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PhD Thesis

Inhibition of SHP1 and SHP2 as a molecular targeted therapy against myeloid leukaemias

PROGRAMA DE DOCTORADO EN FISIOPATOLOGÍA Y FARMACOLOGÍA

ALEJANDRO PÉREZ FERNÁNDEZ

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D. ÁNGEL HERNÁNDEZ HERNÁNDEZ, Profesor Titular de Universidad del Departamento de Bioquímica y Biología Molecular de la Universidad de Salamanca,

CERTIFICA:

Que la presente Memoria de Tesis Doctoral titulada ***“Inhibition of SHP1 and SHP2 as a molecular targeted therapy against myeloid leukaemias”,*** presentada por D. Alejandro Pérez Fernández para optar al Grado de Doctor en Fisiopatología y Farmacología, ha sido realizada bajo su dirección en el Departamento de Bioquímica y Biología Molecular de la Universidad de Salamanca.

Considerando que la Tesis Doctoral se halla concluida, autorizo su presentación para que sea evaluada por el Tribunal correspondiente.

Y, para que así conste, firmo el presente documento en Salamanca, a 29 de octubre de 2019.

Fdo. Ángel Hernández Hernández.

D. ALEJANDRO PÉREZ FERNÁNDEZ, Graduado en Biotecnología y Máster en Biología Celular y Molecular por la Universidad de Salamanca,

DECLARA:

Que es Autor de la presente Tesis Doctoral, titulada ***“Inhibition of SHP1 and SHP2 as a molecular targeted therapy against myeloid leukaemias”,*** realizada en el Departamento de Bioquímica y Biología Molecular de la Universidad de Salamanca bajo la dirección del Profesor Dr. D. Ángel Hernández Hernández.

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LIST OF ABBREVIATIONS

2-HG: 2-hydroxyglutarate.

5-hmC: 5-hydroxymethylcytosine.

5-mC: 5-methylcytosine.

ABC: ATP-binding cassette.

ABCB1: ATP binding cassette subfamily B member 1.

ABL1: ABL proto-onocogene 1, non-receptor tyrosine kinase.

AGM: aorta-gonad-mesonephros.

AKT: protein kinase B.

Allo-HSCT: allogeneic haematopoietic stem cell transplantation.

ALOX5: arachidonate 5-lipoxygenase.

AML: acute myeloid leukaemia.

APC: adenomatous polyposis coli.

AP-CML: accelerated phase of CML.

APL: acute promyelocytic leukaemia.

AraC: arabinocytosine (cytarabine).

ASXL1: ASXL transcriptional regulator 1.

ATO: arsenic trioxide.

ATP5B: ATP synthase F1 subunit beta.

ATRA: all-*trans*-retinoic acid.

AXIN: axis inhibitor.

AXL: AXL receptor tyrosine kinase.

B2M: beta-2-microglobulin.

BC-CML: blast crisis of CML.

BCL2: B cell leukemia/lymphoma-2.

BCR: breakpoint cluster region.

BET: bromodomain extra-terminal domain.

BIRC5: baculoviral IAP repeat containing 5.

BM: bone marrow.

BMI1: BMI1 proto-oncogene, polycomb ring finger.

BRAF: B-Raf proto-oncogene, serine/threonine kinase.

BSA: bovine serum albumin.

CASP3: caspase 3.

CBFB: core-binding factor subunit beta.

CBY1: chibby 1.

CC: coiled-coil domain.

CCL3: C-C motif chemokine ligand 3.

CCND1: cyclin D1.

CCND2: cyclin D2.

CDC73: cell division cycle 73.

CDK: cyclin-dependent kinase.

CDKI: CDK inhibitor.

CDKN1A: cyclin dependent kinase inhibitor 1A.

CDKN1B: cyclin dependent kinase inhibitor 1B.

CDKN1C: cyclin dependent kinase inhibitor 1C.

CEBPA: CCAAT enhancer binding protein alpha.

CFU: colony-forming unit.

CI: combination index.

CITED2: Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2.

CLP: common lymphoid progenitor.

CML: chronic myeloid (or myelogenous) leukaemia.

CMP: common myeloid progenitor.

CP-CML: chronic phase of CML.

CR: complete remission.

CRKL: CRK-like proto-oncogene, adaptor protein.

C_t: threshold cycle.

CTNNB1: catenin beta 1.

CXCL12: C-X-C motif chemokine ligand 12.

CXCR4: C-X-C motif chemokine receptor 4.

DAPI: diaminophenylindol.

DHODH: dihydroorotate dehydrogenase.

DMSO: dimethyl sulfoxide.

DNMT3A: DNA methyltransferase 3 alpha.

DOT1L: DOT1-like histone lysine methyltransferase.

DPBS: Dulbecco’s phosphate-buffered saline.

DPI: diphenylene iodonium.

DVL: dishevelled.

E2A-HLF: transcription factor 3-hepatic leukemia factor.

EC: endothelial cell.

EDTA: ethylenediamine tetraacetic acid.

EF-1α: elongation factor 1 alpha.

EGFP: enhanced green fluorescent protein.

ELN: European leukaemia net.

EMA: European medicines agency.

ENOX2: ecto-NOX disulfide-thiol exchanger 2.

ERK: extracellular regulated kinase.

ESC: embryonic stem cell.

FAB: French-American-British.

FAS: Fas cell surface death receptor.

FBS: foetal bovine serum.

FDA: food and drug administration.

FITC: fluorescein 5-isothiocyanate.

FLT3: fms-like tyrosine kinase 3.

FoxO3a: forkhead box O 3A.

FZD: frizzled.

FZD7: frizzled class receptor 7.

GAB2: GRB2 associated binding protein 2.

GATA2: GATA-binding protein 2.

GFP: green fluorescent protein.

GMP: granulocyte-monocyte progenitor.

GO: gemtuzumab ozogamicin.

GRB2: growth factor receptor-bound protein 2.

GSK3β: glycogen synthase kinase 3 beta.

HBSS: Hank’s balanced salt solution.

HD: healthy donor.

HDAC: histone deacetylase.

HES1: hairy enhancer of split 1.

Hh: hedgehog.

HMA: hypomethylating agent.

HoxA10: homeobox A 10.

HRP: horseradish peroxidase.

HSC: haematopoietic stem cell.

HSPC: haematopoietic stem and progenitor cell.

ICAT: catenin beta interacting protein 1.

IDH: isocitrate dehydrogenase.

IFN: interferon.

IM: imatinib mesylate.

iPSC: induced pluripotent stem cell.

IRF8: interferon regulatory factor 8.

IRIS: International Randomised study of Interferon and cytarabine versus STI-571.

ITD: internal tandem duplication.

ITIM: immuno-receptor tyrosine-based inhibitory motif.

JAK2: janus kinase 2.

KDM1A: histone lysine demethylase 1A.

KIT: KIT proto-onocogene, receptor tyrosine kinase.

KLF4: Kruppel like factor 4.

KMT2A: lysine methyltransferase 2A.

KOSR: knock out serum replacement.

KRAS: KRAS proto-onocogene.

LEF1: lymphoid enhancer binding factor 1.

LMPP: lymphoid-primed multipotent progenitor.

LRP: low density lipoprotein-related protein.

LSC: leukaemic stem cell.

LTC-IC: long term culture-initiating cells.

MCL1: MCL1 apoptosis regulator, BCL2 family member.

MDS: myelodysplastic syndrome.

MegE: megakaryocyte-erythrocyte progenitor.

MK: megakaryocyte.

MkP: megakaryocyte progenitor.

MNC: mononuclear cell.

MOI: multiplicity of infection.

MPP: multi-potent progenitor.

MRD: minimal residual disease.

MSC: mesenchymal stem cell.

MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide.

MYC: MYC proto-oncogene, bHLH transcription factor.

MYH11: myosin heavy chain 11.

MΦ: macrophage.

N/A: not available.

N/D: not detected.

NANOG: Nanog homeobox.

NBs: nuclear bodies.

NDC: no drug control.

NF1: neurofibromin 1.

NGS: next generation sequencing.

NK: normal karyotype / natural killer.

NL: nilotinib.

NOD-SCID: non-obese diabetic-severe combined immunodeficient.

NOX: NADPH oxidase.

NPM1: nucleophospmin 1.

NRAS: NRAS proto-oncogene.

NSC: NSC 87877.

OB: osteoblast.

OCT1: organic cation transporter 1.

OCT3/4: POU class 5 homeobox 1.

OCTN2: organic cation/carnitine transporter 2.

ORF: open reading frame.

OS: overall survival.

PAGE: polyacrlylamide gel electrophoresis.

PB: peripheral blood.

PBS: phosphate-buffered saline.

PDGFR: platelet-derived growth factor receptor.

PDX: patient-derived xenograft.

PE: phycoerythrin.

PEI: polyethyleneimine.

Pen-Strep: penicillin/streptomycin.

PFA: paraformaldehyde.

PI: propidium iodide.

PI3K: phosphatidylinosytol 3-kinase.

PKC: protein kinase C.

PLZF: promyelocytic leukemia zinc finger.

PMA: phorbol 12-myristate-13-acetate.

PML: promyelocytic leukemia.

PORCN: porcupine O-acyltransferase.

PP2A: protein phosphatase 2 phosphatase activator.

PRAME: preferentially expressed antigen in melanoma.

PRC: polycomb repressor complex.

PreE: pre-colony-forming erythroid progenitor.

Pre-GM: pre-granulocyte-monocyte.

PRS: prostratin.

PS: phosphatidylserine.

PTK: protein tyrosine kinase.

PTP: protein tyrosine phposphatase.

PTP1B: protein tyrosine phosphatase non-receptor type 1.

PTPN6: protein tyrosine phosphatase, non-receptor type 6.

PTPN11: protein tyrosine phosphatase non-receptor type 11.

PTPN13: protein tyrosine phosphatase, non-receptor type 13.

pTyr: phoshotyrosine.

PVDF: polyvinylidene fluoride.

r/r AML: relapsed or refractory acute myeloid leukaemia.

RARA: retinoic acid receptor alpha.

Rho/GEF: Ras homolog gene family/guanine nucleotide exchange factor.

RNF20: ring finger protein 20.

ROS: reactive oxygen species.

RT: room temperature.

RTK: receptor tyrosine kinase.

RUNX1: RUNX family transcription factor 1.

RUNX1T1: RUNX1 partner transcriptional co-repressor 1.

S/T-K: serine/threonine kinase domain.

SDS: sodium dodecyl sulphate.

SET: SET nuclear proto-oncogene.

SFKs: SRC family kinases.

SFRP1: secreted frizzled-related protein 1.

SFRP2: secreted frizzled-related protein 2.

SH1: SRC homology domain 1.

SH2: SRC homology domain 2.

SH3: SRC homology domain 3.

SHP1: SH2 domain containing protein tyrosine phosphatase 1.

SHP2: SH2 domain containing protein tyrosine phosphatase 2.

SMO: smoothened.

SOX2: SRY-box transcription factor 2.

SOX6: SRY-box transcription factor 6.

SOX9: SRY-box transcription factor 9.

SOX17: SRY-box transcription factor 17.

SSG: sodium stibogluconate.

STA: specific target amplification.

STAT5: signal transducer and activator of transcription 5.

TBS-T: tris-buffered saline with Tween 20.

TCF/LEF: T cell factor/lymphoid enhancer factor.

TCF7: transcription factor 7.

TCF7L1: transcription factor 7 like 1.

TCF7L2: transcription factor 7 like 2.

TCP: tranylcypromine.

TET2: tet methylcytosine dioxygenase 2.

TGFβ: transforming growth factor beta.

TKD: tyrosine kinase domain.

TKI: tyrosine kinase inhibitor.

TLE1: TLE family member 1, transcriptional corepressor.

TLE2: TLE family member 2, transcriptional corepressor.

TLE3: TLE family member 3, transcriptional corepressor.

TLE4: TLE family member 4, transcriptional corepressor.

T_m: melting temperature.

TP53: tumour protein 53.

TPA: 12-O-tetradecanoylphorbol-13-acetate.

T_{reg}: regulatory T lymphocyte.

TYW1: tRNA-yW synthesizing protein 1 homolog.

UBD: ubiquitin binding domain.

UBE2D2: ubiquitin conjugating enzyme E2 D2.

UDG: uracil-DNA-glycosyase.

VAF: variant allele frequency.

WHO: World Health Organisation.

WNT1: Wnt family member 1.

WNT2: Wnt family member 2.

WNT3A: Wnt family member 3A.

WNT5A: Wnt family member 5A.

WT1: Wilms tumour protein 1.

α-KG: alpha-ketoglutarate.

β-TRC: beta-transducin repeat-containing E3 ubiquitin protein ligase.

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ABSTRACT

Haematopoiesis is a very relevant differentiation process in adult humans where a multipotent cell, the haematopoietic stem cell (HSC), generates a widely varied, fully differentiated progeny, with immune defence, nutrient exchange and volume homeostasis functions. The regulatory cues governing the biology of HSCs must be tightly regulated in order to ensure their own self-renewal, as well as the proper turnover of differentiated cells. These signals are provided by the surrounding environment, known as niche, integrated by both haematopoietic and non-haematopoietic cells. The disruption of this fine equilibrium by alteration of either the external signals or their intracellular transduction in haematopoietic stem and progenitor cells (HSPCs) leads to the development of haematologic malignancies, including leukaemia.

An important blood disorder affecting the myeloid lineage is acute myeloid leukaemia (AML). It is especially recurrent among the elderly, with a median age at diagnosis of 70 years, a fact to be considered due to the increasing life expectancy in Western countries. AML is a highly heterogeneous and aggressive disease with poor prognosis and, in general, no significant therapeutic improvements beyond chemotherapy over the last four decades. An exception to this scenario is the treatment of acute promyelocytic leukaemia (APL), which is highly responsive to pro-differentiative therapy, consisting of all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO). Unlike AML, CML is highly homogeneous in terms of molecular biology, with the expression of the fusion oncokinase breakpoint cluster region-ABL proto-oncogene 1, non-receptor tyrosine kinase (BCR-ABL) as the main pathogenic driver. In the early 2000s, the clinical use of tyrosine kinase inhibitors (TKI) targeting this protein revolutionised the management of CML due to great improvements in treatment response and survival rates. However, this disease remains challenging in particular cases.

Despite its heterogeneous nature, AML displays a differentiation blockage as a hallmark. This feature, together with the example of the differentiation-based APL treatment, has prompted the development of an important line of research focusing on the molecular mechanisms governing cell differentiation during haematopoiesis. This knowledge would lead to a better understanding of the dysregulated processes leading to pathogenesis and their subsequent pharmacological targeting to treat the disease. In line with this, the present work sought to assess in detail the involvement of SRC homology 2 domain containing protein tyrosine phosphatases 1 (SHP1) and 2 (SHP2) in the differentiation of leukaemic cells and the potential of these molecules as pharmacological targets for AML. Herein, it was demonstrated the cooperative function of both phosphatases in phorbol ester-induced cell differentiation, with an enhanced differentiated phenotype of cells subjected to simultaneous downregulation of these proteins. In addition, the kinase SRC was identified as a downstream target of SHP2 in this process, which appeared to influence the extent of the differentiation stimulus triggered by phorbol 12-myristate-13-acetate (PMA). Besides, the role of both phosphatases on cell differentiation showed to be partially overlapping through the regulation of β-catenin protein levels. Based on this evidence, the chemical inhibitor of SHP1 and SHP2 NSC 87877 (NSC) was successfully tested to boost the differentiation-inducing effect of phorbol esters in the AML cell line HL-60. Moreover, this compound synergised with the phorbol ester 13-O-acetyl-12-deoxyphorbol or prostratin (PRS) to prevent proliferation of not only in HL-60 cells, but also additional cell lines used as AML models (NB-4, OCI-AML2 and THP-1). Most importantly, the anti-leukaemic activity of this combination was corroborated *in vivo* with a xenograft mouse model and in primary cells from AML patients *ex vivo.*

In summary, the results described in the present work support a promising therapeutic potential of the chemical inhibitor NSC 87877 in combination with phorbol esters to treat AML. Furthermore, some mechanistic insight on the molecules connected to these phosphatases in both disease biology and pharmacological mode of action of the inhibitor has been provided.

RESUMEN

La hematopoyesis es un proceso de diferenciación muy relevante en el humano adulto, en el cual una célula multipotente, la célula madre hematopoyética (HSC de sus siglas en inglés), genera una amplia variedad de células plenamente diferenciadas con funciones de defensa inmune, intercambio de nutrientes y homeostasis de volumen. Las señales que regulan la biología de las HSCs deben estar estrechamente reguladas para asegurar su capacidad de autorrenovación y el recambio adecuado de células diferenciadas. Estas señales provienen del ambiente que las rodea, conocido como nicho, que está integrado por células hematopoyéticas y de otros linajes. La ruptura de este delicado equilibrio, bien por la alteración de las señales externas o por su transducción intracelular en las células madre y progenitores hematopoyéticos (HSPCs, de sus siglas en inglés) conduce al desarrollo de enfermedades hematológicas, incluyendo la leucemia.

Un importante trastorno hematológico que afecta al linaje mieloide es la leucemia mieloide aguda (LMA). Esta enfermedad es especialmente recurrente entre las personas de edad avanzada, con una mediana de edad de diagnóstico de 70 años, un hecho a tener en cuenta dado el incremento en la esperanza de vida acontecido en países occidentales. La LMA es una enfermedad agresiva y altamente heterogénea con mal pronóstico para la que, en general, no ha habido avances terapéuticos significativos más allá de la quimioterapia en las últimas cuatro décadas. Una excepción a este panorama es el tratamiento de la leucemia promielocítica aguda (LPA), que responde muy bien a la terapia prodiferenciadora con ácido todo-*trans*-retinoico (ATRA, de sus siglas en inglés) y trióxido de arsénico (ATO, de sus siglas en inglés).

A pesar de su naturaleza heterogénea, la LMA presenta un bloqueo en la diferenciación como uno de sus rasgos distintivos. Esta característica, unida al ejemplo del tratamiento de la LPA, basado en la diferenciación, ha impulsado el desarrollo de una importante línea de investigación centrada en los mecanismos moleculares que gobiernan la diferenciación celular durante la hematopoyesis. Este conocimiento daría lugar a una mejor comprensión de las alteraciones patogénicas y su uso como dianas farmacológicas para tratar la enfermedad. En este sentido, el presente trabajo tuvo como objetivo la evaluación detallada del papel de las quinasas con dominios de homología a SRC tipo 2 (SH2) 1 (SHP1) y 2 (SHP2) en la diferenciación de células leucémicas y su potencial como dianas farmacológicas para la LMA. Se demostró la función cooperativa de ambas fosfatasas en la diferenciación celular inducida por ésteres de forbol, con un fenotipo más diferenciado en células donde la expresión de dichas proteínas estaba disminuida simultáneamente. Además, la quinasa SRC fue identificada como una diana aguas abajo de SHP2 en este proceso, lo que parecía influir en la intensidad del estímulo diferenciador iniciado por 12-miristato-13-acetato de forbol (PMA, de sus siglas en inglés). Asimismo, se reveló que el papel de estas fosfatasas en la diferenciación celular se solapaba parcialmente a través de la regulación de los niveles de la proteína β-catenina. Con base en estos resultados, se probó con éxito el inhibidor químico de SHP1 y SHP2 NSC 87877 (NSC) para potenciar el efecto inductor de la diferenciación de los ésteres de forbol en la línea celular de LMA HL-60. Por otro lado, este compuesto mostró un efecto sinérgico con el éster de forbol 13-O-acetil-12-desoxiforbol, o prostratina (PRS), en la disminución de la proliferación no solo de células HL-60, sino también en otras líneas celulares modelo de LMA (NB-4, OCI-AML2 y THP-1). Lo que es más importante, la actividad antileucémica de esta combinación fue corroborada *in vivo,* en un modelo de ratón xenoinjerto, y *ex vivo,* sobre células primarias de pacientes de LMA.

En resumen, los resultados descritos en el presente trabajo apoyan un potencial terapéutico prometedor del inhibidor químico NSC 87877 en combinación con ésteres de forbol para el tratamiento de la LMA. Asimismo, se han revelado algunos detalles de los mecanismos que implican a las moléculas ligadas a estas fosfatasas, tanto en la biología de dicha enfermedad como en el modo de acción del inhibidor.

INTRODUCTION

1. INTRODUCTION

The adult human being is a complex organism composed of myriads of different cell types, each of which has very specific functions that co-ordinately contribute to a correct physiology. However, these differentiated cells lack self-renewal potential, have a limited lifespan and need to be replaced in order to sustain the regular function of different tissues and organs. Adult stem cells are responsible for this goal. These cells remain in an undifferentiated stage, which allows them to undergo cell divisions and originate more stem cells or new committed progenitors, thus ensuring the proper turnover of functional differentiated cells.

Contrary to embryonic stem cells (ESCs), which are pluripotent –they can generate the three embryonic germ layers–, adult stem cells are uni- or multipotent, thus giving rise to only one or a few mature cell types, respectively. There is a restricted group of adult stem cells in the human body, namely haematopoietic, neuronal, intestinal, mesenchymal, satellite and epidermal stem cells (Dulak *et al*, 2015). Among them, haematopoietic stem cells (HSCs) have been extensively studied due to their ability to generate a wide range of mature cell types and their close similarities with leukaemic stem cells (LSCs), which account for drug resistance and disease relapse in blood cancers.

1.1. Relevant aspects of haematopoiesis

1.1.1. Haematopoietic stem cells: the origins of blood

The primitive emergence of HSCs has been described to occur in mammals in a dorsal region of the embryo known as the aorta-gonad-mesonephros (AGM). Although it was initially suggested that the origin of HSCs was the haemangioblast, a cell also capable of generating endothelial cells, this remains nowadays controversial. Regardless of its origin, HSCs migrate from the AGM to several locations during development (the so-called *waves* of haematopoiesis) until they settle in the bone marrow (BM), where they stay throughout the entire lifetime (Orkin & Zon, 2008).

The presence of HSCs in adult human BM was previously demonstrated as a result of the combination of functional assays (comprising long-term culture-initiating cells –LTC-IC– and *in vivo* repopulation assays) and immunophenotype-based selection of restricted populations within the bulk BM through flow cytometry approaches (Sutherland *et al*, 1989; Baum *et al*, 1992; Bhatia *et al*, 1997; Murray *et al*, 1994). These experiments have led to a progressive refinement of the functionally defined HSCs and simultaneously revealed some degree of heterogeneity in terms of immunophenotype, repopulation ability and lineage outputs (Laurenti & Göttgens, 2018).

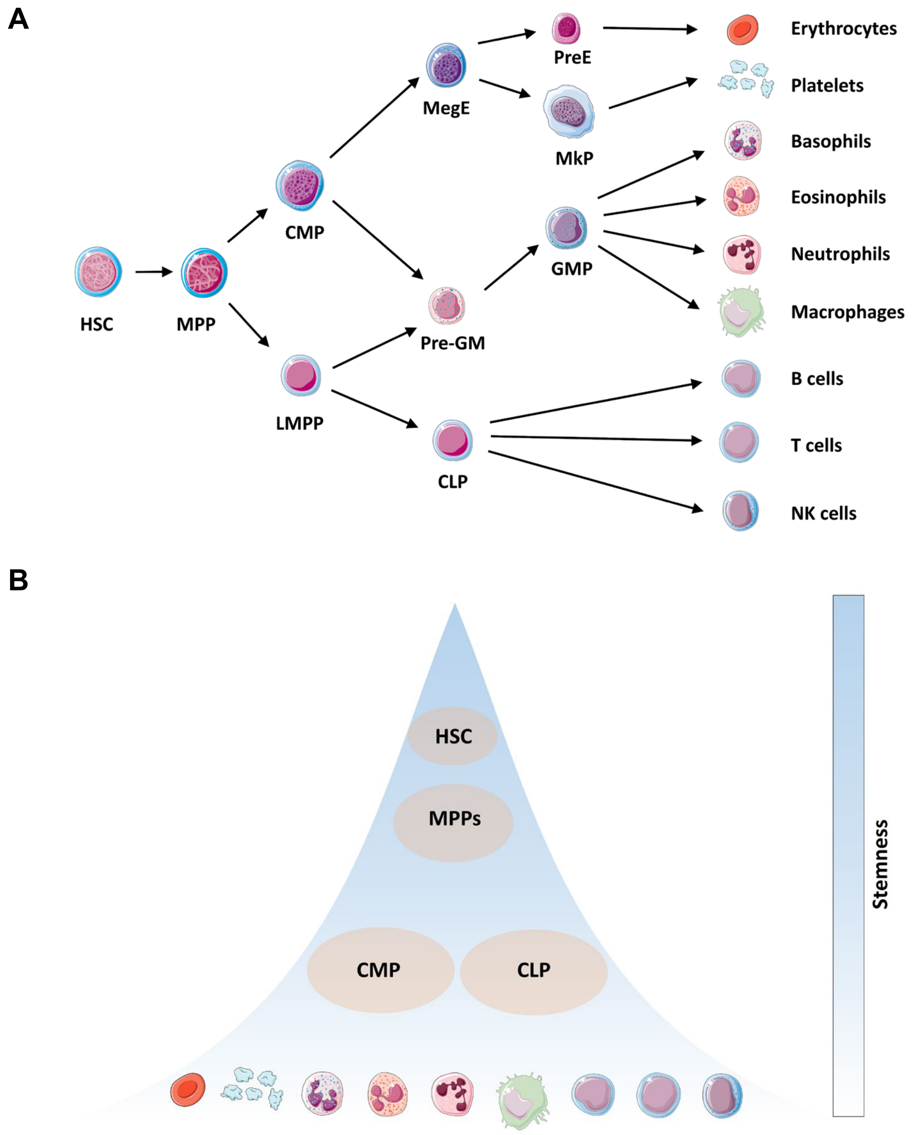
In order to have the HSC status, a cell must be capable of both self-renewing throughout time and generating all blood cell types on a single-cell level (Doulatov *et al*, 2012). Phenotypically, these cells maintain their integrity through a tight regulation of their metabolic activity: they remain highly quiescent, rely on autophagy, have a glycolytic rather than mitochondrial metabolism and keep low levels of protein synthesis (Laurenti & Göttgens, 2018). However, despite all these common features, the previously highlighted heterogeneity and the fact that purification of candidate HSCs has been based on immunophenotypic selection of cell subsets from a complex BM population (Jacobsen & Nerlov, 2019) make the term 'HSC compartment' more accurate.

1.1.2. The haematopoietic hierarchy: revisiting the classical model

The classical model of haematopoiesis situates the HSC compartment at the apex of a hierarchical tree and makes at least three assumptions to explain the whole process: i) lineage decisions are subsequent to a loss of self-renewal capacity, ii) the first step of lineage commitment distinguishes two main branches: lymphoid and myeloid, and iii) lineage decisions occur successively as bifurcations of a given branch of the tree (Doulatov *et al*, 2012).

The first loss of self-renewal potential occurs during the transition of HSC to a multi-potent progenitor (MPP). This compartment can still give rise to all blood lineages, but its repopulation capacity is more limited than that of HSCs (Doulatov *et al*, 2012). After the MPP node, two new compartments arise, namely the common myeloid progenitor (CMP), able to generate all the myelo-erythroid mature lineages, and the lymphoid-primed multipotent progenitor (LMPP), with potential to generate both myeloid and lymphoid cells. The next bifurcation step will lead to the three main branches before unipotent progenitors: the megakaryocyte-erythrocyte progenitor (MegE), the pre-granulocyte-monocyte (Pre-GM) and the common lymphoid progenitor (CLP) compartments. Furthermore, the Pre-GM compartment will experience an additional round of self-renewal ability loss, which generates the granulocyte-monocyte progenitor (GMP) compartment, precursor of all myeloid unipotent progenitors and eventually myeloid cells (**Figure 1.1A**) (Jacobsen & Nerlov, 2019).

The classical model of haematopoiesis has been lately challenged owing to experimental observations supporting (i) the functional heterogeneity found in discrete immunophenotype-based compartments defined to date (Jacobsen & Nerlov, 2019; Laurenti & Göttgens, 2018), (ii) the early lineage commitment observed in immunophenotypical multi- or oligo-potent compartments (Notta *et al*, 2016; Haas *et al*, 2018), (iii) the continuity of the process rather than a tree-like behaviour (Velten *et al*, 2017) and (iv) the differences found between studies under steady-state versus stress conditions (Sun *et al*, 2014; Haas *et al*, 2018). Therefore, new depictions of the process based on these new findings have been proposed


A
HSC
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CMP
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MegE
Pre-GM
CLP
PreE
MkP
GMP
Erythrocytes
Platelets
Basophils
Eosinophils
Neutrophils
Macrophages
B cells
T cells
NK cells
B
HSC
MPPs
CMP
CLP
Stemness

Figure 1.1. ***Different models to explain the haematopoietic differentiation.*** **A)** Traditional model of haematopoiesis with a hierarchical tree structure and strictly defined progenitor subpopulations. PreE: pre-colony-forming erythroid progenitor; MkP: megakaryocyte progenitor. Adapted from Jacobsen & Nerlov, 2019. **B)** Scheme of revisited models of haematopoiesis highlighting the continuous nature of the process and the diffuseness of the transitions between immature progenitors and more committed cells. Adapted from Haas *et al,* 2018.

(Laurenti & Göttgens, 2018; Haas *et al*, 2018) (**Figure 1.1B**). The new paradigm has important consequences when driver events of haematologic malignancies are considered, especially regarding the isolation and pharmacological targeting of putative LSCs.

1.1.3. The HSC niche: the temple of blood integrity

Blood functions comprise gas exchange, volume homoeostasis and defence against pathogens. Therefore, the need of distinct blood cell types is highly variable at different time points. The haematopoietic stem and progenitor cells (HSPCs) must be able to sense all these signals and initiate responses that allow the maintenance of the physiologic condition. Nonetheless, this proliferating status needs to keep a delicate balance with quiescence for two main reasons: to avoid the exhaustion of the progenitor pool that would prevent future responses, and to preserve the genomic integrity, which, if severely altered, can lead to haematologic malignancies (Pinho & Frenette, 2019).

The cues that help to maintain this fine-tuned equilibrium are provided by the surrounding environment, known as *niche.* This term was initially proposed by Schofield in 1978, who suggested that the *stemness* of HSCs was determined by the cells they were in association with. Consequently, leaving this particular environment would commit them to more differentiated progenitors (Schofield, 1978). Ever since, an extensive body of work has confirmed the existence of the niche and the cellular architecture and signalling molecules involved in HSC regulation have been progressively elucidated. Despite that, the distribution of HSCs, their cellular interaction partners and the nature of these relationships are still far from being fully understood.

The evidence available so far is essentially based on transgenic mouse models and surface immunophenotype-based purification of BM cell populations and shows that the BM niche is comprised of both non-hematopoietic and hematopoietic cells.

Within the first group, osteoblasts (OBs) were traditionally considered important promoters of HSC maintenance, although this view has been ultimately questioned in favour of a role on the control of more committed lymphoid progenitors (Frisch, 2019; Pinho & Frenette, 2019). Endothelial cells (ECs), on the other hand, provide regulatory cues for HSC support and mobilisation at different levels (physico-chemical, mechanical and biological) (Sugiyama *et al*, 2019; Frisch, 2019; Pinho & Frenette, 2019). In addition, mesenchymal stem cells (MSCs) secrete different cytokines important for maintenance and release of HSCs to the bloodstream, this latter aspect in cooperation with sympathetic nervous fibres (Frisch, 2019; Pinho & Frenette, 2019). Conversely, non-myelinating Schwann cells regulate HSC quiescence. Finally, adipocytes appear to be essential for emergency haematopoietic reconstitution whereas deleterious if present in unbalanced numbers (Cuminetti & Arranz, 2019).

Among the haematopoietic components supporting HSCs, megakaryocytes (MKs), macrophages (MΦs), neutrophils, regulatory T (T_{reg}) lymphocytes and HSPCs can be named. MKs interact with specific subsets of HSCs to regulate their quiescence through distinct cytokines. MΦs are regulators of HSC quiescence as well, together with their retention in an indirect manner through interaction with OBs and MSCs. On the other hand, neutrophils are positive regulators of HSC self-renewal and engraftment, as well as HSPC expansion in emergency myelopoiesis. Additionally, T_{reg} lymphocytes have shown to favour allogeneic HSC transplantation (allo-HSCT) (Pinho & Frenette, 2019; Cossío *et al*, 2019). Finally, it is worth noting the proliferation-promoting effect of HSPC-HSPC contact by limiting the availability of transforming growth factor beta (TGFβ) for this subset (May *et al*, 2018).

As it can be assumed, the increasing number of niche components depicts a very complex landscape in terms of regulatory networks governing HSC fate. We have previously suggested the possibility that every single HSPC might be located in a unique niche comprised of a particular combination of cellular partners interacting with both HSCs and one another, thereby dictating the individual outcomes of progenitors and stem cells (Pérez-Fernández & Hernández-Hernández, 2016). This idea would be consistent with the HSPC heterogeneity discussed above. In any case, and despite its intricate nature, considerable efforts are still being made to fully elucidate the constituents and functions of the BM niche. This makes the study of the BM niche a continuously flourishing field of research, even more than four decades after the seminal contribution of Schofield.

1.2. Unbalanced equilibrium in haematopoiesis: myeloid leukaemias

The intracellular signalling of HSPCs is a precisely balanced process in which any disturbance may significantly alter the outcome of the cell. When this signalling is perturbed and stops being governed by physiological needs, the proliferation and differentiation status of HSPCs becomes aberrant, thus giving rise to leukaemia.

Among the different types of human leukaemia, myeloid leukaemias comprise alterations in myelopoiesis. However, remarkable differences are found within the same group. According to progression, two sub-groups can be established: chronic myeloid leukaemia (CML), with slower evolution and much better prognosis (Holyoake & Vetrie, 2017), and acute myeloid leukaemia (AML), with a rapid progression (Khwaja *et al*, 2016), especially in elderly patients, and poorer outcome (De Kouchkovsky & Abdul-Hay, 2016).

1.2.1. Acute myeloid leukaemia (AML)

Also known as acute myelogenous leukaemia, this disease is characterized by an aberrant, clonal proliferation of immature myeloid progenitors (blasts) that invade BM and peripheral blood (PB), eventually leading to severe disruptions in physiological haematopoiesis (**Figure 1.2**). In Western countries, it displays an incidence between 3 and 5 cases per 100 000 individuals older than 18 years with increasing rates among mature people and a median age of ~70 years at the time of diagnosis (Döhner *et al*, 2015; Di Nardo & Cortes, 2016; Khwaja *et al*, 2016).

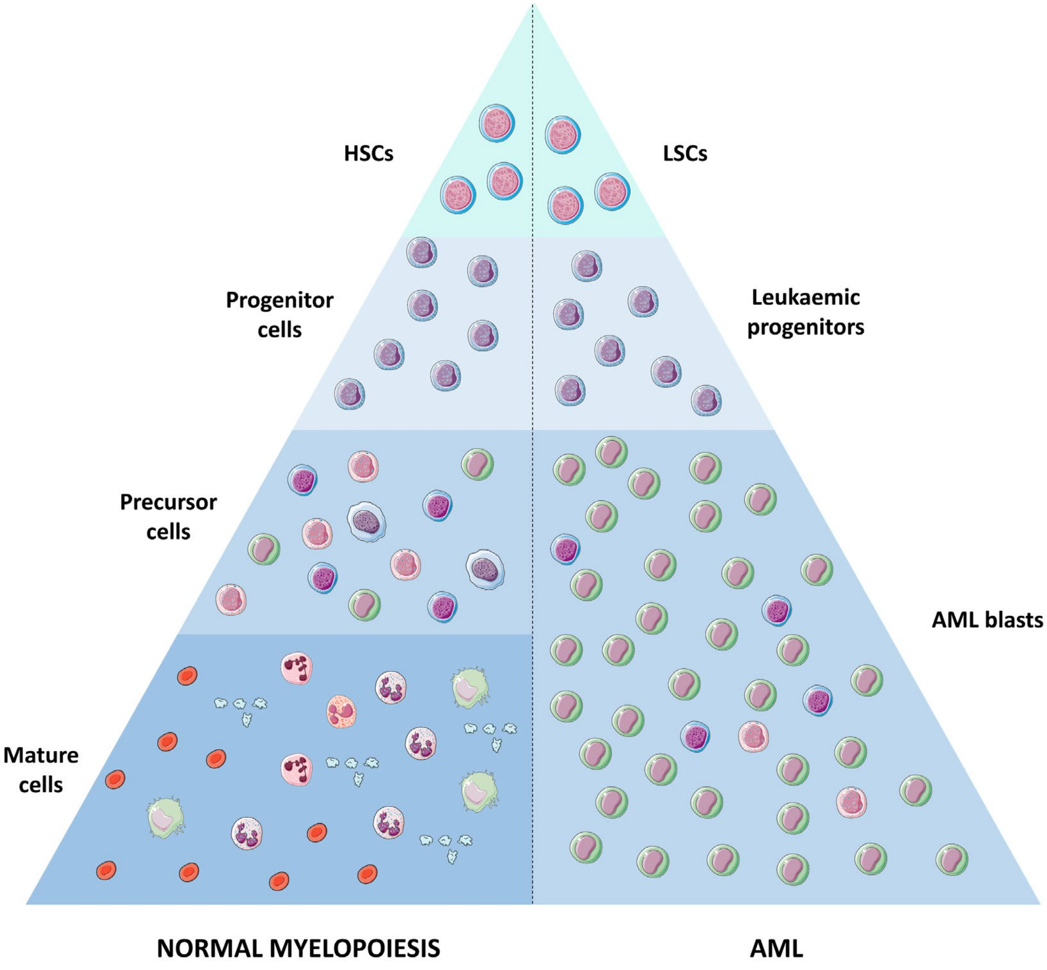

Figure 1.2. ***The pathogenesis of AML.*** The HSC population normally gives rise to gradually more committed progenitors and precursors, which will eventually lead to fully differentiated cells (left side). On the contrary, AML emerges when a driver event generates a LSC population that disturbs the balance between division and differentiation. Leukaemic blasts then outcompete normal cells and populate the BM niche and PB (right side). Adapted from Khwaja *et al,* 2016.

One of the most relevant features of AML is its prominent heterogeneity. Most newly diagnosed AML cases present a founding clone and at least one subclone, a fact that is of capital relevance for relapse and therapy resistance (Döhner *et al*, 2015). The early French-American-British (FAB) classification of 1976 already reflected the varied nature of the disease: eight different subtypes, from M0 to M7, were back then distinguished according to the morphology and maturation of leukaemic blasts (**Table 1.1**) (Miller & Pilichowska, 2014). Ever since, increasing knowledge on cytogenetic and genetic anomalies, as well as clinically relevant features, have been incorporated to FAB criteria by the World Health Organisation (WHO), thus giving rise to a periodically updated classification, being the last version published in 2016 (**Table 1.2**) (Arber *et al*, 2016). The continuous evolution of these grouping criteria further illustrates the complexity of this disease, reflected in terms of diagnosis, prognosis and treatment.

Table 1.1. FAB classification of AML

FAB SUBTYPE	NAME
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Likely due to the abovementioned complexity, the standard therapy for AML has remained almost unchanged for the last four decades. It basically comprises two steps: induction and consolidation. The induction step consists of the so-called 7+3 cycle: 7 days of treatment with the nucleoside analogue arabinocytosine (AraC or cytarabine) followed by 3 days of anthracycline administration (De Kouchkovsky & Abdul-Hay, 2016; Di Nardo & Cortes, 2016). However, patients over 60 years are poor responders to 7+3 cycle and the only possible improvement is the addition of the anti-CD33 calicheamicin-conjugated monoclonal antibody gemtuzumab ozogamicin (GO), which has been recently reintroduced in clinic (Winer & Stone,

2019) and might increase survival of patients who do not display adverse cytogenetic risk (Döhner *et al*, 2015). Fourteen days after the initiation of induction therapy, BM status must be evaluated to make a further decision. In case of unsuccessful response, either additional 7+3 cycles or alternative approaches are undertaken. If complete remission (CR) is achieved, patients can then undergo consolidation therapy, which is needed to avoid short-term relapse (De Kouchkovsky & Abdul-Hay, 2016; Miller & Pilichowska, 2014). At this point, two main alternatives are available: additional chemotherapy with intermediate-dose cytarabine or allo-HSCT. The former is usually the first choice, with different regimens depending on age, genetic profile and additional clinical conditions, whereas the latter is only selected for patients unlikely to respond to chemotherapy (De Kouchkovsky & Abdul-Hay, 2016; Di Nardo & Cortes, 2016; Döhner *et al*, 2015).

Table 1.2. 2016 WHO classification of AML

ACUTE MYELOID LEUKEMIA (AML) AND RELATED NEOPLASMS
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);*RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
APL with *PML-RARA*
AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1);*DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);*GATA2, MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);*RBM15-MKL1*
Provisional entity: AML with BCR-ABL1
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with mutated *RUNX1*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, Not Otherwise Specified
AML with minimal differentiation
AML without maturation
AML with maturation

Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome

Despite the advances achieved since the 1960s, when AML was incurable (Khwaja *et al*, 2016), prognosis after standard treatment is still dismal. The median overall survival (OS) for AML patients is below 1 year and the 5-year survival rate is only ~25% in the United States (US) (Di Nardo & Cortes, 2016; Shallis *et al*, 2019; Miller & Pilichowska, 2014). This clearly illustrates the urgent need for new therapeutic approaches that improve these poor outcomes. Chemotherapy, the cornerstone of the current front-line treatment, is highly unspecific and not well tolerated by all patients. Besides, relapse is very frequent even after CR due to the presence of quiescent, non-responsive LSCs (Zeijlemaker *et al*, 2016). For this reason, considerable efforts are being made in the last years aiming at the development of directed therapies based on differential molecular features of leukaemic cells.

1.2.1.1. Molecular alterations of AML cells

As shown in **Table 1.2,** a proportion of AML cases are diagnosed and stratified on the basis of molecular abnormalities present in leukaemic clone(s). One of the facets that better illustrate the heterogeneity of the disease is the wide range of genomic alterations that can be found. Next generation sequencing (NGS) technologies have revealed at least 1 somatic genetic alteration in more than 95% of AML samples and several genetic alterations, three of them driver mutations, per sample (Di Nardo & Cortes, 2016). Large chromosomal rearrangements and point mutations in relevant genes have shown to be involved in AML pathogenesis and prognosis. Moreover, some of the products of these altered genes are becoming interesting targets for novel directed therapies (see section 1.2.1.2). Some crucial and recurrent genetic abnormalities are the following.

a) Large chromosomal rearrangements

Cytogenetics remains the most important criterion for OS and CR prognosis in AML (De Kouchkovsky & Abdul-Hay, 2016) and was incorporated to WHO classification almost two decades ago (Khwaja *et al*, 2016). Indeed, some AML subtypes are exclusively diagnosed by the presence of chromosomal abnormalities instead of blast percentage. Although hundreds of mutations can be identified in AML samples, the following are listed because of their remarkable frequency or clinical relevance.

- ***RUNX family transcription factor 1 (RUNX1)-RUNX1 partner transcriptional co-repressor 1 (RUNX1T1).*** Also known as *AML1-ETO,* this fusion gene originates from the t(8;21)(q22;q22) rearrangement and represents between 5 and 10% of AML cases. It is especially frequent among young, non-infant individuals and defines a specific entity in the WHO classification of 2016 regardless of blast percentage (**Table 1.2**). *RUNX1-RUNX1T1* gene is used for both diagnosis and minimal residual disease (MRD) monitoring. Its protein product contributes to leukaemogenesis through a wide range of altered functions: gene expression, ribosomal function, DNA repair, exacerbated reactive oxygen species (ROS) production, altered response to haematopoietic growth factors and cell cycle (Reikvam *et al*, 2011). The presence of this translocation at the time of diagnosis is considered a good prognosis factor unless co-occurring specific clinical conditions are present (Yang *et al*, 2017; Reikvam *et al*, 2011).
- ***Promyelocytic leukemia (PML)-Retinoic acid receptor alpha (RARA).*** The translocation t(15;17)(q22;q21) is found in 5-10% of AML-diagnosed individuals and defines the vast majority of APL cases (Yang *et al*, 2017). APL is a paradigm of molecular targeted leukaemia and merits a more detailed description (see section 1.2.1.3).
- ***Core-binding factor subunit beta (CBFB)-Myosin heavy chain 11 (MYH11).*** This fusion gene can be present in different forms coming from the aberrancies inv16 (p13.1q22) or t(16;16)(p13.1;q22). It defines 5-8% of AML cases irrespective of blast count according to 2016 WHO classification (**Table 1.2**). The median age of AML patients with these

rearrangements is 40 years and generally correlates with good prognosis. Co-occurring cytogenetic abnormalities are usual (Yang *et al*, 2017). The leukaemogenic mechanism of the fusion gene product, CBFβ-SMMHC, appears to rely on repression of the haematopoietic transcription factor RUNX1, although the details are yet to be fully elucidated (Castilla & Bushweller, 2017).

- ***Lysine methyltransferase 2A (KMT2A)*** **rearrangements.** Chromosomal abnormalities involving the gene *KMT2A,* located at 11q23, account for about 5% of AML. Gene fusion events can occur with ~80 different partners, which highly influence the outcome. In general, these rearrangements imply a poor prognosis and are found in patients with a median age of 40-60 years. It is also a highly heterogeneous subgroup of AML, with co-operating mutations in *RAS* proto-onocgenes*, B-Raf proto-oncogene, serine/threonine kinase (BRAF)* or *neurofibromin 1 (NF1)*, as well as high expression of of *fms-like tyrosine kinase 3 (FLT3),* being important leukaemogenic factors (Yang *et al*, 2017).

b) Individual gene mutations

The mutational status of AML samples has become a very relevant diagnostic and prognostic tool with the fast development of sequencing technologies over the past few years. Although there are some mutations considered as recurrent –the most frequent ones affecting *FLT3, DNA methyltransferase 3 alpha (DNMT3A)* and *nucleophosmin 1 (NPM1)*–, heterogeneity is still present at this level. Indeed, these mutations only encompass roughly 30% of patients (Di Nardo & Cortes, 2016). Relevant recurrent mutations found in AML have long been grouped as class I (affecting signalling pathways) and class II (affecting cell differentiation), with an emerging class III, including epigenetic modulators (De Kouchkovsky & Abdul-Hay, 2016), and non-classified mutations in the genes *tumour protein 53 (TP53)* and *NPM1*. As it is the case for cytogenetic abnormalities, there is a much wider range of mutations, but only the most recurrent ones will be described herein.

- **Class I mutations**
  - *FLT3.* Affecting to 2/3 AML patients, mutations in this gene can be present as internal tandem duplications of the juxtamembrane domain (FLT3-ITD) or as point mutations within the tyrosine kinase domain (FLT3-TKD). The both of them lead to constitutive activation

of pro-survival signals. They are more frequent in younger adults with normal karyotype (NK) and their variant allele frequency (VAF) is relevant for outcome prediction (Di Nardo & Cortes, 2016).

  - *RAS*-related oncogenes. This group comprises activating mutations in *NRAS proto-oncogene (NRAS), KRAS proto-oncogene (KRAS), protein tyrosine phosphatase non-receptor 11 (PTPN11)* and *NF1* genes. They are found in 10-15% of AML cases and generate aberrant activation of the RAS/RAF/MEK/ERK axis, thus leading to enhanced proliferation. They are associated to poor prognosis when arise during the progression from myelodysplastic syndrome (MDS) to AML, whereas *NRAS* mutations co-occurring with *NPM1* or *DNMT3A* appear to confer a favourable outcome (Di Nardo & Cortes, 2016).
  - *KIT proto-onocogene, receptor tyrosine kinase (KIT).* Mutations in the gene encoding the homonym receptor tyrosine kinase (RTK) are found almost exclusively in the so-called core-binding factor (CBF)-AML, comprising the rearrangements *RUNX1-RUNX1T1* (*RUNX1* codes for the monomer CBF2α of the heterodimeric transcription factor CBF) and *CBFB-MYH11* (involving the β subunit of CBF) (Gu *et al*, 2018a). The missense mutation D816V is particularly recurrent in CBF-AML and commonly associated with poorer outcomes (Di Nardo & Cortes, 2016).

- **Class II mutations.**
  - *CCAAT enhancer binding protein alpha* (*CEBPA*). CEBPA is a master regulator of myeloid differentiation. Leukaemogenic mutations in this gene originate truncations in the N-terminus and insertions/deletions (indels) in the C-terminus that disrupt DNA binding and dimerization. These alterations are mainly found in NK-AML patients, and biallelic CEBPA mutations have become a grouping criterion for 2016 WHO classification as they are linked to the favourable prognosis (**Table 1.2**) (Gu *et al*, 2018a; Di Nardo & Cortes, 2016).
  - *RUNX1.* This transcription factor regulates adult HSC differentiation and homeostasis. Frameshift and missense mutations in this gene are particularly frequent in patients with previous haematological

    disorders and associated to shorter OS and resistance to standard chemotherapy. AML with mutated *RUNX1* has been included into the revised WHO classification of 2016 due to its special features and unfavourable prognosis (**Table 1.2**). Additional co-occurring mutations and mutated *RUNX1* VAF are important contributors to prognosis in this group (Gu *et al*, 2018a; Di Nardo & Cortes, 2016).

  - *GATA-binding protein 2 (GATA2).* GATA2 is a transcription factor with several partners involved in myelopoiesis. Mutations within its coding sequence mainly affect its regulatory region and are frequent co-operating events in *CEBPA*-mutated AML (Di Nardo & Cortes, 2016).

- **Class III mutations.** They are currently acknowledged as a key factor for leukaemogeneis and an important hallmark of clonal haematopoiesis, a pre-leukaemic stage typical in the elderly. This type of mutations is also thought to be dependent on co-operating mutations to initiate a leukaemic transformation. Some of them are:

  - *DNMT3A.* The enzyme encoded by this gene catalyses the methylation of cytosine residues in CpG islands, thus silencing genes participating in HSC differentiation and self-renewal. Mutations in this gene affect ~20% of *de novo* AML, are associated to age and frequently co-occur with alterations in *NPM1, FLT3-ITD* and *isocitrate dehydrogenase 1 (IDH1)* genes. They might also correlate with resistance to chemotherapy and relapse, although there is no consensus in this regard. Apparently, these mutations are early events leading to pre-leukaemic conditions (Gu *et al*, 2018a; Di Nardo & Cortes, 2016).

  - *Tet methylcytosine dioxygenase 2 (TET2).* TET2 performs the catalysis of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), a metabolite important in cytosine demethylation and a recently suggested epigenetic mark important in gene promoters and enhancers. Alterations of this gene described in AML comprise loss-of-function mutations that increase HSC self-renewal and impede differentiation. The prognostic value of these abnormalities is highly dependent on co-operating mutations (Di Nardo & Cortes, 2016; Naoe & Kiyoi, 2013).

  - *IDH1/2.* In non-pathological conditions, IDH1 and IDH2 are enzymes of Krebs cycle that transform isocitrate into α-ketoglutarate (α-KG). *IDH* mutations found in AML usually occur in arginine residues and lead to the generation of 2-hydroxyglutarate (2-HG) instead of α-KG, the natural reaction product (Di Nardo & Cortes, 2016). Although competitive inhibition of TET2 activity by 2-HG and subsequent genome hypermethylation has been suggested as the underlying pathogenic mechanism of mutated IDH, recent work attributes a role for these enzymes on cell differentiation blockage, with methylation patterns resembling normal progenitors (Wiehle *et al*, 2017). The specific mutation site and co-operating events will define the outcome. It has been shown that these mutations arise at the origin of a leukemic clone and, together with additional key events, trigger leukaemogenesis (Di Nardo & Cortes, 2016; Naoe & Kiyoi, 2013).
  - *ASXL transcriptional regulator 1 (ASXL1).* ASXL1 is a chromatin modifier that interacts with polycomb repressor complex 2 (PRC2) and influences the trimethylation of the Lys27 residue of histone 3 (H3K27me3). Alterations in this gene are commonly found together with mutations in *RUNX1* and spliceosome-related genes and are indicators of poor prognosis (Di Nardo & Cortes, 2016).

- ***NPM1.*** Mutations in this gene are found in 1/3 of AML cases, generally leading to dominant-negative forms that block differentiation. They are associated with augmented response to chemotherapy and good prognosis, unless co-occurring with altered *DNMT3A* and *FLT3-ITD,* which correlate with particularly poor outcomes (Di Nardo & Cortes, 2016; Kunchala *et al*, 2018).
- ***TP53.*** This well-known tumour suppressor maintains genomic stability through different mechanisms. Mutations in this gene are frequently related to therapy of AML developed from MDS and associate to lower response to treatment and poor outcomes. Indeed, there is no consensus on optimal treatment for patients with mutated *TP53* (Di Nardo & Cortes, 2016).
- **Spliceosome-related genes.** Mutations in genes coding for splicing factors account for ~10% of AML cases. These alterations lead to aberrant transcriptome and proteome that affect epigenetic regulation, transcription

itself and genome integrity in malignant cells. They associate to worse response to treatment and poor survival (Gu *et al*, 2018a; Di Nardo & Cortes, 2016).

The cytogenetic alterations and gene mutations previously described have become very important criteria for diagnosis and risk stratification. As a proof, the European Leukaemia Net (ELN) includes them among its last recommendations update of 2017 (Döhner *et al*, 2017).

c) Altered expression levels of non-mutated molecules

Apart from the genomic alterations leading to chimeric oncogenes or mutant proteins, there is a group of processes altered owing to differential expression levels of some molecules versus the non-pathologic condition. Although contributing to disease heterogeneity, they constitute potential therapeutic targets, and their understanding has enabled recent and important clinical improvements. Representative examples are the following:

- **Enhanced expression of antiapoptotic proteins.** B cell leukemia/lymphoma-2 (BCL2) is specifically overexpressed in LSCs versus HSCs, thus providing an interesting therapeutic window (Pollyea & Jordan, 2017).
- **Developmental pathways malfunction**. Notch dysregulation has a dual role on AML leukaemogenesis. On one hand, downmodulation of this pathway mediates cell differentiation arrest and expansion of AML LSCs. On the other hand, the overexpression of Jag1 in BM niche triggered by activating mutations of the gene *Ctnnb1* leads to Notch signalling overactivation in HSPCs and leukaemogenesis (Heidel *et al*, 2015). In addition, distinct elements of Hedgehog (Hh) pathway are upregulated in this disease, with involvement in resistance to chemotherapy and radiotherapy (Terao & Minami, 2019). There is also an interaction between Hh and FLT3 signalling in myeloid leukaemia (Winer & Stone, 2019). Finally, Wnt signalling is involved in several aspects of the pathogenesis of AML, most of them through its central player, β-catenin (See section 1.3.2).
- **Differential expression of cellular antigens.** A paradigmatic example is the myeloid-specific surface marker CD33, which led to the development of GO (Godwin *et al*, 2017). Although found in variable intensity on the surface of AML blasts, its expression on LSCs remains controversial. In

addition, its presence in normal myeloid progenitors contributes to the lack of specificity of CD33-based therapies. Other possible targets expressed in normal cells to a lesser extent are Wilms Tumour 1 protein (WT1) and Preferentially Expressed Antigen in Melanoma (PRAME) (Khwaja *et al*, 2016).

1.2.1.2. From molecular features to alternative therapies: a step forward to personalised medicine for AML

Some of the previously outlined molecular alterations, together with many others that can appear in the AML setting, have become potentially helpful pharmacological targets to improve current treatments treatments against this disease. There is a huge number of clinical trials based on compounds targeting such alterations. Illustrative examples recently approved for clinical use are provided below (**Figure 1.3**) (Winer & Stone, 2019):

- **Small molecules restoring aberrant signalling pathways.** A number of FLT3 inhibitors are currently under clinical trials in the FLT3-mutated AML setting. Interestingly, two of them, **midostaurin** (Rydapt) and **gilteritinib** (Xospata) have been commercialised. The former was approved in both US and Europe in combination with standard chemotherapy, whereas the latter achieved the U.S. Food and Drug Administration (FDA) approval for relapsed patients.

- **Drugs targeting epigenetic modulators.** The compounds **enasidenib** (Idhifa, IDH2 inhibitor) and **ivosidenib** (Tibsovo, IDH1 inhibitor) were approved by FDA for relapsed/refractory (r/r) AML and the latter has recently –May 2019– achieved FDA permission for clinical use in patients who are unfit for intensive induction chemotherapy.

- **Pro-apoptotic agents. Venetoclax** (Venclexta) is a BCL2 antagonist that received FDA approval in 2018 for its use in combination with hypomethylating agents (HMA) or low-dose chemotherapy in newly diagnosed AML patients unfit for standard induction chemotherapy.

- **Small molecules targeting developmental pathways.** Up to date, only an inhibitor of Hh signalling has entered the clinic. The small molecule **glasdegib** (Daurismo) obtained FDA approval last year in combination with low dose cytarabine for patients unfit for standard induction chemotherapy.

- **Surface antigen-targeting immunoconjugates.** The paradigm of this strategy is unquestionably the immunoconjugate **GO**. After approval and voluntary withdrawal, it was re-evaluated and approved by FDA in 2017 and by the European Medicines Agency (EMA) in 2018. It is indicated for newly diagnosed or r/r AML either as a monotherapy or in combination with chemotherapy.

Despite the progress of the previously presented approaches after 4 decades of scarce advances, many of them still rely on chemotherapy for proper effectiveness. Besides, although being more directed therapies, clonal heterogeneity remains an obstacle for drug targeting and is a source of relapse (Döhner *et al*, 2015). Therefore, considerable efforts are still needed to (i) refine diagnostic methods aiming at personalised therapies and (ii) identifying universal targets for LSCs that may help at the effective eradication of both founding clones and subclones. A promising avenue for the second purpose is the development of combinatorial therapies targeting at least two relevant molecules involved in pathogenesis, a field of very intense research over the last years.

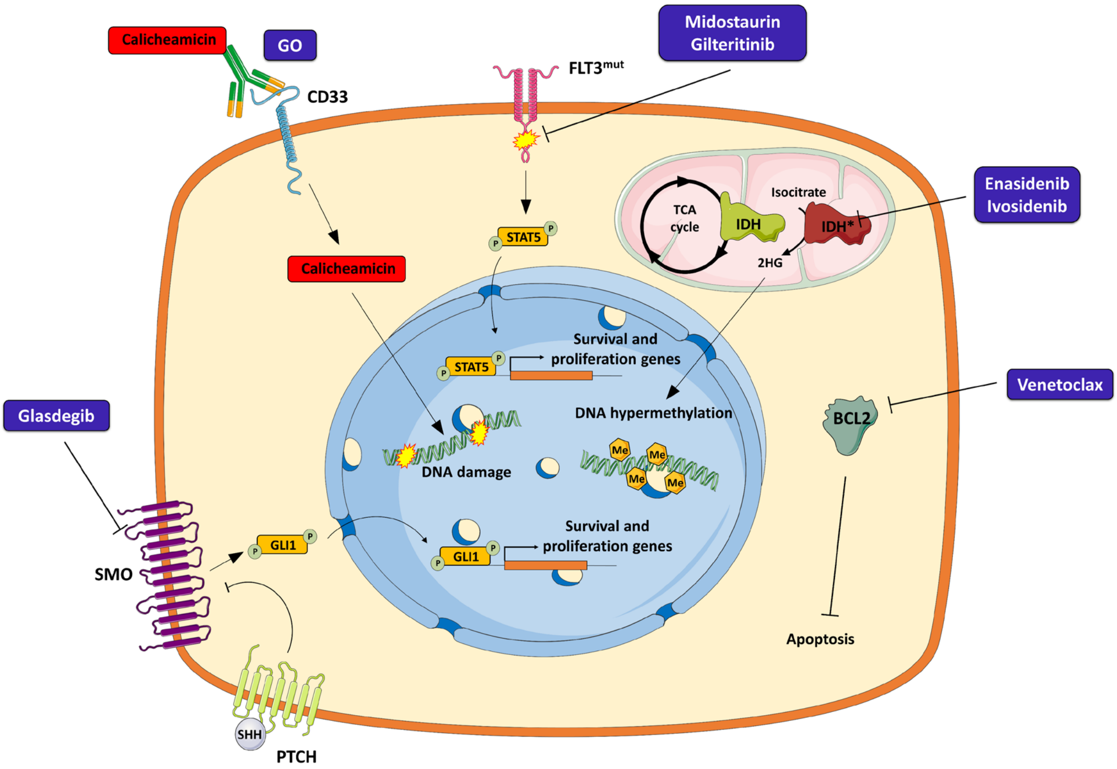
Calicheamicin
GO
CD33
FLT3^{mut}
Midostaurin
Gilteritinib
Enasidenib
Ivosidenib
Isocitrate
TCA cycle
IDH
IDH*
2HG
STAT5
P
Calicheamicin
Survival and proliferation genes
STAT5
DNA hypermethylation
Me
DNA damage
Venetoclax
BCL2
Glasdegib
SMO
GLI1
GLI1
Survival and proliferation genes
Apoptosis
SHH
PTCH

Figure 1.3. ***Drugs approved for clinical use based on distinctive molecular traits of AML cells.*** The diagram shows a simplified depiction of some aberrant processes occurring in AML cells and the different compounds targeting them that are mentioned in this work (see text for details).

1.2.1.3. Differentiation-based therapies for AML: extending the paradigm of APL

APL arises when leukaemic blasts are blocked at the differentiation stage of promyelocytes and undergo uncontrolled proliferation, thereby disturbing normal haematopoiesis. This leads to a fast progressing and deadly malignancy, unless treated, due to severe coagulopathies and bleeding (Ablain & de Thé, 2011; Thomas, 2019). The vast majority of APL cases bear the cytogenetic abnormality t(15;17)(q21;q22), which generates the fusion protein PML-RARα. In contrast to other subtypes of AML, this oncoprotein might be the sole pathogenic driver of the disease. PML-RARα disturbs the normal physiology of myeloid progenitors via transcriptional repression of genes driving myeloid differentiation and stem cell self-renewal, as well as disruption of the so-called nuclear bodies (NBs). These structures, which require the wild-type PML protein, are involved in TP53 activation and senescence (de The *et al*, 2017).

Being a fatal disease until long after 1957, when the first cases of APL were reported, first successful improvements in the management of the disease comprised chemotherapy with anthracyclines, especially daunorubicin. Cytarabine was later combined with daunorubicin as a general AML treatment, together with HSCT. Nevertheless, considerable limitations remained, for instance early death derived from chemotherapy, low rates of disease-free survival and poor outcomes after relapse (Thomas, 2019; Coombs *et al*, 2015).

Undeniably, a new therapeutic era for APL came with the introduction of all-*trans*-retinoic acid (ATRA) into clinical practice in 1985 (Huang *et al*, 1988). After different stages being used as single agent and in combination with chemotherapy (Coombs *et al*, 2015), the results of co-treatment with ATRA and arsenic trioxide (ATO) in APL patients were published in 2004. That work demonstrated a synergistic interaction of between both compounds that led to shorter times to achieve CR, improved reduction of leukemic burden and longer disease-free survival (Shen *et al*, 2004). Thanks to its advantages versus ATRA+chemotherapy, ATRA+ATO has become the new front-line induction regimen for low-intermediate risk APL, with cure rates over 95 % (Coombs *et al*, 2015; De Thé, 2018).

As monotherapy, ATRA propmpts an efficient differentiation of leukaemic blasts into mature granulocytes, which are rapidly cleared by BM macrophages (De Thé, 2018). On the other hand, ATO triggers an initial massive apoptosis followed by blast differentiation, much slower than the one induced by ATRA (Ablain & de Thé, 2011). However, ATRA alone or combined with chemotherapy does not prevent relapse (De Thé *et al*, 2017). The underlying molecular explanation for the success of the co-treatment comes from the separate effect of ATRA and ATO on PML-RARα molecule. The former binds PML-RARα and recruits transcriptional co-activators, thus suppressing its pathogenic transcriptional repression. At the same time, it targets the oncoprotein for proteasome-dependent degradation, thereby allowing the formation of NBs by PML. On the other hand, ATO promotes differentiation through targeting PML-RARα for degradation, thus releasing the previously repressed promoters. Besides, it binds wild-type PML, also restoring the formation of NBs and abrogating the self-renewal advantage of APL cells. The combination of these agents promotes transcriptional de-repression and PML-RARα degradation through different mechanisms, thus acting in a synergistic manner (De Thé, 2018).

APL differentiation therapy has provided a simple conceptual framework for treatments based on overcoming the differentiation blockade common to all AML subtypes. The expression of PML-RARα as the only leukaemic driver might be the main cause of the huge success of ATRA for APL treatment (De Thé, 2018) compared to other AML subtypes. However, widely altered molecules involved in cell differentiation are currently being investigated as general targets for differentiation-based strategies valid beyond APL.

Combinations of ATRA with ATO, chemotherapy or FLT3 inhibitors have proven to be successful in non-APL AML patients. However, a large subset of AML cases is not responsive to ATRA due to epigenetic repression of the *RARA* gene and/or its downstream targets. Drugs targeting epigenetic modulators have shown to improve sensitivity to ATRA in AML cell lines and primary samples (van Gils *et al*, 2017). An interesting example is the lysine demethylase 1A (KDM1A, previously known as LSD1) inhibitor tranylcypromine (TCP). TCP was successfully combined with ATRA in the pre-clinical setting (Schenk *et al*, 2012) and is currently in early-phase clinical trials for AML and MDS treatment (NCT02717884). Moreover, a recent report has demonstrated that the inhibition of SUMOylation, recently acknowledged as an epigenetic mark, re-sensitises non-APL cells to ATRA-mediated differentiation (Baik *et al*, 2018).

Besides, targeting the epigenetic modifiers responsible for the aberrant expression of genetic drivers of differentiation is becoming a powerful rationale for the development of ATRA-independent pro-differentiative therapies.

Different KDM1A inhibitors have demonstrated their pre-clinical efficiency as inductors of AML cell differentiation in a variety of contexts. In line with this, they apparently enhance survival in animal models, although clinical trials need to test their advantage as monotherapy versus the currently approved treatments (Lai *et al*, 2015; Wu *et al*, 2019; Barth *et al*, 2019). Inhibitors of both DOT1-like histone lysine methyltransferase (DOT1L) and bromodomain and extra-terminal domain (BET) epigenetic readers have shown *in vitro* efficacy at inducing differentiation in KMT2A-rearranged AML (Brzezinka *et al*, 2019). Interestingly, the recently approved enasidenib has become a clinical-grade drug inducing AML cell differentiation through mechanisms yet to be fully elucidated (Sun *et al*, 2019).

A very promising target identified through a large-scale compound screening is the metabolic enzyme dihydroorotate dehydrogenase (DHODH), involved in purine metabolism. Different compounds inhibiting its activity have proven efficient differentiation induction both *in vitro* and in patient-derived xenograft (PDX) models, with an encouraging improvement of animal survival upon their administration as monotherapy (Sykes *et al*, 2016; Brzezinka *et al*, 2019; Christian *et al*, 2019). Most importantly, an ongoing clinical trial is evaluating its feasibility for r/r AML (NCT03760666).

Interestingly, there is a group of compounds that have long been known for their effects on differentiation of malignant leukaemic cells: the phorbol esters. They have been traditionally described as potent activators of protein kinase C (PKC) likely through its constitutive anchorage to the cell membrane (Goel *et al*, 2007). They were described back in the late 1970s as inductors of differentiation in cell lines (Huberman & Callaham, 1979) and primary cells of myeloid leukaemia (Koeffler *et al*, 1980). Since then, these compounds have become a widely used tool for the study of cell differentiation processes in both physiological and pathological haematopoiesis, especially the naturally occurring phorbol-12-myristate-13-acetate (PMA, also known as 12-O-tetradecanoylphorbol-13-acetate or TPA). Natural phorbol esters were reported to have antileukaemic potential even before the discovery of their differentiation induction ability (Goel *et al*, 2007), but evaluation of their clinical potential in patients suffering from leukaemia did not take place until the end of the past century (Han *et al*, 1998; Strair *et al*, 2002). An important obstacle hampering the incorporation of phorbol esters to front-line therapies has unquestionably been their carcinogenic potential (Goel *et al*, 2007). For this reason, PKC agonists lacking tumour-promoting activity, like bryostatins, were tested in parallel to phorbol esters from the 1990s as therapeutically feasible compounds to treat haematologic malignancies (van der Hem *et al*, 1995; Roddie *et al*, 2002). Although bryostatin 1 has undergone several clinical trials for the treatment of blood cancers, including AML, it has not been approved for clinical use neither by FDA nor by EMA.

Nonetheless, phorbol esters still serve as an interesting proof of concept for the design of pro-differentiation strategies that may be the basis of future treatments using non-tumour-promoting PKC agonists. In this regard, the phorbol ester prostratin (13-O-acetyl-12-deoxyphorbol, hereafter PRS) emerges as an interesting candidate. Extracts containing this compound have long been used in Samoan traditional medicine without adverse effects. Importantly, it has shown not only a lack of tumour-promoting activity, but also anti-tumoral potential (Miana *et al*, 2015). Moreover, its differentiation-inducing ability in both AML cell lines and primary samples, as well as the potential benefit of its combination with conventional chemotherapy, have been recently reported (Shen *et al*, 2015). Despite these interesting observations, PRS has not been tested *in vivo* in the AML setting.

1.2.2. Chronic myeloid leukaemia (CML)

Chronic myeloid (or myelogenous) leukaemia is a blood disorder affecting approximately 1:100000 individuals per year in Western countries. It accounts for 15% of all annual diagnosed cases of leukaemia and the median age for diagnosis is 65 years (Apperley, 2015; Hanlon & Copland, 2017). It is a clonal disorder arising at the HSC stage in the BM as a consequence of impaired differentiation to mature cells, thereby generating accumulation of immature progenitors in BM and PB (**Figure 1.4**) (Arrigoni *et al*, 2018).

The progression of CML consists of three stages: chronic phase (CP), accelerated phase (AP) and blast crisis (BC). Most patients are diagnosed at CP, where they remain asymptomatic for long time periods as their immune system is still not impaired. Splenomegaly and leucocytosis are highly common features at diagnosis. This stage can be prolonged for up to 5-6 years. If untreated, the disease will progress to AP, when maturation blockage becomes increasingly marked and alterations in blood cell counts are much more evident. This phase typically lasts 4 to 6 months, after which it evolves to BC. This is a very aggressive stage characterised by the presence of ≥ 20% of myeloid or lymphoid blasts in PB and great reluctance to chemotherapy. BC is a deadly condition with a median survival of 3-6 months (Quintás-Cardama & Cortes, 2006).

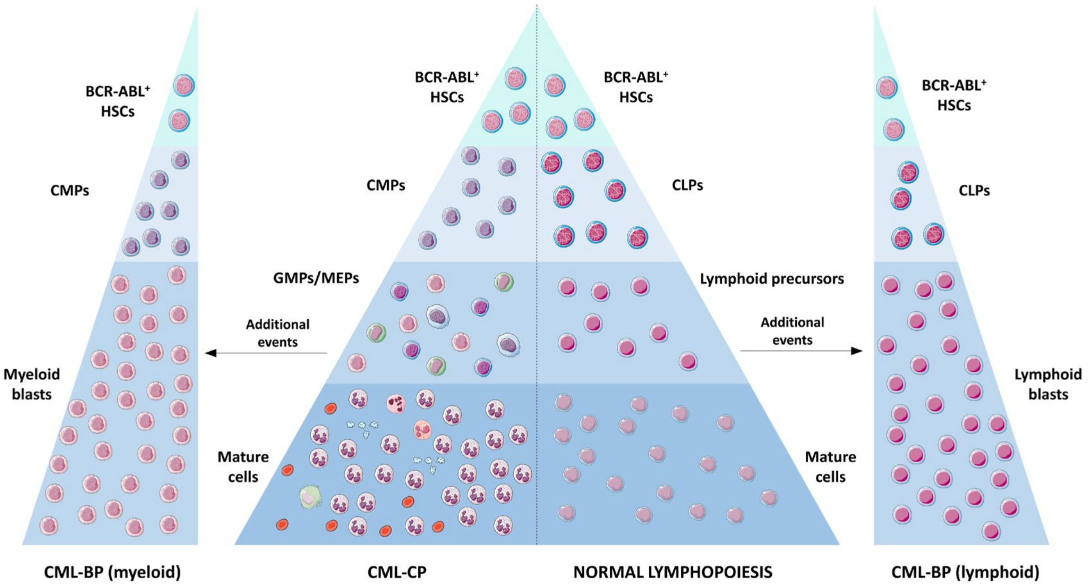
BCR-ABL⁺ HSCs
CMPs
Myeloid blasts
CML-BP (myeloid)
BCR-ABL⁺ HSCs
CMPs
GMPs/MEPs
Additional events
Mature cells
CML-CP
BCR-ABL⁺ HSCs
CLPs
Lymphoid precursors
Additional events
Mature cells
NORMAL LYMPHOPOIESIS
BCR-ABL⁺ HSCs
CLPs
Lymphoid blasts
CML-BP (lymphoid)

Figure 1.4. ***The pathogenesis of CML.*** The emergence of the Ph chromosome containing BCR-ABL in HSCs first leads to an early CP characterised by the accumulation of mature granulocytes in PB. Upon disease progression, further events occur that cooperate with BCR-ABL and give rise to a more severe phenotype: the BP. These events can arise at both myeloid and lymphoid lineages, thus originating two variants of BP: myeloid and lymphoid. Adapted from Ren, 2005.

1.2.2.1. BCR-ABL: a kinase corrupting cell signalling

Contrary to AML, CML is a rather homogeneous disease whose pathogenesis-triggering event is the emergence of the so-called *Philadelphia chromosome* (Ph). This entity arises as a consequence of the chromosomal rearrangement t(9;22)(q34;q11), which generally involves the exon 2 (a2) of the *ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1)* gene, located at chromosome 9, and a variable fragment of the *breakpoint cluster region (BCR)* gene, placed at chromosome 22. This translocation will generate different variants of the fusion gene *BCR-ABL1*, depending on the location of the breakpoint in *BCR* gene. The most common variant involves the exons 13 or 14 of *BCR* and originates the transcripts known as e13a2 or e14a2, respectively. The both of them translate into a 210 kDa protein commonly referred to as p210 BCR-ABL (Flis & Chojnacki, 2019).

The ability of BCR-ABL to disrupt cell signalling derives from its varied multidomain structure (**Figure 1.5**) and cytoplasmic instead of nuclear localisation, contrary to the parental ABL kinase (Hazlehurst *et al*, 2009). The structure of p210 BCR-ABL comprises:

- **N-terminal coiled-coil (CC) domain.** It allows the oligomerisation and subsequent activation of the oncokinase, and is required for its leukaemogenic activity (Ren, 2005).

- **Serine/threonine kinase (S/T-K) domain**. It contains the Y177 autophosphorylation residue, fundamental for the interaction with growth factor receptor-bound protein 2 (GRB2) (Soverini *et al*, 2018).
- **Ras homolog gene family/Guanine nucleotide exchange factors (Rho/GEF) kinase domain** (Soverini *et al*, 2018)**.**
- **SRC homology (SH) domains.** The SH1 domain displays the tyrosine kinase activity essential for the oncogenic activity of the protein. On the other hand, the SH2 and SH3 domains are crucial for interaction with different proteins regulating proliferation, survival, adhesion and migration signalling cascades (Ren, 2005; Soverini *et al*, 2018).
- **C-terminal region.** It contains different regulatory elements of the ABL kinase and proline-rich regions important for interactions with other proteins. An important example is CRK-like proto-oncogene, adaptor protein (CRKL), whose phosphorylation status is a common measurement of ABL kinase activity (Soverini *et al*, 2018).

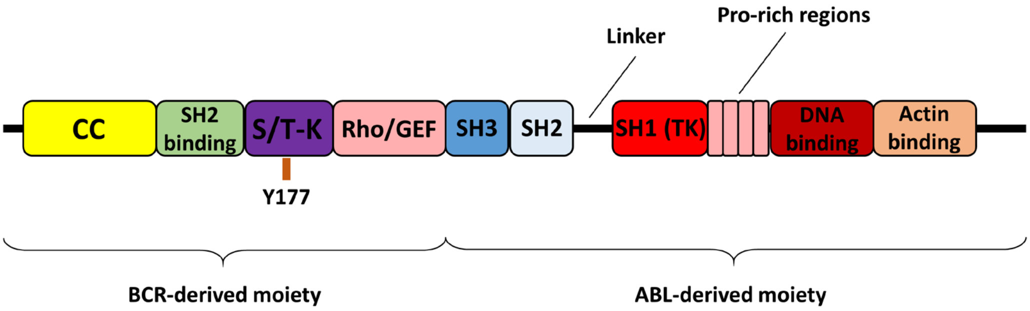

Figure 1.5. ***Structure of the p210 isoform of BCR-ABL.*** Schematic diagram representing the multi-domain nature of the oncokinase (see text for details). Adapted from Soverini *et al,* 2018.

The loss of an N-terminal site of myristoylation in parental ABL kinase (N-cap) and its concomitant fusion with the CC domain and the Y177 of BCR are the main phenomena underlying the constitutive TK activity of BCR-ABL (Soverini *et al*, 2018). Phosphorylated Y177 recruits GRB2, which further interacts with GRB2 associated binding protein 2 (GAB2). CRKL binds BCR-ABL C-terminal domain and initiates a phosphorylation cascade. Altogether, these events lead to phosphatidylinositol 3-kinase (PI3K) activation, which in turn phosphorylates protein kinase B (best known as AKT), thus resulting in the inhibition of pro-apoptotic pathways (Hazlehurst *et al*, 2009).

On the other hand, the interaction between GRB2 and BCR-ABL triggers the recruitment of RAS and SRC homology 2 domain-containing protein tyrosine phosphatase 2 (SHP2). Both are required for the activation of the extracellular regulated kinase (ERK), which activates transcriptional programmes contributing to proliferative and survival advantage (Ren, 2005; Hazlehurst *et al*, 2009).

The signal transducer and activator of transcription 5 (STAT5) transcription factor is also activated via its phosphorylation by BCR-ABL. Apparently, the SH2 and SH3 domains of the fusion oncokinase are required for this event. Besides, it would occur independently of Janus kinase 2 (JAK2), an upstream regulator of this transcription factor in physiological conditions. STAT5 activation provokes the transcription of anti-apoptotic and cell cycle progression genes (Hazlehurst *et al*, 2009).

In line with AML pathogenesis-contributing features, developmental pathways are also de-regulated and promote CML leukaemogenesis. The WNT/β-catenin pathway has been thoroughly studied in this context and evidence supports its key involvement in several aspects of the disease (See section 1.3.2.2). On the other hand, NOTCH and Hh pathways are crucial for LSC expansion and disease progression, the former through the augmented expression of its downstream target *Hairy enhancer of split 1 (HES1)* and the latter via the transmembrane receptor smoothened (SMO) (Arrigoni *et al*, 2018).

1.2.2.2. Targeting BCR-ABL: tyrosine kinase inhibitors and the new era of CML treatment

In a similar way to APL, CML has become a disease with a very good prognosis and a highly efficient front-line treatment thanks to the introduction of tyrosine kinase inhibitors (TKI) targeting BCR-ABL into the clinic.

CML treatment went through different stages until the emergence of imatinib (IM, also known as STI-571 and commercially available as Gleevec) in the late 1990s. The first agent with remarkable improvements in life expectancy was hydroxyurea, used in the 1960s, still unable to prevent progression after 4-5 years from diagnosis and eliminate the Ph^+ cells. Later, prolonged survival (6-7 years) was achieved with the introduction of interferon α (IFNα), although its side-effects hampered long-term usage in patients. Finally, in the early 1990s allo-HSCT truly revolutionized the treatment of the disease and was considered a possible cure, but only eligible patients were benefited from it (Apperley, 2015).

IM was approved by FDA for the treatment of CML in 2002, after the completion of the International Randomised Study of Interferon and cytarabine versus STI-571 (IRIS) clinical trial (Apperley, 2015). IM is a 2-phenylamino pyridine-based that binds the inactive conformation of BCR-ABL and prevents its interaction with ATP, an event required for its activation (An *et al*, 2010). It also inhibits other cellular RTK, such as platelet-derived growth factor receptor (PDGFR) and c-KIT (Soverini *et al*, 2018). From a median survival of 5-7 years before the introduction of IM, patients currently display 5-year survival rates of up to 95% and their life expectancy is nearly the one of general population, with TKI being the front-line therapy (Hanlon & Copland, 2017).

1.2.2.3. Pitfalls of TKI administration: when CML cells evade BCR-ABL inhibition

Despite its efficacy and marked improvement in the prognosis and response, the administration of TKI faces some clinical barriers. First, they do not cure the disease: even when successful molecular response (meaning low or null detection of BCR-ABL expression) is achieved, CML LSCs are still detected in patients treated for more than 4 years with IM (Chu *et al*, 2011; Chomel *et al*, 2011). More than 50% of patients participating in the IRIS trial needed IM discontinuation due to either treatment failure or intolerable adverse effects, which implies that a high proportion of CML patients still require alternative therapies to IM (An *et al*, 2010).

A study performed prior to IM approval for the clinical use reported re-activation of BCR-ABL in BC-CML patients under TKI treatment (Soverini *et al*, 2018). At least 25% of patients show TKI resistance (Arrigoni *et al*, 2018) with increasing probability as the disease progresses, being around 5% in CP-CML and higher than 65% in CML-BC (An *et al*, 2010). There is a number of mechanisms contributing to TKI resistance, which can be BCR-ABL-dependent and independent.

1.2.2.3.1. BCR-ABL-dependent resistance and the use of TKI beyond IM

Among these mechanisms, there are point mutations in particular regions of the oncokinase that impede drug binding. More than 20 different mutations affecting IM sensitivity have been described at the kinase domain, where IM interaction pocket is located.

The most frequent mutation by far (and the first to be characterised) is the amino acid substitution T315I. It affects a crucial hydrogen bond that takes place between IM or second-generation TKI and BCR-ABL. Additionally, the substitution of Thr by Ile originates steric hindrance that physically impedes the TKI to occupy the interaction pocket. Moreover, BCR-ABL keeps the ability to interact with ATP, thus conserving its catalytic activity and oncogenic potential. This mutation has recently been described in 15% of CML patients with IM treatment failure.

On the other hand, IM binds the inactive conformation of BCR-ABL, which is stabilised by two regions of the protein known as the ATP-binding P-loop and the activation loop (A-loop). Mutations in these components hamper BCR-ABL to switch to its inactive conformation, thereby preventing its interaction with TKI. Substitution mutations affecting the P-loop are the most frequent cause of mutation-derived IM resistance, and E255K is an important one. The frequency of P-loop mutations increases with the duration and progression of the disease.

To overcome resistance derived from mutations in BCR-ABL, new TKI were developed. The so-called second generation TKI are nilotinib (NL), dasatinib and bosutinib. NL shares structural features with IM and binds the inactive conformation of BCR-ABL as well, with high specificity and much greater affinity than IM. Dasatinib is even more potent than nilotinib at binding both the inactive and active conformations of BCR-ABL. Last, bosutinib, together with dasatinib, is a dual BCR-ABL/SRC inhibitor. It also binds with great affinity the active conformation of BCR-ABL. The third generation TKI approved for therapy is ponatinib. It is a medium-range specific TKI designed to bind BCR-ABL regardless of mutations, being the only one able to inhibit BCR-ABL^{T315I} (Soverini *et al*, 2018). New TKI are emerging as alternative or complementary options for CML treatment. Radotinib is a NL analogue already approved in South Korea with higher and faster molecular response than IM, as well as a competitive cost for health systems. One promising, recently developed compound is asciminib (ABL001), which interacts with a hydrophobic pocket occupied by the myristoylated N-cap in the native ABL kinase, thereby mimicking its inhibitory effect. Thus, it can bind BCR-ABL irrespective of catalytic site mutations, including T315I. Asciminib is currently under clinical trials evaluating not only its efficacy against CML and toxicity profile, but also the feasibility of its combination with catalytic site-binding TKI as an improved therapy targeting mutations in both binding sites (Massaro *et al*, 2018).

Finally, another BCR-ABL-dependent mechanism of resistance is amplification at the mRNA or protein levels. The cause of resistance in this scenario is the insufficiency of a given concentration of the drug to interact with all the copies of the oncokinase. A possible solution would be the increase of TKI doses, but intolerance could instead become an important issue (An *et al*, 2010; Soverini *et al*, 2018).

1.2.2.3.2. BCR-ABL-independent mechanisms: primary resistance

Multiple BCR-ABL-independent TKI resistance mechanisms have been described. Although still a controversial issue, higher plasma levels of IM have been described in individuals with deep molecular response. The factors controlling this parameter need to be elucidated, although some of them could be adherence, individual pharmacokinetic parameters and cellular uptake of the drug. Incomplete adherence, indeed, is associated to sub-optimal responses. Metabolism by cytochrome P450 enzymes and alpha-1-acid glycoprotein binding have been reported as pharmacokinetic causes of variable IM availability for entry into leukaemic cells (An *et al*, 2010). Finally, cellular uptake is also an important question. HSCs naturally express different members of the ATP-binding cassette (ABC) family to protect themselves from genotoxic stress. These transporters are exploited by CML cells as tools for extracellular release of TKI, hence abrogating their effect. It has been described the increase of ATP binding cassette subfamily B member 1 (ABCB1)-positive cells in AP-CML patients upon IM treatment, which suggests that the administration of TKI selects LSCs expressing these transporters. Concomitantly, CML cells have shown low expression levels of the transporters involved in TKI incorporation organic cation transporter 1 (OCT1) and organic cation/carnitine transporter 2 (OCTN2), which may contribute to TKI resistance through decreased drug uptake (Arrigoni *et al*, 2018).

1.2.2.3.3. Surviving in the presence of TKI: the relevance of targeting CML LSCs

A proportion of patients on TKI treatment cannot be discontinued without assuming a risk of relapse in the long run (Etienne *et al*, 2017; Rea *et al*, 2017). This implies lifelong therapy to keep free of disease. As intolerance can be an obstacle, new strategies aiming at an effective cure are strongly needed. The isolation of CML LSCs (Holyoake *et al*, 1999) and further demonstration of their survival ability in the presence of TKI (Corbin *et al*, 2011; Hamilton *et al*, 2012) established the basis for the understanding of LSC persistence and relapse.

The precise mechanisms of BCR-ABL-independent survival are not completely elucidated, but some reports have shed light on interesting molecules sustaining this process. Some of them are PML, forkhead box O 3A (FoxO3a), SMO, BMI1 proto-oncogene, polycomb ring finger (BMI1), histone deacetylases (HDACs), β-catenin, arachidonate 5-lipoxygenase (ALOX5), BCL2 or protein phosphatase 2 phosphatase activator (PTPA, best known as PP2A). Other important contributors to LSC persistence are the protection provided by the BM niche through IFNα and C-X-C motif chemokine ligand 12 (CXCL12) secretion. The hypoxic microenvironment, which would upregulate hypoxia-inducible factor 1 α (HIF-1α), thereby promoting LSC quiescence and autophagy (Morotti *et al*, 2014). In line with this, great efforts are being made over the last years to develop combinatorial therapies based on the simultaneous targeting of BCR-ABL and some of the abovementioned molecules and processes, which would reduce the chance of CML relapse (Sweet *et al*, 2013).

1.3. The roles of the SRC homology 2 domain-containing protein tyrosine phosphatases 1 (SHP1) and 2 (SHP2) and β-catenin in myeloid leukaemias: therapeutic opportunities

As previously outlined, two very important features shared by both types of myeloid leukaemias are the impaired differentiation and the distinctive properties of LSCs, which account for disease relapse and resistance to therapy. Therefore, research on relevant differentiation mechanisms and discovery of molecules supporting the LSC evasion to current therapies are invaluable tools for the development of new treatments.

1.3.1. SHP1 and SHP2, mediators of redox-controlled cell differentiation and important contributors for leukaemia

Apart from key proteins involved in cell signalling, there is a group of second messengers that also merit attention in the study of the pathophysiological control of those processes. Among the most studied second messengers over the last years, ROS must be highlighted. They were considered detrimental sub-products of aerobic cell metabolism for a long time. Conversely, relevant physiologic functions in different pathways involving GTPases, protein kinases/phosphatases, transcription factors or epigenetic modifiers have been ultimately attributed to these chemical entities (Prieto-Bermejo *et al*, 2018).

In this context, redox signalling is not an exception as a modulator of haematopoietic cell differentiation. Our group described almost a decade ago the need of a regulated production of ROS by NADPH oxidases (NOXs) to fully trigger megakaryocytic differentiation in cell lines and primary HSCs *in vitro* (Sardina *et al*, 2010). This was supported by later reports showing the relevance of NOX function for cell differentiation in other tissues (Yoshikawa *et al*, 2019; Tang *et al*, 2019).

As mentioned before, phosphoprotein phosphatases are targeted by ROS. Particularly, the family of protein tyrosine phosphatases (PTPs) have long been studied as antagonists of protein tyrosine kinases (PTKs) in cell signalling. PTPs share an oxidation prone Cys residue in their catalytic site that makes them sensitive to redox regulation. When the Cys residue becomes oxidised, the PTP loses its catalytic activity. This process is reversible as long as the oxidation is not strong enough to yield sulfinic ($-SO_2H$) or sulfonic acid ($-SO_3H$) (**Figure 1.6**) (Yu & Zhang, 2018).

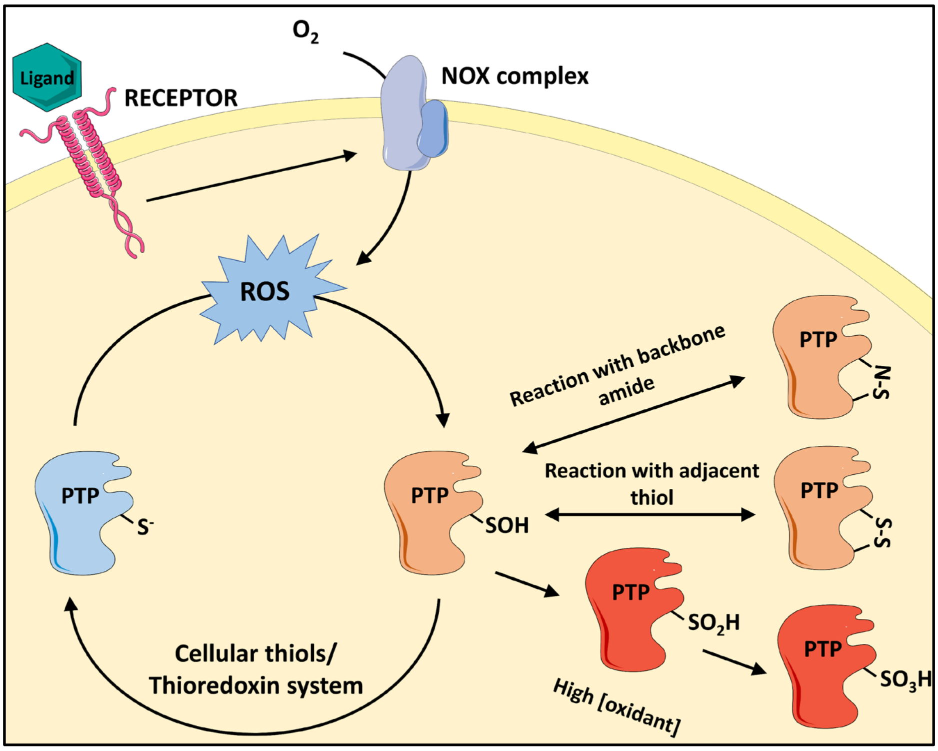
O_2
Ligand
RECEPTOR
NOX complex
ROS
Reaction with backbone amide
Reaction with adjacent thiol
PTP
S^-
SOH
S-N
S-S
SO_2H
SO_3H
Cellular thiols/ Thioredoxin system
High [oxidant]

Figure 1.6. ***Inactivation of PTPs by ROS.*** ROS produced in a regulated manner can reach the catalytic Cys residue of PTPs and oxidise it from its thiolate form. Upon mild oxidation, the thiolate anion turns into sulfenic acid (-SOH), which can form intracellular bonds with other residues. All these variants are reversibly inactive forms (shown in orange). When the oxidation degree is high enough to reach the sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid forms, the inactivation is permanent (shown in red). Adapted from Yu & Zhang, 2018.

SHP1 and SHP2 belong to the non-receptor type subfamily of PTPs. These proteins have an almost identical structure, with two N-terminal SH2 domains (N-SH2 and C-SH2, respectively), a central classical PTP domain and a C-terminal tail that undergoes phosphorylation by different PTKs. The SH2 domains allow the interaction of SHP1 and SHP2 with different proteins, including receptors, scaffolding proteins and immune inhibitory receptors. The interaction takes place through pTyr residues located at the consensus sequence [I/V/L]xY(p)xx[I/V/L], named immuno-receptor tyrosine-based inhibitory motif (ITIM), which is found in most of these interaction partners. These domains also regulate the PTP activity: the N-SH2 domain interacts with the PTP domain at the basal level, whereas the C-SH2 does not (**Figure 1.7A**). Thus, bisphosphorylated partners bind first C-SH2 and then N-SH2, thereby releasing the PTP domain and increasing the phosphatase activity (**Figure 1.7B**) (Neel *et al*, 2003). SHP1 and SHP2 have been widely studied in the context of both physiologic and pathologic haematopoiesis and opposing roles have traditionally been described for them on the literature. SHP1 is classically described as an inhibitor of signals initiated at the cell membrane and SHP2 as required for full activation of such cascades (Abram & Lowell, 2017; Pandey *et al*, 2017).

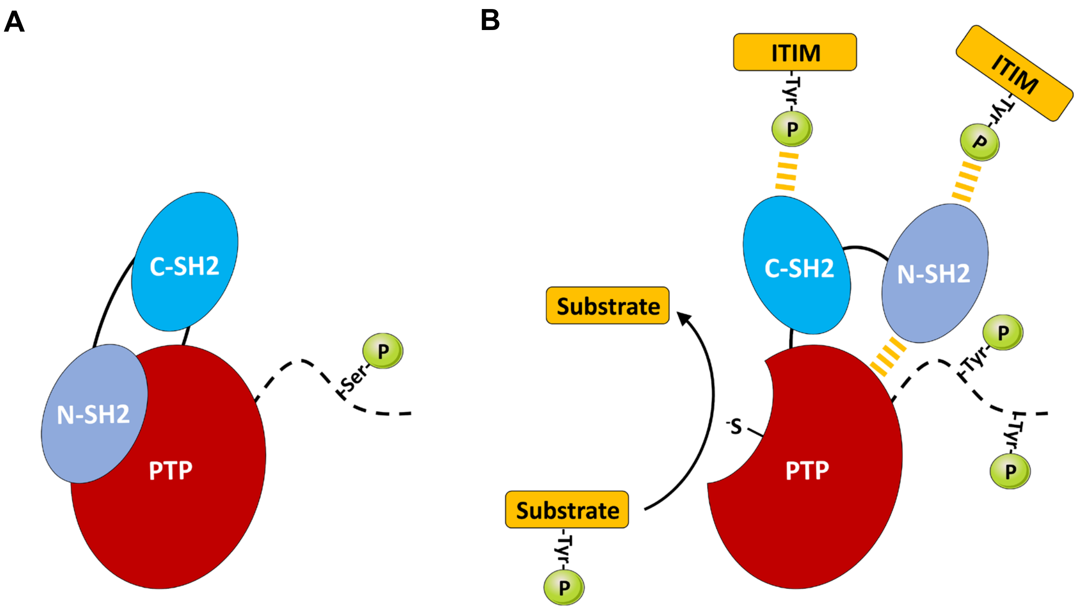

Figure 1.7. ***Structure and mechanism of activation of SH2 domain containing protein tyrosine phosphatases.*** **A)** Diagram showing the three main domains of the PTPs and the interaction of the N-SH2 domain in the inactive basal conformation. **B)** Upon binding phosphorylated partners with ITIM domains, the active site is released from interaction with N-SH2 domain and can catalyse the dephosphorylation reactions on the SHP substrates. Adapted from Abram & Lowel, 2017.

These proteins have been previously investigated in our group as likely targets of NOX-generated ROS in the context of megakaryocytic differentiation triggered by PMA. They were specifically oxidised and inhibited at early time points of this process, whereas the closely related family member PTP1B. Furthermore, this phenomenon was prevented with the addition of the NOX chemical inhibitor diphenylene iodonium (DPI) to the culture medium, supporting the hypothesis that this oxidation occurs in a regulated manner and involves some member(s) of NOX family. The relevance of the specific inhibition of these two PTPs was further tested at the molecular level through RNAi downregulation, which enhanced cell differentiation, contrary to RNAi against PTP1B (López-Ruano, 2015).

In addition to its role in AML cells differentiation, SHP2 overactivation is deeply involved in the pathogenesis of myeloid malignancies. At this respect, conditional expression of the gain-of-function mutant Shp2^{E76K} in murine haematopoietic cells fostered the development of multi-lineage acute leukaemias (Xu *et al*, 2011). Consistently, this same mutant accelerated KMT2A-rearranged AML *in* vivo through the upregulation of the anti-apoptotic molecule MCL1 apoptosis regulator, BCL2 family member (MCL1) (Chen *et al*, 2015) and contributed to homeobox A10 (HoxA10)-mediated induction of AML (Wang *et al*, 2009). The authors of this study suggested the possible involvement of SHP2 mutations in AML sub-clones in disease progression. In line with this, another SHP2 mutant occurring in AML, SHP2^{G503A}, has shown to enhance PTP activity and to accelerate a different subtype of KMT2A-rearranged AML (Fu *et al*, 2017). Furthermore, SHP2 cooperates with the AML driver FLT3/ITD for disease progression (Nabinger *et al*, 2013).

Regarding CML pathogenesis, SHP2 has also recently been reported as an essential molecule for initiation and maintenance of BCR-ABL-induced myeloproliferative neoplasms (MPNs) *in vivo* (Gu *et al*, 2018b), presumably via interaction with GAB2 (Gu *et al*, 2016). This indirect activation of SHP2 by BCR-ABL might be part of the mechanism for JAK2-independent activation of STAT5 in CML (Hjort *et al*, 2016). Indeed, interaction of SHP2 with GAB2 has been proposed as a mechanism contributing to TKI resistance (Wöhrle *et al*, 2013).

Finally, SHP2 malfunction not only contributes to leukaemogenesis in a cell-autonomous manner. A recent report has described that Shp2 mutations in the niche provoke secretion of C-C motif chemokine ligand 3 (CCL3), monocyte recruitment and interleukin 1 beta (IL1B)-mediated overproliferation of HSCs, thus giving rise to MPNs *in vivo* (Dong *et al*, 2016).

Most reports attribute an anti-leukaemogenic role to SHP1 in myeloid malignancies, with lowered expression at the protein level and remarkable promoter methylation in both AML and CML (Abram & Lowell, 2017). This low expression has also been correlated with TKI resistance in CML patients (Esposito *et al*, 2011). Only Kang and colleagues have described a function for SHP1 in the leukocyte associated immunoglobulin like receptor 1 (LAIR1)-mediated promotion of AML. However, SHP1 would act as an adaptor protein, with no role for its catalytic function in this context (Kang *et al*, 2015).

1.3.2. β-catenin: a tightly regulated protein at the forefront of leukaemogenesis

β-catenin is involved in cell adhesion through its interaction with cadherins and the central player of the canonical WNT pathway. In unstimulated cells, free β-catenin levels are modulated by the balanced synthesis and degradation mediated by the multimeric destruction complex, comprising the proteins axis inhibitor (AXIN), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). This complex sequesters and phosphorylates β-catenin, thus priming it for further recognition by β-transducin repeat-containing E3 ubiquitin protein ligase (β-TRC). β-TRC then polyubiquitylates β-catenin, thereby triggering its proteasomal degradation. Upon stimulation by canonical WNT ligands through their interaction with Frizzled (FZD) receptors and low-density lipoprotein-related protein (LRP) co-receptors, dishevelled (DVL) protein is activated and recruits the destruction complex to the proximity of the plasma membrane. At this point, the destruction complex is no longer able to sequester β-catenin and prime it for degradation, hence allowing its nuclear translocation. Once into the nucleus, β-

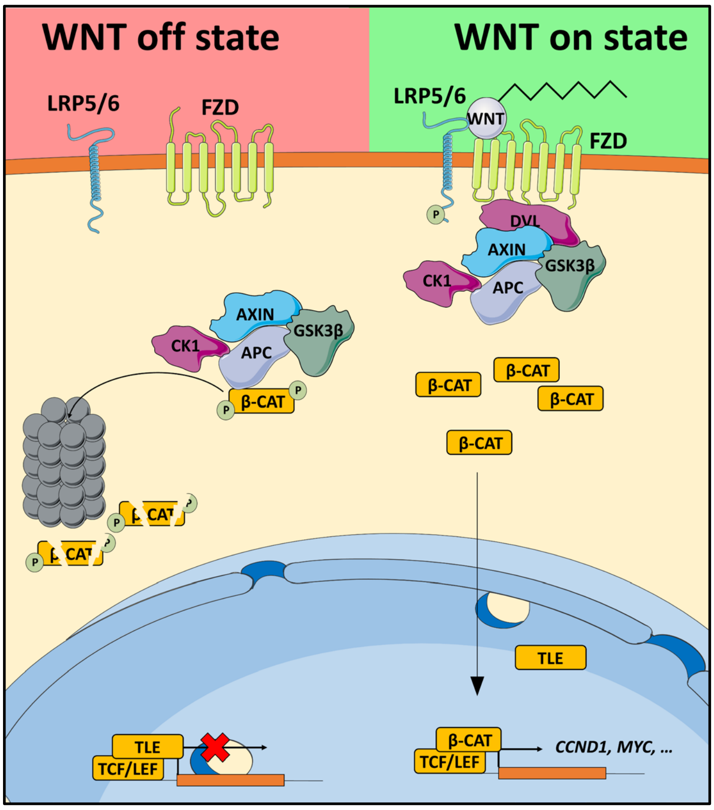

Figure 1.8. *Canonical WNT signalling.* In the absence of WNT ligands, the destruction complex comprised by AXIN, CK1, APC and GSK3β constitutively phosphorylates β-catenin (β-CAT) in different residues, thus priming it for proteasomal degradation. Upon WNT binding to its FZD receptors and LRP co-receptors, the destruction complex is recruited and remains unable to phosphorylate β-CAT, thereby allowing it to translocate into the nucleus and disrupt the interaction between TLE repressors and TCF/LEF transcription factors. In this way, transcription of pro-survival genes can occur.

catenin triggers de-repression of T cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors through competition with Transducin-like enhancer of split (TLE) proteins (**Figure 1.8**) (Staal & Clevers, 2005). This promotes the expression of survival- and proliferation-related genes, for instance *cyclin D1 (CCND1)* and *MYC proto-oncogene, bHLH transcription factor (MYC)*. This protein is involved in several pathogenic mechanisms of different types of cancer, including colon, pancreatic, lung and ovarian cancer, as well as hepatocellular carcinoma (Shang *et al*, 2017).

1.3.2.1. β-catenin in AML pathogenesis and its potential as a pharmacological target

β-catenin contribution to myeloid malignancies is a field of intense research. Ectopic expression of a constitutively active form of this protein in primary human CD34$^+$ cells impaired myeloid differentiation and protect them from apoptosis, a hallmark of leukaemia. In addition, aberrant expression of different components of canonical WNT pathway was found in primary AML samples in the same study (Simon et al, 2005). It is worth noting that β-catenin accounted for the transformation of myeloid-committed progenitors in LSCs and its absence impaired disease development in different murine models of AML (Wang et al, 2010). In agreement with this, expression of β-catenin showed correlation with the clonogenic potential of human primary AML cells *in vitro*, as well as decreased survival of patients (Ysebaert et al, 2006).

One of the mechanisms underlying β-catenin stabilisation and nuclear localisation in AML cells seems to be its cooperation with its closely related partner plakoglobin (also known as γ-catenin), whose overexpression correlated with higher levels of β-catenin and activation of its transcriptional programme (Morgan et al, 2013). Moreover, occurrence of the AML-associated chromosomal translocations RUNX1-RUNX1T1, PML-RARα and promyelocytic leukemia zinc finger (PLZF)-RARα was concomitant with an increase of plakoglobin mRNA and protein levels, β-catenin protein expression and WNT transcriptional targets (Müller-Tidow et al, 2004). FLT3-ITD, another important AML driver, cooperated with β-catenin for its leukaemogeinc activity. It was able to increase β-catenin protein levels in AML samples, likely through enhanced expression of the FZD4 receptor. In addition, FLT3-ITD-mediated cell growth could be dependent on the β-catenin downstream partner TCF4 (Tickenbrock et al, 2005). Interestingly, a very recent report revealed a correlation between the protein levels and nuclear location of β-catenin and the amount of its transcriptional co-activator lymphoid enhancer binding factor 1 (LEF1) in AML primary samples. Furthermore, the authors of the study have provided evidence supporting a regulatory mechanism of LEF1 on the levels and activity of β-catenin in both AML and CML cell lines (Morgan et al, 2019).

Several interesting pre-clinical approaches against AML have exploited the inhibition of β-catenin with other molecular targets. Two recent works reported the use of the small molecule β-catenin inhibitor BC2059, currently under phase I trials to evalulate its safety (NCT03459469). BC2059 was used in combination with the HDAC inhibitor panobinostat (Fiskus et al, 2015), the JAK2 inhibitor ruxolitinib and the BET inhibitor ARV-771 in MPN-derived secondary AML samples *in vitro* and in PDX models *in vivo* with promising results (Saenz et al, 2019). Additionally, the cooperation between FLT3-ITD aberrant signalling and β-catenin has also been used as a rationale for therapy. The combination of the β-catenin inhibitor PRI-724 with sorafenib demonstrated anti-leukaemic activity against AML blasts and stem/progenitor cells *in vitro*, together with prolonged survival versus the individual agents in a PDX model (Jiang et al, 2018). PRI-724 has undergone a clinical trial in combination with cytarabine for AML treatment (NCT01606579). Finally, the β-catenin degradation-promoting compound CWP232291 has recently completed a phase I clinical trial for the treatment of various haematologic malignancies, including r/rAML (NCT01398462).

1.3.2.2. Contributions of β-catenin to CML disease and its co-targeting with BCR-ABL as a therapeutic tool

The role of β-catenin in CML pathogenesis was described before than in AML and has therefore been elucidated in a deeper manner. First evidence of this proposed that the GMP compartment displayed the LSC activity in CML. GMPs from CML patients in the three different stages of the disease (CP, AP, BC) displayed increased levels of β-catenin versus their healthy counterparts, with an important role in their colony-forming ability (Jamieson et al, 2004). Consistently, the murine CML-like disease model induced by ectopic expression of p210 BCR-ABL showed that GMPs had the highest level of β-catenin expression, being the only population able to reproduce the disease in recipients (Minami et al, 2008).

Further studies in the same mouse model demonstrated that BCR-ABL correlated with higher β-catenin expression in HSPCs versus non-transduced cells, a relevant feature for their clonogenic potential, but dispensable for disease progression, probably due to the multiple pathways altered by BCR-ABL expression (Hu et al, 2009; Zhao et al, 2007; Heidel et al, 2012). All these studies revealed the cooperative role of β-catenin with BCR-ABL in leukaemic transformation. In addition, β-catenin levels have shown to correlate with disease progression from CP to BC in different studies (Jamieson et al, 2004; Coluccia et al, 2007; Hu et al, 2016). Indeed, $CD34^+$ CML BC cells displayed differential TCF/LEF transcriptional activity versus CP cells (Sengupta et al, 2007). Functionally, AP-derived GMPs with enforced β-catenin expression augmented their growth ability in re-plating assays (Jamieson et al, 2004).

Finally, β-catenin is also an important player in TKI responsiveness. TKI-resistant cell lines displayed higher β-catenin levels than their sensitive counterparts (Karabay et al, 2018) in a BCR-ABL-independent mechanism (Eiring et al, 2015). Moreover, downregulation of β-catenin led to restoration of TKI responsiveness in several experimental settings (Niu et al, 2013; Pehlivan et al, 2017; Zhou et al, 2017) and the β-catenin-related gene expression signature was different between TKI-responder and non-responder patients (McWeeney et al, 2010). The surrounding niche likely promotes β-catenin stabilisation in CML cells, with some results arguing in favour of an effect on TKI resistance (Zhang et al, 2012) and others against that idea (Eiring et al, 2015; Zhou et al, 2017).

Several molecules involved in β-catenin upregulation have been described in the CML setting. Different models of unresponsiveness to TKI, including primary cells, showed upregulation of genes encoding WNT ligands that promote β-catenin stabilisation (Tsubaki et al, 2017; Chen et al, 2018; Li & Luo, 2018) and FZD receptors (Liu et al, 2015). Promoter hypermethylation of the secreted frizzled-related protein-coding genes *SFRP1* and *SFRP2* has been reported in TKI-resistant CML cells as well (Pehlivan et al, 2009; Li & Luo, 2018).

Interestingly, direct phosphorylation at the Tyr86 and 654 residues by BCR-ABL was reported long time ago as relevant for nuclear accumulation of β-catenin (Coluccia et al, 2007). The N-terminal ubiquitin-binding domain (UBD) of BCR-ABL would be important for the interaction leading to this phosphorylation, with an impact on disease progression (Chen et al, 2013).

Another relevant molecule regarding this issue is interferon-regulatory factor 8 (IRF8), which has emerged as a regulator of β-catenin at different levels. The activation of SHP2 by BCR-ABL would provoke the dephosphorylation of IRF8 in CML cells, thus abrogating its transcriptional repressing ability (Hjort et al, 2016). This, in turn, would release the promoters of *GAS2* and *PTPN13* genes. For the first case, synthesis of GAS2 protein would inhibit calpain, thereby preventing β-catenin degradation (Huang et al, 2010). On the other hand, PTPN13 would be recruited to the destruction complex, where it would dephosphorylate the Tyr216 residue of GSK3β, hence preventing β-catenin degradation through a different mechanism (Huang et al, 2013). The functional crosstalk between IRF8 and β-catenin was validated *in vivo*, thus supporting its relevance in disease progression towards BC and TKI resistance (Scheller et al, 2013).

In addition, chibby 1 (CBY1), a nuclear protein with inhibitory effects in nuclear accumulation and transcriptional activity of β-catenin (Takemaru et al, 2003; Li et al, 2010), has a role in CML. Promoter hypermethylation of *CBY1* with a concomitant protein decrease was reported in CML models. Importantly, an inverse correlation between CBY1 and β-catenin level and transcriptional activity was described in CML cells (Leo et al, 2013). Indeed, IM-mediated degradation of β-catenin would involve *CBY1* promoter demethylation and the formation of the protein complex 14-3-3/β-catenin/CBY1. This tripartite complex would be translocated from nucleus to cytoplasm, eventually leading to β-catenin degradation (Leo et al, 2015a; Mancini et al, 2013; Leo et al, 2015b).

Finally, evidence has provided support for a mechanism of β-catenin modulation with the involvement of phosphatase 2 phosphatase activator (PP2A). The authors of that work proposed a model where the physical kinase-independent interaction of BCR-ABL and JAK2 would lead to SET nuclear proto-oncogene (SET)-mediated inactivation of PP2A, which in turn could not promote GSK3β-induced phosphorylation and subsequent degradation of β-catenin (Neviani et al, 2013).

Despite the vast knowledge on the regulation of β-catenin in CML cells, remarkable pre-clinical or clinical success based on its targeting has been scarce so far. The FDA-approved antihelmintic drug pyrvinum pamoate was tested on TKI-sensitive and resistant cell lines, with anti-leukaemic activity in both cases. However, that work did not assess the effect of combining pyrvinium with TKI in the re-sensitisation of TKI-resistant cells (Zhang *et al*, 2017). Similarly, the small molecule AV65, another β-catenin inhibitor, demonstrated activity against BCR-ABL^{T315I} TKI-resistant CML cell lines and synergy with IM in TKI-sensitive cells. Once again, the authors did not provide evidence of re-sensitisation ability of the drug in TKI-resistant cells (Nagao *et al*, 2011). In the *in vivo* setting, indirect decrease of β-catenin levels through inhibition of PTPN13 by using the Ser-Leu-Val tripeptide or the small molecule quinobene hampered TKI resistance, disease progression and relapse after TKI discontinuation (Huang *et al*, 2016). Additionally, the reduction of secreted WNT ligands through the porcupine O-acyltransferase (PORCN) inhibitor WNT974 markedly enhanced NL activity in different mouse models, including a PDX (Agarwal *et al*, 2017). Most importantly, the abovementioned PRI-724 was recently tested in combination with IM in a PDX model with antileukaemic activity against transplanted BC-CML cells bearing the T315I and E255V mutations (Zhou *et al*, 2017). Not surprisingly, this drug has completed a phase II clinical trial in combination with dasatinib for CML treatment (NCT01606579)

HYPOTHESIS AND AIMS

2. HYPOTHESIS AND AIMS

As highlighted before, a better understanding of the molecular mechanisms governing haematopoietic cell differentiation is key for the development of therapeutic approaches able to overcome the differentiation blockage characteristic of leukaemia. This rationale has become greatly successful in the context of APL (Coombs *et al*, 2015; De Thé, 2018). In this regard, previous work in our lab has unveiled the PTPs SHP1 and SHP2 as targets of ROS that are generated upon PMA stimulation in HEL cells. The oxidation-mediated inactivation of these proteins is required to fully trigger megakaryocytic differentiation in this model (López-Ruano, 2015).

Interestingly, HEL cells were generated from a patient with erythroleukaemia, a subtype of AML (FAB M7). On the other hand, the natural phorbol ester PRS has been proposed as a therapeutic alternative for AML in combination with standard chemotherapy in the pre-clinical setting (Shen *et al*, 2015). The pro-differentiation effects of PRS, together with the side-effects of chemotherapy, make the study of novel compounds targeting other pathways altered in AML in combination with PRS as a differentiation-based therapy a worthy endeavour.

Considering this background, the general goal of this Thesis was to **explore the potential of SHP1 and SHP2 as therapeutic targets against myeloid leukaemias.** This general aim was addressed through the following specific objectives:

1. **Study of the mechanisms involved in the convergent modulation of leukaemia cells differentiation by SHP1 and SHP2.**
2. **Assessment of the use of chemical inhibitors of SHP1 and SHP2 with phorbol esters as a differentiation-based therapy against AML.**

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Cell lines

Table 3.1. Cell lines used in this work

NAME	SUPPLIER	REFERENCE	PURPOSE
HEK-293T	Kindly provided by C. Trigueros PhD (INBIOMED Foundation, Donosti, Spain)	N/A	Lentivirus production
HEL	DSMZ – German Collection of Microorganisms and Cell Cultures GmbH	ACC-11	Model for human megakaryocytic differentiation
HL-60	DSMZ – German Collection of Microorganisms and Cell Cultures GmbH	ACC-3	Model for AML-M2
NB-4	DSMZ – German Collection of Microorganisms and Cell Cultures GmbH	ACC-207	Model for AML-M3
Non-CML iPSCs	Generated at H. Wheadon PhD lab (Toofan *et al*, 2018)	N/A	Model for normal stem cell population
OCI-AML2	Kindly provided by R.I.R. Macias PhD (University of Salamanca, Spain)	N/A	Model for AML-M4
THP-1	Kindly provided by S. Lorenzo (University of Oviedo, Spain)	N/A	Model for AML-M5

3.1.2. Primary bone marrow samples

Table 3.2. AML primary samples used in this work

ID	Age (years)	FAB subtype	Karyotype	Mutations
AML1	53	M0	46, XX, t(3;3)(q21;q21)[20]	*WT1, IDH1*
AML2	59	M0	46, XY	N/D
AML3	57	M0	46, XY	N/A
AML4	52	M1	45, XX, -7[15]	N/D

AML5	71	M0	46, XX, del (5q)(q13q35)[13] 47, XX$_{SL}$, +8[2]	N/A
AML6	39	M4	46, XY	*FLT3*
AML7	50	Secondary AML	No metaphases	N/D
AML8	64	M5	No metaphases	*NPM1*

Table 3.3. HD primary samples used in this work

ID	Age (years)
HD1	26
HD2	27
HD3	39
HD4	36

3.1.3. Drugs

Table 3.4. Pharmacologically active compounds used for this work

ITEM	SUPPLIER	REFERENCE
12-Deoxyphorbol-13-Acetate (Prostratin, PRS)	Santa Cruz Biotechnology	sc-203422A
NSC 87877 (NSC)	Santa Cruz Biotechnology Tocris Bioscience	sc-204139 2613
Phorbol 12-myristate-13-acetate (PMA)	Sigma-Aldrich	P8139
SHP099 hydrochloride	MedChem Express	HY-100388A
Sodium stibogluconate (SSG)	MedChem Express	HY-100595

3.1.4. Flow cytometry antibodies

Table 3.5. Flow cytometry antibodies employed for the present work

ITEM	SUPPLIER	REFERENCE
Annexin V detection kit	Immnuostep	ANXVKPE-100T
Anti CD11b-APC	Miltenyi Biotec	130-191-241

Anti CD41-APC	Immnuostep	41A-100T
Anti CD61-APC	Immnuostep	61A-100T

3.1.5. Western blot antibodies

Table 3.6. Primary antibodies for western blot used in this work

TARGET	SUPPLIER	REFERENCE (Clone)	WORKING DILUTION	BLOCKING SOLUTION
ERK	Santa Cruz Biotechnology	sc-153 (K-23)	1:5000	5% milk in TBS-T
GAPDH	ThermoFisher Scientific	AM4300 (6C5)	1:40000	5% milk in TBS-T
GSK3β	BD Transduction Laboratories	612201 (7/GSK-3b)	1:3000	5% milk in TBS-T
pERK	Santa Cruz Biotechnology	sc-7383 (E-4)	1:3000	2% BSA in TBS-T
pSRC^{Tyr418}	Abcam	ab4816	1:2000	2% BSA in TBS-T
pSTAT5^{Tyr694}	BD Transduction Laboratories	611964 (47)	1:1000	2% BSA in TBS-T
SHP1	Santa Cruz Biotechnology	sc-287 (C-19)	1:2000	5% milk in TBS-T
SHP1	BD Transduction Laboratories	610126 (52/PTP1C/SHP1)	1:1000	5% milk in TBS-T
SHP2	Santa Cruz Biotechnology	sc-280 (C-18)	1:2000	5% milk in TBS-T
SHP2	BD Transduction Laboratories	610622 (79/PTP1D/SHP2)	1:2000	5% milk in TBS-T
SRC	Santa Cruz Biotechnology	sc- 8056 (B-12)	1:2000	5% milk in TBS-T
SRC	Cell Signalling Technology	2108S	1:1000	5% milk in TBS-T
STAT5	Santa Cruz Biotechnology	sc-835 (C-17)	1:1000	5% BSA in TBS-T
β-CATENIN	BD Transduction Laboratories	610153 (14/Beta-Catenin)	1:500	5% milk in TBS-T

Table 3.7. Secondary antibodies for western blot used in this work

TARGET	SUPPLIER	REFERENCE (Clone)	WORKING DILUTION	BLOCKING SOLUTION	DETECTION METHOD
Mouse IgG (H+L)	Bio-Rad	1706516	1:10000	5% milk in TBS-T	Chemiluminescence
Rabbit IgG (H+L)	Invitrogen	A16104	1:5000	5% milk in TBS-T	Chemiluminescence

3.1.6. Oligonucleotides

Table 3.8. Oligonucleotides for RNAi downregulation employed in this work

TARGET	IDENTIFICATION KEY	SEQUENCE (5’-3’ sense)
Firefly luciferase	Luc	CTGACGCGGAATACTTCGA
Human SHP1	SHP1	GAACAAATGCGTCCCATA
Human SHP2	SHP2	TGACAGATCTTGTGGAACA
Human SHP1 and SHP2	SHP1/2	TGACAGAGCTGGTGGAGTA
Human SRC	SRC#1	TCAAGTGCATTAAGAACGA
	SRC#2	TCAAGTGCATTAAGAACGA

3.1.7. Buffers and solutions

- Buffer 1 for protein solubilisation. 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10% (v/v) glycerol, 5 mM EDTA.
- MLB protein solubilisation buffer. 25 mM HEPES pH 7.5, 150 mM NaCl, 1% (v/v) IGEPAL™, 10% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4.
- Phosphate buffered saline (PBS) 1X pH 7.4: 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4.
- Propidium Iodide (PI) working solution for ploidy analysis: 50 µg/mL PI (Acros Organics) and 0.1 mg/ml Ribonuclease A (Thermo Scientific) in PBS 1X.
- Protein loading buffer for western blot 2X. 125 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) glycerol, 0.003% (w/v) bromophenol blue, 1% (v/v) β-mercaptoethanol.
- Stripping solution for western blot membranes: 62.5 mM Tris-HCl pH 6.8; 2% SDS (w/v); 100 mM β-mercaptoethanol.
- Tris buffered saline with Tween 20 (TBS-T). 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05% (v/v) Tween 20.

- Western blot running buffer 1X. 5 mM Tris-HCl pH 8.3, 40 mM glycine, 0.1% (w/v) SDS.
- Western blot running gel buffer. 1.5 M Tris-HCl pH 8.8.
- Western blot stacking gel buffer: 0.5 M Tris-HCl pH 6.8.
- Western blot transfer buffer 1X. 25 mM Tris, 190 mM glycine, 20% (v/v) methanol.

3.1.8. Laboratory equipment

- 40SM-200A (Precisa) analytical scale and Electronic Scale (Want) precision scale.
- Allegra^{TM} 21R (Beckman), 5810R (Eppendorf) and 3-16PK (Sigma) centrifuges.
- Applied Biosystems ProFlex^{TM} PCR system (Thermo Scientific) thermal cycler.
- BFR 25 (Grant Boekel) platform rocker.
- CKX41 (Olympus), TMS (Nikon), Eclipse Ci-L (Nikon) and XL Core (EVOS) inverted light microscopes.
- CS-9000 (Shimadzu) densitometer.
- Cytospin2 centrifuge (Shandon).
- ESCO class II BSC (Labcultures), Telstar Bio IIA class II (Fisher Scientific) and Herasafe^{TM} KS Class II (Thermo Scientific) laminar airflow biosafety cabinets.
- FACSCalibur and FACSCanto II (BD Biosystems) flow cytometers.
- Fluidigm Biomark Analyser (Fluidigm).
- Forma -86 ULT (Thermo Electron Corporation) -80ºC freezer.
- Forma Direct Heat 311 (Thermo Scientific) and New Brunswick Galaxy 170 S (Eppendorf) CO_2 incubators.
- IGNIS -20ºC freezer.
- Liquid nitrogen tank (Thermo Forma).
- Medical X-Ray Processor 102 (Kodak) film processor.
- MicropH 2001 (Crison) pH-meter.
- Minimix (OVAN) magnetic stirrer.
- Mini-PROTEAN® III, Mini-PROTEAN® Tetra (Bio-Rad) and Novex™ XCell™ *SureLock*™ (Invitrogen) vertical electrophoresis and blot transfer systems.
- Mutliskan FC (Thermo Scientific) and Spectramax M5 (Molecular Devices) plate readers.
- Nanodrop 1000 and Nanodrop 2100 (Thermo Scientific) spectrophotometers.
- Odyssey® Fc Dual-Mode Imaging System (LI-COR Biosciences).
- Power Pac HC and Power Pac Basic (Bio-Rad) power suppliers for vertical electrophoresis.
- Roller mix (Ovan).

- SBS TFB serie B (Memmert), Grant sub 28 (Cambridgeshire) and Retostat thermostatic water baths.
- Sorvall Legend Micro 21R (Thermo Scientific), IEC MicroCL 17 (Thermo Electron Corporation) and 2-16 (Sigma) microcentrifuges.
- Steam Sterilizer (Raypa) and Autester-G (Selecta) autoclaves.
- Taqman 7900 (Applied Biosystems) quantitative PCR machine.
- VB85 (Gelaire) laminar airflow hood.
- XL-100K (Beckman) ultracentrifuge.
- XM Full HD Camera (MicrosCopiaDigital).
- Other frequently used laboratory devices.

3.1.9. Software and other informatic tools

- BD CellQuest Pro (BD Biosciences), for cell acquisition in BD FACSCalibur instrument.
- CalcuSyn 2.0.0 (Biosoft) for drug interaction studies and half maximal inhibitory concentration (IC50) determination in cell lines with nearly linear dose-response curves.
- FlowJo v10.5.3 (FlowJo, LLC) and Flowing Software 2 (Turku Centre for Biotechnology) for flow cytometry data analysis.
- GraphPad Prism 6 and Prism 8 (GraphPad Software) for IC50 determination in cell lines with a non-linear dose-response curve and data plotting.
- IBM SPSS Statistics 22 (IBM) for statistical analysis.
- Microsoft Office 365 ProPlus suite (Microsoft) for manuscript writing, data analysis and figure assembly.
- R version 3.4.4 (GNU General Public License) for statistical analysis and data plotting.
- Servier Medical ART (SMART) graphical material was used from the website https://smart.servier.com/ under a Creative Commons Attribution 3.0 Unported License.
- ToupView (ToupTek Photonics) for image acquisition of HL-60 cytospin slides.
- Zotero (Corporation for Digital Scholarship) for citations and reference management throughout this manuscript.

3.2. Experimental procedures

3.2.1. Cell culture

All cell lines used for this work were cultured at 37ºC and 5% CO_2. HEL, HL-60, NB-4 and THP-1 cells were grown in RPMI 1640 supplemented with heat-inactivated foetal bovine serum (FBS) at a final concentration of 10% (v/v) and 1X Penicillin-Streptomycin (Pen-Strep) commercial solution. OCI-AML2 cells were cultured in Alpha MEM with FBS 20% (v/v) and 1X Pen-Strep. Culture medium was replaced every 48 h after cell count with trypan blue exclusion dye and cells were seeded at $2\text{-}2.5 \cdot 10^5$ cells/mL.

HEK-293T cells were grown in DMEM with 10% FBS and 1X Pen-Strep. They were passaged when confluent cultures were observed under the microscope (usually every 3-4 days). Spent medium was removed and culture was washed with 1X PBS. Next, trypsin-EDTA solution pre-warmed at 37ºC was added to detach cells and after brief incubation it was inactivated by adding fresh medium. Cells were then seeded at the desired density for lentiviral production.

Primary bone-marrow mononuclear cells (BM-MNCs) were cultured in RPMI 1640 with 10% FBS and 1X Pen-Strep.

All cell culture reagents were purchased to Biowest or Gibco unless otherwise specified.

3.2.2. Lentivirus production for RNAi

Sequences for RNAi-mediated knockdown of the different proteins studied in this work were designed according to the criteria proposed by Reynolds *et al.* (Reynolds *et al*, 2004). DNA hybridisation and construct introduction into the pLVTHM plasmid was previously performed in the laboratory (López-Ruano, 2015). The HIV-based second-generation shRNA delivery system was kindly provided by D. Trono (Wiznerowicz & Trono, 2003). This system splits the lentiviral genome into three different plasmids: the transfer plasmid (pLVTHM), containing the insertion cassette for stable shRNA expression and a deletion at the 3’ LTR to inactivate lentivirus replication (**Figure 3.1**); the envelope plasmid (pMD2.G), which encodes the envelope glycoprotein dictating the virus tropism (VSV-G is encoded in pMD2.G and allows for wide tropism) and the packaging plasmid (psPAX2), encoding Gag, Pol and accessory proteins required for efficient packaging of the vector.

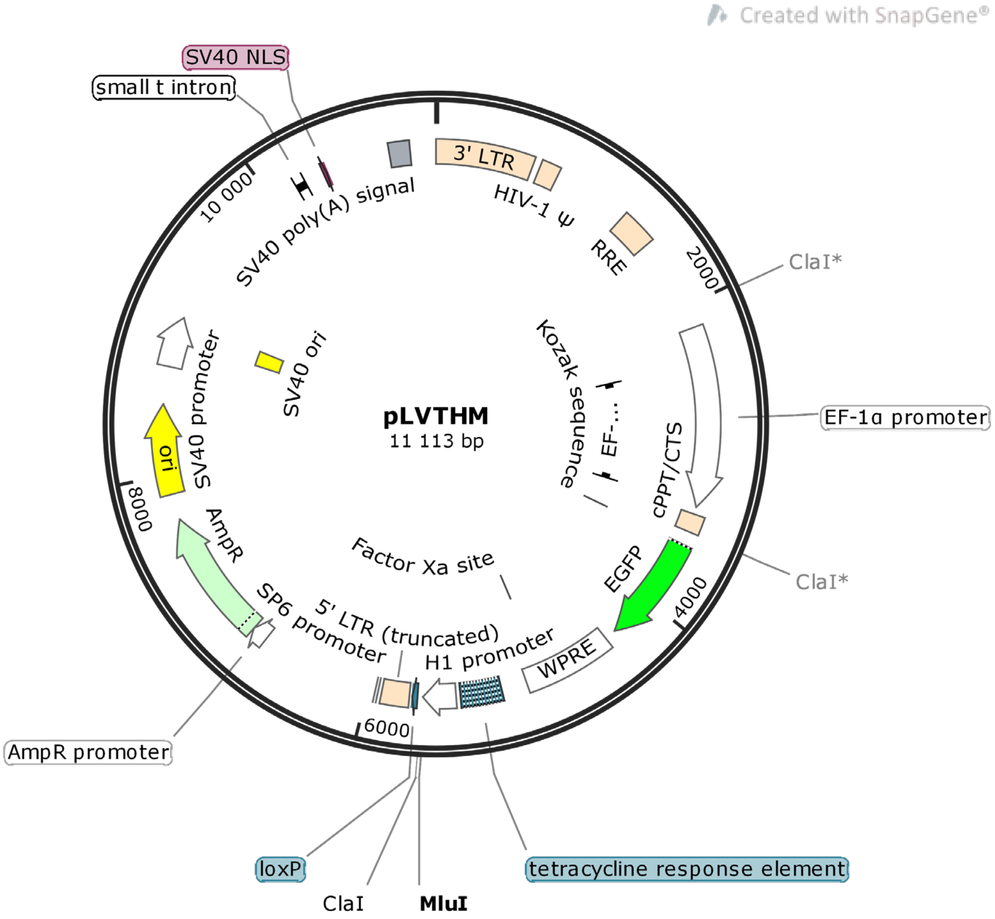
Created with SnapGene®
SV40 NLS
small t intron
3' LTR
HIV-1 ψ
RRE
2000
ClaI*
SV40 poly(A) signal
10 000
SV40 ori
pLVTHM
11 113 bp
Kozak sequence
EF-...
EF-1α promoter
cPPT/CTS
SV40 promoter
ori
8000
AmpR
Factor Xa site
ClaI*
EGFP
4000
SP6 promoter
5' LTR (truncated)
H1 promoter
WPRE
6000
AmpR promoter
loxP
ClaI
MluI
tetracycline response element

Figure 3.1. ***Map of the lentiviral vector for stable RNAi expression pLVTHM.*** Schematic representation of the vector showing its main features. The restriction sites MluI and ClaI downstream of the H1 promoter serve as cloning site for RNAi constructs. The enhanced green fluorescent protein (EGFP) open reading frame (ORF) under the elongation factor 1 alpha (EF-1α) promoter allows the monitoring of transduced cells.

3.2.2.1. Seeding of HEK-293T cells

100 mm-diameter cell culture dishes were coated with 3 mL of 0.1 mg/ml poly-D-lysine (Sigma Aldrich) for 5 min. Coating reagent was then removed and dishes were air-dried prior to cell seeding. After that, HEK-293T suspensions were adjusted to an appropriate volume to achieve 80% confluent cultures in 9.6 mL of complete medium per dish. Once the dishes were dry, cells were plated and incubated at 37ºC for 6 h.

3.2.2.2. Cell transfection

Purified plasmids were mixed in a transfection solution as follows (volumes are given by 100 mm diameter dish of HEK-293T cells at 80% confluence): 1200 µl of 150 mM NaCl, 6 µg of pLVTHM plasmid, 6 µg of psPAX2 plasmid, 4.5 µg of pMD2.G plasmid and 60 µl of 1 mg/ml linear polyethyleneimine (PEI, Polysciences Inc.). The mixture was vortexed and incubated for 20 min at RT to allow the formation of complexes composed of polycationic PEI and polyanionic DNA, which are subsequently endocytosed by the virus producing cells. After that, the mixture was added dropwise onto the previously seeded HEK-293T monolayers. The cultures were incubated overnight with the DNA/PEI mixture. Next day, the transfection medium was safely disposed of and replaced by 8.5 mL of pre-warmed fresh DMEM.

3.2.2.3. Supernatant collection and concentration

Supernatants containing lentiviral particles were harvested at 48h and 72h post-transfection. Lentiviruses were concentrated by ultracentrifugation at 50 000 g for 2h at 16ºC. The liquid fraction was discarded and 200 µl of RPMI with 1% FBS were added to the viral pellet. Tubes containing the concentrated lentiviruses were then incubated for 4 h at 4ºC to allow resuspension and then the concentrates were homogenized by pipetting up and down at least 40 times. Finally, they were aliquoted and frozen at -80ºC prior to use.

3.2.2.4. Titration of viral concentrates

10^5 HEK-293T cells/well were seeded in a 24-well plate pre-coated with poly-D-lysine and air-dried. Cells were incubated for 6 h at 37ºC to allow their attachment to the plate and supernatant volume was adjusted to 350 µl prior to adding viral concentrates. Different volumes of previously made 1:100 dilutions of the lentiviral concentrates were added onto each well. Then, plates were spun down at 1800 g and 32ºC for 90 min to facilitate the virus-cell interaction and incubated overnight at 37ºC. Next day, the lentivirus-containing supernatants were disposed of and replaced by 500 µl of fresh DMEM. After 48 h of incubation at 37ºC to allow cell growth and GFP expression, cells were detached with trypsin-EDTA and acquired in a flow cytometer. The percentage of GFP^+ cells was used to estimate the viral titre with the following equation:

$$Viral\ titre\ \left(\frac{TU}{ml}\right) = \frac{Number\ of\ seeded\ cells\ (10^5) \times \frac{\%\ GFP^{+}cells}{100}}{Added\ volume\ of\ viral\ supernatant\ (ml)}$$

The highest value of viral titre for each shRNA-containing lentivirus was chosen from the previous calculations as theoretically, that would be the number for a situation when one single viral particle infected a single cell.

3.2.3. Lentiviral transduction of cell lines

Cells were cultured for at least 6 h prior to transduction in the appropriate culture medium with 1% FBS at a density of $4\cdot10^5$ cells/mL. After that, different volumes of ice-thawed lentiviral concentrates were added to the desired number of cells in a way that multiplicity of infection (MOI) was enough to yield nearly 100% GFP^+ cultures. Cell density was adjusted to $3\cdot10^5$ cells/mL and cultures were distributed onto 96-well plates with 200 µl/well. Plates were centrifuged at 1800 g and 32ºC for 90 min and subsequently incubated at 37ºC overnight. The day after transduction, cells were harvested, washed twice with PBS and seeded in complete fresh medium with 10% FBS at a density of $3\cdot10^5$ cells/mL for a proper recovery. Two days after lentivirus removal, an aliquot of the culture was acquired in a flow cytometer to monitor the percentage of GFP^+ cells. Simultaneous silencing of SHP1 and SHP2 was performed by transducing SHP2-silenced cells with lentiviruses targeting SHP1 expression. Similarly, simultaneous downregulation of SRC and SH2 domain containing PTPs was performed by adding lentiviruses targeting the expression of those PTPs on SRC-downregulated cells.

3.2.4. Primary bone marrow mononuclear cells (BM-MNCs) isolation

Bone marrow samples from AML patients and healthy donors (HD) were harvested at Hospital Clínico Universitario de Salamanca after informed consent of the participants, according to Helsinki Declaration. To avoid aggregation, samples were sequentially passed several times through 18G and 21G needles. Then, they were transferred into a new tube and diluted 1:3 with Hank's Balanced Salt Solution (HBSS, Lonza). For isolation of mononuclear cells, 4 mL of Ficoll-Paque™ PLUS (GE Healthcare) were carefully added into the bottom of a 15 mL Falcon tube. Next, 6 mL of the diluted BM sample were gently added through the wall to avoid mixing with Ficoll (**Figure 3.2A**). Tubes containing sample + Ficoll were afterwards spun down at 460 g for 30 minutes at minimum acceleration and deceleration to avoid disruption of the different phases. After centrifugation, a cloudy phase appeared on the top of Ficoll, containing the BM-MNCs (**Figure 3.2B**). These cells were sucked up with a Pasteur pipette and transferred into a clean tube. Cells were then washed with HBSS and centrifuged at 300 g for 10 min twice, and finally resuspended in culture medium at a density of 10^6 cells/mL for subsequent treatments.

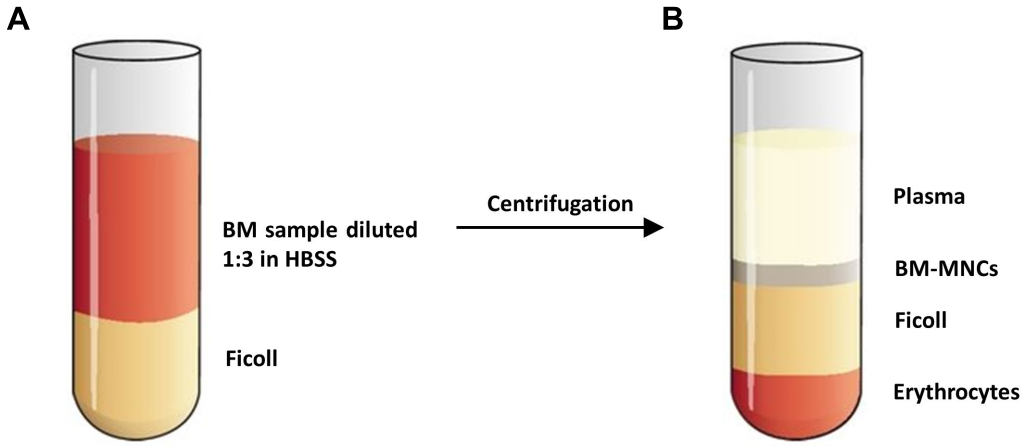

Figure 3.2. ***Isolation of primary BM-MNCs.*** **A)** BM samples diluted in HBSS are gently placed into a tube containing Ficoll. **B)** After proper centrifugation, different phases emerge from the sample, being the BM-MNCs the one of interest for this study. Adapted from (Low & Wan Abbas, 2015).

3.2.5. Primary bone marrow samples thawing and recovery

Liquid nitrogen-frozen AML BM-MNCs were rapidly thawed at 37ºC and washed twice with PBS to remove freezing solution (FBS + 10% DMSO). Then, viable cell number was determined through trypan blue exclusion staining with a Thoma haemocytometer and cells were cultured in complete medium overnight at 37ºC at a density of 10^6 cells/mL prior to treatments.

3.2.6. Drug treatments

3.2.6.1. Stock solutions

PMA and PRS were dissolved in DMSO to a concentration of 2 mM and 20 mM, respectively, aliquoted, and stored at -80ºC prior to use. Thawed aliquots were not re-used. NSC was dissolved in the appropriate volume of dH_2O to a concentration of 50 mM. After thorough vortexing, aliquots were made and stored at -20ºC or -80ºC. They were used up to two freeze-thaw cycles. SHP099 hydrochloride was dissolved in DMSO to a final concentration of and 10 mM with sonication and aliquoted at -80ºC prior to use. The unused fraction of thawed aliquots was discarded. SSG was freshly prepared prior to use to different concentrations depending on the purpose. The desired volume of complete culture medium was added to weighed drug and the mixture was vigorously vortexed. After that, it was incubated at 75ºC with continuous shaking until no turbidity was observed.

3.2.6.2. Drug treatments for cell proliferation studies

For MTT assays, 100 µL of AML cell lines (HL-60, NB-4, OCI-AML2 and THP-1) suspensions at a density of 4-5·10^5 cells/mL were seeded in 96-well plates with three technical replicates per condition. 2X or 4X drug solutions were prepared for single agent or combined treatments, respectively. The different doses were prepared by serial dilution starting from the highest dose tested. 50 or 100 µL of the drug were added to the cells for combinations or individual treatments, respectively, making a final volume of 200 µL/well and a density of 2-2.5·10^5 cells/mL. Cells were incubated for 48 h at 37ºC.

3.2.6.3. Drug treatment for cell differentiation, cell number, viability and colony-forming unit (CFU) ability assessment

Once the interaction between the different drugs was determined, the most interesting drug combinations were selected for further studies. For flow cytometry experiments, HEL and HL-60 cells were seeded at a density of 3.5·10^5 cells/mL in 96-well plates in a final volume of 200 µL. For morphology studies, HL-60 cells were cultured in 6-well plates in a final volume of 5 mL at a density of 3.5·10^5 cells/mL. For cell number, viability and clonogenicity studies, cells were cultured the same way with a final density of 2.5·10^5 cells/mL. Primary BM-MNCs were cultured in the presence of synergistic doses of PRS and NSC in HL-60 cells at a density of 10^6 cells/mL. Cells were pre-incubated with SHP1 and SHP2 inhibitors (SSG, SHP099, NSC) to allow the inhibition of PTPs for 1h at 37ºC prior to addition of phorbol esters (PMA and PRS) at the desired concentrations and the cultures were incubated at 37ºC for 48h.

3.2.7. MTT assay

The reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan by cellular succinate dehydrogenases is a common way to estimate the number of alive cells after a given treatment. MTT-formazan precipitates can be dissolved in DMSO, generating a purple solution whose absorbance is directly related with metabolic activity during the incubation time.

After the incubation period, cells were washed with PBS (twice when NSC was present in the medium, as its intense red colour of the drug in solution may interfere with absorbance measurements). Then, they were resuspended in 100 µL of a 5 mg/mL solution of MTT (Sigma-Aldrich) in PBS and incubated for 75 min at 37ºC. Once the MTT-formazan precipitates were formed, plates were spun down and washed with PBS. Finally, crystals were dissolved with 100 µL/well DMSO (Sigma-Aldrich) and absorbance at 570 nm was measured in a plate reader.

3.2.8. Study of drug interactions using CalcuSyn

The interactions between drugs in MTT or resazurin assays were determined using CalcuSyn software, which utilises the Median Effect method (Chou, 2006) for calculating the so-called combination index (CI). CI values above 1 are indicators of antagonism, around 1 of additivity and below 1 of synergism.

Cells were incubated with a range of doses of the different drugs employed to test their sensitivity. Based on that, different combinations were simultaneously tested with single-agent dose ranges and inhibitory effect was introduced in CalcuSyn software to calculate CIs. Additive or synergistic doses were selected for further studies.

3.2.9. Trypan blue exclusion cell count

Viable cells keeping integrity repel this dye due to its negative charge, which is repelled by the cell membrane, also negatively charged. Only cells with damaged membranes (i.e. dead cells) will incorporate trypan blue molecules and display a blue colour under the microscope. Therefore, trypan blue staining is a very straightforward approach to determine cell viability in a cell culture.

For synergistic combinations in HL-60 obtained through MTT assays, trypan blue exclusion assays were also performed to confirm the enhanced effect through a different method. An aliquot was taken for every treatment and conveniently diluted with PBS. Next, trypan blue 0.4% (w/v) (Gibco) was added to cell suspensions in a volume ratio 1:1 and thoroughly mixed. Then, cells were counted under the microscope by using a Thoma haemocytometer.

3.2.10. Viability tests with Annexin V staining

Annexin V is a protein able to bind phosphatidylserine (PS) residues in the presence of calcium (Ca^{2+}) cations. PS translocation to the outer layer of cytoplasmic membrane occurs at the beginning of apoptosis and is a reliable marker of this process. Fluorophore-conjugated Annexin V is widely used to assess apoptosis induction by flow cytometry.

$5 \cdot 10^5$ HL-60 cells treated for 48 h with PRS and NSC were harvested and washed with PBS prior to 15 min staining with phycoerythrin (PE)-conjugated Annexin V (Immunostep) diluted 1:50 in 1X binding buffer. After the incubation period, cell suspensions were conveniently diluted with binding buffer to stop the reaction and for a proper acquisition speed in a flow cytometer.

3.2.11. Colony-forming unit (CFU) assays

After incubation in the presence of drugs, viable cell number was determined by trypan blue staining in a Thoma haemocytometer. 500 HL-60 cells were resuspended in 500 µL of StemMACS HSC-CFU basic (Miltenyi Biotec) whereas 10^4 AML or $2.5 \cdot 10^4$ HD BM-MNCs were resuspended in 500 µL of StemMACS HSC-CFU complete without Epo (Milteny Biotec). In all cases, cell suspensions in semi-solid medium were seeded in 24-well plates, ensuring proper humidity by filling al the surrounding wells with sterile PBS. Colonies were counted 7 days after seeding for HL-60 cells and 14 days after seeding for BM-MNCs.

3.2.12. Cell differentiation assessment

Cells treated with PMA and PRS become strongly attached to the bottom of the wells. Therefore, an incubation of at least 30 min on ice was carried out to facilitate cell detachment and prevent cell damage during the harvesting process for all the following studies.

3.2.12.1. Measurement of cell surface antigens by flow cytometry

Cells were collected and washed with PBS to remove drugs and cell culture media. They were resuspended in 50-100 µL PBS and incubated with 1:25 dilutions of fluorochrome-conjugated anti CD41, anti CD61 or anti CD11 antibodies for 15 min at RT in the dark. Then, they were washed with PBS to remove the excess of antibodies and conveniently resuspended in PBS to be acquired in a flow cytometer.

3.2.12.2. Determination cell ploidy status

Treated cells were harvested and washed with PBS to remove drugs and culture media. Then, they were fixed with 2.5% paraformaldehyde (PFA, Thermo Scientific) in PBS to maintain GFP fluorescence, washed with PBS and spun down at 1000 x g for 5 min. After that, they were resuspended in 70% ethanol (Thermo Scientific) and incubated overnight at 4ºC. Next day, they were washed and centrifuged as before and incubated at 37ºC for 45 min in a solution containing 50 ng/mL propidium iodide (PI) and 0.5 ng/mL of RNase A in PBS 1X. Cells were washed and centrifuged again and finally resuspended in the proper volume of PBS to be acquired in a flow cytometer. The subsets of interest were gated as shown in **Figure 3.3.**

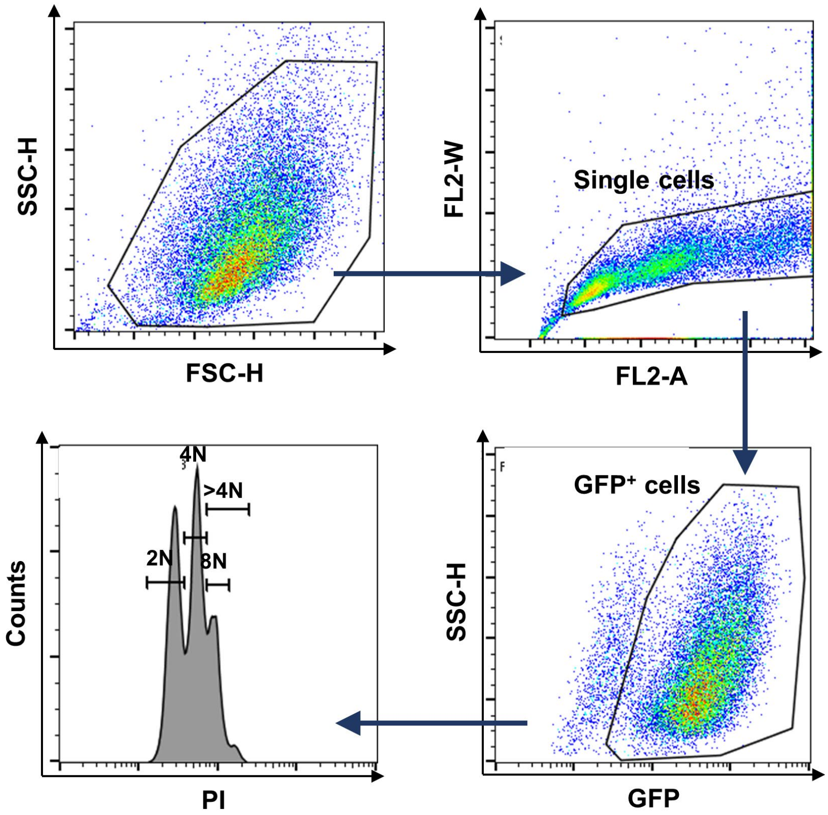

Figure 3.3. ***Gating strategy for ploidy analysis of HEL cells.*** Representative example of the gates and channels used for ploidy analysis in SHP1- and SHP2-silenced HEL cells treated for 7 days with 20 nM PMA.

3.2.12.3. Morphological assessment of cell differentiation

After incubation with drugs and detachment step, cells were collected, and a small aliquot was taken for cell number determination by Trypan blue staining. Then, cells were washed with PBS and centrifuged at 300 g for 5 min. After determination of cell number, cells were resuspended in PBS to a density of 5-7.5·10^5 cells/mL and kept on ice until their use. 100 µL of the cell suspensions were taken per slide to perform cytospins. The cell suspensions were centrifuged at 10 g for 7 min to attach the cells onto the slides and, after visually checking under a microscope, suitable preparations were stained with May-Grünwald-Giemsa solution. Cell morphology and relative differentiation status was assessed by M. Díez-Campelo MD PhD (Hospital Clínico Universitario de Salamanca).

3.2.13. AML xenograft model for drug testing *in vivo*

Female 8-week-old NOD-SCID mice (Charles River) were irradiated with a 2.5 Gy single dose 24 h prior to cell transplant. HL-60 cells were maintained in exponential growth phase (cell density adjusted daily to 3.5·10^5 cells/mL prior to injection). The day of transplantation, they were harvested and washed twice with abundant PBS and then resuspended in RPMI 1640 without serum. Each animal was injected with $5 \cdot 10^6$ HL-60 cells through the lateral tail veins in a maximum volume of 200 µL. Five days after transplantation, treatment with the indicated agents was started. Stock solutions of drugs were diluted in sterile PBS and administered intraperitoneally (i.p.). DMSO concentration was adjusted to be exactly the same in all treatment arms. Animals were daily monitored and treated every two days until humane endpoint, defined by scruffy fur, loss of activity and a loss of weight equal or greater than 25%, was reached. All animal protocols were approved by the University of Salamanca Bioethics Committee.

3.2.14. Study of protein levels by Western blot

All cell lines were seeded at a density of $3.5 \cdot 10^5$ cells/mL the day before protein extraction. When the effects of drug treatments on protein levels were assessed, cells were treated at the specified doses for a 16 h period unless otherwise specified. A minimum of three biological replicates were performed.

3.2.14.1. Protein extraction

Cells were harvested and centrifuged at 700 x g and 4ºC for 7 min and the supernatant was discarded. When the pellets were not immediately processed, they were dried and stored at -20ºC.

For AML cell lines, pellets were resuspended in MLB buffer with a commercial protease inhibitor cocktail (Sigma Aldrich) and incubated on ice for 20 min. Then, samples were centrifuged at 21000 x g and 4ºC for 15 min and the supernatants were recovered for subsequent use. For gel loading, samples were mixed 1:1 with protein loading buffer 2X and denatured by incubation in boiling water for 5 min.

3.2.14.2. Protein quantification

Small aliquots of the soluble fractions of protein extracts were taken and conveniently diluted in water for further quantification using Bradford’s method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard for the curve where sample absorbance values were interpolated. Assays were performed in 96-well plates and samples were measured in triplicate.

3.2.14.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts from AML cell lines were separated in poured gels of a fixed concentration of polyacrylamide in western blot running buffer 1X. The same protein amount was loaded in every lane for comparison purposes. Electrophoresis were performed at a constant voltage of 125 V and RT until the bromophenol blue front reached the edge of the gel.

3.2.14.4. Wet transfer of proteins to membranes

Polyvinylidene difluoride (PVDF) membranes were used for transferring proteins from AML cell lines. Membranes were activated with methanol and then placed in close contact to polyacrylamide gels. The transfer was performed in 1X western blot transfer buffer during 4 or 16 h, depending on the protein(s) of interest, at a constant amperage of 400 or 150 mA, respectively.

The presence of proteins on the membranes was checked by staining with Ponceau S solution. The dye was washed off before incubation with antibodies.

3.2.14.5. Incubation with antibodies

Prior to antibody hybridisation, unspecific binding reactions were blocked by incubating the membranes with the different block solutions indicated in **Tables 3.6 and 3.7.** Block was performed for 1 h at RT.

Blocked membranes were then incubated in the presence of primary antibodies diluted in the block solutions specified in **Table 3.6** overnight at 4ºC. Then, they were washed three times in TBS-T and transferred into appropriate secondary antibody dilutions (**Table 3.7**) for an incubation of 1 h at RT (and protected from light in the case of fluorescent dye-conjugated antibodies). After this step, membranes were washed again three times with TBS-T prior to detection.

3.2.14.6. Signal detection

When horseradish peroxidase (HRP)-conjugated secondary antibodies were used, membranes were incubated with Clarity Western ECL Substrate (Bio-Rad) or Pierce™ ECL Plus Western Blotting Substrate (Thermo Scientific) for band visualisation.

3.2.14.7. Quantification of western blot bands

Signals recorded in X-ray films were quantified by densitometry using a wavelength of 570 nm.

3.2.15. Statistical analysis

For two-group comparisons, unpaired Student’s t test was used. For multiple group comparisons, either ANOVA (data fitting normal distribution) or Kruskal-

Wallis (data not fitting normal distribution) tests were performed. Appropriate *post hoc* tests were subsequently run for pairwise comparisons. For animal survival, a Log-Rank test was conducted. Excel, SPSS and R v3.4.4 were used as statistics software.

Bar graphs represent data average ± standard deviation. Lines above bar diagrams depict statistical significance of pairwise comparison between the bars located below the extremes when a multiple comparison test was performed. Symbols above bars indicate differences versus the corresponding control (untreated cells, control RNAi, etc.). For all tests, significant differences were declared when $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). Non-significant differences were denoted by ‘n.s.’.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. SHP1 and SHP2 show cooperative effects to induce cell differentiation in HEL cells

Previous results from our group showing the role of ROS in haematopoietic cell differentiation (Sardina et al, 2010) led to the interrogation of likely mediators of this process. This led to the finding of SHP1 and SHP2 as direct targets of ROS. By using HEL cells as a model, these molecules were found to be specifically oxidised after PMA exposure, whereas their closely related family member PTP1B did not. The need for this specific inhibition to fully induce differentiation was validated through RNAi mediated downregulation of the three PTPs. Again, an enhancement of the PMA-induced cell differentiation was achieved after silencing SHP1 and SHP2, whereas this effect was absent upon PTP1B downregulation (López-Ruano, 2015). This was the basis for a deeper study of the mechanisms underlying the control of differentiation in HEL cells and the exploration of the therapeutic potential of these observations.

4.1.1. Simultaneous downregulation of SHP1 and SHP2 enhances differentiation of HEL cells over individual silencing

The coincident pro-differentiation effect triggered by individual silencing of SHP1 and SHP2 previously observed in our laboratory (López-Ruano, 2015) raised the question of whether these PTPs were acting through different pathways. To address this issue, SHP1 and SHP2 were simultaneously downregulated in HEL cells (**Figure 4.1**).

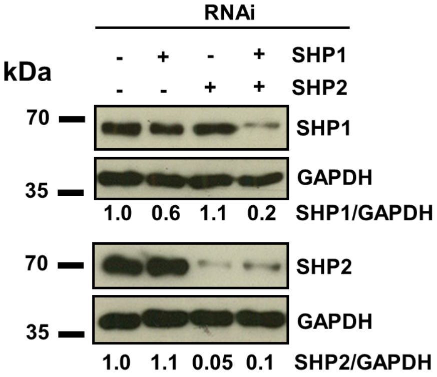

Figure 4.1. ***Individual and simultaneous silencing of SHP1 and SHP2 in HEL cells.*** Immunoblot showing the decrease of SHP1 and SHP2 at the protein level after lentiviral transduction of HEL cells with the RNAi sequences against these proteins. GAPDH was used as loading control. N = 6.

As a cell line with megakaryocytic potential, the differentiation of HEL cells was tracked by measuring the increase of the surface markers CD41 (a protein complex comprised of the glycoproteins GpIIb and GpIIIa) and CD61 (the individual GpIIIa glycoprotein), as previously reported by our group (Sardina *et al*, 2010, 2014).

GpIIb/IIIa is a receptor for fibrinogen and participates in platelet aggregation, and the expression of its constituents progressively augments throughout megakaryocytic differentiation (Block & Poncz, 1995). In the absence of any stimulus, individual silencing of the phosphatases increased the expression of both CD41 and CD61, in agreement with previous observations of our group (López-Ruano, 2015). Moreover, when both PTPs were simultaneously downregulated, the expression of the surface markers was more pronounced than in control and individually silenced cells (**Figure4.2A-B**). A similar trend was observed upon stimulation with 20 nM PMA for 48 h (**Figure 4.2C**).

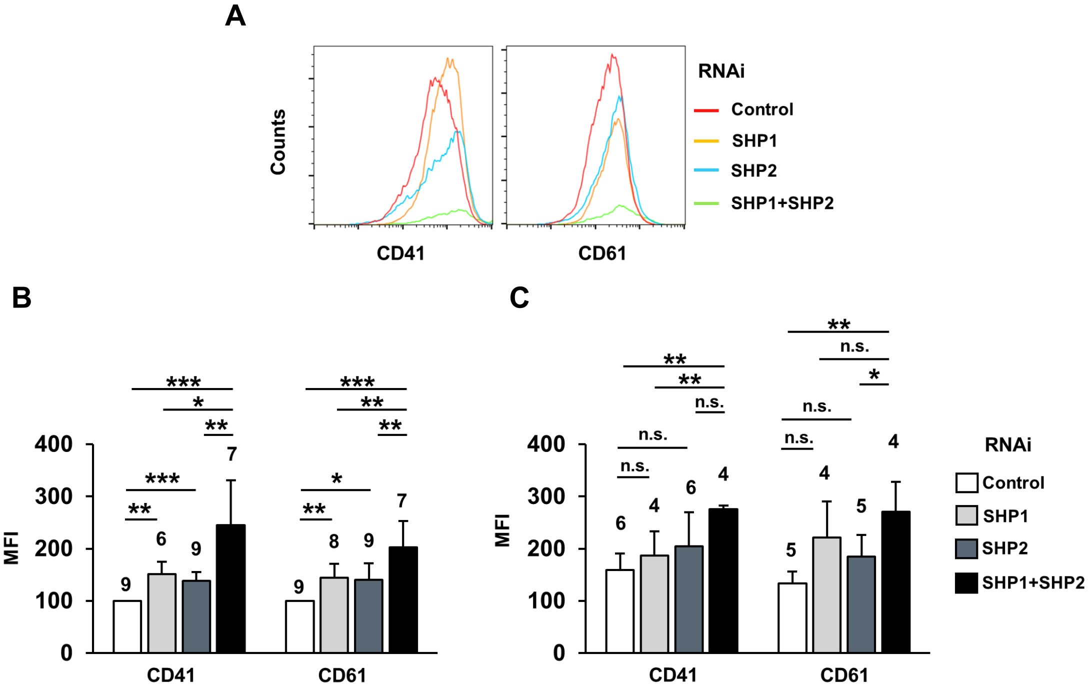

Figure 4.2. ***Simultaneous downregulation of SHP1 and SHP2 enhanced the upregulation of megakaryocytic surface markers.*** **A)** Representative fluorescence histograms of CD41 and CD61 in HEL cells subjected to individual and simultaneous downregulation of SHP1 and SHP2. **B)** Expression levels of the megakaryocytic markers CD41 and CD61 in SHP1- and SHP2-silenced cells without stimulation. **C)** Expression levels of the megakaryocytic markers CD41 and CD61 in SHP1- and SHP2-silenced after induction of cell differentiation with 20 nM PMA for 48 h.

A well-documented process that occurs during megakaryopoiesis is the prominent increase in DNA content due to a process of endomitosis, consisting of DNA synthesis without subsequent cytokinesis. Therefore, an increase of DNA content can be directly associated with the progression in the process of differentiation, and this parameter can be quantified by flow cytometry (Sardina *et al*, 2014). DNA content of HEL cells individually and simultaneously downregulated for SHP1 and SHP2 was measured after a 7-day treatment with 20 nM PMA. Consistent with the expression of surface epitopes, cell ploidy was higher when the expression both PTPs had been reduced over control and individually silenced cells (**Figure 4.3**).

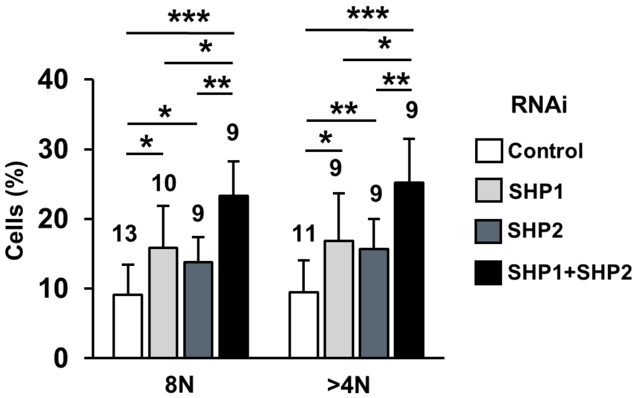

Figure 4.3. ***HEL cells stimulated for cell differentiation displayed increased ploidy upon dual silencing of SHP1 and SHP2.*** Percentage of SHP1- and SHP2-silenced HEL cells with the indicated DNA amount after induction of megakaryocytic differentiation with 20 nM PMA for 7 days.

Taken together, these results confirm a pro-differentiation effect exerted by the downregulation of the PTPs SHP1 and SHP2. Besides, HEL cells show an enhanced acquisition of differentiation traits when the PTPs are downregulated at the same time. This supports the involvement of this proteins in the differentiation of the cell line, at least partially, through different signalling pathways. Although this contrasts with the traditionally opposed roles attributed to these PTPs (Zhang *et al*, 2000; Neel *et al*, 2003), cooperation between them has previously been described in the context of epidermal growth factor (EGF) signalling (Wang *et al*, 2006). Since this convergent role had not been reported so far in the haematopoietic system, the next logical step was to study the different downstream molecules which are targeted by these two PTPs.

4.1.2. SRC levels are decreased in SHP2-silenced HEL cells

SRC family kinases (SFKs) are abundantly expressed in platelets and required for their correct function through activation of phosphorylation cascades initiated at the cell membrane (Senis *et al*, 2014). However, hyperactivation of the founder family member, SRC, in megakaryocytes from patients with myelofibrosis, leads to dysfunctions in platelet generation, arguing in favour of the need for a tight regulation of the activity of this kinase in megakaryopoiesis (Turro *et al*, 2016). It is worth noting that SHP2 has been described as a positive regulator of SRC activation in the mouse cell line 3T3 (Zhang *et al*, 2004). This evidence prompted us to study the activation status of the kinase SRC in our system upon downregulation of the PTPs SHP1 and SHP2.

As shown in **Figure 4.4,** a marked decrease of total SRC levels and, consequently, its active form, pSRC^{Y418}, was observed when SHP2 was downregulated. Of interest, SRC has been reported to be a substrate of the protease calpain in breast cancer cells (Tan *et al*, 2005), and an indirect mechanism of SHP2-mediated inhibition of calpain has been described in BCR-ABL-expressing cells (Hjort *et al*, 2016). Therefore, it is likely that downregulation of SHP2 might be a driving force for calpain activation and subsequent proteolytic degradation of SRC in our system. Nevertheless, this hypothesis has not been experimentally tested and would be an interesting question to address in the future. Contrarily, no remarkable changes of either SRC levels or activation status were detected upon SHP1 downregulation, thus suggesting that the role of this PTP in HEL cells differentiation is not mediated by the kinase.

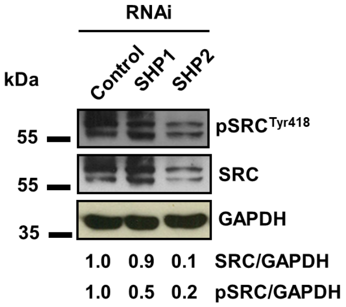

Figure 4.4. ***The downregulation of SHP2, but not SHP1, decreased SRC at the protein level in HEL cells.*** Immunoblot of protein samples from SHP1- and SHP2-silenced HEL cells showing the levels of active and total SRC levels. GAPDH was used as loading control. N = 3.

4.1.3. SRC downregulation increases the responsiveness of HEL cells to PMA

To further explore the relationship between the SHP2 downregulation-induced decrease of SRC levels and its pro-differentiative effect, HEL cells were subjected to transduction with lentiviral particles targeting SRC. Both sequences employed displayed an efficient reduction of SRC protein levels (**Figure 4.5**). Surprisingly, in the absence of differentiation-triggering stimuli, a consistent reduction of the surface

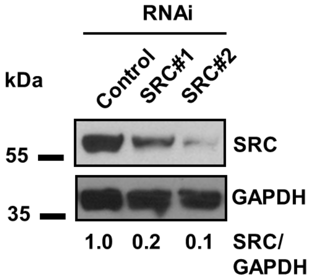

Figure 4.5. ***SRC was efficiently downregulated by the sequences employed HEL cells.*** Immunoblot showing the decrease of SRC at the protein level after lentiviral transduction of HEL cells with the RNAi sequences against these proteins. GAPDH was used as loading control. N = 5.

markers CD41 and CD61 was found for both RNAi sequences (**Figure 4.6A**). Nonetheless, the levels of megakaryocytic markers in SRC-silenced cells closely resembled those of control cells after PMA exposure (**Figure 4.6B**). As a result, the induction of differentiation markers versus the unstimulated condition was clearly higher in SRC-silenced cells (**Figure 4.6C**).

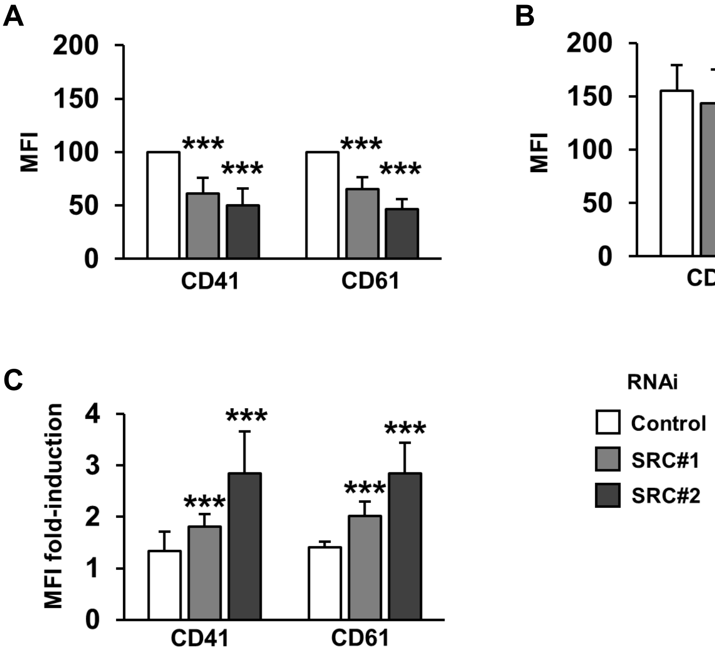

Figure 4.6. ***SRC downregulation primed HEL cells for a greater response to PMA.*** **A)** Expression levels of CD41 and CD61 in unstimulated HEL cells transduced with lentivirus containing RNAi sequences targeting SRC. **B)** Expression levels of CD41 and CD61 in SRC-silenced HEL cells after stimulation with 20 nM PMA for 48 h. **C)** Overall induction of CD41 and CD61 expression by PMA versus untreated SRC-downregulated HEL cells. N = 14.

In order to find a molecular explanation for the observed phenotype, the active forms of ERK and STAT5, two molecules involved in the differentiation process in our system (Sardina *et al*, 2010), were studied. In line with surface markers data, both pSTAT5^{Tyr694} and pERK were lower in unstimulated SRC-silenced versus control cells, whereas their levels not only were restored, but exceeded those of control cells upon stimulation with 20 nM PMA (**Figure 4.7**).

Interestingly, STAT5 has been described as a direct target of SRC, which would contribute to the activation of the former through its tyrosine phosphorylation (Okutani *et al*, 2001). On the other hand, ERK pathway would be subject to indirect regulation by SRC activity (Wu *et al*, 2012). This scenario would be consistent with the reduced levels of active STAT5 and ERK in the absence of stimulation (**Figure 4.7**), which correlated with lower expression of the differentiation markers studied (**Figure 4.6A**). However, PMA stimulation could be triggering a differentiation process mainly relying on PKC activation, a direct target of phorbol esters.

Importantly, PKC phosphorylation by SRC has been reported as an inhibitory post-translational modification in some contexts (Joseloff *et al*, 2002). Under these circumstances, the presence of SRC inside the cell would be hampering PMA-induced differentiation. Therefore, stimulation with this compound in SRC-silenced cells could trigger a stronger activation of STAT5 and ERK pathways due to the absence of SRC as a negative modulator of PKC, in agreement with the observations herein reported (**Figure 4.7**). This would in turn allow cell differentiation to occur in a more pronounced fashion in SRC-silenced versus control cells (**Figure 4.6B-C**).

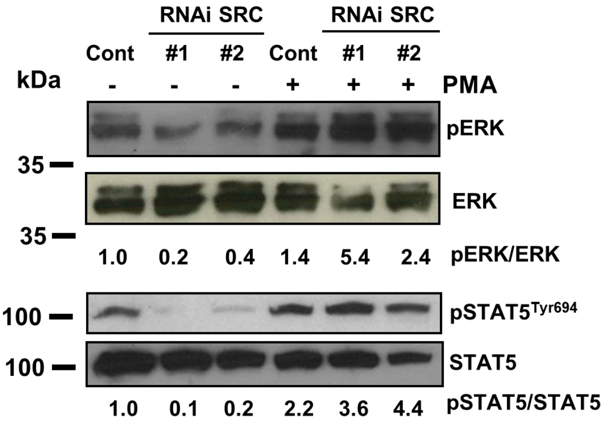
RNAi SRC
RNAi SRC
Cont #1 #2 Cont #1 #2
kDa
- - - + + + PMA
pERK
35
ERK
35
1.0 0.2 0.4 1.4 5.4 2.4 pERK/ERK
100
pSTAT5Tyr694
100
STAT5
1.0 0.1 0.2 2.2 3.6 4.4 pSTAT5/STAT5

Figure 4.7. ***The enhanced response of SRC-downregulated HEL cells to PMA was mediated by activation of ERK and STAT5 pathways.*** Immunoblot showing the active (phosphorylated) and total levels of ERK (upper bands) and STAT5 (lower bands) in SRC-silenced HEL cells before and after stimulation with 20 nM PMA for 48 h. N = 5.

4.1.4. SRC is a downstream target of SHP2 but not of SHP1

As it could be observed on **Figure 4.4,** a relevant reduction of SRC levels was only observed in SHP2-silenced cells. In order to rule out a role of SHP1 and confirm the involvement of SHP2 in the control of SRC protein levels, both PTPs were downregulated in a SRC-silenced background in our system, thus obtaining SRC single-silenced together with SRC/SHP1 and SRC/SHP2 double-silenced

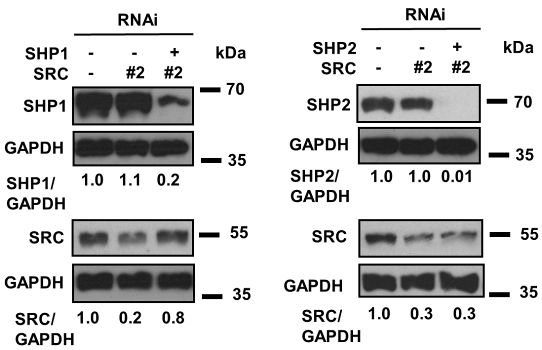
RNAi
SHP1 - - +
SRC - #2 #2
kDa
70
SHP1
GAPDH
35
SHP1/ GAPDH 1.0 1.1 0.2
SRC
55
GAPDH
35
SRC/ GAPDH 1.0 0.2 0.8
RNAi
SHP2 - - +
SRC - #2 #2
kDa
SHP2
70
GAPDH
35
SHP2/ GAPDH 1.0 1.0 0.01
SRC
55
GAPDH
35
SRC/ GAPDH 1.0 0.3 0.3

Figure 4.8. ***Simultaneous downregulation of SRC and either SHP1 or SHP2 was achieved in HEL cells.*** Immunoblot showing reduction of each SH2 domain containing PTP individually and in parallel with SRC in HEL cells. GAPDH was used as loading control. N = 3.

cells (**Figure 4.8**). Without stimulation, surface markers of cell differentiation were again downregulated in SRC single-silenced cells. Moreover, SHP1 downregulation in SRC-silenced cells rescued the expression levels of CD41 and CD61, closely resembling those found in control cells. Interestingly, no differences were observed between SRC-silenced and SRC/SHP2-silenced cells (**Figure 4.9A**). Upon incubation with 20 nM PMA, CD41 and CD61 expression reached similar levels to control cells, in line with the results shown in **Figure 4.6B.** An interesting observation is the slightly increasing trend in the expression of both markers found in SRC/SHP1 double-silenced cells over SRC-silenced cells (**Figure 4.9B**). When the fold-induction of these surface molecules was studied, similar results were observed, with no differences between SRC-silenced and SRC/SHP2 double-silenced cells and significant changes when SHP1 was downregulated after SRC silencing (**Figure 4.9C**). The available knowledge regarding the regulation of SRC activation by SHP1 is not conclusive and seems to be cell type-dependent (Roskoski, 2005). All in all, these results support a role for SHP1 independent of SRC, together with a regulatory role of SRC levels by SHP2 upstream of the kinase in the differentiation of HEL cells.

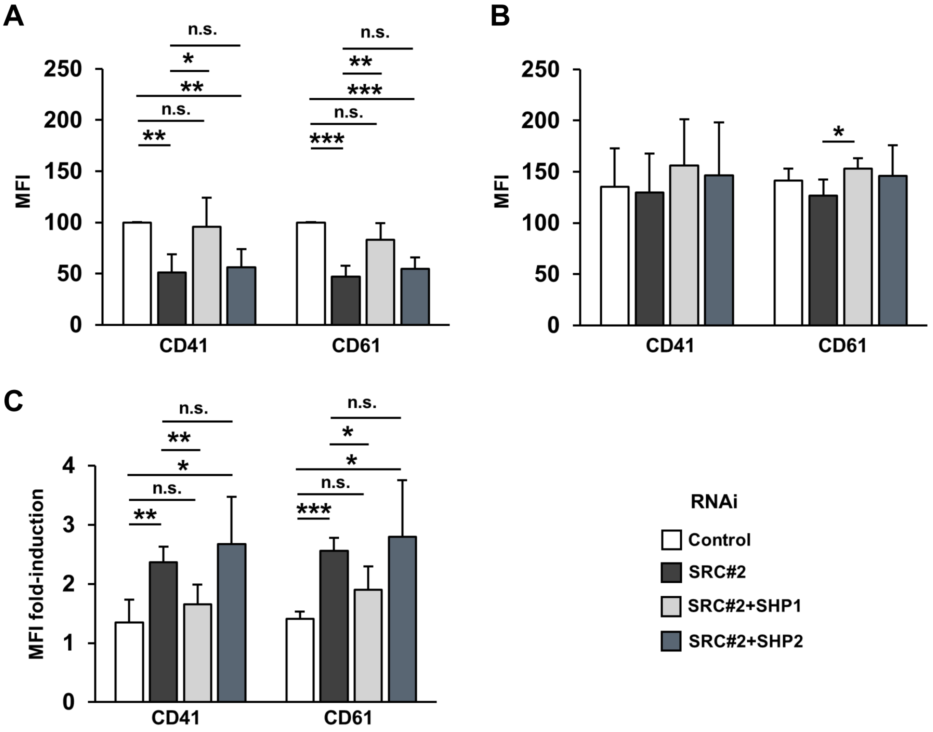

Figure 4.9. ***SHP1 but not SHP2 downregulation reverted the effect of SRC silencing in HEL cells.*** **A)** Expression levels of CD41 and CD61 in unstimulated HEL cells individually silenced for SRC and with simultaneous downregulation of SRC and either SHP1 or SHP2. **B)** Expression levels of CD41 and CD61 in the same cells after stimulation with 20 nM PMA for 48 h. **C)** Overall induction of CD41 and CD61 expression by PMA versus untreated HEL cells with individual SRC silencing and simultaneously with either SHP1 or SHP2. N = 6.

4.1.5. β-catenin levels are decreased upon silencing of SHP1 and SHP2 in HEL cells

The SRC-independent effect of SHP1 downregulation in the differentiation of HEL cells did not explain the cooperative effect found for both PTPs in this process. Previous work performed in our laboratory had shown a relevant function for PTPN13 in the control of β-catenin stability in the same system. Specifically, the downregulation of PTPN13 through RNAi favoured the expression of CD41 and CD61 and the degradation of β-catenin. This molecule was functionally involved in the process of differentiation, since its direct RNAi-mediated knockdown also resulted in an enhanced expression of the surface molecules used for differentiation monitoring (Sardina *et al*, 2014).

The fact that SHP1 and SHP2 are also PTPs provided the rationale to study the levels of β-catenin in HEL cells where these proteins had been knocked down. In line with the data described before, the downregulation of both SHP1 and SHP2 led to a decrease in total β-catenin levels. Nonetheless, double-silenced cells did not display an enhanced effect on this parameter, a fact that *per se* could not explain the stronger differentiation potential of these cells (**Figure 4.10**). SRC intervention is also implied in this process, but additional mechanisms need to be elucidated to explain the increase in differentiation features upon simultaneous knock down of the two PTPs.

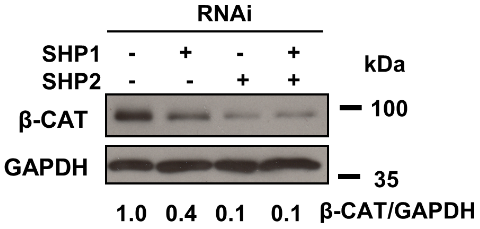

Figure 4.10. ***The downregulation of SHP1 and SHP2 exerted a decrease of β-catenin protein levels*.** Immunoblot showing the levels of β-catenin in HEL cells with individual and simultaneous downregulation of SHP1 and SHP2. GAPDH was used as loading control. N = 4.

Distinct regulatory roles of β-catenin have been attributed to SHP1 function in different cellular systems. For instance, this PTP has been reported to dephosphorylate pGSK3β^{Tyr216} and subsequently inactivate it in MSCs, thereby preventing β-catenin degradation (Jiang *et al*, 2016). These findings would be consistent with the results presented in this work, where a reduction of SHP1 leads to a concomitant decrease of β-catenin (**Figure 4.10**). Contrarily, SHP1 overexpression has been reported to induce β-catenin degradation in HEK-293T cells (Simoneau *et al*, 2011). These findings would be supported by the inverse correlation between SHP1 expression or activity and β-catenin levels found in intestinal epithelial cells (Duchesne *et al*, 2003; Leblanc *et al*, 2017). Altogether, the evidence accumulated so far, together with the data presented here, point to a cell type-dependent regulatory mechanism of β-catenin levels by SHP1.

β-catenin dephosphorylation by SHP2 was previously reported to regulate the interaction of the former with vascular endothelial (VE)-cadherin, but not its protein levels, in epithelial cells (Timmerman *et al*, 2012). On the other hand, SHP2-mediated regulation of the amount of β-catenin by indirect control of pGSK3β$^{\mathrm{Ser9}}$ was described in hepatocellular carcinoma (HCC) stem cells (Xiang *et al*, 2017). Consistently, SHP2 expression and catalytic activity correlated with β-catenin levels in HCC and colorectal cancer cells (Liu *et al*, 2018; Zhang *et al*, 2018). In contrast to the work by Xiang *et al,* SHP2 could indirectly influence β-catenin levels through GAS2-mediated inhibition of calpain and PTPN13-mediated dephosphorylation of pGSK3β$^{\mathrm{Tyr216}}$, as described before (Huang *et al*, 2010, 2013). Interestingly, previous work of our group and others support a relationship between β-catenin phosphorylation in Tyr residues and the stability of the protein (Coluccia *et al*, 2007; Sardina *et al*, 2014).

The elucidation of whether one or several of the previously discussed mechanisms might be operating in our system merits further study and is indeed under current investigation in our group.

4.1.6. Concluding remarks

A deeper understanding of the molecular mechanisms that modulate cell differentiation in haematopoietic cells is paramount to better comprehend how these processes are altered in pathologic conditions, such as leukaemia. Identifying the relevant mediators involved in this regulation is the first step to define pharmacological targets and develop new active compounds for the treatment of blood disorders.

In this regard, the previously described data have contributed to gaining further insights into the regulation of the differentiation process in HEL cells. It has added an additional piece of evidence to the previous work developed in our laboratory, which showed the specific oxidation and inactivation of SHP1 and SHP2 after stimulation of HEL cells with PMA and the relevance of this phenomenon to fully trigger megakaryocytic differentiation (López-Ruano, 2015). The experiments herein described have contributed to the elucidation of a complementary role for both PTPs in the whole process, based on the data showing that their simultaneous downregulation renders a stronger differentiation phenotype than individual knock down (**Figures 4.1 to 4.3**). These results point to, at least, partially non-overlapping functions for the PTPs in the control of cell signalling leading to cell differentiation. To better understand how these phosphatases may act, a rational search of likely downstream molecules based on literature data was performed (Turro *et al*, 2016; Zhang *et al*, 2004; Sardina *et al*, 2014). This led to the finding that SRC is a downstream target of SHP2 involved in HEL cells differentiation (**Figures 4.4 to 4.9**). In addition, β-catenin was revealed as a confluence point for SHP1 and SHP2 in their control of this process (**Figure 4.10**). The precise mechanism of this phenomenon could not be addressed in this work and will be subject to future research in our group.

In a global perspective, the integration of the previously shown results and the scientific evidence available on the literature supports the model depicted in **Figure 4.11.** Upon PMA stimulation, PKC activation would lead to a regulated production

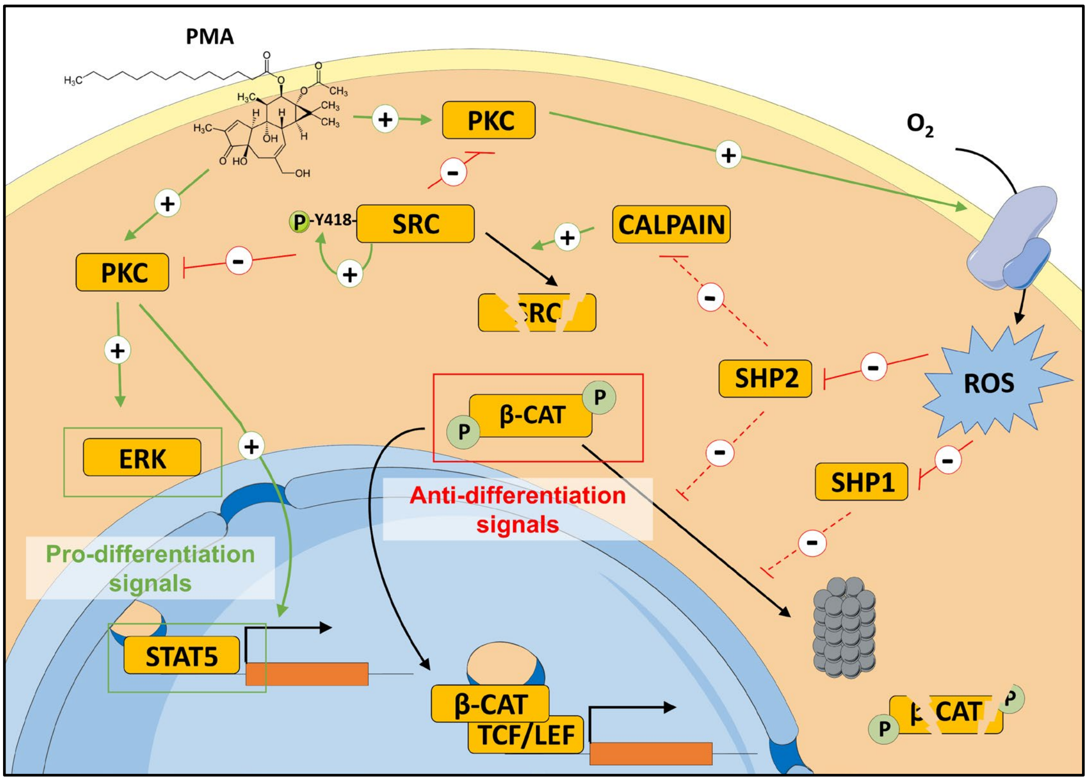

Figure 4.11. ***Schematic model depicting the possible mechanisms underlying the SHP1- and SHP2-mediated regulation of PMA-induced differentiation in HEL cells.*** Stimulation with PMA would lead to the activation of NOX complexes that would in turn produce ROS in a regulated manner. SHP1 and SHP2 molecules located nearby the ROS-producing NOXs would become transiently oxidised and inactivated, thus leading to downmodulation of β-catenin and SRC. These phenomena would thereby preclude the anti-differentiation signals mediated by β-catenin and trigger the pro-differentiation effect exerted by ERK and STAT5 pathways (see text for details).

of ROS by NADPH oxidases. These ROS would transiently oxidise and inactivate SHP1 and SHP2, thus triggering downstream processes. On one hand, SHP2 inactivation would allow calpain to degrade SRC, thereby releasing new PKC molecules previously inhibited by the kinase and amplifying the pro-differentiative stimulus. In addition, β-catenin degradation would also be potentiated through molecular mechanisms yet to be elucidated. On the other hand, inactive SHP1 would also lead to β-catenin decrease by yet unknown phenomena. The elimination of inhibitory signals for STAT5 and ERK activation, together with the reduction of the levels of a negative regulator of cell differentiation, such as β-catenin, would allow the process to fully progress.

In summary, the inhibition of SHP1 and SHP2 is a signal triggering cell differentiation in a leukaemia-derived cell line. This stands as a very relevant finding in the context of a pathology with a differentiation blockage as a hallmark. Consequently, targeting these PTPs was considered as a plausible therapeutic proposal.

4.2. Chemical inhibition of SHP1 and SHP2 as the basis for a pro-differentiation approach targeting AML cells

4.2.1. The downregulation of SHP1 and SHP2 promotes cell differentiation in HL-60 cells

To further validate the enhancement of cell differentiation upon SHP1 and SHP2 downregulation in AML cells, the cell line HL-60 was chosen as a non-APL AML model. This cell line has been widely described on the literature as a cellular system for the study of differentiation processes, especially those involving stimulation with phorbol esters. These cells undergo upregulation of the surface antigen CD11b, a component of the integrin involved in neutrophil adhesion CD11b/CD18 (Mazzone & Ricevuti, 1995), upon exposure to phorbol esters (Shen *et al*, 2015).

HL-60 cells where SHP1 and SHP2 had been knocked down in parallel (**Figure 4.12A**) were treated with 20 nM PMA and 0.5 µM PRS to induce CD11b expression. PMA treatment rendered a stronger induction of the surface epitope in PTP-silenced over control cells, regardless of the incubation time (**Figure 4.12B**). In addition, incubation with PRS for 48 h had also a slightly enhanced effect in this same parameter when SHP1 and SHP2 were downregulated, although it failed to reach statistical significance (**Figure 4.12C**), presumably due to the milder effect of PRS versus the much more powerful one of PMA. Taken together, these results agree with those obtained in HEL cells (**Figure 4.2**) and reinforce the involvement of SHP1 and SHP2 in the phorbol ester-triggered differentiation of AML cells.

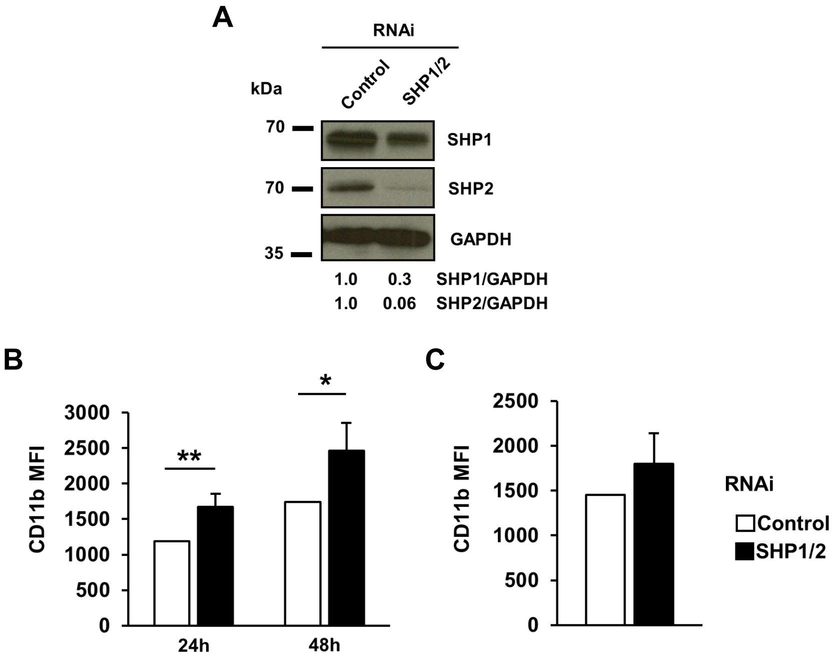

Figure 4.12. ***RNAi-mediated downmodulation of SHP1 and SHP2 enhanced phorbol ester-induced differentiation in HL-60 cells.*** **A)** Immunoblot showing the simultaneous silencing of SHP1 and SHP2 in HL-60 cells. GAPDH was used as loading control. N = 3. **B)** Expression levels of the surface marker CD11b in SHP1/2 silenced-HL-60 cells stimulated with 20 nM PMA for different time periods. N = 5. **C)** Expression levels of the surface marker CD11b in SHP1/2 silenced-HL-60 cells stimulated with 0.5 µM PRS for 48 h. N = 5.

4.2.2. Chemical inhibitors of SHP1 and SHP2 recapitulate the effects of RNAi-mediated downregulation in cell differentiation

The results provided so far strongly support that SHP1 and SHP2 inhibition has a pro-differentiative effect on AML cells differentiation. However, *in vivo* RNAi delivery remains an unfeasible approach when therapeutic purposes are pursued. Therefore, small molecules targeting the phosphatases were tested to assess the differentiation-inducing potential of their RNAi-independent inhibition. Sodium stibogluconate (SSG) (**Figure 4.13A**) has long been used as a therapeutic agent against leishmaniasis. It irreversibly inhibits SHP1, SHP2 and PTP1B *in vitro*, with enhanced sensitivity against the former (Pathak & Yi, 2001). On the other hand, SHP099 (**Figure 4.13B**) is a recently developed allosteric SHP2 inhibitor that has rapidly become very popular due to its specificity and oral bioavailability (Garcia Fortanet *et al*, 2016). Finally, NSC 87877 (NSC) (**Figure 4.13C**) is a non-selective SHP1 and SHP2 inhibitor targeting the catalytic cleft of PTPs through non-covalent interactions (Chen *et al*, 2006). HL-60 cells exposed to only SSG and NSC for 48 h underwent upregulation of CD11b (**Figure 4.14A**).

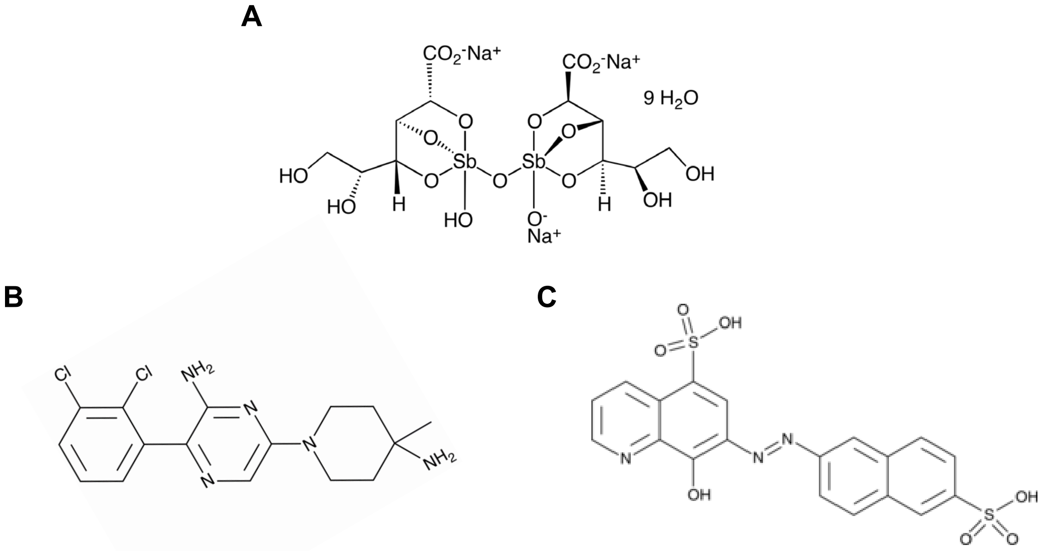

Figure 4.13. ***Chemical inhibitors of SHP1 and SHP2 used throughout this work.*** **A)** Chemical structure of sodium stibogluconate (SSG). **B)** Chemical structure of SHP099. **C)** Chemical structure of NSC 87877 (NSC).

The differentiation ability of SSG on HL-60 cells has been reported before (Pathak *et al*, 2002), although lower doses and longer incubation times were employed herein. Interestingly, SHP099 was the only inhibitor used targeting exclusively one of the PTPs, hence suggesting that inhibition of both PTPs might be needed to trigger differentiation of HL-60 cells under these circumstances. Of note, the fact that its mechanism of action is the stabilisation of the auto-inhibited conformation of SHP2 (**Figure 1.7A**) (Garcia Fortanet *et al*, 2016) brings the possibility that the interaction of SHP2 with activating molecules inside the cell might outcompete its effect. An additional difference between SHP099 and the other inhibitors used relies on the allosteric inhibition as its mode of action, which may account for the distinct observations made with this compound.

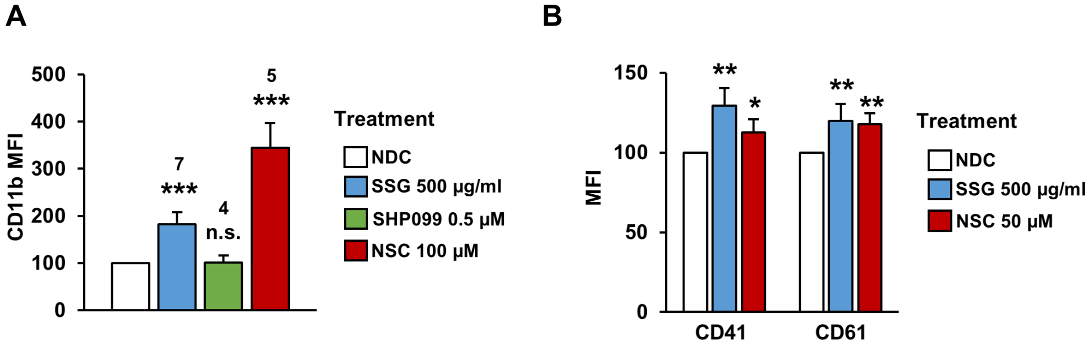

Figure 4.14. ***SSG and NSC promoted the expression of surface markers of differentiation in AML cell lines.*** **A)** Expression levels of the surface marker CD11b in HL-60 cells treated for 48 h with different inhibitors of SHP1 and SHP2 at the indicated doses. **B)** Expression levels of megakaryocytic surface markers in HEL cells after treatment with SSG and NSC at the indicated doses for 48 h. N = 6. NDC: No Drug Control.

To provide evidence that the effect of chemical inhibitors was consistent with the results obtained after RNAi-mediated downregulation, SSG and NSC were also tested on HEL cells, where they induced a significant upregulation of both surface differentiation markers (**Figure 4.14B**). All in all, these results agree with those obtained in RNAi-targeted cells (**Figures 4.2B** and **4.12B**) and support the therapeutic potential of inhibiting SHP1 and SHP2 as a differentiation-based therapeutic strategy against non-APL AML.

4.2.3. Chemical inhibition of SHP1 and SHP2 enhances the differentiation induction of phorbol esters

As highlighted on the Introduction, the the re-discovery of non-tumour promoting phorbol esters that keep their differentiation-inducing effect makes the therapeutic use of these compounds an interesting possibility to be tested (Shen *et al*, 2015). Besides, the pro-differentiative ability of the chemical compounds described in the previous section raised the interesting possibility of an enhanced effect on cell differentiation in cells co-treated with phorbol esters and chemical inhibitors of SHP1 and SHP2.

Despite the effect of SSG as a single agent, its simultaneous incubation with phorbol esters led to a mild and non-significant increase of CD11b versus PMA or PRS alone (**Figure 4.15A**). This might be due to the need for a recovery and subsequent increase of SHP1 and SHP2 activity at long times after PMA stimulation for a correct differentiation (López-Ruano, 2015). As indicated before, SSG irreversibly inhibits its targets, likely hampering the restoration of PTPase activity. Not surprisingly, SHP099 did not display any enhancement of either PMA or PRS

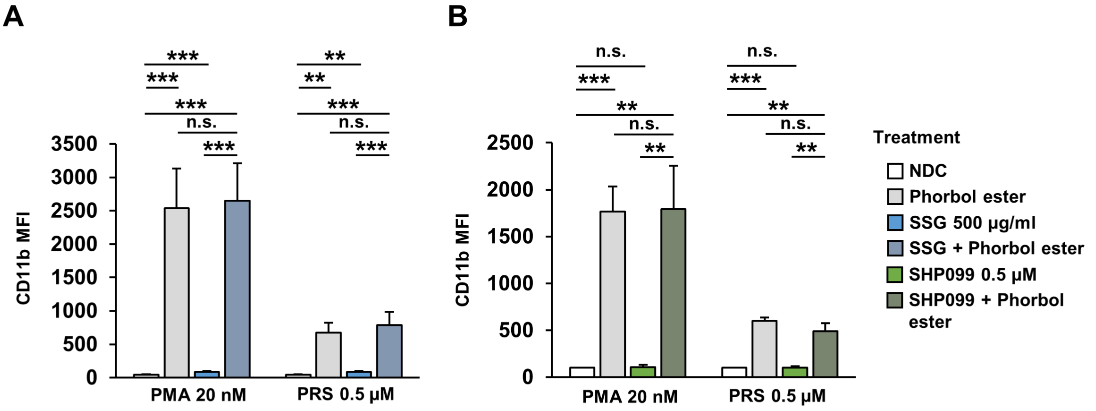

Figure 4.15. ***SSG and SHP did not enhance the pro-differentiative effect of phorbol esters in HL-60 cells*. A)** Expression levels of the surface marker CD11b in HL-60 cells treated for 48 h with PMA or PRS and SSG as single drugs and in combination. N = 5. **B)** Expression levels of the surface marker CD11b in HL-60 cells treated for 48 h with PMA or PRS and SHP as single drugs and in combination. N = 4 for experiments involving PMA and N = 5 for experiments with PRS. NDC: No Drug Control.

induction of cell differentiation, with a slight (but not significant) reduction of PRS effect (**Figure 4.15B**).

As it could be expected, given the strong induction of CD11b expression as a single agent (**Figure 4.14A**), NSC was the only compound capable of boosting both PMA (**Figure 4.16A**) and PRS (**Figure 4.17A**) effects on this surface marker.

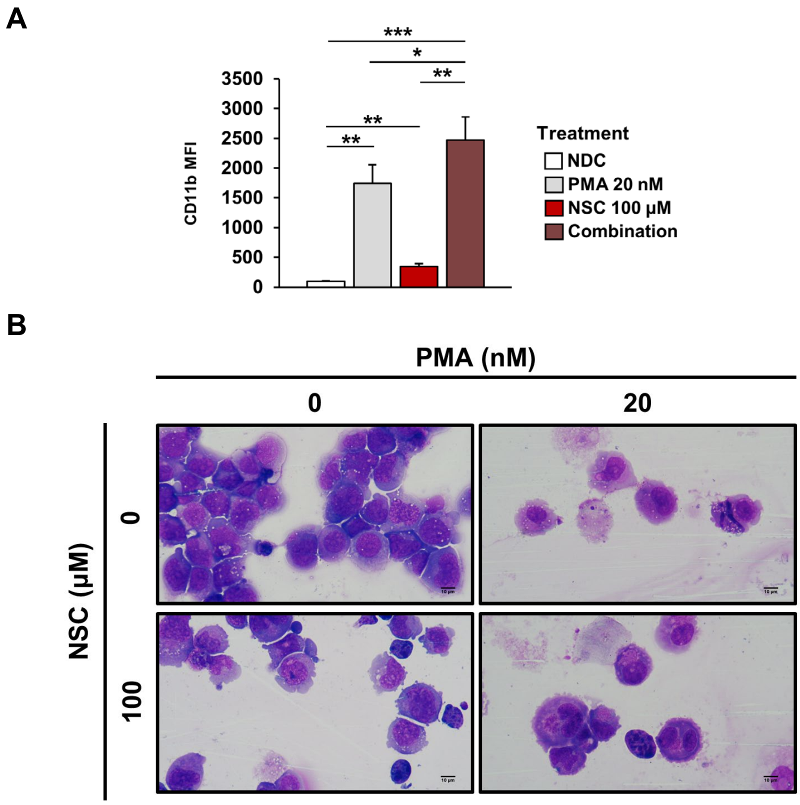

Figure 4.16. ***NSC potentiated the differentiation induced by PMA in HL-60 cells*****. A)** Expression levels of the surface marker CD11b in HL-60 cells treated for 48 h with PMA and NSC as single drugs and in combination. N = 5. NDC: No Drug Control. **B)** Representative images of cytospins from HL-60 under the same conditions of panel A and stained for visualisation under the microscope. N = 3. Scale bar: 10 µm.

Consistently, cells co-treated with either PMA or PRS and NSC displayed typical differentiation features, such as heterochromatic and kidney-shaped nuclei, reduced nucleus/cytoplasm ratio or more eosinophil cytosol, in a more pronounced fashion than control and single agent-treated cells (**Figures 4.16B** and **4.17B**). Finally, the adherent phenotype usually triggered by phorbol ester stimulation was strikingly more evident upon co-treatment with PRS and NSC versus exposure to individual drugs, again supporting an enhancement of the effect of the phorbol ester by the PTP inhibitor (**Figure 4.17C**).

Off-target effects have been attributed to inhibitors of SHP1 and SHP2, including NSC, at the concentration ranges used for this study. These phenomena comprise SHP2-independent alteration of cellular signalling pathways (Tsutsumi *et al*, 2018), as well as inhibition of PTPs other than SHP1 and SHP2 (Shi *et al*, 2015).

However, the consistency observed between the specific inhibition of those PTPs by RNAi and the use of NSC supports the hypothesis that the differentiation effects observed in this work are mediated by the intended targeting of SHP1 and SHP2.

All in all, the combination of the non-tumour promoting phorbol ester PRS with NSC could be a good candidate for therapeutic screening of anti-leukaemic activity based on the induction of cell differentiation in non-APL AML cells.

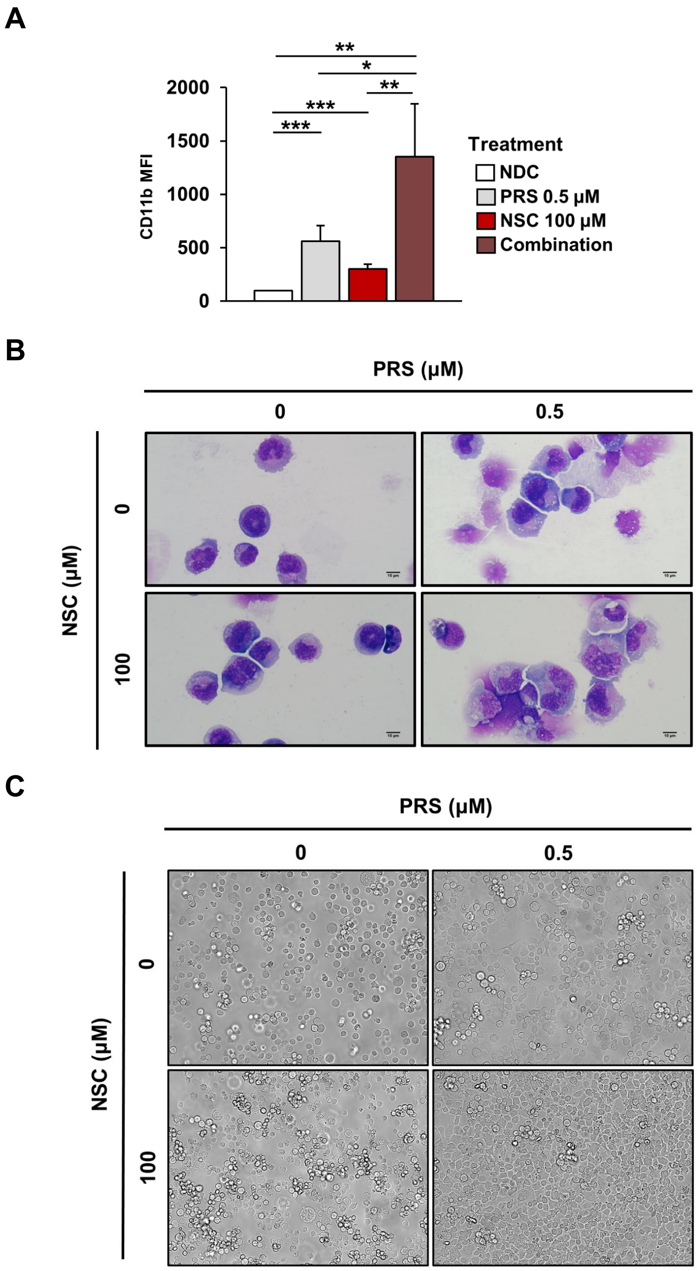

Figure 4.17. ***NSC potentiated the differentiation induced by PRS in HL-60 cells.*** **A)** Expression levels of the surface marker CD11b in HL-60 cells treated for 48 h with PRS and NSC as single drugs and in combination. N = 7. NDC: No Drug Control. **B)** Representative images of cytospins from HL-60 under the same conditions of panel A and stained for visualisation under the microscope. N = 3. Scale bar: 10 µm. **C)** Representative bright field microscopy images of HL-60 cells under the same conditions of panels A and B to show their concomitant attachment to the culture plastic with cell differentiation. N = 7.

4.2.4. Anti-proliferative activity of PRS and chemical inhibitors of SHP1 and SHP2 against AML cells

The previously described effect of PRS+NSC co-treatment was very encouraging and supported its feasibility as a therapeutic differentiation-based strategy for AML. Nonetheless, every anti-cancer therapy should desirably fulfil at least two requirements: to slow down cell proliferation, avoiding tissue invasion, and specifically trigger malignant cell death, decreasing tumour burden. Therefore, the next step in this study was to test whether the combination of chemical inhibition of SHP1 and SHP2 with PRS had anti-leukaemic potential.

4.2.4.1. The growth of HL-60 cells is affected by PRS and chemical inhibition of SHP1 and SHP2

Based on previous reports where the different compounds had been used, distinct dose ranges of PRS and the three PTP inhibitors were tested on HL-60 cells to determine their dose-response curves. PRS had a hampering effect on cell proliferation at the low micromolar range (**Figure 4.18A**), in agreement with published data using the same cell line (Shen *et al*, 2015).

On the other hand, SSG had a remarkable anti-proliferative effect only at high doses (8000 µg/mL), with mild reduction caused by the rest of tested concentrations (**Figure 4.18B**). As it was the case for differentiation, this contrasts with previously reported results (Pathak *et al*, 2002), although the experimental conditions in the present work were different, with an incubation time of 48 h instead of 6 days. This is the most likely cause of the observed dissimilarities.

In line with the lack of effect in CD11b induction, SHP099 displayed a very modest impact on cell proliferation, regardless of the concentration used (**Figure 4.18C**). This is consistent with the data on the literature reporting the lack of effect of this compound on oncokinase-independent cancer cells, such as HL-60 (Chen *et al*, 2016). Our own observations agree well with this oncokinase-based sensitivity to SHP099, since MOLM13 cells, which express FLT3-ITD, were highly responsive to this compound (data not shown).

Lastly, NSC was the only compound showing a factual dose-dependent response in HL-60 cells (**Figure 4.18D**), with an IC50 of 95.8 µM. Anti-tumour potential for NSC has been reported before in neuroblastoma cell lines, although its effects were attributed to the inhibition of DUSP26 instead of SH2 domain containing PTPs (Shi *et al*, 2015). Moreover, the enhanced inhibitory capacity of this molecule against SHP2^{E76K} over the wild-type PTP (Chen *et al*, 2006) makes it a very interesting drug for use against AML cells, where that gain-of-function mutation is frequently expressed (Xu *et al*, 2011; Chen *et al*, 2015). In accordance with cell differentiation data, NSC emerged as the best candidate to be combined with PRS for an efficient impairment of cell proliferation in AML cells.

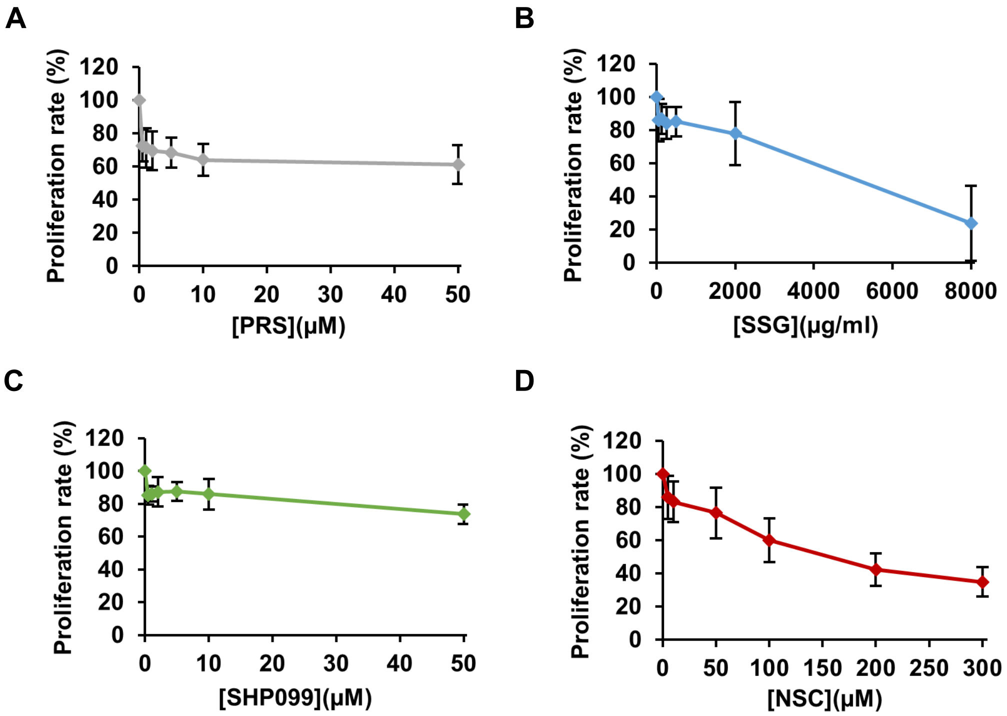

Figure 4.18. ***Dose-response effect of PRS and chemical inhibitors of SHP1 and SHP2 on HL-60 cell proliferation.*** **A)** Dose-response curve of HL-60 cells treated with PRS for 48 h. N = 6. **B)** Dose-response curve of HL-60 cells treated with SSG for 48 h. N = 6. **C)** Dose-response curve of HL-60 cells treated with SHP099 for 48 h. N = 6. **D)** Dose-response curve of HL-60 cells treated with NSC for 48 h. N = 6.

4.2.4.2. NSC and PRS co-treatment synergistically reduces HL-60 cells proliferation

Notwithstanding the mild effects of SSG and SHP099 in cell proliferation as single treatments (**Figure 4.18B-C**), they were tested in combination with PRS to rule out the possibility of a drug interaction. The co-treatments displayed an effect on cell proliferation with a very similar trend to that observed in CD11b expression. SSG only exerted a subtle enhancement of PRS effect at the highest dose tested in combination (**Figure 4.19A**), whereas no increase in cell growth inhibition was observed upon combining PRS and SHP099 versus PRS alone (**Figure 4.19B**).

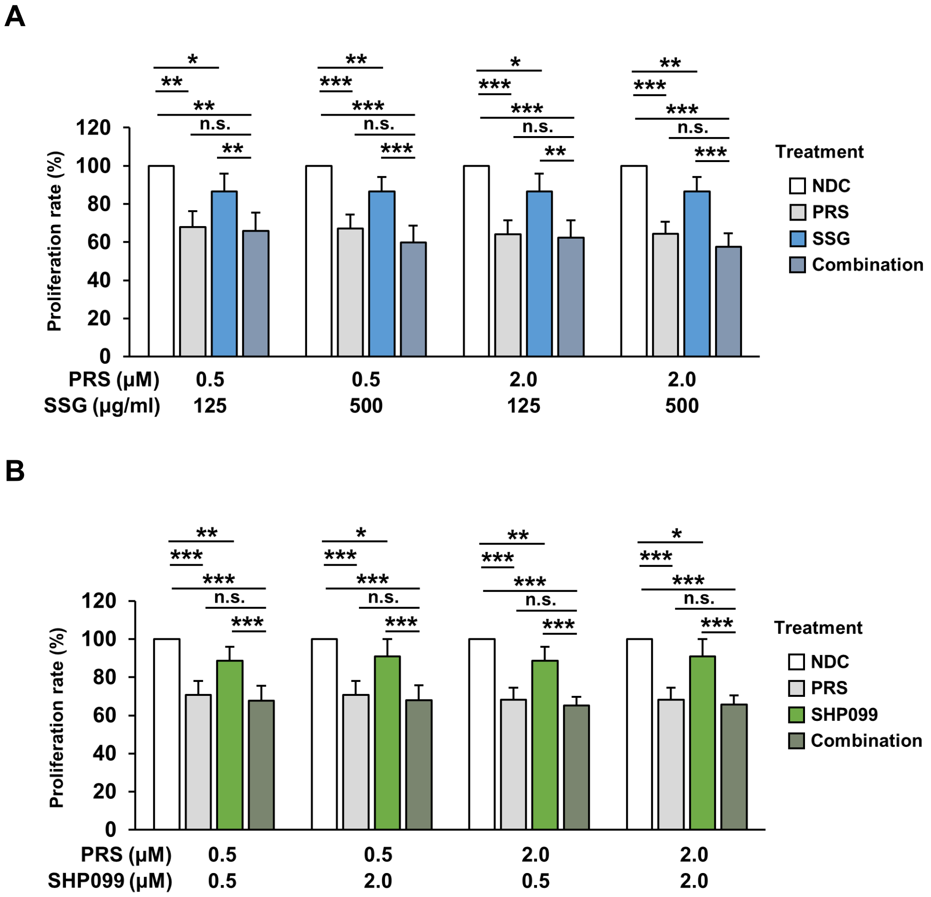

Figure 4.19. ***SSG and SHP did not enhance the effect of PRS on HL-60 cell proliferation.*** **A)** Proliferation rate of HL-60 cells treated with PRS and SSG as single agents and their combination at the specified doses for 48 h. N = 6 for experiments with 0.5 µM PRS and N = 9 for experiments with 2 µM PRS. **B)** Proliferation rate of HL-60 cells treated with PRS and SHP as single agents and their combination at the specified doses for 48 h. N = 9. NDC: No Drug Control.

Again, the combined exposure to PRS and NSC exceeded the effects of single drug treatments (**Figure 4.20A**), with CI values supporting a synergistic interaction between the drugs (**Figure 4.20B**). Since the MTT assay relies on

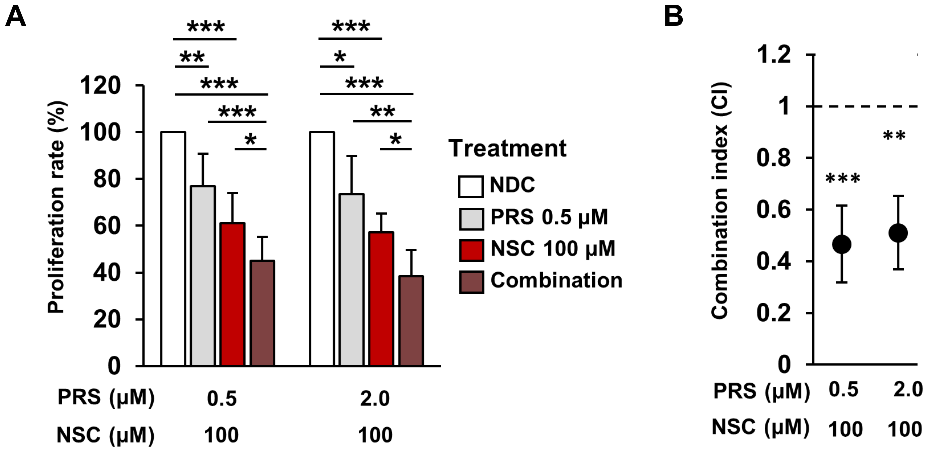

Figure 4.20. ***PRS and NSC synergised to impair the proliferation of HL-60 cells.*** **A)** Proliferation rate of HL-60 cells treated with PRS and NSC as single agents and their combination at the specified doses for 48 h. **B)** Graphical representation of the average CI values obtained for the drug combinations tested. Statistical differences were evaluated between CIs and the value 1. N = 8 for experiments with 0.5 µM PRS and N = 7 for experiments with 2 µM PRS. NDC: No Drug Control.

mitochondrial activity, the possibility of an effect on this parameter without changes in cell proliferation needed to be discarded. Therefore, viable cell count was performed with trypan blue exclusion assays. As shown in **Figure 4.21,** a stronger reduction was obtained when cells were exposed to the drug combination versus the individual treatments. This confirmed the results of MTT assays and supported a synergy between PRS and NSC.

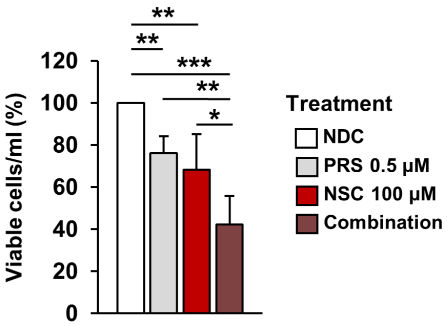

Figure 4.21. ***The synergy between PRS and NSC was also observed in viable cell numbers.*** Cell counts of trypan blue-unstained HL-60 cells treated with PRS and NSC as single agents and in combination for 48 h at the indicated doses. N = 7. NDC: No Drug Control.

Taken together, these results demonstrate that the combined inhibition of SHP1 and SHP2 with the administration of a non-tumour-promoting phorbol ester is an efficient approach to reduce cell proliferation in AML cells.

4.2.4.3. Additional mechanisms to cell differentiation induction contribute to synergistic reduction of cell proliferation by PRS+NSC in HL-60 cells

Cell differentiation and growth might be unlinked in a heterogeneous population of cancer cells due to unresponsive or less sensitive subset of CSCs (de Thé & Chen, 2010). Therefore, differentiation-independent mechanisms could be accounting for the anti-proliferative effect of PRS+NSC treatment on HL-60 cells.

The possible impairment of self-renewal ability was interrogated by performing CFU assays. A mild, non-significant reduction in the number of CFUs was elicited by treatment with PRS. Strikingly, a dramatic effect was observed with NSC treatment, upon which the number of CFUs was reduced in ~2/3 versus the untreated cells. No enhanced decrease was registered upon co-treatment versus NSC as a single drug (**Figure 4.22**). Since the drugs were withdrawn before cell seeding in the semi-solid medium, it can be suggested that cells do not require a continuous exposure to NSC for a long-term effect. Additional wash-out experiments assessing cell proliferation would confirm such hypothesis.

In contrast, when cell death was evaluated by Annexin V staining, a notorious increase of Annexin V$^+$ cells was observed after the treatment with PRS, whereas no changes were exerted by NSC as single drug. In addition, the percentage of cell death was not increased by co-treatment versus PRS alone (**Figure 4.23**). This agrees well with previously reported induction of cell death in HL-60 cells (Shen *et al*, 2015).

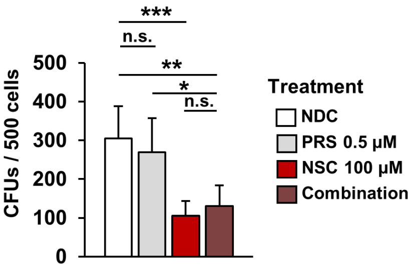

Figure 4.22. ***NSC markedly decreased the clonogenic potential of HL-60 cells.*** Colony-forming unit (CFU) counts of HL-60 cells treated with PRS and NSC as single agents and in combination for 48 h at the indicated doses and subsequently seeded in methylcellulose medium without growth factors for 7 days in the absence of drugs. N = 7. NDC: No Drug Control.

Thus, these results support that, together with cell differentiation enhancement, the drugs employed herein synergise to impair cell proliferation via distinct cellular processes: NSC would drastically reduce self-renewal potential whereas PRS would induce cell death. Combined use of both drugs would have an enhanced effect through triggering both phenomena at the same time.

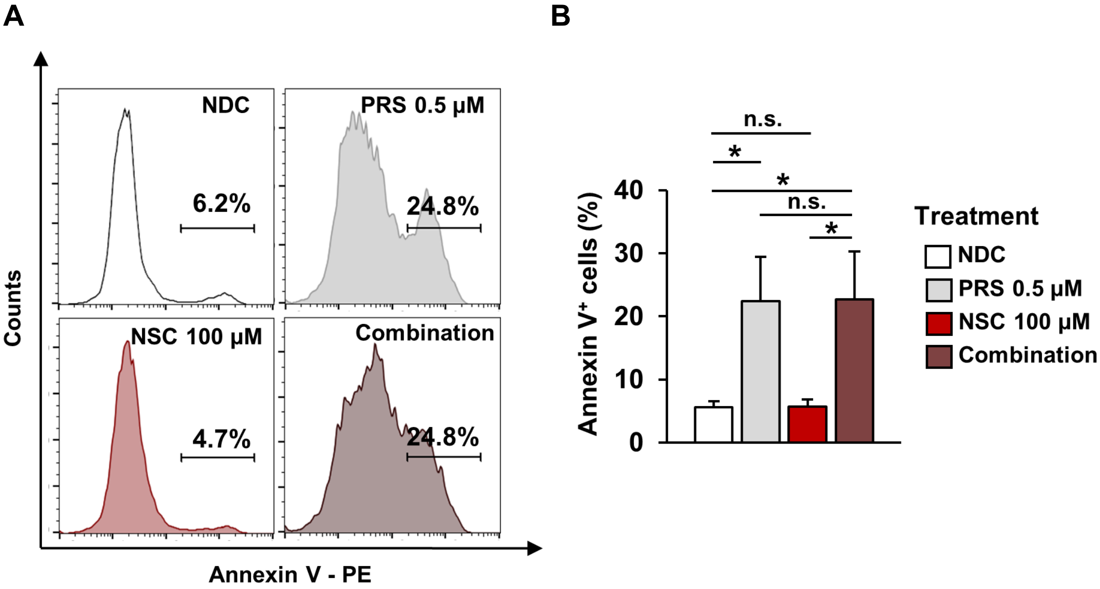

Figure 4.23. ***PRS greatly triggered cell death in HL-60 cells.*** **A)** Representative histograms of fluorescence intensity of HL-60 cells treated for 48 h with PRS and NSC as single agents and in combination at the specified doses and stained with Annexin V – PE. **B)** Summary data of all the experiments performed. N = 4. NDC: No Drug Control.

4.2.4.4. Synergistic effect of PRS+NSC treatment on cell proliferation is reproduced in other AML cell lines

To address whether the anti-leukaemic activity of PRS and NSC was restricted to HL-60 cells, these compounds were tested on additional cell lines used as AML models: NB-4 (PML-RARA$^+$), OCI-AML2 and THP-1 (KMT2A-AF9$^+$). The three cell lines displayed sensitivity to PRS at the micromolar range (**Figure 4.24A**), as well as to NSC at similar dose ranges to that used for HL-60 cells (**Figure 4.24B,** IC50s were 79.0 µM for NB-4, 214.5 µM for OCI-AML2 and 145.9 µM for THP-1 cells). Interestingly, NB-4 cells, which are a model of the differentiation therapy-responsive APL, were the most sensitive ones to both agents. Contrarily, THP-1 cells, which belong to M5 FAB subtype, displayed the lowest sensitivity to both drugs, especially PRS. These results further reinforce an anti-leukaemic effect of PRS+NSC relying on the differentiation induction ability of this combination.

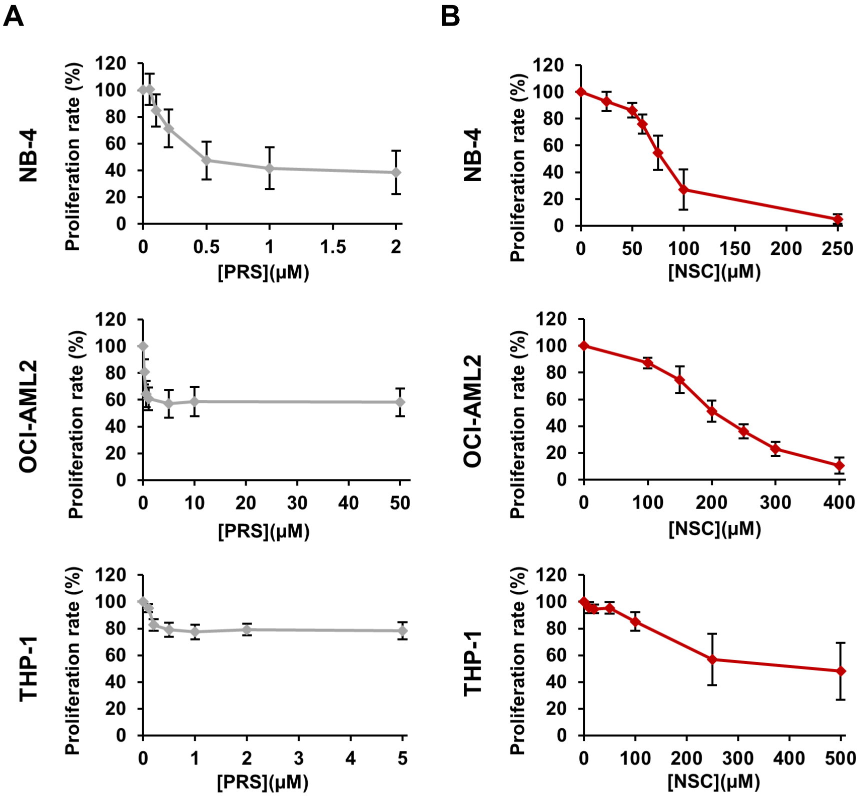

Figure 4.24. ***The anti-leukaemic effect of PRS and NSC held true in AML cell lines other than HL-60.*** **A)** Dose-response curves for different AML cell lines treated for 48 h with PRS. **B)** Dose-response curves for different AML cell lines treated for 48 h with NSC. N = 7 for NB-4 cells and N = 5 for OCI-AML2 and THP-1 cells.

In agreement with the response of HL-60 cells to these drugs (**Figure 4.20A**) co-treatment of the abovementioned cell lines with PRS and NSC displayed enhanced efficiency at reducing cell proliferation versus exposure to single agents at the doses tested (**Figure 4.25A**). CI values again supported a synergistic interaction (**Figure 4.25B**). All things considered, it can be concluded that the anti-leukaemic activity of the combination of PRS and NSC is not restricted to HL-60 cells and can be proposed as a potential wide-range therapy for the treatment of different subtypes of AML.

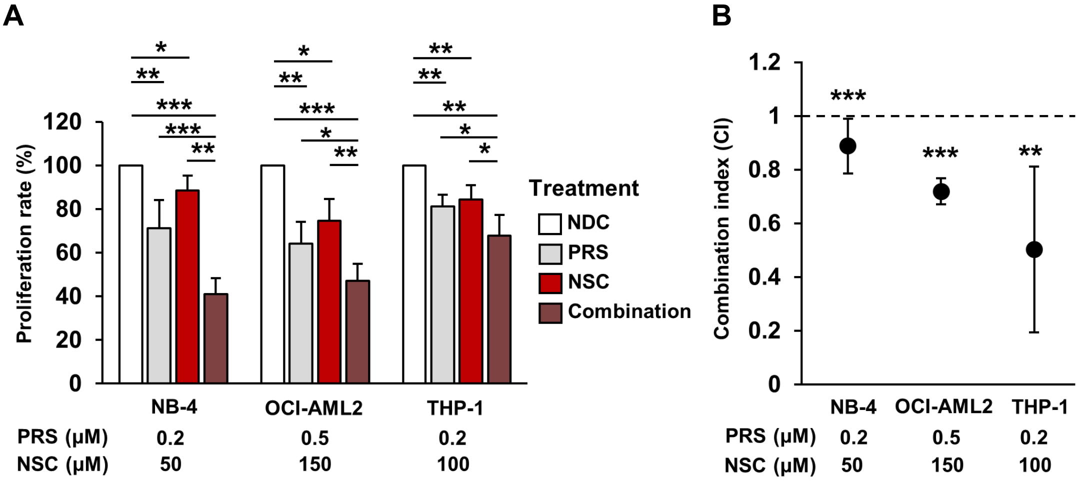

Figure 4.25. ***PRS and NSC synergised to preclude cell proliferation in AML cell lines other than HL-60.*** **A)** Proliferation rate of NB-4, OCI-AML2 and THP-1 cells treated for 48 h with PRS and NSC as single drugs and in combination at the indicated doses. **B)** Graphical representation of the average CI values obtained for the drug combinations indicated in panel A. Statistical differences were evaluated between CIs and the value 1. N = 5 for NB-4 and N = 6 for OCI-AML2 and THP-1 cells. NDC: No Drug Control.

4.2.5. PRS and NSC display anti-leukaemic activity in a xenograft mouse model of AML

The next step after the demonstration of the anti-leukaemic activity of the combined treatment with PRS and NSC was to test their effect in a whole organism. Drug metabolism and microenvironmental protection of leukaemic cells are

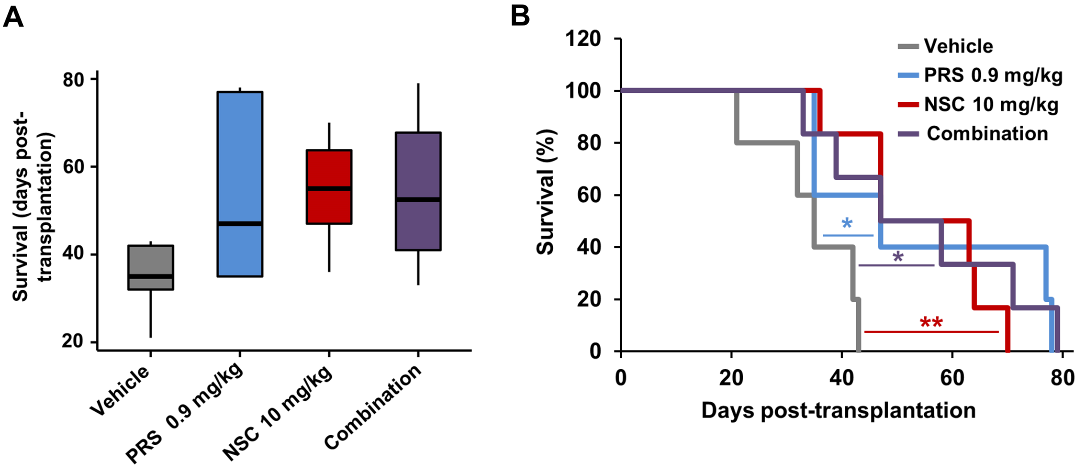

Figure 4.26. ***Treatment with PRS and NSC augmented survival in an*** **in vivo** ***model of AML.*** **A)** Distribution of survival times of NOD-SCID mice transplanted with HL-60 cells under different treatment regimens. **B)** Kaplan-Meier plot displaying the survival curves for the same animals. N = 5 for Vehicle and PRS groups and N = 6 for NSC- and combination-treated mice.

common mechanisms of resistance to therapy, and therefore need to be studied in a pre-clinical setting. The survival of immune-deficient mice transplanted with HL-60 cells was prolonged upon treatment with both PRS, NSC and combination (**Figure 4.26**). In contrast to the interaction found *in vitro,* no enhanced lifespan was achieved when animals were co-treated with both drugs, and no differences between PRS and NSC were observed. Nevertheless, the clear improvement of animal survival with every treatment validates the activity of the compounds *in vivo.* It is likely that dose adjustment could allow the observation of an enhancement of cell survival by the drug combination.

4.2.6. The combination of PRS and NSC affects the CFU ability of primary AML cells *in vitro*

All the results presented so far had been obtained in cell lines, which are very homogeneous populations and do not represent the characteristic sub-clonal and inter-individual heterogeneity of AML. Given the *in vivo* activity of both PRS and NSC, a reasonable step forward in this pre-clinical study was to test their effects on primary cells from patients with AML *ex vivo*. All samples used for this study came from non-APL AML patients (**Table 3.2**).

The treatment with PRS alone displayed a significant reduction of CFU numbers versus untreated cells, with all patient samples showing sensitivity to some extent. On the other hand, most samples (6 out of 8) underwent a reduction of CFU numbers after NSC treatment, although the overall results did not reach statistical significance versus untreated cells. More importantly, the combined treatment decreased the number of CFUs versus the single agents in 7 out of 8 patients, and the overall reduction effect reached statistical significance versus both untreated and NSC-treated cells (**Figure 4.27A**). In contrast, the effect of these drugs on healthy donor-derived BM primary cells was less pronounced, with no overall statistical significance. Most importantly, the drug combination did not display any enhanced effect versus individual treatments (**Figure 4.27B**).

A remarkable observation of these experiments is that the three most responsive patient samples to both PRS and PRS+NSC (AML1, AML3 and AML5) belonged to FAB M0 subtype, commonly associated with poor outcomes (Walter *et al*, 2013). The fact that a pro-differentiative compound targets this AML subtype, also known as minimally differentiated AML, makes sense. Moreover, AML1, showing a chromosomal translocation and two point mutations in *WT1* and *IDH1* (**Table 3.2**), was more sensitive to NSC as well. Contrarily, AML6 and AML8 samples, with *FLT3* and *NPM1* mutations, augmented their CFU capacity upon NSC treatment. Nonetheless, the most important finding was that every single sample underwent a reduction in CFU ability upon combined treatment, further reinforcing the therapeutic potential of this approach. A wider cohort of patient samples would be required to study possible correlations between responsiveness to these compounds and specific molecular alterations.

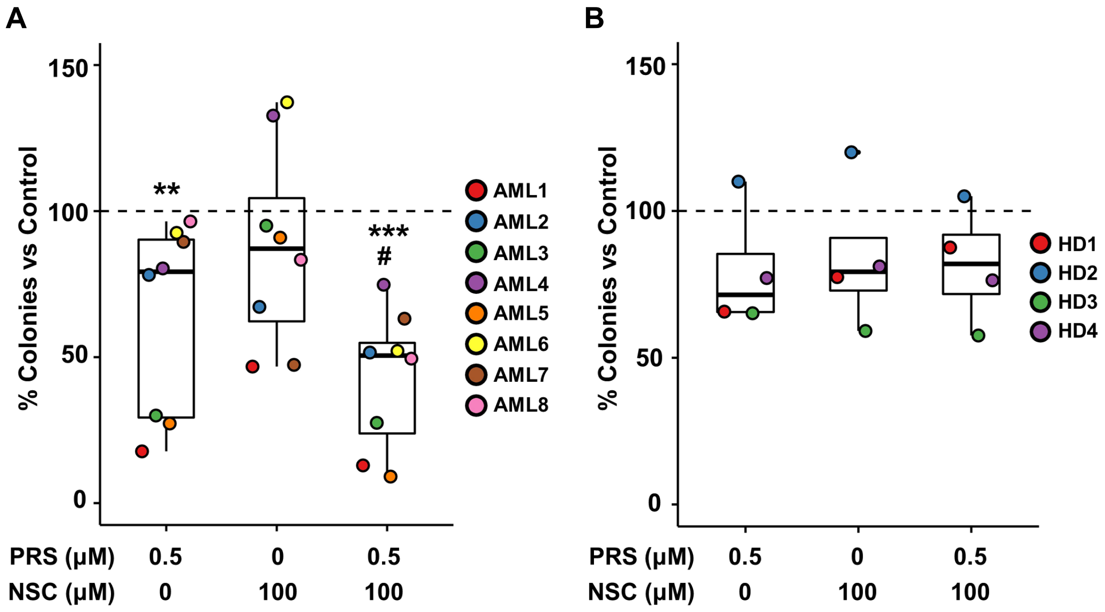

Figure 4.27. ***PRS and NSC co-treatment displayed an enhanced effect at reducing clonogenic potential of patient-derived AML cells while sparing healthy donor-derived cells* in vitro. A)** Relative CFU counts of primary BM-MNCs from AML treated for 48 h with PRS and NSC as single drugs and in combination and seeded for 14 days in methlylcellulose medium with appropriate growth factors in the absence of drugs. **B)** The same representation as in panel A for healthy donor-derived BM-MNCs. Statistical significance is denoted by asterisks (*) versus untreated cells and by hashes (#) versus cells treated with NSC.

4.2.7. Concluding remarks

The astonishing improvement of APL outcomes after the development of differentiation-based therapies has led to an intense research aiming at the implementation of this kind of strategies in non-APL cases (See section 1.2.1.3). All those attempts were based on a deep knowledge of molecular players controlling cell differentiation processes in leukaemic cells.

The findings shown on **Figures 4.2C, 4.3** and **4.12B** were the rationale for testing a therapeutic proposal whose pre-clinical potential has been demonstrated with the set of experiments described throughout this section. First, evidence has been provided that the enhancement of cell differentiation upon SHP1 and SHP2 inhibition was reproduced with the non-tumour-promoting agent PRS, a potential candidate for clinical use much more feasible than PMA (**Figure 4.12C**). Then, the dual inhibitors of SHP1 and SHP2 SSG and NSC have shown differentiation-inducing ability as single agents (**Figure 4.14**), but only NSC enhanced the effect of phorbol ester stimulation in the process (**Figures 4.16** and **4.17**). Additionally, the co-treatment with PRS and NSC has demonstrated to synergise at reducing cell proliferation (**Figures 4.20, 4.21** and **4.25**) likely due to joint effect of cell differentiation induction and differential targeting of self-renewal and apoptosis (**Figures 4.22** and **4.23**). The activity of both compounds has been corroborated in an *in vivo* setting (**Figure 4.26**) and in primary samples from AML patients (**Figure 4.27**). Globally considered, the results presented in this section greatly support the therapeutic feasibility of combining SHP1 and SHP2 inhibitors and phorbol esters as an efficient differentiation-based therapy at the pre-clinical level.

CONCLUSIONS

5. CONCLUSIONS

The results presented and discussed throughout this manuscript allow to make the following conclusions:

1. **The protein tyrosine phosphatases SHP1 and SHP2 cooperate to regulate differentiation in AML cell lines.**
2. **The kinase SRC is a downstream target of SHP2 in the context of HEL cell differentiation.**
3. **β-catenin levels are modulated in the same manner by SHP1 and SHP2 in HEL cells.**
4. **The chemical inhibitor of SHP1 and SHP2 NSC 87877 enhances the differentiation-promoting effect of phorbol esters in AML cell lines.**
5. **Combined administration of prostratin and NSC 87877 exerts a synergistic anti-leukaemic effect against AML *in vitro*.**
6. **Prostratin and NSC are efficacious anti-leukaemic agents in a disperse xenograft AML model *in vivo* and in primary cells from patients with AML *ex vivo.***

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