ALL1 fusion proteins induce deregulation of *EphA7* and ERK phosphorylation in human acute leukemias

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Erythropoietin-producing hepatoma-amplified sequence (Eph) receptor tyrosine kinases and their cell-surface-bound ligands, the ephrins, function as a unique signaling system triggered by cell-to-cell interaction and have been shown to mediate neurodevelopmental processes. In addition, recent studies showed deregulation of some of *Eph/ephrin* genes in human malignancies, suggesting the involvement of this signaling pathway in tumorigenesis. The *ALL1* (also termed *MLL*) gene on human chromosome 11q23 was isolated by virtue of its involvement in recurrent chromosome translocations associated with acute leukemias with poor prognosis. The translocations fuse *ALL1* to any of >50 partner genes and result in production of chimeric proteins composed of the ALL1 N terminus and the C terminus of the partner protein. The most common translocations in *ALL1*-associated leukemias are t(4;11) and t(9;11), which generate ALL1/AF4 and ALL1/AF9 fusion protein, respectively. In the present study, we sought to determine whether ALL1 fusion proteins are involved in regulation of *Eph/ephrin* genes. Screening of K562 cells producing recombinant ALL1/AF4 or ALL1/AF9 fusion protein revealed transcriptional up-regulation of the *EphA7*. Consistent with this finding, siRNA-mediated suppression of ALL1/AF4 in SEMK2 cells carrying the t(4;11) chromosome translocation resulted in down-regulation of *EphA7*. ChIP analysis demonstrated the occupancy of tagged ALL1 fusion proteins on the *EphA7* promoter, pointing to *EphA7* as a direct target of the formers. Further studies demonstrate that EphA7 up-regulation is accompanied by ERK phosphorylation. Finally, we show apoptotic cell death, specific for leukemic cells carrying the t(4;11) chromosome translocation, after treatment of the cells with an ERK phosphorylation blocker.

Eph receptor | gene regulation | oncogene

The erythropoietin-producing hepatoma-amplified sequence (Eph) receptors are a large family of receptor tyrosine kinases comprising eight EphA and six EphB receptors in humans. The distinction between EphA and -B receptors is based on the similarity within each group of the extracellular domain sequences and on the affinity for binding ephrin-A and -B ligands. Thus, EphA receptors bind to the ligands, termed ephrin-A1, -A2, -A3, -A4, and -A5 anchored on cell membrane via glycosylphosphatidylinositol, whereas EphB receptors bind to the ligands termed ephrin-B1, -B2, and -B3, which are transmembrane molecules. Because both the Eph receptors and ephrins localize to the cell surface, the signaling is restricted to the sites of direct cell-to-cell contact and is capable of inducing reciprocal bidirectional events between interacting cells. This unique feature has been shown to play a critical role in establishing topologically organized neuronal connections in many regions of the developing nervous system. Recent studies further unveiled the involvement of Eph–ephrin interaction in a variety of developmental processes including arterial-venous differentiation, cell migration which results in compartmentalizing cell subpopulations in the developing tissue, and cell movement into the appropriate embryonic environment which may determine a particular cell fate and result in cell differentiation and patterning (reviewed in ref.1). Besides such physiological roles, recent studies revealed deregulation of some of the *Eph/ephrin* genes in human malignancies, including up-regulation of *ephrin-A1* or *-B2* in melanoma (2, 3), up-regulation of *EphB2* in stomach cancer (4) and in breast cancer (5), and up-regulation of *EphA2* in prostate (6), breast (7), and esophageal cancers (8), some of which were shown to be associated with tumor invasion or tumor metastasis and therefore associated with poor prognosis. Conversely, mutational inactivation of *EphB2* was detected in prostate (9) and colon cancers (10), suggesting tumor suppressor function of this Eph receptor in the relevant tumors. In contrast to solid tumors, less is known about the role of the Eph/ephrin pathway in the development of hematological malignancies.

The *ALL1* gene (also termed *MLL*) has been isolated by virtue of its involvements in recurrent chromosome translocations occurring in acute leukemias, particularly in infant acute lymphoblastic leukemias and in therapy-related acute myeloid leukemias (11). The chromosome translocation results in the fusion of the *ALL1* gene with 1 of >50 different partner genes and the production of leukemogenic proteins composed of the N-terminal ALL1 sequence and the C terminus of the partner protein (11). The most prevalent *ALL1* rearrangement in acute lymphoblastic leukemia is the *ALL1/AF4* chimeric gene resulting from the t(4;11) chromosome translocation. This rearrangement leads to pro-B cell leukemia and is associated with very poor prognosis in infants and adults (12). The molecular pathways deregulated by ALL1 fusion proteins (14, 21) are only partially defined but are likely to include process(es) involved in proliferation and differentiation of hematopoietic cells. In this context, we noted that whereas EphA7 is expressed in fetal bone marrow pro-B and pre-B cells, it is silenced in the entire series of adult B-lineage cells (13). This finding prompted us to look for the expression of EphA receptors and ligands in *ALL1*-associated leukemias.

In the present study, we systematically searched for expression of EphA receptors in cells producing ALL1/AF4 and ALL1/AF9 fusion proteins and found that both proteins induced *EphA7* transcription. ChIP analysis demonstrated the occupancy of the fusion proteins on the *EphA7* promoter, indicating *EphA7* as a direct target of ALL1 fusion proteins. Consistent with those results, ALL1/AF4-dependent EphA7 expression was demonstrated in a pro-B cell line with the t(4;11). Furthermore, we showed that direct EphA7 knockdown or ALL1/AF4 knock-

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Abbreviations: Eph, erythropoietin-producing hepatoma-amplified sequence; 5-ITU, 5-iodotubercidin; Ct, cycle threshold.

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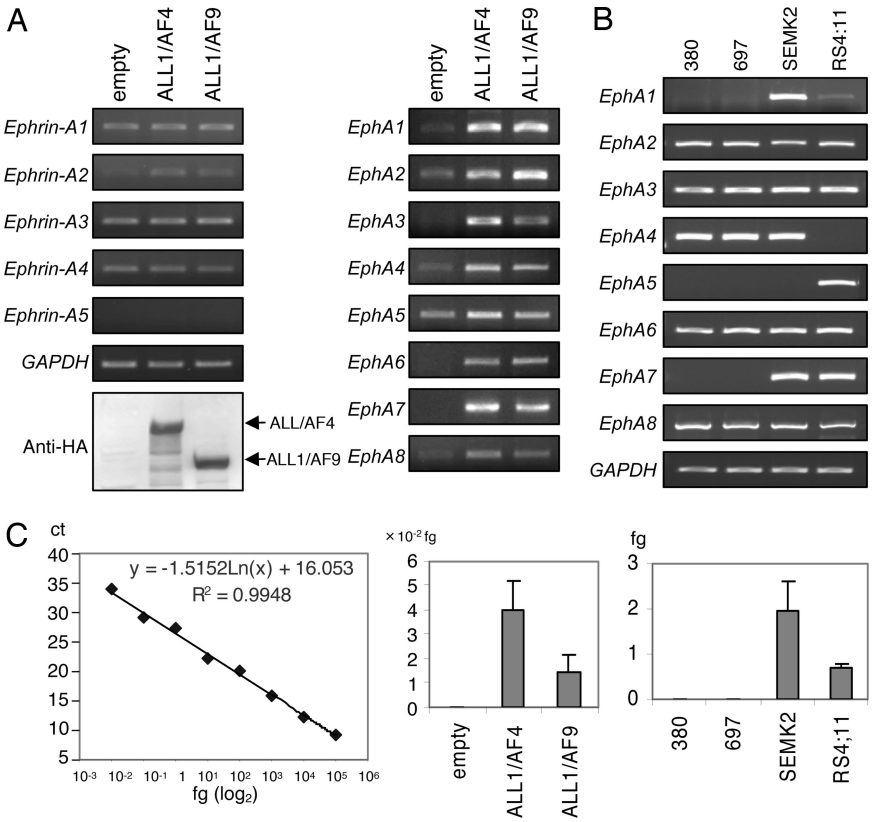

Fig. 1. Detection of *EphA/ephrin-A* transcripts in K562 cells transfected with ALL1 fusion constructs and in leukemic cell lines with the t(4;11) abnormality. (*A* and *B*) K562 cells transfected with ALL1/AF4 or ALL1/AF9 constructs (*A*), pro-B t(4;11) leukemic cell lines SEMK2 and RS4;11, and the pro-B line 380 and pre-B line 697 lacking *ALL1* abnormalities (*B*) were subjected to semiquantitative RT-PCR analysis to determine the expression level of *ephrin-A* and/or *EphA*. "Empty" in *A* indicates transfection with vector. Western blot detection of the recombinant ALL1 fusion proteins with anti-HA mAb in K562 transfectants is shown in *A Left*. (*C*) The amounts of EphA7 transcript in the aforementioned cells were quantified by applying real-time RT-PCR methodology. cDNAs synthesized from the various cell sources were first determined for their Ct values for GAPDH. This procedure gave values of 15.01 ± 0.11 (mean ± SD), indicating similar amounts of total cDNA. The Ct values determined for EphA7 were converted to fg according to a standard curve established by using known amount of EphA7 cDNA (*Left*).

down-mediated EphA7 suppression in the t(4;11) leukemic cells resulted in attenuation of ERK1/2 phosphorylation. Finally, we found that treatment of leukemic cells carrying the t(4;11) with an inhibitor of ERK phosphorylation induced apoptotic cell death. These results indicate that the ALL1 fusion proteins directly up-regulate *EphA7* expression, which apparently results in ERK phosphorylation. The latter modification is likely to contribute to the maintenance of the malignant phenotype.

Results

ALL1 Fusion Proteins Induce *EphA7* Receptor Expression. By applying a transfection strategy to ectopically express ALL1/AF4 and ALL1/AF9 in K562 cells (Fig. 1*A* bottommost blot, left lane), we determined the expression level of the genes encoding eight EphA receptors and five ephrin-A ligands in the transfectants. Semiquantitative RT-PCR analysis showed that both ALL1 fusion proteins induced transcription of all *EphA* receptor genes, whereas they did not exert a noticeable effect on induction of *ephrin-A* genes (Fig. 1*A*). To further extend this finding to leukemic cells with *ALL1* rearrangement, the SEMK2 and RS4;11 pro-B cell lines harboring the t(4;11) translocation, and the 380 pro-B and 697 pre-B cell lines lacking *ALL1* abnormalities, were subjected to similar analysis (Fig. 1*B*). This analysis showed consistent and differential expression of *EphA7* in the t(4;11) leukemic cell lines. Subsequent quantification of *EphA7* transcript by application of real-time RT-PCR methodology enabled estimation of the amounts of the transcript in K562 cells transfected with ALL1/AF4 or ALL1/AF9, in SEMK2 and RS4;11 cells to be 0.04 ± 0.01, 0.014 ± 0.006, 1.97 ± 0.6, and 0.68 ± 0.09 (mean ± SD femtogram), respectively. In parallel, the amount of EphA7 RNA in vector-transfected K562, intact 380 and 697 cells was determined to be <0.001 fg (Fig. 1*C*). These results collectively pointed to *EphA7* as a consistently responsive target of ALL1 fusion proteins and prompted further analysis of its up-regulation. To ascertain that the endogenous ALL1 fusion protein produced in leukemic cells carrying an *ALL1* abnormality regulates EphA7 transcription, we suppressed ALL1/AF4 produced in SEMK2 cells by applying siRNA methodology. SEMj siRNA generated by us (14) and others (15) is designed to target SEMK2 cell-specific *ALL1/AF4* fusion junction; in parallel, MVj siRNA targets the fusion junction produced in other cells (MV4;11), and thus served as a negative control. We first determined the efficiency of SEMj siRNA in suppressing the ALL1/AF4 protein and found ≈80% reduction of the latter, with no effect on the expression level of normal ALL1 or AF4 (Fig. 2*A*). SEMK2 cells treated with SEMj siRNA were then subjected to semiquantitative RT-PCR analysis to determine the expression levels of *EphA7* and *HoxA9*; the latter is a known target of ALL1 fusion proteins (16) and thus served as a positive control to ascertain the effect of elimination of the ALL1 fusion protein. This analysis demonstrated that the suppression of the ALL1/AF4 in SEMK2 cells attenuated expression of *EphA7* and *HoxA9*, supporting the notion of ALL1 fusion protein-mediated transcriptional regulation of *EphA7* (Fig. 2*B*).

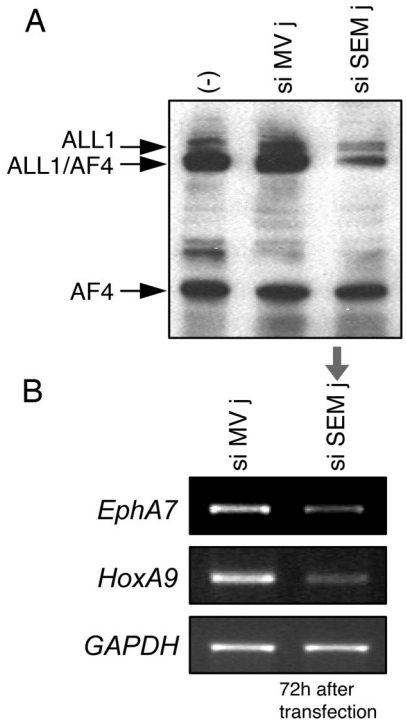

Fig. 2. Suppression of ALL1/AF4 in SEMK2 cells down-regulates *EphA7* expression. SEMK2 cells transfected with either junction-specific (SEMj) or control (MVj) siRNA were subjected to Western blot analysis for the detection of p300 ALL1 (ALL1), ALL1/AF4, and AF4 proteins (*A*) and to semiquantitative RT-PCR analysis for the detection of *EphA7* and *HoxA9* transcripts (*B*). In *A*, a mixture of anti-ALL1 N terminus and anti-AF4 C terminus Abs was used as a probe.

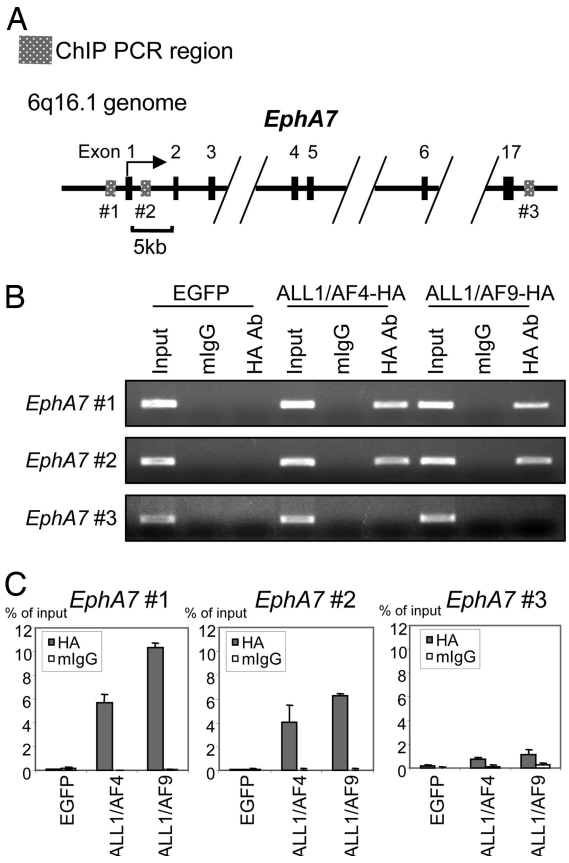

Fig. 3. ChIP analysis of the occupancy of ALL1 fusion proteins on *EphA7* genomic regions. (*A*) Genomic regions within the *EphA7* gene, designated 1, 2, and 3, and used for ChIP analysis are shown. (*B*) ChIP-enriched-DNA (with anti-HA mAb) was PCR-amplified and resolved on 2% agarose gel. The EGFP construct in pMACS 4.1 vector was used as a transfection control. mIgG indicates normal mouse IgG used as a control. (*C*) ChIP-enriched DNA was also subjected to real-time PCR analysis. Ct value determined by using 0.05% of total input set at 100% and the values determined in the assay by using ChIP-enriched DNA are converted to a percentage of the input. Vertical bar above each column indicates SD as determined by triplicate assays.

ALL1 Fusion Proteins Bind to *EphA7* Genomic Locus. By applying ChIP methodology, we determined the occupancy of HA-tagged ALL1/AF4 and ALL1/AF9 exogenously expressed in K562 cells. The *EphA7* genomic regions analyzed by ChIP mapped ≈0.7 kb upstream and ≈0.6 kb downstream from the transcription initiation site and within 3′ noncoding sequence; they were termed regions 1, 2, and 3, respectively (Fig. 3*A*). This analysis showed the binding of the ALL1/AF4 and ALL1/AF9 chimeric proteins to regions 1 and 2, but not to region 3 (Fig. 3*B*). Quantitative real-time PCR analysis supported the previous results (Fig. 3*C*), indicating that *EphA7* is a direct target of the ALL1 fusion proteins.

EphA7 Is an Essential Mediator in Induction of ERK Phosphorylation. Little is known about the signal transduction pathway(s) in which EphA7 is involved. At first, we examined in K562 cells transfected with full-length EphA7 construct the phosphorylation status of several proteins commonly associated with RTK signal transduction pathways. Consistent with previous results (see Fig. 1*A Right*), the EphA7 protein was not observed in intact K562 cells but was detected in a dose-dependent manner in cells transfected with the EphA7 construct (Fig. 4*A Left*). We found no apparent induction of RAF or MEK1/2 phosphorylation, two proteins that are phosphorylated in response to activation of the Ras transduction pathway (Fig. 4*A Left*). In contrast, we found that overexpressing EphA7 correlated with ERK phosphorylation. This correlation was determined by sequential probing of a Western blot with anti-ERK Ab and subsequently with antibody against phosphorylated-ERK which specifically detects *p*-ERK (Fig. 4*A Left*). It was previously shown that MEK1/2 are highly phosphorylated in intact K562 cells (17). Such basic high MEK1/2 phosphorylation may have obscured an additional change in phosphorylation induced by overexpressing EphA7. Therefore, we ectopically expressed EphA7 in the KG1 AML cell line. Here, we found induction of MEK1/2 phosphorylation after expression of recombinant EphA7 (Fig. 4*A Right*). We also noticed that the MEK1/2 phosphorylation in KG1 cells was accompanied by ERK2 as well as ERK1 phosphorylation (Fig. 4*A Right*). Because the prominent function of MEK is to phosphorylate ERK (18), our results indicate that EphA7 is positioned upstream of this cascade.

We next determined whether ALL1 fusion proteins induce ERK phosphorylation as EphA7 does. Indeed, induction of ERK phosphorylation was observed in K562 cells transfected with the ALL1/AF4 and ALL1/AF9 constructs (Fig. 4*B*). To validate that in these transfectants EphA7 is an essential mediator for ERK phosphorylation, SEMK2 cells were treated with siRNAs targeting EphA7 mRNA at two distinct regions within the transcript (siEphA7, -1, and -2 in Fig. 4*C*). Both siRNAs were shown to down-regulate the mRNA at an efficiency of ≈70% (Fig. 4*C Lower*). Suppression of EphA7 resulted in strong reduction of ERK1 and ERK2 phosphorylation, with no effect on the amount of total ERK (Fig. 4*C Upper*). This finding implied that EphA7 is an essential mediator for ERK1/2 phosphorylation in cells expressing ALL1 fusions. Furthermore, we found that ALL1/

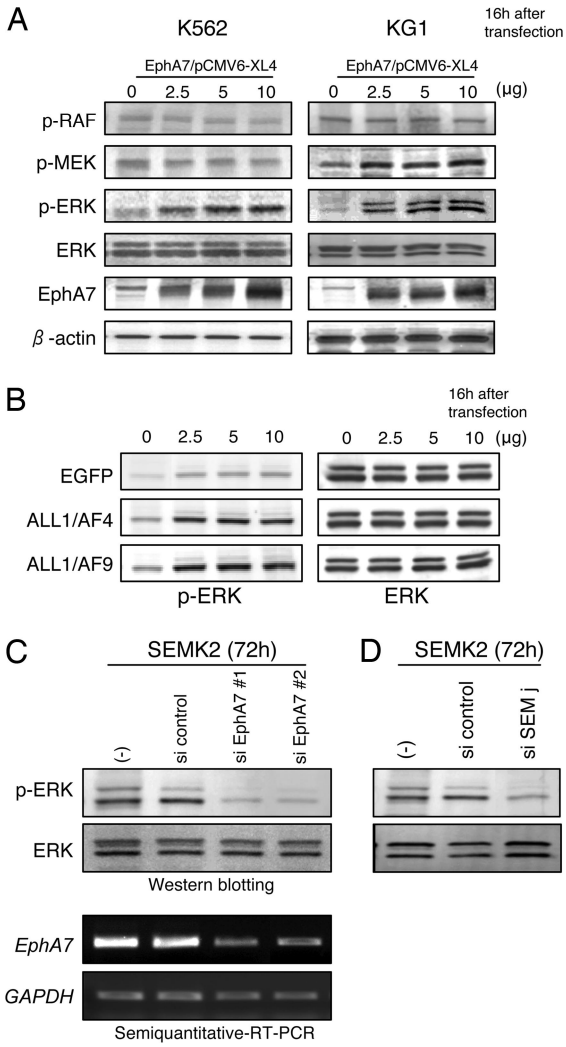

Fig. 4. EphA7 mediates ERK phosphorylation. (*A*) K562 and KG1 cells transfected with the EphA7 construct were analyzed by Western blotting for determining the phosphorylation status of RAF, MEK1/2, and ERK as well as for detection of the construct-derived EphA7 protein. β-Actin served as a loading control. (*B*) The phosphorylation status of ERK was determined in K562 cells transfected with ALL1 fusion construct. (*C* and *D*) SEMK2 cells, suppressed for the expression of EphA7 by treatment with EphA7-specific siRNAs (siEphA7 1 and 2 in *C*) or by treatment with ALL1/AF4-specific siRNA (*D*), were analyzed for ERK phosphorylation status. The efficiency of siEphA7 1 and 2 for EphA7 suppression was determined by semiquantitative RT-PCR (*C Lower*).

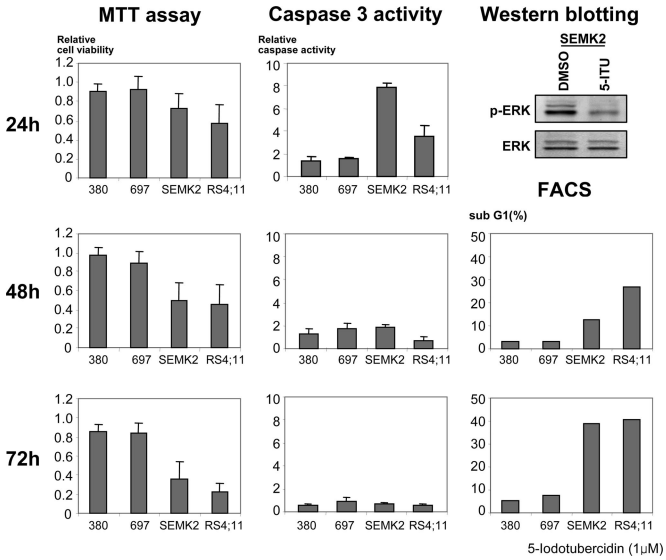

Fig. 5. 5-ITU specifically inhibits ERK phosphorylation and the growth of the cells producing ALL1/AF4 fusion protein. SEMK2 cells with the t(4;11) treated for 24 h with either solvent (DMSO) or 5 μM 5-ITU were subjected to Western blot analysis for the detection of phosphorylated ERK (*Upper Right*). The SEMK2 and RS4;11 pro-B leukemic cell lines with the t(4;11), and the pro-B line 380 and pre-B line 697 lacking *ALL1* abnormality were treated with 1 μM 5-ITU for 24, 48, and 72 h. At each time point, the cells were subjected to MTT assay (*Left*), caspase-3 activity assay (*Center*), and FACS analysis (*Lower Right*). The results of MTT and caspase-3 activity assays are calculated as a ratio against the value for DMSO-treated cells. Vertical bar above each column indicates SD as determined by triplicate assays.

AF4 knockdown in SEMK2 cells similarly caused reduced phosphorylation of ERK1/2 (Fig. 4*D*).

An Inhibitor for ERK2 Phosphorylation, 5-Iodotubercidin (5-ITU), Induces Cell Death of Leukemic Cell Lines with the t(4;11) Abnormality. We examined the effect of 5-ITU, an inhibitor for ERK phosphorylation (19), on cell proliferation. To ascertain the biochemical effect of 5-ITU, SEMK2 cells with the t(4;11) and producing phosphorylated ERK1/2 (see "−" and "si control" lanes in Fig. 4 *C* and *D*) were treated with this compound, and ERK phosphorylation status was determined by Western blot analysis (Fig. 5 *Top Right*). The result indicated inhibition of ERK phosphorylation by 5-ITU. Application of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay indicated reduction in numbers of SEMK2 and RS4;11 cells with the t(4;11) and no diminution in the number of the 380 and 697 control cells (Fig. 5 *Left*). We also found that ALL1/AF4 knockdown in SEMK2 cells, which caused ERK1/2 inactivation (see Fig. 4*D*), resulted in ≈25% reduction in the cell number as compared with the number of cells treated with control siRNA (H.N., unpublished results). To determine the cause for the reduction in cell numbers, the 5-ITU-treated cells were subjected to assays determining apoptotic cell death (caspase-3 activity assay and FACS analysis). Increased caspase 3 activity was demonstrated at 24 h in 5-ITU sensitive leukemic cells (SEMK2 and RS4;11 in Fig. 5 *Center*). In parallel, a high proportion of these cells distributed at the sub-G_1 phase (Fig. 5 *Right*). These results demonstrated dependency of the leukemic cells producing ALL1/AF4 fusion protein on ERK phosphorylation, with the latter preventing apoptotic cell death.

Discussion

In the present study, we show that ectopic expression in K562 cells of the leukemogenic ALL1 fusion proteins ALL1/AF4 and ALL1/AF9 induced transcription of several *EphA* RTKs after a short latency (16 h after transfection). Significantly, applying such an approach to induce *HoxA9* did not work. Thus, we found that ectopic expression of ALL1/AF4 or ALL1/AF9 did not induce *HoxA9* expression, and that further treatment of the transfectants with the HDAC inhibitor trichostatin A was required (T.N., unpublished data). Therefore, it appears that an additional layer(s) of transcriptional regulation is involved in induction of *HoxA9* by ALL1 fusion proteins. We note that deregulation of *EphA7* was not mentioned in previous studies of gene-expression profiling of *ALL1*-associated acute leukemias (20, 21).

A recent review concerning Eph receptors and ephrin signaling (1) showed the involvement of a wide variety of pathways, including examples for activation or inhibition of several different signaling pathways by a single Eph receptor. Presently, however, signaling pathways involved in EphA7 RTK have not

been determined. In this study, we examined the phosphorylation status of the major components of the MAPK/ERK pathway including RAF, MEK1/2, and ERK in K562 cells expressing either of two exogenous ALL1 fusion proteins or EphA7. The three proteins induced ERK phosphorylation. We also noticed ERK phosphorylation in a control transfection with EGFP construct, although the extent of the phosphorylation induced by the latter is less than one-fifth of that induced by the ALL1 fusion proteins (see Fig. 4*B*). Furthermore, siRNA-mediated suppression directed against either ALL1/AF4 or EphA7 in SEMK2 cells resulted in remarkable reduction of ERK phosphorylation (see Fig. 4 *C* and *D*). These results indicate that EphA7 indeed mediates ERK phosphorylation in K562 transfectants and in SEMK2 cells. No induction of RAF and MEK1/2 phosphorylation by EphA7 was observed in K562 cells (see Fig. 4*A*). However, a further study with KG1 cells showed EphA7-mediated MEK1/2 phosphorylation, accompanied with induction of ERK1/2 phosphorylation. It is therefore likely that EphA7 activates MEK1/2, leading to ERK phosphorylation. However, we cannot exclude the possibility that ERK phosphorylation mediated by EphA7 in K562 is executed by a pathway different from the classical MAPK/ERK. Regarding this issue, two studies suggest the presence of MEK-independent pathways (22, 23).

Because of the absence of antibodies for specific detection of phosphorylated EphA7, we could not determine whether EphA7 induced by ectopic ALL1 fusion protein or recombinant EphA7 in K562 and endogenous EphA7 in SEMK2 cells are present in an active phosphorylated form. A study of *in vitro* ephrin-A ligand-receptor binding assay previously showed that EphA7 displays high affinity to ephrin-A3 and -A5 ligands (24). In the present study, we found a steady level of *ephrin-A3* expression in K562 cells transfected with either control empty vector or ALL1 fusion construct (see Fig. 1). It is therefore likely that EphA7 expressed in K562 cells interacts with the ephrin-A3 ligand produced in the same cells and that this interaction affects activation of the Eph/ephrin pathway. Furthermore, ERK phosphorylation caused by overexpression of EphA7 in K562 cells and reduced ERK1/2 phosphorylation caused by suppression of EphA7 in SEMK2 cells suggest that the EphA7 protein in both circumstances is active.

Expression of Eph receptors including EphA7 is tightly regulated. A previous study (13) to determine *EphA7*/*Hek11* expression in human hematopoietic cells showed regulated expression of the gene in fetal bone marrow pro- and pre-B cells but not in adult bone marrow cells of the same differential stages. Consistent with these results, we also found that EphA7 expression level in 380 pro-B and 697 pre-B cell lines as well as in intact K562 cells was below the detection limit (>0.001 fg) as assayed by real-time RT-PCR methodology. The detection of EphA7 in the pro-B cell lines SEMK2 and RS4;11 would further support that ALL1/AF4 up-regulates *EphA7* expression in pro-B cells.

We found that 5-ITU suppressed growth of the leukemic cells producing ALL1/AF4 fusion protein by inducing apoptosis. 5-ITU was originally discovered as a potent inhibitor of adenosine kinase (K_i = 30 nM), Ser/Thr-specific kinases such as casein kinases 1 and 2, and the insulin receptor kinase fragment. The K_i for inhibition of ERK2 phosphorylation was estimated to be 525 nM (19). It is therefore possible that the treatment of the cells with 1 μM 5-ITU not only caused inactivation of ERK but also affected the aforementioned kinases. Regardless of this possibility, the differential apoptotic effect on two cell lines with t(4;11) was clear. Additional studies will be required to determine whether this outcome is restricted to leukemic cells producing ALL1/AF4 or is common to all *ALL1*-associated leukemias. Also, the mechanism underlying phospho-ERK-dependent escape from apoptosis of the t(4;11) leukemic cells should be further investigated considering potential clinical implications.

Materials and Methods

Methods including recombinant protein expression in K562 and KG1 cells, RNA interference, Western blot analysis, MTT assay, caspase-3 activity assay, and FACS analysis are available in the supporting information (SI) *Materials and Methods*.

Cell Lines. The human erythroleukemia cell line K562, the AML cell line KG1, and the pro-B acute lymphoblastic leukemia cell line RS4;11 with the t(4;11) abnormality were obtained from American Type Culture Collection (ATCC). The SEMK2 line was provided by Johann Greil. Other acute lymphoblastic leukemia cell lines selected for this study (380, 697) are maintained in our laboratory. All cell lines were grown in RPMI medium 1640 supplemented with 10% FCS.

Semiquantitative and Real-Time RT-PCR Analysis. Total RNA was isolated from transfectant 16 h after transfection by using RNeasy spin-column kits according to the manufacturer's instructions (Qiagen, Valencia, CA). Five micrograms of total RNA was subjected to cDNA synthesis by using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). At the end of the reaction, 40 μl of TE (10 mM Tris/1 mM EDTA, pH 7.6) was added, and 1 μl of the reaction (1/60 volume) was used as a template for PCR. The following number of cycles, which allowed semiquantitative comparison, were applied to PCR: 35 cycles for *EphA1*, *-2*, *-4*, *-7*, and *-8* and *ephrin-A1*, *-2*, *-3*, *-4*, and *-5*; 40 cycles for *EphA3*, *-5*, and *-6*; 25 cycles for *GAPDH*; and 32 cycles for *HoxA9*. Primer sequences are shown in SI Table 1. EphA7 transcript was quantified by using QuantiTect SYBR Green RT-PCR kit (Qiagen) for the amplification of cDNA (1 μl cDNA of a 60-μl reaction, which corresponds to 0.083 μg RNA) and iCycler real-time PCR detection system (Bio-Rad, Hercules, CA) for the detection of PCR product. Cycle threshold (Ct) values determined by using a known amount of EphA7 cDNA (10-fold serial dilutions starting at 100 pg) were used to construct the standard curve. Ct value of the tested cDNA was converted to weight according to the standard curve.

ChIP Assay. ChIP assay was performed by using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY). Sixteen hours after transfection with the ALL1 fusion constructs, 5×10^6 cells (K562 transfectants) were cross-linked, washed with PBS, resuspended in 1 ml of SDS lysis buffer, and sonicated to generate ≈200–1,000 bp DNA fragments. One-tenth of a volume of this preparation, i.e., 0.1 ml aliquot containing 25 μg DNA, was used per ChIP. Antibodies used were anti-HA mAb clone F-7 and normal mouse IgG purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitated chromatin was reverse cross-linked, deprotenized, and resuspended in 50 μl TE (pH 8.0), and 1-μl aliquot of this preparation was used as a template for PCR. Input indicates PCR with 0.05% of total input chromatin, which corresponds to 0.0125 μg DNA. *EphA7* primer sequences used in ChIP assay are as follows: region 1, 5′-ATGCAGCGAAATGGAAAACT-3′ (forward) and 5′-AAAAGGGAGTGGGAAAGGAA-3′ (reverse); region 2, 5′-TAGTACCTCAGGCGGGTCAC-3′ (forward) and 5′-TTCCGAGCTCATCGAAGTCT-3′ (reverse); region 3, 5′-TTGTCGTTGGACGTTCACAT-3′ (forward) and 5′-CAATAGCGCCTCATCTGACA-3′ (reverse).

ChIP-enriched DNA was also subjected to real-time PCR analysis essentially as described in *Semiquantitative and Real-Time RT-PCR Analysis*. Results were calculated as the percentage of input according to the following formula: $100/2^{\text{Ct of ChIP-enriched DNA} - \text{Ct of input DNA}}$ (%).

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