**Loss of RhoB Expression Promotes Migration and Invasion of Human Bronchial Cells Via Activation of AKT1**

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### Abstract

Lung cancer is the leading cause of cancer-related death worldwide, mainly due to its highly metastatic properties. Previously, we reported an inverse correlation between RhoB expression and the progression of the lung cancer, occurring between preinvasive and invasive tumors. Herein, we mimicked the loss of RhoB observed throughout lung oncogenesis with RNA interference in nontumoral bronchial cell lines and analyzed the consequences on both cell transformation and invasion. Down-regulation of RhoB did not modify the cell growth properties but did promote migration and invasiveness. Furthermore, RhoB depletion was accompanied by modifications of actin and cell adhesion. The specific activation of the Akt1 isoform and Rac1 was found to be critical for this RhoB-mediated regulation of migration.

Lastly, we showed that RhoB down-regulation consecutive to K-RasV12 cell transformation is critical for cell motility but not for cell proliferation. We propose that RhoB loss during lung cancer progression relates to the acquisition of invasiveness mediated by the phosphatidylinositol 3-kinase (PI3K)/AKT and Rac1 pathways rather than to tumor initiation. [Cancer Res 2009;69(15):6092--9]

### Introduction

Lung cancer is the leading cause of cancer-related death worldwide, mainly due to its highly metastatic properties. Despite optimal therapy, disease progression is inevitable in the majority of patients diagnosed at an advanced stage. This highlights the need for a better understanding of the molecular mechanisms involved in lung cancer progression.

The Rho family of small GTPases have been widely implicated in cell transformation, survival, invasion, migration, metastasis, and angiogenesis (1). Analyses in human tumors have shown changes in Rho expression, such as the up-regulation of RhoA in testicular germ cell tumors (2) and RhoC in melanoma (3). RhoB, in contrast with its close relatives RhoA and RhoC, has a negative effect on oncogenesis, thereby likely acting as a tumor suppressor (4). We (5–7) and others (8, 9) have previously shown that ectopic expression of RhoB suppresses cell tumorigenesis. Knockout of the rhoB gene in mice increased the frequency of chemically induced tumors (10). Moreover, we reported in two independent immunohistochemical studies that RhoB protein expression decreased dramatically through lung cancer progression (5). Loss of RhoB expression has also been reported in head and neck carcinomas (11), glioblastomas (12), numerous lung cancer cell lines (13), and lung tumor tissues (14).

The purpose of this work was to decipher the precise role played by RhoB in the acquisition of the tumor phenotype in lung cancer. Our previous results suggest that RhoB might be involved in the processes of proliferation and transformation (5). On the other hand, we reported that the loss of RhoB expression occurred between preinvasive and invasive stages of the tumor (5), suggesting that RhoB might be more involved in the regulation of lung cancer invasiveness than in the processes of proliferation and transformation.

To specifically address this hypothesis, instead of analyzing the effect of forced overexpression of RhoB, as performed until now, we chose to examine the physiopathologic situation. With RNA interference, we mimicked the loss of RhoB expression observed in tumoral tissues in both nontumoral bronchial cell lines (BEAS-2B and HBE-135) and K-RasV12–transformed BEAS-2B cells and analyzed the consequences on cell transformation, migration, and invasion.

### Materials and Methods

**Cell culture and transfection.** Human bronchial epithelial cell lines BEAS-2B (ATCC CRL-9609) and HBE-135 cells (ATCC CRL-2741) were grown in DMEM supplemented with 10% FCS or in keratinocyte serum-free medium (Invitrogen) supplemented with epidermal growth factor, bovine pituitary extract, insulin, and hydrocortisone, respectively, at 37°C in a humidified incubator with 5% CO2. BEAS-2B cells transformed by K-RasV12 were obtained by transfection with pZip-KRasV12 plasmid encoding the constitutively activated mutant of K-Ras (a kind gift of Channing Der, North Carolina) using the Jet PEI method, as indicated by the supplier (Polyplus Transfection), followed by selection with 1 mg/mL G418 (Invitrogen). Transient transfection of small interfering RNA (siRNA; Eurogentec) was performed using Oligofectamine (Invitrogen) following the manufacturer’s instructions. The sequences used were two siRNAs against the 3’ untranslated region of RhoB siB1 (5’GGCAUUCUCUAAAGCUAUG3’) and siB2 (5’GGCAAGAUGGUGUAUUAA3’) at 20 nmol/L, siAkt1-1 (5’GAGCGGG-GAGGAUGGCA3’), siAkt1-2 (5’GCAAGGAUCAGCA3’), and siAkt2-1 (5’GAGCGUAAGAUGCAAG3’), siAkt2-2 (5’CCACAAGCGUGGGAUA3’), siAkt3-1 (5’GCCAAAUGUCCAGAUG3’), and siAkt3-2 (5’AGGGAAGCGCAAGUGGA3’) at 5 nmol/L (all AKT isoform sequences from ref. 15), siRac1 (5’ACCAACGUCCCAACUCUC3’) at 10 nmol/L, and siNeg (5’UGUCACAAAGAUGCAAG3’, a lentiviral sequence from Eurogentec) at a concentration depending on the experiment.

**Adenoviral constructs and transduction protocol.** Replication-defective (ΔE1, E3) adenoviral (Ad) vectors expressing RhoB under the transcriptional control of the cytomegalovirus promoter were constructed with the AdEasy System (MP Biomedicals), as described previously (16). For rescue experiments, 140 × 106 cells were plated on a 35-mm dish 24 h before
transduction with adenoviral vectors at a multiplicity of infection (MOI) of 5:1 for BEAS-2B cells and HBE-135 and 30:1 for K-RasV12–transformed BEAS-2B cells.

Lentiviral vector. LVTHM is a lentiviral vector encoding the short hairpin RNA (SHRNA) RhoB1 (5′-CGGCTGCCCCC-GGCATCTCTTAAACGTCATGCAGAAATGGTCCTTTTGCTGAATTT3′) or RhoB2 (5′-CGGCTGCCCCC-GGCATCTCTTTTTGTGAATTT3′) under the control of the HIV promoter (17). 293T cells were kindly provided by Genethon (France). Generation of 293TLVTHM-shRhoB2 and preparation of high-titer lentiviral vector pseudotyped with VSV-G protein have been described previously. Virus containing supernatants were prepared as described elsewhere (18).

Mouse monoclonal anti-Akt1 (Cell Signaling Technology), rabbit polyclonal anti-AKT (BD Pharmingen), mouse monoclonal anti-RhoA (Santa Cruz Biotechnology), mouse monoclonal anti-Rac1 (Chemicon), and rabbit polyclonal anti-Rac1 (Chemicon). Detection was performed using peroxidase-conjugated secondary antibodies and chemiluminescence detection kit (ECL, Pierce).

Quantitative real-time reverse transcription–PCR. Total RNA was isolated 48 h after transfection by RNeasy kit (Qiagen) according to the manufacturer’s instructions, then reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with an iQ real-time PCR detection system (Bio-Rad) using Q SYBR Green Supermix (Bio-Rad).

Immunofluorescence. Cells were plated onto glass coverslips, precoated with collagen I (5 µg/cm², BD Biosciences). At 48 h after transfection, cells were fixed, permeabilized, and then incubated with phallolidin-Alexa594 (Invitrogen), mouse monoclonal anti-human vimentin (Sigma-Aldrich), or mouse monoclonal anti-vimentin (Dako France SAS) and subsequently Alexa488-coupled donkey anti-mouse antibody (Invitrogen).

Quantification of Rac1 activation. Cells plated on collagen I (5 µg/cm², BD Biosciences) were lysed in lysis buffer (Tris-HCl 50 mM/L, Triton X-100 1%, NaCl 500 mM/L, MgCl₂ 10 mM/L, EGTA 2.5 mM/L, and sodium deoxycholate 0.5%) supplemented with protease and phosphatase inhibitors. Cleared lysates were then incubated with beads glutathione S-transferase (GST)-PAK during 30 min at 4°C. The precipitates were analyzed by Western blotting.

In vivo studies. Cells (10 x 10⁵ or 10⁶) were injected s.c. in the left flank or in the tail vein of 6-wk-old female nu/nu mice (Elevage Janvier). Tumor size on the flank was determined with a caliper twice a week. The results are expressed as the mean size of tumors (mm³) from a group of five mice. At several time points after injection into the tail vein, three mice were sacrificed. Paraaffin-embedded lung tissue was serially sectioned and stained with hemalum-eosin. The presence and size of the metastatic nodules were determined by measuring the volume of the infiltrated paranchyma.

Statistical analysis. The mean ± SE was calculated for each data point. Differences between groups were analyzed by the Student’s t test. Data are representative of three independent experiments at least. *P < 0.05; **P < 0.01; ***P < 0.001.

Results

Down-regulation of RhoB promoted migration and invasiveness but not tumorigenic growth of bronchial cells. To investigate the role of RhoB loss throughout lung oncogenesis, we tested the effects of RhoB knockdown in nontumoral human bronchial epithelial cell lines. A strong silencing of RhoB expression was achieved in BEAS-2B and HBE-135 cells using either transfection with synthetic siRNA or transduction with lentivirus expressing siRNA against RhoB (Fig. 1; Supplementary Fig. S2) without alteration of expression of the highly homologous Rho GT Pases RhoA and RhoC (Supplementary Fig. S1).

We addressed whether the loss of RhoB could initiate cell transformation of the immortalized cells. Stable suppression of RhoB after shRhoB-lentivirus transduction had no effect on the ability of BEAS-2B cells to proliferate in monolayer culture (Supplementary Fig. S1). Similarly, the suppression of RhoB enhanced neither the capability of BEAS-2B cells to grow independently of anchorage in soft agar, in contrast to those cells expressing the oncogene K-RasV12 (Fig. 1B) nor the in vivo tumorigenic growth of cells injected s.c. into immunodeficient mice (data not shown). These results suggest that RhoB loss in transformed cells might be required for other aspects of malignancy, such as the acquisition of a motile and invasive phenotype.

Using the two-dimensional wound-healing assays, we therefore assessed the effect of RhoB down-regulation on cell migration after siRNA transfection. After scratching, cells were allowed to migrate for 8 or 16 hours (for HBE-135 and BEAS-2B cells, respectively) in the presence of mitomycin C to eliminate any contribution of cell...
division to this process. BEAS-2B control cells had covered only 30% to 50% of the wound in this time, compared with ~95% filled by the RhoB-depleted BEAS-2B cells (Fig. 1C and D). Similarly, the RhoB depletion in HBE-135 cells led to a significant increase in the migration rate (Supplementary Fig. S2). This effect was fully abolished by restoration of RhoB expression after adenovirus transduction (Fig. 1C and D; Supplementary Fig. S2). Transwell migration yielded comparable results. The suppression of RhoB expression caused a significant increase in motility of both BEAS-2B cells and HBE-135 cells (Fig. 2A and B; Supplementary Fig. S3).

The invasiveness was then analyzed using in vitro three-dimensional Matrigel assays. As shown in Fig. 2C and D (and Supplementary Fig. S3), inhibition of RhoB led to migration of numerous BEAS-2B and HBE-135 cells in contrast with control cells.

![Figure 1](image1.jpg)  
**Figure 1.** Inhibition of RhoB expression increases migration of BEAS-2B cells, but not transformation. **A,** cells transduced with control lentivirus (cont), coding for shRNA (shB1 or shB2), or transfected with siRNA (siNeg, siB1, or siB2) were then transduced or not with an adenoviral vector encoding RhoB (Rescue). Forty-eight hours later, proteins were extracted and immunoblotted with anti-RhoB or anti-actin. **B,** cells transduced with control lentivirus (cont) or coding for shRNA (shB1 or shB2) or transformed by K-RasV12 were grown in agar. Data are representative of two independent experiments. **C,** a wound healing assay was performed on cells transfected with RhoB siRNA and then transduced or not with RhoB adenovirus (Rescue). **D,** percentage of migration is represented.

![Figure 2](image2.jpg)  
**Figure 2.** RhoB inhibition increases motility and invasion of BEAS-2B cells. After transfection with RhoB siRNA, (A) measurement of motility was performed in transwell system. **B,** percentage of motility is represented. C, invasion was evaluated with Matrigel assays. **D,** percentage invasion is represented.
Same results were obtained with BEAS-2B transduced with lentivirus encoding for shRhoB (Supplementary Fig. S4). Thus, while having no effect on growth properties, the expression of RhoB seems to be critical for migratory and invasive properties of human bronchial cells.

**RhoB depletion participates in epithelio-mesenchymal transition.** Changes in epithelial cell motility and invasiveness during oncogenesis are often the result of a transition from an epithelial-to-mesenchymal (EMT) phenotype. This transition involves profound changes in the cell cytoskeleton, the intercellular junctions, the adhesion to extracellular matrix with modification of focal adhesion, and the expression of mesenchymal proteins. We thus analyzed whether modulation of RhoB expression could influence some of these criteria.

First, we examined the effect of RhoB silencing on cell adhesion to several extracellular matrix proteins. We found that RhoB-depleted BEAS-2B cells displayed a significant reduction in the rate of adhesion to collagen I and IV, fibronectin, and laminin 5, but not to vitronectin and plastic, which was already low in control cells (Fig. 3A,B). Hence, the inhibition of RhoB induced a reduction of cell adhesion to extracellular components, corroborating its effect on cell motility.

Staining of filamentous actin structures showed that BEAS-2B cells displayed a dense filamentous network with thick actin cables, whereas HBE-135 cells bore few thin actin fibers (Fig. 3B). The inhibition of RhoB expression in BEAS-2B cells led to a significant reduction in the number of stress fibers, which were both thinner and shorter than in control cells. On the other hand, the depletion of RhoB in HBE-135 cells induced the polymerization of actin structures at the cell periphery, such as that observed in lamellipodia (Fig. 3B). Both RhoB-depleted BEAS-2B and HBE-135 cells showed a marked decrease in the immunostaining of vimentin, a protein localized in the focal adhesion complexes (Fig. 3B), suggesting that RhoB suppression might induce focal adhesion disassembly.

Because the expression of mesenchymal proteins, such as vimentin, is often increased in invasive cells, we analyzed the expression of this protein in control and RhoB-depleted cells. Western blot analysis revealed no change in vimentin expression in BEAS-2B cells after the inhibition of RhoB expression (data not shown). However, we did observe a significant difference in the subcellular distribution of vimentin by immunofluorescence analysis. In BEAS-2B control cells, the vimentin was found distributed throughout the cell, whereas in RhoB-depleted cells, the vimentin had collapsed to the perinuclear region. In HBE-135 cells, the inhibition of RhoB expression led to a dramatic increase of vimentin expression that was also found localized to the perinuclear region, as illustrated by immunostaining (Fig. 3B).

**The stimulating effect of RhoB depletion on motility is mediated by AKT1 activation.** We next aimed to uncover the molecular mechanisms underlying this RhoB effect on migration and invasion. We reported previously that overexpression of RhoB in transformed cells leads to the inhibition of ERK and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (5, 7), both with well-known involvement in the processes of migration and invasion. We therefore investigated the regulation of ERK and AKT after RhoB repression.

Western blot analysis using a specific anti–phosphorylated ERK antibody showed that the inhibition of RhoB expression by siRNA did not alter the phosphorylation of ERK (Fig. 4A). Moreover, the inhibition of ERK by PD 98059 (Supplementary Fig. S5) did not hamper the migratory capability of RhoB-depleted cells (Fig. 4B). In contrast, the transfection of BEAS-2B cells by RhoB siRNA induced a significant stimulation of AKT phosphorylation on both serine 473 and threonine 308 (two residues implicated in its activation) as observed by Western blot analysis (Fig. 4A). In parallel, the inhibition of the PI3K/AKT pathway by LY294002 (inhibitor of PI3K) or triciribin (inhibitor of AKT; Supplementary Fig. S5) significantly inhibited the migration and invasion of BEAS-2B cells induced by RhoB depletion (Fig. 4B; Supplementary Fig. S6).

The Akt protein kinase family consists of three members Akt1/PKBα, Akt2/PKBβ, and AKT3/PKBγ sharing a high degree of similarity. Some publications have, however, shown that each isoform may function in a distinct manner in the regulation of cell migration and invasion (19, 20). Thus, we examined the Akt isoform active in siRhoB-treated cells. As shown in Fig. 4C, the

![Figure 3](https://www.aacrjournals.org/doi/figure-pdf/10.1158/0008-5472.CAN-08-4147)

**Figure 3.** Effect of RhoB inhibition on certain characteristics of EMT. **A,** adhesion of BEAS-2B cells, transfected with RhoB siRNA, to several extracellular matrix proteins was evaluated. **B,** cells were plated onto coverslips precoated with collagen I and then transfected, and 48 h later, cells were fixed and stained with anti-vinculin or anti-vimentin or with phalloidin.
phosphorylation of Akt1, but not of Akt2 or Akt3, was increased in BEAS-2B cells treated with siRNA to RhoB compared with control cells. We then analyzed the effect of specific siRNAs to each isoform (Supplementary Fig. S5). Only siRNA to Akt1 significantly reduced the migration and the invasion of siRhoB-transfected BEAS-2B cells (Fig. 4D; Supplementary Fig. S6). The same observations were made in HBE-135 cells (Supplementary Fig. S7).

Altogether, these results indicate that RhoB regulates cell migration and invasion via the modulation of Akt1 activity. The stimulating effect of RhoB depletion on motility and invasion is mediated by Rac1 activation.

We have shown that the inhibition of RhoB led to the formation of lamellipodia and activation of Akt (Figs. 3 and 4). These two processes are related to Rac1 activation. To test whether the increase of motility and invasion after RhoB depletion is a Rac1-driven mechanism, we performed a PAK-GST pull-down assay to determine the level of Rac1 activation in BEAS-2B and HBE-135 treated or not by siRhoB. As shown in Fig. 5A and Supplementary Fig. S8, RhoB repression induced an increase of GTP-Rac1 in both cell lines. Moreover the transfection of Rac1 siRNA fully impeded the stimulating effect of RhoB depletion on migration and invasion (Fig. 5B and C; Supplementary Fig. S8).

RhoB is critical for K-Ras–induced migration and metastasis but not oncogenic proliferation of bronchial cells. We previously reported that RhoB expression decreased dramatically through lung cancer progression (5). Moreover, it has been shown that the expression of K-Ras, a major oncogene in lung tumorigenesis, led to an inhibition of RhoB expression in several cell models (8). We therefore investigated the consequences of RhoB loss on both growth and migratory properties of BEAS-2B cells transformed by K-RasV12, a constitutively activated mutant of K-Ras.

As expected, we observed that the expression of K-RasV12 in BEAS-2B cells (BKR) induced a significant inhibition of RhoB protein expression (Supplementary Fig. S9). We examined the effect of reexpression of RhoB at levels comparable with those of BEAS-2B wild-type (WT) RhoB encoding adenovirus transduction (Supplementary Fig. S9). As shown in Fig. 6A, whereas the expression of K-RasV12 decreased the doubling time of BEAS-2B cells, this was not increased by reintroducing RhoB (Fig. 6A). The further inhibition of RhoB expression by shRNA in BEAS-2B–transformed cells did not alter their growth rate (Fig. 6A). Moreover, we observed that the inhibition of RhoB expression by shRNA in BKR cells modified neither their anchorage-independent growth rate nor tumorogenic growth in nude mice (Supplementary Fig. S9).

We did, however, observe that BKR displayed enhanced migratory and invasive properties in comparison with WT BEAS-2B cells (Fig. 6B; Supplementary Fig. S9). It is noteworthy that, in contrast to the lack of effect on proliferation rate, (a) the reexpression of RhoB by adenovirus slowed down the migration

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**Figure 4.** Role of AKT and ERK pathways in RhoB regulation of migration in BEAS-2B cells. A, 48 h after transfection, expression of phosphorylated and total ERK and Akt was examined. B, cells transfected with siRNA were pretreated with the inhibitors and then a wound-healing was performed. C, cells were lysed and immunoprecipitated with antibody against Akt isoforms, followed by immunoblotting of the immunoprecipitates. D, cells were cotransfected with siNeg or siB2 and siRNA against Akt isoforms and a wound-healing assay on collagen I was performed.
and invasion rate of the transformed cells (Fig. 6B; Supplementary Fig. S9) and (b) the further inhibition of RhoB expression by shRNA enhanced their migratory and invasive properties (Fig. 6B; Supplementary Fig. S9). Experiments using another clone of BKR cells (clone 2) gave similar results (Supplementary Fig. S10).

If the oncogene K-Ras increases cell motility and invasion of bronchial cells via RhoB repression, then one can hypothesized a dependence upon Akt1 phosphorylation. We did not show an increase of the activation status of Akt1 in BKR versus BEAS-2B cells (Supplementary Fig. S11). These data suggest that additional mechanisms might limit Akt phosphorylation in K-Ras–transformed cells. Nevertheless, RhoB reexpression inhibited significantly Akt1 phosphorylation (Supplementary Fig. S11). Moreover, the siRNAs against Akt1 lead to an important decrease of migratory and invasive rates of BKR at a level comparable with those of BEAS-2B WT (Supplementary Fig. S11). Thus, Akt1 phosphorylation seems to be critical for migration and invasion and RhoB might control this activation.

To assess whether RhoB inhibition increases metastatic potential in vivo, BKR cells, transduced or not by lentivirus encoding shRNA to RhoB, were injected into the tail vein of athymic mice, and the formation of lung metastases was then determined. BKR cells formed some small lung tumor foci within 5 weeks (Fig. 6C and D).

The inhibition of RhoB expression in BKR cells increased significantly the number and size of lung metastases (Fig. 6C). Whereas the BKR cells induced small perivascular foci, the BKR cells transduced with shRhoB induced a large perivascular and peribronchial carcinomatous infiltration. The volume of lung parenchyma infiltration rapidly reached up to 70% of the lung (Fig. 6D).

Altogether, these results indicate that while RhoB is dispensable for the proliferative aspect of malignancy, it is critical for the acquisition of a motile and invasive phenotype.

Discussion

RhoB loss of expression has been reported in numerous cancer cell lines and lung tumor tissues (5, 13, 14, 21). In this work, we aimed to assess the consequences of RhoB extinction in bronchial cells on various steps of carcinogenesis, including proliferation, transformation, migration, and invasion.

We first showed an absence of RhoB effect on growth properties of normal bronchial cells. Moreover, we showed that, in K-RasV12 transformed cells that displayed a reduction of RhoB levels, neither the slight reexpression of RhoB to levels comparable with WT cells nor a further inhibition by shRNA altered the proliferative rate. These data showed the dispensability of RhoB for proliferation regulation and more specifically for K-RasV12–mediated oncogenic transformation of BEAS-2B cells. Likewise, it was reported previously that reexpression of RhoB with the histone deacetylase inhibitor in lung cancer cells did not reduce tumor cell growth (14).

In contrast, we did observe an association between RhoB loss of expression and the acquisition of a migratory and invasive phenotype of bronchial epithelial cells. As such, we have shown for the first time that RhoB inhibition in human bronchial cells, both immortalized and tumoral, enhances their migratory and invasive abilities. Moreover, we have shown that RhoB is critical for the motile and invasive properties induced by K-Ras transformation. Additionally, in vivo studies revealed that inhibition of RhoB significantly enhanced the capability to induce lung metastases. Interestingly, Liu and colleagues reported a defect of motility and a reduction of adhesion and spreading of rho−/− mouse embryonic fibroblasts (10). The involvement of RhoB in cell migration has also been documented in several developmental studies (22–24). In agreement with our findings, RhoB has been shown to inhibit migration and invasion of Ras-transformed murine fibroblasts (9) and macrophage migration on fibronectin substratum (25).

In addition, here, we have found that the motile and invasive phenotype induced by the inhibition of RhoB is the result of the modification of certain EMT features. At first, the inhibition of RhoB expression led to a reorganization of actin cytoskeleton necessary for cell migration. A RhoB control of actin stress fibers has previously been reported in different cellular models (26, 27), whereas a lack of effect has been reported in others (25). Several studies on human epithelial cell lines have reported an induction of vimentin expression and a role of this intermediate filament protein in migration (28, 29). Indeed, we observed an increase of vimentin expression in HBE-135 cells and a significant change in vimentin subcellular localization in BEAS-2B cells after RhoB inhibition. The homogeneous distribution of vimentin intermediate filaments became dramatically reorganized around the nucleus. Interestingly, similar observations have previously been done after the activation of the small Rho GTPases Cdc42, Rac1, and RhoG (30).

Figure 5. Role of Rac1 in RhoB regulation of migration in BEAS-2B cells. A cells were lysed, and a GST pull-down was performed to precipitate Rac-GTP. B cells were cotransfected with siNeg or siB2 and siRNA against Rac1. A wound-healing assay on collagen I was performed. C, an invasion assay was realized.
Inhibition of RhoB also led to a reduced cell attachment to several matrix proteins. A correlation between reduced cell adhesion and enhanced migration has previously been reported in many models, such as in cells overexpressing Tiam1, a guanine nucleotide exchange factor for Rac1 (31) or R-Ras (32).

Whereas the modulation of cadherin expression has often been implicated in the process of invasion (33), the inhibition of RhoB did not seem to alter E-cadherin or N-cadherin expression in human bronchial cell lines (data not shown). These data raised the possibility that RhoB inhibition is able to induce cell invasion without altering intercellular junctions and without inducing a cadherin switch, one component of EMT. Our observations are corroborated by several lines of evidence suggesting that many invasive carcinomas lack signs of complete EMT (34).

Aside from its role in cell survival, increasing evidence in the present study underlined the critical role of Akt activity in the control of migration, invasion, and metastatic dissemination. We found that RhoB inhibition led to the activation of AKT, but not ERK, in accordance with previous findings that overexpression of RhoB inhibits AKT phosphorylation (5–7, 9). Moreover, we showed that AKT activation was critical for the promotion of RhoB-dependent motility and invasiveness. The detailed analysis of the Akt isoforms showed that inhibition of RhoB led to a selective activation of Akt1, but not of Akt2 or Akt3. The Akt1 activation is critical for bronchial cell migration, as emphasized by experiments of Akt1 down-regulation.

Distinct roles of Akt isoforms in cell motility have been reported. Akt1 has repeatedly been found to promote invasion of fibroblasts (20, 35, 36) and migration of endothelial cells (37). However, several studies have reported that Akt2, but not Akt1, stimulates the motility of breast and ovarian cancer cells (20, 38). Whereas expression of all three Akt isoforms has been found in lung tissue (39), nothing has yet been reported concerning the specific role of each on bronchial cell motility or invasion.

Finally, we propose that the loss of RhoB expression, observed during tumor progression from a noninvasive to highly invasive stage (5), is a crucial event participating in the acquisition of the
invasive phenotype of lung cancer via stimulation of the PI3K/AKT pathway. Therefore, the loss of RhoB might be dispensable for the initiation of the tumor but critical for the invasiveness during the dynamic progression of lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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