UNIVERSITÀ DEGLI STUDI DI MILANO

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DOTTORATO DI RICERCA IN
BIOTECNOLOGIE APPLICATE ALLE SCIENZE MEDICHE - CICLO XXVII
Scuola di Dottorato in Scienze Biomediche Cliniche e Sperimentali

TESI DI DOTTORATO DI RICERCA

CDK5 PLAYS A KEY ROLE IN PROLIFERATION, APOPTOSIS AND *IN VIVO* TUMOR GROWTH OF DIFFUSE LARGE B-CELL LYMPHOMA

Settore BIO/13 – Biologia Applicata

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Anno Accademico 2013/2014

To Gabriele, my mother and my father.

To Ivaldo, who has believed in me.

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ABSTRACT

Diffuse Large B-cell lymphoma (DLBCL) is the most common adult non-Hodgkin lymphoma, whose standard of care is the immunochemotherapy R-CHOP. Chemorefractory patients, still approximately 40%, represent an unmet medical need, requiring novel targets for innovative treatments. One potential target is cyclin-dependent kinase 5 (CDK5), a serine/threonine protein kinase that has been recently linked to tumor development and progression.

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine protein kinase, which forms active complexes with p35 or p39 and it is mostly active in the nervous system, regulating several processes. In the past few years, novel roles for Cdk5 have been proposed in many pathological conditions and cancer, even if its role in DLBCL remains uninvestigated to date.

For the first time, we showed that Cdk5 and p35 are both overexpressed in DLBCL cell lines. Moreover, we highlighted that proliferation and apoptosis are regulated by Cdk5 activity in loss-of-function experiments.

MicroRNAs (miRNAs) are a class of highly conserved short RNAs that regulate diverse cellular processes by degradation or translation inhibition of mRNA targets. Numerous reports have shown that miRNA dysfunction is involved in the development and progression of various human cancers (4).

In particular, miR26a has a tumor suppressor role in different cancers, such as nasopharyngeal carcinoma (5) breast cancer (6) and gastric carcinoma (7), but its role in DLBCL remains still unclear.

In this study we demonstrated that mir-R26 regulates p35 expression and that it involves Cdk5/p35 pathway directly affecting DLBCL cells proliferation and apoptosis. Thus, the clarification of the molecular mechanisms at the base of Cdk5/p35 expression and their role on DLBCL tumor cell proliferation could lead to the identification of innovative therapeutic targets for treatments of DLBCL.

1. INTRODUCTION

1.1 – DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL)

Diffuse Large B-cell lymphoma (DLBCL) is the most common adult B-cell non-Hodgkin lymphoma (NHL), comprising 30–35% of all NHLs and accounting up to 30% newly diagnosed cases in the United States[1]. DLBCL represents a widely heterogeneous group of tumors from a point of view of clinical features, genetic abnormalities, response to treatment and prognosis[2].

It could arise inside the lymph nodes, but also outside of the lymphatic system, in several different locations in the body, such as skin, breast, gastrointestinal tract, testes, thyroid, brain, or bone.

The first sign of the disease is a rapid and painless enlarging lymph nodes, that cause swelling in the neck, groin, or armpit. Sometimes the swelling could be painful. Other symptoms include unexplained fevers, weight loss, and night sweats.

The diagnosis is made on the basis of a surgical biopsy/excisional lymph node or extranodal tissue specimen that are fixed in formalin. In those rare cases requiring emergency treatment, core biopsies could be appropriate as the only diagnostic test. The specimens are analyzed by immunohistochemistry (CD20, CD45, and CD3) and also the fresh frozen material is collected for molecular characterization by gene expression profiling (GEP). To ensure adequate quality, tissue specimens are processed and examined by an experienced pathologist. The diagnosis is given from the histological report according to the classification of the World Health Organization (WHO)[3]. The WHO classification aims to define "real" diseases on the bases of morphology, genetic characteristics, immunophenotype, and clinical features[4].

Through molecular characterization using gene-expression profiling (GEP),

DLBCL can be subdivided into three distinct cell-of-origin subtypes: activated B-cell (ABC), germinal centre B-cell (GCB), and primary mediastinal B-cell lymphoma (PMBL)[5-8] (Figure 1.1). The molecular pattern of each subtypes correspond to the genes overexpressed in the putative B cell developmental stage from which the tumor originated: in the GCB subtype genes associated with the germinal center reaction are overexpressed, such as *LMO2* and *LRMP*[7]; while the ABC subtype is characterized the overexpression of IRF4, FOXP1 and PIM2[7], that are genes that regulate the plasmacytic differentiation, deriving this subtypes from a post-germinal center B cell, and by the constitutive activation of NF-KB pathway; finally, the PMBL subtype derived from the rare post-thymic B cell and its molecular pattern is more similar to which that characterized classic Hodgkin lymphoma, than those of the other DLBCL subtypes[6,9].

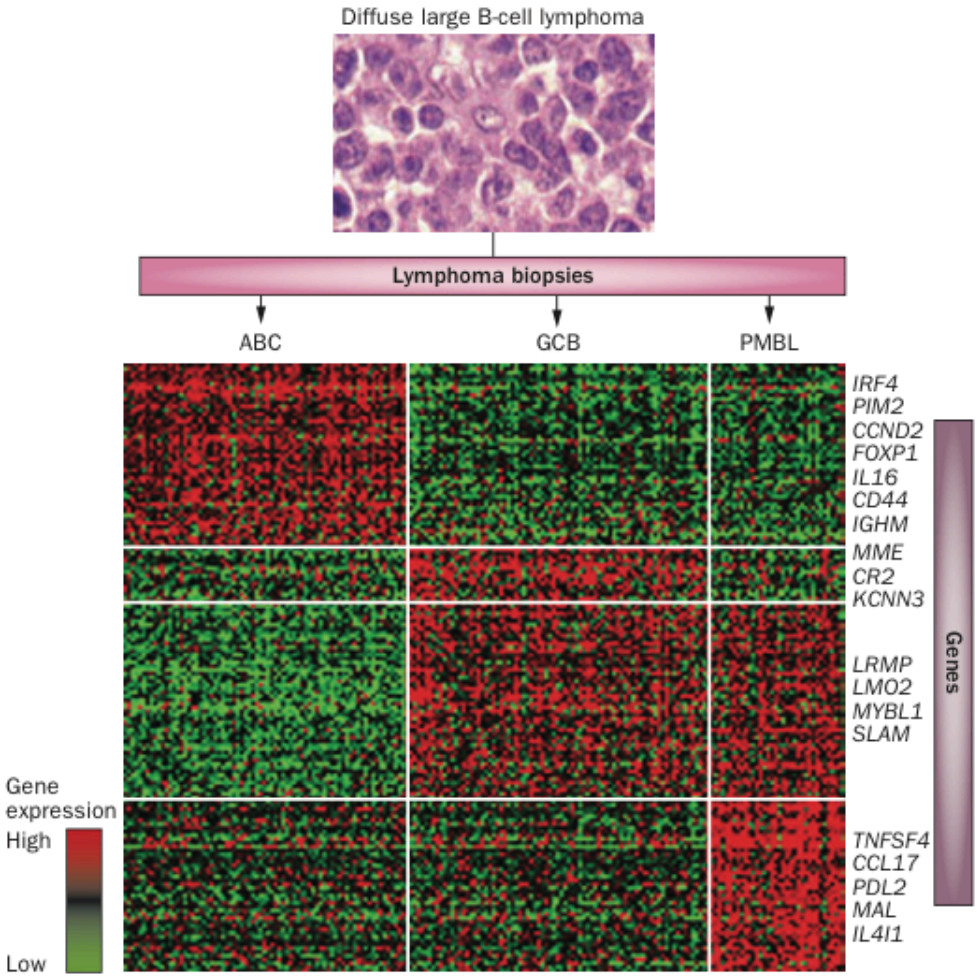

Fig. 1.1 – Morphologically indistinguishable DLBCL tumours can be subdivide by gene-expression profiling into three distinct cell-of-origin subtypes. From Roschewski M. et al., Diffuse Large B-cell lymphoma – treatment approaches in the molecular era. *Nat. Rev. Clin. Oncol*. (2014)

GEP allowed the identification of new potential targets and oncogenic mechanisms (Table 1.1), providing novel therapeutic options and the biological basis for targeted treatment of DLBCL, including the use of different classes of biological agents, and monoclonal antibodies.[10] Targeting specific oncogene that is overexpressed in a specific subtypes allows a more accurate approach to therapy[11] compared with the standard approaches based on chemotherapy. However, to develop personalized treatment for single patient, the identification of mutations driving disease progression and

tumorigenesis is not sufficient for the DLBCL molecular complexity.[12] Characterization of cooperating mutations that lead to drug resistance have to be keep into account.

Table 1.1 | Oncogenic mechanisms and potential targets in DLBCL subtypes. From Roschewski M. et al., Diffuse Large B-cell lymphoma – treatment approaches in the molecular era. *Nat. Rev. Clin. Oncol*. (2014)

DLBCL subtype	Cell of origin	Oncogenic mechanisms	Potential targets
GCB	Germinal centre B-cell	*BCL2* translocation* *EZH2* mutations‡ *PTEN* deletions§ Loss of PTEN expression	BCL6 EZH2 PI3K/Akt
ABC	Post-germinal centre B-cell	NF-κB activation‖ *CARD11* mutations *MYD88* mutations *CD79B* mutations *A20* deletions	BCR CBM complex IRAK-4 JAK–STAT
PMBL	Post-thymic B-cell	NF-κB activation¶ 9p24 amplification¶ *REL* amplification *JAK2* mutations *CIITA* translocations#	JAK–STAT PD-1#

*GCB DLBCL frequently has *BCL2* translocations[13] and most result in activation of BCL-6, the master transcriptional regulator of the germinal centre.[14] ‡Mutations in *EZH2* (21% of GCB DLBCL cases) are specific for this subtype.[15] §Loss of PTEN expression (55% of GCB DLBCL cases)[16] results in activation of the PI3K/Akt pathway for which multiple inhibitors are currently in development. ‖ABC DLBCL is defined by constitutive NF-κB pathway activation[17] and BCR signalling pathways are oncogenically activated in this subtype:[18] mutations in *MYD88, CARD11* and *CD79B* are found in ABC DLBCL along with deletions and mutations of *TNFAIP3*.[19,20] ¶PMBL is characterized by amplification of chromosome 9p24 and NF-κB pathway activation.[21] #*PDL1* and *PDL2* are overexpressed in PMBL[6] making their receptor, PD-1, a potential target; recurrent *CIITA* translocations also result in PD-1 pathway activation in PMBL.[22] Abbreviations: ABC, activated B-cell; BCR, B-cell receptor; CBM, CARD11–MALT1–BCL-10; DLBCL, diffuse large B-cell lymphoma; GCB, germinal centre B-cell; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; PMBL, primary mediastinal B-cell lymphoma.

Staging and risk assessment of DLBCL are based on a complete blood count, a screening test for hepatitis B and C and human immunodeficiency virus as well as routine blood chemistry including uric acid and lactate dehydrogenase (LDH), and protein electrophoresis. Patients sensible to curative therapy have a computed tomography (CT) scan of the abdomen and chest, bone marrow aspirate and biopsy; whereas high-risk patients do a diagnostic spinal tap in addition. The extension of the disease and the evaluation of treatment response in patient, are assessed by

[^{18}F]deoxyglucose positron emission tomography (PET) scanning[23]. Finally, before therapeutic treatment, also the cardiac function (left ventricular ejection fraction) and the performance status of the patient are evalueted.

The staging of DLBCL is established in accordance with the Ann Arbor staging system (Table 1.2)[3]. This system defines the extent of disease progression and helps predict prognosis and direct treatment. Four stages are described followed by the letter A or B, indicating the absence (A) or presence (B) of B symptoms.

Table 1.2	**Ann Arbor staging classification.** From Tilly, H. et al. Diffuse large B-cell lymphoma (DLBCL): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology (2012)
STAGE	**SYMPTOMS**
1	Involvement of a single lymphatic region or localized involvement of single extralymphatic organ or site
2	Involvement of two or more lymphatic regions on the same side of the diaphragm or localized involvement of a single extralymphatic organ or site and of one or more lymphatic regions on the same side of the diaphragm
3	Involvement of lymphatic regions on both sides of the diaphragm
4	Diffuse or disseminated involvement of one or more extralymphatic organs with or without lymphatic involvement

The standard treatment for patients with DLBCL is an immunochemotherapy regimen in which Rituximab, a monoclonal antibody against CD20, is in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).[24] This therapy can cure more than 50% of cases, even in advanced stage, despite DLBCL is biologically aggressive.[25] However, disease in up to one-third of patients will relapse or is refractory to therapy.[26]
The salvage treatment for patients that have relapsed or refractory DLBCL

that are responsive to a second chemotherapy regimen, is autologous stem cell transplantation (ASCT), but the rates of success are poor nowadays[27], requiring the identification of novel targets for innovative treatments.

The International Prognostic Index (IPI) is the clinical prognostic tool commonly used to predict the overall treatment outcome in DLBCL patients, based on the age, serum lactate dehydrogenase (LDH) concentration, tumor stage, number of extranodal disease site and performance[28], but this risk stratification tool does not capture the molecular heterogeneity and the genetic of the disease, leaving a large group of patients poorly-characterized and with an unfavorable course of disease, despite a good prognostic index.[28] The three distinct subtypes of DLBCL identified by GEP have different mechanisms of oncogenic activation as previously described, and also distinct prognosis (Table 1.3).[10]

Table 1.3 | PFS and overall survival for each DLBCL molecular subtype. From Roschewski M. et al., Diffuse Large B-cell lymphoma – treatment approaches in the molecular era. *Nat. Rev. Clin. Oncol*. (2014)

Molecular subtype	Regimen	3-year PFS rate	3-year overall survival rate	Reference
ABC DLBCL	R-CHOP	40%	Approximately 45%	Lenz *et al.* (2008)[29]
GCB DLBCL	R-CHOP	74%	Approximately 80%	Lenz *et al.* (2008)[29]
PMBL	DA-EPOCH-R	100%*	97%*	Dunleavy *et al.* (2013)[16]

*At 5 years. Abbreviations: ABC, activated B-cell; DA-EPOCH-R, dose-adjusted etoposide, doxorubicin and cyclophosphamide with vincristine, prednisone and rituximab; DLBCL, diffuse large B-cell lymphoma; GCB, germinal centre B-cell; PFS, progression-free survival; PMBL, primary mediastinal B-cell lymphoma; R-CHOP, rituximab, cylophosphamide, doxorubicin, vincristine and prednisone.

Patients with PMBL can be treated with another chemotherapeutic regimen, called DA-EPOCH-R.[29] The Lymphoma/Leukemia Molecular Profiling Project (LLMPP) retrospective analysis showed that patients with ABC

DLBCL have worst prognoses than those with GCB DLBCL when treated with R-CHOP.[30] Multiple reports have reveled that the over-expression of Bcl-2 and Myc in DLBCL is associated with poor prognosis. These over-expressions can be verified through immunohistochemistry on formalin-fixed paraffin embedded tissue.[31-33] Nevertheless, it has not been proved if patients with both Bcl-2 and Myc expression are a molecularly different group.

1.2 – CYCLIN-DEPENDENT KINASE 5 (CDK5)

Cdk5 belongs to Cyclin-dependent kinases (Cdks) family. The proteins of this family are kinases that specifically phosphorylate threonine or serine residues in different proteins that are involved in the regulation of the cell cycle. The Cdk family is composed of 9 small serine/threonine kinases (30–35 kDa), numbered from Cdk1 to Cdk9, according to their discovery.[34] Cdks are involved in several biological functions, mitosis, regulation of cellular processes, such as senescence, differentiation and apoptosis, through gene transcription modification.[35] In proliferating cells, dysregulation of Cdks lead to tumor formation, whereas their inhibition in neuronal precursors leads to terminal differentiation.[36] Generally, to be activated, Cdks require the binding of an activator protein, named a cyclin, and the phosphorylation in a loop of activation.[37,38]

Although specific Cdks are associated with different cell cycle phases, their activities can sometimes overlap, depending on the binding with different cyclins.[34,39,40]

1.2.1 – Cdk5, an unusual member of Cdk family

Despite Cdk5 shares a strong sequence homology with other members of Cdks family, it is an unusual member because it's activation does not require either to be phosphorylated or the binding of a cyclin, but of another activator protein, termed p35.[41] However, unlike other cyclin-dependent kinases whose principal function is to regulate the cell cycle progression, most Cdk5 target proteins are not implicated in the control of cell cycle, but are involved in neuronal signaling or cytoskeleton.[42,43] Indeed, in literature there are many

evidences that Cdk5 controls different neuronal processes, such as cell adhesion, pain signaling, neuronal migration, actin dynamics, apoptosis, microtubules stabilization and transport, axonal guidance, synaptic structure and endocytosis.[44-46] The consensus motif phosphorylated by Cdk5 in proteins is (S/T)PX(K/H/R). However also some sequence variations can be phosphorylated, such as the KSPXX motif in the neurofilaments[47] and KSPXK in the microtubule-associated protein 1B.[42] Cdk5 activation requires the binding to its non-cyclin activators p39 or p35.[48,49] When it is bound to Cdk5, p35 adopts a structural conformation comparable to those of a cyclin.[50] The abundance of p39 and p35 in the nervous system cause the prominent activity of Cdk5[51], which has a key role in function and development of the nervous system.[52-54]

In the last ten years, numerous studies have demonstrated that Cdk5 is active also in many different non-neuronal cells[55], such as pancreatic β cells[56], monocytes[57], corneal epithelial cells[58], ovary[59], testis[55]. Recently, its involvement in many other different biological mechanisms, such as activation of immune system, cell migration, angiogenesis, gene expression, apoptosis and proliferation, in many different cell types is raised.

1.2.2 – Regulation of transcription and translation by Cdk5

Cdk1, Cdk2, Cdk6, and Cdk4 have a key role in the cell cycle regulation, providing a check-point system in its progression[38]. Nevertheless, some other members of the family, such as Cdk9, Cdk8, and Cdk7, regulate the cell cycle in an indirect manner by the modulation of the function of many different transcription factors.[60,61] All of these Cdks activated by cyclins can phosphorylate the RNA polymerase II C-terminal domain, thus they

modulate expression of specific genes and have a role in chromatin regulation and RNA processing.[62,63] It is reasonable that also Cdk5 might be involved in gene expression regulation, because Cdk5/p35 complex is found in the nucleus[64], and because some Cdk5 target proteins are transcription factors, such as myocyte enhancer factor 2[65] and p53[66]. In particular, a study has reported that in the nucleus the active Cdk5/p35 complex can phosphorylate mSin3-associated protein (mSds3), a fundamental component of the mSin3–histone deacetylase complex[67], to regulate the function of RNA polymerase II[68], repressing transcription. Interestingly, Cdk5 can also phosphorylate the ubiquitous and the most abundant cromatin-associated non-histone protein, the high mobility group box 1 (HMGB1) at Ser180, inducing its binding to DNA[69] and structural changes that cause the transcription factors recruitment[70]. Finally, in the cytoplasm Cdk5 can phosphorylate a transcription factor activated in hypertonic conditions, the tonicity-responsive enhancer binding protein (TonEBP)[71]. In this condition, TonEBP is phosphorylated and transported to the nucleus, where osmoprotective genes are transcripted[72] (Figure 1.2).

Recently, some studies have been demonstrated that Cdk5 controls also translation.[73] Cdk5 can phosphorylate the glutamyl-prolyl tRNA synthetase (EPRS) at Ser886, after interferon-gamma (IFN-γ) stimulation, which in turn can down-regulate the mRNA translation of encoding pro-inflammatory molecules, binding the GAIT complex in myeloid cells[74,75]. Of note, this is the first study linking Cdk5 role with the regulation of translation (Figure 1.2).

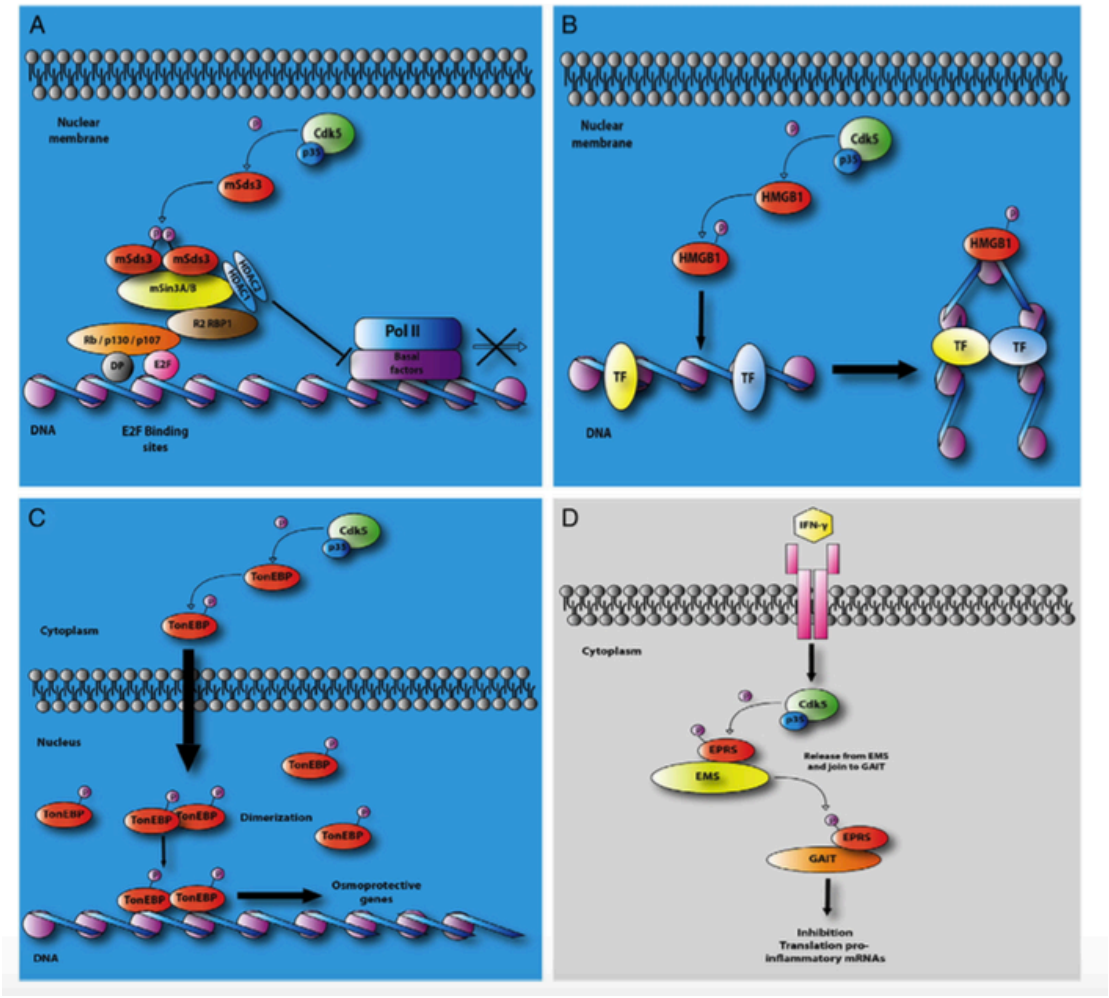

Fig. 1.2 – The role of Cdk5 in controlling transcription (blue background panels) and translation (gray background panel). A) The active Cdk5/p35 complex may be localized in the nucleus where it phosphorylates mSds3 to regulate the function of RNA polymerase II. B) In another cellular context, Cdk5 can phosphorylate HMBG1 to promote transcription. C) Finally, Cdk5 can phosphorylate TonEBP in the cytoplasm. Phosphorylated TonEBP is transported to the nucleus where it can stimulate the transcription of osmoprotective genes. D) Finally, in response to interferon-gamma IFN-g Cdk5 can phosphorylate EPRS which then can bind the GAIT complex to down-regulate the translation of mRNA encoding pro-inflammatory molecules. From Contreras-Vallejos E., et al. Going out of the brain: Non-nervous system physiological and pathological functions of Cdk5. Cellular Signalling (2012)

1.2.3 Cdk5 and the control of the cell cycle

Cdk5 historically is an un common member of the Cdk family, in fact its activation requires a non-cyclin activators and does not need to be phosphorylated at the activation loop, as previously described. More

important, Cdk5 has been supposed to not play any function in the cell cycle control[42,43]. Recently, this view has been modified because it was found that Cdk5 might bind to cyclin I, that in turn may activate the kinase.[76] In addiction, it has been observed that Cdk5 also might interact with other cyclins such as cyclin E, D3 and D1. Nevertheless, the interaction with these cyclins does not affect its kinase activity.[77,78]

Remarkably, cyclin D2 also binds the Cdk5, but in this case, the activity of the Cdk5/p35 complex can be abrogated. A possible molecular explanation can be linked to the differential recognition of the Cip/Kip family members to Cdk5 when it forms a complex with cyclin D2 or p35.[79]

Since the discovery of cyclin I, the classical opinion that the cyclins expression is regulated strictly during the different phases of the cell cycle has been modifying. In fact, cyclin I is not implicated in cell proliferation and continues to be equally expressed during the entire cell cycle.[80,81] Of note, it is expressed mainly in terminally differentiated cells (cardiomyocytes, podocytes and neurons) and when cyclin I is absent these cells are more susceptible to apoptosis.[81,82] The Cdk5/cyclin I complex was hypothesized to activate the MEK-ERK signaling pathway, leading to an augment of Bcl-2 and Bcl-X_L expression in postmitotic cells; these variations in anti-apoptotic proteins are due to the activity of ERK1/2.[83]

The Cdk5/p35 complex might also phosphorylate Bcl-2 at Ser70, inducing stabilization of this protein[84], which indicates that Cdk5 can indeed be able to control apoptosis by two different and maybe independent mechanisms, suggesting that Cdk5 may have a fundamental role in controlling the survival of postmitotic cells. Moreover, Cdk5 also can interacted with cyclin G1,

causing to the phosphorylation of c-Myc at Ser62; this effect has been found primarily in lung carcinoma cells, in which both Cdk5 and cyclin G1 are up-regulated.[85] This study suggests that Cdk5 can have a key function in the regulation of the cell cycle in lung carcinoma cells which overexpress cyclin G1, and thus, it will be interesting to understand if Cdk5 has the same function in other cancers.

In neurons, Cdk5/p35 complex leads to the cell cycle suppression interacting with the transcription factor E2F1 in the nucleus. This interaction impairs the assemblement of the E2F1/DP1 complex that modulates the activation of the transcription of different genes. A similar mechanism has not been still observed in other terminally differentiated cell types, such as cardiomyocytes and podocytes. During cell cycle arrest the CKI inhibitor, p21, represses E2F activity through the inhibition of the phosphorylation of Retinoblastoma protein (pRB)[86]. In this circumstance, Cdk5 can phosphorylate and thus stabilize p53[66], which active the transcription of p21.[87] Therefore, Cdk5 can control both pathways to block the progression of cell cycle in highly differentiated cells.

Another remarkable aspect about the Cdk5 function in the cell cycle is correlated to the localization of p35, Cdk5 and Cdk5rap2, a protein involved in the stabilization of the centrosome and regulation of microtubule dynamics, at the centrosome of HeLa cells. The association between Cdk5rap2 and centrosome is independent from microtubules.[88] Mutated Cdk5rap2 leads to the primary microcephaly development. Interestingly, double knockout p35/p39 mice have a reduction in the body size compared with wild-type mice[89], indicating the possibility that Cdk5 could regulate cell division. Of note, Cdk5rap2 is located in the midbody, an important structure in

cytokinesis. Defects in the midbody results in problems in cell division[90]. Similarly, Cdk5 also is located at the midbody in HeLa cells, but it has been demonstrated that its role in the control of cytokinesis can be independent of kinase activity.[91]

1.2.4 – Role of Cdk5 in apoptosis

Cdk5 involvement in apoptosis mechanisms has been mainly described in central nervous system, but recently also in other cells[92 55].

Sharma and colleagues have shown that Cdk5 is highly expressed in proliferating bovine aortic endothelial cells (EC).[93] After inhibition of EC proliferation and stimulation of apoptosis by Angiotensin[94] a significantly Cdk5 decrease was found. Furthermore, EC proliferation stimulation by basic fibroblast growth factor[95], a pro-angiogenic growth factor, can be inhibited by roscovitine, an inhibitor of Cdk5, which instead can induce EC apoptosis.[93]

Genetic and pharmacological inhibition of Cdk5 function is also linked with increased susceptibility to cell death in several different cell types[96-98].

p35 knockout mice present podocytes without aberrations and with normal morphology. Nevertheless, apoptosis is significantly increased in immortalized podocytes from p35 knockout mice exposed to different stress conditions, including depletion of serum, UV-C radiation, transforming growth factor β1 or puromycin aminonucleoside. Consistently with previous observations, apoptosis increases in podocytes from wild-type mice after transfection with siRNA against p35. Rescue experiments restoring p35 in

podocytes from p35 knockout mice decreases apoptosis.[83] Effects of Cdk5 activity on apoptosis in podocytes may be correlated to increased Bcl-2 expression under the control of the Cdk5/p35 complex or, alternatively, a stabilizing effect of Cdk5 phosphorylation on Bcl-2.

Cdk5 also has a key role in pancreatic β cells, in which extracellular glucose induce an increase in the p35 expression and subsequent Cdk5 activity, indicating that the secretion[99] and transcription[98] of insulin may be dependent on the Cdk5/p35 complex. Nevertheless, p35 overexpression can inhibit the secretion of insulin in pancreatic β cells. Therefore, Cdk5/p35 complex appears to be influenced by glucose levels. In high glucose conditions, p35 is overexpressed and a calpain-dependent p25 fragment of p35 is founded, resulting in apoptosis events and insulin secretion reduction. Both effects can be reversed by the use of the Cdk5 inhibitor.

1.2.5 – Role of Cdk5 in the immune system function and inflammation

Cdk5 seems to have a regulatory function also in the immune system, in which it may be involved in cellular activation and in control of the inflammatory response. Remarkably, Cdk5 can phosphorylate the intermediate filament protein vimentin at Ser56, leading to GTP-dependent secretion of proinflammatory molecules by neutrophils.

Of note, when the Cdk5-dependent phosphorylation of vimentin increased, also the secretion of lactoferrin, matrix metalloprotease-9 and β-hexosaminidase increased. Using Cdk5 siRNA or roscovitine this effect is

reduced considerably, but not abrogated, suggesting that other protein kinases can be implicated in the secretion of these molecules by neutrophils. Considering these evidence, Cdk5 is an attractive target to modulate neutrophil-mediated inflammatory responses by inhibiting the proinflammatory molecules secretion.[100]

In another aspect of inflammation regulation, Cdk5 activation by p35 is requested for migration and proliferation of T cell receptor-dependent activation lymphocytes. Coronin 1a and F-actin are aberrantly polarized in lymphocytes derived from Cdk5 knockout mice, indicating that the Cdk5-mediated phosphorylation of coronin1a is important for modulation of actin dynamics in T cells.

Actin dynamics can have an essential role in T cell activation and CCL19-dependent lymphocyte migration promotion, which are implicated in the pathophysiology of experimental autoimmune encephalomyelitis. Even if this autoimmune model involves the CNS, this Cdk5 novel function in the control of T cells opens new opportunities for innovative therapies for inflammatory diseases treatment.[101]

Recently, it has been demonstrated that Cdk5 presents other functions in the inflammatory response. Berberich et al. demonstrated that pharmacological inhibition of Cdk5 causes the inhibition of the transmigration of leukocytes by blocking gene expression dependent by NFκB. These effects were reproduced by genetic inactivation of both Cdk5 and Cdk9 using siRNAs.[102]

1.2.6 – Cdk5 and cancer

Similar molecular and cellular mechanisms mediate migration of cancer cells during metastasis and of neuronal cells during the nervous system development.[103] Consequently, it is not surprising that Cdk5 can have a role also in the migration of tumor cells.

Feldmann et al. demonstrated that the inhibition of Cdk5 activity, through the use of a pharmacological inhibitors or a dominant negative Cdk5, decreases the migration capacity of pancreatic carcinoma cells.[104] Indeed, Cdk5 inactivation leads morphological changes in these cells that induces to a loss of cell polarity.

Of note, Cdk5 controls not only migration but also invasion through the modulation of the invadopodia formation.[105] Consistent with these effects, Cdk5 inhibition decreases metastasis and tumor growth in pancreatic cancer.[104] Therefore, Feldmann at al. focused on the Ras-dependent signaling pathway, which plays a key role in the development of pancreatic tumor[106], showing a possible involvement of Cdk5, that Cdk5 could be an innovative target for the treatment of pancreatic tumor.[104]

Another observation that link Cdk5 with the onset of tumors is that Cdk5 may phosphorylate and thus modulate the transcriptional activity of STAT3 at Ser727.[57] In medullar thyroid carcinoma cells, STAT3 phosphorylated by Cdk5 leads to an increase in cell proliferation and promotion of malignancy transformation. Moreover, aggregates of calcitonin derived from medullar thyroid carcinoma promote Cdk5 activity inducing cell proliferation. Consistently with these observations, inhibition of Cdk5 in nude mice retards

tumor growth in a STAT3 phosphorylation-dependent manner.[107]

Cdk5 activity can be modulated also by retinoic acid in thyroid neoplastic cells through a plasma membrane glycoprotein that promotes the uptake of iodide.[108] These observations suggest that Cdk5 may be a molecular target for the diagnosis and treatment of medullary thyroid cancer.

In breast cancer, Cdk5 can have a key role in controlling proliferation of tumor cells, because in the presence of either siRNA against Cdk5 or roscovitine, MCF-7 and MDA- MB321 cells show a reduction in proliferation rates. Carboplatin treatment, a known chemotherapeutic drug against breast tumor, leads to Cdk5 activation through increased the ERK activity in response to DNA damage.[107] Increased Cdk5 activity stabilizes p53 leading to cell death.[107] Hence, strict regulation of Cdk5 activity can be fundamental for normal cell physiology because loss or gain of function can result in aberrant cell proliferation.

In cervical carcinoma caused by infection of human papilloma virus (hPV), Cdk5 may phosphorylate p53 at Ser43 and Ser20, hence inhibiting cell proliferation. Nevertheless, hPV may cause p53 degradation. Indeed, HeLa cells infected by hPV do not undergo cell cycle arrest or apoptosis even when p53 is over-expressed. However, changing p53 phosphorylation state, either by inhibiting phosphatases or promoting the protein kinases activity, may induce apoptosis.[109] In this case, Cdk5-dependent p53 phosphorylation should be an essential event that promotes increased stability of p53 and its transcriptional activation.[66]

A previous report demonstrated that Cdk5 activity is fundamental to regulate cell motility and the metastatic potential of prostate tumor cells.[110] Tumor cells

proliferation is increased by the improved stability of androgen receptor, after its phosphorylation by Cdk5/p35. Moreover, Hsu and colleagues found that Cdk5 may enhance both expression and secretion of prostate-specific antigen (PSA), which indicates that Cdk5 may play a key role in the development of prostate tumor.

Cdk5 has been recently proposed to be a risk factor for other types of tumors, although the molecular mechanisms clarifying the relationship between cancer and Cdk5 is still elusive.[111,112] Therefore, in some patients with non-small cell lung cancer, there is a significant correlation between Cdk5/p35 expression levels and the differentiation and metastasis to lymph nodes degree.[113] Moreover, the presence of polymorphisms in the promoter of Cdk5 increases the risk of lung carcinoma in a specific Korean population.[111] In addiction, the overexpression of Cdk5 caused by gene amplification in lung cells may synergize the signaling pathway of epithelial growth factor receptor.[112]

In mantle cell lymphoma, that is a subtype of B cell lymphoma, it has been observed a reduction in methylation of the Cdk5 gene, and this is linked to an increasing in Cdk5 mRNA levels.[114] Although the link between Cdk5 and the mechanism of generation of this tumor remains unknown, the hypothesis that Cdk5 may control cell cycle or DNA repair seems to be an interesting scenario in which to investigate the role of Cdk5. Altogether, these studies suggest that Cdk5 can control the cell cycle via the canonical pathway that involves p35 binding and a key substrates phosphorylation. Nevertheless, also non-canonical mechanisms of Cdk5 role that include the binding of cyclins, such as cyclin G1and cyclin I, can be important. In addiction, important effects not related to the Cdk5 kinase activity can be involved, and in those cases,

protein–protein interactions with the kinase can contribute to control the cell cycle, suggesting the possibility that Cdk5 functions as a scaffold protein.

1.3 – MicroRNA

In the last 20 years, investigators clarify that elements of genome traditionally considered nonfunctional have a gene regulatory capacity.

MicroRNAs were initially discovered in 1993 as negative regulators of lin-14 gene expression in the nematode Caenorhabditis elegans (C. elegans).[115] Then, it has been demonstrated that these essential parts of the uncoding genome play a key role in gene regulation in several important processes in different species, including vertebrates[116] (a list of miRNA databases is reported in Table 1.4[117]).

Table 1.4 – miRNA databases. From Iorio, M.V. and Croce C.M., MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med (2012)

Name	Website	Reference
miRNA map	http://mirnamap.mbc.nctu.edu.tw/	Hsu et al (2006)
miRBASE	http://mirbase.org/	Griffiths-Jones et al (2008)
microRNA	http://www.microrna.org/microrna/home.do	Betel et al (2008)
coGemiR	http://www.cogemir.tigem.it/	Maselli et al (2008)
miRGEN	http://www.diana.pcbi.upenn.edu/miRGen.html	Alexiou et al (2010)
deepBase	http://www.deepbase.sysu.edu.cn	Yang et al (2010)

MicroRNAs are transcribed by RNA polymerase II as long primary transcripts having hairpin structures (pri-microRNAs), and processed into the nucleus into pre-microRNAs of 70–100 nts by RNAse III Drosha.[118] Drosha is a highly conserved protein of 160 kDa, which contains two RNAse III domains and one binding domain for double-strand RNA.

Drosha forms a giant complex called Microprocessor, that is 500 kDa in Drosophila melanogaster (D. melanogaster) and 650 kDa in Homo sapiens. Microprocessor contains a protein called Pasha in C. elegans and D. melanogaster, also known as the co-factor Di George syndrome critical region 8 (DGCR8) in vertebrates.

An alternative biogenesis pathway of miRNA has been discovered among diverse nematodes, drosophila and mammals and its name is miRtron pathway. In this pathway the regulatory RNAs get processed to form pre-miRs by the splicing machinery lacking Drosha-mediated cleavage. [119-121]

The precursor molecules are exported to the cytoplasm by an Exportin 5-mediated mechanism[122] and processed by the complex RNAse III Dicer/transactivating response RNA- binding protein (TRBP), which generates a dsRNA approximately 22 nts long, called miRNA/miRNA*. miRNA/miRNA* contain the mature miRNA guide and its complementary strand, the miRNA*, known also as the passenger strand (star miRNA; many publications distinguish the two strands on the bases of the direction of the functional miRNA, named one strand miR-3p (from the 3' arm) and the other miR-5p (from the 5' arm). Based on the thermodynamic stability, the guide strand or mature miRNA is selected for entry into an RNA-induced silencing complex (RISC), whereas the miRNA* is typically subjected to degradation.[123]

miRNA* was initially thought to be an inactive and carrier strand, instead more recent evidence highlight that it is not a simply non-functional bioproduct of miRNA biogenesis, but it may play significant biological roles.[124]

Dicer is a very large enzyme (≈200 kDa), highly conserved among the species. It contains different domains: two RNAse III catalytic domains, a double strand RNA-binding domain (dsRBD), one PAZ domain, which binds the 3'-end of small RNAs, and other domains with RNA-helicasic and ATPasic activity. Dicer binds the pre-miRNA, recognizing its double strand region. Many other different proteins are involved in this binding: R2D2 e FMR1 (fragile X mental retardation syndrome 1 homolog) in D. melanogaster, RDE-

4 (RNA interference; RNAi defective 4) in C. elegans, and members of the Argonaut family in other species. Argonaute proteins (AGO 1– AGO 4; AGO1 and 2 are the most extensively studied) have a key role in miRNA biogenesis, miRNA effector functions and maturation.[123] These proteins stabilize the complex Dicer-miRNA, but they are not required for the endonucleasic activity of Dicer. In particular, in mammalians the Argonaut 2 (AGO2) protein complex has RNAse H activity and generates an intermediate product, named AGO2-cleaved precursor miRNA (ac-pre-miRNA[125]).

When the processing steps were completed, the mature single stranded miRNA loaded into the Argonaute proteins in the RISC complex, guides RISC to the target mRNAs leading to mRNA degradation or repression of translation (Fig. 1.3).[126,127]

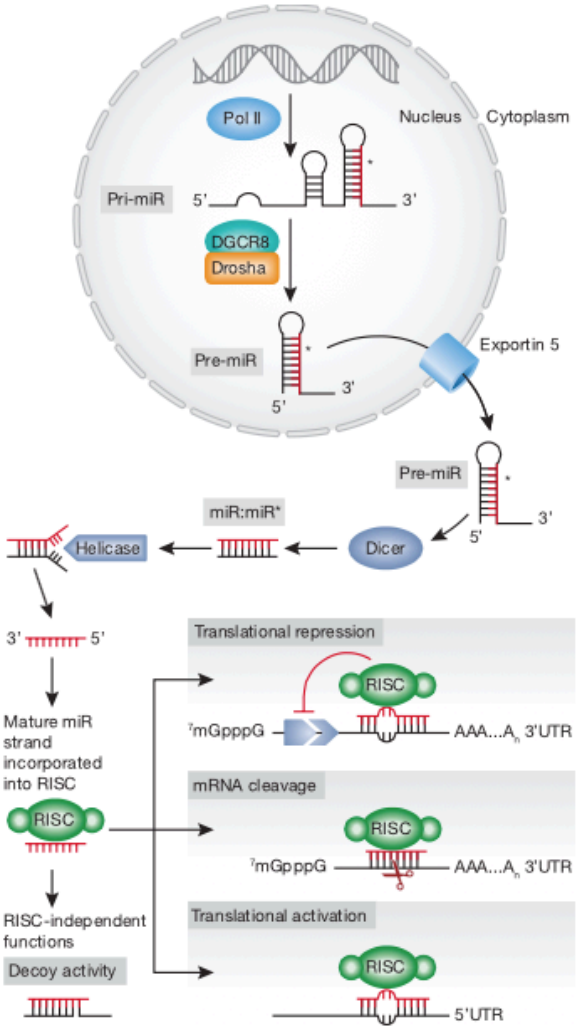

Fig. 1.3 – Biogenesis and mechanisms of action: an overview. From Iorio, M.V. and Croce C.M., MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med (2012)

The specificity of miRNA targeting is due to Watson–Crick complementarities between its seed region, positions 2 – 8 from the 5′ miRNA, with the 3′ untranslated region (UTR) of their target mRNAs.[123] When miRNA and its mRNA target have perfect complementarity, the RISC induces mRNA degradation. If an imperfect miRNA/mRNA target pairing happen,

translation into a protein is blocked.[123] Beside these two mechanisms, there is a site-specific cleavage process, usually defined as RNAi and restricted to miRNAs with a near-perfect or perfect match to the target mRNA. This process is exclusively Ago2 dependent and it is a very rare event in mammals. However, the exact process through which translation can be impaired by miRNAs is still debated. In any case, when miRNAs bind the 3′ UTR of the target mRNAs, the net result is a reduction in the protein quantities encoded by the mRNA targets.[117] Considering the different rules that regulate the microRNA/mRNA target interaction, it is not surprising that each miRNA could potentially target a large number of genes (roughly 500 for each miRNA family). About 60% of the mRNAs have evolutionarily conserved sequences that are in silico predicted to interact with miRNAs.[128-130] Bioinformatical predictions show that the 3′ UTR of the mRNA of a single gene is commonly targeted by several different miRNAs.[131] (Table 1.5 reported a list of bioinformatical tools for miRNA target prediction). A lot of these predictions have been validated experimentally, indicating that miRNAs could cooperate to regulate gene expression.[132]

Table 1.5 – Computational tools for miRNA target prediction. From Iorio, M.V. and Croce C.M., MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med (2012)

Name	Website	Reference
Targetscan	http://www.targetscan.org	Lewis et al (2005)
Pictar	http://www.pictar.org	Krek et al (2005)
RNA22	http://cbcsrv.watson.ibm.com/rna22.html	Miranda et al (2006)
Tarbase	http://diana.cslab.ece.ntua.gr/tarbase/	Sethupathy et al (2006)
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	Kertesz et al (2007)
microRNA	http://www.microrna.org/microrna/home.do	Betel et al (2008)
Diana-microT	http://diana.cslab.ece.ntua.gr/microT/	Maragkakis et al (2009)
miRecords	http://mirecords.biolead.org/	Xiao et al (2009)
Starbase	http://starbase.sysu.edu.cn/	Yang et al (2011)

Beyond the canonical mechanisms of miRNA gene expression modulation through 3′ UTR interactions, other miRNA-mediated mechanisms of mRNA expression regulation are rising in the last years[133-137]. Indeed, it has been

demonstrated that some miRNAs can bind the 5′ UTR or the open reading frame of the mRNAs of the target genes and, in some cases, they activate rather than inhibit gene expression.[133,134] Moreover, Carlo Croce's group has shown that miRNAs can directly bind to ribonucleoproteins in a seed sequence and in a RISC-independent way, preventing the bind of these proteins to their mRNA targets (decoy activity) (Fig. 1.3).[137,138] Three studies have also shown that miRNAs can modulate gene expression at the transcriptional level through direct bind to the DNA.[135,137,139]

All these data highlight the complexity of regulation of gene expression by miRNAs.

1.3.1 – miRNA and cancer

MiRNAs have been demonstrated to control a large variety of biological processes, including proliferation, development, apoptosis, hematopoiesis, differentiation and metabolism.[140] Nowadays more than 880 known mammalian miRNA genes that may encode a mature miRNA are estimated to exist. They regulate the expression of about 30% of gene transcripts.[141-143]

Many researcher groups have shown that the disruption of miRNA regulatory networks is linked with several diseases, including cancer, and is implicated in the initiation and progression of a variety human tumors.[144]

MiRNAs can act as both tumor suppressors and oncogenes to inhibit the expression of cancer-related target genes[145]. Thus, human cancers are characterized by abnormal miRNA expression pattern. Despite miRNAs have

been largely studied in recent years, the molecular mechanisms by which miRNAs regulate gene expression are not fully understood and their role in tumorigenesis remain still largely undetermined.

1.3.2 – Role of miRNAs in the hallmarks of human cancer

The first paper reporting abnormalities in miRNA expression in cancer cells focused on deletion or downregulation of *mir-15a–mir-16-1* cluster in B cells of patients with chronic lymphocytic leukaemia (CLL)[146]; after that, other studies demonstrated that tumor tissues in patients showed distinctive miRNA expression signatures.[147] Genome-wide profiling highlighted that these miRNA expression signatures allowed to discriminate different types of cancer with high accuracy and to identify the tissue of origin of poorly differentiated cancers. Despite, mRNA profiles were highly inaccurate indicators of the type of cancer or tissue.[147]

Nowadays, it is well known that selected groups of distinct miRNAs were concurrently and commonly down-regulated or up-regulated in distinct types of human cancers and were generally associated with well defined cytogenetic abnormalities.[148] Indeed, miR-17 and miR-21 were discovered to be consistently up-regulated in stomach, prostate, colon, pancreatic and lung cancer and miR-155 was shown to be up-regulated in lung, colon, breast tumor[148]; whereas, miR-29 was identified to be down-regulated in acute myeloid leukaemia (AML), rhabdomyosarcom, CLL, lung, cholangiocarcinoma, liver, mantle cell lymphoma and breast cancer.[149-154] Moreover, miR-15a–miR16-1 was discovered to be down-regulated in CLL,

pituitary adenomas and prostate[155,156], and let-7 family members were found to be down-regulated in colon, ovarian, lung, breast, and stomach cancer[122,157-160]. These evidence suggested that up-regulated miRNAs might act as oncogenes and down-regulated miRNAs could act as tumor suppressors (Fig. 1.4). Consequently, it was hypothesized that genes that encode for miRNAs can be subject to genomic alterations as well as the coding genes involved in cancer. For example, amplification or translocations can lead to up-regulation of miRNA expression, whereas insertions, deletions or mutations can lead to the miRNA loss of function (Fig. 1.4).

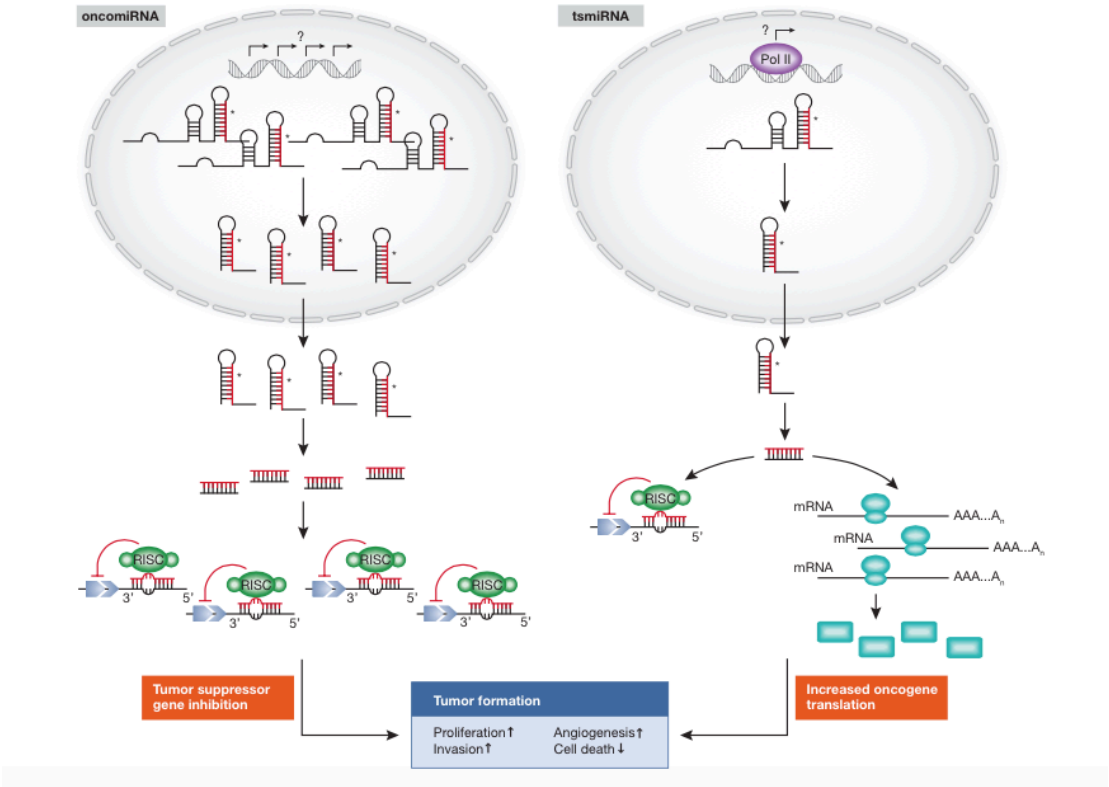

Fig. 1.4 – MicroRNAs as oncogenes or tumour suppressor genes. From Iorio, M.V. and Croce C.M., MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med (2012)

Consistent with this postulate, Carlo Croce's group found that the genes encoding miRNAs are in fact generally located inside or close to fragile sites and in minimal regions of loss of amplification, in minimal regions of heterozygosity and in common breakpoints associated with cancer.[161] For examples, The *mir-17-92* cluster, which includes six miRNAs (*mir-17*, *mir-18a*, *mir-19a*, *mir-19b-1*, *mir-20a* and *mir-92-1*), is situated in an 800 base-pair region of the non-coding gene *C13orf25*, a region commonly amplified in lymphomas. The miR-17-92 cluster has commonly been found overexpressed in hematological malignancies or in solid tumors.[151,153] Instead, the *mir-15a–mir-16-1* cluster, that is situated in the chromosome 13q14 region (between exon 2 and exon 5 of the non-coding gene *LEU2*), is usually down-regulated in patients with CLL because this genomic region is deleted.[146,162]

In addition to structural genetic alterations, miRNA genes could be silenced by DNA promoter histone hypoacetylation and/or hypermethylation; it has been described in hematological malignancies and in solid tumors.[163-165] Saito et al. first demonstrated that miR-127 down-regulation was due to promoter hypermethylation in human bladder cancer. After hypomethylating agents treatment, miR-127 is re-expressed, correlating with the oncogene B-cell lymphoma protein-6 (*BCL-6*) down-regulation, which is a validated target of miR-127.[166]

Aberrant expression of miRNA in cancer might also result from downstream miRNA processing (FIG. 2). Kumar *et al.* demonstrated that miRNA-processing machinery knockdown by short hairpin RNAs against Drosha and Dicer, and the consequent global depletion of miRNAs, stimulates tumorigenesis and cell transformation *in vivo*.[167] Moreover, the conditional loss of *Dicer1* in the lung tissues in a *Kras* mouse model promotes lung tumors

development.[168] The loss of Drosha and Dicer has also been inversely linked with outcome in the ovarian epithelium cancer.[169]

Finally, miRNA expression deregulation can be due to a decreased or increased transcription from their respective miRNA genes through aberrant transcription factor activity. For example, the miR-34a, miR-34b and miR-34c miRNA family was found to be directly induced by the tumor suppressor p53 and it was proposed that some of the p53 effects might be mediated by the transcriptional activation of miRNAs. Chromatin immunoprecipitation experiments demonstrated the p53 binding to the promoter of miR-34.[170,171] Recent work also showed that the oncoprotein MYC negatively regulates transcription of some tumor suppressor miRNAs, such as *let-7* (*let-7a*, *let-7c*, *let-7d*, *let-7f1* and *let-7g*) and *mir-29* family members (*mir-29a*, *mir-29b* and *mir-29c*). Functionally, it was discovered that MYC-induced repression of miRNAs is involved into lymphomagenesis; indeed the restoration of the silenced miRNAs decreases the lymphoma cells tumorigenic potential.[172]

Expression profiling of miRNAs showed that the miRNA signature is associated with tumor diagnosis, classification and progression, as well as response to treatment and prognosis.[149,173] Anyway, the interaction between miRNAs and the classical tumor suppressors and oncogenes is only now beginning to be elucidated.

1.3.3 – miR26a and cancer

MiR-26a, together with miR-26b, belongs to the miR-26 family. The role of miR-26a in cancer cells is controversial nowadays. Some studies have shown

that miR-26a is down-regulated and act as tumor suppressor in breast cancer[174], hepatocellular carcinoma[175], nasopharyngeal carcinoma[176], gastric cancer[177], prostate carcinoma[178] and bladder cancer[179]. Other studies have demonstrated to act as an oncogene in cholangiocarcinoma[180], lung cancer[181] and glioma[182].

Until now, the role of miR-26a in DLBCL was undefined.

1.3.4 – Rational for targeting miRNA

As miRNAs regulate several different pathways and coordinate integrated responses in normal healthy tissues and cells, it's reasonable to think that they also play a key roles in orchestrate cancerous networks. This characteristic makes them excellent candidates for innovative molecular-targeting treatments.

The rationale for utilizing miRNAs as drugs against tumor is based on two main findings: that the expression of miRNAs is deregulated in tumor cells compared with normal cells, and that the targeting of miRNA expression can change tumor phenotype.[146,158,183,184] Indeed, if normal miRNA program is restored in the neoplastic cells, the cell connectivity map might be rewired and cancer phenotypes might be reversed. The therapeutic strategy aims to rescue homeostasis in cancer cells by modifying miRNA expression might be more successful and comprehensive than targeting single proteins or genes, as there are only some miRNAs deregulated in tumor, compared with the high number of perturbations of the proteome and transcriptome in neoplastic cells.

1.3.5 – miRNA-based therapeutic strategies

Two main strategies can be used to target miRNA expression in tumor. Direct strategies implicate the use of virus-based constructs or oligonucleotides to either restore the loss of expression of a tumor suppressor miRNA or to block the expression of an oncogenic miRNA. The indirect strategy implicates the use of drugs to regulate miRNA expression by targeting their processing or transcription. (Fig. 1.5)

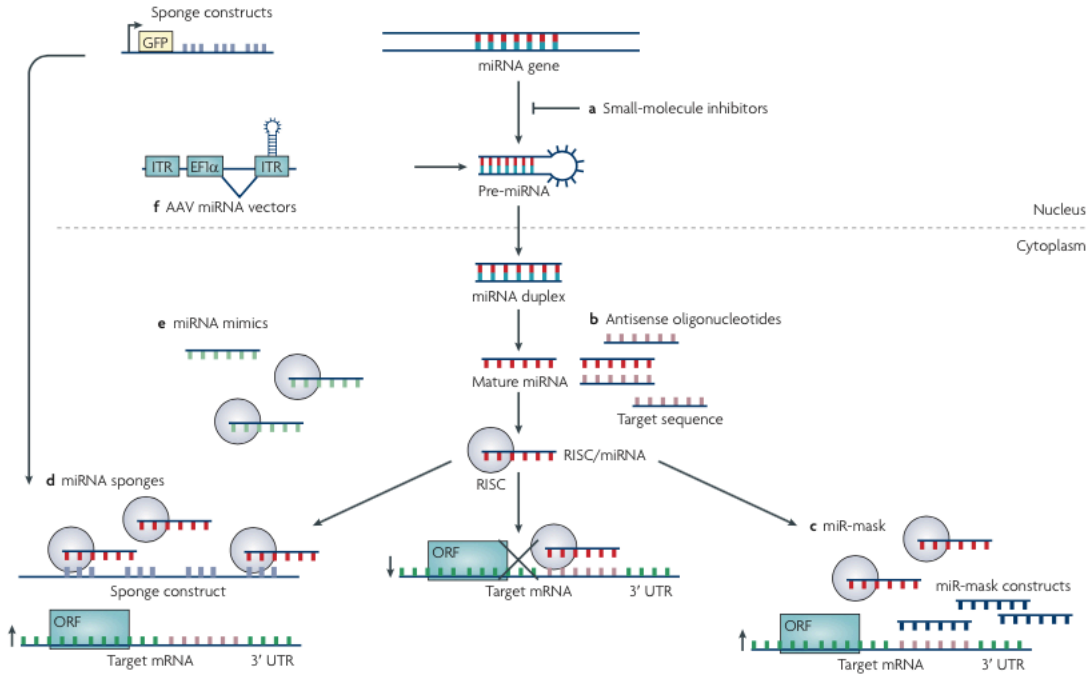

Fig. 1.5 – Blocking oncogenic microRNAs (miRNAs) could be achieved by using a| small RNA inhibitors, b| antisense oligonucleotides, c| miR-mask, and d| miRNA sponges. Restoring downregulated miRNA expression could be achieved by using e| synthetic miRNAs (miRNA mimics) and f| viral constructs containing genes coding for miRNAs. EF1α, elongation factor 1 α; GFP, green fluorescent protein; ITR, inverted terminal repeats; ORF, open reading frame; RISC, RNA-induced silencing complex. From Garzon R., et al. Targeting microRNA in cancer: rational, strategies and challenges. (2010)

To block oncogenic microRNAs (miRNAs) small RNA inhibitors, antisense oligonucleotides, miR-mask, and miRNA sponges can be used (Fig. 1.5).[185-189] Small-molecule miRNA inhibitors might modulate miRNA expression at the transcriptional level[189]; antisense oligonucleotides might bind to the target miRNAs according to the Watson–Crick complementarities and promote either duplex formation or degradation; miR-mask oligonucleotides are synthetic oligonucleotides complementary to the 3′ UTR of the target mRNA that compete with endogenous miRNAs for its target.[190] Hence, the miR-mask can block oncogenic miRNA activities at the target level and promote translation of target mRNAs. The miRNA sponges are oligonucleotide constructs with multiple complementary miRNA binding sites (in tandem) to the target miRNA.[191] After the introduction into the cell, sponges will 'soak up' endogenous miRNAs, diminishing the expression levels of the oncogenic miRNA.

To rescue down-regulated miRNA expression synthetic miRNAs (miRNA mimics) or viral constructs containing genes coding for miRNAs, such as the adenovirus-associated vectors (AAV) can be used.[192,193]

2. AIM

A significant proportion of patients with DLBCL are refractory to first-line chemotherapy or relapsing after autologous transplantation. Thus, novel targets for innovative treatments are needed.

In preliminary experiments, we observed that Cdk5 and p35 protein are overexpressed and that miR26a is down-regulated in several DLBCL cell lines compared to B lymphocytes from healthy donors.

We hypothesize that Cdk5 plays a key role in tumor growth of DLBCL by promoting cell proliferation and by inducing resistance to apoptotic stimuli in neoplastic cells, and that the expression of its activator p35 is directly regulated by miR26a. Therefore, Cdk5 may be a new potential target for treatment of DLBCL.

This study provides a better understanding of DLBCL pathogenesis, important to develop new opportunity of effective treatment.

3. MATERIAL AND METHODS

3.1 – REAGENTS

Recombinant sTRAIL (KillerTRAIL™) was purchased from Alexis Corporation (Lausen, Switzerland, EU); Bortezomib (Velcade; formerly PS-341) was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO.

3.2 – CELL LINES

SU-DHL-4, SU-DHL-6, SU-DHL-16, (Germinal center B-cell like diffuse large B-cell lymphoma), SU-DHL-2, SU-DHL-8, and RCK-8 (Activated B-cell like diffuse large B-cell lymphoma) cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany, EU). Cell lines were cultured in RPMI-1640 supplemented with 20% fetal bovine serum (FBS) and periodically tested for mycoplasma contamination. B lymphocytes derived from human blood samples. B cells were isolated by negative selection using RosetteSep® B cell enrichment cocktail (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions. B cell purity was assessed by flow cytometry by staining for mature B cells (CD20), T cells (CD3) and monocytes (CD14) using fluorescently-labeled monoclonal antibodies, all from BD Biosciences (Oxford, UK). Flow cytometry experiments were conducted with FACSCalibur™ (Becton-Dickinson, San Jose, CA, USA) using BD CellQuest software version 3.3 (BD). The data were analyzed using FlowJo 7.3.5 software version for Windows (Tree Star, Inc. Ashland, OR).

3.3 – GENERATION OF LOSS- AND GAIN-OF-FUNCTION CDK5/P35 OR MIR-26A STABLE DLBCL CELL LINES

Lentiviral CDK5 short hairpin RNA (shRNA) (#66 and #67) and p35-shRNA (#217 and #218) were obtained from The RNAi Consortium (http://www.broadinstitute.org/rnai/trc). A scramble shRNA was used as a control. Lentiviral particles were generated using a three-plasmid system, as previously described[194] For p35-expressing lentiviral particles, a p35-expressing vector containing the human CDS p35 complementary DNA (NM_003885.2) was cloned in a lentiviral plasmids. DLBCL cells were 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 overexpression was validated by immunoblotting and/or qRT-PCR.

3.4 – VIRAL INFECTION OF DLBCL CELL LINES

SU-DHL-4, SU-DHL-8 and SU-DHL-16 (0.4×10^6 per milliliter) were resuspended in lentivirus containing supernatant in the presence of polybrene (8 μg/ml) in a six-well plate. Plates were centrifuged at 1,000 g for 90 min. After centrifugation, cells were washed, resuspended in fresh culture medium, and incubated at 37°C in a CO_2 incubator. Three days after infection, cells were washed in phosphate-buffered saline (PBS) and resuspended in FACS buffer (PBS with 5% FCS). Gene transduction efficiency was determined by cytofluorimetric analysis using the BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed as previously described.

3.5 – APOPTOSIS ASSAYS

The Annexin-V–fluorescein isothiocyanate (FITC) assay (Immunostep, Salamanca, SP, EU) was used to detect cell death by apoptosis or necrosis and was carried out according to the manufacturer's instructions. SU-DHL-8 cells were cultured at 0,4/ml in RPMI 20% FBS and treated with 2.5 or 5 ng/ml TRAIL. In order to detect apoptotic cells, cells were recovered after 24h, double stained and analysed on a FACSCalibur flow cytometry system (Becton-Dickinson, San Jose, CA, USA) as previously described.

3.6 – CELL CYCLE ANALYSIS

Cells (1×10^6) were cultured in the absence or presence of TRAIL for 8, 24, 48 and 72 hours. All of the cells were washed with PBS and fixed in 70% ethanol and kept at 4°C prior to 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.

3.7 – 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 BCA (bicinchoninic acid) 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 using antibodies against the following proteins: CDK5 (1:1000), p35 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:1000) (Cell signaling, Danvers, MA, USA), actin (Sigma-tau, Rome, Italy), STAT3 (1:1000) and pSTAT3 (1:1000) (Cell signaling, Danvers, MA, USA). HRP- conjugated secondary antibodies against mouse or rabbit were used to detect each protein line.

3.8 – RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted using Purezol reagent (Bio-Rad, US) following the manufacturer's instructions. Two micrograms of total RNA was used for cDNA synthesis with Oligo d(T) primer. Real-time PCR was carried out for detection of CDK5, p35 and miR-26a mRNA level on an Applied Biosystems 7900HT Fast Real-Time PCR. PCR reactions were run in duplicate for three independent experiments. The mRNA levels were referred to β-actin, 18s or GAPDH as housekeeping genes and analysed using the ΔΔCt method.

3.9 – LUCIFERASE ASSAY

We performed luciferase reporter experiments in the HEK293T cell line. 3' UTR segment of p35 predicted to interact specifically with *miR-26a* were subcloned by standard procedures into the psiCHECK-2 vector (Promega, Madison, WI, USA) immediately downstream of the stop codon of the renilla gene using the primers p35–5′ (5′-GAGGCTGCTTCGGATGGAGGGA-3′), p35–3′ (5′-TAAGATTTAACATCATCATATT-3′). Seed sequence mutagenesis was performed as described by the manufacturer (Agilent Technologies, Santa Clara, CA, USA). We made short constructs (80–100 bases),

encompassing wild-type or mutated (six point mutations) seed sequences, in order to separately analyse the functional role of each seed. The seed sequences are indicated in figure 4.12. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), cells were transfected with psiCHECK-2-3'UTR plasmid and plasmids containing non-targeting RNA control or *miR-26a*. Renilla luciferase signal is normalized to the firefly Luciferase signal. At 48 h after transfection, cells were lysed and luciferase activity was measured by Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

3.10 – IN VIVO TUMOR GROWTH OF ENGINEERED SU-DHL-8 CELL LINES IN NON-OBESE DIABETIC/SEVERE COMBINED IMMUNODEFICIENT (NOD/SCID) MICE.

Six- to eight-week-old NOD/SCID mice with body weights of 20 to 25 g were purchased from Charles River (Milano, Italy, EU) and used to generate xenografts of SU-DHL-8 cells. The animal experiments were performed according to Italian laws (D.L. 116/92 and following additions) and approved by the institutional Ethical Committee for Animal Experimentation. The engineered tumor cell lines were injected subcutaneously (SC). The cells were inoculated (10×10^6 cells/mouse) into the left flank of each mouse.

The endpoint of the experiment was tumor weight. The tumor cells were fluorescent, thus the tumors were measured with IVIS *in vivo* imaging system (PerkinElmer, Waltham, USA), in addiction to caliper. After caliper measurements, their volumes were calculated using the formula: $(a \times b^2)/2$, where a and b represented the longest and shortest diameters, respectively. Mice were monitored three times weekly and were killed by cervical dislocation 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.

3.11 – HISTOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY

Sections (2 μm) from formalin-fixed, paraffin-embedded tumor nodules were stained with hematoxylin and eosin or processed for tumor necrosis analysis. Tumor necrosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) (Roche, Milano, Italy) according to the manufacturer's instructions. The sections were examined using a light microscope (IX51; Olympus, Tokyo, Japan). Image analysis was performed using open source ImageJ software (http://rsb.info.nih.gov/ij/).

3.12 – BIOINFORMATICS

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.

3.13 – 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 samples and their controls, a two-way analysis of variance was employed, and individual group comparisons were evaluated using the Bonferroni post-test. Terminal deoxynucleotidyl transferase dUTP nick end

labeling data were analysed using one-way analysis of variance, an individual group comparisons were evaluated using the Bonferroni post-test. Differences were considered significant at the level of $P \leq 0.05$. Unpaired Student's t test, paired Student's t test were also used. Data are represented as mean ± SEM unless otherwise stated.

4. RESULTS

4.1 – CDK5 AND ITS ACTIVATOR P35 ARE OVEREXPRESSED IN LYMPHOMA CELL LINES AND REGULATE THE PHOSPHORYLATION AND ACTIVITY OF STAT3

Previous reports have shown that several solid tumors present an over activation of CDK5 that correlates with the progression of the disease. First of all, we investigated if CDK5 could have a role in hematologic malignancies. By using immunoblotting, we found that CDK5 and its activator p35 are overexpressed in several lymphoma and leukemia cell lines compared to B lymphocytes (Figure 4.1 A, B and C).

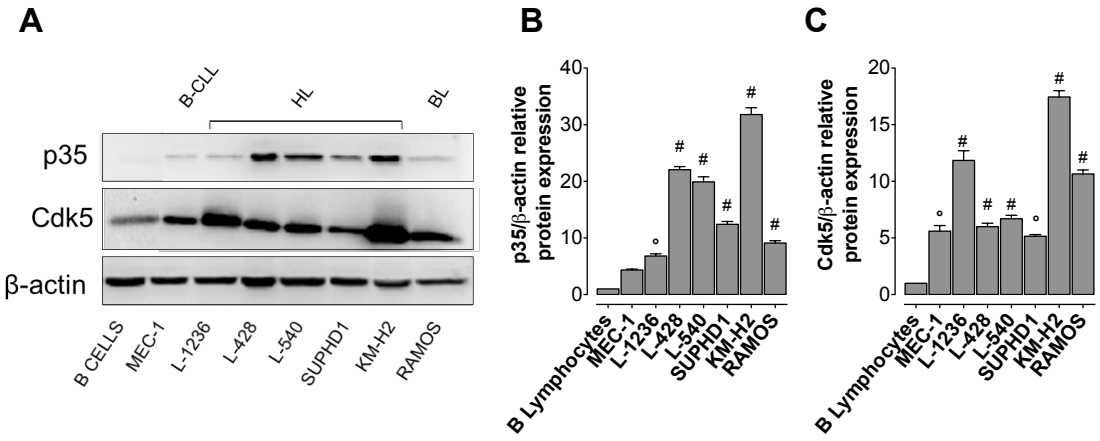

Fig. 4.1 A, B and C – CDK5 and p35 are overexpressed in Lymphoma and Leukemia cell lines. (A) Representative immunoblots of CDK5 and p35 protein expression in primary B lymphocytes, B-cell chronic lymphocytic leukemia (B-CLL), Hodgkin lymphoma (HL) and Burkitt's lymphoma (BL) cell lines. Densitometric analysis of (B) p35 and (C) CDK5 protein levels. Relative concentrations are shown as a ratio normalized to β-actin. The value in the control sample (B cells) was arbitrarily defined as 1. Results are representative of 3 independent experiments. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p < 0.001$

We focused on DLBCL that is the most common form of non-Hodgkin lymphoma and we found that Cdk5 and p35 proteins were overexpressed in all the DLBCL cell lines we analysed (figure 4.2 A, B and C).

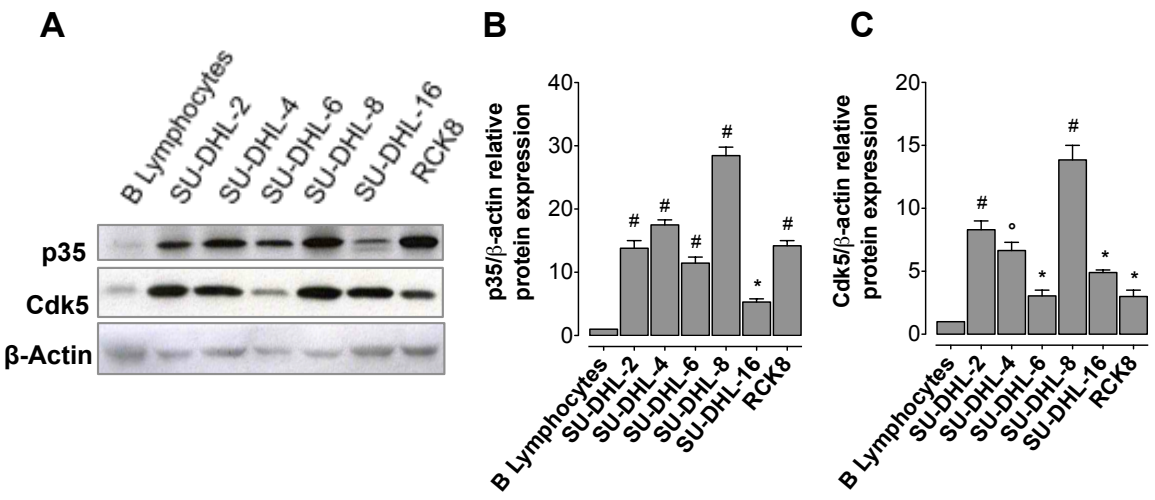

Fig. 4.2 A, B and C – CDK5 and p35 are overexpressed in DLBCL cell lines. (A) Representative immunoblot for CDK5 and p35 proteins in B lymphocytes and DLBCL cell lines. Densitometric analysis of (B) p35 and (C) CDK5 proteins normalized to β-actin. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p< 0.001$.

Moreover, we measured the level of STAT3 phosphorylation, a known target of CDK5[57], at the Serine 727 (S727) in all DLBCL cell lines we collected. By using CDK5-specific shRNAs, we confirmed that phosphorylation level at S727 of STAT3 was dependent on the expression of CDK5 in DLBCL cell lines (Figure 4.3 A, B). As expected, all DLBCL cell lines showed a higher level of phosphorylation (Figure 4.3 C and D).

These results indicate that there is an overexpression of CDK5 and its activator p35 in DLBCL cells.

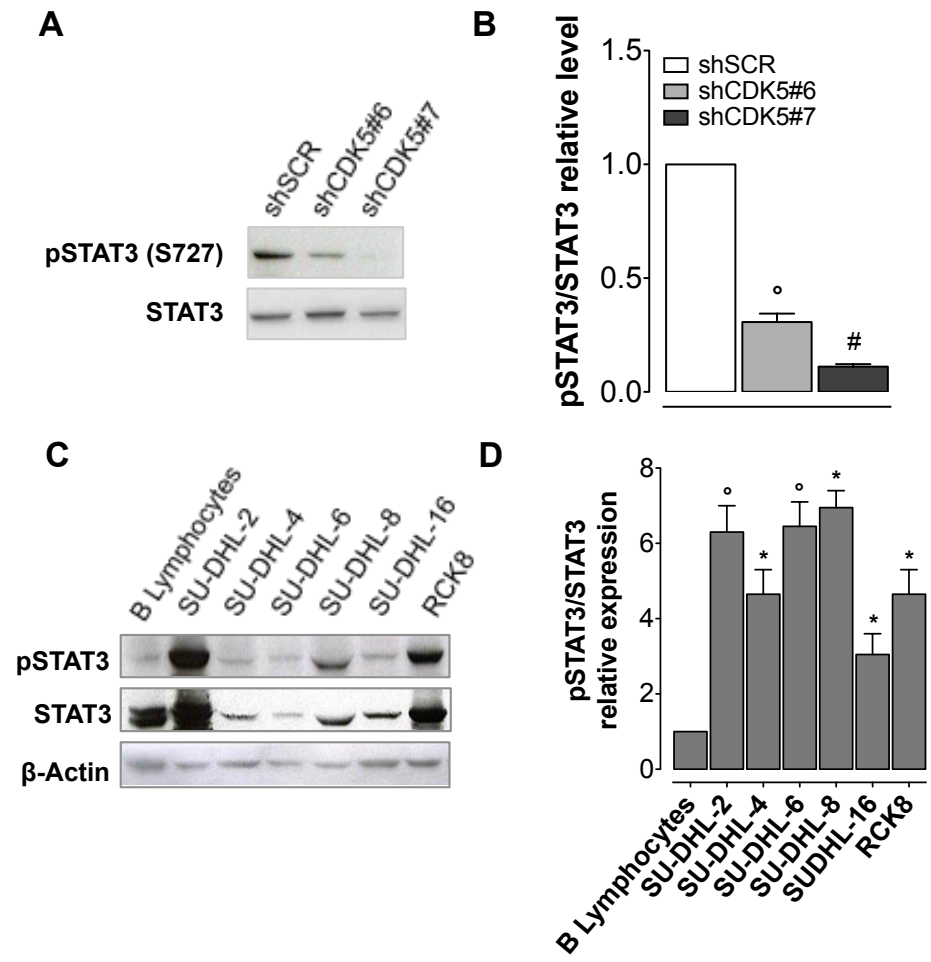

Fig. 4.3 A, B, C and D – (A) Immunoblot of STAT3 S727 phosphorylation level in SU-DHL-8 expressing CDK5-specific shRNAs. (B) Densitometric analysis of normalized pSTAT3 to total STAT3. (C) STAT3 S727 phosphorylation level in B lymphocytes and DLBCL cell lines. (D) Densitometric analysis of pSTAT3 normalized to total STAT3 and β-actin. The value in the control sample (B lymphocytes) was arbitrarily defined as 1. Results are representative of 3 independent experiments. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p< 0.001$.

4.2 – CDK5 ACTIVITY IN DLBCL CELL LINES IMPAIRS PROLIFERATION

To dissect the functional relevance of CDK5 expression and activity in modulating DLBCL cell malignant phenotypes, we studied the effects of CDK5 and p35 loss-of-function and p35 gain-of-function on proliferation and in different DLBCL cell lines. We knocked down CDK5 and p35 using two different lentiviral shRNAs for each gene. We used one Activated B-cell like (ABC) DLBCL cell line, SU-DHL-8, and one Germinal Center B-Cell like

(GCB) DLBCL cell line, SU-DHL-4, both expressing high level of CDK5 and p35. The silencing efficiency was confirmed by qRT-PCR (Figure 4.4 A and B) and the cell growth was monitored at various time points (24, 48, 72 and 96 hours). Stable expression of p35-specific shRNAs inhibited SU-DHL-8 cell proliferation by 40% (average between two shRNAs) (P< 0.001) (Figure 4.4 C).

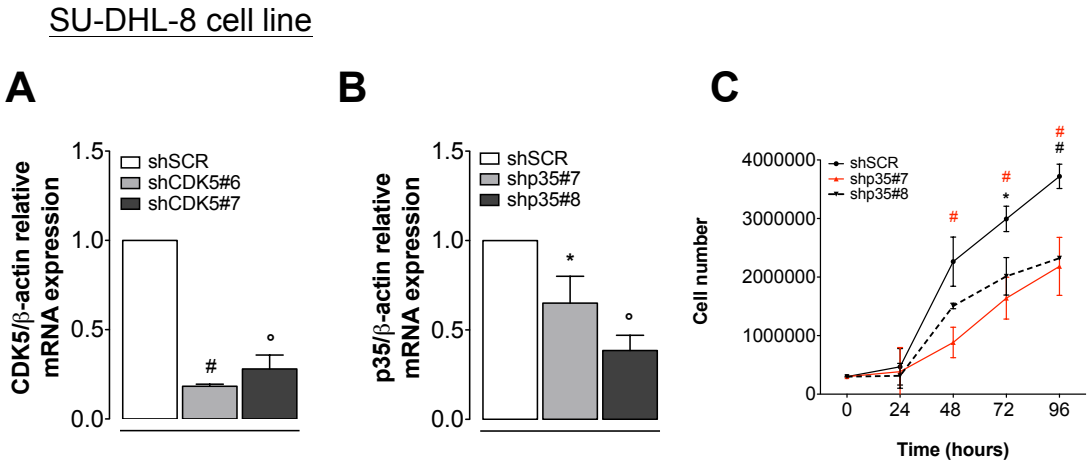

Fig. 4.4 – p35 affects proliferation rate of DLBCL cells. (A) CDK5 and (B) p35 RNA levels in SU-DHL-8 expressing CDK5 or p35-specific shRNAs. (C) Down-regulation of p35 with two different shRNAs significantly inhibited cell proliferation of SU-DHL-8 cells. Results are representative of 3 independent experiments. Significant differences indicated by: *p < 0.05, and °p < 0.01, #p< 0.001.

Similar effects on cell proliferation were observed using CDK5-specific shRNAs in SU-DHL-8 (Figure 4.5 A). Moreover, we created two SU-DHL-8 sub-lines stably expressing the recombinant mouse CDK5 together with each hCDK5-specific shRNAs (#6 and #7). As showed in figure 4.5 B and 4.5 C the expression of exogenous mCDK5 reverted almost completely the effect on the proliferation induced by the hCDK5-specific shRNAs. Similar effects on cell proliferation of CDK5-specific shRNAs were found in SU-DHL-4 cell line (Figure 4.5 D).

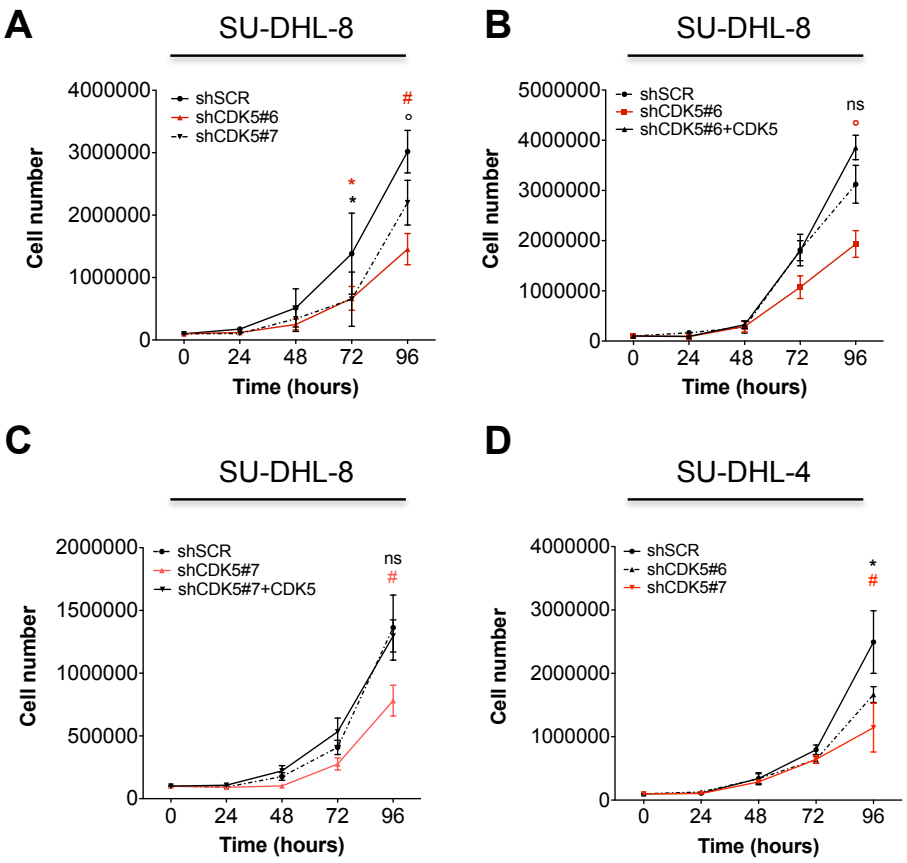

Fig. 4.5 – CDK5 impairs proliferation of DLBCL cells. (A) Down-regulation of CDK5 with two different shRNAs significantly inhibited cell proliferation of SU-DHL-8 cells, while (B and C) concomitant expression of mouse CDK5 increased cell proliferation of SU-DHL-8 cells compared to control level. (D) Down-regulation of CDK5 with two different shRNAs significantly inhibited cell proliferation of SU-DHL-4 cells. Results are representative of 3 independent experiments. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p < 0.001$.

To further strength this result, we performed gain-of-function experiment, expressing the p35 coding DNA sequence (CDS) in the SU-DHL-16 cell line that showed the lowest level of p35 expression (Figure 4.2 A and B) and CDK5 activity (Figure 4.3 C and D). p35 over-expression was confirmed by western blotting and qRT-PCR (Figure 4.6 A and B). Consistently with previous results, stable expression of recombinant p35 increased cell proliferation by 37% ($P<0.001$) (Figure 4.6 C). These results clearly demonstrate that CDK5 and its activator p35 regulate proliferation of DLBCL cells.

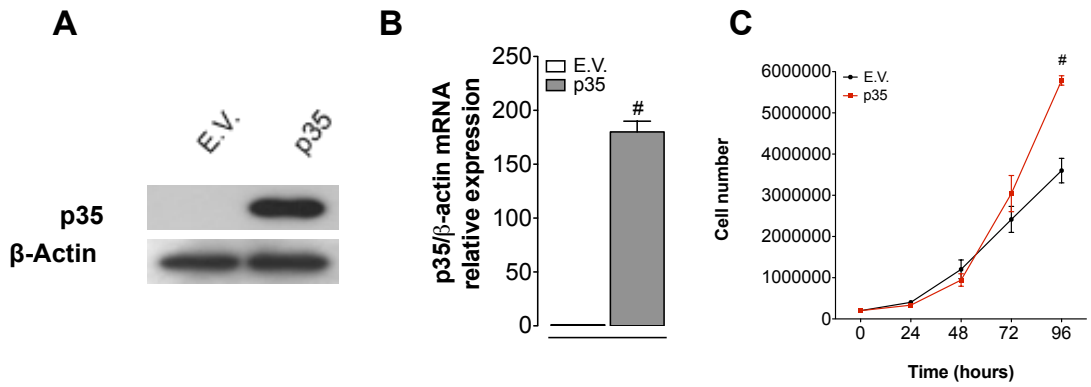

Fig. 4.6 – p35 overespression increases proliferation rate of SU-DHL-16 cells. p35 protein (A) and mRNA (B) levels in SU-DHL-16 expressing recombinant human CDS p35. (C) Up-regulation of p35 significantly increased proliferation of SU-DHL-16 cells. Results are representative of 3 independent experiments. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p < 0.001$.

4.3 – CDK5 ACTIVITY IS REQUIRED TO PROTECT DLBCL CELLS FROM APOPTOSIS

We asked whether CDK5 has a biological role enhancing lymphoma cells survival similar to what has been reported in neurons [195] and in podocytes [76]. SUDHL8 expressing p35-specific shRNAs did not show a significant difference of apoptotic cells compared to cells expressing scrambled sequence (Figure 4.7 A and B). We also investigated whether p35 silencing may enhance sensitivity to pro-apoptotic agents, such as sTRAIL, and found that p35-knockdown SU-DHL-8 cells showed a significantly higher cytotoxicity when exposed to sTRAIL, compared to scrambled-transduced cells (Figure 4.7 A and B).

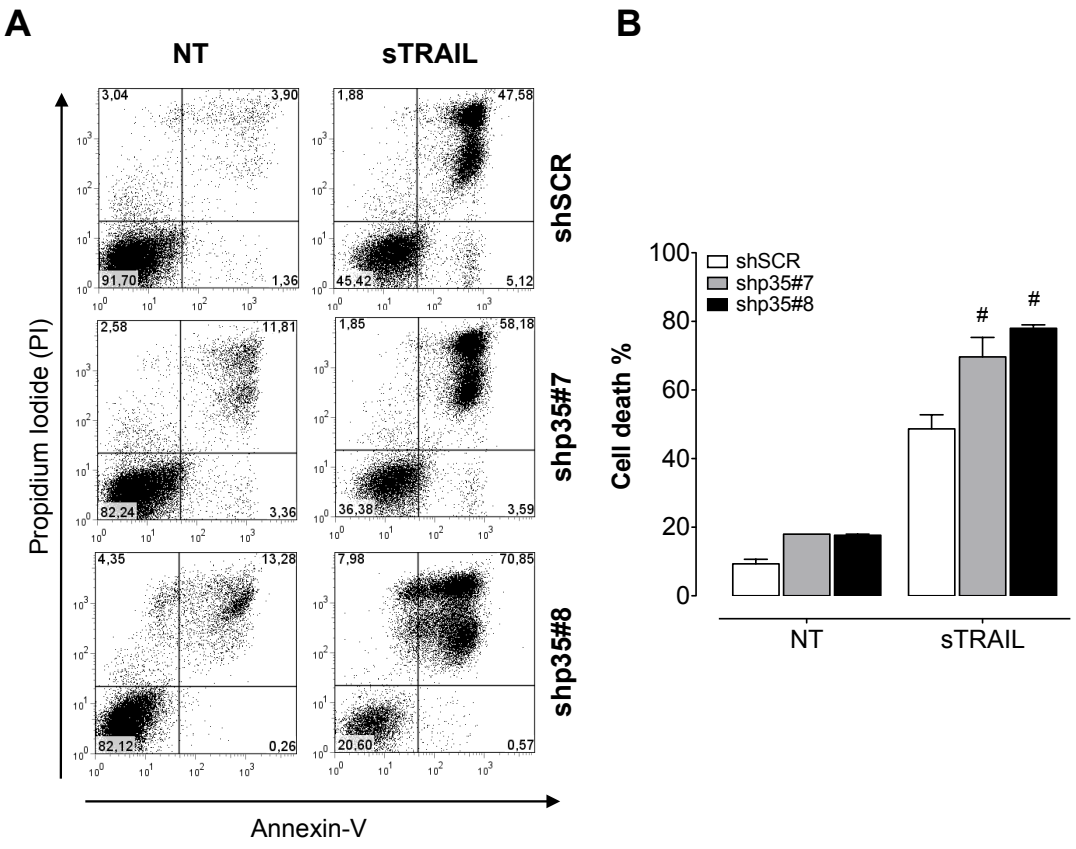

Fig. 4.7 – p35 regulates apoptotic rate of DLBCL cells. (A and B) Down-regulation of p35 in SU-DHL-8 significantly increased apoptotic cell number assessed by Annexin-V/PI staining in the presence of 5 ng/ml Killer sTRAIL. All data are derived from 3 independent experiments with 6 replicates. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p< 0.001$.

Similarly, cells with reduced CDK5 level did not showed increased apoptotic events in the absence of an apoptotic stimulus, but showed an augmented sTRAIL–induced apoptosis (Figure 4.8 A and B), even at the higher concentration of TRAIL used (Figure 4.8 C).

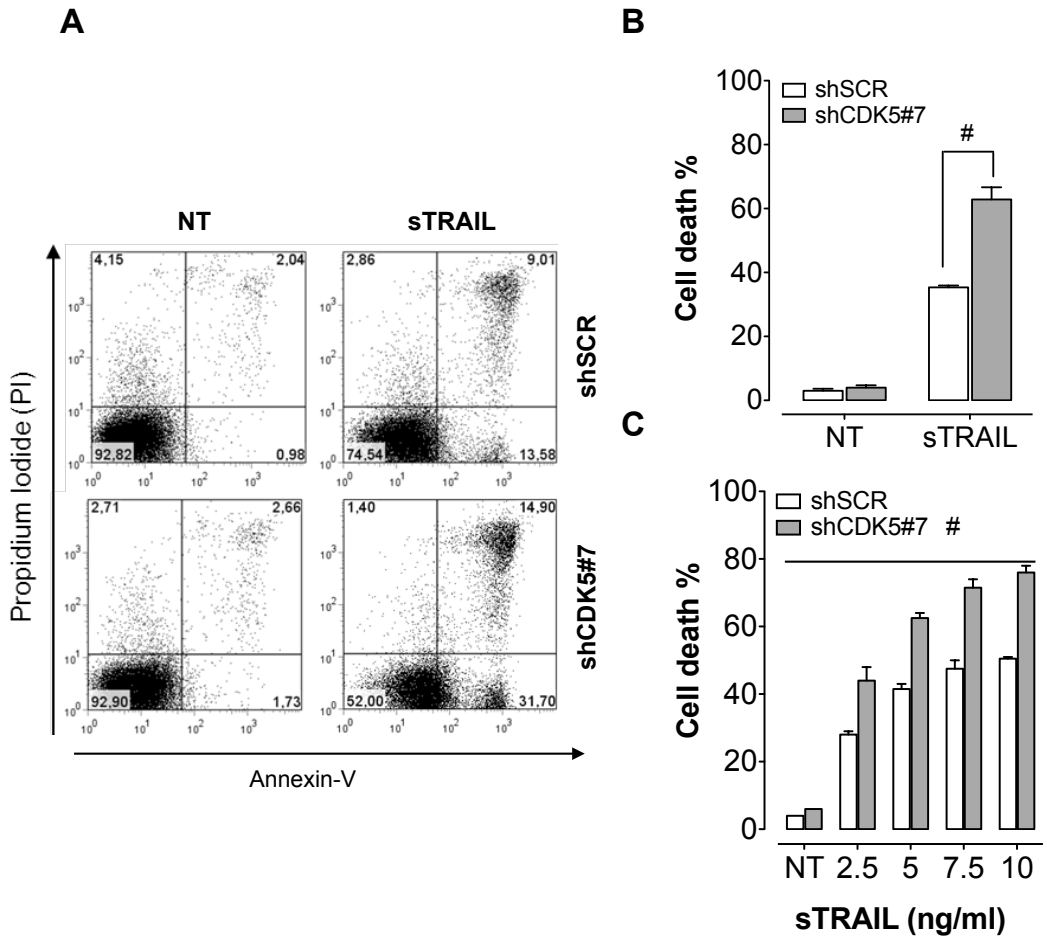

Fig. 4.8 – CDK5 activity protects DLBCL cells from apoptosis. (A and B) Down-regulation of CDK5 significantly increased apoptotic cell number in SU-DHL-8 in the presence of 5 ng/ml Killer sTRAIL. (C) SU-DHL-8 expressing CDK5 specific shRNA treated with different concentrations of apoptotic agent sTRAIL. Results are representative of 3 independent experiments. Significant differences indicated by: *p < 0.05, and °p < 0.01, #p< 0.001.

CDK5 silencing increases apoptotic events in DLBCL cells.

Inhibiting CDK5 activity using chemical compounds further confirmed that CDK5 activity is important to protect cells from apoptosis (Figure 4.9 A, B and C).

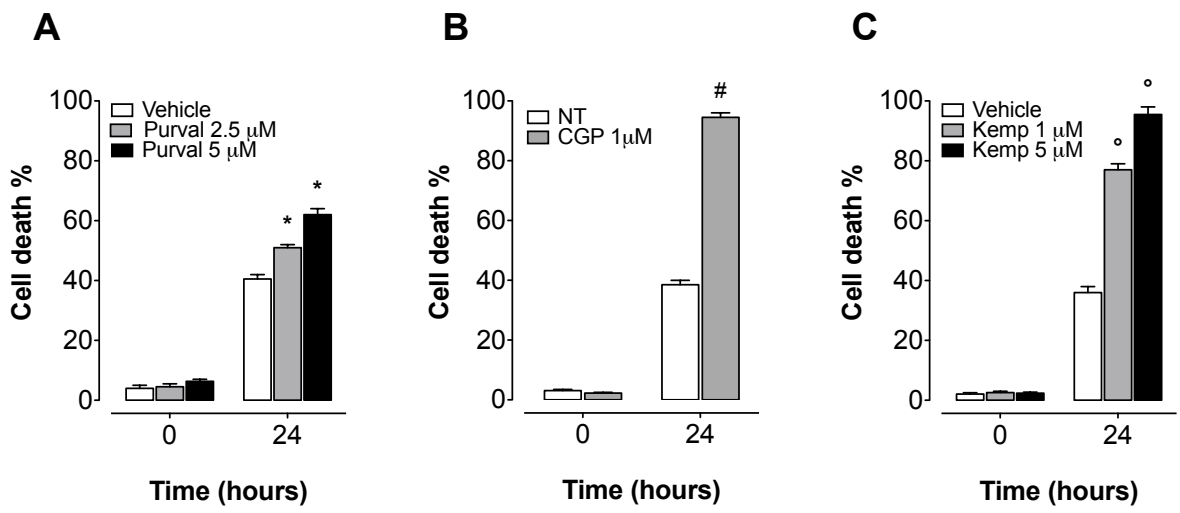

Fig. 4.9 – CDK5 inhibitors regulate apoptotic rate of DLBCL cells. Combination of different CDK5 inhibitors (A) PURVALANOL, (B) CGP, and (C) Kemp with the apoptotic agent sTRAIL. (D) Down-regulation of CDK5 significantly increased apoptotic cell number in SU-DHL-4 in the presence of 5 ng/ml Killer sTRAIL. Results are representative of 3 independent experiments. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p< 0.001$.

Similar results have been obtained also in SU-DHL-4 cell line (Figure 4.10 A and B).

To further strength this result, we measured apoptosis level in SU-DHL-16 overexpressing p35. Consistently with previous results, we detected significant decrease in BTZ–induced apoptosis ratio after p35 overexpression (Figure 4.10 C and D). These results strongly suggest that CDK5 activity is able to prevent apoptotic events in DLBCL cells.

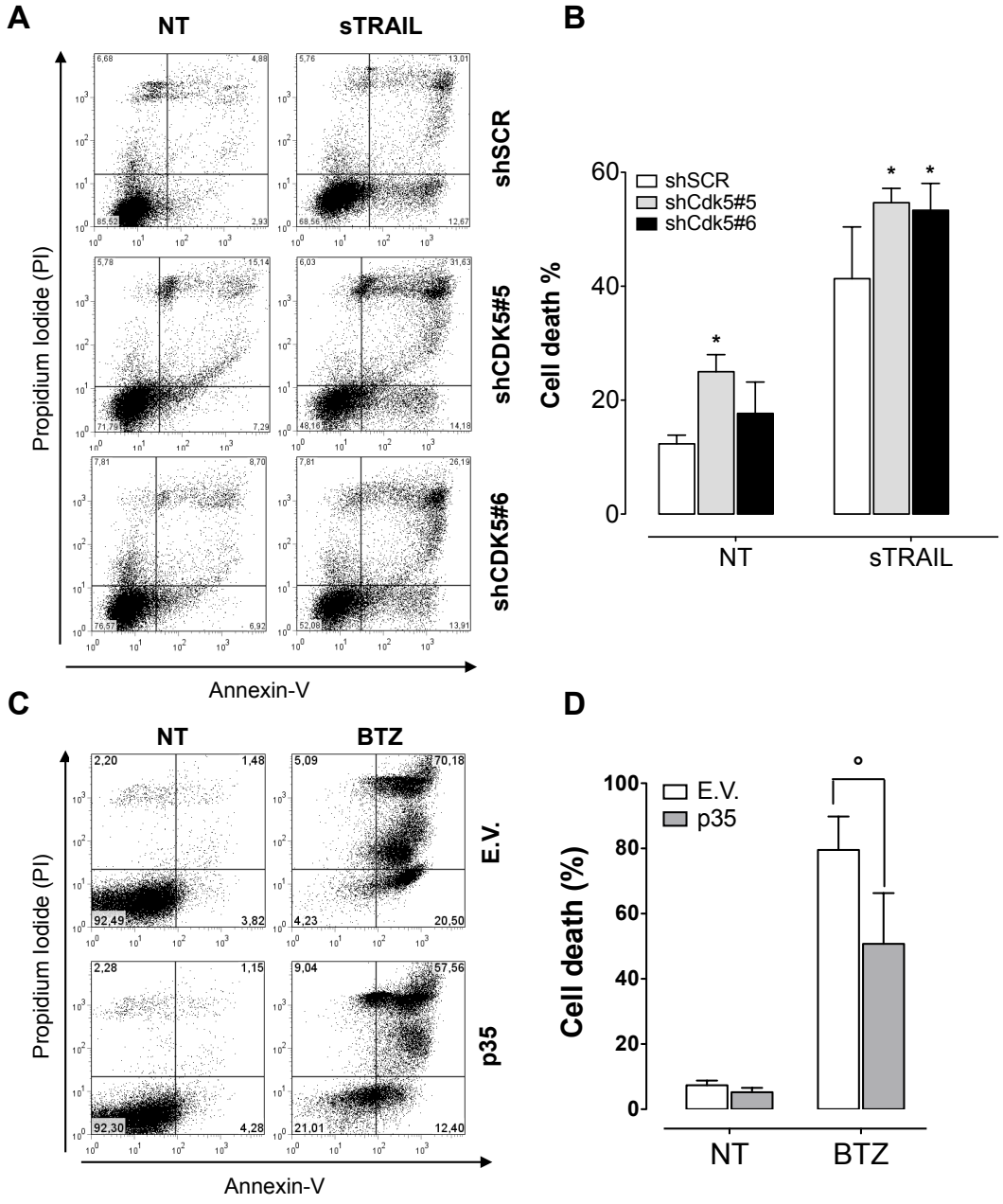

Figure 4.10 – CDK5 regulates apoptotic rate of DLBCL cells. (A and B) Down-regulation of CDK5 significantly increased apoptotic events in SU-DHL-4 in the presence of 2.5 ng/ml Killer sTRAIL. (C and D) Up-regulation of p35 significantly decreased apoptotic cell number of SU-DHL-16 after treatment with Bortezomib. All data are derived from 3 independent experiments with 6 replicates. Significant differences indicated by: *$p < 0.05$, and °$p < 0.01$, #$p< 0.001$.

4.4 – MIR-26A REPRESSES P35 EXPRESSION

While we observed a correlation between CDK5 mRNA and protein levels, in lymphoma cells we did not observe the same correspondence for p35: compared to B lymphocytes, cancer cells showed higher protein level, but lower mRNA levels (Figure 4.2 A and 4.11 A and B).

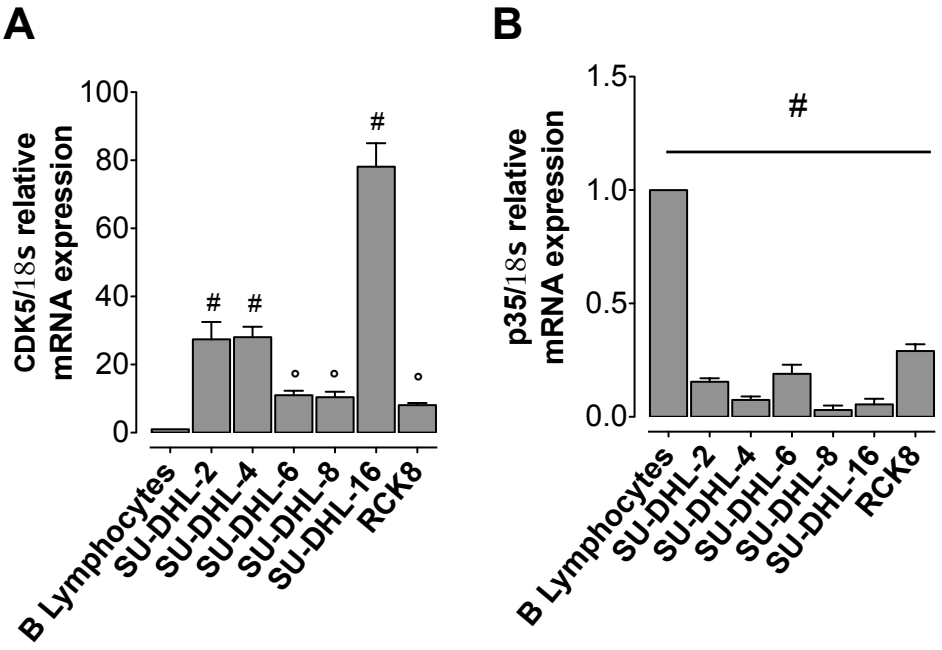

Fig. 4.11 – CDK5 and p35 mRNA are different modulated in DLBCL cells. (A) CDK5 and (B) p35 mRNA expression in DLBCL cell lines.

This led us to investigate potential post-transcriptional mechanisms regulating p35 expression. To elucidate the regulation of p35, we searched for miRNAs that can potentially bind to its 3'–UTR. Bioinformatic analysis showed that miR-26a has a putative binding site in the p35 3'–UTR with seed sequence located at positions 2061-2067 bp (Figure 4.12 A). To test whether p35 was regulated by miR-26a, its precursor (hsa-pre-miR-26a) was expressed into SU-DHL-8 cells. Western blotting of whole lysate of cells overexpressing hsa-miR-26a showed a decrease of the native p35 protein (Figure 4.12 B and C), compared to control. Over-expression efficacy was confirmed by qPCR by

measuring the level of the mature miRNA, demonstrating an increased expression after transfection at 24 hours (Figure 4.12 D).

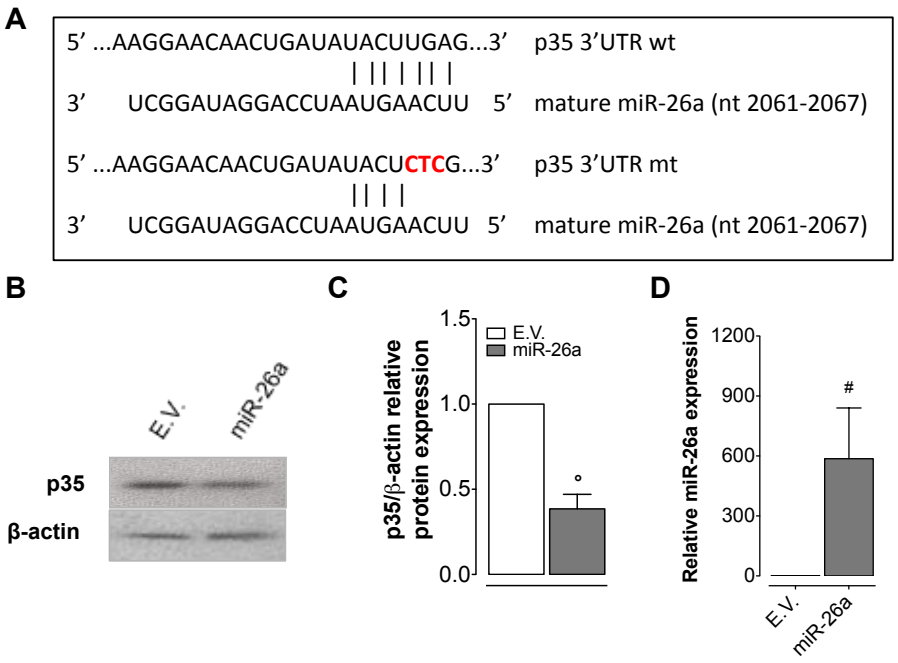

Figure 4.12 – miR-26a affects p35 expression. (A) miR-26a and its putative binding sequence in the 3'-UTR of p35. In red, the mutant seed sequence (wt, wild type; mt, mutant type). (B) Representative immunoblot for p35 in SU-DHL-8 overexpressing miR-26a. (C) Densitometric analysis of p35 protein normalized to β-actin. (D) Expression of miR26a in SU-DHL-8 measured by RT-PCR. The value in the control sample was arbitrarily defined as 1. Results are representative of 3 independent experiments.

To further demonstrate that the observed effect of miR-26a on p35 expression was direct by binding to its 3'–UTR, we performed a luciferase experiment. We fused the p35 3'–UTR sequence to a luciferase reporter gene. Then, by co-transfecting the hsa-miR-26a with this vector, we demonstrated that miR-26a significantly repressed luciferase activity compared to a non-targeting control. Mutagenesis of the seed sequence led to a recovery of the luciferase activity (Figure 4.13). Taken together, these results suggest that p35 is a direct target of miR-26a in DLBCL cells.

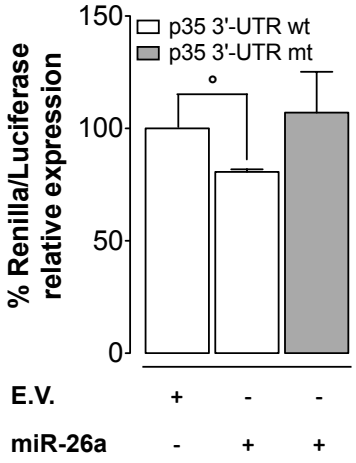

Fig. 4.13 – p35 is a direct target of miR-26a. MiR-26a significantly suppressed luciferase activity of plasmid carrying the wt but not the mt 3'UTR of p35. Values are normalized to the value of control, which is indicated as 100%. Significant differences indicated by: °P <0.01. Error bars represent the mean ± SEM.

4.5 – MIR-26A MODULATES PROLIFERATION AND CELL CYCLE PROGRESSION OF DLBCL CELLS THROUGH P35 REGULATION

First, expression level of miR-26a was measured in DLBCL cell lines by qRT-PCR and normalized against U6 snRNA. MiR-26a was strongly down-regulated in DLBCL cells compared to B lymphocytes (Figure 4.13 A). Next, we investigated the role of miR-26a in lymphoma cells. To this end, we examined the effects of hsa-miR-26a lentivirus expression on proliferation rate of SU-DHL-8 ABC-DLBCL cells. Transduction efficiency was evaluated by qRT-PCR (Figure 4.12 D). Compared to control, hsa-miR-26a expression markedly decreased lymphoma cell proliferation by 41% (P<0.01) (Figure 4.13 B). Moreover, hsa-miR-26a expression substantially augmented sTRAIL–induced apoptosis of SU-DHL-8 cell line (Figure 4.13 C and D).

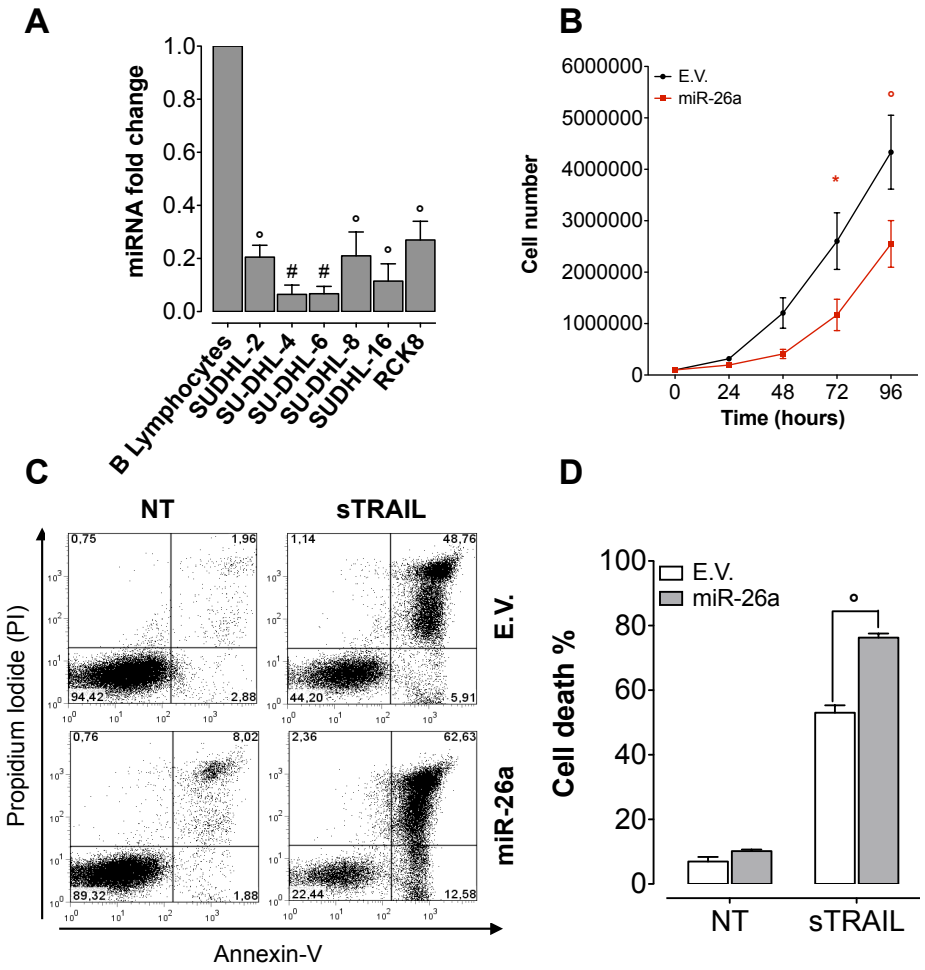

Fig. 4.14 – miR-26a affects proliferation and apoptosis in DLBCL cell lines. (A) Expression levels of miR-26a in DLBCL cell lines. (B) Up-regulation of miR-26a significantly inhibited cell proliferation and (C and D) increased the apoptotic rate compared with controls. Mean (± SEM) values refer to three independent experiments. Significant differences indicated by: *P < 0.01, °P < 0.05 and #P < 0.001.

Then, in order to demonstrate that the effect of miR-26a on proliferation and apoptosis of DLBCL was mediated by p35 and, consequently, by the modulation of CDK5 activity, we generated different new cell lines using lentiviral vectors: starting from SU-DHL-8, we created a control line (E.V.), a cell line overexpressing miR-26a (miR-26/E.V.) and a cell line overexpressing miR-26a together with a vector expressing the human p35 ORF, missing the 3'–UTR (miR-26/p35). Regulation of p35 expression and miR-26a overexpression were confirmed by western blotting and qRT-PCR (Figure 4.14 A, B and C).

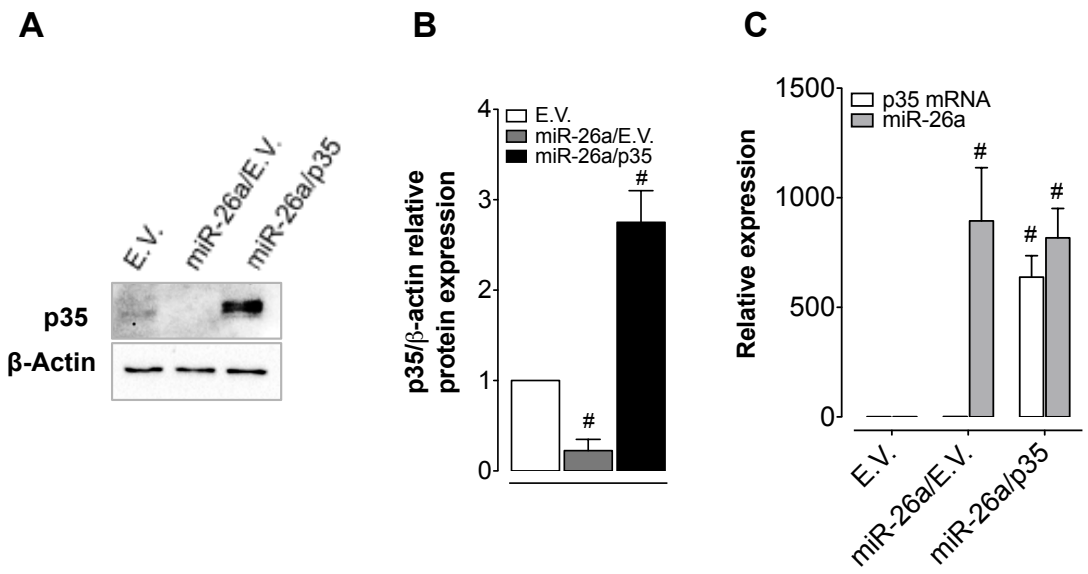

Fig. 4.15 – Expression of miR-26a and p35 in SU-DHL-8. (A and B) Immunoblot for p35 in SU-DHL-8 overexpressing miR-26a and in SU-DHL-8 overexpressing miR-26a together with human p35 missing the 3'–UTR. (B) Densitometric analysis of p35 protein normalized to β-actin. (C) Transduction efficient of the engineered cell lines. Mean (± SEM) values refer to three independent experiments. Significant differences indicated by: *P < 0.01, °P < 0.05 and #P < 0.001.

Compared to miR-26/E.V. cells, miR-26/p35 clone markedly showed an increase on cell proliferation and a decrease on apoptosis, similar to the proliferation and apoptosis index detected in E.V. cells (Figure 4.15 A, B and C). These data demonstrate that miR-26a regulates proliferation and apoptotic event in DLBCL cells and that p35 is an important mediator of these biological events.

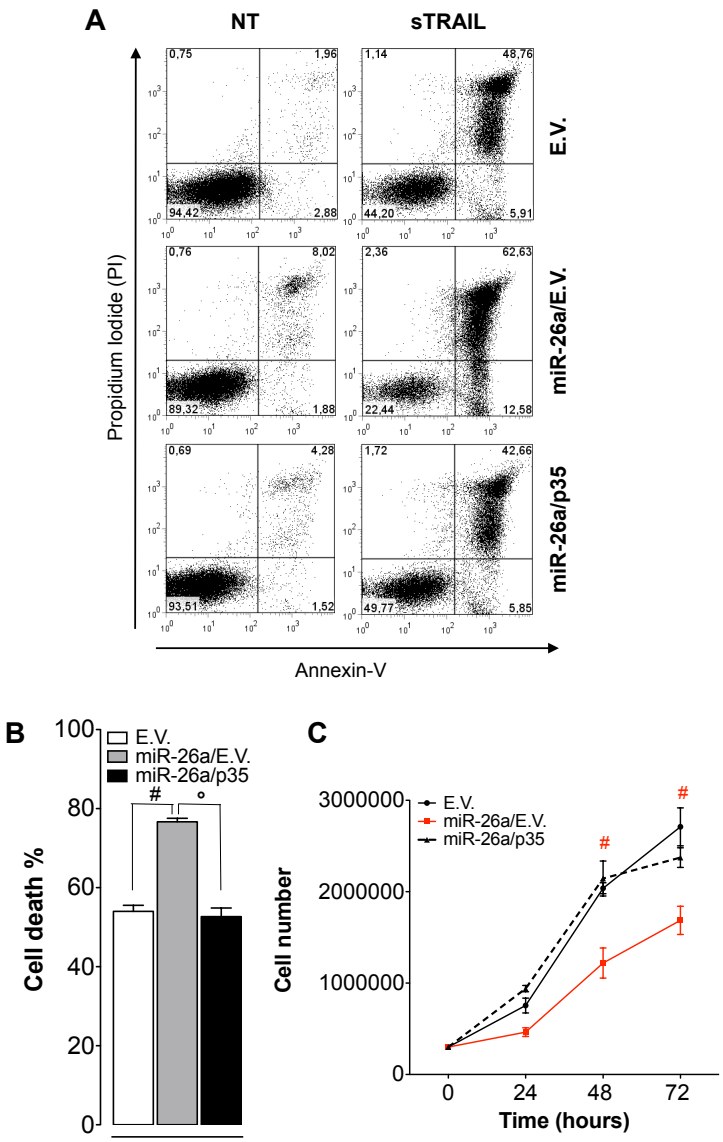

Fig. 4.16 – miR-26a controls proliferation and apoptosis of DLBCL cells through p35 regulation. (A and B) Concomitant expression of a p35 with a 3'UTR truncated form together with miR-26a restored apoptotic rate and (C) cell proliferation rate and to control level compared to miR-26a alone.

4.6 – MIR-26A MODULATES *IN VIVO* TUMOR GROWTH OF DLBCL THROUGH P35 REGULATION

SU-DHL-8 cells stably expressing miR-26a (miR-26/E.V.), cells overexpressing miR-26a together with a vector expressing the human p35 missing the 3'–UTR (miR-26/p35) or control vectors (E.V.) were injected subcutaneously into nude mice. Palpable tumors formed within 2/3 weeks. Tumor volume was monitored for 35 days using a caliper. In addiction, because these cells were GFP positive, we also detected tumor growth by IVIS in vivo imaging scan, calculated the fluorescence intensity of the tumor nodules (Figure 4.16 A). Mice were sacrificed 5 weeks after tumor cell implantation.

The tumors of SU-DHL-8 miR-26/E.V. group were not detectable for almost all the time of the experiment, while SU-DHL-8 (miR-26/p35) and SU-DHL-8 (E.V.) presented an average tumor volume very similar (6/8 cm^3; $P < 0.01$) (Figure 4.16 A, B and C).

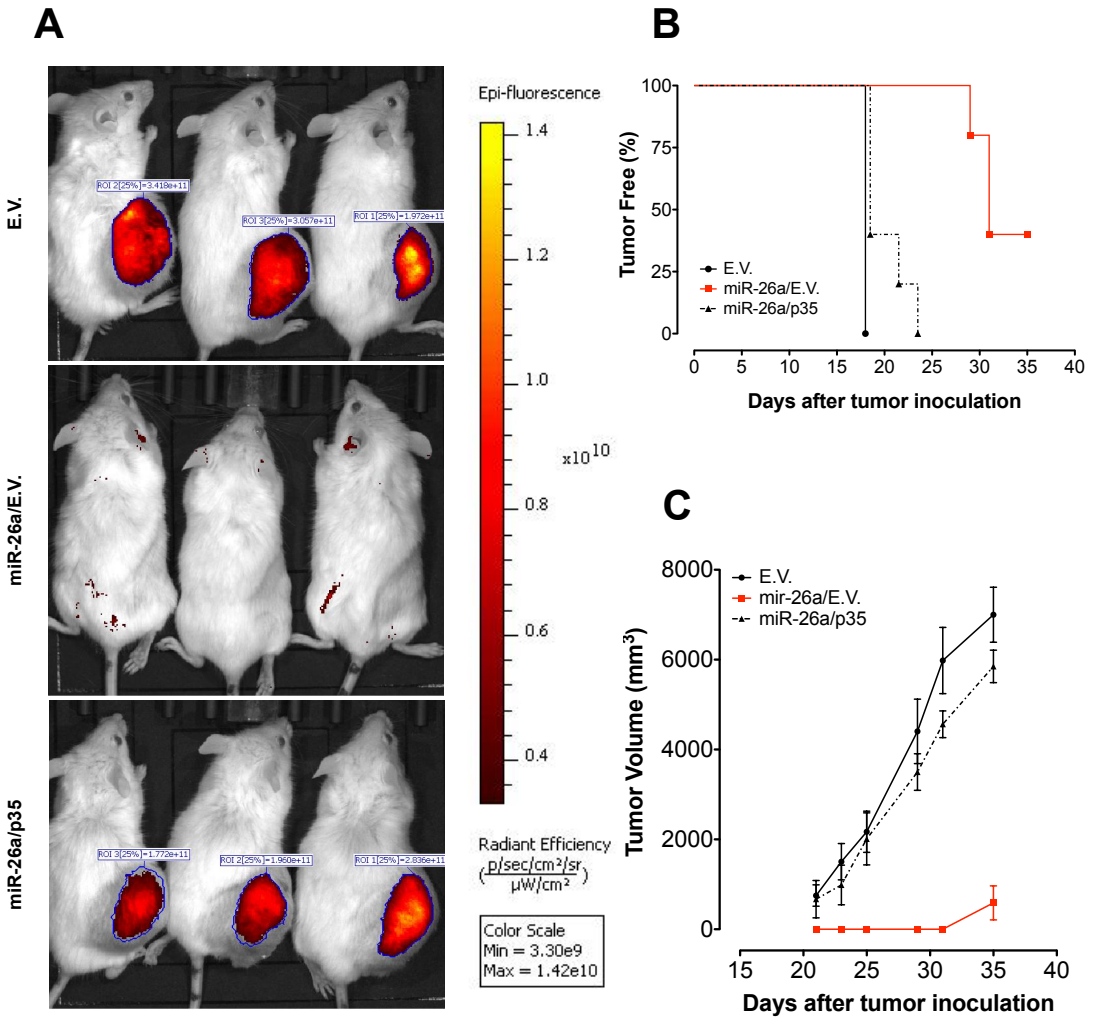

Fig. 4.17 – MiR-26a regulates *in vivo* tumor growth of DLBCL through p35 regulation. miR-26a overexpression suppressed tumor growth in subcutaneous implantation mouse models of SU-DHL-8 cells. (A) *In vivo* fluorescente images (ventral) of three representative mice challenged with DLBCL cells. (B) Tumor growth curves of subcutaneous implantation models of DLBCL are shown. (C) Tumor volumes were monitored until 5 weeks after the challenge. Number of mice per group: 5. Mean (± SEM) values refer to three independent experiments. Significant differences indicated by: *P < 0.01, °P < 0.05 and #P < 0.001 in comparison with single treatments.

Intra-tumoral apoptosis was detected by using tunnel staining and we confirmed an increased level of apoptosis in the group of mice injected with SU-DHL-8 miR-26/E.V. while SU-DHL-8 miR-26/p35 and SU-DHL-8 E.V. presented similar level of cellular dead (Figure 4.17 A and B).

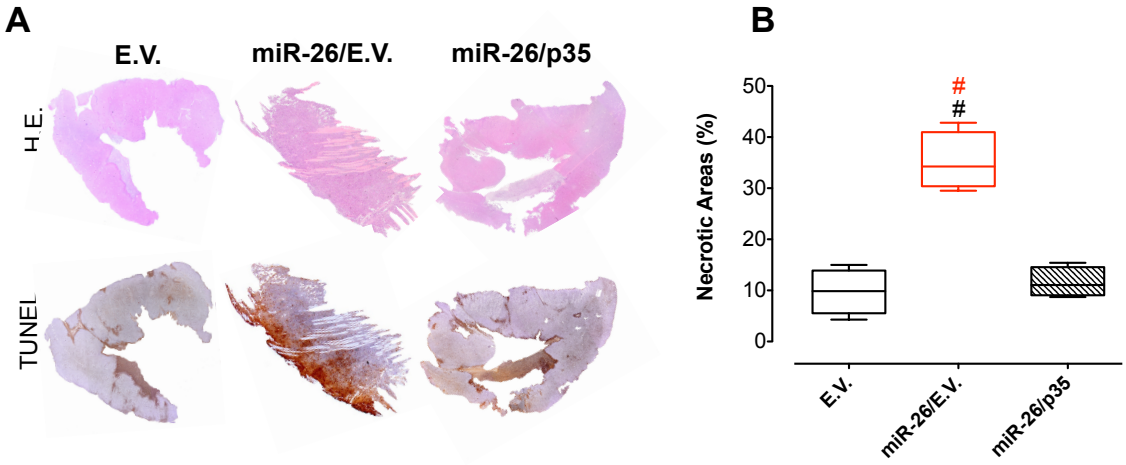

Fig 4.18 – miR-26a *in vivo* promotes tumor necrosis by targeting p35. (A and B) DLBCL tumor sections stained with H&E (upper panel). Apoptotic tumor cells were visualized (lower panels) and quantified using the TUNEL assay.

5. DISCUSSION AND CONCLUSIONS

Diffuse Large B-cell lymphoma (DLBCL) is the most common types of aggressive lymphoma worldwide.[1] The standard of care is the immunochemotherapy R-CHOP, but the chemorefractory and relapsed after treatment patients (approximatively 30-40%) represent an unmet medical need, requiring novel targets for innovative treatments.[196] One potential target is cyclin-dependent kinase 5 (CDK5), a serine/threonine protein kinase that has been recently linked to tumor development and progression.

In the last ten years, several studies have demonstrated that Cdk5 is active not only in neurons, as previously thought, but also in several non-neuronal tissues[55] and it is involved in many different physiological, such as gene transcription, apoptosis and migration, and pathological conditions, such as Alzheimer's disease. Several groups have recently demonstrated a link between CDK5 and a variety of human cancers. In particular, the importance of CDK5 activity for cancer cell migration and invasion and, consequently, for tumor progression, has been well documented for different solid tumors, such as breas[197], prostate[110], lung[198] and pancreatic[104] cancers. Instead, only few studies elucidate CDK5 role in hematological malignancies, such as leukemia[199] and multiple myeloma[200], but no prior studies have shown the role of Cdk5 in lymphoma to date.

In this study, we investigated if CDK5 could play an important role in DLBCL. In preliminary data, we observed that CDK5 protein expression is increased in several hematological malignancies cell lines, such as Hodgkin lymphoma, Burkitt lymphoma and B-Cell Chronic Lymphocytic Leukemia, compared to B lymphocytes from healty donors. Then, we focused on DLBCL

and we showed for the first time that CDK5 expression was increased in all of the human DLBCL cell lines we collected compared to control B lymphocytes, at both protein and mRNA levels, suggesting that an increased transcription of the gene is implicated.

Courapied and colleagues have shown that upon DNA damage, CDK5 phosphorylates STAT3 at Serine 727 and activates the transcription of genes involved in DNA repair, such as Eme1[201], and Lin and colleagues have found that CDK5 regulates cell proliferation of medullary thyroid tumor cells through STAT3 activation[202]. Thus, to investigate a potential link between the expression of CDK5 and the phosphorilation level of STAT3 in DLBCL, we silenced CDK5 by using shRNA and we found a reduction in STAT3 phosphorilation level at S727. In agreement with these data, we observed a higher level of phosphorilation in DLBCL cell lines in which CDK5 is overexpressed compared to B lymphocytes from healthy donors.

To study the function of CDK5 in DLBCL, we silenced the protein and we observed a significant decrease in tumor cell proliferation rate and a promotion of apoptotic events. This effects were rescued when CDK5 protein was restored by the expression of a recombinant CDK5 coding DNA sequence. We obtained similar results when we down-regulated the activator of Cdk5, p35. The overexpression of p35, instead, increased tumor cell proliferation and reduced apoptosis, as expected. These results demonstrated that CDK5 and p35 have a fundamental role in proliferation and apoptosis regulation of DLBCL cells.

Interesting, we found that p35 protein was higher in the cancer cells compared to healthy B lymphocytes, but this difference was not confirmed at the mRNA level. We hypothesized that the discrepancy between the p35 high

protein expression and the low mRNA level was due to the presence of an additional post-transcriptional regulatory process, able to inhibit the translation of p35 in normal cells, but absent on tumor cells. Indeed, we found that miR-26a directly regulates p35 expression in DLBCL cell lines, by binding its 3' UTR, and that the level of miR-26a in tumor cells is inversely correlated with p35 level. In fact, we showed that up-regulation of miR-26a significantly reduced p35 level in DLBCL cells and overexpression of miR-26a decreased the luciferase reporter activity of the p35 wild-type 3'-UTR, but not of the mutant. MiR-26a has a controversial role in cancer. In fact, its expression deregulation has been correlated with tumorigenesis and cancer progression, acting as a oncogene in some types of cancer, but also with tumor suppressor in other types of tumors. Here, for the first time, we studied the role of miR-26a deregulation in DLBCL tumor growth using *in vitro* and *in vivo* assays. We showed that miR-26a level was significantly lower in DLBCL cell lines compared to B lymphocytes from healthy donors, and that miR-26a overexpression suppressed proliferation and promoted apoptosis in tumor cell lines. Moreover, miR-26a inhibits DLBCL tumor growth and promotes tumor necrosis in NOD/SCID mice. These results indicate that miR-26a acts as a tumor suppressor in DLBCL.

The effects of miR-26a modulation on cell proliferation, apoptosis, tumor growth in DLBCL were accompanied by changes in p35 levels and CDK5 activities. Concomitant expression of a recombinant 3'UTR truncated p35 completely abrogated the effects induced by miR-26a. These data revealed that miR-26a directly regulates p35, which is one of the most important downstream mediators of miR-26a function in DLBCL.

All together, 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.

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ACKNOWLEDGEMENTS

First and foremost, I have to thank my research supervisors, Professor Carmelo Carlo-Stella and Dr. Manuela Quintavalle for their support and expert guidance.

A special thanks goes to my colleagues for their assistance and helpful suggestions.

I would like to express my deep appreciation to Nadia for her sincerely support and encouragement.

Last, but not least, I would like to thank my beloved family.