UNIVERSIDAD DE CANTABRIA

INSTITUTO DE BIOMEDICINA Y BIOTECNOLOGÍA DE CANTABRIA
DEPARTAMENTO DE BIOLOGÍA MOLECULAR DE LA FACULTAD DE MEDICINA

Expresión e interacciones Myc-p27 en leucemia linfocítica crónica y en diferenciación de células mieloides

TESIS DOCTORAL PRESENTADA POR
JUAN MANUEL CARABALLO OTERO PARA OPTAR
AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE CANTABRIA
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Javier León Serrano, Catedrático de Bioquímica y Biología Molecular de la Universidad de Cantabria

CERTIFICA:

Que el Ldo. D. Juan Manuel Caraballo Otero ha realizado bajo su dirección el presente trabajo titulado: **"Expresión e interacciones Myc-p27 en leucemia linfocítica crónica y en diferenciación de células mieloide" (Myc-p27 expression and interactions in chronic lymphocytic leukemia and in myeloid cells differentiation)** en el Departamento de Biología Molecular de la Universidad de Cantabria.

Considera que este trabajo reúne los requisitos de originalidad y calidad científica necesarios para su presentación como Memoria de Doctorado por el interesado, al objeto de poder optar al grado de Doctor por la Universidad de Cantabria.

Y para que conste y surta los efectos oportunos, expide y firma el presente certificado.

Santander, a 25 de Marzo de 2013

Fdo. Javier León Serrano

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Para mis padres

When you make the finding yourself - even if you´re the last person on Earth to see the light - you´ll never forget it

Carl Sagan

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Myc-p27 expression and interactions in chronic lymphocytic leukemia and in myeloid cell differentiation

Juan Manuel Caraballo Otero

I.- ABBREVIATIONS

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4-HT = 4-hydroxytamoxifen

µM = micromolar

º C = degree Celsius, also known as centigrade

Aa = amino acid

Ab = antibody

Ala (A) = Alanine

AraC = Arabinofuranosyl Cytodine, also known as Cytarabine or cytosine arabinoside

Annexin V-PE = Annexin V conjugated with PE

ATP = Adenosine triphosphate

BCR = immunoglobulin B cell receptor

bHLH-LZ = basic / helix-loop-helix / leucine zipper

BM = Bone Marrow

bp = base pair

BrdU = Bromodeoxyuridine (5-bromo-2'-deoxyuridine)

BSA = Bovine serum albúmina

CD = cluster of differentiation

CDI =. Cyclin-dependent kinase inhibitor domain

cDNA = complementary DNA

ChIP = Chromatin immunoprecipitation

Cip / Kip = Cdk interacting protein / kinase inhibitor protein

CKI = Cyclin-dependent kinase inhibitor

CLL = Chronic lymphocytic leukemia

CML = Chronic myelogenous leukemia

Ct = cross threshold

C-ter (Ct) = C-terminal region

Ctrol = Control

DAPI = 2´,6-diamidino-2-phenylindole

Del = deletion

DNA = Deoxyribonucleic acid

DTT = Dithiothreitol

DX = Doxycycline

ECL = Enhanced chemiluminescence
EDTA = Ethylenediaminetetraacetic acid
EGTA = Ethyleneglycoltetraacetic acid
E box = Enhancer box
FCS = fetal calf serum
Fig = Figure
FITC = Fluorescein isothiocyanate
FUSE = Far upstream element
G418 = Geneticin
GEF = Guanine nucleotide exchange factor
GFP = Green fluorescent protein
h = hour
H_2O_2 = Hydrogen peroxide
HCl = Hydrochloric acid
HDAC = Histone deacetylase
IB = Immunoblot
IF = immunofluorescence
Ig = immunoglobulin
IgG = immunoglobulin G
IgM = immunoglobulin M
IgV_H = immunoglobulin variable heavy chain
INK4 = Inhibitor of CDK4
Inr = initiator element
IP = immunoprecipitation
IRDye = infrared dyes
Kb = kilobase
kDa = kilodalton
LiCl = Lithium chloride
LNA = Locked nucleid acid
LPL = Lipoprotein lipase
Lymphocyte DT = Lymphocyte doubling time
LRR = Leucine-rich regions
M = Molar
MB = Myc box
MHC = major histocompatibility complex
Min = Minutes
miRNA (miR) = micro-RNA
mL = millilitres
mg = milligram
mm = millimetre

mM = millimolar
nM = nanomolar
mRNA = messenger RNA
MSC = bone marrow mesenchymal stroma cell
NaCl = Socium chloride
$NaHCO_3$ = Sodium bicarbonate
NaOH = Sodium hydroxide
NES = nuclear export signal
NLC = nurse-like cell
NLS = nuclear localization sequence
NP40 = Nonidet P-40 (octyl phenoxylpolyethoxylethanol)
N-ter (Nt) = N-terminal region
PBL = Peripheral blod lymphocytes
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PE = Phycoerythrin
PI =Propidium iodide
PLL = Prolymphocytic leukemia
PMSF = Phenylmethylsuphonyl fluoride
pRb = phospho-retinoblastome
pRS = pRetroSuper
qPCR = quantitative PCR
Rb = Retinoblastome
RNA = Ribonucleic acid
rRNA = ribosomal RNA
Rpm = revolutions per minute
RPMI = Roswel Park Memorial Institute medium
RPS14 = Ribosomal protein S14
RT = Reverse transcription
RT = Room temperature (20 ºC)
RS = Richter syndrome
SDS = sodium dodecyl sulfate
SDS-PAGE = SDS polyacrylamide gel electrophoresis
SEM = Standard error of the mean
Seq = sequencing
Ser (S) = serine
shRNA = short hairpin RNA or small hairpin RNA
T = tonsil
TBS-T = Tris Buffer Saline – Tween20
Thr (T) = Threonine

TPA = 12-O-tetradecanoylphorbol-12-acetate

TSS = Transcription start site

Tyr (Y) = Tyrosine

UTR = untraslated region

V = volt

w/v = weight / volume

Wt = Wild type

YFP = Yellow fluorescent protein

Zn^{2+} = Zinc ion

$ZnSO_4$ = Zinc sulfate

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1.- INTRODUCTION

1 INTRODUCTION

1.1 Cdks and cyclins expression in the cell cycle

The regulation of cell cycle progression is common to all eukaryotic cells and it can be summarized in four principles: First, activation of Cyclin-dependent kinases (Cdks) is driven by the sequential expression and association with cyclin subunits; second, the activity of one Cdk-cyclin sets up the conditions needed for the activation of the next; third, the destruction of cyclins ensures a unidirectional cell cycle; and fourth, the inhibition of assembled Cdk-cyclin complexes, either by phosphorylation or by the binding of inhibitory proteins, delays Cdk activation and slows cell cycle progression in adverse conditions.

Cdks proteins belong to a family of mammalian serine/threonine kinases, and act phosphorylating other proteins required to promote the cell through G_1, initiate S-phase and initiate mitosis (Malumbres and Barbacid, 2005). Its activation is driven predominantly by the periodic expression of the cyclin subunit through a highly coordinated process (Murray, 2004)

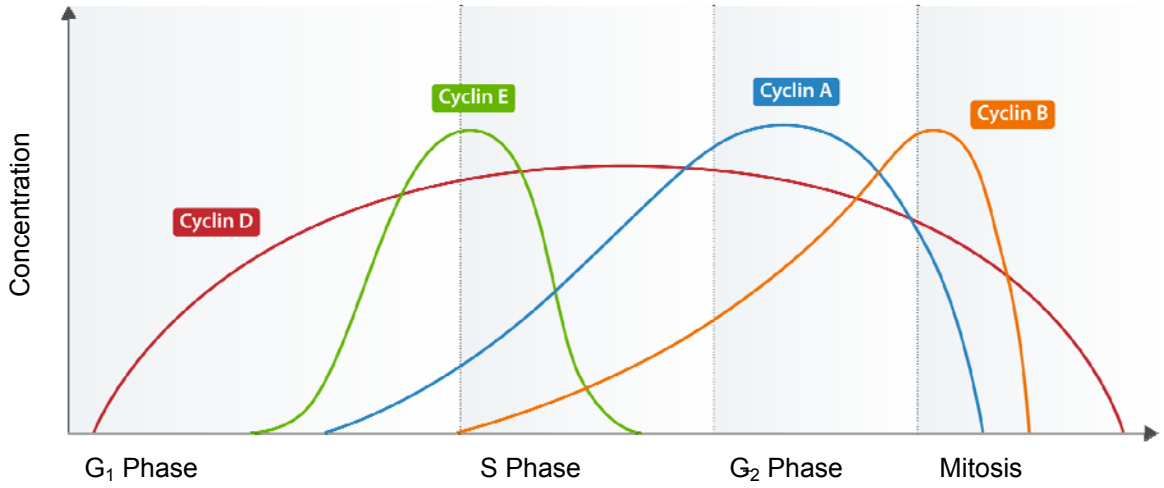


Figure 1.1.- Expression of different cyclins through the cell cycle progression (figure obtained from http://commons.wikimedia.org/wiki/File:Cyclinexpression_waehrend_Zellzyklus.png)

When resting (quiescent) cells are stimulated to enter the cell cycle by mitogenic signaling, D-type cyclins are the first cyclin induced (Fig. 1.1). D-type cyclins assembles with Cdk4 and Cdk6. These complexes are responsible for the phosphorylation of retinoblastoma protein family (Rb, p107 and p130) (Beijersbergen and Bernards, 1996; Xiao et al., 1996), which allows activation of the E2F family of transcription factors and promotes the transcription of

proteins required for G_1 and S-phase, including the E-type cyclins (Botz et al., 1996; Geng et al., 1996), which are thought to be requiered to activate Cdk2 (Resnitzky et al., 1994; Wimmel et al., 1994). Cyclin E-Cdk2 complex mediates the irreversible inactivation of Rb (Malumbres and Barbacid, 2005) and is required for the full activation of the S-phase. A-type cyclins are expressed soon after cyclin E at the G_1-S boundary (Fig. 1.1), mainly due to inactivation of pRb by Cdk2-cyclin E activity (Stevaux and Dyson, 2002), inactivation that also promotes transcription of B-type cyclins, although the syntesis of these cyclins are not evident until the G_2-M transcription (Fig. 1.1)**.** Cyclin A bind and active to Cdk2 and Cdk1 (Elledge et al., 1992; Pines and Hunter, 1990; Tsai et al., 1991) during S- and G_2-phase. In this last phase A-type cyclins are degraded whereas the B-type cyclins are actively synthesized (Fig. 1.1). As a consequence, Cdk1 binds to B-type cyclins, what is essential for triggering mitosis.

1.2 Cell cycle inhibitors CKI, Cip/Kip protein family

Regulation of the Cyclin/Cdk activity has an important role in the regulation of the cell cycle. One of the most important levels of regulation of these cyclin/Cdk complexes is provided by their binding to Cdk inhibitors (CKIs). In mammalians, two CKI gene families have been defined based on their evolutionary origins, structure and CDK specificities. One of them is the INK4 gene family. This family encodes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, all of which bind to Cdk4 and Cdk6 and inhibit their kinase activities by interfering with their association with D-type cyclins (Sherr and Roberts, 1999). Their ectopic expression causes cell cycle arrest in G_1 through block the formation of the Cdk-cyclin complexes by causing allosteric changes in the Cdk that propagate to the cyclin binding site (Pavletich, 1999), and the ATP binding site (Russo et al., 1998), which are distorted and whose affinity is significantly reduced. However, inhibition of cyclin D-dependent kinase activity is not sufficient to cause G_1 arrest. Inhibition of cyclin E-dependent kinases by Cip/Kip family is required in INK4-mediated growth suppression. This inhibition occurs via sequestration of Cdk4 by INK4 family, leaving Cip/Kip family free to associate increasingly with Cyclin E/Cdk2 complexes. (Jiang et al., 1998; McConnell et al., 1999)

The other family is the Cip/Kip (Cdk interacting protein / kinase inhibitor protein) protein family (Fig. 1.2), whose members are $p21^{Cip1/Waf1/Sdi1}$ (p21, encoded by CDKN1A gene) (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), $p27^{Kip1}$ (p27, encoded by CDKN1B gene) (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994) and $p57^{Kip2}$ (p57, encoded by CDKN1C gene) (Lee et al., 1995; Matsuoka et al., 1995). These CKIs share a conserved N-terminal domain termed “Cdk cyclin inhibitor domain” (CDI), that mediates binding to cyclin/Cdk complexes, modulating their activity (Hall et al., 1995; Sherr and Roberts, 1999). However, the remainder of their sequence is different (Fig. 1.2), suggesting that each of these proteins could have distinct functions and regulation. In fact, although it has described the importance of p21, p27 and p57 in different function restraining proliferation during development, differentiation and response to cellular stresses (Sherr and Roberts, 1999), each

one has specific biological functions that distinguish it from the other family members. Thus, different antiproliferative signals tend to cause elevated expression of only a subset of the Cip/Kip proteins. For example, p21 is an imporant transcriptional target of p53 and mediates DNA-damage induced cell-cycle arrest in G_1 and G_2 (el-Deiry et al., 1993; Gartel and Tyner, 1999), in contrast to p27, whose expression is usually elevated in mitogen-starved cells and other quiescent states (Besson et al., 2006; Coats et al., 1996). In other hand, p57 is the only CKI with an important role in the regulation of the cell cyle during embryonic development, as most mice lacking the CDKN1C gene have multiple developmental abonormalities and die at birth (Yan et al., 1997; Zhang et al., 1997).

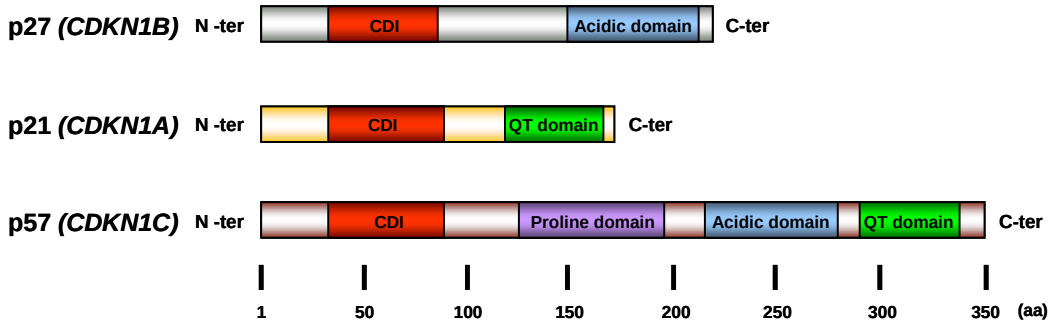


Figure 1.2.- Structural domains of the mammalian Cip/Kip inhibitors. Three members of the CIP/KIP protein family exhibit CDI in their amino-terminal region. Carboxi-terminal region is different in each of the members, although p57 shares homologue domain with p21 and p57 [derived from (Nakayama, 1998)]

1.3 $p27^{Kip1}$

$p27^{Kip1}$ (p27 hereafter) is a protein that in human is encoded by the the *CDKN1B* (Cyclin dependent kinase inhibitor 1b) gene. It is located on locus 12p13.1-p12 of the chromosome 12 (Fig. 1.3) (Bullrich et al., 1995; Pietenpol et al., 1995; Ponce-Castaneda et al., 1995). Identification of p27 was performed through two different avenues: as a Cdk4 interaction protein (Toyoshima and Hunter, 1994), and a gene carrying the CKI region (see below) similar to the other members of the Cip/Kip family, $p21^{Cip1/Waf1}$ and $p57^{Kip2}$ proteins (Polyak et al., 1994b).

1.3.1 p27 structure

The *CDKN1B* gene presents three exons, of which only the first and second are protein-coding (Fig 1.3) (Pietenpol et al., 1995; Polyak et al., 1994b). The sequence of human cDNA is a 90% homologous regarding mice sequence, and both have a similar exon-intron structure. The human p27 protein has 198 amino acids (Hengst and Reed, 1996) and its sequence might be divided into two major regions (Fig. 1.3). One is the amino terminal region where it is localized the domain CDI, which is highly conserved in the Cip/Kip protein family (Lee et al.,

1995) Through this domain, p27 binds to and prevents the activation of cyclin E-Cdk2 complexes.

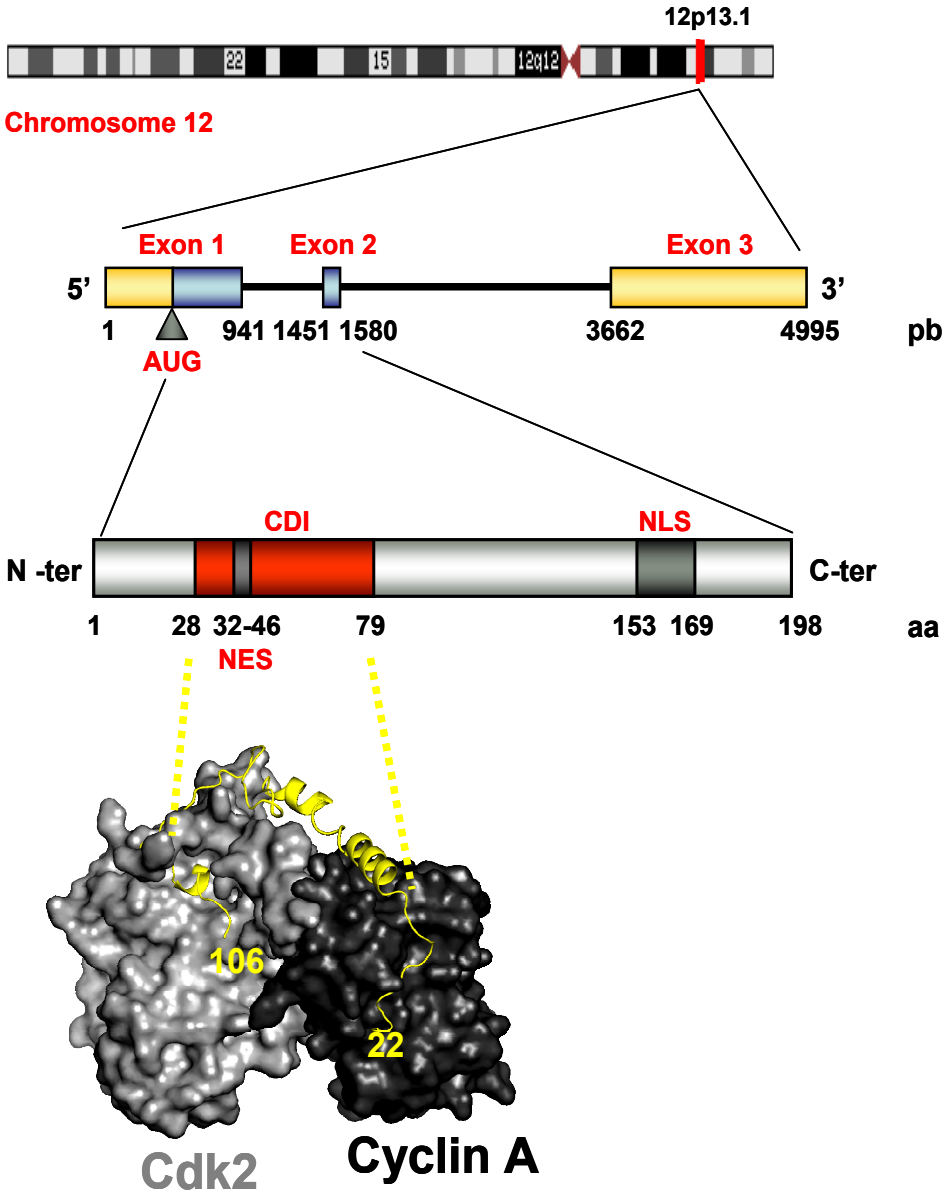


Figure 1.3.- p27 gene and p27 protein structure. In the amino terminal region it is localized the "Cdk cyclin inhibitor domain" (CDI), while the C-terminal region includes a putative nuclear export signal (NES) (Connor et al., 2003) and a bipartite nuclear localization signal (NLS 152-153/166-168) which is recognized by the alpha/beta importins, allowing p27 transport into the nucleus (Sekimoto et al., 2004; Zeng et al., 2000).

With the exception of CDI, p27 is intrinsically unstructured, adopting specific tertiary conformations only after binding to other proteins (Adkins and Lumb, 2002; Esteve et al., 2003; Lacy et al., 2004). In contrast, p27 CDI exhibits a propensity to form preexisting helical structure that corresponds to the R-helix, but not to the 3_{10} helix, that forms upon binding to cyclin A-Cdk2, and stabilization of the helix by alanine mutagenesis down-regulates significantly the rate at which the CKI inhibits cyclin A-Cdk2 complexes (Bienkiewicz et al., 2002), showing the

importance of quaternary interactions in the folding and binding events of p27 and suggesting that a biological advantage of the CKI intrinsic disorder is the speed and flexibility in the molecular identification events (Bienkiewicz et al., 2002; Wright and Dyson, 1999). This conformation may explain why p27 is capable of bind to a wide diversity of proteins.

1.3.2 p27 functions

p27 was initially characterized as inhibitors of all cyclin-Cdk complexes (Polyak et al., 1994a; Slingerland et al., 1994), albeit displaying lower affinity towards cyclin B-Cdk1 (Toyoshima and Hunter, 1994). Nevertheless, in recent years p27 has been involved in other biological functions, such as differentiation, apoptosis and cell migration.

1.3.2.1 Cell cycle regulation induced by p27

Targeted disruption of the murine p27 gene caused a gene dose-dependent increases in animal size. Growth is attributed to an increase in cell number (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), indicating that one of the most important functions of p27 is the cell cycle control. In fact, p27 must be removed of cyclin A/Cdk2 complexes or cell will not be able to progress of the G_1 phase (Nguyen et al., 1999; Pagano et al., 1995; Polyak et al., 1994b; Sherr and Roberts, 1999; Slingerland et al., 1994; Vlach et al., 1997).

The crystal structure of the N-terminal cyclin and Cdk-binding domains of p27 (aa 22-106) bound to cyclin A-Cdk2 revealed that the CKI occludes a groove formed by conserved cyclin box residues on the cyclin subunit and inserts itself in the catalytic cleft of the Cdk (Fig. 1.4), mimicking ATP and preventing ATP loading and catalytic activity (Russo et al., 1996). Thereby, the inhibition of ATP loading blocks activation of the cyclin E/Cdk2 complex, and prevents progression through the cell cycle. However, as detailed below, p27 ability to bind to and inhibit cyclin E/Cdk2 is greatly decreased when is phosphorylated on Tyr-88 (Chu et al., 2007; Grimmler et al., 2007). Moreover, cyclin E-Cdk2 phosphorylates p27 on the Thr-187, what converts p27 in a target for ubiquitilation and degradation by the proteosome (Nguyen et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997).These data indicates that the regulation of cyclin E/Cdk2 complexes is more complicated and it is divided into different levels.

p27 also binds to cyclin D/Cdk4-6 (Polyak et al., 1994b; Toyoshima and Hunter, 1994), but the biochemical results of the interaction of these trimeric complexes is less clear. p27 participates in the assembly of catalytically active cyclin D/Cdk4-6 complexes (Cheng et al., 1999a; LaBaer et al., 1997). Therefore, Cip/Kip proteins could not only promote cyclin D-dependent events, but the sequestration of CKIs into cyclin D/Cdk4-6 complexes could allow the downstream activation of cyclin E/Cdk2 (Cheng et al., 1998; Ladha et al., 1998; Perez-Roger et al., 1999; Reynisdottir et al., 1995) Moreover, cyclin D1 and D2 knockout display a

reduced Cdk2 kinase activity, which is likely due to the increased availability of Cip/Kip proteins to bind to Cdk2 (Geng et al., 2001; Ladha et al., 1998; Perez-Roger et al., 1999) However, this function as essential factor for the formation of Cyclin D/Cdk4-6 complexes is questionable (Bagui et al., 2003; Sugimoto et al., 2002). In vitro experimental studies have shown that Cyclin D/Cdk4-6 complexes are inhibited by stoichiometric concentrations of p27 higher than those needed to inhibit Cyclin A/Cdk2 (Blain et al., 1997). Moreover, further studies showed that Cyclin D/Cdk4-6 complexes are in fact inactive, while the kinase activity resides in the free complexes of p27 (Bagui et al., 2003; Obaya et al., 2002).

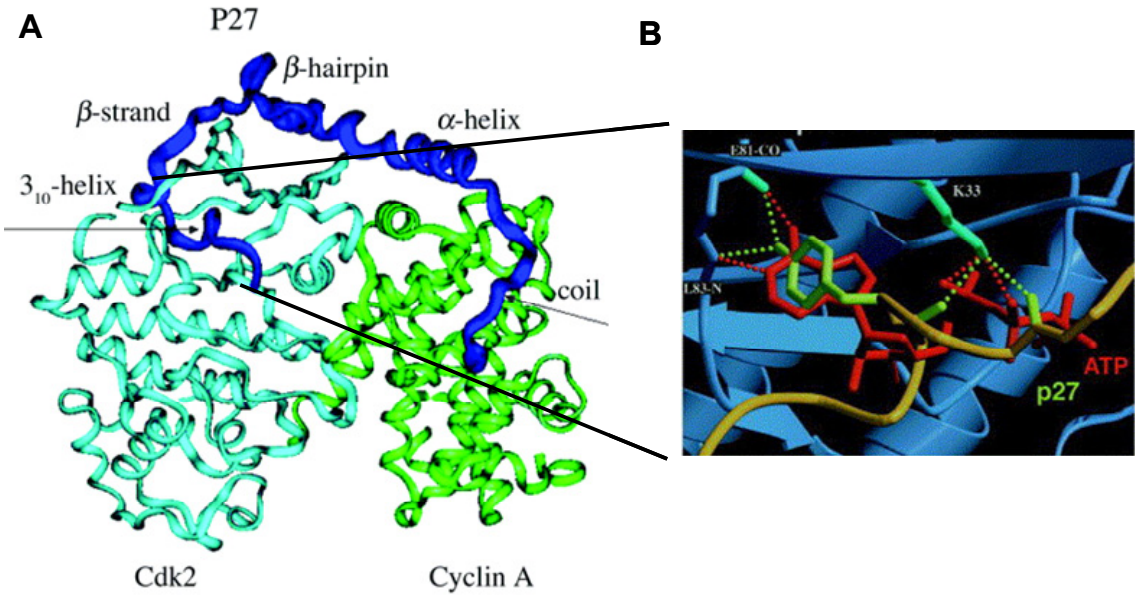


Figure 1.4.- p27 inhibits the Cdk-cyclin complex by changing the shape of the catalytic cleft and by inserting a helix inside the ATP binding site. (a) Protein structure of p27 N-terminal region interacting with Cdk2 and Cyclin A. (from www.cisreg.ca) (b) A close-up view from the superposition of the ATP-Cdk2-cyclinA and p27-Cdk2-cyclinA complexes showing the coincidence in the positions and contacts of the Tyr88 side-chain from p27 and the ATP purine group. Hydrogen bonds are indicated by dotted lines. Side-by-side comparison of the p27-bound N lobe of Cdk2 with the ATP-bound N lobe. Hydrophobic residues involved in the hydrophobic core of the N lobe in the two complexes are shown. (From Pavletich, 1999)

1.3.2.2 Regulation of the cell differentiation by p27

During differentiation process, the cell begins to express genes involved in tissue-specific differentiation. It has been proposed that high expression of these tissue-specific genes required to perform its function is incompatible with the transcription general block that occurs during mitosis. Therefore, a cell usually undergo two parallel processes to differentiate: the output of the progression of cell cycle and entry into G_0 state, and activation of transcription factors that induce the expression of tissue-specific genes (Fig 1.5).

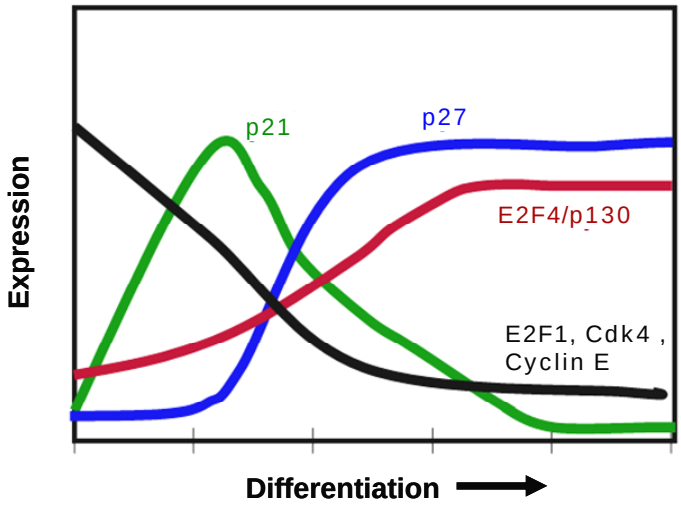


Figure 1.5.- Expression of cell cycle inductors (E2F1, Cdk4, Cyclin E) and cell cycle inhibitors (p21, p27, E2F4, p130) in enterocyte, keranitocyte, adypocyte, myocytes and myeloid differentiation.

In response to this assumption, it has been observed the induction of p27 in a variety of cells and models during their differenciation. Thus, intestinal cells (Tian and Quaroni, 1999; Yamamoto et al., 1999), myeloid cells (Yaroslavskiy et al., 1999), neuronal cells (Nguyen et al., 2006) have shown an increase of p27 expression during their differentiation. It is unknown whether this increase is necessary for the differentiation or occurs as a results of the process itself, but several studies overexpressing p27 have shown that the first assumption is probably correct (Munoz-Alonso et al., 2005; Yamamoto et al., 1999).

p27 is up-regulated in several processes of hematopoietic differentiation (erythroid, myeloid and megakaryocytic) induced by many drugs (Aoki et al., 2004; Gomez-Casares et al., 2012; Munoz-Alonso et al., 2005; Steinman, 2002). Thereby, induction of p27 increase the differentiation of cell lines to monocytic or erythroid pathways (Munoz-Alonso et al., 2005; Zhou et al., 1999) although this increase does not occur in all models of differentiation, even though p27 induced cell cycle inhibition (Drexler and Pebler, 2003; Matushansky et al., 2000a; Tamir et al., 2000). p27 knock-out mice showed that the absence of p27 selectively enhanced proliferation of hematopoietic progenitor cells, but exerts little effects in the terminal hematopoiteic differentiation (Fero et al., 1996). Thus, these mice exhibited a defect in proliferative capacity but not in the terminal differentiation regulation. Subsequent studies in the hematopoietic progenitor cells demonstrated that p27 has an important role in the inhibition of the clonal expansion of the progenitor cells (Cheng et al., 2000).

1.3.2.3 Apoptosis and cell motility regulation by p27

It has been demostrated that apoptosis regulation may be affected by p27 levels, although how p27 modulates this process is unknown. In several cancer cell lines, p27 overexpression facilitates the apoptosis process (An et al., 1998; Drexler and Pebler, 2003; Katayose et al., 1997; Nickeleit et al., 2008). In opposition, forced expression of p27 enhances

the survival of serum-deprived p27 null fibroblast (Hiromura et al., 1999) and it has been shown that the effect of some cytotoxic drugs is reduced when p27 is overexpressed (Dimanche-Boitrel et al., 1998; Eymin and Brambilla, 2004; Gomez-Casares et al., 2012; Le et al., 2010; Yang et al., 2000) Morevoer p27 plays a protective role in safeguarding normal tissues from excessive apoptosis during inflammatory injury (Ophascharoensuk et al., 1998). Therefore, p27 seems to play both the role of apoptosis-promoting and –preventing factor depending on the model. In fact, it has been demostrated that caspase 3 cleaves p27 (Levkau et al., 1998) and that p27 regulates procaspase 3 activation (Levkau et al., 1998; Nickeleit et al., 2008). Although these findings lend support to a cell-cycle –indepedent function of p27 in apoptosis, most of the studies did not explore whether apoptotic regulation mediated by p27 was linked to Cdk regulation. In fact, it is possible that caspasa-mediated cleavage of p27 upregulate Cdk2 activity, thereby enhancing the apoptotic program (Levkau et al., 1998).

Finally, p27 has also been involved in the induction of autophagy. In this case, the energy sensing LKB1-AMP-activated protein kinase pathway appears to regulate p27 phosphorylation and stability, mediating the decision to enter autophagy or to undergo apoptosis (Liang et al., 2007). This may contribute to tumor-cell survival under conditions of growth factor deprivation, disrupted nutrient and energy metabolism, or during stress of chemotherapy.

p27 localization is regulated by different kinases through phosphorylation in several aminoacids (see below). When p27 is located in the cytoplasm, it is able to control cell motility through interaction with extracellular matrix by a mechanims whereby p27 bind to RhoA and interfering with RhoA binding to its GEFs and preventing its activation (Besson et al., 2004) (Larrea et al., 2009), which might lead to an increase in stress fibers stabilization and focal adhesions formation, inducing cell motility.

In contrast to the above reported findings, there are several studies demonstrating that cytosolic p27 inhibits cell motility. The effect has been observed in vascular smooth muscle cells (Diez-Juan and Andres, 2003; Sun et al., 2001), umbilical vein endothelial cells (Goukassian et al., 2001), oral cancer cells (Diez-Juan and Andres, 2003; Supriatno et al., 2003) and glioblastoma cell (Schiappacassi et al., 2008).

1.3.2.4 Transcriptional regulation by p27

p27 is able to regulate transcription of a set of genes through its activity as Cdk/Cyclin repressor. p27 prevents the phosphorylation of Rb-family proteins (Rb, p107 and p130), and Rb hypophosphorylated binds to and sequesters E2F family, thereby inhibiting their trasncription factor function and as result E2F gen targets are repressed (Sherr and Roberts, 1999). In addition, p27 has been implicated in transcripitonal regulation indepedent of its function as cell cycle inhibitor. Neurogenin-2 promotes the differentiation of neuronal progenitors in cortex, and

its stabilization is mediated by p27. p27 protects Neurogenin-2 from degradation and thus promoting the transactivation of neuronal genes (Nguyen et al., 2006).

Recently have been found that p27 has also a role as a transcriptional repressor, which is independent of cyclin-Cdk regulation. This function is carried out through a direct interaction with p130/E2F4. In this context p130 first drives E2F4 to the promoters, then p27 is subsequently loaded by directly interacting by its carboxyl domain with both p130 and E2F4. The presence of p27 on the promoter is required for the subsequent recruitment of HDACs and mSIN3A, whose repressor activity results in repression of transcription (Pippa et al., 2012).

1.3.3 Regulation of p27 expression

Although mRNA regulation has been described in some instances, p27 is mainly regulated by posttranslational modifications. Stability of the protein is critical for the regulation of p27 levels in the cell, and phosphorylation of p27 may induce its degradation through ubiquitin-protosome mechanism.

1.3.3.1 p27 mRNA regulation

Some studies have described a transcriptional regulation of p27. p27 promotor presents different binding sites which can be recognized by several transcripition factors, such as Sp1, CRE, Myb, NFkB and forkhead family (Philipp-Staheli et al., 2001). In particular, forkhead transcription factor family are the most important regulators of p27 transcription. An example is AFX, a member of forkhead family, which induces cell cycle inhibition through an increase on p27 transcription (Medema et al., 2000).

Several models have shown that p27 mRNA translation is also regulated (Agrawal et al., 1996; Hengst and Reed, 1996; Millard et al., 2000). mRNA translation depends on its 5´ untranslated region (5´ UTR), where a sequence rich in uracile is recognized by HuR (ELAV) and hnRNP C1/C2 proteins, regulating the translation of p27 mRNA (Millard et al., 2000). Moreover, it has been described that p27 mRNA can escape the general repression of translational initiation regulated by the recognition of the 5´cap by eukaryotic intiation factor 4E (eIF4E) (Miskimins et al., 2001). On the other hand, two micro-RNAs (miR-221 and miR-222) have been identified as potent regulators of the p27 expression. Two target sites for both micro-RNAs in the 3' untranslated region of p27 mRNA have been found, and their ectopic overexpression directly results in p27 down-regulation. Moreover, when miR-221 and miR-222 are down-regulated through antisense LNA oligonucleotides, levels of p27 are increased in the cell. (Galardi et al., 2007; le Sage et al., 2007b; Visone et al., 2007). Interestingly, high levels of both micro-RNAs are required by certain cancer cell lines to maintain low p27 levels and continuous proliferation, and therefore, their overexpression appears in several tumors, such as

glioblastoma, prostata, thyroid, melanoma, breast, oral and gastric cancers (Felicetti et al., 2008; Galardi et al., 2007; Kim et al., 2009; le Sage et al., 2007a; le Sage et al., 2007b; Miller et al., 2008; Visone et al., 2007; Yang et al., 2011)

1.3.3.2 Phosphorylation of p27

Phosphorylation is one of the most important mechanism through which p27 is regulated [reviewed in (Besson et al., 2008; Vervoorts and Luscher, 2008)]. The conformational flexibility of p27 suggests that phosphorylation events and protein-protein interactions may modify the folding of the CKIs, thereby modulating their ability to inhibit cyclin-Cdk complexes. In fact, the p27 protein possesses multiple tyrosine, serine, or threonine phosphorylation sites. The most important sites are summarized in the Fig. 1.6 along with their effector kinases and their functions. The phosphorylation status of Ser-10 and Thr-187 are studied are tested in this work, and their relevance is detailed below.

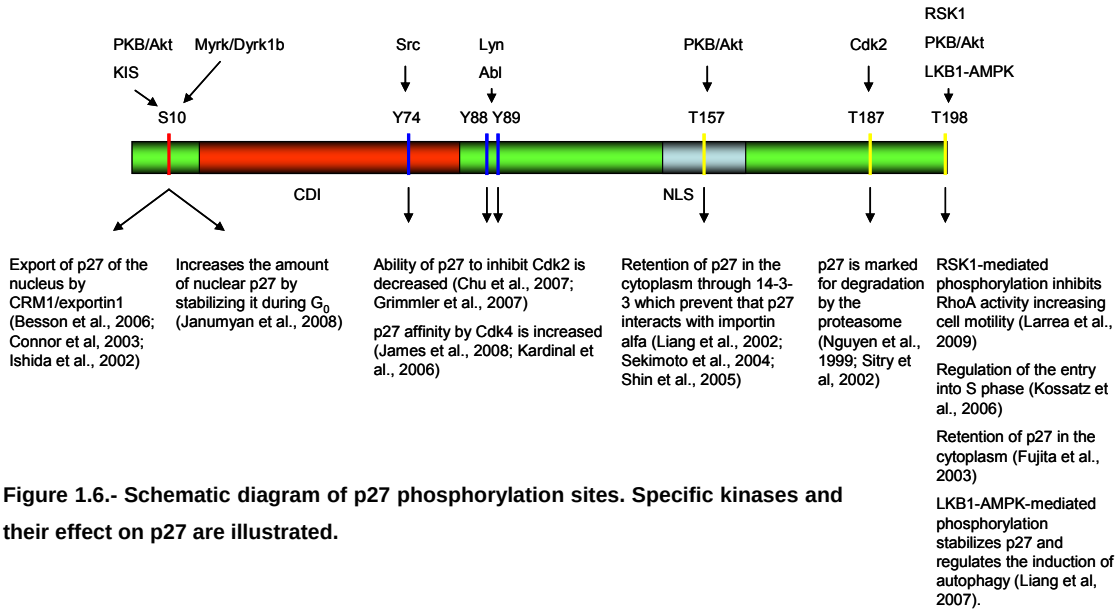


Figure 1.6.- Schematic diagram of p27 phosphorylation sites. Specific kinases and their effect on p27 are illustrated.

Phosphorylation on Ser-10 results in the export of p27 from the nucleus in G1 phase by providing a binding site for CRM1/exportin1 (Besson et al., 2006; Connor et al., 2003; Ishida et al., 2002; Rodier et al., 2001). Thereby, in proliferating cells, stathmin (KIS), PKB/Akt and Erk2 phosphorylate Ser-10 (Boehm et al., 2002; Fujita et al., 2002; Ishida et al., 2000) and providing the export of the nucleus of p27 [reviewed in (Besson et al., 2004; Besson et al., 2008; Besson et al., 2006)], although a recent report proposes that phosphorylation of p27 in Ser-10 is required for efficient C-terminal phosphorylation of its own by Pim and Rock kinases and critically controls the potency of p27 as a Cdk2 inhibitor (Mohanty et al., 2012). On the other hand, in quiescent cells it has been proposed that Mirk/Dyrk phosphorylate Ser-10, but in this case Mirk/Dyrk increases the amount of nuclear p27 by stabilizing it during G_0 (Deng et al., 2004; Ishida et al., 2000; Janumyan et al., 2008; Kotake et al., 2005; Mercer et al., 2005). On

the other hand, cyclin E-Cdk2 also phosphorylates p27 on the Thr-187. This converts p27 to a form that is recognized by an ubiquitin ligase and is targeted for destruction by the proteosome (see below) (Nguyen et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997). However, p27-bound Cdk2 is catalytically inactive due to p27-mediated remodeling of the catalytic cleft and displacement of ATP (Pavletich, 1999). Although this suggested that ubiquitilation by the SCF^{Skp2} complex (see below) may require p27-free cyclin E/Cdk2, it has been observed that Tyr-88 phosphorylated p27 is a poor inhibitor and the associated kinase retained significant catalytic activity (Chu et al., 2007; Grimmler et al., 2007). Futhermore, P-Tyr-88 p27 became an efficient substrate for phosphorylation on Thr-187 by Cdk2 within the trimeric complex, triggering SCF-Skp2 dependent p27 degradation in the absence of free cyclin/Cdk2. Thus, activation of cyclin E-Cdk2 triggers the destruction of its negative regulator.

1.3.3.3 p27 degradation

p27 levels are mostly regulated at the protein level by controlling its degradation. Thus, p27 degradation is critical in the cell cycle progression. p27 is abundant in quiescent G_0 cells, which is able to bind Cdk/cyclin complexes and inhibit their activity. Different mitogenic signals are able to induce cell cycle progression, but for the correct progression of the cell cycle it is necessary a decrease in levels of p27, which allows cyclin E-Cdk2 activation and progression of G_1 to S. In fact, p27 becomes unstable as cells progress toward S phase (Hengst and Reed, 1996; Pagano et al., 1995). Broadly, the expression of p27 is usually poorly regulated (Hengst and Reed, 1996; Pagano et al., 1995). Thus, the levels of p27 in cells are mainly regulated through its degradation.

p27 is degraded by proteosomes upon ubiquitilation by different ubiquitin ligases: Skp2, Kpc and PirH2, being Skp2 the most important (Kamura et al., 2004; Kossatz et al., 2004; Miranda-Carboni et al., 2008; Nakayama et al., 2004). Kpc (formed by two subunits, Kpc1 and Kpc2) is the earlier system that acts in the degradation of p27, given that it takes place in G_0-G_1 transition. When cell is stimulated by mitotic signals, phosphorylation of p27 in Ser-10 (see above) triggers the export of p27 from the nucleus into the cytoplasm by a CRM1/exportin 1 (Besson et al., 2006; Connor et al., 2003; Ishida et al., 2002; Rodier et al., 2001). Upon transport into cytosol, Kpc (Kip1 ubiquitination-promoting complex) poly-ubiquitinates p27 and as results p27 is degradated in G_1 (Fig. 1.7) (Kamura et al., 2004; Kotoshiba et al., 2005).

The second of these mechanisms occurs in the transition from G_1 to S phase, moment in which p27 protein levels significantly decrease (Hengst and Reed, 1996; Pagano et al., 1995). Alteration of the protein stability depends on the phophorylation of Thr-187 (see above) by Cdk2 (Nguyen et al., 1999; Sheaff et al., 1997; Vlach et al., 1997). Phosphorylated p27 creates in the C-terminal region a binding site for Skp2 (Carrano et al., 1999; Montagnoli et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999), a member of the SCF^{SKP2} (Skp-Cullin-F-Box) E3 ubiquitin ligase complex [reviewed in (Nakayama and Nakayama, 2006)]. The result of

this interaction is poly-ubiquitination of p27 and its subsequent proteasomal degradation (Fig. 1.7). The importance of Thr-187 phosphorylation was demonstrated by generating mice that express p27-T187A. This mutant was stable during S and G_2 phases of the cell cycle (Malek et al., 2001), although cell cycle progression was not blocked. Likely, p27 levels in late G_1 are sufficiently low for the activity of Kpc, in addition with phosphorylation in tyrosine residues (see above) of p27 remaining, preventing its bind with Cdk2/cyclin E-A complexes. The knockout of Skp2 resutls in mice that are smaller with a generalized hypoplasia (Nakayama et al., 2000). Interestingly, this is the opposite of the phenotype observed in p27 knockout mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), and the phenotype of $Skp2^{-/-}$ mice and cells is resued by the concominant knock-out of the p27 showed that the phenotype of Skp2 knockout mice is the result of the increased p27 levels (Nakayama et al., 2004)

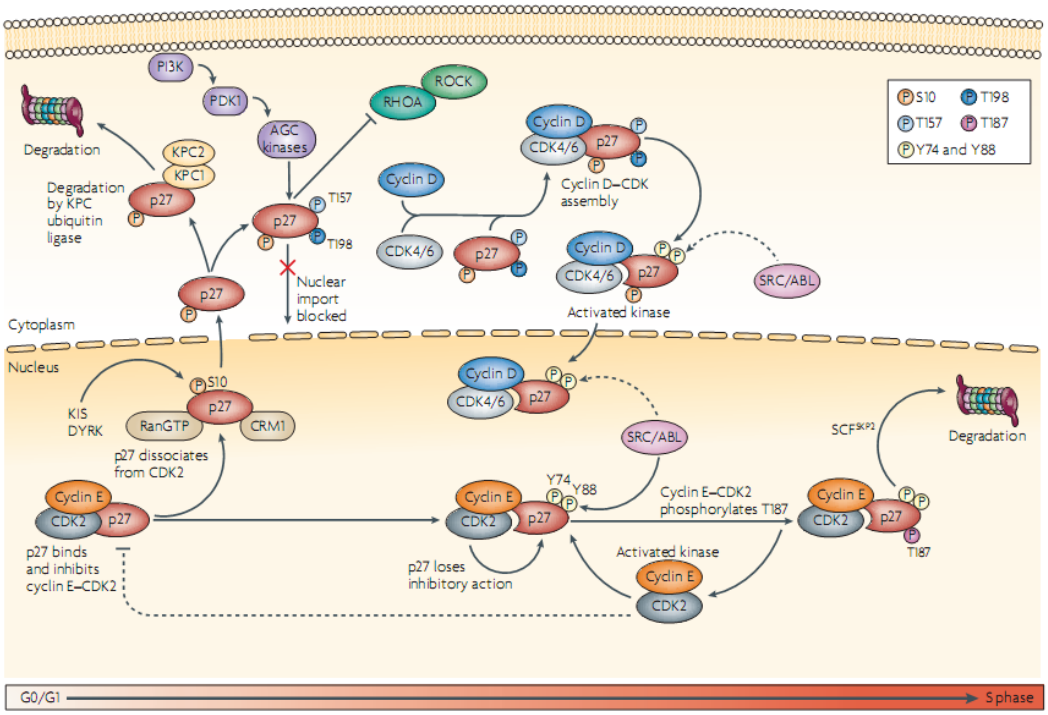


Figure 1.7. Degradation pathways of p27. Kpc acts in early G1 in the cytoplasm. p27 is exported of the nucleus in this phase through phosphorylation in the Ser-10 by KIS and/or PKB/Akt. In transition from G1 to S p27 is phosphorylated in the Thr-187 by Cdk2 and it is recognized by Skp2, a member of the SCF^{Skp2} ubiquitin ligase complex which mediates its degradation via proteosame (from Chu et al., 2008).

Finally, PirH2, other RING-type ubiquitin ligase, also has been identified as a p27-interacting protein, promoting the ubiquitylation and destabilization of p27. Its reduction results in an impairment of p27 degradation (Hattori et al., 2007), while its overexpression have been inversely correlated with p27 and signficantly associated with the poor prognosis of these patients (Shimada et al., 2009).

Other ubiquitin-independent mechanism of degradation has been described for p27. Mutagenesis experiments showed that N-terminal region of p27 can be processed, by a ATP-

dependent mechanism (Shirane et al., 1999). This intermediate had not CDI and it is not able to bind to Cdk2/Cyclin E complexes.

1.3.4 p27 and cancer

The principal function of p27 is the inhibition of cyclin/Cdk complexes, suggesting a role as tumor suppresor. Indeed, many tumors show a marked correlation between reduced p27 levels and poor prognosis, and a reduction in the abundance of p27 is common in many types of human malignancies (Fig. 1.8) (Bloom and Pagano, 2003; Borriello et al., 2011; Chu et al., 2008; Eymin and Brambilla, 2004; Sicinski et al., 2007). However, the available evidence indicates that p27 does not behave as a classical tumor suppressor. In addition to tumor suppressor activities, p27 is involved in the regulation of cellular process such as cell-cycle regulation, apoptosis or cell migration, which may be oncogenic under certain circunstances. The loss or subversion of the regulatory mechanisms governing p27 may lead to the specific loss of the tumor suppressor function while maintaining the oncogenic ones. As a consequence, p27 is regulated in a complex manner to accommodate both anti- and pro-tumorigenic activities (Chu et al., 2008; Kaldis, 2007; Sicinski et al., 2007)

p27 knock-out mice showed hyperplasia in multiple organs, develop adenomas of the intermediate lobe of the pituitary gland and demonstrate and increased susceptibility to tumor formation induced by chemical carcinogens and irradation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Heterozygous mice ($p27^{+/-}$), where the wild-type allele of p27 is retained, are more susceptible to tumor development compared to wild type or, in some cases, to knock-out mouse, leading to the suggestion that p27 is a haploinsufficient tumor suppressor (Fero et al., 1998; Gao et al., 2004; Muraoka et al., 2002).

The analysis of human tumors showed that p27 is not a classical tumor suppressor since both alleles are rarely inactivated (Philipp-Staheli et al., 2001). Thus, although different mutations of the p27 gene have been found recently (Barbieri et al., 2012), it is still considered that the inactivation of p27 is due to posttranscriptional mechanisms (see above) that result in down-regulation and/or aberrant location of the p27 protein and/or its sequestration into cyclin/Cdk complexes (Chu et al., 2008; Sicinski et al., 2007). In fact, in breast cancer low levels of p27 is associated with poor clinical outcome (Catzavelos et al., 1997; Porter et al., 1997; Tan et al., 1997) and more recently, similar conclusions have been reached for a number of additional human tumors including lung, colon, ovary, esophagus, glioma, lymphoma, bladder, hepatoma, thyroid and prostate [reviewed in (Besson et al., 2008; Chu et al., 2008; Philipp-Staheli et al., 2001)]. Moreover, the tumor phenotype induced by the loss of other tumor suppresor genes, such as Rb, PTEN (phosphatase and tensin homolog deleted on chromosome ten), $p16^{INK4a}$ or APC (Adenomatous proliposis coli), are enhanced by the simultaneous loss of p27 (Di Cristofano et al., 2001; Franklin et al., 2000; Malumbres et al., 2000; Martin-Caballero et al., 2004; Park et al., 1999).

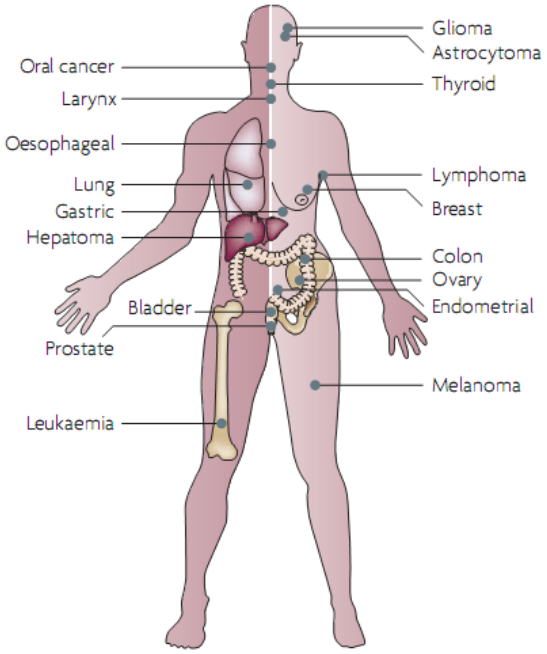


Figure 1.8. Malignancies when p27 protein expression is significantly reduced with respect the healthy tissue (from Chu et al., 2008)

In most human tumors, the loss of p27 results from reduced degradation. In fact, an inverse correlation between reduced p27 and increased Skp2 levels has been observed in many of studies. (Chiarle et al., 2002; Gstaiger et al., 2001; Hershko et al., 2001; Kudo et al., 2001; Latres et al., 2001) However, p27-T187A is down-regulated in lung tumors despite its degradation by Skp2 is inhibited, as the phosphorylation in Thr-187 is blocked. In colon cancer model the frequency of instentinal adenomas is similarly unaffected with p27 mutated on T187A, but in this case the progression of instestinal adenomas to carcinomas is inhibited (Timmerbeul et al., 2006). Activity of Kpc and phosphorylation on tyrosine and serine residues (inactivation or translocation into cytosol) may explain this result (see above). In fact, it has been observed a correlation between SRC family kinase expression, which phosphorylate p27, and activity and a decrease level of p27 in primary breast cancer cell line and in BCR-ABL transformed chronic myelogenous leukemia cells (Andreu et al., 2005; Chu et al., 2007; Grimmler et al., 2007). Inhibition of these tyrosine kinases restored p27 expression and inhibits cell proliferation. In addition, the inhibition of p27 expression by miRNAs in glioblastomas and prostate carcinoma cell lines has been described (Galardi et al., 2007; le Sage et al., 2007a; le Sage et al., 2007b), which might constitute other ways to decrease p27 levels in tumors.

On the other hand, recent evidence supports the hypothesis that p27 may also act as an oncoprotein. Mice with a p27 variant that cannot interact with cyclin/Cdk complexes (termed p27CK-), and thus is unable to interfere with cell cycle progression, develop tumors in multiple organs at frequencies higher than seen with $p27^{+/-}$, leading to conclusion that some function or functions of p27 are enhancing tumorigenesis (Besson et al., 2007). These data suggest that p27 can promote oncogenesis and this occurs independently of its interaction with cyclins and Cdks, most likely in the cell cytoplasm. In agreement with this, aberrant cytoplasmatic location

of p27 has been observed in many tumors, such as primary human breast (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002), colon (Ciaparrone et al., 1998), ovarian (Masciullo et al., 2000; Rosen et al., 2005), thyroid (Motti et al., 2005), melanoma (Denicourt et al., 2007), lymphomas (Qi et al., 2006) and esophageal cancers (Singh et al., 1998), and this localization can be used as a pronostic marker of poor clinical outcome. In this respect, the functions of p27 in the cytoplasm, including its role as a regulator of the cytoskeleton and its functios as an antiapoptotic protein (see above), will be important to understand its activity as an oncogenic factor. It has been observed that both K-Ras and PI3K/Akt pathways regulate the cytoplasmatic localization of p27 in different cancer models (Meuwissen and Berns, 2005; Motti et al., 2005; Viglietto et al., 2002), therefore activation of these pathway may be correlated with the oncoprotein activity of p27. Thus, protein expression and subcellular localization of p27 has both prognostic and therapeutic implications.

1.4 c-Myc

c-Myc (Myc hereinafter) was the first transcription factor identified as an oncogen. Myc was identified as the transforming genes (v-Myc) in a group of avian retrovirus (MC29, MH2, OK10), which induced myeloid leukemia, sarcomas, liver, kidney, and other tumors in chickens (Sheiness and Bishop, 1979). The endogenous c-Myc gene and protein was originally identified in chicken cells (Vennstrom and Bishop, 1982; Vennstrom et al., 1982) and, later, the homologous gene was identified in human and mouse (Crews et al., 1982; Dalla-Favera et al., 1982b).

Myc belongs to the group of Myc family, along with N-Myc and L-Myc. Whereas Myc is ubiquitously expressed, N-Myc is mainly neuronal and L-Myc protein is found in lung. N-Myc is encoded by *MYCN* gene and L-Myc by *MYCL1* gene (DePinho et al., 1987). All members of Myc family present a nuclear localization and a short half life, and their expression is correlated with cell proliferation.

1.4.1 Structure and function of Myc

Myc is a transcription factor of the basic/helix-loop-helix/leucine zipper (bHLH-LZ) protein family (Landschulz et al., 1988; Murre et al., 1989; Prendergast and Ziff, 1989) which elicits a variety of biological responses related to cell cycle control, genomic instability, immortalization, energetic metabolism, ribosome biogenesis, apoptosis, intercellular communication and control of cell differentiation. All these function are important in cancer because deregulation of any of these functions in a cell may lead to tumoral phenotype. In fact, Myc deregulation has been shown in at least 40% of all cancers (www.mycancergene.org) (Dang, 1999; Dang et al., 2006; Nesbit et al., 1999).

Human Myc gene is located in the chromosome 8 (8q24.21) and has three exons (Dalla-Favera et al., 1982a; Dalla-Favera et al., 1982b). Its expression is regulated through four promoters, termed P0, P1, P2 and P3 (Fig. 1.9). However, in normal cells, the majority of transcripts initiate at the P2 promoter (75-90% of Myc transcripts) because of its TATA-box and the presence of two Inr elements in its sequence (Wierstra and Alves, 2008) and references therein).

Two isoforms of Myc can be synthesized depending on the start codon from which translation is initiated. The mainly form is generated from AUG start codon located in the 5´ end of the second exon. Translation from this codon results in a protein of 439 aminoacids and 64 kDa (Persson and Leder, 1984). There is a longer and minoritary form of 454 amino acids termed Myc-1 (in contrast with the mainly form which is known as Myc-2) which is translated from a CUG codon located in the 3´ end of first exon (Fig.1.9) (Hann et al., 1988). Differences in the function between these isoforms are not clear. In some studies, no differences between Myc-1 and Myc-2 were observed (Blackwood et al., 1994), but in other experiments, a difference in the transcriptional activity was suggested (Hann et al., 1994). Other forms can also be found in the cells, such as Myc-S (from Myc-Short) which lacks the N-terminal region (Spotts et al., 1997), or Myc-nick, which lacks the C-terimnal region and it is the results of the cleaved of Myc in the cytoplasm. Myc-Nick form is functional and plays a role in muscle differentiation. (Conacci-Sorrell and Eisenman, 2011; Conacci-Sorrell et al., 2010). In this Thesis we will refer to c-Myc-2 as Myc, which is major and nuclear form.

Two independent functional regions are observed in Myc protein (Fig. 1.9) One of them is the C-terminal region, which presents a bHLH-LZ domain. Myc is bound to DNA through basic domain, whereas HLH and LZ domain allows the dimerization with another bHLH protein, of which the most important is Max (Blackwood and Eisenman, 1991; Grandori et al., 2000; Luscher and Larsson, 1999; Marchetti et al., 1995; Nair and Burley, 2006). Thereby, Myc form a heterodimer with Max and together bind to specific DNA sequences called E box (from Enhancer Box sequences) with the consensus core sequence 5´-CACGTG-3´ (Blackwell et al., 1990; Grandori et al., 2000; Nair and Burley, 2006) (see below)

The other region is the N-terminal region. In this region, several conserved regulatory domains (called Myc boxes, MBI, MBII, MBIII and MBIV) regulate Myc transactivation and/or transrepression functions. MBI and MBII are located from 1 to 143 amino acids (Kato et al., 1990). MB1 contains two important phosphorylation sites, Thr-58 and Ser-62, which have been associated with stability and transforming activity of Myc (Sears et al., 2000; Stone et al., 1987). The transcripition function of Myc is located in the MBII domain. When these regions are mutated, the binding to the most of transcriptional coactivators is impaired, and as result, phenotype induced by overexpression of Myc is repress (Freytag et al., 1990; McMahon et al., 1998; Oster et al., 2003). Finally, MBIII is implicated in transcripitonal repression (Herbst et al., 2005; Kurland and Tansey, 2008) and MBIV regulates DNA binding, apoptosis, transformation and G_2 arrest (Cowling et al., 2006).

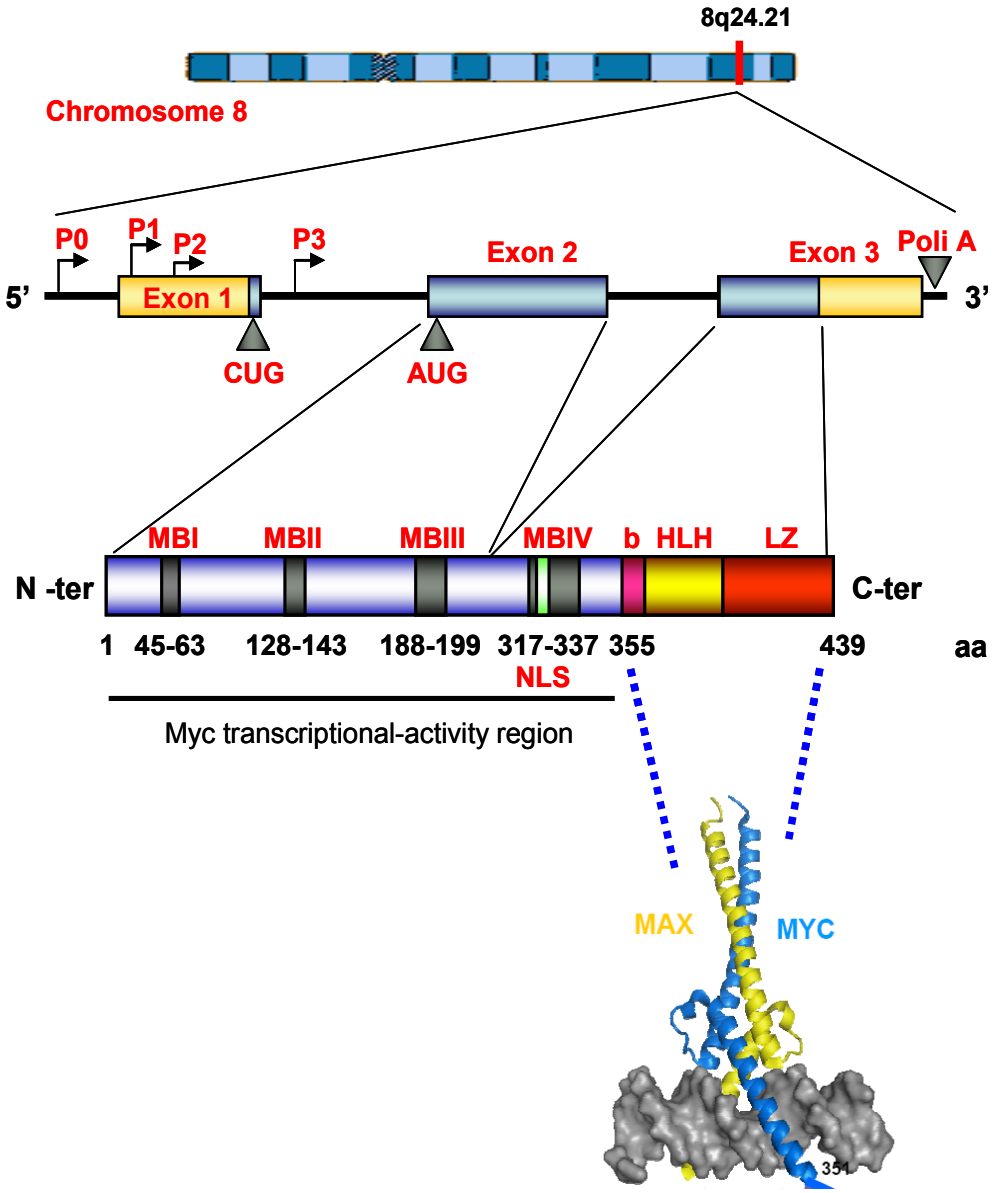


Figure 1.9.- Myc gene and Myc protein structure. Four promotors and two start codon are illustred. Isoform generated from AUG start codon is represented (Myc-2) In the amino terminal region is located four Myc boxes which regulate Myc transactivation and transrepression functions. C-terminal region present a bHLH-LZ domain which allows the dimerization with another bHLH protein (Max) and the bind to DNA.

1.4.2 Regulation of Myc expression

Myc is generally expressed in normal proliferating cells (Marcu et al., 1992; Oster et al., 2002; Spencer and Groudine, 1991). Myc monitors many cell functions and therefore Myc expression and Myc activity have to be tightly controlled. In fact, Myc expression is strongly regulated by transcriptional, posttrancriptional and posttranslational mechanims, although the mechanism of this regulation is yet poorly understood. This is because the regulation of the Myc promoter is extremely complex with a lot of redundancy, many feedback loops, and several cross-regulatory circuits involved (Fig. 1.10) [reviewed in (Wierstra and Alves, 2008)].

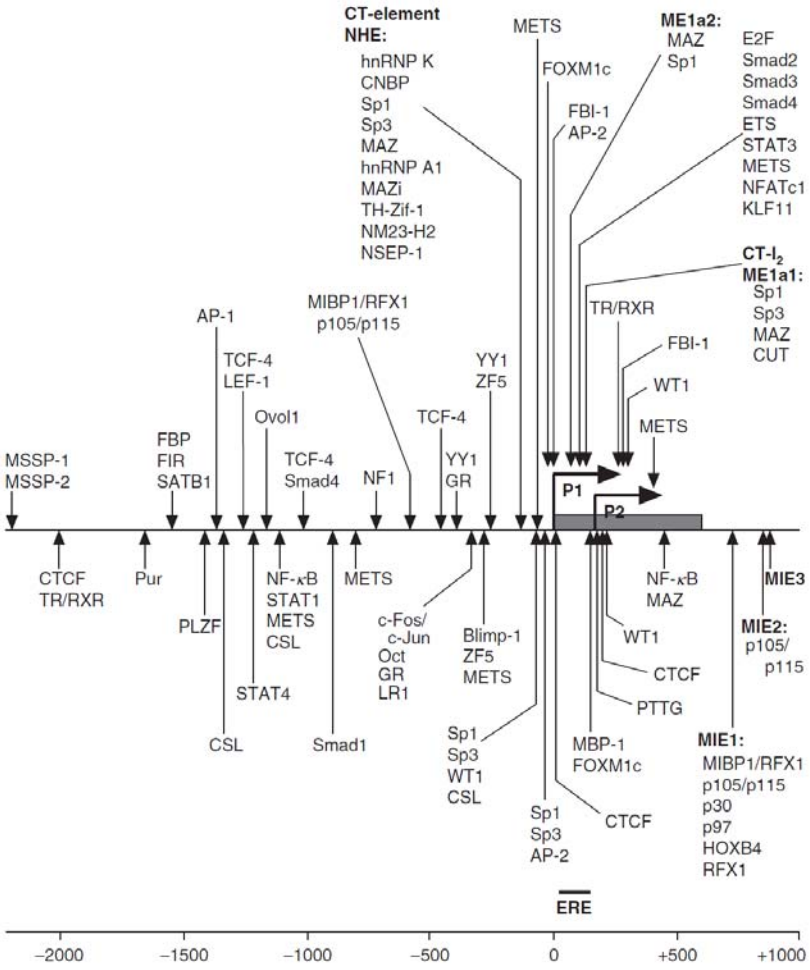


Figure 1.10.- Transcription factor binding sites in the Myc promoter. The horizontal line represents Myc promotor (from -2000 bp to +1000 bp). Exon 1 is illustred by a grey box. P1 and P2 are promotors more used in the majority of transcripts. Vertical arrows represent one or more binding sites for a particular transcription factor (Wierstra and Alves, 2008).

Myc expression correlates with cell proliferation. Thereby, in proliferating cells the Myc mRNA is between 10- to 40-fold higher that in quiescent cells (where Myc is virtually not expressed), and during growth arrest and differentiation, it drops about 90 % (Marcu et al., 1992; Oster et al., 2002; Spencer and Groudine, 1991). In quiescent cells, E2F-1, E2F-2, E2F-4, HDAC and Rb protein family are bound to Myc promoter and its transcription is inhibited (Albert et al., 2001). When Cdk/cyclins complexes are active in proliferant cells, Rb is phosphorylated and removed of Myc promoter. In this moment, other transcription factors, such as TCF3, Sp1, YY1, E2F-1 and E2F-4 bind to the promoter inducing an increase in the transcription. However, this increase is transitory (Hann et al., 1985; Rabbitts et al., 1985a; Rabbitts et al., 1985b; Thompson et al., 1985) because of a regulatory region of Myc called FUSE (Far UpStream Element). Firstly, this region is recognized by FBP (FUSE binding protein) which actives transcription, but later FBP-FIR (FBP interacting repressor) is recruited, resulting in the inhibition of Myc transcription (Chung and Levens, 2005; Liu et al., 2006). Thus, after the myc expression peak due to stimulation of quiescent cells, Myc is expressed at significant but

lower levels in proliferating cells. Moreover, Myc is able to regulate its own expression through a negative feedback loop in which a member of Rb family (p107) is involved (Facchini et al., 1997; Luo et al., 2004; Wierstra and Alves, 2008). This autorepression mechanism is not well understood, but it is known that Polycomb complex is implicated (Goodliffe et al., 2005). The Fig. 1.10 summarizes the main factors controlling Myc transcription.

Myc expression depends on the presence of growth factors. Their removal, stimuli of differentiation or other antipoliferative signals result in immediate downregulation of Myc. In terminally differentiated cells, Myc is no longer expressed. Accordingly, in adults Myc expression is restricted to tissues with proliferating cell types and areas of regenerative proliferation. Myc repression in differentiation is regulated, directly or indirectly, by the same transcription factors that induce the differentiation process. Thus, Myc promotor is repressed by Blimp-1, C/EBPα, C/EBPβ, METS, Ovol1 and GATA-1 through cooperation with E2F and Rb protein family (Berberich-Siebelt et al., 2006; Hester et al., 2007; Iakova et al., 2003; Johansen et al., 2001; Klappacher et al., 2002; Lin et al., 1997; Nair et al., 2006; Rylski et al., 2003).

Myc mRNA stability is also regulated. Myc mRNA has a short half-life because its sequences are rich in A and U which lead a low stability. (Brewer and Ross, 1988; Jones and Cole, 1987). Moreover, other regions of the mRNA which encoding the C-terminal domain of the protein are recognized by several proteins with RNasa activity (Bernstein et al., 1992; Lee et al., 1998; Prokipcak et al., 1994). Myc protein regulation is mediated through phosphorylation of Thr-58 and Ser-62 (localizated in the MBI). Proliferation induction mediated by different signals actives Erk (Extracellular signal-regulaed kinases) through Ras pathway. Erk is a kinase which phosphorylates Myc in the Ser-62. This phosphorylation induces an encrease of Myc stability. However, Myc phosphorylated in Ser-62 is recognized by GSK-3beta, other kinase that phosphorylates Myc in the Thr-58. Both Ser-62 and Thr-58 phosphorylation target to Myc for its degradation through Fbw7 (F-box and WD repeat domain-containing 7), which is a F-box protein family that belongs to SCF (Skp1-cullin-F-box) ubiquitin protein ligase complex. This complex mediate Myc ubiquitination and its subsequend degradation by proteasome (Sears, 2004). In fact, Thr-58 is the most frequent site of Myc mutations in lymphoma cells (Welcker et al., 2004).

1.4.3 Transcriptional regulation by Myc

Transcriptional regulation of Myc carried out as a dimer with Max (Fig. 1.11) (Blackwood and Eisenman, 1991; Grandori et al., 2000; Luscher and Larsson, 1999; Marchetti et al., 1995; Nair and Burley, 2006). Thereby, Myc´s ability to bind to its cognate DNA recognition E-box site (5´-CACGTG-3´) and activate transcription requires its dimerization (Blackwell et al., 1990; Grandori et al., 2000; Nair and Burley, 2006) (see below). In contrast of Myc, Max expression is ubiquitous and constitutive (Berberich et al., 1992). Two spliced forms of Max are possible: Max (21 kDa) and Max9 (22 kDa) (Makela et al., 1992; Vastrik et al., 1993). Max, but not Myc, is

capable of forming homodimers (Littlewood et al., 1992), although the role that they may play remains obscure (Berberich et al., 1992; Kretzner et al., 1992; Yin et al., 1998)

Myc regulates transcription through several mechanisms, including recruitment of histone acetylases, chromatin modulating proteins, basal transcriptional factors and DNA methyltransferase (Brenner et al., 2005; Cheng et al., 1999b; Eberhardy and Farnham, 2002; Fernandez et al., 2003; Kanazawa et al., 2003; McMahon et al., 1998; O'Connell et al., 2003). Transactivation by Myc appears to requiere interactions of Myc´s MBII domain with a member of phosphatitylinositol-3kinase-related kinase protein family called TRRAP (Transformation/ transcription domain-associated protein) (McMahon et al., 1998), which binds the histone acetylase GCN5 which leads an increase of transcription (McMahon et al., 2000). Moreover, Myc´s MBII domain can also recognize to Tip48 and Tip49 proteins, a ATPasa/helicase proteins whose activity is essential for the control of cellular growth and proliferation mediated by Myc (Bellosta et al., 2005; Wood et al., 2000). In addition, Myc´s MBI domain is able to recruite cyclin T1 and Cdk9, both components of the CTD kinase P-TEFb, which induces an increase of transcription through phosphorylation of carboxi-terminal domain of the RNA Pol II (Eberhardy and Farnham, 2001, 2002; Rahl et al., 2010). Interactions through HLH-LZ domain have also been observed. INI1/Snf5, a factor that recruits the Swi-Snf complex, binds to this domain and activates transcription through chromatin remodeling (Amati et al., 2001; Cheng et al., 1999b; Luscher, 2001).

Myc can also mediate trans-repression by a mechanism dependent on initiator (Inr) element of the basal promoters of susceptible genes (Li et al., 1994). Different transcription factor (such as Miz-1, TFII-I or YY1) bound to Inr are isolated by direct interaction with Myc, what triggers the inhibition of transcription (Kleine-Kohlbrecher et al., 2006; Roy et al., 1993; Seoane et al., 2002; Seoane et al., 2001; Shrivastava et al., 1993; Staller et al., 2001; Wanzel et al., 2003; Wu et al., 2003) Moreover, Myc may interact with the DNA methyltransferase Dnmt3a, which inhibits the transcription through DNA methylation. In $p21^{CIP1}$ promoter, Myc forms a complex with Miz-1 and Dnmt3a, and it induces DNA methylation and therefore inhibition of the transcription (Brenner et al., 2005).

Another system of transcriptional regulation by Myc is carried out by Mxd family (Mxd1/Mxd2/Mxi1, Mxd3 and Mxd4). Mxd family are also members of the bHLH-LZ protein family, and they also can bind to Max (Fig. 1.11). Thereby, Mxd family proteins can inhibit Myc through competition for the Max available (Baudino and Cleveland, 2001). Moreover, Mxd/Max heterodimers bind to E-box sequences and compete for binding with Myc/Max heterodimers. Mxd/Max dimers repress transcription by recruiting the chromatin-modifying co-repressor complex what results in deacetylation of histone tails and a closed chromatin conformation, thus preventing the transcriptional activation that occurs through E-boxes (Alland et al., 1997; Ayer et al., 1993; Ayer et al., 1995).

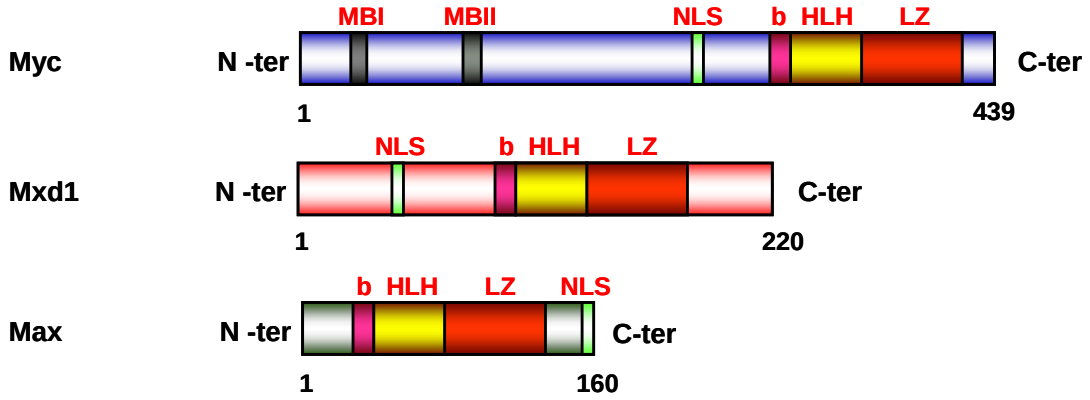


Figure 1.11.- Schematic illustration of a representative Myc, Max and Mxd protein.

1.4.4 Myc target genes and their functions

A lot of studies have attempted to describe how much and which are the genes regulated by Myc. The development of new massive sequencing techniques (such as microarrays, ChIP-on-ChIP, ChIP-PET or ChIP-seq) have elicited a large amount of information about the genes regulated by Myc. It is estimated that 10%-15% of all human genes are recognized by Myc, and at least 1000-4000 genes are regulated by Myc (Dang et al., 2006; Fernandez et al., 2003; Lawlor et al., 2006; O'Connell et al., 2003; Patel et al., 2004; Zeller et al., 2006). However, only a fraction of genes appears to be universally regulated by Myc independent of cell type or species (Zeller et al., 2003). Other genes present differences between the cell type or species of origin. Some of the variations may be attributed to cell-type specific activation of specific sets of genes in response to Myc (Barr et al., 1998; Frye et al., 2003; Yang et al., 1991). Myc responsive genes that appear recurrently in different cell types, systems and species can be indentified from the Myc target gene database (http://www.myccancergene.org). Recently, new reports have gone further and they have proposed that Myc acts as a general amplifier of transcription at promoters already engaged with the transcriptional machinery (Lin et al., 2012; Nie et al., 2012).

Because of the large number of genes regulated by Myc, it is possible that Myc elicits a great variety of functions. We next review the Myc functions with special emphasis in those related to the work carried out in this Thesis.

1.4.4.1 Cell cycle regulation

As it was indicated above, Myc is not expressed in quiescent cells, but it is induced quickly in proliferant cells through addition of growth factor (Marcu et al., 1992; Oster et al., 2002; Spencer and Groudine, 1991). G1 phase is shortened and transition from G_1 to S is enhanced by the presence of Myc (Facchini and Penn, 1998; Karn et al., 1989; Roussel et al.,

1991; Steiner et al., 1995). Consistent with this, reduction in the levels of Myc through delection or antisense oligonucleotides induce cell cycle inhibition and, in some cases, differentiation (Heikkila et al., 1987; Holt et al., 1988; Mateyak et al., 1997) In this context, fibroblasts and B-lymphocytes from Myc conditional knock-out mice present a proliferation rate reduced regarding wild type mice (de Alboran et al., 2001). Myc induce cell cycle proliferation through different mechanisms. Myc represses by an Inr-independent mechanism the transcription of genes such as GADD45 or GADD153 (growth arrest and DNA damaging-inducible 45 and 153), which induces cell cycle inhibition by different factors (Chen et al., 1996; Marhin et al., 1997). Moreover, proteins that decrease Cdks-Cyclins activity are inhibited by Myc (Lutz et al., 2002; Meyer and Penn, 2008). Cdks and cyclins are induced by Myc, such as cyclin D1, cyclin D2, cyclin E1, cyclin A2, cyclin B1 and Cdk4 (Bouchard et al., 2001; Fernandez et al., 2003; Hermeking et al., 2000; Menssen and Hermeking, 2002).

On the other hand, some CKI are directly regulated by Myc. Thereby, Myc prevents p15 and p21 induction by TGF-beta through the inhibition of Miz-1 which is part of the signaling pathway that mediates induction of these genes (Kleine-Kohlbrecher et al., 2006; Seoane et al., 2002; Seoane et al., 2001; Staller et al., 2001; Wanzel et al., 2003; Wu et al., 2003) Moreover, other proteins of great importance for cell cycle progression such as CDC25A, E2F1 and E2F2 are also induced by Myc (Fernandez et al., 2003; Galaktionov et al., 1996; Gartel and Shchors, 2003; Jansen-Durr et al., 1993). Finally, Myc also is implicated in the control of degradation of a lot of cell cycle regulators proteins. Recently, it has been observed that Myc induces the expression of Skp2 (Bretones et al., 2011; Old et al., 2010), a ubiquitin ligase belongs to SCF complex whose function is to regulate the proteolysis of many proteins, between them several proteins implicated in the cell cycle inhibition. Therefore, Myc has a global role in the regulation of the cell cycle, highlighting its role in G_1-S transition.

1.4.4.2 Differentiation

Proliferation and differentiation are antagonistic functions in a lot of models. Thereby, factors that induce differentiation also induce cell cycle inhibition, and factors, such Myc, that induce cell proliferation are strong inhibitors of differentiation [reviewed in (Leon et al., 2009)]. In fact, Myc expression is down-regulated when cell is differentiated (Gonda and Metcalf, 1984; Henriksson and Luscher, 1996; Larsson et al., 1994) and ectopic expression of Myc inhibits cell differentiation in many cell models (see below). Consistent with these assumptions, Mxd expression, which is an antagonist of Myc functions (see above), increases in a lot of models of differentiation (Luscher, 2001; Zhou and Hurlin, 2001). Differentiation is a complex process where cells dramatically change cell´s size, shape, membrane potential, metabolic activity and responsiveness to signals. These changes are largely due to highly controlled modifications in gene expression. However, each specialized cell type expresses a subset of genes different from other cell types. Therefore, effect of Myc in differentiation depends on the cell line. In many

cases, Myc inhibits at “master genes” of the differentiation. C/EBPalfa drives the differentiation of murine cells to adipocytes through directly regulation of GADD45, but Myc antagonized C/EBPalfa-mediated transactivation of GADD45 (Tao and Umek, 1999). In erythroid differentiation of K562 cell line, Myc represses GATA1, which is a transcription factor that is essential for erythroid development (Acosta et al., 2008). In contrast, some models support the idea of Myc as enhancer of differentiation. This occurs in human epidermal stem cells (Flores et al., 2004; Gandarillas and Watt, 1997) or hematopoeitic stem cells and B lymphocytes (Delgado and Leon, 2010; Habib et al., 2007; Wilson et al., 2004), where Myc increases the population of precursors cells of a particular cell lineage. In this case, stem cell requires a step of proliferation where Myc is active before the cells differentiate.

1.4.4.3 Apoptosis

In the absence of growth factors, Myc expression may induce apoptosis (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992). This effect is confirmed by cells that lacks of Myc, which are more resistent to apoptosis than control cells with Myc (de Alboran et al., 2004). Myc induces apopotosis through p53-dependent and -independent mechanisms (Meyer et al., 2006; Sakamuro et al., 1995). In the first case, Myc triggers ARF expression, which induces Mdm2 (a inhibitor of p53) downregulation (Zindy et al., 1998). The p53-independent mechanisms have been associated the Bcl-2 protein family. Myc inhibits the activity of some antiapoptotic members (Bcl-2 or Bcl-X_L) and activates proapoptotic members (Bax) (Eischen et al., 2001; Finch et al., 2006; Maclean et al., 2003; Soucie et al., 2001)

1.4.4.4 Other functions

- **Protein synthesis and cell size:** Myc overexpression is related with new protein synthesis and an increase in the cell size (Iritani and Eisenman, 1999; Mateyak et al., 1997). Myc induces the transcription of genes related with rRNA and ribosomes biosynthesis (Kim et al., 2000; Schlosser et al., 2003) and the RNA polimerase I transcription (Arabi et al., 2005; Grandori et al., 2005) Moreover, Myc activates the promotor of the RNA polimerase III (Felton-Edkins et al., 2003; Gomez-Roman et al., 2003).
- **Cell adhesion and cytoskeleton:** Cell motility and loss of adhesion are regulated by Myc through repression of several genes related with these processes, such as actin, cdc42, alfa3beta1 integrin, cadherin N or several collagens (Barr et al., 1998; Wilson et al., 2004; Yang et al., 1991). Myc-Nick form has also been implicated in these processes through its interaction with alfa-tubulin and GCN5, which results in tubulin

acetylation and in changes in cell morphology (Conacci-Sorrell and Eisenman, 2011; Conacci-Sorrell et al., 2010)

- **Metabolism:** A large number of genes related with the metabolism are regulated by Myc, such as enzymes and glucose, iron and nucleotides transporters (Bello-Fernandez et al., 1993; Osthus et al., 2000; Wu et al., 1999). Thereby, Myc stimulates transcription of the lactate deshidrogenase A (LDH-A) gene (Shim et al., 1997) and many other genes of the glycolitic pathway, as well as a group of nuclear genes involved in mitochondrial function (Morrish and Hockenbery, 2003). Recently, Myc has been shown to promote oxidative phosphorylation as well as glycolysis through coordinated transcriptional control of the mitochondrial metabolic network (Morrish et al., 2008; Zhang et al., 2007)
- **MicroRNAs:** Regulation of several microRNAs is mediated by Myc. One of most important is a group of microRNAs called "*cluster mir-17*". Myc bind to non-canonical E-box localizated in the promotor (O'Donnell et al., 2005)
- **Intracellular signaling:** Factors implicated in vasculogenesis and angiogenesis may be regulated by Myc, such as VEGF and other proangiogenics factors (Baudino et al., 2002)

1.4.5 Myc functions in tumorogenesis

Myc was the first example of oncogenic activation by chromosomal translocation in human cancer. In Burkitt lymphoma, Myc gen is translocated from chromosome 8 to 14, 2 or 22, where heavy or light chain of immunoglobulins genes are located (Dalla-Favera et al., 1982a; Taub et al., 1982). Thereby, Myc expression in this lymphoma is regulated by immunoglobulin promoters, and this results in Myc overexpression. In many different tumors Myc is overexpressed, although its overexpression is achieved through different mechanisms (Nesbit et al., 1999). The first mechanism identified was the retroviral infection (Sheiness and Bishop, 1979), but Myc gene translocations or Myc gene amplifications have been observed (Dalla-Favera et al., 1982a; Taub et al., 1982)**.** Promotor activation through hormones or growth factors, or activation of their receptors, or different signaling pathways or transcriptional effectors activity may trigger Myc overexpression without a chromosomal aberrations or physical defects in the gene [reviewed in (Wierstra and Alves, 2008)]. In other cases, Myc is overexpressed by other factors whose activity trigger an enhancer in the mRNA or protein stability, inducing Myc overexpression (Fig. 1.12) [e.g. (Welcker et al., 2004)]. Myc deregulation is found in many types of human lymphoma and leukemia, and enforced expression of Myc blocks the differentiation in several leukemia-derived cell lines capable of differentiating in culture. In agreement, enforced expression of Myc in mature cells from the lymphoid or myeloid lineages, the result is lymphoma or leukemia [reviewed in (Delgado and Leon, 2010)].

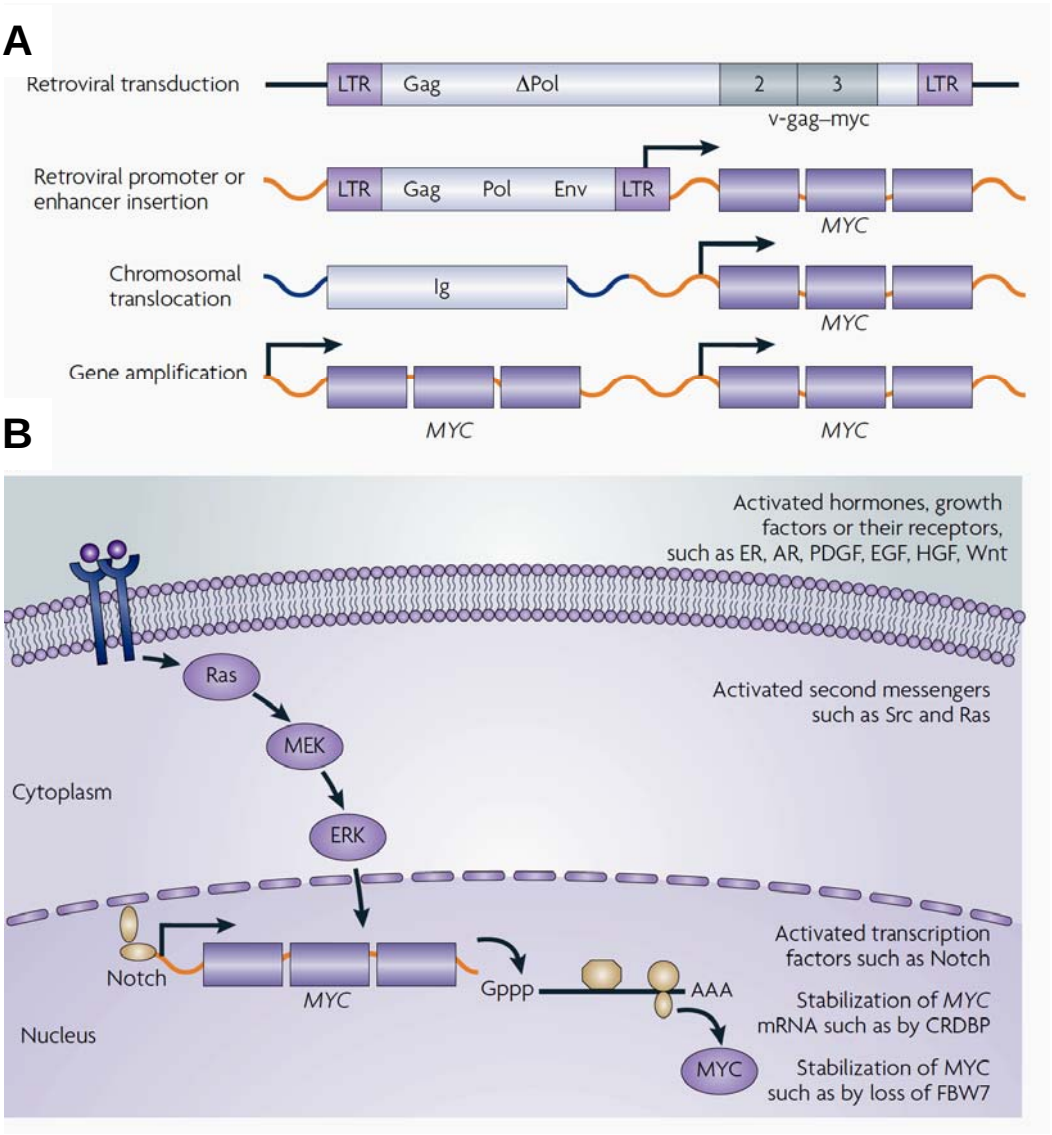


Figure 1.12.- Mechanisms through which Myc is overexpressed in cancer. Deregulation by retroviral or cytogenetic alterations (a), or alteration of pathways that regulates Myc expression (b) (from Meyer et al., 2006)

Different mutations in the Myc sequence have been associated with cancer. These mutations may enhances its stability (Gavine et al., 1999; Salghetti et al., 1999), some of them through mutations in the phosphorylation sites of Myc (Axelson et al., 1995; Bhatia et al., 1993; Gupta et al., 1993). Myc elicits a variety of biological functions whose deregulation lead to tumoral phenotype (see above). Thereby, Myc is a strongly proliferative agent and an inhibitor of differentiation. Myc has the capacity to both stimulate cell growth and abrogate the activity of cell cycle inhibitors, a powerful combination that drives the proliferative potentital. Moreover, it regulates functions such as cell size, metabolism, protein synthesis, immortality and apoptosis (see above), which are very important for tumor development. Other processes such as genomic inestability, cell adhesion, cell transformation and angiogenesis have been observed in different systems where Myc is overexpressed (Henriksson and Luscher, 1996; Nesbit et al., 1999; Oster et al., 2002). Thus, deregulation of Myc function may contribute to various properties of tumor cells, defying a simple assigment to a single functional category (Fig.1.13).

One common feature of many transformed cells is their reduced dependence on external growth factors, and cells expressing deregulated Myc genes share this property (Keath et al., 1984). In many established cell lines, Myc-induced proliferation in the absence of external growth factors is limited solely by Myc-induced apoptosis (Evan et al., 1992). Activation of cyclin E/Cdk2 kinase, or activation of E2F-dependent transcription, or increase in cell mass, all them regulated by Myc (see above), may account for the ability of deregulated Myc to reduce the growth-factor dependence.

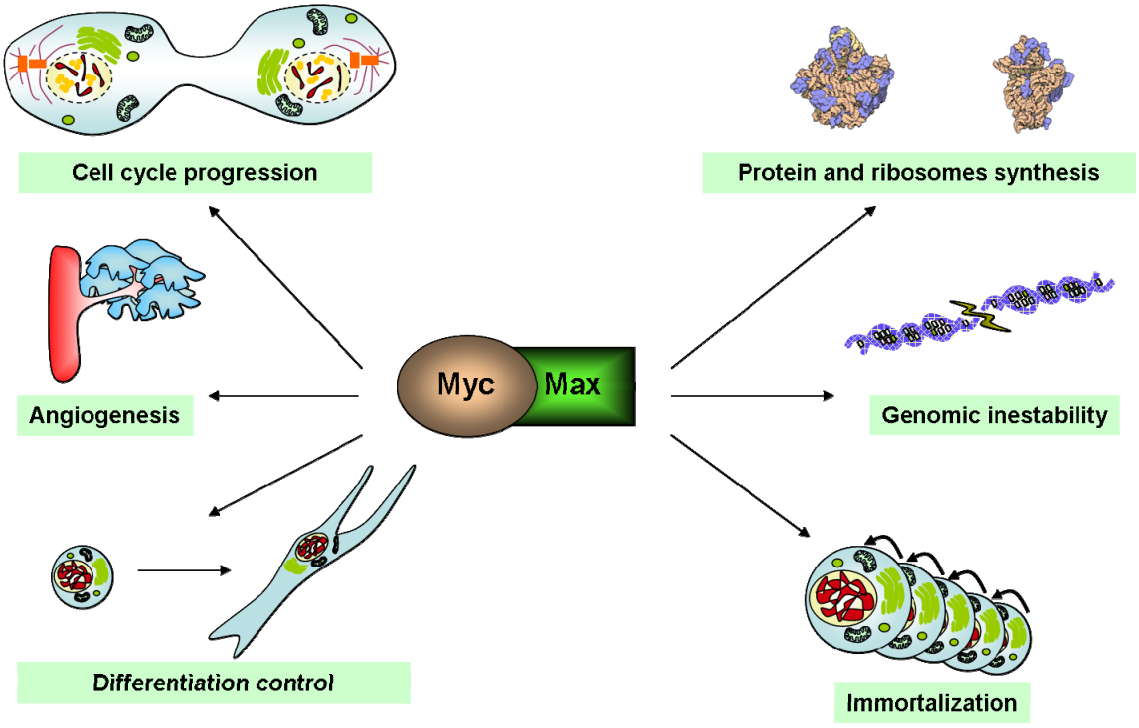


Figure 1.13.- Myc and Cancer

Also, Myc overexpression in several cell lines results in genomic inestability, loss of genomic organization and gene amplification, features characteristic of tumor progression (Felsher and Bishop, 1999; Mai et al., 1996a; Mai et al., 1996b; Prochownik and Li, 2007). As Myc induces genomic inestability is not clear, but some studies have correlated it with reactive oxygen species or with alterations of chromosomal structure through telomeric aggregations (Louis et al., 2005; Prochownik and Li, 2007). Moreover, Myc elicits a direct role in the S phase progression and therefore in the DNA replication induction (Dominguez-Sola et al., 2007; Gutierrez et al., 1988; Iguchi-Ariga et al., 1987), a loss of several cell cycle regulators such as p53 may cooperate with Myc overexpression to promote genomic inestability through deregulation of cell cycle checkpoints and therefore results in the passing of damaged or incomplete DNA on to daughter cells (Yin et al., 1999).

Immortalization is an important characteristic of tumor cells. The lifespan of primary human cells in culture is largely dictated by telomere length, since expression of the catalytic subunit of human telomerase, HTERT, can significantly extend lifespan of human primary cells in culture [reviewed in (Weinberg, 1998)]. The HTERT promoter is activated by Myc, which can

induce HTERT expression and telomerase activity in some primary cells (Greenberg et al., 1999). Moreover, although it has been found that ectopic expression of Myc induces expression of ARF (Zindy et al., 1998), which may induce apoptosis through p53 pathway, recently it has been showed that methylation of ARF promoter, which inhibits its expression, occurs frequently in cells immortalized by Myc (Benanti et al., 2007). Therefore, ectopic expression of Myc is enough to immortalize primary mouse embryo fibroblasts (Land et al., 1983).

Proliferation of cells in vivo is induced by acting growth factors and restrained by anti-mitogenic factors. Leading to resistence against anti-mitogenic factors such as transforming growth factor beta (TGF-beta) contributes to tumorigenesis of colon, skin and breast, among others [reviewed in (Massague and Chen, 2000)]. Ectopic expression of Myc can induce resistence to the anti-mitogenic effects of TGF-beta through, in part, its ability to supress induction of the p15 and p21 genes by TGF-beta (see above) (Claassen and Hann, 2000; Warner et al., 1999).

Finally, angiogenesis process is also regulated by Myc. The ability of Myc to promote the angiogenesis was uncovered in Rat-1a cells in vivo, which showed angiogenesis associated with Myc deregulation (Ngo et al., 2000), although it had already been observed that the reversible activation of conditional alleles of Myc in keratinocytes in vivio induces angiogenesis in a reversible manner (Pelengaris et al., 1999) Later, it would be showed that in same cases the expresion of Myc is crucial for the initiation of angiogenesis through regulation of interleukina 1beta (Shchors et al., 2006). Moreover, Myc is able to regulate this process through the downregulation of thrombospondin or CTGF, which is vital to angionesis, by induction of the miR17-92 microRNA cluster. (Dews et al., 2006; Janz et al., 2000)

1.5 Skp2

Skp2 is a member of the SCF^{Skp2} ubiquitin ligase complex. Ubiquitin ligase complexes belong to ubiquitin-proteasome proteolytic pathway, which comprise the covalent attachment of multiple ubiquitin molecules to the protein substrate and the degradation of the polyubiquitylated protein by the 26S proteasome complex (Pickart, 2004). Ubiquitin-labeling is carried out by a ubiquitin-activating enzyme (E1), which charges to an ubiquitin-conjugating enzyme (E2) with ubiquitin before it can be linked to the substrate, which is captured and presented by a ubiquitin ligase (E3), in such a manner, that its lysines can be efficiently ubiquitinated. Versatility in substrate specificity for the SCF E3 ligases is provided by the large number (more than 70 in humans) of F-box proteins (Nakayama and Nakayama, 2006; van Leuken et al., 2008), which bind distinct substrates. Given the important fuction of one of these F-box proteins (Skp2) in the regulation of proteins implicated in our study, hereafter it will only be reviewed the properties of this specifies F-box protein.

Skp2 is a F-box protein which is part of the SCF complex. This complex consists of the invariable components Skp1, Cul1 (also named Cullin) and Rbx1 (also named ROC) and a

variable component known F-box protein that binds to Skp1 through its F-box motif and it is responsible for substrate recognition (Fig. 1.14) (Deshaies, 1999). In most of cases, F-box protein interacts with their cognate substrates only after the substrates have been posttranslationally modified such as phosphorylation, acetylation, glycosylation or nitration (Guinez et al., 2008; Hwang et al., 2010).

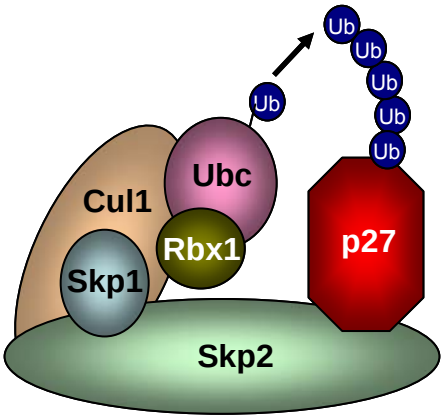


Figure 1.14.- E3 SCF^{SKP2} ubiquitin-ligase complex. Skp2 binds to Skp1 through its F-box motif and it is responsible for substrate recognition, in this case p27 [modified from (Kipreos and Pagano, 2000)]

Skp2, along with Cks1 and Skp1, was discovered as a protein of 45 kDa associated with cyclinA-Cdk2 complexes, and hence its name (S-phase kinase-associated protein 2) (Zhang et al., 1995). Skp2 is characterized by containing a binding motif of approximately 40 residues, termed the F box associated with leucine-rich regions (LRRs) through which binds to Skp1 and its substrate (Deshaies, 1999)

A lot of cell cycle regulators have been implicated as potential substrates of Skp2. These proteins include p27^{Kip1} (Carrano et al., 1999; Sutterluty et al., 1999), p21^{cip1} (Bornstein et al., 2003), p57^{kip1} (Kamura et al., 2003), p130 (Bhattacharya et al., 2003; Tedesco et al., 2002), cyclin A (Nakayama et al., 2000), cyclin D1 (Yu et al., 1998), cyclin E (Nakayama et al., 2000), E2F1 (Marti et al., 1999), Myc (Kim et al., 2003; von der Lehr et al., 2003) between others. However, the fact that most of the cellular and histopathologic defects in Skp2-deficient mice are not observed in Skp2$^{-/-}$;p27$^{-/-}$ mice suggests that p27^{kip1} is the major substrate of Skp2 (Nakayama et al., 2004)

Levels of Skp2 are high during S and G_2 phases, but low in G_1 phase. Therefore, Skp2 regulation is important for the proper cell cycle progression. Interestingly, Skp2 transcription is regulated by several proteins that are targets of itself through a non-canonical E2F site and another well conserved E2F site (Yung et al., 2007; Zhang and Wang, 2006). This regulation by E2F implies the existence of a positive feedback loop between Skp2 and its own targets, also known as “Skp2 autoinduction loop” (Assoian and Yung, 2008). This loop is comprised of Rb-E2F, Skp2, p27 and cyclin E-Cdk2. In this context, it is possible that Myc is implicated in this loop. Cyclin E and E2F1 are regulated by Myc (Fernandez et al., 2003; Galaktionov et al., 1996;

Gartel and Shchors, 2003; Jansen-Durr et al., 1993; Oster et al., 2002), and it has been recently found that Myc induces mRNA and protein expression of Skp2 (Bretones et al., 2011; Old et al., 2010) resulting in the degradation of p27.

1.6 Myc-Skp2-p27 network

Skp2 binds to and mediates the ubiquitylation of the CKI p27 (Carrano et al., 1999; Sutterluty et al., 1999), which was known to be degraded via the ubiquitin-proteasome pathway in G1 phase (Pagano et al., 1995; Shirane et al., 1999). Interestingly, Skp2 was found to target only p27 molecules in which threonine-187 are phosphorylated (Montagnoli et al., 1999; Sheaff et al., 1997; Vlach et al., 1997). This phosphorylation is mediated by Cdk2 (see above) Thereby, Cdk2 mediates the degradation of p27 and its own activation (see above). After, It was described that p27 was accumulated at high levels in mice that lack Skp2 (Nakayama et al., 2000; Nakayama and Nakayama, 2005). According with this, $Skp2^{-/-}$ cells exhibit an abnormal accumulation of p27 during S and G2 phases (Hara et al., 2001). This abnormality is associated with prominent cellular phenotypes, including nuclear enlargement, polyploidy and an increased number of centrosomes, that are likely due to overreplication of chromosomes and centrosomes (Nakayama et al., 2000). Moreover, Skp2 and p27 double-knock-out mice do not exhibit such overreplication phenotypes, suggesting that p27 accumulation in S and G_2 phases is required for their development (Nakayama et al., 2004). Therefore, Skp2 role in the S phase entry is mainly due to p27 destruction. In this destruction, a cofactor named Cks1 is required for the recognition of phosphorylated p27 by Skp2 (Ganoth et al., 2001; Spruck et al., 2001). In summary, Skp2 mediates p27 degradation at the G_1-S transition of the cell cycle to activate cyclin E-Cdk2 kinase complex.

On the other hand, *SKP2* is a Myc target gene. In most systems, the induction of Myc is correlated with an elevation of the mRNA and protein levels of Skp2, and silencing of Skp2 abrogated the Myc-mediated expression resulted in a decrease in p27 protein (Bretones et al., 2011). In fact, elevated levels of Skp2 are a characteristic of Emu-Myc lymphomas and of human Burkitt lymphoma that bear Myc/immunoglobulin chromosomal translocations and, again, Myc-mediated supression of p27 was abolished in Skp2-null Emu-Myc B cells (Old et al., 2010). This Myc-Skp2-p27 network has been observed in vascular smooth muscle proliferation and in chronic myeloid leukemia differentiation (Gomez-Casares et al., 2012; Sicari et al., 2012), stating that this network has an important role in functions such as proliferation or differentiation. Moreover, these networks reveal a new mechanism for the transforming activity of Myc.

Surprisingly, Skp2 also binds to Myc via its MBII and HLH-LZ domains mediating its transcriptional activity and degradation (Kim et al., 2003; von der Lehr et al., 2003). Thus, it is possible that Myc regulates its own expression and levels into the cell through induction of Skp2, and itself. However, Skp2 was also found to enhance Myc-induced S phase transition and increase the transactivation activity of Myc, suggesting a role of Skp2 as a transcriptional

cofactor (see above) (Kim et al., 2003; von der Lehr et al., 2003). Therefore, it is not clear which is the real function of Skp2 in the Myc regulation, and further research will be needed to clarify how this Myc-Skp2-p27 is regulated.

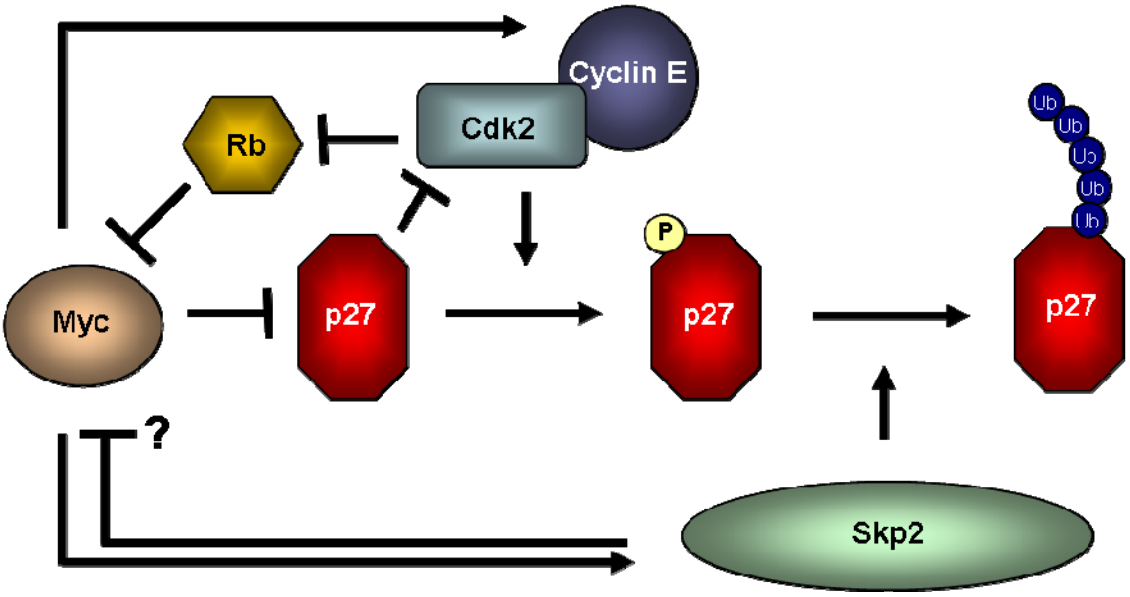


Figure 1.15.- Myc-Skp2-p27 network. Myc repress p27 through three mechanisms: Inhibiting directly its mRNA expression, inducting cyclin E which results in Thr-187 phosphorylation, and inducing Skp2 that recognize Thr-187 phosphorylate p27 and marks it for degradation via proteasome. Skp2 also recognizes Myc, although the function of Myc ubiquitylate by Skp2 is poorly understood. p27 inhibits Cdk2/Cyclin E complexes, which phophorylate Rb protein family and as results Rb is inactivated. Rb hypophosphorylated binds to and inhibits E2F, which induces Myc expression.

1.7 Chronic lymphocytic leukemia

1.7.1 Prevalence and clinical features

Chronic lymphocytic leukemia (CLL hereafter) is the most frequent leukemia in adults meaning almost 25-30% of all leukemia and an annual incidence of about 3-9 new cases per 100.000 in USA and Europe, and less frequent in Asia (Weiss, 1979). CLL is a disease of adults, with a median age at diagnosis of 72 years old, but CLL in young people are not rare (7-10% of patients) (Chiorazzi et al., 2005; Diehl et al., 1999; Espey et al., 2007; Shanshal and Haddad, 2012; Yee and O'Brien, 2006). Gender is also important. Males have twice the risk of developing the disease (Rozman and Montserrat, 1995). CLL is a chronic disease with a slow progression, where the overall five-year survival rate is about 73% and with a median survival of 10 years (Espey et al., 2007).

CLL is defined as a monoclonal leukemia of B cells with a phenotype of mature and activated cells, which accumulate relentlessly because of a faulty apoptotic mechanisms. Thereby, B-CLL cells are accumulated in peripherical blood because of faulty apoptotic mechanisms and this causes constitutional symptoms. Therefore, the main feature is the lymphocytosis in peripheral blood (≥ 5 x 10^9 cell/L) (Binet et al., 2006). This is because clones of B-CLL cells show a characteristic and specific inmunophenotipe (Table 1.1) which can be used to differentiate it from other leukemias (Kurec et al., 1992). Although B cells are accumulated in CLL patients, these B-CLL cells are not functional because they elicit a variety of cellular immune defects, which are linked to different clinically manifested complications such as an increasing rate of infections, autoimmune disorders and secondary tumours (Vitale et al., 2003).

Table 1.1.- Immunophenotype of CLL and other B-cell lymphoproliferative disorders. sIg = surface immunoglobulin; - = not expressed; -/+ = usually is not expressed; + to +++ = varying degrees of strength of expression. Dim indicates that most patients with CLL express CD20 and sIg, albeit, with a low level of expression. (Adapted from Yee and O'Brien, 2006)

DISEASE	sIg	CD5	CD10	CD11c	CD19	CD20	CD22	CD23	CD25	CD79b	CD103	FMC7
Chronic lymphocytic leukemia	Dim	++	-	-/+	++	Dim	-/+	++	-/+	-	-	-/+
Follicular lymphoma	++	-	++	-	++	++	++	-	-	++	-	++
Hairy cell leukemia	+++	-	-	++	+++	+++	+++	-	+++	++	+++	++
Mantle cell lymphoma	++	++	-	-	++	++	++	-	-	++	-	+
Marginal zone lymphoma	++	-	-	+/-	++	++	+/-	+/-	-	++	-	+
Prolymphocytic leukemia	+++	-/+	-/+	-/+	++	++	++	+/-	+/-	++	+/-	++

Most people are diagnosed without symptoms as the result of a routine blood test that returns a high white blood cell count. However, in advanced stages, CLL results in swollen lymph nodes, spleen, and liver, and eventually anemia and infections (Rozman and Montserrat, 1995). Clinical staging is done with the Rai staging system or the Binet classification and they are based on readily available parameters that seem to reflect the biology of the disease (Table 1.2). Thereby, Rai staging system assumes that B-CLL cells first proliferate in the bone marrow and blood, followed by the lymph nodes, then spleen and liver, and finally, anaemia and thrombocytopenia are developed, caused by high tumor burden in the bone marrow. Binet modified his system based on further multivariate analyses of patients. Different parameters accesible to the typicial clinical laboratory have been evaluated as a markers of poor prognosis. Thereby, absolute lymphocyte count and lymphocyte doubling time are independent factors (Krober et al., 2002; Montserrat et al., 1986). A doubling time of less that 12 months identified a population of patients with poor prognosis, even among early stage disease. Another measure of disease burden is the lactate deshidrogenase (LDH), which is commonly elevated in CLL and other lymphomas. Elevated LDH levels have been shown to have prognostic value (Dohner et al., 2000). Beta-2-microglobulin (β_2M) is an extracellular protein nonconvalently associated with the class I major histocompatibility complex (MHC), and expressed by nucleated cells. Serum

levels show positive correlation with clinical staging systems, adverse prognostic features and short survival (Di Giovanni et al., 1989).

Table 1.2.- Staging systems in CLL (adapted from Butler and Gribben, 2010)

Rai stage	**Characteristics**	**Treat in practice?**
0	Lymphocytosis	No
I	Lymphocytosis + lymphadenopathy	Symptomatic patients only
II	As above + splenomegaly + hepatomegaly	Symptomatic patients only
III	As above + anemia Hb < 11 g/dL	Yes
IV	Thrombocytopenia <100 x 10^9 / L	Yes
Binet stage	**Characteristics**	**Treat in practice?**
A	Fewer than 3 areas of lymphadenopathy	Symptomatic patients only
B	3 or more areas of lymphadenopathy	Symptomatic patients only
C	Anaemia Hb <11 g/dL and/or thrombocytopenia <100 x 10^9 / L	Yes

The etiology of disease is unknown. Interestingly, CLL is the only leukemia whose incidence did not increase in people affected by ionizing radiation, drugs or chemical (except in the case of some chemicals used in agriculture) (Rozman and Montserrat, 1995; Shanshal and Haddad, 2012). Viral infection have not been associated with disease (Faguet, 1994). Interestingly, there is a modest genetic susceptibility in CLL. Epidemiological evidences indicate that in 5-10% of cases there is a familial susceptibility to CLL (Goldin et al., 2004; Plass et al., 2007; Yuille et al., 2000). However, different results suggest that one or even two or three loci directly responsible for a sizeable portion of the genetic predisposition to CLL are unlikely to exist. In this case, a part of the inherited susceptibility to the disease is mediated through low-risk alleles (Goldin et al., 2004; Houlston et al., 2003; Sellick et al., 2006).

There is no cure today for CLL, and CLL treatment focuses on controlling the disease and its symptoms rather than on an outright cure. The treatment of late CLL is based in chemotherapeutic agents (chlorambucil, fludarabin, cyclophosphamide, bendamustine, etc), monoclonal antibodies (rituximab, alemtuzumab or ofatumumab) radiation therapy or even bone marrow transplantation (Byrd et al., 2006; Ghia et al., 2007; Wierda and O'Brien, 2006; Yee and O'Brien, 2006). However, the most usual treatment regimen contains fludarabine, cyclophosphamide, bendamustine and rituximab, which has been demonstrated higher overall response rates and complete response rates than those seen with either fludarabine or fludarabine with cyclophosphamide in salvage therapy for patients with preiously treated CLL (Robak et al., 2010)

Several transformations have been described in CLL (reviewed in (Yee and O'Brien, 2006) which is often associated with poor pronogsis:

a. Richter Syndrome (RS): RS is a transformation of CLL to a large cell or immunoblastic lymphoma. It occurs in up to 10% of patients with CLL. RS includes features unusual in CLL, such as systemic symphtoms, rapidly

progressive lymphadenopathy, extranodal disease, monoclonal gammopathy, hepatosplenomegaly, anemia, thrombocytopenia (Yee et al., 2005).

b. Prolymphocytic Leukemia (PLL): PLL transformation of CLL may be recognized when two populations of cells are present and the number of prolymphocytes are greater than 15% admixed with the small round lymphocytes of CLL. Features associated with transformation include leukocytosis, splenomegaly, hepatomegaly, and lymphadenopaty. (Ghani et al., 1986).

1.7.2 Molecular biology of CLL

In general, CLL is a slow progression disease, although it is known that CLL presents a marked variability. About one third of patients shows a more aggressive form of the disease with shorter survival periods (a few years), whereas others present a relative stable form of the disease or show a slow progression leukemia and, actually, most of them do not require treatment (Butler and Gribben, 2010; Chiorazzi et al., 2005). Several molecular features have prognostic value. Thus, a worse prognosis and more rapid progression are associated to the mutation/rearrangement of IgV_H genes; the high expression of the ZAP-70 kinase (kinase implicated in activation of lymphocytes by their surface receptors for antigen), CD38 (one surface molecule that supports B-cell interactions and differentiation) or lipoprotein lipase (LPL) (an enzyme that hydrolyzes triglycerides in lipoproteins) (Butler and Gribben, 2010; Chiorazzi et al., 2005; Damle et al., 1999; Del Principe et al., 2006; Hamblin, 2004; van't Veer et al., 2006). Of all these factors, mutation status is the most powerful predictors of prognosis (Table 1.3). Therefore, according to this features, CLL cases are divisible into two groups (Chiorazzi et al., 2005):

- Patients where B-CLL cells have rearranged its IgV_H genes. In general, these patients show low levels of CD38, Zap-70 or LPL, and this is associated with a less aggressive form of the disease. This group is known as “mutated” CLL.
- Patients where B-CLL cells show few or no mutations (less than 2 percent) in its IgV_H genes. This is correlated with high levels of CD38, Zap-70 or LPL. In these patients, a short time to developing symptoms is observed, requiring therapy and inferior survival when compared to those patients with IgHV mutated status. This group is known as “unmutated” CLL.

Different prognosis of mutated and unmutated CLL may be implicated with the role of the immunoglobulin B Cell Receptor (BCR) mediated signaling. Whereas unmutated CLL express higher levels of surface immunoglobulin, and displays a greater ability to respond to stimulation of the BCR, mutated CLL tending to resemble anergic cells in producing lack of response (Lanham et al., 2003; Nedellec et al., 2005). In fact, the cross-linking of BCR with anti-IgM led to the transcripition of genes which form part of cell-cycle regulation, cytoskeletal organization and proliferation, but only in unmutated CLL (Guarini et al., 2008). Thereby, BCR

ligation in unmutated CLL leads to prodominantly activating and proliferative responses, whereas BCR signalling in mutated CLL favors anergic and antiapoptotic responses. All this led to the hypothesis that CLL may be two distinct entities, one deriving from naive (unmutated) B cells and the other from postgerminal center memory (mutated) B cells. However, this hypothesis is also under discussion because gene expression profiling studies in CLL identified only a relatively small number of gene use differences between unmutated and mutated CLL, as opposed to thousands of differences between normal B lymphocytes and either CLL. The current hypothesis is that both mutated and unmutated CLL have derived from memory B cells (Klein et al., 2001; Rosenwald et al., 2001), but the origin, the causes and the differences between subtypes of CLL have not been solved yet. [for a review see (Chiorazzi and Ferrarini, 2011)].

Other markers have been identified in CLL. In general, cytogenetic aberrations are rare in early CLL and therefore are not likely to be inducing factors. Nevertheless, some appear as the disease progresses. In fact, several comprehensive studies showed that over 50-80 % of CLL samples are characterized by the occurrence of the major chromosomal lesions (Table 1.3) (Dohner et al., 2000; Pfeifer et al., 2007; Plass et al., 2007). The most common is a deletion at 13q14.3, which occurs in 55% of cases. This deleted region contains two micro-RNA genes (Calin et al., 2002), miR15 and miR16, which are deleted or down-regulated in most cases of CLL (Calin et al., 2004). Both micro-RNAs are implicated in the regulation of the anti-apoptotic oncoprotein BCL-2 (Cimmino et al., 2005) which is overexpressed in B-CLL cells (Buggins and Pepper, 2010). 11q22-23 and 17p13 are also frequently deleted (Dohner et al., 2000; Pfeifer et al., 2007; Plass et al., 2007). The ataxia-telangiectasia mutated (ATM) and p53 are included in these deletions respectively. Both genes regulate apoptosis, DNA repair, enhance tumour cell proliferation and confer resistence to chemotherapy. In fact, these deletions are relatively frequent in unmutated CLL cases with a poor outcome (Oscier et al., 1997), and have been associated to disease progression, resistence to conventional therapies and poor survival. (Cordone et al., 1998; Dohner et al., 1995; Dohner et al., 2000; Dohner et al., 1997; Pettitt et al., 2001; Plass et al., 2007). Other chromosomal aberration are also observed. Among them, trisomy 12 is found in about 20% of cases as a result of duplication of one chromosome. This trisomy is usually associated with atypical morphology and the high-risk pathway of the disease (Matutes, 1996), although the molecular mechanism by which this genetic abnoramility contributes to leukemogenesis is unknown. All these markers, together with morphology and immunophenotype features, have an important prognostic impact and they are routinely used in diagnosis of CLL (Matutes et al., 2007).

Recently, a whole genome sequencing has identified a recurrent mutation in Notch1, Xpo1, MyD88 and Klh6 in 12.2%, 2.9%, 2.4% and 1.8% of CLL cases, respectively. Notch1 and Xpo1 mutations are associated with IgV_H unmutated CLL while MyD88 and Khl6 are associated with IgV_H mutated CLL. Both Notch1 and MyD88 mutations appear to be activating mutations. Notch1 mutated cases have been shown to overexpress Notch1 pathway genes and are associated with unfavorable prognosis (Puente et al., 2011). Moreover, investigation of the

coding genome of fludarabine-refractory CLL has also revealed a recurrent mutation in a component of the spliceosome, SF3B1. The mutations were found in 17% of fludarabine refractory cases but in only 5% of CLL at diagnosis (Rossi et al., 2011). This mutation has also been observed in 9.7% of CLL patients by whole-genome sequencing. Moreover, SF3B1 mutations were associated with faster disease progression and poor overall survival (Quesada et al., 2012)

Table 1.3.- Impact of molecular markers on prognosis in CLL. TTT = time to treatment; OS = overall survival (adapted from Butler and Gribben, 2010).

Marker		Frequency, %	TTT, mo	OS, mo
Cytogenetics	del13p	55	92	133
	Normal	19	49	111
	Trisomy 12	16	33	114
	del 11q	13	13	-
	del 17p	9	9	-
IgVH	Mutated	47	110	300
	Unmutated	53	42	115
	ZAP-70	54	110	NS
	Negative	46	35	NS
	Positive			
CD38	Negative	67	94	193
	Positive	33	40	109

The conventional view of CLL has been seen as a disease of failed apoptosis and passive accumulation. This view is supported by the observation that the great majority (<95%) of peripherally circulating CLL cells are arrested in G_0 or the early G_1 phase and have overexpression of antiapoptotic proteins (between them Bcl-2). However, other studies suggest that CLL may have a significant proliferative capacity, which might be in accordance with the fact that unmutated CLL has the capacity for proliferative responses to BCR ligation (see above) (Lanham et al., 2003; Nedellec et al., 2005). Proliferation of CLL cells was evaluated through consume fixed doses of deuterated heavy water (^2H_2O) by different patients. This experiment showed that all patients had a rate of new cell formation of at lest 10^9 new cells per day, and patients with higher proliferation rates were more likely to have symptomatic disease (Messmer et al., 2005). These data suggest that CLL is a dynamic disease comprised of birthing and dying cells.

In agreement with these data, CLL located in the lymphs nodes exhibited a higher percentage of Ki67-positive and ^{3}H-thymidine-labelled cells (both proliferation markers) compared to blood (Smit et al., 2007; Themi et al., 1973). These studies suggested that CLL cell proliferation occurred primarily within solid lymphoid tissues, whereas in peripheral blood CLL cells remain quiescent. Therefore, CLL cells may find in lymph nodes an optimal environment for cell division. This may be explaining by the microenvironment of lymphoid tissues. The tumor microenvironment has been suggested to play an essential role in the survival and progression

of CLL. The tumor microenvironment describes an admixture of malignant cells with host immune cells, stromal elements, and vascular cells that create a niche wherein signals can be transmitted through antigen presentation, cell-cell interactions, and paracrine signalling. In secondary lymphoid organs, $CD14^+$ mononuclear cells called nurse-like cells (NLCs) are present. In contrast, in bone marrow mesenchymal stroma cells (MSCs) may be found. However, both NLCs and MSCs constitutively express the chemokines CXCL12 (also known as Sdf-1), whereas CLL cells express CXCR4 (CD184), the receptor for Sdf-1. Engangement of Sdf-1 with CXCR4 promotes cell survival (Burger et al., 2000; Burger et al., 2005). Moreover, NLCs also secretes B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which induces intracellular NF-kappaB1 and Mcl-1 in CLL cells (Nishio et al., 2005). In fact, expression of Mcl-1 by co-culture of CLL cells with MSCs induces resistence to fludarabine, cyclosphosphamide and dexamethasone, suggesting that the tumor microenvironment enhances CLL cell resistance to chemotherapy-induced apoptosis (Kurtova et al., 2009).

1.7.3 Myc in CLL

Different reports have shown that in most of CLL patients Myc expression is low (Birnie et al., 1984; Drexler et al., 1989; Ferrari et al., 1985; Greil et al., 1991; Korz et al., 2002; Larsson et al., 1991). However, high levels of Myc mRNA have been reported in some patients, and this expression has been associated with more aggressive forms of the disease (Halina et al., 2010; Rechavi et al., 1989) Indeed, some studies have found that patients with advanced Rai stages are characterized by higher Myc mRNA levels (Greil et al., 1991; Zhang et al., 2010). Nevertheless, Myc expression is uncommon in B-CLL cells, and its regulation in this neoplasia poorly understood. However, most of the studies have been performed at the mRNA levels and thus, there is no information on the expression of Myc protein in this leukemia. Myc gene is rarely rearrangement in CLL (Boehm et al., 1987; Nelson et al., 2007; Raghoebier et al., 1991; Rechavi et al., 1989; Wotherspoon et al., 1990) and different alterations, as amplifications or translocations, have been reported in 1% of CLL patients (Boehm et al., 1987; Brown et al., 2012; Crowther-Swanepoel et al., 2010; Edelmann et al., 2012; Fabris et al., 2011; Forconi et al., 2008; Huh et al., 2008; Put et al., 2012; Rechavi et al., 1989; Rimokh et al., 1991; Rinaldi et al., 2011), and most of them was associated with increased prolymphocytes, complex cytogenetic abnormalities and a poor prognosis. Other common cytogenetic alterations, as 17p- or 11q-, have been associated with an increase or a decrease in the levels of Myc, respectively (Kienle et al., 2005). Moreover, it has been reported recently that MGA, a suppressor of Myc, is inactivated in 10,4% of patients with high risk (de Paoli et al., 2012) However, the function of Myc in this subset of CLL is unknown. The low Myc expression is consistent with the finding that CLL cells are resistant to transformation with Myc, even if it is transfected together Ras oncogene (Zheng et al., 1996; Zheng et al., 1992). When B-CLL cells are induced to undergo plasmacytoid differentiation in vitro in response to TPA, IFN-alfa2 or bryostatin1, an increase of

Myc is observed (Einhorn et al., 1988; Hu et al., 1993; Segel et al., 2003). In this process of differentiation, B-CLL cells pass through several intermediate stages of differentiation. Myc is only expressed in these intermediate stages, and its levels are decreased in the last stage, when B-CLL cells have been terminally differentiated to plasma cell (Larsson et al., 1991). Myc has been also associated with induced apoptosis. B-CLL cells are susceptible to irradiation-induced apoptosis, although an aggressive subset of B-CLL is completely resistent. Myc was one of genes whose expression was associated with resistence (Vallat et al., 2003) With regard to drug-induced apoptosis, Myc is able to play both inductor and repressor roles. When B-CLL cells are treated with theophylline and chlorambucil, Myc is induced and this occurs concomitantly with downregulation of Bcl-2 expression. In this case, Myc is associated with its function as an inductor of apotosis (Mentz et al., 1996). In contrast, a subset of CLL patients with resistence to fludarabine-treatment showed higher levels of Myc than sensitive CLL patients (Moussay et al., 2010). These patients showed an accumulation of genomic abnormalities, including 17q- or 8q- gain, which have been associated with high levels of Myc (see above). Moreover, 90% showed 13q- deletion, which has been implicated with Bcl-2 overexpression (Calin et al., 2002; Cimmino et al., 2005). Bcl-2 inhibits the pro-apoptotic effect of Myc (Wagner et al., 1993), and therefore it is very likely that both work together in these resistant patients. Taken together, all these data suggests the possibility that a reduced subset of CLL exhibits Myc deregulation, which are associated with poor prognosis and resistance to induce apoptosis [reviewed (Rossi, 2013)].

1.7.4 p27 in CLL

p27 overexpression in B-CLL cells has been observed in different studies (Pabst et al., 2000; Vrhovac et al., 1998; Winkler et al., 2005; Wolowiec et al., 2001). Contrary to the traditional role of p27 as a tumor suppressor, its overexpression in B-CLL lymphocytes has been associated with poor prognosis (Halina et al., 2010; Vrhovac et al., 1998; Wolowiec et al., 2009). Mechanisms through which p27 colaborates with progression of disease is poor understood, but several indications suggest that p27 expression may be associated with impairment of apoptosis. Thus, CLL-lymphocytes with high levels of p27 have a lower spontaneus cell death ratio in culture (Ricciardi et al., 2001; Vrhovac et al., 1998), and its degradation is a key step in chemotherapy-induced apoptosis by different drugs, such as fludarabine (Sanhes et al., 2003; Vrhovac et al., 1998), flavopiridol (Billard et al., 2003), roscovitine (Zolnierczyk et al., 2009), rituximab (Grdisa, 2004) or hyperforin (Quiney et al., 2006). Because p27 cleavage was detected soon after drug treatment, it is not possible that this occurs due to a late consequence of unspecific degradation by caspases (Sanhes et al., 2003), indicating that p27 degradation may be a key stage in the drug-inducing apoptosis. This idea agrees with the work of Ricciardi et al., who observed that patients with progressive disease showed a higher degree of

quiescence and lower susceptibility to apoptosis than patients with a stable disease (Ricciardi et al., 2001).

1.8 Erythroid differentiation induced by p27 in myeloid cells

The differentiation effect of p27 in the K562 cell line has been demostrated in previous work in our laboratory. K562 are cells derived form chronic myeloid leukemia that are capable of erythroid and megakaryocytic differentiation depending on the agent used or the gene induced (Delgado et al., 1998; Delgado et al., 1995; Delgado et al., 1992; Lerga et al., 1999; Munoz-Alonso et al., 2005) Muñoz-Alonso et al. proved that ectopic expression of p27 in the K562 cell line results in erythroid differentiation (Munoz-Alonso et al., 2005). Moreover, p27 protein expression is up-regulated in the induction of erythroid differentiation of K562 through the treatment with cytosine arabinoside (AraC) or other chemicals (Aoki et al., 2004; Gomez-Casares et al., 2012; Munoz-Alonso et al., 2005). Indeed, inhibition of this endogenic expression through shRNA of p27 leads to a decrease in the ability of differentiation of these drugs (Gomez-Casares et al., 2012). Together, these results showed the important role of p27 in this process of differentiation.

Different transcription factors have been associated with the erythroid differentiation. This “erythroid differentiation master genes” are implicated in the repression and activation of a large number of genes (Higgs, 2005). These master genes can be used as makers of erythroid differentiation along with other specific proteins of erytrocytes.

- Transcription activators of erythroid differentiation :
    - Gata1: a member of the GATA transcription factor family. Gata1 is essential for erythroid development because it plays a role in regulating the switch of fetal hemoglobin to adult hemoblobin.
    - NF-E2: Component of the NF-E2 complex, essential for regulating erythroid maturation and differentiation. This subunit recognizes the TCAT/C sequence of the AP-1-like core palindrome present in a number of erythroid promoters.
    - Stat5A: Signal transducer and activator of transcription 5A (Stat5A) is a member of the STAT family which is activated by erythropietin and other factors associated to erhythroid differentiation.
- Specific proteins of erythrocytes.
    - Glycophorin A: It is a sialoglycoprotein of the membrane of erythropotic cell, which bears the antigenic determinants for the MN and Ss blood groups.
    - Hemoglobin: Hemoglobin or Hb is the iron-containing oxygen-transport metalloportein in the erythrocytes. Hemoglobin carries oxygen and CO_2 in the blood. Hb consists mostly of protein subunits (termed globins). Several globins have been identified, which are synthesized at different stages of development.

- EpoR: Erythropoietin receptor (EpoR) is a 59 kDa peptide which is a member of the cytokine receptor family. At present, the most well-established function of EpoR is to rescue erythroid progenitors from apoptosis.

2.- AIMS

2 AIMS

CLL is a disease with a low proliferative activity because B-CLL cells seem to be arrested in G_0/G_1 phase of the cell cycle. Therefore, and although the pathogenesis of the disease is still unclear, cell cycle pathway seem to be altered in CLL. p27 is a key regulator of the early phase of the cell cycle, and its expression is correlated with the inhibition of cell cycle progression. High levels of p27 indicate low proliferation, and p27 is frequently down-regulated through different pathways in human cancer. However, why p27 is expressed and which its function in B-CLL cells may be is unknown.

Moreover, correlation between high expression of Myc and low levels of p27 has been found in many tumors, and it is consistent with poor prognosis. However, in CLL, p27 is expressed, but it is unknown the expression of p27 and Myc at the protein level in this leukemia and whether p27 can regulate p27 can regulate Myc expression in this disease, and whether this regulation can have some diagnostic significance. Thereby, CLL is an interesting model to study the interaction between Myc and p27.

Functional antagonism in the differentiation process between p27 and Myc is still poorly understood. Our laboratory has reported a p27-inducing erythroid differentation model, where Myc acts as an inhibitor of this process. However, what it is the mechanism through which p27 induces differentiation, and what the regulation is between Myc and p27 in this model are still poor known.

Given the previously results, the aims of this study are:

- To study Myc and p27 mRNA and protein expression in CLL, its cellular localization and whether this expression pattern has some diagnostic significance.
- To examine cell cycle regulation carried out by p27 and Myc in CLL and their role in the high resistance to apoptosis of B-CLL cells, and its association with antiapoptotic protein Bcl-2.
- To study the mechanism through which p27 regulates K562 erythroid differentiation. Dissect the p27 domain involved and the role of Myc downregulation carried out by p27 in this process.

3.- MATERIAL AND METHODS

3 MATERIALS AND METHODS

3.1 Clinical samples

3.1.1 B-CLL cells and normal B-cells

Clinic samples were provided by Dr María Ángeles Cuadrado and Dr Ana Batlle from the Marques de Valdecilla Hospital (Santander), by Dr Teresa Gomez-Casares from Dr. Negrín Hospital (Las Palmas), by Dr Miguel Ángel Cortés from Laredo Hospital (Laredo, Cantabria) and by Dr Dolors Colomer from Hospital Clínic of Barcelona (Barcelona). Samples were provided after diagnosis was made. Informed consent was obtained from every patient.

B-CLL cells samples were from patients with CLL, whereas control samples were from healthy donors (peripheral blood lymphocytes) and from tonsillectomy of patients with tonsillitis (tonsils). B-CLL cells were isolated by Ficoll-Paque or Flux cytometry. Clinical charts of all patients were reviewed and the following data recorded: age, sex, clinical stage according to Rai classification, lymphocyte counts, lymphocyte doubling time (DT), treatment, B symptoms, serum markers (LDH and β_2-microglobulin), Zap70 and CD38 expression, IgV_H status and cytogenetic alterations such as 13q14, trisomy 12, p53 or 11q14. The 70 % of patiets were males and age at diagnosis ranging from 31 to 94 years (their mean age was 69,5 years). Most of patients (63 %) were diagnosed in an early stage of the disease (stages 0 and I). The 46 % of patients showed a progressive form of the disease, of which most of them (86 %) were treated. Active or progressive disease was considered when at least one of the following criteria was present: a) Evidence of progressive marrow failure manifested by the development anemia (Hb <10g/dL) and/or thrombocytopenia (<100x10^9/L); b) massive (i.e., at least 6 centimetre below the left costal margin) or progressive or symptomatic splenomegaly; c) massive nodes (i.e., at least 10 centimetre in longest diameter) or progressive or symptomatic lymphadenopathy; d) progressive clonal lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte DT of less than 6 months. Lymphocyte DT was not used as a single parameter to define a treatment indication; e) autoimmune cytopenia that is poorly responsive to standard therapy; f) constitutional symptoms.

3.1.2 Isolation of mononuclear cells from human peripheral blood by density gradient centrifugation

Blood samples from patients (3.5 mL) were diluted with the same volume of PBS (3.5 mL). Diluted cells were carefully poured over 3 mL of Ficoll-Paque (Linfoprep) avoiding mixing of the two phases. Tube was centrifuged at 2000 rpm for 15 minutes in a swinging-bucket rotor without brake. As a result of centrifugation, samples were separated in three phases and two interphases. From top to bottom, plasma phase, mononuclear cells interphase, ficoll phase, granulocytes interphase and erythrocytes phase were distinguished. The upper layer (plasma phase) were aspirate and mononuclear cells from interphase were transfered to a new tube, washed twice in PBS and centrifuged at 1500 rpm for 5 minutes.

To isolate CD19+ B-cells, it was added 10 µl of CD19+ Antibody (Becton Dickinson) and to isolate B-CLL cells, it was added 10 µl of CD19+ Antibody + 10 µl of CD5+ antibody (Becton Dickinson). Cells were incubated at RT for 15 min (in darkness), washed with PBS and centrifuged (1500 rpm for 5 min). Cells were resuspended in PBS ($20x10^6$ cel/ml) and filtered into a 50 µM filter. Cells were isolated by FaCSAria.

3.1.3 Isolation of CLL cells from lymph node and bone marrow

Single cell suspensions were obtained from the lymph node by physical disruption using a 20 gauge (0.9 mm) needle attached to a sterile plastic syringe. These cells were then isolated in the FACSAria (BD) using the FCS/SSC parameters and CD19+ and CD5+ antibodies (surface markers specific of B-CLL cells).

3.2 Cell culture

3.2.1 Culture and induction of different cell lines

Different cell lines were incubated with RPMI Media 1640 (Gibco) supplemented with 10 % FCS (fetal calf serum), gentamicin (80 µg/mL) and ciprofloxacin (2 µg/mL), at 37ºC and 5% CO_2. Cells were growth in exponential phase ($<5x10^5$ cells/mL).

- K562 cells were the first human immortalised myelogenous leukemia line to be established. The line was derived from a 53 year old female CML patient in blast crisis. The cells are non-adherent and rounded, are positive for the bcr:abl fusion gene and they can be induced to myelocytic, erythrocytic or monocytic differentiation (Lozzio and Lozzio, 1975). Our K562 were purchased from American Type Culture Collection.
- Kp27-5 cell line is a subline of K562 cells, which carry a temporal control of ectopic p27 expression. In this cells, p27 is regulated by the sheep metallothionein promoter for

Zn^{2+}-inducible expression, and therefore p27 is only expressed when cells are growth in presence of Zn^{2+} (Munoz-Alonso et al., 2005). Kp27-5 cell line was treated with 75 µM $ZnSO_4$ for exogenous p27 expression.

- Kp27MER cell line derived from Kp27-5 cell line. Kp27-5 cells were stably transfected with an expression vector for the fusion protein MycER, where Myc activity is activated by 4HT. Thus, addition of Zn^{2+} and/or 4HT to medium allow the conditional expression of p27 and Myc respectively (Acosta et al., 2008). Kp27MER cells were treated with 75 µM $ZnSO_4$ for exogenous p27 expression and with 100 nM 4-hidroxy-tamoxifen (4HT) for exogenous Myc activation.
- P493.6 cell line was stablished by stable transfection of EREB2-5 cells with the construct pmyc-tet. P493.6 cell line is defined as a human EBV-EBNA1 positive B-cell line, in which Myc is expressed under the control of a tetracycline regulated promoter (Pajic et al., 2000). P493.6 cells require tetracyclin-free serum to maintain the expression of Myc and grow. P493.6 cells were treated with 500 nM doxycicline to repress Myc.
- MEC1 cell line was established from a patient with CLL in prolymphocytioid transformation. MEC1 cells are non-adherent and form large clumps. The cells have a complex karyotype, overexpress Bcl-2 and are negative to CD5 (Stacchini et al., 1999).

3.2.2 Transfections

Cells were electroporated with plasmid by Amaxa Nucleofector Technology (using amaxa nuclefector solution V). Millions of cells, concentration of plasmid and program of nucleofector were chosen according to cell&transfection database of Amaxa-Lonza. To transfer MEC1 cells, 8 x 10^6 cells and 10 µg of plasmid were used (Amaxa U-17 program).

To generation of KCK- cell lines, K562 were electroporated with pMT-CB6-p27CK- expression vector. Cells were incubated for 48 hours in RPMI supplemented with 10% FCS. Then, 500 µg/mL of G418 was added until obtaining a pool with resistant cells. Clones were selected by limiting dilution using T96 plates. Clones showed expression of p27CK- after treatment with 75 µM $ZnSO_4$ were selected.

3.3 DNA and RNA analysis

3.3.1 Plasmid and purification

150 ml of bacterial cells was pelleted by centrifugation at 10000 x g for 10 minutes at 4º C. Plasmid DNA was efficiently purificated by QIAGEN Plasmid Midi kit. To precipate the DNA, 10 ml of Isopropanol was added to the eluate. After 5 minutes at room temperature, eluate was

centrifuged at 10000 rpm for 30 minutes at 4ºC. Supernatant was removed and DNA was washed with 2.5 ml of 75%-ethanol and air-dry for 5 minutes. Dry DNA was resuspended in nuclease free-water and preserved at -20ºC.

Digestion of plasmids was carried out by restriction enzymes following the instructions of provider (Roche, Amersham, Fermentas, Invitrogen). Fragments were mixed with loading buffer (0.005% (w:v) bromophenol blue, 30% (v:v) of glycerol) and they was separated by gel agarose electrophoresis (Pronadisa). To run the gel, it was used TAE buffer (0.09 M Tris-acetic acid, 2 mM EDTA) with 0.5 ug/ml of ethidium bromide. Gel run for 30-60 minutes at 100 V. 1 Kb DNA ladder and 100 bp DNA ladder (Fermentas) was used as molecular weight marker.

Table 3.1.- Plasmid used.

Plasmid	Origin	Reference
pCEFL	P. Crespo (Santander)	(Teramoto et al., 1996)
pCEFL p27		(Munoz-Alonso et al., 2005)
pCDNA 3.1	J.M. Roberts and A. Besson	(Besson et al., 2006)
pCDNA 3.1 p27	J.M. Roberts and A. Besson	(Besson et al., 2006)
pCDNA 3.1 p27CK-	J.M. Roberts and A. Besson	(Besson et al.. 2006)
pEYFP	Clontech	
pEYFP p27	G. Bretones	This work
pEYFP p27CK-	G. Bretones	This work
pMT-CB6+	F.J. Rausher	
pMT-CB6 p27CK-		This work
pCS2+	J.M. Roberts and A. Besson	(Besson et al.. 2004b)
pCS2+ p27-Nt.	J.M. Roberts and A. Besson	(Besson et al.. 2004b)
pGL3-Luc	Promega	
-2489-Myc promoter pGL2b	Lee T.C. and Ziff E.B.	(Lee and Ziff, 1999)
pRS	Oligoengine	
pRSshMyc		(Bernard et al., 2003)
pLKO.1	OpenBiosystems	
pLKO.1 shMyc	OpenBiosystems	

3.3.2 DNA subcloning

p27CK- inserts from pCDNA3.1 vector (J.M. Roberts and A. Besson, Howard Hughges Medical inst. Seattle) was subcloning into pMT-CB6 vector (F.J. Rausher, Wistar Inst. Philadelphia). pCDNA3.1 p27CK- and pMT-CB6 vectors were digested with HIND III and XBA1

enzymes (5 µg of DNA in buffer Tango 1x for 2 hours) to obtain compatible overhangs. Digestion was checked running fragments into gel agarosa (insert had a size of 650 kb). A clean blade was used for excising the inserts from gel agarosa. PCR Clean-up System Kit (Promega) was used to elute the DNA from agarosa.

Ligation of inserts with pMT-CB6 vector digested was carried out for 12 hours at room temperature by T4 DNA ligase following the instructions of provider (Invitrogen). DH5α (competent cells, efficiency > 10^8) was used to transformation. Plasmid DNA from DH5α colonies was purified and digested as it was described above. The subcloned insert was confirmed by sequencing.

3.3.3 RNA purification (TRIZOL Reagent)

Cells was centrifuged for 5 min at 1500 rpm. Supernatant was removed and pellet was washed in cold PBS. After a spin at 1500 rpm for 5 min, pellet cells was lysed with TRIZOL Reagent by repetitive pipetting. It was used 1 ml of the reagent per 5-10 x 10^6 of cells. Homogenized sample was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of TRIZOL Reagent was added and samples were vortexed vigorously for 15 seconds and incubated them at room temperature for 2 to 3 minutes. Then samples were centrifuged at 12000 rpm for 15 minutes at 4ºC to separate mixture into three phases: a phenol-chloroform phase, an interphase, and a colorless upper aqueous phase (the volume of the aqueous phase is about 60 % of the volume of TRIZOL Reagent used for homogenization). RNA remains exclusively in the aqueous phase. Upper aqueous phase was transferred carefully into fresh tube and RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used. After 10 minutes at room temperature, samples were centrifuged at 12000 rpm for 10 minutes at 4ºC. Supernatant was removed and RNA was washed once with 1 ml of 75%-ethanol per 1 ml of TRIZOL Reagent. It was added at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization to samples, and then they were centrifuged at 7500 rpm for 5 minutes at 4ºC. Left over ethanol was removed and sampes were air-dry for 5-10 minutes. RNA was dissolved in nuclease free-water through a pippette tip.

3.3.4 Reverse transcription and real-time PCR

Reverse transription was performed with iScript reverse transcription supermix for RT-qPCR kit (Bio-Rad). Supermix was added to 1 µg of RNA and mix was incubated in a thermal cycler using the following protocol and cDNA was stored at -20ºC.

4 Priming: 5 minutes at 25ºC.

5 Reverse transcription: 30 minutes at 42ºC.

6 RT inactivation: 5 minutes at 85ºC.

Quantitative PCR (qPCR) was performed with the IQ™ SYBR Green Supermix kit (Bio-Rad) in a Bio-Rad MyiQ apparatus. Components were added (nuclease free-water, forward and reverse primer, SYBRGreen supermix and cDNA) and they were incubated using the following protocol:

I. Enzime activation and initial denaturation: 2 minutes at 95ºC.
II. PCR protocol (40 cycles):
    A. Denaturing: 15 seconds at 95ºC.
    B. Annealing: 15 seconds at 55ºC
    C. Extension: 20 seconds at 72ºC.
III. Final extension: 5 minutes at 72ºC.
IV. Melt curve: 5 seconds at 55-95º (in 0.5ºC increments)

To quantification, the C_T valous of test samples were normalized by the internal control ribosomal protein RPS14, which was used as a housekeeping gene control samples (gene that was expressed at relatively constant levels in the experimental conditions). Primers sequences and amplicon sizes used in RT-qPCR assays are shown in Table 2.2.

Table 3.2.- Human primers used in the RT-qPCR assays.

Genes	Sequence	Sequence size
RPS14	5'-CAGGTCCAGGGGTCTTGGTCC-3' 5'-GGCAGACCGAGATGAACTCTC-3'	150 bp
GypA	5'-CATTGATCACTTGTCTCTGG-3' 5'-GAGAAAGGGTACAACTTGCC-3'	236 bp
Gata1	5'- CCAAGCTTCGTGGAACTCTC-3' 5'- CCTGCCCGTTTACTGACAAT-3'	202 bp
EpoR	5'-CTGGTCGGAGCCTGTGTC-3' 5'-GAGCACGGTCAGCAGCAC-3'	103 bp
Mxd1	5'-GATGCCTTAAAACGGAGGAAC-3' 5'-ACTGATTCGGGTCCAAGTG-3'	147 bp
Myc	5'-TCGGATTCTCTGCTCTCCTC-3' 5'-GAGCCTGCCTCTTTTCCAC-3'	200 bp
p27	5'-TCACCGCCCTACACATCAAACT-3' 5'-AGAAGAATCGTCGGTTGCAG-3'	120 bp
Skp2	5'-AGCCCGACAGTGAGAACATC-3' 5'-GAAGGGAGTCCCATGAAACA-3'	168 bp
Stat5A	5'-GGACCTTCTTGTTGCGCTTT-3' 5'-GGCGGTCAGGAAACACATAG-3'	287 bp
ε-globin	5'-GCAAGAAGGTGCTGACTTCC-3' 5'-TGCCAAAGTGAGTAGCCAGA-3'	169 bp
LPL	5'-CTGCTCGTGCTGACTCTGG-3' 5'-TGTCCTCAGCTGTGTCTTCAG-3'	330 bp

3.3.5 Chromatin immunoprecipitation (ChIP)

10 x 10^6 cells were fixed in 1% formaldehyde for 10 minutes at RT. 125 mM Glycine was added for 10 minutes at RT. Cells were washed with cold PBS and lysed with lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl pH 8) for 10 minutes. Cell lysate was sonicated once for 30 minutes at 4º C (6 x 30 s pulses) to shear DNA to lengths between 200 and 1000 basepairs. Lysate was centrifuged at 12000 rpm for 10 minutes at 4º C and sobrenadant was aliquoted in 300 µl aliquiots and 50 µl which will be used as imput. The aliquiots used were diluted 10 fold in Dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl pH 8, 167 mM NaCl).

Chromatin immunoprecipitation (ChIP) was performed by using Dynabeads-Protein G (Invitrogen) coupled to anti-p27 antibody and rabbit IgGs as specifity control. To reduce nonspececific background, Dynabeads were wash with 0.2 mg salmon sperm DNA at 4º C for 30 min. After Dynabeads were incubated with lysates overnight at 4ºC and washed for 5 minutes at RT once with low salt immune complex wash buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris Hcl pH 8, 150 mM NaCl), high salt immune complex wash buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), LiCl immunce complex wash buffer (0.25 M LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris HCl pH 8) and twice with TE buffer (10 mM Tris HCl pH 8 and 1 mM EDTA). Chromatin was eluted with 500 µl elution buffer (10 mM Tris HCl pH 8, 0.1 M $NaHCO_3$, 1% SDS), and along with imput was decrosslinked (overnight at 65º C). Then 10 µl of 0.5 M EDTA, 20 µl 1 M Tris HCl pH 6.5 and 2 µl of 10 mg/mL Proteinase K were added and incubated for one hour at 45º C. DNA was purified through Qiaquick columns (Qiagen). PCRs of the eluted DNA were performed with the primers of Myc promoter. The sequences are listed in table 2.3.

Table 3.3.- Human primers from Myc promoter used in the ChIP analysis. TSS = Transcription start site.

Promoter region	Localization from the TSS	Sequence	Sequence size
Distal upstream sequence used as negative control (-5 kb from the TSS)	-6157 -6448	5'-TGTATGTCCAACCACGCAAG-3' 5'-TTCCTTTGTTGAACCCCTTC-3'	291 bp
-2 kb from the TSS	-2070 -1855	5'-AGGAACCGCCTGTCCTTC-3' 5'-ATCGCTATGCTGGATTTTGC-3'	255 bp
-1 kb from the TSS	-1143 -911	5'-CACAAGGGTCTCTGCTGACT-3' 5'-CACACGGAGTTCCCAATTTC-3'	262 bp
-100 bp from the TSS	-343 -100	5'-CCGCCTGCGATGATTTATAC-3' 5'-CAGCCGAGCACTCTAGCTC-3'	243 bp
Distal downstream sequence used as negative control (+10 kb from the TSS)	+10763 +11385	5'-CCAGTGTCCATGTTCTGTG-3' 5'-CCCTGTGGAAACTTGTCTGG-3'	522 bp

3.4 Luciferase reporter assays

Two million of cells were electroporated with 15 µg of pGL3 luciferase reporter vector (Promega) or with pGL2b carrying a fragment of the human Myc promoter upstream of the firefly luciferase (P2-2489-Myc promoter) as specified in transfection paragraph. After 32 hours, cells were washed with PBS and centrifuged at 1500 rpm for 5 minutes. Lysis Solution I (from Dual luciferase reporter assay system kit of Promega) was used for cell lysis and luciferase activity was then determined with Luciferase Substrate (LARII and stop&glo from Dual luciferase reporter assay system kit of Promega) and a GloMax 20/20 luminometer (Promega), following the manufacturer´s instructions. Data were normalized to the values of the Renilla luciferase (0.5 µg of pRL-TK vector in each transfection).

3.5 Protein analysis

3.5.1 Immunoblot

Cells were harvested and centrifuged for 5 minutes at 1500 rpm. Pellet was washed once in PBS and resuspended in lysis buffer (150 mM NaCl, 50 mM tris pH 8, 1mM EDTA, 1 % NP40, 0.2% SDS, 20 mM NaF, 20 % glycerol) supplemented with protease inhibitor cocktail (Set II of Millipore). Cell lysate was sonicated once for 15 seconds to shear DNA and then it was incubated on ice for 30 minutes with occasional mixing. After, lysate was centrifuged at 12000 rpm for 20 minutes at 4ºC. Supernatant was collected and stored at -20ºC.

Protein concentration was measured in duplicate by Bradford protocol (Bradford, 1976). Using a BSA (bovine serum albumine) standard curve.

It was added Laemmli buffer (63 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025 bromophenol blue and 5% Beta-Mercaptoethanol) to 50-80 µg of protein lysate. Lysate with Laemmli was heated to 95º C for 5 minutes. Then, samples were loaded into and runed on an SDS-PAGE minigel at 100 V for 2-3 hours (running buffer: 0.25 M of Tris pH 8.3, 1.92 M Glycine and 1% SDS) until the blue front was at the bottom of the gel. Percentage of the gel was chosen according to the size of the protein to be analyzed (<25 kD, 15%; 25-50 kD, 12%; 50-90 kD, 10%; 90-200, 8%)

Proteins were transferred into nitracellulose membrane (Schleicher and Schuell). Trans-Blot SD Semi-Dry (Bio-Rad) or Mini-Gel Box Electrotransfer (Bio-Rad) were used at 25 V for 40 minutes to transfer or at 300 mA for 1.5-2 hours respectively (transfer buffer: 0.25 M of Tris pH 8.3, 1.92 M glycine and 1% methanol)

Membrane was blocked for 1 hour in 20 ml TBS-T (20 mM Tris pH 7.5, 137 mM NaCl and 0.05 % Tween 20) with 5% non-fat dry milk or with 5% BSA under agitation. Following, the membrane was incubated with primary antibody (1-5 µg/mL) diluted in TBS-T with 1-5 % BSA for 1-3 hours at room temperature under agitation. After three washed (10 minutes each one) at

room temperature with TBS-T, the membrane was incubated with two types of secundary antibodies (both at 1/10000). One of them was conjugated to peroxidase and they was visualized through chemiluminescence with ECL system (GE healthcare), radiographed in Konica-Minolta films. The other type was conjugated to IRDye 680 or IRDye 800 (Li-Cor Biosciences) and was visualized in an Odyssey scanner. Immunoblot quantification and densitometry was carried out by ImageJ software.

Table 3.4.- Primary antibodies used in this work. It is indicated their use in immunoblot (IB), immunofluorescence (IF) or immunoprecipitation (IP)

Antigen	Antibody type	Origin	Used for	Reference
Actin	Goat polyclonal	Santa Cruz Biotech.	IB	I-19, sc-1616
p27	Rabbit polyclonal	Santa Cruz Biotech.	IB, IF, IP	C-19, sc-528
p27	Rabbit polyclonal	Santa Cruz Biotech.	IB, IF	N-20, sc-527
p-p27 (Ser 10)-R	Rabbit polyclonal	Santa Cruz Biotech.	IB	sc-12939-R
p-p27 (Thr-187)	Goat polyclonal	Santa Cruz Biotech.	IB	sc-16324
Cdk2	Rabbit polyclonal	Santa Cruz Biotech.	IB, IP	M-2, sc-163
Cleaved Notch1 (Val1744)	Rabbit polyclonal	Cell Signaling	IB	D3B8, #4147
Cyclin E	Rabbit polyclonal	Santa Cruz Biotech.	IB	M-20, sc-481
Cyclin A	Rabbit polyclonal	Santa Cruz Biotech.	IB	H-432, sc-751
Myc	Rabbit polyclonal	Santa Cruz Biotech.	IB	N-262, sc-764
Max	Rabbit polyclonal	Santa Cruz Biotech.	IB	C-124, sc-765
Skp2 p45	Rabbit polyclonal	Santa Cruz Biotech.	IB	H-435, sc-7164
Active caspase 3	Rabbit polyclonal	Immunostep	IB	CAS3PU

Bcl-2	Mouse monoclonal	BD Transduction Laboratories™	IB	610538
Gata1	Goat polyclonal	Santa Cruz Biotech.	IB	C-20, sc-1233
Mxd1	Rabbit polyclonal	Santa Cruz Biotech.	IB	C-19, sc-222
NF-E2	Rabbit polyclonal	Santa Cruz Biotech.	IB	C-19, sc-291
EpoR	Rabbit polyclonal	Santa Cruz Biotech.	IB	C-20, sc-695

3.5.2 Immunoprecipitation

$1x10^6$ cells was harvested and washed once with PBS. Cells were centrifuged at 1500 rpm for 5 minutes and pellet was resuspended in non-denaturing lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % NP40 and 1 mM EDTA) supplemented with protease inhibitor cocktail (Set II of Millipore). Lysate was sonicated for 20 seconds and centrifuged at 12000 rpm for 20 minutes. Supernantant was collected and distributed in different eppendorfs. The primary antibody (1 µg) was added and incubated overnight at 4ºC under agitation.

Dynabeads protein G (Invitrogene) were used to immunoprecipitation through magnetic separation technology. Dynabeads were washed twice with 1 mL of lysis buffer and they were added to samples after overnight incubation. Protein-antibody-dynabeads mix was incubated for 90 minutes at 4ºC. Following Dynabeads were separated through a magnet and washed four fold (5 minutes at room temperature) with 1 ml of buffer lysis. Dynabeads were resuspended in Laemmli buffer (described above) and heated at 95ºC for 5 minutes. Antibodies and proteins have been removed from Dynabeads because of Laemmly and heat. Dynabeads were removed using a magnet and samples were loaded in SDS page mini-gel.

3.5.3 Gel filtration chromatography

Total cell lysates were prepared in a 0.5% Triton X-100, 50 mM Hepes/NaOH pH 7.4, 150 mM NaCl, lysis buffer containing 1 mM EDTA, 2,5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1/100 protease inhibitor cocktail (Calbiochem) and 10% glycerol (w/v). 2 mg protein samples (200 µl) were applied onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with Chromatography buffer 50 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 1 mM EDTA, 2,5 mM EGTA, 1 mM DTT, 1 mM PMSF and 10% glycerol (w/v) and subjected to fast-performance liquid chromatography in an ÄKTA purifier

apparatus (GE Healthcare) with a flow rate of 0.4 ml/min at 4°C. 500 µl fractions were collected and subjected to immunoblot. Molecular mass standards for the gel filtration column were as follows: Apoferritin (443 kDa), Catalase (232 kDa), BSA (66 kDa) and Ovalbumin (45 kDa).

3.5.4 Immunoflourescence

Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature. Preparations were washed three times with PBS (5 min each wash). Before permeabilized, cells were washed with PBS glycine 0.1 M and permeabilized with 0.5% triton X-100 (15 min). After two washes in PBS and other wash with PBS with 0.05% Tween20 (5 min), we incubated with primary antibody (Table 2.3.) in PBS for 3 hours/room temperature. Then, slides were washed two times in PBS and with PBS with 0.05 % Tween20 (5 min) before at slides were incubated with anti-rabbit FITC diluted 1:100 (Jackson ImmunoResearch) for 45 min at room temperature in dark. Finally, preparations were evaluated by Fluorescence microscope (Zeiss Axioskop)

3.6 Cell cycle and viability analysis

3.6.1 Cell growth and viability

A hemocytometer was used to count the concentration of cells. Dye exclusion method was used for assessment of cell viability. Trypan blue was the vital stain used to selectively colour dead cells.

3.6.2 Flow cytometry

One million cells per sample were washed with PBS and fixed with 85% ethanol for 30 minutes at 4ºC (ice). Following cells were centrifuged at 1500 rpm for 5 minutes, pellet was washed with PBS and resuspended in 0.5 ml of PBS sodium citrate-BSA (0.1 g of sodium citrate and 1g of BSA) with 200 µg/ml of RNasa-DNasa free and 10 µg of propidium iodide. Cells were analysed by flow cytometry on a FACScant (BD Biosciences). Ten thousand events were gated and analysed using CellQuest software (BD Biosciences).

3.6.3 Benzidine test

Benzidine was used to test for erythroid differentation induced in K562 (Rowley et al., 1981). Hemoglobin catalyzes the oxidation of benzidine by hidrogen peroxide, giving a blue color. 10 µl of H_2O_2 30% were added to 0.5 ml of 0.2% benzidine in 0.5 M acetic acid solution

(1.5 ml Acetic acid glacial, 50 ml of water and 0.1 g of hydrochloric benzidine (Sigma B-0386). This mix were added to 20 µl of RPMI with 10.000-20.000 cells. Cells were incubated for 10 minutes at 4ºC (on ice) and protected from light. Finally, blue cells (positives) were counted by hemocytometer. At least 300 cells were counted in each experimantal point.

3.6.4 Apoptosis determination by annexin v assay

Cells were harvested after the apoptosis induction. They were washed once in temparate PBS and then they were centrifuged at 1500 rpm. Supernatant was removed and the cells were resuspended in 1 x Annexin-binding buffer (from Annexin V-PE apoptosis detection kit from Immunostep Research kit) with the Annexin V-PE added. The cells were incubated for 15 minutes in dark at room temperature, and finally they was analyzed by flow cytometry.

3.7 Statistical analysis

Sperman´s rank correlation, Pearson product-moment correlation coefficient, *t*-test, X^2 distribution and One-way ANOVA test were used to determine correlations, dependence and statistical significance. Survival curves were analyzed according to the Kaplan and Meier method. SPSS Statistics 17.0 and GraphPad Prism software were used to different analysis.

4.- RESULTS AND DISCUSSION

4 RESULTS AND DISCUSSION

4.1 Expression of p27 and Myc in B-CLL cells

4.1.1 p27 expression in B-CLL cells

We collected a cohort of more that 100 CLL patients and a small collection of samples from healthy patients which were used as a control. Samples from CLL patients were obtained from peripheral blood, whereas samples from healthy patients were collected from B-lymphocytes CD19+ and from tonsils removed by tonsillectomy. First, the expression of p27 mRNA was measured in our cohort of patients by Northern blot (Fig. 4.1A, determination carried out by M. Angel Cortés and Juan C. Acosta in our laboratory) and RT-qPCR (Fig. 4.1B). Altogether 75 CLL samples were analyzed. Compared with controls, mRNA of p27 is overexpressed in most of the patients. Indeed, most of the patients showed between 2 and 5 fold of mRNA expression, and even some samples showed higher levels at 10 fold (Fig. 4.1C).

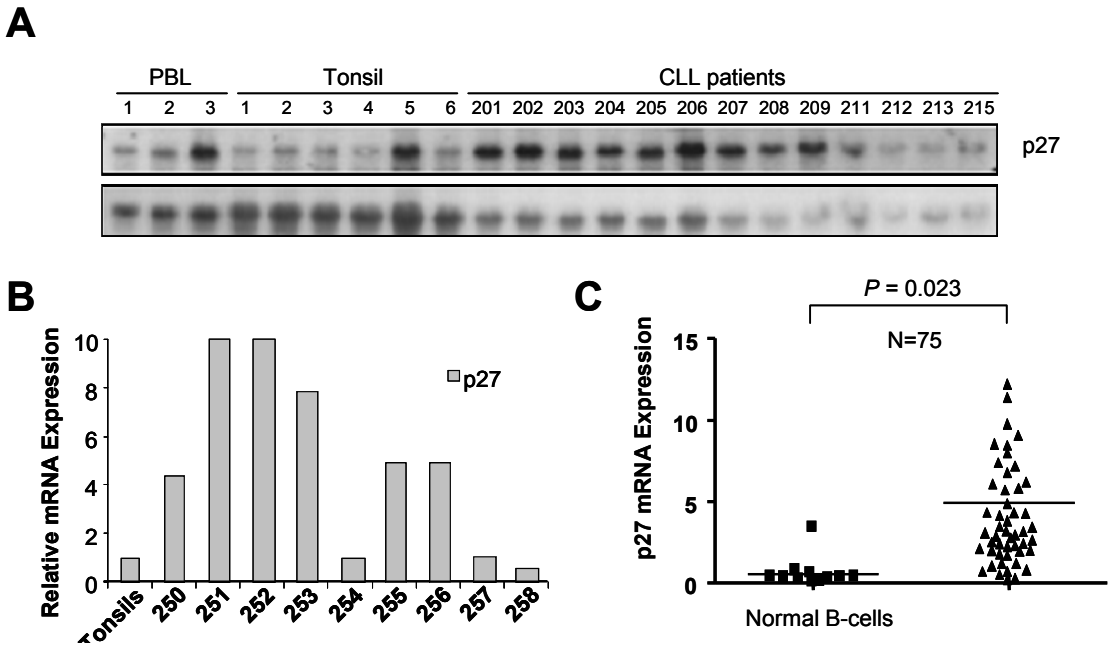
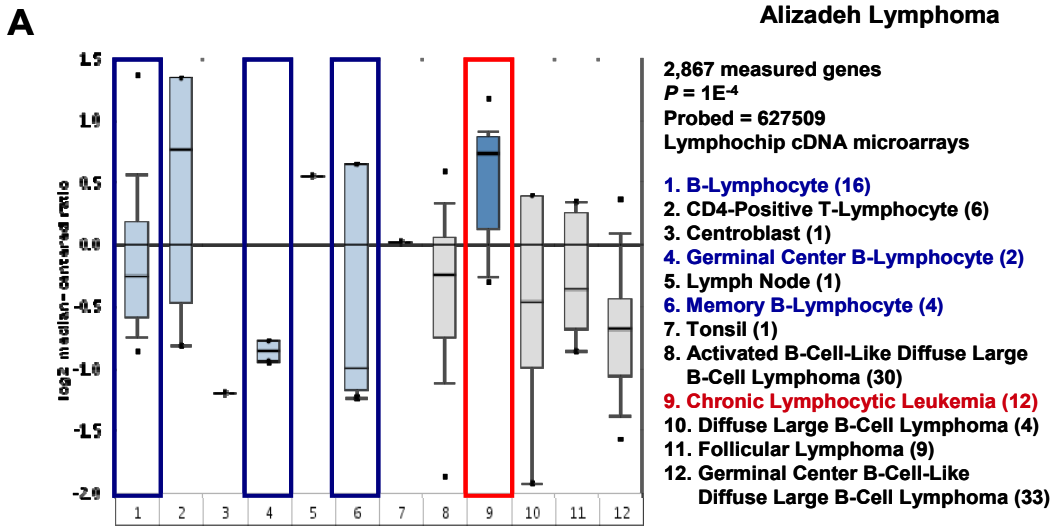
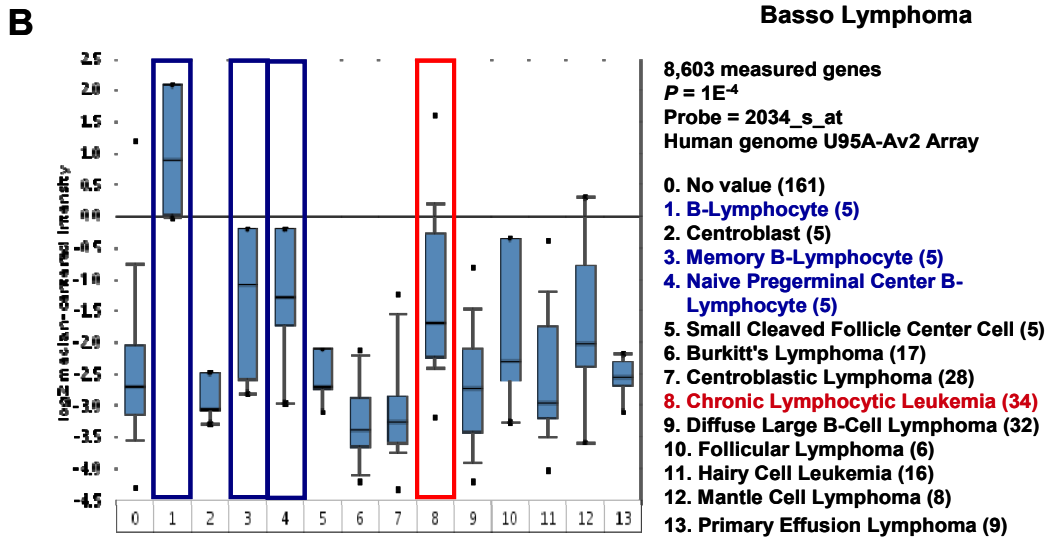


Figure 4.1.- p27 mRNA expression in B-CLL cells. (A) p27 mRNA levels of B-CLL cells in CLL patients were determined by Northern Blot and (B) RT-qPCR. Normal lymphocytes from tonsils and peripheral blood lymphocytes (PBL) were used as control. 18S rRNA levels are shown to asses mRNA loading (Northern blot) and RPS14 mRNA levels were used for normalization (RT-qPCR). (C) The mean levels of p27 mRNA expression (normalized to 18S and RPS14 expression) was significantly higher in B-CLL cells than in normal B-cells.

Although some previous studies have observed high levels of p27 mRNA (Halina et al., 2010), others have reported that p27 mRNA expression in B-CLL cells was identical in all samples that they studied (Vrhovac et al., 1998). Therefore, we decided to confirm our results through Oncomine database, a cancer gene expression database (www.oncomine.org). We found that although CLL samples showed higher levels of p27 than other leukemias, its expression with respect to healthy samples (controls) differs depending on the study. The Fig. 4.2 shows two examples of these studies where p27 mRNA is overexpressed in some studies (Fig. 4.2A), whereas in others the expression was unchanged (Fig. 4.2B).



Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000 Feb 3;403(6769):503-11. PMID: 10676951



Basso K, Margolin AA, Stolovitzky G, Klein U et al. Reverse engineering of regulatory networks in human B cells. Nat Genet 2005 Apr;37(4):382-90. PMID: 15778709

Figure 4.2.- p27 mRNA expression in normal B-cells and primary human leukemias and lymphomas. Two different microarray-based studies from the Oncomine database are shown (OncomineTM, Compedia Bioscience, Ann Arbor, MI, https//www.oncomine.org/). The box plots depict the differential expression of p27 in different lymphomas and leukemias, including CLL (red square). Depending on the study, the expression in CLL was equal or higher than normal B-cells (blue square showed the most suitable controls for CLL). *P*-values from t tests, the probes used in each microarray analysis and the reference are indicated beneath the panels.

However, high levels of p27 mRNA may not correlate with higher protein levels because p27 is mainly regulated at the posttranscriptional level (Hengst and Reed, 1996; Munoz-Alonso et al., 2005; Pagano et al., 1995). Degradation through ubiquitin-proteasome mechanism is critical in the regulation of p27 levels in the cell, even if there are high levels of p27 mRNA (see Introduction). Therefore, we studied the p27 protein expression in B-CLL cells in 118 CLL patients. Immunoblot analysis showed that p27 is significantly overexpressed in most B-CLL samples (Fig. 4.3A and 4.3B), and only 18 % of patients expressed lower levels of p27 than controls (arrows in Fig. 4.3A). The levels of p27 were quantified by film densitometry and normalized against the actin levels. Moreover, in some patients where both p27 mRNA and protein were analyzed, there was not correlation between the intensity of mRNA signal and protein expression (Rho´s spearman = 0,0064; P = 0,9722). This lack of correlation between p27 mRNA and protein expression may indicate that, although both p27 mRNA and p27 protein are overexpressed in B-CLL cells, posttranslational regulation may further modulate the levels of p27 protein in these cells. Regardless of the regulatory mechanisms involved, our results show an over-expression of p27 and therefore, it was conceivable that the presence of high levels of p27 protein may play a role in CLL.

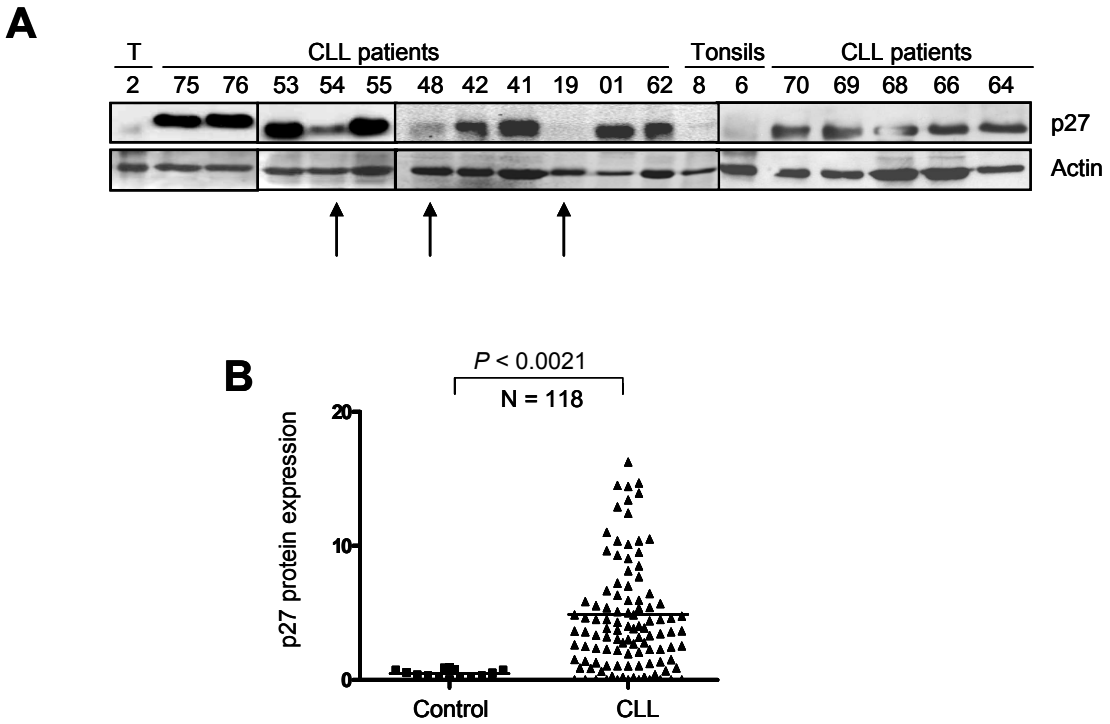


Figure 4.3.- p27 protein expression in CLL samples. (A) Immunoblot showing p27 protein expression in B-CLL cells and normal lymphocytes. Actin levels are shown to asses protein loading. The arrows indicate patients with low p27 protein expression. (B) The mean levels of p27 protein expression (normalized to Actin expression) were significantly higher in B-CLL cells than in normal B-cells.

As described in the Introduction, the clinical course of CLL is highly variable. While some patients show a more aggressive form of the disease with shorter survival periods, other exhibit a relative stable form of the disease which presents slow progression. Patients with a

rearrangement of its IgV_H genes exhibit a less aggressive form of the disease, whereas patients with few or no mutations in its IgV_H genes show an inferior survival (Butler and Gribben, 2010; Chiorazzi et al., 2005). This poor outcome is also associated with the expression of CD38, Zap-70 or LPL (Butler and Gribben, 2010; Chiorazzi et al., 2005; Damle et al., 1999; Del Principe et al., 2006; Hamblin, 2004; van't Veer et al., 2006).

We analyzed the p27 protein expression with the several marker associated with progression of CLL. However, we did not find any difference between p27 protein levels and most of clinical data collected, including the lymphocyte DT, the short overall survival and/or the stage of the disease (see Table 4.1). Other prognosis markers such as serum markers, chromosomal aberrations, B symptoms or LPL expression were also not correlated. In contrast, p27 protein expression was correlated to Zap-70 expression and CD38 expression (Table 4.1 and Fig. 4.4A and 4.4B). However, significance value *P* was close to 0.05, and therefore it would be necessary to analyze a larger number of samples to confirm these association. Moreover, an almost significant correlation between high p27 protein expression and p53 deletion was found (Table 4.1, Fig 4.4c), but it would be necessary to analyze a larger number of samples to confirm these correlations.

Table 4.1.- Correlation between p27 expression and clinical data. * indicate *P* < 0,05.

Prognostic Markers	Correlation with protein	Correlation with mRNA
Treatment	0.42	0.83
Progression	0.29	0.54
B symptoms	0.52	-
Lymphocyte DT	0.40	-
Serum markers	0.66	0.50
Zap-70	**0.03***	0.46
CD38	**0.03***	0.71
IgGV mutation	0.32	0.73
13q14 deletion	0.25	0.14
p53 deletion	**0.08**	0.72
ATM deletion	0.59	0.57
LPL	-	0.28

The same analysis was performed with the mRNA p27 expression. However, in this case we did not find any statically significant correlation (Table 4.1). Because of the lack of correlation between p27 mRNA and protein expression, we suggest that p27 mRNA levels cannot be used as a prognostic marker. Thus, from now on we decided to focus on the p27 protein expression.

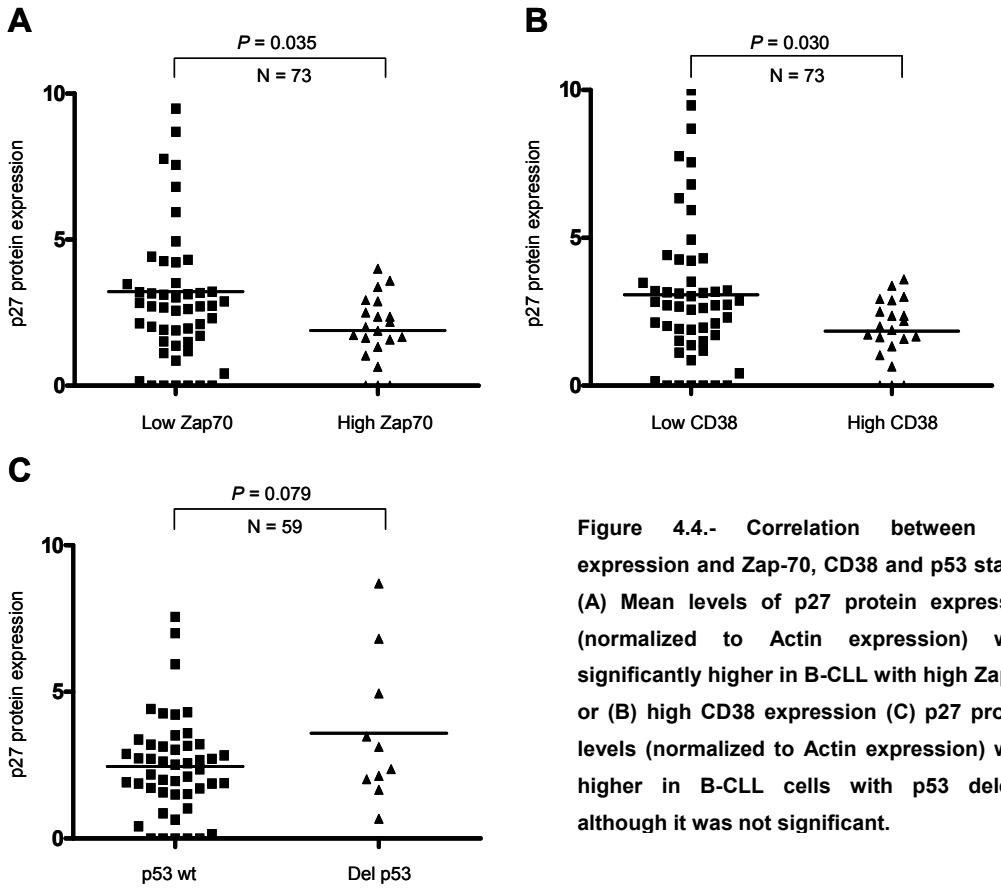


Figure 4.4.- Correlation between p27 expression and Zap-70, CD38 and p53 status. (A) Mean levels of p27 protein expression (normalized to Actin expression) were significantly higher in B-CLL with high Zap-70 or (B) high CD38 expression (C) p27 protein levels (normalized to Actin expression) were higher in B-CLL cells with p53 deleted although it was not significant.

Other prognostic markers, such an absolute lymphocyte count, morphology and immunophenotype, were analyzed for possible correlations with p27 protein expression, but the number of samples tested was not sufficient to allow a statistically significant association between these clinical data and p27 expression (data not shown). On the other hand, the overall survival did not differ significantly between patients with high or low p27 protein expression, shown by Kaplan-Meier analysis (Fig. 4.5).

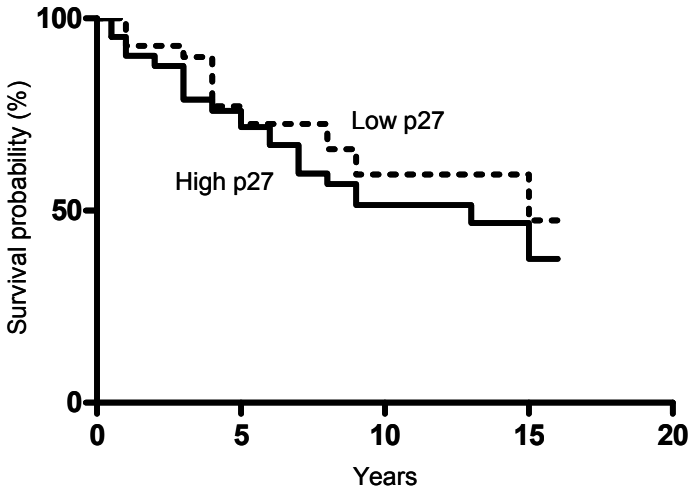


Figure 4.5.- Kaplan-Meier plot for survival of CLL from the time of sampling according to p27 protein expression. Comparision of the two survival curves indicate that Myc expression does not affect the survival time of patients (*P* = 0,2193).

High levels of p27 have been described in some human tumors, as breast, colon, ovarian, melanoma and lymphomas (Ciaparrone et al., 1998; Denicourt et al., 2007; Liang et al., 2002; Masciullo et al., 2000; Motti et al., 2005; Qi et al., 2006; Rosen et al., 2005; Shin et al., 2002; Singh et al., 1998; Viglietto et al., 2002), but in these cases, an aberrant cytoplasmatic expression of p27 was reported, and this localization was correlated with poor clinical outcome. The data can be explained because of the anti-apoptotic functions and cytoskeletal reorganization carried out by p27 in the cytoplasm (Blagosklonny, 2002). Therefore, we explored this possibility in CLL. An immunofluorescence analysis was performed to verify the p27 localization into B-CLL cells (Fig. 4.6). p27 localization was mainly nuclear, although some traces of cytoplasmic p27 could be observed in some samples.

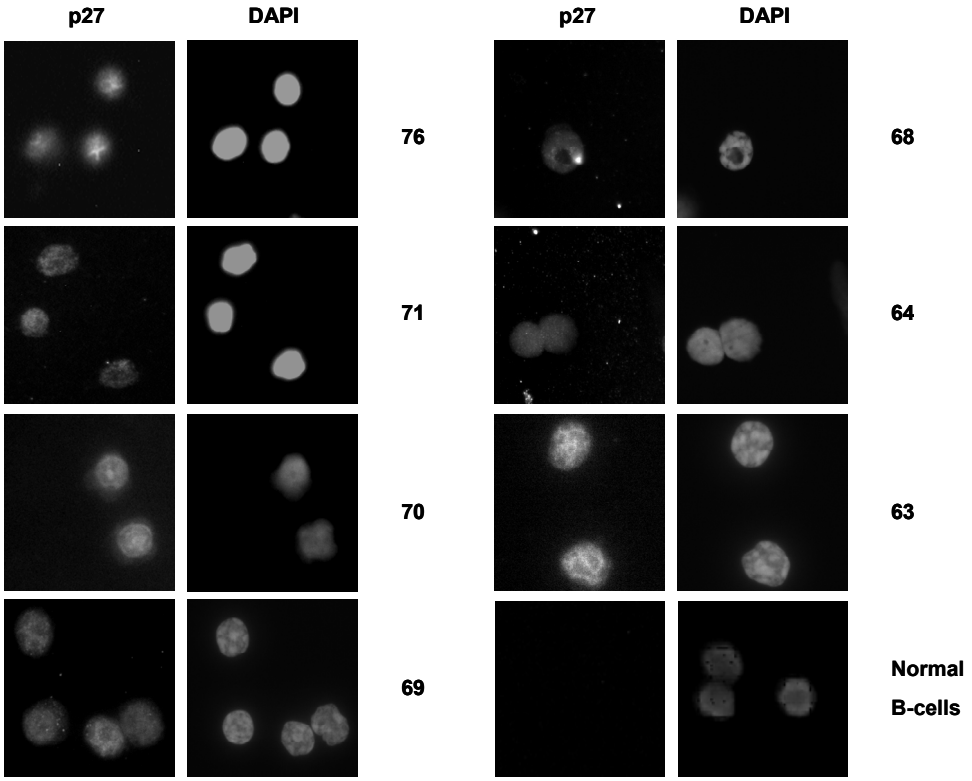


Figure 4.6.- Immunofluorescence analysis of the sub-cellular localization of p27 in B-CLL cells and normal B-cells. Nuclei were counterstained with DAPI.

4.1.2 Myc expression in B-CLL cells

Myc is an oncogen which is deregulated in 40% of human tumors. Its expression is associated with high proliferation, genomic instability, immortalization and less differentiation. Correlation between high levels of Myc and low levels of p27 have been found in a lot of tumors, and the mechanism through which Myc regulates p27 is well described in the literature (see Introduction). Moreover, in most of the studies Myc over-expression and p27 under-expression have been associated with increased proliferation and/or bad prognosis. Although p27 inhibits

the cell cycle proliferation even when Myc is expressed (Acosta et al., 2008), Myc is able to regulate p27 levels through different mechanisms, as described in the Introduction.

Knowing this correlation, and the striking high expression of p27 in CLL, we explored the correlation between Myc and p27 levels in CLL. In general, most studies on Myc expression in CLL patients have reported that in most of the patients, Myc is hardly detectable (Birnie et al., 1984; Drexler et al., 1989; Ferrari et al., 1985; Greil et al., 1991; Korz et al., 2002; Larsson et al., 1991). However, high levels of Myc have been reported in some cases, and its expression has been associated with cytogenetic abnormalities, Myc gene rearrangement and/or with the inactivation of suppressors of Myc as MGA (Boehm et al., 1987; Brown et al., 2012; Crowther-Swanepoel et al., 2010; de Paoli et al., 2012; Edelmann et al., 2012; Fabris et al., 2011; Forconi et al., 2008; Huh et al., 2008; Kienle et al., 2005; Put et al., 2012; Rechavi et al., 1989; Rimokh et al., 1991; Rinaldi et al., 2011). These studies found Myc expression in a small number of cases and recent reports have shown that patients with an advanced stage of the disease are characterized by higher Myc mRNA levels (Greil et al., 1991; Zhang et al., 2010). However, these results were inconsistent with previous studies which had shown that Myc is not expressed in B-CLL cells. Indeed, there is a controversy so as to the expression of Myc in CLL. Data from the Oncomine database (www.oncomine.org) reveal that Myc is underexpressed in some studies while its expression is unchanged in others (Fig. 4.7).

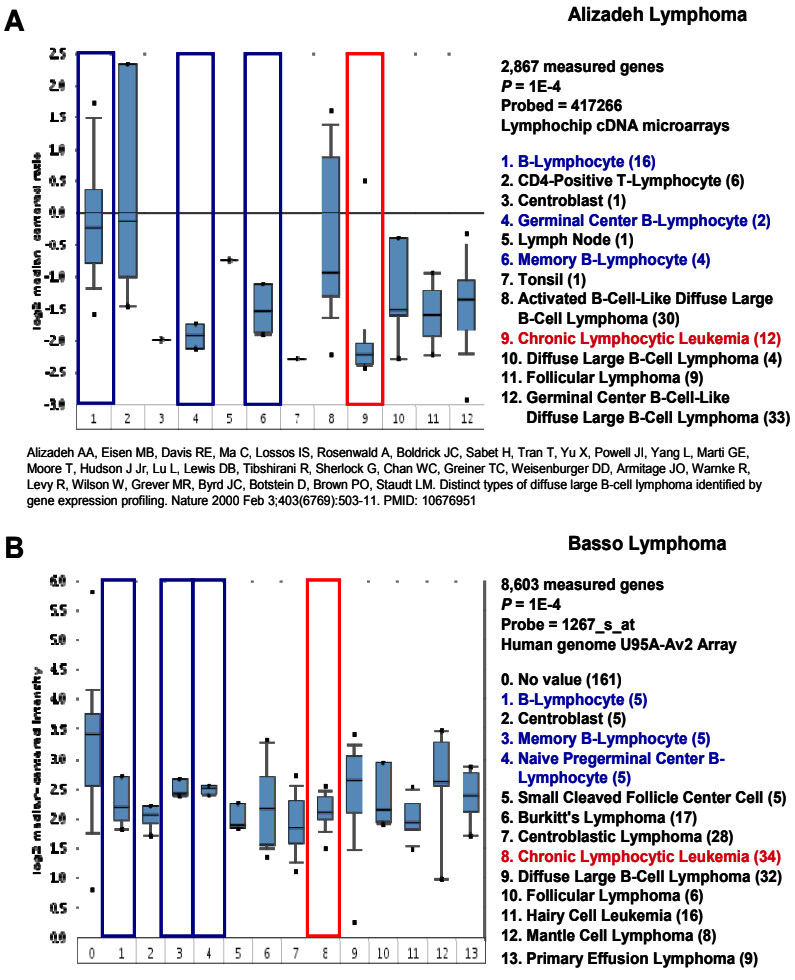


Figure 4.7.- Myc expression in normal B-cells and primary human leukemias and lymphomas. Two different microarray-based studies from the Oncomine database are shown (OncomineTM, Compedia Bioscience, Ann Arbor, MI, https//www.oncomine.org/). The box plots depict the differential expression of Myc in different lymphomas and leukemias, including CLL (red square). Depending on the study, the expression in CLL was equal or lower than normal B-cells (blue square showed the most suitable controls for CLL). *P*-values from t tests, the probes used in each microarray analysis and the reference are indicated beneath the panels.

Owing to contradictory results described, we decided to analyze Myc mRNA expression in our cohort of patients. Our results showed lower levels of Myc mRNA in B-CLL cells than in controls (Fig. 4.8). Northern blot (conducted by Dr. Miguel Angel Cortes y el Dr. Juan Carlos Acosta in our laboratory) and RT-qPCR analysis showed that 98% of 91 patients expressed low levels of Myc (Fig. 4.8). Indeed, we could not detect any Myc mRNA expression by Northern blot in 48 % of our samples (Fig. 4.8A).

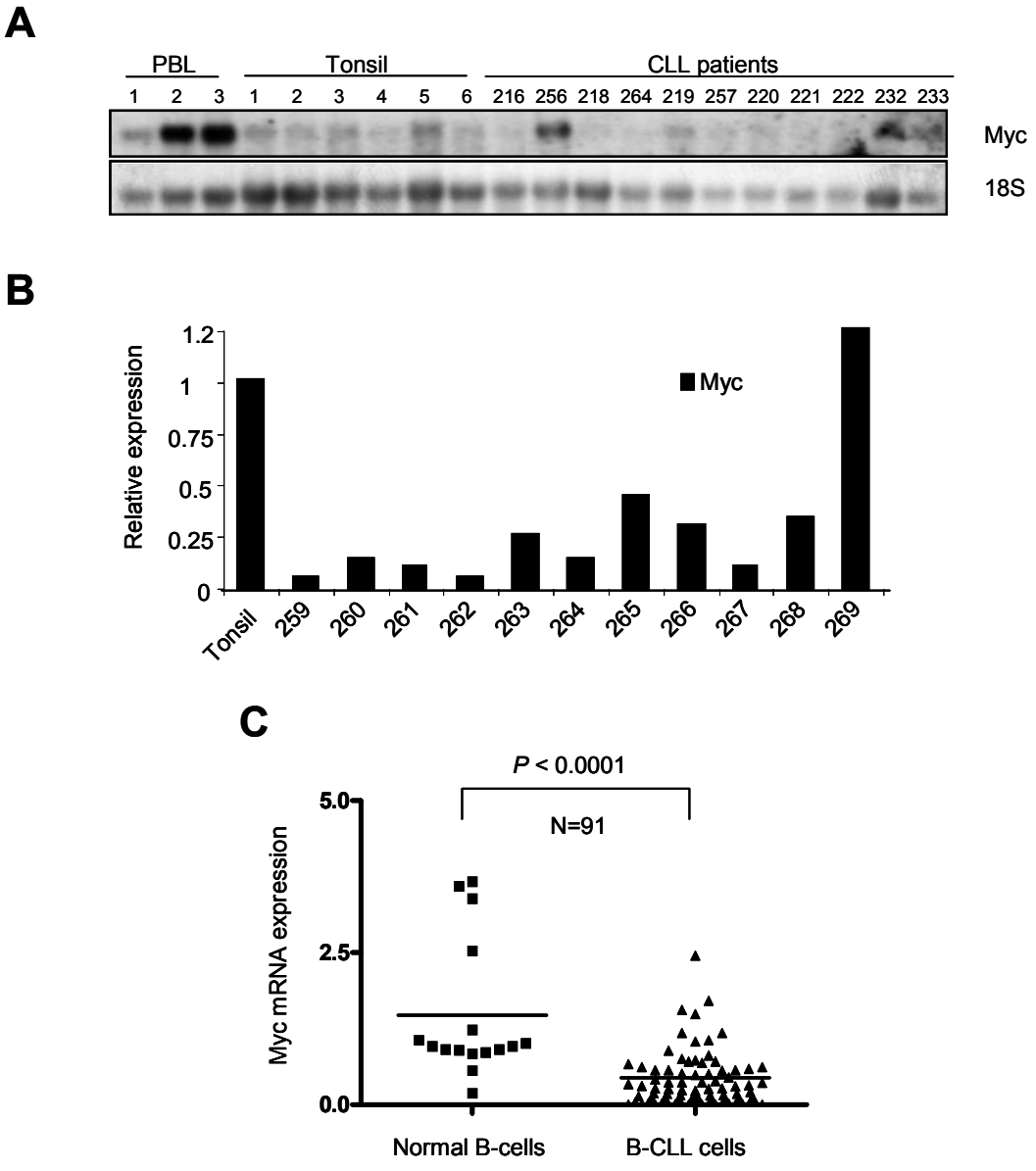


Figure 4.8.- Myc mRNA expression in B-CLL cells. (A) Myc mRNA levels of B-CLL cells in CLL patients were determined by Northern blot and (B) qRT-PCR. Normal lymphocytes from Tonsils and PBL were used as control. 18S rRNA levels are shown to asses mRNA loading (Northern blot), and RPS14 mRNA levels were used for normalization (RT-qPCR) (C) Mean levels of Myc mRNA expression (normalized to 18S rRNA and RPS14 expression) were significantly higher in B-CLL cells than in normal B-cells.

However, similarly to p27, Myc also exhibits both posttranscriptional and post-translational regulation so as to modify their protein levels in the cells. Therefore, we set out to study protein levels of Myc in our patients. 113 patient samples were analyzed (Fig. 4.9), and the Myc protein levels were quantified by film densitometry and normalized against the actin levels. Most of patients did not exhibit detectable levels of Myc protein or the expression was lower in B-CLL cells than controls. However, the percentage of patients that was classified as Myc positive increased to 26%. In this analysis, Myc was defined as positive when the expression was equal to or greater than the expression detected in control samples, Moreover, when Myc mRNA and Myc protein expression were compared with each other in the same samples, both levels did not correlate (Rho´s spearman = 0.1330; *P* = 0.4395). Therefore, similarly to p27, Myc post-translational regulation may be important in the regulation of Myc in B-CLL cells.

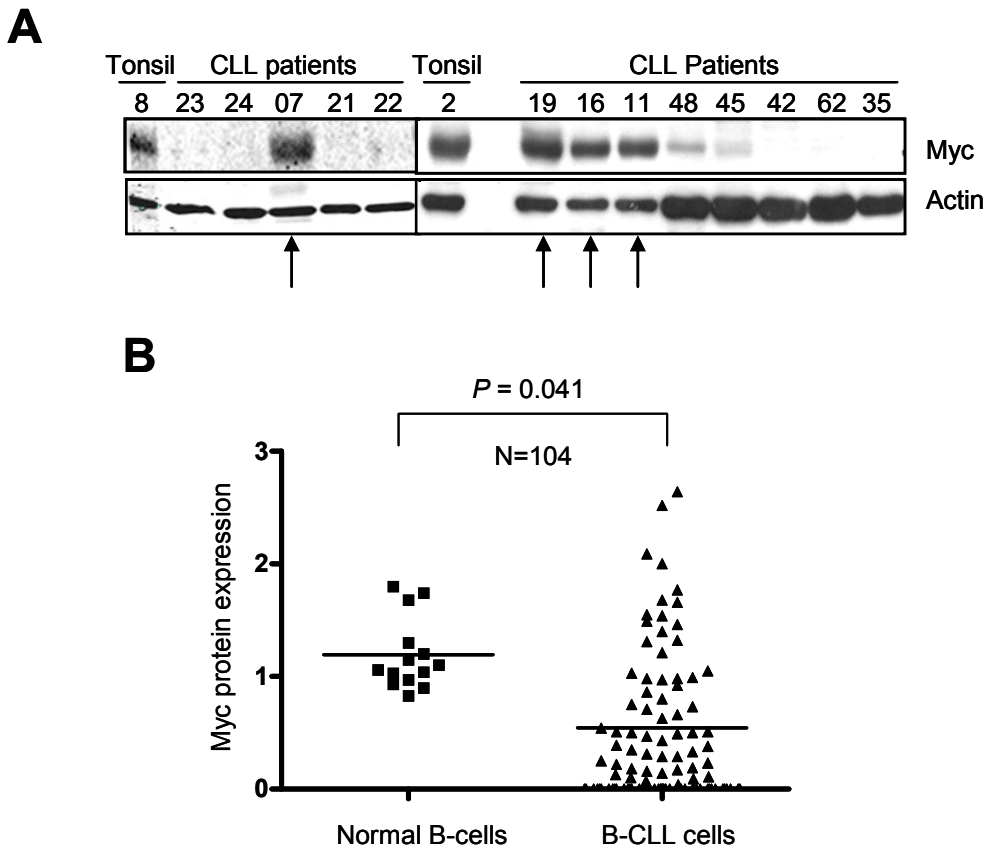


Figure 4.9.- Myc protein expression in CLL samples. (A) Immunoblot showing Myc protein expression in B-CLL cells and normal lymphocytes. Actin levels are shown to asses protein loading. Arrows indicate patients with high Myc protein expression. (B) Mean levels of Myc protein expression (normalized to Actin expression) were significantly higher in B-CLL cells than in normal B-cells.

Although high levels of Myc have been reported in a subset of CLL, we were not able to find this association between Myc and disease progression in our patients (Table 4.2). These contradictory results could be explained if we consider that the most of the previous studies were performed measured only Myc mRNA expression. In our data, a high percentage of our patients exhibited lower levels of Myc than controls, and therefore, we have a low number of

Myc-positive samples (n = 22), which abrogates the generation of statistically significant data. With this handicap, we only found a correlation between Myc mRNA expression and 13q14 deletion. It would be necessary to analyze a larger number of Myc-positive samples to confirm these correlations (Table 4.2, Fig 4.10b). With respect to Myc protein expression, when the groups of Myc-positive and Myc-negative samples were compared with clinical data, we did not find any significant correlation (Table 4.2). We only found an almost significant correlation between Myc protein expression and Zap-70 expression (*P* = 0.09) (Table 4.2, Fig 4.10a), but it would be necessary to analyze a larger number of samples to confirm this correlation. Moreover, the overall survival from the date of determination of Myc protein levels did not significantly differ between patients with a high and a low Myc protein expression (Fig. 4.11).

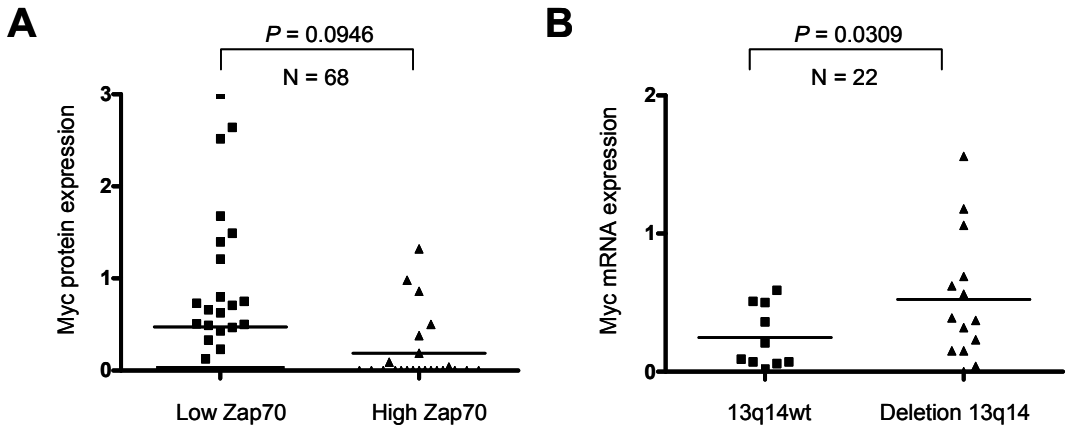


Figure 4.10.- Correlation between Myc expression with clinical data. (A) Myc mRNA levels (normalized to RPS14 expression) were higher in B-CLL cells with lower Zap-70 expression, although this result was not significant. (B) Mean levels of Myc protein expression (normalized to Actin expression) were significantly higher in B-CLL cells with 13q14 deletion.

Table 4.2.- Correlation between Myc expression and clinical data. * indicate *P* < 0,05.

Prognosis Markers	Correlation with protein	Correlation with mRNA
Treatment	0.66	0.49
Progression	0.35	0.96
B symptoms	0.82	-
Lymphocyte DT	0.74	-
Serum markers	0.28	0.38
Zap-70	**0.09**	0.52
CD38	0.41	0.51
IgGV mutation	-	0.22
13q14 deletion	0.60	**0.003***
p53 deletion	0.33	0.35
ATM deletion	0.57	-

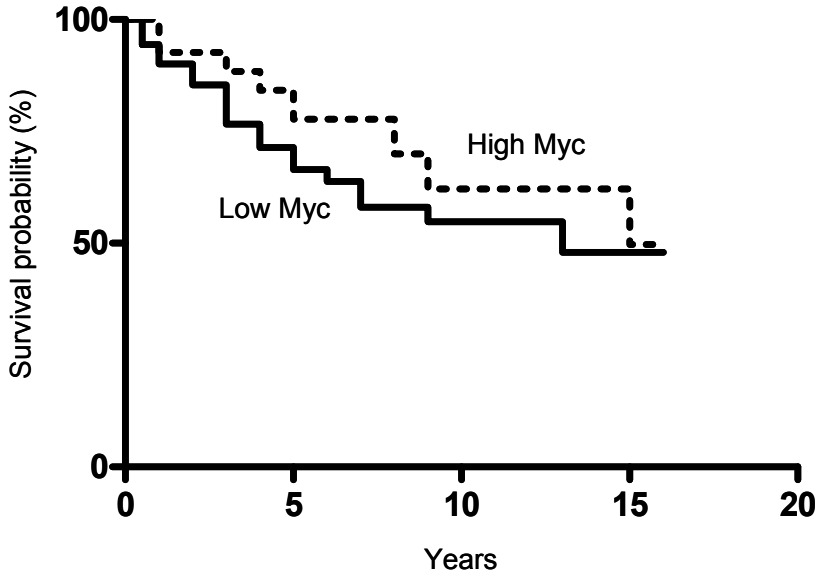


Figure 4.11.- Kaplan-Meier plot for survival of CLL from the time of sampling according to Myc protein expression. Comparison of the two survival curves indicate that Myc expression does not affect the survival time of patients (*P* = 0,3208).

Like p27 analysis, Myc mRNA and protein expression did not correlate (Rho´s spearman = 0.1330; *P* = 0.4395), but Myc protein expression give us a real value of the amount of Myc present in the B-CLL cells. Therefore, we decided to focus on the Myc protein expression.

Recent whole-genome sequencing of CLL patients identified a recurrent mutation in *NOTCH1* in 12% of patients, which results in the accumulation of a more stable and active isoform of the protein (Puente et al., 2011). Notch1 is a single-pass transmembrane receptor of ligands which plays a role in a variety of developmental processes by controlling cell fate decisions. Moreover, it is known that Myc gene is direct downstream transcriptional target of Notch1 (Klinakis et al., 2006; Palomero et al., 2006; Weng et al., 2006). Therefore, we explored the possibility of a correlation between mutational status of Notch1 and the high Myc expression that we found in 26% of our patients. We analyzed the status of Notch1 by Immunoblot in 35 patients with similar o higher Myc levels than B-normal cells. We considered that Notch1 was also active and functional in samples where Notch1 was not mutated but its expression was high (and we named as “active Notch1”). The results showed that mutated (confirmed by sequencing) or active Notch1 was present only in a few of the CLL samples with Myc protein expression (Fig. 4.12). However, we also observed activated Notch1 in patient samples with low expression of Myc (bars under the limit of Myc expression in Fig. 4.12; samples marked with arrows). These results suggest that Myc can be active in some cases when Notch1 expression is altered, but also that Myc can be regulated in B-CLL cells through independent mechanisms from Notch-mediated transactivation.

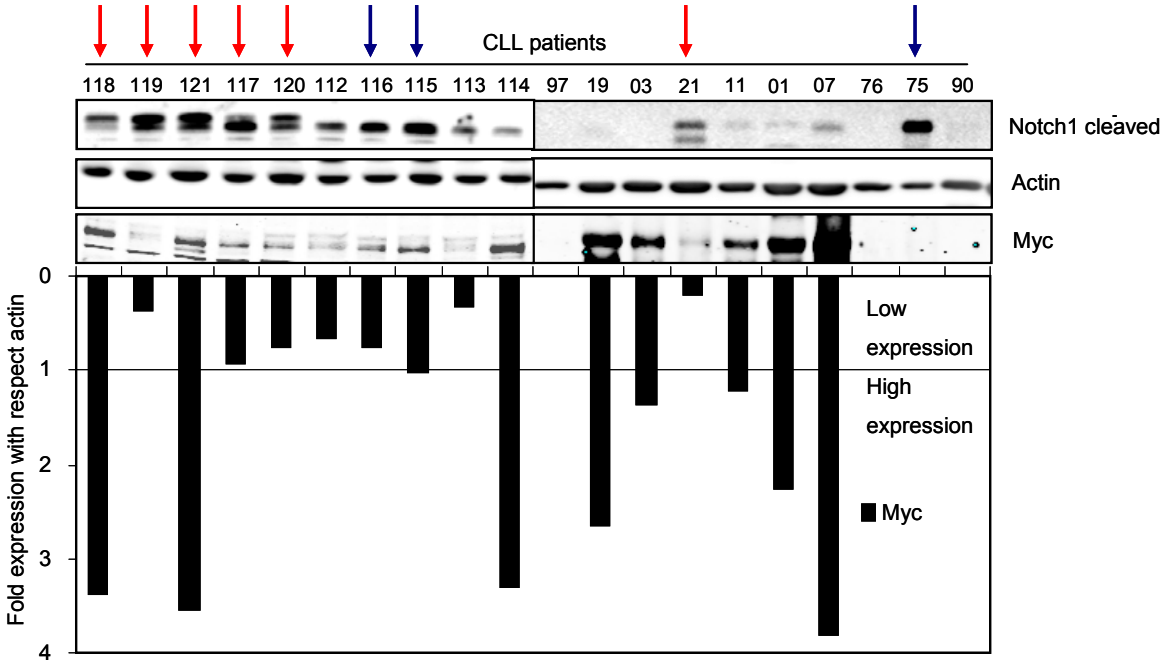


Figure 4.12.- Myc protein expression in patients with mutation in Notch1. Immunoblot showing Myc protein expression in B-CLL cells and normal lymphocytes. Actin levels are shown to asses protein loading. Red arrows indicate samples with mutated Notch1 (confirmed by sequencing) and blue arrows indicate samples with active Notch1 (i.e., samples with high expression of Notch1). Myc expression in samples #118, #121, #115, #114, #19, #03, #11, #01, #07 are found over the limit of high Myc expression.

4.1.3 Expression of other members of the Myc network in B-CLL cells

Myc transcriptional activity requires the dimerization with Max (Blackwood and Eisenman, 1991; Marchetti et al., 1995). In general, Max expression is constitutive, but its expression has not been studied in CLL patients. Due to the low expression of Myc, we wanted to know if Max is also down-regulated in CLL. Max expression analysis of our cohort showed that the most of B-CLL lymphocytes exhibit high levels of mRNA with respect to control (Fig. 4.13A, Northern blot carried out by M. Angel Cortés and Juan C. Acosta in our laboratory). The significance of this high expression of Max was demostrated by a statistical analysis (Fig. 4.13B). Immunoblot analysis also showed that Max protein levels were also higher than control (Fig. 4.13C). The quatification of the signals of the blots confirmed this although the number of samples analyzed were not enough to estimate the significance (Fig. 4.13D).

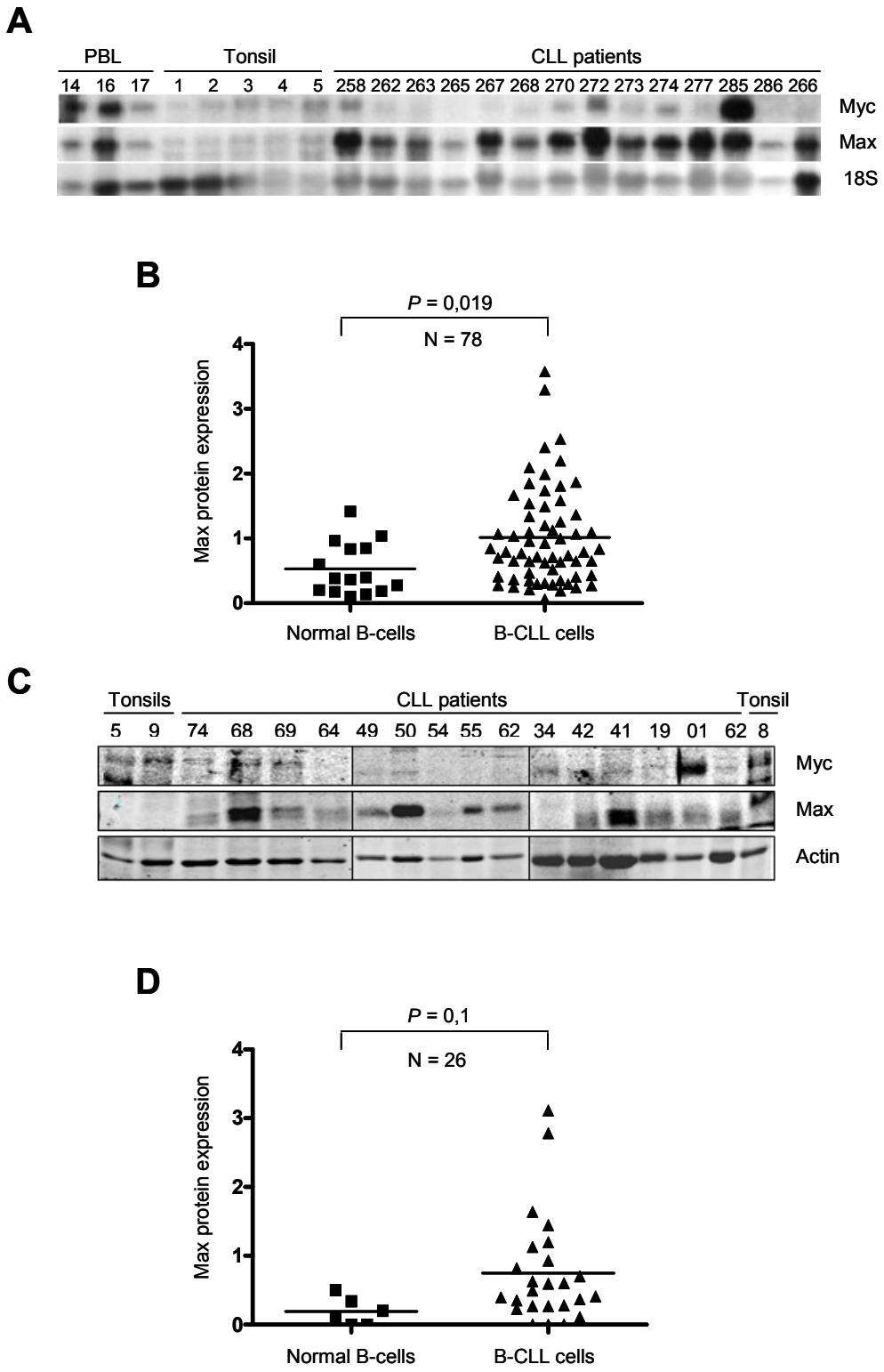


Figure 4.13.- Max expression in B-CLL cells. (A) Max mRNA levels of B-CLL cells in CLL patients were determined by Northern blot. Normal lymphocytes from Tonsils and PBL were used as control. 18S rRNA levels are shown to asses mRNA loading. (B) Mean levels of Max mRNA expression (normalized to 18S rRNA expression) were higher in B-CLL cells than in normal B-cells (C) Immunoblot showing Max protein expression in B-CLL cells and normal lymphocytes. Actin levels are shown to asses protein loading. (D) Max protein levels (normalized to Actin expression) were higher in B-CLL cells than normal B-cells, although this result was not significant.

Mxd (formerly named Mad) family is also able to bind to Max. Mxd/Max compete for binding to E boxes with Myc/Max dimers but repress transcription (see Introduction). We studied the expression of two members of Mxd family, called Mxi1 and Mxd1, in our cohort of patients. We observed that Mxi1 levels were higher than controls in 18 out of 26 patients analyzed (70%) (Fig. 4.14A, by M. Angel Cortés and Juan C. Acosta in our laboratory). The statistical analysis showed that Mxi1 is significantly more expressed in B-CLL samples than in control (Fig. 4.14B). In contrast, 17 out of 20 samples where Mxd1 was analyzed (85%) showed lower levels of B-normal cells used as control (data not shown)

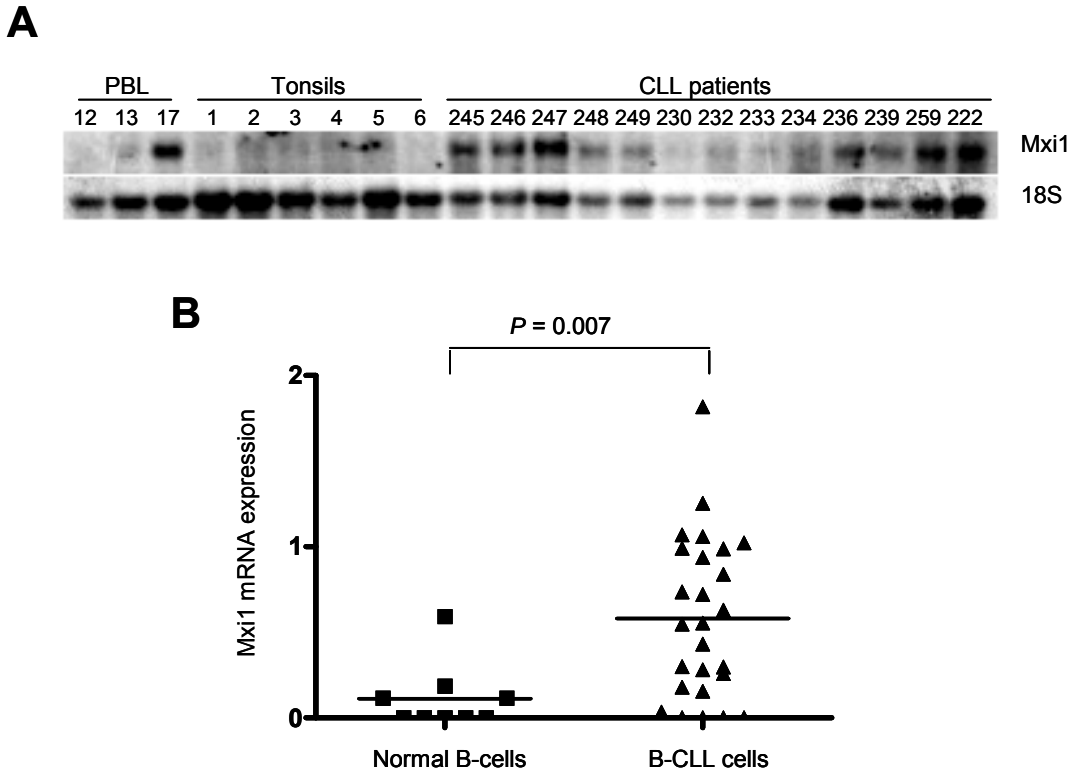


Figure 4.14.- Mxi1 expression in CLL patients. (A) Mxi1 mRNA levels of B-CLL cells in CLL patients were determined by Northern Blot. Normal lymphocytes from Tonsils and PBL were used as control. 18S rRNA levels are shown to asses mRNA loading (B) Mean levels of Mxi1 mRNA expression (normalized to 18S expression) were higher in B-CLL cells than in normal B-cells.

4.1.4 Correlation between Myc and p27 protein expression in B-CLL cells

Once the expression of p27 and Myc were analyzed in our cohort of patients, we studied the expression of both proteins in the same cells to explore the correlation between the levels of both proteins in B-CLL cells. Fig. 4.15 shows protein expression of Myc and p27 in our cohort of CLL patients.

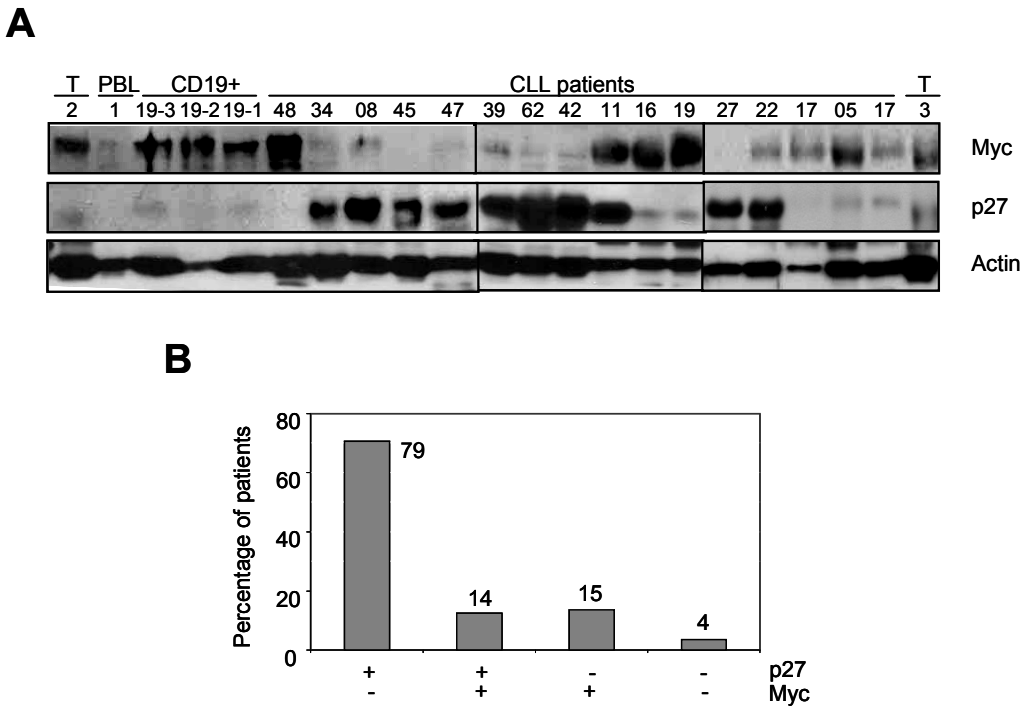


Figure 4.15.- Correlation between Myc and p27 expression in CLL patients. (A) Immunoblot showing p27 and Myc protein expression in B-CLL cells. Normal lymphocytes from tonsils (T), peripheral blood lymphocytes (PBL) and CD19+ B-cells purified by flow cytometry were used as control. Actin levels are shown to asses protein loading. (B) Percentage of patients with p27 and Myc expression are indicated. 102 patients were studied, of which 79 expressed high levels of p27 but low Myc levels, 15 showed high levels of Myc but low p27 levels, and 14 exhibited high levels of both. In 4 patients, it was not possible to find p27 and/or Myc expression.

Four types of patients could be classified according to the Myc and p27 protein expression. First, patients with high levels of p27 and low or undetectable of Myc (the majority, with a 71% of the patients), second, patients with high levels of both p27 and Myc (12.5%) and third, patients with low levels of p27 and high of Myc (13.5%). The fourth type of patients was represented by patients with low levels of both p27 and Myc, but the percentage was very small (3%) and therefore it was not considered for further analyses (Fig. 4.15B). Thus, a large majority of patients (84%) showed an inverse correlation between Myc and p27 protein expression. Since many previous studies had confirmed the relationship between both Myc and p27 in other experimental models, it was possible that the correlation found in CLL may mean that both proteins were regulating one another. In fact, although it was not possible to draw a linear correlation between the two proteins (data not shown), a Spearman´s coefficient showed that both Myc and p27 levels were correlated in CLL (P = 0.0177; Spearman´s rho = - 0.2258)

When patients were classified according to their p27 and Myc protein expression and compared with their clinical data, we observed a relevant association in the disease. Thus, among patients with high p27 levels, leukemic progression was faster in patients with low levels of Myc than in patients with high levels of Myc (Fig. 4.16). However, we did not observe any differences in the progression of the disease when we scored only the Myc expression (independently of the expression of p27) (data not shown). This result is surprising if we consider the oncogenic activity usually attributed to Myc.

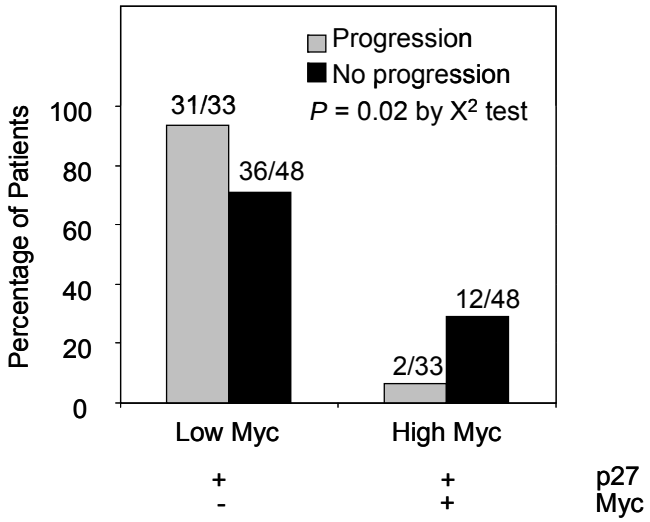


Figure 4.16.- Correlation between the expression of Myc and the percentage of patients with good or bad progression of the disease in patients with high levels of p27. Only a 8% of patients with worse progression showed high levels of Myc, compared to 38% of patients with good prognosis. (*P* = 0.02)

The mechanism through which B-CLL cells accumulate in the blood, despite that most cells are arrested in G_0/G_1, is still an issue under study. The current hypothesis is that B-CLL cells prolifere in solid lymphoid tissues, where these cells may find an optimal environment for cell division. Thereby, the tumor microenvironment could modulate the cell cycle progression in CLL while in peripheral blood these B-CLL cells remain in relative quiescence (Smit et al., 2007; Themi et al., 1973). In this context, it is important to consider that the results shown above have been obtained in cells from peripheral blood. Thus we decided to study p27 and Myc expression in samples from bone marrow and lymph node of patients with B-CLL cells. Conversely to our findings in peripheral blood, Myc expression was high in most of the lymph node and bone marrow samples in agreement with the hypothesis that B-CLL cells may proliferate in these tissues (Fig. 4.17), although the number of cases analyzed was small. However, similarly to peripheral blood, p27 expression was surprisingly high in most of the samples (Fig. 4.17).

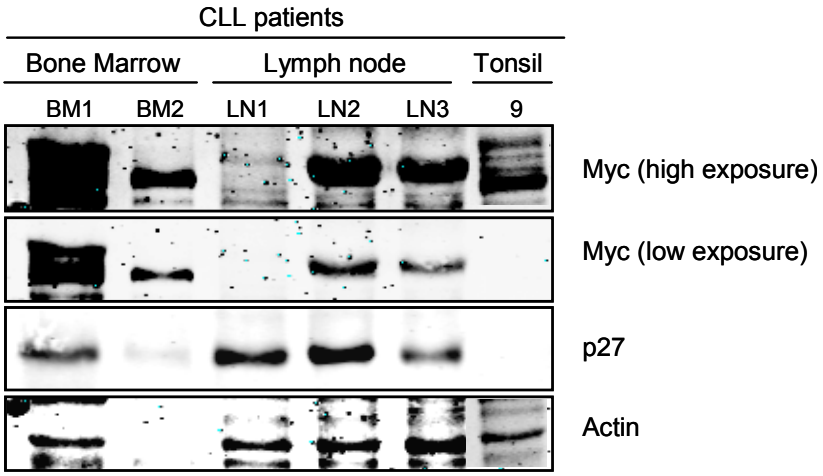


Figure 4.17.- Myc and p27 expression in B-CLL cells from lymphoid tissues. Immunoblot showing p27 and Myc protein expression in B-CLL cells from Lymph node and bone marrow. Tonsil was used as normal lymphocytes control. Actin levels are shown to asses protein loading.

4.1.5 Myc and p27 are mutually regulated

. As described above, most of CLL patients (84%) showed high levels of p27 and low Myc levels, where 16% of these patients presented high levels of Myc and low of p27, in contrast with the scenary with most other tumors where Myc is usually associated with a low expression of p27 (see Introduction). The mechanism by which Myc induces a low expression of p27 occurs through several levels. In same cases Myc is able to down-regulate p27 mRNA expression inhibiting its transcription, but in most cases Myc induces the transcription of Cullin1 (Cul1), Cks1 and Skp2, all components of the SCF^{SKP2} ubiquiting ligase complex that targets p27 for degradation.

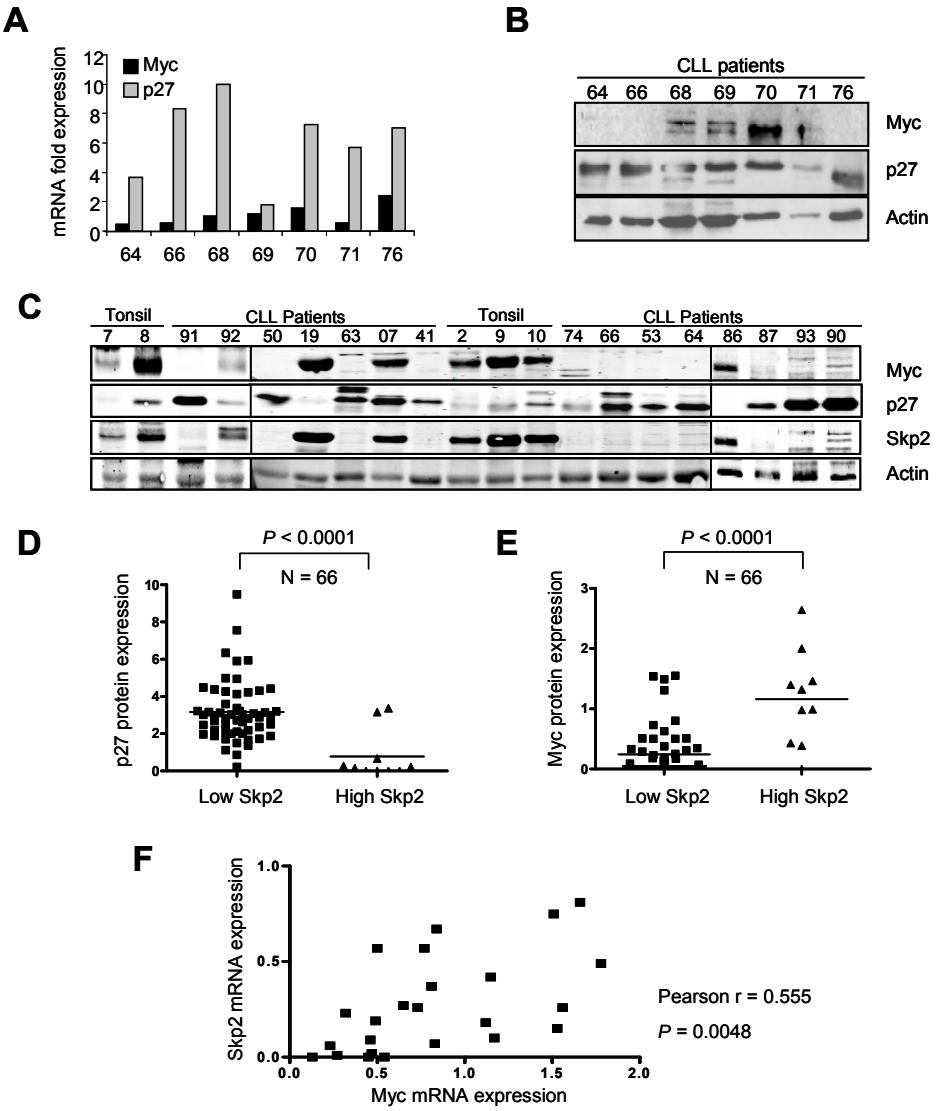


Figure 4.18.- Myc regulates p27 expression. Comparative study of the (A) p27 and Myc mRNA levels of B-CLL cells in CLL patients was determined by RT-qPCR. RPS14 levels were used for normalization. (B) Immunoblot showing p27 and Myc, (C) and Skp2 protein expression in B-CLL cells. Normal lymphocytes from tonsils were used as control. Actin levels are shown to asses protein loading. (D) Mean levels of p27 protein expression and (E) Myc protein expression (normalized to Actin expression) were significantly higher in B-CLL cells than in normal B-cells. (F) Skp2 expression correlated with the Myc expression in B-CLL cells analyzed. The Pearson´s r and *P*-value are indicated.

Although it has been reported that Myc represses the p27 promoter in some models (Chandramohan et al., 2004; Yang et al., 2001). Therefore we decided to compare the p27 and Myc mRNA and protein levels in a subset of our samples. The results (Fig 4.18A and B) showed that p27 mRNA expression in our samples was independent of Myc levels. However, in view that Skp2 is a Myc target gene and that Skp2 controls p27 degradation, we decided to determine Skp2 levels in our samples and analyze their correlation with Myc levels. High Skp2 protein expression strongly correlated with high levels of Myc and low levels of p27 in our patient samples (Fig. 4.18D-F). Moreover, Skp2 mRNA expression also correlated with the expression of Myc mRNA (Fig. 4.18G). Therefore, we concluded that Myc induces p27 degradation through up-regulation of Skp2 mRNA in B-CLL cells. This mechanism could explain, at least in part, the association between high Myc and low p27.

On the other hand, the mechanism through which p27 induces Myc down-regulation is less known, but it is thought that inhibition of G_0/G_1 progression induced by p27 is sufficient to induce Myc repression. In fact, in proliferating cells, the Myc mRNA levels are higher that in quiescent cells, where Myc is virtually not expressed (Wierstra and Alves, 2008). To see the effect of p27 on the levels of Myc in CLL, we used the MEC1 cell line, a cell line derived from B-CLL cells in prolymphocytoid transformation (Stacchini et al., 1999). Unlike B-CLL cells, proliferating MEC1 cells expressed high levels of Myc and low levels of p27 (Fig 4.19A). A p27 expression vector was transiently transfected into MEC1 cells, resulting in a dramatic p27 over-expression with respect to empty vector-transfected cells (Fig. 4.19A.). As expected, ectopic expression of p27 in MEC1 induced cell cycle arrest in G_0/G_1 (Fig. 4.19B) and a decrease in the Myc protein (Fig. 4.19A) and mRNA levels (Fig. 4.19C). These results are consistent with the observations in vivo, where the majority of CLL patients that exhibited high levels of p27 presented low levels of Myc in peripheral blood. In conclusion, Myc and p27 are mutually regulated in CLL.

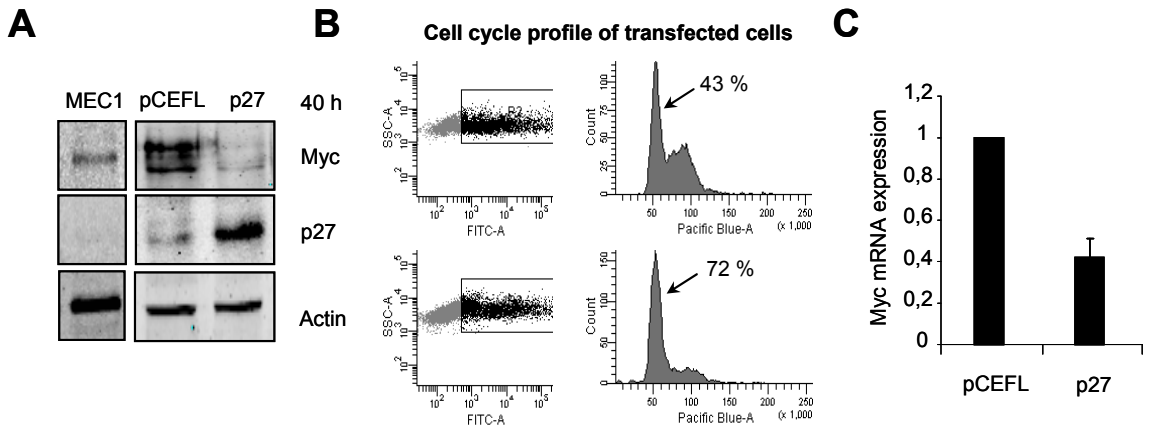


Figure 4.19.- p27 and Myc are mutually regulated in MEC1. (A) Immunoblot showing p27 and Myc expression in MEC1 cell line transfected with pCEFLp27. MEC1 transfected with empty vector was used as control. Proteins were analyzed 40 h after transfection. Actin levels are shown to asses protein loading. (B) Flow cytometry showed cell cycle arrest by p27 in MEC1 cells transfected with YFPp27 vector (YFP vector was used as controls). (C) Myc mRNA levels of MEC1 cell line transfected with pCEFLp27 were determined by RT-qPCR 40 h after transfection. MEC1 cell line transfected with empty vector was used as control. RPS14 mRNA levels were used for normalization. Data are means ± the SEM of three independent experiments.

4.1.6 Expression of p27 and Myc in B-CLL cells: DISCUSSION

We studied p27 mRNA expression in the peripheral blood lymphoid cells from 75 CLL patients respectively. In most of patients, p27 mRNA levels were significantly higher in 75% of the patients with respect to control samples (Fig. 4.1). However, although some previous studies have described high levels of p27 mRNA in CLL, others have reported no variation. The analysis of the Oncomine database revealed that p27 mRNA levels reported greatly differ depending on the study (Fig. 4.2). It must be underlined that most of patients studied by us expressed 2-3 fold more p27 mRNA than controls. In these cases, the sensitivity of the technique and/or the cutoff of differential expression assigned by the investigator may explain the different results. Moreover, these differences may not be relevant as it has been repeatedly reported that p27 mRNA levels do not always correlate to p27 protein levels due to the intense posttranscriptional regulation (Alessandrini et al., 1997; Hengst and Reed, 1996; Munoz-Alonso et al., 2005; Pagano et al., 1995). Most of the data in the literature refers to p27 (and Myc, see later) mRNA. However, the lack of correlation between p27 mRNA and protein means that most of the previous reports and the gene profiling data are meaningless so as to know the roles of p27 in this leukemia. Therefore, we decided to carry out our study on p27 expression at the protein level by immunoblot. Immunoblot analysis performed in 118 CLL samples showed that p27 protein expression is also significantly overexpressed in 82% of the patients (Fig. 4.3). Interestingly, when we studied p27 mRNA and protein expression in the same patients, we found that there was not correlation between both in CLL cells (Rho´s spearman = 0,0064; *P* = 0,9722), indicating that regulation of protein stability may play an important role in the p27 regulation in this leukemia. In fact, there was not correlation between p27 mRNA and any clinical correlation, which suggest that p27 mRNA it have not any prognostic value in CLL. Because this shows the importance of posttranslational regulation of p27 in CLL, we focus on the p27 protein expression.

In contrast to mRNA expression, we observed a significant correlation between p27 protein expression and two molecular markers used in the diagnosis of CLL: Zap-70 and CD38. High p27 protein expression was associated with low Zap-70 and CD38 expression (n = 73) (Table 4.1 and Fig. 4.4). Zap-70 and CD38 are often used as markers of progression. However, we did not find any association between p27 and IgV_H mutational status (Table 4.1), although this marker could only be analyzed in 30 patients. Although low Zap-70 and CD38, together with mutated $IgV_{H,}$ are used as good prognosis markers, we cannot conclude that p27 is a marker of good prognosis because patients with high Zap-70 and CD38 expression (markers of unmutated CLL) also expressed higher levels of p27 than healthy samples (*P* = 0.0122). Mutated CLL cells are less susceptible to be activated resulting in low levels of proliferation rate. Therefore, it is not surprising to find higher levels of p27 in these cells than in unmutated CLL. Thus, p27 seems to give an advantage to the leukemic cell. In this respect, a nearly statistically

significant correlation between p27 protein expression and p53 deletion was found (Table 4.1 and Fig. 4.4C). Deletion of p53 is a strongly indicator of poor prognosis and resistant to chemotherapy treatment in CLL (Cordone et al., 1998; Dohner et al., 1995; Dohner et al., 2000; Pettitt et al., 2001; Plass et al., 2007). Thus, the result suggests that p27 collaborate with the lack of p53 to produce a more aggressive CLL. However, it is important to note that high p27 protein levels did not associate with a better overall survival (Fig. 4.5), the stage of the disease or the cytogenetic abnormalities (table 4.1), which suggests that p27 requires the presence of other factors for contribute to a more aggressive form of the disease.

The cytoplasmatic p27 expression has been correlated with poor prognosis in some tumors. It must be noted that CLL lymphocytes exhibit a very high nucleus/cytoplasmic relation, and therefore it is difficult to assess the amount of cytoplasmic p27 (Fig. 4.7). Nevertheless, our immunofluorescence analysis showed that in most CLL patients p27 is mainly nuclear. As a CKI, p27 should show a tumor suppressor activity, but its high nuclear expression in this leukemia together with its correlation with poor prognosis suggest that p27 over-expression contributes to the increased number of blood B-CLL lymphocytes.

The analysis of Myc mRNA showed that in the 98 % of patients Myc levels were significantly lower than in controls (n = 91). Moreover, 74 % of patients exhibited low Myc protein levels (n = 113) (Fig. 4.9), and in a majority of the case (48 % of total) Myc protein expression was under our level of detection by immunoblot. However, in contrast to Myc mRNA analysis, 26 % of patients expressed equal or higher Myc protein levels than control (Fig. 4.9), but only 5 % showed more of 2-fold of expression with respect to control. Therefore, the over-expression of Myc is a rare event in B-CLL cells, and it is important to note that in most of the patients classified as “positive-Myc”, Myc levels were not higher than in normal tonsil B-cells. It is possible that at least in the patients where it was overexpressed, Myc may play an important role in the molecular biology of B-CLL cells. In our cohort we had a small number of Myc-positive patients, preventing us to generate statically significant data so as to the Myc role in CLL progression (Table 4.2). There are previous reports correlating Myc mRNA expression with poor prognosis, where a former selection of a cohort of patients with high levels of Myc was performed (Halina et al., 2010; Zhang et al., 2010). However, in our cohort of patients, only 2 % exhibited high Myc mRNA expression. Regarding Myc protein expression, although 26 % of patients showed expression of Myc, only six patients (5 % of our samples) showed more of 2-fold of expression with respect to controls. Thus, the number of patients with high levels of Myc analyzed was too low to observe significant correlations.

Despite the low number of Myc-positive samples, we found a correlation between Myc mRNA expression and 13q14 deletion (Table 4.2, Fig 4.10b). Although it would be necessary to analyze a larger number of Myc-positive samples to confirm these correlations, it is possible that 13q14 deletion and Myc expression collaborate in the progression of the disease. It have been found higher levels of Myc in a subset of CLL patients with resistence to fludarabine-treatment than in sensitive CLL patients (Moussay et al., 2010). Most of these patients (90%) showed an accumulation of genomic abnormalities, including 13q- deletion, which has been implicated with

Bcl-2 overexpression (Calin et al., 2002; Cimmino et al., 2005). Bcl-2 inhibits the pro-apoptotic effect of Myc (Wagner et al., 1993), and therefore it is very likely that both work together in these resistant patients.

Since only 2 % of patients showed high Myc mRNA expression, and p27 analysis showed that p27 protein expression has more prognostic value than p27 mRNA expression in CLL, we also decided to focus on the Myc protein expression.

When both Myc and p27 protein expression were analyzed in the same patients, a clear inverse regulation of both genes was found, where most of the patients (84 %) expressed only one of them (Fig. 4.15). Only 13 % of the patients expressed both proteins (above the levels detectable in our immunoblots) and 3% did not express any of them (Fig. 4.15D). The inverse correlation between Myc and p27 protein expression although unreported in CLL, has been observed in other in vitro and in vivo models (Alves et al., 2010; Bagui et al., 2009; Chandramohan et al., 2008; Dvorackova and Uvirova, 2007; Keller et al., 2007; Kim et al., 2010; Maclean et al., 2007; Pathak et al., 2011; Tang et al., 2009; Wang et al., 2011; Wang et al., 2012). However, while in these models Myc is up-regulated and p27 down-regulated, in CLL is p27 which is overexpressed and Myc underexpressed. Moreover, whereas high expression of Myc and low of p27 correlated with poor prognosis in most of the tumors we observed that the Myc expression in CLL patients with high levels of p27 marks no progression and good prognosis (Fig. 4.16). Due to Myc deregulation in many tumor types, and that Myc represses p27 expression, the mechanisms through which Myc down-regulates p27 have been exhaustively studied (see Introduction), (Alves et al., 2010; Bagui et al., 2009; Chandramohan et al., 2008; Dvorackova and Uvirova, 2007; Keller et al., 2007; Kim et al., 2010; Maclean et al., 2007; Pathak et al., 2011; Tang et al., 2009; Wang et al., 2011; Wang et al., 2012). In contrast, whether p27 can regulate Myc expression is still under discussion. Thus, CLL, where p27 is up-regulated, is a perfect model to find a regulation of Myc by p27.

One of the last poor prognosis markers discovered in CLL is the mutation of *NOTCH1* gene (Puente et al., 2011). The mechanisms through which Notch1 expression results in poor prognosis in CLL are poorly understood, but in other models, it has been shown that Myc is one of the genes that are activated by Notch1 (Klinakis et al., 2006; Palomero et al., 2006; Weng et al., 2006). We studied a possible correlation between Myc expression and mutations in *NOTCH1*, which will allow a better understanding of the mechanisms by which Notch1 induces poor prognosis in CLL. However, when we studied Myc in cohorts of CLL with wild-type and mutated Notch1, we only found a coincidence of both in a minority of cases (Fig. 4.14). the absence of Myc overexpression in cases of mutated Notch1 suggests that Myc deregulation is not the major route by which Notch1 induces poor prognosis in CLL.

The Max protein is considered as the center of a network where Myc and Mxd compete for Max to form active heterodimers (Baudino and Cleveland, 2001). Thereby, Max forms transactiviting complexes when associated with Myc but repressive complexes when bound to Mxd. Thus, repression or activation depend on Myc-Max and Mxd-Max equilibrium, and changes in the balance could occur through fluctuations in the expression of Myc or Mxd in

processes such as growth arrest or differentiation (Alland et al., 1997; Ayer et al., 1993; Ayer et al., 1995; Baudino and Cleveland, 2001; Rottmann and Luscher, 2006). When we analyzed Max mRNA and protein expression, we found that Max up-regulated with respect to controls (P = 0.019) (Fig. 4.12). This high expression was not found as statiscally significant at the protein level (P = 0.1, Fig. 4.12D), although, the number of protein samples analyzed for Max protein was small (n = 26). Max upregulation in CLL is a surprising finding as Max is usually reported as a constitutive gene and no significant changes in Max levels have been reported in human cancer. We also found that Mxi1 is significantly up-regulated in CLL (Fig. 4.13), in contrast to Mxd1, which is not expressed in most of the samples analyzed (85%). Although a higher number of patients may be required to confirm this result, the combined up-regulation of Mxi1 and Max would impair the Myc transcriptional activity.

Immunoblot of five samples isolated from lymph node and bone marrow showed high expression of Myc. Although the number of cases is limited, these results suggest that Myc is involved in the proliferarion of B-CLL cells observed in lymphoid tissues (Smit et al., 2007; Themi et al., 1973). Several proteins can induce Myc in lymph node. For example, the expression of BAFF that has been described in these tissues (Nishio et al., 2005) and recently, it has been reported that BAFF induces Myc expression in B-CLL cells (Zhang et al., 2010). Surprisingly, we also detected significant p27 expression in these samples. Given that p27 down-regulation by Myc has been strongly demonstrated in many other models, we think that these results can be explained by the existence of several subpopulations of B-CLL cells within lymphoid node or bone marrow. Thereby, B-CLL cells could proliferate only in a sub-localization of these tissues, and B-CLL cells placed in other location can express p27. Thus, other additional studies are necessary to know the existence of different subpopulations in lymph node of CLL patients.

Myc affects to p27 stability through induction of components of the SCF^{SKP2} ubiquitin ligase complex that targets p27 for the degradation via proteasome (Bretones et al., 2011; Keller et al., 2007; O'Hagan et al., 2000). It has been reported by our laboratory that Skp2 is a Myc target gene (Bretones et al, 2011). We showed that Skp2 protein expression was strongly correlated with high levels of Myc and low levels of p27 in 66 CLL patients analyzed (Fig. 4.18D, 4.18E and 4.18F). This suggests that Myc regulates Skp2 gene transcription also in CLL cells, as it has been recently described for CML in our laboratory (Bretones et al., 2011). 85 % of our patients (57 out of 67) showed low levels of Skp2 and, in most cases, this correlated with high p27 levels. Moreover, only one patient with high levels of Skp2 was not associated with high levels of Myc, which suggests that Skp2 is induced by Myc in B-CLL cells. However, when we consider only patients with Myc expression, Skp2 is expressed only in 60 % of patients and the remaining 40 % with low Skp2 also showed high levels of p27 (Fig. 4.18D). We do not know why Myc did not induce Skp2 in these patients. Nonetheless, as discussed above the progression of the disease was slower in CLL patients that overexpressed both proteins than in patients with high p27 and low Myc (Fig. 4.17), suggesting that Myc has a role which is independent of Skp2.

Why most of our samples (71 %) showed high levels of p27 and low of Myc?. It is possible that this can be due to the down-regulation of Myc because of the lack of external signals that induce its expression, but it is also possible that p27 is able to down-regulate the expression of Myc. It is known that Myc mRNA expression is drastically decreased in quiescent cells through inhibition induced by Rb, which binds to E2F-1 and inhibits its activity as a transcription factor activating the Myc promoter (Albert et al., 2001; Oswald et al., 1994). Rb is only functional when it is hypophosphorylated, and p27 prevents its phosphorylation through the inhibition of Cdk/cyclins complexes, Thus, p27-dependent cell cycle arrest could be sufficient to inhibit Myc expression. This hypothesis is in agreement with transfection of ectopic p27 in MEC1 cell line, which resulted in G_0/G_1 arrest and in a dramatic decrease of the Myc protein and mRNA expression (Fig.4.19b and c). Further work should be carried out to confirm this hypothesis

4.2 Roles of p27 and Myc in B-CLL cells

4.2.1 Cell cycle regulation by p27 and Myc

Next, we explored the functions of Myc and p27 in the biology of B-CLL cells. Although Myc and p27 elicits a variety of biological functions, one of the most important functions is the regulation of the cell cycle progression (see Introduction). Cyclins and Cdks are key pieces to the correct cell cycle progression, and both Myc and p27 regulated the activity of these Cdks. The analysis of the main Cdks of G_1 – S phases (Cdk4, Cdk6 and Cdk2) showed an ubiquitous expression of these Cdks regardless of p27 or Myc expression, as expected (data not shown). In contrast, S-phase Cyclins (Cyclin A and E) were expressed only in patients with high levels of Myc (Fig. 4.20). Therefore, Myc is able to activate cell cycle progression in B-CLL cells through the increase in the levels of S-phase cyclins.

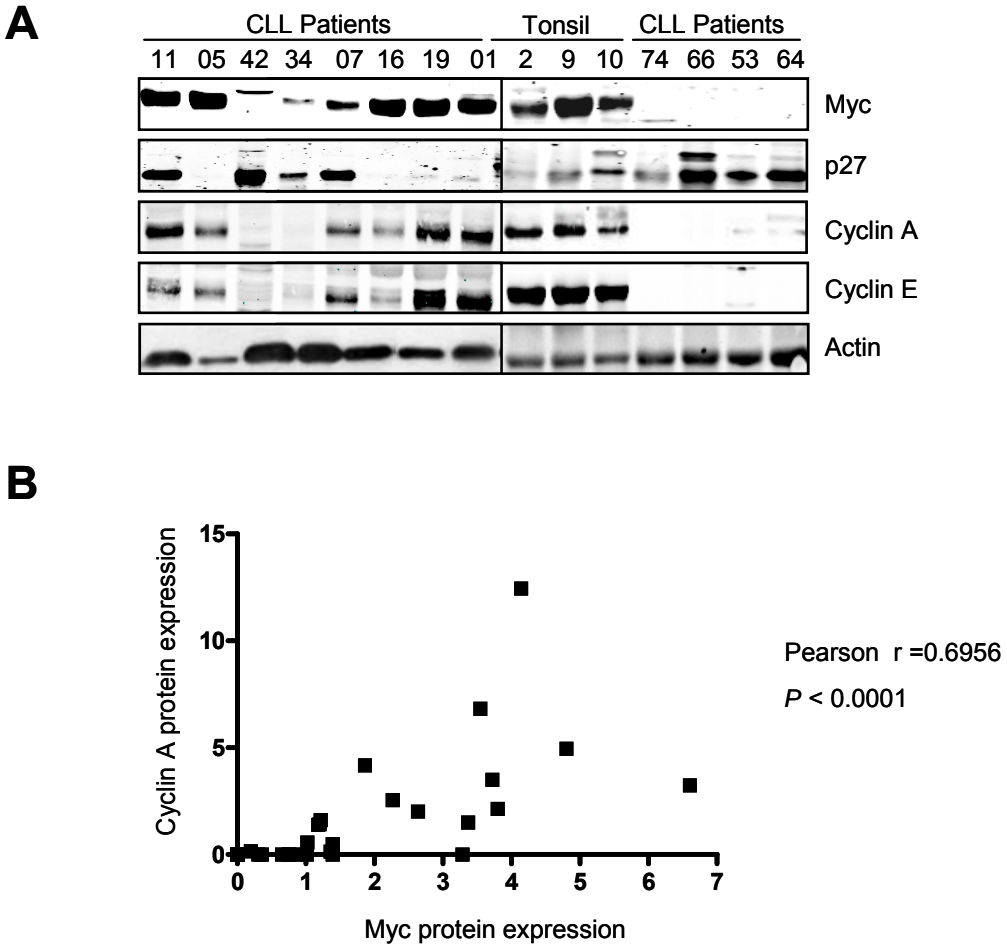


Figure 4.20.- Cell cycle regulation in B-CLL cells. (A) Immunoblot showing Myc, cyclin A and cyclin E protein expression in B-CLL cells and normal lymphocytes. Normal lymphocytes from tonsils were used as control. Actin levels are shown to asses protein loading. (B) The correlation between Myc and S-phase cyclins levels from CLL patients analyzed. The Pearson's r and *P*-value are indicated.

The double positive patients (i.e., with high levels of Myc and p27) also showed high levels of S-phase cyclins (Fig. 4.20). Therefore, Myc expression is sufficient to induce S-phase cyclins regardless of whether the cycle is stopped or not by the presence of p27.

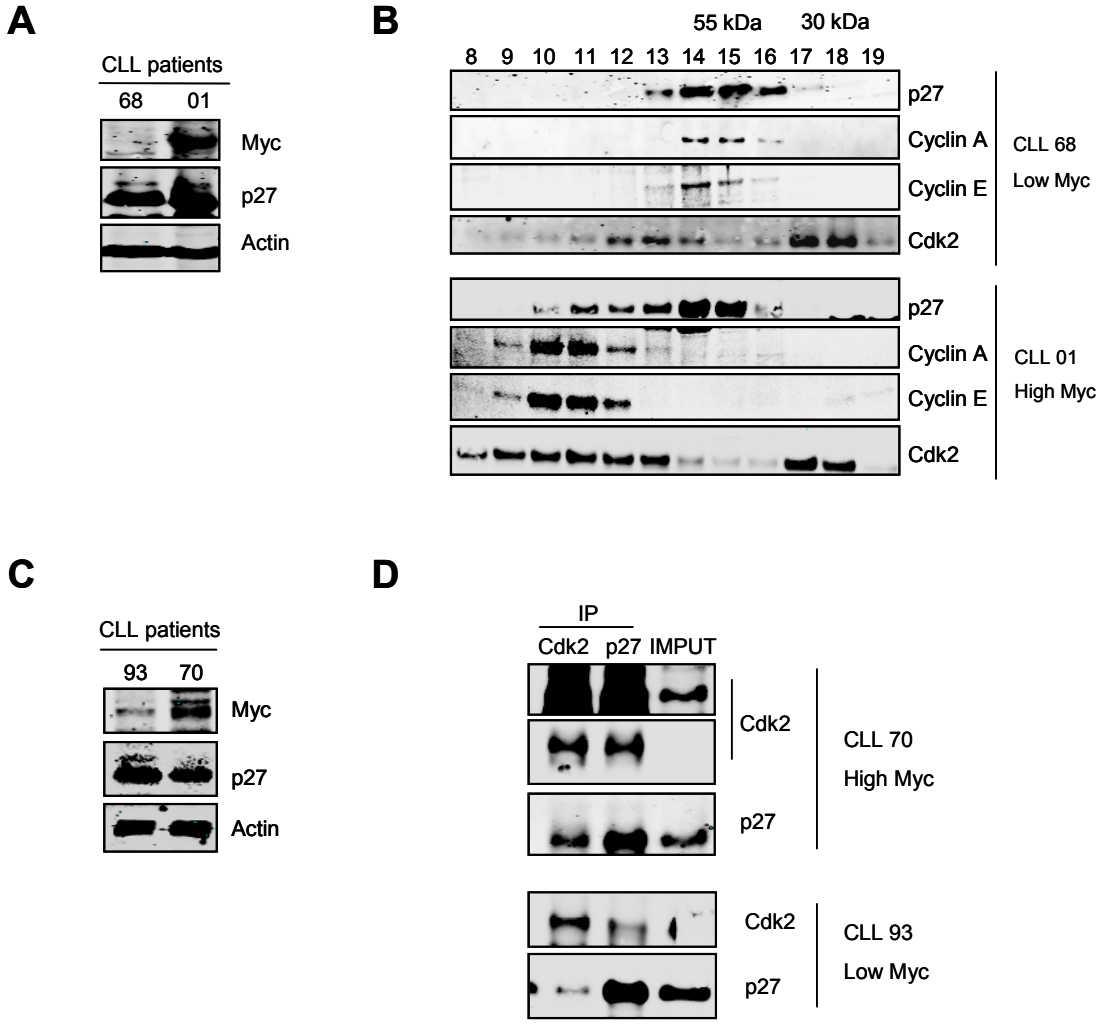


Figure 4.21.- Effect of p27 expression in Myc positive patients. (A) Immunoblot analysis showed Myc and p27 expression of two CLL patients. Actin levels are shown to asses protein loading. (B) CDK-cyclins-p27 complex from these two samples (patient #68 with low expression of Myc and patient #01 with high levels of Myc) were separated by size-exclusion chromatography. From 8 (high-molecular weight) to 19 (low-molecular weight) fractions were tested for p27, cyclin A, cyclin E and Cdk2 by immunoblot. (C) Immunoblot showing p27 and Myc protein expression of two CLL patients. Actin levels are shown to asses protein loading. (D) Lysates of patients analyzed in (C) were immunoprecipitated with Cdk2 and p27 antibodies, and the levels of p27 and Cdk2 in the immunoprecipitates were assayed by immunobloting. Long exposure and short exposure of Cdk2 are showed in patient #74.

In most of our cases, B-CLL cells express high levels of p27, but we wonder if this expression was high enough to prevent cell cycle progression when Myc is expressed. To answer this question, the total lysate of B-CLL cells from two patients, one with high Myc expression and the other with low levels of Myc (Fig. 4.21A), were chromatographed onto a column of gel filtration (Superdex). The results showed that Myc provoked a displacement of p27 from free forms into high-molecular weight fractions containing cyclins and Cdks (Fig.

4.21B). Why p27 is not bound to Cdk/cyclins complexes in the patient where Myc was not expressed (patient #68) could be explained by the low expression of cyclin observed in these samples, so that Cdk-Cyclin complexes cannot be formed. Moreover, we did not find free Cyclin/Cdks complexes without p27, indicating that the levels of p27 were sufficient to bind to and, likely, to inhibit all Cdk/cyclin complexes. To directly confirm these results, we performed an immunoprecipitation of other two CLL patients (Fig. 4.21C). The results showed that Cdk2/p27 complexes were only marginally detectable in B-CLL cells with low levels of Myc (patient #93), but the amount of these complexes was strongly increased in B-CLL cells with high levels of Myc (patient #74) (Fig. 4.21D). Thus, we conclude that Myc increases the association of p27 in Cdk-Cyclin complexes through the induction of S-phase cyclins.

We showed above that Myc expression results in the binding of p27 to Cdk/Cyclins complexes in B-CLL cells. It is known that Cdk2/Cyclin E is able to provoke p27 phosphorylation in Thr-187, which results in the stimulation of p27 degradation through Skp2 pathway (Nguyen et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997). Thus, we next studied the phosphorylation status of p27 in these cells. The immunoblot with phopho-specific antibodies showed that p27 was not phosphorylated in Thr-187 in double positive patients (i.e., expressing Myc and p27) likely because Cdk2/Cyclin E is inactive in the presence of high levels of p27 (Fig. 4.22). On the other hand, p27 phosphorylation in Ser-10 has been shown as an important regulator of the stability of p27 in G_0/G_1 transition. All CLL samples analyzed by immunoblot showed that p27 was phosphorylated on Ser-10 (Fig. 4.22), but immunofluorescence studies showed that p27 localization was mainly nuclear. Although some traces of cytoplasmic p27 could also be observed in some samples, its presence was independent of the levels of Myc. Samples #27, #89, #91, #96 and #101 exhibited low levels of Myc, whereas #93, #94 and #95 presented high levels of Myc. Immunoblot and RT-qPCR of these samples are shown in Fig. 4.18A and 4.18B.

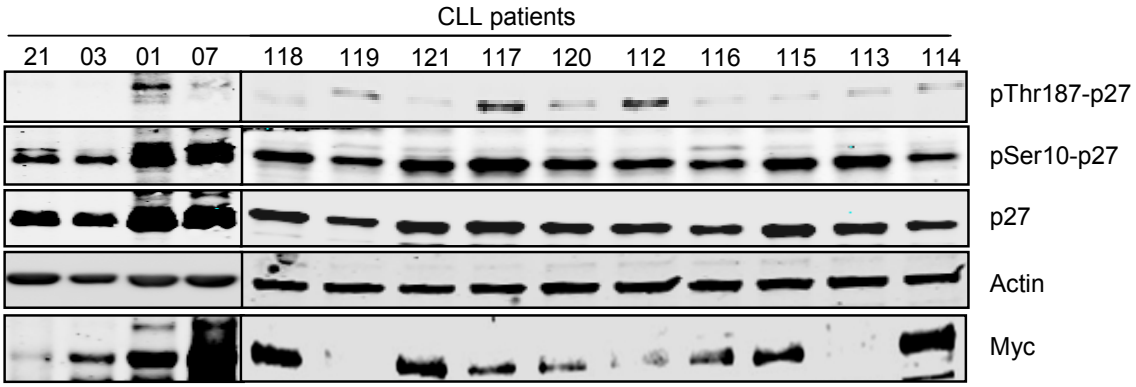


Figure 4.22.- Phosphorylation status of p27 in B-CLL cells. Immunoblot showing Myc and p27 protein expression and p27 phosphorylation status (pThr187 and pSer10) in B-CLL cells. Actin levels are shown to asses protein loading.

4.2.2 p27 and Myc modify the drug-induced apoptosis of B-CLL cells

The accumulation of B lymphocytes in peripheral blood observed in CLL has been ascribed to defective apoptosis (see Introduction). It is thought that resistance to apoptosis of B-CLL cells is due to over-expression of some proteins of Bcl-2 family, which exhibits anti-apoptotic functions (Buggins and Pepper, 2010; Kitada et al., 1998). However, we asked whether p27 over-expression may also play a role in the apoptosis-resistance characteristic of the B-CLL cells. To check out our hypothesis, we used MEC1 cell line again. We previously showed that the ectopic p27 expression induces G_0/G_1 arrest (Fig. 4.19B), and now we explored if these cells could also exhibit resistance to apoptosis. We used fludarabine as the apoptotic agent in our experiments, as fludarabine is the chemotherapy drug most used in the CLL treatment (Byrd et al., 2006; Ghia et al., 2007; Wierda and O'Brien, 2006; Yee and O'Brien, 2006). MEC1 transfected with a p27 expression vector and with empty vector were treated with fludarabine for 24 hours. MEC1 with high levels of p27 was more resistant to fludarabine-induced apoptosis than MEC1 transfected with an empty vector or not transfected (Fig. 4.23). We observed less apoptosis measured by annexin V-positive cells (Fig. 4.23A and 4.23B), less percentage of death (Fig. 4.23C), and less amount of active caspase-3 (Fig. 4.23D) in MEC1 with p27 over-expression. We conclude that p27 induces apoptosis resistance in MEC1 cells.

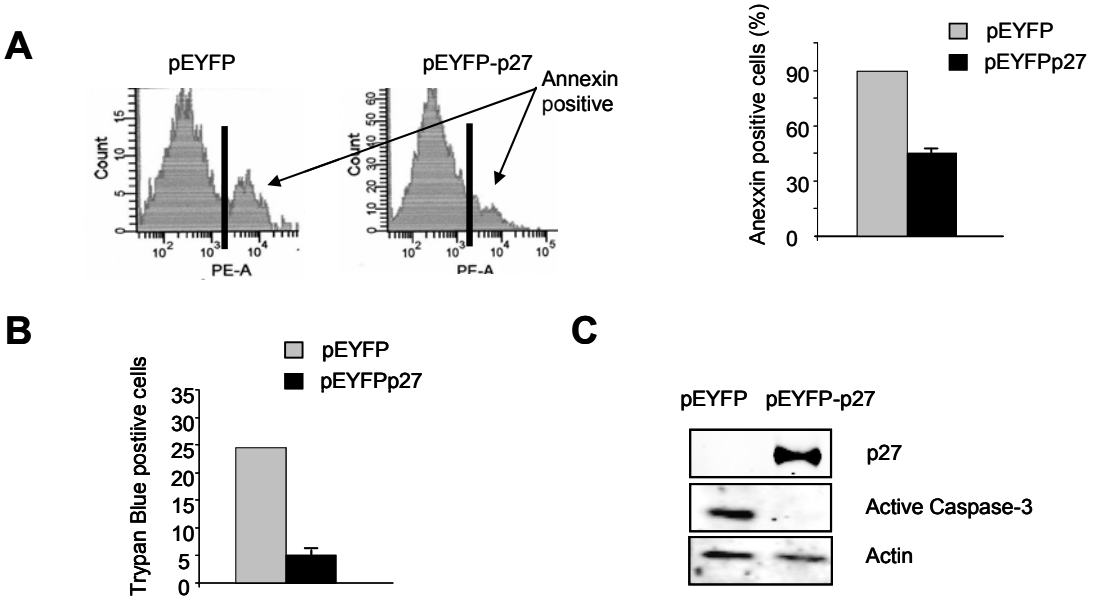


Figure 4.23.- p27 expression rescued fludarabine-induced apoptosis of MEC1. (A) MEC1 cells were transfected with pEYFP or pEYPFp27 and grown for 24 hours. Then they were treated with 10 x 10^6 mol/ml of fludarabine for 24 hours. After treatment, MEC1 were labelled with annexin and analyzed by flow cytometry (left figure). MEC1 cells with high levels of p27 showed significant lower annexin positive cells than Mec1 control (right figure). Data are mean from three independent experiments (B) MEC1 transfected with pEYFP or pEYFPp27 were grown for 24 hours. Then they were treated with fludarabine for 24 hours. The percentage of dead cells was calculated by trypan blue assay. Data are mean from three independent experiments (C) Levels of active caspase-3 in MEC1 overexpressing p27. The cells were transfected with pEYFP or pEYFP-p27 and the levels of active Caspase 3 were assessed by immunoblot. Actin levels are shown to asses protein loading.

Although p27 can play a role in the resistance to fludarabine-induced apoptosis in B-CLL cells, over-expression of other anti-apoptotic proteins has been described in CLL (Buggins and Pepper, 2010; Kitada et al., 1998). Some anti-apoptotic members of Bcl-2 family, as Bcl-2, have been associated with to resistance to apoptosis stimuli characteristic of B-CLL cells. In the last years, expression of this anti-apoptotic protein Bcl-2 has been correlated with the expression of p27 in different systems (Batsi et al., 2009; Greider et al., 2002; Vairo et al., 2000). Thereby, Bcl-2 enhanced p27 levels independent of delayed cell cycle entry induced by itself. In other tumors, p27 is strongly associated with a favorable outcome and therefore can mitigate the oncogenic impact of Bcl-2 through inhibition of cell cycle progression. However, as we showed above, in this case p27 has an impact in the apoptosis function of B-CLL cells (Fig. 4.23) like Bcl-2. Thus, Bcl-2 and p27 could play a same role and work together in the resistance to drug-induced apoptosis.

We studied Bcl-2 expression in our cohort of CLL patients and we observed that 71% of the patients exhibited high levels of Bcl-2 with respect to controls (Fig. 4.24A). When we compared p27 and Bcl-2 expression in our patients, we found a high correlation, where 99% of the patients with high levels of Bcl-2 exhibited p27 over-expression (Fig. 4.24B). This correlation may indicate that p27 collaborates in the Bcl-2 anti-apoptotic function.

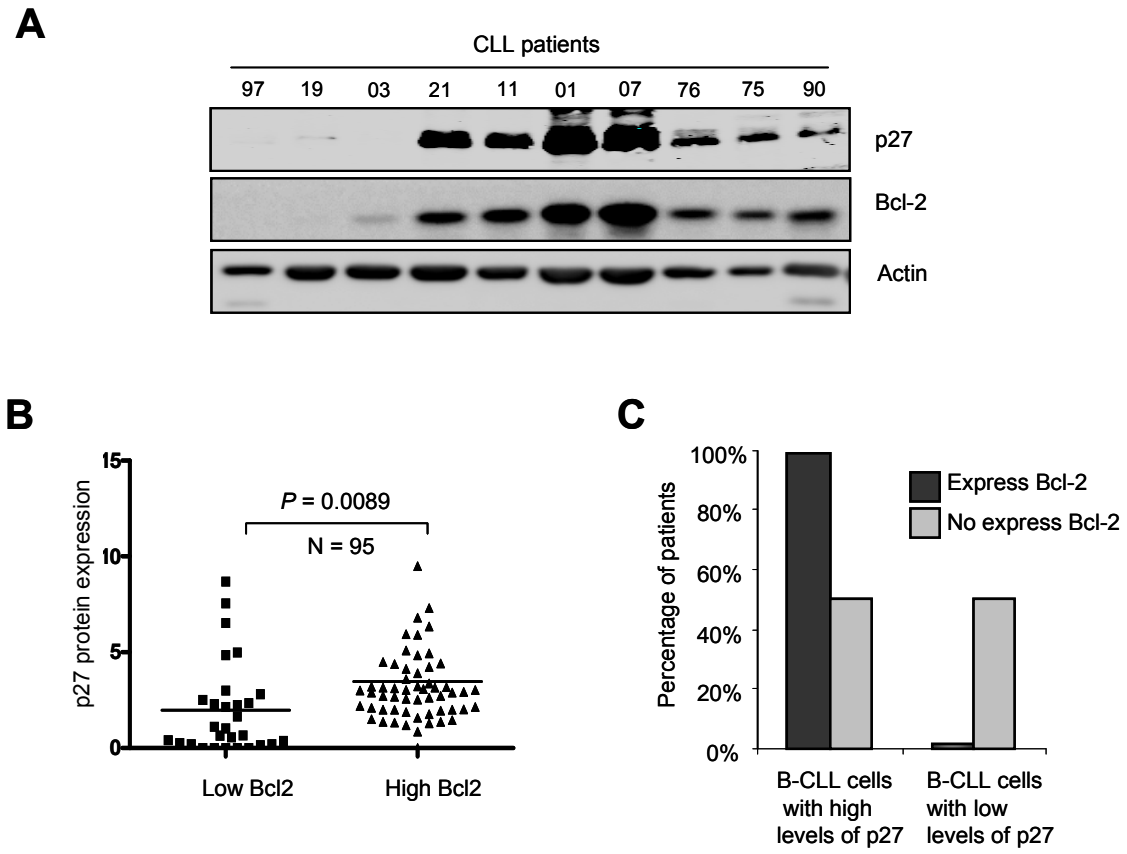


Figure 4.24.- Bcl-2 expression in CLL patients. (A) Immunoblot showing Bcl2 and p27 protein expression in B-CLL cells. Actin levels are shown to asses protein loading. (B) Mean levels of p27 protein expression (normalized to actin expression) in CLL with high and low Bcl2 expression. (C) Fraction of patients expressing Bcl-2 with respect to the p27 levels.

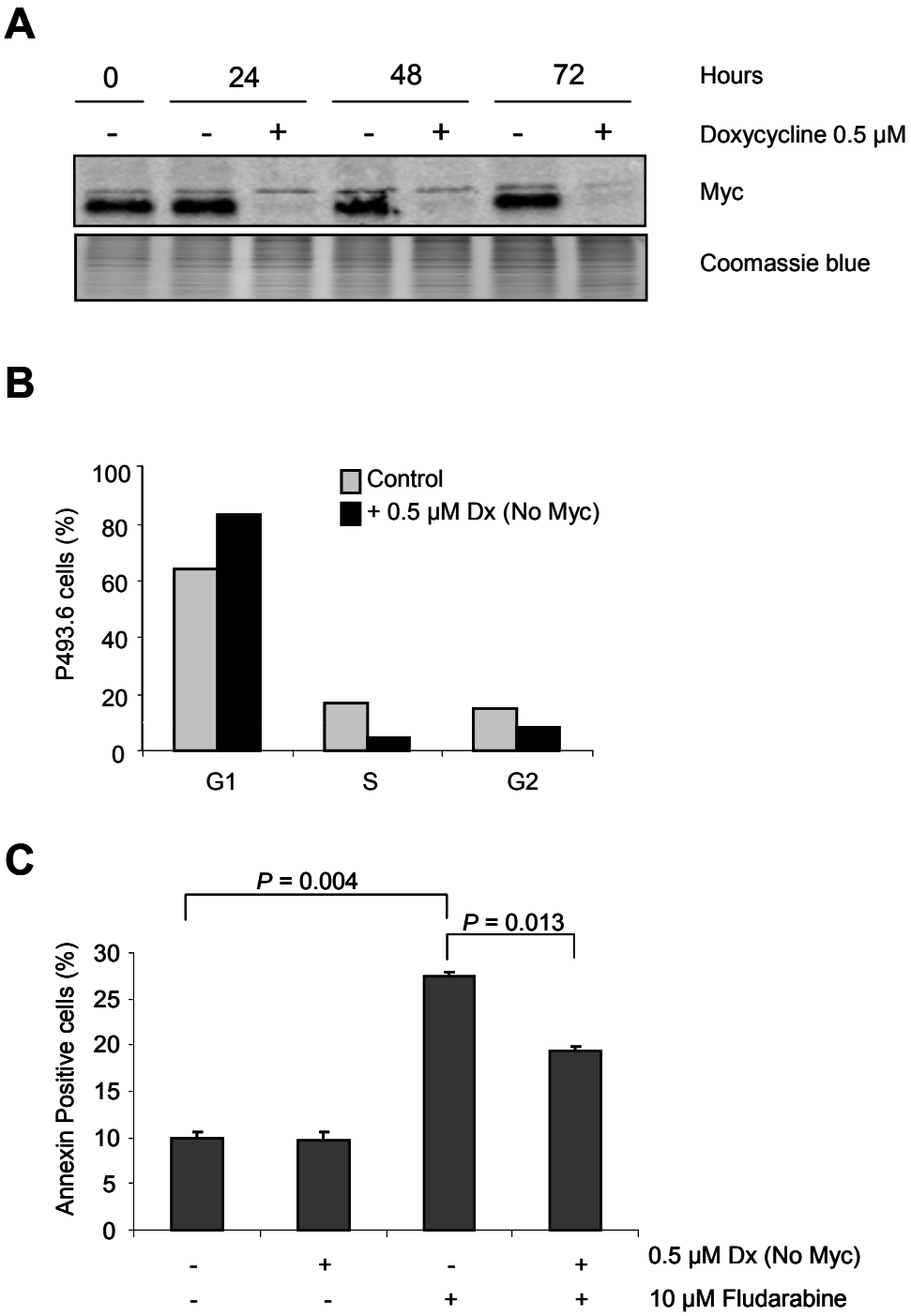


Figure 4.25.- Myc expression promotes fludarabine-induced apoptosis in P493.6 cell line. (A) Myc repression by doxycycline was confirmed by immunoblot. The cells were treated with 0.5 µM doxycycline for the indicated periods of time. A picture of the gel stainied with Coomasie Blue is shown to asses the protein loading (B) Cell cycle analysis by flow cytometry of P493.6 cells treated with doxycycline for 24 h to suppress Myc as indicated. The fraction of cells in each cell cycle phase is shown. (C) Patients with Myc expression exhibited greater sensibility to fludarabine-induced apoptosis. Myc expression of P493.6 cells was repressed by doxycycline treatment for 24 hours. Then, 10 µM of fludarabine was added and cells were incubated for 24 hours. Apoptosis was measured by annexin V assay. Data are means from two independent experiments.

Previously, we had found that high Myc expression is associated to slower disease progression in patients expressing p27 (Fig. 4.17). Given the anti-apoptotic function of p27, we

proposed that Myc, as a strongly S-phase inductor, could be associated with an enhanced apoptosis in these cells. To test this hypothesis we used P493-6 cell line which was derived from human peripheral blood B cells immortalized by Epstein-Barr EBNA gene and carries a tetracycline-repressible Myc transgene (Pajic et al., 2000). Thereby, when P493-6 cells are treated with tetracycline or doxycicline, ectopic Myc is repressed and cells exhibits very low Myc levels (Fig. 4.25A). These low levels of Myc are associated with a decrease in the number of S phase cells (Fig. 4.25B), but not with increased p27 levels (data not shown), in agreement with previous reports (Pajic et al., 2000). When P493-6 cells were treated with fludarabine in the presence or not of doxycycline (i.e., with low or high levels of Myc, respectively), we found a higher number of apoptotic cells in the presence of Myc (Fig. 4.25C). Hence, Myc provokes that these cells were more susceptible to treatment with fludarabine.

4.2.3 Roles of p27 and Myc in B-CLL cells: DISCUSSION

Skp2 has been proposed as a link between p27 and Myc in CLL which it is itself an oncogenic protein (Bretones et al., 2011; Old et al., 2010). In at least 60 % of our patients where Myc is expressed, Skp2 is induced. Skp2 target to degradation important cell cycle inhibitor and tumor suppressors such as p27, p21, p57 or p130 (see Introduction) thus being able to collaborate with the transforming activity of Myc. However, blood B-CLL cells are quiescent cells and arrested in G_0/G_1. We showed that p27 is sufficient by itself to induce cell cycle arrest, and therefore, its over-expression in this leukemia can be a good reason for the low proliferative rate observed. Nevertheless, a low percentage of CLL patients expresses Myc (Fig. 4.9 and Fig. 4.15D). These patients showed higher levels of S-phase cyclins than patients without Myc, which suggests that Myc can play a role as inductor of S-phase in B-CLL cells. Moreover, Myc induced S-phase cyclins despite the presence of p27 (Fig. 4.20), which shows that Myc activity is not suppressed by p27 or by the cell cycle arrest induced by it in patients where both proteins were detected. These results are in agreement with those observed previously in our laboratory by Acosta et al. In myeloid leukemia cells, who found that Myc induced an increase in cyclin A and D-type cyclins despite that p27 is overexpressed (Acosta et al., 2008). However Myc-induced proliferation in p27-arrested cells was dependent on the p27 dose. Thereby, when p27 is expressed at high levels, Myc could not reverse Cdk inhibition by p27 (Acosta et al., 2008). This effect also occurred in our CLL samples that express both Myc and p27. Chromatographic fractionation (Fig. 4.21B) and immunoprecipitation (Fig. 4.21C) showed that the amount of p27 present in the B-CLL cells was sufficient to bind to the S-phase cyclins induced by Myc. Thereby, although Myc induces S-phase cyclins, the cell cycle arrest will continue in the presence of p27.

Cdk2-Cyclin E complexes can phosphorylate p27 on the Thr-187, which converts p27 in an efficient substrate for Skp2 and therefore for ubiquitylation and degradation (Nguyen et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997). Phosphorylation of p27 on the Thr-187 may suggest that cyclin E/Cdk2 complexes are active. The high levels of Cyclin E and Skp2 in patients with high Myc and low p27 indicate that the absence of p27 is likely due to Myc-mediated induction of Cdk2-Cyclin E complexes, resulting in Thr-187 phosphorylation and degradation. However, immunoblot analysis with phospho-specific antibody performed in patients with p27 expression (with high or low Myc levels) showed that Thr-187 phosphorylation is only present in a few patients (independently of the Myc expression) (Fig. 4.22), which suggests that p27 is functional and inhibits Cdk2/cyclin E complexes in patients where Myc is also expressed.

In contrast with the infrequent presence of pThr187-p27, most of our CLL samples expressed pSer10-p27. When the different growth factors stimulate the cell cycle progression, kinases as Akt or Erk2 can phosphorylate p27 in this serine which results in its export of the nucleus (Besson et al., 2006; Boehm et al., 2002; Connor et al., 2003; Fujita et al., 2002; Ishida

et al., 2002; Rodier et al., 2001), followed by its degradation by Kpc complex (Kamura et al., 2004; Kotoshiba et al., 2005). Other kinases as Dyrk1B can phosphorylate Ser-10 in G_0 phases, but in this phase of the cycle, this phosphorylation stabilizes p27 and increases the amount of nuclear p27 (Deng et al., 2004; Ishida et al., 2000; Janumyan et al., 2008; Kotake et al., 2005; Mercer et al., 2005). Although p27 expression remains high in the presence of Myc in these patients, the expression of S-phase cyclins may indicate that these cells may be arrested in G_1 rather than in G_0, and therefore, phosphorylation of Ser-10 may act as an indicator of cytoplasmatic localization. However we showed that p27 was phosphorylated on Ser-10 independently of the presence or absence of Myc (Fig. 4.22), but its localization was nuclear (Fig. 4.6). This suggests that despite the presence of S-phase cyclins in cells expressing p27 and Myc, p27 is stable and active, and therefore, cells are unable to exit the G_0 phase.

The resistance to apoptosis has been reported as a major feature of B-CLL cells. We found in most CLL samples a high Bcl-2 expression, so this could explain the resistance to apoptosis. One of the known but less studied effects of Bcl-2 expression is the inhibition of the cell cycle, which is mediated by p27 expression (Batsi et al., 2009; Greider et al., 2002; Vairo et al., 2000). We demonstrated a strong correlation between both proteins, where patients with Bcl-2 expression exhibited higher levels of p27 than patients where Bcl-2 was not detected. Almost all the patients with Bcl-2 expression showed high levels of p27, although not all cases with high p27 expressed Bcl-2. In contrast, when we compared Myc levels according to Bcl-2 expression, we found that Myc is significantly less expressed in patients where Bcl-2 is expressed (data not shown). In summary, there is a strong correlation between high p27-high Bcl-2-low Myc in our cohort. However, we do not know whether this correlation is a consequence of a direct regulation of Bcl-2 (e.g., repressed by Myc or activated by p27). Studies performed by Greider et al. showed that Bcl-2 can induce cell cycle arrest through the elevation of p27 which in turn results in the inhibition of Myc (Greider et al., 2002). Therefore, it is even possible that Bcl-2 is the primary regulator of p27 in this model. If this pathway is confirmed in B-CLL cells, Bcl-2 may be another factor, together with Mxi1, p27 or Max, that represses Myc expression and/or activity in these cells. Additional studies are necessary to dissect the regulatory network of the three proteins.

p27 can play a role in the apoptotic regulation, as reported in a large number of studies (An et al., 1998; Dimanche-Boitrel et al., 1998; Drexler and Pebler, 2003; Eymin and Brambilla, 2004; Gomez-Casares et al., 2012; Katayose et al., 1997; Le et al., 2010; Nickeleit et al., 2008; Yang et al., 2000). However, p27 can play a dual role in this process, and depending on the drug or the model used, it can be a pro-apoptotic or anti-apoptotic protein (see Introduction). Several data support the idea that p27 can act as an anti-apoptotic protein in CLL: 1) B-CLL cells exhibit a high resistance to apoptosis-inducing drugs, and these cells express high levels of p27; 2) one of the proteins that are cleaved in the early stages of treatment with fludarabine (a first-line anti-CLL drug) is p27 (Sanhes et al., 2003; Vrhovac et al., 1998); 3) patients with a progressive disease showed a higher degree of quiescence accompanied by a much lower susceptibility to apoptosis than patients with a stable disease (Ricciardi et al., 2001).

Upon transfections MEC1 cells with a p27 expression vector, we observed a higher resistance to fludarabine-inducing apoptosis (Fig. 4.23). As a purine analogue, fludarabine blocks S phase and, therefore, it is possible that one of mechanisms through which p27 may induce apoptosis resistance resides in its activity as CKI. This activity requires a nuclear localization of p27, which we confirmed in B-CLL cells (Fig. 4.6). In fact, in the nucleus, p27 expression causes Cdk2 inhibition (Fig. 4.21), whose activity has been linked with apoptosis induction (Levkau et al., 1998). Therefore, expression of nuclear p27 results in G_0/G_1 arrest, preventing the fludarabine acquisition by cells since this occurs in S-phase, and at the same time, p27 inhibits Cdk2 activity blocking its activiy as a apoptosis inductor.

These results explain, at least partly, why over-expression of p27 is selected in B-CLL cells. p27-mediated cell cycle arrest allows first to impair apoptosis and second to prevent S-phase entry, which is required for chemotherapeutic drugs to exert its apoptotic function. If this assumption is true, an S-phase inductor as Myc should have the opposite effect, and actually, we showed that Myc expression can facilitate entry into apoptosis when cells are treated with fludarabine (Fig. 4.26). A similar effect was observed when B-CLL cells were treated with theophylline and chlorambucil (Mentz et al., 1996). All these results suggest that cell cycle regulation is associated with apoptotic resistance, and gives an explanation about why p27 is overexpressed and Myc down-regulated in this leukemia.

4.3 Myc-p27 interaction in the differentiation of myeloid leukemia cells

4.3.1 Region of p27 involved in K562 cell differentiation

Munoz-Alonso et al. showed that ectopic expression of p27 in the CML cell line K562 results in erythroid differentiation (Munoz-Alonso et al., 2005). Moreover, p27 protein expression is up-regulated in the induction of erythroid differentiation of K562 through treatment with cytosine arabinoside (AraC) u other chemicals (Aoki et al., 2004; Gomez-Casares et al., 2012; Munoz-Alonso et al., 2005). Indeed, the inhibition of p27 expression through short-hairpin RNA (shRNA) leads to a decrease in the ability of the differentiation of these drugs (Gomez-Casares et al., 2012). Together, these results showed the important role of p27 in this process of differentiation.

However, the mechanism through which p27 triggers this process of differentiation is still poorly understood. The most important function of p27 (and the most studied) is the inhibition of the cell cycle progression as a Cdk inhibitor. Thereby, in addition to the differentiation, ectopic expression of p27 in K562 cell line results in G_0/G_1 stopped (Munoz-Alonso et al., 2005). Moreover, erythroid differentiation of K562 induced by drugs is often accompanied by cell cycle arrest, and the ectopic expression of other cell cycle inhibitors as p16/INK4 can also induce erythroid differentiation (Munoz-Alonso et al., 2005). Therefore, cell cycle inhibition and differentiation functions elicited by p27 seemed to be part of the same pathway.

To confirm this hypothesis, we used a mutant of p27 which lacks of the C-terminal region, termed p27-Nt (Fig. 4.26A). This C-terminal region has not been involved in the cell cycle inhibition. This mutant also lacks of the NLS which is located in the C-terminal region (Fig. 4.26A). Immunofluorescence of K562 transfected with the mutant p27-Nt showed that, nevertheless, it is located in the nucleus (Fig. 4.26B). PI staining analyzed by flow cytometry confirmed that p27-Nt was able to inhibit the cell cycle (Fig. 4.26C). Therefore, the N-terminal region, where CDI is located, is sufficient to inhibit this process. This mutant also induced erythroid differentiation (Fig. 4.26D), accompanied by a decrease in the levels of Myc (Fig. 4.26E).

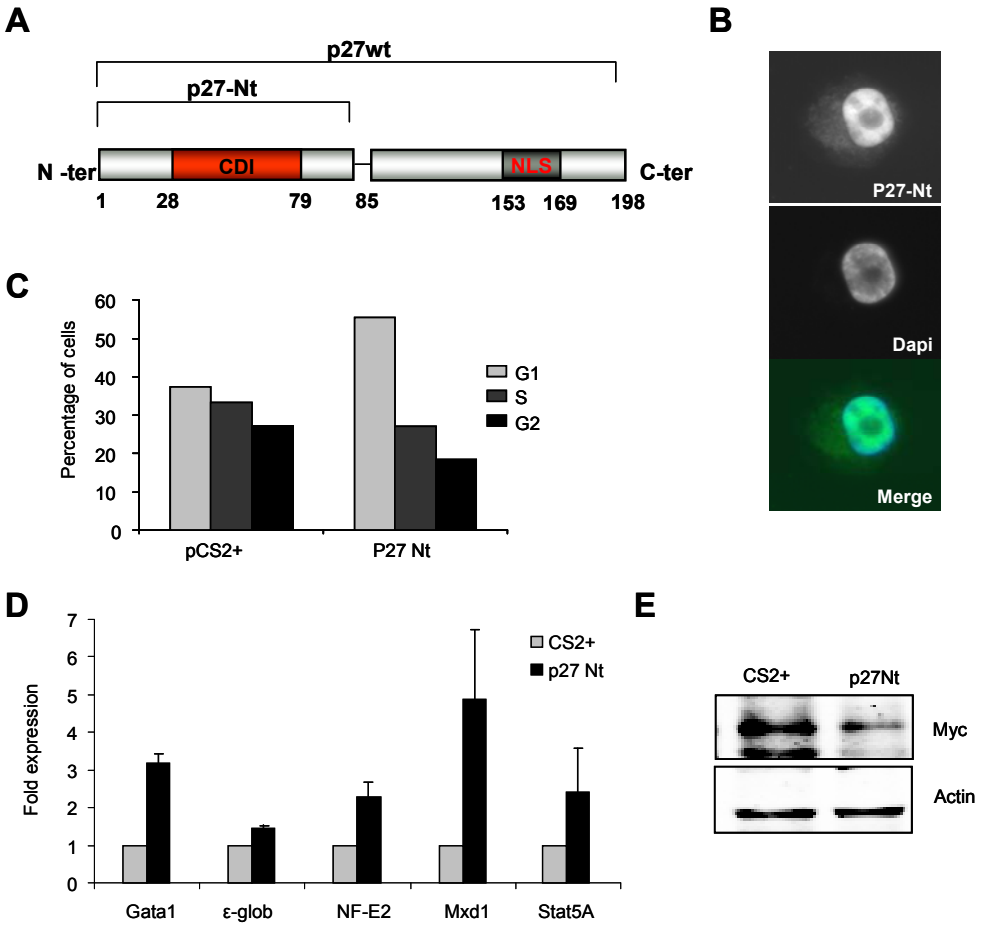


Figure 4.26.- N-terminal region of p27 induced erythroid differentiation. (A) Schematic representation of p27-Nt region. The Cdk-inhibitor domain (CDI) region is present in this mutant. (B) Immunofluorescence analysis showed that p27-Nt is located in the nucleus. (C) Cell cycle analysis by flow cytometry showed that p27-Nt induced G_1 arrest in K562. (D) Gata1, ε-Globin, NF-E2, Mxd1 and Stat5A mRNA levels were determined by RT-qPCR in K562 transfected with p27-Nt. K562 transfected with empty vector was used as control. RPS14 mRNA levels were used for normalization. Data are mean ± SEM of two determinations from three independent experiments (E) Immunoblot showing Myc protein expression in K562 transfected with p27-Nt and with empty vector. Membrane was probed with Actin as load control.

4.3.2 Study of erythroid differentiation induced by p27CK-

Although p27-Nt mutant was able to differentiate K562 cell line, we can not discard a possible function of the C-terminal region in this process. Through the use of a mutant of p27 that is unable to bind Cdk/cyclins complexes, we could ask function of this region in this process. This mutant, called p27CK-, carries four amino acid substitutions in the CDI region (Fig. 4.27A). When we chromatographed extracts of K562 transfected with p27CK- onto a column of gel filtration (Superdex), we found that p27CK- is only found as a free form (Fig. 4.27B). In contrast, p27 wild type (p27wt) was found in the same fractions than Cdks and cyclins, strongly suggesting that they are forming complexes (Fig. 4.27B). Moreover, cell cycle analysis through flow cytometry with PI of K562 transfected with p27wt or p27CK- showed that

while p27wt induced the inhibition of cell cycle progression in the G_0/G_1 step, p27CK- was unable to stop it (Fig. 4.27C). Finally, although p27CK- is not able to stop the cell cycle progression, immunofluorescence of K562 transfected with it showed that p27CK- is located in the nucleus (Fig. 4.27D)

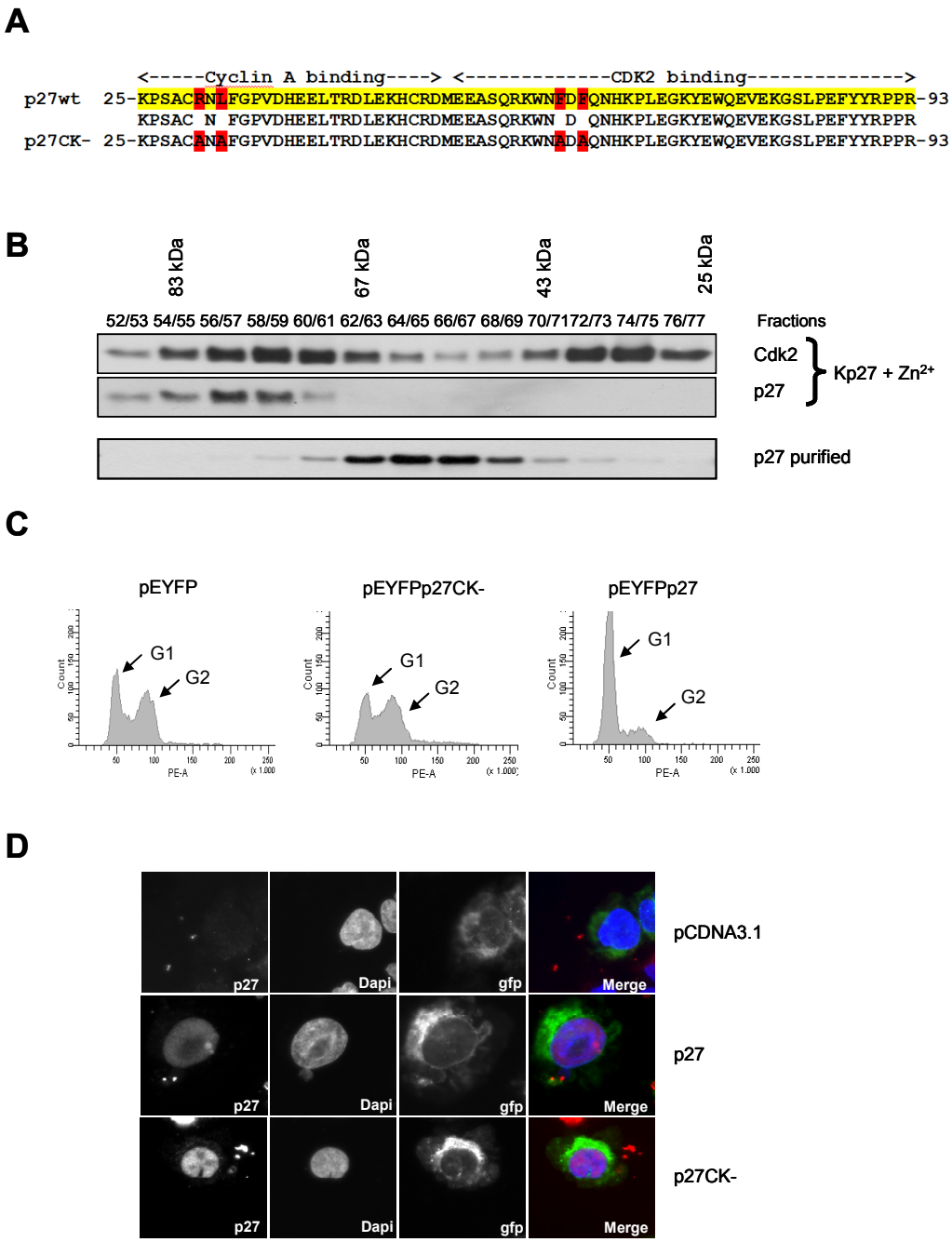


Figure 4.27.- p27CK- mutant. (A) p27CK- is a p27 mutant where four highly conserved amino acids in the Cdk inhibitor domain are substituted by alanines to prevent its interaction with cyclin-CDK complexes. (B) p27 complexes from K562 transfected with p27CK- were isolated by size exclusion chromatography. Kp27 with 50 μM Zn^{+2} (24 h) were used as p27wt control. p27CK- appears only in low molecular forms corresponding to unbound p27, whereas p27wt is present in high-molecular weight fractions, which containing cyclins and Cdks (performed by Gabriel Bretones). (C) Cell cycle of K562 transfected with YFPp27CK- vector was analyzed by flow cytometry using YFP and YFPp27wt vectors as controls. (D) Immunofluorescence analysis showing p27 and p27CK- in the nucleus of K562 cells.

K562 cell line was transfected in transient with a vector which carried the p27CK- cDNA. Cells with ectopic expression of p27CK- showed an increase in the expression of the erythroid marker genes such as Gata1, ε-globin, NF-E2, Mxd1, EpoR, glycophorin A or Stat5A with respect to K562 transfected with the empty vector (Fig. 4.28A and 4.28B). Similarly, and although p27CK- expression did not stop the cell cycle progression, a reduction in the protein levels of Myc was observed (Fig. 4.28C). However, in some transfection experiments, the differentiation was not triggered despite that p27CK- was expressed. Therefore, although these results may imply that Cdk inhibition and the differentiation induction by p27 were carried out by independent routes, we were not able to confirm completely these results.

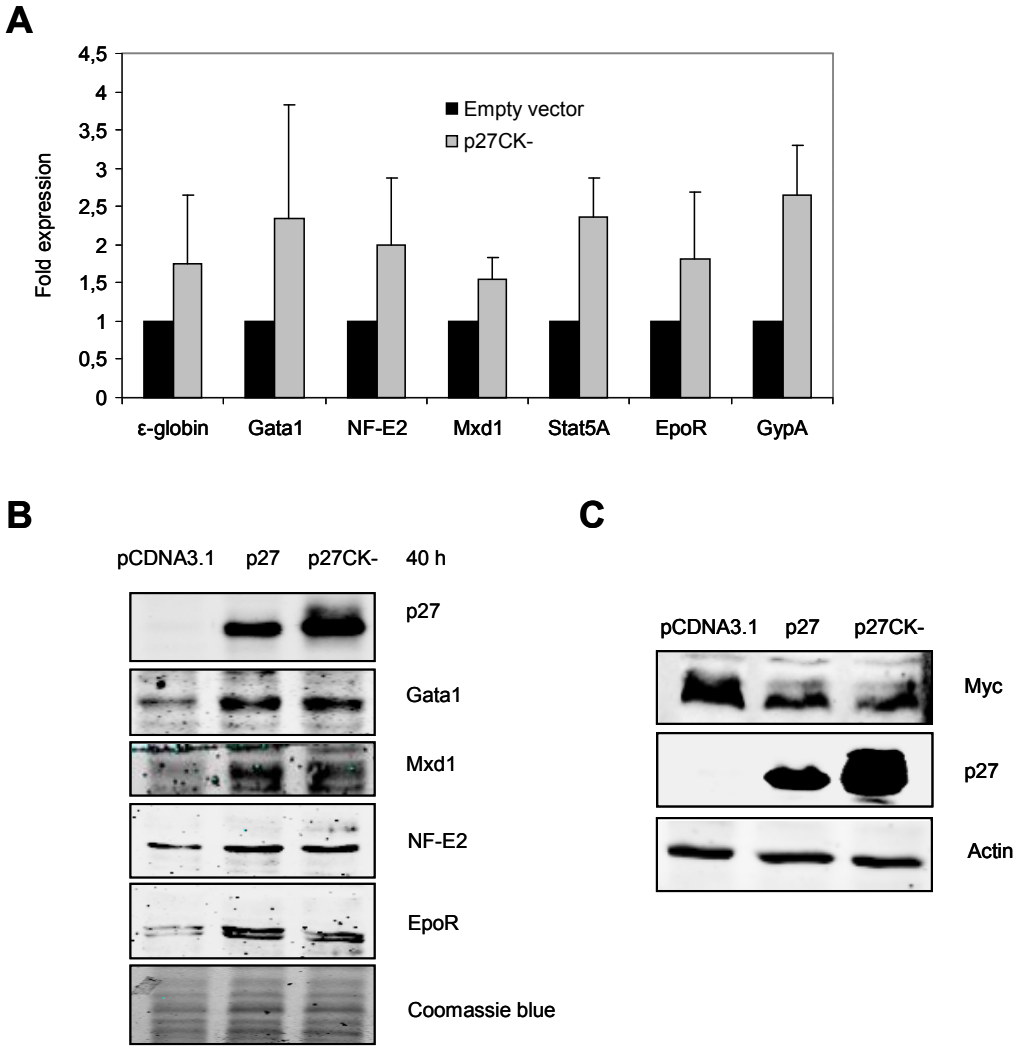


Figure 4.28.- p27CK- induces erythroid differentiation. (A) ε-Globin, Gata1, NF-E2, Mxd1, Stat5A, EpoR and GypA were used as markers of erythroid differentiation of K562 trasfecte with p27CK- (40 h) and its levels were measured by RT-qPCR (RPS14 mRNA levels were used for normalization) and/or (B) immunoblot (Actin levels are shown to asses protein loading). Data are mean ± SEM of two determinations from seven independent experiments (C) Immunoblot showing Myc protein expression in K562 transfected with p27CK-. Actin levels are shown to asses protein loading.

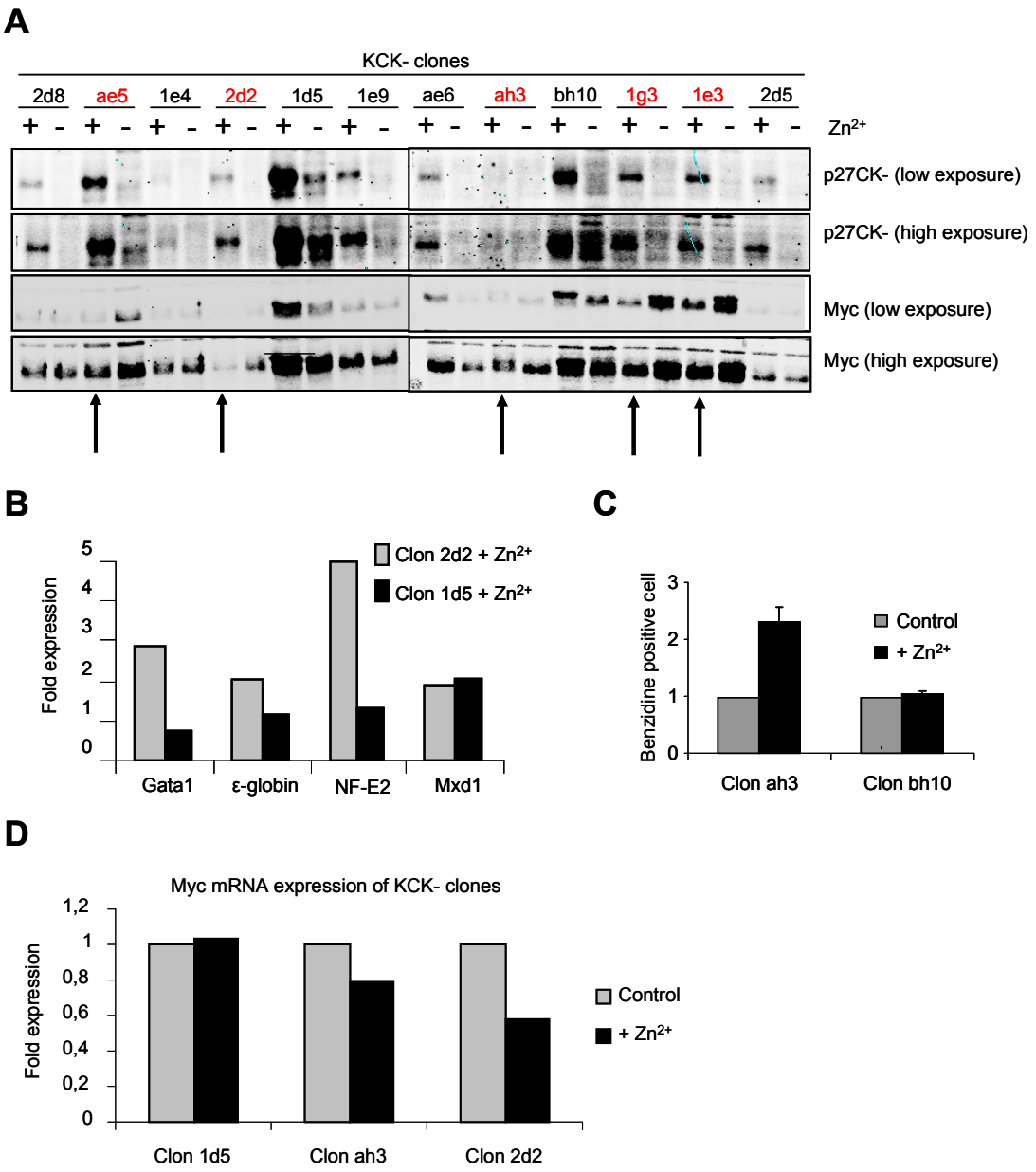


Figure 4.29.- Analysis of erythroid differentiation in KCK- clones induced with 75 µM Zn^{2+}. (A) KCK- clones were treated for 12 hours with 75 µM $ZnSO_4$ and expression of p27CK- was analyzed by immunoblot. Membrane was also probed with Myc. Arrows indicates KCK- clones where Myc was repressed when p27CK- was induced. (B) Erythroid differentiation of KCK- clones induced with 75 µM Zn^{2+} for 48 hours were analyzed through the mRNA expression of Gata1, ε-globin, NF-E2 and Mxd1. mRNA expression was determined by RT-qPCR. RPS14 mRNA levels were used for normalization. (C) The fraction of hemoglobinized KCK- clones induced with 75 µM Zn^{2+} for 48 hours was assayed by the benzidine test. KCK- cells without induction were used as control. Values are means ± SEM of three experiments. (D) Myc mRNA levels of KCK- clones induced with 75 µM Zn^{2+} for 12 hours were determined by RT-qPCR. KCK- cells without induction were used as control. RPS14 mRNA levels were used for normalization..

In order to confirm these preliminary results, we decided to generate a K562 cell line with an inducible expression vector of p27CK-. We used the pMT-CB6 vector, a vector with the metallothionine promoter through which we are able to modulate the expression of our gene, in

this case of p27CK- cDNA, with the addition of Zn^{2+} to the medium. We subcloned the p27CK- cDNA into the Zn^{2+}-inducible pMT-CB6 vector to generate the pMT-p27CK-. K562 cells were transfected with the new pMT-CB6p27CK- vector and selected with geneticin for 2-3 weeks. A dozen clones were selected, termed KCK-. The expression of p27CK- in the KCK- clones in response to 75 μM Zn^{2+} was studied by immunoblot (Fig. 4.29A). Although a great expression of p27CK- was observed in different clones, when we studied erythroid differentiation marker genes in each of them, we found divergent results. Thus, some of clones showed an increase in the expression of erythroid differentiation genes, displaying similar results to those observed in transient expression of p27CK- (Fig. 4.29B). However, the remainder of clones did not differentiate when Zn^{+2} was added (Fig. 4.29B). We asked why some clones differentiated and others did not by the expression of p27CK-. We found that clones with high erythroid differentiation showed a marked decrease in Myc expression associated with the over-expression of p27CK-, whereas Myc was not down-regulated in non-differentiating clones (Fig. 4.29C and arrows in 4.29A).

In order to clarify the contradictory results obtained in KCK- clones, we decided to carry out a more extensive study of the process regulated by p27CK- mutant. We performed a genome-wide RNA-seq of K562 transfected with p27CK-, p27wt and an empty vector. We generated the YFPp27CK- quimera vector. YFP and YFPp27wt was used as a control. GFP-positive (i.e., transfected) K562 cells were isolated by flow cytometry sorting and total RNA was prepared. Comparative analysis showed that while p27wt altered expression of more than one hundred genes (most of the related to S-phase entrance), p27CK- did not alter the expression of any genes more than two-fold with statistically significance ($P < 0.05$) (data not shown). Thus we concluded that p27CK- was unable to differentiate K562, and thereby, another factor was probably involved in triggering the differentiation in KCK- clones, as the decrease in Myc levels.

4.3.3 Role of Myc in K562 erythroid differentiation induced by p27

One of the better-known functions of Myc is the control of differentiation (see Introduction). Many studies have shown that Myc regulates a great number of differentiation genes, and most of the tumors that express Myc are correlated with less differentiation (Leon et al., 2009). In the erythroid differentiation, Acosta et al. found that Myc expression inhibits the p27-induced differentiation (Acosta et al., 2008). However, it was unknown whether Myc decrease was a consequence or, on the contrary, it was necessary for the differentiation process. In the first case, p27 would induce erythroid differentiation through an unknown mechanism, and as a result, Myc expression would be inhibited. An alternative mechanism would be that high expression of p27 induces Myc under-expression, which in turn would trigger the differentiation. By this latter mechanism, Myc would function as a switch of differentiation, which is induced as a result of Myc down-regulation. This mechanism could explain the contradictory results that we obtained in KCK- clones, whole differentiation depended on the

down-regulation of Myc but was independent from the p27CK- expression. The low expression of Myc found in the differentiation of KCK- clones would be due to an indirect mechanism. Since p27CK- was unable to altered the expression of erythroid genes, in these clones an unknown factor would induce the differentiation by an unknown mechanism and this differentiation process would provoke the down-regulation of Myc.

To test this hypothesis, we decreased the Myc levels though two Myc shRNA vectors and we studied the differentiation. We found that the only inhibition of Myc was sufficient to induce the erythroid differentiation (Fig. 4.30).

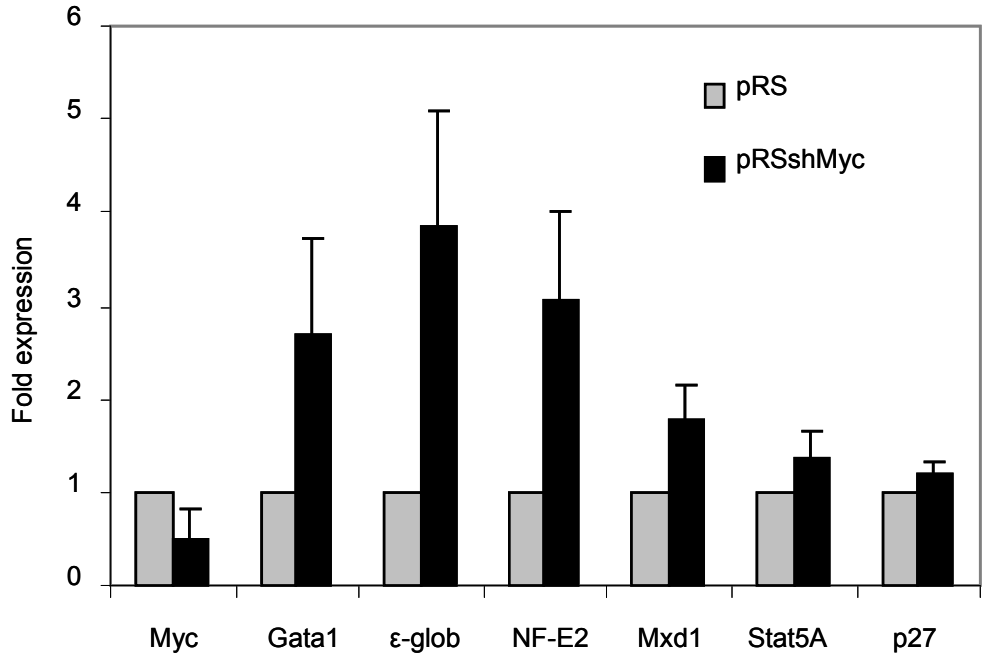


Figure 4.30.- Repression of Myc triggered the up-regulation of erythroid differentiation marker genes. Myc expression was repressed in K562 through the transfection of a short-hairpin vector (pRSshMyc) and the expression of Gata1, ε-Globin, NF-E2, Mxd1 and Stat5A was determined by qRT-PCR 40 h after transfection. RPS14 levels were used as control for equal mRNA loading respectively. Data are mean ± SEM of three determinations from three independent experiments. p27 levels were not affected by Myc repression.

However, how p27 is able to regulate the Myc expression is poorly understood. Luciferase assay of Kp27 cells transfected with a reporter carrying Myc promoter, in the presence or not of Zn^{+2}, showed that Myc promoter is repressed by about 40 % when p27 is expressed in the cell (Fig. 4.31A). The overexpression of p27 in one of these experiments was confirmed by immunoblot (Fig. 4.31B). Next, we studied the possibility that p27 may be bound directly to the Myc promoter acting as a transcriptional repressor, as recently have been shown by other genes (Pippa et al., 2012). ChIP experiments did not show significant binding of p27 on the *MYC* regulatory regions analyzed (Fig. 4.31C). The expression of p27 in the ChIP experiment was confirmed by immunoblot (Fig. 4.31D).

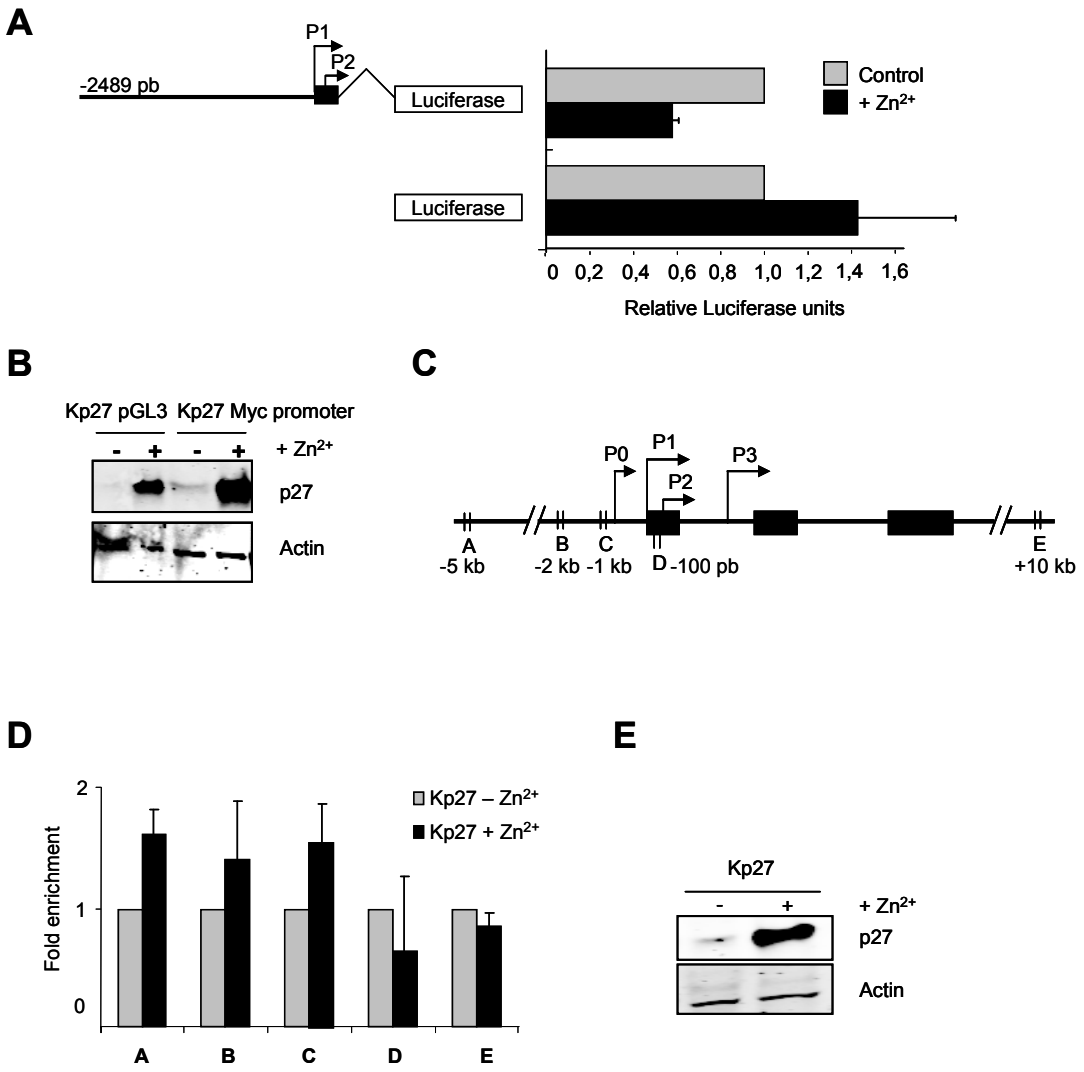


Figure 4.31.- Myc is repressed by p27 through an indirect mechanism. (A) Kp27 cells were electroporated with a luciferase construct of the human Myc promoter. 12 h after transfection cells were splitted in two aliquots and treated or not with 75 µM $ZnSO_4$ and 24 h post-transfection the luciferase activity was determined. Data are means of three different measurements of three duplicate experiments and normalize with respect to the value observed with the empty vector. (B) p27 expression of Kp27 cells induced with 75 µM Zn^{2+} used in (A) was analyzed by immunoblot. (C) Scheme of the human Myc gene. The amplicons (A-E) used for ChIP assays are indicated. (D) Kp27 cells were treated for 10 h with 75 µM Zn^{2+}. Chromatin was immunoprecipitated with anti-p27 antibody or rabbit IgGs and the DNA subjected to quantitative PCR for the amplicons A-E. The results are expressed as fold enrichment of DNA in chromatin immunoprecipitated with anti-p27 (above the signal with anti-rabbit IgGs). Values are mean ±SEM of two determinations from three independent ChIP experiments. (D) p27 expression of ChIP experiment was confirmed by immunoblot.

If p27 induces the differentiation through the inhibition of Myc, this repression should occur before the induction of marker genes of erythroid differentiation. Repression of Myc after the expression of these genes would indicate that this repression is a consequence of p27-induced differentiation. Thus, we determined the Myc mRNA expression at different times after the transfection with p27. Myc repression was observed at 24 h post-transfection, whereas the erythroid genes analyzed were induced after 36-48 h (Fig. 4.32A). This suggests that Myc repression is a prerequisite to p27-induced differentiation. Previous work in our laboratory with

K562 cell lines with zinc-inducible p27 expression shows the expression of marker genes after 12 hours of p27 induction (Acosta et al., 2008; Munoz-Alonso et al., 2005). We analysed Myc expression at different times after p27 induction in Kp27 cells and found a transient Myc down-regulation at 4 h after p27 induction, corresponding to the peak of p27 (Fig. 32B). The differences between Kp27 and K562 transfected with ectopic p27 are probably due to: 1) p27 is induced faster in Kp27 cell line than in K562 transfected, and 2) after transfection with ectopic p27, K562 cells were fed with fresh medium and serum, which can induce Myc expression.

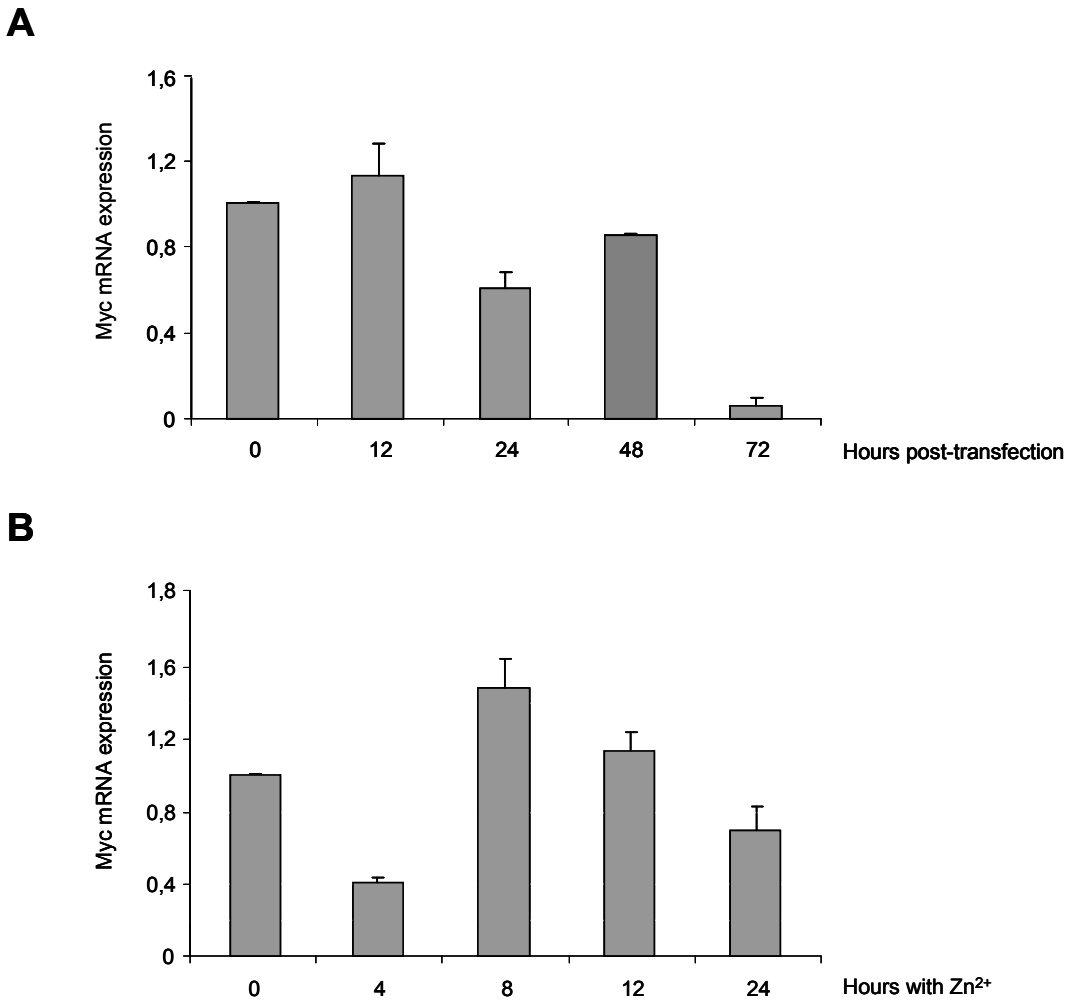


Figure 4.32.- Myc is repressed soon after when p27 is expressed. Myc mRNA levels were measured by RT-qPCR at different times in (A) K562 transfected with pCDNA3.1 vector carrying ectopic p27 and (B) Kp27 treated with 75 µM $ZnSO_4$. RPS14 mRNA levels were used for normalization. Data are mean ± SEM of three determinations from two independent experiments

In both p27-transfected K562 and p27-induced Kp27 models, a second loss of Myc transcripts occured at 72 and 24 hours respectively. This biphasic change of Myc expression after p27 expression is similar to that observed in the erythroid differentiation of K562 induced by AraC, and this differentiation is impaired by Myc (Delgado et al., 1995). Given that one of the effects of AraC is the induction of p27 (Munoz-Alonso et al., 2005), it is possible that the effect of AraC in Myc gene may be mediated by p27, as it occurs in the erythroid differentiation

induced by Imatinib, which is diminished when p27 induction is inhibited (Gomez-Casares et al., 2012).

Thus, p27 expression leads to Myc repression. Next we asked whether the expression of Myc in differentiated cells could induce dedifferentiation. We used Kp27MER cell line, where we could regulate the expression of p27 (through addition of Zn^{+2}) and the activity of ectopic Myc (through the addition of tamoxifen, as the cells express the MycER chimera). We activated Myc at different times after the addition Zn^{+2}, and we observed that in a relatively short period of Myc activation (12 hours), the differentiation process (measured by the percentage of benzidine positive cells) is abrogated, but at longer times (24-48 h), the inhibition is only partial, and finally, in later times (longer than 72 h), we did not find any inhibition in the differentiation process induced by p27 (Fig. 4.33). Thus, Myc is only able to inhibit the differentiation process in the early stages. Once those erythroid marker genes are active, although the expression of Myc is able to inhibit the expression of these genes, this is not sufficient to revert the process of differentiation. Therefore, we conclude that the down-regulation of Myc occurs before the process of differentiation is triggered, and this process will be blocked if the expression of Myc is forced.

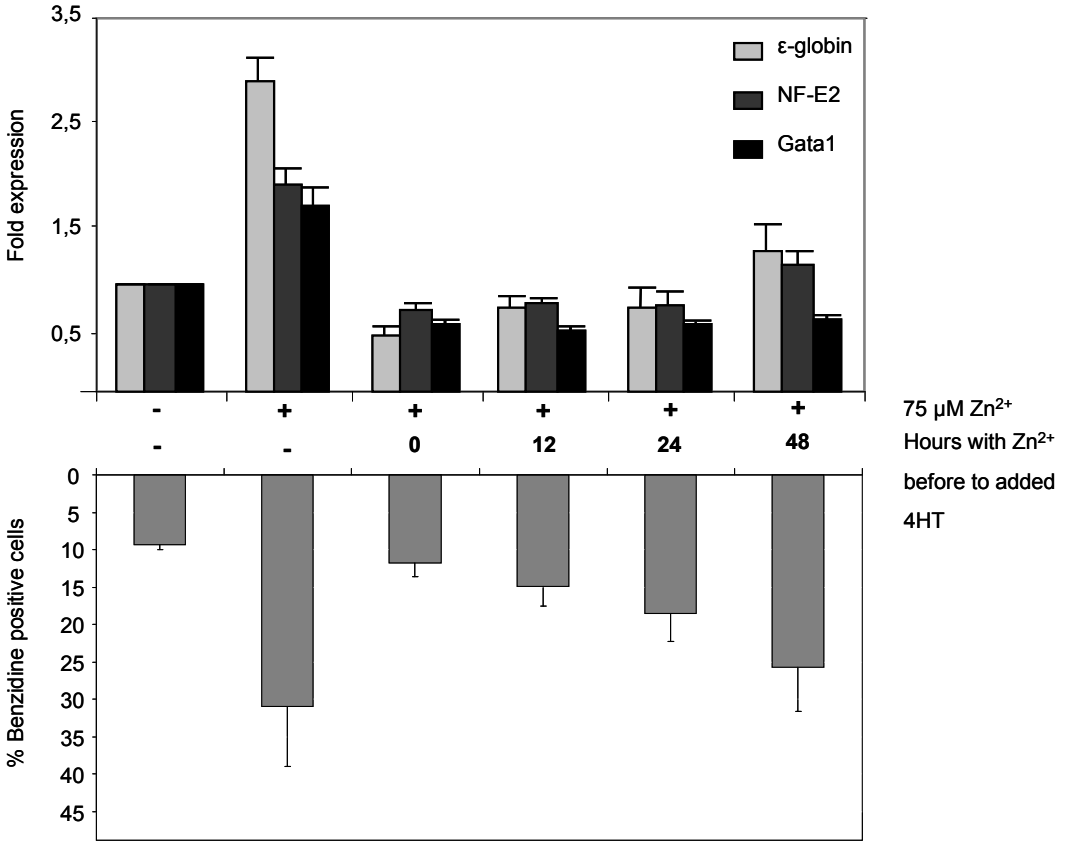


Figure 4.33.- Inhibition of erythroid differentiation by Myc depends on the time elapsed since the differentiation was triggered. Kp27MER cells were treated with $ZnSO_4$ and 4HT were added to different times. Cells were harvested at 96 hours. ε-globin, NF-E2 and Gata1 expression were analyzed by RT-qPCR. RPS14 mRNA levels were used for normalization. Data are mean ± SEM of three determinations from two independent experiments. The fraction of hemoglobinized cells was assayed by the benzidine test. Values are means ± SEM of three experiments.

4.3.4 Myc-p27 interaction in the differentiation of myeloid leukemia cells: DISCUSSION

p27 expression is associated with the differentiation in some cell models, and p27 ectopic expression in different cell lines results in differentiation (see Introduction). One of these models is the hematopoetic system, where the previous work in our laboratory showed that p27 induces erythroid differentiation (Munoz-Alonso et al., 2005). In this model, the induction of p27 results in the binding to and inhibition of Cdk2 and Cdk6 activity, and its down-regulation has been associated in other erythroid differentiation models (Malumbres et al., 2004; Matushansky et al., 2000a, b). This effect is also observed with other CKI such as p16 (Acosta et al., 2008; Minami et al., 2003). All these findings suggested that the cell cycle inhibition and the differentiation are linked. In fact, when we transfected p27-Nt mutant, which lacks of the C-terminal region, we observed an increase of erythroid genes (Fig. 4.26D). This mutant only contains the CDI region, and it is sufficient to induce cell cycle arrest (Fig.4.26C).

If CDI region of p27 is the responsible of the differentiation, p27CK- mutant where CDI region is unable to bind to CDK-cyclins complexes should not be able to induce the erythroid differentiation. Surprisingly, we observed an increase in the expression of erythroid genes upon the transfection of a p27CK- mutant, although lower than with wild-type p27 (Fig. 4.28). p27CK- did not induce cell cycle arrest (Fig. 4.27C), which suggests that p27 was able to induce the differentiation through other independent mechanisms of its role as CKI. To confirm this outcome, we generated clones with Zn-inducible p27CK-, but while some of the clones showed an increase in the expression of erythroid differentiation marker genes, other did not exhibit any differentiation when p27CK- was induced (Fig. 4.29).

Other studies have shown that, in contrast to the tumor suppressor effects of wild-type p27, the mutant p27CK- is involved in tumorogenesis (Besson et al., 2007), although the mechanisms through which p27CK- acted were unknown. Thereby, we decided to study the changes in the gene expression of the K562 cells when the ectopic p27CK- is expressed through a whole genome expression analysis by RNA-seq. However, while the expression of more than one hundred genes was modified in transfected K562 with p27wt, no gene was significantly regulated in K562 transfected with p27CK- with respect to controls (data not shown). The expression of wild-type p27 resulted in the down-regulation of many genes related to cell cycle progression, in agreement with the cell cycle arresting function of p27. This result indicated that most of the functions carried out by p27 depend on its inhibition of CDK activity. Although p27CK- may act at a different level of regulation in the process of tumorogenesis (e.g. posttraductional regulation), it is observed an increase of the differentiation marker genes in K562 differentiation. Therefore, it is likely that p27CK- would not be able to differentiate to K562. However, this result is at odds with the differentiation we observed in several KCK- clones upon the induction of p27CK-. This result could be explained if Myc is down-regulated in these clones.

One of the first biological activities described by Myc was the differentiation inhibition of cultured cell models (Coppola and Cole, 1986; Dmitrovsky et al., 1986; Prochownik and Kukowska, 1986). This is also the general effect of Myc on in vivo differentiation, although in some systems Myc can also enhance differentiation through the expansion of stem cell pools (Leon et al., 2009). Acosta et al. showed that Myc can impair the differentiation through proliferation-independent mechanisms in p27-induced erythroid differentiation of K562 (Acosta et al., 2008). In this model, the differentiation-inhibitory effect of Myc depends on its gene regulatory activity and it is uncoupled from its effect as a proliferation stimulator. However, in this study, they focused exclusively in the role of Myc in the inhibition of differentiation induced by p27. It is an unanswered question whether Myc under-expression is or not essential in the differentiation process, as well as whether p27 regulated or did not regulate the Myc expression.

Thus, we decided to study further the effect of Myc in the erythroid differentiation. We found that, in K562 cells, the mere inhibition of Myc expression was sufficient to induce the erythroid differentiation (Fig. 4.30). A consistent result was observed by Cañelles et al., who found that the expression of two dominant negative Myc mutants in K562 leads an increase in the erythroid differentiation (Canelles et al., 1997). Thereby, Myc appears to be sufficient to inhibit the erythroid differentiation likely by binding and repressing promoters of master genes that regulate the erythroid differentiation, as Gata1 or NF-E2 (Acosta et al., 2008).

If Myc down-regulation is sufficient to induce the differentiation, then a possible mechanism of erythroid differentiation induced by p27 is the inhibition of Myc. The antagonist function and regulation between p27 and Myc have been extensively described in the literature (see Introduction). Acosta et al. showed that Myc is inhibited if p27 is expressed in K562 (Acosta et al., 2008), and we have shown that p27-mediated repression of *MYC* occurs before to the induction of erythroid differentiation genes (Fig. 4.32). The same result was observed in a cell line with a conditional expression of both p27 and Myc (Fig. 4.33). We confirmed that p27 can inhibit the promoter activity of Myc although not through a direct repression of the promoter as we did not detect p27 on Myc promoter by ChIP (Fig. 4.31). The most plausible possibility is that this inhibition results of the activity p27 as a CKI so as the inhibition of G_0/G_1 progression induced by p27 could be sufficient to induce Myc repression. One of the mechanisms proposed to explain Myc low expression in quiescent cells is through Rb, which binds to E2F-1 and repressed Myc promoter (Albert et al., 2001; Oswald et al., 1994). Rb only is functional when it is hypophosphorylated, and p27 prevents its phosphorylation through inhibition of Cdk/cyclins complexes. Indeed, Myc is unable to reverse CDK inhibition and Rb hypophosphorylation induced by p27 (Acosta et al., 2008).

Using cell lines with conditional expression of Myc and p27, Myc was able to inhibit the differentiation induced by p27, but in those experiments Myc and p27 were induced at the same time (Acosta et al., 2008). We asked if the differentiation is reversed when Myc is expressed in cells already differentiated, and we found that Myc can only revert the differentiation process at early times after p27 induction but not once the differentiation is triggered (as detected by increase in the mRNA of erythroid genes) (Fig. 4.33).

In summary, our results in the K562 model demonstrate that the erythroid differentiation induced by p27 is carried out mainly through Myc down-regulation. p27 induction results in Myc repression, which occurs before the process of differentiation is triggered (Fig. 4.34). p27 mediated down-regualtion of Myc ocurrs at the transcriptional level probably because p27 activity leads to Rb hypophosphorylation and to repression of Myc promoter. This mechanism is also available for other inductors of differentiation, such as p16 or several drugs as AraC or Imanitinb which induced G_0/G_1 arrest through p27 induction. Thus, Myc acts as an on / off switch of the erythroid differentiation. An schematic summary of our model is depited in the Fig. 4.34.

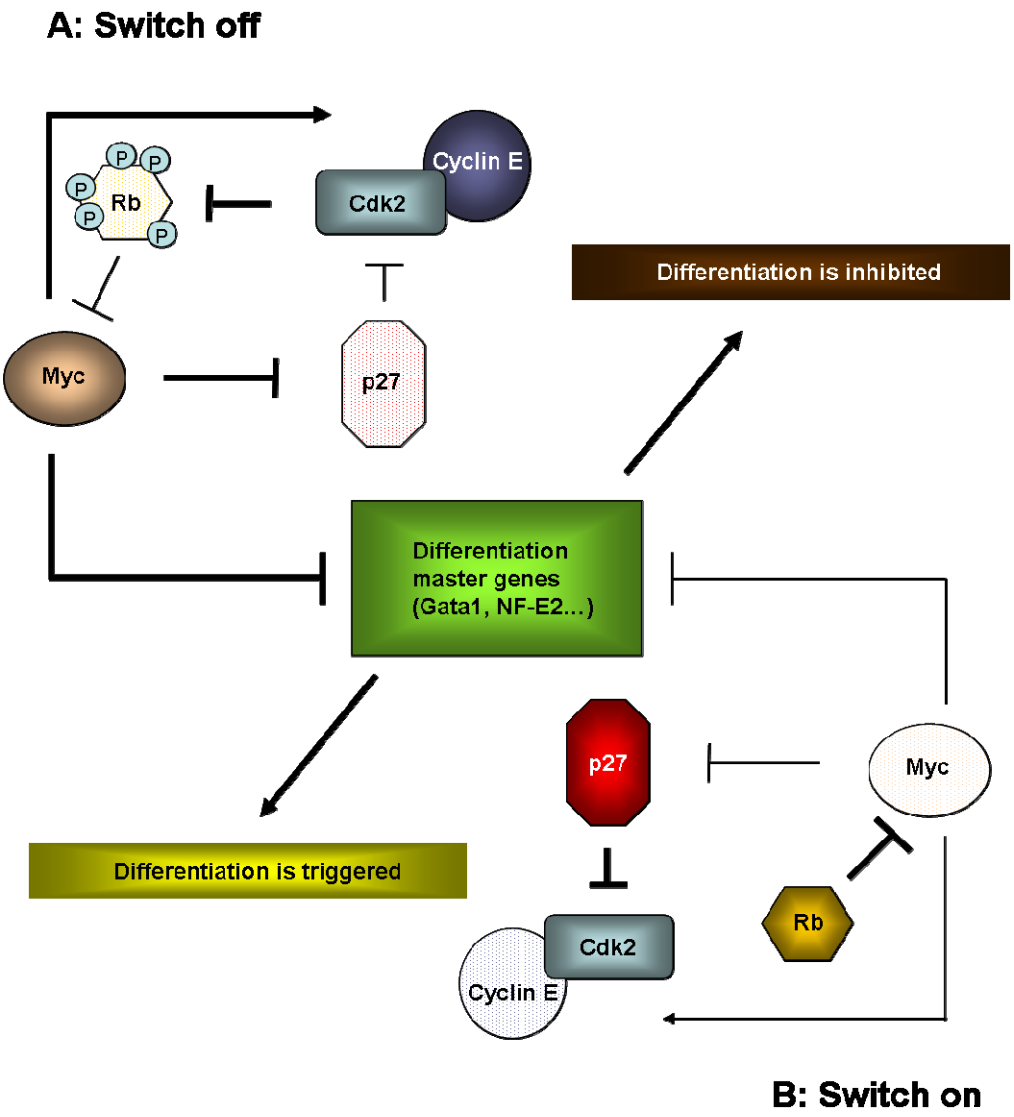


Figure 4.34.- Differentiation switch regulated by Myc. (A) When K562 cells are grown with mitogenic factors, Myc is induced. Myc activation results in p27 repression and cyclin E induction. Cdk2/Cyclin E complexes phosphorylate Rb which results in free E2F1-3 and positive regulation of Myc. Myc inhibits master genes that regulate erythroid differentiation, inhibiting the differentiation of K562 cells. (B) When K562 cells are grown with drugs that triggered differentiation, p27 is induced. p27 inhibits Cdk2/Cyclin E complexes activity and as results Rb is not phosphorylated. Hypophosphorylated Rb binds to and inhibits E2F1-3, preventing its bind to Myc promoter. In this case Myc is repressed and thereby it is unable to inhibit master genes that regulate erythroid differentiation, allowing the differentiation of K562 cells.

5.- CONCLUSIONS

5 CONCLUSIONS

1. A study of p27 and Myc expression at the protein level was performed in 118 samples of CLL complemented with another study at the mRNA level. p27 was overexpressed in most of the CLL samples analyzed (82%), and was nuclear. In contrast, the expression of Myc was low. This result is in agreement with the functional antagonism between Myc and p27, but it is in contrast with the observed expression in the rest of the human tumors.

2. No clinical differences were observed in the progression of the disease between patients with high or low p27 expression. The small fraction of patients with high p27 expression that also expressed Myc showed a slower leukemic progression.

3. The HLH-LZ proteins Max and Mxi1, described as Myc antagonists, are significantly overexpressed in CLL.

4. In patients expressing both p27 and Myc, p27 was not phosphorylated on T187 suggesting that Cdk2 is inactive.

5. In contrast to peripheral blood, Myc is expressed in lymph node and bone marrow in some CLL tested-CLL cells, indicating the impact on tumor microenvironment in Myc expression. However, p27 expression was also high in lymph nodes.

6. Myc expression results in the induction of Skp2 and S-phase cyclins, but it is not associated with Notch1 activation in CLL.

7. p27 mediates resistance to fludarabine-induced apoptosis in B-CLL cells and this is associated to high Bcl2 expression. In contrast, Myc enhances the apoptosis mediated by fludarabin.

8. Erythroid differentiation of K562 cells by p27 depends on its Cdk-binding region. p27 inhibits Myc expression before the induction of erythroid differentiation.

9. Myc downregulation is sufficient and necessary for erythroid differentiation induced by p27.

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Publications

imatinib-mediated apoptosis, suggesting a role of p21 in the apoptosis response to Bcr-Abl inhibition.

2. Materials and methods

2.1. Cell culture and transfections

BV173 [28], KBM5 [29], MEG01 [30] and K562 [31] cell lines derive from human chronic myeloid leukemia in blast crisis. Kp21-4 subline, a K562 derivative with inducible expression of p21 was generated as previously described [32]. The cells were grown in RPMI 1640 medium supplemented with 8% fetal calf serum and antibiotics. Unless otherwise stated, cells at a density of 2.5×10^5 cells/ml were treated with 75 μM $ZnSO_4$ to induce p21 expression. Imatinib mesylate (provided by Novartis, Basel, Switzerland) was added one hour after $ZnSO_4$. K562 were transiently transfected by nucleofection (Amaxa) following the manufacturer indications with 4.75 μg of pCEFL-p21 [32] or empty vector and 0.25 μg of a Green Fluorescent Protein vector (pmaxGFP, Amaxa) to assess transfection efficiency.

2.2. Proliferation, cell cycle and apoptosis assays

Cell proliferation was assayed with a Nucleocounter (Chemometec). For cell cycle analysis, cells were fixed in 90% ethanol at 4 °C and resuspended in PBS-sodium citrate buffer containing 10 μg/ml bovine serum albumin, 200 μg/ml RNAse and 50 μg/ml propidium iodide (Sigma). Stained cells were analyzed by flow cytometry as previously described [14]. Apoptosis was assessed by annexin V binding and TUNEL assays. Annexin V binding was detected by flow cytometry using the BD-Pharmingen kit. TUNEL assays were performed using In situ Cell Death Kit (Roche Applied Science). Apoptosis in each cell cycle phase was determined by incubating cells with Hoescht 33342 (Sigma) (10 μg/ml for 90 min) to stain nuclei, and then annexin V binding was analyzed as above.

2.3. RNA extraction and expression analysis

Total RNA from cell lines and from bone marrow cells was isolated using the RNeasy kit (Qiagen). For reverse transcription and polymerase chain reaction (RT-PCR),

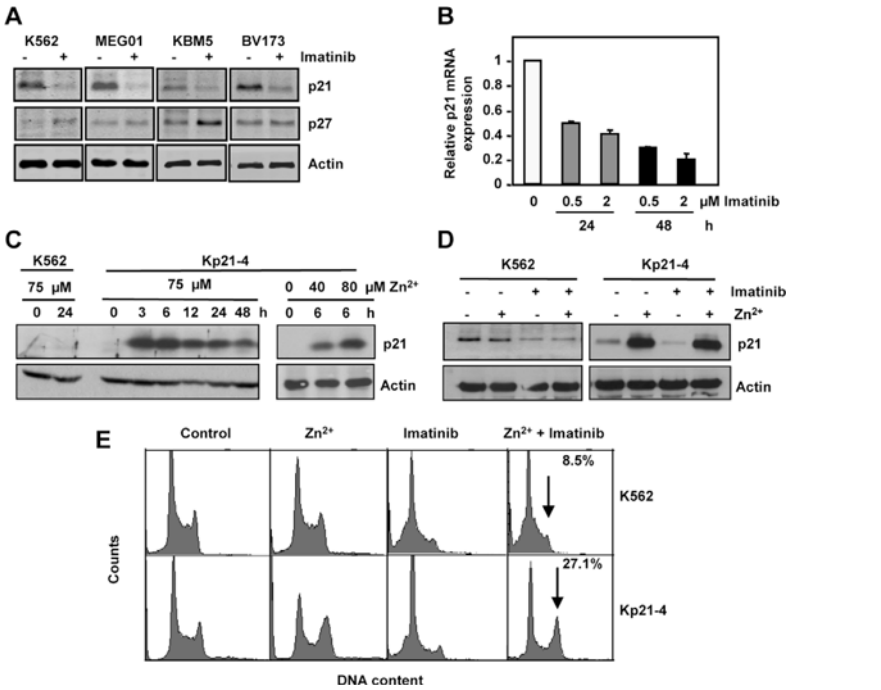

Fig. 1. Effects of imatinib on p21 expression in CML cells. (A) Immunoblot for p21 and p27, and actin as loading control, in CML-derived cell lines treated with 1 μM imatinib for 24 h. (B) qRT-PCR analysis showing the down-regulation of p21 mRNA in K562 cells treated with imatinib. Data are expressed as means ± SEM. (C) Immunoblot analysis showing Inducible p21 expression in Kp21-4 cell line. Cells were treated with 75 μM $ZnSO_4$ and 0.5 μM imatinib for 3 to 48 h (left panel) or 40 and 80 μM $ZnSO_4$ for 6 h (right panel). (D) K562 and Kp21–4 cells were treated for 24 h with 0.5 μM imatinib and 75 μM $ZnSO_4$. The blot for p21 in K562 was overexposed with respect to Kp21–4. (E) Cell cycle analysis of K562 and Kp21–4 cells treated for 48 h with 1 μM imatinib and 75 μM $ZnSO_4$. The arrow indicates the position of G2 phase and the percentage of cells in G2 is indicated in each case.

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p21^{Cip1} Confers resistance to imatinib in human chronic myeloid leukemia cells

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ABSTRACT

Imatinib is a Bcr-Abl inhibitor used as first-line therapy of chronic myeloid leukemia (CML). p21^{Cip1}, initially described as a cell cycle inhibitor, also protects from apoptosis in some models. We describe that imatinib down-regulates p21^{Cip1} expression in CML cells. Using K562 cells with inducible p21 expression and transient transfections we found that p21 confers partial resistance to imatinib-induced apoptosis. This protection is not related to the G2-arrest provoked by p21, a decrease in the imatinib activity against Bcr-Abl or a cytoplasmic localization of p21. The results suggest an involvement of p21^{Cip1} in the response to imatinib in CML.

1. Introduction

p21^{Cip1} (p21 herein after) is a member of the Cip/Kip family of inhibitors of cell cycle progression. The first discovered and so far best studied biochemical activity of p21 is to inhibit Cdks, consistent with its activity as cell cycle inhibitor [1–4]. Also, p21 is a relevant mediator of p53-cell cycle arrest [5–7]. However, more recent studies have shown that p21 has additional functions as differentiation inducer [8], transcriptional co-regulator [9,10] and as an inhibitor of apoptosis induced by DNA-damaging agents [11–13]. The mechanism for p21-mediated apoptosis protection is unclear and is not operative in all cell types [14]. However, since cancer cells can escape death induced by chemotherapeutic drugs, the anti-apoptotic p21 activity might be critically important in human cancer (reviewed in [15]). Actually, p21-null mice develop tumours spontaneously [16] and show increased tumour susceptibility using chemical carcinogenesis [17–20].

Imatinib is the main drug used in chronic myeloid leukemia (CML) treatment. The hallmark of this leukemia is the Bcr-Abl kinase, encoded by a fusion gene produced by the t(9;22) translocation [21,22]. Several previous studies, including ours, have demonstrated that imatinib induced cell death in CML-derived cells, including K562 [23–25].

It has been reported that imatinib down-regulates p21 in mouse cells transfected with Bcr-Abl [26,27]. However, it is unknown whether imatinib regulates p21 expression in human CML cells and the effects of p21 in these cells. In this work we describe that Bcr-Abl inhibition results in low p21 levels and that ectopic p21 expression antagonizes

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were revealed with the ECL system (GE Healthcare). Antibodies used were: anti-actin (I-19), anti-poly(ADP-ribose)polymerase (PARP) (H250), anti-p27 (C-19), anti-UBF (F-9), anti-RhoGDI (A-20) and anti-phospho-tyrosine (PY20) all from Santa Cruz Biotech; anti-c-Abl from BD Pharmingen; anti-p21 (P-184) from Sigma, and anti-α-tubulin antibody kindly provided by Nicholas Cowan (New York University, New York). For cellular fractionation, cells were treated in 0.1% paraformaldehyde in PBS during 5 min and washed with 50 mM glycine in PBS. Nuclear and cytoplasmic extracts were then obtained essentially as described [34] using 50 mM Tris, 1% Nonidet P-40, 0.2% SDS, 1 mM EDTA, 10% glycerol as nuclear lysis buffer.

2.5. CML patients

Bone marrow mononuclear cells from 17 healthy individuals and from 58 CML patients were studied. CML cases included 36 samples collected at the time of diagnosis, 7 in blast crisis and 15 in chronic phase treated with imatinib. In this case samples were taken up to 24 months of treatment. The patients are from two hospitals: Hospital Universitario Marqués de Valdecilla (Santander, Spain) and Hospital Universitario Dr. Negrín (Las Palmas, Spain). The study was approved by the ethics committees according to procedures approved by the two hospitals providing the samples. Statistical analysis was carried out with the SPSS software.

3. Results

We studied the expression of p21 in four CML cell lines (K562, MEG01, KBM5 and BV173) in response to imatinib treatment. p21 levels were low although detectable in growing cells, and decreased upon imatinib treatment (Fig. 1A). In sharp contrast $p27^{Kip1}$ expression, which was also very low, was unchanged or moderately up-regulated by imatinib. p21 down-regulation by imatinib in K562 was also detected at the mRNA level, as assayed by qRT-PCR (Fig. 1B). We next asked whether p21 down-regulation was involved in the cell death mediated by imatinib. To test this hypothesis we used a K562 subline with inducible p21 expression, termed Kp21-4 [32]. In these cells the expression of p21 is induced by $ZnSO_4$ and p21 levels were dramatically increased 3 h after $ZnSO_4$ addition and depended on the inducer concentration (Fig. 1C). Next, we demonstrated that imatinib did not modify the induction of p21 by Zn^{2+} in Kp21-4 cells (Fig. 1D). In these conditions, both p21 and imatinib provoked growth arrest (data not shown). It is reported in many models, including K562, that p21 induces accumulation of cells in the G2 cell cycle phase [32,35]. We found p21 induced G2 accumulation in the presence of imatinib after 48 h of treatment, in contrast to the G1 arrest mediated by imatinib alone (Fig. 1E), thus confirming that the induced p21 was functional in the presence of imatinib. Altogether, the results indicate that imatinib did not impair p21 activity on cell cycle in the K562 model. At longer times (3–5 days) p21 induces polyploidy in K562 cells [32] but imatinib induced extensive apoptosis after long treatment periods [25], which precludes the analysis after long treatments.

We next asked whether p21 modified the apoptosis induced by imatinib. The results showed that p21 significantly reduced the imatinib-mediated apoptosis. This was assessed by several techniques as PARP proteolysis (Fig. 2A), TUNEL assay (Fig. 2B) and the fraction of cells with sub-G0/G1 DNA content (Fig. 2C). The protection that the induction of p21 exerts against the imatinib-mediated apoptosis was also demonstrated by determining the binding of annexin V to cell surface (Fig. 2D). As p21 induced G2 phase accumulation, we asked whether cells in G2 were less sensitive to imatinib-mediated apoptosis. However, in

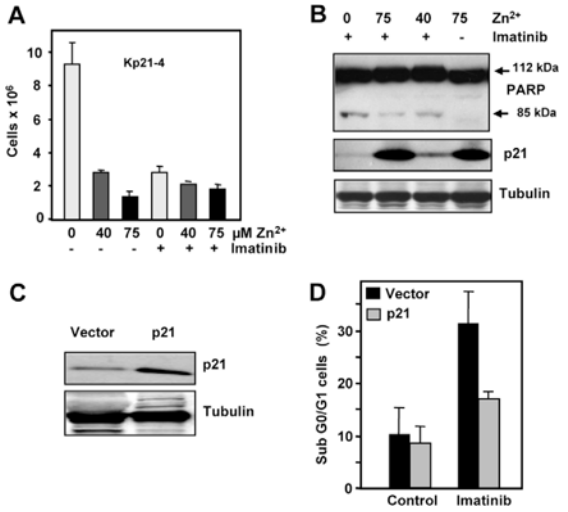

Fig. 3. p21 antagonizes imatinib-induced apoptosis. (A) Cell proliferation analysis of Kp21–4 cells treated for 48 h with 1 μM imatinib and 40 or 75 μM $ZnSO_4$. (B) Apoptosis of Kp21-4 cells assessed by PARP proteolysis in cells treated for 24 h with 1 μM imatinib and 40 or 75 μM $ZnSO_4$. (C) Expression of p21 in K562 cells nucleofected with a p21 expression vector. Cell lysates were prepared 24 h after nucleofection and analyzed by immunoblot. (D) Apoptosis assessed by cytometry in K562 transfected with p21. Imatinib (1 μM) was added 24 h after transfection and cells were further incubated for 24 h. Data are expressed as means ± SEM.

first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RNase reverse transcriptase (Invitrogen) and random primers. Quantitative RT-PCR was performed as described [33] with a QuantiTect™ SYBR green PCR kit (Qiagen). The following primers were used: for p21 5′-AAGACCATGTGGACCTGTCA-3′ and 5′-GGCGTTTGG AGTGGTAGAAA-3′; for ribosomal protein RPS14, 5′-GGCA GACCGAGATGAATCCTC-3′ and 5′-CAGGTCCAGGGGTCTTG GTCC-3′. For each gene, duplicate PCR reactions were performed on the same 96-well plate on an ICycler iQ™ apparatus (Bio-Rad). The expression levels of p21 were normalized by the internal control ribosomal protein RPS14.

2.4. Immunofluorescence staining, immunobloting and cellular fractionation

Cytospin preparations were fixed with paraformaldehyde and the presence of p21 was detected by immunofluorescence using a rabbit polyclonal antibody (C-19 from Santa Cruz Biotech) and Texas Red conjugated secondary antibody (Dako). Actin cytoskeleton was detected using phalloidin-FITC (Sigma). Samples were mounted with Vectashield (Vector) containing 4′-6-diamidino-2-phenylindole (DAPI) to stain nuclei and examined under a fluorescence microscope (Zeiss-Axioskop2). Immunoblots were performed as previously described [14]. Blots

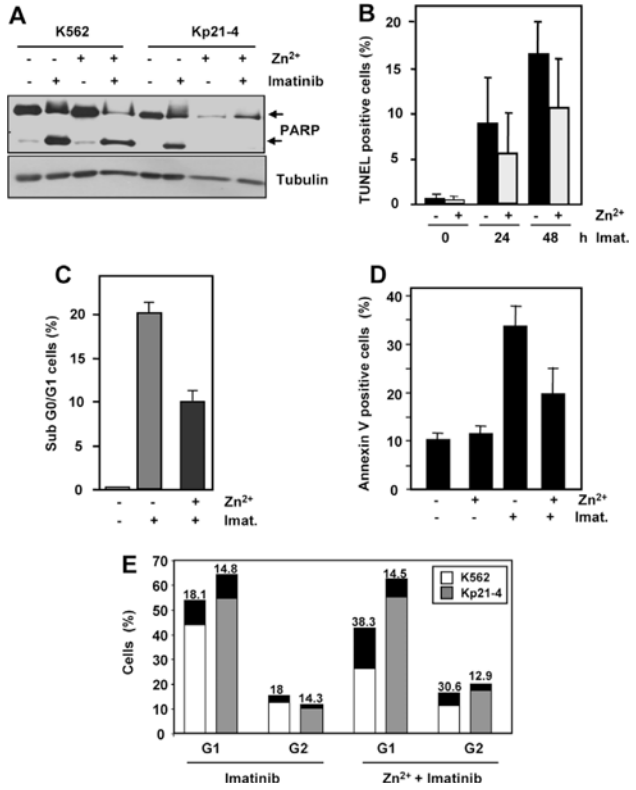

Fig. 2. Ectopic expression of p21 confers partial resistance to imatinib-induced apoptosis. (A) Apoptosis was assessed by proteolytic cleavage of PARP determined by immunoblot in K562 and Kp21-4 cells treated with 75 μM $ZnSO_4$ and 1 μM imatinib for 48 h. (B) Kp21-4 cells were treated as in (A) for 24 and 48 h and TUNEL-positive cells were analyzed by fluorescence microscopy. Data are expressed as means ± SEM. (C) Kp21-4 cells were treated for 48 h as in (A). DNA content was measured by propidium iodide staining and flow cytometry and the fraction of cells with DNA content lower than 2C was scored. (D) Kp21-4 cells were treated for 48 h as in (A) and the fraction of annexin V-positive cells was determined by flow cytometry. Data are expressed as means ± SEM. (E) Cells were treated as in (A) and the apoptosis in each cell cycle phase was assessed by annexin V binding measured by flow cytometry. Black bars correspond to the fraction of annexin V-positive cells, and the percentages of annexin V-positive cells within each cell cycle phase are indicated in each case.

lation in human CML cells. Imatinib represses p21 expression at the transcriptional level. The mechanism is unknown, but it is unrelated to p53 as three of the four cell lines tested (K562, KU812 and MEG01) do not carry active p53 alleles [40]. p21 gene has a complex promoter, which is regulated by many transcription factors [41]. Imatinib effect could be mediated by STAT5, which transactivates p21 [42] and is activated by Bcr-Abl [43,44].

It is striking that inhibition of Bcr-Abl activity results in the down-regulation of a protein known to stop cell cycle. However, p21 exerts apoptosis protection in many models [11–13]. We show here that induction of p21 protects K562 cells from imatinib-induced apoptosis. In our model, p21 did not rescue the growth-arrest induced by imatinib, conversely to the result reported with mouse cells [27]. The reasons for this discrepancy may lie, first, in that we are using human CML cells expressing endogenous Bcr-Abl instead of murine cells transfected with Bcr-Abl gene, and second, that we use an inducible p21 gene and transient p21 transfections, rather than stable selected clones with high constitutive p21 expression. Given the depressing effects of p21 on proliferation it is likely that stable constitutive transfectants have acquired other epigenetic changes.

The induced p21 remains in K562 cell nuclei in the presence of imatinib, ruling out in our system the described anti-apoptotic effect of cytoplasmic p21. In K562, as other cell models, p21 provokes the accumulation of cells in the G2 phase of the cycle, but imatinib did not modify this pattern. Moreover, we ruled out the hypothesis that the drug is less active in G2 cells so as to explain p21-mediated protection. Thus, the mechanism for p21-mediated apoptosis protection in K562 remains unclear, as it is also the case in the other cell models where this effect has been observed [12,13]. However, in K562 the mechanism operates downstream of Bcr-Abl inhibition as there is no significant change in the activity of Bcr-Abl mediated by p21. We also observed an increased level of p21 in cells from blastic crisis CML. This result seems consistent with the refractoriness of these cells to treatment with imatinib, although the difference in expression did not get statistical significance. Regardless of the mechanism involved, our report describes for the first time the p21-mediated protection to apoptosis by the most used drug in CML, suggesting that p21 levels could modulate the response to the drug in CML treatments.

Conflicts of interest

None declared.

Acknowledgements

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double-staining experiments (Hoechst 33342 and annexin V) we found that the fraction of apoptotic cells was similar in the G1 and G2 cell cycle phases (Fig. 2E).

To confirm that p21 was indeed the responsible for the apoptosis protection we first performed a dose–response experiment. Different p21 levels were induced with two $ZnSO_4$ concentrations (40 and 75 μM). As expected, the higher p21 levels correlated with a faster cell growth arrest (Fig. 3A) and with less apoptosis, as assessed by PARP proteolysis (Fig. 3B). The above results were obtained with the p21-inducible cell line Kp21-4. As this cell line was generated by selection of stable transfectants [32], we wanted to rule out the possibility that the p21 effect was due to the specific behavior of the Kp21-4 cell line. Therefore, we transiently transfected parental K562 with an expression vector for p21. p21 overexpression upon transfection was confirmed by immunoblot (Fig. 3C). The results showed that p21-expressed cells were more resistant to imatinib-mediated apoptosis (Fig. 3D). Taken together, these results demonstrate that p21 antagonizes imatinib-induced apoptosis in K562 cells.

It has been reported that cytoplasmic but not nuclear p21, protects leukemia cells from apoptosis [36]. In addition, it is reported that in murine myeloid cells overexpressing Bcr-Abl, p21 is predominantly cytoplasmic and imatinib treatment results in decreased cytoplasmic p21 expression [37]. Thus, we studied the cellular localization of p21 in our model of Kp21-4 cells. Immunofluorescence studies showed that the induced p21 was found in the nuclei and remained nuclear after imatinib treatment (Fig. 4A). We also carried out nucleo-cytoplasmic fractionation studies of Kp21-4 extracts. Immunoblot analysis showed that p21 was predominantly found in the nuclear compartment and that imatinib did not modify this localization (Fig. 4B). p21 was also predominantly nuclear in untreated K562 (not shown). Finally, as imatinib-induced apoptosis depends on Bcr-Abl inhibition we assayed the effect of p21 on Bcr-Abl activity by determining the levels of phospho-tyrosine-Bcr-Abl. The results demonstrated that imatinib efficiently inhibited Bcr-Abl activity, and that the induction of p21 protein levels did not significantly modify this inhibition (Fig. 4C).

Although it is known that p21 is expressed in hematopoietic precursors (reviewed in [38]), the expression of p21 has not been systematically analysed in CML patients. We asked whether p21 levels correlated with CML progression. We determined the expression of p21 by quantitative RT-PCR in bone marrow samples of 36 CML patients at diagnosis, 15 in chronic phase under imatinib treatment and 7 cases of blast crisis (the final and fatal stage of CML) [39] as well as in 17 healthy bone marrow samples as controls. The results indicate that the expression of p21 mRNA was similar in all groups (Fig. 4D). The highest expression was observed in cells from patients in blastic phase. However, the difference was not statistically significant ($P > 0.05$).

4. Discussion

Here we show first that imatinib, the drug almost universally used in CML treatment, results in p21 down-regu-

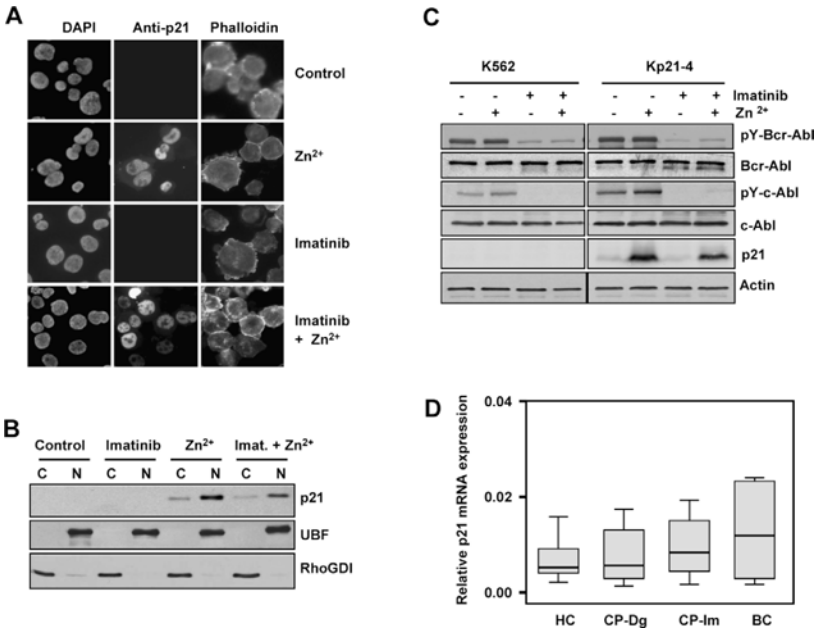

Fig. 4. Nuclear localization of p21 (A) p21 subcellular localization in Kp21-4 cells treated for 24 h with 75 μM $ZnSO_4$ and 1 μM imatinib. Cytospin preparations were subjected to immunofluorescence with anti-p21 and stained with phalloidin-FITC and DAPI to visualize cytoskeleton and nuclei, respectively. (B) Cellular fractionation of Kp21-4. Extracts from nuclear and cytoplasmic fraction were analyzed by immunoblot with anti-p21, anti-UBF (nuclear fraction control) and anti-RhoGDI (cytoplasmatic fraction control). (C) Inhibition of Bcr-Abl activity as determined by phospho-tyrosine-Bcr-Abl. Extracts of K562 and Kp21-4 cells were treated as in (A) and subjected to immunoblot using the antibodies against Bcr-Abl, c-Abl, p21, actin and phospho-tyrosine. The blot for p21 in K562 was overexposed with respect to Kp21-4. (D) p21 mRNA expression assayed by qRT-PCR in samples from CML patients at different stages of the disease. p21 mRNA levels were normalized with RPS14 expression. Each box refers to the range defined by the 25th and the 75th percentiles and the line indicates the median value. HC, healthy controls; CP-Dg, chronic phase at diagnosis; CP-Im, chronic phase treated with imatinib; BC, blastic crisis. In all comparisons the differences were not significant ($P > 0.05$).

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region of p21 is required for its transcriptional effects in leukemia cell. *CCNE2* (cyclin E2) is one of the most potently down-regulated genes and p21 was found to bind and repress the human CCNE2 promoter.

Methods

Cell culture, transfection, viral infection

Primary human keratinocytes wereprepared from discarded human tissue, mostly from reduction abdominoplasties, with Institutional Review Board approval (MGH#2000-P-002418/3) and were grown as described [30]. K562 are human chronic myeloid leukemia cells, and were purchased from ATCC. Kp21-4 cells are K562 carrying a Zn^{2+} -inducible p21 gene; Kp27-5 cells are K562 carrying a Zn^{2+} -inducible p27 gene [31]. K562 and derivatives were grown in RPMI with 8% fetal calf serum. Adenovirus infections of keratinocytes were performed for 1 h in serum and epidermal growth factor-free-low calcium medium as previously described [32]. Keratinocytes were then incubated in fully supplemented medium for 24 h prior to collection for RNA analysis. Adenoviruses expressing full-length, N-terminal, C-terminal p21, p16, and p27 have been previously reported [32]. For transient transfections of K562, ten millions of cells were transfected with 12 µg of p21-WT-GFP, p21-CT-GFP and p21-NT-GFP expression vectors, as well as the GFP empty vector [33] using Lipofectamine 2000 (Invitrogene). 12 h after transfection, GFP-expressing cells were harvested by flow cytometry (FACSAria cell sorter, BD Biosciences). For transient transfections of Kp21-4, one million cells were electroporated with 2 µg pLKO-shCDK2 (Open Biosystems) or pLKO.1 using an Amaxa nucleofector (kit V). 24 h after transfection $ZnSO_4$ was added (75 µM), the cells were further incubated for 12 h and harvested.

Flow cytometry

One million cells per sample were fixed with 80% ethanol and stained with propidium iodide as described previously [31]. Cells were analysed by flow cytometry on a FACScant (BD Biosciences). Ten thousand events were gated and analysed using CellQuest software (BD Biosciences).

siRNA transfection

Human primary keratinocytes were transfected with 200 nM siRNAs for *CDKN1A* gene (p21) (Stealth RNAi, Invitrogen) and control siRNAs (Stealth RNAi negative control, Invitrogen) using Lipofectamine 2000 following the manufacturer's recommendations. 48 h after transfections, cells were analyzed by RT-qPCR (reverse transcriptional-quantitative polymerase chain reaction)

mRNA determination

Total RNA was isolated using TriReagent (Molecular Research Center) kit (Qiagen). Reverse transcription (RT) was performed with iScript reverse transcriptase (BioRad). Quantitative PCR (qPCR) was performed with the SYBRGreen PCR kit (BioRad) in a BioRad MyiQ apparatus. Primers sequences and amplicon sizes used in the RT-qPCR assays are shown in Table S1. Data were normalized to ribosomal protein S14 (RPS14) mRNA levels.

Gene expression profiling

Total RNA was prepared using RNeasy kit (Qiagen). Biotinilated cRNA was obtained from total RNA and hybridized to Affymetrix HG-U133A chip in the Genomic Facility of Centro de Investigación del Cancer (Salamanca, Spain). Data analysis and hierarchical tree clusters were generated using the dChip software [34], http://biosun1.harvard.edu/complab/dchip/]. The expression data was filtered so as to include genes with expression changes ≥2.4-fold. The data were obtained in compliance with the MIAME guidelines and are deposited in the ArrayExpress database. The accession numbers are E-MEXP-3431 for the expression data with Kp21-4 and Kp27-5 cells and E-MEXP-3430 for expression data of human keratinocytes. The analysis was performed with data from two independent experiments and RNA preparations of each experimental condition or adenoviral infection. The interaction network for differentially expressed genes was generated with the Ingenuity Pathways Analysis software.

Immunoblotting and cell extract fractionation

Total cell lysates and immunoblots were carried out as described [31]. Blots were developed with secondary antibodies conjugated to IRDye680 or IRDye800 (Li-Cor Biosciences) and visualized in an Odyssey scanner. Antibodies used were anti-p21 (C-19), anti-UBF (F5), anti-CDK2 (M2) and anti-α-tubulin (H-300). All were polyclonal antibodies from Santa Cruz Biotech. The isolation of chromatin fraction was performed essentially as described [35]. Briefly, the cells were lysed for 20 min with agitation at 4°C in CSK buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Tritón X-100 and protease inhibitors). After centrifugation the supernatant was collected as cytoplasmic plus nucleoplasmic fraction. The pellet was washed at maximum setting with CSK buffer, resuspended in CSK buffer, sonicated and saved as chromatin fraction.

Chromatin Immunoprecipitation (ChIP) assays

ChIP analysis was carried out as described previously [36]. Briefly, 10 millions of K562, Kp21-4 and Kp27-5 cells were fixed with formaldehyde and lysed in SDS lysis buffer. Cross-linked chromatin was fragmented by sonication to an average size of 400 bp. Chromatin was then immunoprecipitated with anti-p21 (C-19, Santa Cruz Biotechnology), anti-p27 (C-19, Santa Cruz Biotechnology), anti-CDK2 (M2, Santa Cruz Biotechnology), anti-histone H3 (FL-136, Santa Cruz Biotecnology) and anti-acetylated-histone H3 (06-559, Millipore). Antibodies and cell lysates were incubated overnight at 4°C, and then with protein G-coupled magnetic beads (Dynabeads, Invitrogen) for 1 h at 4°C. Controls were performed by incubating parallel samples with non-immune IgG. The protein-DNA cross-links were reversed by 4 h incubation at 65°C, and immunoprecipitated DNA was analyzed by quantitative PCR using a BioRad MyiQ apparatus. The signals were normalized to the inputs and the signals obtained with normal rabbit IgG (Santa Cruz Biotechnology) The primers used for PCR reactions are indicated Table S1.

Luciferase reporter assays

Two million of cells were electroporated with 15 µg of pCyCE1-Luc [37,38] carrying a fragment of the human CCNE1 and CCNE2 promoters, respectively, upstream of the firefly luciferase. These luciferase reporters were co-transfected with pCEFL or pCEFL-p21 [39]. Cells were electroporated with the reporters and expression vectors (15 µg) at 260 v and 975 µFa in a BTX electroporator. 24 h alter transfection the cells were treated with 75 µM $ZnSO_4$ for another 24 h. Luciferase activity was then determined with Lysis Solution 1 (Promega), Luciferase Substrate (Promega) and a GloMax 20/20 luminometer (Promega), following the manufacturer's instructions. All transfections were normalized by measuring β-galactosidase activity of the samples. Data are the average of at least three independent experiments and error bars indicate standard deviation.

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p21 as a Transcriptional Co-Repressor of S-Phase and Mitotic Control Genes

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Abstract

It has been previously described that p21 functions not only as a CDK inhibitor but also as a transcriptional co-repressor in some systems. To investigate the roles of p21 in transcriptional control, we studied the gene expression changes in two human cell systems. Using a human leukemia cell line (K562) with inducible p21 expression and human primary keratinocytes with adenoviral-mediated p21 expression, we carried out microarray-based gene expression profiling. We found that p21 rapidly and strongly repressed the mRNA levels of a number of genes involved in cell cycle and mitosis. One of the most strongly down-regulated genes was *CCNE2* (cyclin E2 gene). Mutational analysis in K562 cells showed that the N-terminal region of p21 is required for repression of gene expression of *CCNE2* and other genes. Chromatin immunoprecipitation assays indicated that p21 was bound to human *CCNE2* and other p21-repressed genes gene in the vicinity of the transcription start site. Moreover, p21 repressed human CCNE2 promoter-luciferase constructs in K562 cells. Bioinformatic analysis revealed that the CDE motif is present in most of the promoters of the p21-regulated genes. Altogether, the results suggest that p21 exerts a repressive effect on a relevant number of genes controlling S phase and mitosis. Thus, p21 activity as inhibitor of cell cycle progression would be mediated not only by the inhibition of CDKs but also by the transcriptional down-regulation of key genes.

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Introduction

$p21^{CIP1}$ (p21 herein after) is a member of the Cip/Kip family of inhibitors of cell cycle progression (also including $p27^{KIP1}$ and $p57^{KIP2}$). The first discovered p21 function and so far its best studied biochemical activity was the inhibition of cyclin-dependent kinases [1,2,3,4]. CDKs are protein complexes, composed of a regulatory cyclin and a catalytic CDK subunit, which orchestrate cell cycle transitions. Enforced p21 expression results in cell cycle arrest, which frequently takes place at the G2/M transition and accompanied by polyploidy [5,6,7,8]. p21 is also a p53 target gene that plays a relevant role in p53-induced cell cycle arrest [9,10,11].

However, other studies have shown that p21 has activities in addition to cell cycle arrest. Thus, p21 acts as an inhibitor of apoptosis induced by DNA-damaging agents [12,13,14] and as inducer of senescence [15,16,17] or differentiation (reviewed in [18]). Finally, p21 has been implicated in the control of transcription, through mechanisms that may be coupled to its CDK inhibition activity but also by direct association and modulation of transcription factors. In this way, it has been demonstrated the interaction between p21 and several transcription factors such as CBP, C/EBPα, E2Fs, Myc, Nrf2, STAT3, and others (reviewed in [19,20,21]). p21 has been found to repress several genes through the interaction with the E2F transcription factor [22] or by other mechanisms [15,23,24]. It has also been described as co-activator of the expression of other genes [25,26,27,28]. Nonetheless, there is little information on the biological significance of p21-dependent regulation of gene expression and to what extent it is linked to effects on the cell cycle. It has been shown that CDK2 is not an essential target for p21 in cell cycle inhibition and tumor suppression [29], given further relevance to the gene regulation effects of p21.

To address the relevance of p21-mediated gene regulation we have carried out large-scale expression profiling in two different human systems (keratinocytes and myeloid leukemia cells) upon ectopic expression of p21. p21 provokes a rapid and potent down-regulation of genes involved in the execution and control of mitosis in both models. Mutational analysis revealed that the N-terminal

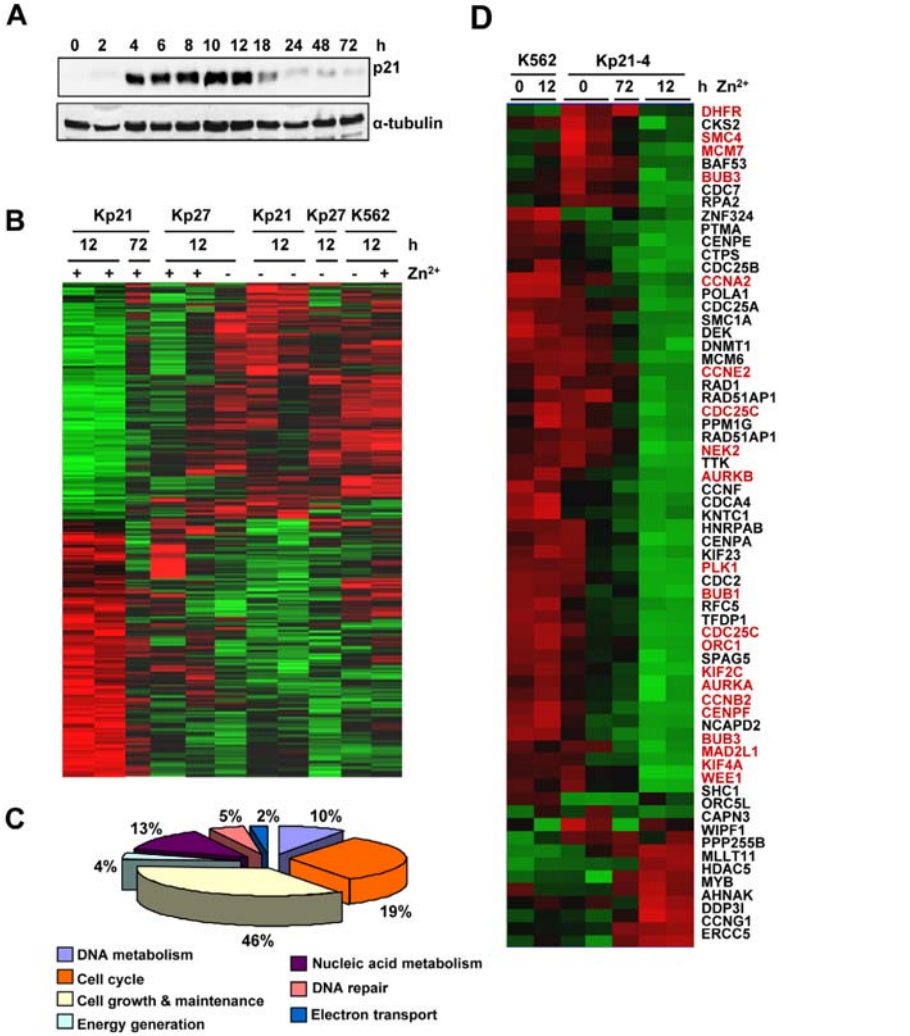

Figure 1. Gene expression changes induced by p21 in K562 cells. A. Expression of p21 in Kp21-4 cells in response to $ZnSO_4$. Cells were treated with 75 μM $ZnSO_4$ for the periods of time indicated. Cell extracts were prepared and the p21 levels analyzed by immunoblot. B. The transcriptome of Kp21-4 cells (Kp21) treated for 12 h with $ZnSO_4$ (to induce p21) were compared to that of cells with induced p27 (Kp27), parental K562 and Kp21-4 treated for 72 h with $ZnSO_4$. The heat map shows the hierarchical clustering with those genes with expression variation ≥2.3-fold between uninduced and p21-induced Kp21-4 cells ($P<0.001$). The heat map shows 350 regulated genes (360 gene probes). C. Distribution of the regulated genes shown in A according with their cell functions. The ontogeny analysis has been carried out with the dChip program. D. Expression changes in 65 genes related to cell cycle and mitosis according to the ontogenic classification. The heat map was obtained as described in B. The genes further validated by RT-qPCR (Figure 2A) are shown in red.
doi:10.1371/journal.pone.0037759.g001

Results

p21 represses mitotic genes in human leukemia cells

In order to find genes regulated by p21 in human primary cells we carried out a gene expression profiling in human myeloid leukemia K562 cells with conditional expression of p21. We previously described a K562 derivative, termed Kp21-4, that carries a zinc-inducible p21 gene [31]. We performed a kinetic study to identify the expression peak of p21 in this system. The immunoblot results showed that treatment of Kp21-4 cells with 75 μM $ZnSO_4$ resulted in induction of p21 that peaked 4–12 h decreasing afterwards (Figure 1A). This transient induction of p21 was accompanied by proliferation arrest and an increase in polyploid cells after 48–72 h [31]. The cell cycle profile did not change over the first 12 h of p21 induction with $ZnSO_4$ but 6–12 h of p21 induction were sufficient to irreversibly trigger proliferation arrest and polyploidy (Figure S1). Therefore, we chose 12 h as the induction time to analyse p21 effects on the transcriptome of these cells, as gene expression changes later on may be indirect due to other phenotypic effects.

We next carried out the gene expression profiling of Kp21-4 cells upon p21 induction by $ZnSO_4$. In order to identify genes specifically modulated by p21 we compared with the cell line Kp27-5, which carries a Zn^{2+}-inducible p27 allele [31]. p27 is a close relative to p21 that also inhibits CDKs and induce cell cycle arrest [40]. Thus, the comparison serves to identify genes specifically regulated by p21 in our analysis. We subtracted the gene expression changes occurring at 72 h in Kp21-4 cells those genes regulated by p27 in the Kp27-5 cells and genes changed by $ZnSO_4$ treatment in parental K562 cells. We found 350 genes whose expression changed ≥2.3 fold in Kp21-4 after 12 h of p21 induction and which were not regulated at this time point in Kp27-5 or K562 cells treated with $ZnSO_4$ (Figure 1B). The list of the genes with their corresponding expression change is shown in the Table S2.

The dataset used for the clustering analysis of Figure 1B was further analyzed with the Ingenuity Pathways software to reveal the network of interactions between differentially regulated genes. The results showed that the two highest-ranked networks were assembled by interactions between genes related to cell cycle control (Figure S2). Gene ontology analysis revealed that cell cycle-related genes accounted for about one fifth of the regulated genes, i.e., 65 genes (Figure 1C). The expression change of these genes is shown as a heat map in Figure 1D, demonstrating that the vast majority of these genes were down-regulated by p21. The former result defined cell cycle and mitosis as the most relevant functional categories of p21-down-regulated genes. We investigated the kinetics of changes in the RNA levels of 19 genes repressed by p21 from those appearing in Figure 1D plus *BIRC5*, *CCNB1* and *CDK2*, because of their involvement in cell cycle. RNA was prepared from Kp21-4 cells and expression was determined by RT-qPCR at different periods of time up to 12 h after $ZnSO_4$ addition, i.e., when p21 expression is maximal and its effect is already irreversible. The results are shown in Fig. 2A and confirmed that most of the down-regulated genes identified by microarray hybridization were down-regulated as soon as 6 h after the addition of the p21 inducer. $ZnSO_4$ did not modify the expression of any of these genes in parental K562 (data not shown). To confirm the repressive effect of p21 we analyzed the level of acetylated-histone H3, a marker of active chromatin [41], in the chromatin region corresponding to the transcriptional start site of several genes. The results showed a dramatic decrease in the fraction of acetylated-histone H3 as soon as 6 h after p21 induction (Fig. 2B). The data also argues that the decrease in mRNA levels is caused by transcriptional switch-off rather than mRNA degradation

The fast kinetics of gene down-regulation caused by p21, before any change in cell cycle profile could be detected, suggested that the repression is a direct consequence of p21 activity, rather than an indirect effect. CDK2 inhibition is the best known biochemical activity of p21, and it also occurs in K562 [31]. Thus, we explored the possibility that the p21-dependent gene expression regulation described above is a consequence of CDK2 inhibition. We conducted two sets of experiments. First, we showed that the depletion of CDK2 in Kp21-4 cells achieved through siRNA did not modify the p21-dependent repression of the assayed genes. This has been demonstrated by transient expression of a short-hairpin CDK2 vector (Fig. 3). Second, we analysed the effects of p27, which also provokes CDK2 inhibition and cell cycle arrest [31], on the expression of genes down-regulated by p21. In contrast to p21, p27 provoked a dramatic G1 arrest already detectable after 6 h of induction in Kp27-5 cells (Figure S1). p27 was induced in Kp27-5 cells for 3–12 h and the expression of 11 genes was assayed by RT-qPCR. The results showed that p27 did not repress cell cycle genes or repressed them with a much slower kinetics than p21 (Figure S3), despite a similar induction level of p21 and p27 in p21-4 and Kp27-5 cells respectively (Figure S1A). Moreover, we performed gene expression profiling in Kp27-5 cells upon 12 h of p27 induction. The results showed that at this induction time p27 elicited weaker effects on gene regulation than p21. After subtraction of p21-regulated genes the analysis revealed that p27 regulated only 180 genes (with an expression change ≥2.3 fold after 12 h of p27 induction) and none of them was annotated as related to cell cycle, according to the Gene Ontology analysis (Figure S4). The comparison to retrieve shared gene expression profiles by p21 and p27 induction revealed 90 common genes that were regulated by both proteins after 12 h of induction. These genes belong to different functional categories, but only 8 genes were related to cell cycle (Figure S4).

The N-terminal region of p21 is sufficient for gene down-regulation

Next, we asked whether the CDK-cyclin binding domain of p21 was involved in the down-regulation of gene expression. We used two p21 deletion constructs carrying the green fluorescent protein. One construct carried the first 91 amino acids, which included the CDK-cyclin binding region and the second construct the C-terminal region, after the codon 91 [33] (Figure 4A). Although the GFP-N-terminal construct lacked the nuclear localization signal in the p21 region, it partly localizes in the cell nuclei, likely due to the GFP domain [33]. We transfected these constructs as well as the full-length p21-GFP construct and selected the GFP-expressing cells by fluorescent-activated cell sorting. We further tested the expression of several genes with rapid (*CCNE2*, *KIF4A*) and slower (*WEE1*) down-regulation kinetics (see Figure 2). The expression of *CCNE2*, *WEE1* and *KIF4A* in GFP-positive cells was determined by RT-qPCR in the transfected cells. The results showed that the N-terminal p21 was sufficient to provoke the gene down-regulation, although it was a less efficient repressor than the full-length p21. In contrast, C-terminal p21 was inactive as repressor of the assayed genes (Figure 4B).

This indicates that the repressive activity of p21 is independent of the protein-protein interaction domain known to reside in the C-terminal region, as PCNA binding [42].

p21 binds to the human CCNE2 promoter

The observation that p21 provokes a rapid mRNA repression of various genes, suggested that p21 might participate in gene

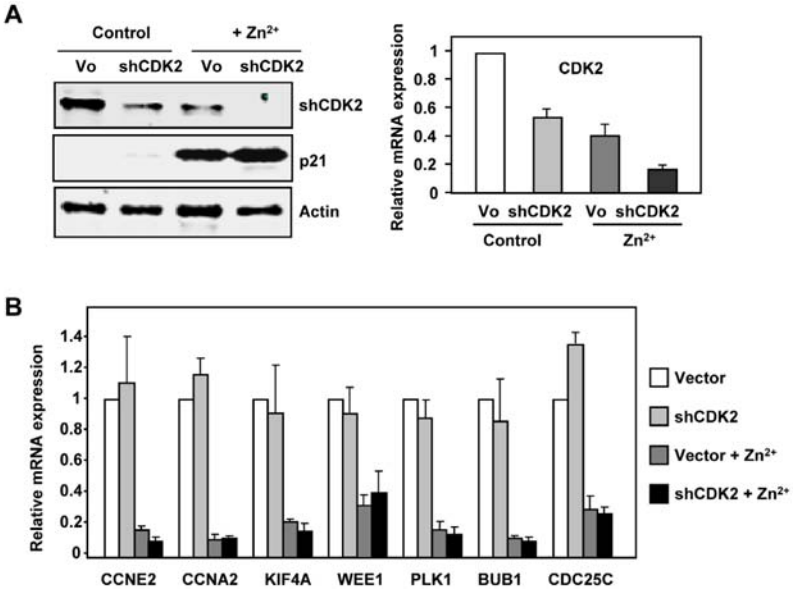

Figure 3. p21-mediated repression of genes is not dependent on CDK2. A. K562 cells were transiently transfected with a short-hairpin CDK2 ("shCDK2") vector and the empty vector (pLKO.1, "Vo"). 24 h after transfection the cells were treated with 75 μM $ZnSO_4$ for 12 h to induce p21 and the silencing of CDK2 was assayed at the protein level by immunoblot (left panel) and at the mRNA level by RT-qPCR (right panel) The expression of p21 and actin was also determined to control the p21 induction by Zn^{2+} and the protein level, respectively. B. The expression of p21 was induced with 75 μM $ZnSO_4$ in K562 transfected with sh-CDK2 and 12 h later, total RNA was prepared and expression of the indicated genes was determined by RT-qPCR. The values are means ±S.E.M. from two independent experiments and two determinations for each RNA.
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repression as a transcriptional modulator, a function already reported for p21 (see Introduction). For further analysis, we focused on the *CCNE2* gene because of its pivotal role in S phase entry and because it showed one of the fastest down-regulation kinetics, with a decrease as soon as 6 h after Zn^{2+} addition (Figure 2). We previously reported that most p21 remains nuclear upon induction with Zn^{2+} in Kp21-4 cells [43]. However, in order to act as co-regulator it is required that at least part of the p21 induced by Zn^{2+} in Kp21-4 cells is bound to chromatin. We first carried out a fractionation of Kp21-4 nuclear extracts into chromatin and nucleoplasmic fractions. The results of the immunoblot show a significant amount of p21 bound to the chromatin fraction in Kp21-4 cells induced with $ZnSO_4$ (Figure 5A). This result led us to ask whether p21 could be bound to the promoter of their regulated genes. We performed chromatin immunoprecipitation (ChIP) in Kp21-4 cells with anti-p21 antibody, and asked for p21 binding in the vicinity of the transcription start site (TSS) of human *CCNE2* and other p21-repressed genes. The results showed that p21 was bound to the region encompassing the TSS in Kp21-4 cells upon p21 induction after 12 h of treatment with $ZnSO_4$ (Figure 4B). We also found p21 binding to the promoters of other p21-target genes as *CDK2*, *KIF4A*, *PLK1* and *WEE1* to similar extent than to *CCNE2* promoter (Figure 5B). As additional controls, we performed ChIP experiments in Kp27-5 cells treated with $ZnSO_4$ to induce p27 [31] and in parental K562 also treated with $ZnSO_4$. No enrichment was found in the chromatin precipitated with anti-p21 in both cases. Also, no specific signal was detected with anti-p27 antibody in Kp21-4 cells and in Kp27-5 cells with induced p27 expression (Figure 5B). As the p21 region required for repression includes the cyclin-CDK binding region (Figure 4) we also performed ChIP assays with anti-CDK2 antibody. The results show that in cells overexpressing p21, CDK2 is recruited to the same site of human *CCNE2* that p21 (Figure 4B), which is in concordance with the presence of p21 on that region of the chromatin.

p21 represses CCNE2 promoter activity

The former results show that CCNE2 is rapidly down-regulated by p21 and that p21 is bound to CCNE2 proximal promoter in Kp21-4 cells. To confirm the hypothesis that p21 may act as a transcriptional modulator we carried out luciferase assays with reporters harbouring the human *CCNE2* and (as a control) *CCNE1* promoters. The results demonstrated a modest but significant and reproducible decrease in *CCNE2* promoter upon transfection of a p21 expression vector (Figure 6A). As a second approach, we next used the Kp21-4 cell line and performed the luciferase assays 24 h after the induction of p21 (Figure 6B). Immunoblot analysis revealed the overexpression of p21 in transfected cells (Figure 6A and 6B, lower panels). Taken collectively the results strongly

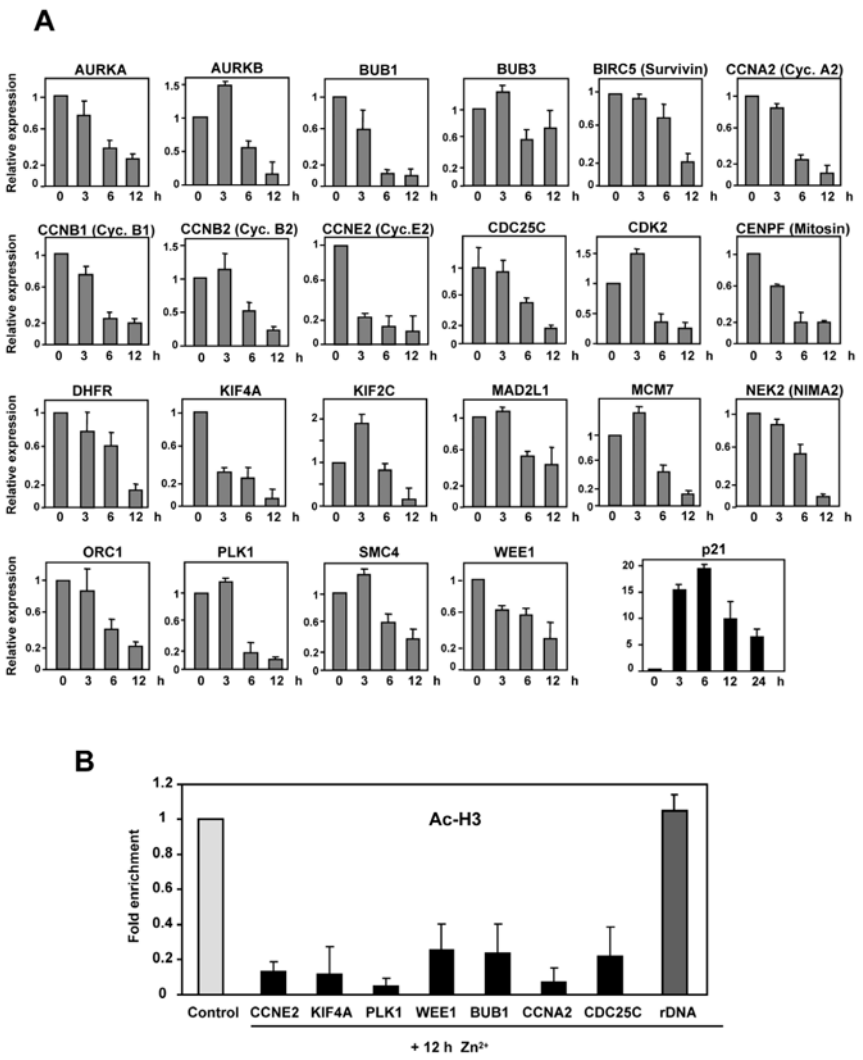

Figure 2. p21-mediated the down-regulation of genes involved in cell cycle. A.The expression of p21 was induced in Kp21-4 cells by 75 μM $ZnSO_4$ and 3, 6 and 12 h later, total RNA was prepared and expression of the indicated genes was determined by RT-qPCR. In some cases an alternative name is given into brackets. Cyc., cyclin. The values are means ±S.E.M. from two independent experiments and two determinations for each RNA. B. Kp21-4 cells were treated for 6 h with 75 μM $ZnSO_4$ to induce p21. Chromatin immunoprecipitation was carried out with anti-histone H3 and anti-acetylated-histone H3 antibodies and (as specificity control) rabbit IgG. The DNA in the immunoprecipitated chromatin was measured by quantitative PCR. The amplicons encompass the transcription start site of the indicated genes. A regulatory sequence of the promoter of rDNA was used as a control. The results are expressed as the ratio of DNA enrichment in chromatin immunoprecipitated with anti-H3 versus acetylated-H3 ("Ac-H3") and normalized to the values obtained in uninduced cells. The values are the means ±S.E.M of two independent ChIP experiments with two PCR determinations each.
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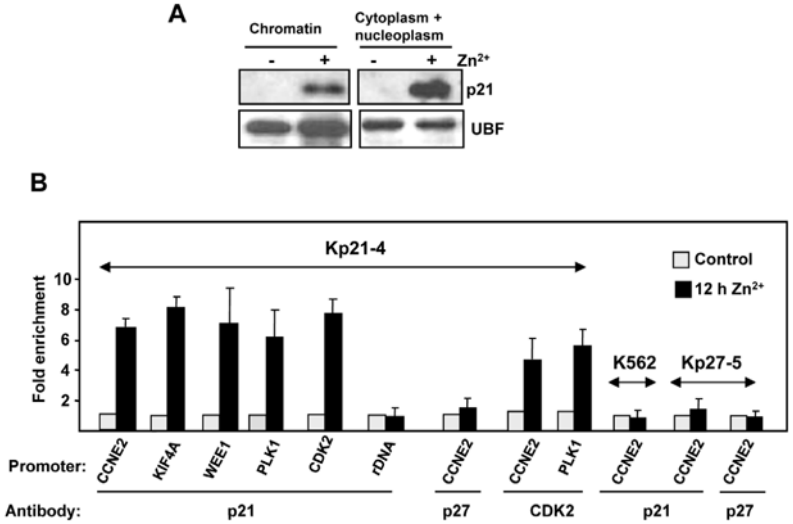

Figure 5. p21 binds to human CCNE2 promoter. A. Kp21-4 cells were treated for 12 h with $ZnSO_4$ and the nuclear extracts were fractionated between insoluble (chromatin) fraction and soluble (nucleoplasmic) fraction. The p21 levels were determined by immunoblot. The transcription factor UBF was used as control of chromatin-bound protein. B. Cells were treated for 12 h $ZnSO_4$ to induce p21 (in Kp21-4 cells), p27 (in Kp27-5 cells), parental K562 cells were also treated with ZnSO4 as negative control. Chromatin immunoprecipitation was performed with anti-p21, anti-p27 and anti-CDK2 antibodies as indicated, and (as specificity control) rabbit IgG. The DNA in the immunoprecipitated chromatin was analysed by quantitative PCR. The amplicons encompass the transcription start site of the indicated genes. rRNA promoter sequences were used negative controls. The results are expressed as enrichment of DNA in chromatin immunoprecipitated with anti-p21 (with respect the signal with anti-rabbit IgG) in cells induced with Zn^{2+}, and normalized to the values obtained in uninduced cells. The values are the means ±S.E.M of three PCR determinations, each from two independent ChIP experiments.
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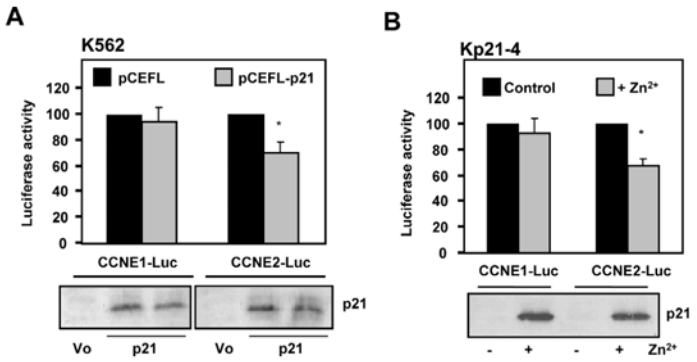

Figure 6. p21 represses the activity of human CCNE2 promoter. A. K562 cells were transfected with luciferase reporters carrying the promoters cyclin E1 and E2 genes (*CCNE1* and *CCNE2*), along with an expression vector for p21 and the corresponding empty vector, and a beta-gal plasmid for transfection efficiency normalization. 24 h after transfection the luciferase activities were determined. The data are normalized to the activity of cells transfected with the empty vector. Lower panel: immunoblot analysis of the transfected cells to assess the expression of p21. B. Kp21-4 cells were transfected with luciferase reporters carrying the promoters cyclin E1 and E2 genes as in (A) and 24 h later the cells were treated with 75 μM $ZnSO_4$ and after 24 h the luciferase activities were determined. The data are normalized to the activity of cells without $ZnSO_4$. Lower panel: immunoblot analysis of the transfected cells to assess the expression of p21.
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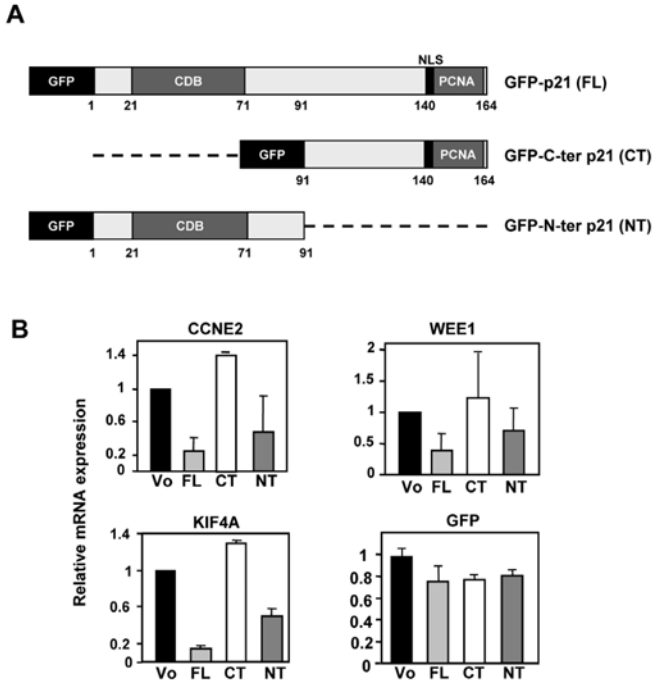

Figure 4. The N-terminal region of p21 is required for its effect as gene repressor. A. Schematic representation of the p21 constructs used. The proteins carry the green fluorescent protein (GFP) in the N-terminal. The position of the CDK binding domain (CDB) and the PCNA binding domain (PCNA) are indicated. B. K562 cells were transfected with expression vectors for the full-length p21 protein (FL), the p21 amino-terminal region (NT) and the p21 carboxy-terminal region (CT). 24 h after transfection the cells were sorted by flow cytometry and the expression of the indicated genes was analysed by RT-qPCR. The values are means ±S.E. from two transfections and two determinations of each mRNA.
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suggest a role of p21 in the negative regulation of *CCNE2* and other genes in the K562 system.

p21 represses mitotic genes in human primary keratinocytes

All the previous results have been obtained in a cell line derived from human myeloid leukemia. In order to confirm these results we studied the p21-dependent repression of mitotic genes in a different cellular system. We chose human primary keratinocytes because they are non-tumorigenic, non-immortalized and epithelial cells, in contrast to K562 cells. Human primary keratinocytes were infected with recombinant adenoviruses expressing the full-length p21 protein. A dramatic increase in p21 in infected keratinocytes was demonstrated by RT-qPCR (Figure 7A). As controls, we also infected the keratinocytes with adenovirus carrying the genes for p27, which overexpression was also confirmed by RT-qPCR (Figure 7A). We prepared RNA 24 h after infection and performed large-scale expression assay using the Affymetrix platform. The clustering analysis revealed that p21 provoked the down-regulation of a number genes involved in cell cycle control not shared by cells expressing p27 (Figure 7B). The list of the genes with their corresponding expression change is shown in the Table S3. We next validated by RT-qPCR the p21-mediated repression of several of these genes involved at various checkpoints of cell cycle (*AURKB*, *BIRC5*, *CDC25C*, *CCNE2* and *WEE1*). The results demonstrated the decreased levels of mRNA for all the tested genes, confirming the data of the microarray hybridization (Figure 8A). To fully confirm the effect of p21 as a repressor of these genes in keratinocytes, we silenced p21 through siRNA transfection (Figure 8B). The expression of the five genes was tested in p21-depleted cells and we found that the five genes were up-regulated to various extents (Figure 8C). The results therefore confirm the regulation of the expression of the analyzed genes by p21.

Bioinformatic analysis of p21-targeted genes

As noted in the Introduction, it has been reported that p21 binds to specific DNA sequences of some p21-regulated genes. As p21 lacks any recognizable DNA-binding domain, the interaction must be indirect, i.e., through another transcription factor or co-factor. We previously reported that p21 can bind genes at E2F-

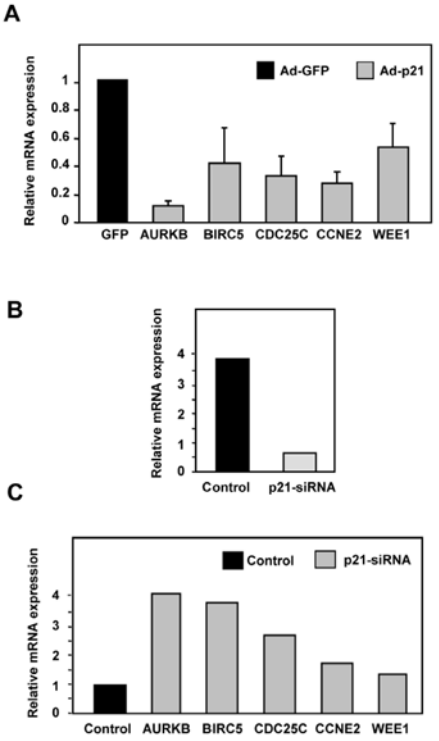

Figure 8. p21-mediated down-regulation of genes involved in mitotic control in human keratinocytes. A. Primary human keratinocytes were infected with adenovirus expressing p21 and ad-GFP as a control. 24 h after infection total RNA was prepared and the mRNA levels of the indicated genes were analysed by RT-qPCR. The values are means ±S.E. from three determinations. B. keratinocytes were transfected with siRNA for p21 and a negative control siRNA. Total RNA was prepared and the mRNA levels of the indicated genes were analysed by RT-qPCR. C. Primary human keratinocytes were transfected with p21 siRNA or control siRNA. 48 h after lipofection total RNA was prepared and the mRNA levels of the indicated genes were analysed by RT-qPCR.
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binding elements [23]. Another report showed the binding of p21 to the CDE/CDH element, composed by the the cell cycle-dependent element (CDE) and the cell cycle genes homology region (CHR) [44]. Therefore, we have performed a bioinformatic analysis of the promoters of the 22 genes where p21-mediated down-regulation was validated by RT-qPCR (shown in Figure 2). The results show that a majority of the genes carried several E2F sites in the 5′ regulatory region and/or the first exon (Figure 9) but there were some exceptions (*BIRC5*, *CCNB2*, *DHFR*, *KIF4A*). More interestingly, all genes but one (*BUB1*) contained at least one CDE site in the vicinity of the TSS (Figure 9). In this context it must be noted that human *CCNE2* promoter and first exon are particularly rich in CDE sequences. Thus, p21 may impair transcription of genes in charge of the execution and control of mitosis through the interaction with transcription factors binding to CDE or CDE/CHR motifs.

Discussion

Despite the original description of p21 as a CDK inhibitor, a number of reports have described a role of p21 as transcriptional modulator for different genes such as *WNT* [23], cyclin D1 [27], polo-like kinase 1 (*PLK1*), topoisomerase IIα [44], *CDC25A*, *MYC* [24] and p53 [45]. p21 has also been described as an antagonist of E2F-dependent transcription [22]. Conversely to previous studies, we have analyzed the genome-wide p21-dependent gene regulation in two different human cellular models: a myeloid leukemia cell line (K562) and primary keratinocytes. p21 overexpression was attained through the induction of a conditional transgene by $ZnSO_4$ (in human myeloid cells) or through acute adenoviral infection (in human keratinocytes).

Our results on the gene expression profiling in both cell models showed that p21 provokes a rapid repression of mRNA of genes involved in cell cycle and mitosis control in the two models. Since p21 elicits cell growth arrest this effect could be indirect, i.e., a consequence of the cell cycle arrest. However, in myeloid cells with inducible p21 (Kp21-4 cells), the kinetics of the mRNA down-regulation of most of the analysed genes was almost parallel to the kinetics of the p21 protein up-regulation. This down-regulation of mRNA was also concomitant with the rapid deacetylation of histone H3 at the TSS. The genes repressed by p21 were up-regulated upon p21 silencing, confirming the p21 activity. Moreover, the early p21-induced down-regulation was not reproduced by p27 induction in K562 cells form most genes, despite that p27 also provokes a cell cycle arrest via CDK inhibition. Furthermore, the depletion of CDK2 does not affect the p21-mediated gene repression. Altogether these data argue against the idea that the p21 effects were a direct consequence of the CDK inhibition or of the cell cycle arrest. Thus, it is conceivable that p21 is directly involved in transcriptional repression of a set of genes involved in cell cycle control. Previous unsuccessful attempts to generate a functional p21 fused to the estrogen receptor (so as to get activated by tamoxifen) [46] precluded the analysis of p21 transcriptional effects in the absence of protein synthesis.

It is remarkable the similarity in the short-term transcriptional effects of p21 overexpression observed in two very different cell types (keratinocytes and myeloid cells), despite the different long-term consequence of p21 overexpression in each cell type. p21 inhibits epidermal differentiation in keratinocytes [25,32] whereas it induces polyploidy and megakaryocytic differentiation in K562 [31]. It is of note however, that the transcriptional changes that we have observed occur before any phenotypic change can be detected in either cell line. In Kp21-4 cells, the polyploidy is detectable 3–4 days after induction and at this time point, the expression of CDC25, cyclin A and cyclin E are recovered, likely to allow DNA synthesis for endoreplication [8]. Previous studies in a fibrosarcoma cell line also showed p21 repressed genes involved in mitosis followed by polyploidy three days after p21 induction [7], suggesting that polyploidization may be a common effect of p21 in tumor cells.

We further analysed the regulation of *CCNE2* (cyclin E2), one of the genes undergoing a fastest down-regulation. ChIP assays revealed that p21 binds in the vicinity of the TSS of human cyclin E2 gene (as well as other four genes: *CDK2*, *KIF4A*, *PLK1* and *WEE1*). The results are consistent with previous literature showing

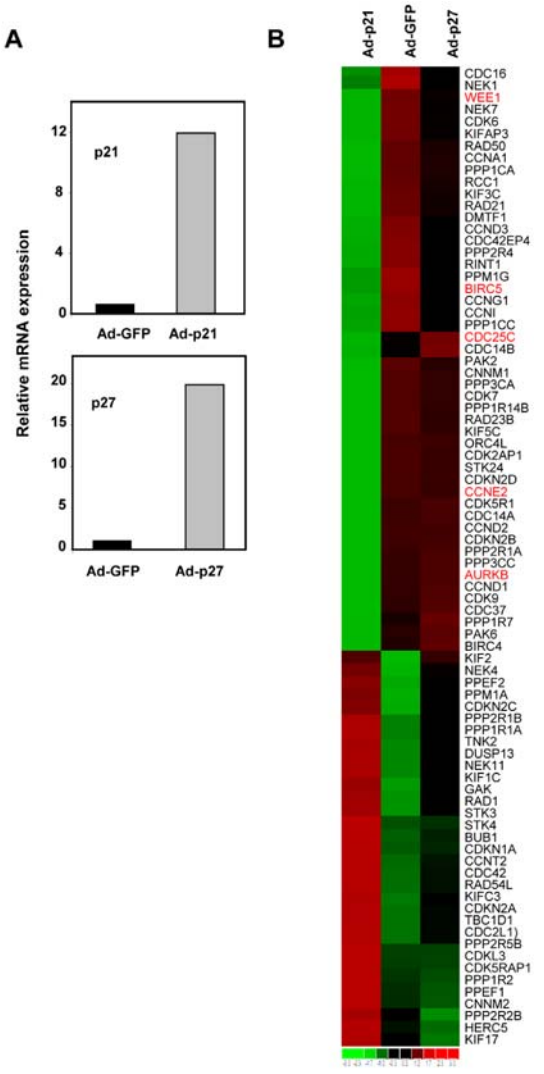

Figure 7. Genes regulated by p21 in human keratinocytes related to cell cycle and cell division. A. Primary human keratinocytes were infected with recombinant adenoviruses expressing p21 (Ad-p21), p27 (Ad-p27). As a control, cells were also infected with adenovirus expressing GFP (Ad-GFP). The mRNA expression of p21 (upper graph) and p27 (lower graph) was determined by RT-qPCR 24 h after infection. Data are represented relative to the expression in Ad-GFP-infected cell. B. Heat-map showing the 82 genes changed by p21 related to cell cycle and cell division with an expression change > 2.3-fold. The names of the regulated genes are indicated at the right. Green indicates genes down-regulated by p21 and red genes up-regulated.
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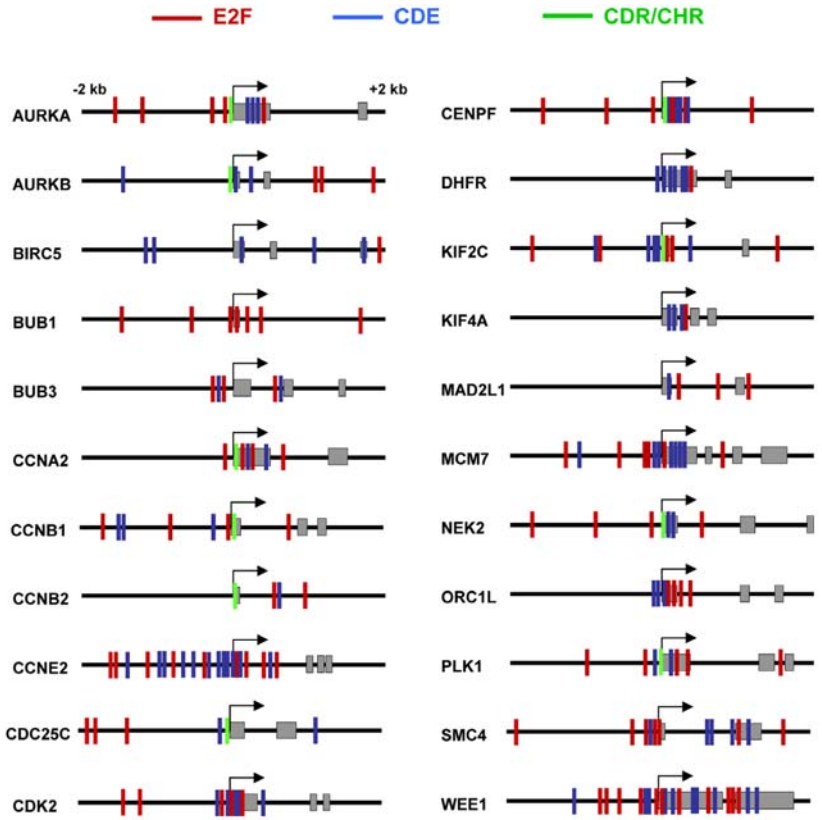

Figure 9. Putative regulatory sites in genes down-regulated by p21. Schematic representation of the E2F (red), CDE (blue) and CDE/CHR (green) sites in the p21-regulated genes analysed in Figure 2. The region analysed encompass 2 kb upstream and downstream the transcription start site (marked by an arrow). Exons are represented as grey boxes. E2F sites: $TTT^{C}/_{G}{}^{C}/_{G}{}^{C}/_{G}{}^{C}/_{G}$. CDE sites: $^{T}/_{G}GGCGG$. CDE/CHR sites: $GCG^{C}/_{G}N_{2-5}\ TT^{A}/_{G}AA$.
doi:10.1371/journal.pone.0037759.g009

chromatin binding of p21 in several models (see Introduction). In contrast, upon induction of p27 for 12 h in Kp27-5 cells, we could not detect significant binding of p27 to CCNE2 promoter.

In full agreement with the results observed at the mRNA level and ChIP assays, luciferase reporter experiments showed that p21 repressed the human *CCNE2* promoter. The mutational analysis revealed that CCNE2 gene repression depends on the N-terminal region of p21, i.e., the region involved in the binding to cyclin-CDK complexes. The C-terminal domain of p21 is not required, but seems to contribute for full activity of p21 as repressor. It is noteworthy that p21 does not repress CCNE1 promoter (if it does) as efficiently as CCNE2, arguing for a differential role of both cyclins.

Taken together the data argue for a role of p21 as co-repressor of gene transcription for genes related to cell cycle. As p21 lacks any recognizable DNA-binding domain, the interaction must be indirect, i.e., through another transcription factor or co-factor. It has been reported that p21 binds to specific DNA sequences of some genes. For instance, we have previously shown that this is the case for *WNT4* repression, which depends on the interaction with E2F1 [23]. Also, it has been reported that p21 repress the mitotic control gene PLK1 through binding to the CDE/CHR element [44]. The CDE/CHR elements control the transcription of genes with maximum expression in G2 phase and in mitosis and are repressed in G0 and G1 phases, although the transcription factor(s) responsible are still unidentified [47]. It has been recently reported that p27 binds to some promoters through E2F4 sites in mouse fibroblasts [48]. Our analysis showed that many of the p21-downregulated genes contained one or several E2F binding sites, but at least four genes did not (*BIRC5*, *CCNB2*, *DHFR*, *KIF4A*),

despite that a relaxed consensus sequence as E2F's binding site was used for the analysis, which includes E2F4 sites (Figure 9). Our analysis does not give information regarding the specific E2F family member involved, so the differential binding of E2F factors on the different genes is an open possibility. More interestingly, we found that all genes but one (*BUB1*) contained at least one CDE site in the vicinity of the TSS. In this context it must be noted that human CCNE2 promoter and first exon are particularly rich in CDE sequences. Thus, p21 may impair transcription of genes in charge of the execution and control of mitosis through the interaction with transcription factors binding to CDE or CDE/CHR motifs.

Altogether, our results indicate that p21 is a multifunctional protein with the capacity to act through at least two mechanisms to control cell cycle: directly inhibiting CDKs and indirectly regulating genes involved in cell cycle control. The relative importance of each mechanism await further investigation, but it is of note that p21 is able to arrest the cell cycle in CDK2-deficient cells [29], arguing for the importance of transcriptional repression in the p21 functions in cell biology. Additional studies are required to dissect out the mechanisms involved in the transcriptional repression mediated by p21.

Supporting Information

Figure S1 Cell cycle alterations mediated by p21 and p27 in K562 cells. A. Immunoblots showing the induction of p21 in Kp21-4 cells and p27 in Kp7-5 cells after 6 and 12 h of treatment with 75 μM $ZnSO_4$. B. Absence of cell cycle profile alteration after short induction times of p21. Cell cycle profile of Kp21-4 and Kp27-5 cells upon induction of p21 and p27 with 75 μM $ZnSO_4$ for 6 and 12 h. The cell cycle profile was determined by flow cytometry of propidium iodide-stained cells. C. p21 induces an irreversible accumulation of G2 and polyploid cells whereas p27 induces a reversible accumulation in G1. Kp21-4 and Kp27-5 were treated with ZnSO4 for 12 h. The cells were then washed to remove the inducers, further incubated for 84 h and the cell cycle profile was determined by flow cytometry (4 days after the induction). The fraction of cells in G1, G2 or polyploidy cells (>G2) is indicated in each case.
(TIF)

Figure S2 Interaction networks of genes regulated by p21 in K562 cells. A knowledge-based database (Ingenuity Pathways Analysis) was seeded with the genes regulated by p21 at 12 h of induction (Table S2). The two networks with the highest score are shown. The program processed 279 genes (137 up-regulated, 142 down-regulated). The ontogeny category of the networks is as indicated at the bottom. Genes in red were up-regulated and those in green were down-regulated. The meanings of node shape and lines are indicated at the bottom.
(TIF)

Figure S3 Comparison of the gene regulation mediated by p21 and p27 in K562 cells. p21 was induced in Kp21-4 cells and p27 was induced in Kp27-5 cells by 75 μM $ZnSO_4$. After 3, 6 and 12 h of induction, total RNA was prepared and expression of the indicated genes was determined by RT-qPCR. The data for Kp21-4 cells are the same than in Figure 2. The values are means ±S.E.M. from two independent experiments and two determinations for each RNA
(TIF)

Figure S4 A. Gene expression regulation mediated by 12 h induction of p27 in K562 cells. The transcriptome of Kp27-5 cells (Kp27) treated for 12 h with $ZnSO_4$ (to induce p27) were compared to that of cells with induced p21 (Kp21) and parental K562 treated for 12 h with $ZnSO_4$. The heat map shows the hierarchical clustering with those genes with expression variation ≥2.3-fold between uninduced and p27-induced Kp27-5 cells after subtraction of the gene expression changes due to p21 in Kp21 cells($P<0.001$). The heatmap shows 179 genes. B. Common genes regulated by both p21 and p27 in K562 cells. Kp21-4 and Kp27-5 cells were treated for 12 h with 75 μM $ZnSO_4$ to induce p21 and p27 respectively. The heat map shows the hierarchical clustering with those genes with expression variation ≥2.3-fold between uninduced cells and Zn^{2+} –treated cells which are regulated in both cell lines($P<0.001$). The heat map shows 90 genes. The genes related to cell cycle according with Gene Ontology are shown in red.
(TIF)

Table S1 Primers used in PCR reactions in this work. The forward primer is in the first line. All correspond to human genes except GFP (Green Fluorescent Protein from *Aequorea victoria*, encoded in the plasmid pEGFP-C1). An alternative common name of the gene is included for some genes.
(DOC)

Table S2 Genes regulated by p21 in K562 cells. The table list the genes showing an expression change in Kp21-4 cells treated with $ZnSO_4$ (p21 inducer) for 12 h, after subtracting those genes changed by $ZnSO_4$ in parental K562 cells (i.e., changed by $ZnSO_4$) and in Kp27-5 cells (i.e., induced by p27). The 253 genes are included in the heat map of Fig. 1B. The table includes genes with ID and with a fold change $\geq \log_2 1.2$ (≥2.3-fold) and with a signal difference ≥50 between both experimental conditions (as defined by dChip program and with Affymetrix U133 biochip data). Values are mean of fold changes (expressed as $\log_2$) of two independent experiments ($P<0.001$). For those genes represented by two or three Affymetrix probes, the fold change is the mean between the values of the probes. A negative fold change indicates down-regulation upon p21 induction.
(DOC)

Table S3 Genes regulated by p21 in primary keratinocytes. The table list the genes showing an expression change in human primary keratinocytes infected with adenovirus-p21 as described in Methods. RNA was analysed 24 h after infection. The 75 genes are included in the heat map of Fig. 7. The table includes genes with a fold change $\geq \log_2 1.2$ (≥2.3-fold) and with a signal difference ≥50 between both experimental conditions (as defined by dChip program and with Affymetrix U133 biochip data). Values are mean of fold changes (expressed as $\log_2$) of two independent experiments ($p<0.001$). For those genes represented by two or three Affymetrix probes, the fold change is the mean between the values of the probes. A negative fold change indicates down-regulation upon p21 induction.
(DOC)

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Author Contributions

Conceived and designed the experiments: NF PD JL. Performed the experiments: NF JMC LGG VD MRP GB AQ MJMA MCL RB JCR. Analyzed the data: NF NA MDD GPD JL. Contributed reagents/materials/analysis tools: VD MDD. Wrote the paper: NF JL.

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differentiation of CML-derived cells. This inhibition is mediated by destabilization of p27, which is achieved in part by the induction of SKP2. Our data suggests that the block in differentiation is a mechanism by which MYC contributes to CML progression.

RESULTS AND DISCUSSION

Treatment of K562 cells with low imatinib concentrations (0.5 μM) abolished cell proliferation as shown by cell counting (Figure 1a) and by [^{3}H]thymidine incorporation (Figure 1b). We examined the imatinib-induced erythroid differentiation in cells treated with 0.5 μM imatinib. The fraction of benzidine-positive (assessed by benzidine cytochemical assay, which detects cells containing hemoglobin) augmented in a dose and time-dependent manner (Figure 1c). The erythroid differentiation was also confirmed by the increased mRNA levels of ε-globin, transferring receptor 2 and erythropoietin receptor (Figure 1d) and glycophorin A as measured by flow cytometry (not shown). Higher imatinib concentrations (2.5 μM) also induced erythroid differentiation but provoked significant apoptosis (Shah *et al*.,[25] Albajar *et al*.[26] and data not shown).

We next investigated the mechanism for imatinib-induced erythroid differentiation. It was reported that p27 induces erythroid differentiation in K562 cells[27] and that BCR-ABL activity decreases p27 levels.[28,29] Thus, we tested the hypothesis that differentiation depended on imatinib-mediated upregulation of p27. Imatinib induced the expression of p27 protein in K562 cells in a time- and concentration-dependent manner (Figure 1e). Importantly, there was a correlation between p27 levels and erythroid differentiation as assessed by the expression of γ-globin (Figure 1e). To confirm p27 involvement we analyzed the imatinib effect in p27 depleted cells. A short-hairpin vector for p27 was transiently transfected into K562, resulting in lower p27 levels in cells treated with imatinib, as compared with empty vector-transfected cells (Figure 1f). Consistently, these cells showed a lower level of differentiation in response to imatinib as assessed by the elevated γ-globin expression (Figure 1f, compare lanes 2 versus 4) and by the increase in the fraction of benzidine-positive cells (Figure 1f, bottom panel).

To investigate whether the imatinib-induced p27 was sufficient to differentiate the cells, we compared the levels of p27 and differentiation obtained by imatinib in K562 with those achieved by Zn^{2+} in Kp27-5 cells. These are K562 cells carrying a zinc-inducible *p27* gene that undergoes erythroid differentiation upon p27 induction.[27] The proliferative arrest induced by p27 in Kp27-5 cells was similar to that achieved by imatinib and the antiproliferative effects of p27 and imatinib were not additive (Figure 1g). Also, p27 did not potentiate the imatinib-induced apoptosis (not shown). The levels of p27 achieved after 24 h of imatinib treatment were similar to those of Kp27-5 treated with Zn^{2+} for the same period of time (Figure 1h) and the concomitant treatment with Zn^{2+} and imatinib did not significantly increase the levels of p27 (Figure 1h, upper panel). Consistently, the extent of differentiation was similar to the differentiation achieved with Zn^{2+} or imatinib alone (Figure 1h, lower panel). In a second approach to elevate p27 levels we used the proteasome inhibitor bortezomib. Bortezomib provoked the accumulation of p27 in a concentration- and time-dependent manner (Supplementary Figure 1a) and induced erythroid differentiation of K562, as shown by the accumulation of hemoglobinized cells and ε-globin expression (Supplementary Figures 1b and c). We conclude that the upregulation of p27 is, at least in part, responsible of the differentiation elicited by imatinib.

As p27 is described as a cyclin-dependent kinases inhibitor, we asked whether differentiation was linked to proliferation arrest using K562-R cell line, a K562 derivative resistant to imatinib mainly due to LYN kinase overexpression.[30] In K562-R imatinib did not provoke growth arrest (Figure 2a) and erythroid differentiation (Figures 2b and c). Consistently, p27 expression was not induced in K562-R cells treated with imatinib concentrations high enough (2.5 μM) to inhibit BCR-ABL, as assessed by CRK-L phosphorylation (Figure 2c). Similar results were observed in KmycBT315I, a imatinib-resistant cell line expressing the BCR-ABL-T315I mutant[19] (not shown). However, imatinib-induced erythroid differentiation is not a mere consequence of the growth arrest because other drugs that provoke growth arrest of K562 (interferon-α, busulfan, TPA, staurosporin) do not induce erythroid differentiation.[20,31,32]

It has been reported that MYC is involved in CML progression.[19] Thus, we investigated whether MYC also impaired the imatinib-induced differentiation. We first showed that the low concentrations of imatinib capable of inducing differentiation also caused MYC downregulation in K562 after 24–72 h of treatment (Figure 2d). This downregulation was also demonstrated at the mRNA level, as shown by northern analysis (Figure 2e). It was noteworthy the correlation between the imatinib-mediated MYC repression and erythroid differentiation, as assessed by globin expression (Figures 2d and e). The decrease in MYC mRNA suggested that imatinib repressed MYC at the transcriptional level. This was confirmed by luciferase assays using a luciferase reporter harboring the human MYC promoter which was inactivated by imatinib (Figure 2f).

The former results prompted us to study whether MYC impaired the differentiation triggered by imatinib. To explore this we used two K562 sublines with conditional MYC expression. KMER4 cells expresses the MycER fusion protein,[19] which is activated by 4-hydroxy-tamoxifen (4HT).[33] The expression of MycER in KMER4 cells treated with imatinib was confirmed by immunoblot (Figure 3a). The benzidine assay indicated that MYC antagonized the imatinib-dependent differentiation in KMER4 cells (Figure 3b). We also demonstrated that the imatinib-mediated induction of ε-globin and transferring receptor 2 mRNA was blunted by MYC (Figure 3c). We sought to confirm the former results in a different system of conditional MYC expression. KmycB cells carry a Zn^{2+}-inducible MYC.[20] KmycB treated with Zn^{2+} in the presence of imatinib expressed significant levels of MYC (Figure 3d). Importantly, the addition of $ZnSO_4$ did not result in supra-physiological levels of MYC but rather restores the levels of control cells. MYC expression in KmycB resulted in a dramatic decrease in the fraction of hemoglobin-containing cells (Figure 3e). We also found that MYC decreased the levels of erythroid markers as ε-globin and transferring receptor 2 mRNA which were induced by imatinib (Figure 3f). Treatment with 4HT or Zn^{2+} did not modify the differentiation of parental K562 (not shown). To further confirm the effect of MYC on imatinib-dependent differentiation we hybridized complementary DNA microarrays containing probes of 4466 genes with labeled complementary DNA from KmycB cells treated with imatinib in the presence or absence of MYC. Out of 30 genes that were downregulated by MYC in imatinib-treated cells, 15 genes are associated to erythroid lineage or erythrocyte markers, including embryonic globins, heme synthesis genes, erythrocyte membrane proteins and erythroid-specific enzyme isoforms (Supplementary Table 1). Altogether, the results demonstrate that MYC impairs the erythroid differentiation mediated by imatinib in K562 cells.

We also tested the effect of dasatinib, a more potent BCR-ABL inhibitor also active against SRC kinase and recently introduced in CML therapy.[34,35] Dasatinib induced differentiation of K562 (Figure 3g), as well as downregulation of MYC and upregulation of p27 (Figure 3h). Next, we asked whether MYC impaired the dasatinib-mediated differentiation. For this purpose we used KMER4 cells, which expressed the MycER protein in the presence of dasatinib and 4HT (Figure 3i). We found that the activation of MYC impaired the dasatinib-induced differentiation of KMER4, as shown by the benzidine test (Figure 3j) and the expression of γ-globin (Figure 3k). We conclude that MYC impairs the differentiation induced by the anti-CML drugs imatinib and dasatinib.

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SHORT COMMUNICATION

MYC antagonizes the differentiation induced by imatinib in chronic myeloid leukemia cells through downregulation of p27^{KIP1}

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Chronic myeloid leukemia (CML) progresses from a chronic to a blastic phase where the leukemic cells are proliferative and undifferentiated. The CML is nowadays successfully treated with BCR-ABL kinase inhibitors as imatinib and dasatinib. In the CML-derived K562 cell line, low concentrations of imatinib induce proliferative arrest and erythroid differentiation. We found that imatinib upregulated the cell cycle inhibitor p27^{KIP1} (p27) in a time- and -concentration dependent manner, and that the extent of imatinib-mediated differentiation was severely decreased in cells with depleted p27. MYC (c-Myc) is a transcription factor frequently deregulated in human cancer. MYC is overexpressed in untreated CML and is associated to poor response to imatinib. Using K562 sublines with conditional MYC expression (induced by Zn^{2+} or activated by 4-hydroxy-tamoxifen) we show that MYC prevented the erythroid differentiation induced by imatinib and dasatinib. The differentiation inhibition is not due to increased proliferation of MYC-expressing clones or enhanced apoptosis of differentiated cells. As p27 overexpression is reported to induce erythroid differentiation in K562, we explored the effect of MYC on imatinib-dependent induction of p27. We show that MYC abrogated the imatinib-induced upregulation of p27 concomitantly with the differentiation inhibition, suggesting that MYC inhibits differentiation by antagonizing the imatinib-mediated upregulation of p27. This effect occurs mainly by p27 protein destabilization. This was in part due to MYC-dependent induction of SKP2, a component of the ubiquitin ligase complex that targets p27 for degradation. The results suggest that, although MYC deregulation does not directly confer resistance to imatinib, it might be a factor that contributes to progression of CML through the inhibition of differentiation.

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Keywords: Myc; imatinib; dasatinib; p27; chronic myeloid leukemia; differentiation

INTRODUCTION

c-Myc (MYC herein after) is an oncogenic transcription factor of the helix–loop–helix/leucine zipper protein family. MYC is a widespread regulator of transcription that regulates about one thousand genes.[1,2] MYC is found deregulated in around half of human tumors and appears frequently associated with tumor progression in solid tumors and leukemia.[3,4] MYC activities include increased proliferative potential, enhanced protein synthesis and energetic metabolism and genomic instability.[2,5] One of the most relevant activities of MYC in carcinogenesis is the impairment of cell differentiation[6].

p27 was originally described as a cyclin-dependent kinases inhibitor, with cyclin E-cyclin-dependent kinases 2 complexes as its primary targets.[7,8] p27 is regulated mainly at the protein stability level and low p27 levels are associated with a poor prognosis in most tumors.[9,10] The degradation of p27 in proteasoma is preceded by ubiquitylation by the SCF^{SKP2} complex, where SKP2 is the p27-recognizing subunit.[11–13] Recently it has been shown that *SKP2* is a MYC target gene, which may explain the inverse correlation between MYC and p27 levels found in many systems.[14]

Chronic myeloid leukemia (CML) is a myeloproliferative malignancy that progresses from a relatively benign chronic phase to a blastic crisis phase.[15] The molecular hallmark of all CML phases is the expression of the BCR-ABL kinase. CML is a hematopoietic stem cell malignancy where BCR-ABL would lead to a progressive block of differentiation and increased genetic instability.[16–18] However, the molecular mechanisms underlying CML progression into the blastic phase are still uncertain. We recently showed that MYC expression was higher in CML patients at diagnosis and that correlated with a poor response to imatinib.[19] Moreover MYC antagonizes the erythroid differentiation of the CML-derived cell line K562 induced by cytosine arabinoside[20] and by p27.[21] Imatinib, a BCR-ABL inhibitor, is the frontline drug in CML therapy.[22–24] Given that differentiation inhibition is a hallmark of the CML progression we explored a possible effect of MYC on imatinib-dependent differentiation. We show here that MYC impairs the imatinib-induced erythroid

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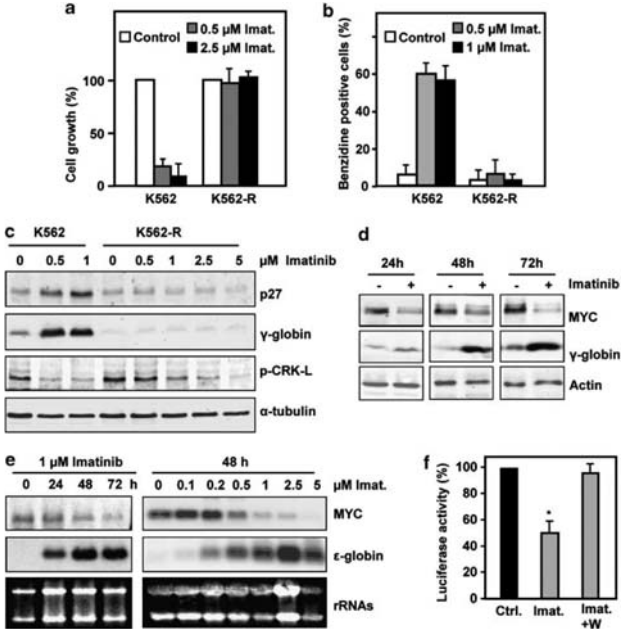

Figure 2. Imatinib-dependent differentiation is associated to proliferative arrest and MYC downregulation. (**a**) K562 and K562-R cells were seeded at a density of 2×10^5 cells/ml and treated for 48 h with the indicated imatinib concentrations and the cells were counted after 72 h. (**b**) K562 and K562-R cells were treated for 48 h with the indicated imatinib concentrations and subjected to the benzidine cytochemical test to count hemoglobin-producing cells. Data are mean values ± s.e.m. of three independent experiments. (**c**) K562-R cells and parental K562 cells were treated for 48 h with the indicated imatinib concentrations and the levels of p27, γ-globin (to assess erythroid differentiation) and phospho-CRK-L (to assess BCR-ABL activity) (anti-phosho-Tyr207-Crk-L, from Cell Signalling, Danvers, MA, USA) determined by immunoblot. α-tubulin levels were also measured to assess the protein loading. (**d**) Concomitant MYC downregulation and γ-globin upregulation induced by imatinib. K562 cells were treated with 0.5 μM imatinib for the indicated periods of time and the MYC and γ-globin protein levels were determined by immunoblot. Levels of actin were determined as a control of protein loading. MYC antibody (N-262, rabbit polyclonal from Santa Cruz Biotech). (**e**) Downregulation of MYC mRNA by imatinib in K562. Cells were treated with the indicated concentrations of imatinib for 48 h and the MYC and ε-globin mRNA levels were determined by Northern analysis. Probe labeling with (α-^{32}P)dCTP and filter hybridization were carried out according to standard procedures. Probes for human MYC and ε-globin were as described.[46] A picture of the filter after transfer showing the ribosomal RNAs stained with ethidium bromide is shown in each case to assess the loading and integrity of the RNAs. (**f**) Imatinib represses the MYC promoter in K562 cells. The activity of a MYC luciferase construct carrying 2.4 kb of human MYC promoter[47] was assayed after 24 h of treatment with 0.5 μM imatinib ('Imat.'), washed and further incubated 24 h without drug ('Imat + W'). Transfections (2×10^6 cells per transfection) were performed at 260 v and 1 mFa in a Bio-Rad electroporator (BioRad). As an internal control, cells were co-transfected with pRL-null plasmid encoding for Renilla luciferase (Promega) and the luciferase activity was normalized to Renilla luciferase. Data are mean values of three independent experiments; bars, s.e.m.; *$P < 0.05$ versus control and washed cells.

So far we showed that (a) imatinib-mediated differentiation depends on the upregulation of p27 and, (b) MYC inhibits this differentiation. As MYC impairs p27-mediated differentiation of K562[21] we determined p27 expression in KMER4 cells treated with 4HT and 0.5 μM imatinib, that is, under conditions where erythroid differentiation was inhibited by MYC. The results of the immunoblot showed that MYC activation blunted the upregulation of p27 by imatinib after 24h and 48 h of treatment (Figure 4a). To assess the erythroid differentiation we determined γ-globin expression levels which correlated with those of p27 (Figure 4a). We confirmed this effect of MYC using the KmycB cells. The induction of MYC antagonized the upregulation of p27 and γ-globin by imatinib in KmycB (Figure 4b). Although MYC decreased p27 protein levels, MYC did not significantly reduced p27 mRNA levels in the presence of imatinib (not shown). Therefore, we concluded that MYC should control p27 stability. To test this hypothesis, we first ruled out that MYC interferes with the inhibition of BCR-ABL activity by imatinib (Supplementary Figure 2a). As BCR-ABL activates SRC kinase[36] and SRC stimulates p27 degradation[37] we asked whether MYC impaired SRC inactivation by imatinib. Imatinib partially inhibited the activity of SRC, as expected (Supplementary Figure 2c) and the specific SRC inhibitor SU6656 induced erythroid differentiation at non-cytotoxic concentrations (Supplementary Figure 2b). Interestingly, MYC activation antagonized the differentiation provoked by SU6656 (Supplementary Figure 2b), in agreement with the inhibition of dasatinib-mediated differentiation shown above. However, MYC did not revert SRC inactivation, either in KmycB cells upon induction of MYC by Zn^{2+} or in KMER4 cells upon activation of MYC by 4HT (Supplementary Figures 2c and d

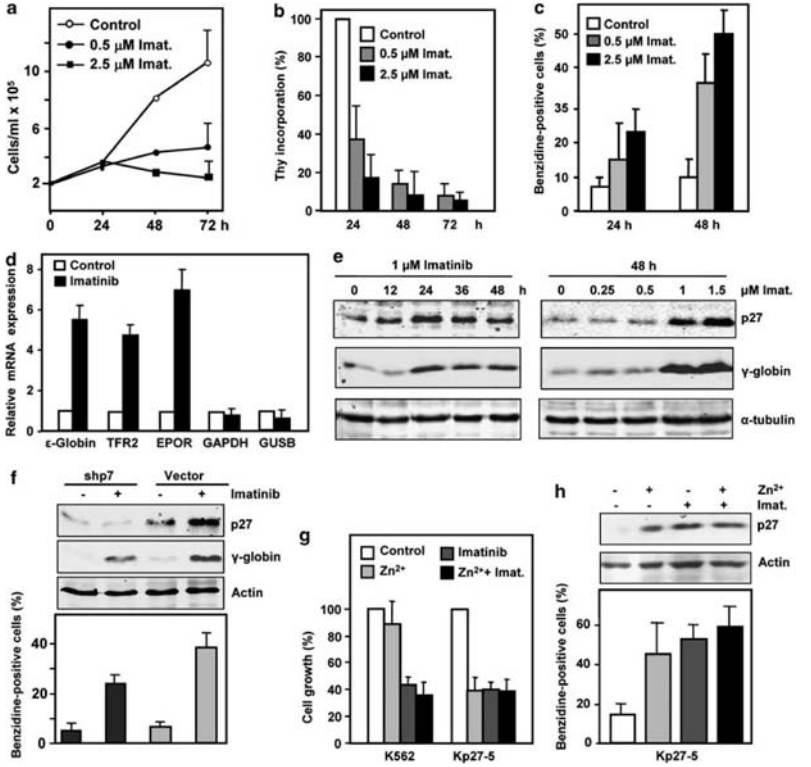

Figure 1. Erythroid differentiation induced by imatinib in K562 cells. (**a**) Inhibition of proliferation mediated by imatinib. Cells were treated with the indicated concentrations of imatinib and proliferation was determined by cell counting, using a hemocytometer. (**b**) DNA synthesis arrest mediated by imatinib. Cells were treated for 24–72 h with imatinib and DNA synthesis was determined by ^{3}H-thymidine incorporation as described.[19] Data are mean values of ^{3}H-thymidine incorporation from three experiments, relative to the thymidine incorporation in untreated cells at each time point; bars, s.e.m. (**c**) Erythroid differentiation induced by imatinib. The fraction of hemoglobinized (benzidine-positive) cells was determined after treatment with 0.5 μM and 2.5 μM imatinib for 24 and 48 h. Benzidine assays were performed essentially as described.[44] At least 300 cells were counted in each determination. Data are mean values from three independent experiments; bars, s.e.m. (**d**) Expression of ε-globin, transferring receptor 2 (TFR2) and erythropoietin receptor (EPOR) mRNA measured by reverse transcription-quantitative PCR (RT–qPCR) in K562 cells treated with 0.5 μM imatinib for 48 h. Expression of the housekeeping genes *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *GUSB* (β-glucuronidase) were also measured as controls of non-erythroid genes. Total RNA extraction was carried out with the Trizol reagent (Life Technologies, Carlsbad, CA, USA). For RT–qPCR, the first-strand complementary DNA was synthesized from 1 μg of total RNA using SuperScript II RNase reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers as described.[26] The data were normalized against the levels of ribosomal protein small 14 (RPS14) mRNA. qPCR were carried out in a MyCycler apparatus (BioRad, Hercules, CA, USA). Primer sequences are available upon request. Data are mean values from three mRNA determinations from two independent experiments; bars, s.e.m. (**e**) Induction of p27 by imatinib in K562. Cells were treated with 1 μM imatinib for 0–48 h (left panel), and for 48 h with 0–1.5 μM imatinib (right panel). The levels of p27 and γ-globin were determined by immunoblot. Tubulin expression was determined to assess protein loading. Cell lysis and protein immunoblotting were performed as described.[21] The antibodies used were: anti-α-tubulin (H-300, rabbit polyclonal), γ-globin (51-7, mouse monoclonal) and p27 (C19, rabbit polyclonal). All antibodies were from Santa Cruz Biotech (Santa Cruz, CA, USA). (**f**) Upper panel: siRNA-mediated depletion of p27 impairs the imatinib-dependent differentiation. A p27 short-hairpin vector[45] was transiently transfected by nucleofection (kit V, Amaxa, Koln, Germany) into K562 cells and 12 h later imatinib (1 μM) was added. After 48 h of imatinib treatment the cells were lysed and the protein levels of p27, γ-globin and actin (as loading control) were determined by immunoblot (upper panel). Anti-actin antibody was I-19, goat polyclonal from Santa Cruz Biotech. The transfection was repeated three times with essentially the same result. Lower panel: fraction of hemoglobinized (benzidine-positive) cells determined after 48 h treatment with 1 μM imatinib and 60 h post-transfection. Data are mean values from two independent experiments; bars, s.e.m. (**g**) Imatinib and p27 do not cooperate in the proliferation arrest of K562 cells. Kp27-5 cells were treated for 72 h with 0.5 μM imatinib or/and 75 μM $ZnSO_4$ as indicated and proliferation was determined by cell counting. (**h**) Upper panel, Kp27-5 cells were treated for 24 h with 75 μM $ZnSO_4$ or 1 μM imatinib and the levels of actin and p27 were determined by immunoblot. Lower panel, the fraction of benzidine-positive cells was also determined in the same samples and data (mean ± s.e.m.) is indicated at the bottom of each lane.

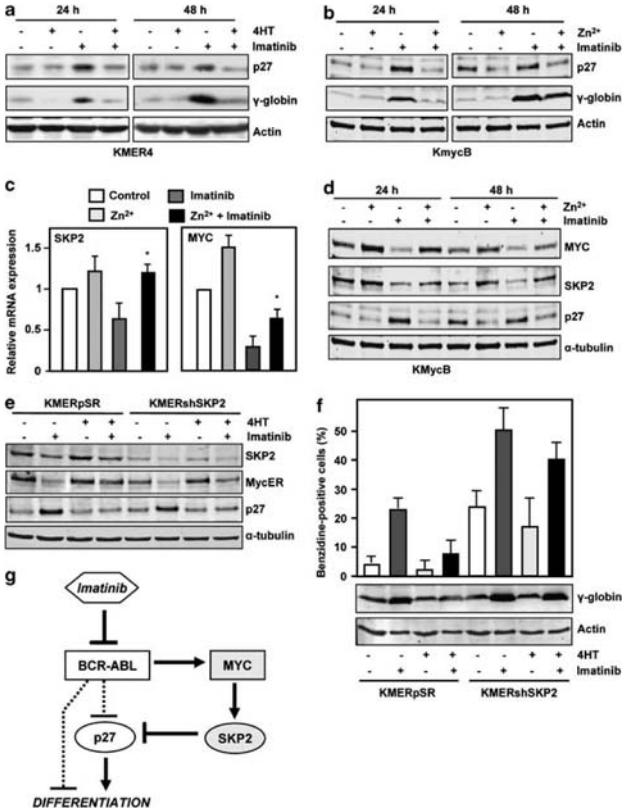

Figure 4. Myc antagonizes the upregulation of p27 induced by imatinib. (**a**) KMER4 cells were treated for 24–48 h with 0.5 μM imatinib and 4HT as indicated. The levels of p27 and γ-globin (as a marker of erythroid differentiation) were determined by immunoblot. Levels of actin were determined as a control of protein loading. (**b**) KmycB cells were treated for 24–48 h with 0.5 μM imatinib and 75 μM $ZnSO_4$ as indicated. The levels of the proteins indicated at the right were determined by immunoblot. (**c**) KmycB cells were treated for 48 h with 0.5 μM imatinib and 75 μM $ZnSO_4$ as indicated and mRNA expression of MYC and SKP2 determined by RT–qPCR. Data are mean values ± s.e.m. of three independent experiments. (**d**) MYC-mediated induction of SKP2 in the presence of imatinib correlates with decreased p27 levels. KmycB cells were treated for 24–48 h with 75 μM $ZnSO_4$ and 0.5 μM imatinib as indicated, and the levels of MYC, SKP2, p27 and α-tubulin (as protein loading control) were assayed by immunoblot. (**e**) KMERshSKP2 and the control cells (KMERpSR) were treated with 1 μM imatinib and 200 nM 4HT for 24 h as indicated, and the levels of SKP2, MycER and p27 were determined by immunoblot, using α-tubulin as protein loading control. (**f**) Enhanced erythroid differentiation in cells with silenced SKP2. KMERshSKP2 cells and control cells (KMERpSR) were treated with 1 μM imatinib and 200 nM 4HT for 72 h and the fraction of differentiated cells was determined by the benzidine assay. Data are mean values from three independent experiments; bars, s.e.m. (upper graph). The levels of γ-globin were determined by immunoblot, using actin as protein loading control (lower panel). (**g**) Model for MYC-mediated inhibition of the differentiation induced by imatinib in CML cells. The model proposes that, in the presence of imatinib, MYC can provoke the downregulation of p27 through the induction of SKP2. Other alternative pathways leading to the downregulation of p27 or the cell differentiation by BCR-ABL cannot be ruled out and are shown in dotted lines.

respectively). Therefore we ruled out SRC activity as a major mechanism for p27 degradation in response to MYC.

SKP2 is the p27-recognizing component of the SCF^{SKP2} ubiquitilation complex and the main regulator of p27 levels *in vivo*.[38,39] In addition, SKP2 is upregulated by BCR-ABL[40,41] and *SKP2* is a MYC target gene.[14] Therefore, we tested the hypothesis that, in the presence of imatinib, MYC downregulates p27 through the induction of SKP2. We first confirmed that the zinc-mediated induction of MYC in the presence of imatinib results in SKP2 mRNA increase in KmycB cells (Figure 4c). The immunoblot of Figure 4d shows that p27 protein levels are decreased upon MYC and SKP2 upregulation. To confirm the SKP2 involvement we next used KMERshSKP2 cells, which are a K562 derivative carrying the MycER construct and in which SKP2 has been partially silenced.[14] The expression of MycER and the reduced levels of SKP2 in KMERshSKP2 were confirmed by immunoblot (Figure 4e). The

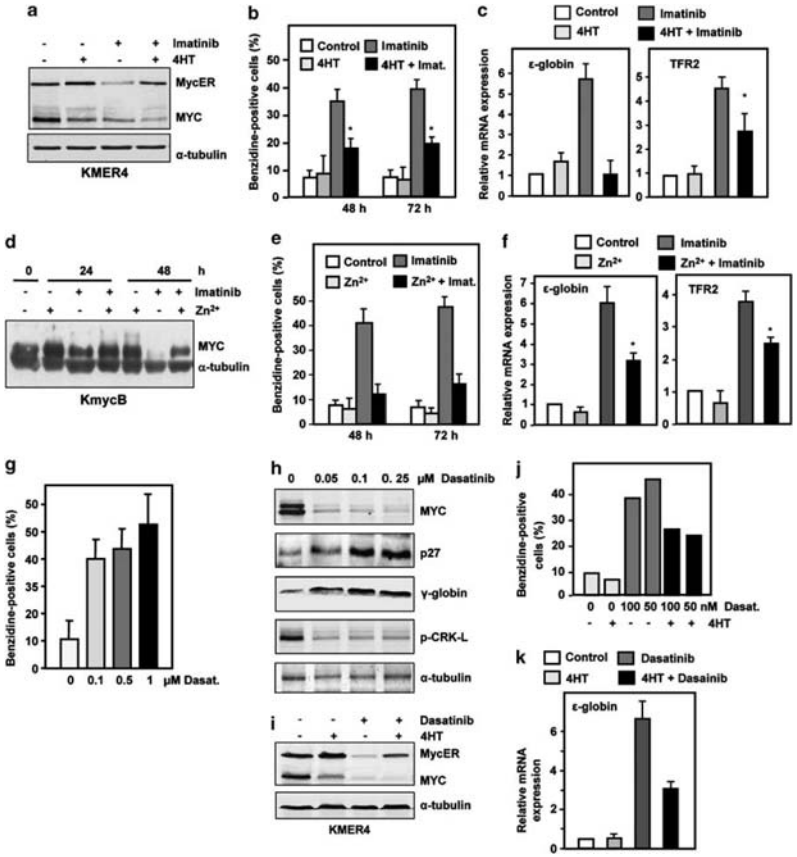

Figure 3. MYC impairs the erythroid differentiation induced by imatinib. (**a**) Immunoblot showing MYC protein levels in KMER4 cells treated for 48 h with 0.5 μM imatinib and 200 nM 4HT. The upper and lower band corresponds to the ectopic MycER and endogenous MYC protein, respectively. Alpha-tubulin levels are shown to assess the protein loading. (**b**) KMER4 cells were treated with 0.5 μM imatinib and 200 nM 4HT for 48–72 h and subjected to the benzidine test to score hemoglobin-producing cells. Data are mean values ± s.e.m. of three independent experiments. *$P < 0.05$ versus imatinib. (**c**) KMER4 cells treated for 48 h with 0.5 μM imatinib and/or 200 nM 4HT. The erythroid differentiation was assessed by ε-globin and transferring receptor 2 (TFR2) mRNA expression. Data are mean values ± s.e.m. of two independent experiments. *$P < 0.05$ versus imatinib treatment. (**d**) Immunoblot showing the MYC protein levels in KmycB cells treated with 0.5 μM imatinib and 75 μM $ZnSO_4$ (to induce MYC) for the indicated periods of time. α-tubulin levels were also determined to assess the protein loading. (**e**) KmycB were treated as in (**b**) and subjected to the benzidine test to score hemoglobin-producing cells. Data are mean values ± s.e.m. of three independent experiments. *$P < 0.05$ versus imatinib. (**f**) KmycB cells were for 48 h with 0.5 μM imatinib and 75 μM $ZnSO_4$ and the erythroid differentiation was assessed by ε-globin and TFR2 mRNA expression. Data are mean values ± s.e.m. of three independent experiments. *$P < 0.05$ versus imatinib treatment. (**g**) Erythroid differentiation induced by dasatinib (purchased from LC Laboratories, Woburn, MA, USA) in K562 cells. The fraction of benzidine-positive cells was determined after treatment with the indicated dasatinib concentrations for 48 h. Data are mean values ± s.e.m. from three independent experiments. (**h**) Immunoblot showing the downregulation of MYC and upregulation of p27 in response to dasatinib. K562 cells were treated for 48 h with the indicated concentrations of dasatinib and the levels of the proteins shown at the right were determined by immunoblot. Globin was determined to assess erythroid differentiation, p-CRK-L to assess the inhibition of BCR-ABL by dasatinib and α-tubulin to assess protein loading. (**i**) Immunoblot showing the levels of MYC protein in KMER4 cells treated for 48 h with 100 nM dasatinib and 200 nM 4HT. The upper and lower band corresponds to the ectopic MycER and endogenous MYC protein, respectively. Alpha-tubulin levels are shown to assess the protein loading. (**j**) KMER4 cells were treated with 50 or 100 nM dasatinib and 200 nM 4HT for 48 h and subjected to the benzidine test to score hemoglobin-producing cells. One representative experiment is shown. (**k**) KMER4 cells were treated for 24 h with 100 nM dasatinib and the erythroid differentiation was assessed by ε-globin mRNA expression. Data are mean values ± s.e.m. of two independent experiments.

extent of differentiation was significantly higher in shSKP2-expressing cells, as assessed by the benzidine assay and the expression of γ-globin (Figure 4f), as well as by the increased ε-globin and erythropoietin receptor mRNA (not shown). As expected, the anti-differentiation effect of MYC was also reduced in SKP2-depleted cells, as compared with control cells (Figure 4f). Therefore, it is likely that the reported induction of SKP2 expression by BCR-ABL is indeed mediated by MYC. Altogether, these results suggest that the induction of SKP2 is at least one mechanism by which MYC antagonizes the differentiation mediated by imatinib.

In summary, we have demonstrated that MYC impairs the differentiation of K562 induced by anti-CML drugs as imatinib and dasatinib. The relevance of our work is based in that (a) the loss of myeloid differentiation is a critical hallmark of CML progression into blastic phase and (b) that MYC has been involved in CML progression. The inhibition of differentiation is not due to overgrowth of MYC-expressing undifferentiated clones or to apoptosis of differentiated clones, as we previously showed that MYC induction does not stimulate cell proliferation and does not enhance the apoptosis due to imatinib ([19] and data not shown). In contrast, our data show that MYC impairs differentiation by blocking p27 upregulation induced by imatinib. We cannot rule out additional mechanisms by which BCR-ABL downregulate p27 (for example, PI3 kinase activation,[28]) or that MYC blocks differentiation through SKP2-independent mechanisms, but the results suggest that MYC opposes imatinib-dependent differentiation through the MYC-SKP2-p27 axis. The model is depicted in Figure 4g. It is reasonable to speculate that p27 upregulation is one of the mechanisms by which imatinib contributes not only to limit the proliferation of leukemic cells but also to prevent the loss of differentiation control. Interestingly, ablation of SKP2 in hematopoietic progenitors attenuates BCR-ABL-induced myeloproliferative disease[42] and erythroid differentiation in bone marrow is associated with accumulation of p27.[43] In conclusion, MYC deregulation may contribute to CML progression through different mechanisms including inhibition of cell differentiation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

SKP2 Oncogene Is a Direct MYC Target Gene and MYC Down-regulates p27KIP1 through SKP2 in Human Leukemia Cells

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Expresión e interacciones Myc-p27 en leucemia linfocítica crónica y en diferenciación de células mieloides

Resumen en castellano

INTRODUCCIÓN

Leucemia linfocítica crónica

La leucemia linfocítica o linfática crónica (LLC a partir de aquí) es la forma de leucemia más frecuente en occidente y supone casi el 25% de todas las leucemias, situándose la mediana de la edad al diagnóstico en los 70 años. Se describe como una leucemia monoclonal de células B con un fenotipo de células maduras diferenciadas con previa exposición a antígeno (expresan CD19, CD5 and CD23) y resistentes a la apoptosis. Es sobre todo una enfermedad de acumulación de células con menor tasa de muerte celular, aunque con una grado de proliferación mayor que el inicialmente reconocido, lo que motiva que la característica principal de la LLC sea la linfocitosis absoluta en sangre periférica. Su tratamiento se basa en quimioterápicos (clorambucilo, ciclofosfamida, doxorrubicina y, sobre todo, fludarabina) y medidas de sostén (transfusiones de hemoderivados, gammaglobulina, tratamientos de las complicaciones autoinmunes y tratamiento antibiótico de las infecciones, etc.) Sin embargo, aunque se trata de una enfermedad indolente con supervivencias medias de diez años, tiene un curso clínico variable: mientras algunos pacientes muestran una enfermedad progresiva totalmente refractaria al tratamiento y con supervivencia más cortas, otros muestran una enfermedad que progresa muy lentamente y no precisaría tratamiento. Los análisis de “gene profiling” con microarrays han identificado que ambos tipos de LLCs difieren en muchos genes, lo que sugiere que puede haber otros marcadores pronósticos por identificar.

La interacción p27 / Myc

p27, proteína producto del gen *CDKN1B* (Cyclin-Dependent Kinase Inhibitor 1B), se une a complejos ciclina E-Cdk2 inhibiendo su actividad y deteniendo el ciclo celular en fase G1. Aunque su papel como inhibidor de Cdks está bien establecido, en los últimos años se han descubiertos nuevas funciones de p27 en diferenciación, motilidad celular, etc. En numerosos tumores se ha demostrado una correlación entre niveles bajos de p27 y mal pronóstico, así como una correlación entre p27 citoplásmica (que no inhibe Cdks) y progresión tumoral. p27 puede ser fosforilada en varias Ser y Thr que pueden ser responsables de su estabilidad y su localización subcelular.

El oncogén Myc (también llamado c-Myc) codifica para un factor de transcripción que se ha encontrado sobre-expresado en muchos tumores humanos. La proteína Myc cuenta con tres dominios bien definidos: un dominio N-terminal de transactivación, uno central y otro C-terminal que incluye una región básica por la que se une a DNA y un dominio hélice-lazo-hélice-cremallera de leucinas (HLH-LZ). La forma activa de Myc es formando un heterodímero a través del HLH-LZ con la proteína MAX, el cual se une a la región reguladora de genes diana (llamada caja E). Se han identificado más de 1000 genes diana de Myc. De ellos

aproximadamente el 60% son inducidos, y el resto reprimidos. Los genes inducidos por Myc se pueden agrupar en aquellos relacionados con la proliferación celular, síntesis de proteínas y ribosomas, el metabolismo glucídico, la comunicación intercelular y otros de función más heterogénea. La sobre-expresión de Myc se correlaciona con la progresión tumoral y peor pronóstico en linfomas

Myc antagoniza las funciones de p27 en el ciclo celular: a) Myc induce la expresión de ciclina D2, que a su vez secuestra e inactiva a p27 b) en algunos tipos celulares Myc reprime la transcripción de p27 en células linfoides c) Myc induce la degradación de p27. Varios genes diana de Myc, tales como Cul1 o SKP2, median esta degradación d) La sobre-expresión de Myc y la ausencia de uno o ambos alelos de p27 cooperan en tumorogenesis en modelos de animales transgénicos.

De esta forma la regulación de p27 llevada a cabo por Myc está muy bien descrita, pero sin embargo la regulación inversa, es decir, el control de los niveles de Myc por parte de p27 está mucho menos estudiado.

OBJETIVOS

La correlación entre altos niveles de Myc y bajos de p27 han sido encontrado en muchos tumores, y se ha asociado frecuentemente con mal pronóstico. Sin embargo, en LLC es un tumor atípico en cuanto a la expresión de estas proteinas se refiere. Así, es todavía desconocido si existe una correlación de la expresión de p27 y de Myc y si p27 puede regular la expresión de Myc en esta enfermedad, así como si esta regulación tiene algun valor pronóstico. Así, LLC es un modelo interesante en el que estudiar la interacción entre estas dos proteínas.

Por otro lado, el antagonismo funcional entre p27 y Myc en el proceso de diferenciación es todavía poco conocido. Nuestro laboratorio ha generado un modelo de diferenciación eritroide a través de la indución transitoria de p27. Sin embargo, cual es el mecanismos a través del cual p27 induce la diferenciación es todavía desconocido, y la interacción entre p27 y Myc en este proceso está poco estudiado.

Dado los antecedentes mencionados, los objetivos de este estudio son:

- Estudiar la expresión de Myc y p27 (RNAm y proteina) en LLC, su localización celular y si el patrón de expresión observado tiene algún valor pronóstico.

- Examinar la regulación del ciclo celular llevada a cabo por p27 y Myc en LLC y su papel en la alta resistencia a la apoptosis típica de esta enfermedad, así como si existe alguna relación con la expresión de la proteina anti-apoptótica Bcl-2.

- Estudiar los mecanismos a través de los cuales p27 regula la diferenciación eritroide en K562. Determinar el dominio de p27 involucrado en dicho proceso y el papel de la bajada de expresión de Myc llevada a cabo por p27 durante la diferenciación

RESULTADOS

1. Correlación de expresión entre el oncogén c-Myc y el inhibidor de ciclo celular p27kip1 en leucemia linfocítica crónica.

Trabajos previos del laboratorio de Javier León (Acosta et al, MCB 2008) demostraron un antagonismo funcional entre Myc y p27 en diferentes modelos celulares tales como K562. Con la idea de si este antagonismo podía ser extrapolado a modelos clínicos se buscó un modelo en el cual se pudiera observar dicha interacción con facilidad. Ciertamente, la correlación entre alta expresión de Myc y baja de p27 había sido observada en una gran número de modelos, pero no había ningún estudio que describiera esta relación en el sentido opuesto, es decir, un modelo tumoral donde se pudiera estudiar in vivo si bajos niveles de Myc vienen acompañados por una alta expresión de p27, y si esto tenía alguna implicación en la progresión de la enfermedad. De todos los tumores, existía un caso particular donde se observaba el patrón de expresión que se buscaba. La leucemia linfocítica crónica.

Para desarrollar este estudio Juan Carlos Acosta y Miguel Ángel Cortes comenzaron el estudio de una limitada cohorte de pacientes en nuestro laboratorio. Se obtuvo el mRNA a partir de muestras de linfocitos de sangre periférica y se analizaron una serie de genes, entre ellos Myc y p27. Como controles de expresión, se utilizaron linfocitos de sangre periférica y extractos de mRNA de amígdalas procedentes de amigdalectomías (las amígdalas inflamadas presentan más de un 80 % de infiltración de linfocitos B activados y por lo tanto suponen un buen control de la expresión de genes en linfocitos B) Las conclusiones de este estudio preliminar fue que Myc se encuentra fuertemente inhibido en estos pacientes, al contrario que p27 cuya expresión es bastante superior a las observadas en los controles.

Mi posterior estudio fue encaminado a confirmar dicha relación mediante la ampliación de las muestras observadas, demostrar que dicha regulación también se establecía a nivel de proteína y establecer una relación entre dichos resultados y la progresión de la enfermedad.

Para ello, en un primer paso aumenté la cohorte de pacientes para confirmar dicha correlación inversa. A su vez, estudiamos trabajos de expresión masivos llevados a cabo por grupos internacionales y diseñados expresamente para cohortes de pacientes con LLC usando la base de datos de Oncomine (www.oncomine.com). En estos últimos observamos una cierta controversia, donde ciertos trabajos confirmaban nuestros resultados mientras que otros pocos no observaban diferencias significativas. Esto indicaba que los cambios a niveles de mRNA dependían de la precisión con la que se desarrollara el estudio.

Teniendo en cuenta que además tanto la expresión de Myc como la de p27 presenta una fuerte regulación postraduccional, optamos por aumentar el rango del estudio y obtener la expresión a nivel de proteína.

Se analizaron más de cien muestras de LLC (procedentes de varios hospitales, en Santander, Laredo, Barcelona y Las Palmas) mediante immunoblot, utilizando como controles tanto linfocitos de sangre periférica como amígdalas. Este nuevo estudio nos proporcionó nuevos datos. Ciertamente confirmó los datos obtenidos a nivel de mRNA, aunque también demostró que la expresión de mRNA y de proteína no siempre es directamente proporcional en estos pacientes, reflejando una regulación postraduccional que debe de ser tenida en cuenta en posteriores estudios. Esta regulación postraduccional se vio reflejada en una pequeña cohorte de pacientes donde se observó una alta expresión de Myc, los cuales normalmente eran correlacionados con una baja expresión de p27. Una vez más estos nuevos resultados sugerían la existencia de una correlación inversa entre los niveles de expresión Myc y de p27.

Para determinar la naturaleza de dicha regulación postraduccional decidimos estudiar la expresión del oncogén Skp2. Skp2 es una proteína F box que funciona como el componente receptor del complejo ubitiquitín ligasa SCF. Trabajos desarrollados por el grupo de Javier León han demostrado que Myc induce la expresión de Skp2, y es conocido que una de las proteínas dianas de Skp2 es p27 (Bretones G et al, JBC, 2011). Puesto que teníamos una serie de pacientes con alta expresión de Myc y baja de p27, hicimos un immunoblot para determinar la expresión de Skp2. Los resultados mostraron una perfecta correlación entre la expresión de Myc y la expresión de Skp2, indicando que con toda probabilidad los bajos niveles de p27 observados en esta pequeña cohorte se deben a la presencia de Myc.

Por otro lado nos quedaba demostrar si la alta expresión de p27 podía regular de alguna forma la expresión de Myc. Para abordar tal cuestión decidimos utilizar el modelo celular MEC1, obtenido a partir de un paciente de LLC. Estas células presentan basalmente una alta expresión de Myc y baja de p27. Para ver el efecto sobre Myc de p27 en estas células, transfectamos un vector que sobre-expresaba p27 y vimos que cuando aumentábamos la expresión de p27, las células se paraban en las fases G_0/G_1 del ciclo y, seguramente como consecuencia de esta parada, disminuían drásticamente los niveles de Myc. Este resultado era importante pues se ha demostrado repetidamente que el ciclo celular está desregulado en las LLC.

Otra vía de regulación de la actividad de Myc es la expresión de otros miembros de la familia bHLH-LZ, entre los cuales se encuentran Max o un miembro de la familia Mxd llamado Mxi1. Max es una proteína con la que Myc forma un heterodímero, el cual es necesario para que Myc pueda unirse a las secuencias específicas que regula. Sin embargo, Mxi1 también

reconoce a Max y se une a las mismas secuencias, regulando también las mismas secuencias de Myc. Por lo tanto, Mxi1 compite directamente con Myc por la unión de Max, lo cual puede ser entendido como una regulación competitiva, de forma que si Myc se une a Max, Mxi1 queda inactivo y viceversa. El estudio de la expresión de Mxi1 y de Max mostró que ambas se encuentras sobre-expresadas en nuestros pacientes de LLC, lo que indica que Myc puede ser reprimido funcionalmente por este factor de transcripción. Además, Mxi1 inhibe la expresión de miembros del complejo ubiquitin ligasa SCF, provocando la regulación positiva de p27 y como consecuencia la posterior desregulación de Myc.

Una de las características más definitorias de la LLC es que las células leucémicas se encuentran detenidas en la fase G_0/G_1 del ciclo, probablemente debido a la alta expresión de p27 descrita en estos pacientes. Además, analizamos por immunoblot la expresión de las ciclinas de fase S (ciclina A y ciclina E) necesarias para el paso de la fase G_1 a S y de la S a G_2 del ciclo. Este estudio mostró que mientras que en los pacientes con baja expresión de Myc dichas ciclinas no se expresaban, en las muestras con alta expresión de Myc estas ciclinas fueron detectadas por immunoblot, estableciendo una clara y significativa relación entre su expresión y la de Myc. Esto indicaba que las muestras con alta expresión de Myc presentaban una mayor tendencia a ciclar, siempre y cuando los niveles de p27 hubieran sido inhibidos, puesto que en aquellos pacientes doble positivos se observó por cromatografía de exclusión molecular e inmunoprecipitación que los complejos Cdk2-ciclinas de fase S se encuentran inhibidos por la actividad de p27.

Una vez bien establecida la correlación, recopilamos los datos clínicos de los pacientes y desarrollamos un estudio estadístico para establecer alguna conexión con nuestros resultados que pudieran ser útiles en clínica. Se encontró una cierta tendencia correlacionando la expresión de p27 y los niveles de Zap70 (p=0.03), CD38 (p=0.03) o la deleción de p53 (p=0.08), indicando que dicha expresión tiene una importante relevancia en la progresión de la enfermedad. Por el contrario, los niveles de Myc sólo pudo ser correlacionado con la del13q14 (0,03). También llegamos a estudiar la expresión de Myc en pacientes con el gen *NOTCH1* mutado, el cual ha sido visto en los últimos años como una de las más frecuentes e importantes alteraciones en esta enfermedad. Aunque está bien documentado que Myc forma parte de la vía de activación de Notch1, no observamos su expresión en todos los casos, lo que indicaba que Myc no era el principal activador de Notch1 en LLC.

Por otro lado si pudimos observar que la relación Myc y p27 podía tener una cierta relevancia en la prognosis de la enfermedad. Si estudiábamos el efecto de la expresión de p27 en la progresión de la enfermedad, esta se veía afectada por la presencia o no de Myc, de forma que aquellos pacientes sin la expresión de Myc presentaban una peor progresión. Esto indicaba que de alguna forma Myc estaba afectando a la capacidad de mal pronóstico de p27.

Al mismo tiempo, tanto el fraccionamiento cromatográfico por exclusión molecular como las inmunoprecipitaciones dejaron entrever que gran parte de la expresión de p27 estaba libre, sin formar complejos, lo que sugería la posibilidad de que pudiera estar realizando otra función independiente de su actividad antiproliferativa. Puesto que p27 fue correlacionado con factores de mal pronóstico, y que la resistencia a la apoptosis es reconocida en las células de LLC, y que en la bibliografía se había descrito una función antiapoptótica de p27, decidimos estudiar si p27 podía de alguna forma proteger a las células de LLC de la apoptosis. Para ello, tratamos a las MEC1 que sobre-expresaban p27 con fludarabina, el quimioterápico más usado hoy día en el tratamiento de la LLC. Los resultados indicaron una reducción de la apoptosis superior al cincuenta por ciento en las MEC1 que sobre-expresaban p27.

Sorprendentemente, el efecto de Myc fue el contrario. Empleamos la línea P493.6, una línea de linfocitos B humanos con expresión condicional de Myc (que puede ser inhibido por la adición de tetraciclina), Tratamos estas células con fludarabina y tetraciclina y pudimos observar que cuando estas células no expresaban Myc obteníamos menos apoptosis.

Estos resultados parecían indicar que la inhibición de ciclo llevada a cabo por p27 protege a las células de LLC de los quimioterápicos que atacan directamente al ciclo celular, mientras que un activador del ciclo como Myc haría más susceptible a estas células de sufrir apoptosis. Esta idea del papel de p27 como un agente anti-apoptótico fue fortalecido al estudiar la expresión de otra proteína anti-apoptótica que había sido implicada en LLC: Bcl-2. El estudio de la expresión de esta proteína en nuestra cohorte mostró una fuerte correlación entre p27 y Bcl-2, lo cual parece indicar que en la mayoría de los pacientes ambas proteínas trabajan juntas en la principal de las características de estas células leucémicas, la alta resistencia a la apoptosis.

2. p27 motiva la diferenciación eritroide en K562 a través de la disminución de los niveles de Myc.

En los últimos años se ha descrito una serie de funciones para p27 a parte de su función clásica como inhibidor de ciclo, entre los cuales se encuentran regulación de la apoptosis, migración celular o diferenciación. El grupo de Javier León ha colaborado en tales progresos demostrando la importancia de p27 como inductor de diferenciación eritroide. Sin embargo, uno de los puntos que queríamos estudiar era si dicha función es dependiente o no de su actividad como inhibidor de ciclo celular. Primero estudiamos la diferenciación con un mutante llamado p27-Nt, el cual carece de región terminal pero que todavía es funcional como inhibidor de ciclo. Estudios de diferenciación en la línea K562 mediante la transfección de este vector mostraron que la región N-terminal de p27 era suficiente para inducir diferenciación.

Esto indicaba que la diferenciación parecía estar asociada a la función de inhibidor de ciclo celular de p27.

Para confirmarlo trabajamos con el mutante de p27 llamado p27CK- (un mutante de p27 incapaz de unirse a los complejos ciclinas-Cdks y por lo tanto incapaz de inhibir el ciclo). Procedimos a subclonar dicho mutante en un vector con el promotor de la metalotionina que permite la regulación del gen deseado mediante la adición de Zn^{2+} (pMT-CB6). Una vez obtenido el nuevo vector, procedimos a obtener líneas estables de dicho vector en K562. Los resultados fueron ambiguos según la línea que fuera analizada, puesto que solo en algunas de ellas se observaba inducción de diferenciación. Con la idea de intentar esclarecer cual era el verdadero efecto del mutante p27CK- realizamos un estudio masivo mediante RNA sequencing en el que determinamos que efectos tenía la sobre-expresión de este mutante con respecto al control p27wt en el transcriptoma de la célula. Sorprendentemente los resultados mostraron que mientras que p27wt regulaba más de un centenar de genes (la mayoría genes que intervienen en el ciclo celular), p27CK- no regulo ningún gen de forma significativa, lo que parecía indicar que la mayor parte de las funciones de p27wt se deben a su función como inhibidor del ciclo celular. Aunque es difícil entender y explicar porque observábamos inducción de diferenciación en algunas de las líneas de p27CK-, todo lo atribuimos a la expresión de otro factor de gran importancia en la diferenciación eritroide, Myc.

Ya que Myc tiene una importancia fundamental en la regulación de la diferenciación eritroide (demostrada por Juan Carlos Acosta en el laboratorio de Javier León) analizamos la expresión de Myc en las líneas obtenidas con el p27CK- inducible y curiosamente observamos que en aquellas cuya expresión de p27CK- inducía diferenciación se correlacionaba con una disminución de los niveles de Myc. En las otras líneas donde no se observaba bajada de Myc la diferenciación no se llevaba a cabo. Pese a que dichos datos confirmaban los datos obtenidos por Juan Carlos Acosta en los que Myc inhibía la diferenciación, lo realmente interesante era que la bajada de expresión de Myc funcionaba como un gatillo que desembocaba en la diferenciación. Si esto fuera cierto, uno de los puntos de control más importantes que regula p27 en los procesos de diferenciación sería la disminución de Myc, que funcionaria como una especie de freno para que la diferenciación se dispare.

Por ello, hemos demostrado que la expresión de p27wt induce una radical bajada de expresión de Myc tanto a nivel de RNAm como de proteína, así como que el promotor luciferasa de Myc se ve reprimido ante la presencia de p27. En un intento de determinar si dicha regulación sobre el promotor era directa o no, mapeamos 2000 pb del promotor en un experimento de ChIP pero no obtuvimos resultados positivos, por lo que es probable que la bajada de Myc se deba a un efecto colateral de la inhibición de ciclo celular en G_0-G_1 desencadenada por p27. Finalmente, silenciamos la expresión de Myc en K562 mediante un vector shMyc y observamos que dicha inhibición es suficiente para que se dispare la expresión

de genes de gran importancia en la diferenciación eritroide, tales como NF-E2, Gata1 o EpoR, así como marcadores de diferenciación como la gamma globina. También observamos aumento de la expresión de Mad1, un reconocido antagonista en las funciones activadoras de Myc y cuya expresión se ha observado estar aumentada en K562 diferenciadas.

Viendo la importancia de Myc a la hora de frenar la diferenciación, nos preguntamos si la diferenciación inducida por p27 era reversible, es decir, si la expresión de Myc era capaz de revertir los procesos de diferenciación llevados a cabo por la expresión de p27. Para ello utilizamos la línea celular Kp27MER donde podemos inducir p27 añadiendo Zn^{2+} gracias a un promotor pMT, y activar la actividad de la proteína de Myc añadiendo Tamoxifeno al medio. Utilizando este sistema, activamos Myc a diferentes tiempos tras la inducción de diferenciación por p27 y observamos que a tiempos largos Myc era incapaz de revocar la diferenciación.

Estos resultados sugieren un rol de Myc como “interruptor” de la diferenciación. De esta forma, cuando Myc es expresado el interruptor esta en posición de “apagado”, debido principalmente a la fuerte inhibición llevado a cabo por Myc de los principales genes reguladores de la diferenciación como son Gata1 o NF-E2. Por el contrario, cuando algún agente como p27 provoca la inhibición de Myc, el interruptor pasaría a estar en posición de “encendido”, lo cual resultaría en la activación de Gata1, NF-E2 y resto de genes reguladores de la diferenciación, y como resultado la diferenciación se activaría.

CONCLUSIONES

1. Un estudio de la expresión de p27 y Myc a nivel de proteína fue realizado en 118 muestras de LLC complementado con otro estudio a nivel de RNAm. p27 fue expresado en la mayoría de las muestras de LLC analizadas (82%), y su localización fue nuclear. Por el contrario, la expresión de Myc fue baja. Este resultado esta en concordancia con el antagonismo funcional entre Myc y p27, pero de una forma inversa a lo observado en otros tumores.

2. Ninguna diferencia fue observada en la progresión de la enfermedad cuando los pacientes fueron clasificados según su expresión de p27 o de Myc. Sin embargo, la fracción de pacientes que expresando p27 también expresaron Myc mostraron una progresión más lenta de la enfermedad.

3. Los niveles de los miembros de la familia bHLH-LZ Max y Mxi1 fueron significativamente más altos en las muestras de LLC que en los controles.

4. En pacientes donde p27 y Myc fueron expresado a la par, p27 no fue fosforilado en la Thr-187 indicando que los complejos Cdk2/ciclinas fase S son inactivos.

5. En contra de lo observado en sangre periférica, Myc es expresado en ganglio linfático y medula ósea en unas pocas muestras obtenidas de pacientes con LLC, indicando la importancia del microambiente tumoral en la expresión de Myc. Sin embargo, la expresión de p27 fue también alta en estas muestras.

6. La expresión de Myc resulta en la inducción de Skp2 y ciclinas de fase S, pero no fue asociada con la activación de Notch1 en nuestras muestras de LLC.

7. p27 media la resistencia a la apoptosis inducida por fludarabina en las células leucémicas de LLC, y esto es asociado con la alta expresión de Bcl-2. Por el contrario, Myc aumenta la apoptosis mediada por fludarabina.

8. La diferenciación eritroide inducida por p27 de la línea celular K562 depende de su región CDI. p27 inhibe a Myc antes de la inducción de la diferenciación.

9. La bajada de los niveles de Myc es suficiente y necesario para la diferenciación eritroide inducida por p27.