Potentiation of Platelet-Derived Growth Factor Receptor-β Signaling Mediated by Integrin-Associated MFG-E8

Sei-ichiro Motegi, Susan Garfield, Xu Feng, Miklós Sárdy, Mark C. Udey

Objective—Pericytes/pericyte precursors produce milk fat globule-associated protein with epidermal growth factor and factor VIII-like domains (MFG-E8) in vivo, and this αv integrin ligand enhances angiogenesis in tumors and in oxygen-induced retinopathy in mice. Inhibition of MFG-E8 production or function attenuates platelet-derived growth factor-BB (PDGF-BB)-induced migration of pericyte/pericyte precursor-like 10T1/2 cells in vitro. Herein, we describe mechanisms by which MFG-E8 modulates PDGFB:PDGF receptor β (PDGFRβ) signaling in 10T1/2 cells.

Methods and Results—Small interfering RNA depletion of MFG-E8 from 10T1/2 cells or antibody inhibition of MFG-E8 action enhanced PDGFB-BB-dependent degradation of PDGFRβ and attenuated signaling. Coimmunoprecipitation revealed transient association of MFG-E8 with PDGFRβ in PDGFB-BB-treated 10T1/2 cells and reduced PDGFRβ-focal adhesion kinase association in MFG-E8-depleted cells. Confocal microscopy demonstrated that MFG-E8 binding to 10T1/2 cells was RGD motif and αv dependent but PDGFB-BB treatment independent, whereas colocalization of MFG-E8 with PDGFRβ was enhanced by PDGFB-BB. Ubiquitination of PDGFRβ was also increased in MFG-E8 small interfering RNA-transfected cells.

Conclusion—Integrin αv-bound MFG-E8 associates with PDGFRβ and focal adhesion kinase after PDGFB-BB treatment, results in cell surface retention of PDGFRβ, delays receptor degradation, potentiates downstream signaling, and enhances migration of 10T1/2 cells. MFG-E8 may promote angiogenesis, in part, via cell autonomous actions on pericytes or pericyte precursors that result in enhanced PDGFB-BB:PDGFRβ signaling mediated via integrin-growth factor receptor cross-talk. (Arterioscler Thromb Vasc Biol. 2011;31:2653-2664.)

Key Words: MFG-E8 ■ PDGF receptor β ■ PDGFB-BB ■ integrin ■ pericyte

Platelet-derived growth factor (PDGF)-BB:PDGF receptor β (PDGFRβ) signaling is an important regulator of angiogenesis. PDGFB-BB is released by endothelial cells (ECs), platelets, vascular smooth muscle cells (VSMCs), and inflammatory cells at sites of neangiogenesis, and it enhances pericyte (PC) and VSMC migration and proliferation.1-6 PDGFB-BB binding to the receptor tyrosine kinase PDGFRβ leads to receptor dimerization, autophosphorylation, and activation of downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway. Ligand-bound receptor undergoes endocytosis and can either recycle to cell surfaces or undergo ubiquitination and proteosomal or lysosomal degradation.1-3,7-9 Pathway activity is influenced by the quantities of receptor that are expressed, and the location of the receptor with respect to ligand and additional proteins that participate in downstream signaling.

Several receptor tyrosine kinases, including vascular endothelial growth factor (VEGF) receptor 2, PDGFRβ, epidermal growth factor receptor, and basic fibroblast growth factor receptor, are known to associate with integrins, and these associations regulate kinase activity.10,11 Integrin αvβ3 complexes with PDGFRβ in vascular ECs, fibroblasts and cancer cells that have been treated with PDGFB-BB.12-14 Integrin αvβ3-associated PDGFRβ subsequently interacts with signaling molecules, including Src, SHP2, phospholipase Cγ, phosphatidylinositol 3-kinase, and focal adhesion kinase (FAK), triggering downstream signaling.12,15-17

The secreted glycoprotein milk fat globule-associated protein with epidermal growth factor and factor VIII-like domains (MFG-E8) (also termed lactadherin and SED1) is composed of 2 N-terminal epidermal growth factor-like domains and 2 C-terminal discoidin-like domains (C1 and C2) that are homologous to blood coagulation factors V and VIII.18-22 One endothelial growth factor-like domain (E2) contains an RGD consensus integrin-binding motif, and MFG-E8 binds to integrin αvβ3.20,21,23,24 Initial reports documented that the carboxy-terminal domains of MFG-E8 bound to negatively charged and oxidized phospholipids,25,26 facilitating opsonization of apoptotic cells for uptake by phagocytes.23,27 Interactions of MFG-E8 with αv integrins...
have also been implicated in regulation of angiogenesis and mammary gland branching,\textsuperscript{24,28} whereas interactions mediated via the C1 domain are important for sperm-egg binding and enhancement of collagen turnover.\textsuperscript{29,30}

With respect to angiogenesis, it has been reported that MFG-E8 enhanced VEGF-induced angiogenesis in an \(\alpha_\beta_\beta/\beta_\alpha\) integrin-dependent manner using a mouse model of acute hindlimb ischemia.\textsuperscript{28} Our group subsequently demonstrated that MFG-E8 enhanced tumor-related angiogenesis and tumor growth in the Rip1-Tag2 mouse pancreatic tumor model, a model in which tumor progression is critically dependent on angiogenesis.\textsuperscript{31} We have additionally characterized the involvement of MFG-E8 in the regulation of angiogenesis in tumors, and in oxygen-induced retinopathy (OIR) in mice.\textsuperscript{32} We determined that PC/PC precursors are important sources of MFG-E8 in vivo, that MFG-E8 may enhance angiogenesis via actions on PC/PC precursors as well as ECs, and that MFG-E8 can be effectively targeted to inhibit MFG-E8 production, 10T1/2 cells (5\times10\(^5\) cells per 60-mm plate) were transfected with 10 nmol/L siRNA using HiPerFect Transfection Reagent (Qiagen). After 48 hours, MFG-E8 mRNA levels were assessed by quantitative reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated with RNeasy Kits (Qiagen) and reverse transcribed using the SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was performed using the following primers: MFG-E8 (forward, 5'-ATCTACTGCCTCTGCGCTA-3' \textsuperscript{3}; reverse, 5'-ACCCAGAACGTGGTGGGAC-3').

To determine whether MFG-E8 could act directly on PDGFR\(\beta\) on PC/PC precursors, we studied C3H 10T1/2 cells in vitro as surrogates. 10T1/2 cells share surface markers with PCs but have multilineage differentiation potential and may be more closely related to PC precursors. Inhibition of MFG-E8 production by 10T1/2 cells rather than ECs, and PDGFR\(\beta\)+ PC/PC precursors purified from tumors contained large amounts of MFG-E8 mRNA. Tumor- and retinopathy-associated angiogenesis was diminished in MFG-E8 knockout mice, and PC coverage of neovessels was also reduced.\textsuperscript{32}

We determined whether MFG-E8 regulates PDGFR\(\beta\) signaling in vitro. Significant, systemic injection of Ab to MFG-E8 that inhibited 10T1/2 cell migration in vitro also resulted in inhibition of pathological neovascularization in the OIR model. These results suggest that MFG-E8 derived from PC/PC precursors enhanced PDGF-BB-induced PC/PC precursor function. However, the precise mechanisms of MFG-E8 action on PC/PC precursors and mechanisms by which PDGF-BB:PDGFR\(\beta\) signaling might be regulated by MFG-E8 have not been determined.

Herein, we describe the results of experiments designed to determine whether MFG-E8 regulates PDGFR\(\beta\):PDGFR\(\beta\) signaling. We found that MFG-E8 that was produced by 10T1/2 cells associated with integrin \(\alpha_\beta\) and PDGFR\(\beta\) on cell surfaces after PDGF-BB treatment, altered the distribution of PDGFR\(\beta\) within cells, increased the ability of PDGFR\(\beta\) to associate with FAK, and inhibited degradation of PDGFR\(\beta\), thereby enhancing PDGFR\(\beta\) signaling and cell function (migration).

**Methods**

Detailed descriptions of additional materials and methods used in this study are available in the supplemental materials, available online at http://atvb.ahajournals.org.

**Cells and Culture Conditions**

Mouse C3H-derived 10T1/2 cells (ATCC) were maintained in BME medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 \(\mu\)g/mL) and were used before passage 10.

**Abs**

Abs and their sources were as follows: rat anti-mouse PDGFR\(\beta\) monoclonal Ab (mAb) (eBioscience), rabbit anti-mouse PDGFR\(\beta\) mAb (Millipore), rabbit anti-integrin \(\alpha_5\) polyclonal Ab (pAb) (intra-cellular C terminus) (Millipore), rat anti-integrin \(\alpha_\beta\) mAb (RMV-7) (Chemicon), anti-phosphotyrosine mAb (pTyr100) (Cell Signaling), anti-phospho-FAK (Tyr397) pAb (Abcam), anti-FAK pAb (Millipore), anti-phospho-Akt (Ser473) pAb (Cell Signaling), anti-Akt pAb (Cell Signaling), anti-phospho-p44/42 MAPK (extracellular signal-regulated kinase 1/2 [ERK1/2], Thr202/Tyr204) pAb (Cell Signaling), anti-p44/42 MAPK (ERK1/2) pAb (Cell Signaling), mouse anti-ubiquitin mAb (P4D1) (Santa Cruz Biotechnology), rabbit anti-Cbl pAb (Santa Cruz Biotechnology), mouse anti-\(\beta\) actin mAb (Sigma), and rabbit anti-collagen types 1 and 4, anti-fibronectin, and anti-laminin mAb (Millipore). Rabbit anti-mouse MFG-E8 pAb and mouse anti-mouse MFG-E8 mAb (clones: 1H6, B10C7) were generated and characterized in our laboratory as described.\textsuperscript{31,32}

**MFG-E8 Knockdown Experiments and Quantification of MFG-E8 mRNA**

siRNA selective for mouse MFG-E8 mRNA were designed using the Qiagen GeneGlobe Search Center (MFG-E8 siRNA) (5'-AACGGTGATTGAGAACAGATGTT-3').

**Assessment of PDGF-Related Signaling Activities via Immunoprecipitation and Immunoblotting**

Relationships between MFG-E8 levels, and PDGFR\(\beta\) expression and PDGFR\(\beta\):PDGFR\(\beta\) signaling were probed using MFG-E8 siRNA. In these experiments, 10T1/2 cells were transfected with siRNA oligonucleotides (MFG-E8 siRNA or control siRNA), shifted to low-serum (0.5% FCS) medium after 24 hours, and then stimulated with PDGF-BB (50 ng/mL) for 0 to 120 minutes after an additional 24 hours (48 hours after transfection). After washing with ice-cold PBS, cells were disrupted in lysis buffer (20 mmol/L Tris-HCl [pH 7.6], 140 mmol/L NaCl, 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche) (complete lysis buffer) on ice. Cell lysates were centrifuged at 10 000g for 15 minutes at 4°C, and the resulting supernatants were subjected to immunoprecipitation using rabbit anti-PDGFR\(\beta\) mAb or rat IgG as a control to SDS-PAGE followed by immunoblot analysis with rabbit anti-PDGFR\(\beta\) mAb, anti-phosphotyrosine mAb (pTyr100), and anti-ubiquitin mAb (P4D1) as indicated. Supernatants were also subjected to SDS-PAGE, followed by immunoblot analysis using anti-phospho-FAK (Tyr397) pAb, anti-FAK pAb, anti-phospho-Akt (Ser473) pAb, anti-Akt pAb, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) pAb, anti-44/42 MAPK (Erk1/2) pAb, and anti-\(\beta\) actin mAb. Anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary Abs (Jackson Laboratory) were used in conjunc-
tion with ECL (Pierce) for immunoblotting. Densitometric analysis of exposed films was accomplished using ImageJ software.

To examine the effect of the MEK/ERK kinase inhibitor PD98059 on PDGF-BB-induced phosphorylation of ERK1/2 and FAK and degradation of PDGFRβ, 10T1/2 cells were preincubated with 50 μmol/L PD98059 (Sigma) or the same amount of dimethyl sulfoxide (0.2%, vehicle control) for 30 minutes and then stimulated with PDGF-BB (50 ng/mL) for 30 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis as described above.

The ability of MFG-E8 to regulate PDGFRβ expression and cell signaling was also assessed using anti-MFG-E8 Abs. In these experiments, 10T1/2 cells were preincubated for 20 minutes with anti-MFG-E8 mAb (B10C7), a rabbit anti-MFG-E8 pAb reactive with the N terminus (including the RGD-containing domain), or corresponding control Abs (all at 20 μg/mL) and then stimulated with PDGF-BB (50 ng/mL) for 0 to 30 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis as described above.

**Signaling Complex Characterization via Coimmunoprecipitation**

10T1/2 cells were incubated with 0.5% FCS medium overnight. On the day of the experiment, cells were pretreated with 100 ng/mL recombinant MFG-E8 for 15 minutes and then incubated with PDGF-BB (50 ng/mL) (PeproTech) for 0 to 30 minutes. After being washed with ice-cold PBS, cells were disrupted on ice in complete lysis buffer, lysates were cleared via centrifugation at 10 000 g for 20 minutes at 4°C, and supernatants were incubated for 3 hours at 4°C with Ab-coated protein G-Sepharose beads (Amersham Biosciences). Rat anti-PDGFRβ mAb (eBioscience) or rabbit anti-α, integrin pAb (C terminus, Intracellular, Millipore) were added as appropriate, and rat IgG or rabbit IgG were used as relevant controls. Beads were then washed 3 times with 1 mL volumes of wash buffer (50 mmol/L HEPES-NaOH [pH 7.6], 150 mmol/L NaCl, 0.1% Triton X-100), suspended in SDS sample buffer, boiled, and subjected to SDS-PAGE followed by immuno blot analysis using the indicated Abs and an ECL detection system (Amersham Bioscience).

**Immunofluorescence Microscopy and Image Analysis**

To assess colocalization/association of PDGFRβ and MFG-E8, and effects of MFG-E8 on PDGFRβ expression by 10T1/2 cells, cells were stained with anti-PDGFRβ and anti-MFG-E8 after treatment with control siRNA and MFG-E8 siRNA. To accomplish this, 10T1/2 cells were transfected with control siRNA or MFG-E8 siRNA oligonucleotides, seeded in 8-well culture slides (BD Falcon) 24 hours after transfection and incubated with 0.5% FCS medium for an additional 24 hours. Forty-eight hours after siRNA treatment, cells were treated with PDGF-BB (50 ng/mL) for 0 to 120 minutes, and then fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 30 minutes. After being blocked with 3% dry milk–PBS (Bio-Rad) supplemented with 5% normal goat serum for 1 hour at room temperature, cells were stained with rabbit anti-MFG-E8 pAb and rat anti-PDGFRβ Ab followed by Alexa 488– or Alexa 568–conjugated secondary Ab and Alexa 647–phalloidin (Invitrogen). Cells were mounted in ProLong Gold antifade reagent (Invitrogen), and immunofluorescence images were collected and visualized with a LSM510 confocal laser-scanning microscope (Zeiss). Quantification of PDGFRβ, MFG-E8, and phosphotyrosine expression levels per cell and extents of colocalization were accomplished with Zeiss AIM (v4.2) software. Each data point represents analysis of 10 randomly chosen cells. Cell borders were determined by visualizing stress fibers with phalloidin as necessary.

Visualization of protein-associated intracellular phosphotyrosine in 10T1/2 cells was achieved via treatment with 0.1% Triton X-100 at room temperature for 30 minutes after fixation with 4% PFA, followed by staining with anti-phosphotyrosine mAb (PY100) and anti-PDGFRβ mAb. To compare images of permeabilized and nonpermeabilized cells, cells were fixed in 4% PFA with or without subsequent 0.1% Triton X-100 treatment before staining with Abs (see Supplemental Figure II). These results strongly support the conclusion that immunofluorescence staining of fixed cells that have not been permeabilized with detergent results in preferential detection of proteins that are extracellular or are located on external cell surfaces.

**MFG-E8-Related Proteins and Assessment of Cell Binding**

MFG-E8-Ig is a fusion protein that is comprised of the N-terminal 148 amino acids of MFG-E8, including the endothelial growth factor–like domains (E1 and E2) and the linker region (L) fused in frame with the hinge-containing Fc region of human IgG1 (Figure 1B). RGE-MFG-E8-Ig is the corresponding point mutant of MFG-E8-Ig in which aspartic acid (D) in the RGD motif was replaced by glutamic acid (E). cDNA fragments encoding MFG-E8-Ig, and RGE-MFG-E8-Ig were subcloned into pCI-neo vector (Promega), and 293F cells (Invitrogen) were transfected with these plasmids. Fusion proteins produced by 293F cells grown in serum-free medium were purified from culture supernatants via affinity chromatography using protein A–Sepharose 4 Fast Flow (GE Healthcare Biosciences) with acid elution followed by rapid neutralization. All fusion proteins migrated as homogenous dimers in size exclusion columns under nondenaturing conditions, in contrast to recombinant MFG-E8, which formed aggregates of various sizes (data not shown).

To test fusion protein binding, 10T1/2 cells were seeded into 8-well culture slides (BD Falcon) and incubated with 0.5% FCS medium overnight. Cells were subsequently treated with MFG-E8-Ig or RGE-MFG-E8-Ig (20 μg/mL) for 20 minutes and then incubated with PDGF-BB (50 ng/mL) for 0 to 60 minutes, followed by fixation with 4% PFA in PBS. After being blocked with 3% dry milk–PBS (Bio-Rad) supplemented with 5% normal goat serum for 1 hour at room temperature, cells were stained with anti-PDGFRβ Ab followed by Alexa 488–conjugated anti-rat Ig, Alexa 568–conjugated anti-human Ig and Alexa 647–phalloidin (Invitrogen), washed and mounted in ProLong Gold before visualization.

To assess effects of anti-integrin αv blocking Ab on binding of MFG-E8-Ig to 10T1/2 cells, 10T1/2 cells were incubated with rat anti-integrin αv blocking mAb (RMV-7) (Chemicon) or control rat Ig (20 μg/mL) for 20 minutes and then treated with MFG-E8-Ig (20 μg/mL) for 20 minutes. After MFG-E8-Ig treatment, cells were incubated with PDGF-BB (50 ng/mL) for 0 or 5 minutes. Cells were subsequently washed, fixed in 4% PFA in PBS, and stained with Alexa 568–conjugated anti-human Ig and Alexa 647–phalloidin (Invitrogen), and immunofluorescence images were collected and analyzed.

**Statistics**

Probability values were calculated using the Student t test (2-sided) or by analysis of 1-way ANOVA followed by the Bonferroni post test as appropriate. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

**Results**

MFG-E8 Enhances PDGF-BB-Induced Phosphorylation of ERK1/2 and FAK

We previously determined that 10T1/2 cells express PC markers, including PDGFRβ, that they produce MFG-E8; and that inhibition of MFG-E8 production using siRNA inhibited PDGF-BB-dependent 10T1/2 cell migration but not proliferation.32 Because PDGF-BB–induced ERK1/2 and FAK activation downstream of PDGFRβ phosphorylation has been reported to be important for VSMC migration, we hypothesized that MFG-E8 might modulate this pathway.1,15,16,33,34 We examined the effect of MFG-E8 on PDGFβ-stimulated signaling in 10T1/2 cells by assessing phosphorylation of MAPK, FAK, and Akt in MFG-E8 siRNA
and control siRNA-transfected cells. MFG-E8 mRNA expression in MFG-E8 siRNA-transfected cells was inhibited by \( \approx 75\% \) relative to that in control siRNA-transfected cells (Figure 2A). We confirmed that the protein level of MFG-E8 was similarly inhibited by MFG-E8 siRNA using immunoblot and ELISA assays (Figure 2A).

In immunoblots of whole cell lysates, MFG-E8 depletion using siRNA significantly reduced phosphorylation of ERK1/2 as early as 2 minutes after PDGF-BB stimulation (Figure 2B). Phosphorylated FAK was also significantly decreased by depletion of MFG-E8 from 10T1/2 cells, but this became evident at later time points (Figure 2B). In contrast, PDGF-dependent Akt phosphorylation was not influenced by modulation of MFG-E8 production, suggesting that MFG-E8 selectively enhances PDGF-BB-induced phosphorylation of ERK1/2 as early as 2 minutes after PDGF-BB stimulation (Figure 2B). Phosphorylated FAK was also significantly decreased by depletion of MFG-E8 from 10T1/2 cells, but this became evident at later time points (Figure 2B). In contrast, PDGF-dependent Akt phosphorylation was not influenced by modulation of MFG-E8 production, suggesting that MFG-E8 selectively enhances PDGF-BB-induced phosphorylation of ERK1/2 as early as 2 minutes after PDGF-BB stimulation. We also examined the effect of the MEK/ERK kinase inhibitor PD98059 on the enhancement of PDGF-BB-induced phosphorylation of ERK1/2 and FAK by MFG-E8. As previously demonstrated, \(^{32}\) inhibition of MFG-E8 production using siRNA inhibited PDGF-BB-dependent 10T1/2 cell migration (Figure 2C). The MEK/ERK kinase inhibitor PD98059 effectively inhibited PDGF-BB-induced control and MFG-E8 siRNA-transfected cell migration. As expected, inhibition of PDGF-BB-induced phosphorylation of ERK1/2 in control and MFG-E8 siRNA-transfected cells by PD98059 was almost complete (Figure 2D). In contrast, the MEK/ERK kinase inhibitor had no effect on PDGF-BB-induced phosphorylation of FAK in control and MFG-E8 siRNA-transfected cells. These results suggest that potentiation of MFG-E8-induced activation of ERK1/2 contributes to the enhancement of PDGF-BB-induced migration and that ERK1/2 activation is downstream of MFG-E8-enhanced FAK phosphorylation.

**MFG-E8 Enhances PDGF-BB-Induced Phosphorylation of PDGFRβ and Inhibits PDGF-BB-Dependent PDGFRβ Degradation**

PDGF-BB-induced PDGFRβ autophosphorylation is required for activation of downstream signaling pathways. In initial anti-phosphotyrosine immunoblots of whole cell lysates, PDGF-BB-induced phosphorylation of a 190-kDa protein, consistent with PDGFRβ based on molecular size (Figure 2B, top panel). Phosphorylation of this protein was significantly attenuated in MFG-E8-depleted cells. To confirm that the 190-kDa protein represented PDGFRβ, we
performed immunoprecipitation/immunoblotting experiments with anti-PDGFRβ and anti-phosphotyrosine Ab. We confirmed that phosphorylation of PDGFRβ was increased after PDGF-BB stimulation, peaking at ∼5 minutes after stimulation and gradually decreasing to basal levels by 60 minutes (Figure 3A, top panel). In MFG-E8-depleted 10T1/2 cells, PDGF-BB-dependent PDGFRβ phosphorylation was decreased relative to controls. Differences between control and MFG-E8 siRNA-treated cells were evident from 5 to 30 minutes after PDGF-BB stimulation and continued up to 120 minutes after stimulation (Figure 3A, top panel). FAK is required for both integrin- and growth factor-stimulated cell migration.15,16,33 It has been reported that FAK associates with activated PDGFRβ through its N-terminal domain and with integrins through its C-terminal domain, thereby promoting PDGFB-stimulated cell migration.15,16 We also detected PDGFRβ-associated FAK in PDGFRβ immunoprecipitates by immunoblotting with anti-FAK Ab (Figure 3A, bottom panel). Interestingly, PDGF-BB-augmented association of FAK with PDGFRβ was strikingly dependent on MFG-E8 (Figure 3A, bottom panel).

The amount of total PDGFRβ that was immunoprecipitated from control 10T1/2 cells increased 2 to 10 minutes after PDGF-BB treatment, and decreased by 30 minutes after...
Figure 3. Milk fat globule-associated protein with epidermal growth factor and factor VIII-like domains (MFG-E8) enhances platelet-derived growth factor (PDGF)-BB-induced phosphorylation of PDGF receptor (PDGFR) β and reduces PDGF-BB-dependent PDGFRβ degradation. A, Control small interfering RNA (siRNA)- or MFG-E8 siRNA-transfected 10T1/2 cells were stimulated with PDGF-BB for 2658 Arterioscler Thromb Vasc Biol November 2011
stimulation (Figure 3A, middle panel). Total PDGFRβ expression in MFG-E8 siRNA-transfected cells was significantly decreased relative to that in control cells from 10 to 60 minutes after PDGF-BB stimulation, suggesting that MFG-E8 might inhibit PDGF-BB-dependent PDGFRβ degradation. Addition of recombinant MFG-E8 to MFG-E8 siRNA-transfected cells reversed the decreases in FAK-PDGFRβ association, and PDGFRβ phosphorylation and levels of PDGFRβ were increased to some extent (Supplemental Figure I). To additionally address the issues of PDGFRβ expression and distribution, we examined effects of MFG-E8 on PDGFRβ and phosphotyrosine levels and assessed colocalization of phosphotyrosine and PDGFRβ in situ via confocal laser immunofluorescence microscopy (Figure 3B). Consistent with the immunoblotting results, PDGFRβ levels were somewhat decreased, and phosphotyrosine levels were dramatically decreased, in MFG-E8 siRNA-transfected cells compared with controls. Colocalization of phosphotyrosine and PDGFRβ (presumably phosphorylated-PDGFRβ) was also decreased in MFG-E8 siRNA-transfected cells, consistent with our association studies. In aggregate, these results suggest that MFG-E8 inhibits PDGFRβ-dependent PDGFRβ degradation and enhances both phosphorylation of PDGFRβ and association of FAK with activated PDGFRβ.

We previously determined that PDGF-BB-induced 10T1/2 cell migration was inhibited by rabbit anti-MFG-E8 pAb, which recognized the N terminus of MFG-E8, as well as mouse anti-MFG-E8 mAb B10C7, which recognized the RGD motif in the E2 domain, and that injection of these Abs also inhibited angiogenesis in OIR in mice.25 Consistent with results that we obtained with MFG-E8 siRNA, both anti-MFG-E8 neutralizing Abs inhibited PDGFRβ-induced phosphorylation of PDGFRβ and led to decreased receptor levels in PDGF-BB-stimulated cells (Figure 3C). These results are consistent with the concept that at least some MFG-E8 that is secreted by 10T1/2 cells acts extracellularly to potentiate PDGFRβ phosphorylation, to attenuate decreases in PDGFRβ levels that occur in PDGF-BB-stimulated cells, and thereby to enhance PDGFB-BB-mediated signaling in 10T1/2 cells.

MFG-E8 Associates With PDGFRβ in PDGF-BB-Stimulated Cells

MFG-E8 has been reported to bind to integrin αvβ3,3 via its RGD motif.20,21,23,24 It has also been reported that integrin αvβ3 associates with phosphorylated PDGFRβ in NIH3T3 cells treated with PDGF-BB, and this association enhances downstream signaling.12,13 We therefore hypothesized that PDGFB-BB induces association of MFG-E8, integrin αv, and PDGFRβ, providing a possible link between MFG-E8 formation and PDGFRβ-mediated signaling. To test this, we characterized PDGF-BB-induced physical interactions between MFG-E8 and PDGFRβ via communoprecipitation. Serum-starved 10T1/2 cells were stimulated with PDGF-BB, lysed, and immunoprecipitated with anti-PDGFRβ mAb. MFG-E8 was detected in PDGFRβ immunoprecipitates; the association of PDGFRβ and MFG-E8 was increased 5 minutes after PDGF-BB stimulation and decreased by 30 minutes after stimulation (Figure 4A, top panels).

To examine relationships between MFG-E8 and PDGFRβ in situ, we assessed colocalization of these proteins on the surfaces of 10T1/2 cells. To test the feasibility of our approach, we performed PDGFRβ and phosphotyrosine immunofluorescence staining in both permeabilized (0.1% Triton X-treated) and nonpermeabilized (Triton X-nontreated) PFA-fixed 10T1/2 cells (see Supplemental Figure II). Thirty minutes after PDGF-BB stimulation, PDGFRβ staining of nonpermeabilized cells was significantly reduced compared with that of permeabilized cells and also compared with that of nonpermeabilized unstimulated cells. In contrast, phosphotyrosine staining in permeabilized cells was significantly increased after PDGF-BB stimulation and was minimal in nonpermeabilized cells in the presence or absence of PDGFB-BB treatment. We interpret these findings to indicate that in the absence of detergent treatment, immunofluorescence staining of PFA-fixed cells detects primarily external surface-associated proteins.

Having established that our experimental approach could be informative, we examined PDGF-BB-induced association of MFG-E8 with PDGFRβ on the surfaces of PDGF-BB-treated nonpermeabilized 10T1/2 cells via immunofluorescence confocal laser microscopy (Figure 4B), and quantified the colocalization of MFG-E8 and PDGFRβ, as well as levels of expression of MFG-E8 and PDGFRβ on the surfaces of 10T1/2 cells (Figure 4C). Before treatment with PDGF-BB, PDGFRβ was diffusely distributed on 10T1/2 cells, and some MFG-E8 was also present on cell surfaces (Figure 4B and 4C; MFG-E8). After treatment with PDGF-BB, PDGFRβ staining became punctate and then decreased dramatically (≈30% by 2 minutes after stimulation and diminishing to ≈40% of initial levels at the end of the 120-minute observation period; Figure 4B and 4C; PDGFRβ). In contrast, surface-associated MFG-E8 increased ≈50% by 2 minutes after PDGF-BB

![Figure 3. (Continued)](http://atvb.ahajournals.org/)
Figure 4. Platelet-derived growth factor (PDGF)-BB induces association of milk fat globule-associated protein with epidermal growth factor and factor VIII-like domains (MFG-E8) with PDGF receptor (PDGFR) on cell surfaces. A, PDGF-BB enhances association of MFG-E8 and PDGFR. Serum-starved 10T1/2 cells were stimulated with PDGF-BB and subjected to immunoprecipitation (IP) with rat
stimulation and continued to increase, reaching levels 2.5 times higher than those on unstimulated cells by 30 minutes after PDGF-BB addition (Figure 4C). At least some of this increase may be attributable to increased MFG-E8 production, because MFG-E8 mRNA levels in 10T1/2 cells were increased within 5 minutes after PDGF-BB was added (Supplemental Figure III).

Interestingly, colocalization of PDGFRβ and MFG-E8 increased after PDGF-BB treatment, and this colocalization persisted for 120 minutes after stimulation (Figure 4B and 4C; colocalization of PDGFRβ and MFG-E8 on cell surfaces). The fraction of PDGFRβ that colocalized with MFG-E8 increased after PDGF-BB stimulation; \( \approx 5\% \) of PDGFRβ colocalized with MFG-E8 before stimulation, increasing to \( \approx 30\% \) colocalization 120 minutes after stimulation (Figure 4C; fraction of PDGFRβ that colocalized with MFG-E8). To determine whether MFG-E8 might modulate PDGFRβ surface levels or distribution, we studied siRNA-treated, MFG-E8-depleted and control siRNA-transfected 10T1/2 cells (Figure 4B and 4C). Surface PDGFRβ expression by control siRNA-treated cells decreased in a time-dependent manner at a slower rate than was observed with MFG-E8 siRNA-treated cells (Figure 4C; PDGFRβ). Differences were evident beginning 5 minutes after PDGF-BB treatment and persisted for up to 120 minutes. These results suggest that 10T1/2 cell-derived MFG-E8 potentiates receptor clustering and that physical association of MFG-E8 with PDGFRβ may lead to retention of PDGFRβ at cell surfaces with concordantly augmented signaling.

**Interactions of MFG-E8 and PDGFRβ Are Mediated by \( \alpha_v \) Integrins**

We predicted that association of MFG-E8 with PDGFRβ was dependent on integrin \( \alpha_v \) and the RGD motif present in the second endothelial growth factor–like domain of MFG-E8. To test this, we immunoprecipitated integrin \( \alpha_v \) from untreated and PDGF-BB-treated 10T1/2 cells and assayed for copurifying MFG-E8 and phosphoproteins of interest by immunoblotting with anti-MFG-E8 and anti-phosphotyrosine, respectively. Association of integrin \( \alpha_v \) and MFG-E8 was observed before stimulation and was slightly increased at 5 minutes after stimulation (Figure 1A, top panels). Anti-phosphotyrosine immunoblots revealed a 190-kDa tyrosine-phosphorylated protein, consistent with phosphorylated PDGFRβ, that associated with \( \alpha_v \) integrin after PDGF-BB stimulation (Figure 1A, middle panels, lane 3). These results indicate that MFG-E8 binds integrin \( \alpha_v \) before PDGF-BB stimulation and that PDGFRβ is recruited into relevant complexes only after PDGF-BB-stimulated receptor autophosphorylation.

To confirm that MFG-E8 binds to cell-associated integrin \( \alpha_v \) via the RGD consensus integrin-binding motif in the E2 domain of MFG-E8, we compared the binding of several MFG-E8 fusion proteins to the surfaces of 10T1/2 cells. MFG-E8-Ig is a fusion protein composed of the N terminus of MFG-E8 (without the C1 and C2 domains) fused in frame to the Fc portion of human IgG1, and RGE-MFG-E8-Ig represents the corresponding RGE point mutant (Figure 1B). These proteins, rather than recombinant MFG-E8, were used because they can easily be distinguished from residual MFG-E8 that is produced by 10T1/2 cells. In addition, these fusion proteins exist in solution as homogenous dimers as compared with the multiple aggregated species that are present in preparations of recombinant full length MFG-E8, making experimental results potentially easier to interpret. Immunofluorescence staining showed that MFG-E8-Ig bound to the surfaces of 10T1/2 cells with or without PDGF-BB treatment (Figure 1C: PDGFRβ in green, MFG-E8-related proteins in red). After stimulation with PDGF-BB, MFG-E8-Ig colocalized in punctate clusters with PDGFRβ. In contrast, we could not detect RGE-MFG-E8-Ig binding to 10T1/2 cells (Figure 1C). Binding of MFG-E8-Ig to 10T1/2 cells was also readily inhibited by anti-integrin \( \alpha_v \) blocking Ab (Figure 1D). These results indicate that MFG-E8 binds to \( \alpha_v \) integrins and that integrin-MFG-E8 binding is RGD dependent, and they suggest that PDGFRβ-induced interactions of MFG-E8 with PDGFRβ are dependent on \( \alpha_v \)-containing integrins.

**MFG-E8 Inhibits PDGF-BB-Dependent Ubiquitination of PDGFRβ**

Levels of PDGFRβ and activity of the PDGF-BB:PDGFRβ signaling axis can be regulated by receptor ubiquitination, and polyubiquitination triggers receptor degradation.\(^7\)\(^-\)\(^9\) To determine whether MFG-E8 depletion of 10T1/2 cells led to increased PDGFRβ ubiquitination, we assayed immunoprecipitated PDGFRβ for associated ubiquitin and polyubiquitin via immunoblotting with anti-ubiquitin Ab. We found that PDGF-BB-induced ubiquitination of PDGFRβ was significantly higher in MFG-E8 siRNA-transfected cells than in control siRNA-transfected cells (Figure 5A, top panel). Quantification of total amounts of PDGFRβ and ubiquinated PDGFRβ 10 minutes after PDGF-BB stimulation revealed that PDGFRβ levels in MFG-E8-depleted cells were \( \approx 40\% \) lower than in control cells (as expected), whereas the proportion of PDGFRβ that was ubiquitinated in MFG-E8-depleted cells was increased by \( \approx 60\% \) compared with that in control cells (Figure 5B). c-Cbl is a ubiquitin E3 ligase that has been implicated as a negative regulator of PDGFRβ, and PDGFRβ is a substrate for c-Cbl.\(^5\)\(^-\)\(^8\) We determined that the association of c-Cbl with PDGFRβ was increased in MFG-E8 siRNA-transfected cells (Figure 5A, middle panel). In addition, we demonstrated that the proteasome inhibitor MG132 inhibited PDGF-BB-induced PDGFRβ degradation in control

---

**Figure 4.** (Continued) anti-PDGFRβ or control antibody (Ab), and SDS-PAGE-resolved proteins were immunoblotted (IB) with rabbit anti-MFG-E8 polyclonal Ab (pAb) and rabbit anti-PDGFRβ monoclonal Ab (mAb). Data are representative of \( n \approx 6 \) experiments. B, Digital fluorescence images depicting colocalization of MFG-E8 (red) and PDGFRβ (green) in PDGF-BB-treated control and MFG-E8-depleted 10T1/2 cells (high-magnification images of the areas indicated by squares in the top images; scale bar=20 \( \mu \)m). C, Quantification of colocalization of PDGFRβ and MFG-E8 on cell surfaces and surface expression of PDGFRβ or MFG-E8 in control and MFG-E8-depleted 10T1/2 cells, and fractions of PDGFRβ that colocalized with MFG-E8 (○, control small interfering RNA [siRNA]; ●, MFG-E8 siRNA treated cell) \( (**P<0.01; \text{data are means±SE of values from 10 randomly chosen cells and representative of } n=2 \text{ experiments}) \). Levels of surface expression are expressed relative to those present on control siRNA-treated cells at time=0.
and MFG-E8 siRNA-transfected cells (Supplemental Figure IV). These results suggest that MFG-E8 reduces the association of c-Cbl with PDGFRβ, PDGFRβ ubiquitination, and receptor degradation via the proteasomal pathway, thereby enhancing cell surface PDGFRβ levels and PDGFRβ-mediated signaling. The MEK/ERK kinase inhibitor PD98059 had no effect on PDGFRβ-mediated signaling (Figure 2D, bottom panel), indicating that ERK activity did not influence regulation of PDGFRβ degradation by MFG-E8.

**Discussion**

MFG-E8 enhances VEGF-induced angiogenesis in a mouse model of acute hindlimb ischemia, tumor-associated angiogenesis in pancreatic β-cell tumors in Rip1-Tag2 mice and melanomas, and angiogenesis in a mouse OIR model as well. In a previous study, we demonstrated that PC/PC precursors as well as ECs are relevant sources of angiogenesis-promoting MFG-E8 and determined that PDGF-BB-induced migration of PC/PC precursor 10T1/2 cells was inhibited by MFG-E8 depletion using siRNA and by anti-MFG-E8 neutralizing Abs (Figure 2C). The present study, we sought to characterize the mechanism(s) by which MFG-E8 regulates PDGF-BB-induced alterations in cellular physiology, focusing on alterations downstream of PDGFRβ activation.

Depletion of MFG-E8 from 10T1/2 cells using siRNA led to decreased PDGFRβ and ERK1/2 phosphorylation after PDGF-BB stimulation, and FAK Y397 phosphorylation became transient. These effects were selective in that Akt phosphorylation was not significantly altered. PDGF-BB-induced ERK1/2 activation is known to be required for smooth muscle cell motility. Phosphorylation of FAK at Y397 promotes recruitment of SH2 domain-containing adaptor proteins, including Src, phosphatidylinositol 3-kinase, and Shc, and subsequently leads to ERK activation that is important for integrin-stimulated cell migration, including PDGF-BB-induced migration. Laser scanning confocal microscopy confirmed a global attenuation of PDGF-BB-stimulated tyrosine phosphorylation in MFG-E8-depleted cells and suggested that whole cell PDGFRβ levels also decreased more rapidly after stimulation in MFG-E8-depleted cells. Immunoprecipitation studies indicated that PDGFRβ entered into complexes with MFG-E8 after receptor ligation, presumably via interactions that are mediated by αv integrin that binds to FAK. This provides a possible link between MFG-E8 production and PDGF-BB/PDGFRβ signaling. Confocal microscopy confirmed colocalization of cell surface-associated MFG-E8 and PDGFRβ that increased over time after PDGF-BB addition, and it demonstrated higher levels of PDGFRβ on surfaces of MFG-E8-producing cells as compared with MFG-E8-depleted cells. Interestingly, ubiquitination of PDGFRβ was also enhanced in MFG-E8-depleted cells. Associations between the E3 ubiquitin ligase c-Cbl and PDGFRβ also increased in MFG-E8-depleted 10T1/2 cells, and inhibition of the proteasome prevented ligand-induced PDGFRβ degradation. Finally, we formally demonstrated that MFG-E8-related proteins interact with 10T1/2 cells via the RGD consensus integrin-binding sequence in the E2 domain of MFG-E8 and αv-containing integrins.

PDGF-BB:PDPGFRβ binding leads to receptor dimerization, receptor autophosphorylation, recruitment of requisite signaling molecules into supramolecular complexes, and receptor internalization. Although receptor internalization is likely to ultimately lead to decreased signaling overall, receptors and associated proteins in endosomal compartments may continue to signal. Some internalized receptors may recycle to cell surfaces where they are available to continue to participate in signaling events, whereas others are degraded in proteasomes and lysosomes. Persistent ubiquitination of PDGFRβ has been reported to target this protein for destruction via the proteasome- and lysosome-dependent pathways. Thus, the activity of the PDGF-BB:PDPGFRβ signaling axis can be regulated via multiple mechanisms. Levels of expression of growth factor, receptor, downstream kinases, phosphatases, and scaffolding proteins are all relevant, but the physical and temporal locations of these components in relationship to one another are also critical.

Several recent studies have focused on regulation of PDGFRβ ubiquitination as an important mechanism by which PDGF-BB:PDPGFRβ signaling can be modulated. The E3 ligase c-Cbl is thought to be primarily responsible for PDGFRβ ubiquitination, and regulation of c-Cbl expression and activity influences PDGF-BB:PDPGFRβ signaling. Low-density lipoprotein receptor-related protein 1 binds to c-Cbl, preventing c-Cbl:PDPGFRβ interaction and PDGFRβ ubiquitination. In the absence of lipoprotein receptor-related protein 1, ubiquitination and degradation of PDGFRβ is
enhanced and PDGF-BB:PDGFRβ signaling is attenuated.39 Endothelin and smooth muscle–derived neuropilin-like protein regulates PDGF-BB:PDGFRβ signaling via a different mechanism. Knockdown of endothelin and smooth muscle–derived neuropilin-like protein in VSMCs using siRNA causes coordinate increases in PDGFRβ signaling, proliferation, and migration that are accompanied by diminished PDGFRβ ubiquitination and turnover, as well as decreased c-Cbl mRNA and protein expression.40

In the present study, we demonstrate the existence of another route by which PDGFRβ ubiquitination may be regulated and PDGF-BB:PDGFRβ signaling can be augmented. 10T1/2 cells secrete MFG-E8 under basal conditions, and MFG-E8 production (as assessed at the mRNA level) was rapidly increased after PDGF-BB treatment. On the basis of its tendency to aggregate in solution and its predominately extracellular location in tissues,29 and because it has previously been reported to bind to collagen,30 we believe that secreted MFG-E8 is readily incorporated into extracellular matrix. Indeed, we detected laminin, fibronectin, and collagen type I that was deposited in close proximity to 10T1/2 cells that were propagated in vitro (Supplemental Figure V). Matrix-associated MFG-E8 binds to αv-containing integrins, and these proteins become incorporated into cell surface-associated supramolecular complexes that include phosphorylated FAK and PDGFRβ (and other proteins) in the presence of growth factor. Binding of MFG-E8 to αv-containing integrins promotes retention of PDGFRβ at cell surfaces, reduces receptor ubiquitination by preventing PDGFRβ from becoming accessible to c-Cbl, and potentiates PDGF-BB-mediated signaling that involves FAK Y397 phosphorylation and subsequent activation of downstream signaling molecules, including ERK1/2, resulting in increased PDGFβ-dependent cellular responses including cell migration.

The scenario that we propose above provides a framework in which to consider the activities of MFG-E8 more globally. Although we have made a strong case for PCs as significant sources of MFG-E8 in situ, other cells (including ECs, VSMCs, macrophages and tumor cells) also have the capacity to produce the protein.20,22,28,31 We have determined that MFG-E8 preferentially regulates PDGF-BB-stimulated migration of PC/PC precursor 10T1/2 cells, whereas others have demonstrated that MFG-E8 augments VEGF-induced EC proliferation and survival.28 Thus, in the setting of reparative angiogenesis, MFG-E8 can be produced by, and act on, several types of cells by potentiating the stimulatory effects of several growth factors. Recent experience with so-called molecular targeting small molecules indicates that drugs that have inhibitory effects on multiple targets (kinases) may be more efficacious than those that are more specific. It is tempting to speculate that, by analogy, inhibition of MFG-E8 production or action may be more advantageous than inhibition of a single signaling pathway, stimulated by VEGF, for example. It will also be interesting to determine whether the mechanisms by which MFG-E8 enhances VEGF signaling are distinct from those that we have described as operative for PDGF-BB.

Acknowledgments

We thank Mallorie Heneghan and Joshua Drago for performing mouse husbandry and genotyping; Michael Lu for purification of fusion proteins; and Drs Thomas Hornyak, Chuanjin Wu, and Stan Lipkowitz for helpful discussions.

Sources of Funding

This work was supported by the Intramural Program of the National Institutes of Health, Center for Cancer Research, National Cancer Institute. Dr Motegi was supported, in part, by project grants from the Japan Foundation for Aging and Health and by a Japan Society for Promotion of Science Research Fellowship for Japanese Biomedical and Behavioral Researchers at the National Institutes of Health.

Disclosures

None.

References


Potentiation of Platelet-Derived Growth Factor Receptor-β Signaling Mediated by Integrin-Associated MFG-E8
Sei-ichiro Motegi, Susan Garfield, Xu Feng, Miklós Sárdy and Mark C. Udey

Arterioscler Thromb Vasc Biol. 2011;31:2653-2664; originally published online August 25, 2011;
doi: 10.1161/ATVBAHA.111.233619
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/11/2653

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Material

Detailed Methods

Quantification of MFG-E8 protein by ELISA

MFG-E8 protein was quantified by ELISA. Mouse anti-mouse MFG-E8 mAb 1H6 (reactive with the C1 domain) was adsorbed (100 ng/well) onto Maxisorp ELISA plates (Nunc) via overnight incubation at 4°C. After blocking with 2%BSA/PBS, conditioned medium was added and incubated for 90 minutes at 37°C. After washing, plates were incubated with rabbit anti-mouse MFG-E8 pAb (reactive with the N-terminus) for 60 minutes at 37°C. Washed plates were then incubated with HRP-conjugated goat anti-rabbit IgG (Jackson) for 60 minutes, developed with TMB substrate solution (Pierce) and enzymatic reactions were halted with 1 N H₂SO₄ before determining the A₄₅₀. Absolute concentrations were determined using appropriate standard curves.

10T1/2 cell migration studies

10T1/2 cells were incubated in low serum (0.5% FCS)-containing media overnight and cell migration was subsequently assayed using FluoroBlok™ 24-Multiwell Insert System transwell culture plates (3 μ pore size; BD Biosciences). Cells were stained with Calcein AM (BD Biosciences), resuspended in 0.5% FCS-containing BME medium, and 10⁵ cells were seeded into upper compartments of the transwell apparatus. Medium containing 0.5% FCS and 50 ng/ml PDGF-BB (PeproTech) was added to the lower compartments. After 4 hours incubation at 37°C, images of cells that were attached to the undersurfaces of transwell filters were acquired with an inverted phase fluorescent microscope equipped with a digital camera. Areas covered by migrated cells in each well were quantified using Image J.

To assess the involvement of MFG-E8 in 10T1/2 cell migration in the transwell assay, MFG-E8 production by 10T1/2 cells was inhibited with siRNAs prior to assessment of migratory ability. To accomplish this, 10T1/2 cells were transfected with appropriate siRNA oligonucleotides, and transfected
cells were used for transwell migration assays 48 hours later.

To examine the effect of the MEK/ERK kinase inhibitor PD98059 on PDGF-BB-induced migration, control siRNA- or MFG-E8 siRNA-transfected 10T1/2 cells were pretreated with 50 μM PD98059 (Sigma) or 0.2% DMSO (vehicle control) for 30 minutes prior to addition to the upper chamber.

Assessment of PDGF-related signaling activities via immunoprecipitation and immunoblotting

Relationships between MFG-E8 levels and PDGFRβ expression and PDGF-BB-related signaling were probed using MFG-E8 siRNA. In these experiments, 10T1/2 cells were transfected with siRNA oligonucleotides (MFG-E8 siRNA or control siRNA), shifted to low serum (0.5% FCS) medium after 24 hours, and then stimulated with PDGF-BB (50 ng/ml) for 0-120 minutes after an additional 24 hours (48 hours after transfection). After washing with ice-cold PBS, cells were disrupted in lysis buffer (20 mM Tris-HCl (pH 7.6) 140 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche) (complete lysis buffer) on ice. Cell lysates were centrifuged at 10,000xg for 15 minutes at 4°C, and the resulting supernatants were subjected to immunoprecipitation using rat anti-PDGFRβ mAb or rat IgG as a control, or SDS-PAGE followed by immunoblot analysis with rabbit anti-PDGFRβ mAb, anti-phosphotyrosine mAb (PY100), and anti-ubiquitin mAb (P4D1) as indicated. Supernatants were also subjected to SDS-PAGE, followed by immunoblot analysis using anti-phospho-FAK (Y397) pAb, anti-FAK pAb, anti-phospho-Akt (Ser473) pAb, anti-Akt pAb, anti-phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) pAb, anti-44/42 MAPK (Erk1/2) pAb, and anti-β actin mAb. Anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Jackson) were used in conjunction with ECL (Pierce) for immunoblotting. Densitometric analysis of exposed films was accomplished using Image J software.

To examine the effect of the MEK/ERK kinase inhibitor PD98059 on PDGF-BB-induced phosphorylation of ERK1/2 and FAK, and degradation of PDGFRβ, 10T1/2 cells were preincubated with 50 μM PD98059 (Sigma) or vehicle control for 30 minutes, and then stimulated with PDGF-BB (50 ng/ml) for 30 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by
immunoblot analysis as described above.

The ability of MFG-E8 to regulate PDGFRβ expression and cell signaling was also assessed using anti-MFG-E8 Abs. In these experiments, 10T1/2 cells were preincubated for 20 minutes with anti-MFG-E8 mAb (B10C7), a rabbit anti-MFG-E8 pAb reactive with the N-terminus (including the RGD-containing domain), or with corresponding control Abs (all at 20 μg/ml), and then stimulated with PDGF-BB (50 ng/ml) for 0-30 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis as described above.

The ability of MFG-E8 to regulate PDGFRβ expression and cell signaling was also assessed by adding recombinant MFG-E8 protein, MFG-E8-Ig, and RGE-MFG-E8-Ig to MFG-E8 siRNA-transfected cells. In these experiments, siRNA transfected 10T1/2 cells were preincubated for 60 minutes with 100 ng/ml recombinant MFG-E8 (R&D systems), and MFG-E8-Ig, or RGE-MFG-E8-Ig (20 μg/ml), and then stimulated with PDGF-BB (50 ng/ml) for 10 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis as described above.

To assess the role of the proteasome in PDGF-BB-induced degradation of PDGFRβ and phosphorylation of ERK1/2 and FAK, control siRNA- or MFG-E8 siRNA-transfected 10T1/2 cells were preincubated with the proteasome inhibitor MG132 (10 μM: EMD Chemicals) or 0.04% DMSO (as a control) for 30 minutes, and subsequently stimulated with PDGF-BB for 30 minutes. Cells were then disrupted in lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis as described above.
Supplemental Figure I. Adding back MFG-E8 protein rescue an siRNA phenotype in 10T1/2 cells. A and B, Control siRNA- or MFG-E8 siRNA-transfected 10T1/2 cells were preincubated for 60 minutes with recombinant MFG-E8 (100 ng/ml), and MFG-E8-Ig or RGE-MFG-E8-Ig (20 μg/ml), and then stimulated with PDGF-BB for 10 minutes, lysed, and then immunoprecipitated with rat anti-PDGFRβ Ab followed by immunoblotting with anti-phosphotyrosine (PY100), anti-PDGFRβ and anti-FAK Ab (A), and cell lysate was immunoblotted with anti-phospho-ERK1/2 and ERK1/2 Ab (B, The relative level of ERK1/2 phosphorylation in PDGF-BB-stimulated control siRNA-transfected cells was assigned a value of 1). Phosphorylated PDGFRβ, total PDGFRβ, PDGFRβ-associated FAK, and relative phosphorylation levels of ERK1/2 was quantified via densitometry using Image J software (**p< 0.01, *p< 0.05; Values determined in 3 independent experiments, NS = not significant).
Supplemental Figure II. PDGFRβ and phosphotyrosine staining in non-permeabilized and permeabilized 10T1/2 cells. 10T1/2 cells were incubated with 0.5% FCS medium overnight in 8-well CultureSlides, and cells were treated with PDGF-BB (50 ng/ml) for 30 minutes. Cells were then fixed in 4% PFA, and treated with blocking buffer with or without 0.1% Triton X-100. After blocking, cells were stained with anti-PDGFRβ Ab and anti-phosphotyrosine Ab, followed by Alexa 488- and Alexa 568-conjugated secondary Ab as appropriate. Fluorescence photomicrographs depicting phosphotyrosine (red) and PDGFRβ (green) in 10T1/2 cells (Bar = 50 µ).
**Supplemental Figure III.** PDGF-BB induced MFG-E8 mRNA expression in 10T1/2 cells as assessed by quantitative RT-PCR.
Supplemental Figure IV. Proteasome inhibitor attenuates the enhancement of PDGF-BB-induced PDGFRβ degradation by depletion of MFG-E8. Control siRNA- or MFG-E8 siRNA-transfected cells were treated with 10 μM MG132 or 0.04% DMSO (as a control) for 30 minutes, and subsequently stimulated with PDGF-BB for 30 minutes. Cells were subjected to SDS-PAGE, followed by immunobloting with anti-PDGFRβ Ab and anti-actin Ab. Quantification of relative levels of PDGFRβ was accomplished via densitometry using Image J software (normalized to Actin protein levels). The amount of PDGFRβ in control siRNA transfected cells cultured in medium with DMSO, and without PDGF-BB was assigned a value of 1 (**p< 0.01 relative to siRNA control; Values determined in 3 independent experiments, NS = not significant).
Supplemental Figure V. Laminin, fibronectin and collagen type 1 in close proximity to 10T1/2 cells. 10T1/2 cells were incubated with 0.5% FCS medium overnight, trypsinized, re-plated into 8-well CultureSlides, and incubated with 0.5% FCS medium. Four hours later, cells were fixed in 4% PFA in PBS. After blocking, cells were stained with anti-laminin, anti-fibronectin, anti-collagen Ab, and anti-PDGFRβ Ab followed by appropriate secondary Ab. Fluorescence photomicrographs depicting laminin (red), fibronectin (red), collagen type 1 (red), collagen type 4 (red) in relationship to 10T1/2 cells stained for PDGFRβ (green)  (Bar = 50 µ).