Loss of RhoB Expression in Human Lung Cancer Progression

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ABSTRACT

Purpose: **RhoB is a low molecular weight GTPase belonging to the Ras protein superfamily. Whereas most Rho proteins have been shown to have a positive role in proliferation and malignant transformation, the specific role of RhoB appears more divergent. We reported previously that RhoB inhibits cell proliferation in various human cancer cells. Here, we studied the specific role played by RhoB in human lung cancer.**

Experimental Design: **We analyzed the expression of RhoB protein by immunostaining in human lung tissues ranging from normal to invasive carcinoma from different histological types in two large independent studies of, respectively, 94 and 45 samples. We then studied the cellular effect of RhoB overexpression in a model of lung cancer (A549, adenocarcinoma) and tumorigenicity in nude mice.**

Results: **We showed in both studies that RhoB protein was expressed in normal lung and decreased dramatically through lung cancer progression ($P < 0.01$). Interestingly, RhoB expression was lost in 96% of invasive tumors and reduced by 86% in poorly differentiated tumors compared with the nonneoplastic epithelium. Moreover, the loss of expression of RhoB correlated significantly with tumor stage and proliferative index, whereas no correlation was found between RhoB and p53 or Bcl-2 expression. We then showed that ectopic expression of RhoB in lung cancer cell line A549 suppressed cell proliferation, anchorage-independent growth, and xenograft tumor growth in nude mice.**

Conclusions: **RhoB loss of expression occurs very frequently in lung carcinogenesis, reinforcing its putative tumor suppressive activity, and raising the value of its potential use in cancer therapy.**

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the world (1). Despite advances in surgery, chemotherapy, and radiation therapy, survival rates have changed little in the last decade, and long-term survival remains dramatically poor. It has been established that lung cancer arises as a consequence of the accumulation of multiple somatic genetic changes involving critical genes of which the protein products control cell motility, proliferation, differentiation, and apoptosis (2). Identification and characterization of these genetic changes that drive lung cancer development and progression is of high interest for helping clinicians in early diagnosis and for developing novel targeted therapy (3).

Ras proteins regulate important cellular functions ranging from cell differentiation to cell proliferation. They function as molecular switches, cycling between the inactive GDP- and the active GTP-bound states (4). The oncogene *ras* plays a pivotal role in malignant transformation. Ras is found mutated to a GTPase-deficient form that lead to constitutive activation of signaling pathways and uncontrolled proliferation in ~50% of lung adenocarcinoma (5). Closely related family members of Ras, such as Ras-homologous (Rho) small guanosine triphosphatases (GTPases) are also involved in the regulation of a variety of cellular processes such as organization of the actin cytoskeleton (6), genotoxic stress-induced signaling (7), and malignant transformation (8). Recent studies additionally confirmed the role of Rho proteins in cancer by showing their involvement in cell transformation, survival, invasion, metastasis, and angiogenesis (9–11). Moreover, analyses in human tumors demonstrate overexpression of several Rho proteins in breast cancers (12), of RhoA in testicular germ cell tumors (13), and of RhoC in pancreatic adenocarcinoma (14), in melanoma (15), and in breast cancer (16).

Whereas most Rho proteins have been shown to have a positive role in proliferation and malignant transformation, the specific role of RhoB in these processes appears more divergent. Among the Rho family members, RhoB displays some particular characteristics such as its localization to early endosomes (17), its role in intracellular transport of cell-surface receptors (18), and its rapid up-regulation by growth factors and genotoxic stress (19) that suggest that RhoB might have a special function in transformed cells. Recent studies indicated that RhoB might exert a tumor-suppressive role in growth control and transformation. We showed that ectopic expression of RhoB in human tumor cells leads to an inhibition of tumor growth in nude mice (20). Furthermore, inactivation of RhoB in knock-out mice

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increased the frequency of tumors (21). Lastly, we reported a decrease in RhoB expression in head and neck carcinoma (22).

The putative tumor-suppressive effect of RhoB in human tumors and the importance of other closely related proteins such as Ras in lung carcinogenesis (23, 24) prompted us to study the role of RhoB in lung oncogenesis. To this end, we analyzed RhoB expression in human lung tissue ranging from normal to invasive carcinoma from different histological types, and we showed in two independent studies that RhoB expression decreases with lung cancer progression. We also confirmed the suppressive role of RhoB in a model of a lung cancer cell line (A549 adenocarcinoma). Thus, we propose that RhoB loss of expression is an important and frequent event that should be considered in the understanding of lung cancer progression.

MATERIALS AND METHODS

Sample Collections

Tissue sections were originated from two different cancer centers, one in France (study 1) and one in the United States (study 2), and were independently analyzed.

The study 1 consisted of a total of 94 cases obtained from the Pathology Department at the Purpan Hospital (Toulouse, France). Tissue sections were placed by the study pathologist in the following categories, 9 normal or inflammatory lung tissues, 9 benign diseases (metaplasia and hyperplasia), 17 preinvasive tumors (squamous dysplasia, *in situ* carcinoma, and bronchioloalveolar carcinoma according to the last WHO classification), 8 low-grade malignant tumors (typical carcinoid), and 51 high-grade malignancy invasive carcinoma (21 adenocarcinoma, 18 squamous cell carcinoma, and 12 large cell carcinoma). All of the specimens were obtained from surgical resection for carcinoid tumors, bronchioloalveolar carcinoma, and all invasive carcinoma, and from biopsies or surgical specimen for preinvasive tumors. Diagnosis was assessed by a lung cancer pathologist by applying the last WHO classification (25), and clinicopathological stage was assigned according to the Tumor-Node-Metastasis classification (26). In all of the cases, presence of normal bronchial epithelium adjacent to the tumor was required as an internal control. We should note that patients did not receive any neoadjuvant treatment (neither chemotherapy nor radiotherapy). Patients were treated and followed-up at the Purpan Hospital to ensure collection of clinical data.

The study 2 consisted of 45 tissue sections of pulmonary tumors obtained from the Pathology Department of the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). Specimens were evaluated in the fresh state by a pathologist who selected a non-necrotic area of the tumor and an adjacent non-neoplastic area of pulmonary parenchyma. Nine bronchioloalveolar carcinoma, 15 adenocarcinoma, 11 squamous cell carcinoma, and 10 undifferentiated carcinoma were included in the study. Diagnoses were established according to standard histopathological criteria (25). Clinical and staging information were obtained from the Cancer Registry at Moffitt Cancer Center.

Immunohistochemistry

Study 1. In study 1 immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections that were cut 4-μm thick. After rehydratation, deparaffinized sections were pretreated by microwave epitope retrieval (750 W during 15 min in citrate buffer 10 mmol; pH 6.0). Before the application of the primary antibody, an endogenous peroxydase activity with 5% hydrogen peroxide was inhibited, and a biotin with bovine albumin blocking step was performed. The tissue sections were incubated with monoclonal antibodies directed against RhoB (C5; Santa Cruz Biotechnology; 1:50), p53 (DO 7; DAKO; 1:50), *Bcl*II (124; DAKO; 1:50), and Ki67 (MiB1; DAKO; 1:50). The primary antibody was detected using secondary biotinylated antibody and a streptavidin-peroxidase conjugate according to the instructions of the manufacturer. Hematoxylin was used as the nuclear counterstain. Adenocarcinoma cell line A549 transfected by RhoB was used as a control of specificity. Cell suspensions handling procedure included the preparation of smears by centrifugation (cytospin, 700 rpm; 15 min) and the confection of paraffin-embedded cellblocks after formalin fixation. Negative controls were done by omitting the primary antibody. The specificity of the RhoB antibody has been demonstrated on nontransfected A549 cells and RhoA-transfected A549 compared with RhoB-transfected cells (data not shown). Immunostaining was evaluated by a semiquantitative method according to the percentage of positive tumors cells, *i.e.*, + 25%, 2+ 25–50%, 3+ > 50%. To exclude equivocal reactions, at least 1% of positive cells were required as a diagnostically relevant positive reaction. Bronchi and bronchioli were strongly positive in all of the cases, serving as consistent internal positive controls. This scoring system has been validated previously (16). A diffuse cytoplasmic staining was scored as positive reactivity for RhoB and *Bcl*II. Only a nuclear labeling with p53 and Ki-67 was considered as a positive reaction.

Study 2. In the study 2, 5 μm sections from formalin-fixed, paraffin-embedded tissues were cut and placed on poly-L-lysine coated slides. Slides were subjected to deparaffination in xylene and hydration through a series of decreasing alcohol concentrations, following standard procedures. Endogenous peroxidase activity was quenched with a 3% solution of H_2O_2, for 20 min at 37°C, and the slides were washed in deionized water for 5 min. Antigen retrieval was performed by placing the slides in a clear plastic container with vented top containing citrate buffer (0.1 M citric acid 4.5 ml, 0.1 M sodium citrate 21.5 ml, and deionized water 225 ml) in a microwave oven set on high, 2 times for 5 min each. The slides were then allowed to cool for 10–20 min, rinsed in deionized water, placed in PBS for 5 min, and drained. Blocking serum was applied and the slides were incubated in a humid chamber, for 20 min at room temperature. After blotting, the slides were incubated with a polyclonal anti-RhoB (119; Santa Cruz Biotechnology) antibody. After 1 h, slides were rinsed with PBS and placed in PBS for 5 min. For detection, the Vectastain ABC kit, Rabbit IgG, Elite series (Vector Laboratories, Inc., Burlingame, CA) was used following the manufacturer's specifications. The biotinylated secondary antibody was applied for 20 min at room temperature in a humid chamber. At the end of this incubation, the slides were rinsed and placed in PBS for 5 min, followed by the addition of the ABC complex. The slides were incubated in a humid chamber for 30 min at room temperature and then rinsed and placed in PBS for 5 min. 3,3′-Diaminobenzidine, prepared according to

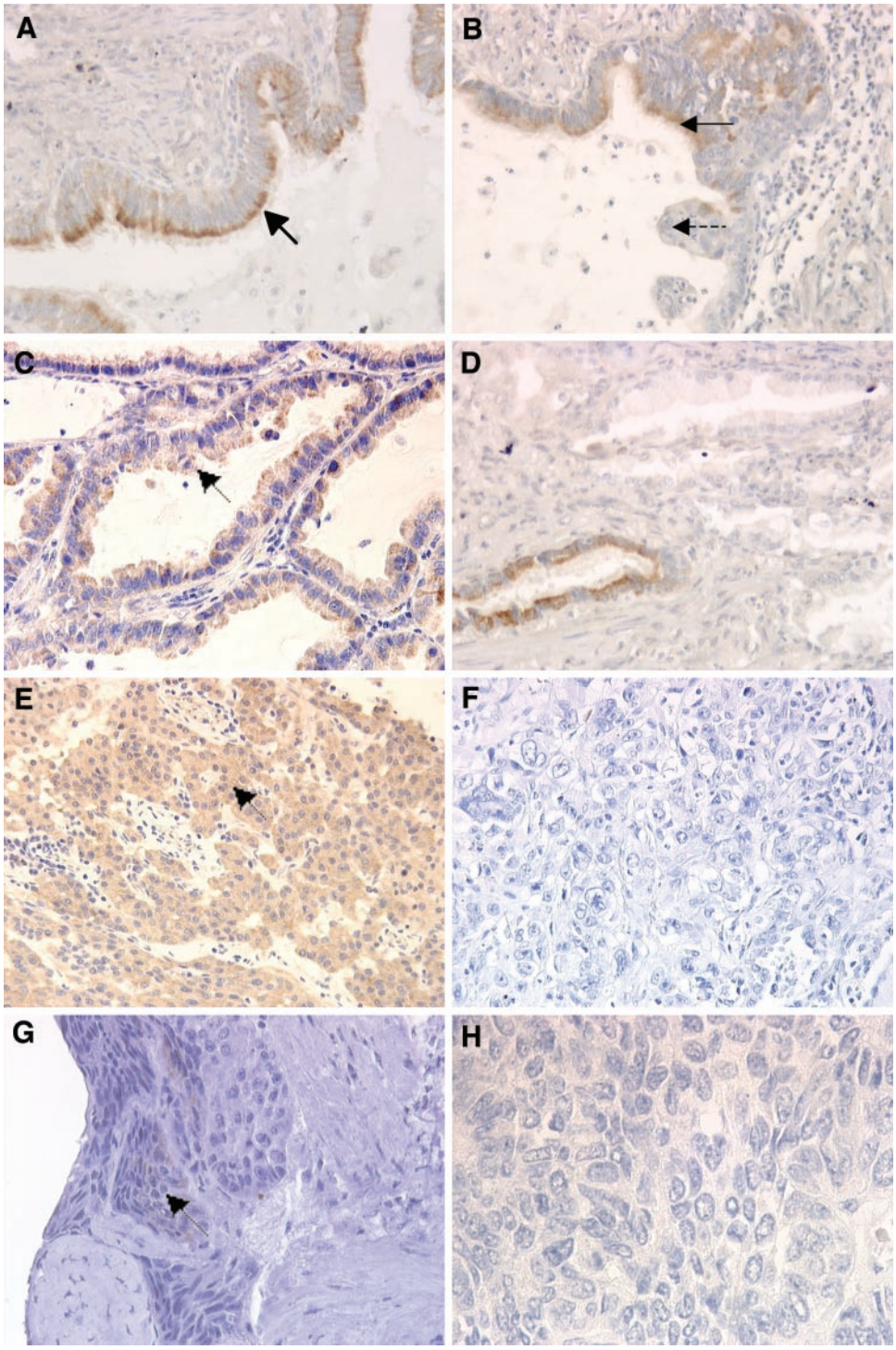

Fig. 1 Analysis of RhoB immunostaining in normal lung, benign lesions, preinvasive lesions, low-grade tumors and invasive lung carcinoma (study 1). *A,* sections from paraffin-embedded tissues were processed and immunostained with anti-RhoB antibody (for details, see "Materials and Methods") in normal lung and in various pathological types. *A,* RhoB immunostaining in normal lung (*arrow*). *B,* staining for RhoB in a partially invaded bronchiole. We can see RhoB positive staining on the upper part (*thick arrow*) and the loss of RhoB expression in the lower part (*dotted arrow*) due to local tumoral invasion. *C,* bronchioloalveolar carcinoma. *D,* RhoB staining in adenocarcinoma (the negative staining of the tumor should be compared with the internal positive control). *E,* carcinoid tumor. *F,* large cell neuroendocrine tumor. *G, in situ* carcinoma. *H,* squamous cell carcinoma.

the manufacturer's specifications, was applied to the slides, and color development monitored. When maximal intensity was reached (~5 min) the slides were rinsed in water and counterstained with modified Mayer's hematoxylin for 30 s. The slides were finally washed in running water for 10 min, dehydrated, cleared, and mounted with resinous mounting medium.

Generation of Stably Transfected A549 Cells

Human lung carcinoma A549 cells (American Type Culture Collection CCL-185), were maintained in RMPI 1640 supplemented with 10% FCS (growth medium) at 37°C in a humidified incubator containing 5% CO_2. One day before transfection, 4×10^5 cells were seeded into 60-mm plates. Cells

were then transfected with pCMV-IRES/Zeo vector expressing a constitutively activated RhoB mutant [for details see (27)] using LipofectAMINE Reagent plus method as indicated by the supplier (Invitrogen). Two days after transfection, cells were collected with trypsin, split to 1:6 ratio, and fed with medium containing selection marker, Zeocin (Cayla S.A., Toulouse, France), at a concentration of 100 μg/ml. Fresh growth medium containing Zeocin was changed every 4 days, until colonies formed (2 weeks later). As soon as colonies reach proper size and cells stop dying under selection marker, single colonies were picked and transferred to 24-well plates. Stably-transfected cell lines were screened for the expression of RhoB by Western blot analysis.

Cell Growth Determination

To determine cell proliferation, the method developed by Skehan *et al.* (28), which measures the cellular protein content of adherent cultures, was used. Cells were seeded in 96-well plates (2000/well) at day 0 in 150 μl of growth medium. At days 1, 2, 3, 4, and 6, cells were fixed by the addition of 50 μl of 50% trichloro-acetic-acid in water at 4°C. Cells were stained for 30 min with 0.4% (w/v) sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of absorbance in a computer-interfaced, 96-well microtiter reader.

Anchorage-Independent Growth Assays

Cells were seeded at 8000 cells/well in 12-well plates in triplicate in 0.3% agar over a 0.6% agar layer as described elsewhere (29). Cells were fed twice weekly until colonies grew to a suitable size for observation (~12 days). Colonies were photographed after 4-h incubation with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in RPMI 1640 at 37°C.

Tumorigenicity in Nude Mice

Female NMRI *nu/nu* were purchased from the CERJ JANVIER (Le Genest Saint-Isle, France). Mice used in the experiments were ear-tagged before injections with tumors. All of the mice used in these experiments were 6–8 weeks old and were housed in barrier cages in the Institut Claudius Regaud animal facilities, according to European guidelines. Mice were injected s.c. with 5×10^6 of cells in 100 μl of PBS in the right flank. Tumor size was determined with a caliper twice a week. The results are expressed as mean size of tumors (mm^3) from groups of 5 mice ± SE.

Western Blot Analysis

Cells were washed in cold PBS and harvested in lysis buffer [HEPES 50 mM (pH 7.5), Triton X100 1%, glycerol 10%, NaCl 10 mM, $MgCl_2$ 5 mM, NaF 25 mM, and EGTA 25 mM], protease inhibitor mixture (Sigma), sodium orthovadanate 2 mM, and paranitrophenylphosphate (6.4 mg/ml). For analysis of extracellular signal-regulated kinase and AKT activation, cells were starved for 24 h (0.1% FCS) and stimulated or not by addition of 20% fetal bovine serum for 15 min. Cellular protein was quantitated by Bradford assay (Bio-Rad), and 10–40 μg of the cleared lysates were separated on a 12.5% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech).

Polyvinylidene difluoride membranes were incubated with polyclonal antibodies against RhoB (119; Santa Cruz Biotechnology), phospho AKT (Ser473; Cell Signaling), AKT1 (BD PharMingen), phospho-extracellular signal-regulated kinase (anti- Active MAPK; Promega) and extracellular signal-regulated kinase (Santa Cruz Biotechnology), respectively. Detection was performed using peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescence detection kit (ECL; Amersham Pharmacia Biotechnology).

Statistical Analysis

The χ^2 test was used to assess differences in RhoB expression between carcinoma of different degree of malignity and of different stages. Fisher's exact test was used to determine the association between RhoB expression and p53, Bcl-2, and Ki-67 ($P < 0.05$ was considered significant). Moreover we applied an interrater agreement test (test κ).

RESULTS

RhoB Expression Decreases in Lung Carcinoma

Study 1. The first study consisted of 94 cases from Purpan Hospital. To address the question of the relevance of RhoB in human lung tumorigenesis, we analyzed RhoB protein levels, respectively, in normal or hyperplastic epithelium, in preinvasive tumors, and in low-malignancy and high-malignancy lung carcinoma (Fig. 1; Table 1). We first studied the expression of RhoB in normal or noncancerous lung biopsies. Moderate or strong cytoplasmic RhoB expression was detected in all of the cases. Staining was well visualized along bronchiolar epithelium and particularly in well-differentiated cells (ciliated and goblets cells), and was weaker in reserve cell. The staining was diffuse with a stronger signal on the apical ciliar membrane. Some other cells were positive with RhoB, chondrocytes and macrophages. In reactive epithelial modifications, RhoB was still expressed by alveolar metaplasia or hyperplasia found in inflammatory process. In preinvasive tumors ranging from atypic hyperplasia or dysplasia to *in situ* carcinoma, RhoB was still largely expressed, but some distinction should be made

Table 1 Positivity of RhoB in normal lung and in various pathological types

RhoB staining for each pathological type.

Tissue	Cases	Positivity for RhoB (*n*, %)	Subgroup (*n*, % positivity)
Normal lung	9	9 (100)	Normal and benign
Benign disease	9	9 (100)	($n = 22$, 95)
Dysplasia	4	3 (75)	
In situ carcinoma	5	3 (60)	Preinvasive tumors
Bronchioalveolar carcinoma	8	8 (100)	($n = 13$, 84)
Carcinoid tumor	8	8 (100)	Low grade tumor ($n = 8$, 100)
Adenocarcinoma	21	2 (9.5)	Invasive tumors
Squamous cell carcinoma	18	0	($n = 51$, 1)
Large cell carcinoma	12	0	$P < 0.001$

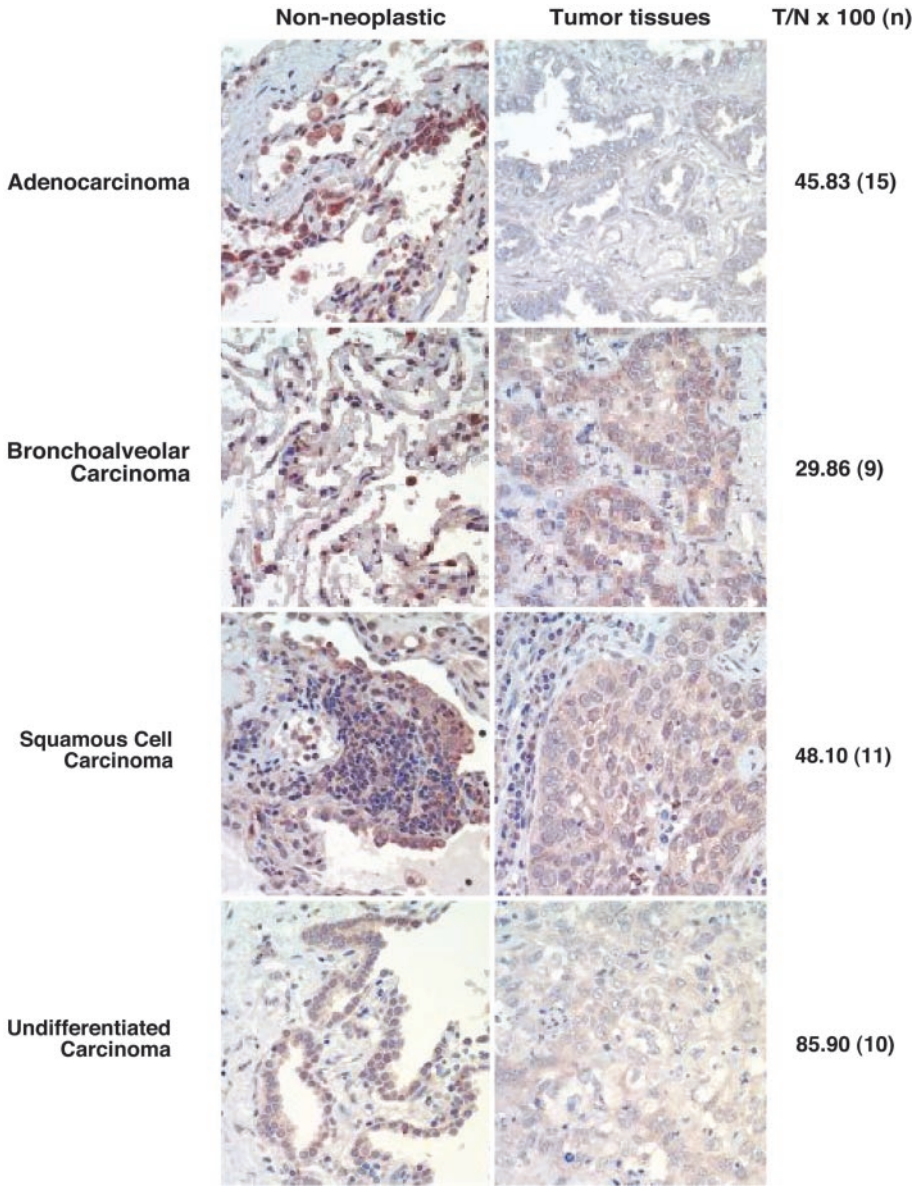

Fig. 2 Analysis of RhoB staining in various lung tumors (study 2). Expression of RhoB in lung tumors. Representative immunostaining of different non-small cell pulmonary carcinoma (adenocarcinoma, bronchoalveolar carcinoma, squamous cell carcinoma, and undifferentiated carcinoma). For any given tumor type, the non-neoplastic and tumor stains were obtained from the same block. Squamous metaplasia in bronchial epithelium was elected as the normal counterpart for the other three tumor types. The values represent the average of three visual determinations. The last column shows the percentage of staining in tumor tissue (*N*) *versus* staining in normal tissues (*T*) and the number of cases.

between bronchi and alveolar events. Positivity of RhoB was inconstant (6 on 9) and mostly weak in squamous dysplasia or *in situ* carcinoma, whereas staining was detected in all of the atypical alveolar hyperplasia and bronchioloalveolar carcinoma. According to the last WHO classification, bronchioloalveolar carcinoma is considered as an *in situ* adenocarcinoma with a good prognosis (30). Moderate and strong RhoB protein expression (scores 2+ and 3+) was detected in all of the cases. Furthermore, we analyzed some composite adenocarcinoma (not retained for our study) with positive immunostaining in their bronchioloalveolar tumors component and negative staining in their invasive component. Carcinoid tumors are low-grade malignant neoplasms of neuroendocrine cells associated with a good prognosis and a low rate of metastatic spread. All of the tested carcinoids showed a diffuse and strong staining with anti-RhoB.

We studied 51 cases of invasive tumors: 21 adenocarcinoma, 18 squamous cell carcinoma, and 12 large cell carcinoma. No RhoB expression was detected in 49 cases (96%), whereas only a faint staining was observed in 2 cases (score 1+). In 2 invasive adenocarcinoma, a faint staining was detected, and they were classified as 1+. It is noteworthy that in these both cases, adenocarcinoma were well differentiated and stage I. Thus, these tumors were quite close to bronchioloalveolar carcinoma. It should be stated that in some tumors (3 squamous cell carcinoma, 2 large cell carcinoma, and 2 adenocarcinoma) a very faint staining was detected in $<1\%$ of the cells and was not considered as positive. No difference was seen between various histological types. All of the cases, however, had consistent and strong RhoB staining of bronchiolar cells, which served as internal positive control. We can, thus, conclude that RhoB expression, which is detected in normal lung, preinvasive le-

sions, and low-grade tumors, decreases significantly in all of the invasive carcinoma ($P<.001$).

Study 2. The second study consisted of 45 patient biopsies from the H. Lee Moffitt Cancer Center, and was carried out simultaneously and independently of the study 1. RhoB expression was analyzed in a non-necrotic area of the tumor and an adjacent non-neoplastic area of pulmonary parenchyma. For any given tumor type, the non-neoplastic and tumor stains were obtained from the same block. Fig. 2 depicts the expression of RhoB in representative adenocarcinoma, bronchoalveolar carcinomas, squamous cell carcinoma, and undifferentiated carcinoma of the lung. Non-neoplastic epithelium expressed RhoB in the cytoplasm. However, the expression of RhoB was lost in the majority of the carcinoma. Poorly differentiated tumors (undifferentiated carcinoma) had a greater reduction in the expression of RhoB (86% reduction) than adenocarcinoma (46%) or squamous cell carcinoma (48%), and than well-differentiated bronchioloalveolar carcinoma (30% reduction).

Expression of RhoB Correlates with Tumor Stage and Ki-67

All of the tumors were assessed according to the Tumor-Node-Metastasis classification (26) and classified in stage I-IV. Expression of RhoB was determined in each stage. Results from study 1 indicate that RhoB expression decreases significantly from stage I to stage III (Fig. 3). This can be partially explained by the fact that bronchioloalveolar carcinoma and carcinoid tumors are most of the time localized tumors without lymph node involvement and belong more frequently to stage I.

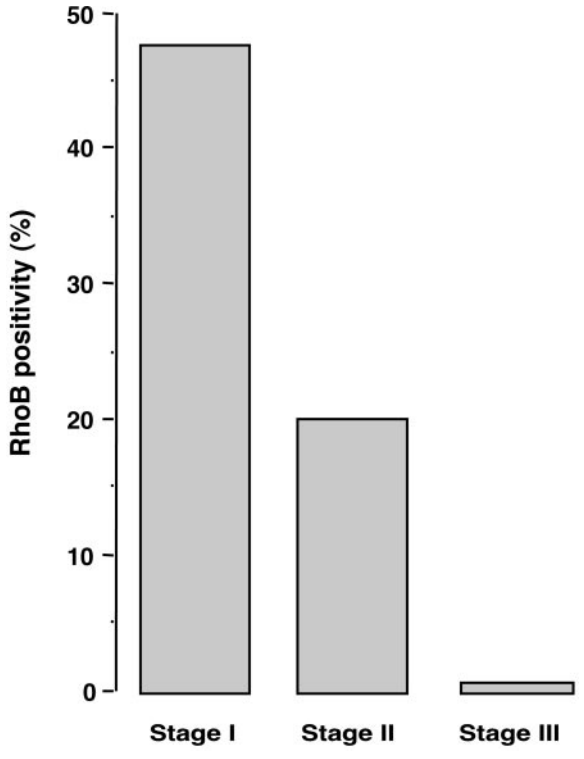

Fig. 3 Decrease of RhoB expression correlates with tumor stage. Invasive carcinomas were classified according to the Tumor-Node-Metastasis classification in stages I to III. Percentage of RhoB-positive staining is reported in each stage in study 1.

Table 2 Relationship between RhoB expression detection by immunohistochemistry and p53, Bcl-2, and Ki67

Immunostaining with antibodies directed against p53, Bcl-2, and Ki-67 was performed for each section. Positive results for each marker are reported. P = fisher exact test and κ is interrater agreement.

	RhoB protein expression		
	Positive	Negative	*P*
P53, *n*			NS[a]
Negative	27	22	
Positive	13	32	
Bcl-2, *n*			NS
Negative	32	34	
Positive	8	20	
Ki67, *n*			$P = 0.001$
Negative	33	9	$\kappa = 0.6337$
Positive	7	45	

[a] NS, not significant.

We also investigated the relationship between RhoB expression and proliferation by analyzing the expression of the tumor proliferative marker Ki-67 antigen that is known to be a strong prognostic marker (31). We found statistical correlation between the loss of expression RhoB and the expression of Ki-67 ($P< 0.01$; Table 2).

The *p53* tumor suppressor gene is the most commonly mutated gene in cancer (32) and is mutated in 50% of non-small cell lung carcinoma. The *Bcl*II gene is involved in the inhibition of programmed cell death, is expressed in 25% of squamous cell carcinoma, and is associated with poor survival (33). Table 2 shows that there is no correlation between RhoB and p53 or *Bcl*II expression.

Ectopic Expression of RhoB Suppresses A549 Cell Tumor Growth *in Vitro* and *in Vivo*

The lung cancer patient biopsies data suggest a tumor suppressive role for RhoB. Therefore, we reasoned that forced expression of RhoB should block malignant transformation. Thus, we evaluated if overexpression of RhoB in the human lung cancer cell line A549 leads to *in vitro* and *in vivo* inhibition of cancer growth. To this end, we transfected the A549 cells with either empty vector pCMV or RhoB and isolated stable transfectants as done previously in Panc-1 cells (20). Fig. 4*A* shows the expression levels of RhoB in 2 of the clones that we expanded. We then evaluated in these clones, the ability of RhoB to affect anchorage-dependent and -independent proliferation, and tumor growth in nude mice.

The analysis of growth properties of pCMV empty vector- and RhoB-transfected A549 cells maintained in growth medium showed that the expression of RhoB induces a significant reduction of growth rate in A549 cells (Fig. 4*B*). When testing on anchorage-independent growth by soft agar cultures, pCMV empty vector-transfected A549 cells grew numerous soft agar colonies (Fig. 4*C*). In contrast, A549 cells stably expressing RhoB grew only a few, barely detectable colonies. These cell lines were then implanted s.c. in nude mice and their tumor sizes

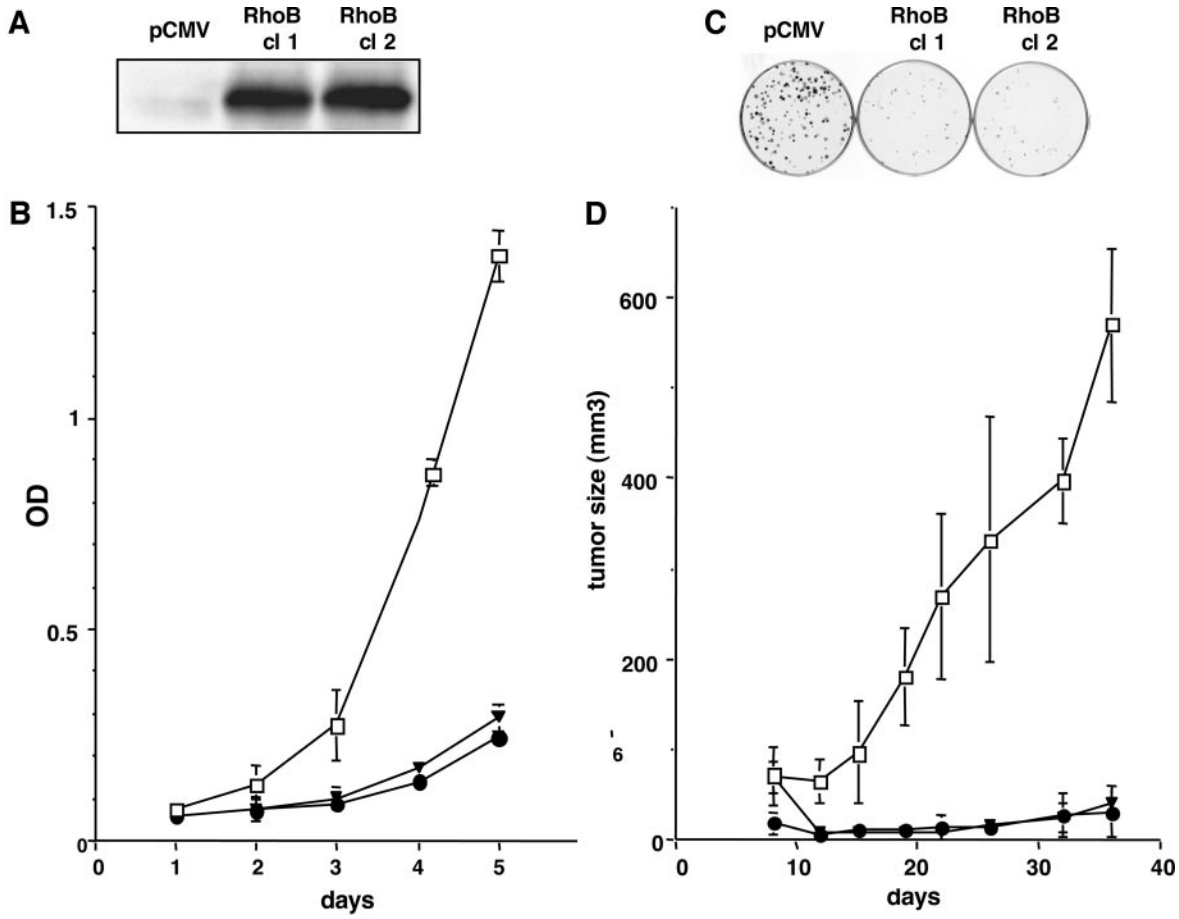

Fig. 4 Ectopic expression of RhoB results in inhibition of A549 tumor growth *in vitro* and *in vivo*. *A*, expression of RhoB in A549 transfected cells. Empty vector pCMV and RhoB transfected A549 were lysed and analyzed for RhoB expression by Western blotting as described in "Materials and Methods." *Lane 1* represents empty vector, and *Lanes 2* and *3* represent two RhoB transfected clones. *B*, the anchorage-dependent growth was determined as described in "Materials and Methods." The data from analysis of empty vector (□), and RhoB-tranfected A549 cells from clone 1 (●) and clone 2 (▼) are representative of three independent experiments; *bars*, ±SE. *C*, anchorage-independent cell growth was assayed by scoring formation of clones after plating on 0.6% agar layer in 0.3% agar containing medium of transfected A549 cells. Data are representative of three independent experiments. Each experiment was done in triplicate. *D*, the effect of RhoB mutants on the tumor growth in nude mice was evaluated as described under "Materials and Methods." Stably transfected A549 cells that express RhoB were implanted s.c. in nude mice and the tumor sizes measured over time as described under "Materials and Methods." The data are representative of three independent experiments and represent empty vector-transfected (□) and RhoB-transfected clones 1 (●) and 2 (▼); *bars*, ±SE.

measured over a period of 36 days. Fig. 4*D* shows that empty vector transfected A549 cells grew to an average tumor size of 617.5 ± 55.3 mm^3, whereas those stably expressing RhoB grew to an average tumor size of only 61.7 ± 22.7 mm^3 in the first clone or 21.5 ± 10.7 mm^3 in the second one.

We then analyzed the effects of RhoB on major signaling pathways as done previously with Panc-1 cells (20), and we showed that RhoB expression decreased the levels of phospho-extracellular signal-regulated kinase 1/2 and phospho-AKT in both serum-stimulated and -unstimulated cells (data not shown). Moreover, some members of the Rho family of GTPases (RhoA, CDC42, and Rac-1) are involved in the regulation of NF-κB-dependent transcription (34) and it has recently been shown that RhoB is a negative regulator of nuclear factor κB (NFκB) signaling (35). Thus, we analyzed the regulation of NFκB-dependent transcription by the RhoB mutants and confirmed that RhoB inhibits NFκB-dependent transcriptional activity in A549 cells (data not shown).

DISCUSSION

Only a few works have previously studied the expression of RhoB in human tumor tissues. In testicular germ cell tumors, RhoB mRNA was detected neither in tumors nor in unaffected tissues (13). In a series of 24 brain tumors, Forget *et al.* (36) reported that RhoB protein expression levels are inversely related to tumor malignancy. In line with these data, we reported recently suppression of RhoB expression in 18 human invasive head and neck carcinoma (22). By contrast, Fritz *et al.* (12) reported an increase in RhoB protein but not mRNA level in 15 breast tumors biopsies compared with normal breast. The present work examined RhoB protein expression in lung cancer in

two large independent studies by different methodologies. In the first one of 94 patients, we compared RhoB expression among a series of normal lung, preinvasive lesions, low-grade tumor, and highly invasive tumor, whereas in the second one we compared RhoB expression between non-neoplastic-adjacent tissue and neoplastic tissue for each of the 45 patients. The conclusions of the two studies are in agreement and show the physiological presence of RhoB protein in normal lung and the dramatic decrease of its expression in invasive carcinoma. These results extend our previous observation on head and neck cancers (22) and suggest that loss of RhoB expression is not limited to squamous cell carcinoma but can be observed on various histological tumor types. Moreover, in contrast with genetic abnormalities such as Ras or p53 mutations, loss of RhoB expression appears to be a common feature of lung cancer.

The mechanism by which RhoB expression decreases in lung carcinoma is not yet elucidated. In a previous study, we did not find RhoB gene deletions or mutations (22), but levels of RhoB RNA were mostly barely detectable, suggesting a transcriptional down-regulation. Fritz *et al.* (12) confirmed that Rho proteins (RhoA, RhoB, and RhoC) are not altered by mutation in breast tumors. We reported recently the presence of a VNTR sequence in the human RhoB promoter that is known to be linked with the penetrance and the development of several cancers (37). An epigenetic regulation has also been proposed by Wang *et al.* (38) by finding that RhoB induction is mediated by histone deacetylase inhibition. RhoB down-regulation thus remains an important issue to address.

We showed here that loss of RhoB expression occurs in lung tumor progression. This could be due to an increased proliferation capability of the cells. This is supported by the inverse correlation between RhoB expression and the proliferative index Ki-67 in tumor tissues, and by the negative role of RhoB on A549 cell proliferation. However, the loss of RhoB expression between preinvasive and invasive carcinoma also suggests that RhoB might be involved in invasiveness. It is well known that Rho proteins regulate various aspects of actin cytoskeleton dynamics in fibroblasts, metalloproteinases gene transcription, phospholipid metabolism, and vesicular transport, and are thus probably involved in tumor cell invasion (reviewed in Ref. 39). Overexpression of Rho family members increases migration and invasiveness in various cell lines and culture models suggesting that such overexpression in carcinoma could affect metastatic potential *in vivo* (40, 41), but most of these findings have been provided for Ras, Cdc42, and RhoA, and data concerning RhoB are lacking.

Many studies propose that RhoB might exert a suppressive activity in cancer cells. We demonstrated previously the ability of RhoB to inhibit malignant transformation in several cancer cell lines (20). It has also been reported that RhoB can act as an activator of apoptosis in transformed cells (35, 42) probably by controlling AKT and NFκB pathways as reported in this study and elsewhere (20, 43, 44). Zeng *et al.* (45) proposed recently that RhoB interferes with the serine-threonine protein kinase PRK, a Rho effector kinase, for mediating RhoB signaling function and its cellular effect on transformation. Thus, we can hypothesize that RhoB acts as a competitor of pro-oncogenic RhoA and RhoC protein perhaps by sequestering common Rho effectors required for cell transformation.

These results provide strong evidence for a tumor-suppressive activity of RhoB. They corroborate previous data obtained in RhoB−/− mice. Liu *et al.* (21) reported that mice receiving i.p. injections of E1A-Ras-transformed RhoB−/− Mouse Embryo Fibroblasts develop tumors more efficiently than the transformed RhoB+/− counterpart. Moreover, RhoB−/− mice subjected to a skin carcinogenesis assay display an elevated rate of papilloma formations (21).

Taken together, these experiments provide new insights into the molecular pathogenesis of lung cancer. Our results coupled with previous findings strongly suggest that RhoB plays a critical role in malignant transformation and progression of human carcinoma. We have identified RhoB loss of expression as a frequent and reproducible event in lung cancer progression, and we, thus, propose that RhoB is a negative modifier in lung cancer enhancing its interest in clinical perspectives.

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