Integrin αVβ1 Regulates Procollagen I Production through a Non-canonical Transforming Growth Factor β Signaling Pathway in Human Hepatic Stellate Cells

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KEYWORDS: hepatic stellate cell (HSC), integrin, transforming growth factor beta (TGF-β), signal transduction, fibrosis
**Abbreviations:** hepatic stellate cell (HSC), integrin, transforming growth factor beta (TGF-β), Compound 8 (C8), Activin receptor-like kinase 5 (Alk5), extracellular matrix (ECM), latency associated peptide (LAP), Arginine-Glycine-Aspartate (RGD), real-time polymerase chain reaction (RT-PCR), Scrambled siRNA Negative Control (SC), Homogeneous Time Resolved Fluorescence (HTRF), Alpha Smooth Muscle Actin (αSMA), Analysis of Variance (ANOVA)
ABSTRACT

Hepatic stellate cells (HSCs) are thought to play key roles in the development of liver fibrosis. Extensive evidence has established the concept that αV integrins are involved in the activation of latent transforming growth factor β (TGF-β), a master regulator of the fibrotic signaling cascade. Based on mRNA and protein expression profiling data, we found that αVβ1 integrin is the most abundant member of the αV integrin family in either quiescent or TGF-β1-activated primary human HSCs. Unexpectedly, either a selective αVβ1 inhibitor, Compound 8 (C8), or a pan-αV integrin inhibitor, GSK3008348, decreased TGF-β1-activated procollagen I production in primary human HSCs, in which the role of β1 integrin was confirmed by ITGB1 siRNA. In contrast to an Activin receptor-like kinase 5 (Alk5) inhibitor, C8 and GSK3008348 failed to inhibit TGF-β1 induced SMAD3 and SMAD2 phosphorylation, but inhibited TGF-β1-induced phosphorylation of ERK1/2 and STAT3, suggesting that αVβ1 integrin is involved in non-canonical TGF-β signaling pathways. Consistently, ITGB1 siRNA significantly decreased phosphorylation of ERK1/2. Furthermore, a selective inhibitor of MEK1/2 blocked TGF-β1 induced phosphorylation of ERK1/2 and decreased TGF-β1 induced procollagen I production, while a specific inhibitor of STAT3 had no effect on TGF-β1 induced procollagen I production. Taken together, current data indicate that αVβ1 integrin can regulate TGF-β signaling independent of its reported role in activating latent TGF-β. Our data further support that αVβ1 inhibition is a promising therapeutic target for the treatment of liver fibrosis.
INTRODUCTION

Liver fibrosis is featured by excessive accumulation of extracellular matrix (ECM) proteins including collagen-1 that occurs in most types of chronic liver diseases (1). Advanced liver fibrosis results in cirrhosis, portal hypertension and ultimately liver failure that are major causes of morbidity and mortality. Currently there are no FDA-approved drugs to treat liver fibrosis and the only medical treatment is liver transplantation (2).

Human hepatic stellate cells (HSCs) are the main ECM-producing cells in the injured liver. In the normal healthy liver, HSCs reside in the perisinusoidal space and serve as the major vitamin A storage sites for the body. Upon the injury that leads to elevated inflammation in the liver, HSCs are activated and transdifferentiated into myofibroblast-like cells, acquiring contractile, proinflammatory, and fibrogenic properties (3). Activated HSCs migrate and accumulate at the sites of tissue repair, synthesizing and secreting large amounts of ECM proteins. When liver injury and inflammation persist in the setting of chronic liver disease, a vicious cycle of mutual stimulations between inflammatory cells and fibrogenic HSCs takes place. On the one hand, inflammatory cells activate HSCs to secrete ECM. In turn, the activated HSCs secrete inflammatory chemokines and cell adhesion molecules that lead to the further activation and recruitment of lymphocytes. If left untreated, the excessive inflammation and fibrosis in the liver leads to cirrhotic condition with functional damages and structural distortions to the liver (3).

TGF-β emerges to be a master regulator of hepatic fibrogenesis and activator of HSCs. TGF-β stimulates the transition of HSCs to myofibroblast-like cells, stimulates the synthesis but
decreases the degradation of ECM proteins (4, 5). Integrins are a family of heterodimeric cell-surface proteins that play a wide range of roles such as cell adhesion, maintenance of tissue integrity and signal transduction. Many different heterodimers can be expressed on a single cell, and each can interact with multiple intracellular signaling cascades (6). In recent years, emerging in vitro and in vivo evidence indicates that αV integrins, a subset of the integrin family, play a key role in the activation of TGF-β1 and TGF-β3 (7-9). Alpha V integrins (αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8) belong to RGD integrin subclass who binds to ligand proteins through a tripeptide Arginine-Glycine-Aspartate (RGD) recognition motif. Both in vitro and cellular model indicate αV integrins bind to the RGD sequence in the latency associated peptide (LAP) of TGF-β1 and TGF-β3. Through a tension- or protease-mediated mechanism, the latent form of TGF-β is converted to its active form (10-13). Animal models either through specific integrin knockout in mice, or through the treatment with inhibitory small molecules or with neutralizing antibodies have established that inhibition of αVβ1, αVβ6 specifically or inhibition of all αV integrins simultaneously (pan- αV integrin inhibition) ameliorate TGF-β activation and liver fibrosis (9, 14, 15). These data strongly indicate that αV integrins are therapeutic targets for the treatment of liver fibrosis.

For small molecule αV integrin inhibitors, Reed et al reported that Compound 8 (C8) is a highly selective αVβ1inhibitor (10). Hall et al reported that GSK3008348 is a potent pan-αV integrin inhibitor (16, 17). In the current study, we have identified that αVβ1 integrin is the major αV integrin member expressed in both quiescent and activated human primary HSCs. When TGF-β1-activated HSCs were treated with C8 and GSK3008348, both compounds diminished TGF-β1 induced procollagen-1 production, indicating that αVβ1 integrin is playing a critical role in the TGF-β mediated signaling cascade. This finding was unexpected in that our
experimental setting used HSCs activated with exogenous active TGF-β1, which bypassed the need for αVβ1 integrin to activate the latent TGF-β. We further explored the signaling mechanism and found that αVβ1 is involved in an ERK1/2 dependent non-canonical TGF-β signaling pathway. Our findings expanded our understanding on the role of αVβ1 and further support that it is a therapeutic target for the treatment of liver fibrosis.

**EXPERIMENTAL PROCEDURES**

**Materials.** Primary human hepatic stellate cells, the cell culture media, fetal bovine serum and antibiotic solution were obtained from ScienCell Research Laboratories. Recombinant human TGFβ1 (240-B), Human Pro-Collagen I α1/COLIA1 (DY6220-05) and human TGF-β1 ELISA kits (DB100B) were from R&D Systems. Active TGF-β1 LEGEND MAX™ ELISA kit was from BioLegend Inc (437707). pSMAD3 (pS423/S425) ELISA kit was from Abcam (ab186038). PathScan® Phospho-p44/42 MAPK (pERK1/2, Thr202/Tyr204) and Phospho-STAT3 (Tyr705) ELISA kits were from Cell Signaling (7177C for pERK1/2, 7300C for pSTAT3). The pTAK1, pJAK2 and pFAK ELISA kits were from RayBiotech (PEL-TAK1-S412 for pTAK1, PEL-JAK2-Y1007 for pJAK2, PEL-FAK-Y397 for pFAK). pSHP2 AlphaLISA assay kit was obtained from Perkin Elmer (ALSU-PSHP2-A-HV).

Recombinant human αVβ1, human αVβ3, human αVβ5, human αVβ6, human αVβ8 integrin proteins were obtained from R&D Systems. Copper Chelate YSi SPA beads were obtained from Perkin Elmer. Pan-αV integrin inhibitor GSK3008348 was obtained from MCE, ALK5 inhibitor SB525334 was from Sigma, MEK1/2 inhibitor U0126 was from Cell Signaling, STAT3 inhibitor S31-201 was from Santa Cruz Biotechnology, C8 and [³H]-CWHM12 were synthesized by Bristol Myers Squibb Company. All Real time-polymerase chain reaction (RT-PCR) probes
were obtained from Thermo-Fisher Scientific. All other chemical reagents were from Sigma-Aldrich.

**Cell line and cell culture.** Primary human HSCs isolated from human donors were cryopreserved immediately after purification by the vendor (ScienCell Reach Laboratories). Written informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution’s human research committee. They were delivered to our research facility under frozen condition. To minimize HSC activation and ensure experimental consistency, the culture of primary human HSCs was carried out in Geltrex coated plates in a 2% serum medium below passage 10. For the treatment by TGF-β1, HSCs were first cultured in a starvation medium that contained 0.02% serum overnight. The serum-starved HSCs were then treated with recombinant active TGF-β1 in a 0.2% serum medium for various indicated times.

**siRNA-mediated gene silencing** The HSCs were seeded at 5x10⁴ cells per well in 24-well Geltrex-coated plates and incubated overnight to reach 30-50% confluence. Then the cells were transfected with two integrin β1 specific siRNA Silencers from ThermoFisher Scientific (siRNA1: Assay ID 109878; siRNA2: Assay ID 288623) at the final concentration of 30 nM using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the forward transfection method described in the manufacturer’s protocol. Meanwhile, a non-targeting scrambled siRNA (SC) (ThermoFisher Scientific, cat# 4390843) was used as a negative control. Medium was changed after 24 hrs of the transfection to starve the cells with 0.02% FBS. After 24 hrs of starvation, cells were treated with 2 ng/ml TGF-β in the assay medium containing 0.2% FBS for 24 hrs before they were lysed with RNA lysis buffer for qPCR analysis. The supernatant was collected for Pro-Collagen 1 measurement. For pERK1/2 quantitation, the starved cells were
treated with TGF-β1 for 10 min and the cell lysates was used to measure pERK1/2 according to the manufacturer's instructions and the results were normalized by total protein levels.

**Real-time polymerase chain reaction (RT-PCR).** Following the treatment of HSCs, cells were lysed with lysis/binding buffer and RNA was extracted using the MagMax-96 Total RNA Isolation kit (Thermo-Fisher Scientific). Complementary DNA (cDNA) was generated from 1 µg of RNA from each sample using Superscript VILO® cDNA Synthesis Kit and Master Mix (Invitrogen). Quantitative RT-PCR was done with Applied Biosystems Taqman PCR master mixture. The Ct values for each target gene and the geometric mean of the Ct values for the three housekeeping genes (UBC, B2m, TBP) were calculated for each RT-PCR reaction sample. ΔCt values were generated by subtracting the Ct of the target gene from the geometric mean of the Ct for the housekeeping genes. For each target gene, the comparative analyses were performed by subtracting the average ΔCt of the samples in the control group from the ΔCt of each sample in the comparison groups to generate ΔΔCt values. For graphical representation, the formula: Fold change = 2^{-ΔΔCt} was used to calculate the relative mRNA expression in each sample normalized to expression in the control group.

**Steady state αVβ1/[3H]-CWHM12 binding assay and determination of αVβ1 inhibitory potency.** The binding assay buffer consisted of 50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.2 mM MnCl₂ and 0.005% CHAPS. For 75 µl binding reactions in each well of a 384-well OptiPlate (Perkin Elmer 6007290), the following reagents were added: 0.75 µl of 100X C8 or GSK3008348 made in DMSO (10-point 1:3 serial dilution, final concentration 1X at 1% DMSO), or 0.75 µl of DMSO for total binding control (final concentration 1% in the assay), or 0.75 µl of 0.3 mM CWHM12 for non-specific binding control (final concentration 3 µM in assay); 15 µl of 15 nM [3H]-CWHM12 ligand (15) (final concentration 3 nM), 15 µl of 37.5 nM
human αVβ1 (final concentration 7.5 nM) and 45 µl of YSi beads at 1.111 mg/ml made in binding assay buffer (final 0.05 mg per well). The reaction mixture was incubated at room temperature overnight. The plate was counted in a TopCount for 1 min/well. Specific binding was calculated by subtracting non-specific binding from the total binding. The percent of specific binding in the presence of C8 or GSK3008348 at various concentrations was calculated relative to the DMSO control. Concentration-response curves were plotted and the binding IC₅₀ was calculated using the “Dose-response variable slope” model in GraphPad Prism. Mean IC₅₀ values were derived from the average of 3 replicated dose response curves.

**Homogeneous Time Resolved Fluorescence (HTRF) assay.** The binding assays for αVβ3, αVβ5, αVβ6, αVβ8 integrins with their optimal RGD-containing ligands were carried out in HTRF assay format according to the manufacturer’s instructions (Cisbio International). The binding pairs used were: biotinylated human αVβ3, 6XHis-tagged vitronectin (Catalog 10424-H08H-100, Sino Biological); biotinylated human αVβ5, 6XHis-tagged vitronectin; biotinylated human αVβ6 and FITC-conjugated fibronectin (Catalog FNR02, Cytoskeleton); biotinylated human αVβ8, latency associated peptide (LAP, Catalog 246-LP-025, R&D Systems). After the incubation of the above binding pairs in the Binding Buffer (20 mM Tris, pH 7.4, 1 mM MgCl₂, 1 mM MnCl₂, 0.01% Tween 20, 0.01% BSA) at room temperature for 1 h, Streptavidin Terbium (610STLB, Cisbio international) and following reagents were added: anti-6HIS mAb Terbium cryptate for αVβ3 and αVβ5, FITC-conjugated anti-LAP antibody (Catalog FAB2463G, R&D Systems) for αVβ8. The mixtures were incubated at room temperature overnight in the dark before the detection of HTRF signals. To test the inhibitory potencies, 10-point 1:3 serial dilutions various concentrations of C8 and GSK3008348 were incubated with the HTRF assay
mixture. IC₅₀ values were calculated using the signal from DMSO control as 100% of activity. All assays were conducted under optimized conditions within the linear range of HTRF signal.

**ELISA assays.** The serum starved HSCs were pretreated with compounds or with DMSO control for 60 min. Then 2 ng/ml TGF-β1 was added and the cells were cultured for 48 hrs. Pro-collagen I protein secreted from HSCs was determined with the Human Pro-Collagen I α1/COLIA1 ELISA. To explore the pathways involved in αVβ1 regulation of TGF-β1 induced procollagen-1 secretion, ELISA or AlphaLISA assays on HSC cell lysates were conducted for p-SMAD3, p-ERK1/2, p-STAT3, pTAK1, pJAK2, pFAK, pSHP2, total TGF-β1 and active TGF-β1. All assays were performed according to the manufacturer’s instructions and the results were normalized by total protein levels.

**Phospho-SMAD2/3 nuclear-translocation immunofluorescence staining.**

HSCs were seeded on Geltrex coated Nunc™ Lab-Tek™ II Chambered Cover glasses (ThermoFisher, Cat# 155409PK) in culture medium (2% serum). After overnight serum-starvation, cells were pre-incubated with selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 or ALK5 inhibitor SB525334 at 10 μM for 1 hr, and then treated with recombinant active TGF-β1 in a 0.2% serum medium for 30 min. Cells were fixed in 4% formaldehyde solution (Electron Microscopy Science, Cat#100496-494) for 10 min, and blocked in 5% BSA solution with 10% normal goat serum (Vector Laboratories, Cat# S-1000-20) and 0.3% Triton X-100 in PBS for 30 min. Cells were incubated with primary antibody in PBS (1% BSA) at 1:100 for 1 hr. Phospho-SMAD3 was obtained from ThermoFisher (Cat# 702292), and Phospho-SMAD2 was obtained from Cell Signaling Technologies (Cat# 18338S). After two times of washing with PBS, cells were incubated with secondary antibody, Alexa Fluor 488 (ThermoFisher, Cat#
A27034) for 1 hr with the presence of Rhodamine Phalloidin (ThermoFisher, Cat# R415). SlowFade™ Gold Anti-fade Mountant with DAPI (ThermoFisher, Cat# S36939) was used to counter stain the nuclei. Images were captured using Leica Application Suite X (LAS X) confocal system.

**Immunoprecipitation and western blot analysis.** HSCs were seeded on 6-well plate in culture medium (2% serum). After overnight serum-starvation, cells were pre-incubated with selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 or ALK5 inhibitor SB525334 at 10 µM for 1 hr, and then treated with recombinant active TGF-β1 in a 0.2% serum medium for 24 hrs. Cells were harvested in 250 µL ice-cold 1X Cell Lysis Buffer (CST, Cat# 9803), sonicated briefly, and centrifuged for 10 min at 14,000 g at 4°C. Supernatant was pre-cleared by incubating with Protein G Magnetic beads (Invitrogen, 10004D) for 20 min, and incubated with 2.5 µg anti-αV integrin HUMAN MAB L230-Mouse antibody (Enzo, 89153-448) or Mouse IgG control (CST, Cat# 5415) with rotation for 1 hr to form immunocomplex. Immunocomplex was added into pre-washed magnetic beads and incubated with rotation for 20 min. After washes, beads coupled with immunocomplex were treated with 25 µl 1X sample buffer with fluorescent dye and boil at 95°C for 5 min. Beads were pelleted using magnetic separation rack and the supernatant was ready for Wes simple western analysis (Protein Simple, SM-004; DM-001; DM-002). Protein levels of αV integrin, β1, 3, 5, 6 integrins in HSCs were determined with anti-αV (ThermoFisher, PA5-29606), anti-β1 (ThermoFisher, 27096-1-AP), anti-β3 (CST, 13166T), anti-β5 (CST, 3629T), anti-β6 (Abcam, ab187155), and anti-Actin (CST, 3700S) primary antibodies with 1:50 dilution.
Data analysis. Data was presented as Mean ± SD. Statistical analyses were conducted with One-way Analysis of Variance (ANOVA) with Dunnett’s post hoc test. P < 0.05 is defined as statistical significance.

Results

Integrin αVβ1 is the major αV integrin expressed in primary human HSCs

In order to understand the αV integrin expression profile in primary human HSCs, RT-PCR was conducted in both quiescent and TGF-β1-treated HSCs. To ensure that the condition of TGF-β1-treatment caused robust HSC activation into a myofibroblast-like phenotype, various cellular densities and TGF-β1 concentrations were tested. It was determined that treatment with TGF-β1 at 2 ng/ml for 18 hrs at a density of 5x10⁵ cells/well in 6 well plates resulted in consistent and robust elevation of gene expression for COL1A1 and ACTA2 as shown in Figure 1A and 1B. The integrin subunit αV is abundantly expressed in the quiescent HSCs and β1 is the predominant β subunit (Figure 1C, 1D and Table 1). In the quiescent HSCs, the level of β1 mRNA is approximately 25X, 14X and 45X comparing to β3, β5, and β8 respectively. The level of mRNA for β6 is negligible; the β1 mRNA level is approximately 455100X higher than β6. After TGF-β1 treatment, αV mRNA level increased by 7.2X. The treatment of TGF-β1 also raised β1 mRNA level by 5.4X, β3 by 4.8X, β5 by 1.4X, and β6 by 3.7X. Nevertheless, the β8 mRNA level was decreases by 40% upon TGF-β1 treatment. Comparing the relative level of expression after the TGF-β1 treatment, the mRNA expression level of β1 is
approximately 28X, 52X, 677000X, 436X higher than β3, β5, β6 and β8 respectively.

Accordingly, TGF-β1 induced significant protein level increase of αV, β1, β3, and β6 integrins (Figure 1E-J) and a trend of increase for β5 integrin (Figure 1I). The β1 and β3 integrins represent the two most abundant integrins, whereas β5 and β6 showed lower abundance (Supplemental Figure 1). The β8 protein was undetectable under the current setting. This result is consistent with literature that integrin αVβ1 is a major integrin in myofibroblasts (18) whereas αVβ6 is selectively elevated in the injured epithelial cells (7). As αVβ1 integrin is the major αV integrin in primary human HSCs, we continued our further exploration of the functional roles of αVβ1 in fibrogenesis using this model system.

Effects of αV integrin inhibitors on the TGFβ1-induced pro-collagen-1 in HSCs

It has been reported that C8 is a highly potent and selective αVβ1 integrin inhibitor (10) and GSK3008348 is pan-αV integrin inhibitor with the highest inhibitory potency against αVβ6 (16). We used a [3H]-CWHM12 binding assay and HTRF assays that detect binding of specific RGD-containing ligands to determine the potencies and selectivity of C8 and GSK300834. Our data confirmed the concept that C8 is a potent and selective αVβ1 inhibitor (Table 2). The IC50 of C8 determined by αVβ1/[3H]-CWHM12 binding assay is 0.70 ± 0.26 nM. C8 has more than 400X selectivity against other αV integrins. In contrast, GSK3008348 is most potent for the inhibition of αVβ6 with an IC50 of 1.50 ± 1.07 nM (Table 2). GSK3008348 also exhibited high inhibitory potencies against αVβ1, αVβ3, αVβ5 and αVβ8 with IC50 values determined to be 2.83 ± 1.28 nM, 12.53 ± 5.29 nM, 4.00 ± 1.93 nM and 2.26 ± 1.68 nM, respectively (Table 2).

Further, we performed a full panel screening covers 44 common human GPCR receptors/Ion channels/Transporters/Enzymes (Supplemental table), and found that C8 showed no notable
activity toward any of these assays, while GSK3008348 has mild activity (>30% inhibition) on 7 out of 44 assays, suggesting both compounds are relatively selective to αVβ1 or Pan-αV integrins. In the following studies, we used C8 as a selective αVβ1 inhibitor and GSK3008348 as a pan-αV integrin inhibitor to explore the effect of αVβ1 on TGF-β1-mediated fibrogenesis.

We first tested the effect of TGF-β1 on the pro-collagen-1 protein secretion in HSCs. TGF-β1 stimulated the pro-collagen-1 protein secretion from basal level (10605 ± 2006 pg/ml) to 26395 ± 1248 pg/ml. As shown in Figure 2A, αVβ1 selective inhibitor (C8), pan-αV inhibitor (GSK3008348), and ALK5 inhibitor (SB525334) all significantly decreased TGF-β1-induced pro-collagen-1 protein levels in a concentration-dependent manner. At 10 μM of C8, GSK3008348 and SB525334, the pro-collagen-1 protein secreted was 8632 ±1932 pg/ml, 8209 ±793 pg/ml, and 5031±2324 pg/ml, respectively. All these values were below the basal level. We conclude that these compounds completely inhibited TGF-β1 induced pro-collagen-1 protein secretion. The IC50 values for C8, GSK3008348 and SB525334 were determined to be 0.64 ± 0.33 μM, 0.30 ± 0.22 μM and 0.21± 0.14 μM respectively.

To determine the role of β1 integrin in this process, two integrin β1 specific siRNAs were used to knock down β1 with knock down rate at 90% and 82% respectively, comparing to scrambled negative control (SC) (Figure 2B). As a result of β1 knock-down by either siRNA, the secreted pro-collagen-1 levels were significantly lower than that of scrambled control under TGFβ treatment (Figure 2C), indicating the important role of β1 integrin in pro-collagen-1 secretion. We also checked mRNA levels of other markers, namely, COL1A1, COL3A1, and ACTA2 following siRNA knock down of integrin β1. Interestingly, we found only ALK5 inhibitor (SB525334), but not αVβ1 selective inhibitor (C8) or pan-αV inhibitor (GSK3008348),
down-regulated the expression of these genes to the basal level (Figure 2D-F), suggesting αVβ1 inhibitors might regulate pro-collagen-1 production at post-translational level.

Effects of αV integrin inhibitors on the TGFβ1-induced total and active TGF-β production

It has been reported that αV integrins are involved in the activation of latent TGF-β form. One of the perpetuating mechanisms for activation of HSCs is through the TGF-β self-amplification loop. In this mechanism, TGF-β transforms quiescent HSCs into activated myofibroblasts, and the activated myofibroblast in turn has enhanced TGF-β synthesis (19). It is possible that the newly synthesized TGF-β in the TGF-β1 activated HSCs is activated by αVβ1. We thus tested effects of C8 and GSK3008348 on the TGF-β1-induced total and active TGF-β production. In quiescent HSCs, total and active TGF-β levels were determined to be 63.20 ± 20.60 pg/ml and 6.70 ± 0.70 pg/ml, respectively. After the cells were treated with active TGF-β1 at 2 ng/ml for 48 hrs, the total and active TGF-β detected in the culture media were 433.90 ± 60.91 pg/ml and 52.60 ± 23 pg/ml, respectively, indicating that there was significant amount of newly synthesized latent TGF-β and the vast majority of active TGF-β added during treatment had already been degraded. As shown in Figure 3A and Figure 3B, the ALK5 inhibitor SB525334 decreased both total and active TGF-β with IC50 value of 0.56 ± 0.36 and 0.36 ± 0.09 μM. In contrast to the effect of SB525334, C8 and GSK3008348 had mild effects on total TGF-β and active TGF-β levels in TGF-β1-activated HSCs if any (Figure 3A and Figure 3B).

Effects of αV integrin inhibitors on the TGF-β1-induced SMAD3 phosphorylation
We next tested whether αVβ1 integrin regulated pro-collagen-1 production in TGF-β1-activated HSCs occurs through downstream SMAD3 phosphorylation. HSC cell lysates were prepared from quiescent and TGF-β1-activated HSCs and SMAD3 phosphorylation levels were determined by an ELISA and immunofluorescence staining (Figure 4). SB525334 significantly decreased TGF-β1-induced p-SMAD3 with the IC₅₀ value of 0.08 ± 0.03 μM, comparable to its IC₅₀ value of 0.21± 0.14 μM for inhibition of procollagen I production. However, C8 and GSK3008348 had little effect on TGF-β1 induced p-SMAD3 levels at concentrations less than 10 μM (Figure 4A). Consistent with the ELISA data, nuclear translocation of p-SMAD3 was induced by TGF-β1 and inhibited by SB525334 (Figure 4B). Neither C8 nor GSK3008348 reversed TGF-β1 induced p-SMAD3 nuclear translocation. These data suggest that αVβ1 integrin-mediated regulation of TGF-β1-activated pro-collagen-1 production in HSCs is SMAD3 independent. Similarly, nuclear translocation of p-SMAD2 was induced by TGF-β1 and inhibited by SB525334 (Supplemental Figure 2). Neither C8 nor GSK3008348 reversed TGF-β1 induced p-SMAD2 nuclear translocation, suggesting SMAD2 is not involved in αVβ1 integrin-mediated inhibition of pro-collagen-1 production in HSCs either.

Effects of αV integrin inhibitors on protein levels of αV, β1, and αVβ1 heterodimer in HSCs

To investigate whether αVβ1 integrin heterodimer is affected by TGF-β1 or three inhibitors treatment, immunoprecipitation was performed with anti-αV antibody, and αVβ1 integrin heterodimer was assessed using anti-β1 antibody. Interestingly, TGF-β1 increased αV integrin protein level (Figure 5A and 5B) and αVβ1 integrin heterodimer (Figure 5C and 5E) which were reversed by SB525334 compound treatment. Despite of the unchanged αV and β1 integrin protein level by C8 and GSK3008348 (Figure 5A-E), both compounds decreased αVβ1
integrin heterodimer (Figure 5D and 5F), probably due to their inhibition on αVβ1 integrin activity.

**Effects of αV integrin inhibitors on the TGF-β1 induced ERK1/2 and STAT3 phosphorylation in HSCs**

We then explored other possible signaling pathways for αVβ1 integrin that could be involved in the regulation of TGF-β signaling in HSCs. We conducted studies at both 30 min and 6 hrs after TGF-β1 treatment because ERK1/2 phosphorylation is a rapid signaling event whereas STAT3 phosphorylation is a more delayed signaling event downstream of the TGF-β receptor. As shown in Figure 6A, both αVβ1 selective inhibitor C8 and pan-αV inhibitor GSK3008348 significantly inhibited TGFβ1 induced ERK1/2 phosphorylation levels at 30 min after TGF-β1 treatment (P < 0.05). To further elucidate the role of αVβ1 in stimulating ERK1/2 phosphorylation, a β1 specific siRNA knock down method was employed. As shown in Figure 6B, the knock down of β1 significantly reduced pERK1/2 levels (P-value < 0.05) by both siRNAs in comparison to the scrambled control. This indicated that β1 was required in TGFβ stimulated ERK1/2 phosphorylation in the non-canonical pathway. C8 and GSK3008348 also significantly inhibited STAT3 phosphorylation in HSCs (P < 0.05) 6 hrs after TGF-β1 treatment (Figure 6C). These data indicate that αVβ1 integrin is involved in the regulation of TGF-β1 non-canonical signaling pathways. Of importance, MEK1/2 inhibitor U0126 and STAT3 inhibitor S31-201 decreased p-ERK1/2 and p-STAT3 levels below the level in basal quiescent HSCs. This difference distinguishes αVβ1 selective inhibitor (C8) and pan-αV inhibitor (GSK3008348) from direct ERK1/2 and STAT3 inhibitors in that the former only decreased the TGF-β1-induced ERK1/2 and STAT3 phosphorylation but had no effects beyond the basal level in quiescent
HSCs (Figure 6A and 6C). Other TGF-β non-canonical signaling molecules such as pTAK1, pJAK2, pFAK and pSHP2 were not stimulated by 2 ng/ml TGF-β treatment in primary HSC cells (Supplemental Figure 3).

Effects of MEK1/2 and STAT3 inhibitors on TGF-β1-induced SMAD3 phosphorylation in HSCs

We further investigated whether MEK1/2 or STAT3 inhibition will affect TGFβ1-induced SMAD3 phosphorylation. As shown in Supplemental Figure 4, throughout the time course from 30 min to 6 hrs after TGF-β1 treatment, SB525334 significantly inhibited TGFβ1-induced SMAD3 phosphorylation. In contrast, the MEK1/2 inhibitor U0126, which inhibits phosphorylation and activation of ERK1/2, and the STAT3 inhibitor S31-201 caused modest effects in SMAD3 phosphorylation, but they failed to show statistically significance. In the same experiment, C8 and GSK3008348 did not show effects on TGF-β1-induced SMAD3 phosphorylation, consistent with the data in Figure 4A.

Effects of ERK1/2 and STAT3 inhibitors on TGF-β1-induced Pro-collagen-1 in HSC

Next we tested if ERK1/2 or STAT3 signaling might be important for the TGF-β1-induced Pro-collagen-1 production in HSCs (Figure 7). At both 1 μM and 10 μM concentrations, MEK1/2 inhibitor U0126 significantly decreased TGF-β1-induced Pro-collagen-1 production in HSCs to a level similar to basal quiescent HSCs (P < 0.05). The effect of U0126 was similar to the effects of αVβ1 selective inhibitor C8 and pan-αV integrin inhibitor GSK3008348 and ALK5 inhibitor SB525334. In contrast, the STAT3 inhibitor failed to show inhibition of TGF-β1 induced procollagen-1 production in HSCs (P > 0.05).
DISCUSSION

In the current study we report that αVβ1 is the major αV integrin expressed in primary human HSCs. In this model system, we used both selective αVβ1 inhibitor C8 and pan-αV integrin inhibitor GSK3008348 to investigate the roles of αVβ1 in fibrogenesis. We found that αVβ1 integrin has profound effects in modulating procollagen-1 production. Treatment with C8 and GSK3008348 at 10 µM completely abolished TGF-β-induced procollagen-1 production. The subsequent mechanistic studies suggested that αVβ1 integrin regulated procollagen-1 production is SMAD3 independent but through the activation of pERK1/2, a non-canonical TGF-β signaling pathway. Of interest, although C8 and GSK3008348 also inhibited TGF-β1-induced p-STAT3, that pathway does not appear to be involved in procollagen-1 production (Figure 8).

Emerging *in vitro* and *in vivo* evidence supports the concept that αV integrins play key roles in the activation of latent TGF-β. Integrins αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8 have all been shown to bind the RGD sequence in the latency associated peptide (LAP) of TGF-β1 and -β3. Through a mechanical tension mechanism or a protease cleavage, αV integrins can activate latent TGF-β under various physiological or pathological conditions in various tissue and cellular contexts (20). In addition to its role in TGF-β activation, current study indicates that αVβ1 is also involved in procollagen-1 production through a TGF-β signaling pathway.

The reason that we chose primary human HSCs as the model system is its potential human relevance. Using the RT-PCR technique, we found that integrin β1 is abundantly expressed in quiescent HSCs. Upon the treatment of TGF-β1, integrin β1 has >20 fold higher expression compared with β3, β5, β6 and β8 (Figure 1). Using selective antibodies for β1, β3,
β5, and β6, we were able to directly quantify and compare the protein content of these integrins, we concluded that αVβ1 is the most abundant αV integrin in HSCs. This notion is also supported by the fact that C8, a selective αVβ1 inhibitor, completely inhibited TGF-β1-induced pro-collagen-I production (Figure 2), thus providing functional data that αVβ1 is the key αV integrin in primary human HSCs.

Zhou et al demonstrated that αVβ3 is abundantly expressed in LX-2 cells, an immortalized hepatic stellate cells (21). The different αV integrin expression profile between primary human HSCs and LX-2 probably reflect the fact that the former is a primary cell line but LX-2 is an immortalized cell line. Despite these differences, using neutralizing antibody to αV integrin and antisense oligonucleotide (ASO) techniques, Zhou et al found that αVβ3, the cell surface integrin in LX-2 regulates proliferation and apoptosis through p-ERK1/2, a very similar signaling cascade that is used in human primary HSCs. STAT3 is a transcription factor that is phosphorylated by Janus tyrosine kinases (Jak) in response to cytokine activation which then dimerizes and moves into the nucleus to activate transcription of cytokine-responsive genes (22). STAT3 plays an important role in the regulation of TGF-β induced connective tissue growth factor (CTGF) expression in HSCs (23) and renal fibrogenesis (24). STAT3 inhibitor S31-201 suppresses activation and proliferation in renal interstitial fibroblasts in a rodent obstructive nephropathy model (24). In our current study, we also identified that αVβ1 is involved in the activation of TGF-β-mediated STAT3 activation. Although S31-201 did not inhibit TGF-β induced pro-collagen-I in HSCs, the TGF-β/ αVβ1/p-STAT3 axis could be an alternative signal transduction pathway important for fibrogenesis, perhaps through the regulation of CTGF in HSCs.
Reed et al demonstrated that C8 not only inhibited the TGF-β activation by αVβ1 expressing cell lines in co-culture assays, but administration of C8 in the CCl4 liver fibrosis model profoundly diminished p-SMAD3 nuclear translocation in the liver fibrotic area (10). These data demonstrate that αVβ1 is involved in TGF-β activation in liver fibrosis. In the current studies of human primary HSCs, both the experimental setting and results bear significant differences from Reed et al. First, we used recombinant TGF-β to activate the primary HSCs to a myofibroblast-like phenotype. This step of active TGF-β treatment bypassed latent TGF-β activation. Thus, our studies only focused on the downstream signaling of TGF-β-mediated effects. Secondly, we found that αVβ1-regulated pro-collagen-1 production is not through p-SMAD3, but rather through the p-ERK1/2 pathway, a non-canonical TGF-β signaling pathway. Current data expanded the understanding on the roles of αVβ1 involved in fibrogenesis and further support the proposal that αVβ1 is a promising therapeutic target for liver fibrosis.

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**Conflict of interest statements** All authors are employees of Bristol Myers Squibb Company and declare no conflict of interest.

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Table 1: Ct Values of Genes Analyzed in the RT-PCR Quantification in HSCs

| Gene      | Quiescent Mean Ct Values | Quiescent S.D. | TGF-β1 Treated Mean Ct Values | TGF-β1 Treated S.D. |
|-----------|--------------------------|----------------|-----------------------------|--------------------|
| αV        | 24.37                    | 0.62           | 20.38                       | 0.31               |
| β1        | 21.13                    | 0.62           | 17.54                       | 0.29               |
| β3        | 25.75                    | 0.49           | 22.35                       | 0.30               |
| β5        | 24.90                    | 0.43           | 23.25                       | 0.37               |
| β6        | 39.93                    | 0.17           | 36.91                       | 0.68               |
| β8        | 26.63                    | 0.67           | 26.31                       | 0.45               |
| COL1A1    | 18.70                    | 0.52           | 15.71                       | 0.35               |
| αSMA      | 29.67                    | 1.22           | 24.51                       | 0.33               |
| UBC       | 24.78                    | 0.30           | 23.59                       | 0.20               |
| B2M       | 21.39                    | 0.35           | 20.27                       | 0.30               |
| TBP       | 27.59                    | 0.40           | 26.50                       | 0.28               |
| Geometric Mean of Housekeeping Genes | 24.46          | 0.31           | 23.31                       | 0.23               |
Table 2. IC$_{50}$ values for C8 and GSK300834 against various αV integrins

| IC$_{50}$ ± SD (nM) | αVβ1   | αVβ3   | αVβ5   | αVβ6   | αVβ8   |
|---------------------|--------|--------|--------|--------|--------|
| C8                  | 0.70 ± 0.26 | 1714.14 ± 1229.76 | 286.04 ± 69.32 | 1106.67 ± 262.39 | 3187.94 ± 0.00 |
| GSK300834           | 2.83 ± 1.28 | 12.53 ± 5.29 | 4.00 ± 1.93 | 1.50 ± 1.07 | 2.26 ± 1.68 |

Inhibitory potencies for αVβ1 were determined using the [³H]-CWHM12 binding assay and inhibitory potencies for αVβ3, αVβ5, αVβ6 and αVβ8 were determined using HTRF assays as described in Materials and Methods.
Figure Legends

Figure 1. Expression profiling of αV and β integrin genes and proteins in HSCs. RT-PCR was conducted in both quiescent and TGF-β1 treated primary human HSCs to evaluate the mRNA expression profile of αV integrin and β integrins. Effects of TGF-β1 on the mRNA expression for A) COL1A1, B) αSMA, C) αV integrin and D) β integrins. E) Wes Western blot was performed to demonstrate protein levels of αV integrin and β integrins in both quiescent and TGF-β1 treated primary human HSCs to evaluate the protein expression profile. Quantified data is presented to show protein levels of (F) αV integrin and (G) β1 integrin, (H) β3 integrin, (I) β5 integrin, and (J) β6 integrin.

Figure 2. Inhibition of αVβ1 and knock-down of β1 decreases TGFβ1-induced procollagen-1 in HSCs. (A) Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 and ALK5 inhibitor SB525334 were tested on TGF-β1-induced pro-collagen-1 protein secretion in HSCs. All compounds were pre-incubated with HSCs for 1 hr before the treatment with TGF-β1 (2 ng/ml) for 48 hr. The culture supernatants were collected and procollagen-1 protein was measured by Human Pro-Collagen Ia1 ELISA kit (R&D Systems) following the manufacturer’s instructions. This experiment was reproduced 3 times with similar results. (B, C) ITGB1 specific siRNAs knocked down significantly decreased pro-collagen 1 secretion upon TGFβ stimulation. ** P<0.01; **** P<0.0001. (D – F): SB525334, but not C8 or GSK3008348 dose-dependently suppressed TGFβ-stimulated up-regulation of collagen 1A1, 3A1 (COL1A1, COL3A1), and alpha smooth muscle actin (ACTA2).

Figure 3. Inhibition of αVβ1 has no effect on TGF β 1-induced total and active TGF-β production. Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348, and
ALK5 inhibitor SB525334 were tested on the TGF β1-induced total TGF-β production in HSCs. All compounds were pre-incubated with HSCs for 1 hr at 0.03, 0.1, 0.3, 1, 3 and 10 µM concentrations before the treatment with TGF-β1 at 2 ng/ml for 48 hrs. The culture medium was collected and total TGF-β (A) and active TGF-β (B) were determined by ELISA assays.

**Figure 4. Inhibition of αVβ1 has little effect on TGFβ1-induced SMAD3 phosphorylation in primary HSCs.** Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 and ALK5 inhibitor SB525334 were tested on the TGF-β1-induced SMAD3 phosphorylation in HSCs. (A) All compounds were pre-incubated with HSCs for 1 hr at 0.03, 0.1, 0.3, 1, 3 and 10 µM concentrations before the treatment of TGF-β1 at 2 ng/ml for 48 hrs. The cell lysates were analyzed to evaluate SMAD3 phosphorylation by an ELISA assay following the manufacturer’s instructions. (B). HSCs were serum-starved overnight and pre-incubated with SB525334, GSK3008348, or C8 at 10 µM for 1 hr followed by TGF-β1 stimulation at 2 ng/ml for 30 min. Cells were fixed and stained with pSMAD3 (green), Actin filaments (red) and nuclei (blue). Images were taken using Leica Application Suite X (LAS X) confocal system. Bars indicate 100 µm.

**Figure 5. Inhibition of αVβ1 decreases αVβ1 heterodimer in HSCs.** Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 and ALK5 inhibitor SB525334 were tested on levels of αVβ1 protein and their heterodimer. HSCs were serum-starved overnight and pre-incubated with SB525334, GSK3008348, or C8 at 10 µM for 1 hr followed by TGF-β1 stimulation at 2 ng/ml for 24 hrs. Cells were collected for (A-C) direct western blot analysis for αV, β1, and Actin (Input), and (D-F) immunoprecipitation analysis with anti- αV antibody and normal mouse IgG control antibody. The αVβ1 heterodimer was detected by Wes simple western
system. Protein bands were quantified relative to control groups from three independent experiments. Symbols with the same color/shape (red circle ●, green square ■, or blue diamond ♦) indicate the same round of experiment. Gel images are representative of three independent experiments with consistent results.

**Figure 6. Inhibition of αVβ1 significantly decreases TGF-β1 induced phosphorylation of ERK1/2 and STAT3 in HSCs.** (A) Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348, and MEK1/2 inhibitor U0126 were tested on the TGF-β1-induced phosphorylation of ERK1/2 in HSCs. All compounds were pre-incubated for 1 hr with HSCs before the treatment with TGF-β1 (2 ng/ml) for 30 min. (B) HSCs were transfected with β1 specific siRNA for 72h and treated with TGF-β1 (2 ng/ml). Cell lysates were collected to measure phosphorylation of ERK1/2. (C) Selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348, and STAT3 inhibitor S31-201 were tested on the TGF-β1-induced phosphorylation of STAT3 in HSCs. All compounds were pre-incubated for 1 hr with HSCs before the treatment with TGF-β1 (2 ng/ml) for 6 hrs. (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

**Figure 7. ERK1/2 inhibition decreases TGF-β1-induced Pro-collagen-1 in HSCs.** MEK1/2 inhibitor U0126 and STAT3 inhibitor S31-201 were compared with selective αVβ1 integrin inhibitor C8, pan-αV integrin inhibitor GSK3008348 and ALK5 inhibitor SB525334 on the procollagen-1 production in TGF-β1 treated HSCs. All compounds were tested at 1 μM and 10 μM concentrations and compounds were pre-incubated with HSCs for 1 hr before the treatment with TGF-β1 (2 ng/ml) for 48 hrs. HSC culture media were collected and the procollagen-1 protein levels were measured by an ELISA assay following the manufacturer’s instructions.
Statistical analysis was conducted using one way ANOVA with Dunnett’s post-hoc analysis. P<0.05 was defined as statistically significant.

**Figure 8. Working hypothesis for the role of αVβ1 integrin in TGF-β induced procollagen-1 production in primary human HSCs.** Integrin αVβ1 is the major member of αV integrin expressed in primary human HSCs. Selective αVβ1 integrin inhibitor C8 and pan-αV integrin inhibitor GSK3008348 decreased TGF-β1 induced procollagen-1. The αVβ1-mediated effects were not through TGF-β/p-SMAD3, a canonical TGF-β pathway, but through TGF-β/p-ERK1/2, a non-canonical TGF-β signaling pathway.
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Figure 2

A) Pro-collagen-1 (pg/ml) levels in response to different compounds.

B) Relative mRNA levels of ITGB1.

C) Pro-collagen-1 (pg/ml) levels for different compounds.

D) Relative mRNA levels of COL1A1.

E) Relative mRNA levels of COL3A1.

F) Relative mRNA levels of ACTA2.

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Figure 3

A

B

Total TGF (pg/ml)

Active TGF (pg/ml)

[Compound] (μM)

SB525334
C8
GSK3008348

SB525334
C8
GSK3008348

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Figure 4

A

![Graph showing pSMAD3 expression levels with concentration of various treatments.](image)

B

![Image of immunofluorescence microscopy showing cellular responses to different treatments.](image)
Figure 5

A. Input

B. αV Input

C. β1 Input

D. IP: αV

E. αV IP

F. β1 IP
Figure 6

A

B

C
Figure 7

Pro-collagen-1 (pg/ml)

TGF-β (2 ng/ml) - + + + + + + + + + + + +
Inhibitor (μM) 0 0 1 10 1 10 1 10 1 10 1 10

SB525334
GSK3008348
C8 U0126
S31-201

* * * * *

* * * * *

* * * * *

* * * * *

* * * * *
Figure 8

Canonical pathway

TGFβ

SMAD4

SMAD2

SMAD3

p-SMAD2

p-SMAD3

p-ERK1/2

Non-Canonical pathway

αβ1 inhibitor

αβ1 inhibitor

ERK1/2

MEK1/2 Inhibitor

ALK5 Inhibitor

Pro-collagen-1 Production

TGFβRI/II