Dysregulation of the C/EBP α Differentiation Pathway in Human Cancer

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Submitted May 2, 2008; accepted October 10, 2008; published online ahead of print at www.jco.org on December 15, 2008.

Supported by National Institutes of Health/National Cancer Institute's "SPORE in Human Lung Cancer" P50 CA90578-04 (B.H. and D.G.T.) and RO1 CA118316 (D.G.T.), the Flight Attendant Medical Research Institute (B.H. and E.L.), the International Association for the Study of Lung Cancer (E.L.), and a grant of the Deutsche Forschungsgemeinschaft (DFG KO2155/1-1 and /2-1; S.K.).

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/09/2704-619/\$20.00

DOI: 10.1200/JCO.2008.17.9812

ABSTRACT

While much is known about aberrant pathways affecting cell growth and apoptosis, our understanding of another critical step of neoplastic transformation, differentiation arrest, remains poor. The differentiation-inducing transcription factor CCAAT enhancer binding protein alpha (C/EBP α) is required for proper control of adipogenesis, glucose metabolism, granulocytic differentiation, and lung development. Studies investigating the function of this protein in hematopoietic malignancies as well as in lung and skin cancer have revealed numerous ways how tumor cells abrogate C/EBP α function. Genetic and global expression analysis of acute myeloid leukemia (AML) cases identifies C/EBP α -deficient AML as a separate entity yielding novel classification schemes. In patients with a dysfunctional C/EBP α pathway, targeted therapies may overcome the block in differentiation, and in combination with conventional chemotherapy, may lead to complete eradication of the malignant clone. Overall, a better understanding of the mechanisms of how C/EBP α dysregulation participates in the neoplastic process has opened new gateways for differentiation biology research.

J Clin Oncol 27:619-628. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Disruption of normal differentiation is a critical component of tumorigenesis, but the genetic mechanisms of this process are still poorly understood. Cells must be capable to enter a quiescent state in a precisely regulated manner, such as during terminal differentiation to maintain tissue homeostasis, or in response to DNA damage or oncogenic stress. Lineage-specific transcription factors control both of these processes and thereby play a pivotal role in the determination of cellular fate.¹ C/EBP α is a critical transcription factor that controls tissue-specific gene expression and proliferation arrest, thereby coordinately regulating two essential features of terminally differentiated cells. Recent studies demonstrate that C/EBP α indeed acts as a bona fide tumor suppressor in a number of tumor types,^{2,3} supporting the view that disruption of normal differentiation and its uncoupling from cell cycle arrest are key components in the development of cancer.⁴ The present review will summarize our current understanding of the role of C/EBP α in neoplasia.

PHYSIOLOGICAL FUNCTION OF C/EBP α

The C/EBP Family of Transcription Factors

C/EBP proteins form a family of basic region leucine zipper (bZIP) transcription factors that includes six members with related sequences and

functions (α , β , γ , δ , ϵ and ζ).⁵⁻¹⁰ C/EBPs are modular proteins consisting of two transactivation domains, a dimerization leucine zipper region, and a DNA-binding domain. Dimerization results in approximation of the basic amino acid residues and is a prerequisite for DNA binding. When the phasing between the basic domain and leucine zipper is altered by nucleotide insertions or deletions as described below in cases of C/EBP α mutations in AML, DNA-binding is altered or eliminated. The x-ray structure of the C/EBP α protein-DNA interaction was recently solved, and several critical basic region residues were identified.¹¹

Structure and Function of C/EBP α

The C/EBP α (C/EBP α) gene is localized to chromosome 19q13.1, is intronless, and two protein isoforms are generated from two inframe AUG codons on the mRNA (Fig 1C). The shorter form, known as p30, contains the same carboxyl terminus as the full-length form, known as p42, but lacks the amino-terminal 117 amino acids.¹² The transactivation domains TE-I and TE-II¹³ (regions 1 to 3 and 5 to 6¹⁴) that interact with the transcriptional apparatus are only present in the p42 isoform, while the domains required for interaction with other transcription factors and the chromatin remodeling complex are present in both isoforms.^{13,14}

C/EBP α is expressed at high levels in a specific subset of tissues and cell types, such as liver, adipose

Fig 1. Physiological function of CCAAT enhancer binding protein alpha (C/EBPα). (A) Major sites of tissue expression of C/EBPα include the liver, the hematopoietic system, white adipose tissue, and the lung. Accordingly, mice with targeted disruption of C/EBPα (C/EBPα-knockout mice) display perinatal hypoglycemia, absence of granulocytes, lack of white adipose tissue, and hyperproliferation of interstitial lung tissue. Modified from Zhang et al²⁴, Yang et al^{24a} and Flodby et al²³ (with permission). (B) Within the hematopoietic system, C/EBPα is expressed at lower levels in hematopoietic stem cells (HSCs), and its expression increases progressively during granulocytic differentiation via common myeloid progenitor cells (CMP) and granulocyte-macrophage progenitor cells (GMP). C/EBPα is not detectable in megakaryocyte-erythrocyte progenitors (MEP), common lymphoid progenitors (CLP), or more mature lymphoid cells. Modified from Akashi et al with permission.¹¹³ (C) The C/EBPα mRNA is comprised of a cap sequence and 5' untranslated region (UTR), the C/EBPα coding sequence derived from a single exon, and the 3' UTR, which is followed by the poly A sequence (upper panel). The letters A, B1, B2, C, and D designate five ATG sites of translational start sites which are highly conserved across the six different species depicted with the exception of the A site, which is missing in the human species (middle panel). Translation from all sites except D produce a specific C/EBPα isoform that can be detected by Western blotting, and omission of each site (ie, -A) leads to the loss of the specific isoform. The major C/EBPα isoforms are produced from the B1 and C sites and are 42 kD and 30 kD in size. From Calhoun et al with permission.¹¹⁴ NK, natural killer; Pro-T, T-cell progenitors; Pro-B, B-cell progenitors; wt, wild type.

tissue, myeloid cells, and airway epithelial cells.^{15,16} In these cells, C/EBPα directly activates transcription from lineage-specific gene promoters (ie, serum albumin, AP2 and stearyl-coenzyme A desaturase (SCD1), granulocyte colony-stimulating factor receptor, or surfactant proteins and CC10). Direct contact with the basal transcriptional apparatus (TBP/TFIIB), interaction with histone acetyltransferases (CBP/p300), and recruitment of chromatin-remodelling complexes (SWI/SNF) have all been implicated in the activation of lineage-specific genes by C/EBPα. The puzzling ability of C/EBPα to direct cellular differentiation in a tissue-specific manner is thought to depend on the presence of specific collaborating transcription factors. In adipocytes C/EBPα mediates growth arrest and terminal differentiation in concert with peroxisome proliferator-activated receptor gamma (PPARγ).¹⁷ In the hematopoietic system, the interplay between C/EBPα and GATA factors is a similar example^{18,19} while in the lung, the tissue-specific regulatory network includes thyroid transcription factor-1, FoxA2 (also called HNF3β), and C/EBPα.²⁰

Much has been learned about the role of C/EBPα in tissue development and differentiation from the study of C/EBPα-/- knockout mice²¹⁻²³ (Fig 1A). These mice die at birth as a result of severe hypoglycemia, and they show disturbed hepatic tissue architecture with proliferative acinar formations resembling pseudoglandular hepatocellular carcinoma. In addition, C/EBPα knockout mice display lung

abnormalities such as a striking hyperproliferation of type II pneumocytes. Moreover, they show a complete lack of granulocytes, while blasts accumulate in the bone marrow, suggestive of an early block in myeloid maturation.²⁴

C/EBPα As a Master Regulator of Hematopoietic Differentiation

The maturation of hematopoietic precursors from hematopoietic stem cells (HSC) to differentiated cells is associated with two fundamental processes: reduction in self-renewal potential and stepwise acquisition of a specific lineage identity.^{1,25} Transcription factors are master regulators of these graduated processes, and aberrations in their regulation/function can lead to loss of lineage identity, increased infidelity, lineage reprogramming, and malignant transformation. The formation of myeloid cells is orchestrated by a small number of critical transcription factors, such as PU-1, C/EBPα/β and δ, IRF-8, RUNX1, SCL, and GFI1. This is well reflected by severe defects seen in myeloid development of mice with targeted deletions of genes encoding these key transcription factors. C/EBPα regulates the expression of many myeloid genes, including genes encoding growth factor receptors (granulocyte colony-stimulating factor, macrophage colony-stimulating factor, granulocyte-macrophage

colony-stimulating factor) and the so-called secondary granule proteins (ie, lactoferrin).²⁶⁻²⁸ C/EBP α has also recently been implicated in the regulation of the microRNA miR223, with C/EBP α -mediated upregulation of miR223 leading to granulocytic differentiation in a myeloid differentiation model.²⁹ Also, the transcription of genes that are normally expressed in other lineages is actively suppressed, driving cells away from granulocytic differentiation toward other differentiation pathways. C/EBP α in the hematopoietic system is expressed in HSCs, myeloid progenitors and granulocytes, but not macrophages.³⁰ C/EBP α -deficient mice have normal numbers of common myeloid progenitors (CMPs) but lack granulocyte-monocyte progenitors (GMPs) and all subsequent granulocytic stages (Fig 1B). Conditional deletion of C/EBP α in GMPs allows for normal granulopoiesis in vitro, indicating that C/EBP α is not required for granulopoietic differentiation beyond the GMP stage.³⁰ C/EBP α also controls stem cell self-renewal properties as C/EBP α -deficient HSCs show increased competitive repopulation activity in mouse transplant studies.³⁰ The increased self-renewal potential of C/EBP α $-/-$ HSCs may, at least in part, be due to elevated expression of the polycomb gene Bmi-1. The mechanism of how altered C/EBP α function ultimately leads to neoplasia may be through failure to induce differentiation in progenitor cells leading to a prolonged and aberrant proliferation phase in these cells followed by random accumulation of additional genetic events and full transformation.

C/EBP α and Proliferation Arrest

C/EBP α possesses the ability to arrest cell proliferation in addition to its ability to drive terminal differentiation and thereby functions as a master switch between proliferating uncommitted and cell cycle-arrested differentiated cells.^{3,31,32} The mechanisms of C/EBP α -mediated growth arrest include interaction with CDK2/CDK4, upregulation of p21^{waf1/cip1} and the SWI/SNF chromatin-remodelling complex³³⁻³⁵ as well as direct inhibition of the E2F complex.^{36,37} Downregulation of *c-myc* is one of the critical steps of C/EBP α -mediated E2F repression and is required for granulocytic differentiation.³¹ Point mutations of the basic region of C/EBP α abrogating C/EBP α -E2F complex formation (BRM-5: Y285A and BRM-2 I294A/R297A) interfere with differentiation in both myeloid and adipocytic differentiation.³⁷ These mutants lack growth inhibitory properties, possibly through diminished binding of E2F and/or abolished DNA-binding, as suggested by structural analyses. Interestingly, the shorter p30 isoform lacks E2F-repression and thus lacks antimitotic activity in adipogenic and granulocytic cell types.

ROLE OF C/EBP α IN HEMATOPOETIC MALIGNANCIES

Deregulation of C/EBP α plays a role in a number of hematologic malignancies, where C/EBP α has been demonstrated to be mutated, involved in chromosomal translocations, or suppressed both at the transcriptional and posttranscriptional level, resulting in different mechanisms of oncogenic events (Fig 2).

Somatic Gene Mutations

Since C/EBP α is essential for granulocytic differentiation and cells from patients with acute myeloid leukemia harbor a differentiation block resulting in an excess of myeloid blasts, it was hypothesized that C/EBP α may be absent or mutated in myeloid leukemias. A

Fig 2. Pathogenetic mechanisms of CCAAT enhancer binding protein alpha (C/EBP α) dysfunction. C/EBP α function is disturbed at various levels in acute leukemias. Promoter methylation leads to silencing of promoter activity and decreased expression of C/EBP α . The fusion protein AML1-ETO suppresses C/EBP α transcription via disturbed autoregulation. Chromosomal translocations involving the immunoglobulin H locus and the *CEBPA* gene (t14;19) occur in B-cell precursor acute lymphoblastic leukemia and have been described to induce aberrant C/EBP α expression in lymphoid cells. Other translocations which are associated with AML (AML1/MDS/Evi1 and CBFB/SMMHC) or chronic myelogenous leukemia (CML; BCR-ABL) have been demonstrated to increase the expression of specific RNA binding proteins which can block proper C/EBP α translation from an intact mRNA. In addition, posttranslational modifications such as Serine-21 phosphorylation by oncogenic Fli3-ITD as well as impairment of C/EBP α DNA binding by PLZF-RAR results in dysfunction of C/EBP α and a block of granulocytic differentiation. Finally, C/EBP α protein degradation is induced by Trib2 whose expression is increased by Notch1 mutations in AML. CBFB, core-binding factor β ; BP, RNA-binding protein; UPD, uniparental disomy.

screen for mutations within the C/EBP α gene coding sequence revealed that C/EBP α was mutated in approximately 7% of all patients with AML,² and the results have since been reproduced and extended by other groups³⁸⁻⁴⁷ (Fig 3A and B). Intriguingly, mutations were predominantly found within the M1 and M2 FAB morphological subtypes, and no mutations were found in patients with a t(8;21), inv(16), or t(15;17) karyotypic abnormality.² When analyses were performed in patients with a normal karyotype (cytogenetically normal [CN]), 10% to 15% of cases harbor a C/EBP α mutation.^{2,42} Clinically, C/EBP α mutations are associated with lower leukocyte counts and lactate dehydrogenase levels and with aberrant expression of T-cell surface markers such as CD7 at presentation.⁴⁸ Moreover, C/EBP α mutations are confined to AML and high-risk myelodysplastic syndrome and have not been found in other diseases. Genome-wide gene expression analysis of blasts from AML patients using unsupervised clustering revealed that C/EBP α mutant cases cluster together, suggesting that they share similar gene expression signatures.⁴⁹ C/EBP α mutations (15% of all CN-AML cases) do not segregate with any other known mutations, such as NPM1, Fli3 or MLL mutations.⁵⁰

Retrospective studies show that patients with C/EBP α mutant AML have an improved prognosis^{38,41,42,45} (Fig 3C). Whether this is counteracted by the negative impact of concurrent Fli3-ITD mutations is controversial.^{38,42} For Fli3-TKD mutations, additional improvement of event-free survival has been described for patients with C/EBP α mutations.⁵¹ Prospective validation of the prognostic impact of C/EBP α mutational status is awaited to determine the clinical impact of C/EBP α mutation analysis. The C/EBP α mutations found

Fig 3. CCAAT enhancer binding protein alpha (C/EBP α) mutations and their effect on prognosis in acute myeloid leukemia (AML). (A) and (B) Mutations of the coding sequence of C/EBP α have been described in approximately 15% of patients with AML and a normal karyotype by conventional cytogenetics. Two thirds of these patients harbor N-terminal frameshift mutations and C-terminal in-frame insertion/deletion mutations, leading to a nonfunctional 20 kD isoform and increased 30 kD isoform expressed from the N-terminally mutated allele as well as a dysfunctional 42 kD isoform expressed from the C-terminally mutated allele (ie, patient 2"). Please see Pabst et al² for more details. (C) Retrospective survival analysis of patients with AML harboring C/EBP α mutations or no C/EBP α mutations showing superior overall survival of patients with C/EBP α mutations. Data from Preudhomme et al³⁸. N'ab, antibody recognizing N-terminal portion; TAD, trans-activation domain; C'ab, antibody recognizing the C-terminal domain; bZip, basic zipper region; wt, wild type.

in AML are typically biallelic and involve frameshift mutations of the 5' region of the coding sequence on one allele and in-frame insertion/deletion mutations within a narrow 3' region on the other allele, leading to so-called N-terminal and C-terminal mutations of the protein, respectively⁵² (Fig 3A and B). Sole N-terminal, sole C-terminal, and other mutations were found in 11.4%, 9%, 12.6% of cases.⁵² C-terminal mutations are usually located at the junction between the basic region and the leucine zipper coding sequence resulting in impaired alignment with the major groove of DNA and disturbed DNA binding by the mutant protein as well as altered dimerization with its partner proteins.⁵³ N-terminal mutations lead to a nonfunctional 20 kD C/EBP α isoform or a shorter additional isoform while the physiological 30 kD isoform generated from an internal translational start site is always preserved² (Fig 3A and B). The ratio of the 30 kD isoform and the residual 42 kD full-length isoform generated from the non-mutated allele is increased in the mutant cells, and functional studies have revealed that the 30 kD isoform shows significantly reduced binding to and transactivation of C/EBP α target promoters.²

The exact mechanism of how the C/EBP α mutants inhibit granulocytic differentiation is not entirely clear at present. N-terminal mutants have been described to exert dominant-negative effects on the unmutated 42 kD C/EBP α protein isoform. This may be due to a change of the ratio of p30 and p42 homo- and heterodimers. Alternatively, an imbalance between p42 and p30 may affect heterodimerization with other C/EBP members and thus interfere with C/EBP-induced cell cycle arrest and differentiation. The mechanism of C/EBP α -mutant leukemogenesis has been addressed in homozygous knock-in mice that express p30 C/EBP α but lack p42 C/EBP α completely. These mice exhibit an initial phase of neutropenia and go on to develop overt AML with full penetrance.⁵⁴ Blasts display increased proliferative activity and harbor a normal karyotype similar to C/EBP α -mutant human AML. Granulocyte-macrophage progenitor cells (GMPs) are still formed in preleukemic mice showing that p30 is sufficient to induce commitment of common myeloid progenitors

(CMPs) toward GMPs.⁵⁴ This is in contrast to C/EBP α knockout mice, which lack all C/EBP α isoforms completely and fail to support the transition from CMPs to GMPs. While bone marrow-derived myeloid progenitors from heterozygous wild-type/p30 mice showed only mildly increased proliferative activity compared with wild-type progenitors, p30/p30 homozygous GMPs showed high proliferative activity, high rates of frequent spontaneous immortalization, and increased self-renewal. Therefore, p30 C/EBP α is required and sufficient for commitment of CMPs to the myeloid lineage, whereas p42 C/EBP α is required for cell cycle arrest and the tumor-suppressing functions in myeloid progenitors. Together with the fact that C/EBP α -mutant AML patients also retain expression of p30 C/EBP α , these data suggest that residual expression is required for commitment of HSC toward the myeloid lineage and for the development of AML⁵⁵ similar to how AML develops in PU.1-hypomorphic but not PU.1 knockout mice.⁵⁶ This concept is supported by the observation that C/EBP α knockout cells are unable to give rise to a myeloid disease on transduction with BCR-ABL but instead develop an acute erythroblastic leukemia, while wild-type C/EBP α cells give rise to a chronic myelogenous leukemia (CML)-like myeloid disease.⁵⁵

Germline C/EBP α Mutations

Germline mutations of C/EBP α have been described and are typically N-terminal mutations that are then joined by a somatic C-terminal mutation on the other allele.^{44,57} The first report described a germline 212delC N-terminal C/EBP α mutation in two siblings and their father with AML.⁴⁴ Additionally, a 36bp C-terminal C/EBP α gene duplication was found in the leukemic samples of one of the siblings.⁴⁴ The next report described five individuals with familial AML harboring a germline 217insC mutation.⁵⁷ Two of these had additional C-terminal insertions at nucleotide 1071. A third report identified a Japanese family with an N-terminal germline mutation and an additional C-terminal mutation in two individuals developing AML.⁵⁸ The germline mutations were N-terminal mutations in all

cases succeeded by a C-terminal somatic mutation in the leukemic blasts suggesting that the combination of these mutations induces AML but is still tolerated by myeloid cells, while a biallelic combination of two C-terminal or two N-terminal mutations is deleterious to the cells and would induce apoptosis instead of AML.

Involvement in Chromosomal Translocations (B-precursor acute lymphoblastic leukemia)

In addition to harboring C/EBP α mutations, the C/EBP α gene as well as other C/EBP members have been demonstrated to be involved in t(14;19)(q32;q13) chromosomal translocations in B-cell precursor acute lymphoblastic leukemia (BP-ALL).^{59,60} The exact consequences of these translocations are unclear. However, high C/EBP α mRNA and protein were demonstrated in several of these BP-ALL cells, which normally do not express any C/EBP α .⁵⁹ These observations suggest opposing functions of C/EBP α dysregulation in myeloid and lymphoid leukemogenesis, with C/EBP α serving as an oncogene in BP-ALL.⁵⁹ This is reminiscent of PU.1 overexpression found in murine erythroleukemias (Friend's erythroleukemia).⁶¹

Uniparental Disomy Involving the C/EBP α Gene

Uniparental disomy (UPD) refers to large regions of acquired homozygosity resulting from mitotic recombination allowing malignant cells to multiply gene mutations and to dispose of wild-type alleles obtaining a selective growth advantage over cells without UPD. UPD was found by genome-wide single nucleotide polymorphism analysis to occur in approximately 20% of AML cases.⁶² Moreover, the authors detected homozygous mutations at four distinct gene loci that have been associated with AML (WT1, FLT3, C/EBP α , and RUNX1). In line with these data, an AML case of UPD on chromosome 19 with a homozygous C-terminal C/EBP α mutation has been reported.⁶³ These results imply that the gene mutation precedes mitotic recombination and depict another means of AML blasts to dispense of the C/EBP α wild-type allele as has already been described for the coincidence of N- and C-terminal C/EBP α mutations.

Suppression of C/EBP α Transcription

Silencing of tumor suppressor genes by hypermethylation is common in human cancers including leukemias.^{64,65} Methylation of the C/EBP α core promoter (−600 to ATG) was found to be an infrequent event in AML,⁶⁶ but more recent studies find that methylation of distal promoter elements (−1600 to −600 from ATG) occurs in 30% of AML patients.⁶⁷ C/EBP α promoter methylation was present in six of 10 cases with undetectable C/EBP α mRNA expression and was associated with activating Notch1 mutations and aberrant T-cell marker expression.⁴⁸ Activation of Notch1 led to overexpression of Trib2, a protein which was found to decrease p42 C/EBP α protein—possibly through enhanced proteasomal degradation—and to induce AML in mice.⁶⁸ Interestingly, these AML cases also clustered together with the C/EBP α mutant samples in the previously mentioned microarray analysis.⁴⁹ C/EBP α mutations have not been described in cases of t(8;21) or inv16 AML. While inversion 16 cases show hypermethylation of the C/EBP α promoter,⁶⁹ t(8;21) cases do not. In these cases, C/EBP α mRNA is downregulated by the oncogene AML1-ETO by a mechanism that is still incompletely understood but appears to involve an altered self-stimulatory transcriptional loop.²

Suppression of Protein Translation

Two main mechanisms of inhibition of C/EBP α protein translation have been reported. C/EBP α translation is inhibited in blasts from patients with CML myeloid blast crisis⁷⁰ via an RNA binding protein, hnRNP-E2, which binds to the upstream open reading frame of the C/EBP α gene and inhibits translation of C/EBP α mRNA. hnRNP-E2 is induced by the oncogenic kinase BCR-ABL via a MAPK-dependent mechanism.⁷¹ Downregulation of hnRNP-E2 by the BCR-ABL inhibitor imatinib restored both C/EBP α protein expression and granulocytic differentiation of the blasts.⁷⁰ Calreticulin, another RNA binding protein, has been demonstrated to be upregulated and to inhibit C/EBP α translation in AML subtypes with CBFB-SMMHC and AML1-MDS-EVI1 translocations.^{72,73}

Posttranslational Modification

Inhibition of C/EBP α activity can also occur through posttranslational modifications. C/EBP α is phosphorylated on serine 21 shifting the ability of C/EBP α to induce differentiation from a granulocytic toward a monocytic phenotype. The oncogenic kinase Flt3-ITD (internal tandem duplication) phosphorylates C/EBP α on ser21 thereby inhibiting granulocytic differentiation.⁷⁴ Another oncogenic protein, PLZF-RARA, has been shown to interfere with C/EBP α transactivating activity on the granulocyte colony-stimulating factor receptor promoter.⁷⁵ In addition, Ubc9 has been described to modify C/EBP α transactivation activity.^{76,77}

THE ROLE OF C/EBP α IN AIRWAY EPITHELIAL DIFFERENTIATION AND LUNG CANCER

C/EBP α As a Master Regulator of Airway Epithelial Differentiation

A number of transcription factors such as thyroid transcription factor-1, FoxA2, and C/EBP α control the intricate process of lung development and airway epithelial differentiation.^{78,20,79,80} Induction of C/EBP α expression occurs in close temporal proximity to the initiation of cellular differentiation in the developing lung. In the adult lung, C/EBP α is expressed in both bronchial epithelial Clara cells as well as type II alveolar cells. C/EBP α can regulate the expression of a number of lung-specific genes, such as Clara cell secretory protein (CCSP), surfactant protein A (SP-A), and surfactant protein D (SP-D). CCSP, SP-A and SP-D.⁸¹ A conditional mouse model of C/EBP α deletion directed to the respiratory epithelium was developed using tissue-specific tetracycline-regulated cre-recombinase mediated excision of C/EBP α (Fig 4).⁸² In these mice, in utero loss of C/EBP α leads to respiratory arrest and death soon after birth due to a differentiation arrest of type II alveolar cells associated with proliferation of immature type II alveolar cells, causing epithelial cell expansion and loss of airspace. Expression analysis of knockout versus wild-type lungs demonstrated that the expression of genes characteristic of differentiated pulmonary cells (AQ5, SPA, ABCA3) were downregulated, and genes associated with proliferation (Rgs2, G0s2), tumor progression (Tiam1, Pcsk6) and cell survival (TGFbeta2, Gli1) were upregulated in CEBP α -deficient cells. Similar findings were noted in a study of Martis et al.⁸³ These observations suggest that modulating C/EBP α function in the lung may be useful for the treatment of different forms of respiratory distress syndrome. Interestingly,

Fig 4. CCAAT enhancer binding protein alpha (C/EBP α) in lung cancer. (A) Generation of tissue-specific, inducible C/EBP α -knockout mice. (Ba) Electron microscopy of airway epithelium in a lung-specific C/EBP α -knockout mouse demonstrates hyperproliferation of immature alveolar cells as well as intracellular accumulation of glycogen. (Bb) Histology of lung tumors in C/EBP α -knockout mice demonstrates bronchoalveolar carcinoma-like histology. (C) Immunohistochemical studies demonstrate downregulation of C/EBP α in non-small-cell lung cancer. The panels shown demonstrate strong expression of C/EBP α in normal bronchial epithelium (a) and show non-small-cell lung tumors with different degrees of expression (panels b, 0; c, 1+; d, 2+; e, 3+). (D) In vitro studies demonstrate growth arrest, (a) apoptosis, and (b) cellular differentiation in lung cancer cell lines as a result of C/EBP α expression in H358 lung cancer cells. Reprinted with permission from Halmos et al,⁸⁴ Costa et al,⁸⁵ and Basseres et al.⁸² H358-MT, H358 cells transfected with a zinc-inducible metallothionein promoter-driven expression plasmid.

C/EBP α is detected at the level of bronchoalveolar stem cells (unpublished data), a cell population that has been recently linked to adenocarcinoma initiation. Postnatal excision of C/EBP α is feasible in these inducible mice, allowing the study of the effects of C/EBP α downregulation in predisposing to lung tumorigenesis. Our data obtained on two different lung conditional models (adult SPC and CC10-driven excision) demonstrate significantly increased numbers of lung adenomas and adenocarcinomas that develop on excision of C/EBP α in the adult lung epithelium, supporting the hypothesis that C/EBP α is a lung tumor suppressor (unpublished data). Our data also indicate that Gli-1, a transcriptional effector of the sonic hedgehog pathway, is highly active only in C/EBP α -deleted pulmonary cells that might represent the cells of origin of the lung adenocarcinomas found in our murine models.

C/EBP α As a Lung Cancer Tumor Suppressor

These observations prompted us to examine the role of C/EBP α in human lung cancer.⁸⁴ A screen of established lung cancer cell lines demonstrated that C/EBP α expression was undetectable or low in 24 of 30 cell lines examined, and immunohistochemical studies confirmed that C/EBP α expression is lost in more than half of all primary tumor specimens. C/EBP α expression is lost more commonly in squamous cell cancers, and there is a trend toward a higher frequency of loss in poorly differentiated and more advanced cancers, suggesting the biologic role of C/EBP α in differentiation and tumor progression, but no prognostic role for C/EBP α expression was found.⁸⁵ In conditional cell line models, induction of C/EBP α led to significant growth reduction attributable to proliferation arrest, morphological changes suggestive of airway epithelial differentiation, and ultimately

apoptosis. A transcriptional profiling study performed using these models identified the forkhead family member and differentiation gene, FoxA2, as directly regulated and induced by C/EBP α in lung cancer cells. FoxA2 appears to be a major mediator of the growth-inhibiting action of C/EBP α .⁸⁶ This finding is particularly intriguing since FoxA2 is a critical transcription factor in foregut development and in airway epithelial differentiation and FoxA2 itself is a candidate tumor suppressor in lung cancer.

The chromosomal region including C/EBP α was found deleted in 50% of non-small-cell lung cancers suggestive of the presence of a tumor suppressor at this locus.⁸⁷ We completed sequencing of 31 established lung cancer cell lines and 50 primary lung cancers and identified mutations of C/EBP α in only three of 81 specimens examined.⁸⁸ Sequencing of the C/EBP α gene by another group in 36 non-small-cell lung cancers failed to identify any coding sequence mutations.³⁹ These findings suggest that mutations of C/EBP α are rare events in lung cancers and that C/EBP α deactivation occurs through alternative mechanisms. The promoter and exonic regions of C/EBP α meet the definition of a CpG island spanning almost 3.4 kb. Interestingly and similar to studies in hematopoietic cells, no methylation was found in the core promoter region. Conversely, hypermethylation of an upstream promoter region of the C/EBP α gene (−1422 to −896 relative to the transcriptional start site) is a very common event in non-small-cell lung cancers (detected in approximately 65% of primary lung tumors) and appears to be the major mechanism for gene silencing.⁸⁹ Overall, promoter methylation of C/EBP α and loss of its expression appears to be one of the most common genetic events in non-small-cell lung cancer and further corroborate that C/EBP α is indeed a tumor suppressor in non-small-cell lung cancer. Moreover, they provide translational opportunities for the reactivation of C/EBP α expression through the use of agents affecting DNA methylation and/or histone acetylation.

THE ROLE OF C/EBP α IN OTHER SOLID TUMORS

C/EBP α is expressed in basal and suprabasal keratinocytes of the epidermis, and forced overexpression of C/EBP α in keratinocytes leads to cell cycle blockade.^{90,91} C/EBP α participates in the p53-mediated G1 cell cycle arrest in response to ultraviolet B radiation or chemotherapy.⁹² Its expression is diminished or undetectable in the majority of human and mouse squamous cell carcinomas and is associated with Ras expression.⁹³ Forced expression of oncogenic Ras leads to downregulation of C/EBP α in immortalized keratinocytes, and C/EBP α expression leads to G1 cell cycle arrest in squamous cell carcinoma cell lines.⁹⁴ These findings suggest that C/EBP α is an important regulator of growth control and DNA damage response in the skin. Epidermal-specific C/EBP α -knockout mice survive and display no detectable abnormalities in epidermal keratinocyte proliferation.⁹⁵ However, C/EBP α -null mice are highly susceptible to dimethylbenzanthracene-induced skin tumor development. These results provide genetic evidence for the tumor suppressive role of C/EBP α in skin carcinogenesis.

C/EBP α is expressed in normal prostate epithelium with strong expression observed in the basal layer.⁹⁶ In most prostate adenocarcinomas it is expressed at low levels. Quite surprisingly, overexpression of C/EBP α in two prostate cancer cell lines led to increased proliferation rates, the mechanism of which was not defined.⁹⁶ Whether these

observations are cell-line specific or represent a more general phenomenon in prostate epithelium remains to be seen.

C/EBP α is expressed at high levels in terminally differentiated, mature liver hepatocytes, while it is virtually absent in regenerating liver and hepatomas¹⁵ suggesting that C/EBP α expression might be inversely correlated with proliferation. Indeed, overexpression of C/EBP α in hepatocytes leads to impaired proliferation and tumorigenicity in human hepatoma cell lines.⁹⁷ Liver-specific C/EBP α -knock-in mice demonstrate partial resistance to diethylnitrosamine-induced hepatocellular carcinoma formation corroborating a tumor suppressive role of C/EBP α in hepatic carcinogenesis.⁹⁸

Downregulation of C/EBP α in squamous cell cancers of the head and neck region has been reported correlating with degree of promoter methylation, while no mutations were found in 59 tumor specimens examined.⁹⁹ C/EBP α expression in cell lines led to growth inhibition. One microarray study identified downregulation of C/EBP α as a marker for extensive lymph node metastases.¹⁰⁰

TARGETING C/EBP α TO FIGHT CANCER

C/EBP α is required for granulocytic differentiation, and restoration of its proper function holds the promise of enabling leukemic stem and progenitor cells from patients with AML and CML myeloid blast crisis to overcome the block of differentiation and undergo maturation into functional effector cells. In mouse models of AML and acute promyelocytic leukemia, overexpression of C/EBP α has resulted in abrogation of the leukemia and in increased survival of the animals.^{101–103} Of course, replacement therapy for a nuclear transcription factor is a serious technical challenge. Gene-therapy utilizing novel vector systems, such as lentiviral vectors or fusion peptides, such as VP22 or TAT to facilitate entry into the cells are potential considerations.^{104–107} Compound nanoparticles that target a TAT-bound protein to specific cell subsets via antibody binding to surface markers, as recently described could be exploited to deliver functional C/EBP α to the cytoplasm and nucleus of leukemic blasts via for example a CD33 antibody targeting myeloid cells in order to induce granulocytic-monocytic differentiation.¹⁰⁸ This approach seems potentially feasible since virtually all AML blasts express CD33, and a calicheamicin-bound anti-CD33 antibody, gemtuzumab is already in clinical use.¹⁰⁹

A more pragmatic approach to restore or activate the C/EBP α differentiation pathway is through small molecule modulators of the expression of C/EBP α , the C/EBP α protein itself or the differentiation pathway governed by C/EBP α . E.g. ATRA upregulates C/EBP α mRNA and protein in myeloid cells.²⁸ ATRA is being used clinically for the treatment of acute promyelocytic leukemia, and recently, it has been employed successfully in AML patients with NPM mutations.¹¹⁰ However, it is not known whether C/EBP α upregulation contributes to the favorable response of these patients to ATRA, as ATRA also greatly increases expression of C/EBP β which can itself induce maturation of acute promyelocytic leukemia cells.¹¹¹ C/EBP α translation can be efficiently stimulated *in vitro* using the triterpenoid antineoplastic drug 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), which increases the translation of p42 CEBP α and the ratio of p42/p30 C/EBP α , leading to granulocytic differentiation in myeloid cells.¹¹² High levels of BCR-ABL, as present in CML blast crisis, induce hnRNP-E2 expression via a MAPK-dependent mechanism. The MEK1/2 inhibitor CI-1040 inhibits hnRNP-E2 expression, and

through its association with C/EBP α mRNA, results in increased C/EBP α protein expression and granulocytic differentiation of BCR-ABL-expressing 32D cells.⁷¹ MEK inhibitors also inhibit serine-21 phosphorylation of C/EBP α , leading to a more active protein and to enhanced granulocytic differentiation.⁷⁴ Dephosphorylation of C/EBP α Ser21 was also seen with a Flt3 tyrosine kinase inhibitor,⁷⁴ and Flt3 kinase inhibitors are the subject of intense clinical study in AML. Our group has also developed luciferase-based assays that are capable of monitoring the modulation of C/EBP α activity in high-throughput assays utilizing a C/EBP-regulated promoter construct. Such assays now allow the screening of millions of small molecules to hopefully lead to the discovery of novel compounds that will indeed restore C/EBP α function and will be lead compounds in further clinical development for the treatment of AML and other cancers where the C/EBP α pathway is involved.

CONCLUSION

Research done over the past several years clearly points out the critical tissue- and cell-specific function of C/EBP α at the crossroads of cell proliferation and differentiation control. Also, evidence now appears overwhelming to document the role of C/EBP α as a bona fide tumor suppressor silenced in multiple different ways in a wide range of leukemias in which its mutational status and transcriptional signature

has led to new diagnostic and prognostic classification schemes. The downregulation and inactivation of C/EBP α might be a required step in the development of several other tumor types indicating a wider role for C/EBP α as a tumor suppressor. Better understanding of this prototypical differentiation factor will likely open doors for novel therapeutic strategies to modulate critical differentiation pathways in cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Acknowledgment

We apologize to colleagues whose original work could not be cited due to space limitations.