Paricalcitol Attenuates Renal Interstitial Fibrosis in Obstructive Nephropathy

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Deficiency in vitamin D and its active metabolites is a pathologic feature of chronic kidney diseases. Despite that tubular epithelial cells are the major sites of active vitamin D synthesis, little is known about the role of vitamin D in maintaining the structural and functional integrity of tubular epithelium. This study investigated the effects of paricalcitol (19-nor-1,25-dihydroxy-vitamin D$_3$), a synthetic vitamin D analogue, on obstructive nephropathy, a model that is characterized by predominant tubulointerstitial lesions. Compared with vehicle controls, paricalcitol significantly attenuated renal interstitial fibrosis in mouse kidney after ureteral obstruction, as demonstrated by a reduced interstitial volume, decreased collagen deposition, and repressed mRNA expression of fibronectin and type I and type III collagens. Paricalcitol largely preserved E-cadherin and reduced α-smooth muscle actin expression in vivo. In addition, paricalcitol suppressed renal TGF-β1 and its type I receptor expression, restored vitamin D receptor abundance, and inhibited cell proliferation and apoptosis after obstructive injury. In vitro, paricalcitol abolished TGF-β1-mediated E-cadherin suppression and α-smooth muscle actin and fibronectin induction in tubular epithelial cells, underscoring its ability to block directly the epithelial to mesenchymal transition (EMT). It is interesting that paricalcitol almost completely suppressed renal induction of Snail, a critical transcription factor that is implicated in EMT programming. Furthermore, paricalcitol inhibited the TGF-β1-mediated Snail induction in vitro, and ectopic expression of Snail repressed E-cadherin promoter activity and downregulated E-cadherin expression in tubular epithelial cells. These studies suggest that paricalcitol is able to ameliorate renal interstitial fibrosis in obstructive nephropathy, possibly by preserving tubular epithelial integrity through suppression of EMT.


Vitamin D, specifically its most active metabolite 1,25-dihydroxyvitamin D$_3$, or calcitriol, plays an important role in a variety of biologic processes such as calcium homeostasis, hormone secretion, cell proliferation, and differentiation (1,2). Most, if not all, pleiotropic actions of vitamin D and its analogues are mediated by the specific vitamin D receptor (VDR), a ligand-dependent transcription factor that belongs to the steroid nuclear receptor gene family (3,4). Clinical studies demonstrate that deficiency of active vitamin D is a common feature in patients with chronic kidney disease (CKD) (5–8). This observation comes as little surprise, because renal proximal tubular epithelial cells, after all, are the major sites of calcitriol synthesis and uptake of its precursor in vivo. Therefore, dysfunction of tubular epithelial cells and/or loss of renal parenchyma as seen in diseased kidney consequently would cause a deficit in the production and supply of active vitamin D. The possibility also exists that vitamin D may be required for maintaining the structural and functional integrity of normal tubular epithelium. Along this line, deficiency of active vitamin D could be a causative factor that contributes to the progressive loss of renal function (6,9). In this context, supplementation of active vitamin D might be a rational strategy to break up the vicious cycle between vitamin D deficiency and CKD progression, thereby leading to a mitigation of renal pathologic lesions and dysfunction (10).

Several studies have shown that vitamin D analogues are renoprotective in experimental animal models of primary glomerular diseases (11). For instance, in rat remnant kidney model after five-sixths nephrectomy, calcitriol is able to reduce albuminuria, prevent podocyte injury, and attenuate glomerulosclerosis (12–14). Likewise, a beneficial effect of active vitamin D is observed in the rat anti–Thy-1 glomerulonephritis model, in which administration of vitamin D analogue prevents albuminuria, matrix accumulation, inflammatory infiltration, and apoptosis (15,16). A recent clinical trial also indicated that paricalcitol, a synthetic vitamin D analogue, reduces proteinuria in patients with CKD (17). The therapeutic effectiveness of active vitamin D could be related to several unique properties of its action, including its anti-inflammatory and antiproliferative effects and its ability to inhibit renin gene expression (18–21). However, relatively little is known about the potential role of vitamin D in tubulointerstitial fibrosis, a lesion that is widely accepted as the common pathway of CKD that leads to end-stage renal failure.

In this study, we examined the effect of paricalcitol (19-nor-1,25-dihydroxyvitamin D$_3$), an active, nonhypercalcemic vitamin D analogue that retains similar, if not better, vitamin D
biologic activity but displays fewer adverse effects and a better tolerance (22,23), in a mouse model of tubulointerstitial fibrosis that was induced by unilateral ureteral obstruction (UO). Our results demonstrate that paricalcitol reduces renal interstitial fibrosis, inhibits the production and accumulation of interstitial matrix components, and suppresses TGF-β1 gene expression. It is interesting that paricalcitol preserves epithelial marker E-cadherin in vivo, directly blocks TGF-β1-mediated tubular epithelial to mesenchymal transition (EMT) in vitro, and represses a key EMT-regulatory gene, Snail. These findings suggest that the renal protective effect of paricalcitol on interstitial fibrosis is mediated, at least in part, by preserving tubular epithelial integrity through suppression of EMT.

Materials and Methods

Animals
Male CD-1 mice that weighed approximately 18 to 22 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). UUO was performed using an approved protocol by the Institutional Animal Care and Use Committee at the University of Pittsburgh, as described previously (24). Paricalcitol (provided by Abbott Laboratories, Abbott Park, IL) was administered by daily subcutaneous injection at the dosages of 0.1 and 0.3 μg/kg body wt, respectively. As a control, a group of UUO mice receive injections of the same volume of vehicle (ethanol). Groups of mice (n = 5) were killed at 7 d after UUO, and the kidneys were removed for various analyses.

Determination of Serum Parathyroid Hormone and Calcium Levels and Intrarenal Calcium Deposition
Mouse serum intact parathyroid hormone (PTH) was determined using a commercially available ELISA kit, according to the protocols specified by the manufacturer (Alpco Diagnostics, Windham, NH). Serum ionized calcium concentration was measured in the Diagnostic Center for Population and Animal Health, Michigan State University (Lansing, MI). Von Kossa’s silver staining was used for detecting tissue calcium deposition. Briefly, formalin-fixed and paraffin-embedded sections were treated with saturated solution of lithium carbonate (Sigma, St. Louis, MO) for 20 min; the slides then were placed in 5% silver nitrate under direct bright light for 30 min, followed by incubation with sodium thiosulfate (Sigma) for 5 min. The slides were counterstained with nuclear fast red. Nondecalcificated mouse bone tissue sections were used as positive control.

Cell Culture and Cytokine Treatment
Human proximal tubular epithelial cells (HKC, clone-8) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cell culture and cytokine treatments were carried out according to the procedures described previously (24). After an overnight serum starvation, HKC were incubated with various concentrations of paricalcitol in the absence or presence of TGF-β1 (2 ng/ml) for 2 d, unless otherwise indicated. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Western Blot Analysis
The preparation of whole-cell lysates and kidney tissue homogenates and Western blot analysis of protein expression were performed according to the procedures described previously (24). The primary antibodies were obtained from the following sources: Anti-TGF-β type I receptor (anti–TβR-I; sc-398), anti-proliferating cell nuclear antigen (anti–PCNA; sc-7907), and anti-vitamin D receptor (anti–VDR; sc-1008; Santa Cruz Biotechnology, Santa Cruz, CA); anti-fibronectin (clone 10) and anti-E-cadherin (clone 36; BD Biosciences Pharmingen, San Jose, CA); anti-α-smooth muscle actin (anti–α-SMA; clone1A4) and anti–α-tubulin (Sigma).

Immunofluorescence and Immunohistochemical Staining
Indirect immunofluorescence staining was carried out using an established procedure (25,26). Cells or kidney cryosections were incubated with the specific primary antibodies described in the previous section, except anti-laminin (T40269R; Biodsign, Saco, ME), followed by staining with secondary antibodies. Some slides were stained for the proximal tubular marker, a fluorescein-conjugated lectin from Tetrarongolobus purpureus (Sigma). Slides also were double stained with 4’,6-diamidino-2-phenylindole, HCl to visualize the nuclei. For immunohistochemical staining of kidney sections, paraffin-embedded slides were stained with anti–TGF-β1, anti–TβR-I, and anti–PCNA antibodies using the Vector M.O.M. immunodetection kit by the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA). Slides were viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY). In each experimental setting, images were captured with identical light exposure times.

Picrosirius Red Staining
For evaluation of the collagen deposition, 3-μm sections of paraffin-embedded tissue were stained with picrosirius red. Sections were deparaffinized by baking at 55°C for 1 h, hydrated, and stained with picrosirius red solution (0.1% Sirius red in saturated picric acid) for 18 h, followed by treatment with 0.01 N HCl for 2 min, dehydration, and coverslip mounting. Sections were examined by Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera.

Table 1. Body and kidney weights and PTH and serum ionized calcium levels among different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (g)</th>
<th>Kidney Weight (g)</th>
<th>PTH (pg/ml)</th>
<th>Serum Calcium (mmol/L)</th>
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<tbody>
<tr>
<td>Sham control</td>
<td>27.09 ± 1.64</td>
<td>0.21 ± 0.08</td>
<td>37.76 ± 10.28</td>
<td>1.37 ± 0.03</td>
</tr>
<tr>
<td>UUO + vehicle</td>
<td>26.27 ± 0.65</td>
<td>0.23 ± 0.03</td>
<td>42.56 ± 8.97</td>
<td>1.25 ± 0.04</td>
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<tr>
<td>UUO + paricalcitol (0.1 μg)</td>
<td>22.76 ± 0.73</td>
<td>0.23 ± 0.04</td>
<td>46.14 ± 14.81</td>
<td>1.22 ± 0.11</td>
</tr>
<tr>
<td>UUO + paricalcitol (0.3 μg)</td>
<td>21.49 ± 0.35</td>
<td>0.22 ± 0.03</td>
<td>31.89 ± 14.14</td>
<td>1.19 ± 0.07</td>
</tr>
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aData are means ± SEM (four to five animals per group). PTH, parathyroid hormone; UUO, unilateral ureteral obstruction.

bSerum ionized calcium concentration was determined.

Paricalcitol was given at 0.1 and 0.3 μg/kg per d, respectively.

*P* < 0.05 versus vehicle control.
Morphometric Analysis of Interstitial Volume and Tubular Damage

For assessment of tubular injury and interstitial volume, computer-aided morphometric analysis was performed in sections that were stained with periodic acid-Schiff and anti-laminin antibody. Briefly, a grid that contained 117 (13 \times 9) sampling points was superimposed on images of cortical high-power field (\times 400). The number of grid points overlying atrophic or necrotic tubular cells (index of tubular cell damage) and interstitial space (interstitial volume index) was counted and expressed as a percentage of all sampling points, as described elsewhere (27). For each kidney, 10 randomly selected, nonoverlapping fields were analyzed in a blinded manner.

Quantitative Determination of Collagen and Total Protein

For quantitative measurement of collagen and total protein, 4-\mu m sections of paraffin-embedded tissue were stained with Sirius red F3BA and Picrosirius red for assessing collagen deposition (middle). Kidney sections from different groups also were double stained with anti-fibronectin antibody (red) and proximal tubular marker, lectin from T. purpureus (green; bottom). Representative micrographs from different groups as indicated were shown. Bar = 25 \mu m. (B and C) Graphic presentations showed renal interstitial volume (B) and tubular damage index (C) in different groups as indicated. (D) Paricalcitol reduced tissue total collagen deposition in the obstructed kidney. Total collagen was measured and expressed as the ratio of collagen per total protein (\mu g/mg). \*P < 0.01 versus sham controls; †P < 0.05 versus vehicle.
and Fast green FCF (Sigma) for collagen and noncollagen protein content. After eluting the dye from tissue sections with sodium hydroxide-methanol, the absorbance of 540 and 605 nm were determined for Sirius red F3BA and Fast green FCF binding protein, respectively. This assay provided a simple, relative measurement of the ratio of collagen/total protein (28,29). The relative amount of collagen in the samples was calculated and expressed as micrograms per milligram of total protein.

Reverse Transcriptase–PCR
A semiquantitative reverse transcriptase–PCR (RT-PCR) was used to determine the steady-state mRNA levels of fibronectin and type I and type III collagens. Briefly, after reverse transcription of renal total RNA, cDNA was used as a template in PCR reactions using gene-specific primer pairs. Generally, 20 to 25 cycles for amplification in the linear range were used. Quantity of PCR products was carried out by using densitometry, and the relative mRNA levels were calculated and compared after normalizing to β-actin. Primers for RT-PCR were synthesized and purchased from Invitrogen (Carlsbad, CA). The sequences were as follows: Snail (human), sense 5'-CAC TAT GCC CCG CTC TTT C-3' and antisense 5'-GTT GAG TCA AGC CAG ACA CA-3'; Snail (mouse), sense 5'-AGC CCA ACT ATA CCG AGC TG-3' and antisense 5'-CCA GGA GAG AGT CCC AGA TG-3'; collagen I, sense 5'-ATC TCC TGG TCG TGA TGG AC-3' and antisense 5'-ACC TTG TTT GCC AGG TTC AC-3'; fibronectin, sense 5'-CGA CCT GAG AGA GAAG CCC AC-3' and antisense 5'-CTG GAG TCA AGC CAG ACA CA-3'; collagen III, sense 5'-AGG CAA CAG TGG TTC TCC TG-3' and antisense 5'-GAC CTC GTG CTC CAG TTA GC-3'; β-actin (human), sense 5'-TCA AGA TCA TTT CTC CTC CTC AGC-3' and antisense 5'-TGC TGT CAC CTT CAC GTG CTC AGT-3'; and β-actin (mouse), sense 5'-CAG CTG AGA GGG AAA TCG TG-3' and antisense 5'-CGT TGC CAA TAG TGA TGA CC-3'.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling
Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was performed by using a colorimetric TUNEL System (Promega, Madison, WI) (30). Briefly, kidney sections were fixed in 4% paraformaldehyde for 15 min and permeabilized with 20 μg/ml proteinase K for 15 min. After washing, slides were incubated with biotinylated nucleotide incorporated at the 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase solution for 1 h at 37°C. Apoptotic cells were counted under light microscope and expressed as apoptotic cells per high-power field.

Transfection and Reporter Gene Assay
HKC cells were transfected with Snail expression vector (pHA-snail; provided by Dr. A. Garcia de Herreros, Universitat Pompeu Fabra, Barcelona, Spain) (31) using Lipofectamine 2000 (Invitrogen). The empty vector pcDNA3 (Invitrogen) was used as a mock-transfection control. A fixed amount (0.1 μg) of internal control reporter Renilla reniformis luciferase driven under thymidine kinase promoter (Promega) was co-transfected for normalizing the transfection efficiency. Luciferase assay was performed using the Dual Luciferase Assay System kit (Promega). Relative luciferase activity (arbitrary unit) was reported after normalizing for transfection efficiency.

Statistical Analyses
Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Kuels test. P < 0.05 was considered significant.

Results
Paricalcitol Reduces Interstitial Fibrosis and Inhibits Matrix Gene Expression
Table 1 shows the general characteristics of mice in different groups. Injections of paricalcitol substantially reduced mouse body weights but preserved the kidney weights after UUO. However, neither UUO nor paricalcitol treatment significantly changed the serum concentrations of PTH and calcium (Table 1). This probably is due to the nature of UUO model, in which one kidney is intact and seems sufficient for supporting renal function. Consistently, no calcium deposition was observed in the obstructed kidney after paricalcitol treatment, as shown in Supplementary Figure 1.

To evaluate the potential role of paricalcitol in renal fibrosis, we first examined its effect on interstitial fibrotic lesions and gene expression.

Figure 2. Paricalcitol inhibits interstitial matrix components fibronectin and type I and type III collagen mRNA expression in the obstructed kidney. (A) Representative reverse transcriptase–PCR (RT-PCR) analysis of renal mRNA levels of fibronectin and type I and type III collagen in different treatment groups as indicated. Numbers (1, 2, and 3) denote each individual animal in a given group. Dosages of paricalcitol were 0.3 and 0.1 μg/kg body wt, respectively. (B) Graphic presentation of the mRNA levels of interstitial matrix components in different groups. Relative mRNA levels were calculated and expressed as fold induction over sham controls (value = 1.0) after normalizing with β-actin. Data are means ± SD of five animals per group (n = 5). *P < 0.05 versus sham controls; †P < 0.05 versus vehicle controls.
matrix accumulation. As shown in Figure 1A, compared with sham controls, UUO caused a dramatic expansion of interstitial volume, as outlined by staining for laminin, a major component of tubular basement membrane. This interstitial expansion was accompanied by a marked increase in collagen deposition, as shown by a collagen-specific Picrosirius red staining. Similarly, immunofluorescence staining revealed an increased fibronectin accumulation and deposition in the expanded interstitium in the obstructed kidney. However, treatment with paricalcitol substantially reduced the interstitial volume expansion and collagen and fibronectin deposition (Figure 1A). Morphometric analysis revealed that paricalcitol at the dosage of 0.3 \( \mu \)g/kg reduced renal interstitial space by 67% in the obstructed kidney at 7 d after UUO (Figure 1B). Paricalcitol also attenuated tubular injury in a dose-dependent manner (Figure 1C). Quantitative determination of tissue collagen abundance also displayed a suppressive effect of paricalcitol on collagen deposition (Figure 1D).

We further investigated the mRNA expression of several major interstitial matrix components in different groups by RT-PCR approach. As shown in Figure 2, marked induction of fibronectin and type I collagen mRNA expression in a dose-dependent manner (Figure 2). Together, it seems clear that paricalcitol is able to ameliorate renal fibrotic lesions and inhibits the mRNA expression of the interstitial collagens and fibronectin in obstructive nephropathy.

Paricalcitol Preserves E-Cadherin and Inhibits \( \alpha \)-SMA Expression

We next examined the expression of epithelial adhesion receptor E-cadherin and \( \alpha \)-SMA, the molecular hallmark of myofibroblasts. Consistent with a previous report (32), ureteral obstruction induced a suppression of E-cadherin and a dramatic induction of \( \alpha \)-SMA (Figure 3), a shift that is in agreement with tubular EMT. It is interesting that paricalcitol preserved E-cadherin expression and inhibited \( \alpha \)-SMA induction in the obstructed kidney (Figure 3, A and C). This suggests that paricalcitol may specifically target tubular EMT, a key event in the pathogenesis of renal interstitial fibrosis. The effects of paricalcitol also were dose dependent (Figure 3, B and D).

Paricalcitol Restores Vitamin D Receptor Expression in Obstructed Kidney

Given that all biologic activities of vitamin D analogues presumably are mediated by its receptor, we sought to examine the VDR expression in the evolution of tubulointerstitial fibrosis. As shown in Figure 4A, VDR protein was almost com-
completely lost in the obstructed kidney after UUO, as demonstrated by Western blot analysis. Quantitative determination revealed that VDR abundance was reduced by 95%, compared with the sham controls (Figure 4C). Remarkably, paricalcitol completely restored VDR abundance in the obstructed kidney (Figure 4C). This action of paricalcitol also was dose dependent; even at the dosage of 0.1 g/kg, paricalcitol substantially increased VDR expression in the obstructed kidney (Figure 4, B and D).

**Paricalcitol Inhibits TGF-β1 and Its Type I Receptor Expression**

TGF-β1 signaling is known to play a crucial role in renal fibrogenesis and mediates several key fibrotic processes, including EMT (32,33). We reasoned whether paricalcitol could influence TGF-β1 and its receptor expression in obstructive nephropathy. Western blot showed that TβR-I was markedly induced in the obstructed kidney (Figure 5A). Quantitative determination indicated a >15-fold induction of renal TβR-I in the obstructed kidney over the sham controls (Figure 5B). However, paricalcitol significantly inhibited TβR-I induction that was caused by ureteral obstruction (Figure 5, A and B). Similar results were obtained when the kidney tissues were immunostained with anti-TβR-I antibody (Figure 5C). In addition, TGF-β1 was markedly induced in the obstructed kidney, as reported previously (32); and paricalcitol abrogated TGF-β1 expression that was induced by ureteral obstruction (Figure 5C). Of note, both TGF-β1 and TβR-I were localized predominantly in the tubular epithelia (Figure 5C), suggesting that tubular epithelial cells are the primary targets of this potent profibrotic cytokine under pathologic conditions.

**Paricalcitol Suppresses Cell Proliferation and Apoptosis**

We also examined the effects of paricalcitol on cell proliferation and apoptosis in the obstructed kidney, in view of its well-known antiproliferative property. Increased cell proliferation was observed in the obstructed kidney, as shown by Western blot analysis and immunostaining for the PCNA (Figure 6, A and C). Paricalcitol at high dosage (0.3 μg/kg) significantly suppressed renal PCNA expression, whereas it had little effect at low dosage (Figure 6B). Meanwhile, paricalcitol also significantly inhibited cell apoptosis in the obstructed kidney, as demonstrated by TUNEL staining (Figure 6, D and E).

**Paricalcitol Inhibits TGF-β1–Mediated EMT In Vitro**

To provide a direct evidence that paricalcitol can target tubular EMT, we used an in vitro cell culture system, in which HKC cells are induced to undergo EMT by TGF-β1, as documented previously (24). HKC cells after treatment with TGF-β1 began to lose epithelial adhesion receptor E-cadherin and gained mesenchymal markers such as α-SMA and fibronectin (Figure 7, A and B). However, simultaneous treatment with paricalcitol inhibited the TGF-β1–mediated EMT in a dose-dependent manner. Western blot exhibited that paricalcitol restored E-cadherin expression, whereas it inhibited TGF-β1–mediated induction of α-SMA and fibronectin (Figure 7, A and B). Similarly, immunofluorescence staining revealed that paricalcitol abolished the TGF-β1–induced α-SMA and fibronectin expression and their assembly (Figure 7C). It therefore is clear that paricalcitol can target EMT directly, thereby leading to a preservation of tubular epithelial integrity.

**Paricalcitol Inhibits Snail Expression, and Ectopic Expression of Snail Represses E-Cadherin**

We further investigated the potential underlying mechanism of paricalcitol blockade of EMT by examining the EMT-regulatory gene, Snail, a zinc-finger transcription factor that represses E-cadherin, is considered as a master gene that plays a critical role in initiating EMT (31,34). Therefore, we tested the hypothesis that paricalcitol might modulate Snail expression. As

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Figure 4. Paricalcitol restores vitamin D receptor (VDR) expression in the obstructed kidney. (A and B) Western blot demonstrated the VDR protein levels in different groups. Whole-kidney lysates were immunoblotted with antibodies against VDR and α-tubulin, respectively. (C and D) Graphic presentation showed the relative VDR protein abundance in different treatment groups. Data are expressed as the ratio of VDR per α-tubulin. *P < 0.01 versus sham controls; †P < 0.01 versus vehicle.
shown in Figure 8, A and B, Snail mRNA was markedly induced in the obstructed kidney and reached to >20-fold over the sham controls. Paricalcitol almost completely inhibited renal Snail mRNA induction after UUO, even at low dosage (Figure 8, A and B). To confirm this finding, we studied the Snail regulation by paricalcitol in cultured HKC cells. Indeed, TGF-β1 induced Snail mRNA expression in HKC cells, and paricalcitol dramatically suppressed Snail induction by TGF-β1 (Figure 8, C and D). Therefore, it seems clear that paricalcitol potently inhibits Snail, a key EMT-regulatory gene in the obstructed kidney and in TGF-β1–stimulated tubular epithelial cells.

To seek the potential relevance of Snail induction to E-cadherin suppression as seen in the obstructed kidney, we investigated the direct effect of Snail on E-cadherin gene transcription and protein expression by co-transfection. As shown in Figure 8E, Snail could repress E-cadherin promoter activity in a dose-dependent manner, as revealed by a promoter-luciferase reporter assay. Similarly, transient transfection of Snail expression vector reduced E-cadherin protein in tubular epithelial cells (Figure 8F). Therefore, Snail induction after UUO may play a critical role in mediating the loss of tubular epithelial E-cadherin.

## Discussion

Vitamin D deficiency long has been recognized to occur early in CKD (5,7,8); however, the cause–effect relationship between vitamin D deficiency and progression of CKD remains ambiguous. In this study, we provide evidence to support the notion that vitamin D deficiency, per se, could be a causative factor that contributes to the progressive destruction of kidney parenchyma and loss of renal function, as supplementation of an active vitamin D analogue, paricalcitol, is renoprotective. We have shown that paricalcitol suppresses interstitial extracellular matrix expression and attenuates renal fibrosis in obstructive nephropathy, a model with predominant tubulointerstitial lesions that are characterized by tubular atrophy and EMT. Paricalcitol is able to target EMT process, as evidenced by preservation of epithelial E-cadherin and inhibition of mesenchymal markers such as α-SMA and fibronectin. Furthermore, paricalcitol potently inhibits Snail, a key EMT-regulatory gene that plays an important role in suppressing E-cadherin expression (31,34,35). These results suggest that endogenous vitamin D could be essential in maintaining the structural and functional integrity of normal tubular epithelium. Our data shed
new light on the mechanism by which active vitamin D elicits its protective actions in renal parenchyma.

One striking observation in this study is the dramatic down-regulation of VDR in the fibrotic kidney, which is completely restored by administration of paricalcitol. This result suggests that the deficiency in vitamin D signaling in the diseased kidney is much greater than originally thought. Because all biologic activities of vitamin D presumably are mediated by its specific receptor, loss of VDR would lead to an eradication of vitamin D signaling, even when no reduction in active vitamin D levels occurs. VDR is a ligand-dependent transcription factor (1,3). Upon activation by its ligand, VDR forms heterodimeric complex with its obligatory partner, retinoid X receptor, and binds to the so-called vitamin D response element, a specific nucleotide sequence that is located in the promoter region of its target genes, and then modulates their transactivation of transcription. Recent studies revealed that VDR also can mediate multiple cross-talks and integrate diverse signaling inputs by virtue of its ability to interact with other transcription regulators such as Smad3 and \( \beta \)-catenin (36–38). Therefore, loss of VDR in the fibrotic kidney would not only obliterate vitamin D signaling but also disrupt the homeostasis of other important signaling networks, leading to a disparaging consequence. Remarkably, restoration of VDR expression in the fibrotic kidney is achieved by administration of paricalcitol. Although exactly how paricalcitol upregulates VDR expression in obstructive nephropathy remains unclear, restoration of VDR would reinstate vitamin D signaling, thereby preserving normal vitamin D function.

Progressive interstitial fibrosis not only is the predominant pathologic feature of obstructive nephropathy but also is considered as a common final pathway of nearly all forms of CKD (39,40). Increasing evidence indicates that tubular epithelial cells, the major constituents of renal parenchyma, are not innocent bystanders but play a decisive role in the evolution of renal interstitial fibrosis (40). Tubular epithelial cells, upon stimulated by profibrotic cues such as TGF-\( \beta \), undergo EMT, a phenotypic conversion process that is characterized by loss of epithelial markers and gain of mesenchymal features (41,42). As a consequence of EMT, tubular cells become the matrix-
producing cells, resulting in overproduction of the interstitial matrix components and tissue scarring. In this context, the ability of paricalcitol to preserve E-cadherin and to suppress \( \alpha \)-SMA, as shown in this study, underscores that vitamin D may be essential for the maintenance of tubular epithelial integrity by targeting EMT. The inhibitory role of paricalcitol in tubular EMT is corroborated further by in vitro studies, in which paricalcitol is able to block directly the TGF-\( \beta \)-mediated epithelial dedifferentiation and mesenchymal transition. These findings also are in line with many studies demonstrating a prodifferentiation property of vitamin D in various experimental systems (2,37). Hence, blockade of EMT by paricalcitol could be an imperative mechanism that accounts for its renoprotective action in the pathogenesis of tubulointerstitial fibrosis.

Among numerous transcription factors that are involved in regulating EMT, the Snail zinc-finger protein is studied most extensively. Snail possesses DNA binding capacity and recognizes the E-box elements in the promoter region of its target genes, including E-cadherin and VDR (43). Binding of Snail to its cognate E-box often leads to the suppression of gene transcription (35,43). Earlier studies showed that induction of Snail is common in malignant carcinomas, and Snail represses epithelial E-cadherin expression (31,34). Snail expression is induced in the fibrotic kidney after ureteral obstruction, and knockout of Smad3 abrogates Snail induction, suggesting that its induction in vivo is dependent on TGF-\( \beta \)/Smad signaling (44). In this study, we found that paricalcitol almost completely abolishes Snail expression in the obstructed kidney in vivo and in cultured tubular epithelial cells induced by TGF-\( \beta \)-1, and ectopic expression of Snail represses E-cadherin promoter and its protein expression (Figure 8). Although the underlying mechanism of Snail inhibition remains to be elucidated, the finding that paricalcitol is able to target a key EMT-regulatory gene, Snail, highlights a fundamental role of vitamin D in controlling EMT, which may vindicate its renoprotection.

Active vitamin D may elicit its renoprotective activities by multiple mechanisms. One relevant finding in our study is that paricalcitol inhibits both TGF-\( \beta \)-1 and its type I receptor expression in the obstructed kidney. Because TGF-\( \beta \)/Smad signaling plays a central role in many fibrogenic processes such as EMT (32,33), downregulation of TGF-\( \beta \)-1 and its receptor by paricalcitol would diminish a major fibrogenic signaling in vivo, leading to an amelioration of fibrotic lesions. However, it remains to be determined whether TGF-\( \beta \)-1 inhibition is a cause or consequence of the reduced fibrosis by paricalcitol in this model.
Another interesting observation is that paricalcitol may exert its beneficial effects by inhibiting cell proliferation and apoptosis in the obstructed kidney (Figure 6), which is consistent with its antiproliferative property (18). Finally, we previously demonstrated that vitamin D is able to block myofibroblast activation from quiescent fibroblast and inhibit matrix production by myofibroblasts (45), and this could be an additional underlying mechanism of the renal protection by paricalcitol. Undoubtedly, elucidating the relative contribution of each individual pathway of paricalcitol to its overall renal protection remains a challenging issue that merits further investigations.

It should be noted that the dosages of paricalcitol that were used in the UUO mice are substantially greater than that used in humans (17,23). This may explain partially the impressive efficacy of paricalcitol in reducing renal fibrosis in this study. Because vitamin D administration may cause several adverse consequences, such as hypercalcemia, hyperphosphatemia, and oversuppression of PTH, development of novel vitamin D an-

Figure 8. Paricalcitol inhibits Snail expression in vivo and in vitro, and ectopic expression of Snail represses E-cadherin promoter and expression. (A and B) Representative RT-PCR analysis of renal mRNA levels of Snail in different treatment groups as indicated. Numbers (1, 2, and 3) denote each individual animal in a given group. Relative mRNA levels were calculated and expressed as fold induction over sham controls (value = 1.0) after normalizing with β-actin (B). Data are means ± SEM of 5 animals per group. *P < 0.01 versus sham controls; †P < 0.01 versus vehicle. (C and D) Paricalcitol inhibited TGF-β1–mediated Snail induction in vitro. HKC cells were treated with paricalcitol in the absence or presence of TGF-β1 for 3 h. Representative RT-PCR results (C) and quantitative determination of Snail mRNA levels (D) are presented. Data are means ± SEM of three experiments. *P < 0.01 versus sham controls; †P < 0.01 versus vehicle. (E) Ectopic expression of Snail repressed E-cadherin promoter activity. HKC cells were transiently transfected with E-cadherin promoter-luciferase reporter and an increasing amount of Snail as indicated. Relative luciferase activity was reported after normalization with transfection efficiency. (F) Expression of Snail suppressed E-cadherin expression in tubular epithelial cells. HKC cells were transiently transfected with pHA-Snail. Cell lysates were immunoblotted with antibodies against E-cadherin, Snail, and α-tubulin, respectively. HKC cells that were treated with TGF-β1 were used as a positive control.
alogs with less adverse effects and a better tolerance is of critical importance. In this aspect, paricalcitol, a synthetic, non-hypercalcemic vitamin D analogue, could have the potential to widen the therapeutic window of vitamin D in ameliorating fibrotic lesions in patients with CKD (22,23).

Conclusion
We have shown that paricalcitol, an active vitamin D analogue, inhibits renal expression and accumulation of the interstitial matrix components, suppresses TGF-β1 and its type I receptor, and restores vitamin D receptor abundance in obstructive nephropathy. Paricalcitol directly blocks TGF-β1–mediated EMT in vitro and represses a key EMT-regulatory gene, Snail, expression in vivo and in vitro, suggesting that the beneficial effect of paricalcitol likely is associated with its ability to preserve tubular epithelial phenotypes through suppression of EMT. Therefore, supplementation of exogenous active vitamin D could be a rational strategy for slowing or halting the progression of chronic kidney fibrotic diseases.

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