GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Platelet-Derived Growth Factor Receptor α Contributes to Human Hepatic Stellate Cell Proliferation and Migration

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Accepted for publication
June 8, 2017.

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Platelet-derived growth factor receptor α (PDGFR α), a tyrosine kinase receptor, is up-regulated in hepatic stellate cells (HSCs) during chronic liver injury. HSCs mediate hepatic fibrosis through their activation from a quiescent state partially in response to profibrotic growth factors. HSC activation entails enhanced expression of profibrotic genes, increase in proliferation, and increase in motility, which facilitates migration within the hepatic lobule. We show colocalization of PDGFR α in murine carbon tetrachloride, bile duct ligation, and 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine models of chronic liver injury, and investigate the role of PDGFR α on proliferation, profibrotic gene expression, and migration in primary human HSCs (HHStECs) using the PDGFR α -specific inhibitory monoclonal antibody olaratumab. Although lacking any effects on HHStEC transdifferentiation assessed by gene expression of *ACTA2*, *TGFB1*, *COL1A1*, *SYP1*, and *FN1*, olaratumab specifically reduced HHStEC proliferation (AlamarBlue assay) and cell migration (transwell migration assays). Using phospho-specific antibodies, we show that olaratumab attenuates PDGFR α activation in response to PDGF-BB, and reduced phosphorylation of extracellular signal-regulated kinase 1 and 2, Elk-1, p38, Akt, focal adhesion kinase, mechanistic target of rapamycin, C10 regulator of kinase II, and C10 regulator of kinase-like, suggesting that PDGFR α contributes to mitogenesis and actin reorganization through diverse downstream effectors. Our findings support a distinct contribution of PDGFR α signaling to HSC proliferation and migration and provide evidence that inhibition of PDGFR α signaling could alter the pathogenesis of hepatic fibrosis. (*Am J Pathol* 2017, 187: 2273–2287; <http://dx.doi.org/10.1016/j.ajpath.2017.06.009>)

Hepatic fibrosis is a manifestation of wound healing in chronic liver disease, resulting from dysregulated and excessive collagen deposition by activated hepatic stellate cells (HSCs). This process is mediated by the release of paracrine and autocrine growth factors, and inflammatory chemokines from injured hepatocytes, infiltrating inflammatory cells, and HSCs themselves.¹ During the process of liver injury, HSCs undergo a process of transdifferentiation toward a myofibroblast phenotype characterized by increased proliferation as well as altered gene transcription.² Also, HSCs become motile and migrate within the hepatic lobule to the sites of injury and inflammation guided by chemotactic stimuli, such as platelet-derived growth factors (PDGFs), transforming growth factor- β 1 (TGF- β 1), and

type I collagen.^{3,4} In the setting of chronic liver injury, HSCs continue to migrate to the areas of injury and facilitate the progression of hepatic fibrosis.⁵

PDGFs form five known dimers (AA, AB, BB, CC, and DD), which bind with varying specificity to PDGF receptors (PDGFRs) that exist as α or β monomers.⁶ On PDGF binding, they form $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ dimers and

Supported by NIH grants 4R01DK095498 (S.P.M.) and 5F30DK107129 (A.K.). Imaging was supported by the Center for Biologic Imaging, Pittsburgh Liver Research Center, University of Pittsburgh.

Disclosures: S.P.M. obtained olaratumab from Eli Lilly under a material transfer agreement. N.L. is an employee of Eli Lilly. S.P.M. has corporate research agreements with Abbvie and Dicerna Pharmaceuticals.

Table 1 List of Primary Antibodies Used in the Study

Primary antibody	Species	Dilution	Block	Company	Catalog no.
Akt	Rabbit	1:1000	5% BSA	CST (Danvers, MA)	4685
P-S473-Akt	Rabbit	1:1000	5% BSA	CST	4060
c-Abl	Rabbit	1:1000	5% BSA	CST	2862
P-Y89-c-Abl	Rabbit	1:1000	5% BSA	CST	3098
P-c-Abl Y412	Rabbit	1:1000	5% BSA	CST	2865
CrkII	Rabbit	1:1000	5% BSA	CST	3492
P-Y221-CrkII	Mouse	1:1000	5% Milk	CST	3491
CrkL	Rabbit	1:1000	5% BSA	CST	3182
P-Y207-CrkL	Rabbit	1:1000	5% BSA	CST	3181
Elk-1	Rabbit	1:1000	5% BSA	CST	9182
P-S383 Elk-1	Rabbit	1:1000	5% BSA	CST	9181
Erk1/2	Rabbit	1:1000	5% BSA	CST	4695
P-T202/Y204-Erk1/2	Rabbit	1:1000	5% BSA	CST	4370
FAK	Rabbit	1:1000	5% BSA	CST	13009
P-Y397-FAK	Rabbit	1:1000	5% BSA	CST	8556
P-Y576/577-FAK	Rabbit	1:1000	5% BSA	CST	3281
P-Y925-FAK	Rabbit	1:1000	5% BSA	CST	3284
P-S2448-mTOR	Rabbit	1:500	5% BSA	CST	2971
P-S2481-mTOR	Rabbit	1:1000	5% BSA	CST	2974
p38	Rabbit	1:1000	5% BSA	CST	8690
P-T180/Y182-p38	Rabbit	1:200	5% Milk	CST	4511
PDGFA	Mouse	1:200	5% Milk	SCBT (Dallas, TX)	sc-9974
PDGFB	Rabbit	1:1000	5% Milk	Abcam (Cambridge, UK)	ab23914
PDGFC	Goat	1:200	3% BSA	R&D Systems (Minneapolis, MN)	AF1560
PDGFD	Goat	1:1000	5% BSA	R&D Systems	AF1159
PDGFR α	Rabbit	1:300	5% Milk	SCBT	sc-338
PDGFR β	Rabbit	1:1000	5% BSA	SCBT	sc-432
P-Y572/574-PDGFR α/β	Rabbit	1:1000	5% BSA	Invitrogen (Carlsbad, CA)	44-1000G
P-Y742-PDGFR α	Rabbit	1:1000	5% BSA	Invitrogen	44-1006
P-Y762-PDGFR α	Rabbit	1:1000	5% BSA	CST	24188
P-Y849/ β Y857-PDGFR α	Rabbit	1:1000	5% BSA	CST	3170
P-Y1018-PDGFR α	Rabbit	1:1000	5% BSA	CST	4547
Pan-phospho-PKC	Rabbit	1:1000	5% BSA	CST	9371

BSA, bovine serum albumin; Crk, C10 regulator of kinase; CrkL, Crk-like; CST, Cell Signaling Technology; Erk1/2, extracellular signal-regulated kinase 1 and 2; FAK, focal adhesion kinase; mTOR, mechanistic target of rapamycin; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PKC, protein kinase C; SCBT, Santa Cruz Biotechnology.

trigger reciprocal tyrosine phosphorylation of specific residues of each receptor.⁷ Phosphorylation of tyrosine residues in the kinase domain increases catalytic efficiency and serves as binding sites for signaling molecules, including other kinases as well as nonenzymatic adaptor molecules. Both PDGFR β ^{1,8,9} and PDGFR α ^{10–13} are expressed in HSCs and play important profibrotic roles in chronic liver injury.

A diverse set of downstream effectors mediate an array of PDGF-induced cellular activities. These include mitogen-activated protein kinases, such as extracellular signal-regulated kinase 1 and 2 (Erk1/2) and p38, which are central in the activation, proliferation, and migration of HSCs.^{14–17} Factors, such as Akt, are also common targets of PDGF signaling and can function downstream of focal adhesion kinase (FAK), which plays a dual role in cell adhesion and PDGF-induced HSC proliferation.¹⁸ Mechanistic target of rapamycin (mTOR) activation can contribute

to cellular processes associated with HSC proliferation¹⁹ and migration.²⁰ Adaptor proteins also play an important function in PDGF signaling. C10 regulator of kinase II (CrkII) and Crk-like (CrkL) form a stable complex with PDGFR α and are one of the few identified distinctions between downstream effectors of PDGFR α and PDGFR β .^{7,21,22}

Although the cellular function and downstream signaling mediators of PDGFR β signaling in HSCs have been extensively studied,^{8,9,23} the precise role of PDGFR α in these cells remains unclear.²⁴ Olaratumab (Eli-Lilly & Co., New York, NY) is a humanized monoclonal antibody that targets PDGFR α with high potency and specificity and has been used in both preclinical and clinical studies.^{25–27} In the liver, PDGFR α was shown to be up-regulated in a subset of hepatocellular cancers and olaratumab treatment showed a notable decrease in hepatoma cell proliferation and survival.²⁸ The availability of olaratumab provides a unique

Table 2 RT-PCR Primers Used in this Study

Gene	Primer sequence	Amplicon, bp
<i>ACTA2</i>		
Forward	5'-TTCATCGGGATGGAGTCTGCTGG-3'	141
Reverse	5'-TCGGTCGGCAATGCCAGGGT-3'	
<i>COL1A1</i>		
Forward	5'-TCGTCACAGATCACGTCATCG-3'	120
Reverse	5'-AATCACCTGCGTACAGAACGG-3'	
<i>PDGFA</i>		
Forward	5'-CACACCTCCTCGCTGTAGTATTTA-3'	220
Reverse	5'-GTTATCGGTGTAAATGTCATCCAA-3'	
<i>PDGFB</i>		
Forward	5'-ACTCGATCCGCTCCTTTGATGA-3'	111
Reverse	5'-GCTCGCCTCCAGAGTGGG-3'	
<i>PDGFC</i>		
Forward	5'-TCACAGCCCAAGTTTCCTC-3'	100
Reverse	5'-CCACACCAGCGCCCTAATAT-3'	
<i>PDGFD</i>		
Forward	5'-GAACAGCTACCCAGGAACC-3'	100
Reverse	5'-CTTGTGTCCACACCATCGTC-3'	
<i>FN1</i>		
Forward	5'-GGCTGACAGAGAAGATTCCCGAGAG-3'	87
Reverse	5'-CCAGTTTAGATGGATCTTGGCAGAGAGAC-3'	
<i>SYP</i>		
Forward	5'-GCAATGGGTCTTCGCCATCT-3'	134
Reverse	5'-GCCTGAAGGGTACTCGAAC-3'	
<i>TGFB1</i>		
Forward	5'-CCCTGGACACCAACTATTGC-3'	75
Reverse	5'-TGCGGAAGTCAATGTACAGC-3'	
<i>GAPDH</i>		
Forward	5'-GAAGGTGAAGGTCGGAGTC-3'	226
Reverse	5'-GAAGATGGTGATGGGATTTC-3'	

opportunity to investigate the specific role of PDGFR α in HSCs as well. In the current study, we first show enhanced expression of PDGFR α in HSCs and myofibroblasts in carbon tetrachloride, bile duct ligation, and 3,5-diethoxycarbonyl-1,4-dihydrocollidine models of chronic liver injury. We then examine the role of PDGFR α in human HSCs and show that selective inhibition of PDGFR α with olaratumab inhibits HSC proliferation and migration *in vitro*, but does not affect profibrotic gene expression. We also show that these cell functions are differentially mediated via multiple downstream signaling pathways, including Erk1/2, p38, Elk-1, Akt, mTOR, FAK, and CrkII/CrkL. Finally, we discuss the implications of using a clinically relevant PDGFR α inhibitor in the treatment of hepatic fibrosis.

Materials and Methods

Mice

Two-month-old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). All animal experiments and procedures were performed under the strict guidelines of the NIH's *Guide for the Care and Use of Laboratory Animals*²⁹

and after approval by the Institutional Animal Use and Care Committees at the University of Pittsburgh (Pittsburgh, PA).

Liver Fibrosis and Injury Models

As a model of toxic liver injury, animals were injected twice weekly for 8 weeks i.p. with 1:3 carbon tetrachloride diluted in corn oil at a volume of 0.5 μ L carbon tetrachloride/g mouse body weight. Controls were injected with corn oil only. Bile duct ligation was performed under anesthesia as a model of cholestatic liver injury by opening the peritoneal cavity to expose the common bile duct, which is cut between two 5-0 silk ligatures. Blood and livers were collected for analysis at 14 days. For a secondary model of cholestatic liver injury and atypical ductular proliferation, mice were fed a special diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Bioserve, Frenchtown, NJ) for 16 days.

Immunofluorescence

Liver sections (6 μ m thick) were stained after detergent permeabilization and 37°C incubation, followed by blocking in 2% bovine serum albumin in phosphate-buffered saline (PBS). Serial confocal images (1 μ m thick) were

obtained using an Olympus FluoView 1000 microscope (Olympus, Center Valley, PA) and FV1000 ASW software version 4.2 (Olympus).

Cell Culture

Human HSCs (HHStECs; catalog number 5300, lot number 10326) were purchased on two occasions from ScienCell Research Laboratories (Carlsbad, CA) and were cultured according to the company's provided protocol. Cells were used before the sixth passage and serum starved 12 hours before treatment by washing cells twice with sterile, cold PBS, followed by serum-free Stellate Cell Medium (ScienCell Research Laboratories). LX-2 cells were kindly provided by Dr. Scott Friedman (Mount Sinai Hospital, New York, NY) and cultured according to a protocol from the commercial provider (Millipore, Billerica, MA). Cells were cultured/expanded in Techno Plastic Product-treated T75 tissue culture flasks (Sigma-Aldrich, St. Louis, MO). Recombinant human PDGF-AA (R&D Systems, Minneapolis, MN) or PDGF-BB (Sigma-Aldrich) and TGF- β 1 (R&D Systems) were diluted with serum-free media and exposed to cells for the indicated time periods after two washes with sterile, cold PBS. For PDGFR α inhibition, PDGFR α -specific human monoclonal antibody inhibitor olaratumab (LY3012207, IMC-3G3) or human IgG (Equitech, Kerrville, TX) was diluted in serum-free media and used at the indicated concentrations after two washes with sterile, cold PBS.

Protein-Binding Assays

PDGFR α and PDGFR β extracellular domains were purchased from R&D Systems. IgGs were evaluated for binding to PDGFRs in a direct binding assay, as previously described.²⁵ Antibody 2C5 serves as a positive control anti-PDGFR β antibody.³⁰

Protein Extraction and Western Blotting

Western blots were performed on cell culture lysates homogenized in radioimmunoprecipitation assay buffer with protease/phosphatase inhibitors (Thermo Scientific, Waltham, MA). Protein was quantified using bicinchoninic acid protein assay, and Western blotting was performed as previously described using commercially available primary antibodies from Santa Cruz Biotechnology (Dallas, TX) and Cell Signaling Technology (Danvers, MA) (Table 1).²⁸ Western blots from pooled samples from three technical replicates from each batch of stellate cells were performed at least twice. Representative Western blots from three pooled technical replicates for each time point are shown. Densitometry was performed on these representative Western blots. For detection of PDGFs in HHStEC media, media were concentrated after 24 or 48 hours of serum starvation using Amicon Ultra-15 Centrifugal Filters (Millipore) and resuspended in sample buffer to perform western blot analysis, as described earlier in this paragraph.

Figure 1 PDGFR α expression in activated hepatic stellate cells in rodent models of liver fibrosis. **A:** Confocal immunofluorescence images of murine livers after 8 weeks of carbon tetrachloride (CCl₄), 16 days of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), or 14 days of bile duct ligation (BDL) show PDGFR α expression colocalizing with desmin. **B:** Confocal immunofluorescence images of murine livers after 8 weeks of CCl₄, 16 days of DDC, or 14 days of BDL show PDGFR α expression colocalizing with α -smooth muscle actin (α -SMA). Non-treated (NT) livers are shown for comparison. Scale bars = 100 μ m (A and B).

Figure 2 PDGFR α expression and activity in hepatic stellate cells. **A:** HHStEC and LX-2 cells show differential expression of PDGFR α at baseline and a corresponding increase in phosphorylated receptor after 10 ng/mL PDGF-BB treatment for 2 minutes (m). **B:** HHStECs stimulated with 10 ng/mL PDGF-AA for the indicated time periods show increased PDGFR α phosphorylation at Y742, Y849, and Y572/574. Associated downstream signaling mediators Akt and protein kinase C (PKC) also showed increased phosphorylation in response to PDGF-AA treatment. **Arrowheads** indicate the correct molecular weight band. **C:** Direct binding of human antibodies to immobilized (coated) human PDGFR α or PDGFR β in an enzyme-linked immunosorbent assay. Antibody 2C5 serves as a positive control anti-PDGFR β antibody. Ponceau staining is included as a loading control. h, hours.

Immunoprecipitation

Coprecipitation studies were performed with 500 μ g protein lysate in radioimmunoprecipitation assay buffer with protease/phosphatase inhibitors. Mouse monoclonal anti-CrkII (Santa Cruz Biotechnology) primary antibody (2 μ g) was incubated with sample for 3 hours, followed by overnight conjugation to Protein G PLUS-Agarose beads (Santa Cruz Biotechnology; sc-2002). Total and phospho-PDGFR α were detected using the same antibodies indicated in Table 1.

RT-PCR

RT-PCR was performed on RNA isolated using TRIzol (Thermo Scientific) RNA extraction from adherent cell cultures. RNA extraction was performed using Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA) and following the manufacturer's protocol. Real-time PCR was performed using Power SYBR Green (Thermo Scientific), 100 ng of cDNA, and 0.2 μ mol/L of forward and reverse primers. Primers used in this study are listed in Table 2. Data were analyzed using the comparative $\Delta\Delta$ Ct method or PCR product was run on 0.8% agarose gel with ethidium bromide and detected by UV lamp. Statistical analysis and graphs were performed in Prism version 7.0a (GraphPad Software, San Diego, CA) using one-way analysis of variance.

Proliferation Assays

For proliferation assays, HHStECs were plated on 48-well plates (BD Falcon; BD Biosciences, Bedford, MA) at an

initial plating density of 50,000 cells/well. Cells were serum starved the following day by gentle washing with chilled PBS, followed by culturing in serum-free media overnight. Cells were treated the next day with serum-free media [nontreated (NT)], PDGF-AA, PDGF-BB, olaratumab, or IgG at the indicated concentrations for 24 hours. Next, 20 μ L of AlamarBlue Cell Viability Reagent (Thermo Scientific) was added to each well and incubated at 37°C for 1 hour before measuring fluorescence readings at 590-nm wavelength. Statistical analysis and graphs were performed in Prism version 7.0a using one- and two-way analysis of variance.

Transwell Migration Assays

All cells were serum starved for 12 hours before use in migration assays. Polycarbonate filters (8- μ m pore; Costar; Fisher Scientific, Waltham, MA) were precoated with 100 μ L collagen I (Sigma-Aldrich) and washed two times with sterile PBS before adding cell suspension. Immediately before addition of cell suspension, olaratumab, control IgG, or PDGF-BB was added to the lower chamber in serum-free culture medium. HHStECs were suspended in serum-free media and plated at 2000 cells in 100 μ L/well. After incubation at 37°C for 3 hours, well inserts were fixed with 4% paraformaldehyde and stained by crystal violet. The number of migrated cells was determined by counting the number of cells per representative $\times 10$ field by light microscopy. Statistical analysis and graphs were performed in Prism Version 7.0a (GraphPad Software, Inc., La Jolla, CA) using nonparametric *t*-tests.

Figure 3 PDGFR α signaling contributes to HHStEC proliferation, but not activation, in the presence of exogenous PDGF stimulation. **A:** Proliferation of HHStECs was measured using AlamarBlue dye after a 24-hour incubation with PDGF-AA, PDGF-BB, olaratumab, or IgG at the indicated concentrations. PDGF-AA and PDGF-BB induced significant proliferation of HHStECs. HHStECs were also exposed simultaneously to PDGF and olaratumab, PDGF and IgG, or PDGF and mitomycin C (DNA synthesis inhibitor). Both olaratumab and mitomycin C cause a reduction in PDGF-induced proliferation compared to IgG control. **B:** PDGFR α inhibition using olaratumab tested over 24 hours at various plating densities shows no significant change in proliferation compared to IgG-treated controls. **C:** RT-PCR shows no significant changes in expression of *ACTA2*, *TGFβ1*, *COL1A1*, *FN1*, or *SYP* in HHStECs after olaratumab treatment at multiple concentrations. TGF-β1 (2 ng/mL) treatment was included as a positive control. All assays were performed in triplicate, and results are normalized to their respective nontreatment (NT) conditions. ** $P < 0.01$, **** $P < 0.0001$.

Results

PDGFR α Expression in Murine and Human Hepatic Stellate Cells

To confirm the expression and pathological relevance of PDGFR α *in vivo*, we examined PDGFR α expression in livers of C56BL/6 mice at baseline as well as after chronic toxic (8 weeks of carbon tetrachloride administration) and cholestatic (14-day bile duct ligation or 16-day 3,5-diethoxycarbonyl-1,4-dihydrocollidine) liver injury. Using confocal microscopy, we observed close colocalization of PDGFR α in desmin-positive and α -smooth muscle actin-positive cells, representing HSC and activated myofibroblast populations, respectively (Figure 1).

Next, to identify suitable HSCs for studies of PDGFR α , we examined its expression and activation in primary HHStECs and LX-2, an immortalized HSC line. Although

PDGFR β was present in both cell types, expression of PDGFR α was more profound in HHStECs (Figure 2A). Furthermore, increased PDGFR α phosphorylation in response to PDGF-BB stimulation was also evident (Figure 2A). LX-2 cells also showed PDGFR α phosphorylation in response to PDGF-BB, albeit to a lesser extent (Figure 2A). Therefore, because of greater basal PDGFR α expression in HHStECs and activation in response to the highly mitogenic and well-characterized profibrotic ligand PDGF-BB, we opt to use these cells as a representative model of PDGFR α -expressing HSCs for further study.

To assess if PDGFR α is functional in HHStECs, these cells were treated with PDGFR α -specific ligand PDGF-AA. This led to phosphorylation of PDGFR α at key tyrosine signaling residues (Figure 2B). Furthermore, PDGF-AA treatment induced phosphorylation of downstream Akt and protein kinase C (Figure 2B).

Figure 4 Olaratumab inhibits PDGFR α activation and downstream proliferative signaling mediators in HHStECs in the presence of exogenous PDGF stimulation. **A** and **B**: HHStECs were pretreated with either 300 nmol/L olaratumab or 300 nmol/L human IgG for 30 minutes (m) before 10 ng/mL PDGF-BB exposure for the indicated time periods. Representative Western blots using pooled samples from each time point from three technical replicates show decrease in phosphorylation of tyrosine residues in PDGFR α in cells pretreated with olaratumab, including Y754, Y762, Y849, and Y1018, but not Y742 and Y572/574. **Arrowheads** indicate the correct molecular weight band. **C** and **D**: Representative Western blots using pooled samples from each time point from three technical replicates also show decreased phosphorylation of p38 MAPK (T180/Y182), FAK (Y397, Y576/Y577, Y925), Akt (Ser473), and mTOR (Ser2448, Ser2481) after PDGFR α blockade with 300 nmol/L olaratumab before 10 ng/mL PDGF-BB treatment. **E**: Decreased phosphorylation of Erk1/2 (T202/Y204) and downstream transcription factor Elk1 (Ser383) was also observed in similar representative Western blots. Ponceau staining is included as a loading control. All Western blots were repeated twice on the same pooled lysates from three technical replicates and performed for the two stellate cell batches. h, hour; NT, nontreated.

To block PDGFR α , we used the fully human anti-PDGFR α antibody olaratumab.^{31,32} To validate the efficacy and specificity of olaratumab, an enzyme-linked immunosorbent assay–based cell-free binding assay was performed. Olaratumab showed a dose-dependent binding to immobilized PDGFR α extracellular

domain (Figure 2C). Furthermore, the antibody concentration required for 50% maximum binding to PDGFR α extracellular domain was calculated to be approximately 0.06 nmol/L, as also reported previously.²⁵ Olaratumab did not cross-react with a human PDGFR β extracellular domain (Figure 2C).

Figure 5 Densitometric quantification of olaratumab-mediated changes in protein phosphorylation of HHStECs in the presence of exogenous PDGF stimulation. Densitometry of key phosphorylated protein changes from representative Western blots using pooled samples from three technical replicates for each time point shown in Figure 4. Values are normalized to total protein levels and shown as the ratio of the signal of olaratumab-treated HHStECs/IgG-treated HHStECs. Dashed lines represent the value at which the relative phosphorylated protein signals of olaratumab-treated HHStECs and IgG-treated HHStECs are equal. h, hours; m, minutes.

PDGF Treatment Induces PDGFR α -Dependent HHStEC Proliferation, but Not Profibrotic Gene Expression

PDGF-BB signaling through PDGFR β is well-known to play a central role in HSC activation, including proliferation and transdifferentiation to an activated myofibroblast state. However, the specific contribution of PDGFR α in these processes is unknown. To test unambiguously the contribution of PDGFR α to HSC proliferation, we used the AlamarBlue cell viability assay after 24-hour exposure to PDGF-AA and PDGF-BB in the presence of PDGFR α -specific blocker olaratumab or control IgG (Figure 3A). We observed an increase in HHStEC proliferation in response to PDGF-AA and PDGF-BB when compared to NT controls.

Although olaratumab alone was insufficient to affect HHStEC proliferation (Figure 3, A and B), incubation of these cells with olaratumab in the presence of either exogenous PDGF-AA or PDGF-BB resulted in a significant decrease in proliferation (Figure 3A).

Next, we examined any effect of olaratumab on profibrotic gene expression. HSC activation is associated with increased expression of genes like collagen (*COL1A1*), *TGFBI*, α -smooth muscle actin (*ACTA2*), fibronectin (*FNI*), and synaptophysin (*SYP*). Olaratumab treatment for 24 hours led to no significant change in expression of any of these genes (Figure 3C). Also, treatment of HHStECs with either PDGF-AA or PDGF-BB led to no change in the profibrotic gene expression (data not shown).

Olaratumab Inhibits PDGF-BB–Mediated HSC Proliferation through Blockade of PDGFR α and Downstream Signaling

We next sought to determine the signaling pathways downstream of PDGFR α affecting HSC proliferation. To assess the most physiologically relevant signaling pathways involved, we tested the effect of olaratumab in the presence of PDGF-BB (a nonselective isoform of PDGF with a central role in HSC mitogenesis, chemotaxis, and activation during hepatic fibrosis).^{1,33} HHStECs were pretreated with 300 nmol/L olaratumab or 300 nmol/L human IgG for 30 minutes before exposure to PDGF-BB for various times (range, 5 minutes to 6 hours).

PDGFR α phosphorylation at all sites tested increased within 5 minutes, peaked at 30 minutes, and decreased at 2 and 6 hours of PDGF-BB treatment (Figure 4, A and B). Pretreatment with olaratumab decreased PDGFR α phosphorylation at Y754, Y849, Y762, and Y1018 but not at Y742 and Y572/574 (Figures 4, A and B, and 5).

We next examined downstream effectors of PDGFR α associated with PDGF-induced proliferation, such as Erk1/2,¹⁶ p38,¹⁴ FAK,¹⁸ Akt, and mTOR,³⁴ using phospho-specific antibodies, including T202/Y204 for Erk1/2, T180/Y182 for p38 mitogen-activated protein kinase (MAPK), Y397/Y576/Y577/Y925 for FAK, S473 for Akt, and S2448/S2481 for mTOR. PDGF-BB treatment increased phosphorylation of Erk1/2, p38 MAPK, Akt, mTOR, and FAK as compared to nontreated cells. The peak phosphorylation of Erk and p38 MAPK occurred at 15 minutes, followed by a gradual decrease up to 6 hours after PDGF-BB (Figure 4, C–E). Although earlier time points were unchanged, a notable decrease in Erk1/2 phosphorylation was evident at 2 to 6 hours in the olaratumab group (Figures 4, C–E, and 5). Decreased phosphorylation of p38 MAPK was evident at only 30 minutes in the olaratumab group (Figures 4, C–E, and 5). Consistent with decreased phosphorylation of Erk1/2 and p38, we observed decreased phosphorylation of Elk-1, a known downstream target of Erk1/2 and p38,³⁵ in olaratumab pretreated cells from 30 minutes to 6 hours (Figure 4E and Figure 5). PDGF-BB

Figure 6 Orlatumab inhibits migration of HHStECs in the absence of exogenous PDGF ligand, because of autocrine baseline signaling. **A:** Representative images from HHStEC transwell migration assays and quantification shows insignificant difference between no treatment (NT) and PDGF-BB treatment. **B:** Decreased HHStEC migration after orlatumab versus IgG-treated controls after 3 hours relative to IgG treatment and quantified. **C:** RT-PCR of cDNA derived from nontreated HHStECs (**left lane**) shows baseline expression of PDGFA, PDGFB, PDGFC, and PDGFD compared to HHStEC RNA in the absence of reverse transcriptase (**middle lane**) or primer only control (**right lane**) (separated because of noninclusion of technical replicates). **D:** Representative Western blot shows PDGF-BB expression in nontreated HHStEC whole cell lysates and hepatocellular carcinoma (HCC) sample as a positive control. Ponceau staining is included as a loading control. **E:** Representative Western blots of PDGF ligands detected in concentrated baseline HHStEC media after 24 or 48 hours of serum starvation indicates autocrine secretion of PDGFB, PDGFC, and PDGFD, but not PDGFA. Concentrated supernatant from TGF- β 1-activated HHStECs is shown for comparison. * $P < 0.05$. Original magnification, $\times 50$ (**A** and **B**).

treatment also led to increased FAK phosphorylation that peaked at 30 minutes to 2 hours. Orlatumab pretreatment modestly affected FAK phosphorylation at Y397 at 15 to 30 minutes and Y925 and Y576/577 at 2 hours (Figures 4, C and D, and 5). PDGF-BB treatment led to a sustained mTOR phosphorylation at both S2481 and S2448, which was reduced by orlatumab for up to 30 minutes (Figures 4, C and D, and 5). Akt phosphorylation after PDGF-BB treatment was also sustained but peaked at 30 minutes to 2 hours (Figure 4, C and D). Orlatumab decreased Akt phosphorylation at all time points, with maximal effect at 2 to 6 hours (Figures 4, C and D, and 5).

Given orlatumab's selective inhibition of PDGFR α , as well as the universal binding of PDGF-BB to both PDGFR α and PDGFR β receptors, our results provide evidence of an important and independent contribution of PDGFR α in regulating multiple proliferative signaling pathways in human HSCs.

PDGFR α Blockade by Orlatumab Inhibits HHStEC Migration, Even in the Absence of Exogenous PDGF Ligand, because of Autocrine Signaling

PDGF is a potent chemoattractant for HSCs.^{16,36} To examine whether PDGFR α signaling contributes to HSC migration, we performed transwell migration assays on HHStECs, which were exposed to orlatumab, PDGF-BB, human IgG, or serum-free medium (NT). No significant change in HHStEC migration was observed after PDGF-BB treatment (Figure 6A). However, orlatumab treatment alone significantly reduced HHStEC migration (Figure 6B). One possibility for the lack of migratory response to exogenous PDGF-BB but inhibition of migration to PDGFR α blockade could be basal PDGFR α activity in HHStECs. We qualitatively assessed basal gene expression of PDGFA, PDGFB, PDGFC, and PDGFD in HHStECs. RT-PCR analysis showed mRNA expression of all PDGFs

Figure 7 Olaratumab inhibits baseline PDGFR α signaling in HHStECs along with downstream effectors. **A:** Representative Western blots from HHStEC treatment with 300 nmol/L olaratumab using pooled lysates from three technical replicates for each time point showing decreased PDGFR α phosphorylation at Y762 and Y849 compared to IgG-treated controls. PDGF-BB treatment included as a positive control. **B:** Similar representative Western blots showing decreased Erk and Elk-1 phosphorylation compared to IgG-treated controls. PDGF-BB treatment serves as a positive control. **C:** Representative Western blots show olaratumab treatment decreases phosphorylation of mTOR at Ser2448 and increases phosphorylation at Ser2481. Olaratumab also decreases p38 phosphorylation. **D:** Similar representative Western blots showing olaratumab treatment increasing Abl expression and phosphorylation at Y412 and Y89 and increasing phosphorylation at inhibitory tyrosine residues of CrkII (Y221) and CrkL (Y207). **Arrowhead** indicates the correct molecular weight band. **E:** Immunoprecipitation of HHStEC lysates using anti-CrkII shows increased binding of CrkII to both total PDGFR α and phospho-PDGFR α Y762 after olaratumab treatment. Ponceau staining is included as a loading control. All Western blots were repeated twice on the same pooled lysates from three technical replicates and performed for the two stellate cell batches. h, hours; IP, immunoprecipitation; m, minutes; WB, Western blotting.

in NT HHStECs (Figure 6C). The presence of PDGF-BB protein in cell lysates of NT HHStECs was also observed (Figure 6D). Secretion of PDGF-BB, PDGF-CC, and PDGF-DD was also confirmed by Western blot analysis of the concentrated cell culture media collected from the NT HHStECs cultured for 24 or 48 hours (Figure 6E). Taken together, these data indicate a role of autocrine PDGFR α signaling in HHStEC migration.

Olaratumab Inhibits Baseline PDGFR α Activation in HHStECs and Downstream Cell Motility Signaling Pathways

Next, downstream signaling affected by olaratumab in the absence of PDGF ligand was assessed to address the mechanism of PDGFR α -mediated HSC migration. Whole cell lysates from HHStECs treated with olaratumab or IgG control for 30 minutes, 1 hour, and 3 hours were examined

for levels of phosphorylated PDGFR α and its downstream effectors.

A notable decrease in PDGFR α phosphorylation at only Y762 and Y849 was observed, whereas other residues, like Y754, remained unaffected after olaratumab treatment (Figures 7A and 8). Relative decreases in phosphorylation of Erk1/2 at 1 and 3 hours, as well as p38 MAPK at all time points, were observed after olaratumab treatment (Figures 7B and 8). Reduced phosphorylation of Elk-1 is consistent with these findings as Elk-1 is a known downstream target of both Erk1/2 and p38 MAPK (Figure 7B).³⁵ Olaratumab treatment decreased mTOR phosphorylation at serine 2448 at 30 minutes and 1 hour (Figures 7C and 8). Intriguingly, olaratumab treatment increased serine 2481—mTOR at 30 minutes and 1 hour (Figures 7C and 8).

Olaratumab's effect on CrkII and its isoform CrkL, which are adaptor proteins with a role in actin reorganization and cell motility, was examined next.³⁷ Phosphorylation of

Figure 8 Densitometric quantification of olaratumab-mediated changes in protein phosphorylation in HHStECs at baseline. **A:** Densitometry of key phosphorylated protein changes from representative Western blots using pooled samples from three technical replicates for each time point shown in Figure 7. Values for each phosphorylated protein signal are normalized to total protein levels and shown as the ratio of the signal of olaratumab-treated HHStECs/IgG-treated HHStECs. **B:** c-Abl—phosphorylated and total proteins are represented separately to highlight the increase in both total and phosphorylated proteins. The dashed line represents the value at which the relative phosphorylated protein signals of olaratumab-treated HHStECs and IgG-treated HHStECs are equal. h, hours; m, minutes.

CrkII (Y221) and CrkL (Y207) by Abl kinase results in sequestration of SH2 and SH3 binding domains of Crk proteins, leading to inhibition of downstream signal activation.³⁸ Olaratumab treatment of HHStECs led to increased phosphorylation of both CrkII and CrkL (Figures 7D and 8). Consistent with inhibition of Crk signaling, we observe activation of Abl kinase, indicated by increased phosphorylation at Y412-Abl³⁹ and Y89^{40,41} (Figures 7D and 8). The

inhibition of Crk signaling downstream of PDGFR α inhibition is also consistent with the specific inhibition of PDGFR α phosphorylation at Y762 (Figures 7A and 8), necessary for PDGF-dependent Crk signaling.²² We also noted olaratumab to strongly induce CrkII protein interaction to both total and Y762-PDGFR α (Figure 7E).

Thus, the overall basis of PDGFR α blockade on human HSC migration appears to be through a cumulative effect on modulation of Erk, p38 MAPK, mTOR complex (mTORC) 1, and CrkII/CrkL.

Discussion

The development of tyrosine kinase inhibitors has been an important avenue for the research of new treatments for fibrosis and advanced liver diseases. Many of the successful inhibitors to date have cotargeted PDGFR α and PDGFR β receptors and have shown promise in preclinical studies of hepatic fibrosis⁴² as well as clinical benefit in the management of diseases like hepatocellular carcinoma.⁴³ However, the development of specific and potent inhibitors, such as olaratumab, that are already in clinical use may present therapeutic and safety advantages.^{31,32} For instance, olaratumab shows approximately 100-fold increased effect on PDGF-mediated cell proliferation compared to the multi-tyrosine kinase inhibitor imatinib.²⁷

The availability of olaratumab offers a unique opportunity to study exclusive PDGFR α function in human HSCs. After confirming the expression of PDGFR α colocalized to HSCs and activated myofibroblasts in multiple models of murine chronic liver injury, we show that human HSCs respond to exogenous or autocrine PDGFs in part through PDGFR α -specific downstream signaling to contribute to proliferation and migration, but not profibrotic gene expression (Figure 9). Baseline blockade of PDGFR α signaling in HHStECs affected migration but not proliferation or profibrotic gene expression. Exogenous PDGF-BB did not induce migration or profibrotic gene expression but induced HHStEC proliferation, which was suppressed by PDGFR α blockade. These findings point to distinct biological outcomes of autocrine versus paracrine PDGF-BB/PDGFR α signaling axis. A possible explanation is a distinct cellular response to dose of ligand available at baseline versus during exogenous treatment. Suffice to say that both distinct and common signaling events were associated with the two biological outcomes of PDGFR α blockade. The major translational significance of the current study is the use of a clinically relevant biological agent in human HSCs and its effectiveness in suppressing two key processes of HSCs for treatment of hepatic fibrosis, which remains a major unmet clinical need.

We found that both PDGF-AA and PDGF-BB promote HHStEC proliferation, which was inhibited by olaratumab. PDGFR β plays an important role in HSC proliferation. Studies in rat HSCs have shown PDGFR β to have higher

Figure 9 Proposed effect of PDGFR α blockade on HSC migration and proliferation through inhibition of specific downstream signaling. **A:** In response to PDGFR α activation by autocrine and/or exogenous PDGF, HSC proliferation and migration are induced through activation of FAK, Erk1/2, Elk-1, p38, Akt, mTOR, and CrkII/CrkL signaling. **B:** Blockade of PDGFR α by olaratumab decreases the above-mentioned signaling mediators, with FAK, Erk1/2, Elk-1, mTOR, and Akt affecting proliferation and Erk1/2, Elk-1, p38, CrkII/CrkL, decreased mTORC1, and increased mTORC2 regulating migration. Decreases in downstream signaling are represented by **dashed arrows**.

mitogenic activity than PDGFR α .^{44,45} Because PDGF-BB is the predominant mitogen for HSCs during hepatic fibrosis, we wanted to address the relative contribution of PDGFR α toward HSC proliferation. In our study, olaratumab blockade before PDGF-BB treatment of HSCs led to decreased HSC proliferation that was associated with inhibition of several phosphorylation sites in PDGFR α and of multiple downstream effectors, including Erk1/2, p38, Elk-1, FAK, mTOR, and Akt. In particular, Erk1/2, Elk-1, FAK, and Akt showed sustained inhibition after olaratumab-mediated PDGFR α inhibition. Erk1/2 is known for its role in HSC proliferation,¹⁶ whereas its downstream target Elk-1 promotes gene expression associated with proliferation⁴⁶ and migration.³⁵ FAK has been shown to serve as a sensor for the detection of integrin-mediated binding to ECM and simultaneously acts as a signaling node for PDGF-induced proliferation in HSCs via the FAK/phosphatidylinositol 3-kinase/Akt pathway.¹⁸ FAK also promotes Erk-mediated cell proliferation through phosphorylation at Y925 and Y397, two of the tyrosine residues affected by olaratumab.⁴⁷ Akt affects cell proliferation through diverse sets of mechanisms.^{48,49} PDGF-BB treatment led to an increase in phosphorylation of PDGFR α at Y754, which was decreased by olaratumab. Y754 is an indicator of formation of the PDGFR $\alpha\beta$ heterodimer,⁵⁰ suggesting that olaratumab mediates part of its effect on HSC proliferation through the inhibition of heterodimer formation. However, because olaratumab inhibits phosphorylation of other signaling residues of PDGFR (which are more specific for PDGFR α homodimer) in the presence of exogenous PDGF-BB, we conclude that there is a definite and unique contribution of PDGFR α to HSC proliferation through Erk1/2, Elk-1, FAK, and Akt (Figure 9). A small

increase in Erk1/2, Akt, mTOR, and FAK after initial olaratumab treatment may be because of its potential transient and partial agonistic activity.

It was intriguing to note that expression of fibrosis-associated genes did not show a response to olaratumab or PDGF-AA treatment in HHStECs, suggesting that PDGFR α may be dispensable in HSC profibrotic gene induction. Thus, our study indicates that PDGFR α has a more narrowly defined function than PDGFR β in HSCs. Indeed, this is consistent with earlier investigations showing less potent activation of HSCs as a result of PDGFR α signaling.^{23,51} Our studies do not, however, preclude a ligand-independent contribution of PDGFR α signaling to HSC activation. This is particularly relevant in light of studies showing that PDGFR α is necessary for TGF- β 1-mediated SMAD activation after interaction and internalization of a PDGFR α monomer/TGF- β receptor II complex.⁵²

Migration of HSCs is an important mechanism of perpetuation of hepatic fibrosis. PDGF ligands, specifically PDGF-BB, are known chemotactic stimuli for HSCs.³³ We did not observe a significant increase in migration of HHStECs after PDGF-BB treatment. However, HHStECs expressed and secreted PDGF ligands at baseline, indicating potential autocrine PDGFR α signaling. Indeed, activation of HSCs as a result of cell culture is well-known in both primary^{53,54} and immortalized HSCs.⁵⁵ Olaratumab blocked HHStEC migration, which was associated with decreased PDGFR α phosphorylation at Y762 and Y849. Among residues not affected was Y754, ruling out the role of the PDGFR $\alpha\beta$ heterodimer in HSC migration. Previous studies have shown that PDGF-AA specific for the PDGFR $\alpha\alpha$ homodimer is not chemoattractive for HSCs, in contrast to other PDGF ligand dimers.^{3,56} However, our study clearly shows a reproducible effect of PDGFR α inhibition on

HHStC migration, which was associated with notable decreases in phosphorylation of well-known mediators of HSC migration, like Erk1/2 and p38 MAPK,⁵⁷ and others, like mTOR (Y2448) and Crk.

Phosphorylation of PDGFR α at Y762 is necessary for direct binding to and phosphorylation of Crk adaptor proteins.²² The prolonged binding of PDGFR α to Crk proteins is considered a major difference in signaling function between PDGFR α and PDGFR β .⁷ On phosphorylation of Y221 on CrkII, and Y207 on CrkL, their SH2 domain is sequestered, leading to inhibition of Crk activity.⁵⁸ During its effect on cell migration, olaratumab reduced phosphorylation of Y762-PDGFR α , which was associated with increased phosphorylation of CrkII and CrkL. Olaratumab treatment increased c-Abl activation, as indicated by increased phosphorylation, which, in turn, is known to phosphorylate and inactivate CrkII/CrkL.³⁸ The relatively stable interaction of CrkII/CrkL and PDGFR α , in contrast to rapid phosphorylation by PDGFR β , has been speculated to lead to prolonged activity of CrkII/CrkL, leading us to hypothesize that inhibition of Y762 phosphorylation by olaratumab may dissociate the Crk/PDGFR α complex and lead to early phosphorylation of CrkII/CrkL by another kinase, like c-Abl.²¹ Surprisingly, olaratumab resulted in a dramatic increase in binding of CrkII to both total and Y762-phosphorylated PDGFR α . It is likely that olaratumab-mediated enhanced phosphorylation and, hence, inhibition of Crk activity occurs despite enhanced PDGFR α -CrkII association. Further studies will be critical to uncover the precise mechanism by which olaratumab alters CrkII/CrkL phosphorylation.

Olaratumab treatment of HHStCs in the setting of both exogenous PDGF and autocrine signaling shared a reduction in Erk1/2 and mTOR. However, in contrast to mTOR phosphorylation changes in the presence of exogenous PDGF, we observed differential changes in mTOR phosphorylation, suggestive of a shift away from mTORC1 signaling to mTORC2 signaling.⁵⁹ Because of the association of autocrine PDGFR α activation with HHStC migration, this implies that mTORC1 is a predominant downstream effector of PDGFR α in HSC chemotaxis. This finding is consistent with studies showing that mTOR inhibitors that primarily affect the rapamycin-sensitive mTORC1 reduce experimental hepatic fibrosis, in part through reduced migration of HSCs.^{60,61} Of particular note, rapamycin has been demonstrated to reduce PDGF-induced migration of HSCs *in vitro*.³⁴

Overall, our study concludes that PDGFR α contributes to human HSC proliferation and migration independent of profibrotic gene expression. These findings suggest that olaratumab, alone or in combination, may have therapeutic activity in the pathogenesis of hepatic fibrosis. However, future research of potential therapeutic approaches aimed at inhibiting the PDGFR α pathway in *in vivo* models, including potential combinations, is unpredictable and would need to be performed before clinical investigation.

Acknowledgments

A.K. and S.M. conceived all experiments, analyzed the data, and drafted the manuscript; A.K. performed experiments; T.P.-S., S.N., and N.L. provided technical assistance for *in vitro* experiments using olaratumab and edited the manuscript; all authors reviewed the results and approved the final version of the manuscript.

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