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Serine-204 in the Linker Region of Smad3 Mediates the Collagen-I Response to TGF-β in a Cell Phenotype-Specific Manner

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Abstract

Regulation of TGF-β1/Smad3 signaling in fibrogenesis is complex. Previous work by our lab suggests that ERK MAP kinase phosphorylates the linker region (LR) of Smad3 to enhance TGF-β-induced collagen-I accumulation. However the roles of the individual Smad3LR phosphorylation sites (T179, S204, S208 and S213) in the collagen-I response to TGF-β are not clear. To address this issue, we tested the ability of Smad3 constructs expressing wild-type Smad3 or Smad3 with mutated LR phosphorylation sites to reconstitute TGF-β-stimulated COL1A2 promoter activity in Smad3-null or -knockdown cells. Blocking ERK in fibroblasts and renal mesangial cells inhibited both S204 phosphorylation and Smad3-mediated COL1A2 promoter activity. Mutations replacing serine at S204 or S208 in the linker region decreased Smad3-mediated COL1A2 promoter activity, whereas mutating T179 enhanced basal COL1A2 promoter activity and did not prevent TGF-β stimulation. Interestingly, mutation of all four Smad3LR sites (T179, S204, S208 and S213) was not inhibitory, suggesting primacy of the two inhibitory sites. These results suggest that in these mesenchymal cells, phosphorylation of the T179 and possibly S213 sites may act as a brake on the signal, whereas S204 phosphorylation by ERK in some manner releases that brake. Renal epithelial cells (HKC) respond differently from MEF or mesangial cells; blocking ERK neither changed TGF-β-stimulated S204 phosphorylation nor prevented Smad3-mediated COL1A2 promoter activity in HKC. Furthermore, re-expression of wild type-Smad3 or the S204A-Smad3 mutant in Smad3-knockdown HKC reconstituted Smad3-mediated COL1A2 promoter activity. Collectively, these data suggest that Serine-204 phosphorylation in the Smad3LR is a critical event by which ERK enhances Smad3-mediated COL1A2 promoter activity in mesenchymal cells.

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Disclosure
No conflicts of interest, financial or otherwise, are declared by the authors(s).

Author contributions
Author contributions: J.A.B., X.L., performed experiments; J.A.B prepared figures; J.A.B., T.H., and H.W.S., drafted manuscript; J.A.B, T.H., X.L. and H.W.S., approved final version of manuscript.

Keywords

Fibrosis; Extracellular Matrix; Fibroblast; Epithelial; mesenchymal; ERK MAP kinase

Introduction

TGF-β1 is a critical mediator of extracellular matrix (ECM) accumulation in fibrogenesis [1]. Canonical signaling begins with TGF-β binding the type II receptor (TβRII) to recruit and phosphorylate the type I receptor (TβRI), which in turn recruits and phosphorylates the receptor-specific transcription factors: Smad2 and Smad3. Smad4 is then included to this complex, which translocates into the nucleus to associate with other transcription factors to regulate the transcription of genes, including those encoding extracellular matrix proteins such as type-I collagen (collagen-I). In addition to this canonical TGF-β/Smad pathway, multiple other signaling pathways appear to regulate the myriad and sometimes opposing effects of TGF-β on cells. Our laboratory has characterized additional, non-canonical mediators that modulate TGF-β/Smad3-mediated fibrogenesis. These mediators include PKC-δ [2], PI3K [3], cytoskeletal components [4], HIF-1α [5] and, of critical importance, ERK MAP kinases [6–9].

Smad3 is essential for most renal fibrosis, confirmed by the suppression of fibrogenesis in Smad3-null mice [10]. Smad3, like Smad2 and Smad4, consists of two highly conserved Mad-homology domains (MH1 and MH2) connected by a less-conserved linker region (LR) [11]. TβRI–mediated phosphorylation of the carboxyl-terminal of Smad3 is critical for downstream Smad3 activity. However the Smad3LR also contains several threonine and serine residues (T179, S204, S208 and S213) that can be differentially phosphorylated by the action of MAP kinases (ERK, JNK and p38), cyclin-dependent kinase (CDK) members and glycogen synthase kinase 3 (GSK3) to regulate Smad3 responses in a highly cell context-dependent manner [12–25]. For example, an early study reported that overexpression of constitutively active Ras that activates MAP kinase phosphorylates the LR to prevent the nuclear accumulation of Smad3 and thus block TGF-β signaling [13]. Consistent with that report, CDK-mediated LR phosphorylation can also inhibit Smad3 transcriptional activity to promote aberrant cell cycle progression, generating resistance to the growth-inhibitory effect of TGF-β [16]. However we and others [26, 27] have proposed that the effect of LR phosphorylation on Smad3 responses is highly cell context-dependent. For example, LR phosphorylation by p38 MAPK and ROCK (S204, S208 and S213) and JNK (S208 and S213) may enhance Smad2/3 transcriptional activity [12, 19, 21, 28]. Consistent with these observations, Smad3LR phosphorylation can also promote extracellular matrix production in cultured rat myofibroblasts [15]. Indeed, it has been reported that the Smad3LR includes a transcriptional activation domain that cooperates with the carboxyl-terminal domain of Smad3 to enhance TGF-β-induced transcriptional activation [29, 30]. Previous work from our lab suggests that ERK enhances Smad3LR phosphorylation and collagen-I synthesis in response to TGF-β in human mesangial cells [6, 7]. We proposed that specific LR phosphorylation sites differentially enhance, inhibit or have no effect on Smad3-mediated collagen-I expression. To test this hypothesis, we examined the effect of site-specific Smad3LR mutants on TGF-β-induced COL1A2 promoter activity. Our results indicate that the collagen-I response to TGF-β is regulated through specific phosphorylations in the linker region of Smad3 in a cell phenotype-specific manner.

EXPERIMENTAL PROCEDURES

Materials

Active, recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) was reconstituted as a 4 μg/ml stock solution in 4 mM HCl with 1 mg/ml bovine serum albumin. Antibodies were purchased from the following vendors: anti-phospho-Smad1/2/3 (H-2), anti-phospho-Smad3-S208 from Santa Cruz Biochemistry (Santa Cruz, CA); anti-phospho Smad3 (S423/S425) from Cell Signaling Technology (Beverly, MA); anti-β-actin, anti-phospho-Smad3-T8, -T179, -S204 and -S213 from Abcam (Cambridge, MA). Puromycin was purchased from Sigma. MEK/ERK inhibitors: PD98059 and PD0325901 were purchased from EMD Millipore (Billerica, MA) and Selleckchem (Houston, TX), respectively.

Expression Plasmids

CS2-Smad3 expression constructs (wild-type, T179V, S204A, S208A and EPSM) were a generous gift from F. Liu (Rutgers, Piscataway, NJ). The -378COL1A2-luc construct containing the 378 base pairs of the α2(I) collagen promoter sequence and 58 base pairs of the transcribed sequence, fused to the luciferase reporter gene was constructed as previously described [31]. The pFA-Elk and pFR-Luc plasmids were purchased from Stratagene (La Jolla, CA).

Cell Culture

Mouse embryonic fibroblasts (wild-type and Smad3-null MEF) and human renal tubular epithelial cells (HKC) were generous gifts of E. Böttinger (Mount Sinai, NY) [32] and L. Racusen (John Hopkins Medical School, Baltimore, MD) [33]. Human dermal fibroblasts were supplied by the Skin Disease Research Center, Northwestern University (Chicago, IL). These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, penicillin-streptomycin, amphotericin B, and HEPES buffer. Human renal mesangial cells were isolated from glomeruli of normal renal cortex as described previously [34] and cultured in DMEM/F12 medium supplemented with 10% heat-inactivated cosmic calf serum obtained from Hyclone (Logan, UT), glutamine, penicillin/streptomycin, sodium pyruvate, HEPES buffer, and 8 μg/ml insulin (Sigma, St. Louis, MO) (used between passages 5 and 8).

Stable Smad3-knockdown HKC

GIPZ clones (empty vector, RHS4349 and human Smad3 shRNA RHS4430-98895790) were obtained from Open Biosystems Products (Huntsville, AL). The constructs were subjected to $CaPO_4$ transfection for lentiviral packaging in HEK293 FT cells (Invitrogen) using psPAX2 and pMD2.G followed by transduction of HKC with the crude viral lysate. Expression of shRNA was visualized at 48 h by expression of GFP that is in tandem with shRNA cassette. Thereafter, infected cells were selected with 2 μg/ml puromycin. Frozen stocks of established cell lines were used for experiments at passages 4–8 after transduction to minimize possible induction of compensatory mechanisms.

Western Blotting Analysis

Following exposure to experimental conditions, cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed on ice in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (1 mM PMSF, 1 mM EDTA, 1 μg/ml of leupeptin, aprotinin, and pepstatin A, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 40 mM 2-glycerophosphate). Lysates were cleared by centrifugation (10,000 × g at 4°C) for 10min and samples of equal protein concentration (determined by protein assay) were resolved by

SDS-PAGE on 8% polyacrylamide gels. Proteins were transferred onto Immobilon-P (PVDF) membranes (Millipore, Bedford, MA), blocked 3% BSA in Tris-buffered saline (TBS: 20 mM Tris/HCl, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T). Membranes were probed with antibodies for target proteins overnight at 4°C. Membranes were washed in TBS-T and probed with the appropriate species of secondary-HRP conjugated antibody (prepared in 3% BSA in TBS-T). Immunoreactive bands were visualized using chemiluminescence reagent according to the manufacturer's instructions (Santa Cruz Biotechnology).

Transient Transfection and Luciferase Assay

Cells were seeded onto 6-well plates (1.0×10^5/well). 18h later the cells were transfected (for 3h) with the indicated plasmids and a β-galactosidase expression vector (transfection efficiency control) in serum-free medium using X-tremeGENE HP DNA Transfection Reagent from Roche (Indianapolis, IN) (3μl: 2μg DNA ratio). Transfected cells were exposed to experimental conditions (described in text) and harvested in reporter lysis buffer (Promega, Madison, WI). Luciferase and β-galactosidase activities were measured as described previously [6, 7]. Each condition was tested in triplicate, and experiments were repeated at least three times. Media from treated cells was also assayed for the activity of lactate dehydrogenase (an enzyme released by dying cells) using the CytoTox96® Non-Radioactive Assay (Promega, Madison, WI).

Statistical analysis

Data is expressed as mean ± SE. GraphPad Prism 6 software was used to analyze statistical differences between experimental groups using analysis of variance and values of $P<0.05$ by Bonferroni post hoc test were considered significant.

Results

Serine-204 in the Linker Region (LR) of Smad3 is a Primary ERK Target in Fibroblasts and Renal Mesangial Cells

First, to confirm the specificity of the phospho-Smad3 linker region (LR) antibodies, we tested their ability to recognize their targets in wild-type murine embryonic fibroblasts (MEF) and Smad3-null MEF (Fig. S1A, Supplemental data). In response to TGF-β-stimulation, Smad3LR phosphorylations at T179, S204, S208 and S213 were detected in wild-type MEF but not in Smad3-null MEF. The upper (60-kD) of the two bands detected by the anti-phospho-Smad3-T179 antibody corresponds to the analogous phosphorylation site (T220) in the Smad2LR (Fig. S1A). To further verify the specificity of these antibodies, we tested their ability to recognize their targets in either HEK or wild-type MEF transfected with constructs expressing wild-type Smad3, Smad3 with mutated LR phosphorylation sites or empty vector (Fig. S1B, C). The transfected MEFs (but not the HEKs) were then stimulated with TGF-β (1 h) (Fig. S1C). Empty vector-transfected cells did not exhibit LR phosphorylations whereas cells transfected with wild-type Smad3 exhibited strong phosphorylations at T179, S204, S208 and S213 in the Smad3LR (Fig. S1B, C). In contrast, the duration of optimal exposure for developing the blots was so short in Smad construct overexpressing cells that the possible phosphorylation of native Smad3-LR was not detected (Fig. S1B, C, first two lanes). Similar to empty vector-transfected cells, phosphorylations at T179, S204, S208 and S213 were not observed in cells transfected with T179V, S204A, S208A or S213A, respectively. Therefore, each of the phospho-Smad3LR antibodies recognized overexpressed wild-type Smad3 and not their corresponding phosphoacceptor-site Smad3 mutant, further validating the specificity of the phospho-Smad3LR antibodies.

We then evaluated how several signaling molecules, which previously have been implicated in Smad signaling, affect specific Smad3LR phosphorylation. TGF-β-stimulated wild-type MEF (Fig. 1A, B), dermal fibroblasts (Fig. 1C) and renal mesangial cells (Fig. 1D) exhibited increased phosphorylation at all four Smad3LR sites: T179, S204, S208 and S213. Threonine-8 (T8) in the MH1 domain of Smad3 was also phosphorylated in TGF-β-treated MEFs (Fig. 1B). Chemical inhibitors of MEK/ERK (PD98059 and PD0325901) were used to evaluate a role for the ERK MAP kinases on these phosphorylations. PD98059 specifically blocked TGF-β-stimulated S204 phosphorylation without changing the phosphorylation of the remaining LR sites (T179, S208 or S213) (Fig. 1A). Consistent with these results, another MEK/ERK inhibitor, PD0325901 also blocked TGF-β-induced S204 phosphorylation without affecting the phosphorylation of the remaining sites (T8, T179, S208, S213 or S423/S425) (Fig. 1B). The ability of PD98059 and PD0325901 to block TGF-β-stimulated ERK phosphorylation was verified (Fig. 1A, B). The MEK/ERK inhibitor, PD98059 also reduced TGF-β-induced S204 phosphorylation without changing the phosphorylation of T179, S208 nor S423/S425 in both human renal mesangial cells (Fig. 1C) and human dermal fibroblasts (Fig. 1D). In contrast, S204 phosphorylation was not inhibited by inhibition of p38 (SB202190) or JNK (SP600125) (data not shown). These results indicate that S204 phosphorylation is ERK-dependent in fibroblasts and renal mesangial cells. In light of previous studies reporting that TGF-β-stimulated S204 phosphorylation is mediated by GSK3 in multiple cell types including MEF [23, 24] we also tested the effect of the GSK3 inhibitor (SB216763) on TGF-β-stimulated Smad3LR phosphorylations. GSK3 inhibition blocked TGF-β-induced S204 phosphorylation without changing the phosphorylation of the remaining sites (T179, S208, S213 nor S423/S425) in MEF (Fig. S1E, Supplemental data).

ERK MAP Kinases are required for Optimal Smad3-mediated TGF-β-induced COL1A2 Promoter Activity in Fibroblasts and Renal Mesangial Cells

Because TGF-β-stimulated Smad activity contributes to the collagen-I response in human renal mesangial cells [6, 7], we examined whether ERK supports Smad3-mediated collagen gene activation in embryonic fibroblasts (MEF) (Fig. 2). TGF-β stimulation (20 h) significantly increased COL1A2 promoter activity in human renal mesangial cells (HMC) (Fig. 2A) or wild-type MEF (Fig. 2B and Fig. 2C), compared to vehicle-treated cells. These responses were attenuated by the MEK/ERK inhibitors: PD98059 (20 μM) in HMC (Fig. 2A) and wild-type MEF (Fig. 2C) and PD0325901 (5 nM) in wild-type MEF (Fig. 2B). As expected, Smad3-null MEF did not show a COL1A2 response to TGF-β compared with a normal response in wild-type MEF (Fig. 2C), and transfection of Smad3-null MEF with wild-type Smad3 reconstituted the COL1A2 response (Fig. 2D). A lactate dehydrogenase (LDH), assay was performed to assess MEF viability. In comparison to vehicle-treated MEFs (wild-type or Smad3-null) there was no change in LDH release (cytotoxicity) following treatment with PD98059 (20 μM) or PD0325901 (5 nM) for 20h (Fig. S2, Supplemental data). This confirmed that the lack of a collagen response in either ERK-inhibitor treated- or Smad3-null MEF was not attributed to cytotoxicity. The absence of Smad3 from the null-MEF and its reconstitution by transfection of the Smad3 expression construct were verified (Fig. 2D). Together, these results demonstrate that optimal TGF-β-induced COL1A2 promoter activity in MEF requires both ERK and Smad3.

Role of the Smad3LR Phosphoacceptor sites in TGF-β-induced COL1A2 Promoter Activity in Fibroblasts

We then tested the role of the specific phosphorylation sites in the Smad3LR on TGF-β-induced COL1A2 promoter activity. Smad3-null MEF were transfected with wild-type Smad3, the single site-specific Smad3LR mutants or Smad3-EPSM (all four LR sites mutated: T179V, S204A, S208A and S213A) (Fig. 3). As was shown in Figure 2D, empty

vector-transfected Smad3-null MEF did not show a COL1A2 promoter response to TGF-β (Fig. 3, bars 1–2). In contrast a 9-fold increase in COL1A2 promoter activity was observed in TGF-β-treated Smad3-null MEF transfected with wild-type Smad3 compared with those treated with vehicle ($P<0.01$, bars 3–4). T179V-expressing Smad3-null MEF exhibited a strong COL1A2 promoter activity response to TGF-β ($P<0.01$, bars 5–6). In contrast, S204A- and S208A-expressing cells failed to reconstitute significant COL1A2 promoter responses to TGF-β (bars 7–10). Smad3-EPSM-expressing cells showed enhanced basal and a strong COL1A2 promoter activity by TGF-β ($P<0.05$, bars 11–12). Interestingly, blocking ERK with PD98059, did not inhibit the COL1A2 promoter response to TGF-β in Smad3-EPSM-expressing cells (data not shown). These results suggest that S204 and S208 in the Smad3LR are necessary for an optimal collagen-I response when the LR phosphoacceptor sites are intact.

Role of ERK and the S204 Smad3LR Phosphoacceptor Site in Smad3-mediated COL1A2 Promoter Activity in Renal Epithelial Cells

We next examined a role for ERK in both Smad3LR phosphorylation and Smad3-mediated COL1A2 promoter activity in renal epithelial cells (HKC). TGF-β treatment (1 h) increased site-specific Smad3LR phosphorylations at T179, S204, S208 and S213 in HKC, as was seen in the fibroblasts and mesangial cells. However, these phosphorylations were unaffected by the MEK/ERK inhibitor, PD98059 (Fig. 4A). TGF-β also significantly stimulated COL1A2 promoter activity in HKC. Unlike our observations in fibroblasts or mesangial cells, PD98059 did not block the collagen response (Fig. 4B), despite blocking Elk-gal transactivation activity (Fig. 4B, lower panel), a downstream readout of ERK-mediated transcriptional activity. A cytotoxicity assay (LDH assay) was performed and confirmed that there was no significant increase in toxicity in HKC treated with PD98059 (20 βM, 20h) compared to vehicle-treated cells (Fig. S3, Supplemental data). Finally, while Smad3 knock down eliminated the COL1A2 response to TGF-β in HKC, re-expression of either wild-type Smad3 ($P<0.0001$) or the S204A mutant ($P<0.001$) reconstituted the response (Fig. 5A). However, the level of the response supported by the S204A mutant was significantly less than that observed in TGF-β treated HKC that had been transfected with wild-type Smad3 (Fig. 5A, bar 4 vs. 6). As a comparison, re-expression of the S204A mutant in Smad3-null MEF failed to reconstitute the response (Fig. 5A, bar 11 vs. 12), as previously shown in Figure 3. Knockdown of Smad3 in HKC was confirmed by western blotting (Fig. 5B). These results suggest that ERK-mediated S204 phosphorylation is less important for the collagen response to TGF-β in epithelial cells than it is in mesenchymal cells.

Discussion

TGF-β/Smad3 signaling plays a central role in renal fibrogenesis. Our laboratory has characterized a number of additional, non-canonical signals that contribute to TGF-β-mediated, type-I collagen expression. Critical among these is ERK-mediated signaling. We previously showed in human glomerular mesangial cells that ERK and Smad3 linker-region (LR) phosphorylation plays a role in TGF-β induction of collagen expression [6, 7]. Because the role of the LR in Smad signaling is variably reported [12–25] the present series of experiments was designed to determine (1) whether and how specific LR phosphorylations occur in human mesangial cells, and (2) the role such phosphorylations plays in the collagen response. Our initial studies confirmed the specificity of the phosphorylation site-specific Smad3LR antibodies. We then determined that inhibiting different known mediators caused various patterns of phosphorylation, but only ERK inhibition consistently blocked the phosphorylation of the serine at amino acid position 204, suggesting that S204 phosphorylation is ERK dependent. Next, we tested whether S204 phosphorylation was

important in the collagen-I response. Because the Smad3-EPSM construct (lacking all four LR phosphoacceptor sites) does not function as a dominant negative mutant, we utilized Smad3-null fibroblasts reconstituted with various Smad3 mutants. By doing this, we were able to test whether each specific phosphoacceptor site of the Smad3LR is required for reestablishing the collagen promoter response. The T179V-Smad3 mutant supported normal or increased responses and in some experiments showed increased basal activity. In contrast, Smad3 mutants -S204A and -S208A did not support the collagen response. Importantly, the Smad3-EPSM construct, in which all four LR phosphoacceptor sites are mutated, did support a response in Smad3-deficient MEFs. Moreover, ERK inhibitor treatment did not prevent TGF-β-stimulated collagen promoter activity in the Smad3-reconstituted cells. Taken together, these data are consistent with a complex role for Smad LR phosphorylation (Fig. 6). In the absence of LR phosphorylation, Smad3 can activate the COL1A2 promoter, likely through the TβR-regulated C-terminal phosphorylation as previously shown [31]. In the presence of intact C-terminal serines and their phosphorylation, phosphorylation of T179 or S213 is likely to be inhibitory, whereas phosphorylation at S204 and S208 enhances responses, likely by releasing the inhibition caused by phosphorylation at the T179 and S213 sites. Since ERK inhibition prevents S204 phosphorylation, we propose that S204 phosphorylation is an important, ERK-mediated step in TGF-β–stimulated collagen expression in fibroblasts and renal mesangial cells. This complex model could explain both our results and the often-contradictory findings reported in the literature.

In contrast, when we performed similar studies in Smad3-knockdown HKC, a renal tubular epithelial line, we did not find a similar set of results. In HKC cells, re-expression of either wild-type Smad3 or the Smad3-S204A mutant reconstituted a strong Smad3-mediated COL1A2 promoter response to TGF-β. This observation is consistent with our finding that ERK inhibition did not block TGF-β-stimulated collagen expression in epithelial cells, despite blocking the transactivational activity of a downstream ERK substrate, Elk. Together, our findings suggest the presence of a mesenchymal cell-specific mechanism regulating Smad3-mediated fibrogenesis.

Several other signaling molecules also affect LR phosphorylation. The ability of CDK inhibitors to block T179, S204 or S208 phosphorylation in TGF-β-stimulated mink lung epithelial (Mv1Lu) cells [23] suggests that CDK family members may target Smad3LR phosphorylation sites in addition to the S204 ERK target. Others have reported TGF-β-stimulated S204 phosphorylation is mediated by GSK3 in epithelial-like cells [23, 24] and in MEF [23]. We also confirmed that GSK3 inhibition (SB216763) reduced TGF-β-induced S204 phosphorylation without changing the phosphorylation of T179, S208 nor S423/S425 in MEF (Fig. S1E, Supplemental data). SB216763 also attenuated TGF-β-stimulated COL1A2 promoter activity in MEFs (data not shown) consistent with the role we have proposed for S204 and suggesting a role for GSK3 as a modulator of Smad3 activity, either in concert with or distinct form the effect of ERK. The ability of TGF-β to phosphorylate S208 in $JNK^{+/+}$ but not in $JNK^{-/-}$ MTE cells implicates JNK in S208 phosphorylation [28]. ROCK or p38 inhibitors block basal- and TGF-β-stimulated S204- and S208-phosphorylation in human mammary carcinoma cells [21], suggesting that ROCK and p38 and may also target these sites.

The mechanism by which S204 phosphorylation enhances Smad3 activity is uncertain. Kretschmar and colleagues reported that LR phosphorylation inhibits Smad3 responses by blocking the nuclear translocation of Smad3 [13]. However, others have described strong nuclear staining for S204 phosphorylation in TGF-β-treated AML12 mouse hepatocytes [24]. Another proposed mechanism of modulated Smad3 signaling is regulation of protein decay. Indeed, CDK8/9-mediated (but not MAPK-mediated) T179 phosphorylation has been shown to be required for the linker region to interact with NEDD4L, an E3 ubiquitin ligase

that targets proteins for degradation [35]. On the other hand, mutations at either S204 or S208 were reported not to alter basal Smad3 degradation [22]. Finally, differential LR phosphorylation could affect Smad3 transactivational activity. The ability of the LR to regulate Smad3 interactions with other transcriptional regulators is supported by work that shows a Smad3 phospho-mimetic (T179E/S204D/S208D) to enhance--and a Smad3-EPSM (all LR sites ablated) to inhibit--the Smad3-Smad4 interaction in alveolar type II epithelial (C10) cells [28]. The authors of that paper also reported that the Smad3-EPSM reduced basal- and TGF-β-stimulated Smad3-binding element (SBE) promoter activities. LR phosphorylation could affect the interaction of Smad3 with the transcriptional coactivator, p300/CBP [29, 30]. Therefore, the S204 and S208 sites could enhance the binding of Smad3 to coactivators involved in the transcriptional regulation of the COL1A2 promoter. However, in the presence of a constitutively active TβRI, the S204A and the S204A/S208A mutants were highly associated with overexpressed CBP compared to wild-type Smad3 [24]. This suggests that S204 and/or S208 inhibit the Smad3-CBP interaction, which fits in their model that shows S204 and S208 to inhibit downstream Smad3 activity [24]. Conversely, S204 and S208 may inhibit the binding of Smad3 with co-repressors of the COL1A2 promoter. These discrepant results further highlight the cell context-dependent role of the Smad3LR.

In summary, ERK-induced S204 phosphorylation in the Smad3LR may be a critical event in mesenchymal cells, to allow ERK to enhance Smad3-mediated COL1A2 promoter activity. This finding may provide new insights into the potential mechanism whereby ERK promotes fibrosis. Further investigation into the mechanism through which S204 and S208 in the Smad3LR enhance TGF-β-stimulated collagen-I accumulation may provide a therapeutic strategy for the treatment of fibrotic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Regulation of TGF-β1/Smad3 signaling in fibrogenesis is complex
- Ser204 in the Smad3 Linker Region is a primary ERK target in mesenchymal cells
- ERK is required for Smad3-mediated TGF-β-induced COL1A2 promoter activity
- Ser204 phosphorylation by ERK is critical for ERK to enhance this collagen response

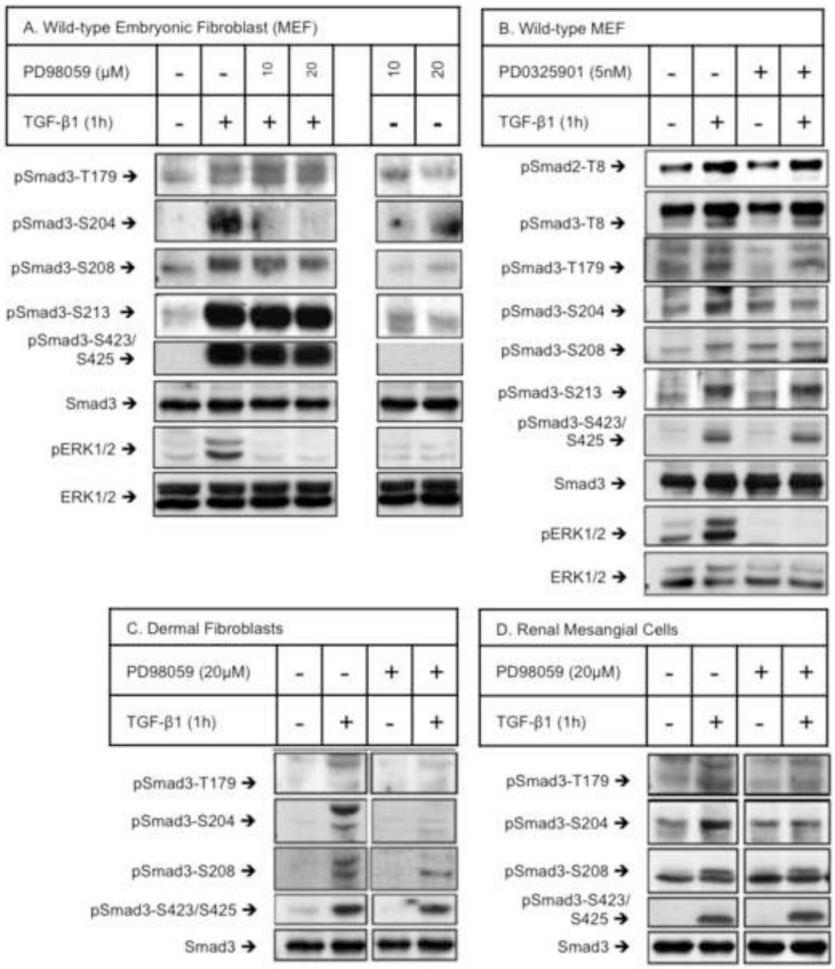

Figure 1. Serine-204 in the Smad3LR is a primary ERK target in fibroblasts and renal mesangial cells

Wild-type murine embryonic fibroblasts (MEF) (A, B), human dermal fibroblasts (C) and human renal mesangial cells were pre-treated (1 h) with vehicle (DMSO), PD98059 (10 and 20 μM) or PD0325901 (5 nM) prior to vehicle- or TGF-β1 (2 ng/ml) stimulation (1 h). Smad3 phosphorylations at T8, T179, S204, S208, S213 and S423/S425 were analyzed by western blotting using site-specific phospho-Smad3LR antibodies. The upper of the two bands detected by the anti-phospho-Smad3-T8 and anti-phospho-Smad3-T179 antibodies represents cross-reaction with the T8 and T220 sites in the Smad2LR, respectively. Shown here are representative experiments of four independent experiments.

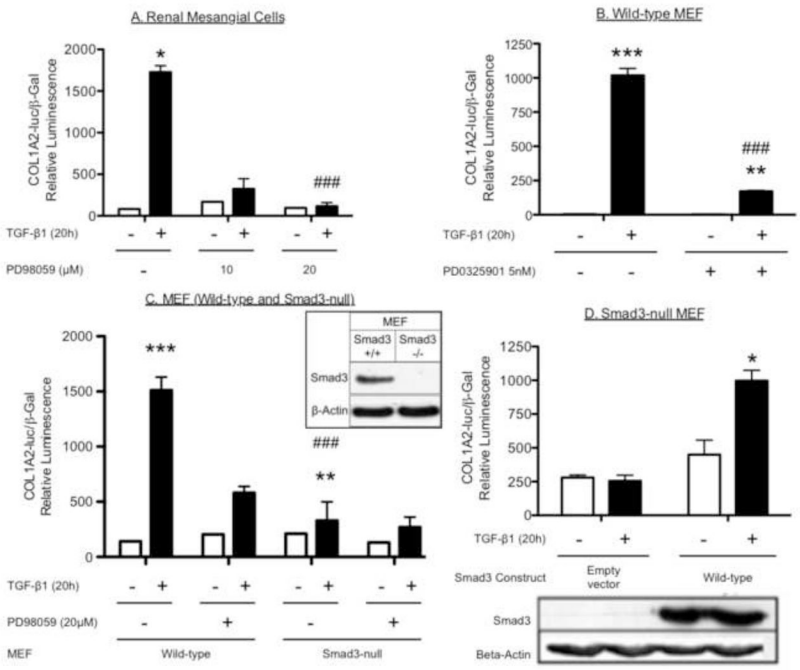

Figure 2. ERK MAP kinases are required for optimal Smad3-mediated TGF-β-induced COL1A2 promoter activity in fibroblasts and renal mesangial cells

A, Blocking ERK in renal mesangial cells inhibits TGF-β-stimulated COL1A2 promoter activity. Renal mesangial cells were transfected with COL1A2-luciferase and β-galactosidase constructs for 3 hours. Transfected cells were then pre-treated with PD98059 (10 and 20 μM) or vehicle (DMSO) for 1 h, before vehicle- or TGF-β- (2 ng/ml) stimulation for a further 20 h. *B*, Blocking ERK in wild-type murine embryonic fibroblasts (MEF) inhibits TGF-β-stimulated COL1A2 promoter activity. MEF were exposed to the same experimental conditions as in (A); however a different MEK/ERK inhibitor, PD0325901 (5 nM), was used. *C*, TGF-β-stimulated COL1A2 promoter activity is both ERK- and Smad3-dependent. Wild-type and Smad3-null MEF were exposed to the same experimental conditions as in (A). *D*, Reconstitution of Smad3-mediated COL1A2 promoter activity. Smad3-null MEFs were transfected with empty vector or pCS2-Smad3-wild-type, including the COL1A2-luciferase and β-galactosidase constructs for 3 hours. Transfected cells were stimulated with TGF-β (2 ng/ml) for a further 20 h. Relative luciferase activity (mean ± S.E.M., n=3) was corrected for β-galactosidase activity. Western blotting shows the absence of Smad3 in Smad3-null MEF versus wild-type MEF. Shown here is a representative experiment of three independent experiments. *P<0.05, **P<0.01 vs. vehicle-treated cells. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. vehicle-treated cells.

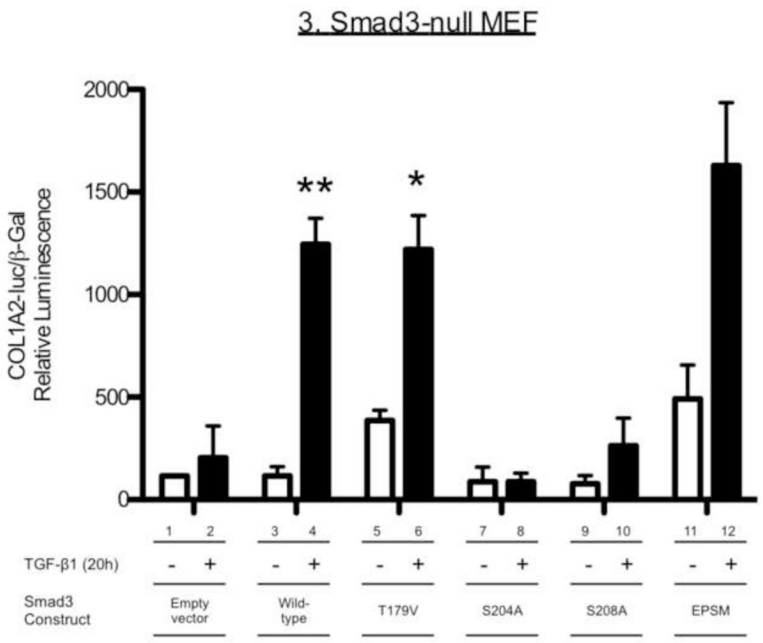

Figure 3. Effect of site-specific Smad3LR mutants (T179V, S204A, S208A or EPSM) on TGF-β-induced COL1A2 promoter activity in Smad3-null murine embryonic fibroblasts

Smad3-null MEFs were transfected with empty vector or pCS2-Smad3 constructs (wild-type, T179V, S204A, S208A, S213A or EPSM) including the COL1A2-luciferase and β-galactosidase constructs for 3 hours. Transfected cells were then stimulated with TGF-β (2 ng/ml) for a further 20 h. Relative luciferase activity (mean ± S.E.M., n=3) was corrected for β-galactosidase activity. Shown here is a representative experiment of five independent experiments. *P<0.05, **P<0.01 vs. vehicle-treated cells expressing the same construct. $^{\#}$P<0.05,

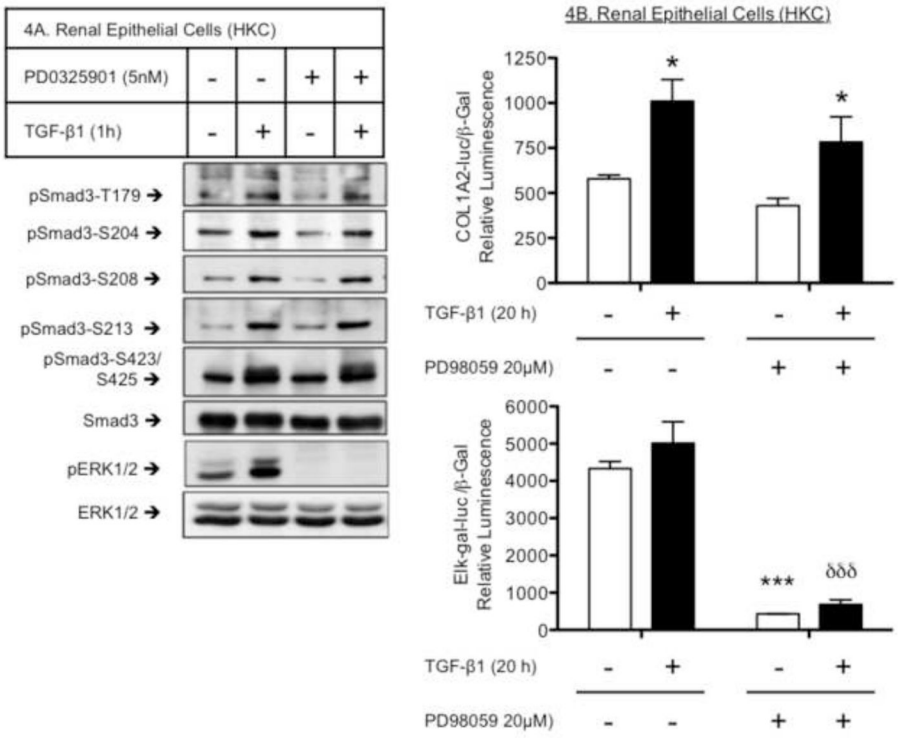

Figure 4. Effects of MEK/ERK inhibition on Smad3LR phosphorylation and Smad3-mediated COL1A2 promoter activity in renal epithelial cells

A, S204 phosphoacceptor is not an ERK target in renal epithelial cells. Renal epithelial cells (HKC) were pre-treated (1 h) with vehicle (DMSO) or PD0325901 (5 nM) prior to vehicle- or TGF-β1 (2 ng/ml) stimulation (1 h). Phosphorylations at T179, S204, S208, S213 and S423/S425 were analyzed by western blotting with specific phospho-peptide Smad3 antibodies. S204 phosphorylation is not prevented by the MEK/ERK inhibitor. *B*, Smad3-mediated COL1A2 promoter activity in renal epithelial cells does not require ERK. HKC were transfected with β-galactosidase, COL1A2-luciferase (upper panel) or Elk-gal (lower panel) and constructs for 3 hours. The pFA-Elk plasmid expresses an activation domain of Elk linked to the DNA-binding region of yeast Gal4, along with a Gal4-luciferase reporter construct. Transfected cells were then pre-treated with vehicle (DMSO) or PD98059 (20 μM) for 1 h, before vehicle- or TGF-β (2 ng/ml) stimulation for a further 20 h. Relative luciferase activity (mean ± S.E.M., n=3) corrected for β-galactosidase activity. Shown here is a representative experiment of three independent experiments. *P<0.05, **P<0.01 vs. vehicle-treated cells. $^{\delta\delta\delta}$P<0.001 vs. TGF-β-treated cells.

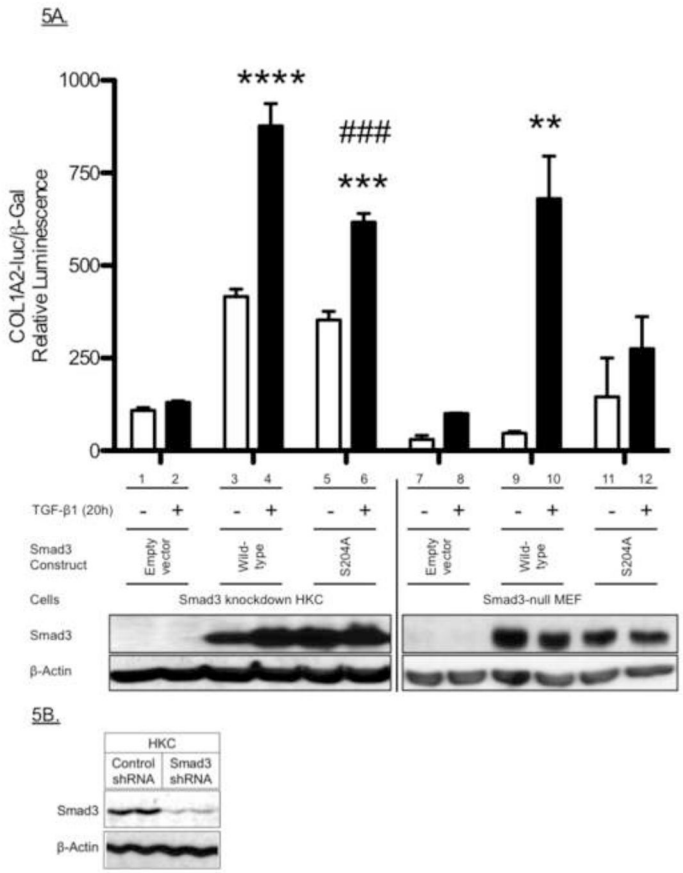

Figure 5. Role of the Serine-204 (S204) Smad3LR Phosphorylation Site in Smad3-mediated renal epithelial cell COL1A2 promoter activity

A, Effect of the S204A-Smad3 mutant on Smad3-mediated COL1A2 promoter activity in renal epithelial cells. Smad3-knockdown HKC (bars 1–6) and Smad3-null MEFs (bars 7–12) were transfected with empty vector or pCS2-Smad3 constructs (wild-type or S204A), including the COL1A2-luciferase and β-galactosidase constructs for 3 hours. The transfected cells were then stimulated with TGF-β (2 ng/ml) for a further 20 h. Relative luciferase activity (mean ± S.E.M., n=3) was corrected for β-galactosidase activity. Shown here is a representative experiment of three independent experiments. *P<0.05, **P<0.01 vs. vehicle-treated cells. *B*, Smad3-knockdown in renal epithelial cells. Western blotting analysis of HKC stably expressing control- or Smad3-pGIPZ lentiviral shRNA.

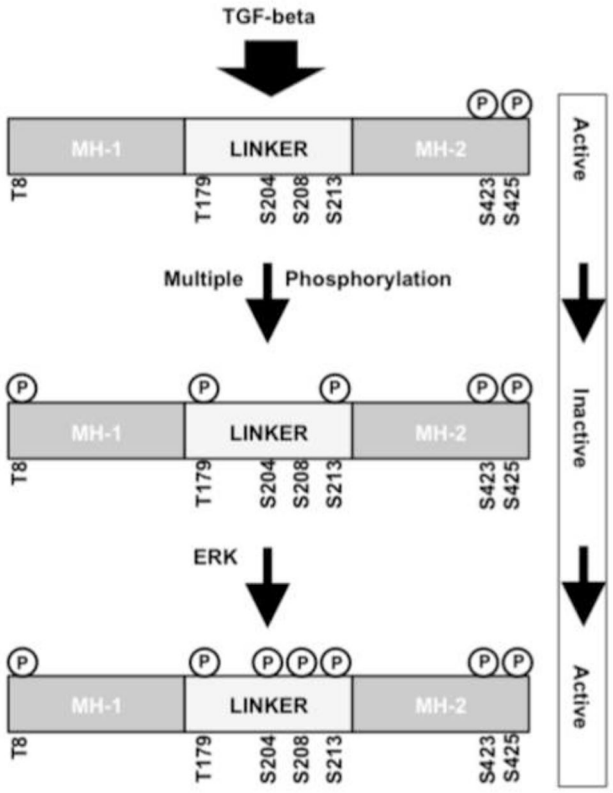

Figure 6. Proposed model for regulation of Smad3 activity in TGF-β-stimulated collagen expression

TGF-β stimulates the TβR to phosphorylate essential serines at the carboxyl terminus of Smad3. In the absence of linker region phosphorylation, Smad3 can activate the COL1A2 promoter. Phosphorylation of T179 and S213 is likely to be inhibitory, whereas ERK-mediated phosphorylation at S204 and possibly S208 enhances responses, likely by releasing the inhibition caused by phosphorylation at the T179 and S213 sites. Since ERK inhibition targets S204 phosphorylation, we propose that S204 phosphorylation is an important, ERK-mediated step in TGF-β–stimulated collagen expression. This complex model could explain both our results and the often-contradictory findings reported in the literature.