Cytoskeletal Regulatory Gene Expression and Migratory Properties of B-cell Progenitors Are Affected by the ETV6–RUNX1 Rearrangement

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Abstract
Although the ETV6–RUNX1 fusion is a frequent initiating event in childhood leukemia, its role in leukemogenesis is only partly understood. The main impact of the fusion itself is to generate and sustain a clone of clinically silent preleukemic B-cell progenitors (BCP). Additional oncogenic hits, occurring even several years later, are required for overt disease. The understanding of the features and interactions of ETV6–RUNX1–positive cells during this “latency” period may explain how these silent cells can persist and whether they could be prone to additional genetic changes. In this study, two in vitro murine models were used to investigate whether ETV6–RUNX1 alters the cellular adhesion and migration properties of BCP. ETV6–RUNX1–expressing cells showed a significant defect in the chemotactic response to CXCL12, caused by a block in CXCR4 signaling, as demonstrated by inhibition of CXCL12-associated calcium flux and lack of ERK phosphorylation. Moreover, the induction of ETV6–RUNX1 caused changes in the expression of cell-surface adhesion molecules. The expression of genes regulating the cytoskeleton was also affected, resulting in a block of CDC42 signaling. The abnormalities described here could alter the interaction of ETV6–RUNX1 preleukemic BCP with the microenvironment and contribute to the pathogenesis of the disease.

Implications: Alterations in the expression of cytoskeletal regulatory genes and migration properties of BCP represent early events in the evolution of the disease, from the preleukemic phase to the clinical onset, and suggest new strategies for effective eradication of leukemia. Mol Cancer Res; 12(12); 1796–806. ©2014 AACR.

Introduction
ETV6–RUNX1, generated by the t(12;21) chromosome translocation, is the most common fusion gene in childhood cancer, selectively associated with B-cell precursor acute lymphoblastic leukemia (BCP-ALL; refs. 1–3). The t (12;21) translocation fuses the protein dimerization domain of ETV6 with essentially all of the DNA binding and activating regions of RUNX1, generating an aberrant transcription factor (2, 4). Observations on clinical samples, normal cord blood (5), monozygotic twins (6), and animal modeling (7–13) indicate that this oncogene induces a preleukemic phenotype which is insufficient for overt leukemogenesis. Indeed, ETV6–RUNX1 fusion generated during fetal hemopoiesis produces a clinically covert preleukemic clone that can persist postnatally for at least 15 years (4). Additional genetic abnormalities observed at diagnosis of ETV6–RUNX1–positive ALL are generally considered to be secondary events associated with the transition of silent preleukemic cells to overt ALL (4).

The understanding of which cellular signaling pathways are corrupted by ETV6–RUNX1 to sustain this persistent preleukemic state might help to explain the vulnerability of its constituent stem cells to secondary genetic changes. We have previously shown evidence that ETV6–RUNX1 compromised the TGFβ signaling pathway, providing a plausible basis for both the persistence and maintenance of covert preleukemic clones in patients and their competitive positive selection in an inflammatory context (12). We and other investigators have also described an increased level of heat shock proteins, survivin, has-mir-125b-2, and erythropoietin receptor in ETV6–RUNX1–positive cells, factors that could provide the survival advantage to the preleukemic clone (14–17).
However, in addition to proliferative advantage and resistance to apoptotic signals, the site of localization and interaction with the microenvironment is crucial to sustain the hematopoietic stem cells in quiescence and the survival of both normal and preleukemic cells (18). Moreover, alterations in adhesive and chemotactic responses to normal stimuli have been described in BCR-ABL1-negative chronic myeloid leukemia (19–21).

Interestingly, some genes involved in cellular adhesion and cytoskeleton organization are listed among the RUNX1-gene target genes (22–23), and changes in the expression of genes belonging to this functional pathway are described in ETV6–RUNX1–positive ALL (24–26).

The aim of this work was to investigate whether the ETV6–RUNX1 preleukemic clone showed alterations in its adhesive and migratory properties that could provide a rationale for its persistence and proliferation.

The preleukemic phase is usually clinically silent, whereas the ETV6–RUNX1 leukemic clone at ALL diagnosis carries additional genetic abnormalities (4); for these reasons, we used 2 alternative murine experimental systems: the Ba/F3 pro-B cell line transduced with a hormone-inducible ETV6–RUNX1 (12) and pre-BI primary cells from fetal liver (27, 28) stably transduced with the pMIGR1–ETV6–RUNX1–IRES–GFP construct.

In both model systems, we found evidence that ETV6–RUNX1 alters the expression of cytoskeletal regulatory genes, resulting in a block of the CDC42 signaling pathway, and compromises the chemoattractive response to CXCL12.

Materials and Methods

Cell culture and ETV6–RUNX1 expression

The GeneSwitch System (Life Technologies), a mifepristone-regulated expression system for mammalian cells, was used to produce inducible expression of the ETV6–RUNX1 gene in the IL3-dependent murine pro-B cell line Ba/F3, as previously described (12). Briefly, cells were transfected with the pSwitch plasmid (Invitrogen) expressing a GAL4 regulatory fusion protein (control cells). Positive clones were then transfected with pGene plasmid (Life Technologies) containing the RUNX1 cDNA fused to c-Myc epitope tag and GFP (MIGR–RUNX1–IRES–GFP construct).

In both model systems, we found evidence that ETV6–RUNX1 alters the expression of cytoskeletal regulatory genes, resulting in a block of the CDC42 signaling pathway, and compromises the chemoattractive response to CXCL12.

Quantitative PCR array and real-time PCR

The RT2 Profiler Assay Cytoskeleton Regulators PCR Array (SuperArray Bioscience) was performed following the manufacturer’s recommendation.

Real-time analysis was done on a Light Cycler 480II with Universal Probe Master System (Roche Diagnostics; Hoffmann-La Roche Ltd.). Optimal primers and probe for amplification were selected by the Roche ProbeFinder software (https://www.roche-appliedscience.com/sis/rtpcr/upl).

Data were expressed using the comparative 2^−ΔΔCt method (30), with the Hprt gene as a reference; for each gene studied, the transcript level was always referred to that of control cells. A fold change of <0.75 or >2.5 was considered as threshold for down- or upregulation, respectively.

Adhesion assays

Plates (96-well) were coated with fibronectin, murine stroma cell line OP9 (kindly provided by Prof. A. Rolink, University of Basel), murine fresh stroma, or murine endothelial cell lines 1G11 and MELC2 (a gift of Prof. A. Vecchi, Instituto Clinico Humanitas, Rozzano, Italy). Details of the coating procedures: 50 μL fibronectin (Sigma-Aldrich) at a concentration of 25 ng/μL in each of the 96 wells, was allow to air dry for 2 hours and the cell lines, and murine fresh stroma were grown in each well until confluent. OP9 were grown in IMDM with 20% FCS, the murine fresh stroma was isolated from bone marrow of a C57BL/6 wt mouse and grown in IMDM with 20% FCS at 33°C and 5% CO2. The cell lines 1G11 and MELC2 were grown on a gelatin coating (Sigma-Aldrich) in DMEM supplemented with 4.5 g/L glucose, FCS (20% for 1G11, 10% for MELC2), 1% nonessential amino acids, 1 mM/L sodium pyruvate, 100 μg/mL endothelial cell growth supplement (ECGS; Sigma-Aldrich), 100 μg/mL heparin (PharmaTex) and, for MELC2 only, 10% murine sarcoma 180 conditioned medium. The endothelial cell lines were stimulated or not for 24 hours with the bicistronic retroviral vectors pMSCV-IRES-GFP (MIGR–GFP) or pMSCV-ETV6-RUNX1-IRES-GFP, which allows the expression of ETV6–RUNX1 cDNA fused to c-Myc epitope tag and GFP (MIGR–ETV6–RUNX1), as previously described (27). At day +3 from transduction, cell sorting for GFP fluorescence was performed by the FACS Aria instrument (BD Biosciences).

The pre-BI cells were cultured on OP9 bone marrow stroma cells in Iscove modified Dulbecco medium (IMDM) supplemented with 2% FCS, 0.03% w/v primatone, and 100 units/mL IL7 (27).

Antibodies and flow cytometry

Phycoerythrin-conjugated antibodies anti-CD18 (M18/2), anti-CD11a (M17/4), anti-CD54 (YN1/1.7.4), anti-CD135 (A2F10), anti-CD29 (HMb 1-1), anti-CD49d (R1-2), anti-CD49e (HMA 5-1: e-Bioscience Inc.), and anti-CXCR7 (8F11-M16; Biologend) were used. Allophycocyanin-conjugated antibodies anti-CD44 (IM7) and anti-CXCR4 (2B11; e-Bioscience Inc.) were used. Data were analyzed using CellQuest Software (BD Biosciences).
inflammatory cytokines IL1β (25 ng/mL; PeproTech), IL6 (20 ng/mL; ImmunoTools), and TNFα (50 ng/mL; ImmunoTools) before being used for the adhesion assay.

After 3 days of induction, control and ETV6–RUNX1–expressing Ba/F3 cells were stained with 12.5 μmol/L Calcein AM (Sigma-Aldrich) and resuspended in adhesion medium (RPMI with 5% FCS and 10nmol/L HEPES). Cells were then added to 96-well plates coated with the substrates indicated above. After 30 minutes of incubation at 37°C and 5% CO₂, nonadherent cells were removed by washing 3 times with the adhesion medium. The adhesion index was measured as ratio of fluorescence detected before and after washing by the fluorescence reader TECAN GENios (Tecan).

Cell adhesion to VCAM1 and ICAM1 recombinant proteins was performed as previously described (28). Briefly, 15-mm round coverslips were coated with recombinant mouse VCAM1-Fc protein (25 μg/mL; R&D Systems) or recombinant mouse ICAM1-Fc protein (25 μg/mL; R&D Systems) and placed in 12-well dishes containing 1.5 × 10⁵ 3-days induced control or ETV6–RUNX1–expressing Ba/F3 cells. After overnight incubation at 37°C and 5% CO₂, the coverslips were washed to eliminate the nonadherent cells and were mounted on slides in the presence of 4’,6-diamidino-2-phenylindole (DAPI). Each coverslip was analyzed by a fluorescence microscope, acquiring 30 representative fields, and the adherent cells were counted.

**Immunoblotting**

Western blot analysis of CDC42 protein was performed by lysing cells in the lysis buffer (Thermo Scientific) with protease inhibitor cocktail (Sigma-Aldrich). Mouse anti-CDC42 antibody was used at working dilution 1:167 (Thermo Scientific), mouse anti-β-actin antibody at 1:1,000 (AC-15, Sigma-Aldrich), and the secondary goat anti-mouse IgG (Fc-specific) peroxidase antibody at working dilution 1:20,000 (Sigma-Aldrich). A StripAblot Stripping Buffer (Euroclone S.p.A.) was used to recover membranes. Densitometry analyses were performed using Alliance instrument and Uvibit software (Uvitec).

For p-ERK, total ERK, p-PAK2, and total PAK2 protein analysis, cells were starved for 1 hour in RPMI without serum, then 2 × 10⁶ cells were stimulated with 100 ng/mL hCXCL12 (PeproTech) in RPMI at 37°C. At different time points, cells were washed with ice-cold PBS and pellets were lysed in 20 mmol/L Tris-HCl/NaCl, pH 7.4, containing 2 mmol/L EDTA, 0.2 mmol/L Na₃VO₄, 1% Triton X-100, 25 mmol/L β-glycerophosphate, 25 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail at 4°C for 30 minutes. Rabbit anti-phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204) antibody, rabbit anti-phospho-PAK1 (Thr423)/PAK2 (Thr402) antibody, rabbit anti-phospho-p44/42 MAPK (ERK1/2) antibody, and rabbit PAK2 (C17A10) antibody were used at working dilution 1:1,000 (Cell Signaling), the mouse anti-GAPDH antibody at 1:200 (6C5, Santa Cruz Biotechnology), and the secondary goat anti-rabbit IgG (H+L) HRP at 1:10,000 dilution (Thermo Scientific).

Western blot analysis of c-Myc epitope tag, fused to ETV6–RUNX1 cDNA in MIGR–ETV6–RUNX1 pre-BI cells, was performed using the rabbit anti-c-Myc (A-14) antibody at working dilution 1:200 (Santa Cruz Biotechnology).

**Migration assay**

Transwell plates (8.0 μm for Ba/F3 cells and 5.0 μm for pre-BI cells) were used; 5 × 10⁵ cells were loaded in the upper chamber in 100 μL of RPMI with 1% FCS (migration medium) with or without 10μg/mL of the anti-CXCR7 antibody (8F11-M16; Biologend). About 600 μL of migration medium with or without hCXCL12 (100 ng/mL), FLT3L (10 ng/mL; ImmunoTools), or 10% FCS was added in the lower chamber. After 3 hours at 37°C and 5% CO₂, cells in the lower chamber were collected and counted by FACS. The migration index was defined as the ratio between the number of cells migrated to the lower chamber of the Transwell in response to the chemokine stimulus and in its absence.

Migration assay in the presence of EGF: migration assay of pre-BI cells toward 100 ng/mL hCXCL12 was performed in presence or absence of 20 ng/mL of EGF (PeproTech) homogeneously present in the upper and lower chambers of the Transwell. After 4 hours, the GFP-positive cells migrated to the lower well were counted by FACS. The migration index was defined as the ratio between the number of cells migrated to the lower chamber of the Transwell in response to CXCL12 and in its absence.

Migration assay in the presence of CCL2: migration assay of Ba/F3 cells toward 100 ng/mL CXCL12 was performed in absence or presence of 100 ng/mL of CCL2 (PeproTech) in the upper chamber of the Transwell. The migration index was defined as the ratio between the number of cells migrated to the lower chamber of the Transwell in response to CXCL12 and in its absence.

**Protein array analysis**

Protein array was performed on cell supernatant, obtained from Ba/F3 control and ETV6–RUNX1–positive cells, using RayBio Cytokine Antibody Arrays—Mouse Array III–IV (Raybiotech Inc), following the manufacturer’s protocol. Densitometry analyses were done using Kodak image station (Kodak SpA).

**Calcium mobilization analysis**

After overnight IL3 starvation, 0.6 × 10⁶ 3-day induced cells were loaded with FluoForté dye loading solution (Enzo Life Sciences) in RPMI with 10% FCS for 45 minutes at 37°C and then 15 minutes at room temperature. Baseline calcium levels were established for about 2 minutes before the addition of 300 ng/mL CXCL12. Data were collected for a total of 512 seconds and analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

**Generation of Ba/F3-overexpressing CDC42 proteins**

The cDNA of wild-type, constitutively active (Q61L), and dominant negative (T17N) CDC42 were cloned into the retroviral vector pMSCV-IRES-GFP using In-Fusion HD Cloning kit (Clontech Laboratories) and following the
manufacturer’s recommendation. Ba/F3 cells were transfected by nucleofection following the Amaza protocol, as previously described (12).

Results

ETV6–RUNX1 alters the expression of cell-surface adhesion molecules and adhesion properties of Ba/F3 cell

After ETV6–RUNX1 expression in Ba/F3 cells, flow cytometric analysis indicated alterations of the cell-surface expression levels of several molecules involved in cell adhesion and migration of BCP (31). In detail, ETV6–RUNX1–positive cells expressed higher levels (MFI ratio) of the following adhesion molecules: CD44 (average of increase in independent experiments: 58%; range: 19%–118%; \( P < 0.05 \)), CD18 (average: 121%; range: 19%–206%; \( P < 0.05 \)), CD11a (average: 182%; range: 54%–334%; \( P < 0.01 \)), and CD54 (average: 145%; range: 80%–207%; \( P < 0.05 \)). On the other hand, they expressed lower levels of the integrin CD29 (average of decrease: 22%; range: 8%–34%; \( P < 0.01 \); Fig. 1A) but did not show a significant difference in the expression of CD49d and CD49e (data not shown).

The transcription level of genes coding some of these antigens tested by RT-PCR confirmed the immunophenotype results (Supplementary Fig. S1).

We observed an increase in the adhesion of ETV6–RUNX1–positive cells to the murine endothelial cell lines 1G11 and MELC2 [adhesion index of ETV6–RUNX1–positive cells vs. control cells: 1.34 ± 0.26 (\( P = 0.0431 \)) on 1G11 and 1.39 ± 0.26 (\( P = 0.0286 \)) on MELC2; Fig. 1B]. The stimulation of the endothelial cell lines with inflammatory cytokines did not modify the adhesion index (data not shown). However, we did not observe any difference in the adhesion abilities to several other substrates: fibronectin, CD54/ICAM1 and VCAM1 molecule, murine stroma cell line OP9, and murine fresh stroma.

ETV6–RUNX1 causes altered expression of genes regulating the cytoskeleton

We explored whether the ETV6–RUNX1 fusion gene affected key genes in the organization of the cytoskeleton. A panel of 84 genes involved in the biogenesis and organization of the cytoskeleton was examined using the RT2 Profiler Assay Cytoskeleton Regulators PCR Array, plus single-candidate gene transcripts tested by RQ-PCR.

We identified 9 genes overexpressed and 7 genes repressed in Ba/F3 ETV6–RUNX1–positive cells compared with control cells (Table 1). These were genes involved in cell shape, formation of pseudopodia, cell migration, actin, and microtubule organization. Interestingly, several of these

![Figure 1](https://example.com/figure1.png)

"Figure 1. Phenotypic and adhesion analyses of control and ETV6–RUNX1–positive Ba/F3 cells. A, overlay analyses of the expression on cell surface of the indicated antigen measured as MFI levels by FACS. The figure shows a representative experiment. B, adhesion analyses of control and ETV6–RUNX1–positive Ba/F3 cells to murine endothelial cell lines 1G11 and MELC2. After 3-day induction, control and ETV6–RUNX1–expressing Ba/F3 cells were stained with Calcein AM and added to 96-well plates coated with the cell lines. After 30 minutes of incubation, nonadherent cells were removed by washing 3 times with the adhesion medium. The adhesion index was measured as ratio of fluorescence detected before and after washing. Ctr, control cells; ER, ETV6–RUNX1–positive cells. t test: *, \( P < 0.05 \)."
differentially expressed genes belonged to the CDC42 pathway, a key element for the regulation of the cytoskeleton and for the directional migration of the cells: Cdc42ep2, Cdc42ep3, Map3k11, Was, Nck2, Nck1, and Mmp9 (32–37). In particular, Cdc42ep2 and Cdc42ep3, negative regulators of Cdc42 (32), were among the most upregulated genes in ETV6–RUNX1–positive cells (Table 1, upregulated genes). Moreover, after induction of the fusion gene (Fig. 2A), we observed a reduction of CDC42 at the transcription and at the protein level by RT-PCR and Western blot analyses (Fig. 2B and C). Consistently, we verified that CDC42 signaling was perturbed by ETV6–RUNX1. As shown in Fig. 2D, the CDC42 downstream effector kinase PAK2 (38) was less expressed in ETV6–RUNX1–positive cells, and cell stimulation with CXCL12 induced a marked increase in the phosphorylation of PAK2 only in Ba/F3 control cells (densitometry analyses of pPAK2 after normalization on the amount of PAK2 total and β-actin: +2.36-fold increase in Ctr cells vs. −0.15-fold decrease in ETV6–RUNX1–positive cells).

**ETV6–RUNX1 impairs migration toward CXCL12**

By applying a Transwell migration assay, we observed a significant defect in the chemotactic response of ETV6–RUNX1–expressing Ba/F3 cells to CXCL12, a potent chemoattractant for BCP (Fig. 3A). In detail, the induction of ETV6–RUNX1 expression caused a decrease in the migration index of 86.2% (average of 5 independent experiments, range: 75.1%–92.6%; P < 0.001), although the expression of the CXCR4 receptor on the cell surface was unaffected or even increased (MFI increase average: Table 1. Upregulated and downregulated genes in ETV6–RUNX1–positive Ba/F3 cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>t test</th>
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<tr>
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<td><strong>Downregulated genes</strong></td>
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Figure 2. Cdc42 pathway analysis in control and ETV6–RUNX1–positive Ba/F3 cells. Ba/F3 cells were induced to express ETV6–RUNX1 for 3 days. A, flow cytometric analysis of the intracellular expression of V5 epitope tag, fused to ETV6–RUNX1 cDNA. B, cDNA was subjected to TaqMan RT-PCR and normalized to Hprt expression. Transcript level of the Cdc42 gene in ETV6–RUNX1–positive cells relative to control cells is shown as an average of triplicates. Ctr, control cells; ER, ETV6–RUNX1–positive cells. t test: **, P < 0.01. C, cell lysates were analyzed by Western blotting with anti-CDC42 antibody. The blot was later stripped and reprobed with an anti-β-actin antibody. CDC42 protein expression level in ETV6–RUNX1–positive cells was quantified by densitometry, normalized to β-actin, and indicated in the figure as the percentage with respect to control cells. M, Marker; − and +, negative and positive controls for CDC42 protein, respectively. D, Western blot analysis of PAK2 phosphorylation, total PAK2, and β-actin after 2 minutes of CXCL12 stimulation.
ETV6–RUNX1 Inhibits CXCL12-Driven Cell Migration

31%, range: 3%–100%, P < 0.05; Supplementary Fig. S2A).

We then explored the possible role of CXCR7 and CCR2 on the migration defect of ETV6–RUNX1–positive cells. These 2 receptors that negatively regulate the CXCL12 signaling cascade in BCP cells (39–41) are both upregulated in Ba/F3 cells after ETV6–RUNX1 induction [MFI increase average of CXCR7: 16%, range: 6%–27%, P < 0.05 (Supplementary Fig. S2B); fold change of the Ccr2 gene: 12.55, P < 0.01 (Supplementary Fig. S3A)].

In Ba/F3 control cells, CXCL12-mediated chemotaxis was modulated by both an anti-CXCR7 antibody and CCL2, the ligand of CCR2 (Supplementary Figs. S4A and S3B). However, in ETV6–RUNX1–positive cells, the migration toward CXCL12 remained defective after CXCR7 blocking (Supplementary Fig. S4A), thus excluding a role of CXCR7 in the inhibition of their migration ability. On the other hand, in presence of CCL2, we observed a more marked decrease in the ability to migrate to CXCL12 in Ba/F3 ETV6–RUNX1–induced cells (Supplementary Fig. S3B). Interestingly, CCL2 is secreted at higher amount by ETV6–RUNX1–positive Ba/F3 cells (Supplementary Fig. S3C). However, no difference in migration toward CXCL12 was observed in control cells upon pretreatment with the ETV6–RUNX1–positive Ba/F3 supernatant, thus also excluded a pivotal role of the secreted CCL2 in the inhibition of the migration ability of ETV6–RUNX1–positive cells.

We previously observed high expression levels of the FLT3L receptor (CD135) in the immature hematopoietic cells from ETV6–RUNX1 transgenic mice (12). FLT3L plays an important role in cellular proliferation and survival, but it also enhances migration toward CXCL12 (42). However, although we confirmed higher level of CD135 protein expression in Ba/F3 cells after ETV6–RUNX1 induction (Supplementary Fig. S2C), we did not observe an increase in their migration ability, unlike the control cells (Supplementary Fig. S4B), thus excluding that FLT3L could recover the ability of ETV6–RUNX1–positive cells to migrate to CXCL12.

Finally, as we showed above that ETV6–RUNX1 deregulated CDC42 signaling, we tested whether the overexpression of Cdc42 could counteract the impaired migration of ETV6–RUNX1–positive cells. In Supplementary Fig. S4C, we showed that while the overexpression of wild-type (WT) or constitutively active (CA) Cdc42 caused a marked increase in the migration toward CXCL12 in control cells, only a slight increase was measured in ETV6–RUNX1–positive cells (MFI increase average in CDC42 CA cells: 3.49 ± 0.13 in Ctr cells vs. 1.29 ± 0.08 in ER cells). This result demonstrated that neither the enhancement of the CDC42 activity was able to recuperate the migration property toward CXCL12 of ETV6–RUNX1–positive cells.

**ETV6–RUNX1–positive cells do not have a general defect of movement**

We wondered whether ETV6–RUNX1–positive Ba/F3 cells were unable to specifically migrate toward CXCL12 or whether they presented any general defect in movement. By Transwell migration assay using 10% FCS as a general stimulus, we found that not only ETV6–RUNX1–positive cells were inhibited in movement, but also they migrated more than control cells (Fig. 3B).

**ETV6–RUNX1–positive cells present a defect in CXCR4 signaling**

Although the expression of CXCR4 receptor on the cell surface was unaffected or even increased (Supplementary Fig. S2A), ETV6–RUNX1 induction inhibited the mobilization of intracellular calcium flux after CXCL12 stimulation in Ba/F3 cells (Fig. 4A). Moreover, the phosphorylation of ERK in response to CXCL12 was absent in ETV6–RUNX1–positive cells (Fig. 4B).

Thus, the expression of ETV6–RUNX1 in Ba/F3 cells resulted in the block of ERK phosphorylation, early downstream to CXCR4 signaling.
ETV6–RUNX1 alters the expression of genes regulating the cytoskeleton and migration properties of primary pre-BI cells

The effect of ETV6–RUNX1 on the expression of genes regulating the cytoskeleton and migration properties was confirmed in primary pre-BI cells, purified from fetal liver of wild-type mice, a more physiological murine model. These cells were stably transduced with a retroviral vector (pMIGR1) containing the ETV6–RUNX1 cDNA upstream of the IRES-GFP and isolated by GFP sorting (Material and Methods; Fig. 5A).

Pre-BI MIGR–ETV6–RUNX1 cells presented a higher cell-surface expression levels (MFI ratio) of adhesion molecules such as CD18 (average of increase: 64%, range: 15%–146%, \( P < 0.01 \)), CD11a (average: 90%, range: 19%–203%, \( P < 0.01 \)), and CD54 (average: 27%, range: 14%–85%, \( P < 0.05 \); Supplementary Fig. S5A–S5C) and lower levels of CD62L (average of decrease: 68%, range: 7%–83%, \( P < 0.05 \); Supplementary Fig. S5D) than MIGR–GFP control cells.

As in Ba/F3 cell line, the expression of ETV6–RUNX1 in pre-BI cells caused alteration in the expression of genes involved in the modulation of the cytoskeleton (Table 2), including the overexpression of Cdc42ep2 and Cdc42ep3, the negative regulators of Cdc42, and a reduction of Cdc42 transcription (Fig. 5B). Moreover, the CDC42 signaling was perturbed in the pre-BI ETV6–RUNX1–positive cells, as the stimulation with CXCL12 induced an increase in PAK2 phosphorylation only in the control MIGR–GFP cells (Fig. 5C).

Interestingly, also pre-BI MIGR–ETV6–RUNX1 cells showed a significant defect in their ability to migrate toward CXCL12, with 73.5% decrease of M.I. (average of 5 independent experiments, range: 57.0%–88.9%, \( P < 0.001 \); Fig. 5D). However, the expression of CXCR4 receptor on the cell surface was unaffected (Supplementary Fig. S5E).

Unlike Ba/F3 cells, pre-BI cells express the EGF receptor (EGFR), a strong activator of the CDC42 pathway (43). As shown in Supplementary Fig. S6, although we observed an...
increased migration index toward CXCL12 in the control cells in presence of EGF stimulus; however, the migration toward CXCL12 remained defective in ETV6–RUNX1–positive cells.

Discussion

Although the t(12;21) translocation is a frequent prenatal initiating mutation in BCP-ALL (1–3, 6), the cellular signaling pathways corrupted by ETV6–RUNX1 in the preleukemic clone remain unknown. In the present article, we consistently showed in 2 in vitro models that ETV6–RUNX1 deregulates the cytoskeleton and compromises the chemotactic response to CXCL12.

It has been increasingly recognized that cancer initiation and progression are not solely a cancer cell autonomous process. Primary tissue cells live in complex microenvironments, characterized by heterotypic signaling between ancillary cells and hematopoietic cells (44). This signaling is considered to play a role in the regulation of the behavior of stem and precursor hematopoietic cells, including their survival, proliferation, and differentiation. For this reason, alterations in the environment or in the abilities of the stem and progenitors cells to interact with the innate niches play a crucial role in tumor initiation and progression.

We have used 2 different model systems to study the ETV6–RUNX1 preleukemic phase and in particular to examine whether ETV6–RUNX1 altered the cellular adhesion and migration properties of BCP. Indeed, this type of study is not feasible in clinical samples at diagnosis of ALL, where the analysis of the fusion function is confounded by the additional genetic abnormalities. We have therefore used a murine progenitor cell line (Ba/F3), with hormone-inducible ETV6–RUNX1 expression, a model developed in the past to demonstrate the impact of the fusion gene on the inhibitory response to TGFβ (12). Next, we confirmed our results in pre-BI cells, primary cells derived from a wild-type mouse fetal liver, already successfully used for functional analysis of BCP-ALL–associated fusion transcripts (27–28).

We observed that the expression of ETV6–RUNX1 in Ba/F3 cell line resulted in changes in the cellular phenotype:

### Table 2. Upregulated and downregulated genes in ETV6–RUNX1–positive pre-BI cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc42ep2</td>
<td>1.46</td>
<td>0.02215</td>
</tr>
<tr>
<td>Arhgef11</td>
<td>1.86</td>
<td>0.00036</td>
</tr>
<tr>
<td>Cdc42ep3</td>
<td>3.1</td>
<td>0.00114</td>
</tr>
<tr>
<td>Downregulated genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rock1</td>
<td>0.66</td>
<td>0.00028</td>
</tr>
<tr>
<td>Stmn1</td>
<td>0.75</td>
<td>0.01874</td>
</tr>
<tr>
<td>Clip1</td>
<td>0.75</td>
<td>0.00008</td>
</tr>
<tr>
<td>Cyfp2</td>
<td>0.78</td>
<td>0.02114</td>
</tr>
<tr>
<td>Mylk</td>
<td>0.47</td>
<td>0.00005</td>
</tr>
<tr>
<td>Mmp9</td>
<td>0.16</td>
<td>0.00022</td>
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<td>Mmp2</td>
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<td>0.00256</td>
</tr>
<tr>
<td>Cdh2</td>
<td>0.3</td>
<td>0.00357</td>
</tr>
</tbody>
</table>

Figure 5. Cdc42 pathway and migration analysis of pre-BI MIGR–GFP and MIGR–ETV6–RUNX1 cells. A, Western blot analysis of c-Myc epitope tag, fused to ETV6–RUNX1 cDNA. B, cDNA of pre-BI cells was subjected to TaqMan RT-PCR and normalized to Hprt expression. Transcript levels of the Cdc42 gene in MIGR–ETV6–RUNX1 (MIGR–ER) relative to MIGR–GFP cells are shown as an average of triplicates. t test: *, P < 0.05. C, Western blot analysis of PAK2 phosphorylation, total PAK2, and β-actin after 2 minutes of CXCL12 stimulation. GFP, pre-BI MIGR–GFP cells; ER, pre-BI MIGR–ETV6–RUNX1 cells. D, migration assays toward 100 ng/mL CXCL12. After 3 hours, the GFP-positive cells that migrated to the lower well were counted by FACS. The migration index was defined as the ratio between the number of cells migrated to the lower chamber of the Transwell in response to CXCL12 and in its absence. Error bars, SD from triplicates of a representative experiment. t test: ***; P < 0.001.
several molecules involved in cell adhesion were deregulated in expression. We observed an increase in the adhesion of ETV6–RUNX1–positive cells to murine endothelial cell lines.

To understand the reason for the reported alterations, we explored the effect of the ETV6–RUNX1 fusion, an aberrant transcription factor, on the transcription of a panel of genes involved in the biogenesis and organization of the cytoskeleton. Indeed, we showed that the expression of ETV6–RUNX1 in Ba/F3 cell line caused alteration in the expression of genes regulating cell shape, formation of pseudopodia, cell migration, actin, and microtubule organization. In particular, among the most overexpressed genes in ETV6–RUNX1–positive cells, we identified 2 negative regulators of CDC42. Moreover, we observed a reduction of CDC42 at the transcription and protein levels and a block of the CDC42 signaling pathway. CDC42 not only has a pivotal role in cell-cycle progression (in agreement with this, we previously described an increase in the proportion of ETV6–RUNX1–expressing cells in G0–G1; ref. 12) but also in cytoskeleton rearrangement during directional migration.

In parallel, we investigated the migration abilities of ETV6–RUNX1–inducible Ba/F3 cells. Interestingly, we found that the fusion gene significantly impaired the chemotactic response to CXCL12, although the cell-surface expression of the receptor CXCR4 was unaffected. Indeed, the CXCL12 chemotaxis defect was not due to a general impairment of movement of ETV6–RUNX1–positive cells, as their migration toward a general stimulus was instead increased.

We then excluded a possible role in this migration defect of several players of the CXCL12/CXCR4 pathway, including CXCR7, a receptor with CXCL12-scavenging activity (39), FLT3L receptor, a positive regulator of CXCL12 migration (42), the GTPase CDC42, as well as the CCL2/CCR2 and EGF/EGFR axes. These 2 axes are both involved in the modulation of CXCL12-mediated chemotaxis. Indeed, it has been reported that the expression of CCR2 negatively regulates the cytoskeletal rearrangement and migration of immature B cells and that the control of B-cell homing by CCR2 is mediated by its ligand, CCL2, which is secreted by B cells and downregulates the CXCL12 signaling cascade (40–41). On the contrary, a synergistic effect of CXCR4 and the EGFR on promoting cancer metastasis has been reported (45). In particular, EGF was described to promote breast cancer cell chemotaxis in CXCL12 gradients, whereas CXCL12 alone failed to stimulate the migration of these cells (46).

We were able to demonstrate that ETV6–RUNX1 impairs the calcium flux, a very proximal CXCR4 signaling event, and the phosphorylation of ERK kinase, as a downstream event.

Further analyses are needed to fully define the mechanism of migration defect and to establish how ETV6–RUNX1 inhibits the CXCL12–CXCR4 signaling pathway. In this regard, it was reported in the literature that ETV6–RUNX1–positive patients with ALL presented at diagnosis lower level of CD9 than the negative patients with ALL (47). Importantly, the tetraspanin CD9 has been described to regulate migration, adhesion, homing of human cord blood CD34+ cells (48). In light of these observations, we believe that it will be important to explore whether the low expression of CD9 is a property not only of the overt leukemic blasts but also of the preleukemic cells and whether this feature may play a key role in the CXCL12 migration defect of ETV6–RUNX1–positive BCP described here.

Noteworthily, the results observed in the Ba/F3 ETV6–RUNX1–inducible expression system were reproducible in primary pre-BI cells. Indeed, after transduction of the chimeric gene, we confirmed an altered expression of genes involved in cytoskeleton modulation, including Cdc42 with its regulators, and several adhesion molecules. Moreover, we consistently observed the same block of CDC42 signaling and the same significant defect of pre-BI ETV6–RUNX1–positive cells to migrate toward CXCL12.

Interestingly, similar alterations in the expression of adhesion molecules and defects in CXCL12 migration have been reported in BCR-ABL1–positive leukemia (19–21). In this context, it has been hypothesized that these aberrations could contribute to the homing and retention defects in the bone marrow typical of immature myeloid cells in chronic myelogenous leukemia (49).

In light of the findings described here, we can sustain the hypothesis that the ETV6–RUNX1 preleukemic clone, compared with its normal counterpart, might have an altered interaction with the bone marrow microenvironment, which results in a greater tendency to migrate to the periphery. A direct demonstration of their ability to leave the bone marrow, despite of their immature status, is represented by the detection of preleukemic clones in peripheral blood at birth (in Guthrie cards, cord blood, and peripheral blood; refs. 5, 50). Appropriate in vivo studies in murine models must be afforded to further exploit the characteristics of the preleukemia phase and to define the role of microenvironmental factors in the preleukemic state induced by ETV6–RUNX1.

In conclusion, the abnormalities we observed in the expression of genes regulating the cytoskeleton and in migration toward CXCL12 may represent early events in the pathogenesis of the disease, not necessary associated to the progress toward overt leukemia unless additional hits occur (4). Indeed, our observation raises the question as to how ETV6–RUNX1 preleukemic cells interact with the microenvironment. We believe that identification of the precise localization of these cells, their cell–cell contacts, and gene regulation are crucial to providing a better understanding of the mechanisms that allow the preleukemic clone to persist covertly in an individual for several years, maybe prone to additional genomic events. This will be decisive in helping develop strategies for their effective eradication and leukemia prevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Cytoskeletal Regulatory Gene Expression and Migratory Properties of B-cell Progenitors Are Affected by the ETV6 –RUNX1 Rearrangement

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