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ARTICLE

# Simvastatin suppresses renal cell carcinoma cells by regulating DDX5/DUSP5

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**ABSTRACT**

**Objectives:** Renal cell carcinoma (RCC) has the highest mortality rate of genitourinary cancers and the incidence of RCC has risen steadily. Simvastatin has been reported to exhibit anti-tumor activity in a variety of cancers; however, its roles and molecular mechanisms in RCC remain unclear. Our aim was to evaluate the inhibitory effect of simvastatin on RCC.

**Methods:** We used a variety of methods to test the changes of RCC cell lines' viability, migration, invasion, cell cycle and apoptosis after treatment with simvastatin.

**Results:** We found that simvastatin not only inhibited RCC cell viability, migration, and invasion, but also regulated the cell cycle and induced apoptosis. We also observed abnormal expression of DDX5 and DUSP5 in RCC cell lines. Mechanistic investigation showed that simvastatin significantly suppressed DDX5 and promoted DUSP5 expression.

**Conclusion:** Together, these results provide a novel mechanism underlying simvastatin-induced inhibition of RCC via regulation of the DDX5/DUSP5 axis.

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**KEYWORDS**
Simvastatin; Renal cell carcinoma; DUSP5; DDX5

## Introduction

Renal cell carcinoma (RCC) accounts for approximately 80% of kidney cancers and its global incidence has increased in recent decades [1]. Surgical resection is the standard treatment for patients with localized RCC [2]. Following nephrectomy, however, high-risk RCC is associated with a 20% and 40% risk of metastasis and recurrence, respectively [3]. The median survival for RCC patients with metastatic disease is 13 months; the 5-year survival rate is less than 10% [1,4]. Despite the advances in diagnosis and systemic therapies, the prognosis is far from satisfactory due to resistance to radiotherapy and chemotherapy [5]. Therefore, adjuvant therapies are being investigated to improve RCC disease-free survival and overall survival.

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are widely used to decrease cholesterol carried in the blood [6]. In addition to mediation of the cholesterol biosynthetic pathway, statins also exhibit a number of additional effects, especially in tumor development and progress [7]. A number of studies have demonstrated that statins may enhance cancer cell apoptosis, regulate metabolism, dissemination, and inhibit tumor proliferation, angiogenesis, and metastasis in diverse cancers [8].

DDX5, also known as p68, is a member of the DEAD-box family of RNA helicases [9]. It was first identified following an immunological cross reaction between monoclonal antibody and simian virus 40 large-T antigen [10]. Studies have demonstrated that DDX5 is involved in transcription and RNA processing activities, and participates in various biological processes, such as cell proliferation and organ differentiation [11,12]. DDX5 has also been implicated in cancer development due to its overexpression or genomic amplification in different types of cancer, including colon, brain, breast, liver and prostate cancer [13,14]. However, to date, no study has focused on the specific role that DDX5 plays in RCC.

Dual-specificity phosphatases (DUSPs), named for their ability to dephosphorylate both tyrosine and serine/threonine residues, play critical roles in the direct or indirect inactivation of different MAP kinases [15]. DUSP5 is a member of the DUSP family and has been implicated in a variety of human cancers over the past decade [16]. However, evidence suggests that DUSP5 is a double-edged sword in tumorigenesis and metastasis, as it regulates different signaling pathways. DUSP5, which acts downstream of DDX5 [14], appears to be a potential therapeutic target, but its effects in different cancers remain to be identified.

The aim of this study was to examine whether simvastatin, the most lipophilic statin, could suppress RCC. In this study, we first detected DDX5 and DUSP5 expression in RCC cell lines, and determined the inhibitory effects of simvastatin on RCC. Further mechanistic experiments demonstrated that the DDX5/DUSP5 axis is involved in simvastatin-induced tumor suppression. Taken together, our findings confirm the anti-tumor potential of simvastatin, which may aid in the development of novel strategies for the treatment of RCC.

## Materials and methods

### Cell culture

The human renal proximal tubular epithelial cell line HK-2, and RCC cell lines A498 and ACHN, were obtained from the

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Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in a 37 °C incubator with 5% $CO_2$ and used for experiments at a relatively low passage number. This study was approved by the ethics committee of Harbin Medical University.

### Reagents and chemicals

Simvastatin (S1792; Selleck Chemicals, Houston, TX) was activated using absolute ethanol and adjusted to final concentrations of 0, 1.25, 2.5, 5 and 10 μM with phosphate-buffered saline (PBS; pH 7.2). Rabbit anti-DDX5 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-DUSP5 (Abcam, Cambridge, MA), and rabbit anti-GAPDH (Abcam) antibodies were obtained for western blotting, immunofluorescent or immunohistochemical assays. Alexa Fluor-488 goat anti-rabbit IgG and DAPI and MTT assay reagents were purchased from Invitrogen (Carlsbad, CA, USA) and Ding Guo Biotech (Beijing, China), respectively. All procedures were performed according to the manufacturer's instructions.

### Quantitative real-time PCR

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen), and approximately 1 μg of RNA was reverse transcribed into cDNA using the Prime Script™ RT reagent kit according to the manufacturer's instructions (Takara Biotechnology Co., Ltd., Dalian, China). RNA was used to synthesize cDNA. Gene expression levels of DDX5 and DUSP5 were determined by quantitative real-time PCR (qRT-PCR) and GAPDH was used as a control. The primers used were as follows: DDX5 sense, 5′-TTTATGAAGCCAATTTCCCTGC-3′; and antisense, 5′-CCACTCCAACCATATCCAATCC-3′; and DUSP5 sense, 5′-CAATGAGGTAGTTGGTTGAAGTAG-3′; and antisense, 5′-CTGAGAAGAGGTGGAATGA-GA-3′.

### Cell proliferation assay

Cells were seeded into 96-well plates (2,000/well). After 48 h incubation, 20 ml of MTT reagent (Beijing Dingguo Changsheng Biotechnology, Beijing, China) was added to each well. Following incubation at 37 °C for 4 h, the culture medium was removed and 100 ml of DMSO was added to allow complete dissolution of purple precipitates. The optical density at 570 nm of each well was determined using a microplate reader (Biotek, Winooski, VT).

### Western blot analysis

HK-2, A498, and ACHN cells were lysed in RIPA buffer (Thermo, Shanghai, China) and the lysates were analyzed using standard western blotting procedures. Generally, lysates (15 μg) were separated on 12.5% SDS-PAGE gels, transferred to PVDF membranes (Millipore, Danvers, MA), blocked in 5% skim milk containing 0.05% Tween 20-Tris-buffered saline for 1 h, and incubated with primary antibodies at 4 °C overnight. After the membranes were incubated with anti-rabbit IgG-HRP secondary antibodies (1:5,000; Santa Cruz Biotechnology), band intensities were determined using ImageJ software.

### Migration and invasion assay

For the wound-healing assay, cells ($1 \times 10^5$ cells/well) were seeded into six-well plates and incubated in serum-free medium for 12 h at 37 °C. The cell monolayer was scratched with a 200-μl sterile pipette tip to form wound gaps. The cells were washed in PBS, incubated for 48 h at 37 °C, and a light microscope was used to monitor wound closure at the indicated time-points.

For the invasion assay, cells ($1 \times 10^5$ cells/well) incubated in serum-free medium were added to the upper chamber of the insert with Matrigel (BD Biosciences, San Jose, CA, USA). After incubation at 37 °C for 48 h, non-invading cells in the upper chamber were scraped with a cotton swab. Invading cells were fixed with 4% polyoxymethylene for 15 min and stained with 0.5% crystal violet (Amresco, Solon, OH) for 15 min. The stained cells were captured and counted under a light microscope (AE31; Motic, Xiamen, China) in six random fields and averaged.

### Immunohistochemical analysis

All tumor tissue samples were fixed in 10% paraformaldehyde for 12 h at 25 °C and subsequently embedded in paraffin and cut into 5-μm-thick sections. The sections were incubated overnight at 4 °C with primary antibodies followed by incubation with a secondary antibody at 37 °C for 1 h and processed with an ImmunoPure Metal Enhanced Diaminobenzidine Substrate Kit (Pierce Biotechnology, Waltham, MA).

### Immunofluorescence staining

RCC cells were co-cultured with 2.5 μM simvastatin for 48 h. Cells ($1 \times 10^5$ cells/well) plated into six-well plates were incubated overnight at 37 °C, fixed with 4% paraformaldehyde for 20 min, permeabilized, blocked with 2% bovine serum albumin and 3% goat serum for 30 min, and incubated with primary antibodies diluted in blocking buffer and secondary antibodies for 1 h. Cell nuclei were stained with DAPI (ZLI-9557; ZSGB-BIO) at 37 °C for 5 min. Immunofluorescence images were captured using a fluorescence microscope (Nikon Corp.).

### Statistical analysis

The difference between experimental groups was analyzed using non-parametric statistical tests. All experiments were repeated $\geq$ 3 times independently with technical replicates and analyzed using Prism 7 (GraphPad, San Diego, CA). The data are presented as the mean ± standard deviation. A value of $p < 0.05$ was considered statistically significant.

## Results

### Simvastatin inhibited RCC cell viability

We first investigated the impact of simvastatin on normal renal proximal tubular epithelial cells (HK-2) and RCC cells (A498 and ACHN). The different cell lines were treated with

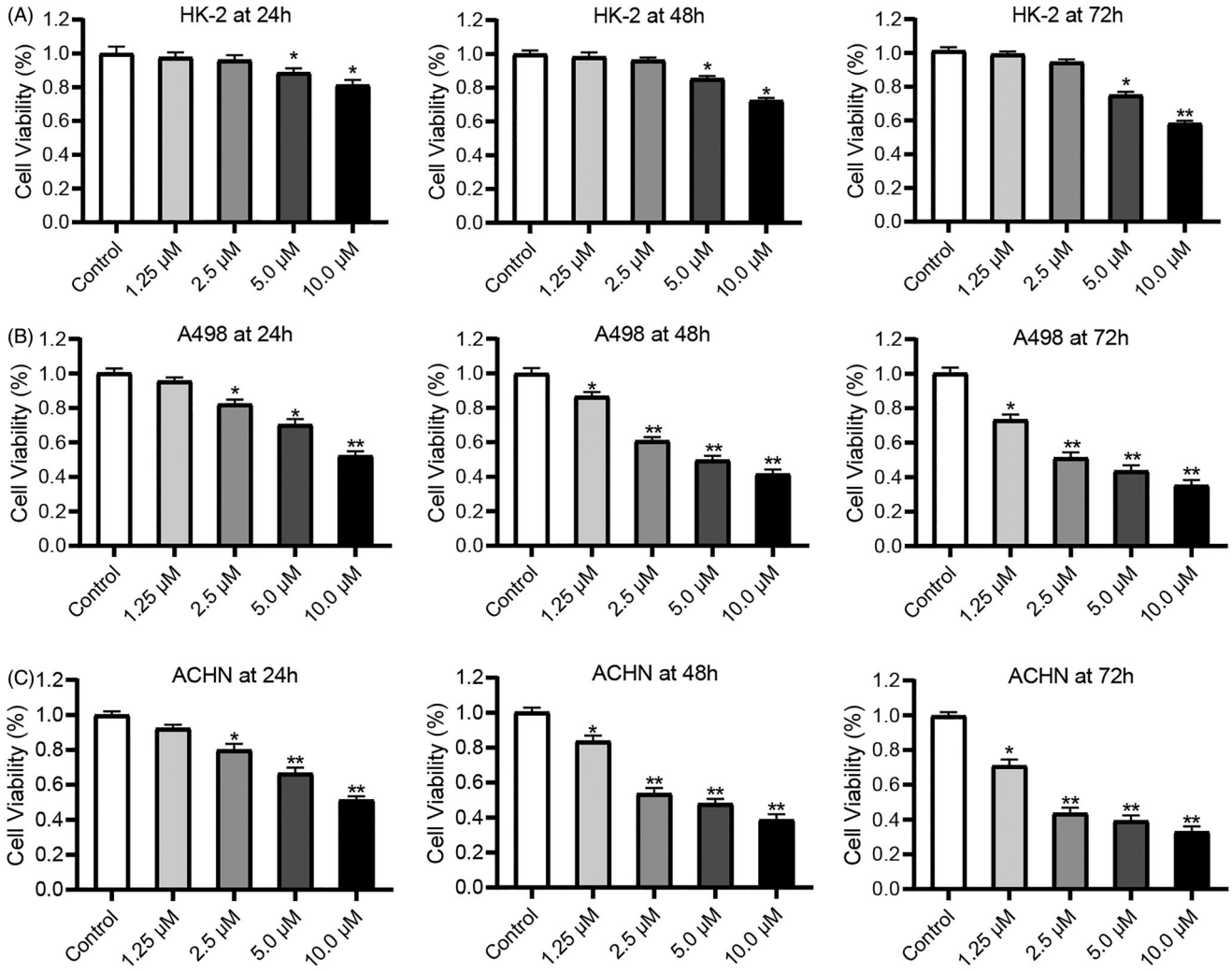


Figure 1. Simvastatin inhibited RCC cell viability. Alterations in the viability of HK-2 (a), A498 (b) and ACHN (c) cells following treatment with different concentrations of simvastatin, as detected by MMT assay and quantitative histogram analysis. $^{*}p < 0.05$ vs Control, $^{**}p < 0.01$ vs Control; $n = 3$.

0, 1.25, 2.5, 5, and 10 μM simvastatin independently for 72 h. For HK-2 cells, 2.5 μM simvastatin did not affect cell viability (Figure 1(a)), and a high concentration ($\geq$ 5 μM) of simvastatin showed limited inhibition of HK-2 cell viability. However, simvastatin significantly suppressed the viability of A498 and ACHN cells. As shown in Figure 1(b), nearly half of A498 cells were inhibited when treated with 2.5 μM simvastatin; a similar result was observed in ACHN cells (Figure 2(c)). The results of the MTT assay showed that simvastatin inhibited the proliferation of A498 and ACHN cells in a time- and dose-dependent manner.

### *Simvastatin suppressed RCC cell migration and invasion*

Wound-healing and Transwell assays were conducted to determine the effects of simvastatin (2.5 μM for 48 h) on migration and invasion in A498 and ACHN cells. The results indicated that both migration (Figure 2(a)) and invasion (Figure 2(c)) of A498 cells were suppressed by simvastatin treatment. Similar results were obtained with ACHN cells (Figures 2(b and d)).

### *Simvastatin inhibited the cell cycle and induced apoptosis of RCC cells*

The cell cycle and apoptosis were analyzed by flow cytometry. There was an increase in S phase (11.1–26.1%, 10.8–31.2%, respectively) with a concomitant decrease in G2/M phase (15.8–9.2%, 13.2–8.7%, respectively) of A498 (Figure 3(a)) and ACHN (Figure 3(b)) cells. There was an obvious difference in the apoptosis ratio between the control and simvastatin-treated groups. The rate of apoptosis was increased in both A498 (2.5–44.7%) and ACHN (3.2–49.1%) cells following treatment with simvastatin (Figures 3(c and d)). In other words, simvastatin inhibits RCC cell division by regulating the cell cycle and promoting apoptosis.

### *Simvastatin suppressed invasion and migration in RCC cells by regulating DDX5/DUSP5*

First, we explored the expression of DDX5 in HK-2, A498 and ACHN cells. Compared with HK-2 cells, expression of DDX5 was increased in A498 and ACHN cells, as determined by

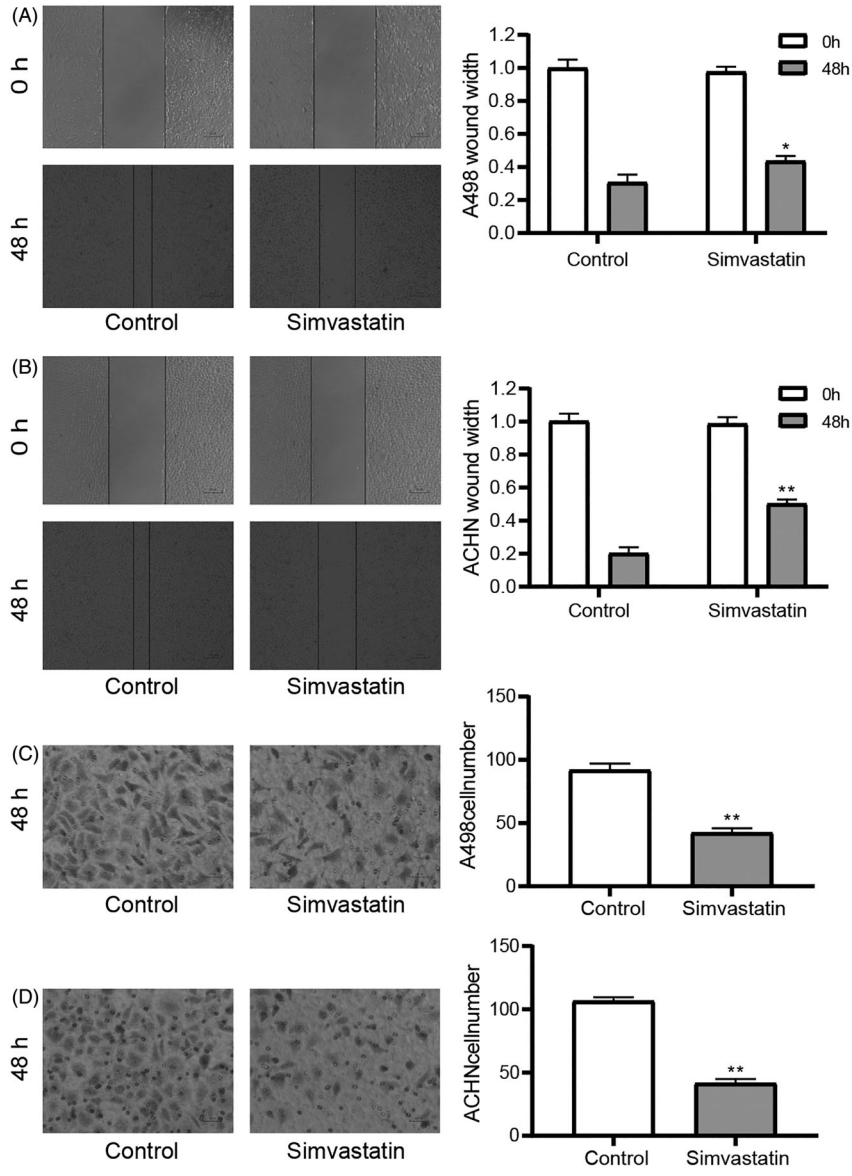


Figure 2. Effect of simvastatin on RCC cell migration and invasion. Migration rate of A498 (a) and ACHN (b) cells treated with 2.5 μM simvastatin for 48 h, as measured by wound-healing assay and quantitative histogram analysis. Invasion rate of A498 (c) and ACHN (d) cells treated with 2.5 μM simvastatin for 48 h, as revealed by Transwell assay and quantitative histogram analysis. $^{*}p < 0.05$ vs Control, $^{**}p < 0.01$ vs Control; $n = 3$.

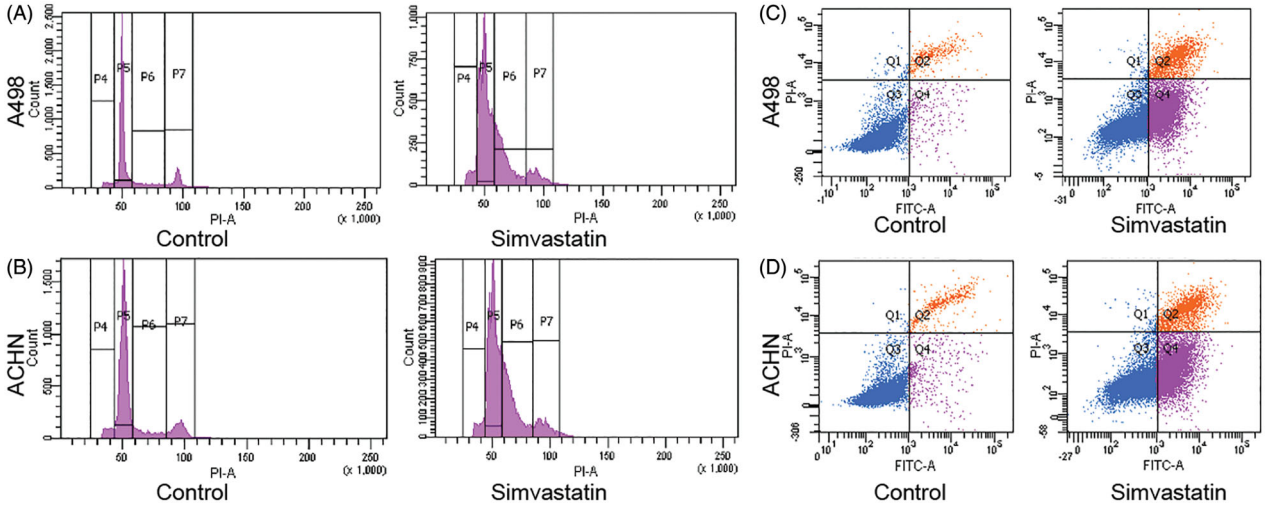


Figure 3. Effect of simvastatin on the cell cycle and apoptosis of RCC cells. A498 and ACHN cells were collected and processed as described in the Materials and methods. The distribution of A498 (a) and ACHN (b) cells in G0/G1 (P5), S (P6) and G2/M (P7) phases was analyzed by flow cytometry. The rate of apoptosis of A498 (c) and ACHN (d) cells was analyzed by flow cytometry.

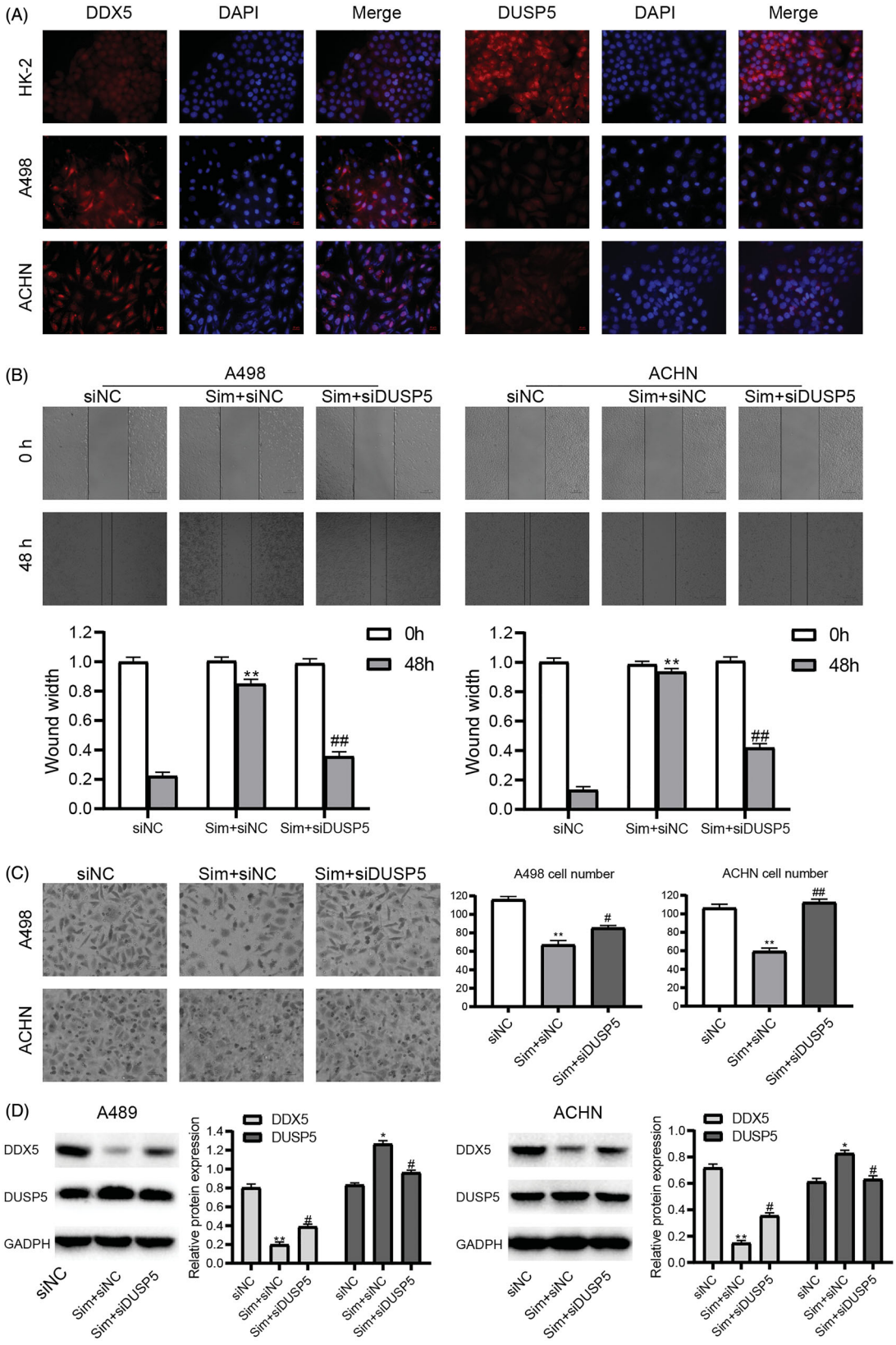


Figure 4. Simvastatin suppressed invasion and migration of RCC cells via DDX5/DUSP5. (a) Immunofluorescence assay of the expression of DDX5 and DUSP5 in HK-2, A498 and ACHN cells. Analysis of cell invasion and migration in RCC cell lines transfected with DUSP5 siRNA. A498 and ACHN cells were transfected with siRNA negative control (si-NC), si-NC + 2.5 μM simvastatin (Sim + siNC) or DUSP5 siRNA + 2.5 μM simvastatin (Sim + siDUSP5) for 48 h. (b) Wound healing assay of A498 and ACHN cells and quantitative histogram analysis. (c) Transwell assay of A498 and ACHN cell migration and quantitative analysis. A498 and ACHN cells were treated with 2.5 μM simvastatin for 48 h. (d) Histogram of relative DDX5 or DUSP5 protein expression levels with relative ratios shown as percentages of DDX5/GAPDH or DUSP5/GAPDH. $^{*}p < 0.05$ vs siNC, $^{**}p < 0.01$ vs siNC, $^{\#}p < 0.05$ vs Sim + siNC, $^{\#\#}p < 0.01$ vs Sim + siNC; $n = 3$.

immunofluorescence (Figure 4(a)). The opposite was observed with DUSP5, which was inhibited in RCC cells, supporting our hypothesis. Following treatment of A498 and ACHN cells with si-NC + 2.5 μM simvastatin, migration and invasion were inhibited, as revealed by wound healing and Transwell assays (Figures 4(b and c)). However, siRNA targeting DUSP5 reversed the simvastatin-mediated suppression of migration and invasion. To investigate the underlying mechanism, we determined the protein expression of DUSP5 and DDX5 after inhibiting DUSP5 via siRNA. In the control group, DDX5 was upregulated and DUSP5 was downregulated. Thus, our results suggest that DDX5 can negatively regulate DUSP5. Following treatment with simvastatin, the expression of DDX5 and DUSP5 was reversed compared with the control group. Thus, simvastatin might exert its inhibitory actions via DDX5/DUSP5. To further confirm this, we used RNA interference to suppress the expression of DUSP5. As expected, the protein expression of both DDX5 and DUSP5 nearly returned to control levels when treated with simvastatin + DUSP5 siRNA in the two cell lines (Figure 4(D)). These data confirmed that simvastatin suppressed migration and invasion of A498 and ACHN cells by regulating DDX5/DUSP5.

## Discussion

RCC, characterized by uncontrolled cell proliferation and evasion of immune surveillance, is not sensitive to conventional radiotherapy and chemotherapy [17]. Most RCC patients benefit from surgical resection, but for patients with metastatic RCC (mRCC), targeted therapy and immunotherapy are recommended. Targeted therapy exerts its anti-tumor effect by inhibiting the signaling molecules related to tumor growth, proliferation, or invasion. For example, vascular endothelial growth factor-targeted therapy is based on tyrosine kinase inhibitors. In terms of immunotherapy, targeting immune checkpoints, which can activate T-cell-induced tumor death, is being extensively investigated for the treatment of RCC [18]. However, only some patients respond to these novel therapies and immunotherapy may cause distinct immune-related adverse events [19]. Therefore, promising therapeutic molecules for RCC are urgently needed. Studies have shown that DDX5 can promote tumor invasion by negatively regulating DUSP5. Our results confirmed that DDX5 was upregulated and DUSP5 was downregulated in RCC cell lines; the DDX5/DUSP5 axis might be a potential therapeutic target.

In addition to treatment of atherosclerotic cardiovascular disease, statins have shown potential for treating an array of cholesterol-independent diseases [20]. Accumulating evidence has demonstrated that a variety of human tumor subtypes can respond to the inhibitory effects of statins. Both *in vitro* and *in vivo* studies have shown that statins exhibit anti-tumor effects against a variety of tumor cells by inhibiting cell proliferation, inducing apoptosis or suppressing angiogenesis [7]. Denoyelle et al. [21] found that statins regulate cell signaling pathways involved in invasiveness and metastasis of highly invasive cancer. Several clinical trials have indicated that simvastatin is associated with a reduced risk of breast cancer mortality and recurrence [22,23]. A large sample size study on statins and all-cancer survival found significantly increased cancer survival among statin users except for lung cancer [24]. Generally, statins have potential for preventing the onset of cancer, suppressing cancer metastasis, and improving patient survival by mediating different mechanistic pathways.

Our results found that simvastatin exerts a strong inhibitory effect on RCC cell viability, migration, and invasion. Moreover, a low concertation of simvastatin could regulate the cell cycle and induce apoptosis; however, the underlying mechanism is unclear. Statins are specific inhibitors of the mevalonate pathway, which is responsible for the de novo synthesis of cholesterol and other, non-sterol isoprenoids. In this pathway, statins inhibit the conversion of HMG68 CoA to MVA by inhibiting the rate-limiting enzyme, HMG-CoA reductase (HMGCR) [25]. Many researches have proved that the mevalonate pathway supports tumorigenesis and is deregulated in human cancers [26]. Meanwhile, the mevalonate pathway is a source of various important biochemical compounds and plays important roles in normal physiology. Therefore, the changes in DXX5/DUSP5 might be one cellular change caused by simvastatin treatment. Considering the abnormal expression of DDX5 and DUSP5 in RCC, we explored whether simvastatin was closely related to DDX5 and DUSP5; our results revealed that simvastatin could inhibit RCC by regulating DDX5/DUSP5. Limitations of our study should also be noted. Firstly, addition of extracellular cholesterol in cell culture is able to rescue the cells from statin-induced growth inhibition. The cholesterol level in cell culture medium should be tested in this study. Secondly, the mechanism of how simvastatin regulates DDX5/DUSP5 should be explored. Further evidence, including animal experiments, is needed to determine the underlying mechanism, which will be the focus of future studies.

## Authors' contributions

YQ and WX conceived and designed the study, and the experiments were performed by YQ, YZ and HW. YQ, WL and CJ analyzed the data and wrote the manuscript. The original text was drafted and modified by YQ and WX. All authors read and approved the final manuscript.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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