

# MUC1, a New Hypoxia Inducible Factor Target Gene, Is an Actor in Clear Renal Cell Carcinoma Tumor Progression

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## Abstract

The hypoxia inducible factor (HIF) signaling pathway is known as the main renal carcinogenetic pathway. MUC1, an *O*-glycoprotein membrane-bound mucin, is overexpressed in clear renal cell carcinomas (cRCC) with correlation to two major prognostic factors: tumor-node-metastasis stage and nuclear Fuhrman grade. We questioned whether there is a direct link between the HIF pathway and MUC1 overexpression in renal tumors. Interestingly, we observed concomitant increase of HIF-1 $\alpha$  and MUC1 in metastatic cRCC group versus nonmetastatic cRCC group. Using different renal cell models and small interfering RNA assays targeting either HIF-1 $\alpha$  or YC-1, a HIF-1 pharmacologic inhibitor, we showed induction of MUC1 expression under hypoxia by a HIF-dependent mechanism. Chromatin immunoprecipitation assay showed a direct binding of HIF-1 $\alpha$  at the MUC1 promoter. In addition, combined site-directed mutagenesis and gel shift assay allowed the identification of two functional putative hypoxia responsive elements at -1488/-1485 and at -1510/-1507 in the promoter. Using a rat kidney model of ischemia/reperfusion, we confirmed *in vivo* that clamping renal pedicle for 1 hour followed by 2 hours of reperfusion induced increased MUC1 expression. Furthermore, MUC1 knockdown induced significant reduction of invasive and migration properties of renal cancer cells under hypoxia. Altogether, these results show that MUC1 is directly regulated by HIF-1 $\alpha$  and affects the invasive and migration properties of renal cancer cells. Thus, MUC1 could serve as a potential therapeutic target in cRCC. [Cancer Res 2009;69(14):5707-15]

## Introduction

Renal cell carcinoma corresponds to 5% of all adult malignancies. The main histologic subtype is represented by clear renal cell carcinoma (cRCC; ref. 1). Ninety percent of cRCC present a biallelic inactivation of the von Hippel Lindau (VHL) tumor suppressor gene (2). In normal cells, in the presence of oxygen, VHL gene product directs the degradation and clearance of various transcription factors such as the hypoxia inducible factor (HIF)-1 $\alpha$ . In the absence of pVHL, HIF is stabilized, accumulates to

supraphysiologic levels, and then activates the transcription of target genes that contribute to the physiology of tumors (3).

MUC1, a membrane-bound mucin, is a large *O*-glycoprotein with an extended, heavily glycosylated extracellular domain that protrudes far away from the apical side of the cell (200–500 nm) and plays a role in signal transduction (4, 5). In adult, its expression is cell- and tissue-specific and is altered during carcinogenesis. MUC1 has been identified as a potential target for immunotherapy and gene-based therapies in breast and prostate cancers (6–8).

In normal kidney, MUC1 is the major mucin expressed in the distal convoluted tubules and in the collecting ducts (9). Previous studies have shown that MUC1 is diffusely overexpressed in cRCC (9–11). In low-grade and low-stage tumors, MUC1 staining is predominantly membranous apical. In high-grade and high-stage tumors, MUC1 staining is cytoplasmic and/or circumferential membranous (10–12). In cRCC, MUC1 overexpression has been found to be associated with metastatic disease and a worse prognosis (10–12). However, the role of MUC1 in renal cancer remains unknown. The main purpose of this article was to determine (a) whether MUC1 could be a downstream target of the pVHL/HIF-1 signaling pathway and (b) whether MUC1 could be involved in renal cancer progression.

## Materials and Methods

**Tissue microarray.** Twenty-seven formalin-fixed and paraffin-embedded primary pT<sub>3</sub> stage (of high metastatic risk) cRCC samples were retrieved from the archives of the Department of Pathology of the University hospital of Lille. A consent form was obtained from each patient. Three core tissue biopsies, 0.6 mm in diameter, were taken from selected morphologically representative regions of each cRCC, distant to necrotic areas, and precisely arrayed using a tissue arrayer (Beecher Instruments). Additional core tissue biopsies were taken from morphologically benign-appearing surrounding renal parenchyma tissue for each tumor.

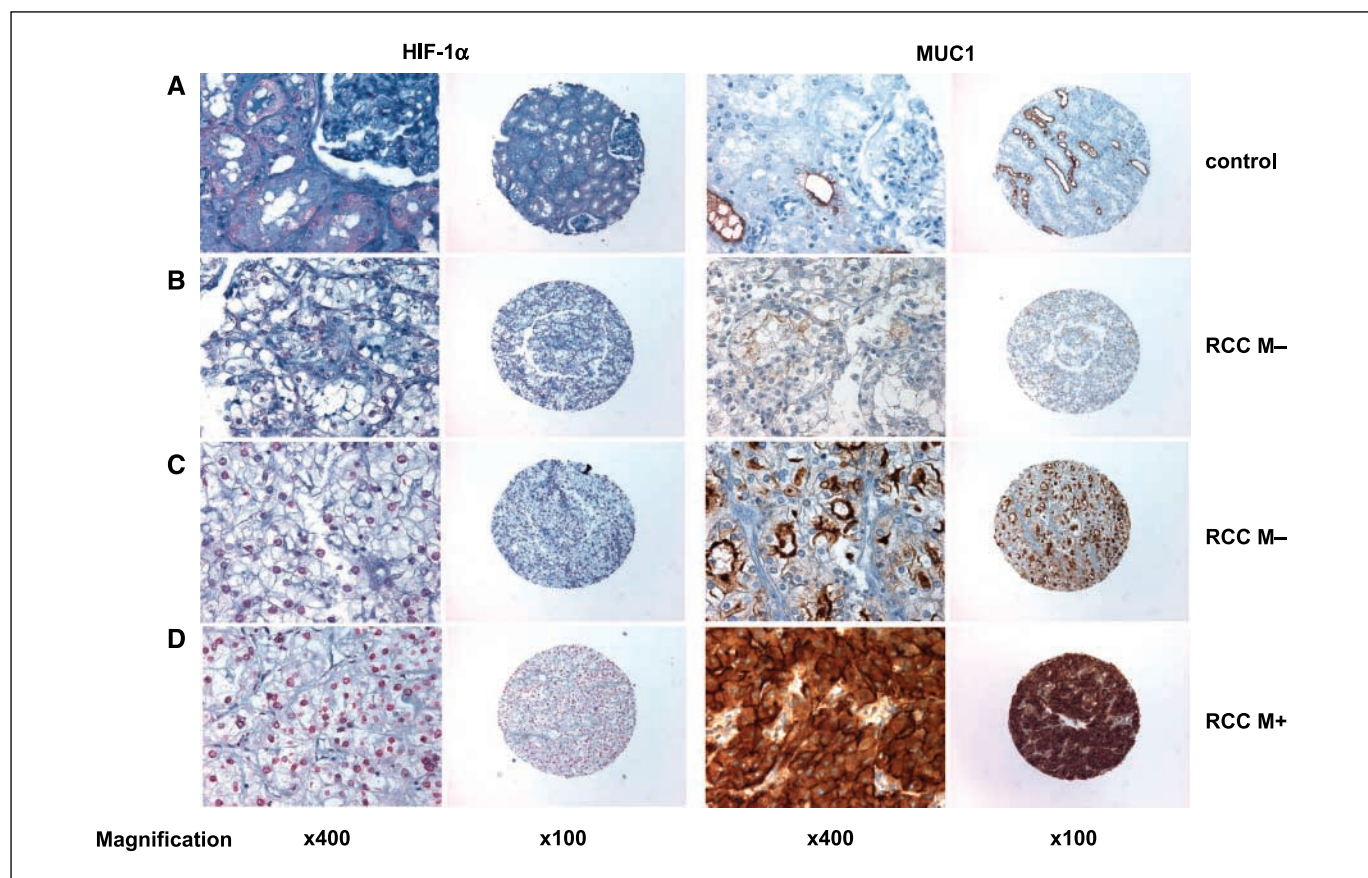
**Immunohistochemistry.** Immunohistochemistry protocols for HIF-1 $\alpha$  (antihuman and antirat: 1/500, H1 $\alpha$ 67, Novus Biological) and MUC1 (antihuman: 1/50, MUC1 clone M8, a gift from D. Swallow; antirat: 1/500, Muc1 Ab-5, Lab Vision Corp.) were followed as previously described (12–14). Tissue sections were evaluated by two pathologists in a blinded manner. Subcellular localization [i.e., membranous (apical or circumferential), cytoplasmic, or nucleic] and degree of reactivity (percentage of positive cells and staining intensity) were assessed. For HIF-1 $\alpha$ , only nuclear staining was taken into account. For tissue microarray analysis, the overall score used for subsequent statistical analysis was the pooled mean of the three spots of the same tumor. Negative controls were done by omitting the primary antibodies.

**Cell culture and treatments.** Renal cell lines ACHN, Caki-2, and HEK293 were obtained from the American Type Culture Collection and cultured in MEM or DMEM supplemented with 10% (v/v) fetal bovine serum. Hypoxic exposure was carried out at 37°C in a humidified incubator (Binder 150, Germany) with 94% N<sub>2</sub>, 5% CO<sub>2</sub>, and 1% O<sub>2</sub>. In inhibition

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-08-4905



**Figure 1.** MUC1 and HIF-1 $\alpha$  are overexpressed in metastatic cRCC compared with nonmetastatic cRCC and normal kidney. Expression of MUC1 (*right*) and HIF-1 $\alpha$  (*left*) by immunohistochemistry in the same renal parenchyma samples. *A*, morphologically normal cortical renal tissue showing an apical membranous MUC1 staining confined to distal convoluted tubules (*right*) and a lack of nuclear HIF-1 $\alpha$  staining (*left*). *B*, nonmetastatic cRCC with rare MUC1 membranous staining (*right*) and very scattered HIF-1 $\alpha$  nuclear staining (*left*). *C*, nonmetastatic cRCC with more diffuse and intense MUC1 staining still confined to the apical membrane (*right*) with well-defined scattered HIF-1 $\alpha$  nuclear staining (*left*). *D*, metastatic cRCC with strong diffuse cytoplasmic and membranous MUC1 staining (*right*) and showing abundant and strongly positive HIF-1 $\alpha$  nuclear expression in tumor cells (*left*).

studies, before hypoxia exposure, cells were pretreated for 30 min with YC-1 (Sigma), a pharmacologic inhibitor of HIF-1 $\alpha$ .

**Western blotting.** Total cellular extracts were prepared using standard procedures. Western blot was done as described previously (15) using specific mouse monoclonal antibodies against  $\beta$ -actin (1/5,000, A5441, Sigma), HIF-1 $\alpha$  (1/200, H1 $\alpha$ 67), and MUC1 (1/500).

**Reverse transcription-PCR.** Preparation of total RNAs and reverse transcription-PCR (RT-PCR) were described in ref. 16. Primer information is given in Supplementary Table S1. Densitometric analysis of DNA bands was carried out using GelAnalyst-GelSmart software (Clara Vision).

**Small interfering RNA assays.** ACHN cells were seeded the day before transfection at a density of  $50 \times 10^3$  per well in antibiotic-free medium. Cells were transfected with 100 nmol/L of HIF-1 $\alpha$  SMARTpool using 1  $\mu$ L of DharmaFECT 4 transfection reagent according to the manufacturer's instructions (Dharmacon). Controls included mock-transfected cells or cells transfected with 100 nmol/L of siCONTROL Non-Targeting Pool siRNA or siCONTROL GAPD Pool siRNA. One day after transfection, ACHN cells were unexposed or exposed to hypoxic conditions during 48 h. Total RNA was isolated 72 h after transfection and RT-PCR was done.

**Site-directed mutagenesis.** Three mutated versions of the *MUC1* promoter construct (−2870/+33) were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The two HIF binding sites identified at −1488/−1485 (HRE2, 5′-CGTG-3′) and −1510/−1507 (HRE1, 5′-CGTG-3′) were mutated in 5′-ATTG-3′ and 5′-CGTA-3′, respectively.

**Transient transfections.** HEK293 cells and Caki-2 cells were transfected with 1  $\mu$ g of pT<sub>7</sub>-MUC1 using Effectene reagent (Qiagen) as previously

described (17). In hypoxic experiments, relative luciferase activity was expressed as fold activation of luciferase activity in hypoxic cells compared with normoxic cells. In cotransfection studies, 1  $\mu$ g of the pT<sub>7</sub>-MUC1 wild-type or mutated for hypoxia responsive element (HRE) was transfected with 0.5  $\mu$ g of the pcDNA3-HIF-1 $\alpha$ , pcDNA3-HIF-1 $\alpha$ (401 $\Delta$ 603) expression vectors or pcDNA3 empty vector. Results were expressed as fold induction of luciferase activity in cells transfected with expression vectors compared with that obtained with the empty vector.

**Chromatin immunoprecipitation.** HEK293 and Caki-2 cells ( $1.0 \times 10^6$ ) were fixed for 10 min at room temperature in 1% (v/v) formaldehyde and processed for chromatin immunoprecipitation analysis as previously described (15). Anti-HIF-1 $\alpha$  antibody (H1 $\alpha$ 67) or normal rabbit IgGs (Upstate Biotechnology) was used. DNA was PCR amplified with primers listed in Supplementary Table S1. The size of the amplified product was 156 bp.

**Nuclear extract preparation and electrophoretic mobility shift assay.** Nuclear extracts were prepared from HEK293 cells as previously described in ref. 17. Binding studies were done using nuclear extracts (8  $\mu$ g) and oligonucleotides recovering HRE1 (5′-TACGCACCT-CACGTGTGCTTTTGC-3′) and HRE2 (5′-GCCCCCGCCTACGTGCC-TACCTGT-3′) present in the *MUC1* promoter. For supershift analyses, 2  $\mu$ L of two anti-HIF-1 $\alpha$  antibodies (BD Biosciences and Novus Biologicals) were added to the proteins and left for 2 h at 4°C before adding the radiolabeled probe.

**Renal ischemia/reperfusion injury.** Protocol for renal ischemia/reperfusion injury experiment was described in ref. 16. The rats were

sacrificed 2 h after the reperfusion period. The kidneys were removed and cut sagittally in half; one half was subdivided into medullary, cortical, or whole kidney fraction and frozen at  $-80^{\circ}\text{C}$  in RNA-Later (Ambion), and the other half was formalin fixed for 16 h and paraffin embedded for immunohistochemistry. All studies were done in accordance with the principles of the Guideline of Animal Experimentation at University of Lille 2.

**Small hairpin RNA.** pRetroSuper.Neo.GFP retroviral vectors encoding small hairpin RNA (shRNA) directed against MUC1 were constructed as described in ref. 18, using two oligonucleotides to target MUC1 (see Supplementary Table S1).

**In vitro invasion, migration, and wound healing assays.** Cell invasion and migration were evaluated using 24-well Matrigel invasion chambers with 10% FCS as chemoattractant and Boyden chamber. In hypoxic conditions, ACHN cells were cultured for 48 h in hypoxia chamber before being seeded onto Matrigel-coated filters or Boyden chambers. For wound healing assay, after 48 h of hypoxia, wounds were created in confluent cells using a pipette tip. The cells were rinsed with PBS to remove any free-floating cells and debris. Medium was then added and culture plates were incubated for an additional 24 h under hypoxic conditions. Wound healings were measured at 0 and 24 h.

**Statistical analysis.** Data are presented as mean  $\pm$  SE. Statistical analyses were done using GraphPad InStat software (GraphPad Software, Inc.).  $P < 0.05$  was considered significant.

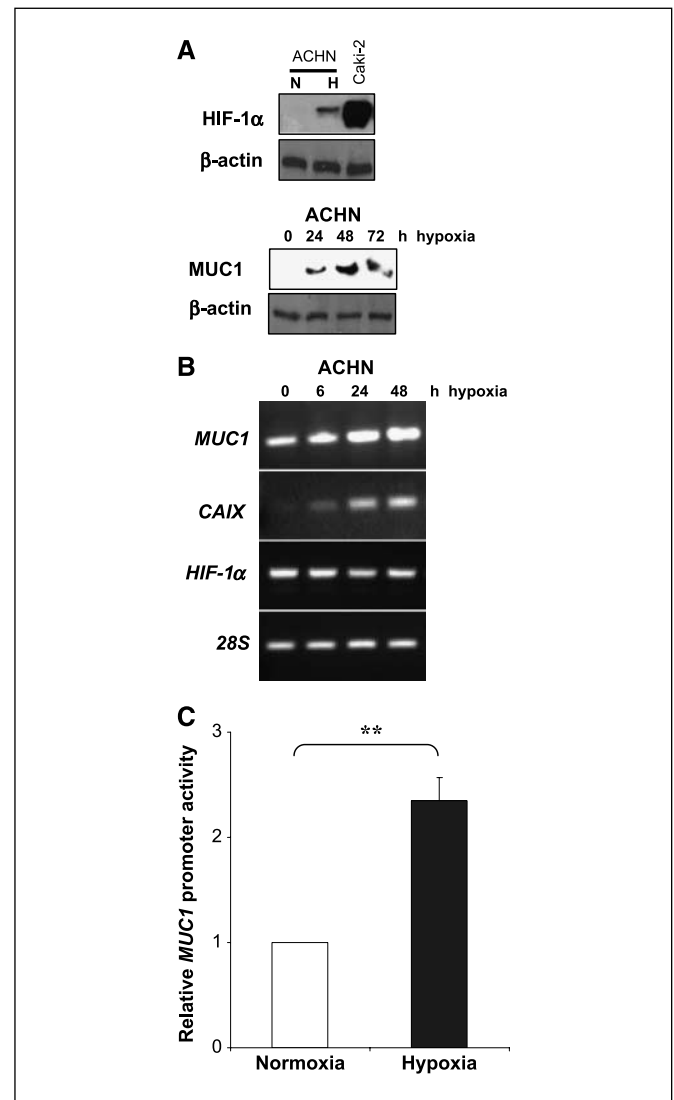
## Results

**MUC1 and HIF-1 $\alpha$  are overexpressed in cRCC.** Using a 27 pT<sub>3</sub> cRCC tissue microarray sampling (16 metastatic versus 11 nonmetastatic), we showed that MUC1 was significantly more expressed in metastatic [M(+)] cRCC compared with nonmetastatic [M(-)] cRCC (mean, 76.1% versus 44.2%;  $P = 0.0223$ , Mann-Whitney test; Fig. 1, right). In M(+) cRCC, MUC1 staining was more commonly cytoplasmic and associated or not with a circumferential membranous staining, whereas in M(-) cRCC, MUC1 staining was commonly restricted to the cytoplasmic membrane with a circumferential or apical pattern. Furthermore, we showed that nuclear HIF-1 $\alpha$  staining was significantly more important in M(+) compared with M(-) cRCC (mean, 77.1% versus 45.5%;  $P = 0.0042$ , Mann-Whitney test; Fig. 1, left). HIF-1 $\alpha$  and MUC1 immunostainings were also stronger in M(+) cRCC than in M(-) cRCC [ $P = 0.0127$  and  $P = 0.0462$ , respectively (Fisher's exact test)] when comparing light with moderate/strong staining intensity. pVHL/HIF pathway being the major carcinogenic pathway in cRCC, these results prompted us to test *in vitro* the hypothesis that MUC1 could be regulated by hypoxia in renal cancer cell models.

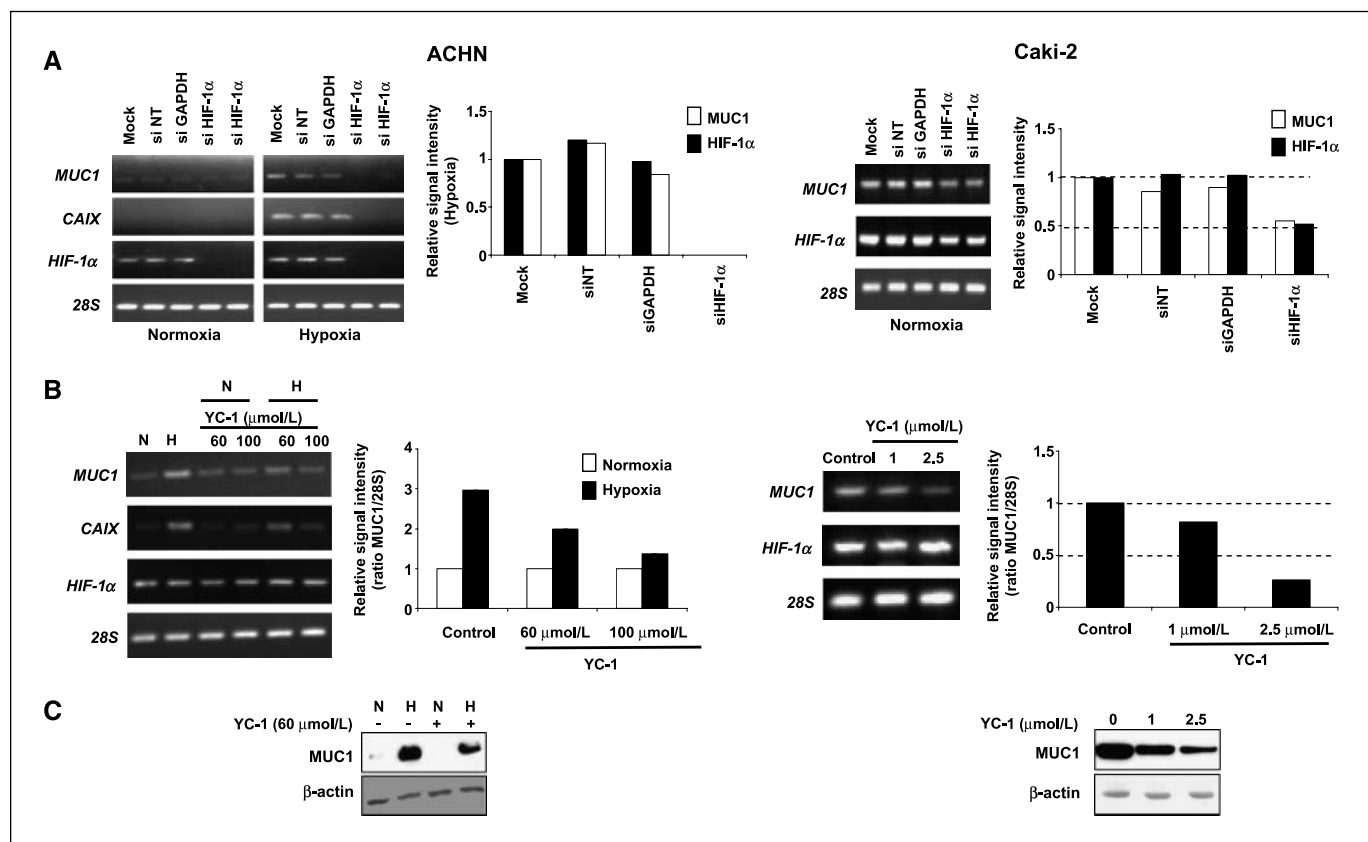
**MUC1 expression is up-regulated in renal cells in response to hypoxia.** To test our hypothesis, we used three renal cell lines. Caki-2 cells are constitutively inactivated for *VHL* gene by mutation and express MUC1 (data not shown) and high levels of HIF-1 $\alpha$  under normoxia (Fig. 2A). The pVHL/ubiquitination/degradation system is functional in ACHN and HEK293 cell lines (19). ACHN cells expressed MUC1 and HIF-1 $\alpha$  at mRNA, but not protein, level under normoxic conditions, whereas under hypoxic conditions, an increase of *MUC1* mRNA level and an induction of MUC1 and HIF-1 $\alpha$  expression at the protein level were observed in a time-dependent manner (Fig. 2A and B). By RT-PCR, we showed that *HIF-1 $\alpha$*  mRNA levels were stable during hypoxia exposure, confirming the posttranscriptionally regulation of HIF-1 $\alpha$  by hypoxia. It is interesting to note that as early as 6 hours following hypoxia induction, expression of *MUC1* mRNA was already increased, similar to *CAIX*, which is a known downstream target gene of the hypoxic pathway (Fig. 2B).

To study the effect of hypoxia on *MUC1* transcriptional activity, we transiently transfected 2.8 kb of the *MUC1* promoter in HEK293 cells. Exposure to hypoxia for 24 hours induced a  $\sim 2.5$ -fold increase of *MUC1* promoter reporter activity compared with normoxia (Fig. 2C). Altogether, these data suggest that MUC1 may be a response gene in hypoxia signaling.

**Increase of MUC1 expression under hypoxia is HIF-1 $\alpha$  dependent.** To assay the importance of HIF-1 $\alpha$  transcription factor in MUC1 increase, we used two strategies: small interfering RNA (siRNA) targeting HIF-1 $\alpha$  and YC-1, a specific pharmacologic inhibitor of HIF-1 $\alpha$  (20). Under normoxia, cell treatment with nontargeting or glyceraldehyde-3-phosphate dehydrogenase



**Figure 2.** MUC1 is overexpressed at transcriptional, mRNA, and protein levels under hypoxia. A and B, ACHN cells were cultured under normoxia (N) or hypoxic (H) conditions for the indicated times or 24 h. Caki-2 cells were cultured under normoxia. A, Western blots were done on cell lysates with anti-HIF-1 $\alpha$ , anti- $\beta$ -actin, and anti-MUC1 antibodies. B, effects of hypoxia on *MUC1*, *CAIX*, and *HIF-1 $\alpha$*  in ACHN cells, measured by RT-PCR. C, HEK293 cells were transfected with the *MUC1* promoter (-2870/+33) and then, 24 h later, cultured under normoxia or hypoxia for 24 h before being processed as described in Materials and Methods. The values obtained in normoxia were referred to as 1. Columns, mean; bars, SE. Representative of five separate experiments in triplicate. \*\*,  $P < 0.01$  (Student's *t* test).



**Figure 3.** MUC1 overexpression under hypoxia is dependent on the HIF signaling pathway in ACHN cells and Caki-2 cells. **A**, 24 h after siRNA transfection, ACHN cells (*left*) were cultured under normoxia or hypoxia for 48 h, and Caki-2 cells (*right*) under normoxia for 48 h. Then, total cellular RNA was isolated and amplified with *MUC1*-, *CAIX*-, *HIF-1α*-, and *28S*-specific primers. The intensities of the signals were determined by densitometric scanning and are expressed as the relative signal intensity compared with that obtained with mock ACHN cells under hypoxia and mock Caki-2 cells under normoxia. **B** and **C**, ACHN cells were exposed to normoxia (*N*) or hypoxia (*H*) in the absence or presence of YC-1 (60 or 100 μmol/L). Caki-2 cells were incubated for 16 h in the absence or presence of YC-1 under normoxia. **B**, cellular mRNA was isolated and amplified with *MUC1*-, *CAIX*-, *HIF-1α*-, and *28S*-specific primers. The intensities of the signals were determined by densitometric scanning and are expressed as the relative signal intensity compared with that obtained with cells under normoxia without treatment. **C**, Western blots were done on cell lysates with anti-MUC1 and anti-β-actin antibodies. *NT*, nontargeting.

(GAPDH) pool siRNA had no effect on the expression of *MUC1*, *CAIX*, *HIF-1α*, and *28S* mRNA in ACHN cells (Fig. 3A). On the contrary, treatment with a pool of siRNA targeting HIF-1α resulted in a significant decrease of *HIF-1α* mRNA. Under hypoxia, HIF-1α knockdown led to an abolition of *MUC1* hypoxia-induced expression as well as *CAIX* expression (Fig. 3A). Treatment of ACHN cells with YC-1 partially or completely inhibited the overexpression of *MUC1* and *CAIX* at mRNA levels in a dose-dependent manner under hypoxia, and YC-1 had no effect on their expression under normoxia (Fig. 3B). The same result was observed at protein level (Fig. 3C). In parallel, Caki-2 cells treated with YC-1 or siRNA targeting HIF-1α resulted also in a decrease of MUC1 expression at mRNA and protein levels. Thus, these results indicate that HIF-1α is involved in hypoxia-induced *MUC1* expression.

**The *MUC1* promoter contains two functional HIF-1α cis-elements.** Then, we investigated a direct involvement of HIF-1α in *MUC1* transcription. Analysis of the 2.8-kb *MUC1* promoter sequence with MathInspector V2.2 software (Genomatix) indicated two putative HRE consensus binding sites (5'-RCGTG-3'; ref. 21) located respectively at positions -1488/-1485 (HRE2) and -1510/-1507 (HRE1) upstream the transcription initiation site. By transient cotransfections assays, we showed that overexpression of HIF-1α in HEK293 cells induced *MUC1* transcriptional activity (~3.2-fold increase; Fig. 4A). In Caki-2 cells, the expression vector

coding HIF-1α had no effect on MUC1 transcription, whereas a HIF-1α dominant negative construct induced 75% inhibition of MUC1 transcriptional activity (Supplementary Fig. S1A). To determine whether these HIF-1 binding sites were indeed essential for mediating *MUC1* activation, we generated three mutants of HRE binding sites within the *MUC1* promoter. In HEK293 cells, directed mutagenesis of HRE1, HRE2, and combined HRE1/HRE2 led to a reduction of 53%, 58%, and 73% of reporter gene induction, respectively (Fig. 4B), whereas in Caki-2 cells, only HRE2 and HRE1/HRE2 mutated constructs led to a decrease of 75% (Supplementary Fig. S1B). These results indicate that both HRE sites are important in hypoxia response and are able to mediate activation of the *MUC1* promoter.

Further, we tested the direct interaction of HIF-1α with HRE sites within the *MUC1* promoter by a chromatin immunoprecipitation assay. As shown in Fig. 4C, under normoxic conditions, the anti-HIF-1α antibody, but not the control rabbit IgG, precipitated the *MUC1* promoter fragment spanning HRE1 and HRE2 in Caki-2 cells in which HIF-1α is constitutively activated. In HEK293 cells, following immunoprecipitation with HIF-1α antibody and PCR, a strong band was observed under hypoxic conditions (~2.9-fold compared with normoxia; Fig. 4C). These data show that HIF-1 directly binds to the *MUC1* promoter. To precisely identify the DNA sequence involved in HIF-1 binding to the *MUC1* promoter,

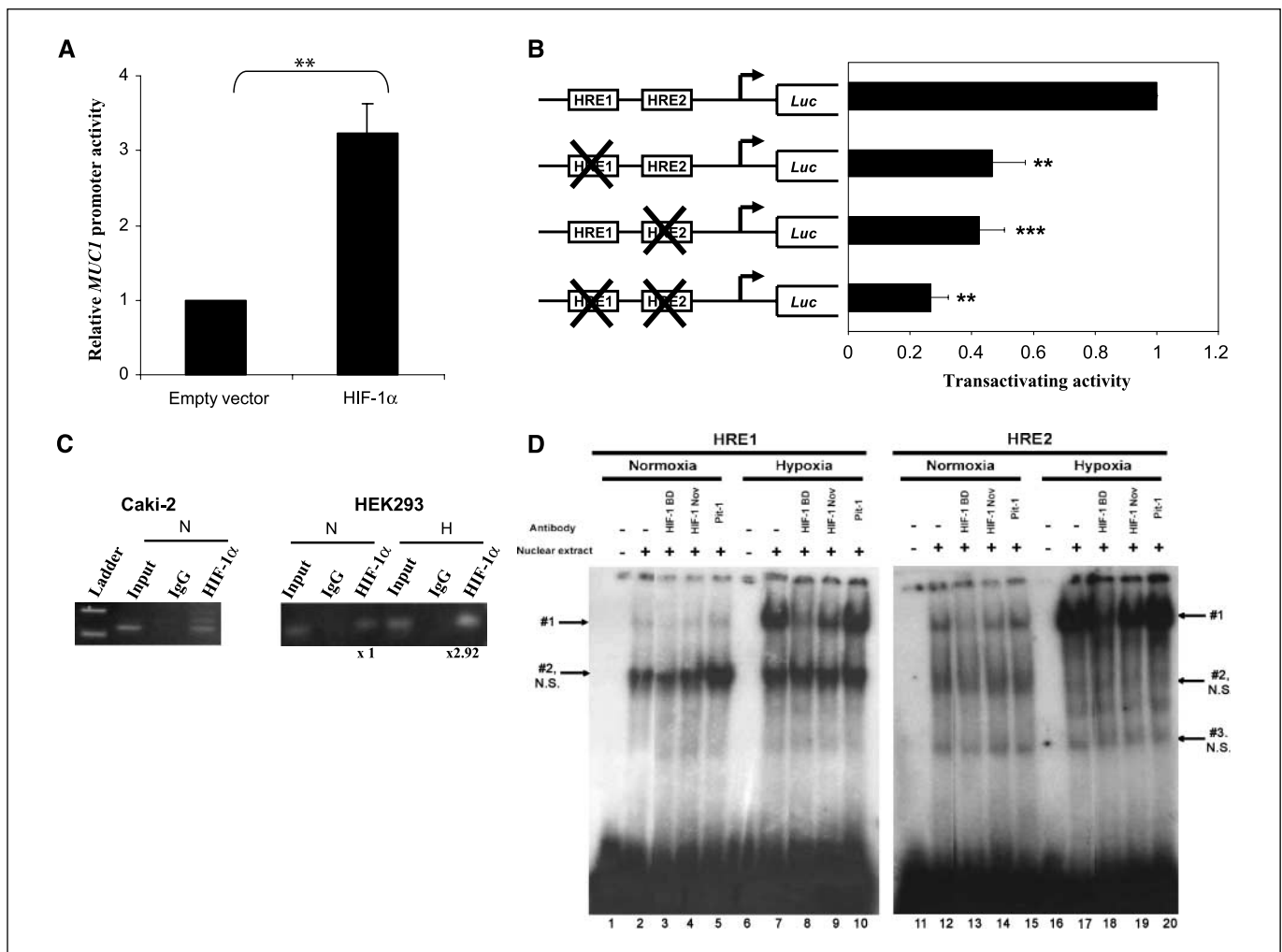
we performed an electrophoretic mobility shift assay. As shown in Fig. 4D, when nuclear extracts from normoxic conditions were incubated with HRE1 (lanes 2–5) and HRE2 (lanes 12–15) probes, shifted bands #1, #2, or/and #3 were visualized. On addition of anti-HIF-1 $\alpha$  antibodies (lanes 3, 4, 13, and 14), no supershifted band was observed for bands #2 and #3. Furthermore, bands #2 and #3 were not specific of hypoxia because on addition of “hypoxic” nuclear extracts, only the intensity of band #1 was very strong (#1, lanes 7 and 17) and decreased in the presence of HIF-1 $\alpha$  antibodies (lanes 8, 9, 18, and 19). An anti-Pit-1 antibody used as negative control has no effect on complex #1 (lanes 10 and 20). As in chromatin immunoprecipitation experiments, we also observed a faint band for complex #1 under normoxic conditions

(lanes 2 and 12) and its intensity decreased in the presence of HIF-1 $\alpha$  antibodies (lanes 3, 4, 13, and 14), suggesting that HIF-1 $\alpha$  transcription factor was translocated to nucleus due to a pericellular hypoxia (22).

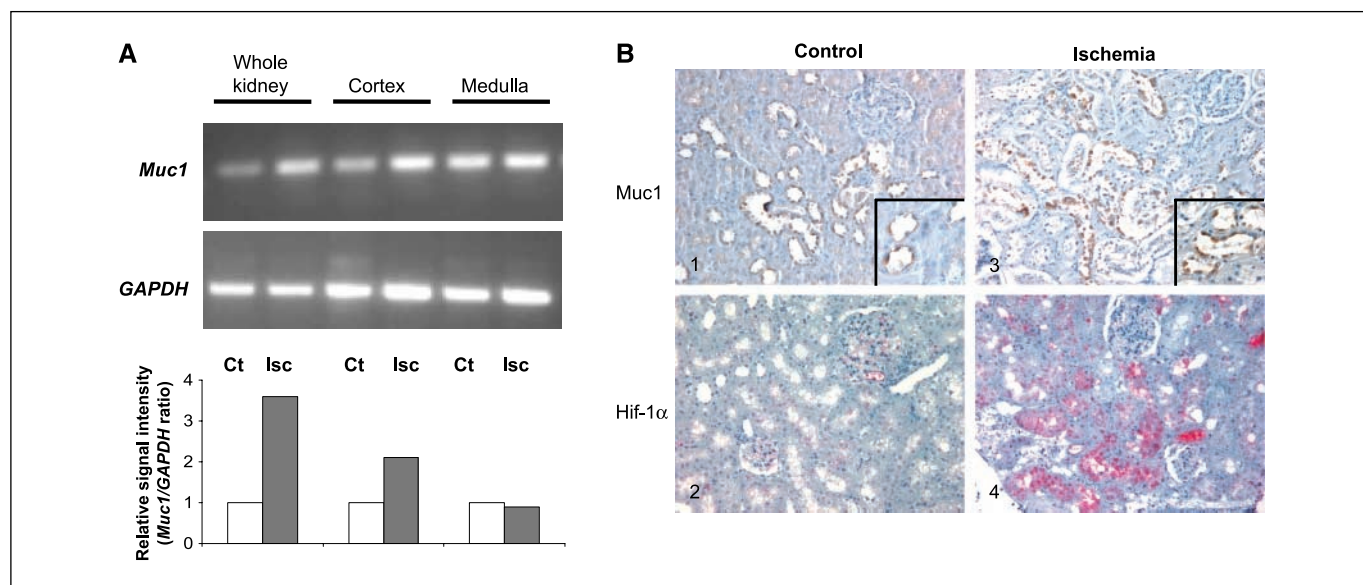
Altogether, these results show that (a) HIF-1 $\alpha$  binds to HRE1 and HRE2 *cis*-elements within the *MUC1* promoter and (b) *MUC1* is a target gene of HIF signaling pathway.

#### MUC1 is induced in an *in vivo* model of kidney ischemia.

To show that *MUC1* is induced in response to hypoxia, we used a rat model of ischemia/reperfusion that mimics a renal hypoxia (16). By RT-PCR, we observed an increase of *Muc1* mRNA expression in ischemic whole kidney (3.6-fold) and in ischemic renal cortex fraction (2.11-fold) compared with control kidney



**Figure 4.** HIF transcription factor directly interacts with the *MUC1* promoter. **A**, cotransfection experiments were done under normoxia with 1  $\mu$ g of the *MUC1* promoter (–2870/+33) and 0.5  $\mu$ g of empty vector or HIF-1 $\alpha$  expression vector in HEK293 cells. The values obtained with the empty vector were referred to as 1. **B**, transient transfection experiments were done with HEK293 cells under normoxia in the presence of 1  $\mu$ g of wild-type or site-directed mutagenesis of HRE sites of the *MUC1* promoter constructs and 0.5  $\mu$ g of HIF-1 $\alpha$  expression vector. The transactivating activity obtained with the wild-type construct was arbitrarily set to 1. **A** and **B**, columns, mean; bars, SE. Representative of five separate experiments in triplicate. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Student's *t* test). **C**, *in vivo* binding of HIF-1 $\alpha$  to chromatin was assayed by chromatin immunoprecipitation technique. Caki-2 cells were cultured under normoxia whereas HEK293 cells were cultured under normoxia and hypoxia. PCR was carried out with a specific pair of primers covering the two HRE sites. Rabbit IgG was used as a negative control. For HEK293 cells, the intensities of the signals were determined by densitometric scanning and are expressed as the relative signal intensity (ratio HIF-1 $\alpha$ /input) compared with that obtained with normoxic HEK293 cells. **D**, autoradiogram of the electrophoretic mobility shift assay. Eight micrograms of nuclear extracts prepared from HEK293 cells cultured under normoxia (lanes 2–5 and 12–15) or hypoxia (lanes 7–10 and 17–20) were incubated with radiolabeled DNA probes recovering HRE1 (lanes 1–10) or HRE2 (lanes 11–20) sites. Radiolabeled probe alone was loaded in first lane of each series (lanes 1, 6, 11, and 16). Supershift experiments were done by adding 2  $\mu$ L of anti-HIF-1 $\alpha$  (BD Biosciences: lanes 3, 8, 13, and 18; Novus Biological: lanes 4, 9, 14, and 19) or anti-Pit-1 (lanes 5, 10, 15, and 20) antibodies. N.S., bands were not specific of hypoxia response.



**Figure 5.** Renal ischemia injury done in a rat model induces MUC1 overexpression. **A**, RNA was isolated from control (Ct) and ischemic (Isc) rat kidneys and then amplified with Muc1- and GAPDH-specific primers. The intensities of the signals were determined by densitometric scanning and are expressed as the relative signal intensity compared with that obtained with control rat kidney. **B**, immunohistochemistry was done on control and ischemic rat kidneys, as described in Materials and Methods, with anti-Muc1 (1 and 3) and anti-HIF-1 $\alpha$  (2 and 4) antibodies. Magnification,  $\times 200$ ;  $\times 400$  (insets).

(Fig. 5A). No difference was noted between renal medullary fractions from ischemic and control kidneys (Fig. 5A). By immunohistochemistry, control rat kidneys were HIF-1 $\alpha$  negative and presented a Muc1 apical membranous expression at the collecting ducts and distal convoluted tubules (Fig. 5B, 1 and 2). We confirmed that clamping induced hypoxia by showing nuclear HIF-1 $\alpha$  staining in clamped kidney compared with control kidney (Fig. 5B, 2 and 4). Importantly, clamping renal pedicle for 1 hour followed by 2 hours of reperfusion induced strong Muc1 overexpression in collecting ducts and distal convoluted tubules with a strong apical and slightly cytoplasmic pattern (Fig. 5B, 1 and 3).

**MUC1 plays a role in migration and invasion properties.** We evaluated the role of hypoxia in the migration and invasive properties of renal cells (Fig. 6A). We showed that hypoxia significantly enhanced invasive and migration properties (4.7- and 3.9-fold induction, respectively). Because MUC1 expression was induced in ACHN cells by hypoxia, we assessed its potential role in hypoxia-induced migration and invasion by generating ACHN cells deficient for MUC1 using a shRNA strategy. By Western blot, after 72 hours of hypoxia, parental ACHN cells and stably transfected ACHN cells with an empty vector expressed MUC1, whereas no expression of MUC1 was detected in ACHN clones 1.1.1, 1.1.6, 1.2.5, and 1.2.8, which were stably transfected with a shRNA targeting MUC1 (Fig. 6B). When tested for invasion and migration properties, these four clones showed a significant reduction of invasive properties under hypoxic conditions compared with control cells ( $P < 0.05$ ; Fig. 6C). Migration and wound closure were also significantly reduced (20–40%) in ACHN clones deficient for MUC1 compared with empty vector ( $P < 0.05$ ; Fig. 6D). These results were confirmed in Caki-2 cells because MUC1 knockdown expression by shRNA retroviral infection (Supplementary Fig. S2A) induced a significant decrease in invasive (50–60%,  $P < 0.05$ ; Supplementary Fig. S2B) and migration (40%,  $P < 0.01$ ; Supplementary Fig. S2C) properties.

These results show that MUC1 is involved in the migration and invasive properties of ACHN renal cancer cells under hypoxia and in Caki-2 cells.

## Discussion

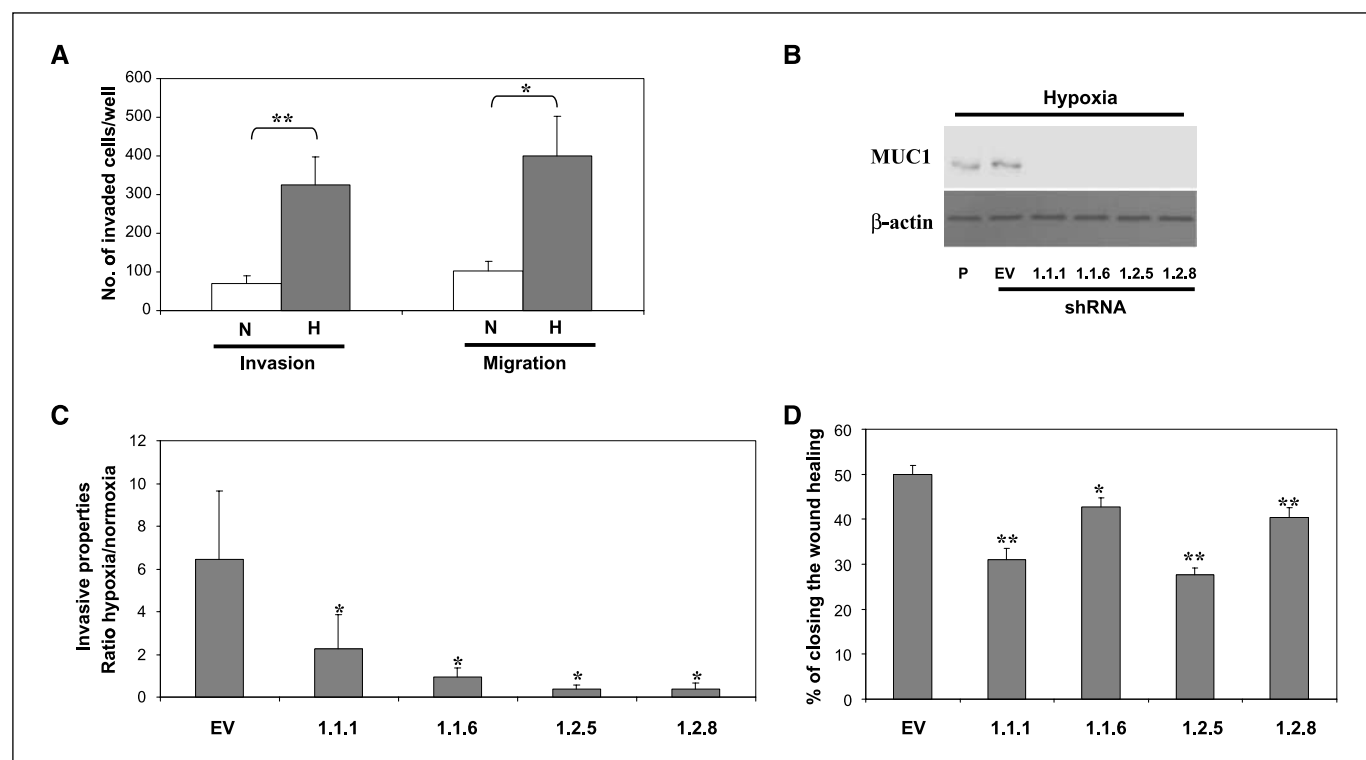
Adaptation to hypoxia is a critical event for tumor cell growth and survival, which is largely accomplished by transcriptional activation of genes facilitating short- and long-term adaptative responses (23). In the current study, we report that hypoxia induces MUC1 in renal cells and show that this up-regulation is directly mediated by HIF-1 $\alpha$  transcription factor. In addition, MUC1 up-regulation represents a critical adaptative mechanism that promotes renal cell invasiveness and migration properties under hypoxic conditions.

MUC1 overexpression has been reported in tumors originating from different tissues, especially in breast and pancreatic tumors (24, 25). Our team and others have reported that MUC1 is consistently overexpressed in cRCC, with a high expression correlated to worse prognosis (9, 10, 12). In the current study, we confirmed the overexpression of MUC1 and HIF-1 $\alpha$  (13) in cRCC and its significant correlation to the metastatic status of patients. Because the HIF pathway is the main renal carcinogenetic pathway, we undertook to study (a) whether MUC1 represents a direct target of hypoxia and (b) whether MUC1 is implicated in cRCC tumor progression as suggested by immunohistochemistry on tumor patient samples. To our knowledge, only two studies reported a potential hypoxia-induced activation of MUC1 (26, 27). Giatromanolaki and colleagues showed in a series of 70 non-small-cell lung carcinomas that MUC1 was coexpressed with CAIX, a well-known target of the hypoxia pathway (26). Second, Leonard and colleagues detected in a global gene expression microarray analysis that 16 hours of hypoxia were able to induce MUC1 gene expression in human tubular proximal renal cells and showed the involvement of HIF-1 $\alpha$  in the hypoxic regulation of MUC1

expression (27). Interestingly, similar results were obtained with MUC3, another member of the membrane-bound mucin family in T84 colon cancer cells (28). Our report shows a link between HIF-1 $\alpha$  and MUC1. Indeed, we confirmed that MUC1 is induced by hypoxic exposure both at the mRNA and protein levels in renal cell lines. MUC1 induction as early as 6 hours under hypoxic conditions suggests a direct mechanism similar to that for CAIX (29). In our renal cell model, MUC1 per se had little, if any, effect on HIF-1 $\alpha$  gene expression and its downstream target CAIX (Fig. 2B). This was confirmed using a pool of siRNA targeting MUC1 where MUC1 silencing did not influence the RNA level expression of HIF-1 $\alpha$  and CAIX under either normoxic or hypoxic exposure as compared with controls (data not shown).

Recently, Yin and colleagues reported that MUC1 was able to attenuate activation of HIF-1 and to promote HIF-1 $\alpha$  degradation in HCT116 colon cancer cells. However, they did not examine the possible role of HIF-1 $\alpha$  in the hypoxic regulation of MUC1 (30). These discrepant results could be related to the use of different cellular models. On the other hand, they may be considered as complementary because up-regulation of MUC1 could represent a feedback to hypoxia-induced activation of HIF-1 $\alpha$  to limit the hypoxic stress effects, as proposed by Yin (30). In this study, MUC1 induction by hypoxia was shown to be HIF-1 $\alpha$  dependent and involved two HRE *cis*-elements located at -1488/1485 and -1510/1507 within the MUC1 promoter. Residual promoter activity despite mutation of the two HRE sites may suggest

requirements of other pathways in the hypoxic setting. Indeed, like other hypoxia target genes such as VEGF (31) and COX-2 (32), HIF-1 $\alpha$  may not be the only regulator of hypoxic induction of MUC1. Indeed, hypoxia induces a generalized stress on cells with activation of multiple pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt by direct hypoxic activation of growth factor receptor (33) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) through hypoxic inactivation of prolyl hydroxylase (34, 35). Using specific pharmacologic inhibitors of the PI3K (LY294002) and NF- $\kappa$ B (BAY11-7082) pathways, we were able to partially inhibit the hypoxia-induced expression of MUC1 at mRNA level in ACHN cells (Supplementary Fig. S3). These data confirmed previous results obtained in the laboratory about the activation of MUC1 expression by the PI3K pathway (36). In addition, the MUC1 promoter has been shown to contain a functional  $\kappa$ B *cis*-element located at -589/-580 (37). However, we could not eliminate the fact that hypoxic activation of the PI3K/Akt and NF- $\kappa$ B pathways may exert their effects directly on HIF-1 $\alpha$  because PI3K is known to control HIF-1 $\alpha$  protein level by hypoxia (38) and that the NF- $\kappa$ B pathway can transcriptionally stimulate HIF-1 $\alpha$  expression (39). The use of a rat model of ischemia (16) allowed us to show that MUC1 was overexpressed not only at mRNA level but also at protein level in the whole renal parenchyma and, more specifically, in the renal cortex. These data confirm that MUC1 and HIF-1 $\alpha$  expression is induced *in vivo* by hypoxia in renal parenchyma and that MUC1 is a target gene of the HIF/ischemia signaling pathway.



**Figure 6.** MUC1 is involved in increase of the migratory and invasive properties of ACHN cells under hypoxia. *A*, cell invasion and migration were evaluated using 24-well Matrigel invasion chambers with 10% FCS as chemoattractant and Boyden chamber, respectively. The graphs show the total number of invasive/migratory cells counted 24 h after seeding. *B*, Western blots were done on cell lysates obtained from parental ACHN cells stably transfected with empty vector (EV) or shRNA targeting MUC1, cultured for 72 h under hypoxic conditions. Lysates were immunoblotted with anti-MUC1 and anti- $\beta$ -actin antibodies. *C*, cell invasion was evaluated using 24-well Matrigel invasion chambers with 10% FCS as chemoattractant. The graphs show the ratio of the total number of invasive cells counted 24 h after seeding in hypoxia to the total number of cells counted in normoxia. *D*, confluent monolayers of ACHN cells stably transfected with empty vector or shRNA targeting MUC1 were scraped from one side of the reference line. Wound healings were measured at 0 and 24 h, and percentage of wound closure was determined. Columns, mean; bars, SE. Representative of five separate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (Student's *t* test).

Because immunohistochemical studies suggested that MUC1 overexpression could be implicated in cRCC tumor progression, we assessed *in vitro* the role of MUC1 in the biological properties of renal cells. We showed that MUC1 silencing significantly decreased the migration and invasive properties of ACHN cells under hypoxia and of Caki-2 cells in normoxia, but effects were less pronounced on migratory properties. These data are in agreement with previous results showing that down-regulation of MUC1 has been accompanied with (a) up-regulation of E-cadherin and  $\beta$ -catenin protein expression with relocation of  $\beta$ -catenin from the nucleus to the cytoplasm (40, 41), (b) an increase in E-cadherin membrane expression and E-cadherin/ $\beta$ -catenin complex formation (41), and (c) a decrease in invasive properties (40). Other authors showed that overexpression of MUC1 in cultured cells inhibits their aggregation possibly not only because of its large, extended, and rigid structure (42) but also by inhibition of E-cadherin-mediated cell-cell adhesion (43). MUC1 expressed in tumors has been hypothesized to function as an anti-adhesion molecule that inhibits cell-cell adhesion, allowing invasion into surrounding tissues (40, 44). Cells with high levels of MUC1 have reduced interaction between integrins and the extracellular matrix (45). Other experimental studies reported that cultured gastric cells acquired increased motility and invasive properties when stably transfected with a MUC1 expressing vector (46). In addition, stable MUC1 expression in Madin-Darby canine kidney cells and breast adenocarcinomatous cells enabled them to polarize and undergo glandular tubulogenesis on type I collagen matrix (47). MUC1 has been implicated in cytoskeletal reorganization and directed cell motility during cell migration through Src-CrkL-Rac1/Cdc42 signaling cascade following intercellular adhesion molecule-1/MUC1 interaction in breast cancer cells (48). Altogether, these

experimental data support a role of MUC1 in the invasive and migratory properties of renal cells. Hypoxia exposure has been shown to promote migration, invasion, and metastasis (49) in several cellular models and, in particular, in renal cells (50). Our study is the first one to establish a direct link between MUC1 and cell migration/invasion through hypoxia in a renal cell model.

In summary, we report that MUC1 is a new target gene of the HIF signaling pathway, which is the main renal carcinogenetic pathway and is an actor in migration and invasive properties in renal cancer cells. Therefore, we postulate that MUC1 could be a new therapeutic target in cRCC because MUC1 has already been identified as a potential target for immunotherapy and gene-based therapies in other carcinomas.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 12/26/08; revised 4/22/09; accepted 5/7/09; published OnlineFirst 6/23/09.

**Grant support:** M. Perrais' work was supported by the "Fondation pour la Recherche Médicale-Région Nord-Pas-de-Calais." D. Bernard's work was supported by the "Association pour la Recherche sur le Cancer" and the "Comité du Pas de Calais de la Ligue Nationale contre le Cancer".

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We thank Marc Samyn (Pôle de Pathologie, CHRU, Lille) for excellent technical help; Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ) for the kind gift of the MUC1 promoter construct; Dr. S. McKnight (Southwestern Medical Center, Dallas, TX) and Dr. F. Bunn (Harvard, Boston, MA) for the kind gift of HIF-1 $\alpha$  constitutive and HIF-1 $\alpha$  dominant-negative expression vectors; and Dr. D. Swallow (Imperial Cancer Research, London, United Kingdom) for the kind gift of MUC1 clone M8 antibody.

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*Cancer Res* 2009;69:5707-5715. Published OnlineFirst June 23, 2009.

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