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DOTTORATO DI RICERCA IN BIOCHIMICA, BIOLOGIA MOLECOLARE E BIOTECNOLOGIE

CICLO XXVIII

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A novel in vivo nanoparticle-mediated delivery for microRNA in a CLL mouse model: identification of miR-26a as a potential therapeutic agent

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ABSTRACT

Purpose. Chronic lymphocytic Leukemia (CLL) is an indolent but incurable disease. MicroRNAs emerged as promising therapeutic molecules. However, the in vivo delivery of microRNAs is challenging. The purpose of this work is to develop an efficient novel delivery system for microRNAs in CLL mouse model and to identify new microRNAs as potential therapeutic agents.

Results. We have successfully developed efficient immune-liposomes formulation composed of anionic lipoplex coupled to antiCD38 (mAb) to specifically target CLL cells. Interestingly, the developed nanoparticles (named CD38-NP) demonstrated approximately 70-fold increase in the level of miR-181b in leukemic splenocytes compared with the cationic polymer PEI. Among tested microRNAs, we successfully identify miR-26a as a potential therapeutic agent in CLL. First, miR-26a selected as a candidate among the group of miR/Anti-miRs tested in vitro and in vivo and viability/apoptosis measured. Second, its anti-leukemic role was validated in a long term treatment in mice with established leukemia. A protocol that included three weeks treatment with miR-26a mimics demonstrated a significant increase in the level of miR-26a in the leukemic splenocytes, accompanied by the down-modulation of the level of its target CDK6 protein. More importantly, a delay in the leukemic cell expansion (LE) was detected at the end of miR-26a treatment.

Conclusions. These results provide a novel delivery approach, which improves efficiency and specificity of delivery to CD38+ leukemic cells. In addition, we report for the first time that miR-26a exhibit a significant activity in reducing leukemic cells expansion.

RIASSUNTO

Scopo. La Leucemia linfocitica cronica (LLC) è una malattia indolente, ma incurabile. I microRNA sono emersi come promettenti molecole terapeutiche. Tuttavia, la veicolazione dei microRNA *in vivo* rappresenta un punto molto critico per il loro utilizzo. Lo scopo di questo lavoro è quello di sviluppare un nuovo efficiente sistema di veicolazione di microRNA in un modello murino di LLC e di individuare nuovi microRNA da utilizzare come potenziali agenti terapeutici.

Risultati. Nel nostro studio abbiamo sviluppato con successo un'efficiente formulazione immuno-lipidica composta da complessi lipidici anionici coniugati con anticorpo antiCD38 (mAb) in grado di riconoscere specificamente le cellule di CLL. È interessante evidenziare come la veicolazione del miR-181b mediante le nanoparticelle sviluppate (denominati CD38-NP) ha determinato un incremento dell'espressione del miR in splenociti leucemici di circa 70 volte in più rispetto al polimero cationico PEI. Tra i microRNA testati, abbiamo identificato con successo il miR-26a come potenziale agente terapeutico nella LLC. Inizialmente, il miR-26a è stato selezionato come possibile candidato in un gruppo di miR / anti-miR i cui effetti sulla vitalità/apoptosi sono stati valutati sia *in vitro* che *in vivo*. Successivamente, il suo ruolo anti-leucemico è stato validato con un trattamento a lungo termine in topi presentanti una leucemia confermata. Un protocollo che comprendeva tre settimane di trattamento con miR-26a ha dimostrato un aumento significativo del livello del miR-26a negli splenociti leucemici, accompagnato dalla riduzione del livello della sua proteina bersaglio CDK6. Ancora più importante, un rallentamento nell'espansione della popolazione cellulare leucemica (LE) è stato rilevato alla fine del trattamento con il miR-26a.

Conclusioni. Questi risultati illustrano un nuovo approccio di veicolazione dei microRNA, che migliora l'efficienza e la specificità della veicolazione nelle cellule leucemiche CD38 +. Inoltre, è emerso per la prima volta che miR-26a mostra una significativa attività nel ridurre l'espansione delle cellule leucemiche.

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DEDICATION

To My Beloved

Mother

&

To the Soul of My Father

&

My Family

LIST of CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

7-AAD	7-aminoactinomycin D
AAV	Adeno Associated Virus
AML	Acute Myeloblastic Leukemia
BCL2	B-cell CLL/lymphoma 2
BCR	B-cell antigen receptor
CDK	Cyclin-dependent kinase
CLL	Chronic Lymphocytic Leukemia
ddPCR	Droplet Digital Polymerase Chain Reaction
DMG-PEG	1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DSPE	Distearoylphosphatidylethanolamine
FVB	Friend Virus B-Type (mouse)
HMW-PEI	High Molecular Weight PEI
HRMs	Hypoxia-regulated microRNAs
IP	Intraperitoneal
LE	Leukemic Expansion
LMW-PEI	Low Molecular Weight PEI
mAb	monoclonal antibody
Mal	Maleimide
MRX34	The liposomal miR-34 mimic
PEG	Poly (ethylene glycol)
PEI	Polyethylenimine
PK/PD	Pharmacokinetic/Pharmacodynamic

PTEN	Phosphoinositide 3-kinase pathway phosphatase and tensin homolog
RES	Reticuloendothelial system
SDS-PAGE	Sodium Dodecyl Polyacrylamide Gel Electrophoresis
ZAP-70	Zeta-chain-associated protein kinase 70

KEY WORDS

Chronic Lymphocytic Leukemia

microRNA Therapy

Eμ-TCL-1 mouse model

Liposomes Nanocarrier

CD38 mAb

miR-26a

1. INTRODUCTION

1.1. CHRONIC LYMPHOCYTIC LEUKEMIA

1.1.1. General Features

Chronic lymphocytic leukemia (CLL) is a B cell neoplastic disease characterized by the accumulation of small mature-appearing lymphocytes in the blood, marrow, and lymphoid tissues. CLL is the commonest form of leukemia, accounting for ~30% of all cases of adults leukemia [1]. Currently, more than 15,000 newly diagnosed cases, and ~ 4,500 deaths estimated in western world. The disease typically occurs in elderly and more male than female patients (1.7:1) are affected [2]. CLL is a characterized by marked clinical heterogeneity due to in part genetic alteration in leukemic cells [3]. It occurs in two forms, indolent and aggressive. Although clinical features and genetic abnormalities in CLL is well documented, molecular details underlying the disease are still not fully understood [1]. The current mainstay of therapy is the cytotoxic chemotherapy; however, CLL is remaining an incurable disease with resistance to therapy developing in the majority of patients [4].

Regarding immunophenotyping, CLL cells co-express the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23 [5]. The levels of surface immunoglobulin (Ig), CD20, and CD79b are characteristically low compared to those found on normal B cells [6], [7]. Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains [6].

1.1.2. Prognosis and Treatment

In chronic lymphocytic leukemia (CLL), genetic markers are well known for their pathogenic and prognostic relevance. CLL is suggested as a model disease for personalized medicine. This is because of several reasons includes the heterogeneity in patients clinical course; accessible malignant cells; prevalent disease, although in western world and genetic aberrations are at high resolution between population. In addition to the diversity in patient characteristics like clinical parameters (e.g., age, comorbidity) and biologic parameters (e.g., genomic aberrations, mutations) [8].

In general, prognostic factors in CLL, includes lymphocyte doubling time (LDT), serum markers, IGHV mutational status, ZAP-70 and CD38 expression, cytogenetic abnormalities and somatic mutations [9], [10]. CLL patients manifest distinct disease courses [11], [12] and prognostic molecular markers identify patients at different risk: leukemic clones presenting few IgHV-gene mutations (U-CLL), many CD38+ or ZAP-70+B-cells, lead to an aggressive disease, resistance to chemotherapy and usually fatal course; clones with mutated IgHV (M-CLL), few CD38+ or ZAP-70+ B-cells, exhibit an indolent asymptomatic course generally responding to therapy [13]. Additionally, T-cell leukemia-1 (TCL1) is an oncogene expressed in almost all CLL patients and high TCL1 protein levels correlate with prognostic markers of aggressive forms, such as unmutated VH status, ZAP70 expression and chromosome 11q22-23 deletions [14]. Accordingly, lower TCL1 levels associate with higher probability of positive response to chemoimmunotherapy [15].

The monoclonal nature of leukemic cells, suggests the existence of genetic lesions in the pathogenesis of CLL. Approximately 80% of individuals with CLL have acquired chromosomal abnormalities [16]. Recurrent cytogenetic aberrations include: deletion at 13q14.3 (55% of cases), associated with the indolent form and loss of miR-15a and miR-16-1 genes [17]; deletions at 17p13 (7%) or 11q22-23 (18%), which associated with the aggressive forms and loss of TP53 at 17p, ATM and miR-34b/miR-34c at 11q; trisomy 12 (16%), associated with an intermediate form of CLL [16].

In addition, genetic mutations in CLL are of prognostic value with many but not all treatment modalities. Nucleotide sequencing have discovered recurrent mutations in a number of genes, like TP53, NOTCH1, SF3B1, BIRC3, ATM and MYD88 indicating the existence of multiple pathways derailed in CLL cells. How these alterations interact to promote CLL has yet to be fully understood [18], [19].

Current treatment includes DNA-damaging chemotherapy such as fludarabine, pentostatin and cladribine [20] and their combinations that results in the development of resistance in virtually all patients on several treatment courses and actually selects for high-risk genetic alterations. Resistance against chemotherapy frequently correlates with loss of function of the DNA damage response pathways,

in CLL as well as in most other cancers with TP53 and ATM genes localized in 17p and 11q, respectively [8]. This is why TP53 is the main biomarker in CLL, which currently drives treatment decisions [21].

As indicated above, microRNAs have been incorporated as novel biomarkers **(Table.1)** in CLL prognostic groups [8]. CLL has been the first entity for which miRNAs have been described as tumor suppressors, such as miR15a, miR16 and miR-34a. The latter, is arguably one of the most interesting tumor-associated miRs not only in CLL, but also in other tumors [22]. A second novel relevant biomarker has been also identified is the epigenetic aberrations [8].

Table (1): microRNA associated with prognosis in CLL (Table adapted from Musilova *et al*) [23].

microRNA	*Expression associated with worse prognosis*	*References*
miR-155	↑ in aggressive CLL	[24]
miR-21	↑ associated with worse prognostic outcome in CLL	[25]
miR-29c	↓ miR-29c associated with shorter OS and TFS in CLL; ↓ miR-29c in CLL with TP53 abnormalities	[26], [27]
miR-34a	↓ associated with shorter OS, ↓ in CLL with TP53 abnormalities	[26], [28] [29], [30]
miR-150	↓ in aggressive CLL, ↓ in CLL associated with shorter OS and TFS	[31]
miR-223	↓ associated with higher tumour burden, disease aggressiveness, and poor prognostic factors	[27]
miR-650	↓ associated with shorter OS and TFS	[32]

Abbreviations: OS, overall survival; TFS, treatment-free survival; ↑, increased;↓, decreased.

Over the last few years, novel targeted therapies have emerged as valuable in CLL treatment, mainly directed at disrupting the B cell receptor pathway and several new immunological drugs, particularly monoclonal antibodies (mAbs) [33]. The most promising newer mAbs are directed against CD20, CD19, CD37 and CD40. In addition, combinations of antibodies with targeted drugs like ibrutinib, idelalisib or lenalidomide may probably replace chemotherapy-based combinations in the near future. For instance, targeting CD20 with Rituximab (Rituxan, Mabthera, F. Hoffmann-La Roche) has been approved by US FDA for the treatment of patients with previously treated and untreated CLL [34]. Therefore,

better understanding of the interplay between genetic and non-genetic factors expected to shed light for further new therapeutic strategies.

CD38

CD38 surface receptor, is a 45 kDa type II transmembrane glycoprotein a able to induce activation, proliferation, and survival of human and mouse lymphocytes; this molecule is expressed on the surface of both mature and immature B cells [35], [36]. Furthermore, CD38 promotes survival and proliferation of B cells on their way to and after neoplastic transformation [37]. Therefore, CD38 is used as a prognostic marker in human CLL. The number of CD38-expressing cells can be considered as a real-time indicator of the proliferative activity of a patient's CLL clone [37]. Similarly, in mouse, CD38 considered as one of the earliest markers in B cell differentiation. The onset of CD38 expression in mice coincides with B220 expression and continues through all stages of differentiation in bone marrow and spleen [38].

1.1.3. Biology and Pathogenesis

Multiple pathogenic mechanisms have been suggested. As indicated above, a number of genetic and cytogenetic lesions have been discovered in CLL cells. The gene expression profile suggests that CLL cells originate from transformed, antigen-stimulated B cells [39]. Therefore, chronic antigenic stimulation via B-cell antigen receptor (BCR) is thought to be involved in CLL pathogenesis [40]. In addition to genetic lesions and signals through the B-cell receptor (BCR), CLL pathogenesis may rise from survival signals of the microenvironment, mediated by integrins, chemokine and cytokine receptors that allow CLL cells to actively proliferate and accumulate [41], [42]. The interaction of these factors with genetic abnormalities promotes survival, proliferation and immune surveillance escape.

Another important role is the over-expression of anti-apoptotic proteins such as BCL2, BCL-XL and BAG1 and under-expression of pro-apoptotic proteins, such as BAX and BCL-XS [43].

1.1.4. Animal Models in CLL

Eμ-TCL-1 mouse model

The availability of mouse models that reproduce leukemia with the distinct immunophenotype and a similar course of the human B-CLL, offers the possibility to better understand pathogenic mechanisms and test novel therapeutic approaches in CLL**.**

A number of CLL mouse models generated and recently reviewed by Simonetti *et al* [44]. It is of great value that several types of mouse model have been produced from TCL1-tg mouse model. The Eμ-TCL1 transgenic mouse (TCL1-tg) is recognized as the best presently available model for CLL. This is because, TCL1-tg mouse shows a form of leukemia that is most similar to the aggressive type of human CLL, in term of immunophenotype, BCR repertoire and disease course. The produced TCL1-tg developed a leukemic with 100% disease penetrance characterized by a distinct clonal expansion of B-cells with B220+/IgM+/CD5+, non-mutated IGHV, increased proliferation and enhanced Akt phosphorylation, which may well represent an aggressive form of CLL [45], [46], [47], [48].

Transplanted mouse model

As indicated above, TCL-1 tg considered as the best animal model for CLL. However, like for human CLL, disease is heterogeneous and TCL1-tg requires approximately12-14 months to establish a clonal leukemic disease. Instead, Bresin *et al* successfully established a transplanted mouse model [49]. A syngeneic transplantation approach on FVB wild type performed and provided a uniform experimental condition was suitable for in vivo testing and validation of microRNAs therapeutic. Leukemia with expected immunophenotyping and other similar features in human CLL leukemia and the TCL-1 tg, developed after 6-8 weeks of intraperitoneal (IP) injection of splenocytes, collected from donors of TCL-1 tg mice [49]. In this study, we follow the same transplantation procedure to establish leukemia in syngeneic FVB mice to have homogeneous disease for testing in vivo microRNAs therapy.

1.2. MicroRNAs

miRNAs are a class of highly conserved small noncoding RNAs that regulate post-transcriptional gene expression [50] . A single miRNA may control the expression of hundreds mRNA targets. Just as miRNAs may control a variety of crucial functions related to normal cell growth, development and differentiation, so their dysregulation is associated with pathological conditions [51].

1.2.1. Biogenesis and Function

MicroRNAs are 20-23 nucleotides in length [52]. In mammalian cells, miRNAs are transcribed by RNA polymerase II as long pri-miRNA molecules from intergenic regions of the genome **(Fig.1)**, but may also be derived from intronic and exonic regions of coding and non-coding genes [53]. In the nucleus, canonical pri-miRNAs are capped with 7-methylguanosine and polyadenylated and cleaved by the RNAse III enzyme Drosha and its cofactor Pasha (or DGCR8) to produce a 60–100 nt precursor miRNA (pre-miRNA) hairpin molecule [54].

RAN-GTP and exportin-5 complex, subsequently transport the pre-miRNAs into the cytoplasm. In cytoplasm, Dicer, also an RNAse III endonuclease, interacts with TRBP (TarRNA Binding Protein) to mediate further processing of pre-miRNA to form a mature 20– 23 nt miRNA–miRNA* duplex [55]. The duplex is unwound by a helicase and the mature miRNA is incorporated into effectors complex known as miRISC (miRNA induced silencing complex) [56], [57], [58], while the miRNA* (read as miRNA “star”) is degraded. Within the RISC complex, miRNAs bind through imperfect base pairing to the 3’untranslated region (3’UTR) of target mRNAs [59].

The binding specificity and efficiency believed to specify by 6–7 nucleotide sequence near 5′ region of miRNA. This sequence is called the “seed sequence” and is the initial binding site of the miRNA to the 3’UTR of the target mRNA [60]. The subsequent procedures depend on the degree of complementarily between the miRNAs and their targets. Imperfect complementarily of the miRNAs and the target mRNA, will cause translational repression of gene targets, while perfect matching, recruits the CAF1–CCR4 mRNA deadenylation complex to initiate mRNA degradation [57].

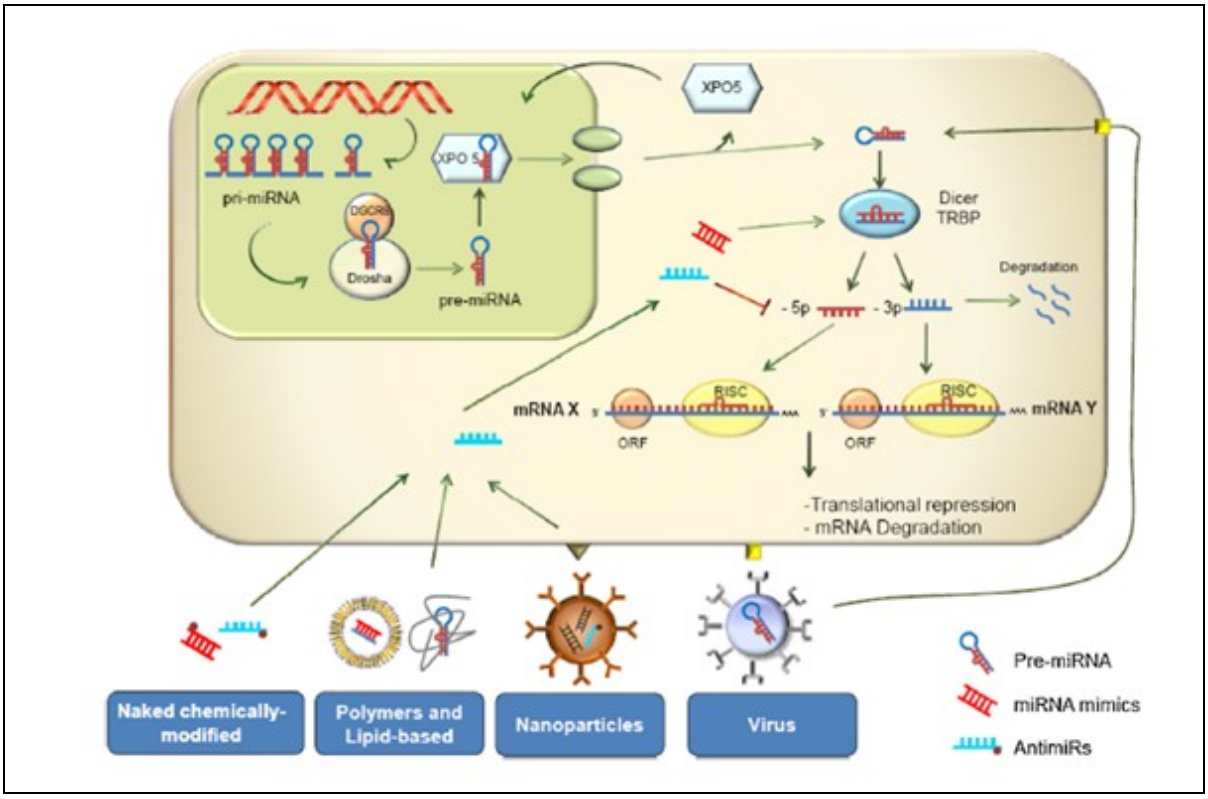

Figure (1): MiRNA biogenesis and delivery options.

Individual or clustered miRNA transcripts (pri-miRNA) are cleaved by the endonuclease complex Drosha-DGCR8 in the nucleus. The resulting pre-miRNA form will be transported to the cytoplasm, where will be further processed by the second endonuclease complex, Dicer-TRBP. Then pre-miRNA becomes a miRNA duplex, composed by two strands. Depending on the miRNA, one or both strands will be directed to the 3'-UTR of their target genes. Synthetic miRNAs can be delivered "naked" with different chemical modifications, conjugated to natural or synthetic polymers, encapsulated in organic nanoparticles (i.e. liposomes) or using inorganic nanoparticles. Precursor RNA molecules can also be delivered using viral particles [61].

1.2.2. MicroRNA Deregulation in CLL

The first evidence of miRNA involvement in human cancer came from a study on CLL [22]. MicroRNAs profiles can be used to distinguish normal B-cells from malignant CLL cells. The most important, is the association with prognosis, progression **(Table 1)** and even drug resistance in CLL [62]. Moreover, microRNAs can differentiate between aggressive and indolent CLL forms, using 13 microRNAs as described by Calin *et al* [63]. It can also discriminate between different cytogenetic subgroups [64].

Table (2): MicroRNAs frequently deregulated in CLL and their targets in B cells (adapted from Musilova *et al*) [23].

MicroRNA	*Expression*	*Target genes validated in B cells*	*Main effect of miRNA deregulation in B cells*	*Reference*
miR-155 (OG)a	↑	SHIP-1, C/EBPβ, HGAL, RTKN2, SMAD5, SOCS1, MAFB, SHANK2, SH3PXD2A, PU.1, AID	↑ PI3K/AKT activity, ↑ B-cell proliferation; ↑ cell motility; ↑ resistance to growth inhibitory effects of TFG-β1 and BMP	[24], [65]
miR-16-1 (TS)b	Deletion	CCND1, BCL2, TP53	↑ proliferation; ↓ apoptosis	[22], [66], [29]
miR-15a (TS)b	↓ Deletion	BCL2, TP53	↓ apoptosis	[22], [66], [29]
miR-29 (TS)b	↓ in aggressive and TP53 aberrant CLL	CDK6, TCL1, MCL1	↑ proliferation; ↓ apoptosis	[26], [14]
miR-34a (TS) b	↓ in TP53 aberrant CLL	FOXP1, BCL2, BCL6, B-MYB, CDK6,ZAP-70, AXL	↑ proliferation, ↑ cell cycle progression, ↓ apoptosis	[26], [28], [67], [68]
miR-150 (TS)b	↓ in aggressive CLL	MYB, FOXP1, CXCR4	↑BCR signalling; ↑ PI3K/AKT	[31]
miR-181b (TS)b	↓ in CLL with 11q deletion; ↓with disease progression	TCL1, BCL2, MCL1, AID	↓ apoptosis	[69], [14], [64]

Abbreviations: ↑, increased; ↓, decreased. (OG)a, oncogenic properties of miRNA. (TS)b, tumour suppressive properties of miRNA.

Furthermore, some specific microRNA can associate with certain course of disease; predict drug resistance, progression or even death [62], [28], [64]. Therefore, microRNAs now play a great role as markers for CLL development and response and prediction for treatment [62] and potential predictors of time to treatment [64]. Currently, both scientific and pharmaceutical field are actively focusing on utilizing microRNAs as therapy, not only in CLL, but also in cancer and benign diseases.

Interestingly, the miR-15/16 cluster, miR-29, miR-181 family members, and miRs-34b/c, were found as the most deregulated microRNAs in CLL. The same microRNAs were found to regulate gene expression patterns. Importantly, this helps to clarify molecular steps that lead to the onset of the disease or drive disease progression. The microRNAs most dysregulated in CLL illustrated in **Table 2**.

1.2.3. MicroRNAs Therapeutics

MiRNAs and miRNA-targeting oligonucleotides have several advantages over traditional small-molecule drugs, most notably the ease with which oligonucleotides can chemically modified to enhance their PK/PD (Pharmacokinetic/Pharmacodynamic) profiles. Moreover, many of microRNAs have the ability to target multiple genes, often within the same pathway. This can enhance their inhibition action to have systematic effects [70]. And this is strongest rationale for exploring the therapeutic potential of miRNAs, which could make it possible to administer one microRNAs that targets multiple oncogenes and oncogenic pathways that are deregulated in cancer disease [71]. Therefore, microRNAs with aberrant expression in diseased tissue could be considered as good therapeutic candidates [70].

On the other hand, synthetic miRs are prone to degradation in bio-fluids and have limited cellular uptake, rendering the clinical development of miR-based therapies relatively difficult [72].

1.3. Delivery System in MicroRNA

1.3.1. Strategies in MicroRNA Therapeutics

In vivo delivery of microRNA facilitates testing of micrRNAs to identify therapeutic candidates, studying their biological effects on tumor cells, besides identifying their protein targets and therefore better understanding pathways to improve disease management. Two strategies are in use to modulate the function of miRNAs in cancers, miRNA antagonists and miRNA mimics.

MiRNA Antagonists

MiRNA antagonists strategy is directed toward a gain of function and aims to inhibit oncogenic miRNAs by using miRNA antagonists, such as anti-miRs, locked-nucleic acids (LNA), or antagomiRs. These miRNA antagonists are oligonucleotides (antagomirs, antimirs) with sequences complementary to the endogenous miRNA, targeting oncomirs. They carry chemical modifications that enhance the affinity for the target miRNA and trap the endogenous miRNA in a

configuration hat is unable to be processed by RISC, or alternatively, leads to degradation of the endogenous miRNA [73].

MiRNA Mimics

MiRNA mimics strategy or also named as 'miRNA replacement therapy' involves introducing a tumor-suppressor miRNA mimic to restore a loss of function. It aims to re-introduce miRNAs into diseased cells that normally expressed in healthy cells. Because of being substantially smaller, miRNAs mimic, unlike proteins, will merely have to enter the cytoplasm of target cells to be active, and they can be delivered by the technologies that are used for siRNAs. Another advantage of miRNA mimics is that miRNA mimic has the same sequence to the depleted, naturally occurring miRNA. Therefore, expected to target the same set of mRNAs that are regulated by the natural miRNA. Another advantage is that nonspecific off-target effects are unlikely when using miRNA mimics, because the replaced miRs is expressed by normal cells [73].

1.3.2. Challenges in MicroRNAs Delivery

Delivery of miRNAs for cancer therapy, either restoring or repressing miRNAs expression holds a great promise [74]. However, despite the early promise and exciting potential, great limitations **(Table 3)** remain to overcome before transition to successful delivery and clinical applications [74]. These limitations include the instability of miRNAs in cellular environments [75], high rates of blood clearance, the risk of systemic toxicity, and an inability to deliver sufficient amounts of miRNAs to the targeted tissues and cells [76], [77]. As a result, high doses are usually required for miRNA-based therapies, which increase the risk for unwanted toxicities and immune response [78].

The Ideal miRNA Carrier

For a successful miRNA delivery, the final destination is the cytoplasm of the target cells [79]. In addition, the delivered molecule should elicit powerful therapeutic effect without causing unwanted side effects. Therefore, the ideal carriers should be able to cross both the kinetic barriers; condense anionic miRNAs via e.g. charge interactions, protect the encapsulated miRNAs from serum degradation by nucleases, accumulate to the disease organs via active

targeting and selectively deliver miRNAs to the target cells, eventually reaching the cytosolic targets [80], [81], [82].

Table (3): Barriers and solutions to the delivery of miRNAs [82]**.**

Barriers	*Solutions*
Degradation and elimination	1. Particle size
1. Degradation by nucleases	2. Surface charge
2. Renal clearance	3. Improved stability
3. Removal by phagocytic immune cell	4. Chemical modification
	5. Local administration
Poor penetration through tissues/cells	
1. Failure to cross capillary endothelium in targeted tissues	1. Targeting ligands
2. Inefficient endocytosis in targeted cells	
	2. Non-specific interactions
	3. Cell penetrating moieties
Intracellular disposition	
1. Ineffective endosomal release	1. Lytic lipids
2. Requirement for intracellular localization	2. Fusogenic peptides
	3. Osmotic lysis
	4. Targeting to RISC

1.3.3. Delivery Vehicles

MiR-delivery for potential cancer therapy currently based on viral and nonviral systems **(Fig. 2)**. Safety concerns of viral delivery systems still have its limitation in human and non-viral approaches seem to be more promising [83].

The most commonly used delivery strategy for macromolecules including miRNA is to take advantage of the receptor-mediated endocytosis **(Fig. 3)**. Second, endocytosed miRNA will need to escape the endosome or caveosome to reach the cellular cytoplasm where its target locates [79]. Owing to similarity to siRNA, microRNAs can be delivered with the same strategies used in delivering siRNA [72].

Viral Delivery System

Plasmid vectors have been successfully used in mammalian cells in vitro by simple transfection. Plasmid vectors need to cross the nuclear membrane for transcription. This is not a serious problem for cells actively growing in culture, because the nuclear membrane disappears during mitosis. For primary cells that are usually, not actively dividing, delivering macromolecules such as miRNAs is more favourable than the plasmid. Adeno-associated virus is non-pathogenic and can achieve long-term gene expression. However, immunogenicity and other safety issues are still great concerns of using viral vectors in human [79].

Therefore, here in current work we prefer to non-viral approach to deliver microRNAs into CLL cells in mouse model.

1.3.4. Non-viral miRNA Delivery System

Non-viral delivery systems have significant advantages including high biocompatibility, amenability to surface functionalization, and a loading capacity independent of the gene size [82].

Different nonviral delivery system have been tried to deliver microRNA in different cancers types **(Fig. 2)**. The most common are anionic naked oligonucleotides, Lipid-based nanoparticles, polymeric vectors (e.g. polyethylenimine (PEI)), Dendrimer, Micelles, Inorganic materials (e.g. gold nanoparticles). As mentioned nonviral vectors are many different types; however, among them, Lipid- based nanocarriers is by far the most popular approach [82].

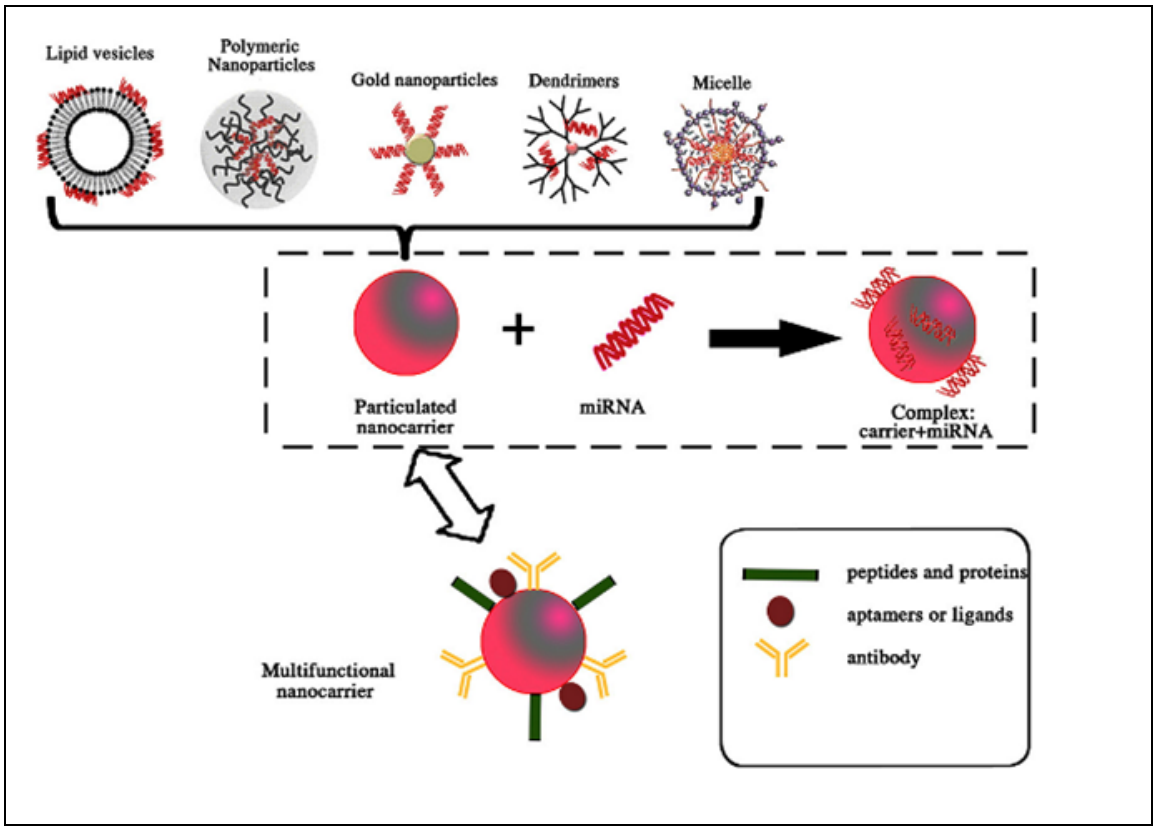

Figure (2): Non-viral delivery systems for miRNAs [82].

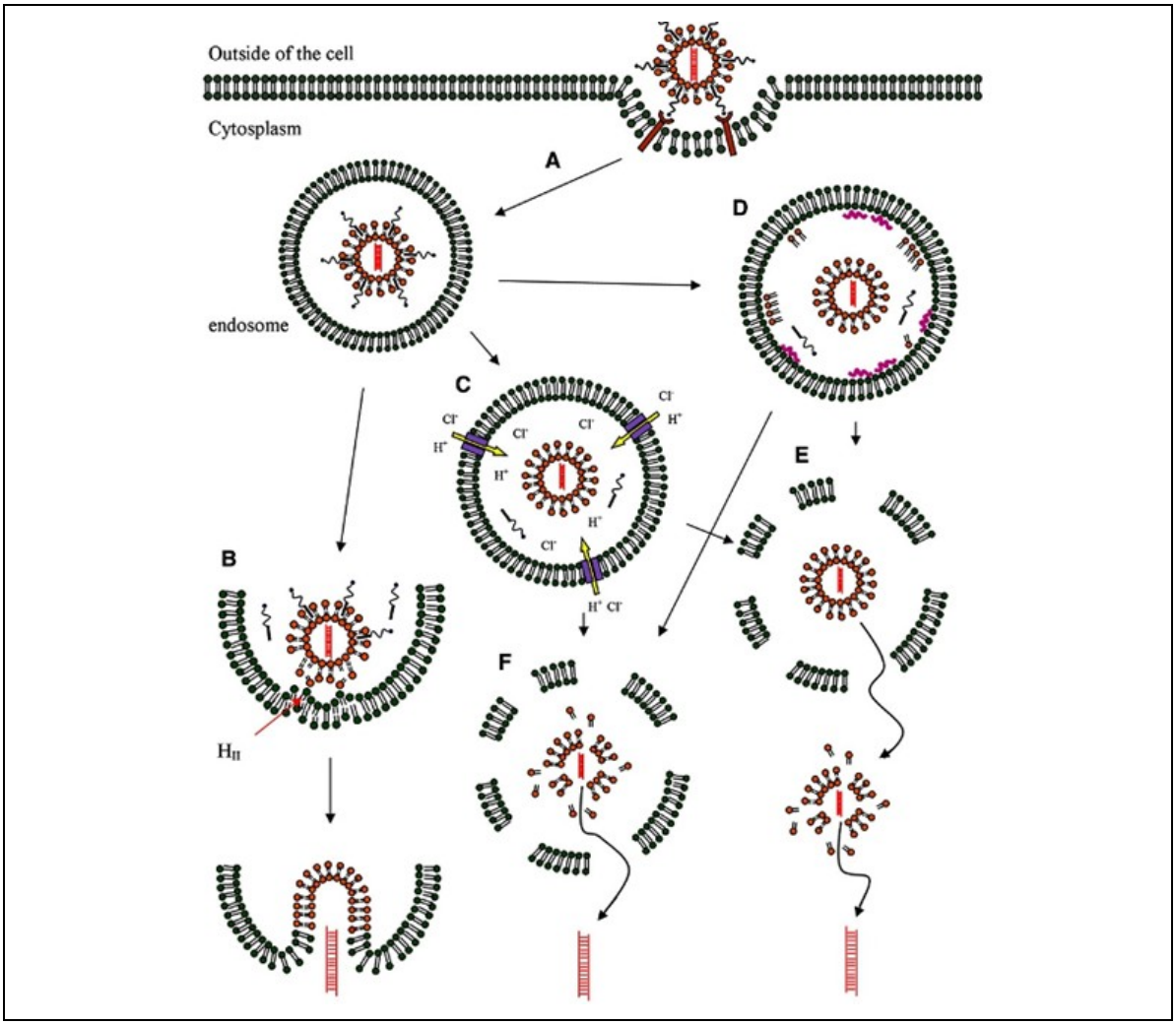

Figure (3): Endosome escape in lipoplex mediated siRNA delivery.

(A): Lipoplex containing siRNA (shown as orange lipid bilayer and red siRNA) with PEG and targeting ligand on the tip (shown as blue circle) are taken up by target cell via receptor mediated endocytosis. (B): The cationic lipid of the lipoplex forms ion pairs with the anionic endosomal lipid (PEG molecules may leave the lipoplex spontaneously or under appropriate design) and can further form the inverted hexagonal phase (HII). This leads to the fusion of the lipoplex with endosomal membrane and release the siRNA into cytoplasm. (C): Lipoplex containing molecules having buffer capacity in endosomal pH range can trigger proton sponge effect that causes the influx of Cl− and swelling of the endosome. (D) Free highly positive charged molecules (shown with orange coloured cationic lipid and purple coloured PEI or oligo-arginine) can interact with anionic endosomal membrane and destabilize it by excluding water. (E): Intact lipoplex may escape from the ruptured endosome and de-assemble in the cytoplasm and release siRNA if the particle is not too large for the “holes” of the ruptured endosome. (F). Lipoplex may also de-assemble inside the endosome and directly release siRNA out of the ruptured endosome [79].

1.3.5. Lipid-based Nanocarriers

Lipid-based nanocarriers are the most frequently used transfection reagents for delivering siRNAs and miRNAs in vitro [84]. In recent years, some successful work has been achieved in modifying the composition and chemical structure of liposomes, to deliver siRNAs [85], [86]. Being nearly identical to siRNAs,

microRNAs have recently attracted the attention to employ the similar approaches of siRNAs such liposomal modification for microRNAs therapeutics [72].

Several lipid-based vectors have been used for miRNA delivery such as cationic lipoplexes, anionic lipopolyplexes, neutral lipid and Liposomes with targeted moiety (DOPE/linoleic acid/DMG-PEG. Even though cationic lipids are the most widely used for lipid-based system, their in vivo application is frequently limited by toxicity and nonspecific response [84]. Their toxicity is directly linked to the positive charge on the surface of the particles necessary for the binding of oligonucleotides. However, they have the advantages of easy chemical modification with the targeting moieties and the availability of fluorescent tracers [87], [82].

On the other hand, materials with an overall neutral or anionic surface charge are more biocompatible with the body compared to their cationic materials [82]. However, neutral or anionic carriers typically contain cationic materials in their core for binding and condensing nucleic acid drugs [82].

Polyethylenimine

Polyethyleneimines (PEIs) are a class of cationic synthetic polymers rich in amine groups with linear or branched structures. The protonatable amino groups on PEIs yield a high positive charge density even at neutral pH, rendering strong electrostatic interactions with the negatively charged nucleic acids (e.g. microRNA), thus forming stable complexes [88].

On the other hand, PEIs have some limitations. A major limitation to the clinical application of PEIs is their severe cytotoxicity, primarily caused by the high positive charge density and poor biodegradation [89]. Another challenge is their dramatically decreased efficiency when exposed to serum. This results from the nonspecific binding of PEIs with serum proteins and their consequent aggregation.

To address these problems, researchers have focused on improving biocompatibility by using low molecular weight PEI (LMW-PEI) that showed reduced toxicity compared with high molecular weight PEI (HMW-PEI).

After comparing different PEIs, researchers found that HMW-PEI caused a higher degree of membrane damage and greater cytotoxicity [90]. This may be due to

differences in charge density and degradability. However, the smaller degree of branching in LMW-PEI is could be one possible reason for its low cytotoxicity compared with branched HMW-PEI [91].

In order to guide the macromolecule to arrive to the cytoplasm, specific lipids, strategically selected with positive charges to formulate the lipid-based vectors to enhance endocytosis **(Fig.3)**. This is achieved by their interaction with the negatively charged cell membrane [82]. As an advantage, PEIs are characterized by a unique property termed as the “proton sponge effect”. Once endocytosis occurs, the protonated PEI will buffer or resist the acidification of the endosomal microenvironment, subsequently facilitating the release of polyplexes into the cytosol [92].

Moreover, this property of PEI can be employed in case of the neutral or anionic carriers because they typically contain cationic materials in their core as we mentioned above. For example, the positively charged LMW-PEI is often selected as a core material to condense the negatively charged miR molecules to form complexes through electrostatic interactions. The miR-PEI core is then coated with the neutral or anionic carrier to improve biocompatibility. This strategy performed to deliver miR-29b in a mouse model of acute myeloblastic leukemia (AML), by conjugation to transferrin protein. The treatment with this type of delivery, efficiently decreased the leukemic activity in mouse model [72]. Another example is the use neutral lipids to deliver let-7 and miR-34a in lung cancer mouse model, which preferentially accumulated in the lung rather than in other organ and was capable to decrease the disease burden [93], [94].

The delivery of nucleic acids by PEIs has been demonstrated in a wide variety of cells as well as a number of animal models. In this study, we employed this characteristic of PEI to develop our nanoparticles to enable endocytosis in vivo mouse mode.

1.3.6. Targeted Delivery in microRNAs

In solid tumors, microRNAs can be administered intratumorally to increase local drug concentration and avoid non-specific biodistribution and other side effects. Despite remain challenging; systematic administration is the way for microRNAs administration in haematological malignancies as leukemias [95]. However, a

number of passive and active targeted delivery systems have been developed to transport miRNAs to target internal tumor cells [82]. Passive targeted delivery is utilized in solid tumors.

1.3.7. Active Targeted Delivery

MiRNAs can target many different mRNAs. As a result, unwanted and potentially toxic off-target effects often occur. When administered systemically, it is important to specifically deliver miRNAs into the desired tissues or cells, thus reducing unwanted exposure to normal organs. Such targeting can be achieved by modifying the miRNA vectors with different moieties such as small molecules as peptides, antibodies or RNA ligands that direct the miRNA to receptors specifically over-expressed in tumors. Active targeting will thus lower the miRNA dosage required for effective treatment. Herein, we will focus on using antibodies as a mean of active targeting, to deliver microRNAs in leukemia that ensure targeting the malignant cells specifically and minimize non-specific utilization of microRNAs molecules.

1.3.8. Antibodies

Antibodies recognize their related cell surface receptors with high affinity and specificity. Antibody-based strategies for binding to specific receptors on tumor cells have been widely applied in the design of targeted delivery systems [82]. As an example, nanoparticles encapsulated with antibodies developed as a carrier of miRNA in lung metastasis in a murine model [96]. However, now no previous is published work regarding the employment of antibodies for active targeting leukemic cells. In this study, we are using liposomes conjugated to CD38 antibodies for active targeting of CLL cells in mouse model.

Despite challenges, successful achievements are there. Among the candidate miRNA drugs that have entered clinical trials is LNA-modified-anti-miR-122, which has already completed the Phase II clinical stage for the treatment of HCV in liver transplants [97], [98]. Another successful case, the liposomal miR-34 mimic, MRX34, for primary liver cancer or other solid cancers with liver involvement, has also entered a multicenter, open-label Phase 1 clinical trial [99], [100].

OBJECTIVES

Over the last few years, microRNAs emerged as potential anticancer agents and there are examples that reached clinical stage. Importantly, a single microRNA can target multiple targets involving different pathways in different cancer types. However, efficient delivery of microRNA molecules into target cells is hindered by some limitations such as low cellular uptake, renal clearance, degradation by serum nucleases, elimination by Reticuloendothelial system (RES), and unwanted side effects. These obstacles may hinder investment in microRNAs therapeutic by pharmaceutical companies. The field is still immature and require further scientific efforts to reach the clinics.

On the other hand, microRNA therapy in CLL is so promising, as recently shown by the first potential therapeutic agent in CLL, miR-181b, identified by Bresin *et al* in a CLL mouse model.

Non-viral systems such as liposomes are preferred approach because of their low immunogenicity and high biocompatibility. To our knowledge, no previous work has been published on microRNA delivery by immune-liposomes in CLL.

One of the main objectives of this study is to synthesize a novel carrier for microRNAs delivery in CLL transplanted mouse model. We used a non-viral antibody-based strategy by employing anionic lipoplex liposomes. Taking the advantage of CD38, being a marker expressed on mouse CLL cells, we introduced the idea to conjugate CD38 (mAbs) to anionic liposomal carrier to achieve an active specific targeting for CLL tumor cells, particularly recapitulated the diseased lymphoid organs. Thereafter, we employed these novel CD38 (mAb) conjugated carriers to identify new unexplored therapeutic candidates in CLL.

Specific objectives are:

1- To develop a novel system for efficient microRNA delivery in CLL cells.

2- To identify microRNAs as new potential therapeutic agent(s) against CLL cells.

2. MATERIALS AND METHODS

2.1. Cell Cultures and Transfections

The human TCL1-expressing B-cell line EHEB was maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum. Mouse splenocytes were freshly isolated from diseased mice, using the following procedure: spleens were explanted from euthanized mice and placed in cold phosphate-buffered saline (PBS) solution. Cell suspension was obtained by squeezing spleen between two slides and washing with PBS. Erythrocytes were degraded by using ammonium chloride (0.8%) with EDTA (0.1 mM) (Sigma). White cells were washed, passed through a 70-µm filter and counted. Leukemic splenocytes were cultured in 24 multi-well plates, using RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES buffer and 1 mM Na pyruvate. Transient transfections were performed with 100 nM pre-miR (Ambion) or single-stranded anti-miR (IDT) or scrambled negative controls, diluted in Opti-MEM serum-free medium (Gibco) and complexed with lipofectamine 2000 transfection reagent (Invitrogen), following the manufacturer's instructions. Cells were harvested 72h after transfection to evaluate apoptosis and miR expression.

2.2. Preparation of Nanoparticles

The synthetic mimic *miR-26a* and *miR-181b* for *in vivo* treatment were purchased from Axolab, while BLOCK-iT^{TM} fluorescent Control, used in vitro experiment, was purchased from Invitrogen (Cat. 2013). The lipid components of nanoparticles were 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (MW~2000; DMG-PEG; Avanti Polar Lipids, Alabaster, AL) and linoleic acid (Sigma-Aldrich, St. Louis, MO). The molar ratio of DOPE/linoleic acid/DMG-PEG was 50/48/2. The preparation of empty nanoparticles was performed as previously described from Huang [72]

The preparation of miR-loaded CD38-conjugated nanoparticles (CD38-NP-miR) is schematically divided in 4 steps:

Step 1: Negatively charged miR molecules were mixed with positively charged polyethylenimine (PEI) at room temperature to form a miR-PEI core structure

Step 2: Empty nanoparticles were formed by injection of a lipid ethanol solvent into 20 mmol/L HEPES buffer. The percentage of ethanol was less than 5%.

Step 3: The miR-PEI were mixed with the empty nanoparticles and sonicated to load the miR-PEI core into the nanoparticles. The mass ratio of lipid to miR was 10/1.

Step 4: NP-miR were modified to incorporate CD38-MAL-PEG-DSPE micelles to form the CD38-NP-miRs.

2.3. Analysis of Apoptosis

The apoptotic effects of mimic or anti-miR molecules on murine tumoral splenocytes treated *in vitro* or *in vivo* were assessed by Muse™ Annexin V and Dead Cell Assay kit (Millipore, cat. MCH100105) according to company's protocols. The Muse™ Annexin V & Dead Cell Assay allows for the quantitative analysis of live, early and late apoptosis, and cell death on both adherent and suspension cell lines on the Muse™ Cell Analyzer. This assay utilizes Annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind them. In this assay a dead cell marker (7-AAD) is also used as an indicator of cell membrane structural integrity. It is excluded from live, healthy cells, as well as early apoptotic cells.

To count viable cells we employed Muse Count & Viability Assay Kit (Millipore, cat. MHC100102) according to company's protocols.

2.4. Mice and Syngeneic Transplantation

Eμ-TCL1 transgenic mice used for these experiments have been previously described, it have been generated by using a construct containing the coding sequence (CDS) of the human TCL1 cDNA under the control of B-cell-specific IgV_H promoter and Ig_H-Eμ enhancer (Eμ-TCL1) [45], [46].

Breeding pairs were provided to our group as a generous gift from C.M.Croce's lab. FVB wild type mice were obtained from Charles River Laboratories. Mice had ad libitum access to water and a pellet diet. The animal room was maintained at

23°C on a 12-h light/12-h dark cycle.

At age of 6 weeks, the WT FVB mice were transplanted with IP injection of $1*10^6$ lymphocytes isolated from spleen of adult TCL-1 transgenic mouse with established leukemia. The engraftment of leukemic cells and progression of disease was monitored over time by Flow Cytometry Analysis (FACS) or absolute quantification human TCL1 gene by digital-PCR in leukemic lymphocytes of peripheral blood. The leukemic lymphocytes from spleen or peripheral blood were detected by FACS analysis according the expression of B220 /CD5.

2.5. Treatment In vivo with mimic or anti-miR oligonucleotides

The FVB transplanted mice were enrolled for treatment when the disease reached between 10-20% of TCL1 positive cells in peripheral blood.

The treatments of mice with mimic or anti-miR oligonucleotides was performed using NP, CD38-NP or using a polyethylenimine (PEI) cationic polymer jetPEI™ according to the protocol of in vivo-jetPEI® DNA & siRNA Delivery Protocol for intraperitoneal injection (polyplus).

To evaluate the bio distribution of miR-181b in vivo by Nanoparticles or jetPEI™, the FVB mice (at least 3 mice each group) were sacrificed after 24 hours from single treatment.

For experiments short term, the FVB mice (at least 3 mice each group) were treated with 100µg of specific single strand mimic/anti-miR and sacrificed after 48 hours, while for long term experiments the mice (at least 6 mice each group) were treated with 100µg of specific single strand mimic miR-26a three times a week for 3 weeks.

The level of the disease was measured by FACS and ddPCR analysis, before and at the end of the long-term treatment of CD38-NP-miR-26a.

All manipulations with mice were conducted according to directive 2010/63/EU of the European Parliament and of the Council and approved by the local ethical committee for animal experimentation.

2.6. RNA and DNA extraction

Total RNA was isolated from EHEB, murine splenocytes or murine tissues using miRNeasy® Mini Kit (Cat. #217004, Qiagen), according to the manufacturer's instructions. DNA was isolated from murine peripheral blood collected in 0.5M EDTA using the QIAamp spin column procedure with QIAamp® DNA Blood Mini kit (Cat. #51304, Qiagen) according to the manufacturer's instructions.

2.7. Real-Time RT-PCR

In EHEB cells and splenocytes transfected *in vitro*, the expression of mature miRNAs was quantified by the Taqman MicroRNA Assays (Applied Biosystems, Grand Island, NY, USA).Reverse transcription reaction was done starting from 5ng of total RNA. Real-Time PCR was performed using the standard Taqman MicroRNA Assay protocol on the CFX Connect™ Real-Time PCR Detection System (Biorad). The reactions were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The ΔΔCt method for relative quantification of gene expression was used to determine miRNA expression levels. Fold change was generated using the equation $2^{-\Delta\Delta Ct}$. Each sample was analyzed in triplicate. To normalize the relative abundance of miRNA from human cells was used the Taqman Assays for U6 RNA (Applied Biosystems). To normalize the relative abundance of miRNA from murine tissues was used the Taqman Assays for SNO412 RNA (Applied Biosystems).

2.8. Reverse Transcription and ddPCR

The ddPCR was performed to measure the expression level of miR in different murine tissues and to quantify the human TCL-1 gene.

For TaqMan assays, miRNAs were reverse-transcripted using TaqMan miRNA Reverse Transcription kits (Life Technologies). Reverse transcription reaction was done starting from 5ng of total RNA. Then, 1.3 µL of the resulting cDNA was prepared for amplification in a 20-µL reaction volume containing 10 µL 2X ddPCR Supermix for Probes (Bio-Rad) and 1 µL 20X TaqMan miRNA PCR primer probe set. Each ddPCR assay mixture (20 µL) was loaded into a disposable droplet generator cartridge (Bio-Rad). Then, 70 µL of droplet generation oil for probes (Bio-Rad) was loaded into each of the eight oil wells. The cartridge was then

placed inside the QX200 droplet generator (Bio-Rad). When droplet generation was completed, the droplets were transferred to a 96-well PCR plate (Eppendorf) using a Rainin multichannel pipet. The plate was heat-sealed with foil and placed in a conventional thermal cycler. Thermal cycling conditions for TaqMan assays were as follows: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (ramping rate reduced to 2%), and a final inactivation step at 98°C for 10 minutes. A negative control for each reverse transcription reaction (RT-neg) were included in every assay.

To assess the progression of leukemia we used different technology FACS and ddPCR, the human TCL-1 gene was quantified in 10ng of genomic DNA extracted from peripheral blood cells of FVB transplanted mice by ddPCR, using 2X ddPCR Supermix and using specific probes / primers as for human TCL1 gene and mouse GAPDH gene (Table 4).

Table (4): probes /primers as for human TCL1 and mouse GAPDH genes.

primers/probe	Sequence
human TCL-1 gene	Forward 5'- GCTCTGGCTCTTGCTTCTTAGGC-3'
	Reverse 5'-CGCAAGAGCACCCGTAACTGTAAC-3'
murine GAPDH	Forward 5'-CCACCCAGAAGACTGTGGAT-3'
	Reverse 5'-CCCAGCTCTCCCCATACATA-3'

The primers/probe specific for murine GAPDH were used as endogenous reference. The probes for TCL-1 and GAPDH are conjugated with different dyes FAM and HEX, respectively. The generation of droplets and thermal cycling conditions was performed as previously described for miR expression.

At the end of the long term treatments with CD38-NP-miR-26a, the progression of CLL was established as delta of TCL1 percent value; this was calculated for each mouse as the difference between percent of TCL-1 before and percent of TCL-1 after treatment.

2.9. Flow Cytometry

Disease progression was monitored by FACS analysis. Flow cytometry was performed on FACSCalibur (BD Bioscience), using CellQuestPro software (BD

Bioscience). Immunophenotyping of PBMCs was performed as follows: blood samples were collected from the retro-orbital plexus of anesthetized mice and placed in a tube containing 0.5 M EDTA as an anticoagulant. Erythrocytes were broken by treatment with ammonium chloride (0.8%) and EDTA (0.1 mM) (Sigma). PBMCs were incubated with FITC-conjugated anti-mouse CD5 and PeCY5-conjugated anti-mouse B220 surface markers (BD Bioscience) for 15 min at room temperature, washed and analyzed. Lymphocytes were recognized by physical parameters and gated on a B220/CD5 plot. Leukemic cells were identified as B220+/CD5dim cells, normal B lymphocytes as B220+/CD5- cells and T lymphocytes as B220-/CD5+ cells. LE was evaluated as the ratio of leukemic cells relative to total lymphocytes LE = B220+/CD5dim cells / (B220+/CD5dim cells + B220+/CD5- + B220-/CD5+).

At the end of the long term treatments with CD38-NP-miR-26a, LE was measured and the delta value was calculated for each mouse as the difference between LE before and after treatment.

2.10. Western Blot Analysis

After 48 hours from last treatment, splenocytes of treated and control mice were isolated to extraction of protein by RIPA buffer (Radio-Immunoprecipitation Assay) (Sigma-Aldrich), supplemented with Protease inhibitors (Sigma-Aldrich), according to Manufacturer's protocol. Protein concentrations were measured using Bradford assay (Bio-Rad), with BSA as the standard. Equal amounts of protein extracts from all samples were applied to SDS-PAGE and then transferred to PVDF membrane (Bio-Rad). The membrane was blocked in Tris Buffered Saline with 0.1% Tween-20 (TBS-T) buffer containing 5% non-fat milk and 0.1% Tween-20 for 1 hour at room temperature followed by incubation with CDK6 (Rabbit, Cat. #sc177, Santa Cruz), diluted 1:200, at 4°C overnight. β-Tubulin H235 (Rabbit, Cat. #sc9104, Santa Cruz), diluted 1:1000, was used as the loading control. After being washed, the membrane was incubated with anti-Rabbit second antibody (Cat. #7074, Cell Signalling) diluted 1:20000 and washed again.

For signal detection Clarity^{TM} Western ECL Substrate (Bio-Rad, Cat.170-5060) was used according to the manufacturer's instructions. Signals were quantified by the ImageJ software and protein expression levels were normalized according to β

-tubulin expression.

2.11. Statistical analysis

Data reported as histograms are expressed as mean ± SD; the P-values, calculated by 2-tailed Student t test, were considered to be statistically significant when less than 0.05. Graphpad Prism 6.0 software was used for statistical data analysis.

3. RESULTS

We employed liposomes to develop novel nanoparticles for efficiently and specifically delivering microRNAs in vivo into CLL cells, using the TCL1-tg CLL mouse model. We will also explain how these particles were validated and how this targeted delivery of microRNAs facilitated their testing and validation as therapeutic agents.

Owing to their instability in bio fluids, their limited cellular uptake, synthetic miRNAs are relatively difficult to develop as miR based therapy [72]. However, recently, Bresin has demonstrated, in collaboration with our research group, that miR-181b could be considered as a potential therapeutic agent for CLL in Eμ-TCL1 mouse model. This was achieved by using the cationic polymer PEI (JET PEI) for in vivo miRNA delivery [49]. In the current study, we aimed at improving the way of delivery to be more specific and efficient in targeting CLL leukemic cells in the diseased organs, mainly spleen and liver. We were capable to develop novel efficient nanoparticles and validate them *in vivo* and *in vitro.* Furthermore, we implemented these particles in testing a selected group of microRNAs as therapeutic agents against CLL cells. MiR-26a was selected as a good candidate and studied for validation as a therapeutic agent against CLL.

In this part, we will focus on the following findings:-

- *Production and validation of novel nanoparticles for microRNA delivery against CLL cells.*
- *Screening of a selected group of miRNAs/Anti-miRNAs as therapeutic candidates in vitro.*
- *Screening of a selected group of miRNAs/Anti-miRNAs as therapeutic candidates in vivo.*
- *Testing of miR-26a as therapeutic agent in a CLL mouse model.*

3.1. Production and Validation of Novel Nanoparticles for microRNA Delivery in CLL

To provide a nonviral delivery for miRNA molecules, novel lypidic nanoparticles were developed. They were firstly prepared in a form of lipopolyplex nanocarrier

as mentioned in materials and methods and as described by Huang *et al* [72]. Because CLL cells in TCL-1 mouse model is not only CD5+, but also CD38+ **(Fig.4)**, we aimed to facilitate an efficient targeted miRNA delivery by coupling these particles with an anti-CD38 antibody (CD38-NP). Thereafter, miR/anti-miRs of interest was encapsulated into the CD38-NPs to be delivered.

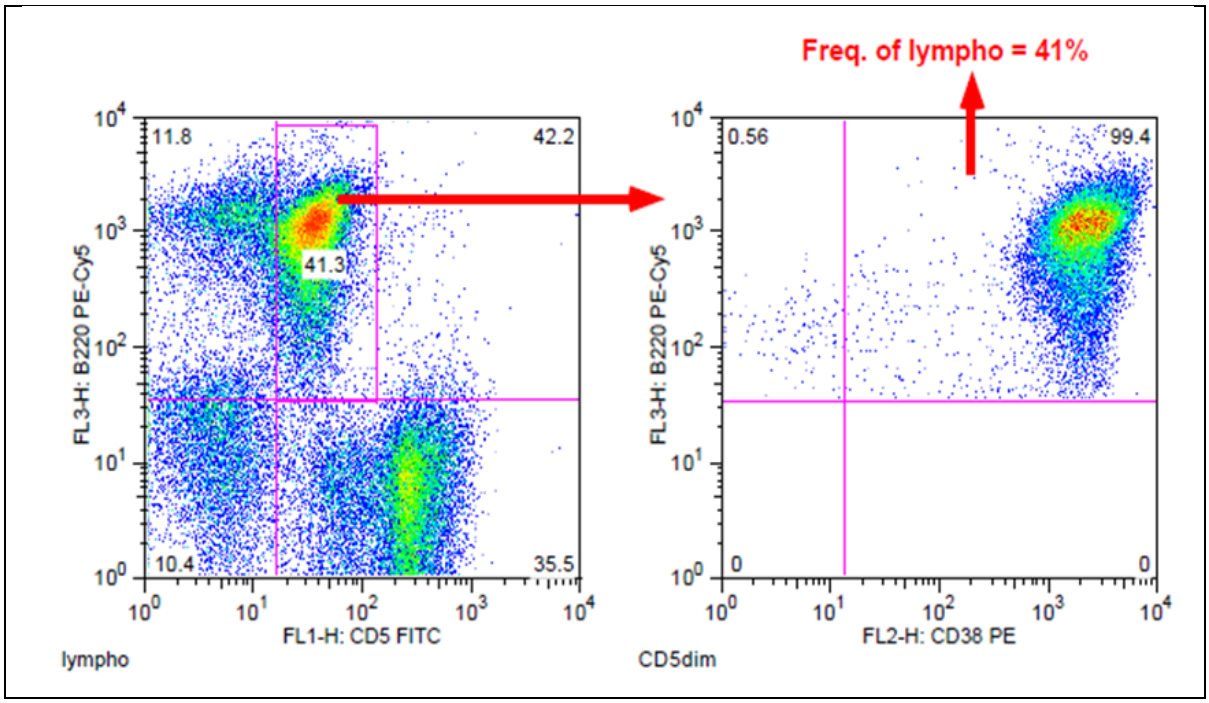

Figure (4): The leukemic population B220+ / CD5dim is CD38+:

The left panel shows the leukemic population in a diseased TCL1-tg mouse. In this mouse, the leukemic population represents the 41% of spleen lymphocytes. On the right panel, the lymphocytes within the gate B220+ / CD5dim was analyzed for CD38 expression: more than 99% of the cells are positive.

3.2. CD38 Nanoparticles were Successfully Taken up by human EHEB cells

To validate the developed nanoparticles in vitro, we assayed the uptake of a fluorescent oligonucleotide by the human EHEB cells. Taking advantage of its fluorescence, the oligonucleotide Block-iT ^{TM} (Ambion) was used to transfect EHEB cells with CD38-NPs to evaluate efficiency of delivery. At the same time, block-iT ^{TM} was also delivered by the use of lipofectamine (Invitrogen) or nanoparticles not coupled to anti-CD38 (NP-). Twenty-four hours later, the cells were examined by fluorescence microscopy. As shown in **Figure (5)**, fluorescence was detected in CD38-NP, NP- as well as in lipofectamine treated cells **(Fig. 5A).**

These results indicated that developed CD38 nanoparticles were able to deliver oligonucleotides into human EHEB cells similarly to other methods. We assessed the level of fluorescence by TECAN Infinite F200Pro plate reader. Results revealed that the fluorescence level in CD38-NP-block-iT was approximately 4-fold higher than in the untreated EHEB cells (P = 0.007) **(Fig. 5B).** Similarly, the unconjugated particles (NP-block-iT) increased by 4-fold compared with untreated cells. No significant difference detected between CD38-NP-Block-iT and NP-Block-iT (P = 0.7). Therefore, the level of fluorescence showed by CD38-NP could demonstrate a successful intracellular uptake of a fluorescent oligonucleotide into human EHEB cells.

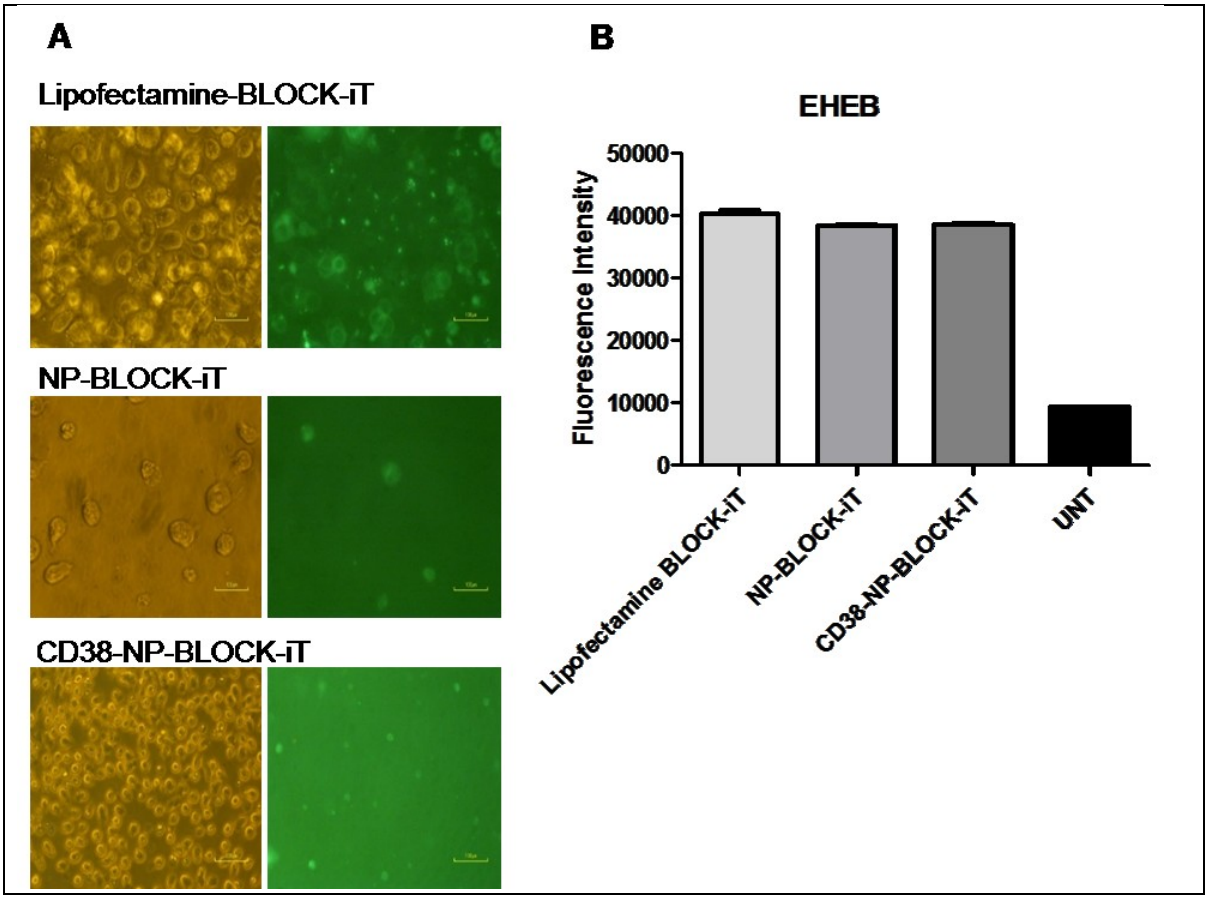

Figure (5): antiCD38 Nanoparticles could deliver Oligonucleotides into human EHEB cells:

(A): EHEB cells were examined by fluorescence microscopy after 24h from transfection with fluorescent BLOCK-iT oligonucleotide (at 100 nM final concentration) using various lipidic carriers: Lipofectamine (Invitrogen) and nanoparticles (NP or CD38-NP) prepared as described in Materials and Methods and in Results. Fluorescence and light microscopy (x10) images are shown. EHEB cells with fluorescence indicated that the BLOCK-iT oligonucleotide was delivered intracellularly. **(B):** Total fluorescence was assessed by TECAN Infinite F200Pro. Total fluorescence level was approximately 4-fold increase for CD38-NP-BLOCK-

iT and NP-BLOCK-iT compared to untreated cells (UNT) (P = 0.007). Lipofectamine BLOCK-iT exhibited about 4.2 fold higher fluorescence than untreated cells (P = 0.0002).

3.3. In vitro Validation for miRNAs Delivery: CD38-NP-miR-34a Increased the Level of miR-34a in Both of human EHEB cell line and Mouse splenocytes

After verification of intracellular uptake, we tested whether CD38-NP could deliver miRNAs into EHEB or mouse CLL cells. We selected miR-34a to assess the efficacy of cellular uptake of the miRNA molecules coupled to CD38-NP. Therefore, we transfected both EHEB cell lines and splenocytes collected from TCL1-tg mouse, with miR-34a. After 24h, cell pellets were collected for RNA extraction and the level of miR-34a expression quantified by qRT-PCR. Results revealed that CD38-NPs were efficiently capable to deliver miR-34a molecules not only into EHEB cells but also into mouse CLL splenocytes. In human EHEB cells, results revealed that the relative expression of miR-34a by CD38-NP was significantly (P = 0.0008) greater than the untreated EHEB **cells (Fig. 6A).** Regarding, mouse splenocytes, results were in concordance with those shown in human EHEB cells. The relative expression of miR-34a delivered by CD38-NP into untreated splenocytes was significantly increased (P = 0.001) compared to untreated splenocytes **(Fig. 6B).** Overall, the developed CD38-NP could successfully increase the level of intracellular mature miR-34a in both of human EHEB and mouse splenocytes.

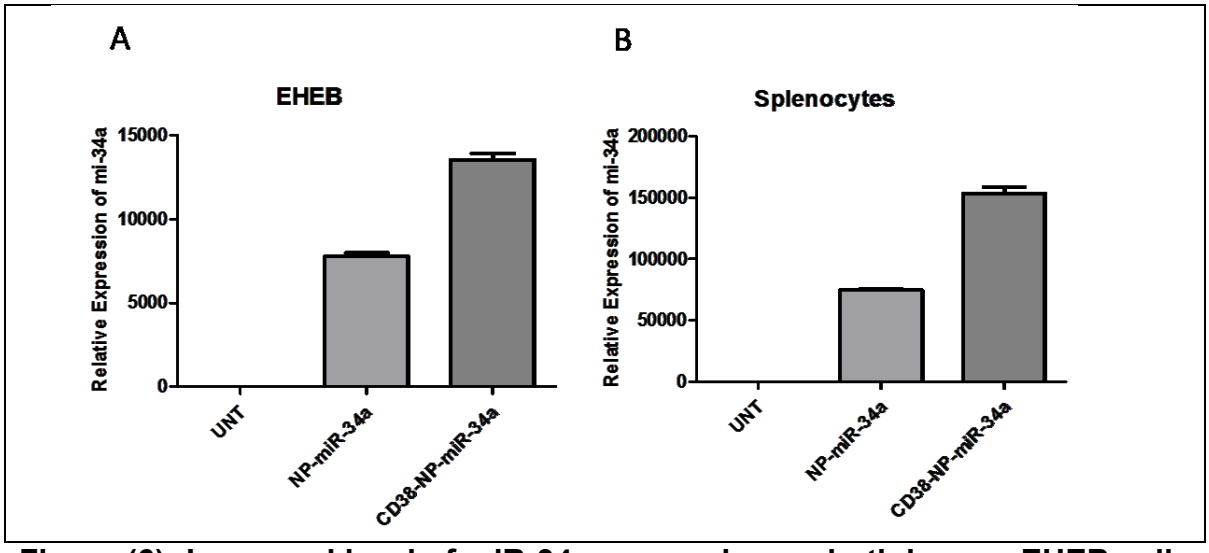

Figure (6): Increased level of miR-34a expression on both human EHEB cells

and CLL mouse splenocytes, using CD38-NP in vitro:

Relative expression of miR-34a versus untreated cells (UNT) at 24 hours from transfection of miR-34a encapsulated into nanoparticles (NP or CD38-NP). **(A):** expression of miR-34a in EHEB cells relative to untreated EHEB cells. MiR-34a in CD38 conjugated nanoparticles (CD38-NP-miR-34a) increased by comparison with untreated EHEB cells (P = 0.0008). **(B):** expression of miR-34a versus untreated splenocytes. CD38-NP-miR-34a increased miR-34a expression by comparison with untreated splenocytes (P = 0.001).

3.4. Spleen and Liver were the Main Organs Targeted by miR-181b Encapsulated CD38-NP in CLL Transplanted Mouse Model

After CD38 nanoparticles were validated *in vitro* for microRNAs intracellular uptake in both human and mouse TCL1 tg cells, we tested them *in vivo* by employing CLL transplanted mice, to examine the biological distribution of CD38-NP and their ability to deliver microRNAs molecules into mice organs. We selected miR-181b to perform the analysis, as it has been previously investigated as a potential therapeutic miRNA in the same CLL mouse model [49] .

To test the capability of the produced CD38-NP to deliver miR-181b into mice organs, we injected (IP) a group of four CLL transplanted mice with 100 µg of miR-181b using either CD38-NP, empty-NP or Jet PEI (the formulation employed in the previous study) (**Fig.7**). After 24 hour, mice were sacrificed and their organs isolated. Total RNA was extracted from liver, spleen, heart, kidneys, thyroid, lungs and brain to estimate the level of miR-181b and assess delivery efficiency and bio-distribution, using digital PCR.

The results revealed that liver and spleen were the most efficiently targeted organs by CD38-NP. As mice treated with CD38-NP, demonstrated the highest relative expression of miR-181b, approximately 3.0- and 1.5-fold increase compared with the uncoupled particles in spleen and liver (**Fig. 7A-B**), respectively. Interestingly, when comparing the level of miR-181b to Jet PEI delivery, CD38-NP presented about 70- and 16-fold increase in spleen and liver, respectively. Therefore, CD38-NPs were capable to efficiently deliver miR-181b into CLL cells, particularly in the spleen of CLL transplanted mice. All other organs exhibited an increased level of miR-181b, generally at lower levels, in particular when delivered by NPs uncoupled to anti-CD38 antibody, as shown by kidney, heart, thyroid gland and

lungs (**Fig. 7B**). This confirms that the developed novel CD38-NPs can be used for a specific and efficient *in vivo* targeting of CD38+ CLL cells.

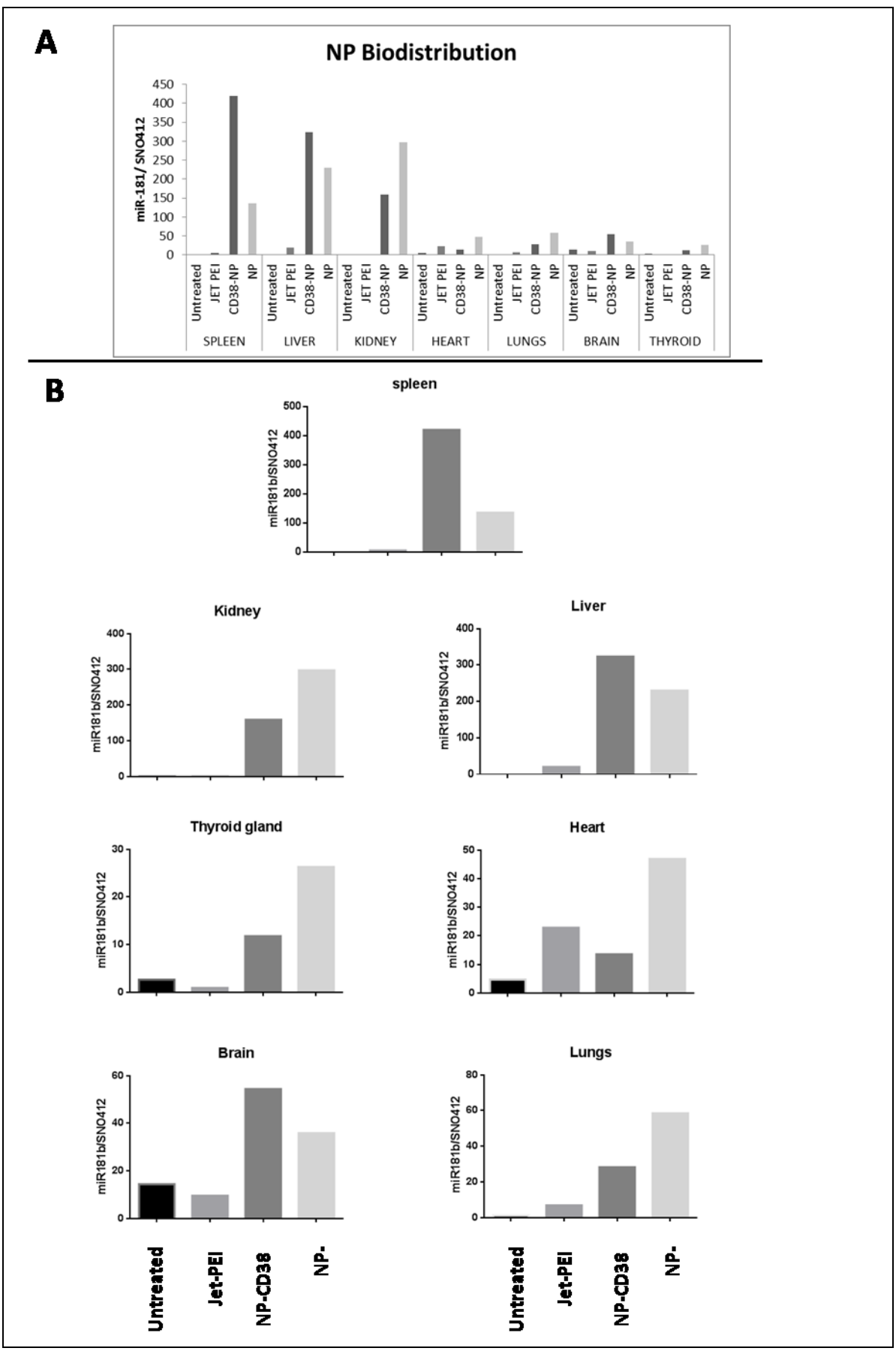

Figure (7): CD38 nanoparticles improve in vivo miR-181b delivery to spleen

cells of TCL1 transgenic mice:

MiR-181b mimics were encapsulated into lipidic CD38-NPs or NPs nanoparticles; as control, the cationic polymer jet PEI was employed for in vivo delivery. Following (IP) injection of 100 ug of miR-181b mimics, mice were sacrificed at 24h from treatment and seven organs were isolated from the treated mice and untreated control. The values described here show the relative expression of miR-181b relative to SNO-412 normalizer. **(A):** The complete panel of seven tissues shows the relative levels of miR-181b in the different tissues. A highly significant increase in the level of miR-181b was achieved by CD38-NPs in the spleen. In the absence of antiCD38, kidney and liver achieved the highest level of miR-181b. **(B):** A detailed effect on the level of miR-181b achieved in the different tissues following injection of NPs. It can be seen that in most of the cases NPs alone or combined with antiCD38 produced a better delivery than Jet-PEI and that, in general, NPs alone were more effective than antiCD38-NPs in delivering miR-181b mimics, with few exceptions the most notable being spleen.

3.5. In vitro Screening of a Selected group of miRNAs/AntimiRNAs as Therapeutic Molecules

After delivery efficacy of CD38-NPs was verified in vivo and in vitro, we turned to test a group of selected miRNAs/anti-miRNAs for anti-leukemic effects against splenocytes of CLL from TCL1-Tg mice. Herein, we transfected miR-15, miR-16, miR-26a, miR-125, miR-130a, miR-34a, miR-181b, antimiR-21, antimiR-155, antimiR-130a, scramble sequence and untreated control. Seventy two hours after transfection, the apoptotic activity was assessed by Muse Cell analyser using the “Annexin v and dead cell” assay. Data analyzed, by comparing the level of total apoptotic cells demonstrated by miRNAs and anti-miRNAs versus untreated cells and scramble oligo, respectively **(Fig. 8A).** The most significant apoptotic activity was present in splenocytes treated with miR-26a, miR-125a, anti-miR-155, miR-16a and miR-130a, as the difference in total apoptosis versus controls was significantly higher **(Fig. 8A)**. In all of the samples, the transfection was efficient and achieved the up- or the down-regulation of the specific miRNA, depending on treatment with mimics or anti-miRNA oligonucleotides **(Fig. 8B).**

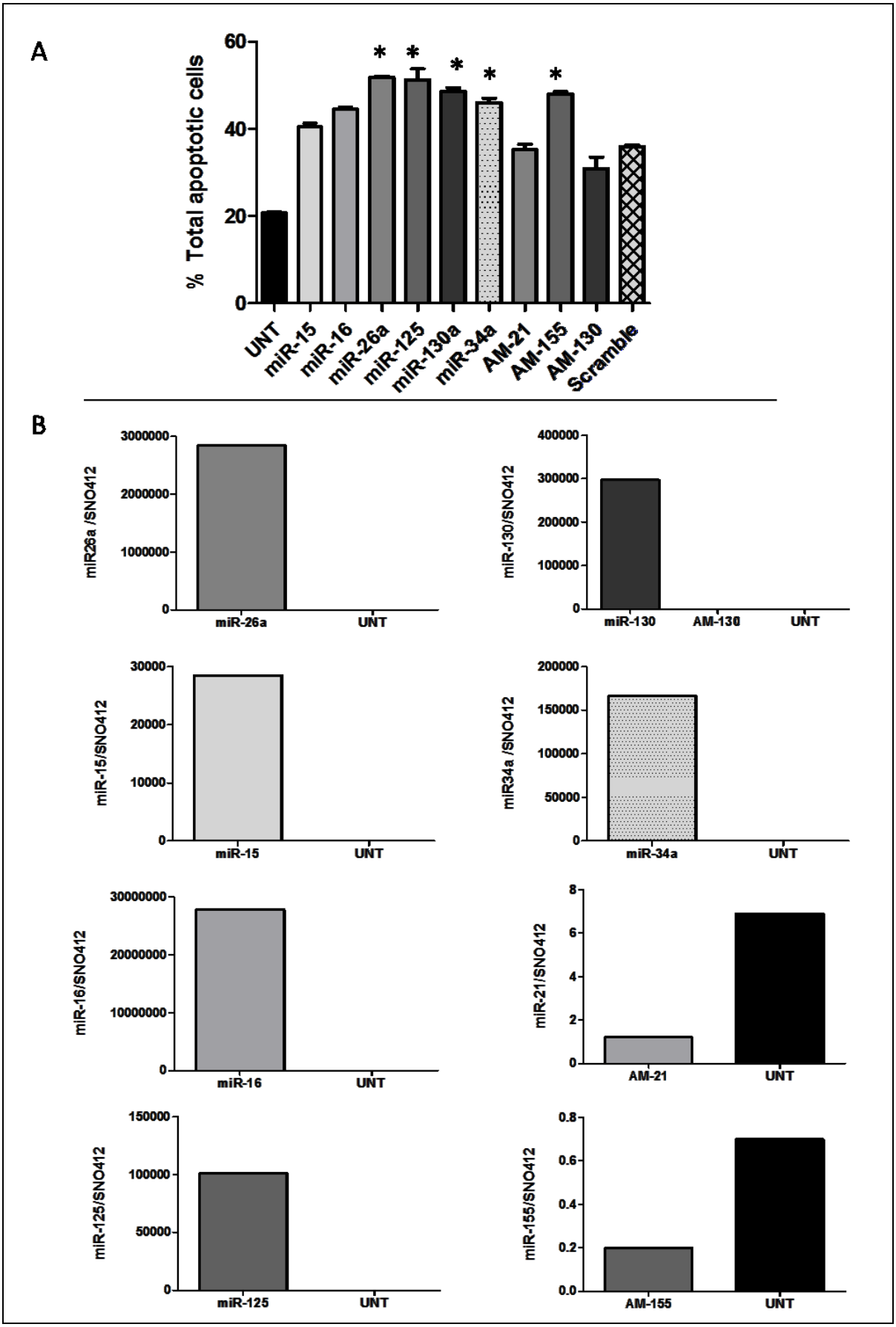

Figure (8): In vitro apoptotic activity induced by some of the selected miRs /

Anti-miRs in CLL leukemic splenocytes.

(A): Total apoptotic activity assessed by Muse Annexin v and dead cell assay analysed by Muse® Cell analyser. Data indicated that the most significant apoptotic activity presented by splenocytes treated with miR-26a, anti-miR-155, miR-16a and miR-130 shown as total apoptotic cells versus scramble control (* *P*: 0.01 < P < 0.05). **(B):** Relative expression of the different miRNAs, normalized to SNO-412, in splenocytes after 72 hours from transfection of selected miRs/anti-miRs.

3.6. Screening of a Selected Group of miRNAs/Anti-miRNAs as Therapeutic Candidates in vivo

To support the previous in vitro findings, that showed an anti-apoptotic effect presented by some miRNA molecules, we further retested some the first selected group in vivo, in CLL transplanted mouse model. For all of the in vivo experiments, we used the newly synthesized CD38-NP to deliver microRNAs into CLL transplanted mice.

We selected miR-26a-5p, miR-16a miR-34a, miR-181b, miR-130a and, anti-miR-21, anti-miR-155 for in vivo testing. A group of transplanted mice were randomized into seven groups. In each group, three mice were treated with miR/antimiR. Using CD38-NP, we treated mice, by intraperitoneal injection of 100ug of miR/anti-miR. After 48h, mice were sacrificed, spleen cells were isolated and tests performed. “Dead cell and Annexin V” assays were performed on collected splenocytes to evaluate the apoptotic activity, whereas the level of miRNAs expression was evaluated by ddPCR. Small nuclear RNA (SNO-412) used as reference *for* ddPCR. Notably, the data analysed from the in vivo 48h short-term experiments revealed variable biological effects on apoptotic activity. Results of miR-26a, anti-miR-155 and miR-130a demonstrated significant pro-apoptotic effects and confirmed the previous in vitro data **(Fig. 9A-B)**.

3.7. MiR-26a-5p as a Potential Therapeutic Agent in CLL

We selected miR-26a-5p for further testing as anti-leukemic therapeutic agent. This is because miR-26 was among the molecules that exhibited a significant pro-apoptotic activity against CLL cells *in vitro* as well as *in vivo*.

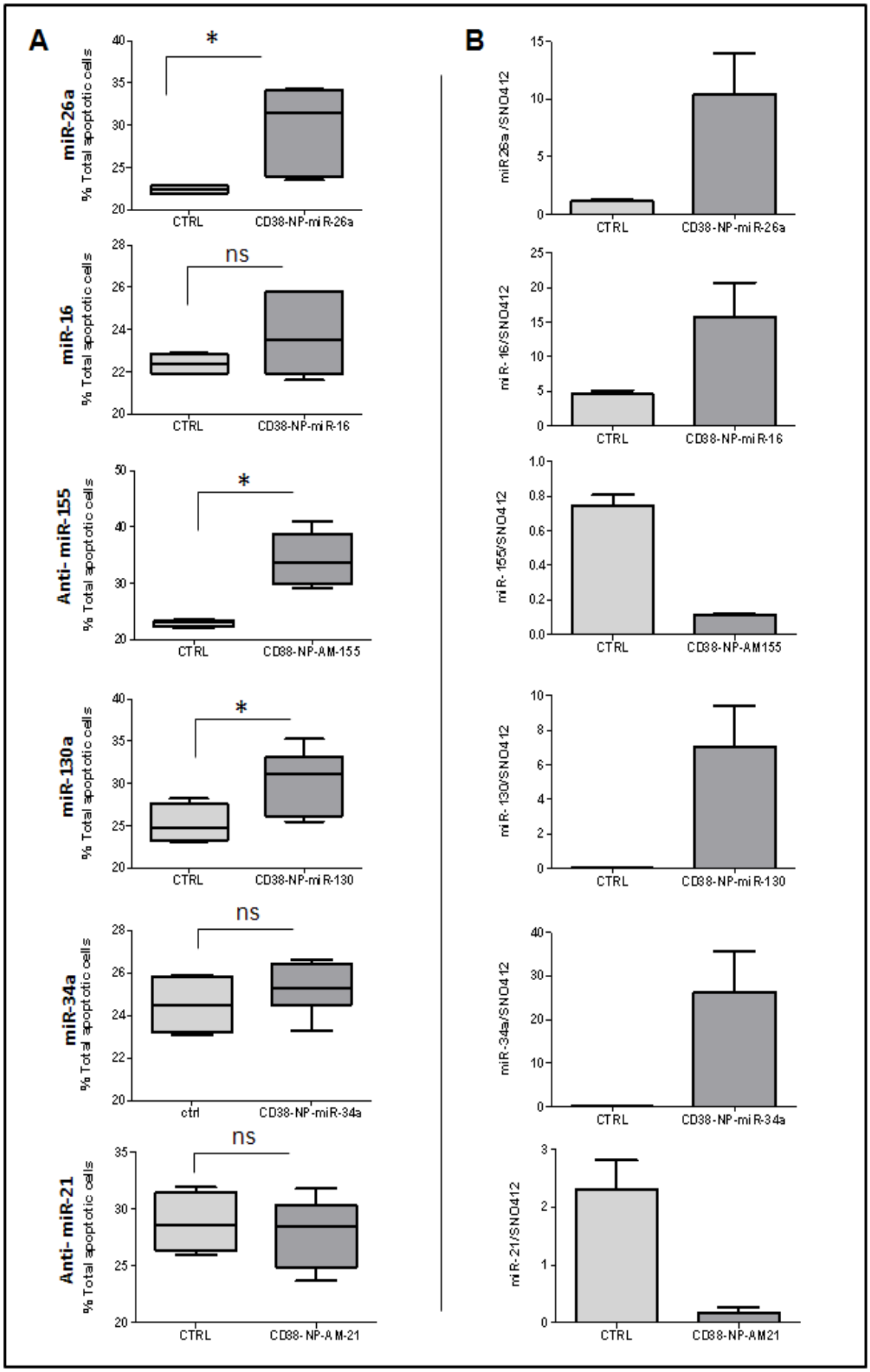
A
B
miR-26a
% Total apoptotic cells
*
CTRL
CD38-NP-miR-26a
miR-16
ns
CD38-NP-miR-16
Anti- miR-155
CD38-NP-AM-155
miR-130a
CD38-NP-miR-130
miR-34a
ctrl
CD38-NP-miR-34a
Anti- miR-21
CD38- NP-AM-21
miR26a /SNO412
miR-16/SNO412
miR-155/SNO412
CD38-NP-AM155
miR-130/SNO412
miR-34a/SNO412
miR-21/SNO412
CD38-NP-AM21

Figure (9): Biological activity of miRNA/anti-miRNA molecules after short in vivo treatment in mice with established leukemia.

Mice with established leukemia (more than 60% leukemic cells in peripheral blood) were sacrificed after 48 hours after a single treatment with miRNA/anti-miRNA molecule and splenocytes were isolated to evaluate apoptosis by Muse apoptosis assay. **(A):** A significant increase of percent of apoptotic cells was observed after treatment with miR-26a (P = 0.01), miR-130a (P = 0.03) mimics oligonucleotides and anti-miR-155 (P = 0.002) oligonucleotides. **(B):** Digital-PCR, expression levels of miR-26a, miR-34a, miR-130, miR-16, miR-21 and miR-155 in splenocytes 48 hours after treatment measured by quantitative RT-ddPCR.

3.8. A Delay in Leukemic Expansion (LE) and Reduction of hTCL1 Following Long term miR-26a-5p Administration in CLL Transplanted mice

The TCL1-tg mouse transgenic model is highly predisposed to development of a leukaemia similar to human CLL immunophenotypically and in disease behaviour. However, because of intrinsic disease heterogeneity and the long time required to develop leukemia, which ranged from 10-12 months, we decided to employ CLL transplanted mouse model instead. On the other hand, CLL transplanted mice have the properties of internal homogeneity, short time (6-8 weeks) to develop leukaemia. Moreover, once cells of TCL1 transgenic mice are transplanted into syngeneic mice, the leukemia engrafts and can grow in a manner similar to the transgenic itself [101], [49]. Here, we used the transplantation approach to perform *in vivo* experiments in more uniform experimental conditions. Syngeneic transplants were performed in 8-weeks-old syngeneic FVB wild-type (wt) mice by intraperitoneal injection of splenocytes, collected from diseased TCL1-tg donors as described by Bresin *et al* [49]. Mice were enrolled for treatment when % TCL1 transgene values ranged from 0.05 to 0.2 (i.e. leukemic cells representing 5-20% of peripheral blood lymphocytes), which were reached at 8 weeks after transplantation. At this point, mice were enrolled in the study of *in vivo* treatment.

We selected a group of 11 CLL transplanted mice, according to the criteria described before. Thereafter, we randomized them into two groups, a group of six were treated with miR-26a-5p and the second group of five mice left untreated as controls. It was previously described by Bresin *et al* [49] that the treatment with a scrambled RNA oligonucleotide has no significant effect on leukemic cells

expansion. So, to reduce the number of animals enrolled in the study, we avoided this control.

We treated mice by injecting (IP) 100ug of miR-26a-5p coupled to CD38-NP, three alternative days a week, for a period of three consecutive weeks. To evaluate the effect of miR-26a-5p, for each mouse we analysed the leukemic expansion (LE) by two different approaches: (1) by analyzing the increase of B220+/CD5 (dim) cells by FACS analysis and (2) by monitoring the increase of the hTCL1 transgene (a marker of leukemic cells) by using a more quantitative ddPCR approach before and following treatment.

In one approach, peripheral blood samples collected and immunophenotypically analysed by Fluorescence-activated cell sorting (FACS). LE was calculated as the number of leukemic cells relative to total lymphocytes (T cells plus normal B cells). The leukaemic cells represented lymphocytes that were expressing both B220+ CD5 (dim), which corresponded to B cells of the CLL transplanted mice. For each mouse, we assessed the difference between LE measured before and after treatment (delta LE). The mean of delta LE was 12.1 after miR-26a-5p treatment, whereas it was 20.46 in the untreated mice (**Fig. 10A**). The differences in delta LE values between miR-26a-5p-treated and untreated mice was statistically significant (P = 0.05). In parallel to LE assessment, we checked the level of hTCL1 in the collected blood samples before and following treatment to monitor the TCL1 level after miR-26a-5p treatment. The level of the hTCL1 transgene assessed pre- and post-treatment, using ddPCR. The reduction in hTCL1 presented as delta TCL1, which was the difference in the % of hTCL1+ cells before and after treatment. Notably, while the two approaches provided a quantitatively similar results, the ddPCR method, which is quantitatively more precise, indicated a statistically more significant reduction in the delta hTCL1 in the treated versus the untreated mice group (P = 0.029, **Fig. 10B**). These data indicate that miR-26a-5p is efficient in reducing leukemic cell expansion. To confirm that the effect could be related to miR-26a increased levels, we assessed the levels of miR-26a as wells as of a known validated target by measuring the level of CDK6 protein at the time of treatment. **Figure 11** shows that indeed the level of miR-26a in the splenocytes of treated animal was significantly higher than controls and the level of CDK6

protein was significantly down modulated by the treatment, indicating that biological effect could be related to the molecular changes induced by miR-26a.

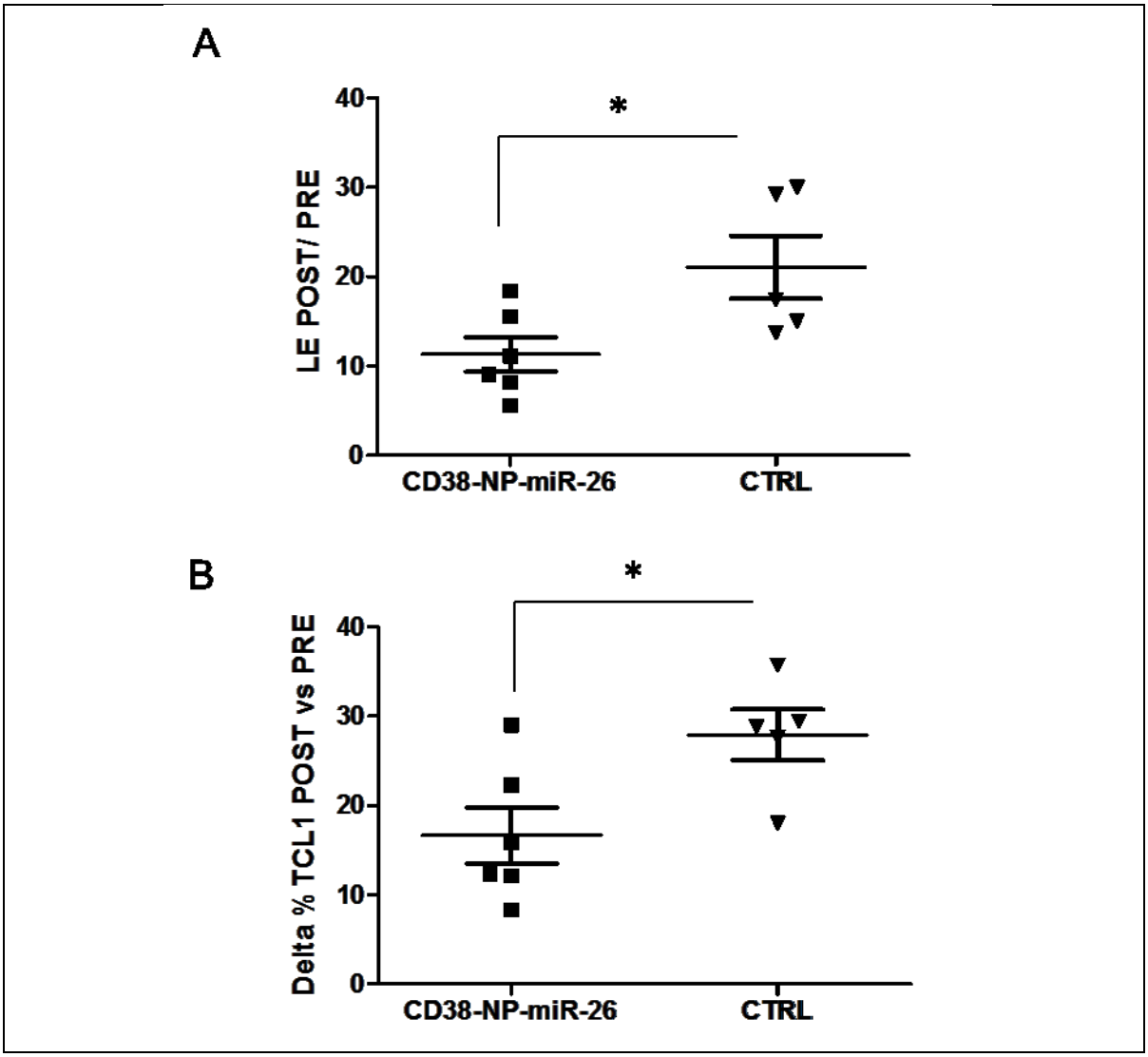

Figure (10): miR-26a-5p delayed leukemic expansion (LE) following long-term miR-26a administration in CLL transplanted mice:

MiR-26a mimics (100 ug) were delivered into transplanted mice using antiCD38-nanoparticles (CD38-NP-miR-26a). A schedule of three times a week for three consecutive weeks was applied. At the beginning and at the end of treatments, the level of circulating leukemic cells were measured by two methods: by FACS analysis, with B CLL leukemic cells gated and identified because they were B220+ and CD5(dim) **(A)**, or by ddPCR analysis **(B)**, based on the percentage of hTCL1+ cells. The scatter plots show the difference post-pre in leukemic cells. Both methods showed that compared to controls, the expansion of leukemic cells was significantly controlled by treatment with miR-26a. *: $P < 0.05$.

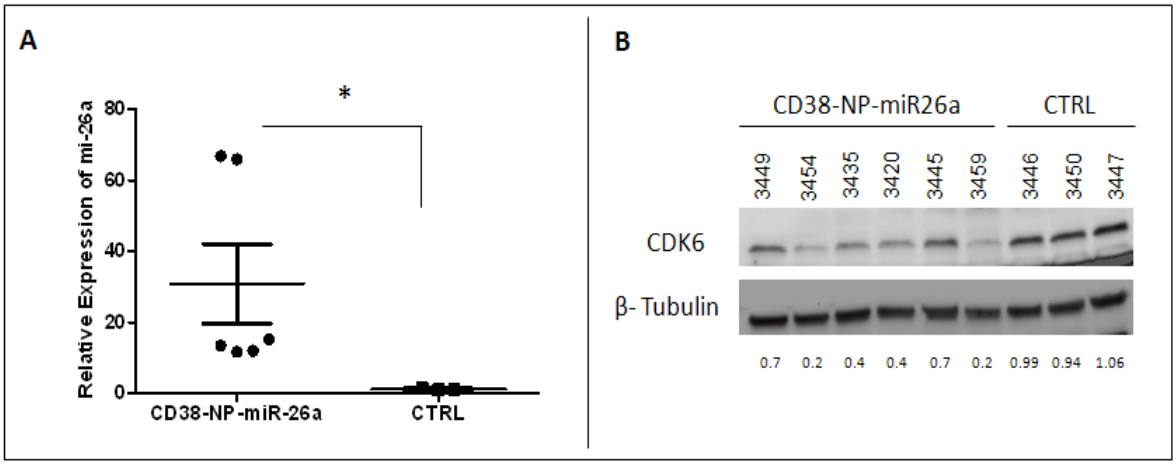

Figure (11): In vivo modulation of CDK6 protein after long-term miR-26a treatment:

Total RNA and proteins were extracted from splenocytes of treated and control mice. The levels of miR-26a and of the target CDK6 protein were evaluated by RT-ddPCR and Western blot analyses, respectively. **(A)** The level of miR-26a was significantly higher (**P* = 0.026) in treated versus control animals, while in (**B**) the target CDK6 protein was down-regulated (**P* = 0.001) in treated versus control animals. The relative level of CDK6 protein was quantified by scanning the visualized fragments with ImageJ software and normalizing the level of CDK6 on the housekeeping β -tubulin protein. The numbers above the WB panels identify the different mice; the numbers below are the relative expression of CDK6 in the different samples.

4. DISCUSSION

The involvement of miRNAs in pathogenesis, progression and drug resistance opened the possibility of designing anti-CLL therapeutic approaches based on miRNAs [102]. The development of smart targeting system such as liposomes that can deliver therapeutic molecule in sustained rate directly to cancer cells may provide better efficacy with less toxicity [103]. In an effort to improve the delivery of microRNAs, we develop a novel immune-liposomes formulation for microRNAs therapy in CLL. We took the advantage of the developed particles to test a group of selected miRs/Anti-miRs to identify new unexplored therapeutic agent(s) in vitro and in vivo CLL transplanted mouse mode. With regard to mouse model, we followed the same procedure by Bresin *et al* to establish leukemia with similar features to human CLL in syngeneic FVB mice to provide disease heterogeneity [49].

Currently, the main objective in gene therapy via a systemic pathway is the development of a stable and nontoxic nonviral vector that can encapsulate and deliver foreign genetic materials into specific cell types with the transfection efficiency of viral vectors [103]. However, there are successful viral delivery approaches such as adeno associated virus (AAV), still safety concerns limit their use in human [83]. Therefore, for targeting CLL cells, a systematic nonviral delivery approach is preferable.

In this study, we successfully developed an efficient specific immuno-liposomes formulation (CD38 mAb) coupled with liposomes) for targeting CLL cells. Since, in vivo delivery by cationic lipids, such as Polyethyleneimine (PEI) is more efficient compared to anionic liposomes, however their application is limited due to toxicity [84]. Herein, we took the PEI advantage to formulate a core of miRNA-PEI and coat it with anionic lipoplex carrier to improve body biocompatibility and avoid toxicity [72]. In addition, one of the components of our particles is PEGylated phospholipids is which is advisable for use, because they increase circulation time in plasma and prevent leakage of particles contents [103]. It is also important to mention that our immune-liposomes are warped with DSPE-PEG to produce NPs with long circulation time [104]. The structure of NPs increases the stability and efficiency and allows the NPs to deliver the encapsulated mimics into its target.

Previous studies utilizing neutral lipid with the miR-PEI core show a decrease in the tumor burden in a lung cancer mouse model, where the particles preferentially accumulated in the lung rather than in other organs, and significantly up-regulated the expression of let-7 and miR-34a [93], [94].

In comparison to results by the cationic polymer PEI, the developed NPs are more efficient than PEI in delivery of microRNA into all the seven collected organs; indeed the spleen represents the greatest efficiency. The recent study by Bresin *et al*, on miR-181b in the same transplanted mouse model, reported that their use of PEI was necessary to significantly increase the efficiency of the delivery to the spleen and that was otherwise negligible in its absence [105]. Thus, the current NPs improve the efficiency of mimic's delivery and therefore give a good opportunity to study microRNA therapeutic in this mouse model and to identify new microRNA agent.

Moreover, synthesized NPs are designed to be more specific because they formulate to particularly target +CD38 leukemic cells as they are coupled with anti-CD38 monoclonal antibodies. To our knowledge, this is the first report using this strategy in CLL. This conjugation improves the bio distribution of the designed particles (CD38-NPs) and this is why they are mainly accumulated and delivered microRNA to spleen more than the other mice organs. They also accumulate on liver and kidney, however with lower level compared to spleen. Other organs presented a negligible miRNA delivery. Our results are in concordance with similar anionic particles, but conjugated to transferrin protein, which successfully delivered miR-29b to minimize disease burden in mouse model of acute myeloid leukemia [72].

Importantly, the developed CD38-NPs are efficient enough to deliver microRNAs into spleen cells, and facilitate microRNA to demonstrate their therapeutic function on tumor cells. This is confirmed by the increase in apoptotic activity associated with up-down regulation of mature miRs according to mimics treatment or antimiR silencing on the treated splenocytes. A previous study using antibodies conjugated nanoparticles to deliver miR-34a in mouse model with neuroblastoma tumor, reported that the systemic delivery of their particles has no adverse effects on kidney or liver tissue. In addition, the tumor-specific delivery of miR-34a successfully increased apoptosis and inhibited the progression of the tumor [106].

With regard to the new therapeutic candidates, the developed particles facilitate suggesting a group of therapeutic candidates that require further validation for long treatment in vivo. However, the in vitro results are in agreement with the short duration treatment in vivo. In the current study, we proceeded for further validation of miR-26a in vivo for three weeks of treatment. The use of CD38-NP was necessary to significantly increase the efficiency of the delivery of miRNA mimics to the spleen and helps to identify and validate miR-26a as a novel potential therapeutic agent in CLL.

The results of miR-26a in vitro and in vivo for short treatment revealed that miR-26a has significant apoptotic activity against CLL cells among tested miR/anti-miRs. This suggests its role in down modulation of some targets related to apoptosis or cell proliferation. In agreement with previous results, miR-26a is strongly associated with progression-free survival (PFS) after analysis of clinical data of CLL patients in relation to miRNA expression [107]. In addition to, our results obtained after three weeks treatment, assessing leukemic expansion, revealed a role of miR-26a in delay of the expansion of CLL leukemic cells. This is confirmed by the significant reduction in number of B CLL cells. These results demonstrate the regression of leukemic activity by miR-26a that was clearly detectable after 3 weeks of treatment. Importantly, western blot results revealed that miR-26a down-modulate CDK6 protein in mice after treatment. The role of miR-26a in apoptosis and reduction in leukemic expansion suggested to be caused by the reduction of the CDK6 protein, which requires gathering with its partner Cyclin D in involvement of G1 phase of cell cycle.

The miR-26 family is composed of miR-26a-1, miR-26a-2 and miR-26b located in chromosomes 3, 12 and 2, respectively [108]. MiR-26 is emerging as critical regulators in oncogenesis and tumor progression by acting as either oncogenes or tumor suppressor genes in various cancers. As a tumor suppressor, the down-regulation of miR-26 family members has implicated in the pathogenesis of multiple malignancies. In some settings like glioma, however, miR-26-mediated repression of PTEN promotes tumorigenesis [109]. Oddly, a work by Zou *et al*, on CLL patients has shown that miR-26a down regulated the expression of PTEN, but not PTEN mutation or promoter methylation [110].

Moreover, miR-26 is one of the HRMs (hypoxia-regulated microRNAs). In hypoxia environment, a proposed mechanism of mir-26 is the direct anti-apoptotic effect by targeting BAK1 (pro-apoptotic protein) [111]. In addition, proliferation, with many cell types undergoing cell cycle arrest during oxygen deprivation process known to be affected by hypoxia. MiR-26a contributes in this process via other predicted targets such as cyclin D2 and cyclin E1 and cdk6 [111].

On the other hand, miR-26a suggested to play a role in phosphorylation and to restore wild-type activity to mutant p53. For example, in pancreatic cancer, miR-26 significantly decreased cell proliferation and inhibited cell cycle at the G0/G1 [112]. Therefore, indicated its involvement in epigenetic reprogramming in cancer [113].

Currently, most of the published work on miR-26, on solid tumors and few studies performed on haematological malignancies. It indicated that miR-26a plays a role as a potential tumor-suppressor in MYC-induced lymphoma. MiR-26 found to be downregulated in primary human Burkitt's lymphoma and MYC-driven lymphoma cell lines. Ectopic expression of miR-26a influences cell cycle progression by targeting the bona fide oncogene EZH2 . MYC found to modulate genes important to oncogenesis via deregulation of miRNAs, miR-26a, contributed to the MYC-driven lymphomagenesis [114].

A recent work conducted on miR-26a transgenic mice, revealed a tumor suppressor role for miR-26 in intestinal cancer that override putative oncogenic activity and, suggested miR-26 as a potential therapeutic agent for these tumors [109].

With regard to its role in CLL pathogenesis, a work by Li *et al* reported that the expression of miR-26a is increased in CLL only in B cells activated with CD40L but does not change in B cells activated with anti-IgM stimulation [115]. This suggests its role in CLL pathogenesis in microenvironment. Interestingly, Lannetti *et al* indicated that that NF-kB2 regulates the expression of CDK4 and CDK6 and EZH2 and all are identified as NF-kB target genes. These together, with other intermediate targets, regulate the stability of p21WAF1 and the tumour suppressor p53 [116]. Interestingly, the activation of NF-kB found to induce EZH2 expression in CD40L stimulated cells from CLL patients. Therefore, it is proposed that this pathway provides a mechanism through which microenvironment induced NF-kB

can inhibit tumor suppressor function and promote tumorigenesis [116]. It is also important to note that both of CDK6 and EZH2 are targets of miR-26a. We found that miR-26a antagonizes CDK6 protein and we suggest that it might inhibit CLL tumorigenesis by interrupting this mechanism. Our results add a novel additional evidence for a potential role of miR-26a as a therapeutic agent in CLL and miR-26a is promising therapy that might soon transfer to clinics.

To sum up, we provide a novel delivery approach, which improves efficiency and specificity of miRNA delivery into CLL cells, which also led to a more effective anti-leukemic activity of microRNAs in vivo and therefore could be suggested as promising approach for its use in CLL therapy. In addition, we report for the first time that miR-26a exhibit a significant activity in reducing leukemic cells expansion. Additional studies might help to better understand the exact contribution of miR-26a in CLL pathogenesis and clarify mechanisms of its anti-leukemic activity.

CONCLUSIONS

- The developed novel delivery approach of CD38 (mAb)-liposomes is efficient and selective for targeting CLL tumor cells.
- miR-26a is a tumor suppressor in CLL
- MiR-26a is a potential therapeutic molecule against CLL, able to slow down leukemic cell growth.

5. REFERENCES

1. Balatti, V., et al., *miR deregulation in CLL.* Adv Exp Med Biol, 2013. **792**: p. 309-25.
2. Siegel, R., et al., *Cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(1): p. 9-29.
3. Chiorazzi, N., K.R. Rai, and M. Ferrarini, *Chronic lymphocytic leukemia.* N Engl J Med, 2005. **352**(8): p. 804-15.
4. Pleyer, L., et al., *Molecular and cellular mechanisms of CLL: novel therapeutic approaches.* Nat Rev Clin Oncol, 2009. **6**(7): p. 405-18.
5. Hallek, M., et al., *Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines.* Blood, 2008. **111**(12): p. 5446-56.
6. Moreau, E.J., et al., *Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b).* Am J Clin Pathol, 1997. **108**(4): p. 378-82.
7. Ginaldi, L., et al., *Levels of expression of CD19 and CD20 in chronic B cell leukaemias.* J Clin Pathol, 1998. **51**(5): p. 364-9.
8. Mertens, D. and S. Stilgenbauer, *Prognostic and predictive factors in patients with chronic lymphocytic leukemia: relevant in the era of novel treatment approaches?* J Clin Oncol, 2014. **32**(9): p. 869-72.
9. Weinberg, J.B., et al., *Clinical and molecular predictors of disease severity and survival in chronic lymphocytic leukemia.* Am J Hematol, 2007. **82**(12): p. 1063-70.
10. Dohner, H., et al., *Genomic aberrations and survival in chronic lymphocytic leukemia.* N Engl J Med, 2000. **343**(26): p. 1910-6.
11. Rai, K.R., et al., *Clinical staging of chronic lymphocytic leukemia.* Blood, 1975. **46**(2): p. 219-34.
12. Binet, J.L., et al., *A clinical staging system for chronic lymphocytic leukemia: prognostic significance.* Cancer, 1977. **40**(2): p. 855-64.
13. Kay, N.E., et al., *The role of prognostic factors in assessing 'high-risk' subgroups of patients with chronic lymphocytic leukemia.* Leukemia, 2007. **21**(9): p. 1885-91.
14. Pekarsky, Y., et al., *Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181.* Cancer Res, 2006. **66**(24): p. 11590-3.
15. Browning, R.L., et al., *Expression of TCL-1 as a potential prognostic factor for treatment outcome in B-cell chronic lymphocytic leukemia.* Leuk Res, 2007. **31**(12): p. 1737-40.
16. Parker, T.L. and M.P. Strout, *Chronic lymphocytic leukemia: prognostic factors and impact on treatment.* Discov Med, 2011. **11**(57): p. 115-23.
17. Calin, G.A., et al., *MiR-15a and miR-16-1 cluster functions in human leukemia.* Proc Natl Acad Sci U S A, 2008. **105**(13): p. 5166-71.

18. Calin, G.A. and C.M. Croce, *Chronic lymphocytic leukemia: interplay between noncoding RNAs and protein-coding genes.* Blood, 2009. **114**(23): p. 4761-70.

19. Schnaiter, A., et al., *NOTCH1, SF3B1, and TP53 mutations in fludarabine-refractory CLL patients treated with alemtuzumab: results from the CLL2H trial of the GCLLSG.* Blood, 2013. **122**(7): p. 1266-70.

20. Moreau, I.B., *Chronic Lymphocytic Leukemia: New Research*. 2008: Nova Biomedical Books.

21. Zenz, T., et al., *Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up.* Blood, 2008. **112**(8): p. 3322-9.

22. Calin, G.A., et al., *Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15524-9.

23. Musilova, K. and M. Mraz, *MicroRNAs in B-cell lymphomas: how a complex biology gets more complex.* Leukemia, 2015. **29**(5): p. 1004-17.

24. Cui, B., et al., *MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia.* Blood, 2014. **124**(4): p. 546-54.

25. Si, M.L., et al., *miR-21-mediated tumor growth.* Oncogene, 2007. **26**(19): p. 2799-803.

26. Mraz, M., et al., *miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities.* Leukemia, 2009. **23**(6): p. 1159-63.

27. Stamatopoulos, B., et al., *microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification.* Blood, 2009. **113**(21): p. 5237-45.

28. Zenz, T., et al., *miR-34a as part of the resistance network in chronic lymphocytic leukemia.* Blood, 2009. **113**(16): p. 3801-8.

29. Fabbri, M., et al., *Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia.* JAMA, 2011. **305**(1): p. 59-67.

30. Asslaber, D., et al., *microRNA-34a expression correlates with MDM2 SNP309 polymorphism and treatment-free survival in chronic lymphocytic leukemia.* Blood, 2010. **115**(21): p. 4191-7.

31. Mraz, M., et al., *miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1.* Blood, 2014. **124**(1): p. 84-95.

32. Mraz, M., et al., *MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia.* Blood, 2012. **119**(9): p. 2110-3.

33. Robak, P., P. Smolewski, and T. Robak, *Emerging immunological drugs for chronic lymphocytic leukemia.* Expert Opin Emerg Drugs, 2015. **20**(3): p. 423-47.

34. Robak, T., *Current and emerging monoclonal antibody treatments for chronic lymphocytic leukemia: state of the art.* Expert Rev Hematol, 2014. **7**(6): p. 841-57.

35. Rodriguez-Alba, J.C., et al., *CD38 induces differentiation of immature transitional 2 B lymphocytes in the spleen.* Blood, 2008. **111**(7): p. 3644-52.

36. Malavasi, F., et al., *Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology.* Physiol Rev, 2008. **88**(3): p. 841-86.

37. Malavasi, F., et al., *CD38 and chronic lymphocytic leukemia: a decade later.* Blood, 2011. **118**(13): p. 3470-8.

38. Donis-Hernandez, F.R., R.M. Parkhouse, and L. Santos-Argumedo, *Ontogeny, distribution and function of CD38-expressing B lymphocytes in mice.* Eur J Immunol, 2001. **31**(4): p. 1261-7.

39. Chiorazzi, N. and M. Ferrarini, *Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities.* Blood, 2011. **117**(6): p. 1781-91.

40. Burger, J.A. and N. Chiorazzi, *B cell receptor signaling in chronic lymphocytic leukemia.* Trends Immunol, 2013. **34**(12): p. 592-601.

41. Burger, J.A., et al., *The microenvironment in mature B-cell malignancies: a target for new treatment strategies.* Blood, 2009. **114**(16): p. 3367-75.

42. Stevenson, F.K., et al., *B-cell receptor signaling in chronic lymphocytic leukemia.* Blood, 2011. **118**(16): p. 4313-20.

43. Packham, G. and F.K. Stevenson, *Bodyguards and assassins: Bcl-2 family proteins and apoptosis control in chronic lymphocytic leukaemia.* Immunology, 2005. **114**(4): p. 441-9.

44. Simonetti, G., et al., *Mouse models in the study of chronic lymphocytic leukemia pathogenesis and therapy.* Blood, 2014. **124**(7): p. 1010-9.

45. Bichi, R., et al., *Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6955-60.

46. Efanov, A., et al., *CD5+CD23+ leukemic cell populations in TCL1 transgenic mice show significantly increased proliferation and Akt phosphorylation.* Leukemia, 2010. **24**(5): p. 970-5.

47. Johnson, A.J., et al., *Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia.* Blood, 2006. **108**(4): p. 1334-8.

48. Yan, X.J., et al., *B cell receptors in TCL1 transgenic mice resemble those of aggressive, treatment-resistant human chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11713-8.

49. Bresin, A., et al., *miR-181b as a therapeutic agent for chronic lymphocytic leukemia in the Emicro-TCL1 mouse model.* Oncotarget, 2015. **6**(23): p. 19807-18.

50. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.

51. Palanichamy, J.K. and D.S. Rao, *miRNA dysregulation in cancer: towards a mechanistic understanding.* Front Genet, 2014. **5**: p. 54.

52. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.

53. Brown, M., et al., *Mammalian miRNA curation through next-generation sequencing.* Front Genet, 2013. **4**: p. 145.

54. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.

55. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.* Genes Dev, 2003. **17**(24): p. 3011-6.

56. Hsu, S.H. and K. Ghoshal, *MicroRNAs in Liver Health and Disease.* Curr Pathobiol Rep, 2013. **1**(1): p. 53-62.

57. Krol, J., I. Loedige, and W. Filipowicz, *The widespread regulation of microRNA biogenesis, function and decay.* Nat Rev Genet, 2010. **11**(9): p. 597-610.

58. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex.* Nature, 2004. **432**(7014): p. 231-5.

59. Tan, G.S., et al., *Clarifying mammalian RISC assembly in vitro.* BMC Mol Biol, 2011. **12**: p. 19.

60. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets.* Cell, 2005. **120**(1): p. 15-20.

61. Soriano, A., et al., *microRNAs as pharmacological targets in cancer.* Pharmacol Res, 2013. **75**: p. 3-14.

62. Ferracin, M., et al., *MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia.* Mol Cancer, 2010. **9**: p. 123.

63. Calin, G.A., et al., *MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias.* Proc Natl Acad Sci U S A, 2004. **101**(32): p. 11755-60.

64. Visone, R., et al., *miR-181b is a biomarker of disease progression in chronic lymphocytic leukemia.* Blood, 2011. **118**(11): p. 3072-9.

65. Vargova, K., et al., *MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia.* Blood, 2011. **117**(14): p. 3816-25.

66. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.

67. Zauli, G., et al., *miR-34a induces the downregulation of both E2F1 and B-Myb oncogenes in leukemic cells.* Clin Cancer Res, 2011. **17**(9): p. 2712-24.

68. Yamakuchi, M., M. Ferlito, and C.J. Lowenstein, *miR-34a repression of SIRT1 regulates apoptosis.* Proc Natl Acad Sci U S A, 2008. **105**(36): p. 13421-6.

69. de Yebenes, V.G., et al., *miR-181b negatively regulates activation-induced cytidine deaminase in B cells.* J Exp Med, 2008. **205**(10): p. 2199-206.

70. Li, Z. and T.M. Rana, *Therapeutic targeting of microRNAs: current status and future challenges.* Nat Rev Drug Discov, 2014. **13**(8): p. 622-38.

71. Esquela-Kerscher, A. and F.J. Slack, *Oncomirs - microRNAs with a role in cancer.* Nat Rev Cancer, 2006. **6**(4): p. 259-69.

72. Huang, X., et al., *Targeted delivery of microRNA-29b by transferrin-conjugated anionic lipopolyplex nanoparticles: a novel therapeutic strategy in acute myeloid leukemia.* Clin Cancer Res, 2013. **19**(9): p. 2355-67.

73. Bader, A.G., D. Brown, and M. Winkler, *The promise of microRNA replacement therapy.* Cancer Res, 2010. **70**(18): p. 7027-30.

74. Zhang, Y., Z. Wang, and R.A. Gemeinhart, *Progress in microRNA delivery.* J Control Release, 2013. **172**(3): p. 962-74.

75. Bravo, V., et al., *Instability of miRNA and cDNAs derivatives in RNA preparations.* Biochemical and Biophysical Research Communications, 2007. **353**(4): p. 1052-1055.

76. Cho, W.C., *Role of miRNAs in lung cancer.* Expert Rev Mol Diagn, 2009. **9**(8): p. 773-6.

77. Pecot, C.V., et al., *RNA interference in the clinic: challenges and future directions.* Nat Rev Cancer, 2011. **11**(1): p. 59-67.

78. Grimm, D., et al., *Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways.* Nature, 2006. **441**(7092): p. 537-41.

79. Tseng, Y.C., S. Mozumdar, and L. Huang, *Lipid-based systemic delivery of siRNA.* Adv Drug Deliv Rev, 2009. **61**(9): p. 721-31.

80. Aagaard, L. and J.J. Rossi, *RNAi therapeutics: principles, prospects and challenges.* Adv Drug Deliv Rev, 2007. **59**(2-3): p. 75-86.

81. Yang, H.W., et al., *Gadolinium-functionalized nanographene oxide for combined drug and microRNA delivery and magnetic resonance imaging.* Biomaterials, 2014. **35**(24): p. 6534-42.

82. Wang, H., et al., *Recent progress in microRNA delivery for cancer therapy by non-viral synthetic vectors.* Adv Drug Deliv Rev, 2015. **81**: p. 142-60.

83. Baumann, V. and J. Winkler, *miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents.* Future Med Chem, 2014. **6**(17): p. 1967-84.

84. Lv, H., et al., *Toxicity of cationic lipids and cationic polymers in gene delivery.* J Control Release, 2006. **114**(1): p. 100-9.

85. Holz-Schietinger, C. and N.O. Reich, *RNA modulation of the human DNA methyltransferase 3A.* Nucleic Acids Res, 2012. **40**(17): p. 8550-7.

86. Akinc, A., et al., *A combinatorial library of lipid-like materials for delivery of RNAi therapeutics.* Nat Biotechnol, 2008. **26**(5): p. 561-9.

87. Hatakeyama, H., et al., *The systemic administration of an anti-miRNA oligonucleotide encapsulated pH-sensitive liposome results in reduced level of hepatic microRNA-122 in mice.* J Control Release, 2014. **173**: p. 43-50.

88. Boussif, O., et al., *A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7297-301.

89. Kunath, K., et al., *Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine.* J Control Release, 2003. **89**(1): p. 113-25.

90. Bieber, T. and H.P. Elsasser, *Preparation of a low molecular weight polyethylenimine for efficient cell transfection.* Biotechniques, 2001. **30**(1): p. 74-7, 80-1.

91. Gebhart, C.L. and A.V. Kabanov, *Evaluation of polyplexes as gene transfer agents.* J Control Release, 2001. **73**(2-3): p. 401-16.

92. Rodl, W., et al., *Synthesis of polyethylenimine-based nanocarriers for systemic tumor targeting of nucleic acids.* Methods Mol Biol, 2013. **948**: p. 105-20.

93. Trang, P., et al., *Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice.* Mol Ther, 2011. **19**(6): p. 1116-22.

94. Wiggins, J.F., et al., *Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34.* Cancer Res, 2010. **70**(14): p. 5923-30.

95. Shi, S., et al., *Systemic delivery of microRNA-34a for cancer stem cell therapy.* Angew Chem Int Ed Engl, 2013. **52**(14): p. 3901-5.

96. Chen, Y., et al., *Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy.* Mol Ther, 2010. **18**(9): p. 1650-6.

97. Obad, S., et al., *Silencing of microRNA families by seed-targeting tiny LNAs.* Nat Genet, 2011. **43**(4): p. 371-8.

98. Guo, J., J.C. Evans, and C.M. O'Driscoll, *Delivering RNAi therapeutics with non-viral technology: a promising strategy for prostate cancer?* Trends Mol Med, 2013. **19**(4): p. 250-61.

99. Bouchie, A., *First microRNA mimic enters clinic.* Nat Biotechnol, 2013. **31**(7): p. 577.

100. Khan, S., et al., *Targeting microRNAs in pancreatic cancer: microplayers in the big game.* Cancer Res, 2013. **73**(22): p. 6541-7.

101. Efanov, A., et al., *CD5+ CD23+ leukemic cell populations in TCL1 transgenic mice show significantly increased proliferation and Akt phosphorylation.* Leukemia, 2010. **24**(5): p. 970-975.

102. Balatti V, P.Y., Rizzotto L, Croce CM. , *mir deregulation in CLL*, in *Advances in chronic lymphocytic leukemia , advances in experimental*

medicine and biology, S.E. Malek, Editor. 2013, Springer: New York. p. 309-325.

103. Caliceti, P., *Targeted Delivery of Small and Macromolecular Drugs. Edited by Ajit S. Narang and Ram I. Mahato.* ChemMedChem, 2011. **6**(12): p. 2323-2325.

104. Zhang, X., et al., *Transferrin receptor targeted lipopolyplexes for delivery of antisense oligonucleotide g3139 in a murine k562 xenograft model.* Pharm Res, 2009. **26**(6): p. 1516-24.

105. Urban-Klein, B., et al., *RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo.* Gene Ther, 2005. **12**(5): p. 461-6.

106. Tivnan, A., et al., *Inhibition of neuroblastoma tumor growth by targeted delivery of microRNA-34a using anti-disialoganglioside GD2 coated nanoparticles.* PLoS One, 2012. **7**(5): p. e38129.

107. Negrini, M., et al., *microRNAome expression in chronic lymphocytic leukemia: comparison with normal B-cell subsets and correlations with prognostic and clinical parameters.* Clin Cancer Res, 2014. **20**(15): p. 4141-53.

108. Han, J., A.M. Denli, and F.H. Gage, *The enemy within: intronic miR-26b represses its host gene, ctdsp2, to regulate neurogenesis.* Genes Dev, 2012. **26**(1): p. 6-10.

109. Zeitels, L.R., et al., *Tumor suppression by miR-26 overrides potential oncogenic activity in intestinal tumorigenesis.* Genes Dev, 2014. **28**(23): p. 2585-90.

110. Zou, Z.J., et al., *miR-26a and miR-214 down-regulate expression of the PTEN gene in chronic lymphocytic leukemia, but not PTEN mutation or promoter methylation.* Oncotarget, 2015. **6**(2): p. 1276-85.

111. Kulshreshtha, R., et al., *A microRNA signature of hypoxia.* Mol Cell Biol, 2007. **27**(5): p. 1859-67.

112. Batchu, R.B., et al., *Enhanced phosphorylation of p53 by microRNA-26a leading to growth inhibition of pancreatic cancer.* Surgery, 2015. **158**(4): p. 981-6; discussion 986-7.

113. Gasque Schoof, C.R., et al., *The Roles of miR-26, miR-29, and miR-203 in the Silencing of the Epigenetic Machinery during Melanocyte Transformation.* Biomed Res Int, 2015. **2015**: p. 634749.

114. Sander, S., et al., *MYC stimulates EZH2 expression by repression of its negative regulator miR-26a.* Blood, 2008. **112**(10): p. 4202-12.

115. Li, S., et al., *MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells.* PLoS One, 2011. **6**(3): p. e16956.

116. Iannetti, A., et al., *Regulation of p53 and Rb links the alternative NF-kappaB pathway to EZH2 expression and cell senescence.* PLoS Genet, 2014. **10**(9): p. e1004642.