# Targeting Transforming Growth Factor (TGF)-βRI Inhibits Activation of β1 Integrin and Blocks Vascular Invasion in Hepatocellular Carcinoma

Emilia Fransvea,[1]* Antonio Mazzocca,[1,2]* Salvatore Antonaci,[1] and Gianluigi Giannelli[1]

**Vascular invasion is one of the major negative prognostic factors in patients with hepatocellular carcinoma (HCC), leading to cancer recurrence. To invade, HCC cells must penetrate the vessel wall, consisting of endothelial cells and extracellular matrix components, including fibronectin and fibrinogen. Employing invasive and noninvasive HCC cells, we studied the mechanism underlying vascular invasion. We show that HCC cells invade blood vessels via α5β1, that is equally expressed in invasive and noninvasive cells. However, in the former, the intracytoplasmic tail of β1 integrin is constitutively phosphorylated at threonine 788-789 and the extracellular part is conformationally activated. In noninvasive cells, β1 integrin is not activated. Transforming growth factor (TGF)-β1 specifically phosphorylates β1 integrin (threonine 788-789) via Smad-2 and Smad-3, causing a conformational change of the extracellular component with an inside-out mechanism. This leads noninvasive HCC cells to behave like invasive cells. A selective TGF-βRI inhibitor inhibits phosphorylation of the β1 integrin intracytoplasmic tail, and blocks invasion of HCC cells, both constitutively invasive and with acquired invasive properties. In human HCC tissues with microvascular invasion, phospho-β1 integrin was detected as well as TGF-β1, p-Smad-2, and E-cadherin. *Conclusion:* TGF-β1 promotes vascular invasion by activating β1 integrin. This suggests a rationale for targeting TGF-βRI in future clinical trials.** (HEPATOLOGY 2009;49:839-850.)

Hepatocellular carcinoma (HCC) poses a major health problem worldwide because of its constantly increasing incidence in developed countries and its poor prognosis.[1,2] The high mortality rate of HCC is attributable to late diagnosis, underlying liver disorders, and cancer recurrence. Only a minority (30% to 40%) of patients is diagnosed early and is eligible for the most effective treatment, including tumor resection, radiofrequency ablation, and, in limited cases, liver transplantation.[3] Nevertheless, even in these cases the prognosis and survival are not satisfactory because the recurrence rate is higher than 70% at 5 years after resection, and the currently available therapies have failed to reduce this rate.[4,5] In cases of HCC recurrence, therapeutic approaches are limited, due to multifocal and intrahepatic spread of the tumor in these advanced and more aggressive cancer forms with an unfavorable prognosis.[6-8] Literature reports show that the most reliable predictor of HCC recurrence is macroscopic or microscopic vascular invasion, which is also an adverse prognostic factor.[9-11] Furthermore, because an extensive histological examination is required to detect microscopic invasion, this problem is underestimated in patients undergoing radiofrequency ablation therapy.

The molecular mechanisms underlying vascular invasion are unknown. Fibronectin (Fn), a major component of the blood vessel wall, is ubiquitously distributed. HCC cells need to engage with Fn to cross the wall, and with fibrinogen (Fg) to migrate into the vascular lumen.[12,13] This migration is mediated by a class of heterodimeric transmembrane receptors named integrins, formed by an

*Abbreviations: Fg, fibrinogen; Fn, fibronectin; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; siRNA, small interfering RNA; TGF, transforming growth factor.*

*From the [1]Department of Internal Medicine, Immunology and Infectious Diseases, Section of Internal Medicine; University of Bari Medical School, Bari, Italy; and the [2]Department of Pathology, Vanderbilt University Medical Center, Nashville, TN.*

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**These authors contributed equally to this work.*

*Address reprint requests to: Gianluigi Giannelli, Dipartimento di Clinica Medica, Immunologia e Malattie Infettive, Sezione di Medicina Interna, Policlinico, piazza G. Cesare 11, 70124 Bari, Italy. E-mail: g.giannelli@intmed.uniba.it; fax: (39)-080-5478-126.*



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α- and β-subunit linked by noncovalent bounds. Integrin receptors αvβ5, αvβ3, α5β1, and αvβ1 have been reported to bind to Fg and Fn, respectively, on epithelial cells.[14-17] Transforming growth factor (TGF)-β1, highly expressed in the biological fluids of HCC patients, is a pleiotropic growth factor that correlates with a worse prognosis and shorter survival.[18-20] We have previously shown that TGF-β1 induces a more aggressive phenotype of HCC cells by decreasing the expression levels of E-cadherin, a cell–cell adhesion molecule that prevents cancer cells from leaving the primary tumor to colonize distant sites.[21,22] In HCC, TGF-β1 triggers migration and invasion of surrounding tissues, up-regulating the expression of α6β1 and α3β1 integrin receptors of different laminin isoforms.[23,24] Whether or not TGF-β1 promotes cancer cell invasion of the blood vessels is still unknown, but statistically significantly higher levels of TGF-β1 have been measured in the serum of patients with gastric cancer and venous invasion as compared with those without blood vessel involvement.[25] Nevertheless, no study has yet reported the role of TGF-β1 in promoting the spread of different malignancies, including HCC, through blood vessels. The aim of this study was to investigate the molecular mechanisms regulating vessel wall invasion and hence intravascular dissemination of HCC cells.

## Materials and Methods

***Cell Culture.*** Human HCC cells, HLE and HLF, previously characterized as invasive, and Alexander and Hep3B cells, characterized as noninvasive, were cultured as reported.[26] To generate HCC cells lines ubiquitously expressing green fluorescent protein (GFP), cultured cells were infected by retroviral vector pLXSN-GFP (BD Clontech) and isolated by neomycin selection without clonal propagation.

***Reagents.*** Fn and Fg were purchased from Calbiochem (La Jolla, CA). Anti-integrin blocking subunit antibodies were obtained as follows: mouse monoclonal P1D6 (anti-α5), mouse monoclonal P4C10 (anti-β1), and PIF6 (anti-αvβ5) were purchased from Chemicon (Temecula, CA); mouse monoclonal 17E6 (anti-αv) was purchased from Abcam (Cambridge, MA); mouse monoclonal LM609 (anti-αvβ3) was purchased from Immunological Sciences (Rome, Italy) and from Chemicon; β1 integrin activating mouse monoclonal antibody TS2/16 was purchased from Immunological Sciences (Rome, Italy); mouse monoclonal antibody 12G10 (anti-β1) integrin was obtained from Abcam; mouse monoclonal 7E3 (anti-β3) was kindly provided by B. Coller (The Rockefeller University, NY); and mouse monoclonal HUHTS-21 against active β1 integrin was purchased from BD Bioscences (San Jose, CA). Recombinant human TGF-β1 was purchased from Calbiochem. LY2109761, a TGF-βRI selective kinase inhibitor, was kindly provided by Eli Lilly (Indianapolis, IN).[27] Antibodies directly against total and phospho–Smad-2 and -3 were purchased from Cell Signaling Technology Inc. (Danvers, MA). α5-Integrin small interfering RNAs (siRNAs) and transfection reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

***Migration Assay.*** Cell migration was performed as described.[26] In the case of LM609, experiments were repeated at least eight times. Blocking antibodies were used at a final concentration of 80 μg/mL in serum-free conditions. TS2/16 was used at a concentration of 40 μg/mL. Cells were cultured with TGF-β1 at a concentration of 3 ng/mL. LY2109761 was used at a final concentration of 0.1 μM.

***α5-Integrin Gene Silencing in HCC Cell Lines.*** At 24 hours after plating, cells were transfected with 10 nM of a pool of three target-specific 20-25 nt siRNAs designed to knock down α5-integrin gene expression or nonsilencing control (control siRNA) in siRNA transfection reagent. After 72 hours from the transfection, cells were harvested, counted, and allowed to migrate toward Fg or Fn as described above. The α5-integrin protein levels were determined in the total cell lysate via western blot analysis using a goat polyclonal antibody directed against the C-terminus of human α5 integrin (Santa Cruz Biotechnology, Inc.).

***HCC Patients and Tissue Preparations.*** We studied 24 HCC patients, 18 with and six without histological evidence of microvascular invasion. Age ranged from 65 to 75 years, and 19 patients were males. In 20 patients the etiology was viral (19 hepatitis C and one hepatitis B), and in four patients it was alcoholic. All the patients had a single nodule (mean size, 5.0 ± 3.5 cm). All the patients had a poorly differentiated histological grade, and six of 18 peritumoral tissues showed the presence of histologically metastatic nodules.

Tumoral and paired peritumoral samples were obtained from surgical patients. Tissues were cut in two pieces; one was processed for routine histology, and the other was immediately snap-frozen in liquid nitrogen.

***Western Blot Analysis.*** Cells or tissues were lysed directly in cell extraction buffer (Biosource International Inc., CA) supplemented with a protease and phosphate inhibitor cocktail (Sigma, Milan, Italy). Protein concentrations were measured using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). TGF-β1 at a 3 ng/mL concentration was added from 30 minutes up to 2 hours to Alexander and Hep3B cells before analysis.

LY2109761 at a 0.1 $\mu$M concentration was added to HLF and HLE cells 48 hours before the analysis and to Alexander and Hep3B cells 1 hour before addition of TGF-$\beta$1. To map the phosphorylation site of $\beta$1 integrin, the following primary antibodies were used: rabbit polyclonal antibody against phospho-tyrosine 795 (Chemicon) and rabbit polyclonal antibody against phospho-serine 785 (Abcam). Two different polyclonal antibodies were used against phospho-threonine 788 and 789 (Abcam and Biosource International, Inc). CAM tissue samples were homogenized, and proteins were extracted using the same extraction buffer described above. After protein concentration measurement, samples were immunoblotted with antibodies against $\beta$1 integrin phosphorylated at T788-789. An anti–$\beta$-actin monoclonal antibody purchased from Sigma (St. Louis, MO) was used as an internal loading control.

***Flow Cytometry.*** Cultured cells were detached with accutase purchased from PAA (Linz, Austria). Nonpermeabilized cells were then incubated with monoclonal antibody against integrins, as already reported, stained with propidium iodide, and analyzed on a Beckman Coulter FACScan flow cytometer (Fulleron, CA).[26] TS2/16 at a concentration of 40 $\mu$g/mL was incubated for 30 minutes before analysis to observe conformational activation of $\beta$1 integrin. TGF-$\beta$1 at a concentration of 3 ng/mL was added from 30 minutes up to 2 hours to Alexander and Hep3B cells before analysis. LY2109761 was used at a 0.1 $\mu$M concentration. A purified mouse immunoglobulin was used as an isotype control.

**In Vivo *Imaging of Chick Embryo CAM.*** For imaging experiments, eggs were prepared according to the shell-less (ex ovo) chick embryo assay procedure. Briefly, fertilized white leghorn chicken eggs (10 per group) were placed in a 38°C humidified incubator for the duration of their development. Prior to incubation and manipulation, the eggs were cleaned and sterilized with 70% ethanol. On day 4 of incubation, the eggs were cracked into plastic 100 $\times$ 100 mm Petri dishes and returned to the incubator. On day 9, GFP-expressing HLE and Alexander cells were placed directly on the CAM, where a primary tumor formed over a period of 7 days. For intravenous delivery of antibodies, injections (50 $\mu$g) were performed the day after tumor cell application using a glass capillary needle. For some experiments, blood vessels in the CAM were visualized via intravenous injection of rhodamine isothiocyanate-lens culinaris agglutinin (rhodamine-LCA; Vector Laboratories, Inc. Burlingame, CA). To achieve real-time imaging of tumor cells invading the tissue stroma and the blood vessels, a chick embryo imaging unit was employed. This unit enabled maintenance of the chick embryo at a proper temperature and humidity during imaging. Invasion of tumor cells within the adjacent stroma and the blood vessels was monitored with a modified epi-illumination Zeiss microscope (Zeiss, Oberkochen, Germany) equipped with a Hamamatsu CCD camera (ORCA-AG; Hamamatsu Photonics, Japan). A series of images of the tumor within the CAM were captured at predetermined time points. Digital images were acquired using AxioVision imaging software (Zeiss, Jena, Germany) and further processed using Photoshop (Adobe, San Jose, CA).

***Pharmacologic Inhibition of the TGF-$\beta$1 Receptor.*** HLE and Alexander cells were seeded on day 0 in 100 $\times$ 100 mm Petri dishes and pretreated in culture with the TGF-$\beta$RI/II kinase inhibitor LY2109761 (0.1 $\mu$M) or dimethyl sulfoxide (DMSO) vehicle on days 1 and 2. On day 1, cell treatment with LY2109761 was performed in RPMI supplemented with 10% FBS; on day 2, cells were incubated with LY2109761 in RPMI without serum. On day 3, cells were harvested, resuspended in PBS, and implanted into the CAM of 9-day-old chick embryos. The developing tumors were treated with 20 mg/kg of LY2109761 every second day for 7 days and imaged as described above. In some experiments, Alexander tumors were stimulated with a single injection of 200 ng hTGF-$\beta$1.

***E-cadherin and TGF-$\beta$1 Measurement.*** E-cadherin and TGF-$\beta$1 were determined via commercial enzyme-linked immunosorbent assay kits purchased from R&D Systems (Minneapolis, MN).

## Results

HCC vascular invasion was investigated in both an *in vitro* and *in vivo* model. For this purpose, we used four different human HCC cell lines previously characterized as invasive, namely HLE and HLF, and noninvasive, namely Alexander and Hep3B.[26] As shown in Supplementary Fig. 1, Hep3B and Alexander cells migrate poorly on Fn and Fg whereas both HLE and HLF cells display a strong migratory phenotype on these substrates ($P < 0.0001$). No migration was observed on blotto used as negative control. To explain this difference, we examined the expression levels of the main integrin receptors for Fn and Fg by fluorescence-activated cell sorting. As reported in Fig. 1A, the nonmigratory Hep3B and Alexander cells express lower levels of $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 1$ compared to the migratory HLE and HLF cells, but all the cell lines express comparable amount of $\alpha 5\beta 1$. To test the functional involvement of these integrins, HCC cell lines were challenged to migrate on Fn and Fg in the presence of functionally blocking antibodies against the integrin subunits $\alpha 5$, $\beta 1$, $\alpha v$, $\beta 3$ and against the complete heterodimers $\alpha v\beta 5$ and $\alpha v\beta 3$ (Fig. 1B). HLE and HLF

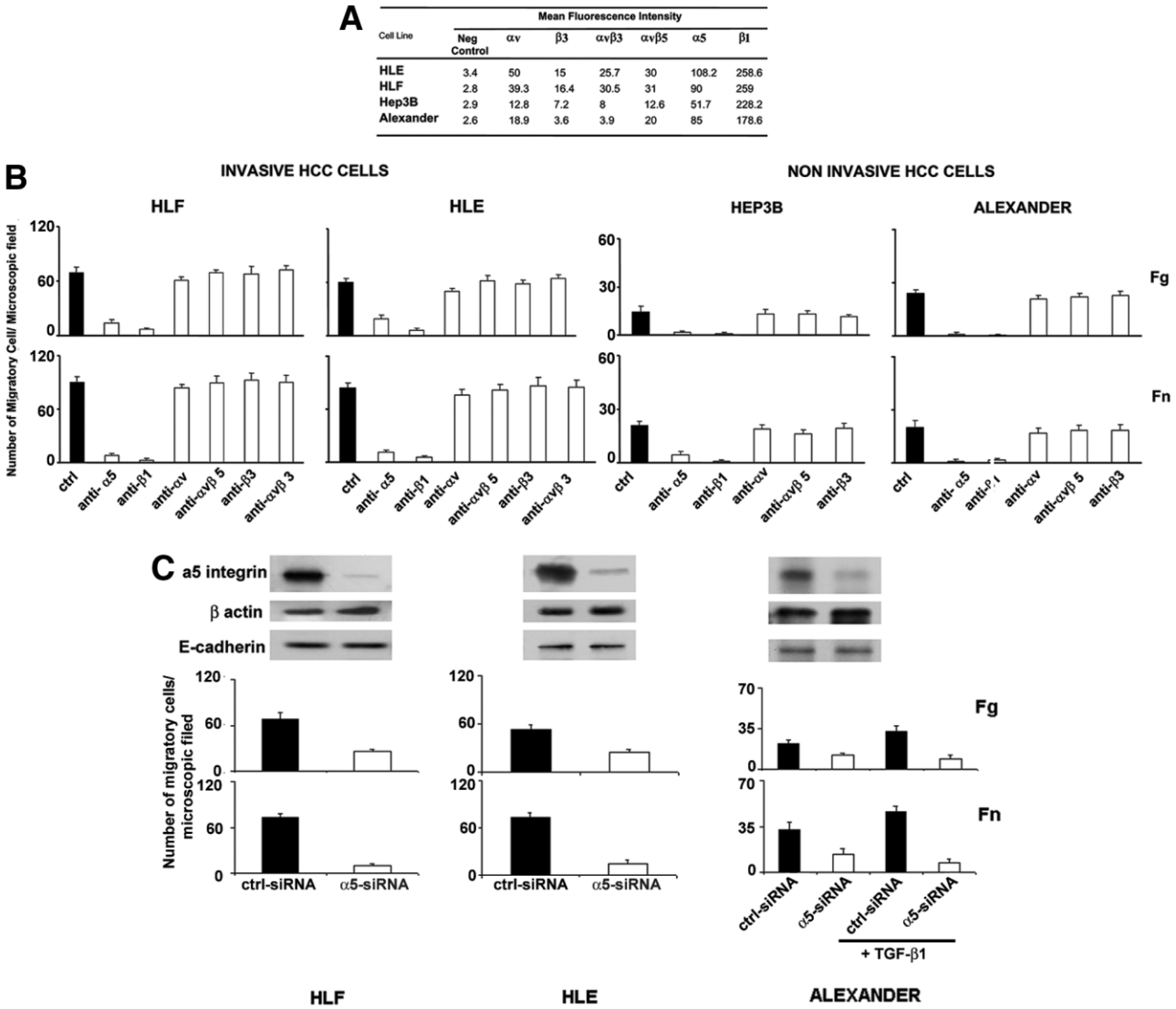


Fig. 1. HCC cell migration on Fn and Fg. (A) Expression level of cell surface integrins measured via fluorescence-activated cell sorting. Invasive HLE and HLF cells express higher levels of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins than noninvasive Hep3B and Alexander cells, but similar levels of $\alpha 5\beta 1$. (B) Blocking antibodies against $\alpha 5$ or $\beta 1$ but not against $\alpha v$, $\beta 3$, $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins inhibit cell migration of invasive and noninvasive cells on Fg and Fn. All conditions were performed in duplicate, and each experiment was repeated at least three times. Results of representative experiments are shown, and values are expressed as the mean $\pm$ standard deviation. (C) Protein expression levels of $\alpha 5$-integrin and E-cadherin in HLF, HLE, and Alexander cell lines following gene silencing of $\alpha 5$-integrin with small interfering RNAs (siRNAs). $\beta$-Actin was used as a loading control. In the bottom part of the panel, the effect of $\alpha 5$-integrin gene silencing on HLF, HLE, and Alexander cell migration is reported. Seventy-two hours after transfection with siRNAs or nonsilencing control (ctrl-siRNA), cells were harvested, counted, and allowed to migrate on Fg- or Fn-coated transwell filters for 16 hours. Results are expressed as the mean number $\pm$ standard deviation of migrated cells obtained from three independent experiments.

cell migration on Fn was completely blocked by anti-$\alpha 5$ and anti-$\beta 1$ antibodies, whereas no effect was observed in the presence of the antibodies against $\alpha v$, $\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 3$. Unexpectedly, similar results were obtained when migration was performed on Fg. In the case of Hep3B and Alexander cells, migration on Fn and Fg was inhibited by anti-$\alpha 5$ and -$\beta 1$ antibodies (Fig. 1B). These results were repeated several times using three different lots of anti-$\alpha v\beta 3$ antibody obtained from two distinct companies. To further prove the role of $\alpha 5\beta 1$ in cell migration across Fg and Fn, the invasive HLE and HLF cells as well as the noninvasive Alexander cells were knocked down for $\alpha 5$ integrin as shown by western blotting, Fig. 1C. No differences were observed with regard to the expression of E-cadherin in control and $\alpha 5$-silenced cells (Fig. 1C). In the migration assay, HCC $\alpha 5$-siRNA cells did not migrate on either Fg or Fg. Collectively, these data show that migration on Fn and Fg is mediated by $\alpha 5\beta 1$ integrin that is similarly expressed on the cell surface of invasive and noninvasive HCC cells.

Based on the above results, we hypothesized that the different ability to migrate on Fn and Fg does not depend

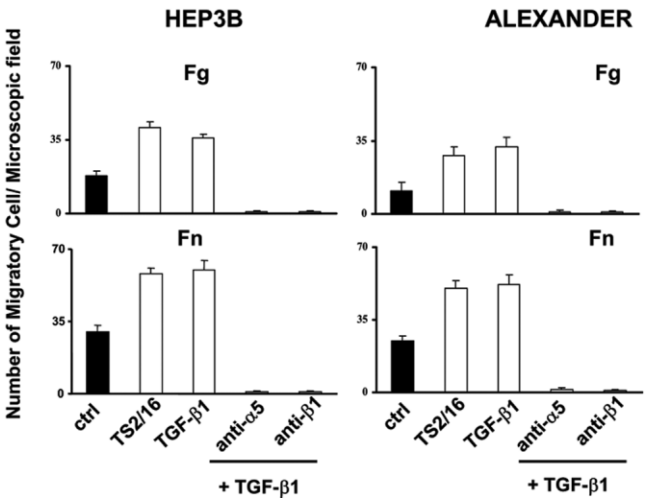


Fig. 2. TGF-$\beta$1 and TS2/16 stimulate migration of HCC noninvasive cells. The activating antibody TS2/16 against $\beta$1 integrin or TGF-$\beta$1 stimulates migration of Hep3B and Alexander cells on Fg and Fn. Blocking antibodies against $\alpha$5 and $\beta$1 inhibit TGF-$\beta$1–stimulated migration. All conditions were performed in duplicate, and each experiment was repeated at least three times. Results of representative experiments are shown, and values are expressed as the mean $\pm$ standard deviation.

on the quantitative expression of $\alpha5\beta1$ but on the different activation status of this integrin. To test this hypothesis, Hep3B and Alexander cells were challenged to migrate on Fn and Fg in the presence of TS2/16, a functional antibody that activates the $\beta$1 integrin subunit by means of a conformational change of the extracellular domain.[28] As expected, nonmigratory HCC cell lines acquired a strong migratory activity on Fn and Fg, and a stronger effect was obtained when Hep3B and Alexander were stimulated with TGF-$\beta$1 (Fig. 2). Intriguingly, TGF-$\beta$1–induced migration was completely inhibited by anti-$\alpha$5 and anti-$\beta$1 functional antibodies. These results suggest that TGF-$\beta$1 activates the integrin subunit $\beta$1 that drives the migration of HCC cells through Fn and Fg.

To gain a better insight into the underlying molecular mechanisms, we investigated the phosphorylation status of the intracytoplasmic tail of $\beta$1 integrin under the same experimental conditions used for migration assays. $\beta$1 integrin, which is minimally phosphorylated in basal conditions, becomes activated upon TGF-$\beta$1 stimulation in Hep3B and Alexander cells. This effect was completely abolished in the presence of LY2109761, a selective TGF-$\beta$RI kinase inhibitor.[27] Conversely, $\beta$1 integrin was found to be constitutively activated in HLE and HLF migratory cells and becomes dephosphorylated following treatment with LY2109761. Using different antibodies, we mapped the phosphorylation site at threonine residues 788 and 789, whereas no differences were observed at serine 785 and tyrosine 795. To investigate the signaling involved downstream to TGF-$\beta$R, we analyzed the phosphorylation status of Smad-2 and -3 in the same experimental conditions via western blotting. As shown, there is a correlation between the activation of $\beta$1 integrin and the phosphorylation of Smad-2 and -3 proteins (Fig. 3A).

To further study the mechanism implicated in $\beta$1 integrin phosphorylation-dependent migration, we performed fluorescence-activated cell sorting to investigate the possible involvement of extracellular conformational changes, using a monoclonal antibody that specifically recognizes the active form of $\beta$1 integrin. Consistently with the western blot findings, $\beta$1 integrin was found to be constitutively activated in HLE and HLF cells but not in Hep3B and Alexander cells (Fig. 3B). However, we found that TGF-$\beta$1 activates $\beta$1 integrin in Hep3B and Alexander cells, whereas LY2109761 blocks this effect and deactivates $\beta$1 integrin in HLE and HLF. These data show that TGF-$\beta$1 activates $\beta$1 integrin through phosphorylation of the intracellular tail at the threonine residues 788 and 789, and this leads to a conformational change of the integrin extracellular domain according to an inside-out mechanism.

To test the reliability of these findings *in vivo*, we developed an imaging system based upon the direct visualization of the chick chorioallantoic membrane (CAM) in an *ex ovo* chick embryo model. As shown in Supplementary Fig. 2A, all the HCC cell lines were able to form tumors 7 days after implantation into the CAM. However, only HLE and HLF tumors were able to invade the surrounding stroma, as demonstrated by the irregular border at the interface with the stroma and the detachment of cells from the primary tumor (Supplementary Fig. 2B, arrowheads).

To evaluate the role of $\alpha5\beta1$ integrin regulating HCC vascular invasion *in vivo*, we labeled HLE cells with GFP, using a retroviral vector, and then implanted them into the CAM. After 24 hours postimplantation, the animals were treated with a control antibody or blocking antibodies directed against anti-$\alpha$5 or anti-$\beta$1 integrin, and the tumors were imaged 5 days later.

HLE tumors treated with the control antibody showed a marked invasion of the surrounding stroma and related blood vessels (Fig. 4A-C, see also Supplementary Movie 1). In contrast, HLE tumors treated with the anti-$\alpha$5 or anti-$\beta$1 integrin antibodies showed a regular border at the tumor–stromal interface (Fig. 4D,G). No GFP-positive cells were present inside the blood vessels, as demonstrated at low and high magnification (Fig. 4E,F and Fig. 4H,I, respectively). Consistently with our previous data, an anti-$\alpha$v blocking antibody did not inhibit HLE invasion of the local stroma (Fig. 4J) nor vascular invasion (Fig. 4K,L), indicating the specific contribution of $\alpha5\beta1$

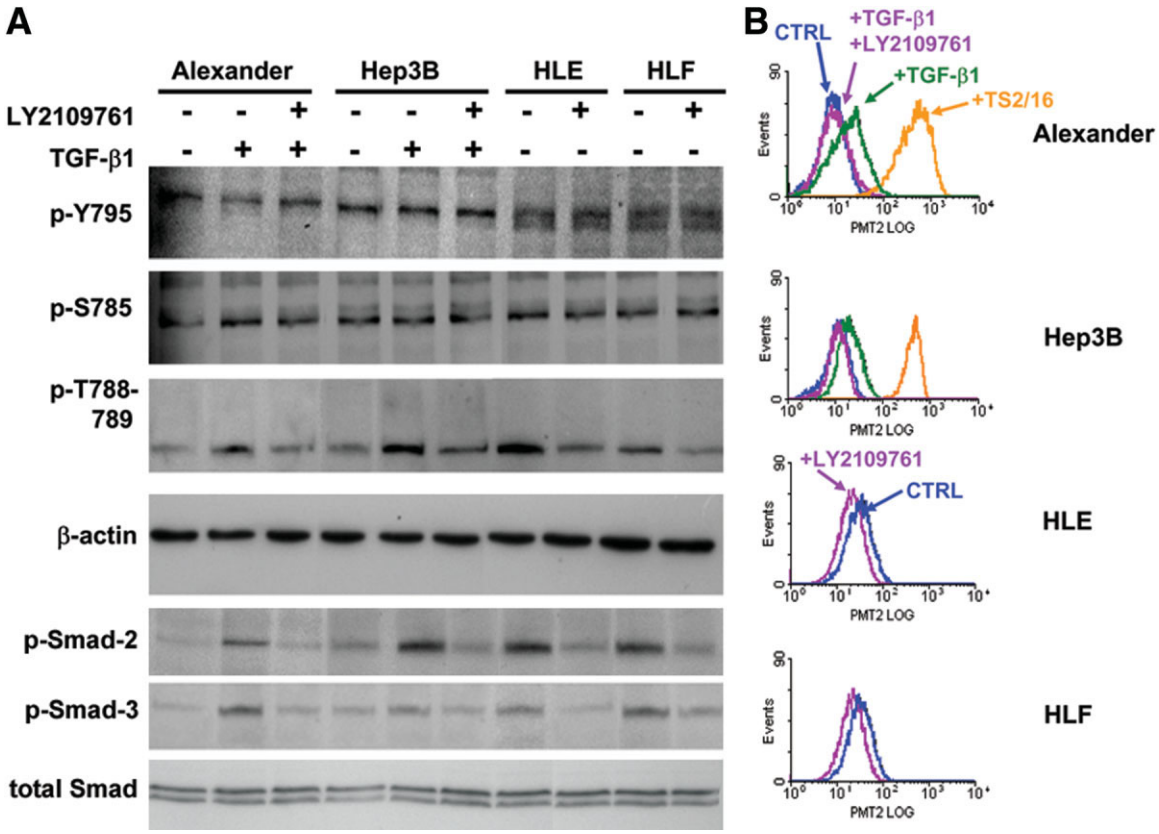


Fig. 3. TGF-$\beta$1 activates $\beta$1 integrin. (A) Western blot analysis of HCC cell lysates probed with anti-phospho–$\beta$1 integrin at threonine 788-789, serine 785, tyrosine 795, phospho–Smad-2, phospho–Smad-3, and total Smad. (B) FACSscan analysis using the HUHTS-21 antibody that recognizes the active form of the extracellular region of the $\beta$1 integrin. TGF-$\beta$1 treatment (green) activates $\beta$1 integrin, while LY2109761 (violet) deactivates it. TS2/16 (orange) was used as positive control.

to this process. Experiments were run in 10 animals for each condition and repeated at least three times. Data on the vascular invasion by HLE tumors were quantified and are expressed as a bar graph in Fig. 4M. Similar results were also obtained with HLF (data not shown).

Alexander tumors, instead, proved incapable of invading the surrounding stroma and blood vessels when implanted into the CAM (Fig. 5A, B, and C). However, activation of $\beta$1 integrin by TS2/16 induced invasion of the surrounding stroma and blood vessels (Fig. 5D-F). Furthermore, TGF-$\beta$1 treatment induced a stronger invasive ability (Fig. 5G-I), whereas blocking antibodies against $\alpha$5 and $\beta$1 completely blocked TGF-$\beta$1–induced invasion (Fig. 5J-L and Fig. M-O, respectively). The quantification of these results is reported in Fig. 5P. Similar results were obtained with Hep3B (data not shown).

To further analyze whether $\alpha5\beta1$ integrin affects the ability of HCC cells to actively invade the vascular capillary bed, both GFP-expressing HLE and Alexander cells were preincubated with anti-$\alpha$5 or anti-$\beta$1 integrin blocking antibody and inoculated subcutaneously into the CAM. The blood vessels were labeled with rhodamine-LCA 3 days after the injection, and the cells were imaged. As shown in Supplementary Fig. 3, HLE and HLF cells treated with anti-$\alpha$5 and anti-$\beta$1 antibodies failed to invade the capillary bed of the CAM (Supplementary Fig. 3c-f) as compared with those treated with a control antibody (Supplementary Fig. 3a,b).

The presence of tumor cells inside the vessels is shown as a merging of the GFP signal with the rhodamine-labeled vasculature (Supplementary Figs. 3 and 4, arrowheads). Consistently with previous data, Alexander cells were incapable of invading the CAM capillary vessels (Supplementary Fig. 4a). However, these cells acquired invasive ability after stimulation with TGF-$\beta$1 and $\beta$1 integrin-activating antibody TS2/16 (Supplementary Fig. 4b,f). More importantly, TGF-$\beta$1–stimulated invasion of the capillary bed was completely inhibited by treatment with anti-$\alpha$5 or anti-$\beta$1 blocking antibodies (Supplementary Fig. 4c,d), whereas no effect was observed using an anti-$\alpha$v integrin blocking antibody (Supplementary Fig. 4e). Similar results were obtained with Hep3B (data not shown). Taken together, these results show that HCC vascular invasion is dependent on $\alpha5\beta1$ activation.

Finally, we investigated whether, *in vivo*, TGF-$\beta$1 plays a role in promoting HCC vascular invasion. GFP-

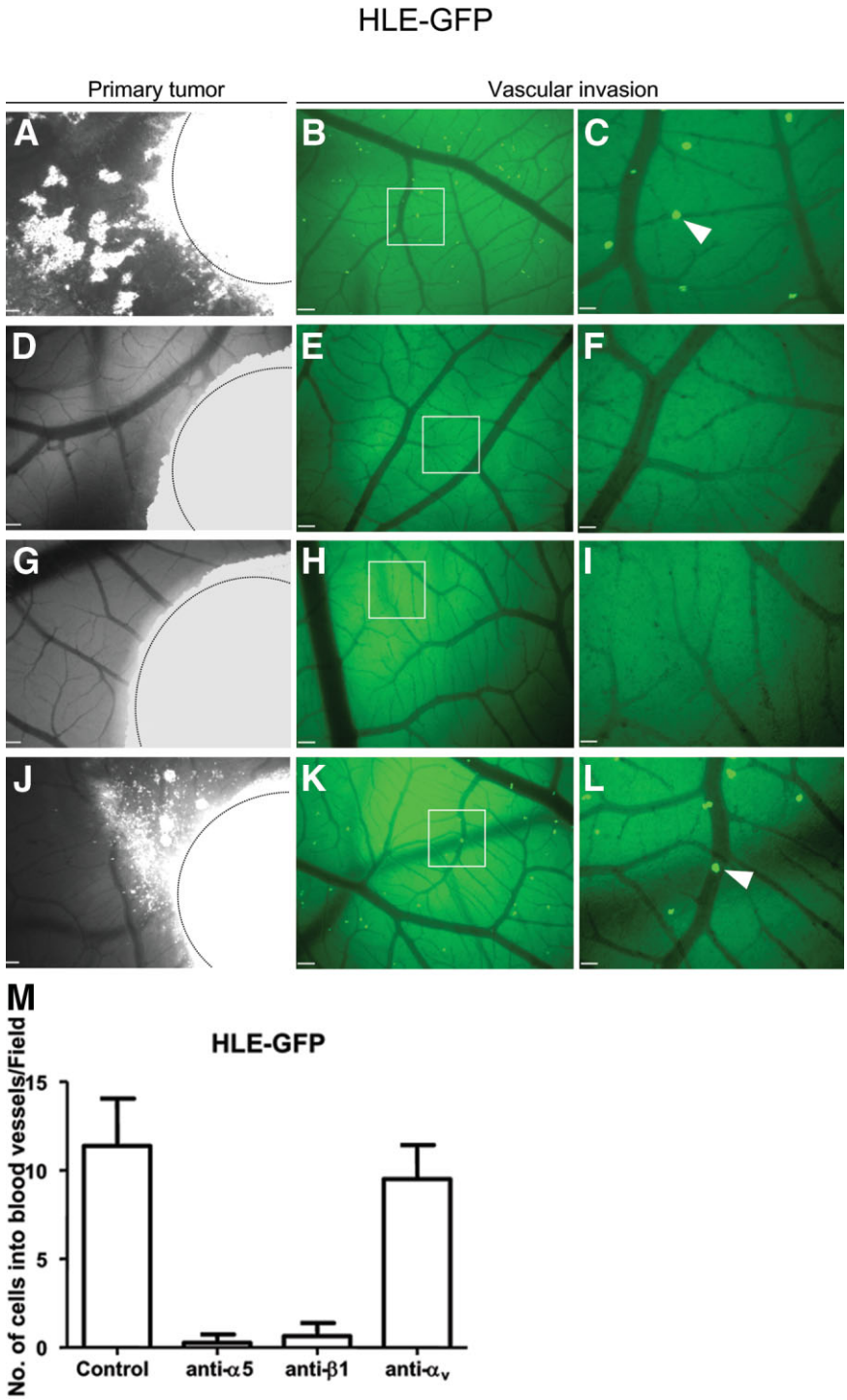


Fig. 4. HLE tumors invade stroma and blood vessels via $\alpha5\beta1$ integrin. (A-C) GFP-labeled HLE cells form tumors in the CAM, invading stroma and blood vessels. (D-F) Anti-$\alpha5$ or (G-I) anti-$\beta1$ integrin blocking antibodies inhibit HLE invasion, while (J-L) anti-$\alpha v$ integrin blocking antibody had no effect. Dotted lines indicate the tumor edge; arrowheads indicate tumor cells inside blood vessels. The boxed regions in the middle panels are magnified $\times100$. Scale bars: left panels, 400 $\mu$m (magnification $\times40$); middle panels, 200 $\mu$m (magnification $\times40$); right panels, 100 $\mu$m (magnification $\times100$). (M) Quantification of the experiment was performed by numbering GFP-expressing HLE cells present in the CAM blood vessels/field (magnification $\times100$). Values are expressed as the mean $\pm$ standard deviation.

expressing HLE and Alexander cells were pretreated with 0.1 $\mu$M of LY2109761 for 48 hours in culture and then implanted into the CAM. The developing tumors were treated with 20 $\mu$M of LY2109761 every second day for 7 days, and the ability to invade the blood vessels was then analyzed. As shown in Fig. 6, HLE tumors treated with the vehicle (DMSO) maintained their ability to invade the surrounding stroma and blood vessels (Fig. 6A-C). Conversely, HLE tumors treated with LY2109761 showed a decreased invasiveness of the embryonic chicken vasculature (Fig. 6D-F). Data relative to vascular invasion were quantified and are expressed as a bar graph in Fig. 6G. To demonstrate the role of TGF-$\beta1$ in phosphorylating $\beta1$ integrin and in promoting HLE invasion of blood vessels, proteins were extracted from the CAM and processed via western blotting. As shown in Fig. 6H, in HLE-invasive cells $\beta1$ integrin was constitutively strongly phosphor-

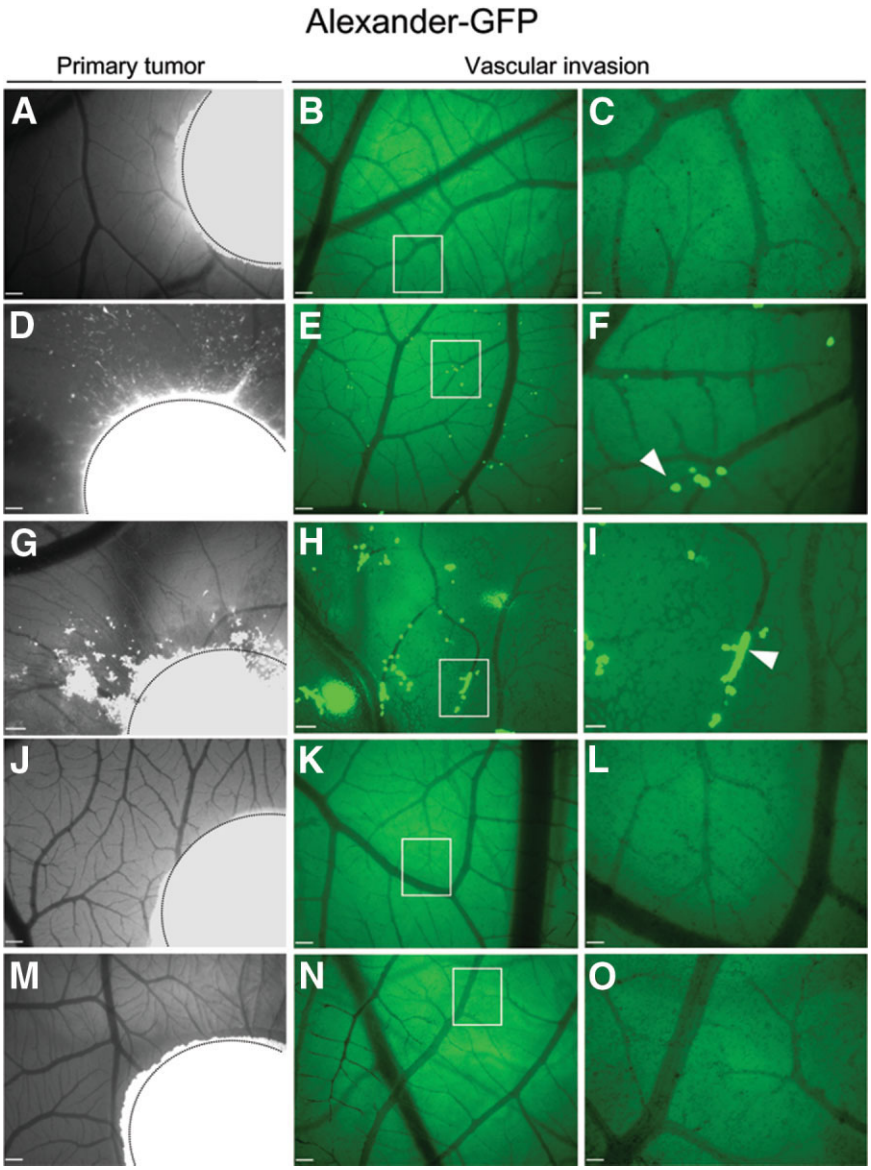


Fig. 5. Alexander tumors stimulated by TGF-$\beta$1 invade stroma and blood vessels via $\alpha$5$\beta$1 integrin. (A-C) Noninvasive GFP-expressing Alexander tumors do not invade the adjacent stroma and blood vessels into the CAM. (D-F) Activation of $\beta$1 integrin followed by TS2/16 incubation promotes Alexander tumor invasion. (G-I) Alexander tumor cells acquire a strong invasive activity followed TGF-$\beta$1 stimulation. This invasion is inhibited by blocking antibodies against $\alpha$5 (J-L) or $\beta$1 integrin (M-O). Dotted lines indicate the tumor edge; arrowheads indicate tumor cells inside blood vessels. The boxed regions in the middle panels are magnified $\times$100. Scale bars: left panels, 400 $\mu$m (magnification $\times$40); middle panels, 200 $\mu$m (magnification $\times$40); right panels, 100 $\mu$m (magnification $\times$100). (P) Quantification of the experiment was perfprmed by numbering GFP–expressing Alexander cells present in the CAM blood vessels/field (magnification $\div$100). Values are expressed as the mean $\pm$ standard deviation.

ylated at T788-789, whereas in LY2109761-treated cells $\beta$1 integrin phosphorylation was inhibited.

Under the same experimental conditions, LY2109761 also inhibited the TGF-$\beta$1–acquired ability of Alexander tumor cells to invade both blood vessels and surrounding stroma (Fig. 7). The quantification of vascular invasion is expressed as a bar graph in Fig. 7J. No toxicity was observed in the developing embryo during and after treatment with LY2109761. The phosphorylation status of $\beta$1 integrin at T788-789 correlates with the modulation of Alexander invasiveness after treatment with TGF-$\beta$1 and LY2109761. In CAM tissue samples, $\beta$1 integrin is phosphorylated by TGF-$\beta$1 and inhibited by LY2109761 (Fig. 7K). These results demonstrate that TGF-$\beta$1 plays a critical role in promoting vascular invasion of HCC phosphorylating $\beta$1 integrin.

Finally, we investigated the expression of $\beta$1 integrin phosphorylated at T788-789 in 18 patients with vascular invasion. Proteins were extracted from tumoral and paired peritumoral specimens. As shown in Fig. 8, phospho-$\beta$1 integrin was detected in all the HCC specimens, albeit with different expression levels, while it was present in peritumoral tissues only when metastatic nodules were histologically observed. Membranes were probed with a monoclonal antibody against $\beta$1 integrin to ensure its presence in all the samples. On the contrary, no phosphorylation of $\beta$1 integrin was observed in the six patients without vascular invasion in either tumoral or peritumoral tissue. In addition, in the same tissue samples showing activation of $\beta$1 integrin, Smad-2 was also phosphorylated. No differences in TGF-$\beta$1 were observed in terms of tissue concentrations between tumoral

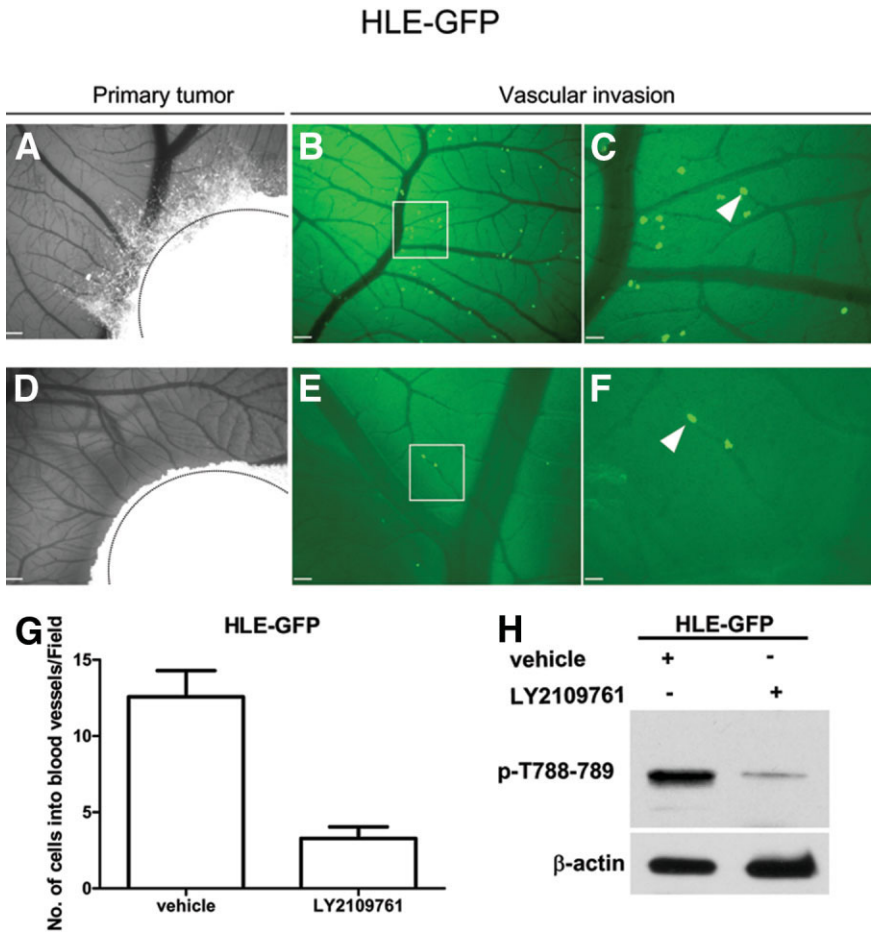


Fig. 6. LY2109761 inhibits HLE tumors invading stroma and blood vessels. GFP-expressing HLE cells were pretreated with vehicle (DMSO) or 0.1 $\mu$M of LY2109761 for 48 hours in culture and then implanted into the CAM. The developing tumors were treated with (A-C) vehicle alone or (D-F) 20 $\mu$M of LY2109761 every second day for 7 days, and then imaged. Each image represents 10 animals imaged in three separate experiments. Dotted lines indicate the tumor edge; arrowheads indicate tumor cells inside blood vessels. The boxed regions in the middle panels are magnified $\times$100. Scale bars: left panels, 400 $\mu$m (magnification $\times$40); middle panels, 200 $\mu$m (magnification $\times$40); right panels, 100 $\mu$m (magnification $\times$100). (G) Vascular invasion was quantified by numbering GFP-expressing HLE cells present in the CAM blood vessels/field (magnification $\times$100). Values are expressed as the mean $\pm$ standard deviation. (H) Proteins extracted from the CAM tissue samples were processed via western blotting, and membranes were probed with antibodies against $\beta$1 integrin phosphorylated at T788-789.

and peritumoral tissues, whereas E-cadherin was more strongly expressed in peritumoral than tumoral tissues. These results demonstrate that $\beta$1 integrin is phosphorylated in HCC patients with vascular invasion, and that there is a correlation with the activation pathway of TGF-$\beta$1.

## Discussion

In patients eligible for surgical resection and therefore with a potentially better prognosis, the occurrence of vascular invasion leads to dissemination of the tumor with a consequently multifocal clinical outcome and a worse prognosis.[10] A clear understanding of the mechanisms that underlie vascular invasion in these patients is of paramount importance to identify new biological targets for therapeutic strategies.

TGF-$\beta$1 plays an intriguing role in cancer, as it has to do with both tumor suppression and tumor promotion. In HCC, it has been reported to be increased in tissues as well as in different biological fluids, supporting its involvement in tumor progression and clinical outcome.[18,19] However, liver cancer cells can escape from the inhibition of TGF-$\beta$ cell proliferation, while still remaining sensitive to its tumor progression effect.[29,30] In this regard, TGF-$\beta$1 is known to trigger the epithelial–mesechymal transition, up-regulating the snail and slug transcription factor in physiological and pathological conditions.[31-33] In HCC human specimens, snail messenger RNA levels are inversely correlated with E-cadherin expression and with tumor invasiveness.[34,35] Together with laminin-5, TGF-$\beta$1 has been shown to promote epithelial–mesechymal transition in a multistep cascade, with a consequent down-regulation of E-cadherin, leading to a more aggressive and invasive phenotype.[21,22] In this study, TGF-$\beta$1 was equally expressed in tumoral and peritumoral tissues, as expected from other studies, from which it was released as a consequence of the proteolytic tissue remodeling occurring at the cancer front edge.[24,36,37] In fact, in the same tissues, Smad-2/3 is phosphorylated and E-cadherin is down-regulated in tumoral as compared with paired peritumoral tissues, allowing cancer cells to move, as suggested in several other studies.[22,38,39] This event commonly occurs as an early step in the cancer invasion cascade in several malignancies, including breast, colon, and stomach, correlating with the onset of metastasis, representing a therapeutic target. These data correspond to the positive late-response to TGF-$\beta$1 recently reported to be directly related

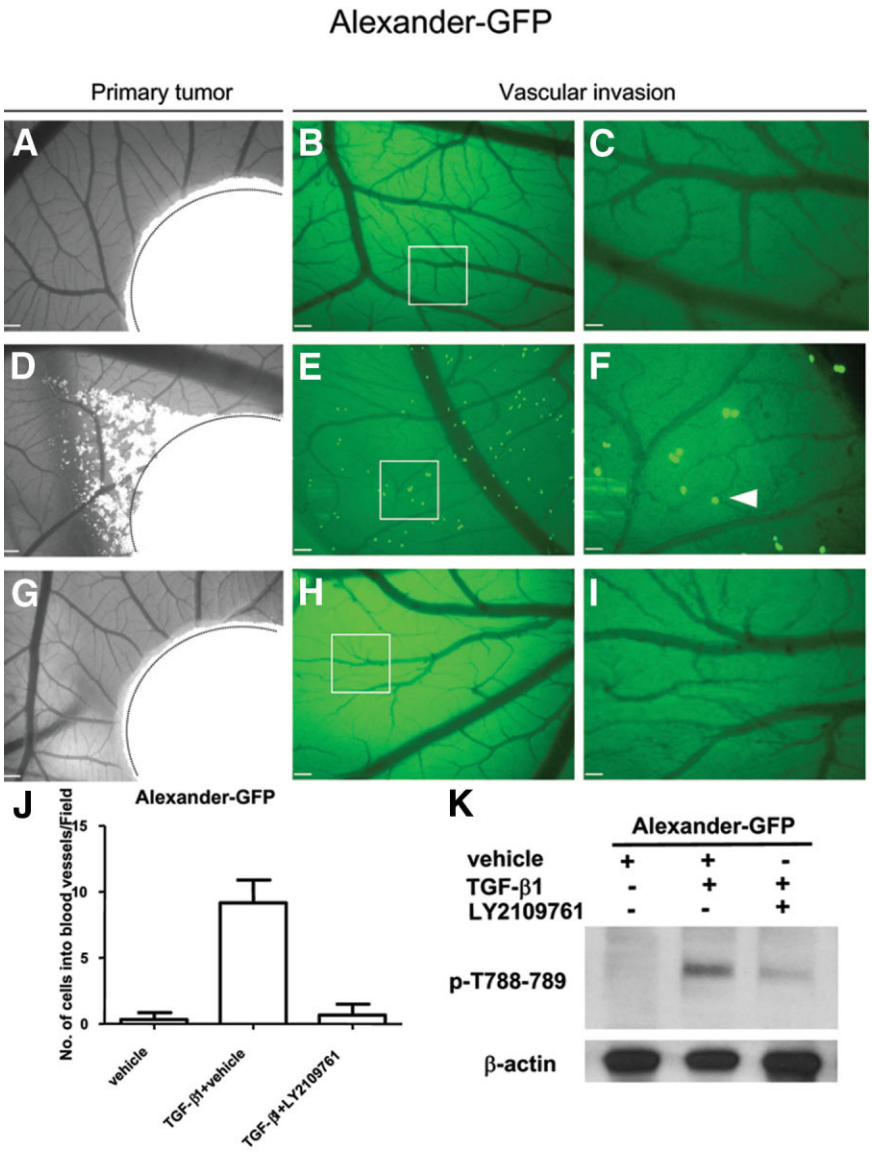


Fig. 7. LY2109761 inhibits Alexander stroma and blood vessel invasion stimulated by TGF-$\beta$1. GFP-expressing Alexander cells were pretreated with vehicle (DMSO) or 0.1 $\mu$M of LY2109761 for 48 hours in culture and then implanted into the CAM. The developing tumors were treated with (A-C) vehicle alone, (D-F) 200 ng of TGF-$\beta$1 + vehicle, or (G-I) 20 $\mu$M LY2109761 every second day for 7 days, and then imaged. Each image represents 10 animals imaged in three separate experiments. Dotted lines indicate the tumor edge. The boxed regions in the middle panels are magnified $\times$100. Scale bars: left panels, 400 $\mu$m (magnification $\times$40); middle panels, 200 $\mu$m (magnification $\times$40); right panels, 100 $\mu$m (magnification $\times$100). (J) Vascular invasion was quantified by numbering the GFP-expressing Alexander cells present in the CAM blood vessels/field (magnification $\times$100). Values are expressed as the mean $\pm$ standard deviation. (K) Proteins extracted from the CAM tissue samples were processed by western blotting and membranes were probed with antibodies against $\beta$1 integrin phosphorylated at T788-789.

to an aggressive HCC phenotype.[40] Furthermore, this late gene signature response also correlates with vascular invasion, although this is not fully explained by gene expression.

In this study, we show a mechanism according to which TGF-$\beta$1 promotes vascular invasion by changing the functional status of integrin $\alpha5\beta1$, thus providing a functional explanation for HCC tumor progression supported by the late TGF-$\beta$ signature genes,[40] although we cannot rule out the possibility that other redundant biological pathways may play a similar role. Nevertheless, the phosphorylation of the $\beta$1 integrin at threonine suggests a specific role of TGF-$\beta$1 through Smad-2/3 signaling, because this residue has been reported to reduce cell adhesion, whereas phosphorylation of serine and tyrosine promotes adhesion localized at podosomes.[41-43] Following intracytoplasmic activation of $\beta$1 integrin, we observed the conformational change reported to occur in the case of threonine but not phosphorylation of other residues.[41] This leads to an inside-out mechanism, corroborated by the reactivity of TGF-$\beta$1–treated noninvasive HCC cells with the HUHTS-21 antibody directed against the active conformation of the $\beta$1 extracellular domain.[44] In our experimental model, we demonstrate that activation of the $\beta$1 integrin subunit is a mechanism that can be turned on and off, which seems to match the clinical observation that vascular invasion occurs in about 50% of patients.

These results are not in contrast with our previous report showing an up-regulation of $\alpha3\beta1$ by TGF-$\beta$1, nor with others reporting an increased expression of $\alpha6\beta1$, both integrins promoting cancer cell invasiveness.[23,24] On the contrary, they support the hypothesis that TGF-$\beta$1 exerts a multistep and pleiotropic effect promoting HCC tumor progression. In such a scenario,

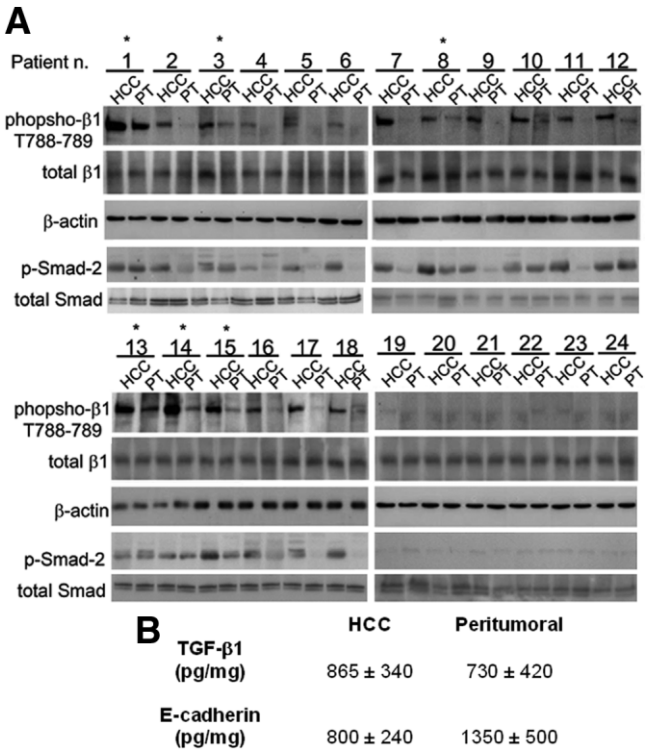


Fig. 8. Phospho-$\beta$1 integrin in HCC tissues. (A) Proteins were extracted from tumoral (HCC) and paired peritumoral (PT) tissues of patients with (1-18) or without (19-24) vascular invasion and processed via western blotting. Membranes were probed with antibodies against the phosphorylated tail at T788-789, against total $\beta$1 integrin, against $\beta$-actin, and against p–Smad-2 and total Smad. *Patients with metastasis in peritumoral tissues. (B) TGF-$\beta$1 and E-cadherin were measured in the same tissue preparations. Values are expressed as the mean $\pm$ standard deviation.

upon TGF-$\beta$1 stimulation, HCC cells can spread to surrounding tissues thanks to $\alpha 3\beta 1$ and $\alpha 6\beta 1$; however, if $\alpha 5\beta 1$ is activated, they can invade the blood vessels.

In conclusion, we show that TGF-$\beta$1 phosphorylates the intracytoplasmic tail of $\beta$1 integrin with an inside-out mechanism causing conformational changes of the extracellular domain. This mechanism ultimately drives HCC invasion of the blood vessels, and represents a new target for the design of clinical trials.

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