TGF-β suppresses the upregulation of MMP-2 by vascular smooth muscle cells in response to PDGF-BB

George M. Risinger, Jr., Dawn L. Updike, Elizabeth C. Bullen, James J. Tomasek, and Eric W. Howard

Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

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Risinger GM Jr, Updike DL, Bullen EC, Tomasek JJ, Howard EW. TGF-β suppresses the upregulation of MMP-2 by vascular smooth muscle cells in response to PDGF-BB. Am J Physiol Cell Physiol 298: C191–C201, 2010. First published October 21, 2009; doi:10.1152/ajpcell.00417.2008.—During platelet-derived growth factor (PDGF)-BB-mediated recruitment to neovascular sprouts, vascular smooth muscle cells (VSMCs) dedifferentiate from a contractile to a migratory phenotype. This involves the downregulation of contractile markers such as smooth muscle (SM) α-actin and the upregulation of promigration genes such as matrix metalloproteinase (MMP)-2. The regulation of MMP-2 in response to PDGF-BB is complex and involves both stimulatory and inhibitory signaling pathways, resulting in a significant delay in upregulation. Here, we provide evidence that the delay in MMP-2 upregulation may be due to the autocrine expression and activation of transforming growth factor (TGF)-β, which is known to promote the contractile phenotype in VSMCs. Whereas PDGF-BB could induce the loss of stress fibers and focal adhesions, TGF-β was able to block or reverse this transition to a noncontractile state. TGF-β did not, however, suppress early signaling events stimulated by PDGF-BB. Over time, though PDGF-BB induced increased TGF-β1 levels, it suppressed TGF-β2 and TGF-β3 expression, leading to a net decrease in the total TGF-β pool, resulting in the upregulation of MMP-2. Together, these findings indicate that MMP-2 expression is suppressed by a threshold level of active TGF-β, which in turn promotes a contractile VSMC phenotype that prevents the upregulation of MMP-2.

THE RECRUITMENT of perivascular cells such as vascular smooth muscle cells (VSMCs) and pericytes is critical for the maturation of nascent vessel sprouts during angiogenesis; failure to do this leads to vessel dysfunction characteristic of diseases such as diabetes (5, 13, 20, 23). This recruitment to angiogenic sprouts is mediated by platelet-derived growth factor (PDGF)-BB expressed by specialized sprout tip endothelial cells (ECs) (1, 5, 11, 24, 29). In addition to serving as a chemoattractant and a mitogen, PDGF-BB induces VSMC dedifferentiation, which is characterized by a program of gene expression changes that includes the downregulation of contractile protein markers (5, 51, 57, 69), and the upregulation of MMP-2 (64). During this process, VSMCs switch from a contractile, quiescent state to a matrix-remodeling, proliferating state (57) and can maintain this dedifferentiated state in the continuous presence of PDGF (58).

Whereas some of the regulatory steps associated with changes in contractile protein marker expression by VSMCs are at least somewhat characterized, little is known about the regulation of markers of VSMC dedifferentiation, particularly MMP-2. MMP-2 is an extracellular matrix-degrading enzyme that plays an important role in vascular remodeling processes. For example, MMP-2 has been linked with VSMC migration/invasion in vitro and in vivo (60, 72, 81) as well as VSMC proliferation (6, 74). MMP-2 is crucial for VSMC migration during atherogenesis (45) but is also important during nonpathological vessel remodeling. The migration of VSMCs toward nascent endothelial tubes is required for vessel maturation (9, 16, 31), and several studies have linked MMP-2 to the angiogenic process. MMP-2 levels peak during the maximal level of angiogenesis in chick chorioallantoic membrane assays (63). Increased MMP-2 levels also correlate with angiogenesis in colorectal carcinoma (42). In a study examining mice with an impaired ability to activate MMP-2, fibroblast growth factor-2-induced angiogenesis was also impaired (83). MMP-2-null mice show decreased angiogenesis in models of experimentally induced neovascularization in the retina (56) and in the cornea (67) and also demonstrate reduced tumor-mediated angiogenesis (38).

We found previously that VSMCs stimulated with PDGF-BB increased MMP-2 expression during the process of phenotypic modulation (64). This MMP-2 upregulation was dependent on the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling axis in response to PDGF-BB but was delayed due to the induction of suppressive signaling pathways that temporally blocked this process (64). In the present study, we asked what signals or factors are responsible for this delay. We have found that PDGF-BB-induced upregulation of MMP-2 is antagonized by a threshold level of endogenously produced TGF-β that acts by maintaining a contractile phenotype. This threshold level of total, active TGF-β is initially surpassed by PDGF-BB-induced expression of TGF-β1, but, in time, decreases due to the subsequent downregulation of TGF-β2 and TGF-β3. These findings demonstrate the precise balance that exists among signals that support VSMC phenotypic switching and those that antagonize it.

EXPERIMENTAL PROCEDURES

Cell culture and reagents. The isolation and culture of S31 VSMCs, which were derived from the heart microvasculature of Wistar-Kyoto rats, has been previously described (61, 64). Cultures were maintained in DMEM containing 10% heat-inactivated fetal bovine serum in the absence of antibiotics-antimycotics. For experiments, S31 VSMCs were grown to postconfluence (57) and then serum starved by rinsing the plates once with PBS and placing cells on fresh serum-free medium (DMEM containing 0.02% fetectalbumin hydrolysate and 1× antibiotic-antimycotic; Invitrogen, Carlsbad, CA) for 48 h. For all treatments, the following growth factors were diluted in serum-starvation medium at the concentrations indi-
cated in the figure legends: recombinant rat PDGF-BB (R&D Systems, Minneapolis, MN), recombinant human macrophage colony-stimulating factor (M-CSF), and recombinant human TGF-β1 (CHO cell-derived, both from PeproTech, Rocky Hill, NJ). In all experiments, “control” treatment was the addition of fresh serum-free medium for the indicated amount of time. In some cases, cells were also treated for 15 min before growth factor stimulation with the following chemical inhibitors at the concentrations indicated in the figure legends: TGF-β Type I Receptor Kinase Inhibitor III (both from Calbiochem, San Diego, CA) and latrunculin (Lat) B, and LY-294002 (both from Biomol, Plymouth Meeting, PA). S31 VSMCs expressing the chimERIC PDGFRβ/CSF-1R were selected and cultured as described previously (64). In most cases, unless noted in the figure legends, all growth factor treatments were continual; that is, additional boluses of the indicated growth factors were added every 24 h. We chose to stimulate cells in this manner due to our observation that PDGF-BB-induced changes in S31 VSMCs begin to revert after 48 h of stimulation if only a single bolus of 25 ng/ml PDGF-BB is used; continual stimulation or a higher dose maintain PDGF-BB-induced changes (Supplemental Fig. 1).

**Immunocytochemistry.** Cells were plated onto coverslips at 50% confluence; after 24 h, cells were serum starved for an additional 48 h and then stimulated as indicated before fixation with 4% paraformaldehyde (Polysciences, Warrington, PA) for 10 min. Cells were permeabilized with 0.5% Triton X-100 in PBS before immunostaining. Rhodamine phalloidin (Invitrogen) was used to visualize F-actin stress fibers; focal adhesions were stained with anti-vinculin (Sigma no. V9131, St. Louis, MO) in combination with goat-anti-mouse IgG-conjugated Alexa-488 (Invitrogen). SM α-actin was stained using anti-SM α-actin (Sigma no. A2547) in combination with goat-anti-mouse IgG-conjugated Alexa-594 (Invitrogen). Coverslips were mounted with Vectashield Mounting Medium (Vectorshields Laboratories, Burlingame, CA); images were captured on an Olympus AX70 fluorescence microscope (Melville, NY) with QCapture 2.68 software (IBM, Armonk, NY).

**RT-PCR, real-time, and semiquantitative PCR.** Total RNA was collected using the Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN). Total RNA (2 μg) was reverse transcribed with oligo(dT) and either Powerscript Reverse Transcriptase (BD Biosciences, Franklin Lakes, NJ) or Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed as described previously (61). Primers used for real-time PCR of tissue inhibitor of metalloproteinase-2 (TIMP-2) and MMP-2 have been previously described (64). The following real-time PCR primers were used: SM α-actin, upper: 5'-CCCGCTCTGTCTCTAGCAC-3' and lower: 5'-ACACGAGTAACAAATCAAAGC-3'; calponin, upper: 5'-CTGGAATGGCAGACTAGTCTACA-3' and lower: 5'-CTGCTGAGGGCGGACAGTGGTGA-3'; SM22α, upper: 5'-TCTGGATCAGGCTCTGGGGTGAA-3' and lower: 5'-GAGGAAAGAGAAAAAGAGACACAGA-3'; c-jun, upper: 5'-TACCAAGGCTAGATTGGCGATGAAC-3' and lower 5'-GCATCGAGGGTGCTTCCTCCTACA-3'; and SM22α upper: 5'-CTATATAGGCTTTGGGCAGTTGG-3' and lower: 5'-AGACAGTGTCCTGCTGGGTA-3'. All semiquantitative PCR images are inverted. TIMP-2 was used as a PCR standard in both real-time and semiquantitative PCR, as we have found that its expression is only modestly changed in response to the growth factors analyzed in the relatively long-term experiments presented here.

**Western blots and zymography.** SDS lysates were collected and sonicated before bicinchoninic acid assay analysis (BCA, Pierce Biotechnology, Rockford IL), and radioimmune precipitation assay (26) lysates were collected and centrifuged to remove insoluble material before BCA analysis. Western blots were performed as described previously (64) using SDS or RIPA lysates. Samples were analyzed by BCA assay, and equal protein amounts were separated on gels before electrotoc transfer to nitrocellulose. Antibodies used included anti-SM α-actin, anti-β-tubulin (both from Sigma; A2547, T7816, respectively), anti-phospho-Akt (T308 and S473), anti-Akt, anti-phospho-JNK, anti-phospho-MAPK, anti-MAPK, anti-phospho-PDGFRβ (all from Cell Signaling Technology, Danvers, MA; nos. 9275, 4051, 9272, 9255, 9106, 9102, and 3166, respectively), and anti-PDGFRβ (R&D Systems; no. AF1042). Zymography was performed on conditioned medium (CM) from S31 VSMCs as described previously (64).

**Proliferation assays.** Analysis of cell proliferation was performed as described previously (62). Briefly, subconfluent (50% confluent) S31 VSMCs were plated into 96-well plates, serum starved for 48 h, and treated with serum-free DMEM containing 0.2% lactalbumin hydrolysate alone, plus 25 ng/ml PDGF, 10 ng/ml TGF-β1, and the combination of both growth factors. Proliferation was assessed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) per manufacturer’s instructions.

**TGF-β analyses.** ELISA was performed on cultured medium collected from cultures for active TGF-β1 and active TGF-β2 using the Emax ImmunnoAssay System (Promega) per manufacturer’s instruction. In cases where P values were calculated, unpaired two-tailed t-tests were performed. To measure the relative levels of functional TGF-β in conditioned media, we introduced a TGF-β-responsive SBE4 LUC construct (Addgene, Cambridge, MA) and a β-galactosidase reporter (SV40 β-gal, Promega) into HEK 293T cells using calcium phosphate. After transfection, cells were trypsinized and plated into 48-well plates. S31 VSMCs were treated with 25 ng/ml PDGF-BB, and conditioned medium was collected at the indicated times (Fig. 4). This medium was added to transfected HEK 293T, and cells were incubated for 6 h. Cell lysates were prepared using the Promega Luciferase Assay System with Reporter Lysis Buffer (Promega) and analyzed for luciferase activity, which was normalized to the β-galactosidase activity of the lysates.
Loss of the contractile phenotype precedes PDGF-BB-induced MMP-2 downregulation. We previously demonstrated that PDGF-BB induces a delayed upregulation of MMP-2 by S31 VSMCs and that this was due to competing signaling pathways (64). When we examined the phenotypic state of S31 VSMC cultures during a time course of PDGF-BB stimulation, we noted that cells stimulated for 8 and 24 h showed only slightly altered morphology compared with nontreated controls and no overt changes in F-actin stress fibers and focal adhesions (Fig. 1A). By 48 h, however, PDGF-BB-stimulated cells contained essentially no actin stress fibers or vinculin-containing focal adhesions (Fig. 1A). This loss of the contractile phenotype was consistent with the progressive reduction of SM α-actin message (Fig. 1B). In contrast, significant changes in MMP-2 expression during the long time courses involved in these studies (64), was used as a control (Fig. 1D). Thus changes in MMP-2 expression occur concomitantly with changes in cell morphology, both of which are delayed in response to PDGF-BB.

Exogenous TGF-β1 blocks PDGF-BB-induced MMP-2 upregulation. TGF-β is known to be a potent inducer of VSMC differentiation (4, 5, 32, 34), so we hypothesized that endogenous TGF-β may be part of a negative feedback loop established by these cells that limits MMP-2 upregulation in response to PDGF-BB. To evaluate this possibility, we asked if exogenous TGF-β1 could reverse the phenotype of fully dedifferentiated VSMCs. The continuous presence of PDGF-BB can suppress SM α-actin expression (12, 58) and promote increased MMP-2 expression by VSMCs over a prolonged period of time (Supplemental Fig. 1). Therefore, we stimulated S31 VSMCs with PDGF-BB every 24 h for a 48-h period, at which time we restimulated cultures with PDGF-BB plus or minus TGF-β1. At T = 72 h, we analyzed the expression of MMP-2 and SM α-actin and found that TGF-β1, even when added to fully dedifferentiated VSMCs, was able to begin reversing the effects of PDGF-BB-induced upregulation of MMP-2 (Fig. 2A). TGF-β1 also reversed the loss of SM α-actin levels (Fig. 2B) and induced the reformation of actin stress fibers (Fig. 2C). When we continuously stimulated S31 VSMCs with both PDGF-BB and TGF-β1 starting at T = 0, we also found that MMP-2 upregulation was completely inhibited (Fig. 3A). Interestingly, TGF-β1 did not completely block PDGF-BB-induced SM α-actin downregulation when both growth factors were used to simultaneously stimulate cells but did suppress it to a significant extent (Fig. 3B). Two other contractile protein genes showed a differential susceptibility to TGF-β1-mediated inhibition of PDGF-BB-induced downregulation (Fig. 3, C and D). Whereas the mechanism associated with this effect is not known, these results do suggest that the way in which TGF-β1 inhibits the actions of PDGF-BB on contractile protein versus MMP-2 gene expression is different.

![Figure 1](http://alpcell.physiology.org/.)
MMP-2 protein expression by cells stimulated with PDGF-BB was notably higher than by cells costimulated with both PDGF-BB plus TGF-β1 (Fig. 3A). As expected, S31 VSMCs stably expressing the chimeric receptor downregulated SM α-actin and upregulated MMP-2 in response to either M-CSF or PDGF-BB (Supplemental Fig. 2B). In both cases, costimulation with TGF-β1 blocked changes in MMP-2 expression.

Several studies, including our own, have linked the PI3K/Akt signaling axis with MMP-2 upregulation in response to growth factors (7, 46, 48, 64). We thus evaluated Akt activation in S31 VSMCs stimulated with both PDGF-BB and TGF-β1 but noted no differences in the phosphorylation of Akt at threonine-308 or serine-473 between PDGF-BB-stimulated and costimulated cells (Supplemental Fig. 2C). Similarly, there were no marked differences in p44/42-MAPK or JNK activation (Supplemental Fig. 2C). TGF-β1 alone did not appear to stimulate any of these signaling pathways. Consistent with an inability to block early signaling pathways, TGF-β1 did not suppress PDGF-BB-mediated mitogenesis (Supplemental Fig. 3A) nor did it block PDGF-BB-induced upregulation of the immediate-early genes, c-fos and c-jun (Supplemental Fig. 3B), or TIMP-1, another gene upregulated by PDGF-BB (Supplemental Fig. 3C). These data indicate that TGF-β1 suppresses only a subset of the genes and cellular processes regulated by PDGF-BB.

The differential regulation of endogenous TGF-β isoforms by PDGF-BB may govern MMP-2 expression by VSMCs. VSMCs are known to express TGF-β isoforms (28), so we sought to determine whether a PDGF-BB-induced increase in endogenous TGF-β expression could transiently suppress MMP-2 upregulation. We costimulated S31 VSMCs with PDGF-BB and an inhibitor of the Type I TGF-β receptor, activin-like kinase (ALK) 5, and found that the inhibitor accelerated MMP-2 upregulation of MMP-2 and SM α-actin downregulation at both early (Fig. 4, A and B, respectively) and later times (Fig. 4, C and D, respectively) post-PDGF-BB stimulation. We also noted that the inhibitor enhanced the ability of PDGF-BB to modify the actin cytoskeleton of S31 VSMCs (Fig. 4F). We next measured the levels of TGF-β isoforms expressed by PDGF-BB-stimulated cells and found that TGF-β1 mRNA was upregulated by 361% by 24 h and 178% by 48 h (Fig. 5A). In contrast, both TGF-β2 and TGF-β3 message levels were significantly downregulated in response to PDGF-BB, with the maximal downregulation of both occurring by 48 h; at this time point, the expression level of TGF-β2 in response to PDGF-BB was only 7% of control values and that of TGF-β3 was 13% of control levels (Fig. 5, B and C). Consistent with message levels, we found that active TGF-β1 protein levels in medium conditioned by S31 VSMCs stimulated with PDGF-BB were increased at 24 and 48 h by 207% and 345%, respectively (Fig. 5D). Again in contrast to TGF-β1, the level of active TGF-β2 decreased in response to PDGF-BB stimulation; by 24 and 48 h poststimulation, TGF-β2 levels were 52% and 16% of control levels, respectively (Fig. 5E). Thus, in nonstimulated cells, the total measurable, activated TGF-β1 and TGF-β2 level was ~150 pg/ml, whereas PDGF-BB-stimulated cultures had ~100 pg/ml; TGF-β3 levels were not detectable by ELISA. To confirm that PDGF-BB transiently increases TGF-β levels in the medium of VSMCs, we stimulated S31 VSMCs with PDGF-BB and then used the resultant medium to stimulate human embryonic kidney (HEK)-293T cells transfected with a TGF-β-responsive luciferase reporter construct. We found that luciferase activity could be stimulated by S31 VSMC-conditioned medium from
PDGF-BB-stimulated cultures and that this stimulation ultimately approached that induced by nonstimulated cell medium over a 24-h period (Fig. 5F). This indicates that the level of activated TGF-β rapidly increased in response to PDGF-BB, reached a maximum level within 20 h, and then decreased thereafter. Together, these data suggest that, whereas PDGF-BB induces the autocrine expression and activation of TGF-β1, it also induces the simultaneous downregulation of the other TGF-β isoforms. The net result is a decrease in TGF-β over time, allowing PDGF-BB-induced upregulation of MMP-2 expression to proceed.

**TGF-β-mediated promotion of stress fibers is critical to its ability to suppress the upregulation of MMP-2.** Given the known importance of the F-actin-to-G-actin ratio to the regulation of contractile protein gene expression (49, 70), we wanted to determine whether the ability of TGF-β to suppress MMP-2 upregulation was related to its ability to maintain stress fibers and focal adhesions in cells exposed to PDGF-BB. To do this, we stimulated cells with PDGF-BB for 24 h, then added increasing concentrations of TGF-β1 plus additional PDGF-BB, and evaluated the expression of MMP-2 and SM α-actin after an additional 24 h (Fig. 6, A and B). We found that TGF-β1 inhibits MMP-2 upregulation and reverses the loss of SM α-actin expression in a highly dose-dependent manner. We also noted that, at the highest dose of TGF-β1 (10 ng/ml), this late addition of TGF-β1 also reversed the downregulation of the contractile genes calponin and SM22α (Fig. 6, D and E, respectively). At this dose of TGF-β1, we also observed that the effect of blocking MMP-2 upregulation was reflected at the level of protein expression as determined by zymography (Fig. 6F). Adding TGF-β1 24 h after the initial PDGF-BB stimulus resulted in a partial rescue of SM α-actin protein levels (Fig. 6F) and appeared to completely block PDGF-BB-induced reduction of vinculin-staining focal adhesions and F-actin stress fibers (Fig. 6G).

This ability of TGF-β1 to block or reverse the effects of PDGF-BB even when added 24 h after an initial PDGF-BB stimulation allowed us to challenge cells with reagents that would normally be cytotoxic during an extended incubation. Using this approach, we stimulated S31 VSMCs with PDGF-BB and rechallenged them 24 h later with fresh PDGF-BB, PDGF-BB plus TGF-β, or TGF-β alone, all in the presence or absence of LatB, a drug that promotes the formation of G-actin. As expected, LatB induced the downregulation of SM α-actin, regardless of other treatments (Fig. 7A). It also blocked the ability of TGF-β to suppress MMP-2 upregulation in response to PDGF-BB (Fig. 7B), and, in all cases, induced a profound loss of stress fibers and focal adhesions (Supplemental Fig. 4).

**DISCUSSION**

It is generally appreciated that PDGF-BB is a potent stimulator of VSMC dedifferentiation and is critical for the recruit-
ment of these cells to developing vessels (30, 33). We have previously shown that PDGF-BB elicits both stimulatory and inhibitory signals that modulate MMP-2 expression by VSMCs (64). Here, we demonstrate that the primary inhibitory signal may be linked to the autocrine expression and activation of TGF-β1, which promotes the maintenance of actin stress fibers and, through a mechanism not yet known, suppresses MMP-2 upregulation. This suppression is temporary in the VSMC model utilized in this study, and this may be linked to the PDGF-BB-induced downregulation of TGF-β2 and TGF-β3, resulting, over time, in a decreased amount of active TGF-β below a critical threshold level necessary to block PDGF-BB upregulation in response to PDGF-BB. In cases of tissue damage, which involves a rapid increase in PDGF-BB levels, this initial increase in TGF-β1 may limit vessel instability, which, in the absence of increased TGF-β might cause an excessive loss of perivascular cells from vessels. During the stabilization phase of angiogenesis, when nascent vessels become ensheathed with perivascular cells, VSMCs and endothelial cells collaborate to produce active TGF-β (4, 19, 34), which induces VSMC differentiation (58). A disruption in TGF-β-induced signaling leads to an impairment in angiogenesis (14, 18, 47), as characterized by undifferentiated mural cells associated with endothelial cell tubes. This is consistent with our findings that exogenously added TGF-β1 will suppress MMP-2 upregulation in response to PDGF-BB. Importantly, we show that an increase in the available, active pool of total TGF-β will completely reverse or block VSMC dedifferentiation, regardless of phenotypic state.

Whereas the detailed mechanisms remain to be elucidated, our findings suggest that the ability of TGF-β to suppress MMP-2 upregulation is linked to the integrity of the actin cytoskeleton. Forcing the disassembly of actin stress fibers, which is known to result in contractile protein downregulation (58), blocked the ability of TGF-β1 to suppress the upregulation of MMP-2 and accelerated this upregulation in response to PDGF-BB. This suggests that the inhibitory signal that delays PDGF-BB-induced MMP-2 upregulation is dependent on intact actin stress fibers or F-actin. Previous studies have suggested an association between F-actin and the contractile protein markers that characterize a differentiated VSMC phenotype. This regulation was linked mechanistically to the disposition of...
myocardin-related transcription factor-A (MRTF-A), which associates with G-actin, thus becoming sequestered away from the promoters of contractile protein genes such as SMα-actin (53, 76, 78). Force generation and stress fiber assembly cause the release of MRTF-A, which then acts as a serum response factor cofactor, stimulating the transcriptional activation of contractile protein markers. MMP-2 and the contractile proteins are regulated reciprocally in VSMCs (64), and it appears that the cytoskeleton plays a role in both processes. It is not clear, however, why the concurrent addition of TGF-β and PDGF-BB to VSMCs prevents stress fiber loss, yet fails to prevent the loss of SMα-actin expression. This may be due to PDGF-BB-induced early signals because the addition of TGF-β 24 or 48 h after the initial PDGF-BB stimulation fully restored SMα-actin expression (Figs. 2 and 6). In all cases, TGF-β prevented the upregulation of MMP-2. It has been demonstrated that loss of force generation or stress relaxation will cause the activation of MMP-2 protein (3, 27, 68, 71), and we previously found in fibroblasts that this was accompanied by modest changes in MMP-2 message levels (71). Our present study suggests that the loss of the contractile phenotype is necessary for transcriptional upregulation of MMP-2 in VSMCs, and that TGF-β acts primarily through its ability to promote F-actin to block this upregulation. An examination of the MMP-2 gene shows that there are Smad-binding elements (CAGACA) (41) present in both the proximal promoter and the first intron; however, when we evaluated ~3500 base pairs of sequence flanking, the 5′ end the rat promoter, and the complete first intron using promoter-reporter assays, we did not observe any suppression by TGF-β, though we did observe PDGF-BB induction (Risinger and Howard, unpublished observation). Future studies will be aimed at determining whether distal elements of the MMP-2 promoter could possibly be involved in regulation elicited by the cytoskeleton or TGF-β signaling.

Whereas PDGF-BB has been previously shown to affect TGF-β isoform expression in SMCs (15, 59, 82) and other cells (75, 77), this is the first demonstration that, in VSMCs, the TGF-β isoforms are reciprocally regulated. It should be noted that the 33% decrease in total active TGF-β levels observed here only reflected the contributions of TGF-β1 and TGF-β2 (Fig. 5). Given that the message level of TGF-β3 decreased much like that of TGF-β2, we assume that a more dramatic change in total active TGF-β levels occur in response to PDGF-BB stimulation. We were unable to measure TGF-β3 protein levels by ELISA, and this could be due to low protein expression or its sequestering to an insoluble pool. Indeed, we were also unable to suppress the ability of endogenous TGF-β to inhibit PDGF-BB-induced MMP-2 upregulation using function-blocking antibodies (Risinger and Howard, unpublished observations), perhaps due to this same sequestration. Previous studies have demonstrated that fibroblasts can sequester TGF-β to the matrix in a form that does not activate receptors but that is nevertheless poised to stimulate TGF-β receptors upon activation (79). In any case, the idea that a net loss of active TGF-β levels facilitates MMP-2 upregulation is supported by the ability of exogenous TGF-β1 to suppress this change in gene expression, regardless of when TGF-β1 was added (Figs. 2, 3, and 6, unpublished observations). It is important to note that our data do not rule out the participation of other TGF-β family members, including the bone morphogenic proteins or activins, in the autocrine suppression of PDGF-BB-induced MMP-2 upregulation, particularly since the ALK inhibitor is known to block ALK5 as well as the activin receptors ALK4 and ALK7 (17). Similarly, the SBE4-Luc construct analyzed may respond to some of these factors as well (37, 44). It is thus possible that TGF-β is one of several factors contributing to the regulation of MMP-2 in these cells.

Finally, our analyses of cellular processes affected by the addition of exogenous TGF-β1 reveal that the combined ef-
The effects of TGF-β1 and PDGF-BB support vessel stabilization. Depending on context, TGF-β1 has been found to have an inhibitory (2, 36, 50, 52, 54, 62, 65, 66, 80) or stimulatory (2, 40, 43, 51, 55, 65, 80) effect on PDGF-induced proliferation in SMCs. In contrast, we demonstrated that TGF-β1 had no significant effect on PDGF-BB-induced VSMC proliferation nor did it affect PDGF-BB-induced PDGFRβ receptor activity or immediate downstream signaling. These findings differ from those observed by others using non-SMC cell systems where TGF-β negatively affected PDGF-induced signaling (22, 25).

Fig. 6. Late addition of exogenous TGF-β1 blocks MMP-2 upregulation in response to PDGF-BB. A–C: S31 VSMCs were grown to postconfluence before 48 h of serum starvation. At T = 0, cells were treated with 25 ng/ml PDGF-BB. At T = 24 h, an additional bolus of 25 ng/ml PDGF-BB was added to the cells, with or without the indicated amount of TGF-β1 (ng/ml). At T = 48 h, total RNA was collected, and expression levels of MMP-2, SM α-actin, and TIMP-2 were analyzed by real-time PCR. Error bars represent means ± SE, and N = 3. D and E: S31 VSMCs were treated as in A–C except only the 10 ng/ml concentration of TGF-β1 was added at T = 24 h. At T = 48 h, total RNA was harvested, and calponin and SM22α expression levels were analyzed by real-time PCR. Expression levels are relative to no-addition control. Error bars represent means ± SE and N = 3. F: S31 VSMCs were cultured as in D and E, except conditioned medium and SDS lysates were collected at T = 48 h. Conditioned medium was analyzed by zymography for MMP-2 expression (top). SDS lysates were analyzed for SM α-actin (middle) and β-tubulin expression by Western blot (bottom). G: cells were cultured on glass coverslips and serum-starved for 48 h before stimulation with 25 ng/ml PDGF-BB. At T = 24 h, an additional 25 ng/ml bolus of PDGF-BB was added with or without 10 ng/ml TGF-β1. Cells were fixed and T = 48 h and analyzed for F-actin stress fibers with rhodamine phalloidin and focal adhesions with an anti-vinculin antibody. Bar = 30 μm.

Fig. 7. Increasing G-actin levels with latrunculin B blocks TGF-β1-mediated inhibition of PDGF-BB-induced MMP-2 expression. A and B: S31 VSMCs were grown to postconfluence and serum-starved for 48 h before stimulation with 25 ng/ml PDGF-BB at T = 0. At T = 24 h, an additional 25 ng/ml bolus of PDGF-BB was added, and as indicated, 10 ng/ml TGF-β1 and/or 1 μM latrunculin B (LatB) were added as well. At T = 48 h, total RNA was collected and analyzed for SM α-actin (A) and MMP-2 (B) expression levels by real-time PCR. Error bars represent means ± SE, and P values were calculated using unpaired, two-tailed t-tests. N = 3.
Previous studies have suggested that TGF-β induces PDGF-A expression, which can antagonize PDGF-B-mediated processes in VSMCs (8, 69). We have previously demonstrated that PDGF-AA does not induce S31 VSMCs to proliferate, does not itself alter MMP-2 or SM α-actin expression, and does not affect the ability of PDGF-BB to upregulate MMP-2 or downregulate SM α-actin (64). Given that PDGF-BB can bind PDGFRα and PDGFRβ hetero- and homodimers (10, 29), we needed to determine whether TGF-β1 somehow alters receptor dimerization. Our studies with receptor chimeras suggest that this is not the case. Mural cell proliferation occurs during vessel maturation (31, 39), and, combined with our observations of the effects of TGF-β on VSMC phenotypic switching, our findings support the idea that the effects of TGF-β on VSMC phenotype are quite selective and are consistent with those events that would promote vessel stabilization.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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