Hypoxic tubular epithelial cells regulating angiogenesis by Rab 7

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This study was supported by the National Natural Scientific Foundation of China (No. 81370868) and Key R & D Program of Jiangsu Province, China (No BE2019712).

Abstract

Aim: The purpose of our study was to discuss Rab 7 effects in chronic kidney disease (CKD).

Methods: Using WT and Rab 7-/- mice as target animal, and HK-2 and HMEC-1 cell co-cultured to make cell model. Measuring kidney tissues were evaluated by Sirius red staining, immunohistochemistry staining to CD 34 protein, Transmission electron microscope (TEM) and gelatin zymography to MMP-2 activities. The cell proliferation were measured by CCK-8 and Ki67 protein expression. Measuring cell invasion and total length were evaluated by transwell and in vitro angiogenesis assay. MMP-2 activities were evaluated by gelatin zymography in cell groups. The relative proteins expression were evaluated by Western blot in kidney tissues and cell groups.

Results: Hypoxia promoted the expression of Rab7 in HMEC-1, and the activity of MMP-2 related with regulatory molecules such as reversion-inducing-cysteine-rich protein with kazal motifs (RECK), negative correlation with membrane-type 1 MMP (MT1-MMP or MMP-14) on the membrane of TECs. In addition, the up-regulation of the expression of Rab7 inhibited the activity of MMP-2 and proliferation and cyclization of endothelial cells, and the inhibitor of MMP-2 partially blocked the effects of Rab7 on angiogenesis. Furthermore, the similar data were also obtained in the fibrosis kidney tissues of mice.

Conclusion: Rab 7 might be an important role in hypoxic TECs regulated angiogenesis, Rab 7 knockdown could improve hypoxic TECs regulated angiogenesis, the relative mechanisms might be correlation with RECK pathway and MMP-2 activities in vivo and vitro study.

Key words: Rab 7; hypoxic; HMEC-1; MMP-2 activity; RECK
Running Title: Rab 7 and kidney fibrosis

Introduction

CKD has already been a serious public health problem with more than 70 million reported cases affected [1]. Although the prevalence of CKD varies in different countries and regions but the prevalence of CKD in high-income countries such as United States and Australia has been around 11%. The etiology of CKD varies such as the deposition of immune complex, hypertension, and hyperglycemia and so on and then it causes kidney cell damage and chronic inflammation with fibroblasts activation and extracellular matrix synthesis increases, eventually results in renal interstitial fibrosis and finally transforms CKD to end-stage renal disease (ESRD). There are still many terrae incognita badly in need of further study on account of complicated renal fibrosis mechanism [2].

Peritubular capillary reduction is a prominent pathological phenomenon in the progression of CKD, but the mechanism is still unclear. The renal tubular epithelial cells (TECs) are the main cells of kidney and extremely sensitive to hypoxia, proteinuria, toxins, metabolic disorders and senescence. It can release large numbers of active substances which are the primary causes of CKD and may perform different functions in status of peritubular capillaries. [3] It is believed that the decrease of peritubular capillaries may related to main factors as follows: Firstly, decreased blood flow through the peritubular capillaries decreased shear force on vascular wall and then weakened survival signals for blood flow-dependent endothelial and in the end caused endothelial cell apoptosis. Secondly, the pericyte cells detached from the wall of the blood vessel because of local inflammation followed by increased capillary permeability and instability and ultimately caused capillary destruction. Thirdly, the microenvironment of peritubular capillaries changed in advanced disease, especially large amount of type I and type III collagen instead of less extracellular matrix and hindered exchange of information between cells and obstructed the status of VEGF [4-6]. It can be seen from above that there are many reasons for decrease of peritubular capillaries and the effects of renal tubular epithelial cells injury on the formation of peritubular capillary remained to be further studied.

Previous studies showed that VEGF and MMP-2 were key factors of angiogenesis [7]. If hypoxic renal tubular epithelial cells were associated with these two molecules, angiogenesis might be affected. Our previous study found that renal tubular epithelial cells in hypoxic condition could express excessive production of VEGF and MMP-2, but MMP-2 activity was decreased [2, 8]. Therefore, the main reason for the decrease of renal fibrosis tissue capillaries might be related to the decrease of the activity of MMP-2. So we started to focus on the regulation of MMP-2 activity.

MMP-2 is a key role in MMPs. A variety of molecules can regulate MMP-2 activity, such as TIMP-2, RECK and MMP-14. TIMP-2 can mediate MMP-14 to bind with pro-MMP-2 to form a complex to promote MMP-2 activity in low concentration; on the other hand, TIMP-2 overexpression could depress MMP-2 activity [9]. RECK protein in cell membrane could down-regulate MMP-2 activity. MMP-2 has a wide range of functions after activation and can be degraded up to 27 extracellular substances. It can also play an anti-inflammatory role by antagonizing phospholipase A2 and monocyte chemotactic protein 3 [10].

Since the activation of MMP-2 is mainly carried out on the surface of plasma membrane, will the membrane structure change affect the activity of this enzyme? Autophagy and endocytosis can induce cell membrane remodeling and regulate
transmembrane signal transduction. Hypoxia can enhance autophagy and endocytosis [2,8], especially membrane receptor-related endocytosis [11]. Endocytosis and autophagy co-occur with sharing functional links in hypoxia condition [2].

Rab7 has been proved to be one of the common molecules of endocytosis and autophagy [11]. Some research had found that Rab7 is a key member in endosomal membrane transportation. In addition, Rab7 has been proved to be related to autophagy vesicles and is essential for the maintenance of physiological autophagy [12]. Rab7 could regulate endocytosis and autophagy, and participated in the fusion of autophagy endocytosis and lysosomes. In conclusion, Rab7 has been proved to be a key regulator of endocytosis and autophagy [13]. The intervention of the shared molecules may affect the activity of MMP-2 and thereby affect angiogenesis in CKD.

The abnormal expression of Rab7 in hypoxic renal tubular epithelial cells may cause remodeling of cell membranes and lead to decreased activation of MMP-2 and thereby interfering with angiogenesis. This work intends to confirm whether Rab7 regulates angiogenesis through MMP-2 and its internal mechanism.

Materials and Methods

Mice and Reagents

Our research was approved by the Animal Care Committee of Southeast University (No.20170306003). Rab7 knockout mice were constructed by using CRISPR/Cas9 technology (SaiyeBiotechnology Co, LTD.). Wild-type mice (C57BL/6) (Qinglongshan Experimental Animal Company, Nanjing, China). Mice were raised in a facility with controlled air, temperature and light. At the beginning of the experiment, the mice weighed 18-22g. The mice were provided with a normal diet (XieTong, Nanjing, China) and tap water throughout the experiment. CsA (Chem Best, Shanghai, China) was diluted to 10 mg/mL in sunflower oil (Arawana, Shanghai, China). Furosemide (ZhaoHui, Shanghai, China) was diluted to10 mg/mL in distilled water.

Experimental Design

Dividing mice as 4 groups of 5 mice as follows: WT, WT model and Rab7-/- model. In order to induce chronic renal fibrosis, mice in model groups received the following interventions: 50mg/kg furosemide 2 days before the experiment, followed by daily administration of 25mg/kg CsA and 50mg/kg furosemide on alternate days for 28 days. Mice in WT Group was untreated for all 28 days. The body weight of each rat was recorded every 4 days and the dosage was adjusted accordingly.

After 28 days, mice were anaesthetized with pentobarbital (Xiya, Linyi, China) and sacrificed. Kidneys were obtained for histology and western blot detection.

Establishing of HK-2-Rab7- cell line (stably lower expression of Rab7)

HK-2 cells were seeded into 96-well plates with $1.0 \times 10^6$ cells per well. Rab7 lentiviral vector was successfully constructed. Then, HK-2 cells were transfected with si-Rab7 or si-NC (negative control). Finally, quantitative real-time PCR and western blot analysis were performed to confirm the expression of Rab7, the data were not shown in study.

Co-culture experiments

HMEC-1 cell density was adjusted to $2\times10^5$/mL. Cell suspension (100 μL) was added to each well of 96-well plate. HMEC-1 cell were co-cultured with HK-2, HK-2 with si-NC or HK-2 with si-Rab7. Cell groups were divided into NC group (HK-2 and HMEC-1 cell were co-cultured in normoxia), Model group (HK-2 and HMEC-1 cell were co-cultured in normoxia)
were co-cultured in hypoxia), Model+si-Rab 7 group (HK-2 with si-Rab 7 and HMEC-1 cell were co-cultured in hypoxia), Model+si-Rab 7+MMP-2 inhibitor (HK-2 with si-Rab 7 and HMEC-1 cell were co-cultured and treated with MMP-2 inhibitor in hypoxia). The hypoxia was 94% N₂/5% CO₂/1% O₂.

CCK-8 Cytotoxicity Experiment
The cell were treated by difference methods, After0.5h, 1h, 2h and 3.5h, CCK-8 dye was added to each well and the plates were incubated for another 2 h at 37 °C, optical density (OD) values were measured at 450nm by a micro plate reader (Biotek, Winooski, VT, USA) and measured the cell proliferation.

Immunofluorescence
After treatment, fixing cells by 4% par formaldehyde at 1 h and permeablizing by 0.5% Triton X-100 at 10 min. Using 10% sheep serum (BOSTER, China) to block nonspecific binding. Incubation by antibodies against Ki67 (1:200) (Proteintech, China) at 4°C overnight, adding secondary antibody(1:1000) (Thermo Fisher Scientific, USA) to apply for 1 h. Using DAPI to stain nuclear for 5 min. Capturing image by laser scanning con focal microscopy (Olympus Corporation, Japan), evaluating Ki67 positive cell number of difference groups.

Transwell assays
The HMEC-1 cell were treated by difference methods for 24 h, Adding medium (50 µL) to upper well of the transwell chamber containing 2.5×10³ cells/well and above medium with 20% FBS in the lower site. Incubation for 48 h, cells in the upper chamber were removed and cells in the lower chamber had been stained with crystal violet at room temperature for 30 min. Finally, cells in the lower chamber were counted under a light microscope. Each experiment had been repeated at least three times.

In vitro angiogenesis assay
Adjusting HMEC-1 cell density to 2×10⁵/mL. Adding Cell suspension (100 μL) to 96-well plate. Culturing cell for 2 h by difference methods. The capillary-like tubule networks were analyzed by measuring total length of the cells per field by Image J Software. The experiment had been repeated for three times.

Western blot (WB)
Collecting kidney tissues or culture cells and extracting protein. Measuring total protein by a BCA protein assay kit (Beyotime). Using Rab7, Collage I, Collage III, α-SMA, GAPDH (1:1000, Proteintech), MMP-14(1:1000, Bioworld, St. Louis Park, MN, USA) and RECK (1:1000, CST) antibodies as primary antibodies and adding second antibody. Protein supernatants were separated via 10% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred to nitrocellulose membranes (placed in ice water with a constant current of 100 mA overnight). The antibody had been blocked with 5% skim milk at 4°C over night. An enhanced chemiluminescence(ECL) imaging method was used to detect the protein bands and the results were analyzed by Image J Software (Version 1.46, NIH, Bethesda, MD, USA). The experiment had been repeated for 3 times.

Gelatin Zymography
Collecting the kidney tissues and HMEC-1 of difference groups. The activity of MMP-2 in the condition media and the supernatants of the endothelial cells was
examined by gelatin zymography (Applygen, China) [16]. The supernatants were collected after centrifuging and the protein content in the supernatants was detected by a BCA assay kit (Beyotime Biotechnology, China). The equal protein amount of supernatants (40μg) were separated by 8% poly acryl amide gel containing 0.1% bovine gelatin (Sigma, USA). After electrophoresis, the gel had been transferred to 2.5% Triton X-100 solution (5 h, twice) at room temperature and then had been incubated in Tris-HCl buffer (pH 7.4) overnight. Next, the gel was treated with 0.1% Coomassie Brilliant Blue R-250, and white bands were visualized after the gel was destained in 30% methanol and 10% acetic acid and the results were analyzed by Image J Software. The experiment had been repeated for 3 times.

**Sirius red stain**

After euthanasia, the kidneys were immediately removed, and a portion of each kidney was removed and frozen in liquid nitrogen. The remaining kidney tissue was fixed in 4% par formaldehyde, embedded in paraffin and sectioned. Sirius red staining was used to evaluate renal fibrosis in different groups.

**Immunohistochemistry stain**

Using anti-CD34 antibody to reveal peritubular capillary density in the interstitium. Incubating Specimens by antibody (1:400; Proteintech, Rosemont, IL, USA) at 4℃ overnight. Using anti-CD34 primary antibody by one-step polymer detection kit (Maixin, Fuzhou, China). After that, observing and evaluating results.

**Transmission electron microscope observation**

Taken the kidney tissues from difference groups, fixing by 2.5% glutaraldehyde (Sigma, USA) (4℃, 24 h), and then post-fixed using 2% osmium tetroxide at room temperature. Specimens were cut into ultrathin sections (50–70 nm) and stained with uranyl acetate/lead citrate. Finally, ultra-structural analysis was performed under the JEM-1011EX instrument (JEOL, Japan).

**In situ gelatinase spectrum**

Frozen sections of fresh kidney tissue, with a thickness of 10nm, were adhered to the in-situ gelatinase membrane, placed in a wet box and incubated at 37℃ for 36h; the frozen sections were taken out and dried at room temperature for 30min, and then dyed in amino black 10B (1% amino black, 70% methanol, 10% ethanol) for 15min, washed with distilled water, immersed in decolorizing solution (70% methanol, 10% acetic acid) for 20min, and then dried. Finally, images were captured by laser scanning confocal microscopy (Olympus Corporation, Japan), evaluating MMP-2 activity of difference groups.

**Statistical analysis**

All experimental data were analyzed using SPSS 18.0 Software (SPSS, Inc., Chicago, IL, USA) and presented as the mean ± standard deviation. Comparisons between groups were performed using one-way ANOVA with Bonferroni’s post hoc correction (SPSS 18.0, IBM, Chicago, IL, USA) for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Fibrosis level and CD34 protein expression in kidney tissues

By Sirius red staining, compared with NC group which was WT mice, the collage
area was significantly increased in kidney fibrosis model mice (P < 0.001, Figure 1A); Compared with Model group, the collagen area of Model+Rab7- which used Rab 7 knockdown mice was significantly depressed compared with that of Model group (P < 0.001, Figure 1A). By IHC assay, compared with NC group, CD34 protein IOD was significantly down-regulation in Model group (P < 0.001, Figure 1B), Compared with Model group, CD34 protein IOD of Model+Rab7- which used Rab 7 knockdown mice was significantly up-regulation in kidney tissues (P < 0.001, Figure 1B).

The ultrastructural changes of kidney tissues

By TEM observation, the ultrastructural was normal in kidney tissue of NC group, after kidney fibrosis molding, the ultrastructural was charge which the mitochondria and nuclei were damaged; however, the mitochondria and nuclei damaging were not found in another model group (Model+Rab 7-) which used Rab 7 knockdown mice’s kidney tissues. The relative data was shown in Figure 2.
MMP-2 activities of difference groups

By In situ gelatinase spectrum, compared with NC group, MMP-2 activity of Model group was significantly depressed in tissues (P < 0.001, Figure 3A), however, with Rab 7 knockdown, compared with Model group, MMP-2 activity of Model+Rab7- group was significantly increased in tissues (P < 0.001, Figure 3A). By Gelatin Zymography, compared with NC group, MMP-2 activity of Model group was significantly depressed in tissues (P < 0.001, Figure 3B), however, with Rab 7 knockdown, compared with Model group, MMP-2 activity of Model+Rab7- group was significantly increased in tissues (P < 0.001, Figure 3B).
Figure 3. MMP-2 activities of difference groups
NC: Normal mice; Model: kidney fibrosis model in normal mice; Model+Rab7-: kidney fibrosis model in Rab 7 knockdown mice
A. MMP-2 activities of difference groups by In situ gelatinase spectrum (200×)
***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group
B. MMP-2 activities of difference groups by Gelatin Zymography
***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group

Relative protein expression in kidney tissues by WB assay
By WB assay, compared with NC group, the fibrosis marker proteins (α-SMA, Collage I and Collage III) and Rab 7 and RECK proteins expression were significantly up-regulation and MMP-14 protein expression was significantly down-regulation in Model group (P < 0.001, respectively, Figure 4), however, using Rab 7 knockdown mice to model kidney fibrosis, the fibrosis marker proteins (α-SMA, Collage I and Collage III) and Rab 7 and RECK proteins expression were significantly depressed and MMP-14 protein expression was significantly increased in Model+Rab 7- group (P <
0.001, respectively, Figure 4).

![Figure 4. Relative proteins expression by WB assay](image)

NC: Normal mice; Model: kidney fibrosis model in normal mice; Model+Rab7-: kidney fibrosis model in Rab 7 knockdown mice

***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group

The cell proliferation and Ki67 positive cell number of difference cell groups

By CCK-8 assay, Compared with NC group, the cell proliferation rate of Model group was significantly depressed in 1 h, 2 h and 3.5 h (P < 0.05, respectively, Figure 5A), with Rab 7 knockdown by si-Rab 7 supplement, the cell proliferation rate of Model+si-Rab 7 group was significantly recovery compared with Model group (P < 0.05, respectively, Figure 5A) in 1 h, 2 h and 3.5 h. Based on Model+si-Rab 7 group treatment, adding MMP-2 inhibitor, compared with Model+si-Rab 7 group, the cell proliferation rate of Model+si-Rab 7+MMP2 inhibitor was significantly down-regulation (P < 0.05, respectively, Figure 5A) in 1 h, 2 h and 3.5 h. In order to observant cell activity, we measured Ki67 protein expression by immunofluorescence, Compared with NC group, Ki67 positive cell number of Model and Model+si-NC groups were significantly down-regulation (P < 0.001, respectively, Figure 5B), however, with si-Rab 7 supplement which knockdown Rab 7, compared with Model group, the Ki67 positive cell number of Model+si-Rab 7 group was significantly up-regulation (P < 0.001, respectively, Figure 5B), meanwhile, with MMP2 inhibitor supplement, compared with Model+si-Rab 7 group, Ki67 positive cell number of Model+si-Rab 7+MMP2 inhibitor group was significantly depressed (P < 0.001, Figure 5B).
Figure 5. Cell proliferation and Ki67 positive cell number

A. Cell proliferation in difference times points
   *: P<0.05, **: P<0.01, ***: P<0.001, compared with NC group; #: P<0.05, ##: P<0.01, ###: P<0.001, compared with Model group; &: P<0.05, &&: P<0.01, &&&: P<0.001, compared with Model+si-Rab 7

B. Ki37 positive cell number of difference groups
   ***: P<0.001, compared with NC group; ###: P<0.001, compared with Model group; &&&: P<0.001, compared with Model+si-Rab 7 group

Rab 7 affect HMEC-1 cell’s migration ability

By transwell assay, compared with NC group, migration cell number of Model and Model+si-NC group were significantly suppressed (P<0.001, respectively, Figure 6); with Rab 7 knockdown, compared with Model group, migration HMEC-1 cell number of Model+si-Rab 7 group was significantly up-regulation (P<0.001, Figure 6); with MMP2 inhibitor supplement, compared with Model+si-Rab 7 group, migration HMEC-1 cell number of Model+si-Rab 7+MMP2 inhibitor group was significantly down-regulation (P<0.001, Figure 6).
Figure 6. Migration cell number of difference groups

***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; &&&: P < 0.001, compared with Model+si-Rab 7 group

Total length of difference groups by vitro angiogenesis

By vitro angiogenesis, compared with NC group, total length of Model and Model+si-NC groups were significantly down-regulation (P < 0.001, respectively, Figure 7), with Rab 7 knockdown, compared with Model group, total length of Model+si-Rab 7 group was significantly up-regulation (P < 0.001, Figure 7), however, with MMP2 inhibitor supplement, compared with Model+si-Rab 7 group, total length of Model+si-Rab 7+MMP2 inhibitor group was significantly down-regulation (P < 0.001, Figure 7).
Figure 7. Total length of difference group

***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group;
&&&: P < 0.001, compared with Model+si-Rab 7 group

Relative proteins expression by WB assay

By WB assay, compared with NC group, fibrosis marker proteins (α-SMA, Collage I and Collage III) and RECK and Rab 7 proteins expressions were significantly increased, and MMP-14 protein expression was significantly depressed (P < 0.001, respectively, Figure 8) in Model and Model+si-NC group; with Rab 7 knockdown, compared with Model group, fibrosis marker proteins (α-SMA, Collage I and Collage III) and RECK and Rab 7 proteins expressions were significantly depressed, and MMP-14 protein expression was significantly increased (P < 0.001, respectively, Figure 8) in Model+si-Rab 7 group; meanwhile, with MMP2 inhibitor supplement, compared with Model+si-Rab 7 group, fibrosis marker proteins (α-SMA, Collage I and Collage III) and RECK and Rab 7 proteins expressions were significantly increased, and MMP-14 protein expression was significantly depressed (P < 0.001, respectively, Figure 8) in Model+si-Rab 7+MMP2 inhibitor group.
Figure 8. Relative protein expression by WB assay

***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; &&&: P < 0.001, compared with Model+si-Rab 7 group

MMP2 activity by Gelatin Zymography in vitro

Compared with NC group, MMP2 activity of Model and Model+si-NC groups were significantly down-regulation (P < 0.001, respectively, Figure 9), with Rab 7 knock down, compared with Model group, MMP2 activity of Model+si-Rab 7 group was significantly up-regulation (P < 0.001, Figure 9); with MMP2 inhibitor, compared with Model+si-Rab 7 group, MMP2 activity of Model+si-Rab 7+MMP2 inhibitor group was significantly down-regulation (P < 0.001, Figure 9).
Discussion

Peritubular capillary rarefaction is an important pathological feature of CKD and the main cause of renal hypoxia and hypoxia promotes the development of CKD\textsuperscript{[17]}. Therefore, promoting the formation of peritubular capillaries and improving tissue oxygen supply are basic strategies to delay or even reverse the development of CKD\textsuperscript{[18]}.

Hypoxia is a prominent pathophysiological feature of renal fibrotic tissue. A series of studies of our group have confirmed that supernatant of hypoxic cultured renal tubular epithelial cells and renal fibrotic tissues of animal model show decreased enzyme activity of MMP-2\textsuperscript{[2,8]}, but the mechanism of decreased enzyme activity under hypoxia condition needs to be clarified.

MMP-2 is mainly released from cells in form of proenzyme and the activation on the surface of cells\textsuperscript{[19,23]}. Since the activation of MMP-2 is mainly on the surface of plasma membrane, we concluded that the changes of cell membrane structure could affect the activity of this enzyme. Autophagy and endocytosis are not only the main factors of cell membrane remodeling, but also play a decisive role in the regulation of transmembrane signal transduction and a variety of metabolic or functional activities\textsuperscript{[24-26]}. Previous study of our group confirmed that autophagy and endocytosis of hypoxic renal tubular epithelial cells do affect the activity of MMP-2.
Previous studies have confirmed that the co-occurrence of endocytosis and autophagy under hypoxia conditions arise from the sharing of some functional links. As a shared molecule of autophagy and endocytosis in renal tubular epithelial cells, Rab7 can control the transport of late endocytosis and autophagy to lysosomes. Rab7 plays an important role in pinocytosis, phagocytosis, exocytosis and autophagy (including mitochondrial autophagy and lipid droplet autophagy). Meanwhile, Rab7 is the basis of lysosomal biosynthesis, localization and function, and also participates in many physiological activities such as apoptosis, neurotrophic factor transport and signal transduction, axon growth and tumor inhibition. Rab7 is a hinge molecule linking various functions inside and outside of the cell.

In this study, the effect of Rab7 expression on angiogenesis in hypoxic renal tubular epithelial cells was studied in vivo and vitro, and we explored whether Rab7 regulated peritubular capillary by regulating the activity of MMP-2 in hypoxic renal tubular epithelial cells. In vivo study, we found that fibrosis level, pathological injury and mitochondrial damage were aggravated in kidney tissues of kidney fibrosis model mice. However, fibrosis level, pathological injury and mitochondrial damage were significantly improved in Rab7 knockdown mice with kidney fibrosis modeling. In vitro study, the results found that endothelial cells (HMEC-1) co-confirmed with HK-2 cell which knock down Rab7 by si-Rab7 were depressed cell proliferation, migration and tubular formation.

In order to confirm whether Rab7 has an effect on angiogenesis through regulating the activity of MMP-2, we used ARP100 to inhibited the activity of MMP-2 and repeated the above experiment. The results of gelatin zymography assay showed that MMP-2 activity was significantly decreased in both normoxia and a hypoxia group, indicating that ARP100 interference was effective. At the same time, it was found that the conditioned media with different expression levels of Rab7 lost its influence on proliferation, migration and tubular formation of endothelial cells in hypoxic and normoxic condition. This study demonstrated that the high expression of Rab7 in hypoxic renal tubular epithelial cells could play an anti-angiogenic role by inhibiting the activity of MMP-2.

In this study, we also found an interesting phenomenon, that is, the cultured supernatant of renal tubular epithelial cells with low expression of Rab7 promotes the proliferation of endothelial cells, which has not been reported, so it is necessary to explore the internal mechanism in subsequent experiments. In recent years, some scholars have carried out some research on renal angiogenesis. Zhu and other found that the mesenchymal stromal cell-derived extracellular vesicles alleviated renal ischemic reperfusion injury and enhance angiogenesis in rats. Chade used a bioengineered polymer-stabilized vascular endothelial growth factor to promote renal angiogenesis. And it was reported that protein kinase LKB1 promoted Rab7-mediated NRP-1 degradation and inhibits tumor angiogenesis. In previous research, we found that endothelial cells co-cultured with renal carcinoma cells significantly reduced RECK expression under chemical hypoxia. As can be seen from these reports, the mechanism of angiogenesis is complex in kidney diseases, perhaps it shares different pathways between tumors and inflammations or between early stage and later stage of the diseases.

In conclusion, we confirmed that the over-expression of Rab7 in hypoxic renal tubular epithelial cells interfered with the generation of peritubular capillaries by regulating the activity of MMP-2 in vivo and vitro experiments. In our opinion, targeted
down-regulation of Rab7 expression may be a candidate for delay the development of chronic kidney disease.

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