UNIVERSITA' DEGLI STUDI DI MILANO BICOCCA

AUDENTES FORTUNA IUVAT

MOLECULAR MECHANISMS FOR THE PROGRESSION OF CHRONIC MYELOID LEUKAEMIA

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ABSTRACT

Chronic myeloid leukaemia (CML) is caused by the BCR-ABL hybrid gene. The molecular mechanisms leading from chronic phase (CP) to blast crisis (BC) are not understood. However, both the presence and the levels of BCR-ABL seem to be important for CML progression. BCR-ABL is under the transcriptional control of BCR promoter. Here we focused on the gene expression control of BCR and BCR-ABL upon myeloid differentiation in healthy donors (HDs), CP and BC patients. As previously reported, BCR-ABL is downregulated during myeloid maturation in CP patients. A similar pattern was detected for BCR (but not for ABL) in CP-CML and in HD, thus suggesting that the two genes may be under a similar transcriptional control. In BC this mechanism is similarly impaired for both BCR-ABL and BCR. These data indicate the presence of an 'in trans' deregulated transcription of both BCR and BCR-ABL promoters, associated with CML progression. The results of the luciferase assay indicate that the region comprised between 420 and 900 bp from the coding ATG site is required to achieve a basal transcription level. Previous studies suggest that a putative SP1 binding site could have a role in the basal promoter activity. In fact, an almost complete absence of transcriptional activity was measured in Δ1041 and Δ1271 constructs, lacking both the main transcription start site and the putative SP1 binding region. We hypothesize that SP1 could be responsible for the basal promoter activity, present in the Δ541 and in longer constructs. The ChIP assay confirmed the SP1-binding to the BCR promoter. The presence of 10 additional putative protein binding sites (PBSs), along the BCR promoter is also known from previous works. Six of these putative PBSs are localized in the region between −1443 to −1202 bp, which appears to be critical from *in silico* studies. In fact, only in presence of a 221 bp region upstream from Δ241, a strong luciferase signal could be detected, suggesting that the promoter region between −1443 and −1202 bp from the coding ATG is indeed critical to achieve the highest level of expression.

General Introduction

Haematopoiesis

Haematopoiesis is the physiological process by which immature precursors cells develop into mature blood cells. All blood cellular components derive from a common haematopoietic stem cell (HSC) (Figure 1). HSCs can undergo self-renewal (the ability of HSC to divide and to maintain the undifferentiated state) and show multipotency (differentiation ability), HSCs proliferate and differentiate into mature blood cell. HSCs localize in the bone marrow niche and constitute 1:10000 of total cells.

The process of haematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and their environment. This interplay determines whether HSCs, progenitors, and mature blood cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis. Pluripotent HSCs origin both the common myeloid progenitor and the common lymphoid progenitors, which will differentiate into myeloid cells and lymphocyte, respectively. During the transition to mature cells, a reduction of differentiation potential characterizes the new cells and they stop to dividing once they became fully differentiated. To satisfy the daily need of cells production, the haematopoietic system constantly generates all the blood elements from pluripotent HSC. The molecular regulation of haematopoiesis is a very complex and tightly controlled process and not all the pathways and mechanism involved are well known. The specific surface cell markers permit the recognition of differentiation stage of maturation: CD133 is known to be a marker for HSCs(1), while the presence of other markers, such as CD34 or CD38, identify the more mature progenitors, CMPs and GMPs.

One of the first differentiated precursors of HSC development is the multipotent progenitor (MPP), which is at the bifurcation between myeloid and lymphoid lineage.

MPP can differentiate into common myeloid progenitors (CMP) or into early lymphoid progenitors (ELPs).

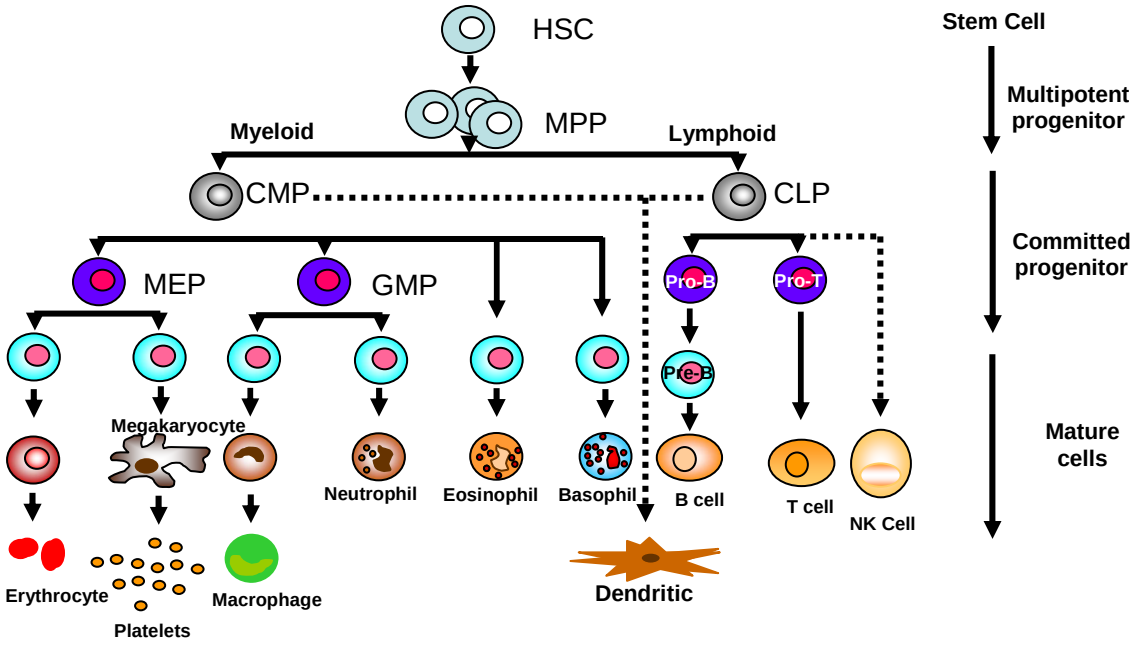

Figure 1: The hierarchy of hematopoietic cells. HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte−macrophage progenitor. Adaptation from [(2)]

Myeloid differentiation

This phenomenon is controlled by a complex network of interactions between cytokines and their receptors. The mechanism of maturation is regulated by the expression of transcriptional factors that coordinates the entire process: the expression of specific transcriptional factors and/or their repression drives the differentiation process.

The specific haematopoietic tissue microenvironment plays an important role in myeloid development: the crosstalk with other components of the niche influences the HSCs fate(3). The microenvironment for HSCs is established by their niche, which is located within the endosteal and perivascular compartments of the bone marrow, and is regulated by an assortment of signaling pathways including (but not limited to) those regulated by Wnt, Notch, c-Kit, BMPs, and osteopontin.

Prevailing models assume the existence of a quiescent population of stem cells residing in a specialized niche of a tissue. Emerging evidence indicates that both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) stem cell subpopulations may coexist in several tissues, in separate yet adjoining locations.Most primitive HSCs are quiescent(4). Using DNA label BrdU incorporation LRCs were found to be predominantly located in the endosteum(5) and subsequently confirmed to be a primitive HSC population(6, 7). The quiescent HSCs have superior long-term reconstitution potential; other studies based on the DNA label BrdU and H2B-GFP incorporation have suggested that the majority of murine HSCs undergo more frequent cycling(6, 8, 9). These seemingly disparate findings may be reconciled by postulating the existence of two subpopulations of HSCs, one being long-term quiescent (“reserved”) and the other being more actively cycling (“primed”). Human bone-forming cells, or osteoblasts, produce important hematopoietic cytokines

(granulocyte colony-stimulating factor or G-CSF, granulocyte macrophage colony-stimulating factor, and leukemia inhibitory factor)(10), can support HSCs *in vitro* and have the ability to maintain long-term culture initiating cells (LTC-ICs), primitive self-renewing hematopoietic cells(11).

In the early stage, the co-regulation of the transcriptional factors PU.1 and GATA1 has been demonstrated to be fundamental for the commitment to CMPs. Their co-expression is important for the differentiation of HSCs into CMPs, but subsequently their mutually exclusive expression coincides with further development into granulocytic/monocytic precursor (GMP) (granulopoiesis) or megakaryocytic/erythroid precursor (EMP) (erythropoiesis). In granulopoiesis, C/EBPα transcriptional factor (CCAAT/enhancer-binding protein α) is the key transcriptional factor. Its expression identifies the passage from CMPs to GMPs, accomplished by the high expression of other characteristic transcriptional factors of the myeloid lineage, for example GATA2.

Chronic Myeloid Leukaemia (CML)

Epidemiology and clinical characteristics

Chronic myeloid leukaemia (CML) is defined as a myeloproliferative disorder derived from the neoplastic transformation of the pluripotent haematopoietic stem cell(12), characterized by excessive accumulation of apparently normal myeloid cells. CML has an annual incidence of 1.0 to 2.0 per 100,000 persons. It occurs rarely in children and account the 15% of leukemia in adults. The median age of onset at presentation is 50/60 years.

Common clinical symptoms may included fatigue, abdominal fullness, anemia, splenomegaly and weight loss, leukocytosis and thrombocytosis, but it's often asymptomatic and about 50% of the diagnoses are a consequence of blood tests performed for unrelated reasons.

CML evolves along a triphasic course: CML is characterized by an initial Chronic Phase, in which the differentiation of leukemic cells in the bone marrow is preserved, but this phase is accomplishes by an increase of mature granulocytes in peripheral blood and an enrichment of differentiated cells into the bone marrow (Table 1). Years after the onset of CP, patients eventually progress to an intermediate phase (Accelerated Phase, AP) and then to the acute, final phase of the disease (Blast Crisis). The accelerated phase characterized by a partial maturation arrest with increased blast number (15-30%), progressive splenomegaly, leukocytosis and thrombocytosis. Accelerated phase eventually transforms into the blastic phase characterized by a further increase in blast numbers(13, 14). Stringent criteria and definitions of accelerated and blastic phases are summarized in Table 2(15). In the latest stage, the differentiation potential of the leukemic cells is lost and blasts cells accumulate in the bone marrow and peripheral blood.

Chronic phase CML
Blasts < 15%
Basophils <20%
No thrombocytemia
Hypercellular bone marrow (myeloid:erythroid ratio from 10:1 to 30:1)

Table 1: In the table are summarized the main common features found in chronic-phase CML patients

Accelerated phase CML
Blast cells in blood or bone marrow 15-30%
Basophils in blood ≥ 20%
Persistent thrombocytopenia (< 100 x 109 /L) unrelated to therapy
Blastic phase CML
> 30% bone marrow or peripheral blasts
Extramedullary haematopoiesis with immature blasts

Table 2: In the table are summarized the criteria proposed by the World Health Organization (WHO) for the definition of accelerated and blastic-phase CML[16]

The Ph Chromosome

CML is characterized by the presence of the Philadelphia Chromosome (Ph chromosome)(17, 18), resulting from the t(9;22)(q34;q11) reciprocal chromosomal translocation. The 5' part of Breakpoint Cluster Region (BCR) gene on chromosome 22q11 recombines with the 3' region of the ABL proto-oncogene, located at 9q34 22, 5', generating the BCR-ABL fusion gene (figure 2) (17, 18). This genetic aberration is needed in order to diagnose CML(15, 19). The transcription of the BCR-ABL fusion gene produces a chimeric BCR-ABL messenger RNA and thus the BCR-ABL fusion protein, that is the molecular event leading to CML.

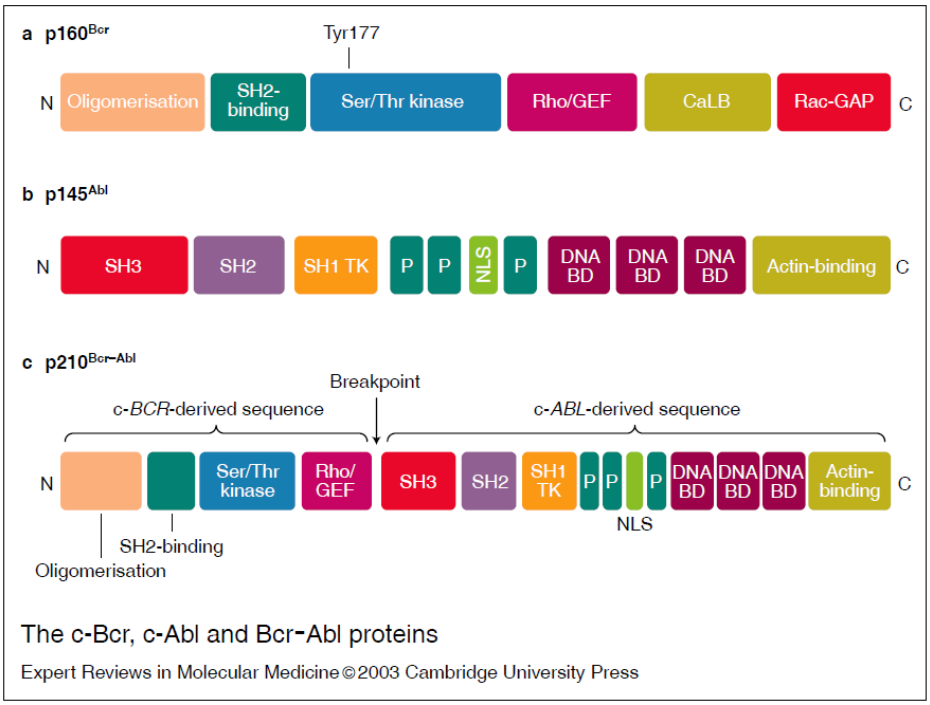

Figure 2: c-BCR, c-ABL and BCR-ABL proteins

The ABL protein

The ABL gene is normally found on chromosome 9(q34) and it is composed of 11 exons (Figure 2). It has two distinct promoters, Pa (proximal) and Pb (distal) that permit the beginning of mRNA transcription from exon 1a or exon 1b respectively. Hence, two distinct mRNAs are produced of 5kb or 6,5kb in length that differ only with respect to their 5' sequence. The ABL type 1b protein contains a myristoylation site at the N-terminus, that allows anchoring to the plasma membrane(20) and stabilizes the inactive conformation of the protein(21). The amino acid numbering of the ABL protein that will be used in this thesis always refers to ABL exon Ia sequence. The whole ABL gene is involved in the translocation, with the more frequent breakpoint located between the exon Ia and the exon Ib. During the translocation, the Pb promoter remains intact but seems to have no influence on BCR-ABL transcription(15). The ABL protein is an ubiquitously expressed non-receptor tyrosine kinase of 145kD, that can shuttle between the nucleus and the cytoplasm thanks to the presence of three nuclear localization signals (NLS) and one nuclear export signal (NES) in its C-terminal region(22).

ABL regulates cytoskeleton remodelling during cell differentiation, cell division and cell adhesion. It localizes to dynamic actin structures, and phosphorylates CRK and CRKL, DOK1, and other proteins controlling cytoskeleton dynamics. ABL regulates DNA repair potentially by activating the proapoptotic pathway when the DNA damage is too severe to be repaired. Phosphorylated PSMA7 by ABL leads to an inhibition of proteasomal activity and cell cycle transition blocks(23-25).

The functions of the ABL protein are many and complex(26). Nuclear ABL has been implicated in the regulation of the cell cycle, inducing cell cycle arrest in the G1 phase(27, 28) and it has been shown to have a role in the cellular response to genotoxic

stress(29). Cytoplasmic ABL is instead involved in the transduction of signals from membrane integrins(30). The ABL-deficient mice present multiple defects including high postnatal mortality, runting, morphological abnormalities, susceptibility to infections, and reductions in lymphocytes and their precursors. ABL-null mice show high neonatal mortality and decreased B lymphocytes(31).

The ABL N-terminus portion is characterized by the presence of three domains with a high degree of homology to domains found within the Src non-receptor tyrosine kinases. Src 13 homology 1 (SH1) is the ABL kinase domain, Src homology 2 (SH2) and Src homology 3 (SH3) are non catalytic domains and act as binding sites for other proteins(32). The SH2-ABL domain binds to regions containing phosphotyrosines and can also bind to phosphoserine containing sites within BCR(33), while the SH3 domain is essential for the regulation of SH1. The central region of the ABL protein contains proline-rich sequences functioning as binding sites for the SH3 domains of other proteins, such as Crk(34) and Crkl(35). The ABL C-terminal region contains, in addition to the nuclear localization and nuclear export signals reported above, other functional domains such as motifs that allow the association with actin filaments (G-actin and F-actin binding domain)(36) and the binding to DNA sequences (DNA binding domain)(27) (Figure 3). Different studies have shown that despite the structural homology between ABL and Src kinases, the mechanisms responsible for the regulation of ABL kinase activity are not entirely comparable to that of Src. The mechanism that inhibits the kinase activity of the SH1 Src domain has been shown to require phosphorylation of Tyrosine-527(37), an amino acid that has no homologue in the ABL C-terminus. Mutational studies have been useful to determine the role of ABL domains in the control of its activation: the last exon of ABL, encoding the C-terminus, was found

to be dispensable for its kinase domain activation, while the N-terminus has proven to function as an inhibitory "cap" that clamps intramolecular domains into a conformation that suppresses ABL kinase activity(38). It has also been shown that the myristoyl group present in the ABL Ib isoform helps to stabilize the protein in its inactive form(21). Myristate binds(39) within a hydrophobic pocket at the base of the c-Abl kinase domain, docking the SH2 (40) domain against the kinase domain in such a way that it prevents activation of the kinase (41) by phosphotyrosine ligands. Despite of these differences, some features of the Src regulatory mechanisms are shared by ABL. The SH3 domain is required in both ABL and Src for the inhibition of SH1 activity and its inhibitory-influence is dependent upon its position within the molecule. In fact it has been demonstrated that the insertion of three amino acids between the SH3 and SH2 domains is sufficient to block the repression of the SH1 region(42).

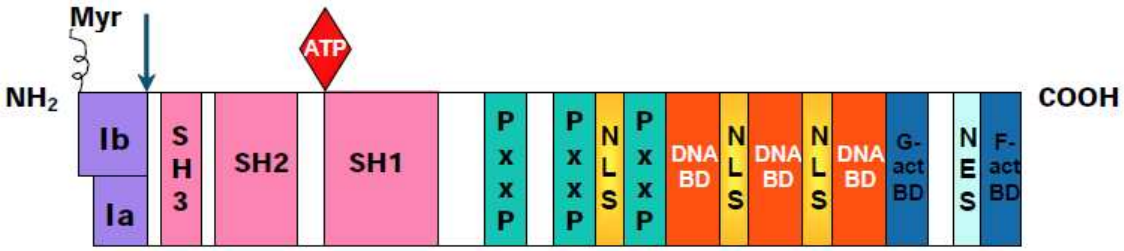

Figure 3: Structure of the ABL protein. The two alternative exons Ia and Ib are represented. The ABL Ib isoform is myristoylated and larger than the Ia. Downstream of the blue arrow are the sequences present in the BCR-ABL fusion protein. In the figure are highlighted the Src homology domains (SH1, SH2, SH3), the proline rich regions (PxxxP), the nuclear localization signals (NLS), the single nuclear export signal (NES), the DNA binding domain (DNA-BD) and the actin binding domain (G-actBD and F-actBD).

The BCR protein

The BCR gene (Breakpoint Cluster Region) is ubiquitously expressed as a 160 kDa cytoplasmic protein (Figure 4) and is located on chromosome 22(q11). Results obtained from Bcr knockout mice suggests that the functions of BCR are not essential for normal cell physiology(43). In fact Bcr-null mice are viable and characterized only by an increased neutrophil respiratory burst.

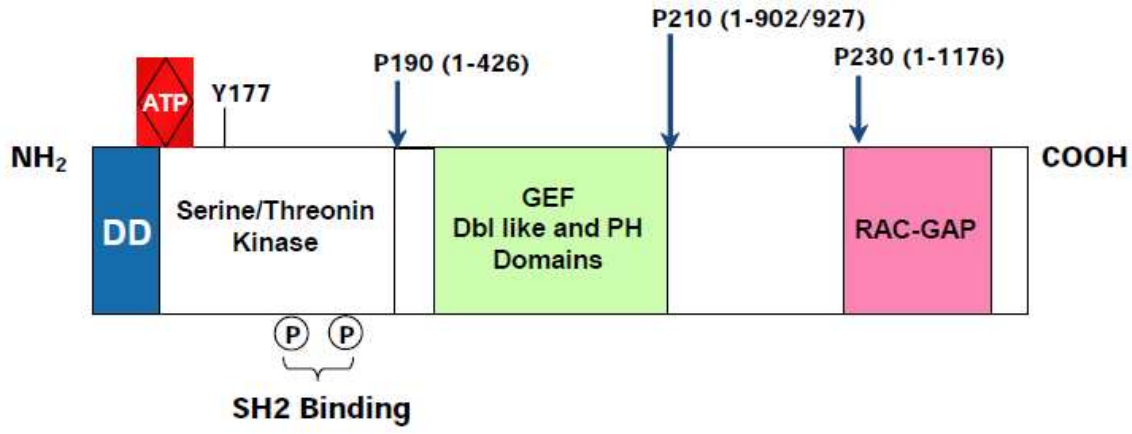

Figure 4: Structure of the BCR protein. An oligomerisation domain (DD) is located at the N-terminus of the protein. In the figure are also shown the serine/threonine kinase domains, Tyr177 (Y177) and the region with homology to guanine nucleotide exchange factors (GEFs) known also as dbl-like and pleckstrin homology domain (PH). The C-terminus of the protein can function as a GTPase-activating protein for the GTP-binding protein Rac (RAC-GAP). The arrows indicate the position of the main BCR breakpoints in the BCR-ABL fusion protein.

The BCR region consisting of the first 426 amino acids is encoded by the first exon (e1) of the gene and it is present in all the BCR-ABL isoforms derived from the reciprocal translocation. The N-terminal region of the protein contains a serine-threonine kinase domain and a Tyrosine in position 177 (Tyr177). The Tyr177 is a key residue since, when phosphorylated, it forms a binding site for Grb-2, an adapter protein that links BCR to the Ras pathway(44). In exon1 (Figure 4) there are also two serine-threonine-rich domains (aa176-242 and aa298-413) that could bind to SH2 domains of other proteins

and have an important role for the transforming activity of BCR-ABL(33). The first 72 amino acids of the protein consist of a coiled-coil oligomerization domain. This region is essential for BCR-ABL oncogenicity, allowing the formation of oncoprotein oligomers that leads to the activation of BCR-ABL and to its interaction with F-actin(45, 46). The central region of the protein (from exon 3 to exon 10) shares homology with several guanine nucleotide exchange factors (GEFs) that (28) normally are able to stimulate the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP). This region is also called dbl-like (Dbl) and pleckstrin homology domain (PH). The C-terminus of BCR contains a GTPase-activating domain (GAP) for Rac(p21Rac) known as GAPRac that catalyses the hydrolysis of GTP to GDP. It thus seems that BCR has a dual function in guanine nucleotide-binding protein signalling, with an excitatory role for the central domain and an inhibitory one from the C-terminus domain.

The BCR-ABL oncoprotein

The reciprocal translocation t(9;22)(q34;q11) between ABL and BCR leads to the formation of the BCR-ABL fusion gene on chromosome 22, generating the Philadelphia chromosome. The mechanisms responsible for this genetic rearrangement remain elusive. It has been suggested that the localization of chromosome 9 and 22 in the nucleus could favour the fusion between these two genes during the transition between the S and the G2 phase of the cell cycle. In fact fluorescent in situ hybridisation (FISH) and confocal microscopy showed a juxtaposition of ABL and BCR during this phase(47, 48). It has also been suggested that homology regions, such as Alu sequences, between the two chromosomes, may have a role in the BCR-ABL recombination that initiates CML(49). Epidemiological studies suggest that environmental causes, such as ionising radiation and chemical compounds (e.g. benzene(50)), are risk factors for CML(51-54). It is in fact

known that ionising radiation (IR) is able to induce DNA double-strand breaks and it has been shown that a high dose of IR can lead to the in-vitro formation of the BCR-ABL fusion. Actually, chromosomal translocations could occur as a consequence of DNA double strand breaks on non-homologous chromosomes(55) repaired erroneously by genomic repair enzymes. Although BCR-ABL contains nuclear localization signals, it is restricted to the cytoplasm where it activates mitogenic and anti-apoptotic pathways. It has been demonstrated that this localization is critical for its oncogenic potential, since the nuclear expression of BCR-ABL activates apoptosis(56).

Different breakpoint regions on ABL and BCR gene can create various BCR-ABL isoforms. On chromosome 9, breaks on the ABL gene can occur in three different regions, but more frequently between the exon Ib and the exon Ia (Figure 5). In every one of the three cases there are no differences in the fusion protein because the ABL exon I is never transcribed and the BCR-ABL protein consists of BCR exons fused directly to ABL exon a2. The breakpoints within the BCR gene are also found in three different regions but, unlike those in ABL, they have a functional impact on BCR-ABL protein, giving rise to three oncoprotein isoforms. The breakpoint region on BCR gene are known as Minor Breakpoint Cluster Region (m-BCR), Major Breakpoint Cluster Region (M-BCR) and Micro Breakpoint Cluster Region (μ-BCR) (Figure 6) and lead to the formation of BCR-ABL isoforms. Since the ABL component of the oncoprotein is invariant, the variability in disease phenotype is due to different length of protein sequences encoded by BCR.

There are three main BCR-ABL isoform proteins (Figure 5):

- p230, the longest form with less oncogenic potential than other isoforms;
- p210, the responsible of CML;

- p190, the smallest form, associated to Acute Lymphoblastic Leukemia (ALL).

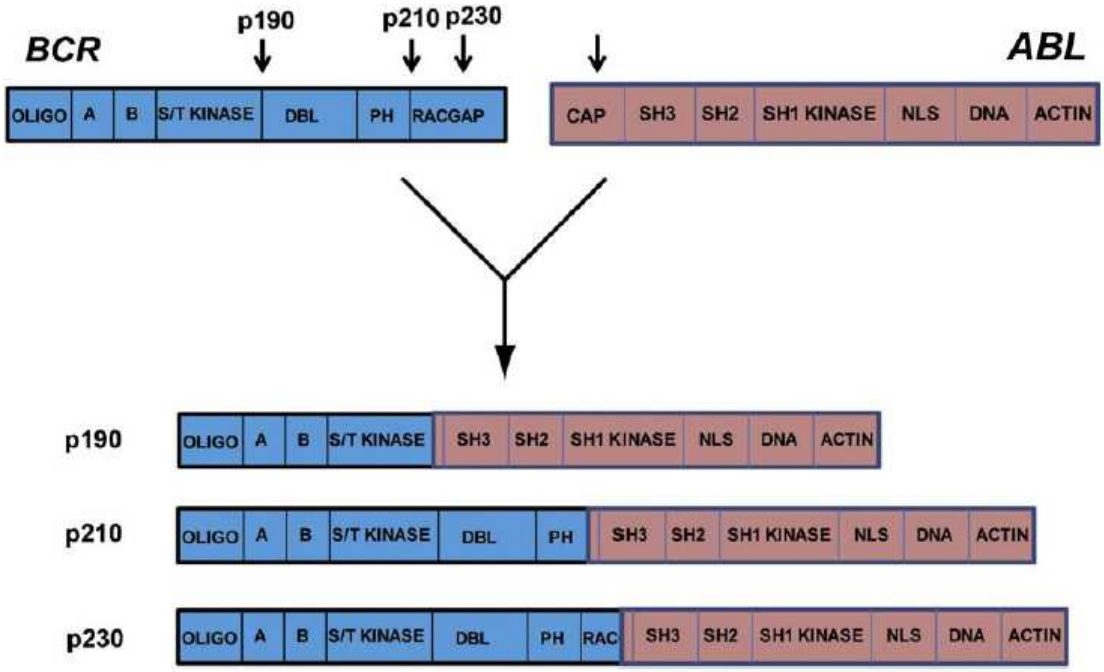

Figure 5: Distinct breakpoints in the BCR gene can lead to formation of different BCR-ABL oncogenes, each with unique leukemic features. The oncogene encoding the p190 isoform is associated with B-ALL, while the oncogene encoding the isoform for p210 is associated with CML. The oncogene encoding the p230 isoform has weaker kinase activity, and its leukemic phenotype is more closely associated with leukemias of myeloid origins.

The largest BCR-ABL isoform p230BCR-ABL has been described mainly in patients affected by Ph+ chronic neutrophilic leukaemia (CNL)(57). CNL has a milder clinical course in relation to CML. It is characterized by lower total white blood cells count, less severe anaemia, less prominent splenomegaly and a delay in the progression to blastic transformation. Hence, p230BCR-ABL seems to have less oncogenic potential than p210BCR-ABL. The p230 isoform contains over 90% of the BCR protein sequence and lacks only the last 2/3 of the GAPRac homology domain(58). It has been proposed that the N-terminal region of the GAPRac domain contained in the p230BCR-ABL could be functionally active and that this "gain of function" could result in a milder myeloproliferative phenotype(58).

p190 is the smallest BCR-ABL isoform. Since the alternative BCR exons e1' and e2' are not present in the mature chimeric mRNA due to alternative splicing, the contribution of BCR to the oncogenicity of the p190 fusion protein is limited to the sequence encoded by the exon e1. Consequently, the BCR domains present in the oncoprotein are the oligomerisation domain, the serine-threonine kinase domain, the serine-threonine rich domains and Tyr177 (Figure 3). Acute Lymphoblastic Leukaemia (ALL) is the major disease phenotype associated with p190 BCR-ABL although it is present also in a minority of CML patients characterized by prominent monocytosis. It has been proposed that the oncogenic potential of this form of oncoprotein is restricted to the lymphoid and monocytic lineages, suggesting that p190-induced transformation is mediated by interference with pathways involved in lymphoid(59) and monocytic development(33). In fact, myeloid differentiation seems to be influenced by Dbl and PH domains that are not present in the p190 isoform(60). Further evidence for the different activities of p190BCR-ABL and p210BCR-ABL derives from the finding that the ABL SH2 domain is necessary for the transforming activity of the oncoprotein p210 but not for that of p190. This result confirms that the two oncoprotein isoforms activate distinct signalling pathways(46).

BCR-ABL p210 has the Dbl homology and pleckstrin homology (PH) domains from BCR, while the smaller form p190 doesn't have these domains. Thus, a complete understanding of the biological mechanisms underlying the origin of ALL and CML requires the characterization of the signaling activities that reside within BCR. Recently, PH domain has been shown to bind various phospholipids and to take part in protein-protein interactions(61). Moreover, the BCR-ABL protein has predominantly cytoplasmic localization generating cortical-F-actin ring(62) due to actin-binding domain

in Abl moiety of BCR-ABL hybrid protein(63). PH domain has been shown to bind to the membrane of Golgi complex, in particular with $PIP_{(3)}$ and $PIP_{(4)}$. The different patterns of the intracellular localization of BCR and BCR-ABL may be attributable to the different domains interacting with different multiprotein complexes. PDZ-binding domain in C-terminal part of BCR provides for its interaction with AF-6 protein facilitating Ras binding and producing the multimeric complex. As a result, Ras and ERK are down-regulated(64). Besides, PDZ-binding domain provides for the interaction between BCR and the apical PDZ-K-1 and Mint 3, the latter being the component of the vesicular trafficking in the secretory pathway(65). For understanding the role of BCR in cell biology, the fact of its displacement to the membrane upon the effects of growth factors is of high importance(66). Thus, from experimental evidence, in K562 CML cells BCR-ABL is predominantly localized to the cell periphery. This fact seems to be explained by the presence of actin binding domain in BCR-ABL hybrid protein. On the other hand, the localization of BCR may be partly explained by the presence of PH and PDZ-binding domains. Recently, Telegeever demonstrated that in K562 CML cells BCR-ABL is predominantly localized to the cell periphery. This fact seems to be explained by the presence of actin binding domain in BCR-ABL hybrid protein. Signalling network analysis indicated that PH domain of BCR-ABL protein could be involved in signalling cascades regulating metabolic processes, DNA integrity, cell proliferation, cell motility and cell adhesion(67).

Figure 6: Schematic representation of ABL exons (yellow). The breakpoint regions are indicated with blue arrows.

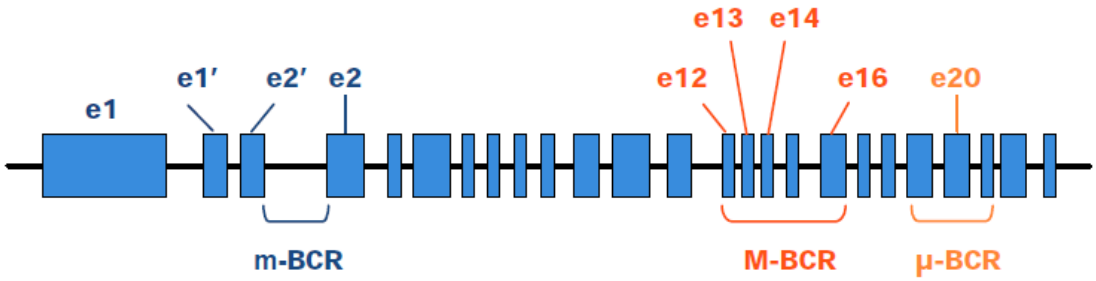

Figure 7: Schematic representation of BCR exons (light blue). Breakpoint regions are indicated: Minor Breakpoint Cluster Region (m-BCR), Major Breakpoint Cluster Region (M-BCR) and Micro Breakpoint Cluster Region (µ-BCR). e1' and e2' are the alternative BCR exons not present in the mature mRNA.

Interaction between BCR and BCR-ABL

In Ph^+ cells, BCR-ABL phosphorylates BCR on tyrosine residues (Y177) reducing its kinase activity. It is know that the overexpression of BCR in BCR-ABL cells produces a phosphoserine form of BCR, which inhibits the oncogenic effects of BCR-ABL (68). In fact, BCR becomes resistant to tyrosine phosphorylation in BCR-ABL cells when its expression is in molar excess. In this condition, the excess BCR protein is mostly in the phosphoserine form and reduces the phosphotyrosine content of BCR-ABL, strongly inhibiting its oncogenic activity(68, 69). This phosphoserine form of BCR is also predominant after overexpression of BCR in soft agar clones of the CML K562 line, a Ph^+ cell line, containing an inducible BCR gene. As a consequence, these clones have reduced ability to induce extramedullary leukemia(69). On the basis of these results, the authors proposed that the BCR protein plays two roles in CML(70). In the tyrosine-phosphorylated form, BCR would be neutralized as an inhibitor of BCR-ABL effects and would serve as an important facilitator of BCR-ABL-induced leukemia, possibly in the form of a heterotetramer structure with BCR-ABL(45). On the other hand, in the serine/threonine-phosphorylated form, BCR would function as an inhibitor of BCR-ABL oncogenic ability. Perazzona et al. propose that BCR may play a role in generating the myeloid phenotype caused by BCR-ABL in CML patients and may be an important player in the chronic phase of CML by down-modulating BCR-ABL. The investigation of the mechanism of the interaction between BCR and BCR-ABL proteins, based on a TonB210 cells model, in which BCR-ABL expression is controlled by a tetracycline-inducible promoter and BCR is stably transduced by lentivirus infection, the levels of BCR-ABL expression increased the levels of the BCR protein(71). After imatinib treatment of TonB210 cells the levels of BCR-ABL and surprisingly the BCR protein as

well decreased, indicating that the tyrosine kinase function of BCR-ABL is required to up-regulate BCR protein expression. In addition, withdrawal of doxycycline also reduced BCR-ABL and BCR protein levels, confirming that BCR-ABL is required for increased expression of the BCR protein. In BaF3 and 32D cells, BCR was expressed at extremely low levels, but the treatment with the proteasome inhibitor calpain inhibitor I restored BCR expression. Forced expression of BCR-ABL in BCR-transduced cells restored high expression of BCR protein, confirming that BCR-ABL is required for preventing degradation of the BCR protein. Together these findings indicate that BCR-ABL up-regulated BCR expression by interfering with proteasome-mediated degradation of the BCR protein(71).

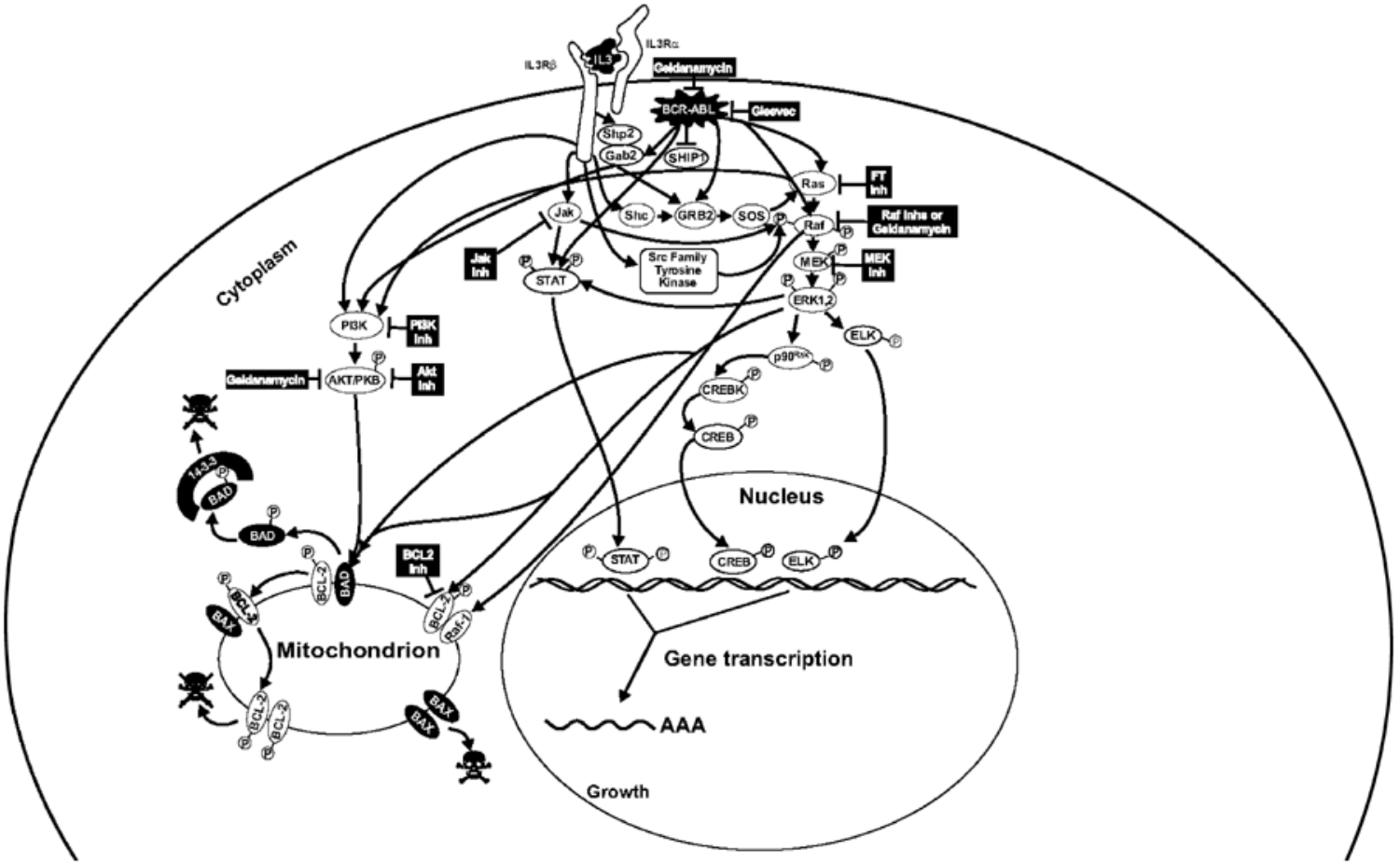

Figure 8: BCR-ABL activation of cytokine signal transduction and sites of inhibition. BCR-ABL is a potent oncogene that can affect many downstream signaling pathways including JAK/STAT, PI3K/Akt and Raf/MEK/ERK. Furthermore, BCR-ABL can affect adaptor proteins such as Gab2 and phosphatases such as SHIP1, which further fine-tune these pathways. Many sites of inhibition of cell growth have been discussed in this review and some of them are indicated in the figure. As chemotherapeutic drugs such as imatinib are used more frequently to treat CML, more imatinib resistance will be observed. Potential methods to circumvent this drug resistance include treatment of the cells with inhibitors that target other molecules in the pathway dysregulated by BCR-ABL (eg, Ras, Raf, MEK, PI3K, BCL-2) or affect BCR-ABL function by different mechanism (eg, geldanamycin).

BCR-ABL activity

BCR-ABL is a constitutively activated tyrosine kinase, with high activity compared to ABL(72). BCR-ABL lacks ABL first exon sequences and myristoylation but retain the SH domains and has gained sequences from N-terminus of BCR, including BCR first exon-derived sequence common to all BCR-ABL isoforms and determinig the oligomerization of the protein. Theoretically, dysregulation of ABL could be due to either loss of the ABL first exon or gain of the BCR first exon polypeptides. In type Ib c-ABL, recent studies demonstrated that sequences N-terminal to the SH3 domain (the N-terminal "cap") bind to the ABL catalytic domain(38) and are required along with the myristoyl group(73) for proper regulation of ABL kinase activity upon overexpression in vivo. In agreement, a crystallographic study demonstrated a physical interaction of the myristoyl group with the C-terminal lobe of the ABL catalytic domain(74). Binding of the myristoyl group induces a conformational change that permits the docking of the SH2 domain with the non-catalytic face of the C-lobe in a fashion that closely resembles the inactive conformation of Src(75). These observations suggest that loss of the myristoly group and the portion of the cap donated by the ABL first exon might contribute to dysregulation of BCR-ABL. However, the c-ABL Ia isoform and several c-ABL mutants are not constitutively active despite lacking the type Ib cap and myristoyl group, indicating that ABL dysregulation is not an inevitable consequence of deletion of the first exon(76).

BCR-ABL is active in a high variety of cell pathway and it can influence cell cycle, inducing proliferation in absence of physiological stimuli, and also promotes the survival and inhibits apoptosis. BCR-ABL is able to alter the cell adhesion and to cause a modification in cell homing in the bone niche. A summary of the BCR-ABL influenced pathways is represented in the figure 8.

RAS pathway

Ras proteins are small GTPases that act as molecular switches, transducing signals from activated receptors to downstream effectors to regulate cell proliferation, survival and differentiation. Ras small GTPases are activated in many hematopoietic growth factor signalling and in hematological malignancies, but their role in haematopoiesis and leukemogenesis is not completely known. The BCR portion is fundamental for the interaction of BCR-ABL with the growth factor receptor-binding protein (GRB2)/Gab2 complex, via the GRB2-binding Y177 site in the BCR portion of BCR-ABL that shows a high affinity-binding for the Grb2 SH2 domain when phosphorylated. The interaction of BCR-ABL with GRB2/Gab2 and the phosphorylation of SHC lead to enhanced activity of the guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor SOS, which promotes the accumulation of the active GTP-bound form of RAS. Activated Ras recruits phosphotidylinositol-3 kinase (PI3-K) and thus the downstream pathway. The importance of RAS-dependent signalling for the phenotype of BCR-ABL/expressing cells is supported by the observation that by the use of antisense strategies, expression of dominant negative molecules or chemical inhibitors causing a downregulation of RAS pathway suppresses proliferation and sensitizes cells to apoptosis stimuli. Additionally, in a mouse model of CML, in which the disease is induced by injection of cells transfected with BCR-ABL in a retroviral vector(77, 78), the mutation of Y177F induced a T cell leukemia and lymphoma after a prolonged latent period(79-81). The importance of Y177, linking BCR-ABL to the Ras signaling, suggests that Ras plays a critical role in the pathogenesis of CML.

PI-3K/Akt pathway

The PI3k/Akt pathway is constitutively active in CML cells and plays a major role in cell survival. It has been demonstrated that PI3 kinase activity is regulated by BCR-ABL and is required for the growth of CML cells(82). On activation, Akt phosphorylates key survival proteins such as the transcription factor regulator glycogen synthase kinase 3b (GSK3b) which in turn regulates Mcl-1 and other downstream proteins, resulting in decreased cell susceptibility to apoptosis(83). The BCR-ABL oncoprotein forms multimeric complexes with PI3 kinase and the adapter proteins Cbl and Crk(35) that ultimately lead to the activation of the serine-threonine kinase Akt(84). Activation of Akt causes a proliferative effect down-regulating the cell cycle inhibitor p27(85) and causes also an anti-apoptotic effect inducing the phosphorylation of the pro-apoptotic protein Bad, thus preventing Bad-mediated inactivation of the anti-apoptotic protein Bcl-XL. Survival signals mediated by the cytokine IL-3 are transduced into the cell via the PI3/Akt kinase pathway. BCR-ABL activity can thus substitute for the survival signals provided by IL-3 supply.

The MAP Kinase pathway

MAPK signalling consists of a three-kinase cascade module composed of a MAPK kinase kinase (MAPKKK or MEKK) that activates a MAPK kinase (MAPKK, MKK or MEK) which ultimately activates a MAPK enzyme. In mammals, it is possible to define four distinctly regulated groups of MAPKs regulated by specific MAPKKs: ERK1/2 regulated by MEK1/2, p38 by MKK3/6, JNKs by MKK4/7 (JNKK1/2) and ERK5 by MEK578. BCR/ABL can activate the MEK1/2- ERK1/2 signalling pathway thanks to the autophosphorylation of tyrosine 177 that generates a binding site for the adapter

molecule Grb-227,(40, 41). Grb-2 associates with the Sos protein stimulating the conversion of the GDPRas to the active GTP-Ras(40). Ras is thus able to activate Raf-1 which can act on the MEK1/2-ERK1/2 proteins (Figure 8) that ultimately activate gene transcription. Additionally, it has been shown that Ras can be activated by Shc, CrkL and Dok adapter proteins, which are known to be BCR/ABL substrates(40). The relevance of Ras Crkl-dependent activation is questionable because it has been shown that it is important for BCR/ABL transformation of fibroblasts, but it is not required for myeloid cells transformation(86, 87). Although ERK2 activation has not been detected in Ba/F3 cells transfected with a temperature sensitive p210BCR/ABL molecule(88), another study demonstrated that BCR/ABL activates the Ras-Raf-MEK1/2-ERK1/2 pathway in haematopoietic cells(41). The importance of the MAP-kinase pathway in the BCR/ABL-mediated transformation is emphasized by the discovery that the oncoprotein activates also the JNK/SAPK-MAPK pathway(89). Interestingly, the p38-MAPK pathway has been shown to be activated by IFN-α in primary cells from CML patients and in BCR/ABL-positive cells(90). It is therefore possible that the oncoprotein negatively regulate this pathway and that IFN-α, which is used as a pharmacological treatment for CML could overcome this inhibition leading to an antiproliferative effect.

The JAK-STAT pathway

Constitutive phosphorylation of the STAT family of transcription factors has been detected in BCR/ABL-positive cell lines(91) and in primary CML cells(92). In physiological conditions STAT proteins are phosphorylated downstream of Janus kinase (JAK) activation(93), while BCR/ABL appears to phosphorylate directly multiple STAT family members(94). It has been demonstrated that the activation of STAT5 by p210BCR/ABL contributes to the malignant (36) transformation of haematological cell

lines(95), being responsible for the enhanced transcription of the anti-apoptotic BCL-XL protein(96, 97).

The MYC pathway

The MYC gene is over-expressed in many human malignancies(32). In physiological conditions MYC is a transcription factor; its targets were recently identified(98) and include genes involved in cell cycle regulation (e.g. cyclin-dependent kinase 4 known as CDK4) and genes involved in apoptosis (e.g. prohibitin known as PHB).

The link between BCR-ABL and MYC induction is not well understood, although the induction of MYC expression caused by v-Abl has been already described. This model proposes that v-Abl initiates a cascade of phosphorylation involving Ras, Raf, cyclindependent kinases (cdks) and the transcription factor E2F that ultimately activates and binds to MYC promoter(99).

Experiments with rat fibroblasts showed that MYC could be activated downstream of BCR-ABL and that it was involved in the cellular transformation(100). Moreover, in mouse bone marrow cells the expression of a dominant negative form of MYC blocked the BCR/ABL transforming potential(100), confirming the role of MYC activation in CML oncogenesis.

More recently, BCR-ABL has been shown to activate survivin, an important regulator of cell growth and survival(39, 101), but the precise molecular mechanisms behind its expression and consequences thereof in CML cells remain unclear. BCR-ABL promotes survivin expression and its cytoplasmic accumulation. The increase of survivin was largely controlled at the transcriptional level through a mechanism mediated by JAK2/PI3K signal pathways that activated c-Myc, leading to transactivation of survivin promoter. Dynamic down-regulation of survivin was a key event involved in imatinib-

induced cell death while forced expression of survivin partially counteracted imatinib's effect on cell survival. Additionally, short hairpin RNA-mediated silencing of survivin or c-Myc inhibits the colony formation of K562 cells in soft agar culture system, suggesting an important role for c-myc transcriptional network in BCR-ABL-mediated cell transformation and survival(102).

Altered cellular adhesion

In CML, HSCs exhibit reduced adhesion to stromal cells and to the extracellular matrix of the bone marrow(103), causing a loss of regulatory signals that are normally supplied to haematopoietic progenitors. Cell surface receptors of the integrin family have an important role in transmitting signals from the extracellular environment. In particular the β1-integrins, during haematopoiesis, function as negative regulators of cell proliferation(104, 105). It has been shown that in CML cells, the activity of β1-integrins is diminished, in part explaining the impaired binding of oncogenic cells to the bone marrow stroma(103, 106). It is also known that integrins are connected, via their cytoplasmic tail, with various cytoskeletal proteins including the F-actin which can also complex with BCR-ABL(36). Since BCR-ABL has been shown to interact with cytoskeletal proteins, it is thus possible that these interactions could influence cytoskeletal and integrin functions. All these effects could also lead to the release of premature CML cells from the bone marrow, contributing to the accumulation of undifferentiated cells in the peripheral blood.

Apoptosis and Autophagy

When the cells acquire DNA damage, the damage-sensiting proteins recognize the error and stimulate repair, but when the damage is too great the cell may instead signal to undergo apoptosis. This prevents the replication of potentially harmful mutations, and evading apoptosis is one of the hallmarks of cancer. Another of the known roles of BCR-ABL is the inhibition of apoptosis. The resistance of K562 cells to apoptosis is a consequence of BCR-ABL expression, as antisense oligonucleotide treatments against BCR-ABL induce apoptosis(107, 108).

Growth-factor-dependent human or murine cell lines transfected with exogenous BCR-ABL do not undergo apoptosis after growth factor withdrawal(98, 109). Furthermore, cell lines transfected with BCR-ABL have been reported to show an increased resistance to DNA-damage induced apoptosis(110, 111). Cells expressing a high level of BCR-ABL expressed constitutively high level of p53, p21 and Bax, and low level of Bcl-2, and cytotoxic insult did not alter these levels. The reason is that high levels of BCR-ABL prevent the translocation of the pro-apoptotic proteins Bax and Bad to the mitochondria, the late mitochondrial depolarization and the Caspase 9 and 3 processing(112). It has been shown that the cytochrome-C release from mitochondria is apparently blocked in BCR-ABL expressing cell lines(113). In agreement with this, there is over-expression of the anti-apoptotic protein BCL-2 (84, 114) and silencing of the pro-apoptotic BCL-2 family member BIM(115). It seems that the inhibition of BIM expression is directly dependent upon the phosphorylation of the transcription factor FoxO3A. FoxO3A was found to be inhibited through phosphorylation in the presence of the active BCR-ABL, thus being unable to activate BIM transcription(115, 116).

It has been demonstrated that BCR-ABL localization is critical for its anti-apoptotic

potential(56). The oncoprotein has a different subcellular localization compared to the normal counterpart ABL. In fact ABL is found in both the nucleus and the cytoplasm, shuttling between these two components, while BCR-ABL is exclusively cytoplasmic. Interestingly, nuclear ABL is a positive inducer of apoptosis, while cytoplasmic BCR-ABL induces proliferation. It has been demonstrated that nuclear entrapment of BCR-ABL, by treating cells with the nuclear export inhibitor Leptomycin B (LMB), results in apoptosis induction, suggesting an important role of BCR-ABL localization for tumorigenic potential(56).

Autophagy is a highly conserved catabolic process for the elimination and recycling of organelles and macromolecules, characterized by the formation of double-membrane vesicles called autophagosomes. To date, the function of autophagy in cell differentiation is poorly documented. The treatment of BCR-ABL cells with specific anti-cancer agents, INNO-406, a second-generation BCR-ABL TK inhibitor, induced the BCR-ABL cells death by autophagy(117), representing an alternative to the apoptosis in the cases in which the mechanisms of programmed cell death is not functionally active.

BCR-ABL and the DNA damage and repair

The appearance of chromosomal abnormalities in patients with BC-CML has led to many attempts to elucidate the mechanisms by which BCR-ABL affects DNA damage and repair.

Several common themes emerge from previous studies(84, 118): the presence of BCR-ABL increases the genomic and chromosomal instability in cells, although the increase is modest. But after a long period of BCR-ABL expression, the frequency of appearance of DNA damage increases. BCR-ABL has been shown to induce the production of reactive oxygen species, which cause oxidative damage and mutation(119-121).

Briefly, DNA damage mechanisms could lead to single-nucleotide alterations, single-strand breaks or double-strand breaks (DBSs). Single-strand breaks are prone to generate DSBs and the two will be considered together here. Single-nucleotide alterations are repaired by mismatch repair (MMR) or by nucleotide excision repair (NER) mechanisms. Strand breaks are repaired by either high-fidelity homologous recombination when a sister chromatin is available as a template (during the S or G2 phase of the cell cycle), or by non-homologous end joining (NHEJ), which may lead to short deletions in the repaired strands. Figure 9 summarizes the influence of BCR-ABL on the DNA repair mechanism.

DNA mutations can occur as the result of several situations; when there are mutations in single-nucleotide repair pathways, mutations in proteins necessary for the complex process of DNA DSB recognition and repair or, alternatively, when there is a failure of cell cycle checkpoints that allows subsequent replication of damaged DNA. The latter may occur because of defects in sensing DNA damage or defects in proteins necessary to execute the cell cycle arrest. Effects of BCR–ABL on all of these pathways have been described and summarized in the figure from Burke and Carroll's review(122).

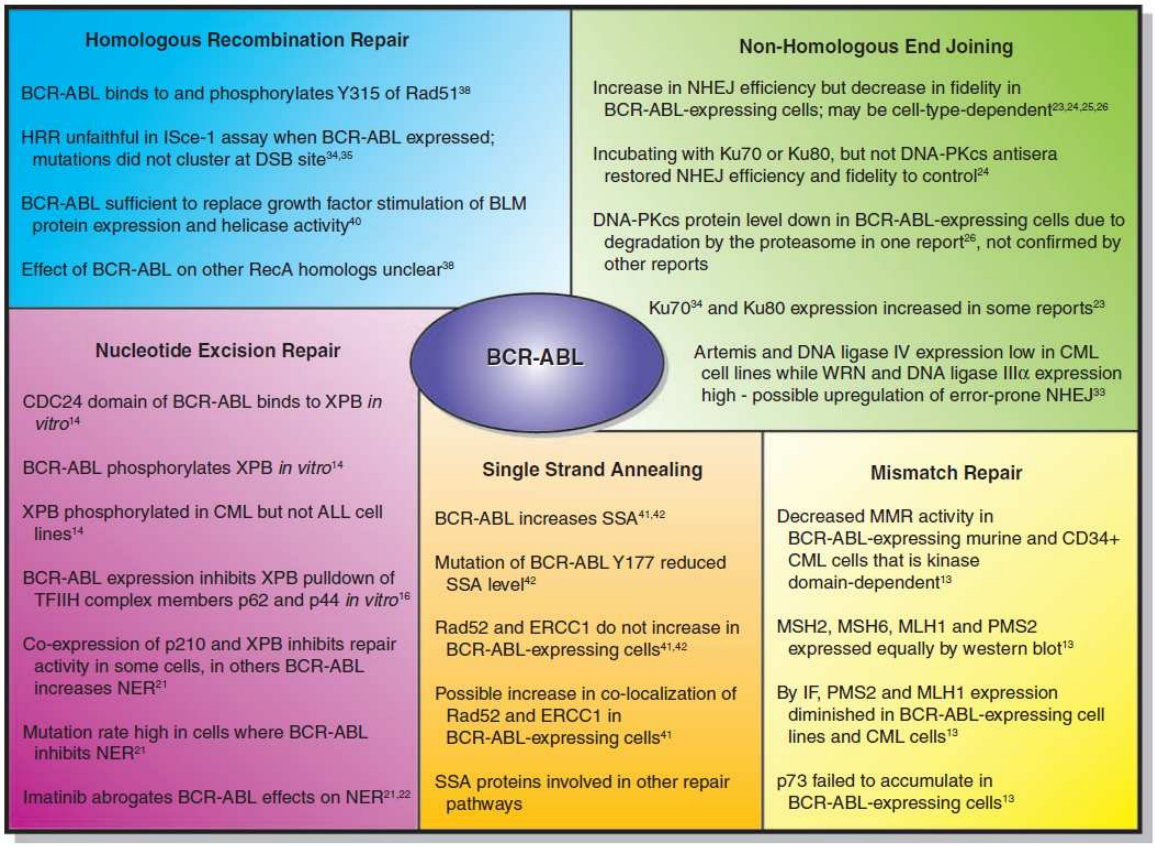

Figure 9: BCR-ABL affects the DNA repair mechanisms[(122)].

CML treatment

In CML it is possible to distinguish three different levels of response to the therapy:

- Haematologic response: a complete hematologic response (CHR) means that the numbers of white cells, red cells and platelets are normal or near normal; partial haematologic response (PHR) means that the numbers of blood cells is altered;
- Cytogenetic response: complete cytogenetic response (CCyR) means that there are no Ph + cells that can be detected; the complete response is obtained by the cytogenetic analysis, based on a standard karyotype with 20 metaphases, shows the absence of BCR-ABL positive cells; major cytogenetic remission (MCyR) by the same analysis shows the presence of less than 33% of BCR-ABL positive cells; minor cytogenetic remission (MyCyR) shows the presence of more than 33% and less than 66% of BCR-ABL positive cells; absence of cytogenetic remission shows the presence of more than 90% of BCR-ABL positive cells;
- Molecular response: a partial molecular response means that there is a reduction in the number of cells with the BCR-ABL cancer gene. A major molecular response (MMR) means that there is a 1,000-fold decrease in the level of cells with the BCR-ABL gene from the level measured at the start of treatment. A complete molecular response (CMR) means that the BCR-ABL cancer gene cannot be detected by PCR.

If not effectively treated, CML follows an inexorable course that results in the progression to the advanced phases and eventually in the death of the patient. The finding of BCR-ABL as the main causative factor of CML led to the design of a specific therapeutic agent able to inhibit the enzymatic activity of the oncogenic protein.

During the 1970s, the main drug for the CML treatment was interferon-α (IFN-α). This

drug was able to cause a sustained reduction in Ph+ marrow cells, with some patients able to achieve a MCR or a CCyR(123). Unfortunately, prolonged administration of high dose IFN-α was not well tolerated in most of the cases(124).

Until the introduction of stem cell transplantation(125), CML treatments were purely palliative. Currently, only allogeneic stem cell transplantation has been shown to provide a long term eradication of the disease (126) but only a small minority of CML patients are eligible for this therapeutic option. Limitations relate to the availability of suitable donors and the treatment related mortality due to acute/chronic graft-versus-host disease or infections(126). It has been demonstrated that the transplantation related mortality ranges from 5 to 50%, and that the prognosis is better if the transplantation is performed during the chronic phase CML(15).

In the 1990s a small molecule known as Imatinib (CGP57148B, STI571; Glivec, Novartis Pharmaceuticals) was found to inhibit specifically BCR/ABL kinase activity(127-129).

Imatinib is a member of the class of 2-phenylaminopyrimidine compounds, derived from a random screening for inhibitors against PKC-α(130). It has an high specificity of action against c-ABL and the activated ABL fusion proteins like p210BCR-ABL. Imatinib is also able to inhibit c-Kit, the platelet derived growth factor receptor (PDGFR-β), the ABL Related Gene (ARG) and Lck, but it has no significant activity against other kinases(131). The molecular mechanism of BCR-ABL inhibition by imatinib is represented by the binding of the inhibitor to the SH1 domain of the protein, corresponding to the kinase domain of ABL, in particular imatinib binds the ATP site (ATP pocket) in the inactive conformation.

Imatinib is now established as the front-line therapy for CML(132, 133), despite the fact it does not completely eliminate BCR-ABL expressing cells(134, 135).

The patients treated with imatinib can develop resistance to this inhibitor, especially if treated in advanced phase. The development of resistance can became a serious issue, with rates between 0.2 and 4% per year, depending on the phase of disease(136). Resistance most often originates as a consequence on mutations in the kinase domain of BCR-ABL, with one survey noting a 90% correlation(137). These mutations are thought to induce resistance by disruption of amino acids that contact imatinib or by prevention of the formation of the inactive conformational state(136, 137). The most intractable of these occurs at the threonine residue 315. Missense mutation in this gatekeeper residue most often convert this amino acid into an isoleucine. The T315I mutation is responsible for 20% of all cases of imatinib resistance(138). This gatekeeper threonine is common to many tyrosine kinases and its mutation has been shown to enhance their kinase and transformation activities(138).

The eventual selection of imatinib-resistant mutants in combination with its diminished efficacy in patients in CML blast crisis has made the development of alternative therapeutic approaches necessary.

Another resistance mechanism is represented by the upregulation of the BCR-ABL kinase in association with amplification of the BCR-ABL gene and was first reported in the Ba/F3 BCR-ABL+, LAMA84, and AR230 imatinib-resistant cell lines in the absence of mutations within the BCR-ABL kinase domain(139).

Dasatinib (Sprycel) is at the forefront of the second generation of anti-leukemia drugs, it has broader anti-kinase activity than imatinib, dually inhibiting both BCR-ABL and downstream SCR family kinases and has proven utility against imatinib-resistant leukemias (excluding T315I) as well as Ph+ CML in accelerated phase and blast crisis(140). Dasatinib has a higher binding affinity for BCR-ABL kinase ATP pocket and is able to interact with BCR-ABL in multiple conformation state (active and inactive).

Conversely, imatinib binds only the inactive conformation of BCR-ABL(141).

Nilotinib (Tasigna) is a second-generation tyrosine-kinase inhibitor designed to improve upon imatinib(142). Nilotinib, similarly to imatinib, binds the inactive conformation of BCR-ABL kinase, but the inclusion of alternative binding groups to the N-methyl-piperazine moyiety to improve the interaction between the drug and kinase binding site.

Bosutinib (SKI-606) is a 7-alkoxy-3-quinolinecarbonitrile, which functions as a dual inhibitor of Src and ABL kinases. Unlike imatinib and dasatinib, bosutinib does not significantly inhibit PDGFR or c-kit. In phase 1/2 studies, bosutinib proved very safe, with the most frequently reported adverse effects(143). Bosutinib showed activity across a wide range of BCR-ABL mutations, and it has also shown activity in advanced phase CML, though the activity is more modest than in CP CML [48]. The efficacy and remarkably favorable toxicity profile of bosutinib may lead to the approval of this agent to treat CP CML after failure of prior TKI therapy(144).

The third-generation of BCR-ABL inhibitors are represented by the drugs that show an activities against BCR-ABL^{T315I} mutant kinase, like AP24534.

AP24534 is a potent pan-inhibitor of BCR-ABL, including BCR-ABL^{T315I}. At the presence, AP24534 is in phase 1 of clinical trial. Kinase selectivity studies showed that AP24534 does not inhibit Aurora kinases, clearly distinguishing it from other T315I inhibitors in development. These studies also revealed inhibition of SRC, LYN, PDGFRα, and c-KIT with <10-fold selectivity compared to ABL^{T315I}. Several of these kinases are important clinical targets of imatinib, nilotinib and/or dasatinib, although only dasatinib has been reported to inhibit SRC family kinases(145).

Progression of CML from CP to BC

At present, the reasons and the mechanisms underlying the disease progression are still unknown, but they most likely involve the blockage of differentiation.

Chronic phase CML usually does not represent a major clinical problem because cytotoxic agents are able to control it. However, if not effectively treated, the disease (37) progresses to the more aggressive and treatment refractory accelerated and blast-crisis phases, often within 5 years from the diagnosis(146). Cytogenetic and molecular evidences suggest that this evolution results from progressive changes in the clone that supported the chronic phase.

These changes could be due to the presence of the BCR-ABL protein that causes genetic instability, predisposing the cells to an increased probability of secondary genetic changes that contribute to the more malignant phenotype of advanced-phase clones. It has been demonstrated that the oncoprotein induces an over-expression of the DNA-polymerase-β, one of the most inaccurate of the mammalian DNA polymerases(147), leading to inaccuracy of DNA replication. Furthermore, BCR-ABL is able to down-regulate the DNA repair protein PKcs(118). All these features account for the establishment of a "mutator" phenotype in BCR-ABL positive cells, increasing the probability of the appearance of additional genetic defects. Additional genetic and cytogenetic changes are frequently found in blast crisis patients. In 60-80% of them, chromosomal aberrations are evident, including second Ph chromosome, trisomy 8, isochromosome 17q and trisomy 19(148). Some of the genetic changes include mutations in the tumour suppressors p53 and p16 or overexpression of genes such as EVI1 and MYC55.

The progression of CML to blast crisis has also been associated with an increased level

of nuclear β-catenin. The β-catenin signalling pathway is involved in cell renewal capacity of haematopoietic cells. CML granulocyte macrophage progenitors (GMPs) have self renewal capacity in contrast to normal GMPs. It has been demonstrated that the GMP population of blast crisis patients show elevated amounts of nuclear β-catenin in relation to their normal counterparts, which could account for their enhanced self renewal ability(149).

It's known that BCR-ABL controls β-catenin protein stabilization through tyrosine phosphorylation: BCR-ABL physically interacts with β-catenin, and its oncogenic tyrosine kinase activity is required to phosphorylate β-catenin at Y86 and Y654 residues(150). Phosphotyrosine β-catenin is resistant to APC-mediated degradation and binds to the TCF4 transcription factor, thus representing a transcriptionally active pool(150).

Differentiation block in CML

The arrest of myeloid differentiation is a common feature in haematological diseases: the block in myeloid differentiation of bone marrow progenitors and the acquired inability to differentiate in mature cells are the main characteristics of the blastic phase. In CP, the myeloid precursors are still able to mature to granulocytes, but when the disease progresses the ability is lost.

It's known that myeloid differentiation is controlled by a network of transcription factors that regulate the expression of important differentiation-related genes. C/EBPα represents the principal inducer of granulocytic differentiation; its key role is the differentiation of multipotent myeloid progenitors into granulocytic precursors, a process that depends, in part, on the C/EBPα-mediated transcriptional regulation of genes essential for granulocytic differentiation (e.g., G-CSF receptor, myeloperoxidase, and neutrophil

elastase).

Previous works showed that the BCR-ABL expression level is critical for this differentiation block (151). C/EBPα represents the principal inducer of granulocytic differentiation; in BC primary cells, C/EBPα protein is almost undetectable, although its mRNA is expressed at high levels (152), suggesting the presence of an important post-transcriptional control mechanism. The central role of loss of C/EBPα activity in the differentiation arrest of myeloid blasts is supported by two lines of evidence:

1. ectopic C/EBPα expression induces maturation of differentiation-arrested BCR-ABL myeloid precursors;
2. a CML-BC–like process emerges in mice that receive a transplant of BCR-ABL transduced C/EBPα -null, but not heterozygous or wild-type fetal liver cells.

The downmodulation of C/EBPα protein expression is the results of BCR-ABL involvement: it inhibits granulocytic differentiation, possibly promoting the progression of CML, by the decrease of C/EBPα. This effect seems to involve translation inhibition mediated by enhanced expression of hnRNP-E2 (PCBP2; poly(rC)-binding protein 2), an mRNA-binding protein that regulates translation (Figure 10)

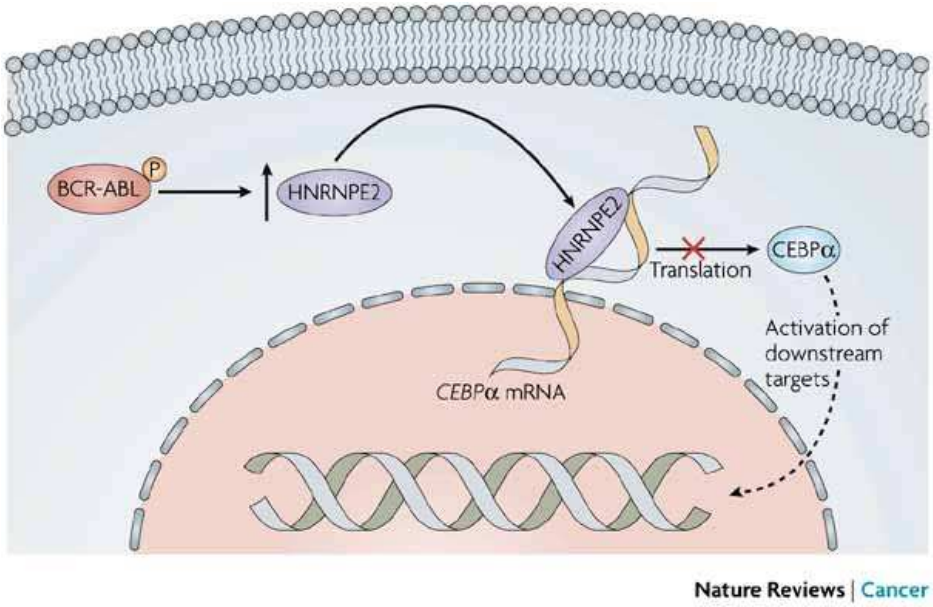

Figure 10: high expression of BCR-ABL stabilizes the hnRNP-E2, which binds C/EBPα mRNA. The binding of hnRNP-E2 to C/EBPα mRNA inhibits its translation, leading to the transcriptional downregulation of one of its target genes, CSF3R, which encodes the granulocyte colony-stimulating factor receptor (G-CSFR) required for granulocytic differentiation.

In CP myeloid precursors, when BCR-ABL expression is low, hnRNPE2 is downmodulated and C/EBPα protein is expressed. C/EBPα is thus able to induce the differentiation of the myeloid precursors into mature cells.

In blast crisis, the mechanisms that cause BCR-ABL downmodulation are lost and BCR-ABL is detectable at high levels (149). This leads to hnRNP-E2 upregulation, that upon interaction with the 5′ untranslated region of C/EBPα mRNA, causes the inhibition of CEBPα expression, upregulation of activated β-catenin and, eventually, to the loss of the differentiation potential of the BC myeloid precursors. This hypothesis is further supported by the ability of BCR-ABL to block myeloid differentiation (151, 152).

C/EBPα protein but not mRNA expression is downmodulated in primary bone marrow cells from BC patients and inversely correlates with BCR-ABL levels. Accordingly, hnRNP-E2 expression inversely correlates with that of C/EBPα, as hnRNP-E2 levels are abundant in BC but undetectable in CP mononuclear marrow cells. Furthermore, in

myeloid precursors expressing high levels of p210-BCR-ABL, hnRNP-E2 levels are downmodulated by imatinib treatment, suggesting that BCR-ABL-generated signals suppress differentiation by affecting hnRNP-E2 expression/function.

hnRNP-E2 expression is induced by BCR-ABL in a dose- and kinase-dependent manner through constitutive activation of $MAPK^{ERK1/2}$. This, in turn, post-translationally increases hnRNP-E2 protein stability. Furthermore, *in vitro* and *in vivo* suppression of hnRNP-E2 phosphorylation/expression by inhibition of $MAPK^{ERK1/2}$ activity restores C/EBPα expression and the myeloid differentiation.

These data suggest that the "oncogene dosage" is a determinant factor for the differentiation block in BC-CML and that the progression to BC could be linked to an abnormal control of BCR-ABL expression during myeloid differentiation with ensuing maturation block. Unfortunately, the mechanisms leading to the deregulated expression of BCR-ABL in BC are not known.

microRNA

MicroRNAs are small non-coding RNAs that act at the post-transcriptional level, regulating protein expression by repressing translation or destabilizing mRNA target. MicroRNAs have been associated with almost every normal cell function, including proliferation, differentiation and apoptosis. They usually recognize target sites in the 3'-untranslated regions (UTRs) of mRNAs and probably in the 5'-UTRs through perfect (in plants) or imperfect (in mammals) base-pairing. They bind to the target transcript by their nucleotides 2–8, counted from the 5' end. These nucleotides constitute the 'seed' region of the microRNA.

Recently, it has been demonstrated that also microRNAs operate in the haematopoiesis process (Figure 11).

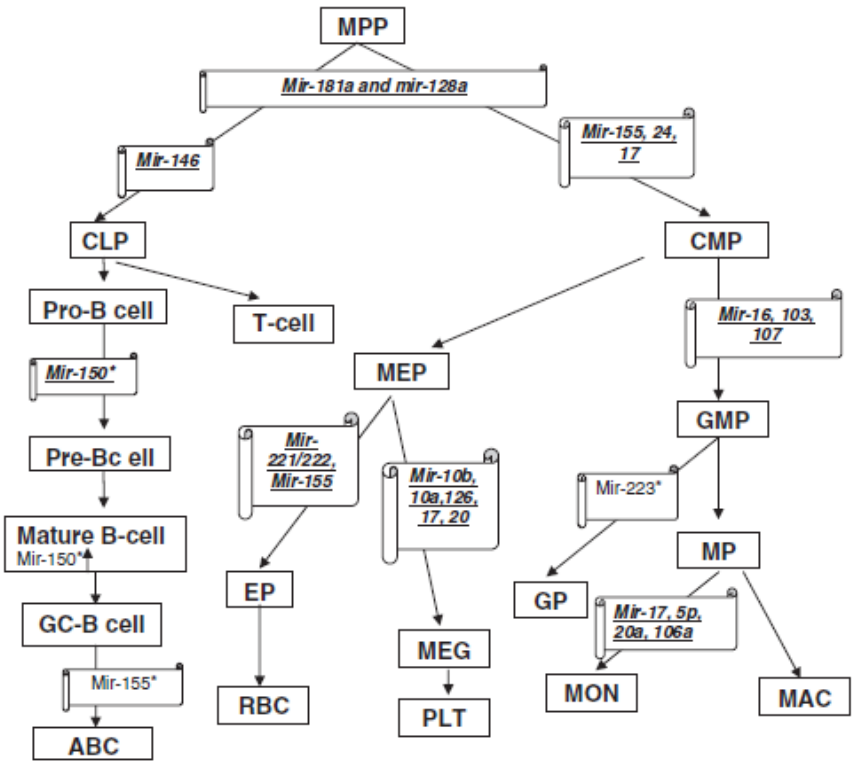

Figure 11: MicroRNA expression in normal haematopoiesis.

MicroRNAs in granulocyte and monocyte differentiation

A microRNA that plays an important role in myeloid differentiation is mir-223(153). Fazi et al. studied myeloid differentiation in acute promyelocytic leukemia (APL) cells and demonstrated that mir-223 activates mouse granulopoiesis, together with the transcription factors NF-IA (negative nuclear factor IA) and C/EBPα. Both transcription factors are able to bind to mir-223 promoter. NF-IA down-regulates while C/EBPα up-regulates mir-223 expression. Treatment of APL cells with retinoic acid (RA) resulted in C/EBPα replacement of NF-IA, up-regulation of mir-223 and enhanced granulocytic differentiation. Interestingly, mir-223 was found to inhibit translation of NF-IA, resulting in a negative-feedback loop that favours granulocytic differentiation(153).

However, two recent studies do not confirm the previous results. In the first study, Fukao et al. found that myeloid expression of mir-223 might be specified by the conserved 5' proximal cis-regulatory element where transcription factors PU.1 and C⁄EBP cooperatively act on. In addition, by studying the APL cell model that was used by Fazi et al., they found that RA induced differentiation of APL cells, repressed PU.1 and resulted in down regulation of mir-223(154). In the second study, Johnnidis et al. found that mir-223 negatively regulates progenitor proliferation and granulocyte differentiation and activation in KO mice. In addition, they showed that mir-223 targets Mef2c, a transcription factor that promotes myeloid progenitor proliferation and that genetic ablation of Mef2c suppresses progenitor expansion and corrects the neutrophilic phenotype in mir-223 null mice(155). Further investigations are required to shed light on the real mechanisms that regulate mir-223 expression.

Fontana et al. investigated the role of mir-17-5p, mir-20a and mir-106a in monocytic differentiation and maturation. In unilineage monocytic cultured cells these microRNAs

are down-regulated while acute myeloid leukemia 1 (AML1) transcription factor, which promotes M-CSFR transcription (M-CSF receptor) is up-regulated at the protein but not at the mRNA level. Transfection with mir-17-5p, mir-20a and mir-106a caused the opposite results in AML-1 protein expression followed by enhanced blast proliferation and inhibition of monocytic differentiation and maturation. Further experiments confirmed that these microRNAs target AML-1. In addition, AML1 binds the microRNA 17-5p-92 cluster and 106a-92 cluster promoters and inhibits the expression of mir-17-5p-20a-106a as a negative feedback, indicating that monocytopoiesis is controlled by a circuitry involving mir-17-5p, mir-20a, mir-106a, AML-1 and M-CSF(156). Finally, according to model of Georgantas et al.(157), mir-155, mir-24a and mir-17 may inhibit the differentiation of MPP into common myeloid progenitor (CMP), while mir-16, mir-103 and mir-107 may inhibit the differentiation of CMP into granulocytic-macrophage progenitor (GMP).

MicroRNA expression in chronic myeloid leukemia (CML)

The scenario of BC-CML biology is further complicated by the evidence that miRNA takes part in the regulation of ABL and BCR-ABL.

It is known that miR-203 is able to downmodulate ABL and BCR-ABL expression through a direct interaction with ABL and BCR-ABL 3'UTR, causing a decrease in ABL and BCR-ABL mRNA half-life (158). Moreover, the mechanisms involved in the physiological regulation of BCR upon myeloid differentiation are yet unknown (159, 160). Mir-203 is silenced in some hematopoietic malignancies including CML. However, re-expression of this miRNA reduces ABL and BCR-ABL fusion protein levels and inhibits tumor cell proliferation(158). Venturini et al. screened for BCR-ABL and c-MYC-dependent microRNA expression using microarrays and quantitative real-time

reverse transcriptase PCR. Treatment of K562 cells with imatinib (to inhibit BCR-ABL kinase activity), with specific anti-BCR-ABL RNA interference (to knock-down BCR-ABL gene expression) or anti c-MYC RNA interference resulted in the down-regulation of mir-17-92 cluster. The results were confirmed by quantitative Real Time-Polymerase Chain Reaction (Q-PCR). Finally, it was demonstrated by the same group that mir-17-92 cluster is over-expressed in primary CML CD34+ cells in chronic phase but not in blast crisis, compared with normal CD34+ cells(161). These results suggest that microRNA expression in CP of CML is under the control of a BCR-ABL–c-MYC pathway.

Recently, Eiring et al. showed that miRNA-328 is reduced in CD34+ CML blast crisis (CML-BC) progenitors(162). In the figure 12 the double function of miRNA-328 is summarized: for its decoy activity mir-328 binds hnRNPE2 and rescue the translation of C/EBPα and restores C/EBPα-dependent maturation of myeloid CML-BC progenitors. By canonical pathway, it represses one of its target: PIM1. Its repression causes a decrease in cell-survival.

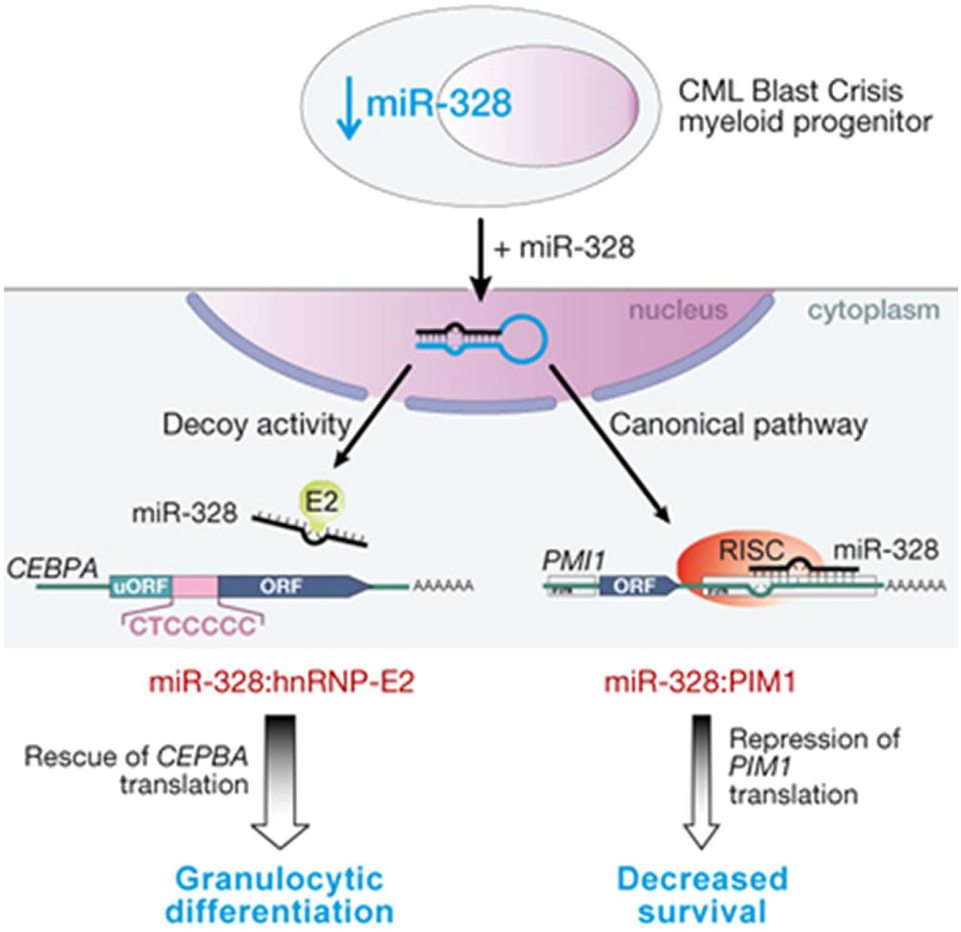

Figure 12: summary of miRNA-328 activity from Eiring et al.[162]

BCR-ABL expression level during the CML progression

Several lines of evidence suggest that BCR-ABL is crucial for the maintenance of the disease even during progression (163). The central role of the oncogenic fusion protein BCR-ABL in the transition towards the blastic phase has been pinpointed also by three different studies (164-166), showing an association between increased levels of BCR-ABL and advanced phase.

In few works, BCR-ABL expression level was analyzed during the passage from CP to BC. Also BCR was analyzed and both genes showed an increase during the progression in a pool of mononuclear cells, suggesting the involvement of a common mechanism of regulation. However the interpretation of these analyses is complicated by the fact that mononuclear cells don't constitute a pure myeloid subpopulation.

Recently, Jamieson and coll. investigated the expression level of BCR-ABL in sorted myeloid population, distinguishing the different subpopulation: Haematopoietic Stem Cells (HSCs), Common Myeloid Precursors (CMPs) and Granulocytes and Monocytes Precursors (GMPs). They analyzed the expression of BCR-ABL in myeloid cells from CP and BC samples. They demonstrated that in chronic phase the level of BCR-ABL mRNA decreases during differentiation from CD34+ hematopoietic stem cells to myeloid progenitors. Conversely, in samples from patients in blast crisis (BC), no downregulation of BCR-ABL is detectable during myeloid maturation (Figure 13).

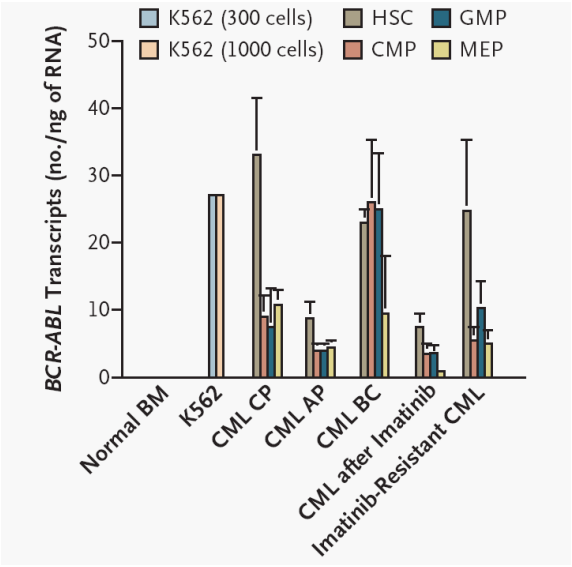

Figure 13: Downregulation of BCR-ABL in CP and the loss of this regulation with the passage to BC[149]

This correlates with an expansion of the myeloid precursors pool and with a marked increase of activated β-catenin and an enhanced self-renewal capacity of the same precursors.

The role in self-renewal of β-catenin is known and its pathway is normally active in HCSs. It is known that β-catenin is regulated by BCR-ABL in CML: BCR-ABL levels control the degree of β-catenin protein stabilization by affecting its Y/S/T-phospho content in CML cells. BCR-ABL physically interacts with β-catenin, and its oncogenic tyrosine kinase activity is required to phosphorylate β-catenin at Y86 and Y654 residues. This Y-phospho β-catenin binds to the TCF4 transcription factor, thus representing a transcriptionally active pool(150). The figure 14 summarized the hypothesized mechanism.

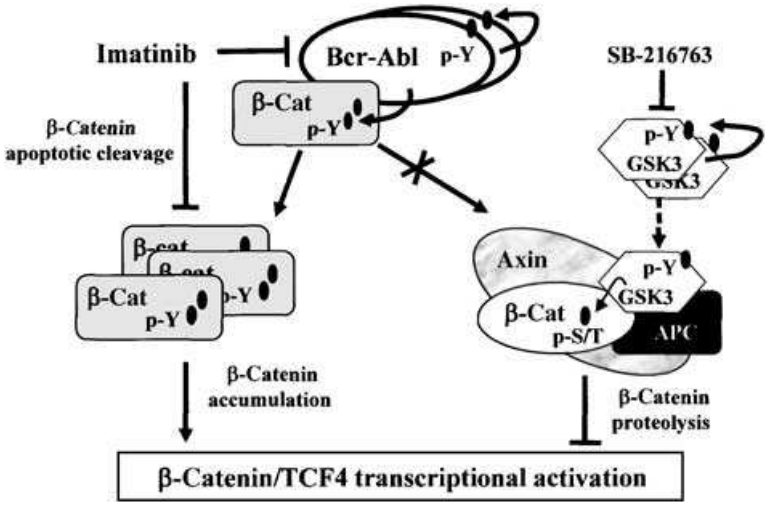

Figure 14: A model of β-catenin/TCF4 transcriptional activation in BCR-ABL CML cells. The figure summarizes the effects of BCR-ABL, Imatinib and SB-216763 on b-catenin protein stabilization and nuclear signaling.

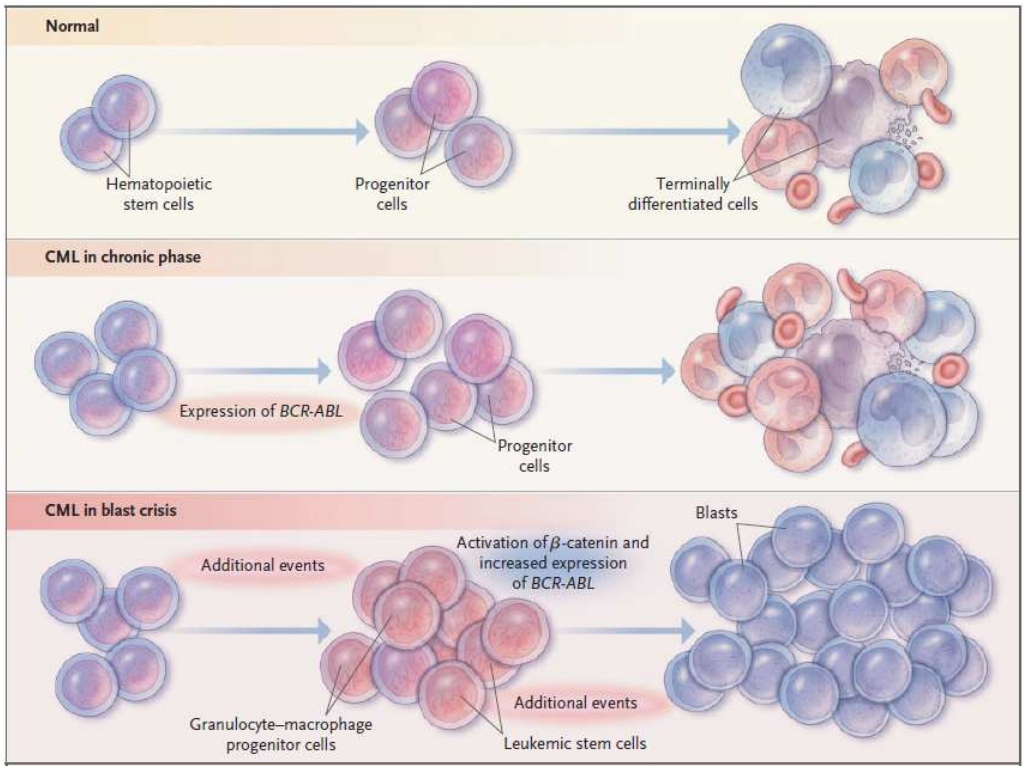

Figure 15: a representation of CML phase and their characteristic compared with the normal myeloid maturation.

In the figure 15 the hypothesis of Jamieson and colleagues is reported: in CP the expression of BCR-ABL produces an expansion of the mature cells; the subsequent activation of β-catenin, causes the differentiation block and thus leads to the blastic phenotype.

BCR promoter

The promoter of the human BCR gene regulates the transcription of the chimeric BCR-ABL mRNA in leukemia. At present, just few works focused on BCR promoter(159, 160). The isolation and partial characterization has been done for a region of 1.1 kb immediately 5' to the main transcription start site(159). Another work identified as functional promoter a more restricted region about 1 kb 5’ of BCR exon 1 coding sequences by using a chloramphenicol acetyltransferase reporter gene assay(160). In our work, we considered the extended region to study the BCR promoter, in fact, along the sequence several putative protein binding sites have been identified by DNase protection assay and gel retardation. Moreover, nucleotide sequence analysis was performed on the 5’ region of BCR promoter and many consensus binding sites for transcription factor (TF) SP1 has been revealed. This TF is ubiquitinated and shows a double role: it’s a repressor and also an activator and binds with high affinity to GC-rich motifs. The target genes of SP1 are involved in a high variety of processes such as cell growth, apoptosis, differentiation and immune responses.

The BCR promoter sequence is rich in GC motifs: the GC content of the whole promoter is about 78%. This region could form a secondary DNA structure. In other promoters, the formation of secondary structure is important for the activity of promoter and permits the interaction with specific proteins: *Hox* promoter forms a secondary structure which is important for Hox gene regulation(167) by *Fbxl10*.

In figure 16, the 10 putative binding site for TF, identified by DNase protection analysis are shown. Of these, one is found in an inverted repeat in the 3' coding and splice donor region of BCR exon 1: the 3' coding and splice donor region of exon 1 is present in the 5' region of the gene in an inverted orientation. From mouse DNA analysis emerged that

the repeat sequence is a relatively novel addition to the gene in evolutionary terms, because only part of this region is detectable in the mouse genome. The inverted 5' repeat appears to have some function in the BCR promoter: within it, the complement of the sequence 5' CACGATGGTGGCCTC7UACACGA 3' is protected against DNase I digestion by protein factors in both K562 and A498 extracts. Interestingly, the sense strand of the 3' repeat located in the 3 'coding and splice donor region of exon is also protected, although this sequence contains one base-pair difference compared to the 5' repeat. In both areas, the digestion patterns are very similar; the protected areas are also similar except that in the 3' repeat the protected area extends further to the 5' side.

Additionally to the main transcription initiation site, many others have been identified.

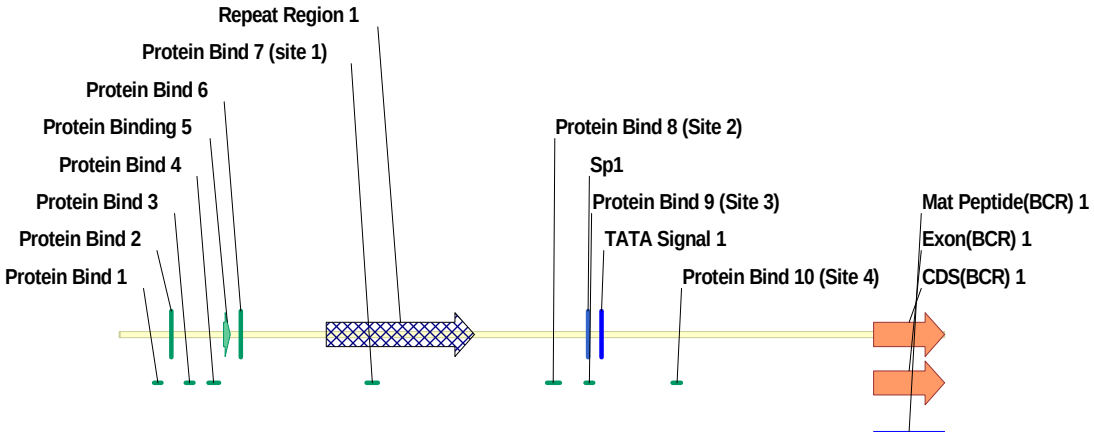

Figure 16: representation of BCR promoter

In silico analysis may lead to the identification of putative transcription factors that bind BCR promoter and regulate its expression and also the expression of BCR-ABL. BCR and ABL promoters are structurally similar, but the experimental evidences about the expression level of BCR-ABL in myeloid subpopulation, together with the observation that the expression of both BCR and BCR-ABL increases during the evolution from CP to BC, suggest the involvement of a mechanism that could act on both genes.

Biological Complexity of CML-BC

The evolution of BC from CP CML could be a multistep, time-dependent process: during CP, DNA repair mechanisms are able to repair the DNA damage that occurs in the cells. One possibility for the progression is that after a prolonged exposure to the BCR-ABL expression, the DNA repair mechanisms become inefficient and the DNA damages increase, leading to the selection of one or more blastic clones(168).

Even if many chromosomal abnormalities have been associated with disease progression (169, 170), none has been proved to be causative for this phenomenon. Moreover, several mutations are identified as associated to the BC phase: the tumor suppressor genes p53 is mutated in 20%–30% of cases; Runt-related transcription factor gene (RUNX1) in 38% in myeloid BC(171-176), but also this evidence don’t represent a cause for the progression.

However, the genomic instability is just one of the cell processes that the increased BCR-ABL expression levels perturbs (figure 17).

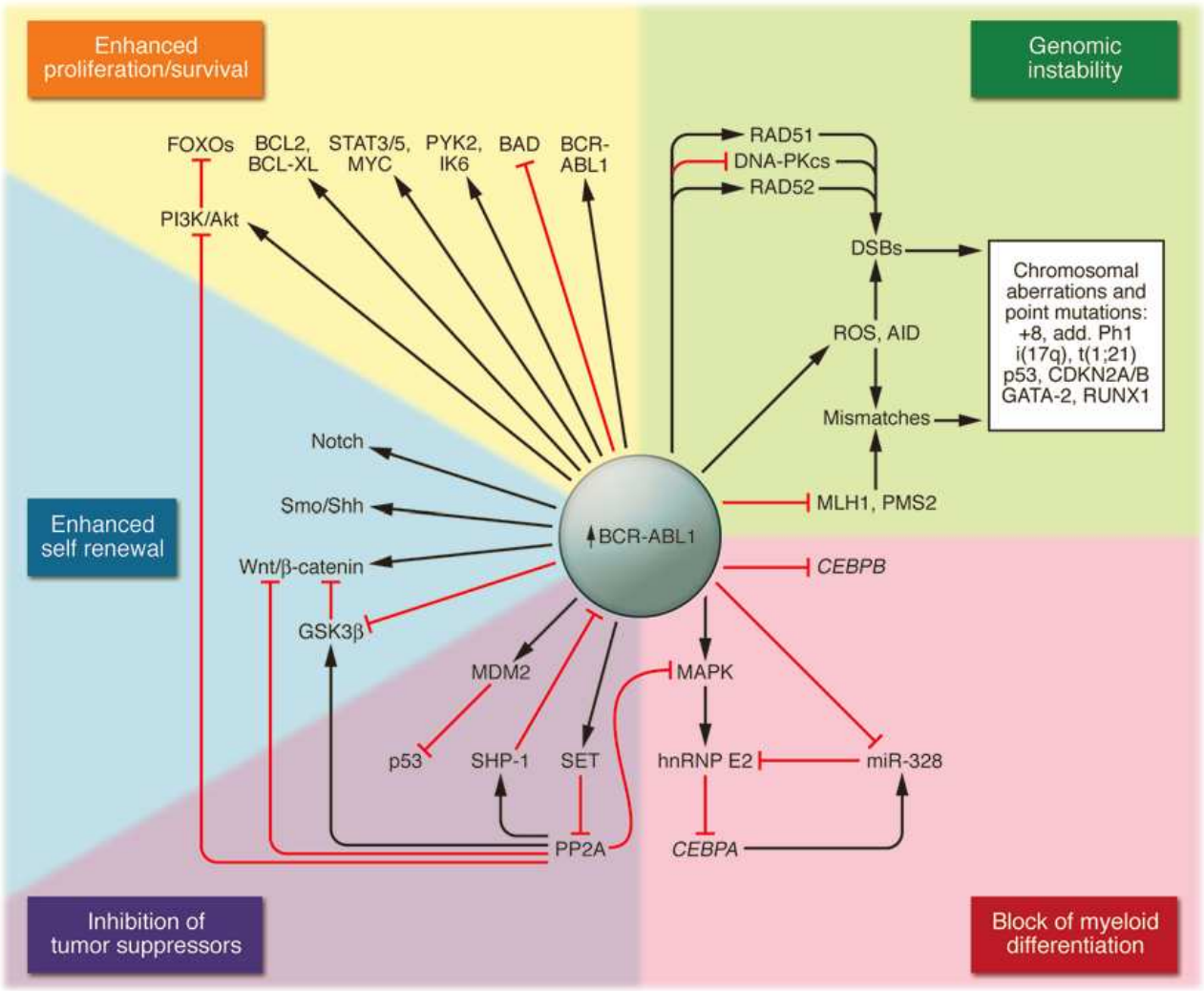

Figure 17: Overview on the effects of BCR-ABL expression[168].

The central role in the progression is held by BCR-ABL expression level. The increase of BCR-ABL levels in BC is known(177) and the effects of this feature is extensive, from cell survival to self renewal ability. The common feature of the effects of BCR-ABL is that all of these are closely related to the expression level of fusion protein.

Epigenetic changes are dependent mostly on the pleiotropic effect of constitutive BCR-ABL1 activity(178, 179), the levels of which start to increase in AP(177). In support of this suggestion, expression studies revealed that upregulation of BCR-ABL dramatically perturbs the CML transcriptome(180), resulting in altered expression of genes, some of which (e.g., PRAME, MZF1, EVI-1, WT1, and JUN-B) might play a role in BC(178,

181, 182). Nonetheless, the post-transcriptional, translational, and post-translational effects of high BCR-ABL levels result in the constitutive activation of factors with reported mitogenic, antiapoptotic, and antidifferentiation activity (e.g., MAPKERK1/2, MYC, JAK2, YES-1, LYN, hnRNP-E2, MDM2, STAT5, BMI-1, and BCL-2) and in the inhibition of key regulators of cellular processes, such as those regulated by the tumor suppressors p53, C/EBPα, and PP2A(149, 178, 179).

The changes in the activity of the tumor suppressor PP2A, protein phosphatase 2, may be involved in the pathogenesis of CML progression. In general, PP2A activity is involved in regulating proliferation, survival, and differentiation, and is involved in the proteosomal degradation of BCR-ABL. Increasing BCR-ABL levels (induced *in vitro* or in human leukemia) increase the expression of the phosphoprotein SET, a negative regulator of PP2A(183). Thus, progression may set up a feedback loop whereby increasing BCR-ABL increases SET, decreasing PP2A, which further ensures the persistence of BCR-ABL. In *in vitro* experiments and in mouse models, restoration of PP2A activity by the activator forskolin appeared to decrease BCR-ABL leukemic potential, thus suggesting a potential therapeutic target to arrest or regress CML progression(183).

The direct correlation between the tumorigenicity of induced disease in nude mice and BCR-ABL expression levels emerges from murine models studies. Issaad and colleagues investigated how different levels of BCR-ABL cause a CML-like disease in nude mice with different tumorigenic effects(184). They used the UT-7 cell line, a pluripotent cell line, trasfected with a BCR-ABL retrovirus vector and selected UT-7 clones with different BCR-ABL expression: some of the clones showed a very low level of BCR-ABL, while other clones showed a significant high level of BCR-ABL. The UT-7 clones were injected into nude mice and only the mice with the high level of BCR-ABL UT-7

clones showed a CML-like disease; in particular, the UT-7 clones with very high level of BCR-ABL induced in nude mice an acute phase model of CML as a progressive increase of BCR-ABL levels has been shown in patients progressing to the BC. In contrast, the clones with low level of BCR-ABL could be comparable to the earlier stage of CML, CP. The progression of CML is usually accomplished by the resistance to the therapy. The amplification of Ph chromosome represent one of the possibility to develop the imatinib resistance. Several study reported the amplification of BCR-ABL gene in BC patients that determines the increase of BCR-ABL expression, that characterized this phase of disease. Moreover, the main characteristic of BC is the differentiation block from C/EBPα suppression and it's known the "oncogene dosage" effects of BCR-ABL expression levels. C/EBPα suppression is dependent by the inhibitory action of hnRNPE2 and BCR/ABL up-regulates hnRNP-E2 expression is post-translational and depends on increased hnRNP-E2 protein stability. This enhanced expression of RNA binding proteins correlates with the levels of BCR/ABL and is sensitive to imatinib treatment(152, 183, 185).

The reactivation of β-catenin in committed progenitors is a consequence of its Y-phosphorylation by BCR-ABL that causes an increase of its stability. The accumulation of b-catenin in the nucleus of GMPs causes the re-acquisition of the self renewal ability and contributes to the complexity of BC. During the passage in BC the level of BCR-ABL increases, in the GMPs the high expression of fusion gene is accomplished by an enhanced activity of b-catenin and an increase of leukemic potential of these precursors cells.

All the main characteristics of BC seem to derive from the expression level of BCR-ABL and the progression of CML is indissolubly connected to the increase of expression during the passage from CP to BC. The understanding of the reason underline of this

increase represents an important goal in the study of CML and its development.

Aim

Despite the great progress in our understanding of the basic mechanism controlling the oncogenic potential of BCR-ABL, still little is known about the molecular events that drive the progression of CML from the initial chronic phase (CP) towards the late acute blastic crisis (BC). Even if many chromosomal abnormalities have been associated with disease progression, none has been proved to be causative for this phenomenon. Notably, several lines of evidence suggest that BCR-ABL is crucial for the maintenance of the disease even during progression. The central role of the oncogenic fusion protein BCR-ABL in the transition towards the blastic phase has been pinpointed also by several studies, showing an association between increased levels of BCR-ABL and advanced phase.

More recently, Jamieson C. and co-workers analysed the expression levels of *BCR/ABL* in sorted bone marrow (BM) subpopulations in patients in chronic as well as accelerated and blastic phase. They demonstrated that in chronic phase the level of *BCR/ABL* mRNA decreases during differentiation from CD34+ hematopoietic stem cells to myeloid progenitors. Conversely, in samples from patients in blast crisis (BC), no downregulation of *BCR/ABL* is detectable during myeloid maturation. This correlates with an expansion of the myeloid precursors pool and with a marked increase of activated β-catenin and an enhanced self-renewal capacity of the same precursors.

These data suggest that the progression to BC could be linked to an abnormal control of BCR-ABL expression at the transcription level, most notably within the myeloid precursors. This observation opens new questions about BCR/ABL expression control and it's important to underline that BCR-ABL is under the control of the *Bcr* promoter. However, the analysis of these mechanisms is hampered by the absence of information about the physiological regulation of BCR during myeloid differentiation. Up to date

only few studies have been focused on the characterization of *Bcr* promoter, so little is known about the transcriptional regulation of this gene.

The objectives of this study are the characterization of the molecular mechanisms regulating BCR expression during normal myeloid differentiation and the definition of the transcriptional regulation of BCR-ABL in chronic myeloid leukemia (CML) during chronic as well as blastic phase of the disease. Here we focused on the gene expression control of BCR and BCR-ABL upon myeloid differentiation in healthy donors (HD), CP and BC patients and also on the characterization of the BCR promoter in order to identify possible factors modulating BCR-ABL expression during myeloid differentiation in CP and BC.

Materials and Methods

Patient samples and isolation of myeloid subpopulation: normal bone marrow (BM) or peripheral blood (PB) samples were obtained after written informed consent in agreement with the Declaration of Helsinki. Mononuclear cell were isolated by Ficoll gradient.Hematopoietic Stem Cell (HSCs), Common Myeloid Progenitors (CMPs) and Granulocyte-Macrophage Progenitors (GMPs) were isolated from mononuclear cells by Fluorescence-Activated Cell Sorting (FACS) and Magnetic Cell Sorting (MACS) techniques. Samples were subjected to a purification/enrichment using MACS systems (MACS Lineage Cell Depletion Kit, Miltenyi Biotech, Germany) the following cocktail of antibodies: CD2, CD3, CD10, CD11b, CD14, CD15, CD16, CD19, CD56, CD123 and Glycophorin A. Subsequently, the myeloid subpopulation was sorted by FACS (FACS ARIA, Becton-Dickinson) in the following subpopulations: HSCs (Lin-, CD34+/CD38-/Thy+/-); CMPs (Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA-) and GMPs (Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA+) as described by Manz(186) starting from 7 healthy donors (HD), 10 CP (not in cytogenetic remission) and 7 BC patients.

RNA extraction and Q-PCR: total RNA was extracted by two different techniques, in accordance with the cell number. RNA was extracted from cells with Trizol reagent (Invitrogen, UK). Extraction and centrifugation steps were carried out according to manufacturer's instructions. The concentrations of total RNA was estimated using a spectrophotometer at 260 nm wavelength, and the purity of RNA was determined by calculating the ratio of absorbance at 260/280 nm. Alternatively, when the number of cells was very low, RNA was isolated by RNeasy Micro Kit (QIAGEN, Hilden, Germany). Then, 1 μg of total RNA was retrotranscribed ('MultiScribe™ Reverse Transcriptase', Applied Biosystems, CA, USA). Quantitative polymerase-chain-reaction analysis (Q-PCR) for BCR, ABL, BCR-ABL and GUS was performed using a specific sets of primers reported in table 1 (187, 188). BCR, ABL and BCR-ABL levels were

normalized against GUS and analyzed with the statistical software package Prism 4 (GraphPAD).

Cell line and cell culture: the BCR-ABL positive K562 cell line was maintained at 37 °C as a suspension in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 80 μg/mL gentamycin and 20 mmol/L HEPES in humidified atmosphere containing 5% CO2. For the experiments, the cells were washed by centrifugation 5 minutes at 1500 rpm for 2 times with saline phosphate buffer (PBS, 20 mM sodium phosphate, 140 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, and 5 mM glucose, pH7.4).

RNA stability assay: Actinomycin D (5 μM) was added to cultures for up to 24 hours. Total RNA was prepared at predefined time points. BCR and BCR-ABL expression levels were analyzed by Q-PCR.

Construction of Different BCR promoter Plasmids: the human BCR promoter was cloned by PCR from human genomic DNA extracted from a HD with the following strategy: a reverse primer carrying an artificial HindIII site was designed to bind to the 3' region of the promoter, in correspondence to the first coding ATG sequence, which defines the coding protein region. This primer was used to amplify all the constructs. The sequence of the forward primers, carrying an artificial NheI restriction site at their 5', changed accordingly with the target region (see table 2 for sequence details). The two restriction sites were added to allow for the directional cloning into the pGL3 basic luciferase vector. We produced different constructs of BCR promoter with specific length, in order to identify the most transcriptionally active regions.

Transfection: The Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) was used to measure the Firefly and Renilla Luciferase activities according to the manufacturer's instruction. The Firefly luminescence was normalized

with Renilla Luciferase signal. Luciferase and Renilla signals were detected by MicroBeta (Perkin Elmers, Waltham, Massachusetts, USA) system. The experiments were performed in triplicate. For the transient transfection, K562 cells were resuspended in RPMI 1640, and the plasmids were added. Cells were incubated on ice for 2 minutes and then electroporated (270 V, 975 μF) using a GenePulser XCell (Bio-Rad, Hercules, CA). Cells were kept on ice for 2 minutes and then transferred in RPMI 1640 with FBS (10%)**.**

Statistical analysis: All the statistical analyses (unpaired two-tailed T-test, mRNA half-life) were performed by the GraphPad Prism (GraphPad, CA, USA) statistical package. A p value < 0.005 was regarded as statistically significant.

Site-direct mutagenesis: Mutation in the BCR promoter were introduced using the Quick Change site-directed mutagenesis kit (Stratagene, LA Jolla, CA, USA). The starting template was the recombinant plasmid pGL3/BCR promoter longest form. Mutagenesis reactions were performed in a working volume containing 1X Pfu reaction buffer (10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 0.1% Triton X-100, and 0.1 mg/mL BSA), 20 ng template plasmid DNA, 6 pmol of each primer, 200 μM of each dNTP, and 1 unit of PfuTurbo DNA polymerase (QuikChange II Site-Directed Mutagenesis kit, Qiagen, Valencia, CA). Reactions were thermal cycled: 95ºC for 5 min, followed by 16 cycles of 95ºC for 30 sec, 60ºC for 1 min, and 68ºC for 10 min, then a final incubation of 68ºC for 10 min. Larger plasmids may require longer extension times. Reactions were cooled on ice and digested with 5 units of Dpn I for 1 h at 37ºC to cleave methylated and hemimethylated parental DNA, but not the newly synthesized mutant DNA molecules. Primers for the mutagenesis were reported in table 3.

Transformation of competent cells: Reactions were used to transform the chemically competent TOP10 *E. coli*. Cells were thawed on ice, part of reaction added, mixed

gently, then incubated on ice for 30 minutes. Cells were heat-shocked at 42ºC for 45 seconds, then placed on ice again for 2 minutes. Next, 250 µL of SOC media (Sigma Chemical, St. Louis, MO) was added, and transformed cells incubated at 37°C with shaking for 1 h. Finally, the competent was plated onto LB agar plates containing ampicillin (100µg/mL) and incubated overnight at 37ºC.

In silico analysis: To identify involved transcription factors in the regulation of BCR expression, Transcription Element Search System (TESS) was used to obtain preliminarily data; additionally, other program, TFSEARCH, Searching Transcription Factor Binding Sites implemented the putative transcriptional factor list. The expression data during myeloid differentiation for each TF were obtained from literature and were compared to the *in silico* data to screening the most interesting putative transcriptional factor.

Isolation of Transcriptional Factors SP1: the SP1 binding site present in the BCR promoter was synthesized as a DNA oligonucleotides, adding the nucleotides that formed a sequence of a biotinylated capture DNA (the complete sequence was reported in table). Mutated SP1 binding sites were synthesized, with two or four mutated bases, in order to confirm the real binding of transcription factor to the sequence present in the BCR promoter. For all binding analysis the protein extract from K562S was incubated for 10 minutes at room temperature with the biotinylated DNA, and the protein-DNA complexes were subsequently bound to µMACS Streptavidin MicroBeads (Miltenyi Biotec, Germany). The iron filings contained within a µ column trapped the paramagnetic complexes, and the pure native SP1 was eluted after the column was stringently washed to remove unbound polypeptides according to the manufacturer's instructions.

Chromatin ImmunoPrecipitation (ChIP): Cells were crosslinked with formaldehyde 0.4% (Sigma, St.Louis, MO, USA) for 10 min at room temperature and the reaction was stopped by adding glycine to final concentration 0.2M for 10 minutes at room temperature. Fixed cells were rinsed twice with PBS and resuspended in lysis buffer (10mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 0.1% SDS, 1% Triton X-100, 10% Glycerol). Lysate was sonicated using Labsonic Braun sonicator and centrifuged at 13000 rpm for 10 min. The sonicated chromatin was diluted 10 times in ChIP Dilution Buffer (1% SDS, 0.1M NaHCO3). 50 µL of each samples were stored at -20°C as the INPUT. The sonicated chromatin were pre-cleared for 1 hour, rotating at 4°C, with 80µL of Salmon Sperm DNA/ProteinA agarose (Upstate, Lake Placid, USA), before the overnight incubation with 2 µg of specific antibody. The bound material was recovered after a 2 hours incubation, rotating at 4°C, with 60 µL of Salmon Sperm DNA/ProteinA agarose. The agarose was washed, for 5 minutes, once in Low Salt Buffer (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 150 mM NaCl), once in High Salt Buffer (pH 8.1) (0.1% SDS, 1% Triton X-100, 2mM EDTA, 10mM Tris-HCl, 500 mM NaCl), once in LiCl Buffer (pH 8.1) (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl) and once in TE (pH 8) (10mM Tris-HCl, 1mM EDTA). ChIPed material was eluted by two 15 minute incubations at room temperature with 250 ul Elution Buffer (SDS 1%, 0.1 M NaHCO3). Chromatin was reverse-crosslinked by adding 20 ul of NaCl 5M and incubated at 65°C for 4 hours minimum. Primers for the Q-PCR analysis of ChIP samples were reported in table 4.

Western Blot Analysis: Antibodies against SP1 transcriptional factor (Santa Cruz Biotech, Palo Alto, CA, USA) were used in this study. The fractions from the TF experiment were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted on nitrocellulose membrane.

Western blots were performed using previously described antibodies at a 1:500 dilution, followed by incubation with rabbit peroxidase-conjugated secondary antibody (Biorad) at a 1:2000 dilution.

Table 1. Primers Sequences for TaqMan Q-PCR

BCR taqman For	TTCTCCCTGACATCCGTGG
BCR taqman Rev	ACCTCCAGGGTGCAGTACAGAT
BCR probe	TGAAACTCCAGACTGTCCACAGCATTC
ABL taqman For	TGGAGATAACACTCTAAGCATAACTAAAGGT
ABL taqman Rev	CCATTTTTGGTTTGGGCTTCACACCATT
ABL probe	GATGTAGTTGCTTGGGACCCA
BCR/ABL taqman For	TCCGCTGACCATCAATAAGGA
BCR/ABL taqman Rev	CACTCAGACCCTGAGGCTCAA
BCR/ABL probe	CCCTTCAGCGGCCAGTAGCATCTGA
GUS taqman For	GAAAATATGTGGTTGGAGAGCTCATT
GUS taqman Rev	CCGAGTGAAGATCCCCTTTTTA
GUS probe	CCAGCACTCTCGTCGGTGACTGTTCA

Table 2. Primers Sequences for BCR promoter constructs

Primer reverse (for all constructs)	AATAAAGCTTGGCGCGGCCGGCCTTACCTG
Primer forward BCR promoter	AATAGCTAGCTTAGAGGGAGGCTAATCAGGGAA
Primer forward BCR promoter Δ241	AATAGCTAGCAAGGGTGCTGGCATTGGTTACTCAG
Primer forward BCR promoter Δ541	AATAGCTAGCTTGCTTCTGTCGGAGGACTGCTG
Primer forward BCR promoter Δ1041	AATAGCTAGCCTGGCTGAGCTTAGCGTCCGAG
Primer forward BCR promoter Δ1271	AATAGCTAGCCTCCGCCTCACCTGCCACCAG

Table 3. Primers Sequences for TaqMan Q-PCR of SP1 mutagenesis

BCRPromSP1mtFor	CCGCCTCCAGGGGGCCTCCCTGCCTGTGCCCACGGC
BCRPromSP1mtRev	GCCGTGGGCACAGGCAGGGAGGCCCCCTGGAGGCGG

Table 4. Primers Sequences for TaqMan Q-PCR of SP1 ChIP

BCR-SP1-FOR	CACAGTCCAATTCGCTGTTGTTAGGG
BCR-SP1-REV	CGATGACGGAGCCAGGCGGG

Results

BCR and BCR-ABL expression in mononuclear cells from CP and BC patients

It's known that BCR-ABL expression level is fundamental for the progression of CML, and also for the main characteristics of BC: differentiation block and re-activation of self renewal properties in myeloid progenitors. The re-activation of self renewal abilities, demonstrated by Jamieson et al., is explained as a consequence of BCR-ABL levels(149). BCR-ABL changes its expression during myeloid maturation: in CP the chimeric mRNA is downregulated; conversely, in BC this regulation disappears during myeloid maturation: BCR-ABL expression is detected at a high level in all myeloid precursor cells(149). Jamieson and coll. didn't define any mechanism that could cause the change of BCR-ABL during CML phases, but it's known that BCR promoter controls the expression of chimeric BCR-ABL in CML disease. At the presents the mechanism that regulates BCR-ABL expression during myeloid maturation in CP and in BC is unknown and the transcriptional regulation could be one of the possible responsible for BCR-ABL increase in expression during BC.

The transcriptional regulation of BCR-ABL could involve also BCR: both genes are regulated by the same promoter, and this observation permits us to discriminate between the two possible scenarios. The mechanism that causes the increase of BCR-ABL could be an *in cis* or *in trans* transcriptional regulator. Whether the mechanism acts *in trans*, both BCR and BCR-ABL should show a similar increase in expression during the progression from CP to BC. Conversely, whether the mechanism should be an *in cis* mechanism, only BCR-ABL expression should show the increase during the progression of CML, while BCR should be independent from the regulation of BCR-ABL.

In Figure 1 the two possibilities above are shown.

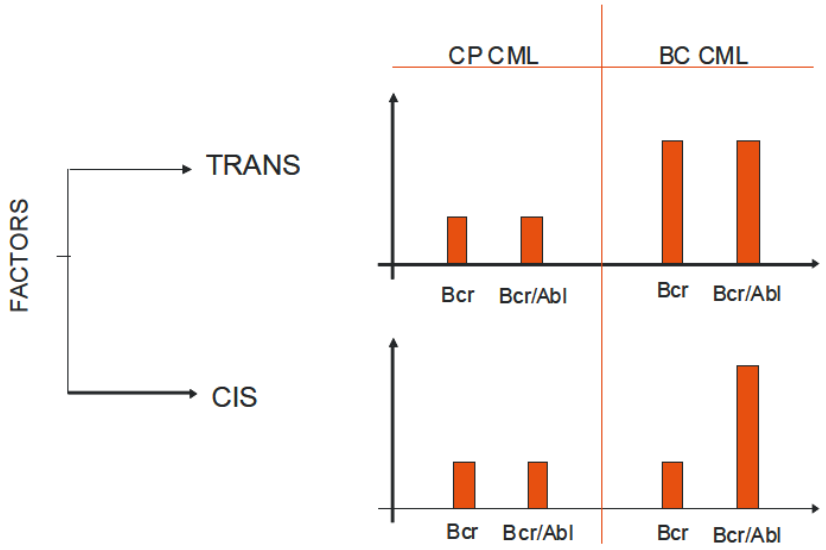

Figure 1: The hypothesis of mechanism that could influence the expression of BCR-ABL during the progression of CML.

To discriminate between these hypotheses and to define the transcriptional mechanism of regulation, we started to analyze a set of patients in CP and other in BC: we compared the expression of BCR and BCR-ABL in the mononuclear cells to discriminate the possible mechanisms controlling the BCR-ABL expression by Q-PCR. We introduced in the analysis a housekeeping gene, GUS (β-glucuronidase), validated as a reference gene for the BCR-ABL quantification in a previous study(187). The analysis with Q-PCR is reported in Figure 2 and 3.

In Figure 2 the comparison of expression levels of BCR and BCR-ABL analyzed by Wilcoxon matched pairs test is shown: the p_{values} (0.01 and 1 respectively for CP and BC) indicate that the level of both transcript is comparable in both phases of disease. In CP, the levels of BCR compared to BCR-ABL appeared very similar, and it's possible to observe a similar pattern also in BC, indicating that the expression of BCR and BCR-ABL are very similar in both phases of CML disease.

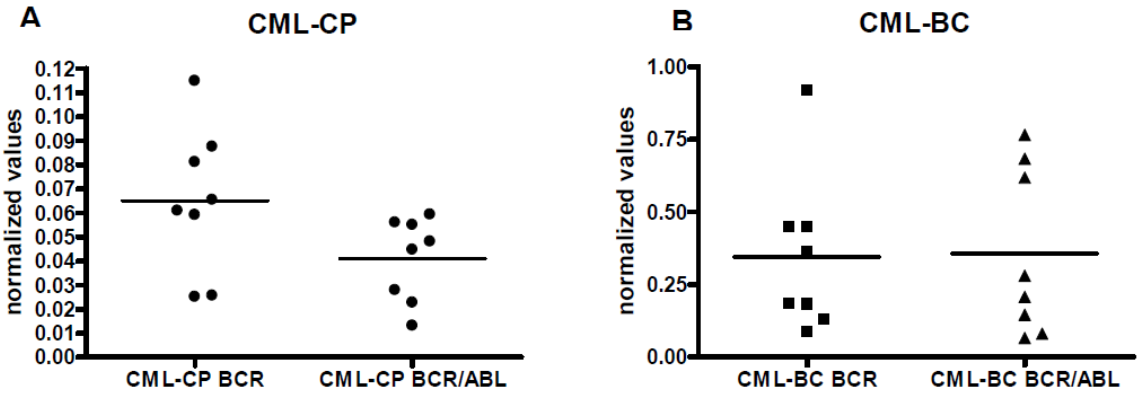

Figure 2: analysis of BCR and BCR-ABL in CP and in BC: in both CML phase the expression level of genes are similar in the mononuclear cells.

In the Figure 3A the expression of BCR in CP is compared with BCR expression in BC. In the Figure 3B is represented the same analysis for the BCR-ABL transcripts. From these analyses, it's clear that both genes show a similar increase during the progression of CML. The increase in expression of BCR-ABL and BCR is similar during the transition from CP to BC (p_{value}=0.0002 for BCR-ABL and p_{value}=0.0006 for the BCR).

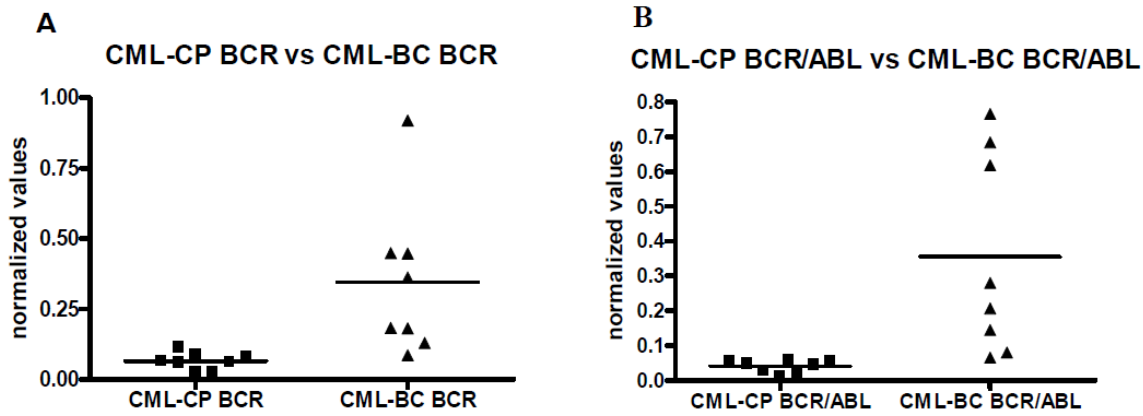

Figure 3: in 3A is represented the comparison of BCR expression in CP and BC, in 3B the comparison of BCR-ABL expression

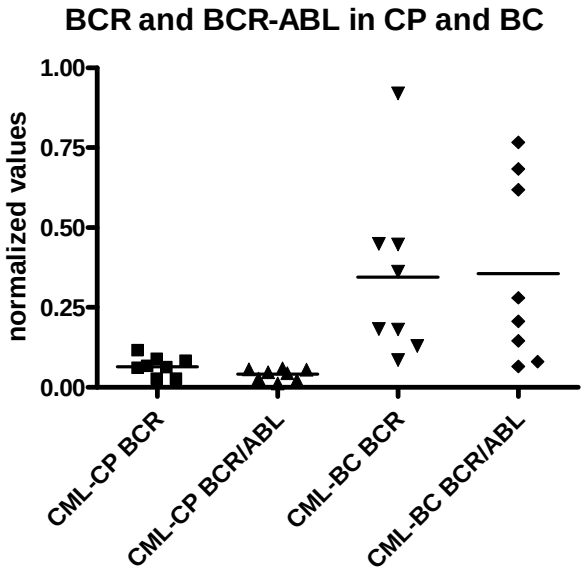

Figure 4: summary of data sets

The Figure 4 summarized the data obtained from BCR and BCR-ABL analysis. From these comparison clearly emerges that BCR-ABL increases during the passage from CP to BC and also BCR shows a similar increase during the progression. These results suggest that BCR and BCR-ABL could be regulated by the same mechanism, possibly involving the BCR promoter, that controls both genes. An *in trans* mechanism could cause a similar increase in expression of BCR-ABL and BCR during the evolution from CP to BC.

BCR and BCR-ABL expression during myeloid maturation in HD, CP-CML and BC-CML

The BCR promoter could have a central role in the increase of BCR-ABL during the progression of CML, but the activity of this promoter is unknown. It is known that BCR-ABL is downregulated during differentiation in CP(149), but in BC the fusion gene is expressed at high levels in all considered myeloid subpopulations. The first step of this project is the definition of the regulation of BCR in physiological condition, in order to verify the normal activity of BCR promoter and to compare with CP and BC activity.

To analyze the gene expression in different myeloid subpopulation, we set a protocol based on molecular surface markers, in order to isolate the different progenitors. Based on a prospective isolation of myeloid subpopulation we obtained the haematopoietic stem cells and early progenitors, the common myeloid progenitors and their downstream progeny, the granulocyte-macrophage and megakaryocyte-erythrocyte progenitors(186). All the populations reside in the lineage-negative (Lin-), the common myeloid progenitors and granulocyte-macrophage progenitors reside in CD34+CD38+ fraction of adult bone marrow. They are distinguishable by the expression of the IL-3Rα chain, the receptor of an early-acting hematopoietic cytokine, and CD45RA, an isoform of a phosphotyrosine phosphatase involved in negative regulation of cytokine signalling.

In our study, the myeloid subpopulations, Hematopoietic Stem Cell (HSCs), Common Myeloid Progenitors (CMPs) and Granulocyte-Macrophage Progenitors (GMPs), were isolated from mononuclear cells by a combination of Fluorescence-Activated Cell Sorting (FACS) and Magnetic Cell Sorting (MACS) techniques. Samples from healthy donors and patients in CML, in both phases of disease, were subjected to a purification/enrichment using MACS systems (MACS Lineage Cell Depletion Kit, Miltenyi Biotech, Germany) the following cocktail of antibodies: CD2, CD3, CD10,

CD11b, CD14, CD15, CD16, CD19, CD56, CD123 and Glycophorin A, to obtain the Lin- cells. Subsequently, the myeloid subpopulation was sorted by FACS (FACS ARIA, Becton-Dickinson) in the following subpopulations: HSCs (*Lin-, CD34+/CD38-/Thy+/-*); CMPs (*Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA-*) and GMPs (*Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA+)* as described by Manz(186).

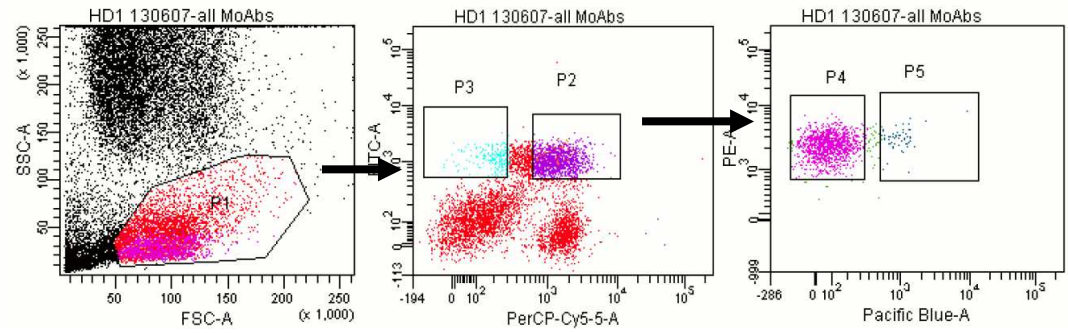

Figure 5: A report from a FACS sorting of HD. P1 represents the interested population, then P3 corresponded to HSCs (*Lin-, CD34+/CD38-/Thy+/-*), while P2 was sorted into P4, CMPs (*Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA-*) and P5 was GMPs (*Lin-, CD34+/CD38+/IL-3Rαlo/CD45RA+).*

After the setting of FACS sorting (Figure 5), the first analysis was focused on the expression of BCR in a set of healthy donors (HD): the definition of the physiological regulation of this gene, permits the definition of the BCR promoter activity and the interpretation of the data about BCR and BCR-ABL expression during the CP and BC.

From HD analysis, BCR levels are significantly downregulated upon myeloid maturation (Figure 8A): they are higher in HSCs (0.834±0.115) than in CMPs and GMPs subpopulations (CMPs 0.156±0.025, GMPs 0.142±0.035 HSCs vs CMPs, p_{value}=0.0006; HSCs vs GMPs, p_{value}=0.0012).

The analysis of BCR expression was then extended to CP-CML patients. The FACS analysis showed an increase in GMPs population compared to the HD samples (Figure 6).

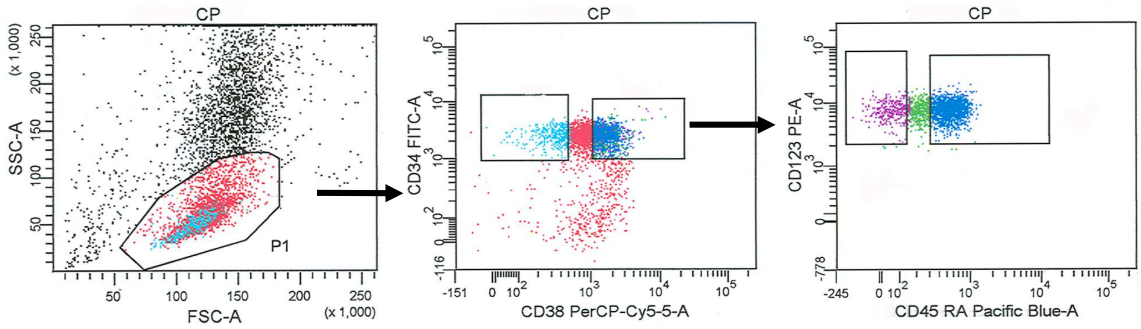

Figure 6: A report from a FACS sorting of CP. The myeloid subpopulations were sorted: P1 is the started subpopulation, then HSCs was isolated in the second panel, CMPs and GMPs were sorted in the third based on CD45RA marker.

We analyzed the myeloid subpopulation in CP BCR. Here we showed a similar downregulation upon differentiation to the more mature phenotype (Figure 8C, HSCs 0.301±0.045; CMPs 0.102±0.011; GMPs 0.043±0.015; HSCs vs CMPs, p_{value}=0.0039; HSCs vs GMPs, p_{value}=0.0003). The higher levels of BCR in HD than in CP samples (2.8 fold in HSCs, 1.5 fold in CMPs and 3.1 fold in GMPs) could be due to the presence of a single copy of the BCR gene in CML cells.

The evidence of a downmodulation of BCR upon myeloid differentiation suggests that the mechanisms controlling the expression of BCR in the normal haematopoiesis are conserved in CP. In line with previously published results(149), the analysis of BCR-ABL expression in our CP population, showed the downregulation of BCR-ABL mRNA expression upon myeloid differentiation (Figure 8D, HSCs 3.155±0.338; CMPs 0.806±0.084; GMPs 0.485±0.108; HSCs vs CMPs, p_{value}=0.0003; HSCs vs GMPs, p_{value}<0.0001).

To assess if ABL is similarly regulated during differentiation, we analyzed ABL expression in 5 HDs. We failed to detect ABL downmodulation upon differentiation in these samples (Figure 8B, HSCs 0.22±0.17, CMPs 0.16±0.13 and GMPs 0.152±0.135, HSCs vs CMPs p_{value}=0.42 and HSCs vs GMPs p_{value}=0.55). These data suggest that the mechanisms controlling BCR and ABL expression during myeloid differentiation are

different. Therefore the mechanisms active specifically on ABL (like miRNA 203) should not be operative in determining the observed downregulation of BCR-ABL.

The analysis was then extended to BC.

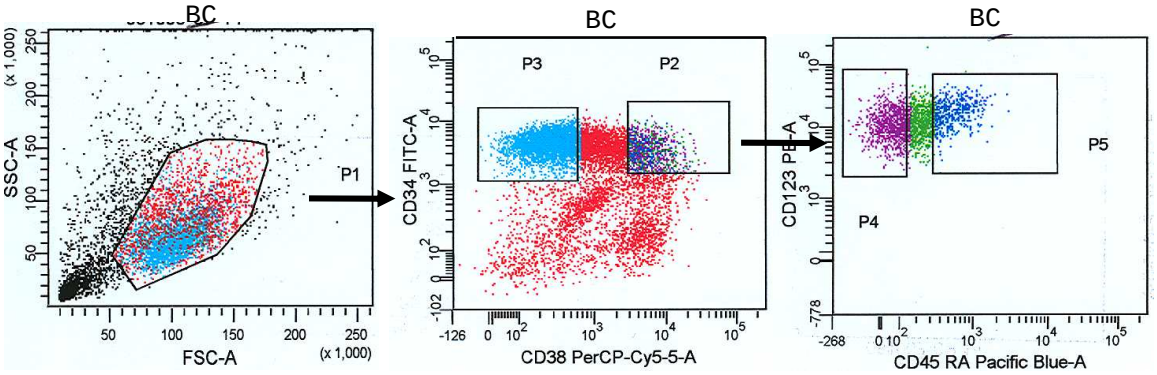

Figure 7: A report from a FACS sorting of BC cells. P1 is the myeloid subpopulation; panel 2 shows the purification of HSCs and the third panel shows the sorting strategy used for CMPs and GMPs enrichment.

The downregulation of BCR-ABL upon myeloid commitment is modest and fails to reach statistical significance (Figure 8F, HSCs 5.319±1.223; CMPs 3.285±1.260; GMPs 2.205±0.578). This correlates with the finding that the expression of BCR-ABL in the GMPs subpopulation is significantly higher in BC than in CP (2.205±0.578 vs 0.485±0.108, respectively; p_{value}=0.0115).

The analysis of BCR expression in the same set of BC patients showed a similar pattern, with a loss of BCR downmodulation in the GMPs population (Figure 8E, HSCs 0.226±0.048; CMPs 0.081±0.019; GMPs 0.296±0.1205). Similarly to BCR-ABL, the expression of BCR was significantly higher in BC than in CP in the GMPs subpopulation (0.296±0.1205 vs 0.043±0.015, respectively; p_{value}=0.0226). These findings indicate that the loss of BCR-ABL downmodulation in BC is caused by an *in trans* mechanism, affecting also the expression of BCR.

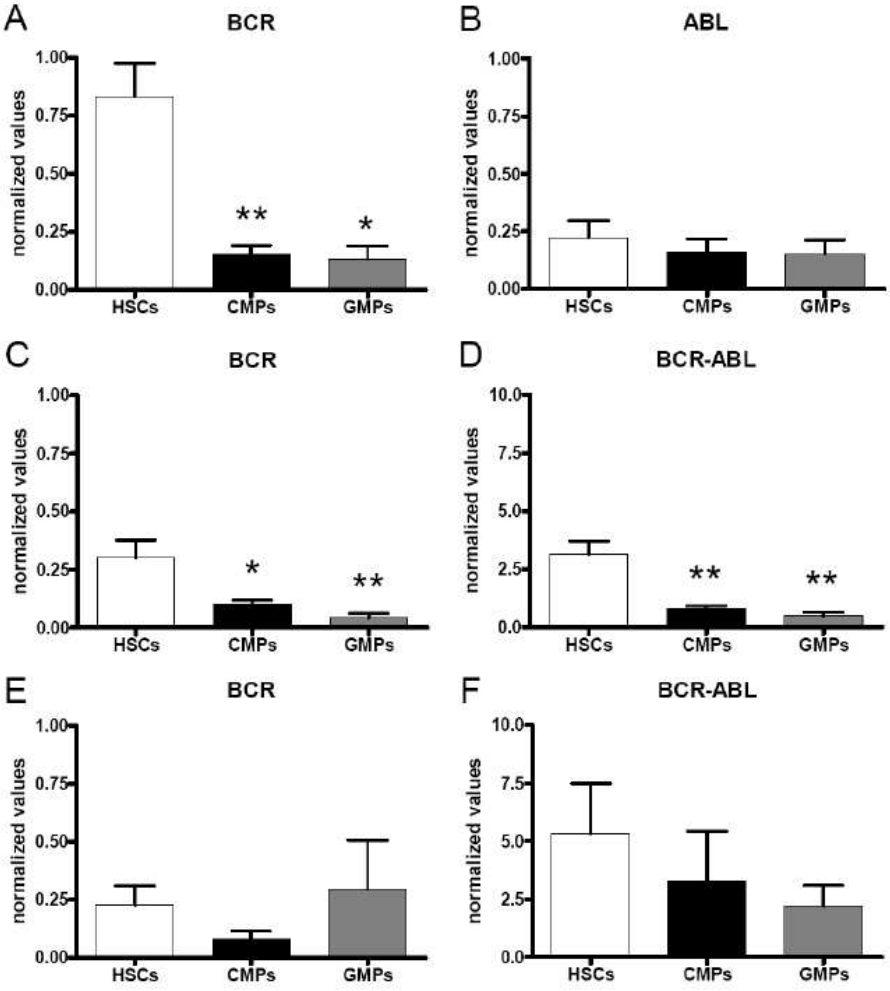

Figure 8: panel **A** shows the downregulation of BCR during the myeloid maturation; in panel **B** ABL expression is shown; panel **C** and **D** show the expression of BCR and BCR-ABL in CP, while in panel **E** and **F** the BCR and BCR-ABL expression level in CP is reported.

mRNA stability of BCR and BCR-ABL

To investigate if these findings could be due to differences in mRNA stability, like those caused by miR203(158), BCR and BCR-ABL mRNA half-life were determined (Figure 9). No significant differences in BCR-ABL mRNA stability could be detected (CP BCR half-life is 3.2 hours, while BCR-ABL is 2.0; p_{value}=0.0523; BC BCR half-life is 1.8 hours, BCR-ABL is 2.2; p_{value}=0.774; in K562 BCR and BCR-ABL half-lives are 3.0 and 2.3 hours; p_{value}=0.956).

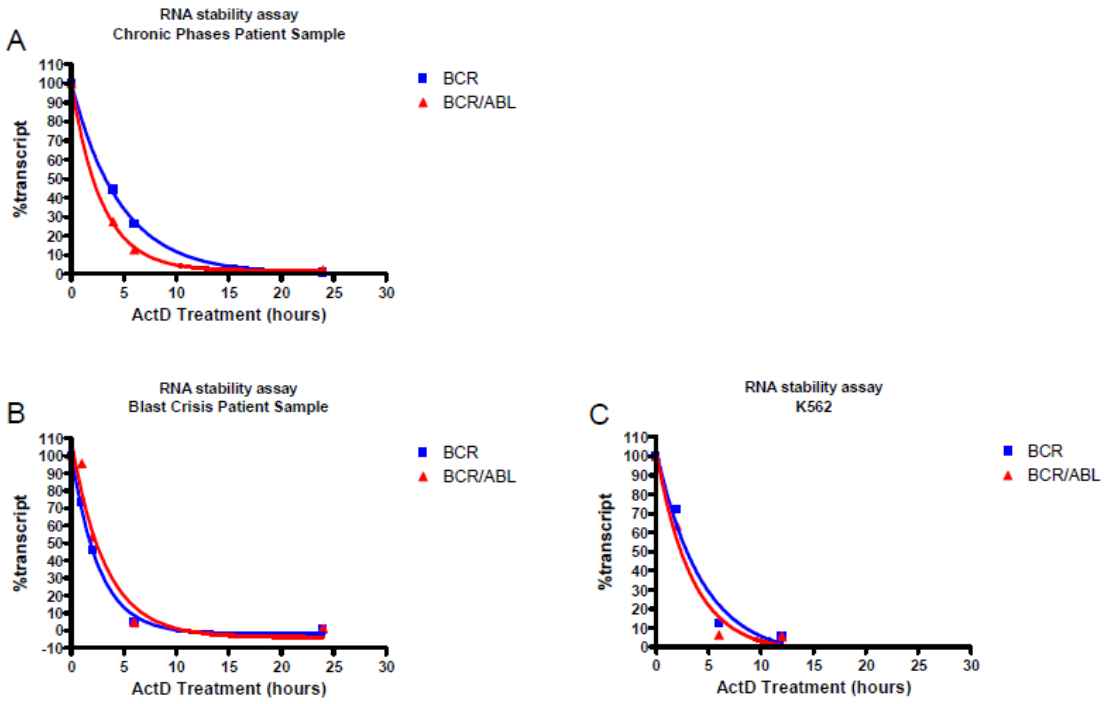

Figure 9: BCR and BCR-ABL mRNA half-life, during treatment with actinomycin D in K562 cells and one CP and BC patient samples.

BCR promoter activity

To identify the regions of BCR promoter involved in the activation of BCR transcription, we cloned different BCR promoter constructs in a luciferase reporter vector and we performed a gene report assay to study the transcriptional activity of BCR promoter in the K562 cell line, starting from a genomic DNA of healthy donors. We based our construct on the sequence of BCR promoter present in GenBank (X52828.1).

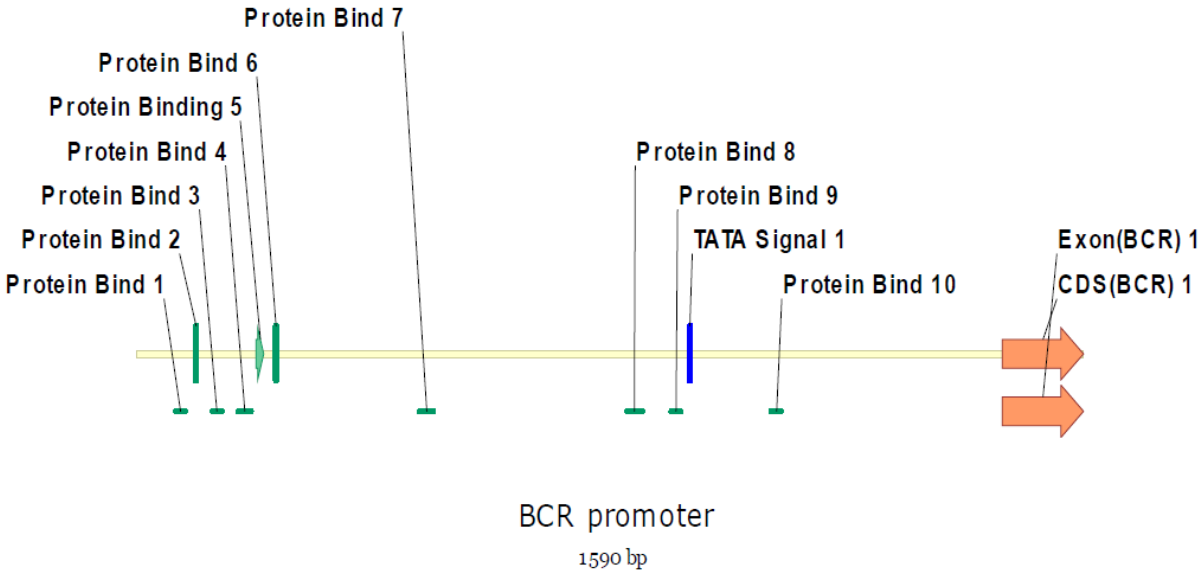

Figure 10: BCR promoter representation (GenBank: X52828.1)

A minimal promoter was previously identified, corresponding to a 1 kb region immediately upstream of exon 1 coding sequence. We extended our study to 452 bp upstream from this minimal promoter, because several putative protein binding sites have been identified in this region by DNase protection studies, to a total of 1443 bp, corresponding to the longest construct used here (pGL3/BCR promoter). The importance or the transcriptional influence of these regions is yet unknown. The main transcription start site was identified at -471 bp from the ATG sequence and other minor transcription start sites have been identified in the region between the main transcription start site and

the coding sequence (Figure 10). Based on this information, we cloned the Δ1041 and Δ1271 constructs of BCR promoter, corresponding to 418 and 170 bp upstream from the coding sequence, containing 3 minor transcription start sites (indicated in Figure 12) but not the main start site, to analyze the contribution of these regions to the transcriptional activity. To investigate the region upstream from the main transcriptional start site, three additional constructs, Δ541, Δ241 and the pGL3/BCR promoter were generated, spanning 896, 1203 and 1443 bp upstream from the ATG sequence. In these 3 constructs the main transcription start site and a putative SP1 binding site, located 69 bp upstream from the main transcription start site, are present. All constructs were cloned in pGL3-basic vector (Figure 11) in order to detect the luciferase expression level and thus the activity of the promoter.

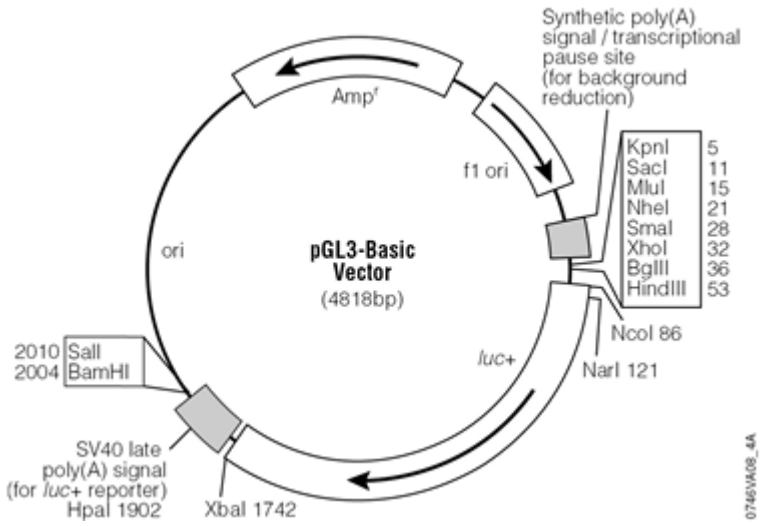

Figure 11: we inserted the BCR promoter and the truncated forms in the pGL3 basic vector using Nhe I and Hind III restriction sites

We performed the Luciferase assay to verify the activity of the BCR promoter constructs. The shorter Δ1041 and Δ1271 BCR promoter showed a very low level of luciferase activity, comparable to that of the empty vector (Figure 12), as expected. This is in line with previous reports showing that only minor transcriptional start sites could be found in this region and suggesting that the DNase protected regions identified in this part of the BCR promoter do not play a major role in the activation of BCR transcription. Additionally, it's interesting to observe that in this region also the site for SP1 is absent. In contrast, when we analyzed the Δ541 construct, the luciferase activity was significantly increased over the empty vector (19 fold increase, p_{value}=0.0011). This finding was not unexpected, because in Δ541 the presence of the main transcription site and of a putative SP1 binding site has been previously reported. Δ241 construct showed a level of luciferase activity (13 fold increase, p_{value}=0.0061) similar to Δ541. The luciferase activity of the longest BCR promoter (pGL3/BCR promoter) showed a 49 fold increase in the luciferase activity in comparison to the empty vector (p_{value}=0.0006), significantly higher than Δ541 and Δ241 (p_{value}=0.0048 and p_{value}=0.0023). These data indicate that the region between -1443 and -1202 bp from the ATG site could be critical for the transcriptional activity of the BCR promoter.

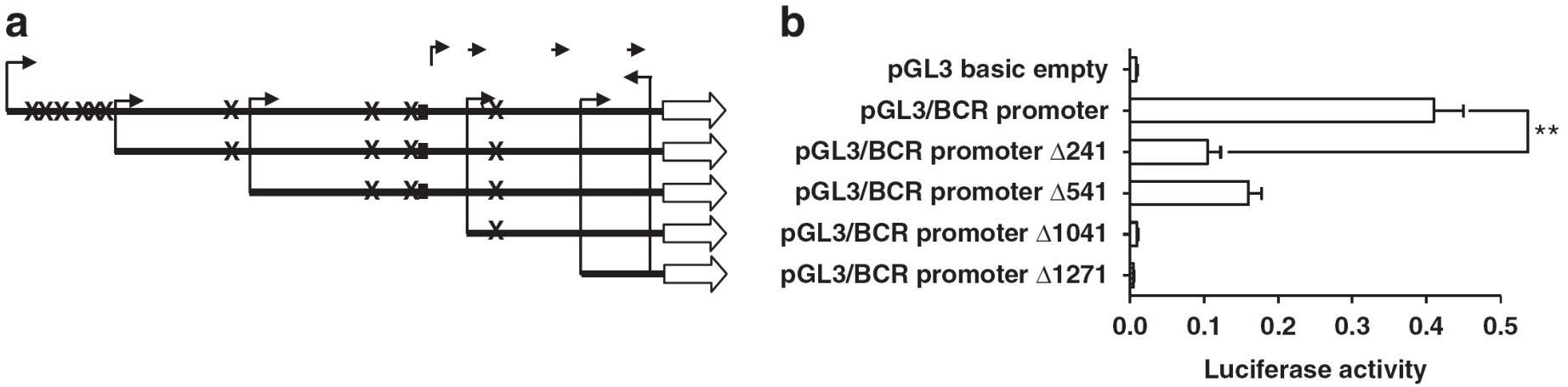

Figure 12: In A the BCR promoter structure is shown: the mutant lacking the upstream promoter sequence was constructed in a promoter-devoid luciferase vector, pGL3-Basic. The forward primers are indicated by thin arrows, the reverse primer is the same for all BCR promoter forms (Sequences in Table 2). The black boxes represent the main SP1 putative binding site, present in the Δ541 and Δ241 constructs, together with the main transcription start site, indicated in the Figure by the bold arrow. Other minor transcriptional start sites are reported in the Figure as arrowheads. The 'x' label indicates the putative binding sites along the promoter. The upper line corresponds to the longest BCR promoter, and the following ones represent the progressively deleted constructs. In all forms the open arrows represent the coding DNA sequence.
In B, the BCR promoter activity is reported: the Firefly luciferase reporter constructs containing the previously indicated parts of the BCR promoter were transiently transfected into K562 cell line and the luciferase activity was measured. Results are presented as the average fold of Firefly luciferase activity versus Renilla control vector. Value reported in the graph represented the average of three separate experiments.

** $p_{value} = 0.0023$ pGL3/BCR promoter Δ241 compared with the pGL3/BCR promoter

We carried on the identification of transcriptional factors that could be involved in the regulation of BCR and BCR-ABL using an *in silico* approach. Using TESS (Transcription Element Search System), available on http://www.cbil.upenn.edu/tess, or TFSearch available on http://www.cbrc.jp/research/db/TFSEARCH.html and the Genome Browser from the University of California Santa Cruz (UCSC, http://genome.ucsc.edu/), we identified a high number of putative TFs that could bind the BCR promoter. The data from the *in silico* analysis were compared with the data from literature in order to exclude TFs that didn't have a expression profile similar to BCR and that are up or downmodulated in BC.

TFs	PBS
c-myc	2
MAX	2
c-myb	1
YY1	6
E47	3
AML-1	4
GATA2	6
GATA1	6
SP1	9

Table 1: in the table are reported the identified putative transcriptional factors. TF= transcriptional factor; PBS=putative binding site.

The identification of the first region of BCR promoter, corresponding to the first 241 bp, as a critical regulator of BCR expression, led us to further investigate this region and the PBSs present in this region. We decided to clone other shorter different constructs of BCR promoter, to further characterize this region and to improve the analysis of PBSs.

We generated pGL3/BCR promoter Δ110 and pGL3/BCR promoter Δ180. In pGL3/BCR promoter Δ110 are excluded PBS1 and PBS2, while pGL3/BCR promoter Δ180 additionally exclude PBS3 and PBS4. With this approach it should be possible to identify

the critical PBS present in this region. At the writing time of this thesis, some preliminary experiments are in progress.

SP1 binding in BCR promoter

A basal activity of BCR promoter seems to be conserved in Δ241 and Δ541 BCR promoter constructs, while in the Δ1041 construct this activity is almost completely lost. Probably, in this region, between -418 and -1201 bp, upstream from the ATG sequence, localize the PBSs for the TFs that are responsible for this basal activity of BCR promoter. In this critical region there are 3 PBSs, from PBS7 to PBS9. From previous studies, one of these, PBS9, should be a binding site for SP1. SP1 is an ubiquitinated transcriptional factor that interacts with several different proteins. It is involved in chromatin remodelling and can activate or repress transcription in response to physiological and pathological stimuli(189, 190). To check whether SP1 was bound to BCR promoter we used the FactorFinder kit from Miltenyi Biotech.

First of all, we optimized the experiment, changing the concentration of the probe used in the incubation step. The optimal concentration was about 40 pmol of oligonucleotide probes showed the better signal in elution fraction for SP1. High concentration of probes permits to obtain good signal, but already the use of 40 pmol produce a clear and good signal (Figure 13).

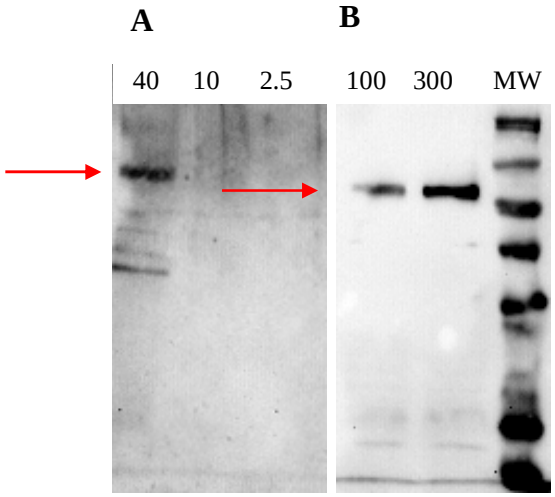

Figure 13: Western Blot against SP1, elution fractions: in A different concentration of probes were used 40, 10 and 2.5 pmol, in B the concentration were 100 and 300 pmol: the best concentration resulted 40 pmol, sufficient to have a good and clear signal about SP1

We used three different biotinylated probes: the wild type, in which the SP1 binding site is complete, KO2 probe, in which two bases are modified and KO4 probe, in which 4 basis are different from wild type.

In Figure 14, the wild type probe for SP1 showed a strong signal, indicating the presence of SP1 TF, while the KO2 probe is only partially able to capture the SP1 TF. This evidence confirms the specific binding of SP1 to its binding site present in the BCR promoter.

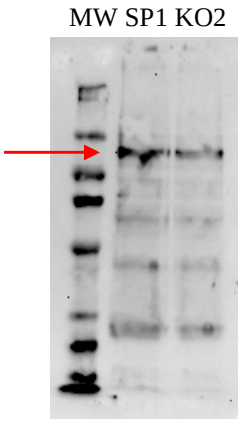

Figure 14: Western Blot against SP1, elution fractions: the SP1 signal in the elution fraction from KO2 sample was less than the SP1 signal of wild type probe. MW=molecular weight, SP1=wild type probe, KO2=mutated probe

The experiment was repeated with the KO4 probe, with the same condition (Figure 15).

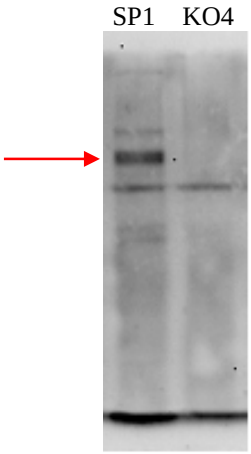

Figure 15: Western Blot against SP1, elution fractions: the signal about the TF is present only in the lane SP1 eluate, in which the probe was wild type. KO4 eluate represents the mutated probe and in fact the signal was completely absent. SP1=wild type probe, KO4=mutated probe

In Figure 15, the signal of SP1 was present when the probe was wild type, while KO4, the mutated probe in 4 bases, didn't show the SP1 signal. This result demonstrated that the binding of SP1 to the sequence present in BCR promoter used in the wild type probe was specific.

ChIP to detect the binding of SP1 to the BCR promoter

We performed a ChIP analysis to verify the binding of SP1 to the PBS9 in the BCR promoter *in vivo*.

Using LAMA cell line as starting material, we performed the ChIP assay and we analyzed the results by Q-PCR, amplifying the region closed to PBS9. The dihydrofolate reductase promoter was used as a positive control. It is known in fact that SP1 strongly binds to the dihydrofolate reductase promoter, being one of its master regulators. We demonstrated that SP1 binds the BCR promoter (Figure 15) with a 5 fold-enrichment when compared with the GAPDH promoter. The difference between BCR region PBS9 and GAPDH region samples showed a statistical significant difference, p_{value}= 0.0024.

This difference is conserved also between BCR region PBS9 and the IgG control (p_{value}= 0.0414), confirming the binding of SP1 to PBS9 and the specificity of this interaction.

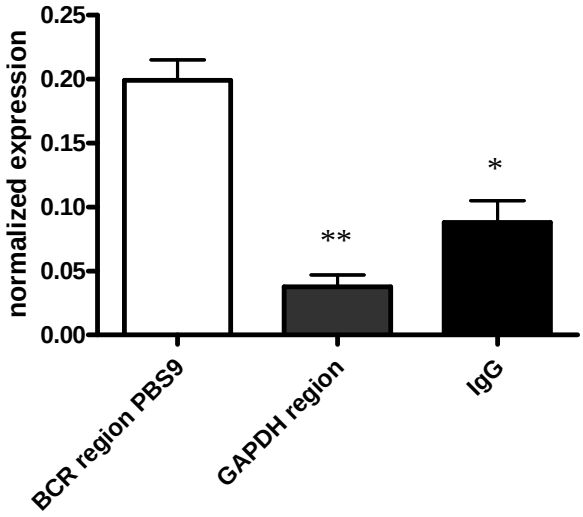

Figure 16: ChIP against SP1 to verify the binding of this TF to BCR promoter PBS9

SP1 overexpression

We performed a transient transfection with RSV-SP1, a plasmid encoding SP1, in order to verify the influence of this TF in the expression of BCR and BCR-ABL; the experiment was done in duplicate in LAMA cell line and the mRNA level of both genes was subsequently analysed by Q-PCR.

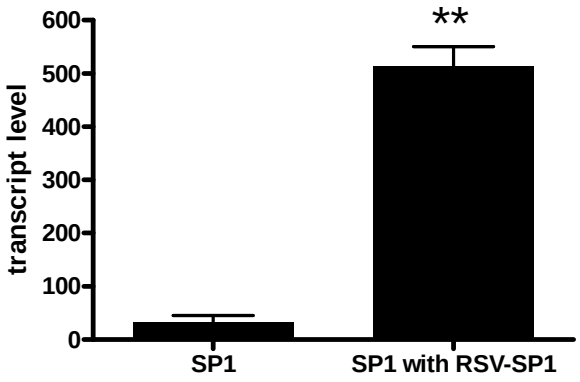

Figure 17: the transfection with the plasmid RSV-SP1 showed a significant increase in the expression of SP1 in LAMA cell line.

We preliminarily showed that the transfection with the RSV vector increased SP1 expression (p_{value}=0.0065), as expected (Figure 17). Then we verified the level of BCR and BCR-ABL: in presence of RSV-SP1 both BCR and BCR-ABL showed an increase in the expression level, although this increase failed to reach statistical significance (Figure 18, p_{value}= 0,1021 for BCR and p_{value}= 0,7166).

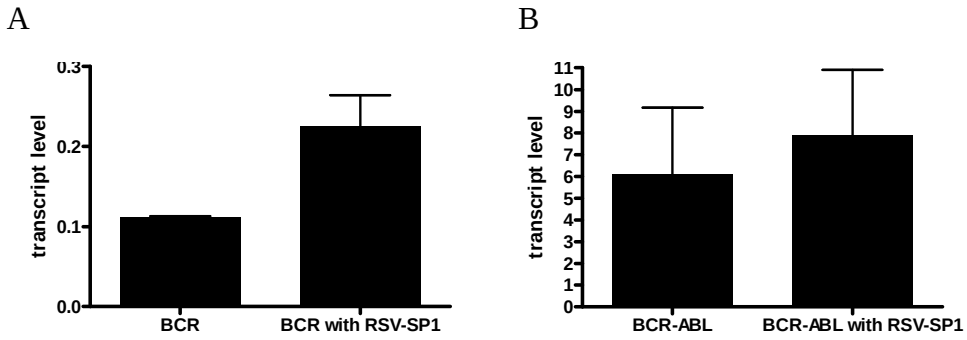

Figure 18: in **A** BCR expression in untransfected cells and in cells transfected with RSV-SP1; in **B** BCR-ABL expression in untransfected cell and in cells transfected with RSV-SP1.

We are now cloning the BCR promoter constructs mutated in the PBS9 to verify the influence of the loss of SP1 binding on the BCR promoter activity.

Discussion

At present, the reason of CML progression from the chronic phase to the blastic phase is unknown. Several works have been tried to dissect the key events for this phenomenon, using different approaches.

The use of microarray technology in CML has increased the understanding of the biology of this disease. In the initial study, mononuclear cells (MNC) from CML patients were compared with the MNC from healthy donors, which led to the characterization of the transcriptional role of BCR-ABL. The major problem in this analysis is the heterogeneity of the biological material under study. As microarray technology is able to identify small changes in the gene expression profile, the pitfall in using unfractionated MNC is that the proportion of leukemic blasts, and other differentiated cell populations may vary between patients, which consequently hampers the interpretation of results(191).

Subsequent profiling studies to differentiate the molecular phenotype of CML cells from that of healthy individuals used $CD34^+$ progenitors(192, 193). This approach allowed the identification of the molecular signature of CML samples and of the normal counterpart. Moreover, the comparison of CML samples at different stages, led to the identification of the molecular signature of CP, AP and BC: for example, SOCS-2 (suppressor of cytokine signalling-2) was the most upregulated gene in BC samples compared with CP using Affymetrix HG-U133A microarray chips, which cover the majority of the well-annotated genes available(194). Among the different groups of CML samples, the gene expression pattern of BC cells was also found to be very similar to that of normal $CD34^+$ cells, with aberrations in genes involved in the WNT/β-catenin pathway, cytoskeletal and adhesion molecules(181). Reacquisition of self-renewal capability happens in GMPs with persistence of high BCR-ABL mRNA levels and accumulation of a nuclear S/T-nonphospho β-catenin(149, 150). In these progenitors β-catenin appears to enhance the self-renewal activity and also the leukemic potential(149). In general, in the absence of

Wnt ligands, the β-catenin protein is degraded through the action of a multiprotein β-catenin turnover complex. β-Catenin accumulates in CML as a Y-phosphoprotein coupled to BCR-ABL and the fusion protein phosphorylates β-catenin at Y86 and Y654 and promotes its protein stabilization. Y-phosphorylated β-catenin does not interact with the Axin/GSK3β complex and is resistant to APC-mediated degradation permitting the binding of β-catenin to the TCF4 transcription factor and thus representing a transcriptionally active pool(150). The re-activation of the β-catenin pathway and its transcriptional effects are a consequence of the high level of BCR-ABL, measured in BC: only in presence of high BCR-ABL expression levels, β-catenin is phosphorylated and stabilized. Therefore, BCR-ABL is a critical factor in the process of β-catenin stabilization and accumulation, which is typically observed in BC. It is thus possible to speculate that probably BCR-ABL upregulation is one of the driving forces leading to CML progression.

In this scenario, in which all the main feature of BC seem to be dependent from BCR-ABL expression levels, the definition of the transcriptional regulation of the fusion gene appears very important in the understanding of the CML progression. We and others demonstrated that the expression of the chimeric BCR-ABL mRNA changes during CP and BC(149, 195). This leads to two possibility: BCR-ABL expression can be regulated *in cis* or *in trans*. It's possible to discriminate between these alternative hypotheses because BCR-ABL is under the control of the same promoter of BCR gene. The comparison of BCR and BCR-ABL could address what is the mechanism that acts on the transcription of the fusion gene. If the mechanism acts *in cis*, only the BCR-ABL expression level should change during the passage from CP to BC; conversely, if the mechanism acts *in trans*, BCR and BCR-ABL should be co-regulated and thus they should show a similar upregulation from CP to BC. Therefore, the first step of this

project was to analyze the expression analysis level of BCR and BCR-ABL in MNC from CP and BC patient samples.

This analysis allows us to discriminate between the two previous hypothesis and thus may give important indications about the mechanism involved in CML progression.

The analysis showed a similar increase for BCR and BCR-ABL from CP to BC: in MNC the level of both genes increased, comparing the expression levels in the two set of samples. Therefore, in CP and BC patients, BCR and BCR-ABL seem to be regulated in a similar way, suggesting the presence of a common mechanism, acting *in trans*.

It is known that the percentage of the different cell subpopulations present in MNC may vary greatly from sample to sample. The study of gene expression on MNC samples may thus be hampered by the limited homogeneity of the cell populations. To overcome this limitation, we decided to study BCR and BCR-ABL regulation in myeloid subpopulations. In fact, it's known that BCR-ABL expression levels change not only during the passage from CP to BC, but also during the maturation of myeloid cells: in CP, BCR-ABL showed a higher level in HSCs than CMPs and GMPs. Conversely, in BC the expression of BCR-ABL in high in all myeloid progenitors, from HSCs to GMPs. These evidences take us to analyze the expression level of BCR as well as BCR-ABL in HSCs, CMPs and GMPs subpopulation.

An important indication of the activity of BCR promoter comes from the analysis of HD samples: in this set of samples, BCR is physiologically regulated and thus the analysis of its expression allows us to gain insight into the normal mechanism of BCR promoter regulation. Therefore BCR mRNA was analyzed by Q-PCR in the myeloid subpopulations as previous published(149). The results showed that BCR is physiologically downregulated upon myeloid maturation from HSCs to CMPs and GMPs.

The analysis of CP samples reveals that BCR transcriptional regulation observed in HD samples is conserved during the initial phase of CML disease: BCR and also BCR-ABL showed a similar pattern of expression during the myeloid maturation. Both genes are progressively downregulated from HSCs to CMPs and GMPs and this observation suggests the presence of an *in trans* mechanism, that is still active on both genes during CP.

It is known from previous studies that miRNA 203 is able to target the 3'UTR of ABL, thus controlling ABL mRNA stability and modulating ABL expression. Notably, the sequence of the 3'UTR is shared between ABL and BCR-ABL. Therefore, we speculated that ABL and BCR-ABL could be similarly coregulated through the action od miRNA 203(158). To test this hypothesis, we analyzed ABL expression, in the HSCs, CMPs and GMPs subpopulations. In sharp contrast with the pattern of BCR and BCR-ABL, here we failed to detect evidence of ABL downmodulation upon differentiation. This evidence suggests that BCR-ABL and ABL expression are not coregulated during myeloid differentiation.

The analysis was then extended to BC-CML. The downregulation of BCR-ABL upon myeloid commitment was modest and failed to reach statistical significance. This correlated with the finding that BCR-ABL expression in the GMPs subpopulation is significantly higher in BC than in CP. The analysis of BCR expression in the same set of BC patients showed a similar pattern, with a loss of BCR downmodulation in the GMPs population. Similarly to BCR-ABL, the expression of BCR was significantly higher in BC than in CP in the GMPs subpopulation. These findings suggest that the loss of BCR-ABL downmodulation in BC is caused by an *in trans* mechanism, affecting also the expression of BCR and selectively impairing the downmodulation of the two genes in the more mature myeloid subpopulations (GMPs). To investigate if these findings could be

due to differences in mRNA stability, like those caused by miR203(158), BCR and BCR-ABL mRNA half-life was determined. No significant differences in BCR-ABL mRNA stability could be detected.

The evidence of BCR and BCR-ABL coregulation in both phases of CML strongly suggested that the mechanism causing the increase of BCR-ABL expression acts *in trans*. For this reason, to further characterize this mechanism, we concentred our attention on the BCR promoter, which regulates both BCR and BCR-ABL expression. To identify the most critical region for the activity of BCR promoter, we put the luciferase gene under the control of the full BCR promoter and of progressively shorter region.

The results of the luciferase assay indicate that the region comprised between 420 and 900 bp from the coding ATG site is required to achieve a basal transcription level. In this region a consensus binding site for SP1 was previously identified in the BCR promoter (160). The putative SP1 binding site could play a role in the basal promoter activity. In fact, an almost complete absence of transcriptional activity was measured in Δ1041 and Δ1271 constructs, lacking both the main transcription start site and the putative SP1 binding region. We hypothesize that SP1 locus, which present in Δ541 and in longer constructs, could be one o the activator of the basal BCR promoter activity. ChIP analyses confirmed the binding of SP1 to its PBS, thus indicating that SP1 physically interacts with BCR promoter. The Luciferase Assay with mutated SP1 PBS constructs of BCR promoter could be confirm or not if the SP1 binding to the PBS in the promoter has importance for the transcriptional activity. At the present the cloning of these constructs is ongoing.

In addition to the SP1 PBS, along the BCR promoter, the presence of ten additional putative PBSs is also known from previous works(159, 160). Six of these putative PBSs are localized in the region between -1443 to -1202 bp, which appears to be critical from

in vitro studies. In fact, only in presence of a 221 bp region upstream from Δ241, a strong luciferase signal could be detected, suggesting that the promoter region between -1443 and -1202 bp from the coding ATG is indeed critical to achieve the highest level of expression. *In silico* analysis showed that different TFs could be involved in the regulation of BCR promoter activity in the -1443 to -1202 region: c-myc and its co-factor Max could localize in PBS2; also C/EBP box motif (CCAAT) is predicted to bind in the same region. Putative E47 and AML1 binding sites are present in PBS3; finally USF could bind in PBS4. The interactions between the TF and BCR-ABL could however be more complicated than expected. For example, c-myc, has been identified as being regulated by β-catenin and this evidence supports a possible role of this TF in the progression of CML, when the β-catenin is overexpressed in BC(196). Moreover, BCR-ABL protein also induces the expression of c-myc (197) through the MAPK-dependent regulation of HNRPK translation regulatory activity. An alteration of the transcriptional activity present in this region could theoretically be involved in disease progression, in accordance with the results of BCR and BCR-ABL expression in normal individuals and in CP and BC patients.

The role of *in silico* identified TFs in the transcriptional activity of BCR promoter is now being experimentally investigated, including the presence of methylated CpGs islands that could modify the binding of TFs. Moreover, the investigation of the causes of BCR deregulation in BC is presently being addressed by High-Throughput Sequencing (including ChIP-Seq and mRNA-Seq), Protein/DNA Arrays techniques and additional *in silico* analysis of putative binding sites present in the BCR promoter.

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