

Original Article

MEX3A suppresses proliferation and EMT via inhibiting Akt signaling pathway in cervical cancer

Yichi Xu*, Shuya Pan*, Hong Chen, Hongfei Qian, Zhiwei Wang, Xueqiong Zhu

Center for Uterine Cancer Diagnosis & Therapy Research of Zhejiang Province, Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, People's Republic of China.
*Equal contributors.

Received November 30, 2020; Accepted February 11, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: MEX3A, one member of the human MEX3 gene family, exerts different effects on a variety of human cancer cells. However, the biological functions and regulatory mechanism have not been explored in cervical cancer. In our study, we used multiple approaches to determine the functions and underlying molecular mechanism of MEX3A in cervical tumorigenesis, including CCK-8 assay, BrdU assay, FACS for cell cycle and apoptosis, wound healing assay, Transwell migration and invasion assays, immunohistochemistry (IHC) assay, Transfection, real-time RT-PCR and Western blotting analysis. IHC results showed that the expression levels of MEX3A were decreased in cervical cancer patients with advanced clinical stages and lymph node involvement. Moreover, upregulation of MEX3A attenuated cell proliferation, migration and invasion and induced cell cycle arrest at G0/G1 phase in human cervical cancer cells, whereas knockdown of MEX3A exhibited the opposite effects. Mechanistically, MEX3A exerted its tumor suppressive functions via inactivation of Akt signaling pathway and inhibiting epithelial to mesenchymal transition (EMT). Importantly, Akt activation by its activator SC79 reversed the biological functions of MEX3A overexpression. Furthermore, MEX3A inhibited tumor growth in xenograft models. Overall, our investigation suggested that MEX3A participated in antitumor activity in cervical cancer by inhibition of the Akt signaling pathway and EMT. Hence, targeting MEX3A might have a therapeutic potential to treat cervical cancer.

Keywords: MEX3A, cervical cancer, EMT, Akt, proliferation, invasion

Introduction

Cervical cancer ranked as the fourth place in both cancer incidence and mortality rates in 2018 [1]. In the year of 2020, it is estimated that there will be approximately 4,290 deaths cases, and 13,800 new cases of cervical cancer in the United States [2]. Due to the extensive use of cytology screening and the availability of HPV vaccines, the incidence rate has remarkably decreased in patients with cervical cancer [3]. However, it is concerned that HPV-associated cervical cancers still lack an effective, specific treatment strategy. The standard treatments are surgery or a combination of chemotherapy and radiation therapy for the majority of cervical cancer cases, which contribute to improving the prognosis of patients [4, 5]. Unfortunately, in patients with the advanced stage, chemoresistance remains a severe challenge for the better outcomes of

cervical cancer patients [6, 7]. Hence, we need to develop a valid therapeutic agent for the prevention and treatment of patients with cervical cancer.

MEX3A belongs to a family of human MEX3 genes, which comprises four members: MEX3A, MEX3B, MEX3C and MEX3D. MEX3 genes contain two heterogeneous nuclear ribonucleoprotein K homology (KH) domains (named as KH1 and KH2), and the conserved regions of these two amino acids bind RNA via the KH domains [8]. Existing evidence indicates that MEX-3 protein is involved in regulation of core biological processes, including embryonic development, epithelial homeostasis, immune function, metabolism and tumorigenesis [9]. By bioinformatics analysis of Oncomine database, it has been unveiled that MEX3 is altered in many types of human cancers at transcriptional level [9]. A recent study demonstrated that

MEX3A interacts with miR126-5p through KH1 and KH2 domains and this complex prevents the dimerization of caspase 3 and the formation of its active sites, and then inhibits the proteolytic activity of caspase 3 expression, leading to hindering cell apoptosis [10]. *In vivo* studies have shown that calcitriol triggers stemness-related genes such as MEX3A in mice, which is a marker of slow-dividing subsets of LGR5-positive stem cells in normal organoids [11]. Currently, numerous investigations have indicated that MEX3A plays complex and diverse roles in the development of various cancers. MEX3A plays a pivotal role in the post-transcriptional procession of mRNA and gene expression [8]. MEX3A gene expression may be closely connected with malignant tumor development and progression. One group unveiled that MEX3A knockdown suppresses the growth and migration of human gastric cancer cell; however, the specific biological mechanisms are still unclear [12]. Similarly, another study uncovered that MEX3A plays vital roles in intestinal differentiation, polarity, and stemness features, which may cause carcinogenesis by inhibition of CDX2 [13]. Moreover, it has been revealed that the expression of the cyclin-dependent kinase inhibitors p21 (CDKN1A) and p57 (CDKN1C) is increased in intestinal stem cells with high MEX3A expression [11]. These studies suggest that MEX3A may exert bidirectional modulatory functions in different types of cancer cells. However, the biological effects and underlying mechanisms of MEX3A in cervical carcinogenesis remain ambiguous until now.

Due to no available literatures regarding the effects of MEX3A on cervical cancer, we explored the functions and molecular mechanisms of MEX3A in the process of cervical tumorigenesis. The present study demonstrated the expression levels of MEX3A gene in cervical cancer cells and tissues. Moreover, the biological functions of MEX3A in cervical cancer, including cell viability, cell cycle, apoptosis, migration and invasion, were investigated by *in vitro* and *in vivo* experiments. Furthermore, we verified whether Akt signaling pathway participated into MEX3A-mediated cervical tumorigenesis. Our study might provide a novel mechanism by which MEX3A regulates the cervical tumorigenesis, suggesting that MEX3A might be a target for cervical cancer therapy.

Materials and methods

Cell culture and reagents

The human cervical cancer cells C33A, SiHa, Caski and MS751 cells, as well as normal cervical cancer cells H8 were obtained from American Type Culture Collection (ATCC, USA). Caski cells were cultured with Roswell Park Memorial Institute (RPMI, Gibco, USA) 1640 and five other types of cells were cultivated with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA), contained with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Thermo Scientific, USA) at 37°C with 5% CO₂ in a humidified incubator. The Akt phosphorylation activator SC79 (HY-18749, MCE, New Jersey, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to 10 μM with complete medium before use.

Tissue samples

A tissue microarray was purchased from Shanghai Outdo Biotech with 44 cases of cervical cancer samples. The tumor xenografts were resected and fixed with 4% formalin, paraffin-embedded and cut into 4 μm thick sections. The slices were deparaffinized with xylene and rehydrated with graded ethanol. In addition, we collected five paired fresh cervical cancer tissues and their corresponding adjacent normal tissues which were obtained from the patients who underwent tumor resection surgery in the Second Affiliated Hospital of Wenzhou Medical University between February 2017 and January 2019. None of the patients had received chemotherapy or radiotherapy preoperatively. The tissues samples were frozen in liquid nitrogen and stored at -80°C for protein detection. This study was approved by the ethics committee of the Second Affiliated Hospital of Wenzhou Medical University.

Immunohistochemistry (IHC) analysis

The tissue microarray sections were incubated with anti-MEX3A rabbit antibody (1:400, Abcam, ab79046), anti-E-cadherin mouse antibody (1:100, CST, #14472S), and anti-β-catenin rabbit antibody (1:100, CST, #8480S) at 4°C overnight. After that, slides were probed with HRP-labeled universal anti-mouse or anti-rabbit IgG for 20 min and then stained with 3,3-diaminobenzidine tetrahydrochloride. Ulti-

MEX3A targets Akt in cervical cancer

mately, the slides were counterstained with hematoxylin. Intensity score was in accordance with the following criteria: 0, no appreciable staining; 1, weak staining; 2, moderate staining; and 3, strong staining. And the percentage score was based on the percentage of cervical cancer positive cells (0-100%). The IHC staining was scored by two independent pathologists and the final score was calculated by multiplying the staining intensity score and positive staining percentage score.

Real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cervical cancer cells by use of TRIzol reagent (Invitrogen, CA). After that, the cDNA was produced by reverse transcription (RT) and oligo (dT) primers following the instructions. By qPCR using SYBR Premix ExTaq (Takara), the mRNA expressions of MEX3A were assessed. The PCR reaction was carried out at 95°C for 5 min, then repeated 40 times of 95°C for 15 s and 65°C for 40 s. The data was analyzed using the Rotor-Gene 6000 software following the $\Delta\Delta C_t$ method [14]. The sequences of primers are listed as follows: MEX3A, forward primer (TGG AGA ACT AGG ATG TTT CGG G) and reverse primer (GAG GCA GAG TTG ATC GAG AGC); GAPDH, forward primer (TGT GGG CAT CAA TGG ATT TGG) and reverse primer (ACA CCA TGT ATT CCG GGT CAA T).

Western blotting analysis

Cells were lysed in lysis buffer with protease inhibitors. After that, an equal amount of protein (40 μ g) was loaded on each lane for analysis and then resolved by SDS/PAGE, subsequently transferred onto the PVDF membranes. The membranes were blocked in 5% milk and incubated with the primary antibodies at 4°C overnight. Primary antibodies contain: anti-MEX3A polyclonal rabbit antibody (1:1000, Abcam, ab79046), anti-bactin monoclonal mouse antibody (1:5000, Abcam, ab6276), anti-Akt monoclonal rabbit antibody (1:1000, CST, #4691S), anti-p-Akt monoclonal rabbit antibody (1:1000, CST, #4060S), anti-E-cadherin monoclonal mouse antibody (1:1000, CST, #14472S), anti-Vimentin monoclonal rabbit antibody (1:1000, CST, #5741S), anti- β -catenin monoclonal rabbit antibody (1:1000, CST, #8480S) and anti-Snail monoclonal rabbit

antibody (1:1000, CST, #3879S). Then, the membranes were washed with TBST, and were incubated with secondary antibodies at 37°C for one and a half hours. Finally, bands were visualized by using enzyme-linked chemiluminescence detection kit (ECL) assay and imaging by using the BioSpectrum Imaging System.

Cell viability assay

The C33A (1×10^4 cells/well) and SiHa cells (2×10^3 cells/well) were seeded in 96-well flat-bottomed plates and 100 μ l supplement medium was provided for growth. Cell Counting Kit-8 (CCK-8, Sigma, USA) assay was performed to determine cell viability. The absorbance of 450 nm wavelength was determined by a Microplate Reader (Bio Tek Instruments, Winooski, VT, USA).

Wound healing assay

In order to define migration ability of the human cervical cancer cells, cells were seeded onto 6-well plates and allowed to grow until to more than 90% confluence. The artificial gap was scratched by a 200 μ l yellow pipette tip to create. The wound area was photographed by a phase-contrast microscope (Olympus CK30, Tokyo, Japan) at 0 h and 24 h after scratching, respectively. Then, the images were analyzed by ImageJ software 1.35 (NIH, Bethesda, MD, USA). The migratory rate was calculated by use of the following formula: (scratch width at 0 h-scratch width at 24 h)/scratch width at 0 h.

Cell migration and invasion assays

For migration and invasion assays, cells were seeded in 24-well plates ($3-10 \times 10^4$ cells) coated with Matrigel (BD Biosciences, CA) (invasion) or without Matrigel (migration) in the upper compartment with 8 μ m pore polycarbonate membranes containing 100 μ l FBS-free DMEM, and the lower compartment was supplemented medium with 20% FBS. After 24 h of incubation, non-migrated or non-invaded cells across the membranes were carefully removed with cotton swabs, while cells across the membrane were fixed with 4% paraformaldehyde and then stained by using the crystal violet dye. After that, cells were calculated under a microscope. It is noted that more than five randomly non-overlapping fields of view were selected.

BrdU proliferation assay

Briefly, 7×10^5 cells were cultured into 6-well plates. When the cells were attached to the wall, BrdU was added and maintained in a humidified atmosphere of 37°C and 5% CO₂ for 8 h. Then the cells were fixed by using 4% paraformaldehyde for half an hour at room temperature and treated with 0.2% TritonX-100 for 10 min. The cells were blocked with 3% BSA for 1 h at room temperature. After that, cervical cancer cells were incubated with the anti-BrdU antibody (CST, 1:200, 5292S) at 4°C overnight. The second day, cells were cultured with the second antibody for 90 min at room temperature. After that, the cells were stained with DAB for 5 min and hematoxylin to counterstain for 2-4 min. Positive cervical cancer cells were observed under the microscope, and the brown nuclei in the cells indicated that the cells were in the proliferation phase. The proportion of positive cells was calculated (total number of positive cells per field/total number of living cells).

Flow cytometry assay

Cell apoptosis and cell cycle distribution were measured by flow cytometry analysis. For the apoptosis detection, cells were collected and resuspended in binding buffer and stained with Annexin V-PE/7-Amino actinomycin D (AAD) for 15 minutes at room temperature in dark. Then the percentage of apoptotic cell was analyzed by flow cytometry assay according to manufacturer's instructions. For the analysis of cell cycle distribution, the cells were stained with propidium iodide (PI) and RNase in the dark at room temperature for 30 min. Then, samples were inspected by BD FACS flow cytometer.

Lentivirus and transfection

C33A cells were infected with lentivirus carrying MEX3A shRNA and SiHa cells were infected with lentivirus carrying MEX3A cDNA. Lentiviruses were transfected into cells in the presence of 8 µg/ml polybrene. After 48 h, the cells were added puromycin dihydrochloride (Sigma, 2 µg/ml) into the medium for 7 days in order to select cells by drug screening. After that, overexpression or knockdown efficiency of MEX3A was assessed by real-time PCR and western blotting, respectively. The C33A and SiHa cells were transfected with shRNA-MEX3A or MEX3A

cDNA and their respective controls by Lipo-2000 (Thermo Fisher Scientific) accordance with the manufacturer's instructions. The RNAi target sites were designed and then synthesized by Sigma-Aldrich (Shanghai, China) and were cloned into a lentiviral pLKO.1 vector. The sequences of shRNAs were listed as below. MEX3A-shRNA-1: 5'-CCG GCA CGC AAG CCA TCC GAA TAT TCT CGA GAA TAT TCG GAT GGC TTG CGT GTT TTT G-3'; MEX3A-shRNA-2: 5'-CCG GAG GCA AGG CTG CAA GAT TAA GCT CGA GCT TAA TCT TGC AGC CTT GCC TTT TTT G-3'. Additionally, plasmid MEX3A (NM_001093-725)-pCDH-GFP+Puro-3xFlag and its control were purchased from Changsha Youbio Biosciences Inc. In order to get virus particles, the plasmid was transfected into 293T cells using the Polyethylenimine (PEI) method together with two infectious viral particles psPAX2 and pMD2G to generate lentivirus. To produce cells with MEX3A downregulation, the C33A cells were infected with recombinant lentiviral particle containing MEX3A-pGFP-C-shLenti and its control vector. SiHa cells were infected with lentiviral particle containing MEX3A-pCDH or empty vector, and the stable MEX3A-overexpressing cell lines and its control were obtained. The primers used as below: MEX3A-F-(EcoRI): CCG GAA TTC ATG CCT AGT CTA GTG GTA TC; MEX3A-R-(BamHI): CGC GGA TCC TTA GGA GAA TAT TCG GAT GG.

Animal studies

Four to six weeks old female athymic BALB/c nude mice were purchased from the Department of Experimental Animal Centre of Wenzhou Medical University. After one week to acclimatize, 20 mice were randomly divided into four groups (each group had 5 mice): model C33A group (injection of C33A cells with blankvector transfection, and MEX3A shRNA-transfected C33A cells) and model SiHa group (injection of control cDNA-transfected SiHa cells and MEX3A cDNA-transfected SiHa cells). C33A (3×10^6) and SiHa cells (2×10^6) were dispersed in 100 µL PBS and the mouse model was generated by subcutaneous implanted of tumor cells into the right flank. The growth of tumor size (width and length) was measured with caliper and monitored every two days. The tumor volume (mm³) was counted using the formula of (length × width²)/2. Thirty days after injection, the nude mice were sacrificed and

MEX3A targets Akt in cervical cancer

the tumors were excised and fixed in 4% formaldehyde overnight, and embedded for further investigation. The xenograft tumors of mice were examined by hematoxylin-eosin (H&E) staining. And slides were stained with MEX3A (1:100 dilution), E-cadherin (1:100 dilution) and β -catenin (1:100 dilution) antibodies at 4°C overnight. And then incubated with the secondary antibody for half an hour at room temperature, the slides were stained with DAB for 1 min. The experimental methods and procedures were showed in the IHC analysis section of the method. The animal studies were performed in accordance with the guidelines for animal experiments, which was approved by Institutional Animal Care and Use Committee of Wenzhou Medical University.

Statistical analysis

All experiments were performed at least three times, and the results are displayed as the mean \pm standard deviation (SD). All the statistical data were analyzed by utilizing Statistical Product and Service Solutions (SPSS, Version 15.0.1; SPSS Inc., Chicago, IL, USA). The two-sided Student's t-test was applied to compare between two groups. Comparison of mean in multiple groups was conducted using ANOVA. Further pairwise comparison among groups was detected by Fisher's Least Significant Difference (LSD) test (with equal variance) and Mann-Whitney U-test (with unequal variance). $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered to be statistically significant.

Results

MEX3A expression is associated with better clinicopathological feature in cervical cancer

In order to figure out the role of MEX3A in cervical cancer development and progression, we explored MEX3A expression in 44 cervical cancer specimens by IHC. The MEX3A protein was differentially expressed in cervical cancer tissues (**Figure 1A**). Moreover, high MEX3A protein expression was negatively correlated with advanced clinical stage (**Figure 1B**). Notably, the expression of MEX3A was negatively correlated with lymph node involvement (**Figure 1B**). These results indicated that MEX3A may be a biomarker for prediction of a better prognosis in cervical cancer patients.

The expression of MEX3A is decreased in cervical cancer cell lines

Next, we measured the expression of MEX3A in normal cervical H8 cells and several cervical cancer cell lines including C33A, SiHa, MS751 and CaSki cells by real-time RT-PCR and Western blotting. We found that the expressions of MEX3A at the transcriptional and translational levels were lower compared with normal cervical H8 cells (**Figure 1C**). Among these cancer cell lines, MEX3A expression is highest in C33A cells (**Figure 1C**).

MEX3A overexpression inhibits cell viability and proliferation

The efficacy of MEX3A knockdown by shRNA transfection was determined by Western blotting analysis in C33A cells because this cell line had the highest expression of MEX3A among four cervical cancer cell lines (**Figure 1C**). We found that both shRNA1 and shRNA2 transfections downregulated the expression of MEX3A in C33A cells (**Figure 1D**). In order to investigate the effects of MEX3A expression on cell viability and proliferation, the CCK-8 and BrdU assay were performed in C33A cells after MEX3A knockdown. We discovered that downregulation of MEX3A promoted viability and proliferation of C33A cells (**Figure 2A** and **2B**). Moreover, MEX3A expression was increased in SiHa cells after MEX3A cDNA transfection (**Figure 1D**). Overexpression of MEX3A suppressed viability and proliferation of SiHa cells (**Figure 2A** and **2B**). These results uncovered that MEX3A plays a suppressive role in viability and proliferation of cervical cancer cells.

MEX3A overexpression induces cell cycle arrest at G0/G1 phase

We studied the effect of MEX3A modulation on cell cycle in C33A and SiHa cells. Cell cycle analysis data showed that MEX3A knockdown decreased the percentage of G0/G1 phase but increased cell number at S phase in C33A cells (**Figure 2C**). Additionally, MEX3A overexpression induced cell cycle arrest at G0/G1 phase and decreased the percentage of S phase in SiHa cells (**Figure 2C**). However, flow cytometry result demonstrated that MEX3A knockdown or overexpression did not affect apoptosis of C33A and SiHa cells (**Supplementary Figure 1**).

MEX3A targets Akt in cervical cancer

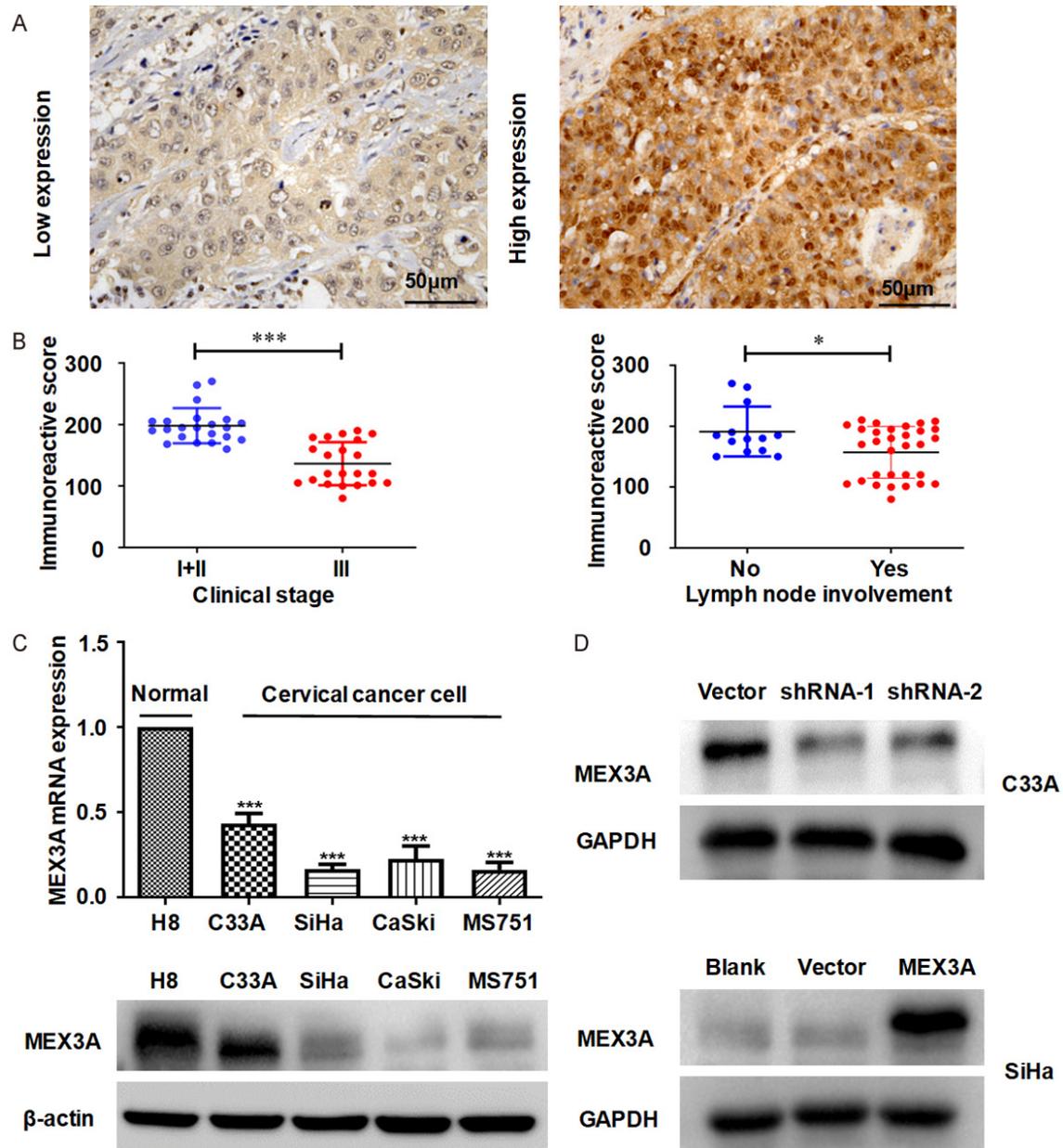


Figure 1. The expression level of MEX3A protein in tissues and its functions on viability. A. Representative MEX3A staining figure (high or low expression) in tissue microarray. Scale bars: 50 µm. B. The correlation between MEX3A expression with clinical stage and lymph node involvement. C. MEX3A expression levels in cervical cancer cells and normal cervical epithelial cells at transcriptional and translational level. D. The efficacy of MEX3A knockdown in C33A and MEX3A overexpression in SiHa were measured by Western blotting.

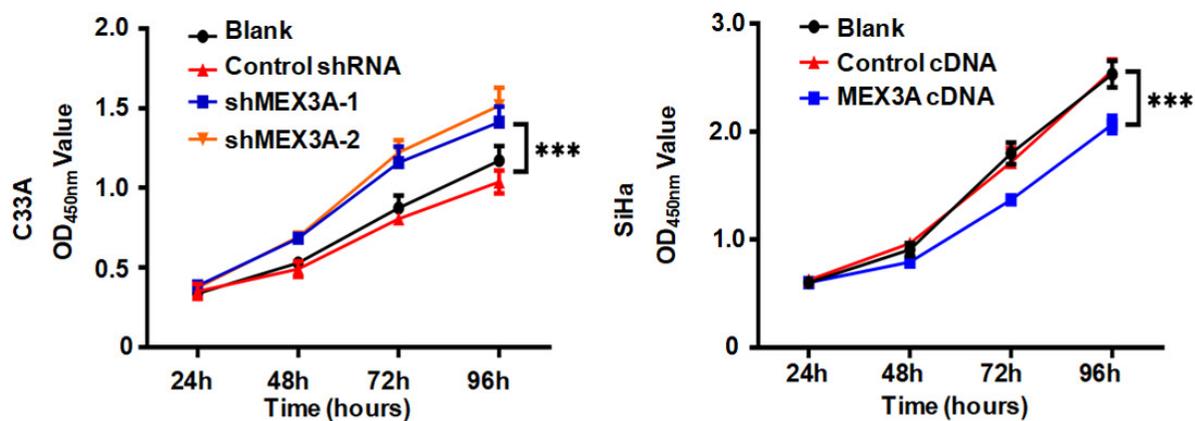
MEX3A overexpression inhibits cell migration and invasion

We explored whether MEX3A modulation affected the capabilities of cell migration and invasion in cervical cancer cells. The cell migration abilities were explored by wound healing assays and transwell assays in C33A and SiHa cells after MEX3A knockdown or overexpression.

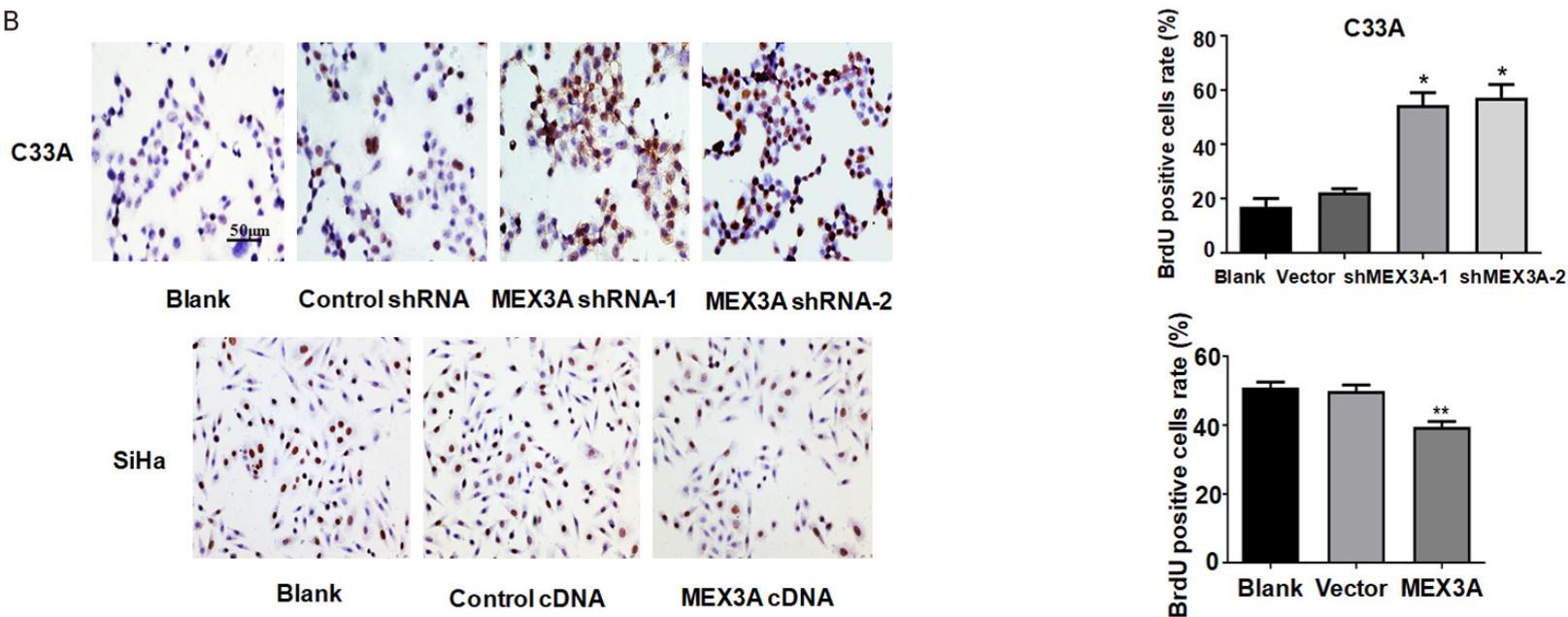
MEX3A downregulation led to greater migratory ability to close the wound in C33A cells at 24 h (Figure 3A). Consistently, cells with overexpression of MEX3A showed a decrease in wound closure in SiHa cells at 24 h (Figure 3A). In line with this finding, our data from transwell migration analysis demonstrated that downregulation of MEX3A promoted cell migration in C33A cells, whereas upregulation of

MEX3A targets Akt in cervical cancer

A



B



MEX3A targets Akt in cervical cancer

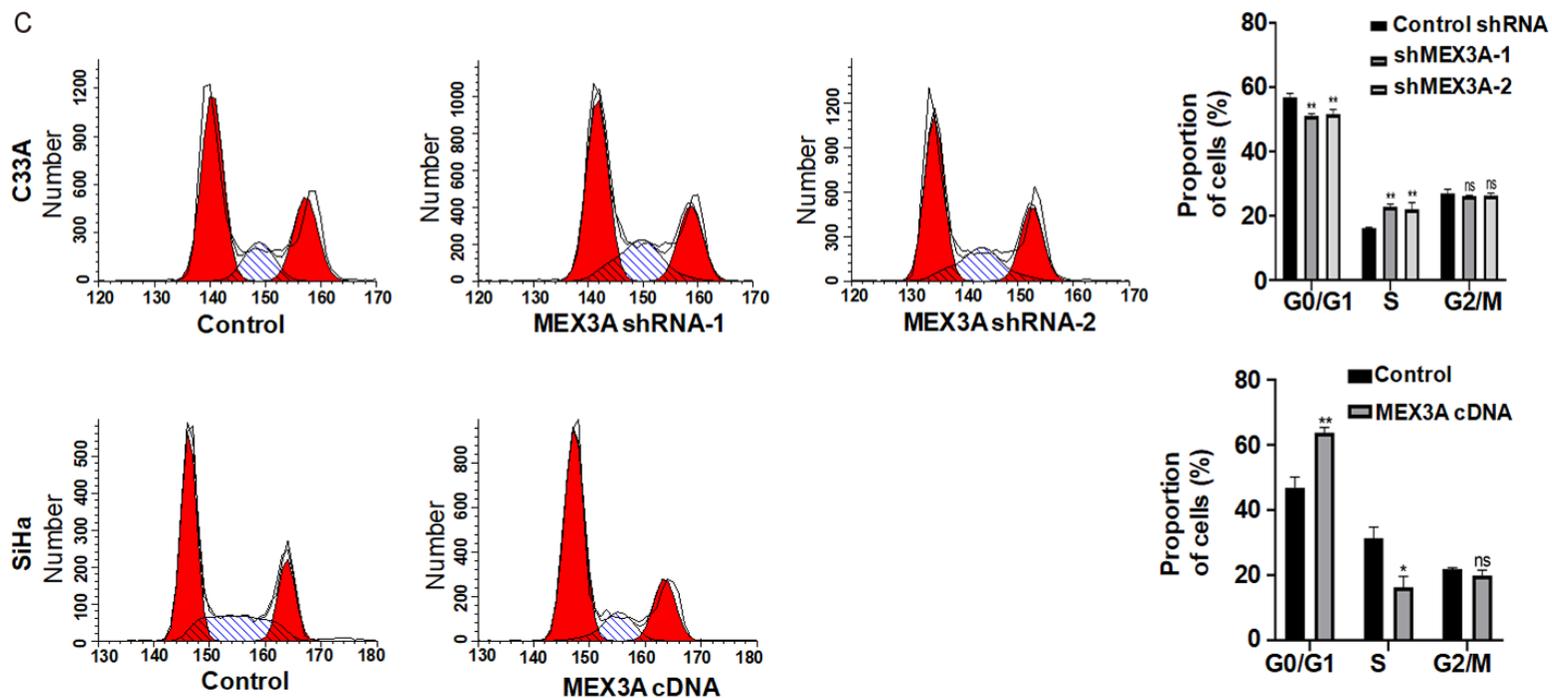


Figure 2. MEX3A inhibits the viability and induced cell cycle arrest. A. MEX3A knockdown promoted viability of C33A cells, while MEX3A overexpression suppressed viability of SiHa cells by CCK-8 assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. B. Left panel: MEX3A knockdown promoted proliferation of C33A cells, and MEX3A overexpression suppressed proliferation of SiHa cells by BrdU assay. Scale bar: 50 μm . Right panel: Quantitative results were also performed. C. MEX3A knockdown decreased the percentage of G0/G1 phase but increased cells at S phase in C33A cells, while MEX3A overexpression showed the opposite results in SiHa cells by detection of flow cytometry.

MEX3A targets Akt in cervical cancer

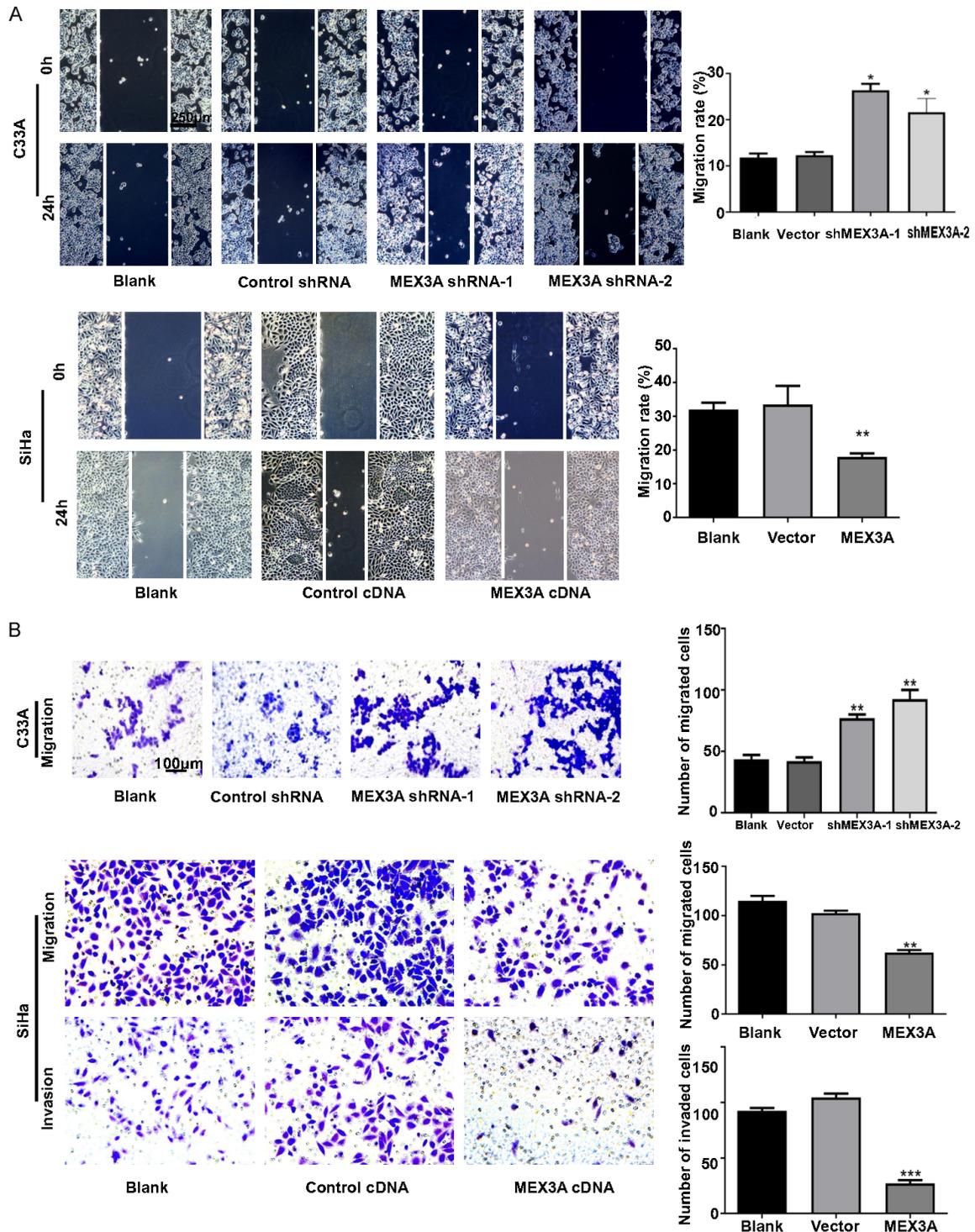


Figure 3. MEX3A inhibits the migration and invasion of cervical cancer cells. A. Left panel: Cell migration ability was detected by wound healing assay in C33A and SiHa cells after transfection with MEX3A shRNA or MEX3A cDNA. Right panel: Quantitative data was shown for left panel. B. Transwell assays were performed in cells with MEX3A downregulation or upregulation. Right panel: Quantitative analysis for left panel. ** $P < 0.01$, *** $P < 0.001$.

MEX3A repressed migration of SiHa cells (Figure 3B). Furthermore, we observed that MEX3A overexpression suppressed cell inva-

sive ability in SiHa cells (Figure 3B). Our results indicated that MEX3A regulates cell migration and invasion in cervical cancer.

MEX3A overexpression inhibits EMT via regulation of Akt

One study showed that MEX3A could influence EMT in pancreatic cancer cells [15]. Therefore, we determined whether MEX3A plays a role in regulation of EMT in cervical cancer cells. We observed that C33A cells displayed epithelioid features, while C33A cells with downregulation of MEX3A exhibited a spindle-like shape (**Figure 4A**). SiHa cells displayed mesenchymal phenotype, whereas overexpression of MEX3A led to round shape of SiHa cells (**Figure 4A**). Moreover, we measured the expression of EMT markers, including E-cadherin, Vimentin, β -catenin and Snail, in cells with MEX3A knock-down or overexpression. Western blotting analysis data uncovered that MEX3A knockdown elevated the expression of Vimentin, β -catenin and Snail, but reduced E-cadherin expression in C33A cells (**Figure 4B-D**). Consistently, the expression of E-cadherin was elevated in SiHa cells after MEX3A overexpression, while Vimentin, β -catenin and Snail expressions were decreased in MEX3A-overexpressing SiHa cells (**Figure 4B-D**). Several studies have revealed that MEX3A participated in tumorigenesis via regulating Akt signaling pathway [15-17]. Next, we measured whether Akt signaling pathway is changed in cervical cancer cells after MEX3A modulation. We found that downregulation of MEX3A activated p-Akt in C33A cells, while upregulation of MEX3A inhibits p-Akt expression level in SiHa cells (**Figure 4B-D**). To further validate the association between MEX3A and p-Akt in cervical cancer, we measured the expression of MEX3A and p-Akt in cervical cancer tissues. The expression of MEX3A protein was downregulated in cervical cancer tissues compared with their paired normal tissues (**Supplementary Figure 2**). Moreover, MEX3A expression was negatively associated with p-Akt expression in cervical cancer tissues (**Supplementary Figure 2**). Therefore, MEX3A regulated Akt signaling pathway in cervical cancer.

Activation of Akt signaling pathway reverses MEX3A-mediated inhibition of motility

In order to further validate role of Akt pathway in MEX3A-mediated suppression of motility, we performed a reversal experiment via upregulation of Akt pathway by its activator SC79 in

SiHa cells. The results from wound healing and Transwell assays demonstrated that SC79 treatments increased migration and invasion of SiHa cells (**Figure 5A** and **5B**). Importantly, activation of Akt pathway by SC79 exposures abolished MEX3A-induced suppression of migration and invasion in SiHa cells (**Figure 5A** and **5B**). Therefore, Akt signaling pathway participated in MEX3A-triggered repression of motility in cervical cancer cells.

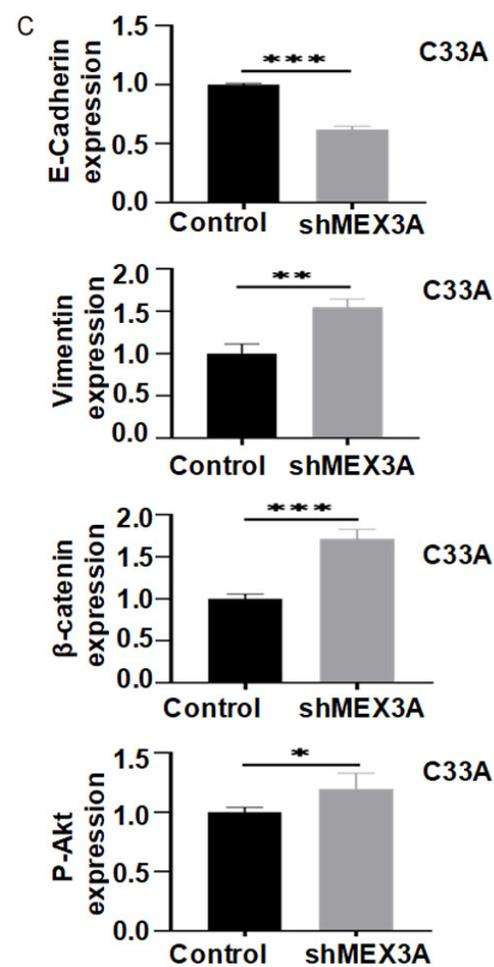
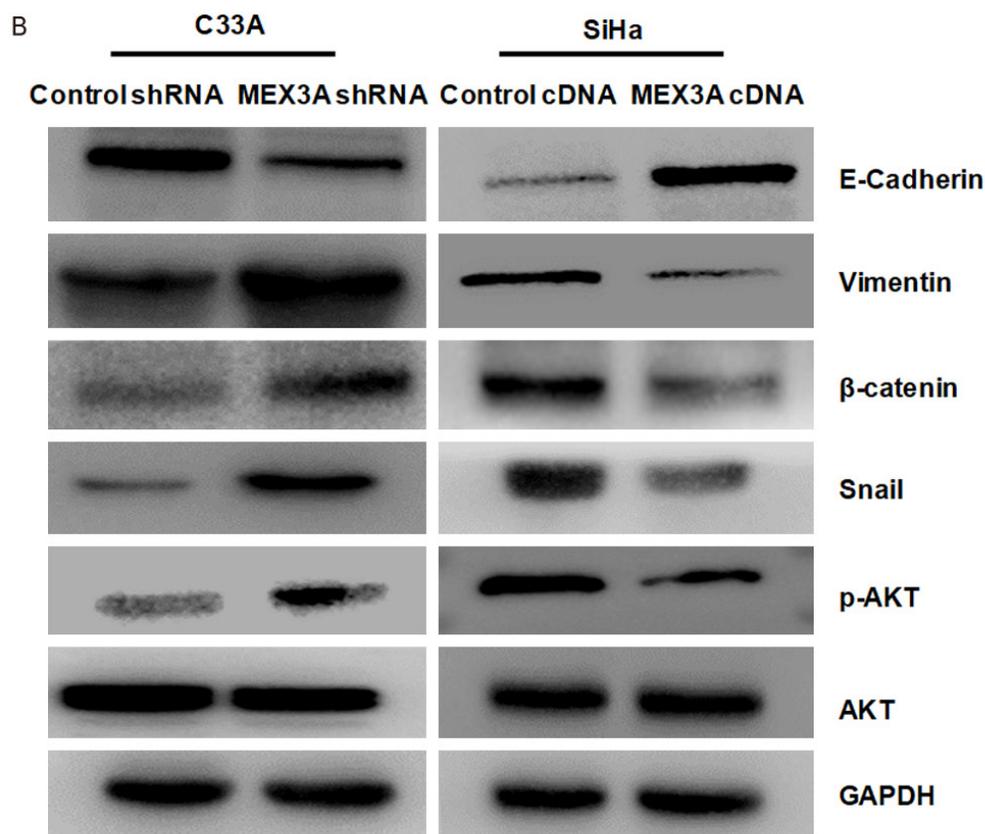
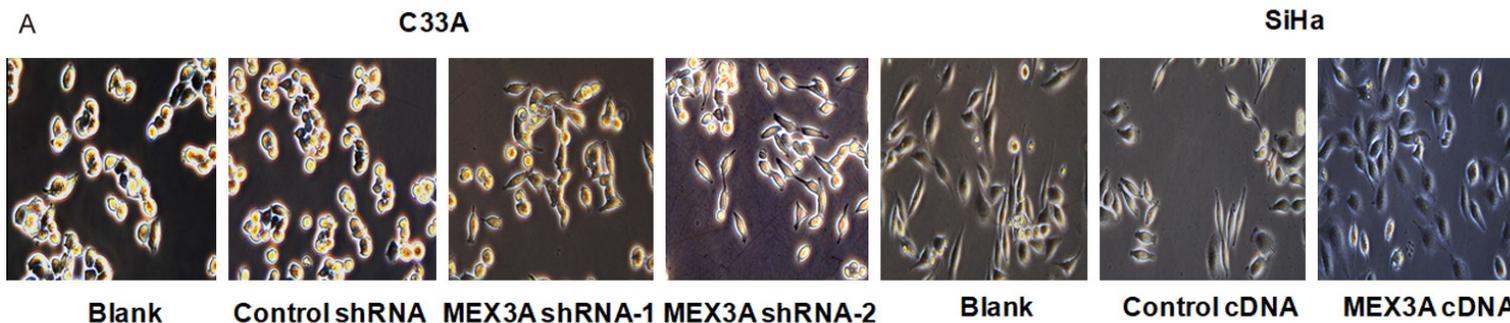
Activation of Akt signaling pathway abolishes MEX3A-mediated inhibition of viability and proliferation

We also detected whether activation of Akt signaling pathway is associated with MEX3A-mediated inhibition of viability in SiHa cells. Our CCK-8 assay data showed that 10 μ M SC79 treatments enhanced viability of SiHa cells (**Figure 5C**). Moreover, activation of Akt pathway by SC79 exposures rescued the suppression of viability in MEX3A-overexpressing SiHa cells (**Figure 5C**). In keeping with this finding, our data from BrdU assays revealed that SC79 treatments increased proliferation of SiHa cells and abrogated MEX3A-involved suppression of proliferation (**Figure 5D**). These results suggested that MEX3A inhibits cell viability and proliferation via inactivation of Akt pathway in cervical cancer cells.

MEX3A overexpression suppresses tumor growth in nude mice

To test the anticancer activity of MEX3A *in vivo*, the nude mouse model was established by subcutaneous injection with tumor cells. The tumor xenograft models were obtained via injection of C33A cells with MEX3A knockdown and MEX3A-overexpressing SiHa cells. The tumor xenografts from nude mice were collected at 35 day after injection, and the weight and volume of tumors were measured as displayed in **Figure 6**. As shown in **Figure 6A-C**, tumor weights and volumes were increased in MEX3A-shRNA transfection group. Conversely, a dramatically decrease of tumor weights and volumes in MEX3A-overexpressing group was observed in comparison with its matching group (**Figure 6A-C**). In addition, our IHC data showed that β -catenin expression was obviously upregulated whereas E-cadherin expression was downregulated after MEX3A knock-down in C33A cells (**Figure 6D**). Consistent with

MEX3A targets Akt in cervical cancer



MEX3A targets Akt in cervical cancer

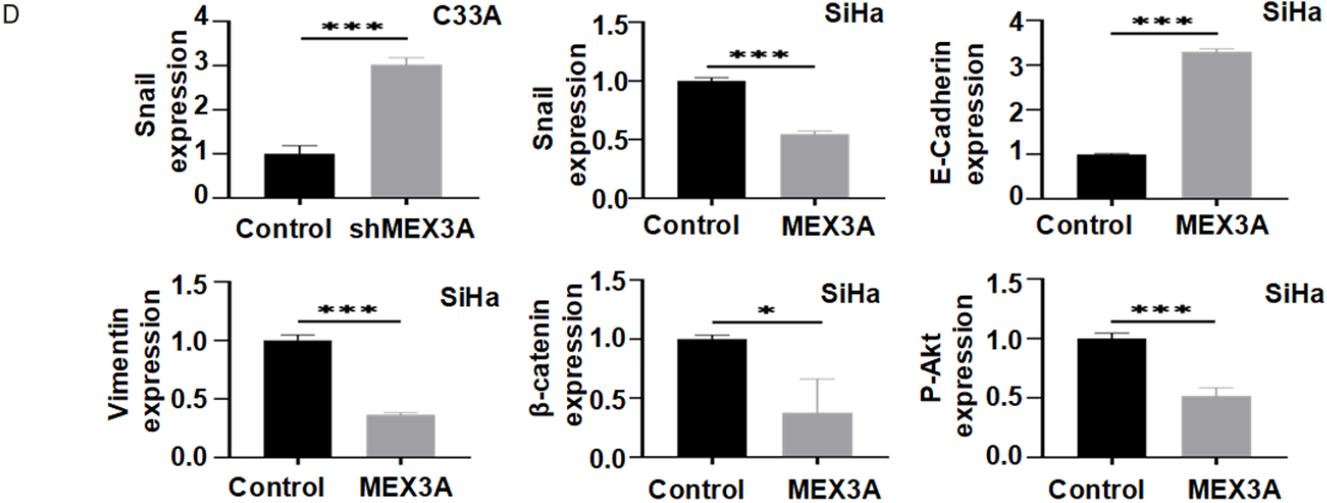


Figure 4. MEX3A modulates EMT and pAkt in cervical cancer cells. (A) Bright-field microscopy images showing phenotypical changes in cervical cancer cells after MEX3A knockdown or overexpression. (B) Western blotting analysis of Akt pathway and EMT-related marker expressions after MEX3A modulation. (C, D) Quantitative results for (B). *P < 0.05, **P < 0.01, ***P < 0.001.

MEX3A targets Akt in cervical cancer

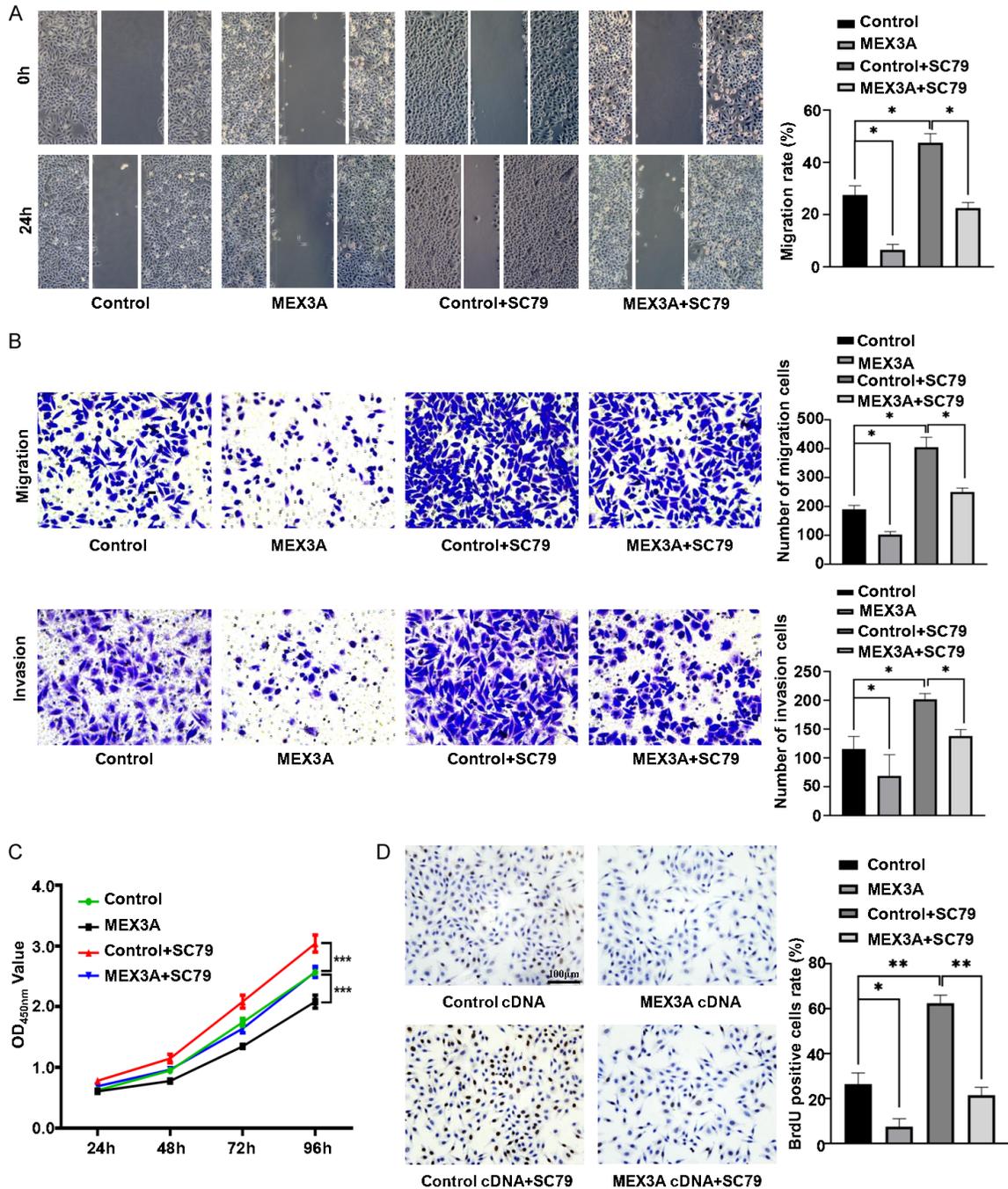


Figure 5. MEX3A modulates EMT by activating Akt signaling pathway in SiHa cells. A. Left panel: Wound healing assay showed that SC79 treatment rescued MEX3A-induced suppression of migration SiHa cells. Right panel: Quantitative analysis for left panel. B. Left panel: Transwell assay indicated the suppression of motility ability by MEX3A overexpression was rescued after SC79 treatment. Right panel: Quantitative analysis for left panel. C. CCK8 assay data showed that SC79 treatment rescued the inhibition of viability by MEX3A overexpression. D. BrdU assay indicated that SC79 treatment rescued the suppression of proliferation by MEX3A overexpression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

this result, the expression of β -catenin was decreased while E-cadherin was increased as a result of MEX3A overexpression in SiHa

cells (Figure 6E). These results indicated that MEX3A expression inhibited tumor growth and EMT in vivo.

MEX3A targets Akt in cervical cancer

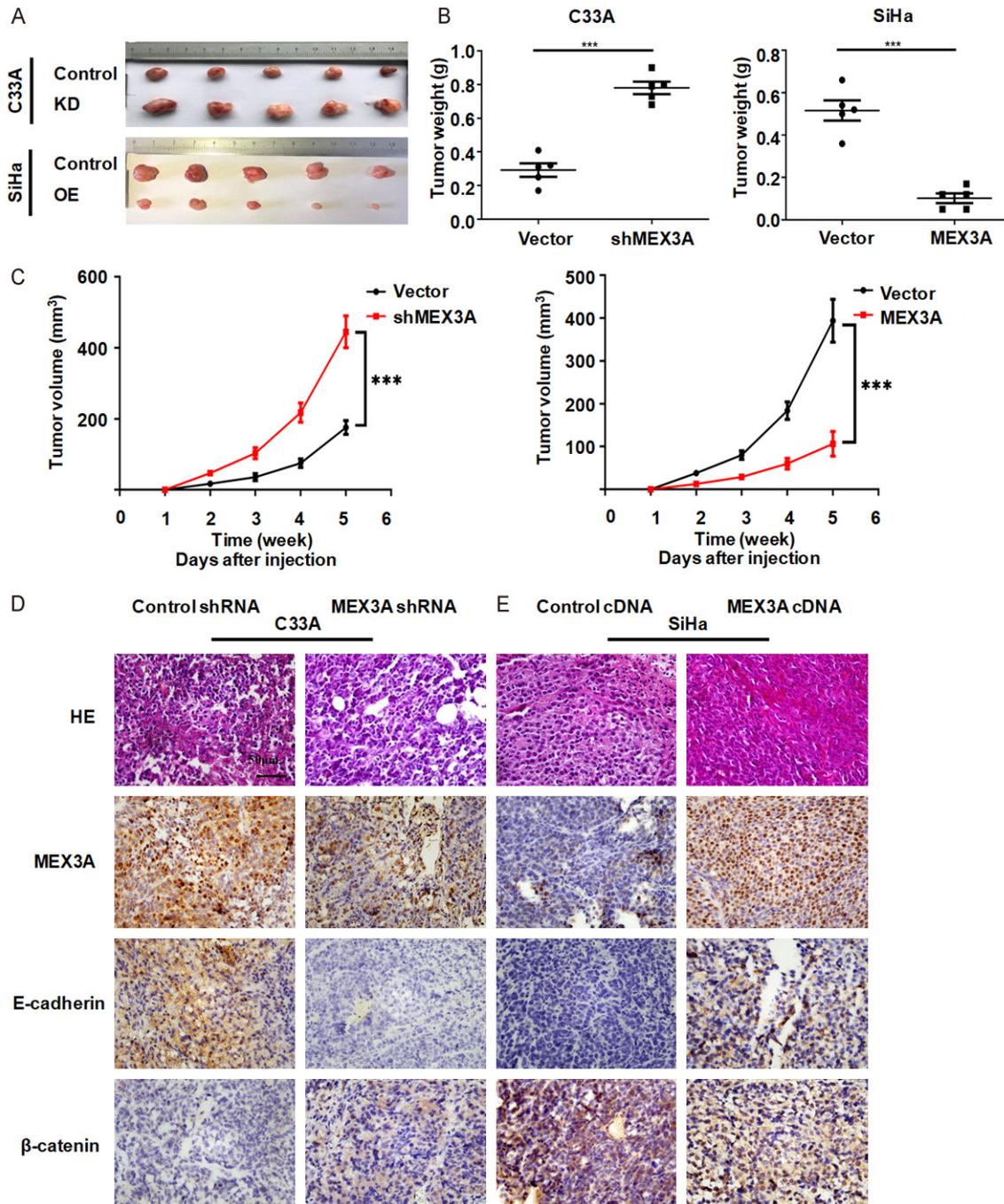


Figure 6. MEX3A suppresses tumor growth in vivo. (A) The picture of xenografts was obtained at 35 day after injection of transfected C33A and SiHa cells in nude mice. (B) Tumors were collected and each tumor weight was measured. (C) The tumor volume was assessed and calculated according to the formula $(L \times W^2)/2$. The data were displayed as mean \pm SD. (D, E) The representative pictures for H&E staining (top) and immunohistochemical staining of E-cadherin, β -catenin of C33A (D) and SiHa (E) subcutaneous tumor samples from the different groups. Scale bar: 50 μ m. *** $P < 0.001$.

Discussion

Evidence has unveiled that MEX3A has a crucial role in tumorigenesis and malignant pro-

gression. For example, MEX3A expression was highly upregulated in Wilms renal cancer, bladder cancer, gastric cancer, pancreatic cancer, glioblastoma, breast cancer, and liver cancer

tissues [12, 15, 16, 18-22]. In liver cancer patients, survival analysis revealed that MEX3A expression was obviously correlated with poor OS and PFS, suggesting that MEX3A might be an independent prognosis biomarker in liver cancer [20]. In addition, the higher expression of MEX3A was observed in pancreatic cancer patients and associated with tumor grade [15, 23]. Similarly, overexpression of MEX3A was associated with malignancy and poor prognosis in triple negative breast cancer (TNBC) patients [16]. The high expression of MEX3A was associated with the mortality of breast cancer patients [22]. Consistently, increased expression of MEX3A was correlated with poor prognosis in lung adenocarcinoma [17]. In bladder urothelial carcinoma patients, the expression of MEX3A was not correlated with overall survival rate [24]. In the current work, we displayed that MEX3A was downregulated in cervical cancer tissues and was negatively correlated with advanced clinical stage. Our findings indicated that the role of MEX3A may be different in various types of human cancers.

One study reported that MEX3A knockdown inhibited cell viability and colony formation ability and modulated cell cycle in gastric cancer cells [12]. In bladder cancer cells, knockdown of MEX3A remarkably inhibited cell proliferation and promoted apoptosis [19]. MEX3A upregulation enhanced cell growth in glioblastoma cells [21]. Downregulation of MEX3A inhibited cell growth and induced apoptosis and cell cycle arrest in pancreatic cancer cells [15, 23]. MEX3A knockdown repressed cell growth, while overexpression of MEX3A elevated growth of TNBC cells [16]. These studies suggested that MEX3A exerted promotion of cell proliferation in several types of human cancers. Our data demonstrated that MEX3A overexpression suppressed viability and proliferation of cervical cancer cells and caused G0/G1 phase arrest, while MEX3A downregulation exhibited the opposite effects, demonstrating that MEX3A has an anti-proliferation function in cervical cancer. MEX3A has been characterized to regulate the cell motility in multiple types of human cancers, including TNBC, gastric cancer, and lung cancer [12, 16, 17]. MEX3A downregulation inhibited migration of pancreatic cancer cells via influencing EMT [15]. MEX3A knockdown suppressed metastasis in lung cancer cells and in nude mice [17].

Here, we reported that MEX3A overexpression suppressed migration and invasion of cervical cancer cells. Notably, MEX3A overexpression inhibited EMT and modulated the expression of EMT molecular biomarkers in cervical cancer cells.

Mechanistically, MEX3A knockdown exerted its anticancer activity via suppression of p-Akt, PIK3CA, CDK6 and MAPK9 in pancreatic cancer [15]. MEX3A promoted migratory and invasive ability and metastasis in TNBC cells via downregulation of laminin subunit alpha 2 (LAMA2) expression [17]. MEX3A overexpression promoted ubiquitylation and degradation of RIG-1 in glioblastoma cells, resulting in cell growth promotion [21]. Elevated expression of p21 and p57 was identified in cells with high MEX3A expression [11]. The Akt pathway is one of the key signaling pathways involved in tumor growth and metastasis [25]. In cervical cancer, Akt pathway is dysregulated and has been identified to be a prospective target for cervical cancer therapy [26]. In breast cancer cells, MEX3A has been discovered to modulate PI3K/Akt signaling pathway [16, 17]. We also found that Akt signaling pathway was activated by MEX3A knockdown in cervical cancer cells. Numerous investigations have revealed that SC79, an activator of Akt pathway, could trigger Akt phosphorylation, cell proliferation and motility ability [27, 28]. Consistently, we observed that SC79 treatment restored cells proliferation and motility in MEX3A-overexpressing cells. These findings indicated that Akt pathway might be a key driver in MEX3A-mediated tumorigenesis.

Our results demonstrated the tumor suppressive role of MEX3A in cervical cancer cells via targeting Akt signaling pathway (**Figure 7**). However, there are multiple limitations in our current study. It is necessary to enlarge the numbers of cervical cancer patients to detect the expression of MEX3A in tissues and define its association with clinicopathological feature. The detailed mechanisms how MEX3A modulates Akt activation in cervical cancer need to be clarified. MEX3A depletion enhanced gemcitabine sensitivity in pancreatic cancer cells, indicating that MEX3A is involved in drug resistance in human cancer [23]. It is required to determine whether MEX3A modulates drug sensitivity in cervical cancer cells. Moreover, conditional MEX3A transgenic or knockout mouse models are ideal tools for exploration of

MEX3A targets Akt in cervical cancer

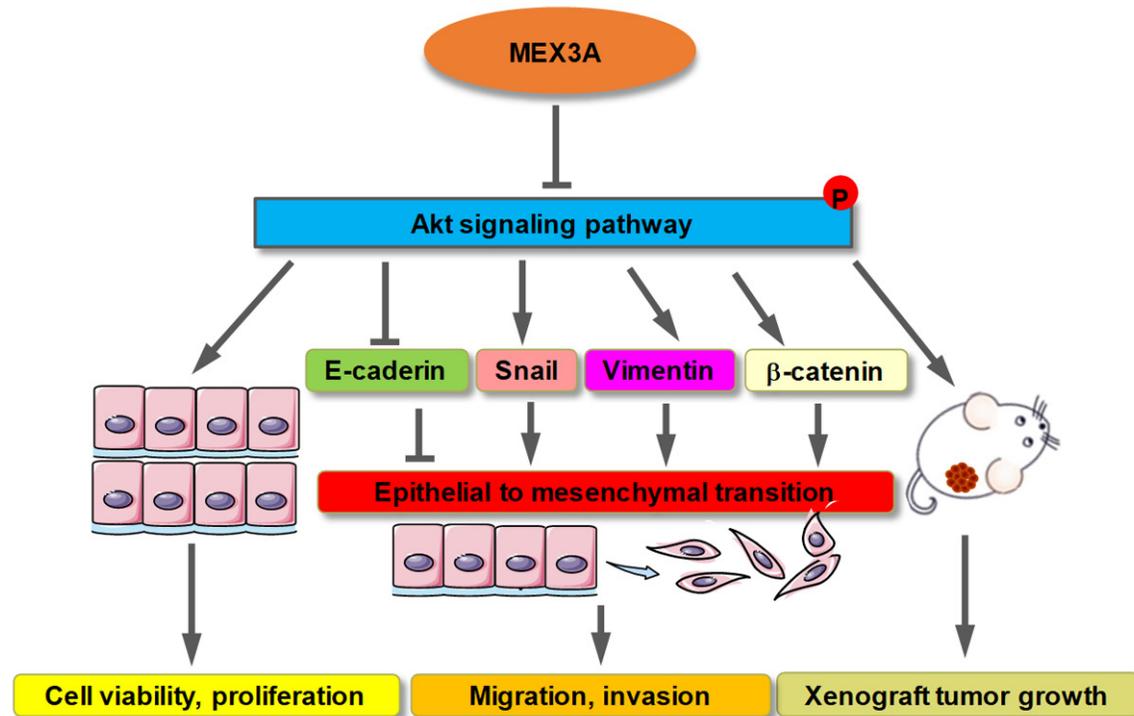


Figure 7. A schematic illustration shows how MEX3A suppresses cell viability, migration and invasion by targeting Akt signaling pathway in cervical cancer.

MEX3A functions in cervical cancer in the future.

Acknowledgements

This work was supported by grants from Exploration project from Natural Science Foundation of Zhejiang Province (No. LY21H160060). The sponsor had no involvement in the collection, analysis, and interpretation of data or in the writing of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Xueqiong Zhu and Zhiwei Wang, Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Wenzhou Medical University, No. 109 Xueyuan Xi Road, Wenzhou 325027, Zhejiang, People's Republic of China. Tel: +86-577-88002796; Fax: +86-577-88002796; E-mail: zjwzzxq@163.com (XQZ); zhiwei-china@126.com (ZWW)

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics

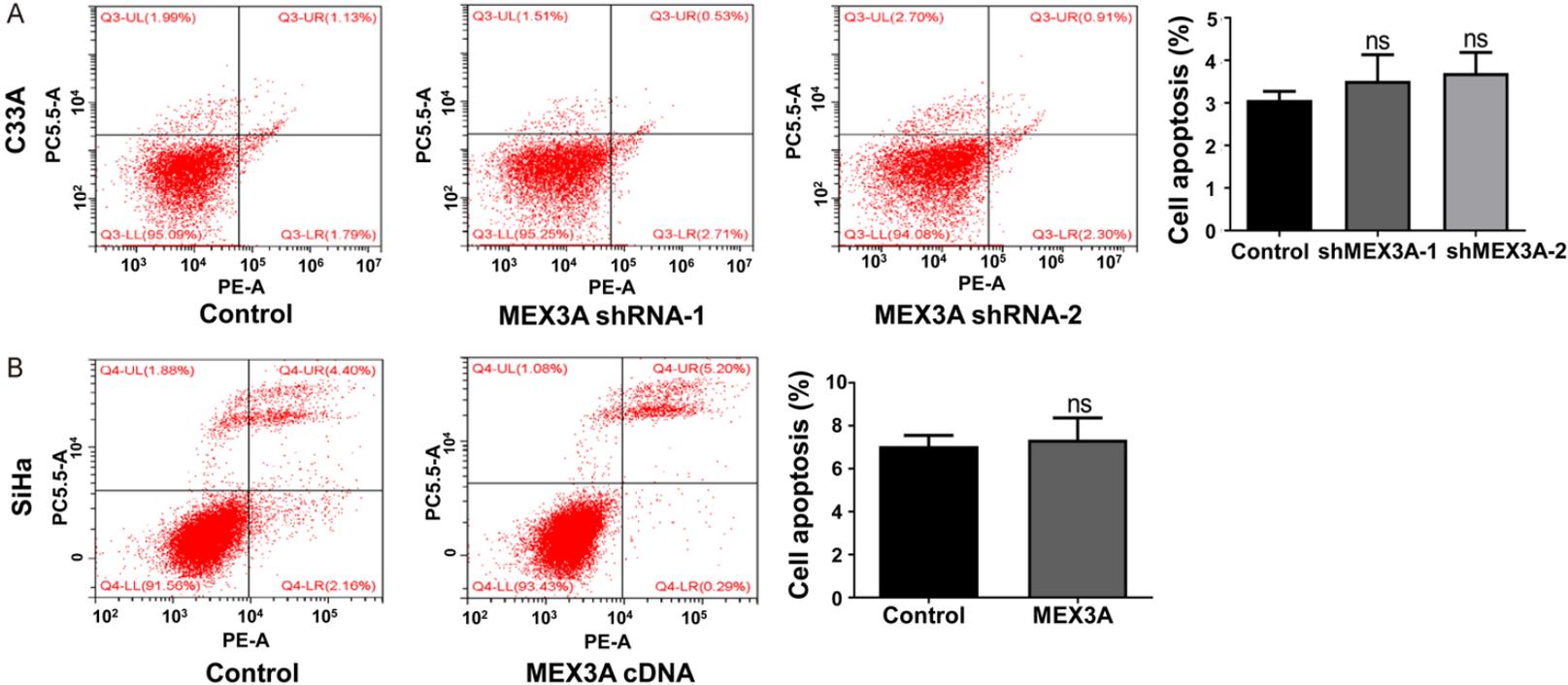
2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394-424.

- [2] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; 70: 7-30.
- [3] Eun TJ and Perkins RB. Screening for cervical cancer. *Med Clin North Am* 2020; 104: 1063-1078.
- [4] Gadducci A and Cosio S. Neoadjuvant chemotherapy in locally advanced cervical cancer: review of the literature and perspectives of clinical research. *Anticancer Res* 2020; 40: 4819-4828.
- [5] Gurram L, Kalra B and Mahantshetty U. Meeting the global need for radiation therapy in cervical cancer-an overview. *Semin Radiat Oncol* 2020; 30: 348-354.
- [6] Small W Jr, Bacon MA, Bajaj A, Chuang LT, Fisher BJ, Harkenrider MM, Jhingran A, Kitchener HC, Mileskin LR, Viswanathan AN and Gaffney DK. Cervical cancer: a global health crisis. *Cancer* 2017; 123: 2404-2412.
- [7] Zhu H, Luo H, Zhang W, Shen Z, Hu X and Zhu X. Molecular mechanisms of cisplatin resistance in cervical cancer. *Drug Des Devel Ther* 2016; 10: 1885-1895.
- [8] Buchet-Poyau K, Courchet J, Le Hir H, Seraphin B, Scoazec JY, Duret L, Domon-Dell C, Freund JN and Billaud M. Identification and character-

MEX3A targets Akt in cervical cancer

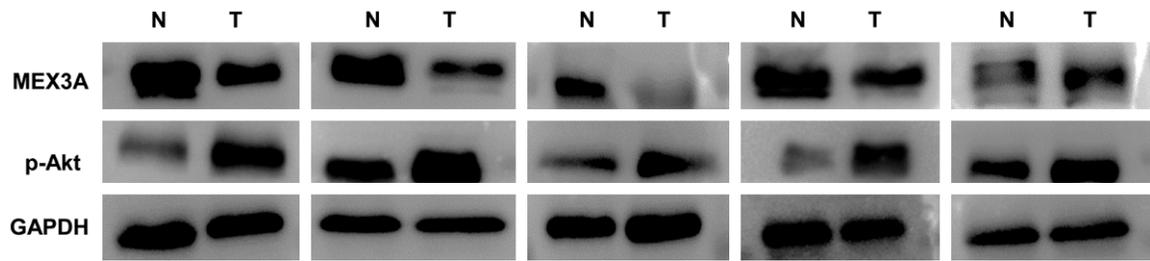
- ization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Res* 2007; 35: 1289-1300.
- [9] Pereira B, Le Borgne M, Chartier NT, Billaud M and Almeida R. MEX-3 proteins: recent insights on novel post-transcriptional regulators. *Trends Biochem Sci* 2013; 38: 477-479.
- [10] Santovito D, Egea V, Bidzhekov K, Ntarelli L, Mourao A, Blanchet X, Wichapong K, Aslani M, Brunssen C, Horckmans M, Hristov M, Geerlof A, Lutgens E, Daemen M, Hackeng T, Ries C, Chavakis T, Morawietz H, Naumann R, Hundelshausen PV, Steffens S, Duchene J, Megens RTA, Sattler M and Weber C. Autophagy unleashes noncanonical microRNA functions. *Autophagy* 2020; 16: 2294-2296.
- [11] Barriga FM, Montagni E, Mana M, Mendez-Lago M, Hernando-Mombona X, Sevillano M, Guillaumet-Adkins A, Rodriguez-Esteban G, Buczacki SJA, Gut M, Heyn H, Winton DJ, Yilmaz OH, Attolini CS, Gut I and Batlle E. Mex3a marks a slowly dividing subpopulation of lgr5+ intestinal stem cells. *Cell Stem Cell* 2017; 20: 801-816, e807.
- [12] Jiang H, Zhang X, Luo J, Dong C, Xue J, Wei W, Chen J, Zhou J, Gao Y and Yang C. Knockdown of hMex-3A by small RNA interference suppresses cell proliferation and migration in human gastric cancer cells. *Mol Med Rep* 2012; 6: 575-580.
- [13] Pereira B, Sousa S, Barros R, Carreto L, Oliveira P, Oliveira C, Chartier NT, Plateroti M, Rouault JP, Freund JN, Billaud M and Almeida R. CDX2 regulation by the RNA-binding protein MEX3A: impact on intestinal differentiation and stemness. *Nucleic Acids Res* 2013; 41: 3986-3999.
- [14] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 2001; 25: 402-408.
- [15] Wang X, Shan YQ, Tan QQ, Tan CL, Zhang H, Liu JH, Ke NW, Chen YH and Liu XB. MEX3A knockdown inhibits the development of pancreatic ductal adenocarcinoma. *Cancer Cell Int* 2020; 20: 63.
- [16] Jiang S, Meng L, Chen X, Liu H, Zhang J, Chen F, Zheng J, Liu H, Wang F, Hu J and Li Z. MEX3A promotes triple negative breast cancer proliferation and migration via the PI3K/AKT signaling pathway. *Exp Cell Res* 2020; 395: 112191.
- [17] Liang J, Li H, Han J, Jiang J, Wang J, Li Y, Feng Z, Zhao R, Sun Z, Lv B and Tian H. Mex3a interacts with LAMA2 to promote lung adenocarcinoma metastasis via PI3K/AKT pathway. *Cell Death Dis* 2020; 11: 614.
- [18] Krepischi ACV, Maschietto M, Ferreira EN, Silva AG, Costa SS, da Cunha IW, Barros BDF, Grun- dy PE, Rosenberg C and Carraro DM. Genomic imbalances pinpoint potential oncogenes and tumor suppressors in Wilms tumors. *Mol Cyto- genet* 2016; 9: 20.
- [19] Huang Y, Fang C, Shi JW, Wen Y and Liu D. Identification of hMex-3A and its effect on hu- man bladder cancer cell proliferation. *Oncotar- get* 2017; 8: 61215-61225.
- [20] Yang D, Jiao Y, Li Y and Fang X. Clinical charac- teristics and prognostic value of MEX3A mRNA in liver cancer. *PeerJ* 2020; 8: e8252.
- [21] Bufalieri F, Caimano M, Lospinoso Severini L, Basili I, Paglia F, Sampirisi L, Loricchio E, Petro- ni M, Canettieri G, Santoro A, D'Angelo L, In- fante P and Di Marcotullio L. The RNA-binding ubiquitin ligase MEX3A Affects Glioblastoma Tumorigenesis by inducing ubiquitylation and degradation of RIG-I. *Cancers* 2020; 12: 321.
- [22] Ning S, Li H, Qiao K, Wang Q, Shen M, Kang Y, Yin Y, Liu J, Liu L, Hou S, Wang J, Xu S and Pang D. Identification of long-term survival-associat- ed gene in breast cancer. *Aging* 2020; 12: 20332-20349.
- [23] Panzeri V, Manni I, Capone A, Naro C, Sacconi A, Di Agostino S, de Latouliere L, Montori A, Pi- lozzi E, Piaggio G, Capurso G and Sette C. The RNA binding protein MEX3A is a prognostic fac- tor and regulator of resistance to gemcitabine in Pancreatic Ductal Adenocarcinoma. *Mol On- col* 2020; 15: 579-595.
- [24] Shi JW and Huang Y. Mex3a expression and survival analysis of bladder urothelial carcino- ma. *Oncotarget* 2017; 8: 54764-54774.
- [25] Hoxhaj G and Manning BD. The PI3K-AKT net- work at the interface of oncogenic signalling and cancer metabolism. *Nat Rev Cancer* 2020; 20: 74-88.
- [26] Bahrami A, Hasanzadeh M, Hassanian SM, ShahidSales S, Ghayour-Mobarhan M, Ferns GA and Avan A. The potential value of the PI3K/Akt/mTOR signaling pathway for assess- ing prognosis in cervical cancer and as a target for therapy. *J Cell Biochem* 2017; 118: 4163- 4169.
- [27] Li X, Chen W, Liu K, Zhang S, Yang R, Liu K, Li D and Huang Y. Oridonin sensitizes hepatocel- lular carcinoma to the anticancer effect of sorafenib by targeting the Akt pathway. *Cancer Manag Res* 2020; 12: 8081-8091.
- [28] Li X, Song Q, Guo X, Wang L, Zhang Q, Cao L, Ren Y, Wu X, Meng Z and Xu K. The metastasis potential promoting capacity of cancer-associ- ated fibroblasts was attenuated by cisplatin via modulating KRT8. *Onco Targets Ther* 2020; 13: 2711-2723.

MEX3A targets Akt in cervical cancer



Supplementary Figure 1. Flow cytometry result showed that MEX3A knockdown (A) or overexpression (B) did not affect apoptosis of c33a and SiHa cells.

MEX3A targets Akt in cervical cancer



Supplementary Figure 2. The expression of MEX3A protein was downregulated in cervical cancer tissues compared with their paired normal tissues. MEX3A expression was negatively associated with p-Akt expression in cervical cancer tissues. N: normal tissues. T: cervical cancer tissues.