Transcription factor nuclear factor erythroid-2 mediates expression of the cytokine interleukin 8, a known predictor of inferior outcome in patients with myeloproliferative neoplasms

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ABSTRACT

The transcription factor nuclear factor erythroid-2 is over-expressed in patients with myeloproliferative neoplasms irrespective of the presence of the JAK2^{V617F} mutation. Our transgenic mouse model over-expressing nuclear factor erythroid-2, which recapitulates many features of myeloproliferative neoplasms including transformation to acute myeloid leukemia, clearly implicates this transcription factor in the pathophysiology of myeloproliferative neoplasms. Because the targets mediating nuclear factor erythroid-2 effects are not well characterized, we conducted microarray analysis of CD34$^+$ cells lentivirally transduced to over-express nuclear factor erythroid-2 or to silence this transcription factor via shRNA, in order to identify novel target genes. Here, we report that the cytokine interleukin 8 is a novel target gene. Nuclear factor erythroid-2 directly binds the interleukin 8 promoter *in vivo*, and these binding sites are required for promoter activity. Serum levels of interleukin 8 are known to be elevated in both polycythemia vera and primary myelofibrosis patients. Recently, increased interleukin 8 levels have been shown to be predictive of inferior survival in primary myelofibrosis patients in multivariate analysis. Therefore, one of the mechanisms by which nuclear factor erythroid-2 contributes to myeloproliferative neoplasm pathology may be increased interleukin 8 expression.

Introduction

The molecular etiology of myeloproliferative neoplasms (MPN) remains incompletely understood despite recent advances achieved through the detection of various mutations in MPN patients, especially the JAK2^{V617F} (Janus Kinase 2^{V617F}) mutation found in a large majority of affected individuals.[1] However, there are several lines of evidence to suggest that somatic changes in MPN patients precede the acquisition of the JAK2^{V617F} mutation and that several alterations may coincide to contribute to the MPN phenotype.[2-7] In addition, a large proportion of patients with primary myelofibrosis (PMF) and essential thrombocythemia (ET) display no known molecular aberration, hence, their molecular pathology remains unknown.

We have recently reported that the transcription factor 'nuclear factor erythroid 2' (NF-E2), is over-expressed in the vast majority of patients with all three MPN subtypes.[8,9] NF-E2 is a tissue specific transcription factor expressed in hematopoietic stem cells as well as in the myeloid, erythroid and megakaryocytic lineage.[10] NF-E2 overexpression in polycythemia vera (PV), ET and PMF is independent of the presence or absence of the JAK2^{V617F} mutation.[8,9] In a murine model, NF-E2 overexpression causes an MPN phenotype including thrombocytosis, leukocytosis, erythropoietin (Epo)-independent colony formation, characteristic bone marrow histology, expansion of stem- and progenitor compartments, and spontaneous transformation to acute myeloid leukemia.[11] This observation establishes a role for aberrant NF-E2 expression in the pathophysiology of MPN.

Despite the well documented functions of NF-E2 in chromatin remodeling and gene transcription,[12-15] few direct target genes of this transcription factor are known. We, therefore, conducted gene expression analysis of CD34$^+$ cells, lentivirally transduced to over-express NF-E2 or to silence endogenous NF-E2 expression via shRNA, in order to characterize novel NF-E2 target genes.

Design and Methods

Lentiviral constructs

The lentiviral pLeGO-iG vector[16] was modified by exchanging the murine U6 promoter for its human counterpart, as previously described for the pLeGO-G plasmid.[17] Briefly, the following primers were used to amplify the human U6 promoter: forward primer: 5' atc tag aga ggg cct att tcc cat g 3'; reverse primer 5' agt taa cgt cct ttc cac aag ata t 3'. The resulting fragment was digested with XbaI and HpaI and used to replace the murine U6 promoter in pLeGO-iG. The modified pLeGO-iG was named pLEGO-iG-hU6.

Lentiviral transduction of CD34$^+$ cells

Buffy coats of healthy volunteer blood donors were obtained from the University Hospital Freiburg Center for Blood Transfusion. The study protocol was approved by the local ethics committees (University Hospital Freiburg). CD34$^+$ cells were isolated by immunoselection (MACS Miltenyi). Lentiviral transduction of CD34$^+$ cells using either the empty pLEGO-iG-hU6 or a vector expressing the human NF-E2 cDNA was performed as previously described.[17] Briefly, cells were pre-stimulated for 2 h in serum-free medium (StemSpan SFEM, 09650, Stem Cell Technologies, Vancouver, BC, Canada) con-

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taining 100 ng/mL rhSCF (300-07, PeproTech, Rocky Hill, NJ, USA), 100 ng/mL rhFLT-3 ligand (300-19, PeproTech), 20 ng/mL rhIL6 (200-06 PeproTech) as well as 200 ng/mL rhTPO (300-18, PeproTech). Subsequently, CD34+ cells were lentivirally infected using MOIs between 7 and 10 without addition of a transduction facilitator. Forty-eight hours after transduction, GFP expressing cells were FACS sorted to obtain pure populations.

RNA extraction

RNA was extracted using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany) and subsequently analyzed for quality and integrity on an Agilent Bio-Analyzer 2100 (RNA 6000 Nano Assay). Total cellular RNA samples with an RIN (RNA integrity number) over 7.4 were subjected to purification of mRNA using the Ribo-Minus-Kit (Invitrogen, Carlsbad, USA; #K1550-01) according to standard protocols (Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay Manual).

Microarray analysis

Gene expression analyses were conducted using the Human Exon Array 1.0 ST (Affymetrix Santa Clara, CA, USA). Hybridization, washing, and scanning were performed according to standard protocol (Affymetrix Human Exon 1.0 ST).

The raw microarray data were pre-processed using Affymetrix Power Tools (APT, version 1.10.2, Affymetrix) by applying the GC Robust Multi-array Average (GC-RMA) and the detection above background (DABG) methods. Metaprobe sets included in the core annotation (HuEx1.0/v2/hg18) were further analyzed with the R (R-project for statistical computing, version: 2.10.1; *http://www.r-project.org*[18]) and Bioconductor (version 2.6.1; *http://www.bioconductor.org*[19]) softwares. The analysis of differential expression was performed using the Linear Models for Microarray Data Package (limma)[20] after applying a filter for the DABG *P* value of 0.01 or under. A linear model representing the matched paired structure of the experiment was fitted for each gene by applying the lmFit function. Differences in gene expression were ranked according to the eBayes function. To control for multiple testing, the *P* values obtained were adjusted by calculating the false discovery rate (fdr) using the method of Benjamini and Hochberg.[21] Genes were considered to be differentially expressed if the fdr-adjusted *P* value was less than 0.05.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) experiments were performed using the following Assay on Demand (Applied Biosystems, Foster City, CA, USA) products for gene expression analysis:

Human IL-8 Assay on demand (Hs00187842_m1)

Human Beta-2-Microglobulin Assay on demand (Hs00187842_m1)

Human NF-E2 Assay on demand (Hs00232351_m1)

Reverse transcription of 50 ng of total RNA of CD34+ cells was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative PCR assays were conducted in duplicate in an ABI PRISM 7000 Cycler. Relative quantification of IL-8 mRNA expression was performed using the $\Delta\Delta C_T$-method employing beta 2 (β2)-microglobulin as the reference gene.[22]

Lentiviral transduction of cell lines

Human erythroleukemia (HEL) cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (all from Invitrogen). UKE-1 cells were cultured in Iscove's modified Dulbecco medium (Invitrogen) supplemented with 10% FBS, 10% horse serum (Biological Industries), 1 μM hydrocortisone (Sigma-Aldrich), and penicillin/streptomycin. Culture medium for SET-2 cells consisted of RPMI 1640 medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS) and penicillin/streptomycin (all from Invitrogen).

Cell lines were transduced with lentiviral particles at an MOI of 4, resulting in more than 97% GFP-positive cells.

Intracellular IL-8 FACS-staining

Cells were treated with brefeldin A (BFA, 10ug/ml) for 4 h and subsequently labeled using the FastImmune Anti-Hu–IL-8 PE Detection Kit (#340510, Becton-Dickinson Biosciences, San Jose, CA, USA) according to the manufacturer's recommendations. Intracellular cytokine expression was analyzed on a FACSCalibur (BD Biosciences) using the FlowJo software (Tree Star).

IL-8 ELISA

Cell supernatents were assayed for IL-8 using the Quantikine IL-8-ELISA (R&D Systems, #D8000C).

Plasmid constructs

The IL-8 promoter constructs were generated by PCR amplification of the -5kb upstream enhancer and the IL-8 promoter region from total cellular DNA of purified granulocytes from healthy donors by using the following primers:

-5k-enhancer:
fwd 5'-GCTGGTACCCCAAGGCCAAAGTGAGGA-3',
rev 5'-GCTGCTAGCGAAGTTTAGGTTTAGGGGAAGAC-3'
280bp-promoter-region:
fwd 5'-CCGGCTAGCCAAATTGT-3'
rev 5'-CGCCTCGAGCTTGTGTG-3'

The promoter and enhancer elements were cloned into the pGL4.10 luciferase reporter vector (Promega, Mannheim, Germany). Mutations of the NF-E2 binding site constructs were performed using GeneArt Site-Directed Mutagenesis system (Invitrogen, Karlsruhe, Germany). NF-E2 binding sites at position -5k (TTAGTCA / from -5235bp to -5228bp relative to TTS) and at -120bp (TGACTCA) were mutated to GTCGGAC and TTGCGAC, respectively.

Transient transfections and luciferase assays

293T cells were transiently transfected using the $CaCl_2$-BES Method. A total of $3x10^5$ 293T cells were transfected using 0.05 μg of the various IL-8-promoter-luciferase reporter constructs. In addition, 0.68 μg of either an NF-E2-pRC-CMV expression vector or an empty pRC-CMV control vector and 0.17 μg of a MafG pCMV6XL4 (Origene) or an empty pCMVXL4 control vector were co-transfected. As an internal control, 0.1 μg of pRL-TK-Renilla vector (E2241, Promega) were used for each reaction. Cells were harvested 24 h after transfection and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega) within a Microplate Luminometer LB 96 V (Berthold). Luciferase activity was normalized to the Renilla internal control to compensate for variations in transfection efficiency.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed following a modified protocol previously described by Shang *et al.*[23] HEL cells were treated with formaldehyde (1%) for 10 min at room temperature and subsequently washed two times with cold phosphate buffered saline (PBS). The cell pellets were flash frozen in liquid nitrogen and stored at -80°C. For the immunoprecipitation, cell pellets were thawed on ice and resuspended in 200 μL lysis buffer (50mM Tris pH 8.0; 10mM EDTA; 1% SDS) per $2x10^6$ cells. Subsequently, lysate was incubated for 10 min on ice and sonicated five times at 30 s each at maximal output. The lysate

was centrifuged 10 min at 13,000 rpm and 4°C, 180 mL of the lysate were diluted in 720 mL dilution buffer (20 mM Tris pH 8.0; 2 mM EDTA; 150 mM NaCl; 0.01% SDS; 1% Triton X100). The pre-clearing reaction was performed at 4°C for 2 h, using 50 μL of a protein-A agarose slurry including salmon sperm DNA (16-157; Milipore). Antibody binding was obtained by an over-night incubation at 4°C using 2 μg of anti-NF-E2 antibody (sc-291-X, Santa Cruz) as well as an anti-IgG control (#2729; Cell Signaling). Immuncomplexes were precipitated at 4°C for 2 h, using 50 μL of a protein-A agarose slurry including salmon sperm DNA (16-157; Milipore). The sepharose beads were washed once using 1 mL dilution buffer, two times with 1 mL low salt buffer (20 mM Tris pH 8.0; 2 mM EDTA; 150 mM NaCl; 0.1% SDS; 1% Triton X100), once with 1 mL high salt (20 mM Tris pH 8.0; 2 mM EDTA; 500 mM NaCl; 0.1% SDS; 1% Triton X100) as well as LiCl buffer (10 mM Tris pH 8.0; 1 mM EDTA; 250 mM LiCl; 1% NP40; 1% Na Deoxycholat) followed by two washes using 1 mL TE Buffer (10 mM Tris pH 8.0; 1 mM EDTA). The extraction was performed two times, using 250 μL of elution buffer (0.1 M $NaHCO_3$; 1% SDS). Eluates were pooled and heated at 65°C for 4h after addition of 0.2 M NaCl. Following elution, a Proteinase K digestion was performed at 45°C for 30 min, using 20 μg Proteinase K. The DNA was purified by phenol-chloroform extraction and precipitated overnight at -20°C. The DNA pellets were washed in 70% ethanol and resuspended in water. The following primer pairs were used for DNA amplification:

–5.3kb to -5.1kb of the human IL-8 promoter:
sense 5'-ACATATCACTACAGAATCATAAC-3'
antisense 5'-AAGTTGCTATGTTAGAAATGAC-3'

bp -240bp to -30bp of the human IL-8 promoter:
sense 5'-TCACCAAATTGTGGAGCTTCAG-3'
antisense 5'-AGAGAACTTATGCACCCTCATC-3'

-2.7kb to -2.5 kb of the human IL-8 promoter:
sense 5'- GAATGGGACGTAAATAAACAG -3',
antisense 5'- GAGTCCTTGCATAATGAGTAG -3'

-13.4kb to -13.2kb of the human IL-8 promoter:
sense 5'- CTAGGAAGGGGAAGGGTCTC -3',
antisense 5'- CTGCCTATCTCACTTTCCAATCTC -3'

human myogenin control:
sense 5'-AGGGGCTGCTGAGAAATGAAAAC-3'
antisense 5'-ATATAGCCAACGCCACAGAAACCT-3'

Statistical analysis

Student's one sample t-test and paired t-test were applied to determine whether there was a significant ($P<0.05$) difference between two groups. When comparing more than two groups, a one-way ANOVA with *post hoc* Tukey's Multiple Comparison Test was conducted. These analyses were performed using the Graphpad Prism software (version 5.3 for Windows, Graphpad Software).

Results

Novel NF-E2 target genes

Because NF-E2 has been demonstrated to play a substantial role in the pathogenesis of MPN, but few target genes of this transcription factor are known, we sought to identify novel NF-E2 targets. To this end, we transduced purified healthy control $CD34^+$ hematopoietic stem cells with three different lentiviruses, an empty control (pLeGO-iG-hU6), a pLeGO-iG expressing the NF-E2 cDNA (pLeGO-iG-hU6-NF-E2) and a pLeGO-iG expressing an shRNA against NF-E2 (pLeGO-iG-hU6-shNF-E2), whose efficiency we have previously demonstrated.[17] Following FACS purification of transduced $CD34^+$ cells, RNA was harvested and subjected to microarray analysis on an Affymetrix human exon array (Figure 1A). For each viral construct, 4 independent, match-paired replicates were analyzed. Quantitation of NF-E2 mRNA demonstrated that the transcription factor was indeed over-expressed in $CD34^+$ cells transduced with the cDNA and suppressed in cells transduced with the NF-E2 shRNA (Figure 1B).

Gene expression analysis revealed a set of genes inversely regulated by elevated NF-E2 levels and diminished NF-E2 levels (*Online Supplementary Table S1*). In this group, mRNA expression of the cytokine IL-8 was increased 2.9-fold by NF-E2 cDNA expression and decreased 2-fold by NF-E2-shRNA expression (Figure 1C and D). Because abnormally elevated IL-8 levels have been reported in MPN patients,[24] we chose to analyze this potential novel NF-E2 target in more detail.

To verify the microarray gene expression data, we used qRT-PCR to measure $CD34^+$ cells independently transduced with the same three lentiviruses. Again, transduction of $CD34^+$ cells with an NF-E2 cDNA statistically significantly elevated IL-8 mRNA expression by a mean of 3.1-fold over the empty control virus, whereas the NF-E2 shRNA decreased IL-8 mRNA expression below the empty virus control (Figure 1D).

NF-E2 expression modulates IL-8 protein levels

In order to study the effect of NF-E2 on IL-8 protein levels, we used the myeloid cell line U937, which expresses low amounts of NF-E2 and elaborates very low levels of endogenous IL-8.[25] U937 cells were lentivirally transduced with the empty pLeGO-iG vector or the vector expressing the NF-E2 cDNA construct. Western blot analysis demonstrated that NF-E2 is indeed over-expressed in cDNA transduced U937 cells (Figure 2A). Intracellular IL-8 levels were detected by intracellular FACS staining. While untransduced and empty virus transduced U937 cells display very little intracellular IL-8, transduction with the NF-E2 cDNA caused a statistically significant increase in intracellular IL-8 staining (Figure 2B and C). Supernatants from transduced U937 cells were investigated for IL-8 secretion by ELISA. Again, untransduced or empty virus transduced cells elaborate very low levels of IL-8 protein; however, expression of the NF-E2 cDNA is sufficient to induce a significant, 5.3-fold increase in IL-8 protein secretion (Figure 2D).

Having demonstrated that elevated NF-E2 levels are sufficient to increase IL-8 expression, we investigated whether a decrease in NF-E2 expression similarly causes a reduction in IL-8 protein expression. We used three different hematopoietic cell lines, HEL, SET2 and UKE1, the latter two of which are derived form MPN patients. All three cell lines express high levels of NF-E2[9] and secrete high levels of IL-8 when transduced with an empty control vector or a vector expressing a scrambled, inactive shRNA (Figure 3A-C). Transduction with the NF-E2 shRNA significantly reduces IL-8 protein secretion in all three lines (Figure 3A-C). These data strongly suggest that NF-E2 is required for IL-8 expression.

NF-E2 binds and transactivates the IL-8 promoter

In silico analysis of whole genome NF-E2 chromatin

immunoprecipitation experiments[26] revealed two potential NF-E2 binding sites in the IL-8 promoter region and upstream sequences, one at bp -120 and one 5 kb upstream of the transcriptional start site. We, therefore, cloned these regions upstream of a luciferase reporter gene (Figure 4A) and transfected the resulting construct into 293T cells in the presence or absence of expression vectors encoding NF-E2 and MafG. As previously shown for other NF-E2 target genes, expression of NF-E2 or MafG alone is insufficient to induce transcriptional activation (Figure 4B). Co-transfection of both NF-E2 and MafG, however, resulted in a 7-fold increase in IL-8-promoter-driven luciferase activity (Figure 4B), demonstrating that NF-E2 is able to transactivate the IL-8 promoter.

In order to delineate the contribution of the two potential binding sites to NF-E2 mediated IL-8 promoter activation, we mutated each of the two putative NF-E2 binding sites in the IL-8 promoter, individually or *in trans*. The resulting constructs were again evaluated in luciferase assays following transient transfection into 293T cells in the presence of expression vectors for NF-E2 and MafG (Figure 4C).

While mutation of each of the putative NF-E2 binding sites individually reduced IL-8 promoter transactivation by around 45%, mutation of both sites simultaneously virtual-

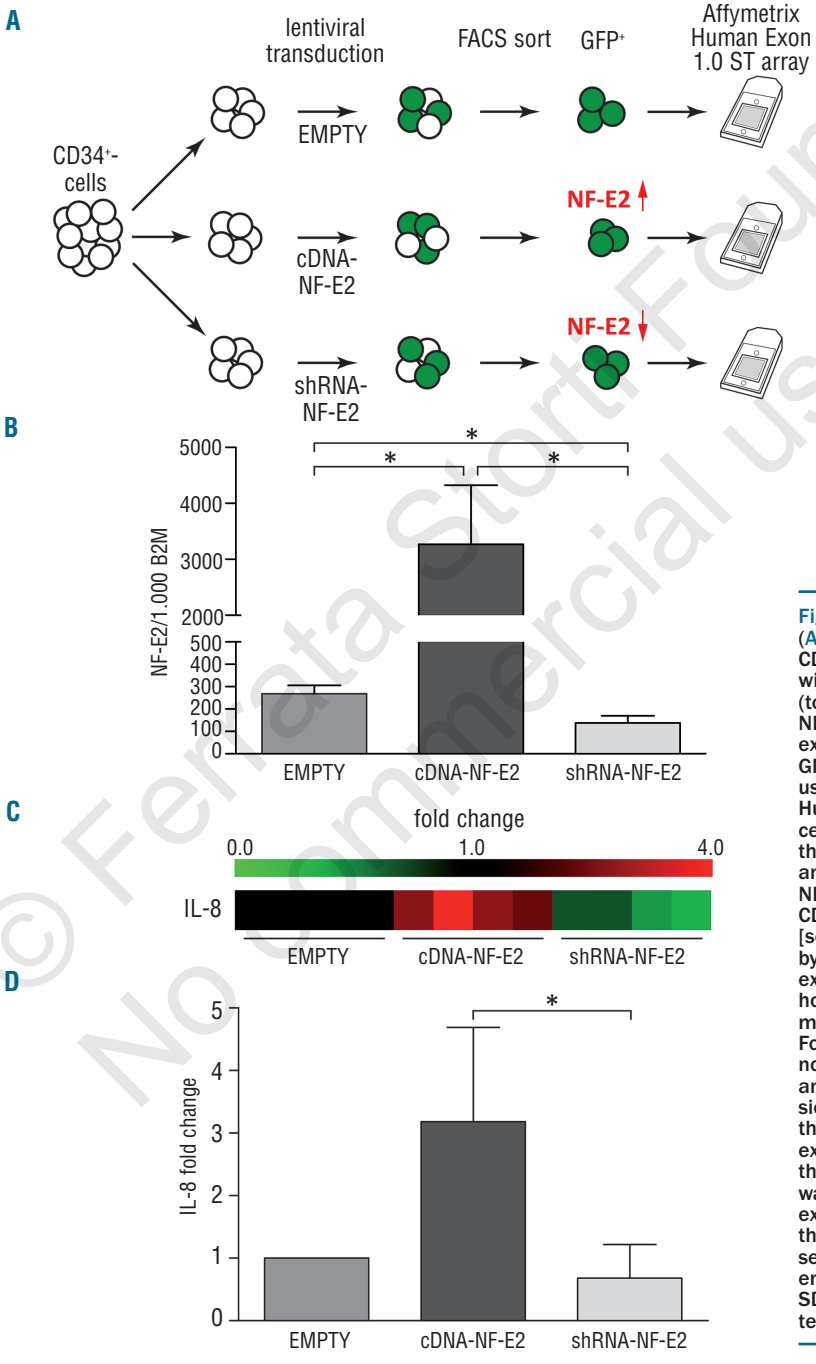

Figure 1. Identification of novel NF-E2 target genes. (A) Experimental Design. Purified peripheral blood CD34+-cells from healthy donors, were transduced with either the empty lentiviral vector, pLeGo-iG-hU6 (top), or pLEGO-iG-hU6 engineered to express human NF-E2 cDNA (middle) or a shRNA silencing NF-E2 expression (bottom). 84 h following transduction, GFP+-cells were FACS-sorted, RNA extracted, and used for microarray analysis on an Affymetrix Human Exon Array. Four separate pools of CD34+ cells were transduced in parallel with each of the three vectors, yielding a total of 12 microarrays for analysis, four independent arrays for each vector. (B) NF-E2 mRNA expression in lentivirally transduced CD34+ cells. The RNA used for microarray analysis [see (A) above] was quantitated for NF-E2 expression by qRT-PCR. Results are reported as copies NF-E2 expressed per 10^3 copies of the beta-2-microglobulin housekeeping gene; n = 4 each; *P<0.05. (C) IL-8 mRNA expression in microarray analysis. (Top) Following data analysis, mRNA expression values are normalized, assigned to a color on a green-red scale and displayed as a color field. (Bottom) IL-8 expression for each of the four independent microarrays in the three conditions is depicted. (D) IL-8 mRNA expression by qRT-PCR. mRNA expression of IL-8 and the housekeeping gene beta-2-microglobulin (β-2-M) was quantified in CD34+ cells by qRT-PCR. IL-8 expression was normalized to β-2-M expression and the expression in empty virus transduced CD34+ cells set at 1. Values are reported as fold change over empty virus transduced cells and depict mean and SD of 4 individual experiments. *P≤0.05 by paired t-test.

ly abolished NF-E2-mediated IL-8 promoter activation (Figure 4C). Both of the NF-E2 binding sites identified *in silico* thus contribute functionally to induction of IL-8 promoter activity by NF-E2.

Because the two NF-E2 binding sites are functional in the IL-8 promoter, we sought to investigate whether they are occupied by NF-E2 *in vivo*. To this end, we used chromatin immunoprecipitation (ChIP) to interrogate NF-E2 binding to the IL-8 locus in HEL cells (Figure 5A). Both the proximal -120 bp site as well as the distal -5kb site of the IL-8 locus are bound by NF-E2 in intact chromatin in HEL cells (Figure 5B), whereas other sites within the IL8 locus as well as sites in the myogenin control gene are unaffected (Figure 5B). Taken together these data establish a role for NF-E2 in regulating expression of the pro-inflammatory cytokine IL-8, thereby identifying IL-8 as a novel NF-E2 target gene.

Discussion

Plasma and serum levels of the pro-inflammatory cytokine IL-8 are elevated in patients with PV and PMF, displaying a considerable variation in plasma concentrations.[24,27] In PMF, where only half of the patients carry the JAK2^{V617F} mutation, increased IL-8 expression is independent of the presence or absence of the JAK2 mutation.[28] Elevated IL-8 levels have been implicated in the altered megakaryocyte growth and differentiation observed in PMF, as either inhibition of IL-8 by neutralizing antibodies or reduction of IL-8 expression by RNAi technology restored normal megakaryocyte proliferation and differentiation to PMF cells.[27] Moreover, addition of neutralizing anti–IL-8 receptor antibodies to PMF cells in liquid culture increased proliferation and differentiation of CD41^{+}

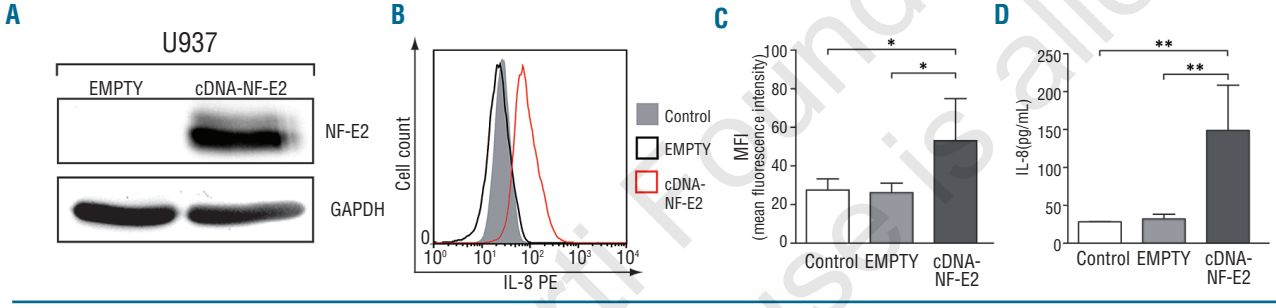

Figure 2. IL-8 protein expression following NF-E2 expression in U937 cells. U937 cells were transduced either with the empty lentivirus or with a virus expressing the NF-E2 cDNA. (A) Total cell extracts were interrogated with an antibody against NF-E2 (top). Equal loading was assured by reprobing with an antibody against GAPDH (bottom). (B-C) Lentivirally transduced U937 cells were cultured for 4 h in medium containing brefeldin A (10 ng/mL) to block the secretion of IL-8. Subsequently, intracellular IL-8 was stained and analyzed by FACS. (B) Representative histogram of n=5, depicting intracellular IL-8 staining of untreated cells (control) as well as cells infected with empty vector (empty) or with NF-E2 expressing virus (NF-E2), as indicated. (C) Mean and SD of the mean fluorescence intensity (MFI) of IL-8 staining in n=5 independent experiments are displayed, *$P \leq 0.05$ by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. (D) Lentivirally transduced U937 cells were cultivated for 24 h and the supernatant subsequently removed for detection of IL-8 protein secretion by ELISA. Bars depict the mean and SD IL-8 concentration of 3 independent experiments from untreated cells (control) or from cells transduced with empty virus (empty) or with virus expressing the NF-E2 cDNA.

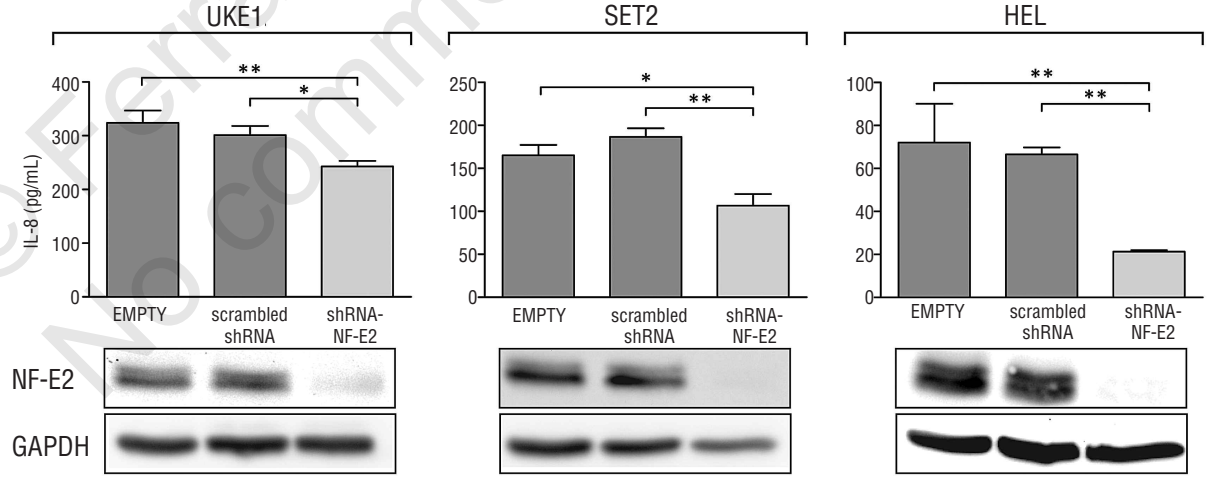

Figure 3. IL-8 protein expression following knockdown of NF-E2 in MPN cell lines. (A) UKE1 cells (B) SET2 cells and (C) HEL cells were transduced with the empty lentiviral vector as well as a virus expressing a shRNA against NF-E2 (shRNA-NF-E2). 24 h after transduction, the supernatant was removed and IL-8 concentration determined by ELISA (top). Bars depict the mean and SD IL-8 concentration of 3 independent experiments measured in duplicate. *$P \leq 0.05$ by t-test. Total cell extracts were interrogated with an antibody against NF-E2 (top). Equal loading was assured by reprobing with an antibody against GAPDH (bottom).

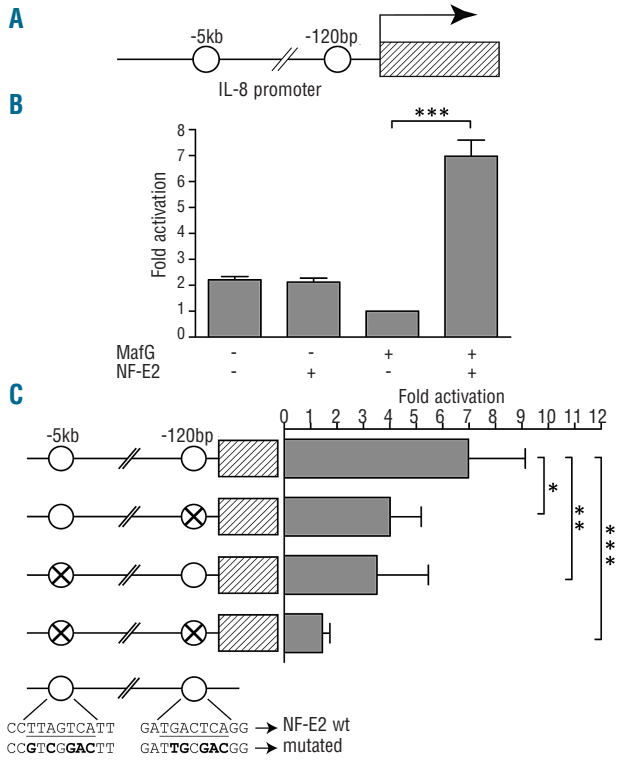

Figure 4. NF-E2 transactivation of the IL-8 promoter. (A) Schematic representation of the IL-8 promoter/enhancer reporter gene construct. The construct includes a 1 kb upstream enhancer region, from -5.5kb to -4.5kb, and the proximal 262 bp of the IL 8 promoter. NF-E2 sites predicted by *in silico* analysis are indicated by open circles. (B) The IL-8 promoter/enhancer luciferase vector was transfected into 293T cells together with plasmids encoding NF-E2 and/or MafG as indicated. Bar graphs represent the mean and SD of at least 5 independent experiments, each performed in duplicate. Data are shown normalized to the IL-8 reporter construct co-transfected with MafG alone, which was set at 1. ***P<0.001 by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. (C) The potential NF-E2 binding sites were altered by site-directed mutagenesis. Presence of the mutations is indicated by crosses in the circles. Sequences of the wt and the mutated sites are depicted below (mutated bases in bold). The IL-8 reporter constructs shown were co-transfected into 293T cells together with expression plasmids for NF-E2 and MafG. Bar graphs represent the mean and SD of 4 independent experiments, each performed in duplicate. Data are normalized to the wt IL-8 reporter construct transfected with MafG alone, which was set at 1. ***P<0.001, **P<0.01, *P<0.05 by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test.

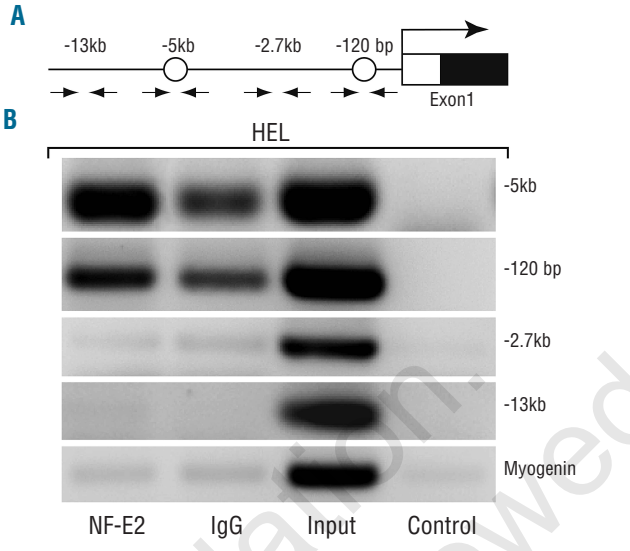

Figure 5. Chromatin immunoprecipitation (ChIP) analysis of NF-E2 binding sites on the IL-8 promoter and enhancer. (A) Schematic representation of the primers used to ChIP the two NF-E2 binding sites. (B) HEL cell lysates were chromatin immunoprecipitated (ChIPed) either with an antibody to NF-E2 or with an unrelated IgG control, as indicated. ChIPed DNA was amplified by PCR using either primers covering the NF-E2 binding sites in the IL-8 promoter and enhancer or with control primers from other regions within the IL8 locus and from the myogenin promoter, as indicated. In lane 3 (Input), a 1:50 dilution of the input DNA was used, lane 4 (Control) shows control PCR reactions without DNA.

megakaryocytic cells and restored their polyploidization.[27] These data implicate pathologically elevated IL-8 levels in the altered megakaryocyte maturation of PMF.

Low doses of IL-8, which is produced by bone marrow stromal cells,[29] have been shown to stimulate CD34$^+$ proliferation and increase cell survival.[30,31] In mice, exogenous IL-8 administration causes neutrophilia and stem cell mobilization.[32] Increased numbers of circulating CD34$^+$ hematopoietic stem cells are consistently observed in all MPN subtypes, with the highest efflux seen in PMF patients.[33] By stimulating IL-8 production, increased NF-E2 levels may thus contribute to MPN stem cell mobilization.

Growth of erythroid colonies in the absence of Epo (so called endogenous erythroid colonies, EECs) is a pathognomonic feature of PV which can be used diagnostically.[34] Treatment of healthy bone marrow with a combination of IL-8 and SCF allows for Epo-independent EEC growth.[35] Hence, the elevated IL-8 levels observed in PV and PMF may contribute to several prominent disease features, including Epo-independent erythroid growth and stem cell efflux.

Discovery of the JAK2^{V617F} mutation in MPN patients spurred the development of several specific JAK2 inhibitors. These were initially assessed for clinical efficacy in patients with PMF; patients who demonstrate the highest medical need. Surprisingly, several trials have now demonstrated that the response rate to JAK2 inhibitors is similar among PMF patients carrying the JAK2^{V617F} mutation and those with wild-type JAK2.[36,37] One possible explanation for this observation is that the recently FDA approved JAK1/JAK2 inhibitor ruxolitinib (INC424) has been shown to lower the levels of inflammatory cytokines, including IL-8. Increased cytokine levels are independent of the JAK2 status.[28,36,38] The reductions in spleen size observed with ruxolitinib treatment may thus reflect an anti-inflammatory effect, mediated in part by the observed reduction of IL-8 levels.

IL-8 was the only cytokine that appeared statistically significantly altered by changes in NF-E2 levels (*Online Supplementary Table S1*). However, we analyzed changes in expression levels as soon as successful transduction could be observed by GFP positivity in order to capture early, primary events. It is entirely possible that other cytokines may also constitute NF-E2 targets that are up-regulated at a later time point, or that these genes may be regulated indirectly through secondary effects following NF-E2 upregulation.

Tefferi and colleagues have recently correlated plasma inflammatory cytokine levels with clinical outcome.[39] In a

multivariate analysis of treatment-naïve patients, elevated IL-8 levels independently predicted inferior outcome. Moreover, IL-8 levels remained a significant predictor of inferior survival, even when risk stratification according to the recently up-dated Dynamic International Prognostic Scoring System (DIPSS plus) was included in the analysis.[39] IL-8 was the only cytokine analyzed which was associated with an elevated level of circulating blasts (> 1%) and predicted leukemia-free survival. In addition, increased IL-8 levels were associated with both leukocytosis and constitutional symptoms.[39] JAK2 inhibitors decrease white blood cell counts and lower IL8 levels. Since neutrophils and monocytes are the main sources of IL8, JAK2 inhibitor treatment, may relieve constitutional symptoms ultimately by decreasing IL-8 levels.[36] Likewise, in a recent phase I trial, elevated IL-8 levels were found to predict a poor anemia response to palidomide in PMF.[28] IL-8 may thus contribute to several pathophysiological processes in PMF patients, changes that are reversible with novel treatment strategies.

Taken together, our data demonstrate that the pro-inflammatory cytokine IL-8 is a novel NF-E2 target gene and that augmentation of NF-E2 levels increases IL-8 production. The elevated IL-8 levels observed in PV and PMF patients have been demonstrated to contribute to various aspects of disease pathophysiology. Therefore, one mechanism by which NF-E2 overexpression contributes to MPN pathology may constitute increased IL-8 expression.

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