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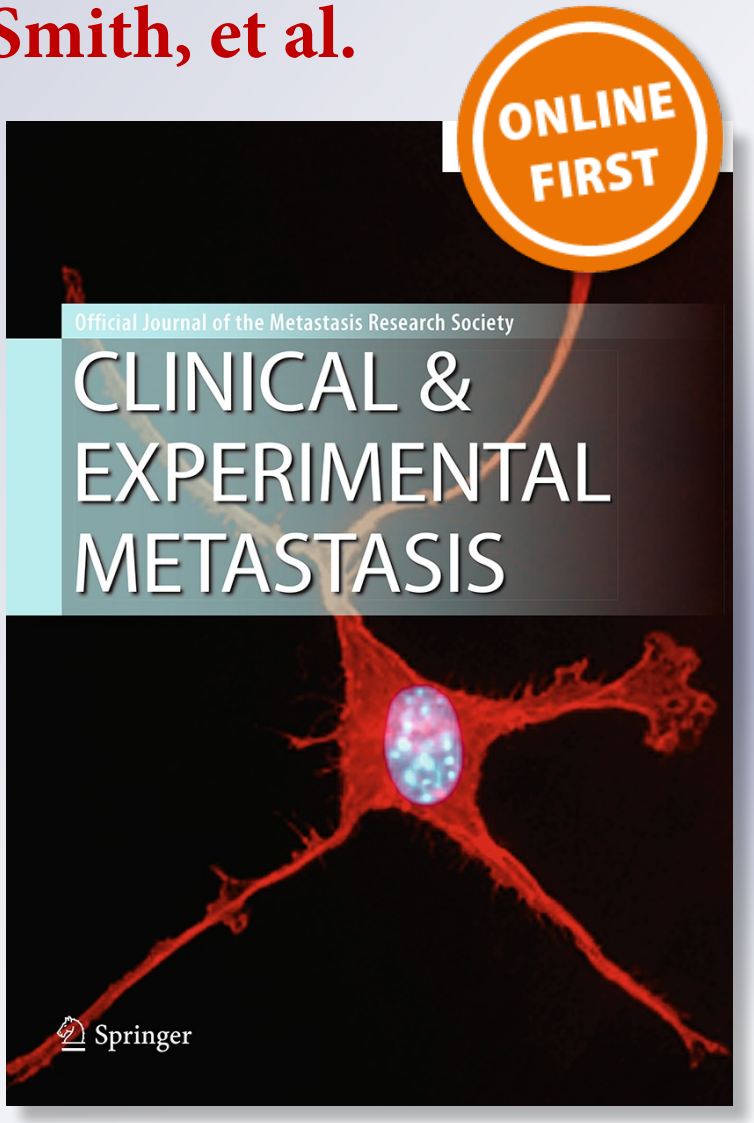

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RESEARCH PAPER

TGF-beta specifically enhances the metastatic attributes of murine lung adenocarcinoma: implications for human non-small cell lung cancer

Paula Fernanda Vázquez · María José Carlini · María Cecilia Daroqui · Lucas Colombo · Mercedes Liliana Dalurzo · David Eduardo Smith · Julieta Grasselli · María Guadalupe Pallotta · Marcelo Ehrlich · Elisa Dora Bal de Kier Joffé · Lydia Puricelli

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Abstract Lung cancer is the most frequent and one of the most deadly cancer types and is classified into small cell lung cancer and non-small cell lung cancer (NSCLC). Transforming growth factor beta (TGFβ) regulates a wide array of cell functions and plays a major role in lung diseases, including NSCLC. TGFβ signals through the complex of TGFβ type I and type II receptors, triggering Smad and non-Smad signaling pathways such as PI3K/Akt and MEK1/ERK. We investigated the role of TGFβ1 on the progression of the murine lung adenocarcinoma cell line LP07. Furthermore, we undertook a retrospective study with tissue samples from stage I and II NSCLC patients to assess the clinical pathologic role and prognostic significance of TβRI expression. We demonstrated that although lung cancer cell monolayers responded to TGFβ1 anti-mitogenic effects and TGFβ1 pulse (24 h treatment) delayed tumor growth at primary site; a switch towards malignant progression upon TGFβ1 treatment was observed at the metastatic site. In our model, TGFβ1 modulated in vitro clonogenicity, protected against stress-induced apoptosis and increased adhesion, spreading, lung retention and metastatic outgrowth. PI3K and MEK1 signaling pathways were involved in TGFβ1-mediated metastasis stimulation. Several of these TGFβ responses were also observed in human NSCLC cell lines. In addition, we found that a higher expression of TβRI in human lung tumors is associated with poor patient's overall survival by univariate analysis, while multivariate analysis did not reach statistical significance. Although additional detailed analysis of the endogenous signaling in vivo and in vitro is needed, these studies may provide novel molecular targets for the treatment of lung cancer.

Keywords TGFβ1 · TβR · Metastasis · Non-small cell lung cancer

Abbreviations

NSCLC	Non-small cell lung cancer
TGFβ	Transforming growth factor beta
SCC	Squamous cell carcinoma
ADC	Adenocarcinoma
TβRI	TGFβ type I receptor
TβRII	TGFβ type II receptor
EMT	Epithelial to mesenchymal transition
FAK	Focal adhesion kinase

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Introduction

Lung cancer is the most frequent and one of the most deadly cancer types. In Argentina, the age-standardized mortality rates for the 1990–2005 period were 31.9 among men and 7.5 among women per 100,000 inhabitants per year, being the leading cause of cancer-related death in men and the fourth leading cause in women [1]. Lung cancer is classified into small cell lung cancer and non-small cell lung cancer (NSCLC). The latter accounts for 80 % of all cases and represents a heterogeneous group which includes squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma. Over the past decades, the 5-year survival rate of lung cancer ($\sim$10 % in most countries) has not increased significantly, and surgery remains the standard treatment for resectable NSCLC [2].

TGFβ is a conspicuous member of a cytokine superfamily that regulates a wide array of cell functions and therefore has a pivotal role in normal physiological and disease processes [3]. TGFβ initiates intracellular signaling by binding to the complex of TGFβ type I (TβRI) and type II (TβRII) receptors, transmembrane glycoproteins with serine-threonine kinase activity [4]. Upon ligand binding, TβRII phosphorylates TβRI activating the TβRI kinase, which in turn phosphorylates and activates Smad transcription factors. Receptor-associated Smad2 and Smad3 (R-Smads) and co-mediator Smad4 translocate to the nucleus, where they regulate the transcription of TGFβ target genes. In addition, TGFβ can activate non-Smad signaling pathways, like MAP kinases, p21 activated kinase 2 and PI3K-Akt signaling, among others [5, 6]. Furthermore, TGFβ signaling is engaged in RhoA–Rock1 signaling that is required for the epithelial to mesenchymal transition (EMT) [7]. Although being referred as non-Smad, there is often cross-talk in these activities with the Smad proteins to obtain a full biological response. The variety of R-Smad/co-Smad/co-factor combinations regulates the transcription of a large amount of target genes, such as the transcription factors Snail, Slug, Zeb and Twist [8]. The combinatorial usage of these pathways can explain, at least in part, the diversity and cell type-specificity of TGFβ signaling responses.

TGFβ plays a major role in lung diseases including lung cancer. Certain TGFβ1 genetic polymorphisms have been associated with increased risk for developing NSCLC [9] and with greater likelihood of lymph node metastasis in ADC of the lung [10]. Increasing TGFβ1 levels in plasma were found in patients with lung cancer than those with nonmalignant pulmonary disease and in healthy individuals [11]. An increased level of TGFβ1 in plasma has been associated with poor outcome after chemotherapy [12] and radiotherapy [13] in NSCLC patients. Furthermore, a significant correlation between TGFβ1 protein level in primary tumors and prognosis was detected by multivariate analysis in patients with NSCLC [14]. In addition, human NSCLC-derived primary cell lines were shown to produce TGFβ1 [15] and levels of tumor-derived TGFβ1 are higher within sentinel lymph nodes than in non-sentinel nodes from patients with NSCLC [16]. Regarding non-tumor cells, a subpopulation of highly suppressive regulatory T cell population that express high levels of TGFβ1 has been identified in the peripheral blood of NSCLC patients [17]. Tumor associated fibroblasts isolated from primary NSCLC tumors also produce TGFβ1 [18]. Moreover, differentially expressed genes, encoding for proteins that are significantly enriched for extracellular proteins regulated by the TGFβ1 signaling pathway have been identified in tumor associated fibroblasts compared with matched normal fibroblast cell lines [19]. Also, another TGFβRII ligand has been found to be overexpressed in lung cancer and to modulate EMT and cancer progression [20]. TGFβ1-induced EMT has been associated with reduced sensitivity to cisplatin and paclitaxel in A549 NSCLC cells. Moreover, it has been shown that the disease free survival rate of patients with EMT changes as response to chemo-radiotherapy was significantly lower than that of patients without EMT changes [21].

In the murine Lewis lung carcinoma model (3LL) maintained either in vitro or in vivo only the TGFβ1 gene was found to be expressed at higher levels in highly metastasizing cells, while 3LL metastatic variants expressed similar levels of several growth-regulated genes and oncogenes [22].

These compiling evidences show that TGFβ1, to varying degrees, is associated with tumor progression in lung cancer. Therefore, we investigated the regulatory role of TGFβ1 on the metastatic potential of lung cancer cells taking advantage of the murine ADC cell line LP07, a syngeneic lung cancer model that was derived from a spontaneously arising BALB/c lung tumor (P07) [23]. Furthermore, we undertook a retrospective study with tissue samples from 73 patients with stage I and II NSCLC to assess the clinical pathologic role and prognostic significance of TβRI expression in this pathology.

Materials and methods

Cells and cell culture conditions

The LP07 cell line was established in our laboratory from primary cultures of BALB/c-transplantable lung tumor P07 [23]. LP07, A549 and H125 cells were maintained in Minimum Essential Medium (MEM) (GIBCO BRL, Grand Island, NY, USA), F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC, Manassas, VA, USA) and

RPMI (GIBCO BRL, Grand Island, NY, USA), respectively and supplemented with 10 % fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine and 80 μg/ml gentamycin at 37 °C in a humidified 5 % CO_2-air atmosphere.

Mice

2–4 month-old, weight-matched female BALB/c mice were obtained from our Animal Care Division. Mice were housed under a 12:12 h light–dark cycle with access to water and food ad libitum. All animals studies were conducted in accordance with the highest standards of animal care as outlined in the NIH guide for the Care and Use of Laboratory Animals.

Cell treatments

Cells were treated with TGFβ1 (4 ng/ml) (Calbiochem, CA, USA), TβR type I and type II dual inhibitor LY2109761 (TβR inh; 2 μM) that was kindly provided by Lilly Research Laboratories, PI3K inhibitor LY294002 (10 μM) (Lilly, Indiana, USA) and MEK1 inhibitor PD98059 (50 μM) (Millipore, Billerica, MA, USA). PD98059 and LY294002 were dissolved in dimethylsulfoxide (DMSO) (Merck, New Jersey, USA). The final concentration of DMSO was below than 0.01 %. Treatments were done for 24 h, unless otherwise indicated.

Western blot

70–80 % confluent control or treated monolayers were washed with phosphate buffered saline (PBS) and lysed with RIPA buffer (150 mM NaCl, 1 % NP-40, 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5 % deoxycholate) with protease and phosphatase inhibitors for 30 min on ice, centrifuged at 4 °C for 15 min, and equal amounts (50 μg) of the supernatant proteins were analyzed by Western blotting. Antibodies to TGFβ1, TβRI, TβRII, β1 integrin, pERK1/2, ERK1/2, pAkt1/2/3 and Akt1/2/3 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies to Smad2/3, pSmad3 and Smad3 were from Cell Signaling Technologies Inc. (Beverly, MA, USA). Antibodies to pSmad2, pFAK and FAK were from Millipore (Billerica, MA, USA). Antibody to E-cadherin was from BD Biosciences (Franklin Lakes, NJ, USA) and antibody to actin was from Sigma (St. Louis, MO, USA). Proteins were separated by SDS-PAGE and transferred onto Amersham Hybond-P GE Healthcare PVDF membrane (Buckinghamshire, UK). The membranes were blocked for non-specific binding for 1 h in 5 % milk (w/v) diluted in TBS-Tween-20. Then, the blots were incubated overnight with primary antibodies. Subsequently, the blots were washed and incubated either with anti-rabbit IgG or anti-mouse IgG antibodies linked with horseradish peroxidase (Millipore, Billerica, MA, USA). After further washing, the blots were developed using Enhanced Chemiluminescence Detection System (ECL Plus, GE Healthcare).

Cell proliferation assays

For tritiated thymidine ([^{3}H]dThd) incorporation assays, 6×10^3 cells per well were cultured in 96-well plates in 10 % FBS-enriched media for 24 h, washed with PBS and then treated with various doses of TGFβ1 (0.1, 1, 10 ng/ml) for 48 h. Cells were incubated with 1 mCi/ml [^{3}H]dThd for 8 h and then 5 % trichloroacetic acid was added. Subsequently, the TCA insoluble fraction was dissolved in 0.5 N sodium hydroxide. [^{3}H]dThd concentration was measured using a liquid scintillation counter.

Alternatively, cells were treated with 4 ng/ml TGFβ1. After 72 h, with a medium change at 48 h, cell proliferation was assessed by the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA).

Cell proliferation was also assessed by manual counting using an improved Neubauer hemocytometer. 2×10^4 cells in MEM containing 10 % FBS were seeded in 24-well plates and allowed to attach overnight, washed with PBS and treated with 4 ng/ml TGFβ1 in 2 % FBS MEM for 72 h, with a medium change at 48 h. Then, cells were trypsinized and counted.

Cell cycle analysis

Cells were seeded in triplicates in 60 mm plates and treated with 4 ng/ml TGFβ1 for 48 h. Cells were trypsinized and gently pipetted to obtain a monodispersed cell suspension. Cells were centrifuged, resuspended in 0.5 ml of PBS and fixed by transferring into centrifuge tubes containing 4.5 ml of 70 % ethanol, on ice. Ethanol-suspended cells were centrifuged for 5 min at 300 g; the cell pellet was suspended in 5 ml of PBS and centrifuged again. Cells were stained by suspending in 1 ml of propidium iodide (PI) staining solution (0.1 % v/v Triton X-100, 50 μg/ml PI (Molecular Probes, Eugene, OR, USA), and 100 μg/mL DNase-free RNase A in PBS) and kept in the dark at room temperature for 30 min. Samples were transferred to the flow cytometer and cell fluorescence was measured. The percentage of cells in G0/G1, S and G2/M phases was calculated.

Adhesion assay

35 mm dishes were coated with fibronectin (2 μg/cm^2) (Millipore, Billerica, MA, USA) in 0.2 ml of PBS at room temperature for 60 min. Coated wells were further incubated

for 60 min with MEM plus 1 mg/ml bovine serum albumin (BSA) to saturate any substrate-free space. Dishes coated with 1 % BSA alone were used as a substrate control. 1×10^5 control or TGFβ1 treated cells per coated well were seeded in 0.5 ml complete MEM after recovery of membrane proteins and incubated for 15 min or 45 min at 37 °C. Then, adherent cells were washed twice with PBS, trypsinized and counted using an improved Neubauer hemocytometer. Alternatively, cells were fixed for 20 min with methanol:acetic acid (3:1), washed 3 times with PBS, stained with crystal violet solution (0.5 % in 2 % ethanol) for 5 min and washed with water. Adhered cells were highlighted in black and the percentage of pixels in the image was calculated using ImageJ (area fraction).

Cell-spreading assay

LP07 cells, treated or not with TGFβ1, were seeded at 8×10^4 cells on BSA or fibronectin-coated 35 mm dishes and allowed to adhere for 120 min. Non-adherent cells were removed through washing with PBS, the remaining cells were fixed with 4 % formaldehyde in PBS and the percentage of spreading cells was determined, scoring 400 cells under a phase-contrast microscope (Nikon, Tokyo, Japan). For each condition, data were collected by random observation and at least by triplicate.

Immunofluorescence

LP07 cells (1.2×10^4) were cultured in glass cover slips and treated or not with TGFβ1. After 72 h cells were fixed with 4 % formaldehyde in PBS, permeabilized with 0.2 % Triton-X100, and blocked using 2 % BSA in PBS. Primary antibodies were incubated overnight at 4 °C and followed by FITC- or TRITC- conjugated secondary antibodies. Fibronectin antibody was from Gibco (Grand Island, NY, USA) and vimentin antibody from Abcam (Cambridge, UK). To detect actin, TRITC-phalloidin was used (Sigma, St. Louis, MO, USA).

In vitro migration

LP07 subconfluent monolayers were manually scratched with a pipette tip to create scratches in the center of the 35 mm dishes. The detached cells were removed by washing the cells once with PBS and 2 % FBS MEM with or without TGFβ1 was added to each dish. Phase contrast images were taken immediately after adding the treatments and 7 h later using Nikon Eclipse E2000-S (Nikon, Tokyo, Japan) microscope. Cell-free areas were analyzed using Image Pro Plus image analysis software by measuring the area at 0 and 6 h and cell advancement area was derived for each treatment.

Evaluation of apoptotic cells by nuclear staining

LP07 cells cultured in glass cover slips for 24 h in the presence or absence of TGFβ, were serum starved for 72 h, fixed with 4 % formaldehyde in PBS, stained with DAPI and mounted on slides with PBS:Glycerol (1:1). Cells were observed for nuclear condensation/fragmentation indicative of apoptosis under a fluorescent microscope and photographed using a high resolution camera. Four fields were photographed and the percentage of total apoptotic cells compared to the total number of cells (at least 300 cells) was determined. Each condition was assayed in triplicate and the mean and standard deviation were calculated and graphically represented.

Clonogenic assay

LP07 (5×10^3), A549 or H125 (3×10^3) monodispersed control or treated cells were seeded on six-well plates in complete culture medium. Medium was changed every 48 h. After 7 days of culturing, plates were washed, fixed with 5 % acetic acid in methanol, stained with Giemsa or crystal violet solution and the number of colonies was counted under a dissecting microscope. Cloning efficiency was defined as the percentage of cells able to grow as colonies with more than 10 cells.

Lung retention assay

Mice were injected with 1.5×10^5 cells/300 μl culture medium that were pretreated or not with TGFβ1 and labeled with PKH67 fluorescent cell linker kit (Sigma, St. Louis, MO, USA). Lungs were removed 24 h later, embedded in OCT and frozen at −80 °C. 5–15 μm thick tissue sections were cut using a cryostat. The number of fluorescent cells in 30 random fields was assessed for each of three different lungs per group.

Experimental metastasis assay

Two groups of mice were injected into the tail vein with 1.5×10^5 LP07 cells/300 μl culture medium that were pretreated or not with TGFβ1, LY294002 and PD98059 either alone or in combination. 3 weeks later, mice were sacrificed and their lungs dissected and fixed in Bouin overnight. Metastatic tumors were counted under a dissecting microscope.

Subcutaneous tumor growth

Control or TGFβ1- treated cells (3×10^5/200 μl culture medium) were inoculated subcutaneously in the right flank of mice. Tumors were measured twice a week in 2

perpendicular diameters using a Vernier caliper. Tumor volume (mm^3) was calculated as ($a \times b^2$)/2, where a and b are the largest and smallest diameters, respectively.

Patients and tumor specimens

Seventy three samples from patients with Stage I or II NSCLC were obtained from the "Hospital Italiano de Buenos Aires" in Argentina for this retrospective analysis. The study included Caucasian individuals (median age: 63 years, range: 45–83; median survival: 41 months, range: 1–116). All tissue specimens were obtained by surgery from untreated patients and were classified morphologically according to the established WHO criteria (2004). Data on pathological findings and clinical follow-up were obtained from the medical charts. All patients who died had clear evidence of uncontrolled tumor growth at the time of death. The Ethical Committees of the Institute of Oncology "Ángel H. Roffo" and the "Hospital Italiano de Buenos Aires" approved this study, being the Helsinki Declaration carefully followed. All patients gave their informed consent for the stored samples to be used for academic research. Immediately after removal, tumor specimens were fixed in 4 % formaldehyde in PBS and embedded in paraffin.

Immunohistochemistry

5-μm tissue sections were obtained from each patient tumor block on polylisine-coated slides. All sections were routinely deparaffinized and rehydrated before staining procedures. Sections were treated with 7 % hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity, microwaved for 5 min in citrate buffer (pH 6.0) for antigen retrieval, and incubated with 5 % milk in distilled water for 45 min to block nonspecific binding, washing thoroughly in PBS between steps. Immunohistochemical staining to detect TβRI expression was performed using a Santa Cruz antibody (Santa Cruz, CA, USA) and a streptavidin–biotin amplification method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). After washing, secondary anti-mouse biotinilated antibody (Gibco BRL, Grand Island, NY, USA) was added diluted 1:1000 for 1 h at room temperature. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution, counterstained with Mayer's hematoxylin, rehydrated, and then mounted with Canada synthetic balsam. Negative controls missing the primary antibody showed no immunoreactivity.

Statistical analysis

All experiments were performed in triplicate and repeated at least twice with similar results. Data were expressed as means and standard deviation. Statistical comparisons were performed by *t* test or one-way ANOVA with the indicated post-tests using Prism v3.00 for Windows (GraphPad Software, San Diego, CA, USA). Two-way ANOVA and repeated-measures two-way ANOVA followed by Bonferroni's post-tests were performed when indicated. The level of statistical significance for all tests was $p < 0.05$. For patient data, dependence among the variables in study and the clinical pathologic parameters was evaluated by Chi square test. Global survival was measured from the date of surgery to the time of the last follow up visit or death. Survival curves were plotted according to the Kaplan–Meier method and differences were assessed by log rank test. To identify independent prognostic factors we used Cox proportional hazard model. *p* values are based in two sided tests and significance was set at $p \leq 0.05$.

Results

LP07 cells have functional TGFβ1 Smad- mediated signaling

We first analyzed TGFβ1 signaling pathway components. The presence of TGFβ1 protein in LP07 cells was shown by Western blotting (Fig. 1a). TβRI and TβRII were also found to be expressed by LP07 cells (Fig. 1b). Phosphorylation of Smad2 (range of response 3.9- to 29.3-fold) and Smad3 (range of response 3.7- to 11.8-fold) (Fig. 1c) and nuclear translocation of Smad4 (data not shown) were induced in response to TGFβ1 stimulation, indicating a functional TGFβ1-Smad-signaling pathway. Moreover, inclusion of a TβR inhibitor completely blocked this effect. The expression of endogenous TGFβ1 together with the detectable levels of phosphorylated Smad2 in unstimulated cells likely reflects autocrine TGFβ1 activity (Fig. 1a, c). In addition, LP07 conditioned media proved to be active in an Mv1Lu mink lung epithelial inhibition assay indicating that cells secrete biologically active TGFβ1 in vitro (data not shown).

TGFβ1 inhibits in vitro proliferation of LP07 cells

The effect of TGFβ1 on the proliferation of LP07 cells was first examined by a [^{3}H]dThd incorporation assay. Mv1Lu cells were used as control cultures (data not shown). As shown in Fig. 2a, DNA synthetic activity was inhibited by TGFβ1. We found that 1 ng/ml TGFβ1 was sufficient to inhibit growth of the cells. Growth inhibition was also detected by the MTS assay (data not shown) and manual cell counting, using an intermediate TGFβ1 concentration (4 ng/ml) and was reversed when adding TβR inhibitor

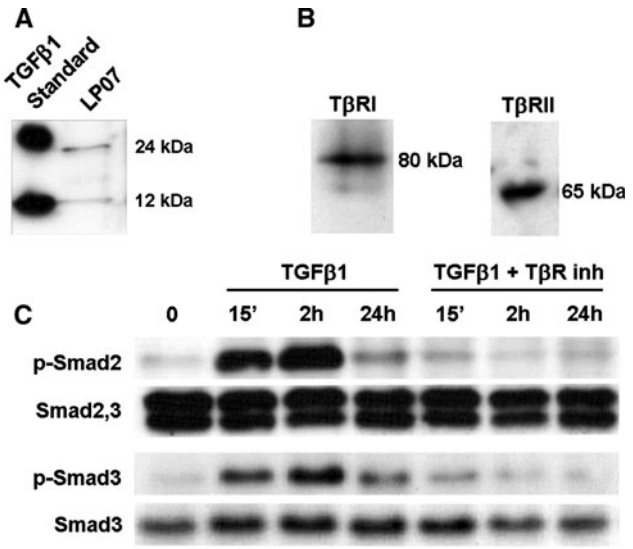

Fig. 1 TGFβ1 signaling pathway in LP07 cells **a** Western blot analysis for TGFβ1 standard and endogenous protein from the cell lysates isolated from LP07 cells **b** Western blot analysis for the TGFβ1 receptors TβRI and TβRII from the same lysates **c** LP07 cells were incubated with 4 ng/ml TGFβ1 alone or in combination with the TβR type I and type II dual inhibitor LY2109761 (TβR inh, 2 μM) for 15 min, 2 h and 24 h. At the end of incubations, Smad2/3 and phosphorylated Smad2/3 protein levels were analysed by western blot

(Fig. 2b). All subsequent experiments were done with 4 ng/ml TGFβ1.

Next, we examined TGFβ1 effect on cell cycle progression. Exposure of LP07 cells to TGFβ1 increased the percentage of cells in G0/G1 phase of the cell cycle at the expense of S phase population after 48 h of treatment, as assessed by propidium iodide staining of DNA and flow cytometry analysis (Fig. 2c). This effect was completely prevented by inclusion of the TβR inhibitor. Consistently, treatment with TGFβ1 resulted in the increased expression of $p21^{Waf1/Cip1}$, a universal inhibitor of cell cycle progression, with maximum expression after 6 h of cytokine exposure (Fig. 2d). Furthermore, when we examined the protein level of $p27^{Kip1}$, which is one of the key players in the regulation of G0/G1 phase of cell cycle, western blot analysis showed that TGFβ1 induces sustained $p27^{Kip1}$ protein expression up to 24 h (Fig. 2d). Taken together, these results show a possible involvement of $p27^{Kip}$ induction in TGFβ1-induced G0/G1 arrest in LP07 cells.

TGFβ1 increases LP07 cell adhesion and spreading on fibronectin

The ability of TGFβ1 pretreated LP07 cells to attach to fibronectin-coated tissue culture dishes was tested. 24 h pretreatment with TGFβ1 promoted the adhesiveness of LP07 cells to fibronectin and inclusion of a TβR inhibitor completely blocked this effect (Fig. 3a). Because the MAPK/ERK signaling pathway has been involved in cell attachment to fibronectin, we tested whether TGFβ1 could activate this pathway. Figure 3b shows that the treatment with TGFβ1 induced ERK phosphorylation, indicating that this non-Smad signaling pathway is also responsive to TGFβ1 in LP07 cells. However, in experiments including the MEK1 inhibitor PD98059, the increase of LP07 cells adhesive properties mediated by TGFβ1 was not reversed (Fig. 3b).

Next, we investigated the effect of TGFβ1 pretreatment on cell spreading on fibronectin coatings and found that it significantly increased the number of completely spread cells at 1 h (Fig. 3c). No differences were found in β1 integrin expression, the major cellular receptor for fibronectin, between control and TGFβ1-treated cells as assessed by western blot (data not shown). Therefore, we explored the effect of TGFβ1 on the activation of focal adhesion kinase (FAK), another signal transduction pathway downstream integrin-dependent cell attachment and spreading. Figure 3d shows that FAK activity was higher in TGFβ1-treated cells compared to control cells and the inclusion of the TβR inhibitor partially blocked this effect at 24 h. These results suggest that the TGFβ1 induced increase of adhesion and spreading on fibronectin is mediated by FAK signaling pathway.

LP07 cells display traits of an EMT program even in the absence of exogenous TGFβ1

It is well-known that TGFβ1 is a potent EMT inducer; then we asked whether an EMT program was taking place on TGFβ1-treated LP07 cells. LP07 cells exhibited phenotypical changes upon TGFβ1 treatment. While control untreated cells exhibited epithelial morphology in culture, TGFβ1-treated cells showed elongated, fibroblast-like morphology at 72 h (Fig. 4a). TRITC-phalloidin staining revealed that LP07 cells already displayed cytoskeletal actin arranged as stress fibers. The mesenchymal marker vimentin was not organized in filaments as suggested by a punctate staining pattern in indirect immunofluorescence. Surprisingly, TGFβ1 treatment did not introduce substantial changes either on the distribution or expression of these molecules (Fig. 4b). LP07 cells expressed the epithelial marker E-cadherin and its pattern of expression was similarly unchanged by TGFβ1 treatment (data not shown). The effect of TGFβ1 treatment on the synthesis of the mesenchymal marker fibronectin was also examined. Immunofluorescence revealed that the fibrillar network of fibronectin was more profuse in TGFβ1 pretreated cells and this effect was blocked in the presence of TβR inhibitor (Fig. 4c).

Then, we assessed whether TGFβ1 was a regulator of LP07 cell migration, a process highly associated with

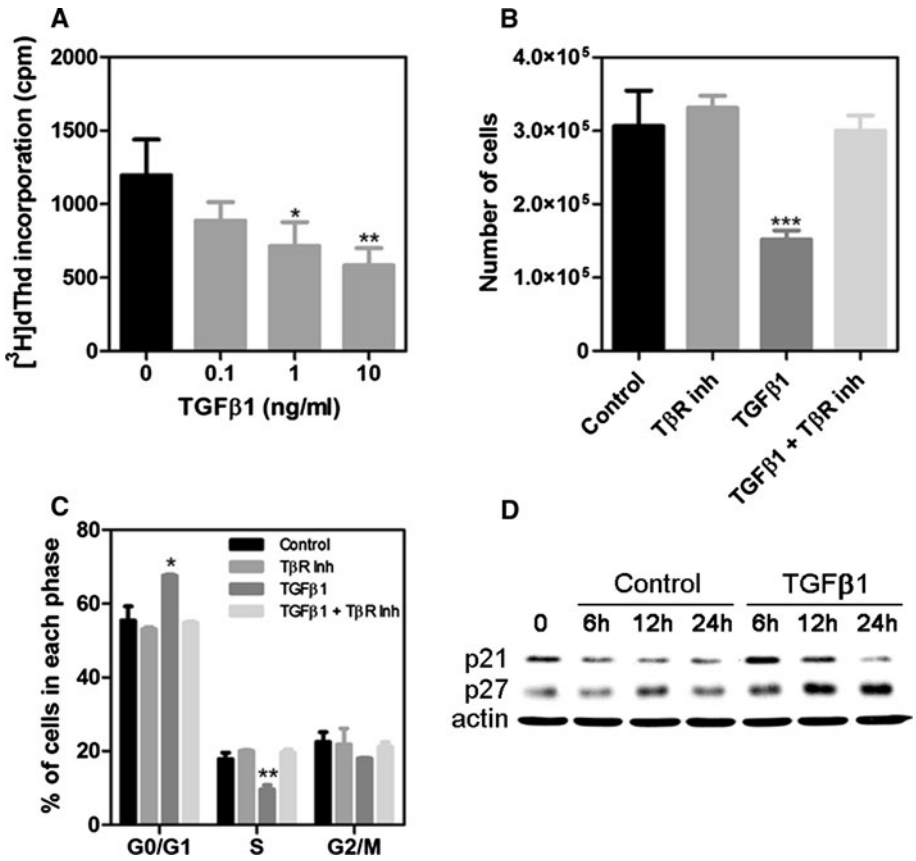

Fig. 2 Effect of TGFβ1 on in vitro proliferation of LP07 cells **a** DNA synthesis in response to 0.1, 1 or 10 ng/ml TGFβ1. Incorporated [^{3}H]-thymidine was measured by liquid scintillation spectrometry at 48 h **b** Proliferation was assessed by cell count 72 h after adding vehicle, 4 ng/ml TGFβ1 or 2 μM TβR inhibitor LY2109761 (TβR inh) alone or in combination **c** Cell cycle distribution was analyzed 48 h following the same treatments as in (**b**). The propidium iodide fluorescence profile was divided into three curves representing cells in G0/G1, S or G2/M. On the basis of surface contribution of each curve, the fraction of cells in the different cell cycle phases has been calculated **d** Effect of TGFβ1 on protein expression of cell cycle-associated components in LP07 cells was analyzed by western blotting. The expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ was detected after 6, 12 and 24 h treatment with vehicle or TGFβ1. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control One-way ANOVA, Dunnett's Multiple Comparison Test

EMT. Using a wound healing assay, we found that cell migration was not modified by TGFβ1 treatment, while TβR inhibitor blocked basal migration in these cells (Fig. 4d).

These results suggest that LP07 cells have partially acquired an EMT program even in the absence of exogenous TGFβ1 treatment, showing some mesenchymal traits while continuing to express epithelial traits. Addition of exogenous TGFβ1 did not shift the balance towards full acquisition of an EMT program.

TGFβ1 favors clonogenic ability at 24 h and protects LP07 cells from apoptosis

The effect of TGFβ1 treatment on long-term cellular proliferation was evaluated with a clonogenic assay. Interestingly, we found that a short TGFβ1 pretreatment increased the percentage of colonies by 2-fold. However, if treated with TGFβ1 continuously, LP07 cells were found to be extremely sensitive to inhibition by TGFβ1 with more than 83 % inhibition of colony formation (Fig. 5a).

To assess TGFβ1 effect on survival, nuclear morphological changes in LP07 cells that were serum-starved for 72 h were examined by fluorescence microscopy after staining with DAPI. Brightly stained chromatin-condensed nuclei and some pyknotic nuclei were apparent in ~20 % of control cells, indicating apoptotic nuclear morphology. As shown in Fig. 5b, TGFβ1 pretreatment efficaciously protected LP07 cells from apoptosis induced by serum starvation. To gain insights into the molecular mechanisms by which the TGFβ1-dependent signaling pathway protected LP07 cells from programmed cell death, the involvement of PI3K- mediated signaling pathway was investigated. Blocking PI3K pathway with LY294002 prevented TGFβ1- mediated protection from apoptosis, indicating that this pathway is involved in TGFβ1 anti-apoptotic action (Fig. 5b). Consistently, Fig. 5c shows that phospho-Akt, which relays signals downstream PI3K, was

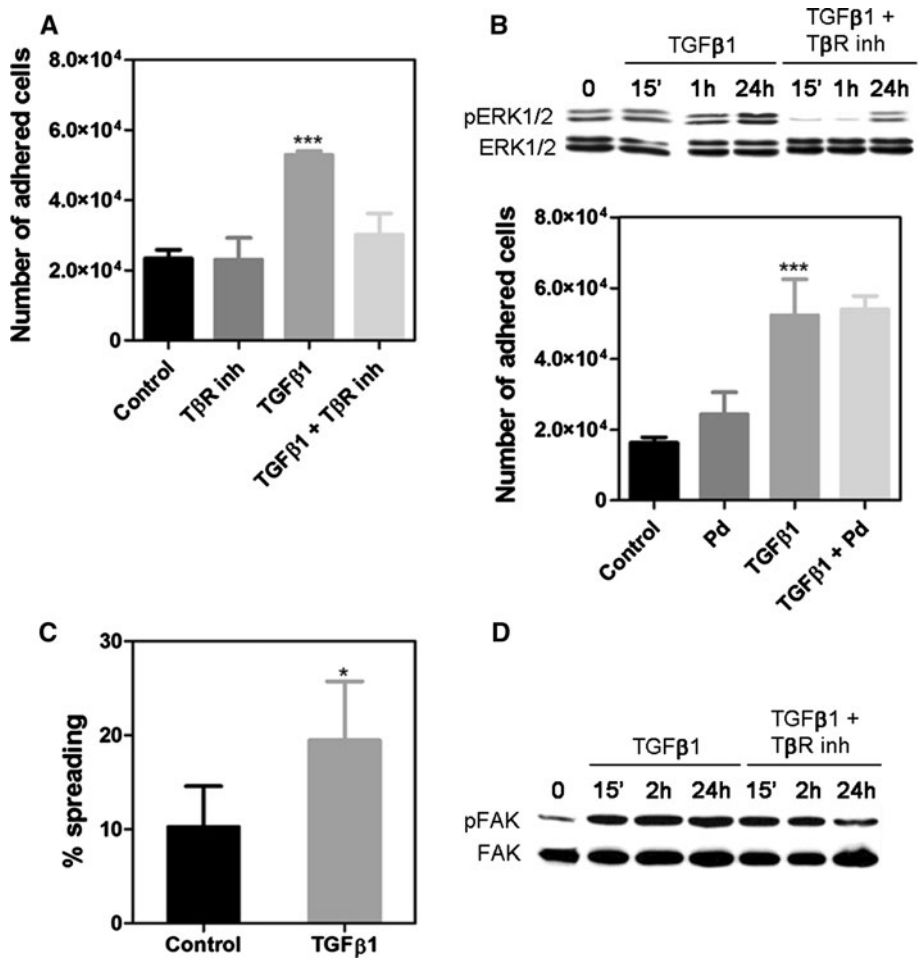

Fig. 3 TGFβ1 effect on adhesion and spreading on fibronectin of LP07 cells **a** Cells were treated with 4 ng/ml TGFβ1 or 2 μM TβR inhibitor LY2109761 (TβR inh) either alone or in combination for 24 h and then allowed to adhere to fibronectin-coated wells. After 15 min adherent cells were tripsinized and counted **b** Upper panel, LP07 cells were treated as in (a) for 15 min, 1 h and 24 h. At the end of incubations, ERK1/2 and phosphorylated ERK1/2 protein expression were analysed by western blot. Bottom panel, adhesion assay was performed as in (**a**) but including the MEK1 inhibitor PD98059 (Pd) to block signal upstream ERK1/2 **c** 24 h vehicle or TGFβ1 treated cells were allowed to adhere and spread on fibronectin-coated wells for 2 h and the percentage of completely spread cells was determined, scoring 400 cells under a phase-contrast microscope (**c**) LP07 cells were treated as in (**a**) for 15 min, 1 h and 24 h. At the end of incubations, FAK and phosphorylated FAK protein expression were analysed by western blot. ***$p < 0.001$ versus control One-way ANOVA, Bonferroni's Multiple Comparison Test, *$p < 0.05$ versus control, Unpaired *t* test

readily induced by TGFβ1 treatment and this activation was reversed by the TβR inhibitor.

NSCLC human cell lines and murine LP07 cells show similar responses to TGFβ1 treatment

To extend our study of TGFβ1 effects on NSCLC, we performed selected experiments on human NSCLC cell lines. We assessed the effect of TGFβ1 on proliferation by MTS assay with A549 and H125 human cells. Inhibition in proliferation was (31.77 ± 1.10) % in A549 cells and (21.75 ± 1.56) % in H125 cells ($p < 0.05$ TGFß1 treated cells vs. control, *t* test). We further tested in these human cell lines the effect of TGFβ1 on adhesion to fibronectin, that was significantly increased by 2.06 ± 0.58 fold in A549 cells and 1.60 ± 0.05 fold in H125 cells ($p < 0.05$ TGFß1 treated cells vs. control, *t* test). Then, we analyzed the effect of TGFβ1 treatment on long-term cellular proliferation with a clonogenic assay in A549 cells and found that a short TGFβ1 pretreatment did not affect the percentage of colonies. However, if treated with TGFβ1 continuously, A549 cells were found to be extremely sensitive to inhibition by TGFβ1 with more than (74.64 ± 4.22) % inhibition of colony formation ($p < 0.05$, one-way ANOVA, Dunnett's Multiple Comparison Test). As we had observed for murine LP07 cells, in human H125 cell line continuous treatment with TGFβ1 significantly diminished clonogenic ability [(36.77 ± 22.50) % inhibition], while a short treatment or pulse of TGFβ1 for 24 h caused a significant increase in the number of colonies (1.96 ± 0.08 fold increase, $p < 0.05$,

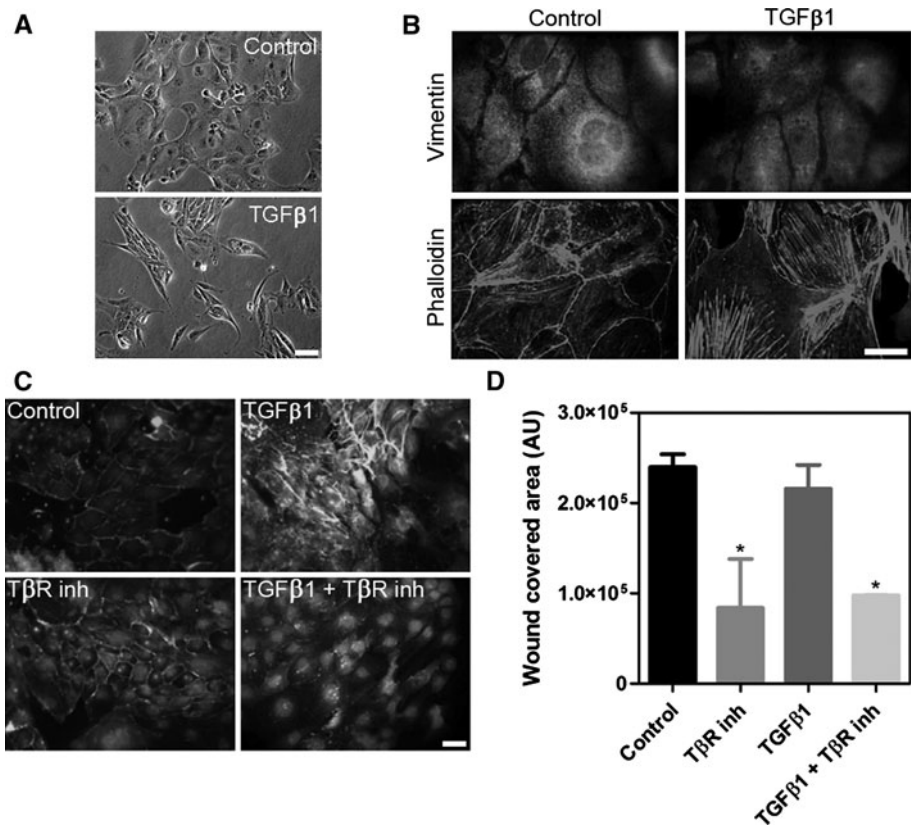

Fig. 4 TGFβ1 modulation of epithelial to mesenchymal transition in LP07 cells **a** Phase contrast microphotographs of control and 4 ng/ml TGFβ1-treated LP07 cells at 72 h. *Bar*, 25 μm **b** LP07 cells were treated as in (**a**) and stained using TRITC-phalloidin to assess actin stress fiber distribution, or indirect immunofluorescence to detect vimentin. *Bar*, 25 μm **c** Cells were treated with 4 ng/ml TGFβ1 or 2 μM TβR inhibitor LY294002 (TβR inh) either alone or in combination for 72 h and the mesenchymal marker fibronectin was detected using indirect immunofluorescence. *Bar*, 25 μm **d** LP07 cells were treated as in (**c**) for 6 h and their migration capacity was assessed by wound healing assay. *AU* arbitrary units. $^{*}p < 0.05$ versus control One-way ANOVA, Tukey's Multiple Comparison Test

one-way ANOVA, Dunnett's Multiple Comparison Test). Therefore, the responses to TGFβ1 of murine LP07 and human A549 and H125 cell lines, regarding proliferation, adhesion to fibronectin and clonogenic ability were similar to a large extent.

TGFβ1 enhances LP07 cell retention and metastatic outgrowth in the lung while inhibiting subcutaneous tumor growth

When LP07 cells are introduced via the tail vein, metastases are apparent in the lungs after 3 weeks [24]. Fluorescence labeled-TGFβ1 treated cells or control cells were injected into the tail vein of mice to test whether TGFβ1 could endow tumor cells with an improved ability to seed the lungs. Interestingly, this short pretreatment with TGFβ1 significantly increased the retention of tumor cells in the lungs 24 h after inoculation (Fig. 6a). Then, we tested if the initial advantage provided by a transient exposure to TGFβ1 was sustained during the subsequent outgrowth of metastatic colonies by means of an experimental metastasis assay. Pretreatment of LP07 cells for 24 h with TGFβ1 increased the number of lung metastases 1.8- to 4-fold, 3 weeks after inoculation in syngeneic mice (Fig. 6b and 6c). Combining TGFβ1 pretreatment with either the PI3K inhibitor LY294002 (Fig. 6b) or the MEK1 inhibitor PD98059 (Fig. 6c) prevented this effect, indicating that these non-Smad signaling pathways are involved in TGFβ1-mediated metastasis stimulation.

Then, we subcutaneously injected LP07 control- or TGFβ1- treated cells to determine the effects of TGFβ1 on murine lung ADC tumor growth. After tumors became palpable, we measured tumor size throughout a 33-day period. As shown in Fig. 6d, TGFβ1 pretreatment of LP07 cells decreased primary tumor growth when compared to the control group. Upon subcutaneous or tail vein injection LP07 cells developed into poorly differentiated adenocarcinomas comprised of masses of polyhedric cells surrounded by spindle cells and scarce structures with glandular aspect. No morphological changes were observed in primary tumors (Fig. 6d) or metastasis (data not shown) derived from control- or TGFβ1- treated cells, as assessed by H&E stain.

TβRI expression in NSCLC tumor specimens is associated with poor survival

Table 1 resumes the main clinical pathological characteristics of the patients under study. Immunohistochemichal studies showed that TβRI staining was predominantly cytoplasmic, although membrane staining was also observed in some cases. We found that 20/73 (27.4 %) tumor specimens showed positive TβRI cytoplasmic staining in more than 10 % of the tumor cells, and this

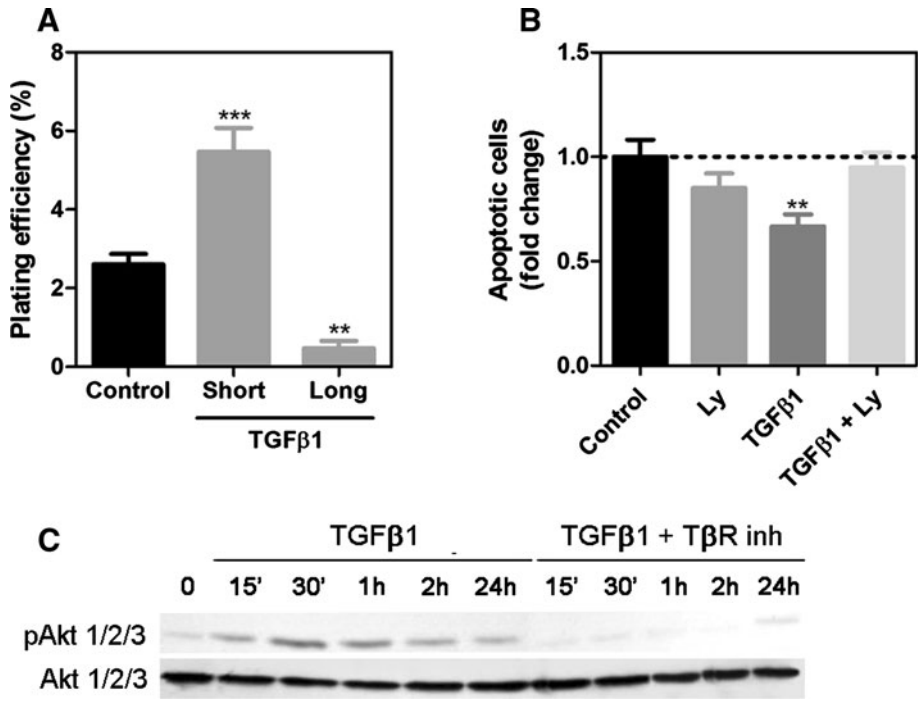

Fig. 5 TGFβ1 modulation of clonogenic ability and survival in LP07 cells **a** LP07 cells were treated with 4 ng/ml TGFβ1 for a short (24 h) or long (continuous) period and their plating efficiency was assessed as described in "Materials and methods" **b** Cells were treated with 4 ng/ml TGFβ1 or the PI3K inhibitor LY294002 (Ly, 10 μM) either alone or in combination and then serum- starved for 72 h to induce apoptosis. Nuclei were stained with DAPI and examined for nuclear condensation/fragmentation indicative of apoptosis **c** Cells were treated with 4 ng/ml TGFβ1 or 2 μM TβR inhibitor LY2109761 (TβR inh) either alone or in combination for 15 min, 30 min, 1 h, 2 h and 24 h. At the end of incubations, Akt1/2/3 and phosphorylated Akt1/2/3 protein expression were analyzed by western blot. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control. One-way ANOVA, Dunnett's Multiple Comparison Test

percentage was set as the cut-off point to dichotomize patients for survival analysis. The overall survival was shorter in patients with high TβRI cytoplasmic expression compared with patients with low expression, a difference that did not reach statistical significance (Fig. 7a). Then, we carried out a more exhaustive analysis by stratifying patients based on their clinical pathologic characteristics. When the analysis was made among stage I patients with ADC or SCC, high TβRI cytoplasmic expression was associated with decreased 5-year survival (Fig. 7b), but not with other clinicopathologic parameters (Table 2). When we evaluated the independence of clinical pathologic parameters on global survival by multivariate Cox test we found that stage remains an independent prognostic factor when adding the remaining variables one by one (data not shown). However, a multivariate Cox test including TβRI cytoplasmic expression revealed that this variable does not constitute a factor of prognostic significance.

Discussion

Lung cancer is a world health problem, not only due to its high incidence rate, but also to the lack of therapeutic response in advanced patients [25]. In addition, it is difficult to stratify lung cancer patients based on histopathology due to its heterogeneous nature. More recently, the outcome and survival of lung cancer patients has improved with the advent of novel molecular targeted agents. Nevertheless, a better understanding of the molecular biology of the group of diseases covered by lung cancer is needed to help improve prognostic tools and treatment approaches.

It has been suggested that TGFβ pathway has a tumor suppressive role that needs to be circumvented to allow tumorigenesis [26]. However, in different tumor settings, TGFβ plays a dual role, acting as a suppressor early in tumor progression, while later shifting to a promoter of tumor cell invasion, dissemination, and immune evasion [27].

Thus, TGFβ directly and differentially regulates distinct cellular programs, such as proliferation, apoptosis, and the differentiation/de-differentiation equilibrium [28], all critical factors in tumor progression. Such diversity stems from the ability of TGFβ to concomitantly activate different signaling cascades, termed canonical (Smad-dependent) and non-canonical (Smad-independent) TGFβ signaling pathways, and regulate the expression of a broad repertoire of genes [3, 6]. The complexity of this scenario dictates that the dissection of the influence of TGFβ in a

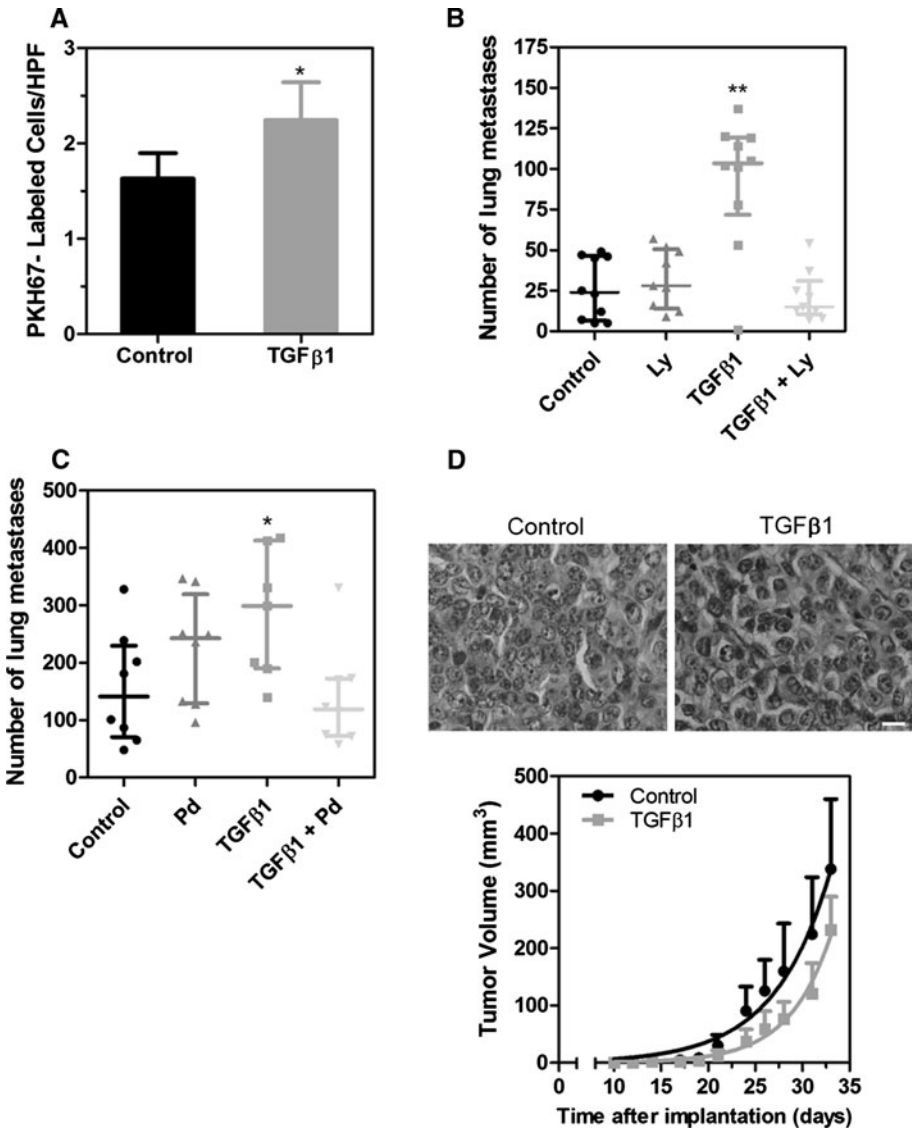

Fig. 6 TGFβ1 modulation of in vivo behavior of LP07 cells **a** LP07 cells were treated with 4 ng/ml TGFβ1 for 24 h, fluorescent-labeled and introduced via tail vein in syngeneic mice. Frozen sections of the lungs excised 24 h later were examined for fluorescent cell count. $*p < 0.05$ versus control, Mann–Whitney test **b** Cells were treated with 4 ng/ml TGFβ1 or the PI3K inhibitor LY294002 (Ly, 10 μM) either alone or in combination and introduced via tail vein in singeneic mice. Lungs were excised 3 weeks later and were examined for metastatic lesions count. Data are expressed as median with interquartile range. $*p < 0.05$ versus control One-way ANOVA, Dunnett's Multiple Comparison Test **c** The same experiment as in (**c**) was performed, but including the MEK1 inhibitor PD98059 (Pd, 50 μM). Data are expressed as median with interquartile range $**p < 0.01$ and $***p < 0.001$ versus control. One-way ANOVA, Dunnett's Multiple Comparison Test **d** LP07 cells were treated with 4 ng/ml TGFβ1 for 24 h, inoculated subcutaneously in the right flank of mice and tumor growth was assessed during 33 days. Primary tumors from LP07 control or treated cells showed no morphological differences, as shown in H&E images, *Bar:* 25 μM. Curves are statistically different as assessed by two-way ANOVA and exponential growth curve fits comparison. Bonferroni post-test of two-way ANOVA was statistically significant from day 28 of the study, with p value <0.05 on day 28 and $p < 0.01$ on days 31 and 33

particular cellular setting, such as lung ADC, requires the quantitative assessment of its influence on multiple parameters. It is in the context of such requirement that the present study was devised and performed.

Employing the murine lung ADC LP07 cell line, a model that resembles human tumors, we show that TGFβ1 activated Smad2 and Smad3 and the non-Smad pathways of MAPK, FAK and PI3K. Moreover, TGFβ signaling translated into a blockade of cell cycle progression at G1 phase, with the upregulation of the G1/S transition regulatory proteins, $p27^{Kip1}$ and $p21^{Waf1/Cip1}$, resulting in the suppression of proliferation of unsynchronized LP07 cells. Therefore, as found in other models, TGFβ seems to exert its cytostatic effect by targeting primarily the G1 phase [29]. However, TGFβ1- treated LP07 cells were more resistant to cell death induced by serum starvation than

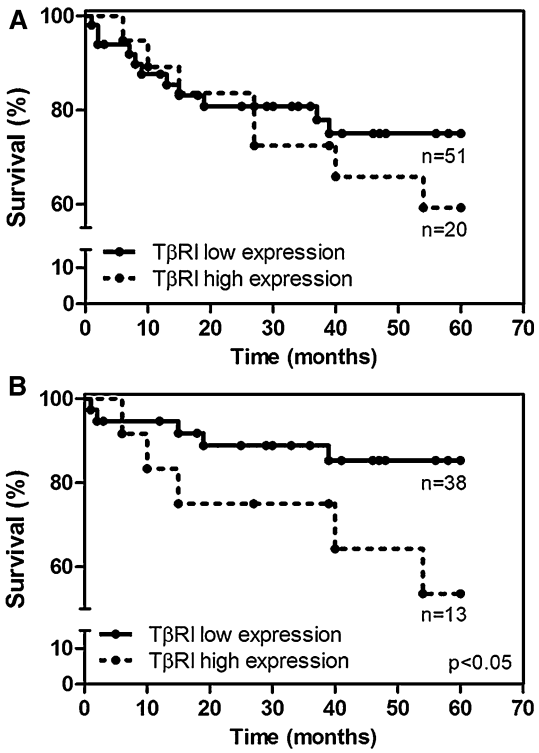

Fig. 7 Kaplan-Meier survival curves for cytoplasmic TβRI in non-small cell lung cancer patients **a** 5-year survival was shorter in patients with high TβRI cytoplasmic expression compared with patients with low expression, although it did not reach statistical significance **b** High TβRI cytoplasmic expression was associated with decreased 5-year survival among stage I patients with ADC or SCC. *$p < 0.05$ log rank test

control cells, an effect that depended on a functional PI3K/Akt pathway. Similarly, in A549 human lung carcinoma cells it was reported that TGFβ1 blocked apoptosis resulting from serum withdrawal [30]. Thus, given that tumor growth is determined by the resulting balance between cell proliferation and apoptosis, we propose that the presence or absence of additional intrinsic or extrinsic stimuli promoting these processes at different stages of lung ADC, contribute to the differential effects of TGFβ to this malignancy.

Cell adhesion and migration correlate with metastatic potential and TGFβ was proposed to modulate these parameters [31, 32]. Here too, TGFβ1 increased the adhesiveness and spreading of LP07 cells on fibronectin, and mediated the activation of FAK, suggesting the involvement of integrins and focal adhesions in these effects. Interestingly, the activation of FAK by TGFβ was only partially suppressed by an inhibitor of TβR kinase (at late but not early timepoints of activation), suggesting a ligand-dependent kinase-independent mechanism of induction of FAK activity by TGFβ. Such mechanism was recently proposed to mediate the activation of the non-canonical TAK1 arm of TGFβ signaling in mesangial cells [33]. Interestingly, Horowitz et al. [34] demonstrated TGFβ-mediated FAK activation, which was dependent on Smad-3, in normal primary human fetal lung fibroblasts. In addition to the substantiation of the notion of the sensitivity of LP07 to exogenously added TGFβ1, observed through the ligand-mediated activation of Smads, FAK, PI3K; our data support a scenario of autocrine TGFβ signaling with potential implications in the regulation of cell-matix interactions. Thus, TGFβ is readily detected in the medium of LP07 cells, phosphorylated Smads were detected prior to exogenous stimulation with TGFβ1, and the basal activation of Erk1/2 presented by unstimulated LP07 cells was sharply reduced by a TβR kinase inhibitor. Interestingly, an analogous scenario was observed when assessing cell migration, a process in which MAPKs play a well documented role [35] where TGFβ1 addition failed to augment migration, which was blocked by the TβR inhibitor. In accord with their intrinsic pronounced migratory abilities, unstimulated LP07 cells exhibited mesenchymal traits, such as an actin stress fiber network and vimentin and fibronectin expression. However, these cells also express E-cadherin, a marker and determinant of the epithelial phenotype. Such convoluted scenario is suggestive of an incomplete phenotypic transition, such as EMT. In accord with this complexity, TGFβ stimulation increased the fibrillar network of the mesenchymal protein fibronectin and the expression of transcriptional regulators such as Zeb1 and Sp1 (our unpublished data), while failing to alter the levels of expression of E-cadherin. We speculate that within an in vivo tumor scenario, such partiality of effects may be overcome by prolonged exposure to TGFβ, or by crosstalk between the signaling induced by TGFβ and by other ligands present in the tumor microenvironment. An elaborate role for TGFβ in EMT induction and tumor progression is exemplified by the variability in results obtained with different experimental systems. For instance, in a primary cell line obtained from tissue sample after surgery from a NSCLC patient, TGFβ induced EMT and promoted xenograft tumor growth [36]; contrastingly, Brown & Moses who studied normal and cancer cell lines found that in vitro TGFβ1 induced-EMT is an infrequent phenomenon [37].

TGFβ1 presented a dual effect on influencing the potential for clonogenic growth of LP07 cells, an important indicator of cell malignancy. While continuous treatment with TGFβ1 blocked LP07 clonogenic ability, a short treatment or pulse of TGFβ1 for 24 h caused a significant increase in the number of colonies. This may be related to our observations of increased adhesive properties and pro-survival effect of TGFβ1 on LP07 cells. Moreover, we observed that LP07 cells exposed in vitro to TGFβ1 pulse prior to tail vein injection had increased metastatic ability. Furthermore, we showed that TGFβ1-mediated metastasis

Table 1 Clinical pathological data of patients with NSCLC ($n = 73$)

	Number of cases (%)[a]
Sex	
Male	50 (68.5)
Female	23 (31.5)
Stage	
Ia	22 (30.1)
Ib	36 (49.3)
II	14 (19.1)
T	
T1	27 (37.0)
T2	43 (58.9)
T3	2 (2.7)
Node status	
Positive	12 (16.4)
Negative	60 (82.2)
Tumor histopathology	
Adenocarcinoma	40 (54.8)
Squamous cell carcinoma	24 (32.9)
Other	7 (9.6)

[a] where columns do not sum to the total, data were missing or unknown

Table 2 Associations between the TβRI cytoplasmic expression and the main relevant parameters in lung cancer

	TβRI cytoplasmic expression[a]		Pearson χ^2
	Low	High	p
Sex			
Male	35 (66 %)	15 (75 %)	0.462
Female	18 (34 %)	5 (25 %)	
Stage			
I	42 (80.8 %)	16 (80.0 %)	0.941
II	10 (19.2 %)	4 (20.0 %)	
T			
T1	19 (36.5 %)	8 (40 %)	0.725
T2	32 (61.5 %)	11 (55 %)	
Node status			
Positive	43 (82.7 %)	17 (85 %)	0.814
Negative	9 (17.3 %)	3 (15 %)	
Tumor histopathology			
Adenocarcinoma	29 (56.9 %)	11 (55 %)	0.725
Squamous cell carcinoma	18 (35.3 %)	6 (30 %)	
Age			
≤59	15 (30 %)	6 (30 %)	0.678
60–69	23 (46 %)	11 (55 %)	
≥70	12 (24 %)	3 (15 %)	

[a] where columns do not sum to the total, data were missing or unknown

stimulation was dependent on MEK1 and PI3K/Akt signaling pathways. Enhancement of experimental metastasis may be correlated to our observation that TGFβ1- treated LP07 cells have increased retention in the metastatic target organ. In addition to the described effects on tumor cells, TGFβ1 treatment of LP07 cells may promote tumor progression as a result of paracrine effects on the host tumor microenvironment [38]. In our model, immune suppression through galectin-1 may be involved in TGFβ- treated cells enhanced metastasis [39]. Consistent with our findings, several reports have stressed that many TGFβ responses are dependent on time exposure. For instance the short-term stimulation with TGFβ stimulates metastasis formation, while persistent stimulation decreases the metastatic spread to the lungs [40–42]. Paradoxically, we also found that the TGFβ1 pulse decreased the growth of primary tumors growing subcutaneously in mice indicating that TGFβ response is context-dependent, as it has been suggested by others [43]. In this regard, different microenvironments may determine the interplay of TGFβ activated pathways and consequently, the tumor cells fate. It is broadly accepted that during carcinogenesis, the Smad-dependent pathway correlates with the anti-proliferative or tumor suppressor functions of TGFβ, and that the Smad-independent pathways are involved in TGFβ pro-malignant functions [44]. In our model, TGFβ growth inhibitory responses may be selectively replaced by metastatic responses that signal towards the Smad-independent pathways of MEK and PI3K in the metastatic scenario. Furthermore, TGFβ signaling may be non-uniformly active in tumors. For example, live tumor imaging of TGFβ signaling has revealed that fast moving single cells showed high levels of TGFβ signaling while cells moving collectively did not display TGFβ signaling. TGFβ signaling enabled invasion into blood vessels but was downregulated at distant sites to permit growth of secondary tumors [40]. Consistent with our finding, Itoh et al. [45] have reported that activated TGFβ signaling increases the latency of Neu-induced mammary tumor formation but also enhances the incidence of lung metastasis. Also, we have found that the responses to TGFβ of murine LP07 and human A549 and H125 cell lines were similar to a large extent regarding proliferation, adhesion to fibronectin and clonogenic ability.

While TGFβ1 [14] and TβRII [46] have been widely studied in NSCLC tumor specimens, only a few works have focused on TβRI. Colasante et al. [47] showed that immunoreactive TβRI protein expression was significantly higher in tumors than in non-lesional tissues. A similar observation was reported by Zhao et al. [48] who in addition found no association with clinical pathologic parameters. Takanami et al. [49] studied the association between TβRI expression and overall survival in lung ADC and found no significant association by univariate and

multivariate analysis. In our population of NSCLC patients, TβRI high expression in tumor specimens was associated with worse prognosis by a univariate study among stage I patients with ADC or SCC, suggesting a pro tumorigenic role of the TGFβ/TβR system and the potential value of TβRI expression as a prognostic factor in lung cancer. However, the Cox multivariate analysis revealed that other covariates were contributing to survival. To further determine whether TβRI expression can be considered a biomarker of value in the prognosis of NSCLC patients, extended analysis of patient's tumor samples is needed.

Although widely studied in other cancer types, the role of TGFβ in lung cancer is not well-known. We have demonstrated that even though lung cancer cell monolayers responded to TGFβ1 anti-mitogenic effects and TGFβ1 pulse delayed tumor growth at primary site, a switch towards malignant progression upon TGFβ1 treatment was observed at the metastatic site. In our model, TGFβ1 modulated in vitro clonogenicity, protected against stress-induced apoptosis and increased adhesion, spreading, lung retention and metastatic outgrowth. In addition, we found that a higher expression of TβRI in human lung tumors is associated with poor patient's survival. Although further detailed analysis of the endogenous signaling in vivo and in vitro is needed to achieve a better comprehension as to how TGFβ shapes a biological cell response, these studies may provide novel molecular targets for treatment of lung cancer.

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