Activation of Integrin-Linked Kinase Is a Critical Prosurvival Pathway Induced in Leukemic Cells by Bone Marrow–Derived Stromal Cells

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Abstract

Integrin-linked kinase (ILK) directly interacts with β integrins and phosphorylates Akt in a phosphatidylinositol 3-kinase (PI3K)–dependent manner. In this study, we examined the functional role of ILK activation in leukemic and bone marrow stromal cells on their direct contact. Coculture of leukemic NB4 cells with bone marrow–derived stromal mesenchymal stem cells (MSC) resulted in robust activation of multiple signaling pathways, including ILK/Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducers and activators of transcription 3 (STAT3), and Notch1/Hes. Blockade of PI3K or ILK signaling with pharmacologic inhibitors LY294002 or QLT0267 specifically inhibited stroma-induced phosphorylation of Akt and glycogen synthase kinase 3β, suppressed STAT3 and ERK1/2 activation, and decreased Notch1 and Hes1 expression in leukemic cells. This resulted in induction of apoptosis in both leukemic cell lines and in primary acute myelogenous leukemia samples that was not abrogated by MSC coculture. In turn, leukemic cells growing in direct contact with bone marrow stromal elements induce activation of Akt, ERK1/2, and STAT3 signaling in MSC, accompanied by significant increase in Hes1 and Bcl-2 proteins, which were all suppressed by QLT0267 and LY294002. In summary, our results indicate reciprocal activation of ILK/Akt in both leukemic and bone marrow stromal cells. We propose that ILK/Akt is a proximal signaling pathway critical for survival of leukemic cells within the bone marrow microenvironment. Hence, disruption of these interactions by ILK inhibitors represents a potential novel therapeutic strategy to eradicate leukemia in the bone marrow microenvironment by simultaneous targeting of both leukemic cells and activated bone marrow stromal cells. [Cancer Res 2007;67(2):684–94]

Introduction

The bone marrow microenvironment plays a crucial role in the pathogenesis of acute myelogenous leukemia (AML) by promoting tumor cell growth and survival as well as drug resistance (1). Integrins represent an essential component of the bone marrow microenvironment that regulates cell survival by interacting with the extracellular matrix. AML cells bind to bone marrow stromal cells through combined β_1 and β_2 integrin mechanisms (2). Recent data suggest that the interaction between VLA-4 ($\alpha_4\beta_1$ integrin) on leukemic blasts and fibronectin on stromal cells activates phosphatidylinositol 3-kinase (PI3K)/Akt/Bcl-2 signaling, an important determinant of AML chemosensitivity and the level of minimal residual disease of AML patients (3). Thus, activation of the signaling cascades downstream of integrin engagement may play a critical role in the well-documented chemoresistance of bone marrow–resident AML cells.

Integrin-linked kinase (ILK) was proposed to play a central role in integrin activation and signaling (4). ILK is a highly conserved ankyrin repeat–containing serine-threonine protein kinase that links the cell adhesion receptors, integrins, and growth factors to the downstream signaling pathways. The activity of ILK that is modulated by integrin ligation in a PI3K-dependent manner (5) stimulates the phosphorylation of Akt^{Ser473} (6). Activated Akt phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β), resulting in the nuclear localization of β-catenin and activated transcription, cell cycle progression, and cell proliferation (5). Furthermore, ILK directly phosphorylates GSK3β and inhibits its activity in neuroblastoma cells (7). Recent studies indicate that ILK expression and activity are significantly up-regulated in several types of cancers (8–11), hence providing a potential target for cancer therapy.

The active cross-talk between signaling pathways responsible for cell survival and apoptosis has recently been elucidated. In particular, inhibition of GSK3β in the Wnt signaling system induces Hes1 (12), which encodes a basic helix-loop-helix transcription factor downstream of Notch and positively regulates the self-renewal of hematopoietic stem cells (13). Cross-talk between the Notch/Hes1 pathway and the Janus-activated kinase (JAK) 2/signal transducers and activators of transcription 3 (STAT3) pathway has also been shown (14). Further, extracellular signal-regulated kinase (ERK) 1/2 has recently been identified as a target of ILK signaling in a cell culture model of renal epithelial morphogenesis (15). Thus, it is possible that ILK functions upstream in the hierarchy of these pathways such that its inhibition in turn inhibits the activation of multiple pathways involved in tumor cell survival and proliferation.

In this study, we used pharmacologic inhibitors of PI3K and ILK to determine the role of the respective pathways in bone marrow stroma–supported AML cell survival and examined the mechanisms that coordinate PI3K/ILK/Akt signaling with stroma-induced activation of other pathways. Our results elucidated the prominent role of ILK in the interaction of bone marrow stromal cells and leukemic cells that leads to the survival of leukemic cells. In turn, pharmacologic blockade of PI3K/ILK axis inhibited activation not only of Akt and GSK3β signaling pathways but also

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of multiple prosurvival signaling pathways in both leukemic and stromal cells, indicating its proximal role in the leukemia/stroma interactions.

Materials and Methods

Cell cultures. Human promyelocytic leukemic NB4 cells were kindly provided by Dr. M. Lanotte (Hôpital St. Louis, Paris, France; ref. 16). Human monocyte U937 cells, human lymphoma Raji cells, human pre-B lymphocyte REH cells, and NB4 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and penicillin-streptomycin at 37°C in 5% CO_2 in a humidified incubator. Human megakaryocytic leukemia MO7e cells (provided by Dr. H. Broxmeyer, Indiana University, Indianapolis, IN) were supplemented with granulocyte macrophage colony-stimulating factor (37 units/mL; Schering-Plough, Kenilworth, NJ) and maintained under conditions similar to those described for the other cell lines. Mesenchymal stem cells (MSC) obtained from a normal bone marrow donor were cultured at a density of $0.2 \times 10^5/cm^2$ in MEMα with 20% FBS, 1% L-glutamine, and penicillin-streptomycin. Passage 3 or 4 MSCs were used for the coculture experiments.

Leukemic cells were cultured at a starting concentration of 5×10^5/mL with and without $0.2 \times 10^5/cm^2$ MSC stromal layer in serum-free (for NB4, U937, Raji, and REH cells) or cytokine-free conditions (for MO7e cells). To study the effects of the soluble factors produced by MSCs, conditioned serum-free medium from the culture of 1-day-old MSCs was added to the NB4 cell culture.

Samples of bone marrow or peripheral blood were obtained for *in vitro* studies from patients with newly diagnosed or recurrent AML with high (>70%) blast count after informed consent according to the institutional guidelines (Table 1). Mononuclear cells were purified by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation and cocultured with MSC for 24 h in MEMα with 10% FBS, 1% L-glutamine, and penicillin-streptomycin, after which they were exposed to LY294002 (20 μmol/L) or QLT0267 (10 μmol/L) for 48 h.

Cocultured leukemic cells were separated from MSC monolayer by careful pipetting with ice-cold PBS (repeated twice). After collecting the leukemic cells, MSC monolayer was observed under microscopy (×100) to confirm that MSC monolayer was not damaged and that <10 leukemic cells per vision field remained attached. MSC monolayer was then trypsinized, and cells were counted using the trypan blue dye in a hemocytometer.

To verify lack of significant contamination in collected leukemic cells and MSC fractions and to evaluate cell type–specific phosphorylation, the expression of phosphorylated (p)-STAT3 and p-ERK1/2 in cocultured leukemic cells and trypsinized MSCs was measured by two-color flow cytometry using CD45 as a discriminator between leukemic cells and MSCs. Briefly, cells were counterstained with allophycocyanin-conjugated anti-CD45 and isotype control IgG1 antibody to distinguish $CD45^+$ leukemic cells and $CD45^-$ MSCs. Cells were then fixed using 2% formaldehyde at 37°C for 10 min and then resuspended in 90% ice-cold methanol for 30 min. Next, cell suspensions were centrifuged and washed once with PBS containing 4% bovine serum albumin and labeled with the FITC-conjugated p-$STAT3^{Tyr705}$, p-$ERK1/2^{Thr202/Tyr204}$, or isotype control IgG1 antibody (BD Biosciences, San Jose, CA) for 30 min at 4°C. After one wash, samples were analyzed immediately by a FACSCalibur flow cytometer. Data acquisition and analysis were done using the CellQuest software.

Reagents. The ILK inhibitor QLT0267 was obtained from QLT, Inc. (Vancouver, British Columbia, Canada; refs. 10, 11). QLT0267 is a submicromolar inhibitor of the phosphotransferase activity of ILK toward a specific peptide substrate, which was identified in the high-throughput screening of a rationally designed small-molecule library against the target ILK. QLT0267 has been shown to inhibit the kinase activity at 26 nmol/L and possess 1,000-fold selectivity over other kinases, including CK2, CSK, DNA-PK, PIM1, protein kinase B or Akt kinase, and protein kinase C; and 100-fold selectivity over ERK1, GSK3β, LCK, protein kinase A, p70S6K, and RSK1 (QLT; refs. 8, 10, 17). Recently, the specificity of QLT0267 as the ILK inhibitor has been confirmed using the constitutively active mutant of ILK, which protected cells from the inhibitory effect of QLT0267 (9). The PI3K inhibitor LY294002, mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor PD98059, JAK2 inhibitor tyrphostin AG490, GSK3 inhibitor IX (2′*Z*,3′*E*)-6-bromoindirubin-3′-oxime (BIO), and γ-secretase inhibitor XII (Z-IL-CHO) were purchased from Calbiochem (Novabiochem Corp., La Jolla, CA). The STAT3 inhibitor WP1066 (18), QLT0267 (10 μmol/L), LY294002 (20 μmol/L), WP1066 (2 μmol/L), AG490 (20 μmol/L), PD98059 (20 μmol/L), BIO (100 nmol/L), and γ-secretase inhibitor XII (10 μmol/L) were prepared by diluting to the appropriate concentration in DMSO and stored at −20°C with the culture medium before the *in vitro* exposure of cells.

Cell viability assay. Viable cells were identified by using the trypan blue dye exclusion method and were counted in a hemocytometer.

Apoptosis and cell cycle analysis. For the propidium iodide/Annexin V binding studies done to analyze apoptosis, leukemic cells were washed twice with PBS and then counterstained with allophycocyanin-conjugated anti-CD45 (NB4 cells) or anti-CD90 (primary AML cells) and isotype control IgG1 antibody to exclude contamination of MSCs. Cells were then resuspended in binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$; all from Sigma-Aldrich, St. Louis, MO], and FITC-conjugated Annexin V (Roche Diagnostics Co., Indianapolis, IN) was added at a final concentration of 1 μg/mL. The mixture was incubated at room temperature for 15 min in the dark before flow cytometric analysis. Membrane integrity was simultaneously assessed by propidium iodide staining (0.25 μg/mL). Flow cytometry done using Annexin V fluorescence was determined using a FACSCalibur flow cytometer (BD Biosciences), and

Table 1. Clinical data for patients

Sample no.	Status at date of sampling	Source	% Blasts	Cytogenetics	FAB	FLT3-ITD/D835	Ras mutation
1	Primary refractory	PB	96	del(11),t(18;19)del(11),t(18;19)	UNK	No	A mutation (CAA to AAA) is observed in codon 61 of NRAS
2	Primary refractory	PB	61	t(11;19), del(7),t(11;19)	M1	No	No
3	Primary refractory	PB	87	del(X),-3,add(4),-5,del(7),add(12),-17	M1	No	No
4	Primary refractory	BM	71	del(2),del(6)	UNK	No	No
5	Newly diagnosed	PB	46	del(5), del(7)(q31),dup(8), −12, −14, −17,−21	M1	No	No
6	Relapsed refractory	BM	84	inv(5)	UNK	No	No
7	Primary refractory	BM	71	del(2),del(6)	UNK	No	No

Abbreviations: PB, peripheral blood; BM, bone marrow; UNK, unknown (outside diagnosis); FAB, French-American-British classification.

data acquisition and analysis were done with CellQuest software (BD Biosciences).

The cell cycle distribution was determined by flow cytometry analysis of propidium iodide–stained nuclei. Briefly, NB4 cells were washed twice with PBS, fixed in ice-cold ethanol [70% (v/v) in water], and stained with the propidium iodide solution [25 μg/mL propidium iodide, 180 units/mL RNase, 0.15% Triton X-100, and 30 mg/mL polyethylene glycol in 4 mmol/L citrate buffer (pH 7.8); all from Sigma-Aldrich]. The DNA content was determined using a FACSCalibur flow cytometer, and the ModFit computer program (Verity Software House, Topsham, ME) was used for cell cycle analysis. Cells with a sub-G_1 DNA content were considered apoptotic.

Western blot analysis. NB4 cells and trypsinized MSCs were washed twice with PBS and lysed in cell lysis buffer (10 mmol/L NaF, 1 mmol/L Na_3VO_4, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.1% NaN_3, 10 mmol/L iodoacetamide, 3 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche Diagnostics). Equal amounts of lysate (equivalent to 30 μg protein) were separated on 10% polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) and immunoblotted with primary antibodies to the following: ILK (Upstate, Lake Placid, NY); Akt, p-Akt^{Ser473}, p-GSK3α/β$^{Ser9/12}$, STAT3, p-STAT3^{Tyr705}, p-STAT3^{Ser727}, and p-MAPK$^{Thr202/Tyr204}$ (p-ERK1/2; Cell Signaling Technology, Danvers, MA); p42^{MAPK} (ERK1/2) and Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA); Hes1 (provided by Dr. Tetsuo Sudo, Toray Research Center, Tsukuba, Japan; ref. 19); and β-actin (Abcam, Cambridge, MA). Membranes were then probed with a horseradish peroxidase–conjugated secondary antibody and reacted with electrochemiluminescence reagent (Amersham Biosciences, Baie d'Urfe, Quebec, Canada). Signals were detected by a luminescent image analyzer (LAS-100 Plus, Fujifilm, Tokyo, Japan) and quantified by Image Gauge (Fujifilm).

ILK kinase assay. ILK kinase assay was done using nonradioactive Akt kinase kit (Cell Signaling Technology) as described previously (10). Briefly, ILK was immunoprecipitated from 200 μg of cell lysate using 1 μg of a specific anti-ILK antibody and protein A agarose (Life Technologies, Rockville, MD). The ILK-containing agarose pellet was washed twice in assay buffer and twice in kinase buffer (both from Cell Signaling Technology) followed by incubation with 200 μmol/L ATP and 1 μg of

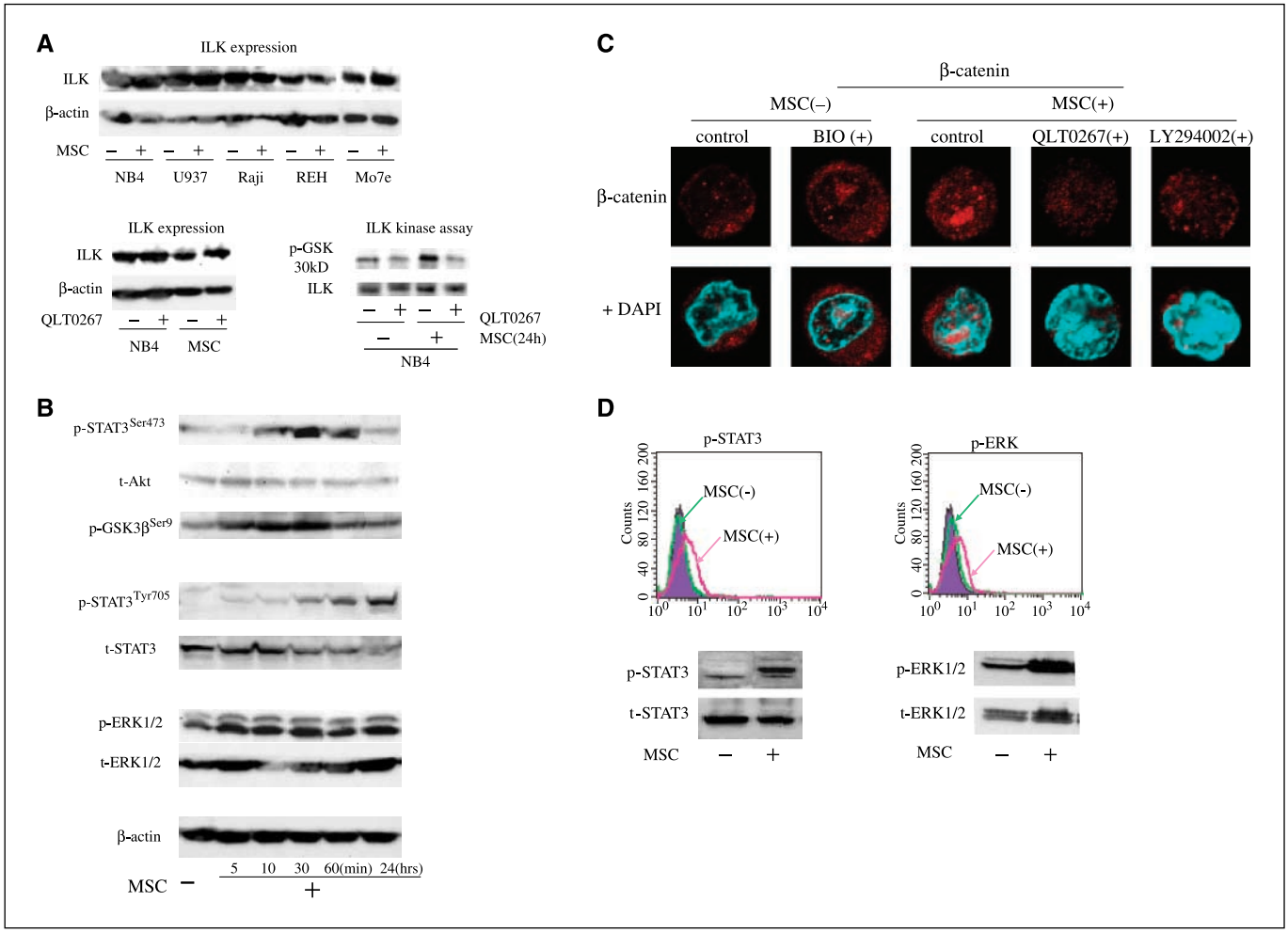


Figure 1. *A,* expression and kinase activity of ILK in leukemic and bone marrow stromal cells. Analysis of ILK expression by immunoblotting in NB4, U937, Raji, REH, and MO7e leukemic cells cultured with or without MSCs (24 h) and in NB4 and MSC cells treated with QLT0267 (10 μmol/L, 24 h). QLT0267 (10 μmol/L) inhibits basal and MSC-induced ILK kinase activity: NB4 cells were cultured with and without MSCs for 24 h, and total cell lysates were immunoprecipitated with a polyclonal ILK antibody. Kinase assay was done using the synthetic GSK3 protein as a substrate. *B,* induction of Akt, GSK3, STAT3, and MAPK phosphorylation by MSCs. Western blot analysis of the protein levels of p-Akt^{Ser473}, p-GSK3α/β$^{Ser9/21}$, p-STAT3^{Tyr705}, and p-ERK1/2$^{Thr202/Tyr204}$ in NB4 cells after coculture with MSCs for increasing durations. *t-*, total. β-Actin showed equal loading of samples. Results are representative of three independent experiments. *C,* translocation of β-catenin into the nucleus of NB4 cells cocultured with MSCs. Immunofluorescence confocal microscopic analysis of β-catenin expression in NB4 cells cultured alone [*MSC*(−)] or after coculture with MSCs for 60 min [*MSC*(+)], alone or exposed to the indicated inhibitors. Cells were stained with antibody against human β-catenin (*red*), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). *D,* examples of flow cytometric and Western blot analyses of p-STAT3^{Tyr705} and p-ERK1/2$^{Thr202/Tyr204}$ in NB4 cells with or without MSCs coculture for 24 h after electronic gating on CD45$^+$ leukemic cells in two-color flow cytometric analysis.

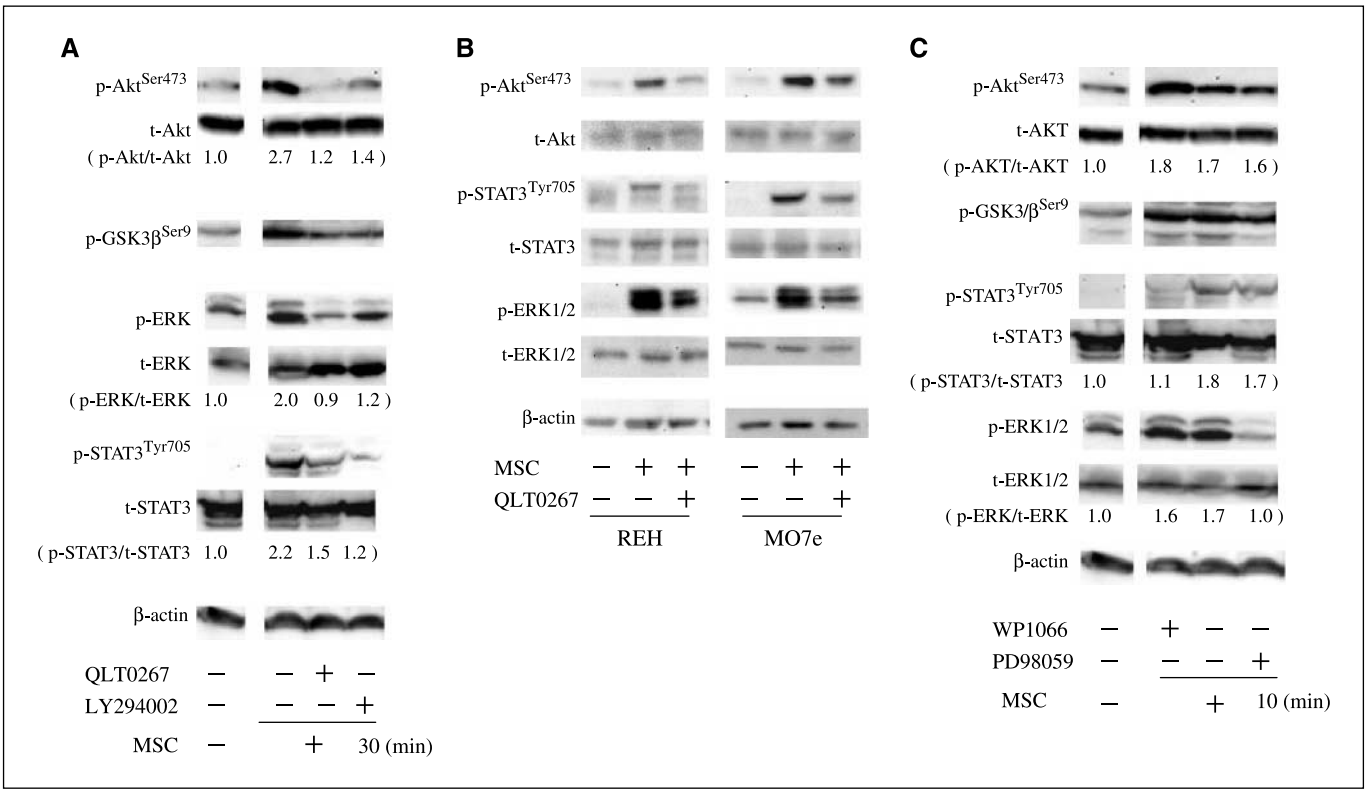


Figure 2. Molecular effects of QLT0267, LY294002, WP1066, and PD98059 on leukemic cells. Results are representative of three independent experiments. *A,* Western blot analysis of the protein levels of p-Akt^{Ser473}, p-GSK3α/β$^{Ser9/21}$, and p-ERK1/2$^{Thr202/Tyr204}$ (after 30 min) and p-STAT3^{Tyr705} (after 60 min) after treatment with QLT0267 and LY294002 in NB4 cells cocultured with MSCs. *B,* Western blot analysis of p-Akt^{Ser473}, p-STAT3^{Tyr705}, and p-MAPK$^{Thr202/Tyr204}$ in REH and MO7e cells after 60 min of QLT0267 treatment in MSC coculture. *C,* WP1066 and PD98059 do not affect the phosphorylation of Akt and GSK3. Western blot analysis of the protein levels of p-Akt^{Ser473}, p-GSK3α/β$^{Ser9/21}$, p-STAT3^{Tyr705}, and MAPK$^{Thr202/Tyr204}$ in NB4 cells after 10 min of treatment with WP1066 and PD98059 in MSC coculture.

GSK3 fusion protein for 30 min at 30°C. The reaction was terminated with 20 μL of 3× SDS sample buffer, and the supernatants were boiled for 5 min and loaded onto 10% SDS-polyacrylamide gel. Phosphorylation of the substrate was detected by Western blot analysis with the anti–p-GSK3α/β$^{Ser9/12}$ antibody. The amount of ILK immunoprecipitated from 30 μg of the cell lysate was determined by immunoblot with anti-ILK antibody.

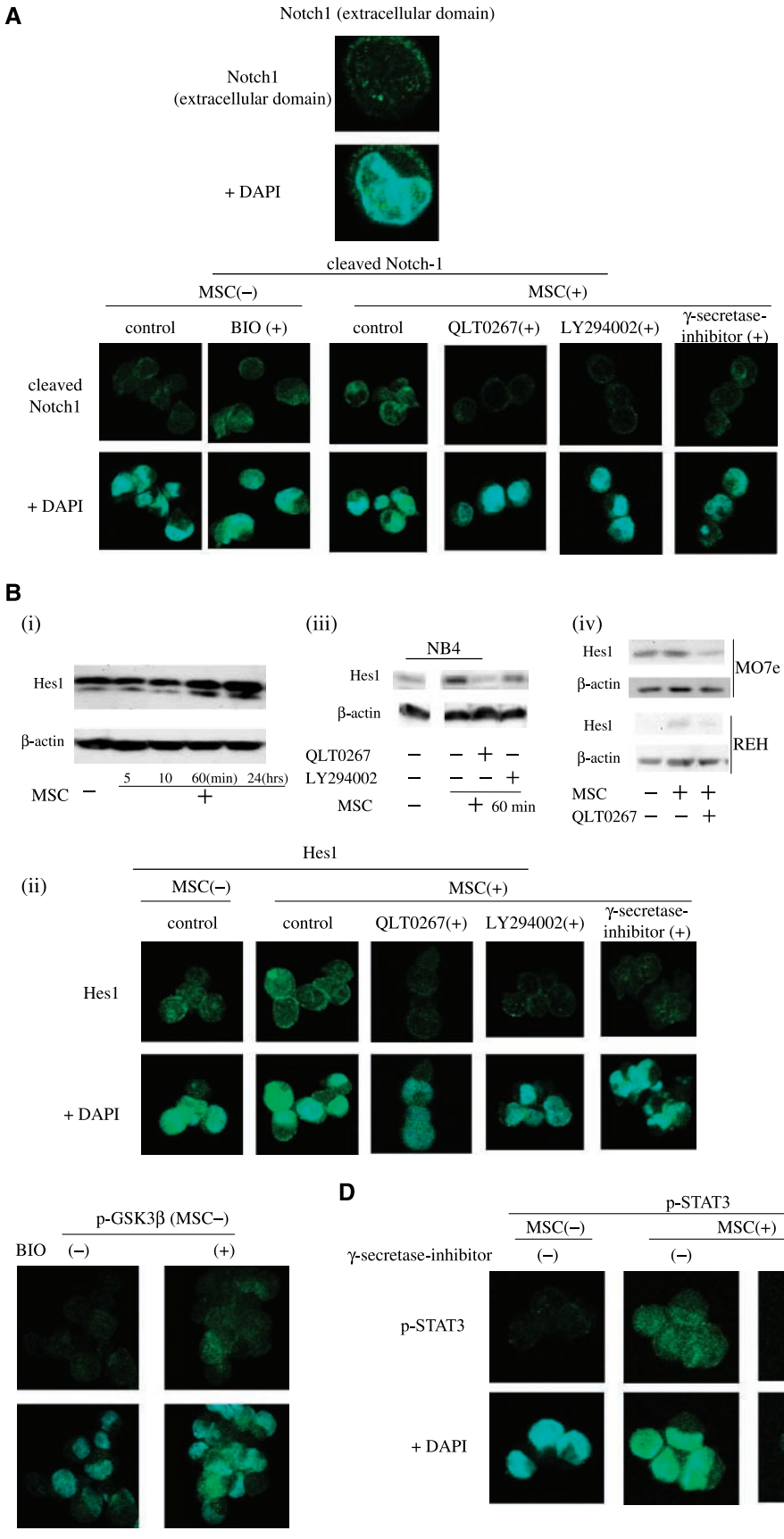
Immunofluorescence staining. Cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 90% methanol in TBST buffer (10 mmol/L Tris, 15 mmol/L NaCl, 0.1% Triton X-100) for 15 min, and rinsed with PBS for 5 min. Next, cells were blocked with 5% goat serum in PBS for 30 min and incubated with anti–β-catenin (Cell Signaling Technology), Notch1 (extracellular domain), cleaved Notch1 (intracellular domain), Hes1, p-Akt^{Ser473}, p-GSK3β^{Ser9} (Cell Signaling Technology), p-STAT3^{Tyr705}, and p-ERK1/2$^{Thr202/Tyr204}$ antibodies (1:100) overnight at 4°C. Excess antibody was removed by washing with PBS. Cells were then incubated with secondary antibody, either FITC-labeled anti-rabbit or anti-mouse IgG (H+L; 1:250; Caltag, Burlingame, CA), for 30 min at 37°C. Cells were washed with PBS, mounted on slides, and analyzed under a Zeiss LSM 510 laser confocal microscope (Carl Zeiss, Thornwood, NY).

Statistical analysis. Statistical analysis was done using ANOVA and Fisher's post hoc tests, and $P < 0.05$ was considered statistically significant. The results are expressed as the mean ± SD or mean ± SE.

Results

PI3K/ILK/Akt, JAK/STAT3, MEK/ERK, and Notch/Hes pathways are activated in NB4 cells cocultured with MSCs. First, we did Western blot analysis to assess ILK protein expression in five leukemic cell lines, NB4, U937, Raji, REH, and MO7e. As shown in Fig. 1*A*, ILK protein was highly expressed in all cell lines, and the expression was not affected by 24-h coculture of the leukemic cells with MSCs. ILK was also expressed in MSCs. In addition, ILK protein levels were unchanged following treatment with ILK inhibitor QLT0267 (9) in both NB4 cells and MSCs (Fig. 1*A*). In contrast, coculture with MSC resulted in increased ILK kinase activity in leukemic cells as determined in an *in vitro* kinase activity assay (ILK kinase activity; Fig. 1*A, lanes 1* and *3*). QLT0267 moderately reduced basal ILK kinase activity in NB4 cells but potently abrogated stroma-induced ILK activation (Fig. 1*A, lanes 2* and *4*).

Because the regulation of intracellular signaling molecules through ILK involves the phosphorylation of Akt and GSK3β (6, 8, 9) and the direct phosphorylation of Akt is cell type dependent (20), we next examined the phosphorylation levels of Akt and GSK3β in NB4 leukemic cells cultured with and without MSCs. Nonadherent NB4 cells were separated from MSC as described in Materials and Methods, and the purity of leukemic cells was assured by flow cytometry using anti-CD45 antibody (>95% CD45$^+$ cells; data not shown). NB4 cells incubated in serum-free medium without MSCs expressed low levels of p-Akt^{Ser473} and p-GSK3β^{Ser9}, but phosphorylation was significantly increased by the 5- to 10-min coculture with MSCs (Fig. 1*B*). Further, the expression of β-catenin, which interacts with GSK3β (21), was induced with concomitant translocation into the nucleus where it normally activates gene expression (MSC$^-$ control versus MSC$^+$ control; Fig. 1*C*; ref. 22).



Because the β_1 integrin cytoplasmic domain was reported to activate ERK1/2 (23) and the mammalian target of rapamycin (mTOR) pathway downstream of Akt can phosphorylate STAT3 on Ser^{727} (24), we next examined changes in the activation of STAT3 and MAPK in NB4 cells cultured with and without MSCs. The NB4 cells cultured with MSCs showed a considerable increase in p-ERK1/2 and p-$STAT3^{Tyr705}$ (Fig. 1*B*) but not p-$STAT3^{Ser727}$ (data not shown). Phosphorylation of ERK1/2 was induced within the first 5 min, whereas $STAT3^{Tyr705}$ phosphorylation was delayed and remained at markedly high levels at 24 h of cocultivation with MSCs (Fig. 1*B*). We confirmed specific induction of p-ERK and p-STAT3 in leukemic cells identified by CD45 immunofluorescence in two-color flow cytometry analysis (Fig. 1*D*). Thus, from this, it seems that multiple prosurvival signaling cascades, including the PI3K/ILK/Akt/GSK3β, with the nuclear localization of β-catenin, JAK2/STAT3, and MEK/ERK1/2 pathways are activated in leukemic cells cultured in direct contact with bone marrow stromal cells.

Pharmacologic PI3K or ILK inhibitors block activation of PI3K/ILK/Akt, JAK/STAT3, and MAPK/ERK pathways in NB4 cells cultured with MSCs. Because multiple prosurvival pathways are activated in leukemic cells by virtue of their interactions with MSC, we next attempted to dissect a functional role of each of these pathways by use of the respective pharmacologic inhibitors. To determine whether the stroma-induced phosphorylation of Akt and GSK3β is ILK dependent, we first assessed the effects of the specific ILK inhibitor QLT0267 on p-Akt and p-GSK3β levels in NB4 cells cocultured with MSCs. QLT0267 suppressed MSC-induced p-Akt^{Ser473} and p-$GSK3\beta^{Ser9}$ (Fig. 2*A*), which inhibited the translocation of β-catenin into the nucleus (MSC^+, compare control and QLT0267; Fig. 1*C*). No effect on phosphorylation of Thr^{308} of Akt (PDK-1 site) was noted (data not shown). Unexpectedly, QLT0267 also decreased MSC-triggered p-ERK1/2 and p-$STAT3^{Tyr705}$ (Fig. 2*A*). Similar changes were induced by the PI3K inhibitor LY294002, suggesting that ILK induces Akt and GSK3β phosphorylation and β-catenin translocation into cell nuclei in a PI3K-dependent manner. Likewise, QLT0267 abrogated MSC-induced p-Akt^{Ser473}, p-ERK1/2, and p-$STAT3^{Tyr705}$ expression in two other leukemic cell lines, REH and MO7e cells (Fig. 2*B*).

We next examined the putative feedback cross-talk among STAT3, MAPK, and ILK/Akt pathways by use of STAT3 inhibitor WP1066 and MEK inhibitor PD98059. These inhibitors effectively blocked MSC-induced activation of their respective targets, STAT3 and ERK1/2, but failed to suppress the MSC-induced phosphorylation of the ILK downstream targets, Akt and GSK3β (Fig. 2*C*). Tyrphostin AG490, an established inhibitor of the JAK2/STAT3 pathway, likewise had no effect on the phosphorylation of Akt and GSK3β (data not shown). Collectively, these results indicate that ILK is a proximal kinase activated by MSC in leukemic cells that in turn regulates activation of MAPK and STAT3 signaling.

Cross-talk among ILK/Akt/GSK3β, Notch/Hes, and JAK/STAT3 signaling. Wnt and Notch signaling pathways are critical regulators of normal and leukemia stem cell survival (12, 25). To determine if coculture with MSCs activates Notch signaling, we examined the expression of the intracellular form of active (cleaved) Notch1 and of Hes1, a transcriptional target of Notch1 (26), in NB4 cells by Western blot and confocal microscopic analyses. The endogenous Notch1 was expressed in NB4 cells as determined by immunofluorescence staining with antibody that recognizes extracellular domain of Notch1 (Fig. 3*A*). MSC coculture activated Notch signaling as shown by increased expression of cleaved intracellular Notch1 (MSC^- control versus MSC^+ control; Fig. 3*A*) and Hes1 (MSC^- control versus MSC^+ control; Fig. 3*B, i* and *ii*), which was evident after 60 min of MSC coculture (Fig. 3*B, i*).

Hes1, a Notch target gene, can be induced by GSK3β inhibition (phosphorylation of GSK3β) through up-regulation of Notch-dependent transcription of the Hes1 promoter (12, 26). A recent report documented the cross-talk between Notch and Wnt signaling pathways (27, 28). Because ILK is activated upstream of GSK3β in our system, we examined the effects of ILK inhibition on Notch signaling. Our results showed that QLT0267 diminished MSC-induced induction of Hes1 (Fig. 3*B, ii* and *iii*) possibly via the inhibitory phosphorylation of ILK direct target GSK3β. Similar results were obtained in REH and MO7e cells (Fig. 3*B, iv*). To confirm the role of GSK3β as a critical intermediate between ILK and Hes1/Notch induction, we investigated the molecular effects of the direct GSK3 inhibitor BIO. Treatment with 100 nmol/L BIO increased p-GSK3β in NB4 cells growing without stromal support (Fig. 3*C*), which was accompanied by the nuclear translocation of β-catenin (Fig. 1*C*) and increased expression of cleaved Notch1 (Fig. 3*A*) as shown by confocal microscopy. The GSK3 inhibition might therefore contribute to NB4 cell survival in part through activation of the Notch signaling. Recently, it has been reported that Hes1 protein binds directly to STAT3 and activates JAK2/STAT3 signaling (14). We therefore investigated the effects of Notch blockade on STAT3 phosphorylation. Treatment with the Notch inhibitor, γ-secretase inhibitor, prevented MSC-induced cleaved Notch1 (Fig. 3*A*) and Hes1 (Fig. 3*B, ii*) and significantly decreased p-$STAT3^{Tyr705}$ expression (Fig. 3*D*), indicating contribution of Notch in the activation of JAK2/STAT3 signaling. In contrast, blockade of STAT3 or MAPK activation by WP1066 or PD98059, respectively, did not affect Hes1 expression (data not shown).

MSCs prevent serum deprivation–induced apoptosis but not QLT0267-induced apoptosis in NB4 cells. We next examined the ability of MSCs to support survival of leukemic cells under conditions of serum deprivation. NB4 cells cultured without serum rapidly undergo apoptosis at 48 h as determined by Annexin V positivity. In contrast, coculture with MSC significantly protected NB4 cells from apoptosis (Fig. 4*A*). This finding was confirmed by

Figure 3. Expression of Notch1 and Hes1 proteins and GSK3β phosphorylation in leukemic cells. *A,* immunofluorescence confocal microscopic analysis of Notch1 (extracellular domain) and of cleaved intracellular Notch1 in NB4 cells cultured alone [*MSC*(−)] or in coculture with MSCs for 24 h [*MSC*(+)] with indicated pharmacologic inhibitors. Cells were stained with the antibodies recognizing the extracellular or intracellular (cleaved) Notch1 domains (*green*), and nuclei were counterstained with DAPI (*blue*). *B, i,* Western blot analysis of the Hes1 protein expression and β-actin in NB4 cells cocultured with MSCs for the indicated times. *ii,* immunofluorescence confocal microscopic analysis of Hes1 expression in NB4 cells cultured alone [*MSC*(−)] or in coculture with MSCs for 24 h [*MSC*(+)] exposed to the indicated pharmacologic inhibitors. Cells were stained with the antibody recognizing the cleaved Notch1 (*green*), and nuclei were counterstained with DAPI (*blue*). *iii,* Western blot analysis of the Hes1 protein expression and β-actin in NB4 cells after 60 min of treatment with QLT0267 and LY294002 in coculture with MSCs. *iv,* Western blot analysis of the Hes1 protein expression and β-actin in REH and MO7e cells after 60 min of treatment with QLT0267 in coculture with MSCs. *C,* induction of GSK3β phosphorylation by BIO (60 min) detected by immunofluorescence confocal microscopic analysis in NB4 cells cultured without MSC support. Results are representative of three independent experiments. *Green,* cells were stained for p-$GSK3\beta^{Ser9}$; *blue,* nuclei were counterstained with DAPI. *D,* molecular effects of γ-secretase inhibitor on p-$STAT3^{Tyr705}$ expression in NB4 cells detected by immunofluorescence confocal microscopic analysis after coculture with MSCs for 24 h. Results are representative of three independent experiments. Cells were stained with antibody that specifically recognized p-STAT3 (*green*), and nuclei were counterstained with DAPI (*blue*).

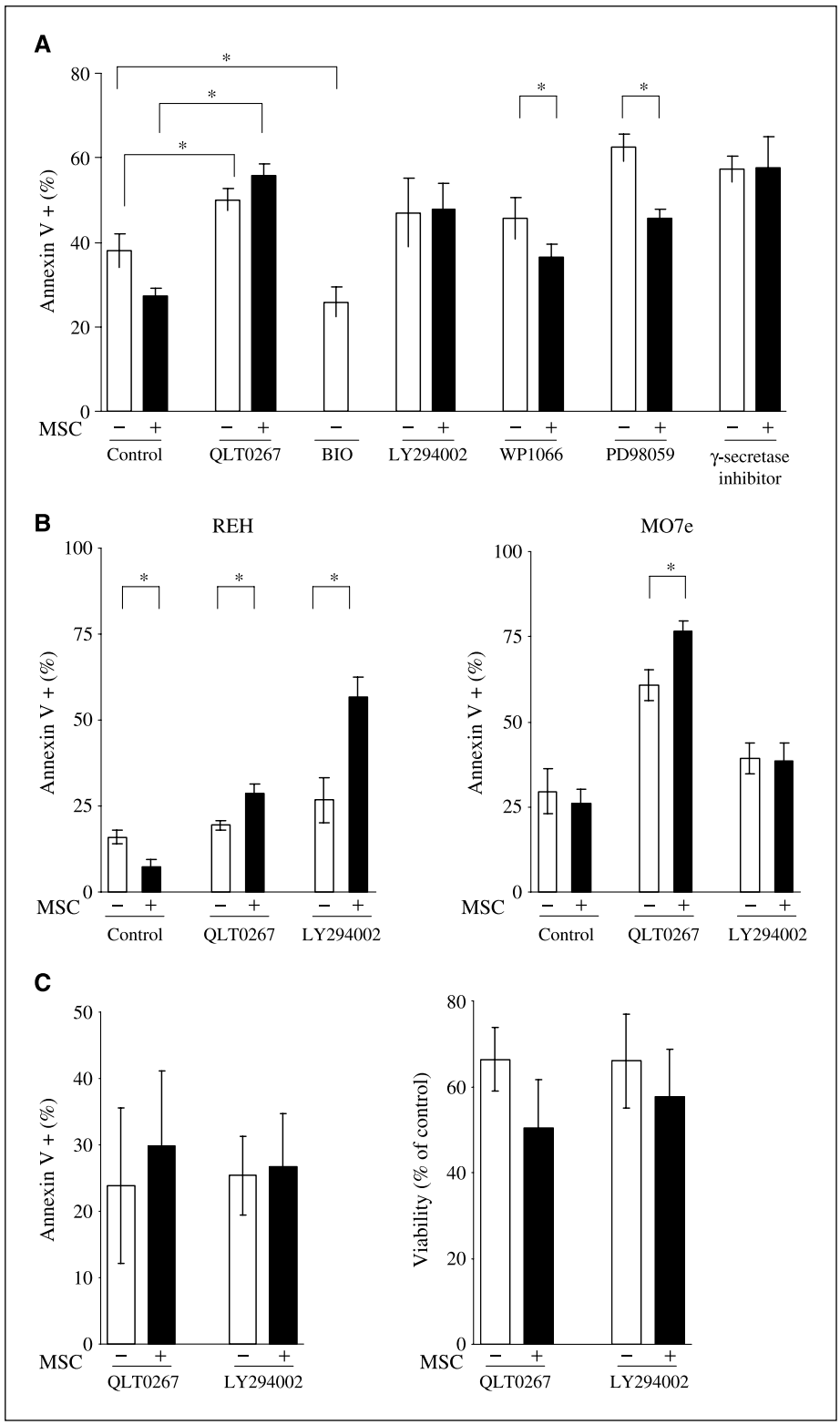


Figure 4. Apoptotic effects of QLT0267 and LY294002 are not prevented by MSCs. *A,* percentage of Annexin V$^+$ NB4 cells treated with the indicated agents for 48 h with and without coculture with MSCs. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$. *B,* percentage of Annexin V$^+$ REH and MO7e cells treated with QLT0267 or LY294002 for 48 h with and without coculture with MSCs. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$. *C,* AML primary samples were treated with QLT0267 or LY294002 in the presence or absence of MSC coculture. Induction of apoptosis in primary AML cells was measured by Annexin V$^+$ flow cytometry after electronic gating on CD90$^-$ cells. Data are averaged percentage specific apoptosis calculated as (test − control) × 100 / (100 − control). Loss of viability was detected by cell count using the trypan blue dye exclusion method, and the results are expressed as the percentage of the viable cell number in an untreated group. *Columns,* mean ($n = 7$); *bars,* SE.

DNA flow cytometry, in which the number of sub-G_1 cells among the NB4 cells cultured with MSCs was diminished compared with cells cultured alone (MSC$^-$ versus MSC$^+$: 39.5 ± 3.4% versus 28.6 ± 5.9%; $P < 0.05$; data not shown). These data suggest that the MSCs inhibit serum deprivation–induced apoptosis in the NB4 cells in confirmation of our reported findings (29).

To determine whether ILK inhibition affects leukemic cell survival, we next studied the extent of apoptosis induced by QLT0267 in NB4 cells cultured with and without MSCs. QLT0267 only moderately affected apoptosis of serum-deprived NB4 cells (12 ± 3.5%). Remarkably, QLT0267-induced apoptosis was enhanced by coculture with MSC, with an average of 29% more

cells undergoing apoptosis compared with control NB4 cells growing in MSC coculture (Fig. 4*A*). On the contrary, mimicking ILK-induced inactivation of GSK3 with direct GSK3 inhibitor BIO prevented serum withdrawal–induced apoptosis in stroma-free conditions (Fig. 4*A*), in support of the antiapoptotic role of ILK-GSK3β pathway in the context of bone marrow microenvironment.

Next, we examined the effects of LY294002, PD98059, and WP1066 in NB4/MSC cocultures. Whereas MSC cocultivation did not protect NB4 cells from LY294002-induced apoptosis, WP1066- and PD98059-induced apoptosis was partially but significantly inhibited by MSCs (Fig. 4*A*). The effect of the JAK2 inhibitor AG490 was similar to that of WP1066 (AG490-induced Annexin V positivity in NB4 cells: MSC^- 49.3 ± 5.4% versus MSC^+ 40.9 ± 3.2%; $P = 0.04$; data not shown). These data indicate that inhibition of stroma-induced PI3K/ILK, but not of ERK1/2 or STAT3 signaling, is able to overcome the protective effects of MSCs on survival of leukemic cells. On the contrary, blockade of Notch signaling by γ-secretase inhibitor promoted apoptosis of NB4 cells both in MSC^- and MSC^+ conditions (Fig. 4*A*) similar to the effects of PI3K/ILK inhibition.

The effects of MSCs and QLT0267 on serum deprivation– or cytokine deprivation–induced apoptosis in REH and MO7e cells were further examined. As shown in Fig. 4*B*, MSC coculture protected viability in REH cells; however, MSC enhanced QLT0267- and LY294002-induced apoptosis. Similarly, QLT0267-induced apoptosis was enhanced when MSCs were cocultured with cytokine-deprived MO7e cells, and modest apoptosis induced by LY294002 was not inhibited by MSC.

We next investigated the effects of QLT0267 and LY294002 in seven primary AML samples cocultured with MSC (Fig. 4*C*; Table 1). In five of seven primary AML samples, MSC coculture protected AML cells from spontaneous apoptosis (% $CD90^-$ Annexin V^+ cells, AML control in medium only, 35.8 ± 5.9%; AML + MSC, 21.0 ± 3.5%; $P = 0.03$), whereas in two additional samples no spontaneous apoptosis was observed. To correct for differences in spontaneous apoptosis, we calculated percentage specific apoptosis as [test (inhibitor-induced apoptosis) − control (spontaneous apoptosis)] × 100 / (100 − control). As shown in Fig. 4*C*, inhibition of PI3K/ILK signaling induced apoptosis in both suspension and MSC cocultured AML cells. Importantly, QLT0267 induced a higher degree of specific apoptosis in five of seven and LY294002 in four of seven AML samples cocultured with MSC compared with AML cells cultured in medium. This resulted in corresponding loss of viability as determined by cell counts with trypan blue exclusion (Fig. 4*C*).

NB4 cells prevent serum deprivation–induced apoptosis in MSCs and up-regulate p-Akt, p-STAT3, Hes1, and Bcl-2 protein in MSCs in part through activation of PI3K/ILK/Akt signaling. The phenomenon of "tumor-associated fibroblasts" that are activated by tumor cells and acquire the ability to further promote tumor growth has been recently characterized (30); however, no data are available on the activation of bone marrow stroma in leukemias. Notably, ILK activation was reported in tumor-associated fibroblasts (11) and endothelial cells (31). We therefore hypothesized that cell-to-cell contact with NB4 cells activates the β_1 integrin-ILK/PI3K/Akt signaling pathway in MSCs, which may contribute to their ability to support growth of leukemic cells. The phosphorylation levels of Akt, GSK3β, STAT3, and ERK1/2 and the expression of Hes1 and antiapoptotic protein Bcl-2 were examined in MSCs isolated after coculture with or without NB4 cells. The purity of isolated MSCs was assured by flow cytometry using anti-CD45 antibody (>94% $CD45^-$ cells; data not shown). We found a time-dependent increase in the phosphorylation of Akt, GSK3β, ERK1/2, and $STAT3^{Tyr705}$ that was induced within the first 10 min and remained at markedly high levels at 24 h of cocultivation with NB4 (Fig. 5*A*). The p-Akt^{Ser473}, p-$STAT3^{Tyr705}$, and p-ERK1/2 up-regulation in MSCs cultured with NB4 cells was confirmed by flow cytometric analyses using cell size and CD45 staining as a discriminator of leukemic cells from MSC (data not shown).

We assessed next the effects of QLT0267 and LY294002 on p-Akt, p-ERK1/2, Hes1, and Bcl-2 levels in MSCs cultured with NB4 cells. QLT0267 significantly suppressed the NB4-induced p-Akt^{Ser473}, p-ERK1/2, and Hes1 levels but only slightly decreased the p-$STAT3^{Tyr705}$ and Bcl-2 levels in MSCs (Fig. 5*B*). On the other hand, LY294002 specifically inhibited Bcl-2 expression and abrogated p-Akt^{Ser473}, p-ERK1/2, and Hes1 along with only moderately suppressed p-$STAT3^{Tyr705}$ (Fig. 5*B*). These data suggest that leukemic cells activate the PI3K/ILK/Akt pathway in MSCs, which induces Notch/Hes1 signaling, and phosphorylate ERK1/2 along with increase in Bcl-2 expression.

Discussion

In this study, we characterized signaling cascades activated on direct contact of leukemic cells with bone marrow–derived stromal cells. Our findings indicate stroma-induced activation of ILK with phosphorylation of Akt and GSK3β. Because p-$GSK3\beta^{Ser9}$ is a known cellular target of ILK (7), these results are consistent with evidence of ILK activation induced by growth factors or engagement of the integrins by stroma. We further showed that the nuclear accumulation of β-catenin, a downstream GSK3β target, was induced in NB4 cells by coculture with MSCs and that this accumulation was abrogated when the PI3K/ILK pathway was inhibited. In contrast, the GSK3 inhibitor BIO, which prevented the serum withdrawal-induced apoptosis of NB4 cells, stimulated the translocation of β-catenin to the nucleus in cells growing without stromal support. Altogether, these findings provide evidence that stroma-induced ILK activation results in GSK3 inhibition and up-regulation of nuclear β-catenin consistent with similar observations in transformed mammary epithelial cells (32).

Although the constitutive activation of ILK is known to promote cell survival by stimulating Akt^{Ser473} phosphorylation (6, 8, 9), the mechanisms that coordinate PI3K/ILK/Akt signaling with other signaling pathways are poorly understood. Our data provide evidence of cross-talk between the PI3K/ILK/Akt pathway and other signaling pathways that have overlapping roles in cell survival (i.e., the Notch/Hes1, JAK2/STAT3, and MEK/ERK pathways; Fig. 5*C*). In particular, the Notch/Hes1 signaling pathway plays a critical role in cell proliferation, differentiation, and apoptosis (33). Wnt signaling, by repressing GSK3, was shown to cause accumulation of β-catenin and intracellular fragments of Notch in the nucleus, which results in the activation of Notch targets, such as Hes1 (26). Notably, ILK activity has been shown to modulate Wnt-mediated β-catenin stabilization and nuclear translocation via inhibitory GSK3β phosphorylation (17). Moreover, GSK3 inhibition can modulate gene targets of Wnt, Hedgehog, and Notch pathways in primitive hematopoietic stem cells (28). Other studies using the genetic and pharmacologic inhibitors of PI3K and/or Akt have identified Akt as an intermediate critical to Notch1 signaling (34). In our coculture system, the marked increase in cleaved Notch1 and Hes1 expression in NB4 cells induced by MSCs was blocked by QLT0267 and LY294002. Conversely, the GSK3 inhibitor BIO induced reciprocal increase in cleaved Notch1

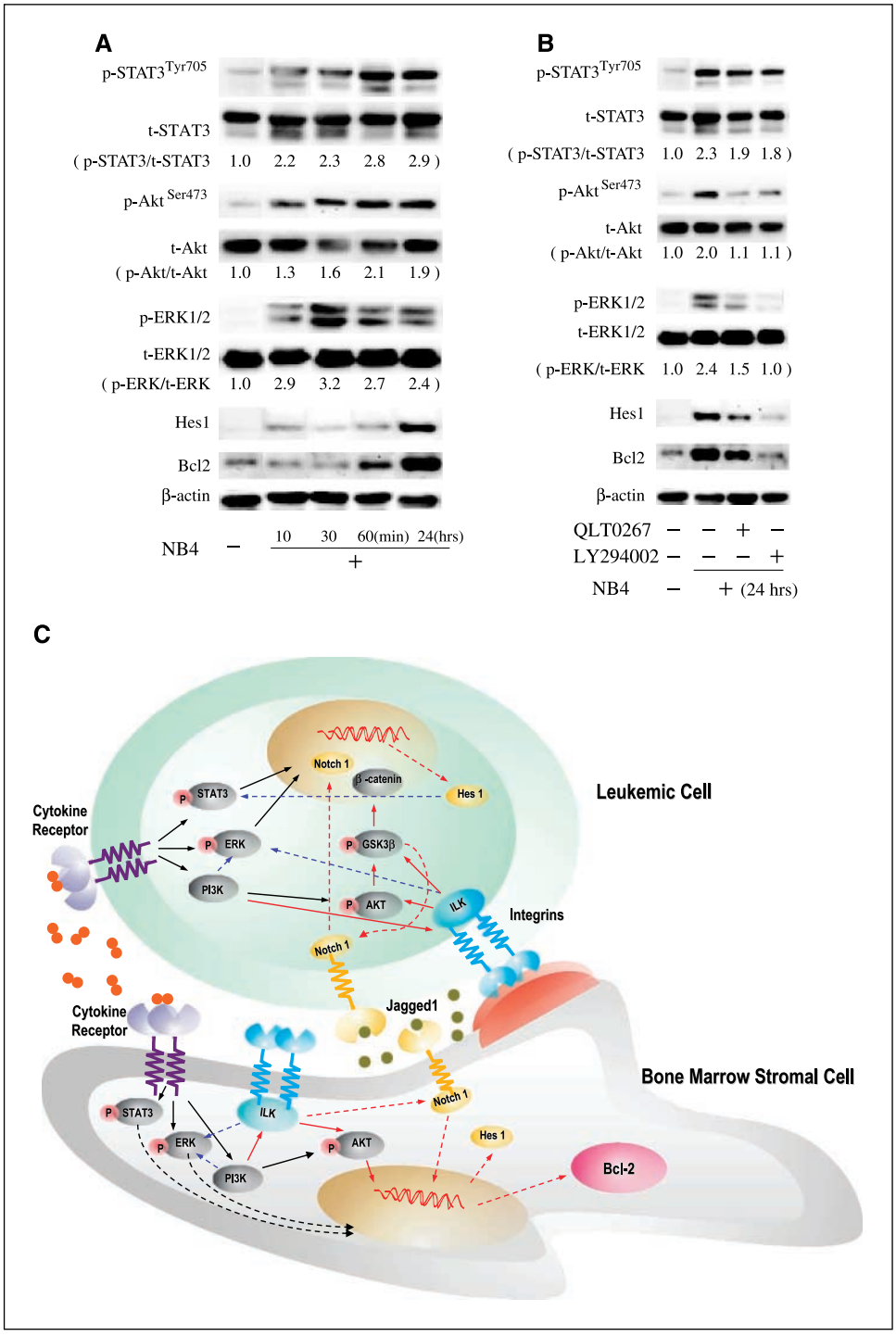


Figure 5. NB4 cells protect MSCs from spontaneous and QLT0267- and LY294002-induced cell death. *A,* coculture of NB4 cells with MSC results in induction of Akt and STAT3 phosphorylation and increase in Hes1 and Bcl-2 expression in MSCs. Western blot analysis of the protein levels of p-Akt^{Ser473}, p-STAT3^{Tyr705}, Hes1, and Bcl-2 in MSCs after coculture with NB4 cells for increasing durations. β-Actin showed equal loading of samples. *B,* QLT0267 and LY294002 inhibit p-Akt, p-STAT3, Hes1, and Bcl-2 expression in MSCs. Western blot analysis of the protein levels of p-Akt^{Ser473}, p-STAT3^{Tyr705}, Hes1, and Bcl-2 in MSCs after 24 h of treatment with QLT0267 and LY294002 in coculture with NB4 cells. *C,* activation of multiple prosurvival pathways by bone marrow stromal cells in leukemic cells: Cross-talk between ILK/Akt/GSK3β, Notch/Hes, and JAK/STAT3 signaling. In leukemic cells, ILK is activated through integrin-mediated cell adhesion to the bone marrow stromal cells. *Red arrow,* ILK induces its direct targets p-Akt and p-GSK3α/β$^{Ser9/21}$ in a PI3K-regulatable fashion. Inhibitory phosphorylation of GSK3 (induction of p-GSK3α/β$^{Ser9/21}$) results in up-regulation of the nuclear β-catenin signaling (*red arrow*) and promotes the accumulation of the intracellular fragment of Notch1 in the nucleus and induction of the Notch1 target Hes1 (*dashed red arrow*). *Dashed blue arrow,* activated Notch/Hes1 signaling induces phosphorylation of STAT3^{Tyr705}. PI3K/ILK/Akt pathway also regulates ERK activation. In MSC, the direct contact with leukemic cells activates ILK and PI3K signaling, which induces p-Akt, p-ERK, and p-STAT3^{Tyr705}, accompanied by increased Hes1 and Bcl-2 proteins (*dashed red arrow*).

expression. These data indicate that stroma-activated PI3K and ILK, the upstream kinases of GSK3, trigger activation of Notch signaling, which may be one of the critical mediators of leukemia cell survival in the bone marrow microenvironment. Notch receptors (Notch1, Notch2, Notch3, and Notch4) and ligands (Delta-like 1, Delta-like 4, and Jagged1) are expressed on both hematopoietic progenitors and stromal cells (35, 36), and the overexpression of the intracellular domain of Notch promotes their self-renewal (37) through up-regulation of the expression of Hes1, a direct target of Notch (12, 13, 26, 27). On the other hand, Notch signaling was reported to induce cell cycle arrest (38, 39) and bone marrow stroma–mediated activation of Notch1 signaling was reported to up-regulate p21, resulting in growth inhibition and protection from chemotherapy-induced apoptosis of myeloma cells (40). Curiously, both the oncogenic and tumor-suppressive properties of Notch1 are known to take place at the same time, and the final outcome is dependent on the cellular context (41). Notably, Notch signaling blockade by γ-secretase inhibitor promoted apoptosis of NB4 cells both in MSC$^-$ and MSC$^+$ conditions, perhaps recapitulating the effects of PI3K/ILK inhibition.

The studies elucidating detailed molecular mechanisms and functional role of activation of Notch signaling in leukemic cells by bone marrow stroma are ongoing.

We found that MSCs induced the phosphorylation of $STAT3^{Tyr705}$ but not $STAT3^{Ser727}$ in NB4 cells, which was inhibited by QLT0267 and LY294002. This observation is concordant with findings in pancreatic cancer cells in which ILK inhibition was shown to block phosphorylation of $STAT3^{Tyr705}$ (42). The mTOR pathway downstream of Akt is responsible for $STAT3^{Ser727}$ phosphorylation, whereas $STAT3^{Tyr705}$ is phosphorylated through JAK2 or receptor and nonreceptor tyrosine kinases at the plasma membrane before dimerization and translocation to the nucleus for phosphorylation of the serine site by mTOR (42). The recently reported cross-talk between Notch/Hes1 and JAK2/STAT3 showed that the Hes1 protein functions as a nonreceptor scaffold protein that allows JAK2 to phosphorylate $STAT3^{Tyr705}$ (14). It is possible that the phosphorylation of $STAT3^{Tyr705}$ is indirectly induced by ILK/Akt through activation of Notch/Hes1 signaling. Consistent with this hypothesis, blockade of Notch signaling by γ-secretase inhibitor abrogated $STAT3^{Tyr705}$ phosphorylation in NB4 cells cocultured with MSC.

PI3K/mTOR and MEK signaling pathways have been reported to synergistically cooperate in the ability of stroma to support survival of leukemic cells (43). Notably, transgenic mice expressing ILK in the mammary epithelium (mouse mammary tumor virus/ILK mice) developed a hyperplastic mammary phenotype accompanied by the constitutive phosphorylation of protein kinase B/Akt, GSK3β, and MAPK (44). In the coculture system, MSCs stimulated the phosphorylation of ERK1/2 in leukemic cells, and this effect was efficiently blocked by QLT0267 or LY294002. Inhibition of ERK activity by PD98059 or inhibition of JAK2/STAT3 signaling by WP1066 or AG490 did not affect MSC-dependent stimulation of p-Akt and p-GSK3β. These data indicate that stroma-activated ILK can at least partially regulate ERK1/2 and STAT3 activation. Recently, the direct interplay between tumor cell and neighboring endothelial cells through MAPK and Notch signaling pathways was characterized (45). Further studies are required to fully dissect the contribution of ERK1/2 and STAT3 signaling in the supportive role of stromal cells in leukemic cell survival.

Our data therefore place ILK upstream of Notch1/Hes1, JAK2/STAT3, and MEK/ERK1/2 signaling in the leukemia/MSC interplay and suggest that bone marrow stromal cells activate ILK in a PI3K-dependent manner at a point where the stimulatory signals converge (Fig. 5*C*). In agreement with these results, the small-molecule ILK inhibitor QLT0267 and PI3K inhibitor LY294002 abrogated the ability of stroma to protect NB4 cells from apoptosis; this was accompanied by the specific inhibition of stroma-induced phosphorylation of Akt and GSK3β. Analogous results were obtained in MO7e and REH leukemic cells and in a subset of primary AML samples. In contrast to well-documented inhibition of chemotherapy-induced apoptosis (29), MSC failed to protect primary AML cells from apoptosis triggered in response to PI3K/ILK activation and in fact enhanced cell death in approximately half of the case studied. These findings indicate that the proapoptotic responses of AML cells to ILK and/or PI3K inhibition are preserved in the context of bone marrow microenvironment due to specific activation of this pathway as a consequence of leukemia/stroma cell interactions.

Consistent with the recent finding that β_1 integrin–activated ILK in fibroblasts regulates cell viability via an Akt-dependent mechanism (46), we found that leukemic cells induce a "leukemia-associated stroma" phenotype in MSC, with phosphorylation and increases of $p\text{-}Akt^{Ser473}$, p-GSK3β, Hes1, p-ERK1/2, and Bcl-2, which were abrogated by ILK and PI3K inhibitors. Although we did not investigate in detail the molecular mechanisms underlying induction of antiapoptotic protein Bcl-2 in MSCs, ILK-induced Akt activation has been reported to contribute to the integrin-dependent up-regulation of Bcl-2 (47). Alternatively, Notch overexpression was shown to increase Notch-dependent Hes1 transcription in stromal cells (48), and the intracellular domain of Notch1 increases the expression of antiapoptotic Bcl-2 protein (49). Notably, Notch1 and its ligand Jagged1 proteins are widely expressed in AML cells (50), providing a plausible explanation for this phenomenon. As such, leukemic cells may activate stromal cells as it was shown in the models of solid tumors (43), and this possibly results in reciprocal secretion of cytokines by stroma and further supports survival of leukemic cells.

In summary, our data suggest that the PI3K/ILK/Akt pathway is a critical prosurvival pathway activated in both leukemic and bone marrow stromal cells on their interactions. In turn, inhibition of ILK/Akt signaling may overcome the protective effects of the bone marrow microenvironment on leukemic cells and thereby potentially ameliorate chemoresistance. Hence, targeting both leukemic and stromal cells via selective blockade of ILK represents a novel therapeutic approach to eradicate leukemia in the bone marrow microenvironment. Although further studies are required for the elucidation of the role of the bone marrow microenvironment and its ability to activate specific signaling pathways in the pathogenesis of leukemias, focus on this stroma-leukemia cross-talk may result in the development of strategies that alleviate the acquisition of a chemoresistant phenotype and enhance the efficacy of therapies in hematologic malignancies.

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