Down-Regulation of Connective Tissue Growth Factor by Inhibition of Transforming Growth Factor β Blocks the Tumor–Stroma Cross-Talk and Tumor Progression in Hepatocellular Carcinoma

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Tumor–stroma interactions in hepatocellular carcinoma (HCC) are of key importance to tumor progression. In this study, we show that HCC invasive cells produce high levels of connective tissue growth factor (CTGF) and generate tumors with a high stromal component in a xenograft model. A transforming growth factor β (TGF-β) receptor inhibitor, LY2109761, inhibited the synthesis and release of CTGF, as well as reducing the stromal component of the tumors. In addition, the TGF-β–dependent down-regulation of CTGF diminished tumor growth, intravasation, and metastatic dissemination of HCC cells by inhibiting cancer-associated fibroblast proliferation. By contrast, noninvasive HCC cells were found to produce low levels of CTGF. Upon TGF-β1 stimulation, noninvasive HCC cells form tumors with a high stromal content and CTGF expression, which is inhibited by treatment with LY2109761. In addition, the acquired intravasation and metastatic spread of noninvasive HCC cells after TGF-β1 stimulation was blocked by LY2109761. LY2109761 interrupts the cross-talk between cancer cells and cancer-associated fibroblasts, leading to a significant reduction of HCC growth and dissemination. Interestingly, patients with high CTGF expression had poor prognosis, suggesting that treatment aimed at reducing TGF-β–dependent CTGF expression may offer clinical benefits. *Conclusion:* Taken together, our preclinical results indicate that LY2109761 targets the cross-talk between HCC and the stroma and provide a rationale for future clinical trials. (HEPATOLOGY **2009;50:000-000.**)

Drug-based treatments to cure patients with hepatocellular carcinoma (HCC) remain a highly desirable goal in drug development because so-called curative therapies are successful in only few patients.[1] The approval of sorafenib for the treatment of patients with advanced-stage disease has stimulated the investigation of new therapeutic strategies.[2] However, the development of new drugs is hampered by the heterogeneity of HCC and the underlying liver disease type, which limit the recognition of useful therapeutic targets.[3]

Because HCC generally develops in fibrotic tissue, the growth of tumor cells is dependent on an intricate cross-talk between the tumor and the stroma. Due to this complexity and interaction with fibrotic liver, HCC cells develop a cross-talk with adjacent components of the tissue microenvironment, including extracellular matrix proteins and various growth factors. A growing body of evidence suggests that the cross-talk affects the malignant phenotype and is a key factor of poor clinical outcome. Cancer-associated fibroblasts (CAFs), a heterogeneous population of stromal cells, play a central role in this stroma–tumor interaction, involving the production of growth factors, which in turn may reduce the effect of chemotherapy and radiotherapy.[4,5] CAFs can form after stimulation with transforming growth factor β-1 (TGF-

Abbreviations: CAF, cancer-associated fibroblast; CAM, chorioallantoic membrane; CM, conditioned media; CTGF, connective tissue growth factor; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; mRNA, messenger RNA; PCR, polymerase chain reaction; shRNA, short hairpin RNA; TGF-β, transforming growth factor β.

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β1), which plays an important role in HCC by stimulating fibrogenic remodeling of the liver and contributing to tumor progression.[6] In this scenario, the connective tissue growth factor (CTGF), a multifunctional protein implicated in several distinct functions, can directly interact with TGF-β1.[7] CTGF expression has been correlated with shorter survival in various cancers, such as pancreatic cancer, which like HCC is characterized by extensive fibrotic remodeling.[8]

Recently, we have reported on a molecule, LY2109761, that selectively blocks the kinase activity of TGF-β receptor type I, and inhibits tumor growth and progression of HCC in an experimental in vivo model.[9,10] We have also demonstrated that LY2109761 inhibits neo-angiogenesis to the same extent as bevacizumab and can inhibit the growth of HCC. Unlike bevacizumab, LY2109761 does not have any effect on physiological angiogenesis in a xenograft model of HCC.[11] The present study further investigates the biological and molecular mechanisms of LY2109761 to better identify HCC patients who could be candidates for future clinical trials using TGF-β inhibitors.

Materials and Methods

Cells, Antibody, and Reagents. The human HCC cell lines HLE and Alexander were cultured as described.[12] For some experiments, HCC cells lines expressing green fluorescent protein (GFP) were generated. Cultured cells were infected by the retroviral vector pLXSN-GFP (BD Clontech) and isolated by neomycin selection without clonal propagation. TGF-β1 receptor kinase inhibitor LY2109761 was kindly provided by Eli Lilly (Indianapolis, IN).

Chorioallantoic Membrane Tumor Formation and Cell Growth Assays. A chorioallantoic membrane (CAM) assay was performed as described, the difference being that this time, at the indicated time points the CAMs were excised and analyzed using quantitative Alu polymerase chain reaction (PCR) for numbers of human cells.[11] Cell growth assays were performed as described.[9]

[3H]Thymidine Incorporation Assay. DNA synthesis was measured as the amount of [methyl-^{3}H]thymidine ([^{3}H]thymidine) incorporated into trichloroacetic acid–precipitable material. Subconfluent HLE in 24-well dishes were incubated in serum-free/insulin-free medium for 24 hours in the presence or absence of 1 μM of LY2109761. Cells were then incubated with or without 3 ng/mL of TGF-β1 for 20 hours and then pulsed for an additional 4 hours with 1.0 μCi/mL [^{3}H]TdR. At the end of the pulsing period, medium was carefully aspirated, ice-cold 5% tricholoroacetic acid was added, and dishes were kept on ice for 15 minutes. After two additional washes with 5% tricholoroacetic acid, cells were solubilized by adding 750 μL of 0.25 N NaOH, 0.1% sodium dodecyl sulfate. 0.5 mL of the solubilized cell solution was then neutralized with 50 μL of 6 N HCl and counted in a scintillation counter. Cell number was determined in three separate wells from each dish and results were expressed as cpm/10^5 cells.

DNA Fragmentation. To detect DNA fragmentation, cellular DNA was prepared using the blood and cell culture mini-DNA kit (QIAGEN, Valencia, CA). Purified DNA was then analyzed on 1.5% agarose gel. DNA was visualized by ethidium bromide staining.

Histology, Immunohistochemistry, and Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling Assay. HLE tumors were dissected from the CAMs and fixed in 4% buffered formalin. The tissues were frozen and 5-μm thick sections were cut and stained with hematoxylin-eosin. Stromal tissue was visualized by way of Azan-Mallory staining. Immunohistochemistry was performed as described.[12] Briefly, sections were fixed in a cold chloroform/acetone mixture for 10 minutes, air-dried, and incubated with a rabbit polyclonal antibody directed against CTGF (ab6992; Abcam, Cambridge, UK). Reactions were developed using red fuchsin as chromogen and abundantly washed for 20 minutes. Finally, randomly chosen microscopic fields were captured, and positive staining was defined according to an image analysis software-assisted system (Lucia, Nikon Corp.). Apoptosis in HLE tumor tissue sections was detected using the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling enzyme reagent according to the manufacturer's instructions.

Isolation of Cancer-Associated Fibroblasts. Samples from tumor resections were placed in Dulbecco's modified Eagle's medium (Sigma Chemical Corporation, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco Invitrogen Corporation). The tissue was finely minced into 1 mm fragments, washed twice in phosphate-buffered saline supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1.5 g/mL fungizone), and disaggregated with 0.1% collagenase III (Worthington Biochemical Corp., Lakewood, NJ) at 37°C on a rotator. After 24 hours, the epithelial cells were separated from stromal cells by differential centrifugation, as described.[13] Fibroblasts were washed twice in phosphate-buffered saline and plated in 60-mm dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified chamber containing 5% CO_2. Primary cultures of chick embryo CAFs isolated from HCC tumors growth in the CAM were characterized by positive immunostaining for vimentin and distinguished from tumor cells by negative staining for pan-

cytokeratin (Supporting Fig. 1). Cells from passages 3-10 were used for all experiments.

Soft Agar Colony Formation Assay. We mixed 2×10^4 cells in culture medium with a twice-higher volume of 0.5% top agar and seeded them in 60-mm plates onto a base layer of complete medium containing 0.5% agar in the presence of medium from untreated CAFs or treated them with TGF-β1, LY2109761, or TGF-β1 in combination with LY2109761. The cells were incubated at 37°C in 5% CO_2 in air. After 2 to 3 weeks, the number of colonies was counted and statistically analyzed. The data represent the mean ± standard error of the mean of three independent experiments.

In Vivo Imaging of the Chick Embryo CAM. For imaging experiments, eggs were prepared according to the shell-less (ex ovo) chick embryo assay procedure as described.[10] For quantification of the stroma in untreated and treated tumors, imaging was performed using a proprietary software combining automatic and manual counting.

Measurement of Intravasation and Spontaneous Lung Metastasis Using the Chick Embryo Chorioallantoic Membrane Assay. Fertilized SPAFAS White Leghorn eggs (Charles River, North Franklin, CT) were incubated in a humidified rotary incubator at 38°C for 10 days. The CAM was gently dropped as described, and a single-cell suspension of tumor cells was prepared at 0.1 to 8×10^6 cells in 25 μL of phosphate-buffered saline and inoculated on the dropped CAM. The embryos were incubated for 1 to 9 days in a humidified stationary incubator at 38°C. Where indicated, the TGF-β1 receptor kinase inhibitor LY2109761 (50 mg/kg) or dimethyl sulfoxide (control) were applied topically onto the upper CAM on days 3 and 5. At the indicated time points, the upper CAM with the developing tumor, the portions of the lower CAM, and the lungs were excised from the individual embryos and collected for further analyses.

Real-Time Quantitative Detection of Human HCC Cells (Alu PCR) and CTGF. Real-time PCR was performed as described.[11] Briefly, the Alu primer sequences were 5′-ACGCCTGTAATCCCAGCACTT-3′ (sense) and 5′-TCGCCCAGGCTGGGTGCA-3′ (anti-sense). The actual number of tumor cells present in each tissue sample was determined using a standard curve generated by serial dilutions of human tumor cells. With regard to CTGF messenger RNA (mRNA) quantification, the sequences were 5′-GCATCCGTACTCCCAAAATCTC-3′ (sense) and 5′-GGCAGGGTGGTGGTTCTGT-3′ (anti-sense).

Gene Silencing. At 24 hours after plating, HLE cells were transfected with a pool of three target-specific lentiviral vector plasmids, each encoding 19-25 nt short hairpin RNAs (shRNAs) designed to knock down CTGF gene expression (Santa Cruz Biotechnology, Inc.) or nonsilencing control (control-shRNA). Twenty-four hours after the transfection, cells were grown in medium containing 2 μg/mL puromycin to select the stably-transfected clones.

Western Blot Analysis. Cells were lysed as described.[11] The primary antibody directed against CTGF (Abcam) was incubated at 4°C overnight. An anti–β-actin monoclonal antibody purchased from Sigma was used as an internal loading control.

Human HCC Tissues. We determined the expression of CTGF in tumoral and paired peritumoral tissues from 21 patients undergoing surgery by way of immunohistochemical analysis. Patients with comparable clinical characteristics (etiology, age, stage of disease assessed by the Barcelona classification) were divided into two groups, good (n = 13) and poor (n = 8) prognosis, depending on cancer recurrence over a 5-year follow-up period.

Data Analysis and Statistics. Data processing and statistical analysis were performed using the GraphPad Prizm (GraphPad Software, Inc., San Diego, CA). All experiments were performed at least twice, and the total number of experiments and samples is indicated in the text or figure legends. Data are presented as the mean ± standard error of a representative experiment or of normalized data of pooled experiments. A Student *t* test ($P < 0.05$) was used to compare differences between the data for the two cell variants or the conditions used in ex vivo and in vivo models. The Wilcoxon test was used to analyze CTGF expression in HCC tissues. Two-way analysis of variance was used to analyze tumor growth, intravasation, and metastasis experiments.

Results

We used two invasive (HLE and HLF) and two noninvasive (Alexander and Hep3B) human HCC cell lines to characterize the role of TGF-β–dependent HCC growth.[12] As recently reported for HLE cells,[11] HLE and HLF cells were inoculated in the CAM of the chick embryo model to generate HCC tumors within 1 week. The treatment of such HCC tumors with LY2109761, a selective inhibitor of TGF-β receptor type I, at days 3 and 5 after the implantation of the HLE cells, strongly reduced the tumor development as compared with vehicle (dimethyl sulfoxide)-treated animals after the second administration (Fig. 1A). By contrast, the noninvasive HCC cells Alexander and Hep3B (data not shown) form small tumors when implanted in the chick embryo CAM. In the presence of TGF-β1, Alexander cells formed larger tumors, but this effect was inhibited by LY2109761 treatment (Fig. 1B). Images of the LY2109761-treated HLE tumors show a significant decrease in tumor size over

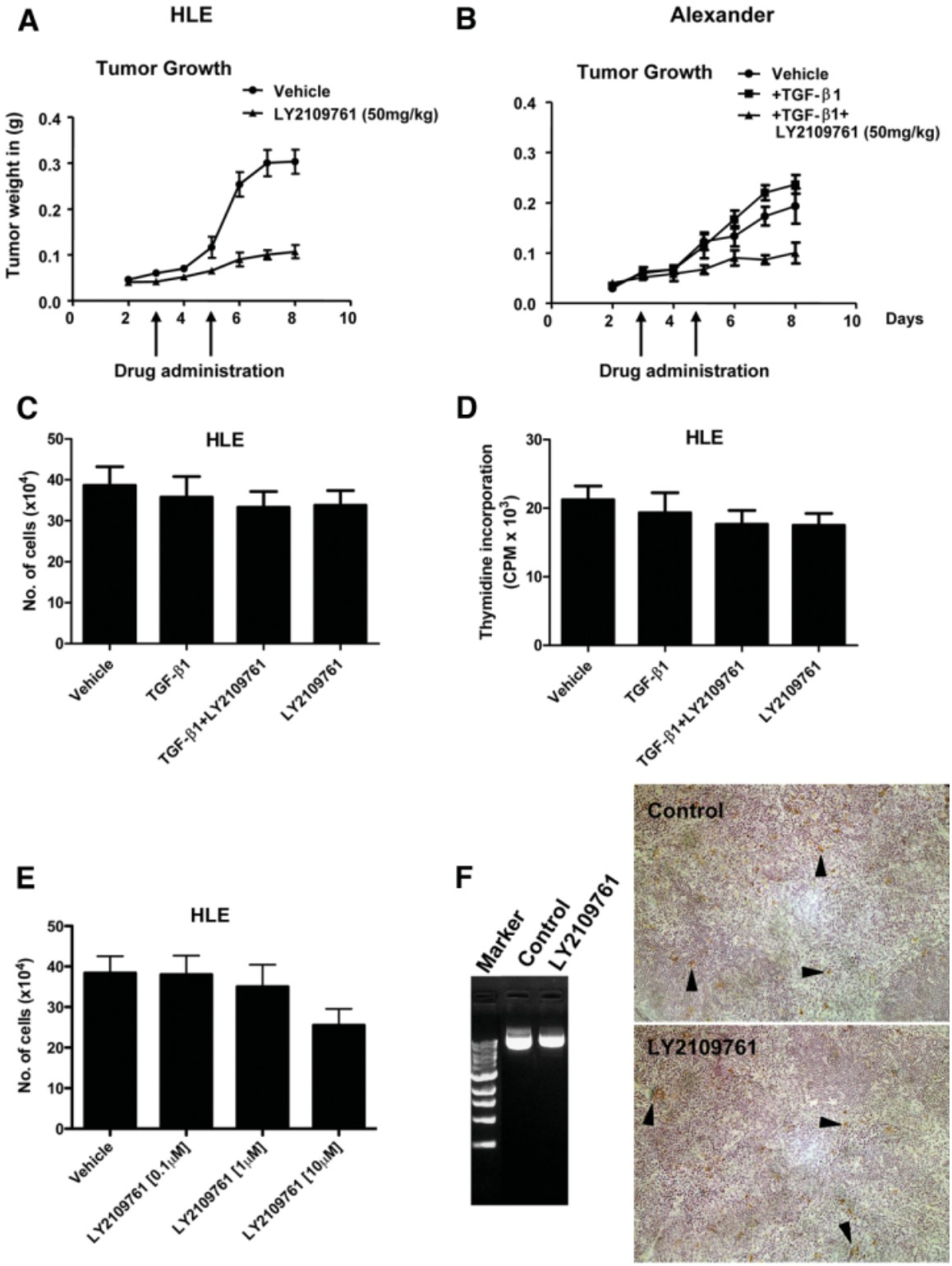

Fig. 1. LY2109761 inhibits HCC growth without affecting cell proliferation and/or apoptosis. (A,B) Kinetics of tumor growth of (A) HLE cells and (B) Alexander cells in the presence or absence (vehicle) of TGF-β1 in the chick embryo CAM. A total of 5×10^5 HLE or Alexander cells were grafted on the dropped CAM of 10-day chick embryos. LY2109761 was administered on days 3 and 5. (C) Differences of tumor weights were statistically significant after the second drug administration ($P < 0.0001$). The effect of LY2109761 and (D) dose-dependent assay on HLE cell proliferation was determined by cell counting. (E) The effect of LY2109761 on DNA synthesis in HLE cells was evaluated using a [3H]Thymidine incorporation assay. (F) No effect on apoptosis was detected in HCC treated with LY2109761 as evaluated by way of DNA fragmentation and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay. The results shown represent the mean $\pm$ standard error of the mean of three separate experiments (magnification $\times$200). Arrows indicate brown, positive-stained cells.

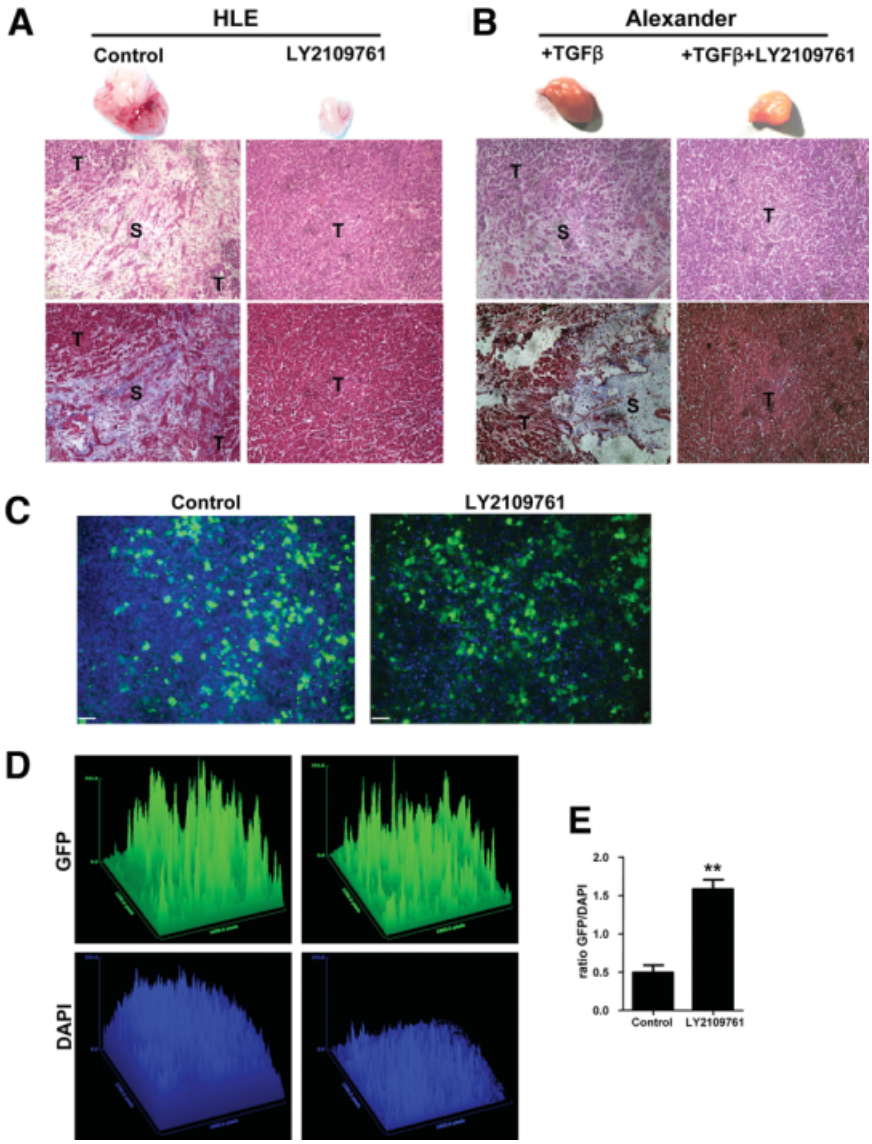

Fig. 2. LY2109761 reduces the associated stromal component in HCC tumors. (A) Representative sizes and histology of HLE tumors in each group of animals treated with dimethyl sulfoxide (controls) or LY2109761 (50 mg/kg). (B) Representative sizes and histology of Alexander tumors in each group of animals treated with TGF-β1 alone or in combination with LY2109761 (50 mg/kg). Tumors were excised from the CAM and photographed. For histology, tissues were collected after treatment with LY2109761 and the stromal component was visualized by hematoxylin-eosin and Azan-Mallory staining. (C) The effect of LY2109761 on tumor stroma was evaluated by GFP/DAPI staining in LY2109761-treated tumors and compared with controls. (D) Images selected for the same amount of GFP showed different levels of DAPI staining. (E) The ratio between GFP and DAPI was quantified. S, stroma; T, tumor. $^{**}P < 0.005$.

time, whereas control-treated animals had progressively increasing tumors (Supporting Fig. 2A). In Alexander-formed tumors, the addition of LY2109761 at days 3 and 5 blocks this TGF-β1–mediated tumor growth and also leads to a reduction of tumor size (Supporting Fig. 2B). Thus, LY2109761 appears to have an effect on both invasive and noninvasive HCC cells by blocking tumor growth in the former and preventing TGF-β1–dependent growth in the latter type of HCC cells.

To gain a better insight into this dual molecular mechanism of TGF-β–dependent tumor growth in HCC cells, we first tested the hypothesis that LY2109761 may have an antiproliferative effect on HLE cells. Neither the manual counting assay nor thymidine incorporation (Fig. 1C,D) indicated that this antiproliferative effect was based on a cytotoxic effect, even though a significant inhibition of HLE proliferation was obtained at higher doses of LY2109761 (Fig. 1E). We had already observed this cytotoxic effect, which may not be TGF-β–specific, in a previous study.[9] Similar data were obtained with HLF cells (data not shown). Moreover, we did not observe any DNA fragmentation in HLE cells incubated with the drug or any increase in the number of apoptotic cells, as assessed by way of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay (Fig. 1F).

Because the biological approaches described above failed to explain the effectiveness of LY2109761 on HCC growth, we investigated tumor tissues in greater detail, in view of the obvious difference in tumor size of LY2109761-treated tumors versus controls. Surprisingly, histological examination of the tumors revealed that the stromal component was dramatically reduced in LY2109761-treated tumors (Fig. 2A). Tumors from noninvasive Alexander cells had little stroma (data not

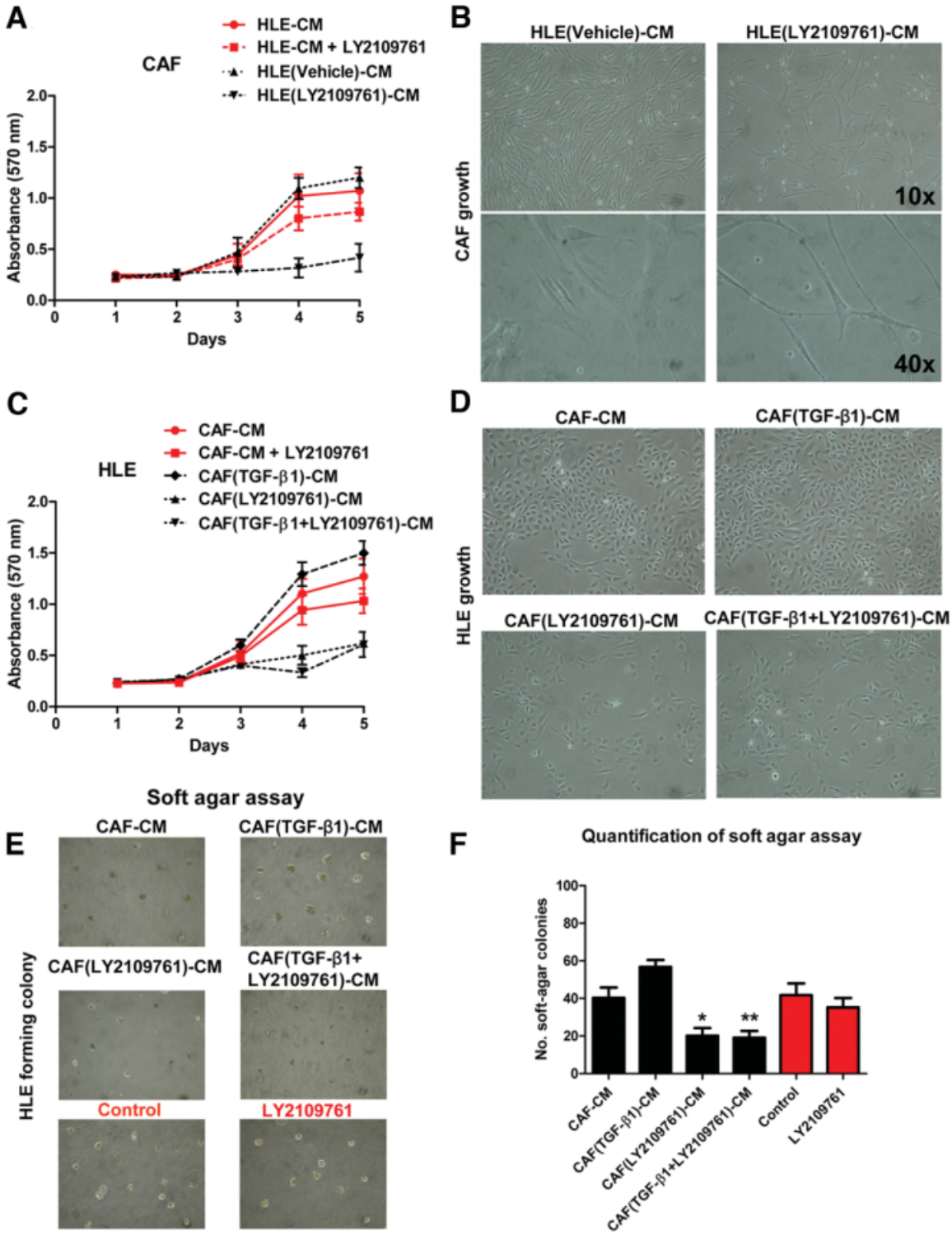

Fig. 3. LY2109761 inhibits the cross-talk between HCC cells and CAFs. (A,B) Effects of CM from HLE cells treated with LY2109761 on (A) CAF proliferation and (B) CAF morphology. (C,D) Reciprocal effects of CM from CAFs treated with LY2109761 on (C) HLE cell proliferation and (D) HLE cell morphology. (E) Effects of CM from CAFs treated with LY2109761 on the anchorage-independent growth of HLE cells, measured as number of colonies in a soft agar. (F) Quantification of the soft agar assay. The anchorage-independent growth of HLE cells was inhibited by CM from CAFs treated with LY2109761 alone or in combination with TGF-β1. *$P < 0.05$ versus control. **$P < 0.005$ versus control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

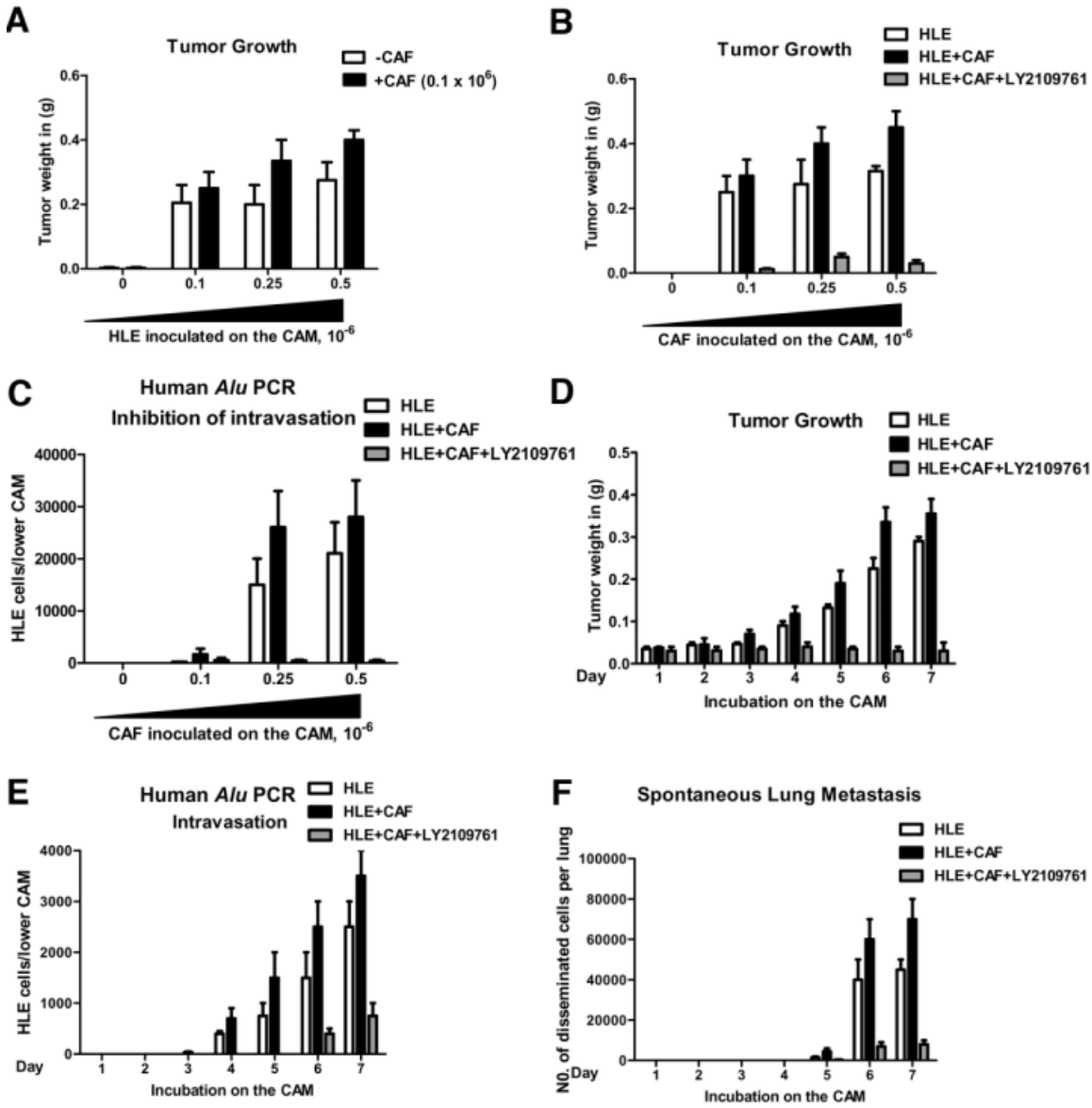

Fig. 4. LY2109761 inhibits the cross-talk between HCC cells and CAFs, reducing the capacity of HCC cells to intravasate and metastasize. (A) Effects of coinjection of CAF with an increasing number of HLE cells on tumor growth ($P < 0.05$). (B) Effects of LY2109761 on tumor growth following coinjection of HLE cells with an increasing number of CAFs ($P < 0.0001$). (C) Inhibition of HLE cell intravasation by LY2109761 treatment with an increasing number of CAFs evaluated by way of human Alu PCR ($P < 0.005$). (D-F) Kinetics of (D) tumor growth, (E) intravasation, and (F) metastatization to the lung of HLE cells coinjected with CAFs onto chick embryo CAM. Bars represent the mean ± standard error of the mean based on one of three independent experiments ($P < 0.0001$).

shown), but when treated with TGF-β1 these cells developed a high stromal content. Treatment of these noninvasive HCC cells with TGF-β1 and LY2109761 caused the development of small tumors comparable with controls (Fig. 2B). Similar data were obtained with Hep3B (data not shown). To further investigate this difference, HLE cells were transfected with a GFP expression vector and all other cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Fig. 2C). Randomly selected images of HLE and nontumor cells were quantified and calibrated for the same number of tumor cells, based on green fluorescence intensity. DAPI staining revealed that nontumor cells were reduced in LY2109761-treated tumors (Fig. 2D). This difference is further illustrated by the ratio of GFP/DAPI-stained cells (Fig. 2E).

Stimulated by this interesting finding, we used the chick embryo CAF model to test the hypothesis that the interaction between CAFs and HLE stimulates the proliferation of each cell type. We found that CAFs proliferated in the presence of HLE conditioned media (CM) with or without added LY2109761 or control vehicle, but did not proliferate in the presence of CM from HLE pretreated

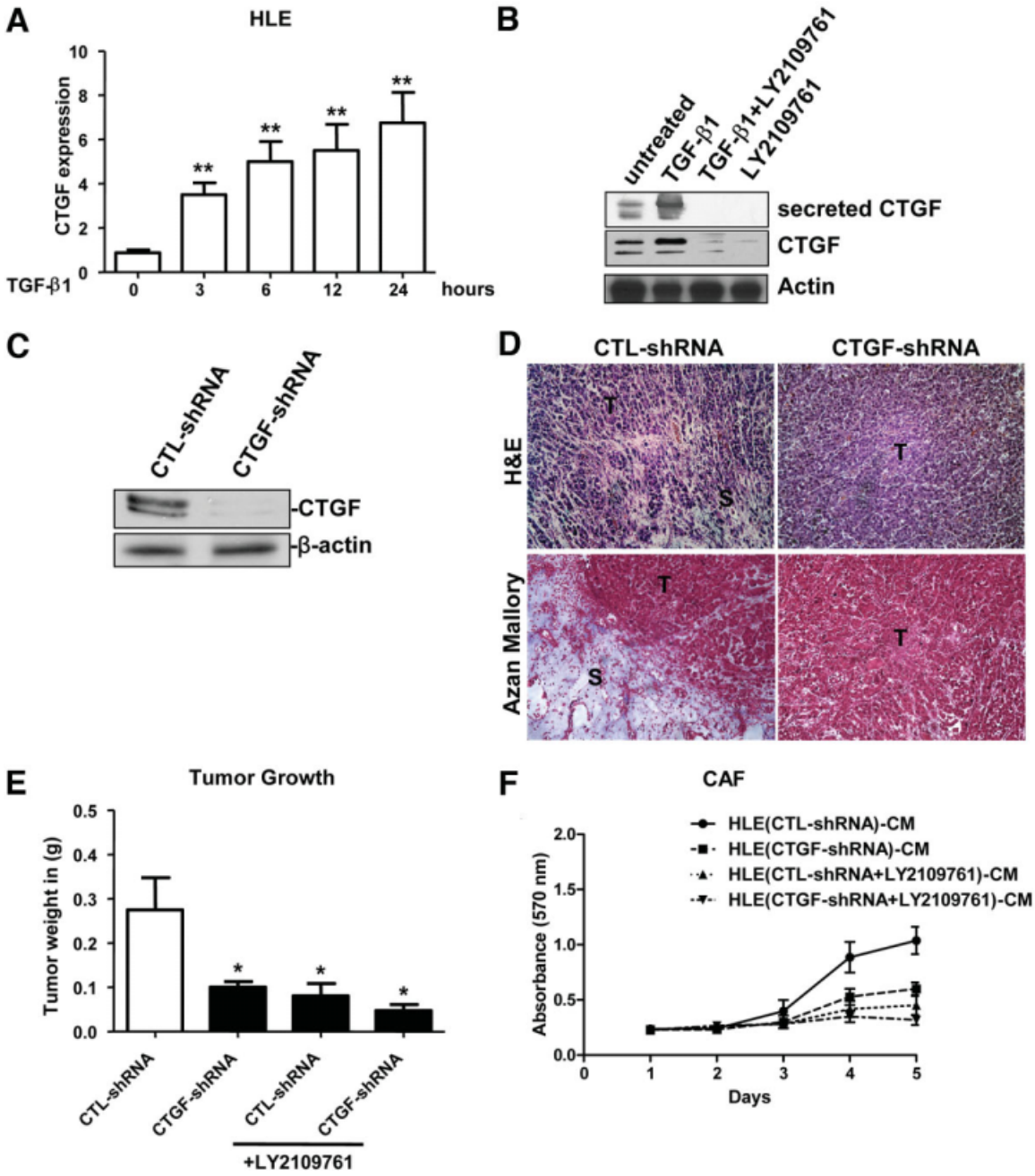

Fig. 5. LY2109761 treatment inhibits the expression of CTGF in HCC. (A) TGF-β1 up-regulates the endogenous production of CTGF mRNA in a time-dependent manner in HLE cells as evaluated using real-time PCR. The mean $\pm$ standard error of the mean of three separate experiments are shown. (B) Expression and secretion of CTGF is increased by TGF-β1 and completely blocked by LY2109761 treatment in HLE, as shown by western blot experiments. (C) CTGF silencing by shRNA in HLE was evaluated by way of western blot analysis. (D) Azan-Mallory staining of sections of HCC tumors generated by CTGF shRNA transfectants. CTGF shRNA transfectants showed a minimal stromal component in the histological architecture as compared with those formed by HLE control shRNA. (E) In vivo, HCC growth of CTGF transfectants alone or treated with LY2109761. (F) CAF proliferation of HLE cells incubated with CM from CTGF-shRNA transfectants alone or treated with LY2109761. S, stroma; T, tumor. *$P < 0.05$ versus control. **$P < 0.005$ versus control.

with LY2109761 (Fig. 3A,B). Similarly, HLE proliferated in the presence of CM from CAFs pretreated with TGF-β1, whereas they failed to proliferate in the presence of CM from CAFs pretreated with LY2109761 alone or in combination with TGF-β1 (Fig. 3C,D). To exclude a direct role of LY2109761 on cell proliferation, we challenged HLE with CM of CAFs with added LY2109761 and observed no proliferation (Fig. 3C). In conclusion, both CAFs and HLE reciprocally stimulated cell proliferation through the secretion of factors, and this effect was inhibited by pretreatment with LY2109761. Interestingly, fibroblasts isolated from the chick embryo weakly

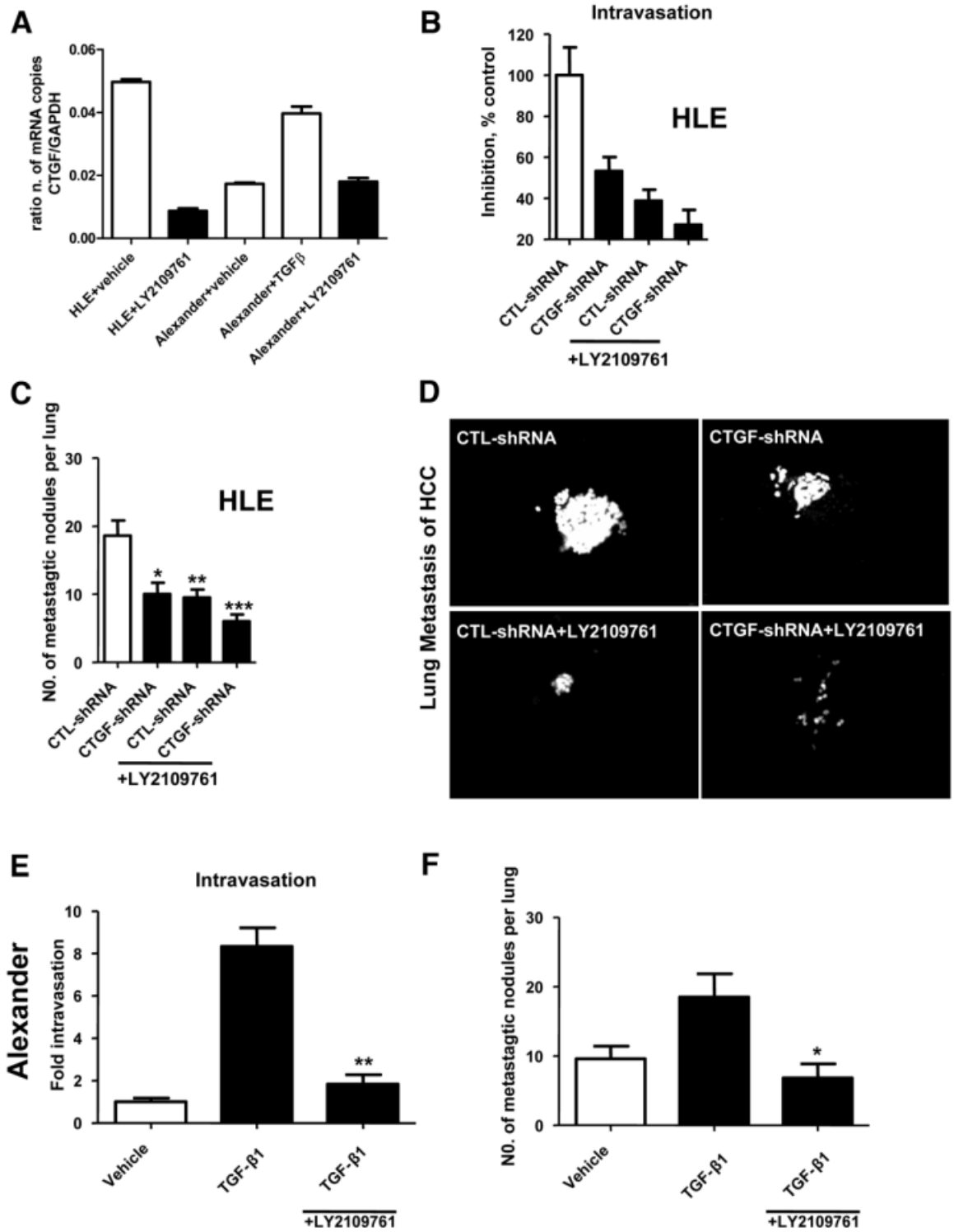

Fig. 6. Knocking down CTGF expression in combination with LY2109761 treatment strongly inhibits intravasation and the metastatic dissemination of HCC. (A) Analysis of CTGF mRNA expression levels was performed in both HLE and Alexander cells (stimulated or not with TGF-β1) treated with LY210976 or control (vehicle). Levels of CTGF mRNA are expressed as the ratio of the number of CTGF/GAPDH copies. (B) Analysis of intravasation and (C) metastatic growth of CTGF shRNA transfectants. Intravasation of transfectants was evaluated using real-time Alu PCR of genomic DNA extracted at day 7 after injection of CTGF transfectants alone or treated with LY210976. (C) HCC dissemination to the lung was confirmed by fluorescent microscopy of GFP-expressing HLE cells during experimental colonization of the chick lung at 7 days postinjection (magnification $\times$200). (E) Analysis of intravasation and (F) metastatic growth of Alexander cells implanted onto the chick embryo CAM. Intravasation of Alexander cells was evaluated by way of real-time Alu PCR of genomic DNA extracted at day 7 after injection of cells treated with vehicle, TGF-β1 alone, or in combination with LY210976. (F) Dissemination of Alexander cells to the lung was measured as the number of metastatic nodules per lung. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ versus control.

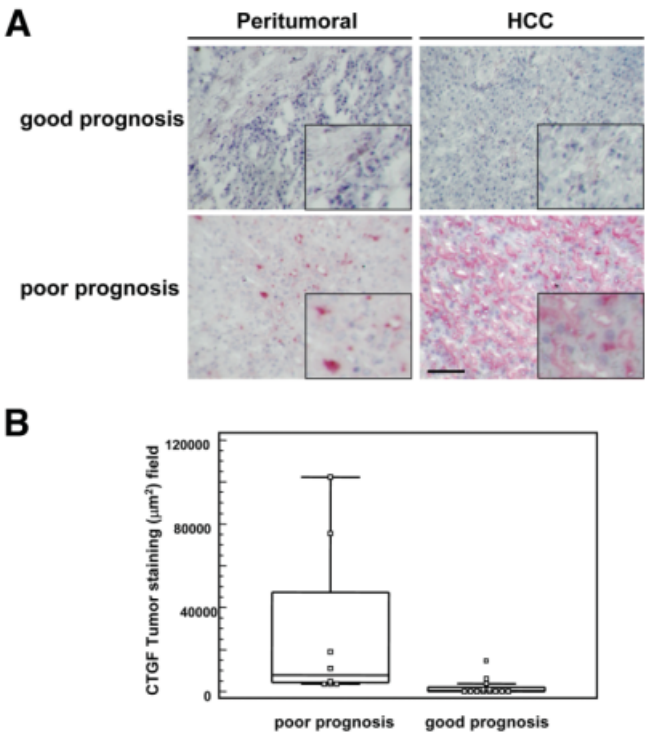

Fig. 7. CTGF expression in patients with HCC. (A) CTGF expression was evaluated through immunohistochemical analysis in HCC patients with good (upper) and poor (bottom) prognosis. CTGF expression was stronger in tumoral (right) compared with peritumoral (left) tissues. (B) To better quantify the difference, appropriate software for imaging analysis was used. Ten random microscopic fields were chosen from each section and captured, and CTGF expression was measured as the mean stained area. The overall expression was much stronger in patients with worse than with good prognosis. The box plot shows the statistically significant difference ($P < 0.005$). Bar = 100 μm (magnification $\times$200; inset, $\times$400).

proliferate in the presence of CM from HLE as compared with CAF ($P < 0.05$) (Supporting Fig. 3). The anchorage-independent growth of HLE cells, measured as the number of colonies in a soft agar assay, was also inhibited when the cells were incubated with CM from CAFs treated with LY2109761 alone or in combination with TGF-β1 (Fig. 3E,F).

In vivo, HLE cells formed larger tumors if inoculated together with CAFs. In fact, coinjection of an increasing number of HLE cells with a constant number of CAFs led to the formation of large HCC tumors (Fig. 4A). Similarly, coinjection of the same number of HLE cells with an increasing number of CAFs led to large tumor growth. By contrast, LY2109761 treatment led to the formation of only small tumors (Fig. 4B). Furthermore, increasing numbers of CAFs stimulated intravasation of HLE cells, as determined by human Alu-PCR, whereas LY2109761 pretreatment completely blocked the spread of tumor cells (Fig. 4C). Moreover, CAFs accelerated HCC growth, intravasation and metastatic colonization of the lung, whereas pretreatment of HLE with LY2109761 blocked the metastatic spread (Fig. 4D,F). In conclusion, CAFs exert a key role in modulating the biological activities of invasive HCC cells.

In this scenario of interaction of tumor and fibrotic tissue by way of CAFs, we investigated CTGF as a potential mediator of this type of cross-talk. TGF-β1 up-regulated the endogenous gene expression of CTGF in a time-dependent manner in HLE cells (Fig. 5A). At protein level, TGF-β1 also increased CTGF levels whereas coincubation of TGF-β1 with LY2109761 blocked the production of CTGF (Fig. 5B). To demonstrate the functional role of CTGF, HLE cells were silenced with shRNA to block the production of CTGF (Fig. 5C). Tumors with CTGF–shRNA transfectants had a minimal stromal component as compared with controls (Fig. 5D). HCC growth was reduced in CTGF–shRNA transfectants, and this effect was boosted by LY2109761 treatment (Fig. 5E). Finally, CAF proliferation was blocked when cells were incubated with CM from CTGF–shRNA transfectants (Fig. 5F). As compared with the invasive HLE cells, the noninvasive Alexander cells expressed lower levels of CTGF mRNA. TGF-β1 stimulated the expression of CTGF in Alexander cells, whereas incubation with LY2109761 blocked TGF-β1–dependent CTGF gene expression in these cells (Fig. 6A).

In vivo, CTGF inhibition after LY2109761 treatment resulted in a strong reduction of tumor cell intravasation and the development of metastatic lung nodules (Fig. 6B,C). Although the development was not completely blocked, these metastatic nodules were smaller than in controls (Fig. 6D). Alexander cells only intravasated and metastasized to the lung when treated by TGF-β1 stimulation. This metastatic effect was completely inhibited by LY2109761 (Fig. 6E,F). These data indicate that CTGF modulates the cross-talk between stroma and tumor, which in turn influences HCC growth and progression. Because of this important role of CTGF, we examined human HCC tumor tissue to determine whether CTGF expression was correlated with poor prognosis. In HCC human tissues, CTGF was highly expressed in cancer cells as compared with peritumoral tissue. CTGF was more abundantly ($P < 0.005$) expressed in the tissues of patients with poor prognosis (Fig. 7A,B).

Discussion

In this study, we report for the first time that LY2109761, a kinase inhibitor of TGF-β receptor type I, inhibits the production of CTGF, interrupts the cross-talk between tumor and stroma, and blocks the progression of HCC. Our findings can be summarized as follows: (1) LY2109761 inhibits the production of CTGF; (2)

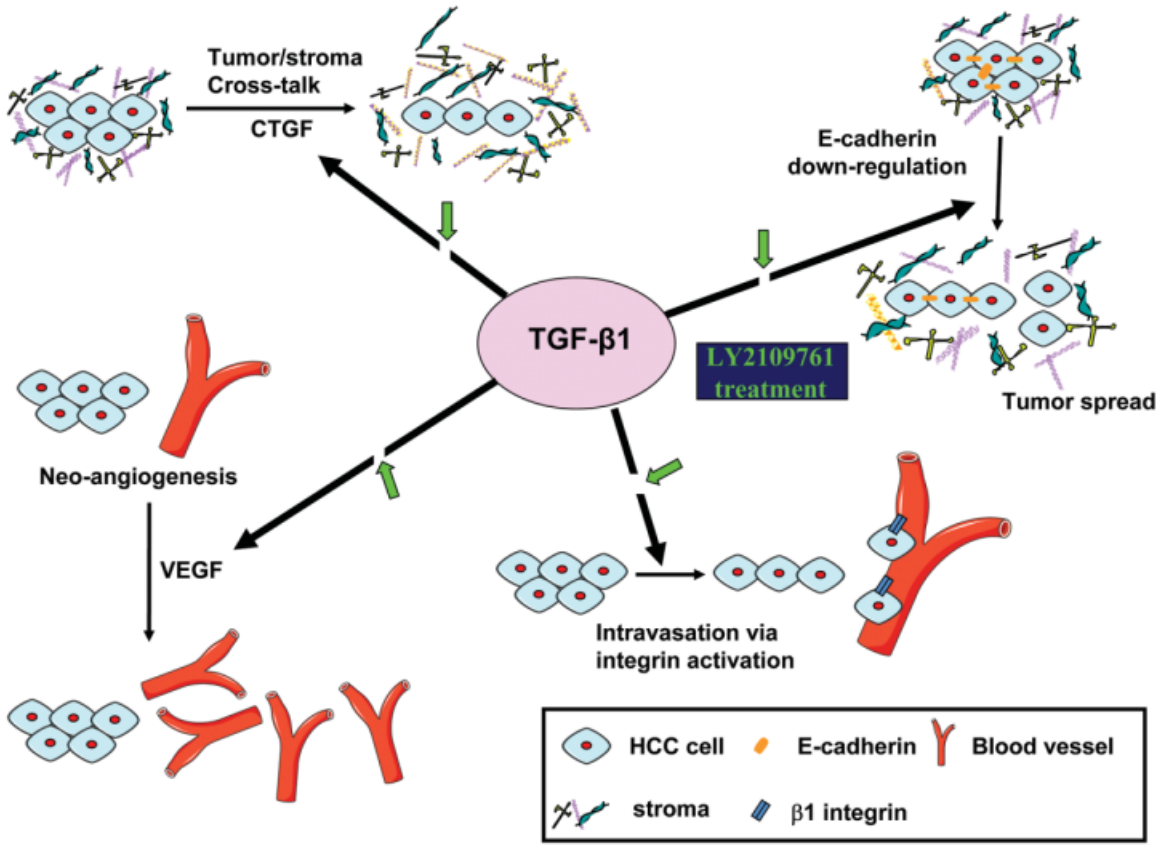

Fig. 8. Schematic representation of LY2109761 effectiveness in HCC. The blockade of the TGF-β1 kinase receptor by LY2109761 results in an inhibition of various biological HCC steps underlying tumor progression: tumor spread,[9] intravasation,[10] neo-angiogenesis,[11] and tumor–stroma cross-talk.

LY2109761 treatment reduces the stromal component of HCC; (3) HCC growth and metastatic ability depend upon the presence of CAF; (4) HLE knocked down for CTGF generates HCC with a minimal stromal component; (5) CTGF silencing inhibits HCC growth, intravasation, and metastasis; and (6) in noninvasive HCC cells, TGF-β1 up-regulates CTGF, resulting in the formation of tumor stroma and the acquisition of a metastatic capability; this is inhibited by LY2109761.

TGF-β1 is an intriguing pleiotropic factor in cancer because of its dual function as a tumor suppressor and promoter. In colon cancer, TGF-β1 promotes cell migration, invasion, and anchorage-dependent growth in an experimental and in vivo preclinical model. LY2109761 treatment reduces the development of liver metastases and increases survival in an in vivo experimental model.[14] In our study, LY2109761 interrupts the cross-talk between HCC and stromal cells that has recently been recognized as an important factor for the regulation of HCC growth, invasion, and differentiation.[15-17] However, because TGF-β is a profibrotic factor, and at the same time a promoter of HCC tumor progression,[18,19] inhibition of TGF-β pathways may have an important role in early carcinogenesis.

We have recently reported that LY2109761 displays several different antitumoral activities in HCC preclinical models (Fig. 8). For instance, the spread of HCC cells in the surrounding tissue is inhibited by the up-regulation of E-cadherin.[9] As illustrated in Fig. 8, LY2109761 inhibits intravasation of HCC cells by deactivating β1 integrin,[10] and also inhibits tumor growth thanks to inhibiting neo-angiogenesis by reducing vascular endothelial growth factor production.[11] In our previous studies, we showed that these different mechanisms are selectively dependent on the TGF-β1/Smad-2 pathway, in agreement with other studies,[14,20] even though an additional effect on other kinases cannot be excluded at higher concentrations than those used in our studies. Therefore, we propose a novel therapeutic strategy based on the use of a single selective kinase inhibitor that can target multiple HCC biological activities, such as sorafenib, although the latter is a multi-tyrosine kinase inhibitor designed to target different molecular pathways that has been shown to improve overall survival in HCC patients.[2,21]

In this study, we have demonstrated that LY2109761 targets the cross-talk between HCC cells and stromal cells that has been indicated as an important factor in HCC progression.[22] Another advantage of this drug is that it targets nontumoral cells of the microenvironment (such as endothelial cells and fibroblasts) that are genetically more stable and likely more responsive to therapies with lesser toxicity, unlike other novel drugs directed against tumor cells, such as sorafenib.[23,24]

In particular, TGF-β1 stimulates CAFs, which in turn leads to enhanced tumor spread. The mechanism is dependent on the up-regulation of CTGF, as suggested in different types of cancer including pancreatic and mammary cancers.[8,25,26] CTGF is a key regulator of fibrotic processes in the liver and kidney.[27,28] In addition, CTGF has been reported to promote tumor progression in several different cancers,[29] providing the rationale for the use of a humanized antibody against CTGF in ongoing clinical trails. Therefore, LY2109761 represents the first biological drug that can inhibit CTGF production, and so may prove to be a useful additional weapon to fight HCC.

In conclusion, we have found that targeting TGF-β receptor type I yields an antistromal therapy that may have domino effects on different mechanisms responsible for the progression of HCC. The present findings, together with our previous observations, suggest that targeting TGF-β in HCC may offer a novel way to improve the clinical outcome of HCC treatment.

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