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Hypoxia-induced downregulation of miR-30c promotes epithelial-mesenchymal transition in human renal cell carcinoma

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MicroRNAs (miRNAs), which negatively regulate protein expression by binding protein-coding mRNAs, have been integrated into cancer development and progression as either oncogenes or tumor suppressor genes. miR-30c was reported to be downregulated in several types of cancer. However, its role in human renal cell carcinoma (RCC) remains largely unknown. Here, we show that miR-30c is significantly downregulated in human RCC tissues and cell lines. We found that miR-30c downregulation could be induced by hypoxia in RCC cells in a hypoxia-inducible factors (HIFs) dependent manner. Repression of miR-30c through its inhibitor resulted in reduction of E-cadherin production and promotion of epithelial-mesenchymal transition (EMT), while overexpression of miR-30c inhibited EMT in RCC cells. We identified Slug as a direct target of miR-30c in RCC cells. Slug was upregulated in RCC tissues and its expression could be induced by hypoxia, which is consistent with downregulation of miR-30c by hypoxia. Forced overexpression of Slug in 786-O cells reduced E-cadherin production, and promoted EMT as well as cell migration. Moreover, Slug overexpression abrogated the inhibitory role of miR-30c in regulating EMT and cell migration, indicating miR-30c regulates EMT through Slug in RCC cells. Our findings propose a model that hypoxia induces EMT in RCC cells through downregulation of miR-30c, which leads to subsequent increase of Slug expression and repression of E-cadherin production, and suggest a potential application of miR-30c in RCC treatment. (*Cancer Sci* 2013; 104: 1609–1617)

Renal cell carcinoma (RCC), the most common type of kidney cancer, is one of the leading causes of cancer deaths in western countries, and the overall incidence of RCC is steadily increasing.[1] Despite more than 50% of renal tumors being detected incidentally due to advances in diagnosis, especially improved imaging techniques, about 20–30% of all patients are diagnosed with metastatic disease. In addition, another 20% of patients undergoing nephrectomy will develop metastatic RCC during follow-up. For patients with metastatic RCC, the prognosis is extremely poor, despite multimodal treatment.[2] Therefore, a major challenge for improving clinical outcomes is to understand molecular mechanisms of RCC in detail and search for molecular therapeutic targets.

MicroRNAs (miRNAs) are small non-coding RNAs in the size range of 19–25 nucleotides that are cleaved from hairpin pre-miRNA precursors. By binding to imperfect complementary sequences in the 3′-untranslated region (3′UTR) of target mRNAs, miRNAs cause translational repression or mRNA degradation. miRNAs play critical roles in a wide variety of biological process including cell differentiation, proliferation, apoptosis and metabolism.[3,4] Notably, dysregulation of miRNAs has been extensively implicated in cancer pathogenesis in various tumor types.[5] They can act as potential oncogenes or tumor suppressor genes during cancer initiation and progression.[6,7] Accumulating evidence shows that miRNAs play roles in the pathogenesis of RCC,[8] and contribute to RCC pathogenesis at different levels.[9] Recently, several groups have reported the expression profiles of miRNA in RCC.[10,11]

miR-30c is involved in many biologic events, including cell apoptosis, growth and differentiation.[12] It has been reported that miR-30c is downregulated in several types of cancer, such as bladder cancer, invasive micropapillary carcinoma and malignant peripheral nerve sheath tumors.[13,14] miR-30c expression level can also serve as an independent predictor of clinical benefit of endocrine therapy in advanced estrogen receptor positive breast cancer.[15] miR-30c is generally considered as a tumor suppressor miRNA, and it inhibits the proliferative, migratory and invasive abilities of many cancer cells,[16] and sensitizes cancer cells to chemical treatments.[17,18] miR-30c can also trigger upregulation of transmembrane tumor necrosis factor-α expression and enhance NK cell cytotoxicity against hepatoma cell lines.[19] Recently, there were several targets of miR-30c that were closely related to its tumor suppressor roles in cancer. For instance, miR-30c negatively regulates MTA1 in endometrial cancer cells and downregulates the oncogene BCL9 in ovarian cancer cells.[20] Twinfilin 1 and vimentin involved in tumor invasion are the targets of miR-30c in human breast cancer cells.[21] Breast cancer cell growth was suppressed by miR-30c through inhibition of KRAS expression and KRAS signaling.[22] miR-30c targets DLL4 and regulates Notch signaling to modulate endothelial cell behavior during angiogenesis.[23]

In this study, we found the downregulation of miR-30c in RCC, and that miR-30c expression was inhibited by hypoxia in a HIFs-dependent manner. Moreover, we demonstrated that miR-30c regulated EMT through directly targeting Slug in RCC cells. We propose a model that hypoxia induces EMT in RCC cells through downregulation of miR-30c, which leads to subsequent increase of Slug expression and repression of E-cadherin production.

Materials and Methods

Clinical specimens. Human renal cell carcinoma specimens, including adjacent non-tumor tissues, were obtained from the tissue bank of Renji Hospital. The Institutional Ethics Committee approved the study protocol and the use of clinical specimens. Written, voluntary, informed consent was taken from all the patients. The specimens were obtained after surgical resection, immediately frozen in liquid nitrogen, and stored in liquid nitrogen until use.

Cell culture and transfection. All cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences

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(Shanghai, China). Human RCC cell lines ACHN, Caki-1 and 786-O were maintained in RPMI-1640 media (Invitrogen, Carlsbad, CA, USA). Human proximal tubule epithelial cell line HK-2 and RCC cell line A498 were maintained in RPMI-1640 and MEM media (Invitrogen) respectively. All media were supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cell culture was conducted at 37°C in a humidified 5% CO_2 incubator. Cells were subjected to time controlled normoxic (21% oxygen) or hypoxic (0.5% oxygen) conditions for indicated times. Cell transfection was performed with a Lipofectamine 2000 regent (Invitrogen) following the manufacturer's protocol.

Quantification of miR-30c expression. Total RNA was isolated from RCC tissues and cell lines using the TRizol regent (Invitrogen), and reverse-transcribed into cDNA. Expression of miR-30c was detected by quantitative RT-PCR based TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA) followed by manufacturer's instructions. U6 snRNA was used as an endogenous control. The miRNA levels were determined using the 7500 Fast System SDS software (Applied Biosystems). The ddCt algorithm was used to calculate the relative quantification. Each reaction was repeated independently at least three times in triplicate.

Mutation analysis of VHL. Genomic DNA was isolated from RCC tissues. Mutation screening of the VHL was performed using PCR and sequencing. The following primers were used: VHLexon1F: 5′-CGAAGACTACGGAGGTCGAC-3′; VHLexon1R: 5′-GGCTTCAGACCGTGCTAT CG-3′; VHLexon2F: 5′-GTGTGGCTCTTTAACAACC-3′; VHLexon2R: 5′-CTGT A CTTACCACAACAACC-3′; VHLexon3F: 5′-TCCTTGTA-C T GAGACCCTAG-3′; and VHLexon3R: 5′-AGCTGAGAT-G-A AACAGTCTA-3′. The amplified PCR products were used for DNA sequencing.

***In situ* hybridization.** The locked nucleic acid (LNA) based *in situ* hybridization was performed. The digoxigenin-labeled LNA probes (Exiqon, Vedbaek, Denmark) were used to detect miR-30c. The probes were visualized by using a horseradish peroxidase (HRP) conjugated anti-digoxigenin antibody (Abcam, Cambridge, MA, USA) and enzymatically reacted with 3,3′-diaminobenzidine (DAB) substrate. The nuclei were stained using hematoxylin. The signal was evaluated by assessing staining intensity using a BX51 microscope (Olympus, Tokyo, Japan).

miR-30c overexpression and inhibition, knockdown of HIF-1α and HIF-2α. Overexpression of miR-30c was conducted by transfection of miR-30c mimics (Ambion, Austin, TX, USA). Inhibition of miR-30c was conducted by transfection of miR-30c inhibitor (Ambion). Knockdown of HIF-1α and HIF-2α were conducted by transfection of siRNAs against HIF-1α and HIF-2α (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blotting. Cells were lysed in a RAPI lysis buffer and solubilized in SDS loading buffer. Equal amounts of protein extracts were separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Quebec, Canada). Protein expression was analyzed using standard procedures for western blotting. The membranes were incubated with a primary antibody (anti-HIF-1α, Cell Signaling Technology [Beverly, MA, USA]; anti-HIF-2α, Cell Signaling Technology; anti-E-cadherin, Cell Signaling Technology; anti-VHL, Abcam; anti-vimentin, Abcam; anti-Slug, Abcam; anti-α-SMA, Sigma Aldrich [St. Louis, MO, USA]; anti-tubulin, Sigma Aldrich). After incubation with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), the membranes were treated with an enhanced chemiluminescence reagent (Thermo Scientific, Dreieich, Germany), and exposed to X-ray film (Kodak, Rochester, NY, USA). The relative intensity of bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell migration assay. Cells were plated in serum-free medium after transfection to the top of 8-lm transwell inserts (BD Biosciences) in 24-well companion plates (BD Biosciences, San Diego, CA, USA) containing 10% FBS. After 24 h, cells were fixed with methanol and stained with crystal violet (Invitrogen). Cells on the top of inserts were removed using cotton swabs and migrated cells were dissociated in a Triton-X100 buffer from the bottom side of inserts and read at 595 nm.

Construction of expression vectors. VHL mRNA was amplified by PCR using the following primers: forward: 5′-CCGC TCGAGATGCCCCGGAGGGCGGAGAAC-3′; and reverse: 5′-GGGGCCCTCAATCT CCCATCCGTTGATG-3′. The PCR products were digested with *Xho*I and *Apa*I enzymes and inserted into a pcDNA3-Flag vector to construct VHL overexpression vector.

The 1.1 kb 3′UTR of human Slug from a human cDNA library was amplified using PCR, digested with *Nhe*I and *Xho*I enzymes, and cloned into a pGL3 vector (Promega, Madison, WI, USA) to generate a Luc-WT-3′UTR vector. The following primers were used to clone Slug 3′UTR: forward: 5′-CTAGCTAGCGTGACGCAATCAATGTTTACTC-3′; and reverse: 5′-CCGCTCGAGTAACAAACAATTCTTTGTA-CAG-3′. Deletion of the miR-30c target site in the Slug 3′ UTR was performed using a QuikChange mutagenesis kit (Stratagene, Heidelberg, Germany) to generate the Luc-Del-3′ UTR vector.

Dual-luciferase reporter assay. Dual-luciferase reporter assay was performed in ACHN and 786-O cells followed by manufacturer's instructions (Promega). Cells were co-transfected with pRL-TK vector, wild-type (Luc-WT-3′UTR) or deletion (Luc-Del-3′UTR) reporter vectors, along with miR-30c mimics or control miRNA. Forty-eight hours after transfection, cells were rinsed with PBS, and performed with dual-luciferase assay. Luciferase activity was measured using a Victor Luminometer (Perkin Elmer, Waltham, MA, USA). The firefly luciferase activity was normalized using co-transfected Renilla luciferase for transfection efficiency. All experiments were performed in triplicate.

Immunohistochemistry. Immunohistochemistry was performed by using standard protocols. The tissue sections were incubated with anti-Slug antibody (1:100 dilution) for 3 h. After incubation with the horseradish peroxidase-conjugated secondary antibody (1:100 dilution), the signal was visualized using 3,3′-diaminobenzidine (DAB) substrate. The sections were counterstained with hematoxylin and the staining was observed by BX51 microscope (Olympus).

Establishment of stable cells overexpressing Slug. Slug overexpression was conducted by lentiviral expression system. Slug mRNA was amplified by PCR using the following primers: forward: 5′-CTAGCTAGCATGCC GCGCTCCTTCCTGGTC -3′; and reverse: 5′-GGAATTCTCAGTGTGCTACACAGCA GCCAG-3′. The PCR products were digested with *Nhe*I and *Eco*RI enzymes, and inserted into a pLV vector to construct pLV-Slug vector. pLV-Slug, pVSVG and delta 8.91 vectors were co-transfected into 293T cells to generate Slug overexpression lentivirus. 786-O cells were infected with the lentivirus to establish stable cells overexpressing Slug.

Statistical analysis. Data were analyzed using the SPSS software (SPSS Inc., Chicago, IL, USA). Quantitative data were presented as the mean ± standard deviation. Differences between miR-30c expression in tumor tissues and adjacent non-tumor tissues were analyzed by the Wilcoxon matched pairs test. Statistical differences between groups were determined by the Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

miR-30c is downregulated in RCC tissues. We used microarray analysis to identify the dysregulated miRNAs in RCC, and

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found miR-30c as one of the downregulated miRNAs in RCC. Considering the downregulation and potential tumor suppressive roles of miR-30c in multiple cancer types, we further investigated the implication and roles of miR-30c in RCC. To confirm miR-30c downregulation in RCC, we used quantitative RT-PCR analysis to measure miR-30c levels in 32 pairs of RCC tissues. Compared to matched adjacent non-tumor tissues, miR-30c expression was downregulated in 23/32 of the tested RCC tissues ($P < 0.01$, Fig. 1a). To further confirm the downregulation of miR-30c, we examined miR-30c expression in RCC tissues using *in situ* hybridization assay. We found that the staining signal of miR-30c was much lower in RCC tissue than matched non-tumor counterpart (Fig. 1b). Our results together indicated that miR-30c was downregulated in RCC tissues.

We further analyzed the correlation between the downregulated miR-30c and several clinicopathological parameters among the 32 patients. There were 20 men and 12 women included with age ranging from 35 to 78 years old (median, 56 years). The 32 of RCC can be divided in three types including 12 of clear cell renal cell carcinoma (CCRCC), 14 of papillary renal cell carcinoma (PRCC) and six of chromophobe renal cell carcinoma (ChRCC). The results showed that miR-30c correlated with the parameters of age and tumor type, but not with gender (Table 1). We also subdivided tumors in terms of histological grades and TNM (tumor-lymph node-metastasis) stages. Among these 32 cases, 12 (37.5%) were well differentiated (nine grade I, and three grade I–II), 20 (62.5%) were moderately-to poorly differentiated (14 grade II, four grade II–III and two grade III). According to TNM cancer staging system, the 32 RCC cases were classified into TNM stage I ($n = 23$; 71.9%), II ($n = 8$; 25.0%), and III ($n = 1$; 3.1%). The results showed no significant correlation between miR-30c and histological grade; however, week expression of miR-30c tended to be in late stages rather than in early stages ($P = 0.017$, Table 1), suggesting that downregulation of miR-30c is implicated in RCC progression.

Hypoxia induces downregulation of miR-30c in RCC cells. Because hypoxia takes an important part in RCC pathogenesis, we investigated whether miR-30c expression would be regulated by hypoxia. ACHN cells were cultured under hypoxia conditions, and miR-30c expression was examined by quantitative RT-PCR analysis. The results showed that miR-30c levels were significantly downregulated by hypoxia after culture for 12 and 24 h compared to normoxia treatment ($P < 0.01$, Fig. 2a). To investigate whether hypoxia induced downregulation of miR-30c could also happen in other RCC cell lines, we examined miR-30c expression of ACHN, Caki-1, A498 and 786-O cells after culture under hypoxia conditions for 24 h. Significant reduction of miR-30c expression by hypoxia was observed in all the tested RCC cell lines ($P < 0.01$, *vs* control, Fig. 2b). These results indicated that miR-30c is downregulated by hypoxia in RCC cells.

Table 1. Statistical correlations between miR-30c expression and each clinicopathological parameter in 32 patients with renal cell carcinoma (RCC)

	Total	miR-30c expression			*P*-value
		Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)	
Age (years)					
≤56	15	5 (33.1)	8 (53.3)	2 (13.3)	0.031*
>56	17	8 (47.0)	2 (11.8)	7 (41.2)	
Gender					
Male	20	9 (45.0)	5 (25.0)	6 (30.0)	0.495
Female	12	4 (33.3)	5 (41.7)	3 (25.0)	
Tumor type					
CCRCC	12	5 (41.7)	2 (16.7)	5 (41.6)	0.042*
PRCC	14	5 (35.8)	8 (57.1)	1 (7.1)	
ChRCC	6	3 (50.0)	0 (0.0)	3 (50.0)	
Differentiation					
Well	12	5 (41.7)	2 (16.7)	5 (41.6)	0.278
Moderate to poor	20	8 (40.0)	8 (40.0)	4 (20.0)	
TNM stage					
I	23	6 (26.1)	8 (34.8)	9 (39.1)	0.017*
II and III	9	7 (77.8)	2 (22.2)	0 (0.00)	

*$P < 0.05$. *P*-values were derived using the chi-square test. CCRCC, clear cell renal cell carcinoma; ChRCC, chromophobe renal cell carcinoma; PRCC, papillary renal cell carcinoma; TNM, tumor-lymph node-metastasis.

Hypoxia induces activation of hypoxia-inducible factors (HIFs) to regulate expression of many genes. We suppressed HIFs expression to examine whether downregulation of miR-30c is induced by hypoxia in HIF-dependent manner. Expression of HIF-1α and HIF-2α was suppressed by transfection of the corresponding siRNAs (siHIF-1α and siHIF-2α) and evaluated by western blotting. The results showed that expression of HIF-1α and HIF-2α was efficiently suppressed (Fig. 2c). We then transfected combined siRNAs of siHIF-1α and siHIF-2α in RCC cells, and examined miR-30c under hypoxia conditions. The knockdown of HIF-1α and HIF-2α was confirmed by western blotting analysis (Fig. 2d). Our results showed that siHIF-1/2α transfection rescued downregulation of miR-30c induced by hypoxia (Fig. 2e). These results indicated that hypoxia induced downregulation of miR-30c was regulated in HIF-dependent manner.

miR-30c expression is correlated with VHL status in RCC cells. We measured miR-30c levels in human RCC cell lines ACHN, Caki-1, A498, 786-O, and human proximal tubule epithelial cell line HK-2 derived from normal kidney. Consistent

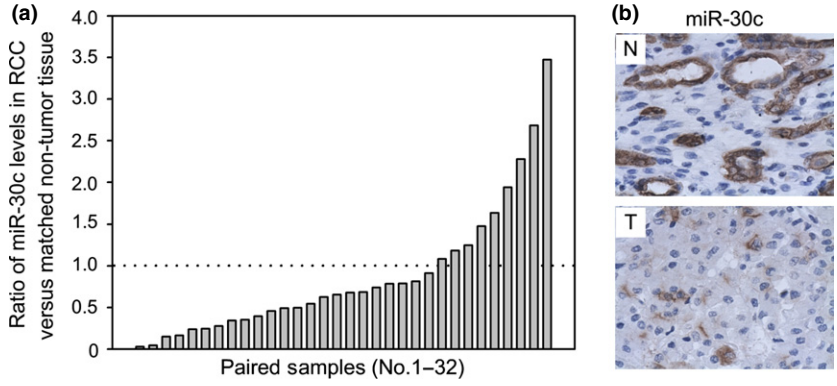

Fig. 1. miR-30c is downregulated in renal cell carcinoma (RCC)tissues. (a) Expression of miR-30c in 32 pairs of RCC tissues (T) and matched adjacent non-tumor tissues (N) was detected by TaqMan MicroRNA assay. U6 snRNA was used as an endogenous control. The ratio of miR-30c level in RCC tissue to that in non-tumor tissue (T/N) in each case is indicated by a column. miR-30c levels were significantly downregulated in RCC tissues as determined by the Wilcoxon matched pairs test, $P < 0.01$. (b) Detection of miR-30c by *in situ* hybridization in paired RCC tissue and its matched non-tumor tissue. N, non-tumor tissue; T, tumor tissue.

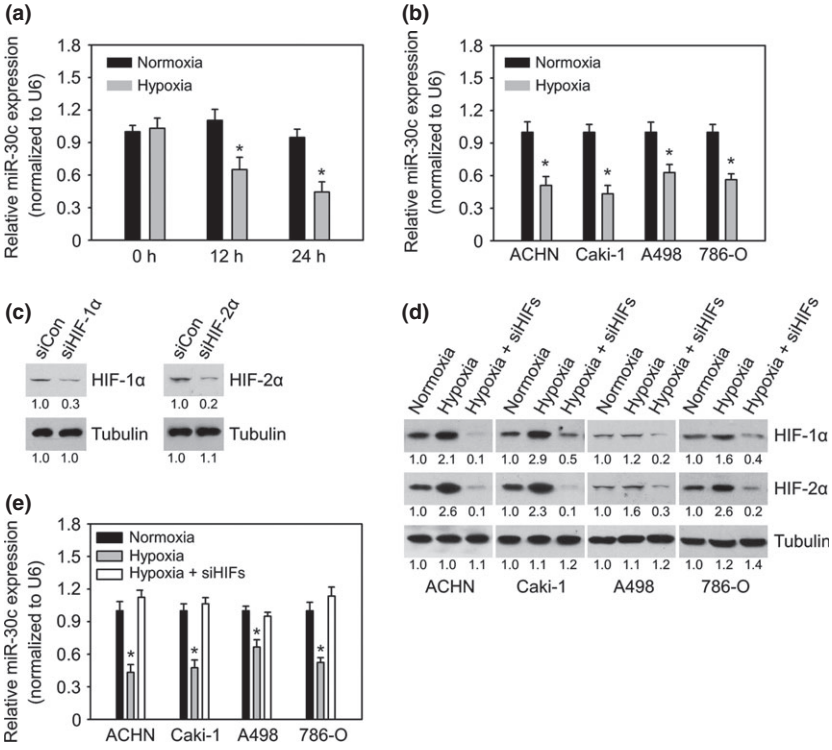

Fig. 2. Hypoxia induces downregulation of miR-30c in renal cell carcinoma (RCC) cells. (a) ACHN cells were incubated under normoxia or hypoxia conditions for 12 and 24 h. Levels of miR-30c were measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The relative expression of miR-30c normalized to U6 snRNA is shown. Values are given as means ± SD of three experiments. $*P < 0.01$ *vs* normoxia group (Student's *t*-test). (b) ACHN, Caki-1, A498 and 786-O cells were incubated under normoxia or hypoxia conditions for 24 h, and miR-30c levels were measured by quantitative RT-PCR. The relative expression of miR-30c normalized to U6 snRNA is shown as means ± SD of three experiments. $*P < 0.01$ *vs* normoxia group (Student's *t*-test). (c) Caki-1 cells were transfected with siRNA against HIF-1α or HIF-2α. At 3 days post-transfection, proteins levels of HIF-1α and HIF-2α were examined by western blotting. (d) ACHN, Caki-1, A498 and 786-O cells were transfected with siRNA against HIF-1α and HIF-2α. At 2 days post-transfection, cells were incubated under normoxia or hypoxia conditions for 24 h, and proteins levels of HIF-1α and HIF-2α were examined by western blotting. (e) ACHN, Caki-1, A498 and 786-O cells were treated as (d), and miR-30c levels were measured by quantitative RT-PCR. The relative expression of miR-30c normalized to U6 snRNA is shown as means ± SD of three experiments. $*P < 0.01$ *vs* Hypoxia + siHIF-1/2α group (Student's *t*-test).

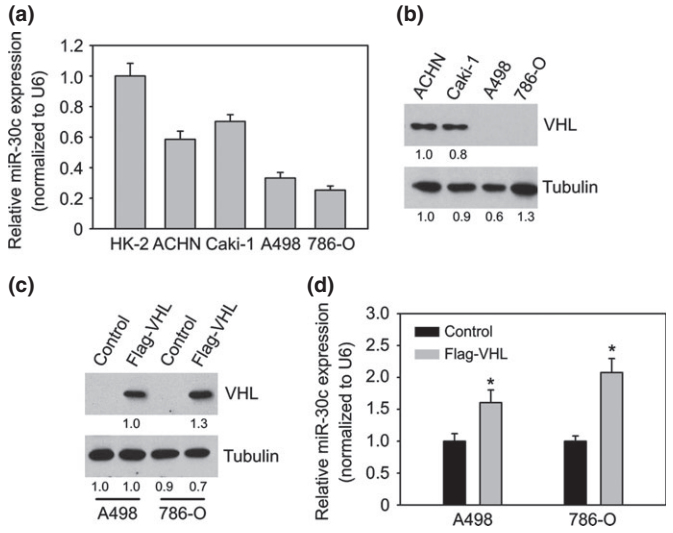

Fig. 3. miR-30c expression correlates with VHL status in renal cell carcinoma (RCC) cells. (a) miR-30c expression in HK-2, ACHN, Caki-1, A498 and 786-O cell lines was measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The relative expression of miR-30c normalized to U6 snRNA is shown as means ± SD of three experiments. (b) Western blotting analysis was performed to detect VHL expression in RCC cell lines ACHN, Caki-1, A498 and 786-O. Tubulin was used as an inner control. (c) pcDNA3-Flag-VHL or pcDNA3-Flag control vector was transfected into A498 and 786-O cells. VHL protein levels were measured by western blotting using VHL antibody at 72 h post-transfection. (d) A498 and 786-O cells were treated as (c). miR-30c expression was measured by quantitative RT-PCR at 72 h post-transfection and normalized to U6 snRNA. Data were shown as means ± SD of three experiments. $*P < 0.01$ *vs* control (Student's *t*-test).

with downregulation of miR-30c in RCC tissues, all the tested RCC cell lines showed significantly reduced expression of miR-30c compared to HK-2 cells ($P < 0.01$, Fig. 3a). Intriguingly, miR-30c levels in A498 and 786-O cells were relatively lower than that in ACHN and Caki-1 cells. It has been reported that Von Hippel-Lindau (*VHL*) gene is often mutated or lost in RCC, and A498 as well as 786-O cells were reported to be different from ACHN and Caki-1 cells for their VHL lost status. Our results of western blotting also confirmed VHL loss in A498 and 786-O cells but not in ACHN or Caki-1 cells (Fig. 3b). The lower levels of miR-30c in VHL-deficient RCC cell lines A498 and 786-O indicated that VHL might regulate miR-30c expression. To assess this hypothesis, we transfected Flag-VHL into VHL-deficient cells and examined miR-30c expression. The forced overexpression of VHL in A498 and 786-O cells was confirmed by western blotting (Fig. 3c). We found that there was a robust increase of miR-30c expression in Flag-VHL transfected A498 and 786-O cells compared to their control counterparts (Fig. 3d). In A498 cells, VHL overexpression produced a 1.6-fold increase of miR-30c expression compared to control ($P < 0.01$). In 786-O cells, VHL overexpression led to 2.0-fold increase of miR-30c expression ($P < 0.01$, *vs* control). To better investigate the correlation between miR-30c expression and VHL status, we examined the VHL status in 32 of our human RCC tissues using DNA sequencing. Our results showed that there was mutation or loss of VHL in 12 of 32 RCC tissues (Table S1). Moreover, there was a significant correlation between miR-30c expression and VHL status in RCC tissues ($P < 0.01$, Table 2).

Downregulation of miR-30c promotes EMT in RCC cells. Because miR-30c expression can be regulated by VHL whose loss results in an epithelial-mesenchymal transition (EMT), we examined whether miR-30c downregulation in VHL positive RCC cell lines would affect EMT. miR-30c downregulation was achieved by miR-30c inhibitor and detected by quantitative RT-PCR. The results showed that miR-30c inhibitor successfully caused a significant reduction of miR-30c in ACHN and Caki-1 cells (Fig. 4a). miR-30c expression was reduced to 26% and 31% in ACHN and Caki-1 cells, respectively

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Table 2. Statistical correlations between miR-30c expression and VHL status in 32 patients with renal cell carcinoma (RCC)

	Total	miR-30c expression			*P*-value
		Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)	
VHL status					
Mutant or loss	12	9 (75.0)	2 (16.7)	1 (8.3)	0.008*
Normal	20	4 (20.0)	8 (40.0)	8 (40.0)	

*$P < 0.05$. *P*-values were derived using the chi-square test.

($P < 0.01$, *vs* control). The effect of miR-30c downregulation on EMT was examined through western blotting analysis of E-cadherin and EMT target genes such as α-SMA and vimentin. The results showed that E-cadherin expression was significantly inhibited by miR-30c downregulation in ACHN and Caki-1 cells, and expression of α-SMA as well as vimentin was highly induced, indicating that EMT was promoted by miR-30c downregulation (Fig. 4b). We also examined the effect of miR-30c downregulation on EMT using transwell assay. The results showed that miR-30c inhibitor promoted migration of RCC cells (Fig. 4c). A 2.6- and 3.6-fold increase of migrated cells was observed in miR-30c downregulated ACHN and Caki-1 cells respectively ($P < 0.01$, *vs* control). Taken together, our results indicate that downregulation of miR-30c promotes EMT in RCC cells.

We also overexpressed miR-30c in A498 and 786-O cells to further investigate the roles of miR-30c in EMT of RCC cells. Overexpression of miR-30c was achieved by transfection of miR-30c mimics, and confirmed by quantitative RT-PCR (Fig. 4d). The effect of miR-30c overexpression on EMT was examined through western blotting analysis of indicated proteins. Our results showed that E-cadherin expression was promoted by miR-30c overexpression, expression of α-SMA and vimentin was significantly inhibited in A498 and 786-O cells, indicating that miR-30c overexpression inhibited EMT (Fig. 4e). We also found that miR-30c inhibited migration of A498 and 786-O cells using transwell assay (Fig. 4f). The number of migrated cells was reduced to 54% and 35% in A498 and 786-O respectively. These results indicated that miR-30c could inhibit EMT of RCC cells.

miR-30c directly targets Slug in RCC cells. To investigate how miR-30c regulates EMT in RCC cells, we searched for its target genes related to EMT using PicTar, TargetScan and miRBase Targets. Slug was found to be a potential target gene of miR-30c as 3′UTR of Slug mRNA contains a putative binding site of miR-30c (Fig. 5a). We integrated wide type Slug 3′UTR or Slug 3′UTR with deletion of miR-30c binding site into a luciferase reporter vector and tested the effect of miR-30c on luciferase activity using dual-luciferase assay. The results showed that miR-30c significantly repressed

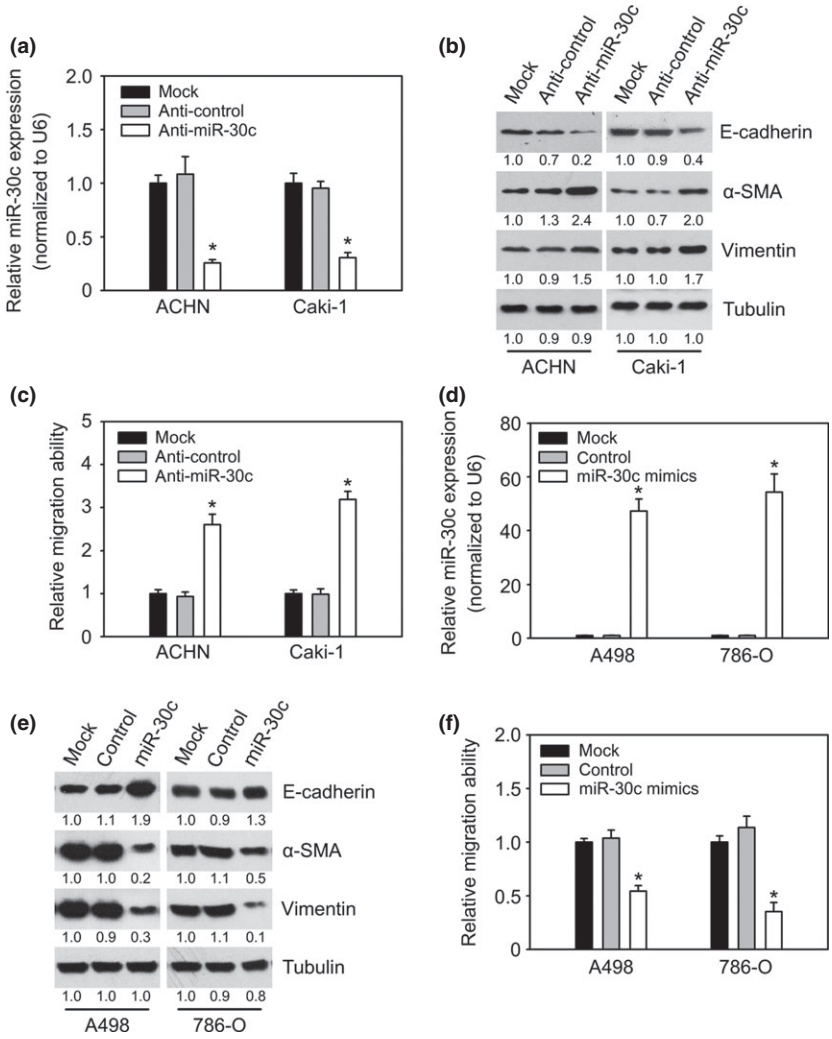

Fig. 4. Downregulation of miR-30c promotes EMT in renal cell carcinoma (RCC) cells. (a) ACHN and Caki-1 cells were transfected with miR-30c inhibitor (anti-miR-30c) or control miRNA inhibitor (anti-control). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to measure miR-30c levels at 48 h post-transfection. The relative expression of miR-30c normalized to U6 snRNA is shown as means ± SD of three experiments. *$P < 0.01$ *vs* control (Student's *t*-test). (b) Cells were treated as (a). Western blotting analysis was performed to examine expression of E-cadherin, α-SMA and vimentin. Tubulin was used as an inner control. (c) Cells were treated as (a). At 48 h post-transfection, cell migration was examined by transwell assay. The relative numbers of migrated cells were shown as means ± SD of three experiments. *$P < 0.01$ *vs* control (Student's *t*-test). (d) A498 and 786-O cells were transfected with miR-30c mimics or control miRNA. Quantitative RT-PCR was used to measure miR-30c levels at 48 h post-transfection. The relative expression of miR-30c normalized to U6 snRNA is shown as means ± SD of three experiments. *$P < 0.01$ *vs* control (Student's *t*-test). (e) Western blotting analysis was performed to examine expression of E-cadherin, α-SMA and vimentin. Tubulin was used as an inner control. (f) At 48 h post-transfection, cell migration was examined by transwell assay. The relative numbers of migrated cells were shown as means ± SD of three experiments. *$P < 0.01$ *vs* control (Student's *t*-test).

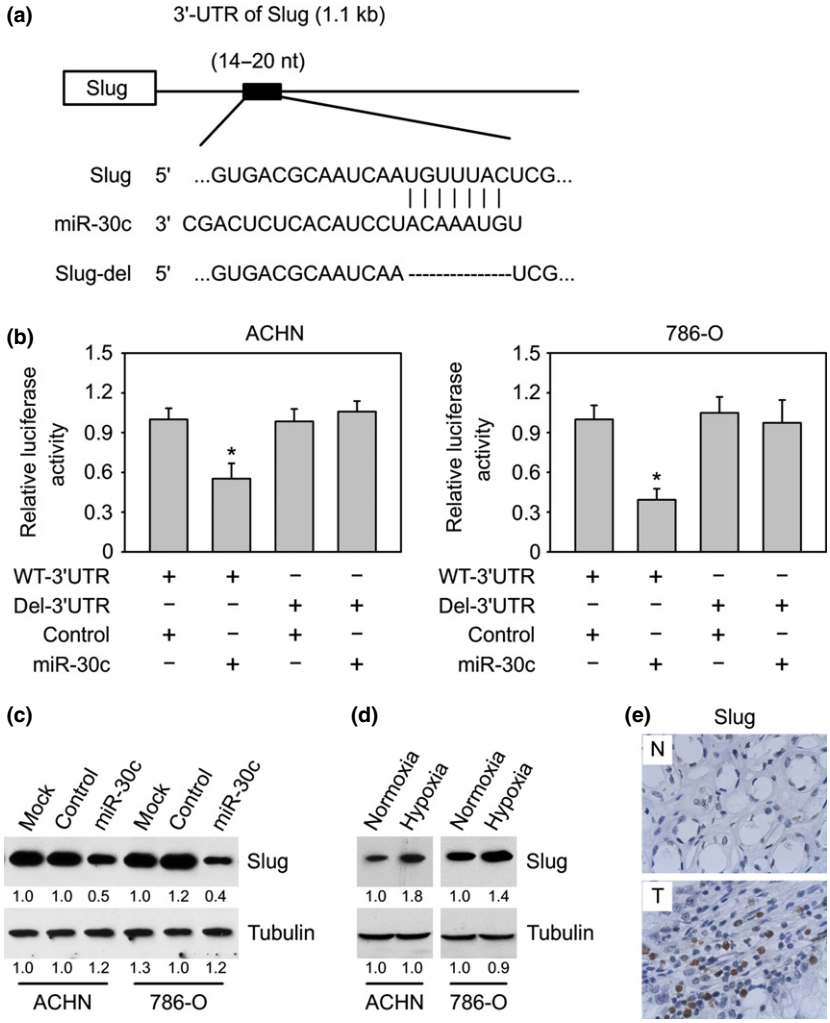

Fig. 5. miR-30c directly targets Slug in renal cell carcinoma (RCC) cells. (a) Schematic of the putative miR-30c binding site in Slug 3′UTR region, as detected by TargetScan. Slug-del indicates the Slug 3′UTR with deletion of miR-30c binding site. (b) The dual-luciferase reporter assay was performed in ACHN and 786-O cells. Cells were co-transfected with pRL-TK vector, the Slug 3′UTR luciferase reporter vector (WT-3′UTR) or the reporter vector of Slug 3′UTR with deletion of miR-30c binding site (Del-3′UTR), as well as miR-30c or control miRNA mimics. The ratio of firefly activity to renilla activity represents luciferase activity. Bars indicate the relative luciferase activities of three experimens. $*P < 0.01$, *vs* control (Student's *t*-test). (c) miR-30c inhibits Slug expression in ACHN and 786-O cells. Cells were transfected with miR-30c mimics or the control miRNA. Slug protein was examined by western blotting at 72 h post-transfection. (d) ACHN and 786-O cells were incubated under normoxia or hypoxia conditions for 24 h. Slug expression was examined by western blotting. (e) Detection of Slug by immunohistochemistry in the same RCC tissue and its matched non-tumor tissue. N, non-tumor tissue; T, tumor tissue.

the luciferase activity of WT-3′UTR reporter vector compared to control miRNA ($P < 0.01$), but barely affect the luciferase activity of Del-3′UTR reporter vector (Fig. 5b). The same results were obtained in both ACHN and 786-O cells. Interestingly, we found that the miR-30c family members miR-30a, miR-30b and miR-30d, which bind the same site on Slug mRNA as miR-30c can also repress the luciferase activity of WT-3′UTR reporter vector compared to control miRNA (Fig. S1).

We also examined the effect of miR-30c on endogenous Slug in ACHN and 786-O cells using western blotting. The results showed that Slug expression was significantly inhibited by miR-30c, indicating that miR-30c directly targets Slug in RCC cells (Fig. 5c). We further investigated whether hypoxia would affect Slug expression in RCC cells. Our results showed that hypoxia induced a robust upregulation of Slug in ACHN and 786-O cells (Fig. 5d). This result was consistent with downregulation of miR-30c induced by hypoxia and further confirmed that Slug is a direct target of miR-30c in RCC cells. We also examined Slug expression in RCC tissues using immunohistochemistry. Our results showed that Slug was upregulated in RCC tissue compared to its matched non-tumor tissue (Fig. 5e). These observations together indicated that miR-30c directly targeted Slug in RCC cells.

miR-30c regulates EMT through Slug in RCC cells. To investigate whether Slug could abrogate miR-30c regulated EMT in RCC cells, we overexpressed Slug and then examined miR-30c regulated EMT in 786-O cells (Fig. 6a). A significant increase of Slug was observed in Slug overexpressed 786-O cells (786-O-Slug) compared to control cells (786-O-control), and the increased Slug was barely affected by miR-30c because the introduced Slug cDNA carries no 3′UTR for miR-30c binding. 786-O-Slug cells showed increased EMT compared to 786-O-control cells, which was consistent with other reports that Slug promotes EMT. More importantly, we found that 786-O-Slug cells were resistant to the inhibitory role of miR-30c on EMT, while miR-30c can normally inhibit EMT in 786-O-control cells. We also performed transwell assay to examine the effect of Slug on miR-30c regulated cell migration (Fig. 6b). 786-O-Slug cells showed a threefold increase of migrated cells compared to 786-O-control cells ($P < 0.01$), indicating that Slug expression promoted cell migration. miR-30c transfection resulted in 52% reduction of cell migration in 786-O-control cells ($P < 0.01$, *vs* control miRNA), but failed to inhibit Slug induced cell migration in 786-O-Slug cells. These results indicate that miR-30c regulates EMT through Slug in RCC cells. We further examined the expression of Slug, E-cadherin and vimentin using immunohistochemistry in miR-30c downregulated RCC tissues. Our results showed that there was a downregulation of E-cadherin but an upregulation of Slug and vimentin in miR-30c downregulated RCC tissue (Fig. 6c), indicating that there was a close correlation between miR-30c and EMT-related genes.

In conclusion, we found that miR-30c expression was downregulated in RCC and inhibited by hypoxia. Moreover, we

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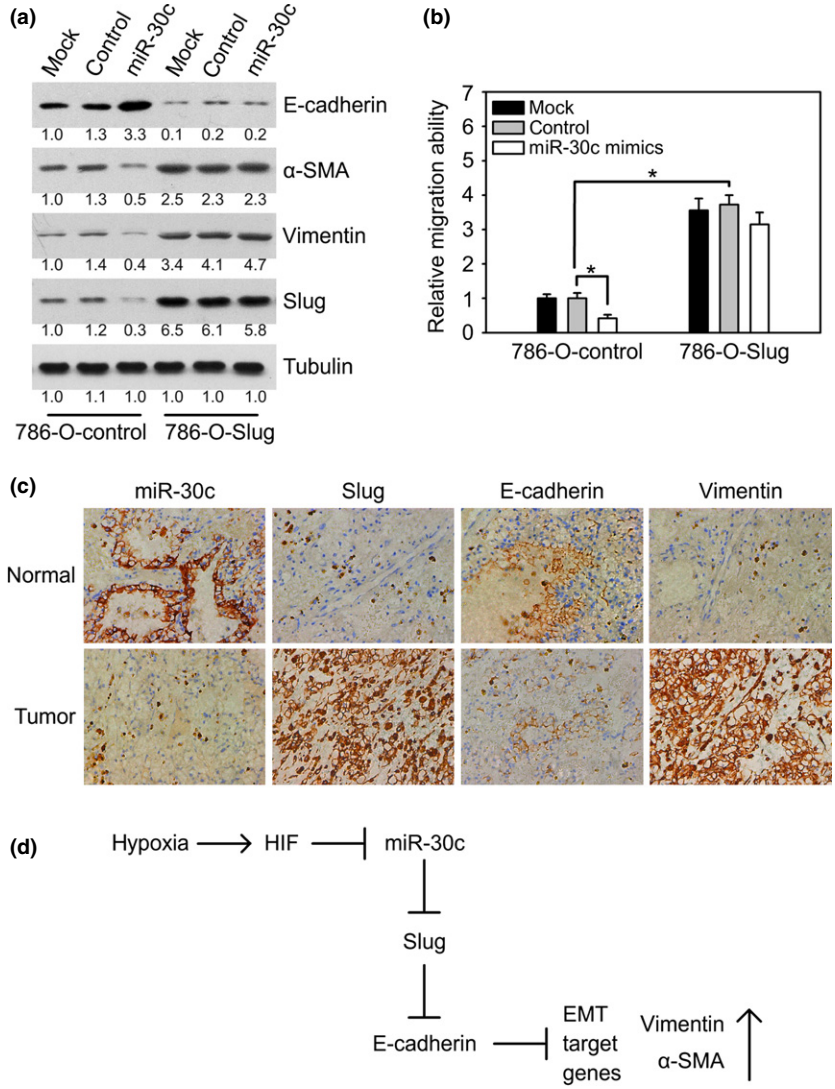

Fig. 6. Slug overexpression abrogates the effect of miR-30c downregulation on epithelial-mesenchymal transition (EMT). (a) Slug overexpressed 786-O cells (786-O-Slug) and control cells (786-O-control) were generated by using lentiviral expression system. 786-O-control and 786-O-Slug cells were transfected with miR-30c mimics or control miRNA. Western blotting analysis was performed to examine expression of E-cadherin, α-SMA and vimentin at 48 h post-transfection. (b) Cells were treated as (a). Cell migration was examined by transwell assay at 48 h post-transfection. The relative number of migrated cells was shown as means ± SD of three experiments. $*P < 0.01$ *vs* control (Student's *t*-test). (c) Detection of miR-30c, Slug, E-cadherin and vimentin in the same renal cell carcinoma (RCC) tissue and its matched non-tumor tissue. N, non-tumor tissue; T, tumor tissue. (d) Schematic diagram showing the hypoxia-induced EMT regulated by miR-30c. A model proposed to illustrate that hypoxia induces the expression of Slug through downregulation of miR-30c and thus represses E-cadherin production, which lead to promoted EMT in RCC cells.

showed that miR-30c targeted Slug and inhibited EMT in RCC cells, and miR-30c-inhibited EMT was rescued by Slug overexpression. We propose a model that hypoxia induced downregulation of miR-30c causing upregulation of Slug and thus repressing E-cadherin production, which leads to increased EMT in RCC cells (Fig. 6d).

Discussion

Hypoxia is a common feature that occurs in rapidly growing malignant tumors and their metastases as a consequence of inadequate blood supply. Hypoxia causes induction of selection processes for gene mutations, stress adaptation, tumor cell apoptosis and tumor angiogenesis, and eventually contributes to tumor aggressiveness and metastasis. It is well established that hypoxia functions through HIFs to regulate expression of numerous genes in cancer. However, there were few studies of hypoxia-regulated microRNAs (miRNAs) in cancer biology especially in human RCC. Here, we show that hypoxia induces downregulation of miR-30c and thus promotes EMT in RCC.

The downregulation of miR-30c has been reported in several types of cancer. We examined miR-30c expression in RCC, and found that miR-30c is downregulated in tumor tissues and cell lines compared to their non-tumor counterparts. Moreover, we found that hypoxia could induce downregulation of miR-30c in RCC cell lines. miR-30c expression has been reported to be regulated by hypoxia in retinoblastoma cells,[24] which is consistent with our results. The exposure of cells to hypoxia normally leads to coordinated regulation of many genes by HIFs. Several miRNAs have been reported to be downstream effectors of HIFs. Here, we found that miR-30c was regulated by hypoxia in HIFs-dependent manner. In the hypoxia process, the VHL protein functions as an E3 ubiquitin ligase, which recognizes and binds to HIFs and facilitates ubiquitination, leading to rapid proteasomal degradation. The VHL defect caused by mutation or deletion is observed in approximately 60% of RCC. In hypoxic conditions or with *VHL* gene defects, HIF-α is stabilized, allowing for the expression of a large panel of target genes involved in growth, motility, metabolism and angiogenesis, such as glucose transporters, parathyroid hormone-related protein (PTHrP), and vascular endothelium growth factor (VEGF), all shown to contribute to RCC progression. Besides VHL regulated genes, certain miRNAs can also be regulated by VHL in either a HIFs-dependent or HIFs independent manner in RCC.[25]

Dysregulation of miR-30c in RCC suggests that miR-30c may play important roles in RCC. We found that miR-30c downregulation promoted EMT in RCC cells that is consistent with the tumor suppressive roles of miR-30c. Epithelial-

mesenchymal transition is a biologic process that allows an epithelial cell to acquire a mesenchymal cell phenotype, including fibroblastoid morphology, enhanced migratory capacity, and elevated resistance to apoptosis.[26] In the case of cancer cells, EMT means acquisition of increased invasion, metastasis and resistance to chemotherapy. The hallmark of EMT in cancer cells is downregulation of E-cadherin, which is regarded as a repressor of invasion and metastasis. We showed that downregulation of miR-30c led to reduction of E-cadherin and increased expression of EMT target genes including α-SMA and vimentin. It has been reported that miR-30c is downregulated in TGF-β-induced EMT in rat tubular epithelial cells.[27] Our results further indicated the role of miR-30c in EMT.

To determine how miR-30c regulates EMT, we searched for its physiological targets using bioinformatics analysis. Several targets of miR-30c in different cells have been reported, such as PAI-1, ALK2, BCL9, MTA1, HMBOX1, DLL4, REDD1 and twinfilin 1. Here, we show that miR-30c regulates EMT through targeting Slug in RCC cells. miR-30c significantly repressed the luciferase activity of Slug WT-3′ UTR reporter vector and inhibited expression of endogenous Slug in RCC cells. Moreover, forced overexpression of Slug abrogated the inhibitory role of miR-30c in regulating EMT. Slug (coding by *SNAI2* gene), as well as another member of Snail family, Snail (coding by *SNAI1* gene) are zinc-finger transcription factors which play critical roles in regulation of EMT by suppressing several epithelial markers and adhesion molecules such as E-cadherin. Transgenic mice overexpressing Slug are morphologically normal at birth and develop mesenchymal tumors later, showing an oncogenic ability. Overexpression of Slug is often observed in numerous cancers and is associated with poor therapeutic prognosis.[28] Both transcriptional and posttranscriptional regulation of Slug by many proteins contributes to the overexpression of Slug.[29] Recently, miRNA regulated expression of Slug has also been reported. For example, miR-124 can directly target Slug in breast cancer cells.[30]

In this study, our results imply that downregulation of miR-30c caused by hypoxia or VHL defect may result in gained expression of Slug, which allows RCC cells to acquire enhanced ability of migration and eventually favors tumor progression. Our findings suggest that miR-30c might serve as a potential target miRNA for RCC therapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. The dual-luciferase reporter assay was performed in ACHN cells.

Table S1. VHL mutation in examined 32 patients with renal cell carcinoma (RCC).