDK5 involvement in breast cancer specimens.**

Firstly, in order to describe CDK5 clinical relevance in human breast cancer patients, we analysed the Overall Survival (OS; n=1402), taking advantage of public available TCGA Datasets (https://portal.gdc.cancer.gov/). CDK5 is highly over-expressed in tumor tissues compared to healthy controls (Figure 1.1A). Further breast cancer patients were divided in “high” and “low” CDK5 expression, according to median. The Kaplan-Meier curves suggest that higher levels of CDK5 are associated with shorter OS (p = 0.027; Figure1.1B).

![Figure 1. CDK5 in breast cancer patients. Box plot comparing CDK5 expression in healthy individuals and breast cancer patients derived from the TCGA database. TCGA analysis also revealed that CDK5 was significantly upregulated in breast cancer (BC) specimens compared to healthy controls (HC) (A) (n=1247). Survival assay of CDK5 (B) and CDK5R1 (C) in breast cancer patient using Kaplan–Meier plotter (n=3955). Analysis is shown for relapse-free survival (RFS). Survival curves were derived from Kaplan–Meier plotter and p<0.01 was considered statistically significant. (FPKM= Fragments Per Kilobase Million)
2. **CDK5 is expressed in the tumor microenvironment of human breast cancer specimens.**

We took advantage of several human breast cancer samples availability in-house. Breast cancer section were compared to section obtained from mammoplasty intervention (Figure 2.1A), to better correlate CDK5 expression in a neoplastic context. Through immunohistochemical analysis, we detected CDK5 expression not only in the tumor core (T), but also in the tumor microenvironment (TME) (Figure 2.1B).

![Figure 2.1: Immunohistochemical analysis of CDK5 in human specimens](image)

**Figure 2.1.** Immunohistochemical analysis of CDK5 in human specimens. Representative CDK5 staining of FFPE sample from healthy controls, obtained from mammoplasty intervention (A) and breast carcinoma (B), at 20X magnification. Representative images were chosen for similarity to the global quantification. Data normality was calculated with Kolmogorov-Smirnov (K-S) test. Scale bars: 100 μm.

Further, to better characterize CDK5 involvement in the TME, consecutive sections were stained for either CDK5 or CD68. TME is a complex and dynamic structure, involved in tumor growth and metastatization. Within the TME, Tumour Associate Macrophages (TAMs) are the main cellular type involved in tumor sustenance and in cancer-related inflammation [123]. In light of this, TAMs represent an attractive target of novel biological therapies of tumors. In particular, no previous report describes CDK5 involvement in macrophage biology. Interestingly, IHC analysis showed that macrophages sub-population, especially TAMs, were positive for both markers (Figure 2.2).
Figure 2.2. CDK5 correlates with CD68 expression. Representative CDK5 and CD68 staining of FFPE sample from breast carcinoma, at 20X magnification. Representative images were chosen for similarity to the global quantification. Representative images chosen for similarity to the global quantification. Data normality was calculated with Kolmogorov-Smirnov (K-S) test.

To further define CDK5 positive macrophages we stained macrophages also for CD163 marker. CD163 is a scavenger receptor, a member of the cysteine-rich family, which is considered a common monocyte/macrophage-specific membrane marker. Specifically, CD163 is considered a specific marker for the staining of alternatively activated macrophages. IHC analysis confirmed that CDK5 positive macrophages display a M2 phenotype (Figure 2.3).

Figure 2.3. CDK5 correlates with CD163 expression. Representative CDK5 and CD163 staining of FFPE sample from breast carcinoma, at 20X magnification. Representative images were chosen for similarity to the global quantification. Representative images chosen for similarity to the global quantification. Data normality was calculated with Kolmogorov-Smirnov (K-S) test.
In addition, to further confirm that CDK5 expression within the microenvironment was specifically related to TAMs, we performed a double immunofluorescence (IF) staining (CDK5/CD68). Intriguingly, IF analysis confirmed that breast cancer associated macrophages, were positive for both markers (Figure 2.4).

![Figure 2.4](image.png)

**Figure 2.4.** CDK5 correlates with CD68 expression. Representative staining of breast cancer specimens to detect CD68 (red) and CDK5 (green) colocalization. Representative images chosen for similarity to the global quantification. Data normality was calculated with Kolmogorov-Smirnov (K-S) test. Scale bars: 100 μm.
3. **CDK5 involvement in Tumor Associated Macrophages.**

To better characterize CDK5 involvement in breast cancer microenvironment, we decided to analyse CDK5 expression in human macrophages differentiated from peripheral blood monocytes, taking advantage of public datasets (Figure 3.1A). We noticed that CDK5 is strongly upregulated after macrophage M0 differentiation in vitro and its expression is modulated after macrophage polarization from human peripheral blood macrophages, indicating that CDK5 could be involved in macrophage biology.

In addition, we measured CDK5 protein level in THP1-derived macrophages during polarization induction. We observed that CDK5 is down-regulated in inflammatory macrophages after LPS stimulation, instead we did not notice any statistical difference under IL-4 stimulation (6h, 24h; Figure 3.1B).

**Figure 3.1** CDK5 expression is modulated during macrophage polarization in vitro. CDK5 expression in human primary differentiated and polarized cells (A), monocytes (M), macrophages (Mφ), pro-inflammatory macrophages (M1, 20ng/ml LPS and 100ng/ml IFNγ treated cells) and alternatively-activated macrophages (M2, 20ng/ml Il-4 treated cells) analysed from a public available dataset (E-MTAB-3827, n=5). Representative immunoblot and quantification of THP1-derived macrophages differentiated 72h with PMA, then polarized toward pro-inflammatory macrophages (M1, 20ng/ml LPS and 100ng/ml IFNγ treated cells) and alternatively-activated macrophages (M2, 20ng/ml Il-4 treated cells) for 4h and 24h. Results are representative of three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: ***P<0.001, **P<0.01.

To further study CDK5 role in the tumor microenvironment, THP1 cell lines were used as a CDK5 loss-of-function model, via shRNAs approach. CDK5 silencing was measured via both qPCR (Figure 3.2A) and at protein level (Figure 3.2B).
Figure 3.2. CDK5 silencing in THP-1 cells. CDK5 mRNA expression was measured after CDK5 silencing in THP-1 cells (A). Representative immunoblot and quantification of THP1-derived macrophages lacking CDK5 (B). Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, Student’s t-test was used. Significant differences are indicated by: *** $P<0.001$ and **$P<0.01$. 
4. **CDK5 involvement in podosome formation in vitro.**

During wound healing process, macrophages are key player cells able to coordinate immune defence and tissue repair, by a fine regulation of epithelial migration, matrix remodelling, and angiogenesis [124]. Within the tumor microenvironment, macrophages, instead can provide support for the aberrant proliferation of neoplastic cells. Indeed, cancer cells are able to recruit macrophages to suppress immune functions, providing a free escape to continue growing and invading surrounding tissues. Podosomes are protrusive structures implicated in macrophage extracellular matrix degradation and 3D-spatial migration through cell barriers within the stroma. Previous report showed how CDK5 could modulate cancer cell invasiveness in vitro, by regulating caldesmon phosphorylation rate [125], therefore we measured podosome formation rate in shCDK5 THP-1 cells.

Accordingly, we observed that shCDK5 induced a reduction in podosomes formation (Figure 4.1), indicating that it could regulate tumor associated macrophages infiltration within the tumor.

![Figure 4.1](image)

**Figure 4.1** CDK5 loss induces an impairment in podosome formation. THP-1 cells were stained for F-actin (green) DAPI. The percentage of podosome positive cells (indicated with arrows) were quantified. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, Student’s t-test was used. Significant differences are indicated by: **P<0.01.
5. **p35 role in TAMs.**

To better understand p35/CDK5 role in macrophage biology, we decided to study p35 modulation during macrophage M1 polarization by using the same experimental strategy used for CDK5 investigation. Intriguingly, the results obtained for p35 are in agreement with those obtained for CDK5 (Figure 5.1A). To further study p35 role in tumor microenvironment, also in this case THP1 cell lines were used as a p35 gain-of-function model, via lentiviral approach. p35 overexpression was measured via both qPCR (Figure 5.1B) and protein level (Figure 5.1C).

**Figure 5.1** p35 expression in THP-1 polarization. Representative immunoblot and quantification of THP1-derived macrophages differentiated 72h with PMA, then polarized toward pro-inflammatory macrophages (M1, 20ng/ml LPS and 100ng/ml IFNγ treated cells) and alternatively-activated macrophages (M2, 20ng/ml Il-4 treated cells) for 4h and 24h (A). p35 mRNA expression was measured after p35 overexpression in THP-1 cells (B). Representative immunoblot and quantification of THP1-derived macrophages overexpressing p35 (C). Results are representative of three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used in A, Student’s t-test was used in B and C. Significant differences are indicated by: ***P<0.001, **P<0.01.
6. **p35/CDK5 axis modulation induced a pro-inflammatory phenotype in THP-1 cells.**

LPS treated macrophages produce pro-inflammatory cytokines (e.g., TNFα and IL-1β), that are critical for the immune responses. Since CDK5 expression was reduced by M1 stimuli, we hypothesized that this reduction might be important for the pro-inflammatory phenotype. In response to IFNγ/LPS stimulation, CDK5 inhibition induced a marked enhancement in the expression of several M1 genes, as shown in Figure 6.1A-D. Coherently, p35 over-expression displayed an impairment in pro-inflammatory cytokines transcription (Figure 6.1E-H).

![Figure 6.1 CDK5 modulation induces pro-inflammatory cytokines transcription.](image_url)

**Figure 6.1** CDK5 modulation induces pro-inflammatory cytokines transcription. Change in classical M1 cytokines mRNA expression after M1 polarization, in CDK5-lacking THP-1 (A-D) and in p35 overexpressing cells (E-H). THP-1 cells were treated with 20ng/ml LPS and 100ng/ml IFNγ for 24h. Classical M1 cytokines expression was measured (IL-1β, IL-6, IL-23, and TNF-α). Results are representative of three independent experiments. The data are presented as the means±SD. To compare means Student’s t-test was used. Significant differences are indicated by: **P<0.01.

Since human published datasets indicate a significant increase in CDK5 expression in M2-polarized macrophages, we decided to better characterize CDK5 involvement in M2 polarization. Thus, we decided to measure CDK5 expression in different “M2-like” conditions. Due to the fact that we can’t observe differences in CDK5 protein expression in THP-1 cells, we decided to use murine Bone Marrow Derived Macrophages (BMDM). We treated BMDM with several M2 stimuli for 24h (IL-4, IL-13, IL-4+IL-13, IL-10, and DEX).

We observed that all those stimuli induced a mild increase in CDK5 expression after 24h of treatment, in all condition and especially with the combination of both IL-4 and IL-13 (Figure 7.1).

![CDK5 expression in M2-polarized BMDM](image)

**Figure 7.1** CDK5 expression in M2-polarized BMDM. Change in CDK5 mRNA expression after M2 polarization, in BMDM cells specifically treated with recombinant murine IL-4, IL-13, the combination of IL-4 and IL-13, dexamethasone (DEX), and IL-10, detected by qPCR. The data are shown as fold changes, normalized to NT BMDMs. Results are representative of minimum three independent experiments. The data are presented as the means±SD. To compare means, one-way ANOVA was used. Significant differences are indicated by: ***P<0.001, **P<0.01, and *p < 0.05.

Therefore, we decided to quantify M2 cytokines production in IL-4/IL-13 treated BMDM. To do this we generated CDK5-silenced BMDM via lentiviral transduction.

Interestingly, we noticed that CDK5 abrogation is able to inhibit the production of interleukin 10 (IL-10; Figure 7.2A), indicating a low tumoricidal activity. Furthermore, CDK5-silenced M2 macrophages showed low CCL22 mRNA level.
(Figure 7.2B), suggesting a possible impairment in lymphocytes recruitment. Overall these results show a possible correlation between CDK5 activity and M2 polarization in murine macrophages.

![Graph A: IL-10 mRNA expression](image)

**Figure 7.2** M2-cytokines production in shCDK5 BMDM. Change in classical M2 cytokines mRNA expression after M2 polarization, in CDK5-lacking BMDM. BMDM cells were treated with 20ng/ml IL-4 and 20ng/ml IL-13 for 6h and 24h. Classical M2 cytokines expression was measured (IL-10 and TNF-α). Results are representative of three independent experiments. The data are presented as the means±SD. To compare means Student’s t-test was used. Significant differences are indicated by: **P<0.01.
In this thesis we decided to examine CDK5 involvement in cancer progression and in the tumor microenvironment.

In particular, the first part of the thesis is focused on the role of CDK5 in Diffuse Large B cell Lymphoma.

In the second part, we decided to unravel CDK5 involvement in breast cancer related macrophages.

In the two last decades, several studies described how the activity of cyclin dependent kinase “CDK5” contributes to the oncogenic initiation and progression of several types of malignancies. Several reports described the importance of CDK5 activity in several solid tumors like breast [126], prostate [127], lung [128] and pancreatic [129] cancers. Only few groups investigated CDK5 role in haematological malignancies, such as leukaemia [130] and multiple myeloma [131], while its role in lymphoma has been completely neglected. In this scenario, new discoveries regarding the molecular implications of CDK5 involvement in tumor development and progression could be very helpful in the clinics, to improve possible targeted therapies in patients.

In light of this, in the first part of the thesis, we decided to dissect CDK5 involvement in haematological malignancies.

On this line, we found that CDK5 is broadly expressed at the protein level in cancer cells in several haematological malignancies, compared to B lymphocytes from healthy donors. Thus, we decided to better investigate CDK5 involvement in lymphoma development and progression. We showed for the first time that DLBCL markedly over-expressed CDK5 at both protein and mRNA levels compared to normal lymphocytes. Coherently with our results, it was previously demonstrated that CDK5 is one of the most hypomethylated genes in mantle cell lymphoma [132], therefore its high expression could be induced by epigenetic modification that can lead to an aberrant transcription.

In addition, being pSTAT3 (S727) reported to be a direct CDK5 target [133], we observed that STAT3 phosphorylation rate is strongly increased in DLBCL cell lines in which p35/CDK5 were up regulated. Thereby, we designed loss-of-function experiments on DLBCL cells that revealed, for the first time, that STAT3 phosphorylation correlates with CDK5 expression in SU-DHL-8 cells and that CDK5 loss directly impairs cellular proliferation and promotes apoptotic events, by affecting STAT3 downstream pathway and in particular EME1 transcription.

Further, since CDK5 activity is mainly conferred by the association with its primary activator p35 [134], we decided to examine p35 role in this context. p35 has a fast turnover, finely regulated both at transcription level [135] or through post-translational modification via ubiquitin-proteasome degradation [136]. We observed that p35 is almost absent in healthy controls, instead it is strongly upregulated in cancer cells at protein level. We noticed an inverse tendency in p35 expression among mRNA and protein expression in DLBCL. Therefore, we
looked for post-transcriptional regulatory modification able to inhibit p35 translation of healthy cells that could be abrogated in cancer cells. Thereby, we thought that a mechanism involving microRNAs, that have been associated with tumorigenesis [137], might be the answer. Several reports described miR-26 involvement in cancer development and progression. Nonetheless, miR-26 role as a tumor suppressor [85, 87-89], or as an oncogene [90-92] was unclear. miR-26a expression inversely correlated with p35 mRNA level in DLBCL, indicating that cancer cells can adopt possible mechanisms that abrogate miR-26 transcription in order to gain survival and self-renewing capacity. The binding capacity was confirmed by reduced luciferase reporter activity of the p35 WT 3’UTR under miR-26a overexpression. In addition, we demonstrated that miR-26a can be considered a tumor suppressive mediator in DLBCL, since its over-expression inhibits DLBCL tumor growth and promotes tumor necrosis in NOD/SCID mice. Altogether, these findings support the notion that the CDK5/p35 complex mediates the tumor-suppressive function of miR-26a, and that p35 deregulation through miR-26a plays an important role in tumor growth and may be considered as a novel prognostic marker and a potential therapeutic target for DLBCL.

The second part of this PhD thesis was focused to gain a comprehensive picture of CDK5 role in the tumor microenvironment. In particular, we decided to study CDK5 involvement in breast cancer progression. First of all, we found some case reports in which it was described a fine correlation between DLBCL and breast cancer development [138-140]. In the past decades breast cancer treatment was improved by targeted therapies, and especially the development CDK inhibitors, targeting CDK4/6 like abemaciclib (Verzenio) and palbociclib (Ibrance) are now commonly used in clinics and are now of great importance in the management of hormone receptor-positive (HR+) metastatic breast cancer [141]. However, treatment strategies for advanced and metastatic breast cancer as well as HER2-positive and especially triple-negative breast cancer are not yet satisfying [142, 143]. Therefore, possible identification of molecular targets that could modulate cancer progression is crucial. CDK5 was previously reported to be a potential molecular target in the clinical practice of breast cancer patients [66]. Therefore, we investigated CDK5 clinical relevance in human breast cancer patients. We observed that CDK5 expression is strictly related to patient outcome and correlates with poor overall survival. In addition, taking advantages of human specimens available in-house we discovered that, in our court of patients, CDK5 expression was not only localized at the tumor level, but it was present in the periphery. In particular, CDK5 expression co-localized with CD68+/CD163+ TAMs.

The understanding of how recruitment and migration of leukocytes occur into the TME can represents a key step to fulfil their role in cancer response. Macrophage
are able to migrate requiring well organized contractile system, including F-actin based protrusive elements, actin/myosin contractility apparatus, and retractile properties. Podosomes were firstly described in osteoclasts [144] and have been characterized in both mononuclear phagocytes [145] and lymphocytes [146]. Several reports described their importance, in comparison with invadopodia, that are podosome-like structure involved in neoplastic cells adhesion and ECM degradation [147]. To this purpose we demonstrated for the first time that CDK5 impairment could partially abrogate podosome formation in macrophage-like cells.

Moreover, we observed that CDK5 is modulated in human macrophages differnetiated in vitro, from circulating monocytes. In addition, we confirmed that CDK5 expression is markedly expressed in THP1-derived macrophages at basal level. Its expression is down regulated after LPS/IFNγ polarization in vitro. On the contrary, we did not observe any significant increase of CDK5 protein level in alternatively-activated macrophages obtained from THP-1 cells, following IL-4/IL-13 stimulation.

To further study CDK5 role in tumor microenvironment, THP1 cell lines were used as a CDK5 loss-of-function model, via shRNAs approach. We noticed a reduction in podosomes formation, indicating that CDK5-silenced macrophages can display an impairment in tissue infiltration. In addition, CDK5-silenced THP-1 cells display a pro-inflammatory phenotype. From our results CDK5 seems to promote pro-inflammatory cytokines over-expression both in not-treated cells, but also after LPS stimulation.

To better understand CDK5 involvement in the tumor microenvironment we analysed the expression of CDK5 in tumors and we found that its expression is correlated with TAM present in breast cancer periphery.

Through gain- and loss-of-function experiments, we discovered that p35/CDK5 axis is fundamental for macrophages pro-inflammatory cytokines production in vitro. M1 macrophages have been extensively studied in cancer and they display an anti-cancer phenotype. M2 polarized macrophages, instead, promote anti-inflammatory responses, and display pro-tumorigenic properties. Accordingly, few years ago Dorand et al. [148] reported in Science that CDK5 could mediate the anti-tumor immune response, by regulating programmed cell death ligand 1 (PD-L1). In particular, they proposed that CDK5 depletion induced a lower PD-L1 expression and, as a consequence, an increased number of CD4+ T cells, which mediated tumor rejection.

Future experiments are aimed at better characterizing tumor infiltrating macrophages in vivo, via shRNA approach and taking advantage of available CDK5 inhibitors. In particular, in vivo experiments, incuding the orthotopic injection of 4T1-breast cancer cells with CDK5 silencing BMDM could give a clear evidence on how CDK5 could affect breast cancer progression in vivo.
In addition, possible molecular mechanisms can elucidate if CDK5 modulation in the fine regulation of macrophages polarization may represent a novel breakthrough for macrophage-directed cancer immunotherapy. Due to the practice of pan-CDKs inhibitor as innovative therapeutic strategies, it could be very helpful to discover possible CDK5 specific inhibitors, in order to specifically target its activity in patients in which common therapy is useless.


