Hypoxia-inducible factor-1α induces twist expression in tubular epithelial cells subjected to hypoxia leading to epithelial-to-mesenchymal transition

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Epithelial-to-mesenchymal transition (EMT) induced by chronic hypoxia is one of the critical causes of renal fibrosis. Twist, a basic helix-loop-helix (bHLH) transcription factor, is believed to be important in promoting the EMT. We found that the expression of Twist was increased in human tubule cell lines (HK-2 and HKC) grown under hypoxic conditions. This was accompanied by a knockdown of Twist by short interfering RNA (siRNA) and overexpression of Twist by transfection in cells subjected to hypoxia. Electrophoretic mobility shift and chromatin immunoprecipitation assays identified the presence of a functional Twist binding site in the proximal Twist gene promoter. In an in vivo assay using the rat remnant kidney we found that both Twist and HIF-1α were overexpressed in tubular epithelial cells showing EMT. These studies suggest that twist expression is involved in EMT during renal fibrogenesis.

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Chronic hypoxia is one of the final pathways that lead to end-stage renal failure.1 The development of renal interstitial fibrosis in chronic kidney disease is aggravated by chronic tubulointerstitial hypoxia.2,3 Emerging evidence has suggested a crucial role for hypoxia in the tubulointerstitium before structural microvasculature damage in the corresponding region, emphasizing the pathogenic role of this condition from an early stage of kidney disease.1,4 An important mechanism underlying the development of renal interstitial fibrosis was epithelial-to-mesenchymal transition (EMT).5–8 Recent studies have suggested that hypoxia, a critical mediator of kidney disease progression,2 could induce transdifferentiation of tubular cells into myofibroblasts, which was characterized by the expression of mesenchymal markers, phenotypic alterations, and acquisition of mobility.9 The myofibroblasts transdifferentiated from tubular cells migrated to the tubular interstitium and sequentially induced renal fibrogenesis.10,11 However, the molecular mechanisms by which hypoxia induced tubular EMT remain largely unknown.

Twist, a basic helix-loop-helix transcription factor, was newly identified as an important factor of promoting EMT involved in cancer progression and metastasis.12,13 Twist transcriptionally repressed the expression of E-cadherin,14 leading to loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and gain of cell motility.15 Besides the relation to tumor metastasis, Twist was also found to contribute to the promotion of EMT in renal epithelial cells. Yang et al.12,13 showed that the ectopic expression of Twist might potently induce EMT in MDCK cells (dog kidney epithelial cells). Kida et al.16 further confirmed that Twist was related to tubular EMT, proliferation of myofibroblasts, and subsequent renal fibrosis in obstructed kidneys. Taken together, Twist was an important EMT regulator, and might play important roles in mediating the invasion, migration, and metastatic activity of different carcinoma cells, possibly resulting in renal fibrosis. However, it was still not well defined how Twist expression and activity were regulated. Hypoxia was an important micro-environmental factor that induced the expression of certain EMT regulators,
such as Snail, Zeb1, SIP1, E47/TCF3, CTGF, and LOX (lysyl oxidase), and coordinated the interaction between these EMT regulators.\textsuperscript{17-20} Recent reports have shown that various kinds of stress, such as hypoxic state, might induce Twist expression in dilated tubules.\textsuperscript{16} After exposure to hypoxia, Twist was activated in all the 5 pancreatic cancer cell lines with no expression of Twist originally.\textsuperscript{21} Given the known features of Twist, we hypothesized that hypoxia states of renal tubular cells might affect Twist activity and further mediate renal tubular EMT.

In this study, we characterized the effect of Twist on the hypoxia-induced tubular EMT and investigated the underlying mechanisms. In vitro and in vivo studies revealed that hypoxia, through activation of Twist, promoted renal tubular EMT phenotype and that Twist expression might be induced by hypoxia-inducible factor-1\(\alpha\) (HIF-1\(\alpha\)) through the HRE located in the Twist proximal promoter. Taken together, these data suggest that hypoxia-induced Twist expression mediated by HIF-1\(\alpha\) activation might contribute to the pathogenesis of progressive renal fibrosis.

RESULTS

Twist was critical for hypoxia-induced EMT in human tubular epithelial cells

Epithelial cells undergoing EMT typically lost expression of epithelial markers such as E-cadherin and ZO-1 and obtained expression of mesenchymal markers including \(\alpha\)-SMA and vimentin. We found that human tubular epithelial cell lines (HKC and HK-2) cultured under hypoxic conditions (1\% O\(_2\) and 5\% CO\(_2\)) had reduced expression of E-cadherin and epithelial junction protein ZO-1 and increased expression of vimentin and \(\alpha\)-SMA when compared with cells under normoxic condition (Figure 1a). The hypoxic response was confirmed by a check of the known hypoxia-regulated molecule CTGF expression.\textsuperscript{22} These findings suggest that the epithelial cells have undergone EMT with a more fibroblast-like cell type, and further reveal that hypoxia might promote EMT, one of the major mechanisms that mediate renal fibrosis. Furthermore, Twist expression was also found increased in HK-2 and HKC cells under hypoxic conditions, and was negatively correlated to expression of E-cadherin and ZO-1 (Figure 1a).

Immunofluorescence staining was employed to confirm EMT phenotype changes of renal tubular cells under hypoxic condition. It was shown that Twist staining in hypoxic induced cells was greatly increased and mainly located in the nucleus compared with that of normoxic cells (Figure 1b). Meanwhile, the expression of E-cadherin decreased and the expression of vimentin increased when compared with those in normoxic HK-2 cells, respectively. In addition, HIF-1\(\alpha\), an important marker of hypoxia pathway, was found intensely expressed in the cytoplasm and nucleus of tubular cells, mainly in the nucleus, at the 48th hour under hypoxic induction (Figure 1b). All these results suggest that Twist may be involved in the hypoxia-induced EMT of tubular cells.

To show whether Twist is required for hypoxia-induced EMT, upregulation and downregulation of Twist through sense and short interfering RNA (siRNA) transfection were performed in HK-2 and HKC cells. Both HKC and HK-2 cells with Twist overexpression had gained vimentin expression and reduced E-cadherin expression, suggesting transformation from epithelial to mesenchymal phenotype (Figure 1c). Forty-eight hours after exposure to hypoxia, the expression of E-cadherin was markedly increased in siRNA-Twist transfected cells compared with control cells that were transfected with scramble DNA (Figure 1d). In contrast, the protein vimentin was greatly upregulated in both parental cells and pSilencer-transfected cells under hypoxia state, whereas lower vimentin level was detected in hypoxic siRNA-Twist transfected cells (Figure 1d). Our results show that Twist could cause EMT of tubular cells directly and at least partly mediate hypoxia-induced EMT phenotypes.

Hypoxia increased the expression of twist at the transcriptional level

Western blot analysis was used to evaluate the changes of Twist protein expression in HKC and HK-2 cells under normoxia and hypoxia. It was shown that Twist was markedly upregulated 12 h after hypoxic stimulation (1\% O\(_2\)) (Figure 2a). Twist remained upregulated for more than 48 h and was consistent with the increase of HIF-1\(\alpha\), which was accompanied by reduced expression of E-cadherin and enhanced expression of the vimentin (Figure 2a).
**S Sun et al.: HIF-1α induces twist expression**

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**Figure a**

<table>
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<tr>
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**Figure b**

- **Normoxia**
  - Twist
  - E-cadherin
  - ZO-1
  - Vimentin
  - α-SMA
  - CTGF
  - β-Actin

- **Hypoxia**
  - Twist
  - E-cadherin
  - ZO-1
  - Vimentin
  - α-SMA
  - CTGF
  - β-Actin

**Figure c**

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<td>pcDNA-Twist</td>
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**Figure d**

- **HKC cell**
  - HIF-1α

- **HK-2 cell**
  - Parental
  - pcDNA3.1
  - pcDNA-Twist
  - siTwist

**Graphs**

- **HKC cell**
  - Relative expression level
  - Twist, E-cadherin, ZO-1, Vimentin, α-SMA, CTGF, β-Actin

- **HK-2 cell**
  - Relative expression level
  - Twist, E-cadherin, ZO-1, Vimentin, α-SMA, CTGF, β-Actin

- **HKC cell**
  - Hypoxia for 48 h

- **HK-2 cell**
  - Hypoxia for 48 h

**Statistical Significance**

- *p < 0.05
- **p < 0.01
- ***p < 0.001
normoxic control cells, there was little expression of Twist and HIF-1α (Figure 2a).

In addition, we applied semiquantitative reverse transcription-PCR to investigate the transcriptional mRNA level of Twist. As shown in Figure 2b, hypoxia induced the Twist mRNA transcripts in a time-dependent manner compared with those under normoxia in HKC and HK-2 cells, respectively (mean ± s.e., n = 3). Vascular endothelial growth factor, a known HIF-1 target gene, as a positive internal control was upregulated under hypoxic condition. To further clarify whether hypoxia augmented Twist mRNA at the stage of mRNA transcription or stability, HKC and HK-2 cells were exposed to normoxia or hypoxia in the absence or presence of actinomycin D. Treatment with actinomycin D, which blocked the function of the transcriptional machinery, almost completely abolished the hypoxia-induced upregulation of Twist in both cell lines tested (Figure 2b), indicating that hypoxia regulates Twist by a transcriptional mechanism.

**Figure 2** Effect of hypoxia on Twist and HIF-1α expression. (a) Western blot analysis of HIF-1α, Twist, E-cadherin, and vimentin in HKC and HK-2 cells after 12, 24, and 48 h of hypoxia. A representative blot from three independent experiments was shown. (b) Semiquantitative RT-PCR was used for analysis of Twist mRNA levels in cells under hypoxic conditions and the impact of actinomycin D treatment on hypoxic induction of Twist (VEGF as positive internal control). HKC and HK-2 cells were cultured under hypoxic conditions for 0, 12, 24, and 48 h, without or with actinomycin D at a concentration of 5 μg/ml medium. Cycle numbers were optimized in several experiments for determination of linear phase PCR. The results shown were representative of three independent experiments.

Twist expression and transcriptional activation were directly induced by HIF-1α

To explore whether hypoxia-induced Twist is mediated by HIF-1α, which is an important transcription factor involved in the hypoxia signal pathway, HK-2 cells were transiently transfected with an siRNA plasmid containing an HIF-1α targeting sequence. Repression of endogenous HIF-1α by siRNA in HK-2 cells under hypoxic condition showed that the mRNA and protein levels of Twist were decreased (Figure 2a and b), showing that HIF-1α might regulate Twist expression at the transcriptional level.

As the hypoxic induction pattern of Twist appeared to be regulated by HIF-1α, we further investigated whether Twist was a target of HIF-1α. When examining the Twist promoter for putative HIF-1-binding sites (HBSs), we found three sites sharing homology with the HIF-1α-binding consensus sequence BDCGTV (B = C/T/G, D = A/G/T, V = G/C/A). To identify the transcriptional regulation of Twist by HIF-1α, luciferase reporter constructs containing varied lengths of Twist promoter or mutated HRE were constructed (Figure 3c), and the effects of HIF-1α on their activity were tested. As shown in Figure 3d, after HK-2 cells were transiently co-transfected with the Twist wild-type promoter vector (from −488 to +152 bp) and HIF-1α encoding cDNA, the luciferase activity increased by 6.4 ± 1.89-fold compared with that of the controls (P < 0.01). However, the reporters that contained the HRE1 deletion or mutation, HRE2 and/or HRE3 promoter region of Twist showed lower luciferase activity compared with Twist wild-type promoter vector transfected cells. It was very interesting that the Twist promoter containing only HRE1 resulted in a significant increase of luciferase activity (82.9%) as compared with the activity of the control. These findings suggest that hypoxia-induced expression of Twist was transcriptionally dependent on HIF-1α, and that HRE1 region of Twist should be the major target-binding site for HIF1α.

**Confirmation of HIF-1α-binding sites in Twist promoter**

Next, we performed chromatin immunoprecipitation (ChIP) assay to examine whether HIF-1α associates with the HRE sites in Twist promoter. As shown in Figure 4, ChIP analysis of nuclei derived from HK-2 cells revealed a dominant band of 204 bp containing the first possible binding (−317 to −312) site in hypoxic condition. No bands were evident in the other two possible binding sites and the control IgG immunoprecipitates. These results suggest that the proximal HRE at −317 was the main HIF-1α-binding site in the Twist promoter (Figure 4).

This conclusion was further confirmed by the electrophoresis mobility shift assay. Three oligonucleotides corresponding to nt −317 to −312 (HRE1), −171 to −166 (HRE2), and +63 to +68 (HRE3) of the Twist promoter were incubated with nuclei derived from HK-2 cells exposed to hypoxia for 48 h. Of the three examined putative HBSs within the Twist gene promoter, only one site located at −317 to −312 (CACGTC) displayed specific HIF-1 binding in...
Figure 3 | HIF-1α directly induced Twist expression and transcriptional activation. HK-2 cells were transiently transfected with either a pSilencer vector or a plasmid containing a HIF-1α targeting sequence 24 h before incubation under hypoxic condition for 48 h. (a) Lysates were then prepared and immunoblotted for HIF-1α and Twist. *P<0.05 compared with the parental cells and the pSilencer empty vector cells. (b) Semiquantitative RT-PCR was used for analysis of Twist and VEGF mRNA levels in HK-2 cells, respectively. *P<0.05 compared with the parental cells and the pSilencer empty vector cells. (c) Twist promoter luciferase reporter assay was used for analysis of HIF-1α-induced Twist transcriptional activation. Schematic diagram of wild-type, truncated, or mutated Twist promoter with three consensus HREs constructs was shown. (d) Relative luciferase activity of the Twist promoter report gene. HK-2 cells were transfected with 20 ng of reporter constructs and 1 μg of pCDNA3.1-HIF-1α expression vector for 24 h. To correct for transfection efficiency variation, cells were co-transfected with 0.2 ng of pRLTK vector. The luciferase results were reported as relative light units of firefly luciferase activity normalized with respect to the Renilla luciferase activity. The mean values from three independent experiments were shown. *P<0.05 compared with the control.

Figure 4 | Identification of HRE in the Twist promoter. Chromatin immunoprecipitation was used to examine HIF-1α binding to the Twist promoter in hypoxic HK-2 cells. Reaction controls included immunoprecipitations performed by using a nonspecific IgG monoclonal antibody (Cont IP) and PCR was performed by using whole cell genomic DNA (Input). A representative example of three independent experiments was shown.

Figure 5 | Analysis of HIF-1α-binding site in the Twist promoter by EMSA. Biotin-labeled HRE1, HRE2, and HRE3 oligonucleotides were used as a probe. (a) Of the three examined putative HIF-1-binding sites within the Twist gene promoter, only one site (HRE1) displayed specific HIF-1 binding. (b) For the competition assays with HRE1, 200-fold molar excess of the cold HRE1 and mutant HRE1 was used. For the supershift assay, anti-HIF-1α antibody (1 μg) was added to the binding reaction. All results were representative of at least three independent repeated experiments.
together, these findings indicated that the Twist promoter was directly activated by HIF-1 and that the HRE at −317 to −312 bp was essential for the transcriptional activation of the Twist promoter.

**Twist is involved in hypoxia-induced renal fibrosis in vivo**

Collectively, the above in vitro study has shown that Twist is involved in hypoxia-induced EMT confirmed by morphological and functional changes. Thus, we further verified this result by an in vivo study. Hence, we chose the classic fibrosis model – the 5/6 subtotal nephrectomy rat model – to investigate hypoxia-related renal fibrogenesis.23–25 As shown in Table 1, there was no significant difference between the body weights of 5/6-nephrectomized rats and the sham-operated group. However, serum urea nitrogen and serum creatinine were significantly elevated in 5/6-nephrectomized rats compared with sham-operated rats. In addition, light microscopic examination verified interstitial fibrosis in 5/6-nephrectomized rats (data not shown). Immunohistochemistry staining showed that Twist was mainly expressed in the cytoplasm of renal tubules cells in 5/6-nephrectomized rats, whereas there was almost no staining in sham-operated kidneys (Figure 6a). HIF-1α staining was also observed to be markedly increased in many of the transdifferentiated tubular cells, especially in the nucleus (Figure 6a). Meanwhile, in renal tubular epithelial cells, the staining of E-cadherin was found decreased, whereas the staining of α-SMA was increased after subtotal nephrectomy. Western blot study also revealed similar results: Twist and HIF-1α protein expression were significantly elevated in the kidney of 5/6-nephrectomized rats compared with the control kidneys (Figure 6b). These results confirm that chronic hypoxia-induced EMT occurs in 5/6 subtotal nephrectomy of the rat. In linear regression analysis of Western blot bands of Twist and HIF-1α in 5/6-nephrectomized rats (n = 11), the Pearson correlation coefficient was 0.892, indicating a positive correlation (P<0.01) between the expression of HIF-1α and Twist (Figure 6b). Taken together with the in vitro findings, it was suggested that Twist is involved in hypoxia-induced renal fibrosis through HIF-1α-dependent mechanism.

**DISCUSSION**

Epithelial-to-mesenchymal transition of tubular epithelial cells is a crucial process in renal fibrogenesis.10,26 During EMT, epithelial cells dedifferentiate, lose cell polarity and epithelial surface markers, induce expression of mesenchymal markers and display phenotypic alterations, and subsequently lead to the formation of more motile fibroblast-like cells.27

Any renal injuries are potentially able to induce renal tubular cells to go through the various steps of EMT and generate myofibroblasts. Accumulating evidence suggests that hypoxia could induce transdifferentiation of cultured tubular cells into myofibroblasts, further leading to renal fibrosis.9 Although it had been proposed that hypoxia by itself could be profibrogenic, the underlying molecular mechanisms were

![Image 310x370 to 542x703](image)

**Figure 6 | Analysis of Twist and HIF-1α expression in hypoxia-induced rat renal fibrosis.** (a) Immunohistochemical analysis for HIF-1α, Twist, E-cadherin, and α-SMA in the kidney tissue of 5/6-nephrectomized rats (right panels) and sham-operated rats (left panels). Increased staining for HIF-1α, Twist, and α-SMA and reduced staining for the E-cadherin. Original magnification × 200. (b) Western blot analysis of HIF-1α and Twist in kidney tissue of sham-operated and 5/6-nephrectomized rats. A representative blot from six pairs of rats is shown. The histogram shows the average volume density corrected for the loading control, β-actin (n = 6). *P<0.05 compared with the sham-operated rats.

**Table 1 | Renal function in sham-operated rats and 5/6-nephrectomized rats**

<table>
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<th>Groups</th>
<th>n</th>
<th>Bun (mmol/l) ±</th>
<th>Scr (μmol/l) ±</th>
<th>BW (g) ±</th>
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<td>Sham-operated</td>
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<td>5.92 ± 1.03</td>
<td>26.41 ± 8.83</td>
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</tr>
<tr>
<td>5/6-nephrectomized</td>
<td>11</td>
<td>29.61 ± 14.31</td>
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<td>468 ± 12</td>
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</table>

Bun, serum urea nitrogen; Scr, BW, body weight; serum creatinine.

*P<0.01 compared with sham-operated rats.
not fully clear. In this report, we show the occurrence of EMT of renal tubular cells after hypoxia through in vitro and in vivo studies and have found that Twist is involved in hypoxia induced EMT in renal tubular cells. Furthermore, we investigated how Twist function and is regulated in hypoxia-induced renal tubular EMT.

Twist is a basic helix-loop-helix transcription factor that was firstly described as a master regulator in multiple developmental processes and was associated with more aggressive phenotypes and metastasis of tumors. It had been suggested that Twist transcriptional repression of E-cadherin might be mediated through the E-box elements on the E-cadherin promoter. E-cadherin is required for the formation of stable adheren junctions and thus the maintenance of the epithelial phenotype. Loss of E-cadherin expression is emerging as one of the most common indicators of EMT onset. Twist induces EMT and promotes cancer cell invasion and metastasis. Recently, a few studies also indicated that Twist might be involved in EMT in renal epithelial cells under pathological conditions. Yang et al. reported that the expression of Twist in MDCK cells (dog kidney epithelial cells) might lead to loss of cell-cell contacts and cell scattering. They observed complete loss of E-cadherin (typical epithelial marker) and the appearance of mesenchymal markers, including fibronectin, vimentin, and α-SMA. Hence, both the morphological and molecular changes in the Twist-expressing MDCK cells showed that these cells had undergone EMT. Kida et al. further evaluated Twist expression level in the unilateral ureteral obstruction (UUO) model, and found that in UUO day 7 kidneys, some tubular cells co-expressed Twist and fibroblast-specific protein-1, indicating that Twist was related to tubular cell EMT of obstructed kidneys. However, it was unclear whether Twist was related to the occurrence of EMT induced by hypoxia in renal tubular cells and how Twist expression was regulated. Our data suggest that Twist also mediated hypoxia-induced EMT in human tubular epithelial cells. This notion was based on the findings as follows: (1) HK-2 cell and HKC cell exposed to hypoxia presented myofibroblast-like characters. Downregulated level of E-cadherin expression and upregulated vimentin protein levels were observed in both cells after hypoxia exposition, which were consistent with the previous reports. (2) The mRNA and protein levels of Twist were upregulated in HK-2 cells and HKC cells after induction of hypoxia. Furthermore, knockdown of Twist expression suppressed the hypoxic mesenchymal phenotype, presenting an increased expression level of E-cadherin protein and reduced vimentin expression. The result further confirmed that Twist might have been an inducer of EMT in tubular epithelial cells. (3) Twist expression level was negatively correlated to that of E-cadherin, suggesting that the potential mechanism of Twist-induced EMT might be that Twist transcriptionally repressed the expression of E-cadherin, leading to loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and gain of cell motility. It had been suggested that Twist, together with Snail and SIP1, might act as a repressor of E-cadherin. Whether there were any other molecules besides Twist or other unknown signal pathways functioning in hypoxic-mediated EMT remained to be further investigated.

How is Twist expression regulated under hypoxic conditions? It has been well established that HIF-1α is involved in hypoxic response. HIF-1α binds to the hypoxia-responsive element in the cis-regulatory regions of its target genes, and transcriptionally activates genes encoding proteins that mediate adaptive responses to reduced oxygen availability. A recent study by the Haase group showed that hypoxia promotes fibrogenesis through HIF-1 stimulation of EMT. In this report, we show that the Twist gene was transcriptionally activated in response to hypoxia through its binding to HIF-1α at sites located within the promoter region. The conclusion is based on the following observations. First, the expression of Twist was upregulated by hypoxia or HIF-1α overexpression in renal tubular cells. Knockdown of HIF-1α resulted in a marked blockade of Twist induction acquired by hypoxia. Second, the blockade of the transcriptional machinery by actinomycin D treatment completely disrupted the hypoxia-induced Twist expression in tubular cells. Third, the hypoxia-responsive region of Twist promoter was clarified by luciferase reporter system. Fourth, the HRE-1 region of Twist was identified by both ChIP and electrophoresis mobility shift assay. The results from these studies nailed down the sequence −317 to −312 as a classic HRE, and the mutation of this HBS results in a complete blockage of HIF-1α-binding activity. In vivo, we show that the distribution pattern of Twist is highly congruent with that of HIF-1α in hypoxic renal fibrosis, further supporting the results that the Twist-induced fibrogenic effect is regulated by HIF-1α activation.

In summary, the data presented here provide an explanation for EMT of hypoxic renal tubular cells through the upregulation of Twist. The HIF-1α-dependent induction of Twist in hypoxic tubular cells offer new insight into the antifibrogenesis strategies.

MATERIALS AND METHODS

Cell culture

Human proximal tubular epithelial cell line (HK-2) was used as described earlier. The human kidney proximal tubular epithelial cell line (HKC) was preserved in our laboratory. HK-2 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen Inc.), supplemented with 10% fetal calf serum. HKC cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum. For hypoxic culture, cells were placed in a hypoxic (1% O2, 5% CO2, 37°C) incubator (Precision Scientific, Winchester, VA, USA) for 12, 24, or 48 h. Control cells (normoxic cells) were incubated for equivalent periods under normoxic conditions (21% O2, 5% CO2, 37°C).

Animal model

Male Sprague-Dawley rats weighing 160–180 g were obtained from the laboratory animal center of our university (Xi’an, China). The chronic renal hypoxia model was induced by 5/6 subtotal nephrectomy. All rats were killed at 12 week 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 snap-frozen in liquid nitrogen until analysis. Kidney sections were stained by the periodic acid-Schiff method and examined by light microscopy. Animal handling conformed to the guidelines for care and use of experimental animals established by the Ethical Committee of Animal Experiments.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry was carried out as described earlier using the Avidin–Biotin Complex (ABC) Vectastain Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. Briefly, 2-μm thick tissue slides were dewaxed, rehydrated, incubated 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 antibodies including anti-HIF-1α (1:500; Chemicon, USA), anti-Twist antibody (1:200; Santa Cruz, CA, USA), E-cadherin (1:100; Santa Cruz, CA, USA), vimentin (1:200; Santa Cruz Biotechnology), CTGF (1:400; Chemicon), α-SMA (1:100; Santa Cruz Biotechnology) at 4°C overnight, respectively. The sections were incubated with biotinylated goat anti-rabbit or anti-mouse Ig antibody as the secondary antibody, and the antibody reactions were visualized using diamino benzidine (DAKO, Tokyo, Japan). The non-immune goat IgG or rabbit IgG were used as negative controls. The slides were counterstained with hematoxylin, then dehydrated and mounted.

For immunocytochemical analysis, HK-2 cells were cultured on sterile glass coverslips in six-well plates. For hypoxic culture, cells were placed in a hypoxic (1% O₂, 5% CO₂, 37°C) incubator for 48 h, and control cells (normoxic cells) were incubated for equivalent periods under normoxic conditions (21% O₂, 5% CO₂, 37°C). The slides were fixed with 95% alcohol for 20 min at room temperature. The coverslips were washed with phosphate-buffered saline and permeabilized for 10 min with 0.5% Triton X-100 in phosphate-buffered saline. The cells were then incubated with primary antibodies including anti-HIF-1α (1:500; Chemicon), anti-Twist antibody (1:200; Santa Cruz), E-cadherin (1:100; Santa Cruz Biotechnology), and vimentin (1:100; Santa Cruz Biotechnology) after blocking with 10% normal goat or rabbit serum for 1 h. The slides were incubated with FITC-conjugated goat anti-mouse or anti-rabbit IgG as the secondary antibody at room temperature for 1 h. Finally, slides were analyzed by confocal laser scanning microscopy.

Western blotting

The treated cells were harvested and lysed on ice for 30 min in lysis buffer (50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 0.2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride and 1% NP-40), then quantified by Bradford method. A measure of 100 μg of whole cellular lysates were electrophoresed in 10% SDS-polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. The membranes were blocked with 10% fat-free milk at room temperature for 2 h and incubated with primary antibodies including anti-HIF-1α (1:1000; Chemicon), anti-Twist antibody (1:200; Santa Cruz), E-cadherin (1:200; Santa Cruz Biotechnology), ZO-1 (1:400; Chemicon), α-SMA (1:100; Santa Cruz Biotechnology), and vimentin (1:200; Santa Cruz Biotechnology), CTGF (1:100; Santa Cruz Biotechnology) or anti-β-actin (1:4000; Sigma) at 4°C overnight. After three washes for 15 min in Tris-buffered saline supplemented with 0.1% Tween 20 (TBST), the membrane was incubated with the peroxidase-conjugated secondary antibody (Amersham-Pharmacia Biotech, Beijing, China) for 2 h at room temperature. Enhanced chemiluminescence (Amersham, Freiburg, Germany) was used for detection.

Semiquantitative reverse transcription-PCR assays

Total RNA was extracted from treated cells using TRIzol reagent (Invitrogen Corp.). Aliquots (5 μg) of RNA were reverse transcribed to cDNA using Superscribe First-Strand Synthesis System (Invitrogen Corp.). Aliquots (1, 2, and 4 μl) of cDNA were used as template for PCR of Twist (GenBank accession NM-000474) and β-actin. The following oligonucleotides were used as primers. Twist, 5'-GGGAATCCCGAGTCGTTCA-3' and 5'-TCAAGAAACAGGCGGTGG-3'; β-actin, 5'-ATCTGCTGAGTAGCATTG-3' and 5'-ACCGGTGTTGTTGATCTG-3'. Each primer set was amplified using an optimized number of PCR cycles: 30 s at 95°C, 30 s at 58°C, and 40 s at 72°C. The PCR reactions were then visualized on a 1.0% agarose gel containing 5 μg/ml of ethidium bromide.

Plasmid constructs and cell transfection

The recombinant sense expression vector for HIF-1α and the siRNA expression vectors for HIF-1α and Twist were constructed as described earlier.37,38 In brief, recombinant sense expression vector pcDNA3.1-HIF-1α was constructed by subcloning the HIF-1α cDNA that contained the complete coding sequence between KpnI and BamHI of the multiple cloning sites in eukaryotic expression vector pcDNA3.1(+). The target siRNA was determined using the siRNA design tool (Invitrogen). The HIF-1α siRNA-specific targeting sequence was 5'-aaagaggttacctgcgtg-3' and Twist siRNA specific targeting sequence was 5'-aatgcaacgtacgtacat-3'; a scramble sequence (5'-tcagctgcctagctgac-3') was used as control. The sequences were checked against the database to confirm specificity and were cloned into the pSilencerTM2.1-U6neo siRNA expression vector (Ambion, respectively). The pcDNA3.1-Twist plasmid was the gift from Professor Carlotta A Glackin (National Medical Center and Beckman Research Institute).

Cells were plated and grown to 70-90% confluency without antibiotics. Transfections were performed with Lipofectamine 2000 (Invitrogen AB., Lidinggo, Sweden), as directed by the manufacturer. The cells were cultured 48 h after transfection and then exposed to hypoxia for 12-48 h.

Bioinformatic analysis of HIF-1 binding site

A genomic region of ~800 bp upstream of the Twist transcriptional initiation site was determined using the NCBI Genomic BLAST program. This DNA sequence was then pasted into DNA Strider 1.0 software, which was employed to locate putative HBSs. The search was based on compilations of functional HBSs and the HIF-1-binding consensus sequence BDGCTV (B=D/G/T; V=G/C/A), in turn established by following definitions of consensus sequences.39,40

Dual-luciferase reporter gene assay

To assay the transcriptional activity of Twist under hypoxic condition, a set of Twist promoter-driven luciferase reporter vectors were constructed by inserting the wide-type sequence (from –488 to +152 bp), two truncated sequences (from –256 to +152 bp and from –20 to +152 bp, respectively), HRE1 mutants (CACGTG mutated to CATAAC and –317 to –312 bp were deleted), HRE2 mutants (TGCGTC mutated to TGAAGC), and HRE3 (CAGTCG mutated to CGAAGC) of the Twist promoter into a pGL3-basic vector (Promega, WI, USA), respectively.
HK-2 cells in a 24-well plate (50,000 cells per well) were co-transfected with pcDNA3.1-HIF-1x and the reporter plasmid using Lipofectamine 2000 (Invitrogen), with PRL-TK as a control for transfection efficiency in Dulbecco’s modified Eagle’s medium without serum. Forty-eight hours post-transfection, cells were exposed to hypoxic conditions for 48 h. The luciferase activity was measured and quantitated in a luminometer using the Dual-Luciferase Reporter Assay System (Promega). Experiments were performed in triplicates. Results were expressed as means of the ratio between the firefly luciferase activity and the renilla luciferase activity.

**Chromatin immunoprecipitation assay**

Hypoxia-inducible factor-1 binding to Twist promoter was analyzed by ChIP on HK-2 cells, using methodologies described earlier. HK-2 cells were fixed with 1% paraformaldehyde, and chromatin derived from isolated nuclei was sheared by using an F550 microtip cell sonicator (Fisher Scientific). After centrifugation, supernatants containing sheared chromatin were incubated with an anti-HIF-1x antibody or control IgG. Protein A sepharose was then added before the overnight incubation, and the immune complexes were subsequently eluted. Complexes were next treated with RNase and proteinase K and were extracted with phenol/chloroform and then with chloroform. DNA was precipitated, washed, dried, resuspended in water, and analyzed by PCR. The primers used in this analysis spanned 204 bp around the first possibility of the HBS located -317 bp from the translation start site (sense, 5′-AGCAATTTGTAGACCCGGACTC-3′) or spanned 193 bp around the second possibility of the HIF-1x-binding site located -171 bp from the translation start site within the Twist promoter (sense, 5′-AGGCCCAATGACACTGCTGGCCAAAATCT-3′) and antisense, 5′-AGCGGTTGGAATGCAGGAGCTCAAC-3′) or spanned 201 bp around the third possibility of the HIF-1x-binding site located +63 bp from the translation start site within the Twist promoter (sense, 5′-TCCGCTCTCCTCCTCTCCTGT3-3′) and antisense, 5′-TTCCTCGTGTTGCTCGGGCTGTC3-3′).

**Electrophoresis mobility shift assay**

The nuclear extracts were analyzed for HIF-1x binding to a HRE on the Twist promoter by gel mobility shift assays as described earlier. The double-stranded DNA probes used in the electrophoresis mobility shift assay experiments contained the following sequences: 5′-CTCCTCTCAAGTCAGGGCAAT-3′ for the first possibility wild-type HIF-1x-binding site and 5′-CTCCTCTGCAAGCGGCAAT-3′ for the first possibility mutant HIF-1x-binding site. 5′-CGGGGTGTGGGTCCAGGCGGT-3′ for the second possibility wild-type HIF-1x-binding site and 5′-CGGGGTGTAAAGCCGAGGCTG-3′ for the second possibility mutant HIF-1x-binding site and 5′-CGGGGTGTAAGCCGAGGCTG-3′ for the second possibility wild-type HIF-1x-binding site and 5′-CGGGGTGTAAAGCCGAGGCTG-3′ for the second possibility mutant HIF-1x-binding site. An unrelated double-stranded oligonucleotide 5′-AGTGGGGGTGGGAGGCTGCTCACT-3′ was used (ISRE (insulin receptor responsive element)) as a nonspecific competitor. The oligonucleotides were end-labeled with Biotin. HK-2 cells incubated under hypoxic or normoxic conditions were harvested and lysed in extraction buffer (20 mM HEPES (pH 7.9), 1 mM EDTA, 400 mM NaCl (25%), 0.1% NP-40, 1 x protease inhibitors cocktail, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM dithiothreitol). An equal amount of protein (1 μg) from the nuclear extract was used for binding reactions with the biotin-labeled wild-type or mutant Twist probe for 20 min at room temperature in binding buffer (8 mM HEPES (pH 7.4), 80 mM KCl, 0.8 mM EDTA, 1 mM dithiothreitol) at a 20 μl final volume. For supershift experiments, 1 μg of monoclonal antibody against HIF-1x was added to the reaction mixture before the addition of labeled oligonucleotides. Equivalent amounts of Biotin-labeled probe were used for all samples. For the binding competition experiment, unlabeled oligonucleotides were added into the reaction mixture in a 200-fold excess. DNA-protein complexes were analyzed in a 4% polyacrylamide gel with 0.5 x Tris-borate-EDTA at 100 V. Then, the binding reaction was transferred to nylon membrane at 380 mA for 30 min and to crosslink for 15 min on a transilluminator equipped with 312 nm. The biotin-labeled DNA was detected by chemiluminescence.

**Statistical analysis**

Data were analyzed by standard statistical methods, including linear regression, the t-test, and one-way analysis of variance using SPSS (version 14.0). Data are expressed as the mean ± s.e.m. Significance was assessed at P < 0.05.

**DISCLOSURE**

All the authors declared no competing interests.

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