Effects of CoCl$_2$-simulated hypoxia on the expression levels of matrix metalloproteinases in renal adenocarcinoma cells and renal tubular epithelial cells

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Abstract. Renal cell carcinoma (RCC) and chronic kidney disease (CKD) are associated with hypoxia, but the effects of hypoxia on the process of angiogenesis in the two diseases are dramatically different. Some of matrix metalloproteinases (MMPs), such as MMP2 and MMP9, may have a role because they represent the most prominent family of proteinases associated with angiogenesis. In the present study, the differential response of human renal cell cancer cells (786-0), human renal tubular epithelial cells (HK-2) and human microvascular endothelial cells (HMEC-1) to hypoxia with regards to the expression of MMP2, MMP9, MMP14, TIMP2, RECK was investigated. Cobalt chloride (CoCl$_2$) treatment was used to simulate the hypoxia environment in RCC and CKD. The expression levels of HIF-1α, RECK, MMP2, MMP9, MMP14 and TIMP2 in HK2, 786-0 and HMEC-1 cells were determined by western blot analysis after incubation with varying concentrations of CoCl$_2$ for 24 h. It was indicated that the effects of hypoxia on the endogenous expression of RECK and MMP2 differed depending on the considered cell type. Notably, the RECK expression was significantly decreased in 786-0 cells under hypoxia, whereas this expression was slightly increased in HK2 and HMEC-1 cells. Furthermore, the MMP2 expression was significantly increased in HMEC-1 cells under hypoxia, whereas the expression was slightly decreased in HK2 and 786-0 cells. These results demonstrate that 786-0, HK2 and HMEC-1 cells respond differently under hypoxic conditions. Furthermore, MMP2 and RECK may serve divergent roles in HK2 and HMEC-1 cells under hypoxic conditions.

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Introduction

Renal cell carcinoma (RCC) usually has an abundant number of vessels, but they suffer from hypoxia and nutrient deprivation because abnormal vessel structure and function. The hypoxic tumour environment creates a favourable condition for tumour progression and metastasis, and it also leads to the resistance of tumour cells to radiation therapy and many chemotherapeutics (1). Hypoxia is also one of the most important mechanisms that accelerate chronic kidney disease (CKD). Compared with RCC, the angiogenic response to hypoxia in CKD is seriously inadequate, which is followed by progressive rarefaction of the peritubular capillary (PTC) network (2). Chronic tissue hypoxia can generate both an angiogenic and a fibrogenic response. However, in CKD, the balance is strongly tipped toward fibrogenesis. It has been suggested that failed angiogenesis and hypoxia may be central to progressive CKD, but the mechanisms that lead to the apparent imbalance between angiogenesis and fibrosis under hypoxia are as yet poorly understood.

The angiogenic switch refers to a process during tumor progression where the balance between pro- and anti-angiogenic factors tilts towards a pro-angiogenic outcome, resulting in normally quiescent vasculature continually sprout new vessels (3). In response to hypoxia, the expression of VEGF is increased via hypoxia-inducible factor 1α (HIF-1α) that is usually considered to be a pro-angiogenic factor. However, in CKD, hypoxia and HIF-1α activation are observed, but there is essentially no angiogenic response, the balance of angiogenic switch is strongly tipped towards an anti-angiogenic outcome. Taken together, there may be one or more crucial molecular mediators in RCC and CKD, which control the transition from capillary vessel hyperplasia to regression. The changes of these moleculars lead to sustained vascular loss in the face of hypoxia during CKD and excessive angiogenesis during RCC.

The matrix metalloproteinases (MMPs) represent the most prominent family of proteinases associated with angiogenesis. In vascular tissues, MMPs can be stimulated and activated by various stimuli, including hemodynamics, oxidative stress, inflammation, hormonal factors, and hypoxia (4). Activated MMPs degrade ECM to liberate ECs from basement membrane and facilitate migration and invasion of ECs. MMPs

can liberate angiogenic molecules that are sequestered in the ECM. On the other hand, they may generate new fragments that inhibit angiogenesis. Moreover, vascular cell proliferation and apoptosis can also be regulated by MMPs. The vessel maturation involves recruitment of mural cells and the establishment of a basement membrane. During this process, the activities of MMPs need to be tightly regulated to avoid excessive ECM degradation and tissue damage. Tissue inhibitors of metalloproteinases (TIMPs) and RECK are endogenous protein regulators of the MMPs family. TIMPs are traditionally thought to control extracellular matrix (ECM) proteolysis through direct inhibition of MMP-dependent ECM proteolysis. TIMPs also have biological activities that are independent of metalloproteinases. RECK, a membrane-bound glycoprotein, is normally expressed in all cells and plays an important role in ECM remodeling and angiogenesis by negatively regulating at least three MMPs: MMP2, MMP9, and MMP14.

In this study, we hypothesized that there exist some crucial molecular mediators in RCC and CKD under hypoxic condition, which controls the transition from capillary vessel hyperplasia to regression. It is also the reason why angiogenic responses to hypoxia are significantly different in renal cancer and fibrosis. Therefore, in this study, we used $CoCl_2$-simulated hypoxia to investigate the differential effect of hypoxia on MMPs expression between 786-0, HK2 and HMEC-1 cells.

Materials and methods

Cell culture and treatment. Human renal cell cancer cells (786-0), human renal tubular epithelial cells (HK-2) and human microvascular endothelial cells (HMEC-1) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal calf serum at 37˚C in a humidified atmosphere at 5% CO_2. The culture medium was changed every two days and cells were subcultured about once a week. For treatment with cobalt chloride ($CoCl_2.6H_2O$; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), cells were seeded into 6-well plates at a density of $1x10^5$/well. After 90% confluence was reached, they were treated with varying concentrations of $CoCl_2$ for 24 h.

Western blot analysis. Cells were lysed in lysis buffer. After centrifugation, the supernatant was collected and quantified. The same amount of total protein was separated by 12% SDS-PAGE and then transferred to a PVDF membrane. The membrane was then incubated overnight at 4˚C with antibodies against RECK, HIF-1α, MMP2, MMP9, MMP14, TIMP2 or β-tubulin. After incubation with the secondary antibody labelled with HRP at 37˚C for 1 h, protein bands were visualized using enhanced chemiluminescence (ECL). The relative protein levels were calculated by comparison to the amount of β-tubulin protein. The source and dilution of unconjugated antibodies used in this study are as follows: HIF-1α (rabbit pAb; NB100-134SS, 1:1,000; Novus Biologicals, Ltd., Cambridge, UK), RECK (goat pAb; AF1734, 1:1,000; R&D Systems, Inc., Minneapolis, MN, USA), MMP2 (rabbit pAb; NB200-193, 1:1,000; Novus Biologicals), MMP9 (rabbit mAb; ab76003, 1:1,000; Abcam, Cambridge, UK), MMP14 (rabbit mAb; ab51074, 1:1,000; Abcam), TIMP2 (rabbit pAb; BS1366, 1:500; Bioworld Technology, Inc., St. Louis Park, MN, USA), VEGF-A (rabbit pAb; BS2431, 1:500; Bioworld Technology, Inc.), β-Tubulin (Mouse pAb; BS1482 M, 1:1,000; Bioworld Technology, Inc.), Fluorescein (FITC)-conjugated Goat Anti-Rabbit IgG (BS10950, 1:200; Bioworld Technology, Inc.), Horseradish Peroxidase (HRP)-conjugated Goat Anti-Rabbit IgG (BS13278, 1:3,000; Bioworld Technology, Inc.), Horseradish Peroxidase (HRP)-conjugated Goat Anti-Mouse IgG (BS12478, 1:3,000; Bioworld Technology, Inc.), Horseradish Peroxidase (HRP)-conjugated Rabbit Anti-Goat IgG (BS30503, 1:3,000; Bioworld Technology, Inc.).

Cell proliferation assay. HK2, 786-0 and HMEC-1 cells were seeded into 96-well plates at a density of $5x10^3$/well. After adherence of cells, they were treated with $CoCl_2$ at different concentrations for various periods of time as indicated. Six duplicate wells were set up for each sample. MTT (5 mg/ml) was added to the medium at 10 ml/well followed by incubation at 37˚C for 4 h. After the medium was removed, cells were lysed with 150 μl dimethyl sulfoxide (DMSO). Absorbance of each well was measured by a microplate reader at 490-nm wavelength.

Immunofluorescent staining. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and immersed in 0.2% Triton X-100 for 10 min at 4˚C. After blocking with 4% BSA for 1 h at 37˚C, cells were incubated overnight at 4˚C with primary antibody (1:50) in PBS. Cells were then incubated with fluorophore-conjugated secondary antibody (1:200) in 4% BSA for 1 h at 37˚C, and then stained DAPI (1:500) for 5 min at room temperature. For the negative control, PBS was used in place of the primary antibody. Cells were viewed under the fluorescence microscope equipped with a digital camera.

Statistical analysis. Each experiment was performed at least three times. All results are presented as the mean ± standard deviation. All analyses were performed using GraphPad Prism 5 software. Statistical analysis was performed using one-way analysis of variance with Tukey' s post-hoc test. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chemical hypoxia model. $CoCl_2$ is a chemical hypoxia agent which leads to the stabilization of HIF1-α and the expression of hypoxia responsive genes. Indeed, we observed the up-regulation of HIF-1α in HK2 and HMEC-1 cells after incubation with varying concentrations of $CoCl_2$ from 0 to 800 μmol/l for 24 h (Fig. 1A). The protein expression level of HIF-1α was rapidly up-regulated in a dose-dependent manner with the increase of $CoCl_2$ concentration, and with a peak response occurring at 150 μmol/l $CoCl_2$ groups. Then the HIF-1α expression began to gradually decrease when concentration of $CoCl_2$ was higher than 150 μmol/l. It should be noted that, in 786-0 cells, HIF-1α was continuously expressed and maintained at a relatively high level under normoxia, which was associated with the
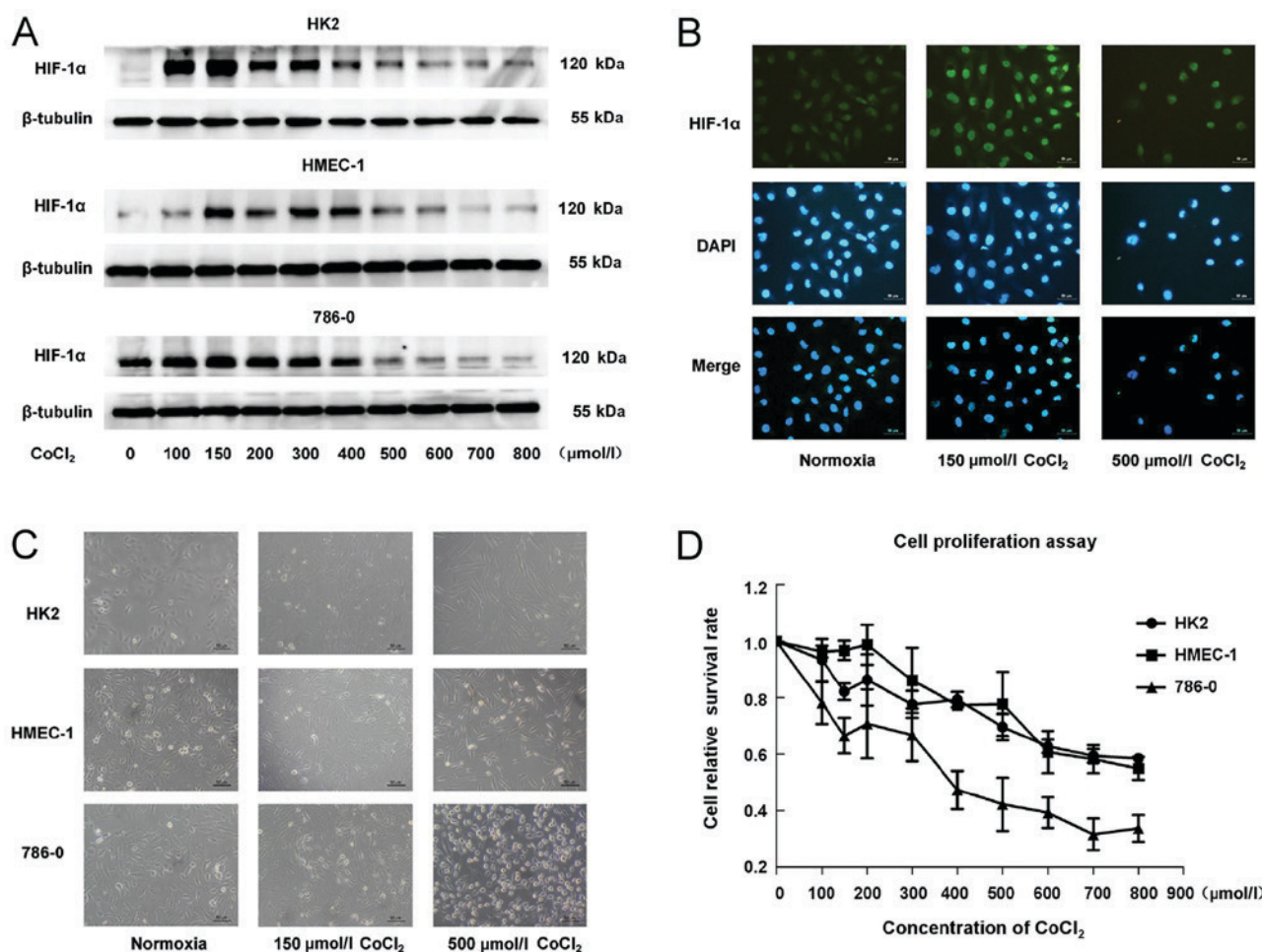
Figure 1. Establishment of chemical hypoxia models. (A) Expression levels of HIF-1α in HK2, 786-0 and HMEC-1 cells were determined by western blot analysis after incubation with varying concentrations of CoCl₂ from 0 to 800 µmol/l for 24 h. (B) Representative images from immunohistochemical staining for HIF-1α in HK2 cells after treatment with varying concentrations of CoCl₂ from 0 to 800 µmol/l for 24 h were indicated (magnification, x200). (C) To examine the effects of varying concentrations of CoCl₂ on cells, cell morphological observation was performed under a microscope (magnification, x200). (D) Effects of CoCl₂ on cell survival rate of HMEC-1, HK2 and 786-0 cells. In all cases, at least three independent experiments were performed. HIF-1α, hypoxia-inducible factor 1α; HK2, human renal tubular epithelial cells; 786-0, human renal cell cancer cells; HMEC-1, human microvascular endothelial cells.

loss of the von Hippel-Lindau (VHL) gene product in RCC. At low CoCl₂ concentration (150 µmol/l), HIF-1α expression was slightly up-regulated in 786-0 cells (P=0.0086), whereas when the concentration of CoCl₂ was higher than 400 µmol/l (P=0.0415), reversely, it was significantly decreased compared with normoxic group (Fig. 1A). We also observed the distribution of HIF-1α in HK2 cells treated with CoCl₂ by using cellular immunofluorescence staining technique. The data in Fig. 1B suggested that HIF-1α molecule always distributed in the nucleus, not in the cytoplasm, at a relatively uniform density when HK2 cells were treated with varying concentrations of CoCl₂ from 0 to 800 µmol/l for 24 h. Exposure to CoCl₂ at 150 µmol/l for 24 h considerably enhanced the mean fluorescent intensity (MFI) of FITC stain in HK2 cells (P=0.0008).

Chemical hypoxia affects cellular morphology and proliferation. To examine the effects of varying concentrations of CoCl₂ on cells, morphological observation was firstly performed under microscope (Fig. 1C). At low CoCl₂ concentration (150 µmol/l), there was no significant change in cell morphology except that some vacuoles was observed in the cytoplasm, whereas when concentration of CoCl₂ was

higher than 500 µmol/l, cells were gradually elongated. The cells treated by high CoCl₂ concentration (500 µmol/l) for 24 h began to shrink and were easier to die. In order to further evaluate the cytotoxicity of CoCl₂ at different concentrations, MTT assay was used to observe cell proliferation and cell survival rate was calculated (Fig. 1D). The data revealed that CoCl₂ treatment caused the loss of cell viability in a dose-dependent manner.

Effects of chemical hypoxia on the expression of MMPs are dramatically different. In the present study, the HMEC-1, HK2 and 786-0 cells treated with varying concentrations of CoCl₂ were used to further explore the responses of MMPs to hypoxia in RCC and CKD. Their endogenous MMPs protein levels under normoxia and chemical hypoxia conditions were examined by western blot. After treated with low CoCl₂ concentration (150 µmol/l) for 24 h, we found that RECK expression was down-regulated in 786-0 cells (P=0.0325). However, in HK2 and HMEC-1 cells, RECK expression was obviously up-regulated (P=0.0106 and 0.0083; Fig. 2). The MMP2 expression was increased in HMEC-1 cells in chemical hypoxia (P=0.0092). However, it was decreased in
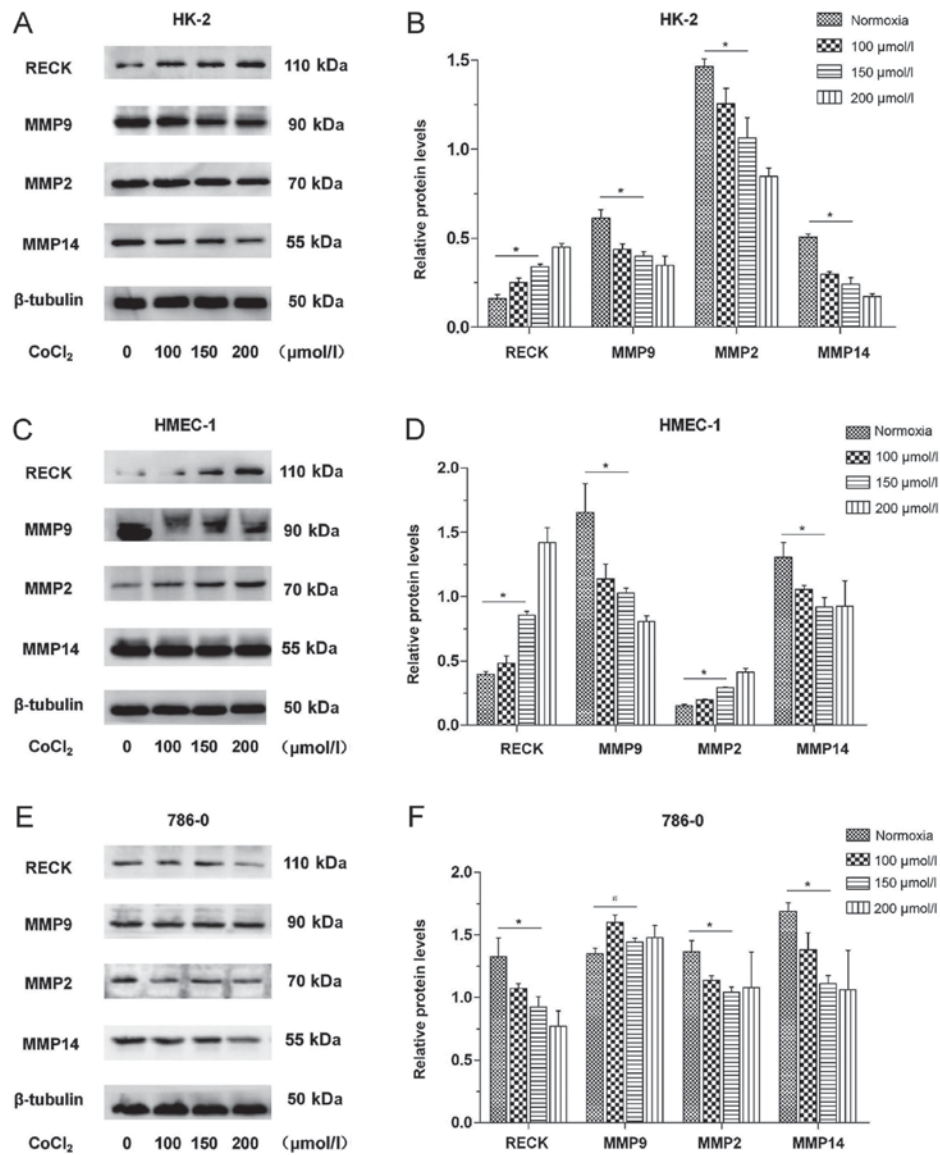
Figure 2. (A-F) Effects of chemical hypoxia on the expression levels of MMPs differed. (A, C and E) Expression levels of RECK, MMP2, MMP9 and MMP14 in HK2, 786-0 and HMEC-1 cells were determined by western blot analysis after incubation with varying concentrations of CoCl$_2$ from 0 to 200 μmol/l for 24 h. (B, D and F) Normalized densitometric quantitation. *P<0.05, #P>0.05 as indicated. In all cases, at least three independent experiments were performed. MMP, matrix metalloproteinase; HK2, human renal tubular epithelial cells; 786-0, human renal cell cancer cells; HMEC-1, human microvascular endothelial cells.

HK2 and 786-0 cells (P=0.0018 and 0.0216; Fig. 2). We also noticed that MMP9 expression was gradually decreased after up-regulation of HIF-1α by CoCl$_2$ in HMEC-1 and HK2 cells. Compared with control group, we did not find significant change of MMP14 expression in 786-0 cells in chemical hypoxia (P>0.05; Fig. 2). We also found that MMP14 expression was slightly decreased in HK2, HMEC-1 and 786-0 cells (Fig. 2).

TIMP2 selectively interacts with MMP14 to facilitate the cell-surface activation of proMMP2. At lower concentrations of TIMP-2, the activation is enhanced while at higher concentrations the activation is inhibited. The ratio between TIMP-2 and MMP-2 is critical to the activity of MMP-2. The expression levels of TIMP2 and RECK were also determined by western blot. The data in Fig. 3 suggested that the TIMP2 expression was obviously decreased in HMEC-1 cells at low concentration of CoCl$_2$ (150 μmol/l). However, at high

concentration of CoCl$_2$ (>500 μmol/l) group, the expression of TIMP2 was increased significantly in HMEC-1 cells although cells began to shrink at the moment. Similar results were also found in HK2 and 786-0 cells.

Discussion

In the present study, we investigated the differential response of 786-0, HK2 and HMEC-1 cells to hypoxia on expression of MMPs. Following exposure to low concentration of CoCl$_2$ (150 μmol/l), cells induce hypoxia adaptive responses via HIF-1α up-regulation (Fig. 1). However, high concentration of CoCl$_2$ (>500 μmol/l) cause cells injury or atrophy, probably by cobalt ion (Co^{2+}), injured cells lose the ability to furthest up-regulate HIF-1α expression.

Fig. 2 showed that RECK expression was down-regulated in 786-0 cells after treated with low concentration of CoCl$_2$.
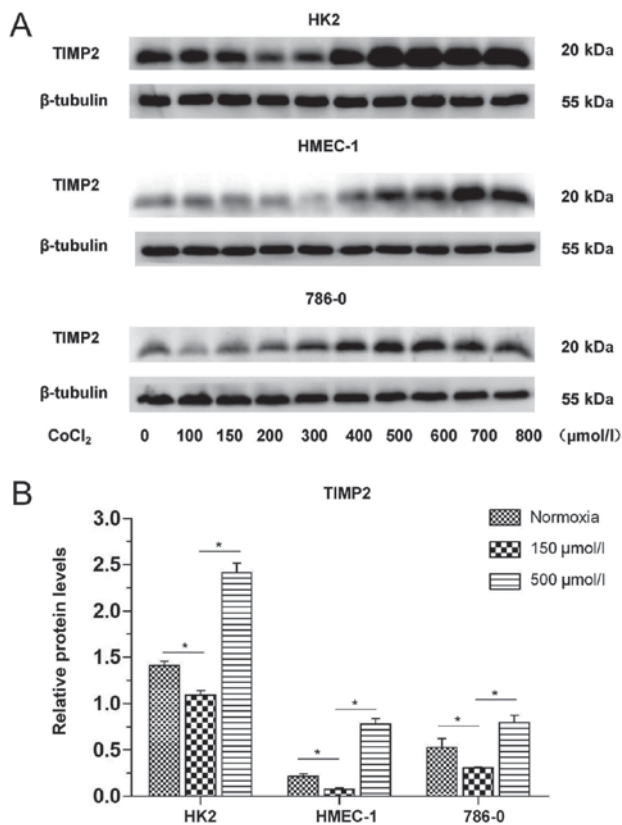
Figure 3. Effects of chemical hypoxia on the expression levels of TIMP2 in 786-0, HK2 and HMEC-1 cells. (A) Expression levels of TIMP2 in HK2, 786-0 and HMEC-1 cells were determined by western blot analysis after incubation with varying concentrations of CoCl$_2$ from 0 to 800 μmol/l for 24 h. (B) Normalized densitometric quantitation. *P<0.05. In all cases, at least three independent experiments were performed. TIMP, tissue inhibitors of metalloproteinase; HK2, human renal tubular epithelial cells; 786-0, human renal cell cancer cells; HMEC-1, human microvascular endothelial cells.

Recent studies have provided a potential mechanism by which some tumor cells reduce RECK expression in response to hypoxia. It is that RECK is a target of at least three groups of miRNAs (miR-15b/16, miR-21 and miR-372/373) and miR-372/373 are up-regulated in response to hypoxia through HIF-1α and TWIST1, whereas miR-21 is up-regulated by RAS signaling pathway. Hypoxia and RAS signaling pathway cooperatively down-regulate this tumor suppressor and thereby promote malignant cell behavior (5). However, we also observed that, in HK2 and HMEC-1 cells, RECK expression was obviously upregulated in chemical hypoxia induced CoCl$_2$. This different phenomenon suggested that the mechanism of RECK modulation within 786-0 and HK2 cells may be different though they receive the same hypoxic signal.

We observed that endogenous MMP2 protein level was increased in HMEC-1 cells at low concentration of CoCl$_2$ (150 μmol/l). In recent studies, normoxic HMEC-1 cells are found to constitutively release MMP2 proteins by ELISA and gelatin zymography analyses, whereas hypoxic HMEC-1 cells enhance the secretion of latent form of MMP2, but not the activated form (6). Similar results were observed in human macrovascular ECs (EAhy 926) in ischemia reperfusion (I/R) injury. Hypoxia of macrovascular ECs enhance MMP-2 production and secretion, whereas reoxygenation

that take place in reperfusion further increases its level (7). In general, hypoxia results in an upward tendency of vascular MMP-2 expression and secretion *in vitro* However, we also observed the slightly decrease of MMP2 expression in HK2 cells in chemical hypoxia. Previous studies about ischemic kidneys have reported this different phenomenon. Ischemia and hypoxia in this organ led to a decrease in MMPs and an elevation in TIMP expression accompanied by fibrosis. In this study, however, increased TIMP2 expression did not occur in hypoxic renal epithelial cells but in atrophic ones treated with high concentration of CoCl$_2$ (Fig. 3). These results suggest that the decrease in MMPs and elevation in TIMP expression may do not occur simultaneously in ischemic kidney.

There are four homologous members of the TIMP family from TIMP1 to TIMP4. TIMP2 is constitutively expressed in all normal tissues but demonstrates reduced expression in human cancer cells (8). In the present study, we found that endogenous TIMP2 expression in HMEC-1 cells was obviously decreased at low concentration of CoCl$_2$ (150 umol/l). Some studies have shown that hypoxia reduces secretion of TIMP-2 protein by HUVEC and EaHy926 cells and reduced TIMP-2 levels enhance endothelial cell migration and proliferation (9). However, others have found that normoxic HMEC-1 cells constitutively released TIMP2 proteins and the secretion of TIMP-2 is not altered in response to hypoxia. Interestingly, hypoxic HMEC-1 cells display lower abilities to migrate and organize in tube-like structures compared to normoxic cells, despite increased MMP2 levels (7). A possible explanation for this different phenomenon is that ECs of different origin may respond differently to environmental stimuli. In this study, we also found that, at high concentration of CoCl$_2$ (500 umol/l), the expression of TIMP2 was increased significantly in HMEC-1 cells although cells began to shrink at the moment (Fig. 3). These results suggest that increased TIMP-2 levels in injured endothelial cells may play a different role in endothelial cell migration and proliferation compared with that in hypoxic HMEC-1 cells. Further study will be required to test whether these multiple functions of TIMP2 are truly implicated in CKD in renal fibrosis and cancer.

We observed that endogenous MMP9 expression was gradually decreased after up-regulation of HIF-1α by CoCl$_2$ in HMEC-1 and HK2 cells. Previous studies have shown that the amount of secreted MMP9 proteins by normoxic HMEC-1 cells is so small that they are undetectable in cell supernatants by ELISA or gelatin zymography analyses (6). Many studies suggest that MMP-9 has a distinct role in angiogenic switch by regulating the bioavailability of VEGF-A in tumors. Macrophages, neutrophils and mast cells initiate angiogenesis by MMP9-mediated activation of VEGF. MMP9 has also been reported to be a negative prognostic factor in RCC. Recent studies have shown that protein expressions of MMP9 in 786-0 cells are significantly higher than that in HK-2 cells and MMP9 overexpression in RCC tissues is correlated with vasculogenic mimicry that is one of the modes of vessel formation in tumors (10). However, in other cancer models, MMP9 generates ECM fragments like tumstatin, a potent suppressor of tumor vasculature formation, resulting in increased tumor growth and pathological vascularization in MMP9-deficient mice (11). Taken together, MMPs can generate both angiogenesis-inhibiting as well as promoting

signals. Depending on the time frame of MMP expression and the availability of substrates, the effects of MMPs on angiogenesis might be diverse. This illustrates that one MMP can have opposing effects in different tumor types. In addition, in MMP9-deficient mice, the PTC rarefaction is significantly attenuated following Ischemia-reperfusion injury (12).

MMP14 is a central regulator of cellular migration and involved in inflammation, angiogenesis, cancer cell invasion, epithelial morphogenesis and so on. We observed that MMP14 expression was slightly decreased under chemical hypoxia in HMEC-1 cells. Others have shown that hypoxia inhibits the mRNA expression of MMP14 in human macrovascular ECs (EAhy 926), whereas reoxygenation that take place in reperfusion upregulates MMP14 mRNA expression (7). It should be noted that ECs of different origin may respond differently to environmental stimuli. *In vitro* wound healing migration model, human umbilical vein endothelial cells (HUVECs) are stimulated to migrate by byphorbol 12-myristate 13-acetate (PMA), the expression of MMP14 is up-regulated and its activity can modulate endothelial migration, invasion, and formation of capillary tubes (13). It was found that MMP14 is expressed selectively at the tip of growing vessels where ECM degradation is needed (14). Furthermore, MMP14 performs not only as a matrix-degrading enzyme, but also a signaling molecule on ECs (15). We also observed that the MMP14 expression had a decreasing tendency in 786-0 cells with the up-regulation of HIF-1α. Actually, many studies have reported that hypoxia induce MMP14 expression in tumor cell lines such as retinal glial cells and hepatocellular carcinoma cells (16,17). Whereas some other studies showed that hypoxia does not induce MMP14 mRNA and protein expression in breast cancer TICs. Instead, hypoxia results in trafficking of preexisting MMP14 from cytoplasmic storage pools to cell surface (18). MMP14 expression was also decreased in HK2 cells when treated with low concentration of $CoCl_2$. This result suggested that the effects of hypoxia on MMP14 in renal epithelial cells may have an important role in renal fibrosis. The mechanisms of MMPs regulation by hypoxia are not fully understood. Recent studies suggested that the expression and activity could be regulated by hypoxia via some microRNAs (19). Taken together, activity and function of MMPs in RCC and CKD seem not to be uniform. It is necessary to develop valid experimental models that allow the further study of MMPs involved in RCC and CKD.

In summary, in this study, we found that the effects of hypoxia on the endogenous expression of RECK and MMP2 are dramatically different depending on the considered cell type. These differences may contribute to their different angiogenesis response. It is important to note that these results are found in single cells *in vitro*. We need more effective experimental models for mimicking *in vivo* tissue environment, such as co-culture systems or animal model. In fact, in our other study, co-culture systems of HMEC-1 with 786-0 cells or HK2 cells were established to explore the influence of different parenchymal cells on RECK expression, proliferation and angiogenesis of adjacent endothelial cell (20). These results suggest that RECK or MMP2 protein may be a key molecule involved in the regulation of angiogenesis in RCC and chronic renal disease. It may be a potential target for the clinical treatment of these two diseases.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LC and XZ conceived and designed the experiments. XZ performed the experiments. LC and XZ jointly analyzed the results, drafted the manuscript and performed critical revisions.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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