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TGF-β Signaling Preserves RECK Expression in Activated Pancreatic Stellate Cells

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Abstract Activated pancreatic stellate cells (PSCs) play a pivotal role in the pathogenesis of pancreatic fibrosis, but the detailed mechanism for dysregulated accumulation of extracellular matrix (ECM) remains unclear. Cultured rat PSCs become activated by profibrogenic mediators, but these mediators failed to alter the expression levels of matrix metalloproteinases (MMPs) to the endogenous tissue inhibitors of metalloproteinases (TIMPs). Here, we examined the expression of RECK, a novel membrane-anchored MMP inhibitor, in PSCs. Although RECK mRNA levels were largely unchanged, RECK protein expression was barely detected at 2, 5 days after plating PSCs, but appeared following continued in vitro culture and cell passage which result in PSC activation. When PSCs at 5 days after plating (PSCs-5d) were treated with pepstatin A, an aspartic protease inhibitor, or TGF-β1, a profibrogenic mediator, RECK protein was detected in whole cell lysates. Conversely, Smad7 overexpression or suppression of Smad3 expression in PSCs after passage 2 (PSCs-P2) led to the loss of RECK protein expression. These findings suggest that RECK is post-translationally processed in pre-activated PSCs but protected from proteolytic degradation by TGF-β signaling. Furthermore, collagenolytic activity of PSCs-5d was greatly reduced by TGF-β1, whereas that of PSCs-P2 was increased by anti-RECK antibody. Increased RECK levels were also observed in cerulein-induced acute pancreatitis. Therefore, our results suggest for the first time proteolytic processing of RECK as a mechanism regulating RECK activity, and demonstrate that TGF-β signaling in activated PSCs may promote ECM accumulation via a mechanism that preserves the protease inhibitory activity of RECK. J. Cell. Biochem. 104: 1065–1074, 2008.

Key words: pancreatic stellate cells; fibrosis; RECK; TGF-β

The extracellular matrix (ECM) undergoes continuous remodeling during growth and development [Vu and Werb, 2000]. The dysregulation of ECM turnover (synthesis, secretion, and degradation) is often associated with pathologic processes. Pancreatic fibrosis, seen in chronic pancreatitis and pancreatic cancer, is characterized by excessive production and deposition of ECM components, mainly type I collagen [Apte and Wilson, 2004]. Numerous studies suggest that activated pancreatic stellate cells (PSCs) are the primary source of the excess type I collagen observed in the fibrotic pancreas and play an important role in

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pancreatic fibrogenesis [Haber et al., 1999; Jaster, 2004].

PSCs, in their quiescent state, can be identified by their angular appearance and the presence of vitamin A-containing lipid droplets in their cytoplasm [Bachem et al., 1998]. When activated by profibrogenic mediators, they transform into myofibroblast-like cells characterized by positive staining for α-smooth muscle actin (α-SMA), reduction in vitamin A lipid droplets, and greatly increased synthesis of the ECM proteins, including type I collagen [Apte et al., 1998]. PSCs not only produce ECM components, but also secrete matrix degrading enzymes of the matrix metalloproteinase (MMP) family, such as MMP-2, MMP-9, MMP-13, and MT1-MMP, as well as their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 [Shek et al., 2002; Phillips et al., 2003]. Despite studies on the effects of profibrogenic mediators on PSCs which leads to excessive ECM accumulation, the detailed mechanism responsible remains largely unknown, in particular the role of matrix proteases and protease inhibitors.

Takahashi and coworkers recently described a novel membrane-anchored MMP regulator RECK (110 kDa). In contrast to the mild phenotype of null TIMP animals [Jiang et al., 2002], homozygous loss of RECK in mice resulted in embryonic lethality and caused severe disruption of tissue integrity due to the excessive MMP activity, documenting its crucial role in maintaining ECM integrity in vivo [Oh et al., 2001]. Several clinical studies describe RECK as a tumor suppressor and a potential prognostic indicator, with its presence correlating with increased patient survival [Noda and Takahashi, 2007].

In this study, we included an examination of RECK expression, along with examination of other effectors of ECM turnover, in the process of PSC activation. Our findings suggest that RECK is expressed but proteolytically degraded in pre-activated PSCs. As PSCs become activated, proteolytic modification of RECK is reduced, at least in part by TGF-β/Smad3 mediated signaling, resulting in expression of the fully active 110 kDa form.

MATERIALS AND METHODS

Pancreatic Stellate Cell Isolation and Culture

Rat PSCs were isolated as described previously [Apte et al., 1998], suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and plated on non-coated plastic dishes. After reaching confluence in the primary culture, serial passages were obtained always applying a 1:3 split.

Analysis of Gene Expression Using Real-Time RT-PCR

Total RNA was isolated from PSCs using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 1.0 μg total RNA with GeneAmp RNA PCR (Applied Biosystems, Foster city, CA) using random hexamers. Real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM of the upstream and downstream PCR primers, and 2 μl of the first strand cDNA as a termplate. To control for variations in the reactions, all PCRs were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primers used were as follows: 5′-GAA CGG GAA GCT CAC TGG C-3′ (forward primer) and 5′-GCA TGT CAG ATC CAC AAC GG-3′ (reverse primer) for GAPDH; 5′-CCT AAC CTC TGC CCC AA-3′ (forward primer) and 5′-ACG GGC CTC TGT AGG A-3′ (reverse primer) for RECK; 5′-AAG AAG TCA CCC GCG TGC TA-3′ (forward primer) and 5′-TGT GTG ATG TCT TTG GTT TTG TCA-3′ (reverse primer) for TGF-β; 5′-TGG AGA GCC TCT GTG GAT ATG TC-3′ (forward primer) and 5′-AAC GGC CCG CGA TGA-3′ (reverse primer) for TIMP-1; 5′-TGC CCT ATG ATC CCA TGC TA-3′ (forward primer) and 5′-TCT GTG ACC CAG TCC ATC CA-3′ (reverse primer) for TIMP-2; 5′-GAT CTG CAA GCA AGA CAT TGT CTT-3′ (forward primer) and 5′-GCC AAA TAA ACC GAT CCT TGA A-3′ (reverse primer) for MMP-2; 5′-TGG AAC TAA AGA ACA TGG TGA CTT CTA-3′ (forward primer) and 5′-CCC CGC CAA GGT TTG G-3′ (reverse primer) for MMP-13; 5′-GCA GCG GAG CCG TGA GT-3′ (forward primer) and 5′-GTG TCC CAT GGC GTC TAA AGA-3′ (reverse primer) for MMP-14.

Western Blot Analysis

Cells were rinsed in ice-cold PBS and harvested by scraping in the lysis buffer [Oh et al., 2004]. Equivalent amount of protein was separated by SDS-PAGE, followed by immunoblot

detection using the primary antibody. Antibodies against RECK, α-SMA, and β-actin were purchased from BD Transduction Laboratories (San Diego, CA), Sigma (St. Louis, MO), and Santa Cruz (Santa Cruz, CA), respectively; antibodies against α-tubulin and Smad2 from Cell Signaling (Beverly, MA); antibodies against Smad3, Smad6, and Smad7 from Zymed (San Francisco, CA).

Immunofluorescence

PSCs were plated onto glass coverslips in 12-well plates coated with gelatin. Samples were fixed in paraformaldehyde and incubated with anti-RECK monoclonal antibody overnight at 4°C in a moist chamber, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Cells were washed with PBS, and nuclei were stained with DAPI (1 mg/ml in methanol for 15 min at 37°C) to localize all nuclei before mounting with Vector shield (Vector Laboratories, Burlingame, CA). Stained cells were visualized on a Zeiss LSM 5 PASCAL confocal microscope equipped for fluorescence microscopy.

Northern Blot Analysis

Total RNA was isolated from PSCs using an RNeasy kit (Qiagen, Valencia, CA). Total RNA (15 μg) was resolved by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled, rat specific RECK probe that was prepared by PCR using primers 5′-CTG GGA TGC TAC GGG TTT TA-3′ and 5′-GTG GGA GTA CTG CCG AGT GT-3′. Nylon membranes were washed, dried, and exposed to X-ray films at −80°C.

Transfection of PSCs and siRNA

Expression vector pEF-Flag, containing cDNA for Smad7, were kindly provided by Dr. H.-J. Lee, KRIBB. PSCs after passage 2 were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and subject to analysis after 36 h.

Sequences for Smad2 and Smad3 siRNA are as follows: 5′-CAC AGU ACU CGU GUG UGA U(dTdT)-3′ (sense) and 5′-AUC ACA CAC GAG UAC UGU G(dTdT)-3′ (anti-sense) for Smad2; 5′-GUG AUG UGU AGU UCC UAG U(dTdT)-3′ (sense) and 5′-ACU AGG AAC UAC ACA UCA C(dTdT)-3′ (anti-sense) for Smad3. PSCs were transfected with siRNA at 30% confluence using Lipofectamine 2000 according to the manufacturer's instructions and subject to analysis after 72 h.

Collagenolysis Assays

A pre-cooled coverglass of LabTek chambered coverglass system (Nalge Nunc International, Rochester, NY) was coated with 25 μg/ml of the quenched fluorescent substrate DQ-collagen I (Molecular Probes, Carlsbad, CA) suspended in Matrigel, 10 mg/ml (Becton Dickinson, Bedford, MA), for 1 h at 37°C. Plated cells were grown for a period of 48 h. A Zeiss LSM 5 PASCAL confocal microscope was used to observe the cells for fluorescent degradation products of DQ-collagen I.

Soluble Collagen Assay

The amount of collagen in the culture supernatants was determined with a Sircol collagen assay kit (Biocolor Ltd., Newtown Abbey, UK) according to the manufacturer's instructions.

Histological Analysis in Samples of Normal and Acute Pancreatitis

Acute pancreatitis was induced in Male Sprague–Dawley rats (weighing 125–200 g; $n = 3$) by two hourly intraperitoneal injection of cerulean (200 μg/kg). Animals were sacrificed 72 h after last cerulean injections and pancreas were rapidly harvested for histology. Sections of paraformaldehyde-fixed, paraffin-embedded rat pancreatic tissues were prepared and stained as described elsewhere [Oh et al., 2001]. The primary antibodies used for immunostaining (visualized in brown color) were α-SMA (DAKO, Carpinteria, CA) and RECK (BD Transduction Laboratories). Finally, the sections were counterstained in Harris' hematoxylin, dehydrated through graded alcohol, and mounted.

Statistical Analysis

Results are expressed as mean ±Ystandard deviation (SD). Paired statistical analysis was done using *t*-tests. Comparisons were considered significant at $P < 0.05$ and P values were two tailed.

RESULTS

Effects of Profibrogenic Mediators on MMPs and TIMPs in Rat PSCs

As in hepatic fibrosis [Hemmann et al., 2007] and pulmonary fibrosis [Gauldie et al., 2006], there is some evidence to support a role for enhanced TIMP expression in pancreatic fibrosis. However, studies in TIMP-1 deficient mice

revealed that lack of this endogenous inhibitor does not necessarily prevent renal [Eddy et al., 2000; Kim et al., 2001] or hepatic fibrosis [Vaillant et al., 2001], suggesting that the role of TIMPs and MMPs in fibrotic disease may be more complex. Thus we first examined the effects of the profibrogenic mediators, such as FGF, TGF-β1, ethanol and its metabolite acetaldehyde, on the expression of MMP-2, MMP-13, MMP-14 (MT1-MMP), TIMP-1, TIMP-2, and TGF-β in cultured rat PSCs by real-time PCR. PSC activation is directly correlated with the duration in in vitro culture and the expression of α-SMA, a marker for the activated PSC phenotype [Kruse et al., 2001]. We therefore studied PSCs at two different stages of activation; PSCs at 5 days after plating (PSCs-5d) and PSCs after passage 2 (PSCs-P2). Unlike humans, rats express only one interstitial collagenase, MMP-13 [Krane et al., 1996]. TGF-β autoinduction was observed in both PSCs-5d and PSCs-P2 (Fig. 1A,B), which is consistent with previous reports [Ohnishi et al., 2004]. TGF-β1 treatment also led to a significant increase in mRNA levels of TIMP-1 and MMP-13 in PSCs-5d and of MMP-13 alone in PSCs-P2. In addition all other profibrogenic agents also activated MMP-13 mRNA expression in PSCs-5d, but not in PSCs-P2, suggesting that these effects are restricted to PSCs-5d. Interestingly, all of the other profibrogenic mediators tested (FGF, ethanol, acetaldehyde) failed to show any significant effects on TIMP-1, TIMP-2, MMP-2, or MMP-14 mRNA expression. These findings suggest that profibrogenic mediators do not increase the mRNA ratio of TIMPs to MMPs in PSCs and that the role of these genes in pancreatic fibrosis may be secondary.

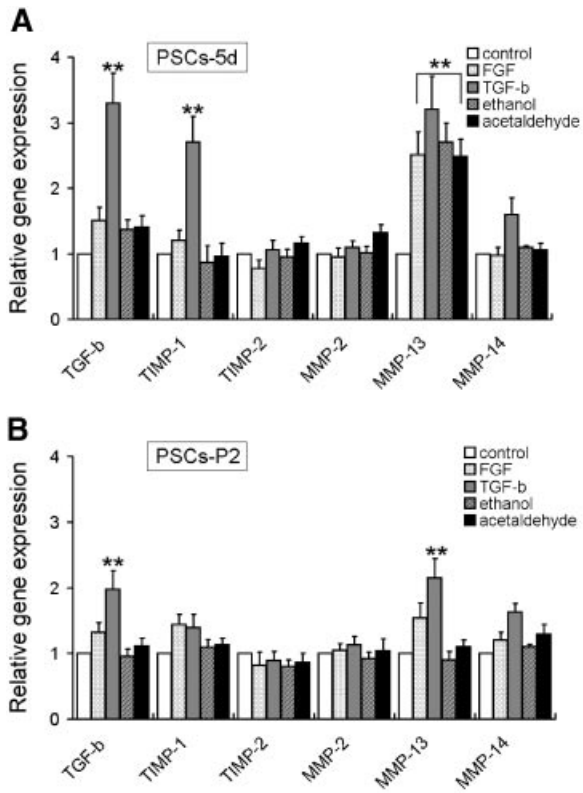

Fig. 1. Effects of profibrogenic mediators on mRNA expression levels of MMPs and TIMPs. Following serum starvation for 24 h, PSCs at 5 days after plating (**A**) and PSCs after passage 2 (**B**) were treated with b-FGF (20 ng/ml), TGF-β1 (5 ng/ml), ethanol (50 mM), or acetaldehyde (200 μM) for 6 h and analyzed by real-time PCR. The data are presented as the foldness of the baseline levels and represent means ± SD for three independent experiments from three separate RNA preparations. $^{**}P < 0.05$ compared untreated baseline.

RECK is Expressed in Rat PSCs

As we have demonstrated that RECK is essential for the maintenance of tissue integrity during mouse embryonic development [Oh et al., 2001], the expression of RECK was examined in the process of PSC activation. Whole cell lysates were prepared from PSCs at different stages of activation in culture for 2 or 5 days after plating, and after passage 1, 2, or >3, and analyzed by Western blotting. The activation status of the PSC cultures was assessed by the expression of α-SMA (Fig. 2A, middle panel). RECK protein signal was barely detected in PSCs at 2, 5 days after plating (PSCs-2d, -5d), but appeared in PSCs after passage 1 (PSCs-P1) which display myofibroblast-like cell morphology (Fig. 2A, upper panel). RECK signal was found increased in PSCs-P2. A similar pattern of RECK protein expression was observed using a different monoclonal anti-RECK antibody (5B11D12 [Takahashi et al., 1998] data not shown). These findings show a significant change in the RECK protein expression during the course of PSC activation, although real-time PCR analysis revealed no statistically significant changes in RECK mRNA levels during this time period of culture (Fig. 2B). It is interesting to note that both protein and mRNA levels of RECK decrease after several passages (passage >3).

RECK was reported to be linked to the cell surface by a glycosyl-phosphotidylinositol (GPI) anchor [Takahashi et al., 1998]. Both PSCs-5d and PSCs-P2 were subject to immunofluores-

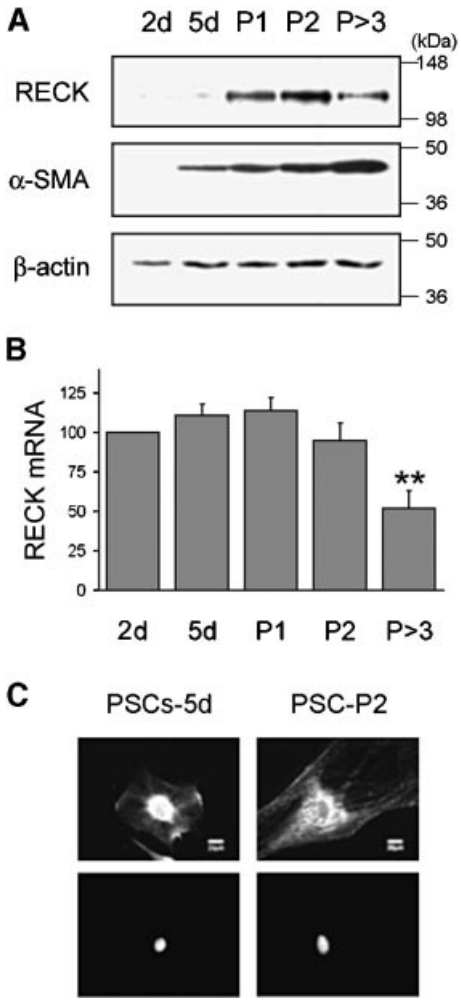

Fig. 2. RECK expression during the culture activation of rat PSCs. **A**: Cell lysates were prepared from PSCs at 2 (2d), 5 (5d) days after plating and from PSCs after passage 1 (P1), 2 (P2), >3 (P > 3) and analyzed by Western blotting. α-SMA was used as a marker for PSC activation and β-actin used as a loading control. This figure is representative of three independent experiments from separate cell preparations. **B**: Total RNA was isolated from PSCs at different stages of activation and analyzed for RECK by real-time PCR. The data are expressed as the percent of PSCs-2d and represent means ±YSD for three independent experiments. $^{**}P < 0.05$ compared with PSCs-2d. **C**: PSCs-5d and PSCs-P2 were fixed, and immunofluorescent staining for RECK was performed. Nuclei were stained with DAPI (blue).

cence study to investigate the cellular distribution of RECK. PSCs-5d demonstrate a very strong perinuclear signal for RECK staining, as well as some cell membrane staining (Fig. 2C). In contrast, PSCs-P2 show cell membrane RECK localization that is associated with cellular stellate protrusions, as well as a more diffuse cytoplasmic staining pattern. These observations suggest that in PSCs-P2 RECK may be more readily translocated from perinuclear stores to the cell surface.

Regulation of RECK Expression in PSCs

To test the possibility of RNA splicing, total RNA samples were prepared from PSCs at different stages of activation and analyzed for RECK by Northern blotting. As seen in Fig. 3A, RECK mRNA was detected as a single transcript of the reported molecular size (~4.3 kb), excluding alternative splicing as the mechanism responsible for the loss of RECK protein signal. To examine whether RECK was post-translationally cleaved, PSCs-5d were incubated in serum-free medium containing inhibitor of serine, cysteine, aspartic, or MMPs for 24 h and analyzed for RECK by Western blotting. As described above, RECK protein signal was not detectable in PSCs-5d, but treatment with pepstatin A, an inhibitor of aspartic proteinases, led to the appearance of RECK protein band (Fig. 3B). On the other hand, inhibitors of seryl-(PMSF), cysteinyl-(E64), and MMPs (BB94) all failed to protect RECK from proteolytic degradation. These

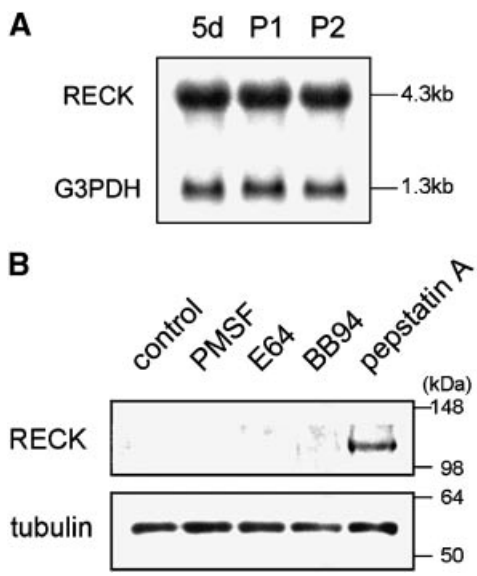

Fig. 3. Regulation of RECK expression in PSCs. **A**: Total RNAs were isolated from PSCs-5d, PSCs-P1, and PSCs-P2, and analyzed for RECK by Northern blotting. G3PDH was used as loading control. **B**: PSCs-5d were incubated in serum-free medium containing no inhibitor, PMSF (1 mM), E64 (10 μM), BB94 (100 nM), or pepstatin A (100 μM) for 24 h, and analyzed for RECK by Western blotting. Tubulin was used as loading control.

findings suggest that RECK is post-translationally processed, either directly or indirectly by aspartic proteinase(s), in pre-activated PSCs.

TGF-β Signaling Provides Proteolytic Protection to RECK Protein

In order to examine the effects of profibrogenic mediators on RECK expression, serum-starved PSCs-5d were treated with FGF, TGF-β1, ethanol, or acetaldehyde for 24 h and analyzed by Western blotting. Only treatment with TGF-β1, a potent PSC activator [Kordes et al., 2005], induced the appearance of 110 kDa RECK band (Fig. 4A). At 6 h post-treatment with profibrogenic mediators, we also measured RECK mRNA levels by real-time PCR but found no significant changes (Fig. 4B). This finding suggests that the effects of TGF-β1 on RECK expression are downstream of transcriptional regulation and are consistent with our earlier finding of proteolytic processing (Fig. 3B). On the other hand, when myofibroblast-like PSCs-P2 were treated with profibrogenic mediators, no significant change in RECK expression was found (Fig. 4C, D).

Regulation of RECK Expression and Post-Transcriptional Processing by SMADs

To elucidate the molecular mechanism for the TGF-β effects on RECK, we carried out two different experiments. First, PSCs-P2 were transiently transfected with the expression vector for inhibitory Smads, Smad6 or Smad7 [Schiller et al., 2004], and analyzed by Western blotting. Unlike the case with Smad6 expression, Smad7 overexpression led to the loss of RECK protein expression (Fig. 5A). Second, we employed siRNA to suppress the expression of Smad2 or Smad3 in PSCs-P2 and measured the effects by Western blotting. As seen with Smad7 overexpression, lack of Smad3 expression resulted in a similar loss of RECK protein band (Fig. 5B). Thus, these findings suggest that

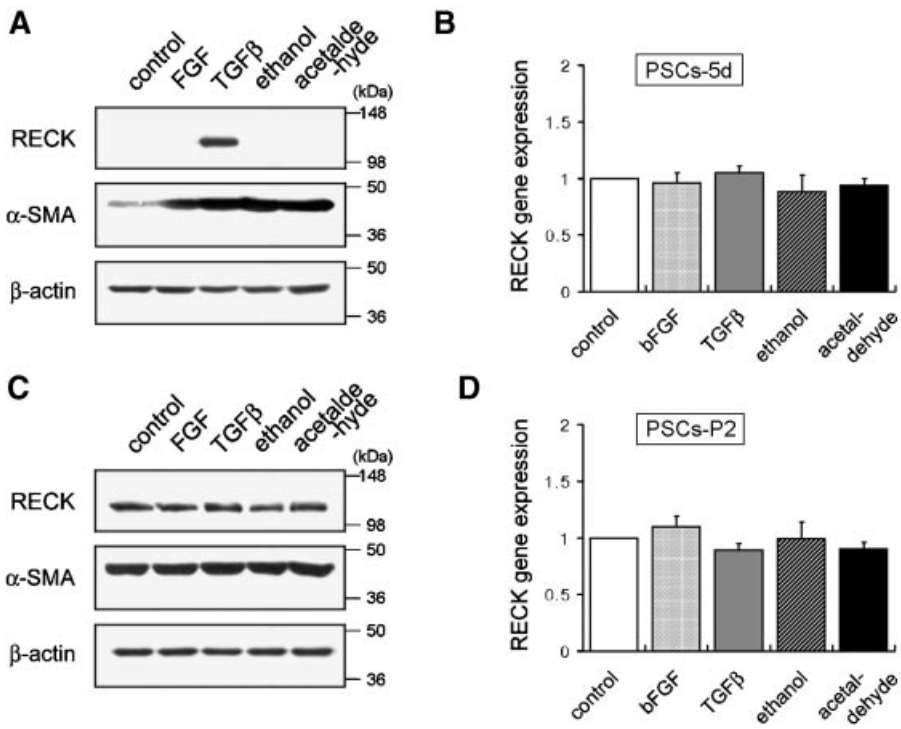

Fig. 4. Effects of profibrogenic mediators on RECK expression. Following serum starvation for 24 h, PSCs-5d were treated with b-FGF (20 ng/ml), TGF-β1 (5 ng/ml), ethanol (50 mM), or acetaldehyde (200 μM), and analyzed for RECK by Western blotting at 24 h post-treatment (**A**) and by real-time PCR at 6 h post-treatment (**B**). PSCs-P2 were treated same as above and analyzed by Western blotting (**C**) and real-time PCR (**D**). Data from real-time PCR are presented as the foldness of the baseline levels and represent means ± SD for three independent experiments. α-SMA was used as a marker for PSC activation.

TGF-β signaling may propagate via Smad3 pathway and protect RECK from proteolytic degradation.

Role of RECK as an ECM Protector in PSCs

We next sought to examine a functional role for RECK as a modulator of ECM remodeling in PSCs by several different experiments. First, PSCs-5d were plated on quenched fluorescent substrate DQ-type I collagen mixed with Matrigel, and fluorescent products resulting from cleavage of DQ-type I collagen were visualized at 48 h after plating (Fig. 6A). PSCs-5d exhibited strong collagenolytic activity and such activity was greatly reduced by TGF-β1 treatment. This TGF-β1 specific effects was abolished by co-incubation of RECK neutralizing antibody (Fig. 6B). Second, we measured collagenolytic activity of PSCs-P2 and found that addition of anti-RECK antibody promotes collagenolytic activity by ~2-fold (Fig. 6C). Third, PSCs-P1 which produce type I collagen were incubated in serum-free medium containing anti-RECK antibody, TGF-β1 or both for 36 h. Total soluble secreted collagen was measured in culture supernatants using Sircol collagen assay. Collagen levels were found decreased by ~30% with addition of RECK neutralizing antibody (Fig. 6D). TGFβ1 treatment led to an increase in collagen levels, but co-incubation of RECK neutralizing antibody reduced collagen levels to basal levels. These findings suggest that TGF-β1 induces expression of the functionally active RECK (110 kDa), which correlates with cell-associated collagenolytic activity, and that expression of RECK promotes the collagen deposition.

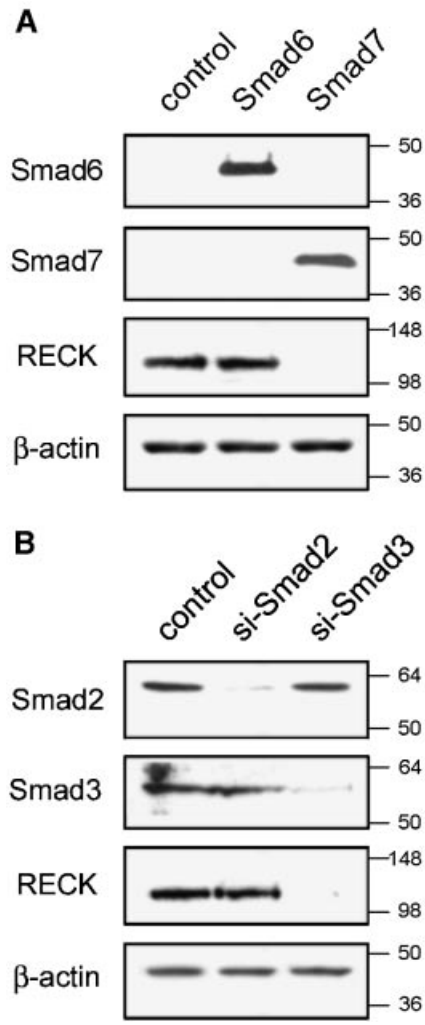

Fig. 5. Involvement of TGF-β signaling in the proteolytic protection of RECK. **A**: PSCs-P2 were transiently transfected with pEF-Flag Smad6 or pEF-Flag Smad7 and after 36 h analyzed by Western blotting. **B**: PSCs-P2 were transfected with si-Smad2 or si-Smad3, and after 72 h the effects of siRNAs were measured by Western blotting. These figures are representative of at least two independent experiments from separate cell preparations.

Lastly, in order to confirm that the pattern of RECK expression observed in cultured PSCs reflects expression by injured and fibrotic pancreas, cerulein-induced acute pancreatitis and normal rat controls underwent immunohistochemical staining for RECK and α-SMA. As compared with the normal controls, acute pancreatitis exhibited intense RECK expression within the areas of abnormal periacinar fibrosis, where α-SMA consistent with activated PSCs was clear localized (Fig. 6E).

DISCUSSION

Our data suggest that RECK is post-transciptionally processed in pre-activated PSCs and TGF-β prevents proteolytic degradation of RECK, preserving the full length 110 kDa species. Further study using DQ-collagen showed that the proteolytic processing of RECK abolishes its MMP inhibitory activity and inhibition of RECK by adding anti-RECK antibody restores collagenolytic activity in PSCs-P2. RECK gene expression was reportedly regulated by several oncogenes, including *ras*, at the transcriptional level [Takahashi et al., 1998], and it is the first demonstration of RECK protein degradation. This processing appears to involve aspartic proteinase(s) and inversely correlates with PSC activation and the fibrogenic phenotype.

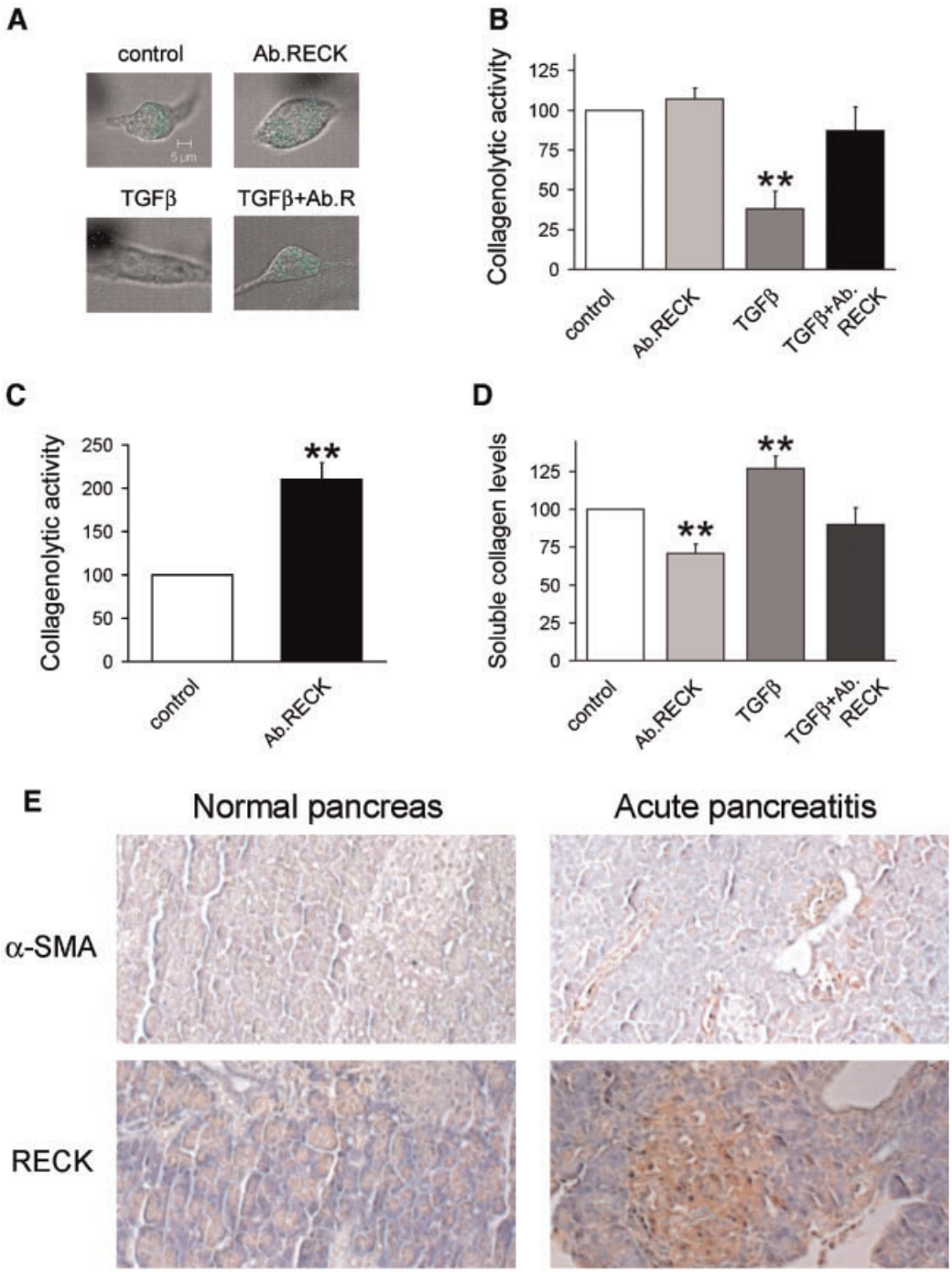

Fig. 6. Role of RECK as an ECM modulator in PSCs. **A**,**B**: PSCs-5d were plated on DQ-type I collagen mixed with Matrigel in the presence of indicated factors. After 24 h of incubation, fluorescence images of collagen degradation products (green) were taken at an extended depth of focus and superimposed on phase images of PSCs (A), and fluorescence intensities were quantified from confocal fluorescent images and expressed as the percent of the control levels (B). Each bar represents the standard deviation of triplicate assays. $^{**}P < 0.05$ compared with the untreated control. **C**: Collagenolytic activity of PSCs-P2 was measured in the presence or absence of anti-RECK antibody. **D**: PSCs-P1 were incubated in serum-free medium containing TGF-β1 (5 ng/ml), anti-RECK antibody (5 μg/ml), or both for 36 h, and the amounts of secreted type I collagen in culture supernatants were measured. **E**: Representative sections of normal rat pancreas and cerulean-induced acute pancreatitis were immunostained for RECK and α-SMA, respectively. (E) Original magnifications, ×100.

Following the receptor activation, TGF-β signals are transduced intracellularly by Smad proteins [Schiller et al., 2004]. Smad family is divided into three functional groups: receptor-associated Smads, including Smad2 and Smad3, co-Smads, such as Smad4, and inhibitory Smads, including Smad7. We showed that suppression of Smad3 expression, as well as overexpression of Smad7 expression, in PSCs-P2 leads to the disappearance of 110 kDa RECK band (Fig. 5b), suggesting that TGF-β signaling responsible for the proteolytic protection of RECK propagates via Smad3 pathway. Smad3 has been reported as a mediator of TGF-β-induced fibrotic response [Schnabl et al., 2001; Flanders, 2004; Uemura et al., 2005]. It remains to be clarified how TGF-β/Smad3 signaling protects 110 kDa RECK from proteolytic degradation. To examine whether the observed, TGF-β-mediated regulation of RECK expression can be seen in other fibroblast cell lines, we examined the effects of TGF-β1 treatment and Smad3 or Smad7 overexpression in NIH3T3 cells but found no significant changes in RECK protein levels (data not shown).

Alteration in the balance between ECM protein synthesis and degradation can result in pathological increases in ECM deposition (e.g., fibrosis), but the detailed molecular mechanism responsible remained unclear. Culture rat PSCs become activated when exposed to profibrogenic mediators, but these mediators failed to alter the expression levels of MMPs to TIMPs (Fig. 1), the balance of which is thought to regulate ECM turnover and/or accumulation (fibrosis). Our results showed that as PSCs become activated, RECK processing is reduced, resulting in expression of the full length 110 kDa species with known protease inhibitory activity. Together with our recent report showing that increased production of RECK facilitates deposition of matrix proteins following non-ablative laser therapy [Oh et al., 2007], these findings certainly support the role of RECK as a key ECM modulator and help to understand the mechanism of pancreatic fibrogenesis. As hepatic stellate cells (HSCs) display similar cellular behavior as PCAs [Benyon and Iredale, 2000], RECK expression in HSCs also needs to be examined.

Previous report demonstrated that Ras and TGF-β exert antagonistic effects on ECM gene expression [Verrecchia and Mauviel, 2002; Wisdom et al., 2005]. It is interesting to note that the activity of RECK, a key ECM modulator, is down-regulated by *ras* oncogene at the transcriptional level [Takahashi et al., 1998] but up-regulated via TGF-β/Smad3 mediated proteolytic protection. Considering the role of RECK as a tumor and metastasis suppressor, it would be worthwhile to examine the possibility that RECK activity may be modulated by proteolytic degradation, as well as by transcriptional suppression, during carcinogenesis.

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