Biochimica et Biophysica Acta 1833 (2013) 1454–1462

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Hypoxia-inducible factor prolyl-hydroxylase-2 mediates transforming growth factor beta 1-induced epithelial–mesenchymal transition in renal tubular cells

Wei-Qing Han [a,b], Qing Zhu [a], Junping Hu [a], Pin-Lan Li [a], Fan Zhang [a], Ningjun Li [a,*]

[a] Department of Pharmacology & Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA, USA
[b] Shanghai Key Laboratory of Hypertension, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, PR China

ARTICLE INFO

Article history:
Received 4 November 2012
Received in revised form 20 February 2013
Accepted 22 February 2013
Available online 1 March 2013

Keywords:
Prolyl hydroxylase
domain-containing protein
Smad signaling pathway
Renal fibrosis

ABSTRACT

Transforming growth factor beta 1 (TGF-β1)-induced epithelial–mesenchymal transition (EMT) in kidney epithelial cells plays a key role in renal tubulointerstitial fibrosis in chronic kidney diseases. As hypoxia-inducible factor (HIF)-1α is found to mediate TGF-β1-induced signaling pathway, we tested the hypothesis that HIF-1α and its upstream regulator prolyl hydroxylase domain-containing proteins (PHDs) are involved in TGF-β1-induced EMT using cultured renal tubular cells. Our results showed that TGF-β1 stimulated EMT in renal tubular cells as indicated by the significant decrease in epithelial marker P-cadherin, and the increase in mesenchymal markers α-smooth muscle actin (α-SMA) and fibroblast-specific protein 1 (FSP-1). Meanwhile, we found that TGF-β1 time-dependently increased HIF-1α and that HIF-1α siRNA significantly inhibited TGF-β1-induced EMT, suggesting that HIF-1α mediated TGF-β1 induced-EMT. Real-time PCR showed that PHD1 and PHD2, rather than PHD3, could be detected, with PHD2 as the predominant form of PHDs (PHD1:PHD2 = 0.21:1.0). Importantly, PHD2 mRNA and protein, but not PHD1, were decreased by TGF-β1. Furthermore, over-expression of PHD2 transgene almost fully prevented TGF-β1-induced HIF-1α accumulation and EMT marker changes, indicating that PHD2 is involved in TGF-β1-induced EMT. Finally, Smad2/3 inhibitor SB431542 prevented TGF-β1-induced PHD2 decrease, suggesting that Smad2/3 may mediate TGF-β1-induced EMT through PHD2/HIF-1α pathway. It is concluded that TGF-β1 decreased PHD2 expression via an Smad-dependent signaling pathway, thereby leading to HIF-1α accumulation and then EMT in renal tubular cells. The present study suggests that PHD2/HIF-1α is a novel signaling pathway mediating the fibrogenic effect of TGF-β1, and may be a new therapeutic target in chronic kidney diseases.

1. Introduction

Renal tubulointerstitial fibrosis, characterized by accumulation of extracellular matrix, is the key underlying pathology in the progression of chronic kidney diseases and is the final common pathway for end-stage renal disease [1,2]. Epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells lose their epithelial specific markers, undergo cytoskeletal remodeling, and gain a mesenchymal phenotype. More and more studies show that tubular EMT is an important resource of fibrogenic myofibroblasts and plays a central role in tubulointerstitial fibrosis [3,4], such as in diabetes nephropathy [5,6]. Furthermore, there is overwhelming evidence implicating that transforming growth factor-beta 1 (TGF-β1) may act as the key mediator of tubular EMT [2,7]. For example, TGF-β1 was found to induce EMT in a rat tubular epithelial cell line (NRK-52E), and streptozotocin-induced diabetic rats exhibited increased gene expression of TGF-β1, enhanced mesenchymal markers α-smooth muscle actin (α-SMA) and collagen with a concomitant decrease in epithelial marker such as E-cadherin in the kidneys [8]. Yeh et al. reported that TGFβ1-induced EMT plays a critical role during chronic tubulointerstitial fibrosis [9]. Moreover, transgenic mice with increased expression of TGF-β1 develop renal fibrosis [10].

Hypoxia-inducible factor 1α (HIF-1α) is a transcriptional factor that has been recently associated with the progression of chronic renal injuries [11–13]. Notably, HIF-1α has been shown to play a critical role in EMT. Higgins et al. reported that hypoxia induced significant increase in EMT marker fibroblast-specific protein 1 (FSP-1) and cell migration in murine primary tubular epithelial cells. However, this hypoxia-induced EMT was not observed in HIF-1α-deficient cells [13]. In vivo study showed that genetic ablation of epithelial HIF-1α inhibited the development of tubulointerstitial fibrosis and FSP-1-positive cells in unilateral ureteral obstruction kidney [13]. Hypoxia induced significant EMT in hepatocytes from wild type mouse, but failed to induce EMT in hepatocytes isolated

Abbreviations: α-SMA, α-smooth muscle actin; EMT, epithelial-to-mesenchymal transition; FSP-1, fibroblast-specific protein 1; HIF-1α, hypoxia-inducible factor 1α; PHD, prolyl hydroxylase domain; TFIID, transcription factor II D; TGF-β1, transforming growth factor beta 1

* Corresponding author at: Department of Pharmacology & Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, P.O. Box 980613, USA. Tel.: +1 804 828 2071; fax: +1 804 828 4794.
E-mail address: nli@vcu.edu (N. Li).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.02.029

from HIF-1α-deficient mice [14]. Moon et al. demonstrated that in bile duct ligations-induced liver fibrosis animal model, liver underwent hypoxia, and HIF-1α was activated. After HIF-1α was deleted, bile duct ligations-induced collagen I and α-SMA expressions in the liver were significantly decreased [15]. Another study in alveolar epithelial cells showed that hypoxia-induced EMT was significantly attenuated by HIF-1α shRNA [16]. All these data demonstrated an important role of HIF-1α in EMT.

Interestingly, it has been shown that TGF-β1 stimulates HIF-1α accumulation and that HIF-1α functions as a mediator in many TGF-β1-induced actions [17–19]. For example, in colorectal cancer cells, HIF-1α was found to mediate TGF-β1-induced glutathione peroxidase-1 and protects from H_2O_2-induced cell death in colorectal cancer cells [17]. It was also reported that HIF-1α mediated TGF-β1-induced fibrogenic protein such as collagen and plasminogen activator inhibitor expression in renal epithelial cells and in alveolar macrophages [18,19]. However, the contribution of HIF-1α to TGF-β1-induced EMT in renal epithelial cells has not been evidenced. In addition, the role of HIF prolyl-hydroxylases, the enzymes that promote the degradation of HIF-1α [20–22], in EMT process has not been investigated. A recent study reported that TGF-β1 inhibited PHD2 level via Smad2/3-dependent mechanism in tumor cells [23], indicating a possible role of HIF prolyl-hydroxylases in TGF-β1-induced EMT.

HIF prolyl-hydroxylases hydroxylate HIF-1α at the specific proline site using oxygen as a cofactor and the prolyl-hydroxylated HIF-1α is then recognized and targeted for degradation by the ubiquitin–proteasome pathway [20,21]. Under hypoxia, prolyl hydroxylase activity is inhibited and HIF-1α becomes stabilized to induce the transcription of its target genes. In addition to oxygen homeostasis-related regulation, PHDs also respond to non-hypoxic stimulations including TGF-β1 and regulate HIF-1α via oxygen-independent mechanisms [23,24]. Three isoforms of HIF prolyl hydroxylase, including *p*rolyl *h*ydroxylase *d*omain-containing proteins (PHDs) 1, 2, and 3, have been identified and PHD2 is the primary PHD in the kidneys [25–28]. Given the fact that (1) TGF-β1 induces HIF1-α accumulation, (2) HIF1-α mediates EMT, and (3) HIF prolyl-hydroxylases are present in the kidneys and regulate HIF-1α levels in renal cells [25–28], the present study tested the hypothesis that PHD2/HIF-1α pathway mediates TGF-β1-induced EMT thereby leading to fibrogenesis in renal tubular cells.

2. Materials and methods

2.1. Cell culture

NRK-52E cells, a rat renal tubular cell line, were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium with 4 mmol/l l-glutamine adjusted to contain DMEM/Ham's F12 (DMEM/F12) medium, supplemented with 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air [29]. For EMT experiments, cells were treated with 5 ng/ml TGF-β1 for 48 h.

2.2. Transfection of HIF-1α siRNA

Transfection of siRNA was performed using the siLentFect lipid reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions as we described previously [30]. For a 10 cm dish, 200 pmol of siRNA was used. After 6 h incubation in transfection reagent, the cells were then switched to normal medium. The sequence of HIF-1α siRNA was: sense, 5′-GGAAAGAGACUCAUAGAAA-3′ and antisense, 5′-UUUCUAUGACUCUCUUUCC-3′ (Sigma-Aldrich, St Louis, MO). A scrambled small RNA (Qiagen, Valencia, CA), which was confirmed as non-silencing double-stranded RNA, was used as control for siRNA experiments.

2.3. Transfection of plasmids expressing rat PHD2 into the cells

Plasmid transfections were performed using lipids (DOTAP/DOPE; Avanti Polar Lipids, Alabaster, AL) according to the manufacturer's instructions as we described previously [30]. In brief, 5 μg of DNA was mixed with lipid solution in a ratio of 1:10 (DNA/lipid, w/w) in serum-free culture medium (5 ml for a 10 cm dish). Cells were incubated with this transfection medium for 6 h and switched to normal medium for another 16 h. The cells were then ready for experiment. In preliminary experiments, almost all cells were positive after transfection with luciferase plasmids when detected by bioluminescent imaging (IVIS200; Caliper Life Sciences, Hopkinton, MA), demonstrating a high transfection efficiency (data not shown). Plasmids encoding full-length rat PHD2 were generous gifts from Dr Frank S. Lee (University of Pennsylvania). The expression and function of rat PHD2 protein by this plasmid have been validated by Dr Lee [31,32] and in our previous study [28,33,34]. Luciferase plasmids (Promega, Madison, WI) were used as control for PHD2 expression vector transfection experiments.

2.4. RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted using TRIzol solution (Life Technologies, Inc., Rockville, MD) and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad, Hercules, CA). The RT products were amplified using a TaqMan Gene Expression Assays kit (Applied Biosystems). A kit for detecting the levels of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

2.5. Western blot analysis

Cytosolic protein and nuclear protein preparation, as well as western blotting, were performed as we described previously [28,30,35]. Briefly, after boiling for 5 min at 95 °C in a 5× loading buffer, cytosolic protein and nuclear protein were subjected to SDS-PAGE, transferred onto a PVDF membrane and blocked by solution with dry milk respectively. For cytosolic protein, the membrane was probed with primary antibodies of anti-P-cadherin (1:500, R&D System), anti-α-SMA (1:5000, R&D System), anti-FSP-1 (1:500, Abcam) and anti-PHD2 (1:500, Novus) overnight at 4 °C followed by incubation with horseradish peroxidase-labeled secondary antibody (1:5000); β-actin was detected by using horseradish peroxidase-labeled anti-β-actin antibody (1:5000, Santa Cruz Biotechnology) as a loading control. For nuclear protein, HIF-1α was detected using anti-HIF-1α antibody (1:500, GeneTex) followed by incubation with horseradish peroxidase-labeled secondary antibody (1:3000). Transcription factor II D (TFIID) was detected using anti-TFIID antibody (1:100, Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-labeled secondary antibody (1:3000) as a loading control for nuclear protein [36]. The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat X-ray films. The densitometry analyses of the blots were performed using an ImageJ software (free download from National Institutes of Health http://rsbweb.nih.gov/ij/download.html). To calculate the relative values of blot intensities, band intensities in control group were averaged and then all the band intensities were normalized to the mean value of control group. The normalized values in different groups were averaged and expressed as fold change with the mean value of control group as 1.

2.6. Immunofluorescent microscopy

Immunofluorescent staining was performed using cultured renal tubular cells on cover slips. After fixation, the cells were incubated with goat anti-FSP-1 (1:50 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat anti-P-cadherin (1:25 dilution), or mouse

anti-α-SMA (1:300 dilution) (R&D System, Minneapolis, MN, USA) antibodies, respectively, at 4 °C overnight. After washing, the slides were incubated with corresponding Alex-555-labeled secondary antibodies and then mounted and subjected to examinations using a confocal laser scanning microscope (FluoView FV1000, Olympus, Japan). These experiments were performed to observe the changes of EMT markers in renal tubular cells. Integrated optical intensity (IOD) was calculated by using an Image-Pro Plus v6.0 software (Media Cybernetics, Silver Spring, MD). The IOD values in control group were averaged, and all the IOD values were normalized to the mean value of the control group. The normalized values in different groups were averaged and expressed as fold change with the mean value of control group as 1.

2.7. Statistics

Data are presented as means ± S.E.M. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. Student's *t* test was used to evaluate the significance of differences between two groups of experiments. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HIF-1α siRNA blocked TGF-β1-induced EMT

We first evaluated whether TGF-β1 would have any effect on HIF-1α protein levels in renal tubular cells. As shown in Fig. 1A & B, TGF-β1 increased HIF-1α protein level when cells were treated for 16 h, and HIF-1α reached the highest level after 24 h and 48 h treatment. Interestingly, epithelial marker P-cadherin was decreased when cells were treated with TGF-β1 for 16 h, and the protein level reached the lowest level after 24 and 48 h treatment. In contrast, mesenchymal marker α-SMA was increased when cells were treated with TGF-β1 for 16 h, 24 h and 48 h (Fig. 1C & D).

Previous studies showed that HIF-1α was increased in response to hypoxia, and the increased HIF-1α was involved in hypoxia-induced EMT. We then determined whether the increased HIF-1α in response to TGF-β1 stimulation mediated TGF-β1-induced EMT. As shown in

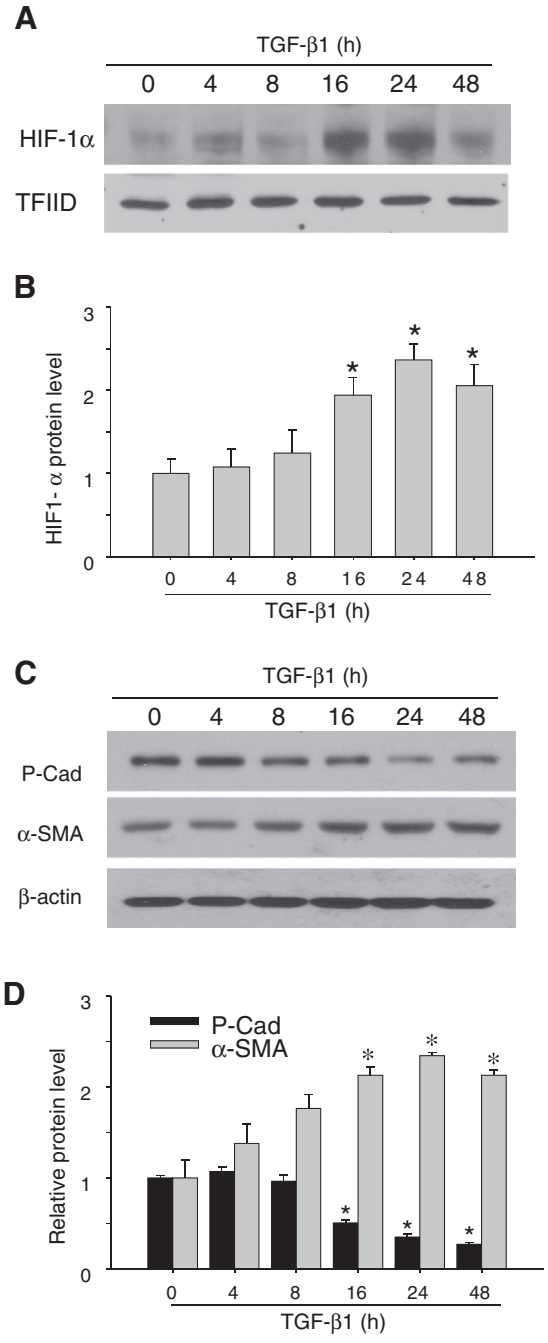

Fig. 1. Effect of TGF-β1 on HIF-1α and EMT marker protein content. Representative gel documents (A) and summarized data (B) showing the effect of TGF-β1 on HIF-1α protein level. Transcription factor II D (TFIID) was used as a loading control for nuclear protein. Representative gel documents (C) and summarized data (D) showing the effect of TGF-β1 on epithelial marker P-cadherin and mesenchymal marker α-SMA protein level. All band densities were normalized to the mean value of 0h group and the normalized values were then calculated and presented in the figure. P-cad: P-cadherin. n = 4 batches of cells. *$P < 0.05$ vs. 0h TGF-β1 treatment.

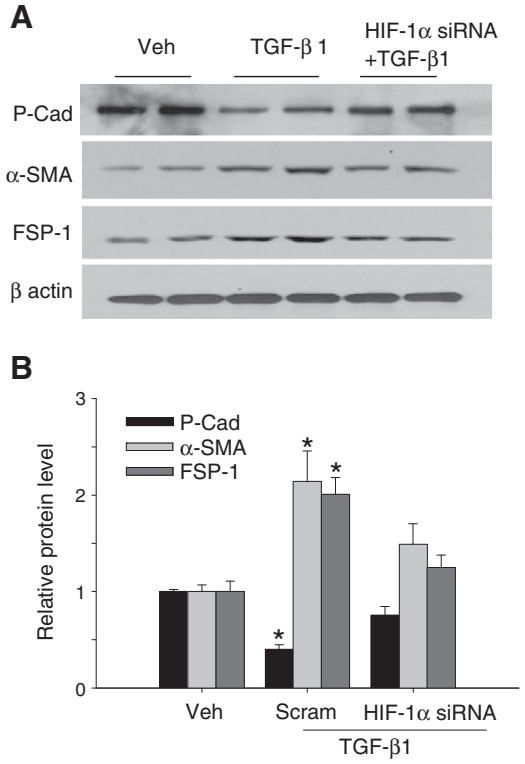

Fig. 2. HIF-1α siRNA blocked TGF-β1-induced changes in P-cad, α-SMA and FSP-1. Representative gel documents (A) and summarized data (B) showing the effect of HIF-1α siRNA on TGF-β1-induced decrease in epithelial marker P-cadherin, and the increase in mesenchymal markers α-SMA and FSP-1. Veh: vehicle; Scram: scrambled RNA; P-cad: P-cadherin. The values were normalized to Veh. n = 6 batches of cells, *$P < 0.05$ vs. other groups.

Fig. 2, TGF-β1 significantly decreased epithelial marker P-cadherin, and increased mesenchymal markers including cytoskeletal protein α-SMA and signal transduction protein FSP-1, indicating that EMT occurred in response to TGF-β1 stimulation. In cells pretreated with HIF-1α siRNA, TGF-β1-induced EMT was significantly inhibited as indicated by the increase in epithelial marker P-cadherin, and decrease in mesenchymal markers α-SMA and FSP-1 compared with TGF-β1-treated group. These results indicate that HIF-1α mediates TGF-β1-induced EMT in renal tubular cells.

3.2. TGF-β1 decreased PHD2 mRNA and protein levels

It has been shown that prolyl hydroxylase is the main regulator of HIF-1α in cells. We then determined the expression and abundance of PHD subtypes in renal tubular cells by real-time PCR. Among three different PHDs (PHD1, PHD2, PHD3), only PHD1 and PHD2 were detected. The ΔCt value of PHD1 and PHD2 was 15.6 and 13.4 respectively, and the calculated ratio of PHD1 to PHD2 was 0.21:1.0 ($n = 6$), indicating that PHD2 is the predominant form of PHDs in renal tubular cells.

Next, we evaluated whether TGF-β1 had any effect on mRNA/protein levels of PHD1 and PHD2. As presented in Fig. 3, TGF-β1 had no significant effect on PHD1 mRNA (Fig. 3A) and protein levels (Fig. 3B & C). In contrast, TGF-β1 dramatically decreased PHD2 mRNA levels with sustained effect from 16 h (Fig. 4A). Similarly, TGF-β1 time-dependently decreased PHD2 protein level, which reached its maximum value from 1.0 ± 0.08 to 0.26 ± 0.08 ($P < 0.05$) at 24 h as shown in western blot assay (Fig. 4B & C).

3.3. PHD2 transgene prevented TGF-β1-induced HIF-1α increase and EMT

To determine whether the decreased PHD2 contributes to the HIF-1α increase and consequent EMT after TGF-β1 stimulation, PHD2 overexpression plasmid was transfected into renal tubular cells, and then the effect on TGF-β1-induced changes in PHD2 and HIF-1α, as well as EMT, was evaluated. The gene transfection efficiency was validated by western blot showing that TGF-β1 treatment decreased PHD2 protein level, and this decrease was reversed when cells were transfected with PHD2 overexpression plasmid (Fig. 5A & B). As shown in Fig. 5C & D, PHD2 transgene effectively prevented TGF-β1-induced HIF-1α increase. In Fig. 6, TGF-β1 induced significant EMT, as indicted by the decrease in epithelial marker P-cadherin, and the increase in mesenchymal markers α-SMA and FSP-1. When the cells were pretreated with PHD2 overexpression plasmid, TGF-β1-induced EMT was significantly inhibited, as shown by the increase in epithelial marker P-cadherin, and decrease in mesenchymal markers α-SMA and FSP-1 compared with TGF-β1-treated group. These results indicate that PHD2 mediates TGF-β1-induced HIF-1α increase and EMT.

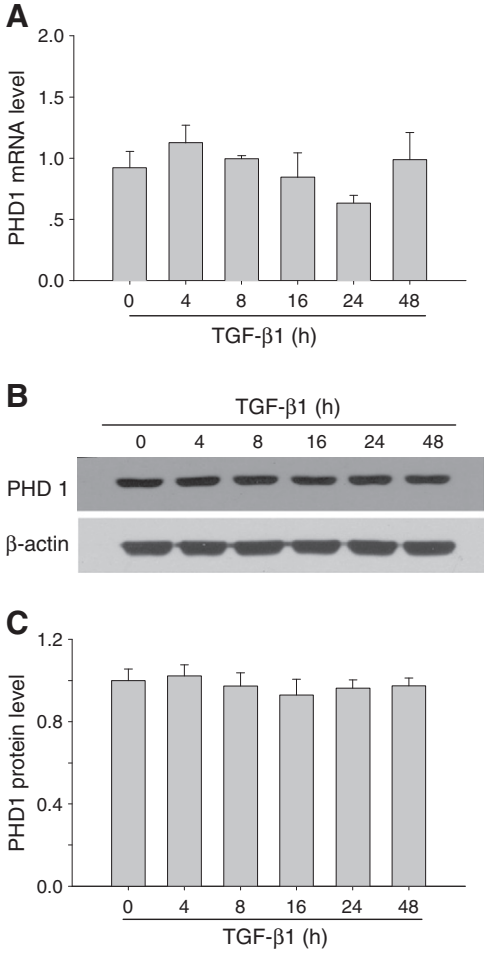

Fig. 3. Effect of TGF-β1 on PHD1 mRNA and protein levels. (A) Summarized data showing the effect of TGF-β1 on PHD1 mRNA level. Representative gel documents (B) and summarized data (C) showing the effect of TGF-β1 on PHD1 protein level. The values were normalized to 0h. n = 4–6 batches of cells.

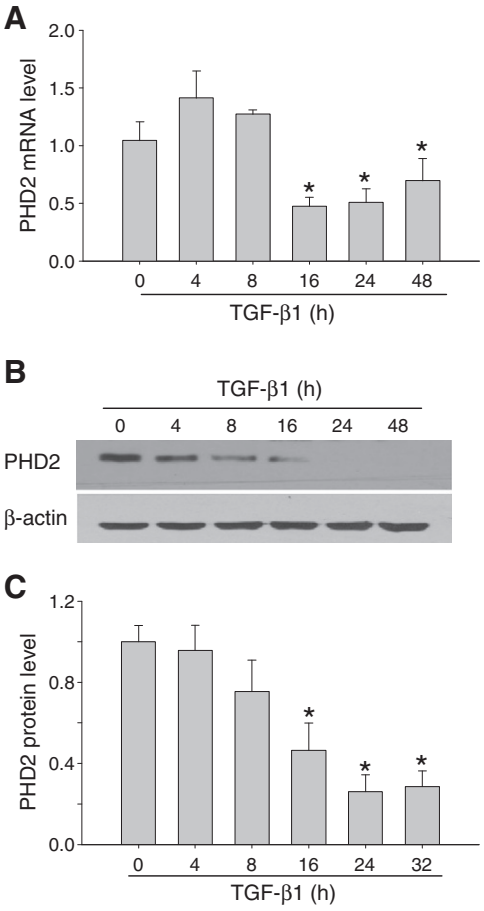

Fig. 4. TGF-β1 decreased PHD2 mRNA and protein levels. (A) Summarized data showing the effect of TGF-β1 on PHD2 mRNA level. Representative gel documents (B) and summarized data (C) showing the effect of TGF-β1 on PHD2 protein level. The values were normalized to 0h. n = 4–5 batches of cells, *$P < 0.05$ vs. 0h TGF-β1 treatment.

3.4. HIF-1α siRNA and PHD2 transgene prevented TGF-β1-induced EMT as detected by fluorescence microscopy

The involvement of HIF-1α and PHD2 in TGF-β1-induced EMT was further investigated by fluorescence confocal assay. Immunostaining analysis of EMT markers was performed in cells with or without stimulation of TGF-β1. As shown in Fig. 7, under basal condition renal tubular cells were enriched with epithelial marker P-cadherin, and it is mainly located on the plasma membrane. When these renal tubular cells were treated with TGF-β1, the expression of P-cadherin was markedly reduced as shown in decreased red fluorescence in TGF-β1-treated cells, and P-cadherin was delocalized from plasma membrane (Fig. 7). When cells were pre-treated with HIF-1α siRNA or transfected with PHD2 overexpression plasmid, TGF-β1-induced P-cadherin delocalization was blocked.

In contrast, the abundance of two mesenchymal markers, α-SMA and FSP-1 was very low in renal tubular cells under control condition as indicated by the weak fluorescence in confocal images. When these cells were stimulated by TGF-β1, the expression of both α-SMA and FSP-1 was remarkably increased as shown by the increased fluorescence in confocal images. When renal tubular cells were pretreated with HIF-1α siRNA or transfected with PHD2 overexpression plasmid, TGF-β1 failed to increase α-SMA and FSP-1 expressions (Fig. 7). These results further confirmed that HIF-1α and PHD2 mediate TGF-β1-induced EMT.

3.5. HIF-1α siRNA and PHD2 transgene prevented TGF-induced collagen I expression

It has been shown that collagen expression is increased in cells undergoing EMT, which is pathologically related to fibrosis. We then evaluated whether PHD2/HIF-1α mediated TGF-β1-induced collagen expression upon EMT. Fig. 8 shows that TGF-β1 induced significant increase in collagen I expression in renal tubular cells. After cells were pretreated with HIF-1α siRNA or PHD2 overexpression plasmid, TGF-β1-induced collagen increase was almost fully inhibited, indicating that HIF-1α/PHD2 mediates TGF-β1-induced collagen expression, and this change may be caused by HIF-1α/PHD2-mediated EMT.

3.6. Smad pathway mediates TGF-β1-induced PHD2 supression

Finally, we investigate whether TGF-β1-induced PHD2 change is mediated by Smads signaling pathway, since previous studies show that TGF-β1 decreased PHD2 level in an Smad-dependent manner. As shown in Fig. 9, TGF-β1 induced significant PHD2 decrease in PT cells, this effect was abolished in the presence of Smad2/3 inhibitor SB431542, indicating that Smad signaling pathway was involved in TGF-β1-induced PHD2 decrease.

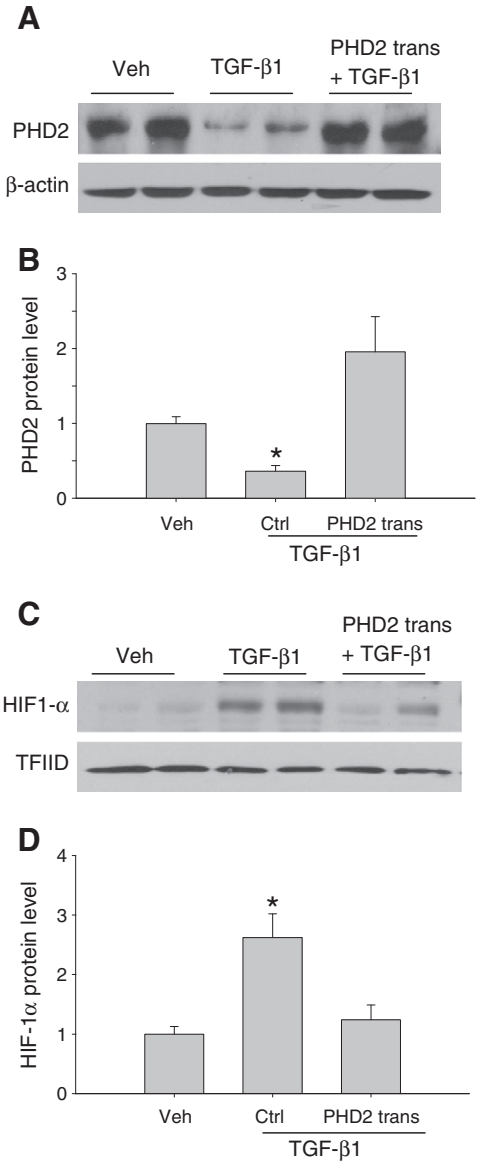

Fig. 5. PHD2 transgene blocked TGF-β1-induced HIF-1α changes. Representative gel documents (A) and summarized data (B) showing the effect of PHD2 transgene on TGF-β1-induced PHD2 decrease. Representative gel documents (C) and summarized data (D) showing the effect of PHD2 transgene on TGF-β1-induced HIF-1α increase. Veh: vehicle; Ctrl: control plasmid; trans: transgene. TFIID was used as a loading control for nuclear protein. The values were normalized to Veh. n = 4–6 batches of cells, $^{*}P < 0.05$ vs. other groups.

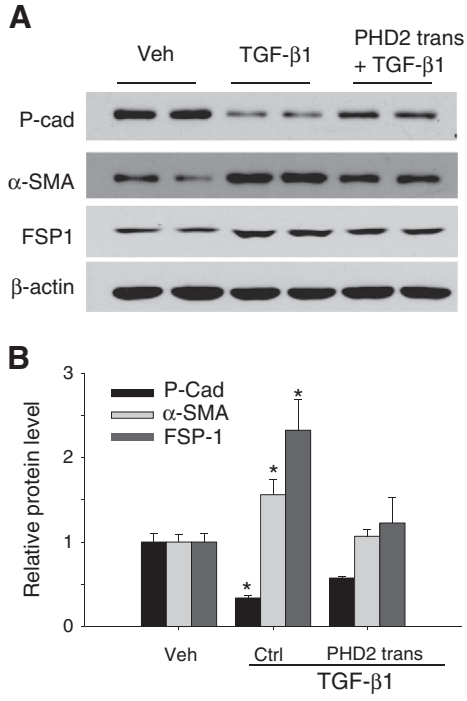

Fig. 6. PHD2 transgene blocked TGF-β1-induced changes in EMT markers. Representative gel documents (A) and summarized data (B) showing the effect of PHD2 transgene on TGF-β1-induced decrease in epithelial marker P-cadherin, as well as the increase in mesenchymal markers α-SMA and FSP-1. Veh: vehicle; Ctrl: control plasmid; trans: transgene; P-cad: P-cadherin. The values were normalized to Veh. n = 4–6 batches of cells, $^{*}P < 0.05$ vs. other groups.

4. Discussion

The present study demonstrated that TGF-β1 decreased PHD2 expression, thereby leading to HIF-1α accumulation. It was found that this PHD2/HIF-1α signaling pathway mediates TGF-β1-induced EMT, since both HIF-1α siRNA and PHD2 overexpression blocked TGF-β1-induced lose of epithelial marker P-cadherin and gain of mesenchymal markers α-SMA and FSP-1. In addition, Smad2/3 inhibitor SB431542 prevented TGF-β1-induced PHD2 decrease. These data support a direct role for PHD2/HIF-1α pathway as a crucial mediator in TGF-β1-induced EMT via an Smad-dependent mechanism in renal tubular cells.

As epithelial cells transdifferentiate into mesenchymal cells during EMT, levels of cytoskeletal proteins (e.g. α-SMA) [37,38], and signal transduction proteins (e.g. FSP-1) are increased [37,38], and the expression of epithelial genes, including P-cadherin is repressed [39,40]. Morphologically, the epithelial marker cadherin protein is delocalized from cell membrane during EMT [41]. Therefore, the changes in α-SMA, FSP-1 and P-cadherin have been widely used as indicators for EMT [40,42]. In the present study, exposure of proximal tubular cells to TGF-β1 stimulated the expression of α-SMA and FSP-1, and inhibited P-cadherin expression, demonstrating that TGF-β1 stimulated EMT in tubular cells, which is consistent with previous reports [43,44]. It has been shown that TGF-β1 induces HIF-1α accumulation under normoxic

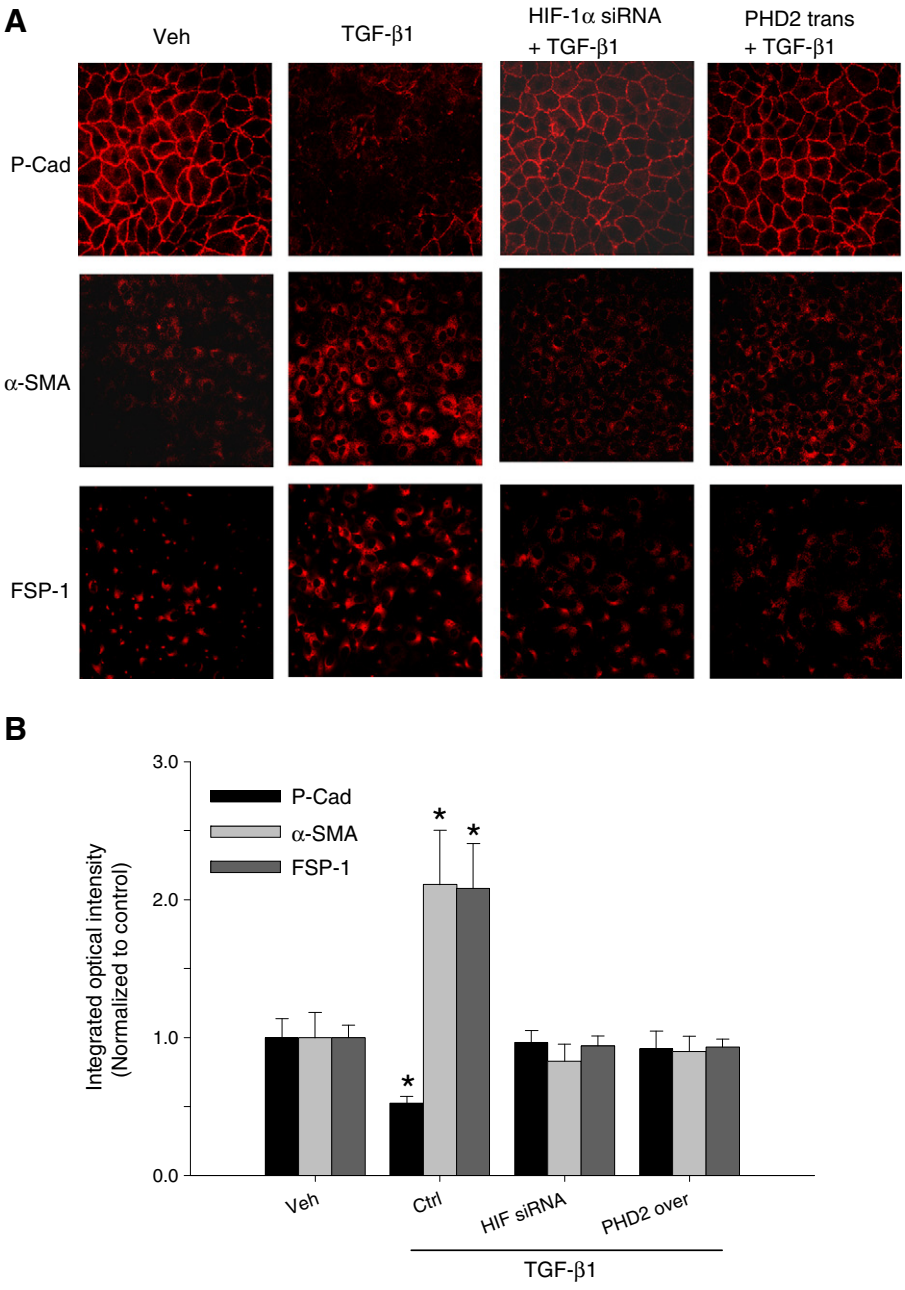

Fig. 7. HIF-1α siRNA and PHD2 transgene blocked TGF-β1-induced changes in P-cad, α-SMA and FSP-1 by immunofluorescent microscopy assay. (A) Representative images showing the inhibitory effect of HIF-1α siRNA and PHD2 transgene on TGF-β1-induced decrease in epithelial marker P-cadherin, and increases in mesenchymal markers α-SMA and FSP-1. (B) Summarized data show the inhibitory effect of HIF-1α siRNA and PHD2 transgene on integrated optical intensity of α-SMA and FSP-1. Veh: vehicle; Ctrl: scrambled RNA as control for HIF-1α siRNA treatment, and control plasmid for PHD2 transgene treatment; trans: transgene; P-cad: P-cadherin. Integrated optical intensity (IOD) was calculated by using Image-Pro Plus v6.0 software. All the IOD values were normalized to the mean value of Veh group and then the normalized values were calculated and presented. n = 5 batches of cells, $^{*}P < 0.05$ vs. other groups.

conditions in different cells such as HT1080 and vascular smooth muscle cells and that HIF-1α accumulation is involved in TGF-β1-induced various effects, such as fibrosis, apoptosis and tumor angiogenesis [17,19,23,45,46]. In the present study, we demonstrated that TGF-β1 increased HIF-1α protein level in renal tubular cells, and provided novel data by showing that this HIF-1α accumulation mediated EMT in response to a non-hypoxic stimulator TGF-β1 [13,14,16].

The role of PHDs in EMT was also evaluated in the present study since it represents the most important signaling pathway in regulating HIF-1α. The present study found that PHD2 is the major form of PHDs in renal tubular cells, which is consistent with previous studies showing that PHDs are present in the kidneys, with PHD2 as the predominant isoform of PHDs, although PHD1 and PHD3 can also be detected [26–28,47,48]. Furthermore, we found that PHD2, but not PHD1, was decreased in response to TGF-β1 stimulation, and that this PHD2 decrease was responsible for the increased HIF-1α accumulation, suggesting divergences in their regulation [23,49–51]. It has been well documented that PHDs are emerging as an important regulator of HIF-1α in response to both hypoxic and non-hypoxic stimulation, and are involved in many physiological and pathological processes such as collagen expression, cell death, tumor suppression, and blood pressure regulation [17–19,52]. By using both Western blot and confocal staining, the present study shows novel finding by demonstrating that PHD2, as an upstream regulator of HIF-1α, mediates non-hypoxic agonist TGF-β1-induced EMT in renal tubular cells.

It has been shown that myofibroblasts are activated fibroblasts and the main source for extracellular matrix, thus contributing to kidney fibrosis [53,54]. In particular, it has been demonstrated that the tubular epithelial cells undergoing EMT contribute to one-third of myofibroblast during kidney fibrosis [4,55]. Consistently, the present study shows that PHD2/HIF-1α mediated TGF-β1-induced collagen I expression, which is even considered as an EMT marker in previous studies [53]. Considering the critical roles of TGF-β1 and EMT in tubulointerstitial fibrosis, the findings in the present study suggest that PHD2/HIF-1α may be an important signaling pathway in mediating this pathological change.

Smad signaling pathway, especially Smad2 and Smad3, plays a central role in TGF-β1-induced effects including EMT in various cell types, such as human bronchial epithelial cells [56,57], murine cloned corneal progenitor cells [58], and panc1 cells [59]. Furthermore, Smad2/3 signaling pathway has been reported to mediate EMT in response to hypoxia in hepatocytes [14]. In animal studies, targeted disruption of TGF-β1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction [60]. It has been suggested that Smad2/3 can regulate the expression of EMT-related genes after interacting with other proteins [2,61]. The present study provided novel mechanisms underlying Smad-mediated EMT by showing that Smad2/3 mediated TGF-β1-induced PHD2 decrease, which then leads to HIF-1α increase and the subsequent EMT.

It should be noted that although EMT has been well acknowledged, there are debates about the involvement of EMT in renal fibrosis in vivo [54,62–65]. Evidence supporting EMT in vivo includes the loss of epithelial markers, acquisition of mesenchymal markers and collagen synthesis by epithelial cells in diseased kidneys from both human and animal studies, as well as effective strategies to treat experimental fibrosis based on EMT mechanism. However, studies using lineage-tracing techniques to detect tubular epithelial cell-derived fibroblasts show controversial results. An early experiment showed that labeled tubular cells gained EMT markers and migrated into the peritubular interstitium, while several recent similar fate-tracing studies did not detect the EMT markers in labeled tubular cells and failed to find labeled cells in peritubular interstitium. One of the arguments for the conflicting results from these in vivo cell lineage-tracking studies is that the technical differences may account for the disparity. One opinion is that detection of intermediate stages of EMT in injured kidney is straightforward and the current gold standard, and to observe EMT process as well as cell migration in real time in vivo is not feasible with current technology.

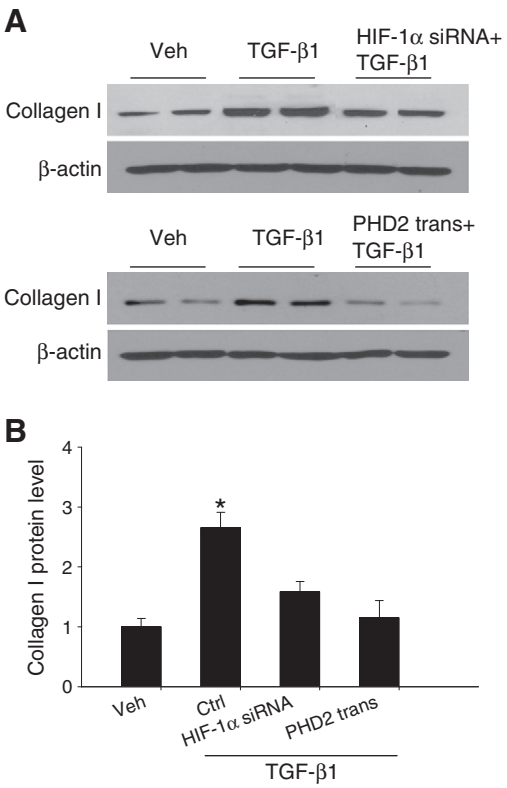

Fig. 8. HIF-1α siRNA and PHD2 transgene blocked TGF-β1-induced collagen I increase. Representative gel documents (A) and summarized data (B) showing the effect of HIF-1α siRNA and PHD2 transgene on TGF-β1-induced collagen I changes in renal tubular cells. Veh: vehicle; Ctrl: scrambled RNA as control for HIF-1α siRNA treatment, and control plasmid for PHD2 transgene treatment; trans: transgene. The values were normalized to Veh. n = 4–6 batches of cells, $^{*}P < 0.05$ vs. other groups.

Nevertheless, our current study revealed a novel mechanism for EMT in renal tubular cells.

In summary, the present study demonstrated that TGF-β1 decreased PHD2 expression via an Smad-dependent mechanism, thereby leading to HIF-1α accumulation, and that this PHD2/HIF-1α signaling pathway

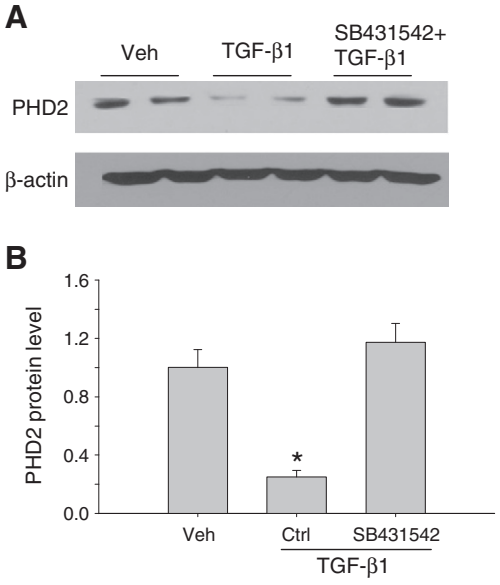

Fig. 9. Smad inhibition blocked TGF-β1-induced PHD2 decreases. Representative gel documents (A) and summarized data (B) showing the effect of Smad inhibitor SB431542 on TGF-β1-induced PHD2 decrease. Veh: vehicle; Ctrl: control. The values were normalized to Veh. n = 6 batches of cells, $^{*}P < 0.05$ vs. other groups.

mediated TGF-β1-induced EMT in renal tubular cells. This novel signaling pathway in renal tubular cells may contribute to renal tubulointerstitial fibrosis in chronic kidney diseases.

Disclosures

None.

Acknowledgement

This study was supported by grants from the US National Institutes of Health (HL89563 and HL106042).

References

[1] Y. Liu, Cellular and molecular mechanisms of renal fibrosis, Nat. Rev. Nephrol. 7 (2011) 684–696.
[2] C.E. Hills, P.E. Squires, The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy, Cytokine Growth Factor Rev. 22 (2011) 131–139.
[3] C.E. Hills, P.E. Squires, TGF-beta1-induced epithelial-to-mesenchymal transition and therapeutic intervention in diabetic nephropathy, Am. J. Nephrol. 31 (2010) 68–74.
[4] M. Iwano, D. Plieth, T.M. Danoff, C. Xue, H. Okada, E.G. Neilson, Evidence that fibroblasts derive from epithelium during tissue fibrosis, J. Clin. Invest. 110 (2002) 341–350.
[5] Y.S. Kanwar, J. Wada, L. Sun, P. Xie, E.I. Wallner, S. Chen, S. Chugh, F.R. Danesh, Diabetic nephropathy: mechanisms of renal disease progression, Exp. Biol. Med. (Maywood) 233 (2008) 4–11.
[6] K. Sharma, F.N. Ziyadeh, Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator, Diabetes 44 (1995) 1139–1146.
[7] R.M. Carew, B. Wang, P. Kantharidis, The role of EMT in renal fibrosis, Cell Tissue Res. 347 (2012) 103–116.
[8] W.C. Burns, S.M. Twigg, J.M. Forbes, J. Pete, C. Tikellis, V. Thallas-Bonke, M.C. Thomas, M.E. Cooper, P. Kantharidis, Connective tissue growth factor plays an important role in advanced glycation end product-induced tubular epithelial-to-mesenchymal transition: implications for diabetic renal disease, J. Am. Soc. Nephrol. 17 (2006) 2484–2494.
[9] Y.C. Yeh, W.C. Wei, Y.K. Wang, S.C. Lin, J.M. Sung, M.J. Tang, Transforming growth factor-{beta}1 induces Smad3-dependent {beta}1 integrin gene expression in epithelial-to-mesenchymal transition during chronic tubulointerstitial fibrosis, Am. J. Pathol. 177 (2010) 1743–1754.
[10] H.W. Schnaper, T. Hayashida, S.C. Hubchak, A.C. Poncelet, TGF-beta signal transduction and mesangial cell fibrogenesis, Am. J. Physiol. Renal. Physiol. 284 (2003) F243–F252.
[11] M. Nangaku, T. Fujita, Activation of the renin–angiotensin system and chronic hypoxia of the kidney, Hypertens. Res. 31 (2008) 175–184.
[12] M. Nangaku, Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure, J. Am. Soc. Nephrol. 17 (2006) 17–25.
[13] D.F. Higgins, K. Kimura, W.M. Bernhardt, N. Shrimanker, Y. Akai, B. Hohenstein, Y. Saito, R.S. Johnson, M. Kretzler, C.D. Cohen, K.U. Eckardt, M. Iwano, V.H. Haase, Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition, J. Clin. Invest. 117 (2007) 3810–3820.
[14] B.L. Copple, Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-beta-dependent mechanisms, Liver Int. 30 (2010) 669–682.
[15] J.O. Moon, T.P. Welch, F.J. Gonzalez, B.L. Copple, Reduced liver fibrosis in hypoxia-inducible factor-1alpha-deficient mice, Am. J. Physiol. Gastrointest. Liver Physiol. 296 (2009) G582–G592.
[16] G. Zhou, L.A. Dada, M. Wu, A. Kelly, H. Trejo, Q. Zhou, J. Varga, J.I. Sznajder, Hypoxia-induced alveolar epithelial–mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1, Am. J. Physiol. Lung Cell Mol. Physiol. 297 (2009) L1120–L1130.
[17] Y. Huang, W. Fang, Y. Wang, W. Yang, B. Xiong, Transforming growth factor-beta1 induces glutathione peroxidase-1 and protects from H_2O_2-induced cell death in colon cancer cells via the Smad2/ERK1/2/HIF-1alpha pathway, Int. J. Mol. Med. 29 (2012) 906–912.
[18] R.K. Basu, S. Hubchak, T. Hayashida, C.E. Runyan, P.T. Schumacker, H.W. Schnaper, Interdependence of HIF-1alpha and TGF-beta/Smad3 signaling in normoxic and hypoxic renal epithelial cell collagen expression, Am. J. Physiol. Renal. Physiol. 300 (2011) F898–F905.
[19] M. Ueno, T. Maeno, M. Nomura, K. Aoyagi-Ikeda, H. Matsui, K. Hara, T. Tanaka, T. Iso, T. Suga, M. Kurabayashi, Hypoxia-inducible factor-1alpha mediates TGF-beta-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis, Am. J. Physiol. Lung Cell Mol. Physiol. 300 (2011) L740–L752.
[20] R.K. Bruick, S.L. McKnight, A conserved family of prolyl-4-hydroxylases that modify HIF, Science 294 (2001) 1337–1340.
[21] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing, Science 292 (2001) 464–468.
[22] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.
[23] S. McMahon, M. Charbonneau, S. Grandmont, D.E. Richard, C.M. Dubois, Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression, J. Biol. Chem. 281 (2006) 24171–24181.
[24] S. Tug, B. Delos Reyes, J. Fandrey, U. Berchner-Pfannschmidt, Non-hypoxic activation of the negative regulatory feedback loop of prolyl-hydroxylase oxygen sensors, Biochem. Biophys. Res. Commun. 384 (2009) 519–523.
[25] K. Takeda, A. Cowan, G.-H. Fong, Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system, Circulation 116 (2007) 774–781.
[26] C. Rosenberger, S. Rosen, A. Shina, U. Frei, K.U. Eckardt, L.A. Flippin, M. Arend, S.J. Klaus, S.N. Heyman, Activation of hypoxia-inducible factors ameliorates hypoxic distal tubular injury in the isolated perfused rat kidney, Nephrol. Dial. Transplant. 23 (2008) 3472–3478.
[27] K. Takeda, H.L. Aguila, N.S. Parikh, X. Li, K. Lamothe, L.J. Duan, H. Takeda, F.S. Lee, G.H. Fong, Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins, Blood 111 (2008) 3229–3235.
[28] N. Li, F. Yi, C.M. Sundy, L. Chen, M.L. Hilliker, D.K. Donley, D.B. Muldoon, P.L. Li, Expression and actions of HIF prolyl-4-hydroxylase in the rat kidneys, Am. J. Physiol. Renal Physiol. 292 (2007) F207–F216.
[29] C.E. Hills, N. Al-Rasheed, N. Al-Rasheed, G.B. Willars, N.J. Brunskill, C-peptide reverses TGF-beta1-induced changes in renal proximal tubular cells: implications for treatment of diabetic nephropathy, Am. J. Physiol. Renal. Physiol. 296 (2009) F614–F621.
[30] Z. Wang, L. Tang, Q. Zhu, F. Yi, F. Zhang, P.L. Li, N. Li, Hypoxia-inducible factor-1alpha contributes to the profibrotic action of angiotensin II in renal medullary interstitial cells, Kidney Int. 79 (2011) 300–310.
[31] J. Huang, Q. Zhao, S.M. Mooney, F.S. Lee, Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3, J. Biol. Chem. 277 (2002) 39792–39800.
[32] M.J. Percy, Q. Zhao, A. Flores, C. Harrison, T.R. Lappin, P.H. Maxwell, M.F. McMullin, F.S. Lee, A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 654–659.
[33] Q. Zhu, M. Liu, W. Han, P.L. Li, Z. Wang, N. Li, Overexpression of HIF prolyl-hydoxylase-2 transgene in the renal medulla induced a salt-sensitive hypertension, J. Cell Mol. Med. (2012).
[34] Q. Zhu, Z. Wang, M. Xia, P.L. Li, B.W. Van Tassell, A. Abbate, R. Dhaduk, N. Li, Silencing of hypoxia-inducible factor-1alpha gene attenuated angiotensin II-induced renal injury in Sprague–Dawley rats, Hypertension 58 (2011) 657–664.
[35] Z. Wang, Q. Zhu, M. Xia, P.L. Li, S.J. Hinton, N. Li, Hypoxia-inducible factor prolyl-hydroxylase 2 senses high-salt intake to increase hypoxia inducible factor 1alpha levels in the renal medulla, Hypertension 55 (2010) 1129–1136.
[36] L. Tacchini, E. Gammella, C. De Ponti, S. Recalcati, G. Cairo, Role of HIF-1 and NF-kappaB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals, J. Biol. Chem. 283 (2008) 20674–20686.
[37] H. Peinado, D. Olmeda, A. Cano, Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7 (2007) 415–428.
[38] A. Masszi, C. Di Ciano, G. Sirokmany, W.T. Arthur, O.D. Rotstein, J. Wang, C.A. McCulloch, L. Rosivall, I. Mucsi, A. Kapus, Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial–mesenchymal transition, Am. J. Physiol. Renal. Physiol. 284 (2003) F911–F924.
[39] G. Robert, C. Gaggioli, O. Bailet, C. Chavey, P. Abbe, E. Aberdam, E. Sabatie, A. Cano, A. Garcia de Herreros, R. Ballotti, S. Tartare-Deckert, SPARC represses E-cadherin and induces mesenchymal transition during melanoma development, Cancer Res. 66 (2006) 7516–7523.
[40] C.X. Li, M. Xia, W.Q. Han, X.X. Li, C. Zhang, K.M. Boini, X.C. Liu, P.L. Li, Reversal by growth hormone of homocysteine-induced epithelial-to-mesenchymal transition through membrane raft-redox signaling in podocytes, Cell. Physiol. Biochem. 27 (2011) 691–702.
[41] L. Larue, A. Bellacosa, Epithelial–mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways, Oncogene 24 (2005) 7443–7454.
[42] S. Tamiya, L. Liu, H.J. Kaplan, Epithelial–mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell–cell contact, Invest. Ophthalmol. Vis. Sci. 51 (2010) 2755–2763.
[43] Q. Zhou, R. Zeng, C. Xu, L. Liu, L. Chen, P. Kou, G. Pei, S. Bai, Y. Zhang, C. Li, S. Rong, M. Han, G. Xu, Erbin inhibits TGF-beta1-induced EMT in renal tubular epithelial cells through an ERK-dependent pathway, J. Mol. Med. (Berl) 90 (2012) 563–574.
[44] J.B. Kopp, TGF-beta signaling and the renal tubular epithelial cell: too much, too little, and just right, J. Am. Soc. Nephrol. 21 (2010) 1241–1243.
[45] S.C. Shih, K.P. Claffey, Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression, Growth Factors 19 (2001) 19–34.
[46] K.S. Chae, M.J. Kang, J.H. Lee, B.K. Ryu, M.G. Lee, N.G. Her, T.K. Ha, J. Han, Y.K. Kim, S.G. Chi, Opposite functions of HIF-alpha isoforms in VEGF induction by TGF-beta1 under non-hypoxic conditions, Oncogene 30 (2011) 1213–1228.
[47] K. Takeda, A. Cowan, G.H. Fong, Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system, Circulation 116 (2007) 774–781.
[48] J. Schodel, B. Klanke, A. Weidemann, B. Buchholz, W. Bernhardt, M. Bertog, K. Amann, C. Korbmacher, M. Wiesener, C. Warnecke, A. Kurtz, K.U. Eckardt, C. Willam, HIF-prolyl hydroxylases in the rat kidney: physiologic expression patterns and regulation in acute kidney injury, Am. J. Pathol. 174 (2009) 1663–1674.
[49] O. Aprelikova, G.V. Chandramouli, M. Wood, J.R. Vasselli, J. Riss, J.K. Maranchie, W.M. Linehan, J.C. Barrett, Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors, J. Cell. Biochem. 92 (2004) 491–501.
[50] J.H. Marxsen, P. Stengel, K. Doege, P. Heikkinen, T. Jokilehto, T. Wagner, W. Jelkmann, P. Jaakkola, E. Metzen, Hypoxia-inducible factor-1 (HIF-1) promotes

its degradation by induction of HIF-alpha-prolyl-4-hydroxylases, Biochem. J. 381 (2004) 761–767.
[51] H.L. Janssen, K.M. Haustermans, D. Sprong, G. Blommestijn, I. Hofland, F.J. Hoebers, E. Blijweert, J.A. Raleigh, G.L. Semenza, M.A. Varia, A.J. Balm, M.L. van Velthuysen, P. Delaere, R. Sciot, A.C. Begg, HIF-1A, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors, Int. J. Radiat. Oncol. Biol. Phys. 54 (2002) 1537–1549.
[52] Y. Su, M. Loos, N. Giese, E. Metzen, M.W. Buchler, H. Friess, A. Kornberg, P. Buchler, Prolyl hydroxylase-2 (PHD2) exerts tumor-suppressive activity in pancreatic cancer, Cancer 118 (2012) 960–972.
[53] A.C. Mackinnon, M.A. Gibbons, S.L. Farnworth, H. Leffler, U.J. Nilsson, T. Delaine, A.J. Simpson, S.J. Forbes, N. Hirani, J. Gauldie, T. Sethi, Regulation of transforming growth factor-beta1-driven lung fibrosis by galectin-3, Am. J. Respir. Crit. Care Med. 185 (2012) 537–546.
[54] M. Zeisberg, J.S. Duffield, Resolved: EMT produces fibroblasts in the kidney, J. Am. Soc. Nephrol. 21 (2010) 1247–1253.
[55] F. Strutz, H. Okada, C.W. Lo, T. Danoff, R.L. Carone, J.E. Tomaszewski, E.G. Neilson, Identification and characterization of a fibroblast marker: FSP1, J. Cell Biol. 130 (1995) 393–405.
[56] J. Camara, G. Jarai, Epithelial–mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha, Fibrogenesis Tissue Repair 3 (2010) 2.
[57] U. Valcourt, M. Kowanetz, H. Niimi, C.H. Heldin, A. Moustakas, TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial–mesenchymal cell transition, Mol. Biol. Cell 16 (2005) 1987–2002.
[58] T. Kawakita, E.M. Espana, K. Higa, N. Kato, W. Li, S.C. Tseng, Activation of Smad-mediated TGF-beta signaling triggers epithelial–mesenchymal transitions in murine cloned corneal progenitor cells, J. Cell. Physiol. 228 (2012) 225–234.
[59] M. Brandl, B. Seidler, F. Haller, J. Adamski, R.M. Schmid, D. Saur, G. Schneider, IKK(alpha) controls canonical TGF(ss)-SMAD signaling to regulate genes expressing SNAIL and SLUG during EMT in panc1 cells, J. Cell Sci. 123 (2010) 4231–4239.
[60] M. Sato, Y. Muragaki, S. Saika, A.B. Roberts, A. Ooshima, Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction, J. Clin. Invest. 112 (2003) 1486–1494.
[61] J. Xu, S. Lamouille, R. Derynck, TGF-beta-induced epithelial to mesenchymal transition, Cell Res. 19 (2009) 156–172.
[62] R. Kalluri, R.A. Weinberg, The basics of epithelial–mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428.
[63] W. Kriz, B. Kaissling, M. Le Hir, Epithelial–mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy? J. Clin. Invest. 121 (2011) 468–474.
[64] M. Fragiadaki, R.M. Mason, Epithelial–mesenchymal transition in renal fibrosis — evidence for and against, Int. J. Exp. Pathol. 92 (2011) 143–150.
[65] M. Zeisberg, E.G. Neilson, Mechanisms of tubulointerstitial fibrosis, J. Am. Soc. Nephrol. 21 (2010) 1819–1834.