CD21/CR2 as a possible Myc target gene in hematopoietic cell lines.

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Index

Abstract

c-Myc is a transcription factor which belongs to the family of basic-helix/loop/helix-leucine zipper proteins. It is found deregulated in more than 50% of human tumors, including Burkitt lymphoma, a human B-cell tumor, where Myc is chromosomally translocated. There is an African epidemic version of Burkitt lymphoma associated with the infection of Epstein-Barr virus (EBV). CD21/CR2 is the co-receptor of B-cell receptor (BCR) that serves as receptor for this virus. We explored whether CR2 could be a potential Myc target gene, since previous results in our laboratory showed that CR2 and Myc were correlated. We have used several hematopoietic cell lines (Raji and K562) in which we have measured CR2 mRNA levels by qPCR upon different conditions of Myc expression. Our results indicate that Myc overexpression in K562 cells results in CR2 overexpression and this effect is reproduced in the absence of protein synthesis. Moreover, in Burkitt cells, inhibition of Myc through the drug JQ1 (which impairs Myc transcription) resulted in CR2 mRNA decrease. The bioinformatic analysis of the ChIP-seq data of the ENCODE project revealed that Myc is bound to the CR2 promoter. Altogether the data strongly suggest that CR2 is a novel Myc target gene and this may explain the association of Burkitt lymphoma in EBV infection.

Introduction

Myc gene belongs to a family that includes MYCL (L-Myc) and MYCN (N-Myc). While L-Myc is less understood, N-Myc expression is tissue-restricted and it could be substitute for c-Myc in murine development (Dang, 2012). The proto-oncogene MYC was the first oncogenic transcription factor discovered in the late seventies (Duesberg and Vogt, 1979; Hu*, et al.*, 1979; Sheiness and Bishop, 1979). The studies on fulminant chicken tumors caused by oncogenic retroviruses, leaded to the identification of the v-Myc oncogene that causes **my**elo**c**ytomatosis (leukemia and sarcoma) (Dang, 1999). The c-Myc gene (Myc hereafter) was discovered as the cellular homolog of the retroviral v-myc oncogene (Sheiness et al., 1979). Until then, it was found highly amplified in many different human cancers (Beroukhim et al., 2010) and also frequently translocated like in Burkitt lymphoma (Dalla-Favera et al., 1982).

Myc protein is included in the family of basic-helix/loop/helix-leucine zipper proteins. The basic domain is rich in basic amino acids and directly contacts specific DNA sequences within the DNA major groove. The HLH/LZ domain allows Myc to dimerize with its binding partner Max. Myc-Max heterodimer binds to DNA binding motifs (Figure 1), called E-boxes (canonical 5'-CACGTG-3' or non-canonical) (Eilers, et al., 2008).

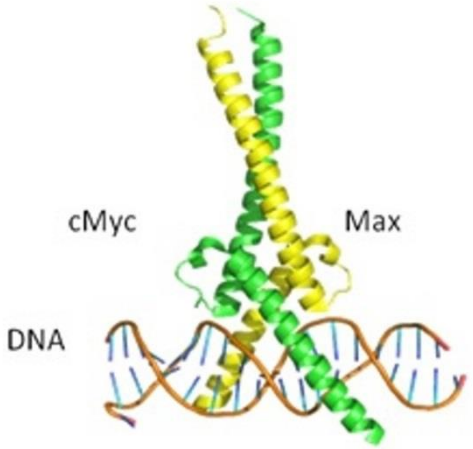

Figure 1. Myc-Max dimer recognizing DNA (Paris et al. 2009. J. Med. Chem. 52 (5), 1247-50).

The canonical Myc E-box is the most frequently occurring DNA binding motifs in the human genome. However, there are different transcription factors that could bind in these sites. In non-proliferating cells, these transcriptions factors regulate basal metabolism to maintain cellular structural and functional integrity. But when cells are stimulated to proliferate, the available amount of Myc increase and could occupy these binding motifs, activating proliferation programs (biomass accumulation and enhanced cellular bioenergetics). It is reasonable to hypothesize that constitutive Myc expression could cause Myc to promiscuously activate E-box driven genes that would be regulated by other E-box transcription factors in normal non-proliferative cells (Dang, 2012).

Myc protein activity is involved in many functional cellular pathways such as cell cycle regulation, metabolism or apoptosis. It is not surprising that deregulation of Myc expression affects cellular homeostasis at different levels. It is described that overexpression of Myc promote cellular proliferation independently on extracellular signals which contributes to deregulated DNA synthesis and genomic instability (Kuzyc and Mai, 2014). This genomic instability includes gene amplifications, an increase in the rate development of aneuploidy or affects the cellular pathways responsible of eliminate reactive oxygen species to avoid DNA damage (Egler, et al., 2005).

Therefore, Myc is in the crossroads of many different and important biological pathways that in physiological conditions are fine-tuned regulated but it aberrant expression affects many processes. Myc is documented to be involved broadly in many cancers, in which its expression is estimated to be elevated or deregulated in up to 70% of human cancers. In murine models is demonstrated that deregulation of Myc expression is sufficient to drive tumorigenesis, although *in vivo*, Myc requires another gene alterations for tumor formation (Eilers et al., 2008)). In mammary carcinoma with Myc expression deregulated, mutations in k-ras make the tumor more aggressive. Acute overexpression of Myc in normal cells triggers the ARF or p53 pro-apoptotic pathway, although Myc-induced transgenic lymphomas lack functional ARF of p53 (Dang, 2012).

On the other hand, CD21 or CR2 (complement receptor type 2) is the cellular receptor for the C3dg fragment of the complement system and is part of the co-receptor of B-cell receptor (Roberts et al., 1996). It is also the membrane glycoprotein that recognizes the Epstein-Barr virus (EBV) a human herpesvirus with oncogenic potential. C3dg fragment and the major EBV glycoprotein gp350/220 are epitopes and both binds to the two amino-terminal short consensus repeats (SCRs) of CR2. After binding, endocytosis of the virus followed by movements of the nucleocapsid to the nucleus occurs in few minutes (Nemerow et al., 1989).

CR2 regulation is under control of different transcription factors. In other study, using bioinformatic analyses revealed that CR2 proximal promoter includes transcription factor binding sites which may regulate chromatin structure and CR2 gene expression (Figure 2). They scan the variations of CR2 proximal promoter accessibility to different transcription factor, among different B cell lines, in order to determine if the selective accessibility to the promoter correlates with B cell differentiation (Cruickshank et al., 2009). So, CR2, as other surface membrane proteins, is used to classified different maturation stages of B cells.

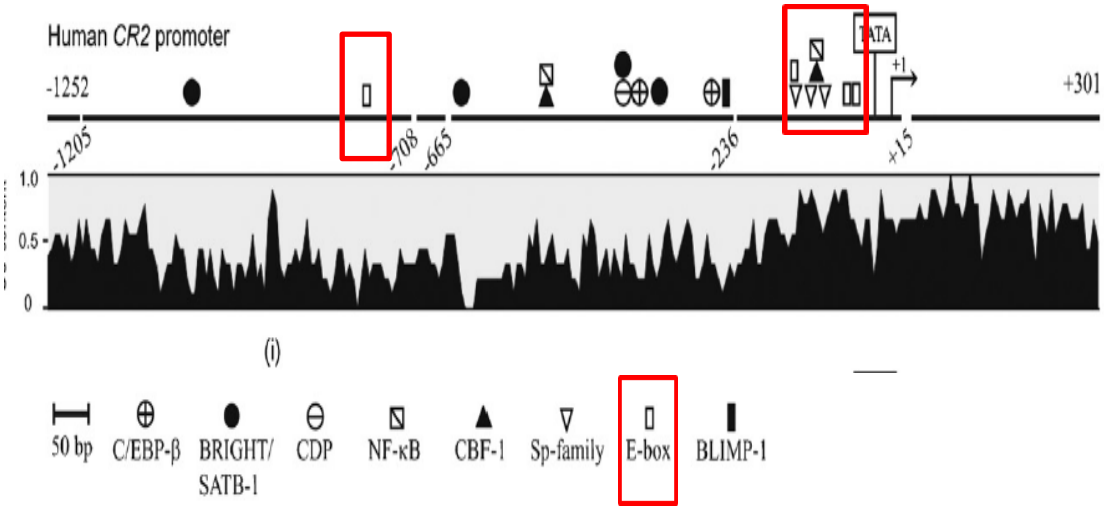

Figure 2. Scheme representation of CR2 human promoter. The symbols represent different transcription factors binding sites along CR2 promoter. The graph below represents the GC content. The squares in red highlight Myc binding sites (Roberts et al., 1996).

The physiological function of CR2 is almost unknown, but there are some intracellular signals initiated after binding with B-cell receptor (BCR) and its related proteins. EBV interaction with normal B cells has also been reported to rapidly activate phospholipase C and protein kinase C (PKC). CD23, another CR2 ligand, induced immunoglobulin E (IgE) production in B-cells and recues germinal center B cells from apoptosis together with interleukin-1 (IL-1α) (Roberts et al., 1996). In B-cell membrane CR2 is occasionally associated with other membrane proteins as CD19 (Figure 3), although its function in intracellular signaling pathways due to CR2 activation has not been completely elucidated (Roberts, et al., 1996).

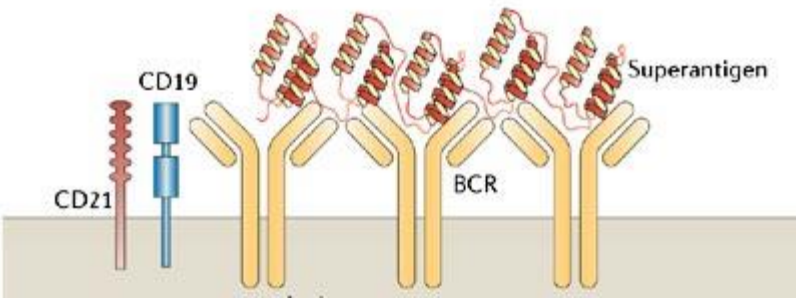

Figure 3. Scheme representation of CD21 and CD19 and their relation with B-cell receptor (Silverman et al., 2006. *Nature Reviews Immunology* **6**, 465-475).

CD19/CD21 complex is an important co-receptor that plays a critical role in B cell responses to T-cell dependent antigens (Ags). This complex functions synergistically with the BCR to reduce threshold for B cell activation. Certain studies relate CD19 with tyrosine kinase Lyn, and recruits Vav, resulting in the activation of mitogen-activated protein kinases and phosphatidylinositol 3-kinase (PI3K) (Figure 4) (Cherukuri et al., 2001). The importance of CD19/CD21 complex as B cell co-receptor *in vivo* has been demonstrated by the reduced formation of germinal centers and reduced primary antibodies responses to T cell-dependent Ags in mice that lack CD19 or CD21 (Cherukuri et al., 2001).

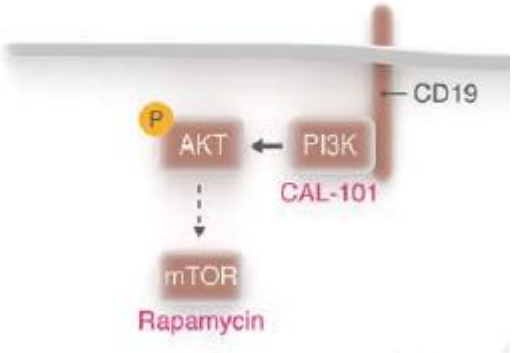

Figura 4. Principal signalling pathway activated downstream CD19. CD19-mediated PI3K-mTOR activity (Vaqué et al., 2014. Haematologica. 99 (2)).

EBV is a ubiquitous B-lymphotropic herpesvirus that after a usually asymptomatic infection is carried by more than 90% of adults for the rest of their lives (Shlee, et al., 2004). As noted above, this virus has an oncogenic potential and is closely associated with several malignant tumors, like Burkitt's lymphoma, Hodgkin's disease, certain forms of T-cell lymphomas, and some lymphoepithelial tumors, as nasopharyngeal carcinoma and a proportion of gastric cancers (Figure 5). Indeed, infection of primary B-cells with EBV *in vitro* activate B-cells and begin to proliferate, in an immortalization process which were used to establish cell lines, called lymphoblastoid cell lines (LCLs) (Shlee et al., 2004).

EBV infects resting B-cells while other viruses infect activated cells. One study demonstrated that the intracellular signaling pathway trigger after binding of EBV with CR2 leading the expression of certain genes as CD23, as well as the endogenous viral genes, and enables EBV to infect resting B-cells (Roberts et al., 1996).

Moreover, EBV depend on cellular machinery for the expression of viral latent genes (EBNA1, EBNA2 or LMP1 are the most important and first endogenous EBV latent genes expressed after infection). EBNA2 targets cellular genes such as Myc, CD21 and CD23. This EBV-induced growth program is also inititated during primary infection with EBV *in vivo*. In individuals with an intact immune system, proliferation of these highly immunogenic cells is controlled by cytotoxic T cells specific for EBV antigens. However, it represents a life-threatening danger for immunosuppressed individuals, like AIDS patients or recent transplant patients (Shlee et al., 2004).

In healthy individuals, EBV escapes the immune system by persisting in resting memory B cells. For establish a stable viral latency, B-cells require an active cell proliferation programs. Overexpression of Myc to a highest level is sufficient to support B-cell proliferation independently of endogenous viral latency genes as EBNA2 (Shlee et al., 2004).

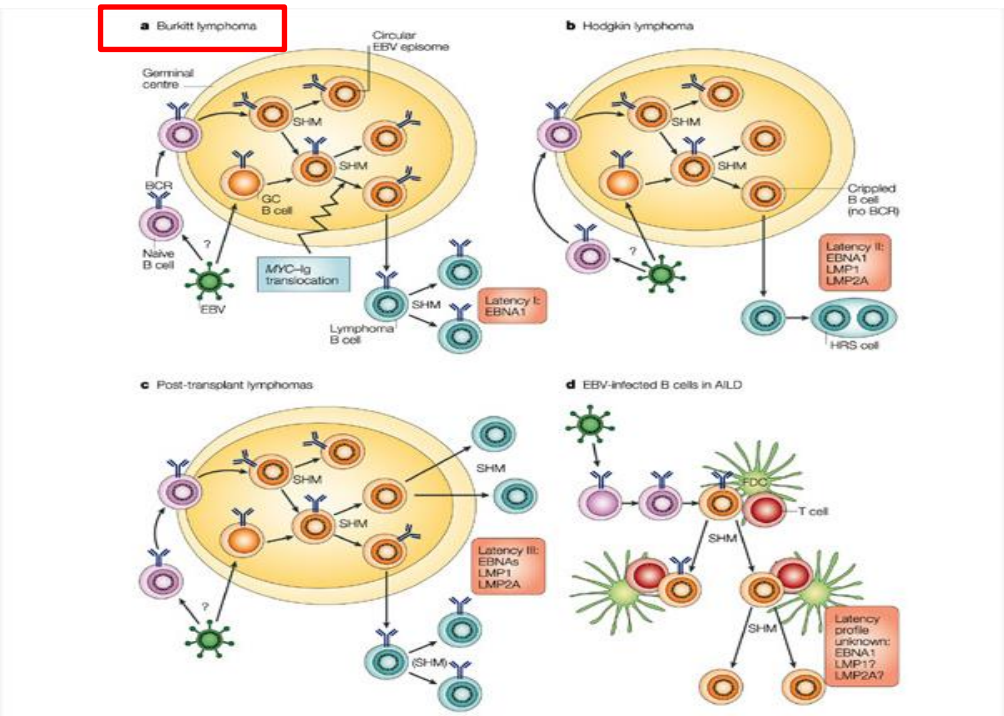

Figure 5. Scheme representation of different mechanims of EBV infection in different B-cell lines. The red square highlight the mechanism proposes for Burkitt's lymphoma cells (Kuppers, 2003. Nature rewievs immunology 3, 801-812).

Studies from several laboratories using different approaches have demonstrated that infection of EBV of resting B-cells is initiated by the specific binding between EBV gp350/220 to CD21 (Roberts et al., 1996). Some approaches indicate that CD21 is a component of an incompletely characterized B-cell activation pathway which modulates intracellular signaling, particularly when co-ligated with the BCR or CD19. However, the role of the CD21 signaling pathway in EBV induced B cell activation leading to EBV infection, still remain unknown.

We focus our work in Burkitt's lymphoma, which is a B-cell lymphoma that carries a Myc 8q24 translocation and certain subtype of this lymphoma is associated with EBV infection (Dalla-Favera et al., 1982; Schmitz et al., 2012). BL is an aggressive germinal centre- derived B cell lymphoma, more common in children, comprising one third of pediatric non-Hodgkin lymphoma cases. Myc translocation is under control of the immunoglobulin gene enhancers which provoke Myc overexpression and cause aberrant cell proliferation, metabolic reprograming and genomic instability (Dos Santos Ferreira et al., 2014).

Translocation involving Myc is characteristic of BL but not specific, so there are many studies trying to identify some standards patterns for better diagnosis. Neither EBV infection nor Myc translocation is sufficient to cause BL, so with new generation sequence techniques it is possible to know essential pathways that can play an important role in this pathogenesis.

There are 3 clinical variants of BL. Endemic BL usually occurs in equatorial Africa and is associated with EBV in almost all cases. The sub-Saharan Burkitt belt extends across equatorial Africa and New Guinea that affects more usually children (more often boys) and the sporadic BL in immunocompetent patients is encountered mainly in Western Europe and the United States, where it affects approximately 3 per million person per year. EBV is positive in about 30% of the cases (Said et al., 2014).

There are many studies focus on identify different morphology, cytogenetic or surface markers features to classify this pathogenesis, but there is a great variability between different BL cells. Although the diagnosis is usually easy when the characteristic features are present, it may be difficult in cases which are not entirely typical. For example, BL may be negative for BCL6, particularly in EBV-positive cases in which the BCL6 is downregulated. Although absence of BCL2 is characteristic of BL, a subset may be weakly positive. Moreover, as we noted above, the typical translocation of Myc 8q24 occurs in more than 80% of the cases, there are some variants translocation located at IgL at chromosome 22q11 or IgK at chromosome 2p12. These additional chromosome abnormalities suggest that there is some biological heterogeinity within the category of BL and that select abnormalities may impact the biological behavior and clinical outcome in BL (Said et al., 2014).

Although Myc inhibits cell differentiation and induces proliferation in these cells, it also makes the cells enter into apoptosis under some circumstances. Unlike other B-cell lymphomas, BL do not exhibit constitutive activity of the pro-survival factor NF-$\varkappa$B, so recently studies consider the possibility that it could be activating the PI3K pathway as a pro-survival downstream pathway depending on BCR activation in these cells (Sander et al., 2012).

There is a study in which engineered mice expressing deregulated Myc and constituvely active PI3K in germinal center B cells develops lymphomas with histology, phenotype, cell surface markers and activating pathways/transcription factors which resemble human BL. Coactivation of Myc-PI3K selects for stabilizing mutations in cyclin D3, which is a key regulator of the cell cycle in germinal center B cells. Abrogating PI3K signaling or cyclin D3 leads to BL-cell death, highlighting the importance of this pathway in Burkitt tumorigenesis (Said et al., 2014).

Up to 70% of BL show constitutively active PI3K signaling, that derived from a somatic mutations of the transcription factor TCF3 or through inactivation of ID3 (Said et al., 2014). Other study performs a genetic prolife of different BL and diffuse large B-cell lymphoma cells and conclude that BL is pathogenetically distinct from other germinal centre-derived lymphomas. Concretely highly recurrent mutations in TCF3 and its negative regulator ID3 indicated that TCF3 has a central role in BL pathogenesis (Shmitz et al., 2012).

TCF3 knockdown decreased phosphor-AKT levels in all BCR-dependent cell lines that were tested in this study and also ID3 overexpression, perhaps due to decreased cell-surface BCR expression following TCF3 depletion. This contributes to PI3K activation in BL. Treatment of BL cell lines with PI3K inhibitors in clinical trials, or rapamycin, inhibitor of the mTORC1 complex (which is engage with AKT-PI3K pathway), was toxic to most BL lines (Figure 6)(Shmitz et al., 2012).

Consequently, they concluded that the majority of BL tumors acquire mutations that release TCF3 of ID3 inhibition. These mutations activate TCF3 transcriptional program that is characteristic of germinal centre B cells and distinguishes BL from other aggressive lymphomas. BL cell lines require TCF3 to increase PI3K signaling through BCR receptor and finally increase the survival of these cells. Moreover, the synergy between PI3K and Myc pathways is supported by the generation of a mice model with BL-like tumors which carries both pathways deregulated (Shmitz et al., 2012).

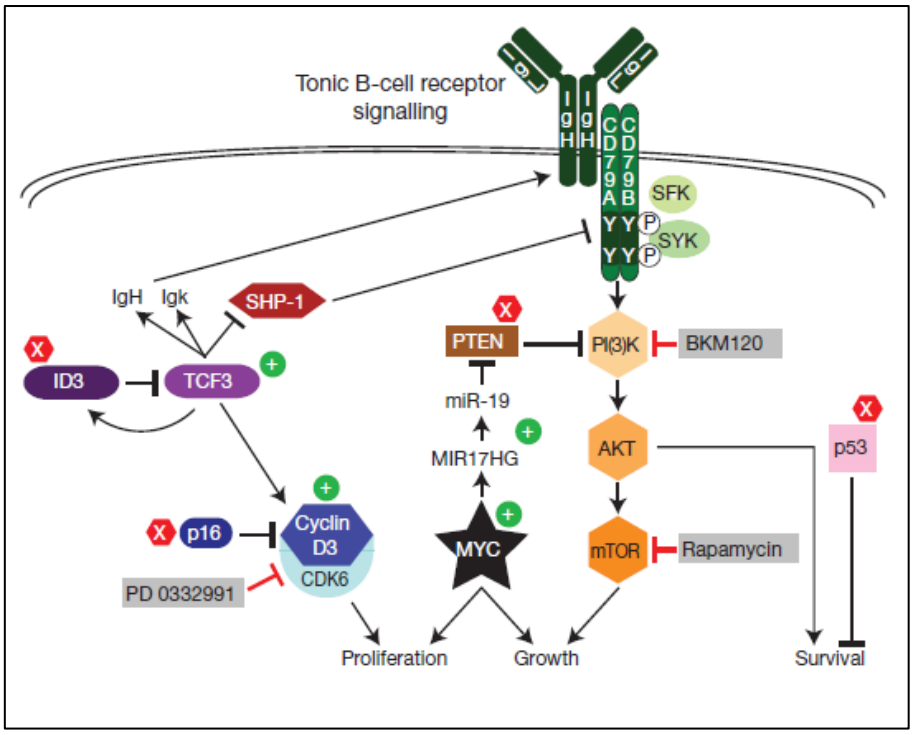

Figura 6. Schematic of recurrent oncogenic pathways in Burkitt's lymphoma. Gain-of-function and loss-of-function aberrations are indicated by + signs and by X signs, respectively. Grey boxes indicate drugs that block these deregulated pathways (Shmitz et al., 2012).

Although today BL can be cured by relatively short course of intensive chemotherapy, the toxicity, expense and complications of this treatment hamper its use in the elderly and in developing countries. Molecular findings as this of Myc/PI3K synergistic pathway could improve treatment and diagnostic strategies for this disease (Said et al., 2014).

Preliminar data of our laboratory suggested that CR2 could be a potential target gene of Myc. One study on CR2 promoter analyzes the relative expression of this gene along the B-cell differentiation process. CR2 as a membrane protein could be used as a surface marker and it is express only in certain subtypes of B-cells. Pre-B cells express less amount of CR2 than cells in later stages of differentiation. Thereby, Raji cells comprise a population of cells expressing high surface levels of CR2 and also high levels of CR2 mRNA (Cruickshank, et al., 2009). Raji cell line derived from a Nigerian 40 years old patient with Burkitt's lymphoma and presented Myc 8q24 translocation and is Epstein Barr positive, although they cannot produce viral particles due to a mutation into the EBV endogenous genes. So Raji cell line becomes an ideal model to explore CR2 regulation by Myc gene expression.

In addition to Raji cells, we used two different cells lines derived from K562 establish cell line. KMycJ cells carry a zinc-inducible Myc gene. This cell line is susceptible to be growth arrested by adding TPA. As we mentioned before Myc expression induces cell proliferation. Therefore, there are many genes affected indirectly due to the physiological change trigger by Myc overexpression or deregulation. Induce cell growth arrest and overexpress Myc at the same time rule out the possibility of Myc regulation over CR2 due to indirect mechanism.

Finally we used KMER cell line to figure out if CR2 is a direct Myc target gene or Myc regulates a second gene which expression induced CR2. KMER cell line has a construction with Myc-ER protein activation depend on tamoxifen addition. Treatment with cycloheximide inhibit protein synthesis at ribosome level (Baliga et al., 1969), therefore activation of Myc-ER in presence of cycloheximide avoids the activation of any gene which are not under Myc control.

Our data show that Myc expression induces CR2 expression and binds CR2 promoter, suggesting that CR2 could be a novel Myc target gene.

Material and methods

Culture cells and treatments. Cells were grown in RPMI 1640 medium (supplemented with 10% FBS, 10μg/ml Ciprofloxacin and 500μg/ml Gentamicin) at 37ºC in 5% CO_2.

Raji and Raji6B cells were treated with JQ1 which is a BET inhibitor, a small molecule that inhibits the individual BET family bromodomains. The BET protein family employs tandem bromodomains to recognize specific acetylated lysine residues in the N-terminal tails of histone proteins. JQ1 competitively occupy the acetyl-binding pockets of BET bromodomains, resulting in the release of BET proteins from chromatin. It has been proved that BET-Bromodomain inhibition potently suppressed Myc gene expression (Mertz et al., 2011).

Raji and Raji6B treatment with JQ1: we treated these cells with a range of concentrations between 0 and 1μM of JQ1. The cells were counted in Nucleocounter and we used $2x10^6$ cells per condition in Petri dishes of 100mm. Control cells were treated with DMSO using the half maximal amount JQ1 (1.5μl). The cells were harvested after 6 and 12 hours, washed with PBS 1X, centrifuged and after removed PBS were conserved at -80ºC. The results are the mean of two measures with the same RNA.

KMycJ is a K562 derived cell line with an exogenous Myc inducible by $ZnSO_4$ (Bretones et al., 2011). Zn^{2+} was added to the cells at a final concentration of 75μM to induce Myc expression in the absence of cell proliferation (maintaining TPA treatment 10nM) and harvested at different hours to study CR2 mRNA expression (0, 12, 24 and 48 hours), washed with PBS 1X, centrifuged and after removed PBS were conserved at -80ºC. The results are the mean of two different measures with the same RNA, but different cDNA.

KMycER is a K562 derived cell line that expresses the exogenous Myc dependent on Tamoxifen. Myc-ER is a fusion protein in which the ligand-binding domain of the estrogen receptor is fused with the carboxyl terminus of Myc (Littlewood et al., 1995). ER lacks intrinsic transactivation activity. These cells expressed Myc-ER constitutively but it is inactive until we add 4-hidroxitamoxifen (4HT) to the media.

KMycER treatment with Cycloheximide: the cells were harvested and divided in four Petri dishes 100mm. Control cells were treated using the same amount of Tamoxifen (8.5µl). The cells were harvested after 6h of treatment, washed with PBS 1X and conserved at -80ºC. The results are the mean of four different measure of two independent experiments.

Relative quantification of mRNA by Real Time-PCR. Total RNA was extracted using Trizol®.A two-step RT-PCR was performed with Bio-Rad iScript Advance cDNA Kit for RT–qPCR using 1µg RNA in a total volume of 40µl. The following primers were used: Myc-157: 5'TCGGATTCTCTGCTCTCCTC; reverse primer: 5'CCTGCCTCTTTTCCACAGAA. CR2: 5'CCGACACGACTACCAACCTG; reverse primer: 5'GACAATCCTGGAGCAATGGA. PCR conditions: 95ºC 3 min, 95ºC 10sec-56ºC 30sec-72ºC 10sec; 40 cycles; 30sec 72ºC, 5sec 55ºC. Real-time PCR was performed in duplicates with 2µl of RT products equivalent of 50ng total RNA, 150nM of each primer and SYBR 3x. Relative quantification was done using the standard curve method.

Western Blot analysis. The cells extracts were lysate with NP-40 0,2% SDS (buffer recommended to analyze whole cell protein amount), sonicated for 10sec and centrifuged 12000 rpm 20min. The total protein concentration of each sample was quantified with Bio-Rad Bradford Protein Assay. To denature proteins it was used the loading buffer Laemmli 5X with 50µg of lysated proteins. The samples were heated at 100ºC 10min and running in a 10% acrylamide gel (recommended gel to see protein size between 15-100 kDa). Electrophoresis was performed at 175V during 1 hour. Electrophoretic transfer of proteins onto nitrocellulose membrane (NC) was made with a current of 395 mA for 75min and the temperature was maintained below 35ºC by placing an ice pack inside the electrophoresis chamber. The NC membranes were blocked with 1% BSA in TTBS (Tris-HCl pH 7.5 1M, NaCl 5M and Tween) during 40min. After removed BSA, membranes were washed three times with TTBS during 10min. NC membranes were incubated with primary polyclonal antibodies overnight at room temperature of 4ºC (anti-actin I-19 goat-polyclonal dilution 1:1000 in TTBS with 1% BSA and anti-Myc N-262 rabbit-polyclonal dilution 1:2000 in TTBS with 2% BSA). After removed primary antibodies washed again three times with TTBS during 10 min and incubated with secondary antibodies (anti-goat 1:10000 in TTBS with 1% BSA; anti-rabbit 1:10000 in TTBS with 1% BSA).

Results

Previous results

Kp27MER cell line is a K562 derived cell line with a conditional induced of p27 by Zn^{2+} and Myc-ER activated by 4HT (Acosta et al., 2008). Overexpression of p27 induced growth arrest in this cell line. The Northern-blot assay showed that CR2 gene expression was up-regulated in presence of 4HT even with p27 expression induced by Zn^{2+} (Figure 7), suggesting that CR2 could be a potential target gene of Myc.

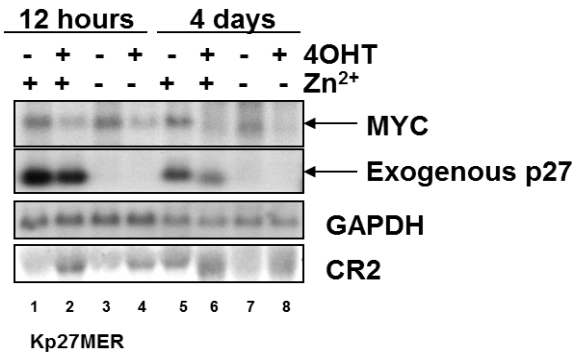

Figure 7. Kp27MER derived from K562 cell line with chimeric protein Myc-ER inducible by Tamoxifen and p27 condicional induction by $ZnSO_4$. Control RNAs of microarray experiment and Myc effect over CR2 gene expression. Northern blot of Kp27MER cells treated with $ZnSO_4$ and 4HT during 12 hours and 4 days with probes of Myc and CR2. GADPH were used as loading control. (J. C. Acosta, PhD Dissertation).

Myc downregulation after treatment with JQ1 repress CR2 expression.

JQ1 is a small molecule that inhibits BET-bromodomains with anti-proliferative activity in many different cell lines. Previous works confirmed that cells treated with JQ1 increase the percentage of cells in G_0/G_1 phase suggesting that BET-bromodomains inhibition affect cells either during mitosis or at M/G_1 border, leading to a failure to enter the next cell cycle (Mertz et al., 2011). One of the mechanisms that could explain this phenotypic response is the repression of Myc-MAX binding motif by BET inhibitors.

Raji and Raji derived cell line were treated with four different concentrations of JQ1 and harvested after 6 and 12 hours. The qPCR analysis showed that Myc gene expression is repressed by JQ1 in a dose-dependent manner in Raji cells (Figure 8A). This data are agreed with previous results obtained in different cell lines, harboring Myc translocations, including Raji cells, in which Myc expression was markedly suppressed after treatment with JQ1 (Mertz et al., 2011). Furthermore, CR2 expression correlates with Myc expression levels.

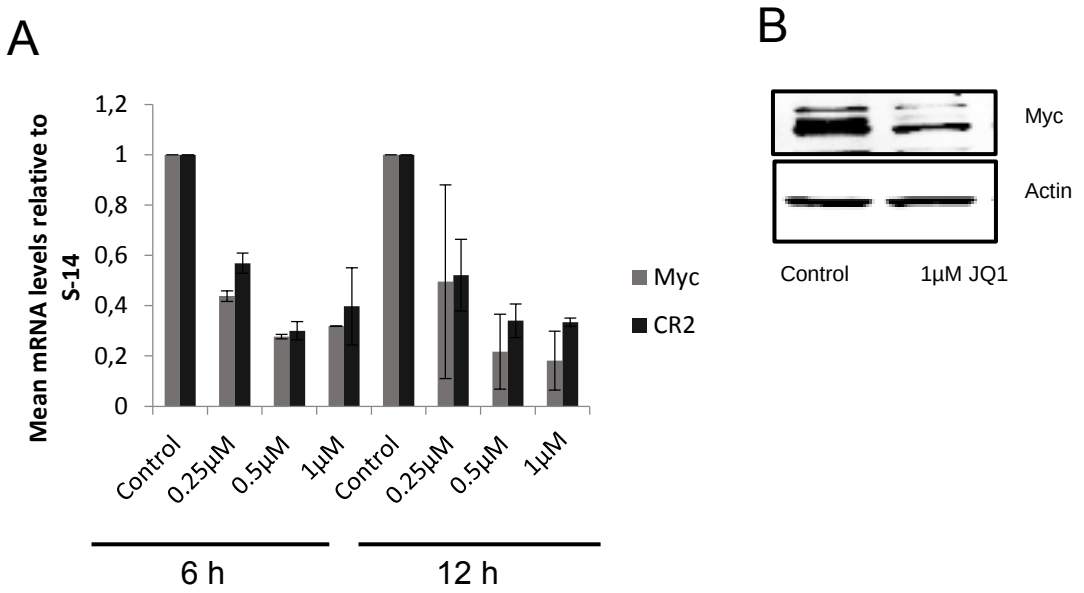

Figure 8. Raji cells treated with 0.25 μM of JQ1 showed decreased Myc expression after 6 hours. This decrease is more significant as we increase the concentration JQ1, although the difference between 0.5 and 1 μM is less obvious than between 0.25 and 0.5 μM. After 12 hours of treatment the cells showed the same gradually decrease of Myc expression and so did CR2. The highest concentration of JQ1 is consistent with lower levels of Myc as confirmed by qPCR A) and WB B).

Raji6B cell line was also treated with JQ1 (Figure 9A). This is a Raji subline with higher Myc expression. After 6 hours of treatment the qPCR analysis showed a decrease in Myc expression similar to that in Raji cells.
Although at longer times of JQ1 exposures Myc expression apparently tends to recover, probably due to the expression of exogenous Myc. Importantly, CR2 expression also correlates with Myc expression.

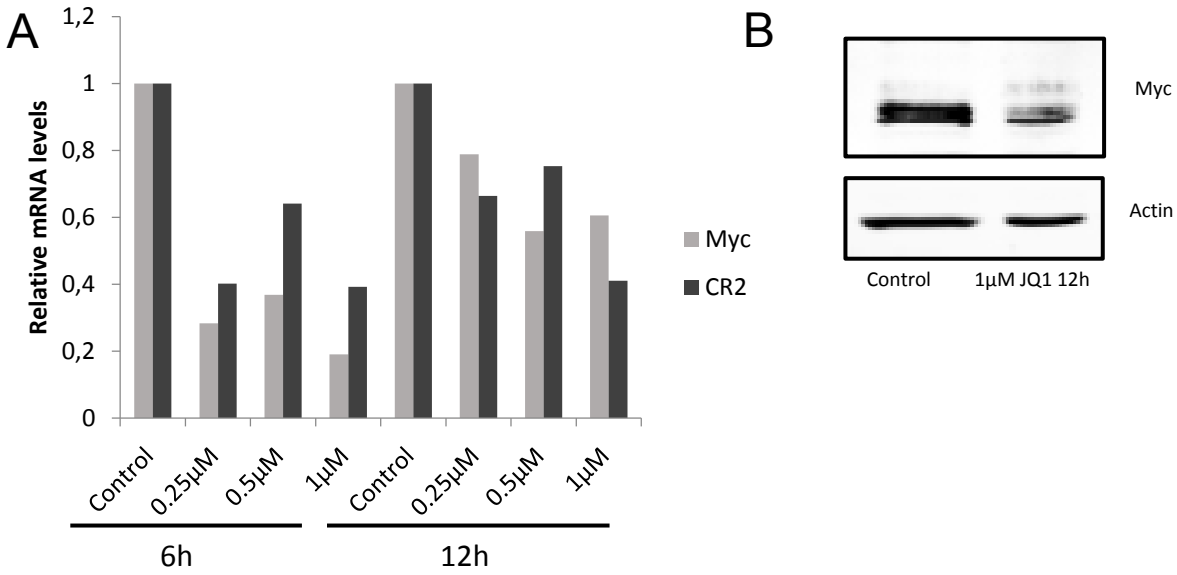

Figure 9. Raji6B cells were treated with JQ1. It also showed similar inhibition of the exogenous Myc transcription as Raji parental cells at 6 hours, although after 12 hours of treatment Myc expression tends to recover, independently of the concentration. CR2 expression correlates with Myc downregulation at 6 hours and appears to stabilize at 12 hours as shown in A). B) WB shows Myc downregulation at the protein level after JQ1 treatment.

The results indicate that, in both cell lines, exposed to low JQ1 concentrations for 6 hours of treatment, the inhibition of Myc gene expression is followed by a downregulation of CR2 expression. High concentration of the compound is consistent with lower expression of gene Myc as confirmed with the qPCR and protein expression in Western-blot (Figure 8B and 9B). That correlates with the minimal expression of CR2, suggesting that Myc inhibition repress CR2 expression at transcriptional level.

CR2 expression is regulated by Myc independently of cell proliferation.

Myc downregulation leads to cell cycle arrest, so it is not possible to conclude that changes in CR2 mRNA expression are directly due to Myc transcriptional activity or whether it is a consequence of the proliferative arrest caused by Myc downregulation. To address this issue, KMycJ cells (a K562 derivative cell line containing an exogenous Myc gene inducible by Zn^{2+}) were pre-treated 12 hours with TPA to indude growth arrest as previous studies demonstrated that treatment of K562 cells with 10nM of TPA induced growth arrest and terminal differentiation in this cell line (Lerga et al., 1999).

KMycJ cells were treated with TPA and then with Zn^{2+} for 0, 12, 24 and 48 hours and were counted to analyze the growing pattern of each condition (Figure 10). Whereas control cells and cells treated only with Zn^{2+}, grew at the expected rate cells treated with TPA were arrested and Myc overexpression induced by Zn^{2+} in presence of TPA, could not rescue the proliferative arrest of the cells.

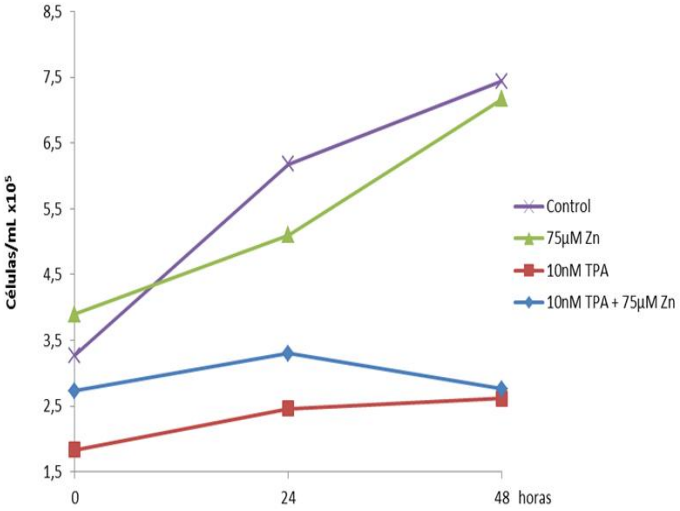

Figure 10. Control cells and cells treated with Zn2+ presented the same growing pattern; however TPA treatment produced growth arrest in control cells. Myc induction by Zn2+ is not able to restore the antiproliferative arrest caused by TPA, therefore this is an ideal system to study Myc target genes independently of cell proliferation.

Thus, Zn^{2+} was added to the cells to induce Myc expression in the absence of cell proliferation (maintaining TPA treatment), although Myc induction is not able to restore the antiproliferative arrest caused by TPA in this cell line (Figure 11). The results suggest that CR2 expression correlates with Myc overexpression independently of cell proliferation in this cell line.

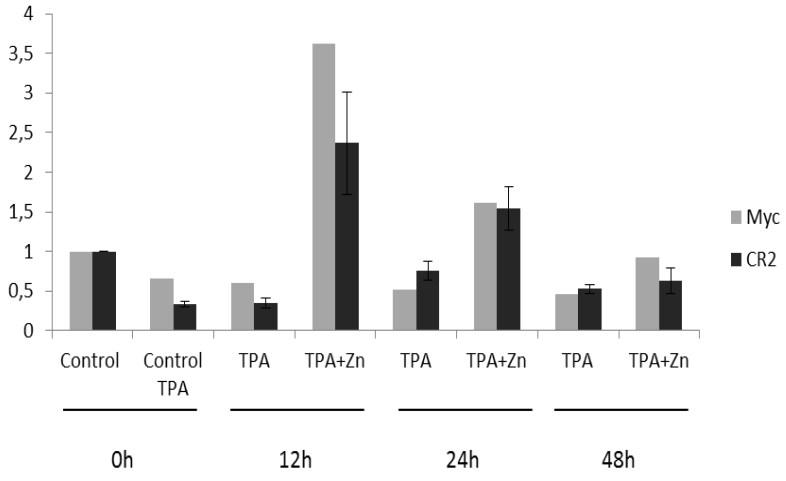

Figure 11. KMycJ cells treated with TPA showed decreased Myc levels, maintained a long time. Cells treated with ZnSO4 in presence of TPA showed an increase of CR2 expression which decreases gradually at 24 and 48 hours after treatment, consistent with Myc induction.

Myc binds CR2 promoter

The previous results indicate that Myc regulates CR2 expression at transcriptional level, but to ensure that Myc could bind to CR2 promoter we used the ChIP-Seq database from ENCODE/HAIB project were downloaded from the University of California Santa Cruz database (UCSC, http://genome.ucsc.edu/). This search reveals that Myc binds to CR2 promoter at the Transcription Start Site (TSS) (Figure 12). This data also showed that Max, Myc binding partner protein, co-localize in the same place. Moreover, human CR2 promoter has two canonical E-boxes (5'-CACGTG-3'), where Myc-Max binds, the first one 17 bp upstream the TSS and the second one in the first intron.

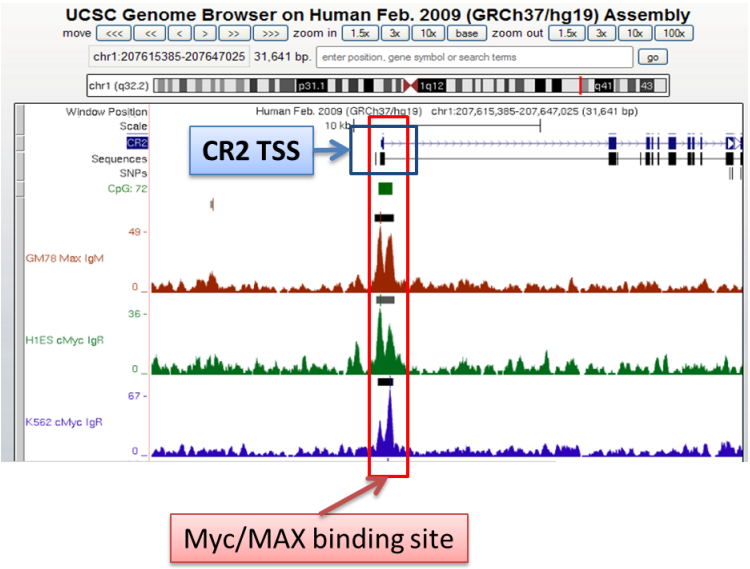

Figure 12. According to the Chip-Seq data from the "ENCODE" project Myc binds the CR2 promoter al the Transcription Start Site (TSS) as shown in the figure. These data also shown that Myc partner (Max) is bound in the same region. Besides CR2 promoter contain two canonical E-boxes, one at the TSS (17bp upstream the TSS) and the other one in the first intron.

All this data suggest that Myc is able to bind at CR2 promoter and regulate its function at transcriptional level.

Myc regulates directly CR2 expression.

A direct target gene is one whose expression is altered by direct interaction of the Myc protein with the gene regulatory elements or with *trans*-acting factors that bind *cis*-elements. Myc target genes regulation should closely follow Myc expression. The Myc-estrogen receptor (Myc-ER) fusion protein system has become an established system to detect a candidate target gene by Myc (Dang, 1999). In this system the Myc-ER protein is retained in the cytoplasm via chaperone proteins and after exposure to estrogenic ligands the fusion protein is translocate to the nucleus. Thereby, the exposure of estrogen compounds to Myc-ER cell lines simultaneously with cycloheximide (an antibiotic that inhibits the initiation of new peptide chains and the elongation of nascent peptides on ribosomes by different mechanisms) will result in the activation or repression of Myc target genes.

KMER cells were treated with 4HT and cycloheximide and were harvested after 6 hours of treatment with both compounds. Although Myc endogenous protein is repress after treatment with cycloheximide, as expected, the cells treated with 4HT shown an increase of Myc-ER protein due to the activation of Myc-ER even in presence of cycloheximide (Figure 13).

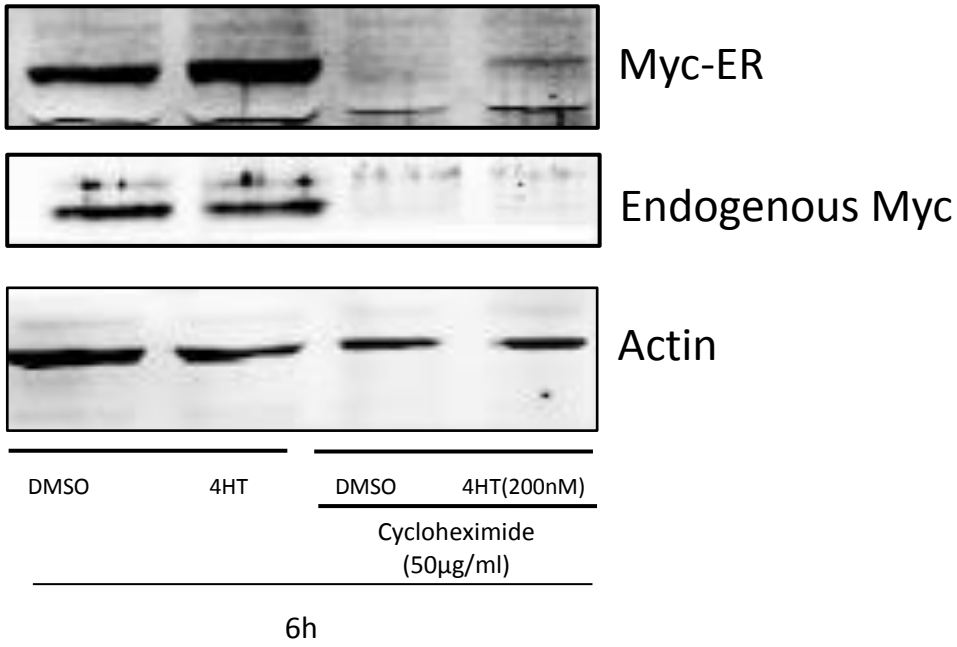

Figure 13. KMycER cells were harvested after 6 hours of treatment with 4HT and Cycloheximide. Control cells showed stabilization of Myc-ER protein after 6 hours of treatment with 4HT by WB. The cells treated with cycloheximide showed the inhibition of protein synthesis as expected, although Myc-ER activation by 4HT makes it more stable even in presence of Cycloheximide.

Q-PCR analysis showed that this correlates with an increase of CR2 expression at transcriptional level. Although after treatment with cycloheximide the protein synthesis was inhibited there was transcriptional activity of CR2, due to Myc-ER activation with 4HT (Figure 14). Thus, the inhibition of protein synthesis avoids the expression of CR2 due to a second protein, confirming the hypothesis that CR2 is a direct target of Myc.

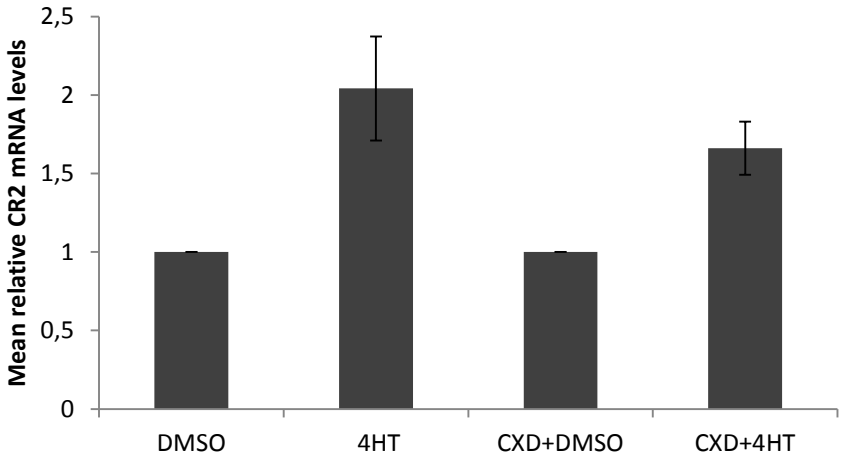

Figure 14. qPCR analysis showed that activation of Myc-ER by 4HT correlates with an increase of CR2 expression at transcriptional level. In presence of cycloheximide we could still see an increase in CR2 levels after Myc-ER activation.

Discussion

Myc inhibition by JQ1 represses CR2 expression

It is demonstrated that Myc gene expression can be potently and reversibly abrogated by BET bromodomains inhibitors. Although BET inhibitors affects great variety of genes, Myc transcriptome heavily dominates the gene expression changes observed upon BET-bromodomain inhibition (Mertz, et al., 2011). BET family members include BRD2, BRD3, BRD4 and BRDT that modulate gene expression by recruiting transcriptional regulators to specific genomic locations. BRD4 is a well-established regulator of P-TEF, a complex consisting of cyclin-dependent kinase (CDK). P-TEF is recruited to promoters to phosphorylate the carboxy-terminal domain of the large subunit of RNA polymerase II (RNAPII). Many studies suggested that BRD4 plays an important role in growth associated genes involve in M/G_1 boundary by retaining P-TEF at the promoters of key regulatory genes throughout mitosis (Mertz et al., 2011).

Myc is chromosomically translocated in all patients of BL and in a significant fraction of multiple myeloma and diffuse large B-cell lymphoma. Several cell lines harboring Myc translocations treated with BET inhibitors are very sensitive to this treatment probably due to the phenomenon known as "addiction to the oncogene". Therefore, decrease the levels (or enzymatically inactivate the oncoproteins), may results in death of the tumor cell. These findings suggest an opportunity to use BET-bromodomain inhibitors to fine-tune key regulatory pathways for therapeutic benefit (Mertz et al., 2011).

Our data demonstrated that BL cell lines sensitive to JQ1 treatment showed Myc inhibition at lower concentrations and short times of exposure. This inhibition affects Raji cells in a dose-dependent manner. Raji6B showed also Myc repression after treatment with JQ1, but only at shorter times. After 12 hours of treatment Myc expression tends to recover. This increase might be explain due to the exogenous Myc that harbor this cell line, that it is not affected as the same level as the endogenous Myc. As we mentioned before, BET-bromodomains affects many genes, although Myc promoter is very sensitive to this treatment. Exogenous Myc promoter could be less sensitive to BET inhibitors, so after 12 hours the increase of Myc expression probably is due to the expression of the exogenous Myc. Myc gene repression is translated into a decrease in Myc protein synthesis in both cell lines.

Raji and Raji6B cells showed CR2 repression that correlates with Myc inhibition. All this data suggest that JQ1 inhibitor could repress Myc expression and this provoke a downregulation of its potential target genes in this cell line. The inhibition of Myc expression by JQ1 affects CR2 expression, but because JQ1 affects other genes it is possible that CR2 repression is not directly related with Myc downregulation.

Although, we showed that CR2 expression are related with Myc gene downregulation, it is necessary to verify this relation through other techniques. For this purpose, in the final experiments of my Master laboratory work, I tried to silence Myc expression with siRNAs. siRNAs are a subtype of small RNA (20-30 nucleotide non-coding RNAs that regulate genes and genomes). This regulation can occur at some of the most important levels of genome function, as chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability and translation. siRNAs are synthesized as short hairpin vectors and processed by different proteins (DICER, Argonaute family proteins), to form a ssRNA that inhibit the expression of its target by base-pairing complementarity (Carthew and Sontheimer, 2009).

I tried to inhibit Myc expression in Raji cells through transfection of siRNA. We used Amaxa electroporation protocol of transfection following manufacturer's instructions. The protein synthesis of Myc was decreased with increasing concentration siRNA Myc but not in cells transfected with control (scrambled) siRNAs (Figure 15).

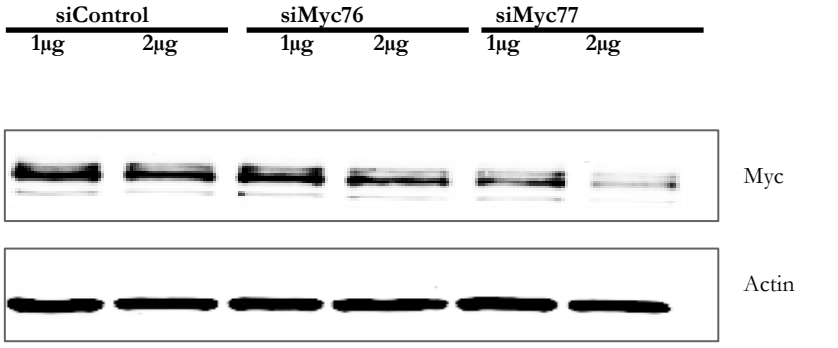

Figure 15. Western-blot analysis of Raji cells transfected with two different siRNAs. For both siRNAs the amount of Myc protein decreases with higher amount of siRNA. We used siControl as transfection control and actin was used as loading control.

The results showed that, we are able to silence Myc protein expression, but we could not measure RNA expression of CR2. Because lack of time, we could not reproduce this experiment, so this require further investigations.

CR2 regulation by Myc is independent on the physiological state of the cell.

Myc activity is involved in many processes in cell biology; thereby inhibition of Myc expression necessarily affects indirectly the expression of many genes. One of the main features of Myc expression is the increase of cell proliferation, consequently many genes involve in cell cycle regulation are affected when Myc is deregulated. To asses that CR2 regulation by Myc is independent of the proliferative state of the cell, we induced growth arrest to KMycJ cells and upregulated Myc expression, so that only Myc target genes could increase its expression, despite of the proliferation inhibition.

We treated KMycJ cell with TPA to induced growth arrest in this cell line. TPA has been used in other studies to induced growth arrest in cancer cells like human breast cancer skBr3 or LNCaP human prostate cancer cell lines in which TPA induced the expression of the cell cycle inhibitor p21. p21 inhibits cell cycle progression by binding cyclin-cyclin dependent kinases and inhibiting their kinase activity as well as by binding to the proliferating cell nuclear antigen PCNA, thereby inhibiting processive DNA synthesis (Mitchell and El-Deiry, 1999).

KMycJ cells treated with TPA stop growing, as expected, and the induction of Myc expression is unable to recover the normal rate proliferation of control cells. When we added Zn^{2+} to induce Myc overexpression CR2 mRNA expression increase in the same manner than Myc, suggesting that CR2 is regulated by Myc independently of the proliferation state of the cell. This means that CR2 expression is not related with the cell cycle state of the cell and also that genes involved in cell cycle regulation are not acting over CR2 expression.

CR2 is a direct Myc target gene

According to the data of ChIP-Seq "ENCODE" project Myc is able to bind CR2 promoter near the TSS as well as Max protein. As we mentioned before Myc binds DNA almost always when it is bind to Max protein forming a dimer and also in specific binding sites called E-boxes. CR2 promoter has two canonical E-boxes, thereby these data strongly support the hypothesis that Myc could bind to CR2 promoter.

Myc directly regulates a large number of genes, which in turn are involved in most of the biological processes of the cell. But, at the same time, regulates many genes indirectly subject to the Myc activity. Find out if a gene is a direct target of Myc is a complicated task.

There are few techniques that allow us to discover a possible Myc target gene. Inhibiting protein synthesis in a model system which carries Myc-ER inducible activation is one of them. Myc protein has a short mean life (it is estimated in 20 minutes), although Myc-ER protein remain stable after treatment with 4HT in presence of cycloheximide, probably because the activation stabilize the protein and impair its degradation. The activation of Myc-ER protein in the absence of protein synthesis allows us to measure the expression of our possible Myc target gene and be sure that the changes in its expression necessarily are due to Myc-ER activity.

In conclusion, Myc is able to bind CR2 promoter and regulates CR2 expression without a second intermediate, indicating that CR2 is a direct Myc gene target.

Biological propose model of Myc/CR2 function

CR2 is a membrane protein that interacts with other proteins such as CD19 to activate different intracellular pathways. It also acts as the BCR co-receptor modulating its activity. Finally, it is the protein responsible for recognizing the EBV and activates T-dependent complement system. That occurs in physiological conditions.

In a cancer model cell many pathways are deregulated. As we mentioned before Myc is deregulated in many cancer cells, and it provokes an increase in cell growth and proliferation. Normally it is associated with an increase in activity of pro-survival pathways like NF-kB pathway. Altogether increase chromosome abnormalities and genome instability.

Burkitt's lymphoma is an aggressive lymphoma with many features that increases the complexity of this pathogenesis and impair, in many cases, a properly diagnosis and effective therapeutical solutions. One of its main characteristic is a Myc translocation on one of the immunoglobulin gene enhancers and in some cases is associated with EBV infection. But, neither Myc translocation nor EBV infection are sufficient to provoke the BL pathogenesis.

Recent studies discovered new pathways involve in BL pathogenesis that probably collaborates in its complexity and make more difficult the diagnosis and impairs proper clinical treatments. On the other hand this new findings allows us to know better the pathogenesis and improve new therapeutical targets.

TCF3 deregulation has been demonstrated an altered pathway that activates AKT-PI3K pathway and provokes an increase in surveillance of BL cells. Other studies relate CD19 with the activation of this pathway.

Our results provide new information about a possible new target of Myc gene and its importance in Burkitt lymphoma pathogenesis. If CR2 is a direct Myc target is possible that Myc overexpression in BL cell lines enhances CR2 expression. This overexpression increases the amount of this membrane receptor and makes the cell more susceptible to be activated by CR2 ligands. As we mentioned before CR2 occasionally is associated with other transmembrane proteins, as CD19, and is the co-receptor of the BCR (Figure 16). CD19 and BCR activate intracellular signaling pathways, that in the case of CD19 is involved with AKT-PI3K pro-survival pathway.

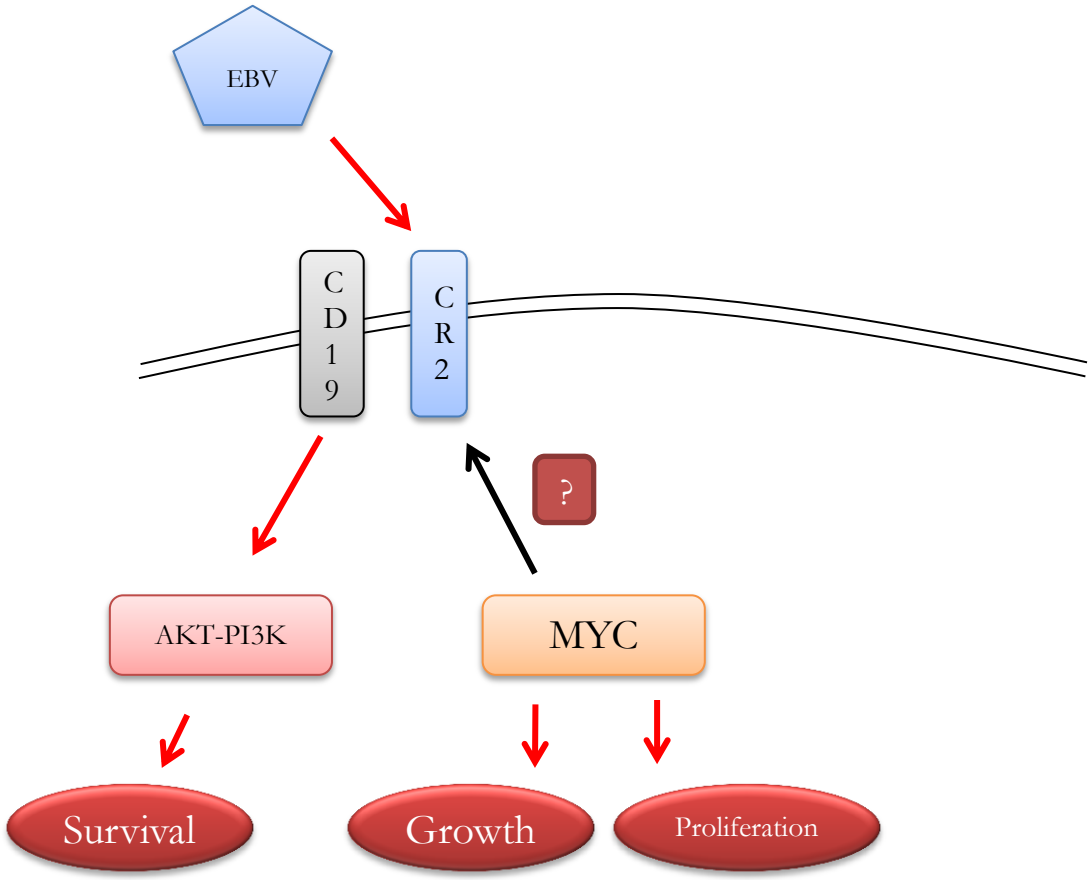

Figure 16. Schematic representation of different pathways related with Burkitt lymphoma pathogenesis. The red arrows represent known pathways. The black arrow represents a possible new model pathway.

Although CR2 expression is regulated by Myc, the implication in BL lymphoma or other hematopoietic pathogenesis have to be clear. The biological consequences of these findings require further investigation.

Conclusions

Myc is a proto-oncogene involved in many process in cell biology. Although its deregulation is related with many human cancers, it is not possible to use Myc as a therapeutical target. Instead of that many efforts are focused on investigate Myc related pathways to discover possible new targets.

Myc activity is widely spread over cell physiology, so that it is difficult to distinguish between Myc direct target genes and genes regulated indirectly because of Myc deregulation. In this work, we demonstrated that CR2 expression is under Myc gene expression control.

Myc downregulation with BET-bromodomains inhibitors, also inhibits CR2 expression. Although, it is necessary to confirm this relation through other techniques, that allow us to knockdown only Myc expression, as siRNAs or lentiviral infection with shMyc. It is necessary as well, extend these observations to other hematopoietic BL cell lines.

Finally, the biological implications of these findings are still unknown, mostly because of the lack of information about CR2 activity. But the relation of CR2 with CD19 co-receptor, and BCR, suggests that it has an important role in B cell antigens response, and could trigger signaling pathways that could determine the response of B cell activity. Besides, it is the main link between EBV and Myc, both of them with a central role in BL pathogenesis.

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