THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 14, pp. 9426–9432, April 3, 2009

RGC-32 Mediates Transforming Growth Factor-β-induced Epithelial-Mesenchymal Transition in Human Renal Proximal Tubular Cells*[S]

Received for publication, January 5, 2009, and in revised form, January 21, 2009 Published, JBC Papers in Press, January 21, 2009, DOI 10.1074/jbc.M900039200

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Epithelial-mesenchymal transition (EMT) occurs in several disease states, including renal fibrosis and carcinogenesis. Myofibroblasts produced from EMT of renal tubular cells are responsible for the deposition of extracellular matrix components in a large portion of renal interstitial fibrosis. Transforming growth factor-β (TGF-β) plays an essential role in the EMT of renal tubular cells, but the molecular mechanism governing this process remains largely unknown. In this study, we found that RGC-32 (response gene to complement 32) is critical for TGF-β-induced EMT of human renal proximal tubular cells (HPTCs). RGC-32 is not normally expressed in the HPTCs. However, TGF-β stimulation markedly activates RGC-32 while inducing an EMT, as shown by the induction of smooth muscle α-actin (α-SMA) and extracellular matrix proteins collagen I and fibronectin, as well as the reduction of epithelial marker E-cadherin. TGF-β function is mediated by several signaling pathways, but RGC-32 expression in HPTCs appears to be mainly regulated by Smad. Functionally, RGC-32 appears to mediate TGF-β-induced EMT of HPTCs. Blockage of RGC-32 using short hairpin interfering RNA significantly inhibits TGF-β induction of myofibroblast marker gene α-SMA while repressing the expression of E-cadherin. In contrast, overexpression of RGC-32 induces α-SMA expression while restoring E-cadherin. RGC-32 also inhibits the expression of another adherens junction protein, N-cadherin, suggesting that RGC-32 alone induces the phenotypic conversion of renal epithelial cells to myofibroblasts. Additional studies show that RGC-32 stimulates the production of extracellular matrix components fibronectin and collagen I. Mechanistically, RGC-32 induces EMT via the activation of other transcription factors such as Snail and Slug. RGC-32 knockdown inhibits the expression of Snail and Slug during TGF-β-induced EMT. Taken together, our data demonstrate for the first time that RGC-32 plays a critical role in TGF-β-induced EMT of renal tubular cells.

Epithelial-mesenchymal transition (EMT),[2] the conversion from an epithelial to a mesenchymal phenotype, is a normal process during embryonic development such as mesoderm and neural tube formation. EMT is also a process in several disease states, including carcinogenesis and renal fibrosis. Renal tubulointerstitial fibrosis is the final inevitable common consequence of an excessive accumulation and deposition of extracellular matrix (ECM) components in the tubulointerstitium that occurs in virtually every type of chronic kidney disease; the degree of renal tubulointerstitial fibrosis correlates closely with the decline in renal function of progressive chronic kidney disease (1–3). Regardless of the initial causes, the striking feature of tubulointerstitial fibrosis is the activation of smooth muscle α-actin (α-SMA)-positive myofibroblasts. It is thought that these cells are the central effectors responsible for ECM deposition in the pathogenesis of renal disease (3–5).

Although the exact origins of myofibroblasts remain largely unclear and controversial, accumulating evidence has demonstrated that the major sources for the new myofibroblasts in advanced fibrosis originate from renal tubular epithelial cells via EMT (3, 6, 7). These myofibroblasts are morphological intermediates between fibroblasts and smooth muscle cells characterized by loss of epithelial function and the cell marker E-cadherin or N-cadherin and gain of the ability to produce ECM components such as collagen I, collagen III, and fibronectin, as well as smooth muscle cell phenotype expressing α-SMA (8, 9).

Transforming growth factor-β (TGF-β) and its downstream signaling molecules have been shown to play an essential role in EMT. Both *in vitro* and *in vivo* studies have demonstrated that TGF-β, by itself, can initiate and complete the entire EMT

* This work was supported, in whole or in part, by National Institutes of Health Grants HL093429 (to S.-Y. C.) and DK39308 and HL23081 (to P. A. J.). This work was also supported by American Heart Association Beginning Grant-in-aid 0765265U (to S.-Y. C.), Chinese National Nature Science Foundation Grant 30871177 (to W. -Y. H.), and a Veterans Administration Merit Award (to H. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: EMT, epithelial-mesenchymal transition; MAPK, mitogen-activated protein kinase; HPTC, human renal proximal tubular cell; α-SMA, smooth muscle α-actin; ECM, extracellular matrix; RT-PCR, reverse transcription-PCR; ERK, extracellular signal-regulated kinase; shRNA, short hairpin RNA; PI3K, phosphatidylinositol 3-kinase; TGF-β, transforming growth factor-β.

process (10, 11). The TGF-β signal is transduced by its transmembrane serine/threonine kinase receptors type I and type II. Binding of TGF-β to receptor type II leads to the recruitment and phosphorylation of receptor type I, which further activates its downstream signaling mediators, Smad2 and Smad3. Phosphorylated Smad2/3 then binds to the common partner Smad4 and is subsequently translocated into the nucleus, where it controls the transcription of TGF-β-responsive genes (12–14). Overexpression of inhibitory Smad7 abolishes Smad2 phosphorylation and tubular cell phenotypic conversion (15, 16). Smad3 knock-out mice are protected from unilateral ureter obstruction-induced tubulointerstitial fibrosis, as shown by reduced EMT and collagen deposition (17). Although much progress has been made to demonstrate the importance of TGF-β and its Smad mediators in EMT, the downstream effectors of Smad signaling that mediate EMT remain largely unknown.

RGC-32 is found in many adult human tissues, including heart, brain, liver, skeletal muscle, placenta, kidney, and pancreas (18). It is overexpressed in colon cancer and many tumors (19). RGC-32 plays a role in cell cycle activation. It is a substrate and regulator of cyclin-dependent kinase $p34^{cdc2}$ (18, 20). Our previous studies have shown that RGC-32 is important in TGF-β-induced smooth muscle cell differentiation from neural crest cells (21). In the present study, we found that RGC-32, acting downstream of Smad, plays an important role in mediating TGF-β-induced EMT of HPTCs. RGC-32 appears to induce EMT by activating other regulators.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HPTCs were cultured as described (22). Briefly, normal HPTCs were grown in Dulbecco's modified Eagle's/F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum, 5 ng/ml selenium, 5 mg/ml insulin, 5 mg/ml transferrin, 36 ng/ml hydrocortisone, 4 pg/ml triiodothyronine, and 10 ng/ml epidermal growth factor at 37 °C in a 95% O_2 and 5% CO_2 incubator. NRK-52E cells (rat kidney epithelial cell line) (23) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 4 mM L-glutamine. TGF-β1 was obtained from R&D Systems (Minneapolis, MN). α-SMA, α-tubulin, collagen type I monoclonal antibodies, and fibronectin polyclonal antibody were purchased from Sigma. E-cadherin monoclonal antibody was from BD Biosciences.

Preparation of RGC-32 Antibody—RGC-32 antigen (peptide sequence vtprkaklgdtkeled) was synthesized, and polyclonal antibody was produced by Proteintech Group, Inc. (Chicago, IL). The antibody was purified by immunoaffinity chromatography using RGC-32 peptides. Antibody specificity was confirmed by examining the expression of T7-tagged RGC-32 cDNA.

RGC-32 Expression and Short Hairpin Interfering RNA (shRNA) Constructs—The RGC-32 expression plasmid was described previously (21). For the construction of RGC-32 shRNA plasmids, double-stranded DNA oligonucleotides for RGC-32 and scrambled (control) shRNA were designed using siRNA Target Designer (Promega). The RGC-32 shRNA sequence is CGGCCATTCTTGGTTCACTATTCAAGAGATAGTGAACCAAGAATGGCCCT; the scrambled shRNA sequence is CGCCTCTCTCTTAGTGAGATTTCAAGAGAATCTCACTAAGAGAGAGGCCT. shRNA DNA templates were inserted into pGeneClip™ vectors using GeneClip™ U1 hairpin cloning systems (Promega) following the manufacturer's recommendations. The inserts were verified by sequencing.

Transient Transfection—HPTCs or NRK-52E cells were plated at 3×10^5/well in 6-well plates and incubated at 37 °C in a CO_2 incubator until they reached 80% confluency. Cells were then transiently transfected in triplicate with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. 4.0 μg of plasmid DNA and 10 μl of Lipofectamine 2000 were diluted separately in Opti-MEM I medium and incubated for 5 min. They were then combined and incubated for 30 min at room temperature. 24 h after transfection, cells were starved in serum-free Dulbecco's modified Eagle's/F-12 medium for 6 h, followed by treatment with 5 ng/ml TGF-β1 or vehicle for the indicated times.

Reverse Transcription-PCR (RT-PCR)—Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). PCR was performed as described previously (24). mRNA expression of the genes of interest was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase or cyclophilin. The primers used in PCR are listed in supplemental Table 1.

Western Blot Analysis—HPTC protein extraction and Western blotting were performed as described using α-SMA, collagen I, fibronectin, E-cadherin, RGC-32, or α-tubulin antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody (25). The blots were visualized by enhanced chemiluminescence (Pierce) and analyzed using a Fuji imaging system.

Immunofluorescent Staining—NRK-52E epithelial cells were fixed in 1% paraformaldehyde in phosphate-buffered saline at room temperature for 1 min, followed by methanol (−20 °C) for 10 min. Cells were then blocked with 3% bovine serum albumin and incubated with RGC-32 and E-cadherin antibodies. Fluorescent dye-conjugated anti-mouse and anti-rabbit secondary antibodies were used for detecting E-cadherin and RGC-32 staining, respectively. Antibody localizations were visualized by fluorescence microscopy.

Statistical Analyses—Data are expressed as mean ± S.E. All experiments were repeated independently three or four times. Analysis of variance was used to assess the differences among multiple groups. A t test was used to assess the differences between pairwise groups. $p < 0.05$ was considered a statistically significant difference.

RESULTS

TGF-β is known to induce EMT from renal tubular cell lines isolated from human, rat, mouse, and pig (26–29). RGC-32 has been shown to be a downstream target of TGF-β. To determine whether RGC-32 plays a role in TGF-β-induced EMT of renal tubular cells, we first developed an EMT cell model by using TGF-β to treat an HPTC line isolated from human kidney (22). HPTCs were treated with TGF-β for 0, 0.5, 2, 8, or 24 h to induce EMT. The expression of EMT-related genes such as

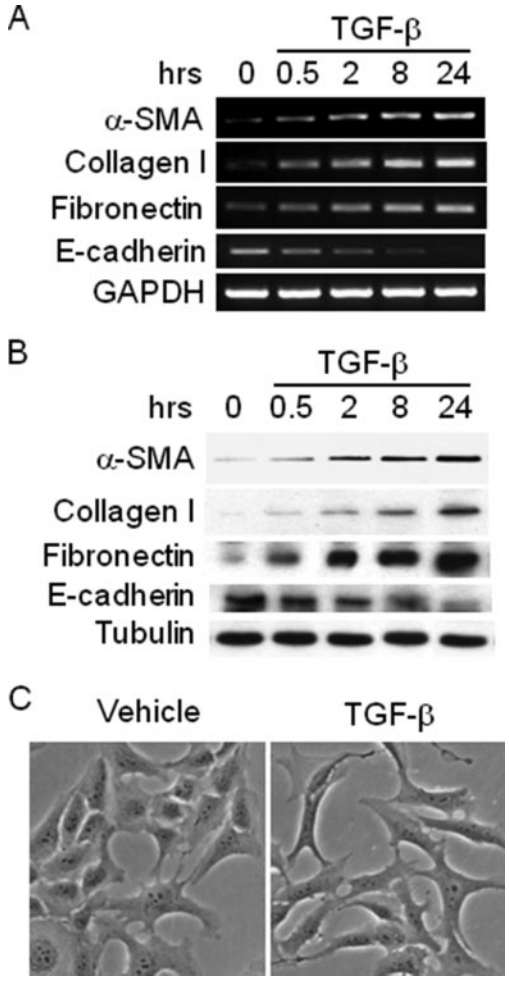

FIGURE 1. **TGF-β induces EMT of HPTCs.** HPTCs were cultured and treated with 5 ng/ml TGF-β1 for the times indicated. mRNA and protein expression of α-SMA, collagen I, fibronectin, and E-cadherin were examined by RT-PCR and Western blotting, respectively. *A*, mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is an internal control. *B*, protein expression. Tubulin is an internal control. *C*, morphology alteration. TGF-β induced the typical cobblestone shape of cells to become elongated and spindle-like in appearance.

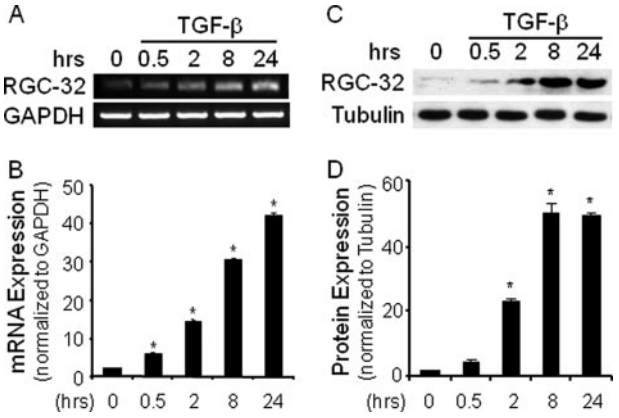

FIGURE 2. **TGF-β induces activation of RGC-32 in the EMT of HPTCs.** HPTCs were cultured and treated with 5 ng/ml TGF-β1 for the times indicated. RGC-32 mRNA expression was quantified by RT-PCR (*A*) and normalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (*B*). RGC-32 protein expression was quantified by Western blotting (*C*) and normalized by tubulin (*D*). *, $p < 0.01$ compared with vehicle-treated groups (0 h). TGF-β induced RGC-32 expression in a time-dependent manner.

α-SMA and ECM proteins collagen I and fibronectin was examined by RT-PCR and Western blotting. As shown in Fig. 1, TGF-β treatment markedly increased both mRNA and protein expression of α-SMA and ECM components in a time-dependent manner. TGF-β also blocked the expression of the epithelial marker E-cadherin (Fig. 1), suggesting that HPTCs were converted to myofibroblasts by TGF-β.

TGF-β induces RGC-32 expression in neural crest cells and mesenchymal C3H10T1/2 fibroblast cells, which results in smooth muscle cell differentiation (21). However, RGC-32 expression in renal cells and its importance in renal diseases have not been studied. In view of the important role of TGF-β in renal tubular EMT and RGC-32 being a TGF-β downstream target, we sought to determine whether or not RGC-32 plays a role in TGF-β-induced EMT of human renal tubular cells. We first examined whether RGC-32 is expressed in HPTCs and whether RGC-32 expression is regulated by TGF-β. As shown in Fig. 2 (*A* and *C*), RGC-32 is normally expressed at a very low level in HPTCs. After 2 h of TGF-β stimulation, both mRNA and protein expression were significantly increased. After 24 h of treatment, both RGC-32 mRNA and protein expression levels were robustly increased 20- and 39-fold, respectively, compared with vehicle treatment (Fig. 2, *B* and *D*). These data suggest that TGF-β is a strong inducer of RGC-32 in HPTCs.

Smad proteins are the major intermediates for TGF-β function. Other signaling pathways also mediate TGF-β function, including RhoA, p38 MAPK, p44/42 MAPK, and PI3K (30, 31). To determine how RGC-32 is regulated in HPTCs, we used specific inhibitors to block individual signaling pathways and tested whether these pathways are important for RGC-32 activation. As shown in Fig. 3*A*, inhibitors of PI3K (wortmannin), Rho kinase (Y27632), p44/42 MAPK (U0126), and p38 MAPK (SB203580) did not block RGC-32 expression, suggesting that these signaling pathways are not involved in RGC-32 induction. To test whether Smad proteins are important for RGC-32 activation, we used a dominant-negative Smad4 (ΔSmad4) in which the Smad4 activation domain in the middle linker region is deleted. This deletion abolishes the function of Smad signaling (32). We found that ΔSmad4 completely inhibited RGC-32 induction (Fig. 3, *B* and *C*), suggesting that RGC-32 is regulated by Smad signaling.

To test whether RGC-32 is involved in TGF-β-induced EMT of HPTCs, we first determined whether RGC-32 is essential for myofibroblast marker α-SMA expression. We used shRNA to block RGC-32 expression and then determined whether or not TGF-β is still able to induce α-SMA in the absence of RGC-32. As shown in Fig. 4*A*, RGC-32 shRNA significantly blocked RGC-32 expression. RGC-32 knockdown resulted in a significant reduction of α-SMA. To test whether RGC-32 alone induces myofibroblast transdifferentiation, we transfected empty vector or RGC-32 cDNA into HPTCs and tested whether RGC-32 induces the myofibroblast marker gene. As shown in Fig. 4*B*, RGC-32 overexpression induced a strong expression of α-SMA, suggesting that RGC-32 mimics TGF-β function in the EMT of HPTCs.

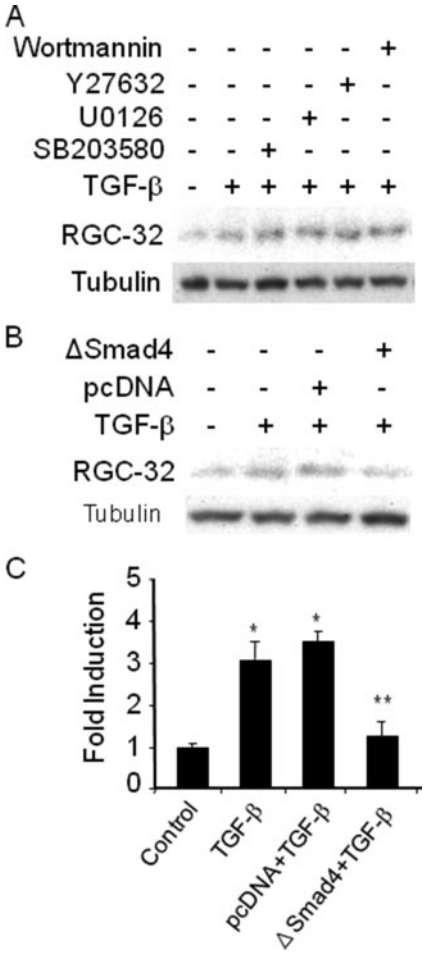

FIGURE 3. **RGC-32 expression is mediated by Smad signaling.** *A*, RGC-32 expression is not regulated by the p38, ERK MAPK, Rho kinase, or PI3K signal pathway. HPTCs were treated with pathway-specific inhibitors for PI3K (wortmannin, 10 nM), Rho kinase (Y27632, 10 μM), ERK (U0126, 10 μM), and p38 MAPK (SB203580, 10 μM), followed by vehicle or TGF-β induction for 24 h as indicated. RGC-32 expression was examined by Western blotting and normalized by tubulin. Pathway-specific inhibitors did not alter RGC-32 expression induced by TGF-β. *B*, dominant-negative Smad4 (ΔSmad4) inhibits RGC-32 induction. HPTCs were transfected with empty vector (pcDNA3) or ΔSmad4 cDNA, followed by TGF-β1 (5 ng/ml) treatment for 24 h. RGC-32 expression was examined by Western blotting. *C*, quantification of RGC-32 expression normalized by tubulin. *, $p < 0.01$ compared with vehicle-treated group (control); **, $p < 0.01$ compared with TGF-β-treated or pcDNA-transfected group ($n = 3$). ΔSmad4 significantly inhibited TGF-β-induced RGC-32 expression.

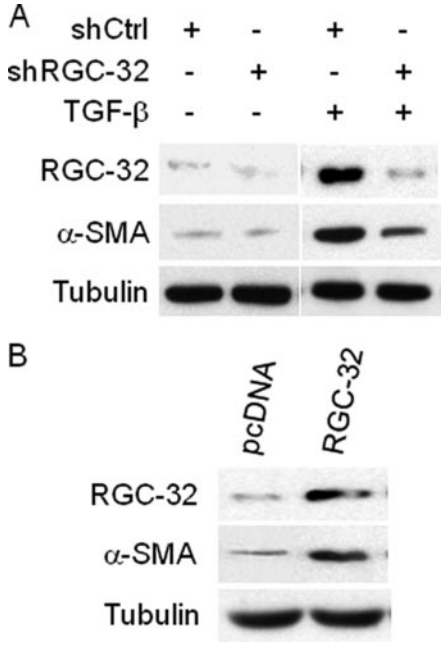

FIGURE 4. **RGC-32 plays a role in myofibroblast transdifferentiation from HPTCs.** *A*, RGC-32 is essential for TGF-β-induced EMT of HPTCs. HPTCs were transfected with RGC-32 (*shRGC-32*) or scrambled (*shCtrl*) shRNA for 24 h, followed by treatment with 5 ng/ml TGF-β1 or vehicle for additional 24 h. The knockdown efficiency of RGC-32 shRNA was determined by examining RGC-32 protein expression. The expression of the myofibroblast marker α-SMA was examined by Western blotting. Knockdown of RGC-32 significantly blocked α-SMA expression. *B*, RGC-32 induces α-SMA expression. HPTCs were transiently transfected with empty vector (pcDNA) or RGC-32 cDNA for 24 h. The expression of α-SMA was determined by Western blotting. RGC-32 alone can induce α-SMA expression.

During the EMT, renal tubular cells lose their epithelial phenotype and acquire new features characteristic of mesenchyme. A hallmark of this process is the loss of epithelial markers. To confirm RGC-32 function in renal tubular EMT, we determined whether RGC-32 regulates the expression of the epithelial marker E-cadherin. TGF-β blocked E-cadherin expression in HPTCs (Figs. 1 and 5*A*). However, RGC-32 knockdown restored its expression (Fig. 5*A*), indicating that RGC-32 is critical for phenotypic conversion. To test whether RGC-32 alone affects E-cadherin expression, we overexpressed RGC-32 in HPTCs and found that RGC-32 inhibited E-cadherin expression in the absence of TGF-β (Fig. 5*B*). Additional studies in rat kidney epithelial cells using immunocytochemistry showed that RGC-32 expression disrupted E-cadherin localization at the plasma membrane (Fig. 5*C*). RGC-32 also altered the distribution of F-actin and significantly increased stress fiber formation. Phase-contrast microscopy revealed that the cells expressing RGC-32 underwent a morphologic change, from the cobblestone-like epithelial cell morphology to an elongated mesenchymal morphology (Fig. 5*C*). These data demonstrate that RGC-32 plays a critical role in renal tubular EMT.

Although the loss of E-cadherin expression is a hallmark of tubular EMT, N-cadherin appears to be the predominant classic cadherin in human and rat proximal tubules *in vivo* (33, 34). HPTCs used in this study express N-cadherin. TGF-β blocked N-cadherin expression (Fig. 6*A*). To determine whether RGC-32 is involved in N-cadherin expression during EMT, we manipulated RGC-32 expression by transfecting RGC-32 shRNA or cDNA into HPTCs. We found that RGC-32 knockdown by shRNA enhanced N-cadherin expression (Fig. 6*B*), whereas RGC-32 overexpression inhibited N-cadherin expression (Fig. 6*C*). These data indicate that RGC-32 mediates the loss of the epithelial phenotype of HPTCs by blocking the expression of both adherens junction proteins E-cadherin and N-cadherin.

EMT is not only characterized by the repression of epithelial proteins but by the emergence of the EMT proteome as well. The latter includes the expression of ECM components. To determine whether RGC-32 mediates ECM protein production, both mRNA and protein expression of collagen I and fibronectin were examined in cells where RGC-32 was blocked or overexpressed. As shown in Fig. 7 (*A* and *B*), knockdown of RGC-32 blocked TGF-β-induced mRNA and protein expres-

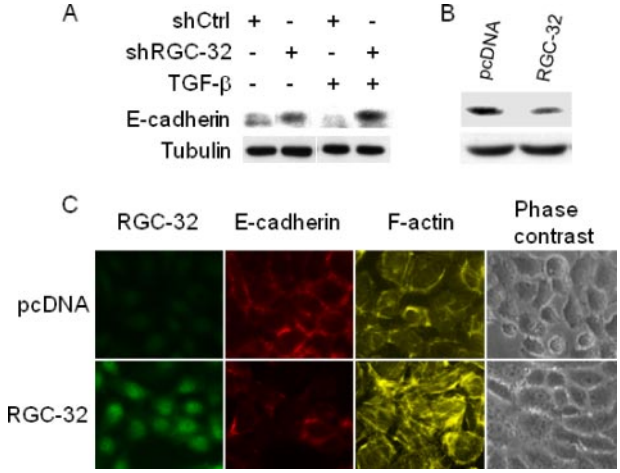

FIGURE 5. **RGC-32 inhibits epithelial marker E-cadherin during TGF-β-induced EMT of HPTCs.** *A*, knockdown of RGC-32 reverses TGF-β-induced phenotypic conversion. HPTCs were transfected with RGC-32 (*shRGC-32*) or scrambled (*shCtrl*) shRNA as indicated for 24 h, followed by treatment with 5 ng/ml TGF-β1 or vehicle for an additional 24 h. The expression of the epithelial marker E-cadherin was assessed by Western blotting. Knockdown of RGC-32 by shRNA restored the expression of E-cadherin that was blocked by TGF-β. *B*, RGC-32 inhibits epithelial marker expression. HPTCs were transiently transfected with empty vector (pcDNA) or RGC-32 cDNA for 24 h. E-cadherin expression was determined. RGC-32 inhibited E-cadherin expression. *C*, RGC-32 disrupts E-cadherin and F-actin localization at the cell-cell junctions. NRK-52E cells were transfected with empty vector (pcDNA) or RGC-32 cDNA for 24 h as indicated. Immunostaining was performed by using RGC-32 (*green*) or E-cadherin (*red*) antibody. F-actin was stained with phalloidin (*yellow*). Phase-contrast images (magnification ×200) were captured to analyze cell morphology. RGC-32 overexpression diminished the membrane staining of E-cadherin, promoted F-actin stress fiber formation, and altered cell morphology.

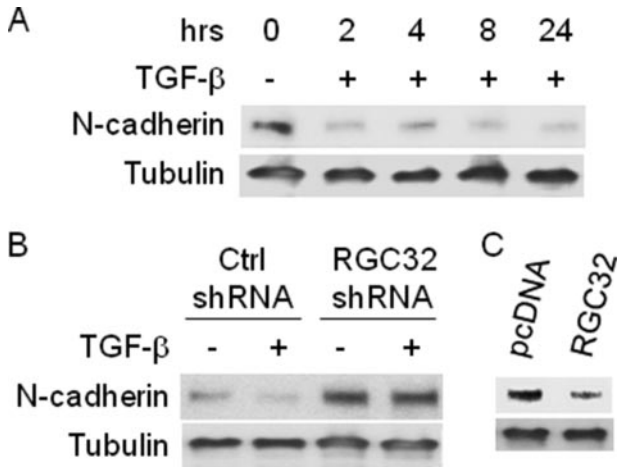

FIGURE 6. **RGC-32 inhibits N-cadherin expression in HPTCs.** *A*, TGF-β blocks N-cadherin expression in HPTCs. HPTCs were treated with vehicle (*0*) or TGF-β for the times indicated. N-cadherin expression was determined by Western blotting. *B*, knockdown of RGC-32 increases N-cadherin expression. HPTCs were transfected with RGC-32 or scrambled shRNA (control (*Ctrl*)) for 24 h, followed by treatment with 5 ng/ml TGF-β1 or vehicle for an additional 24 h. N-cadherin expression was examined by Western blotting. Block of RGC-32 significantly increased N-cadherin expression. *C*, RGC-32 overexpression inhibits N-cadherin expression. HPTCs were transiently transfected with empty vector (pcDNA) or RGC-32 cDNA for 24 h. N-cadherin expression was determined. RGC-32 significantly inhibited N-cadherin expression.

sion of collagen I and fibronectin. RGC-32 overexpression stimulated their expression (Fig. 7, *C* and *D*). These data demonstrate that RGC-32 plays an important role in ECM protein production in the myofibroblasts converted from renal tubular cells.

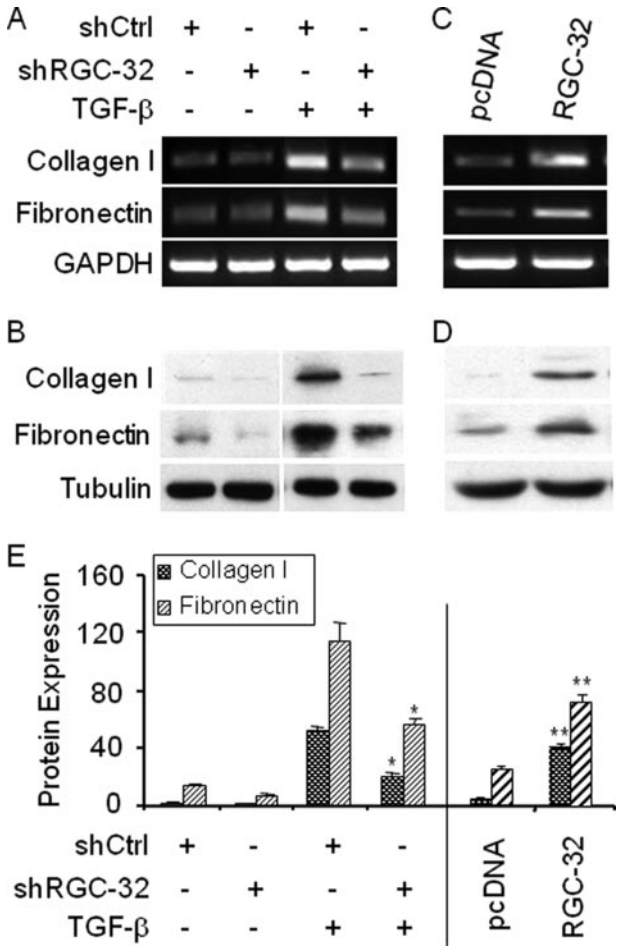

FIGURE 7. **RGC-32 mediates ECM protein production.** *A* and *B*, RGC-32 is essential for TGF-β-induced ECM production. HPTCs were transfected with RGC-32 (*shRGC-32*) or scrambled (*shCtrl*) shRNA for 24 h, followed by treatment with 5 ng/ml TGF-β1 or vehicle for an additional 24 h. The mRNA (*A*) and protein (*B*) expression of ECM collagen I and fibronectin were examined by RT-PCR and Western blotting, respectively. Knockdown of RGC-32 significantly inhibited both mRNA and protein expression of collagen I and fibronectin. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C* and *D*, RGC-32 promotes ECM protein production. HPTCs were transfected with empty vector (pcDNA) or RGC-32 cDNA for 24 h. Collagen I and fibronectin mRNA (*C*) and protein (*D*) expression were determined. RGC-32 stimulated ECM protein expression. *E*, quantification of ECM protein expression. Collagen I and fibronectin expression were normalized to tubulin. *, $p < 0.01$ compared with scrambled shRNA- and TGF-β-treated groups; **, $p < 0.01$ compared with pcDNA-transfected groups.

Fibronectin has been shown to be regulated by the Smad-independent pathway (35), although it was reported to require Smad signaling in the same MDA468 cells from a different study (32). To determine whether fibronectin is regulated by Smad or Smad-independent pathways in HPTCs during TGF-β-induced EMT, we used specific inhibitors to block PI3K, RhoA, p44/42 ERK, or p38 MAPK pathways while using Smad7 to block Smad signaling. Western blotting showed that blockade of RhoA, ERK, or p38 MAPK signaling completely inhibited TGF-β-induced fibronectin expression (Fig. 8*A*). However, the PI3K inhibitor wortmannin had no effect on expression (Fig. 8*A*), suggesting that the PI3K pathway is not involved. Smad7 also significantly inhibited fibronectin expression (Fig. 8*B*), indicating that Smad signaling is important for TGF-β-induced fibronectin production. These data demonstrate that fibronec-

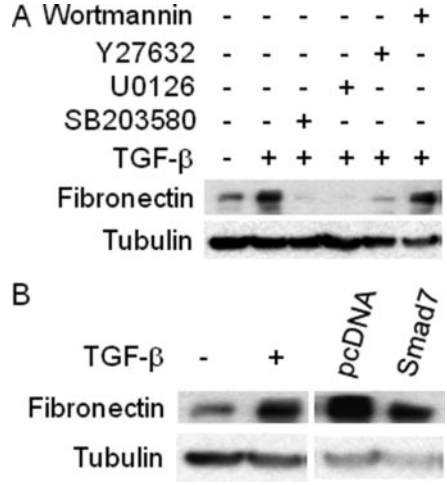

FIGURE 8. **Fibronectin is regulated by both Smad and Smad-independent pathways in HPTCs.** *A*, fibronectin is regulated by Smad-independent pathways. HPTCs were treated with pathway-specific inhibitors for PI3K (wortmannin, 10 nM), Rho kinase (Y27632, 10 μM), ERK (U0126, 10 μM), and p38 MAPK (SB203580, 10 μM), followed by vehicle or TGF-β induction for 24 h as indicated. Fibronectin expression was assessed by Western blotting. MAPK and Rho, but not PI3K, pathways appeared to be important for fibronectin expression. *B*, Smad regulates fibronectin expression. HPTCs were transfected with empty vector (pcDNA) or Smad7 cDNA for 24 h. Fibronectin expression was examined. Smad7 blocked fibronectin expression, indicating that Smad pathway regulates fibronectin expression.

tin is regulated by both Smad and Smad-independent pathways during TGF-β-induced EMT of HPTCs.

Previous studies have shown that transcription factors Snail, Slug, SIP1, ZEB1, and Twist1 play important roles in TGF-β-mediated EMT (36–42). We sought to determine whether RGC-32 function in renal tubular EMT is related to these factors. Twist is not expressed in HPTCs. SIP1 and ZEB1 are both expressed in these cells. However, TGF-β does not alter the expression of Twist, SIP1, or ZEB1 in HPTCs (data not shown). Snail and Slug were very weakly expressed in HPTCs. However, TGF-β treatment significantly up-regulated their expression (Fig. 9). To determine whether RGC-32 acts downstream or upstream of Snail or Slug in mediating TGF-β-induced EMT, we blocked RGC-32 expression using shRNA and determined Snail and Slug expression in HPTCs. We found that blockade of RGC-32 significantly inhibited TGF-β-induced Snail and Slug expression. These data indicate that RGC-32 acts as an upstream regulator of Snail and Slug in TGF-β-induced renal tubular EMT.

DISCUSSION

Although TGF-β/Smad signaling plays critical roles in renal tubular EMT, TGF-β downstream targets important for EMT remain largely unknown. RGC-32 appears to be one of the TGF-β downstream targets important for EMT of HPTCs. Several lines of evidence support this conclusion. First, TGF-β activates RGC-32 expression when inducing EMT (Fig. 2). Second, RGC-32 knockdown blocks TGF-β-induced expression of the myofibroblast marker α-SMA, whereas RGC-32 overexpression induces α-SMA in HPTCs (Fig. 4). Third, RGC-32 blocks the epithelial marker E-cadherin, a hallmark of EMT (Fig. 5). Finally, RGC-32 induces the production of the ECM proteins collagen I and fibronectin (Fig. 7), which are markers characteristic of a mesenchymal cell.

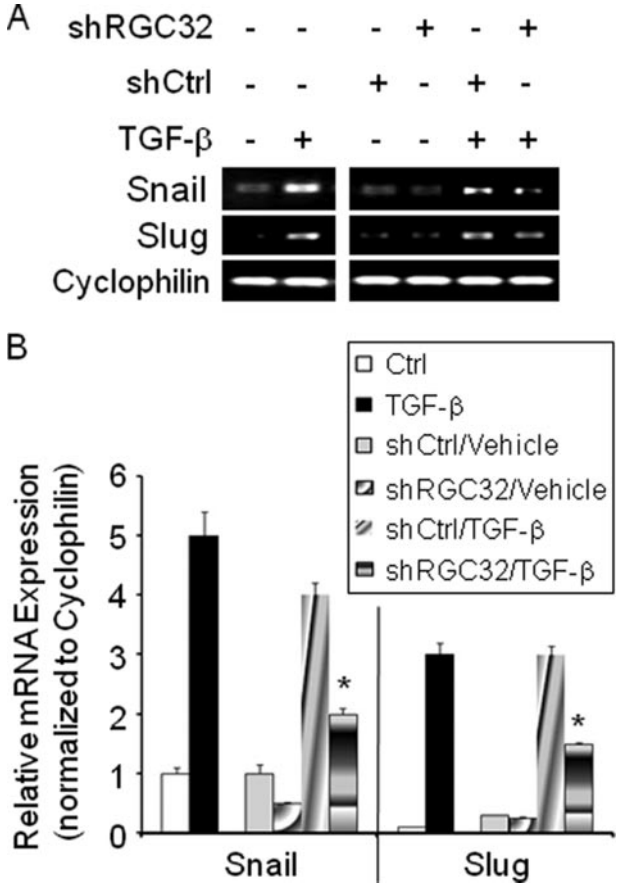

FIGURE 9. **RGC-32 regulates Snail and Slug expression in TGF-β-induced EMT of HPTCs.** *A*, mRNA expression of Snail and Slug. HPTCs were transfected with RGC-32 (*shRGC32*) or scrambled (*shCtrl*) shRNA as indicated for 24 h, followed by 5 ng/ml TGF-β1 or vehicle (*Ctrl*) treatment for an additional 24 h. Expression of Snail and Slug was assessed by RT-PCR. TGF-β induced the expression of Snail and Slug. However, knockdown of RGC-2, significantly inhibited Snail and Slug expression. *B*, quantification of Snail and Slug expression normalized to cyclophilin. The value for Snail in control cells was set as 1. *, $p < 0.05$ compared with scrambled shRNA- and vehicle-treated groups.

TGF-β function is mediated by several different signaling pathways. RGC-32 activation in HPTCs appears to be regulated by Smad proteins because RGC-32 expression is inhibited only when Smad signaling is blocked by mutant Smad4. The specific blockade of other signaling pathways does not affect RGC-32 activation. Previous studies show that RGC-32 is regulated by both Smad and RhoA in TGF-β-induced smooth muscle differentiation from neural crest cells, indicating that RGC-32 activation in normal development and pathological conditions is controlled by different mechanisms (21).

The mechanisms underlying renal tubular EMT appear to be different from the EMT in carcinogenesis. Loss of E-cadherin is a hallmark for both processes. However, in the EMT of cancer cells, loss of E-cadherin is accompanied by increased expression of N-cadherin. This E- to N-cadherin switch is correlated with the increase of invasion and metastasis of cancer cells (40, 43, 44). N-cadherin is expressed in HPTCs. However, in the EMT of HPTCs, instead of an increase, N-cadherin expression is blocked by TGF-β (Fig. 6*A*). Identification of the regulators responsible for the differential regulation of N-cadherin will help elucidate the different mechanisms governing renal tubu-

lar EMT and carcinogenesis. RGC-32 appears to play a role in the repression of N-cadherin (Fig. 6, *B* and *C*) in the EMT of HPTCs.

In the cancer cell line MDA468, TGF-β induces fibronectin expression via a Smad-independent pathway (35). In HPTCs, however, fibronectin is regulated by both Smad and Smad-independent pathways, including RhoA, p44/42 ERK, and p38 MAPK pathways. These data further indicate the different mechanisms of renal tubular EMT from that of EMT in carcinogenesis. Because RGC-32 expression in HPTCs is regulated only by Smad (Fig. 3), RhoA, p44/42 ERK, or p38 may regulate fibronectin expression through different factors.

Several transcription factors have been identified to mediate TGF-β-induced EMT, including Snail, Slug, ZEB1, SIP1, and Twist (36–42). Among them, Snail and Slug have been shown to be important for renal tubular EMT and fibrosis (45, 46). Interestingly, although Snail, Slug, ZEB1, and SIP1 are all present in HPTCs, only Snail and Slug are highly activated by TGF-β, consistent with their functional importance in the renal tubular EMT. Snail and Slug appear to be downstream targets of RGC-32 because knockdown of RGC-32 significantly inhibits Snail and Slug mRNA expression.

Taken together, our studies demonstrate that RGC-32, as a downstream target of TGF-β, activated by Smad signaling, is a novel activator of EMT in renal tubular cells. RGC-32 induces EMT through activating the transcription of other EMT-related genes.

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