Up-regulation of homeodomain genes, *DLX1* and *DLX2*, by *FLT3* signaling

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ABSTRACT

Background

Activating mutations in fms-like tyrosine kinase-3 (FLT3) are frequent in acute myeloid leukemia and represent both a poor prognostic feature and a therapeutic target. We have identified a previously unrecognized downstream effect of FLT3 activation, namely up-regulation of the homeodomain genes, *DLX1* and *DLX2*.

Design and Methods

MV4;11 cells with *FLT3*-internal tandem duplication mutation, RS4;11 cells with wild-type *FLT3* and blasts from patients with acute myeloid leukemia were used to pursue the relation between FLT3, DLX1/2 and transforming growth factor-β (TGFβ). Real-time quantitative reverse transcriptase polymerase chain reaction, western blot and reverse-phase protein array were performed to detect changes in gene and protein expression. RNA interference and MTS assays were used to study the interaction of PKC412, FLT3 inhibitor and TGFβ1.

Results

A direct relationship between FLT3 activity and DLX1/2 expression was revealed by both inhibition and up-regulation of FLT3 signaling in MV4;11 and RS4;11 cell lines, respectively, in isolated blast cells from patients with acute myeloid leukemia, and in reverse-phase protein array assays of samples from patients with acute myeloid leukemia. Mechanistically, the link between FLT3 and DLX1 expression appears to involve MAPK signaling through the ERK and JNK pathways. To determine whether elevated DLX1 had a functional consequence, we explored the reported inhibition by DLX1 on TGFβ/Smad signaling. Indeed, TGFβ responses were blunted by FLT3 activation in a DLX1-dependent manner and FLT3 inhibition resulted in a time-dependent increase in nuclear phospho-Smad2.

Conclusions

These findings suggest that alterations in DLX1/2 contribute to the biological consequences of FLT3 activation.

Key words: acute myeloid leukemia, FLT3, *DLX1, DLX2*, TGFβ.

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The onlive version of this article has a Supplementary Appendix.

Introduction

Homeodomain genes encode transcription factors that affect pattern formation, differentiation and proliferation during development. In humans, four clusters of homeodomain genes are distributed on chromosomes 7p15 (*HOXA*), 17q21 (*HOXB*), 12q13 (*HOXC*) and 2q31 (*HOXD*). Non-clustered homeodomain genes are distributed throughout the genome.[1] For simplicity, all homeodomain-containing genes, clustered and non-clustered, will hereafter be referred to as *HOX*.

HOX deregulation is involved in the development of leukemia, as evidenced by recurrent chromosomal translocations that fuse the nucleoporin gene, *NUP98*, with *HOXA9*, *HOXA11*, *HOXA13*, *PMX1*, *HOXC11*, *HOXC13*, *HOXD11* or *HOXD13*.[2-5] Rearrangements of *MLL*, which normally functions to maintain proper HOX levels,[6] result in overexpression of *HOXA9* and *MEIS1*.[7] In addition, overexpression of other *HOX* genes such as *HOXA5*,[8] *HOXB3*[9] and *HOXB4*[10,11] have been shown to affect myeloid proliferation and differentiation, suggesting that their deregulation may contribute to the leukemogenic process or phenotype. Other examples of *Hox* involvement include murine BXH-2 leukemia, in which *HoxA7* and *HoxA9* are frequent targets of retroviral integration,[12] and are necessary for the development of leukemia in mice carrying a *MLL-ENL* rearrangement.[13,14]

We previously reported that *HOX* expression patterns correlated with major cytogenetic subtypes in human acute myeloid leukemia (AML)[15] and that cases of AML with the highest levels of *HOX* expression represented a subset of AML with intermediate-risk cytogenetics with elevated levels of fms-like tyrosine kinase-3 (FLT3), a higher rate of FLT3 mutations, and a lower incidence of CEBPα mutations.[16] More recently, we identified a common *HOX* expression signature in cases of prognostically favorable AML,[17] which may influence their biological behavior.

FLT3 is a tyrosine kinase expressed in early hematopoiesis.[18] The FLT3 pathway affects proliferation, differentiation and apoptosis of hematopoietic cells. FLT3 signaling leads to activation of RAS, STAT5 and PI3K.[19,20] It is constitutively activated in about 30% of AML[21] and the *FLT3*-internal tandem duplication (ITD) mutation is predictive of increased relapse rates and higher mortality.[22-24] The association between *FLT3* mutations and certain patterns of *HOX* expression prompted us to ask whether there is a direct connection between FLT3 signaling and any particular *HOX* gene. As described here, we identified a novel interaction between FLT3 activity and regulation of the homeodomain transcription factors, DLX1 and DLX2, at both the mRNA and protein levels. The *DLX* genes are part of the *Drosophila distal-less* family that play a role in the control of craniofacial patterning and the differentiation and survival of inhibitory neurons in the forebrain.[25] Moreover, the resulting changes in DLX1 expression appear to be functionally significant, as evidenced by effects on the transforming growth factor-β (TGFβ) signaling pathway.

Design and Methods

Cell lines and reagents

We used the following cell lines obtained from the American Type Culture Collection (ATCC; VA, USA): MV4;11, derived from an acute monocytic leukemia with a *FLT3*/ITD mutation and RS4;11, an acute lymphoid leukemia with wild-type *FLT3*. We verified the cell lines by polymorphic microsatellite genome profiling (*data not shown*). We performed *in vitro* treatment with the following agents: PKC412 (Novartis, Switzerland), imatinib mesylate (Novartis), staurosporine (Sigma, St. Louis, MO, USA), human recombinant TGFβ1, human recombinant FLT ligand (R&D Biosystems, Minneapolis, MN, USA), U0126, SP600125, SB203580 and LY294002 (Sigma). Antibodies used for western blotting were: monoclonal DLX1 (M01, clone 2H3; Abnova), monoclonal β-actin (Sigma), phospho-FLT3 (Tyr 591, Cell Signaling, Danvers, MA, USA), monoclonal CREB-1 (X-12; Santa Cruz), monoclonal GAPDH (Ambion), polyclonal phospho-Smad2 (Ser465/467), and monoclonal Smad2 (L16D3) (Cell Signaling).

Treatment of leukemic cell lines with different compounds

To measure the expression of *HOX* and TGFβ1 target genes, MV4;11 and RS4;11 cells (5×10^5 cells/mL) were treated for 2, 5 and 24 h with PKC412 (0.1 μM), TGFβ1 (1, 2, 3, or 5 ng/mL), FLT ligand (100 ng/mL), or with the following kinase inhibitors as described in the text: U0126 (MEK1/2 inhibitor, SP600125 (JNK inhibitor), LY294002 (PI3K inhibitor) and SB203580 (p38 inhibitor), all at 10 μM. Cells were seeded into six-well plates containing RPMI 1640 (HyClone, UT, USA), 10% fetal calf serum (HyClone), 100 U/mL penicillin/streptomycin (HyClone) 24 h before the drug was added. Cells were harvested by centrifugation and washed with phosphate-buffered saline (HyClone) prior to preparation of protein lysates and RNA using standard methods. All experiments were performed in independent triplicates.

To measure changes in phospho-Smad2, cells were first incubated in serum-free RPMI 1640 medium for 7 h. PKC412 (0.1 μM) was added for 5 h (to achieve a decrease in DLX1), and cells were then incubated with two different low concentrations of TGFβ1 (1.2 and 0.6 ng/mL) for 2 h. Control samples were treated with TGFβ1 only (in serum-free medium).

Patients' samples

Eighteen RNA samples from AML patients at diagnosis were used for *DLX1* analysis. Patients were divided into two groups according to *FLT3* mutation status. *FLT3*/ITD mutation was detected by polymerase chain reaction (PCR) amplification and DNA agarose gel electrophoresis. Differences in blast counts in diagnostic samples did not correlate with *DLX1* expression. Five hundred and seventy-six samples from patients with AML were analyzed by reverse phase protein assays (RPPA). All experiments were conducted according to the principles expressed in the Declaration of Helsinki. The ethical committee and institutional review board approved the project and all samples were analyzed with written informed consent of the subjects or their guardians.

Treatment of patients samples ex vivo with PKC412

AML blast cells isolated from patients' samples containing a *FLT3*/ITD mutation (n=3) or wild-type *FLT3* (n=1) were incubated *ex vivo* in RPMI 1640, 10% fetal bovine serum enriched with a cytokine cocktail (10 μg/mL FLT3, 10 μg/mL interleukin-6 and 10 μg/mL stem cell factor-1) for 24 h, then PKC412 was added and the cells incubated for another 24 h prior to harvest.

Quantitative real-time polymerase chain reaction

Total RNA was isolated using an RNeasy Plus mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Intact RNA was verified by 1% agarose gel electrophoresis and cDNA was prepared using RT SuperScript III (Invitrogen, Carlsbad, CA, USA) and random-hexamer priming. Gene expression was measured on an ABI 7500 fast real-time PCR

machine using a 2X Power SybrGreen PCR Master kit (ABI, Foster City, CA, USA). The panel of *HOX* genes has been previously described and the assays validated.[17] All genes were analyzed in the same PCR conditions, i.e., 95°C for 10 min. followed by 35 cycles of: 95°C for 15 s then 60°C for 1 min. Primer sequences of the genes utilized are provided in *Online Supplementary Table S1*. Raw data were normalized against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), that is, $\Delta C_t = C_{t\,\text{specific gene}} - C_{t\,\text{GAPDH}}$. Expression data are reported as the number of transcripts per 1,000 copies of *GAPDH*.

Western blotting, nuclear and cytoplasmic fractions

Cells for protein extraction were washed with phosphate-buffered saline and the cell pellets kept at -80°C before use. Whole-cell protein lysates were prepared in 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 0.5% NP-40, 1 mM DTT, 2 mM magnesium chloride, together with 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthosodium vanadate, 10 μg/mL pepstatin A, 10 μg/mL aprotinin and 10 μg/mL leupeptin, followed by sonication for 15 s and centrifugation at 4,000 rpm for 5 min. Protein concentrations were measured by the Bradford assay using a Synergy 2 plate reader (BioTek Instruments, VT, USA). Expression of DLX1, phospho-FLT3 (Tyr 591) and Smad2 was measured in whole lysates. β-actin was used as a loading control.

A NePer kit (Pierce, Rockford, IL, USA) was used, according to the manufacturer's instructions, for nuclear-cytoplasmic fractionation. Protein concentrations of nuclear and cytoplasmic extracts were adjusted to the same ratio. Phospho-Smad2 was measured in both fractions; CREB-1 was used as a loading control for the nuclear fractions and GAPDH for the cytoplasmic fractions.

Cell cycle analysis

DNA was analyzed using a BD FACSCalibur cytometer. Cells were washed in phosphate-buffered saline and digested with 0.3% saponin, 50 μg/mL propidium iodide (Sigma), 0.1 mM EDTA, RNase A (Sigma) in 1x Dulbecco's phosphate-buffered saline (HyClone) and left overnight at 4°C in the dark before analysis.

Transfection and RNA interference

MV4;11 and RS4;11 cells were transfected by electroporation using an EPI2500 instrument (Dr. L. Fischer, Heidelberg, Germany) and 0.4 cm gap Gene Pulser/MicroPulser Cuvettes (Bio-Rad, Hercules, CA, USA) under the following conditions: 10^6 cells/100 μL of culture medium, 500 nM short interfereing (si) RNA, and 1 pulse of 15 ms, 250 V and 1200 μF. The siRNA transfection efficiency was monitored by flow cytometry. DLX1 targeting ON-TARGETplus SMARTpool (siDLX1) was used for the DLX1 knock-down in MV4;11 and RS4;11 cells with ON-TARGETplus Non-targeting Pool (sinonT; Dharmacon) as a negative control.

MTS assay

MTS is a colorimetric mitochondrial-based cell proliferation assay based on measurement of soluble formazan, which is proportional to the number of live cells. MV4;11 cells were seeded at 8×10^3 cells/well 24 h before treatment with either PKC412 (25, 12.5, or 6.25 nM), TGFβ1 (0.3125, 0.625, or 1.25 ng/mL), or the combination. After 96 h, 14 μL of MTS reagent (Promega, Madison, WI, USA) were added to the cells and incubated for 3 h. Absorbance at 490 nm was measured in a Synergy 2 plate-reader (BioTek Instruments).

Reverse phase protein array analysis

RPPA allows measurement of protein expression levels in a large number of biological samples simultaneously in a quantitative manner.[26] A RPPA dataset (576 samples printed in replicate with five serial 1:2 dilutions in 48 rows and 144 columns) was generated by using blood or marrow samples from patients with AML. Samples had been acquired during routine diagnostic assessments in accordance with the regulations and protocols approved by the MDACC Investigational Review Board. Informed consent had been obtained in accordance with the Declaration of Helsinki. We compared 383 *FLT3*/ITD-negative patients and 76 *FLT3*/ITD-positive ones, analyzing DLX1 and 187 other proteins. The samples were normalized to a concentration of 1×10^4 cells/μL and a whole-cell lysate was prepared. Briefly, patients' samples were printed in five serial dilutions onto slides along with normalization and expression controls. Slides were probed with a strictly validated primary antibody against ARC (Imgenex, San Diego, CA, USA) and a secondary antibody to amplify the signal, and finally a stable dye was precipitated. The stained slides were analyzed using MicroVigene® software (Vigene Tech, Carlisle, MA, USA) to produce quantified data. The RPPA data were properly normalized. ANOVA analysis of protein levels was used to assess the association of protein level with categorical clinical variables.

Statistical analysis

Non-parametric Mann-Whitney tests were performed to assess differences of gene expression measured by quantitative reverse transcriptase (RT)-PCR.

Results

Changes in DLX1/2 expression by FLT3 signaling

To search for possible interactions between FLT3 signaling and *HOX* gene expression, we first used MV4;11 cells, which were derived from a monocytic leukemia containing a *FLT3*/ITD gain of function mutation. These cells were treated with PKC412, a FLT3 inhibitor, and real-time quantitative RT-PCR was used to examine *HOX* expression (see *Design and Methods* section). Although we expected to find down-regulation of the *HOXA* or *HOXB* genes, only *DLX1* and *DLX2* expression was reproducibly affected by FLT3 inhibition ($\Delta\Delta C_t$ values are shown in *Online Supplementary Table S2*). Other *DLX* genes were not expressed in these cell lines. At 24 h, *DLX1* and *DLX2* mRNA levels were decreased by 4.8 and 3.3-fold, respectively (Figure 1A). The mRNA changes were mirrored at the protein level and reduced DLX1 protein expression could be observed as early as 5 h after addition of PKC412 (Figure 1A).

To demonstrate that the change in DLX1 was unrelated to non-specific toxicity, we analyzed MV4;11 cells by flow cytometry following treatment with PKC412. No effects on cell-cycle parameters were evident after 2 or 5 h (*Online Supplementary Figure S1A*), although by 24 h the cells were arrested in G0/G1. Furthermore, there was no evidence of a sub-G1 peak (i.e., DNA fragmentation), nor did we see any significant change in viability as assessed by trypan blue exclusion. To further test for non-specific effects, we treated MV4;11 cells with 0.1 μM imatinib mesylate, an ABL inhibitor; no significant change in *DLX1* expression was observed after 24 h (*Online Supplementary Figure S1B*). In contrast, treatment of cells with staurosporine, a general tyrosine kinase inhibitor, led to widespread changes in most *HOX* genes examined (*data not shown*).

To confirm that FLT3 signaling affects *DLX1* levels, we used the lymphoid leukemic cell line, RS4;11, which con-

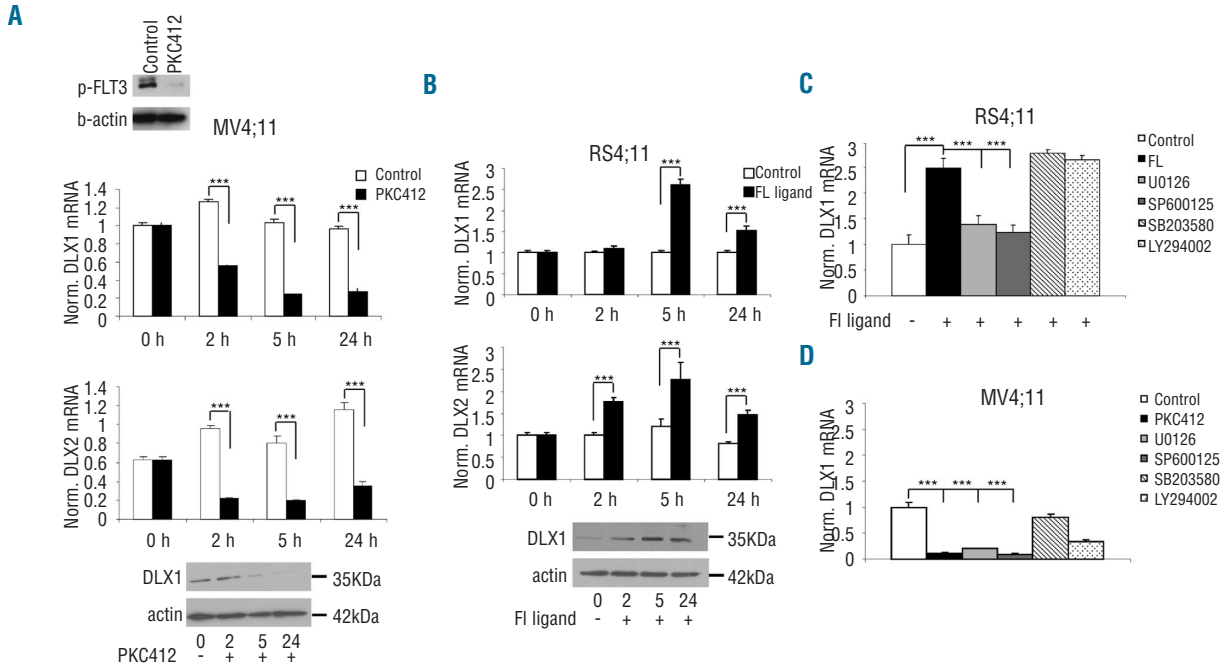

Figure 1. Effect of PKC412 on *DLX1* and *DLX2* gene expression (A). The Y axis shows the fold-change of gene expression of *DLX1* and *DLX2* in samples treated for 0, 2, 5 and 24 h with kinase inhibitors. Western blot shows effectiveness of PKC412 on p-FLT3 inhibition and DLX1 protein expression in the MV4;11 cell line. (B) Fold-change of *DLX1* and *DLX2* expression in RS4;11 cells treated for 2, 5 and 24 h with FLT ligand (100 ng/mL) *DLX1* and *DLX2* mRNA levels were detected. The western blot shows DLX1 protein expression in the RS4;11 cell line. (C) Normalized *DLX1* expression in RS4;11 (5 h) and (D) MV4;11 (5 h) cell lines after treatment with ERK1/2 (U0126, 10 μM), JNK1/2 (SP600125, 10 μM), PI3K (LY294002, 10 μM) and p38 (SB203580, 10 μM) inhibitors in FLT3 activated pathway. Asterisks correspond to statistically significant change, ***P<0.0001.

tains the same t(4;11) *MLL/AF4* fusion gene as MV4;11 cells but has wild-type *FLT3* and low *DLX1* levels. FLT3 signaling was activated by the addition of FLT ligand (100 ng/mL) for 2, 5 and 24 h and DLX1/2 expression was examined. Increased DLX1 protein was evident within 5 h; corresponding *DLX1* mRNA levels were elevated 2.6-fold and *DLX2* expression was increased by 2.4-fold (Figure 1B).

To demonstrate that FLT3 activation affects DLX1 levels in patients, we first examined 469 AML patients for DLX1 protein levels by RPPA. The group of 383 patients negative for *FLT3*/ITD had significantly lower expression of DLX1 than 76 patients positive for *FLT3*/ITD (P=0.0004; Figure 2A). At the mRNA level, we examined *DLX1* expression in diagnostic samples from 18 additional AML patients with or without *FLT3*/ITD mutations (the patients' characteristics are shown in *Online Supplementary Table S3*). Patients with *FLT3*/ITD mutations had significantly higher expression of *DLX1* compared to patients with wild-type *FLT3* (Figure 2B). Lastly, we isolated bone marrow cells from patients with AML with wild-type *FLT3* or the *FLT3*/ITD alteration. After 24 h of *ex vivo* treatment with PKC412, *DLX1* was decreased, by approximately 3.1-fold, only in the blasts from *FLT3*/ITD-positive patients (Figure 2C). We, therefore, conclude that FLT3 signaling affects expression of DLX1 and DLX2 in both cell lines and patients' samples.

Regulation of DLX1 downstream of FLT3 signaling

FLT3 mediates signaling through three known pathways (RAS, PI3K and STAT5). We used specific inhibitors affecting ERK1/2 (U0126, a MEK1/2 inhibitor), JNK1/2 (SP600125), p38 MAPK (SB203580) and PI3K (LY294002) to look for effects on DLX expression in the RS4;11 and MV4;11 cell lines. For RS4;11, the cells were first incubated with the various inhibitors for 1 h, then treated with FLT ligand for up to 24 h with measurement of mRNA levels at 2, 5 and 24 h time-points. FLT ligand treatment of RS4;11 cells caused an approximate 2.5-fold induction of *DLX1*, whereas U0126 and SP600125 reduced expression back to basal levels (Figure 1C). In contrast, neither the p38 or PI3K inhibitors had any significant effect. For MV4;11 (with constitutively activated FLT3), *DLX1* expression was monitored at 2, 5 and 24 h after addition of the inhibitors. Similar to the results in RS4;11 cells, blocking ERK1/2 and JNK1/2 activation with U0126 and SP600125 inhibited *DLX1* expression and, moreover, the degree of inhibition was similar to that following FLT3 inhibition with PKC412 (i.e., a 4-fold change; Figure 1D). LY294002 also inhibited *DLX1* expression, although not to the same degree as the ERK1/2 and JNK1/2 inhibitors, and only at the 5 h time-point. Blocking p38 had no effect on *DLX1*. These results demonstrate that both ERK1/2 and JNK1/2 participate in the regulation of *DLX1* mRNA expression in both RS4;11 and MV4;11 cell lines. For DLX2, only U0126 inhibited expression consistently in both cell lines (*data not shown*).

To search for an ERK/JNK responsive transcription factor binding site in DLX1, we used multiple-sequence alignment analysis, i.e., Mulan (*http://mulan.dcode.org/mulan*), which identifies local sequence conservation for the detection of evolutionarily conserved transcription factor binding sites. Activation of JNK and ERK pathways results in up-regulation of the c-Jun and c-Fos gene products, which

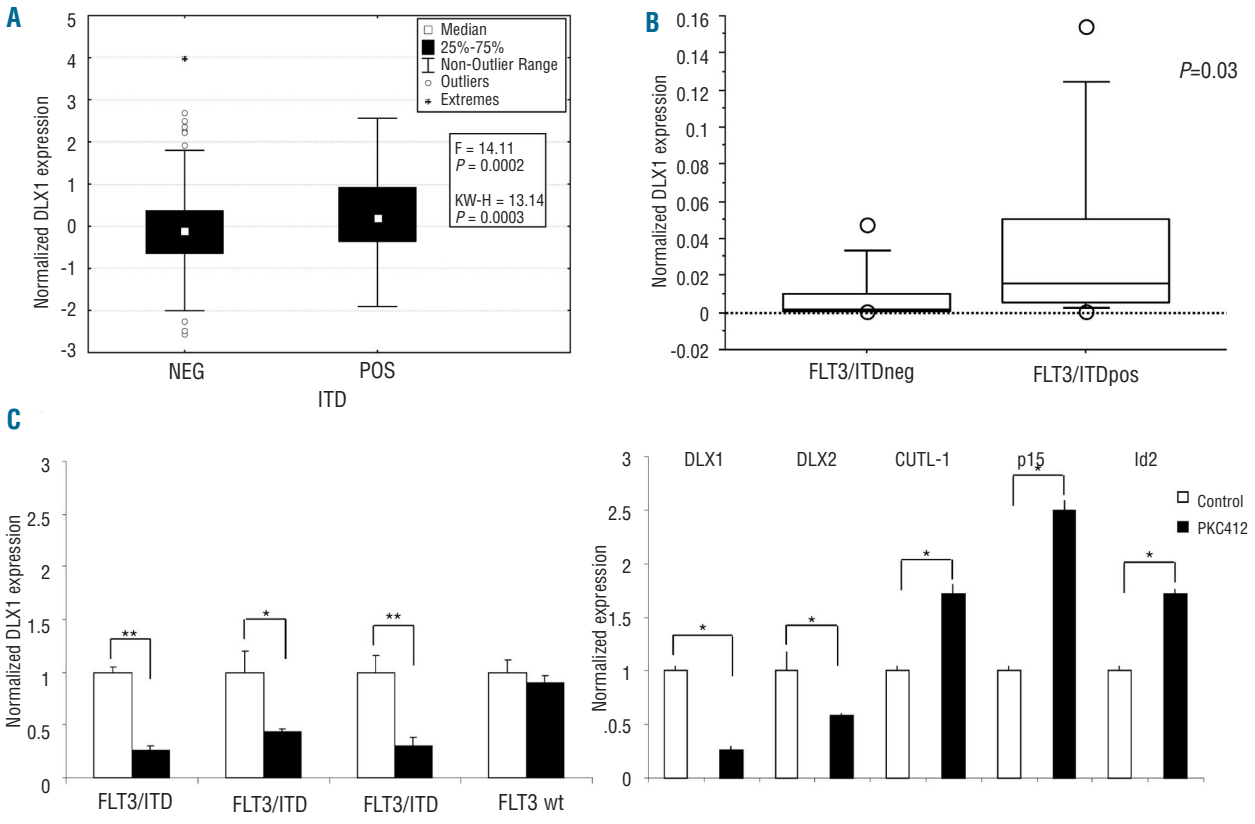

Figure 2. ***DLX1*** **expression in samples from AML patients and effect of PKC42** ***ex vivo*** **(A) Box plots showing results of RPPA analysis in AML patients characterized according to FLT3 status (NEG –** ***FLT3*****wt; POS –** ***FLT3*****/ITD) using the ANOVA test (B) Box plot illustrating the results of the Mann-Whitney statistical test for** ***DLXI*** **expression in diagnostic samples from AML patients with (FLT3/ITDpos) or without FLT3/ITD (FLT3/ITDneg) (C) Relative expression of** ***DLX1*** **presented as fold-change in leukemic blasts isolated from bone marrow cells of AML patients with** ***FLT3*** **wild-type or** ***FLT3*****/ITD mutation treated with PKC412. The expression of** ***DLX2***, ***CUTL-1***, ***p15*** **and** ***Id2*** **was also studied in leukemic blasts isolated from the bone marrow of AML patients with** ***FLT3*****/ITD and treated with PKC412 for 24 h. Asterisks correspond to statistically significant changes, *$P \leq 0.02$, **$P \leq 0.001$.**

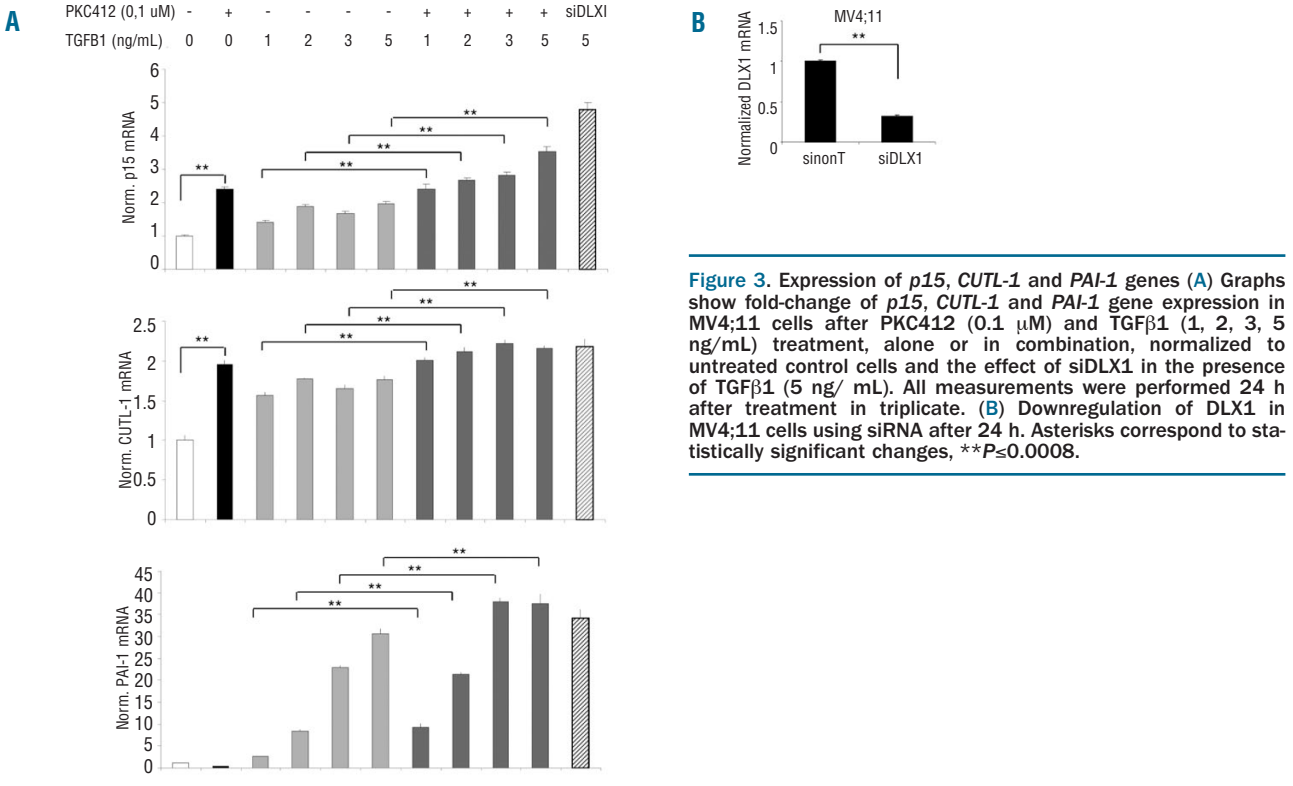

Figure 3. Expression of ***p15***, ***CUTL-1*** **and** ***PAI-1*** **genes (A) Graphs show fold-change of** ***p15***, ***CUTL-1*** **and** ***PAI-1*** **gene expression in MV4;11 cells after PKC412 (0.1 μM) and TGFβ1 (1, 2, 3, 5 ng/mL) treatment, alone or in combination, normalized to untreated control cells and the effect of siDLX1 in the presence of TGFβ1 (5 ng/ mL). All measurements were performed 24 h after treatment in triplicate. (B) Downregulation of DLX1 in MV4;11 cells using siRNA after 24 h. Asterisks correspond to statistically significant changes, **$P \leq 0.0008$.**

together comprise the AP-1 transcription factor. Of note, a conserved AP-1 dimer binding site, TGAGTCA, was found 205 bp upstream from the DLX1 transcription start site (*Online Supplementary Figure S2*).

Repressive effect of DLX1 on transforming growth factor-β signaling

It was previously shown that DLX1 is able to bind Smad4 and block TGFβ signaling.[27] To determine whether FLT3 inhibition with PKC412 and the down-regulation of DLX1 affected TGFβ target genes, we treated MV4;11 cells with threshold levels of TGFβ1 (1-5 ng/mL) and PKC412 (0.1 μM) separately, or in combination, and analyzed the expression of selected TGFβ target genes (*p15*, *CUTL-1*, *p21*, *Id-2* and *PAI-1*) using quantitative RT-PCR. TGFβ1 alone induced *p15* by 2-fold, *CUTL-1* by 1.7-fold, and caused a 30-fold increase in *PAI-1*. PKC412 modestly increased the expression of *p15* and *CUTL-1* by 2.4-fold and 2.0-fold, respectively, but had no effect on *PAI-1*. The combination of PKC412 and TGFβ1 led to a further modest increase in target gene expression, i.e., *PAI-1* (37-fold induction), *p15* (3.5-fold induction) and *CUTl-1* (2.2-fold induction, Figure 3A). In addition, we isolated bone marrow cells from a patient with AML harboring a *FLT3*/ITD and treated these with PKC412 (Figure 2B). *p15* levels were up-regulated by 2.5-fold, as were levels of *CUTL-1* (1.7-fold) and *Id2* (1.7-fold).

Although the effects of PKC412 on TGFβ gene expression were reproducible, this agent is not specific for FLT3. To confirm that DLX1 levels could be responsible for the effects observed with PKC412, we used RNA interference to inhibit *DLX1* and treated MV4;11 cells with threshold levels of TGFβ1 (5 ng/mL for 24 h). The siRNA against DLX1 reduced its level by 3-fold (Figure 3B) and caused a significant enhancement in the expression of *p15*, *CUTL-1*, *PAI-1* and *Id2* compared to TGFβ1 alone. Moreover, these changes were quite similar to the effects observed with PKC412 (Figure 3A).

We then investigated whether FLT3 stimulation would have the opposite effect in RS4;11 cells, which are otherwise responsive to TGFβ1 as evidenced by increased levels of *CUTL-1*, *PAI-1* and *Id2*. As anticipated, the addition of FLT ligand reduced the expression of *CUTL-1* (2-fold), *PAI-1* (1.5-fold) and *Id2* (1.5-fold) at 24 hours (Figure 4A), whereas *p15* was not expressed in this cell line. Importantly, silencing *DLX1* abrogated the effect of FLT ligand and brought TGFβ target genes back to their basal level (Figure 4B).

Since TGFβ can induce a G1/G0 cell-cycle and growth arrest, we used MTS assays to monitor the growth of MV4;11 cells following treatment with three different concentrations of TGFβ1, either alone [based on the half-maximal inhibitory concentration (IC_{50})] or in combination with varying concentrations of PKC412 (6.25 to 100 nM). At higher concentrations, TGFβ1 and PKC412 alone had a cytostatic effect on the leukemic cells. However, at threshold concentrations of TGFβ1 (0.3 and 0.6 ng/mL), we observed an enhanced cytostatic effect when the TGFβ1 was combined with a low concentration (6.25 nM) of PKC412 (Figure 5).

PKC412 affects levels of nuclear phospho-Smad2

Activation of the TGFβ pathway results from ligand binding to the type II receptor and phosphorylation of a regulatory (R) Smad (e.g., Smad2, Smad3) by the type I receptor. The phosphorylated R-Smad then associates with Smad4 and the complex translocates to the nucleus to regulate gene expression.[28] We examined the phosphorylation and nuclear translocation of Smad2/Smad3 in MV4;11 cells treated with TGFβ1 and PKC412. The addition of TGFβ1 (1.2 and 0.6 ng/mL) in serum-starved conditions led to increased nuclear Smad2 phosphorylation, as expected. Although PKC412 as a single agent did not increase nuclear phospho-Smad2, it reproducibly enhanced its levels at 2 h after stimulation with TGFβ1 (Figure 6A), while total Smad2 remained unchanged (Figure 6B). These results are consistent with the observed

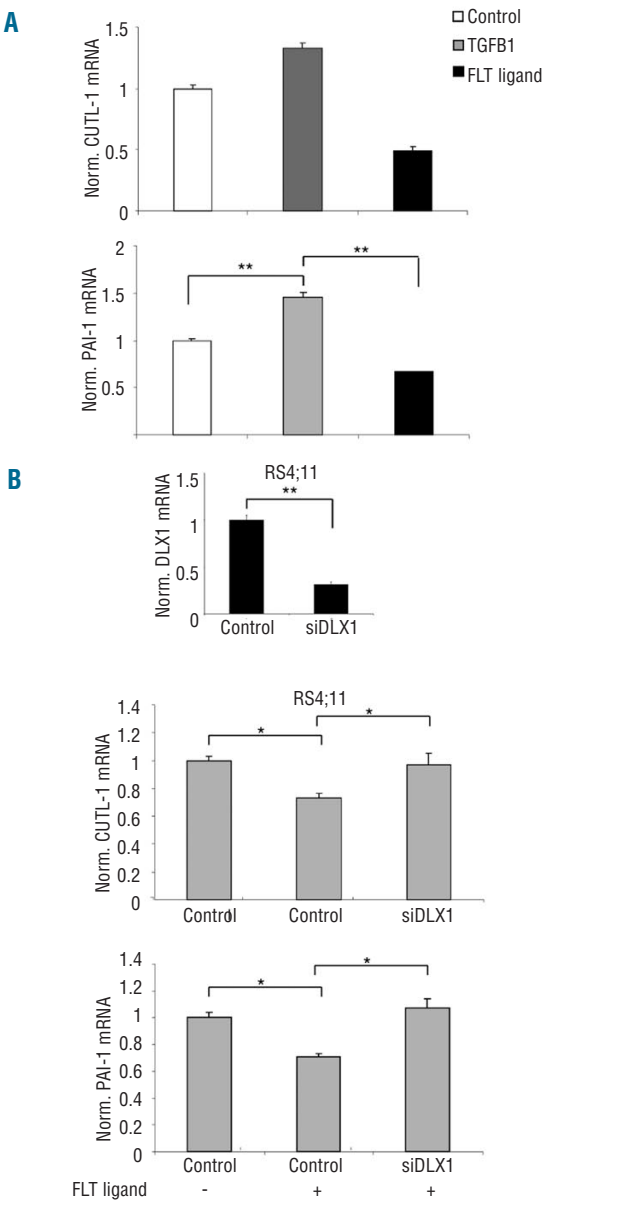

Figure 4. Knockdown of *DLX1* (siDLX1) alters response to TGFβ1 and FLT3 ligand. (A) Relative expression of *CUTL-1* and *PAI-1* in RS4;11 cells treated with TGFβ1 (5 ng/mL) or FLT ligand (100 ng/mL) for 24 h compared to untreated control cells; (B) Down-regulation of *DLX1* in RS4;11 cells using siRNA after 24 h blocks FLT3 ligand-mediated suppression of *CUTL-1* and *PAI-1*. Cells were transfected with either non-targeted siRNA (control) or siDLX1 and exposed to FLT3 (100 ng/mL for 24 h). Asterisks correspond to statistically significant changes, **P*<0.05; ***P*≤0.0004.

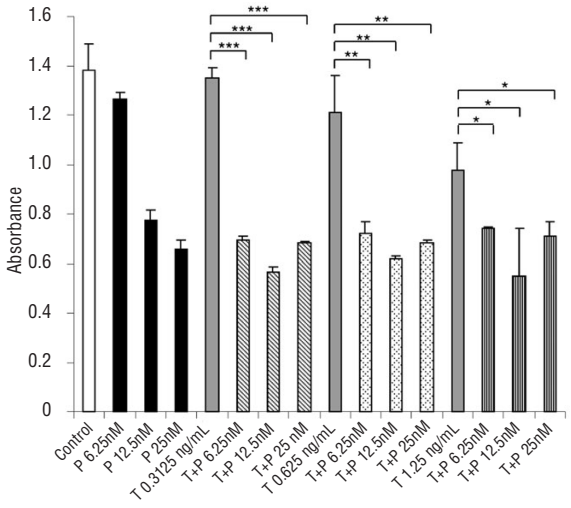

Figure 5. Cytostatic effect of PKC412, TGFβ1 and their combination. Results of MTS assay using threshold concentrations of TGFβ1 (T) and PKC412 (P) and their combination (T+P) were monitored 72 h after incubation. Experiments were done in triplicate and the standard deviation was calculated. Asterisks correspond to statistically significant changes, **P*≤0.03; ***P*≤0.005; ****P*<0.0001.

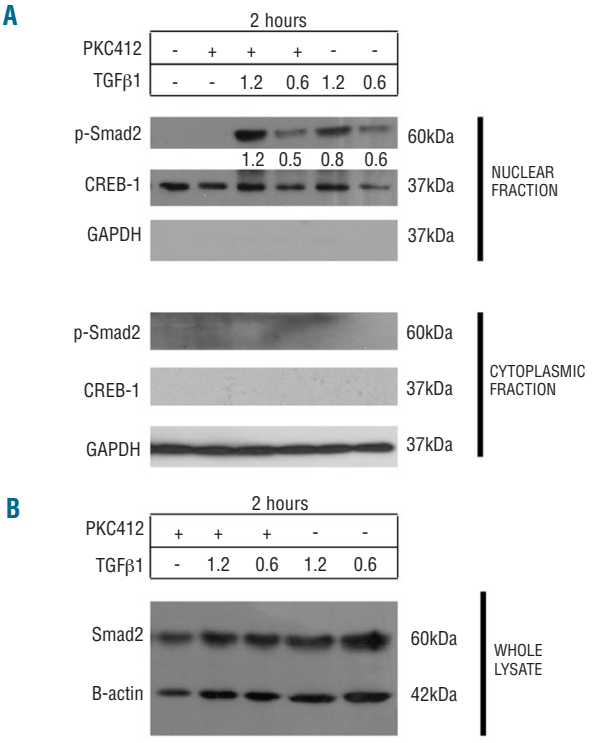

Figure 6. Effect of PKC412 on phosphorylation of Smad2. (A) Phospho-Smad2 detected by western blot in MV4;11 cells treated with PKC412 (0.1 μM) and TGFβ1 (0.6 and 1.2 ng/mL) alone, or in combination, in nuclear and cytoplasmic fractions at 2 h. CREB-1 (nuclear protein) and GAPDH (cytoplasmic protein) were used as controls. Values from densitometry are shown. (B) Total Smad2 was detected in whole lysates. All experiments were done in independent triplicates. Representative examples are shown.

changes in TGFβ target gene expression following inhibition (or stimulation). Whether the changes in nuclear phospho-Smad2 result directly from the reported interaction between DLX1 and Smad4 or involve another mechanism is unknown.

Discussion

We describe here a novel and specific relationship between the homeodomain genes, *DLX1* and *DLX2*, and FLT3 kinase activity. This relationship was observed both in leukemic cell lines and in patients' samples. Furthermore, at least *in vitro*, this relationship appears to have functional consequences on TGFβ target gene expression and signaling (*Online Supplementary Figure S3*).

We initiated this study based on previous results in patients' samples suggesting that the expression of certain *HOX* genes correlated with FLT3 mRNA levels.[16] To investigate this further, we utilized two t(4;11) translocation-containing cell lines, MV4;11 and RS4;11, which express constitutively active and wild-type FLT3 receptors, respectively. Among many homeodomain genes examined by quantitative RT-PCR following FLT3 manipulation, only *DLX1* and *DLX2* mRNA were consistently affected. Short-term inhibition of mutated FLT3 in MV4;11 cells by PKC412 caused down-regulation of DLX1/2, while exposure of RS4;11 cells to FLT3 ligand resulted in DLX up-regulation. Although the survival of MV4;11 cells is dependent on FLT3 signaling,[29] down-regulation of DLX1/2 preceded any measurable evidence of PKC412-mediated toxicity using trypan blue exclusion and sub-G1 DNA content as indices. While these indices might have been insufficiently sensitive, the corroborative results from FLT3 stimulation in RS4;11 cells strongly support the proposed regulatory interaction. Moreover, DLX1 levels were also down-regulated by PKC412 in blast cells isolated from patients with *FLT3*/ITD-positive AML, and our reverse phase arrays demonstrated a highly significant correlation between the presence of activating *FLT3* mutations (ITD) and DLX1 protein expression. Mechanistically, a link between DLX1 regulation and FLT3 signaling appears to involve the ERK and JNK pathways, which are known to affect AP-1 levels, and a conserved AP-1 binding site was identified in the promoter region of *DLX1*.

The *DLX* genes were originally identified in the forebrain of the developing mouse embryo. Although individual *DLX1* and *DLX2* knockouts do not cause forebrain deformities, the double knockout exhibits abnormalities in facial structures and is neonatally fatal.[30,31] In addition, DLX1 was shown to be a negative regulator of definitive erythropoiesis.[27] Aberrant expression of DLX family members has also been associated with breast, ovarian and lung cancer progression/invasiveness.[32-34]

Previous studies have demonstrated that DLX1 negatively affects TGFβ signaling by direct interactions with Smad4,[27] a key downstream effector of TGFβ/BMP signaling. Interactions between Smads and *HOX* genes were also shown in other studies.[35,36] We observed that the decrease in DLX expression by either PKC412 treatment or following siRNA-mediated knockdown of DLX1 was functionally significant, as evidenced by the up-regulation of *p15*, *CUTL-1* and *Id2*, known TGFβ target genes.[37-39] In contrast, FLT3 stimulation in RS4;11 cells led to a repression of *CUTL-1*, *Id2* and *PAI-1* mRNA. This effect was blocked by siRNA-mediated DLX1 knockdown, thus confirming its direct role in the regulation. The *p15*, *CUTL-1*,

Id2 and *PAI-1* genes were not affected equally; however, the changes were reproducible and in the expected direction (up-regulation *versus* down-regulation). There are several possible explanations for the lack of response in *PAI-1* expression after PKC412 treatment in MV4;11 cells, as well as in patients' blasts. The transcriptional effects of Smad are known to be dependent on the presence of other co-activators or co-repressors.[40] PKC412 is also not specific for FLT3 signaling and potentially could affect other pathways that interfere with *PAI-1* activation. In addition, a novel type of ITD mutation not localized in the juxtamembrane region of the FLT3 receptor was recently discovered by Breitenbuecher *et al.*[41] This mutation was detected in about 29% of *FLT3*/ITD-positive patients and was associated with persistence of phosphorylated-ERK1/2 despite PKC412 treatment.

In addition, TGFβ signaling is known to inhibit proliferation of hematopoietic cells, and mutations in this pathway contribute to the growth of AML blasts.[28,42] We found that the combination of both TGFβ and PKC412, at threshold levels, caused more growth inhibition than either agent alone, which is consistent with an enhanced TGFβ response, although other interpretations are not excluded.

Recent data indicate that Smad may repress gene expression by interacting with co-repressor complexes associated with TGIF, HOXC8 and ATF2.[43-45] Using nuclear-cytoplasmic fractionation and western blots, we found that PKC412 reproducibly enhanced the level of nuclear phospho-Smad2 induced by TGFβ1 at 2 h, while total Smad2 was unchanged. Mechanistically, because DLX1 has been reported to bind and sequester Smad4, reducing the level of DLX1 might result in more translocation of a p-Smad2/p-Smad3/Smad4 complex to the nucleus. In addition, it has been reported that ERK-mediated phosphorylation of Smad1-3 impairs the nuclear transport and signaling activity of these proteins.[40-42] Thus, inhibition of FLT3, which is known to signal in part by phospho-ERK1/2, would be predicted to enhance nuclear transport of phosphorylated Smad2. However, further studies are required to reveal this mechanism.

In summary, our results demonstrate that FLT3 signaling leads to specific up-regulation of DLX1/2 expression at both the RNA and protein levels in leukemic cell lines and patients' samples. Although the full consequences of this up-regulation remain undetermined, we suggest that effects on TGFβ target gene expression and cell growth may be involved. Thus, FLT3 signaling in leukemia may function in part through DLX1/2 to overcome the growth inhibitory effects of TGFβ. Given the frequency of FLT3 alterations in AML, more investigation into the role of DLX1/2 in the disease process seems warranted.

Authorship and Disclosures

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