Loss of vitamin D receptor in chronic kidney disease: a potential mechanism linking inflammation to epithelial-to-mesenchymal transition

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1Department of Pathology, Nankai University, Medical School, Tianjin, China; 2Tianjin Key Laboratory of Modern Drug Delivery and High Efficiency, Tianjin University, Tianjin, China; and 3Division of Cellular and Molecular Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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Xiong M, Gong J, Liu Y, Xiang R, Tan X. Loss of vitamin D receptor in chronic kidney disease: a potential mechanism linking inflammation to epithelial-to-mesenchymal transition. Am J Physiol Renal Physiol 303: F1107–F1115, 2012. First published July 11, 2012; doi:10.1152/ajprenal.00151.2012.—Both peritubular inflammation and tubular epithelial-to-mesenchymal transition (EMT) are critical events during the pathogenesis of renal fibrosis. However, the relationship between these two processes is unclear. Here, we investigated the potential role of the vitamin D receptor (VDR) in coupling peritubular inflammation and EMT. In a mouse model of unilateral ureteral obstruction (UUO), loss of VDR was observed as early as 1 day after surgery. In cultured proximal tubular epithelial HK-2 cells, proinflammatory TNF-α inhibited the expression of VDR in a dose- and time-dependent manner. Treatment with TNF-α sensitized HK-2 cells to EMT stimulated by transforming growth factor (TGF)-β1. Ectopic expression of VDR counteracted the synergistic effect of TNF-α and TGF-β1 on EMT. Furthermore, knockdown of VDR using a small interfering RNA strategy mimicked the effect of TNF-α on facilitating EMT. Either TNF-α treatment or a loss of VDR induced β-catenin activation and its nuclear translocation. The VDR ligand calcitriol reversed the VDR loss and inhibited EMT in the mouse UUO model. Previous studies from our laboratory and others demonstrated that proinflammatory TNF-α, which renders tubular cells susceptible to EMT, contributes to the matrix-producing cell population, leading to an excessive production of interstitial matrix and disruption of the dynamic balance between matrix production and degradation (17). It has been reported that the pro-fibrogenic effect of inflammation depends, at least partially, on triggering EMT (27). Several studies have indicated that sustained stimulation of inflammatory cytokines TNF-α or IL-1 can induce EMT in epithelial cell lines (24, 28). Moreover, Kamitani et al. (11) reported that in bronchial epithelial cells, TNF-α enhances the effect of transforming growth factor (TGF-β1) on EMT induction. Although the relationship between inflammation and EMT is well established, the detailed mechanisms coupling inflammation with EMT, as well as the subsequent renal fibrosis, are poorly understood.

The vitamin D receptor (VDR) is a ligand-dependent transcription factor (15), which is activated upon binding to vitamin D (or vitamin D analogs) followed by the recruitment of cofactors such as the retinoid X receptor (RXR) (7). This process results in the formation of the VDR-RXR cofactor complex. Such a complex then binds to the vitamin D response element (VDRE) in the promoter region of its target genes, thereby regulating gene transcription (6, 20). Under normal physiological conditions, tubular cells express a significant amount of VDR. Although renal tubular cells of VDR-null mice are normal, Li and colleagues (33) reported that VDR deletion leads to more severe phenotype transition of tubular cells in the mouse unilateral ureteral obstruction (UUO) model. Previous studies from our laboratory and others demonstrate that vitamin D administration has therapeutic effects in obstructive nephropathy, which are brought about by preventing renal interstitial fibrosis (25, 33). The mechanism underlying these effects may be related to the preservation of renal tubular integrity by preventing EMT. It has also been reported that TNF-α, an important proinflammatory mediator, suppresses the expression of VDR in various cell types (18), which raises the question as to whether an intrinsic connection exists between renal inflammation, VDR suppression, EMT, and fibrosis.

In this study, we show that loss of VDR is an early event in an animal model of renal fibrosis and in human biopsies from patients with kidney diseases. The loss of VDR in renal tubular epithelium could be mediated by proinflammatory cytokines such as TNF-α. In addition, loss of VDR sensitizes tubular epithelial cells to TGF-β1-triggered EMT. These findings suggest that loss of VDR links inflammation to EMT, two of the major pathological events that occur during fibrosis.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice, weighing 18–22 g, were obtained from the Academy of Military Medical Sciences, Laboratory Animal Center (Peking, China). The mice were housed in the animal facilities...
of Nankai University, with free access to food and water and were treated in accordance with guidelines outlined by the Institutional Animal Use and Care Committee at Nankai University. The UUO experiments were performed according to established procedures (26). In brief, left ureters were exposed via a left lateral incision after the mice were anesthetized with pentobarbital sodium. Then, double ligation of the left ureter was performed with 3-0 silk. Groups of mice (n = 5) were euthanized at days 1, 3, 7, and 14 after UUO, respectively. For the 1,25-dihydroxyvitamin D$_3$ (Bio Basic, Markham, Ontario) administration experiments, vitamin D was administered from day 5 after UUO by daily intraperitoneal injection at a dose of 0.3 µg/kg body wt, respectively. For the control group, the UUO mice received injections of the same volume of vehicle (ethanol). The mice were euthanized 7 or 14 days after UUO, and the kidneys were removed for various analyses. One portion of the kidneys was fixed in 3.7% paraformaldehyde, followed by paraffin embedding for immunohistochemistry. The remaining kidneys were snap-frozen in liquid nitrogen and stored at −80°C until RNA and protein extractions.

**Western blot analysis.** Protein expression was detected by Western blotting, which was carried out as described previously (29). The primary antibodies consisted of anti-VDR (sc-1008), anti-α-smooth muscle actin (sc-71113), and anti-β-catenin (sc-1496, Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-SMA (clone 1A4, Sigma-Aldrich, St. Louis, MO), and anti-E-cadherin (3195, Cell Signaling Technology, Danvers, MA). Quantification was performed by using Image J software (National Institutes of Health, Bethesda, MD) to measuring the intensity of the signals.

**RNA isolation and real-time PCR.** Total RNA was extracted from kidney tissue using TRIzol reagent in accordance with the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Next, first-strand cDNA synthesis was carried out by using a Reverse Transcription System Kit in accordance with the manufacturer’s instructions (Promega, Madison, WI). Real-time PCR was performed with the CFX96 Real-Time System (Bio-Rad, Berkeley, CA). Briefly, the PCR reaction mixture consisted of a 20-µl volume containing 10 µl of 2× SYBR Green PCR super Mix (TransGen Biotech, Beijing, CN), 10 µl of diluted RT product (1:10), and 0.5 µM sense and antisense primer. The PCR reaction was run under standard conditions. Arrays of triplicates of amplifications at 95°C for 15 s, 60°C for 1 min, respectively, the amplification protocol consisted of 40 cycles of denaturing at 95°C for 15 s and annealing and extension at 60°C for 60 s. The mRNA expression of the respective genes was calculated after normalizing with GAPDH. The primer sequences are listed as follows: collagen I (sense) 5'-GGG CGA GTG CTG TCG TTG CTG-3' and (antisense) 5'-GGC ATA GGACAT CGG AGA AGC AA-3'; β-catenin (sense) 5'-TCA GAG GGT CGG ACC TGC CA-3' and (antisense) 5'-TGT CAG CTC AGG AAT TGC AC-3'; TNF-α (sense) 5'-CAA GGG ACA AGG CCT CCC GG-3' and (antisense) 5'-GGC GGC GGT CCT GAC GGC AG-3'; TGF-β1 (sense) 5'-TGC TCC CAC TCC CGG TTC TT-3' and (antisense) 5'-TTG GGG GAG TGG CGA GCC TT-3'; α-smooth muscle actin (SMA; sense) 5'-GGT GTC GAG TTG GCT GCC CG-3' and (antisense) 5'-GCG GCC GTG GCC ATC TCA TCA TT-3'; and GAPDH (sense) 5'-GCA CAG TCA AGG CCG AGA AT-3' and (antisense) 5'-GCG TTC TCC ATG GTG GTG AA-3'.

**Immunofluorescence and immunohistochemical staining.** Immunofluorescence staining was carried out using methods previously described (14, 30). The cultured cells were fixed in 3.7% solution of paraformaldehyde and then incubated with the β-catenin primary antibody. The slides also were double stained with 4'6-diamidino-2-phenylindole-HCl to visualize the nuclei. For immunohistochemical staining, the paraformaldehyde-fixed kidney sections from the UUO and sham-operated mice were paraffin embedded, sectioned (3 µm), and stained with the specific primary antibodies against VDR, β-catenin and β-catenin, as described above. In the negative control, normal rabbit IgG was used to replace the primary antibody. In these samples, no staining was observed. The slides were viewed on an Olympus X51 fluorescence microscope equipped with a digital camera (Shinjuku, Tokyo, Japan). In each experimental setting, images were captured with identical light exposure times.

**Cell culture, treatment, and transient transfection.** The human normal proximal tubular cell line HK-2 was purchased from ATCC (Manassas, VA). The cells were cultured in DMEM/F-12 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and were typically seeded at ~70% confluence in complete medium that contained 10% FBS for 24 h. After overnight serum starvation, the HK-2 cells were incubated with various concentrations of TNF-α and/or TGF-β1 and for 2 days with or without pretreatment with vitamin D (unless otherwise indicated). Recombinant human TGF-β1 and TNF-α were purchased from R&D Systems (Minneapolis, MN). For the VDR-overexpressing stable cell line, parental HK-2 cells were infected with the Lv-EF1α-VDR-IRE3-Bsd using a lentivirus delivery system (Bioseittia, San Diego, CA), followed by clonal selection using 10 g/ml Blasticidin to generate a polyclone of HK-2 cells with stable overexpression of VDR (HKC-VDR). The HK-2 cells transfected with mock lentivirus were subjected to identical clone selection procedures to generate the stable control cell line (HKC-Con). For VDR silencing, the HK-2 cells were transiently transfected with VDR small interfering (si) RNA (sc-106692, Santa Cruz Biotechnology) for 36 h and then treated with recombinant human TGF-β1 (R&D Systems). Control siRNA was also transfected as a negative control (sc-37007, Santa Cruz Biotechnology).

**Statistical analyses.** Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups were made using one-way ANOVA, followed by a t-test. P < 0.05 was considered to be significant.

**RESULTS**

**Loss of VDR occurs early in chronic kidney diseases.** To investigate changes in VDR expression during renal fibrosis, we measured VDR expression in a mouse model of renal fibrosis induced by UUO at different time points. As shown in Fig. 1A, VDR protein was down-regulated at very early stages following UUO. Compared with the sham controls, VDR protein abundance drops to nearly one-third at 1 day after ureteral obstruction. A further decrease in VDR was observed when the duration of ureteral obstruction was prolonged. By 14 days after obstruction, the level of VDR protein was almost undetectable (Fig. 1B). Immunohistochemical staining also showed the time-dependent loss of VDR in the tubular epithelium of the obstructed kidneys (Fig. 1C). At the same time, we detected the mRNA expression of TNF-α, TGF-β1, and the marker for fibrogenetic cells, α-SMA, at various periods of UUO. Real-time PCR data showed that up-regulation of TNF-α occurred earlier than increased expression of TGF-β1 and α-SMA (Fig. 1, D and E).

To study the clinical relevance of VDR regulation in renal fibrosis, we further investigated VDR expression in renal biopsy samples collected from patients with minimal change disease, diabetic nephropathy, and hypertensive nephropathy by immunohistochemical staining. As shown in Fig. 2, loss of VDR expression was clearly evident in renal tubular epithelia of the kidneys with diabetic and hypertensive nephropathy, while VDR abundance was relatively intact in the kidneys with minimal change disease. Of particular interest, loss of VDR was only seen in the focal area characterized by a significant infiltration of inflammatory cells, suggesting a close correlation between renal inflammation and loss of VDR.
TNF-α inhibits tubular VDR expression in vitro. The close relationship between peritubular inflammation and the loss of tubular VDRs in human CKD may imply a potential connection of these two events. To test this, we further investigated the possibility that proinflammatory cytokines regulate VDR expression in the obstructed kidneys. As shown in Fig. 3, A and B, treatment of human proximal tubular epithelial cells (HK-2) with the proinflammatory cytokine TNF-α resulted in a dose- and time-dependent suppression of VDR expression. Quantitative data on VDR abundance after various treatments are presented in Fig. 3, C and D. Therefore, it is conceivable that renal infiltrating cells, via cells secreting proinflammatory cytokines, are instrumental in mediating VDR suppression in tubular epithelium of the injured kidneys.

Fig. 1. Downregulation of vitamin D receptor (VDR) expression and upregulation of TNF-α occurred at an early stage after unilateral ureteral obstruction (UUO) in obstructed kidneys. A: whole tissue homogenates were prepared from sham and obstructed kidneys at days 1, 3, 7, and 14 after UUO, respectively. Each individual mouse in a given group is indicated by a number (1–3). Western blot analysis demonstrated the downregulation of VDR protein in obstructive nephropathy. B: graphic representation of the relative abundance of VDR protein levels in different groups as indicated after normalization with β-actin. Note that the downregulation of VDR expression was significant even from day 1 after UUO. C: immunohistochemical staining showing VDR expression in the different groups. Scale bar = 50 μm. D: semiquantitative PCR demonstrated the upregulation of TNF-α, transforming growth factor (TGF)-β1, and α-smooth muscle actin (SMA) mRNA expression in obstructive nephropathy. E: graphic representation of renal mRNA level of TNF-α, TGF-β1, and α-SMA in different groups. Relative mRNA levels were calculated and expressed as fold-induction over sham control after normalization with GAPDH. Note that the upregulation of TNF-α became significant at day 1 after UUO, while increased expression of TGF-β1 and α-SMA were not significant until day 3 after UUO. Values are means ± SE of 5 animals/group (n = 5). *P < 0.01 vs. sham control. **P < 0.05 vs. sham control.

Fig. 2. Downregulation of the VDR is associated with the severity of inflammatory infiltration. Renal biopsy samples from patients with minimal change nephropathy, diabetic nephropathy, and hypertensive tubular necrosis were evaluated using immunohistochemistry with an anti-VDR antibody. The results suggest that the loss of VDR expression is largely colocalized to areas characterized by the presence of a significant degree of inflammatory cell infiltration. Scale bar = 50 μm.
TNF-α sensitizes tubular cells to undergo EMT. EMT is a critical event leading to renal fibrosis. Thus we next investigated the role of TNF-α with respect to the onset of EMT. As shown in Fig. 4, TNF-α alone (5 ng/ml) was not able to induce EMT in HK-2 cells. However, pretreatment with 5 ng/ml TNF-α potentiated TGF-β1-induced fibronectin and α-SMA expression and suppression of E-cadherin in HK-2 cells (Fig. 4, A and B). Previous studies suggest that plasminogen activator inhibitor-1 (PAI-1) is an active participant in mediating EMT (32). Our results show that either 5 ng/ml TNF-α or relative low dosages of TGF-β1 had no effect on the induction of PAI-1. However, 0.2 and 0.5 ng/ml TGF-β1 significantly induced PAI-1 expression when HK-2 cells were pretreated with 5 ng/ml TNF-α (Fig. 4C). We also determined the effect of TGF-β1 and TNF-α on VDR expression. Western blotting shows that 5 ng/ml TNF-α significantly inhibits VDR expression in the presence or absence of TGF-β1.

Fig. 3. TNF-α inhibits VDR expression of human epithelial cells in a time- and dose-dependent manner. A and B: HK-2 cells were treated with TNF-α for 48 h at different concentrations (0, 1, 2, 5, 10 ng/ml) or treated with 5 ng/ml TNF-α for various periods of time as indicated. Whole cell lysates were immunoblotted with antibodies against VDR and β-actin, respectively. Numbers (1 and 2) indicate a time-independent experiment(s) in a given group. C and D: graphic representation of the relative abundance of VDR protein levels in the different groups after normalization with β-actin. Values are means ± SE of 3 time-independent experiments. *P < 0.05 vs. control group.

Fig. 4. Pretreatment with TNF-α potentiates the TGF-β1-mediated epithelial-to-mesenchymal transition (EMT) in HK-2 cells. HK-2 cells were treated with different doses of TGF-β1 (0.1, 0.2, or 0.5 ng/ml) for 48 h with or without pretreatment with 5 ng/ml TNF-α for 3 h. Whole cell lysates were immunoblotted with antibodies against α-SMA, fibronectin, E-cadherin, plasminogen activator inhibitor-1 (PAI-1), VDR, and β-actin, respectively. A: Western blot analysis showed that compared with the groups treated with TGF-β1 alone, pretreatment with 5 ng/ml TNF-α significantly induced fibronectin and α-SMA expression. B and C: simultaneously, pretreatment with 5 ng/ml TNF-α significantly inhibited E-cadherin and induced PAI-1 expression compared with the TGF-β1 only-treated groups. D: Western blotting showed that low-dose treatment with TGF-β1 had no obvious effect on VDR expression while pretreatment with TNF-α significantly inhibited VDR.
mcs VDR overexpression

PAI-1 expression as well as inhibiting E-cadherin expression.

TNF-α/H9251 or PLv-EF-vdr-Bsd plasmid. Thus we constructed a stable cell line which overexpressed VDR to explore whether VDR contributed to the synergistic effect of TNF-α and TGF-β1 on the EMT. A: the control cell lines and VDR-overexpressing cell line were treated with control medium, 0.2 ng/ml TGF-β1, 5 ng/ml TNF-α, or 0.2 ng/ml TGF-β1 plus pretreatment with 5 ng/ml TNF-α, respectively. Western blot analysis showed that compared with control cell lines transfected with the control virus, VDR expression was much higher in the VDR-overexpressing stable cell line, even in the groups treated with 5 ng/ml TNF-α. Moreover, VDR overexpression was resistant to the decrease in E-cadherin expression induced by TGF-β1 following transfection with VDR siRNA, compared with the control group, 0.2 or 0.5 ng/ml TGF-β1 significantly inhibited E-cadherin expression in the VDR siRNA group (Fig. 5, B–D). However, in the VDR-overexpressing cell lines, the induction of EMT by TNF-α plus TGF-β1 was effectively abrogated (Fig. 5, B–D).

Using an opposite strategy, we then knocked down VDR expression in HK-2 cells by transfecting with VDR siRNA. After transfection with either control siRNA or VDR-specific siRNA, HK-2 cells were then treated with 0.2 or 0.5 ng/ml TGF-β1 to determine whether knockdown of VDR can mimic the effect of TNF-α on potentiating EMT. Western blotting showed that compared with the control group, 0.2 or 0.5 ng/ml TGF-β1 significantly induced the expression of fibronectin, α-SMA, and PAI-1 in HK-2 cells deficient in VDR (Fig. 6, A–C).

Active vitamin D restores VDR expression and prevents EMT. As active vitamin D is the endogenous ligand for VDR, we...
then studied whether the administration of vitamin D could protect VDR and prevent EMT induced by TGF-β1 plus TNF-α. Western blotting showed that treatment with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or calcitriol, the endogenous, hormonally active form of vitamin D, had the ability to preserve VDR expression in a dose-dependent manner after TNF-α treatment (Fig. 7A). At the same time, incubation of HK-2 cells with calcitriol also blocked the induction of α-SMA as well as fibronectin and restored E-cadherin following TNF-α and TGF-β1 treatment (Fig. 7, B and C).

Late administration of active vitamin D restores VDR in vivo. To evaluate the regulation and action of VDR in renal fibrogenesis, we performed a new series of in vivo experiments, in which calcitriol was administered at day 5 after UUO, a time point when significant kidney injury and fibrosis are already established (4, 5). As shown in Fig. 8, A–C, Western blotting and immunostaining showed that administration of vitamin D at day 5 after UUO significantly restored VDR expression compared with the vehicle control group. We further evaluated the changes in EMT markers along with the restoration of VDR expression. Compared with vehicle control, expression of α-SMA in the calcitriol-treated group significantly decreased at day 14 after UUO while the expression of E-cadherin was preserved with the late administration of vitamin D (Fig. 8, D–F). Meanwhile, real-time PCR and immunostaining showed a significant suppression of collagen I and fibronectin at 14 days after UUO compared with vehicle controls (Fig. 8, G and H).

VDR exerts its antifibrotic action by blocking β-catenin signaling in HK-2 cells. Recent studies demonstrate that activation of Wnt/β-catenin signaling is implicated in the pathogenesis of EMT and renal fibrosis (9, 21). In a mouse model of UUO, a significant induction of β-catenin expression and its nuclear localization were evident at 14 days after UUO as shown by real-time RT-PCR and immunostaining. Interestingly, late administration of calcitriol significantly suppressed renal expression of β-catenin as well as its nuclear translocation (Fig. 9, A and B). Furthermore, we investigated the potential role of VDR in regulating β-catenin signaling in cultured HK-2 cells. As shown in Fig. 9C, treatment with TNF-α at 5 ng/ml induced β-catenin expression, but this effect could be abrogated by the ectopic expression of VDR. Similarly, knockdown of VDR by an siRNA strategy in HK-2 cells could mimic the effect of TNF-α on β-catenin activation, as a significant induction of β-catenin expression was observed in the group transfected with VDR siRNA (Fig. 9D). Immunostaining also demonstrated that loss of VDR not only induced β-catenin expression but also promoted its nuclear translocation (Fig. 9E).

DISCUSSION

It is well known that the severity of renal fibrosis in CKD patients often correlates closely with the level of inflammation (13). On one hand, inflammatory cells can produce a wide array of chemokines and cytokines (22), thereby creating a vicious feedback loop of inflammation. On the other hand, the production of profibrotic cytokines such as TGF-β1 by inflammatory cells induces matrix-producing myofibroblast activation, affects tubular epithelial cell behavior and differentiation status, and promotes EMT. Studies show that TGF-β receptors are upregulated predominantly in renal tubular epithelial cells, suggesting that they are the primary target of this profibrotic cytokine after tubulointerstitial injury. However, whether and how peritubular inflammation and EMT are connected remain largely unknown. Chen et al. (3) reported that 1,25-dihydroxyvitamin D₃ suppresses PAI-1 induced by TNF-α, which implies that vitamin D regulates the intrinsic interaction between inflammation and EMT. In this study, we found that both the loss of VDR and upregulation of TNF-α were early events that preceded EMT and fibrosis in obstructive nephropathy. The proinflammatory mediator TNF-α strongly inhibited VDR expression. Moreover, in biopsy samples obtained from patients with different renal diseases, the loss of VDR is often observed in the focal areas characterized by severe inflammatory infiltration. Together, these observations suggest that loss of VDR is an early event in renal fibrogenesis and plays a crucial role in linking peritubular inflammation to tubular EMT and renal fibrosis.

Tubular EMT is a phenotypic conversion that is increasingly recognized to be one of the key events in renal fibrogenesis (19). It is reported that TNF-α working in concert with TGF-β1 accelerates the EMT in cultured colonic organoids (1). Similarly, data from Takahashi et al. (24) reveal that in retinal pigment epithelial cells TNF-α induces an interaction between hyaluronan-CD44-moesin, implying that TNF-α cooperates with TGF-β2 to trigger EMT. Our present results demonstrate that TNF-α renders renal tubular cells susceptible to TGF-β1-

![Fig. 7. Vitamin D rescues VDR inhibited by TNF-α and abolishes the EMT induced by TGF-β1 coupled with NF-α in HK-2 cells. HK-2 cells were treated with 5 ng/ml TNF-α alone or were pretreated with 10⁻⁶, 10⁻⁵, 10⁻⁴, or 10⁻³ M active vitamin D plus 5 ng/ml of TNF-α. Whole cell lysates were immunoblotted with antibodies against VDR, α-SMA, and E-cadherin, respectively. A: Western blotting results showed that vitamin D restored the VDR expression inhibited by TNF-α alone in a dose-dependent manner. HK-2 cells were treated with 5 ng/ml TNF-α plus 0.2 ng/ml TGF-β1 or pretreated with 10⁻⁸, 10⁻⁷, or 10⁻⁶ M vitamin D, then treated with 5 ng/ml TNF-α plus 0.2 ng/ml TGF-β1. B and C: Western blotting results showed that vitamin D restored the inhibited E-cadherin expression and decreased the expression of α-SMA and fibronectin induced by TGF-β1 and TNF-α.](http://ajprenal.physiology.org/DownloadedFrom)
induced EMT. This finding is significant given that the concentration of TGF-β1 is progressively accumulated in the kidney and the sensitivity of tubular cells to the stimuli of TGF-β1 toward EMT largely determines the extent of renal fibrosis.

One novel finding in the present study is that loss of VDR mediates the effect of TNF-α on rendering tubular cells susceptible to EMT. Our data suggest that silencing VDR mimics the effect of TNF-α on facilitating the EMT driven by TGF-β1. Consistently, forced expression of VDR in tubular epithelial cells prevents the phenotype changes induced by TGF-β1 and TNF-α. This suggests that the loss of VDR couples peritubular inflammation and tubular EMT. Interestingly, overexpression and silencing of the VDR affect EMT in a ligand-independent manner. Ellison et al. (8) has reported the calcitriol-independent transactivation of the VDR in keratinocytes. It is not hard to speculate that the ligand-independent transactivation might also contribute to the effect of VDR on EMT. Another possi-

Fig. 8. Administration of vitamin D after UUO restored VDR expression and significantly alleviated the EMT as well as fibrotic injury. Male CD-1 mice were divided into 3 groups: 1) sham control (n = 5); 2) UUO vehicle control (n = 10); and 3) UUO mice receiving 0.3 μg/kg body wt vitamin D from day 5 after the operation (n = 5). The animals were euthanized at 7 or 14 days after the operation. A: Western blotting showed the expression of VDR in the different groups. B: graphic representation of the relative abundance of VDR protein levels in different groups as indicated after normalization with β-actin. Values are means ± SE (n = 5 mice/group). **P < 0.01 relative to the vehicle control group. C: results of immunohistochemistry using an antibody against VDR. D and E: whole tissue homogenates were immunoblotted with antibodies against α-SMA and E-cadherin. Compared with the vehicle control group, late administration of vitamin D significantly inhibits the expression of α-SMA induced by UUO and restores the expression of E-cadherin 14 days after the operation. F: graphic representation of the relative abundance of α-SMA and E-cadherin in different groups, as indicated after normalization with β-actin. Values are means ± SE (n = 5 mice/group). *P < 0.05 relative to the vehicle control group. G: graphic representation of the relative abundance of collagen I mRNA expression in the different groups. Values are means ± SE (n = 5 mice/group). **P < 0.01 relative to the vehicle control group. H: immunohistochemical staining showing the expression of fibronectin in different groups.
bility for the ability of the VDR to block EMT induction could be regulation of the receptors for TNF-α and TGF-β. Although no significant change in mRNA level of the receptors for TNF-α and TGF-β can be detected after administration with different dosages of calcitriol (data not shown), further evidence is still needed to figure out the exact mechanism underlying the non-ligand-dependent effect of VDR on EMT. Moreover, knockdown of VDR in cultured tubular cell increased the expression and nuclear translocation of β-catenin, a transcription regulator which has been widely implicated in the pathogenesis of EMT and renal fibrosis. Recent studies show that overexpression of β-catenin induced expression of the EMT-related transcription factor, Snail, in addition to promoting phenotype changes in the cultured tubular cells (9). Similarly, Larriba et al. (12) also reported that VDR knockdown enhances the expression of β-catenin in the nuclei in addition to enhancing target gene expression in cultured human colon cancer cells. Infiltrated inflammatory cells are likely responsible for the suppression of tubular VDR by TNF-α. In this context, downregulation of VDR following renal inflammation would potentiate β-catenin signaling, thereby sensitizing tubular cells to TGF-β1-induced EMT. Taken together, these studies identify the loss of VDR as a potential linkage between renal inflammation and tubular EMT, as well as subsequent renal fibrosis.

Our data in this study have clearly shown that the late administration of active vitamin D after UUO can reverse the suppression of VDR and inhibit EMT-like phenotype changes. At the same time, β-catenin expression and nuclear translocation after UUO is also inhibited by active vitamin D. Similar findings have been reported by He et al. (10), whereby in a mouse model of adriamycin nephropathy, a vitamin D analog inhibits β-catenin expression in tubular cells as well as podocytes. Almost all of the biological effects of active vitamin D are mediated by VDR (6). Because the expression of VDR is largely induced by vitamin D, this explains why the administration of vitamin D restores VDR expression in vivo.

In conclusion, our study suggests that loss of VDR is an early event in renal fibrogenesis, which is likely mediated by renal inflammation via TNF-α. Such a loss of VDR renders tubular epithelial cells susceptible to EMT and renal fibrosis by derepression of β-catenin signaling. Therefore, administration of active vitamin D is able to restore VDR and ameliorate renal fibrosis by blocking EMT, even at a late time point when significant kidney injury is already established.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: M.X. and J.G. performed experiments; M.X. and X.T. prepared figures; J.G. and X.T. analyzed data; Y.L. and X.T. provided conception and design of research; Y.L. and R.X. approved final version of manuscript; X.T. interpreted prepared figures; J.G. and X.T. edited and revised manuscript; Y.L. and R.X. approved final version of manuscript; X.T. interpreted results of experiments; X.T. drafted manuscript.

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