Transforming Growth Factor-β and Substrate Stiffness Regulate Portal Fibroblast Activation in Culture

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Myofibroblasts derived from portal fibroblasts are important fibrogenic cells in the early stages of biliary fibrosis. In contrast to hepatic stellate cells, portal fibroblasts have not been well studied *in vitro,* and little is known about their myofibroblastic differentiation. In this article we report the isolation and characterization of rat portal fibroblasts in culture. We demonstrate that primary portal fibroblasts undergo differentiation to α-smooth muscle actin–expressing myofibroblasts over 10–14 days. Marker analysis comparing portal fibroblasts to hepatic stellate cells demonstrated that these are distinct populations and that staining with elastin and desmin can differentiate between them. Portal fibroblasts expressed elastin at all stages in culture but never expressed desmin, whereas hepatic stellate cells consistently expressed desmin but never elastin. Immunostaining of rat liver tissue confirmed these results *in vivo.* Characterization of portal fibroblast differentiation in culture demonstrated that these cells required transforming growth factor-β (TGF-β): cells remained quiescent in the presence of a TGF-β receptor kinase inhibitor, whereas exogenous TGF-β1 enhanced portal fibroblast α-smooth muscle actin expression and stress fiber formation. In contrast, platelet-derived growth factor inhibited myofibroblastic differentiation. Portal fibroblasts were also dependent on mechanical tension for myofibroblastic differentiation, and cells cultured on polyacrylamide supports of variable stiffness demonstrated an increasingly myofibroblastic phenotype as stiffness increased. *Conclusion:* Portal fibroblasts are morphologically and functionally distinct from hepatic stellate cells. Portal fibroblast myofibroblastic differentiation can be modeled in culture and requires both TGF-β and mechanical tension. (HEPATOLOGY 2007;46:1246-1256.)

Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) proteins, the result of a combination of increased expression and decreased degradation of matrix components.[1] Abnormal ECM in liver fibrosis is produced by myofibroblasts, defined as fibrogenic, α-smooth muscle actin (α-SMA)–expressing cells. The source of myofibroblasts in liver fibrosis is the subject of some controversy. Although in chronic liver injury hepatic stellate cells (HSCs) differentiate into myofibroblasts (also termed activation) and produce ECM,[2] the myofibroblast population of the diseased liver is heterogeneous, and fibrogenic myofibroblasts also arise from other cells, including portal fibroblasts (PFs).[3-10] PFs are fibroblasts surrounding the biliary tree; their differentiation into myofibroblasts in bile duct ligation (BDL) models of fibrosis precedes HSC activation, and they may function as first responders after biliary injury.[11-14]

Isolation of rat liver myofibroblast precursor cells distinct from HSCs was first reported in 1984. These cells were shown by electron microscopy to have the features of myofibroblasts after 1 month in culture, and in retrospect it is likely they were PFs.[15] PFs have also been isolated by outgrowth from dissected, HSC-free segments of bile duct.[16] Cells isolated by this technique and cultured by standard methods rapidly expressed α-SMA and type I collagen although they were clearly distinct from HSCs

Abbreviations: α-SMA, α-smooth muscle actin; FBS, fetal bovine serum; CRBP, cellular retinol–binding protein; HSC, hepatic stellate cell; NTPDase2, nucleoside triphosphate diphosphohydrolase-2; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PF, portal fibroblast; TGF-β, transforming growth factor.

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by marker analysis.[17] Isolation of PFs from rat liver by perfusion and size selection has been described, and the ecto-ATPase nucleoside triphosphate diphosphohydrolase-2 (NTPDase2) has been shown to be a specific marker for PFs before myofibroblastic differentiation, distinguishing them from quiescent HSCs.[18,19]

Little is known about the process by which PFs undergo myofibroblastic differentiation, although soluble factors appear to play an important role. Exposure to platelet-derived growth factor (PDGF) enhanced α-SMA expression in PFs treated on day 4 and studied on day 7.[17] The chemokine monocyte chemotactic protein-1 was shown to induce α-SMA expression and procollagen I transcription 1 day after isolation, although it had no effect at later times.[20] Transforming growth factor β (TGF-β) is likely to be important in PF myofibroblastic differentiation given its well-established role in inducing α-SMA expression in fibroblasts derived from multiple tissue types. The role of TGF-β in HSC activation is limited—it regulates α-SMA organization but not α-SMA expression[21]—but passaged HSCs and PFs respond differently to TGF-β, and thus TGF-β may also act differently during their differentiation into myofibroblasts.[22] Importantly, biliary injury induces bile duct cells to release large amounts of TGF-β in the direct vicinity of PFs.[23]

The role of other factors, specifically mechanical factors, in PF activation is also not known. Mechanical tension as a mediator of cell phenotype has recently received considerable study. Pioneering work from Pelham and Wang demonstrated that 3T3 fibroblasts seeded on a soft surface were less well spread, with fewer actin stress fibers, than were cells seeded on stiff surfaces, and this work has since been extended by others using multiple cell types.[24-28] The importance of mechanical factors specifically for myofibroblast differentiation has been demonstrated as well: human gingival fibroblasts differentiate into myofibroblasts on stiff as opposed to soft collagen substrates, and the number of α-SMA-containing stress fibers is correlated with matrix stiffness.[29] We have shown that activation of HSCs increases as supports become stiffer and have suggested that understanding mechanical changes in the chronically injured liver may be key to understanding fibrosis, particularly its early stages (manuscript submitted).

Given the potential importance of PFs in biliary fibrosis, our goal was to characterize a culture model of PF myofibroblastic differentiation to mirror the widely used *in vitro* model of HSC differentiation and to determine the relative importance of soluble and mechanical factors in determining PF phenotype.

Materials and Methods

Materials. Cell culture reagents were obtained from Gibco BRL (Carlsbad, CA), fetal bovine serum (FBS) from Gemini Bio-Products (West Sacramento, CA), and type I collagen from BD Biosciences (Bedford, MA). The antibodies used were α-SMA (clone 1A4) and desmin (Sigma, St. Louis, MO), elastin (Cedarlane Laboratories Limited, Hornby, Ontario, Canada), glial fibrillary acidic protein (GFAP) and cytokeratin wide-spectrum screening antibody (pan-CK; Dakocytomation Inc., Carpinteria, CA), CD34 and CD68 (BD Biosciences), retinol–binding protein (CRBP; clone G4E4, Lab Vision Corp., Fremont, CA), Ki-67 (Vector Laboratories, Burlingame, CA), and Cy-2- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Rabbit NTPDase2 antibodies were generated as described.[30,31] The TGF-β type I receptor kinase inhibitor NPC-34016 was a kind gift from David Liu (Scios Inc., Fremont, CA), and the PDGF receptor kinase inhibitor AG-1295 was obtained from Calbiochem (San Diego, CA). Growth factors were obtained from R&D Systems (Minneapolis, MN), all-*trans*-retinol from Fluka (Buchs, Switzerland), Oil Red O 0.5% solution in propylene glycol from Poly Scientific (Bay Shore, NY), and acrylamide solutions from National Diagnostics (Atlanta, GA).

Isolation of PFs and HSCs. PFs were isolated from male retired breeder Sprague-Dawley rats (500–750 g).[19] Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg), the livers were perfused *in situ* with collagenase (300 mg/L, Worthington, Lakewood, NJ), and the hepatic hila were removed manually from dissociated parenchymal elements and then digested serially with a 0.033% pronase (Roche, Chicago, IL) and 0.036% hyaluronidase (Sigma) solution. Cell suspensions were filtered through a 30-μm-pore mesh (Sefar America, Inc., Kansas City, MO). The resulting suspension of nonparenchymal cells was plated in Dulbecco's modified medium/F-12 with 10% FBS. Primary HSCs were isolated from the male retired breeder Sprague-Dawley rats and cultured as described previously.[21,32] All work with animals was approved by the University of Pennsylvania Institutional Animal Care and Use Committee and done in accordance with National Institutes of Health guidelines. All the cells used in any given experiment in which PFs or HSCs were studied over time were isolated from a single animal.

Growth Factor Treatment. PFs were isolated as described in the previous section. After overnight incubation, cells were washed with serum-free medium and then incubated with 100 pM TGFβ1, 5 μM type I TGF-β kinase inhibitor (NPC-34016), 20 ng/mL PDGF-BB, or 10 μM PDGF receptor kinase inhibitor AG-1295, all in

cell culture medium with 3% FBS. The medium was changed and additives replenished every 2 days. On day 7 the cells were either fixed for immunostaining or harvested for real-time PCR. Washout experiments demonstrated that the inhibitors were nontoxic to PFs at the concentrations used (data not shown).

Preparation of Polyacrylamide Gels. Polyacrylamide gels of variable stiffness were prepared on glass coverslips using a modification of a method previously described.[24,25] Gels were a mixture of 7.5% acrylamide and 0.01%–0.3% *bis*-acrylamide (with a final thickness of approximately 100 μm) impregnated with 0.5 mg/mL sulfosuccinimidyl-6-(4′-azido-2′-nitrophenyl-amino) hexanoate (Sulfo-SANPAH; Pierce Biotechnology, Rockford, IL). A thin layer of type I collagen (0.2 mg/mL) was crosslinked to the polyacrylamide gels at 4°C overnight. Excess matrix proteins were washed off with 50 mM Hepes, and the crosslinker was blocked with 1% ethanolamine. Gels were soaked in serum-free culture medium for at least 2 hours at 37°C before cells were plated. It has previously been demonstrated that the layer of matrix protein does not alter the overall stiffness of the system, that these concentrations of matrix protein allow maximal cell adhesion, and that cells deposit minimal amounts of additional matrix on the supports.[25,33]

Microscopy and Morphological Measurements. Phase-contrast images of cells were taken using an inverted microscope with IPLab software (Scanalytics, Fairfax, VA) and a Hamamatsu digital camera. Stained cells were visualized using an inverted microscope with a QICAM digital camera (QImaging, Burnaby, British Columbia, Canada). Cell area was calculated after tracing cell boundaries manually using IPLab software.

Immunostaining. PFs cultured on chamber slides were fixed with acetone/methanol at −20°C for 8 minutes and were permeabilized in 0.1% Triton X-100 for 3 minutes. After blocking with 1% bovine serum albumin (BSA) at room temperature for 30 minutes, slides were incubated with primary antibodies against elastin (1:200), NTPDase2 (1:200), desmin (1:1,000), CD34 (ready-to-use solution), CD68 (ready-to-use solution), α-SMA (1:1,600), GFAP (1:500), CRBP (1:200), or pan-CK (1:200) either at 4°C overnight or at 37°C for 45 minutes, then incubated with Cy3-conjugated secondary antibodies (1:400) for 1 hour at room temperature, followed by 4,6-diamidino-2-phenylindole (DAPI, 1:10,000; Molecular Probes, Eugene, OR) for nuclear staining.

PFs cultured on polyacrylamide gels were fixed in 1% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature and permeabilized in 0.1% Triton X-100 for 3 minutes followed by immunofluorescence staining.

Immunostaining Staining of Rat Tissue. Sprague-Dawley rats weighing 250–300 g underwent BDL with placement of double ligatures. Normal animals, animals 6 days post-BDL, and animals 4 weeks post-BDL were euthanized and the livers removed, fixed in formalin, and embedded in paraffin. For immunological staining, sections were deparaffinized, treated with 0.5% hyaluronidase for 1 hour at room temperature, and incubated with elastin antibody (1:200) overnight at 4°C. This was followed by a 20-minute treatment with 1.5% H_2O_2 to inhibit endogenous peroxidases, incubation with a second antibody for 30 minutes at 37°C, and then incubation with an avidin-biotin-peroxidase complex (Vectastain ABC-HP-kit, Vector Laboratories, Burlingame, CA). Slides were developed with diaminobenzidine and counterstained with hematoxylin. Control specimens stained with secondary antibody but not primary antibody were used to confirm the specificity of the antibody (data not shown).

For immunofluorescence staining, rat livers were embedded in OCT compound, snap-frozen in liquid nitrogen, and cut into 5-mm-thick cryostat sections. Slides were fixed in ice-cold acetone for 10 minutes at −20°C, blocked, and then labeled with primary antibody (NTPdase2, 1:500, or elastin, 1:200) at 4°C overnight. Blocking was with 50 mM NH_4Cl and 3% (v/v) goat serum in PBS for NTPDase2. For elastin, antigen retrieval was carried out with 0.5% hyaluronidase for 1 hour at room temperature, followed by blocking with 3% BSA and 0.9% sodium chloride in Tris buffer (pH 7.6). After washing with PBS, samples were labeled with desmin (1:1,000) at 37°C for 45 minutes, then washed and incubated with a mixture of secondary antibodies (Cy3-conjugated antirabbit and Cy2-conjugated antimouse antibodies, 1:400) at 37°C for 30 minutes.

Treatment with Retinol and Staining with Oil Red O. HSCs or PFs were isolated and plated as already described. After 10 days of culture in standard media, cells were treated for 24 hours with 25 μM all-*trans*-retinol as described previously.[34] Cells were then washed with PBS, fixed with 10% formalin for 15 minutes, incubated with oil red O solution for 1 hour at room temperature, and treated with 85% propylene glycol for 2 minutes.

Real-Time PCR. mRNA expression was determined using 1-step real-time PCR (FullVelocity SYBR Green master mix, Stratagene, La Jolla, CA). PFs were seeded in 6-well dishes and harvested at the times noted. Harvesting of cells on polyacrylamide gels was done on day 10. RNA was isolated using a Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA). Primers (Supplementary Table 1)

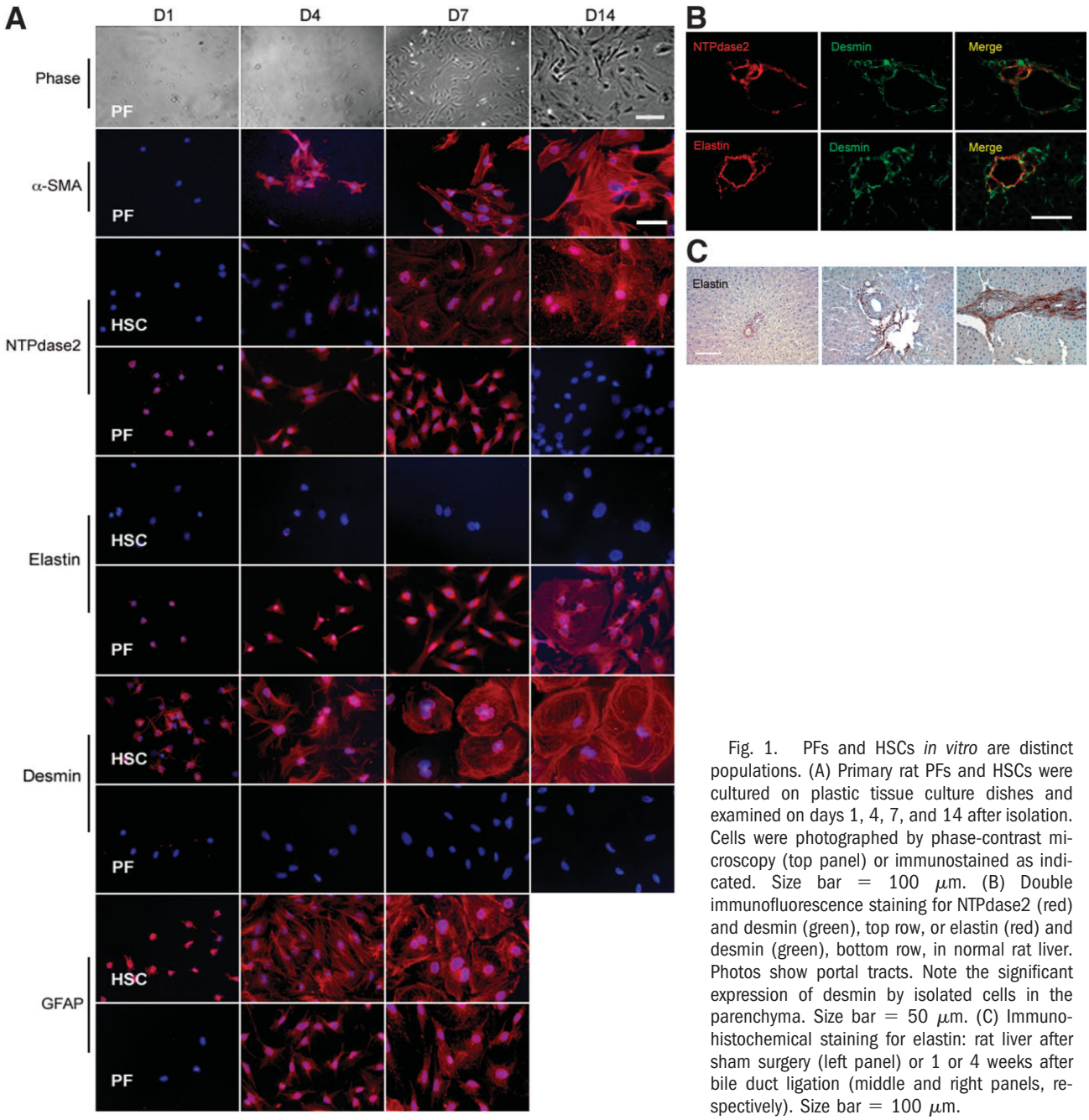

Fig. 1. PFs and HSCs *in vitro* are distinct populations. (A) Primary rat PFs and HSCs were cultured on plastic tissue culture dishes and examined on days 1, 4, 7, and 14 after isolation. Cells were photographed by phase-contrast microscopy (top panel) or immunostained as indicated. Size bar = 100 μm. (B) Double immunofluorescence staining for NTPdase2 (red) and desmin (green), top row, or elastin (red) and desmin (green), bottom row, in normal rat liver. Photos show portal tracts. Note the significant expression of desmin by isolated cells in the parenchyma. Size bar = 50 μm. (C) Immunohistochemical staining for elastin: rat liver after sham surgery (left panel) or 1 or 4 weeks after bile duct ligation (middle and right panels, respectively). Size bar = 100 μm.

were obtained from Invitrogen (Carlsbad, CA). The cycle number (C_T) at which amplification entered the exponential phase was determined and used as an indicator of the amount of target RNA.

Measurement of Proliferation. The proliferation of PFs on polyacrylamide gels was measured by Ki-67 immunostaining. Cells were fixed in 1% formaldehyde in PBS for 15 minutes at room temperature and permeabilized in 0.1% Triton X-100 for 3 minutes, then denatured in 2 N HCl for 30 minutes. After extensive washing, cells were incubated with Ki-67 antibody (1:5000) at 4°C, followed by incubation with Cy3-conjugated secondary antibodies. Nuclei were stained with DAPI. To measure the effects of growth factors on PF proliferation on polyacrylamide gels, cells were first cultured on 12-kPa gels for 4 days, starved overnight in serum-free medium, treated with TGF-β1, PDGF, and the kinase inhibitors for 24 hours, and then fixed and stained as already described. Ki-67-positive cells were counted in 5 random fields (200×); the proliferation index was calculated as the number of Ki-67-positive cells per 100 DAPI-stained nuclei.

Statistical Analysis. Data are expressed as mean ± standard deviation (SD). Means of different groups were

Table 1. Summary of Markers Expressed in PFs and HSCs in Culture

	PF				HSC			
	d1	d4	d7	d14	d1	d4	d7	d14
NTPdase2	+	+	+	−	−	±	+	+
Desmin	−	−	−	−	+	+	+	+
Elastin	+	+	+	+	−	−	−	−
GFAP	−	+	+	ND	+	+	+	ND
CRBP-1	+	+	+	ND	+	+	+	ND

ND, not done.

compared using 1-way analysis of variance. Statistical analysis was performed using the unpaired Student *t* test.

Results

Portal Fibroblasts Are Distinct from HSCs. To study PF myofibroblastic differentiation *in vitro* and to demonstrate that PFs and HSCs generate distinct populations of myofibroblasts, we first determined which markers were expressed in our cells. Freshly isolated PFs were cultured on standard plastic tissue culture dishes for 14 days. Cells were round on day 1 after isolation but adopted a progressively spread-out morphology and expressed α-SMA (Fig. 1A, top 2 rows).

Cells were labeled with antibodies against NTPDase2. This was previously shown to be a specific marker for PFs early after isolation, although known to be lost as they activate.[19] In culture, PFs expressed NTPDase2 through day 7, but not on day 14. GFAP was expressed by PFs from day 4 onward (Fig. 1A). Elastin, however, appeared to be a marker for PFs at all stages, as has been suggested by histological staining.[35] In contrast, HSCs did not express elastin at any point in culture, although they expressed desmin and GFAP (Fig. 1A). HSCs expressed NTPdase2 beginning on day 4, indicating that NTPDase2 is specific for PFs only when comparing PFs and HSCs early after isolation. The marker analysis for PFs and HSCs is summarized in Table 1. The analysis suggested that elastin is specific for PFs and desmin for HSCs and that staining with both markers can differentiate between the 2 cell types at all stages in culture. The data also demonstrated clearly that PFs and HSCs in culture are distinct and nonoverlapping populations. We confirmed by antibody staining that PFs failed to express a variety of nonparenchymal cell markers including CD68 (a marker of Kupffer cells), pan-CK (which stains rodent biliary epithelial cells), and the endothelial cell marker CD34 (data not shown). PFs did not contain lipid droplets at any time. From the marker analysis, we estimated our cultures to be 90%–92% pure on day 1 and nearly 100% pure by the 4th day after isolation.

Immunostaining of normal rat liver confirmed *in vivo* that PFs are distinct from HSCs. Expression of elastin and NTPDase2 was limited to the portal tract of the normal liver, whereas desmin was found in the parenchyma as well as in connective tissue of the portal tract (Fig. 1B). Although there was some colocalization of desmin and NTPDase2, no colocalization was observed after double staining with antibodies against desmin and elastin. This is consistent with our *in vitro* results. *In vivo* as in culture, NTPDase2 appears to be less specific than elastin.[18,36,37] In fibrotic rat liver, elastin immunoreactivity was observed only in the portal region and in the fibrotic scar, even 1 and 4 weeks after bile duct ligation, with none observed in the parenchyma (Fig. 1C).

PFs Do Not Take Up Retinol. To further compare HSCs and PFs and to evaluate the possibility that PFs represent a subpopulation of HSCs lacking vitamin A, cells were stained for CRBP, a protein involved in retinoid metabolism. Both HSCs and PFs expressed CRBP and this expression increased with differentiation (Fig. 2A). This was confirmed by real-time PCR (Fig. 2B). Peroxisome proliferator–activated receptor γ (PPAR-γ, a regulator of adipogenic differentiation, was down-regulated in HSCs but unchanged in PFs during the course of myofibroblastic differentiation, although the 2 cell types had similar absolute levels of mRNA by day 14 (Fig. 2B). When HSCs and PFs were cultured in media containing 25 μM all-*trans*-retinol for 24 hours, HSCs but not PFs developed lipid droplets (Fig. 2C), clearly demonstrating that only HSCs take up retinol.

PFs Differentiate to Myofibroblasts in Culture. PFs undergo myofibroblastic differentiation in culture, as shown by staining with the myofibroblast marker α-SMA (Fig. 1A). PFs demonstrated progressively increased α-SMA protein expression beginning on day 4; by day 14, α-SMA expression was significant, and the protein was organized in stress fibers. Real-time PCR also illustrated the development of α-SMA expression with time in culture (Fig. 3A). Synthesis of procollagen I (Fig. 3B) and procollagen III (Fig. 3C) increased as PFs acquired a myofibroblast phenotype, but expression of collagen IV mRNA decreased significantly (Fig. 3D). Collectively, the preceding data show that PFs can be isolated in pure cultures, that they are distinct from HSCs, and that their myofibroblastic differentiation, like that of HSCs, might be useful for understanding the cellular basis of fibrosis.

PFs Require TGF-β for Myofibroblastic Differentiation. To determine whether TGF-β is required for PF activation, we treated cells from day 1 to day 7 with TGF-β1 or a TGF-β receptor kinase inhibitor. TGF-β1-treated cells spread out more rapidly and demonstrated more pronounced α-SMA stress fiber organization than

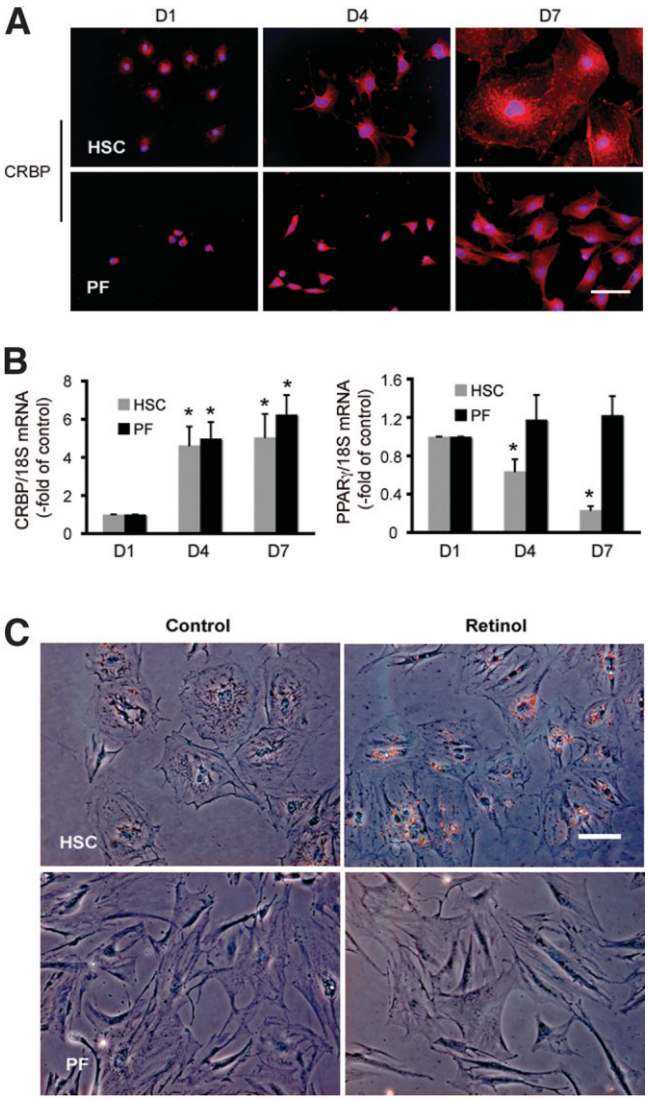

Fig. 2. HSCs, but not PFs, took up retinol. (A) Primary rat PFs and HSCs were cultured on plastic tissue culture dishes and stained with an antibody against CRBP on days 1, 4, and 7 after isolation. Size bar = 100 μm. (B) HSCs and PFs on days 1, 4, 7, and 14 were lysed and analyzed by real-time PCR for expression of CRBP and PPAR-γ. Data represent the mean $\pm$ SD of 2 independent experiments in which each sample was analyzed twice ($^*P < 0.01$ compared with HSCs or PFs on day 1, respectively). (C) HSCs and PFs were isolated and cultured for 10 days in growth medium, then incubated in media with or without 25 μM all-*trans*-retinol for 24 hours. Cells were stained with Oil Red O to detect lipid droplets and then photographed by phase=contrast microscopy. Size bar = 100 μm.

did untreated cells (Fig. 4A). Cells treated with TGF-β2 responded similarly (data not shown). Cells treated with the TGF-β receptor kinase inhibitor, which inhibits autocrine TGF-β signaling, failed to differentiate into myofibroblasts, as assessed by morphology and α-SMA expression (Fig. 4B).

PDGF Inhibits Myofibroblastic Differentiation. Given previous reports that PDGF enhances activation of PFs,[17] we treated primary cells with either PDGF or a PDGF receptor kinase inhibitor, akin to the experiments with TGF-β. Surprisingly, we observed that PDGF inhibited PF differentiation into myofibroblasts and that differentiation was enhanced in the presence of the kinase inhibitor (Fig. 4C). Real-time PCR confirmed these results, demonstrating that expression of α-SMA mRNA increased with inhibitor treatment and decreased with PDGF treatment (Fig. 4D).

PFs Undergo Myofibroblastic Differentiation as Substrate Stiffness Increases. We previously demonstrated that HSC myofibroblastic differentiation is dependent on substrate stiffness (manuscript submitted). To determine whether PFs respond similarly, we cultured primary PFs as well as HSCs on polyacrylamide supports of variable stiffness coated with thin layers of type I collagen. Similar culture systems have been used to investigate the effects of substrate stiffness on many other types of cells,[24,25] and the range of stiffness employed in the present study (from 400 Pa to 12 kPa) mirrored the range of stiffness seen in normal and cirrhotic human and rat livers (data not shown). After 10 days of culture, the HSCs and PFs on the softest supports (400 Pa) both retained the phenotypic appearance of freshly isolated cells (Fig. 5A). Ki-67 staining confirmed that the cells were viable and proliferative, with a proliferation index of greater than 40% for PFs in the presence of 10% FBS (Fig. 6A and data not shown). Cells on the stiffest supports appeared myofibroblastic, whereas cells on supports of intermediate stiffness showed a stable intermediate morphology. Perimeter tracing showed that the average cell area of both PFs and HSCs increased with the stiffness of the support (Fig. 4B). α-SMA immunostaining and real-time PCR confirmed that PFs on increasingly stiff

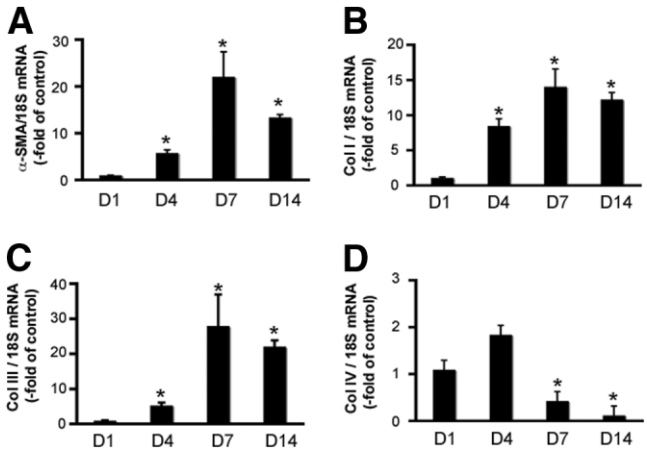

Fig. 3. PF undergo activation in culture. PFs on days 1, 4, 7, and 14 were lysed and analyzed by real-time PCR for expression of (A) α-SMA, (B) procollagen I, (C) procollagen III, and (D) procollagen IV. Data represent the mean $\pm$ SD of 3 independent experiments in which each sample was analyzed twice ($^*P < 0.01$ compared with cells on day 1).

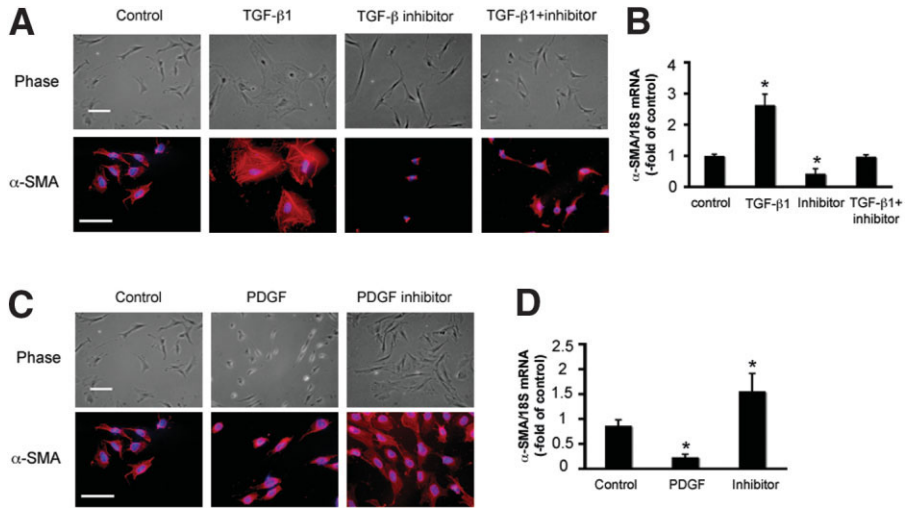

Fig. 4. TGF-β and PDGF have opposing effects on myofibroblastic differentiation. PFs were isolated and cultured on plastic tissue culture dishes in standard media. After overnight incubation, cells were washed with serum-free medium and then incubated in cell culture medium with 3% FBS plus either 100 pM TGFβ1, 5 μM TGF-β receptor kinase inhibitor, 20 ng/mL PDGF, or 10 μM PDGF receptor kinase inhibitor. (A, C, upper panels) After 6 days of treatment, cells were photographed by phase-contrast microscopy and then either (A, C, lower panels) stained for α-SMA or (B, D) lysed and analyzed for α-SMA mRNA expression by real-time PCR (where data represent the mean $\pm$ SD of 2 and 3 independent experiments in which each sample was analyzed twice; $^{*}P < 0.05$ compared with untreated control). Size bar = 100 μm.

gels had increased α-SMA expression and stress fiber organization (Fig. 5C,D), and functional analysis demonstrated that cells on stiff supports were highly fibrogenic (Fig. 6B–D). These changes with increasing matrix stiffness are similar to those seen with time as PFs differentiate on plastic (compare Fig. 6B–D with Fig. 3B–D). Thus, like HSCs, PFs appear to differentiate as a function of mechanical tension.

TGF-β1 and Mechanical Stiffness Are Both Required for PF Differentiation. To test the relative contributions of mechanical stiffness and TGF-β to PF activation, after isolation and plating, cells on polyacrylamide supports were treated with either TGF-β or the TGF-β receptor kinase inhibitor for 10 days. Cells treated with TGF-β expressed α-SMA, even in cells cultured on the softest supports (400 Pa), although both α-SMA expression and cell spreading were minimal, and α-SMA was not organized in stress fibers (Fig. 7A, top row left; Fig. 7B). On gels of increasing stiffness, TGF-β treatment resulted in greater α-SMA expression, stress fiber organization, and cell spreading (Fig. 7A) than in untreated controls on similar gels (Fig. 5C). When cells were treated with the TGF-β receptor kinase inhibitor, myofibroblastic differentiation was completely prevented independent of gel stiffness (Fig. 7A, bottom row). TGF-β treatment of cells on stiff gels also resulted in a more significant increase in expression of procollagens I and III mRNA (Fig. 7C,D).

PDGF Up-Regulates Proliferation on Stiff Supports. To test the role of TGF-β and PDGF on the proliferation of myofibroblastic PFs, PFs seeded on stiff supports

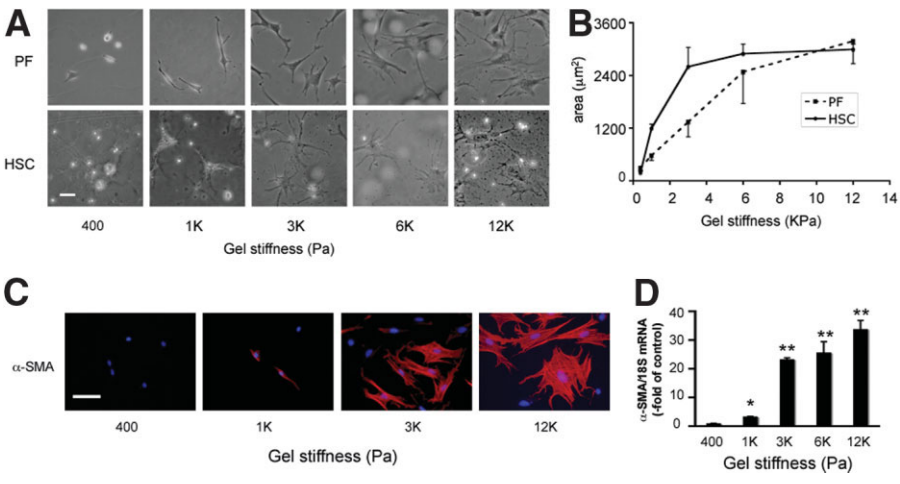

Fig. 5. PFs activate as a function of matrix stiffness. Primary PFs and HSCs were cultured on polyacrylamide supports of increasing stiffness (G′, in pascals). (A) Cells photographed after 10 days in culture. (B) Area calculated by perimeter tracing after 19 days in culture. For each point, 5 cells were traced in each of 3 preparations. Size bar = 50 μm. (C) Cells immunostained for α-SMA expression (red) with nuclei stained with DAPI (blue). (D) Cells lysed for real-time PCR analysis; data represent the mean $\pm$ SD of 3 independent experiments in which each sample was analyzed twice. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with cells on 400-Pa gels. Size bar = 100 μm.

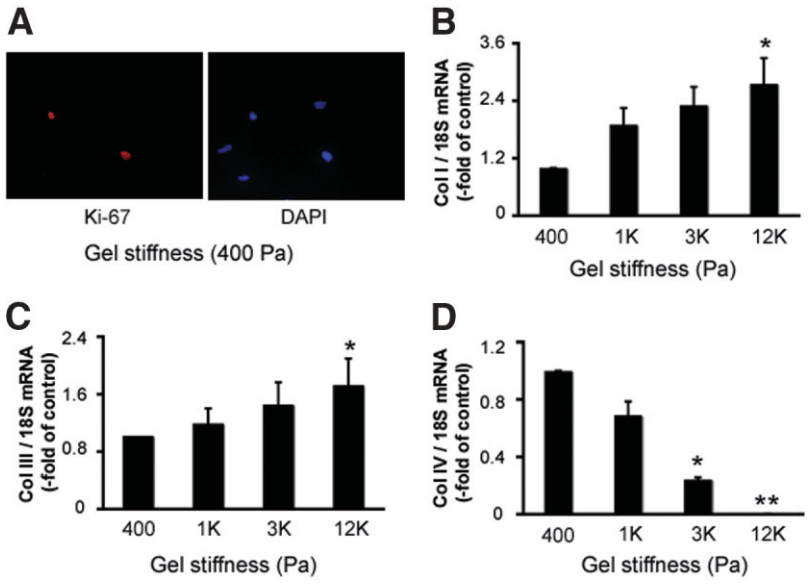

Fig. 6. PFs on stiff supports are fibrogenic. (A) PFs were cultured for 5 days on 400-Pa polyacrylamide supports coated with collagen I and then stained with antibodies against the proliferation marker Ki-67 (red). (B–D) Real-time PCR analysis of cells cultured on gels of different stiffness. Data represent the mean ± SD of 3 independent experiments in which each sample was analyzed twice ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with cells on 400-Pa gels).

(12 kPa) were treated for 10 days with TGF-β1, PDGF-BB, or inhibitors of either receptor kinase. Cells were then labeled with Ki-67 to determine the proliferation index. Although PDGF inhibited myofibroblastic differentiation, as shown in Fig. 4C,D, it up-regulated PF proliferation 3.2-fold over that in untreated cells. Similarly, the PDGF kinase inhibitor down-regulated proliferation 1.8-fold. TGF-β1 and the TGF-β kinase inhibitor did not significant affect the proliferation index (Fig. 8).

Discussion

Our data demonstrate that: (1) PFs can be isolated and, like HSCs, undergo myofibroblastic differentiation in culture; (2) elastin is expressed in both quiescent and myofibroblastic PFs and appears to be a specific marker for these cells in culture and *in vivo*, clearly establishing them as a population distinct from HSCs; and (3) PF myofibroblastic differentiation, unlike that of HSCs, is dependent on both TGF-β and matrix stiffness.

Although HSCs are believed to be the major fibrogenic cell type in liver fibrosis, increasing evidence suggests that PFs are also important prefibrogenic cells, particularly in biliary fibrosis.[10] These cells have been studied in culture, although generally after outgrowth from fragments of the biliary tree or late after isolation[16,17]; the characteristics of PF myofibroblastic differentiation have not been studied. We suggest that myofibroblastic PFs are fibrogenic and that PFs in culture can be studied in much the same way as HSCs.

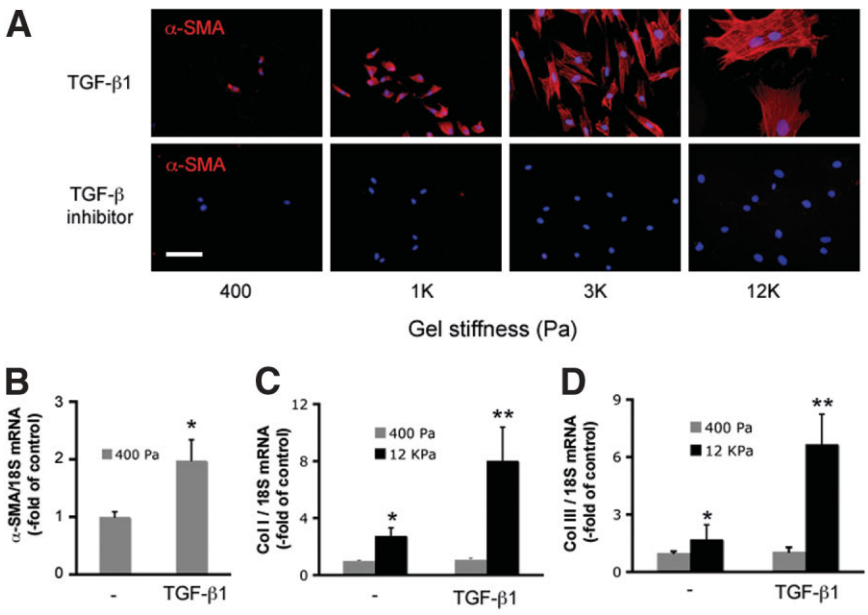

Fig. 7. TGF-β1 regulates differentiation of PFs cultured on polyacrylamide gels. (A) PFs cultured on polyacrylamide supports of increasing stiffness coated with collagen I were treated with either 100 pM TGF-β1 or 5 μM TβRI kinase inhibitor for 10 days, then stained for α-SMA expression (red). Nuclei were stained with DAPI (blue). Size bar = 100 μm. (B) Real-time PCR for α-SMA; cells were cultured on 400-Pa supports with and without TGF-β1 treatment. (C, D) Real-time PCR for procollagens I and III. Cells were cultured on 400-Pa and 12-kPa supports with and without TGF-β1 treatment. Data represent the mean ± SD of 3 analyses and are representative of 2 independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ compared with control).

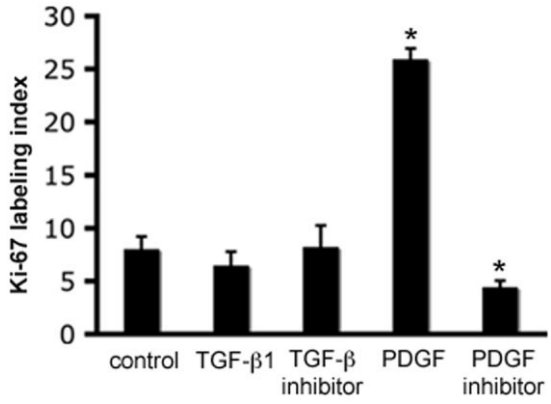

Fig. 8. PFs proliferate in response to PDGF. PFs cultured on stiff supports (12 kPa) were serum-starved overnight and treated for 24 hours with either 100 pM TGF-β1, 5 μM TGF-β receptor kinase inhibitor, 20 ng/mL PDGF-BB, or 10 μM PDGF receptor kinase inhibitor. Cells were stained with antibodies against Ki-67. Note that these cells were serum-starved compared with the Ki-67-stained cells shown in Fig. 5A ($^*P <$ 0.01 compared with control).

NTPDase2 has previously been used as a specific marker for PFs, and we confirmed here that our cultures were NTPDase2 positive. With differentiation in culture, however, as in biliary fibrosis *in vivo*, NTPDase2 expression is lost, implying that it is a suboptimal marker for the total PF population.[38] Histological staining has suggested that elastin is a specific marker for PFs.[36,39] Our data are consistent with this idea: our PF population was uniformly elastin positive, independent of differentiation state. In primary cultures of cells, we never observed elastin staining of HSCs (Fig. 1A); similarly, we never observed elastin staining in the parenchyma of normal or fibrotic rat liver (where there were multiple cells expressing the HSC-specific marker desmin; Figs. 1B,C) and there was minimal costaining of desmin and elastin (Fig. 1B). Thus, we provide both *in vivo* and *in vitro* evidence that HSCs are elastin negative and that PFs are elastin positive. Our findings with primary cells differ from those reported by Kanta et al., who found that primary HSCs expressed elastin mRNA and, as assayed by immunoblotting, elastin protein.[40] Although they did not immunostain cells in culture, they observed elastin reactivity in normal liver to be limited to the portal tract and central vein and to fibrous septa in fibrotic liver. In agreement with our immunostaining results, they observed no elastin reactivity in the sinusoids.

CRBP-1 expression increased in both PFs and HSCs as they differentiated, consistent with the results of other studies suggesting a role for CRBP-1 in smooth muscle cell activation.[41] However, our findings differ from published reports that showed that PFs express CRBP-1 only after myofibroblastic differentiation.[7,16] We cannot explain this difference. Nonetheless, although PFs and HSCs in our study showed similar CRBP-1 profiles, PFs did not show the changes in PPAR-γ expression seen in HSCs and failed to form lipid droplets in response to retinol treatment. In combination with the desmin and elastin marker analysis, this clearly demonstrates that PFs are distinct from HSCs.

TGF-β is well established as one of the most important mediators of liver fibrosis. Overexpression of TGF-β in the liver induces fibrosis in the absence of injury, and multiple studies in the literature reported that anti-TGF-β agents inhibited fibrosis in animal models.[42,43] In this context, it is interesting that TGF-β is not required for α-SMA expression by HSCs but, as our data indicate, it is essential for PF activation.[21,44,45] We previously demonstrated that activated PFs produce large amounts of TGF-β2, and we suggest in this article that autocrine production of TGF-β is an important component of an activation loop.[22,44,45] Although TGF-β clearly plays a role in the functioning of activated HSCs, the data suggest that anti-TGF-β agents have significant effects on non-HSC populations and that these agents might be particularly effective early in biliary fibrosis.

Interestingly, we observed that PDGF inhibited rather than enhanced α-SMA expression in PFs, in contrast with previously published data.[17] The different conditions used may explain this; in a study by Kinnman et al.,[17] PFs were treated with PDGF when established in culture and expressing baseline levels of α-SMA. This point deserves further study given other evidence from rodent models that PDGF increases and PDGF receptor kinase inhibitor decreases peribiliary fibrosis.[17,46,47]

Mechanical tension has recently emerged as an important factor determining cell phenotype. Mechanical tension can affect cellular locomotion, morphology, adhesion, and cytoskeletal protein expression.[24,25] Our previous work showed that mechanical properties are a major determinant of HSC activation *in vitro* (manuscript submitted). In the present study we demonstrated that activation of PFs is also substrate stiffness–dependent. On soft supports (400 Pa, similar to the stiffness of normal rat and human livers), PFs remained quiescent, whereas on stiff supports (12 kPa, similar to the stiffness of a fibrotic liver), they underwent myofibroblastic differentiation. Interestingly PFs, like HSCs, demonstrate stable intermediate phenotypes at intermediate stiffness; we did not observe an all-or-nothing phenomenon with activation. The relevance of these data to fibrosis *in vivo* has not yet been studied, although the suggestion that mechanical as well as soluble factors mediate myofibroblast activation and could therefore regulate fibrosis has interesting implications for understanding its pathogenesis and for therapy. It is conceivable that mechanical changes precede fibrosis,

for example, because of collagen crosslinking rather than collagen deposition. Potential mechanosensors such as integrins along with their downstream signaling pathways might be attractive therapeutic targets.[48,49]

In summary, we have characterized portal fibroblast activation *in vitro* and have demonstrated that both TGF-β and matrix stiffness are critical for activation to occur. The demonstration that PFs and HSCs are morphologically, geographically, and functionally distinct may have important implications for both the study and treatment of fibrosis.

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