Università degli Studi di Padova

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Salute della Donna e del Bambino – SDB

SCUOLA DI DOTTORATO DI RICERCA

in

MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE

INDIRIZZO

EMATOONCOLOGIA, IMMUNOLOGIA E GENETICA

CICLO XXV

NOVEL MOLECULAR STRATEGIES TO OVERCOME CREB OVEREXPRESSION IN ACUTE MYELOID LEUKEMIA

Direttore della Scuola: Ch.mo Prof. Giuseppe Basso

Coordinatore d'indirizzo: Ch.mo Prof. Giuseppe Basso

Supervisore: Dott.ssa Martina Pigazzi

Dottorando: Claudia Tregnago

INDICE

SUMMARY

Leukemias are the most common form of pediatric cancers, and whereas Acute Lymphoblastic Leukemia (ALL) treatment has made significant progresses over the past decades reaching up to 86% of survival, Acute Myeloid Leukemia (AML) survival rate reaches the 60%, and 30% of those patients relapsed within 1/2 years from remission. The cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) has been found to be overexpressed in the leukemic blast cells of 66% of patients with AML and in the 84% of patients with ALL, compared to normal bone marrow or remission samples. CREB overexpression in AML patients was also correlated with a worse prognosis and increased risk of relapse. Nevertheless, CREB's role in AML pathogenesis is still debated. In this work I identified three main ways in order to target CREB overexpression: ICER, its endogenous repressor; the miR-34b which targets its 3'UTR; the zebrafish as an *in vivo* model where new molecules can be tested.

CREB level has been shown to be controlled by the inducible cAMP early repressor (ICER), an endogenous CREB competitor able to recognize cAMP responsive element (CRE) sequences and stop transcriptional activity. ICER level was found downregulated in AML, failing to control CREB transcriptional activity, thus sustaining leukemia. Restoration of ICER expression in leukemic cell lines and in AML primary cultures, led to a decreased expression of CREB and its target genes. Among the genes affected by ICER restoration, impairment of the dual-specificity phosphatases DUSP1 and DUSP4 was found. The decreased level of DUSPs1/4 directly sustained the p38-mediated apoptotic pathway, which in turn triggered caspase-dependent apoptosis. This important ICER role in cell death, led us to focus the attention on combining its action together with specific drug treatment. AML cell lines reduced cell growth and enhanced apoptotic behavior after chemotherapy treatment if ICER was expressed. A significant lowered expression of CREB target genes involved in cell cycle control (CyA1, B1, D1), and in mitogen-activated protein kinase signaling pathway (ERK, AKT, DUSP1/4) was found after Etoposide (a topoisomerase inhibitor used in therapy of AML) treatment. To verify the involvement of DUSPs on p38 activation, we silenced DUSPs1/4 in HL60 cell lines, and we confirmed the same enhanced drug sensitivity induced by ICER. Moreover, DUSPs1/4 silencing or ICER restoration in primary AML cultures showed DUSP1 downregulation and p38 activation. ICER has been demonstrated to mediate chemotherapy activity by DUSP1-p38 pathway activation, thus reprogramming leukemic cells from survival to apoptosis. These results supported important effects of ICER restoration which mediated CREB activity, remarking the involvement of CREB in sustaining leukemia.

Thus we argued to seek out the cause of CREB overexpression in AML, and giving that it was found overexpressed in its proteic form, we looked at translational control, focusing on microRNAs. We found that miR-34b targeted the 3'UTR of CREB gene, and was downregulated in AML. We dissected its role in inducing CREB overexpression. We used AML cell lines and revealed the

hypermethylation of its promoter. Thus, we analyzed miR-34b expression and methylation status of its promoter in cells from patients diagnosed with myeloid malignancies. We found that miR-34b was upregulated, since it was not hypermethylated at its promoter, in pediatric patients affected by preleukemic hematopoietic disease as juvenile myelomonocytic leukemia (JMML, n=17) and myelodysplastic syndrome (MDS n=28) with the concomitant low CREB expression. On the other hand, miR-34b was downregulated in AML patients at diagnosis (n=112). Our results showed that hypermethylation of the miR-34b promoter occurred in 66% of acute myeloid leukemia, explaining the low miR-34b levels and CREB overexpression, whereas preleukemic MDS and JMML patients were not associated with hypermethylation nor CREB overexpression. Interestingly, in paired samples of MDS evolved into AML, we confirmed miR-34b promoter hypermethylation at leukemia onset, and gene expression profile revealed 103 CREB target genes differentially expressed between the two disease stages. This subset of CREB targets was confirmed to associate with high risk MDS in a separate cohort of patients (n=20), identifying a signature of myeloid transformation To confirm this hypothesis, we evaluated miR-34b tumor suppressor potential *in vitro* and *in vivo*, finding that miR-34b restoration decreased AML engraftment and progression through CREB-dependent pathways. These results confirmed that miR-34b controlled CREB expression and contributed to myeloid transformation. Furthermore, we identified a subset of CREB target genes that represents a novel transcriptional network involved in tumor formation, and their functional activity may be further pursued.

We considered to test CREB and its target genes' role in mediating MDS and AML in a suitable *in vivo* model, such as zebrafish. We chose this model organism for its numerous advantages that led to its widespread use in both developmental studies and cancer research, such as high regenerative ability, rapid and transparent embryonic development, and easy genetic manipulation. We integrated a plasmid coding human *CREB* under *pu.1* promoter into fertilized egg at one cell stage to overexpress CREB in the myeloid lineage. Results showed that CREB-overexpression led to hematopoiesis perturbation in embryos at 24 and 48 hours post fertilization, with an altered CREB target gene expression, that conferred an enhanced cell cycle progression. Since 6 months after CREB enforced expression, zebrafish kidney marrow showed dysmyelopoiesis, with increased monocytes and myelocytes rates. At the age of 9 months, CREB-overexpressing zebrafish developed a sick phenotype with abdominal and dorsal enlargement that at morphological evaluation resembled an abdominal mass formed by clonal cells with infiltrating capability. The mass cells were found to originate from the cells overexpressing human CREB, to rapidly proliferate and to migrate into gills, adipose tissue and muscles. Mass cell morphology was similar to that of kidney marrow with a complete lack of myelocytes, and a preponderance of monocytes, confirming a myeloproliferative disease. These results demonstrated that zebrafish overexpressing CREB resembled the same dysmyelopoiesis already seen *in vitro* and in CREB transgenic mice, with a relevant perturbed CREB target gene expression. The long latency of disease onset suggested that additional events may occur.

Neverthless, CREB-overexpressing zebrafish model recapitulated a CREB-mediated myeloproliferative disease: it represents a useful model with high potentialities in dissecting CREB role on malignant transformation. Moreover, the use of ICER and miR-34b as tumor suppressors, as well as the screen of novel compounds which may be confidently tested, will open for further therapeutic opportunities.

SOMMARIO

La leucemia è il cancro più diffuso nell'età pediatrica. Oggigiorno, le cure per la Leucemia Linfoide Acuta (LAL) hanno portato a progressi molto significativi, con una sopravvivenza dei pazienti che raggiunge l'86%, mentre il trattamento contro la Leucemia Mieloide Acuta (LAM) ha raggiunto una quota di sopravvivenza del 60%, ma entro 1/2 anni dalla remissione il 30% di questi ultimi ricade. Uno dei principali scopi della ricerca nelle LAM è quello di identificare nuovi marcatori molecolari che siano in grado di identificare geneticamente alcuni sottogruppi di LAM, e anche di comprendere la leucemogenesi. A questo scopo, negli ultimi anni è stato identificato il gene *CREB* (cyclic adenosine monophosphate (cAMP) responsive element binding protein), che codifica per un fattore di trascrizione implicato nelle principali attività cellulari. Nel 66% dei pazienti affetti da LAM, e nell'84% dei pazienti affetti da LAL, è stata trovata una sovra espressione della proteina CREB rispetto alla espressione fisiologica dello stesso riscontrata in cellule di midollo osseo di donatori sani o di pazienti in remissione. Inoltre è stata trovata una correlazione tra la sovra espressione di CREB nelle LAM, ed una prognosi peggiore con un aumentato rischio di ricaduta. Ciononostante, il ruolo di CREB nella patogenesi delle LAM rimane ancora dibattuto. In questo lavoro, ho utilizzato tre diverse strade per agire sulla sovra espressione di CREB: ICER, il suo repressore endogeno; miR-34b, microRNA che si lega alla sua regione 3'UTR; lo zebrafish, come modello *in vivo* per poter testare nuove molecole.

In lavori precedenti è stato dimostrato che l'espressione di CREB è controllata dalla presenza di ICER (inducible cAMP early repressor), un inibitore endogeno di CREB. ICER infatti compete con CREB per legare le sequenza responsive CRE che si trovano nel promotore dei geni target, e blocca l'attività trascrizionale. Il livello di ICER è stato trovato sotto espresso nelle LAM, con una conseguente perdita di inibizione sull'attività trascrizionale di CREB, promuovendo così l'avanzare della leucemia. In questo lavoro siamo andati a studiare il ruolo di ICER introducendolo in forma esogena nelle linee cellulari leucemiche e nelle culture primarie di LAM. La riespressione di ICER portava ad una diminuzione dell'espressione di CREB e dei suoi geni target. Tra questi ultimi, in particolare, era stata trovata una diminuzione dell'espressione di DUSP1 e DUSP4 (dual-specificity phosphatases). Abbiamo verificato che la riduzione delle DUSPs1/4 andava ad attivare direttamente p38, che a sua volta induceva l'apoptosi caspasi-dipendente. Dato il coinvolgimento di ICER nella morte cellulare, abbiamo cercato di combinare la sua azione al trattamento farmacologico chemioterapico. Nel trattamento delle linee cellulari leucemiche, questa combinazione ha portato ad una riduzione della crescita cellulare e ad un aumento dell'apoptosi. In seguito al trattamento con Etoposide (un inibitore delle topoisomerasi usato nella terapia delle LAM), si riscontrava una diminuzione significativa dell'espressione dei geni target di CREB coinvolti nel ciclo cellulare (CyA1, B1,D1), e nei geni cha fanno parte della via di segnale MAPK (ERK, AKT, DUSP1/4). Per verificare

che l'attivazione di p38 fosse realmente indotta dall'abbassamento delle DUSPs, abbiamo silenziato DUSPs1/4 nella linea cellulare leucemica HL60, confermando lo stesso aumento di sensibilità al trattamento chemioterapico provocata dalla reintroduzione di ICER. Inoltre, il silenziamento delle DUSPs1/4 o l'espressione esogena di ICER nelle culture primarie di LAM, portavano all'abbassamento di DUSP1 e all'attivazione di p38. In questo lavoro abbiamo dimostrato che ICER aiuta l'attività chemioterapica attraverso l'attivazione della via di segnale DUSP-p38, riprogrammando quindi le cellule leucemiche dalla sopravvivenza all'apoptosi. Questi risultati suggeriscono che ICER ha un importante ruolo di regolatore di CREB.

Ci siamo poi interessati a scoprire la causa della sovra espressione di CREB nelle LAM. Dato che CREB risulta sovra espresso in forma proteica, siamo andati a valutare la sua regolazione traslazionale, focalizzandoci sui microRNA. E' stato dimostrato che uno di questi, il miR-34b, si lega alla la regione 3'UTR del gene *CREB*, ed è sotto espresso nelle LAM. Con studi *in vitro* su linee cellulari di LAM, abbiamo scoperto che la down regolazione del miR-34b era dovuta all'ipermetilazione del suo promotore. Quindi siamo andati a valutare la funzione del miR-34b come modulatore di CREB. analizzando la sua espressione e la metilazione del suo promotore nei pazienti affetti da patologie mieloidi. Nei pazienti pediatrici affetti da disordini ematopoietici preleucemici, come la JMML (juvenile myelomonocytic leucemia, n=17) e MDS (myelodysplastic sindrome, n=28) abbiamo trovato una normale espressione di miR-34b, unitamente ad una bassa espressione di CREB. Al contrario, nei pazienti alla diagnosi di LAM (n=112), il miR-34b è stato trovato sotto espresso. Nel 66% delle LAM, è stata riscontrata l'ipermetilazione del suo promotore, spiegando così la bassa espressione di miR-34b e la sovra espressione di CREB, mentre nei pazienti affetti da malattie preleucemiche come JMML e MDS, non è stata trovata la metilazione del promotore di miR-34b, e l'espressione di CREB è risultata infatti normale.

Dall'analisi di alcuni pazienti affetti da MDS e successivamente evoluti in LAM, abbiamo confermato che l'ipermetilazione del promotore di miR-34b insorgeva al momento dell'evoluzione in LAM. Inoltre, tracciando un profilo di espressione genica dei suddetti pazienti, abbiamo scoperto che 103 geni target di CREB erano differenzialmente espressi tra le due fasi di malattia. In una coorte separata di pazienti (n=20) è stato confermato che lo stesso sottogruppo di geni target di CREB era associato ad MDS ad alto rischio di evoluzione in LAM, identificando così uno specifico profilo di trasformazione leucemica. Per verificare ulteriormente la nostra ipotesi, abbiamo valutato il miR-34b come potenziale oncosoppressore *in vitro* e *in vivo*, trovando che la sua espressione esogena portava alla diminuzione sia della progressione tumorale che dell'invasione dei tessuti, inibendo l'azione di CREB e l'espressione dei suoi geni target. Questi risultati hanno confermato che il miR-34b controlla l'espressione di CREB, e che la metilazione del suo promotore contribuisce alla trasformazione leucemica mieloide. Inoltre abbiamo identificato un sottogruppo di geni target di CREB che rappresenta un nuovo network trascrizionale coinvolto nella trasformazione tumorale, aprendo la strada a nuovi possibili studi funzionali su questi geni.

Con queste premesse, abbiamo deciso di sviluppare un modello *in vivo* di zebrafish per valutare il ruolo di CREB e dei suoi geni target nell'insorgenza della leucemia. La scelta di questo organismo modello è stata dettata dai numerosi vantaggi che presenta, che negli ultimi anni lo hanno portato ad essere ampiamente usato sia in studi di sviluppo che nella ricerca sul cancro. Infatti, lo zebrafish possiede alta capacità rigenerativa, ha uno sviluppo embrionico rapido, allo stadio di embrione è trasparente, e ha numerose possibilità di manipolazioni genetiche. Nel presente studio, per sovra esprimere CREB nella linea mieloide di zebrafish, abbiamo iniettato un plasmide codificante CREB umano sotto il controllo del promotore *pu.1* all'interno di uova fertilizzate allo stadio unicellulare, L'analisi dei geni che regolano l'ematopoiesi, ha dimostrato che negli embrioni a 24 e 48 ore di vita, la sovra espressione di CREB portava ad una perturbazione ematopoietica, e ad un'alterazione di espressione genica dei target di CREB, conferendo una progressione del ciclo cellulare. A partire dai 6 mesi dall'induzione dell'espressione di CREB, le popolazioni cellulari del midollo mostravano una lieve dismielopoiesi, caratterizzata prevalentemente da un'aumentata quota di mielociti e monociti.Tra i 9 e i 14 mesi dall'induzione esogena di CREB, i pesci manifestavano un'espansione addominale e dorsale, che ad una valutazione morfologica si è rivelata una massa addominale formata da cellule clonali con capacità infiltranti. E' stato provato che le cellule della massa originavano dalle cellule che sovra esprimevano CREB umano, e possedevano alta capacità proliferativa. Queste cellule avevano infiltrato tessuti ed organi, quali branchie, tessuto adiposo e muscoli. Ad un esame morfologico, le cellule della massa apparivano del tutto simili alle cellule che popolavano il midollo dei pesci malati, con una completa perdita dei mielociti ed una preponderanza di monociti, confermando un disordine mieloproliferativo. Questi risultati mostrano che gli zebrafish sovra esprimenti CREB hanno manifestato la stessa dismielopoiesi già descritta *in vitro* e nei topi transgenici sovra esprimenti CREB, probabilmente mediata da un'elevata espressione dei geni target di CREB. La lunga latenza dell'insorgere della malattia suggerisce che potrebbero esserci ulteriori eventi che inducono la trasformazione tumorale.

In conclusione, il modello di zebrafish creato in questo lavoro descrive una patologia mieloproliferativa indotta da CREB, e può rappresentare uno strumento utile per poter studiare il ruolo di CREB nella leucemogenesi. Inoltre, l'utilizzo di ICER e del miR-34b come oncosoppressori, e lo screening di nuovi composti che interferiscano con l'attività di CREB e con l'espressione dei suoi geni target, può aprire la strada a nuove opportunità terapeutiche.

CHAPTER 1

INTRODUCTION

HEMATOPOIESIS

The earliest cell in hematopoietic hierarchy is the pluripotent hematopoietic stem cell (HSC), which has self-renewal capacity and multi-lineage hematopoietic potential, giving rise to all hematopoietic lineages. HSC existence was demonstrated by serial bone marrow transplantation (BMT) experiments, where all hematopoietic lineages could be reconstituted in lethally irradiated mice [1]. According to the current model of hematopoiesis, maturing HSCs give rise to multipotent progenitors, which also possess stem cell-like properties, but exhibit a progressive restriction of cellular fate, that in turn differentiate into either common myeloid or common lymphoid progenitor cells (CMPs and CLPs, respectively), which are lineage-restricted. CMPs differentiate into either granulocyte–macrophage progenitors (GMP) or megakaryocyte-erythrocyte progenitors (MEP), which eventually differentiate into functional end cells, such as granulocytes, macrophage, platelets and erythrocytes. CLPs further differentiate into pro-B and pro-T cells, which give rise to terminally differentiated B cells and T cells, respectively (figure 1). Elucidation of the cellular hierarchy in human hematopoiesis provides a valuable resource for mapping the key regulatory networks that control blood differentiation and lineage commitment. Lineage commitment could be induced either by extracellular factors, including cytokines, direct cell-cell interactions, or other environmental signals. Alternatively, it could be induced by intrinsic mechanisms: among these, transcription factors (TFs), owing to their capacity to simultaneously activate and repress hundreds of genes, have been shown to be master regulators of lineage commitment fates.

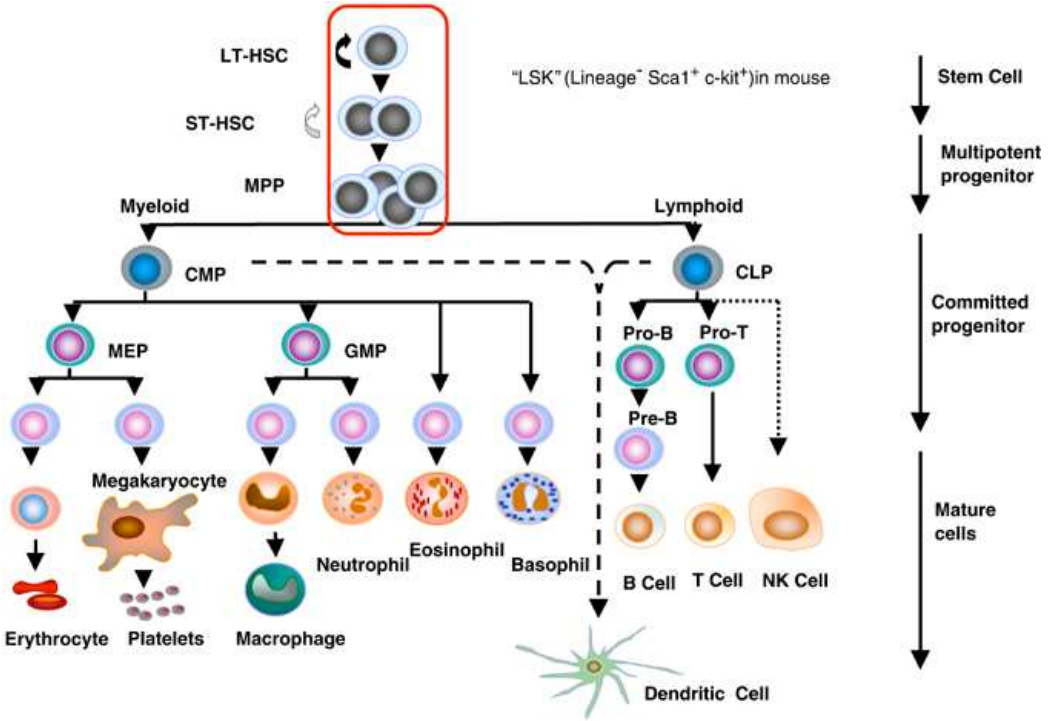


Figure 1. Hematopoiesis development: from a totipotent stem cell to mature hematopoietic cells, through multipotent progenitors and committed progenitors stages. Legend: LT-HSC: long term hematopoietic stem cell; ST-HSC: short term hematopoietic stem cell; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; MEP: megakaryocyte-erythrocyte progenitor; GMP: granulocyte–macrophage progenitor [2]

HEMATOPOIETIC DISORDERS AND IMPAIRED MYELOPOIESIS

Hematopoiesis is a tightly regulated process; studies on the development of hematopoietic cells led to the discovery of proteins that regulate cell viability, growth and differentiation of diverse lineages and to the identification of the molecular basis underlying normal and aberrant cell development in blood-forming tissues. These regulators include principally cytokines, colony stimulating factors and interleukins. The evolution process needs a spatial and temporal regulation of cytokines in order to induce the right lineage commitment. Cytokines can induce the expression of transcription factors and can thus ensure the autoregulation and transregulation of this maturation process. Genetic abnormalities involving regulators of hematopoiesis can impair the proliferation or block the differentiation of subtypes of hematopoietic cells. So, the alteration of specific gene and protein expression programs, may lead to the development of a hematologic disease.

The pathogenetic event that occurs during hematopoiesis can affect stem, precursors, or committed cells at the lymphoid or the myeloid lineage, leading to a different disease with a different outcome.

The interest of this work is addresses to myeloid disorders.

According to the 2008 World Health Organization (WHO), the classification of hematopoietic disease includes 5 subgroups of myeloid neoplasms:

1- Myeloproliferative neoplasms (MPNs), which are characterized by excessive cell proliferation, 10-20% of blast count and risk of AML evolution. They originate from genetically transformed hematopoietic stem cells that retain the capacity for multilineage differentiation and hematopoiesis [3].

2- Myeloid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1, that are featured by blood and bone marrow eosinophilia in which the tumor cells harbor a gene fusion between *PDGFRA* and an adjacent gene locus termed *FIP1L1*, manifested as overexpression of PDGFRA receptor tyrosine kinase activity, which leads to cell proliferation.

3- Myelodysplastic/Myeloproliferative neoplasms (MDS/MPNs), which are rare de novo myeloid neoplasms that exhibit some clinical, laboratory, or morphologic findings of myelodysplastic syndrome, and others typical of myeloproliferative neoplasm. They present cytopenias and cell dysplasia of at least 1 lineage. The bone marrow of patients with MDS/MPNs is characteristically hypercellular. By definition, the percentage of blasts in the blood and the bone marrow must be less than 20% [4].

4- Myelodysplastic syndrome (MDS), a clonal disorder characterized by inefficient dysplastic hematopoiesis, peripheral blood cytopenias and risk of progression to AML. It is featured by defective differentiation of hematopoietic cells, which in turn lead to peripheral blood cytopenia and, in case of AML evolution, to the expansion of the abnormal clone. A variety of molecular abnormalities have been demonstrated in MDS, reflected as phenotypic heterogeneity. MDS is called either primary or secondary MDS: primary MDS is much more common than secondary MDS and may also be called *de novo* MDS; secondary MDS occurs because of damage to the DNA from chemotherapy or radiation therapy previously given to treat another medical condition and it is often associated with more complex chromosomal abnormalities.

5- Acute Myeloid Leukemia (AML), which represents a group of heterogenic hematopoietic cancers characterized by clonal proliferation of neoplastic myeloblasts arrested in diverse differentiation stages, with loss of normal hematopoietic function and accumulation of leukemic blasts or immature forms in the bone marrow (> 20% blast count), peripheral blood and occasionally in other tissues. The leukemic blasts accumulation leads to a number of systemic consequences including anemia, bleeding and increased risk of infection. In the WHO scheme, a myeloid neoplasm with 20% or more blasts in the bone marrow (BM) or peripheral blood (PB) is considered to be acute myeloid leukemia (AML) when it occurs *de novo*, evolution to AML when it occurs in the setting of a previously diagnosed myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN), or blast transformation in a previously diagnosed myeloproliferative neoplasm (MPN) [5].

CELLULAR AND MOLECULAR ORIGINS OF AML

AML accounts for approximately 20% of acute leukemias in childhood; significant progresses have been made in its treatment over the past 40 years, achieving survival rates increase from < 20% in 1970 to approximately 60% nowadays, considering children treated on clinical trials in developed countries [6]. The advance in survival rate is due to a better risk classification, adaptation of treatment based on patient's response to therapy and improvements in allogenic hematopoietic stem cell transplantation. However, most of the AML cases continue to pose a therapeutic challenge, therefore understanding the pathogenesis of this disease and the identification of molecular abnormalities would lead to the development of a better rationally designed therapy [7]. Myeloid disorders present a heterogeneous nature reflected by differences in morphology, immunophenotype, as well as cytogenetic and molecular aberration; this is due to the diversity of myeloid precursors susceptible to malignant transformation, and the multiplicity of genetic or epigenetic events that can lead to the transformation. Despite this heterogeneity, the various subtypes seem to share the activation of

common pathways leading to leukemogenesis. It has been established that AML development requires at least two types of cooperative genetic events: type I and type II aberrations. Type I aberrations concern mutations in hotspots of specific genes involved in signal transduction pathways, resulting in uncontrolled cell proliferation and/or survival of leukemic cells, including *FLT3, KIT, NRAS, KRAS, PTPN11* mutations. Type II aberrations concern chromosomal rearrangements of transcription factors resulting in impaired differentiation of leukemic cells, including *PML-RARα* [t(15;17)(q22;q21)], *AML1-ETO* [t(8;21)(q22;q22)], *CBFB-MYH11* [inv(16)(p13q22)/t(16;16)(p13;q22)] and 11q23/*MLL*-rearrangements [8]. Moreover, cell that undergoes transformation may arise from a self-renewing hematopoietic stem cell, or it may come from a more differentiated myeloid progenitor that acquires the ability to self-renew upon leukemogenic event.

Identifying specific leukemogenic aberrations may guide the development of targeted therapy approaches for selected patient groups.

Genetic aberrations

Genetic lesions consist of gene mutations and chromosome modifications, which mainly include entire or partial chromosomal amplifications, deletions, inversion, or translocation, as well as, point mutations. Nowadays we can consider that more than 90% of pediatric AML patients have at least one known genomic alteration (figure 2). In particular, chromosomal aberrations are molecularly detectable in about half of AML pediatric cases, and are often balanced translocations. These genetic lesions create fusion proteins that often results in loss-of-functions or gain-of-function mutations. Depending on which pathway is affected, they can cause a differentiation block (preventing physiological hematopoietic development), or an aberrant enhanced proliferation [7]. Identification of chromosomal abnormalities has been largely demonstrated to have diagnostic and therapeutic implications. In particular, patients affected by t(8;21), t(15;17), or inv(16) have usually a better prognosis than patients affected with deletion of chromosome 5, 7 (5-,7-), del(5q) [9]. Interestingly, *MLL* gene located at 11q23 has been found rearranged with a variety of partner genes, bearing to the most heterogeneous of all genetic subtypes of leukemia.

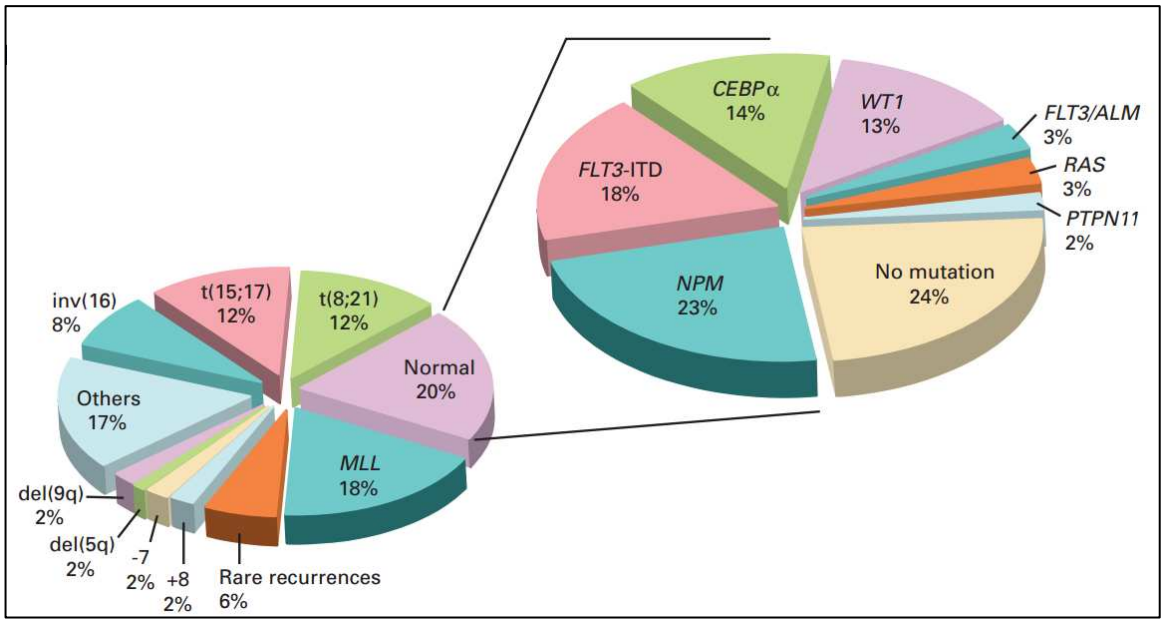


Figure 2. Estimated frequency of genetic abnormalities in childhood AML. Left panel demonstrates the most common karyotypic alterations. Right panel shows mutation profile in patients without cytogenetic abnormalities (normal karyotype). 67% of those in the normal karyotype population have one of the known mutations; thus, more than 90% of all children with AML have at least one known genomic abnormality [8].

In the last decade, the molecular detection of several mutations have increased the knowledge of the AML genetic leading to a considerable improvement of therapy protocols and patient survival. Of relevance there are mutations at tyrosine kinase receptors, that often impart proliferative and survival signals. *FLT3-ITD* is the most important one because it occurs in up to 15% of pediatric AML *de novo*. This mutation involves a duplication of the internal juxtamembrane domain of the FLT3, leading to constitutive receptor activation (incidence of the 5 to 10 % in 5-to-10 year old patients), and it is correlated with high risk stratification for its poor prognostic value.. NPM1 mutation accounts up to 20% of childhood leukemia with normal karyotypes, resulting in aberrant cytoplasmic translocation of this nucleolar phosphoprotein, which normally is involved in ribosome biogenesis and control of centrosome duplication [10]; subgroup characterized by this mutation as single aberrancy is associated with a favorable prognosis, whereas it has poor prognostic value if associated to FLT3 mutations. Loss-of-function mutation in CEBPA, a key leucine zipper transcription factor critical for regulate the differentiation of myeloid precursors, accounts for 15% to 20% of pediatric AML, and predicts a favorable prognosis in AML patients with normal cytogenetics [11]. Inactivating mutation of Wilms tumor (WT1) transcription factor gene is reported in 8% to 12% of AML patients, but without independent prognostic significance in predicting outcome in pediatric AML [8, 12].

Recent genome-wide and sequencing analyses are revealing new chromosomal and point mutations respectively, opening for a lot of new data to be analyzed and prognostically

classified. First results indicate that AML contains fewer genetic alterations than do other malignancies. Pediatric AML in particular contains few genomic alterations, with only 2.4 somatic copy-number alterations per leukemia and no copy-number alterations in about one-third of cases, suggesting that the development of AML may require fewer genetic alterations than other malignancies [13]. Interestingly, genome sequencing data are mainly from adult AML, whose mutations frequency is demonstrated to be different from those found in pediatric cases, such as *IDH1*, *IDH2*, *DNMT3A*, nucleophosmin *NPM1* [14-16].

miRNA

microRNAs are highly conserved genes of 70-80 nucleotides in length, coding for mature 18-25 oligonucleotides, not proteins. Although miRNA genes represent approximately 1% of the genome [17], there have been estimated that 30% of all human genes are regulated by at least one miRNA [18]. miRNAs achieve this regulation by binding to the 3'-untranslated region (UTR) of their target mRNA with partial complementarity, inducing translational repression [17]. MiRNAs are processed from a larger stem-loop precursor (pre-miRNA) by the enzyme Dicer, generating double-stranded RNA (dsRNA) intermediates. One strand encodes the mature miRNA that binds to 3'-UTR region of its target-mRNA (figure 3). In contrast, the other strand (denoted as miRNA*- strand) is believed to be non-functional and is degraded upon its release [19].

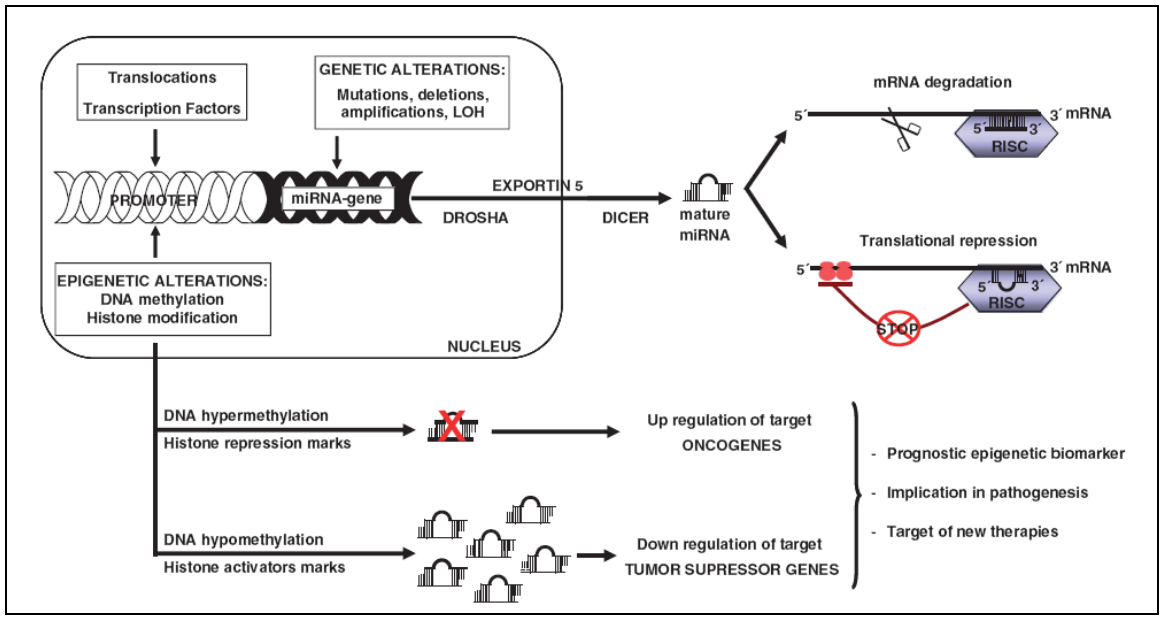


Figure 3. Biogenesis and epigenetic regulation of the expression of miRNAs. miRNA genes are processed by DROSHA and DICER enzymes, and one strand of mature miRNA binds to 3'-UTR region of its target mRNA, whereas the other strand is degraded. miRNAs expression can be affected by transcription factors, genetic alterations or epigenetic alterations [20].

miRNAs have an important role in cell processes such as development, proliferation, differentiation, apoptosis, and tumorigenesis as well [20]. miRNAs manage the control of

multiple pathways, including both lymphoid and myeloid lineages commitment in hematopoietic system. For example, miR-181 was one of the first miRNA demonstrated to be preferentially expressed in hematopietic tissue, whereas miR-328 has been found to stimulate differentiation of the myeloid lineage through C/EBPα promoted expression. Having such important role in hematopoiesis homeostasis, deregulation of miRNAs is involved in altered hematopoiesis. The first evidence of the involvement of miRNA in human hematopoietic cancer was described by Calin et al., who showed the downregulation of miR-15a/16-1 because of a genomic deletion in CLL patients [21].

To unveil which miRNAs are dysregulated in acute leukemia, miRNA genome wide-expression studies have been conducted, revealing that miRNA expression patterns of acute leukemia patient's are different from that of normal hematopoietic cells, and also within diverse acute leukemia subtypes. In fact miRNA expression levels are associated to gene expression profile and to AML mutational status. In fact in 2009 Zhang et al [22], performing a genome-wide miRNA expression analysis, demonstrated that the miRNA expression signature could accurately discriminate pediatric AML. They identified significantly 17 up-regulated and 18 down-regulated miRNAs; among the up-regulated miRNAs, the highly expressed ones were miR-100, miR-125b, miR-335, miR-146a and miR-99a, which were diverse from those highly expressed observed in pediatric ALL or adult AML analysis. Moreover, the clustering based on these miRNAs expression in pediatric AML identified three groups that coincided exactly with the morphologic French-American-British (FAB) classification of AML lineage subtypes: miR-355 up-regulation correlated with FAB-M1, miR-126 up-regulation correlated with FAB-M2, and miR-125a up-regulation correlated with FAB-M3.

Epigenetics

Although gene and microRNAs expression signatures can accurately discriminate cases with specific chromosomal translocations and identify novel subsets of AML, there are evidences that knowledge of genetic profile is not sufficient to fully understand cancer biology. Some of the differences observed in gene expression studies are beginning to be understood in terms of epigenetic regulation of chromatin function.

Epigenetic mechanisms are described as heritable changes in cell phenotype without alteration of cell genotype. The two most widely studied epigenetic mechanisms are DNA methylation and histone modification. DNA methylation is a covalent modification that typically occurs at CpG dinucleotides, located in small genome regions known as CpG islands, and can be catalyzed by different enzymes, including DNMT1, DMNT3a/b. Most CpG islands are located in the promoter region of almost half of the genes, and are unmethylated in normal cells, whereas they can be methylated in tumor cells, driving an inappropriate transcriptional

silencing of these genes, by direct interference of transcription factors binding to target sites [20]. Histone modification consists of post-translational modification of histone tails, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation [20]; these alterations impact chromatin structure and regulatory factor accessibility hereby altering chromatin structure, and can lead either to a closed chromatin conformation, thus blocking the access of regulatory proteins to cognate DNA elements, or to the disruption of contacts between nucleosomes to increase chromatin accessibility [23].

Altered epigenetic modifications are important players in the pathogenesis of AML. Aberrant activity of post-translational modification enzymes probably causes abnormal gene expression in hematopoietic precursors that contribute to the cell differentiation arrest of AML [24]. A central role for epigenetic dysfunction in AML is supported by the observation of mutations and translocations in genes involved in these processes, such as *TET2, DNMT3, IDH1, IDH2*, and the histone H3K4 methyltransferase gene (*MLL*), even if there is a difference between mutation found in adult and pediatric AML. An excellent example are *MLL* rearrangements that are frequent in children younger than 2 years, then decreasing in adultness; whereas *IDH1, IDH2, DNMT3A* mutations are more common in adult AML than in childhood [6]. Several studies revealed that aberrant epigenetic modifications, particularly DNA hypermethylation of genes promoters, may affect miRNAs expression in acute leukemias. In B-ALL an extensive analysis of genomic sequences of miRNA genes has shown that around half of them are frequently within CpG islands rich DNA regions,, suggesting that they could be subjected to this regulatory mechanism. Analysis from different laboratories showed that miRNAs subjected to aberrant hypermethylation were associated with miRNA downregulated expression, both in ALL and AML,, and that their expression could be reverted by treating with demethylating agents such as 5-Aza-2'-deoxycytidine. Importantly, miRNAs methylation profile has demonstrated to be an important prognostic factor in ALL patients (the methylated patients showed a significantly higher relapse and mortality rate), and miRNA hypermethylation to be an independent adverse prognostic factor for disease free survival and overall survival, also by multivariate analysis [25].

Transcription Factor alterations

Tanscription factors (TFs) are proteins that contain one or more DNA-binding domains (DBDs), through which they bind to specific sequences of DNA in the promote region of the genes that they regulate, in response to various stimuli. Through this interaction, TFs control gene transcription by activating or repressing the transcription, either by direct recruitment of RNA polymerase, or by forming multiprotein enzymatic complexes together with other cofactors, which facilitate or inhibit gene transcription by modification of chromatin [26]. TFs have a crucial role in physiological cell activity, including hematopoiesis, development,

proliferation, apoptosis, survival and differentiation, and they respond to intracellular stimuli or environmental signals controlling transcription of diverse genes. Because of the importance of their role, they are tightly spatially and temporally regulated. Disruption of this strict control may lead to neoplastic transformation and tumor onset: numerous genetic and epigenetic aberrations result in alteration of transcription factors critical for normal hematopoiesis, supporting their key role in hematopoietic homeostasis maintenance. For example, the CCAAT/enhancer binding protein alpha (C/EBPα) transcription factor, which is fundamental for granulocytic differentiation, regulation of myeloid gene expression and growth arrest, has been found impaired in AML. There are diverse mechanisms through which C/EBPα function is reduced or disrupted: suppression of transcription, post-transcriptional regulation, post-translational modification or gene mutation. All of these events, lead to both affected cell cycle regulation and granulocyte differentiation, driving malignant transformation [27].

cAMP RESPONSIVE ELEMENT BINDING PROTEIN (CREB)

Among the variety of TFs, the cAMP response element (CRE) binding protein (CREB) has been well characterized. *CREB* has been localized to human chromosome 2q32.3-q34 [28] and encodes a nuclear transcription factor of the CREB/CREM/ATF1-1 basic leucine zipper (bZIP) family. It regulates gene expression principally following activation of cAMP-dependent cell signal transduction pathways. It binds to a specific regulatory sequence on its target genes promoter, called CRE sequence, a palindromic consensus sequence 5'-TGACGTCA-3', or to the half CRE site (CGTCA/TGACG). CREB is a modular protein that contains a conserved structural motif formed by the leucine zipper at the C-terminus of the protein, through which CREB homodimerizes or heterodimerizes on its DNA target sequence; a basic lysine- and arginine-rich domain amino-terminal to the leucine zipper, through which DNA binding is mediated; two glutamine-rich domains (Q1 and Q2); a kinase-inducible domain (KID, residue 100-160), that contains serine 133 residue (Ser133), a pivotal residue whose stimulus-induced phosphorylation mediates activation of CREB [29] (figure 4).

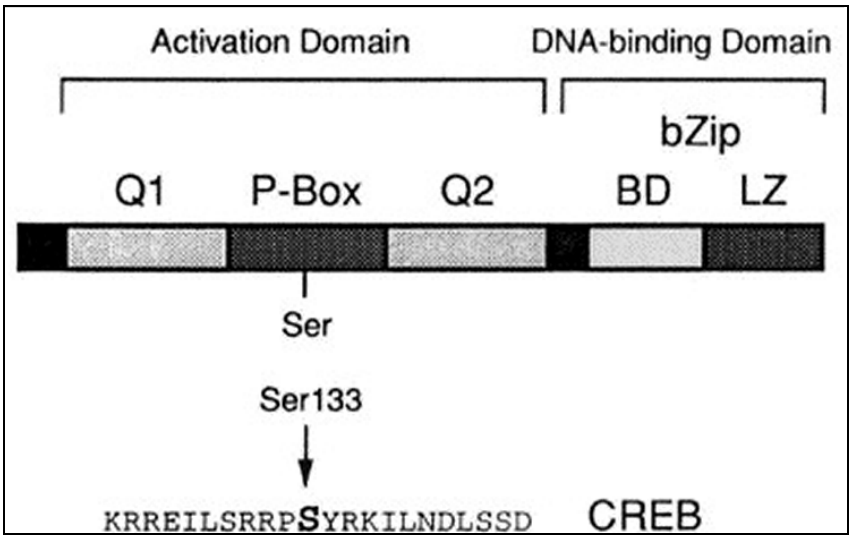


Figure 4. Structure of CREB. The glutamine-rich domains (Q1 and Q2) and the bZip region (BD and LZ) are indicated in addition to the P-box (the KID domain). Phosphorylation at this serine residue turns CREB into activate form, through the interaction with the co-activator CBP. Ser-133 has been shown to be phosphor-acceptor site for various kinases [30].

Ser133 is phosphorylated by various kinases, mostly by cAMP-dependent protein kinase A (PKA), in response to various stimuli such as growth factors (mast/stem cell growth factor, basic fibroblast growth factor and granulocyte-macrophage colony-stimulating factor), neurotransmitters, stress signals that increase intracellular cAMP or calcium levels [31, 32]. CREB phospholylation promotes its interaction with a number of transcription coactivators, especially the CREB-binding protein (CBP), and p300 [33, 34]. CBP and p300 are nuclear proteins that serve as molecular bridge and allow transcription factor to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box. In addition, CBP and p300 through their acetyltransferase (HAT) activity contribute to CREB-mediated transcription by affecting chromatine structure, acetylating histone lysine residues, yielding a more accessible DNA template to the transcriptional machinery. CREB activity is also regulated via phosphorylation independent mechanisms, through a family of cytoplasmic coactivators known as transducers of regulated CREB activity (TORCs), which bind to the bZip domain and strongly activate CREB transcription [35]. Interestingly, mammalian CREB has CREs in its promoter region, giving rise to a positive feedback loop through which CREB enhance its own transcription [36].

CREB physiological role

Studies have demonstrated that CREB is activated in response to different stimuli, and it has the ability to enhance the expression of up to 5000 putative genes. It is ubiquitously expressed in somatic cells of human tissues, and is critical for a variety of cellular processes including proliferation, differentiation and adaptive response [37, 38]. Among different tissues in which it is activated, CREB has an important role in the brain, where it controls learning and memory formation, and contributes to neuronal adaptation to drug of abuse. In the liver,

CREB activity is important for hormonal control of metabolic processes, including gluconeogenesis, by regulating glucagon and insulin hormones [39]. In pancreas β-cells, CREB is a key transcriptional element for the maintenance of an efficient glucose sensing, insulin exocytosis and β-cell survival [40]. In endocrine tissues, cAMP pathway is crucial for normal development of both the thyroid and pituitary glands, thus a regular CREB activity is required for the development and homeostasis of these endocrine tissues [41]. In pinealocytes, CREB phosphorylation regulates the stimulation of melatonin biosynthesis: the connection between CREB phosphorylation and melatonin concentration suggests a relevant CREB's role in the circadian clock [42]. Another important role of CREB is found in Sertoli cells, the somatic cells that populate the seminiferous tubules in testis. CREB fluctuating levels regulate transcription of genes essential for proper germ cell differentiation [43]. In hematopoiesis, CREB is highly expressed in stem cells and uncommitted progenitors of both human and murine bone marrow cells [26]. Indeed, transduction of primary HSCs with lentiviral CREB shRNA resulted in decreased proliferation, cell cycle abnormalities and inhibition of CREB transcription. Nevertheless, transplantation of bone marrow cells transduced with CREB shRNA in irradiated mice led to decreased committed progenitors, without effects on long-term engraftment, suggesting that CREB is important but not essential for normal HSCs' functions [26]. Importantly, CREB has a complex action on cellular proliferation and cell cycle control: it has been shown to positive regulate proliferation alterating cell cycle through cyclins A1 and D1 upregulation, to enhance S-phase entry and to promote growth-factor-independent proliferation [26, 44].

CREB in cancer

One unifying theme in oncogenesis is that apparently unrelated tumors can activate the same oncogenic signaling pathways using different strategies. CREB has a significant impact on cellular growth, proliferation and survival, and to overturn the cellular strict control of these processes, tumor cells have developed various mechanisms to achieve constitutive activation of CREB, including chromosome translocations, gene amplification, interaction with viral oncoproteins, and inactivation of tumor suppressor genes. Direct CREB implication in cancer was first demonstrated in clear-cell sarcomas of the soft tissues (CSSTs), where it has been identified a *CREB*-involving translocation, Ewing's sarcoma gene *EWS-CREB* fusion gene, which lead to a constitutive activation of CREB [45]. Many other evidences about indirect CREB upregulation and tumor onset have been found. Among these, two oncogenic retroviruses, such as human T-cell leukemia virus (HTLV-I) and Hepatitis B virus (HBV), which cause T-ALL and hepatocellular carcinoma respectively, have been shown to promote cellular transformation by constitutively activating CREB, thus enhancing CREB target gene expression [46, 47]. Recently, evidences of CREB involvement in the neural stem/progenitor

cell (NSPC) survival, differentiation and proliferation have been demonstrated, and a new role for CREB in development and growth of brain tumors has been established [48]. Considering endocrine tissues, cAMP levels in adrenocortical carcinoma, characterized by mutations in the gene encoding phosphodiesterase (PDE) 11A4, resulted elevated, and consequently a constitutive CREB phosphorylation was found. Recently, despite the absence of mutations in known genes, abnormalities of cAMP signaling have been found in both bilateral adrenal hyperplasias (BAHs) and cortisol-producing adenomas (CPAs): these findings suggest that constitutive activation of CREB signaling might be one contributory factor in the initiation and development of adrenocortical tumors [49]. CREB plays a role also in prostate cancer, where p21-activated kinase 4 (PAK4) has been shown to enhance the transcriptional activity of CREB independent of Ser133 phosphorylation. This study also indicated a significant correlation between impaired tumorigenicity and decreased expression of CREB together with its targets, included *BCL-2* and *CYCLIN A1* [50]. In non-small cell lung carcinoma (NSCLC) lines, expression levels of mRNA and protein of CREB and phosphorylated CREB (p-CREB) were significantly higher than in the normal human tracheobronchial epithelial cells [51]. Moreover, a relation between nicotine treatment and upregulation of α-4 and α-7 subunits of nicotine acetylcholine receptors (nAChRs) has been found, highlighting that nicotine abuse brings to activation of cAMP signaling, and in turn to CREB activation, promoting lung cancer growth [52]. Moreover, CREB coactivators TORCs have also been found deregulated in diverse type of cancer. In salivary gland tumors a recurring t(11;19)(p21;p13) translocation generates a *TORC1-Mastermind-like 2* (*MAML2*) fusion gene, which activates the transcription of CREB target gene, which in turn leads to cell transformation. Importantly, the transforming activity of TORC1-MALM2 was markedly reduced by blocking CREB binding to DNA [53]. TORC1-MAML2 was also found in other types of cancer related to salivary gland tumor: Warthin's tumor [54] and clear cell hidradenoma [55].

CREB in AML

Among the variety of cancers, CREB has been shown to have a role in transformation of hematopoietic cells. In 2005 Shankar et al. demonstrated that the majority of ALL and AML patients overexpressed both protein and mRNA levels of CREB two- to threefold in the blast cells of the bone marrow at diagnosis and relapse, but not in remission and non-leukemic controls. Moreover AML patients that overexpress CREB were associated with an increased risk of relapse and a decreased event-free survival compared to patients with normal expression of CREB [44]. Further studies demonstrated that enforced expression of CREB promoted growth and survival in leukemia cells, whereas its downregulation lead to suppression of myeloid cell proliferation and survival. Transgenic mice overexpressing CREB in macrophage/monocyte lineage were shown to develop a MDS/MPN with higher white

blood counts and aberrant myelopoiesis in both the bone marrow and spleen after one year, but not leukemia, suggesting that CREB contributed to leukemic phenotype through upregulation of its downstream target genes, but was not sufficient to complete transformation into leukemia [44].The involvement of CREB in leukemia was then confirmed by a study performed on a large cohort of pediatric ALL and AML patients at diagnosis. This study revealed that 84% of patients with ALL and 66% patients with AML significantly overexpressed phosphorylated and thus active CREB protein at diagnosis, but not during remission nor in nonleukemic controls [56]. Furthermore, CREB was found to bind CRE region exclusively in samples at diagnosis of AML because of the lack of its competitor at CRE sites, the inducible cyclic AMP early repressor (ICER).

In mammals, ICER is a basic leucine zipper (bZIP) family member, generated by alternative splicing of cAMP responsive element modulator gene (CREM). ICER is a transcription repressor, because it conserves the DNA-binding domain but lacks the transactivation domain [57]. As a result, it recognizes and binds the CRE consensus region, and homodimerizes or heterodimerizes with CREB repressing transcription of target genes. *ICER* promoter region contains tandemly repeated CREs, resulting in rapid and strong induction upon activation of cAMP pathway. ICER competes with CREB for its own promoter occupancy, generating a negative feedback that is important for correct cAMP-dependent gene expression in different tissues [58]. Analysis of ICER expression revealed that it was downregulated at diagnosis but was significantly increased in remission and control samples [56]. Further studies showed that ICER restoration in leukemic cell lines gave rise to a significant lower number of colonies in *in vitro*, compared to control cells. In these transfected cell lines, chromatin immunoprecipitation experiments showed that ICER recognized CRE elements and thus contrasted CREB on activating gene expression [59]. *In vivo* experiments revealed that ICER-transfected leukemic cells lowered invasion capability when inoculated in mice, and decreased bone marrow angiogenic potential [59].

Although CREB is overexpressed in leukemia, the underlying mechanisms that lead to the increased CREB levels remain largely unknown. Recently, miRNAs have emerged as regulators of multiple transcription factors and pathways, with 30% of human genes that possess a conserved miRNA binding site. The analysis of *CREB 3'UTR* region has highlighted a miRNA consensus sequence, confirmed to be the miR-34b binding site. Indeed, analysis of miR-34b expression in AML cell lines revealed low expression levels compared to healthy bone marrow, in agreement with low of the miR-34 family members expression in other human cancers [60]. miR-34b downregulation was also found in AML patients at diagnosis, and correlated with CREB upregulation. The cause of miR-34b low expression was found to be the hypermethylation of its promoter, which in contrast was not found in normal bone marrow samples, indicating this epigenetic event an AML associated phenomenon. The

restoration of miR-34b in leukemic cell lines showed a direct interaction with the *CREB-3'UTR*, leading to a modulation of CREB protein and consequently to a lowered CREB target gene expression *in vitro*, which in turn inhibited cell growth, proliferation and clonogenic potential [61].

ZEBRAFISH in vivo MODEL

In order to decipher pathogenesis of various cancer types, included leukemia, a number of *in vivo* models have been developed. Among these, the mouse provides a powerful model system that recapitulates many aspects of human genetics, anatomy, physiology and biochemistry; however its capacity for the assessment of embryonic hematopoiesis, as well as for large-scale chemical screens remains limited. On the contrary, simple organisms, such as yeasts and flies, allow an extraordinary amount of genetic manipulations, facilitating gene discovery and pathway analysis, but they have obvious limitations given to disparate genetics from vertebrates and humans. Furthermore, cancer malignancies are not seen in flies and worms. In contrast, a full spectrum of malignant disease is found to occur naturally in teleost, including leukemia, making these organisms interesting as a model for studying vertebrate biology [62].

The zebrafish, *Danio rerio*, is a tropical freshwater fish that possesses approximately 75% conservation to human genome [63]. The zebrafish within the past three decades has emerged as an excellent vertebrate organism in basic research because of its numerous advantages. Principally, it has a high regenerative ability, laying hundreds of eggs in each clutch, which are fertilized externally, becoming transparent, allowing easy genetic manipulation, a characteristic that makes zebrafish a convenient research model species. Fertilized eggs, kept at the ideal temperature of 28.5 °C, immediately begin their rapid embryonic development, which in 36 hours post fertilization (hpf) leads to appearance of precursors to all major organs. Embryos transparency permits direct visualization of organogenesis and tissue formation. Sex determinants are not clearly understood, and the sex of juveniles cannot be distinguished except by dissection [64]. The zebrafish possesses a great ability of genetic manipulations, facilitating novel gene discovery as well as detailed genetic pathway studies, chemical screens and assessment of organogenesis. Gene knockdown or gene overexpression studies can be rapidly performed by morpholinos (chemically modified oligonucleotides that block transcription or translation), or DNA plasmid injection into one-cell stage embryos, respectively [62]. Due to the relatively short history of the use of zebrafish as a model system, some key tools are still missing: one of the main limitations is the absence of hematopoietic cell surface markers. In some cases this lack can be compensated by using fluorescent transgenic reporter lines [65].

Zebrafish hematopoiesis

Several studies of hematopoiesis in zebrafish have provided insights into the conservation of essential pathways and genes for normal blood cell development. Zebrafish hematopoiesis is

similar to that of mammals and other higher vertebrates, with the formation of analogous blood lineages [66]. Zebrafish and mammals have a similar transcription profile in hematopoietic tissues, in which highly conserved transcriptional factors regulate the blood development. As in mammals, zebrafish demonstrate waves of hematopoiesis. The first or primitive wave predominantly produces erythrocytes and some primitive macrophages, and arises in two intraembryonic regions: the posterior lateral mesoderm (PLM), that later forms the intermediate cell mass (ICM), and the anterior lateral mesoderm (ALM). At 10 hpf, both ALM and PLM coexpress hematopoietic and vascular transcription factors, indicating initiation of HSC and angioblast (vascular precursor), proving the existence of a bipotent hemangioblast. By 18 hpf in ICM region these bipotent cells differentiate in proerythroblasts and endothelial cells; in the erythropoietic process, the zinc-finger transcription factor *gata1* have a crucial role [67]. By 24 hpf approximately 300 *gata1*-positive proerythroblasts in the ICM enter in the circulation and mature into erythrocytes. All zebrafish erythrocytes are nucleated, opposite to mammalian ones. Similar to those in the ICM, the cells in the ALM contribute to both blood and vascular development. By 11 hpf the myeloid-specific transcription factor *pu.1* is detected in ALM cells, indicating the myeloid commitment of these cells, and by 22-24 hpf they mature into macrophages and granulocytes and migrate across the yolk sac [64, 68]. The primitive hematopoietic wave is followed by a transient wave in the posterior blood island (PBI) site also known as the caudal hematopoietic tissue (CHT), where from 24 to 36 hpf erythro-myeloid progenitors arise, which are the first cells that give rise to multiple blood lineages. The second or definitive hematopoietic wave begins at 32 hpf, and demonstrates an anatomical location shift towards the ventral wall of the dorsal aorta, the equivalent of the aorta-gonad-mesonephros (AGM), where the first HSCs arise. Next, HSCs are found in the CHT, the equivalent of mammalian fetal liver, and finally by 4 to 5 days post fertilization (dpf), HSCs migrate from the AGM and CHT to seed the kidney marrow, the equivalent of the mammalian bone marrow [69-71], and thymus for lymphopoiesis. The transcription factor *runx1* is a key regulator of definitive hematopoiesis and is expressed in HSCs [67] (figure 5).

Many of the morphologic and cytologic features of hematopoiesis are conserved between zebrafish and mammals. In the matter of myelopoiesis, zebrafish have two granulocyte lineages: the first resembles mammalian neutrophils, in spite of two or three lobes segmented nuclei instead of four or five lobes found in human cells; the other lineage possesses features of both mammalian basophils and eosinophils, with very large granules, but without bilobed nuclei characteristics of mammals cells. Monocytes/macrophages show similar morphology to mammalian cells, with large nucleus and basophilic cytoplasm, demonstrating phagocytosis ability [72].

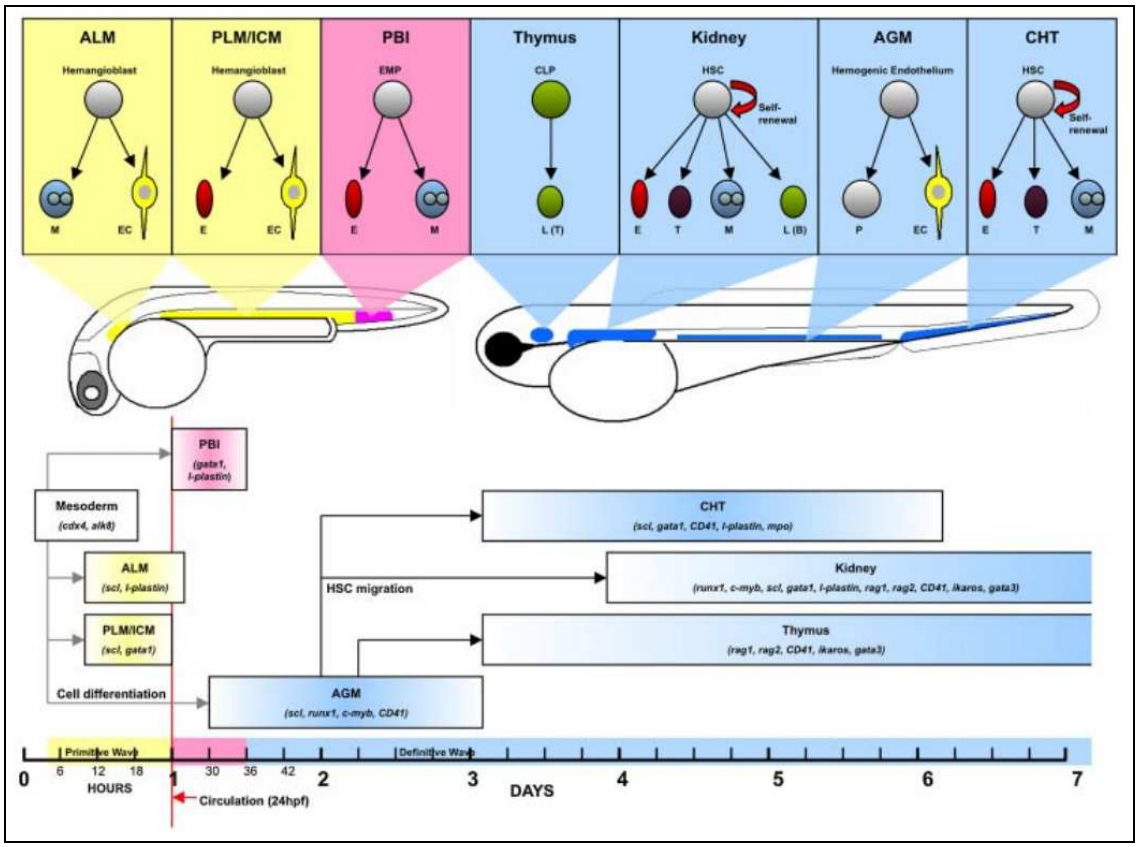


Figure 5. Spatial and temporal representation of zebrafish hematopoiesis. Different sites of hematopoiesis are represented in the top panel. The primitive wave of hematopoiesis (in yellow) consists of hemangioblasts that give rise to myeloid (M) and endothelial cells (EC) in the anterior lateral mesoderm (ALM) and to erythroid (E) and ECs in the posterior lateral mesoderm (PLM), which later becomes the intermediate cell mass (ICM). The first hematopoietic progenitor cells with multilineage potential are found in the posterior blood island (PBI); these erythromyeloid progenitors (EMPs) give rise to erythroid and myeloid cells in a transient definitive wave (in pink). The definitive wave of hematopoiesis (in blue) that contains long-term self-renewing hematopoietic stem cells (HSCs) originates in the aorta-gonad-mesonephros (AGM). The AGM progenitor cells seed the caudal hematopoietic tissue (CHT) and kidney and proliferate to population of HSCs with self-renewal potential. Based on the expression patterns of blood-specific markers, erythroid, myeloid, and thromboid (T) lineages are found in the CHT. Kidney marrow contains all the different blood lineages. The common lymphoic progenitors (CLP) mature into lymphoid T cells (L(T)) in the thymus. The locations of different sites of hematopoiesis are depicted in a 24 h post-fertilization (hpf) embryo (left) and a 72 hpf larva (right). The timeline for expression of blood-specific markers at different sites is shown in the bottom panel. Important blood-specific transcription factors and markers are listed in each box. Mesoderm cells (white box and gray arrows) differentiate into four independent populations of blood progenitor cells in the ALM, PLM/ICM, PBI, and AGM. Blood circulation begins around 24 hpf (red line and arrow). The AGM progenitors begin to enter the circulation around 33 hpf, and by 48 hpf, the CHT and kidney are simultaneously seeded by these progenitors (black arrows). The CLPs from the AGM begin to migrate to the thymus around 54 hpf. The CHT serves as an intermediate site of definitive blood development, then from larval stage and into adulthood the kidney is the primary site of hematopoiesis. The thymus is the site of maturation of lymphoid T cells, however the CLPs, like other blood progenitors in the adult, originate in the kidney. ALM, anterior lateral mesoderm; PLM, posterior lateral mesoderm; ICM, intermediate cell mass; PBI, posterior blood island; AGM, aorta-gonad-mesonephros; CHT, caudal hematopoietic tissue; HSC, hematopoietic stem cell; M, myeloid; EC, endothelial cell; E, erythroid; EMP, erythromyeloid progenitor; CLP, common lymphoic progenitor; L(T), lymphoid T cell; L(B), lymphoid B cell; T, thromboid; P, hematopoietic progenitor cell; hpf, hour post-fertilization [67].

Zebrafish leukemia models

In the last decade, in addition to hematopoiesis studies, the zebrafish has been increasingly used as a vertebrate model for studying hematological disorders. So far, three different lymphoblastic leukemia models have been generated in zebrafish. Thomas Look's group was the first to develop a T-ALL model in 44-52 dpf by expressing the mouse *c-Myc* gene fused to the eGFP under the zebrafish recombinase-activating 2 (*rag2*) promoter, specific for lymphocytes. In this model, T-ALL first emerged as a thymic expansion before hematologic dissemination [73]. Chen et al. developed a T-ALL model by overexpressing the intracellular domain of human Notch1 (ICN1) fused to eGFP. Transgenic fish ICN1-overexpressing developed an oligoclonal T-ALL in 11 months, and showed increased expression of Notch targets [74]. The only model for B cell precursor ALL was generated by Sabaawy et al. TEL-AML1 fusion protein has been expressed under an ubiquitous promoter, and B-ALL developed in 3% of transgenic zebrafish after a latency of 8-12 months [75]. Among myeloid malignancies, only one zebrafish model has been developed so far. Zhuravleva et al. constructed a zebrafish model in which a member of MYST histone acetyltransferases (MOZ) was fused to TIF2, a member of the p160 nuclear receptor transcriptional coactivator family, resembling a rare genetic lesion found in AML. MOZ-TIF2 fusion protein driven by the *pu.1* promoter, developed AML characterized by immature myeloid cells invasion of the kidney marrow [76] in only 2 out of 180 injected-embryos after a long latency (14 and 26 months after injections), suggesting a likely additional needed event. Others zebrafish models created by insertion of fusion genes and gene mutations, led to aberrant myelopoiesis, without develop an overt AML. Human AML1-ETO induction in zebrafish led to immature blasts accumulation and redirection of erythroid progenitors towards a myeloid cell fate, even if this fusion protein alone was insufficient for leukemia induction [77]. Zebrafish *tel-jak2* fusion gene introduction driven by the *pu.1* promoter, resulted in disruption of embryonic hematopoiesis as well as expansion of the myeloid compartment [78]. The N-terminal of nucleoporin fused to C-terminal of homeobox A9 (NUP98-HOXA9) fusion gene, when injected in zebrafish led to a myeloprolifearative disorder with malignant infiltration of myeloid cells between 19 and 23 months of age, without developing AML [79]. Mutated nucleophosmin (NPM), often found in human AML, when expressed in zebrafish resembled aberrant localization to cytoplasm, and took to aberrant hematopoiesis, but failed to develop AML [80]. A model to study KRAS was created by Le et al., in which the active form of human KRAS (KRASG12D) was expressed ubiquitously in the zebrafish embryos. With a short latency, 8% of zebrafish developed a myeloproliferative disorder characterized by an expansion of the kidney, with abundant myeloid cells at various differentiation stages [81].

6. REFERENCES

1. Zhu, J. and S.G. Emerson, Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene, 2002. 21(21): p. 3295-313.
2. Larsson, J. and S. Karlsson, The role of Smad signaling in hematopoiesis. Oncogene, 2005. 24(37): p. 5676-92.
3. Tefferi, A., Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia, 2010. 24(6): p. 1128-38.
4. Foucar, K., Myelodysplastic/myeloproliferative neoplasms. Am J Clin Pathol, 2009. 132(2): p. 281-9.
5. Vardiman, J.W., et al., The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood, 2009. 114(5): p. 937-51.
6. Rubnitz, J.E. and H. Inaba, Childhood acute myeloid leukaemia. Br J Haematol, 2012. 159(3): p. 259-76.
7. Rubnitz, J.E., How I treat pediatric acute myeloid leukemia. Blood, 2012. 119(25): p. 5980-8.
8. Pui, C.H., et al., Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol, 2011. 29(5): p. 551-65.
9. Mrozek, K., N.A. Heerema, and C.D. Bloomfield, Cytogenetics in acute leukemia. Blood Rev, 2004. 18(2): p. 115-36.
10. Grisendi, S., et al., Nucleophosmin and cancer. Nat Rev Cancer, 2006. 6(7): p. 493-505.
11. Ho, P.A., et al., Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood, 2009. 113(26): p. 6558-66.
12. Ho, P.A., et al., Prevalence and prognostic implications of WT1 mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood, 2010. 116(5): p. 702-10.
13. Radtke, I., et al., Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A, 2009. 106(31): p. 12944-9.
14. Paganin, M., et al., DNA methyltransferase 3a hot-spot locus is not mutated in pediatric patients affected by acute myeloid or T-cell acute lymphoblastic leukemia: an Italian study. Haematologica, 2011. 96(12): p. 1886-7.
15. Ley, T.J., et al., DNMT3A mutations in acute myeloid leukemia. N Engl J Med, 2010. 363(25): p. 2424-33.
16. Pigazzi, M., et al., Low prevalence of IDH1 gene mutation in childhood AML in Italy. Leukemia, 2011. 25(1): p. 173-4.
17. He, L. and G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet, 2004. 5(7): p. 522-31.
18. Bartel, D.P., MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 2004. 116(2): p. 281-97.
19. Schwarz, D.S., et al., Asymmetry in the assembly of the RNAi enzyme complex. Cell, 2003. 115(2): p. 199-208.
20. Agirre, X., et al., Epigenetic regulation of miRNA genes in acute leukemia. Leukemia, 2012. 26(3): p. 395-403.
21. Calin, G.A., et al., Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A, 2002. 99(24): p. 15524-9.
22. Zhang, H., et al., MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. PLoS One, 2009. 4(11): p. e7826.
23. Zaidi, S.K., et al., Epigenetic mechanisms in leukemia. Adv Biol Regul, 2012. 52(3): p. 369-76.
24. Lokken, A.A. and N.J. Zeleznik-Le, Breaking the LSD1/KDM1A addiction: therapeutic targeting of the epigenetic modifier in AML. Cancer Cell, 2012. 21(4): p. 451-3.
25. Roman-Gomez, J., et al., Epigenetic regulation of microRNAs in acute lymphoblastic leukemia. J Clin Oncol, 2009. 27(8): p. 1316-22.
26. Cheng, J.C., et al., CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood, 2008. 111(3): p. 1182-92.
27. Reckzeh, K. and J. Cammenga, Molecular mechanisms underlying deregulation of C/EBPalpha in acute myeloid leukemia. Int J Hematol, 2010. 91(4): p. 557-68.
28. Taylor, A.K., et al., Assignment of the human gene for CREB1 to chromosome 2q32.3-q34. Genomics, 1990. 7(3): p. 416-21.
29. Nakajima, T., et al., Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. Genes Dev, 1997. 11(6): p. 738-47.
30. Fimia, G.M., et al., Transcriptional cascades during spermatogenesis: pivotal role of CREM and ACT. Mol Cell Endocrinol, 2001. 179(1-2): p. 17-23.

31. Xing, J., D.D. Ginty, and M.E. Greenberg, Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science, 1996. 273(5277): p. 959-63.
32. Kwon, E.M., et al., Granulocyte-macrophage colony-stimulating factor stimulation results in phosphorylation of cAMP response element-binding protein through activation of pp90RSK. Blood, 2000. 95(8): p. 2552-8.
33. Chrivia, J.C., et al., Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature, 1993. 365(6449): p. 855-9.
34. Kundu, T.K., et al., Activator-dependent transcription from chromatin in vitro involving targeted histone acetylation by p300. Mol Cell, 2000. 6(3): p. 551-61.
35. Conkright, M.D., et al., TORCs: transducers of regulated CREB activity. Mol Cell, 2003. 12(2): p. 413-23.
36. Meyer, T.E., et al., The promoter of the gene encoding 3',5'-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription. Endocrinology, 1993. 132(2): p. 770-80.
37. Johannessen, M., M.P. Delghandi, and U. Moens, What turns CREB on? Cell Signal, 2004. 16(11): p. 1211-27.
38. Zhang, X., et al., Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A, 2005. 102(12): p. 4459-64.
39. Shaywitz, A.J. and M.E. Greenberg, CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem, 1999. 68: p. 821-61.
40. Dalle, S., et al., Roles and regulation of the transcription factor CREB in pancreatic beta -cells. Curr Mol Pharmacol, 2011. 4(3): p. 187-95.
41. Rosenberg, D., et al., Role of the PKA-regulated transcription factor CREB in development and tumorigenesis of endocrine tissues. Ann N Y Acad Sci, 2002. 968: p. 65-74.
42. Maronde, E., et al., Control of CREB phosphorylation and its role for induction of melatonin synthesis in rat pinealocytes. Biol Cell, 1997. 89(8): p. 505-11.
43. Don, J. and G. Stelzer, The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. Mol Cell Endocrinol, 2002. 187(1-2): p. 115-24.
44. Shankar, D.B., et al., The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell, 2005. 7(4): p. 351-62.
45. Antonescu, C.R., et al., EWS-CREB1: a recurrent variant fusion in clear cell sarcoma--association with gastrointestinal location and absence of melanocytic differentiation. Clin Cancer Res, 2006. 12(18): p. 5356-62.
46. Yin, M.J., et al., Protein domains involved in both in vivo and in vitro interactions between human T-cell leukemia virus type I tax and CREB. J Virol, 1995. 69(6): p. 3420-32.
47. Williams, J.S. and O.M. Andrisani, The hepatitis B virus X protein targets the basic region-leucine zipper domain of CREB. Proc Natl Acad Sci U S A, 1995. 92(9): p. 3819-23.
48. Mantamadiotis, T., N. Papalexis, and S. Dworkin, CREB signalling in neural stem/progenitor cells: recent developments and the implications for brain tumour biology. Bioessays, 2012. 34(4): p. 293-300.
49. Bimpaki, E.I., M. Nesterova, and C.A. Stratakis, Abnormalities of cAMP signaling are present in adrenocortical lesions associated with ACTH-independent Cushing syndrome despite the absence of mutations in known genes. Eur J Endocrinol, 2009. 161(1): p. 153-61.
50. Park, M.H., et al., p21-Activated kinase 4 promotes prostate cancer progression through CREB. Oncogene, 2012.
51. Seo, H.S., et al., Cyclic AMP response element-binding protein overexpression: a feature associated with negative prognosis in never smokers with non-small cell lung cancer. Cancer Res, 2008. 68(15): p. 6065-73.
52. Al-Wadei, H.A., et al., Gamma-amino butyric acid inhibits the nicotine-imposed stimulatory challenge in xenograft models of non-small cell lung carcinoma. Curr Cancer Drug Targets, 2012. 12(2): p. 97-106.
53. Wu, L., et al., Transforming activity of MECT1-MAML2 fusion oncoprotein is mediated by constitutive CREB activation. EMBO J, 2005. 24(13): p. 2391-402.
54. Enlund, F., et al., Altered Notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. Exp Cell Res, 2004. 292(1): p. 21-8.
55. Behboudi, A., et al., Clear cell hidradenoma of the skin-a third tumor type with a t(11;19)--associated TORC1-MAML2 gene fusion. Genes Chromosomes Cancer, 2005. 43(2): p. 202-5.
56. Pigazzi, M., et al., cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression. Haematologica, 2007. 92(10): p. 1435-7.

57. Molina, C.A., et al., Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell, 1993. 75(5): p. 875-86.
58. Razavi, R., et al., ICER-IIgamma is a tumor suppressor that mediates the antiproliferative activity of cAMP. Oncogene, 1998. 17(23): p. 3015-9.
59. Pigazzi, M., et al., ICER expression inhibits leukemia phenotype and controls tumor progression. Leukemia, 2008. 22(12): p. 2217-25.
60. Fabbri, M., C.M. Croce, and G.A. Calin, MicroRNAs. Cancer J, 2008. 14(1): p. 1-6.
61. Pigazzi, M., et al., miR-34b targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. Cancer Res, 2009. 69(6): p. 2471-8.
62. Payne, E. and T. Look, Zebrafish modelling of leukaemias. Br J Haematol, 2009. 146(3): p. 247-56.
63. Hsu, C.H., et al., The zebrafish model: use in studying cellular mechanisms for a spectrum of clinical disease entities. Curr Neurovasc Res, 2007. 4(2): p. 111-20.
64. Berman, J.N., J.P. Kanki, and A.T. Look, Zebrafish as a model for myelopoiesis during embryogenesis. Exp Hematol, 2005. 33(9): p. 997-1006.
65. Teittinen, K.J., et al., The zebrafish as a tool in leukemia research. Leuk Res, 2012. 36(9): p. 1082-8.
66. Mead, P.E. and L.I. Zon, Molecular insights into early hematopoiesis. Curr Opin Hematol, 1998. 5(2): p. 156-60.
67. Chen, A.T. and L.I. Zon, Zebrafish blood stem cells. J Cell Biochem, 2009. 108(1): p. 35-42.
68. Hsia, N. and L.I. Zon, Transcriptional regulation of hematopoietic stem cell development in zebrafish. Exp Hematol, 2005. 33(9): p. 1007-14.
69. Thompson, M.A., et al., The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol, 1998. 197(2): p. 248-69.
70. Zapata, A., Ultrastructural study of the teleost fish kidney. Dev Comp Immunol, 1979. 3(1): p. 55-65.
71. Scott, E.W., et al., Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science, 1994. 265(5178): p. 1573-7.
72. Herbomel, P., B. Thisse, and C. Thisse, Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development, 1999. 126(17): p. 3735-45.
73. Langenau, D.M., et al., Myc-induced T cell leukemia in transgenic zebrafish. Science, 2003. 299(5608): p. 887-90.
74. Chen, J., et al., NOTCH1-induced T-cell leukemia in transgenic zebrafish. Leukemia, 2007. 21(3): p. 462-71.
75. Sabaawy, H.E., et al., TEL-AML1 transgenic zebrafish model of precursor B cell acute lymphoblastic leukemia. Proc Natl Acad Sci U S A, 2006. 103(41): p. 15166-71.
76. Zhuravleva, J., et al., MOZ/TIF2-induced acute myeloid leukaemia in transgenic fish. Br J Haematol, 2008. 143(3): p. 378-82.
77. Yeh, J.R., et al., Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. Nat Chem Biol, 2009. 5(4): p. 236-43.
78. Onnebo, S.M., et al., Hematopoietic perturbation in zebrafish expressing a tel-jak2a fusion. Exp Hematol, 2005. 33(2): p. 182-8.
79. Forrester, A.M., et al., NUP98-HOXA9-transgenic zebrafish develop a myeloproliferative neoplasm and provide new insight into mechanisms of myeloid leukaemogenesis. Br J Haematol, 2011. 155(2): p. 167-81.
80. Bolli, N., et al., Expression of the cytoplasmic NPM1 mutant (NPMc+) causes the expansion of hematopoietic cells in zebrafish. Blood, 2010. 115(16): p. 3329-40.
81. Le, X., et al., Heat shock-inducible Cre/Lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish. Proc Natl Acad Sci U S A, 2007. 104(22): p. 9410-5.

CHAPTER 2

ICER EVOKES DUSP1-P38 PATHWAY ENHANCING CHEMOTHERAPY SENSITIVITY IN MYELOID LEUKEMIA

Martina Pigazzi[1], Elena Manara[1,2], Alessandra Beghin[1], Emma Baron[1], Claudia Tregnago[1] Giuseppe Basso[1]

[1] Department of Pediatrics, Laboratory of Hematology-Oncology, University of Padova, Padova, Italy

[2] Division of Hematology-Oncology, Gwynne Hazen Cherry Memorial Laboratories, Mattel Children's Hospital UCLA, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA.

Clinical Cancer Research; 17(4) February 15, 2011.

ABSTRACT

Purpose: The Inducible cAMP early repressor (ICER) is found downregulated in acute myeloid leukemia (AML), failing to control cAMP response element binding protein (CREB) transcriptional activity, recently demonstrated to mediate AML progression. We aimed to characterize ICER's role in drug sensitivity by treating myeloid cell lines and primary AML with chemotherapics.

Experimental design: The effects on CREB target genes induced by ICER restoration and drug treatment were studied by Real Quantitative-PCR (RQ-PCR) and Western blot. Cell cycle and apoptosis analysis were performed. Possible ICER evoked pathways were investigated *in vitro*. The mechanism involved in enhanced drug sensitivity was described in primary AML cultures by silencing ICER main target genes.

Results: AML cell lines reduced cell growth and enhanced apoptotic behavior after chemotherapy treatment if ICER was expressed. A significant lowered expression of CREB target genes involved in cell cycle control (CyA1,B1,D1), and in the mitogen-activated protein kinase (MAPK) signaling pathway (ERK, AKT, DUSP1/4) was found after Etoposide treatment. The dual-specificity phosphatases DUSP1 and DUSP4, directly repressed by ICER, activated p38 pathway which triggered an enhanced caspase-dependent apoptosis. The silencing of DUSP1/4 in HL60 confirmed the same enhanced drug sensitivity induced by ICER. Primary AML cultures, silenced for DUSP1 as well as restored of ICER expression, showed DUSP1 downregulation and p38 activation.

Conclusion: ICER mediates chemotherapy anticancer activity through DUSP1-p38 pathway activation and drives cell program from survival to apoptosis. ICER restoration or DUSP1 inhibition might be possible strategies to sensitize AML cancer cells to conventional chemotherapy and to inhibit tumor growth.

INTRODUCTION

Transcriptional regulation via the cyclic adenosine monophosphate (cAMP) dependent pathway is controlled principally by the cAMP response element binding protein (CREB) and by the cAMP response element modulator (CREM) (1,2). CREB is a transcriptional activator of the downstream target of hematopoietic growth factor signaling, and its role in leukemogenesis was recently described (3). CREB was found to be overexpressed in myeloid leukemia cell lines and in patients at diagnosis, contributing to disease progression, and to improve tumor proliferation and survival *in vitro* (4-6). The CREM gene generates positive and negative transcription regulators. In particular, ICER (inducible cAMP early repressor) is driven by an alternative promoter (P2), which directs the transcription of a truncated product (7). Through its bZIP domain, it can either recognize cAMP response element (CRE) consensus elements on gene promoters, impeding their transcription, or it can dimerize with CREB, impeding CREB phosphorylation of the residue of Serine 133, triggering gene repression and CREB destabilization (8-10). Previous studies demonstrated that exogenous ICER expression decreased CREB protein levels and induced a lowered clonogenic potential *in vitro* and *in vivo*, demonstrating its potential role as tumor suppressor in leukemia as well as in prostate tumors. ICER was shown to repress many target genes upregulated by CREB in acute myeloid leukemia (AML), restoring the normal regulation of the main survival cellular pathways (11-13). The impact of the restored ICER on leukemic cell activity and its ability to suppress tumors is under investigation here. Considerable attention has been focused on the role played by ICER in different kinase cascades, specifically in the control of apoptosis. We focused on the MAPK family members which included numerous cellular signaling, such as extracellular signal regulated kinase (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 known to transmit different types of signals (14,15). Erk1/2 acts through mitogenic stimuli promoting cell proliferation, whereas p38 and JNK are stress factors related to cell growth inhibition and apoptosis (16,17). The outcome of MAPK activation depends on the level and period of the phosphorylation status of the proteins involved, which are mostly controlled by a specific family of phosphatases with negative regulatory control ability, called dual specificity phosphatases (DUSPs) (18,19). The DUSP proteins family contains several members with substrate and subcellular localization specificity (20). In particular, DUSP1/4 target principally p38, and many different stimuli are able to activate their activity (21,22). The balance between the activation or inactivation of the MAPK mediated by DUSPs modulates the proliferative or apoptotic cell phenotype in several tissues. Increased levels of DUSP1 have been found in ovarian carcinoma, breast and prostate cancer (23-25). On the other end, we previously demonstrated that CREB stabilized extracellular signal regulated kinase (ERK), which is responsible for lowered ICER expression levels (11) by driving it to the proteasome (26), permitting overexpression of DUSP1/4 and thus influencing MAPKs. These facts highlight an intricate interplay between CREB/ICER transcription factors and the signaling of MAPK in the control of myeloid leukemia cell fate (27-29).

The aim of this study was to elucidate whether and how the restoration of ICER expression leads to increased sensitivity of myeloid leukemic cells to chemotherapy treatment, resulting in apoptosis.

MATERIAL AND METHODS

Cell culture and transfection. HL60, ML2, THP-1 cell lines (American Type Culture Collection, MD, USA) was cultured in DMEM (Invitrogen-Gibco, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco). Cell lines were grown in suspension diluted to 500X106/ml , and after 24 h were transiently transfected by Nucleofector system from Amaxa GmbH (Cologne, Germany) with 1.5 μg of pEGFPΔ-N1_FLAG-ICERγ or pEGFPΔ-N1-EV (the Empty Vector) used as control. We used the stable HL60+ICER and HL60+EV cell lines previously described in Pigazzi et al. (11).

Primary cell cultures. Three untreated AML patients were included in the present study. The study was approved by the local Ethics Committee, and samples collected after obtaining written informed consent. AML cells were isolated from BM by hemolysis and cultured in DMEM (Invitrogen-Gibco) supplemented with 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen-Gibco), IL-3, IL-6, Flt-3 ligand and TPO (Sigma). After 24 h of incubation at 37 ° C, cells were added to 10% heat-inactivated fetal calf serum (FCS) to be transiently transfected by nucleofection (Amaxa) with 3.5 μg of pEGFPΔ-N1_FLAG-ICERγ or pEGFPΔ-N1-EV. Silencing of DUSP1 was also performed in the same cells by using 400 nMol (in 2 ml of total volume) of oligonucleotides (Dharmacon Industries, Lafayette, CO), as well as the scramble negative Sirna (Sir-sc) used as control at the same concentration. To establish the time of experiments, we observed cell viability. The three patients were pediatric, age < 18 years; the FAB classifications were one M4, one M1 and one M5. Two of them had normal karyotype; the M4 was inv16-CBFB-MYH11 rearranged. BM infiltration was up to 70%.

3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay. We performed dose-dependent studies of VP-16, doxorubicin (doxo) and staurosporine (stauro) by growing cells in the presence or absence of increasing concentrations of the drug at 37 °C for up to 72 hours, and reduction of the MTT (Sigma-Aldrich, MO, USA) salt was measured. The subtoxic drug dose at which the proliferation of cell lines was not significantly affected was 1 μM for VP-16, 0.03 μM for doxorubicin and 0.24 μM for staurosporine.

Fluorescence Microscopy. HL60+ICER and HL60+EV were serum-starved overnight before adding 1 μM VP-16 for up to 72 hours. To assess time-dependent nuclear morphology perturbations of VP-16-treated and untreated cells, $5x10^5$ cells were spotted every 24 hours and incubated in 10% FCS-PBS with DAPI nucleic acid stain (1:1000). The cells were observed at 63/0.75 numerical aperture with a Leica DMBL microscope; images were obtained with a Leica DC 300F digital camera (Leica Microsystems Ltd., Germany).

Cell cycle analysis. HL60+ICER and HL60+EV cell lines were serum-starved overnight and then continuously treated with 1 μM VP-16, 0.03 μM Doxo and 0.24 μM Stauro. After 6, 24 and 48 hours, 5x105 cells were washed twice with PBS, lysed and treated with 50 μg/ml Propidium Iodide (PI) in 1 ml PBS overnight at 4 °C. Cells were analyzed by using Cytomics FC500 (Beckman Coulter, FL, USA). Cycle analyses were performed using Multicycle Wincycle software (Phoenix Flow Systems, CA, USA).

Apoptosis assays. Cell lines were serum-starved overnight and then continuously treated with 1 μM VP-16, 0.03 μM Doxo and 0.24 μM Stauro over a 48 hours period. Drugs were solubilized in DMSO, which was found to induce less than 0.5 % apoptosis. Cells were stained with annexinV conjugated to fluorescein-isothiocyanate (FITC) and PI, according to the manufacturer's instruction (Boehringer, Mannheim, Germany) and analyzed by Cytomics FC500 (Beckman Coulter). To determine caspase activation, we used 10 μM Z-VAD-fmk (Sigma-Aldrich) to study the apoptosis induction pathway 120 minutes before VP-16 treatment. We administered the specific p38 inhibitor SB203580 (4-(4´-fluorophenyl)-2-(4´-methylsulfinylphenyl)-5-(4´-pyridyl) imidazole) (20 μM) and a selective inhibitor of MAPK/ERK kinase 1 inhibitor PD98059 (2´-amino-3´-methoxyflavone) (Sigma-Aldrich) 2 hours before VP-16 to selectively block different signals and measured apoptosis by Annexin V/PI staining. Cell lines with empty vector (EV) expression were treated at same drug concentration and during the same period. In apoptosis assay cell death of EV was subtracted to ICER expressing cells, whereas in western blot the same antibodies were evaluated in the EV protein lysates.

RNA isolation and SYBR green quantitative real-time RT-PCR (QRT-PCR) assays. Total RNA was isolated using TRIzol (Invitrogen) from cell lines after being treated with VP-16 up to 48 hours or without being treated. 1 μg of RNA was transcribed using the Superscript II system (Invitrogen-Gibco) in 25 μl final volume following manufacturer's instructions. RQ-PCR was performed with 1 μl cDNA in 20 μl using the Sybr Green method (Invitrogen-Gibco) and analyzed on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems).

Western Blot (WB). 20 μg from the total protein fraction (Buffer-Biosource International, CA, USA), obtained from HL60+ICER and HL60+EV cell lines, were used to perform protein analyses. Protein concentration was determined using the BCA method (Pierce, IL, USA). Samples were subjected to 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.2 μm PVDF membranes (GE-healthcare, IL, USA) for immunodetection with a series of antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse Ig (Upstate Biotechnology, NY, USA). Antibodies used included anti-β-Actin, anti-FLAG and anti-CyclinA1 (Sigma-Aldrich); anti-BCL-2(C2), anti-DUSP1, anti-DUSP4, anti-IL6 (Santa Cruz Biotechnology, CA, USA); anti-CREB, anti-PhosphoCREB, anti-CIP1-p21, anti-Bak (Upstate Biotechnology); anti-CdK2 (78B2), anti-BAX, anti-Bcl-xl, anti-Phospho-p44/42 Map kinase (Thr202/Tyr204), anti-Phospho-p38 (Thr180/Tyr182), anti-JNK (T183/Y185), anti-AKT (S473), anti PARP, anti-RB (Cell Signaling technology, MA, USA); anti-Caspase8 and anti-Caspase3 (Alexis Biochemicals, CA, USA); anti-CyclinE (Oncogene Research

Products, MA, USA); anti-CyclinB, anti-CyclinD1 AND ANTI-Kip1-p27 (BD Bioscience, NY, USA). The specific bands of target proteins were visualized by enhanced chemiluminescence (ECL advance) according to manufacturer's instructions (GE-Healthcare), and the density of bands were quantified using ScionImage software.

Chromatin immunoprecipitation assay. HL60+ICER and HL60+EV cell lines were processed for chromatin immunoprecipitation assay (Upstate Cell Signaling Solutions, VA, USA) following the manufacturer's instructions. The immunoprecipitation was performed overnight at 4 °C with rotation by using an antibody of interest (CREB, ICER, FLAGM2, RNAPOL) and without antibody selection (NoAb) and Immunoglobulin (Ig), as controls. Imput DNA was used as positive control. DNA was recovered and used to perform PCR. Agarose gel electrophoresis was performed to observe promoter activity. RQ-PCR was also performed by the SYBR Green method for amplification and detection (Invitrogen-Gibco) by 7900HT technology (Applied Biosystems) and analyzed by comparative threshold cycle (Ct) method.

Anisomycin treatment. The HL60 parental cell line was treated with anisomycin (Sigma-Aldrich), a p38 activator, 2 µM final concentration up to 3 hours. Cell proliferation and apoptosis were measured as described above. For rescue experiments, the p38 mitogen-activated protein kinase phosporylation inhibitor SB203580 (Sigma-Aldrich) was used at 20µM concentration 2 hours before anisomycin exposure or VP-16 treatment.

siRNA experiments. Exogenous small interfering RNAs (siRNAs) specific for the DUSP1 and DUSP4 genes (Dharmacon Industries, Lafayette, CO) were introduced in the HL60 cell line (100 nM in 2 ml of medium) by nucleofection. Scramble Sirna (Sc-Sir) were used as negative control. mRNA and protein expression were performed to monitor silencing. Apoptosis was measured after VP-16 treatment. VP-16 1 µM was added after 14 hours of silencing and continuously treated for 24 hours.

Data analysis. Values are presented as mean ± s.d. Significance between experimental values was determined by Student's unpaired t-test, and one-way ANOVA was used to test differences in repeated measures across experiments. $p < 0.05$ was considered significant.

RESULTS

ICER expression enhances chemotherapy susceptibility of leukemic cell lines.

In the present study, we used myeloid leukemic cell lines (HL60, THP-1, ML2,) that were confirmed to express CREB at high levels and ICER at nondetectable levels by Pigazzi and colleagues (11). We induced ICER transient (t-ICER) expression in these cell lines (Figure 1A) and we treated them with a chemotherapeutic agent to investigate the cellular response. We found that exogenous ICER expression in HL60, THP-1 and ML2 mediated an increase in apoptosis with respect to EV (whose % apoptosis was subtracted from the value presented in the figure) after 24 (13 %, 2.2 % and 2.1 %, $n = 3$, $p < 0.05$ for HL60) and 48 hours (32.2 %, 14 % and 13 % $n = 3$, $p < 0.05$ for all cell lines) of VP-16 treatment (Figure 1B). HL60+ICER was found to be more sensible to VP-16, its lowered cell

proliferation was confirmed up to 72 hours of treatment (Figure 1C). The cell morphology showed an increased number of apoptotic nuclei after VP-16 treatment in ICER expressing cell lines, confirming the HL60 to be more sensitive to the drug (Figure 1C). To evaluate if the increase in apoptosis was due exclusively to VP-16, and not to ICER overexpression, two different compounds, Staurosporine (stauro) and Doxorubicin (doxo), were also used. The annexin assay showed an increase in apoptosis after treatment in all of the cell lines that overexpress ICER. At 24 hours, the increase in apoptosis for HL60, THP-1 and ML2 overexpressing ICER was 20.8 %, 8 %, 10.8 % when exposed to doxo; 30 %, 19.3 %, 10.7 % when exposed to stauro. At 48 hours of treatment 35 %, 8.9 %, 6.4 % for doxo and 41 %, 40 %, 32 % for stauro. Results are obtained subtracting apoptosis drug mediated in cell line transfected with EV. Results showed that all drugs significantly increased cell death in the ICER expressing cell lines, demonstrating that ICER role in cell death was regardless of the type of drug used (Figure 1D, n = 3, * $p < 0.05$).

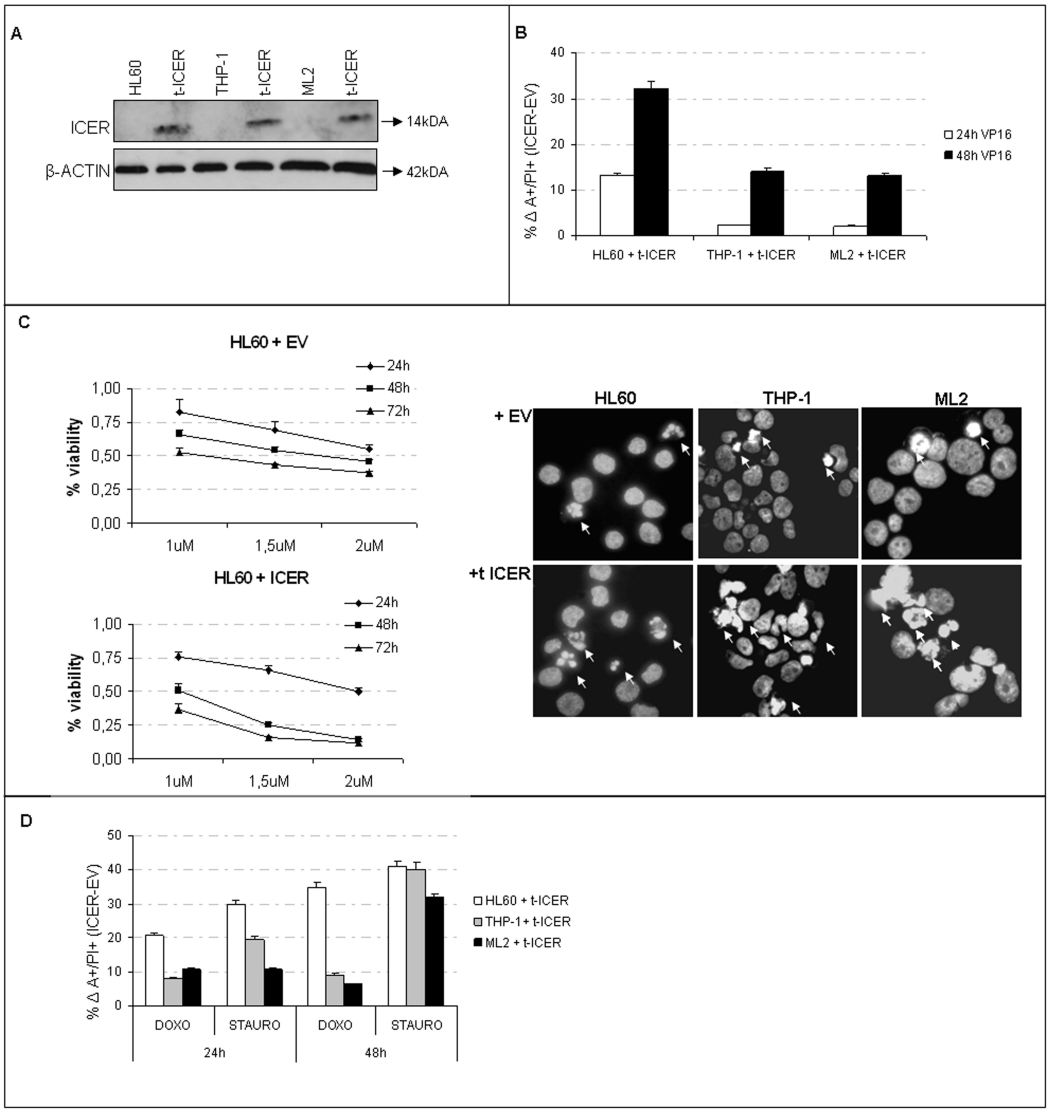


Figure 1. ICER enhances chemotherapy induced cell death. A) Western blot analysis of ICER transient expression (t-ICER) induced in HL60, THP-1 and ML2 48h post transfection. B) The increase in apoptotic of ICER expressing cell lines after VP-16 treatment is shown with respect to cells transfected with EV in the same treatment conditions (its value has been

subtracted in the figure, n = 3, * $p < 0.05$). C) Proliferation of ICER or EV transfected cell lines after VP16 treatment (with respect to transfected but untreated cell lines). Treatment and ICER expression conferred the lowest proliferation values after 48 and 72 h (n = 3). On the right, DAPI nuclear staining after VP-16 treatment in cell lines +ICER reveals chromatin condensation, and the number of rounded and fragmented nuclei increased with respect to cells +EV in the same conditions. D) Apoptosis of cell lines +EV and +ICER after different drug treatments is shown. Histogram represents the percentages of A+/PI+ (to which the value of apoptosis induced by DMSO was subtracted) after 48 h of drug treatments. Apoptosis is significantly increased at 24 and 48 h of treatment in cell lines overexpressing ICER (n = 3, * $p < 0.05$).

ICER counteracts CREB gene expression.

By studying the ICER transcriptional repressor activity by RQ-PCR (11), we revealed that the MAP-phosphatases DUSP1 and DUSP4, the survival molecules ERK1/2 and AKT, as well as controllers of the cell cycle progression, Cyclin A1, B1 and D1, were significantly less expressed in HL60+ICER cells with respect to HL60+EV cells, or HL60 after VP-16 treatment (Figure 2A, n = 3, * $p < 0.05$). The mRNA repression correlated with protein reduced levels, particularly after 48 hours of VP-16 treatment, which were not found in the HL60+EV cells treated at the same conditions (Figure 2B). To further investigate the role of cyclin down regulation, cell cycle analysis was performed. Results revealed mainly an accumulation of sub G0 (apoptotic) HL60+ICER cells after drug treatment. A block in the G2-M phase was evidenced mainly after VP-16 and doxo treatment; whereas a rapid ongoing to apoptosis mainly after stauro treatment was shown. We documented cell cycle regulators p21 and Cdk2 activation up to 24 hours of VP-16 treatment, supporting the G2 block of cell cycle progression; their reduction at 48 hours established the maximum effect of treatment linked to higher cell death (Figure 2C, n = 2, $p < 0.05$, ctr = untreated cells).

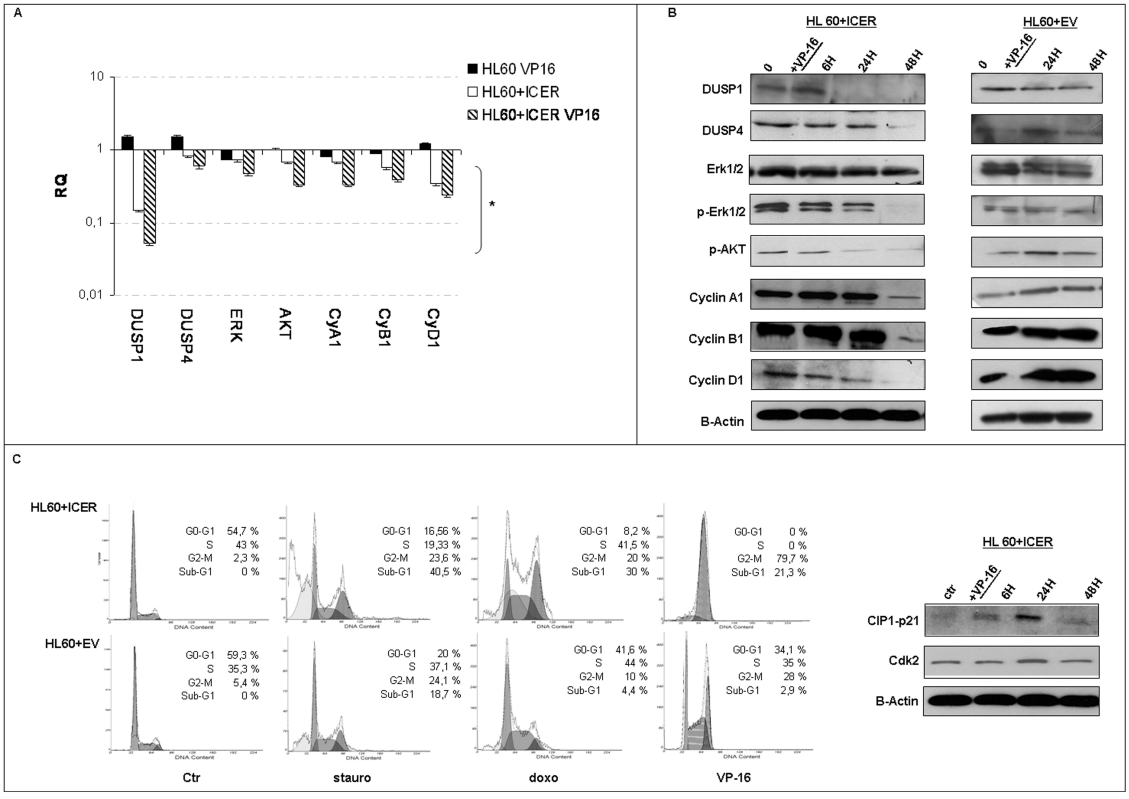


Figure 2. ICER controls gene expression influencing cell cycle and MAP Kinases. A) Histogram shows the relative quantitation (RQ) of CREB/ICER target genes of HL60+EV and of HL60+ICER at 48 h of VP-16 treatment compared to the untreated HL60+EV cell line used as calibrator (RQ = 1 in the figure, n = 2, * $p < 0.05$). B) Western blot analysis of target proteins which were found decreased in HL60+ICER treated with VP16. HL60+EV cell line, treated at same conditions, did not show reduction in protein levels. C) Cell cycle analysis of HL60+ICER and HL60+EV cell lines, treated with VP-16, doxo, or stauro, were performed after 48h of treatment. Histograms show that HL60+ICER cells treated with drugs present a increased sub-G1 phase or block in G2 phase compared to the untreated cells (ctr). Western blot analysis: p21 and Cdk2 are activated 24 h post treatment in HL60+ICER, consistently with the block in cell cycle progression and with the reduction of cyclins observed in panel 2B.

ICER enhances chemotherapy induced apoptosis by DUSP1/4-p38 pathway.

The apoptosis previously discussed was confirmed in the HL60+ICER cell line after 24 hours of treatment (Figure 3A) by the increase of expression of the following: active caspase-3 (active fragments p20, p19 and p17), caspase 8 (active fragments p43/41) and cleaved poly (ADP-ribose) polymerase (PARP) (cleaved fragment p89).

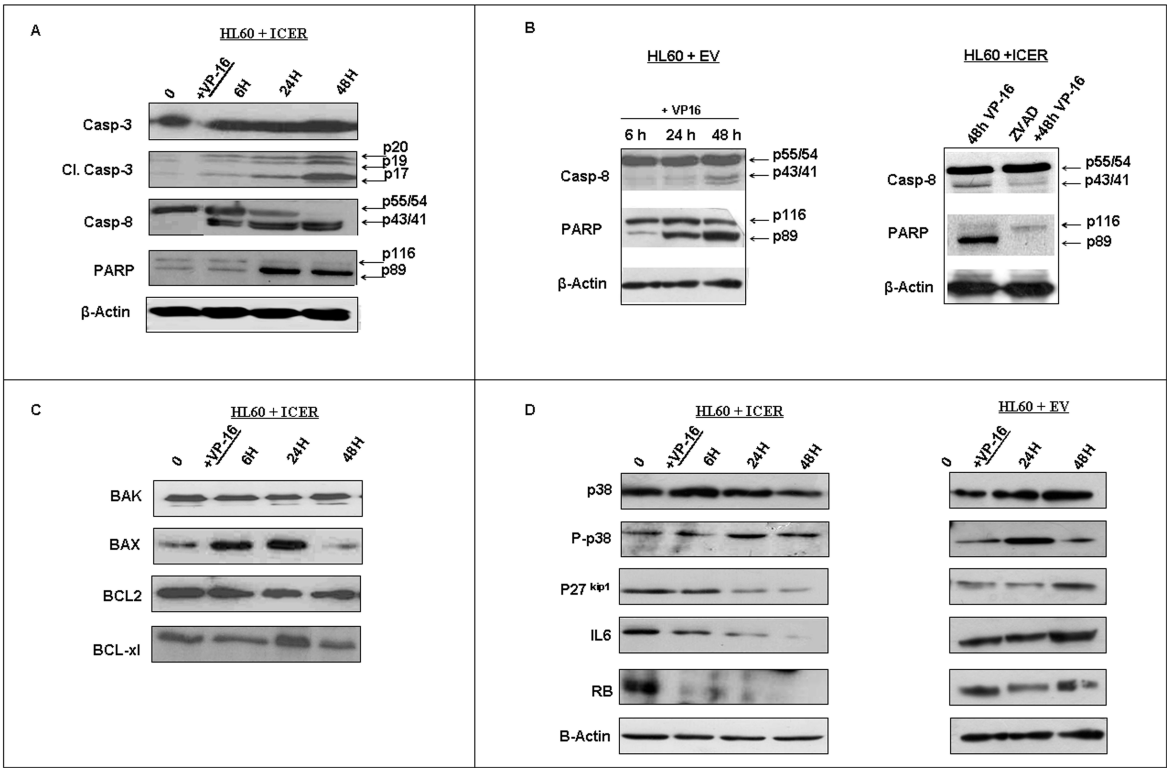


Figure 3. ICER evokes caspases mediated external apoptosis by p38 pathway. A) Western blot shows the activation cleavage mediated of inactive proteins into mature fragments of caspase 3 (p17, p19, p20), caspase 8 (p43, p41) and PARP (p89) starting from 24 h of treatment and up to 48 h. B) Western blot analysis showed that apoptosis enforced by ICER was found to be caspase-mediated (increase of fragments p43, p41 of caspase 8 and of p89 fragment of PARP). By the use of ZVAD as caspase inhibitor, delayed caspase 8 and PARP activation was shown. C) Western blot analysis was conducted on the mitochondrial protein fraction (Mt). The presence of BAK confirm that mitochondria are undamaged. The expression of antiapoptotic BCL-2 and BCL-XL over the treatment period is not influenced by VP-16 in HL60+ICER cell line. D) DUSP 1 and 4 are shown to be repressed over treatment (Figure 2B), whereas p38 was kept phosphorylated during the same period of

time. The impaired expression of downstream p38 substrates, such as p27, IL6 and RB is documented. Same proteins reduction was not observed in HL60+EV treated at same conditions.

We observed that caspase 8 and PARP activation was markedly induced in HL60+ICER with respect to the HL60+EV cell line treated in the same conditions. Caspase 8 and PARP activation was controlled by the use of the broad inhibitor z-VAD, confirming that ICER evoked caspases to trigger apoptosis (Figure 3B). In an attempt to determine the sequence of events involved in ICER mediated apoptosis in HL60, we excluded mitochondrial involvement by demonstrating the maintenance of BAK, BCL-2, BAX, and BCL-XL protein expression during VP-16 treatment. We checked the mitochondrial protein fraction for purity and as loading control, and the presence of BCL-2/BCL-XL demonstrated the mitochondrial membrane integrity up to 48 hours (Figure 3C). We then investigated the role of the phosphatases DUSP1/4 impaired gene and protein expression mediated by ICER repression, and investigated their main target, the proapoptotic p38 protein. Results showed the upregulation and maintenance of the phosphorylated form of p38 expression during VP-16 treatment of HL60 + ICER. The activation of p38 was confirmed by looking at specific substrates, p27, RB and IL6, whose expression was found severely compromised, supporting the observed cell apoptosis (Figure 3D).

ICER activated p38 by repressing DUSP1/4 transcription triggering cell apoptosis in HL60 and primary cultures.

The chromatin at the DUSP1/4 promoter was immunoprecipitated. Pulled-down DNA showed the binding of CREB in the HL60+EV cell line partially or totally substituted by ICER when exogenously expressed in the HL60+ICER cell line (Figure 4A). Results of RQ-PCR were interpreted by the ΔΔct method considering HL60+EV as calibrator (RQ = 1). The amount of DNA immunoprecipitated by CREB was significantly decreased for DUSP1 (RQ = 0.46) and for DUSP4 (RQ = 0.80) in HL60 + ICER with respect to HL60 + EV, confirming that ICER might work preferentially on DUSP1 promoter ($p < 0.05$) (data not shown). To further emphasize the importance of p38 in regulating stress-induced AML cell death, HL60 was treated with anisomycin a specific activator of p38. Western blot showed that increased phospho-p38 levels contributed to higher apoptosis in the HL60 cell line. By using the specific p38 inhibitor SB203580 we prevented apoptosis in the same context (Figure 4B, $n = 3$, * $p < 0.05$).

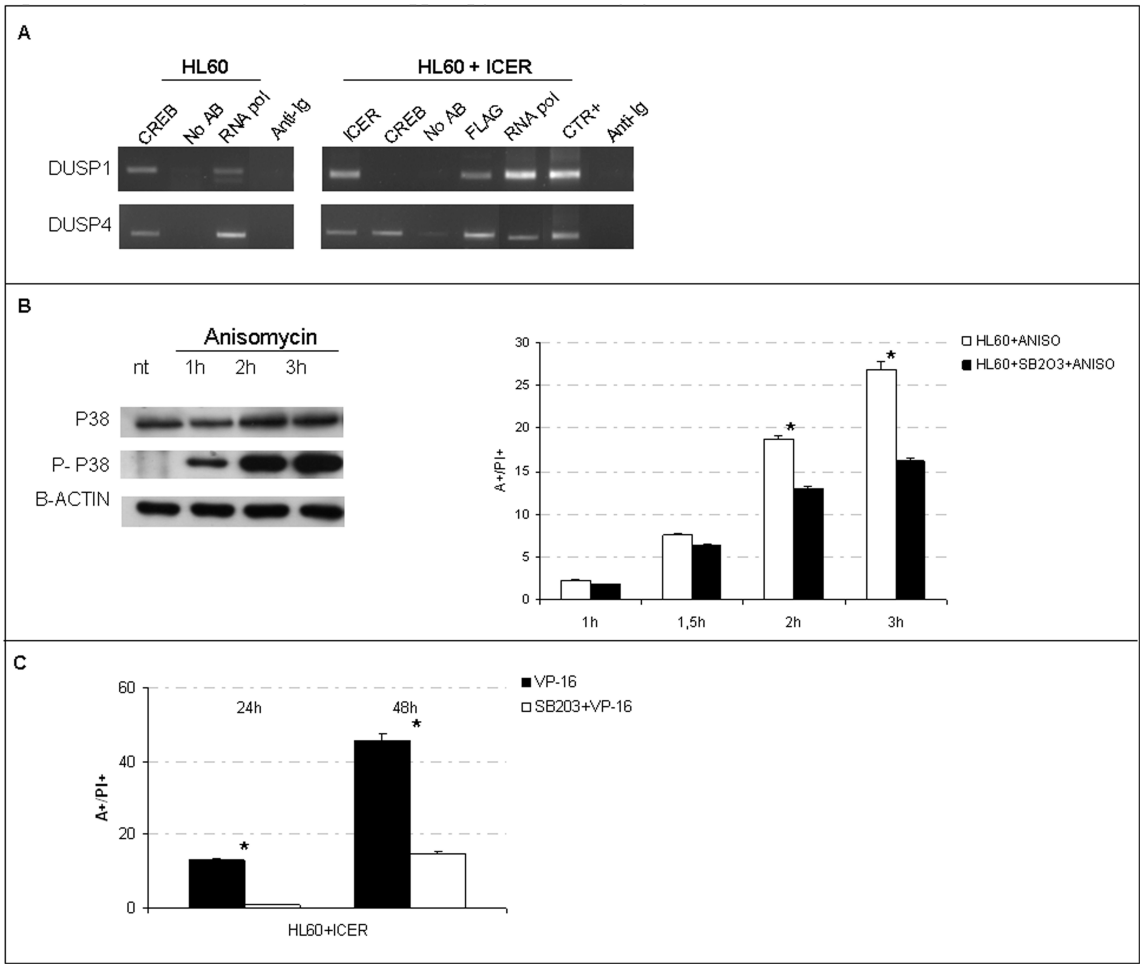


Figure 4. ICER controls DUSP1/4 promoter triggering p38 mediated apoptosis. A) Chromatin immunoprecipitation was performed using CREB, ICER or FLAGM2 antibody in both cell lines. FLAG antibody was used to pull down ICER exogenous protein, while RNAPOL was used to assess active promoters. Positive control is the imput DNA (ctr+) while negative control is obtained without any antibody (NoAb) or using an anti-IgG antibody. B) Western blot shows the increase of phosphorylated p38 (p P38) expression after the use of the specific drug anisomycin (aniso). The treatment increases HL60 apoptosis (A+/PI+) as the consequence of specific p38 induction. By using p38 inhibitor (SB203580), apoptosis was rescued ($n = 3$, $*p < 0.05$). C) Apoptosis assay (% of annexin V and PI positive cells) was performed in HL60+ICER or +EV cell after VP-16 treatment with or without SB203580 pretreatment. Results show a rescue in apoptosis when cells are pretreated with SB203.

To prove that the VP-16 enhanced sensitivity induced by ICER expression was mediated by the p38 pathway, a specific inhibitor of all p38 homologues (p38α, p38β and p38β2), SB203580, was used. Apoptosis was found reduced from 13.1 % to 1 % in HL60+ICER 24 hours post treatment and from 45.7 % to 14.8 % at 48 hours (Figure 4C, $n = 3$, $p < 0.05$), suggesting that p38 activation was directly involved in the apoptosis mediated by ICER restoration. Apoptosis was rescued by SB20358 also in HL60 + EV treated with VP-16, but with lower efficacy (data not shown).

DUSP1 and DUSP4 silencing phenocopies ICER's role in parental HL60 cell line.

The silencing of both DUSP1 and DUSP4 was induced in the HL60 cell line by using small interfering RNAs. To evaluate their ability their ability to induce the same effect mediated by ICER restoration in

the HL60+ICER cell line. The decreasing expression of DUSP1 and DUSP4 mRNA was measured with the ΔΔCt method considering scramble siRNA as calibrator (RQ = 1). After 16 hours of DUSP1 silencing, the RQ was strongly decreased (RQ = 0.58), whereas DUSP 4 silencing was inefficient (RQ = 0.87). After 30 hours, DUSP1 mRNA was maintained reduced (RQ = 0.65), whereas DUSP4 was strongly diminished (RQ = 0.37). Protein levels followed the same trend of mRNA. Protein levels followed the same trend of mRNA. DUSP1/4 silencing was documented to increase phospho-p38 levels (p-p38) after 20 hours; apoptosis was slightly improved as well (data not shown). We then treated the DUSP1/4-silenced HL60 cell line with VP-16. Results showed that silenced cells had a decreased proliferation and an increased apoptosis with respect to the scRNA used as control. Therefore, the parental DUSP1/4-silenced HL60 cell line became more sensitive to the drug treatment, as established in the HL60+ICER cell line (Figure 5B, n = 3, * $p < 0.05$).

DUSP1 silencing or ICER exogenous expression promote apoptosis in AML primary cultures.

We silenced DUSP1 in primary BM cultures of AML at diagnosis. In the same cultures, we also restored ICER exogenous expression. DUSP1 silencing was more intense after 24 hours (RQ = 0.27) (calibrated to Sc-Sir, RQ = 1), with respect to 48 hours (RQ = 0.72).

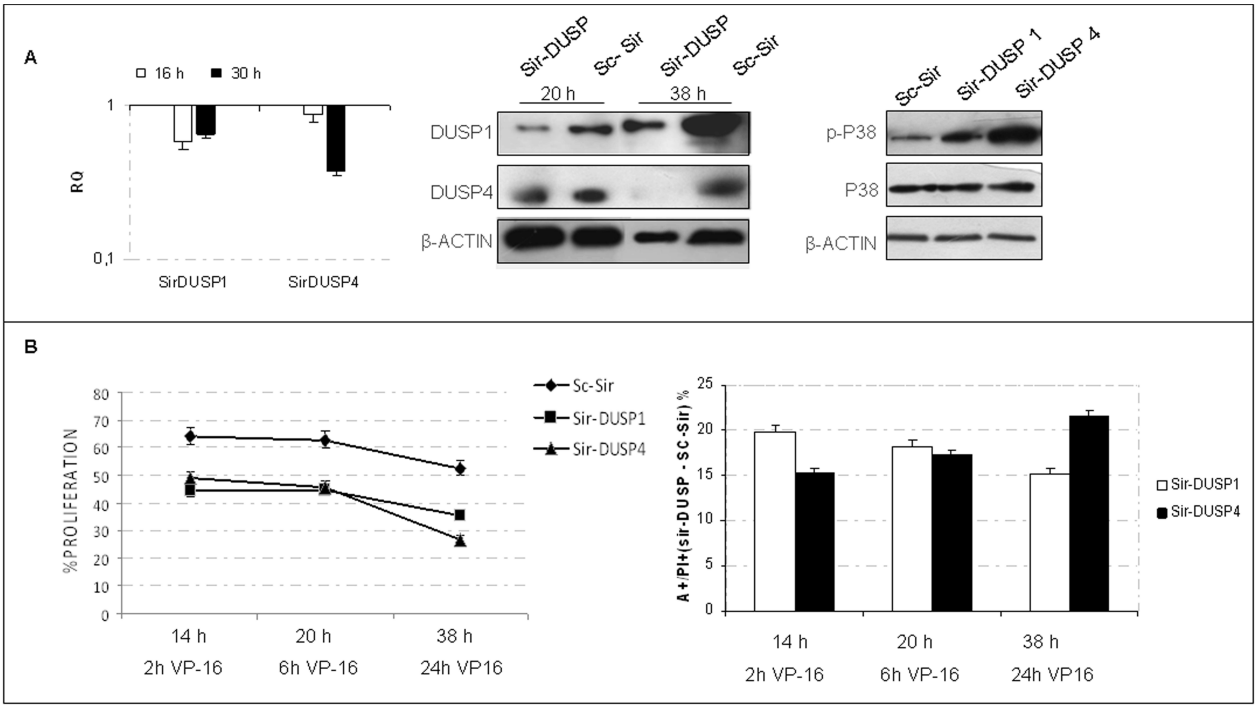


Figure 5. DUSP1/4 silencing phenocopies ICER role in enhancing drug sensitivity. A) RQ of DUSP1/4 after 16 and 30 h of silencing is shown, results are calibrated to Sc-Sir mRNA (RQ = 1) used as control. Western blot shows that silencing of DUSP1 and DUSP4 in HL60 cell line decreased their protein expression compared to the scramble siRNA oligonucleotide used as control (Sc-Sir) at 20 and 48 h post transfection. An increase in phospho-p38 (p P38) was also found concomitantly with silencing of DUSP proteins. B) HL60 cells after 14 h of silencing were treated with VP-16. Their proliferation rate decreases over time. The % of apoptosis significantly increases after DUSPs silencing with respect to cells transfected with Sc-Sir (sc-Sir value has been subctrated) and treated at the same VP-16 concentration (n = 3, * $p < 0.05$ for all time points presented).

Protein expression was impaired as well. We monitored the effect on p38 levels and found its phosphorylation increased, confirming the pathway activation after DUSP1 silencing (Figure 6A). We restored ICER expression in the same primary AML cultures by transiently transfecting the pEGFPΔ-N1_FLAG-ICERγ plasmid as well as the Empty Vector as control (EV). DUSP1 mRNA and protein expression were found decreased after transfection, confirming that DUSP1 is a downstream target of ICER in myeloid leukemia cells. p38 phosphorylation increased (Figure 6B) promoting cell death and supporting the same activation pathway in patients of the myeloid cell lines. The scheme in figure 6C summarizes a new view of how CREB might influence the survival signaling in myeloid leukemia.

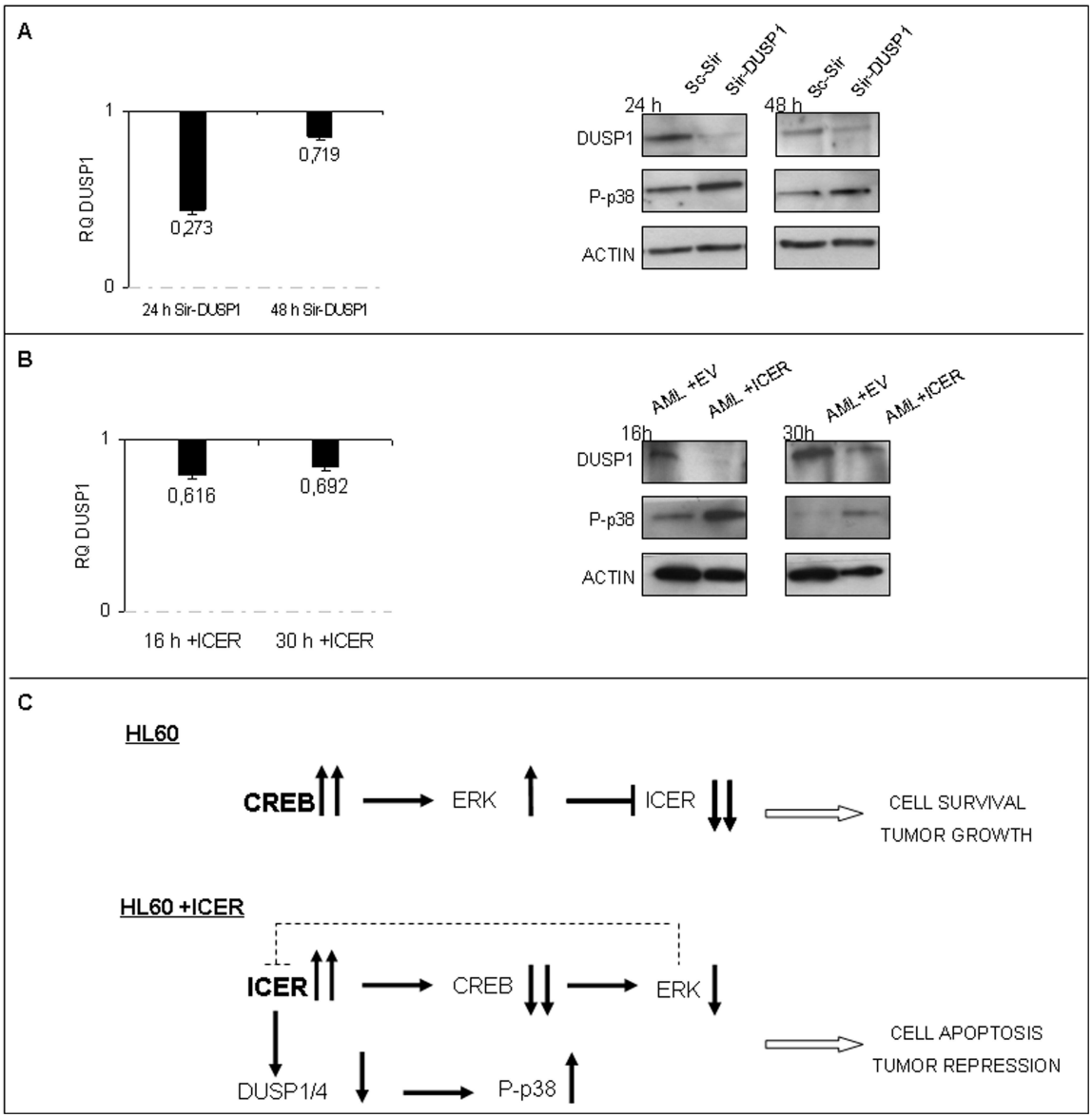


Figure 6. DUSP1 silencing or ICER expression promote apoptosis in AML primary cultures. A) Primary cultures were used to silence DUSP1. A decrease of DUSP1 mRNA after 24 and 48 h (ScSir used as calibrator, RQ = 1) is shown. DUSP1 protein levels were lowered and phospho-p38 (P-p38) activated after silencing. B) ICER expression was introduced in primary AML bone marrow: DUSP1 mRNA was found reduced (RQ = 0.6 after being calibrated to EV) and protein levels as

well. p38 activation was confirmed (P-p38). C) Proposed scheme of the cross-talk between CREB/ICER and the DUSPs/p38 pathways in leukemic myeloid cells.

DISCUSSION

AML is a heterogeneous tumor, specifically for its clinical outcome and molecular features. Although many chromosome abnormalities have been recently characterized, such as gene mutations, expression profiles and microRNAs, alternative treatment is needed because resistance to therapy and relapse still occur (30). Several approaches are under experimentation to increase cell apoptosis and a large number of kinases and phosphatases are under examination in the hematological field. Sensitizing cell to drugs could help to improve treatment response, and the characterization of novel molecules is urgent (31–33). We previously reported that ICER expression in leukemic cell lines induces significant antiproliferative effects (6,11). In this regard, results presented here show that restored ICER expression confers an enhanced drug susceptibility to leukemic cells. We described DUSP1 expression to be considered as a crucial target in AML treatment response. Focusing on DUSP1 and DUSP4 repression as ICER's main targets, we established their role in p38 activation, and elucidated the apoptotic signaling evoked by ICER in myeloid leukemia.

Multiple pathways might be addressed to be responsive to ICER restoration in leukemia. In this article, ICER-dependent regulation of pro- and antiapoptotic genes are demonstrated to enhance apoptosis with respect to that observed in leukemia cells without ICER. Cyclins and genes of the MAPK signaling pathway were specifically found severely downregulated by both ICER and chemotherapy treatment leading myeloid leukemia to a different predisposition to cell death. In particular, we found a novel link between ICER and DUSP1/4 phosphatases, whose expression has already been reported to be high in different types of tumors, though never previously discussed in the leukemia field (34). ICER-mediated transcriptional DUSP1/4 repression was demonstrated to contribute to increase p38 phosphorylation, triggering proapoptotic signals. The involvement of p38 in mediating apoptosis was confirmed by the use of p38 inhibitor SB203580, which interrupts the ongoing process of apoptosis. The induction of DUSP downregulation and p38 activation have been demonstrated to activate a cascade of different stimuli (35-37). The downregulation of the cell survival signaling of ERK and AKT might concur to enforce the p38-mediated apoptosis (23,38). Furthermore, the severe decrease of other downstream factors, such as p27, IL6 and RB, confers the final anti-survival cellular response. The fact that IL6 and RB transcription depends on CREB activity, whereas their phosphorylation on MAPK, confers to these two intricate pathways the ability to converge and collaborate for cell growth and AML progression (39,40). The finding of CREB and ICER transcriptional regulation of DUSP1/4 reveals a novel role in maintaining the balance between the activity of stress and survival kinases, which modulate leukemia cell fate.

DUSP1 and 4 repression appeared to be the crucial event for drug response of leukemia cell lines. The silencing strategy was also used in primary bone marrow cultures of AML at diagnosis to assume the same condition of the HL60+ICER cell line. DUSP1/4 down regulation lowered cell proliferation, phospho-p38 activation and an increased apoptosis of AML patients phenotyping the HL60+ICER cell line behavior. The proposed pathway was confirmed to modulate chemotherapeutic

susceptibility of myeloid leukemia. The *in vivo* strategy was also used to exogenously reintroduce ICER expression in primary AML giving the same results as DUSP1 silencing. ICER as controller of DUSP1 expression and of p38 pathway activation in AML patients confirmed our hypothesis, opening for further investigation in future therapy strategy.

Taken together, our results describe a novel apoptotic pathway in myeloid leukemia, summarized as a working model in Figure 6C: in HL60, CREB overexpression maintains high ERK levels, which takes ICER to degradation (11); DUSP1 and 4 dephosphorylate p38, supporting survival and leukemia growth. In the HL60+ICER cell line, ICER is highly expressed. It decreases CREB expression and promotes gene repression, in particular of DUSP1/4, which in turn allows p38 to remain phosphorylated and to trigger apoptosis. Lowered CREB levels and high p38 levels maintain ERK downregulated, preventing ICER degradation and contributing to tumor suppression (41,42). The identification of this pathway, confirmed in AML patients at diagnosis, offers novel targets to be considered in leukemia treatment. In particular, we support the idea that DUSP1 inhibition by ICER is a good strategy to sensitize cancer cells to conventional chemotherapy and to inhibit tumor growth. With regards to leukemogenesis, blocking CREB or inducing ICER might be further considered as phenomena involved in malignant transformation.

ACKNOWLEDGMENT

This work was supported by research funding from "Fondazione Città della Speranza" and University of Padua. We thank Dr. Paolo Bonvini Dr. Giuseppe Germano, and Cristina Zanon. We thank Nancy Jenkins, a scientific writer who provided editorial assistance during preparation of this manuscript.

REFERENCES

1. Haus-Seuffert P, Meisterernst M. Mechanism of transcriptional activation of cAMP-responsive element binding protein CREB. Mol Cell Biochem 2005;212:5-9.
2. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2001;2:599-609.
3. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, et al. The role of CREB as proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell 2005;7:351-62.
4. Pigazzi M, Ricotti E, Germano G, Faggian D, Aricò M, Basso G. cAMP response element binding protein (CREB) overexpression in childhood acute lymphoblastic and myeloid leukemia. Haematologica 2007;92:1435-37.
5. Cheng JC, Esparza S, Sandoval S, Shankar D, Fu C, Sakamoto KM. Potential role of CREB as a prognostic marker in acute myeloid leukemia. Future Oncol 2007;3:475-80.
6. Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I et al. CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood 2008;111:1182-92.
7. Molina CA, Foulkes NS, Lalli E, Sassone-Corsi P. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell 1993;75:875-86.
8. Razavi R, Ramos JC, Yehia G, Schlotter F, Molina CA. ICER-IIg is a tumor suppressor that mediates the antiproliferative activity of cAMP. Oncogene 1998;17:3015-19.
9. Jaworski J, Mioduszewska B, Sánchez-Capelo A, Figiel I, Habas A, Gozdz A, et al. Inducible cAMP early repressor, an endogenous antagonist of cAMP responsive element-binding protein, evokes neuronal apoptosis *in vitro*. J Neurosci 2003;23:4519-26.
10. Mouravlev A, Young D, During MJ. Phosphorylation-dependent degradation of transgenic CREB protein initiated by heterodimerization. Brain Res 2007;1130:31-37.
11. Pigazzi M, Manara E, Baron E, Basso G. ICER expression inhibits leukemia phenotype and controls tumor progression. Leukemia 2008;2:2217-25.
12. Memin E, Yehia G, Razavi R, Molina CA. ICER reverses tumorigenesis of rat prostate tumor cells without affecting cell growth. Prostate 2002;53:225-31.
13. Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, et al. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 2005;102:4459-64.
14. Franklin RA, McCubrey JA. Kinases: positive and negative regulators of apoptosis. Leukemia 2000;14:2019-2034.
15. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 2002;12:9-18.
16. Han J, Sun P. The pathways to tumor suppression via route p38. Trends Biochem Sci 2007;32:364-71.
17. Ahn YH, Jung JM, Hong SH. 8-Cloro-cyclic AMP-induced growth inhibition and apoptosis is mediated by p38 mitogen-activated protein kinase activation in HL60 cells. Cancer Res 2005;65:4896-901.
18. Salojin K, Oravecz T. Regulation of innate immunity by MAPK dual-specificity phosphatases: knockout models reveal new tricks of old genes. J Leukoc Biol 2007;81:860-69.
19. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene 2007;26:3204-13.
20. Patterson KI, Brummer T, O'Brien PM, Daly RJ. Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J 2009;418:475-89.
21. Ewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res 1998;74:49-60.
22. Xia Z, Dickens M, Raingeaud J, Davis R, Greenberg M. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326-31.
23. Chattopadhyay S, Machado-Pinilla R, Manguan-Garcia C, Belda-Iniesta C, Moratilla C, Cejas P,et al. MKP1/CL100 controls tunor growth and sensitivity to cisplatin in non-small cell lung cancer. Oncogene 2006;25:3335-45.
24. Birkenkamp KU, Dokter WH, Esselink MT, Jonk LJ, Kruijer W, Vellenga E. A dual function for p38 MAP kinase in hematopoietic cells: involvement in apoptosis and cell activation. Leukemia 1999;13:1037-45.
25. Schaar DG, Liu H, Sharma S, Ting Y, Martin J, Krier C, et al. 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced dual specificity phosphatase expression and AML cell survival. Leuk Res 2005;29:1171-79.
26. Folco EJ, Koren G. Degradation of the inducible cAMP early repressor (ICER) by the ubiquitin-proteasome pathway. Biochem J 1997,328:37-43.
27. Ananieva O, Darragh J, Johansen C, Carr JM, McIlrath J, Park JM, et al. The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol 2008;9:1028-36.
28. Arthur JS. MSK activation and physiological roles. Front Biosci 2008;13:5866-79.
29. Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cell Sign 2005;17:1343-51.
30. Litzow MR. Progress and strategies for patients with relapsed and refractory acute myeloid leukemia. Curr Opin Hematol 2007;14:130-37.
31. McCubrey JA, Abrams SL, Ligresti G, Misaghian N, Wong EW, Steelman LS, et al. Involvement of p53 and Raf/MEK/ERK pathways in hematopoietic drug resistance. Leukemia 2008;22:2080-90.
32. Nishioka C, Ikezoe T, Yang J, Yokoyama A. Inhibition of MEK signaling enhances the ability of cytarabine to induce growth arrest and apoptosis of acute myelogenous leukemia cells. Apoptosis 2009;14:1108-20.
33. Morel C, Carlson SM, White FM, Davis RJ. Mcl-1 integrates the opposing actions of signaling pathways that mediate survival and apoptosis. Mol Cell Biol 2009;14:3845-52.

34. Ono K, Han J. The p38 signal transduction transduction pathway: activation and function. Cell Signal 2000;12:1-13.
35. Siu YT, Jin DY. CREB-a real culprit in oncogenesis. FEBS J 2007;274:3224-32.
36. Bulavin DV, Fornace AJ. P38 MAP-kinase's emerging role as a tumor suppressor. Adv Cancer Res 2004;92:95-118.
37. Ashwell JD. The many paths to p38 mitogen-activating protein kinase activation in the immune system. Nat Rev Immunol 2006;281:6087-95.
38. Kawauchi K, Ogasawara T, Yasuyama M, Otsuka K, Yamada O. The PI3K/Akt pathway as a target in the treatment of hematologic malignancies.Anticancer Agents Med Chem 2009;9:550-59.
39. Khidr L, Chen PL. RB, the conductor that orchestrates life, death and differentiation. Oncogene 2006;25:5210-19.
40. Cavarretta IT, Neuwirt H, Untergasser G, Moser PL, Zaki MH, Steiner H et al. The antiapoptotic effect of IL-6 autocrine loop in a cellular model of advanced prostate cancer is mediated by Mcl-1. Oncogene 2007;26:2822-32.
41. Shi Y. Gaestel M. In the cellular garden of forking paths: how p38 MAPK signal for downstream assistance. Biol Chem 2002;383:1519-36.
42. Pigazzi M, Manara E, Baron E, Basso G. Mir-34b targets cAMP response element binding protein (CREB) in acute leukemia. Cancer Res 2009;69:2471-78.

CHAPTER 3

HYPERMETHYLATION OF MIR-34B IS ASSOCIATED WITH CREB OVEREXPRESSION AND MYELOID CELL TRANSFORMATION

Martina Pigazzi, PhD[1*], Elena Manara, PhD[1,4*], Silvia Bresolin[1], Claudia Tregnago[1], Alessandra Beghin[1], Emma Baron, PhD[1], Emanuela Giarin, PhD[1], Er-Chieh Cho, PhD[4], Riccardo Masetti, MD[2], Dinesh S. Rao, MD, PhD[3], Kathleen M. Sakamoto, MD, PhD[4,5#], Giuseppe Basso, MD[1#]

**contributed equally to the manuscript*

#co-senior authors

1) Women and Child Health Department- Hematology-Oncology laboratory, University of Padova, Padova, Italy
2) Department of Pediatrics, "Lalla Seràgnoli", Hematology-Oncology Unit, University of Bologna, Bologna, Italy
3) Department of Pathology & Laboratory Medicine, Jonsson Comprehensive Cancer Center, Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine at UCLA, Los Angeles, California
4) Department of Pediatrics, Gwynne Hazen Cherry Memorial Laboratories, Department of Pathology & Laboratory Medicine, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, and California Nanosystems Institute, Los Angeles, California
5) Department of Pediatrics, Stanford University School of Medicine, Stanford, California.

Haematologica. 2012 Oct 25. [Epub ahead of print]

ABSTRACT

Background. MiR-34b downregulation in acute myeloid leukemia was previously shown to induce CREB overexpression thereby causing leukemia proliferation *in vitro* and *in vivo*. The role of miR-34b and CREB in patients with myeloid malignancies were never evaluated.

Design and Methods. We examined miR-34b expression and the methylation status of its promoter in cells from patients diagnosed with myeloid malignancies. We used gene expression profile to identify signatures of myeloid transformation. We established miR-34b suppressor ability and CREB oncogenic potential in primary bone marrow cell cultures and *in vivo*.

Results. MiR-34b was found to be upregulated in pediatric patients with juvenile myelomonocytic leukemia (n=17) and myelodysplastic syndromes (n=28), but was downregulated in acute myeloid leukemia patients at diagnosis (n=112). Our results showed that hypermethylation of the miR-34b promoter occurred in 66% of acute myeloid leukemia explaining the low miR-34b levels and CREB overexpression, whereas preleukemic myelodysplastic syndromes and juvenile myelomonocytic leukemia patients was not associated with hypermethylation or CREB overexpression. In paired samples of myelodysplastic syndromes and subsequent acute myeloid leukemia from the same patients, we confirmed miR-34b promoter hypermethylation at leukemia onset, with 103 CREB target genes differentially expressed between the two disease stages. This subset of CREB targets was confirmed to associate with high risk myelodysplastic syndromes in a separate cohort of patients (n=20). 78 of these 103 CREB targets were also differentially expressed between healthy samples (n=11) and *de novo* acute myeloid leukemia (n=72). Further, low miR-34b and high CREB expression levels induced aberrant myelopoiesis through CREB-dependent pathways *in vitro* and *in vivo*.

Conclusions. We suggest that miR-34b controls CREB expression and contributes to myeloid transformation from both healthy bone marrow and myelodysplastic syndromes. We identified a subset of CREB target genes that represents a novel transcriptional network that may control myeloid transformation.

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression at a post-transcriptional level. MiRNA expression patterns are regulated during development and differentiation of the hematopoietic system and have an important role in cell proliferation, apoptosis, differentiation and even tumorigenesis (1). In acute leukemia, various miRNAs and their functions have been intensively studied, but the precise mechanisms that control their expression and critical targets are largely unknown for the majority of aberrantly expressed miRNAs (2). The discovery of miRNAs as a new class of post-transcriptional regulators that act via interactions with their target messenger RNAs has revealed an important pathway for controlling gene expression (3, 4).

Previously, we reported that miR-34b regulates CREB expression levels in myeloid cell lines by directly binding to its 3'untranslated region (UTR) (5). MiR-34b/c has been implicated in colon (6), ovarian (7) and oral oncogenesis (8). Here, we further studied the role of miR-34b in the pathogenesis of myeloid malignancies. One mechanism by which miRNAs are frequently silenced in human tumors is aberrant hypermethylation of CpG islands that encompass or lie adjacent to their genes or by histone modifications (9, 10). DNA methylation is in fact increasingly recognized as important in the regulation of normal and tumor cells (11, 12), the latter being characterized by specific hypermethylation of CpG islands in the promoters of tumor suppressor genes, resulting in transcriptional repression and gene inactivation (1, 3, 4, 9-12).

We identified hypermethylation as a cause of decreased miR-34b levels in leukemia cell lines, which in turn directly controls the expression of the proto-oncogene cAMP-response-element-binding protein (CREB)(5). CREB is known to regulate a wide range of cellular processes such as growth, proliferation, differentiation and apoptosis. Furthermore, it plays a crucial role in normal (13) and neoplastic hematopoiesis (14-17). CREB knockdown decreases proliferation and survival of myeloid progenitor cells and regulates the differentiation of committed progenitors (13). CREB is overexpressed in bone marrow from AML patients and increases AML cell proliferation *in vitro* and *in vivo*, through upregulation of specific target genes (13, 15). CREB overexpressing transgenic mice develop a myeloproliferative neoplasm with splenomegaly but not AML, highlighting a causative role for CREB in myeloid cell transformation (13). However, the consequences of abnormal expression of both CREB and miR-34b in AML are not well defined, and the role of both CREB and miR-34b in transition from MDS to AML has not been examined.

In this report, we studied a cohort of patients with JMML and MDS as well as de novo AML at diagnosis to study CREB and miR-34b. We investigated the role of their abnormal expression in the development of a malignant phenotype using *in vitro* and *in* vivo assays. Our results suggest that miR-34b promoter hypermethylation leads to CREB derepression and MDS evolution into AML through upregulation of a subset of CREB target genes.

DESIGN AND METHODS

Patients. We studied bone marrow (BM) samples from 112 patients with AML at diagnosis enrolled in the AIEOP-2002 AML pediatric protocol. The cohort of 28 pediatric MDS samples was composed of 6 refractory cytopenia of childhood (RCC), 22 refractory anemia with excess blasts (RAEB) according to the MDS classification proposed for pediatric patients (ages 0-18, age mean 10.26 ± 4.43). Seventeen pediatric patients (ages 0-18, age mean 2.51 ± 3.76) affected by juvenile myelomonocytic leukemia (JMML) according to differential diagnostic criteria, as previously published, were also included in the study. The enrollment criteria of the study are described in Supplementary Online Design and methods (18, 19). DNA and RNA were extracted and analyzed for methylation of miR-34b promoter, and for miR-34b levels. CD19-CD3- from 17 pediatric healthy BM

(HL-BM) (ages 0-18, age mean 11.86 ± 4.84) were used as control samples in the assessment of miR-34b expression in AMLs.

RNA isolation and SYBR Green real-time-PCR (RQ-PCR) assays. Total RNA was isolated using Trizol (Invitrogen). One microgram (μg) of RNA was reverse-transcribed into cDNA using the SuperScript II (Invitrogen) according to the manufacturer's instructions. RQ-PCR was performed with SYBR Green method (Invitrogen) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) (20). Experiments were carried out in triplicate and gene expression, relative to GUS, was calculated by the comparative ΔΔCt method (21).

RNA isolation and Real Time PCR (RQ-PCR) for miRNA analysis. Ten nanograms (ng) of total RNA were used as starting material for the stem-loop RQ-PCR method to detect the expression level of mature miR-34b (Applied Biosystems). All PCRs were run in triplicate and gene expression relative to RNU6B, was calculated by the comparative ΔΔCt method (21).

Methylation analysis. One μg of genomic DNA extracted from pediatric samples of 112 AML, 28 MDS, 17 JMML, 3 HL-BM, from sorted CD34+ of MDS and HL-BM samples according to the manufacturer's instructions (Gentra Autopure LS, Qiagen) was treated with sodium bisulfite using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH). A methylation-specific (MS) and unmethylation-specific (UMS) PCR reaction to detect the methylation status of miR-34b promoter was performed as previously described by Lujambo et al. (22). PCR amplicon obtained by MS-PCR was sequenced by an ABI PRISMTM310 Genetic Analyser sequencer (Applied Biosystems). (see Supplementary Online Design and Methods).

Gene expression analysis. Seventy-two bone marrow samples from patients with de novo AML at diagnosis, 24 MDS at diagnosis, 4 AML that evolved from MDS, and 11 healthy bone marrows were subjected to gene expression analysis. This series of de novo AML patients encompasses 16 patients with Core Binding factor aberrations, 23 MLL-rearranged positive patients, 30 patients with normal karyotype and 3 with complex karyotype. Promyelocytic AML with t(15;17) were *a priori* excluded from this series as they constitute an independent group. RNA quality was assessed on an Agilent2100 Bioanalyzer (Agilent Technologies). The GeneChip Human Genome U133 Plus 2.0 was used for the microarray experiments as previously described (23-26). (Supplementary Online Design and Methods).

Gene Set Enrichment Analysis (GSEA). Gene expression profiles obtained from 19 out of 72 AML patients at diagnosis were analyzed by using GSEA (http://www.broadinstitute.org/gsea/index.jsp) (see Supplementary Online Design and Methods).

Principal Component Analysis (PCA). We studied gene expression for an independent cohort of 20 samples from MDS patients at diagnosis, selecting the top 103 genes among CREB targets. PCA was performed using Partek Genomic Suite Software.

Primary cell culture and transfection. Primary cell cultures were obtained from BM from healthy donors. CD34+ sorted cells from human fetal liver were also used (27). Cell transfection was

performed using a Nucleofector (Amaxa Biosystems) according to the manufacturer's guidelines and efficiency was up to 40%. Cell cycle analysis and colony assays were performed (see Supplementary Online Design and Methods).

Constructs. pEGFP-N1-ΔGFP-CREB plasmid was obtained by cloning between the NotI and EcoRI cloning site a full length cDNA for CREB into the pEGFP-N1 vector (Invitrogen). Lentiviral constructs were constructed as previously described by Gentner et al.(28,29) (see Supplementary Online Design and Methods).

Western blot analysis (WB). WB were performed as previously described (30). Antibodies used were anti-Actin (Sigma-Aldrich), anti-PU.1, anti-GATA1, anti-MEIS1/2, anti-cMET, anti-cMYB (Santa Cruz Biotechnology), anti-cMYC, anti-CDK6 (GeneTex), anti-CREB, anti-P-CREB, the horseradish peroxidase–conjugated secondary antibody was goat anti-rabbit or mouse IgG (Upstate Biotechnology).

Flank injection xenograft experiments in NOD-SCID IL-2receptor gamma null (NSG) mice. Ten NSG mice between 6 to 8 weeks of age were injected subcutaneously with 5×106 HL60-miR-34b/LUC (or EV/LUC as a control) or K562-miR-34b/LUC (or EV/LUC as a control) cells. All mice were euthanized when tumors reached a volume of 1.5cm3. The mice were treated according to the NIH Guidelines for Animal Care and as approved by the UCLA Institutional Animal Care and Use Committee.

Xenograft experiments by bioluminescence Imaging in NSG mice. Ten mice were injected with 5×106 HL60-34b/LUC (or EV/LUC as a control) cells through the tail vein. Mice were imaged (IVIS100 bioluminescence/optical imaging system Xenogen) every week to monitor tumor engraftment and growth since 21 day post transplant. Three mg/mouse of D-Luciferin (Xenogen) in PBS was injected intraperitoneally 15 minutes before measurement (Relative Intensity units (RIU) = photons/sec/cm2). General anesthesia was induced with isoflurane. RIU for regions of interest were measured in triplicate and averaged.

Data analysis. Statistical analyses were performed by using Mann-Whitney or unpaired two-tailed t test. A p value of < 0.05 was considered significant.

RESULTS

MiR-34b promoter is hypermethylated in AML.

MiR-34b expression was already reported to be downregulated in 78 patients with AML at diagnosis (15). We enlarged this cohort of patients to 112, confirming its downregulation with respect to HL-BM (RQ_{AML}= 0.178, $RQ_{HL\text{-}BM}$= 1). We previously correlated this decrease in miR-34b expression with promoter hypermethylation in leukemic cell lines (15). Here, we investigated miR-34b promoter hypermethylation directly in 112 AML patients. MS-PCR revealed 74/112 (66%) AML patients demonstrated miR-34b promoter hypermethylation (Figure 1A). We sequenced the amplicons and confirmed the presence of CpG islands in patients with miR-34b promoter hypermethylation, whereas

the TG nucleotides at same position were found in unmethylated cases after bisulfite treatment. The 74 patients with hypermethylation of miR-34b promoter had significantly lower miR-34b expression and higher CREB protein levels, compared to the 38 patients without hypermethylation of miR-34b promoter where miR-34b was highly expressed (RQ_{Meth}= 0.075, RQ_{Unmeth}= 0.373, $p< 0.05$) and CREB protein was undetectable by Western blot analysis. Moreover, 19 AML patients were analyzed for gene expression profile using GSEA. Results showed that patients with hypermethylation of miR-34b promoter, and therefore miR-34b lowered levels, had a positive enrichment of genes predicted to be miR-34b targets (Figure 1S). These results suggested that hypermethylation of miR-34b promoter is a common feature in pediatric AML. Then, we studied the Event free Survival (EFS) of this AML patient cohort (74 AML patients with miR-34b promoter hypermethylation vs 38 patients not methylated at same promoter). There was a trend towards lower overall survival of AML patients with methylated miR-34b promoter (69 % at 4y) compared to patients with unmethylated promoter (61 % at 4y), but these results did not reach statistical significance ($p = 0.34$). Still, the methylation of miR-34b promoter might have negative prognostic significance in AML. There was not a significant independent correlation among clinical and biological parameters with the methylation of miR-34b promoter (Figure 2S). These findings suggest that cells from a large group of AML patients (66%) have miR-34b promoter hypermethylation (15), suggesting that this may be contributing to leukemogenesis.

Mir-34b expression is higher in MDS or JMML since miR-34b promoter is not hypermethylated.

We examined miR-34b expression in other rare pediatric hematopoietic disorders such as MDS (22 RAEB, 6 RCC) and 17 JMML, which can sometimes transform into AML. MiR-34b expression was higher in patients with RAEB (RQ_{mean}= 5.74), RCC (RQ_{mean}= 8.79) and JMML (RQ_{mean}= 8.86) compared to AML at diagnosis (RQ= 1). We then investigated miR-34b promoter methylation status in this cohort of myeloid malignancies. MS-PCR revealed that miR-34b promoter was never methylated in this cohort of samples, and sequencing always showed TG nucleotides at the CpG islands after bisulfite treatment (Figure 1C). However, since the expression of miR-34b is heterogeneous and some patients have low miR-34b expression, a nested PCR was performed to increase the sensitivity of the target amplicon detection (sensibility up to 10^{-6} as calculated with serial dilution of MS-PCR positive AML samples). These results confirmed the absence of methylation in all the MDS and JMML samples at diagnosis. Moreover, we sorted CD34+ hematopoietic cells from HL-BM or MDS patient samples to verify if the methylation of miR-34b promoter was found only in stem cells and therefore not detected in the whole bone marrow of patients. Neither HL-BM nor MDS sorted CD34+ cells showed hypermethylation of miR-34b promoter (Figure 1C). Furthermore, CREB expression was undetectable in MDS samples (Figure 1D).

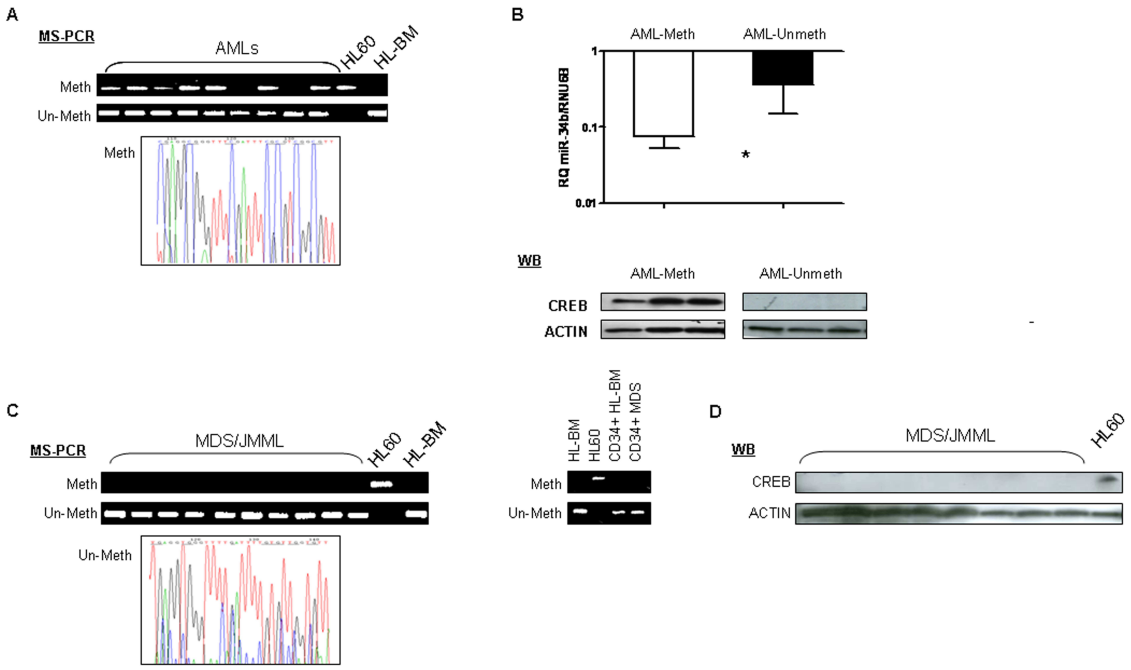


Figure 1. Methylation status of miR-34b promoter in AML, MDS or JMML patients. A. Methylation Specific PCR (MS-PCR) analysis of the miR-34b/c promoter in a representative set of AML patients samples is shown. HL60 cell line and healthy bone marrow samples (HL-BM) were used as positive and negative controls respectively. Direct sequencing of the miR-34b/c promoter amplicon after bisulfite treatment confirmed the methylation of GC nucleotides in the miR-34b promoter of patients. B. RQ-PCR analysis of this group of patients with the hypermethylation of miR-34b promoter (Meth, RQ=0.075) and without the hypermethylation of the promoter (Un-Meth, RQ=0.373, * $p<0.05$). CREB expression by WB in AML patients with or without methylated promoter. . C. MS-PCR analysis of the methylation of the miR-34b/c promote CpG island in a representative set of MDS patient samples. HL60 cell line and healthy bone marrow samples (HL-BM) were used as positive and negative controls, respectively. Direct sequencing of the miR-34b promoter amplicon confirmed the unmethylation of GpC islands shown as T nucleotides after bisulfite treatment. HL-BM and MDS CD34+ sorted cells did not show, as well, a methylation specific band. D. WB of a representative cohort of MDS patients shows a non-detectable levels of CREB expression in MDS samples. HL60 cells were used as positive controls

MiR-34b promoter hypermethylation occurs during transformation from MDS to AML.

MDS is a disease known to evolve to AML in 30% of cases. We examined miR-34b expression in 3 patients where paired samples were available at diagnosis of MDS and at the time of progression to AML. MiR-34b expression decreased following transformation into AML (RQmeanMDS= 0.61 vs RQmeanAML= 0.26). MS-PCR on the same paired disease samples revealed hypermethylation of miR-34b promoter exclusively in the AML specimens (Figure 2A). We then studied the gene expression profiling (GEP) in paired RNA samples at both MDS and AML diagnosis that were available for 4 patients. Using a supervised analysis we found a set of genes (175, see Supplementary table 1S) that were significantly ($p< 0.05$) differentially expressed between the two diseases. To interrogate the transcriptional programs associated with the aberrant expression of miR-34b and CREB, we intersected our gene expression data with CREB-target genes identified by performing ChIP on Chip analysis in human tissue (table S4 at http://natural.salk.edu/CREB/) (31). This analysis

revealed that 103/175(59%) genes differentially expressed were CREB target genes. We used the 103 CREB target genes as a new subset to perform hierarchical clustering analysis. Results revealed that CREB targets were able to distinguish the MDS from their own AML evolution (Figure 2C). Then, by PCA analysis we validated the 103 CREB target genes in an independent cohort of 20 MDS patients at diagnosis. This analysis showed that two distinct groups of MDS were generated. One included four high risk MDS patients (who are known to be evolved into AML with a median interval between diagnosis and evolution = 225 days, range 59-714 days; mean age difference between the two groups it is not statistically significant) and the other included low risk MDS patients (that were not evolved at the time of the study from 3-10 years from diagnosis) (26) (Figure 2D). Therefore, differential expression of CREB target genes marks MDS, high risk MDS (MDS that evolved) and AML, supporting the role of miR-34b and CREB in the myeloid transformation process. To identify the mechanism of miR-34b downregulation, we performed MS-PCR on the four evolved MDS, finding that miR-34b promoter hypermethylation became evident at the onset of AML, supporting the idea that promoter hypermethylation occurred during the transition from MDS to AML.

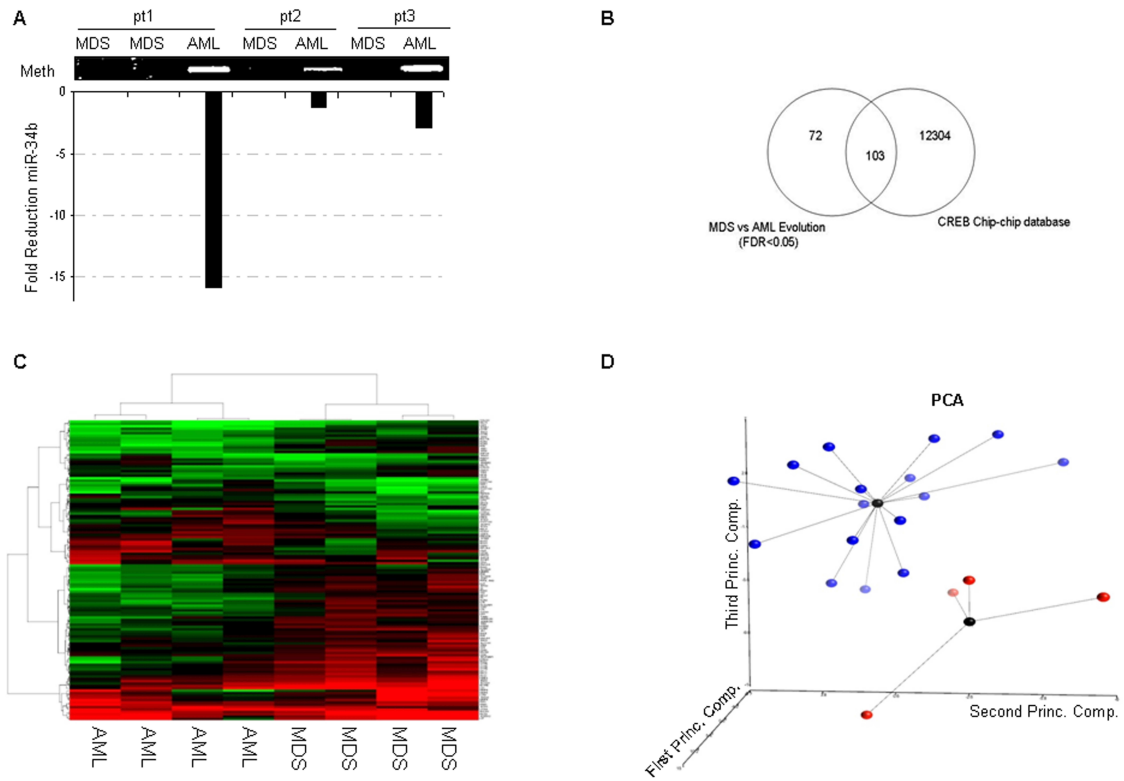


Figure 2. Characterization of MDS patients and their evolution into AML. A. MS-PCR analysis of the miR-34b promoter in 3 MDS patients evolved to AML. Methylation of promoter is present just at the onset of AML. Fold reduction of miR-34b expression for each patients samples was 15.9, 1.16, 2.9. B. Venn diagram shows the overlap (103 genes) between genes differentially expressed by GEP and human CREB targets (by ChIP-chip database, (29)) among the MDS and

evolution into AML. C. Hierarchical clustering analysis of 4 patient pairs at diagnosis of MDS and their evolution into AML using the 103 differentially expressed CREB-target genes divided the MDS from their evolution into two separate clusters. D. Principal Component Analysis using 103 CREB target genes that distinguishes MDS from their evolution to AML in an independent cohort of 20 MDS patients. Patients are divided between those known to be evolved (red), and those not evolved (blue). In black there is the centroid of the two groups of patients**.**

CREB overexpression in HL-BM induces dismyelopoiesis through its targets upregulation.

To study the effect of CREB overexpression in myeloid transformation, we transiently transfected healthy bone marrow cultures with a full-length plasmid containing CREB cDNA. We confirmed increased protein levels post CREB transfection (Figure 3A). Cell cycle analysis showed a slight increase in S phase cells after CREB overexpression (Figure 3B) (cell apoptosis was similar in cells transfected with CREB or empty vector, data not shown). The clonogenic growth of bone marrow cells overexpressing CREB was enhanced as measured by methylcellulose colony assays (Figure 3C). By FACS analysis we identified increased numbers of erythroid precursors (CD45 negative), an increased number of cells positive for erythroid and megakaryocyte markers (CD36, Glycophorin A, and CD61), and for granulocyte/monocyte marker (CD15) in CREB overexpressing cells (Figure 3D, ns). We investigated CREB targets of the myeloid lineage expression, and found GATA-1 and PU.1 proteins being upregulated by Western blot analysis (32, 33). MEIS-1, a known CREB target gene in AML, was also found increased (34, 35) (Figure 3E). We performed RQ-PCR for previously confirmed CREB targets (20) finding that most of them were increased in normal bone marrow cultures with enforced CREB expression (Figure 3F). Next, we investigated the expression of a series of CREB target genes that were extrapolated from the analysis of MDS evolved into AML (see supplementary table 1S). These novel CREB target genes were upregulated after CREB exogenous transfection (Figure 3G). To better dissect the mechanism of transformation, we analyzed the gene expression profile of 72 AML at diagnosis versus 11 healthy volunteers. We found 15597 present calls by Affymetrix parameters expressed in all the 11 HL-BM analyzed and intersected this result to the 12407 genes present in CREB-database (that we previously used in our gene expression analysis (31) . We found 3281/12407 CREB target genes expressed in HL-BM, that represent the 25% of expressed genes in normal bone marrow. When we compared gene expression from HL-BM and de novo AML, we found 2618 genes differentially expressed (FDR< 0.05) between the two groups, and among them 1195/2618 (46%) were CREB targets (previously found by human Chip-chip analysis) (31). The differences highlighted by these probe sets are not due to age difference of HL-BM and AML, since their mean is not statistically different. We therefore defined a crucial subset of CREB targets that are expressed at AML onset versus HL-BM. We intersected these data with the gene expression data regarding CREB genes in the transition of MDS to AML, in the above section, and found that 78 out of 103 genes (76%) were common between the two data sets (Figure 3H, See supplementary table 1S). Furthermore, if one examines all genes (CREB targets and non-targets) that are significantly

differentially expressed and in common between the two processes, CREB targets are enriched (78/131 CREB targets (60%)) compared with non-targets (53/131,40%).

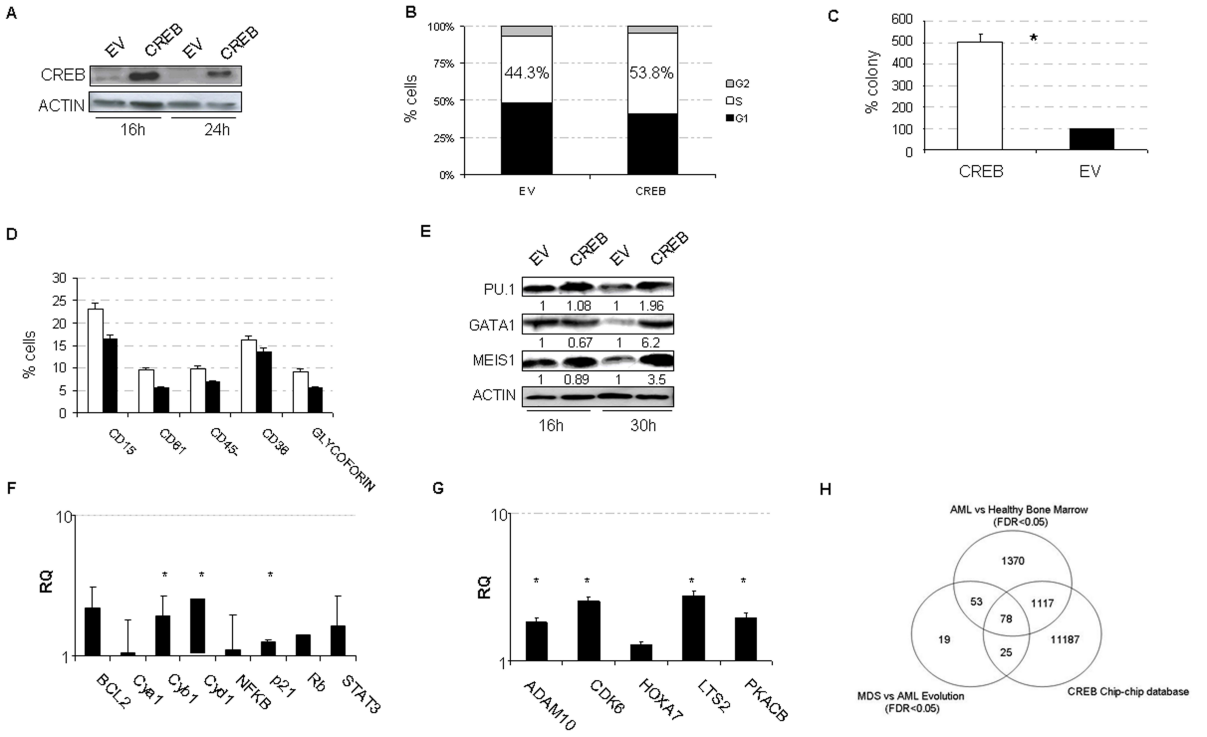


Figure 3. CREB overexpression in normal bone marrow cultures. Healthy bone marrow transiently transfected with a CREB overexpressing vector (CREB) or a control (EV). A. WB 16h and 24h post transfection: induced CREB protein expression is shown in CREB transfected cells. B. Cell cycle analysis in transfected healthy bone marrow showed an increased S-phase in CREB transfected HL-BM (n = 2). C. Colonies growth in methocult were counted after 14 days of culture and histograms represent the number of colony formed by CREB expressing cells (white bar) with respect to those formed by cells transfected with control vector (EV = 100%, black bar,* p< 0.05). D. FACS analysis of myeloid and erythroid markers (CD15, 61, CD45-, CD36, Glycoforin) in healthy primary culture transfected with CREB (white bar) compared to EV (black bar) showed an increase in all myeloid markers. E. WB of PU.1, GATA-1 and MEIS-1 16 and 30h post transfection showed an increase in expression of those protein after CREB exogenous expression in healthy bone marrow after 30h. Densitometry measured the expression levels normalized to total amount of Actin. F. Expression of CREB target genes 24h post transfection was RQ BCL2= 2.20, CyA1= 1.04, CyB1= 1.93, CyD1= 2.4, NFKBp50= 1.11, p21= 1.26, STAT3= 1.63. Each gene quantification has been normalized to the RQ of HL-BM transfected with EV (RQ = 1) (n= 3). G. Expression of genes extrapolated in Figure 2B (see Supplementary table 1S).was RQ ADAM10= 1.82, CDK6= 2.54, HOXA7= 1.26, LTS2= 2.75, PKACB= 1.97. Each gene quantification has been normalized to the RQ of HL-BM transfected with EV (RQ= 1) (n= 3). H. Venn diagram representation shows the overlap between genes differentially expressed between the MDS and their evolution, genes differentially expressed between AML and HLBM and CREB targets by ChIP-chip database (29).

MiR-34b inhibition phenocopies CREB upregulation in healthy fetal liver (FL) cells.

Since decreased expression of miR-34b leads to CREB overexpression, we measured miR-34b levels in whole HL-BM (RQmean=13.66, n=17), in CD34+ sorted HL-BM cells (RQmean= 9.08, n= 3) and CD34+ sorted FL cells (RQmean= 43.14, n= 6). Our results demonstrated that miR-34b expression

was higher in healthy hematopoietic tissues than in bone marrow from patients with AML (n= 3, p< 0.05). To study the role of miR-34b in human fetal liver cells, we knocked down miR-34b using a lentiviral sponge vector containing four in tandem miR-34b target sequences (28, 29). Infected cells were sorted and analyzed. MiR-34b expression decreased by 75% compared to negative control (RQ= 0.25), whereas CREB levels were increased (RQ= 4.48) in addition to CREB target genes known to influence myeloid leukemia (Figure 4A). We next analyzed cell morphology and phenotype of miR-34b knockdown human fetal liver cells. MGG staining revealed a larger number of myeloid precursors and differentiated cells (examples are highlighted in the 60X square), indicating an aberrant expansion of the myeloid compartment (Figure 4B). A difference in the percentage of myeloid markers by flow cytometry was also documented (Figure 4C). Since decrease of miR-34b expression can affect CREB expression, we examined the clonogenic properties in methylcellulose colony assays. Enhanced colony formation in the miR-34b knockdown human fetal liver cells was observed compared to the scrambled miR-target (miR-Neg, Figure 4D). Therefore, miR-34b is critical for normal hematopoietic proliferation and differentiation.

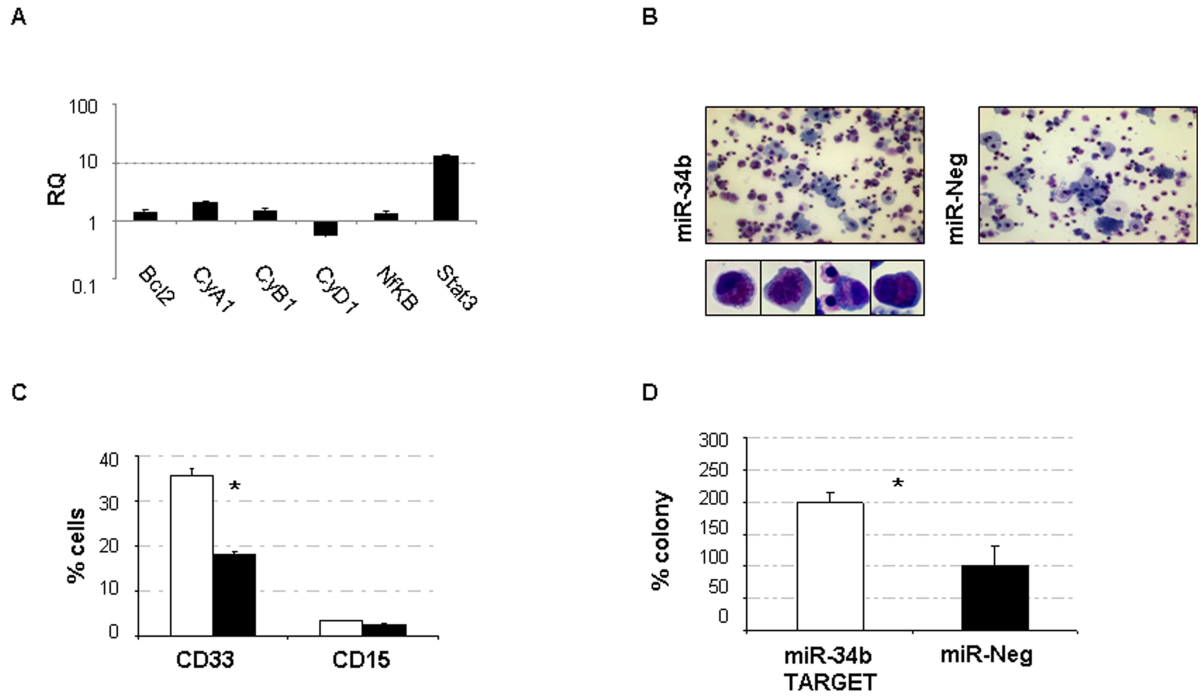


Figure 4. MiR-34b knockdown in CD34+ fetal liver cells. A.Expression of known CREB targets was BCL2= 1.44, CyA1= 2.09, CyB1= 1.51, CyD1= 0.54, NFKBp50= 1.37, STAT3= 13.5. Each gene quantification has been normalized to the RQ of fetal liver transduced with EV (RQ= 1) (n= 3). B. Representative microscope field of a May-Grünwald-Giemsa (MGG) staining (20X) of fetal liver cell with a knock down in miR-34b shows an increase in all the myeloid populations compared to EV. Example of myeloid cells not found in the controls are reported in the enlargement (60X). C. By FACS analysis we showed an increase in myeloid markers (CD33 and CD15) in cells of fetal liver transduced with miR-TARGET (white bar) compared to control (black bar) (CD33= 35.4% vs 18%, CD15= 3.3% vs 2.5%, * p< 0.05). D. Histogram represents the 2-fold increase in number of colony formed after knockdown of miR-34b (white bar) with respect to those formed by cells transfected with miR-NEG (miR-NEG= 100%, black bar,* p< 0.05).

MiR-34b is an AML tumor suppressor *in vivo*.

To confirm the hypothesis that miR-34b is a tumor suppressor in AML, we transduced HL60 and K562 leukemic cell lines with lentivirus for miR-34b or miR-Neg (RQmiR-34b up to 10-3 fold increase). MiR-34b overexpression decreases CREB expression as shown by Western blot analysis (Figure 5A). The introduction of miR-34b in K562 did not affect cell proliferation *in vitro* (data not shown). We next injected HL-60 and K562 cells into NSG mice and monitored tumor formation. We observed that cells injected into the flank of NSG mice with K562+miR-34b developed smaller tumors (0.68g) 21 days post-injection compared to K562 + empty vector (1.18g) (Figure 5B, mice n= 10 in each cohort, $p< 0.05$). The same result was observed for HL60+miR-34b (0.79g) compared to HL60+empty vector (1.39g) (Figure 5C, mice n=10 in each cohort, $p< 0.05$). Furthermore, 5x106 HL60+miR-34b or HL60+empty vector cells were injected into the tail veins of 5 mice per group to analyze the effect of miR-34b overexpression in leukemia cell engraftment and progression. Results showed a decreased engraftment and disease progression in mice injected with HL60+miR-34b compared to the empty vector controls monitored by acquiring *in vivo* bioluminescence 2 and 3 weeks after inoculation. After two weeks, mice injected with cell lines+miR-34b did not show a significant difference in tumor size compared to cell lines+empty vector. However, after 3 weeks, the mice injected with cell lines+miR-34b had a significant decrease in tumor progression due to miR-34b overexpression ($p< 0.01$, Figure 5C). Other miR-34b targets, such as C-MYB, CDK6 and MET (22, 36), were not found significantly reduced in the transplanted cell lines (data not shown). These experiments suggest that miR-34b is a tumor suppressor in AML principally by inhibiting CREB expression.

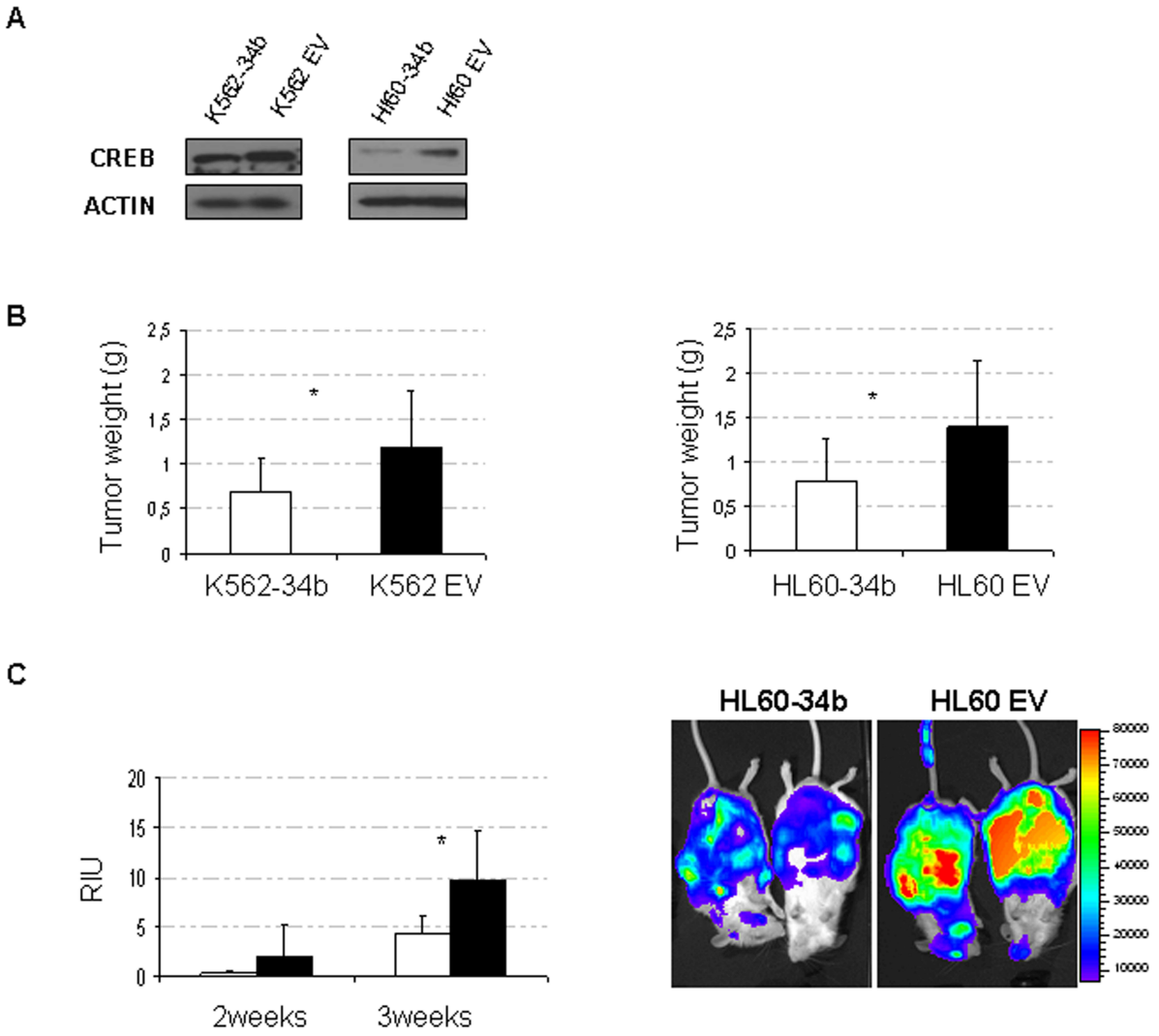


Figure 5. MiR-34b suppresses CREB expression and AML progression in primary cultures and *in vivo*. A. Western blot analysis confirmed the reduction of CREB protein levels in leukemic cell lines (K562 and HL60) after infection with Fugw-miR-34b with respect to empty vector (EV). Densitometric analysis: HL-60 = 0.7±0.15 vs. 1, and K562 = 0.22±0.24 vs. 1, n = 3, $p < 0.05$. B. Tumor weight of the xenograft model obtained with a flank injection of K562 or HL60 leukemic cell lines overexpressing miR-34b in NSG mice was reduced in comparison to EV injected cells. Error bars represent standard deviation. * $p< 0.05$. C. *In vivo* imaging with IVIS 100 bioluminescence/optical imaging system (Xenogen) of mice tail vein injected with $5x10^6$ HL60-34b or EV transduced cells 2 or 3 weeks post injection. The histogram shows a decrease in the Relative Intensity Units (RIU) of mice injected with leukemic cell line overexpressing miR-34b compared to those injected with the control cell line (n= 5 each group, p= 0.01).

DISCUSSION

In this paper, we show that AML patients have decreased miR-34b expression through aberrant hypermethylation of the miR-34b promoter CpG island. Interestingly, hypermethylation of miR-34b promoter was completely absent in primary samples of HL-BM as well as in MDS or JMML patients.

We considered the childhood myelodysplastic syndromes because of their propensity to evolve into acute myeloid leukemia in approximately 30-40% of cases (19, 37, 38). Primary bone marrow samples from MDS and its corresponding, transformed AML allowed us to show that cells acquired miR-34b promoter hypermethylation during the evolution to AML. Epigenetic modification of miR-34b promoter results in aberrant CREB levels. We previously showed a direct interaction of miR-34b with the CREB 3'-untranslated region using a reporter assay, with the subsequent reduction of the CREB protein levels *in vitro*. We also documented that miR-34b overexpression caused cell cycle abnormalities, reduced colony assay growth, and altered CREB target gene expression in leukemic cell lines (5). Here, we demonstrate a direct link between miR-34b and CREB expression in pediatric myeloid malignancies. By using an integrative bioinformatic approach, we identified a subset of genes that could potentially mediate this transformation. We extrapolated a series of genes that were able to significantly cluster MDS from their paired AML samples. CREB target genes represented 59% of differently expressed genes, and were also able to distinguish high risk MDS from the low risk MDS, therefore being a marker of disease evolution into AML. The differentially expressed genes that were non-CREB targets, did not predict correctly all the four cases of MDS that evolved into AML, supporting the idea that CREB targets play a main role in triggering the disease evolution. To further support the hypothesis that CREB, through its targets, contributes to myeloid transformation, we used *de novo* AML and healthy bone marrows and performed gene expression analysis intersecting those results with the CREB target genes database. Fifty percent of CREB target genes, which are two-fold more than typical CREB targets expressed in normal bone marrow, were found to be differentially expressed between healthy bone marrow and bone marrow from AML patients, suggesting that CREB overexpression activates a pathway that is involved in the pathogenesis of AML de novo. We intersected gene expression profiles of MDS evolved into AML and healthy bone marrow into de novo AML, extrapolating 78/103 genes in common with known CREB target genes. A relative enrichment of CREB targets with respect to non-targets supports our hypothesis that CREB controls a key pathway involved in the myeloid transformation. These results suggest that during transformation from normal bone marrow as well as from MDS, CREB target genes may represent a common involved pathway. Among the targets there were several genes already known to play a role in tumor process, such as RAB7L1, CDK6, HOXA7 and PKACβ, the catalytic subunit beta of the protein kinase A which posphorylates CREB on Ser133, enhancing its transcriptional activation. We propose that CREB is one of the regulators of the transcriptional program associated with myeloid leukemia, and moreover is critical to the pathogenesis of myeloid transformation. The fact that CREB driven expression profile “signature” is also found in MDS samples that are prone to evolve into AML even if miR-34b promoter is not methylated, might be due to PKACβ expression. This gene encodes for the catalytic subunit beta of protein kinase A, that leads to CREB phosphorylation, and consequently CREB transcriptional activation. We found that PKACβ expression increased from HL-BM, to MDS to AML. In this scenario, we suppose that it triggers CREB activation during high risk MDS, this

condition is then stabilized and worsened with the hypermethylation of miR-34b promoter at the onset of leukemia leading to AML. Previous results documented that PKACβ is activated in proliferating cells also in a cAMP independent mechanism, leading to cell transformation (39), supporting its role in our model. Nevertheless, our data suggest that future studies may shed light to this mechanism.

Finally, we examined the role of miR-34b inhibition and CREB overexpression *in vitro* and *in vivo*. The inhibition of miR-34b levels in healthy bone marrow and fetal liver cells enhances cell proliferation and clonogenic potential, leading to aberrant myelopoiesis. Our results showed that miR-34b rescue had a minor effect on cell growth in leukemic cell lines. However, miR34b overexpression suppressed tumor growth *in vivo*, suggesting that the microenvironment may play a role in the tumor suppressive activity of miR-34b. Moreover, in an attempt to demonstrate that miR-34b acts solely through CREB, we rescued CREB expression on K562 stably expressing miR-34b. Our data did not show a significant increase in cell growth after CREB rescue, and this may be due to the fact that leukemia cell line have a high proliferative rate and multiple mutations affecting various signaling pathways. *In vivo* rescue experiments are needed in the future to prove this hypothesis, and to eventually identify other miR-34b targets.

These data implicate CREB overexpression, together with the alteration of its target genes, acting as a driving force in AML transformation from both healthy bone marrow and MDS. This work highlights the oncogenic circuitry that is fed by the hypermethylation of miR-34b, increased CREB levels, and a potential downstream mechanism involved in the pathogenesis of AML transformation. Furthermore, the CREB subset of genes may be considered a novel transcriptional network that controls the leukemia phenotype, which can guide further functional studies and therapeutic opportunities, and represents a novel approach to evaluate risk stratification of MDS patients.

Acknowledgements. We thank Dr. Hee-Don Chae, Dr. Sabrina Gelain, Dr. Samuela Francescato, Dr. Sanja Aveic, Dr. Marco Giordan, Dr. Cristina Zanon, Prof. Andrea Pession, Dr. Alan Ikeda, Maria Grazia Giacometti, Silvia Disarò and Katia Polato for their collaboration. This work was supported by the National Institutes of Health (NIH) R01 grants HL75826 and HL83077 (KMS).

Funding support: This study was supported by grants from the Fondazione Città della Speranza-Padova-Italy (MP, EG, GB), from EU FP7-228971 (EM, GB), University of Padova, Fondazione Veneto Banca and AIL (AB, EB, SG, CT). D.S.R. is supported by a career development award from the National Institutes of Health (NIH) CA133521 and is a Scholar of the Sidney Kimmel Foundation for Cancer Research. K.M.S. is supported by the National Institutes of Health (NIH) HL75826 and HL83077, William Lawrence and Blanche Hughes Foundation, and the St. Baldrick's Foundation. K.M.S. is also a Scholar of the Leukemia and Lymphoma Society of America.

Authorship Contributions: MP and EM performed experiments, interpreted results, and wrote the manuscript; EG and RM contributed to patient samples and clinical data; SB was responsible for gene expression analyses and statistical analysis; AB, EB, CT, ECC performed experiments; KMS, DR and GB interpreted results and revised the manuscript.

REFERENCES

1. Davalos V, Esteller M. MicroRNAs and cancer epigenetics: a macrorevolution. Curr Opin Oncol. 2010;22(1):35-45.
2. Agirre X, Martinez-Climent JA, Odero MD, Prosper F. Epigenetic regulation of miRNA genes in acute leukemia. Leukemia. 2012;26(3):395-403.
3. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol. 2009;27(34):5848-56.
4. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet. 2009;10(10):704-14.
5. Pigazzi M, Manara E, Baron E, Basso G. miR-34b targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. Cancer Res. 2009;69(6):2471-8.
6. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res. 2008;68(11):4123-32.
7. Suzuki H, Yamamoto E, Nojima M, Kai M, Yamano HO, Yoshikawa K, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis. 2010;31(12):2066-73.
8. Corney DC, Hwang CI, Matoso A, Vogt M, Flesken-Nikitin A, Godwin AK, et al. Frequent downregulation of miR-34 family in human ovarian cancers. Clin Cancer Res. 2010;16(4):1119-28.
9. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006;6(4):259-69.
10. Kunej T, Godnic I, Ferdin J, Horvat S, Dovc P, Calin GA. Epigenetic regulation of microRNAs in cancer: An integrated review of literature. Mutat Res. 2011.
11. Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wijermans P, et al. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood. 2002;100(8):2957-64.
12. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol. 2002;20(10):2429-40.
13. Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I, et al. CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood. 2008;111(3):1182-92.
14. Mora-Garcia P, Cheng J, Crans-Vargas HN, Countouriotis A, Shankar D, Sakamoto KM. Transcriptional regulators and myelopoiesis: the role of serum response factor and CREB as targets of cytokine signaling. Stem Cells. 2003;21(2):123-30.
15. Pigazzi M, Ricotti E, Germano G, Faggian D, Arico M, Basso G. cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression. Haematologica. 2007;92(10):1435-7.
16. Sandoval S, Pigazzi M, Sakamoto KM. CREB: A Key Regulator of Normal and Neoplastic Hematopoiesis. Adv Hematol. 2009;2009:634292.
17. Sakamoto KM, Frank DA. CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy. Clin Cancer Res. 2009;15(8):2583-7.
18. Pession A, C. R, MC. P, Masetti R, Casale F, Fagioli F, et al. Results of the AIEOP AML 2002/01 Study for Treatment of Children with Acute Myeloid Leukemia. 51st ASH annual meeting and exposition; 2009; Orlando: Blood; 2009.
19. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia. 2003;17(2):277-82.
20. Pigazzi M, Manara E, Baron E, Basso G. ICER expression inhibits leukemia phenotype and controls tumor progression. Leukemia. 2008;22(12):2217-25.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
22. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, et al. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A. 2008;105(36):13556-61.
23. Mills KI, Kohlmann A, Williams PM, Wieczorek L, Liu WM, Li R, et al. Microarray-based classifiers and prognosis models identify subgroups with distinct clinical outcomes and high risk of AML transformation of myelodysplastic syndrome. Blood. 2009;114(5):1063-72.
24. Bresolin S, Zecca M, Flotho C, Trentin L, Zangrando A, Sainati L, et al. Gene expression-based classification as an independent predictor of clinical outcome in juvenile myelomonocytic leukemia. J Clin Oncol. 2010;28(11):1919-27.
25. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Kronnie GT, Bene MC, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. J Clin Oncol. 2010;28(15):2529-37.
26. Bresolin S, Trentin L, Zecca M, Giordan M, Sainati L, Locatelli F, et al. Gene expression signatures of pediatric myelodysplastic syndromes are associated with risk of evolution into acute myeloid leukemia. Leukemia. 2012.
27. Shimizu S, Hong P, Arumugam B, Pokomo L, Boyer J, Koizumi N, et al. A highly efficient short hairpin RNA potently down-regulates CCR5 expression in systemic lymphoid organs in the hu-BLT mouse model. Blood. 2010;115(8):1534-44.
28. Rao DS, O'Connell RM, Chaudhuri AA, Garcia-Flores Y, Geiger TL, Baltimore D. MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. Immunity. 2010;33(1):48-59.
29. Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol. 2007;25(12):1457-67.
30. Hermeking H. The miR-34 family in cancer and apoptosis. Cell Death Differ. 2010;17(2):193-9.

31. Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, et al. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A. 2005;102(12):4459-64.
32. Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, et al. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. Dev Cell. 2005;8(1):97-108.
33. Liew CW, Rand KD, Simpson RJ, Yung WW, Mansfield RE, Crossley M, et al. Molecular analysis of the interaction between the hematopoietic master transcription factors GATA-1 and PU.1. J Biol Chem. 2006;281(38):28296-306.
34. Esparza SD, Chang J, Shankar DB, Zhang B, Nelson SF, Sakamoto KM. CREB regulates Meis1 expression in normal and malignant hematopoietic cells. Leukemia. 2008 Mar;22(3):665-7.
35. Wang Z, Iwasaki M, Ficara F, Lin C, Matheny C, Wong SH, et al. GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. Cancer Cell. 2010;17(6):597-608.
36. Migliore C, Petrelli A, Ghiso E, Corso S, Capparuccia L, Eramo A, et al. MicroRNAs impair MET-mediated invasive growth. Cancer Res. 2008;68(24):10128-36.
37. Hasle H, Niemeyer CM. Advances in the prognostication and management of advanced MDS in children. Br J Haematol. 2011;154(2):185-95.
38. Niemeyer CM, Kratz CP. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia: molecular classification and treatment options. Br J Haematol. 2008;140(6):610-24.
39. Wu KJ, Mattioli M, Morse HC, 3rd, Dalla-Favera R. c-MYC activates protein kinase A (PKA) by direct transcriptional activation of the PKA catalytic subunit beta (PKA-Cbeta) gene. Oncogene. 2002;21(51):7872-82.

CHAPTER 4

THE CAMP RESPONSE ELEMENT BINDING PROTEIN (CREB) OVEREXPRESSION INDUCES MYELOID TRANSFORMATION IN ZEBRAFISH

Claudia Tregnago[1], Sanja Aveic[1], Elena Manara[1], Valeria Bisio[1], Martina Pigazzi[1], Giuseppe Basso[1]

1) Department of Woman and Child Health, Laboratory of Hematology-Oncology, University of Padova, Padova, Italy

(Manuscript in preparation)

ABSTRACT

Leukemias are the most common form of pediatric cancers, and whereas Acute Lymphoblastic Leukemia treatment has made significant progresses over the past decades reaching up to 86 % of survival, Acute Myeloid Leukemia (AML) survival reaches the 60 % of patients, and 30 % of AML patients relapse within 2 years from remission. The cAMP responsive element binding protein (CREB) has been found overexpressed in pediatric AML patients, correlating with an increased risk of relapse, but the pathogenesis of CREB's role in AML is still debated. To better understand CREB's mechanism of transformation and its possible therapeutic targeting, we developed a zebrafish *in vivo* model that overexpress human CREB in myeloid precursors. Results showed that CREB-overexpression led to hematopoiesis perturbation from larvae until adultness, with an altered CREB target gene expression, which conferred an enhanced cell cycle progression to myeloid cells. After 6 months of CREB enforced expression, zebrafish kidney marrow showed dysmyelopoiesis, with increased myelocytes and monocytes counts. At the age of 9 months, CREB-overexpressing zebrafish developed a sick phenotype with abdominal and dorsal enlargement that morphologically resembled an abdominal mass formed by clonal cells with infiltrating capability. The mass cells were found to originate from the cells overexpressing human CREB, to rapidly proliferate and to migrate into gills, adipose tissue and muscles. Mass cell morphology was similar to that of kidney marrow with a complete lack of myelocytes, and a preponderance of monocytes, confirming a myeloproliferative disease. The long latency of disease onset suggests that additional events may occur. Finally, by using this zebrafish model, CREB molecular pathways associated to transformation may be identified, and subsequent genetic screens to identify novel therapeutic targets will be pursued.

INTRODUCTION

Leukemias are defined as a group of disorders characterized by the uncontrolled proliferation and the block of the differentiation of hematopoietic cells. Such malignancies arise from abnormal precursors cells that have acquired a series of mutation, or have lost their physiological regulating mechanism [1]. Overall leukemia is the most common form of pediatric cancer (30%), nowadays Acute Lymphoblastic Leukemia (ALL) and Acute Myeloblastic Leukemia (AML) can be cured in 86% and 63% of patients respectively, but challenges remain [2]. The causes that leads to leukemia development in pediatric patients are largely unknown, as still debated are the causes to treatment failure and relapse. Risk adapted therapy has been the cornerstone of ALL therapy, whereas improvements in AML therapy have been limited being generally associated with escalation of induction therapies, improvements of intensive supportive care measures, and extreme intensification of chemotherapy finalized to bone marrow transplantation. Nowadays, up to 25% of AML pediatric patients experienced relapse within 1 years of remission with a cure rate survival less than 30%. This may be due to the inability of drugs to effectively eradicate the leukemic stem cells, or to target the cooperating mutations that occur to transform cells. For leukemia, several genetic lesions of transcription factors, tyrosine kinases, and transcriptional regulators that derive from genetic lesions or from aberrant chromosomal translocations, have been established. The cAMP response element-binding protein (CREB) is a 43-kDa nuclear transcription factor of the basic leucine zipper (bZIP) family. It recognizes the conserved cAMP-responsive elements (CRE) at gene promoters, leading to the transcription of its target genes in response to various stimuli that activate protein kinases, mainly protein kinase A (PKA), that activates its transcriptional activity phosphorylating CREB at its Ser133 residue [3, 4]. Through activation of its target genes, it controls crucial physiological function as cell growth, survival and proliferation [5]. CREB has been found overexpressed in adult and pediatric AML, and it has been established a proto-oncogene in AML since it leads to abnormal cell proliferation, cell cycle progression and higher clonogenic potential *in vitro* and *in vivo*. CREB overexpressing transgenic mice have been shown to develop myeloproliferative disorder between the age of 14 and 20 months [6]. Its overexpression has also been associated with high risk AML patients [6, 7]. Nevertheless the mechanism underlying CREB-mediated hematopoietic disorders remains unknown. Here, we take advantages of zebrafish (*Danio rerio*) *in vivo* model to depict the processes altered by CREB aberrant expression. Zebrafish model is useful to study human cancer with a genetic approach, such as mutagenesis and screening, and its optical transparency during embryonic stages allows the monitoring of biological processes throughout development [8, 9]. Furthermore, zebrafish are well-established useful for studying hematopoiesis: erythroid, myeloid and lymphoid lineages have been characterized, and the molecular pathways governing their development are largely conserved between mammals and zebrafish [8]. Moreover, several human oncogenes involved in leukemogenesis have been shown to disrupt normal hematopoiesis in zebrafish, and to resemble human hematological disease [10-14]. Zebrafish *Creb1* is located at chromosome 1. It encodes a 311 amino acids protein, sharing 88.7 % identity with human CREB, conserving the activation domain (kinase inducible domain (KID), DNA-binding (DBD) and leucine zipper dimerization domains), that implies functional conservation [15]. We established a zebrafish model with human CREB enforced expression under the control of *pu.1* promoter to investigate its

role in hematopoiesis and in leukemogenesis, Pu.1 is an Ets family transcription factor that plays a restrictive role in hematopoietic cell lineage, both myeloid and lymphoid. During hematopoiesis, pu.1 is upregulated with myeloid commitment, being strongly expressed in myeloid precursor cells [16]. We assessed embryos and larvae for defects in primitive and definitive hematopoiesis, respectively, and monitored CREB overexpressing zebrafish for incidence of disease. Perturbations in the hematopoietic compartment and on CREB target genes were seeked. Here, we revealed that CREB overexpression in zebrafish triggered dismyelopoiesis that led to an abnormal phenotype in adult zebrafish, with clear features of a myeloid disorder. This model emerged to be useful to study the events driven by CREB to enhance cell transformation and for further therapeutic screening.

MATERIALS AND METHODS

Plasmid construction. The human CREB1 was amplified using F: AGTCGAATTCAATGACCATGG and R AGTCCTGCAGTTTCCTCATTT primers, then cloned in frame to eGPF into pME-eGFP (383, Tol2kit) [17]between, between EcoRI and PstI restriction sites.

The p5'-228-pu.1 promoter plasmid was obtained by excising the 9kb pu.1 promoter sequence from z*pu.1*p-GFP plasmid using SalI and SacII restriction enzyme (NEB), and by cloning it into p5E MCS (228) [16, 17].

The Invitrogen MultiSite Gateway Technology was used to generate the plasmids for zebrafish embryos injection. The Gateway strategy allows modular assembly of promoter, coding sequence and 3'tag in a Tol2 transposon backbone. Transposon elements enable random plasmid integration into genome, through transposase enzymatic reaction. The plasmids were recombinated through LR reaction (Gateway Technology) according to manufacturer's recommendation (Invitrogen), using LR plus clonase enzyme and performing the reaction for 16 h at 25°C. After LR reactions, 2 µl of the sample was transformed into TOP 10 bacteria. Clones were selected on carbenicillin plates and screened by PCR, miniprep and DNA sequencing. Entry and destination vectors used from Tol2kit were: 299 (p5E-bactin2), 228 (p5E-MCS), 383 (pME-EGFP), 302 (p3E-polyA), 394 (pDestTol2pA2) [17]. Plasmid for RNA probes synthesis were obtained by PCR fragments generated from a pool of cDNAs from zebrafish embryos at different stages (at 24 and at 48 hpf) and cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA). Probes for rb1 and jun were designed from the published NCBI sequence (NM_001077780.1) (F: cgtgtctcctctgaaaagca; R: ctgcccaggaagaacgtaa) and from NCBI sequence of reference was NM_199987.1 (F: caccgctctctcctatcgac; R:tgaggtgctcagaaaacaaaca), respectively. Pu.1, gata.1 and mpo plasmids used to synthesize riboprobes were kindly provided from Professor Felix CA, bcl2 from Dr. Forrester AM [14]; p21 from Dr. Chen J [18]; ccnD1 from Dr. Bessa J [19].

Zebrafish maintenance and microinjection. Embryos were grown at 28.5 C, and developmental staging of injected zebrafish embryos was determined by hours post fertilization (hpf), according to Kimmel et al. [20]. At established time points single cell plasmid injection was performed. Purified vectors were diluted at a final concentration of 30 ng/µl, and trasposase mRNA at a final concentration of 25 ng/µl in Danieau solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5 mM HEPES (pH 7.6)], with 10% phenol red. Trasposase mRNA synthesis was performed using mMESSAGE mMACHINE T7/SP6 kit (Ambion, Austin, TX), using pCS-TP plasmid, kindly provided by Dr. Koichi Kawasakami.

The plasmids and the trasposase mRNA (25 ng/µl) was diluited in Danieau solution with 10% phenol red. Microinjections of 2 nl of solution were performed into the yolk of fertilized one-cell stage embryos. Fluorescence was monitored on a Nikon SMZ1500 zoom stereomicroscope (Nikon Instruments Inc).

Whole-mount in situ hybridization (WISH). Digoxogenin (DIG) -labelled antisense zebrafish RNA probes were synthesized according to manufacturers' instruction. Briefly, plasmids were linearized with NotI or BamHI restriction enzyme (NEB) and all antisense riboprobe were synthesized using DIG RNA Labeling Kit (SP6/T7) (Roche). Whole-mount in situ hybridization (WISH) for mRNA expression were performed as previously published [21]. Embryos were fixed in 4% paraformaldehyde (PFA) and the DIG probes were detected using anti-DIG-alkaline phosphatase (AP) and NBT/BCIP. After in situ hybridization, embryos

were put in 4% paraformaldehyde in PBS and mounted in 85% glycerol/PBS. Embryos were visualized and imaged with Nikon SMZ1500 zoom stereomicroscope (Nikon Instruments Inc).

Luciferase assay. For luciferase activity detection, pDest-βactin:eGFP_CREB-polyA or pDest-βactin:eGFP-polyA together with pAd C6-6xCRE_luciferase plasmids were injected at equimolar ratios of 20 ng/µl. Renilla plasmid was used at 1:10 ratio (2 ng/µl). After 24 and 48 h of injection proteins were extracted and analyzed by Dual Luciferase Assay System (Promega).

RNA extraction and SYBR Green quantitative real-time reverse transcription-PCR assays. Total RNA from zebrafish embryos at 24 and 48 hpf was extracted using Trizol (Invitrogen). RNA (1 µg) was transcribed using the SuperScript II system (Invitrogen) following the manufacturer's instructions. RQ-PCR was performed with 1 µL cDNA in 20 µL using the SYBR Green method (Invitrogen) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Expression values were normalised to zf β-actin and expressed as fold change relative to the control injected zebrafish group.

bactin F	TGCTCCCCGAGCTGTCTT	***jun R***	CAGACATCAAGCCCCTGAAGTT
bactin R	ACCAACCATGACACCCTGATG	***dusp1 F***	CGAGGCCATCGAATTTATTG
scl F	GGGAGCGCGGATATAAAAGG	***dusp1 R***	CGCATCAGATAAGCCAGACA
scl R	GAGGCAGTGGCAAGGGAAT	***dusp4 F***	GAGGACATCAGCTCCTGGTT
gata-1 F	CAGTTCAGCAGCGCTCTATTCA	***dusp4 R***	TGGAATCCTTGACGGAGTCTAT
gata-1 R	AGCCTCAGGTGGCGAAAGT	***rb1 F***	CCCACAGAAGAAAGAGCTGAA
pu.1 F	AGAGAGGGTAACCTGGACTG	***rb1 R***	GGATCCTTGTCCTTGTGTCG
pu.1 R	AAGTCCACTGGATGAATGTG	***stat3 F***	TCTCCCGGTGGTGGTCAT
l-plastin F	GAAGCTCTGATCGCTCTGCT	***stat3 R***	AATTCACGTTCTTGGGATGGTT
l-plastin R	GTTGTTGATTTTGGGGCATC	***jak3 F***	AACTGGCCTGTGCTCTTAACTT
Runx1 F	CCGACTCCCGGCAGATG	***jak3 R***	TCTTTGCACAAATATTCCCATGT
Runx1 R	AGATCGGGCCCAGATATGG	***gata2 F***	CCACTGCAAGAATGGACGAA
c-myb F	CAGTTTGGGACACCGTGATG	***gata2 R***	GCCAAGCTTCCCCGAAGA
c-myb R	GGAGAGATGTGACAGTGGTTTGC	***ccnA1 F***	GACGGCTGCAATACTCCTG
jun F	CTGCCAGCTCATGTTGACACA	***ccnA1 R***	CGTGATGTAGACAAACTCATCCA

Table 1. RQ-PCR primer sequences.

FACS cell sorting of kidney tissue or abdominal mass. Kidney or abdominal mass isolated from wild-type fish or leukemic fish were homogenized in protease solution (Collagenase/Dispase 1:200, DNAse 1:1000, MgCl2 1:1000 in phosphate-buffered saline) at 37°C for 30 minutes and manually dissected. The whole suspension was filtered through a 45 μm cell strainer. before being analyzed at BD Facs Aria III (Becton Dickinson), as previously described [10].

Hisytology, immunohistochemistry and cytology. Whole adult zebrafish were euthanized with 2 mg/ml MS-222 at pH 7 (Tricaine; Sigma-Aldrich), fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 μm serial sections were obtained. Hematoxylin and eosin (H/E) staining, and immunohistochemical analyses were performed. For this purpose, sections were re-hydrated and then antigen retrieval was performed by incubation with citrate buffer 0.01 M pH6 at 95 °C for 20 min. For immunohistochemistry, slides were incubated with 1:400 dilution anti-GFP (B-2) Ab (Santa Cruz Biotechnology), 1:500 dilution anti-CREB (Millipore), 1:500 dilution anti-PCNA (FL-261) Ab (Santa Cruz Biotechnology) for 1 hour and then developed with the DAKO Envision Detection Kit. Kidney or abdominal mass isolated from zebrafish were imprinted on glass, and stained with Wright-Giemsa and Alpha-naphtyl acetate esterase (ANAE) with standard procedures. Images were captured on a Nikon SMZ1500 zoom stereomicroscope (Nikon Instruments Inc), or on a ViCo Nikon eclipse 80i microscope.

Data analysis. Values are presented as mean ± standard deviation (sd). Significance between experimental values was determined by Student's unpaired *t*-test, and Fisher's exact test. $P < 0.05$ (*) was considered significant.

RESULTS

CREB is induced in myeloid cell lineage and works as a transcription factor

To investigate the oncogenic properties of CREB, we created a CREB-overexpressing *in vivo* model by injecting eGFP_CREB fusion gene under the control of *pu.1* promoter into 1-cell stage fertilized embryos. CREB exogenous expression was monitored by eGFP expression. The fluorescent signal was observed from 20 hpf in the anterior lateral mesoderm (ALM) and intermediate cell mass (ICM), both sites of primitive myelopoiesis. eGFP expression was found also in muscle, indicating ectopic *pu.1* promoter expression as previously observed (figure 1) [16].

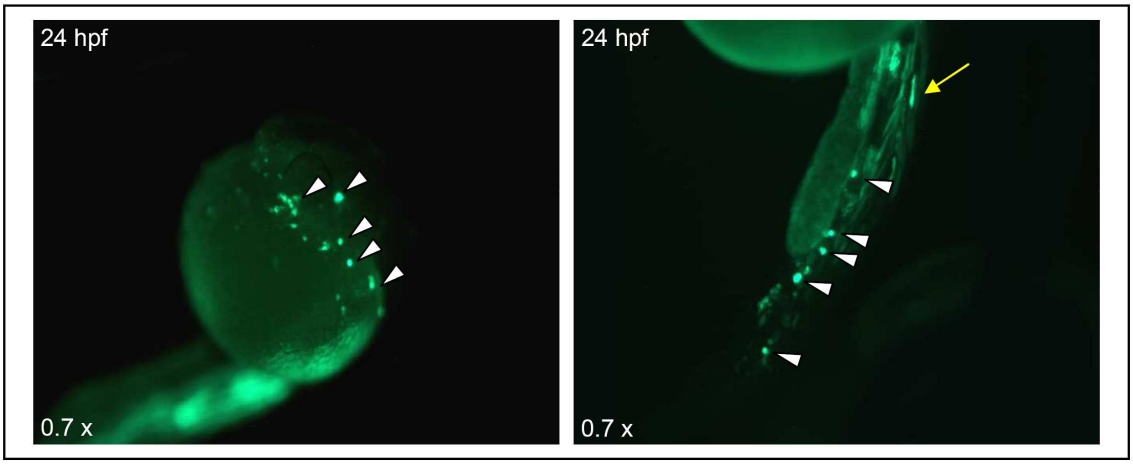


Figure 1. pu.1:eGFP_CREB injected embryos expressed *CREB* gene under the control of the 9.1 kb zebrafish pu.1 promoter. At 24 hpf eGFP_CREB expressing cells were detected in ALM (left picture, white arrowhead), and in ICM (right picture, white arrowhead). Ectopic eGFP expression in musculature were detected as well (right picture, yellow arrow). 0.7 x original magnification

Then, we aimed to confirm CREB activity as transcription factor. In human, it recognizes the cAMP response elements (CREs) consensus regions and activates the transcription of a large number of genes. To verify if its activity is maintained in zebrafish, we co-injected pu.1:eGFP-CREB and a reporter plasmid, made up of 6xCRE repeats upstream to a red fluorescent mCherry protein. At 24 hpf we observed a co-expression of eGFP and mCherry in CREB-injected embryos indicating that CREB recognized the consensus regions, and activated the transcription of mCherry gene. In the control zebrafish injected with the reporter plasmid and the empty vector pu.1:eGFP we did not observe the co-localization of signals (figure 2).

Furthermore, to confirm the transcriptional activity of CREB in zebrafish embryos, we co-injected a ubiquitous plasmid βactin:eGFP-CREB together with a reporter construct containing 6xCRE repeats located upstream to a luciferase reporter gene. Luciferase activity was measured 26 hpf, and was found significantly increased of 2.7 fold in CREB-injected zebrafish respect to control-injected zebrafish (mean luciferase activity βact:eGFP = 7.5, sd = 4.6, n = 3; mean luciferase activity βact:eGFP_CREB = 20.2, sd = 5.2, n = 5; p= 0.01). At 48 hpf the luciferase activity was also found increased of 2.7 fold in CREB-injected zebrafish respect to control-injected zebrafish, but results were not significant

probably due to the relative luciferase plasmid dilution during time following the high cell proliferation in early embriogenesis (mean luciferase activity βact:eGFP = 7.5, sd = 5.4, n = 4; mean luciferase activity βact:eGFP_CREB = 20.6, sd = 12.4, n = 3; p = 0.1). These results indicated that exogenous CREB recognized the same CRE consensus regions, and therefore may increase the transcription of zebrafish target genes.

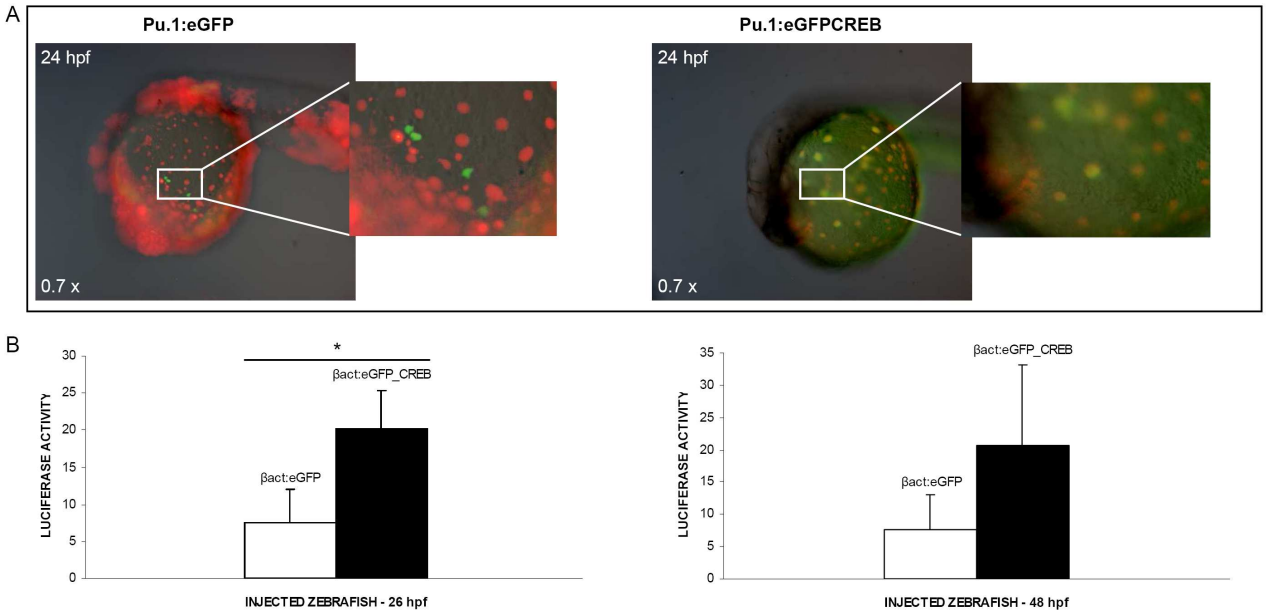


Figure 2. Increased trascriptional activity in CREB-injected zebrafish. A) Co-expression of eGFP and mCherry signal was detected in pu.1:eGFP_CREB and 6xCRE_mCherry co-injected embryos (right panel), but not in pu.1:eGFP and 6xCRE_mCherry co-injected embryos (left panel). 0.7 x original magnification B) An increased luciferase activity was observed in βactin:eGFP-CREB-injected zebrafish compared to βactin:eGFP-injected zebrafish at 26 hpf (left panel, p = 0.01), and at 48 hpf (right panel, p = 0.1). * p < 0.05

CREB overexpression perturbs gene expression of primitive and definitive zebrafish hematopoiesis

To study the effects exerted by CREB overexpression in myeloid cells, we focus on the expression of specific genes by RQ-PCR on the whole embryos till 48 hpf. We selected genes associated with early primitive hematopoietic precursors (*scl)*, erythroid (*gata1*), early and late myelomonocytic lineages (*pu.1* and *lcp*). Genes that regulate hematopoietic stem cells (HSCs) onset during definitive hematopoiesis such as *runx1* and *c-myb* were also studied. In CREB-overexpressing zebrafish, genes involved in primitive hematopoiesis were found lightly upregulated at 24 hpf (*scl* fold change:1.4 ± 0.3, p = 0.2, *gata1* fold change: 1.1 ± 0.3, p = 0.6; *pu.1* fold change: 1.1± 0.4, p = 0.7; *lcp* fold change: 1.2 ± 0.3, p = 0.4) as well as genes that regulates definitive hematopoiesis (*runx1* fold change: 1.6 ± 0.4, p = 0.1; *c-myb* fold change: 1.4 ± 0.2, p = 0.1). At 48 hpf genes expressed by hematopoietic precursors were found still upregulated (*scl* fold change: 1.1 ± 0.1, p = 0.5; *gata1* fold change: 1.3 ± 0.4, p = 0.4; *pu.1* fold change: 1.7 ± 0.9, p = 0.4), as well as gene of myelomonocitic lineage (*lcp* fold change: 1.1 ± 0.1, p = 0.1). Viceversa, *runx1* was found significantly decreased (fold change: 0.9 ±

0.01, p = 0.006), while *c-myb* remained upregulated (fold change: 1.6 ± 0.4, p = 0.1) (Figure 3A). These results indicated a broad aberrant gene expression of all the hematopoietic cell lineages analyzed. Next, we investigated the expression of a series of previously confirmed CREB targets found to be aberrantly expressed in human bone marrow cells by CREB enforced expression [22]. Results revelaed that *jun* expression was not affected (fold change at 24 hpf: 1.1 ± 0.1, p = 0.4; at 48 hpf: 1.1 ± 0.4, p = 0.8), whereas genes encoding *dusp* and *jak3* were not differently expressed with respect to control (*dusp1* fold change at 24 hpf: 0.9 ± 0.03, p = 0.02; at 48 hpf: 1 ± 0.1, p = 0.9; *dusp4* fold changes at 24 hpf: 0.8 ±0.1, p = 0.1; 48 hpf: 0.8 ± 0.3, p = 0.4; *jak3* at 24 hpf: 0.8 ± 0.4, p = 0.5; 48 hpf: 0.7 ± 0.04, p = 0.09). In contrast *rb1*, *stat3*, *gata2* and *cyclin A1* expression were found increased (*rb1* fold change:1.4 ± 0.04 at 24 hpf, p = 0.005, 1.4 ± 0.1 at 48 hpf , p = 0.07; *stat3* fold change at 24 hpf: 2.1 ± 0.8, p = 0.2; at 48 hpf: 2.1 ± 1.1, p = 0.3; *gata2* fold change at 24 hpf: 1.7 ± 0.2, p = 0.04, at 48 hpf: 1.8 ±1.3, p = 0.4; *cyclinA1* fold change at 24 hpf: 4 ± 1.6, p = 0.1; at 48 hpf: 2.5 ± 1.6, p =0., figure 3B). These results revealed an aberrant upregulation of main oncogenic transcription and cell cycle factors.

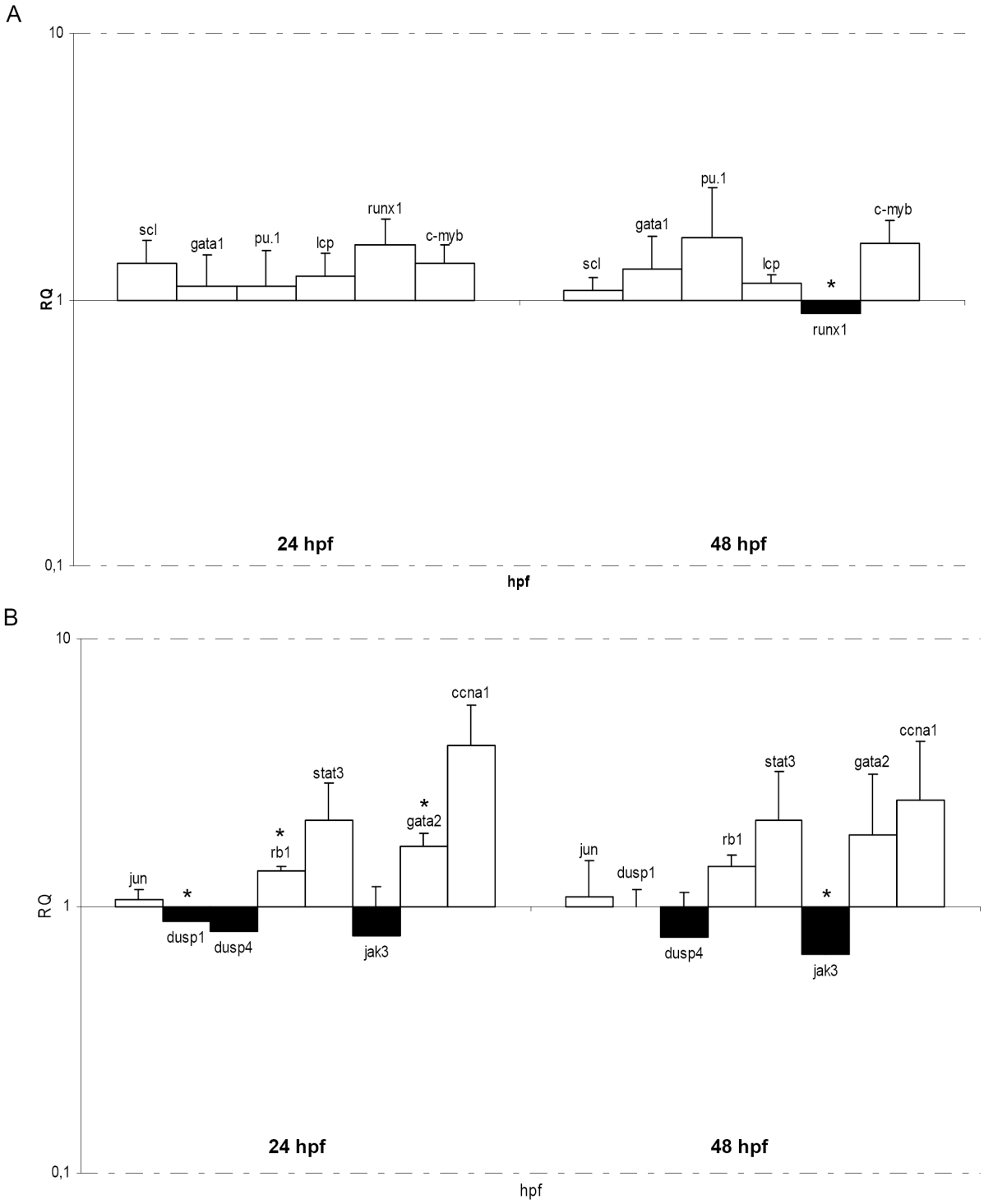


Figure 3. Gene expression analysis investigated by RQ-PCR of CREB-overexpressing zebrafish compare to control-injected zebrafish (used as calibrator, RQ = 1). A) Fold changes of genes involved in zebrafish primitive hematopoiesis (*scl, gata1, pu.1, lcp*) and genes that mark HSCs during zebrafish definitive hematopoiesis (*runx1, c-myb*), at 24 hpf and 48 hpf. B) Fold changes of known human CREB target genes that are important in different cell pathways: MAPK signaling pathway (*jun, dusp1, dusp4*), cell-cycle apoptosis pathway (*rb1*), *stat3, jak3, gata1, ccna1*. * $p < 0.05$.

In order to monitor CREB effects on spatial and temporal foci of hematopoiesis, we performed RNA whole mount in situ hybridization (WISH) on CREB-overexpressing zebrafish. At 20 hpf 100% (n =

30) of CREB-overexpressing embryos displayed high pu.1 positive cell number in ALM, whereas 17 out of 20 control zebrafish showed the same expression (85%, p = 0.05); 100% of CREB-overexpressing embryos displayed a strong gata1 expression in ICM (n = 33), compared to 78% of control zebrafish injected with the empty vector (n = 23, p = 0.009, Figure 4A). At 24 hpf, the expression of myeloperoxidase, feature of more mature myeloid granulocytes, was seen in 36% (n = 25) of CREB-overexpressing embryos compared to 4% of the control zebrafish (N = 26, p = 0.005, Figure 4B, left panel). These data suggested that CREB exogenous expression altered the early hematopoietic compartment development in zebrafish, by influencing both the myeloid and erythroid lineages commitment during primitive hematopoiesis, even if this perturbation was not sufficient to induce embryogenic defect or altered larvae phenotype.

We then analyzed the first wave of definitive hematopoiesis, which occurs between 1 to 2 day post fertilization (dpf), when erythromyeloid progenitors (EMPs) have been described to be in the posterior blood island (PBI) [23]. We looked at *gata1* and *pu.1* expression, finding that *gata1* signal in 40% (n = 10) of CREB-overexpressing zebrafish at 30 hpf was increased, whereas none of control zebrafish showed this strong *gata1* expression (n = 5, p = 0.2, Figure 4B, right panel). These results were in agreement with our previous findings in humans, where CREB-induced overexpression in healthy bone marrow cells caused an increased number of cells positive for erythroid and granulocyte/monocyte markers [24].

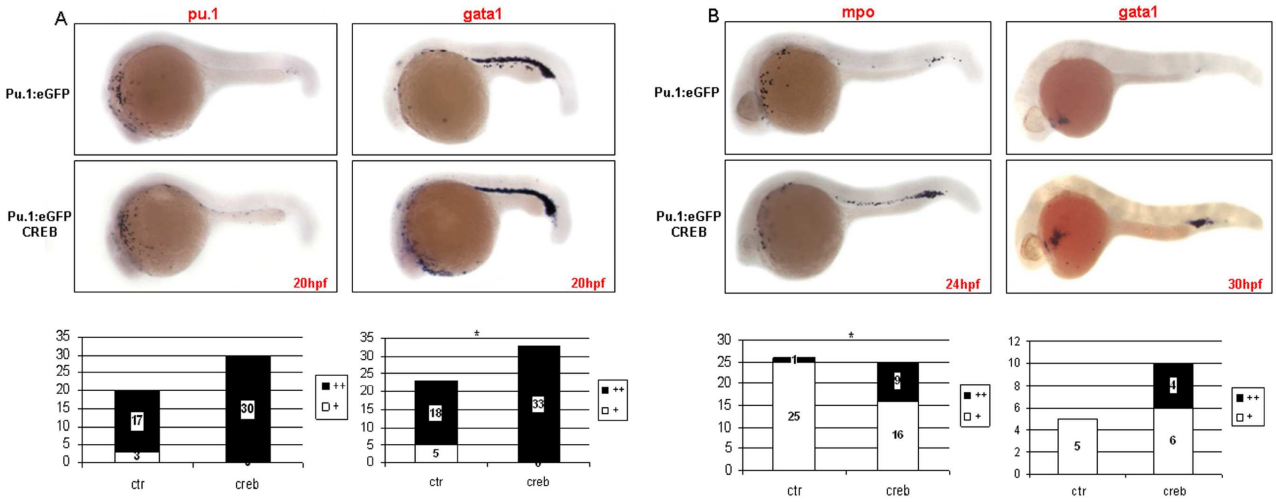


Figure 4. WISH for primitive hematopoietic genes. A) CREB-overexpressing larvae at 20 hpf showed that there are increased number of *pu.1* positive cells: 100 % (n = 30) of CREB-overexpressing embryos compared to 85 % (n = 20) of controls (P= 0.05); significant *gata1* increase was found: strong signal in 100 % (n = 33) of CREB-overexpressing embryos compared to 78 % (n = 23) of control zebrafish (p = 0.009, right panel). B) *mpo* expression at 24hpf showed a significant augmented positive cell number: 36 % of CREB overexpressing zebrafish (n = 25) compared to 4 % (n = 26) of control embryos (p = 0.005, left panel). WISH for *gata1* expression in PBI during transient hematopoiesis at 30hpf showed an increased *gata1* signal in 40% (n = 10) of CREB-overexpressing embryos compared to none of controls (n = 5. p = 0.2, right panel).* $p < 0.05$.

We then performed WISH using specific RNA probes against CREB target genes to evaluate effects on its downstream pathways. At 24 hpf in ALM, CREB-overexpressing zebrafish showed an increased expression of *bcl2* in 17% of embryos (n = 12) compared to 0% of controls (n = 12, p = 0.5, figure 5A, left panel), and of *jun* in 58% of embryos (n = 24) compared to 6% (n = 17) of controls (p < 0.001, figure 5A, right panel). Moreover, when CREB target genes involved in cell cycle control have been considered, we found a detectable expression of *p21* in 24% (n = 21) of CREB-overexpressing embryos, compared to 59% (n = 17) of controls, resulting in a significant decrease of *p21* expression in CREB-overexpressing zebrafish (p = 0.046, figure 5B, left panel), and an detectable *ccnd1* expression in 42% (n = 19) of CREB-overexpressing embryos, with respect to 25% (n = 20) of controls (p = 0.3, figure 5B, right panel). These results suggested a dysregulation of cell cycle towards an enhanced cell proliferation.

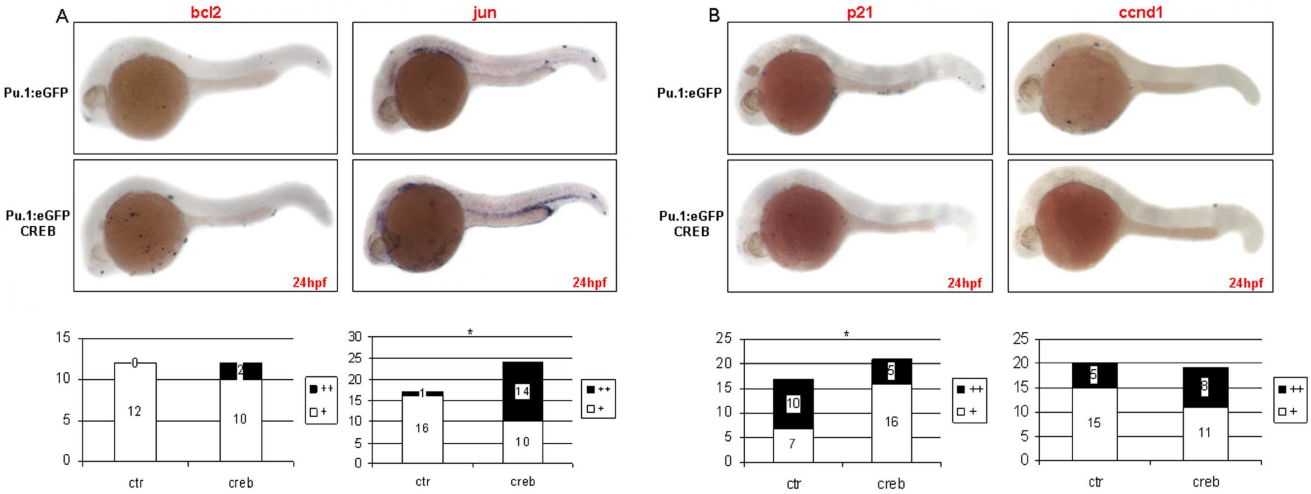


Figure 5. WISH for CREB target genes. A) WISH for *bcl2* and *jun* performed on CREB-overexpressing zebrafish and control ones at 24hpf shows increased *bcl2* signal in 17 % (n = 12) of CREB-overexpressing embryos compared to none (n = 12) of control ones (p = 0.5, left panel), and significant increased *jun* signal: 58 % (n = 24) of CREB-overexpressing embryos compared to 6 % (n = 17) of control ones showed strong *jun* signal. (p < 0.001, right panel). B) WISH for genes involved in cell cycle regulation displays that expression of *p21*, that negatively control G1/S transition, is significantly decreased: its expression is found in 24 % (n = 21) of CREB-overexpressing zebrafish with respect to 59 % (n = 17) of control ones (p = 0.046,left panel); *ccnd1* expression is detected in 42 % (n = 19) of CREB-overexpressing zebrafish compared to 25 % (n = 20) of control embryos (p = 0.3, right panel).* p < 0.05.

CREB alters myelopoiesis during zebrafish development

To observe hematopoietic perturbation during zebrafish development we monitored hematopoietic organs of injected zebrafish during time. First group of 3 CREB-overexpressing and 3 control zebrafish at the age of 3 months were sacrificed. Sagittal sections of paraffined CREB zebrafish did not show morphological abnormalities in kidney marrow characters, neither in other tissues such as muscles, adipose tissue, gills and skin (Figure 6).

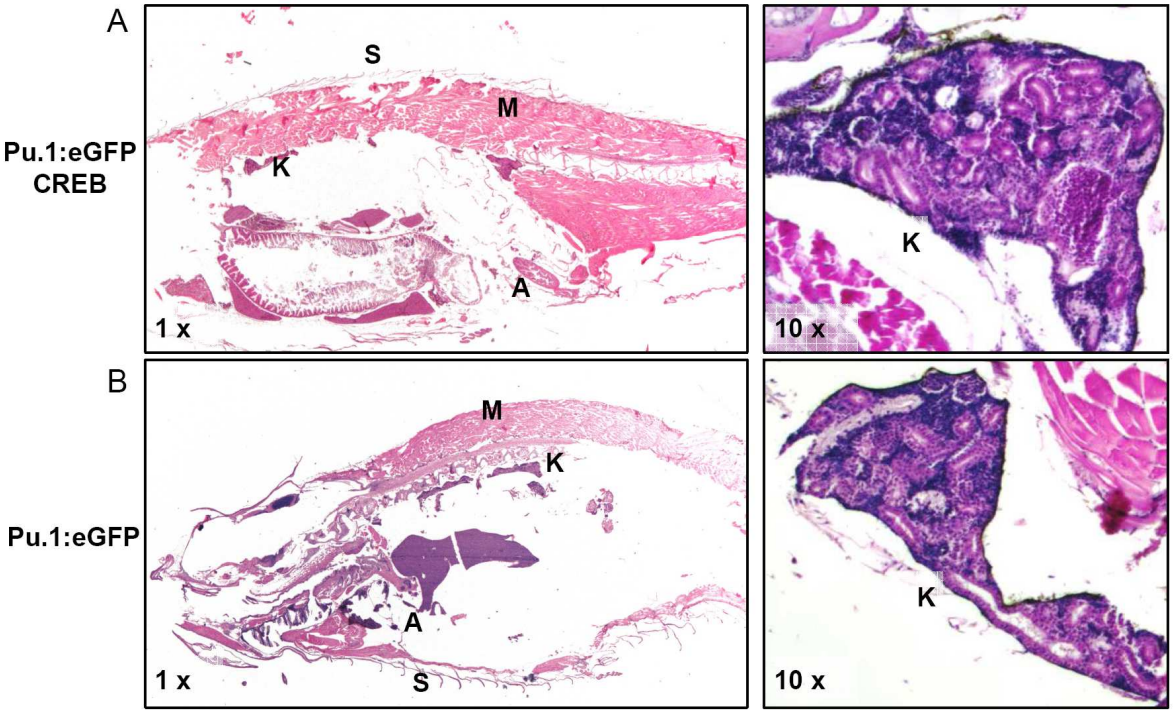


Figure 6. H&E staining of sagittal slides of pu.1:eGFP_CREB and pu.1:eGFP control zebrafish 3 months aged. A) Pu.1:eGFP-injected zebrafish showed normal phenotype at the main organs. B) pu.1:eGFP_CREB-injected zebrafish showed no evident abnormalities in organs size nor tissues. Legend: A: adipose tissue; K: kidney; M: muscle; S: skin.

The second group was sacrificed at the age of 6 months, and kidney dissected. We performed slides and stained for cell morphology analysis. As described in figure 7, pu.1:eGFP_CREB # 12 zebrafish showed significant impairment in number of lymphocytes (22 ± 2%, compared to 41 ± 11 % in control zebrafish, $p = 0.04$) and a significant increase in number of monocytes (22 ± 1% compared to 14 ± 2% in control zebrafish, $p < 0.01$), whereas no significant differences in number of myelocytes (35 ± 6% compared to 26 ± 9% in control zebrafish, $p = 0.22$) and erythroblasts (22 ± 10% compared to 19 ± 4% in control zebrafish, $p = 0.65$) were found. Pu.1:eGFP_CREB # 16 zebrafish showed a significant difference in number of erythroblasts (9 ± 4% compared to 19 ± 4% in control zebrafish, $p = 0.04$), but no significant variation in number of lymphocytes (39 ± 7% compared to 41 ± 11% of control zebrafish, $p = 0.80$), myelocytes count (32 ± 9% compared to 26 ± 9% of control zebrafish, $p = 0.46$) and monocytes count (19 ± 7% compared to 14 ± 2% of control zebrafish, $p = 0.30$) was found (Figure 7 A, B).

A

imprints of kidney from 6 months aged zebrafish	% lymphocytes	% myelocytes	% erythroblasts	% monocytes
pu.1:eGFP	41 ± 11	26 ± 9	19 ±4	14 ± 2
pu.1:eGFP_CREB # 12	22 ± 2	35 ± 6	22 ± 10	22 ± 1
p value	0.04	0.22	0.65	<0.01
pu.1:eGFP_CREB # 16	39 ± 7	32 ± 9	9 ± 4	19 ± 7
p value	0.80	0.46	0.04	0.30

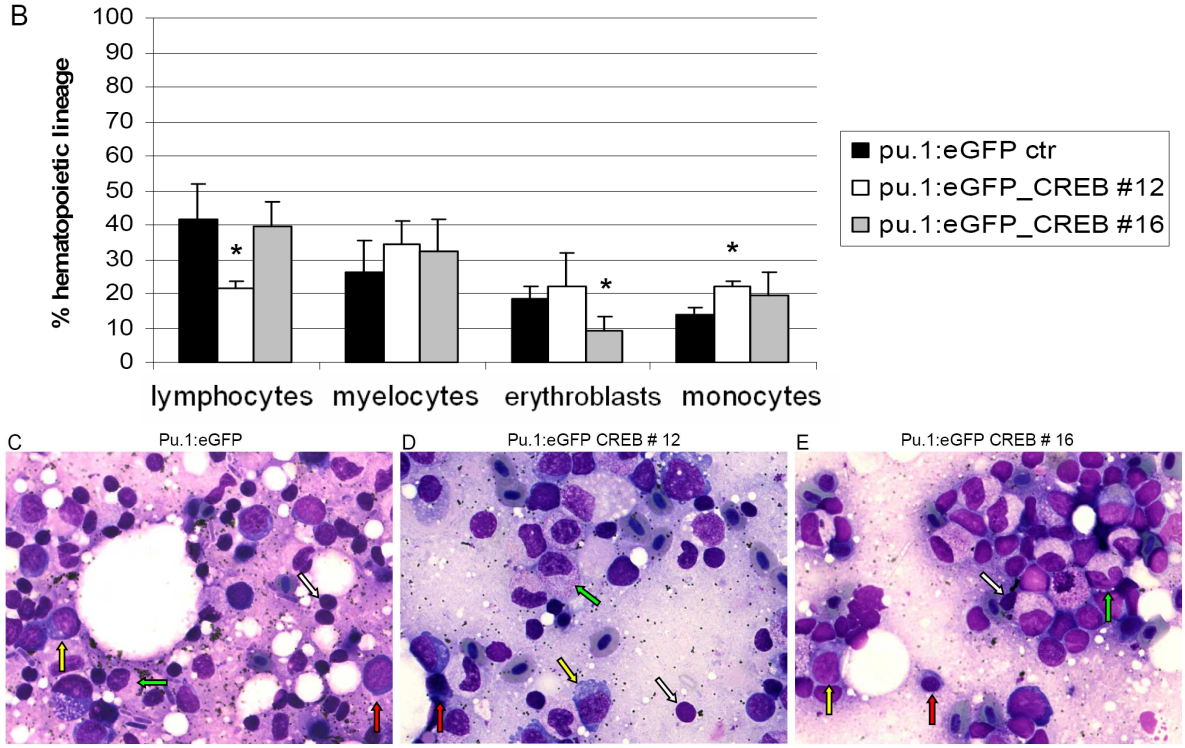
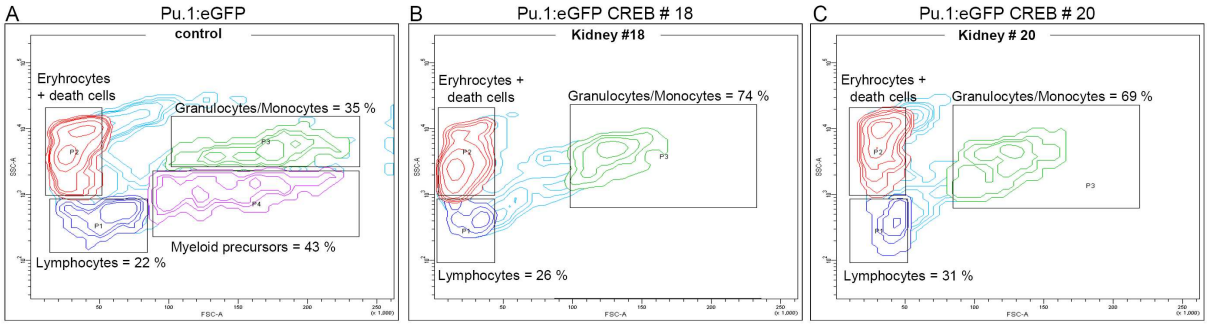


Figure 7. Blood cell count rating of kidney imprints of 6 months aged CREB-overexpressing or control zebrafish. A) Cell counts with standard deviation (n counts = 3). B) Histogram representation of the hematopoietic lineage rate. Bars represent the mean of 3 independent measurement for each sample. * means statistical significant = $p < 0.05$. C) Wright-Giemsa staining of representative slides. Legend: white arrow: lymphocytes; green arrow: myelocytes; red arrow: erythroblasts; yellow arrow: monocytes. 60x original magnification. Pu.1:eGFP control zebrafish, with normal kidney population of lymphocytes, myelocytes, erythroblasts and monocytes. D) Pu.1:eGFP_CREB # 12 showed an unbalanced blood cell count with higher monocytes rate at the expense of lymphoid lineage E) Pu.1:eGFP_CREB # 16 showed an unbalanced blood cell count with an impairment of erythroblasts and a predominance of monocytes and myelocytes.

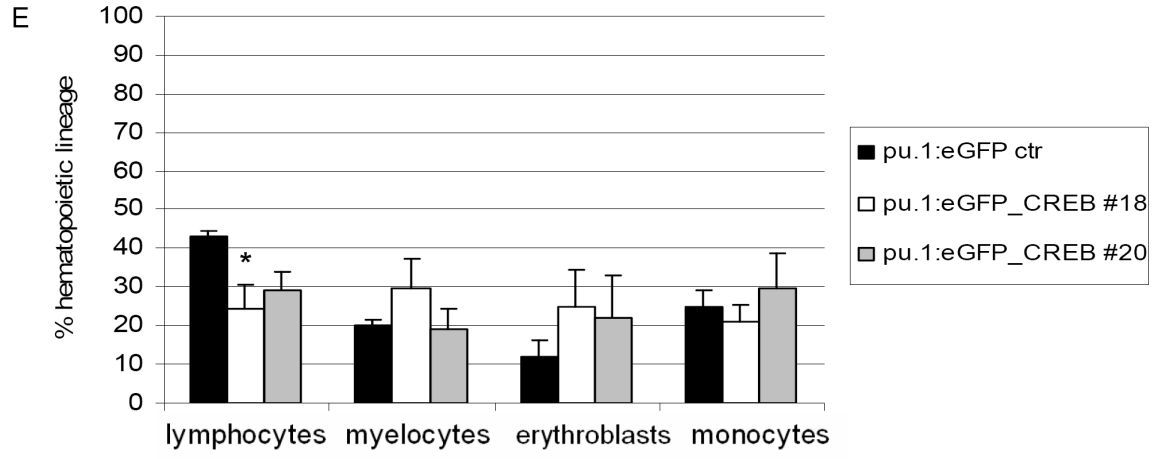
Then, we sacrificed three adult zebrafish 8 months post CREB injection. Sorting of dissociated cells of kidney from control zebrafish revealed that there were 4 distinct population of cells with different physical parameters. In contrast, the same analysis performed in CREB-overexpressing zebrafish showed the absence of the myeloid precursors compartment, with an increase of granulocytes/monocytes rate, confirming an impaired lineage commitment. We considered to exclude mature erythocytes and dead cells, and try to measure the hematopoietic lineages distribution after CREB expression. In particular, pu.1:eGFP_CREB # 18 zebrafish showed that most of cells (74%) were matured into granulocyte/monocytes population. Lymphocytes were similar (28% instead of

22%, figure 8 A, B). pu.1:eGFP_CREB # 20 zebrafish showed the same phenotype, with the 69% of mature granulocyte/monocytes population, whereas lymphocytes rate hasn't found affected (31 % instead of 22 %, figure 8 A, C). These data suggested an aberrant maturation of cells into myeloid lineage. Kidney imprints of CREB-injected zebrafish and same age controls were analyzed for blood cell counts. As illustrated in figure 8, pu.1:eGFP_CREB # 18 zebrafish showed a decrease of lymphocytes (25 ± 6%, compared to 43 ± 1% in control zebrafish, p = 0.01); an increase in number of myelocytes (30 ± 8% compared to 20 ± 1% in control zebrafish, p =0.10), in erythroblasts (29 ± 9% compared to 12 ± 4% in control zebrafish, p = 0.08) even if statistical significance was not reached, and no significant variation in monocytes was found (21 ± 4% compared to 25 ± 4% in control zebrafish, p = 0.29). The same phenomenon of dysmyelopoiesis was observed in the pu.1:eGFP_CREB # 20 zebrafish: a significant lowered number of lymphocytes (29 ± 5% compared to 43 ± 1% of control zebrafish, p = 0.01), an increase in erythroblasts (22 ± 11% compared to 12 ± 4% in control zebrafish, p = 0.21), and no relevant differences in myelocytes (19 ± 6% compared to 20 ± 1% of control zebrafish, p = 0.79), or monocytes (30 ± 9% compared to 25 ± 4% of control zebrafish, p = 0.43, Figure 8 D, E).



D

imprints of kidney from 8 months aged zebrafish	% lymphocytes	% myelocytes	% erythroblasts	% monocytes
pu.1:eGFP	43 ± 1	20 ± 1	12 ± 4	25 ± 4
pu.1:eGFP_CREB # 18	25 ± 6	30 ± 8	29 ± 9	21 ± 4
p value	0.01	0.10	0.08	0.29
pu.1:eGFP_CREB # 20	29 ± 5	19 ± 6	22 ± 11	30 ± 9
p value	0.01	0.79	0.21	0.43



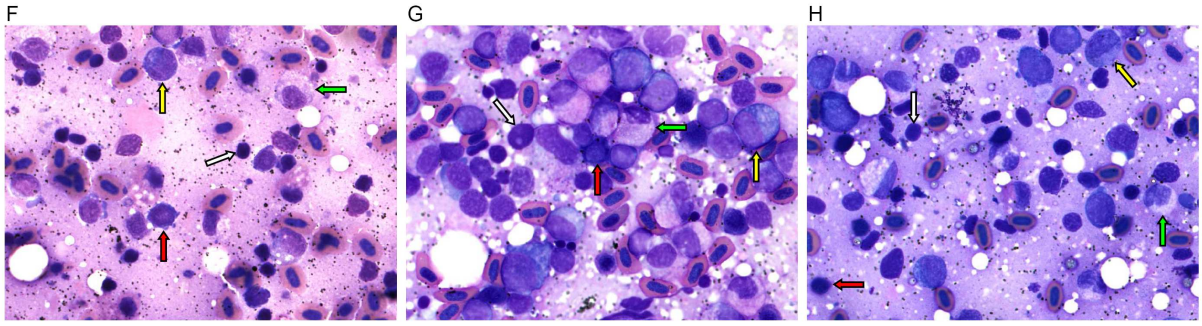


Figure 8. Blood cell analysis of 8 months aged CREB-overexpressing or control zebrafish kidney. A) Sorting plot of dissociated cells of kidney from control zebrafish B) and C) Sorting plot of dissociated cells of kidney from CREB-overexpressing embryos D) table of percentage of the diverse hematopoietic lineage cell number (n = 3). E) Histogram representation of the different hematopoietic lineages. Bars represents the mean of 3 independent measurement for each sample. * means statistical significance = $p < 0.05$ Representative pictures of Wright-Giemsa staining of 8 months aged zebrafish kidney imprints. F) pu.1:eGFP control zebrafish with normal rate of lymphocytes, myelocytes, erythroblasts and monocytes. G and H) pu.1:eGFP_CREB # 18 and pu.1:eGFP_CREB # 20 showed an increase in myeloid, eryhroid and monocytic lineages at the expense of a lowered lymphocytes number. Legend: white arrow: lymphocytes; green arrow: myelocytes; red arrow: erythroblasts; yellow arrow: monocytes. 60x original magnification.

Furthermore, the third zebrafish pu.1:eGFP_CREB # 19 sacrificed at 8 months from injection, revealed an acute lymphoblastic leukemia with a monomorphous cell population infiltration (Figure 9 C). Sorting of dissociated cells from kidney showed 90% of lymphocytes, whereas in the control zebrafish all the hematologic lineages were represented (22% lymphocytes, 35 % granulocytes/monocytes and 43 % myeloid precursors populations, figure 9 A, B).

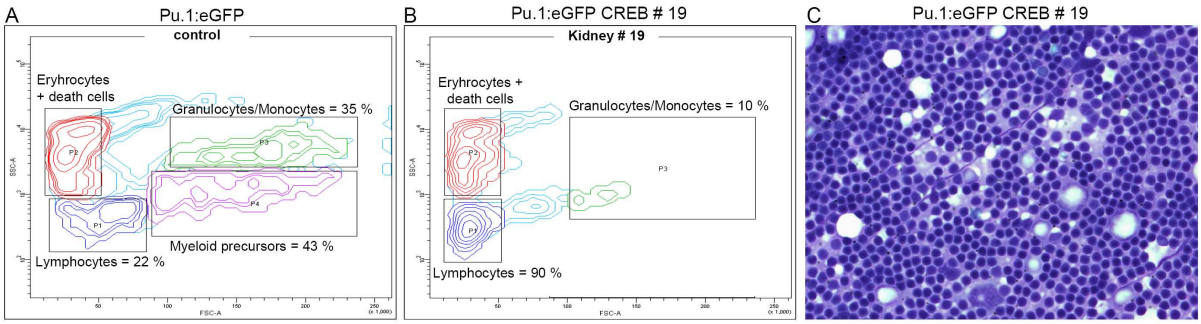


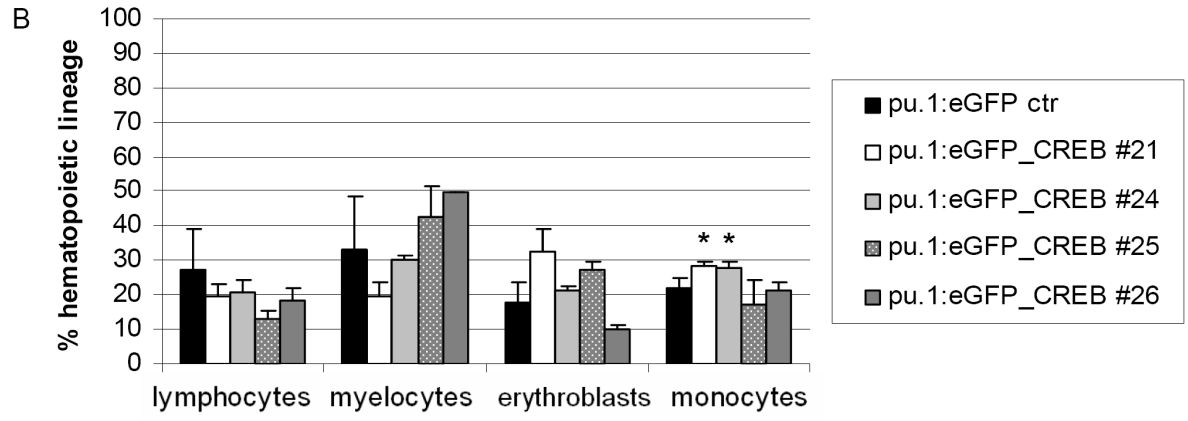
Figure 9. Blood cell analysis of 8 months aged CREB-overexpressing or control zebrafish kidney. A) Sorting plot of dissociated cells of kidney from control zebrafish B) Sorting plot of dissociated cells of kidney from pu.1:eGFP_CREB # 19 embryo. C) Representative pictures of Wright-Giemsa staining of 8 months aged zebrafish kidney imprints. Kidney showed lymphoblasts totality, with the complete absence of the rest of hematopoietic cell lineages. 60x magnification.

Giving that zebrafish were developing a hematopoietic disorder,, we considered to monitor our adult injected zebrafish cohort more frequently. We sacrificed four zebrafish at 9 month of age. Kidney imprints from CREB-overexpressing zebrafish and controls were analyzed for blood cell counts. The pu.1:eGFP_CREB # 21 presented a significant increase in monocytes rate (28 ± 2%, compared to 22 ± 3% in control zebrafish, p = 0.04), as well as in erythroblasts (32 ± 7% compared to 18 ± 6% in control zebrafish, p = 0.06), whereas myelocytes and lymphocytes number was slightly decreased (20 ± 4% compared to 33 ± 15% in control zebrafish, p = 0.22 and 20 ± 3% compared to 27 ± 12% in control zebrafish, p =0.38, respectively). Pu.1:eGFP_CREB # 24 zebrafish showed a significative increase in number of monocytes (28 ± 1% compared to 22 ± 3% in control zebrafish, p = 0.03), but not significant dissimilarities in number of lymphocytes (21 ± 4% compared to 27 ± 12% in control zebrafish, p =0.46), myelocytes (30 ± 1% compared to 33 ± 15% in control zebrafish, p = 0.75) and erythroblasts (22 ± 1% compared to 18 ± 6% in control zebrafish, p = 0.32). Pu.1:eGFP_CREB # 25 and Pu.1:eGFP_CREB # 26 zebrafish showed no significant differences in any hematopoietic lineages even if a trend towards an increase in myeloid cell amount was observed (# 25 = lymphocytes 13 ± 3% compared to 27 ± 12% in control zebrafish, p =0.12; myelocytes 43 ± 9% compared to 33 ± 15% in control zebrafish, p = 0.38; erythroblasts 27 ± 3% compared to 18 ± 6% in control zebrafish, p = 0.08; monocytes 17 ± 7% compared to 22 ± 3% in control zebrafish, p = 0.32. # 26 = lymphocytes 9 ± 4% compared to 27 ± 12% in control zebrafish, p =0.33; myelocytes 50 ± 2 % compared to 33 ± 15% in control zebrafish, p = 0.12; erythroblasts was 10 ± 1% compared to 18 ± 6% in control zebrafish, p = 0.09; monocytes 22 ± 2% compared to 22 ± 3% in control zebrafish, p = 1, Figure 10).

A

imprints of kidney from 9 months aged zebrafish	% lymphocytes	% myelocytes	% erythroblasts	% monocytes
pu.1:eGFP	27 ± 12	33 ± 15	18 ± 6	22 ± 3
pu.1:eGFP_CREB # 21	20 ± 3	20 ± 4	32 ± 7	28 ± 2
p value	0.38	0.22	0.06	0.04
pu.1:eGFP_CREB # 24	21 ± 4	30 ± 1	22 ± 1	28 ±1
p value	0.46	0.75	0.32	0.03
pu.1:eGFP_CREB # 25	13 ± 3	43 ± 9	27 ± 3	17 ± 7
p value	0.12	0.38	0.08	0.32
pu.1:eGFP_CREB # 26	19 ± 4	50 ± 0	10 ± 1	22 ± 2
p value	0.33	0.12	0.09	1

B



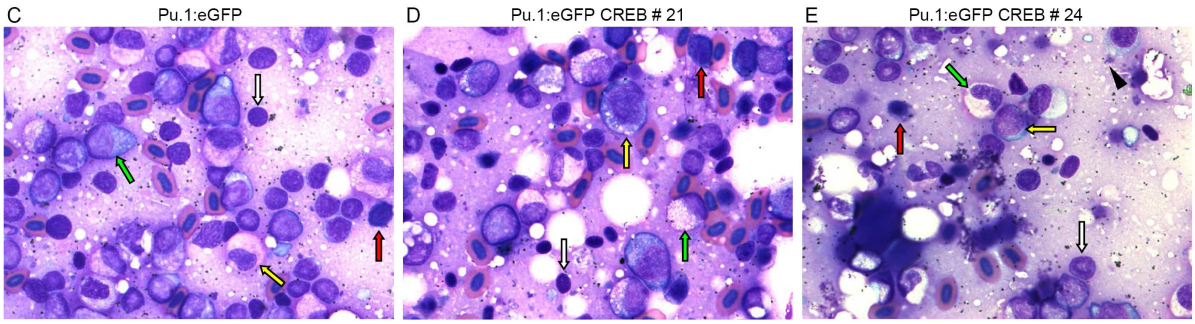


Figure 10. Blood cell count rating of kidney imprints of 9 months aged CREB-overexpressing and control zebrafish. A) percentage of the diverse hematopoietic lineage cells. B) Histogram representation of the hematopoietic lineages. Bars represents the mean of 3 independent measurement for each sample. * means statistical significance = $p < 0.05$. Representative pictures of Wright-Giemsa staining of 9 months aged zebrafish kidney imprints. C) pu.1:eGFP control zebrafish showed normal rate of lymphocytes, myelocytes, erythroblasts and monocytes. D and E) pu.1:eGFP_CREB # 21 and pu.1:eGFP_CREB # 24 showed an unbalanced blood cell count with higher monocytes number. Legend: white arrow: lymphocytes; green arrow: myelocytes; red arrow: erythroblasts; yellow arrow: monocytes. 60x original magnification.

CREB induces myeloproliferative neoplasms in zebrafish

9 months post CREB injection we observed atypical zebrafish phenotypes and behaviour. From 9 to 14 months, ten CREB-injected zebrafish with abnormal phenotype presented an evident

abdominal/dorsal mass. In particular, 4 zebrafish were found dead, and 6 zebrafish were sacrificed because they presented a sick phenotype (Figure 11).

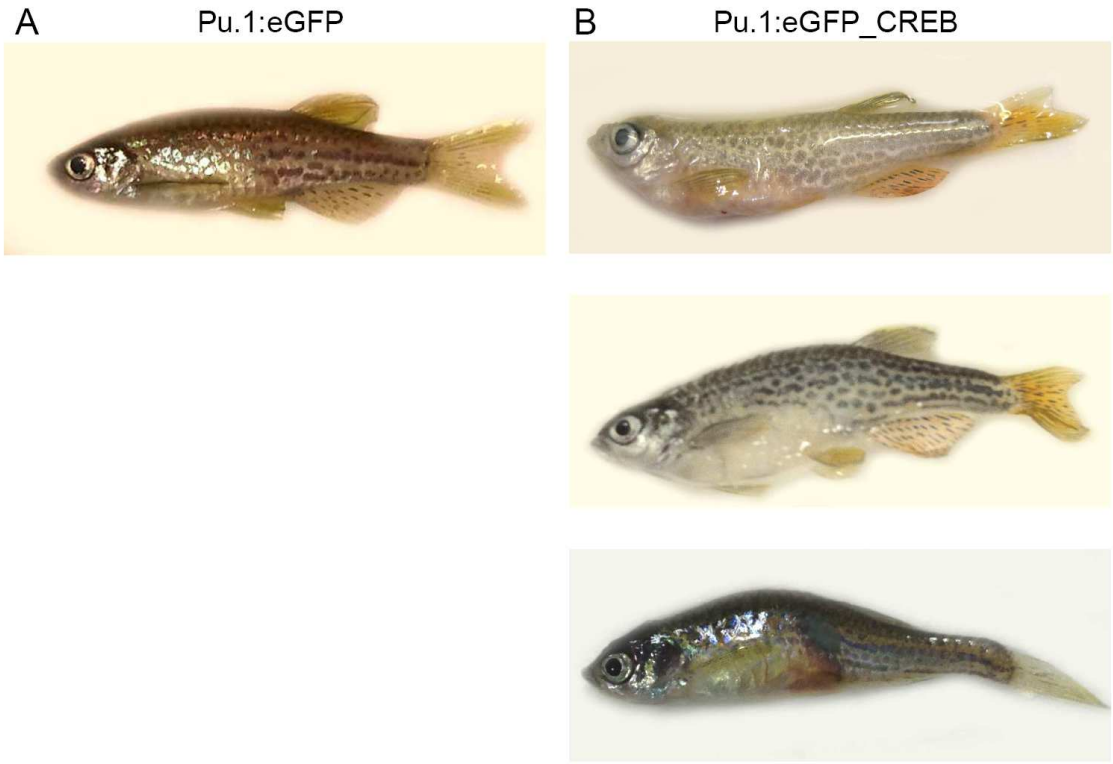


Figure 11. Abnormal phenotypes developed in pu.1:eGFP_CREB zebrafish after a latency period. A) pu.1:eGFP injected zebrafish showed a normal phenotype. B) pu.1:eGFP_CREB injected zebrafish showed atypical phenotypes with abdominal or dorsal prominence.

To investigate about the nature of the observed mass, we sectioned the paraffine-embedded zebrafish. Whole fish histological sagittal sections stained with H&E revealed an evident abdominal mass composed by clonal cells, with open chromatin texture (Figure 12 A, B). In particular, we noted infiltrations of clonal myeloid cells presenting the same morphology of the mass in gills, muscles and adipose tissue (Figure 12F-L).

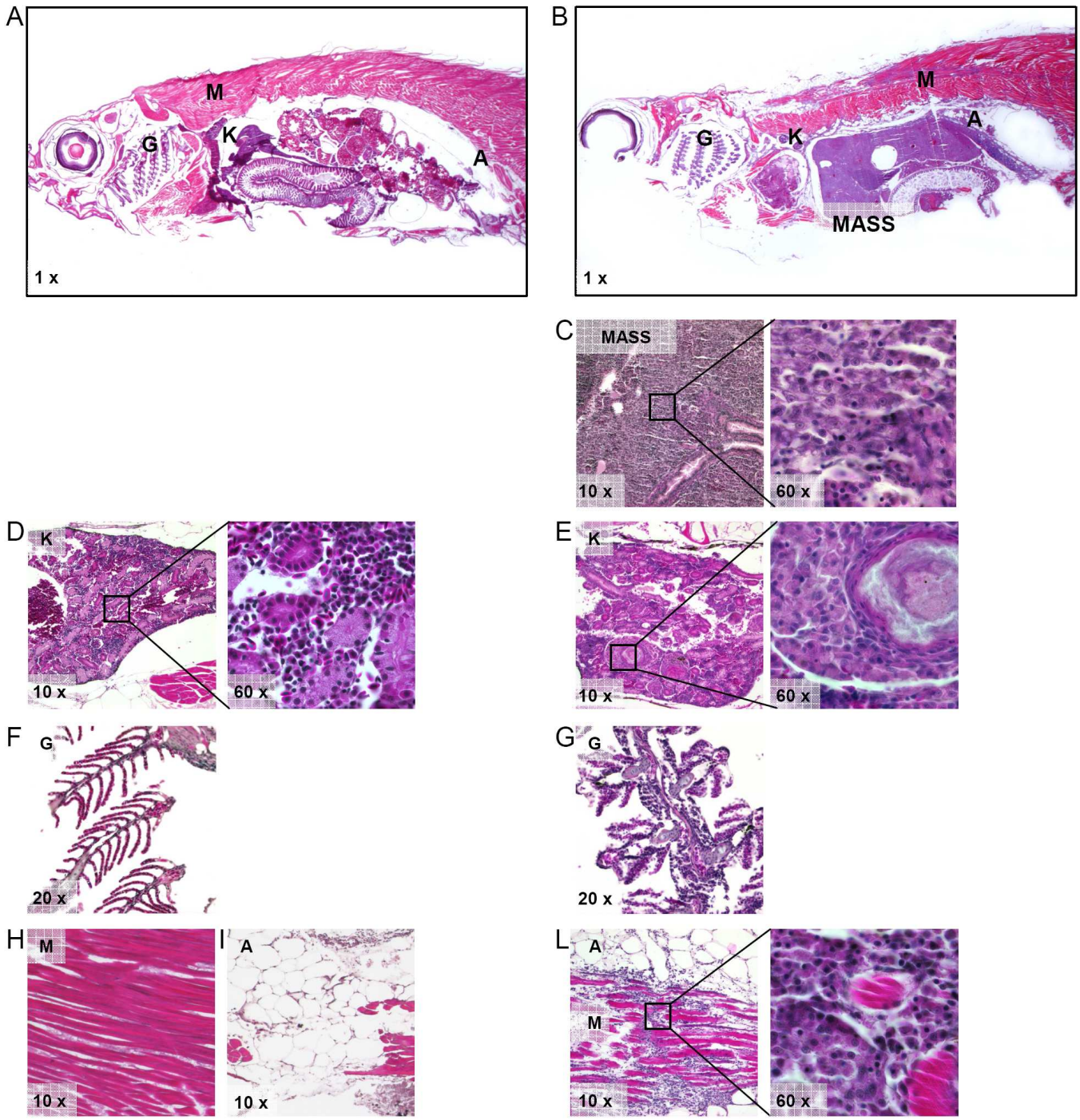


Figure 12. H&E histochemical staining of paraffined sections of pu.1:eGFP and pu.1:eGFP_CREB. A) H&E staining of a sagittal section of 1 year aged pu.1:eGFP injected zebrafish. Regular organs and tissues were evident. B) H&E staining of a sagittal section of 1 year aged pu.1:eGFP_CREB injected zebrafish revealed a prominent mass that filled the abdominal cavity. C) 10x and higher 60 x original magnification of the mass found in CREB-overexpressing zebrafish showed clonal cells with open chromatin texture. The presence of cells throughout gills, muscles and adipose tissues. D) higher magnification of kidney of the control fish showed a normal tubular structure, with hematopoietic cells within the proper kidney tissue. E) higher magnification of kidney of the CREB-overexpressing fish revealed a disorganized structure, with the presence of blobs containing clonal cells with open chromatin texture. F) 20x original magnification of gills of the control fish showed a regular shape. G) 20x original magnification of gills of the CREB-overexpressing zebrafish revealed a disrupted gills shape, with infiltration of invading cells. H) muscles of control fish showed normal fibers, with no cells between fibers. I) adipose tissue of control zebrafish presenting a regular morphology without cells infiltration within adipose

cells. L) muscle and adipose tissue of CREB-overexpressing zebrafish revealed high infiltration degree of invading cells, which colonized muscle fibers and interstice within adipose cells. High magnification showed the same cell type as those found in the abdominal mass (C).

We found that the clonal abnormal infiltrating cells of the abdominal mass were derived from myeloid eGFP-CREB expressing cells by immunohistochemical assay for CREB and for GFP, that were expressed throughout the mass. Moreover, we revealed that these cells were highly proliferating because of strong positivity of the PCNA antibody (Figure 13).

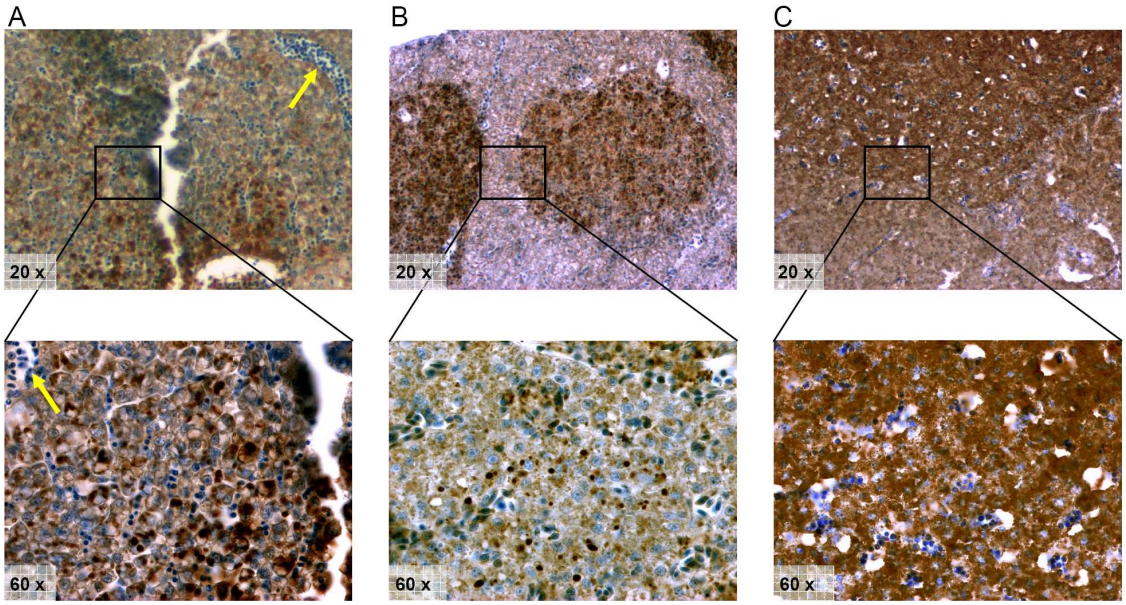


Figure 13. Characterization of abdominal mass cells by IHC. A) IHC for CREB showed a strong positivity of infiltrating cells, opposite of vessel forming erythrocytes, which were negative stained (yellow arrow). B) IHC for GFP showed a high positivity of mass cells. C) IHC for PCNA revealed a broad positive signal throughout the mass. 60 x original magnification.

To further investigate the nature of these aberrant cells, mass cell sorting has been performed. Based on forward scattering (FSC) and side scattering (SSC) physical parameters, the mass forming cells were localized in the mono-myeloid compartment. Cytospin of these cells was done, and by Wright-Giemsa staining we proved that the totality of cells were monocytes. Moreover, most of sorted cells were in proliferation, as pointed out by chromatin condensation in the nucleus or by the presence of just divided cells (data not shown).

To confirm that mass forming cells were monocytes, and to explore if the kidney marrow of sick CREB-overexpressing zebrafish may have a role in the origin of the mass, we performed a series of imprints of abdominal mass and kidney of sick zebrafish and stained them with Wright-Giemsa. By microscope we verified that both, the mass and the kidney cells, had monocytic features, with basophilic cytoplasm and eccentric nucleus. Kidney marrow was completely populated by monocytes, to the detriment of other hematopoietic lineages. To examine the magnitude of this imbalance, blood cell counts of the imprints from kidney and mass of sick CREB-overexpressing zebrafish and controls were executed. As illustrated in figure 14, kidney and mass of pu.1:eGFP_CREB zebrafish showed no

variation in lymphocytes number (31 ± 4% and 32 ± 8% compared to 30 ± 2% in control zebrafish, p = 0.76 and p = 0.70, respectively) and a high significant impairment of myelocytes (1 ± 1% and 3 ± 1% compared to 26 ± 1% in control zebrafish, both p < 0.01). Erytroblasts showed to be increased in sick kidney, whereas decreased in the mass (38 ± 4% in kidney of CREB-overexpressing zebrafish, and 6 ± 6% in abdominal mass compared to 26 ± 1% in control zebrafish, p = 0.05 and p = 0.01 respectively). Monocytes revealed a huge increase, confirming to be almost the unique cell type in the abdominal mass (32 ± 6% in kidney of CREB-overexpressing zebrafish, and 60 ± 14% in abdominal mass compared to 19 ±1 % in control zebrafish, p = 0.10 and p < 0.01, respectively).

A

imprints of kidney from 1 year aged zebrafish	% lymphocytes	% myelocytes	% erythroblasts	% monocytes
pu.1:eGFP	30 ± 2	26 ± 1	26 ± 1	19 ± 1
kidney pu.1:eGFP_CREB # 8	31 ± 4	1 ± 1	38 ± 4	32 ± 6
p value	0.76	<0.01	0.05	0.10
abdominal mass pu.1:eGFP_CREB # 8	32 ± 8	3 ± 1	6 ± 6	60 ±14
p value	0.70	<0.01	<0.01	<0.01

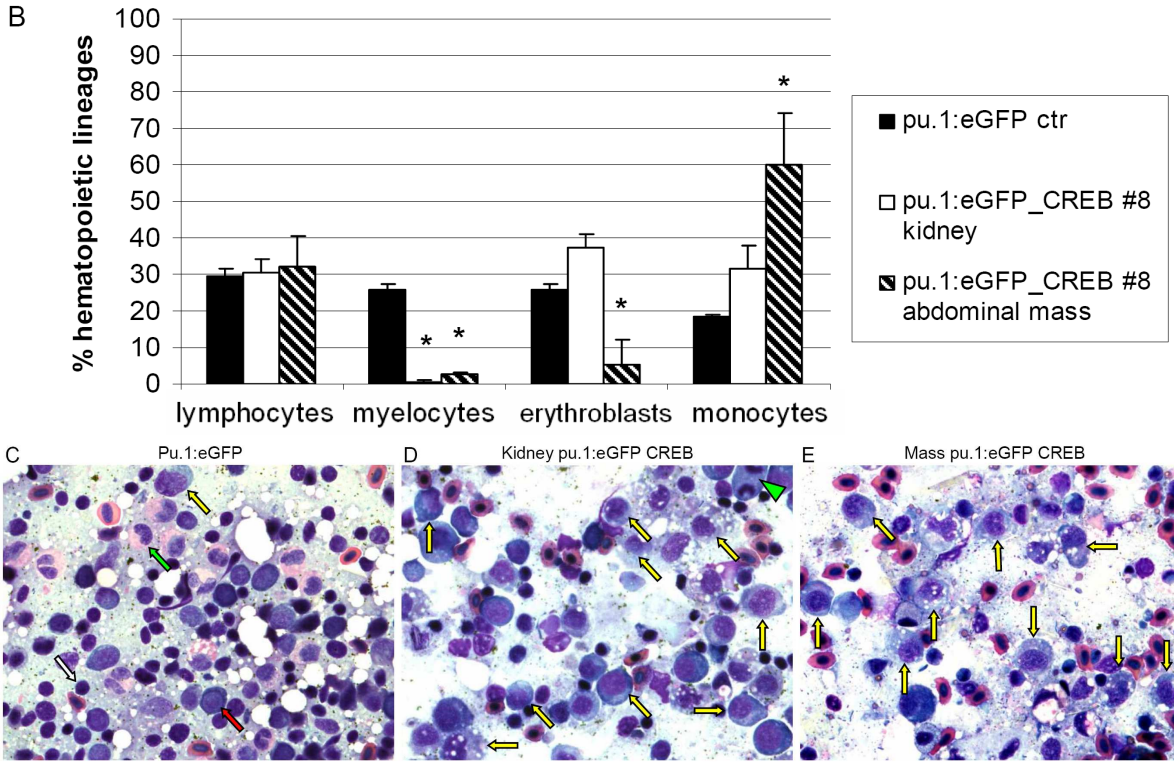


Figure 14. Blood cell count of kidney imprints of 1 year aged sick CREB-overexpressing or control zebrafish and representative pictures of Wright-Giemsa staining of 1 year aged zebrafish kidney imprints. A) Table of percentage of the diverse hematopoietic lineage cell number (n = 3). B) Histogram representation of the different hematopoietic lineages. Bars represents the mean of 3 independent measurement for each sample. * means statistical significance = p < 0.05. C) pu.1:eGFP control zebrafish with normal rate of lymphocytes, myelocytes, erythroblasts and monocytes. D and E) kidney and mass of sick pu.1:eGFP_CREB showed disrupted hematopoietic lineage balance, with a predominance of monocytes at the

expense of myeloid cells. CREB-overexpressing kidney imprint showed cells in mitosis phase (green arrowhead). Legend: white arrow: lymphocytes; green arrow: myelocytes; red arrow: erythroblasts; yellow arrow: monocytes. 60x original magnification.

We further characterized that the mass infiltration was composed principally of monocytes for their high activity of alpha-naphthyl acetate esterase (ANAE) as shown in Figure 15.

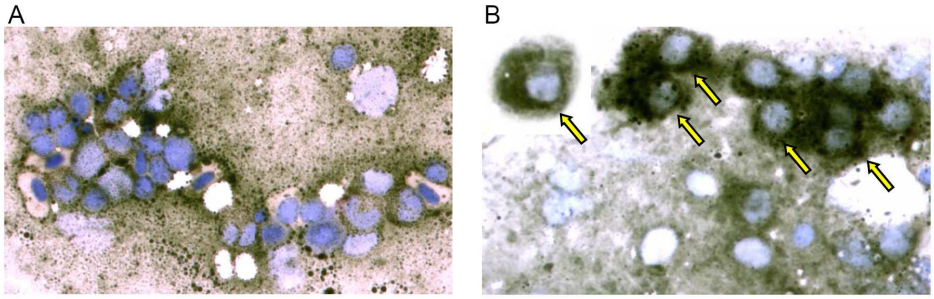


Figure 15. ANAE assay on imprints of control zebrafih kidney and CREB zebrafish mass. A) ANAE staining was positive in few cells (monocytes) due to heterogeneity of the normal kidney marrow. B) ANAE staining on mass imprint showed strong positive activity into cytoplasm of the most of cells, being monocytes. 60 x original magnification.

DISCUSSION

CREB has been found to be overexpressed in pediatric and adult acute myeloid leukemia. It played a role in abnormal cell proliferation, cell cycle progression and conferred high clonogenic potential *in vitro* and *in vivo*. Since it was established to be a proto-oncogene in AML, the molecular pathway of transformation has not been identified yet [6, 7].

Here, the enforced CREB overexpression in myeloid lineage of zebrafish embryos provided evidence that it enhanced an aberrant myelopoiesis since 20 hpf, leading to a myeloproliferative disease in the adult zebrafish. Therefore we revealed that CREB overexpression mediates a myeloid disease in zebrafish model, as previously described in human cell lines and mice [6, 7].

Zebrafish Creb1 shared 88.7% identity with human CREB gene, including the functional domains [15]. We demonstrated, that CREB worked as a transcription factor in zebrafish myeloid cells increasing expression of genes leading to the perturbation of primitive hematopoiesis. The analysis of CREB target genes expression, that were previously found to be aberrantly induced upon CREB higher expression in human bone marrow cells [22], revealed that there was a trend towards upregulation of genes that positively control proliferation, in contrast to a down-regulation of genes that inhibit cell cycle progression. *Ccna1* expression, which controls advance in cell cycle and is already known to be expressed in several leukemic cell lines, as well as *ccnd1* expression, which normally regulates cell cycle G1/S transition and whose overexpression was observed in a variety of tumors, were found more expressed in CREB-injected zebrafish. On the contrary, *p21*, which normally encodes a cyclin-dependent kinase inhibitor protein functioning as a negative regulator of cell cycle progression at G1 advance signals [25], was found decreased in CREB-overexpressing zebrafish. Other genes involved in leukemia, such as *stat3, gata2 and bcl-2* were found upregulated by CREB overexpression. Despite this perturbation of gene expression, zebrafish at larvae stage did not show aberrant phenotypes. Then, when we monitored the effects of prolonged CREB overexpression in myeloid lineage, we revealed that from 6 months after CREB plasmid injection, the kidney marrow of adult zebrafish had increased the myeloid and monocytic compartment, at the expense of the lymphoid one. This finding was in agreement with previous studies *in vitro* and *in vivo*, where enforced CREB expression promoted an increased myeloid cell proliferation and survival *in vitro*, as well as an aberrant myelopoiesis in both the bone marrow and spleen of transgenic mice that recapitulated a myeloproliferative disease after 1 year [6].

Starting from 9 months after injection, CREB-overexpressing zebrafish developed a sick phenotype, with an evident abdominal and dorsal swelling. The animals died with a latency period that was variable from 9 to 15 months, suggesting that further events may occur to take to disease. The analysis of sick animals revealed the presence of a mass composed by clonal cells, with large nucleus and open chromatin texture characters that resemble cancer cells. These features caused infiltration of mass-like cells throughout the zebrafish tissues and organs that ultimately led to fish death. By morphological analysis, we found that tumor cells expressed eGFP_CREB and therefore derived from

the kidney marrow. They acquired the ability to rapidly expand due to they high proliferation rate and to migrate. The paired mass and kidney showed to have the same cell morphology, such as a complete absence of myelocytes opposed to an overt preponderance of monocytes, disclosing a severe disrupted myelopoiesis.

Nevertheless, these results supported that CREB overexpression in myeloid compartment might be considered a driving force of cell cycle dysregulation and proliferation advantages, that when subjected to additional hits is able to induce myeloid transformation. Here, we described one single adult CREB-injected zebrafish developing an acute lymphoblastic leukemia (ALL), characterized by a total lymphocytes invasion of the kidney marrow. In fact, *pu.1* promoter has been used to drive expression specifically in early myeloid cells [13, 14, 16, 26], it also plays a pivotal role in the differentiation of lymphoid cells, and it has been found expressed in most of the hematopoietic cells including B lymphocytes, macrophages, neutrophils, mast cells, early erythroid and megakaryocytes [27, 28]. Of relevance, CREB was found to be overexpressed in both adult and pediatric ALL as well [6, 7]. This result, supported our hypothesis that CREB is a proto-oncogene, and additional events and the time of occurrence may have a crucial role in causing different cell transformation.

Finally, we recapitulated a multistep model for CREB-associated myeloid disorders, where CREB overexpression triggered proliferation advantages and gene expression alteration that conferred pre-oncogenic features to clones that in turn, by accumulating mutations and/or epigenetic changes, took to hematologic disease. This is the first evidence that a zebrafish model may constitute a valid model to test the role of CREB in leukemogenesis as well as the possibility to test new therapeutic molecules associated to hematopoietic/leukemic disease. The further identification of CREB overexpression driven pathways may unravel the leukemic process, and identified new targets that, together with new compounds directed to block CREB-transcriptional activation [29, 30], would open for novel therapeutic opportunities.

REFERENCES

1. Dash, A. and D.G. Gilliland, Molecular genetics of acute myeloid leukaemia. Best Pract Res Clin Haematol, 2001. 14(1): p. 49-64.
2. Pui, C.H., et al., Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol, 2011. 29(5): p. 551-65.
3. Sandoval, S., M. Pigazzi, and K.M. Sakamoto, CREB: A Key Regulator of Normal and Neoplastic Hematopoiesis. Adv Hematol, 2009. 2009: p. 634292.
4. Shaywitz, A.J. and M.E. Greenberg, CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem, 1999. 68: p. 821-61.
5. Impey, S., et al., Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell, 2004. 119(7): p. 1041-54.
6. Shankar, D.B., et al., The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell, 2005. 7(4): p. 351-62.
7. Pigazzi, M., et al., cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression. Haematologica, 2007. 92(10): p. 1435-7.
8. Hsia, N. and L.I. Zon, Transcriptional regulation of hematopoietic stem cell development in zebrafish. Exp Hematol, 2005. 33(9): p. 1007-14.
9. Driever, W. and M.C. Fishman, The zebrafish: heritable disorders in transparent embryos. J Clin Invest, 1996. 97(8): p. 1788-94.
10. Langenau, D.M., et al., Myc-induced T cell leukemia in transgenic zebrafish. Science, 2003. 299(5608): p. 887-90.
11. Bertrand, J.Y. and D. Traver, Hematopoietic cell development in the zebrafish embryo. Curr Opin Hematol, 2009. 16(4): p. 243-8.
12. Payne, E. and T. Look, Zebrafish modelling of leukaemias. Br J Haematol, 2009. 146(3): p. 247-56.
13. Zhuravleva, J., et al., MOZ/TIF2-induced acute myeloid leukaemia in transgenic fish. Br J Haematol, 2008. 143(3): p. 378-82.
14. Forrester, A.M., et al., NUP98-HOXA9-transgenic zebrafish develop a myeloproliferative neoplasm and provide new insight into mechanisms of myeloid leukaemogenesis. Br J Haematol, 2011. 155(2): p. 167-81.
15. Dworkin, S., et al., CREB activity modulates neural cell proliferation, midbrain-hindbrain organization and patterning in zebrafish. Dev Biol, 2007. 307(1): p. 127-41.
16. Hsu, K., et al., The pu.1 promoter drives myeloid gene expression in zebrafish. Blood, 2004. 104(5): p. 1291-7.
17. Kwan, K.M., et al., The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn, 2007. 236(11): p. 3088-99.
18. Chen, J., et al., Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish. Genes Dev, 2005. 19(23): p. 2900-11.
19. Bessa, J., et al., meis1 regulates cyclin D1 and c-myc expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye. Development, 2008. 135(5): p. 799-803.
20. Kimmel, C.B., et al., Stages of embryonic development of the zebrafish. Dev Dyn, 1995. 203(3): p. 253-310.
21. Thisse, C., et al., Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development, 1993. 119(4): p. 1203-15.
22. Pigazzi, M., et al., ICER expression inhibits leukemia phenotype and controls tumor progression. Leukemia, 2008. 22(12): p. 2217-25.
23. Chen, A.T. and L.I. Zon, Zebrafish blood stem cells. J Cell Biochem, 2009. 108(1): p. 35-42.
24. Pigazzi, M., et al., MiR-34b promoter hypermethylation induces CREB overexpression and contributes to myeloid transformation. Haematologica, 2012.
25. Coqueret, O., New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol, 2003. 13(2): p. 65-70.
26. Onnebo, S.M., et al., Hematopoietic perturbation in zebrafish expressing a tel-jak2a fusion. Exp Hematol, 2005. 33(2): p. 182-8.
27. Lloberas, J., C. Soler, and A. Celada, The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. Immunol Today, 1999. 20(4): p. 184-9.
28. Klemsz, M.J., et al., The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell, 1990. 61(1): p. 113-24.
29. Best, J.L., et al., Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. Proc Natl Acad Sci U S A, 2004. 101(51): p. 17622-7.
30. Zhao, J., et al., P6981, an arylstibonic acid, is a novel low nanomolar inhibitor of cAMP response element-binding protein binding to DNA. Mol Pharmacol, 2012. 82(5): p. 814-23.

CHAPTER 5

CONCLUSIONS

During my PhD, we definitively ascertained that CREB was a proto-oncogene, and for this reason we considered the possibility that targeting its expression might have great relevance in leukemia therapy.

We firstly verified the effects that CREB levels reduction might have on leukemia cell proliferation and disease progression. The first hypothesis that we formulated was that ICER, the endogenous CREB competitor able to recognize CRE sequences and stop its transcriptional activity, was maintained at low levels in AML. This led to CREB overexpression and to the aberrant activation of its targets in order to sustain leukemia. So, we restored ICER expression in leukemic cell lines and in AML primary cultures, and verified that CREB protein levels decreased together with its target genes expression. We then considered identifying the mechanism that was activated by this modification. We unveiled that ICER restoration had the main effect on two particular target genes: the dual-specificity phosphatases, DUSP1 and DUSP4. DUSP1 and DUSP4, directly repressed by ICER, activated the p38 apoptotic mediated pathway, which in turn, triggered caspase-dependent apoptosis. Being ICER restoration involved in leukemia cell death, we considered testing its role in drug sensitivity, and we revealed that when ICER was restored, it enhanced the chemotherapeutic response. Finally, we revealed that ICER downregulation in AML had a crucial role in sustaining leukemia proliferation, and that ICER may be considered a tumor-supressor to be further validated. We also identified that ICER restoration mediated chemotherapy anticancer activity and drove leukemia cell program from survival to apoptosis. It is reasonable that the strategy to target CREB overexpression through ICER may improve conventional chemotherapy and inhibit leukemia progression.

Next, we aimed to understand the cause of CREB overexpression in AML. It was previously shown that CREB overexpression was dependent on miR-34b, a micro RNA which physiologically targets the 3'UTR of CREB gene. We revealed that miR-34b expression was maintained low in AML to support CREB overexpression. We dissected that miR-34b promoter was hypermethylated in AML cell lines, causing miR-34b downregulation and in turn CREB overexpression, as well as in patients diagnosed with AML. Then, we found that miR-34b was upregulated (since it was not hypermethylated at its promoter) in pediatric patients affected by preleukemic hematopoietic disease as juvenile myelomonocytic leukemia (JMML) and myelodysplastic syndrome (MDS) with the concomitant low CREB expression. In paired samples of myelodysplastic syndromes and subsequent acute myeloid leukemia from the same patients, we confirmed miR-34b promoter hypermethylation at leukemia onset, with a strong CREB target genes dataset differentially expressed between the two disease stages. This subset of CREB targets was confirmed to associate with high risk myelodysplastic syndrome. These results confirmed that miR-34b played a crucial role on CREB expression controlling myeloid transformation. To support this hypothesis we evaluated miR-34b tumor

suppressor potential *in vitro* and *in vivo*, finding that miR-34b restoration decreased AML engraftment and progression. Finally, we point out the possibility of evaluating the miR-34b as a therapeutic agent to be tested in an *in vivo* model,. This is supported by recent experimental evidences which demonstrated that correction of specific miRNA alterations using miRNA mimics or antagomirs, vehiculated both as short double stranded oligonucleotides or in form of viral vectors, can normalize the gene regulatory network and signaling pathways, and reverse the phenotype in cancerous cells. Thus miRNA-based gene therapy provides an attractive anti-leukemic approach.

The identification of miR-34b tumor suppressor ability, and the discovery of a dataset of CREB target genes representing a novel transcriptional network that may control myeloid transformation, drove us to look at an ideal *in vivo* model to better interpret our findings. We considered the zebrafish model for its numerous advantages that led to its widespread use in both developmental studies and cancer research, such as high regenerative ability, rapid and transparent embryonic development, and easy genetic manipulation. To mimic the condition found in AML patients, we integrated a plasmid coding human CREB under *pu.1* promoter into fertilized egg at one cell stage to overexpress CREB in the myeloid lineage. Up to now, this is the first time that a CREB-overexpressing zebrafish model was performed. Moreover, zebrafish overexpressing CREB resembled the same dysmyelopoiesis already seen *in vitro* and in CREB transgenic mice, with a relevant perturbed CREB target gene expression. CREB-overexpressing zebrafish recapitulated myeloproliferative disease after one year, with more features of a leukemia than previously seen in mice. Finally, we consider that this model represents the best way to evaluate the main effects of CREB overexpression on its downstream effectors *in vivo*, with the aim to unravel the leukemogenic mechanism.

Due to the availability of a variety of fluorescent transgenic reporter lines and the easy genetic manipulations, we intend to test ICER and miR-34b as targets to reduce CREB expression. Moreover, because of new therapeutic compounds directed to CREB-transcriptional activity arrest has been created, we aim to test them alone and in combination with conventional chemotherapy in this zebrafish model.

Finally, during my PhD, I confirmed CREB to play a crucial role in supporting AML. I set up an *in vivo* model that recapitulates myeloid disease and thus represents a valid approach to delineate the mechanism of leukemia induction, as well as an interesting model where the additional events that probably follow CREB overexpression will be further addressed. Moreover, the zebrafish model will be useful to test the efficacy of new molecules that target CREB, with the perspective to hit this transcription factor. In fact it was demonstrated to have a broad role in many cellular processes, and its aberrant regulation is increasingly found to be involved in many cancer types, suggesting that there might be an underpinning CREB-regulated common pathway that urgently need to be identified. I believe that by targeting CREB, we can contribute to support and improve AML therapy.