

TSU-68 Ameliorates Hepatocellular Carcinoma Growth by Inhibiting Microenvironmental Platelet-derived Growth Factor Signaling

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Abstract. *Background:* TSU-68 is a multikinase inhibitor that targets platelet-derived growth factor receptors (PDGFRs). *In the present study, we evaluated the effect of TSU-68 on the tumor-microenvironment interaction in hepatocellular carcinoma (HCC). Materials and Methods:* HCC and fibroblast cell lines (HuH7, Hep3B, HuH1 and WI-38) were used to evaluate their interactions. Cancer characteristics were evaluated by spheroid formation and tumorigenicity in immunodeficient mice. Time-lapse image analysis was performed to monitor cell motility. *Results:* Although PDGFA was abundantly expressed, PDGFR- α was predominantly located in the cytoplasm and was not functional in HuH7 cells. Co-culture experiments demonstrated that HCC cells induced phosphorylation of PDGFR- α in WI-38 fibroblasts and that stimulated fibroblasts, in turn, boosted the spheroid formation capacity of HCC cells. TSU-68 inhibited phosphorylation of PDGFR- α in WI-38 cells and suppressed the growth of subcutaneously co-injected HuH7/WI-38 tumor xenografts. *Conclusion:* TSU-68 inhibits stromal PDGF signaling activated by cancer cells and suppresses HCC growth.

Hepatocellular carcinoma (HCC), one of the most common malignancies worldwide, has a dismal outcome (1). HCC is considered a heterogeneous disease in terms of morphology, clinical behavior and response to treatment

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(2). This heterogeneity has been considered to be related to the diversity of the genetic/epigenetic alterations that accumulate in the cancer cells; however, recent evidence has shed new light on the role of the microenvironment in the regulation of the heterogeneous nature of HCC, potentially through paracrine activation of various signaling pathways (3). The HCC microenvironment is composed of diverse stromal cells including lymphocytes, macrophages, stellate cells, fibroblasts and endothelial cells. These cells produce various cytokines, chemokines and growth factors, such as interleukin (IL)-6, IL-8, Wnt, fibroblast growth factors, platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGFs) and transforming growth factor (TGF)- β that activate downstream signaling pathways in cancer cells.

Sorafenib is a multikinase inhibitor targeting the RAF, RET, VEGFR and c-Kit pathways. It has been shown in two large independent clinical cohorts to enhance overall survival (by a median of approximately 3 months) in advanced HCC patients compared to placebo control (4, 5). Because tumor shrinkage has rarely been observed in these trials, the effectiveness of sorafenib is considered to be mainly related to anti-angiogenesis effects through inhibition of VEGFR2 signaling in tumor endothelial cells. However, recent evidence has demonstrated that some HCC patients exhibit complete/partial responses after sorafenib treatment. Interestingly, some of these HCC specimens showed an amplification of fibroblast growth factors 3/4 or VEGFA that may potentially activate stromal cells, such as endothelial cells or fibroblasts, which, in turn, may activate growth signaling in cancer cells through production of growth factors, such as hepatocyte growth factor (6, 7). Therefore, the effectiveness of a molecular-targeted therapy in HCC may be determined, at least in part, by an interaction between the tumor and its microenvironment; however, the role of the microenvironment in HCC chemosensitivity remains to be elucidated.

The PDGF signaling system contributes to tumor angiogenesis and vascular remodeling in solid tumors (8). TSU-68 is a multikinase inhibitor that targets PDGFRs, as well as VEGFR2. The efficacy of TSU-68 is currently being investigated in various solid cancers, including breast, gastric, lung and colorectal (9-12). In addition, a recent phase I/II study has suggested promising preliminary efficacy with a high safety profile in patients with advanced HCC (13). The inhibitory effect of TSU-68 on PDGF receptor phosphorylation at low concentrations suggests its utility in HCC with activated PDGF signaling. Indeed, PDGFRs are expressed in a subset of HCCs (14). However, the role of PDGF signaling in HCC development remains to be fully elucidated (15). Furthermore, it is unclear how and in which type of cells PDGF signaling is activated and how these cells participate in the pathogenesis of HCC (16). Accordingly, in the present study, we evaluated the expression of PDGF and PDGFRs in HCC and fibroblast cell lines to clarify the role of PDGF signaling in HCC. We further evaluated the effects of TSU-68 on the interaction between cancer cells and fibroblasts *in vivo* and used an *in vitro* co-culture system to evaluate the molecular mechanisms of TSU-68 action on HCC.

Materials and Methods

Cell lines and reagents. The human HCC cell lines HuH1 and HuH7 were obtained from the Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan). Hep3B was obtained from the American Type Culture Collection (Manassas, VA, USA). WI-38 fibroblasts were also obtained from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). TSU-68 was provided by Taiho Pharmaceutical (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The expression of selected genes was determined in triplicate using an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The following probes were used for qRT-PCR: *PDGFA*, Hs00964426_m1; *PDGFB*, Hs00234042_m1; *PDGFC*, Hs00910225_s1; *PDGFRA*, Hs00183486_m1; *PDGFRB*, Hs01019589_m1; *TGFB1*, Hs00171257_m1; and *18S*, Hs99999901_s1 (Applied Biosystems).

Western blotting. Whole-cell lysates were prepared using RIPA lysis buffer. Mouse monoclonal antibody to human PDGFR- α clone D13C6 (Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibody to human PDGFR- β clone C82A3 (Cell Signaling Technology), mouse monoclonal antibody to human phospho-PDGFR- α (Thr754) clone 23B2 (Cell Signaling Technology) and mouse monoclonal anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO, USA) were used. Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ, USA), as described previously (17).

Cell proliferation assay. Single-cell suspensions of 2×10^3 cells were seeded in 96-well plates and cell proliferation was evaluated in quadruplicate using a Cell Titer 96 Aqueous kit (Promega, Madison, WI, USA). TSU-68 was dissolved in DMSO and cells were treated with 0.1% DMSO (control) or 10 μ M TSU-68 (0.1% final concentration) for 72 h.

Fluorescence-activated cell sorting analyses. For fluorescence-activated cell sorting (FACS), HuH7 cells were trypsinized, washed and re-suspended in Hank's Balanced Salt Solution (Lonza, Basel, Switzerland) supplemented with 1% HEPES and 2% FBS. Intracellular PDGFR- α protein levels were examined using a Cytotfix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA, USA), as described previously (18). Permeabilized or untreated cells were incubated with phycoerythrin (PE)-conjugated anti-human CD140a (PDGFR- α) antibody clone 16A1 (BioLegend, San Diego, CA, USA) on ice for 30 min. Labeled cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Spheroid formation assay. For co-culture experiments, 1×10^5 HuH7 or WI-38 cells were harvested in Cell Culture Inserts (BD Biosciences) for 24 h. Inserts were then washed twice with phosphate-buffered saline (PBS). Single-cell suspensions of 2×10^3 HuH7 cells were seeded in 6-well Ultra-Low Attachment Plates (Corning, Corning, NY, USA). Cells were incubated with culture media containing 0.1% DMSO (control) or 10 μ M TSU-68 (0.1% final concentration). Co-culture cell inserts were transferred to Ultra-Low Attachment Plates (Corning) and incubated for 14 days. The number of spheroids (>200 μ m in diameter) was measured in triplicate in each group.

Wound-healing assay and time-lapse image analysis. A wound-healing assay was performed using μ -Slide 8-well chambers and culture inserts (Ibidi, Martinsried, Germany) as described previously (19). Briefly, 2×10^3 HuH7 and WI-38 cells were labeled with the lipophilic fluorescence tracer DiO (indicated as green) or DiD (indicated as blue) and incubated in a culture insert in μ -Slide 8-well chambers overnight. Silicone inserts were detached and the culture media was replaced with DMEM containing 10% FBS and 0.1% DMSO (control) or 10 μ M TSU-68. Immediately after the medium change, cells were cultured at 37°C in 5% CO₂ and time-lapse images were captured for 48 h using a CSU-X1 spinning disk confocal (Yokogawa, Tokyo, Japan) and Andor iXon3 EMCCD camera system (Andor Technology, Belfast, UK). Images were analyzed by iQ Software (Andor Technology).

Tumorigenicity in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Cells (1×10^5 HuH7 and 1×10^5 WI-38 cells) were mixed and suspended in 200 μ l of 1:1 DMEM and Matrigel (BD Biosciences) and subcutaneously injected into 6-week-old NOD/SCID mice (NOD/NCR1-Prkdc^{scid}) (Charles River Laboratories, Wilmington, MA, USA). Control vehicle (aqueous carboxymethylcellulose containing 0.9% (w/v) benzyl alcohol, 0.4% (w/v) Tween 80, and 9 mg/ml sodium chloride) or TSU-68 solution (400 mg/kg) was orally ingested twice daily on days 16-20 and 23-28. The sizes and incidence of subcutaneous tumors were recorded as previously described (18). All mice were euthanized on day 31 and tumors were formalin-fixed and paraffin-embedded and used for immunohistochemistry. The protocol was approved by the Kanazawa University Animal Care and Use Committee.

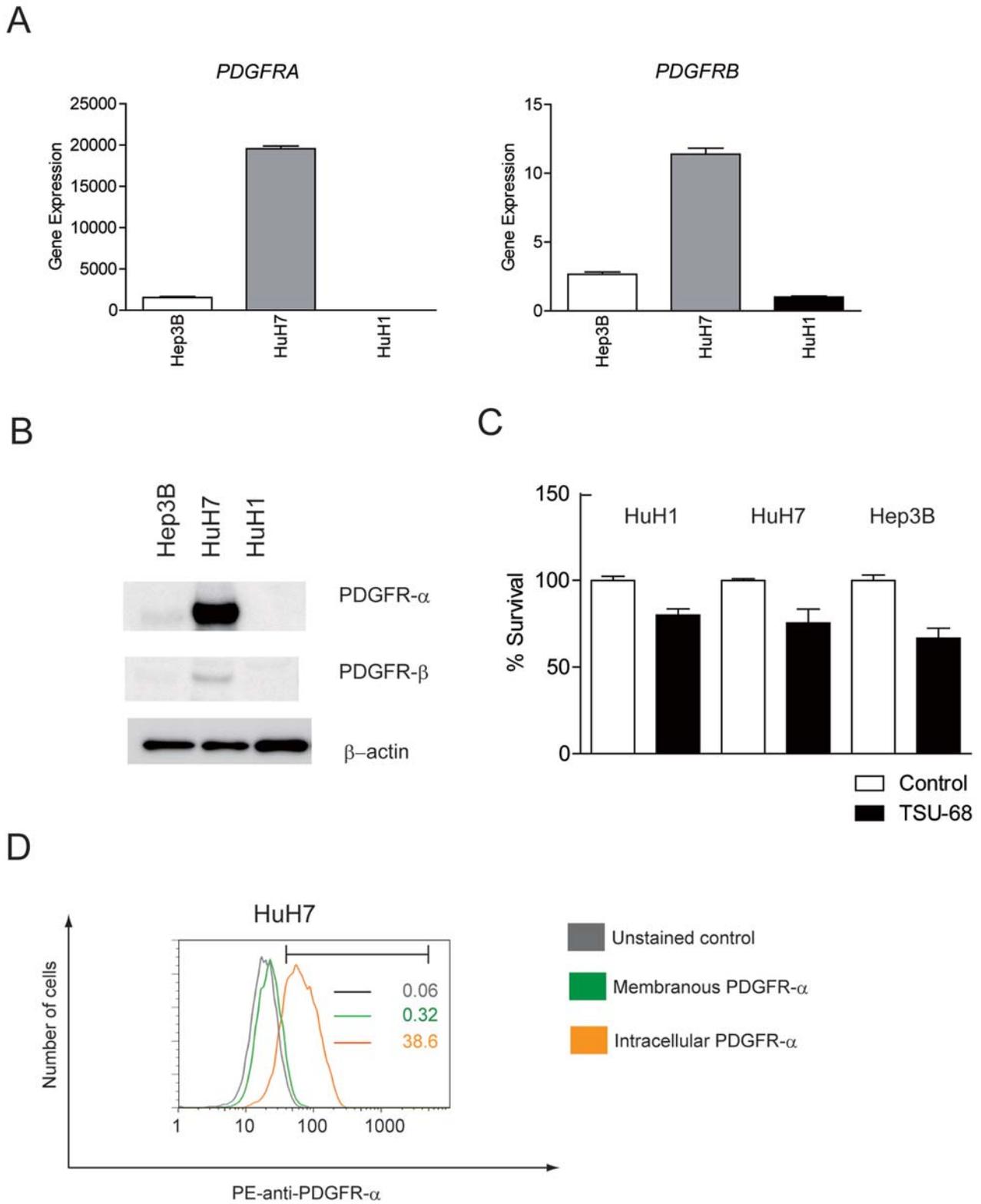


Figure 1. *PDGFR* expression in HCC. **A**, qRT-PCR analysis of *PDGFRA* and *PDGFRB* expression in Hep3B, HuH7 and HuH1 cells. **B**, Western blotting analysis of *PDGFR-α* and *-β* in Hep3B, HuH7 and HuH1 cells. **C**, MTS assay of HuH1, HuH7 and Hep3B cells treated with 0.1% DMSO or 10 μ M TSU-68. **D**, FACS analysis of HuH7 cells stained with PE-conjugated anti-*PDGFR-α* antibodies. Cells were fixed and permeabilized to stain intracellular *PDGFR-α*.

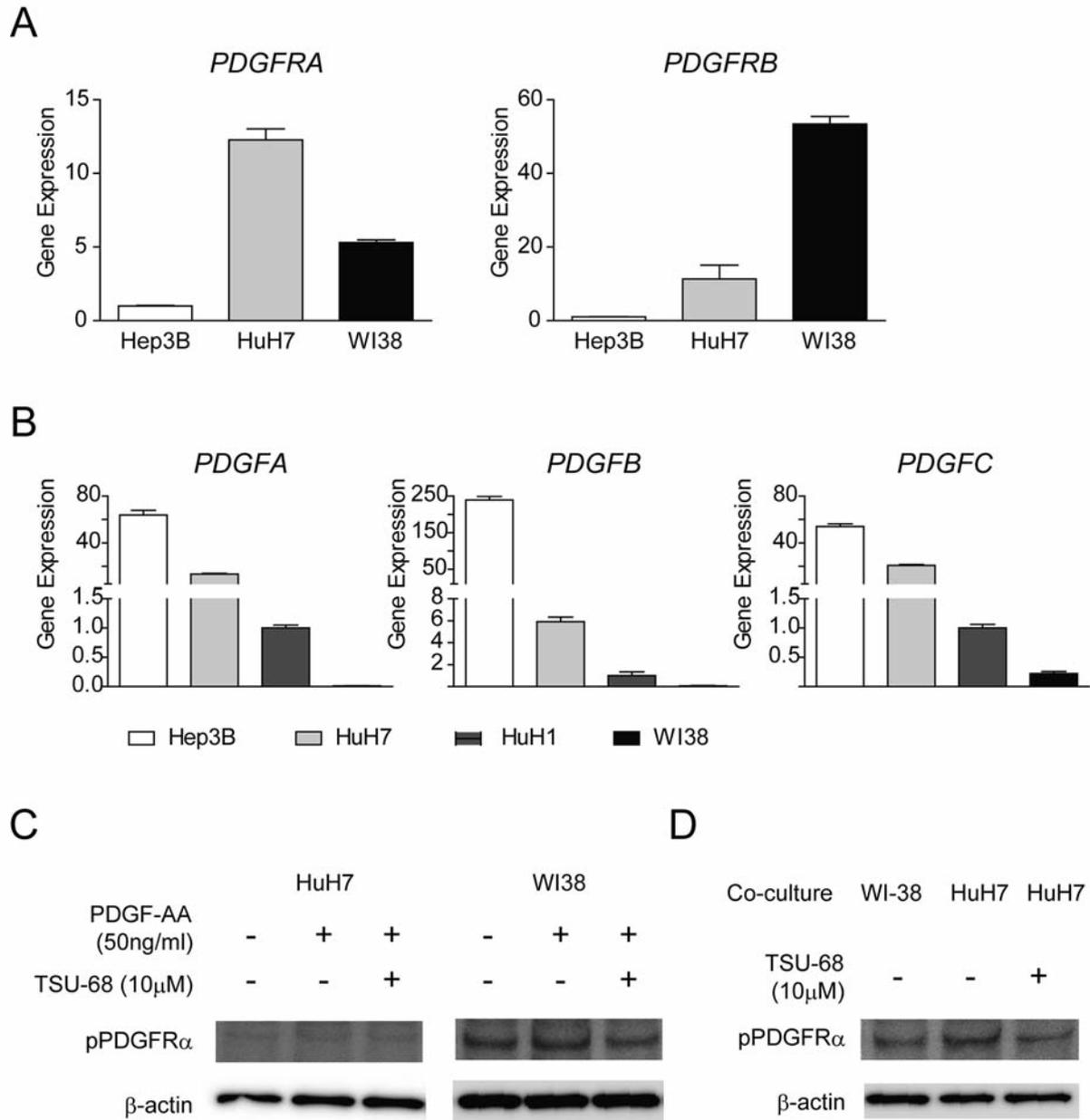


Figure 2. Activation of PDGF signaling in fibroblasts by HCC co-culture. A, qRT-PCR analysis of *PDGFRA* and *PDGFRB* expression in Hep3B, HuH7 and WI-38 fibroblasts. B, qRT-PCR analysis of *PDGFA*, *PDGFB* and *PDGFC* expression in Hep3B, HuH7 and HuH1 HCC cells, as well as WI-38 fibroblasts. C, Western blot analysis of HuH7 and WI-38 cells treated with 50 ng/ml PDGF-AA with or without 10 μ M TSU-68. D, Western blot analysis of WI-38 cells co-cultured with WI-38 or HuH7 cells treated with or without 10 μ M TSU-68 for 48 h.

Immunohistochemistry analysis. Immunohistochemistry (IHC) was performed using mouse anti-Smad3L (Ser 208/213 phosphorylated) rabbit antibodies (Takara Bio Inc., Ohtsu, Japan) and Envision+ kits (Dako USA, Carpinteria, CA, USA), as described previously (18).

Statistical analyses. Student's *t*-tests were performed with the GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA, USA) to compare the various test groups assayed by spheroid assays and qRT-PCR analysis.

Results

PDGFs are expressed but their receptors are absent or not functional in HCC cell lines. To explore the role of PDGF signaling in HCC, we evaluated the expression of genes encoding PDGF receptors (*PDGFRA* and *PDGFRB*) in three human HCC cell lines (Hep3B, Huh1 and Huh7) by qRT-PCR (Figure 1A). We identified a marked expression of

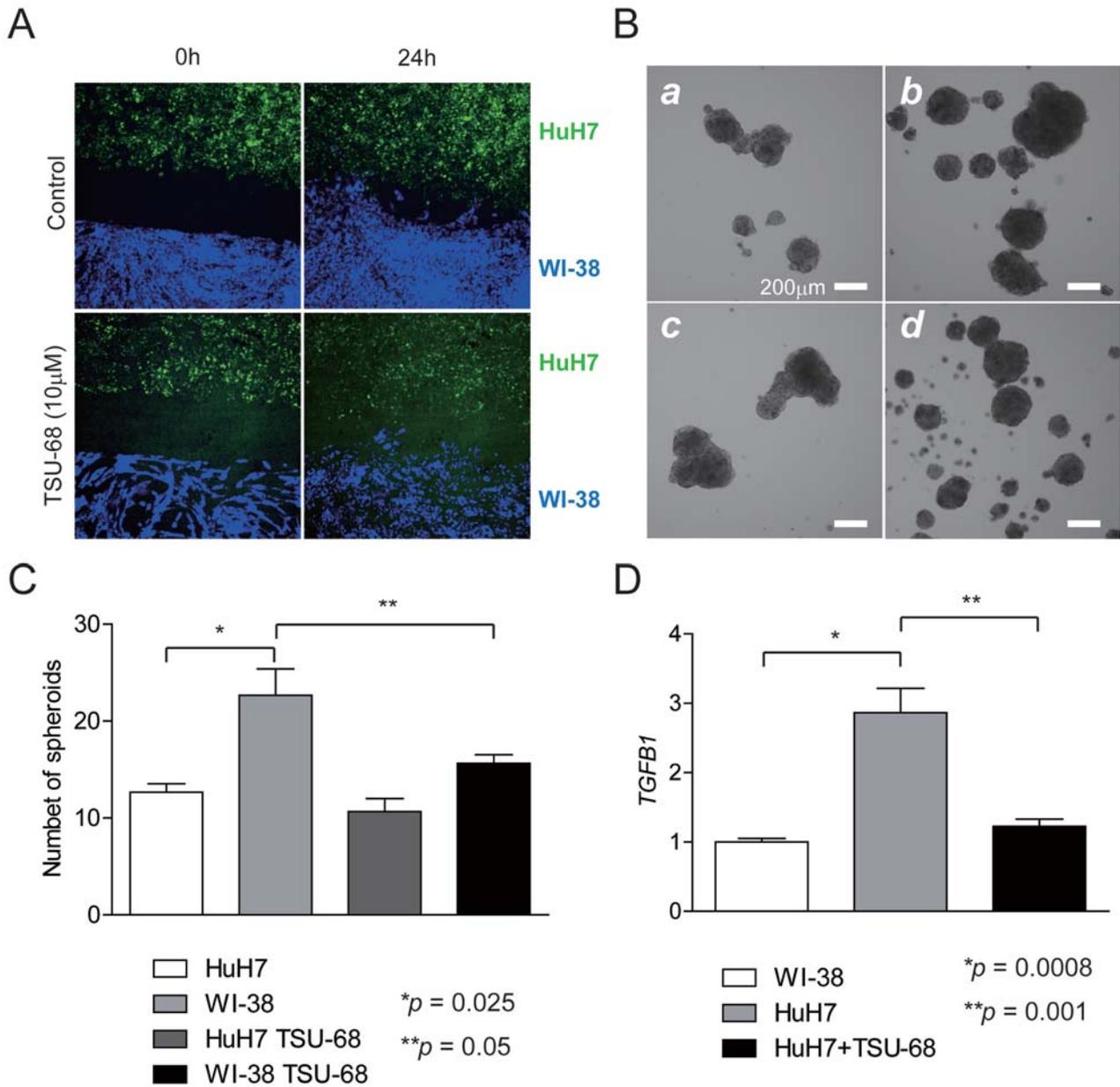


Figure 3. TSU-68 ameliorates malignant features of HCC induced by fibroblasts with activation of PDGF signaling. A, Wound-healing assay of HuH7 (green) and WI-38 cells (blue) treated with or without 10 μM TSU-68 for 48 h. Note the high green background signals of TSU-68-treated cells due to the autofluorescence of TSU-68. B, Spheroid formation assay of HuH7 cells co-cultured with HuH7 cells (panels a and c) or WI-38 fibroblasts (panels b and d) treated with (panels c and d) or without (panels a and b) 10 μM TSU-68 (scale bar: 200 μm). C, Number of spheroids obtained from 2,000 HuH7 cells co-cultured with HuH7 or WI-38 cells and treated with or without 10 μM TSU-68. The number of spheroids with a size of 200 μm or more was counted. D, qRT-PCR analysis of TGFB1 in WI-38 cells co-cultured with WI-38 or HuH7 cells and treated with or without 10 μM TSU-68.

PDGFRA and PDGFRB genes in HuH7 cells compared to HuH1 and Hep3B cells. We further evaluated the protein expression of PDGFR-α and PDGFR-β by western blotting, finding that PDGFR-α and PDGFR-β protein was only detectable in HuH7 cells (Figure 1B). PDGFR-α was more abundantly expressed than PDGFR-β in HuH7 cells,

consistent with a previous report showing the dominant expression of PDGFR-α in primary HCCs (14). We evaluated the effect of TSU-68 on these cell lines, expecting strong chemosensitivity to TSU-68, specifically in HuH7 cells, because TSU-68 inhibits phosphorylation of PDGFRs. However, we identified a reduction in cell proliferation of up

to 20% when all cell lines were treated with 10 μ M TSU-68 (Figure 1C). We could not detect differential chemosensitivity to TSU-68 between HuH1 and HuH7 cells irrespective of a 20,000-fold difference in *PDGFRA* expression and a 10-fold difference in *PDGFRB* expression.

We investigated the subcellular localization of PDGFR- α in HuH7 cells by FACS analysis. Surprisingly, most PDGFR- α expressed in HuH7 cells was located in the cytoplasm and not in the cell membrane (Figure 1D) suggesting that most PDGFR- α expressed in HuH7 cells cannot bind to PDGF ligands. Similar results were obtained for PDGFR- β in HuH7 cells (data not shown). These data suggest that even if PDGFRs are detected in some HCCs, PDGFRs may not always be located at the cell membrane and be in a functioning state.

HCC cell lines produce PDGFs and activate PDGF signaling in fibroblasts in a paracrine manner. We next examined the expression of PDGFRs in WI-38 fibroblasts because fibroblasts are the major stromal cells in liver cirrhosis tissues and HCC. By qRT-PCR, *PDGFRA* expression was detected at a comparable level to that of HuH7 cells, while *PDGFRB* was more abundantly expressed in WI-38 cells (Figure 2A). We also evaluated the expression of PDGF ligands (*PDGFA*, *PDGFB* and *PDGFC*) in HCC cell lines and WI-38 cells. Interestingly, *PDGFA*, *PDGFB* and *PDGFC* were strongly expressed in Hep3B and HuH7 cells compared to HuH1 cells (Figure 2B), even though PDGFRs were not detected or were not functional in these cells. Furthermore, the expression of PDGF ligand genes was extremely low in WI-38 cells, irrespective of the abundant expression of PDGFRs. These data prompted us to hypothesize that HCC cells produce PDGF ligands but do not stimulate themselves in an autocrine manner. Rather, PDGF ligands produced by HCC activate stromal fibroblasts in a paracrine manner and stimulated fibroblasts, in turn, produce growth factors that may enhance the malignant nature of HCC cells.

We evaluated the effect of recombinant PDGF ligands on the phosphorylation of PDGFRs in HuH7 and WI-38 cells. Enhancement of PDGFR- α phosphorylation was clear in WI-38 cells but not in HuH7 cells (Figure 2C). PDGF-AA (50 ng/ml) could not augment the proliferation of HuH7 cells (data not shown). TSU-68 suppressed PDGFR- α phosphorylation in WI-38 cells but had little effect in HuH7 cells. We could not appropriately evaluate PDGFR- β phosphorylation by western blotting in HuH7 and WI-38 cells (data not shown) due to a lack of antibodies suitable for this approach. These data are in line with our hypothesis described above, namely, that PDGF ligands expressed in HCC may mainly activate stromal fibroblasts. We further evaluated the interaction of HuH7 and WI-38 cells using a co-culture system. Phosphorylation of PDGFR- α was enhanced in WI-38 fibroblasts when they were co-cultured

with HuH7 cells compared to those co-cultured with WI-38 cells (Figure 2D). This enhanced phosphorylation was attenuated by TSU-68 treatment.

Fibroblasts stimulated by PDGFs produce TGF- β and regulate hepatocellular carcinoma growth. We evaluated the motility of WI-38 fibroblasts co-cultured with HuH7 cells treated with DMSO control or 10 μ M TSU-68 using a wound-healing assay and time-lapse image analysis (Figure 3A). Cell motility of WI-38 fibroblasts was suppressed by TSU-68 treatment compared to control suggesting a role for PDGF signaling in the activation of fibroblast cell motility. We further evaluated the role of fibroblasts on the spheroid formation of HuH7 cells using a co-culture system. Spheroid formation capacity was significantly enhanced when HuH7 cells were co-cultured with WI-38 cells compared with those co-cultured with HuH7 cells ($p=0.025$) (Figure 3B panels a and b and Figure 3C) suggesting that WI-38 secretes certain growth factors that enhance the spheroid formation capacity of HuH7 cells. TSU-68 treatment had no effect on the suppression of spheroid formation in HuH7 cells when co-cultured with HuH7 cells (Figure 3B panel c). However, TSU-68 treatment modestly suppressed the spheroid formation capacity of HuH7 cells boosted by co-culture with WI-38 fibroblasts ($p=0.05$) (Figure 3B panel d and Figure 3C).

As a candidate growth factor secreted by stimulated fibroblasts, we evaluated the expression of *TGFBI* because we have previously found that endogenous *TGFBI* expression is very low in HuH7 cells and because exogenous supplementation with TGF- β enhances cell motility and distant organ metastasis of HuH7 cells (19). Interestingly, a three-fold increase in *TGFBI* expression was observed when WI-38 cells were co-cultured with HuH7 cells compared with those co-cultured with WI-38 cells (Figure 3D). Furthermore, this enhancement was abolished by TSU-68 treatment suggesting that PDGF ligands secreted by HuH7 cells may be responsible for this induction of *TGFBI* in WI-38 cells.

TSU-68 inhibits PDGF signaling in fibroblasts and suppresses hepatocellular carcinoma growth. Finally, we explored the effect of TSU-68 *in vivo* using a subcutaneous xenotransplantation model in immunodeficient mice. We injected HuH7 and WI-38 cells (1×10^5 each per mice) together into a subcutaneous lesion of NOD/SCID mice to clarify the role of PDGF signaling in the HCC-fibroblast interaction. We initiated oral ingestion of TSU-68 ($n=4$) or control vehicle ($n=4$) 2 weeks after inoculation to clarify the effect of TSU-68 in the early phase of subcutaneous tumor formation. By day 31, three out of four control mice had developed subcutaneous tumors, whereas only one out of four TSU-68-treated mice had developed tumors. Moreover,

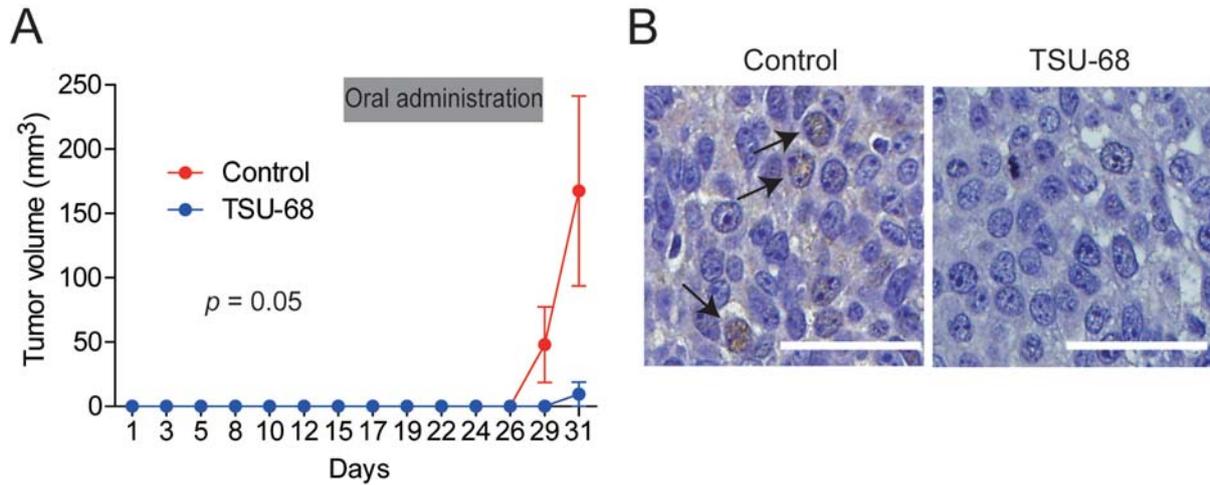


Figure 4. *TSU-68 suppressed the HCC growth in vivo. A, Tumorigenicity of 1×10^5 HuH7 cells co-injected with 1×10^5 WI-38 cells in NOD/SCID mice. Mice were treated with TSU-68 (400 mg/kg, twice daily) or control vehicle for 2 weeks. B, Representative photomicrographs of subcutaneous tumors stained with anti-phospho-Smad3 antibodies (scale bar: 100 μ m). The black arrow indicates nuclear accumulation of phospho-Smad3 in HuH7 cells.*

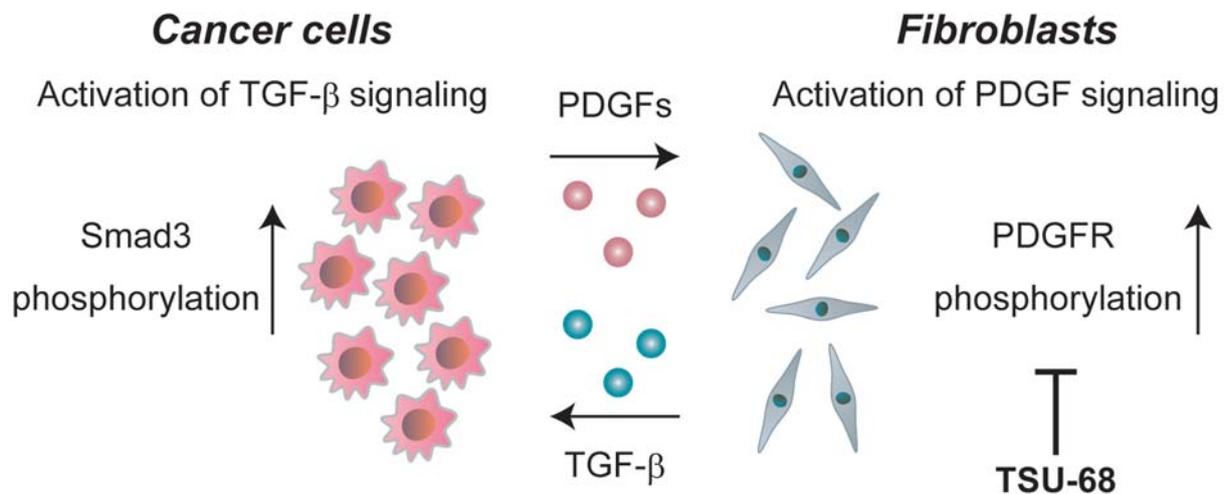


Figure 5. *Schematic of the cancer cell-fibroblast interaction and PDGF signaling in HCC. HCC cells produce PDGF ligands and phosphorylate PDGFRs in fibroblasts. Activated fibroblasts produce TGF- β and phosphorylate Smad3 in HCC cells. TSU-68 inhibits PDGFR phosphorylation in fibroblasts, suppresses the expression of TGF- β and inhibits HCC growth.*

the tumor volume differences were statistically significant ($p=0.05$) (Figure 4A).

We identified nuclear accumulation of phosphorylated Smad3 in the tumor in the control group suggesting activation of TGF- β signaling in HuH7 cells when co-injected with WI-38 (Figure 4B, left panel). We could not detect nuclear accumulation of phosphorylated Smad3 in the tumors that developed in TSU-68-treated mice (Figure 4B, right panel). Taken together, these data suggest a role for PDGF signaling in HCC (Figure 5). HCC cells secrete PDGF ligands and PDGF signaling is activated in stromal fibroblasts to produce

TGF- β , which, in turn, activates TGF- β signaling in HCC in a paracrine manner. TSU-68 may target the activated stromal fibroblasts and, therefore, may be most effective in HCC with abundant stromal fibroblasts such as scirrhous HCC.

Discussion

Recent evidence suggests the pivotal roles of stromal cells in tumor initiation, promotion, progression and chemoresistance in several cancers (3). PDGFs and PDGFRs are frequently expressed in various cancers and may regulate tumor growth,

invasiveness and chemoresistance (8). In this study, we found that, although PDGFs are abundantly expressed, PDGFRs are expressed at quite low levels or are not functional in three HCC cell lines. PDGFs activate PDGFRs in WI-38 fibroblasts and stimulated fibroblasts produce TGF- β and regulate the aggressive nature of HuH7 cells. This is the first study to demonstrate that TSU-68, a kinase inhibitor of PDGFRs, mainly acts on stromal fibroblasts to block the production of TGF- β and inhibit HCC growth.

The PDGF family consists of four structurally-related single polypeptides, encoded by *PDGFA*, *PDGFB*, *PDGFC* and *PDGFD* that form five functional homo- or heterodimers: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD. PDGFRs are cell surface tyrosine kinase receptors composed of PDGFR- α and PDGFR- β , encoded by *PDGFRA* and *PDGFRB*, that form homo- or heterodimers: PDGFR- $\alpha\alpha$, PDGFR- $\alpha\beta$ and PDGFR- $\beta\beta$. A previous study investigated the expression of PDGFR- α , - β and its ligand genes *PDGFA*, *PDGFB* and *PDGFC* in fetal liver and HCC, finding the expression of PDGFR- α in 10 of 22 HCCs by western blotting and 17 of 21 HCCs by IHC (14). Although we could not reliably evaluate the activation of PDGFR- β (phospho-PDGFR- β), we identified the over-expression of PDGFR- α in one out of three HCC cell lines. However, most PDGFR- α protein was located at the cytoplasm and was not functional in HuH7 cells. Indeed, the photomicrographs of the previous report also showed cytoplasmic PDGFR- α staining in human HCC tissues (14).

Because we evaluated the expression of PDGFR- α and PDGFR- β in only three HCC cell lines and one fibroblast cell line, we cannot conclude that PDGF signaling is generally defective in human HCC. Besides, we could not reliably evaluate the PDGFR- β status due to the lack of available appropriate antibodies, including those used in the previous publication. Further studies are required to evaluate the function of PDGFRs expressed in a subset of HCCs.

Our data suggest that HCC cell lines produce PDGF ligands and activate stromal fibroblasts. TSU-68 suppressed PDGFR- α phosphorylation, inhibited the expression of *TGFB1* *in vitro* and suppressed Smad3 phosphorylation and tumor growth *in vivo*. Although the tumor suppressive effects of TSU-68 may be mainly related to anti-angiogenic effects exerted by inhibiting VEGFR2 *in vivo*, our data demonstrated that TSU-68 also suppressed PDGFR phosphorylation in stromal fibroblasts and inhibited TGF- β production. A previous study demonstrated the activation of TGF- β signaling in scirrhous HCC with abundant fibrous stromal component and poor prognosis (20). TSU-68 may, thus, effectively treat this HCC subtype; however, further studies are required to evaluate the efficacy of TSU-68 in various HCC subtypes with distinct molecular features and prognosis.

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