ETV6-PDGFRB and FIP1L1-PDGFRA stimulate human hematopoietic progenitor cell proliferation and differentiation into eosinophils: the role of nuclear factor-κB

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ABSTRACT

Background

ETV6-PDGFRB (also called *TEL-PDGFRB*) and *FIP1L1-PDGFRA* are receptor-tyrosine kinase fusion genes that cause chronic myeloid malignancies associated with hypereosinophilia. The aim of this work was to gain insight into the mechanisms whereby fusion genes affect human hematopoietic cells and in particular the eosinophil lineage.

Design and Methods

We introduced *ETV6-PDGFRB* and *FIP1L1-PDGFRA* into human CD34$^+$ hematopoietic progenitor and stem cells isolated from umbilical cord blood.

Results

Cells transduced with these oncogenes formed hematopoietic colonies even in the absence of cytokines. Both oncogenes also stimulated the proliferation of cells in liquid culture and their differentiation into eosinophils. This model thus recapitulated key features of the myeloid neoplasms induced by *ETV6-PDGFRB* and *FIP1L1-PDGFRA*. We next showed that both fusion genes activated the transcription factors STAT1, STAT3, STAT5 and nuclear factor-κB. Phosphatidylinositol-3 kinase inhibition blocked nuclear factor-κB activation in transduced progenitor cells and patients' cells. Nuclear factor-κB was also activated in the human *FIP1L1-PDGFRA*-positive leukemia cell line EOL1, the proliferation of which was blocked by bortezomib and the IκB kinase inhibitor BMS-345541. A mutant IκB that prevents nuclear translocation of nuclear factor-κB inhibited cell growth and the expression of eosinophil markers, such as the interleukin-5 receptor and eosinophil peroxidase, in progenitors transduced with *ETV6-PDGFRB*. In addition, several potential regulators of this process, including HES6, MYC and FOXO3 were identified using expression microarrays.

Conclusions

We show that human CD34$^+$ cells expressing *PDGFR* fusion oncogenes proliferate autonomously and differentiate towards the eosinophil lineage in a process that requires nuclear factor-κB. These results suggest new treatment possibilities for imatinib-resistant myeloid neoplasms associated with *PDGFR* mutations.

Key words: PDGF receptor, eosinophils, hematopoietic progenitors, myeloproliferative neoplasms, signal transduction, tyrosine kinase inhibitors.

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The online version of this article has a Supplementary Appendix.

Introduction

Chromosomal rearrangements of *PDGFRA* and *PDGFRB*, the genes encoding the platelet-derived growth factor receptors (PDGFR) α and β, respectively, have been identified in patients with a range of rare myeloid disorders such as chronic eosinophilic leukemia, atypical chronic myeloid leukemia and chronic myelomonocytic leukemia.[1,2] These patients have now been grouped into a single clinical entity named myeloid neoplasms associated with eosinophilia and abnormalities of *PDGFRA* or *PDGFRB*.[2] Most patients are males and feature hypereosinophilia, which can sometimes lead to severe tissue damage. Left untreated, the disease can evolve towards secondary acute myeloid leukemia. However, patients with these myeloid neoplasms are highly sensitive to imatinib mesylate (Gleevec/Glivec®) and other tyrosine kinase inhibitors that target PDGFR. Resistance to this treatment can occur through the acquisition of secondary mutations.[3]

More than 20 different PDGFR fusions have been described in hematopoietic malignancies so far.[1,2,4,5] The t(5;12) translocation between *PDGFRB* and *TEL* (now renamed *ETV6*) produces the TEL-PDGFRβ (TPβ) fusion protein, in which the extracellular ligand-binding domain of PDGFR is replaced by the pointed (PNT, also named SAM) domain of TEL. The expression of the fusion transcript is driven by the TEL/ETV6 gene promoter. The fusion protein was first studied in the murine hematopoietic Ba/F3 cell line. It was shown that the pointed domain of TEL induced oncoprotein oligomerization, mimicking ligand-induced dimerization and increasing the tyrosine kinase activity of the receptor.[6] TPβ stimulates the proliferation of Ba/F3 cells in the absence of growth factors. In addition, TPβ, which resides in the cytosol despite the presence of a transmembrane domain,[7] escapes the efficient degradation by lysosomes to which activated PDGFR are normally targeted.[8] Several studies showed that TPβ activates multiple signal transduction pathways in these cells, including phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinases (MAPK) and the transcription factors STAT1, STAT5 as well as nuclear factor-κB (NF-κB).[9-12] Mouse transplantation models have demonstrated that TPβ also stimulates hematopoietic cell proliferation *in vivo*, leading to a myeloproliferative disease, in a process that requires the activation of STAT5.[13] However, this model imperfectly mimics the human disease as mice do not develop eosinophilia in these conditions.

FIP1L1-PDGFRα (FPα, also named FIP1L1-PDGFRA) is another recurrent fusion protein, which has been identified in patients with hypereosinophilic syndromes.[1,2,4] This fusion protein is not activated by oligomerization but by an alternative mechanism involving the deletion of the inhibitory PDGFR juxtamembrane domain, which normally keeps the kinase domain inactive.[14] Even though FPα was reported to impose eosinophil lineage commitment on murine hematopoietic stem/progenitor cells *in vitro*,[15] mice transplanted with bone marrow cells expressing FPα do not develop eosinophilia. In human hematopoietic progenitor cells, FPα induces autonomous formation of colonies of various lineages, including neutrophils, erythrocytes and eosinophils, in the absence of cytokines.[16] The mechanism by which FPα specifically favors eosinophil development remains elusive and it was suggested that a second alteration may be required.[17,18]

Design and Methods

Isolation, culture and viral infection of human CD34+ cells

The isolation, culture and viral infection of human CD34+ cells are described in more detail in the *Online Supplementary Design and Methods*. Briefly, umbilical cord blood units unsuitable for preservation were used following a procedure approved by the ethics committee of the medical faculty (reference B40320108411) within 24 h of collection. Leukocytes were isolated from fresh cord blood by centrifugation over a Ficoll-Paque density-gradient (GE Healthcare). CD34+ cells were purified using the EasySep kit (StemCell Technologies) and transduced with lentiviral particles as described previously.[19] The oncogene expression was checked by western blotting with anti-PDGFR-α and -β (Santa Cruz, sc951 and sc958, respectively) as described elsewhere.[8] To study differentiation and proliferation, 3×10^4 transduced cells were seeded into 24-well plates in 1 mL in the presence of FMS-like tyrosine kinase-3 ligand (FLT3L; 25 ng/mL), stem cell factor (SCF; 25 ng/mL), interleukin (IL) 3 (20 ng/mL), IL5 (20 ng/mL), IL6 (1 ng/mL) and thrombopoietin (10 ng/mL). All recombinant human cytokines were purchased from PeproTech except IL6, which was a kind gift from Prof. Jacques Van Snick (Brussels, Belgium). Half of the medium was renewed every 3 days.

Cell morphology was assessed as follows. Cells (3×10^4/200 μL) were deposited on a glass slide using a Shandon Cytospin 3 device and were then fixed with methanol and stained with May-Grünwald-Giemsa (1/20 dilution, Sigma). Slides were observed using an Axiovision microscope (Zeiss, 1000x magnification with oil).

Colony-forming unit assay

Twenty-four hours after the final transduction, cells were washed and plated in duplicate in methylcellulose semisolid medium: 10^4 cells were plated in the absence of cytokines (Methocult H4230, Stem Cell Technologies Inc.) and 10^3 cells were plated in presence of SCF (50 ng/mL), granulocyte-monocyte colony-stimulating factor (GM-CSF; 10 ng/mL), IL3 (10 ng/mL) and erythropoietin (3 U/mL) (Methocult GF H4434). After 12 days, the colonies were identified morphologically by light microscopy. Over 50 cells in a cluster were scored as a colony.

Flow cytometry, signaling assays and quantitative real-time polymerase chain reaction analysis

Flow cytometry and quantitative real-time polymerase chain reaction (PCR) analysis are described in more detail in the *Online Supplementary Design and Methods*. NF-κB phosphorylation (p-Ser536 p65) was also measured using an AlphaScreen SureFire assay (Perkin Elmer) in thawed FPα-positive blasts cells from a patient who has been described previously (ethics committee approval ref # F/2005/02).[3,8] Thawed cells were cultured in Iscove's modified Dulbecco's medium supplemented with fetal bovine serum and imatinib (10 μM) or LY294002 (25 μM) for 4 h. Cells were lysed (150,000 cells/20 μL) and processed according to the manufacturer's instructions to quantify NF-κB phosphorylation in an Envision plate reader (Perkin Elmer). The assay was performed in triplicate in 384-well plates.

RNA isolation and microarray

TPβ-transduced CD34+ cells were treated with imatinib (500 nM) or vehicle for 4 h. Total RNA was extracted using Trizol reagent (Invitrogen) and purified with the RNeasy kit (Qiagen). Samples were analyzed using HG U133A 2 PLUS expression array (Affymetrix) as previously described.[20-22] The MAS5 algo-

rithm in GeneChip® Operating Software (Affymetrix) was used to normalize each replicate condition (untreated cells) against its baseline (imatinib treatment). We selected genes that were significantly increased or decreased in the three biological replicates. The data were submitted to the Gene Expression Omnibus (#GSE28698). The expression of the same genes in EOL-1 was retrieved from previously published data (#GSE15237).[23] Prior to gene set enrichment analysis (GSEA), probe sets marked as absent in all conditions were discarded. Intensities of all other probe sets in all the conditions were submitted for enrichment analysis using the curated GSEA catalogue.

Statistical analysis

All the statistical analyses were performed using Microsoft Excel or R. The results were analyzed using Student's t-test or ANOVA (*P<0.05; **P<0.01; ***P<0.001). All experiments were repeated at least three times.

Results

TEL-PDGFRβ and FIP1L1-PDGFRα stimulate the proliferation of human hematopoietic cells in the absence of growth factors

Human CD34$^+$ hematopoietic progenitor cells isolated from cord blood were transduced using lentiviral particles encoding TPβ, FPα or green fluorescent protein (GFP) alone. The pTM895 lentiviral vector was preferred to a retroviral one because it is more efficient in transducing slowly dividing CD34$^+$ cells with fewer side effects related to the genomic insertion site.[24] Expression of the fusion *PDGFR* oncogenes was confirmed by western blot (Figure 1A) and by quantitative PCR, which showed that TPβ was expressed at a level comparable to that of endogenous PDGFRβ in human fibroblasts (*data not shown*).

We first analyzed the ability of transduced cells to generate colony-forming units (CFU) in semisolid medium. In the presence of an optimal cytokine cocktail, the various types of myeloid colonies were observed with normal frequency, except CFU-GEMM, the number of which was significantly increased (Figure 1B). This suggested that TPβ and FPα may increase the proliferation of multipotent myeloid progenitors but does not block cell differentiation. When cells were washed and plated without cytokines, expression of a fusion oncogene allowed the development of colonies, mostly CFU-GM, in sharp contrast to control cells, which did not form colonies in these conditions. Thus TPβ and FPα could replace hematopoietic growth factors and favored differentiation into the granulocyte/macrophage lineage. This is consistent with a published report of Buitenhuis and colleagues, who analyzed CD34$^+$ cells expressing FPα.[16]

To further characterize the impact of *PDGFR* fusion genes on myeloid cell differentiation, we performed liquid cell cultures, which greatly facilitated the analysis of the differentiated cells by flow cytometry and other techniques. In the absence of growth factors, CD34$^+$ cells transduced with an oncogene proliferated significantly, while control cells remained mostly quiescent (Figure 1C), in agreement with our CFU results. TPβ and FPα also stimulated the growth of cells cultured with cytokines known to promote progenitor cell expansion, i.e. SCF, FLT3L, thrombopoietin and IL6.[15]

TPβ and FPα induce eosinophil differentiation

As *PDGFR* fusion oncogenes are associated with hypereosinophilia, we next carried out cell cultures with IL3 and IL5, which favor eosinophil development. In the presence of saturating amounts of these cytokines, TPβ and FPα still enhanced cell growth (Figure 1C and *Online Supplementary Figure S1*). Minor differences between the two fusion oncogenes were not reproducible. We next assessed the presence of eosinophil lineage markers. Both oncogenes increased the expression of the IL5 receptor α chain (IL5Rα) independently of the culture conditions, as shown by flow cytometry (Figure 2A,B). As the presence of IL5 in the culture medium was reported to down-regulate IL5Rα surface expression,[25] we also performed quantitative reverse transcriptase PCR, which confirmed the increased IL5Rα expression in cells expressing PDGFR fusion proteins (Figure 2C,D). Similarly, the expression of eosinophil peroxidase, a specific eosinophil marker, was enhanced by TPβ and FPα (Figure 2C,D). The expression of eosinophil markers was also increased in cells cultured with SCF, FLT3L, IL6 and thrombopoietin (*data not shown*). After 14 days of culture, a significant proportion of cells transduced with TPβ or FPα had eosinophilic granules and a characteristically shape nuclei (Figure 2E, F). Many cells presented morphological features that have been described in eosinophilic leukemia, such as vacuolization, cytoplasmic inclusions and the presence of immature cells.[26] Altogether these data strongly suggested that TPβ and FPα favor hematopoietic cell commitment towards the eosinophil lineage. Remarkably, no significant difference was observed between these two fusion oncogenes.

Signal transduction and gene regulation by TPβ and FPα in human hematopoietic cells

To investigate the mechanism by which TPβ and FPα interfere with human hematopoietic cell proliferation and differentiation, we analyzed the gene expression response downstream of these two oncogenes. CD34$^+$ cells were transduced with TPβ and cultured for 7 days without cytokines. Using Affymetrix microarrays, we compared gene expression in these cells and in cells treated for 4 h with imatinib to switch off TPβ signaling. Imatinib was used at a concentration of 0.5 μM, which efficiently inhibits *PDGFR* but not *ABL*.[4] We identified 79 probe sets that were consistently regulated in three independent experiments (Figure 3A). Interestingly, the expression of most of these transcripts is also regulated by imatinib in the EOL-1 cell line, which is derived from a human eosinophilic leukemia positive for FPα.[23,27] In addition *CD69*, *EGR1*, aquaporin-3 (*AQP3*), *DUSP-5* and *-6* have been shown to be expressed in human eosinophils and up-regulated by IL5.[28] The regulation of DUSP5 and CD69 was confirmed by quantitative PCR (*Online Supplementary Figure S2*). Taking the whole transcriptome into consideration, GSEA indicated that the TPβ transcriptional profile was significantly enriched in eosinophil-specific transcripts, compared to the profile of cells treated with imatinib (P<0.001).[29,30] In conclusion, the transcriptome of CD34$^+$ cells expressing TPβ significantly matches human eosinophils and the eosinophilic leukemia cell line EOL-1.

The transcriptional regulator *HES6* is one of the genes that was regulated to the greatest extent by imatinib in our analysis. Quantitative PCR confirmed its regulation in EOL-1 and cells expressing TPβ and showed that this gene is also regulated in cells transduced with FPα but not in

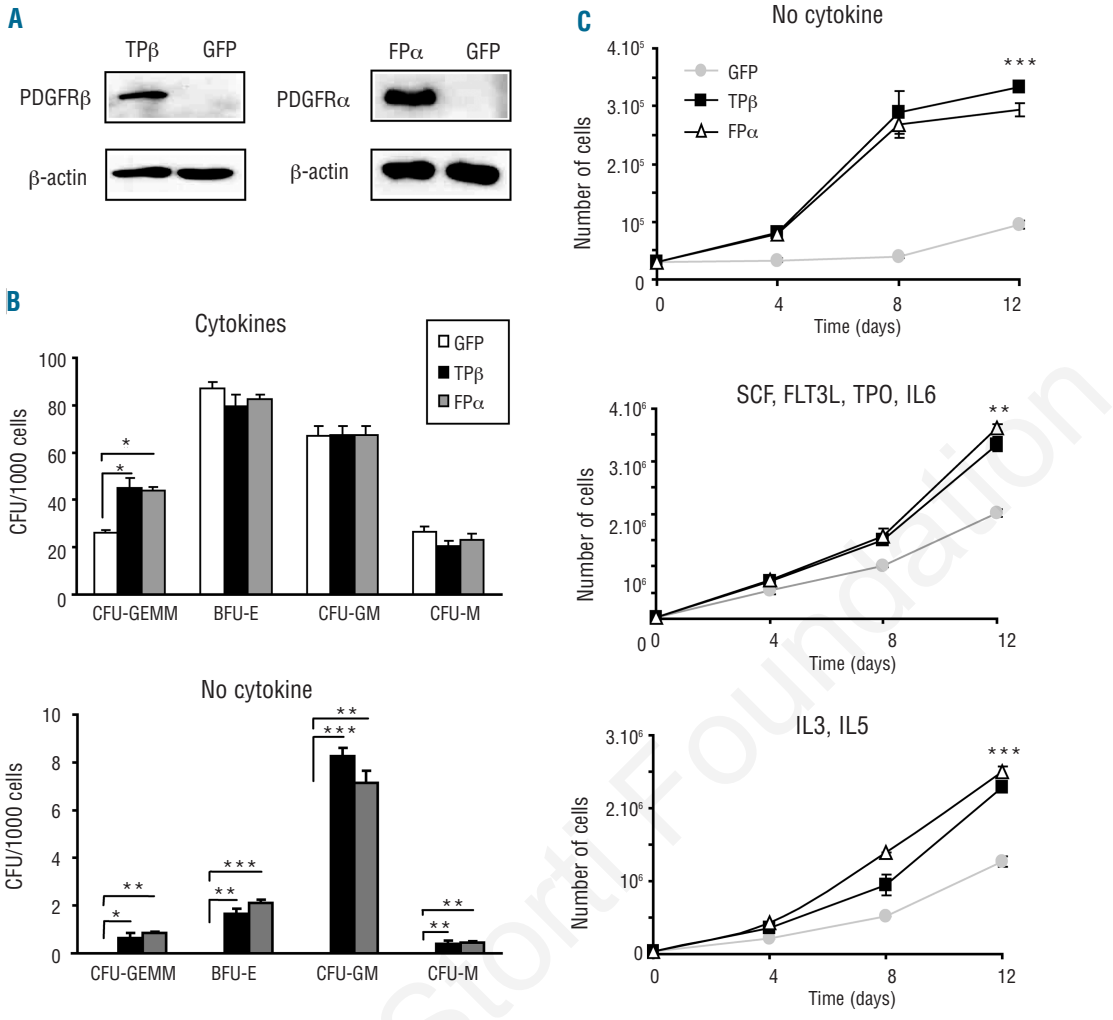

Figure 1. TPβ and FPα expression in human CD34⁺ progenitor cells stimulates cell proliferation and the formation of cytokine-independent colonies. (A) CD34⁺ cells were isolated from umbilical cord blood and transduced with TPβ, FPα or GFP lentiviral particles. Cells were cultured with SCF and FLT3L for 7 days. Total cell lysates were prepared and analyzed by western blot with anti-PDGFRβ and anti-PDGFRα antibodies. Western blot against β–actin is shown as a loading control. (B) CD34⁺ cells were transduced with TPβ (black bars), FPα (gray bars) or GFP (white bars). Ten thousand cells were plated in methylcellulose in the absence of cytokines. Alternatively, 1,000 cells were plated with SCF, GM-CSF, IL3 and erythropoietin. The number of colonies was scored after 12 days. The average of three independent experiments with standard errors of the mean is shown. CFU, colony forming unit; CFU-GEMM, granulocyte-erythrocyte-macrophage-megakaryocyte colonies; CFU-GM, granulocyte-macrophage colonies; CFU-M, macrophage colonies; BFU-E, burst-forming unit-erythrocytes. (C) Transduced CD34⁺ cells were seeded at 3x10⁴ cells/well in presence of the indicated cytokines in duplicate. Viable cells were counted in the presence of trypan blue every 4 days. The difference between cells expressing GFP (solid gray circles) and TPβ (solid black squares) or FPα (open triangles) was significant in all cases (ANOVA). One representative experiment is shown.

control GFP cells (*Online Supplementary Figure S2*). HES6 promotes neuronal differentiation and acts as an inhibitor of the transcriptional repressor HES1, an effector of the NOTCH pathway.[31]

To gain further insight into the transcription factors that are regulated by TPβ, we used TFactS, a bioinformatics tool that predicts the regulation of transcription factors from microarray data using a database of experimentally validated target genes.[23] Based on the list of genes regulated by imatinib in TPβ-expressing cells, TFactS predicted the activation of STAT1, STAT3, STAT5, NF-κB, MYC and the inhibition of FOXO3 (Figure 3B). MYC expression was regulated by imatinib in CD34⁺ cells expressing TPβ and in EOL-1, according to the microarray results (Figure 3A). Genes belonging to the JAK-STAT pathway were also found to be enriched in our gene list according to the DAVID computational method (Figure 3B) and GSEA (*data not shown*). To confirm the regulation of these transcription factors, transduced cells were analyzed by flow cytometry using activation-specific antibodies raised against key phosphorylated sites. We used cells either cultured in the absence of cytokines or grown for 7 days with SCF and FLT3L. The latter cells were washed extensively and starved for 16 h in cytokine-free medium before staining to switch off signaling by SCF and FLT3L. *Online Supplementary Figure S3* shows that TPβ and FPα strongly induced the phosphorylation of STAT5, STAT3 and STAT1 on the tyrosine residue that is required for dimerization and activation. We also observed the phosphorylation of PKB (also known as AKT), the kinase that inacti-

vates FOXO3 (*Online Supplementary Figure S3*), in agreement with the bioinformatics predictions.

TFactS also predicted the activation of NF-κB, which was illustrated by the strong up-regulation of its target gene CCL2 (*Online Supplementary Figure S2*). Accordingly, the phosphorylation of the p65 NF-κB subunit on serine 536, which is mediated by IκB kinase (IKK),[32] was enhanced by the fusion oncogenes (Figure 4). NF-κB activation by PDGFR was suggested to depend on the phosphorylation of IKK by PKB.[33] Cell treatment with LY294002, an inhibitor of the PI3K-PKB pathway, was as efficient as imatinib in blocking p65 phosphorylation (Figure 4). Similar results were obtained in EOL-1 cells (Figure 4C and *Online Supplementary Figure S4*). In control experiments, LY294002 did not affect phospho-STAT5 staining, as expected (*Online Supplementary Figure S4*). These results suggest that *PDGFR* fusion oncogenes activate the PI3K/PKB/NF-κB pathway in human hematopoietic cells. Collectively, our bioinformatics and experimental data supported the activation of MYC, STATs and NF-κB downstream of FPα and TPβ.

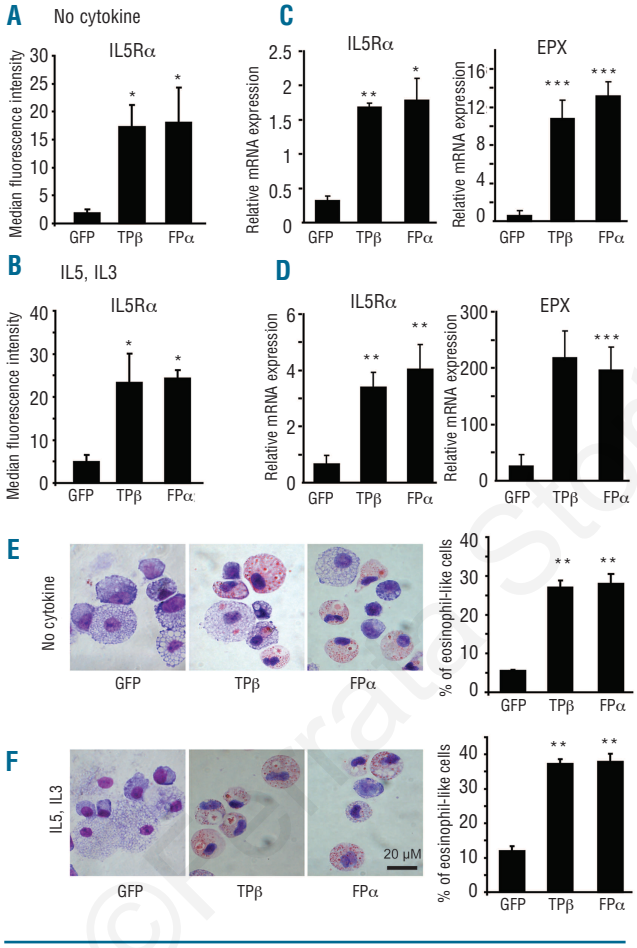

Figure 2. TPβ and FPα induce eosinophil differentiation. Transduced CD34⁺ cells were cultured in the absence of cytokines (A, C, E) or with IL3 and IL5 (B, D, F). (A-B) After 7 days of culture, cells were analyzed by flow cytometry after staining with anti-IL5Rα antibodies. The median fluorescence intensity is shown after background subtraction. IL5Rα expression was likely underestimated in cells exposed to IL5 due to ligand-induced receptor down-regulation.[31] (C-D) RNA was extracted from cells cultured for 7 days to measure the expression of IL5Rα and eosinophil peroxidase (EPX) by quantitative RT-PCR. Expression of the ribosomal protein RPLP0 was used as a control to normalize the results. One representative experiment is shown with standard deviations. (E-F) Cells cultured for 14 days with the indicated cytokines were stained using May-Grünwald-Giemsa reagent. The nuclear morphology and the presence of eosinophilic granules were indicative of cell differentiation into the eosinophil lineage. The percentage of eosinophil-like cells was calculated from randomly selected fields (right panels). Results are representative of four independent experiments.

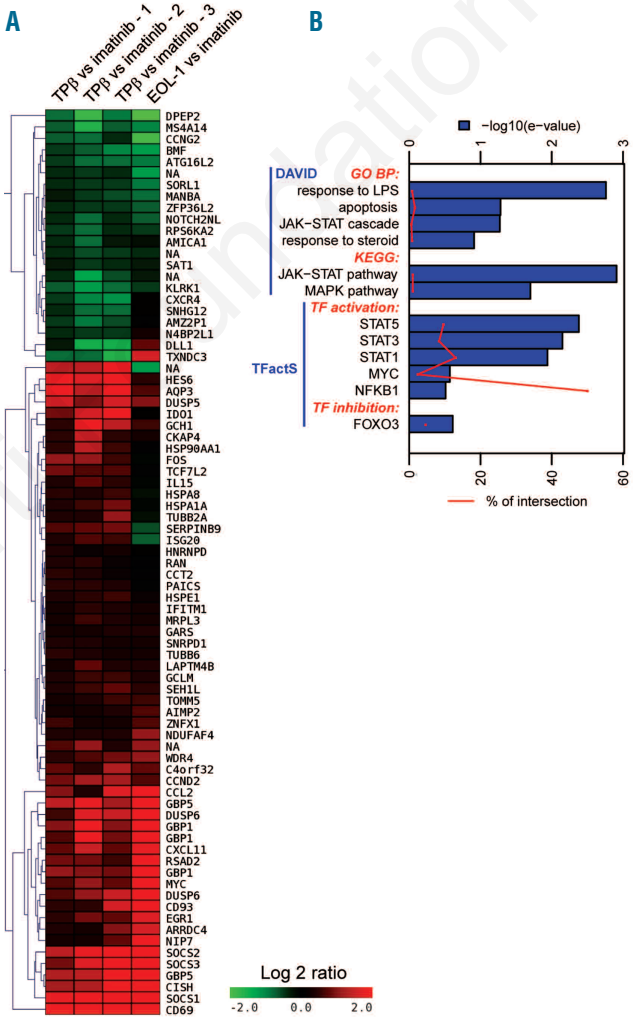

Figure 3. Genes and transcription factors regulated by TPβ and FPα. (A) CD34⁺ cells were transduced with TPβ and cultured for 7 days without cytokines. To switch off TPβ kinase activity, cells were treated for 4 h with imatinib (500 nM) or left untreated as a control. RNA was extracted and used to hybridize expression microarrays. Results were expressed as a $\log_2$ ratio between the untreated condition and the imatinib condition. Probe sets that were consistently regulated in three independent biological replicates are shown (imatinib-induced genes, which are thus predicted to be repressed by TPβ, are represented in green). Data from a similar experiment performed with EOL-1 cells are also shown.[23] (B) The list of imatinib-regulated probe sets shown in (A) was analyzed using DAVID and TFactS. The corrected probability of enrichment (e-value) is shown in blue and the percentage of targets in the intersection between our gene list and the list of genes included in a particular pathway or targeted by a given transcription factor (TF) is shown in red. For clarity, only statistically significant results are shown. See text for details.

Nuclear factor-κB plays a role in TPβ-induced hematopoietic cell proliferation and differentiation

The role of STAT transcription factors in hematopoietic cell transformation by FPα and TPβ has been well established in various model systems including human cells.[13,16] By contrast, NF-κB activation by TPβ has only been reported in the murine Ba/F3 cell line.[11] We sought to confirm NF-κB activation in patients' cells. However, patients carrying a FPα or TPβ fusion are rare and frozen eosinophils did not recover after thawing in our hands. We have previously described a patient with FPα-positive blasts cells,[8] which could be analyzed using a sensitive assay that relies on the simultaneous binding of anti-p65 and anti-phospho-p65 to the same target in cell lysate (Figure 4C). A significant specific signal was observed in these cells, and was blocked by treatment with LY294002 or imatinib, in line with our results in EOL-1 and CD34+ cells.

To further assess the function of NF-κB, we first tested the proteasome inhibitor bortezomib (PS-341, Velcade®)

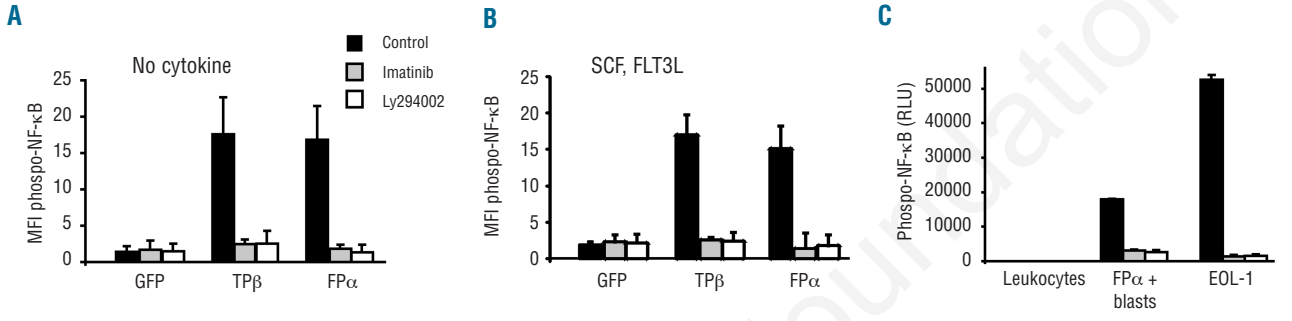

Figure 4. TPβ and FPα activate NF-κB in a PI3K-dependent manner. Transduced CD34+ cells were cultured for 7 days either in the absence of cytokine (A) or with SCF and FLT3L and then starved as described in *Online Supplementary Figure S3* (B). Cells were permeabilized, stained with labeled anti-phospho-S536-p65 antibodies and analyzed by flow cytometry. When indicated, the PI3K inhibitor LY294002 (25 μM, white bars) or imatinib (500 nM, gray bars) was added to the cultures for 4 h before harvest. The median fluorescence intensity (MFI) is shown after background subtraction. (C) FPα+ blasts cells from a patient and control leukocytes from a healthy donor were isolated from blood samples.[9] Cells treated with imatinib or LY294002. p65 phosphorylation on ser536 was quantified using a SureFire assay as described in the *Design and Methods* section. EOL-1 cells were treated similarly. One representative experiment out of three is shown with standard deviations.

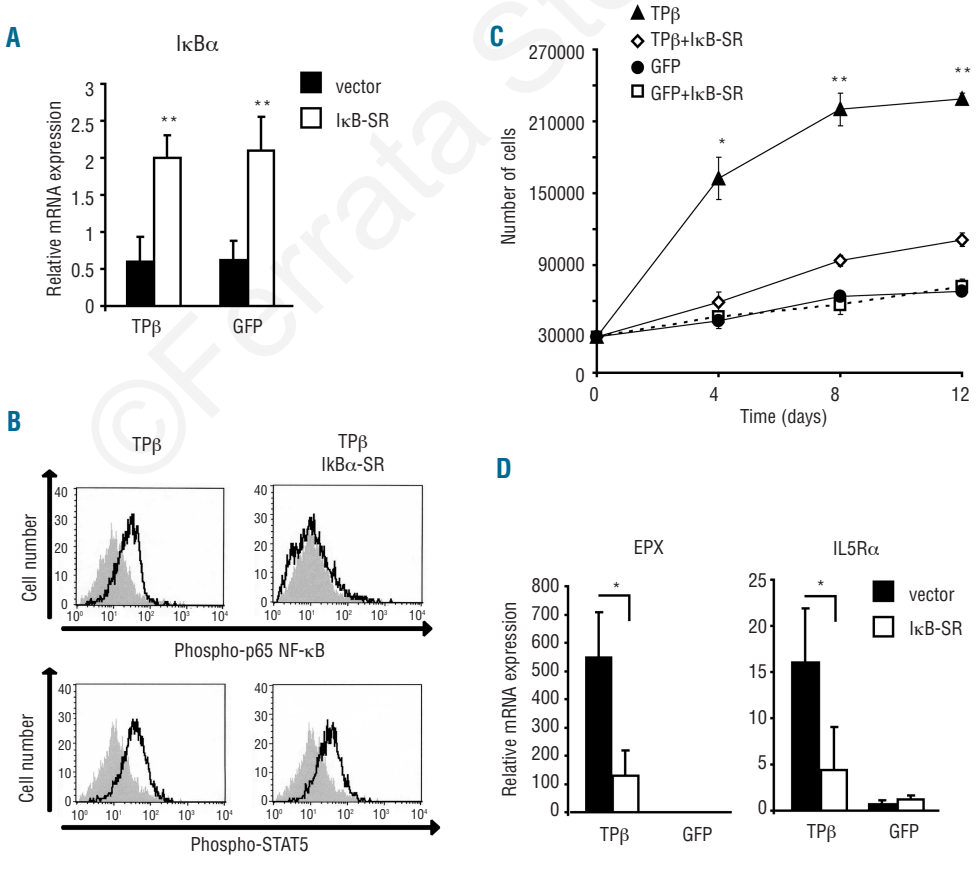

Figure 5. NF-κB activation is essential for TPβ-induced hematopoietic cell proliferation and differentiation. CD34+ cells were transduced to express TPβ and/or IkBα-SR, a potent inhibitor of NF-κB activation, or the corresponding empty vectors. Cells were cultured for 7 days in the absence of cytokine. (A) RNA was extracted to measure IkBα expression by quantitative RT-PCR using oligonucleotides that amplified both endogenous IkBα and the transduced IkBα-SR mutant. (B) Cells were permeabilized, stained with antibodies specific to phosphorylated p65 or STAT5 and analyzed by flow cytometry (black lines). Control cells are shown in gray. (C) Cells expressing IkBα-SR (open symbols) or the empty vector (black symbols) were seeded in duplicate cultures in the absence of cytokines and counted by trypan blue exclusion. (D) Expression of IL5Rα and eosinophil peroxidase (EPX) was evaluated by quantitative RT-PCR. Expression levels were normalized to RPLP0 expression. The average of two experiments is shown.

and the IKK inhibitor BMS-345541, which prevent NF-κB activation.[34] Both molecules blocked NF-κB phosphorylation and cell proliferation, but also affected STAT5 signaling (*Online Supplementary Figure S5* and *data not shown*). BMS-345541 also blocked colony formation from transduced CD34+ cells in the absence of cytokines (*Online Supplementary Figure S5B*). To inhibit NF-κB specifically, we transduced cells with an IκBα super-repressor (SR) mutant, which is resistant to IKK-induced degradation and prevents NF-κB translocation to the nucleus.[11] Using a retroviral vector, IκBα-SR was over-expressed in CD34+ cells, as determined by quantitative PCR (Figure 5A). As expected, cells transduced with both TPβ and IκBα-SR showed a marked decrease in p65 phosphorylation compared to cells expressing TPβ alone (Figure 5B). The phosphorylation of STAT5 was not affected by IκBα-SR expression, thus confirming the specific inhibition of NF-κB activation. IκBα-SR expression blunted the proliferation of cells transduced with TPβ (Figure 5C). Expression of two eosinophil markers, eosinophil peroxidase and IL5Rα, was also significantly inhibited (Figure 5D). These experiments suggested that NF-κB is an important mediator of the effects of TPβ on human hematopoietic cell growth and differentiation.

Discussion

Our results show that the introduction of FPα and TPβ into primary human CD34+ hematopoietic progenitor cells *in vitro* is sufficient to recapitulate several key features of the myeloproliferative neoplasm associated with these oncogenes. Indeed, these oncogenes induced cell proliferation in the absence of cytokine with a bias towards the eosinophil lineage. The CFU analysis also showed that the granulocyte-macrophage lineage (CFU-GM) was strongly expanded. This was consistent with the reported increase in granulocytes and monocytes in some patients. Importantly, *PDGFR* fusion genes did not block differentiation into other lineages in the presence of hematopoietic growth factors, as shown by the CFU analysis. No additional alterations have been described in this disease so far.[35] Although it remains possible that other mutations contribute to the disease, our results indicate that these oncogenes are major players in the development of hypereosinophilia.

Importantly, FPα and TPβ stimulated the production of eosinophil-like cells in the absence or in the presence of added IL3 and/or IL5 in the culture media. This result contrasted with the previous observation that transduction of FPα in CD34+ cells does not increase the number of eosinophilic colonies in the presence of IL3 and IL5.[16] However, this previous study focused on colony formation, which depends on the number of progenitors, while we counted the total number of cells in liquid culture. In patients, an *IL5* gene polymorphism may be associated with the severity of FPα-positive hypereosinophilia.[36] This is consistent with our observation that FPα and IL5 have an additive effect.

The discrepancy with mouse models, in which TPβ does not promote eosinophil differentiation[13,15] and FPα only amplifies hypereosinophilia induced by IL5 overexpression *in vivo*,[18] may be ascribed to the documented differences between mice and humans in the development of eosinophils.[37] Overall, lentivirally transduced CD34+ cell cultures appear as an attractive alternative model to study the effect of *PDGFR*-derived oncogenes, compared to mouse hematopoietic cells.

FPα-associated myeloproliferative neoplasms, which are often referred to as chronic eosinophilic leukemia, affect the eosinophil lineage more specifically than does TPβ, which also induces the expansion of monocytes. However, we did not observe any significant difference between FPα and TPβ in transduced CD34+ cells. A more thorough and direct comparison of the transcriptome of cells expressing the two oncogenes should be performed to pinpoint such differences. Notably, this model does not recapitulate all aspects of the chromosomal rearrangement that occurs in patients' cells. Indeed, there might be significant differences in the expression patterns of the fusion genes, which are controlled by different gene promoters in patients.[1] The fusion also affects neighboring genes, particularly in the case of the deletion that fuses *FIP1L1* to *PDGFRA*. In addition, one normal allele of *ETV6* or *FIP1L1* is lost in the fusion process. In the case of *ETV6*, this was suggested to contribute to the disease.[1] Finally, the *in vivo* human environment, in particular the bone marrow niche, may affect cells expressing FPα or TPβ differently.

We detected the activation of transcription factors of the STAT and NF-κB families. The role of STAT5 in myeloproliferative diseases and PDGFR fusion signaling is well established.[13,16] In addition, STAT5 is required but not sufficient to induce human CD34+ cell differentiation into eosinophils.[38,39] Using pharmacological inhibitors and a mutant IκB, we show here that NF-κB also contributes to the proliferation and differentiation of CD34+ cells transduced with TPβ. It is likely to play a similar role downstream of FPα. The function of NF-κB in normal hematopoiesis remains elusive.[40] Constitutive activation of NF-κB alone does not seem to induce eosinophil differentiation of CD34+ cells,[40] so it is likely that a combination of several transcription factors is needed. In this respect, NF-κB and STAT5 regulate several target genes in a synergistic manner, including *CCL2*, which we found highly regulated by both PDGFR fusions in CD34+ cells. Future studies will analyze whether the autocrine production of CCL2 and other cytokines could mediate the effects of NF-κB on hematopoietic progenitors. Additional transcriptional regulators, such as MYC, FOXO3 and HES6, may also support cell proliferation and differentiation in synergy with STAT5 and NF-κB.

NF-κB was reported to prevent apoptosis in human granulocytes and eosinophils.[41,42] In Ba/F3 cells, activation of NF-κB by TPβ was also shown to block apoptosis.[11] Accordingly, we observed that EOL-1 cell viability was decreased by IKK inhibition (*data not shown*). By contrast, there was no difference in primary CD34+ cell survival upon expression of the IκB super-repressor (*data not shown*). It is well established that immortalized cell lines usually undergo apoptosis upon cell cycle arrest while primary cells can enter a quiescent state. Nevertheless, a minor role of NF-κB in CD34+ cell survival cannot be ruled out.

Activation of NF-κB by PDGF in adherent cells was reported to rely on IKK phosphorylation by PKB,[33] although this has been a matter of debate.[43,44] NF-κB contributes to cell transformation by oncogenic PKB.[32] We observed that PKB was phosphorylated in CD34+ cells transduced with TPβ and that NF-κB activation was sensitive to PI3K inhibition, which is compatible with a PI3K/PKB/NF-κB pathway. This is in agreement with pre-

viously published studies showing that PI3K inhibition reduces TPβ-induced Ba/F3 cell proliferation and colony formation from CD34+ cells transduced with FPα.[9,16]

Constitutive NF-κB activation is found in other hematologic neoplasms, including lymphoid malignancies and acute myeloid leukemia.[45,46] Remarkably, constitutive activation of NF-κB in acute myeloid leukemia is also sensitive to PI3K inhibition.[46] Ongoing studies are testing whether anti-NF-κB therapy can be useful in these diseases. In this respect, we observed that the IKK inhibitor BMS-345541 strongly inhibited the proliferation of EOL1 and CD34+ cells transduced with a *PDGFR* fusion. We speculate that PI3K and NF-κB inhibitors may also be relevant in myeloid neoplasms associated with *PDGFR* fusions, in combination with tyrosine kinase inhibitors or in patients resistant to this first-line treatment.

Finally, our results may apply to other tyrosine kinase fusion genes that have been found in atypical chronic myeloid disorders associated with eosinophilia, such as *FGFR1* fusions, *PCM1-JAK2* and *ETV6-FLT3*.[2,47,48]

Authorship and Disclosures

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