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Mechanisms of Chemoresistance and miRNA Regulation in Myeloid Hematologic Malignancies

IRENE GAÑÁN GÓMEZ

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DIRECTORA: MARÍA DEL CARMEN BOYANO ADÁNEZ

DEPARTAMENTO DE BIOLOGÍA DE SISTEMAS

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RESUMEN

Los cánceres hematológicos mieloides son aquellos que afectan específicamente a la producción de células del linaje mieloide, comenzando generalmente en médula ósea (MO) y alterando el número y la funcionalidad de dichas células. Las leucemias mieloides se caracterizan por la infiltración de células hematopoyéticas tumorales en sangre, MO y otros tejidos. Generalmente, estas patologías presentan translocaciones cromosómicas y, por consiguiente, expresan proteínas de fusión oncogénicas. Frecuentemente, las leucemias mieloides también muestran deficiencias funcionales, mutaciones o pérdida del locus del gen supresor tumoral P53, como ocurre en muchos casos de leucemia promielocítica aguda (LPA) y leucemia mieloide crónica (LMC). Ambos tipos de leucemia expresan proteínas de fusión características, lo que ha permitido el desarrollo de terapias dirigidas. Sin embargo, entre los problemas que presentan las terapias actuales se encuentran la necesidad de asociación con otros fármacos, las elevadas tasas de recaída en algunos grupos de pacientes o la aparición de resistencias. Por ello, nos propusimos analizar el efecto de una combinación de fármacos alternativa que pudiera resultar eficaz en el tratamiento de LPA y LMC, así como investigar el origen y los mecanismos de la resistencia a fármacos en estas patologías.

El decualinio (DQA) es una molécula de pequeño tamaño que, dadas sus características, se dirige de manera selectiva a la mitocondria de las células cancerosas y desencadena estrés oxidativo. Además, ha demostrado ser eficaz en la inducción de apoptosis en células leucémicas. Los inhibidores del proteosoma, cuya efectividad se ha descrito en cánceres hematológicos, cuando se administran a dosis bajas parecen sensibilizar a las células a la quimioterapia. Además, la inhibición del proteosoma contribuye al estrés oxidativo y de retículo endoplasmático, e induce apoptosis a través de un mecanismo que parece ser, principalmente, independiente de p53. Por las razones expuestas, en este trabajo se investigaron los efectos citotóxicos y citostáticos de la combinación del DQA con dosis sub-letales del inhibidor del proteosoma MG-132 en las líneas celulares NB4, derivada de LPA, y K562, derivada de LMC. Para ello, se emplearon técnicas de cultivos celulares, citometría de flujo, PCR cuantitativa (qPCR), Western blot y microscopía confocal.

La combinación de MG-132 y DQA detuvo la proliferación, tanto en células NB4 como en K562, sin potenciar los efectos pro-apoptóticos individuales de cada uno de los compuestos. Es más, en células NB4, el MG-132 antagonizó la apoptosis inducida por el DQA, al menos en parte, a través la reducción de los niveles de estrés oxidativo, que podría estar relacionado con la activación de la respuesta anti-oxidante desencadenada por el factor de transcripción Nrf2. En estas células, también identificamos dos mecanismos de resistencia intrínseca a la apoptosis. Así, esta línea celular expresa

una proteína p53 mutante, $p53^{R248Q}$, que parece inhibir o dificultar la apoptosis mediante un mecanismo de ganancia de función relacionado con sus efectos a nivel mitocondrial. Además, nuestros resultados indican que los niveles elevados de autofagia constituyen otro mecanismo intrínseco de protección frente a la muerte celular en la línea celular NB4. El DQA supera ambos mecanismos de resistencia, al menos en parte, gracias a la inhibición de la autofagia y a la inducción de la translocación nuclear de $p53^{R248Q}$.

En las células K562, el MG-132, solo y en combinación con el DQA, indujo la expresión de p21, un regulador del ciclo celular. Esta sobreexpresión de p21 se acompañó de una estabilización de su mRNA sostenida en el tiempo. Ya que se ha descrito que los niveles elevados de p21 inhiben la apoptosis e inducen resistencia a fármacos en varios tipos de cáncer, nos propusimos investigar los mecanismos moleculares implicados en la inducción de la expresión de p21 en células K562.

Los microRNAs (miRNAs) son reguladores post-transcripcionales de una amplia variedad de genes implicados en muchos procesos fisiológicos. Modulan, entre otros, la expresión de genes implicados en la regulación del ciclo celular, incluido P21. Por otro lado, la expresión de muchos miRNAs se encuentra alterada en el cáncer, y parece participar en la tumorigénesis y en la resistencia a fármacos. Por ello, se investigó la posible implicación de uno o más miRNAs en la inducción de la expresión de p21 por el MG-132 en células K562, haciendo uso de algoritmos bioinformáticos, empleando técnicas de cultivos celulares, qPCR, clonaje y transfección, y realizando ensayos del gen reportero de la luciferasa. Así, identificamos un miRNA, el miR-22, cuya expresión aumenta significativamente tras el tratamiento con MG-132. El miR-22 interacciona de forma específica con la región 3'UTR del mRNA de p21 e induce su acumulación. Este miRNA podría ser, al menos en parte, el responsable del efecto del MG-132 en la expresión de p21 en las células K562.

Por otra parte, los síndromes mielodisplásicos (SMD) son un grupo de cánceres hematológicos mieloides caracterizados por una hematopoyesis deficiente y por la apoptosis intramedular. Estas patologías son muy heterogéneas, tanto morfológica como citogenéticamente, lo cual complica su diagnóstico. Además, los pacientes son particularmente resistentes a la quimioterapia, que no es curativa debido a la falta de dianas farmacológicas específicas. De manera importante, en cáncer se ha descrito que los miRNAs se expresan siguiendo patrones específicos de cada tumor, incluidos los SMD, y, por ello, se han propuesto como biomarcadores en varios tipos de cáncer. Por estas razones, nos planteamos como siguiente objetivo estudiar la participación de los miRNAs en la patogénesis y la progresión de los SMD y su potencial utilidad como biomarcadores y dianas terapéuticas. Para ello, se emplearon técnicas de cultivos celulares, qPCR, transfección y silenciamiento de miRNAs, y se llevaron a cabo ensayos del gen reportero de la luciferasa y de diferenciación celular.

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Los resultados obtenidos muestran que los pacientes de SMD sobreexpresan miRNAs del clúster miR-99b/let-7e/miR-125a y el miembro del clúster parálogo miR-125b. Los datos sugieren que estos miRNAs podrían participar en la activación del factor de transcripción NF-κB, que se encuentra frecuentemente hiperactivado en SMD y parece conferir a las células de MO una ventaja de supervivencia. Además, la expresión de un miembro de este clúster, el miR-125a, parece estar regulada por mecanismos independientes del resto del clúster, y su sobreexpresión en células progenitoras de MO de pacientes con SMD se correlaciona con un peor pronóstico. Según estos resultados, el miR-125a podría participar en la patogénesis de los SMD a través de la desregulación de la actividad de NF-κB por un mecanismo dependiente del estado de activación de la vía de señalización pro-inflamatoria de los "*toll-like receptors*" (TLR), y también mediante una posible contribución al bloqueo de la diferenciación celular. Además, nuestros resultados indican que el TLR7 se sobreexpresa en MO de pacientes con SMD, lo que se correlaciona positivamente con un mejor pronóstico de la enfermedad. Por tanto, el miR-125a y el TLR7 podrían ser biomarcadores y dianas farmacológicas de interés en los SMD.

RESUMEN

SUMMARY

Myeloid hematologic malignancies are cancers that specifically affect the production of blood cells from the myeloid lineage, generally starting in bone marrow (BM) and altering the number and functionality of these cells. Myeloid leukemias are characterized by the infiltration of neoplastic hematopoietic cells in blood, BM and other tissues. These diseases frequently present with recurrent chromosomal translocations and the subsequent expression of oncogenic fusion proteins. Myeloid leukemias also often exhibit functional deficiencies, mutations or ablation of the tumor suppressor protein p53, as it occurs in many cases of acute promyelocytic leukemia (APL) and chronic myeloid leukemia (CML). Both types of leukemia express characteristic fusion proteins which have allowed the development of targeted therapies. However, the existing therapies have the problems of needing association with other drugs, presenting high relapse rates in some subsets of patients or inducing the acquisition of resistance. Thus, we sought to explore an alternative drug combination which might be effective in APL and CML, and to investigate the origin and mechanisms of chemoresistance in these types of myeloid leukemias.

Dequalinium (DQA) is a small molecule which selectively targets the mitochondria of cancer cells and triggers oxidative stress, and which has been proved to effectively induce apoptosis in leukemic cells. Proteasome inhibitors, in turn, have been reported to sensitize cells to chemotherapy when used at low doses and have been repeatedly demonstrated to be effective in hematologic malignancies. Remarkably, proteasome inhibition contributes to oxidative and endoplasmic reticulum stress, and induces apoptosis through a mechanism that appears to be mainly p53-independent. For the aforementioned reasons, we investigated the cytostatic and cytotoxic effects of a combination of DQA with sub-lethal doses of the proteasome inhibitor MG-132 in two cell lines: NB4 (derived from APL) and K562 (derived from CML). For this purpose, we used cell culturing, flow cytometry, quantitative PCR (qPCR), Western blot analysis and confocal microscopy thechniques.

Our results show that this combination triggers a strong proliferative arrest in both leukemic cell lines without enhancing the individual pro-apoptotic effects of the two drugs. Moreover, MG-132 antagonized apoptosis induced by DQA in NB4 cells through a mechanism that involves the reduction of oxidative stress and which could be related to the activation of the anti-oxidant response driven by the transcription factor Nrf2. We also identified two mechanisms of intrinsic resistance to apoptosis in NB4 cells. This line expresses the p53 hot-spot mutant $p53^{R248Q}$, which appears to inhibit or hinder apoptosis through a gain-of-function mechanism related to its mitochondrial activity. In addition, our findings indicate that elevated autophagy is another intrinsic mechanism of protection

from cell death in the NB4 line. DQA overcomes these two mechanisms by inhibiting autophagy and inducing the nuclear translocation of $p53^{R248Q}$.

In K562 cells, we observed a strong overexpression of the cell cycle regulator p21 after the treatment with MG-132, alone and in combination with DQA. This upregulation was accompanied by a sustained stabilization of p21 mRNA over time, triggered by MG-132. Because it has been reported that p21 overexpression inhibits apoptosis and induces chemoresistance in several types of cancer, we sought to investigate the molecular mechanism involved in this upregulation.

microRNAs (miRNAs) are post-transcriptional regulators of a plethora of genes, involved in many physiological processes. Among others, they have been reported to modulate the expression of genes involved in the regulation of cell cycle, including p21. Importantly, miRNAs are frequently deregulated in cancer and appear to participate in tumorigenesis and chemoresistance. For this reason, we explored the possible involvement of one or more miRNAs in the upregulation of p21 triggered by MG-132 in K562 cells, searching bioinformatic algorithms and using cell culturing, qPCR, vector cloning and transfection techniques and performing luciferase-reporter gene assays. We identified one miRNA, miR-22, which is significantly upregulated upon treatment with MG-132. miR-22 induces the positive regulation of p21 expression through a specific interaction with the 3'UTR of its mRNA and might be, at least in part, responsible for MG-132-induced p21 upregulation in K562 cells.

On the other hand, myelodysplastic syndromes (MDS) are a group of myeloid hematologic malignancies characterized by defective hematopoiesis and intramedullary apoptosis. These malignancies are highly heterogeneous, morphologically and cytogenetically, which complicates their diagnosis. Furthermore, patients are particularly refractory to chemotherapy, which is non-curative owing to the lack of specific pharmacological targets. Interestingly, miRNAs have been reported to follow tumor-specific expression patterns in cancer, including MDS, and have been proposed as biomarkers in many types of malignancies. For these reasons, we aimed to study the participation of miRNAs in the pathogenesis and progression of MDS and their potential utility as biomarkers and therapeutic targets. To fulfill this aim, we used cell culturing, qPCR, transfection and miRNA inhibition techniques and performed luciferase-reporter gene and differentiation assays.

We found that MDS patients overexpress miRNAs belonging to the miR-99b/let-7e/miR-125a cluster and the member of the paralogous cluster miR-125b. Our results suggest that these miRNAs could participate in the activation of the transcription factor NF-κB, which is frequently hyperactivated in MDS and thought to provide BM cells a survival advantage. Furthermore, one member of this cluster,

miR-125a, appears to be regulated through cluster-independent mechanisms and its overexpression in BM progenitor cells is strongly correlated with a poorer prognosis in MDS. miR-125a could participate in the pathogenesis of MDS by deregulating NF-κB activity through a mechanism that depends on the activation state of the pro-inflammatory signaling pathway of toll-like receptors (TLRs), and through a possible contribution to the blockade of differentiation. In addition, our results indicate that TLR7 is overexpressed in MDS BM and positively correlated to a better prognosis of the disease. Therefore, miR-125a and TLR7 are potentially valuable prognostic biomarkers and therapeutic targets in MDS.

SUMMARY

ABBREVIATIONS

3-MA – 3-methyladenine
AGO – Argonaute
AIF – Apotosis-Inducing Factor
ALL – Acute Lymphoblastic Leukemia
AML – Acute Myeloid Leukemia
AMPK – AMP-dependent protein kinase
AO – Acridine Orange
Apaf-1 – Apoptotic Protease-Activating Factor 1
APC – Antigen-Presenting Cell
APC/C – Anaphase-promoting complex/ cyclosome
APL – Acute Promyelocytic Leukemia
Ara-C – Arabinosyl cytosine or cytarabine
ASK-1 – Apoptosis signal-regulating kinase 1
ASO – Anti-Sense Oligonucleotide
Atg – Autophagy-related gene
ATO – Arsenic Trioxide
ATRA – All-Trans Retinoid Acid
AVO – Autophagic Vesicular Organelle
BAFFR – B-cell-Activating Factor belonging to TNF Family Receptor
Bcn-1 – Beclin-1
BCP – B-Cell Progenitor
BCR – B-Cell Receptor
BH – Bcl-2 Homology
BM – Bone Marrow
bp – Base Pair
BrdU - 5-bromo-2'-deoxyuridine
BSA – Bovine Serum Albumina
Cdk – Cyclin-dependent kinase
CDKI – Cdk inhibitor
cIAP – Inhibitor of Apoptosis homolog C
CLL – Chronic Lymphocytic Leukemia
CLP – Common Lymphoid Progenitor
CLR – C-type Lectin Receptor
CML – Chronic Myelogenous Leukemia
CMP – Common Myeloid Progenitor
COX-2 – Cytochrome c Oxydase subunit II
CR – Complete Remission
DAMP – Damage-Associated Molecular Pattern
DD – Death Domain
DHE – Dihydroethidium
DISC – Death-Inducing Signal Complex
DLC – Delocalized Lipophilic Cation
DMSO – Dimethylsulfoxide
DQA – Dequalinium (Español: Decualinio)
ELAM - Endothelial Leukocyte Adhesion Molecule
Endo G – Endonuclease G
EP – Erythroid Progenitor
EPO – Erythropoietin
EPO-R – EPO Receptor
ER – Endoplasmic Reticulum
FAB – French-American-British classification
FADD – FAS-Associated Death Domain
FBS – Fetal Bovine Serum
FLIP – Flice-Inhibitory Protein
FXR1 – Fragile X mental retardation-related protein 1
G-CSF – Granulocyte Colony-Stimulating Factor

ABBREVIATIONS

GM-CSF – Granulocyte/Monocyte Colony Stimulating Factor
GMP – Granulocyte/Macrophage Progenitor
GP – Granulocyte Progenitor
GPX – Glutathione Peroxidase
GR – Glutathione Reductase
GRx – Glutaredoxin
GSH – Glutathione (reduced)
GSSG – Glutathione (oxidized)
GST – Glutathione S-Transferase
GYPA – Glycophorin A
HIF – Hypoxia-induced factor
HMGB1 – High-Mobility Group Box 1
HSC – Hematopoietic Stem Cell
HSP – Heat Shock Protein
IAP – Inhibitor-of-Apoptosis
IC_{50} – Inhibitory Concentration 50
ICAM – InterCellular Adhesion Molecule
IFN – Interferon
IκB – Inhibitory κB protein
IKK - IκB Kinase
IL – Interleukin
ING4 – Inhibitor of Growth family 4
iNOS – Inducible Nitric Oxyde Synthase
IPSS – International Prognostic Scoring System
IPSS-R – Revised-International Prognostic Scoring System
IRAK – IL-1R-Associated Kinase
IRF – Interferon Regulatory Factor
ITGAM – Integrin alpha M
LB – Lysogeny Broth
LC3 – Light-Chain protein-3
LMC – Leucemia Mieloide Crónica
LPA – Leucemia Promielocítica Aguda
LPS – Lipopolysaccharide
LSC – Leukemic Stem Cell
LTβR – Lymphotoxin β Receptor
MAPK – Mitogen-Activated Protein Kinase
MC – Methylcellulose
M-CSF – Monocyte Colony-Stimulating Factor
MDC – Monodansylcadaverine
MDR – Multi-Drug Resistance
MDS – Myelodysplastic Syndromes
MEP – Megakaryocytic/Erythroid Progenitor
miRNA – microRNA
MkP – Megakaryocyte Progenitor
MO – Médula Ósea
MOMP – Mitochondria Outer-Membrane Permeabilization
MP – Monocyte Progenitor
MPN – Myeloproliferative Neoplasm
mTOR – Mammalian Target-of-Rapamycin
MTT – Methylthiazoltetrazolium
mut – Mutant
MyD88 – Myeloid Differentiation primary response gene 88
NAC – N-acetyl-L-cysteine
NCBI – National Center for Biotechnology Information (USA)
NEMO – NF-κB Essential Modulator
NF-κB – Nuclear Factor Kappa B
NIK – NF-κB-Inducing Kinase
NK – Natural Killer
NKP – NK Cell Progenitor
NLR – NOD-Like Receptor
NOX – NAD(P)H Oxidase

NQO1 – NAD(P)H–quinone oxidoreductase 1
NR – NCBI Reference sequence number
Nrf2 – Nuclear factor E2-related factor 2
OS – Overall Survival
PAM3 - PAM(3)CysSK(4)
PAMP – Pathogen-Associated Molecular Pattern
PAZ – Piwi-Argonaute-Zwille/Pinhead
P-Bodies – Processing Bodies
PBS – Phosphate Buffered Saline
PCD – Programmed Cell Death
PCNA – Proliferating-cell nuclear antigen
PFT-α/μ - Pifithrin-α/μ
PI – Propidium Iodide
PI3K - PhosphatidylinositoI 3-kinase
piRNA – Piwi-Interfering RNA
PML – Promyelocytic Leukemia (gene)
PMSF - Phenylmethanesulfonyl fluoride
Pol II – RNA Polymerase II
Pre-miRNA – Precursor miRNA
Pri-miRNA – Primary miRNA Transcript
PRR – Pattern Recognition Receptor
Prx – Peroxiredoxin
PS – Phosphatidilserine
qPCR – Quantitative-Polymerase Chain Reaction
RAIDD – RIP-Associated ICH/CED-3 homologous Death Domain
RANK – Receptor Activator for NF-κB
RAPA – Rapamycin
RARα – Retinoid Acid Receptor Alpha
RAS – Retinoid Acid Syndrome
RE – Response Element
RHD – Rel Homology Domain
RIG – Retinoic Acid-Inducible Gene
RIP – Receptor-Interactive Protein
RISC – RNA-induced Silencing Complex
RLR – RIG-I-Like Receptor
RNase – Ribonuclease
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
RT – Reverse Transcription
RXR – Retinoid X Receptor
SCF – Stem Cell Factor
SCT – Stem Cell Transplantation
SG – Stress Granule
siRNA – Short Interfering RNA
Smac/DIABLO – Second Mitochondria-derived Activator of Caspases/ Direct IAP-Binding protein with Low pI
SMD – Síndromes Mielodisplásicos
SOD – Superoxide Dismutase
ssRNA – Single-Stranded RNA
TBS – Tris Buffered Saline
TCP – T-Cell Progenitor
TCR – T-Cell Receptor
TF – Transcription Factor
TIR – Toll/IL-1R
TLR – Toll-Like Receptor
tMDS – Therapy-related MDS
TNF – Tumor Necrosis Factor
TNFR – TNF Receptor
TNK – T-cell/NK Progenitor
TPO – Thrombopoietin
TRADD – TNFR-Associated Death Domain
TRAF – TNF Receptor-Associated Factor
TRAM – TRIF-Related Adaptor Molecule

ABBREVIATIONS

TRIF – TIR domain-containing adaptor inducing IFN-β

Trx – Thioredoxin

TTBS – Tween 20-TBS

ULK – Unc-51-Like Kinase

UPS – Ubiquitin-Proteasome System

UTR – Untranslated Region

VCAM – Vascular Cell Adhesion Molecule

VMP1 – Vacuole Membrane Protein 1

WHO – World Health Organization

wt – Wild type

XIAP – X-linked Inhibitor of Apoptosis

XO – Xanthine Oxidase

$\Delta\Psi_m$ – Mitochondrial transmembrane potential

γGCS – Gamma Glutamyl-Cysteinyl Synthetase

PROLOGUE

The present Doctoral Thesis has been written according to the regular structure of a research article, including the main sections *Introduction*, *Materials and Methods*, *Results and Discussion* and *References*. In addition, this Thesis contains a *General Hypotheses and Aims* section which clarifies the general objectives of this research work, a *General Discussion* section, in which additional aspects of our research have been further addressed, and a *Conclusions* section that reports the inferences drawn from our results in a concise and informative manner.

The results compiled in this Thesis reflect the work of four years during which our own results have led us through divergent paths, and the initial aims have evolved with the progress of our research. For this reason, results presented in this work have been sub-divided in three blocks, each of which addresses a different question and is focused on a distinct disease from one common group of pathologies.

In order to facilitate the understanding of the three different topics, the *Introduction* section covers a wide spectrum of basic concepts that support the foundations of this research work and, based on those foundations, the *General Hypotheses and Aims* section summarizes the overall aims of this project. The *Materials and Methods* section details the protocols used in an intuitive order (cells and reagents first, then methods used in more than one block and, last, more specific methods, in order of appearance). Nevertheless, each of the three separate *Results and Discussion* blocks includes a brief but more specific Introduction or *Background*, its own *Hypothesis and Aims* and a shortened *Materials and Methods* sub-section, in addition to the corresponding *Results* and *Discussion*. Finally, the *General Discussion* and *Conclusions* sections bring all the information from the three previous blocks together and transmit the take-home message.

I. INTRODUCTION

INTRODUCTION

1. Differentiation and Hematopoiesis

1.1. Differentiation

Differentiation is a specialization process in which the developmental potential of cells is progressively restricted. It starts with ***stem cells***, unspecialized cells that can reproduce themselves indefinitely, and culminates in the formation of highly-specialized, ***terminally differentiated cells***, which often cannot divide but carry out their functions for varying lengths of time and then die [1].

Stem cells are characterized by their **self-renewal and differentiation potential**. *Self-renewal ability* allows stem cells to reproduce themselves indefinitely through a mechanism of ***symmetric division***, which yields two identical daughter stem cells. *Differentiation* takes place via epigenetic change during a process of ***asymmetric cell division***, which generates two different daughter cells: one copy of themselves and a derivative stem cell (a *progenitor* cell) that has more restricted capabilities, such as a limited proliferative ability, or fewer types of progeny [1, 2]. When both daughter cells are progenitors, this division is termed ***symmetric differentiation*** [2].

The event that allows cells to differentiate further along one or more particular lineage, but not along other lineages, is called ***lineage commitment*** and is generally irreversible [2]. ***Pluripotent (or multipotent) stem cells*** have the capability of generating a number of different cell types, but not all. This is the case of ***hematopoietic stem cells (HSC)***, which can give rise to any blood cell lineage but not to cells from other types of tissue [1]. Differentiation of a pluripotent/multipotent stem cell can generate a ***progenitor cell***, which commits to a specific path of differentiation, or a ***unipotent cell***, which can just divide to form a copy of itself and a cell that can only give rise to one cell type. Further differentiation of progenitors and unipotent cells eventually generates *terminally differentiated* cells [1]. Therefore, the process of differentiation occurs in **consecutive steps** through lineage commitment, and during these steps **the volume of cell populations is stabilized by the fine regulation of proliferation and cell death**. Both cell cycle regulation and cell death are key processes which will be referred to further on in the *Introduction* section.

1.2. Hematopoiesis

Hematopoiesis is the physiological process through which functionally mature blood cells are produced from a limited number of immature progenitor cells, allowing the replacement of senescent cells with new specialized cells. This continuous production of cells is mediated by ***differentiation***, ***proliferation and death*** mechanisms and results in a hierarchically organized blood

cell tissue, which can be divided in different sub-populations of cells with different degrees of maturation (Figure 1.1) [3].

Mature blood cells can be classified in different groups according to their function [4]:

- ***Red blood cells*** *or* ***erythrocytes***, which transport O_2 and CO_2 bound to hemoglobin.
- ***Platelets***, which are cell fragments from larger cells (***megakaryocytes***) that participate in blood clotting.
- ***White blood cells*** *or* ***leukocytes***, which carry out the immune defense.
  In turn, leukocytes can be classified in two groups based on their lineage, and these can be classified in sub-populations according to their morphology:
  - ***Lymphocytes*** participate in different types of immune responses; ***B cells*** synthesize antibodies, and ***T cells*** kill virus-infected cells and regulate the activity of other leukocytes. ***Natural Killer (NK) cells*** eliminate some types of tumor cells and virus-infected cells.
  - ***Myelocytes*** are divided in:
    - ***Granulocytes***: their cytoplasm contains numerous lysosomes and secretory granules which vary in function and chemistry. *Neutrophils* phagocytose and destroy microorganisms; *basophils* secrete histamine and mediate inflammatory reactions; and *eosinophils* help to destroy parasites and modulate allergic responses.
    - ***Monocytes and macrophages***: monocytes are circulating white cells that localize to infected or damaged tissues and mature to macrophages, which are the main phagocytes in the organism. They can recognize and digest senescent and dying cells as well as large pathogens.

Hematopoiesis is a **cell-type specific** process, that is, the production of each cell type is individually regulated to fulfill changing needs of the organism [4]. Regulation of hematopoiesis is carried out by extracellular growth factors called ***cytokines***, which modulate proliferation and differentiation of the stem and precursor cells towards the myeloid or lymphoid lineages (Figure 1.1) [1].

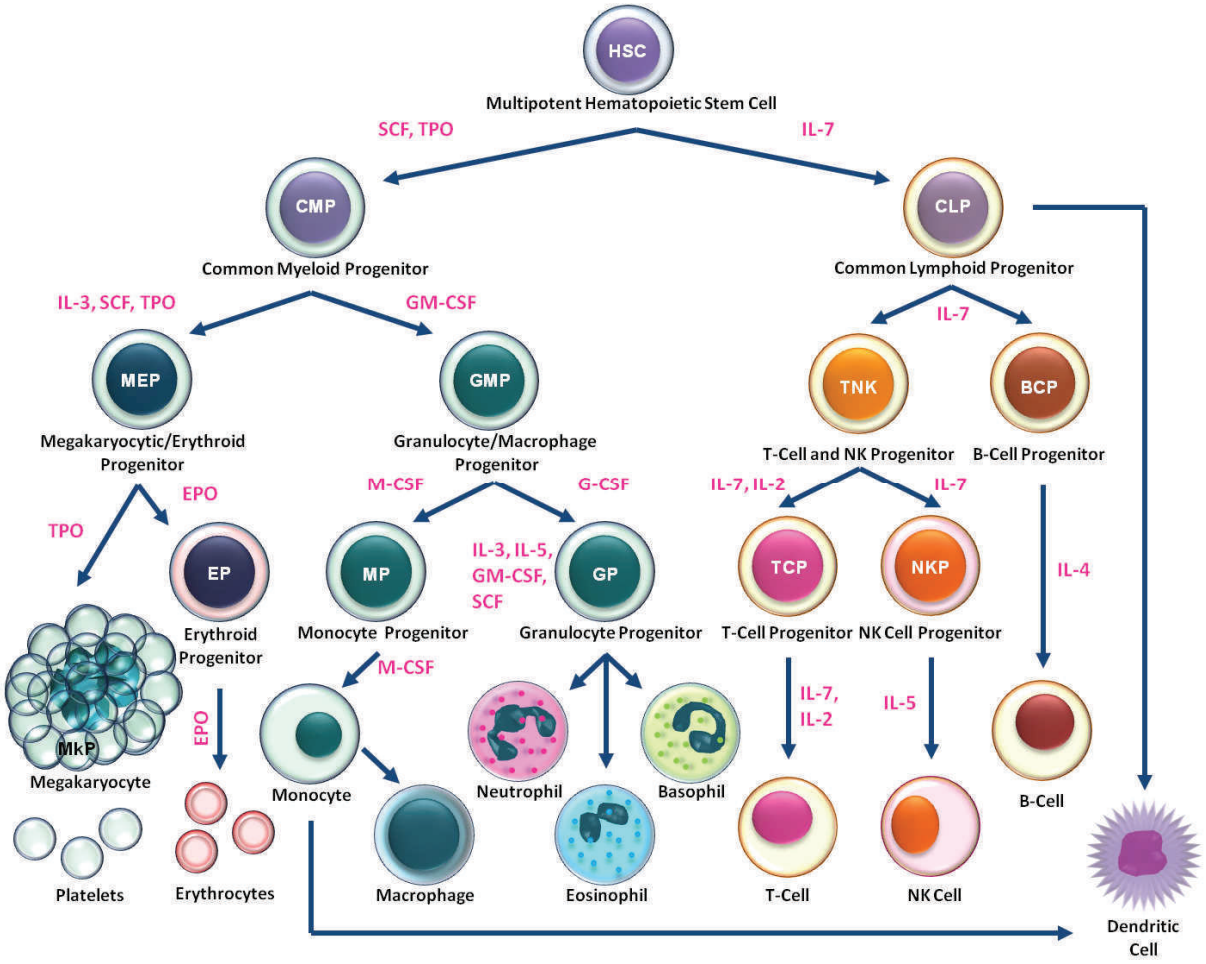

Figure 1.1. Scheme of hematopoietic differentiation. Hematopoietic cytokines are indicated in pink. SCF: Stem Cell Factor, TPO: Thrombopoietin, IL: Interleukin, CSF: Colony-Stimulating Factor (GM- Granulocyte/Monocyte; M- Monocyte, G- Granulocyte), EPO: Erythropoietin. Remaining abbreviations are defined within the figure text.

In the post-natal period, hematopoiesis takes place in ***bone marrow (BM)*** for most types of blood cells, with the exception of lymphocytes and macrophages. BM is necessary because survival, proliferation and differentiation of HSC depend on contact signals from stromal cells (*osteoblasts*) [4]. As explained above, differentiation is a step-by-step process. During hematopoiesis, HSC located in a BM *niche* are stimulated by specific cytokines and, accordingly, give rise to myeloid or lymphoid progenitors which further commit to the production of just one cell type. These steps correlate with changes in the **expression of specific transcription factors** that regulate the protein expression patterns which determine cell specialization [4, 5]. However, differentiation is not controlled by one particular lineage-specific transcription factor alone, but multiple transcription factors simultaneously act together to direct lineage commitment in complex combinatorial networks [2, 5,

6]. The expression, or its loss, of those transcription factors is a **characteristic signature of each stage of differentiation and hematopoietic lineage** (Table 1.1).

Table 1.1. Some transcription factors involved in hematopoiesis.

Factor	Functions in hematopoiesis
PU.1	Balance with other transcription factors determines myeloid or lymphoid commitment. Within the myeloid path, it directs differentiation to the granulocytic/monocytic lineage.
GATA1	Balance with PU.1 in myeloid progenitors determines commitment towards megakaryocytic/erythroid differentiation.
SCL	Necessary for erythroid commitment.
GATA2	Regulates commitment towards the megakaryocytic lineage and participates in basophils/mast cells differentiation.
NF-E2	Specific of megakaryocytic differentiation and platelet production.
c/EBPα	Modulates granulocytic/monocytic differentiation towards the neutrophil lineage and participates in B-cell commitment.
Gfi-1	Directs granulocytic differentiation towards neutrophils.
Egr-1/2 c-Jun	Determine the monocytic/macrophage lineage.
RUNX1	Directs lymphocytic differentiation and also regulates megakaryocytic commitment.
Fli-1 IKAROS	Specific of the lymphoid lineage.
GATA3	Necessary for T-cell differentiation.
Notch1	Determines commitment towards T or B-cell lineages.
E2A/EBF Pax-5	Specific of B-cell development.
Ets-1	Necessary for NK cell commitment.

The expression and activity of this intricate network of transcription factors are in turn associated with **epigenetic changes**, mainly with the modulation of DNA methylation and histone acetylation by specific enzymes [2]. Moreover, transcription factors may also interact with other proteins or undergo **postranslational modifications** [6]. **Regulation by microRNAs (*miRNA*)** also appears to be essential for the expression of gene profiles that are determinant of cell identity during hematopoiesis [2, 7]. Regulation of protein expression by miRNA will be described in detail further on in the *Introduction* section (Chapter 6).

Another characteristic of great importance for the identification of cells in different stages of differentiation is the acquisition and loss of expression of **specific membrane antigens,** or surface markers. One example of special interest is **CD34**, a membrane protein involved in cellular adhesion processes that is typical from a fraction of **multipotent progenitors localized in BM** [3]. Other surface

antigens characteristic of hematopoietic progenitors or mature blood cells are summarized in Table 1.2 [5].

Table 1.2. Some surface markers characteristic of different blood cell lineages.

Myeloid		Lymphoid	
Granulocytic/ Monocytic	GM-CSFR IL3R IL5R CD11b CD33	**Common**	IL7R
Granulocytic	CD11b CD15	**T-cell**	TCR CD90 CD4 CD8
Monocytic	CD14 CD64		
Megakaryocytic	CD41 CD42 CD61 PF4	**B-cell**	CD25 CD45 CD19
Erythroid	CD242 (ICAM-4) EPO-R	**NK-cell**	CD122 IL15R

To sum up, hematopoiesis is the physiological process of blood cell production, which takes place through the modulation of proliferation, differentiation and programmed cell death of cells that form the hematopoietic tissue. These processes are regulated by cytokines, which induce changes in the epigenetic control and transcriptional program of cells, ultimately resulting in the modification of protein expression patterns towards a more specialized phenotype. The **tight modulation** of all these processes is, nevertheless, **susceptible of being altered** by various factors (i.e. mutations) which would cause **errors in the production of one or more types of blood cells**, eventually giving rise to pathology.

INTRODUCTION

2. Hematologic Malignancies

Hematologic or hematopoietic malignancies are cancers that affect the **production and function of blood cells** [8]. The *World Health Organization* (WHO) defines ***cancer*** as a generic term for a large group of diseases characterized by the rapid creation of abnormal cells that grow beyond their usual boundaries and which can invade adjacent parts of the body and spread to other organs. Nearly all types of cancer arise from a single cell, through a multistage process in which multiple events accumulate to transform a normal cell into a malignant tumor [9]. This process is called ***tumorigenesis*** and is driven by DNA mutations and frequently also by epigenetic deregulation, which finally result in changes in gene expression.

There are two major classes of genes participating in cancer: those which directly affect cell growth and survival, either positively (*oncogenes*) or negatively (*tumor suppressors*), and those which do not directly affect cell division or death but regulate the ability of cells to maintain the integrity of their genome (*caretaker* or *housekeeping* genes) [10].

Most blood cancers start in BM, where normal hematopoiesis is interrupted by the uncontrolled growth of an abnormal type of blood cell. This process generally affects number and functionality of one or more types of mature blood cells and prevents them from performing some of their functions, like immune defense or coagulation [8].

The American Society of Hematology defines three main types of blood cancer [8]:

- ***Leukemias*** are blood stream and BM cancers caused by the rapid production of abnormal white blood cells. These malignant cells are unable to exert their regular functions in the *Immune System* and their high number impairs normal hematopoiesis of the rest of blood cells. Based on the white blood cell lineage which is mainly affected, they can be classified in two sub-types: *lymphoid leukemias* and *myeloid leukemias*.

  The GLOBOCAN project, from the International Agency for Research on Cancer, provides epidemiological statistics from major types of cancer in 184 countries of the world. GLOBOCAN 2008 estimates that, among all the types of cancer classified by the WHO, leukemias occupy the 12th and the 8th position on the incidence and mortality rankings, respectively, in more developed regions. Leukemias are especially prevalent in infants, having the second-highest incidence and the highest mortality rates in children aged 0 to 14 years [11].

- ***Lymphomas*** are blood cancers affecting the *Lymphatic System*, in which transformed lymphocytes proliferate abnormally and accumulate in lymph nodes and other tissues, hampering the immune response.
- ***Myelomas*** are cancers specifically targeting plasma cells and thus preventing normal production of antibodies.

In the present work, we will focus on **myeloid malignancies**, which affect the myeloid lineage; specifically, in *myeloid leukemias and myelodysplastic syndromes (MDS)*. The definition, clinical characterization, epidemiology and current frontline treatments of both groups of diseases will be addressed below.

2.1. Myeloid Leukemias

Myeloid leukemias are a group of diseases characterized by the **infiltration of neoplastic hematopoietic cells from the myeloid lineage** in blood, BM and other tissues. Myeloid leukemias are classified as *acute* or *chronic*, based on the course of the untreated disease [10].

2.1.1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a *clonal malignancy* (arising from the clonal expansion of a single progenitor cell) [6] which is clinically characterized by the presence of ≥20% myeloblasts in blood and/or BM. Most of the patients present nonspecific symptoms that are mainly the consequence of anemia, which can be severe, and also of leukocytosis, leukopenia or leukocyte dysfunction, or thrombocytopenia (Figure 2.1) [10].

Over the past fifteen years, AML has suffered a significant increase in incidence, which is age-dependent [10]. The development of AML has been associated with genetic heredity of certain syndromes or diseases, excessive chromatin fragility, radiation, exposure to benzene, tobacco smoke or other toxics and with the use of chemotherapy and other drugs. The latter is the main cause of the so-called treatment-associated AML, for which a successful therapeutic approach is extremely difficult [10]. Advanced age of the patient is associated with a poorer prognosis (higher risk), since AML is biologically distinct in older patients and they have fewer chances to survive chemotherapy and achieve *complete remission* (CR) [10].

Treatment of AML is usually divided into two phases: *induction* of CR, which is an aggressive and quick step, and *consolidation or post-remission management*, which has the objective to prolong survival of the patient and achieve the cure of the disease. **Supplementing frontline induction therapies with other agents** appears to be effective in younger patients (<60 years); as for the elderly, the benefits of intensive therapy are not so clear and **novel consolidation therapies are currently being explored** [10].

Post-remission therapy is designed to eradicate any residual leukemic cells, and it is essential to prevent relapse, being critical to long-term disease-free survival in AML. For young patients, intensive chemotherapy and stem cell transplantation (SCT) seem to be the best approaches; for older patients, attenuated intensive therapy has been carried out but new agents are currently being investigated. Despite these efforts, **relapse still occurs in a percentage of patients**, who are then rarely cured with standard-dose chemotherapy. Allogeneic SCT is the first-line treatment for relapse; however, because of the poor outcome of patients, it is **necessary to explore innovative alternatives**, either new drugs or immunotherapy, with clinical activity in AML [10].

Acute leukemias can be classified into different sub-groups based on morphology, cytochemistry, immunophenotype, cytogenetics or molecular characteristics of the cells.

- The *French-American-British classification* (FAB) divides AML in eight groups (M0-7) according to the type of cell from which the leukemia developed and its degree of differentiation. Generally, FAB types correspond to distinct cytogenetic groups [10].
- The *WHO classification* is more clinically focused and contains numerous descriptive subcategories [12].

In the present work, we will focus on one of these subtypes:

- **Acute Promyelocytic Leukemia**

Acute promyelocytic leukemia (APL) is a distinct type of AML which is morphologically and cytochemically classified by FAB as M3 due to the high presence of cytoplasmic granules in promyeloblasts [10]. In contrast to other AMLs, which have been demonstrated to be originated from clonal expansion of a single stem cell ("leukemic stem cell" or LSC), APL appears to arise from cells which are more differentiated [6].

The most distinctive characteristic of APL is a recurrent genetic abnormality: the **chromosomal translocation t(15;17)(q22;q12)** [13]. WHO classifies APL based on this translocation and its multiple variants, which do not exceed 2% of APL cases [12]. This characteristic is frequently associated with

younger patients (10-30 years) [14] and is generally a marker for a better prognosis [10]. t(15;17) fusion transcript is not only necessary for diagnosis, but also a valuable tool to evaluate treatment effectiveness and detect early relapse [15].

Translocation t(15;17) encodes the **fusion protein PML-RARα**, composed of the *retinoid acid receptor alpha* (RARα), encoded in chromosome 17, and the *promyelocytic leukemia gene* (PML), in chromosome 15 [16]. PML is a tumor suppressor gene [17] and RARα is a nuclear hormone receptor that initiates transcription of a variety of genes and participates in hematopoiesis [18]. Normal activity of PML and RARα is disrupted by the **dual dominant-negative activity** of the chimeric protein [17, 18]. Thus, PML-RARα forms homodimers that sequester *retinoid X receptors* (RXR) and/or PML proteins, forming complexes that bind to *retinoid acid response elements* in the regulatory region of genes involved in granulocytic differentiation [18, 19]. These complexes recruit co-repressors like histone deacetylases and DNA methyltransferases, resulting in an effective inhibition of gene expression [20]. This repression results in the **blockade of cell differentiation at the promyelocytic stage** and promotes **proliferation and survival of tumor cells** (Figure 2.1) [21]. Other RAR fusion proteins resulting from different translocation variants also share the common characteristic of forming homodimers and having high affinity for the recruitment of co-repressors [22], demonstrating that the epigenetic deregulation of genes that are necessary for differentiation by PML-RARα is crucial for the pathogenesis of APL [23].

APL is not a frequent condition but is probably **the most malignant form of AML** due to a very rapid fatal course dominated by severe haemorrhages [24] ascribed to *disseminated intravascular coagulation* [10]. Fortunately, APL is relatively sensitive to chemotherapy, as it was demonstrated by the combined treatment with anthracyclines (DNA intercalators) and the nucleotide analog *arabinosyl cytosine* (Ara-C), which was used as frontline therapy for a long time [25]. However, chemotherapy was demonstrated to **aggravate bleeding syndromes and lead to high early death rates** [26].

Later on, it was discovered that APL cells respond to the differentiating drug *tretinoin* or *all-trans retinoic acid* (ATRA), which is a RARα ligand. Treatment with pharmacological doses of ATRA induces a conformational change in PML-RARα that causes the dissociation of the co-repressor complex and allows the recruitment of transcriptional co-activators. Moreover, ATRA also induces proteasome-mediated PML-RARα degradation [26]. Overall, ATRA leads to the expression of genes involved in the initiation or promotion of granulocytic commitment, overcoming the differentiation block. The introduction of ATRA greatly improved clinical outcomes (CR and long-term survival) in low-risk

patients and refractory or relapsed APL cases [27, 28]. However, continuous treatment with ATRA originates **resistance and decreased drug availability in plasma**, inducing relapse within a few months [26], and causes a fatal complication, the *retinoid acid syndrome* (RAS), which is related to adhesion of differentiated neoplastic cells to the pulmonary endothelium and has a mortality rate of about 10% [10, 26].

Combinations of ATRA with chemotherapy (anthracyclines and Ara-C) improved patient response decreasing relapse rates [29] and reduced the number of RAS cases [30]. However, more recent therapies have replaced chemotherapy with *arsenic trioxide* (ATO), which induces both mitochondria-mediated apoptosis and differentiation of APL cells [31, 32] through an apparently selective mechanism targeting PML-RARα and wild type (wt)PML for degradation [33]. ATO significantly improves long-term survival in APL patients [33, 34] and several treatment strategies using ATRA/ATO, usually in combination with chemotherapy, have demonstrated to be very effective in the treatment of APL [35, 36]. Nevertheless, ATO-based treatments are associated with several electrolyte abnormalities which can lead to **fatal ventricular arrhythmias** [37].

ATRA/ATO and ATRA/chemotherapy combinations have constituted the frontline induction therapy for APL since the 2000s [26, 38]. In high-risk patients, protocols including Ara-C in combination with ATRA or chemotherapy are especially useful to lower the risk of relapse [38]. These risk-adapted therapies have made significant improvements, achieving 80% survival rates [39]. However, this excellent clinical outcome may lead to underestimation of important aspects of APL treatment [38]. Chemotherapy causes a **10% incidence of disseminated intravascular coagulation** [10] and older age remains a prominent negative prognostic factor, so patients with **high-risk still need better and safer upfront therapies** [40]. Furthermore, some chemotherapy-free clinical trials have suggested that **chemotherapy might not be essential and could be replaced by other types of drugs** [26, 39, 41].

To sum up, despite APL is a type of AML which is relatively easy to treat and very specific therapies are available nowadays, the history of this disease has demonstrated that, no matter how specific a treatment might be, it can always benefit from the discovery and combination of new drugs. Therefore, we should not settle for the current clinical results and should **aim to improve the safety and success rates of APL therapies** (see Table 2.1).

2.1.2. Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm characterized by the clonal expansion of hematopoietic stem cells bearing an acrocentric chromosome that was named the *Philadelphia chromosome* [12, 42] and that is generated by the **reciprocal chromosomal translocation t(9;22)(q34;q11)** [43]. This abnormality results in the expression of the **fusion protein Bcr-Abl** [44, 45].

c-Abl is a proto-oncogene with tightly-regulated nuclear kinase activity [46] which participates in the DNA damage-response pathway. In this chimerical protein, **Abl kinase gains constitutive activation** and activates downstream pro-survival signalling pathways that transform hematopoietic progenitors in malignant cells [47]. The mechanism of transformation has not been fully characterized yet, but includes **increased proliferation or decreased apoptosis** [48] **of HSC or progenitors** leading to a massive increase in myeloid cells, premature release of these cells into circulation and genetic instability, which results in disease progression [49]. In spite of the complexity of the pathways that are involved in the pathogenesis of CML, it is accepted that Bcr-Abl is responsible for the transformation of HSC, since its silencing induces apoptosis, cell cycle arrest and differentiation of CML cells [48]. Thus, Bcr-Abl is considered the oncogenic event responsible for leukemogenesis in CML [47, 50].

The incidence of CML is higher in men than in women and increases slowly until the middle forties, when it starts to rise. Unlike AML, there is no evidence of a correlation between the development of CML and exposure to cytotoxic drugs, and only high doses of radiation have been proved to induce it. However, smoking habits seem to accelerate progression to blast phase [10].

Untreated CML is clinically characterized by the transition, after an average of 5 years, from the so-called ***chronic phase*** to an ***accelerated phase*** and further ***blast crisis***, which is a fatal acute lymphocytic leukemia (ALL) [51]. The chronic or stable phase presents with excess of myeloid and megakaryocytic cells that differentiate and function normally. CML patients on the chronic phase can be initially asymptomatic or present with very general symptoms (headache, fatigue, sickness, etc.), which worsen upon progression to the acute or blastic phases [10]. These present with increasing anemia and high counts of blood or BM blasts (Figure 2.1) [10]. The acquisition of additional genetic aberrations, such as trisomy 8, **17p$^-$ (loss of p53)**, and concomitant deletions on chromosome 9 and alterations in RAS or MYC genes, leads to a progressive loss of the capacity of terminal differentiation that is **critical for the phenotypic transformation to the acute phase** [10, 51].

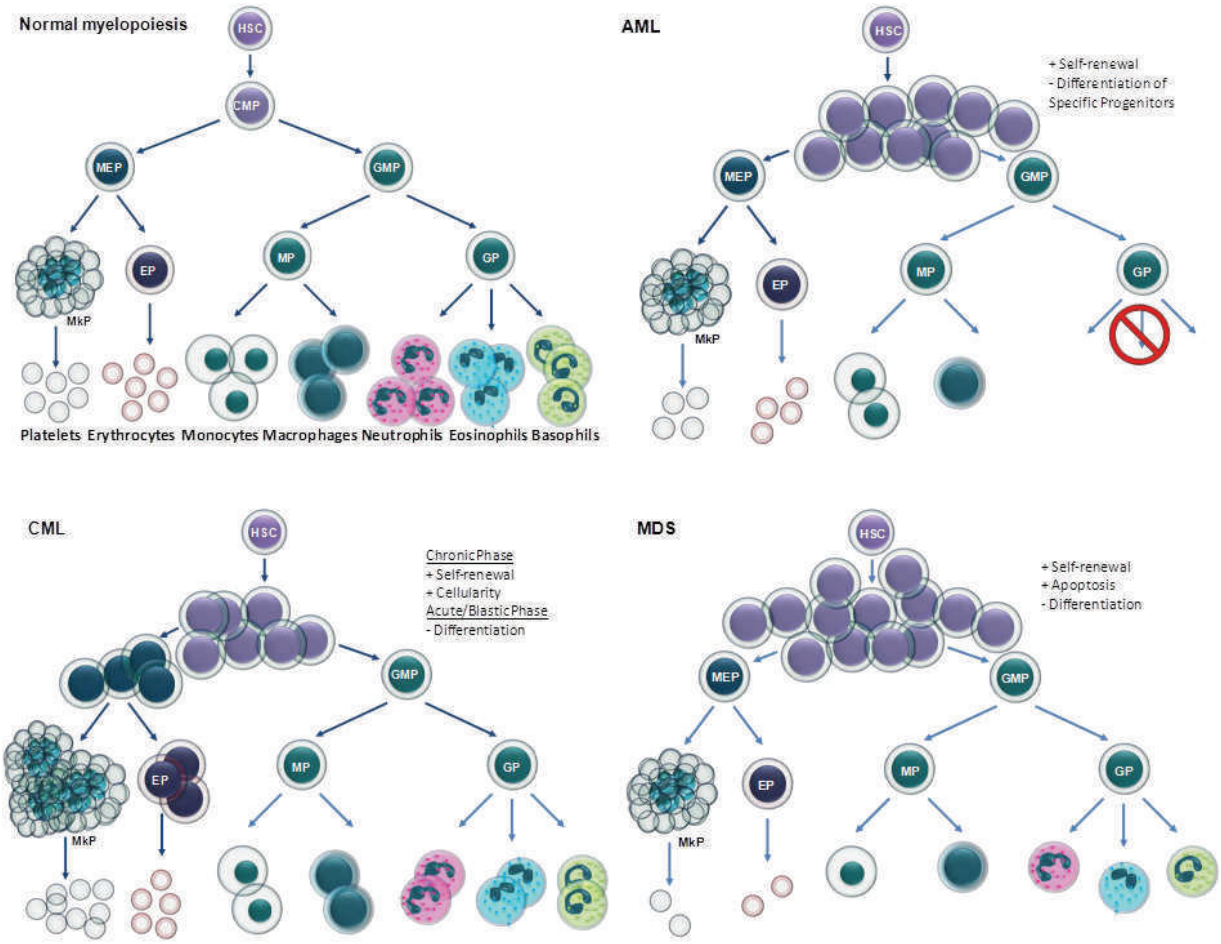

Figure 2.1. Defective myelopoiesis in AML, CML and MDS.
Upper-left image represents hematopoiesis of the myeloid lineage (myelopoiesis) in normal cells. HSC are capable of maturing into all myeloid lineages by first committing to the CMP and then to either MEP or GMP. AML, CML and MDS are clonal malignancies which can be sustained by a small population of LSC. In AML (upper-right), mutated HSC or CMP gain increased self-renewal (proliferative) capacity and differentiation towards specific lineages is blocked. In CML (lower-left) mutated progenitors increase their self-renewal capacity and megakaryocytic/erythroid blast number increases while differentiation towards other myeloid lineages is decreased. Upon progression, differentiation is impaired and mature cells are lost, with a similar cellularity to that in AML. In MDS (lower-right), proliferation increases in mutated HSC or CMP and differentiation towards all myeloid lineages is reduced.

The goal of the treatment of CML is to achieve CR and cure. Allogeneic SCT is curative but it has a high mortality rate and was rapidly replaced as the first choice therapy [10]. Nowadays, first line treatment is the specific pharmacological inhibitor of c-Abl kinase activity *imatinib mesylate* [52], which competitively binds the ATP-binding site of c-Abl [53]. This targeted therapy efficiently induces apoptosis in CML cells and suppresses their proliferation [52].

Imatinib has obtained extraordinary results in chronic phase patients, which have substantially increased survival rates, with a generally well-tolerated therapy [54]. However, it shows **no or poor**

response in a subset of ~10% Bcr-Abl$^+$ chronic phase patients, a 3% of which suffer progression to accelerated or blastic phases [10]. Furthermore, **treatment of accelerated phase and blast crisis is usually ineffective**, with only 21% patients achieving CR [10], and the majority of them relapsing during the first year of therapy [54]. Most patients who respond to imatinib relapse through a mechanism of reactivation of the Bcr-Abl tyrosine kinase activity, either by preventing imatinib to reach the target (i.e. through expression of drug efflux pumps or protein binding of imatinib) or by desensitizing the target (i.e. via Bcr-Abl mutations) [10, 54]. Indeed, at least 50% of patients who relapse have mutations in the Abl kinase domain [54, 55]. For this reason, second and third-generation Bcr-Abl inhibitors have been developed. *Nilotinib* or *dasatinib* are approved in the United States for patients with resistance or intolerance to imatinib and achieve a better response [54]. However, a number of patients always escape CR, mainly due to the appearance of clones with more than one mutation or with mutations in residue 315 of Abl, which result in a kinase that cannot be inhibited [54, 56]. Nilotinib has significant efficacy in patients in blastic phase, but **novel agents are needed to increase the limited long-term survival** of these patients [57]. Additionally, **residual resistant stem or progenitor cells** are an important reservoir for leukemia that needs to be eradicated for a successful treatment [58].

In conclusion, there is a **need of better therapies in CML for cases of relapse, slow responders, blast phase progression, late diagnosis and for patients who have intolerance** to imatinib and/or show a bad adherence to the treatment [59]. Moreover, residual CML stem cells express very low levels of Bcr-Abl [58], so it is **indispensable to acquire a better understanding of non-Bcr-Abl mechanisms of resistance in CML** to be able to develop effective protocols to definitively cure CML [60].

2.2. Myelodysplastic syndromes

MDS are a heterogenous group of clonal hematologic stem cell malignancies characterized by cytopenias associated with defective hematopoiesis, dysplasia of one or more of the major myeloid cell lines and generlly hypercellular BM, which lead to impaired cell proliferation and differentiation (Figure 2.1) [10, 61]. One distinctive feature of this group of hematologic malignancies is the presence of **increased apoptosis in BM**, mainly due to increased death receptor signaling [62].

MDS is a relatively common form of BM failure. It can be caused by environmental exposures to radiation and toxics and *secondary MDS (therapy-related, or tMDS)* may occur as late toxicity of cancer chemotherapy. Idiopathic MDS is age-related and usually appears at ages starting on 70 years [10]. **Incidence rates have increased over time**, due to the better knowledge of the disease, the

aging of the population and the rising number of patients who survive high-dose chemotherapy [10, 62].

The most important clinical feature of MDS is anemia, which is present in the majority of the patients, as well as general (unspecific) symptoms. There are **no single characteristic features of BM morphology that distinguish MDS**; BM can be normal, hypercellular and hypocellular [10, 62], complicating the diagnosis. **MDS naturally progresses to AML** with an especially higher frequency in tMDS cases, which have a poorer prognosis. Anyway, most patients die of complications of a pancytopenia instead of leukemic transformation [10, 61].

MDS are **particularly refractory to chemotherapy**, although the old age of patients is probably related to the low efficiency of treatments [10]. These are assigned based on the clinical classification of the disease. Due to the heterogeneity of MDS, several clinical classifications exist, such as the FAB or the WHO classifications [12]. Since most hematologic manifestations of MDS result from the **accumulation of multiple genetic lesions** like loss of tumor suppressor genes, activating oncogene mutations and other aberrations [10], one of the most used classifications is the *International Prognostic Scoring System* (IPSS), which was revised (IPSS-R) in 2012 [63]. IPSS-R classification is based on cell counts and cytopenias, as well as cytogenetic abnormalities and ranges of karyotypes [62].

Cytogenetic alterations are frequent and related to survival in MDS; their type and number has prognostic significance and striking effects in risk, strongly correlating with the probability of leukemic transformation [10, 62]. However, a current problem for the establishment of a good diagnosis and prognosis of MDS is that **cytogenetics are very heterogeneous** and none of the most frequent alterations are exclusively associated with MDS [64, 65]. Among them, **mutations in chromosome 17p (P53 gene) are rather frequent and related to a poor prognosis** [66]. Thus, there is a need to refine diagnosis and prognosis classifications of MDS to provide the patients with more specific treatments, taking especial care of molecular genetics [61, 65].

Cure of MDS can only be achieved with SCT but advanced age and low donor availability limit the eligibility for this therapy [64]. For this reason, **combination therapies are being currently studied**. Three drugs, the immunomodulator *lenalidomide* and the demethylating and differentiating agents *5-azacytidine* and *decitabine,* have been approved for the treatment of MDS [62]. Despite they improve the symptoms, treatment with these agents is **not curative** [61, 64]. In low-risk MDS, frontline therapies with 5-azacytidine and decitabine might be enhanced by the use of ion chelators,

cytokines or lenalidomide to improve blood counts [61, 62, 64]; however, elderly patients tend to develop treatment-related myelosupression and mortality [10, 64].

Progress in MDS research has been hampered by the **lack of established cell lines** from patients and the **difficulties in developing mouse engrafting models** [62]. Future approaches will need to overcome these impediments in order to achieve the breakthroughs that are necessary for the **establishment of good prognostic models and personalized therapies, which are vital** for a successful treatment of MDS (see Table 2.1).

Table 2.1. Current clinical needs in AML, CML and MDS.

Malignancy	Current needs
APL	• Dose reduction of chemotherapy or replacement with novel drugs. • Overcoming resistance in ~20% refractory patients.
CML	• Overcoming resistance to Bcr-Abl inhibitors (new combinatorial therapies).
MDS	• Discovery of new diagnosis/ prognosis markers. • Validation of specific pharmacological targets.

3. Innate Immunity and Inflammation

3.1. Innate Immunity

Innate immunity is the group of **first-line and unspecific protective responses** of the organism against microbial infections or tissue damage. For these functions, the Innate Immune System relies on the recognition of particular types of molecules by ***pattern recognition receptors*** **(PRRs)** localized in the so-called **antigen-presenting cells (APCs)**, which are "professional" immune cells from the granulocytic and monocytic myeloid lineages, and in other "non-professional" cells like fibroblasts, epithelial and endothelial cells. Molecules recognized by PRRs can be conserved exogenous structures present in pathogens and called ***pathogen-associated molecular patterns*** **(PAMPs),** or endogenous molecules released by damaged host cells and termed ***damage-associated molecular patterns*** **(DAMPs)** [67-69].

There are various types of PRRs. **Cell-associated PPRs** are *membrane proteins*, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), or *cytoplasmic proteins* such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the NOD-like receptors (NLRs) [68]. There are also **PRRs that are soluble in blood**, like the components of the complement system [4].

- *The complement system* consists of about twenty soluble interacting proteins that circulate in blood and extracellular fluids. The early components are proenzymes, which are sequentially activated by proteolytic cleavage and initiate an amplifying, proteolytic cascade [4]. Proteins of the complement are generally involved in the adaptive immune response, but some of them can be directly activated by molecules in the surface of pathogens and initiate the so-called alternative pathway of innate immunity.
- *The Toll-like receptor (TLR) family* is the most important type of cell-associated mammalian PRRs. They were termed "Toll-like" because of their similarity to the *Drosophila* protein Toll, which is a transmembrane protein with a large extracellular domain comprised of a series of leucine-rich repeats. TLRs also contain a cytosolic *Toll/interleukin(IL)-1R (TIR) homology* domain, through which they interact with adaptor proteins that participate in their downstream pathways. In mammals, **TLRs activate many intracellular signaling pathways and stimulate gene transcription, promoting inflammatory responses** and helping the induction of adaptive immune responses. Of note, intracellular TLRs should not be confused with NLRs, which also have leucine-rich repeats. NLRs are functionally similar to TLRs but recognize a different set of ligands [4, 68, 69].

With the exception of some NLRs, recognition of PAMPs or DAMPs by PRRs triggers two types of innate immune responses: *inflammatory responses* and *phagocytosis* by neutrophils, macrophages, and also by dendritic cells, which subsequently activate the adaptive immune response [4, 68].

3.2. Inflammation

Inflammation occurs through an increase in permeability of the vascular endothelium, which allows the extravasation of serum proteins and immune cells into the affected tissue. This process is mediated by the liberation of lipid signaling molecules, ***prostaglandins***, and protein (or peptide) signaling molecules, such as ***proinflammatory cytokines and chemokines***, which activate specific receptors. Some proinflammatory cytokines are tumor necrosis factor (TNF), IL-1 and IL-6 [4, 68].

The induction of an inflammatory response removes or sequesters the detrimental stimuli and ultimately **restores functionality and homeostasis** to the tissue, even functioning as a **healing process** in damaged tissue. However, if tissue damage or stress cannot be overcome, inflammation **may trigger cell death** by apoptosis or necrosis (reviewed in [70]). Therefore, depending on the type, intensity and duration of the insult that triggers inflammation, this process may lead to cell recovery and proliferation in order to replenish the damaged tissue, or to cell death.

In the context of cancer, **tumors can induce inflammation** through active secretion of pro-inflammatory cytokines that confer cancer cells renewal and survival abilities. Inflammation can also be induced by the cells surrounding the tumor as a mechanism of defense of the organism. The **tumor-promoting or anti-tumoral effects** of inflammation secondary to a tumor seem to depend on the expression of different immune mediators by tumor cells and on the abundance and activation state of the immune cells surrounding the tumor. On the other hand, pre-existing **inflammation can promote tumor initiation and progression** *per se*, promoting the acquisition of mutations and enhancing the proliferation of mutated cells through any of the following mechanisms [71]:

- Production of *reactive oxygen species (ROS) and reactive nitrogen species (RNS),* which are liberated to the extracellular space and may enter other cells; and *secretion of TNF-α,* which can directly stimulate ROS production in neighboring cells. ROS/RNS, in turn, can induce DNA damage and inactivate mismatch repair enzymes, favoring mutagenesis.
- Induction of *epigenetic changes*, like modifications in histone methylation/acetylation or DNA methylation, which may contribute to tumor initiation.
- Secretion of *growth factors and cytokines* that can stimulate stem cell expansion and confer tumor cells a stem cell-like phenotype.

- Activation of *transcription factors like NF-κB, AP-1 or STAT3*, which regulate the expression of genes that control cell survival, proliferation, growth, angiogenesis, invasiveness, mobility and cytokine or chemokine production.

3.3. TLR signaling pathways

There are ten different TLRs in humans (Table 3.1), all of which recognize different **microorganism ligands and self-components** including heat shock proteins, DNA/RNA from dying cells, fibronectin fragments, and fibrinogen released in response to stress, tissue damage and cell death. Most TLRs are localized **on cell surfaces**, being especially abundant in macrophages, dendritic cells, and neutrophils, as well as in epithelial cells from the respiratory and intestinal tracts. Other TLRs are associated with **intracellular membranes** from organelles such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes. Cytoplasmic membrane-bound TLRs usually recognize lipids, lipoproteins and proteins, while intracellular TLRs detect nucleic acids, generally from intracellular pathogens [68, 69, 72].

Table 3.1. Human TLRs and their ligands. Modified from *Takeuchi et al. 2010 [68]*.

TLR	Localization	Canonical ligand	Origin of ligand
TLR1	Plasma membrane	Triacyl lipoproteins	Bacteria
TLR2	Plasma membrane	Lipoproteins (dimerizes with TLR1 or TLR6)	Bacteria, viruses, parasites, self
TLR3	Endolysosomes	dsRNA	Viruses
TLR4	Plasma membrane	Lipopolysaccharide (LPS)	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoproteins	Bacteria, viruses
TLR7	Endolysosomes	ssRNA and small purine analogs	Bacteria, viruses, self
TLR8	Endolysosomes	ssRNA	Bacteria, viruses, self
TLR9	Endolysosomes	CpG-DNA	Bacteria, viruses, protozoa, self
TLR10	Plasma membrane	Unknown	Unknown

Ligand recognition by TLRs leads to transcriptional activation of a subset of genes that depends on the type of TLR and the adaptor molecules recruited, which transduce the signal and ultimately determine the pathway followed (reviewed in [68, 69, 73]). The two most-immediate TLR adaptors, which directly interact with the corresponding intracellular TIR domains of TLRs, are the ***myeloid differentiation primary response gene 88 (MyD88)*** and ***TIR domain-containing adaptor inducing***

IFN-β (TRIF). TLR-induced signaling pathways are divided in two types of responses, based on the type of mediator which associates with the corresponding receptor:

- *MyD88-dependent signaling pathway* is essential for the downstream signaling of all TLRs except for TLR3 and it mediates a **rapid and acute pro-inflammatory response**. After initial activation by the ligand-TLR complex, MyD88 triggers the activation cascade of various serine/threonine kinases from the IL-1-R associated kinase (IRAK) family, which then interact with TNFR-associated factors (TRAFs) and their downstream proteins. The MyD88 pathway ultimately results in the activation of transcription factors NF-κB and AP-1, which induce the expression of pro-inflammatory cytokines (Figure 3.1). The intracellular TLR7/8 and TLR9 additionally activate interferon-regulatory factor (IRF) 7, which activates the expression of type I interferon (IFN) (Figure 3.2).

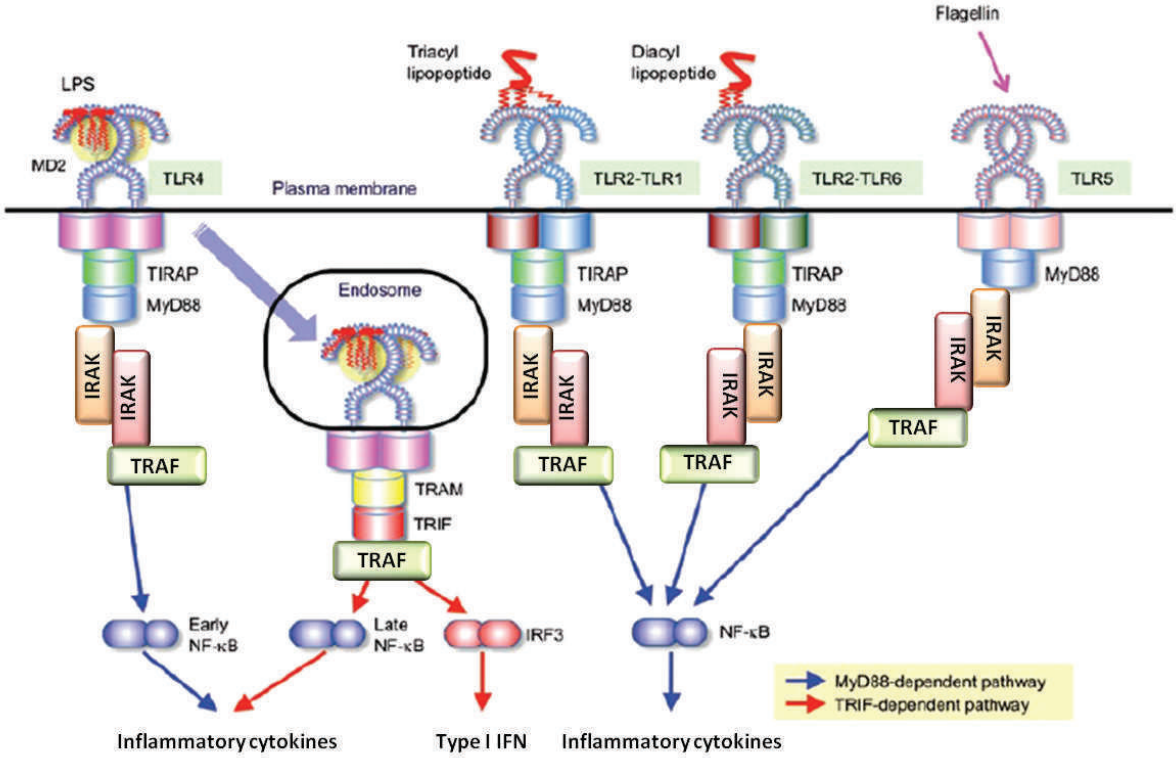

Figure 3.1. Signaling pathways activated by cell surface TLR. Modified from *Kawai and Akira (2010)* [69].

- *TRIF-dependent signaling pathway* can only be activated by TLR3 and TLR4 and triggers a **delayed pro-inflammatory response**. TLR4 is the only TLR which can activate both MyD88 and TRIF pathways, but it requires an additional adaptor for activating TRIF, named TRIF-related adaptor molecule (TRAM). When TRIF is activated, it associates with TRAFs, which results in the activation of other downstream proteins. Overall, TRIF pathway triggers NF-κB-mediated

activation of inflammatory cytokines and IRF3-dependent type I IFN expression (Figures 3.1 and 3.2), although it can also activate proteins from the death receptor apoptosis pathway (see Chapter 4 of the *Introduction* section).

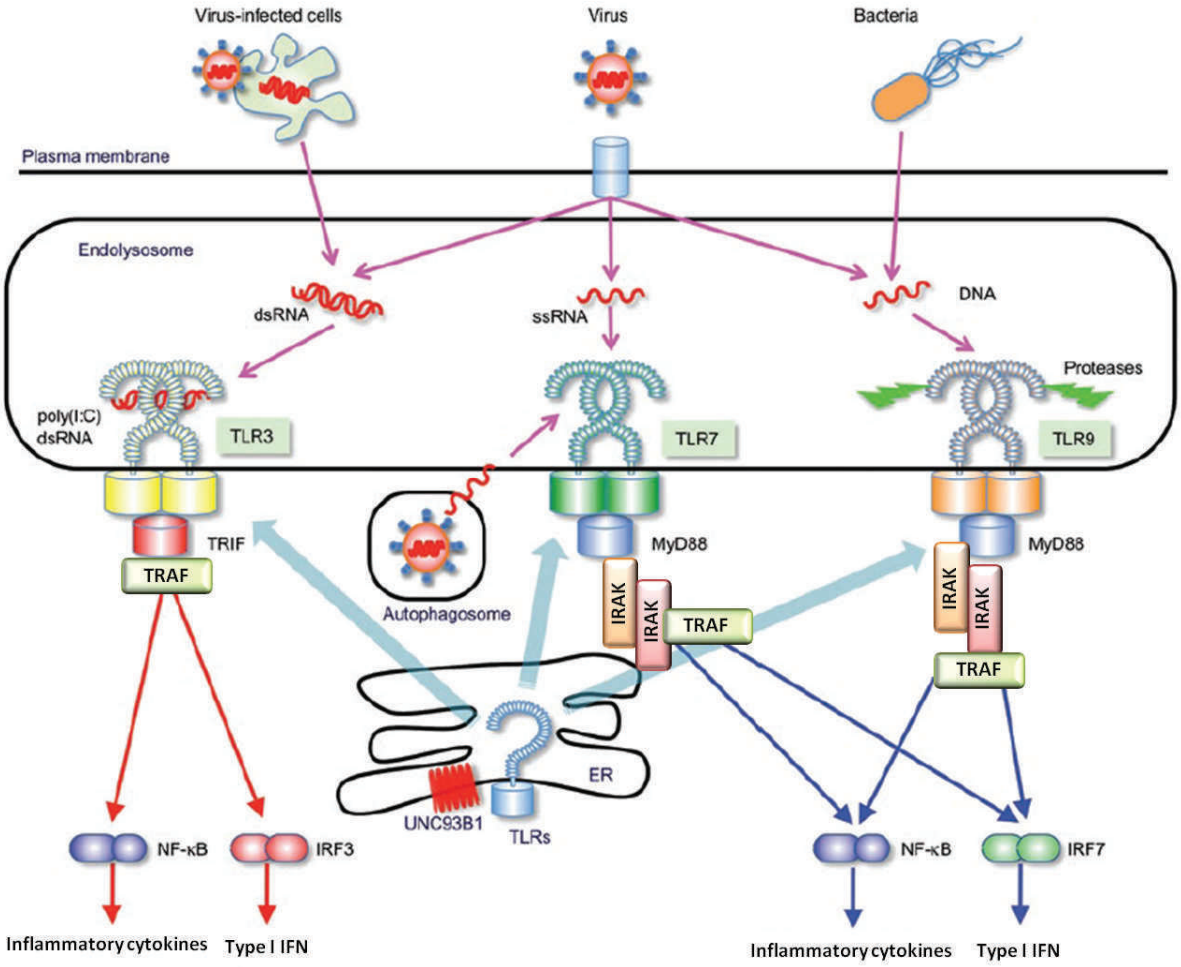

Figure 3.2. Signaling pathways activated by endogenous TLR. Modified from *Kawai and Akira (2010)* [69].

To sum up, activation of TLR signaling pathways leads to the activation of transcription factors, like **NF-κB, AP-1 and IRFs**, which cooperatively regulate the transcription of **IFNs**, **pro-inflammatory cytokines and chemokines** like tumor necrosis factor (TNF)-α, pro-IL-1β, IL-6, IL-12p40, G-CSF, M-CSF and IL-8. Moreover, in monocytes/macrophages, TLRs upregulate the expression of hundreds of other genes that might be involved in the **antimicrobial defense, metabolic changes, tissue repair and even differentiation** (referred to in Chapter 1 of the *Introduction* section) [68, 73, 74]. Interestingly, TLR signaling also induces the expression of microRNAs (reviewed in Chapter 6 of *Introduction*) like miR-146a/b, miR-147, miR-155, miR-181 and miR-21, which participate in the fine-tuning of the inflammatory response [68, 73].

As it was mentioned before, TLRs can recognize DAMPs, **endogenous host molecules,** besides PAMPs. Most of these ligands are produced upon **cell death and injury** or can even be **released by tumor cells**. They include degradation products of the extracellular matrix, heat-shock proteins (HSPs) and high-mobility group box 1 (HMGB1) proteins released from necrotic cells, which are all recognized by **TLR2 and TLR4**; as well as DNA and ribonucleoprotein complexes released by dying cells and recognized by **TLR7/8 and TLR9**. Therefore, these TLRs mediate inflammatory responses in non-infectious situations and may contribute to the pathogenesis of non-infectious inflammatory and auto-immune diseases, such as systemic lupus erythematosus or chronic polyarthritis [68, 69].

3.4. TLRs in hematopoiesis and hematologic malignancies

Because stimulation of TLRs triggers an immunostimulatory response, some TLR agonists have been approved for clinical use or are under investigation in open clinical trials for their use in several types of cancer, in which they help the host immune system to fight the tumor [75]. However, their hyperstimulation might be detrimental in some types of cancer which present with local inflammation, or in those which overexpress TLRs; thus, the theory that TLRs might contribute to tumorigenesis is gaining strength [76].

The presence of all types of TLRs in BM $CD34^+$ precursor cells has been extensively reported [77]. When some of those TLRs localized in hematopoietic precursors are activated by their ligands, both *in vivo* and *in vitro*, they initiate a transcriptional response that mediates **growth factor-independent differentiation towards the myeloid lineage**. Interestingly, participation in myeloid commitment has only been described for those TLRs which are **susceptible of being activated by endogenous molecules**, namely TLR4, the TLR2-TLR1 dimer and TLR7/8 [74, 78, 79]. It is therefore possible that the augmented expression of BM TLRs or their aberrant stimulation by endogenous ligands participate in the pathogenesis of hematologic diseases.

In agreement, abnormalities in the expression of TLRs appear to be related with different types of hematologic malignancies [73, 80-84]. Moreover, there is a growing body of evidence that **TLR signaling is especially important in the pathogenesis of MDS**. It has been hypothesized that the elevated levels of pro-inflammatory cytokines, like IL-1β and TNF-α, present in MDS BMs are a consequence of increased TLR signaling [82]. Indeed, **TLR2, TLR1, TLR4 and TLR9** have been reported to be overexpressed in BM cells of MDS patients. Moreover, expression of TLR2 and TLR9 is positively **correlated with TNF-α levels in MDS** [82, 83, 85]. Thus, it has been suggested that TLR signaling pathways are involved in the intramedullary apoptotic cell death that has been proposed as the

cause of MDS-associated cytopenias and which is thought to be mediated by inflammatory cytokines. For instance, TNF-α receptors are increased in MDS BM cells and correlated with the progression of the disease [84].

Overall, it seems that innate immunity responses initiated by receptors from the **TLR family in MDS BM progenitors, and mediated by transcription factor NF-κB, induce the production of pro-inflammatory cytokines like TNF-α**, which in turn **may have proapoptotic effects** on the surrounding cells. This hypothesis is in agreement with a report that shows that TLR2-TLR6 stimulation induces NF-κB-mediated apoptosis [86].

3.5. Transcription factor NF-κB

Nuclear factor (NF)-κB is the term used to define the proteins from the superfamily comprised of Rel and NF-κB proteins, which share a highly conserved DNA-binding and dimerization domain, the Rel homology domain. The **Rel subfamily proteins (c-Rel, RelA, RelB)** have C-terminal transactivation domains, and the members of the **NF-κB subfamily (p100 and p105 in humans)** contain long inhibitory C-terminal domains, which are eliminated by proteolysis or arrested translation, generating **shorter DNA-binding proteins (p52 and p50, respectively)** [87].

Proteins from the NF-κB family dimerize to become active transcription factors, forming **homodimers or heterodimers.** NF-κB is also, and more frequently, the name used to denominate the best-characterized dimer from this family, formed by proteins RelA (also called p65) and p50. NF-κB dimers selectively bind to **9-10 bp DNA sites (κB sites)**, which have rather variable sequences, to activate transcription of their target genes. This combinatorial diversity confers NF-κB transcription factors a **great potential to regulate different sets of genes** [87].

In homeostatic conditions, NF-κB dimers are present in the cytoplasm as inactive complexes, and need to be activated either by the degradation of an inhibitor or by cleavage of an inhibitory domain. The NF-κB pathway is activated as a mechanism of **adaptation to stress** in response to a variety of stimuli, such as inflammatory cytokines (like TNF-α), growth factors, pathogen antigens (activation of TLRs and other PRRs), physiologic stress, physical and chemical hazards, oxidative stress, DNA damage, drugs and apoptotic mediators [88, 89].

Two different NF-κB activation pathways have been exhaustively characterized so far, although there is evidence that other pathways involving different kinase activities exist. According to the ***classical or canonical pathway***, latent NF-κB dimers are bound to **inhibitory proteins from the IκB family** in

the cytoplasm, forming an inactive complex. There are different IκB proteins (IκBα, IκBβ, IκBγ or NF-κB essential modulator –NEMO- and IκBε), each of which has differential tissue-specific patterns, mechanisms of regulation and affinities for the NF-κB dimers. In this pathway, activation of NF-κB requires the phosphorylation-mediated degradation of the IκB protein. This phosphorylation is carried out by the so-called **IκB kinase (IKK)**, which is an enzyme complex comprised of a catalytic subunit (IKKα and/or IKKβ) and a sensing subunit (IKKγ or NEMO). As shown in Figure 3.3, upon an activating signal, IKK phosphorylates IκB and this protein is degraded by the proteasome, releasing the NF-κB dimer (most commonly, a p50-p65 dimer) and allowing it to translocate to the nucleus, where it binds κB sites and activates the expression of its target genes [87].

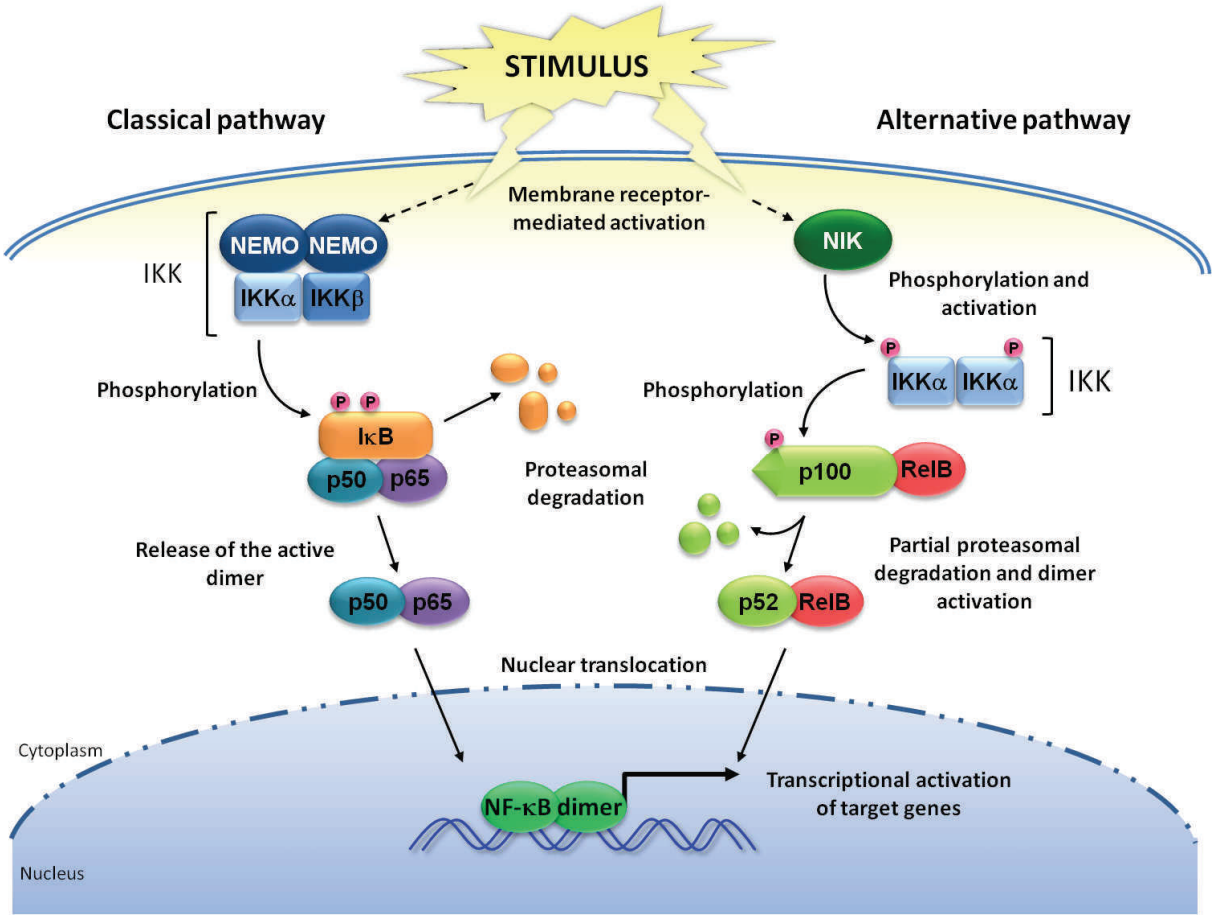

Figure 3.3. Canonical (classical) and non-canonical (alternative) NF-κB activation pathways. Discontinuous lines represent indirect processes.

In the ***alternative or non-canonical pathway***, IKK contains two IKKα subunits but no NEMO, which means that it requires an external activator. This activator is called **NF-κB-inducing kinase (NIK)**, which acts as a signal integrator and phosphorylates the IKK complex, after membrane receptor stimulation. In this pathway, NF-κB dimers are comprised of a large NF-κB protein (generally, p100)

and a Rel protein (RelB). The NF-κB protein exerts the inhibitory action and needs to be phosphorylated by an IKK complex to be cleaved into its active form, p52 in the case of p100. Finally, the activated dimer tranlsocates to the nucleus and transactivates its target genes (Figure 3.3) [87, 90].

In addition to the source of inhibition and the kinases involved, the classical and alternative pathways differ in the **types of receptor involved in the initiation of the signal**. In example, TLRs, TNF receptor (TNFR), B-cell receptor (BCR) or T-cell receptor (TCR) are generally thought to signal through the classical pathway responding to numerous stimuli and carrying out diverse functions; while those receptors activating the alternative pathway are a subset of receptors from the TNFR family, such as B-cell-activating factor belonging to TNF family receptor (BAFFR), the B-cell surface antigen CD40, lymphotoxin β-receptor (LTβR) or receptor activator for NF-κB (RANK), which trigger responses with more specific functions [90]. Of note, **intracellular stimuli** like the accumulation of ROS or DNA damage can also activate NF-kB through *signaling loops,* which are considered as a third activation pathway [89].

As mentioned above, there is a great **variability of gene expression patterns** activated by NF-κB, which mainly depend on the initiation stimulus and the activation pathway followed (that is, the type of NF-κB dimer that is formed), but also on the association of those dimers with other transcription factors [91, 92]. NF-κB regulates the expression of a number of **components of the immune system**, such as inflammatory cytokines and enzymes (TNF, IL-1, IL-6, COX-2, iNOS), chemokines (IL-8), adhesion molecules (ICAM-1, VCAM-1, ELAM-1) and proteins regulating both innate and acquired immune responses; **anti-apoptotic proteins** (Bcl-XL, XIAP, cIAP1/2, A20) **and pro-survival and proliferation factors** (cyclin D1, c-Myc, metalloproteinases, telomerase, etc.) [92-94].

3.6. NF-κB in hematopoiesis and hematologic malignancies

Provided the type of gene sets that are transcriptionally regulated by NF-κB, it is obvious that this transcription factor is involved in **inflammation and initiation or modulation of the immune response** (reviewed in [95]). Indeed, some members of the NF-κB protein family like c-Rel, RelB and p52 are predominantly expressed in blood cells and most receptors activating the alternative pathway are specifically expressed in lymphocytes [90, 96]. Moreover, there is multiple evidence that the **NF-κB pathway participates in myeloid and lymphoid differentiation** from early progenitors and also at different levels of maturation of the granulocytic/monocytic, erythroid and B/T-cell lineages, and is involved in B-cell activation. Interestingly, NF-κB has been implicated in both the

differentiation process and the survival of these cells, which is important for their functionality in peripheral blood [95, 96].

Accordingly with its functions in differentiation, NF-κB appears to play an important **role in hematologic malignancies** [94, 97-99]. Furthermore, important **pro-oncogenic functions** have been reported for this factor as a result of its inflammatory, anti-apoptotic and pro-survival effects. For instance, the connection between NF-κB, chronic inflammation and cancer is well established [88, 100, 101]. Furthermore, a number of oncogenes, such as Bcr-Abl, Ras and ErbB2, have been demonstrated to induce constitutive NF-κB activation [96, 101]. In agreement with this pro-oncogenic nature, constitutive or elevated NF-κB activity has been found in AML, MDS, acute lymphoblastic leukemia, Hodgkin lymphomas, B-cell lymphomas, CML and multiple myeloma [94, 98, 101-103]. Importantly, in MDS, NF-κB activity has been reported to be correlated with the progression of the disease [104]. On the other hand, NF-κB activity is significantly elevated in MDS BM progenitors and cell lines, and its blockade results in apoptosis, suggesting that constitutive NF-κB signaling in MDS provides cells with a **survival advantage** (reviewed in [73]). It is therefore clear that NF-κB plays a role in the pathogenesis of MDS, but its effects are still a matter of discussion.

For all the aforementioned, **NF-κB inhibition has become a frequent therapeutic approach** in anti-inflammatory and antitumor treatments, mainly through IKK inhibition [92, 94, 99, 101]. This is a double-hit strategy, since IKK has been demonstrated to have NF-κB-independent tumorigenic functions [105].

However, a growing body of evidence that **members of the NF-κB family can participate in tumor suppression** needs to be taken into account. These functions of NF-κB are not as well-understood as its tumor promoter functions, but it seems that they are connected with the **transcriptional regulation and recruitment of certain NF-κB subunits by tumor suppressor proteins** like p53, $p16^{INK4A}$ or the inhibitor of growth family member 4 (ING4). They have also been related to the **induction of apoptosis by RelA** through a p50-independent transcriptional induction or inhibition of proapoptotic and antiapoptotic proteins, respectively. In most cases, these pro-apoptotic effects of NF-κB occur in response to atypical inducers [92]. Nevertheless, the complexity of this pathway claims for caution when interpreting the role that NF-κB plays in each particular malignancy.

4. Cell Death

4.1. Concept of cell death

Just like every other living organism, cells have a limited lifespan that ends through a process of *death*. In multicellular organisms, this process is required for a correct **development and maintenance of tissue homeostasis**. Cell death may also occur as a natural response to irreversible or massive damage caused by chemical compounds, physical insults or other biological systems, all of which can be termed ***cytotoxic stimuli*** due to their cell-killing properties.

The *Nomenclature Committee on Cell Death* defines a dead cell as a cell in which any of the following molecular or morphological conditions is met [106]:

a) The cell has **lost the integrity of its plasma membrane.**
b) The cell has undergone **complete cytosolic and nuclear fragmentation** into discrete bodies.
c) The cell or its fragments have been **engulfed by another cell.**

4.2. Types of cell death

Cell death can occur through various biochemically different pathways and with morphologically distinct events, according to which four typical modes of cell death have been defined: necrosis, apoptosis, autophagy and cornification [106].

4.2.1. Necrosis

Necrosis, also named *type III cell death* [107], was initially defined as a "violent" form of cell death which results from the rapid disruption of homeostasis [108]. Thus, it has been traditionally considered as an **accidental or uncontrolled** type of cell death, although there is increasing evidence that this might in fact be a **regulated** process. Despite the causative elements of necrosis being unclear, it has been shown that stimulation of **death-domain receptors and TLRs** can trigger this process through a pathway that depends on the excessive activation of the cytotoxic **kinase RIPK1**. To distinguish between accidental and programmed forms of necrosis, some authors have suggested the term "*necroptosis*" to name this more regulated type of necrosis [106, 109].

Morphologically, necrosis is characterized by a **gain in cell volume** (*oncosis*), **organelle (mitochondria, ER) swelling**, **rupture of plasma membrane** and the subsequent loss of the intracellular content. The lack of consensus has lead to the negative definition of necrosis as the absence of apoptotic or autophagic markers. Although there is not a common biochemical change

that characterizes necrosis, this process is generally accompanied by mitochondrial alterations, lysosomal abnormalities, lipid degradation and increases in the cytosolic levels of calcium, which result in mitochondrial overload and activation of proteases such as calpains and cathepsins (Figure 4.1), while the nucleus becomes distended and remains largely intact [109, 110]. Because cells ultimately release their content to the intracellular space, **necrosis is typically followed by inflammatory reactions**, although this may also occur under certain apoptotic conditions [111].

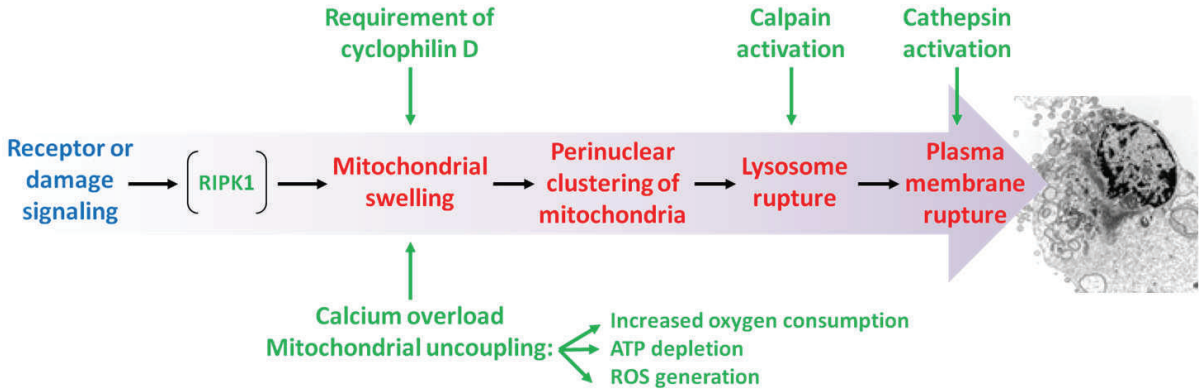

Figure 4.1. Tentative scheme of events occurring during necrotic cell death. Modified from *Golstein et al. (2007)* [109] and *Nikoletopoulou et al. (2013)* [110]. Molecular events are represented in green; morphological events, in red and signaling events, in blue.

4.2.2. Apoptosis

The term *apoptosis*, which in Greek is used to describe the falling off of petals from flowers, or leaves from trees, was proposed in 1972 by Kerr et al. [108] to name a distinct mode of cellular death which was observed to appear spontaneously or in response to physiological stimuli and occur in an **active an controlled manner**. It is therefore, a ***programmed cell death (PCD)***; however, apoptosis and PCD are not synonyms, since other types of PCD different to apoptosis have been described [106]. Those will be referred to below.

Apoptosis, also called *type I cell death*, can be identified by the coexistence of several among the following morphological features: **rounding-up** of the cell, with retraction of pseudopodes and detachment from the neighbouring cells, **reduction of cellular volume** (*pyknosis*) owing to the condensation of the cytoplasm, **chromatin condensation, nuclear fragmentation** (*karyorrhesis*), **little or no structural modifications of cytoplasmic organelles, plasma membrane blebbing** (although integrity is maintained until the final stages of the process) **and engulfment by resident phagocytes**. Apoptosis has been called a “clean death” because it usually occurs in a non-inmunogenic fashion, although some stimuli may lead to the exposure or the secretion of proteins that can induce antigen presentation by phagocytic cells and stimulation of an immune response (Figure 4.2) [106].

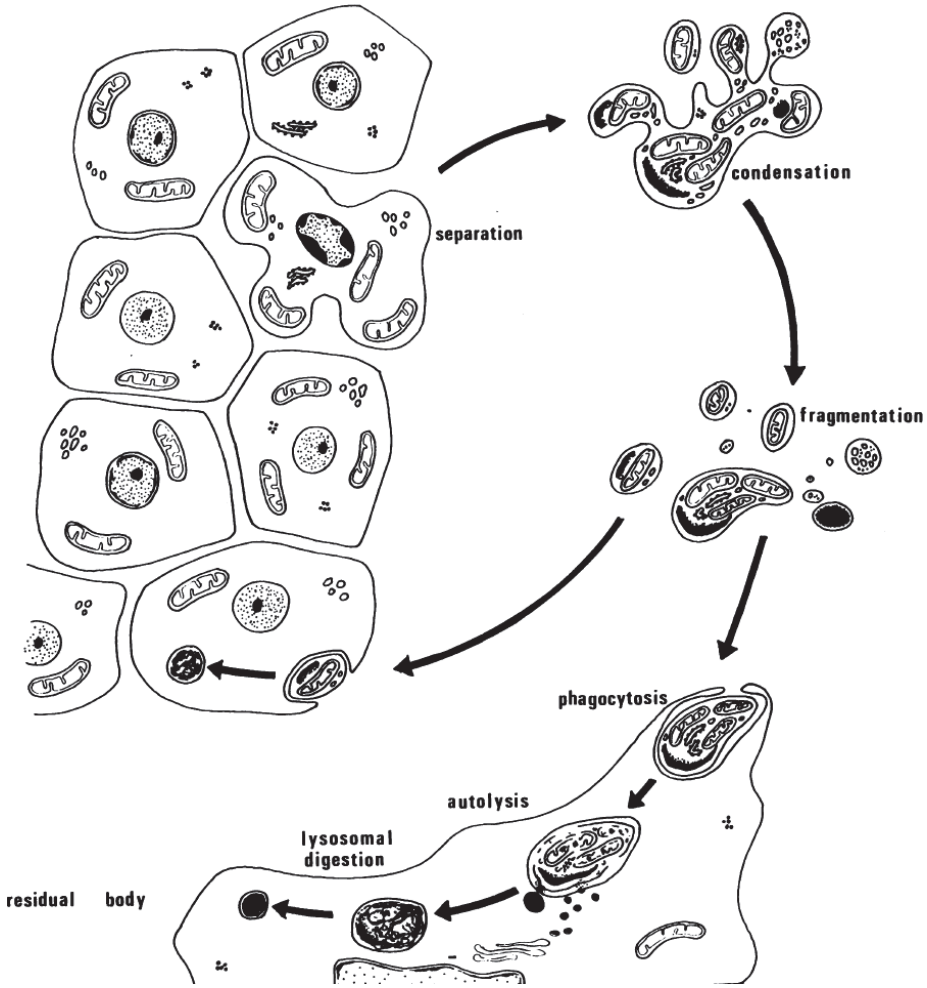

Figure 4.2. Morphological features of apoptosis in a parenchymal cell, as illustrated by *Kerr et al. (1972)*[108].

In regard of the signalling pathways that regulate the process, there are distinct subtypes of apoptosis, of which biochemical features will be further detailed in this section.

4.2.3. Autophagic cell death

Autophagy or *macroautophagy* is a process through which **cytoplasmic material**, such as degenerating organelles, of one cell is **sequestered within two-membraned vesicles** called ***autophagosomes* or *autophagic vesicular organelles* (AVO).** After the engulfment of the cytoplasmic content, autophagosomes fuse with lysosomes and generate *autolysosomes* or *autophagolysosomes*, in which both the autophagosome inner membrane and its content are **degraded by lysosomal enzymes**. Biochemical characteristics of autophagy will be further described in this section.

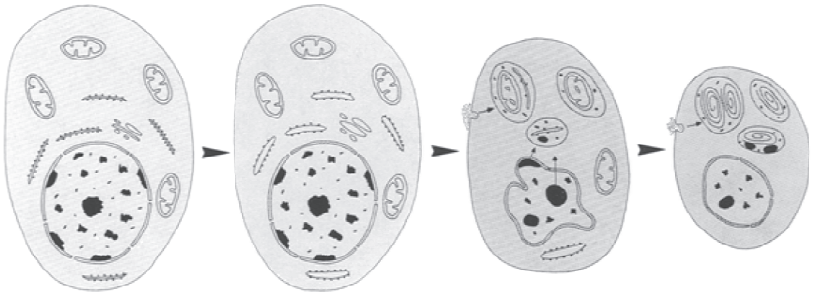

Figure 4.3. Schema of autophagic cell death, as illustrated by *Clarke et al. (1990)* [107].

Paradoxically, autophagy is a process that in many occasions has been shown to **promote cell survival** and allow cells to recover after the withdrawal of the death stimulus. However, in specific contexts, autophagy may result in the destruction of cells. Thus, *autophagic cell death,* also termed *type II cell death* [107], is the type of PCD that is accompanied by massive autophagy. In contrast to apoptosis (or *type I*), autophagic cell death occurs in the **absence of chromatin condensation and interaction with surrounding phagocytes** [106].

4.2.4. Cornification

Cornification or *keratinization* is a cell-type specific form of **PCD which occurs exclusively in the keratinocytes** of epidermis. This process is morphologically and biochemically different from apoptosis and autophagy and follows a **specific terminal differentiation** mechanism, during which cells express enzymes and synthesize lipids which are necessary for the function of the epidermal barrier. This is distinct from other terminal differentiation programs because it ultimately leads to the formation of dead cells or *corneocytes*, which form the *cornified skin layer* [106, 112].

4.2.5. Atypical cell death modalities

The following types of cell death are diffusely used in the bibliography and present with features that overlap with one or more of the major types of cell death. For these reasons, the use of these terms should be limited or avoided, when possible [106].

- Anoikis: subtype of apoptosis induced by the loss of attachment to the substrate or to other cells [113].
- Paraptosis: is a PCD which is irresponsive to caspase inhibitors or anti-apoptotic proteins and is frequently triggered by the insulin-like growth factor receptor 1 through a signalling pathway involving the activation of mitogen-activated protein kinases (MAPKs) [114].

- Pyroptosis: occurs after infection with certain microorganisms that activate distinct routes towards caspase-1, but not caspase-3, activation (*caspases* are defined below). This conducts to the release of the pyrogens IL-1β and of IL-18, which induce local and systemic inflammatory reactions [115].
- Pyronecrosis: is a caspase-1-independent type of pyroptosis [116].
- Mitotic catastrophe: cell death that occurs during or shortly after failed cell division and that can be accompanied by *micronucleation* (uneven distribution of chromosomes and their fragments between daughter cells) and *multinucleation* (acquisition of more than one nuclei by one daughter cell) [117].
- Entosis or "cellular cannibalism": is a type of death reported in lymphocytes of patients with Huntington's disease and in some types of cancer cells, in which an apparently healthy cell is engulfed by one of its neighbours and dies inside its phagosome through lysosomal degradation [118].
- Wallerian degeneration: alternative form of cellular catabolism that takes place in the Nervous System and involves the degeneration of a part of a neuron without affecting the cell body [119].
- Excitotoxicity: is a term used in neurons which die after mitochondrial membrane permeabilization induced by electrolyte deregulation and activation of lethal signalling pathways that follow the overstimulation of the cell with excitatory aminoacids [120].

4.3. Biochemical and molecular features of apoptosis

A common feature of apoptosis in vertebrates is that this process is specifically regulated by effector proteins from the family of ***caspases***. These are **intracellular cysteinyl-aspartate proteases** that are present in cells as *zymogens* (inactive forms) called ***procaspases***, which require proteolytic processing for activation. Mammals have 14 different caspases, which can be classified in two groups based on their function: *inflammatory caspases* (like caspase-1) and *apoptotic caspases,* which can in turn be ***initiator caspases*** (like caspase-2, -8, -9 and -10) and ***effector caspases*** (like caspase-3, -6 and -7), depending on their structural levels (Figure 4.4). Procaspases contain a highly homologous *protease domain*, which can be further divided into a large and a small subunit. They also contain *amino-terminal pro-domains* of variable length which mediate homophilic interactions with caspase adaptors and play an important role in caspase activation [121, 122].

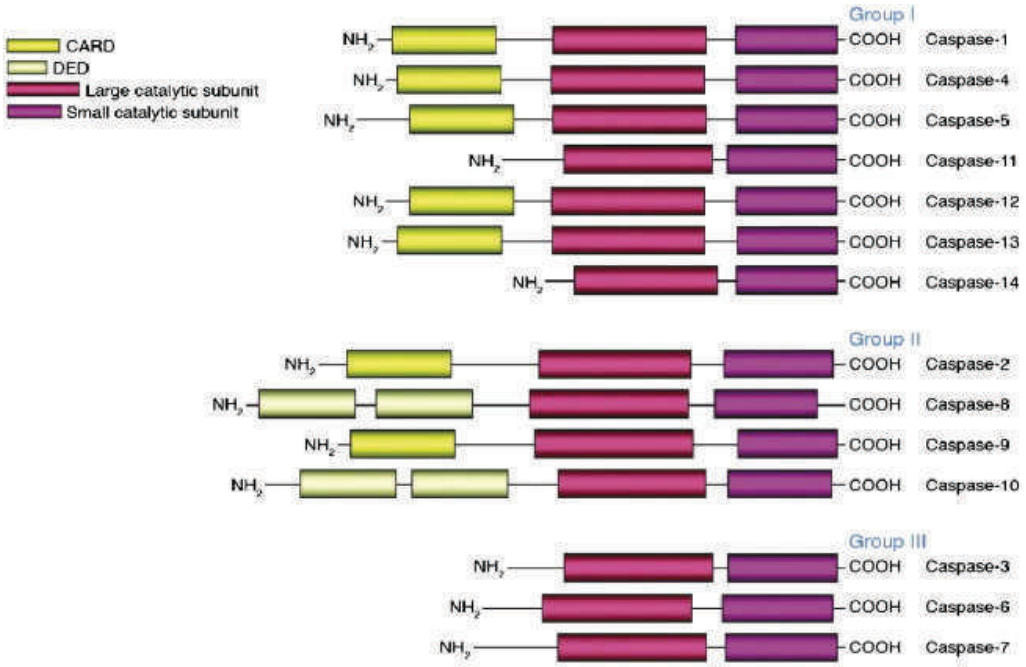

Figure 4.4. Basic structure of mammalian caspases. Modified from *Rupinder et al. (2007)* [122]. All caspases contain a conserved long pro-domain which is responsible for their adaptor-driven activation, and a small catalytic subunit which contains the cysteine-protease activity. CARD: caspase activation and recruitment domain; DED: death effector domains. Group I: Inflammatory caspases; Group II: Initiator caspases; Group III: Effector caspases.

Procaspases can be activated by three different mechanisms: by proteolytic cleavage by a previously-activated upstream caspase, by induced proximity of several molecules of procaspase recruited by adaptor proteins, and by association with a regulatory subunit or cofactor. Generally, initiator caspases are activated by autoproteolysis via oligomerization with regulatory proteins (although caspase-8 can be activated by induced proximity), and in turn they cleave and activate downstream effector caspases, resulting in a rapid amplification of total caspase activity. Active effector **caspases eventually trigger the proteolytic cleavage of a great variety of key components of cellular structure**, such as nuclear lamins or cytoskeletal proteins, and activate other factors, such as DNases and kinases, causing multiple cellular damage that ultimately lead to cell death [122, 123].

Because caspases are critical for many events in apoptosis and for the acquisition of the apoptotic morphology, they are considered as the **central executioners of apoptosis** [123]. However, it is important to consider that **caspase-independent apoptosis** may occur owing to the mediation of other proteins that can kill cells after their mitochondrial release, such as *apoptosis-inducing factor (AIF)* or *endonuclease G (endo-G)* [1, 106].

There are two pathways through which initiator and effector caspases can be activated: the *extrinsic* or *death-receptor* pathway, and the *intrinsic* or *mitochondrial pathway*.

4.3.1. The extrinsic or death receptor pathway

The *death receptor pathway* is so-called because it is activated by **ligand-binding to "death" receptors located on the cell membrane**. Death receptors are cell surface receptors from the *tumor necrosis factor (TNF) gene superfamily* and contain a *"death domain" (DD)* in their intracellular region which recruits downstream apoptotic proteins. There are four characterized death receptors: **CD95/Fas** or Apo1, **TNFR-1** (but not TNFR-2), **DR3/TRAMP** and DR4, DR5 or **TRAIL-R2**/killer [122]. A list of their specific ligands and adaptor proteins is summarized in Table 4.1.

Table 4.1. Death receptors and their associated proteins.

Death receptor	Ligand	Adaptors	Pro-apoptotic modulators	Anti-apoptotic modulators
TNFR-1	TNF-α	TRADD, FADD	RAIDD (RIP-associated ICH/CED-3 homologous death domain)	RIP (Receptor-interactive protein) TRAF2 (TNFR-associated factor 2) FLIP (Flice-inhibitory protein)
CD95/Fas	FasL	FADD		
DR3/TRAMP	TRAIL	FADD		
DR4/DR5/TRAIL-R2	TRAIL	TRADD, FADD		

Briefly, when specific ligands bind to death receptors, these undergo a process of **trimerization and clustering of their intracellular DD.** This allows the binding of the cytosolic adaptor protein ***FADD*** *(Fas-associated death domain)*, which can in turn be mediated (only in the case of TNFR-1 and TRAIL-R2) by the previous binding of the adaptor ***TRADD*** *(TNFR-associated death domain)*. FADD then recruits an **initiator caspase** (generally, procaspase-8 or -10) through a homologous death effector domain (DED), and they form the *death-inducing signal complex* ***(DISC)***. Formation of DISC allows **the cleavage and complete activation of the initiator caspases**, which are released from the DISC and activate **the downstream effector caspase-3, -6 and -7**. These, especially caspase-3, are responsible for the cleavage of a series of death substrates that results in the acquisition of the morphological and biochemical features of apoptosis by the dying cell (Figure 4.6) [122, 124, 125].

4.3.2. The intrinsic or mitochondrial pathway

The mitochondrial pathway is activated by several **intracellular and extracellular types of stress** such as growth factor deprivation, DNA damage, oncogene induction, hypoxia and cytotoxic drugs, among others. Signals originated by these stimuli through different mechanisms eventually converge in the **mitochondria**, which acts as a **signal integrator** [126].

The mitochondrial pathway begins with the activation of the pro-apoptotic members of the **Bcl-2 family**, which comprises several proteins which can be functionally classified into three groups: *Group I* proteins are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1-BH4) and a hydrophobic C-terminal tail which allows them to localize to the outer surface of the mitochondria. These are all **anti-apoptotic proteins, such as Bcl-2 and Bcl-XL**. In contrast, *Group II* consists of **pro-apoptotic or pro-survival proteins, like Bax and Bak,** which are very similar in structure and localization to their anti-apoptotic counterparts but lack the BH4 domain (Figure 4.5). Last, *Group III* proteins is comprised by a large number of proteins which only share the BH3 domain, such as **Bid, Bim, Bad or Bik.** These are called ***BH3-only proteins***, and their function is pro-apoptotic but they need proteins from Group II to trigger apoptosis. BH3-only proteins play a regulatory role upstream from the two other groups, having high affinity for the anti-apoptotic members of the family; some of them, such as Bim, Bid or Puma, bind to any of the pro-survival proteins, but others exert more specific functions. As an example, Bad only binds to Bcl-2, Bcl-XL and Bcl-w, but not to Mcl-1 or A1, whereas Noxa only binds to Mcl-1 and A1 but not to the others [123, 127-129].

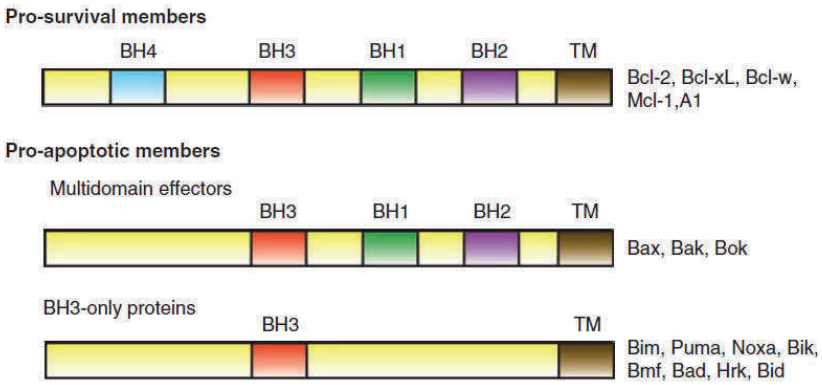

Figure 4. 5. Mammalian Bcl-2 family members. From *Giam et al. (2009)* [129]. TM: trans-membrane domain. It might not be present in some of the BH3-only proteins.

The pro-apoptotic members **Bax and Bak are the main effectors of the mitochondrial pathway**. Whereas Bak is an integral membrane protein, Bax is mainly cytosolic because its C-terminal domain is hidden into a hydrophobic pocket when the protein is inactive. Activation of Bax induces a conformational change that allows its translocation to the mitochondria, where it undergoes oligomerization, just as well as activated Bak [130, 131].

Two models have been proposed for Bax and Bak activation (reviewed in [129]). The *direct activation model* postulates that, after a stimulus, “sensitizer” BH3-only proteins bind to anti-apoptotic proteins and allow the activation of a second group of BH3-only proteins, “activators”, which directly interact

with Bax and Bak and induce their oligomerization in the mitochondria outer membrane. The *indirect activation model* is based on the observation that Bcl-2 can interact with Bax [132]; it proposes that Bax and Bak are directly repressed by the anti-apoptotic proteins, and that the BH3-only proteins displace this association and favour the release of Bax or Bak, without the need for an interaction between these and the BH3-only proteins.

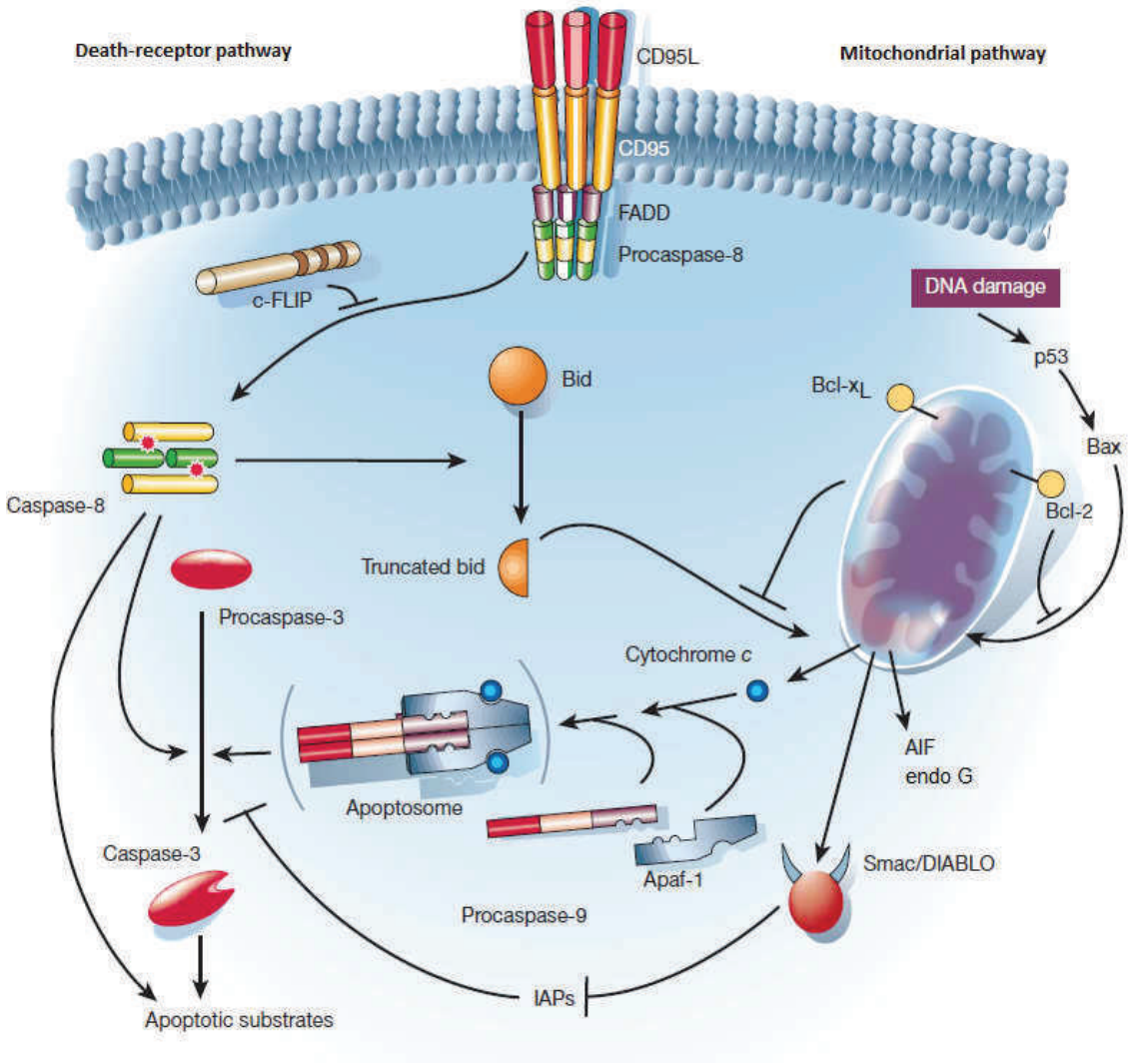

Figure 4.6. Major apoptotic pathways. Modified from *Hengartner et al. (2000)* [123].

Because neither of the models explains all the experimental observations, it is likely that the reality is a combination of both of them. Either way, the result is that active Bax or Bak **homo-oligomerize in the outer membrane of the mitochondria** [133, 134]. It is not yet clear if Bax and Bak form channels by themselves or if they recruit other proteins for this purpose, but the result is the opening of pores that induce ***mitochondria outer-membrane permeabilization (MOMP)*** and allow the **release of apoptogenic proteins** into the cytoplasm. MOMP is considered as the **no-return point** of apoptosis,

since it is known to be accompanied by the fragmentation of the mitochondrial network, mitochondrial fission events and changes in the morphology of mitochondrial *cristae* (*cristae* junction opening). These processes disrupt the functionality of the mitochondria and, more importantly, allow and enhance the **release of cytochrome c to the cytoplasm** [126, 129, 135].

Cytochrome c is required for the formation of the **apoptosome** in the cytosol, which is comprised of cytochrome c, the *apoptotic protease-activating factor 1 (Apaf-1)* and procaspase-9. The recruitment of **procaspase-9** leads to its **activation**, which will trigger the subsequent activation of effector caspases [122, 126]. At this point, apoptosis is modulated by the presence of a series of cytosolic proteins called ***inhibitors-of-apoptosis (IAP)***, which are thought to act as buffers that avoid spontaneous caspase activation. Human IAPs are **NAIP, XIAP, c-IAP1, CIAP-2 and survivin**. They prevent feedback mechanisms involved in the activation of caspase-8, and XIAP, c-IAP1 and CIAP-2 are even capable of inhibiting effector caspases and cytochrome c-mediated caspase-9 activation [122, 123].

However, cytochrome c is not the only apoptogenic molecule that escapes from mitochondria after MOMP. Other released proteins include **AIF, endo G,** the *second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI* ***(Smac/DIABLO),*** and several procaspases (Figure 4.6). AIF and endo G translocate to the nucleus, where they seem to carry out functions of DNA fragmentation and chromatin condensation in a nuclease-independent and –dependent manner, respectively. Smac/DIABLO, in turn, antagonizes IAP-mediated caspase inhibition [123, 126].

4.4. Biochemical and molecular features of autophagy

Autophagy is induced by a variety of stress stimuli, such as nutrient deprivation, *ER* stress, PAMPs, DAMPs, hypoxia, redox stress and mitochondrial damage. It is initiated with the **formation of the double-layered membrane**, called *phagophore*, which will give rise to autophagosomes. This membrane can be generated from different sources, such as the ER, the outer mitochondria membrane or the plasma membrane [136].

The process begins with the stress-mediated activation of the *autophagy-related gene 1 (Atg1)/unc-51-like kinase (ULK)* protein complex. Under basal conditions, Atg1/ULK complexes reside in the cytoplasm in association with other protein complex containing *the mammalian target-of-rapamycin* ***(mTOR).*** This is a serine-threonine kinase that phosphorylates Atg1/ULK and other Atgs like Atg13, keeping them inactive and blocking their association. Inhibition of mTOR induces its dissociation from the Atg1/ULK/Atg13 complex and the subsequent dephosphorylation and activation of ULK and other

proteins from the complex (Figure 4.7; step 1). This activation might also occur in an mTOR-independent manner, through interactions with the *AMP-dependent protein kinase* ***(AMPK)***. The Atg1/ULK complex starts the recruitment of proteins to the phagophore and facilitates the activation of the next protein complex that comes into play, the *beclin-1 (Bcn-1)/ class III phosphatidylinositol 3-kinase (PI3K)* complex or ***Bcn-1 core complex***. This multiprotein complex generates phosphatidylinositol-3-phosphate, which recruits effectors that will mediate the initial stages of **vesicle nucleation** (Figure 4.7; step 2). Bcn-1 is also recruited to the phagophore by the *vacuole membrane protein 1 (VMP1)* and, along with the Atg1/ULK complex, induces cycling of the transmembrane protein Atg9 between subcellular compartments for the **recruitment of lipids** that will form the phagophore [136, 137].

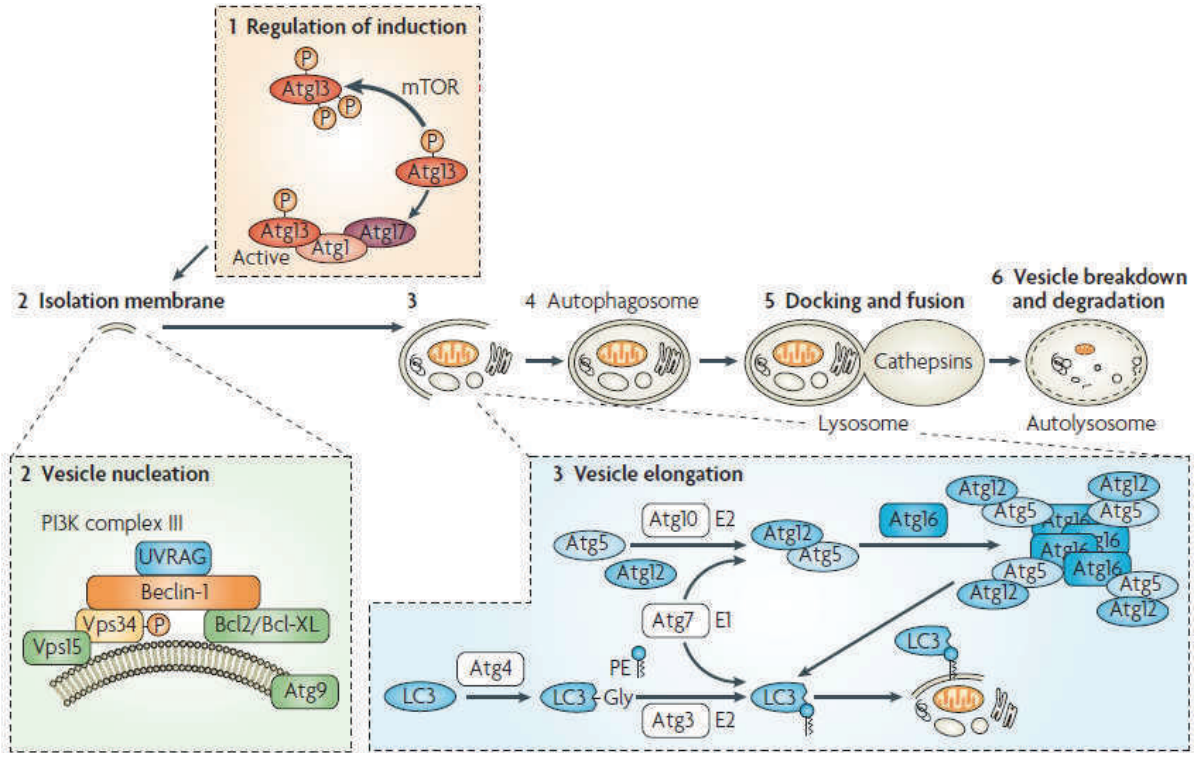

Figure 4.7. Autophagosome formation in mammals. Modified from *Maiuri et al. (2007)* [137].

As a central mediator of the autophagosome formation [138], there are numerous proteins that interact with Bcn-1 and induce or inhibit autophagy. For instance, anti-apoptotic members of the Bcl-2 family such as **Bcl-2, Bcl-XL and Mcl-1 directly inhibit Bcn-1** through their BH3 domain and therefore block autophagy [136].

Once the phagophore is formed, it needs to be elongated (Figure 4.7; step 3). **Vesicle elongation** is mediated by the conversion of the soluble form of *light-chain protein-3 (LC3)*, the mammal analogue of yeast Atg8, to the autophagic vesicle-associated form **(LC3-I to LC3-II)** [139]. Two ubiquitin-like

protein conjugation systems carry out this process. The first pathway is initiated by the covalent conjugation of Atg12 to Atg5 and the non-covalent association of Atg16 to form the multimeric complex Atg12-Atg5-Atg16, which functions as an LC3-E3 ligase. This pathway is regulated by cytosolic calcium concentrations, since Atg5 levels depend on its calcium-dependent cleavage by calpains. The second pathway involves the conjugation of phosphatidyletanolamine to LC3 by the sequential action of various Atg proteins. This lipid conjugation of LC3-I leads to its conversion to LC3-II, which stably associates with the autophagosome membrane. Conversion of LC3-I to LC3-II is another critical step for autophagosome formation and, therefore, it is **tightly regulated** by stress signals [136].

When the autophagosome is fully mature (Figure 4.7; step 4), it is transported through the cytoplasm by structural proteins called *dyneins* and finally fuses with lysosomes to create autophagolysosomes, in which the inner membrane as well as the luminal content of the autophagic vacuoles is enzymatically degraded (Figure 4.7; steps 5 and 6) [136, 137].

4.5. Implication of apoptosis and autophagy in cancer

Evasion of cell death is a hallmark of cancer. Transformed cells that escape apoptosis mechanisms proliferate or survive inappropriately, which favours tumorigenesis and tumour progression. Moreover, the great majority of current cancer treatment approaches rely on the stimulation of cell death programs, and deficiencies in the pathways that lead to apoptosis are associated with resistance to anti-cancer therapies [140]. **Impaired apoptosis in human cancers** is usually a consequence of mutational loss-of-function or deletion of apoptosis regulators, such as Bax; increased expression of pro-survival molecules, such as IAPs; or a result of the deregulation of one or more signalling pathways leading to apoptosis. Additionally, the presence of chromosomal translocations that enhance the expression of genes that block apoptosis or lead to the expression of fusion proteins that cause cell death resistance, such as PML-RARα and Bcr-Abl, is a characteristic of leukemias and lymphomas. Proper regulation of programmed cell death is especially relevant in **hematologic malignancies** because the hematopoietic system is a cellular compartment with high intrinsic cell proliferation and turnover rates and therefore depends on a **tight balance between proliferation and cell death** [140, 141].

On the other hand, **autophagy malfunction has been exhaustively reported in cancer** and there is evidence that signalling pathways that regulate autophagy are closely related to oncogenic signalling. Because several oncogenes hyperactivated in cancer inhibit autophagy and various tumour

suppressor genes, which are commonly mutated or epigenetically silenced, stimulate it, autophagy has been **traditionally considered as a tumour suppressor mechanism**. Moreover, autophagic cell death appears to occur with high frequency in apoptosis-deficient cells and in the presence of caspase inhibitors, suggesting that this type of PCD is some sort of last resort to protect the organism [142-144]. Paradoxically, this non-apoptotic cell death has been reported to be dependent on the anti-apoptotic members of the Bcl-2 family, which were previously mentioned to be inhibitors of Bcn-1, in Bax or Bak-null cells [145]. This apparently contradictory role may in fact reflect the function of autophagic cell death as the "last chance" in conditions in which apoptosis is not possible. According to these data, **apoptosis and autophagy complement each other** in the elimination of damaged cells. Furthermore, this complementary functionality seems to work in both directions, since it is known that calpain-mediated Atg5 cleavage, which inhibits autophagosome maturation, generates a pro-apoptotic fragment that functions in the intrinsic pathway of apoptosis, which suggests that failure to adapt to stress conditions leads to cell death by apoptosis [137, 143]. In agreement, autophagy inhibition has been shown to sensitize cells to stress-induced apoptosis [146].

However, there is a growing body of evidence that **autophagy and apoptosis may not always be complementary to each other** but have opposing effects owing to the protective functions of autophagy. Bulk protein and organelle degradation induced by autophagy can lead to recycling of cellular components through catabolism of macromolecules and metabolic substrates and therefore lead to **adaptation of cell metabolism** to starvation or stress. As an example, the cell cycle inhibitor p27 (see next section) has been shown to inhibit apoptosis in response to starvation and allow them to recover through autophagy. Moreover, autophagy can **specifically target damaged or overabundant organelles**, like mitochondria in *mitophagy* or ER in *reticulophagy* and prevent cell death [137]. For instance, protection from caspase-independent cell death by autophagy has been reported and is thought to occur through protection of cells from mitochondrial damage [147]. In agreement with these reports, there is accumulated evidence that **autophagy may lead to pro-survival effects** [137, 148]. In the context of cancer, these effects might be **potentially tumorigenic**. Moreover, there is a great number of reports showing that the cytoprotective role of autophagy might render cancer cells **resistant to chemotherapy** [143, 144, 149].

To sum up, **balance between autophagy and apoptosis is critical for cell fate** in many physiological processes and also in diseases like cancer. Both pathways share common inducers but also cross-inhibitory reactions that may cause polarization between them. The predominance of one over the other might be determined by the type of stress stimulus and by the cellular context.

INTRODUCTION

5. Cell Cycle

As it was commented in Chapter 1, cells proliferate through an orderly sequence of events that ultimately leads to the production of two daughter cells. This process is called **cell cycle**, and is essential for the continuity of life. The two fundamental steps of cell cycle are the **duplication** of the parental chromosomes and the segregation or **division** into two new cells. These two steps are basically similar in eukaryotic cells, which have developed a complex regulatory system based on a series of *switches or* ***checkpoints*** that ensure the fidelity of DNA duplication and cell division [150, 151]. Regulation of cell cycle is vital for the normal development of multicellular organisms, for stem cell function, and for tissue renewal. For this reason, the malfunctioning of the control systems of cell cycle frequently leads to disease [152-154].

5.1. Phases of cell cycle

The eukaryotic cell cycle is divided into four consecutive phases (Figure 5.1) [1, 150, 155]:

- ***G_1 phase****:* replicating somatic cells start preparing for division by synthesizing RNA and proteins, duplicating their organelles and increasing in size. During this phase cells increase their metabolic requirements and undergo a process called *metabolic reprogramming*, during which they change their metabolic pathways to allow a more efficient synthesis of macromolecules [156].
- ***S (synthesis) phase****:* in this phase, cells undergo DNA replication to duplicate their genomes.
- ***G_2 phase****:* cells duplicate their centrosomes and prepare for division or mitosis.

  These three phases together comprise the *interphase*, so-called because it is the period between the end of one mitosis and the beginning of the next.
- ***M (mitosis) phase****:* cells divide into two daughter cells. This is a complex process which, in turn, can be divided in various steps (Figure 5.2):

  - *Prophase*: involves chromosome condensation into symmetric structures formed by two sister chromatides linked by a centromere. In this step, the nuclear membrane retracts into the endoplasmic ER and Golgi and breaks down into vesicles, and structural proteins of the cell rearrange into a structure of microtubules radiating from two poles, called mitotic spindle.
  - *Metaphase:* condensed chromosomes associate with microtubules coming from opposite spindle poles through their centromere and arrange in the centre of the cell.
  - *Anaphase:* sister chromatides separate and are segregated to opposite spindle poles.
  - *Telophase:* the mitotic spindle disappears, chromosomes decondense and the nuclear envelope is formed again.

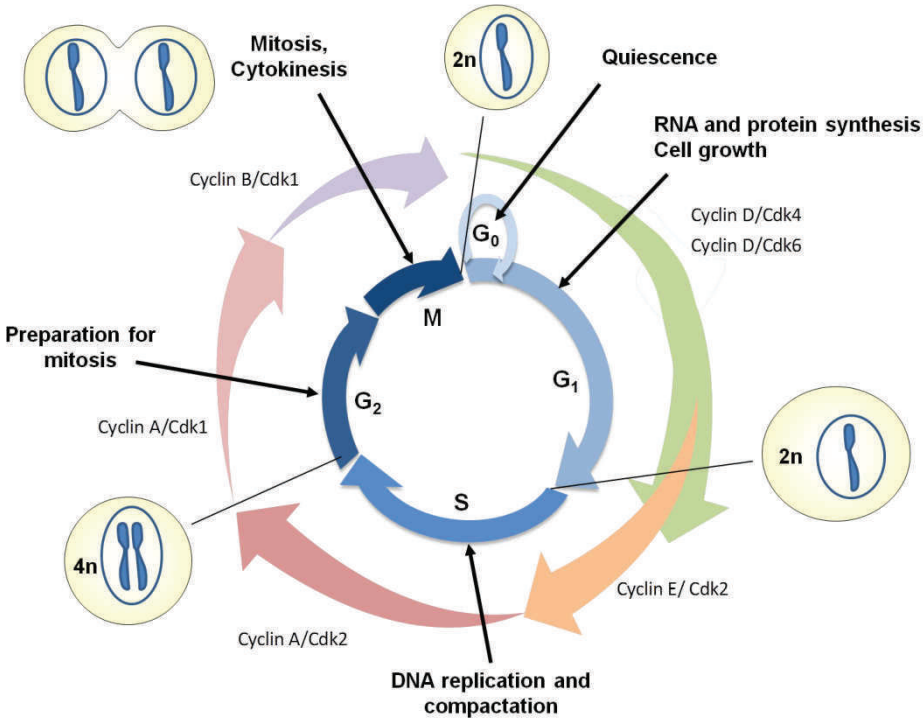

Figure 5. 1. Cell cycle phases and their regulation. The inner circles (blue arrows) represent cell cycle phases. Black arrows point the main events occurring on each phase. Colored arrows (other than blue) represent changes in cell cycle regulating complexes throughout the progression of the cycle. Schematic representations of the DNA content and morphological changes of the cell are distributed around the diagram.

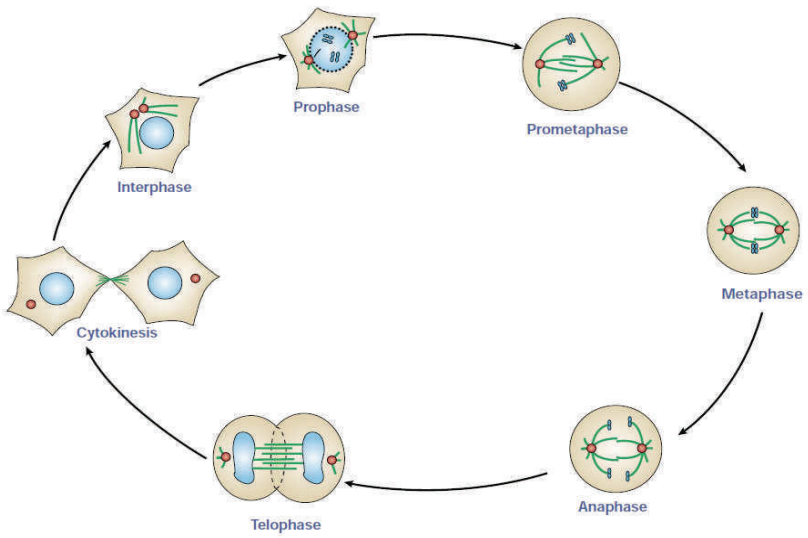

Figure 5.2. Stages of mitosis. Modified from *Nigg et al. (2001)* [155]. Prometaphase is a transition phase between prophase and metaphase, distinguished by some authors.

The M phase culminates with *cytokinesis*, which is the division of the cytoplasm into two daughter cells. These cells can re-enter cell cycle to continue replicating or enter a resting or quiescent state called G_0 phase [1, 150, 155].

Quiescence or G_0 is the common state of most somatic cells. It is a non-proliferative state; however, it is functionally different from growth arrest. Cells enter quiescence in response to an initiating signal that triggers the expression of a quiescence transcriptional program, which varies among cell types and tissues. Importantly, quiescence is a reversible state and cells can re-enter cell cycle upon specific stimulation by external signals called *mitogens* [152, 157].

5.2. Regulation of cell cycle

As it was mentioned before, malfunctioning of cell cycle frequently leads to the development of disease. A clear example is cancer, which can be favoured by uncontrolled division or by the production of genetically aberrant cells during DNA replication [158, 159]. Moreover, death and survival pathways and cell cycle are connected by common regulatory mechanisms, showing the relevance of the coordinated regulation of these processes in cell fate [160]. For these reasons, cell cycle progression is tightly modulated in physiological conditions.

There are three steps of the cell cycle that are determinant for its progression, called **cell cycle checkpoints.** These checkpoints are located at the mid-end of the G_1 and G_2 phases and in the middle of the M phase, and control the adequacy of the conditions in the cell before allowing it to continue to the following phase [1, 150]. Cell cycle checkpoints are basally blocked and need a positive signal to be overcome. In eukaryotes, this positive signal is triggered by the activation of heterodimeric serine/threonine protein kinase complexes that are comprised of two subunits: the catalytic subunit, or ***cyclin-dependent kinase*** **(Cdk),** and the regulatory subunit called ***cyclin,*** which confers Cdk its substrate-specific activity. In vertebrates, there are four main cyclin-Cdk complexes (Table 5.1) [151, 155, 161]:

Table 5. 1. Vertebrate cyclin-Cdk complexes

Complex name	Cyclin	Cyclin-dependent kinase
G_1-Cdk	Cyclin D	Cdk4/Cdk6
G_1/S-Cdk	Cyclin E	Cdk2
S-Cdk	Cyclin A	Cdk2/Cdk1
M-Cdk	Cyclin B	Cdk1

Each complex has a specific substrate and plays a specific role along the phases of the cell cycle. Their activity throughout cell cycle (Figure 5.1) is regulated by different mechanisms [150, 155, 161]:

- *Start of a new cycle (G_1 entry)*

  After the end of one cycle, there is temporarily no cyclin-Cdk activity. At this point, the DNA pre-replication complex (origin recognition complexes, helicases and initiator factors) starts re-assembling at the origin of replication. After re-assembly, a mitogenic signal triggers a gene expression cascade that allows the synthesis of cyclins and the formation of the G_1-Cdk and G_1/S-Cdk complexes to enter in a new cycle. Otherwise, the cell will stay in G_0, maintaining its size and DNA content [162].

- *Start of DNA replication (G_1 → S transition)*

  When G_1-Cdk and G_1/S-Cdk complexes, which are active throughout the G_1 phase, reach an activity threshold, they activate the synthesis of the S phase cyclin, and S-Cdk complexes start accumulating. The entry in the S phase is regulated by the **G_1 checkpoint** through the activity of ***Cdk inhibitors (CDKIs)*** [163], which block the activity of S-Cdk complexes to prevent cells from entering another cycle after certain conditions, such as completed differentiation or *DNA damage*. If the conditions are optimal, the CDKIs are phosphorylated by the G_1-cdk and G_1/S-cdk complexes and degraded by the proteasome, allowing the activation of the S-Cdk complex. Then, the active G_1/S-Cdk and S-Cdk complexes phosphorylate the pre-replication complex bound to the origin of replication and initiate DNA synthesis. The formation of other replication complexes during this phase is tightly regulated to protect cells from synthesizing more than one copy of the genome. First, proteins that form the pre-replication complexes can only be phosphorylated once, and they are not dephosphorylated until the end of the cycle; second, the timely and sequential activation of G_1-, G_1/S- and S-Cdk complexes ensures that the prevailing kinase activity after the initiation of replication, S-Cdk activity, specifically phosphorylates proteins that inhibit the re-assembly of the pre-replication complexes at the origin of replication [161, 164]. It is also essential for the progression to S phase that the G_1-Cdk, G_1/S-Cdk and S-Cdk complexes sequentially phosphorylate and inactivate the tumor suppressor protein *Rb* and its family members, which results in the release of the E2F family of transcription factors and in the subsequent transactivation of genes involved in DNA replication [162].

- *Initiation of mitosis (G_2 → M transition)*

  After DNA replication in S phase and before entering mitosis, cells must overcome the **G_2 checkpoint**. This is a *DNA-structure checkpoint* that delays or blocks mitosis in the presence of *mis- or unreplicated DNA or DNA damage* through the modulation of the activity of Cdk1. If no

errors occur during replication of chromosomal DNA and duplication of the centromeres, inactive M-Cdk complexes that accumulate during G_2 [165] are activated by a series of phosphorylation/ dephosphorylation reactions, in many of which Cdk1 is involved. The kinase activity of the M-Cdk complex then mediates the events that take place in prophase and metaphase [155].

- *End of mitosis (exit of M phase)*

  The transition to anaphase is controlled by the **M checkpoint,** which is a spindle assembly checkpoint that prevents anaphase if the chromosomes are not properly attached and aligned to the mitotic spindle. The regulation of this checkpoint relies predominantly on ubiquitin-dependent proteolytic events that trigger the transition between the sub-phases of mitosis [166]. The M-Cdk complex, which is activated during the G_2-M transition, regulates the phosphorylation, accumulation and activation of a ubiquitin-ligase complex called anaphase-promoting complex/cyclosome (APC/C). APC/C associates in two different types of complexes that are sequentially activated and carry out specific functions throughout mitosis. The ubiquitination of a series of mitotic proteins by APC/C and their subsequent proteasomal degradation triggers the progression of cell cycle through anaphase and telophase. After the chromatides are correctly segregated, the cell can exit mitosis through the APC/C-mediated proteasomal degradation of the M-Cdk complex [155].

- *Cytokinesis*

  This event is not considered as a cell cycle phase; however, it is also regulated by kinase activity. Namely, cytokinesis is thought to be regulated by two families of kinases, *Polo* and *Aurora,* which also mediate some of the events occurring during mitosis [155].

5.3. DNA-damage checkpoint regulators

The G_1 and G_2 checkpoints are both DNA-structure and DNA-damage checkpoints that block progression through the cell cycle until the damage is repaired to prevent the transmission of mutations or unrepaired chromosomal rearrangements to daughter cells. These are abnormalities that can contribute to the onset of cancer [159]. For this reason, proteins encoded by several *tumor-suppressor genes* normally participate in the DNA-damage checkpoints. Herein, we will focus on the roles in cell cycle regulation and cancer pathogenesis of the **tumor suppressor protein p53** and the **CDKI p21$^{CIP1/WAF1}$.**

5.3.1. Tumour suppressor gene P53

Protein p53 is a **highly-conserved transcription factor** which is considered as a tumor suppressor due to its ability to integrate signals from pathways activated by diverse harmful stimuli such as *DNA damage, hypoxia, oxidative stress and oncogene activation*. These stimuli lead to p53 activation, which triggers a series of transcriptional responses that may result in **cell cycle arrest, metabolism, senescence, differentiation, DNA repair and apoptosis** (Figure 5.3) [167, 168]. Interestingly, p53 can also function as a transcriptional repressor for some sets of genes (reviewed in [168]).

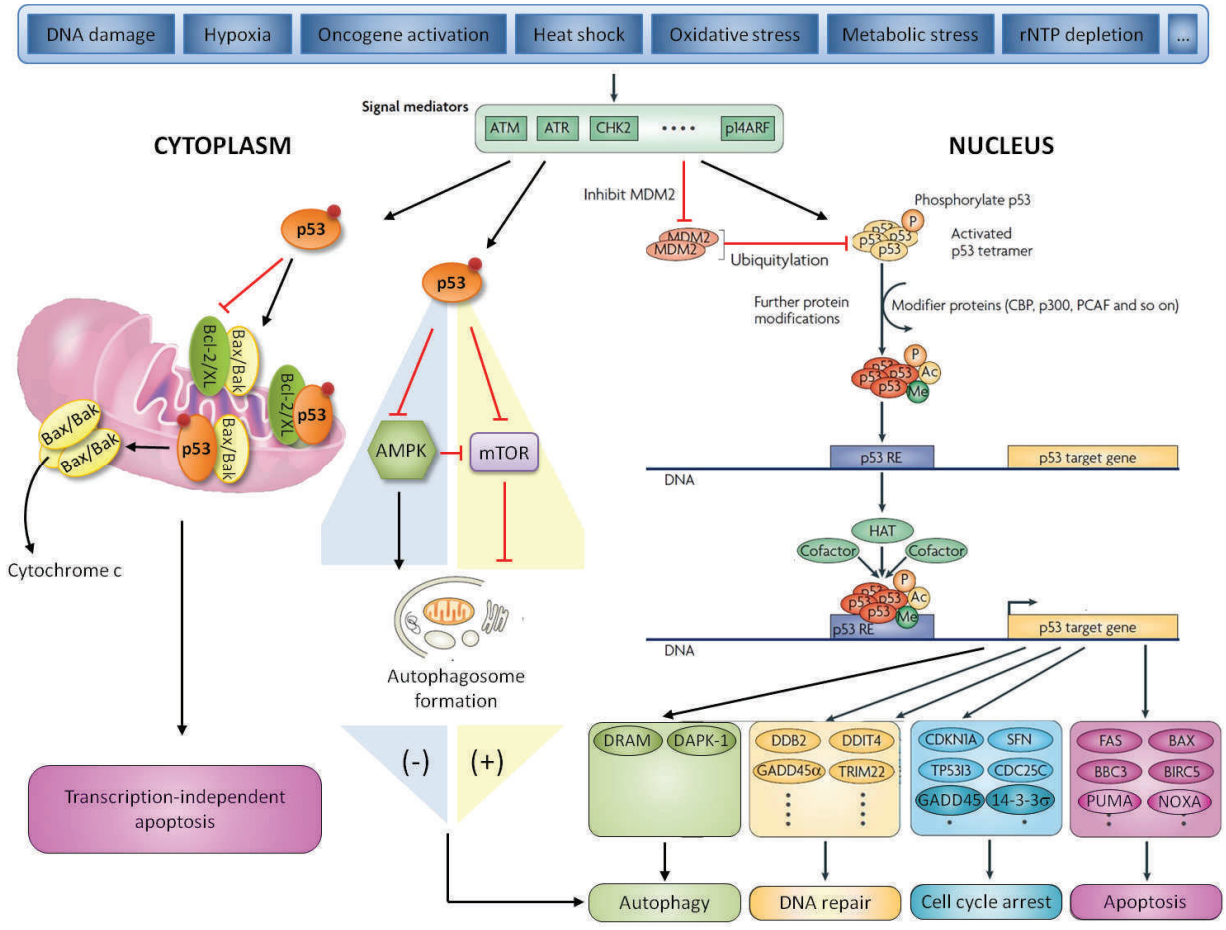

Figure 5.3. Nuclear and cytoplasmic functions of p53. Modified from *Riley et al. (2008)* [168], *Holley et al. (2009)* [169] and *Maiuri et al. (2007)* [137]. Under cellular stress, signal mediator proteins activate p53 through the inhibition of the ubiquitination mediated by Mdm2 and/or phosphorylation of certain residues. Both processes increase the half-life of p53 and activate it. **From left to right:** In the cytoplasm, p53 can localize to the outer mitochondrial membrane and act as a BH3-only protein, inhibiting Bcl-2/Bcl-XL/Mcl-1 and activating Bax/Bak, which leads to the formation of the Bax/Bak pore, release of cytochrome c and MOMP. These processes eventually lead to transcription-independent apoptosis. Cytosolic p53 may also play a role in autophagy through the inhibition of AMPK and subsequent inhibition of autophagy, or through the inhibition of mTOR, which induces autophagy. The balance between activation and inhibition is probably determined by cell context (proliferative state and induction of other p53-dependent mechanisms). Nuclear functions of p53 are triggered by the tetramerization and postranslational modifications of this transcription factor. Activated p53 binds to response elements localized in the promoter of its target genes and activates various transcriptional programs that may lead to the induction of apoptosis, cell cycle arrest, DNA repair or autophagy.

In basal conditions, when active p53 is not required, this protein is expressed at low but constant levels to allow a rapid activation upon a cellular stress stimulus. The main regulator of p53 is the E3 ubiquitin-ligase **Mdm2** [170], which binds to nuclear p53 and induces its ubiquitination, nuclear export and subsequent **proteasomal degradation** in the cytoplasm. Mdm2 does not only trigger p53 degradation in the cytoplasm, but can also polyubiquitinate nuclear p53 and induce its degradation in the nucleus. Thus, under physiologic conditions, Mdm2 keeps p53 basally repressed by limiting its half-life. Moreover, the association with Mdm2 induces a conformational change in p53 that may contribute to the inactivation of its transcriptional functions (reviewed in [171]). This is part of a negative feedback loop in which p53 induces the transcription of Mdm2 to self-regulate [170].

As a DNA-damage checkpoint regulator, p53 controls G_1-S and G_2-M transitions (Figure 5.4) [172]. Under genotoxic stress, p53 is phosphorylated at its Mdm2-binding site by cell cycle kinases and Mdm2 is ubiquitinated and degraded by the proteasome [173], allowing the **stabilization of the p53 protein.** Mdm2 activity can also be regulated by other **post-translational modifications** such as phosphorylation, acetylation or sumoylation, as well as by **proteins that bind to Mdm2 or p53** and modulate their association. After dissociation from Mdm2, cytosolic p53 is in turn activated by post-translational modifications and translocates to the nucleus [171]. There, p53 forms **tetramers ("dimers of dimers")** [174] that bind to DNA and activate the transcription of genes involved in DNA repair or cell cycle arrest (Figure 5.3) [168], such as the mitotic regulator 14-3-3σ [175] and the CDKI $p21^{CIP1/WAF1}$ [176], which will be referred to below in this section.

On the other hand, under critical circumstances such as extensive or irreversible DNA damage, p53 also activates the **expression of genes that lead to programmed cell death**, like Bax [177], BH3-only Bcl-2 family members [178, 179] or components of the death receptor pathways [180, 181]. Moreover, there is a cytoplasmic pool of p53 which localizes to the mitochondria [182, 183] and is able to induce cell death-independent transcriptional functions (Figure 5.3) [184]. When cytoplasmic p53 is activated by phosphorylation in serine 15 (Ser^{15}) [185] after a death signal, it rapidly translocates to **mitochondria** and induces **outer membrane permeabilization**, triggering the release of Bcl-2 family proteins and the subsequent apoptosis. It has been postulated that p53 functions as a BH3-only protein, binding to Bcl-2, Bcl-XL and Mcl-1 and de-repressing Bax and Bak by displacing their interactions. Moreover, p53 also appears to directly interact and activate Bax/Bak [186-189]. Although this direct induction of apoptosis is **independent of its transcriptional activity**, it seems that the transcriptional activation of certain genes contributes in some extent to the cytoplasmic activity of p53, resulting in a synergistic proapoptotic effect [184].

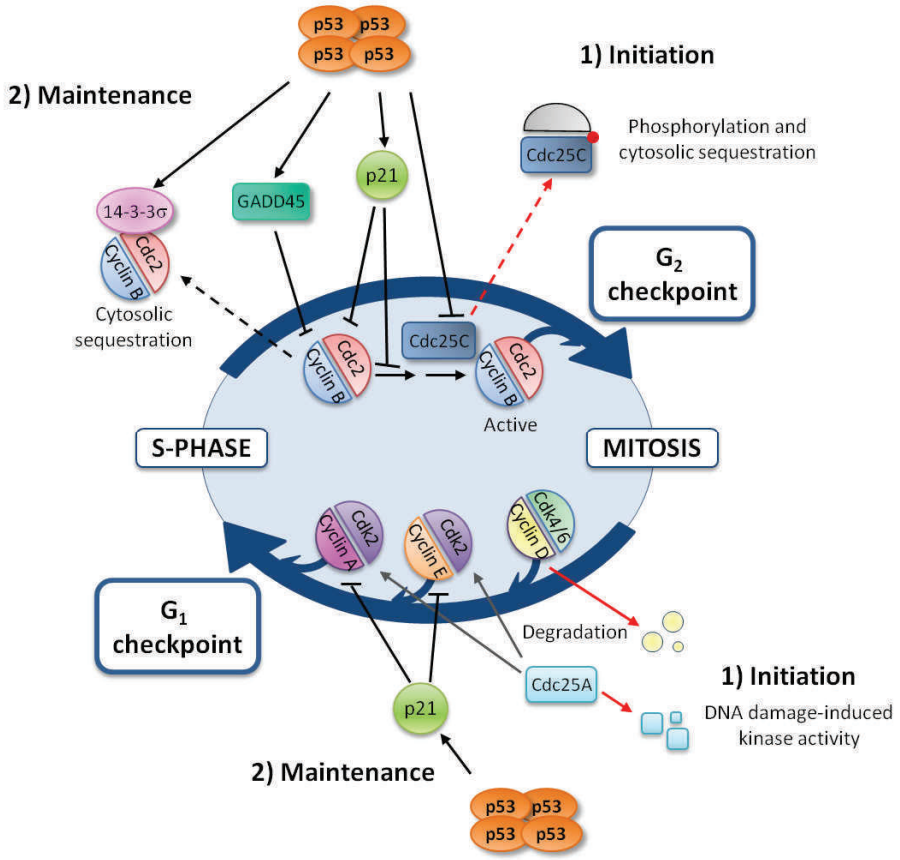

Figure 5.4. Cell cycle control by p53. Modified from *Giono et al. (2006)* [172]. Growth arrest at the G_1 checkpoint is initiated by the destruction of cyclin D and the Cdk2 activator Cdc25A by ATM/Chk2 kinases (not in the figure). p53 maintains cell cycle arrest by inducing the expression of the CDKI $p21^{CIP1/WAF1}$, which blocks progression through the inhibition of the Cdk2 complexes (G_1/S-Cdk and S-Cdk). The G_2/M arrest is initiated by ATM/ATR/Chk1/Chk2 kinases (not in the figure) through the phosphorylation and cytoplasmic sequestration of the M-Cdk complex activator Cdc25C. p53 participates in the maintenance of G_2 arrest through the transactivation of p21, which inhibits the Cyclin B-Cdc2 complex and its activating kinase; GADD45, which promotes dissociation of the complex; and 14-3-3σ, which sequesters the complex in the cytoplasm. In addition, p53 can also repress the expression of Cdc25C.

In addition to induction of apoptosis, DNA damage has been reported to induce **p53-dependent autophagy** through the transactivation of the pro-autophagic gene DRAM and the inhibition of the negative regulator mTOR [137, 190]. This p53-induced autophagy may contribute to cell death or be a defense against it [190]. On the other hand, cytosolic p53 has been related to **transcription-independent autophagy inhibition** [191-193], which appears to be mediated by the inhibition of the positive autophagy regulator AMPK (Figure 5.3) [192]. It is not known what determines if p53 induces or inhibits autophagy, although it seems that **autophagy inhibition depends on the proliferative state** of the cell [191]. Whether these functions occur coordinately with apoptosis induction or if they are regulated by different stimuli is still unclear [184].

Because of its role in protecting the cell from diverse types of cellular stress, p53 is an important tumor suppressor and its activity plays an essential role in preventing the onset of cancer [167]. Thus, this protein and many downstream effectors of p53-dependent pathways are **frequently mutated or absent in cancer** [194, 195]. Hematologic malignancies rank eighth in number of P53 mutations and, among them**, myeloid leukemias and MDS present a high mutation prevalence**, only exceeded by lymphomas (Figure 5.5) [196]. Besides P53 mutations, hematologic malignancies may also have mutations in p53 target genes or in other genes encoding proteins involved in p53 regulation, such as Mdm2 [197].

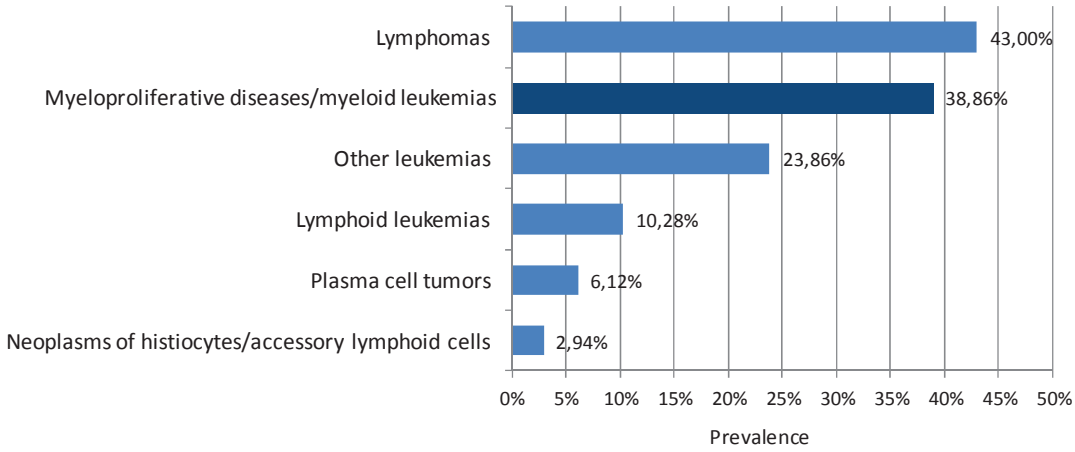

Figure 5.5. Prevalence of P53 mutations in hematologic malignancies. Data obtained from WHO [196].

CML is frequently associated to a reduced or absent p53 expression due to either gene inactivation, especially during the blastic phase of the disease, to locus alterations or to a Bcr-Abl-mediated increase in the expression of Mdm2. Whereas Mdm2 overexpression seems to induce a delay in myeloid differentiation, the loss of the wild type allele of p53 confers cells a more aggressive leukemogenic potential [197]. **In AML and MDS,** the most frequent alterations of p53 occur in the **therapy-related** diseases but there is also a ~20% of the *de novo* AML and MDS patients who bear **deletions at the p53 locus**. Interestingly, most of these patients (~64%) carry **mutations** in the remaining allele and therefore are p53 null ($p53^{-/-}$). AML and MDS cases with p53 mutations have been shown to have a **worse prognosis and significantly worse responses to chemotherapy** [198]. **In APL, the ability of p53 to arrest cell cycle and induce apoptosis is generally impaired** by the loss of functionality of PML protein, which is a p53 co-activator that sequesters Mdm2 [199] and associates with p53 in the nucleus during senescence, proliferation arrest and cell death induction [200]. PML directly binds the DNA-binding domain of p53, enhancing its affinity for its target genes, particularly for Bax and $p21^{CIP1/WAF1}$ [201], and its transactivation activity. Thus, in leukemias presenting the PML-

RARα fusion protein, p53 has reduced functionality as a transcription factor, irrespective of the possible mutations that cells may bear.

5.3.2. Cyclin-dependent kinase inhibitor 1A ($p21^{CIP1/WAF1}$ or CDKN1A)

Protein $p21^{CIP1/WAF1}$ (hereinafter referred to as p21) is a **mammalian cyclin-Cdk inhibitor and a transcriptional target of p53.** Two families of CDKIs have been described in mammals: the *INK4 inhibitors* and the *Cip/Kip inhibitors*, which target a broader spectrum of Cdks. P21 belongs to the latter, and is able to inhibit all cyclin-Cdk complexes as well as the *proliferating-cell nuclear antigen (PCNA)*, **arresting cells in G_1 and G_2/M**. The versatility of p21 in controlling cell cycle makes it one of the most important regulators of cell proliferation [176, 202, 203].

The presence of p21 is not only necessary to mediate p53-dependent cell cycle arrest, but is also **sufficient in the absence of p53** to arrest cells in G_1 or G_2/M. For instance, the initiation of cell cycle arrest in G_1 has been reported to be mediated by p21 through p53-independent inhibition of Cdk2 after the proteasomal degradation of phosphatase *Cdc25A* and cyclin D, which is triggered by DNA-damage kinases. This step is followed by a second phase in which extension of DNA damage activates p53-dependent maintenance of the cycle arrest (Figure 5.4) [172]. This evidences that p21 expression is not only transcriptionally induced by p53 but also by **other transcription factors**, such as *E2F, NF-κB, STAT, c/EBP* or *Sp1/3* [204, 205]. Furthermore, **post-translational regulation** of p21 by proteolysis and/or phosphorylation appears to be equally important for its functions as the transcriptional control of this gene (Figure 5.6) [206].

In addition to its function as a kinase inhibitor, it was long ago reported that p21 participates in the **regulation of gene expression** by modulating the activity of the transcription factor E2F throughout the course of the cell cycle [207]. For instance, it was recently unveiled that this occurs through the interaction with cell cycle-dependent elements located in the transcription factor, and through the direct binding to the promoter of its target genes [208]. p21 mediates the repression of genes related to cell cycle progression, including Cyclin E2 and Cdk2, [208] and the activation of genes involved in differentiation [209] and senescence [210]. This indicates that p21 blocks cell cycle progression in G_1 through both Cdk inhibition and transcriptional regulation mechanisms (Figure 5.6), and that it participates in the regulation of other cellular processes.

Because of the important role that it plays in the regulation of cell cycle, p21 has been *considered a tumor suppressor protein* [206, 211]. However, there is growing evidence that p21 is not necessary for the tumor suppressor activity of p53 and, furthermore, that it is a **negative regulator of p53-**

dependent apoptosis [206, 212-214]. This inhibition of apoptosis can be interpreted as an additional mechanism to protect cells from stress or DNA damage; thus, p21-defective cells that escape cell cycle arrest after p53 activation are redirected towards apoptosis (Figure 5.6) [212]. Nevertheless, this is not the only function of apoptosis inhibition by p21, since this protein has also emerged as a **negative regulator of p53-independent apoptosis** [212, 213].

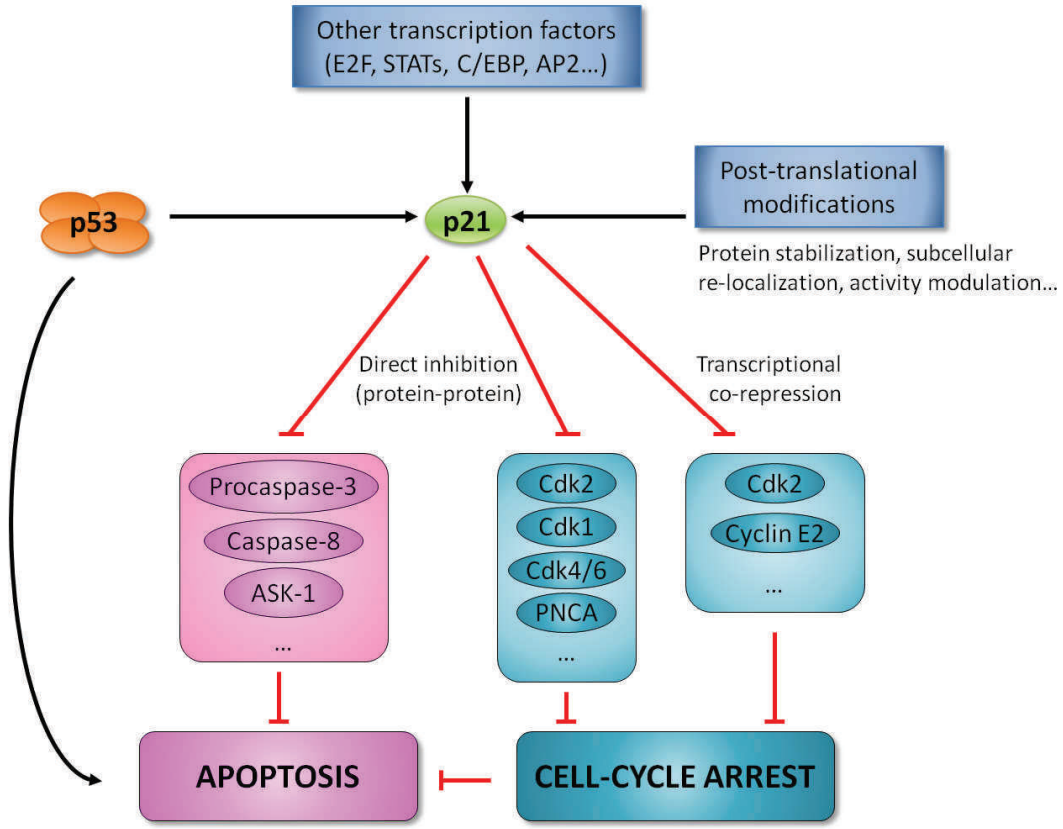

Figure 5.6. Effects of p21 activation on cell cycle progression and apoptosis. Activation of p21 is transcriptionally mediated by p53 after cell stress but can also be induced by other transcription factors. Post-translational modifications of the short-lived protein can also prolong its half-life, activate it and trigger its subcellular re-localization, which will eventually determine its effects. Through p21 activation, p53 protects cells from DNA damage or other types of insults by arresting cell cycle; however, if the damage is massive or irreversible, p53 may also induce cell death by apoptosis. Functional p21 inhibits p53-mediated (and p53-independent) apoptosis to modulate the intensity of the response and to provide an additional protective mechanism: in case of mutation or loss of p21 and inability to arrest cell cycle, p53-mediated response is re-directed towards apoptosis. p21 exerts its functions through the direct inhibition of cell cycle kinases (nucleus) and pro-apoptotic proteins (cytoplasm), and through the transcriptional co-repression of genes involved in cell cycle progression (nucleus).

Two types of mechanisms have been suggested as mediators of apoptosis inhibition by p21. On one hand, the anti-apoptotic effect of p21 could be a **consequence of cell cycle arrest**, which would allow DNA repair or prevent DNA damage and therefore prevent apoptosis [206, 212]. It has also been proposed that cell cycle arrest protects cells from apoptosis because an active proliferative state is necessary to detect genotoxic insults and microtubule-destabilizing agents [206]. Moreover, caspase-

3-mediated cleavage of p21 and subsequent upregulation of Cyclin A/Cdk2 is necessary for apoptotic death in different cell types [212]. On the other hand, direct, **cell cycle-independent anti-apoptotic mechanisms** have been described for p21, such as the inhibition of *procaspase-3*, *caspase-8* and the *apoptosis signal-regulating kinase 1 (ASK-1)* through cytosolic protein-protein interactions [206, 212, 213]. p21 may also act as an anti-apoptotic factor from the nucleus, where it can co-activate the expression of anti-apoptotic proteins [210] and suppress the induction of pro-apoptotic genes by the transcription factors Myc and E2F [206].

The anti-apoptotic effects of p21 suggest that this protein may act as an **oncogene**. In agreement, p21 expression is frequently increased in human cancer [206] and an overwhelming number of reports show that p21 reduces the sensitivity of many types of tumors to chemotherapeutic drugs, such as cisplatin, adriamycin, genistein or taxol, indicating that this gene may function as a **determinant of cancer chemoresistance** [212, 215-221].

Conversely, there is also a number of works that indicate that p21 has **pro-apoptotic functions** under certain conditions. However, the mechanisms through which p21 may promote apoptosis are not well understood, and may only be active in the absence of functional p53 and/or the tumor suppressor protein Rb [212, 213]. Furthermore, the number of reports describing p21 as an anti-apoptotic gene is much higher than that of those suggesting a pro-apoptotic role [212].

The role of p21 as a tumor suppressor or an oncogene appears to be affected by **post-translational modifications** of the protein and its **sub-cellular location** [206], being the *anti-apoptotic effects of p21 related to a cytoplasmic accumulation* [218, 220, 222]. The effects of p21 also seem to depend on the **type of death stimulus**, as it was shown in myeloma cells that constitutive expression of p21 can protect them from ROS-induced apoptosis but not from stimulation with a Fas agonist [217], but more importantly they **largely depend on the cellular context and genetic background** [206, 213]. In this regard, it has been suggested that p21 acts as a tumor suppressor in the context of a genomically unstable background where its loss, which is a rare event in human cancer, may promote transformation. However, as it was mentioned before, a variety of human cancers overexpress p21, which functions as an oncogene. In spite of existing little evidence of the contribution of p21 to cancer onset [223, 224], p21 seems to participate in tumor progression and chemoresistance since its overexpression has been found to **positively correlate with tumor grade, invasiveness, aggressiveness and lack of responsiveness** to chemotherapy and radiotherapy [206].

In **AMLs**, p21 has been reported to participate in both the **initiation** of the disease and the **maintenance of the leukemic phenotype** in BM progenitors. For instance, the AML-specific

oncogene AML1-ETO induces the expression of p21, which has been shown to be essential for leukemogenesis [225, 226]. In APL, p21 is upregulated by PML-RARα and protects BM HSCs of a previously established leukemia from exhaustion [226].

In **CML**, reports about p21 expression are contradictory. Whereas it was postulated that the fusion protein Bcr-Abl represses p21 expression and maintains low levels of this protein in human cell lines [48], more recent work reported that the specific Bcr-Abl inhibitor imatinib decreases p21 levels, which suggests that p21 expression in CML is upregulated by Bcr-Abl [227]. In agreement with the latter results, Bcr-Abl kinase activity in mouse BM cells has been reported to induce the expression of p21 [228], suggesting that p21 acts as an oncogene in CML. Importantly, and consistent with the role of p21 as an oncogene, these and other works have reported p21 to **confer CML cells resistance to apoptosis** induced by chemotherapeutic agents such as imatinib or taxol [227-229].

INTRODUCTION

6. miRNAs

miRNAs are **conserved non-coding RNAs with a short-sequence** (~20-23 nucleotides), that participate in the **translational regulation** of many genes involved in the control of important biological processes by selectively binding to their mRNAs and, generally, blocking their protein expression [230, 231].

miRNAs belong a group of ***small RNAs*** discovered in the last decade but not fully-characterized yet, along with ***short interfering RNAs (siRNAs)*** and the less-known ***piwi-interacting RNAs (piRNAs)*** [231]. These small RNAs exert their functions by directly binding to a matching sequence in their respective target mRNAs by Watson-Crick base pairing. miRNAs and siRNAs differ from piRNAs (reviewed in [232]) in the double-stranded nature of their precursors and the proteins that they associate with (referred to further on in this section). Despite those similarities between miRNAs and siRNAs, these two classes of small RNAs can be distinguished from each other, mainly by their origin but also by their evolutionary conservation and the types of genes they target [230, 231]. The main differences between miRNAs and siRNAs are summarized Table 6.1.

Table 6.1. Differences between miRNAs and siRNAs.

	miRNA	siRNA
Origin	Endogenous: genomic loci independent from their targets	Exogenous: mRNA, trasposons, viruses, heterochromatic DNA
Structure of primary transcript	Short hairpin (stem loop) structures	Long bimolecular RNA duplexes or extended hairpins
Number of mature molecules	One miRNA duplex per precursor	Many siRNA duplexes per precursor
Ontology	Conserved	Rarely conserved
Relation with target genes	"Hetero-silencing" (mRNA from other loci)	"Auto-silencing" (the originary sequence)

6.1. Biogenesis and canonical activity of miRNAs

Most miRNA genes are coded in **intergenic regions** of the genome that are distant from other genes, meaning that they have their own transcriptional regulatory elements and derive from independent transcription units [230, 233, 234]. Nevertheless, genomic studies have revealed that ~30% miRNAs are included in the **introns of pre-mRNA** of protein-coding genes and, frequently, in the same orientation, implying that they are transcribed in the same mRNA primary transcript (although they could also be independently transcribed through an alternative promoter) [233].

The majority of human miRNA genes are **isolated** from each other within the genome, but others can be **clustered** together (within a distance no longer than 50 kb) and transcribed as a polycistronic primary transcript [230, 233]. Since miRNAs within a genomic cluster have similar or the same expression patterns, they are often, but not always, functionally related to each other. Similarly, functionally related miRNAs are sometimes clustered together [230]. This feature suggests that several miRNAs collaborate in the regulation of the same processes. In agreement, miRNAs show cell-type and tissue-specific expression patterns, as well as distinct expression signatures in different developmental stages [230, 233], which is indicative of their collaborative mechanism of action.

Primary miRNA transcripts, *Pri-miRNAs*, are typically processed by **RNA polymerase II (RNA Pol II)** and they are capped and polyadenylated sequences, sometimes longer than 1 kb, fold into an imperfectly-paired stem loop structure with unpaired flanking segments (Figure 6.1. step A) [230, 231, 235]. miRNAs that do not reside in introns of protein-coding genes are probably an exception and share their primary transcripts with their pre-mRNA host genes [230]. Pri-miRNAs mature through nuclear cleavage by the RNase III endonuclease **Drosha**, which cleaves both strands of the pri-miRNA near the base of the primary stem loop (Figure 6.1. step B), leaving a ~2-3 nucleotide 3' overhang and liberating a ~60-100 nucleotide stem loop intermediate known as **precursor miRNA (*Pre-miRNA*)** [230, 235].

Pre-miRNAs are actively transported from the nucleus to the cytoplasm, where the other end (the loop) is cleaved by another RNase III endonuclease, **Dicer**, which recognizes and associates with the base of the stem loop cleaved by Drosha through a **PAZ domain** (identified in Piwi, Argonaute and Zwille/Pinhead proteins) that weakly binds to double-stranded RNA and single-stranded RNAs of at least 5 nucleotides in length. Dicer then cuts both RNA strands at about 2 helical turns away, leaving again a ~2-3 nucleotide 3' overhang and a 5' monophosphate (Figure 6.1. step C) [230, 231, 235]. Besides pre-miRNA, Dicer also cleaves siRNA precursors, and therefore is one of the signature components that miRNAs and siRNAs biogenesis have in common [231]. Of note, Dicer-independent miRNA biogenesis has also been reported, although it seems to function in very a specific context [236].

The **miRNA duplex** generated after cleavage is comprised by the mature miRNA and a similar-sized complementary fragment (miRNA*), but this is a short-lived structure which is rapidly released and degraded upon entering of the other strand into a protein complex named **RNA-induced silencing complex (RISC)** (Figure 6.1. step D) [230, 231]. The mechanism of miRNA duplex unwinding and entering in the RISC is still not fully understood [231]. It is believed that the strand that binds to the RISC is determined by the thermodynamic stability of its 5' end, since this is the end of the miRNA

that will bind to the target [237]; the strand with a 5' end which is more easily released from the other strand will enter the RISC in first place, while the other strand is subsequently degraded. In miRNA duplexes with ends with similar stabilities, both strands of the duplex might accumulate in RISCs and either only one or both complexes might be functional [230, 235]. This process occurs so rapidly in mammals due to the direct interaction between Dicer and some protein components of the RISC [231].

RISC is the second feature that the mechanisms of action of miRNA and siRNA have in common. One of its core components is a member of the *Argonaute* protein superfamily, **Ago**, which contains a PAZ domain and the characteristic domain PIWI, which in some cases can catalyze guide strand-directed endonucleolytic cleavage of the target [230, 231]. However, most species express multiple Ago genes and not all Ago proteins have endonucleolytic activity. Among the four mammalian Ago proteins, **only Ago2 bears cleavage activity** [238, 239]. Other proteins contained in RISC are regulatory factors that will determine the final effect of the RISC/miRNA complex. One remarkable component of this complex is *GW182*, which is necessary and sufficient for Ago/miRNA-mediated silencing of gene expression [231].

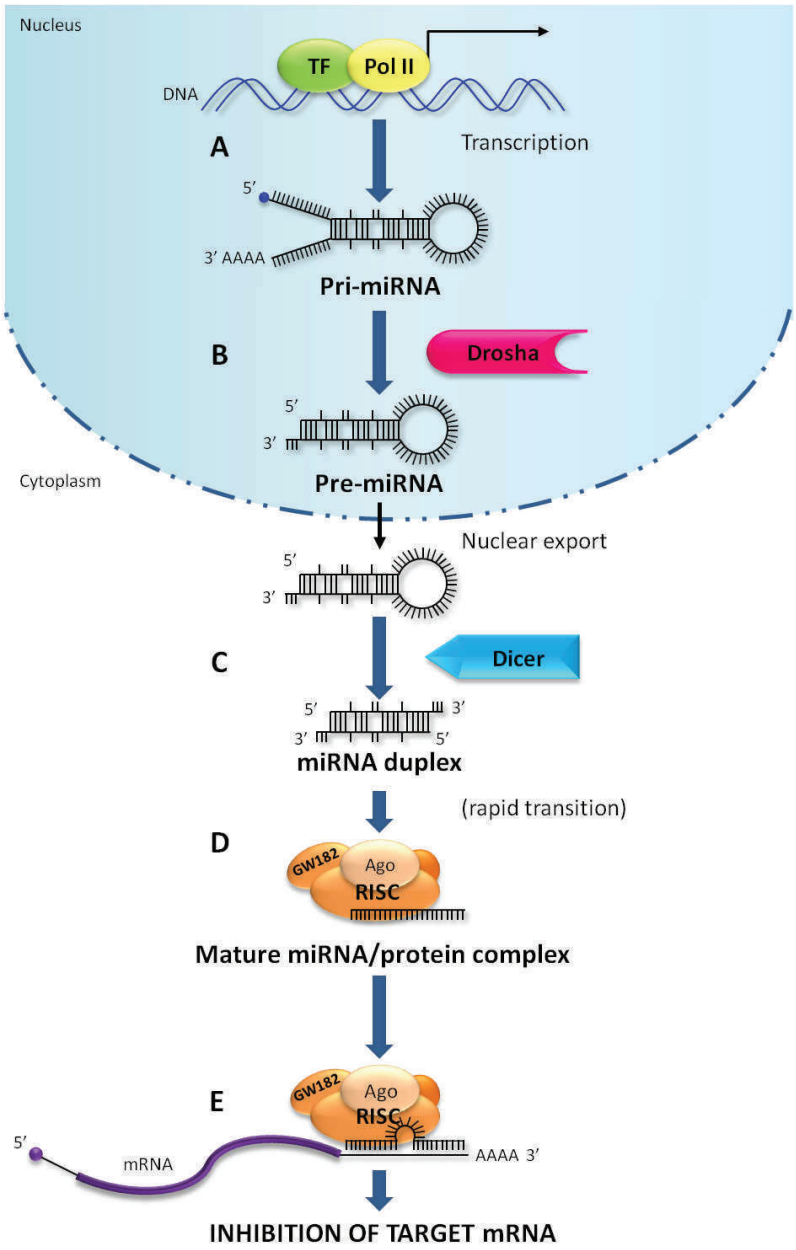

Figure 6.1. miRNA biogenesis

According to the prevailing model, **miRNA/RISC complexes bind to target mRNAs**, most generally accepted to **more than one binding site located in their 3' untranslated region (3'UTR)**, and post-transcriptionally regulate their expression either by cleavage or by repression of translation (Figure 6.1. step E). Perfect complementarity between the miRNA and the mRNA binding site is determinant for cleavage, while translational repression appears to be irrespective of the degree of pairing and can be complementary to mRNA degradation [230, 231].

Cleavage of mRNAs occurs by deadenylation, decapping and exonucleolytic digestion of the mRNA [240, 241], rather than by Ago-mediated cleavage, as it was initially thought. However, it requires Ago and GW182, besides the endogenous decapping and deadenylating machinery [240]. As it has been mentioned before, cleavage needs perfect or almost-perfect complementarity between the miRNA and the mRNA target, and may be coupled with translational repression [231].

Nevertheless, cleavage and translational repression appear to be independent processes and the number, type and position of mismatches seem to determine which of them will be triggered [231]. The mechanism of **translational repression** is not clear yet, and existing studies have yielded discrepant results, but data suggest that the repression may occur either at the initiation stage [242, 243] or at some subsequent step [244] and may involve competence with translation initiation factors, blockade of the circularization of the mRNA, premature dissociation of the ribosome [231] or early polypeptide degradation [231, 245]. Apparently, the most efficient translational inhibition is achieved with the cooperative action of multiple RISCs [230], which supports the aforementioned idea that different miRNAs cooperate in the regulation of the same targets.

Of note, the type of repression that miRNAs exert is not as strong as that induced by transcriptional repressors or epigenetic modifications, since it can be easily counterbalanced by the production of a higher number of mRNA molecules. Thus, it has been suggested that miRNAs work as "buffers" that increase the threshold signal required for the activation of a certain gene [246].

6.2. Regulation of miRNA biogenesis and activity

One remarkable characteristic of miRNAs is that they, and their associated proteins, are one of the **most abundant ribonucleoprotein complexes** in the cell. However, some of them can be abundantly expressed while others are present in a much smaller number in the same cell [230]. Those differences in basal concentration within the same cell are probably related to the repression requirements of their target genes, since the level of **silencing depends on the proportion between**

the number of target mRNA and miRNA molecules [246]. In consequence, different organs present characteristic miRNA expression patterns [233].

Regulation of miRNA activity is still a rather unexplored field. It has been postulated that it may be modulated through the following mechanisms [246]:

- Transcriptional activation or repression of the miRNA by common transcriptional regulators, which control the expression of miRNAs sets in a cell-specific manner [247].
- Transcriptional activation of the target gene, which decreases the ratio miRNA/mRNA and, therefore, the effectiveness of translation repression.
- Transcriptional activation of other targets ("target mimics") that compete for the existing miRNA.
- Binding of different effector proteins that can modulate the activity of the RISC.
- Occasional subcellular relocalization and association with different regulators (see point 6.3).
- Regulation of miRNA decay (despite their usually-high stability).
- Changes in the availability or nature of a miRNA binding site by RNA editing, alternative splicing or alternative polyadenylation sites.

Regarding their transcriptional regulation, miRNAs frequently participate in **positive and negative feedback loops** and it is known that a striking number of miRNA genes are under the control of their own targets, participating in a **double-negative feedback loop** [231, 246].

6.3. Alternative cell contexts and functions

Most miRNAs and their mRNA targets and associated proteins **diffusely accumulate in the cytoplasm**. However, a small subpopulation is localized in subcellular structures called **cytoplasmic granules**, either basally or upon different types of stress conditions. These granules differ in their RNA-interacting protein composition, and it has been postulated that they can participate in the modulation of miRNA activity during response to stress [246].

It is generally assumed that miRNAs always direct the inhibition of gene expression, or gene silencing. However, in the past few years, evidence that **miRNAs can carry out translational activation** has started to arise, as it was demonstrated in the context of cell cycle arrest [248]. Interestingly, this alternative regulation seems to be related to the fact that miRNAs and RNA-binding proteins, namely Ago2 and FXR1 (fragile X mental retardation-related protein 1), which are essential for translational activation, can associate in cytoplasmic granules [249]. It is not known which type of cytoplasmic granules is involved in the positive translational regulation exerted by miRNA, but two types of

granules have been proposed based on the presence of Ago2 in their composition [249]: **GW1282 bodies (GW bodies or mammal P-bodies)**, of which size and number depend on cell cycle progression [250], and **stress granules (SGs)**, formed upon stress conditions [251]. Both types are comprised of mRNA and miRNA-binding proteins, which suggests that modulation of miRNA activity takes place, at least in part, in these structures [252].

Other studies in human cells have reported translational activation functions for miRNAs which have binding sites located in the 5' UTR of their targets [253, 254]. Additionally, it has been postulated in *Drosophila melanogaster* that miRNA-mediated translational activation depends on Ago2 and the degree of polyadenylation of the target mRNA [255]. Nevertheless, evidence of miRNA-directed translational activation is still a mostly unexplored field.

6.4. Target genes and biological effects

At present, **more than 2500 mature miRNA sequences** have been reported in humans [256], and it is believed that over **30% of the human genome** is under the translational regulation of miRNAs [237]. Each miRNA can bind to and silence **hundreds of target mRNAs with great specificity**, just like one mRNA can bear **binding sites for many different miRNAs** [230, 257]. The ability of miRNA to specifically interact with a target mRNA without a need for a larger overlap is probably conferred by its highly-exact ends [231]. The exactitude of both ends of the mature miRNA duplex is determined by Drosha through the specificity of the first cleavage, since Dicer just binds to the loop-free end and cleaves the pre-miRNA, irrespective of its sequence [230]. The quality and stability of base pairing is also thought to be a determinant of specificity. Indeed, residues 2-8 of the miRNA are the most conserved and a perfect or near-perfect complementarity of 5-7 nucleotides near the 5' end of the miRNA appears to be required for a good target prediction [231, 237, 258].

Computational methods have been used to identify miRNA targets [237], searching for multiple conserved regions within the 3' UTR of genes, which are complementary to miRNAs. Despite this is a method with a high number of false positives in animal genes due to their lower miRNA/mRNA complementarity, a good number of target genes have been identified with confidence, allowing the study of the types of processes regulated by miRNA [230].

miRNA target genes participate at many levels in the regulation of a vast diversity of biological processes, such as **cell cycle, differentiation, apoptosis and development** [234, 259], although predicted target genes involved in development are just a minority, contrary to what could be supposed from their conserved nature [230]. miRNA also appear to play key roles in the **mediation of**

stress responses and inflammation [246, 259]. Indeed, some miRNAs that seemed dispensable in homeostatic conditions have been demonstrated to be necessary upon cell stress [246].

Importantly, among miRNA targets there are a high number of **transcription factors**, meaning that miRNAs mediate cellular processes not only through direct translational inhibition of genes involved in those processes, but also by indirectly modulating entire gene expression programs through the translational control of other regulators of gene expression [230].

In regard of miRNA-mediated control of *cell cycle*, it is remarkable that most cell cycle regulators (cyclins, Cdks and other kinases, CDKIs, phosphatases and transcription factors involved in the control of DNA replication) have been reported to be regulated by one or more miRNAs. Furthermore, some transcription factors that are critical for the progression of cell cycle, like the E2F family and p53, have been demonstrated to control the expression of miRNA sets involved in cell cycle regulation, creating positive and negative feedback loops that form an intricate regulatory network [260].

Considering the relevance of the physiological processes regulated by miRNA target genes, it is not surprising that the deregulation of miRNA expression and function has been related to disease [261-263]. In this section, we will focus on the role of miRNA deregulation in cancer.

6.5. miRNAs in cancer

In the same way that different types of tissue show characteristic miRNA expression profiles [233], miRNAs are **differentially expressed in tumor tissues** and this miRNA signatures even allow the distinction between primary tumors and metastatic tissues (reviewed in [234]). This finding suggests that **miRNAs participate in the pathogenesis or development of cancer**. Indeed, it has been proven that miRNAs like miR-155, miR-21, miR-221 and miR-222, as well as the miR-106b-93-25 and miR-17-92 clusters can act as *oncogenes* in a vast number of tumors, while miR-15, miR-16, let-7, miR-34, miR-29 and miR-122 function as *tumor suppressors* that are frequently mutated or deleted in cancer [234, 259].

For all these reasons, miRNAs are **promising therapeutic targets and useful diagnostic and prognostic markers** in cancer [263]. Two strategies for miRNA targeting have been proposed so far: the use of miRNA mimics to compensate for downregulation, deletion or malfunctioning of tumor suppressor miRNAs, and the treatment with different types of inhibitors to block the effects of overexpression of oncogenic miRNAs (reviewed in [234]). It is worth mentioning that one of the

existing types of miRNA inhibitors called anti-sense oligonucleotides or *antagomiR* (referred to in the *Materials & Methods* section), have yielded excellent results *in vivo* [264].

6.6. miRNA in hematopoiesis and hematologic malignancies

Similarly to other tissues, a number of miRNAs have been reported to be prominently expressed in hematopoietic cells, and this differential expression appears to be involved in the **regulation of hematopoietic differentiation** [7]. Accordingly, hematologic malignancies are one of the types of cancer with the highest number of reported miRNA deregulations, especially lymphomas, myelomas and chronic lymphocytic leukemia (CLL). Besides miRNAs involved in hematopoiesis and **abnormally expressed in hematologic cancers** (summarized in Table 6.2.), other miRNAs with roles in cell cycle, apoptosis and survival, such as miR-15, miR-16, miR-29, miR-34, let-7, miR-143, miR-195 and the miR-17-92 cluster, may be deregulated in hematologic malignancies [7, 234, 259, 265-270]. Thus, specific miRNA expression patterns have been suggested as good **diagnostic and prognostic markers** for this group of diseases [270-273].

Table 6. 2. miRNAs with roles in hematopoiesis and their possible involvement in cancer

miRNA	Functions in hematopoietic cells	Involvement in hematologic malignancies
miR-106-363 cluster	Regulates monocytopoiesis	Overexpressed in T-cell leukemias and downregulated in CML; overexpressed in multiple myeloma
miR-17-92 cluster	Involved in monocytic differentiation, preventing transition to macrophages	Upregulated in B-cell lymphoma, multiple myeloma and MDS
miR-125b	Enhances proliferation and blocks differentiation of hematopoietic progenitors	Mutated in AML, overexpressed in AML, CML, MPN, APL, MDS and ALL
miR-133	Promotes myeloblast differentiation	Unknown
miR-142	Involved in B-cell commitment and myeloid maturation	Mutated in aggressive B-cell leukemia
miR-146	Modulates inflammatory response	Downregulated in $5q^-$ MDS, mutated in AML
miR-150	Controls B-cell differentiation and is highly expressed in mature B and T cells	Unknown
miR-155	Regulates cytokine expression in B and T cells and is involved in the maintenance of erythroblasts	Upregulated in Burkitt's lymphoma, Hodgkin's disease, non-Hodking lymphoma, CLL and AML
miR-181a	Involved in commitment of the B-cell lineage and highly expressed in mature T cells	Loss of expression in aggressive B-cell chronic lymphoma, downregulated in imatinib-resistant CML cells
miR-196	Participates in myeloid differentiation	Downregulated in CML
miR-221/222	Maintains progenitor cells in erythropoiesis	Upregulated in CLL
miR-223	Regulates granulocytic differentiation	Silenced in leukemia

MPN: Myeloproliferative Neoplasms

7. Oxidative Stress

Oxidative stress can be defined as a **disruption of redox homeostasis** that induces an overall increase of intracellular levels of *reactive oxygen species (ROS) or nitrogen species (RNS)*. **ROS** is a collective term that refers to **oxygen-derived *free radicals*** such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl ($HO^{\cdot}$), peroxyl ($RO_2^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals, as well as oxygen-derived **non-radical species** such as hydrogen peroxide (H_2O_2) and hypochlorite anion (ClO^-). **RNS** include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$) [274, 275].

A *free radical* is a molecule or molecular fragment that contains one or more unpaired electrons in atomic or molecular orbitals. For this reason, radicals are **very reactive species,** and extremely toxic to cellular and sub-cellular structures. Oxygen-derived radicals are the most important class of radicals generated in living systems [276]; therefore, in this section we will focus on ROS as the main source of oxidative stress.

Intracellular ROS, as well as RNS, are **products of normal metabolism** which can be formed from both endogenous and exogenous sources. The main **endogenous** source of ROS is the **electron transport chain,** localized in the inner membrane of the **mitochondria.** There, $O_2^{\cdot-}$ is generated from molecular oxygen through the acquisition of one electron originated by electron leakage from complexes I and III. Other endogenous sources include *cytochrome P450 complexes* embedded in microsomal membranes [277], *peroxisomal oxidases*, the membrane-associated *NAD(P)H oxidase (NOX)*, *cytochrome c oxidase*, *xanthine oxidase (XO)* and *redox-active metals* like Fe^{2+} and Cu^{2+}. **Exogenous inductors** of ROS production are radiation, atmospheric pollutants and chemicals [274, 276].

7.1. ROS in homeostasis

Under physiologic conditions, cells maintain redox balance through the **elimination of reactive species by enzymatic and non-enzymatic anti-oxidant systems**. Enzymatic reactions that eliminate ROS are started by the family of *superoxide dismutases (SOD)*, which transform the "primary" ROS, $O_2^{\cdot-}$, into H_2O_2. This molecule is further converted into H_2O and O_2 by the peroxisome enzyme *catalas*e, or by coupled reactions that involve the conversion of reduced *glutathione (GSH)* to *oxidized glutathione (GSSG),* which are catalyzed by *glutathione peroxidases (GPX).* GSSG is recycled (reduced back) by glutathione *reductases (GR),* which use NADPH as a source of electrons (Figure 7.1) [274].

Non-enzymatic anti-oxidants include **vitamins C (*ascorbic acid*) and E (*α-tocopherol*), *carotenoids, flavonoids*** and thiol anti-oxidants. The latter carry out **thiol-disulfide exchange reactions** that are essential for the reduction of oxidized thiol-containing proteins. These anti-oxidants include the *GSH*, *thioredoxin (Trx)*, *glutaredoxin (GRx)* and the *peroxiredoxin (Prx)* systems. All of them are based on the presence of cysteine-containing redox active centers that are used to reduce protein disulfides [274, 276].

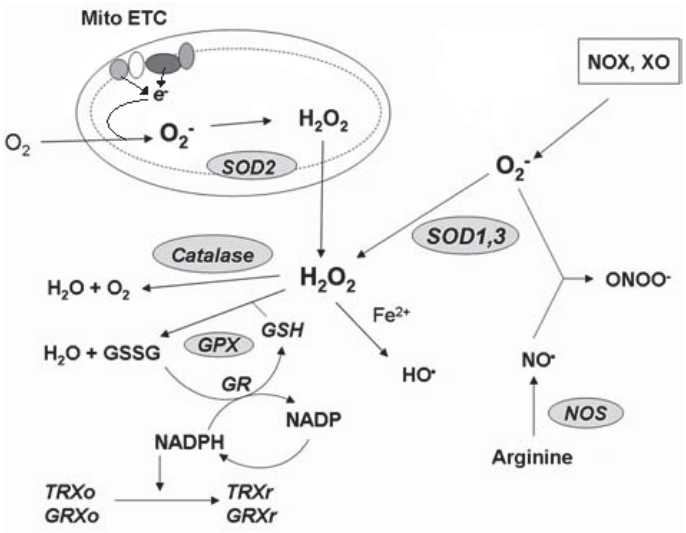

Figure 7.1. Main enzymatic reactions involved in redox homeostasis. Modified from *Trachootham et al. (2008)* [274]. Major ROS-scavenging enzymes are highlighted in grey. ETC: electron transport chain; NOS: nitric oxide sintase.

As explained above, the balance between ROS generation and elimination is thinly regulated by complex mechanisms. Any dysfunction in those mechanisms could lead to oxidative stress.

7.2. Biochemical consequences of oxidative stress

Under physiological conditions, the presence of low levels of ROS allows their reversible **incorporation into the structure of macromolecules**. This has beneficial effects in the function of a number of signalling systems, in defense against pathogens and in the induction of a mitogenic response (reviewed in [276]), events which are regulated through transcriptional modulation, direct oxidative modification of protein functions, regulation of redox-sensitive interacting proteins and enzymes, and through the regulation of protein turnover (for a review, refer to [274]). However, under oxidative stress conditions, ROS accumulate in excess and attack cellular components by reacting with the main classes of macromolecules in an **irreversible manner**, which has a harmful effect [274, 276].

The macromolecules which are most susceptible to oxidative modification are lipids, preferentially **poly-unsaturated fatty acids**. Their **oxidation generates lipid radicals** that can rapidly propagate the

oxidation reactions to other lipid molecules by reacting with oxygen and becoming **peroxyl radicals**. Finally, peroxidation of phospholipids can lead to cell death through both damage of cell membranes and the accumulation of the peroxidation product 4-hydroxy-2-nonenal. This aldehyde can interact with proteins and lead to functional alterations which are consequence of the disruption of signalling pathways [274, 276].

Protein oxidation can be reversible or irreversible depending on the target and the oxidative species. Proteins containing residues of arginine, histidine, proline, lysine and threonine are especially sensitive to oxidation, which produces carbonyl derivates. Moreover, severe oxidative stress can induce disulfide bond cross-linkage of different proteins containing cysteine and methionine residues and secondary modifications such as **adduct formation** between proteins and lipid peroxides or glucides. These protein cross-links and adducts may inactivate proteasomal degradation and lead to the accumulation of damaged-protein aggregates and subsequent cell death [274, 276].

Last, and more importantly, **oxidative stress is the main source of DNA damage.** ROS can damage DNA in nearly all of its components, inducing single- and double-strand breaks. As explained in the previous section, moderate levels of DNA damage can trigger cell-cycle arrest and DNA repair, but excessive damage or failure in DNA repair induces apoptosis. Mitochondrial DNA is even more susceptible to DNA damage than nuclear DNA, because of its proximity to the electron transport chain, one of the main sources of ROS, and because of its limited repair capacity [274].

Therefore, sustained or excessive levels of ROS can induce **structural and functional damage** in almost every cellular component. For this reason, stress-sensing mechanisms and the subsequent response are essential to protect cells.

7.3. Oxidative stress in cell proliferation, survival and death

Importantly, two of the signalling pathways affected by the redox state are cell death and proliferation, which relates oxidative stress with various pathological conditions, including cancer.

Oxidative stress modulates several **transcription factors which regulate cell survival** [274, 276]:

- As it was explained before, **NF-κB** is a redox-sensitive transcription factor that generally promotes cell survival. Activation or inhibition of this factor by oxidative stress depends on the type of stimuli that induces stress, the cellular levels of ROS (low levels appear to activate it whereas high levels inhibit it) and cell context.

- Closely related to NF-κB functions is the transcription factor **AP-1**, which controls both cell growth and apoptosis. Upon oxidative stress, AP-1 may promote one or the other depending on the balance between positive and negative regulators of apoptosis.
- Another transcription factor which is regulated by ROS levels **is nuclear factor E2-related factor 2** (**Nrf2**), which mediates cytoprotection from oxidative and electrophilic stress through the transcriptional control of a wide variety of genes. Nrf2 target genes are mainly anti-oxidant and phase-II enzymes such as NAD(P)H–quinone oxidoreductase 1 (NQO1), heme oxygenase-1, glutathione S-transferases (GSTs), γ-glutamyl-cysteinyl synthetase (γGCS), GPXs, thioredoxin reductase 1, peroxiredoxin 1, aldehyde oxygenase, and other genes regulating the response to oxidative stress. Nrf2 also activates the transcription of some genes of the multidrug resistance family such as MRP1, MRP2, MRP3, and MRP4. Overall, Nrf2 regulates the expression, both basal and inducible, of enzymes and other proteins involved in cell protection from physical/chemical insults, detoxification, and restoration of homeostasis [278, 279]. This factor appears to promote cell survival under stress conditions and there is increasing evidence that it plays an important role in cancer progression and chemoresistance (reviewed in [280]; see *Appendix-I*).
- Activity of *hypoxia-induced factor (**HIF**)* can also be modulated by ROS. HIF normally regulates metabolism and cell survival under hypoxic stress and can activate and suppress the expression of many genes involved in metabolism, angiogenesis, invasion and survival.
- **Tumour suppressor p53** does not only upregulate genes that participate in ROS-mediated apoptosis, but is also sensitive to oxidative stress and triggers specific transcriptional and non-transcriptional responses to variations in ROS levels [276]. Under low ROS levels, p53 induces the expression of anti-oxidant genes and protects cells from oxidative DNA damage, whereas under severe stress, p53 promotes the expression of pro-apoptotic genes and plays a direct pro-apoptotic function in mitochondria, as it was described in Chapter 5 of the *Introduction* section.

In addition to redox-sensitive transcription factors, changes in ROS levels can also regulate signal transduction systems involved in proliferation, like **MAPK and PI3K/Akt pathways**, as well as components of death-execution routes like **caspases, Bcl-2 and cytochrome c**. Similarly to transcription factors, low or moderate levels of ROS trigger survival mechanism by inducing NF-κB-mediated Bcl-2 expression and by direct caspase inhibition. However, higher levels of oxidative stress induce inactivation of Bcl-2 and direct caspase activation that promote cell death. Moreover, accumulation of endogenous ROS may induce MOMP *per se* and trigger procaspase-9 activation and cytochrome c release [274].

Cytochrome c* is a highly redox-sensitive molecule** which participates in electron transfer between complexes III and IV. Increased endogenous ROS interact with proteins of the mitochondrial permeability transition pore complex, interfering with anion fluxes and disrupting mitochondrial electron transport chain. This leads to the loss of mitochondrial intermembrane potential and to cytochrome c release from the mitochondrial inner membrane into the intermembrane space, event which is mediated by the oxidation of the anionic phospholipid ***cardiolipin. Further mitochondrial damage triggers MOMP, which eventually allows cytochrome c release into the cytoplasm and apoptosome formation. Importantly, peroxidized cardiolipin facilitates MOMP by migrating to the mitochondria outer membrane and interacting with the BH3-only protein Bid. Thus, cardiolipin also acts as a mitochondrial oxidative stress sensor and a modulator of apoptosis [274, 275].

Because it has been suggested that the **degree of permeabilization** may determine the extension of the cellular damage as to reversible (autophagy) or irreversible (apoptosis) [126], mitochondrial redox-sensing systems like cardiolipin play a pivotal role in the decision of cell fate. For instance, when only a fraction of the mitochondria are damaged, they are targeted for autophagy (**mitophagy**) to rescue the cell from apoptosis. The autophagic recognition of depolarized mitochondria is mediated by the mitochondrial kinase PINK1, which facilitates the ubiquitination of mitochondrial substrates like the outer membrane protein VDAC1 or *p62*/*SQSTM1*, involved in the selective aggregation of ubiquitin-positive molecules into autophagosomes [136, 281].

Oxidative stress can also **induce autophagy** through the direct activation of Atg4 proteases, which accelerates the maturation of LC3, the direct and indirect inhibition of mTOR and activation of AMPK, the activation of certain kinase routes and the induction of p53-mediated transactivation of pro-autophagic genes [136].

7.4. Oxidative stress in cancer

Genetically unstable cells like **tumour cells can adapt to live with stress** by adjusting their metabolism, and therefore ROS levels, to an extent that promotes cell survival. Moreover, malignant cells are frequently deficient in anti-oxidant systems. As a consequence, tumor cells frequently have **increased levels of ROS** as compared to normal cells. This is often related to the overexpression of oncogenes and loss of functional p53 and, thus, is considered to play an important role in cancer-related **increased proliferation and evasion of cell death** (Figure 7.2; step 1) [274].

In addition, as it has already been explained, oxidative damage can induce **permanent DNA mutations** which play a critical role in carcinogenesis (Figure 7.2; step 2). For instance, high levels of

oxidative DNA lesions have been found in a number of tumours, suggesting a correlation between high oxidative stress levels and cancer initiation. Thus, **tobacco smoke** is a source of ROS that increases oxidative DNA damage rates and is a well-characterized carcinogen. Moreover, several **redox metals** like iron, cadmium or chromium have been involved in the mechanisms of carcinogenesis owing to their ability to generate free radicals, as well as **non-redox metals** like arsenic, which is capable of binding thiol groups and inactivate enzymes involved in the metabolism of glutathione. Moreover, there is also evidence of the involvement of oxidative **mitochondrial DNA damage** in the process of carcinogenesis. As mentioned above, this DNA has a greater susceptibility to oxidative damage. Consistent with this, various mutations in mitochondrial DNA genes encoding proteins from the electron transport chain and in hypervariable regions of this DNA have been identified in cancer [276].

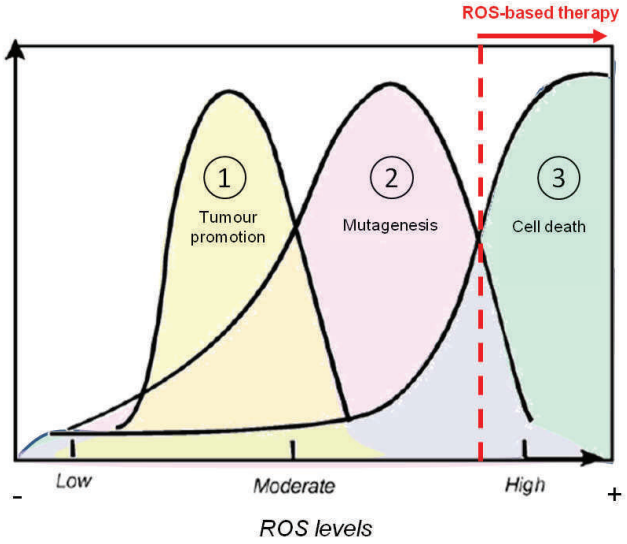

Figure 7.2. Dose-dependent effects of oxidative stress in cancer. Modified from *Valko et al. (2007)* [276].

As explained before, **dramatic increases in ROS can cause lethal damage and kill cells** (Figure 7.2; step 3). Because cancer cells have increased ROS levels compared to normal cells, and frequently exhibit deficiencies in their anti-oxidant defense mechanisms, they have a lower threshold of oxidative damage. Thus, **ROS-mediated therapeutic strategies can selectively target cancer cells**. Moreover, depletion of anti-oxidant systems may be especially useful to avoid chemoresistance acquired through increased levels of cytoprotective enzymes and defective apoptosis [274, 280].

7.5. Mitochondria as a pharmacological target: delocalized lipophilic cations

From what was discussed in this and previous sections, it can be deduced that mitochondria are not only a source of energy for the cell, but also play a central role in the integration of cell death signals and are **key regulators of the balance between death and survival**. This central role makes

mitochondrial function a good pharmacological target to induce programmed cell death in cancer. In agreement, the **higher sensitivity of cancer cells** to the deliberate disruption of the electron transport chain or to the induction of the endogenous production of ROS has been demonstrated before [282]. Moreover, because mitochondria can regulate p53 accumulation and activity, many p53-targeting drugs used in cancer do so by altering the mitochondrial function [169].

Delocalized lipophilic cations (DLCs) are a family of compounds which has been demonstrated to be efficient in selective death induction in cancer cells, *in vivo* and *in vitro,* owing to their affinity for the mitochondria [283-287].

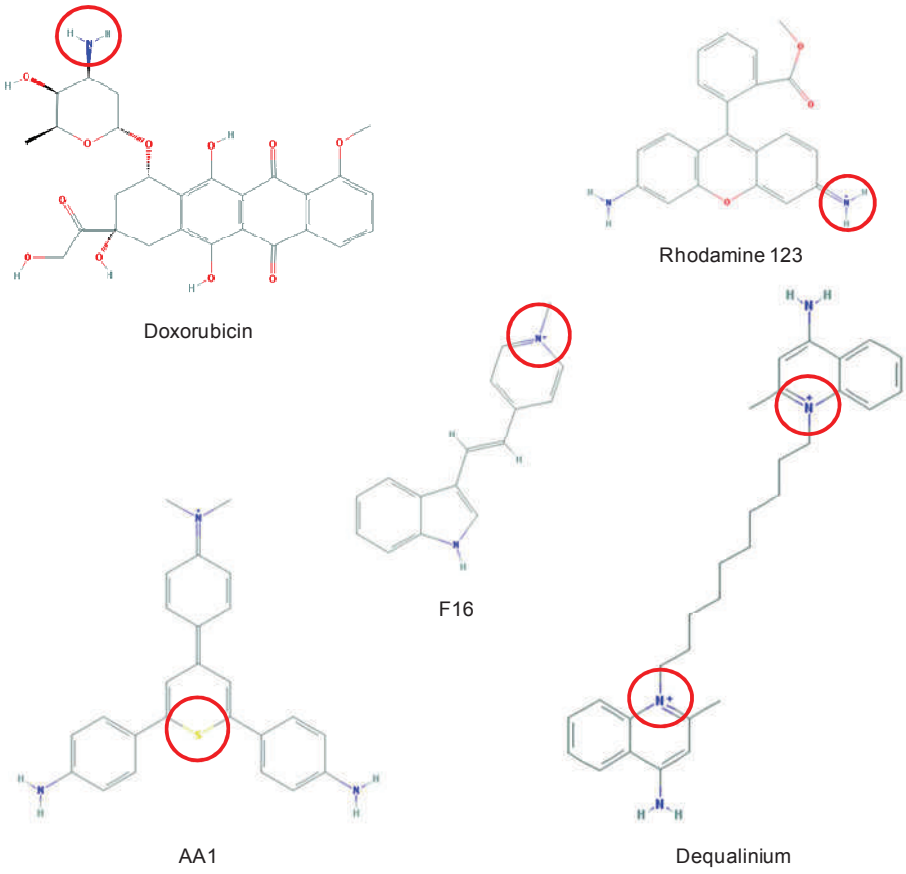

Figure 7.3. Chemical structure of anthracyclins (doxorubicin) and DLCs. Red circles indicate positive charges. Oxygen molecules are highlighted in red; nitrogen, in blue and sulphur, in yellow. Doxorubicin contains a NH_2 group which is protonated at physiologic pH, whereas DLCs (rhodamine 123, AA1 [284], F16 [286] and dequalinium [288]) carry a delocalized positive charge. One further difference is the size of the carbon backbone, which is generally smaller in DLCs (except for dequalinium, but this is a flexible molecule) [287].

Structurally, DLCs are small, amphiphilic molecules, generally containing more than one benzene ring that confer them a highly lipophilic character (Figure 7.3). They are characterized by a delocalized positive charge that renders them attracted by the negative mitochondrial transmembrane potential. For this reason, DLCs selectively **localize to the mitochondrial inner membrane of living cells,**

preferentially of cancer cells. Apparently, the delocalized nature of the positive charge plays an important role in determining mitochondrial versus nuclear localization and is what makes them distinct from other compounds such as anthracyclines [287]. Interestingly, one of the firstly-described DLCs was *rhodamine 123* [283], which is nowadays used as a fluorescent mitochondrial probe in the determination of mitochondrial transmembrane potential. Although all DLCs follow the same mechanism for mitochondrial accumulation, their mechanisms for cytotoxicity are different. Some DLCs, like rhodamine 123 or *AA1*, inhibit F_0F_1-ATPase whereas *dequalinium (DQA)* inhibits NADH-ubiquinone reductase activity of complex I. In both cases, the accumulation of sufficient concentration of DLCs in the mitochondria results in **the disruption of the electron transport chain** and therefore in a decrease in the production of ATP. Despite DLCs having demonstrated to be selective and effective as single agents, molecules from this family have a **promising utility in combination therapies** to enhance their anti-carcinoma activity [285].

DQA is a DLC with anti-microbial properties [289, 290] which has been reported to induce a 100-fold higher inhibition of clonal growth in carcinoma versus control epithelial cells in culture, and to have significant **anti-tumour activity** *in vivo* [288, 291]. This molecule has a characteristic symmetrical shape with two charged centers separated by a carbon chain, which allows it to group in micelles in an aqueous solution. DQA micelles or *DQAsomes* lack an internal aqueous compartment, reason why they have been suggested as drug and gene delivery systems for cancer cells [292, 293].

Remarkably, DQA **induces apoptosis in human myeloid leukemia cell lines**, subsequent to the depolarization of mitochondrial membrane, release of cytochrome c, overproduction of ROS and a decrease in cellular ATP content [294-296]. Moreover, this apoptosis induction appears to be enhanced by DQA-mediated downregulation of ERK and PI3K/Akt signaling pathways [297]. Therefore, DQA is an interesting pharmacological tool to selectively induce ROS-mediated cell death in leukemic cells.

8. The Ubiquitin-Proteasome System

The *ubiquitin-proteasome system (UPS)* is a **major protein degradation system**, characterized by the fact that it is targeted to a specific fraction of short-lived proteins. The core machinery of the UPS is the **26S proteasome**, which is a multimeric protein complex that identifies and degrades proteins that have been tagged by a covalent bond with a chain of at least four molecules of **ubiquitin** (although it has been unveiled that ubiquitination is not always a requisite for proteasomal degradation) [298, 299].

The UPS generally degrades **proteins that have been damaged or misfolded** and prevents their accumulation and the subsequent induction of ER stress. Therefore, UPS is considered as the degrading machinery of the ER-associated degradation pathway [300]. On the other hand, the UPS plays an important role in cell signalling and the ubiquitin tag is known to target proteins for cleavage-mediated activation or inactivation, cellular trafficking and other activities. Therefore, the 26S proteasome plays a central role in de turnover of waste proteins but is also involved in many **signal transduction pathways** [298, 301].

8.1. The 26S proteasome

8.1.1. Structure and mode of action

The *26S proteasome* is a large **ATP-dependent proteolytic complex** in eukaryotic cells which can be localized in the cytoplasm and in the nucleus, depending on the cell type. It consists of a catalytic cylindrical core, the ***20S proteasome***, which is capped by two regulatory subunits, the ***19S regulatory complexes*** (Figure 8.1) [298].

The 20S proteasome is comprised of 28 protein subunits that are organized in four rings that create a cylindrical structure. The peptides forming the top and bottom rings are called α-subunits, and those forming the two inner rings are called β-subunits. β1, β2 and β5 contain the proteolitically active sites of the proteasome, which are facing the cavity inside the cylinder. The β1 subunit has a *caspase-like or post-acidic activity*, the β2 subunit has a *trypsin-like* activity and the β5 subunit has a *chymotrypsin-like activity.* β-subunits from the 26S proteasome are distinct from other proteases because their catalytic nucleophiles are N-terminal threonine residues, while these are normally serines, cysteine and aspartic residues or metals.

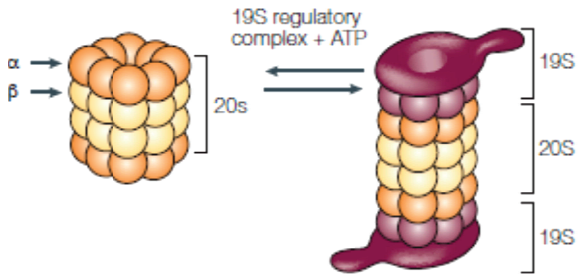

Figure 8.1. Structure of 26S proteasome. From *Adams J (2004)* [298].

The 19S regulatory complex or *19S cap* is a 20 polypeptide complex that binds to both ends of the proteasome 20S. It can be divided in two subcomplexes, the "base", which contains 6 ATPases, and the "lid". The "base" alone is able to bind to proteasome 20S and degrade peptides and non-ubiquitinated proteins, but the "lid" is necessary for the specific recognition of ubiquitinated proteins [298, 302]. After substrate recognition by the "lid", the ATPases that form the "base" direct substrate unfolding and translocation into the 20S subunit, with the corresponding hydrolysis of ATP [303]. Finally, the 20S subunit cleaves the unfolded substrate and releases short peptides and ubiquitin, which can be subsequently recycled. It is noteworthy that, under certain conditions, 20S proteasomes can form complexes with non-ATPase activators, as in the case of PA28, which forms what have been called *immune proteasomes* [302].

8.1.2. Functions and relevance in cancer: the 26S proteasome as a therapeutic target

Many substrates of the 26S proteasome are mediators of pathways that are known to be deregulated in cancer. For example, proteasomal degradation mediates **cell cycle regulation** through the proteolytic inactivation of cyclins and CDKIs, and it participates in the regulation of **apoptosis** through its effects on caspase levels, Bim stabilization and p53 or NF-κB activation [298, 304].

In spite of the fact that the proteasome is crucial for many cellular functions and seems to be a poorly specific pharmacological target, empirical evidence has shown that **cancer cells are more sensitive** to proteasome inhibition than non-cancer cells. The biological basis for the higher susceptibility of cancer cells appear to be related to the **abnormal regulation of signalling pathways** that require proteasomal degradation for their correct functioning, and to their **increased proliferation rate** [298].

Thus, proteasome inhibition would have a greater impact on cells with constitutive activation of oncogenic signalling pathways like NF-κB, which is frequently upregulated in cancer and requires proteasomal degradation to be activated (see Chapter 3 of *Introduction*). Similarly, the inhibition of

the degradation of certain proteins, like cyclins or CDKIs such as p21 and $p27^{KIP1}$, would suppress uncontrolled proliferation of tumour cells, and the upregulation of p53 and pro-apoptotic proteins from the BH-3 only family would facilitate apoptosis [298, 304].

On the other hand, by having one or more aberrant cell cycle checkpoints, tumour cells accumulate defective proteins (that fail to be folded or post-translationally modified) at much higher rate than normal cells and therefore are more dependent on the ubiquitin-proteasome system for their turnover. As it was previously commented, if misfolded or unfolded proteins fail to be degraded by the proteasome, they form aggregates and lead to **ER stress** [298, 302]. If ER-stress is too severe or sustained, it could eventually trigger apoptosis [305] or activate alternative protein degradation by autophagy, since ER-stress and autophagy are functionally coupled degradation pathways [136, 301].

Proteasomal degradation is not only important in response to ER-stress but also appears to be essential for the elimination of oxidized proteins and proteins accumulated in the mitochondria under **moderate oxidative stress** [306, 307]. Therefore, failure in proteasomal protein turnover may affect the cellular response to different types of stress and, consequently, cell survival. For instance, it has been reported that proteasome inhibitors induce the formation of cytoplasmic granules, which generally appear upon exposure of cells to various types of stress such as UV irradiation, hypoxia, oxidative stress and viral infections [308, 309], suggesting that inhibition of the proteasomal function does not only **impair cellular response to stress** but may **constitute a stress stimulus** by itself.

8.2. Proteasome inhibitors

Proteasome inhibitors are a family of drugs, from natural or synthetic origin, that have the ability to block targeted protein degradation by the 26S proteasome. For the reasons argued above, ever since their introduction in cancer research twenty years ago, proteasome inhibitors have demonstrated to be efficient antitumoral agents *in vivo* and *in vitro*, with a great clinical potential in cancer [302, 310, 311].

Most synthetic inhibitors are **short peptides that mimic proteolytic substrates**, binding to and inhibiting threonine residues in the 20S active sites. Extensive mechanistic and structural studies of proteases have led to the development of highly efficient and specific cell-permeable inhibitors, which can be classified into five families: *peptide aldehydes, peptide vinyl-sulfones, peptide boronates, peptide epoxyketones and β-lactones* (Figure 8.2) [302, 312].

Although the precise mechanisms of action of each compound are still not clearly understood and seem to diverge in different cell types, proteasome inhibitors **effectively prevent replication of DNA**

lesions and induce cell cycle arrest and subsequent apoptosis in cancer cells [310, 313-315]. Furthermore, low doses of proteasome inhibitors have been demonstrated to sensitize cells to chemotherapy [316-318].

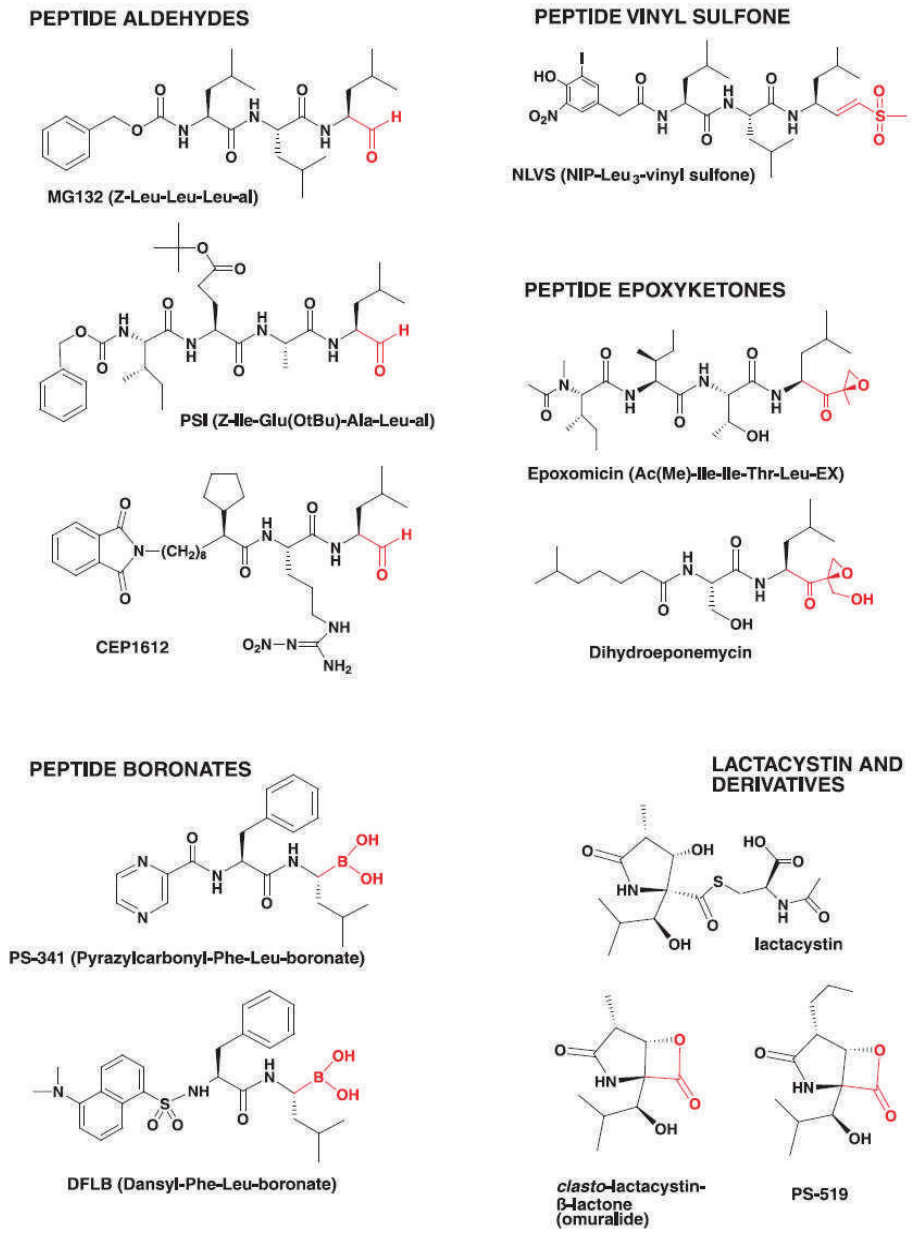

Figure 8.2. Major families of proteasome inhibitors. From *Kisselev et al. (2001)* [312]. Pharmacophores are indicated in red.

Interestingly, whereas some works have reported that proteasome inhibitors induce mitochondria-dependent apoptosis and production of ROS [319-321], many others postulate that they sensitize cells to death receptor-mediated apoptosis [316, 322, 323]. There is indeed a growing body of evidence that both **intrinsic and extrinsic apoptosis** pathways contribute to proteasome inhibitor-

induced apoptosis [305, 318, 324, 325]. Importantly, most reports agree that this apoptosis is **irrespective of the p53 status** [310, 319, 323, 326], which makes proteasome inhibitors a good choice for the treatment of cancer cells bearing p53 mutations.

One more common effect of proteasome inhibitors is **autophagy induction**. This event has been reported for inhibitors of different nature and in several cell types, suggesting that it is a direct consequence of proteasome inhibition, and this is consistent with the notion that proteasome inhibition induces ER-stress and that this might trigger autophagy [301, 327]. Nevertheless, the effects of autophagy induced by this drug family in tumor cells are not clear. It has been reported that autophagy has pro-survival effects [301, 327, 328], whereas in other works it seems to be an anti-tumor mechanism [329, 330]. Provided that the effects of autophagy in cancer cells largely depend on the initiating stimulus and on cell context [137], the consequences of proteasome inhibitor-induced autophagy will vary in each cancer type, stage and cellular environment.

With the approval of the boronic peptide bortezomib (previously known as PS-341) for the clinical treatment of multiple myeloma in 2003 [331], proteasome inhibitors have been demonstrated to be **useful for the treatment of hematologic malignancies** [332]. Interestingly, they appear to be innocuous to normal blood cells at therapeutic concentrations [322, 333-336], probably because **leukemic cells have a higher number of proteasomes** [337, 338]. Moreover, proteasome inhibitors appear to be effective in resistant and non-resistant CML cells [339] and even in refractory or relapsed AML patients [340, 341], in whom a phase II clinical study is currently open [342]. For these reasons, proteasome inhibitors appear to be a good choice to improve current frontline therapies for leukemia; consistent with this, compounds from this family have proved **utility in killing leukemia cells in combination with other drugs** [335, 336, 343, 344].

In this work, we have focused on the pharmacological properties of a proteasome inhibitor from the family of **peptide aldehyde inhibitors.** These compounds are characterized by their rapid and reversible inhibition of the chymotrypsin-like activity of the proteasome. **MG-132**, or *Z-Leu-Leu-Leucinal* (Figure 8.2, upper-left corner) is **one of the most potent inhibitors of its kind and much more selective** than others, since it requires a 10-fold higher concentration to inhibit calpains and cathepsines, which are cysteine- and serine-proteases that can be secondarily affected by proteasome inhibitors [312]. Remarkably, there is evidence that MG-132 effectively induces **p53-independent apoptosis** in human cancer cells, including leukemias, and that it **sensitizes them to chemotherapy** [305, 321, 334, 345, 346]. To sum up, MG-132 is an interesting pharmacological and research tool to improve the response of leukemia cells to current therapies and its combination with other cytotoxic drugs could be a good approach to prevent chemoresistance.

INTRODUCTION

II. GENERAL HYPOTHESES AND AIMS

GENERAL HYPOTHESES AND AIMS

1. Foundations

The discovery of recurrent chromosomal translocations in leukemias was a breakthrough for the understanding of this group of diseases and an important milestone in cancer therapy. Hematologic malignancies with a characteristic cytogenetic profile, like APL and CML, have substantially benefited from the development of targeted therapies. However, as it was explained before, current therapies are not perfect. In APL, ATRA and ATO have improved long-term survival but the acquisition of resistance to the former and the toxicity of the latter make them need to be associated with chemotherapy in order to reduce their dosages [26, 37]. Although the introduction of Ara-C in frontline therapies significantly improved survival rates, high-risk patients still need better and safer therapies, and some studies suggest that chemotherapy could be replaced by safer types of drugs [39, 40]. As for CML, in which imatinib has proved to be an extraordinarily efficient and rather safe treatment, it exhibits the problem of presenting a permanent subset of non-responders and a high relapse rate in the accelerated phase and blast crisis [54]. Second and third-generation derivates of imatinib have improved clinical outcomes in non-responders, but they are still subjected to resistance due to the mutation of the BCR-ABL gene and to the existence of leukemic stem or progenitor cell reservoirs [54, 56, 58]. **It is therefore clear that the discovery of new agents that could be effective alone or in combination with the existing therapies in leukemia is a current need and a continuous challenge.**

On the other hand, MDS is a group of diseases which is gaining relevance as its incidence rates increase [62]. However, these malignancies are still largely unknown, with the disadvantage of being highly heterogeneous, morphologically and cytogenetically [62, 64, 65]. Furthermore, the lack of established molecular targets in MDS impedes the use of targeted therapies and these syndromes are particularly refractory to chemotherapy and, up to now, non-curable [61, 64]. **For these reasons, it is essential to identify useful diagnostic and prognostic markers and pharmacological targets that allow the development of better therapies for MDS patients.**

As it was explained in the introduction section, oxidative stress is frequently increased in cancer cells [274]. For this reason and because cancer cells often have deficient anti-oxidant systems, they generally have a lower threshold of ROS-induced death. Thus, ROS-based cytotoxic strategies, such as the **treatment with DQA, selectively target cancer cells, including leukemic cells** [282, 285, 294-296]. However, DQA and its family of compounds appear to be more effective in combination with other types of drugs [285, 287, 297]. In this regard, the drug family of **proteasome inhibitors** attracted our attention as **selective inducers of growth arrest and apoptosis** in cancer cells [310,

313-315] and, more importantly, in **hematologic malignancies** [322, 333-336, 339-341]. Interestingly, the proteasome participates in the eradication of **oxidative stress** [306, 307] and prevention of **ER stress** [300], events which are deregulated upon proteasome inhibition. Furthermore, proteasome inhibitors have been reported to **sensitize cancer cells to chemotherapy** when used at low doses [316-318], and cell death induced by these compounds is **p53-independent** [323, 326]. Therefore, therapeutic combinations including these drugs are particularly interesting in the treatment of p53-deficient cancers, such as many types of AML and CML [197, 198].

In addition, as it has been emphasized in the *Introduction section*, **miRNAs** play multiple roles in the regulation of differentiation, cell growth and cell death [234, 259], and mediate stress responses and inflammation [246, 259], cellular processes which are frequently deregulated in cancer. Accordingly, they seem to participate in the **pathogenesis and development of tumors**, along with the **appearance of chemoresistance** [234, 259]. miRNAs have been reported to follow tumor-specific expression patterns in many types of cancer (reviewed in [234]), including **hematologic malignancies** [271, 272] and have emerged as **useful biomarkers and prospective therapeutic targets** [234, 263].

2. General Hypotheses

Considering all the aforementioned, this work has been founded on the following hypotheses:

1. "Cytostatic but non-lethal doses of the proteasome inhibitor MG-132 could sensitize leukemic cells to ROS-mediated apoptosis induced by DQA through the blockade of the degradation of oxidized proteins, which would exacerbate oxidative stress and cause ER stress, eventually triggering cell death".

2. "Altered miRNA regulation may participate in the events that lead to the appearance of chemoresistance in leukemic cells, as well as in the pathogenesis and progression of MDS, and therefore miRNAs could be interesting biomarkers and therapeutic targets in these hematologic malignancies".

3. General Aims

Based on these two pillars, three general aims were established for the development of this work:

I. To analyze the effects of the combination of low doses of the proteasome inhibitor MG-132 and DQA on cell viability, proliferation and death in leukemic cell lines.

II. To investigate the mechanisms of resistance to chemotherapy that may arise from the combination of MG-132 and DQA, and explore the involvement of miRNA regulation in the molecular mechanisms that contribute to chemoresistance in leukemic cell lines.

III. To study the participation of miRNAs in the pathogenesis and progression of MDS and their potential utility as biomarkers and therapeutic targets in this group of diseases.

GENERAL HYPOTHESES AND AIMS

III. MATERIALS AND METHODS

MATERIALS AND METHODS

1. Cell Cultures

All cell lines utilized in the present work are derived from humans and were maintained at 37°C in a humidified atmosphere with 5% CO_2.

For long-term storage, cells were re-suspended at a cell density of $3x10^6$ cells/mL in 2 mL of freezing medium, composed of the corresponding culture medium, containing 20% heat-inactivated[1] fetal bovine serum (FBS; Gibco®, Life Technologies, Paisley, UK) and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA). After re-suspension, cells were kept on ice and rapidly frozen at -80°C for a minimum of 24 hours and a maximum of one month. After this time, cells were transferred into a liquid phase nitrogen storage vessel.

When required, cells were thawed by warming up the cryogenic vials for a short time in a water bath at 37°C and, as soon as they detached from the walls of the vial, transferring them into 10 mL of pre-warmed (at 37°C) complete medium to dilute DMSO. Cells were then centrifuged for 3 minutes at ~160 g to remove the freezing medium and they were finally re-suspended in 10 mL of fresh, complete growth medium, plated and placed in the culturing conditions described above. Growth medium was replaced 16-24 hours after thawing, to remove death cells and cell debris.

1.1. AML cell lines

- The **NB4 cell line** was isolated from long-term cultures of APL blast cells of a relapsed patient on BM-stromal fibroblasts. They are positive for t(15;17) [347] and present the point mutation R248Q in the P53 gene [348]. NB4 cells were provided by Dr. Delgado and Dr. León (University of Cantabria, Santander, Spain). They were grown in RPMI 1640-GlutaMAX™ culture medium (Gibco®, Life Technologies, Paisley, UK), supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (Gibco®, Life Technologies, Paisley, UK). Cells were passaged every 2 days at a cell density of 3 x 10^5 cells/mL, or every 3 days at 1.5 x 10^5 cells/mL. Cells were treated at a density of 2.5-3 x 10^5 cells/mL.
- The **KG-1 cell line** is a BM-derived AML line [349] with a point mutation and a 5-base insertion in the P53 gene that affect mRNA processing [350]. They were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in IMDM medium supplemented with 20% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (HyClone, Thermo Fisher

[1] For inactivation, FBS was incubated for 30 minutes in a water bath at 56°C; then, it was allowed to cool down and used conveniently or aliquoted and stored at -20°C.

Scientific, Waltham, MA, USA). Cells were passaged every 2 days at a cell density of 3 x 10^5 cells/mL, or every 3 days at 1.5 x 10^5 cells/mL.

- Other cell lines: the APL cell line **HL-60** [351] and the myelomonocytic leukemia cell lines **THP-1** [352] and **OCI/AML3** [353] were used exclusively for nucleic acids extraction to assess their miRNA levels (see Block III of *Results and Discussion*). HL-60 and THP-1 cells were obtained from the ATCC (Manassas, VA, USA) and grown in RPMI medium, supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin; OCI/AML3 were provided by Dr. Bueso-Ramos (Department of Hemopathology, MD Anderson Cancer Center, Houston, TX, USA) and cultured in RPMI medium, supplemented with 20% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (all from HyClone, Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged every 2 days at a cell density of 3 x 10^5 cells/mL, or every 3 days at 1.5 x 10^5 cells/mL.

1.2. CML cell lines

- The **K562 cell line** is an erythroleukemic cell line derived from a patient in the acute phase of CML. It is positive for the Philadelphia chromosome (Bcr-Abl$^+$) [354] and presents a mutational inactivation of P53 gene [355]. K562 cells were provided by Dr. Delgado and Dr. León (University of Cantabria, Santander, Spain) and purchased from ATCC (Manassas, VA, USA)[2]. Cells were cultured in RPMI 1640-GlutaMAX™ (Gibco®, Life Technologies, Paisley, UK) or RPMI 1640 (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) culture medium, supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (all from Gibco®, Life Technologies, Paisley, UK or from HyClone, Thermo Fisher Scientific, Waltham, MA, USA)[3]. Cells were passaged every 2 at a cell density of 3 x 10^5 cells/mL, or every 3 days at 1.5 x 10^5 cells/mL. Prior treatment, cells were plated at a density of 2-3 x 10^5 cells/mL.
- The **Meg-01 cell line** was established from BM of a patient with CML in blast crisis. They are positive for Philadelphia chromosome (Bcr-Abl$^+$) [356] and bear a mutated P53 gene [357]. Cells were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI medium, supplemented

[2] Experiments using K562 cells were performed in two different laboratories. Cell viability assays, cell cycle analysis and gene/protein expression experiments –results Block II- were carried out in Dr. Boyano Adánez's laboratory (University of Alcalá, Madrid, Spain) whereas luciferase reporter gene and differentiation assays –results Blocks II and III- were performed in Dr. García-Manero's laboratory (MD Anderson Cancer Center, Houston, TX, USA). The two different origins for K562 cells correspond to these two locations, respectively.

[3] Variations between growth medium manufacturers used in the different laboratories did not cause significant changes in cell growth rates or viability of K562 cells.

with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (all from HyClone, Thermo Fisher Scientific, Waltham, MA, USA).

Among all the leukemia cell lines used, Meg-01 was the only one not growing in suspension, but in an *adherent fashion*. Cells were passaged whenever confluent, to maintain a logarithmic growth rate. For passaging, growth medium was removed and cells were washed with pre-warmed phosphate buffered saline (PBS; HyClone, Thermo Fisher Scientific, Waltham, MA, USA); then, PBS was removed and cells were detached from the culture dishes through incubation with Trypsin (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) for one minute at 37°C. Trypsin was rapidly diluted with pre-warmed growth medium and removed by centrifugation (3 minutes at ~160 g). Cells were then re-suspended in fresh growth medium at the appropriate density and plated in new culture dishes.

1.3. MDS cell line

- The **MDS-L cell line** is a myeloblastic sub-line derived from a myelodysplastic cell line, MDS92, which was established from BM of an MDS patient with the deletion del(5q) [358]. MDS-L cells were provided by Dr. Starczynowski (Cincinnati Children's Hospital, Cincinnati, OH, USA) and cultured in RPMI medium, supplemented with 20% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin (all from HyClone, Thermo Fisher Scientific, Waltham, MA, USA) and 10 ng/mL human IL-3 (Stem Cell Technology, Vancouver, CA, USA). MDS-L cells grow in an adherent fashion and have a rather low proliferation rate. They were passaged whenever confluent, following the same protocol explained above for Meg-01 cells, but were centrifuged at a lower speed (~50 g for 5-10 minutes) and plated at a higher density to allow an exponential growth rate.

2. Primary Samples

Control BM samples from 5 healthy individuals were obtained from AllCells (Emeryville, CA, USA). Human BM samples from 48 MDS patients referred to the Department of Leukemia of MD Anderson Cancer Center (Houston, TX, USA) were collected following institutional guidelines. Samples were obtained from the Department of Hematology, in which they had been previously processed with a MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany) for the isolation of $CD34^+$ cells. Primary BM $CD34^+$ cells were centrifuged at ~1500-2300 g for 5-7 minutes to remove the buffer from the isolation kit, and prepared for RNA extraction.

3. Treatments

All drugs and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise specified.

3.1. DQA

DQA was administered as a micellar solution, prepared as described by D'Souza et al. [293] with minor modifications. Five milliliters of a 10 mM dequalinium chloride (MW = 527,6 g/mol) stock solution in methanol was prepared in a round bottom flask. The organic solvent and soluble contaminants were removed with a rotary evaporator, rotating for 5-10 minutes at 37°C. The DQA precipitate obtained was re-suspended in 5 mM HEPES, pH 7.4, for a final concentration of 5 mM DQA. Micelles were formed by sonicating the suspension in an ultrasonic liquid processor (Misonix, Newtown, CT, USA) at maximum power for 30-60 seconds or until the resulting micellar solution was transparent and slightly opalescent. Micellar solutions were filtered using a 0.2 μM sterile filter (Merk-Millipore, Darmstadt, Germany).

Because product can be partially lost during rotation, re-suspension and sonication, the final concentration of DQA solutions was determined in a spectrofluorimeter (Perkin-Elmer LS-50 B, Waltham, MA, USA). Briefly, a series of DQA dilutions in methanol were prepared, with increasing concentrations ranking between 0.5 and 4 μM to create a standard DQA curve. The micellar solution was diluted in methanol in different ratios (1:1000, 1:2000, and so on). Fluorescence was measured with the following settings: excitation λ = 335 nm; emission λ = 360 nm; cut-off λ= 350 nm. Methanol was used as blank. Every dilution was analyzed twice and the mean fluorescence intensity was used to calculate the concentration of the stock solution. The final result was expressed as the mean value resulting from all the dilutions.

DQA stock solutions were stored at room temperature, protected from light, for no longer than one month. Aliquots were stored for longer time periods at -80°C and rapidly thawed in a water bath at 50°C to avoid precipitation of the micellar solution, which occurs if the solution is kept at temperatures lower than 10-15°C. Final concentrations used in cell cultures were 10 μM and 20 μM.

3.2. MG-132

The proteasome inhibitor MG-132 was purchased as a 10 mM DMSO solution (Calbiochem, Billerica, MA, USA) and stored at -20°C. Before use, MG-132 was freshly diluted to a working concentration of

100 μM in supplemented RPMI medium. Final concentrations used in cell cultures ranged between 0.1-1 μM. In combined treatments, cells were treated with MG-132 for 2 hours prior addition of DQA to growth medium.

3.3. Pharmacological inhibitors of p53

- **Pifithrin-α (PFT-α)** is a reversible inhibitor of p53-mediated gene transcription and p53-dependent apoptosis, which has been proposed to function by inhibiting nuclear transport of p53 [359]. PFT-α was dissolved in DMSO for a stock concentration of 10 mg/mL; aliquots were stored at -20°C and 10 mM working solution was prepared in sterile PBS before use. Final concentration used in cell cultures was 30 μM. In combined treatments, cells were treated with PFT-α one hour after addition of MG-132 and/or one hour prior addition of DQA to growth medium.
- **Pifithrin-μ (PFT-μ)** is a more specific inhibitor of p53 which shuts down the mitochondrial pathway without affecting its transcriptional activity [360]. PFT-μ was dissolved in DMSO to a stock concentration of 25 mM and stored at 4°C. Before treatment, a 10 mM working solution was prepared in sterile PBS. Final concentration used in cell cultures was 5 μM. In combined treatments, cells were treated with PFT-α one hour after addition of MG-132 and/or one hour prior addition of DQA to growth medium.

3.4. Autophagy modulators

- The autophagy inhibitor **3-methyladenine (3-MA)** blocks vesicle nucleation during the formation of autophagosomes [361, 362]. 3-MA was dissolved at 37°C in sterile PBS to a final working concentration of 30 mg/mL, aliquoted and stored at room temperature for short-term storage and at 4°C for longer periods. 3-MA precipitates rapidly upon cooling; to prevent this from occuring, working solutions were warmed up to 37°C immediately before treatment. Final concentration used in cell cultures was 5 mM. In combined treatments, cells were treated with 3-MA one hour after treatment with MG-132 and/or one hour prior addition of DQA.
- **Rapamycin (RAPA)** is an inhibitor of the kinase mTOR which induces autophagy [362, 363]. Stock solutions (supplied at 2.5 mg/mL in DMSO) were stored at -20°C; before use, RAPA was diluted to 100 μM working solutions in fresh PBS. Final concentration used in cell cultures was 1 μM. In combined treatments, cells were treated with RAPA one hour after addition of MG-132 and/or one hour prior addition of DQA to growth medium.

3.5. Other chemicals

- **N-acetyl-L-cysteine (NAC)** is a glutathione precursor used in research as an antioxidant [364]. NAC was dissolved in DMSO to obtain a stock solution with a concentration of 3 M, which was further diluted before use to a 300 mM working solution in fresh, serum-free medium. Final concentration used in cell cultures was 10 mM. In combined treatments, NAC was added to cell growth medium 3 hours before treatment with DQA.
- **LPS** and **PAM(3)CysSK(4) (PAM3)** (both from Invivogen, San Diego, CA, USA) are specific ligands (agonists) of TLR4/TLR2 [365] and TLR2 [79], respectively. LPS and PAM3 were dissolved in sterile PBS for a working concentration of 1 mg/mL and stored at -20°C. Final concentrations in cell cultures were 1 μg/mL for LPS and 0.1 μg/mL for PAM3.
- **Cytarabine or Ara-C** is a nucleotide analog frequently used in the treatment of leukemia and a well-known differentiation inducer at low doses [366]. Ara-C was dissolved in sterile PBS to obtain a 1 mM stock solution. Aliquots were stored at -20°C. Final concentration in cell cultures was 1 μM. In combined treatments, Ara-C was added to growth medium 6 hours after the treatment with miRNA inhibitors (see point 12).
- **The MyD88 inhibitory peptide (Pepinh-MYD)** is a 26-aminoacid peptide that blocks MyD88 signaling by binding to it and inhibiting its homodimerization. Pepinh-MyD88 and a **control peptide (Pepinh-Control;** both from Invivogen, San Diego, CA, USA) were dissolved in sterile PBS to prepare 1 mM stock solutions, which were stored at -20°C. Final concentration in cultured cells was 5 μM. In combined treatments, peptides were added to growth medium 2 hours after the treatment with miRNA inhibitors (see point 12).

4. Assessment of Cell Viability, Proliferation and Death

4.1. Cell viability and proliferation

Cell viability and proliferation are two parameters used to measure the health of a cell population. *Cell viability* can be defined as the number of healthy cells in a sample, and it does not distinguish between dividing or quiescent cells. On the other hand, *cell proliferation* measures the number of cells that are dividing in a culture. Neither of these techniques is adequate to assess cell death; however they are illustrative of the effect of drugs or other stimuli on the vital functions of cells [367]. Accordingly, in this work they have been utilized as a guide to estimate the cytotoxicity of

certain treatments, calculate the *inhibitory concentration 50* (IC_{50}) of compounds or analyze their effect on the number of actively dividing cells. In this context, IC_{50} is defined as the drug concentration which causes a loss of 50% of cell viability.

4.1.1. Analysis of metabolic activity of cell populations by the MTT assay

Metabolic assays are based on the premise that cellular damage results in the loss of the ability of the cell to provide energy for its metabolic functions and growth. Metabolic activity of a cell population can be measured by adding to the cell culture a chemical substrate susceptible of being metabolized by the cells and which renders a colored product whose concentration can be spectrophotometrically detected. The metabolic substrate utilized in this work is the tetrazolium salt *methylthiazoltetrazolium* (*MTT)*, which is reduced by viable cells to a dark-purple, water-insoluble formazan salt (Figure 1). When this salt is solubilized in the cell growth medium, it can be quantified by colorimetry, presenting the maximum absorbance at 570 nm [367]. Metabolically incompetent cells produce less colored product than viable (healthy) cells and this is detected as a decreased spectrophotometric signal.

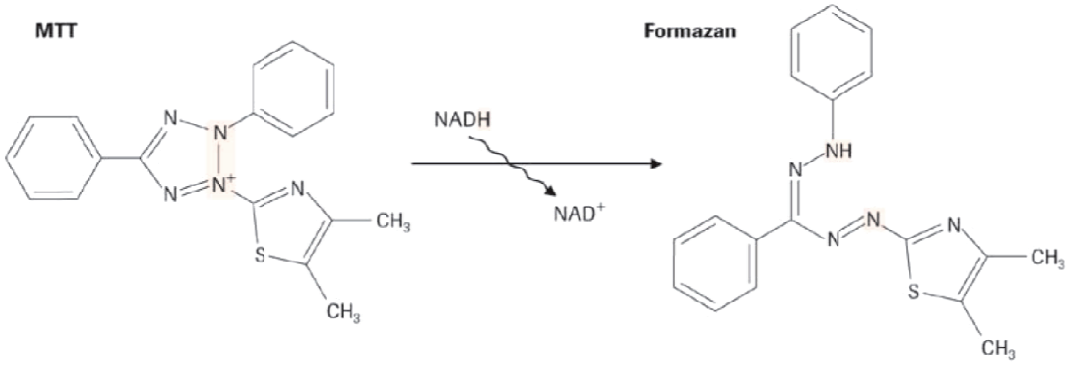

Figure 1. Molecular structure of MTT and its reaction product. From *Schulze-Osthoff (2008)* [367].

To estimate the metabolic activity of NB4 and K562 cells after treatment with MG-132, we used Cell Proliferation Kit I (MTT; Roche Applied Science, Manheim, Germany). For this assay, cells were cultured in 96-well plates in a total of 100 µL of growth medium per well, at a density of 1.2 x 10^5 cells/mL, in the presence of increasing concentrations of MG-132. Three replicates were plated for each experimental condition, as well as for a blank sample, which consisted of fresh growth medium alone. After 24 or 48 hours of treatment, 10 µL of the MTT solution were added to each well and incubated at 37°C in a humidified atmosphere for at least 4 hours to allow the reduction reaction to occur. After this incubation period, dark-purple salts could be observed at the bottom of some wells. Then, 100 µL of the solubilization solution were added to each well, and the plate was incubated at

37°C in a humidified atmosphere for a minimum of 6 hours before the spectrophotometrical quantification of the solubilized formazan. The absorbance of control cells was assigned a 100% of metabolic activity, and that of the remaining samples was calculated accordingly.

4.1.2. Study of membrane integrity (permeability assays)

Cells with damaged plasma membrane can be discriminated from viable cells through the *dye exclusion method*, which consists on the addition of a colored or fluorescent membrane-impermeable dye to the cell population. The dye penetrates and stains only those cells with altered plasma membrane permeability, whereas it is excluded from the membrane of healthy cells [367]. In this work, we used the colored exclusion dye *trypan blue* to assess the cytotoxic effects of a miRNA inhibitor (see point 12 of this section). Of note, an exclusion dye-based method was also used to complement an apoptosis-specific assay (see point 4.2.2).

To investigate the cytotoxic effects of miRNA inhibitors in K562 and MDS-L cells through the study of membrane integrity, cells were stained 1:1 with a 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) and placed in a hemocytometer. Healthy cells were visualized as unstained, bright spheres whereas non-viable cells were visualized as dark-blue spheres. Both cell populations were counted to calculate the percentage of viable cells of each sample. An example cell count is shown in figure 2. Every condition was counted twice and the mean value was used for the final statistical analysis of all the experiments.

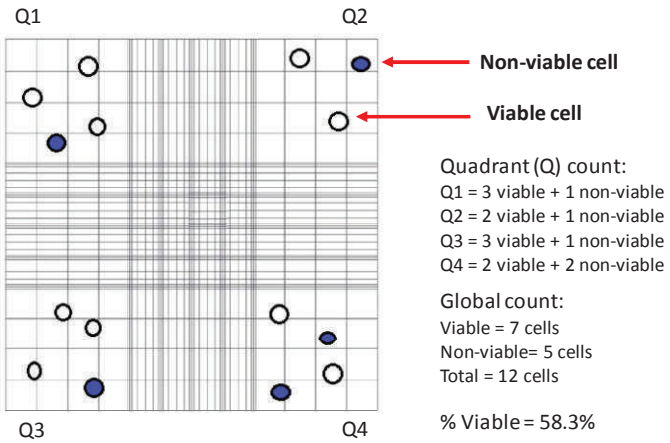

Figure 2. Example cell count with trypan blue in a hemocytometer. Modified from *Schulze-Osthoff (2008)* [367].

4.1.3. Proliferation assay through the measurement of DNA content

The analysis of cell cycle phase distribution in a cell population can be performed through the detection of the DNA content of each single cell. Cells in G_0/G_1 phase have a single copy of their genome (*2n*, since they have two chromosomes of each kind), whereas cells in G_2/M have two copies (*4n*) and therefore will have double DNA content. Any cell with DNA content between that of 2n and 4n is in the S phase. Importantly, the detection of DNA content allows the simultaneous analysis of DNA fragmentation in dead cells, and therefore can be used to estimate the proportion of apoptotic cells within a population (see point 4.2.1) [367].

DNA content of individual cells within a cell population can be determined by staining cells with a DNA intercalator which can be subsequently detected by a specific method. *Propidium iodide* (PI) is a small, fluorescent DNA intercalator which accumulates in the cell nucleus in amounts which are proportional to the DNA content of each cell. Therefore, the fluorescence intensity of PI bound to DNA (excitation λ=535 nm, emission λ=617 nm) within each cell is proportional to the DNA content of each single cell. Fluorescence of individual cells within a population can be detected by flow cytometry, and the DNA content of the cell population can be plotted in a histogram, as illustrated in Figure 3. Importantly, PI is impermeable to cell membrane and therefore needs the presence of a permeabilizing agent in the medium to open pores in the membrane which allow its internalization. This property of PI has been used to utilize it as an exclusion dye (see points 4.1.2 and 4.2.2).

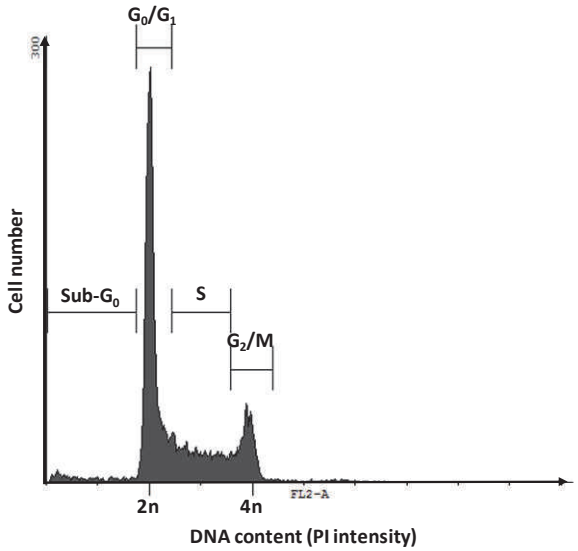

Figure 3. Example DNA content histogram of cells stained with propidium iodide (PI).

In this work, we utilized this method to analyze the cell cycle profile in NB4 and K562 cells after treatment with MG-132. For this assay, cells were cultured in 12- or 24-well plates, at a cell density of 1.5 x 10^5 cells/mL, and were allowed to grow for 12-16 hours before treatment. Following incubation

with the corresponding treatment, cells were collected and centrifuged at ~160 g for 3 minutes. When growth medium was removed, cells were washed by adding 0.5 mL of ice-cold PBS and centrifuging again[4]. Following PBS removal, cells were permeabilized at room temperature with a PBS solution containing 0.1% NP-40 (Boehringer-Ingelheim GmbH., Mannheim, Germany) and 0.1 mg/mL Ribonuclease (RNase)A (Sigma-Aldrich St. Louis, MO, USA; stock concentration 1 mg/mL in PBS). After 30 minutes of incubation, PI (Biotium, Hayward, CA, USA) was added (final concentration 20 μg/mL) and flow cytometry analysis was immediately performed in a FACScalibur cytometer[5] (Becton-Dickinson, Franklin Lakes, NJ, USA; excitation laser λ=488 nm, detector FL-2 λ=585 nm).

4.1.4. Proliferation assay through the measurement of DNA synthesis

DNA synthesis is a reliable indicator of cell growth. It can be detected using radioactive or non-radioactive nucleotide analogs, which are incorporated into DNA of actively proliferating cells and measured by a specific assay [367]. We assessed active DNA synthesis for an in-depth study of changes in cell cycle progression in K562 cells. For this purpose, we utilized the DNA analog *5-bromo-2'-deoxyuridine (BrdU)*, which is incorporated into DNA like thymidine. Because BrdU is not a fluorescent molecule, it needs to be labeled after incorporation to DNA. In the method used in our experiments, BrdU was labeled using a specific anti-BrdU antibody bound to the fluorochrome FITC, which emits green fluorescence (excitation λ=493 nm, emission λ=525 nm) and can be detected by flow cytometry. In this assay, the measurement of BrdU uptake was coupled to the detection of DNA content, as explained in point 4.1.3, which allowed us to obtain an accurate cell cycle distribution profile.

Cells were cultured as explained in point 4.1.3 and incubated with the corresponding treatments. After 24 or 48 hours, cells were treated with 5 μg/mL BrdU (Roche Applied Science, Basel, Switzerland; stock concentration 3 mg/mL in PBS) and incubated for 1 hour at 37°C in a humidified atmosphere to allow the incorporation of BrdU into the cells. A BrdU-free well was included as a control of the unspecific activity of the antibody. After incubation, cells were collected and washed with 1 mL PBS as indicated in point 4.1.3. Dry cell pellets were fixated with ice-cold 80% ethanol for 1 hour at 4°C. Because the antibody recognizes and binds to single-stranded DNA, cellular DNA was denatured with 1 mL of 2 M HCl for 30 minutes at room temperature, mixing sporadically. HCl was

[4] This culturing, collection and washing method was used for every flow cytometry experiment preformed in this work and therefore will not be explained again in this section.

[5] This equipment was used in every flow cytometry experiment, so it will not be referred to in the subsequent points.

removed and cells were washed three times with PBS and one last time with a PBS/0.1% bovine-serum albumin (BSA)/0.2% Tween 20 solution. Following removal of the supernatant, cells were incubated for 30 minutes, in the dark, with PBS/BSA/Tween 20 containing 5 μg/mL of anti-BrdU-FITC (Beckton-Dickinson Biosciences, San Diego, CA, USA). Cells were then washed with the PBS/BSA/Tween 20 solution twice more and, after removal of the washing solution, they were incubated with 0.5 mL of 0.1 mg/mL RNase A for 15 minutes, at room temperature. Last, PI was added extemporaneously (final concentration 20 μg/mL) before flow cytometry analysis (FITC excitation laser λ=488 nm, detector FL-1 λ=530 nm; PI excitation laser λ=488 nm, detector FL-3 λ=650 nm).

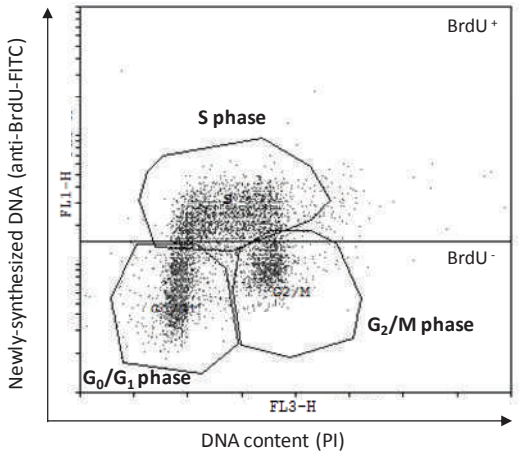

Figure 4. Example plot of DNA synthesis analysis with BrdU and PI

Results from the flow cytometry analysis of the fluorescence of anti-BrdU-FITC and PI can be represented in a dot plot graph (Figure 4), in which each cell is visualized as a point in the graph and cell populations corresponding to the different stages of cell cycle can be identified and quantified.

4.1.5. Clonogenic assays

Clonogenic assays determine long-term viability of cultured cells, in terms of their ability to form colonies *in vitro*. They are also useful to detect signs of differentiation in a viable cell population, since differentiating cells progressively lose their proliferative capacity, as it was explained in Chapter 1 of the *Introduction* section. We used clonogenic assays to test the loss of self-renewal potential of K562 and MDS-L cells.

As a semi-solid medium for these assays, the ready-to-use *methylcellulose (MC)* MethoCult GF H4434 (Stem Cell Technologies, Vancouver, BC, Canada) was used. This medium was stored at -20°C and required to slowly thaw at 4°C or room temperature. After thawing, MC was vigorously vortexed for

2 minutes to re-homogenize it and let stand at room temperature for at least 20 minutes, or until all the air bubbles had moved to the surface.

When MC was ready, 1 mL aliquots were transferred to as many conical sterile tubes as needed (one for each experimental condition), using a 6 mL syringe with a 16-gauge blunt-end needle. Cells, incubated for 48 hours with the respective treatments, were counted as indicated in point 4.1.2 and a portion of cell-containing growth medium was diluted with the required volume of fresh medium to obtain a cell suspension with a density of 2500 cells/mL. Two hundred microliters of cell suspension from each experimental condition were transferred into each MC tube, which was then vortexed at very low speed for 5 seconds to allow the homogenization of the MC-cell medium mix and allowed to settle for 5 minutes. After this time, MC from each tube was evenly distributed in 2 wells of a 12-well plate (0.5 mL each) using the syringe and making sure that no air bubbles formed within the MC drop. Thus, each experimental condition was plated in duplicates. A variable number of wells (3-4) from every plate were left empty and, afterwards, filled up with pre-warmed PBS to preserve humidity in the plate and prevent MC from dehydration. Colonies were allowed to grow at 37°C and 5% CO_2 for at least 4 days for K562 cells and 7 days for MDS-L cells, time after which they were counted in a phase-contrast microscope and subsequently collected for RNA extraction by repeatedly washing the wells with ice-cold PBS and collecting the dissolved MC.

4.2. Cell death

In this project, we have focused on the study of *apoptosis* as the main mechanism of cell death induction. Therefore, we have used techniques that allowed us to estimate the percentage of apoptotic cells in a population:

4.2.1. Measurement of DNA fragmentation by detection of hypodiploid DNA

The internucleosomal cleavage of DNA by nucleases is a classical feature of apoptosis [123] and can be studied through the detection of nuclei with DNA content lower than *2n* (hyplodiploid or sub-G_0 DNA content; see Figure 3). The number of cells with hypodiploid DNA content was determined by flow cytometry of permeabilized cells stained with PI. This method was previously explained in point 4.1.3.

4.2.2. Detection of phosphatidylserine (PS) externalization and membrane breakup

The exposure of the phospholipid PS to the outer cell membrane is another characteristic feature of apoptosis, and is a signal for circulating macrophagues to phagocyte dying cells *in vivo* [368]. PS externalization can be detected by flow cytometry through the incubation of cells with the calcium-dependent phospholipid-binding protein Annexin-V, which has high affinity for PS [367]. In this work, apoptosis was analyzed through the detection of externalized PS-bound Annexin-V, labeled with FITC. This analysis was completed with the simultaneous study of the compromise of membrane integrity (see point 4.1.2) by double-staining the cells with PI, which emits red fluorescence.

Results from the Annexin-V/PI double-staining assay are generally represented in a *dot plot* graph, in which four sub-populations of cells can be identified according to their dye incorporation (Figure 5): cells with negative Annexin-V and PI staining (-/-) are considered as *healthy cells*, with intact membrane integrity and no signs of PS exposure; cells positive for Annexin-V and negative for PI (+/-) are purely apoptotic cells, also called *early apoptotic*; cells positive for both markers (+/+) are cells undergoing *late apoptosis, also called secondary necrosis* because, in an *in vitro* model, apoptotic cells cannot be removed from the medium by phagocytosis and the process of self-destruction culminates with the disruption of plasma membrane, which allows PI to stain the dying cell. Last, cells which are negative for Annexin-V and positive for PI (-/+) are purely *necrotic cells*. Of note, during analysis of flow cytometry data, cell populations are selected according to their size and cytoplasmic complexity to exclude cellular particles or fragments (cell debris).

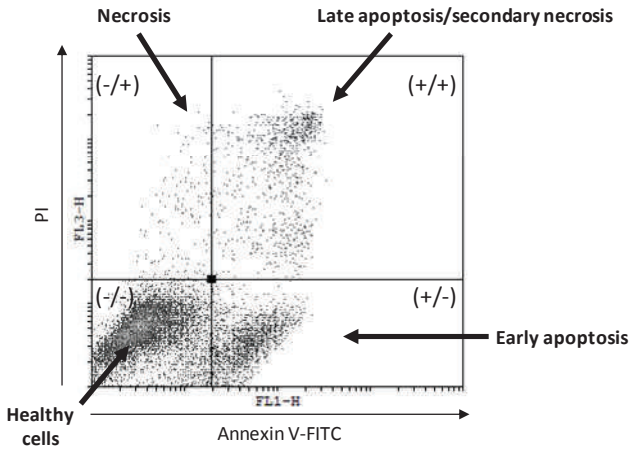

Figure 5. Example of a dot plot analysis of an Annexin V-FITC/PI double-staining assay.

For this assay, cells were cultured, collected and washed with PBS as described in point 4.1.3, and subsequently incubated for 5 minutes, in the dark, with 0.5 mL of a specific Annexin V-binding buffer from the Annexin V-FITC apoptosis kit (BioVision Inc., Milpitas, CA, USA), containing 1 µL of Annexin

V-FITC. After this time, PI was added extemporaneously at a final concentration of 20 µg/mL and flow cytometry analysis was performed (Annexin V-FITC excitation laser λ=488 nm, detector FL-1 λ=530 nm; PI excitation laser λ=488 nm, detector FL-3 λ=650 nm).

4.2.3. Analysis of the loss of the mitochondrial transmembrane potential

One of the distinctive features of the intrinsic or mitochondrial pathway of apoptosis is the permeabilization of the mitochondrial outer membrane by pro-apoptotic members of the Bcl-2 protein family, and the subsequent release of cytochrome c into the cytosol and loss of **the** mitochondrial transmembrane potential ($\Delta\Psi_m$) [123]. This last event is a good marker of apoptosis, and can also allow the estimation of mitochondrial damage induced by oxidative stress [367].

In this work, the measurement of the loss of $\Delta\Psi_m$ at early and late times after treatment was used to study the induction of apoptosis through the intrinsic pathway and also the direct targeting of mitochondria by oxidative stressors. For a detailed description of the technique, see point 5.1.

5. Determination of Intracellular Oxidative Stress

5.1. Analysis of the loss of the mitochondrial transmembrane potential

To evaluate $\Delta\Psi_m$, cells were plated as described in point 4.1.3 for other flow cytometry-based analyses and incubated with the corresponding treatments for 3, 6, 12, 24 or 48 hours. For the last 20 minutes of treatment, cells were stained with the fluorescent lipophilic cation *rhodamine 123*, which localizes to the inner membrane of functional mitochondria (see Chapter 7 of *Introduction*).

Rhodamine 123 (Sigma-Aldrich, St. Louis, MO, USA; stock solution 1.25 mg/mL in ethanol) was added at a final concentration of 1 µg/mL and incubated for 20 minutes at 37°C, in the dark. Cells were then collected and washed as indicated in point 4.1.3 to remove the fluorescent probe from the medium. This process was performed protecting cells from the dark in every possible moment. Last, cells were re-suspended in 0.5 mL of PBS and fluorescence was measured by flow cytometry (excitation λ = 511 nm; emission λ = 534 nm; detector FL-1 λ = 530 nm). Loss of $\Delta\Psi_m$ was interpreted as a loss of rhodamine 123-binding to the inner mitochondrial membrane and therefore as a decrease in fluorescence intensity. The fluorescence intensity value of control was assigned a 100% $\Delta\Psi_m$, and that of the remaining experimental conditions were calculated accordingly.

5.2. Assessment of superoxide anion levels

The intracellular accumulation of $O_2^{\cdot-}$ was determined using the fluorescent probe *dihydroethidium (DHE),* which is permeable to cell membrane and emits blue fluorescence in the cytoplasm. When DHE is oxidized by intracellular $O_2^{\cdot-}$, it intercalates with cellular DNA and emits a bright red fluorescence. In this technique, the red fluorescence of oxidized DHE can be detected and is proportional to the intracellular amount of oxidants, namely $O_2^{\cdot-}$ [369].

Similarly to the $\Delta\Psi_m$ assays, cells were cultured and incubated with the corresponding treatments for 3, 6, 12, 24 or 48 hours, times after which they were collected, washed and re-suspended in 0.5 mL of a PBS solution containing 2 μM DHE (Molecular Probes, Life Technologies, Paisley, UK). Samples were incubated for 20 minutes at 37°C, in the dark, and then fluorescence intensity was immediately measured by flow cytometry (red fluorescence: excitation λ = 535 nm; emission λ = 610 nm; detector FL-2 λ = 585 nm). The fluorescence intensity value of control was assigned a 100% $O_2^{\cdot-}$ concentration (in terms of basal levels), and the levels of the remaining experimental conditions were calculated accordingly.

6. Gene and Protein Expression Assays

For all gene and protein expression assays, cells were plated in 6-well plates at a cell density of 1.5 x 10^5 cells/mL and were allowed to grow for 12-16 hours before treatment. After the corresponding incubation times, cells were collected and centrifuged for 3 minutes at ~160 g. Growth medium was removed and cells were washed with PBS and centrifuged prior lysis with the appropriate method.

6.1. Analysis of gene expression by quantitative PCR

A) RNA isolation

Dry cell pellets were lysed with Trizol (Sigma-Aldrich St. Louis, MO, USA or Invitrogen, Carlsbad, CA[6]), in a variable volume (0.5-1 mL), depending on the size of the pellet. Trizol homogenates were incubated on ice for 5 minutes and then 200 μL of chloroform were added per 1 mL of Trizol to extract the lipophilic fraction of cell components. Tubes were immediately vortexed at high speed for 15 seconds, and allowed to stand on ice for 3 minutes. Lipophilic/aqueous phases were separated by centrifugation at ~14500 g for 15 minutes at 4°C. The aqueous phase, containing nucleic acids, was

[6] Different manufacturers were used in the different laboratories where these experiments were carried out.

carefully extracted and RNA was precipitated by adding 0.5 mL of isopropyl alcohol per 1 mL of Trizol used. Tubes were mixed by inversion, incubated for 10 minutes at room temperature and then centrifuged at ~14500 g and 4°C for 15 minutes. After removal of the supernatant, RNA pellets were washed twice with 1 mL of 75% ethanol for each 1 mL of Trizol used and subsequently centrifuged for 5 minutes at ~19800 g. This step was performed twice to gain RNA purity. As much supernatant as possible was removed and, afterwards, RNA pellets were allowed to air-dry in a chemical hood for 5-10 minutes and dissolved in DNase- and RNase-free sterile water. Final concentration and purity of the RNA were measured using the spectrophotometer Nanodrop (Thermo Fisher Scientific, Waltham, MA). If not immediately used, RNA was stored at -80°C.

B) Reverse transcription

cDNA was synthesized using High Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's indications. For assays involving protein-coding genes, 1 μg of RNA was used per reaction. For assays involving miRNA genes, 400 ng of RNA were used per reaction, and random primers included in the RT kit were replaced with specific TaqMan® miRNA-Expression Assays (Applied Biosystems, Life Technologies, Carlsbad, CA, USA; detailed in step C). Because in the case of miRNA gene RT it is essential to include a control RNA gene, two miRNA-specific RT primers were included in each reaction tube: one corresponding to the miRNA under investigation, and one expression control. In our experiments, the small nuclear U6 (snU6) RNA was utilized as an expression control for miRNA genes. In both cases (protein-coding and miRNA genes), the final volume per tube was adjusted to 15 μL. Table 1 summarizes the conditions used for RT in the PCR thermal block:

Table 1. RT conditions

Protein-coding genes	miRNA genes
25°C for 10 minutes	16°C for 30 minutes
37°C for 2 hours	42°C for 30 minutes
85°C for 5 minutes	85°C for 5 minutes
Keep at 4°C until end of run	Keep at 4°C until end of run
Estimated time: 2 hours 15 minutes	Estimated time: 1 hour 5 minutes

If not immediately used, cDNA was stored at -20°C.

C) Quantitative PCR

Quantitative *polymerase-chain reaction (qPCR)* or real-time PCR is a technique that amplifies DNA *in vitro* to allow the detection and quantification of cDNA (and therefore, mRNA) from a set of samples. It was termed "real-time" PCR because it allows data collection during the progress of the DNA amplification and not only at the end, providing an accurate comparison between the initial cDNA levels in each sample. qPCR allows the relative quantification of a target sequence relative to a control sample. The quantification is based on the detection of a fluorescent probe or dye that binds to DNA [370]. In this work, the TaqMan® probe system from Applied Biosystems (Life Technologies, Carlsbad, CA, USA) was utilized.

qPCR was performed in a 7500 FAST® Real Time PCR System using TaqMan® Universal PCR Mastermix and TaqMan® probes for the set of genes detailed in Table 2, (all from Applied Biosystems, Life Technologies, Carlsbad, CA, USA), according to the protocol from the manufacturer. Expression levels of protein-coding genes and miRNAs were normalized to those of GAPDH and snU6, respectively.

Table 2. List of genes assayed by qPCR

Protein-coding genes		
Gene symbol	**Gene name**	
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	
BAX	BCL2-associated X protein	
DRAM1	DNA-damage regulated autophagy modulator 1	
TLR2	Toll-like receptor 2	
TLR7	Toll-like receptor 7	
MYD88	Myeloid differentiation primary response gene (88)	
KDM6B	Lysine (K)-specific demethylase 6B (JMJD3)	
EPOR	Erythropoietin receptor	
GYPA	Glycophorin A	
TFRC	Transferrin receptor (p90, CD71)	
SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1 (PU.1)	
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit) (CD11b)	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	
miRNA genes (assay name)		
Hsa-miR-17-5p	Hsa-miR-106b	Hsa-let-7e
Hsa-miR-20a	Hsa-miR-125a	U6 snRNA (snU6)
Hsa-miR-22	Hsa-miR-125b	
Hsa-miR-93	Hsa-miR-99b	

Every qPCR reaction was adjusted to a final volume of 15 µL, containing 1 µL of cDNA. Prior amplification, total cDNA (from protein-coding genes) was diluted 1:5 or 1:10 in sterile water; cDNA

from miRNA genes was not further diluted[7]. Each condition was run in triplicates to minimize variability. The detailed cycles of the qPCR run are indicated in Table 3. The number of cycles was 40 for protein-coding genes and 50 for miRNA genes.

Table 3. qPCR conditions

Stage	Temperature	Time
Hold	95°C	10 min
Cycles (each)	95°C	15 sec
	60°C	1 min

6.2. Study of protein expression by Western blot

A) Preparation of protein lysates and protein quantification

- For whole-protein extracts, cell pellets were re-suspended in 50-100 µL (depending on pellet size) of NP-40-based lysis buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 20 mM NaF, 1% NP-40 (Boehringer-Ingelheim GmbH., Mannheim, Germany) and 1 mM EDTA pH 8.0. The protease inhibitors sodium ortovanadate (1 mM), phenylmethanesulfonyl fluoride (PMSF; 0.1 mg/mL), leupeptin (10 µg/mL) and aprotinin (10 µg/mL; all from Sigma-Aldrich St. Louis, MO, USA) were added extemporaneously. Samples were incubated on ice for 30 minutes, with occasional vortexing. After this time, they were centrifuged at ~19800 g for 20 minutes at 4°C and the supernatant was collected. If not immediately used, protein lysates were stored at -20°C.
- Nuclear protein fractions were obtained using the Nuclear/Cytosol Fractionation Kit (BioVision Inc., Milpitas, CA, USA), according to the manufacturer's instructions. If not immediately used, protein lysates were stored at -20°C.
- Protein quantification was performed by Bradford assay with a protein-assay reagent concentrate (Bio-Rad, Hercules, CA, USA). Briefly, a protein concentration standard curve was prepared in triplicates with increasing concentrations of BSA (Sigma-Aldrich St. Louis, MO, USA), ranging from 0.2 to 1 mg/mL. Five microliters of BSA were pipetted per well in a 96-well plate, including a BSA-free (water-only) point as a blank. Next, protein lysates were diluted 1:5 or 1:10 with distilled water and 5 µL of the dilutions were pipetted per well, also in triplicates. Then, 200 µL of Bradford reagent were added to each well and the plate was incubated for 10 minutes at room temperature with shaking. Spectrophotometry was performed in a plate reader at 595 nm.

[7] Of note, although more than one miRNA RT primers were mixed in the same RT reaction, qPCR reactions must be performed in different wells to detect both genes separately.

B) Western blot analysis

Protein samples were prepared by diluting 5x Laemmli buffer with 30-40 μg of protein lysates and the corresponding volume of distilled water, for a final composition of 62.5 mM Tris pH 6.5, 2% SDS and 10% glycerol. β-mercaptoethanol (4%) and bromophenol blue (0.1%) were added extemporaneously (all from Sigma-Aldrich St. Louis, MO, USA).

Briefly, protein samples were incubated for 5 minutes at 100°C, spinned down and loaded into acrylamide-polyacrilamide (30:1.6) gels at the required concentration (10%, 12.5% or 15%). SDS-PAGE was run for 1 hour at 120 V in a Mini-PROTEAN® electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins were then transferred onto nitrocellulose membranes by wet electroblotting using a Mini Trans-Blot® Cell system (Bio-Rad, Hercules, CA, USA), for 90 minutes at 75 V, on ice.

Membranes were blocked with a 5% non-fat milk-Tris buffered saline (TBS) solution for 1 hour, at room temperature with shaking, and then incubated overnight with the respective primary antibodies (see Table 4), at 4°C with shaking. After this time, membranes were washed with a Tween 20-TBS (TTBS) solution (five times, 5 minutes each) and incubated with the corresponding secondary IRDye® antibodies (anti-mouse or anti-rabbit; LI-COR Biosciences, Lincoln, NE, USA) for 1 hour, at room temperature, in the dark and with shaking. Both primary and secondary antibodies were dissolved in 5% non-fat milk-TTBS. Prior protein detection, membranes were washed again with TTBS four times (5 minutes each), and one last time with PBS to remove Tween 20 from the medium.

Last, membranes were scanned and protein bands were visualized using Oddysey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). α-tubulin and SAM68 were used as loading controls of whole-protein and nuclear protein lysates, respectively.

Table 4. List of antibodies used in Western blot

Target protein	Dilution	Manufacturer
p53	1:10000	Sigma-Aldrich, St. Louis, MO, USA
Nrf2	1:1000	Abcam, Cambridge, MA, USA
SOD-2	1:2000	Santa Cruz Biotechnology, Santa Cruz, CA, USA
γGCSc	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Bcn-1	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA, USA
LC3α	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA, USA
p21	1:300	Sigma-Aldrich, St. Louis, MO, USA
Cyclin B1	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA, USA
p27	1:1500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
α-tubulin	1:10000	Sigma-Aldrich, St. Louis, MO, USA
SAM68	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA, USA

7. Autophagy-related assays

7.1. Fluorescence microscopy

Autophagy levels in individual cells can be estimated through the staining of AVO with specific fluorescent molecules [362]. The lysosomotropic agent *acridine orange (AO)* has been widely used for the detection of autophagy due to its accumulation and selective red fluorescence in acidic vesicles such as lysosomes and autophagolysosomes. AO also binds to cellular DNA, yielding green fluorescence which is brighter upon chromatin condensation and which can be used to visualize apoptotic cells [371, 372]. *Monodansylcadaverine (MDC)* stains autophagolysosomes due to ion trapping in acidic compartments and increased relative fluorescence in hydrophobic environments [373, 374]. In this work, we combined the staining of fresh cells with both agents to visualize autophagosomes.

For fluorescence microscopy assays, cells were cultured, collected and washed as reported in point 4.1.3. Following centrifugation and removal of PBS, cells were re-suspended in fresh PBS and stained with either 1 μg/mL AO (stock solution, 0.1 mg/mL in PBS) or 50 μM MDC (stock solution, 50 mM in methanol; working solution, 5 mM in PBS). Both stains were purchased to Sigma-Aldrich (St. Louis, MO, USA). Samples were incubated for 20 or 30 minutes, respectively, at 37°C (in the dark) and then they were centrifuged, washed with PBS and re-suspended in a small volume of PBS to concentrate them (volume varied with pellet size). Microscope slides were prepared with one drop of freshly stained cells and coverslips were sealed. Slides were immediately visualized in the confocal microscope Leica TCS-SL (Leica Microsystems, Wetzlar, Germany) using an argon laser (excitation λ = 488 nm).

7.2. Detection of proteins involved in the initiation and progression of autophagy

The visualization of AVO was complemented with the detection of the autophagy-related proteins LC3α and Bcn-1 by Western blot. The corresponding method is described in point 6.2.

8. Bioinformatics

Potential miRNA interactions with the 3'UTR of human P21 mRNA were screened *in silico* using the miRNA target prediction database DIANA miRGen [375] and the independent target prediction algorithms miRanda (microrna.org) [376], PicTar [377] and TargetScanS [378]. An individual search

was carried out using each algorithm, and the final candidate lists were compared to each other and to the integrated list by DIANA miRGen. Those miRNAs which appeared as high-score hits in more than one algorithm were selected as possible candidates. More information is provided in the *Results and Discussion* section (Block II).

9. Vector construction

The 3'UTR of the p21 mRNA is a 1539 bp fragment covering bp 9342-10880 of the mRNA. To construct a reporter plasmid containing p21 3'UTR for luciferase assays, a 776 bp fragment covering bp 9742-10518 of the whole primary assembly (bp 401-1176 in the 3'UTR) and containing the predicted miR-22 binding site (bp 10292-10298 in the whole assemby; bp 951-957 in the 3'UTR) was cloned from human genomic DNA. The whole 3'UTR sequence, the fragment cloned and miR-22 binding site are detailed in Figure 6A. For cloning, we designed specific primers using the Primer 3 Input online tool [379, 380] and acquired them in Sigma-Aldrich (St. Louis, MO, USA). Each primer contained restriction sites for XbaI downstream of the reporter gene. Cloning primer sequences are detailed in Figure 6B.

Cloning was performed by PCR using Phusion® High-Fidelity PCR Mastermix with the HF Buffer (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's specifications and using 200 ng DNA per reaction. Cloning products (inserts) were separated by electrophoresis in 1% agarose gels and purified using QIAquick® Gel Extraction kit (Qiagen, Hilden, Germany). Inserts were amplified by cloning them into a pCR®-Blunt cloning vector (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, and by subsequent transformation of the supercompetent *Escherichia coli* bacteriae TOP10 OneShot® (Invitrogen, Carlsbad, CA, USA). Following incubation of the transfected bacteriae, a few colonies were selected and allowed to grow in lysogeny broth (LB) medium (10 mg/mL tryptone, 5 mg/mL yeast extract, 10 mg/mL NaCl) for 24 hours, at 37 °C and with shaking. After this time, plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced to check the fidelity of cloning[8]. Sequencing primers were designed using the Primer 3 Input online tool and purchased to Sigma-Aldrich (St. Louis, MO, USA).

Next, pCR®-Blunt vectors were digested with the restriction enzyme XbaI and the restriction buffer NEBuffer 4 (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's specifications, and were gel-purified using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). Simultaneously, the reporter plasmid pGL3 (Promega, Madison, WI, USA), which contains a firefly

[8] Sequencing was performed by Lone Star Labs Inc. (Houston, TX, USA).

luciferase gene (*luc+*), was digested with the same restriction enzyme and purified as well. The insert and the pGL3 vector were ligated using Roche™ Rapid DNA Ligation kit (Roche Applied Science, Manheim, Germany), following the manufacturer's instructions and using 150 ng of the insert, in a ratio 1:3 with the vector. Both pGL3 vector and the product of ligation are represented in Figure 7.

A

```
   1 TCCGCCCACAGGAAGCCTGCAGTCCTGGAAGCGCGAGGGCCTCAAAGGCCCGCTCTACAT
  61 CTTCTGCCTTAGTCTCAGTTTGTGTGTCTTAATTATTATTTGTGTTTTAATTTAAACACC
 121 TCCTCATGTACATACCCTGGCCGCCCCCTGCCCCCCAGCCTCTGGCATTAGAATTATTTA
 181 AACAAAAACTAGGCGGTTGAATGAGAGGTTCCTAAGAGTGCTGGGCATTTTTATTTTATG
 241 AAATACTATTTAAAGCCTCCTCATCCCGTGTTCTCCTTTTCCTCTCTCCCGGAGGTTGGG
 301 TGGGCCGGCTTCATGCCAGCTACTTCCTCCTCCCCACTTGTCCGCTGGGTGGTACCCTCT
 361 GGAGGGGTGTGGCTCCTTCCCATCGCTGTCACAGGCGGTTATGAAATTCACCCCCTTTCC
 421 TGGACACTCAGACCTGAATTCTTTTTCATTTGAGAAGTAAACAGATGGCACTTTGAAGGG
 481 GCCTCACCGAGTGGGGGCATCATCAAAAACTTTGGAGTCCCCTCACCTCCTCTAAGGTTG
 541 GGCAGGGTGACCCTGAAGTGAGCACAGCCTAGGGCTGAGCTGGGGACCTGGTACCCTCCT
 601 GGCTCTTGATACCCCCCTCTGTCTTGTGAAGGCAGGGGGAAGGTGGGGTCCTGGAGCAGA
 661 CCACCCCGCCTGCCCTCATGGCCCCTCTGACCTGCACTGGGGAGCCCGTCTCAGTGTTGA
 721 GCCTTTTCCCTCTTTGGCTCCCCTGTACCTTTTGAGGAGCCCCAGCTACCCTTTTTCTCC
 781 AGCTGGGCTCTGCAATTCCCCTCTGCTGCTGTCCCTCCCCCTTGTCCTTTCCCTTCAGTA
 841 CCCTCTCAGCTCCAGGTGGCTCTGAGGTGCCTGTCCCACCCCCACCCCCAGCTCAATGGA
 901 CTGGAAGGGGAAGGGACACACAAGAAGAAGGGCACCCTAGTTCTACCTCAGGCAGCTCAA
 961 GCAGCGACCGCCCCCTCCTCTAGCTGTGGGGGTGAGGGTCCCATGTGGTGGCACAGGCCC
1021 CCTTGAGTGGGGTTATCTCTGTGTTAGGGGTATATGATGGGGGAGTAGATCTTTCTAGGA
1081 GGGAGACACTGGCCCCTCAAATCGTCCAGCGACCTTCCTCATCCACCCCATCCCTCCCCA
1141 GTTCATTGCACTTTGATTAGCAGCGGAACAAGGAGTCAGACATTTTAAGATGGTGGCAGT
1201 AGAGGCTATGGACAGGGCATGCCACGTGGGCTCATATGGGGCTGGGAGTAGTTGTCTTTC
1261 CTGGCACTAACGTTGAGCCCCTGGAGGCACTGAAGTGCTTAGTGTACTTGGAGTATTGGG
1321 GTCTGACCCCAAACACCTTCCAGCTCCTGTAACATACTGGCCTGGACTGTTTTCTCTCGG
1381 CTCCCCATGTGTCCTGGTTCCCGTTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAGGG
1441 ACCACACCCTGTACTGTTCTGTGTCTTTCACAGCTCCTCCCACAATGCTGAATATACAGC
1501 AGGTGCTCAATAAATGATTCTTAGTGACTTTACTTGTAA
```

B

Left primer (fw)	TCTAGAATGAAATTCACCCCCTTTCC (26pb)
Right primer (rv)	TCTAGAACTCCTTGTTCCGCTGCTAA (26pb)

Figure 6. (A) p21 3'UTR sequence. The region highlighted in grey corresponds to the fragment cloned in the luciferase vector, with the cloning primers binding sites highlighted in yellow. The 7-mer highlighted in blue is the predicted binding site for miR-22. **(B) Sequences of the cloning primers for the p21 3'UTR fragment.** Fragments highlighted in pink indicate restriction sites for XbaI**.**

Following ligation, vectors were amplified by supercompetent cell transformation and purified by miniprep, as explained before. Because the insertion site was not surrounded by a selection gene, insertion was confirmed by digestion with XbaI and visualization in an agarose gel, as previously described. To confirm that the 3'UTR fragment was inserted in the correct direction, vectors were sequenced with primers specially designed and synthesized for this purpose, as explained above. When the sequence was confirmed, a bigger amount of the luciferase-p21 3'UTR vector was produced by supercompetent cell transformation, inoculation of high volumes of LB medium and subsequent maxiprep, using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany).

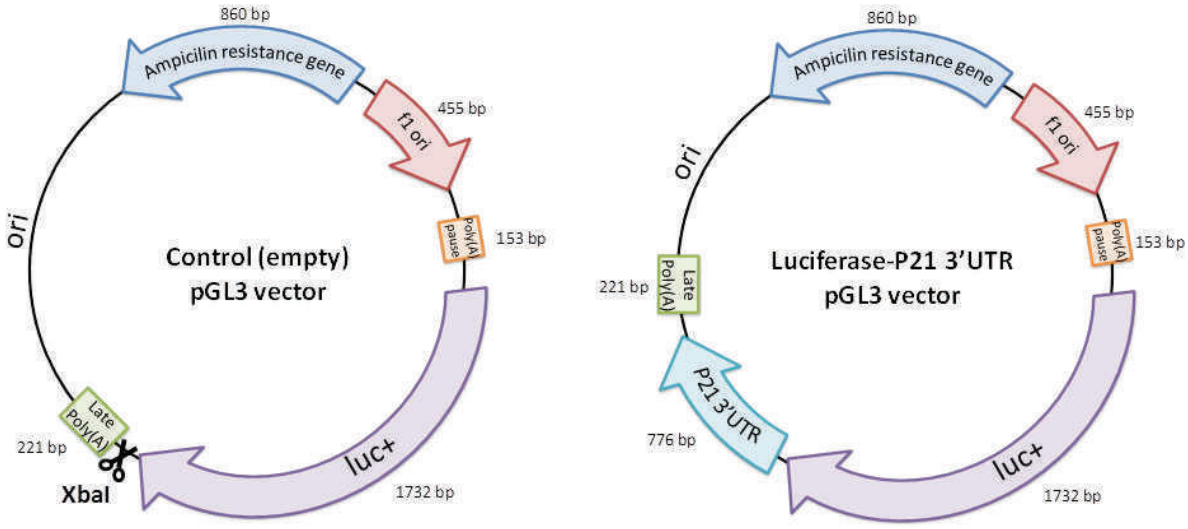

Figure 7. pGL3 reporter plasmids. Left, "empty" pGL3 vector; right, pGL3 vector containing a fragment of p21 mRNA 3'UTR.

10. Transfection

Prior luciferase-reporter gene assays, cells were transfected with the corresponding vector or its "empty" control, and co-transfected with miRNA precursors/mimics or their respective "mock" (scramble) controls, as indicated below. Every experimental condition was co-transfected with the endogenous control vector pRL-TK, which contains the reporter gene *Renilla* luciferase (*Rluc).* All vectors (except for pGL3 luciferase-P21 3'UTR, which was constructed as described in point 9) were purchased to Promega (Madison, WI, USA), and all miRNA precursors and controls were acquired from Applied Biosystems (Life Technologies, Carlsbad, CA, USA). All the experimental conditions were plated in duplicates and each sample was measured twice to minimize the high variability inherent to this technique. Table 5 summarizes the vectors and miRNA precursors or mimics used to transfect the cells (pRL-TK was included in every experimental condition and does not appear in the table).

Table 5. Vector-RNA combinations transfected for luciferase-reporter gene assays

Assay	**Vectors** (in addition to pRL-TK)	**miRNA precursors or mimics and controls**
Affinity for p21 mRNA 3'UTR	pGL3/ pGL3 luciferase-p21 3'UTR	hsa-miR-22 Pre-miR™ miRNA precursor/ mirVana™ miRNA mimic control #1
NF-κB activity (activation of NF-κB response elements)	pGL4.32/ pGL4.32[luc2P/NF-κB-RE/Hygro]	hsa-miR-99b Pre-miR™ miRNA Precursor/ hsa-miR-125a-5p mirVana™ miRNA mimic/ mirVana™ miRNA mimic control #1

10.1. Lipid-based transfection

Meg-01 cells were transfected with Lipofectamine® 2000 in the presence of OptiMEM medium (both from Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations for the vectors, RNAs and transfecting reagent. Briefly, one day before transfection, cells were cultured in a 24-well plate in antibiotic-free medium and were allowed to grow until they reached a confluence of 90-95%. Each well was transfected with a pre-incubated (20 minutes, room temperature) mix of the vectors and RNA in OptiMEM medium. Cells were incubated overnight at 37°C and then growth medium was replaced with complete fresh medium, containing antibiotics. Cells were allowed to grow for 24 hours prior collection and lysis.

10.2. Electroporation

K562 and KG1 cells were transfected by electroporation with an Amaxa® Nucleofector system (Lonza, Basel, Switzerland), according to the manufacturer's instructions. Nucleofected cells were collected for assaying 24 hours after transfection.

- K562 cells were nucleofected with Cell Line Nucleofector™ Solution V and the corresponding reagent (Lonza, Basel, Switzerland), using program T-016 of Amaxa® Nucleofector. Six hours after nucleofection, K562 cells were treated with 0.25 μM MG-132 (see point 3.2).
- KG1 cells were nucleofected with Cell Line Nucleofector™ Solution R and the corresponding reagent (Lonza, Basel, Switzerland), using program V-001 of Amaxa® Nucleofector. Two hours after nucleofection, KG1 cells were treated with anti-sense oligonucleotides (see point 12) and, after two more hours, with LPS or PAM3 (see point 3.5).

11. Luciferase reporter gene assays

Cells transfected with the corresponding combinations of vectors and RNAs (indicated in Table 5) were collected, washed with PBS and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA) according to the manufacturer's protocol for Active Lysis. Luciferase activity of the lysates was then measured with the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) in a Monolight™ 3010 luminometer (Pharmingen, Beckton-Dickinson Biosciences, San Diego, CA, USA), using 5-10 μL of lysate per test (the same volume for all samples from the same experiment), in triplicates. Mean firefly luciferase activity of each sample (RLU1) was normalized to that of *Renilla* luciferase (RLU2).

The normalized value (RLU1/RLU2) of controls was assigned a 100% luciferase activity, and the activity of the remaining experimental conditions was calculated accordingly.

12. miRNA inhibition with anti-sense oligonucleotides

Anti-sense oligonucleotides (ASOs) are efficient biological tools for the inhibition of miRNA activity, *in vivo* and *in vitro* [264].

ASOs bind to their target mature miRNA in RISCs and block their function, or even induce their degradation. They consist of a core RNA sequence that is perfectly complementary to a mature miRNA sequence and includes diverse chemical modifications that confer it a stronger inhibitory power [381]. In this work, we designed an ASO to selectively inhibit the function of miR-125a, along with a specific control ASO, following the guidelines by Horwich and Zamore [381]. In our design, 2'-O-methyl modifications (m) were added to every nucleotide, as well as phosphotiorate bonds (*) on the first two and the last four nucleotides, and a 3'-cholesterol modification (3'-Chl) that made the ASO membrane-permeable. Thus, miRNA inhibition with ASO did not require any transfection method. The control ASO was designed by replacing four nucleotides of the miR-125a-ASO in such way that they resulted in purine–purine mismatches with the target sequence (in Table 6, replaced nucleotides are highlighted in blue). ASOs were synthesized by Dharmacon (Thermo Scientific, Waltham, MA, USA) and dissolved in the provided buffer for a stock concentration of 100 µM.

Table 6. ASOs designed and used for miR-125a-inhibition experiments

ASO name	Sequence
Control ASO	mU*mC*mUmUmAmUmCmA**mA**mAmGmGmU**mG**mAmAmAmGmGmG**mG**mCmU**mA**mAmGmGmGmA mAmC*mC*mU*mU*3'-Chl
miR-125a ASO	mU*mC*mUmUmAmUmCmA**mC**mAmGmGmU**mU**mAmAmAmGmGmG**mU**mCmU**mC**mAmGmGmGmA mAmC*mC*mU*mU*3'-Chl

For miRNA inhibition dose-response assays, KG1, K562 and MDS-L cells were cultured at a cell density of $5x10^4$ cells/mL, treated with ASO concentrations between 100 nM and 1 µM and incubated for 48 hours prior analysis of miRNA levels and cytotoxicity. For colony formation assays (see point 4.1.5), K562 were treated either with Ara-C (see point 3.5) or with Pepinh-MYD and its control (see point 3.5), 6 or 2 hours after the treatment with 1µM ASO, respectively.

13. Differentiation studies

13.1. Benzidine staining

Benzidine is an aromatic amine that forms a blue precipitate upon oxidation by the heme group of hemoglobin, in the presence of H_2O_2. Such characteristic makes it a good dye for the detection of erythroid-like differentiated cells [382]. In order to study differentiation in K562 cells cultured in MC (see point 4.1.5), a solution containing 0.2% benzidine (Sigma-Aldrich, St. Louis, MO, USA), 0.5% acetic acid and 0.6% H_2O_2 was added in a 1:1 ratio to each MC well. Benzidine-acetic acid solutions were stored at 4°C. Of note, H_2O_2 was added extemporaneously. Colonies were incubated for 1-3 minutes with the benzidine stain and heme-positive (blue) colonies were counted in a phase-contrast microscope.

13.2. Detection of differentiation surface markers

The study of differentiation in MC cultures was completed with the detection of changes in the expression of genes involved in erythroid (EPO receptor, glycophorin A and transferrin receptor/CD71) and myeloid differentiation (the transcription factor PU.1 and integrin αM) by qPCR. This technique is detailed in point 6.1.

14. Data analysis

14.1. Raw data analysis

Flow cytometry data were analyzed with Cyflogic (CyFlo Ltd.), Western blot band densitometry was performed with the Odyssey 3.0 software (LI-COR Biosciences, Lincoln, NE, USA) and qPCR data were analyzed using the 7500 FAST® Real Time PCR System software (Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

14.2. Statistical analysis

14.2.1. Cell lines-based experiments

Data were analyzed using GraphPad Prism 6™ (GraphPad Software Inc.). Statistical significance was calculated by one-way ANOVA or two-way ANOVA, when appropriate, correcting for multiple

comparisons with Bonferroni post-test. Data are presented as mean ± SEM of at least three independent experiments. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

14.2.2. Patient data

Overall survival (OS) of a patient was defined from date of sample to date of death, or date last known alive. To investigate associations between gene expression and OS, we considered splitting expression levels at the 25th, 50th and 75th percentiles, generating three possible binary variables[9].

Differences in miRNA expression levels between patients and controls were analyzed using the non-parametric Mann-Whitney test. Linear correlations between miRNA levels or between TLR pathway proteins levels were determined using the Pearson or Spearman correlation tests, where appropriate. In other cases, two cohorts of patients were established, based on relative miRNA levels, as “higher” or “lower” than the mean of all patients. Mann-Whitney test was used to analyze the differences between the two groups. Outliers were removed using Grubb’s or ROUT methods.

The rest of the statistical analyses were carried out by individual Student t tests, one- or two-way ANOVA, as required. Data are presented as mean ± SEM of at least three independent experiments. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

[9] Survival data were analyzed by S. Pierce (Research Nurse Manager, Department of Leukemia, MD Anderson Cancer Center, Houston, TX, USA)

MATERIALS AND METHODS

IV. RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

BLOCK I

Intrinsic Mechanisms of Chemoresistance in the APL Cell Line NB4

RESULTS AND DISCUSSION

1. Background

Cancer cells have *intrinsic* and extrinsic, or *acquired*, mechanisms of resistance to cytotoxicity. Intrinsic mechanisms allow "normal" members of the cell population to tolerate a particular drug or adverse stimulus. They include *drug-unspecific mechanisms* like lower drug uptake, higher detoxification capacity, increased stress responses or changes in PCD pathways; and *drug-specific mechanisms*, such as the absence or modification of a target site [383].

In current targeted therapies for APL, ATO is used for targeting PML-RARα for degradation [33]. ATO is thought to trigger apoptosis in leukemia, at least in part, through the induction of oxidative stress [282], although it needs to be used at low doses and requires to be combined with a sensitizing agent, such as other oxidative-stress inducers [384-386], chemotherapy or ATRA. Combined treatments of ATRA and ATO successfully achieve survival rates of up to 80% [39]. However, there are still a 20% of patients who relapse due to resistance to conventional treatments. In order to decrease relapse rates in high-risk patients, Ara-C is used in high doses in combination with ATRA and/or chemotherapy [38]. Nevertheless, patients with high-risk still need safer upfront therapies that avoid relapse and allow a dose reduction or replacement of chemotherapy [38, 39]. It is therefore important to understand the intrinsic mechanisms that confer chemoresistance to APL cells and to develop new combinatory therapeutic strategies to approach relapse to frontline therapies.

As explained in the *Introduction* section, it is well established that the P53 gene status influences tumor responsiveness to radiation and chemotherapy *in vivo* [387], and this is the reason why this factor is so frequently mutated in human cancer [196]. The great majority of p53 mutations found in cancer are located in a conserved region comprising codons 110-307 [194, 196], which contains the DNA binding domain of p53 [388]. These "hot-spot" mutations decrease transcriptional activity of p53 [389, 390] and some of them have been found to acquire new $p53^{wt}$-independent oncogenic properties ("gain-of-function" alterations) that reduce responsiveness of tumors to chemotherapy [391-394], including Ara-C [395], and favor invasiveness and migration [394, 396, 397]. In spite of being more frequent in solid cancers, 6% of known somatic mutations of p53 have been found in hematological malignancies and are related to a bad prognosis [196]. In AML, p53 mutations are more frequent in older patients and have been associated with a poorer survival [398]. Additionally, cell lines with the most abundant p53 mutations are those derived from hematological tumors, bearing 17% of all mutations [196]. p53 mutants can be found in AML cell lines of all differentiation stages, including APL [348]. Thus, some APL cell lines and patients may have inherent resistance to chemotherapy through the expression of p53 mutants.

The APL cell line NB4 bears the hot-spot p53 mutation R248Q ($p53^{R248Q}$) [348], which is one of the most frequent in human cancers [196] and reduces the DNA-binding ability of p53 [390, 399, 400]. This mutation is thought to enhance the proliferative capacity of NB4 cells, since the expression of exogenous wild type p53 ($p53^{wt}$) in these cells decreases proliferation and significantly induces cell death [348]. Therefore, $p53^{R248Q}$ could not only participate in the malignification of NB4 cells but also inhibit apoptosis and confer cells resistance to death. For this reason, the NB4 cell line is a good model to study the possible implication of p53 mutants in chemoresistance in APL.

Interference with mitochondrial respiration and the subsequent induction of oxidative stress has been postulated to be an effective way of inducing programmed cell death in cancer cells [126]. Consistent with this, DQA induces mitochondria-mediated cytotoxicity in NB4 cells, triggering MOMP, production of $O_2^{\cdot-}$, cytochrome c release and ATP depletion, followed by the activation of caspase-3 and caspase-9 [294-296]. However, DQA seems to be more effective in combination with other types of drugs [285, 287, 297].

Proteasome inhibitors are a group of drugs known to effectively induce cell cycle arrest and, eventually, apoptosis in cancer cells [310, 401], and low doses have been demonstrated to sensitize cells to chemotherapy [316-318, 335]. Both intrinsic and extrinsic pathways of apoptosis appear to contribute to proteasome inhibitor-induced apoptosis [305, 318, 324, 325] and, importantly, most reports agree that this cell death is irrespective of the p53 status [310, 319, 323, 326], which makes proteasome inhibitors a good choice for the treatment of cancer cells bearing p53 mutations. Furthermore, proteasome inhibitors have been demonstrated to be especially useful for the treatment of hematologic malignancies [331] since they are innocuous to normal blood cells [322, 335, 336, 338], and they appear to be effective to some extent even in refractory or relapsed AML patients [340, 341]. Interestingly, proteasomes are important turnover centers for oxidized proteins that accumulate after oxidative stress, which suggests that proteasome inhibition could not only induce ER stress but also worsen the effects of oxidative stress [307].

2. Hypothesis and Aims

For all these reasons, we hypothesized that the combination of low doses of the proteasome inhibitor MG-132 with DQA would synergize in the induction of oxidative stress and additionally induce ER stress, events that would eventually lead to apoptosis in NB4 cells.

Thus, following specific aims were proposed:

- To investigate the combined effect of low doses of MG-132 and DQA on cell viability and proliferation of NB4 cells.
- To study intrinsic mechanisms of chemoresistance in NB4 cells, focusing on those related to $p53^{R248Q}$.

3. Materials and Methods

3.1. Cell lines

- NB4 cells

3.2. Treatments

- DQA
- MG-132
- PFT-α and PFT-μ
- 3-MA
- RAPA
- NAC

3.3. Methods

- MTT assay
- Analysis of DNA content
- Detection of PS externalization and permeability to PI
- Analysis of $\Delta\Psi_m$ and assessment of $O_2^{\cdot-}$ levels
- Western blot (for p53, Nrf2, SOD-2, γGCSc, Bcn-1 and LC3α).
- qPCR (P21, BAX and DRAM1)
- Study of autophagy by fluorescence microscopy

4. Results

4.1. MG-132 induces cell death and cell cycle arrest in NB4 cells

In order to determine the most adequate working concentrations of MG-132 in NB4 cells, they were treated with increasing doses of this compound and changes in metabolic activity were determined by MTT. Results showed that MG-132 decreases metabolic activity of NB4 cells in a time- and dose-

dependent manner, with IC_{50} values of 0.74 µM and 0.20 µM at 24 and 48 hours, respectively (data not shown). Next, we analyzed the effects of different doses of MG-132 on NB4 cells proliferation and DNA fragmentation. In agreement with the extended notion that proteasome inhibitors arrest cell cycle in phase G_1 or G_2/M depending on the p53 status [402], MG-132 induced cell cycle arrest in phase G_2/M in this cell line at doses higher than 0.1 µM (Figure 1). DNA fragmentation (sub-G_0/G_1 population) could be detected at doses higher than 0.15 µM.

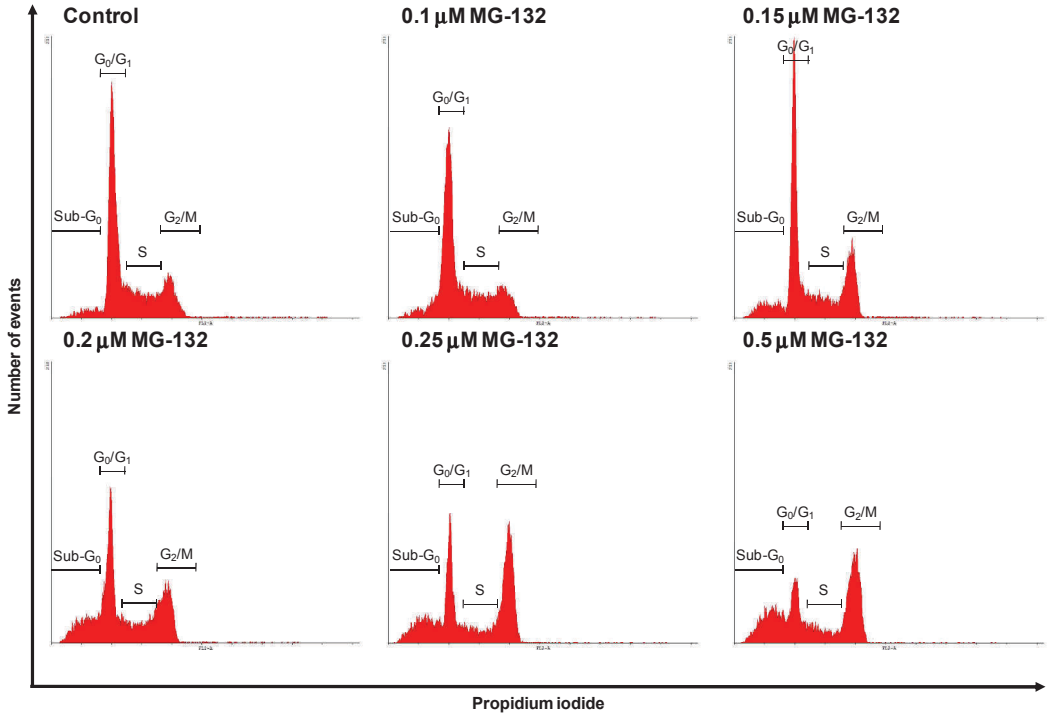

Figure 1. Cell cycle profile of NB4 cells treated with MG-132. NB4 cells were treated for 24 hours with increasing doses of MG-132 (0-0.5 µM). Flow cytometry histograms are representative of three independent experiments.

To study if proteasome inhibition at sub-lethal doses would potentiate the effects of DQA in NB4 cells, we selected for the following experiments the dose of 0.15 µM MG-132, at which proteasome inhibition was patent in terms of cell cycle arrest and which corresponded to a decrease of ~11% and ~37% in the rate of metabolic activity at 24 and 48 hours, respectively, as determined by the MTT assay (results not shown).

4.2. A low dose of MG-132 induces resistance to apoptosis in NB4 cells treated with DQA

To address the effect of MG-132 on DQA-induced apoptosis, NB4 cells were treated with 0.15 µM MG-132 2 hours prior treatment with DQA (10 µM or 20 µM) for 24 or 48 hours (Figure 2). Interestingly, cells co-treated with MG-132 and 10 µM DQA showed lower levels of apoptosis as

compared to cells treated with DQA alone. These differences were especially significant after 48 hours of treatment (Figure 2B) and could not be appreciated with the dose of 20 µM DQA at the time points studied. These results suggest that MG-132, which has apoptotic activity in several cell types as a single agent at similar [322] and higher doses [305, 321, 322, 338, 403], induces dose-dependent resistance to apoptosis triggered by DQA in our model.

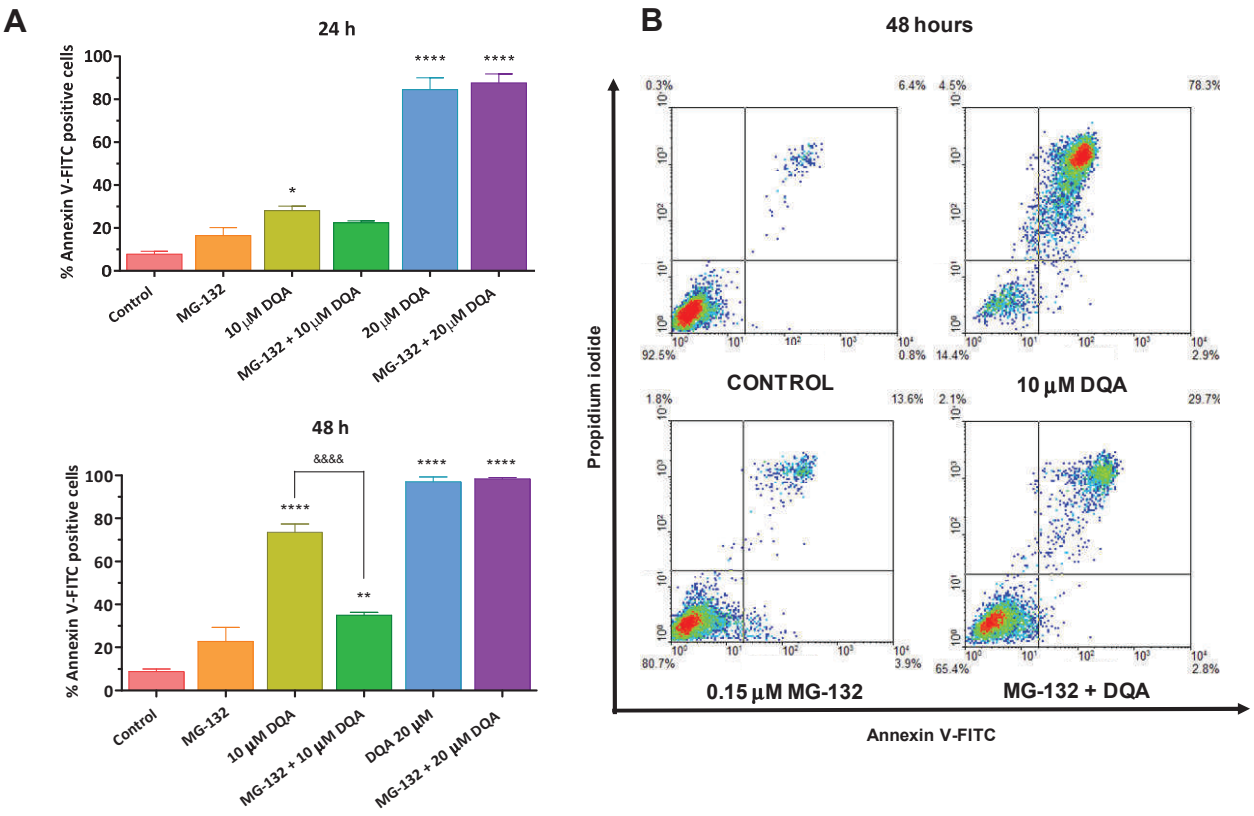

Figure 2. Cell death by apoptosis of NB4 cells treated with MG-132 and/or DQA. (A) Bar graphs represent the percentage of Annexin V-positive NB4 cells after 24 and 48 hours of treatment with 0.15 µM MG-132 ± DQA. Data reflect mean ± SEM of three independent experiments. Statistical significance versus control: *P<0.05; **P<0.01; ****P<0.0001. Statistical significance between DQA and MG-132 ± DQA: $^{\&\&\&\&}$P<0.0001 **(B)** Representative Annexin V-FITC/PI dot-plot of a 48-hour treatment of NB4 cells with MG-132 ± DQA.

Because DQA is known to target mitochondria of cancer cells [285, 293] and our group previously reported that it decreases $\Delta\Psi_m$ and induces the synthesis of $O_2^{\cdot-}$ in NB4 cells [294-296], we sought to determine if these parameters were affected by MG-132. As shown in Figures 3A-D, MG-132 did not significantly affect $\Delta\Psi_m$ or $O_2^{\cdot-}$ levels *per se*. However, when it was combined with DQA, it prevented the accumulation of $O_2^{\cdot-}$ triggered by DQA after 48 hours of treatment (Figure 3D). Consistent with the Annexin-V results (Figure 2), 20 µM DQA could efficiently overcome the resistance induced by MG-132, showing a direct relationship among the dose of DQA, ROS levels and the degree of cell death. However, DQA-induced $\Delta\Psi_m$ decrease was not disrupted by MG-132.

Therefore, it appears that MG-132 does not prevent DQA from targeting the mitochondria but could instead interfere at some point downstream of it, either blocking the pathway or restoring ROS levels after 48 hours.

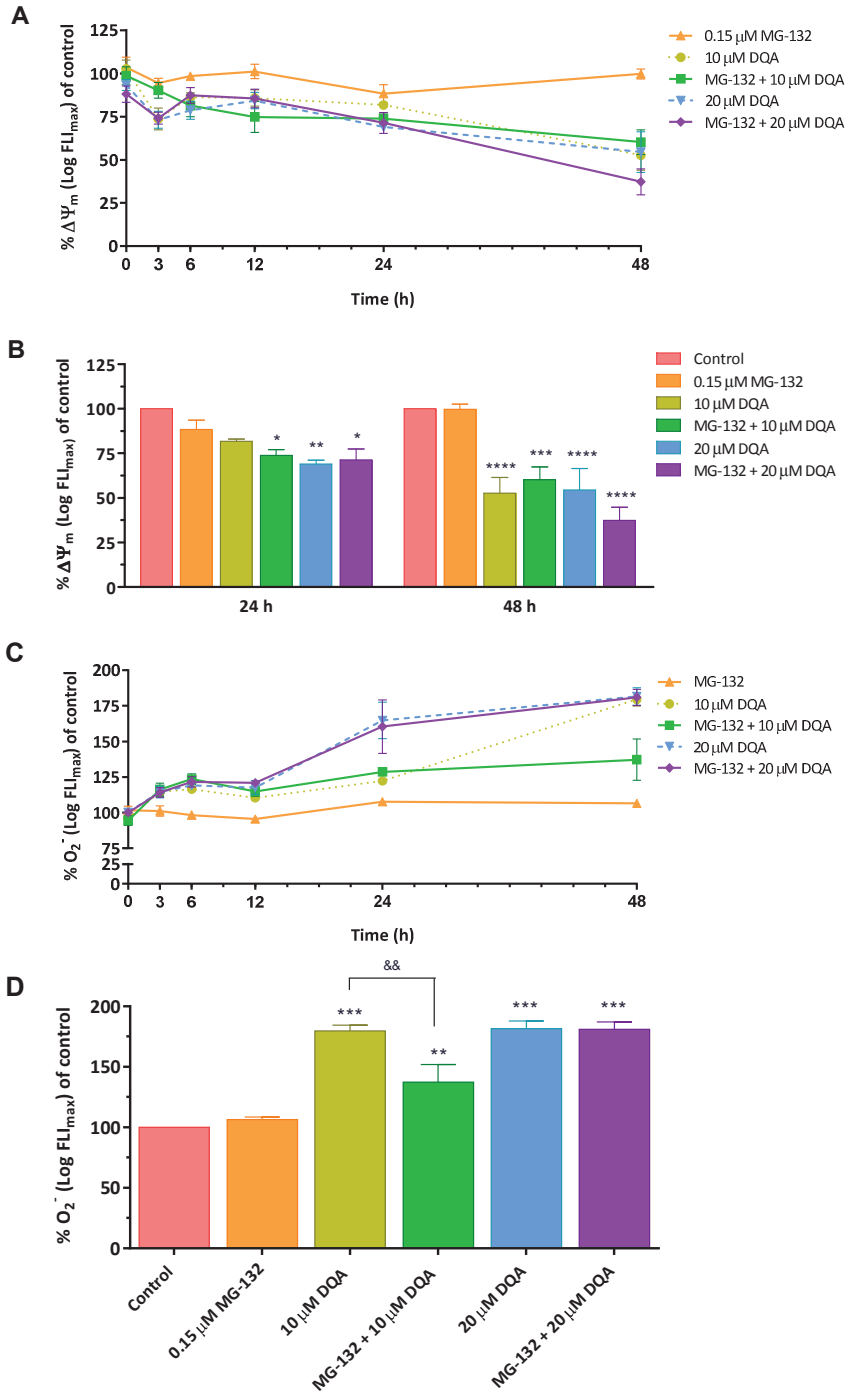

Figure 3. Changes in $\Delta\Psi_m$ and $O_2^{\cdot-}$ levels of NB4 cells treated with MG-132 and/or DQA. (A) Linear graph represents $\Delta\Psi_m$ for each treatment as the logarithm of maximum intensity of the fluorescence peak (Log FLI_{max}) at the different times, expressed as the percentage from control (mean ± SEM of n=3). **(B)** $\Delta\Psi_m$ after 24 and 48 hours of treatment. Statistical significance versus control: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. **(C)** Percentage of $O_2^{\cdot-}$ levels, represented as Log FLI_{max}, relative to control cells (mean ± SEM of n=4). **(D)** $O_2^{\cdot-}$ levels after a 48-hour treatment. Statistical significances versus control: **P<0.01; ***P<0.001. Statistical significance between DQA and MG-132± DQA: $^{\&\&}$P<0.01.

4.3. MG-132 enhances the anti-oxidant response in NB4 cells

Hyperactivation of the transcription factor Nrf2 has been reported to be involved in chemoresistance of a wide number of solid cancers and AML (reviewed in [280]). Interestingly, the family of proteasome inhibitors is a well-known inducer of the expression of this factor in normal cells [280, 404, 405]. Thus, we next sought to determine if low doses of MG-132 could activate Nrf2 in NB4 cells and induce an anti-oxidant response that could account for the inhibition of DQA-induced apoptosis and accumulation of ROS. Provided that the rescue from apoptosis was only detected in cells treated with 10 μM DQA, the following experiments were only performed with this dose.

Under basal conditions, Nrf2 is negatively regulated by proteasomal degradation and, upon stimulation by an oxidative or electrophilic insult, it is rapidly activated by protein stabilization and translocates to the nucleus, where it activates the transcription of a large pool of anti-oxidant enzymes [279]. To determine the activation and functionality of Nrf2 in NB4 cells, we detected nuclear levels of the Nrf2 protein and total protein levels of its transcriptional targets SOD-2 [406] and the catalytic subunit of γGCS (γGCSc) [407], which have been previously reported to be expressed in AML [408, 409].

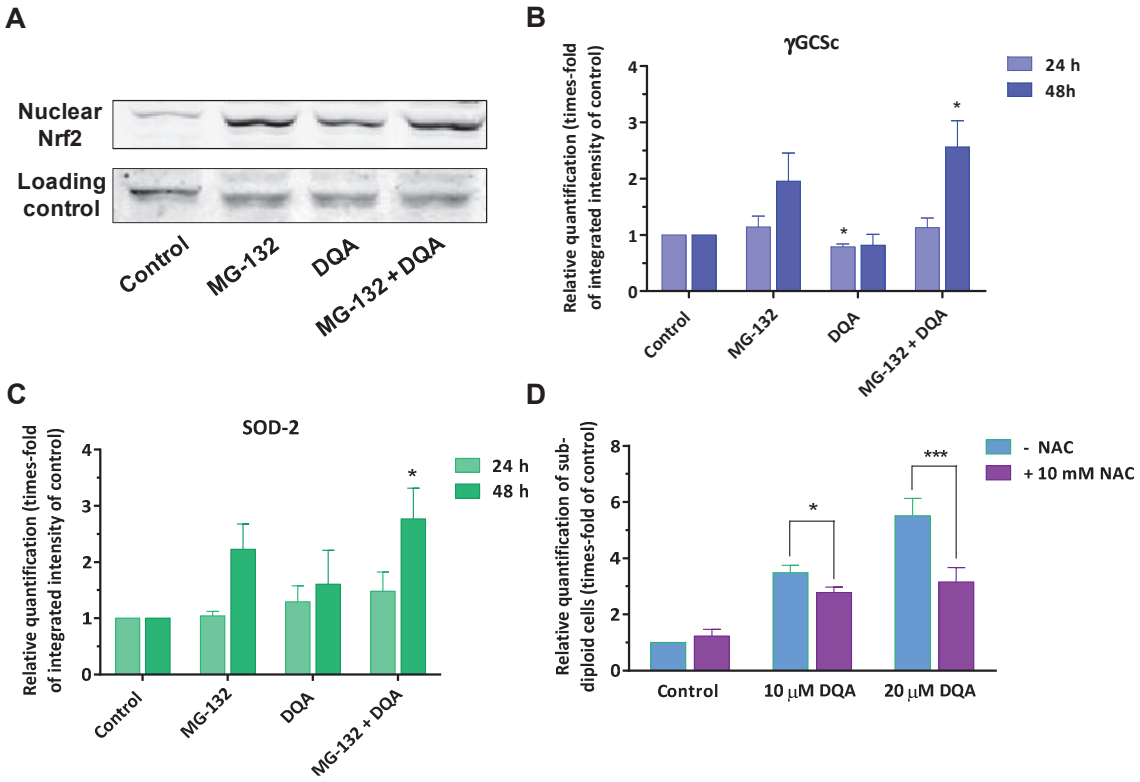

Figure 4. Effect of MG-132 and/or DQA on Nrf2-mediated anti-oxidant response in NB4 cells. (A) Nuclear protein levels of Nrf2 after 6 hours of treatment with 0.15 μM MG-132 ± 10 μM DQA; a representative blot is shown (n>3). **(B-C)** Total protein expression of γGCSc and SOD-2, determined after 24 and 48 hours of treatment (mean ± SEM of n≥3 independent experiments). Statistical significance versus control: *P<0.05. **(D)** Apoptotic cell death in NB4 cells pre-treated with 10 mM NAC for 3 hours and subsequently treated with DQA at the indicated doses for 24 hours (mean ± SEM of n=3). Statistical significance: *P<0.05; ***P<0.001.

As expected, treatment with MG-132 induced nuclear translocation of Nrf2 after 6 hours (Figure 4A) and a subsequent increase in the protein expression of its transcriptional targets γGCSc and SOD-2 after 48 hours (Figure 4B and 4C). Remarkably, total Nrf2 protein levels remained unchanged (data not shown).

These results indicate that sub-lethal doses of MG-132 can induce an anti-oxidant response triggered by Nrf2 activation. Of note, DQA induced a slight increase in nuclear Nrf2 protein levels (Figure 4A). However, this effect did not add to the increase induced by MG-132; moreover, the expression of γGCSc or SOD-2 was not significantly augmented in the presence of DQA.

The expression of Nrf2-dependent anti-oxidant enzymes explains the reduction in $O_2^{\cdot-}$ levels by MG-132 observed in co-treatment with DQA, and this might be the mechanism responsible for the resistance to DQA-induced apoptosis. To confirm that anti-oxidant mechanisms could counteract the apoptotic effect of DQA at the dose utilized, we pre-treated NB4 cells with the glutathione precursor NAC, 3 hours prior treatment with DQA and measured changes in apoptosis after 24 hours (Figure 4D). As expected, the enhancement of the anti-oxidant response by NAC significantly inhibited DQA-induced cell death, even at the dose of 20 μM DQA. This efficiency in a higher dose of DQA is probably due to the early presence of NAC molecules in the cytoplasm, in contrast with the transcriptional response triggered by Nrf2, which needs at least 48 hours to induce a significant increase in the expression of anti-oxidant proteins. It could be argued that, after such long time, the amounts of ROS generated by 20 μM DQA have already induced considerable damage and cell death.

4.4. DQA induces activation and nuclear translocation of $p53^{R248Q}$

Because proteasome inhibitors are known to induce stabilization of the p53 protein [402] and NB4 cells express the p53 mutant $p53^{R248Q}$ [348], which has impaired DNA-binding ability [390, 399] and has been shown to confer cells invasive potential and chemoresistance [392, 410], we then decided to analyze the effects of the treatment with MG-132 on the expression and nuclear import of $p53^{R248Q}$ protein in these cells.

Western blot data analysis of whole-protein lysates of cells treated with MG-132 and/or DQA for 6, 24 and 48 hours did not reveal any significant change in $p53^{R248Q}$ expression (results not shown). Strikingly, immunoblotting of nuclear extracts showed a 2-fold enrichment of the nuclear fraction in $p53^{R248Q}$ after 24 hours of treatment with DQA, both alone and in co-treatment with MG-132 (Figure 5). These results suggest that DQA induces post-translational activation of cytosolic $p53^{R248Q}$ and its nuclear translocation.

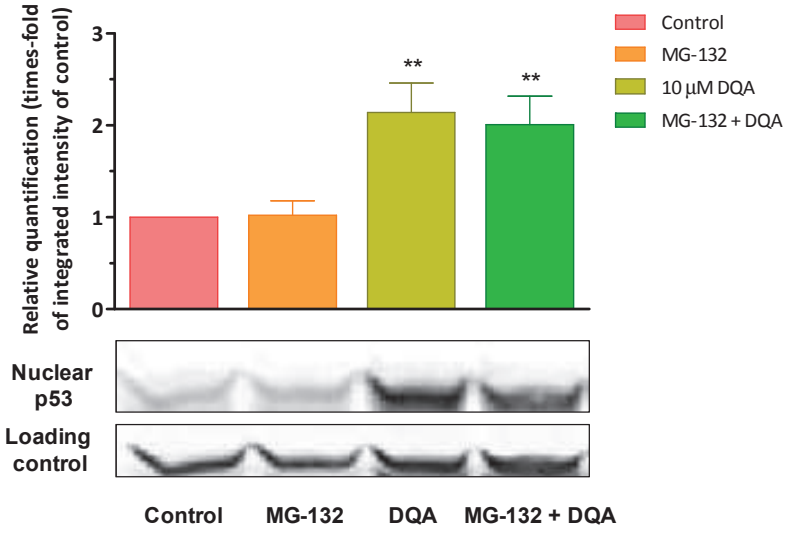

Figure 5. Effect of MG-132 and/or DQA on p53 nuclear translocation in NB4 cells. Nuclear translocation of p53 was studied in NB4 cells treated for 24 hours with MG-132 ± DQA; a representative blot and mean ± SEM of n>3 are shown. Statistical significance versus control: **P<0.01.

4.5. Cytosolic p53^{R248Q} is involved in resistance to apoptosis in NB4 cells

Provided the mutant nature of p53 in NB4 cells, it remained to be determined if this protein preserves its functionality. To shed light on this issue, we treated the cells with two pharmacological inhibitors of p53 with different affinities for it: PFT-α, a reversible inhibitor of p53-mediated gene transcription and p53-dependent apoptosis [359], and PFT-μ, a more specific inhibitor of p53 which shuts down the mitochondrial pathway without affecting its transcriptional activity [360].

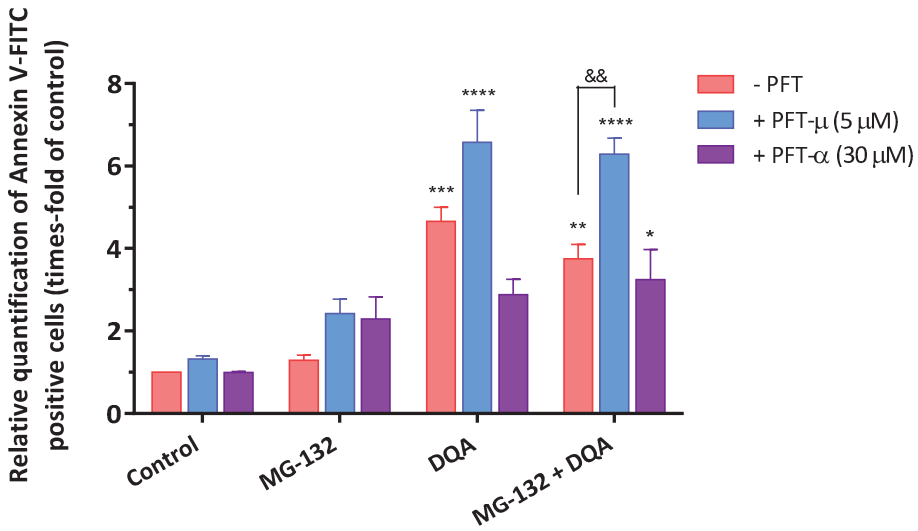

Figure 6. Changes in cell viability of NB4 cells induced by pharmacological inhibition of p53. Bar graph represents apoptotic (Annexin V-FITC positive) NB4 cells treated with 0.15 μM MG-132 ± 10 μM DQA for 24 hours and pre-treated with the p53 inhibitors PFT-α and PFT-μ. Data reflect mean ± SEM of 3 independent experiments. Statistical significance versus control: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Statistical significance between the indicated groups: $^{\&\&}$P<0.01.

As shown in Figure 6, neither of the inhibitors had a significant apoptotic effect by themselves. In combination with MG-132, both PFT seemed to enhance apoptosis, although this increase was not statistically significant. In contrast, they triggered a differential response in cells treated with DQA. On one hand, PFT-μ significantly enhanced DQA-induced apoptosis, even overcoming resistance induced by MG-132, which suggests that the p53/mitochondrial pathway might be involved in resistance to apoptosis in NB4 cells. On the other hand, PFT-α inhibited DQA-induced apoptosis, mimicking the decrease induced by MG-132 in combination with DQA, which may indicate that the nuclear activity of $p53^{R248Q}$ is functional and necessary for DQA-induced apoptosis.

Interestingly, a recent study demonstrated that hot-spot mutants $p53^{R273H}$ and $p53^{R248Q}$ reduce the inducible expression of Nrf2 target phase-II detoxifying enzymes in several cell line models through a gain-of-function mechanism [411]. According to this, inducible Nrf2 activation could be partially inhibited in NB4 cells by $p53^{R248Q}$. In light of the results obtained by pre-treatment with PFT-α, we questioned if nuclear $p53^{R248Q}$ inhibition was able to antagonize DQA-induced apoptosis through the de-repression of Nrf2 and consequent activation of its transcriptional program. Thus, we determined the protein expression of Nrf2 and its targets γGCSc and SOD-2 in NB4 cells pre-treated with both PFT and treated with MG-132 ± DQA. No significant changes were detected (results not shown) as compared to non-pretreated cells, indicating that the effect of nuclear $p53^{R248Q}$ on apoptosis is not mediated by the Nrf2 pathway.

4.6. Expression of the p53 targets BAX, DRAM1 and P21 is not increased after nuclear translocation of $p53^{R248Q}$

Since nuclear $p53^{R248Q}$ appeared to be necessary for the effectiveness of DQA, we next aimed to determine if this protein preserves any of its transcriptional activity in NB4 cells and if the expression of p53 target genes could be responsible for DQA-induced apoptosis. For this purpose, we analyzed the expression of three well-known p53 target genes: P21 [211], BAX [177] and DRAM1 [190]. Relative mRNA levels were determined by qPCR after 6, 12 and 24 hours of exposure of NB4 cells to MG-132 and/or DQA in order to investigate early and late responses.

DQA induced no significant effects on P21 expression, although a moderate increase in P21 mRNA levels was detected after 6 hours for all treatments (Figure 7A). However, after 12-24 hours this increase only persisted in cells treated with MG-132, both alone and in combination with DQA. Nuclear import of $p53^{R248Q}$ could not account for this event since $p53^{R248Q}$ was not augmented in the

nucleus of cells treated with only MG-132 (Figure 5). In agreement, p53-independent p21 induction has been previously described to be a typical effect of proteasome inhibitors [310, 326, 401].

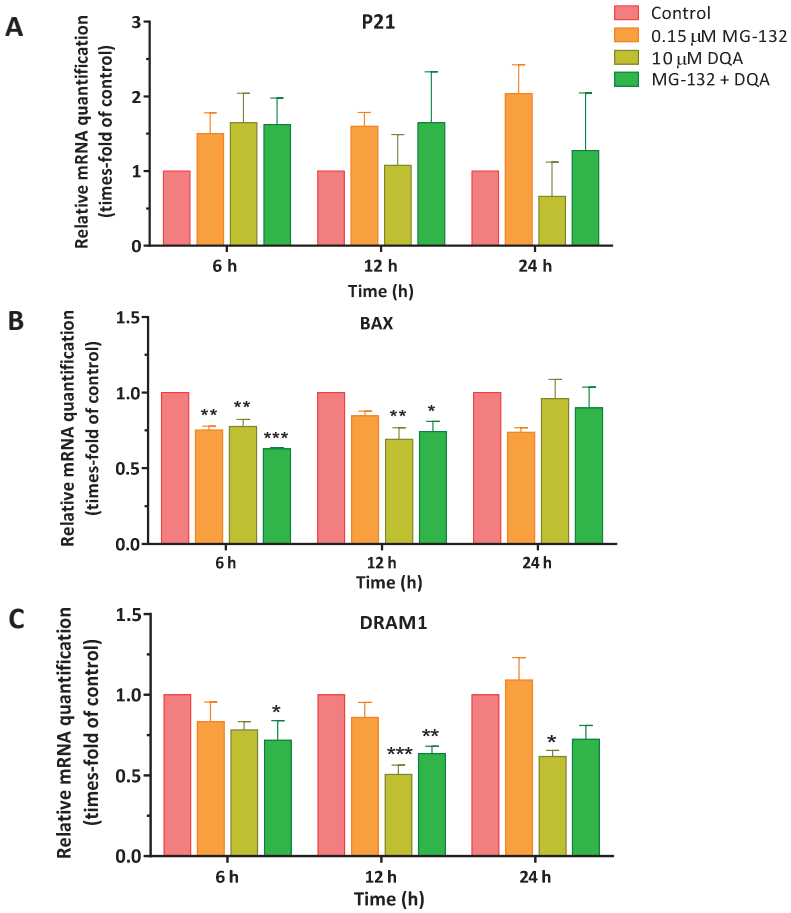

Figure 7. Effect of MG-132 and/or DQA on P21, BAX and DRAM gene expression in NB4 cells. Changes in the expression of p21 **(A)**, Bax **(B)** and DRAM **(C)** mRNA after 6, 12 and 24 hours of treatment with MG-132 ± DQA. Bar graphs represent relative mRNA quantification (mean ± SEM of n=3 experiments). Statistical significance versus control: *P<0.05; **P<0.01; ***P<0.001.

Figure 7B shows a decrease in BAX mRNA levels by both MG-132 and DQA, alone and in combination, at 6 and 12 hours. After 24 hours of treatment, BAX mRNA levels returned to basal conditions in cells treated with DQA but not in those treated with MG-132 alone. These results suggest that the early response to both treatments generates an anti-apoptotic signal which later on disappears in DQA-treated cells, enabling cell death. Anyway, these experiments failed to show an increase in the expression of this gene which is high enough to justify the contribution of nuclear $p53^{R248Q}$ to apoptosis.

Similarly, treatment with DQA, alone and in combination with MG-132, significantly inhibited the expression of the pro-autophagic gene DRAM1 [190, 412] (Figure 7C) while MG-132 did not show any significant effect by itself. Whether the sustained inhibition of DRAM1 is connected or not with the

nuclear translocation of $p53^{R248Q}$, this result points to the inhibition of autophagy by DQA, which could be a secondary mechanism of apoptosis induction by this drug.

4.7. DQA inhibits autophagy in NB4 cells

Provided that DQA seemed to have an inhibitory effect on DRAM1 expression in NB4 cells, our next objective was to determine the role that autophagy plays in DQA-induced cell death and its inhibition by MG-132. For this purpose, we studied the presence of autophagic vesicles in NB4 cells by fluorescence microscopy after 24 and 48 hours of treatment. Staining AVO with AO showed high basal levels of autophagy in NB4 cells (Figures 8A and 8B). In agreement with the inhibition of DRAM1 by DQA, cells treated with either DQA or DQA and MG-132 underwent an almost complete loss of AVO and a subsequent increase in chromatin aggregation, typical from apoptosis [372]. MG-132 did not appear to have any effect on autophagy; however, co-treated cells preserved a slightly higher proportion of AVO than those treated only with DQA. This was confirmed by staining with MDC (Figures 8C and 8D). Therefore, data demonstrate that DQA inhibits autophagy while it induces apoptosis in NB4 cells and suggest that MG-132 could protect them from the loss of AVO.

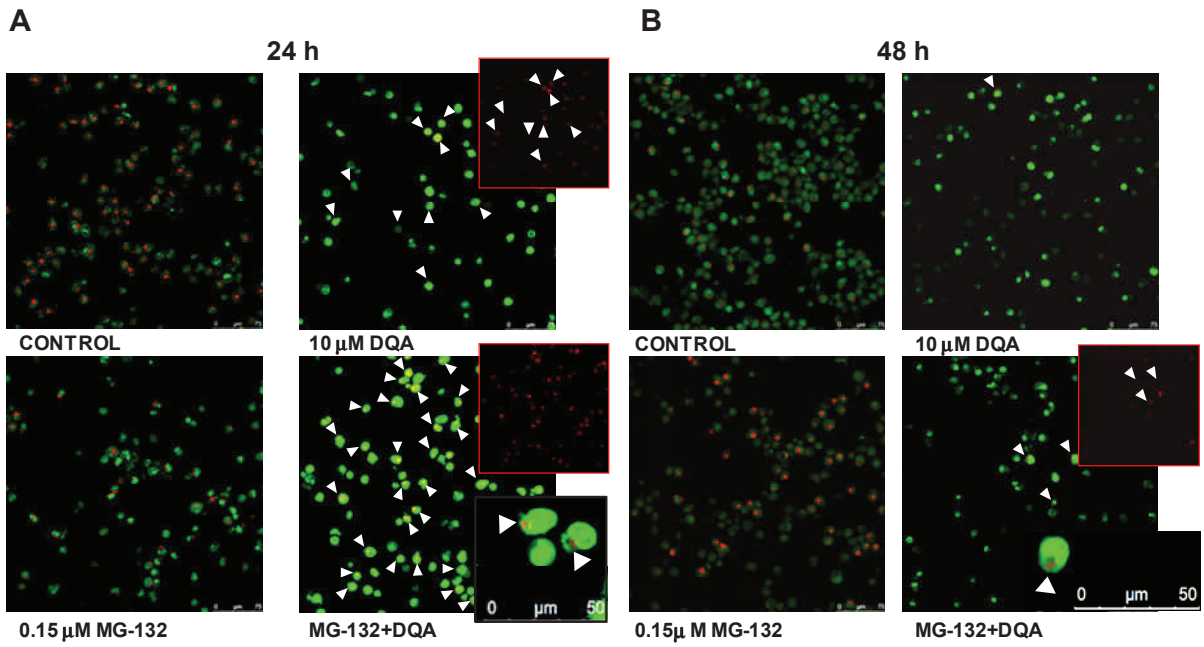

Figure 8. (Continues in the next page)

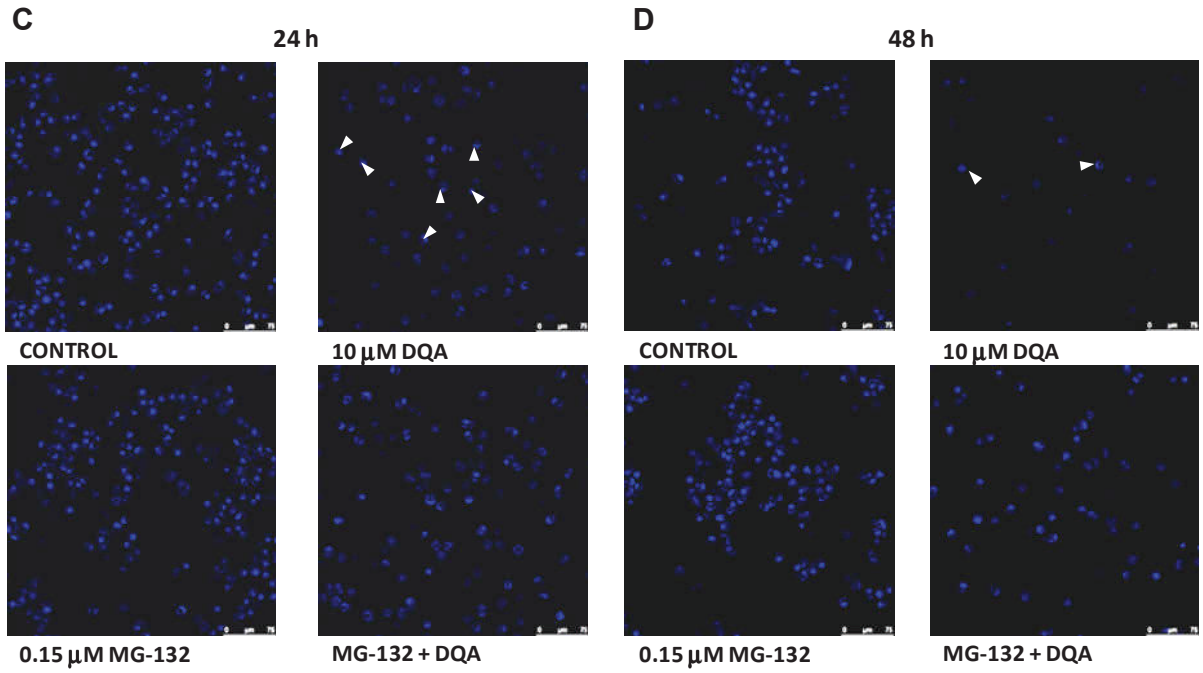

Figure 8. Autophagy in NB4 cells treated with MG-132 and/or DQA. Representative confocal microscopy captures of NB4 cells treated with 0.15 µM MG-132 ± 10 µM DQA (n>3 experiments). After 24 or 48 hours of treatment, fresh cells were stained with AO **(A,B,** see the previous page**)** and MDC **(C,D)** to visualize AVO (small groups of red or blue fluorescent vesicles, respectively). White arrows indicate the location of AVO, when needed. Emerging smaller-sized windows represent independent (not merged) AVO captures and zoomed cells. In the presence of AO, apoptotic nuclei can be visualized as intensely green fluorescent extensive areas of condensed chromatin. Upon disappearance of AVO, MDC is detected as a weaker fluorescence, diffusely distributed in the cytoplasm.

4.8. Autophagy protects NB4 cells against apoptosis

As mentioned in the *Introduction* section, the relationship between autophagy and apoptosis is a controversial topic. In our model, apoptosis and autophagy seemed to be incompatible processes; however, it remained unclear if the inhibition of autophagy is necessary for the induction of apoptosis or if it is a secondary event triggered by DQA. To address this question, we co-treated the cells with the pharmacological inhibitor of autophagy 3-MA and the autophagy inductor RAPA and studied their effects on apoptosis.

Inhibition of autophagy by 3-MA (5mM) in control cells induced high levels of apoptosis (Figure 9), suggesting that the high basal autophagy detected in NB4 cells is a cytoprotective mechanism *per se.* Similar results were obtained when 3-MA was combined with MG-132. Induction of autophagy by RAPA (1 µM) did not show any changes in viability, supporting the non-lethal role of autophagy in these cells. By contrast, RAPA decreased DQA-induced apoptosis, mimicking the effect of pre-treatment with MG-132.

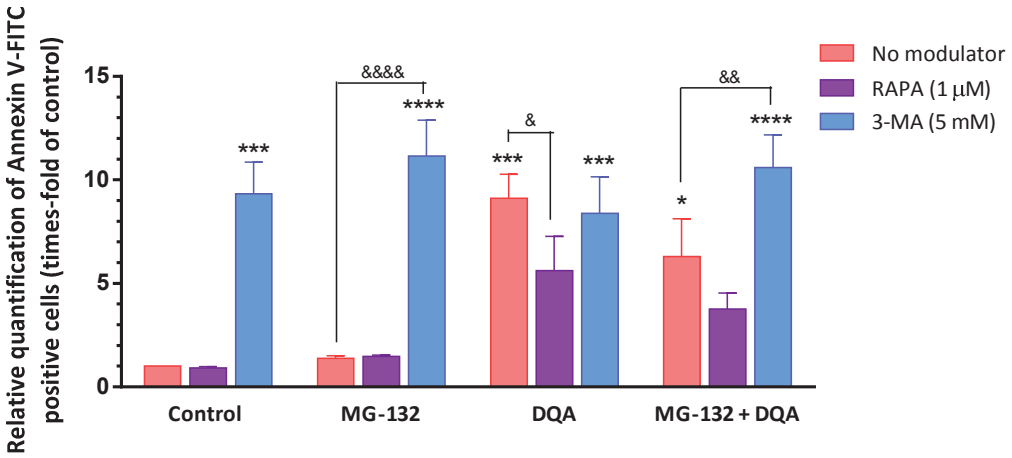

Figure 9. Effect of selective autophagy modulators on apoptosis of NB4 cells. Data represent mean ± SEM (n=4) of the relative number of apoptotic (Annexin V-positive) cells after 24 hours of treatment with 0.15 μM MG-132 ± 10 μM DQA and pre-treatment with 1 μM RAPA or 5 mM 3-MA. Statistical significance versus control: *P<0.05; ***P<0.001; ****P<0.0001. Statistical significance between the indicated groups: $^{\&}$P<0.05; $^{\&\&}$P<0.01; $^{\&\&\&\&}$P<0.0001.

These results show that the inhibition of autophagy is necessary for DQA to effectively trigger apoptosis and is not a secondary effect of cell death. Therefore, our data confirm the opposite roles of apoptosis and autophagy in NB4 cells. These data also suggest that, although MG-132 did not increase the number of AVO, it may in fact generate additional resistance to DQA-induced apoptosis in these cells via protection of autophagy.

Remarkably, 3-MA seemed to induce slightly higher apoptosis levels in cells treated with MG-132, which might occur due to the addition of the intrinsic apoptotic activity of MG-132 to the blockade of autophagy by 3-MA. In contrast, autophagy inhibition in DQA-treated cells did not induce any change in apoptosis in addition to levels induced by DQA, indicating that autophagy inhibition by DQA is sufficient for the cells to undergo apoptosis.

4.9. Regulation of autophagy in NB4 cells is independent of the regulation of apoptosis by p53^{R248Q}

It has been demonstrated that proteasome inhibitors can induce autophagy [301, 413], and this induction has been related to the stabilization of p53 in the cytosol [414]. Thus, we sought to study more deeply the possible induction of autophagy by MG-132 and the role that cytosolic p53^{R248Q} might play in this process and/or in the autophagy inhibition induced by DQA. For this purpose, we pre-treated NB4 cells with PFT-α and PFT-μ and then measured the expression of the pro-autophagic proteins Bcn-1 and LC3α [138, 139].

In agreement with our previous fluorescence microscopy results, control cells presented detectable levels of LC3α-II, indicative of elevated basal levels of autophagy (Figure 10A). MG-132 alone failed to significantly affect the levels of either Bcn-1 or LC3α kinase proteins but slightly reverted changes induced by DQA, which in turn significantly decreased the levels of Bcn-1 and LC3α-II proteins (Figures 10A-C) without affecting the levels of LC3α-I. These data confirm our previous results showing that DQA inhibits autophagy and MG-132 partially protects it.

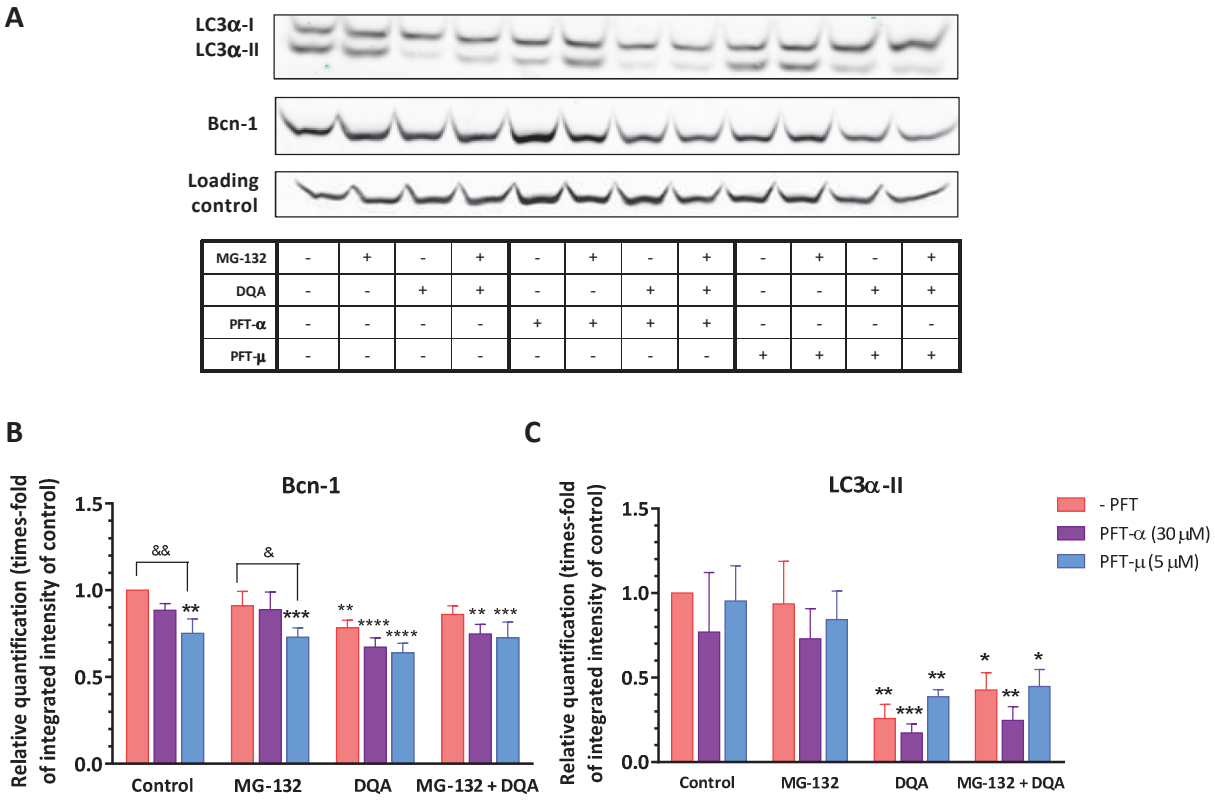

Figure 10. Effect of the pharmacological inhibition of p53 on autophagy in NB4 cells. Changes in autophagy in NB4 cells treated with 0.15 μM MG-132 ± 10 μM DQA for 24 hours and pre-treated with PFT-α or PFT-μ were detected by Western blot. **(A)** A representative blot is shown for each protein (n≥3). **(B)** and **(C)** Densitometric quantification of Bcn-1 **(B)** and LC3α-II **(C)** levels. Data show relative quantification (mean ± SEM) of n≥3 experiments. Statistical significance versus control: *P<0.05;**P<0.01; ***P<0.001; ****P<0.0001. Statistical significance between the indicated groups: $^{\&}$P<0.05; $^{\&\&}$P<0.01.

Effects of p53 inhibition on autophagy were mild. Overall, PFTs seemed to slightly decrease Bcn-1 and LC3α-II levels in control cells but none of them induce major differences in MG-132 and/or DQA-treated cells, except for PFT-μ, which significantly decreased Bcn-1 also in MG-132-treated cells. However, this inhibitor did not induce significant changes on LC3α-II levels and even seemed to induce a slight increase in DQA-treated cells. These opposing effects on the expression of both proteins are contradictory and most likely do not lead to changes in autophagy. Accordingly, confocal

microscopy analysis in PFT-co-treated cells did not show any change in number or fluorescence intensity of AVO (data not shown). Therefore, it is not likely that p53 plays a major role in the regulation of autophagy in NB4 cells.

5. Discussion

The present study was initially designed to determine the combined effects of proteasome inhibition and ROS-mediated cell death in the APL cell line NB4. Due to the widely-demonstrated clinical utility of proteasome inhibitors in hematologic malignancies [322, 331, 334-336, 338, 340, 341], and the selectivity and specificity of the lipophilic cation DQA in APL cells [294-296], we hypothesized that their combination would enhance apoptosis in an additive or synergistic manner. In fact, sub-apoptotic doses of MG-132 have previously been reported to sensitize myeloid leukemia cells to chemotherapy [321, 343]. Unexpectedly, we found that the proteasome inhibitor MG-132 decreased DQA-induced apoptosis, interfering downstream of mitochondria.

According to our results, low doses of MG-132 induce Nrf2-mediated anti-oxidant response in NB4 cells that is probably the mechanism which counteracts the production and accumulation of ROS triggered by DQA, therefore protecting cells from apoptosis. This finding differs from previous works reporting that proteasome inhibitors induce ROS-mediated apoptosis [319, 321, 325, 415] and disagrees with the notion that low-dose proteasome inhibition sensitizes cancer cells to chemotherapy [316-318, 335]. However, the induction of an adaptive response to cell stress by low doses of proteasome inhibitors has been extensively documented in non-cancerous vascular endothelial cells [404, 405, 416, 417], supporting our results. Moreover, Rushworth et al. [418, 419] demonstrated that Nrf2 is overexpressed in AML primary cells and in various cell lines due to aberrant NF-κB signaling, and thus reduces cell sensitivity to chemotherapy and to proteasome inhibitors. These, in turn, enhanced the cytoprotection by inducing Nrf2 through ROS accumulation [418]. However, in such study APL cells and patients did not show increased levels of Nrf2, neither in the absence or presence of bortezomib, and were shown to be more sensitive to this drug than other AML subtypes or cell lines [418]. In contrast, we have shown that very low doses of proteasome inhibitor, which do not generate ROS accumulation, are able to induce Nrf2 activation in APL cells and counteract DQA-induced apoptosis. Proteasome inhibition at higher doses (over 10-fold of 0.15 μM) has been related to resistance to apoptosis before, generally due to the direct interference with the proteolysis of a specific substrate required for apoptosis induction by the second drug [221, 420, 421]. In other occasions proteasome inhibition induced the transcription of proteins that interfered with apoptosis, such as Mcl-1 [422] or HSP72 [423]; but doses as low as the one presented here have never been related to the inhibition of apoptosis in cancer cells.

Although this study should be extended to other Nrf2 target genes such as NQO1, GSTs or GPx, our results show that the combination of low doses of proteasome inhibitors and drugs which induce oxidative stress is not effective to improve their pro-apoptotic effects and therefore caution is recommended when new combined therapies are designed.

In addition to these findings, this work has characterized two other important mechanisms of resistance to apoptosis in NB4 cells: high basal levels of autophagy and gain-of-function of the p53 hot-spot mutant $p53^{R248Q}$.

As it has been mentioned before, autophagy is a context-specific mediator of cell death [137]. Altogether, our results showed that autophagy acts as an anti-apoptotic process in NB4 cells. Moreover, this cell line basally expresses pro-autophagic proteins and presents AVO in the cytoplasm, signs of high basal levels of autophagy. Therefore, autophagy can be considered an intrinsic mechanism of resistance to cell death in NB4 cells. Accordingly, autophagy inhibition appears to be necessary, at least partially, for DQA-induced apoptosis. Provided that DQA selectively targets mitochondria [285] and induces $\Delta\Psi_m$ disruption and ROS production, it is reasonable to hypothesize that autophagy needs to be inhibited because the presence of AVO could protect cells from mitochondrial damage and cytochrome c release and therefore hamper apoptosis [147]. Because we observed that pro-apoptotic effects of 3-MA and DQA did not add to each other, it is likely that apoptosis and autophagy inhibition are simultaneously regulated by DQA in NB4 cells as a part of its mechanism of action. In agreement with our results, it was found that selenite, which also induces apoptosis after ROS accumulation in APL cells [185], decreases autophagy [424].

The finding of autophagy being a pro-survival process in NB4 cells might be of great utility in current APL therapies, which could benefit from autophagy inhibition. Furthermore, it has been reported that both ATO and ATRA treatments induce autophagy *per se* [425, 426], which could be an important self-limitating effect of frontline therapies, supporting the use of autophagy inhibitors to improve clinical outcomes. The possible involvement of autophagy in resistance to apoptosis in leukemia should be more deeply explored, and additional cell lines need to be studied to obtain more robust conclusions, for these findings could have important implications in terms of the development of new therapies for APL. Similarly, we did not fully confirm that MG-132 protects autophagy. However, this notion is consistent with numerous reports demonstrating that proteasome inhibitors induce autophagy [301, 327, 427] as a mechanism to alleviate the endoplasmic reticulum stress caused by the accumulation of non-degraded proteins [301, 305, 327]. The fact that we did not observe significant changes in the LC3-α kinase and Bcn-1 levels or in the amount of AVO is probably due to the high levels of autophagy that are present in these cells in basal conditions, and the low dose of MG-132 used. On the other hand,

there is a possibility that we did not observe significant effects on autophagy by MG-132 because autophagy induction by proteasome inhibitors is a p53-mediated event and, thus, it might be impaired in NB4 cells [414]. Anyway, although the mild effects of MG-132 on autophagy cannot account for the overall anti-apoptotic response observed in co-treated cells, this and other drugs from the proteasome inhibitor family should be used with caution in tumors in which increased autophagy might work as a mechanism of chemoresistance. As a matter of fact, proteasome inhibitor-based therapies might benefit from the combination with autophagy inhibitors [328, 414].

Another major implication of the present work is the notion that acquired aberrant activity of $p53^{R248Q}$, and not only the loss of its normal functions, is involved in resistance to apoptosis in NB4 cells. According to our research, nuclear functions of $p53^{R248Q}$ are necessary for DQA-induced apoptosis while cytosolic $p53^{R248Q}$ (susceptible to interact with the mitochondrial membrane) appears to inhibit cell death through a gain-of-function mechanism. Consequences of gain-of-function of p53 mutations have been widely explored in cell lines and mice exogenously expressing hot-spot mutant forms of p53 [391-394, 396, 397] and few cell lines endogenously expressing p53 mutants have been studied [394, 428] but this is, to the best of our knowledge, the first time that gain-of-function anti-apoptotic effects of p53 are described in NB4 cells. In agreement with our finding, the oncogenic gain-of-function of exogenous R248Q mutants has been previously reported to promote cell invasiveness and chemoresistance *in vitro* [392, 410] and a recent report demonstrates that mice harboring the R248Q mutation have accelerated tumor growth and reduced tumor-free survival [429]. Therefore, the data obtained from the pharmacological inhibition of endogenous p53 by PFT are encouraging and provide a good basis to further study this topic.

Interestingly, PFT-α has been previously shown to inhibit apoptosis in cells that preserved their p53 transcriptional activity, but not in those in which the functionality of mutant p53 as a transcriptional activator was lost [430]. In our model, PFT-α was able to decrease DQA-induced cell death, suggesting that $p53^{R248Q}$, which translocates to the nucleus upon treatment with DQA, preserves its transcriptional activity. However, in agreement with reports that postulate that p53 hot-spot mutations are unable to correctly bind DNA and transcriptionally activate its target genes [399, 400], we failed to demonstrate active transcription of P21, BAX or DRAM1 by nuclear $p53^{R248Q}$. According to these results, $p53^{R248Q}$ is probably not transcriptionally active. Then, why did PFT-α reduce DQA-induced apoptosis? A possible explanation is that this inhibitor seems to act over nuclear import/export of p53, blocking its nuclear accumulation [359]. This block would increase cytosolic levels of $p53^{R248Q}$, which would subsequently inhibit apoptosis through a gain-of-function mechanism, in agreement with the results obtained with PFT-μ. It is therefore reasonable that nuclear $p53^{R248Q}$ is not transcriptionally active and it could be

hypothesized that DQA-induced $p53^{R248Q}$ nuclear translocation facilitates apoptosis by favoring the withdrawal of this protein from the cytosol. Of note, a previous study of cell death induced by selenite in NB4 cells reported that PFT-α inhibited the apoptosis-preceding loss of $\Delta\Psi_m$ and ROS accumulation [185]. These results agree with our findings; however, authors interpreted them as proof of the direct participation of p53 in mitochondria-mediated cell death, omitting both the mechanism of action of PFT-α and the mutant nature of p53 in these cells. Indeed, an earlier report investigating selenite-induced apoptosis in prostate cancer cells with different functional statuses of p53 postulated that ROS accumulation precedes p53 activation [431]; therefore inhibition of $\Delta\Psi_m$ loss and ROS accumulation by PFT-α [185] responded to an effect unrelated to p53 inhibition. Moreover, this report showed that caspase-independent (mitochondria-mediated) cell death is delayed and less efficient in cells with mutated p53 due to the lack of a p53-mitochondria feedback loop [431], supporting our hypothesis that cytosolic (that is, mitochondrial) functions of mutant p53 are altered in NB4 cells.

On the other hand, there is a possibility that the enhancement of apoptosis observed in combination of MG-132 and/or DQA with PFT-μ did not respond to the inhibition of the cytosolic activity of $p53^{R248Q}$ but to a secondary effect of this molecule. In addition to p53 inhibition, PFT-μ appears to inhibit HSP70 [432]. The induction of a dose-dependent inhibition of proliferation and a decrease in viability by PFT-μ in the AML cell line KG-1 was attributed to this mechanism in one study in which the inhibitor synergistically induced apoptosis in combination with drugs frequently used in leukemia [433]. However, sensitivity of KG-1 cells to PFT-μ was considerably lower than that of the rest of the leukemia cells studied in this work, and the dose that was used was twice as the dose that we utilized for pre-treatment of NB4 cells (10 μM and 5 μM, respectively) [433], which suggests that HSP70 inhibition could be a secondary effect of PFT-μ at a high dose. Remarkably, the KG-1 cell line, similarly to NB4 cells, bears a mutation in the DNA-binding domain of p53 [350], so the effects observed in apoptosis in the aforementioned work could also be interpreted as a result of the inhibition of mutant p53. That is, although it cannot be ruled out that the effects observed in cells pre-treated with PFT-μ in our model are caused by the inhibition of HSP70, it is more likely that they respond to $p53^{R248Q}$ inhibition due to the low dose that we used in NB4 cells. A further study of the effect of PFT-μ on HSP70 expression in NB4 cells could help assessing the specificity and efficiency of p53 inhibition by this molecule and would be essential to validate our results. Anyway, the present work demonstrates that PFT-μ has great usefulness and therapeutic potential in combination therapies in leukemia.

To sum up, this work has explored three mechanisms that may lead to resistance to apoptosis in the APL cell line NB4. More work will be needed to determine the repercussions of Nrf2 signaling activation, high basal autophagy levels and gain-of-function of p53 mutants on chemoresistance in this

disease. However, we have gone some way towards enhancing our understanding of the mechanisms that may protect cancer cells from apoptosis. While preliminary, our data point to the possible use of selective autophagy and p53 inhibitors to improve the efficiency of current protocols and reduce the doses of chemotherapy. Results presented here are encouraging and support further investigations that may allow the development of better therapies, especially needed in high-risk APL patients [39].

BLOCK II

miRNA-mediated Gene Upregulation Induced by Proteasome Inhibition in CML Cells

RESULTS AND DISCUSSION

1. Background

In the previous section we studied three cell-specific mechanisms that confer APL cells resistance to apoptosis. Two of them, the gain-of-function activity of mutant p53 and the elevated basal levels of autophagy, were intrinsic mechanisms in NB4 cells and protected them from cytotoxicity induced by different stimuli. On the other hand, the activation of the anti-oxidant response specifically affected death signals mediated by the generation of ROS and was directly triggered by the proteasome inhibitor MG-132. Remarkably, even though the introduction of proteasome inhibitors in the clinics represented a breakthrough in the therapy of multiple myeloma and other hematologic diseases [331, 332, 336, 434], this drug family produces a complete response in less than half of the patients and drug resistance is a patent problem of current therapies involving proteasome inhibitors (reviewed in [304]). In light of the results obtained after treatment of NB4 cells with MG-132 and DQA, we determined to continue the study of proteasome inhibitor-related chemoresistance and test the effectiveness of the combination of MG-132 and DQA in a different cell context, in which the gain-of-function of mutant p53 and elevated basal autophagy did not interfere with our research. For this purposes, we chose a CML model.

As it was extensively described in the *Introduction* section, CML is a clonal malignancy characterized by the expression of the fusion protein Bcr-Abl, a constitutively activated kinase [44]. This oncogene confers CML blasts resistance to apoptosis and most types of drugs [435, 436]. Nowadays, first line therapy for CML is the specific Bcr-Abl inhibitor imatinib mesylate [52], which has achieved excellent results in chronic phase patients. However, the problem of this disease continues to be the high rate of resistance, which is especially high in the accelerated and blast phases [54, 57]. For this reason, there is a need for novel agents that are efficient in these subsets of patients and that can kill resistant stem or progenitor cells**,** which are the main source of residual disease [58]. The use of proteasome inhibitors in CML has been especially studied in patients in the blastic phase, who have developed a secondary AML. There are two currently open phase 1 studies of the use of proteasome inhibitors in CML, and several others have been conducted in the past ten years, with those including patients in the blastic phase having reached phase 2 [342]. This points to the prospective use of drugs from this family in the treatment of CML and underlines the relevance of the study of proteasome inhibitor-related chemoresistance in this disease.

K562 cells were originated from human bone marrow of a CML patient in the blastic phase and, accordingly, express Bcr-Abl [354]. The p53 gene is inactivated in these cells [355], similarly to the case of a great number of CML patients in blast crisis [51]. In K562 cells, autophagy seems to be involved in megakaryocytic differentiation [437] and its induction favours the downregulation of Bcr-

Abl [438] and sensitizes cells to apoptosis [439]. For all these reasons, we considered K562 cells as good subjects of study for our purposes. Similarly to primary CML cells in blastic phase, K562 cells are more resistant to apoptosis than other types of leukemic cells and show a lower sensitivity to most kinds of chemotherapy [440], including DQA, which has a higher IC_{50} in this cell line than in NB4 cells [294, 295], and proteasome inhibitors [441], in spite of existing evidence that the proteasome inhibitor bortezomib sensitizes Bcr-Abl^+ cells to imatinib [339].

Interestingly, resistance to distinct types of drugs [212, 215-221], including proteasome inhibitors [442], has been recently related to the accumulation of the cyclin-dependent kinase inhibitor p21, which is a well-known gene product upregulated after treatment with this family of drugs [173, 441, 443]. In addition, protein p21 is overexpressed in Bcr-Abl positive cells owing to the aberrant kinase activity of this fusion protein [228] and there is increasing evidence that high p21 levels interfere with apoptosis in Bcr-Abl positive cells, including K562 [227-229]. Thus, it needs to be taken into account that the use of proteasome inhibitors to potentiate the efficiency of chemotherapy in CML may be hampered by the resulting upregulation of p21. Moreover, the mechanisms involved in such upregulation and p21 itself could be interesting therapeutic targets to improve response to proteasome inhibitors.

Importantly, miRNAs are well-known modulators of the expression of several cell cycle regulators, including p21 [260]. In particular, there are numerous studies about the p21-inhibitory function of miRNAs belonging to the miR-106 family [444-447]; moreover, the inhibition of those miRNAs has proved to render cancer cells resistant to chemotherapy through the upregulation of p21 [220]. It is therefore possible that intrinsic or proteasome inhibitor-induced changes in the expression of p21-regulatory miRNAs lead to the overexpression of this protein.

2. Hypothesis and Aims

Based on the aforementioned, we hypothesized that the combination of non-lethal doses of the proteasome inhibitor MG-132 would sensitize K562 cells to ROS-mediated apoptosis induced by DQA by enhancing oxidative and ER stress and by activating p53-independent mechanisms of apoptosis; furthermore, we expected that the use of a low dose of MG-132 would prevent p21-associated chemoresistance in this cell line.

To investigate our theory, we established the following aims:

- To study the effect of the combination of DQA and a low dose of MG-132 on proliferation and death in K562 cells.
- To investigate the effects of MG-132 and DQA on p21 expression in this cell line and the possible regulation of this protein by miRNAs in a p53-deficient model.

3. Materials and Methods

3.1. Cell lines

- K562
- Meg-01

3.2. Treatments

- MG-132
- DQA

3.3. Methods

- MTT assay
- Study of apoptosis through the detection of PS externalization and permeability to PI
- Assessment of $\Delta\Psi_m$ and detection of $O_2^{\cdot -}$
- Analysis of DNA content and BrdU uptake
- Western blot analysis (p21, cyclin B1 and p27)
- qPCR (P21, miR-17-5p, miR-20a, miR-22, miR-93 and miR-106b)
- Bioinformatic search of p21 3'UTR-interacting miRNAs
- Reporter plasmid construction
- Transfection (lipid-based and electroporation)
- Luciferase reporter gene assays ("affinity for p21 3'UTR" assays)

4. Results

4.1. MG-132 induces apoptosis and cell cycle arrest in G_2/M in K562 cells

Following the same approach as in NB4 cells, we first tested the effects of incremental doses of MG-132 on viability and cell cycle progression of K562 cells. MTT analysis showed a time- and dose-dependent decrease in metabolic activity of K562 cells, with IC_{50} values of 4.6 μM and 0.35 μM at the times of 24 and 48 hours, respectively (data not shown). MG-132 induced a significant increase in the

number of sub-diploid cells starting at the dose of 0.15 µM (data not shown), indicating cell death by apoptosis. As shown in Figure 1, effective cell cycle arrest in G_2/M phase occurred at doses higher than 0.25 µM MG-132. Because we aimed to use this proteasome inhibitor at cytostatic but non-lethal doses to combine it with DQA, we selected the dose of 0.25 µM for the following experiments, which corresponds to a decrease of ~6% and ~35% in the metabolic activity rate at the times of 24 and 48 hours, respectively, as observed in MTT assays (data not shown).

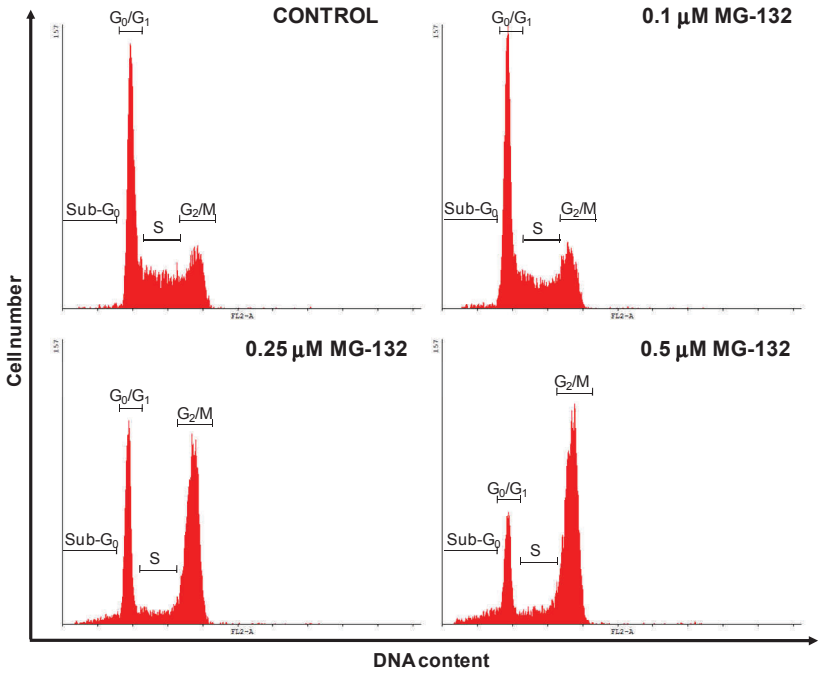

Figure 1. Effects of MG-132 on cell cycle progression in K562 cells. Histograms represent the DNA content, determined by flow cytometry with PI, after a 24-hour treatment with MG-132 at the indicated doses. Results are representative of n=3 independent experiments.

4.2. MG-132 and DQA do not cooperate in the induction of apoptosis in K562 cells

In order to explore the effect of the combination of MG-132 and DQA, K562 cells were pre-treated with MG-132 2 hours before treatment with DQA. As expected from the previous cell cycle experiments, MG-132 alone moderately but significantly induced apoptosis in K562 cells, whereas DQA only induced a mild increase in the apoptosis rate at 20 µM and after 48 hours of exposure (Figure 2), confirming that the effectiveness of this compound is much lesser in K562 cells than in NB4 cells. When both compounds were used in combination, no significant differences in the rate of apoptotic cells were observed as compared to the effects of MG-132 alone (Figure 2). Of note, the cytotoxic effects of DQA after 48 hours of treatment appeared to add to those of MG-132, although differences were not statistically significant.

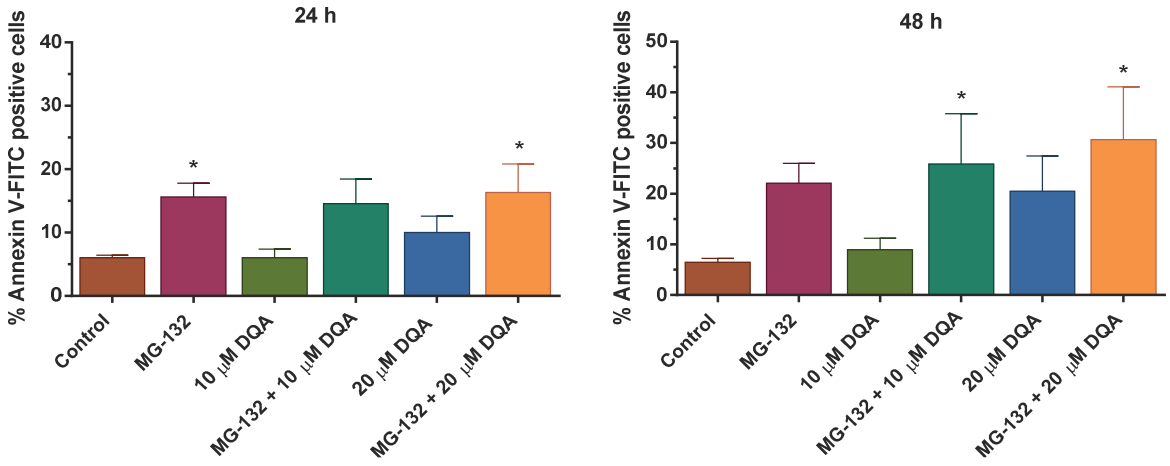

Figure 2. Cytotoxic effects of MG-132, DQA and their combinations in K562 cells. K562 cells were pre-treated with 0.25 μM for 2 hours and then treated with 10 μM and 20 μM DQA for 24 and 48 hours. Bar graphs represent apoptotic (Annexin V-FITC positive) cells (mean ± SEM of n=3). Statistical significance: *P<0.05.

4.3. MG-132 does not interfere with the generation of oxidative stress by DQA in K562 cells

In NB4 cells, we had previously observed a decrease in the DQA-induced accumulation of ROS which was a result of the stimulation of the anti-oxidant response by MG-132. For this reason, we analyzed the changes in $\Delta\Psi_m$ and $O_2^{\cdot-}$ accumulation in K562 cells to study the effect of MG-132 and DQA on the levels of intracellular oxidative stress.

Figures 3A and 3B show that the treatment with MG-132 slightly, but not significantly, decreased $\Delta\Psi_m$ after 48 hours of treatment whereas DQA induced a ~25% decrease which was statistically significant at the dose of 20 μM after 12 hours of treatment. This indicates that DQA affects mitochondrial membrane permeability, although this is not sufficient to efficiently induce apoptosis. This drug also induced a mild but significant increase in $O_2^{\cdot-}$ levels after 24 and 48 hours (Figures 3C and 3D).

As expected, MG-132 did not increase $O_2^{\cdot-}$ levels. These results agree with those obtained in NB4 cells and confirm that low doses of MG-132 do not affect the oxidative state of these cell lines and therefore induce cytotoxicity through other mechanisms. More importantly, the fact that MG-132 did not counterbalance the accumulation of $O_2^{\cdot-}$ mediated by DQA (Figures 3C and 3D) in K562 cells suggests that it does not stimulate the anti-oxidant response in these cells, at least in the conditions studied.

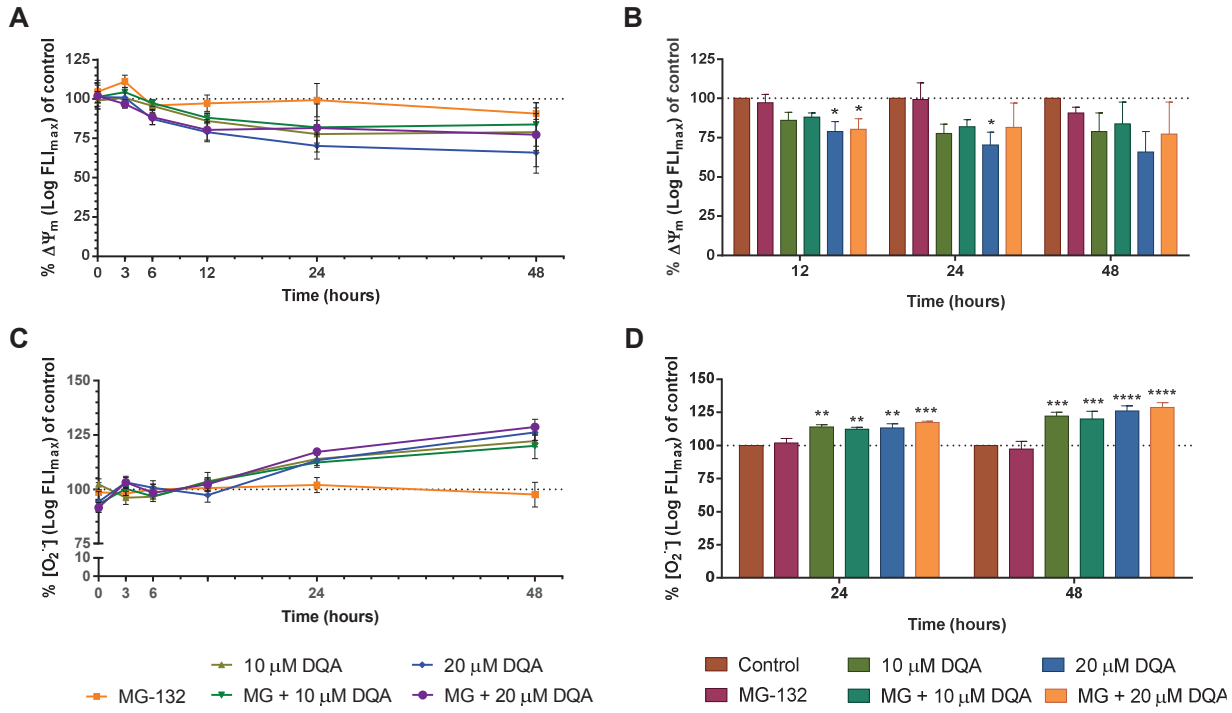

Figure 3. Effect of MG-132 ± DQA on mitochondrial function and oxidative stress in K562 cells. **(A)** Time-course of changes in $\Delta\Psi_m$ induced by 0.25 μM MG-132 ± 10 μM or 20 μM DQA. **(B)** Changes in $\Delta\Psi_m$ induced by MG-132 ± DQA after 12, 24 and 48 hours. **(C)** Time-course of changes in the levels of $O_2^{\bullet-}$ induced by MG-132 ± DQA. **(D)** Changes in the levels of $O_2^{\bullet-}$ induced by MG-132 ± DQA after 24 and 48 hours. **(A-D)** Results are expressed as the percentage of the logarithm of maximum fluorescence intensity ($logFLI_{max}$) of controls and represent mean ± SEM of n=4 independent experiments. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

4.4. The combination of DQA with MG-132 strongly decreases cell proliferation rate

Because the combination of MG-132 and DQA failed to enhance apoptosis in K562 cells and an interaction between both drugs in the generation of oxidative stress was not found, we next determined to explore more deeply the effects of both compounds on cell proliferation. For this purpose, we analyzed cell cycle progression and DNA synthesis, as well as the protein levels of cell cycle regulators in the presence of MG-132 and/or DQA.

In agreement with our previous experiments (Figure 1), MG-132 induced cell cycle arrest in G_2/M phase after 24 hours of treatment (Figure 4). Moreover, this drug also induced a significant decrease in cells in the S phase, which suggests that it also arrests proliferation in G_0/G_1, although no accumulation of cells in this phase was observed probably due to the strong arrest in mitosis. Interestingly, DQA alone arrested cell cycle in G_0/G_1. As a consequence, the combination of both compounds strongly decreased the number of cells actively replicating DNA. It is noteworthy that the significant cell cycle arrest in G_2/M was prolonged after 48 hours only in cells treated with both MG-132 and DQA, reaching dramatically low levels of actively proliferating cells.

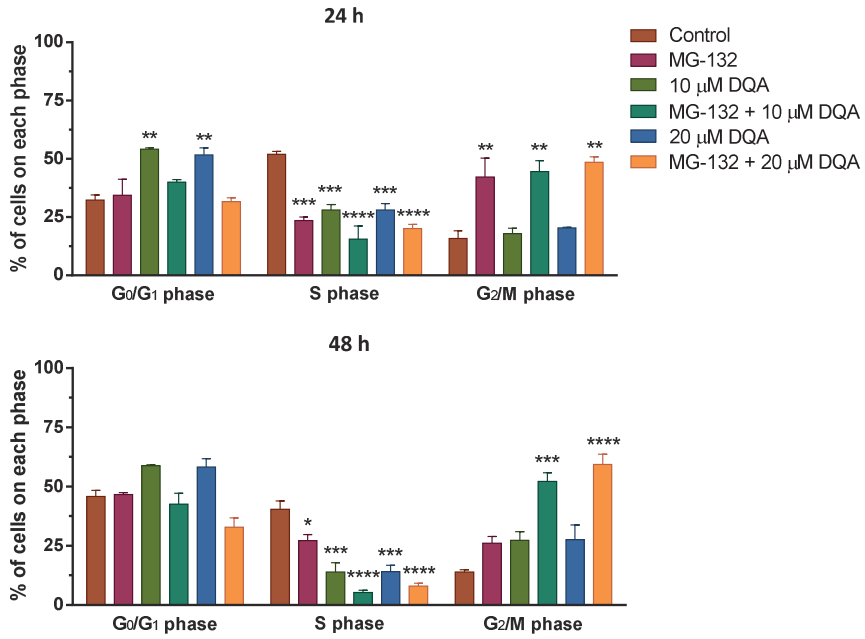

Figure 4. Cell cycle distribution of K562 cells treated with MG-132 ± DQA for 24 and 48 hours. Bar graphs represent the proportion of cells on each cycle phase, determined through the analysis of DNA content and BrdU uptake. Results are presented as mean ± SEM of n=3 experiments. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

As explained in Chapter 5 of *Introduction*, mitosis is regulated by the levels of the mitotic cyclin, cyclin B in humans, which increase during the initiation of this process [448]. Before the cell exits mitosis, cyclin B needs to be degraded by the proteasome to allow the depolymerization of spindle microtubules and chromosome decondensation [166, 448]. As a proteasome inhibitor, MG-132 induced the accumulation of cyclin B1 after 24 hours (Figure 5). This could be, at least in part, the mechanism through which MG-132 induces cell cycle arrest. Interestingly, there was a trend towards a reduction in cyclin B1 levels in DQA-treated cells, significant after 48 hours.

Because DQA appeared to arrest cell cycle in G_0/G_1 *per se*, we also examined the expression of the CDKIs $p27^{KIP1}$ (herein referred to as p27), which is a regulator of the $G_1 \rightarrow S$ transition and is also degraded by the proteasome [449], and p21 [443]. As it was extensively explained before, p21 is a cell cycle regulator but it is also involved in resistance to apoptosis (reviewed in [212]).

Whereas the protein expression of p27 remained unchanged (results not shown), levels of protein p21 strongly increased after treatment with MG-132, both in single treatments and in combination with DQA (Figure 6A), in agreement with numerous reports describing the induction of p21 expression by proteasome inhibitors [173, 443]. However, what attracted our attention was that DQA moderately increased (~2-3-fold) p21 expression after 24 hours *per se*. More interestingly, pre-

treatment with MG-132 strongly enhanced p21 expression in cells treated with DQA (Figure 6A), suggesting a synergistic effect between MG-132 and DQA after 24 hours of treatment.

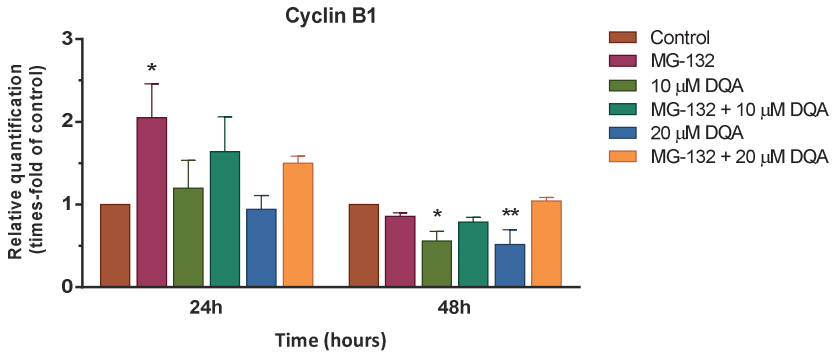

Figure 5. Protein levels of cyclin B1 after the treatment of K562 cells with MG-132 ± DQA. Cyclin B1 levels were determined by Western blot and are expressed as relative to controls. Data represent mean ± SEM of n=3 independent experiments. Statistical significance: *P<0.05; **P<0.01.

4.5. MG-132 strongly enhances p21 expression in the presence of DQA

Because overexpression of p21 has been extensively reported to play an important role in cancer chemoresistance [215-221], including CML [227-229], we determined to investigate in depth the origin of the strong increase in p21 protein levels in cells treated with both MG-132 and DQA.

The analysis of p21 mRNA levels (Figure 6B) confirmed that p21 expression is dramatically enhanced by the combination of MG-132 and DQA. On one hand, MG-132 alone induced a 2-fold increase in p21 mRNA levels. These levels remained constant over time, consistent with protein data. On the other hand, DQA alone appeared to induce a strong transcriptional activation of p21 starting 24 hours after treatment and exponentially increasing over time. The combination of both drugs yielded extremely high levels of p21 mRNA that remained high even after 48 hours, time point at which, apparently, p21 protein turnover had already started to occur (Figures 6A and 6B).

We next focused on the study of the mechanism through which p21 mRNA levels were so highly augmented. Because p21 mRNA has a short half-life, of about 1.5 hours, and its levels were constant in cells treated with MG-132, we hypothesized that this drug stabilizes p21 mRNA, preventing its decay and increasing its half-life, which would explain the massive accumulation observed after the combination of MG-132 and DQA. Taking into account the aforementioned role of miRNAs in posttranscriptional regulation, it could be hypothesized that this mRNA stabilization results from the loss of miRNA-mediated mRNA degradation. Thus, we sought to study the possible involvement of one or more miRNAs in the stabilization of p21 mRNA during treatment with MG-132.

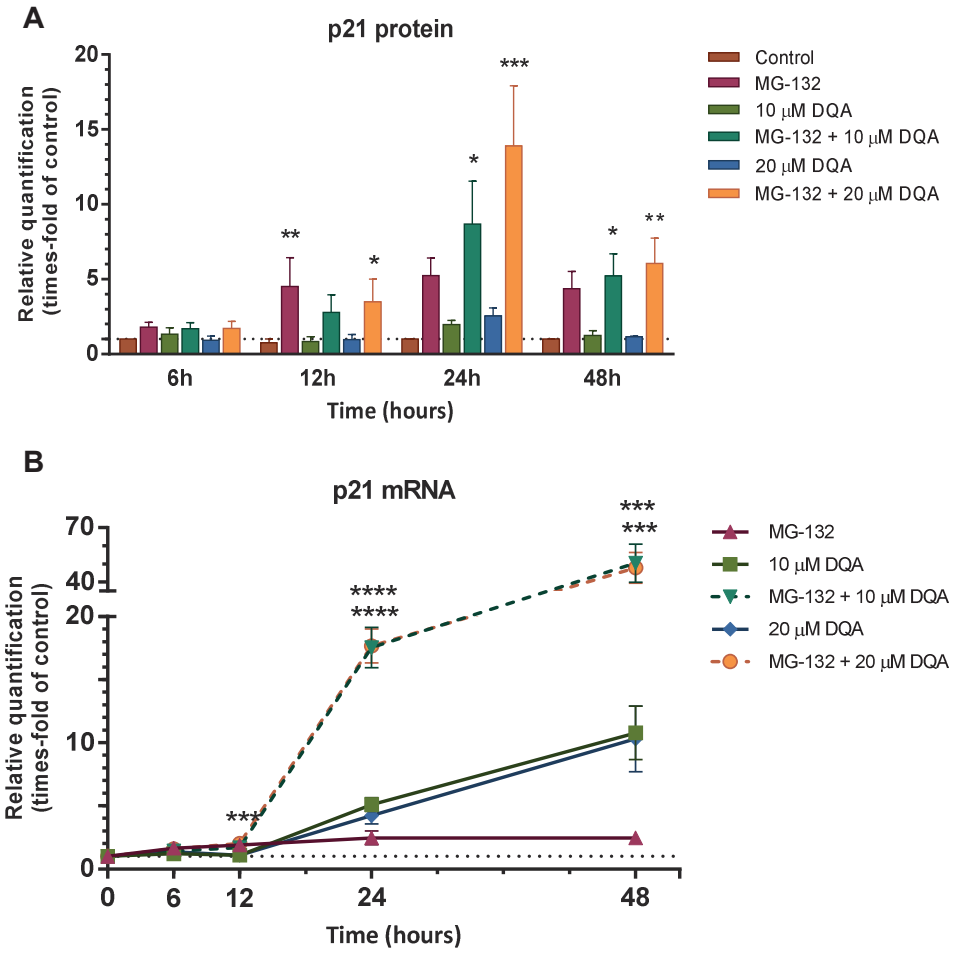

Figure 6. Expression of the cell cycle regulator p21 after the treatment of K562 cells with MG-132 ± DQA. (A) Protein levels of p21, determined by Western blot. **(B)** mRNA levels of p21, measured by qPCR. Continuous lines represent single treatments, and discontinuous lines represent MG-132 + DQA combinations. **(A-B)** Graphs represent mean ± SEM of n=4 and n=3 independent experiments, respectively. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

4.6. Selection of potential p21-regulatory miRNAs

In order to screen potential miRNA interactions with the 3'UTR of human p21 mRNA, we used the miRNA target prediction database DIANA miRGen, which integrates information from four widely used target prediction algorithms: DIANA-microT, miRanda (microrna.org and miRBase), PicTar and TargetScanS. The search by miRGen yielded 47 results, from which we selected 5 miRNA that had been predicted by more than one program: hsa-miR-106b, hsa-miR-17-5p, hsa-miR-20a, hsa-miR-20b and hsa-miR-22; as well as 8 miRNAs which had been predicted twice by the same algorithm in the miRGen screening: hsa-miR-224, hsa-miR-368, hsa-miR-371, hsa-miR-372, hsa-miR-409-5p, hsa-miR-505, hsa-miR-608, hsa-miR-93 (Table 1).

Additionally, the miRanda, PicTar and TargetScanS algorithms were analyzed individually to confirm the suitability of the miRNA selection according to the prediction scores obtained by each algorithm.

Remarkably, the integrated search yielded some hits which did not appear among the results obtained independently with miRanda while some of the results yielded with the individual search were not included among the 47 hits obtained with miRGen.

Table 1. Potential p21 3'UTR-interacting miRNAs predicted by bioinformatic algorithms. Search hits are ordered according to their score ranking position in DIANA miRGen.

	DIANA miRGen database		miRanda (microrna.org)	PicTar (4-way)	TargetScan
miRNA	Score ranking position (N=47)	Algorithm	Score ranking position (N=35)	Score ranking position (N=12)	Score ranking position (N=15)
hsa-miR-106b	2-4	PicTar (4-way), TargetScanS	7	7-8	4
hsa-miR-17/17-5p	5-7	PicTar (4-way), TargetScanS	8	9-10	4
hsa-miR-20a	10-12	PicTar (4-way), TargetScanS	5	3-4	4
hsa-miR-20b	13-15	PicTar (4-way), TargetScanS	9	3-4	4
hsa-miR-22	17-21	PicTar (4-way), TargetScanS, miRanda (microrna.org)	35	11-12	3
hsa-miR-224	22-23	miRanda (microrna.org)	1	-	-
hsa-miR-368	25-26	miRanda (microrna.org)	-	-	-
hsa-miR-371	27-28	miRanda (microrna.org)	-	-	-
hsa-miR-372	29-30	PicTar (4-way)	-	2	-
hsa-miR-409-5p	31-32	miRanda (microrna.org)	-	-	-
hsa-miR-505	35-36	miRanda (microrna.org)	-	-	-
hsa-miR-608	41-42	miRanda (microrna.org)	-	-	-
hsa-miR-93	45-46	PicTar (4-way)	3	1	4

The combination of the results obtained by the three different algorithms after the initial screening led to the selection of six mature miRNAs, predicted by all of the algorithms (Figure 7A): homo sapiens (hsa)-miR-106, hsa-miR-17-5p, hsa-miR-20a, hsa-miR-20b, hsa-miR-22 and hsa-miR-93. Human miR-106b, miR-17-5p, miR-20a, miR-20b and miR-93 share seed sequences (Figure 7B) and belong to the miR-106b family. miR-22, on the other hand, has a completely different seed sequence (Figure 7B) [256]. miR-22 has been reported to downregulate p21 [450] and c-Myc binding protein [451], although it is better known as a PTEN inhibitor [452, 453].

Because miR-20a and miR-20b are homologous miRNAs, to further reduce our list we only performed expression assays for miR-20a, which ranked first in the prediction scores. Therefore, our final miRNA selection consisted of 5 miRNAs: hsa-miR-106b, hsa-miR-17-5p, hsa-miR-20a, hsa-miR-22 and hsa-miR-93.

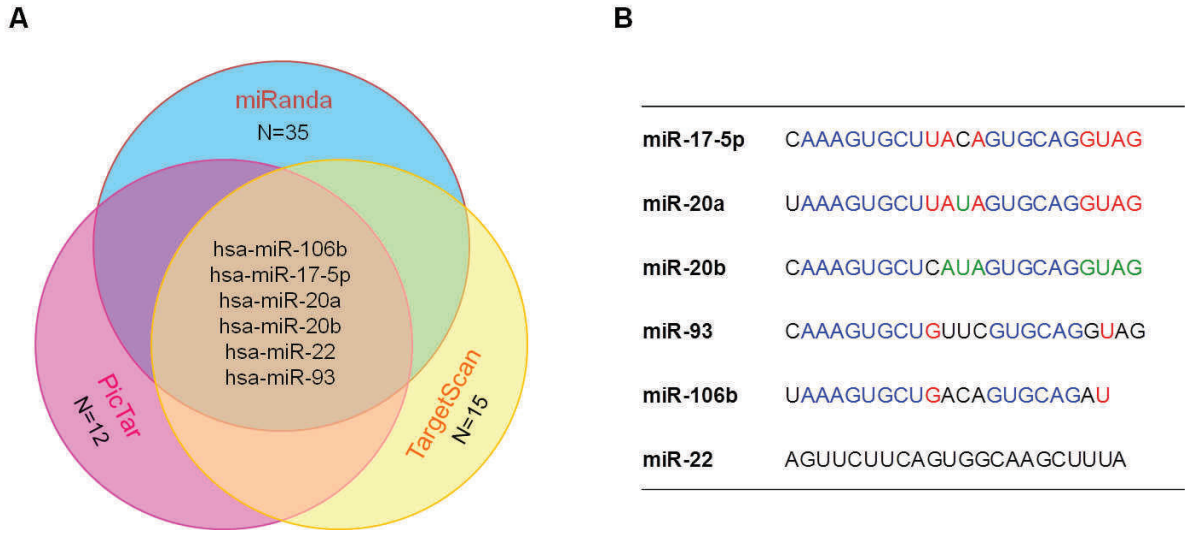

Figure 7. **Potential p21 3'UTR-interacting miRNAs. (A)** Summary of the prediction by the bioinformatic algorithms miRanda, PicTar and TargetScan. **(B)** Comparison of the seed sequences (5p[1]) of the 6 miRNAs predicted by the three algorithms. Sequences highlighted in blue are shared between all members of the miR-106b family; red highlights indicate similarities between members of the same cluster; green indicates similarities between homologs.

4.7. miR-22 is upregulated in K562 cells treated with MG-132

After the screening, we analyzed changes in the expression levels of the selected miRNAs in K562 cells treated with MG-132 and/or DQA. Because no significant differences in p21 mRNA levels were detected between the two doses of DQA utilized (Figure 6B), for the next experiments we only treated cells with 10 µM DQA.

Time-course expression assays revealed no major changes in the expression of miR-17-5p, miR-20a, miR-93 and miR-106b (Figure 8). miR-17-5p, miR-93 and miR-106b levels underwent a mild increase 6 hours after treatment with MG-132 and DQA, which was statistically significant in the case of miR-93, but they rapidly recovered and decreased up to a 50% by the time of 48 hours, as well as miR-20a levels. This decrease was significant for miR-17-5p, miR-20a and miR-93.

Conversely, when we analyzed changes in miR-22 expression, we found that this miRNA was significantly upregulated upon treatment with MG-132 (Figure 9A), whereas DQA did not appear to exert any effect on its expression.

These data suggest that miRNAs from the miR-106 family, which negatively regulate the expression of p21, could be releasing p21 mRNA from repression after treatment, and therefore allowing its

[1] In the nomenclature used to distinguish the two mature miRNAs that are potentially obtained form a miRNA duplex, the "5p" suffix refers to the mature miRNA originated from the 5' arm of the pre-miRNA, and the "3p" suffix refers to the mature miRNA originated from the 3' arm.

upregulation. After all, some miRNAs from the miR-106 family are well-known cell cycle regulators [260]. However, the kinetics of the changes in miR-106 family members do not match the changes observed in p21 expression, since they only reach significantly low levels after 48 hours. More importantly, these changes are not specific of MG-132 and there are no significant differences between treatments. Therefore, neither of these miRNAs plays an important role in the upregulation of p21 after treatment with MG-132.

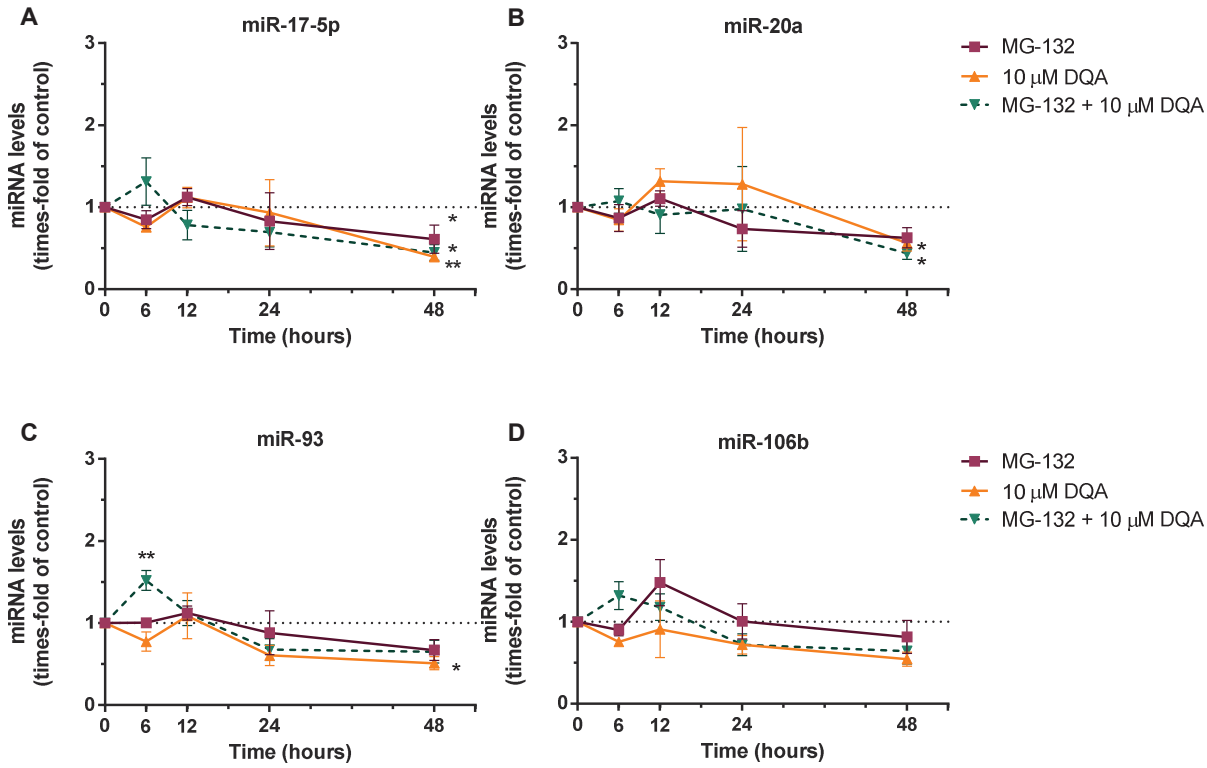

Figure 8. **Expression levels of potential p21-regulatory miRNAs in K562 cells after treatment with MG-132 ± DQA**. **(A)** miR-17-5p **(B)** miR-20a **(C)** miR-93 **(D)** miR-106b. Results represent relative miRNA expression, determined by qPCR (mean ± SEM of n=3 independent experiments). Statistical significance: *P<0.05; **P<0.01.

Expression of miR-22, in turn, appeared to be strongly related to proteasome inhibition. Despite miRNAs are typically considered as negative regulators of protein expression, evidence that they may also participate in the positive regulation of some genes has started to arise [248, 253-255]. Because our results suggested that miR-22 could be involved in the upregulation of p21, we next sought to explore the possible role of this miRNA as a positive post-transcriptional regulator.

4.8. Upregulation of miR-22 increases p21 mRNA levels

In order to investigate the connection between miR-22 and p21 expression, we used a miRNA precursor to transfect K562 cells and compared its effects with those of MG-132. The miR-22 precursor induced a significant increase in endogenous p21 mRNA levels after 48 hours from transfection (Figure 9B). This increase was equivalent to that induced by MG-132 alone. Moreover, treatment with MG-132 in cells transfected with hsa-miR-22 did not induce any significant change in comparison to untreated cells (Figure 9B). These results indicate that miR-22 participates in the stabilization of p21 mRNA and, importantly, suggest that MG-132 and miR-22 increase p21 mRNA levels through the same mechanism. That is, MG-132 could increase p21 expression, at least in part, through miR-22-mediated mRNA stabilization.

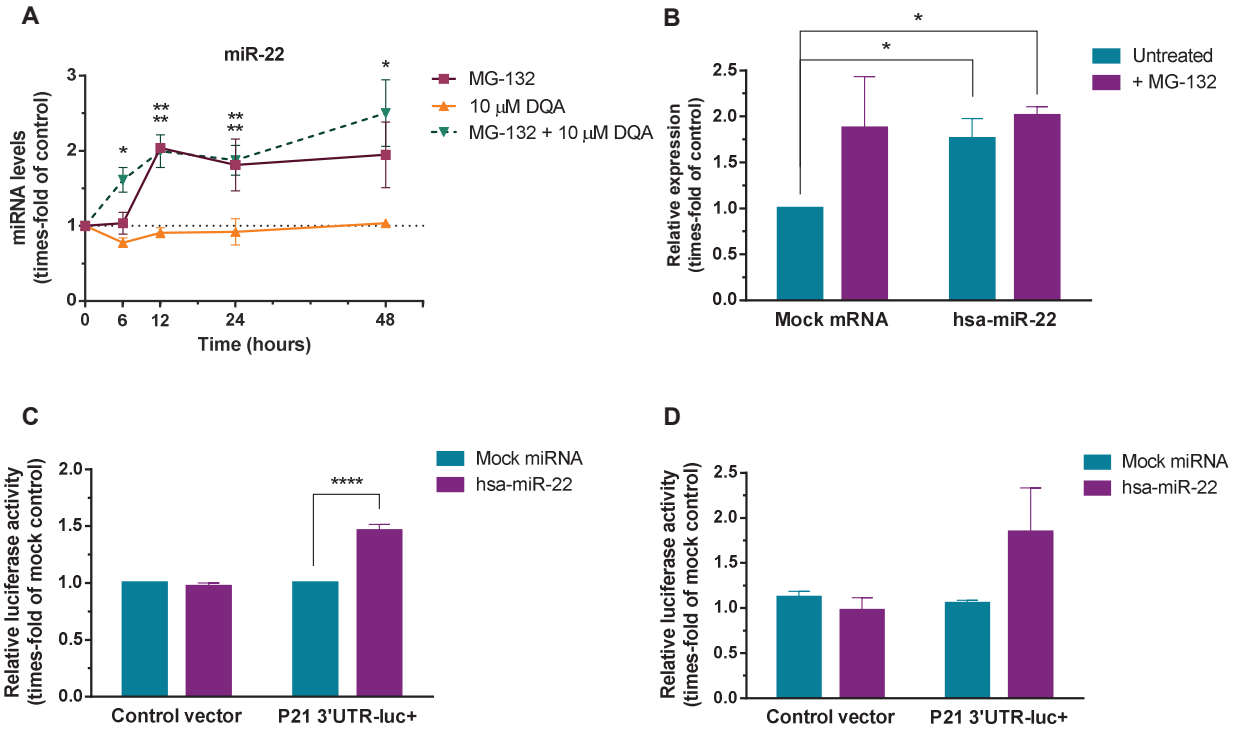

Figure 9. Role of miR-22 in MG-132-induced p21 expression. (A) Expression levels of miR-22 in K562 cells after treatment with MG-132 ± DQA at the indicated times, determined by qPCR and expressed as relative to control. **(B)** Effect of the ectopic expression of miR-22 and treatment with MG-132 on p21 mRNA levels in K562 cells, detected by qPCR after 24 hours. **(C)** Effect of the ectopic expression of miR-22 on p21 3'UTR-driven luciferase activity in Meg-01 cells. **(D)** Effect of ectopic expression of miR-22 on p21 3'UTR-driven luciferase activity in K562 cells treated with MG-132. **(A-D)** Graphs represent mean ± SEM of n=3 independent experiments. Statistical significance: *P<0.05; **P<0.01; ****P<0.0001.

4.9. miR-22 specifically binds p21 mRNA 3'UTR and increases protein expression

Next, we sought to confirm that miR-22 can specifically target p21 mRNA 3'UTR. For this purpose, we carried out luciferase reporter gene assays with a reporter vector carrying a fragment of the 3'UTR of p21 mRNA (see the *Materials and Methods* section). First, we analyzed the effect of miR-22 on Meg-01 cells, which are also Bcr-Abl positive lymphoblasts from CML like K562 cells but, in contrast, are more easily transfectable. Ectopic expression of miR-22 significantly increased luciferase activity on a ~1.5-fold (Figure 9C), suggesting that this miRNA can specifically bind to p21 mRNA 3'UTR and enhance protein expression of its target gene.

To verify these results, we performed the same experiment in K562 cells. However, co-transfection with the miR-22 analog did not lead to any changes in the luciferase activity in these cells (results not shown). Surprisingly, when cells were treated with MG-132 after transfection, miR-22 induced a ~2-fold increase in the expression of the luciferase gene when compared to the respective vector and RNA controls also treated with MG-132 (Figure 9D).

These data confirm the 3'UTR-mediated induction of p21 expression by miR-22 and point to a mechanism which is MG-132-dependent in K562 cells. If we assume that the p21 3'UTR-luciferase transcript accumulates with the same effectiveness as p21 mRNA, these results could indicate that K562 cells need additional stimuli for the complete translation of this protein.

5. Discussion

The initial aim of this work was to explore the combined effects of the proteasome inhibitor MG-132 and the lipophilic cation DQA in of the human leukemic cell line K562, going in depth in the mechanisms of chemoresistance associated to proteasome inhibition and especially focusing on the upregulation of p21.

As our group had reported before [294, 295], K562 cells were less sensitive to DQA than NB4 cells, showing an intrinsic resistance to the induction of oxidative stress. Probably for this reason, the treatment with MG-132 did not trigger a biologically significant anti-oxidant response in K562 cells, in contrast to what was observed in the cell line NB4. Despite MG-132 not interfering with the mechanism of action of DQA, effects of both drugs did not enhance each other's cytotoxicity when they were combined but, surprisingly, this combination had a strong anti-proliferative effect as a result of the simultaneous cell cycle arrest in G_0/G_1 and G_2/M phases directed by DQA and MG-132, respectively. Because K562 cells are deficient in the cyclin-dependent kinase inhibitors $p15^{INK4b}$ and $p16^{INK4a}$ [454], and since we did not detect any changes in protein levels of p27, the mechanism

through which DQA induces cell cycle arrest in phase G_0/G_1 remains unclear. On the other hand, the study of cell cycle regulators allowed us to attribute G_2/M arrest to the accumulation of cyclin B1 and p21 induced by MG-132. It is important to note that, in spite of p21 being one of the best-known transcriptional targets of p53 [176], its expression is not exclusively dependent on p53-mediated transactivation [204] and numerous studies have shown that functional p53 might not be necessary for p21 accumulation after proteasome inhibition [173, 326, 443]. For instance, it has been reported that p21 induces cell cycle arrest in phase G_0/G_1 only in cells expressing functional p53 while it arrests cycle in phase G_2/M in p53-deficient cells like K562 [402].

As it was mentioned above, it is well-documented that proteasome inhibitors augment p21 protein expression [173, 441, 443], and this has been generally interpreted as a consequence of reduced protein decay due to proteasome inhibition [173]. Despite our results do not allow us to rule out the theory of increased protein half-life, in this work we have shown that p21 mRNA levels also remain constant after treatment with a proteasome inhibitor, and this might be the consequence of a miRNA-mediated stabilization. Therefore, MG-132 may maintain high levels of p21 protein through a complex mechanism involving both mRNA and protein stabilization. As a consequence, the combination of MG-132 and DQA, which strongly increases p21 mRNA levels, results in a dramatic accumulation of the transcript that leads to a high p21 protein expression. Therefore, it can be deduced that the joint use of proteasome inhibitors and other drugs which transcriptionally induce p21 may result in the sustained overexpression of this protein. This notion is useful for the design of combined therapies, since high p21 levels have been demonstrated to lead to resistance to cell death in numerous types of cancer and a significant overexpression if this protein could be detrimental for the clinical outcomes of the therapy [215-218, 220, 222, 227-229].

Nevertheless, the most important finding of this work is that MG-132 induces the expression of miR-22 and that this miRNA upregulates p21 in a 3'UTR-specific manner.

miR-22 had been previously described as a p21 inhibitor in colorectal cancer [450]. In that work, miR-22 was shown to inhibit cell proliferation through the repression of p21 expression, inducing apoptosis in a p53-dependent manner. Interestingly, in p53-deficient cells ($p53^{-/-}$) or cells bearing p53 mutations ($p53^{mut}$), miR-22 was unable to induce apoptosis and induced cell cycle arrest in G_2/M instead. Researchers suggested a number of miR-22 target genes alternative to p21 through which miR-22 could potentially induce p53-independent cell cycle arrest; unfortunately the effects of miR-22 on p21 expression in $p53^{-/-}$ or $p53^{mut}$ cells were not investigated [450]. These results suggested that the activity of miR-22 depends on the cellular context and lead us to hypothesize that in cells with absent or mutated p53, like the K562 [355] or Meg-01 [357] lines, respectively, miR-22 may

have different effects. For this reason, it should be taken into account that the results presented in this work could be exclusive of cells with p53 deficiencies.

On the other hand, bibliography suggests that the upregulation of p21 expression by miR-22 is more likely the consequence of a stress-induced mechanism. The positive regulation of protein expression by a miRNA is still a quite unexplored field but was reported for the first time some years ago by Vasudevan et al. [248]. That group postulated that miRNA regulation can switch from translational repression to translational activation upon growth arrest conditions. This switch depends on the direct interaction of the miRNA with AU-rich elements localized in the 3'UTR region of mRNAs, which in turn must interact with Ago2 and FXR1 (see Chapter 8 of *Introduction*) [248, 249]. These complexes co-localize in polysomes upon growth arrest in G_0, activating translation of the mRNA [248, 249]. Other hints of miRNA-dependent protein upregulation in the literature have suggested the loss of the poly(A) tail [255] and the miRNA-5'UTR complementarity [253, 254] as possible mechanisms of positive regulation mediated by miRNAs.

Consistent with the report by Vasudevan et al. [248], our results show that miR-22 can induce 3'UTR-dependent p21 upregulation. Because proteasome inhibition blocks protein decay, we could not analyze changes in the protein/mRNA ratio after treatment with MG-132 to study variations in the translation rate and therefore we cannot strictly conclude that this drug induces a direct translational activation. However, our luciferase data show that the expression of ectopic miR-22 induces the synthesis of the target protein (Figures 9C and 9D), suggesting that this miRNA does not only increase transcript levels but also induces protein expression. Anyway, this is probably not the main effect of miR-22, since we observed that p21 mRNA levels after the treatment with MG-132 and DQA were disproportionately higher than protein levels.

Interestingly, the need for growth arrest described by Vasudevan et al. [248] provides an explanation for our observations that treatment with MG-132 was required in K562 cells to appreciate the effect of miR-22 in the luciferase assays (Figure 9D). Translational activation was only observed by that group in cells arrested in G_0, while it did not occur in those arrested in G_2 because the process appears to need GW body disassembly [248, 249]. Although MG-132 typically induces G_2/M arrest, we detected a significant decrease in actively proliferating cells (S phase) upon treatment with this drug (Figure 4), suggesting that, in an asynchronous population like ours, MG-132 may induce a simultaneous G_0/G_1 arrest. This would allow translation in K562 cells, according to the Vasudevan model. As for Meg-01 cells, in which MG-132 was not necessary to detect protein expression, it is worth noting that this line grows in a semi-adherent fashion and that for the luciferase experiments cells were cultured for 48 hours until confluent. Because cell-to-cell contact induces quiescence

[157], it is possible that growth of confluent Meg-01 cells was arrested by the time of collection for the experiment and therefore these cells would not need MG-132 to undergo translational activation. Thus, the differences found in the conditions needed for the expression of the luciferase protein might be a consequence of the proliferative state of the two cell lines during the experimental protocols used. Furthermore, the need to overcome mitosis to allow protein translation explains why in K562 cells treated with MG-132 and DQA p21 protein levels did not correlate to mRNA levels.

Nevertheless, the need for MG-132 in K562 cells may also be interpreted as a consequence of the collaborative and cell-specific mechanism of action of miRNAs [230, 257]: the single expression of miR-22 may not be sufficient to induce the accumulation of the p21 3'UTR-luciferase mRNA transcript in K562 cells, and treatment with MG-132 may induce the upregulation of other miRNAs involved in this process that could help miR-22 to stabilize p21 mRNA.

To sum up, independently of the mechanisms involved, in this work we have demonstrated for the first time that miR-22 is upregulated by treatment with MG-132 and that this miRNA can behave as a specific and positive regulator of the expression of p21. This could be an exclusive function of miR-22 in p53-deficient cells, but it is possible that the positive translational regulation is a general stress-mediated mechanism. Moreover, this might be, at least in part, the mechanism through which MG-132 induces the long-term expression of p21. The results presented here point to miR-22 and other unidentified players involved in this process as potential targets to prevent chemotherapy-associated p21 upregulation and the associated chemoresistance. The process of miRNA-mediated p21 mRNA stabilization should be studied in depth for a better understanding of the complex role that miRNAs play in post-transcriptional regulation of gene expression.

RESULTS AND DISCUSSION

BLOCK III

Involvement of miRNA Regulation and Innate Immunity Signaling in the Pathogenesis of MDS

RESULTS AND DISCUSSION

1. Background

The main biological, physiopathological and clinical features of MDS have been reviewed in the *Introduction* section. As it was emphasized during the presentation of the general aims of this work, increasing incidence and significant mortality rates in MDS, especially in higher-risk cases [61, 62, 64], make it necessary to establish good prognostic factors and more selective therapies. However, the high clinical and cytogenetic heterogeneity and the not-fully-understood molecular mechanisms of these malignancies complicate this task [62, 64, 65]. Furthermore, progress in MDS research has been hampered up to now by the lack of fully-characterized cell lines from patients and the difficulties in the development of mouse engrafting models [62].

The presence of TLRs in BM stem and progenitor ($CD34^+$) cells and their involvement in the modulation of myeloid differentiation has been extensively reported [74, 78, 79] and there is compelling evidence that TLR signaling is important in the pathogenesis of MDS [73]. Moreover, TLR1, TLR2, TLR4 and TLR9 have been found to be overexpressed in BM of MDS patients [82-84, 455], as it was pointed out in Chapter 3 of the *Introduction* section. TLR overexpression appears to be correlated with the progression of the disease, and with the levels of the cytokine TNF-α, which is thought to be responsible of intramedullary cell death [82-84, 455]. Furthermore, MyD88 overexpression was recently reported in MDS $CD34^+$ cells [456], as well as a gain-of-function mutation of TLR2 [85, 455]. Both alterations are involved in a positive feedback loop which results in the constitutive expression of NF-κB and ultimately leads to the production of inflammatory cytokines, the increase of the cell proliferation rate and the blockade of differentiation of BM MDS cells [85, 455, 456]. Besides mediating the production of cytokines, NF-κB is involved in the regulation of cell survival, although its role in MDS has been a matter of discussion. It was initially suggested that NF-κB induced apoptosis in MDS cells [457], however, a growing body of evidence suggests that this factor provides BM cells with a survival advantage [73, 104, 458, 459].

In addition to the expression of proteins involved in inflammation, TLR signaling also induces the expression of miRNAs, which participate in the fine-tuning of the inflammatory response [68, 73]. miRNAs participate in the control of hematopoiesis and their deregulation might be involved in the pathogenesis of several hematopoietic diseases [7]. In agreement, many miRNAs have been reported to be abnormally expressed in hematologic cancers, especially in lymphomas, myelomas and CLL [234, 259, 266-270]. Moreover, specific miRNA expression profiles have been proposed as good diagnostic and prognostic markers in various hematologic malignancies [268, 271, 273], including MDS [460-462]. Therefore, abnormal TLR signaling in MDS could be related to the expression of a

specific miRNA signature, which in turn could participate in the pathogenesis of the disease through the modulation of hematopoiesis and the inflammatory response.

The majority of human miRNA genes are isolated from each other within the genome, but others can be clustered together and transcribed as a polycistronic primary miRNA transcript, as it was described in Chapter 6 of the *Introduction* section [230, 233]. Because miRNAs within a genomic cluster have similar or the same expression patterns, they are often functionally related to each other [230]. miR-125a and miR-125b are two of the most-studied miRNAs participating in the regulation of hematopoiesis and cell differentiation [265, 463-465] and there is novel evidence of their involvement in lymphoid and myeloid diseases [265, 466-468]. They are located in homologous miRNA clusters encoded in chromosomes 19 and 11, respectively. Recurrent chromosomal translocations affecting the locus of miR-125b and resulting in a strong overexpression of this miRNA have been reported in MDS and AML [466, 467]. However, little is still known about the participation of miR-125a in the pathogenesis of MDS. Interestingly, NF-κB-activating ability has been described for both miR-125a/b [469], and miR-125b promoter contains an NF-κB binding-site [470] and is upregulated by NF-κB activation [471], which suggests the participation of miR-125b in a positive feedback loop within the NF-κB pathway. This fact, along with the recent finding that segregated miRNAs can bind and directly activate TLRs [472], make miR-125a and miR-125b good candidates to take part in the regulation of the TLR/MyD88/NF-κB axis and therefore participate in the molecular mechanisms involved in MDS.

2. Hypothesis and Aims

For the reasons mentioned above, it was hypothesized that miR-125a and/or miR-125b could be involved in the pathogenesis of MDS as a result of the deregulation of TLR signaling pathways in BM cells. Consequently, the aims of the present work were the following:

- To analyze the expression of miR-125a/b in MDS $CD34^+$ cells and their utility as prognostic markers for the disease.
- To study the possible connection between miR-125a/b and innate immunity pathways.
- To explore the role of miR-125a/b in differentiation and their utility as therapeutic targets in MDS.

3. Materials and Methods

3.1. Cell lines

- CML: K562, Meg-01
- AML: KG1, HL-60, THP-1, OCI/AML3
- MDS: MDS-L

3.2. Primary samples

- BM $CD34^+$ cells from healthy individuals (N=5)
- BM $CD34^+$ cells from MDS patients (N=48)

3.3. Treatments

- TLR ligands: LPS and PAM3
- Ara-C
- MyD88 inhibitory peptide (Pepinh-MYD) and control peptide (Pepinh-Control)
- ASOs

3.4. Methods

- qPCR (TLR7, EPO-R, GYPA, CD71, PU.1, ITGAM, miR-125a, miR-125b, miR-99b and let-7e).
- Transfection (lipid-based and electroporation)
- Luciferase reporter gene assay (NF-κB activity assay)
- Determination of cell number and viability
- Clonogenic assays
- Benzidine staining

4. Results

4.1. miR-125a and miR-125b are overexpressed in MDS $CD34^+$ cells

In order to find out if miR-125a and/or miR-125b play a role in the pathogenesis of MDS, their relative expression in $CD34^+$ cells from BM of MDS patients was analyzed by qPCR and compared to their expression in BM $CD34^+$ cells from healthy donors.

As shown in Figure 1A, miR-125a was strongly overexpressed in MDS patients when compared to healthy controls, with relative expression levels higher than 2-fold in 70.83% of the patients (data not shown). miR-125b also had a clear trend towards overexpression in MDS patients, with a relative

expression higher than 2-fold in 48.94% of them (data not shown); however the differences with healthy controls were not statistically significant (Figure 1B).

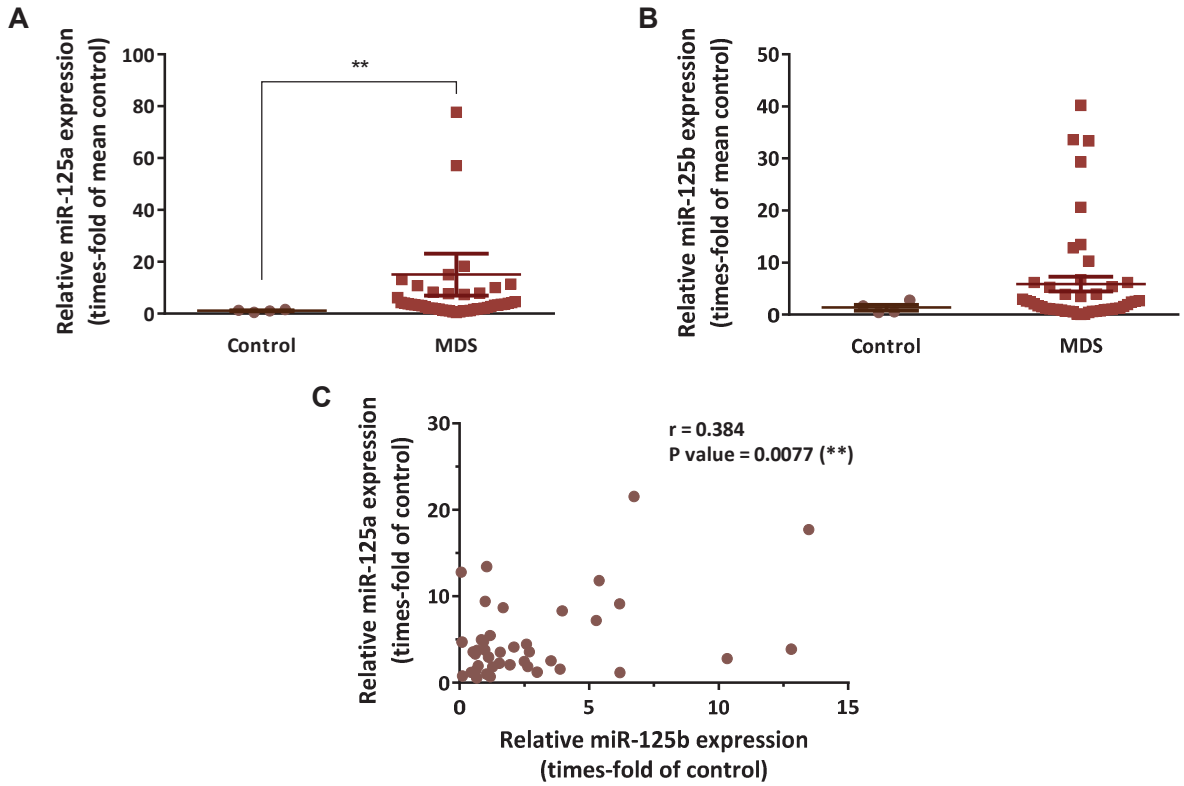

Figure 1. Expression of miR-125a and miR-125b in BM CD34$^+$ cells. (A-B) Relative expression of miR-125a and miR-125b, respectively, in CD34$^+$ cells from MDS patients (N=48/47, respectively) and healthy donors. In **(A),** one data point (Y=386.31) is outside the axis limits. **(C)** Correlation between relative expression of miR-125a and miR-125b in MDS CD34$^+$ cells. Six data points are outside the axis limits. Statistical significance versus healthy donors: **P<0.01.

Interestingly, levels of both miRNAs were significantly correlated in a direct fashion (Figure 1C), which suggests that they might be at least partially subjected to the same regulatory mechanisms. Of note, miR-125a expression levels were twice as high as miR-125b expression levels in MDS patients and healthy controls (data not shown), which might be indicative of a more important function for miR-125a.

4.2. miR-125a, but not miR-125b, is significantly correlated with the progression of MDS

To determine if the overexpression of these two miRNAs is clinically relevant in MDS, we next studied the correlation between patient survival and levels (“high” or “low”) of miR-125a and miR-125b.

Our data showed that the expression of miR-125a was significantly correlated to the progression of the disease (Figure 2), in which patients with higher miRNA levels showed a poorer prognosis.

Interestingly, despite of being directly correlated to miR-125a levels, miR-125b expression did not appear to have a significant impact on overall survival of MDS patients (data not shown), which agrees with the surmise that its functions are not as important in MDS as those of miR-125a.

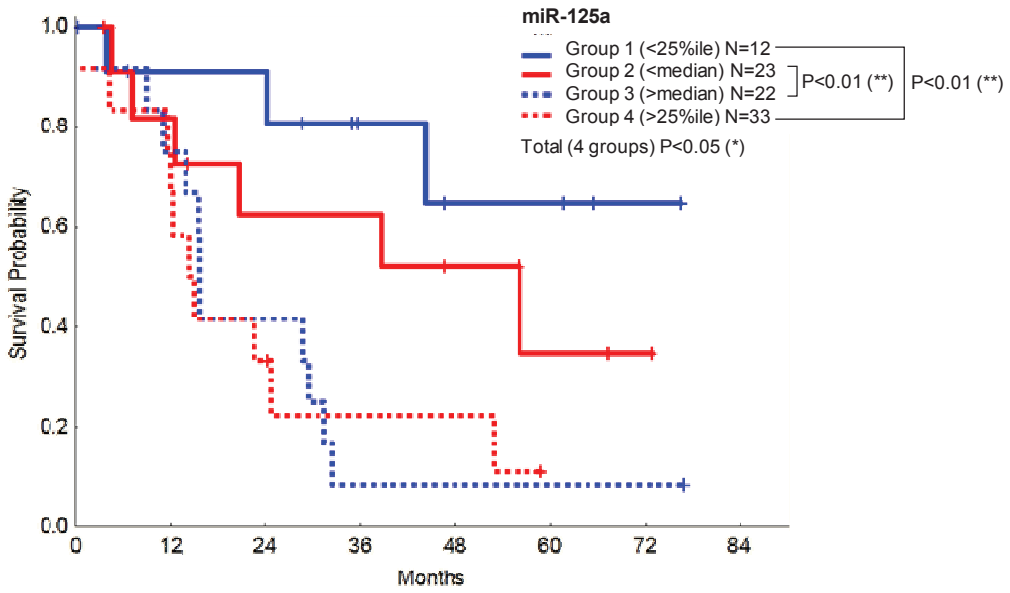

Figure 2. Correlation between the relative expression of miR-125a and OS in MDS.

4.3. The miR-99b/let-7e/miR-125a cluster may be involved in the pathogenesis of MDS

In light of the clinical data, we resolved to focus on the study of miR-125a. This miRNA is encoded in a conserved DNA cluster comprising the sequence of two more miRNAs: miR-99b and let-7e (Figure 3). In order to determine if the other members of the cluster are involved in MDS, we also analyzed their relative expression in patients $CD34^{+}$ cells.

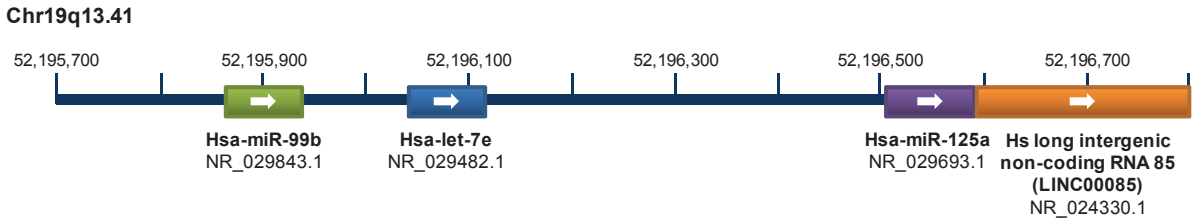

Figure 3. miR-125a cluster in humans. miR-125a, miR-99b and let-7e are clustered together in a ~750 bp intergenic region located in chromosome 19q13.41. NR: National Center for Biotechnology Information (NCBI; USA) Reference sequence number.

Although there were not statistically significant differences with the control group (Figure 4A), miR-99b showed levels over 2-fold of controls in 29.71% of patients (data not shown) and strongly correlated to miR-125a expression (Figure 4C), indicating co-expression of both cluster members.

let-7e was the least abundant miRNA of the cluster (data not shown), with low expression in controls and barely detectable levels in most patients. It is unclear if this fact is a result of an inefficient detection of the miRNA by our qPCR experiments or of a defective miRNA processing or a faster decay. Again, there were not significant differences between patients and healthy donors (Figure 4B) and this time only 17.78% of patients expressed let-7e over 2-fold of controls (data not shown). However, let-7e levels directly and strongly correlated with the expression of both miR-125a (Figure 4D) and miR-99b (P=0.0075; graph not shown).

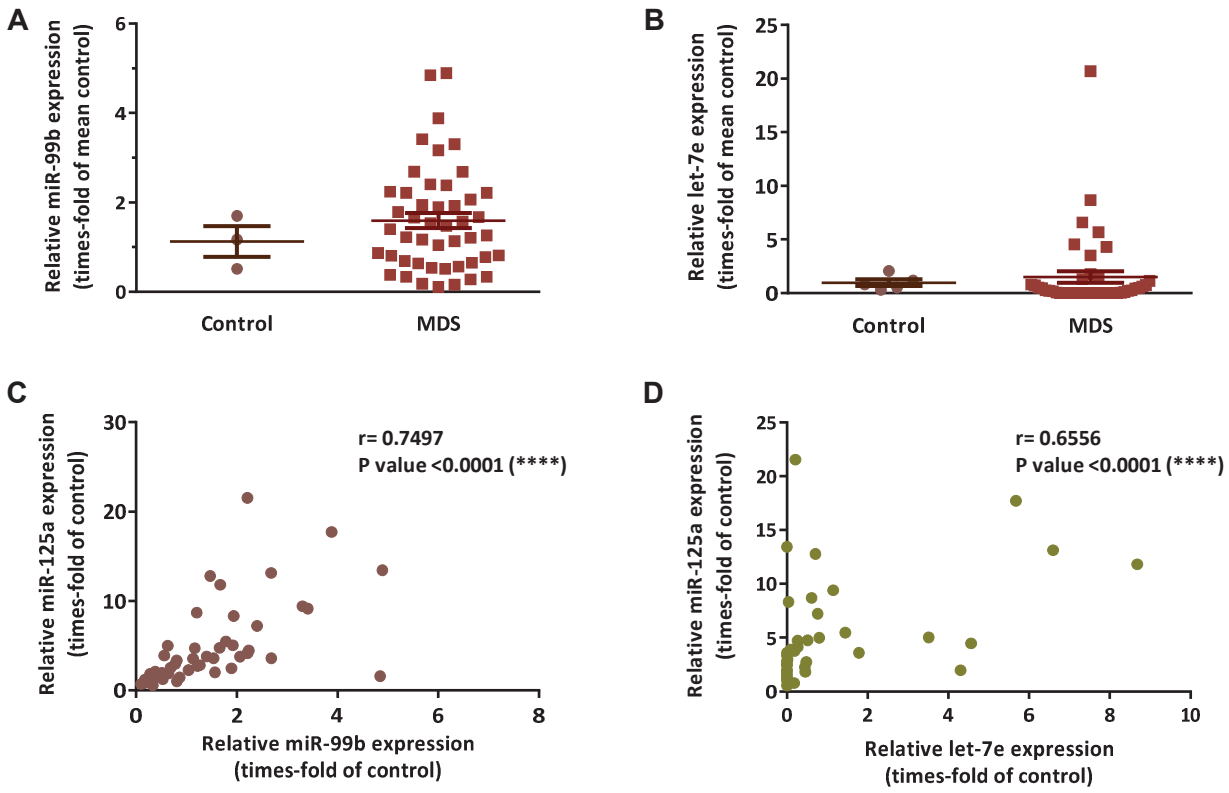

Figure 4. Expression of miR-99b and let-7e in BM CD34$^+$ cells. (A-B) Relative expression of miR-99b and let-7e, respectively, in CD34$^+$ cells from MDS patients (N=48/45, respectively) and healthy donors. One outlier removed by Grubb's method (α=0.05) in each case. **(C-D)** Correlation between the relative expression of miR-125a and miR-99b or let-7e, respectively, in MDS CD34$^+$ cells. In (C) and (D), three data points are outside the axis limits.

Altogether, these data show that the expression of the members of the miR-99b/let-7e/miR-125a cluster is regulated by a common mechanism and involved, at least in part, in the progression of MDS. However, the considerably higher expression levels of miR-125a suggest that this miRNA has alternative regulation mechanisms independent of the rest of the cluster and that this could be the only member which is relevant in MDS.

Interestingly, survival studies revealed that miR-99b (Figure 5), but not let7-e (data not shown), is significantly correlated with the progression of the disease. However, it needs to be taken into account that this could be a consequence of the strong correlation existing between the relative expression of miR-125a and miR-99b.

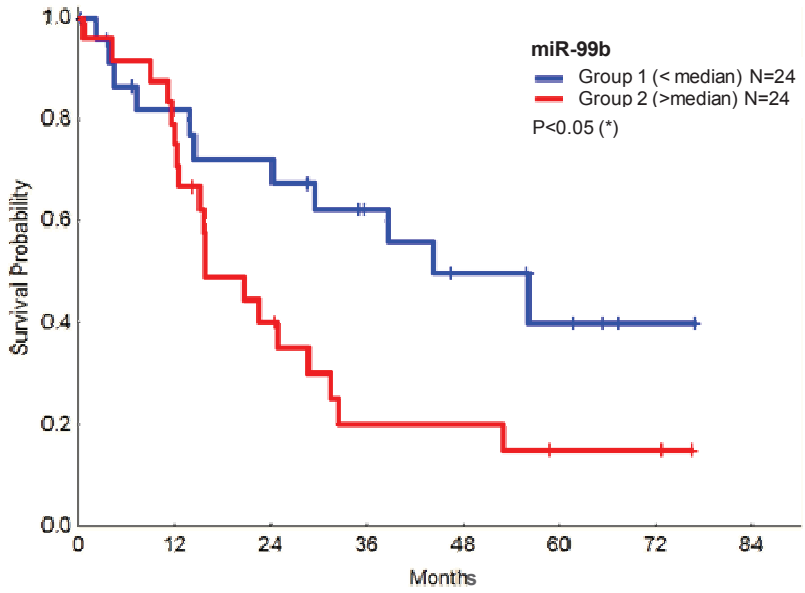

Figure 5. Correlation between the relative expression of miR-99b and OS in MDS.

4.4. miR-125a and miR-99b are positive regulators of NF-κB activity in vitro

Since both miR-125a and miR-99b seemed to be clinically relevant in MDS, we sought to investigate their possible connection with the TLR/NF-κB pathway. For this purpose, we co-transfected Meg-01 cells with a reporter vector containing NF-κB response elements and with synthetic analogs of both miRNAs, and determined their activity on NF-κB activation by the luciferase reporter gene assay.

As shown in Figure 6, ectopic expression of miR-99b was only able to induce a slight increase on NF-κB activity, while miR-125a significantly augmented it. Because we showed before that it is likely that both miRNAs are co-expressed in MDS cells, the combination of both of them was also studied. Interestingly, ectopic expression of miR-125a and miR-99b induced a more powerful activation of NF-

κB when co-transfected together, indicating that they can cooperate in a synergistic manner. Nevertheless, miR-125a appears to drive most of the NF-κB-activating activity.

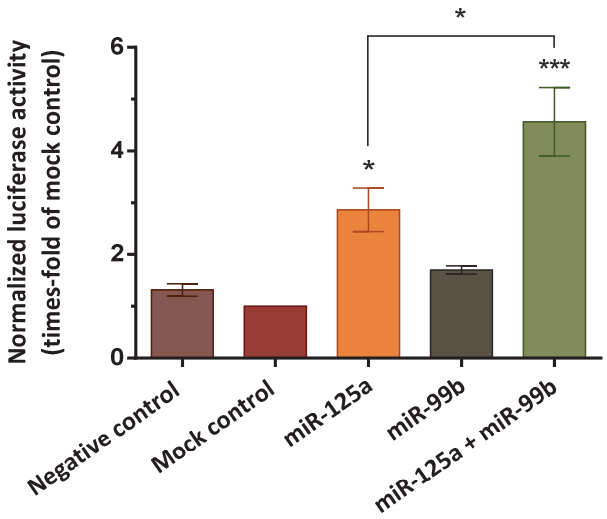

Figure 6. Effect of ectopic expression of miR-125a and/or miR-99b on NF-κB activity. Transactivating activity of NF-κB was determined after 48 hours from transfection of Meg-01 cells with miRNA mimics and luciferase vectors. Results are relative to cells transfected with mock RNA and expressed as mean ± SEM of n=3 independent experiments. Statistical significance: *P<0.05; ***P<0.001.

4.5. Expression of miR-99b and miR-125b is inversely correlated with the levels of TLR2/NF-κB pathway members

The hyperactivation of the TLR2/MyD88/NF-κB pathway in MDS $CD34^+$ cells has been recently reported [85, 455, 456]. Provided that miR-125a and miR-125b are potential NF-κB regulators [469] and in light of the results presented above, we next studied the connections between miR-125b or the miR-99b/let-7e/miR-125a cluster and the expression of TLR2, MyD88 and the histone demethylase JMJD3, which was recently identified as a positive regulator of inflammation downstream of NF-κB that could participate in a positive feedback loop resulting in the hyperactivation of this factor [455].

As shown on figures 7A-E, miR-99b and miR-125b levels are negatively correlated to TLR2 and MYD88 expression, and miR-125b also correlates with JMJD3 levels. No significant negative correlation was found between miR-125a and most of the proteins studied (data not shown). These differences with miR-99b support our previous hypothesis that miR-125a undergoes different mechanisms of regulation than the rest of the members of the cluster and miR-125b.

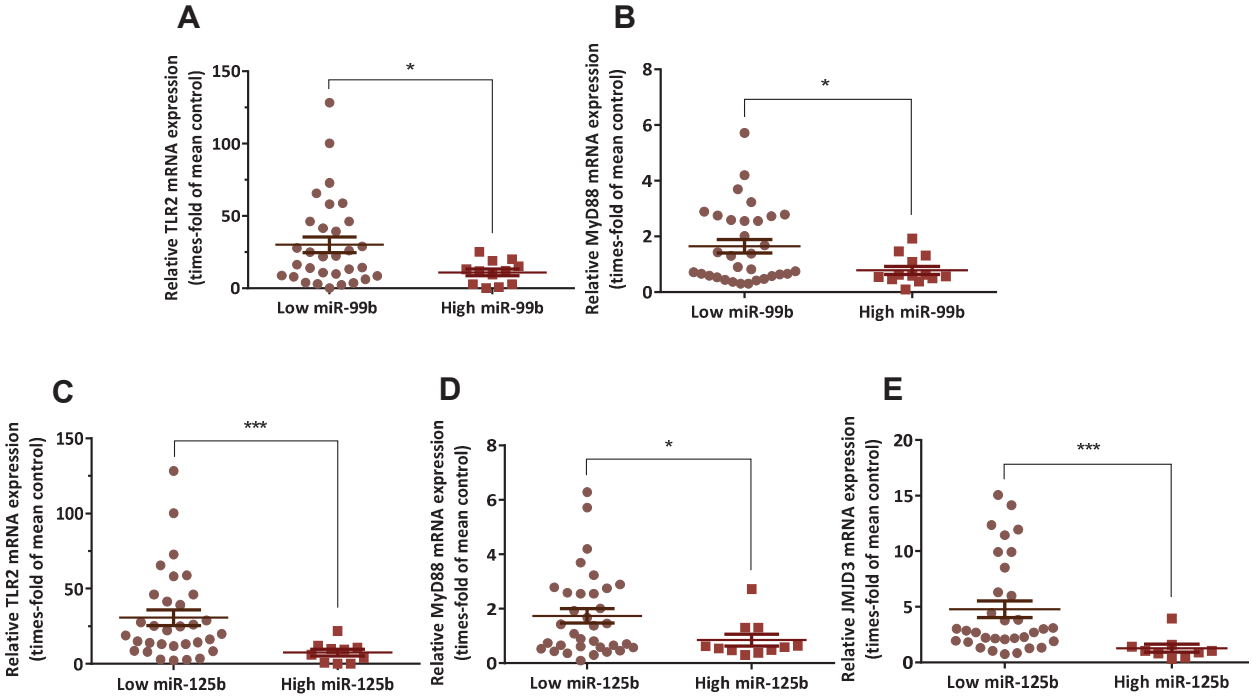

Figure 7. Relationship between miR-99b/let-7e/miR-125a cluster members and proteins from the TLR2-NF-κB pathway in MDS. "Low" and "high" expression cohorts were established based on comparison with the mean relative expression value. **(A-B)** Correlation between relative expression of miR-99b and TLR2 or MyD88, respectively, in MDS $CD34^+$ cells. In (B), one outlier removed by ROUT method. **(C-D)** Correlation between relative expression of miR-125b and TLR2, MyD88 or JMJD3, respectively in MDS $CD34^+$ cells. In (E), five outliers removed by ROUT method. Statistical significance: *P<0.05; ***P<0.001.

It is worth noting that a significant positive correlation and a similar trend were found between JMJD3 levels and the expression of miR-125a (Figure 8A) and miR-99b (Figure 8B), respectively.

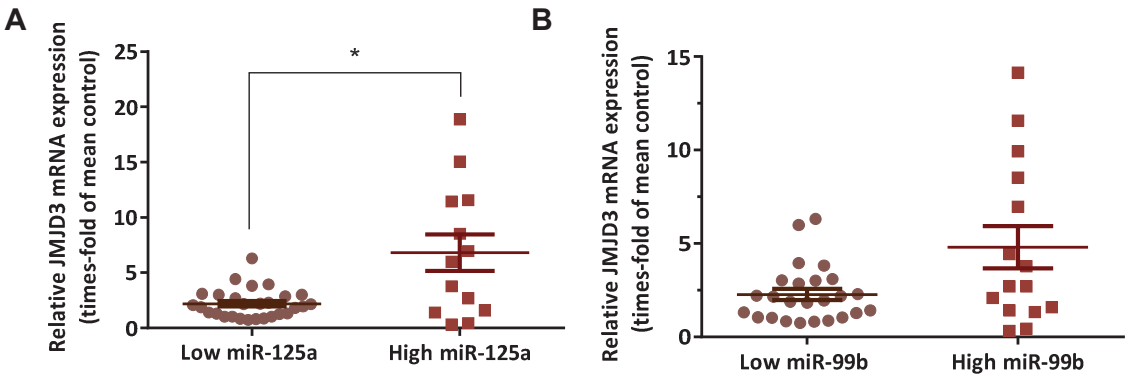

Figure 8. Correlation between relative expression of JMJD3 and (A) miR-125a or (B) miR-99b in MDS $CD34^+$ cells. "Low" and "high" expression cohorts were established based on comparison with the mean relative expression value. In (A), eight outliers removed by ROUT method. Statistical significance: *P<0.05.

4.6. TLR7 is also deregulated and correlates with a better prognosis in MDS

Because it was recently postulated that miRNAs can bind and activate endogenous TLRs such as TLR7 and TLR8 [472], and a mild overexpression of TLR7 in a small cohort of MDS CD34$^+$ patients was recently detected [85], we determined to investigate if the regulation of this TLR is also altered in MDS and if it is connected to miR-125a/miR-99b expression.

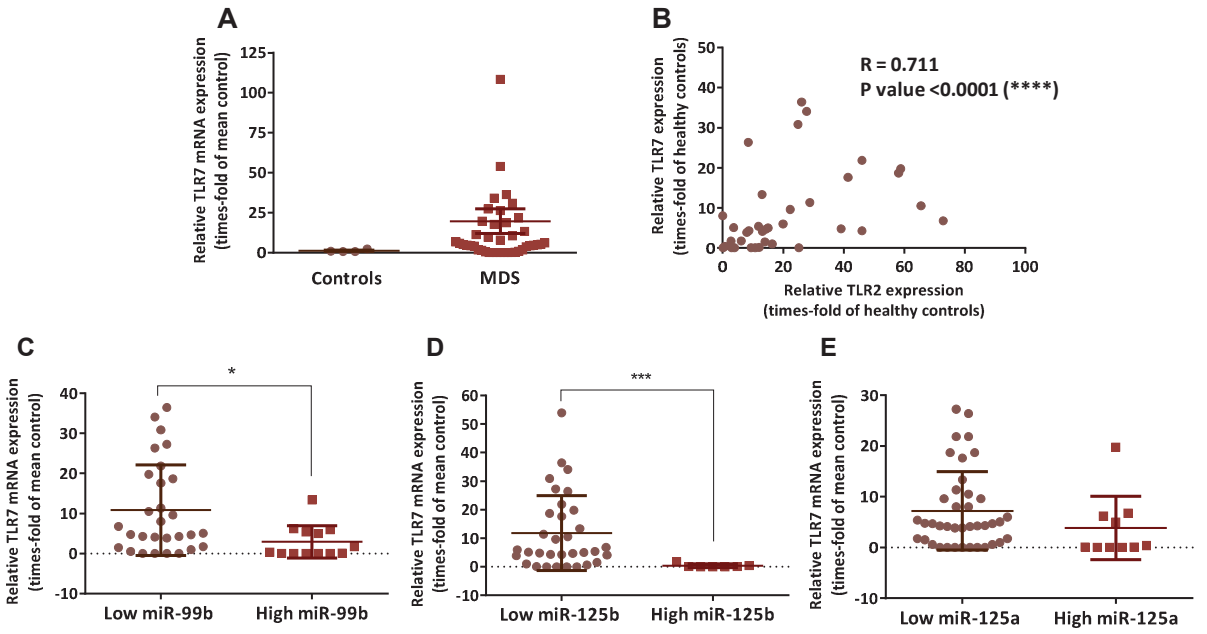

Figure 9. Expression of TLR7 in MDS. (A) Relative expression of TLR7 in BM CD34$^+$ cells. mRNA levels of TLR7 in CD34$^+$ cells from MDS patients (N=42) and healthy donors were measured by qPCR. **(B-E)** Correlation between the relative expression of TLR7 and TLR2, miR-99b, miR-125b or miR-125a, respectively, in MDS CD34$^+$ cells. **"**Low" and "high" expression cohorts were established based on comparison with the mean relative expression value. In (B), two data points are outside the axis limits; in (C), three outliers removed by ROUT method; in (D), four outliers removed by ROUT method; in (E), seven outliers removed by ROUT method. Statistical significance: *P<0.05; ***P<0.001.

Relative expression levels of TLR7 in MDS CD34$^+$ cells were higher than 2-fold of healthy controls in 64.44% of patients; however, differences between groups were not statistically significant (P=0.0932) (Figure 9A). Remarkably, we found a strong direct correlation between the relative expression levels of TLR7 and TLR2 in these patients (Figure 9B), as well as between TLR7 and MYD88 (P=0.017) or JMJD3 (P=0.022) (results not shown), which suggests that TLR2 and TLR7 pathways may be simultaneously upregulated in MDS.

As for the correlation with the miRNAs, miR-99b and miR-125b showed a significant negative correlation with TLR7 levels (Figures 9C and 9D), in agreement with what was observed for TLR2. Again, no relationship was observed for miR-125a; however, there was a trend towards a negative

correlation too (Figure 9E). Although these data might be an indirect consequence of the strong correlation between TLR2 and TLR7, results support the idea that the expression of the cluster is related to the TLR/NF-κB pathway. Moreover, the expression of the miR-99b/let-7e/miR-125a cluster might be linked to the paralogous cluster miR-125b/miR-99a/let-7c through the TLR/NF-κB pathway.

To confirm the relevance of TLR7 in MDS we also performed a survival analysis in MDS patients with high or low levels of TLR7. Strikingly, high TLR7 levels appeared to be beneficial for overall survival of the patients (Figure 10), in contrast with data obtained for TLR2 [85].

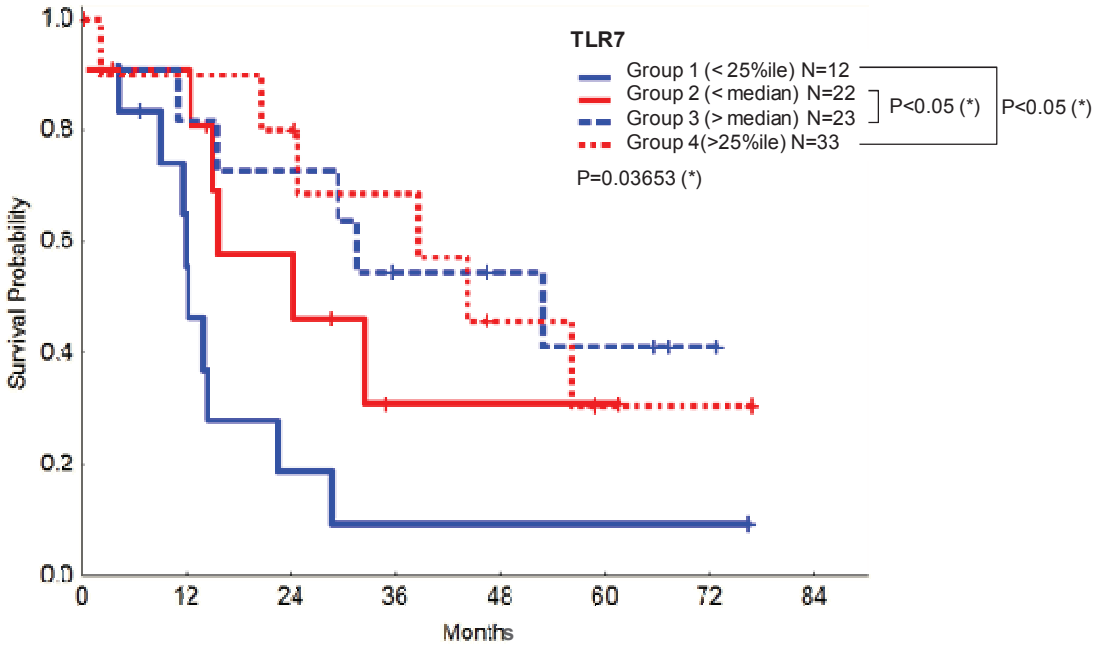

Figure 10. Correlation between the relative expression of TLR7 and OS in MDS.

4.7. High endogenous levels of miR-125a prevent cells from NF-κB activation

The reverse correlation between miR-99b/miR-125b and the expression of the TLR pathway members, along with the fact that the combination of ectopic miR-99b and miR-125a activates NF-κB lead us to speculate that the deregulation of the miR-99b/let-7e/miR-125a cluster (and its paralog) and aberrations in the TLR pathway could be mutually exclusive mechanisms that released selective pressure towards each other. However, because miR-125a appeared to undergo alternative mechanisms of regulation that allowed its accumulation and its levels did not correlate to TLRs and their downstream proteins, we reasoned that the expression of this miRNA could be elevated also in cells with hyperactivated TLR signaling. Therefore, our next step was to elucidate the activity of high endogenous levels of miR-125a on NF-κB activation in the presence of TLR signaling.

First, we screened several human AML cell lines for miR-125a expression in order to find a good model in which miR-125a levels were high. AML cell lines were utilized due to the lack of an easily transfectable MDS cell line. The AML myeloblastic cell line KG1 was selected for the experiments for having the most similar miRNA expression pattern to MDS cells (Figure 11A) and because MyD88 is overexpressed in this cell line [456], which guarantees a high TLR activity. To explore the effect of endogenous miR-125a on NF-κB activity, KG1 cells were transfected with the same reporter vector used before and treated with a miR-125a ASO to specifically inhibit the expression and functionality of this miRNA. To reproduce the conditions of TLR activation, the TLR/NF-κB pathway was simultaneously stimulated with the specific TLR4/TLR2 and TLR2 agonists LPS and PAM3, respectively.

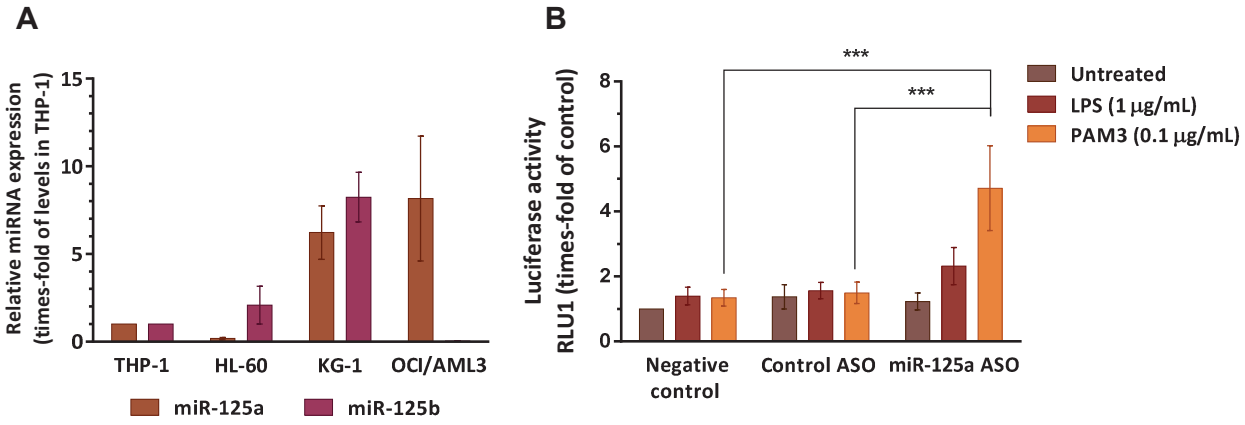

Figure 11. (A) Basal expression levels of miR-125a and miR-125b in AML cell lines. miRNA levels are represented as the relative value to expression in THP-1 cells (note that relative levels of miR-125a are calculated independently from those of miR-125b). Data represent the mean ± SEM of n=3. **(B) Effect of the simultaneous activation of TLR2 pathway and inhibition of miR-125a on NF-κB activity in KG1 cells.** Transactivating activity of NF-κB was measured after 24 hours from nucleofection of KG1 cells with the luciferase vectors and treatment with ASO. Results are expressed as relative to cells transfected with mock and represent mean ± SEM of n=4 independent experiments[2]. Statistical significance: ***P<0.001.

As determined by luciferase reporter assay, miR-125a inhibition does not have a significant effect on NF-κB activity in basal conditions. However, if cells are simultaneously stimulated with TLR agonists, miR-125a inhibition results in the enhancement of NF-κB activation (Figure 11B). Importantly, this synergy is especially strong in the case of TLR2, pointing out the importance of miR-125a in the modulation of the effects of this receptor and its downstream effectors. These results contradict our hypothesis that miR-125a collaborates with the TLR pathways on NF-κB activation and unveil a

[2] Method disclosure: technical problems regarding the endogenous *Renilla* control were experienced during these luciferase assays; only one experiment out of four efficiently expressed the *Renilla* luciferase and could be properly normalized. Because normalized results were almost identical to non-normalized data, we conducted a joint statistical analysis of the four experiments.

potential inhibitory activity of this miRNA in the presence of TLR signaling, which will be further discussed below.

4.8. miR-125a inhibition in K562 cells favours Ara-C-induced erythroid differentiation

The different regulation of the expression of miR-125a compared to the rest of the cluster members suggested by our previous results and its reported involvement in the control of hematopoiesis [463] lead us to focus on the effects of this miRNA on differentiation, which might be responsible for its participation in the pathogenesis of MDS.

It was recently demonstrated that MDS $CD34^+$ primary cells can differentiate towards the erythroid lineage [456]. To explore the potential role of miR-125a on differentiation, we first studied its effects on the erythroleukemia cell line K562, which undergoes erythroid differentiation upon stimulation with several agents, including low doses of Ara-C [366].

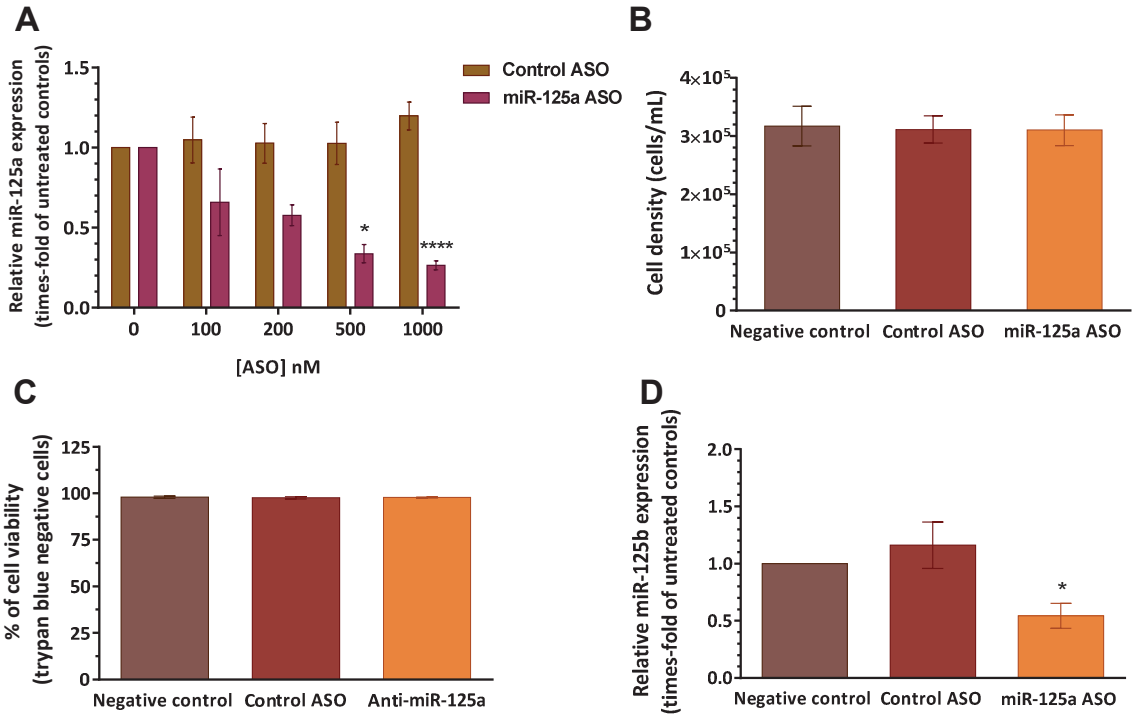

Figure 12. Efficiency of miR-125a inhibition in K562 cells. (A) Dose-response of miR-125a ASO. K562 cells were treated with increasing doses of ASO (100-1000 nM) for 48 hours and miR-125a expression was assessed by qPCR (n>2). **(B-C)** Effect of miR-125a inhibition on cell density and viability, respectively, in K562 cells after 48 hours of treatment with 1 µM ASO (n=6). Negative controls are untreated cells. **(D)** Unspecific effect of 1µM ASO on miR-125b expression in K562 cells treated for 48 hours (n=4). **(A-D)** Data represent mean ± SEM. Statistical significance: *P<0.05; ****P<0.0001.

Prior performing colony formation assays for the differentiation study, the effects of miR-125a ASO on cell proliferation and viability were tested in order to avoid changes in the colony number caused

by the cytotoxicity of the treatment. Forty-eight hour-treatments of K562 cells with ASO at an effective dose were demonstrated to be safe (Figures 12A-C); however a significant unspecific inhibition of miR-125b (which shares the seed sequence with miR-125a) by 1 µM ASO was detected (Figure 12D). For this reason, results presented herein could also be attributed, at least in part, to the inhibition of miR-125b, although the inhibition of miR-125a at the same dose was clearly stronger (Figure 12A).

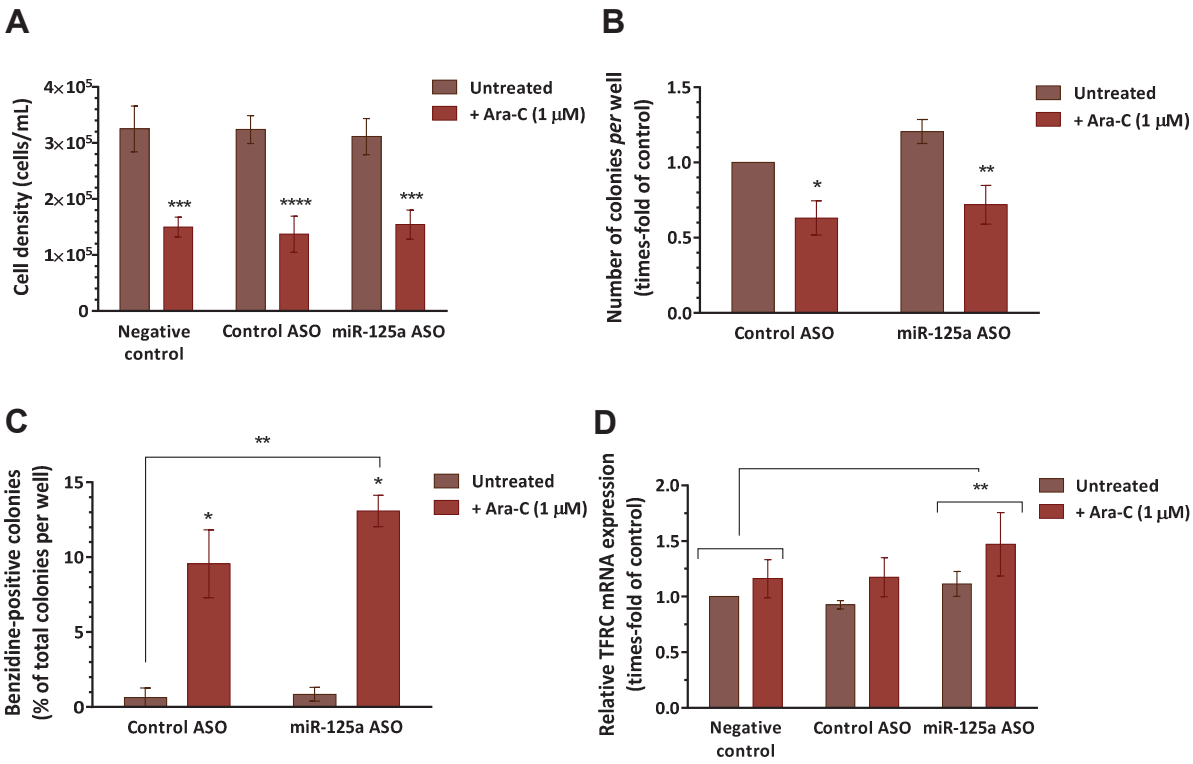

Figure 13. Effect of miR-125a inhibition on Ara-C-stimulated erythroid differentiation of K562 cells. K562 cells treated with ASO and Ara-C for 48 hours were plated in MC for 4 days before being counted and collected for the corresponding assays. **(A)** Effect of on cell density (n=5). **(B)** Colony formation assay (n=5). **(C)** Benzidine-positive colony count (n=3). **(D)** Expression of CD71 (TFRC). Statistical analysis represents a grouped analysis of the main effect of ASO (n=4). **(A-D)** Data represent mean ± SEM. Statistical significance: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Treatment of K562 cells with 1 µM Ara-C induced a significant decrease on cell number after 48 hours (Figure 13A) without affecting viability (results not shown), suggesting the induction of a proliferative arrest. No differences in cell number or viability were found when cells were co-treated with ASO. As expected, after 4 days of incubation of colonies, Ara-C reduced colony number (Figure 13B) while it increased the number of benzidine-positive (heme group-forming or erythroid-like) colonies (Figure 13C). miR-125a inhibition alone did not induce significant changes in colony number or benzidine-positive K562 cells (Figures 13B-C). When combined with Ara-C, miR-125a inhibition did not affect colony formation ability either (Figure 13B); however, it induced an increase on the

number of erythroid-like colonies (Figures 13C). This suggests that miR-125a inhibition does not trigger differentiation *per se* but favors differentiation started by other stimuli. Thus, miR-125a might participate in the blockade of erythroid differentiation.

In order to confirm the effects of miR-125a on Ara-C-induced differentiation in K562 cells, we also analyzed the expression of the common erythroid markers glycophorin A (GYPA), erythropoietin receptor (EPO-R), and CD71/transferrin receptor (TFRC). Levels of EPO-R and GYPA mRNA (mature erythroid markers) increased with the treatment with Ara-C but did not show any changes upon co-treatment with miR-125a ASO (results not shown), whereas the early marker CD71 was significantly higher in all ASO-treated cells (Figure 13D), confirming that miR-125a inhibition favors Ara-C-stimulated erythroid differentiation in K562 cells.

4.9. MDS-L cells express high levels of miR-125a/miR-125b

We then sought to confirm the effects of miR-125a on differentiation using a model that was more approximate to MDS. For this purpose, we utilized the human cell line MDS-L, which has been established from a MDS patient and is not fully characterized yet [358]. For this reason, we first analyzed miR-125a and miR-125b levels in this cell line and compared them to those in K562 cells. As shown on Figure 14, miR-125a and miR-125b expression in MDS-L cells was higher than in K562 cells and similar to that on KG1 cells, which agrees with the overexpression observed in MDS patients and allows the study by inhibition of the miRNA with ASO.

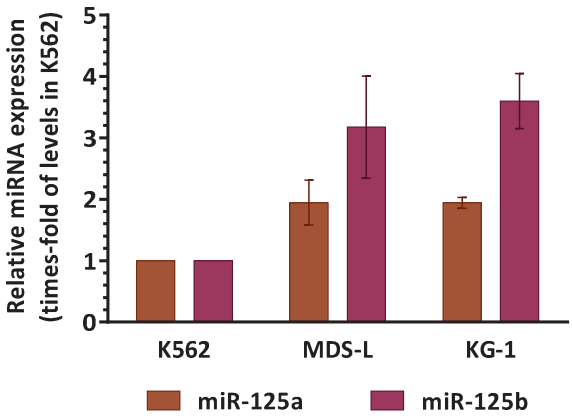

Figure 14. Basal expression levels of miR-125a and miR-125b in myeloid leukemia and MDS cell lines. miRNA levels are represented as the relative value to expression in K562 cells. Expression of miR-125a in MDS-L cells was 5-10 times higher than that of miR-125b (note that relative levels of miR-125a are calculated independently from those of miR-125b). Data represent mean ± SEM of n=4 independent experiments.

Cytotoxicity and effectiveness of the ASOs, as well as their specificity, were tested prior proceeding to the colony formation assays. Treatment with 1 µM ASO inhibited approximately 80% of the relative expression of miR-125a and did not affect cell density or viability (Figures 15A and 15C-D). Unspecific inhibition of miR-125b was not statistically significant in this cell line but should also be taken into account (Figure 15B).

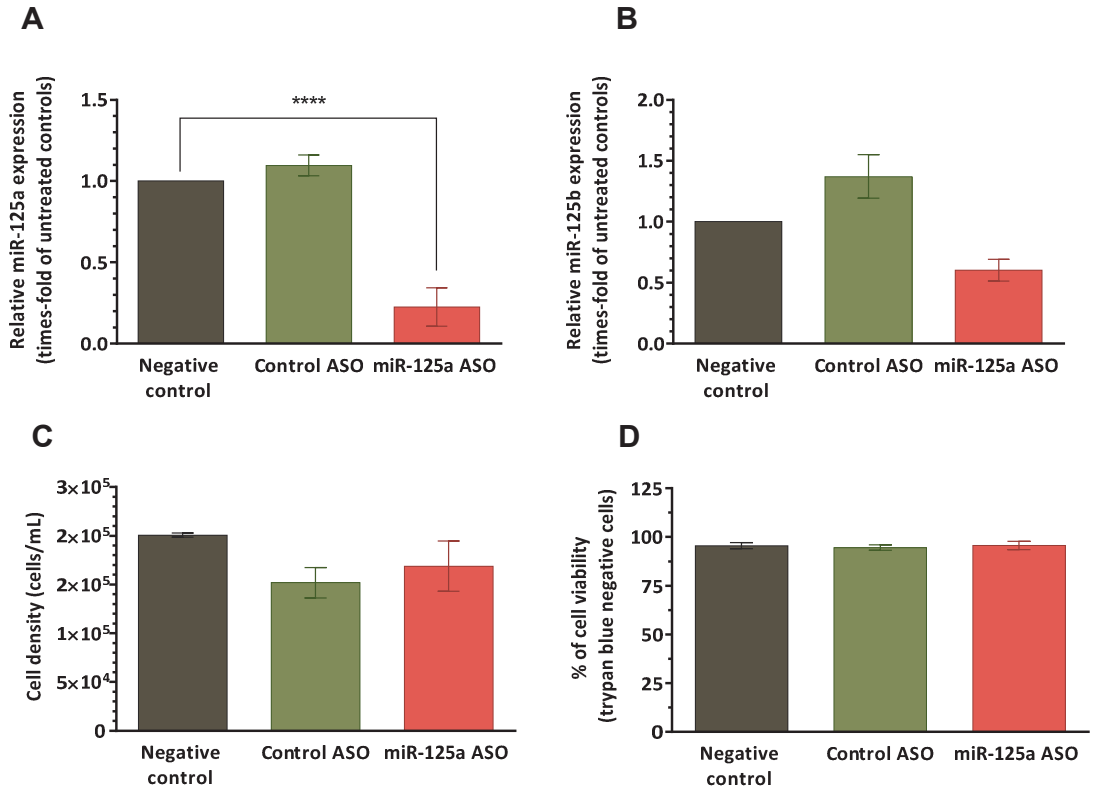

Figure 15. Efficiency of miR-125a inhibition in MDS-L cells. (A) Changes in miR-125a expression**.** MDS-L cells were treated with 1 µM ASO for 48 hours. **(B)** Unspecific effect of miR-125a inhibition on miR-125b expression in MDS-L cells. **(C-D)** Effect of miR-125a inhibition on cell density and viability, respectively, of MDS-L cells. **(A-D)** Data represent mean ± SEM of n=3 experiments. Statistical significance: ****P<0.0001.

4.10. MDS-L cells can spontaneously differentiate towards the myeloid lineage in methoculture

Similarly to what was observed on K562 cells, miR-125a inhibition alone did not significantly affect colony formation ability of MDS-L cells or the expression of any of the erythroid markers of study (results not shown). Moreover, levels of the myeloid markers PU.1 and CD11b were also determined and no significant changes were detected, although there was a trend towards an increase on CD11b levels (Figure 16).

Because we ignored the behavior of MDS-L cells in MC cultures, next we plated untreated cells and allowed them to grow for one week in order to determine if they would undergo spontaneous

differentiation upon stimulation with the growth factors present in the MC medium. After this time, levels of the differentiation markers studied before were analyzed by qPCR.

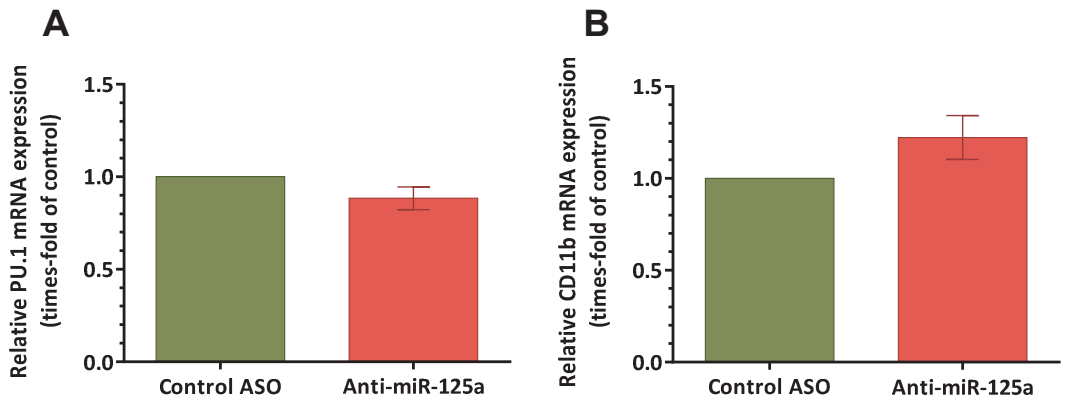

Figure 16. Effect of miR-125a inhibition on MDS-L differentiation. (A) PU.1 expression (B) CD11b expression. PU.1 and CD11b levels were determined by qPCR after treatment with ASO for 48 hours and colony incubation for 7 days. Data represent mean ± SEM of n=8 independent experiments.

Interestingly, colonies were significantly enriched on CD11b (Figure 17), a mature myeloid surface marker, suggesting that untreated MDS-L cells can undergo certain degree of spontaneous myeloid differentiation in methoculture. Surprisingly, cells also underwent significant changes in the expression of the erythroid markers EPO-R and GYPA, which increased and decreased, respectively. It is therefore not clear if MDS-L acquire a myeloid phenotype in methylcellulose cultures; anyway, this spontaneous differentiation could be the reason why the inhibition of miR-125a alone did not result in any observable change in the differentiation state of these cells.

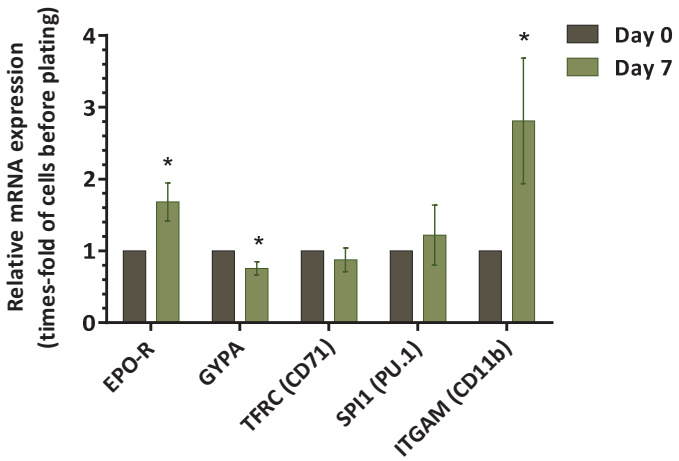

Figure 17. Spontaneous differentiation of MDS-L cells after a 7-day culture in MC. Relative expression of differentiation markers was determined by qPCR. Data represent mean ± SEM of n=3 independent experiments. Statistical significance: *P<0.05.

4.11. miR-125a inhibition enhances erythroid differentiation of MDS-L cells induced by MyD88 inhibition

Because we could not address the effects of miR-125a on differentiation in untreated MDS-L cells, we sought to study the activity of this miRNA in the presence of a differentiating stimulus, as it was done with K562 cells. As shown in a recent publication, inhibition of MyD88 induced erythroid differentiation in MDS $CD34^+$ primary cells [456]. For this reason, we decided to examine differentiation of MDS-L cells in the presence of a specific inhibitory peptide of MyD88.

Interestingly, MyD88 inhibition itself slightly decreased miR-125a levels (Figure 18A) after 48 hours of treatment, which could be again indicative of a link between miR-125a regulation and the TLR/NF-κB pathway.

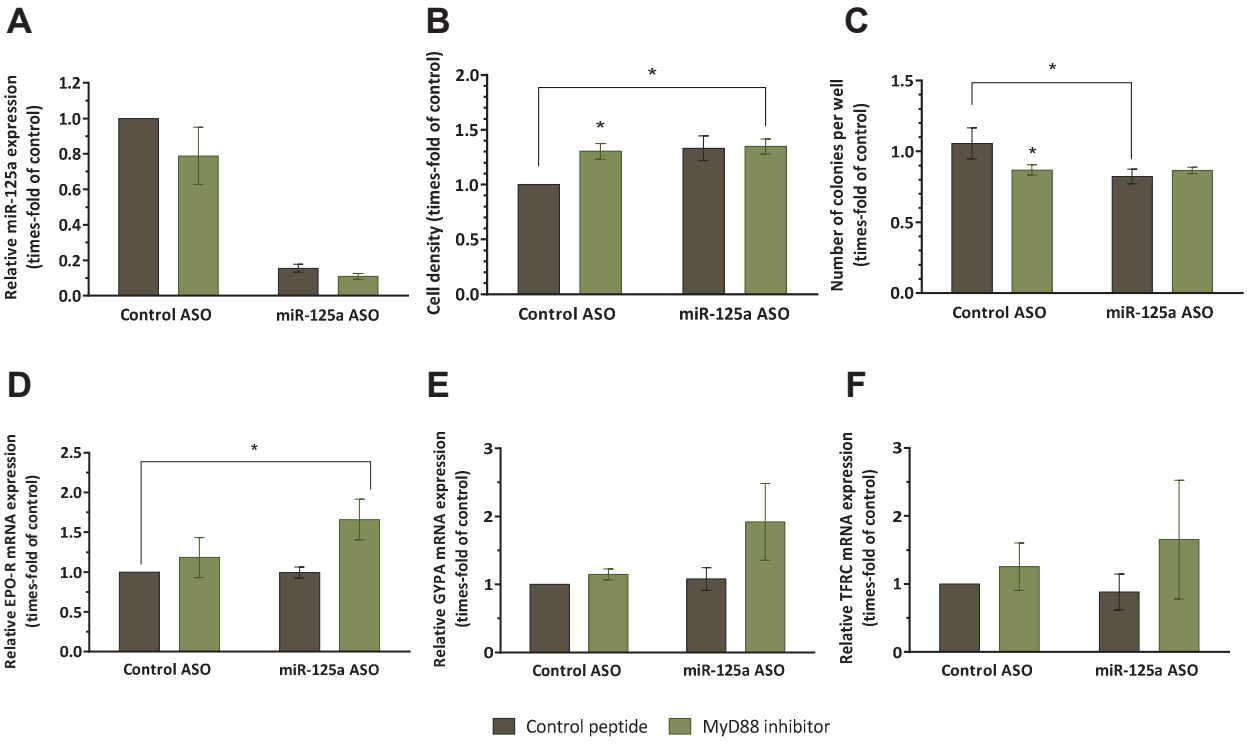

Figure 18. Effect of the inhibition of miR-125a and TLR-NF-κB pathway in MDS-L cells. (A) Effectiveness of miR-125a ASO in MDS-L cells, expressed as relative miR-125a expression levels, determined by qPCR. **(B)** Cell density after 48 hours of treatment. **(C)** Colony formation assay 7 days after plating in methylcellulose**. (D-F)** Relative expression of EPO-R, GYPA and CD71, respectively. **(A-F)** Data represent mean ± SEM of n=3 independent experiments. Statistical significance: *P<0.05.

The MyD88 inhibitor peptide and miR-125a ASO slightly induced an increase in MDS-L cell number, both separately and in combination (Figure 18B). Similarly, both treatments induced a mild but significant decrease in colony number (Figure 18C). These effects on the proliferating activity and colony formation of MDS-L cells were quite moderate. The analysis of the expression of erythroid

differentiation markers, however, revealed that miR-125a inhibition potentiates differentiation of MDS-L cells towards the erythroid lineage (Figures 18D-F). Accordingly, no differences were detected in the expression of myeloid markers (results not shown).

5. Discussion

In the present work we aimed to study the involvement of the homologous miRNAs miR-125a and miR-125b in the pathogenesis of MDS and their possible participation in the regulation of innate immunity pathways in and the modulation of hematopoietic differentiation. Our results show that both miRNAs are overexpressed in BM $CD34^+$ cells of MDS patients and that their levels are strongly correlated, which may indicate that they share common mechanisms of regulation that could be altered in the pathogenesis of this disease. However, miR-125a was expressed in significantly higher levels than miR-125b, both in MDS patients and in healthy donors. This suggests that, despite miR-125b having been more deeply studied in hematopoiesis [265, 464, 466, 467], miR-125a may in fact play a more important role in hematopoietic malignancies. For instance, high miR-125a levels were found to be directly correlated with the progression of MDS, while no relation was found for miR-125b levels. These results confirm the relevance of miR-125a in this group of diseases.

Nevertheless, it needs to be noted that the fact that we did not find a significant correlation between miR-125b levels and patient survival does not necessarily mean that this miRNA is not important for the pathogenesis of MDS. The key might be that miR-125b overexpression and its tumorigenic activity have been reported exclusively in MDS patients with translocations in chromosome 11 [466, 467]. Because cytogenetic data of our cohort of patients were not available, we could not group patients accordingly and this could have masked significant differences or clinical relevance of miR-125b in a subset of patients bearing the translocation. Likewise, previous studies of potential miRNA signatures in MDS had reported that miR-125a [272] and miR-125b [272, 466] are especially upregulated in MDS with associated 5q deletion. Although this type of array-based studies usually have little concordance when compared to similar ones [268, 271], data point out that it would be interesting to expand our study to concrete cytogenetic aberrations.

The expression of the other members of the miR-99b/let-7e/miR-125a cluster was also found to be strongly correlated with that of miR-125a but, similarly to miR-125b, no statistically significant differences were found between MDS patients and healthy controls and their total levels were also considerably lower. Of note, let-7e had previously been identified as a miRNA which is differentially expressed in MDS [462]. Although we found evidence of a coordinated regulation of miR-125b and

the three components of the miR-99b/let-7e/miR-125a cluster, our results suggest that miR-125a undergoes alternative mechanisms of regulation which are independent of the transcriptional activation of its cluster and the homologous miR-99a/let-7c/miR-125b. The higher levels of miR-125a in comparison to other related miRNAs might be explained by a higher stability (reduced decay) or by increased transcription, which could occur independently from the rest of the cluster since miR-125a is localized at the 3' end (Figure 3). Moreover, conserved binding sites for various transcription factors, which could be responsible for the independent transcription of miR-125a, exist in the intergenic region between miR-99b and let-7e [473]. It is possible therefore to hypothesize that miR-125a is independently transcribed under certain stimuli and carries out alternative functions.

Provided the strong relationship between miR-125a and MDS, we studied more deeply the mechanisms through which this miRNA could be participating in the pathogenesis and/or progression of the disease. Two important functions were explored: its regulatory role in the TLR/MyD88/NF-κB pathway and its participation in the modulation of hematopoietic differentiation.

As for the regulation of the TLR/MyD88/NF-κB pathway, we found opposing activities of miR-125a in different activation states of the pathway. Exogenous expression of miR-125a in non-stimulated cells induced the transactivating activity of NF-κB, effect which was enhanced by the co-expression of miR-99b (Figure 6); while the blockade of miR-125a's functionality in TLR2-stimulated cells unveiled an inhibitory role for this miRNA on NF-κB activation (Figure 11B). These results seem contradictory and need a cautious interpretation because different effects on NF-κB activation may respond to different molecular mechanisms active in the two cell lines utilized; however, it can be hypothesized that the dual role of miR-125a in NF-κB modulation responds to a fine-tuning mechanism of this pathway, which would explain the two types of regulation of miR-125a expression (cluster-associated and cluster-independent) reported in this work. This topic will be further discussed in the *General Discussion* section.

Nevertheless, it has been postulated that NF-κB activation in $CD34^+$ cells is not enough to induce changes in stem and progenitor cell growth and differentiation [474]. Because there is evidence of miR-125a playing a role in the regulation of hematopoietic differentiation [463, 465], it was hypothesized that this miRNA contributes to MDS in other ways in addition to NF-κB modulation. For instance, we showed that the inhibition of miR-125a in K562 cells and MDS-L cells enhances erythroid differentiation induced by Ara-C and a MyD88 inhibitor, respectively. These results suggest that miR-125a contributes to the blockade of differentiation and that its overexpression in MDS patients might contribute to retain BM cells in an undifferentiated state, in agreement with reports

describing the involvement of miR-125a in the maintenance and self-renewal of the hematopoietic stem/progenitor state in normal BM cells [463-465]. Furthermore, our results fully agree with two recently published works in which constitutive expression of miR-125a in BM transplanted into irradiated mice induced various phenotypes indicative of myeloproliferative neoplasms [465, 468]. Importantly, one group of authors [468] excluded miR-125b from the experiments because it induced weaker phenotypes than miR-125a, supporting our conclusion that miR-125b is not as relevant as miR-125a in the pathogenesis of MDS. Nevertheless, it needs to be kept in mind that miR-125b was also partially inhibited in our experiments using ASOs, so we cannot rule out the cooperation or redundancy of function of this miRNA in the regulation of differentiation in MDS.

To the best of our knowledge, this is the first time that miR-125a levels have been reported to be directly connected to the progression of MDS. Overall, our results indicate that the deregulation of miR-125a expression may contribute in various ways to the disease and that this molecule is a therapeutic target of interest that should be further explored. Moreover, miR-125a is a potential prognosis marker of great utility in the clinical practice, since miRNAs have been reported to be secreted to peripheral blood and serve as circulating cancer biomarkers [475, 476], enabling an easy and safe sample extraction. As a matter of fact, miR-125a, miR-99b and let-7e have been recently reported to be present in peripheral blood and suggested as biomarkers for different diseases [273, 477, 478]. Of note, in two studies of miRNA signatures in AML, it was found that both miR-125a and miR-125b are downregulated when compared to normal $CD34^+$ cells [479, 480]. It was suggested that this might be a differentiation-related and not a pathological event (loss of expression in more differentiated cells) [271]. It would be of great interest to determine if this is only the case in *de novo* AML or if it also occurs in MDS-derived AML, since these two miRNAs could also be used as diagnostic markers to differentiate the two subtypes of AML.

Last, we have found that TLR7 is not only overexpressed in MDS and strongly correlated with TLR2 expression, but also that it is significantly correlated with a better prognosis, the opposite as for TLR2. It is unclear how TLR7 may contribute to patient survival in MDS, but the mechanism may be related to the fact that its stimulation appears to induce myeloid differentiation in $CD34^+$ progenitors [78]. On the other hand, the correlation between the expression levels of TLR7 and TLR2 suggests that they share regulatory mechanisms. However, the overexpression of TLR7, which is lower, could also be indirectly induced by TLR2 via NF-κB activation or through other mechanisms [481]. Because TLR7 recognizes single stranded RNA molecules [482], it is possible that its activation is triggered by miRNAs in response to TLR2. For instance, it has been recently reported that TLR7/TLR8 can be activated by secreted miRNAs. Importantly, TLR7 activation by such miRNAs did not result in NF-κB

activation [472], although this is the typical consequence of TLR7 stimulation [483-485]. Due to the lack of significant correlations with the expression of TLR7, it is not likely that the miRNAs of study in this work participate in its modulation. Nevertheless, our data suggest that TLR7 could also be a good prognosis marker in MDS and confirm that innate immunity signaling is involved in the pathogenesis and/or progression of MDS.

V. GENERAL DISCUSSION

Because the three blocks of results have been discussed in the previous section, in the *General Discussion* we have addressed additional questions that might have arisen during the analysis of our results and sought to join the three blocks together.

I. Intrinsic Mechanisms of Chemoresistance in the APL Cell Line NB4

In the previous section, we reported that the hot-spot mutant $p53^{R248Q}$ participates in the blockade or inhibition of apoptosis in NB4 cells. Gain-of-function of $p53^{R248Q}$ is particularly interesting since this mutant has been demonstrated to be more powerful on inducing invasiveness [410] and survival advantage [392] than others, and it has been recently reported that it strongly induces the expression of a cancer-related gene signature [486].

One of the issues for future research that emerges from this finding is the mechanism through which $p53^{R248Q}$ hinders apoptosis. Several mechanisms of oncogenicity have been proposed for gain-of-function mutations, including aberrant modulation of gene transcription (both activation and inhibition) through direct interactions with other transcription factors [487] or through epigenetic modulation [397], and regulation of non-transcriptional processes [487]. Moreover, it has been postulated that some p53 hot-spot mutants defectively bind proteins from the Bcl-2 family, therefore showing impaired mitochondrial apoptogenic functions like cytochrome c release [182, 189]. However, this field still remains unclear and the fact that the oncogenic characteristics acquired appear to be mutant-specific complicates the identification of the precise mechanisms [410, 486, 487].

Because it seems to be the cytosolic form of $p53^{R248Q}$ which bears most of the anti-apoptotic activity, it could be speculated that gain-of-function is connected with abnormal interactions between the mutant and mitochondrial membrane proteins involved in apoptosis. A study of mitochondria-mediated apoptosis in cells endogenously expressing p53 mutants revealed that they preserved their ability to translocate to mitochondria upon a death stimulus but they failed to effectively induce apoptosis, in part through a gain-of-function mechanism [428]. Moreover, it has been demonstrated that both mutant and wild-type p53 are present in the mitochondria independently of apoptotic signals [182, 183]. Therefore, it is possible that mitochondrially-localized $p53^{R248Q}$ aberrantly binds Bcl-2, Bcl-XL [182, 189] or Bak [187] and consequently hinders apoptosis. Figure 1 shows a graphical representation of the gain-of-function model that we propose for $p53^{R248Q}$.

Future research on this mechanism is suggested for a better understanding of the consequences of DNA-contact mutations in the p53 gene. The involvement of these mutations in tumorigenesis or chemoresistance of leukemias, and specially APL, ought to be confirmed in future studies exploring other cell lines and patient samples. Nevertheless, these results broaden our understanding of the behavior of mutant p53 and suggest that this protein could become an important target for selective inhibition in cancer, not only in APL but also in a number of other hematologic malignancies and solid tumors with p53 mutations.

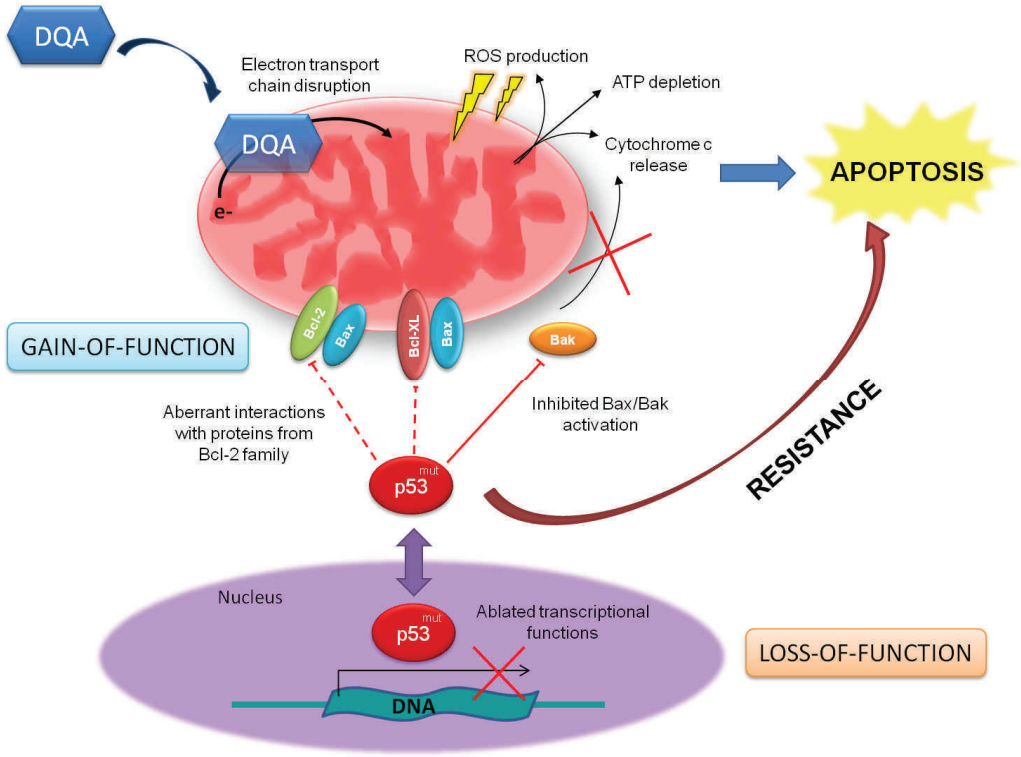

Figure 1. Suggested model for p53 gain-of-function effects on apoptosis in NB4 cells. When cells receive a stress stimulus, p53 hot-spot mutants ($p53^{R248Q}$) may translocate to the nucleus or to the mitochondria, where they localize in the outer membrane. Because $p53^{R248Q}$ has reduced DNA-binding ability, its transcriptional functions are impaired and therefore nuclear $p53^{R248Q}$ is unable to transactivate its target genes (loss-of-function). $p53^{R248Q}$ localized in the mitochondria, in turn, may fail to interact and inhibit the pro-survival members of the Bcl-2 family (in the figure, Bcl-2 and Bcl-XL) or may be unable to interact and activate the pro-apoptotic members Bax and Bak, hampering their dimerization. These aberrant mitochondrial functions could hinder the formation of pores in the outer membrane and therefore inhibit the induction of apoptosis (gain-of-function). When DQA enters the cell and localizes to the inner mitochondrial membrane, it disrupts the electron transport chain and triggers MOMP and the subsequent production of ROS, ATP depletion and cytochrome c release. ROS, in turn, can damage the cellular components, including the mitochondria, and amplify the apoptotic response. When this occurs in NB4 cells, these events might be hindered, or delayed by the gain-of-function of $p53^{R248Q}$.

II. miRNA-mediated Gene Upregulation Induced by Proteasome Inhibition in CML Cells

In regard of the positive regulation of the expression of p21 by miR-22, an obvious question that remained unsolved is how miRNAs can stabilize and/or induce translation of one mRNA. As we commented before, only a few works reporting positive gene regulation by miRNAs exist, and the mechanisms proposed in them are varied [248, 253-255].

One of those reports related translational activation with the interaction between the miRNA, the 3'UTR of the mRNA and the RISC components Ago2 and FXR1 and, interestingly, suggested that this

occurs in cytoplasmic granules [248, 249]. As it was explained in Chapter 6 of the *Introduction* section, these are subcellular structures comprised of mRNA and RNA-binding proteins that have been proposed as key controllers of posttranscriptional gene regulation processes and epigenetic changes [252]. It has been suggested that, due to their composition, cytoplasmic granules could participate in the modulation of miRNA activity [246].

For instance, p21 mRNA has been recently reported to be isolated into SGs granules and stabilized, escaping from degradation and prolonging its half-life [442, 488]. As it was explained before, SGs are cytoplasmic granules formed under stress conditions and function as hotspots for post-transcriptional regulation of certain genes [251]. Upon SGs formation, mRNAs are sequestered, retained and protected from their degradation enzymes [251]. SGs have been reported to be comprised of RNA transcripts and several miRNA-binding proteins, including Ago2 and other RISC components [251, 489], and they lack GX128 [246], which is essential for miRNA-mediated transcriptional repression [231]. This allows SGs to retain mRNAs and protect them from turnover. Moreover, miRNAs have also been reported to be components of SGs [489, 490]. It could be therefore speculated that miRNAs mediate the stabilization and/or positive translational regulation of their target genes by sequestering the mRNAs into SGs. Interestingly, proteasome inhibitors like MG-132 can induce the formation of SGs [308, 309]. Because we observed a sustained p21 mRNA accumulation over time in cells treated with MG-132, which we attributed to mRNA stabilization by miR-22, we initially hypothesized that this phenomenon took place in SGs. However, these structures have short half-lives since they form and disappear within a period of ~6 hours after treatment [308], which does not explain why in our experimental conditions p21 mRNA levels are stable after much longer periods of time (24 and 48 hours). In addition, miR-22 levels are significantly increased at the time of 6 hours but continue increasing after 12 hours, and then remain stable up to 48 hours after treatment; thus, it is not likely that this miRNA drives p21 mRNA stabilization in SGs.

Nevertheless, because it has been reported that SGs establish direct and dynamic interactions with other types of cytoplasmic granules, allowing RNA trafficking [489, 491], the mRNA-protein complexes could be transferred to other longer-lived structures before SG disassembly. Trafficking of mRNA-protein complexes among the different types of granules and the translation machinery under certain types of stimuli is a rapid mechanism which depends on the RNAs and proteins associated to each transcript, including miRNAs [252, 490, 492]. The trans-acting factors bound to mRNAs eventually determine their fate: transcription, degradation and/or stabilization. Thus, it is possible to speculate that miR-22 mediates the transference of p21 mRNA into a different type of cytoplasmic granule upon SGs disassembly.

Besides SGs, two other types of granules have been described so far in somatic cells: neural granules and processing bodies (P-bodies) [493], which in mammals have been named GW bodies due to the presence of GW128 [250]. After SGs disassembly, most mRNAs and proteins are recruited back to the translation machinery [308], but it has also been reported that SGs establish direct interactions with GW bodies before disassembling and transfer them some of their components, including selected transcripts [491]. GW bodies are dynamic foci comprised of factors involved in mRNA degradation, storage and translation repression [492]. They share several common components with SGs, including miRNAs and miRNA-binding proteins [250, 492], but they are basally expressed and increase their number and size upon stress conditions and during cell cycle, reaching their largest size in the late S and G_2 phase [491, 494, 495]. Most of them are disassembled in mitosis and start to reassemble again in early G_1 [494, 495]. GW bodies are generally considered decay sites but mRNA stored in these structures is not necessarily degraded, but they can accumulate and be released for translation under certain conditions [496, 497]. Thus, GW bodies are good candidates to retain mRNAs after long-term treatments with proteasome inhibitors. Moreover, these structures were reported by Vasudevan et al. [249] to participate in miRNA-mediated positive regulation of gene expression, hosting Ago2 and FXR1 in basal conditions and releasing them upon G_0 arrest.

In light of the observations by Gareau et al. [442] and Vasudevan et al. [248, 249], herein we suggest that the p21 mRNA could be first retained in SGs after treatment with MG-132 and, upon SGs disassembly after ~6 hours, binding of miR-22 to the 3'UTR could mediate its transference into GW bodies, which are numerous and increased in size during G_2 arrest.

On the other hand, it has been demonstrated that a large number of miRNAs and Ago proteins diffusely reside in the cytoplasm [245, 489], so the participation of cytoplasmic granules in the stabilization of p21 mRNA is a hypothesis that should be further investigated.

III. Involvement of miRNA Regulation and Innate Immunity Signaling in the Pathogenesis of MDS

There are many aspects of the involvement of miR-125a in MDS that have remained unknown. First, it should be elucidated which other miRNAs collaborate with miR-125a in the blockade or hindering of differentiation. We believe that miR-125b is a good candidate to exert a collaborative function, and that it would be of extreme interest for the completion of this work to investigate the effect of the combined inhibition of both miRNAs in MDS-L and, especially, in primary samples.

A second aspect that needs further research is definitely the role that miR-125a plays in the regulation of NF-κB activity. According to our results, this miRNA may either activate the transactivating activity of NF-κB or inhibit it. In the discussion of results in Block III, we speculated that our observations could be an artifact resulting from the different cell lines and experimental approaches utilized; however, the differences found in the activity of miR-125a may respond to a finely regulated mechanism of modulation of NF-κB which depends on the state of activation of the TLR pathway. Herein, we go one step further and suggest a mechanism in which the expression of miR-125a can be differentially regulated (cluster-dependent and cluster-independently) based on the degree of activation of the TLR pathway.

On one hand, the synergistic effect of miR-99b and miR-125a on NF-κB activation suggests that NF-κB activation in the absence of TLR stimulation is a cluster-specific process, and could explain the significant correlation between miR-99b levels and OS in MDS patients. NF-κB activation could occur through the translational repression of one or more NF-κB inhibitor molecules, since it was reported that both miR-125a/125b can constitutively activate NF-κB through the inhibition of the TNF-activated inhibitor of NF-κB TNFAIP3 [469]. Furthermore, it has been recently postulated that certain miRNAs can activate NF-κB in immune cells via the TLR1/TLR2 pathway and that those miRNAs are upregulated in cancers with low levels of members of the (TLR1/TLR2)/NF-κB pathway [498]. This agrees with the negative correlation that we found between the TLR/NF-κB pathway members and miR-99b/miR-125b in MDS patients, which indicates that the miR-99b/let-7e/125a cluster and its paralogous are only highly expressed in the absence of TLR/MyD88 hyperactivation. The fact that no significant negative correlation was found for miR-125a suggests, as we said before, that this miRNA can be expressed independently of the cluster. According to this theory, in cells expressing normal levels of TLR proteins/effectors, such as the Meg-01 cells that we used for our first luciferase experiment, the expression of the miR-99b/let-7e/125a cluster would trigger NF-κB activation. The suggested model is depicted in Figure 2A.

On the other hand, miR-125a expression appears to undergo a cluster-independent regulation. According to our previous explanation, in cells with hyperactivated TLR signaling, the miR-99b/let-7e/125a cluster would be downregulated, except for miR-125a, which does not correlate with the expression of TLR proteins/effectors. Under these conditions, attenuation of NF-κB activity by miR-125a could be the result of a higher affinity for the mRNAs of one or more members of the TLR pathway, which would lead to a preferential inhibition of TLR signaling and a consequent attenuation of NF-κB activity. It would be of especial interest for the understanding of this negative regulation to investigate the potential miR-125a target within the TLR pathway. Because no correlation was found

between miR-125a and TLR2 and MyD88, it is unlikely that these are specific targets of this miRNA. However, a downstream adaptor protein would be a better candidate. Herein, we suggest TRAF6 since the 3'UTR of its mRNA contains a conserved miR-125a binding site [378] and it has been postulated that this molecule is tightly regulated by a miRNA feedback loop in hematopoietic progenitors and stem cells [73]. The proposed model is represented in Figure 2B.

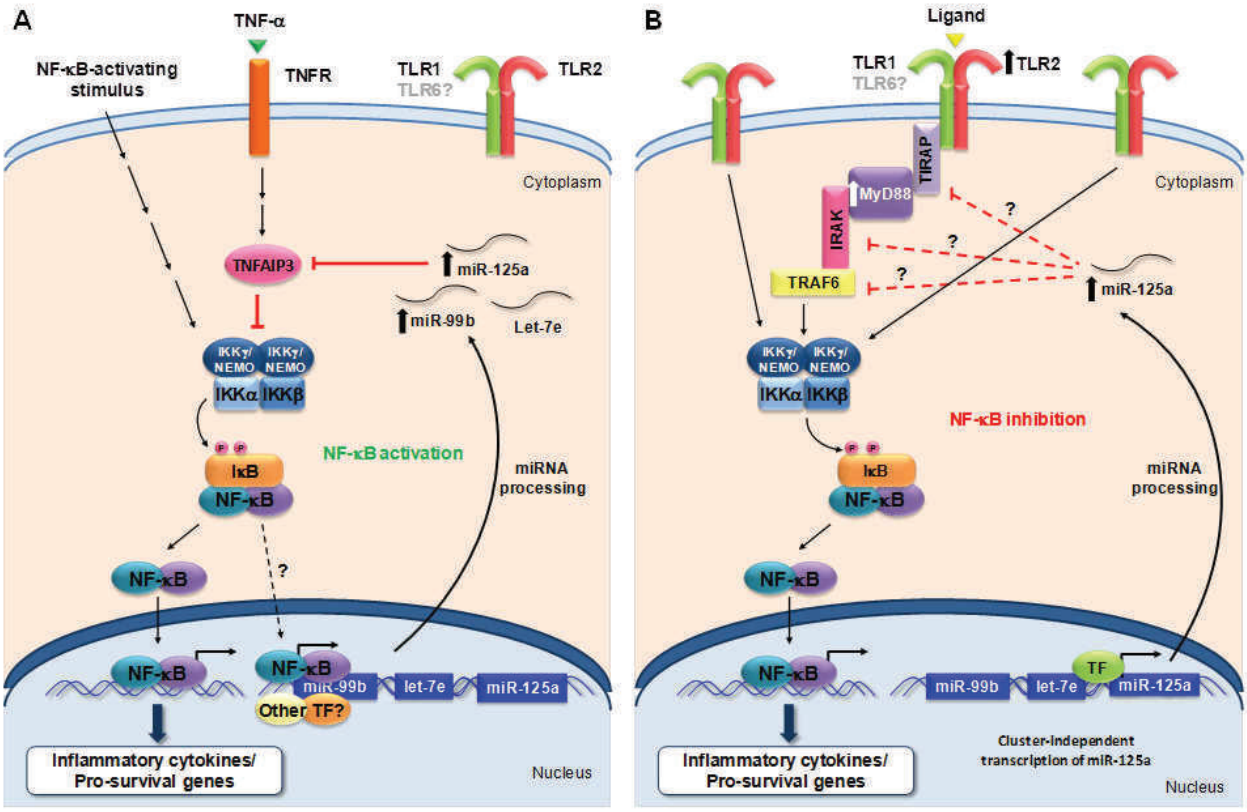

Figure 2. Proposed model for miR-125a regulation in MDS cells. Discontinuous arrows represent speculations and unknown mechanisms; consecutive arrows represent known pathways that do not need to be explained for the understanding of the figure. **(A)** In the absence of TLR signaling, the miR-99b/let-7e/miR-125a cluster (and very likely also miR-125b) is coordinately upregulated, via transcriptional activation by either NF-κB as a part of a positive feedback loop or by other transcription factors (TF). miR-125a, presumably in collaboration with miR-99b, enhances NF-κB activation through the inhibition of the TNF-induced NF-κB inhibitor TNFAIP3 [469] and/or other inhibitors. Thus, the expression of the miR-99b/let-7e/miR-125a cluster may favor survival of hematopoietic cells, protecting them from the deadly effects of TNF-α. **(B)** Upon TLR signaling, NF-κB is activated through a cascade of adaptor proteins. Under these conditions, the miR-99b/let-7e/miR-125a cluster is not expressed (or expressed at low levels) but the expression of miR-125a might be independently induced by unknown (maybe disease-related) mechanisms. Highly increased levels of this miRNA could preferentially target the mRNA of a TLR adaptor molecule and participate in the negative modulation of the TLR signaling towards inflammation. In MDS, elevated miR-125a levels in cells with normal TLR signaling (A) would favor sustained NF-κB activation and pro-survival effects; while in those cells with increased TLR/MyD88 levels and activation (B), high miR-125a levels would negatively modulate NF-κB activation. However, in this case, the hyperactivation of the TLR/MyD88/NF-κB pathway would probably mask the inhibitory effects of miR-125a. Additionally, in both cases, (A) and (B), overexpression of miR-125a in MDS patients would favor differentiation arrest so its effects in either case would be detrimental for the evolution of the disease.

Although the mechanisms of activation or inhibition of the NF-κB pathway were not further explored, the model proposed points to an important role for miR-125a in the modulation of NF-κB activation, as it was suggested before for this [469] and other miRNAs [498, 499]. According to our model, both cluster-associated and cluster-independent regulation mechanisms could be altered in MDS in such way that miR-125a is upregulated in most patients. In those subjects without alterations in the expression or activation of the TLR pathway, miR-125a might be enhancing NF-κB activation; while in those patients with hyperactivated TLR pathway, miR-125a would be counteracting the activation of NF-κB, although its effects could be masked by the strong overexpression of the members of the pathway and continuous stimulation of the signal by positive feedback loops, such as the one reported for the histone-demethylase JMJD3 [85, 455]. Thus, NF-κB signaling would be constitutively activated in patients with and without TLR/MyD88 hyperactivation.

IV. Implications of this Work and Future Perspectives

Leaving cell type- and molecular context-specific mechanisms aside, in this work we have shown that leukemic cells bear intrinsic mechanisms of resistance to apoptosis, such as the expression of mutated tumor suppressors or augmented levels of autophagy. As it has been shown, these factors may hamper the efficiency of a cytotoxic treatment and therefore, in a clinical context, may reduce the outcomes of the therapy. For this reason, for the optimization of therapies, it would be interesting to characterize this sort of intrinsic factors in tumor cells before treatment. Unfortunately, personalized therapies are still a long way from this ideal. Anyway, our results point to a very interesting prospective use of PFT-μ or inhibitors of its kind to reduce chemoresistance in cancers that are known to bear hot-spot p53 mutations that have gain-of-function properties. Similarly, they suggest a potential use of autophagy inhibitors in combination with other drugs.

Regarding chemoresistance, it has also been shown that the treatment of leukemic cells with proteasome inhibitors may trigger the activation of alternative mechanisms that eventually lead to resistance to cell death. Specifically, in the course of our research we found that a compound from this family, MG-132, when used at low doses, induces an anti-oxidant response that could antagonize ROS-mediated apoptosis. This finding calls for caution when combining proteasome inhibitors with cytotoxic drugs which attack the mitochondria or induce oxidative stress. Furthermore, we reported that MG-132 upregulates a miRNA, miR-22, that is directly involved in the overexpression of protein p21, which in turn is known to render cells resistant to chemotherapy. The identification of miR-22 as a mediator of proteasome inhibitor-induced p21 overexpression has two important implications: on

one hand, it contributes to the emerging, but still naive, notion that miRNAs may function as positive regulators of gene expression; on the other hand, it suggests that miR-22 could be a useful target to block p21 overexpression upon treatment with proteasome inhibitors and therefore to improve their effectiveness. This could be achieved by using ASOs which, as we mentioned, are effective miRNA inhibitors *in vitro* and *in vivo*.

Continuing with the utility of miRNAs in the clinics, we have unveiled the role that miR-125a plays in the pathogenesis of MDS. The notion that this miRNA is strongly upregulated in most patients and that its expression levels are positively correlated with the progression of the disease, suggest that this miR-125a is a good biomarker for the prognosis of MDS. It will be very interesting for this potential use of miR-125a to determine if it is a circulating miRNA in MDS patients, which would facilitate sample collection. This is extremely important in a disease in which tumor cells are mainly located in bone marrow. In addition, future research on the functions of this miRNA is needed to fully address its participation in the mechanisms that lead to the development of MDS and to confirm the model herein proposed. If the participation of miR-125a in the blockade of differentiation was confirmed and other participating miRNAs were identified, this miRNA regulatory system would become a greatly interesting therapeutic target in MDS. Along with the progresses currently being made on personalized therapies, this could lead to a breakthrough in the treatment of MDS.

VI. CONCLUSIONES/CONCLUSIONS

CONCLUSIONES/CONLCUSIONS

Conclusiones

I. La combinación de dosis bajas del inhibidor del proteosoma MG-132 y de decualinio, el cual induce estrés oxidativo intracelular, provoca un fuerte bloqueo en la proliferación de las líneas celulares leucémicas humanas NB4 y K562, sin potenciar los efectos pro-apoptóticos individuales de ninguno de estos compuestos.

II. El MG-132, administrado a una dosis sub-letal, induce resistencia a la apoptosis provocada por el decualinio al antagonizar el efecto pro-apoptótico de este compuesto en células NB4. La reducción de los niveles de estrés oxidativo intracelular parece ser responsable de esta resistencia y podría estar relacionada con la activación de la respuesta anti-oxidante mediada por Nrf2.

III. Los niveles elevados de autofagia y la ganancia de función del mutante de p53, $p53^{R248Q}$, parecen conferir a las células NB4 una resistencia intrínseca a la apoptosis. El decualinio supera dicha resistencia, al menos en parte, mediante la inhibición de la autofagia y la inducción de la translocación nuclear de $p53^{R248Q}$.

IV. En las células K562, el MG-132 incrementa de forma sostenida la expresión de p21 y potencia considerablemente la acumulación del mRNA de p21 en presencia de decualinio, lo cual parece resultar de un efecto estabilizador del mRNA.

V. La estabilización del mRNA de p21 por el MG-132 podría estar mediada por la inducción de la expresión del miR-22, que interacciona selectivamente con la región 3'UTR del del mRNA de p21 y favorece su acumulación.

VI. La expresión del clúster de miRNAs miR-99b/let-7e/miR-125a y del miembro del clúster parálogo miR-125b aumenta en células progenitoras de médula ósea de pacientes con síndromes mielodisplásicos y podría participar en la estimulación de la actividad de NF-κB.

VII. La expresión de miR-125a en los síndromes mielodisplásicos parece estar regulada por mecanismos dependientes e independientes de su clúster, y su sobreexpresión en células progenitoras de médula ósea se correlaciona con un mal pronóstico de la enfermedad. Por tanto, el miR-125a es un potencial biomarcador para este grupo de patologías.

VIII. El miR-125a podría participar en la patogénesis de los síndromes mielodisplásicos a través de la desregulación de la actividad de NF-κB, mediante un mecanismo que parece depender del estado de activación de la vía de señalización de los "*toll-like receptors*", y de su contribución al bloqueo de la diferenciación celular.

IX. La expresión del *toll-like receptor 7* está aumentada en células progenitoras de médula ósea de pacientes con síndromes mielodisplásicos y se correlaciona con un mejor pronóstico de la enfermedad, por lo que se podría utilizar como un biomarcador de pronóstico en este grupo de enfermedades.

CONCLUSIONES/CONLCUSIONS

Conclusions

I. The combination of low doses of the proteasome inhibitor MG-132 and dequalinium, which induces oxidative stress, triggers a strong proliferative arrest in the human leukemic cell lines NB4 and K562, without enhancing the individual pro-apoptotic effects of neither of these drugs.

II. A sub-lethal dose of MG-132 antagonizes apoptosis induced by dequalinium in NB4 cells, indicating that MG-132 induces resistance to dequalinium-induced apoptosis. The responsible mechanism involves the reduction of intracellular oxidative stress and could be related to the activation of the Nrf2-mediated anti-oxidant response.

III. Elevated autophagy levels and gain-of-function of the mutant protein $p53^{R248Q}$ appear to confer intrinsic resistance to apoptosis to NB4 cells. Dequalinium overcomes this intrinsic resistance, at least in part, by inhibiting autophagy and inducing the nuclear translocation of $p53^{R248Q}$.

IV. MG-132 induces a sustained increase in p21 expression in K562 cells and strongly enhances p21 mRNA accumulation in the presence of dequalinium, which appears to be the result of an mRNA-stabilizing effect.

V. The mechanism of p21 mRNA stabilization by MG-132 could be mediated by the upregulation of miR-22, which selectively interacts with the 3'UTR of this mRNA and induces its accumulation.

VI. Expression levels of the miR-99b/let-7e/miR-125a cluster and the member of the paralogous cluster miR-125b are augmented in bone marrow progenitor cells of myelodysplastic syndrome patients and could participate in the stimulation of NF-κB activity.

VII. Expression of miR-125a appears to be regulated through cluster-dependent and independent mechanisms in myelodysplastic syndromes, and its overexpression in bone marrow progenitor cells is strongly correlated with a poorer prognosis. Therefore, miR-125a is a potentially valuable prognostic biomarker in this group of diseases.

VIII. miR-125a could participate in the pathogenesis of myelodysplastic syndromes by deregulating NF-κB activity through a mechanism that appears to depend on the activation state of toll-like receptors pathways, and by contributing to the blockade of differentiation.

IX. Toll-like receptor 7 is overexpressed in bone marrow progenitors of myelodysplastic syndrome patients and its expression is correlated with a better prognosis, indicating that this receptor could be used as a prognostic biomarker in this group of pathologies.

CONCLUSIONES/CONLCUSIONS

VII. REFERENCES

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VIII. APPENDIX-I

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APPENDIX-I

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Review Article

Oncogenic functions of the transcription factor Nrf2

Irene Gañán-Gómez [a,*], Yue Wei [b], Hui Yang [b], María Carmen Boyano-Adánez [a], Guillermo García-Manero [b]

[a] Department of System Biology, Biochemistry and Molecular Biology Unit, University of Alcalá, 28871 Alcalá de Henares (Madrid), Spain
[b] Department of Leukemia, University of Texas M.D. Anderson Cancer Center, 77030 Houston, TX, USA

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ABSTRACT

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that controls the expression of a large pool of antioxidant and cytoprotective genes regulating the cellular response to oxidative and electrophilic stress. Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) and, upon stimulation by an oxidative or electrophilic insult, is rapidly activated by protein stabilization. Owing to its cytoprotective functions, Nrf2 has been traditionally studied in the field of chemoprevention; however, there is accumulated evidence that Keap1/Nrf2 mutations or unbalanced regulation that leads to overexpression or hyperactivation of Nrf2 may participate in tumorigenesis and be involved in chemoresistance of a wide number of solid cancers and leukemias. In addition to protecting cells from reactive oxygen species, Nrf2 seems to play a direct role in cell growth control and is related to apoptosis-regulating pathways. Moreover, Nrf2 activity is connected with oncogenic kinase pathways, structural proteins, hormonal regulation, other transcription factors, and epigenetic enzymes involved in the pathogenesis of various types of tumors. The aim of this review is to compile and summarize existing knowledge of the oncogenic functions of Nrf2 to provide a solid basis for its potential use as a molecular marker and pharmacological target in cancer.

Contents

Abbreviations: b-ZIP, leucine-zipper protein; ARE, antioxidant response element; PT, primary tumor; LOH, loss of heterogeneity; NSCLC, non-small-cell lung carcinoma; EOC, epithelial ovarian carcinoma; ESCC, esophagus squamous cell carcinoma; SCC, squamous cell carcinoma; STC, stomach carcinoma; HCC, hepatocellular carcinoma; COL, colorectal carcinoma; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; ER, estrogen receptor; MGMT, methylguanine DNA-methyltransferase; ERK, extracellular-regulated MAP kinase; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase; MEK, MAP kinase-ERK kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; GSK-3β, glycogen synthase kinase 3β; LC3α, kinase light chain 3α; MDR, multidrug resistance; CHOP, C/EBP homologous protein; SAHA, suberoylanilide hydroxamic acid; mTOR, mammalian target of rapamycin.

* Corresponding author.
E-mail address: irene.ganan@edu.uah.es (I. Gañán-Gómez).

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Introduction

Nrf2, or nuclear factor E2-related factor 2, is a transcription factor belonging to the cap′n'collar family of leucine-zipper (b-ZIP) proteins [1], which regulates cell response to oxidative and electrophilic insults.

Under homeostatic conditions, Nrf2 is generally localized in the cytoplasm, where it is sequestered by its inhibitor, Keap1, or Kelch-like ECH-associated protein 1 (Fig. 1A). Keap1 interacts with the Nrf2 N-terminal domain Neh2 [2,3] through two binding sites located on its Kelch-like domains and facilitates the association of Cul3, a Cullin-3-based E3 ubiquitin ligase [4–6]. Cul3 then mediates the ubiquitination and subsequent degradation of Nrf2 by 26 S proteasome [7,8]. Thus, under basal conditions, Nrf2 undergoes a rapid physiological turnover triggered by Keap1.

Under oxidative or electrophilic stress conditions (Fig. 1B), however, Keap1 acts as a molecular sensor and undergoes chemical modifications in a series of reactive cysteine residues (reviewed in [9,10]), allowing the release of Nrf2 [11,12], which escapes from degradation and translocates to the nucleus. Therefore, protein stabilization is the main mechanism for the activation of the Nrf2 response [13,14].

Once in the nucleus, Nrf2 heterodimerizes with proteins from a family of b-ZIP oncogenes called small Maf (musculoaponeurotic fibrosarcoma) proteins [15] and binds antioxidant response elements (AREs) localized in the promoter region of its target genes. Nrf2 target genes are mainly antioxidant and phase II enzymes such as heme oxygenase-1 (HO-1) [16], NAD(P)H–quinone oxidoreductase 1 (NQO1) [15,17], glutathione *S*-transferases (GSTs) [18], γ-glutamylcysteinyl synthetase [19], glutathione peroxidases [20], thioredoxin reductase 1 (TrxR1) [21,22], peroxiredoxin 1 [23], aldehyde oxygenase [24], and other genes regulating the response to oxidative stress [25]. Nrf2 also activates the transcription of some genes of the multidrug resistance (MDR) family such as MRP1 [26], MRP2 [27,28], MRP3, and MRP4 [28]. Overall, Nrf2 regulates the expression, both basal and inducible [29], of enzymes and other proteins involved in cell protection from physical/chemical insults, detoxification, and restoration of homeostasis. For this reason, Nrf2 is considered a cytoprotective transcription factor.

Owing to its role in protecting the cell from cytotoxicity associated with reactive oxygen species (ROS) and electrophilic stressors, Nrf2 is especially important in chemoprevention of diseases. The role of Nrf2 in this field has been widely studied and a great number of Nrf2 inductors, mainly natural compounds present in vegetables, have been described (reviewed in [30,31]). Moreover, there are currently several active clinical trials for activators of the Nrf2 pathway with potential utility in chemoprevention of various pathologies that are generally characterized by the production of intracellular ROS and eventual cell death [32]. Among those pathologies, the role of Nrf2 has been deeply studied in cancer, in which the response of cells to physical (radiation) and chemical (pollution, toxins, drugs) insults is especially important. Consequently, the number of citations relating Nrf2 and cancer has exponentially increased in the past decade [33].

As a cytoprotective gene, Nrf2 has been traditionally considered to be a tumor suppressor. For instance, Nrf2-deficient mice seem to be more sensitive to carcinogenesis [34,35] and Nrf2 loss has been related to enhanced metastasis [36,37]. Accordingly, there are multiple reports describing the beneficial effects of Nrf2 signaling in cancer chemoprevention (reviewed in [30]). However, in the past few years, mounting evidence that the activation of the Nrf2 pathway might not be beneficial in all cancer types and stages has started to arise. In fact, there are many reports that support the idea that Nrf2 activation in malignant cells could be detrimental for the evolution of the disease as well as for the outcomes of the treatment, and findings of several mutations and aberrant signaling of the Nrf2 pathway in cancer reveal a new role for this factor beyond its functions in chemoprevention. Thus, the beneficial effects of the activation of Nrf2 signaling in cancer have become a controversial issue (reviewed in [38]).

From either perspective, it seems to be clear that Nrf2 is an interesting pharmacological target for the prevention or treatment of malignant diseases. There is therefore an increasing need to define the limits between Nrf2's positive and negative effects in cancer and establish the basis for rational Nrf2-targeted therapies. In this article, we attempt to address this issue, focusing on Nrf2 as a potential oncogene and reviewing the most recent advances in this field to help provide a solid basis for the use of Nrf2 as a molecular marker and pharmacological target in cancer.

Nrf2 as a proto-oncogene

Nrf2 signaling in physiological conditions acts as a switch that is turned on by the presence of stressors in the cellular microenvironment and that is rapidly deactivated when the insult is withdrawn and homeostasis is restored. However, under pathological conditions, the tight regulation of Nrf2 by rapid protein turnover is highly susceptible to being altered. This could result in the loss of responsiveness to cell stressors and subsequent vulnerability of the cell to various insults. For instance, $Nrf2^{-/-}$ mouse models have shown a high sensitivity to chemical and physical insults [39–41].

On the other hand, Nrf2 regulation could be unbalanced toward the loss of the inducible nature of Nrf2 signaling and the acquisition of a constitutively active phenotype. Constitutive signaling toward the expression of cytoprotective enzymes would confer cells a survival advantage under adverse conditions. This advantage would become a serious drawback in the context of cancer pathogenesis and treatment. Therefore, constitutive activation or augmented signaling of the Nrf2 pathway might be decisive for cell fate during tumorigenesis and affect the response to chemotherapy. Under these conditions, Nrf2 can be defined as a proto-oncogene [42].

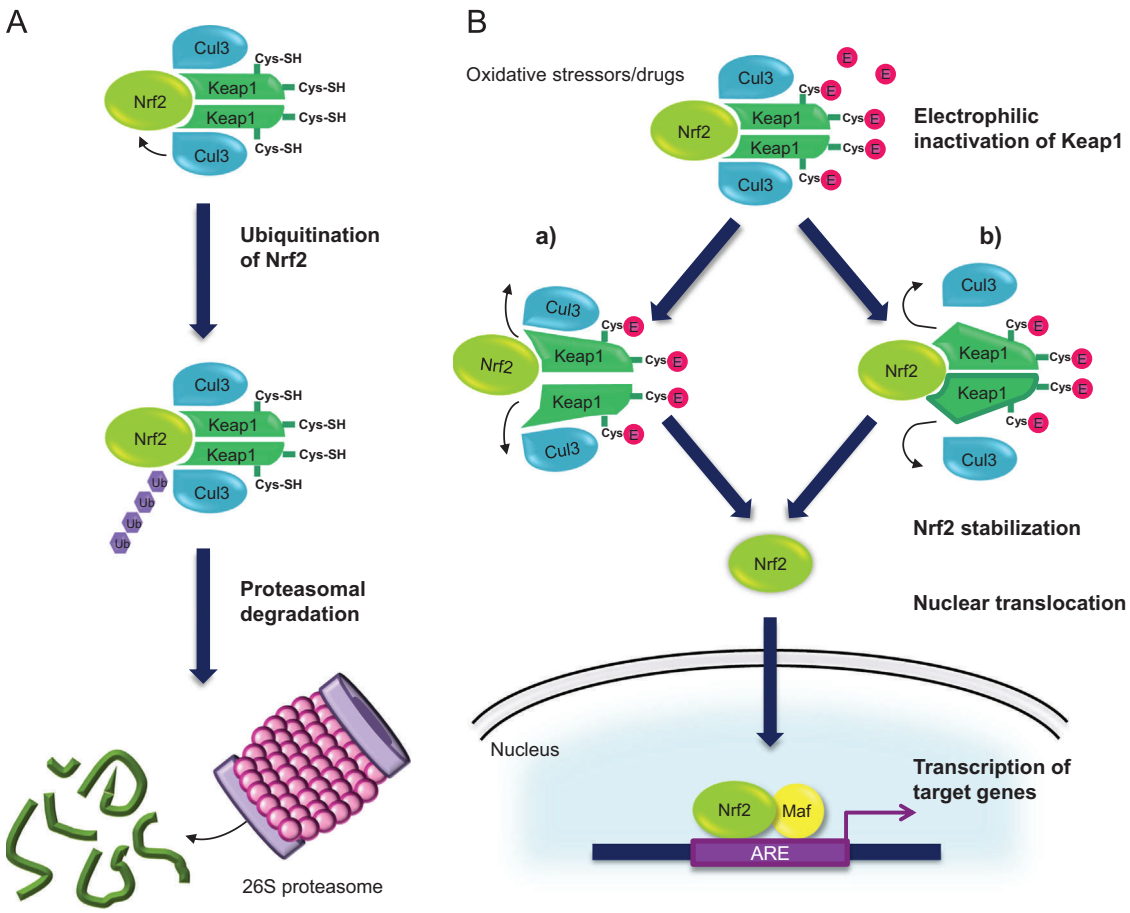

Fig. 1. Regulation of Nrf2 by Keap1. (A) Basal state. Under homeostatic conditions, Keap1 dimers bind Nrf2 in the cytoplasm and recruit Cul3 [5], which mediates Nrf2 ubiquitination. Ubiquitin-labeled Nrf2 is then rapidly degraded by 26 S proteasome. Thus, in the absence of oxidative or electrophilic stimuli, Nrf2 activity is regulated by a quick turnover. (B) Nrf2 activation by oxidative or electrophilic stressors. During oxidative stress, electrophiles (E) bind sulfhydryl groups of cysteine residues exposed on the surface of Keap1 and trigger a conformational change in this protein that ultimately leads to the stabilization of Nrf2. Two different mechanisms have been proposed: the first, or the "hinge and latch" model (a), suggests that Keap1 dissociates from Nrf2, impeding its ubiquitination and proteasomal degradation [11]; the second, or the "Cul3 dissociation" model (b), postulates that the conformational change disrupts Cul3 and Keap1 association and therefore prevents Cul3 from ubiquitinating Nrf2 and tagging it for turnover [12]. In both cases, the result is the escape of Nrf2 protein from degradation and its stabilization. Free cytosolic Nrf2, not bound to Keap1, translocates into the nucleus, where it dimerizes with small Maf proteins, binds to the ARE, and activates the transcription of its target genes.

Nrf2 in tumorigenesis

The participation of Nrf2 in cancer pathogenesis is a controversial topic, provided a number of reports that still assign Nrf2 a role in cancer chemoprevention from genotoxic agents [43–45] or inflammation [46]. However, some reports have shown that drugs that activate Nrf2 can promote cell growth [47–49] and an increasing number of works point to a potential role for Nrf2 and its transcriptional target genes in tumorigenesis.

Nrf2-induced antioxidant enzymes in cancer

Many Nrf2 target genes have been profusely described to play a role in cell growth and tumorigenesis.

HO-1 is one of the best-known Nrf2 targets [16] and its involvement in cell growth and cancer development has been widely documented. HO-1 is overexpressed in a variety of solid tumors [50–52] and has been reported to play a role in metastasis of prostate cancer by accelerating angiogenesis [53]. Accordingly, HO-1 has been recently demonstrated to cooperate with phosphatase PTEN deletions to increase the invasiveness of this type of tumor [54]. This finding has also been confirmed in lung cancer [54] and is of great importance because PTEN is frequently inactivated in human cancers [55].

Furthermore, in chronic myelogenous leukemia (CML), in which the oncoprotein Bcr/Abl induces constitutive expression of HO-1 [56,57], this enzyme was shown to inhibit apoptosis in cell lines as well as in primary samples [56]. Moreover, expression of HO-1 seems to correlate with the progression of CML to blastic (terminal) phase [57]. Interestingly, results suggested that the mechanism is not related to its enzymatic activity [56].

Similarly, NQO1 was long ago found to be expressed in a variety of solid tumors, such as hepatoblastoma [58,59], colon cancer [58], breast cancer [58,60], and non-small-cell lung cancer (NSCLC) [58,60,61]. Surprisingly, the role of this enzyme in cancer chemoprevention has always been the focus of attention of most of the studies (reviewed in [62]). However, its involvement in cancer development has been recently unveiled in melanoma [63,64] and estrogen-dependent breast cancer [65,66].

TrxR1 is also known to be overexpressed in a number of human cancers [67] and seems to influence the aggressiveness of the tumor, because knockdown or downregulation of TrxR1 has been proven to decrease tumor cell growth in human hepatocarcinoma [68] and deplete invasiveness and metastatic potential of human lung cancer cells, reducing the levels of cancer-related growth factors [69]. Importantly, it has been suggested that TrxR1 is essential for tumor growth *in vivo* but not for proliferation of cultured cells [69], which may indicate that interactions with the tumor microenvironment are necessary for the proliferative effect of this enzyme.

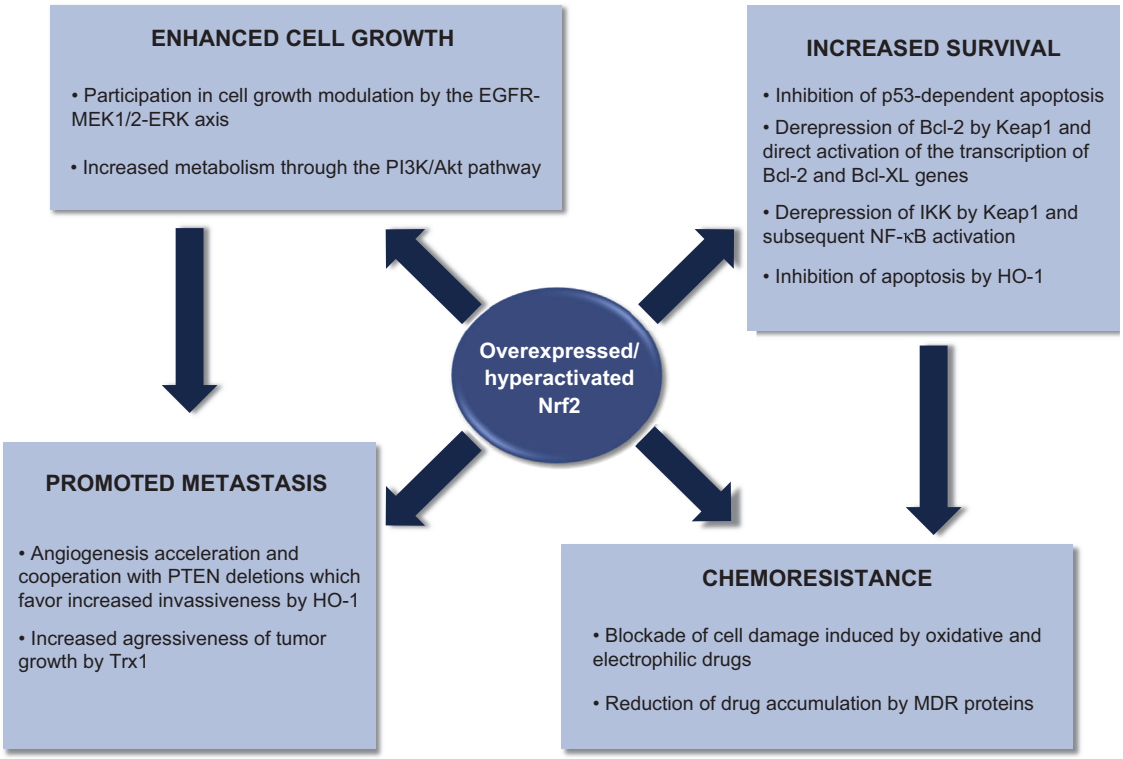

Fig. 2. Proto-oncogenic activity of Nrf2.

GSTπ levels have been suggested as tumor markers in several types of cancer [70–75]. The finding that GSTπ expression is induced by mutant K-Ras in colon cancer [75] connects the expression of this enzyme with oncogenic signaling. In this particular work, the authors suggested the involvement of AP-1-driven transcription in GSTπ overexpression; however, the participation of Nrf2 was never studied and cannot be ruled out, especially considering a more recent report that will be referred to later on in this review [76]. Moreover, recent findings point to an important role for glutathione (GSH) in the regulation of cell growth by Nrf2, because Nrf2-induced GSH-synthesizing enzymes are strongly activated in the presence of proliferative signaling [77] and GSH itself seems to be necessary for the transcription of several genes involved in cell growth, such as receptors, growth factors, kinases, and transcriptional regulators [78].

Role of Nrf2 in cell growth

Nevertheless, effects of Nrf2 activation are not limited to the expression of enzymes involved in cytoprotection from oxidative or electrophilic insults. There is evidence of its direct participation in the regulation of other transcription factors [29] and genes involved in cell growth and survival [78–80] and it was recently reported that Nrf2 partially contributes to the regulation of cell proliferation by the epidermal growth factor receptor (EGFR)–MEK1/2–ERK axis [81].

The role that this factor plays in metabolic reprogramming is particularly relevant. This is the process through which quiescent cells, which generally invest their energy in maintenance and self-protection from exogenous insults, change their metabolism to enter a proliferative status in which they need to take up abundant nutrients to utilize their metabolites in anabolic pathways [82]. A recent study of the relevance of Nrf2 in metabolism of proliferating cells showed that Nrf2 collaborates with the PI3K/Akt signaling pathway to increase metabolism via the pentose phosphate pathway and divert glucose metabolites toward *de novo* nucleotide synthesis [77]. Nrf2 also induces GSH and NADPH synthesis [77] and regulates genes involved in lipid metabolism [83,84]. Increased metabolite availability facilitates cell proliferation, which can account in part for Nrf2-induced tumor growth.

Importantly, induction of metabolic genes by Nrf2 has two major requirements that, under physiologic conditions, limit its effects to tissues with a certain proliferative potential: first, the PI3K/Akt pathway needs to be active; second, Nrf2 needs to accumulate in levels higher than those needed for the transcription of antioxidant target genes [77]. These conditions allow Nrf2 to control only the inducible expression of metabolic genes involved in cell growth. It is therefore reasonable that aberrant Nrf2 overexpression or hyperactivation in cells with high PI3K/Akt activity may lead to a hyperproliferative phenotype and, eventually, to tumor growth.

Also remarkable is a recently described crosslink between Nrf2 signaling and E-cadherin expression [85]. According to this work, E-cadherin acts as a corepressor of Nrf2 in the presence of β-catenin, binding the Keap1/Nrf2 tandem near the cell membrane and preventing Nrf2 from translocating to the nucleus. In agreement, enhanced resistance to treatment with doxorubicin (presumably due to Nrf2 activation) was observed in hepatocellular carcinoma cell lines with low expression of E-cadherin [85]. These findings are extremely interesting, considering that loss of E-cadherin expression or its aberrant localization is a common feature during tumor progression and correlates with a higher invasive potential of malignant cells [86]. Loss of E-cadherin functionality may favor increased Nrf2 nuclear translocation and therefore confer cells additional survival and/or proliferative mechanisms that could contribute to tumor progression. The potential role of Nrf2 in the acquisition of the invasive phenotype in solid tumors is a field that should be further explored.

Nrf2 and the regulation of apoptosis

During tumorigenesis, cells not only increase their proliferative potential but also need to develop mechanisms that allow them to escape their own tumor suppressor systems. Impairment or

deregulation of the main apoptotic pathways is a major characteristic of cancer cells [87]. In this regard, a crosslink between Nrf2 and some effectors of the main apoptotic pathways has been proposed on several occasions.

Tumor suppressor p53, which induces apoptosis upon DNA damage, partially in a ROS-dependent fashion [88], has been shown to inhibit the transcriptional activation of Nrf2 target genes in various cancer cell lines [89]. This finding is supported by another report in which mice with decreased p53 levels showed enhanced expression of Nrf2 target genes after treatment with a genotoxic agent [90]. Despite it being initially hypothesized that the inhibition of Nrf2 targets was carried out by direct binding of p53 to the ARE, a more recent work demonstrates that p53 does not interact with AREs and in turn suggests that the inhibition is mediated by its transcriptional target p21 [91] and also involves changes in Nrf2 protein levels [92]. In agreement with these data, a recent study demonstrated that the hot-spot mutants $p53^{R273H}$ and $p53^{R248Q}$ reduce the inducible expression of Nrf2 target phase II detoxifying enzymes in several cell line models through a gain-of-function mechanism involving the decrease of Nrf2 protein stabilization [93]. These data suggest that Nrf2 inhibition is needed for p53-dependent apoptosis. Conversely, in this case p53 mutants also inhibited apoptosis [93], but this is more likely to be a consequence of the aberrant activity of the mutants rather than an event related to increased ROS levels.

Regulation of Nrf2 by p53 seems to be even more complicated than what was initially thought. A two-phase model has been proposed, in which subtoxic doses of agents inducing ROS activate Nrf2 and maintain low p53 expression levels, whereas higher doses of these compounds induce a stronger activation of p53, which results in the inhibition of Nrf2 [92]. ERK and p38 kinase might be involved and compete with each other in this regulation of p53 upon stimulus of varying intensity or kinetics [94]. The notion that p53 not only inhibits Nrf2 upon cytotoxicity but also is important in maintaining basal and inducible (under low risk) Nrf2 levels suggests a key role for p53 in the crosslink between apoptosis and Nrf2 signaling and demands a deeper study of this regulation.

Taken together, these reports indicate that p53-dependent apoptosis induced by various stressors (genotoxic and oxidizing agents) requires the inhibition or silencing of Nrf2-regulated antioxidant genes. Therefore, an increase in the expression of the antioxidant gene battery could interfere with p53-dependent apoptosis induced after DNA damage and thus favor the development of cancer.

Another important connection between Nrf2 activity and apoptotic pathways is the Nrf2-mediated regulation of proteins from the Bcl-2 family [95–97]. Keap1 directly interacts with Bcl-2 and induces its ubiquitination by Cul3 in a conserved lysine residue, which then triggers Bcl-2 proteasomal degradation [95]. Keap1 appears to bind the BH2 domain of Bcl-2, which participates in complex formation with the proapoptotic protein Bax [98]. Therefore, Keap1 competes with Bax for its Bcl-2 binding site, induces Bcl-2 turnover, and stabilizes Bax, facilitating apoptosis. Accordingly, the well-known Keap1/Nrf2 dimer disruptor and Nrf2 activator *tert*-butylhydroquinone (tBHQ) also inhibits Keap1/Bcl-2 interaction and decreases cell death [95]. Moreover, the human lung carcinoma cell line A549, which bears a mutation in the same domain of Keap1 that binds Bcl-2, was shown to be unable to downregulate Bcl-2 and stabilize Bax [95]. These results suggest that the decreased interaction of Keap1 and Bcl-2 accounts, at least in part, for the radio- and chemoresistance observed in A549 cells and bring up the question as to what extent Bcl-2 might be responsible for other antiapoptotic effects observed in Keap1 mutants.

The relation between Nrf2 and p53 or Bcl-2 connects Nrf2 inhibition with the activation of apoptosis. In agreement, recent data demonstrate that Nrf2 is able to directly activate the transcription of the antiapoptotic proteins Bcl-2 and Bcl-XL and therefore increase cell survival and drug resistance [97,99]. Altogether, data show that Nrf2 activation and apoptosis are antagonistic events. Apoptosis is inhibited in a context of Nrf2 activation and, in turn, high levels of functional Keap1 (and therefore inactive Nrf2) result in its induction by proapoptotic proteins.

It is worth mentioning that Keap1 has also been reported to inhibit IKKβ (IκB kinase-β), the activator kinase of NF-κB [100]. Provided the role that NF-κB plays in inflammation and apoptosis and the NF-κB-independent tumorigenic activity of IKK [101,102], Keap1 could be acting as a tumor suppressor at many levels in addition to Nrf2 regulation.

Overall, the interplay between the Keap1/Nrf2 pathway and the major regulators of apoptosis, such as p53 and Bcl-2, gives an idea of the importance of Nrf2 signaling in cell death control and how the deregulation of any of the pieces of this puzzle could affect cell fate and contribute to cancer pathogenesis.

Nrf2 in chemoresistance

The aforementioned ability of Nrf2 to counterbalance proapoptotic signals and favor cell survival, along with its cytoprotective nature, makes this factor not only a good promoter of tumorigenesis but also an important antagonist of the effects of chemotherapy.

High expression of some antioxidant Nrf2 target genes has been related to chemoresistance before. In myeloid leukemias, both acute and chronic, HO-1 seems to be an especially important effector of Nrf2-induced chemoresistance and the treatment of these diseases seems to benefit from the inhibition of either Nrf2 or HO-1 [57,103–106]. Similarly, it has been reported that inhibition of GSTπ in colon cancer cells sensitizes them to several types of chemotherapy [107]. This enzyme is also overexpressed in head and neck cancer owing to a gain of copy number in these tumors and its expression levels correlate with the lack of sensitivity to conventional chemotherapy [108].

In addition to target overexpression, genetic aberrations in Keap1/Nrf2 genes were also found to be correlated with a reduced response of cancer cells to chemotherapy [109–114]. Interestingly, most of these reports study the response to platinum-based chemotherapy, which generates electrophilic molecules that damage DNA. Similar results have been published for etoposide [109,115], doxorubicin [115–117], or doxycycline [118], drugs that can induce the production of free radicals that interact with DNA. Likewise, because of the direct relationship between Nrf2 signaling and protection from oxidative stressors, resistance to radiation and drugs that induce the accumulation of ROS or nitric oxide species as a part of their mechanism of action has been more deeply studied and widely described in cancers showing Nrf2 overexpression or hyperactivation [97,106,119–122].

However, the proapoptotic effects of other compounds not directly targeting redox balance can also be hampered by Nrf2 signaling. One of the proposed mechanisms is the induction of the expression of proteins from the drug efflux pump family ABC or MDR [26,96,123], which prevent intracellular drug accumulation. In fact, members of this family have been found to be overexpressed in Keap1/Nrf2-mutated cancer cells [124,125].

Cytoprotective effects of Nrf2 against chemotherapy are determined not only by the endogenous levels of this factor, but also by the drug itself. Certain types of drugs, such as the family of histone-deacetylase inhibitors (HDACI) [119,126], which will be referred to further on in this review, can specifically induce the activation of Nrf2 and thus decrease their own effectiveness.

Another drug family that has drawn attention to its Nrf2-activating potential is the group of proteasome inhibitors. These compounds have been reported to induce Nrf2-dependent expression of antioxidant proteins such as HO-1, SOD-1 (superoxide dismutase 1), cyclo-

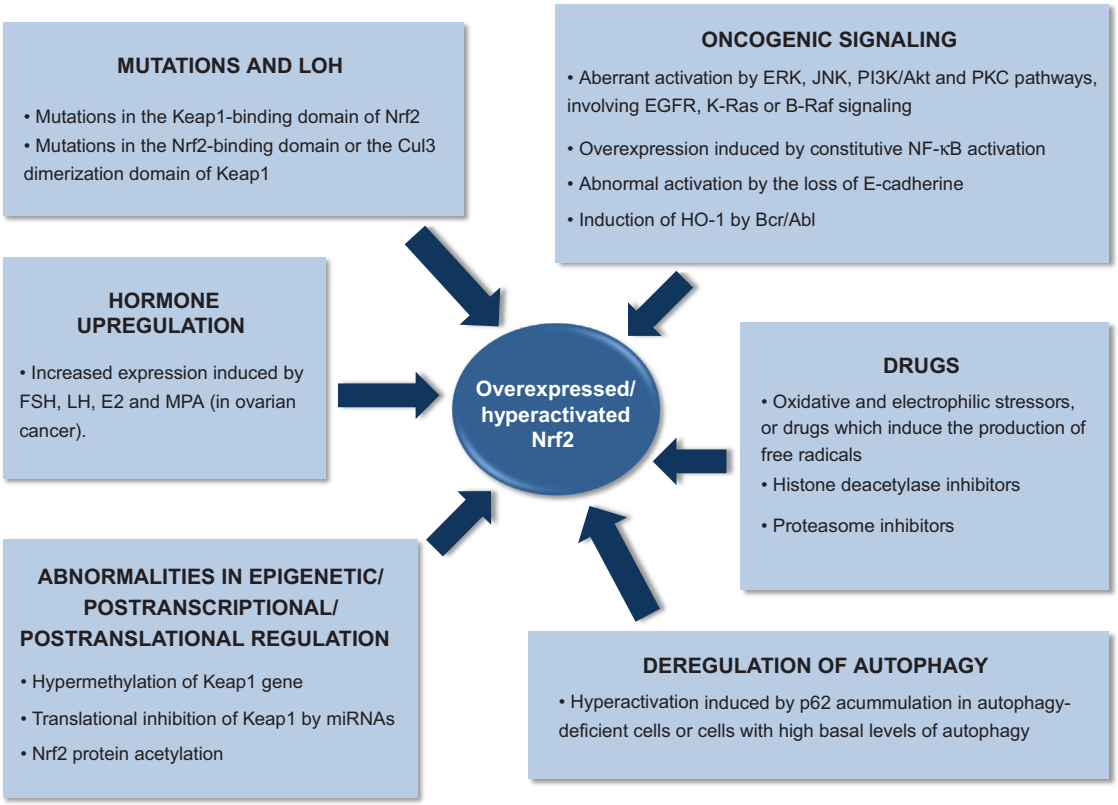

Fig. 3. Nrf2 deregulation in cancer.

oxygenase 2, and AKR (aldo-ketoreductases) [127–129] and a consequent resistance to chemotherapy in cancer cells [130,131]. Moreover, recent work in thyroid cancer cells shows that proteasome inhibition not only induces the transcriptional activation of Nrf2 targets but also generates a Nrf2-dependent antiapoptotic response via p38 kinase and the inhibition of the transcription factor C/EBP homologous protein (CHOP) [132,133], which suggests that these two pathways are closely related.

Despite the fact that the most generally accepted mechanism for Nrf2 activation by proteasome inhibitors is their ability to produce oxidative stress [131,134], it has been demonstrated that Nrf2 regulates the expression of several proteins necessary for the functionality of proteasomes [130,135]. Provided that proteasomal degradation is a process that contributes to the maintenance of cell homeostasis and inhibits endoplasmic reticulum and oxidative stress, it seems reasonable that Nrf2 participates in its regulation to protect cells from harmful stimuli [135,136].

It is therefore particularly important to take into account the possible Nrf2 induction by drugs frequently used in chemotherapy, whether they are ROS inductors or not, because this side effect may reduce the efficiency of such other compounds used in combination.

Deregulation of the Nrf2 pathway in cancer

Consistent with its role in tumorigenesis and chemoresistance, the hyperactivation and/or aberrant expression of Nrf2 and its transcriptional targets is well documented in cancer (summarized in Figs. 2 and 3).

Mutations of Keap1/Nrf2 in the pathogenesis of cancer

Nrf2/Keap1 mutations in lung cancer

Lung cancer is undoubtedly the type of tumor with the largest number of reports addressing aberrant expression or activation of the members of the Nrf2 signaling pathway [58,60,61,137]. This overexpression or constitutive activation and the subsequent increase in the antioxidant defense in lung cancer cells are mainly consequences of somatic mutations in the Nrf2 and Keap1 genes, which seem to be more prevalent in this type of cancer than in any other kind of tumor.

The first described somatic mutations in the Nrf2 pathway were reported in lung cancer in 2006 as two-point mutations on the Kelch-like repeat domain of Keap1 [138], which is a Nrf2-binding domain. In that work, it was shown that point mutations are enough to significantly decrease the binding ability of Keap1 to Nrf2, hampering its repressive function and subsequently increasing the expression of Nrf2 target genes.

After that, many others have broadened the knowledge of Nrf2/Keap1 mutations in lung cancer. In a more extensive study, Keap1 mutations reducing Nrf2 inhibition and resulting in Nrf2 nuclear overexpression and increased target protein levels were described all over the gene, and not only in the Nrf2-interacting region [109]. The fact that mutations in Keap1 that do not affect its Nrf2-binding activity might as well result in an increased Nrf2 half-life is an important finding; this can occur through a decrease in Keap1-promoted Nrf2 ubiquitination by Cul3 [112,139] or through the impairment of nuclear export of Nrf2 by Keap1/Cul3 complexes [140]. In both cases, after a cellular stress signal, Nrf2 would escape degradation and be free to translocate and activate the expression of its target gene battery. In contrast, other Keap1 mutations might result just in a truncated form of Keap1 protein [141].

Among lung cancers, Nrf2 has been more deeply studied in NSCLC, which is the most common type and is characterized by a lower sensitivity to chemotherapy compared to small-cell carcinomas. A study identified mutations in the Keap1 gene in 25% of NSCLC patients analyzed and, importantly, a loss of heterozygosity (LOH) of 41% for this gene [109]. Similar results were found in cell lines in the same study and in different cohorts of patients in

Table 1
Keap1/Nrf2 mutations in cancer.

Type of cancer	Domain	Type of mutation, zygosity	Frequency, other characteristics	Ref.
Keap1				
Biliary tract cancer	All along the gene	Somatic, 33.33% heterozygous and 66.6% homozygous; 100% of homozygous presented with LOH	11.3% PT, more frequent (30.7%) in gallbladder cancer	[110]
Breast	BTB domain[a]	N/A[b]	N/A[b]	[139,144]
Breast	DC domain[a]	Somatic, N/A[b]	2% PT (1/51)	[145]
Colon (COL)	All along the gene	Somatic, N/A[b]	7.8% PT (4/51); 50% (2/4) LOH	[145]
Gastric (STC)	All along the gene	Somatic, N/A[b]	11.1% PT (6/54); 33.3% (2/6) LOH	[145]
Liver (HCC)	All along the gene	Somatic, N/A[b]	8.9% PT (4/45); 25% (1/4) LOH	[145]
Lung (NSCLC)	All along the gene	Somatic, N/A[b]	4.6% PT (6/130); 66.6% (4/6) LOH; more frequent in adenocarcinoma (6.1%) vs SCC (3.7%)	[145]
Lung (NSCLC)	DC domain[a]	Somatic, N/A[b]	18.5% PT showed mutations, more frequent in adenocarcinoma (60%)	[141]
Lung (NSCLC)	DGR motif, DC domain[a]	N/A[b]	3.2% PT (1/31), all SCC	[113]
Lung (NSCLC)	DGR motif, DC domain[a]	N/A[b]	5.1% PT, more frequent in adenocarcinoma (75%); 100% history of smoking	[142]
Lung (various subtypes)	All along the gene	Somatic, 33.33% NSCLC cell lines were homozygous, of which 75% showed LOH (44% in total PT)	18% PT; 83.3% mutated PT showed LOH	[109]
Lung (various subtypes)	DGR motif, DC domain[a]	Somatic (patient), homozygous (cell lines)	N/A[b]	[138]
Lung (various subtypes)	DGR motif, DC domain/IVR domain[a]	Somatic, 20% PT were homozygous; 41% of total PT presented with LOH	8% PT, more frequent in adenocarcinoma	[112]
Ovary (EOC)	All along the gene	Somatic, 100% heterozygous	18.5% PT (5/27), more frequent in clear cell carcinoma (28.6%) vs non-clear (8%)	[114]
Prostate	IVR domain[a]	Somatic, N/A[b]	8.3% PT (1/12)	[146]
Prostate	IVR domain[a]	Somatic, N/A[b]	1.3% PT (1/75)	[145]
Nrf2				
Esophagus (ESCC)	Neh2 domain[c]	Somatic, N/A[b]	11.4% PT (8/70); 87.5% (1/8) history of smoking	[143]
Head and neck	Neh2 domain[c]	Somatic, 100% heterozygous	25% PT (3/12), 66.6% (2/3) history of smoking	[111]
Head and neck, larynx	Neh2 domain[c]	Somatic, N/A[b]	13% PT (3/23); 100% history of smoking	[143]
Lung (NSCLC)	Neh2 domain[c]	Somatic, 90.9% heterozygous	10.7% PT (11/103), 90.9% (10/11) history of smoking; no concomitant Keap1/Nrf2 mutations	[111]
Lung (NSCLC)	Neh2 domain[c]	Somatic, N/A[b]	8.0% PT (10/125), more frequent in SCC (15.8%) than in adenocarcinoma (1.6%); 90% history of smoking	[143]
Lung (NSCLC)	Neh2 domain[c]	N/A[b]	6.9% PT (2/29), all SCC	[113]
Skin (SCC)	Neh2 domain[c]	Somatic, N/A[b]	6.3% PT (1/17); nonsmoker	[143]

[a] Keap1 domains: DC (Kelch-like domain), Nrf2-binding domain; IVR, low-affinity Nrf2-binding domain; BTB, Cul3-dimerization domain.
[b] Not available.
[c] Nrf2 domains: Neh2, Keap1-binding domain.

other works [112,113,142]. More interestingly, practically all homozygous mutants presented with LOH [109,112], meaning that most patients presented with biallelic deactivation.

These results suggest that the two alleles of Keap1 are necessary to maintain an effective repression of Nrf2. However, the loss of the two copies of the Keap1 gene is not essential to provide the cells with a selective advantage. As proof, it has been postulated that Keap1 proteins harboring mutations on the Kelch-like domain repress wild-type Keap1 in a dominant-negative manner, forming nonfunctional heterodimers that fail to associate with the two binding sites of Nrf2 [112]. This hypothesis would explain why heterozygous mutations are enough to induce Nrf2 hyperactivation. Moreover, a comprehensive study of Nrf2 and Keap1 expression and genetic aberrations in NSCLC [113] showed that most tumors have either a decreased Keap1 expression or mutations in Keap1 and that both conditions coexist in only a small subset of patients, confirming that only one type of abnormality in Keap1 is necessary to induce aberrant Nrf2 expression and reduces biological pressure toward the acquisition of another mutation.

Mutations in the coding region of the Nrf2 gene were also first described in lung cancer and are clustered in the Keap1-binding domain Neh2, which consists of the motifs ETGE and DLG, affecting Keap1-binding affinity [111]. Interestingly, mutations on the ETGE motif had a higher impact on Nrf2 activity than mutations on the DLG motif, because the first can support Keap1 binding by itself (for a review on Nrf2 and Keap1 gene structures, refer to [10]). It should also be pointed out from this study [111] that mutations of Nrf2 and Keap1 found in lung cancer were not concomitant, which would indicate again that these abnormalities are powerful enough to be mutually exclusive.

Why lung tissue is especially prone to the hyperactivation of the Nrf2 pathway is still a matter of discussion. The constant exposure of respiratory tissues to oxygen and chemicals present in the air has been suggested as a possible explanation. For instance, high NQO1 levels comparable to those present in lung cancer have been found in normal respiratory epithelium [137]. According to this hypothesis, normal lung cells would initially benefit from a permanently activated protective response against oxidative stress conferred by mutations on Nrf2 or Keap1; however, upon malignant transformation, mutated cells would have a selective advantage to escape endogenous tumor suppression mechanisms. In agreement with this, overexpression and aberrant nuclear localization of Nrf2 have also been described in a variety of squamous cell carcinomas (SCCs) located in tissues of the external surfaces of the body and the upper aerodigestive tracts, areas with a high exposure to oxygen and environmental pollution [143].

Nrf2/Keap1 mutations in other types of cancer

In addition to lung cancer, mutations in the Nrf2 and Keap1 genes have been found to be involved in the pathogenesis of a plethora of other solid tumors (Table 1). For instance, at the same time as the discovery of Keap1 mutations in NSCLC in 2006, a genetic profiling study identified a point mutation in the N-terminal domain of Keap1 in breast cancer cells [144]. Such mutation was later on described to reduce the efficiency of Nrf2 repression by decreasing Keap1's ability to induce Nrf2 ubiquitination [139].

Other somatic Keap1 mutations have been identified in gastric, colorectal, and hepatocellular carcinomas, prostate tumor [145,146], a small cohort of patients of gallbladder cancer [110], and ovarian cancer [114]. These mutations are generally heterozygous or present "two-hit" alterations (homozygous mutations with LOH) [110,145]. Furthermore, mutations were not found in patients with a lack of Keap1 protein expression [114], reinforcing the hypothesis that only one alteration is sufficient to deregulate Nrf2 signaling.

Mutations in the Nrf2 gene have also been found in head and neck cancer [111], larynx, esophagus, and skin SCC [143]. As well as in lung cancer, all mutations of the Nrf2 gene are clustered within or near the Neh2 domain [111,143].

It is noteworthy that, among the tumors with the highest prevalence of mutations, there are several in which exposure to tobacco smoke is correlated with the prevalence of the disease. Despite the fact that a significant correlation between smoking habits in patients and Nrf2/Keap1 mutations has not been established yet, available data strongly suggest a trend toward a higher frequency in smokers [111,142,143]. Considering the number of carcinogenic agents present in tobacco smoke, it is reasonable to think that some of the mutations found in those patients could have been induced by these chemicals. Moreover, there is recent evidence that cigarette smoke extract is able to induce Nrf2 activation by itself and promotes cell growth and survival [81,147]. On the other hand, mutations could have also preceded exposure to tobacco. Anyway, irrespective of their origin, Nrf2 mutations would have provided tumor cells a protective advantage against exposure to oxidative stress-inducers present in tobacco smoke [81], and this advantage could have eventually become detrimental and favored the evolution of the disease.

Aberrant Nrf2 expression or activation not related to mutations

Regulation of Nrf2 by oncogenic pathways

It is interesting to point out that mutations in either Nrf2 or Keap1 have been found only in certain solid tumors and there is no evidence so far that they occur in other cancers such as leukemias [145]. However, it has been demonstrated by us and others that Nrf2 also plays an important role in hematological diseases [103,119]. In acute myeloid leukemia (AML), increased NF-κB activity induces increased Nrf2 transcription and overexpression [148]. The proposed increase in Nrf2 mRNA levels as a mechanism of upregulation versus the traditional mechanism of decreased turnover is a quite novel concept [76] and might gain importance in the future. Highly expressed Nrf2 then seems to aberrantly localize in the nucleus in a "primed" status, which triggers a strong antioxidant response upon activation by an external stressor [148]. This model of increased Nrf2 pools in the nucleus agrees with an alternative model for Nrf2 activation, in which Nrf2 can direct its own translocation independent of Keap1 [9]. According to this model, "floating" Nrf2 proteins (the pool of Nrf2 not bound to Keap1) are ready-to-react probes, and their relative abundance determines the intensity of the antioxidant response.

Increased Nrf2 response confers AML cells resistance to chemical insults such as treatment with frontline drugs [148]. In a recent clinical trial, protein levels of Nrf2 and its target gene manganese superoxide dismutase (MnSOD or SOD-2) [149] have been found to increase in AML patients treated with a combination of cytarabine, idarubicin, and vorinostat or SAHA [150]. These results agree with the role of Nrf2 in chemoresistance; moreover, they suggest that not only "primed" Nrf2 is activated after the treatment but also new Nrf2 is expressed, pointing to the participation of this factor in a positive feedback loop [150]. Controversially, this same study showed a more favorable prognosis in those patients who suffered a bigger increase in Nrf2 levels. One possible explanation is that patients with preexisting high basal levels of Nrf2 would not experience a significant increase in the inducible expression of this gene after chemotherapy and therefore would have been classified in the group with no changes, masking the actual high basal Nrf2 activity. In agreement with this hypothesis, it was recently reported in breast cancer that only cells with low basal levels of Nrf2 undergo an increase in this factor after stimulation with a GSH depletor but conversely achieve a higher response to chemotherapy than those with more elevated basal levels, which remain resistant [121]. This is an interesting finding that should be confirmed in a bigger cohort of patients and studied in depth to avoid misleading conclusions after clinical studies involving Nrf2 activation.

Overall, data show that the disruption of Nrf2 regulation is possible without the presence of mutations affecting the Keap1/Nrf2 interaction, as has also been pointed out for some solid tumors [81,110,113,143,145,151]. For instance, it has been demonstrated that deficiencies in Keap1 are not enough to initiate cancer development *per se* [10], despite most of the mutations found in cancer being located in critical binding sites of Nrf2 to Keap1. This finding reinforces the relevance of the Keap1-independent model of Nrf2 activation [9] and draws attention to alternative Nrf2 regulation mechanisms.

Leukemias are not the only type of cancer in which the Nrf2 pathway has been found to be hyperactivated; constitutive activation of Nrf2 has been reported by many groups. Because of their role in the canonical activation of Nrf2 via acetylation of Keap1, high intracellular ROS levels and other stressors that chemically interact with Keap1 have been investigated as the main cause of Nrf2 hyperactivation. However, it has been demonstrated that elevated endogenous ROS levels are not necessary in tumorigenic Nrf2 activation. Thus, alternative regulation pathways involving oncogenic signaling have also started to gain relevance.

It was described long ago that some Nrf2 targets, such as NQO1, could be regulated by kinase pathways [152]. There is now accumulated evidence that the MAP kinases ERK and JNK [153–159], PI3K/Akt [96,159,160], and PKC [161,162] can activate the Nrf2-mediated antioxidant response, whereas p38 kinase [163,164], GSK-3β [165,166], and PTEN [167] can inhibit it.

Latest findings link the aberrant activation of Nrf2 with oncogenic deregulation of some of these kinase pathways. A recent study in lung cancer cell lines [81] reports that EGFR, which is a frequent activator of the ERK and PI3K/Akt pathways in cancer, activates Nrf2 under normal conditions (upon ligand activation) via the MEK1/2 pathway and mutations on EGFR that confer on it constitutive activation are able to induce a permanent Nrf2 activation in these cells.

Similarly, endogenous mutated K-Ras and B-Raf have been shown to activate expression of Nrf2 via MEK/ERK and Jun kinases, resulting in an augmented antioxidant and cytoprotective response and increased resistance of cancer cells to conventional treatments [76]. Moreover, this report suggests that Nrf2 participates in K-Ras-induced tumorigenesis and cell proliferation in pancreatic and lung cancers. The identification of this crosslink between Nrf2 regulation and aberrant MEK/ERK signaling is of an extremely high relevance in cancer and definitively assigns Nrf2 a proto-oncogenic nature.

As was mentioned before, the oncogenic fusion protein kinase Bcr/Abl is another possible activator of the Nrf2 antioxidant

signaling, because it has been reported to activate HO-1 expression in CML cells through a ROS-dependent mechanism [56,57].

Last, certain proteins with relevance to the pathogenesis of cancer have been shown to directly interact with Nrf2 and therefore participate in its regulation, such as Src3 [96] or the aforementioned E-cadherin, of which a loss in solid tumors might be another mechanism for Nrf2 hyperactivation [85].

Nrf2 in hormone-related cancers

The Nrf2 pathway is aberrantly activated in a number of cancers in which hormones play a fundamental role, such as breast [118,121,139,145], prostate [145,146], endometrial [120,168], and ovarian cancer [114,169]. In some cases, mutations in Keap1 have been found to account for the deregulation of Nrf2 [139,145], but genetic aberrations are not as frequent in hormone-related tumors as in other types of cancer.

Interestingly, it was recently reported that the hormones follicle-stimulating hormone, luteinizing hormone, estradiol (E2), and medroxy-progesterone acetate significantly upregulate Nrf2 in ovarian cancer cell lines [169]. Thus, it has been postulated that Nrf2 overexpression, induced by the characteristically augmented hormonal levels, might participate in the pathogenesis of ovarian epithelial carcinoma [169].

The activation of Nrf2 by E2 has also been described in the neuroblastoma cell line SH-SY5Y via PI3K/Akt [96], suggesting that hormonal regulation of the antioxidant defense could be a common mechanism also in other tissues. Provided that E2 plays a master role in the pathogenesis of breast cancer and induces oxidative stress [170], it seems reasonable to think that Nrf2 activity could increase in response to E2-induced ROS accumulation. Moreover, it is possible that Nrf2 participates in a positive feedback loop regulating response to E2, because it seems to play a regulatory role in the overexpression of the estrogen receptor β2 in ovarian cancer [122].

Despite studies showing that Nrf2 is either upregulated or hyperactivated in breast cancer MCF-7 cells [118,121], recent data show that treatment of rat breast cancer cells with E2 inhibits Nrf2 binding to the ARE localized in the promoter of SOD-3, the extracellular form of SOD, increasing oxidative DNA damage [171]. In contrast, treatment with antioxidant drugs significantly increased Nrf2 expression and nuclear translocation, enhancing Nrf2 activity on the SOD-3 promoter and increasing SOD-3 protein levels and enzymatic activity. According to these data, Nrf2-induced SOD-3 activity protects cells from oxidative stress and tumor formation and is inhibited by E2. This protective role of Nrf2 in breast cancer tumorigenesis agrees with an older study that postulated that several cancer cell lines, as well as 70% of breast cancer patient tissues studied ($n = 10$), have low Nrf2 expression levels due to overexpression of the ubiquitin-ligase Cul3 [172]. Interestingly, another work investigating Keap1 regulation by microRNAs (miRNAs) reported that E2 inhibits the expression of a Keap1-regulating miRNA, inducing an increase in Keap1 protein levels and, subsequently, a decrease in Nrf2 expression [173]. As of the date of this review, breast cancer is the only type of tumor in which Nrf2 regulation by miRNAs has been described.

In light of these data, it is possible that estrogen receptor-positive (ER^+) cells MCF-7 and ER^+ patient samples express low levels of Nrf2 due to increased Cul3 or to the loss of the regulatory axis miRNA/Keap1/Nrf2 during initiation of tumorigenesis. This fact would make cells more sensitive to oxidative stressors and may favor the progression of the disease.

It seems, therefore, that Nrf2 activation by E2 could participate in ovarian cancer tumorigenesis but, in contrast, Nrf2 downregulation might be important for the development of breast cancer. Nevertheless, there are aspects of the aforementioned studies that should be further explored. As an example, the same report showed that, in addition to inhibiting SOD-3 expression, treatment with E2 induces the expression of SOD-2, which could participate in tumorigenesis [171]. The involvement of Nrf2 in the regulation of the various forms of SOD in this type of cancer and the effects of SOD-2 expression should be clarified to determine the role that Nrf2 plays in breast cancer and its connection with hormone regulation. Likewise, expression levels of Nrf2 should be analyzed in a larger cohort of patients to confirm downregulation in breast cancer, and the relationship between Nrf2 levels and E2 responsiveness or the stage of the disease should be elucidated.

Nrf2 and autophagy

One of the Nrf2 target genes, P62 [174], encodes a protein that binds ubiquitin and LC3α kinase during autophagy, inducing aggregation of ubiquitin-positive proteins in cytosolic inclusions that are subsequently degraded [175]. Protein p62 is also a Nrf2 activator that directly interacts with the DC (Nrf2-interacting) domain of Keap1, leading to decreased Nrf2 ubiquitination and to its stabilization [176].

In autophagy-deficient mice, protein aggregates escape degradation and accumulate in the cytosol, along with high protein levels of p62 [177,178]. Accumulated p62 then competes with Nrf2 bound to Keap1 [176], causing a substantial induction in the expression of enzymes belonging to the Nrf2-controlled gene battery [176–178]. Strikingly, this activation of the Nrf2-dependent antioxidant response seems to be responsible for the liver injury observed in autophagy-deficient mice [176]. Interestingly, high p62 and/or $p62^+$-inclusions have been found to accumulate in hepatocellular carcinoma (HCC) cells [178–180], where autophagy is inhibited [181]. Moreover, mice deficient in autophagy-related protein 7, which is essential for autophagy, develop liver tumors that can be pathologically classified as hepatocellular adenomas, and this tumorigenesis seems to be associated with chronic inflammation, blockade of mitochondrial activity, and genomic instability [178].

Overall, data show that deficiencies in autophagy, and therefore p62 overexpression and Nrf2 activation, may participate in the development of hepatic tumors [182]. Despite growing lines of evidence suggesting that proto-oncogenic activity of p62 is responsible for most tumorigenic alterations [183–185], it seems reasonable that Nrf2 and/or its transcriptional targets are also involved in the tumorigenesis of autophagy-deficient cells. Indeed, a study in human HCC cell lines showed that p62-induced Nrf2 activation contributes to tumor growth [178,182]. Likewise, Nrf2 hyperactivation might participate in the development of other types of tumors in tissues with loss of autophagy. In these cases, autophagy induction might be beneficial to preventing abnormal cell proliferation; furthermore, it was recently reported that an mTOR inhibitor prevented Nrf2 activation through the inhibition of its nuclear translocation [186]. Thus, autophagy induction could be doubly positive thanks to inhibition of aberrant Nrf2 activation by p62.

Controversially, whereas numerous reports suggest a tumor suppressor role for autophagy, many others show the participation of this process in tumor growth and chemoresistance (reviewed in [187]). In these cases, tumors with increased levels of autophagy may benefit from treatment with autophagy inhibitors. Considering the potential role of Nrf2 activation in chemoresistance, caution is needed when using inhibitors that disrupt degradation of protein inclusions downstream of p62, because they may favor the accumulation of this protein and therefore activate a Nrf2-dependent antioxidant response. Moreover, Nrf2 constitutive activation has also been reported in renal cell carcinoma cell lines with high basal autophagy [186], probably owing to permanent p62 expression. Therefore, the status of Nrf2 signaling pathways should not be deduced from basal levels of autophagy in a certain type of cancer. Furthermore, an extensive

study of p62 and Nrf2 activation in NSCLC revealed that p62 expression does not necessarily correlate with Nrf2 expression levels [188], demonstrating that additional mechanisms, which might depend on the type of tissue, participate in the activation of this factor.

Epigenetic abnormalities and posttranslational regulation of Nrf2

In addition to aberrant activation of Nrf2 by oncogenic signaling and mutation-induced overexpression of this factor, epigenetic regulation needs to be taken into account. However, this field still remains mostly unexplored.

In one study aiming to gain insights into the low Keap1 expression found in certain lung cancer cell lines and a small cohort of patient tissues, an aberrantly hypermethylated CpG island was identified within the promoter region of the Keap1 gene [189]. Treatment with the hypomethylating agent 5′-azacytidine in those cell lines restored Keap1 expression levels to various extents, suggesting that the hypermethylation of CpG islands might be an important mechanism for Keap1 downregulation in lung cancer. A later study in a bigger cohort of NSCLC primary tumors confirmed these data and unveiled that aberrant hypermethylation of the Keap1 promoter is actually much more frequent than Keap1 gene mutations and LOH (47% versus 15 and 21%, respectively) [190] and could be the main alteration responsible for the reduced or absent Keap1 protein expression previously reported in NSCLC [113]. Remarkably, the combined study of genetic and epigenetic alterations in NSCLC also showed that they can coexist in the same tumor and, moreover, that the combination of two or more of them correlates with a poor prognosis [190]. These results evidence the importance of epigenetic regulation of Keap1 in lung cancer tumorigenesis.

A second study by the same group in malignant glioma patients confirmed hypermethylation of the Keap1 gene on the same CpG island described before, among others, and reported a consequent strong inverse correlation with Keap1 expression [191], indicating that aberrant methylation of the Keap1 promoter is a mechanism of deregulation that might also be common in other types of cancer. Unexpectedly, in this study methylation of the Keap1 promoter was statistically associated with a lower risk of progression of the disease but it needs to be clarified that this result was obtained in a cohort of patients with a hypermethylated MGMT (methylguanine DNA-methyltransferase) gene [191], which is generally a marker of good prognosis in malignant glioma [192].

It is worth mentioning that, despite aberrant Keap1 methylation and subsequent Nrf2 hyperactivation being significantly higher in tumor tissues than in normal samples, a recent study in colon cancer revealed that hypermethylation of the Keap1 promoter is also considerably high (25%) in healthy patients [193]. Unfortunately, no prognosis or drug-response studies were carried out in this report.

It is also interesting to note that Keap1 gene hypermethylation can coexist with other postranscriptional aberrations such as alternative splicing, which can lead to the expression of a truncated and nonfunctional form of Keap1 and thus contribute to deregulation of Nrf2 [146].

On the other hand, the Nrf2 gene was also reported to be hypermethylated in one study of transgenic adenocarcinoma of mouse prostate, resulting in an inhibited transcription and consequent Nrf2 downregulation [194]. Despite there being no evidence so far that Nrf2 is hypermethylated in human tumors, this option should not be completely ruled out and will need further study.

Taken together, these results demonstrate that Keap1/Nrf2 expression is also epigenetically controlled and therefore highlight the importance of investigating this type of regulation in cancer.

Histone acetyltransferases (HATs) are enzymes that generally act as epigenetic transcriptional coactivators but can also acetylate nonhistone proteins. HATs such as p300 and CBP have been reported to directly acetylate Nrf2 protein on its Neh1 and Neh3 C-terminal domains, respectively, in response to oxidative stress [195,196]. In both cases, Nrf2 protein acetylation results in the transcriptional activation of its target genes but the effects appear to be HAT-specific: p300 seems to bind Nrf2 in the nucleus, where they colocalize in the ARE [195], whereas CBP binds Nrf2 in the cytoplasm and induces both nuclear translocation and transcriptional activation [196]. Moreover, these two reports show that the pool of antioxidant genes that is expressed after Nrf2 activation is different depending on the activator HAT, providing an insight into how thinly this pathway is regulated.

Physiological positive regulation of Nrf2 by HAT is supported by the fact that endogenous histone deacetylases (HDACs) negatively control this process [196]. In agreement, there is evidence that HDACIs activate Nrf2 [119,126], although the mechanism has not been elucidated yet. One group recently suggested that HDACIs may control Nrf2 activation by directly inhibiting Keap1 expression [126]; however, they failed to detect Keap1 protein acetylation after treatment with an HDACI, whereas they did detect Nrf2 acetylation, which proves that activation of Nrf2 is acetylation mediated and indicates that the decrease in Keap1 expression is an indirect effect. The key might be the regulation of Keap1 expression by miRNAs studied in breast cancer and referred to earlier in this review [173]. Apparently, HDACIs can inhibit Keap1 via miRNA and induce Nrf2 expression, nuclear localization, and ARE binding. This miRNA inhibition would explain why Keap1 protein acetylation did not seem to be directly involved in its downregulation. Moreover, the specific blockade of miRNA activity could not completely reverse HDACI-induced Nrf2 activation [173], supporting the idea that other mechanisms, such as Nrf2 protein acetylation, collaborate with Keap1 inhibition. Further studies on this regulation would be needed to elucidate the precise mechanisms involved.

Overall, these data suggest that Nrf2 activity can also be finely regulated by posttranslational modifications including acetylation by HAT and inhibition by miRNAs. The involvement of the deregulation of HAT and miRNAs in many types of cancer is well documented; therefore, it is reasonable to think that aberrant acetylation or miRNA inhibition of Nrf2 could participate in its oncogenic activation. However, it is worth mentioning one report that describes a positive modulation of Nrf2 by human HDAC2 [197], suggesting that this regulation is more complicated than it seems.

Conclusions and perspectives

In this review, we have presented compelling evidence that the transcription factor Nrf2 can function as a proto-oncogene in plenty of solid tumors and leukemias.

Nrf2 can be activated by numerous compounds and is also frequently deregulated in a wide variety of cancers by mutations, aberrant epigenetic or posttranslational regulation, or hyperactivation of oncogenic signaling pathways involving other transcription factors such as NF-κB, various protein kinases, structural proteins such as E-cadherin, or other regulators such as p62.

Overexpressed or hyperactivated Nrf2 can participate in tumorigenesis by helping cells escape from diverse forms of stress through the induction of antioxidant target genes or by directly promoting cell survival, proliferation, and even metastasis. Moreover, we have shown that Nrf2 plays an important role in the chemoresistance of malignant tissues to several types of drugs, not only by protecting cells from the production of ROS or electrophiles, but also by preventing the intracellular accumulation of drugs and actively inhibiting apoptosis. The most important implication of this is the possible use of Keap1/Nrf2 and/or Nrf2 target genes as biomarkers and therapeutic targets in cancer.

Use of Nrf2 as a biological marker

In light of the prevalence of Keap1/Nrf2 mutations or hyperactivation in certain types of tumors, it seems reasonable to think that these signatures could be good biomarkers for the early detection or prognosis of cancer.

In agreement, a mutant Nrf2 signature has been suggested and validated as a prognostic marker in lung and head and neck cancer [80], whereas Keap1 mutations have been suggested as prediction tools of poor prognosis, high risk for metastasis and/or recurrence, and increased resistance to chemotherapy in NSCLC [142]. However, caution is needed because there are significant differences in the types of mutations found in lung cancer between studies that took place in Asia (Korea and Japan) [112,145] and those carried out in the United States [109], which indicates that etiologic divergences may exist between races or populations.

In this regard, it would also be interesting to study why there are tumors in which Keap1/Nrf2 mutations have not been found, such as pancreas cancer [110] or leukemias [145]. The high incidence of mutations in tumors located in tissues highly exposed to oxygen and air pollution or tobacco smoke is also an important characteristic worthy of further investigation.

Data presented in this review also suggest that Nrf2 is a biological marker of interest in cancers with positive or negative deregulation of autophagy and/or p62. Likewise, it may be interesting to determine the status of p62 in Nrf2-overexpressing tumors, because Nrf2 might activate p62 expression through a positive feedback loop [174] and this protein could enhance tumorigenesis, irrespective of autophagy levels. In support of this, a study of the clinical relevance of p62 and Nrf2 expression in NSCLC found that they are both independent indicators of a poor survival and their combined accumulation in a tumor is a marker of a significantly worse prognosis [188].

Overexpression of Nrf2 has also been suggested as a good diagnostic marker in endometrial serous carcinoma [168] and could be used to predict response to chemotherapy in a variety of other tumors [110,118,121,143,145,146,151,169]. Nevertheless, it needs to be taken into account that, in some types of cancer, Nrf2 upregulation [150] or absence of Keap1 [191] correlated with a better prognosis. In this case, the association occurs only in patients with a methylated MGMT gene, which is, to date, the main prediction marker for good prognosis and response to chemotherapy in malignant gliomas. However, further studies are required in this field to be able to validate the use of the upregulation of the Nrf2 pathway as a marker.

Similarly, some Nrf2 targets could be proposed as prognostic markers; however, the different expression patterns in different tissues should be taken into account to establish good criteria. That is, the use of a specific enzyme as a biomarker should be first validated. For example, HO-1 overexpression has been studied in brain, prostate, and kidney tumors [50–52] and a recent report validated AKR, in particular AKR1C1, as a highly specific molecular marker of Nrf2 activity in normal adult kidney and leukemia cells [198]. In this particular study, AKR expression in peripheral blood cells was suggested as a good marker of individual susceptibility to oxidative or electrophilic damage [198]. It needs to be clarified that such information would not reflect Nrf2 status at the tumor site, which could be altered by somatic mutations or aberrant expression related to oncogenic processes, as shown in this review. Therefore, it would be advisable that biomarker expression was directly determined in cancer cells for a guaranteed success.

Use of Nrf2 as a pharmacological target

The balance between health and disease is sometimes fragile and factors that previously protected homeostasis may favor growth and survival of malignant cells upon a change in the cellular context. Activation of Nrf2 by dietary compounds has been traditionally considered to help prevent cancer development, as was shown in a clinical trial concluded 20 years ago [199]. However, current findings indicate that the benefit of supplementation depends on the stage of carcinogenesis, preventing initiation of cancer in healthy cells at young age, whereas it may be harmful and support tumor growth of already initiated cells [200].

Thus, there is a thin line between chemoprevention and chemoresistance: whether cell survival is positive for the evolution of the pathology will be determined by the state of the cell at the moment of receiving the exogenous or endogenous insult. For this reason, there is currently a concern that sustained administration or high doses of chemopreventive agents could have detrimental side effects such as increased cell proliferation and, eventually, tumorigenesis [201].

In light of data presented in this review, caution is recommended when using chemopreventive agents that act through the activation of Nrf2 and its target genes. It would be advisable to analyze the cytogenetic background of the patient before starting any treatment and not use Nrf2-activating drugs in already initiated cancers or tumors in advanced stages.

In contrast, this work provides enough evidence that Nrf2 is a greatly interesting novel target for pharmacological inhibition of cancer. Efficient Nrf2 inhibition would favor cell growth arrest and sensitize cells to apoptosis [57,103,104,106,109,115–118], with promising results in combination with current frontline therapies. Moreover, Nrf2-dependent protection against chemotherapeutic agents is not specific to those that kill cancer cells by generating ROS or nitric oxide species [119,126,130,131], so many different types of chemotherapy may benefit from combination with Nrf2 inhibitors.

Importantly, knockdown experiments of Nrf2 in endometrial serous carcinoma, a highly resistant type of cancer, sensitized to chemotherapy only those cells with high basal levels of this protein, compared to their controls [120], suggesting that Nrf2 inhibition treatments would be harmless for noncancerous cells (expressing lower basal levels of Nrf2) and therefore allow safer and more specific combination therapies.

However, Nrf2 inhibition presents the limitation that there are no or few selective inhibitors for this factor. Nrf2-inhibitory drugs described so far are basically electrophiles with a nonspecific activity that have a high risk of inducing off-target toxic effects by binding cysteine residues in other proteins such as enzymes [31].

During a high-throughput screening performed in search of natural specific Nrf2 activators, one plant extract from the species *Brucea javanica* was identified as a Nrf2 inhibitor [202]. Brusatol, the active compound, was fractionated and it was demonstrated that this molecule is able to reversibly inhibit ARE-dependent gene expression by inducing Nrf2 degradation. Brusatol decreased intracellular GSH levels and sensitized cancer cell lines and xenografts to chemotherapy; however, it presents the disadvantage of increasing Nrf2 protein levels 2 h after withdrawal [202]. The potential consequences of this rebound effect should be further investigated to predict the results of *in vivo* drug elimination from the tumor site, because the experiments carried out in this report measured accumulated effects on tumor size only after repeated injections.

A more recent screening of a chemical library containing 8000 small molecules identified IM3829, a Nrf2 inhibitor that significantly reduced Nrf2 and HO-1 mRNA and protein expression and even prevented Nrf2 nuclear translocation upon activation by tBHQ and ionizing radiation [147]. IM3829 sensitized cells to apoptosis and, importantly, Nrf2 inhibition occurred independent of Keap1 status [147], which would enable the use of this inhibitor also in mutants or cancers not expressing Keap1. Despite the promising effects of this inhibitor in combination therapies, our group could not reproduce

these results in AML cell lines (results not published). More studies will be required to fully validate the effectiveness and selectivity of this molecule in different cancer types.

In addition to finding good and selective inhibitors, before using Nrf2 as a pharmacological target, it needs to be kept in mind that differences in basal levels of Nrf2 expression or activation even in cancers in advanced stages may be an important determinant of the response to Nrf2 inhibition, as occurs in breast cancer [172,173]. Additional caution would also be required in those cancers in which autophagy plays a significant role or that are being treated with autophagy modulators; as it was discussed before, the combined evaluation of the status of Nrf2 and p62 in those cases would be highly recommended for a successful therapy.

Last, Nrf2 may play additional roles in cancer different from cytoprotection or tumorigenesis that will need to be fully characterized before implementing inhibitory therapies. For example, it has been postulated that Nrf2 participates in vitamin D-induced differentiation of AML cells [203] and that Keap1 inhibits PPARγ-dependent differentiation [204]. Thus, Nrf2 inhibition could hamper the positive effects of differentiating agents frequently used in cancer therapies.

To summarize, Nrf2 is a pharmacological target of great potential in cancer but its therapeutic utility depends on the molecular and clinical context, the type of cancer and the stage of carcinogenesis being important factors to take into account, as well as the state of other pathways that may contribute to Nrf2 activation. It will be necessary therefore to determine which patients will benefit from Nrf2 inhibition before using it to optimize existing chemotherapy.

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