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URG11 mediates hypoxia-induced epithelial-to-mesenchymal transition by modulation of E-cadherin and β-catenin

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ABSTRACT

Upregulated gene 11 (URG11), recently identified as a new HBx-upregulated gene that may activate β-catenin and Wnt signaling, was found to be upregulated in a human tubule cell line under low oxygen. Here, we investigated the potential role of URG11 in hypoxia-induced renal tubular epithelial-to-mesenchymal (EMT). Overexpression of URG11 in a human proximal tubule cell line (HK2) promoted a mesenchymal phenotype accompanied by reduced expression of the epithelial marker E-cadherin and increased expression of the mesenchymal markers vimentin and α-SMA, while URG11 knockdown by siRNA effectively reversed hypoxia-induced EMT. URG11 promoted the expression of β-catenin and increased its nuclear accumulation under normoxic conditions through transactivation of the β-catenin promoter. This in turn upregulated β-catenin/T-cell factor (TCF) and its downstream effector genes, vimentin, and α-SMA. In vivo, strong expression of URG11 was observed in the tubular epithelia of 5/6-nephrectomized rats, and a Western blot analysis demonstrated a close correlation between HIF-1α and URG11 protein levels. Altogether, our results indicate that URG11 mediates hypoxia-induced EMT through the suppression of E-cadherin and the activation of the β-catenin/TCF pathway.

Introduction

URG11 (upregulated gene 11) has recently been identified as a HBx-upregulated gene that plays a key role in HBx-related hepatocellular carcinoma (HCC) [1]. Further studies showed that URG11 was essential in mediating HCC tumorigenesis through the transactivation of β-catenin [2]. Recently, a monoclonal antibody recognizing URG11 was established in our laboratory [3]. Using this specific antibody, we found that the expression of URG11 and β-catenin was correlated in metastatic gastric cancer tissues. Further studies demonstrated that URG11 activated transcription via the β-catenin promoter in gastric cancer cells, leading to the accumulation of β-catenin in nucleus, where it binds to members of the T-cell factor (TCF) family of transcription factors to increase the expression of target genes and thereby promote gastric cancer cell invasion and metastasis [4]. These findings strongly suggest that URG11 may be a regulatory element in the β-catenin signaling pathway.

β-Catenin is a key component of the adherens junctions that link the actin cytoskeleton to cadherins (transmembrane cell–cell adhesion receptors that are critical for establishment and maintenance of epithelial layers) [5]. In addition, β-catenin is a central and essential component in the Wnt signaling cascade [6]. In normal cells, free cytosolic β-catenin is rapidly phosphorylated by a multiprotein complex that includes adenomatous polyposis coli (APC), axin/conductin, and GSK-3β. The latter phosphorylates serine and threonine residues on β-catenin and promotes its degradation [7]. While the Wnt signal may be aberrantly activated when APC, β-catenin, or axin/conductin have been mutated, or Wnt ligands are improperly regulated, the degradation complex can be inhibited, leading to the stabilization of cytoplasmic β-catenin. This, in turn, results in the nuclear accumulation of β-catenin, which can bind to members of the T-cell factor (TCF) family of transcription factors (including LEF-1) [8,9]. These β-catenin–LEF-1 transcription complexes then cause the increased expression of target genes (vimentin, fibronectin, α-SMA) that drive the development of the mesenchymal phenotype [10,11].

As URG11 has been shown to activate β-catenin in tumor cells, it is of great interest to determine whether URG11 may cause epithelial-to-mesenchymal transition (EMT) via the β-catenin pathway. In this study, we showed that hypoxia induced the expression of HIF-1α and URG11 in HK2 cells. Moreover, URG11 mediated hypoxia-induced kidney tubular EMT through the suppression of E-cadherin and the activation of Wnt/β-catenin signaling.

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Materials and methods

Cell culture and experimental conditions. The human proximal tubular epithelial cell line HK2, described previously [12], was cultured in DMEM/F12 medium (Invitrogen Inc.) supplemented with 10% fetal calf serum (Gibco–BRL Life Technologies, Burlington, Ontario, Canada). The cells were seeded at 1.5×106 cells/10 cm diameter dish for 3–4 days at 37 °C in a humidified atmosphere containing 5% CO_2. Hypoxic conditions were attained by incubation of cells in an anaerobic box. The oxygen was maintained at 1% by a compact gas oxygen controller incubator (Precision Scientific, Winchester, VA, USA) with a residual gas mixture composed of 94% N_2 and 5% CO_2.

Plasmid constructs and transfection. Sense expression and siRNA vectors for URG11 and a pGL3-β-catenin reporter vector containing a fragment (−298 to +139) of the β-catenin promoter were constructed as described [4]. All plasmids were verified by direct DNA sequencing. Synthetic siRNAs for β-catenin and nonspecific control pools were purchased from Upstate Biotechnology (Lake Placid, NY). Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Total RNA and protein were prepared 48 h after transfection and were used for quantitative reverse transcription PCR or Western blotting analysis.

Quantitative reverse transcription PCR. Total RNA was extracted from the cells using Trizol (Invitrogen) according to the manufacturer's protocol. Aliquots (5 mg) of RNA were reverse transcribed to cDNA using Superscribe First-Strand Synthesis System (Invitrogen Corporation). Aliquots (1, 2, and 4 μL) of cDNA were used as templates for PCR with primers specific to URG11, β-catenin, E-cadherin, α-SMA, vimentin, and GAPDH. The sequences of the PCR primers are listed in Table 1.

Immunohistochemistry. For immunohistochemistry, 2–3 μm sections of formalin-fixed paraffin-embedded specimens were taken. Sections on slides were deparaffinized in xylene and rehydrated serially with alcohol and water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 min, and blocked in 10% normal goat or rabbit serum for 1 h. The slides were then incubated with primary anti-URG11 [3], anti-HIF-1α, anti-β-catenin, anti-α-SMA, or anti-E-cadherin antibodies (1:100 Santa Cruz, Inc., Santa Cruz, CA, USA) at 4 °C overnight. The sections were incubated with biotinylated goat anti-rabbit/mouse Ig secondary antibody, and the antibody reactions were visualized using diaminobenzidine (DAKO, Tokyo, Japan). Goat or rabbit IgG (irrelevant isotypes) were used as negative controls. Four to five sections per rat were assessed for immunoreactive areas ($n = 6$).

Protein preparation and Western blot. Protein was extracted from kidney tissues (0.1 mg) and cells (2×10^6) with lysis buffer as described [4]. Protein concentration was determined using the Bradford method. For Western blot, 60 μg of total protein or a nuclear protein fraction were electrophoresed on 10% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 10% fat-free milk in TBS (20 mmol/L Tris, 0.15 mol/L NaCl (pH 7.0), 0.1% Tween 20), the membranes were incubated with primary antibody: anti-URG11, anti-β-catenin (both 1:500), anti-HIF-1α, anti-E-cadherin, anti-α-SMA, or anti-vimentin (each at 1:200). After several washes, the membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) at a 1:2000 dilution. Protein bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and exposed on Kodak X-OMAT film (Rochester, New York, USA). A Western blot for β-actin (mouse monoclonal antibody, 1:5000, Sigma Chemical Co.) was performed as an internal control. Autoradiograms were quantified by densitometry with Bio Image IQ software. Relative protein levels were calculated as a ratio to the level of β-actin in the sample.

Dual-luciferase reporter assay. HK2 cells were plated at a density of 1.5×10^5/35 mm dish at 24 h before transfection. The plasmid pGL3-β-catenin (0.2 μg) was transfected in every case and the pRL-TK vector (Promega, 0.04 μg) was used as an internal control. Cotransfection experiments were performed with 0.2, 0.4, and 0.8 μg of pCDNA-URG11 plasmid. After cultivation for 48 h, the transfected cells were harvested, lysed, and subjected to the luciferase assay. Luciferase activity was measured as chemiluminescence in a luminometer (Perkin-Elmer, Norwalk, CT, USA) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

The TCF reporter plasmid kit, which contained the TOPflash, FOPflash, and TK vectors, was purchased from Upstate Biotechnology (Lake Placid, NY). HK2 cells were cotransfected transiently with 0.8 μg URG11 (or pcDNA3.1), 0.2 μg TOPflash (or FOPflash), and 0.04 μg TK vectors using Lipofectamine 2000. Cells were collected 24 h later and analyzed using the dual-luciferase reporter assay system. The ratio of firefly luciferase activity (TOPflash or FOPflash) to *Renilla* luciferase activity (TK vector) was used to estimate changes in β-catenin-mediated transcription.

Animal model. Male Sprague–Dawley rats weighing 150–180 g were obtained from our University's Laboratory Animal Center (Xi'an, China). Chronic renal hypoxia was induced by 5/6 subtotal nephrectomy. All rats were sacrificed 12 weeks after nephrectomy, and serum was collected for determination of creatinine and urea nitrogen. The samples were immediately excised; some were fixed with 4% paraformaldehyde and others were stored in −80 °C until analysis. Kidney sections were stained by the periodic acid–Schiff method and examined by light microscopy. All animal handling conformed to the guidelines for care and use of experimental animals established by the Ethical Committee of Animal Experiments.

Statistical analysis. Each experiment was repeated at least three times. Bands from Western blots were quantified with Quantity One software (Bio-Rad, Hercules, CA, USA). Relative protein and mRNA levels were calculated in comparison to internal β-actin or GAPDH standards. Numerical data are presented as means ± SD. The difference between means was analyzed with ANOVA. Differences were considered significant when $p < 0.05$.

Table 1
PCR primers for URG11 and Wnt/β-catenin pathway genes.

Gene	Primers
URG11	5′-TGAATCAAGGAGTCGCTGGAC-3′ 5′-GCATCTCACTGGAACACAAG-3′
β-Catenin	5′-ATTTGATGGAGTTGGACATGG-3′ 5′-TGTTCTTGAGTGAAGGACTGA-3′
E-cadherin	5′-CAGGATTACAAGTTCCCGCCA-3′ 5′-CACTGTCCGCTGCCTTCAG-3′
Vimentin	5′-TGTCCTCGTCCTCCTACCGC-3′ 5′-AGCTGCTCGAGCTCAGCCAGC-3′
α-SMA	5′-AGGCGGTGCTGTCTCTCTAT-3′ 5′-GACATTGTGGGTGACACCAT-3′
GAPDH	5′-CAA CGGATTTGGTCGTATTGGG-3′ 5′-CCTGGAAGATGGTGATGGGATT-3′

Results

Hypoxia induces URG11 mRNA and protein in vitro

We induced hypoxia in vitro by incubating HK2 cells in 1% O_2 and 5% CO_2 from 2 to 72 h. As shown in Fig. 1, the expression of URG11 mRNA and protein was markedly upregulated at 2 h and

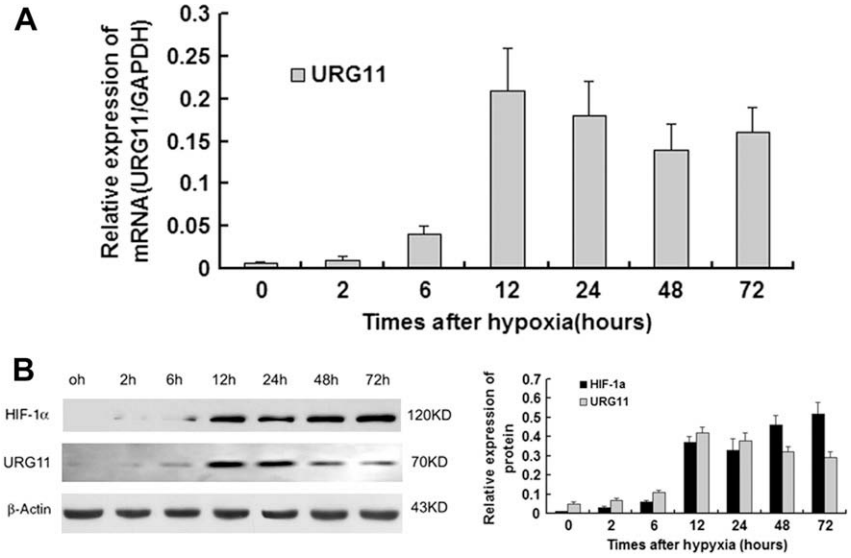

Fig. 1. Hypoxia induced URG11 mRNA and protein expression in HK2. (A) qRT-PCR analysis of URG11 mRNA expression after 2, 6, 12, 24, 48, and 72 h of hypoxia. (B) Western blot analysis of HIF-1α and URG11 protein expression after different times of hypoxia.

reached a maximum level at 12 h under low oxygen stimulation. URG11 remained upregulated for more than 72 h, consistent with the expression pattern of hypoxia-inducible factor 1α (HIF-1α; Fig. 1B). In normoxic control cells, only trace URG11 and HIF-1α expression was detected.

URG11 promotes EMT in HK2 cells under low oxygen

To investigate the effect of URG11 on EMT, HK2 cells were transfected with a vector containing full-length URG11 to overexpress this protein under normoxia. At 48 h after transfection, the expression of E-cadherin, vimentin, and α-SMA were detected by qRT-PCR. As shown in Fig. 2A, the epithelial marker E-cadherin was downregulated in URG11-transfected cells compared with control cells. In contrast, the mesenchymal markers vimentin and α-SMA were upregulated in URG11-transfected cells compared with control cells. Western blots were performed to confirm that the EMT phenotype is induced in renal tubular cells by URG11. Decreased E-cadherin and increased vimentin and α-SMA staining was observed in URG11-transfected HK2 cells relative to control cells (Fig. 2B). Light microscopy revealed that parental cells and empty vector-transfected cells formed a cobblestone-like monolayer, which is typical of epithelia. URG11-transfected cells also formed an epithelial monolayer, but there were larger gaps between cells and the cells were larger and more elongated than control cells (Fig. 2C).

Next, we asked whether URG11 was involved in the EMT of hypoxia-induced HK2 cells. HK2 cells were transfected with siRNA targeting URG11 or a control siRNA after 48 h of growth under low oxygen. After another 48 h, the cells were collected and the expression of E-cadherin, vimentin, and α-SMA were evaluated by Western blot. After 48 h of hypoxia, the level of E-cadherin protein was significantly reduced, whereas vimentin and α-SMA were upregulated in these cells (Fig. 2D). Transfection with siRNA against URG11 abrogated this increase in E-cadherin such that levels were similar in hypoxic and normoxic cells (Fig. 2D). The expression of vimentin and α-SMA were correspondingly reduced in hypoxic cells transfected with URG11 siRNA (Fig. 2D). These experimental data show that URG11 is involved in the EMT of hypoxia-induced tubular cells.

URG11 activates β-catenin and β-catenin/TCF transcription

URG11 has been demonstrated to activate the β-catenin/TCF pathway in HCC and GC cells, and β-catenin/TCF is sufficient to promote EMT in HK2 cells [2,3,13]. Here, we investigated whether URG11 could activate β-catenin and β-catenin/TCF signaling in HK2 cells. First, we measured the level of β-catenin mRNA in URG11-transfected and control HK2 cells. qRT-PCR showed that β-catenin mRNA was markedly upregulated in URG11-transfected HK2 cells compared with control HK2 cells (Fig. 3A, left panel). Next, Western blotting showed that both the total and nuclear levels of β-catenin protein were increased in URG11-transfected HK2 cells (Fig. 3A, right panel). In addition, we investigated whether β-catenin was transcriptionally activated by URG11 via a dual-luciferase reporter assay. HK2 cells were transiently cotransfected with a pGL3-β-catenin reporter vector and an URG11 or pcDNA3.1 (control) plasmid. Cotransfection with different amounts of URG11 resulted in a 3.8- to 5.5-fold increase in relative luciferase activity compared to transfection with pcDNA3.1 (Fig. 3B). These results indicated that URG11 may upregulate β-catenin by activating its transcription (i.e., through its promoter).

It is well known that β-catenin interacts with the TCF/LEF family of transcription factors upon activation of the Wnt signaling pathway. To determine whether URG11 had a role in β-catenin/TCF signal gene regulation, HK2 cells were transiently transfected with a TOPflash vector that contained wild-type TCF/LEF binding sites upstream of a luciferase reporter gene. Changes in β-catenin-mediated transcription were examined after URG11 transfection. An increase in luciferase activity was observed, probably reflecting a steady increase in the nuclear accumulation of β-catenin (Fig. 3C). In contrast, luciferase activity remained at the background level in cells transfected with a FOPflash vector containing mutated TCF/LEF binding sites. Therefore, we can conclude that URG11 promotes the activation of TCF/LEF transcription factors by transactivating β-catenin.

Next, we sought to investigate whether β-catenin/TCF was required for the URG11-mediated increase in EMT. HK2 cells were transiently cotransfected with URG11 or a control plasmid and siRNA targeting β-catenin or a control siRNA sequence, and the effects on cell EMT were determined as described above. Fig. 3D shows

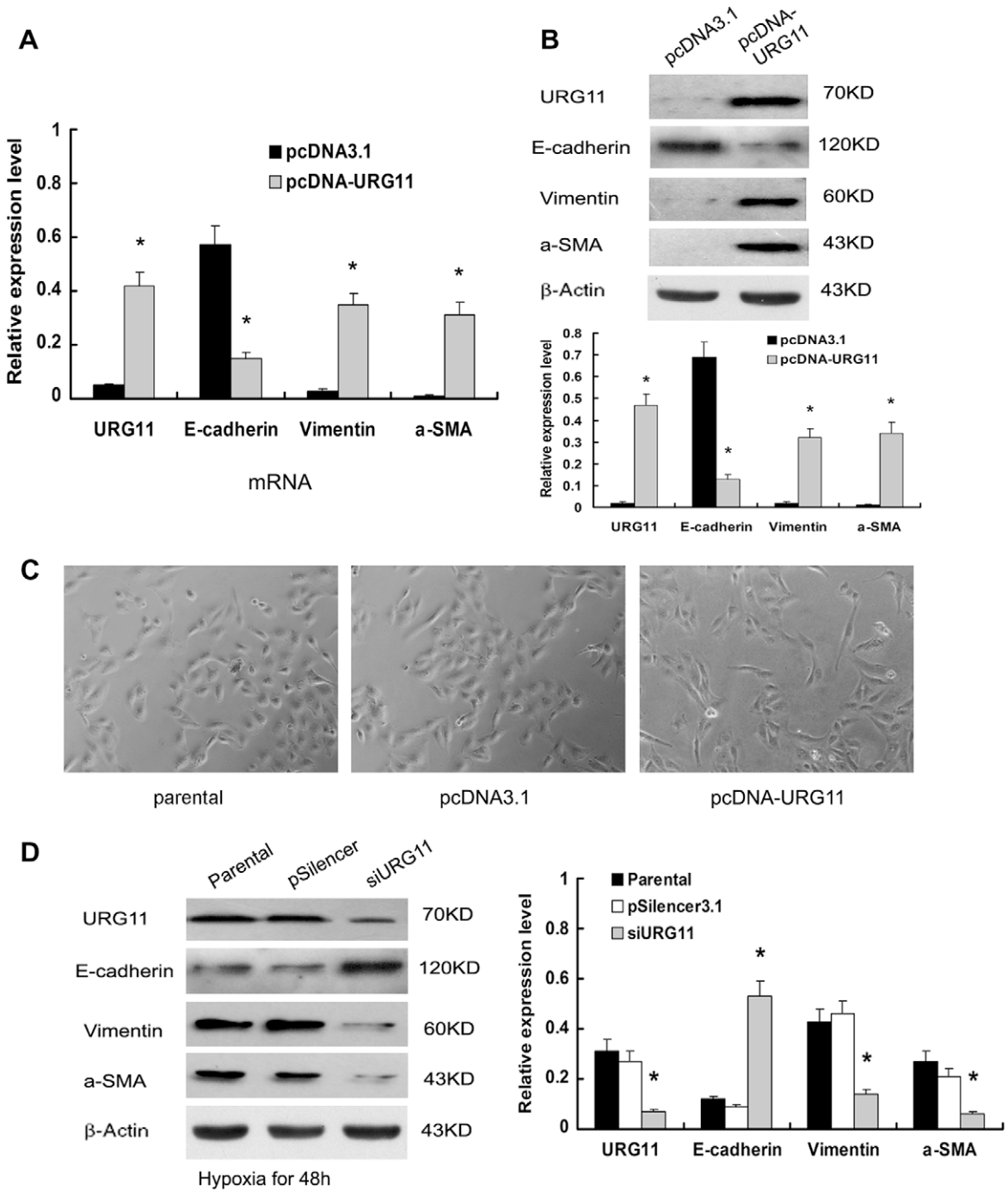

Fig. 2. URG11-induced EMT in HK2 cells under hypoxic conditions. (A) qRT-PCR analysis of E-cadherin, α-SMA, vimentin, and URG11 expression in URG11-transfected and pcDNA3.1 (control vector)-transfected cells. The histogram shows the average volume density normalized to the loading control, β-actin ($n = 3$). (B) Western blot analysis of E-cadherin, α-SMA, and vimentin expression in URG11-transfected and pcDNA3.1-transfected cells. (C) Morphological changes in cells. Both parental and pcDNA3.1-transfected cells showed a typical cuboidal epithelial shape, whereas URG11-transfected cells were elongated and larger than control cells, consistent with the morphology of myofibroblasts. Magnification: 200×. (D) Western blot analysis of URG11, E-cadherin, and vimentin expression in parental cells, pSilencer3.1 empty vector-transfected cells, and URG11 siRNA transfected cells after 48 h under hypoxic conditions. $^{*}p < 0.05$ compared with the parental cells and the pSilencer empty vector cells.

that transfection with β-catenin siRNA reversed the URG11-mediated increase in β-catenin and restored β-catenin levels to those present in parental cells. The expression of vimentin and α-SMA was correspondingly reduced in URG11-transfected cells after treat with β-catenin siRNA. These results indicated that URG11 promotes the mesenchymal transition of HK2 cells through the β-catenin/TCF pathway.

URG11 participates in hypoxia-induced renal fibrosis in vivo

The 5/6 nephrectomy is the classic fibrosis model, and is also used as a model of hypoxia [14]. Hence, we used this model to investigate hypoxia-related renal fibrogenesis. As described in our previous work [12], light microscopic examination revealed glomerular sclerosis and interstitial fibrosis in 5/6-nephrectomized

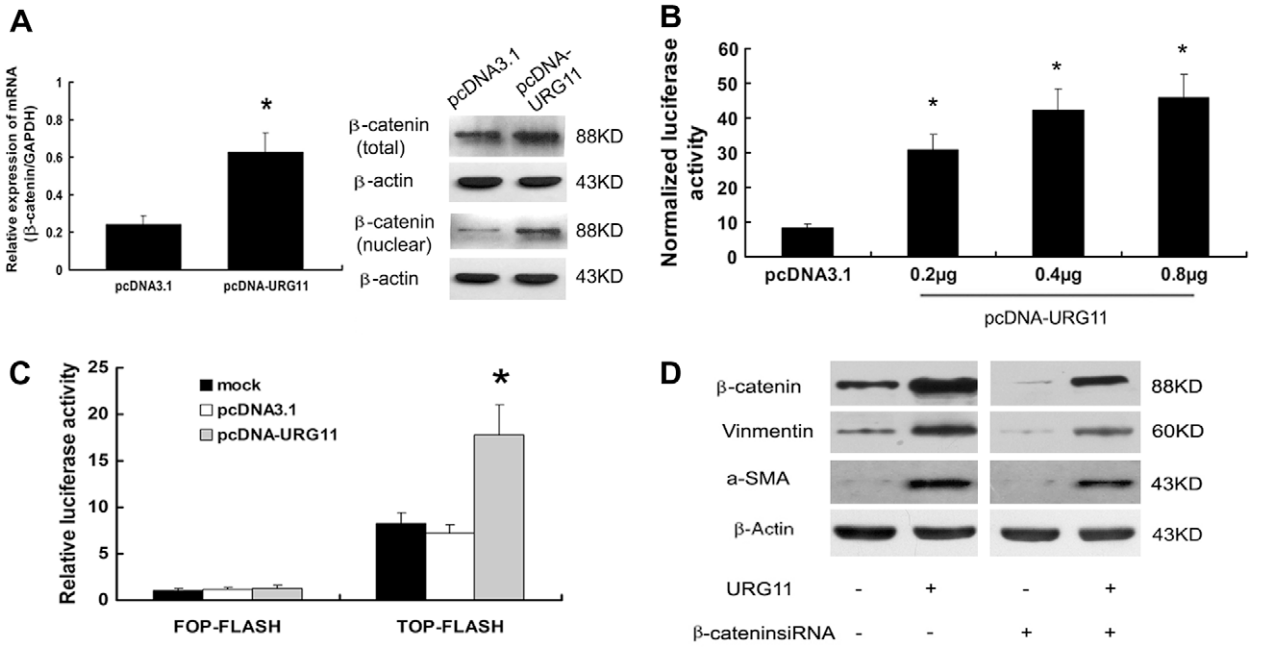

Fig. 3. URG11 activates β-catenin and promotes β-catenin/TCF mediated transcription. (A) The expression of β-catenin mRNA in transfected HK2 cells was examined by RT-PCR (left panel). The total and nuclear protein levels of β-catenin in transfected HK2 cells were evaluated by Western blot (right panel). β-Actin was used as an internal control. (B) The relative activity of the β-catenin promoter in HK2 cells cotransfected with URG11 or pcDNA3.1 was evaluated with a dual-luciferase reporter assay. $^{*}p < 0.01$ vs. cells transfected with pcDNA3.1. (C) Activation of β-catenin-mediated transcription in HK2 cells by URG11. A dual luciferase assay was performed in HK2 cells transfected with a TOPflash or FOPflash vector alone or cotransfected with URG11 or pcDNA3.1. The ratio of firefly luciferase activity (TOPflash or FOPflash) to *Renilla* luciferase activity (TK vector) was used to estimate changes in β-catenin-mediated transcription. $^{*}p < 0.01$ vs. cells transfected with pcDNA3.1. (D) β-Catenin-specific siRNA abolished URG11-induced vimentin and α-SMA expression. URG11 and β-catenin-specific siRNAs were cotransfected into HK2 cells with pcDNA3.1 or scrambled siRNA as control. Western blot analysis was performed to detect the expression of β-catenin, vimentin, and α-SMA proteins 48 h later.

rats. Immunohistochemical staining showed that URG11 was mainly expressed in the cytoplasm of renal tubule cells in 5/6-nephrectomized rats, whereas there was almost no staining in sham-operated kidneys (Fig. 4A). HIF-1α staining was also markedly increased in many of the transdifferentiated tubular cells (Fig. 4A). Meanwhile, decreased E-cadherin and increased α-SMA staining were observed in renal tubular epithelial cells after subtotal nephrectomy. Western blots also revealed that URG11 and HIF-1α protein expression were significantly elevated in the kidneys of 5/6-nephrectomized rats compared with control kidneys (Fig. 4B). In light of our in vitro findings, this result suggests that URG11 is involved in hypoxia-induced renal fibrosis.

Discussion

Chronic hypoxia may represent an early and potentially initiating event in the development and progression of renal disease, including renal fibrogenesis [15–17]. Increasing evidence from biopsies and animal models suggests that chronic tubulointerstitial hypoxia induced by the distortion and loss of peritubular capillaries aggravates the development of tubulointerstitial fibrosis [15,18]. Recent studies have suggested that chronic hypoxia could induce tubular cells to transdifferentiate and become myofibroblasts [19]. These myofibroblasts could then migrate to the tubular interstitium and sequentially induce renal fibrogenesis [20]. However, the mechanisms underlying hypoxia-induced tubular EMT remain to be explored. In this paper, we have demonstrated that chronic hypoxia can induce the expression of URG11 in renal tubular cells and remnant kidneys. Furthermore, we investigated the effects of URG11 on hypoxia-induced renal tubular EMT on two cellular mechanisms: (a) suppression of E-cadherin expression, and (b) activation of β-catenin/TCF and downstream genes.

URG11 is a key Wnt/β-catenin pathway regulator, and it can be induced by exogenous stimuli [2]. Moreover, URG11 expression is inversely correlated with E-cadherin expression in liver cancer tissues. Although there is no direct evidence that URG11 can directly downregulate expression of E-cadherin, URG11 was suspected to be responsible for HBx-induced E-cadherin suppression in liver cancers [21]. E-cadherin is an epithelial cell–cell adhesion protein that is essential for the formation and maintenance of epithelia in the embryonic stage, and for homeostasis and maintenance of epithelial tissue architecture in adult tissues [22–24]. In the kidney, E-cadherin plays an essential role in the maintenance of structural integrity and cell polarity in the normal tubular epithelium [25,26]. Therefore, the loss of E-cadherin would lead to the destabilization of the renal epithelium, causing tubular cells to dissociate from their neighbors and lose polarity. Indeed, the loss of E-cadherin is an early event in EMT, which occurs in four characteristic steps: (1) loss of epithelial adhesion, followed by (2) α-SMA induction and actin cytoskeleton reorganization, (3) tubular basement membrane destruction, and (4) enhanced migration and invasion [27]. In this study, we found that URG11 almost completely abolished E-cadherin expression in the tubular epithelia of 5/6-nephrectomized rats, as well as in tubular epithelial cells cultured under hypoxic conditions. Although the underlying mechanism of E-cadherin inhibition remains to be elucidated, the finding that URG11 was able to target this key EMT gene highlights the fundamental role of URG11 in controlling first step of EMT.

Another important mechanism of URG11-induced EMT is the β-catenin-mediated transcription program. Our previous work suggested that URG11 could transactivate β-catenin and promote β-catenin-mediated transcription [2,3]. β-Catenin functions as a component of adherent cell–cell junctions. β-Catenin promotes cell adhesion by binding to the intracellular domain of E-cadherin and

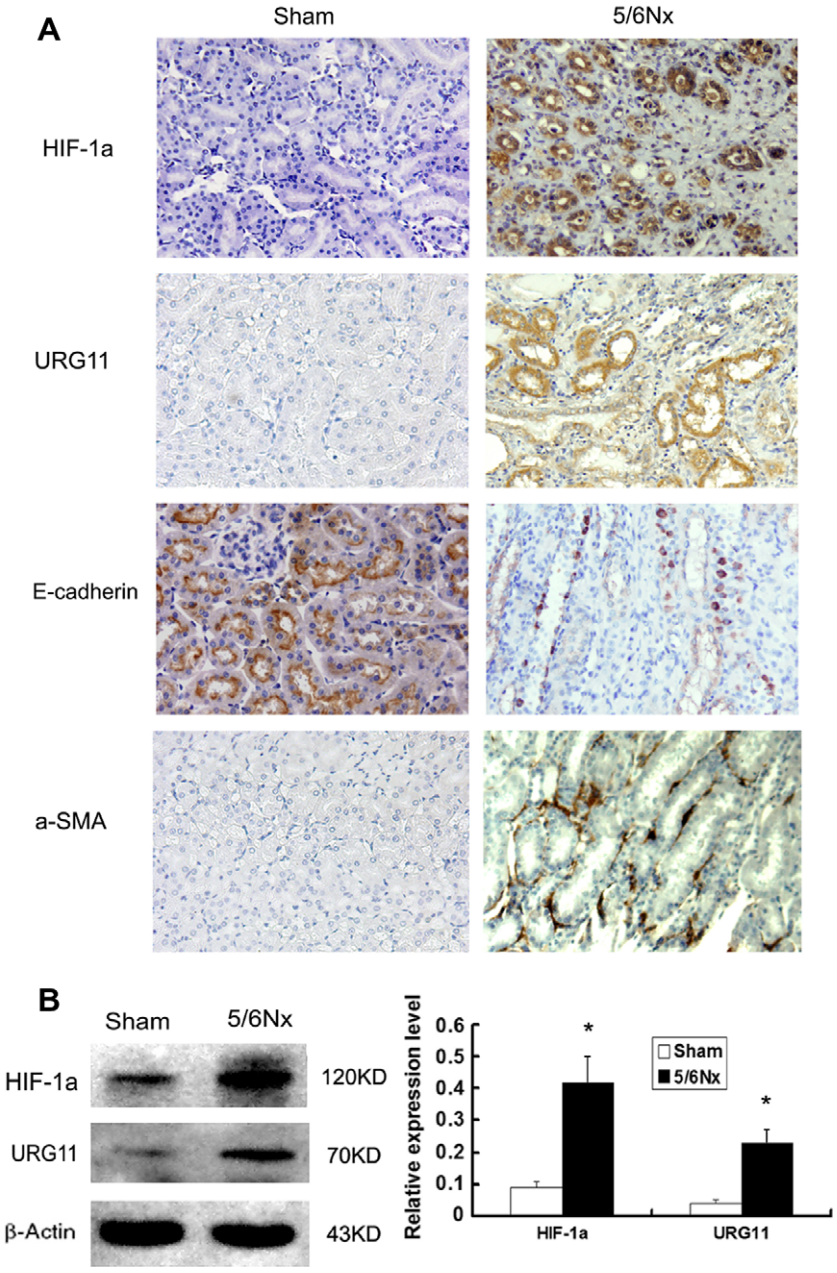

Fig. 4. Analysis of URG11 and HIF-1α expression in hypoxia-induced rat renal fibrosis. (A) Immunohistochemical analysis of HIF-1α, URG11, E-cadherin, and α-SMA in the kidney tissue of 5/6-nephrectomized rats and sham-operated rats. Increased HIF-1α, URG11, and α-SMA staining and reduced E-cadherin staining was observed in tissues of 5/6-nephrectomized rats. Original magnification: 200×. (B) Western blot analysis of HIF-1α and URG11 expression in kidney tissue from 5/6-nephrectomized and sham-operated rats. $^{*}p < 0.05$ compared to sham-operated rats.

also acts as a transcriptional co-activator by binding to members of the TCF/LEF family of transcription factors [8,28]. Indeed, several studies suggest that β-catenin-mediated transcription can induce the expression of Slug [29] and Twist1 [30], thereby contributing to the EMT program. A recent study by the Medici group [13] showed that β-catenin/TCF-4-dependent expression of TGFβ3 is responsible for snail-induced EMT [13]. In this study, we found that URG11 overexpression increased β-catenin transcription, promoting β-catenin/TCF activity and in turn inducing the expression of the mesenchymal markers α-SMA and vimentin. We therefore concluded that URG11 overexpression trans-activated β-catenin and promoted its nuclear translocation, thus leading to the upregulation of β-catenin/TCF target genes that subsequently cause mesenchymal transition.

Next, we investigated the underlying mechanisms through which URG11 expression is regulated by hypoxia. Significant evidence has demonstrated that hypoxia-inducible factor (HIF) is crucial in hypoxia-induced EMT. Work from the Haase group and our lab has shown that hypoxia aggravates renal fibrogenesis through the activation of HIF-1 and its target genes [12,16]. HIF-1 is a transcription factor that is activated in mammalian cells cultured under low oxygen [31]. It binds a core sequence of the HRE (hypoxia responsive element) in the promoters of hypoxia-responsive genes and induces their expression [32]. Here, we identified URG11 as a putative HIF-1α target gene. The expression of URG11 was upregulated by hypoxia in renal tubular cells and was highly consistent with that of HIF-1α in hypoxic renal fibrosis. Furthermore, bioinformatics reveals the presence of six hypoxia-

responsive regions in the URG11 promoter. However, more evidence is required to confirm whether URG11 expression is directly regulated by HIF-1α.

In conclusion, our findings revealed that URG11 is a hypoxia responsive factor that is critical for the induction of EMT in renal tubular cells. The experimental data indicated that URG11-induced EMT, at least in part, by blocking E-cadherin and promoting β-catenin/TCF activity.

Acknowledgments

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References

[1] Z. Lian, J. Liu, L. Li, X. Li, N.L. Tufan, M. Clayton, M.C. Wu, H.Y. Wang, P. Arbuthnot, M. Kew, M.A. Feitelson, Upregulated expression of a unique gene by hepatitis B × antigen promotes hepatocellular growth and tumorigenesis, Neoplasia 5 (2003) 229–244.
[2] Z. Lian, J. Liu, L. Li, et al., Enhanced cell survival of Hep3B cells by the hepatitis B × antigen effector, URG11, is associated with upregulation of beta-catenin, Hepatology 43 (2006) 415–424.
[3] X. Zou, X. Li, J. Liu, et al., Preparation and characterization of a specific monoclonal antibody against a new gene product: URG11, Hybridoma 25 (2006) 378–381.
[4] R. Du, L. Xia, S. Sun, et al., URG11 promotes gastric cancer growth and invasion by activation of beta-catenin signaling pathway, J. Cell. Mol. Med. (2008) (Epub ahead of print).
[5] C. Jamora, E. Fuchs, Intercellular adhesion, signalling and the cytoskeleton, Nat. Cell Biol. 4 (2002) 101–108.
[6] R. Fodde, T. Brabletz, Wnt/beta-catenin signaling in cancer stemness and malignant behavior, Curr. Opin. Cell Biol. 19 (2007) 150–158.
[7] J. Nelson, R. Nusse, Convergence of Wnt, beta-catenin, and cadherin pathways, Science 303 (2004) 1483–1487.
[8] P. Morin, A.B. Sparks, V. Korinek, et al., Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC, Science 275 (1997) 1787–1790.
[9] J. Behrens, J.P. von Kries, M. Kuhl, et al., Functional interaction of beta-catenin with the transcription factor LEF-1, Nature 382 (1996) 638–642.
[10] K. Kim, Z. Lu, E.D. Hay, Direct evidence for a role of beta-catenin/LEF-1 signalling pathway in the induction of EMT, Cell Biol. Int. 26 (2002) 463–476.
[11] Y. Jing, A.W. Robert, Epithelial–mesenchymal transition: at the crossroads of development and tumor metastasis, Dev. Cell 14 (2008) 818–829.
[12] S. Sun, X. Ning, Y. Zhang, et al., Hypoxia-inducible factor-1alpha induces Twist expression in tubular epithelial cells subjected to hypoxia, leading to epithelial-to-mesenchymal transition, Kidney Int. 75 (2009) 1278–1287.
[13] D. Medici, E.D. Hay, B.R. Olsen, Snail and Slug promote epithelial–mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3, Mol. Biol. Cell 19 (2008) 4875–4887.
[14] R. Zeng, Y. Yao, M. Han, et al., Biliverdin reductase mediates hypoxia-induced EMT via PI3-kinase and Akt, J. Am. Soc. Nephrol. 19 (2008) 380–387.
[15] K. Manotham, T. Tanaka, M. Matsumoto, et al., Evidence of tubular hypoxia in the early phase in the remnant kidney model, J. Am. Soc. Nephrol. 15 (2004) 1277–1288.
[16] D.F. Higgins, K. Kimura, W.M. Bernhardt, et al., Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition, J. Clin. Invest. 117 (2007) 3810–3820.
[17] L.G. Fine, D. Bandyopadhay, J.T. Norman, Is there a common mechanism for the progression of different types of renal diseases other than proteinuria? Towards the unifying theme of chronic hypoxia, Kidney Int. 75 (2000) S22–S26.
[18] T. Tanaka, T. Miyata, R. Inagi, et al., Hypoxia in renal disease with proteinuria and/or glomerular hypertension, Am. J. Pathol. 165 (2004) 1979–1992.
[19] K. Manotham, T. Tanaka, M. Matsumoto, et al., Transdifferentiation of cultured tubular cells induced by hypoxia, Kidney Int. 65 (2004) 871–880.
[20] Y.Y. Ng, T.P. Huang, W.C. Yang, et al., Tubular epithelial myofibroblast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats, Kidney Int. 54 (1998) 864–876.
[21] J. Liu, Z. Lian, S. Han, et al., Downregulation of E-cadherin by hepatitis B virus X antigen in hepatocellullar carcinoma, Oncogene 25 (2006) 1008–1017.
[22] E. Levine, C.H. Lee, C. Kintner, et al., Selective disruption of E-cadherin function in early *Xenopus* embryos by a dominant negative mutant, Development 120 (1994) 901–909.
[23] L. Larue, M. Ohsugi, J. Hirchenhain, et al., E-cadherin null mutant embryos fail to form a trophoectoderm epithelium, Proc. Natl. Acad. Sci. USA 91 (1994) 8263–8267.
[24] D. Riethmacher, V. Brinkmann, C. Birchmeier, A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development, Proc. Natl. Acad. Sci. USA 92 (1995) 855–859.
[25] M.F. Horster, G.S. Braun, S.M. Huber, Embryonic renal epithelia: Induction, nephrogenesis, and cell differentiation, Physiol. Rev. 79 (1999) 1157–1191.
[26] M.A. Huber, N. Kraut, H. Beug, Molecular requirements for epithelial–mesenchymal transition during tumor progression, Curr. Opin. Cell Biol. 17 (2005) 548–558.
[27] Y. Liu, Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention, J. Am. Soc. Nephrol. 15 (2004) 1–12.
[28] C. Jamora, E. Fuchs, Intercellular adhesion, signalling and the cytoskeleton, Nat. Cell. Biol. 4 (2002) 101–108.
[29] M. Conacci-Sorrell, I. Simcha, T. Ben-Yedidia, et al., Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK, J. Cell Biol. 163 (2003) 847–857.
[30] T.T. Onder, P.B. Gupta, S.A. Mani, et al., Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways, Cancer Res. 68 (2008) 3645–3654.
[31] G.L. Wang, B.-H. Jiang, E.A. Rue, et al., Hypoxia-inducible factor 1 is a basic-helix–loop–helix–PAS heterodimer regulated by cellular O(2) tension, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[32] M. Nangaku, K.U. Eckardt, Hypoxia and the HIF system in kidney disease, J. Mol. Med. 85 (2007) 1325–1330.