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Ang-(1-7) promotes the migration and invasion of human renal cell carcinoma cells via Mas-mediated AKT signaling pathway

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ABSTRACT

Ang-(1-7) is an active peptide component of renin-angiotensin system and endogenous ligand for Mas receptor. In the current study, we showed that Ang-(1-7) enhanced migratory and invasive abilities of renal cell carcinoma cells 786-O and Caki-1 by wound-healing, transwell migration and transwell invasion assays. Mas antagonist A779 pretreatment or shRNA-mediated Mas knockdown abolished the stimulatory effect of Ang-(1-7). Furthermore, Ang-(1-7)-stimulated AKT activation was inhibited by either A779 pretreatment or Mas knockdown. Blockage of AKT signaling by AKT inhibitor VIII inhibited Ang-(1-7)-induced migration and invasion in 786-O cells. Taken together, our results provided the first evidence for the pro-metastatic role of Ang-(1-7) in RCC, which may help to better understand the molecular mechanism underlying the progression of this tumor.

1. Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults and accounts for approximately 3% of all malignancies [1]. At diagnosis, 20–30% of patients present with locally advanced or metastatic disease [2]. Although surgical resection can be curative in localized RCC, 30% of patients will eventually develop distant metastases at a median of 15–18 months after nephrectomy. A 5-year survival rate of advanced RCC is less than 10% [3,4].

In the past two decades, increasing evidence has demonstrated that the development of RCC is linked to hyperactivation of tyrosine kinase receptors and mTOR signaling [5]. The recent application of targeted agents against these pathways indeed improved cancer-specific survival. Unfortunately, their effect appears to be rather limited, with only 10–30% of cases exhibiting a complete or partial response [6,7]. Therefore, better understanding of molecular mechanisms underlying metastasis of RCC is critical to improve the clinical management and prognosis of these patients.

Ang-(1-7) is a biologically active heptapeptide component of renin-angiotensin system (RAS). It has been identified as the endogenous ligand for Mas, a G protein-coupled receptor (GPCR), which is expressed in many tissues including kidney, brain, heart, liver and lung [8,9]. In general, Ang-(1-7) is widely thought to counterbalance the vasoconstrictive and pro-proliferative action of angiotensin II (AngII), the most well-characterized component within the RAS [10]. Although recent work on biological function of Ang-(1-7) in cancer has made out a new vision of the RAS, the data to date remain conflicting. In some studies, Ang-(1-7) reduced the growth and angiogenesis of human lung adenocarcinoma xenografts [11,12]. The same group also observed that Ang-(1-7) inhibited the growth of orthotopic human estrogen receptor positive breast tumor xenografts and tumor fibrosis [13]. In contrast to these anti-cancer functions, Ang-(1-7) exerted growth-stimulatory effect on glioma cells [14]. In mesangial cells, Ang-(1-7) activated MAPK phosphorylation via Mas receptor, leading to production of TGF-β1 and extracellular matrix proteins [15]. Additionally, Ang-(1-7)/Mas axis has also been shown to mediate AngII-stimulated epithelial-to-mesenchymal transformation (EMT) in tubule cells

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[16]. These contradictory findings indicate pleiotropic roles for Ang-(1-7) in tumor progression. Ang-(1-7) is generated in the kidney via enzymatic pathways including ACE2, and maintains a relatively high intrarenal level. The kidney has recently emerged as an important target for the actions of Ang-(1-7) [17]. However, the role of Ang-(1-7) in pathophysiology of kidney cancer still remains elusive.

In the present study, we investigated the stimulatory effect of Ang-(1-7) on the migration and invasion of RCC cells, and explored related signaling pathway which mediated its function.

2. Materials and methods

2.1. Antibodies and reagents

Anti-PS^{473}AKT and anti-AKT antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Anti-Mas antibody was obtained from NOVUS Biologicals (Littleton, CA). Anti-β-actin and HRP-conjugated secondary antibodies were obtained from ZSGB-BIO (Beijing, China).

Fibronectin and matrigel were purchased from BD Biosciences (Franklin Lakes, NJ). Ang-(1-7), A779 and AKT inhibitor VIII were obtained from Calbiochem (Darmstadt, Germany).

2.2. Cell culture

The human renal carcinoma cell lines 786-O and Caki-1 were from Institute of Basic Medical Sciences, Cell resource center, China Academy of Medical Sciences (Beijing, China). Cells were grown in RPMI-1640 (786-O) or McCoy'5A medium (Caki-1) supplemented with 10% fetal bovine serum (FBS) (complete medium) and 1% penicillin/streptomycin, and maintained at 37 °C in 5% CO_2 at constant humidity. All cell culture reagents were purchased from HyClone (Logan, UT).

2.3. shRNA and transfection

shRNA and their sources were as follows. The human Mas shRNA 1 targeted the sequence 5′-GGGUCAAACGUGACAUCAU-3′. The human Mas shRNA 2 targeted sequence was 5′-GGAUGAGAA-GAAAUCCCUU-3′. The control shRNA was 5′-UUCUCCGAACGUGU-CACGU-3′. The shRNA sequences were subcloned into pGV248 vector (GeneChem Inc, Shanghai, China) following the manufacturer's instructions.

For stable knockdown of Mas expression, 786-O cells were seeded into a 6-well plate and transfected with 2 μg various pGV248 constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Selection was started on bulk cultures 48 h after transfection using 300 ng/mL puromycin (Sigma, St Louis, MO).

2.4. Wound-healing assay

A total of 3×10^5 786-O or Caki-1 cells were seeded into 6-well plates and allowed to reach 90% confluence. The cells were then incubated in corresponding medium containing 1% FBS (starving medium) with or without Ang-(1-7) for 24 h. In inhibitor study, A779 or AKT inhibitor VIII was added 30 min prior to Ang-(1-7) treatment. Cell monolayer was carefully scratched using a sterile 200 μL pipette tip across the center of the well. The detached cells were removed by washing twice with PBS and then the cells were maintained in above fresh medium for indicated time. Images of the wound were recorded under a phase contrast microscope at different time. The widths of three different wound surfaces in each group were noted and measured using NIH Image J 1.62.

2.5. Transwell migration and invasion assays

Cell migration or invasion assays was performed using modified Boyden chambers in 24-well plate with 8 μM pore inserts (BD Biosciences) coated with 50 μg/mL fibronectin or 1 mg/mL matrigel, respectively. Cells were cultured for 24 h as described in *wound-healing assay*. Then, 4×10^4 cells were plated in 100 μL of starving medium containing different reagents in the upper chamber. The lower chamber contained 600 μL complete medium. After 16–20 h (for migration assay) or 20–24 h (for invasion assay) of incubation at 37 °C, the migrated or invaded cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. For each experiment, the numbers of cells were counted at × 200 magnification in five separate fields by light microscopy.

2.6. Western blotting

Cell lysis and western blotting were performed as described previously [18]. Immunoreactive bands were visualized by enhanced chemiluminescence detection reagents and analyzed with NIH Image J 1.62.

For cell signaling study, 2×10^5 cells were seeded into 6-well plates and allowed to settle for 24 h. Then the cells were starved in serum-free medium for 24 h before stimulation with Ang-(1-7) for indicated time. In some experiments, cells were treated with A779 or AKT inhibitor VIII for 30 min before Ang-(1-7) was added.

2.7. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5 software (La Jolla, CA) and SPSS 18.0 (SPSS Inc, Chicago, IL). The data were expressed as the mean ± SD. Group distributions were compared using the Student's *t* test or repeated measurement ANOVA. A value of $P < 0.05$ was considered statistically significant. Each assay was repeated at least 3 times.

3. Results

3.1. Ang-(1-7) promotes migration and invasion in RCC cells

Cell migration and invasion are important factors for tumor metastasis which is correlated with the prognosis of RCC patients. To investigate the role of Ang-(1-7) in RCC progression, we first evaluated its effect on migration of RCC cells 786-O by wound-healing assay. Confluent monolayers of 786-O cells were scraped with a micropipette tip, and exposed to 1–100 nM Ang-(1-7). The wound closure was monitored for 12 h. As shown in Fig. 1A, Ang-(1-7) enhanced the ability of cells to migrate into the wounded area as compared with the control group (cell in media alone) and its stimulatory effect was observed in a dose-dependent manner. The cells were then cultured in the presence or absence of 100 nM Ang-(1-7), and the migration distances were measured at different time after wounds were made. We found that the migration rate of cells was dramatically promoted in response to Ang-(1-7) (Fig. 1B). Likewise, Ang-(1-7) also promoted wound closure in Caki-1 cells (Fig. 1C). To further substantiate these findings, a transwell migration assay was performed with both RCC cells. Compared with control group, Ang-(1-7)-treated 786-O and Caki-1 cells increased the ability to migrate through Boyden chamber by 1.6- and 2-fold, respectively (Fig. 1D).

We next addressed whether Ang-(1-7) would affect cell invasion. In transwell invasion assay, Ang-(1-7) treatment increased invasive ability of 786-O and Caki-1 cells in matrigel-coated Boyden chamber by approximately 1.5-fold (Fig. 1E).

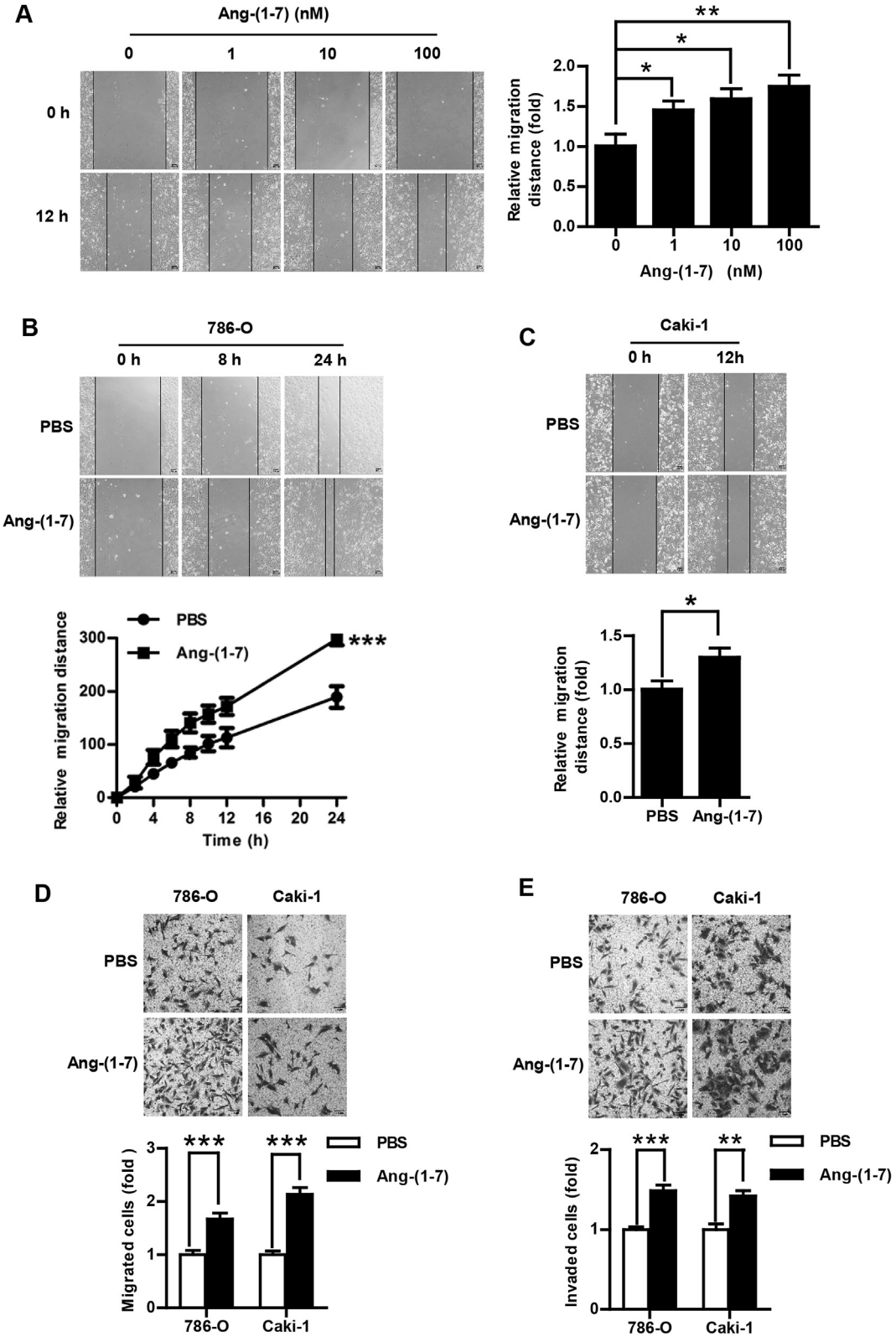

Fig. 1. Ang-(1-7) enhances migration and invasion of RCC cells. (A–C) Effects of Ang-(1-7) on the migration of 786-O and Caki-1 cells in wound-healing assays. (A) Confluent monolayers of 786-O cells treated with 1–100 nM Ang-(1-7) were scraped. The photographs were taken at 0 and 12 h after wounds were made. Values are expressed relative to the migration distance in the absence of Ang-(1-7). (B) Time course of effect of Ang-(1-7) on migration of 786-O cells. 786-O cells were treated with or without 100 nM Ang-(1-7). The photographs were taken at indicated time after scraped. (C) Scratch wounds were created in monolayers of Caki-1 cells treated with or without 100 nM Ang-(1-7) at 0 and 12 h after scraped. (D) Effects of Ang-(1-7) on the migration of 786-O and Caki-1 cells in transwell migration assays. Cells treated with or without 100 nM Ang-(1-7) were plated in Boyden chambers coated with fibronectin. The migrated cells were counted after 20 h (786-O) or 16 h (Caki-1) of culture. Values are presented relative to migrated cells in PBS-treated cells. (E) Effects of Ang-(1-7) on the invasion of 786-O and Caki-1 cells in transwell invasion assays. The cells treated with or without 100 nM Ang-(1-7) were plated in Boyden chambers coated with matrigel. The invaded cells were counted after 24 h (786-O) or 20 h (Caki-1) of culture. Values are presented relative to invaded cells in PBS-treated cells. In (A–E), images are representative of three individual experiments and data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

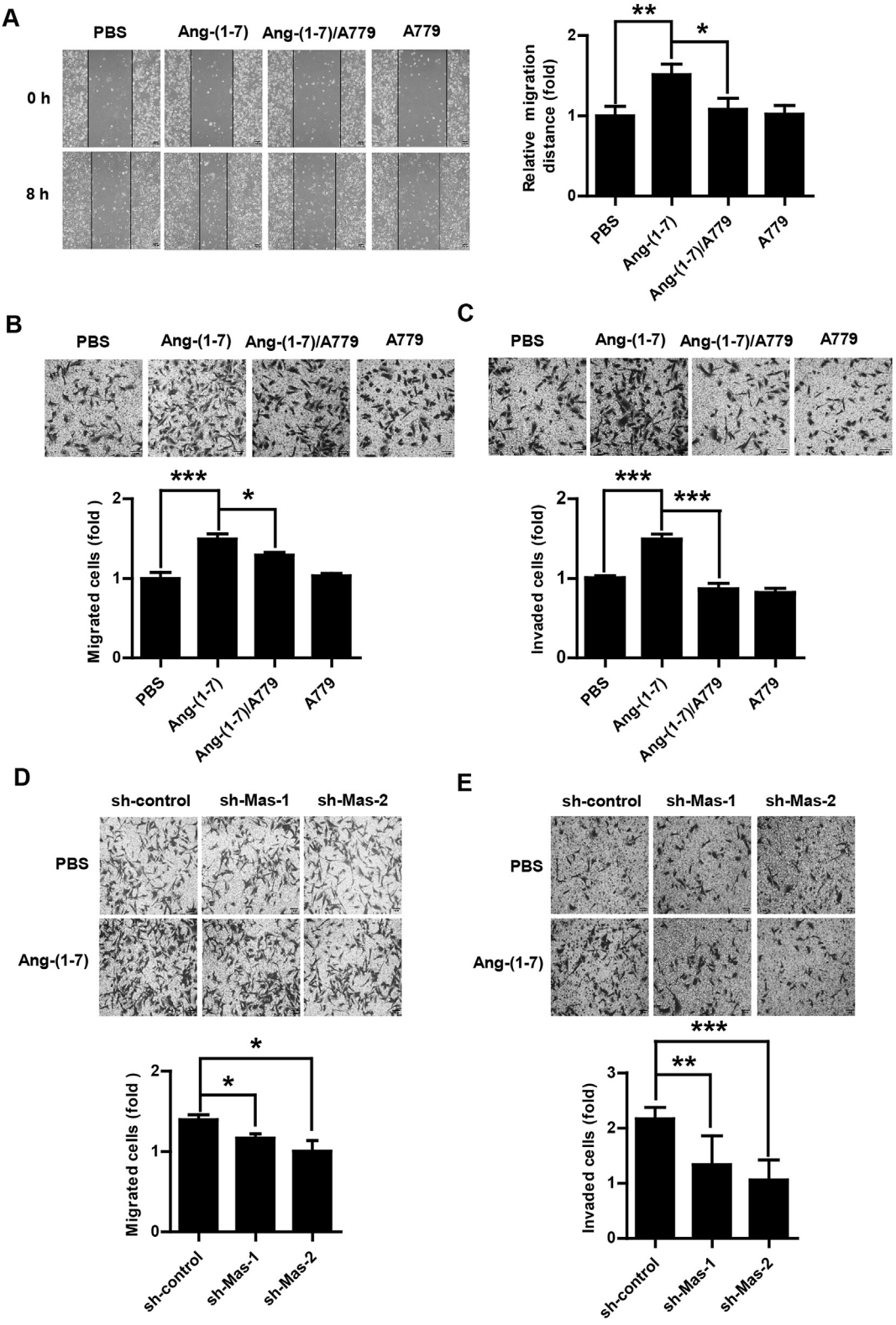

Fig. 2. Ang-(1-7) stimulates the migration and invasion of 786-O cells via Mas receptor. (A–C) Mas receptor selective antagonist A779 attenuated Ang-(1-7)-mediated increase in the migration and invasion of 786-O cells. 786-O cells were treated with PBS, 100 nM Ang-(1-7), 100 nM Ang-(1-7)/1 μM A779 or 1 μM A779 for 24 h before wound-healing assay (A), transwell migration assay (B) or transwell invasion assay (C) were performed as described in "Materials and Methods" section. (D–E) shRNA-mediated Mas knockdown inhibited effect of Ang-(1-7) on cell migration and invasion. 786-O cells transfected with sh-control or sh-Mas were treated with or without 100 nM Ang-(1-7) for 24 h before transwell migration assay (D) and transwell invasion assay (E) were done. Values are expressed relative to the migration distance (A), migrated (B, D) or invaded cells (C, E) in PBS-treated cells. In (A–E), images are representative of three individual experiments and data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

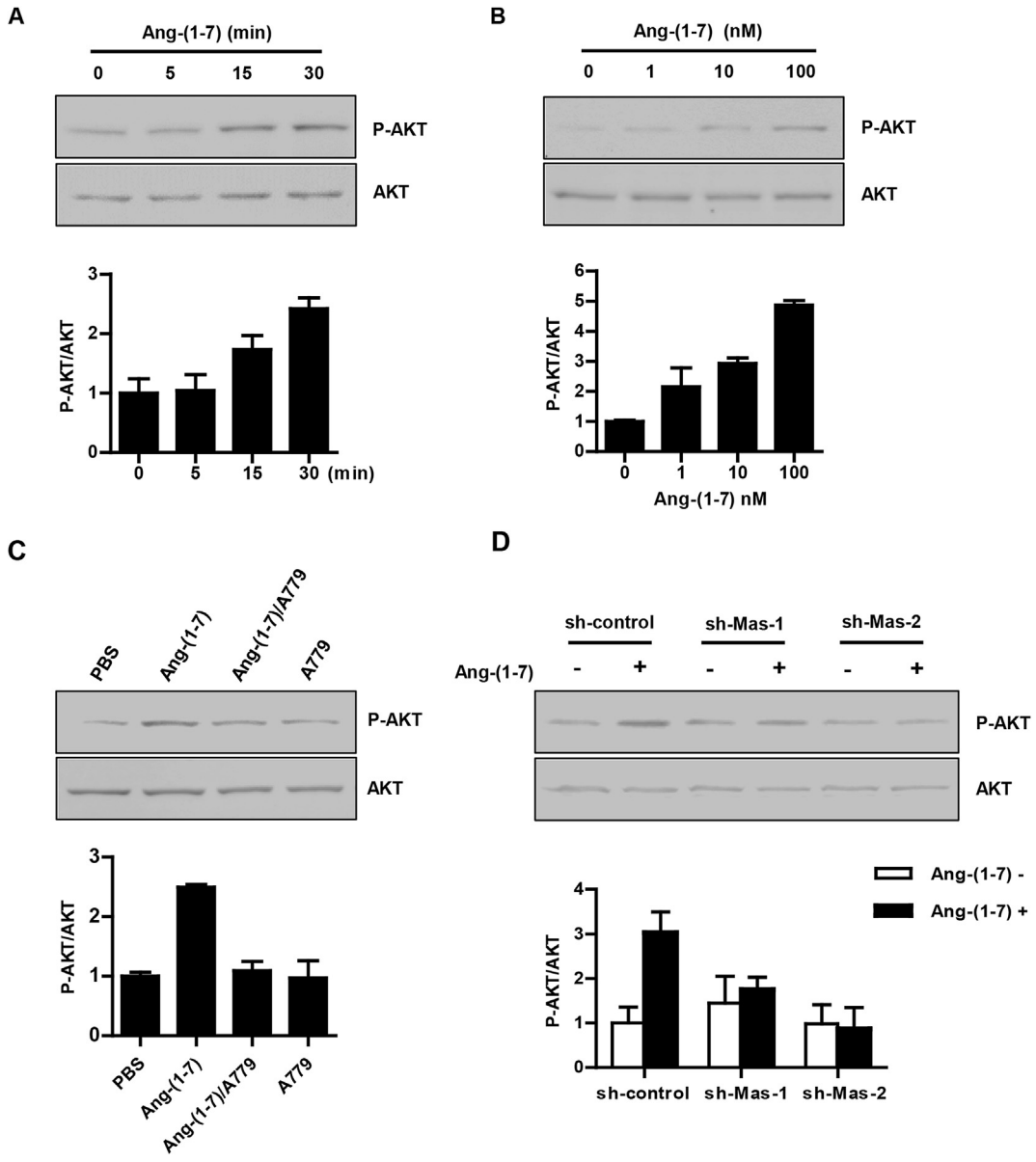

Fig. 3. Ang-(1-7) stimulates AKT phosphorylation via Mas receptor. (A) Time course of Ang-(1-7)-induced AKT phosphorylation. 786-O cells were serum starved for 24 h before stimulated with 100 nM Ang-(1-7) for indicated time. (B) Dose-dependent AKT phosphorylation stimulated by Ang-(1-7). Starved 786-O cells were stimulated with 0–100 nM Ang-(1-7) for 30 min. (C) A779 attenuated Ang-(1-7)-mediated AKT activation. Starved 786-O cells were pretreated with or without 1 μM A779 for 30 min before incubated with PBS or 100 nM Ang-(1-7) for 30 min. (D) Mas knockdown attenuated Ang-(1-7)-induced AKT activation. Starved sh-control or sh-Mas 786-O cells were stimulated with or without 100 nM Ang-(1-7) for 30 min. In (A–D), phosphorylation of AKT was analyzed by western blotting with anti-P-AKT antibody. The blot was reprobed with anti-AKT antibody as loading control. The results are representative of 3 independent experiments.

3.2. Mas receptor mediates Ang-(1-7)-induced promotion of migration and invasion

Since Ang-(1-7) was reported as an endogenous ligand for Mas receptor [8], we then investigated whether it exerted its effect on cell migration and invasion via activation of Mas signaling. Migratory and invasive abilities were evaluated in the 786-O cells pretreated with A779, a selective Mas receptor antagonist. In wound-healing or transwell migration assays, A779 inhibited the pro-migratory action of Ang-(1-7) (Fig. 2A and B). Furthermore, Ang-(1-7)-mediated promotion of invasion was completely abolished by A779 in 786-O cells (Fig. 2C). As expected, neither migration nor invasion was affected by A779 alone. The similar results were also observed in Caki-1 cells (Fig. S1). To further demonstrate Mas as a functional receptor for Ang-(1-7), Mas expression was knocked down using two independent shRNAs (Fig. S2), and migration and invasion of 786-O cells were analyzed. ShRNA-mediated Mas knockdown led to dramatic decrease in the migration and invasion of Ang-(1-7)-treated cells. Of note, in line with the effects of shRNA on Mas expression, Mas shRNA 2 inhibited Ang-(1-7) function to a greater extent than Mas shRNA 1 (Fig. 2D and E). Altogether, these data demonstrate that Ang-(1-7) drives migration and invasion of RCC cells through Mas signaling.

3.3. Ang-(1-7)-induced cell migration and invasion is dependent on AKT activation

AKT signaling pathway plays an important role in the migration and invasion of cancer cells [19]. To understand the molecular mechanism by which Ang-(1-7) promoted the migration and

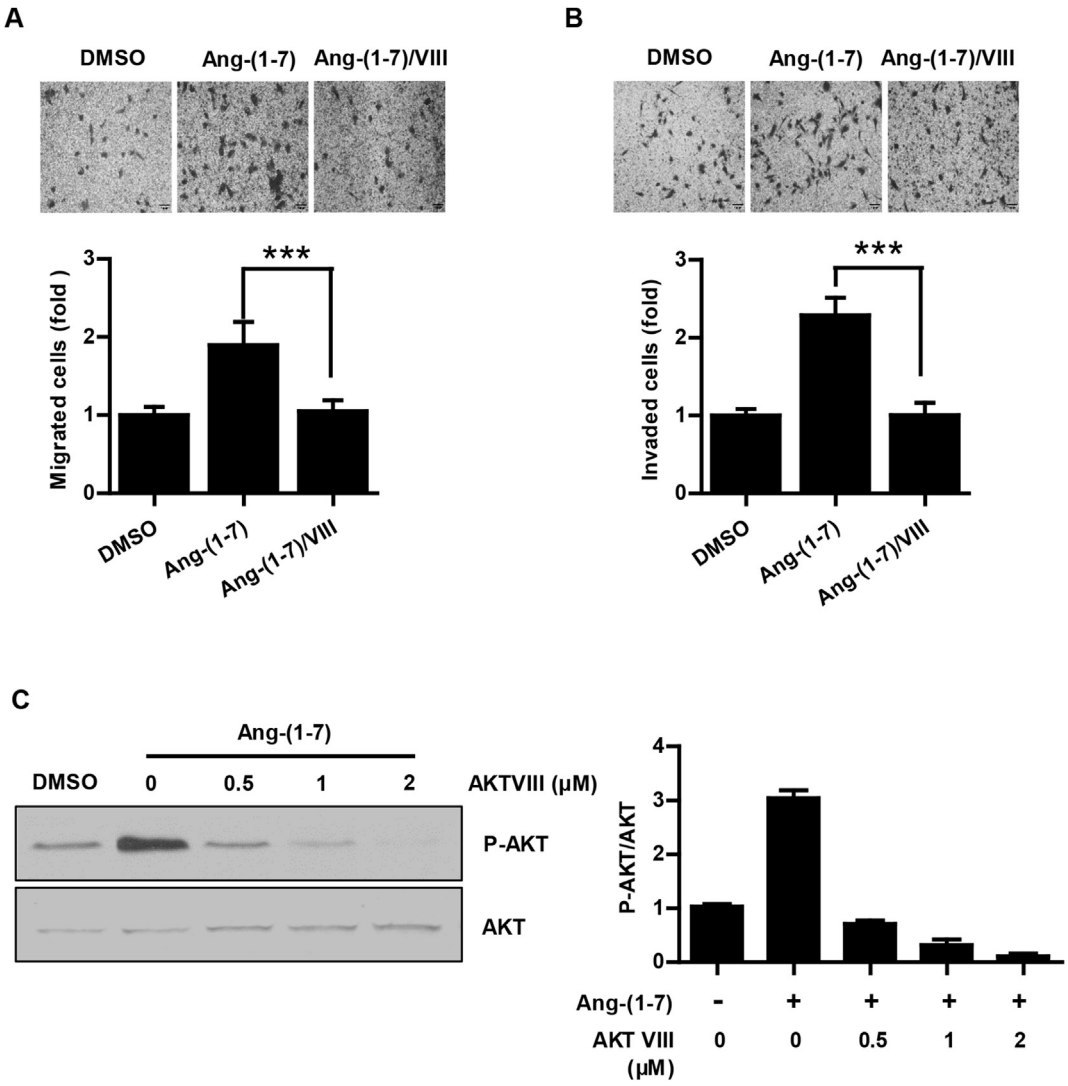

Fig. 4. Ang-(1-7)-induced migration and invasion of 786-O cells is dependent on AKT activation. (A–B) AKT inhibitor VIII blocked Ang-(1-7)-induced migration and invasion of 786-O cells. 786-O cells were treated with DMSO, 100 nM Ang-(1-7) or 100 nM Ang-(1-7)/1 μM AKT inhibitor VIII for 24 h before transwell migration assay (A) or transwell invasion assay (B) were performed as described in "Materials and Methods" section. Values are expressed relative to the migrated (A) or invaded cells (B) in DMSO-treated cells. Data is presented as mean ± SD of three independent experiments. ***$P < 0.001$. (C) AKT inhibitor VIII abolished Ang-(1-7)-stimulated AKT activation. Starved 786-O cells were pretreated with or without AKT inhibitor VIII for 30 min before incubated with 100 nM Ang-(1-7) for 30 min. Phosphorylation of AKT was analyzed by western blotting with anti-P-AKT antibody. The blot was reprobed with anti-AKT antibody as loading control.

invasion of RCC cells, we tested whether AKT activation was involved in Ang-(1-7) signaling pathway. 786-O cells were stimulated with 100 nM Ang-(1-7) for different time and status of AKT phosphorylation was assessed by western blot analysis. At basal condition, weak and leaky phospho-AKT signals were detected, which was in agreement with previous study [20]. Although the total level of AKT protein had no detectable change with Ang-(1-7) stimulation, phospho-AKT signals were significantly enhanced at 15-min and peaked at 30 min (Fig. 3A). Meanwhile, Ang-(1-7)-stimulated AKT phosphorylation was also observed in Caki-1 cells (Fig. S3). To further evaluate the dose-response relationship of Ang-(1-7) on AKT activation, the cells were then exposed to 0–100 nM Ang-(1-7) for 30 min, and Ang-(1-7)-induced a dose-dependent increase in AKT phosphorylation was observed (Fig. 3B). Meanwhile, Ang-(1-7)-induced AKT activation was abolished by pretreatment with A779 (Fig. 3C). Moreover, knockdown of Mas expression significantly retarded Ang-(1-7)-induced AKT phosphorylation (Fig. 3D). These data suggest that the stimulatory effect of Ang-(1-7) on AKT signaling was mainly dependent on Mas receptor.

To investigate whether Ang-(1-7)-induced the migration and invasion of RCC cells is mediated via AKT pathway, 786-O cells were pretreated with AKT inhibitor VIII, a highly specific allosteric AKT1/2 inhibitor [21]. 1 μM AKT inhibitor VIII completely abolished Ang-(1-7)-induced the migratory and invasive abilities of 786-O cells (Fig. 4A and B). Biochemical analysis confirmed a potent inhibitory effect of AKT inhibitor VIII on Ang-(1-7)-induced AKT activation (Fig. 4C). These data indicate that AKT activation is required for Ang-(1-7)-induced the migration and invasion in RCC cells.

4. Discussion

The Renin-angiotensin System (RAS) has been classically recognized as a principal determinant of arterial blood pressure, and hydroelectrolytic balance. Recently, increasing evidences indicate that several components of RAS might play an important

role in the progression of tumor [22,23]. Here, we show that Ang-(1-7) enhances the migration and invasion of RCC cells through activation of AKT signaling pathway. The stimulatory effects of Ang-(1-7) are abolished by the Mas receptor selective antagonist A779, Mas knockdown or AKT inhibitor VIII, which indicates that the stimulatory effect of Ang-(1-7) is mediated by Mas receptor and downstream AKT signaling. Our findings provide the first evidence for pro-metastatic role of Ang-(1-7) in RCC.

Following the identification of Mas receptor and characterization of ACE2 in the kidney, more attention has been given to the involvement of Ang-(1-7) in the context of renal disease. Some studies pointed to a deleterious role for Ang-(1-7) at renal system. Esteban et al. reported that Ang-(1-7)/Mas axis exerted proinflammatory effect in the kidney through local activation of the NF-κB pathway as well as upregulation of proinflammatory genes, rather than through systemic stimulation of bone marrow-based progenitors of inflammatory cells. Ang-(1-7)/Mas axis-induced activation of NF-κB pathway and upregulation of proinflammatory genes were then confirmed in cultured renal tubule-epithelial cells [24]. Meanwhile, Ang-(1-7) also has profibrotic action. Chronic administration of Ang-(1-7) increased renal expression of TGF-β1 and renal injury in STZ-induced diabetic rats [25]. Ang-(1-7) treatment induced EMT of renal tubular cells as well as tubular expression of matrix proteins in the kidney [16]. It is now evident that inflammation and fibrosis favor metastasis of tumor through modifying tumor micro-environment and promoting EMT [26–28]. These combined data raise the possibility that Ang-(1-7) might be a driving force in development of kidney cancer. This hypothesis could be supported by the observation that Ang-(1-7) significantly potentiated the migration and invasion of RCC cells 786-O and Caki-1 (Fig. 1). Furthermore, Mas receptor mediated the pro-migratory and pro-invasive effects of Ang-(1-7), as evidenced by the findings that Mas antagonist A779 or Mas knockdown inhibited these effects (Fig. 2). This is consistent with the role of Mas which was initially identified as a proto-oncogene by its ability to transform NIH-3T3 cells [29].

In this study, Ang-(1-7) treatment resulted in an increased AKT phosphorylation through Mas receptor (Fig. 3), and AKT inhibitor completely blocked pro-migratory and pro-invasive effects of Ang-(1-7) on RCC cells (Fig. 4). These findings agree with the requirement of AKT activation in these effects of Ang-(1-7). Indeed, elevated activity of AKT pathway was significantly associated with tumor grade, metastatic disease and adverse clinical outcome in RCC patients [30,31]. Various downstream effectors of AKT pathway have been implicated in cell migration and invasion [19]. Further studies should be performed to elucidate the involvement of these molecules in Ang-(1-7)-mediated function.

While our results argue for a pro-metastatic role of Ang-(1-7) in RCC, several recent studies regarding other cancers indicate the opposite. Krishnan et al. reported that Ang-(1-7) reduced the prostate cancer cell migration and metastatic prostate cancer growth in mice [32]. Another group found that Ang-(1-7) inhibited the migration and invasion of A549 human lung adenocarcinoma cells through inactivation of PI3K/AKT and MAPK signaling pathway [33]. This discrepancy may be explained by differences in experimental condition or cell-specific signaling, either through selective expression of positive regulator in RCC cell lineage or via a negative regulation pathway in other tumor cell lineage. These data may give rise to a novel issue concerning the clinical application of ACE inhibitors in human tumors. Previous studies suggest that blockade of the RAS with these inhibitors may inhibit tumor growth in several tumor types, such as pancreatic cancer and lung cancer [34,35]. However, treatment of patients or animals with ACE inhibitors led to a significant increase in the circulating and tissue levels of Ang-(1-7) [36,37]. Therefore, it should be prudent to use these medications on RCC patients with pre-existing hypertension.

Taken together, our results demonstrated that Ang-(1-7) promoted migration and invasion of RCC cells through activation of Mas receptor and AKT signaling, which may open a new avenue in the understanding of RCC metastasis and provide a novel therapeutic target for RCC.

Conflict of interest

There is no conflict of interest.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.035.

Transparency document

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