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DEVELOPMENT OF A LENTIVIRAL TRANSDUCTION TOOL FOR THE STUDY OF miR-28 THERAPEUTIC POTENTIAL IN DIFFUSE LARGE B CELL LYMPHOMA

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ABSTRACT

- **Fundamentals**

R-CHOP is the standard therapeutic regimen for aggressive B-cell lymphomas, which include DLBCL. However, aside from being highly toxic, a fraction of patients -about 40% with DLBCL- are refractory or relapse after treatment with this scheme, making the development of alternative therapies a necessity. Potential approaches include the administration of tumor-suppressor miRNAs, such as miR-28, in combination with targeted-therapies, among which is the BTK inhibitor ibrutinib. As the study of miR-28-ibrutinib therapeutic suitability involves preclinical testing in PDX models, we sought to develop an efficient PDX miR-28 transduction tool.

- **Material and methods**

pCDH plasmid was modified to encode for the pre-miR-28 sequence. High titer lentiviral supernatants were used to transduce the MD901 and SUDHL4 ABC-DLBCL cell lines as well as the U2932 GC-DLBCL cell line in the presence or absence of ibrutinib to subsequently perform miR-28 antitumoral and miR-28-ibrutinib synergy assays.

- **Results**

The pCDH plasmid allows the generation of high titer lentiviral supernatants which efficiently transduce DLBCL cell lines. However, pCDH-miR-28 reexpression does not reproduce previous MD901 miR-28-ibrutib synergy results from the laboratory obtained after inducible lentiviral transduction of miR-28, as pCDH-transduced miR-28 reexpression only slightly potentiates ibrutinib effect in preventing MD901 cell growth and does not exert any effect of synergy with ibrutinib in SUDLH4 and U2932 cell growth impairment.

- **Conclusions**

Results from this work discard pCDH as a suitable vector for miR-28 reexpression in DLBCL therapeutic assays and raise the need to develop new strategies for this purpose.

Keywords: B cell lymphoma, chemotherapy, ibrutinib, microRNAs, plasmids

RESUMEN

- **Fundamentos**

R-CHOP es el régimen terapéutico estándar de los linfomas B agresivos, incluyendo DLBCL. Sin embargo, además de acarrear elevada toxicidad, una fracción de pacientes -el 40% en DLBCL- son refractarios o recaen tras este tratamiento, siendo necesario desarrollar alternativas terapéuticas más eficaces y seguras. Entre los potenciales candidatos destacan los miRNAs oncosupresores, como miR-28, administrados en combinación con terapias dirigidas, como el inhibidor de BTK ibrutinib. Dado que el estudio de la idoneidad terapéutica de ibrutinib-miR-28 implica ensayos preclínicos en modelos PDX, hemos buscado desarrollar una herramienta de transducción eficiente de miR-28 para PDX.

- **Material y métodos**

Se introdujo la secuencia pre-miR-28 en el plásmido pCDH. Los sobrenadantes lentivirales de alto título se emplearon para transducir las líneas celulares de ABC-DLBCL MD901 y SUDHL4 y la línea de GC-DLBCL U2932 en presencia o ausencia de ibrutinib para posteriormente realizar experimentos de actividad antitumoral y sinergia.

- **Resultados**

El plásmido pCDH permite la generación de sobrenadantes lentivirales de alto título que transducen con eficiencia líneas celulares de DLBCL. Sin embargo, los resultados de sinergia con ibrutinib obtenidos previamente tras la transducción lentiviral inducible de miR-28 no se reproducen con pCDH. La reexpresión de miR-28 mediada por pCDH potencia ligeramente la prevención de proliferación celular de ibrutinib en MD901, pero no sinergiza con ibrutinib en la limitación de la proliferación en SUDHL4 o U2932.

- **Conclusiones**

Estos resultados descartan la utilización de pCDH como vector de reexpresión de miR-28 en ensayos de actividad terapéutica en DLBCL, siendo preciso desarrollar nuevas estrategias para este propósito.

Palabras clave: linfomas B, quimioterapia, ibrutinib, microRNAs, plásmidos

Abbreviation list from the abstract:

- R-CHOP: rituximab, cychophosphamide, doxorubicin, vincristine, prednisone
- DLBCL: diffuse large B cell lymphoma
- miRNAs: microRNAs
- BTK: Bruton's tyrosine kinase
- PDX: patient-derived xenografts

Lista de abreviaturas del resumen:

- R-CHOP: rituximab, ciclofosfamida, doxorrubicina, vincristina, prednisona
- DLBCL: linfoma B difuso de célula grande
- miRNAs: microRNAs
- BTK: tirosina quinasa de Bruton
- PDX: xenografts derivados de pacientes

1. INTRODUCTION AND OBJECTIVES

Non-Hodgkin lymphomas (NHL) are the most frequent hematologic malignancy and the 13th neoplasm overall with a highest incidence globally[1]. Within them, mature B-cell lymphomas represent the largely predominant entity. Mature B-cell lymphomas derive from B-cells that have undergone somatic hypermutation and class-switch recombination, which allow the generation of antibodies with increased antigen affinity but involve a molecular process that generates mutations and double strand breaks that can trigger oncogenic events. These processes take place in the germinal centres (GCs), temporary microstructures located in secondary lymphoid organs[2]. Therefore, most B-cell lymphomas are GC-experienced.

However, mature B-cell lymphomas are a very heterogeneous group that comprises a wide variety of indolent and, for the most part, aggressive pathological entities. Diffuse large B-cell lymphoma (DLBCL) is the most representative one, accounting for 30 to 40% of the aggressive B-cell neoplasms -others include Burkitt lymphoma, mantle cell lymphoma and high-grade B cell lymphoma. DLBCL has been traditionally classified into three subtypes depending on the gene expression pattern: germinal centre-B cell (GCB), which presents with the gene expression pattern of normal GC B-cells; activated B-cell (ABC), whose cells resemble post-germinal centre plasmablasts; and an unclassified subtype[3].

Cyclophosphamide, doxorubicin, vincristine and prednisone, also known as CHOP chemotherapy regimen, has been the standard treatment for aggressive B-cell lymphomas for many years, with only a significant modification in the recent years after the development of the targeted therapy rituximab and its inclusion as part of the first-line therapeutic scheme (R-CHOP) in 2006[4]. Rituximab is a human/murine chimeric monoclonal antibody that targets

CD20 antigen, which is widely expressed by B cells (from pre-B to the immunoblast stage) and, therefore, by a vast majority of Non-Hodgkin lymphomas[5]. Accordantly, Rituximab is effective in most Non-Hodgkin malignancies, including DLBCL[6].

Targeted therapies are a therapeutic group that targets specific genes or proteins that are required for the growth and survival of cancer cells[7], as opposed to chemotherapy which has a much broader and non-specific cytotoxic effect. Nevertheless, R-CHOP, while being an effective regimen which cures most DLBCL patients[4], presents with the not negligible side effect of cardiotoxicity -which is a shared characteristic of all anthracycline-containing chemotherapy schemes[8]. Chemotherapy-derived side effects also cause post-administration hospitalization to be required on a relatively frequent basis. Furthermore, R-CHOP success rates vary upon DLBCL molecular subtypes. While GC-DLBCL patients exhibit a 5-year overall survival rate of around 70% and a 5-year progression-free survival rate of around 80%, these values decrease to approximately 40% and 50%, respectively, for the ABC-DLBCL subtype[9, 10]. Such disparity in prognosis is due to the difference in refractory and relapsed disease rates, which are ultimately related to the mutational pattern found in the tumor[11]. Altogether, these data outline the need to develop new less toxic and more effective specific alternatives for aggressive B-cell lymphoma treatment, especially for currently-bad-prognosis molecular variants such as ABC-DLBCL. In this sense, targeted therapies arise as the alternative of choice.

Findings from recent years regarding the molecular characterization of dysregulated signaling pathways have set the basis for the development of novel targeted therapies[4]. Constitutive activation of antiapoptotic and proliferative pathways are common pathogenic mechanisms underlying neoplastic transformation. As for B-cell malignancies, PI3K and NFkB pathways -which embody the intracellular effectors of the B-cell receptor (BCR)- are

the most commonly affected (Figure 1 in annex). It is no surprise, therefore, that most B-cell malignancies maintain surface expression of BCR[3].

Both the predominant dysregulation of either PI3K or NFkB and the oncogenic mutations that lead to their constitutive activation are a differential characteristic of each NHL type, and the molecular subtypes of DLBCL are no exception. NFkB constitutive activation is a hallmark of ABC-DLBCL and usually arises due to the overlap of several genetic alterations, the main ones being point mutations in CD79a/b, MYD88 and CARD1, and biallelic inactivation of TNFAIP3[4, 12, 13]. Mutations in CD79a/b BCR co-receptor result in chronically active BCR-dependent signaling, whereas MYD88, CARD1 and TNFAIP3 are downstream effectors and therefore cause BCR-independent NFkB constitutive activation. As for the GC-DLBCL molecular subtype, PI3K dysregulation is the most common feature. This event is usually caused by PTEN deletions -or other alterations that ultimately end in PTEN expression loss. Other relevant pathogenic mutations often found in the GC-DLBCL subtype, such as those affecting the EZH2 and BCL-2 genes, promote cell proliferation and survival via non-PI3K-related mechanisms.

Different drugs targeting specific NFkB and PI3K effectors have been developed to date (Figure 1 in annex). Among them, ibrutinib -an irreversible inhibitor of NFkB-effector Bruton's tyrosine kinase (BTK)- has shown promising results as a therapeutic alternative in DLBCL, as shown by phase I and II studies carried out in patients with relapsed or refractory disease[4, 13]. Ibrutinib-responding patients mostly belonged to the ABC-DLBCL subtype, a finding that is consistent with ABC-DLBCL characteristic NFkB activation. This has led to a currently ongoing phase III clinical trial to test the efficacy of ibrutinib + R-CHOP therapy as opposed to the R-CHOP standard regimen in nontreated patients with non-GC DLBCL[14]. However, the ulterior objective is to develop directed-therapy-based regimens which do not rely on chemotherapy.

The high relapse rates found in DLBCL patients after first-line treatment (30-40%)[11] are a consequence of the acquisition of resistance mutations and the subsequent treatment-induced selection of the resistant mutant subclones[15]. Another potential contributor is the rebound activation of alternative oncogenic pathways, which has been observed in DLBCL cell lines after treatment with a directed agent in monotherapy regimen[16]. Thus, it is crucial to identify drugs that target different oncogenic pathways and prevent the generation of resistant subclones when administered together. This is the main principle of combined therapy, the current focus in DLBCL therapeutics research.

MicroRNAs (miRNAs) present as an attractive candidate to be part of combined therapeutic schemes. micro-RNAs are 21-24-nucleotide non-coding RNA molecules, processed from longer pre-mi-RNA precursors by Drosha and Dicer enzymes, that regulate gene expression by causing target-mRNA destruction or translational blockade, and have lately emerged as key GC regulators[17]. Several miRNAs have been proven to exert oncogenic or tumor-suppressant roles in different neoplasms, including B-cell lymphomas. Among the tumor-suppressant miRNAs is miR-28. Its antitumoral role was studied after observing that various NHL cell lines, including BL, DLBCL, follicular lymphoma and chronic lymphocytic leukemia, showed diminished expression levels of miR-28 when compared to its normal cell counterpart. Indeed, previous work of our lab revealed that miR-28 reexpression shows antitumoral activity *in vitro* in several NHL cell lines, including BL and DLBCL human cell lines by targeting a BCR-signaling protein-coding gene network comprising AKT, ERK, BCL-2 and effectors from the NFkB pathway[18].

NFkB-targeting makes ABC-DLBCL neoplasms presumably especially amenable to miR-28-targeted therapies. This idea is also supported by *in vivo* experiments carried out in mouse primary[18] and human DLBCL patient-derived xenograft (PDX) tumors (unpublished previous results from host laboratory) that show antitumoral effect of miR-28 after lentivirally-

delivered miRNA reexpression or intravenous administration of a miR-28 synthetic mimic, respectively.

PDX tumors are human primary neoplasms which are engrafted in immunodeficient mice in order to create models that accurately resemble the original tumor. PDX models allow both tumoral molecular characterization and preclinical development and testing of antineoplastic therapeutic agents[19]. They therefore offer a significant advantage compared to mouse primary tumor models, representing a useful tool that could be of much help in testing the therapeutic potential of miR-28-based therapies.

The objective of this work is centered in identifying, developing and testing a lentiviral-based tool that would allow high-efficiency transduction of DLBCL PDX samples and DLBCL cell lines for further testing of miR-28 reexpression therapeutic potential in combination with other agents, namely ibrutinib. For this purpose, a set of GC- and ABC-DLBCL cell lines will be transduced with the newly generated vector in order to assay miR-28 antitumoral activity and miR-28-ibrutinib synergistic potential.

2. MATERIAL AND METHODS

Material	Source	Reference number
pCDH-EF1a-eFFly-mCherry plasmid	Addgene	104833
pTRIPZ plasmid	Thermo Scientific	RHS4697
Ibrutinib	Selleck Chemicals (Fisher Scientific)	S2680
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650
Phosphate-buffered saline (PBS)	Lonza Biosciences	BE17-517Q
Polybrene	Sigma Aldrich	TR-1003
CellTiter-Glo	Promega	G7570
DAPI	Sigma Aldrich	10236276001
DMEM culture medium	Gibco	41965-039
RPMI 1640 culture medium	Gibco	21875-034
Fetal bovine serum (FBS)	Gibco	1027-106
HEPES	Hyclone	SH30237.01
Penicillin/Streptomycin	Sigma Aldrich	P4333

Table 1. Materials and reagents utilized to perform the experiments in this work. Source and reference number is listed for each element.

2.1. Cell culture

The DLBCL lines MD901, SUDHL4 and U2932, as well as the CD4 T cell line Jurkat, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. HEK-293 cell line was cultured

in DMEM medium supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were incubated at 37ºC, 5%CO_2.

2.2. pCDH lentiviral vector generation

pCDH-EF1a-eFFly-mCherry plasmids were obtained from Addgene. For the control (empty) condition, no modifications were performed on the purchased plasmids. For the miR-28 condition, the eFFly sequence was replaced by the pre-miR-28 sequence. This process was carried out prior to the initiation of this work.

293T cells were seeded in 18 15-cm dishes (9 for each condition: empty and miR-28) in DMEM 20% FBS culture medium. When cells were 70-80% confluent, calcium phosphate transfection was performed with Δ9.8, VSVG and pCDH plasmids. Lentiviral supernatants were then concentrated by ultracentrifugation (4ºC, 2 hours at 26000 revolutions per minute/RPM) and each viral pellet was resuspended in 200 µl phosphate-buffered saline (PBS). 20 µl lentiviral supernatant aliquots were made and frozen at -80ºC for subsequent cell transduction.

2.3. pCDH lentiviral vector titration in Jurkat cells

50 000 Jurkat cells were seeded in a 100 µl volume in 96-well plates and transduced with three serial ten-fold dilutions of empty and miR-28-expressing viral suspension using 10µl, 1 µl and 0.1 µl of the concentrated lentiviral supernatants in the presence of 8 µg/ml polybrene. Expression levels of the fluorescent protein mCherry were measured by flow cytometry 48 hours after transfection (flow cytometry details and protocol below).

2.4. Cell transduction

MD901, SUDHL4 and U2932 cells were seeded at a concentration of 300 000 cells/µl and transduced with ≥ $8.5x10^5$ PFU/million cells of the empty/miR-28 pCDH lentiviral supernatants in the presence of 8 µg/ml polybrene. Effective transduction was evaluated by flow cytometry and experiments were performed 24 hours after transduction.

2.5. miR-28 antitumoral activity assays

MD901 cells were transduced as previously stated with miR-28-pCDH lentiviral supernatants, and empty-pCDH lentiviral supernatants as a negative control. Cells were seeded in RPMI 10% FBS at a concentration of 300 000/ml and cultured for 11 days. A 1/8 dilution was carried out every 48 hours in order to maintain cell concentration. Effective transduction was evaluated by flow cytometry 24, 48 and 72 hours after transduction and cell viability measurements were performed by flow cytometry and ATP luminometry (details and protocol below) in both conditions at days 8 and 11.

2.6. MiR-28-ibrutinib synergy assays

MD901, SUDHL4 and U2932 cells were transduced as previously stated with miR-28-pCDH lentiviral supernatants, and empty-pCDH lentiviral supernatants as a negative control. Cells were seeded in RPMI 10% FBS at a concentration of 300 000/ml and cultured for 3 days in the presence 8 µg/ml polybrene and the following ibrutinib concentrations:

- MD901: ibrutinib 12 µM, 25 µM, 37,5 µM, 50 µM and DMSO as a negative control.
- SUDHL4: ibrutinib 1,5 µM, 3 µM, 6µM and DMSO as a negative control.
- U2932: ibrutinib 6 µM, 12 µM, 25 µM and DMSO as a negative control.

Effective transduction was evaluated by flow cytometry 24, 48 and 72 hours after transduction. Cell viability measurements were performed by flow cytometry and ATP luminometry in all conditions 72 hours after transduction.

2.7. Flow cytometry

Flow cytometry analyzes single particles or cells suspended in a buffered salt-based solution as they flow past single or multiple lasers. Particles or cells are analyzed for various parameters that include visible light scatter and single or multiple fluorescence parameters, which in turn allows the measurement of their physical or chemical characteristics. Among other applications, fluorescent cells can then be separated from the rest of the population in a process called cell sorting.

Light scatter, which is independent from fluorescence, reports the relative size of the cell when measured in the “forward” direction (Forward Scatter, FSC) and the complexity of the cell when measured at 90º (Side Scatter, SSC). Besides, cells can be labeled with fluorescent reporters that emit light at a characteristic and known wavelength. Different techniques are used for this purpose, including transfection and expression of fluorescent proteins, fluorescent dye staining (both of which were performed in this work), or fluorescently conjugated antibodies staining[20].

In this work, flow cytometry assays were performed to analyze cell viability and to measure miR-28 transduction by means of RFP or mCherry fluorescent proteins analysis. Fluorescent proteins emit fluorescence when excited by light at a specific wavelength spectrum. The emission wavelength is also protein-specific. Accordingly, mCherry shows an excitation peak at 587 nm and an emission peak at 610 nm, whilst the RFP protein excitation and emission peaks are 555 and 583 nm respectively.

To stain dead cells for subsequent performance of flow cytometry analysis, 2 µl of DAPI were added to 100 µl of each cell condition. After 5 minutes incubation at room temperature, flow cytometry data were collected using a LSRFortesssa flow cytometer (BD Biosciences).

2.8. ATP luminometry

Adenosine triphosphate (ATP) is the main energy source in all living organisms and its intracellular concentration is the fundamental indicator of the viability of a cell. Given that intracellular ATP concentration of a cell suspension is proportional to the amount of metabolically active (thus, alive) cells, determination of intracellular ATP concentration allows an estimation of cell viability.

The bioluminescence ATP assay, among all methods available for this task, is the most rapid and sensitive. Its main principle is the requirement of ATP for the conversion of D-luciferin in oxyluciferin by firefly luciferase. Oxyluciferin is produced in an electronically excited state and its transfer to the stationery state emits a photon which can be detected by a luminometer. The amount of photons emitted is proportional to the ATP content of the sample, and the result is presented in relative luminescence units (RLU). Since luciferase is not able to penetrate cell membranes, ATP luminometry technique requires the use of agents that increase cell permeability or disrupt the cell membrane[21].

In this work we utilized CellTiterGlo® reagent. For cell viability assays, 100 µl of each cell condition -and 100 µl of RPMI 10% FBS as negative control- were seeded in an opaque-walled multiwell plate. 100 µl of CellTiter-Glo® reagent were added to each well and the content of the wells was mixed on an orbital shaker for 2 minutes. The plate was then incubated at room temperature for 10 minutes and luminescence was recorded in an Orion luminometer (Berthold).

2.9. Data analysis

Results obtained from the DNA-dye exclusion flow cytometry assays were analyzed with FlowJo v10.7.1 (BD Biosciences) and Prism 8 (GraphPad Software, San Diego, CA). Results obtained from ATP luminometry were analyzed with Excel 2019 (Microsoft Office) and Prism 8. Statistical tests were performed with Prism 8 and statistical significance was evaluated using the unpaired Student t-test. Differences were considered statistically significant when $p<0,05$ (*). ** indicates $p<0,01$.

3. RESULTS

3.1. miR-28 synergizes with ibrutinib in the inhibition of MD901 DLBCL cell growth *in vitro*

miR-28 exhibits antitumoral activity in several human BL and DLBCL cell lines, as well as in mouse primary[22] and human PDX tumors (unpublished previous results from host laboratory) when administrated as a synthetic mRNA molecule or expressed by inducible lentiviral transduction. However, whether miR-28 can enhance the antitumoral activity of other lymphoma-targeted therapies remains unknown. For this purpose, several experiments were previously carried out in the laboratory to test the effect of miR-28 reexpression when combined with ibrutinib treatment in DLBCL growth. MD901 ABC-DLBCL cells were transduced using a pTRIPZ doxycycline-inducible lentiviral vector carrying the miR-28 precursor sequence or a scramble control and RFP as reporter sequence (Figure 1A). Transduced cells were selected with puromycin. Expression of miR-28 (or scramble control) was induced with 0,5 µg/ml doxycycline for 2 days before treatment with sensitivity-determined ibrutinib concentration conditions (0 µM/DMSO, 12 µM, 25 µM, 37.5 µM, 50 µM) for 3 days. Cell viability was then analyzed by luminometry using ATP CellTiterGlo reagent, as well as by DNA-dye exclusion flow cytometry, both 72 hours after ibrutinib administration in the presence or absence of miR-28 re-expression (Figure 1B). MD901 metabolic activity was notably reduced in the miR-28 conditions when compared to the scramble controls in all ibrutinib concentrations, therefore showing a disadvantage in cell growth when cells that express miR-28 were exposed to ibrutinib. Accordantly, flow-cytometry-assayed cell viability was found reduced in ibrutinib conditions after miR-28 reexpression. Thus, we conclude that inducible miR-28 expression synergizes with ibrutinib in the inhibition of pTRIPZ lentivirally transduced MD901 DLBCL cell growth.

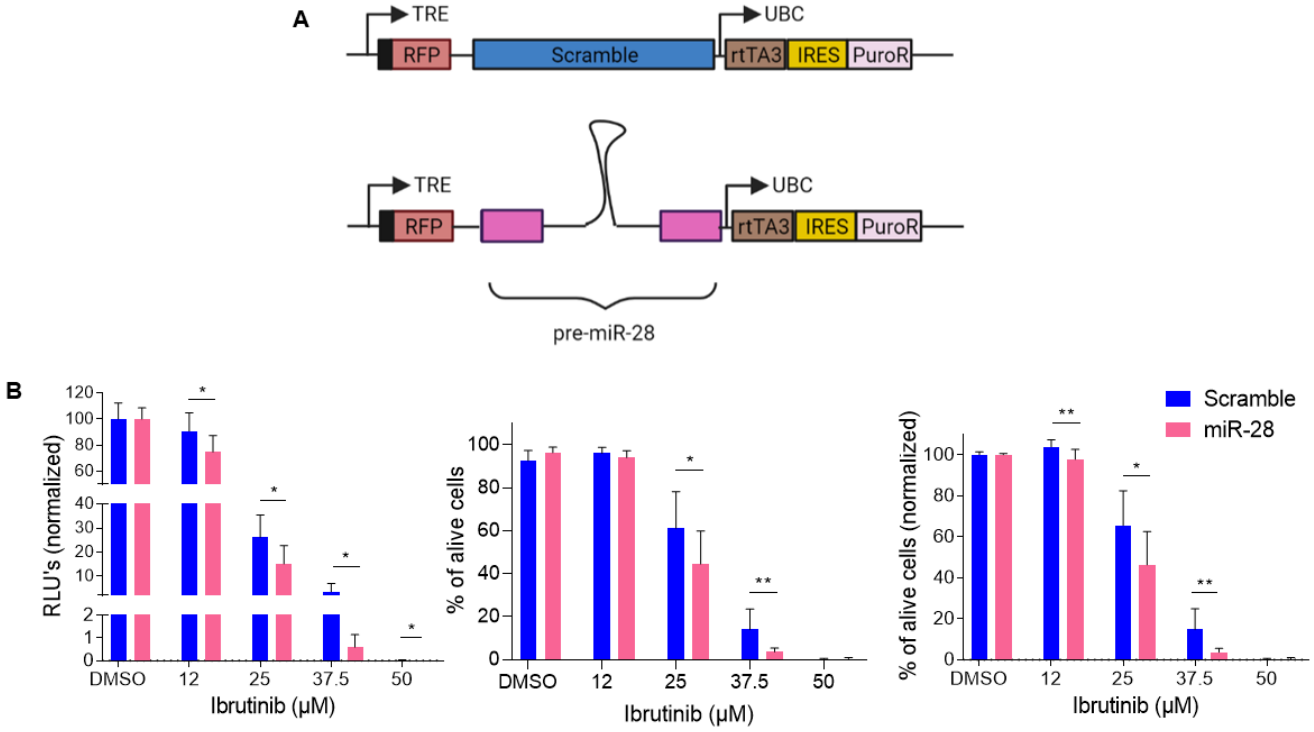

Figure 1. miR-28 enhances the antitumoral effect of ibrutinib in DLBCL cell growth (A) Schematic representation of pTRIPZ lentiviral constructs: scramble (top panel) and miR-28 (down panel). (B) The MD901 cell line was transduced with pTRIPZ vectors encoding scramble RNA or pre-miR-28 sequence. miR-28 expression was induced with 0,5 μg/ml doxycycline for 2 days before treatment with different ibrutinib concentrations. Cells were cultured for 3 days in the miR-28/scramble + ibrutinib combined regime and cell viability was analyzed by ATP cell viability assay by luminometry (left panel) and by DNA-dye exclusion by flow cytometry (medium and right panels), performing normalization to control (DMSO) conditions. Data are from 4 independent experiments. *P<0.05, **P<0.01, unpaired Student t test.

3.2. Generation of high-titer miR-28 pCDH lentiviral supernatants

In order to generate a lentiviral tool that allows the efficient transduction of DLBCL PDX samples[23, 24] and to transduce with high efficiency DLBCL cell lines, the lab cloned miR-28 in the pCDH-eF1a-eFFly-mCherry lentiviral vector. pCDH carries two reporter sequences for transduction assessment: eFFly luciferase for *in vivo* imaging and mCherry red fluorochrome for flow cytometry analysis and cell sorting. For the “empty” control condition, pCDH vector was utilized without sequence modifications, whereas for the miR-28 construct, eFFly sequence was replaced by miR-28 precursor sequence (Figure 2A). In contrast to the

previously used pTRIPZ doxycycline-inducible vector, sequences contained in the pCDH vector are constitutively expressed. We then generated the lentiviral supernatants by calcium-phosphate transfection of 293T cells with a VSVG-pseudotyped packaging and viral media was concentrated by ultracentrifugation and resuspended in 200 µl PBS.

In order to test the efficiency of the newly generated vector, 50 000 Jurkat T cell acute lymphocytic leukemia (T-ALL) cells were transduced with three serial ten-fold dilutions of empty and miR-28-expressing viral suspension (using 10 µl, 1 µl and 0.1 µl of the concentrated lentiviral supernatants). mCherry expression was measured by flow cytometry 48 hours after transduction, showing close to 100% efficiency in all conditions (Figure 2B). Thereby, viral titration resulted greater than $5x10^5$ plaque forming units per µl (PFU/µl). Next, the expression of miR-28 was verified by qPCR amplification of the mature miRNA and no mutations were identified after sequencing by Sanger the vector. These results thus demonstrate that concentrated pCDH lentiviral supernatants are suitable for miR-28 transduction of lymphoblastic leukaemia and lymphoma cell lines.

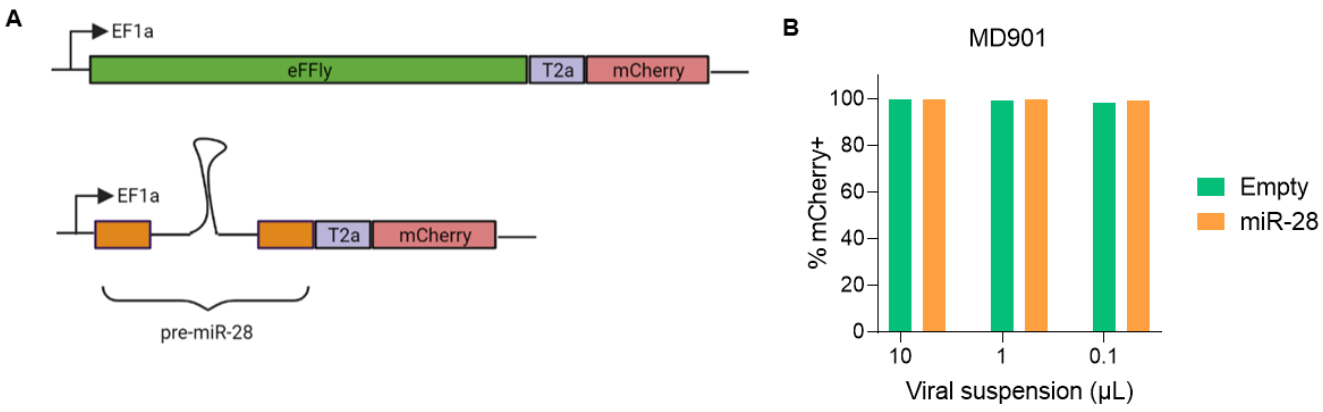

Figure 2. Concentrated pCDH lentiviral supernatants enable high transduction efficiency. (A) Schematic representation of pCDH lentiviral constructs: empty (top panel) and miR-28 (down panel). (B) 50.000 Jurkat cells were transduced with 10µl, 1 µl and 0,1 µl of the concentrated lentiviral supernatants. Cells were cultured for 48 hours and mCherry expression was measured by flow cytometry, showing close to 100% transduction efficiency in all conditions.

3.3. Re-expression of miR-28 by pCDH lentiviral transduction of the MD901 ABC-DLBCL cell line promotes an enhancement of the effect of ibrutinib in the inhibition of MD901 cell growth

To determine if the effect of miR-28 in the potentiation of ibrutinib-induced antitumoral effect is induced by miR-28-pCDH transduction, we transduced MD901 ABC-DLBCL cells with empty or miR-28 pCDH lentiviral constructs and cultured them for three days. Percentages of both empty and miR-28 mCherry+ cells remained above 70% within 72 hours post-transduction, therefore providing strong additional data supporting pCDH as a suitable vector for DLBCL cell line transduction (Figure 3A). mCherry+ percentage of MD901 cells was slightly higher for the miR-28 condition compared to empty, presumably due to a shorter-length transcription advantage of the miR-28 sequence compared to eFFly.

Once an adequate transduction efficiency was proven, we proceeded to test the antitumoral effect of miR-28 when delivered by the pCDH vector. Transduced MD901 cells were cultured for 11 days with regular measurements of cell viability performed by flow cytometry and luminometry on days 8 and 11. We did not observe an effect of miR-28 in the inhibition of MD901 cell growth. This outcome differs from previous results published by the research group that demonstrated antitumoral effect of miR-28 in the MD901 cell line after miR-28 re-expression with pTRIPZ-miR-28 lentiviral transduction as well as after miR-28 mimic administration *in vivo*[22], and suggests a limitation in miR-28 expression levels achieved by miR-28-pCDH transduction.

To determine whether the reason behind this inconsistency was a lack of antitumoral effect in pCDH-transduced miR-28-reexpressing cells or a measuring method resolution difference, we next tested the synergistic effect of miR-28 reexpression when combined with ibrutinib treatment by performing an experiment in the same conditions as the previously described with pTRIPZ vector. MD901 cells were transduced with empty or miR-28 pCDH

vector and were cultured for 3 days with the same Ibrutinib concentrations in the culture medium as those utilized in the previous MD901 synergy experiments. Results showed a small but significant reduction in miR-28-transduced cell growth in luminometry-measured ATP cell viability in the 12 μM ibrutinib condition, and certain impairment (although not statistically significant) in the 37.5 μM and 50 μM ibrutinib conditions (Figure 3C). Thus, these results show that reexpression of miR-28 by pCDH lentiviral transduction of the MD901 cell line synergizes, albeit slightly, with ibrutinib in cell growth inhibition.

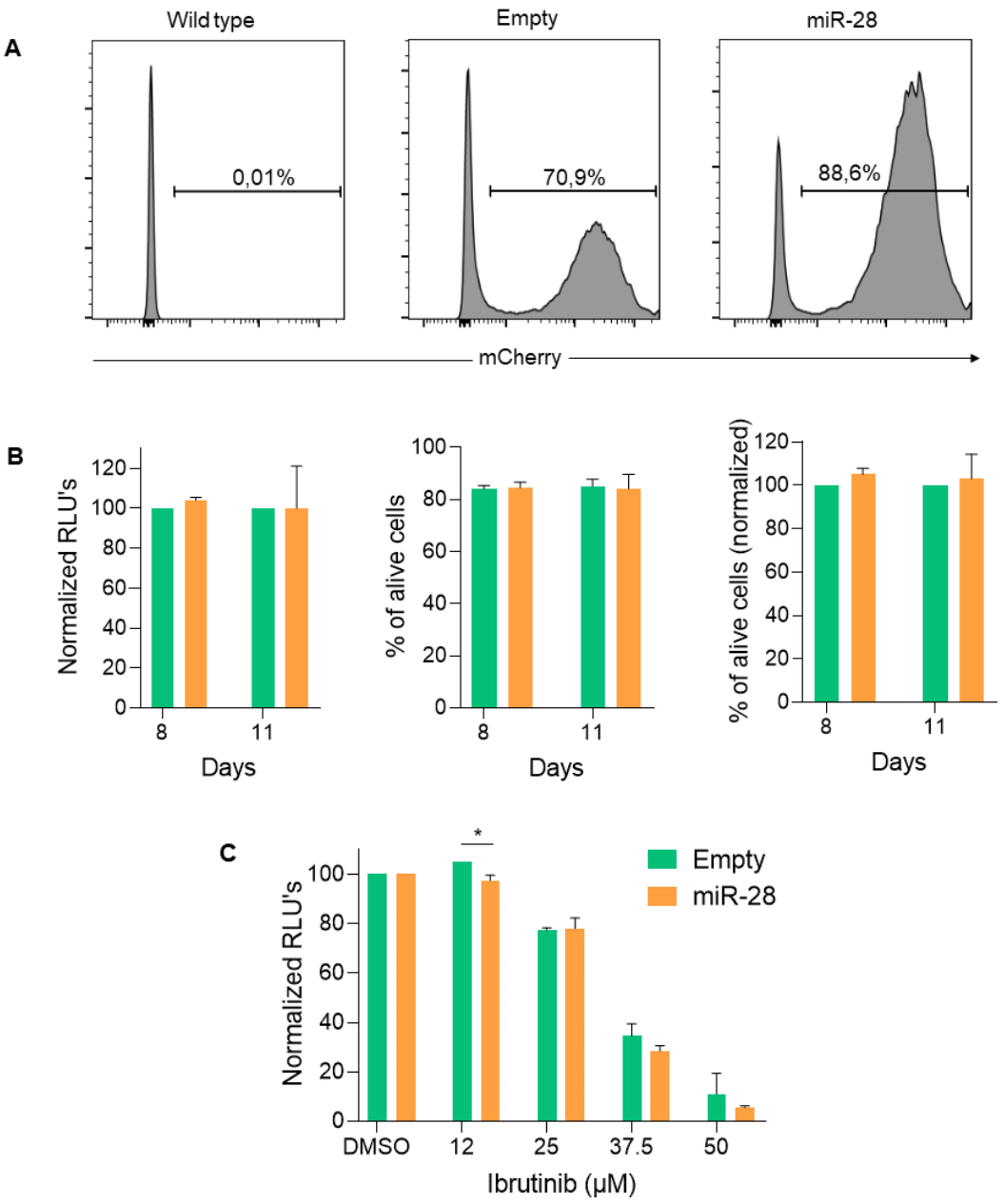

Figure 3. Re-expression of miR-28 by pCDH lentiviral transduction of the MD901 ABC-DLBCL cell line promotes a slight enhancement of the effect of ibrutinib in the inhibition of MD901 cell growth. (A) Flow cytometry histograms of mCherry+ MD901 cells measured 72 hours after empty/miR-28 pCDH transduction. (B) Transduced MD901 cells were cultured for 11 days and cell viability was assessed by ATP luminometry (left panel) and DNA-dye exclusion flow cytometry (middle and right panels), performing normalization to empty conditions. (C) Synergystic effect of miR-28 was assessed in combination with different ibrutinib concentrations by cell viability 72 hours after transduction. Results are displayed after normalization to control (DMSO) conditions. *P<0.05, unpaired Student t test.

3.4. miR-28-pCDH-transduction of SUDHL4 and U2932 DLCBL cell lines does not potentiate ibrutinib antitumoral effect.

To further expand the previous findings, ibrutinib synergy experiments were carried out in another ABC-DLBCL cell line (U2932) and a GC-DLBCL cell line (SUDHL4). Both cell lines were transduced with empty/miR-28 pCDH vectors and cultured for 3 days with previously sensitivity-determined ibrutinib concentration conditions. SUDHL4 cells transduction showed similar efficiency to MD901, while this was lower for U2932 cells (Figure 4A). The transduction advantage for the miR-28 construct observed in MD901 cells was also present in SUDHL4 and U2932 cells. As for miR-28 synergistic activity, none of the tested miR-28-ibrutinib conditions showed a statistically significant positive result for ATP luminometry-measured growth impairment when compared to the empty controls (Figure 4B), whereas a slight decrease in miR-28 alive U2932 cells was determined by DNA-dye exclusion by flow cytometry in all ibrutinib concentrations. However, no statistical analysis could be performed due to single-sample testing. Overall, these results raise the need to characterize in more detail the effect pCDH-mediated miR-28 reexpression exerts in the potentiation of ibrutinib anti-tumoral effect in DLBCL cells and to compare the levels of miR-28 expression achieved in DLCBL cells after pTRIPZ and pCDH lentiviral transduction.

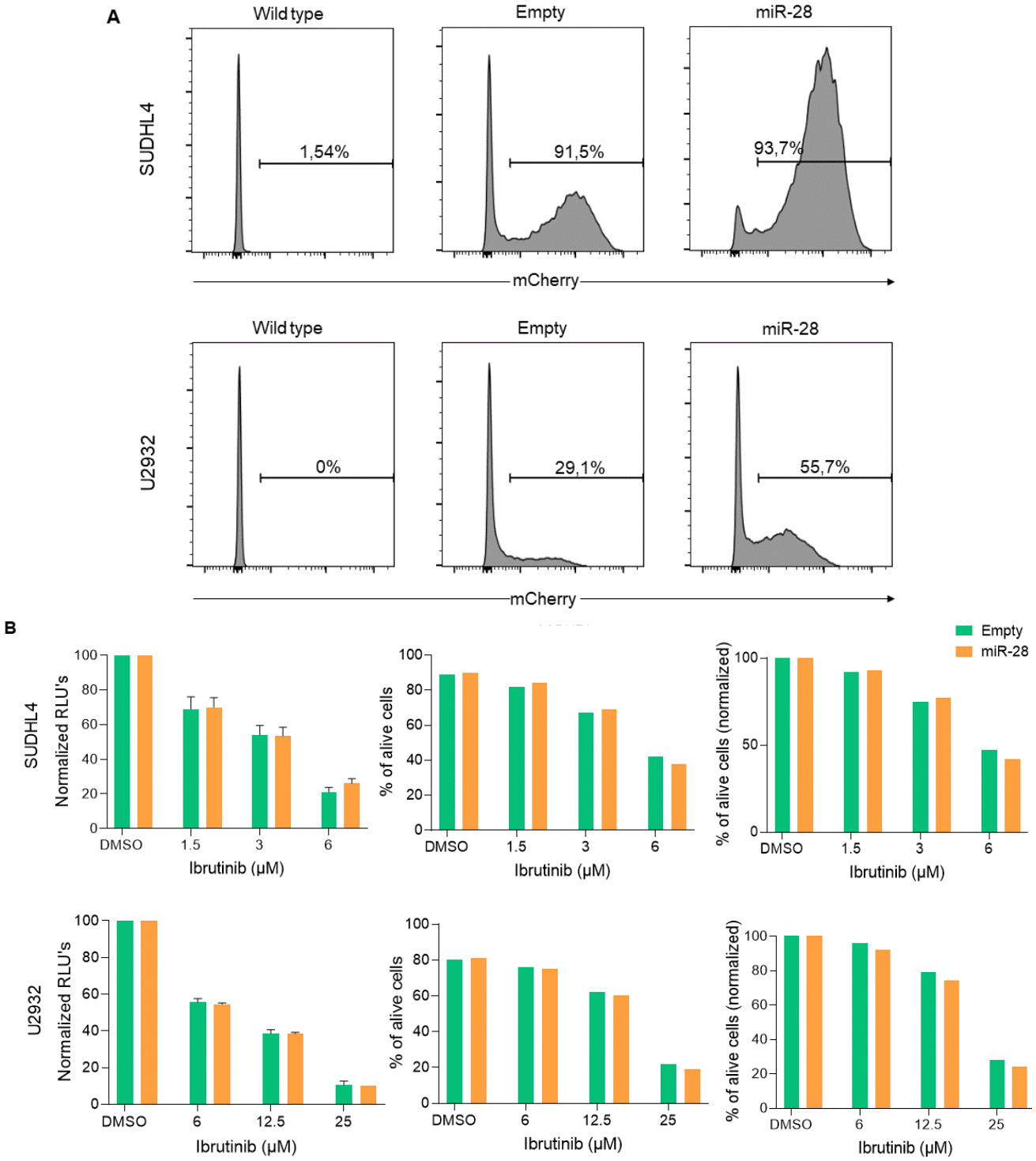

Figure 4. Re-expression of miR-28 by pCDH lentiviral transduction in U2932 ABC-DLBCL and SUDHL4 GC-DLBCL cell lines does not enhance ibrutinib antitumoral effect in the inhibition of DLBCL cell growth. (A) Flow cytometry histograms of mCherry+ SUDHL4 and U2932 cells measured 72 hours after empty/miR-28 pCDH transduction. (B) Synergystic effect of miR-28 was assessed in combination with different ibrutinib concentrations by measuring cell viability 72 hours after transduction. Left panels represent ATP cell viability luminometry results displayed after normalization to control (DMSO) conditions, whereas middle and right panels represent DNA-dye exclusion flow cytometry results before and after normalization to control (DMSO) conditions. No statistical analyses were performed for flow cytometry results due to single-sample testing.

4. DISCUSSION

The need to find novel therapeutic approaches remains a pending task to achieve successful and long-lasting tumoral response in some NHL types, especially in bad-prognosis subtypes such as ABC-DLBCL. In an attempt to overcome both obstacles and increase response rates while avoiding chemotherapy-related adverse effects, targeted-directed therapies have arisen as the alternative of choice. Among them, ibrutinib presents as a suitable agent due to its mechanism of action inhibiting BTK, a downstream effector in the NFkB pathway -the main dysregulated pathogenic pathway in ABC-DLBCL. Nevertheless, since monotherapy has been proven a suboptimal therapeutic regime, current research is centered in the development of combined therapeutic schemes.

miR-28 is a microRNA with proven antitumoral activity in various NHL types, including DLBCL. miR-28 regulates the BCR signaling network at different levels, including NFkB and PI3K -the other commonly dysregulated pathway in DLBCL. Accordantly, our lab previously hypothesized that combined ibrutinib-miR-28 based therapies could be a suitable therapeutic option, as both agents target effectors from the main DLBCL-related pathways.

Supporting this idea, unpublished results from previous experiments carried out in the lab showed a significant synergistic effect of miR-28 when combined *in vitro* with ibrutinib treatment in MD901 ABC-DLBCL cells. These findings, which were observed after miR-28 reexpression by pTRIPZ inducible lentiviral vector, merited further research regarding the potential of ibrutinib+miR-28 as a combined therapy. PDX mouse models represented the ideal alternative for this purpose since they allow both basic and preclinical testing[19] and would facilitate the extrapolation of results to clinical practice.

Since no published data was found regarding the use of pTRIPZ vector for PDX transduction and previous experiments carried out in the lab had proven pTRIPZ unsuccessful for such task, our group settled on designing and developing a new lentiviral tool that would assure efficient PDX transduction of miR-28. The pCDH vector was thought to be the best choice, according to Proxe's luciferization protocol and published references from other groups stating its previous successful use in lymphoid neoplasia PDX models[23,24].

After the pCDH plasmid was designed, purchased and modified to carry the miR-28 sequence, the first challenge to overcome was achieving successful transduction rates. Given that obtaining a high lentiviral titer is a main requisite for this purpose, we performed VSVG-pseudotyping of the lentiviral constructs, which enabled concentration by ultracentrifugation and therefore allowed the generation of high titer lentiviral supernatants (greater than $5x10^5$ PFU/µl, as tested in the Jurkat cell line). Subsequent pCDH transduction of the MD901 ABC-DLBCL cell line was proven highly efficient as well.

However, we found no antitumoral effect after pCDH-mediated miR-28 reexpression, and subsequent synergy experiments carried out in the MD901 cell line showed a notably lower potentiation of ibrutinib's cell growth impairment when compared to inducible miR-28 pTRIPZ-based expression. These findings raised a first concern regarding pCDH adequacy and conditioned the course of the subsequent research activity, making it necessary to perform additional experiments to test whether this result was shared among other DLBCL cell lines. This hypothesis was next confirmed, as no synergistic effect was identified for miR-28-pCDH mediated reexpression when combined with ibrutinib treatment in either the U2932 ABC-DLBCL or the SUDHL4 GC-DLBCL cell line.

Since lack of miR-28 expression and miR-28 sequence mutation were ruled out as possible explanations behind this phenomenon -expression of miR-28 was verified by qPCR amplification of the mature miRNA and no mutations were identified after sequencing by

Sanger the vector-, the most likely reason for this finding was limited miR-28 expression levels after pCDH transduction.

Supporting this idea, pTRIPZ lentiviral tool had specifically been designed to optimize miRNA transduction and expression -as stated in pTRIPZ technical manual-, in contrast to pCDH. Indeed, pTRIPZ shRNA constructs are expressed as human miRNA primary transcripts due to the miR-30 based hairpin design, which adds a processing site for the Drosha enzyme. The hairpin, which consists of a 19-nucleotide miR-30 loop, results in a tenfold rise in processing by Drosha and Dicer when compared to standard shRNA designs[25]. As Drosha and Dicer are the main enzymes responsible for miRNA maturation, this feature makes the pTRIPZ construct a reasonable candidate to achieve higher expression levels of human miRNAs than other constructs including pCDH.

Promoters used for pCDH and pTRIPZ expression also differ. The pCDH vector carries the constitutive EF1a promoter and the pTRIPZ vector carries two promoters of which TRE is in charge of miR-28 expression. In contrast to EF1a, TRE is activated by the rtTA transcriptional activator when induced by doxycycline. In this sense, a difference in promoter strength could account for a subsequent variation in expression levels. Supporting this, a systematic comparison of the most commonly used promoters in mammalian cell systems reported certain variations in promoter strength depending on which cell line was transduced. However, this study also revealed both EF1a and TRE promoters to have a notable and relatively consistent strength in most human cells[26], making this hypothesis plausible but not as likely as the aforementioned.

Overall, the absence of subsequent experiments to prove or discard a difference in pCDH- and pTRIPZ-miR-28 expression levels is the main limitation of this work. Towards that end, qPCR determination of mature miR-28 expression in two conditions of the same cell line

respectively transduced with pCDH and pTRIPZ would shed light on this issue and provide a definitive explanation.

Anyhow, regardless of the outcome of the aforementioned experiment, data obtained in this work proved pCDH unsuitable for miR-28-ibrutinib synergy testing in DLBCL cell lines and, presumably, DLBCL-PDX models. Consequently, further research on this matter should draw on alternative tools. Treatment of PDX models with a miR-28 synthetic mimic, as performed in previous *in vivo* miR-28 antitumoral activity experiments carried out by our group, is a suitable and safe alternative to lentivirally-induced miR-28 reexpression -although of a significantly higher cost. As for *in vitro* experiments, pTRIPZ-mediated transduction provides an adequate opportunity to expand the previous miR-28-ibrutinib positive synergy results obtained in the MD901 cell line to additional human DLBCL cell lines. Indeed, after the completion of this work, miR-28-ibrutinib synergy assays were performed in pTRIPZ-transduced SUDHL4 and U2932 cells, obtaining positive results which support the promising future of ibrutinib-miR28 therapeutics for DLBCL and merit further research to be pursued.

5. CONCLUSIONS

1. We have verified the correct cloning of miR-28 precursor in the pCDH vector by sequencing and the expression of the mature miR-28 miRNA molecule expression in miR-28-pCDH transfected cells by qPCR.
2. The VSVG pseudotyped empty and miR-28 pCDH constructs allow the generation of lentiviral supernatants at a high titer (greater than $5x10^5$ PFU/µl) and the efficient transduction of MD901, SUDHL4 and U2932 human DLBCL cell lines.
3. pCDH-mediated miR-28 reexpression in DLBCL cell lines shows a limited enhancement of ibrutinib antitumoral activity when compared to other miR-28 lentiviral based expression systems.

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7. ANNEX

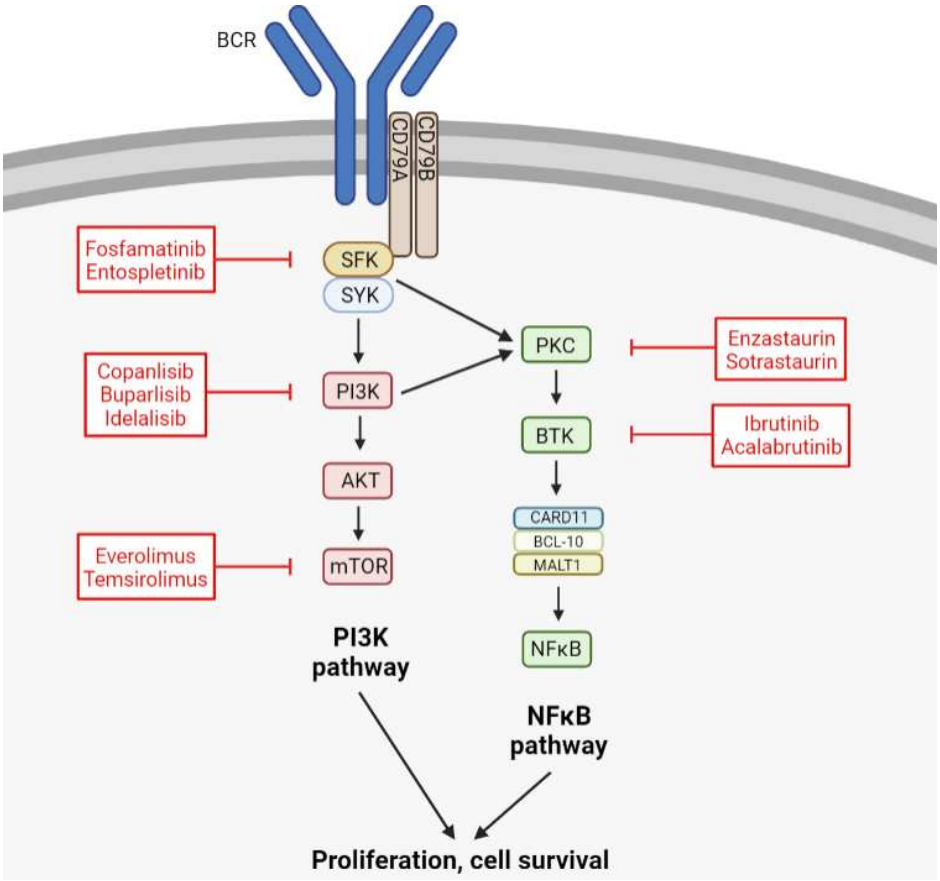

Figure 1 (annex): Most commonly dysregulated pathways leading to B-cell neoplastic transformation and directed therapies developed to date against different effectors from both pathways. Adapted from: Dunleavy K, Erdmann T, Lenz G. Targeting the B-cell receptor pathway in diffuse large B-cell lymphoma. Cancer Treat Rev. 2018;65:41-46.

PUBLICATIONS:

- Fuertes T, **Salgado I**, de Yébenes VG. microRNA fine-tuning of the germinal center response. Front Immunol. 2021;12:660450.

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