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OLFM4 Inhibits Epithelial–Mesenchymal Transition and Metastatic Potential of Cervical Cancer Cells

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OLFM4 has been shown to play an important role in tumor initiation and progression. This study aims to investigate the role of OLFM4 in metastatic cervical cancer and its underlying mechanism. Here we discover that OLFM4 expression is significantly reduced in metastatic cervical cancer. Accordingly, overexpression of OLFM4 inhibits epithelial–mesenchymal transition (EMT), migration, and invasion in human cervical cancer cells. To further explore its molecular mechanisms, we reveal that OLFM4 augmentation interferes with mTOR signaling pathway, and the suppressive effects of OLFM4 on cell migration and invasion are largely weakened by phosphatidic acid (PA)-induced mTOR signal activation, which implicates the potential role of the mTOR pathway in OLFM4-related cervical metastasis. In conclusion, our results confirm OLFM4 as a tumor suppressor that inhibits cervical cancer metastasis by regulating mTOR signal pathway.

Key words: OLFM4; Epithelial–mesenchymal transition (EMT); Cervical cancer; mTOR; Metastasis

INTRODUCTION

Cervical cancer is the third most common cancer in the female reproductive system and leads to a high mortality in many developing countries[1]. According to the reports from the International Agency for Research on Cancer (IARC) in 2012, 61,776 out of 528,000 (11.7%) cases occurred in China with mortality rate ranking second[2]. Although several combined approaches (including radical surgery, radiotherapy, and chemotherapy) have been implemented to improve clinical outcomes, the treatment options for patients with advanced metastasis are limited and ineffective[3]. Thus, it is crucial to identify the molecular mechanisms of cervical cancer development and metastasis to develop more effective treatments for advanced cervical cancer patients in the future.

OLFM4 (olfactomedin 4), also known as hGC-1 (human granulocyte colony-stimulating factor-stimulated clone 1), is an olfactomedin-domain-containing protein that is proved to regulate important cellular processes such as cell growth and apoptosis. OLFMF4 has also been shown to play distinctive roles in regulating tumor initiation and progression depending on different cancerous tissues and tumor stages[4]. For instance, OLFM4 is upregulated in gastric cancer[5] and pancreatic cancer[6], but in advanced prostate cancer[7] and colorectal cancer[8] its expression is hindered. Besides, Yu et al. have reported that OLFM4 expression is increased with the progression of cervical neoplasia and decreased in poorly differentiated cervical cancers[9]. However, the correlation between OLFM4 expression and metastatic cervical cancer remains unclear. The potential biological implication of OLFM4 in cervical cancer metastasis remains to be elucidated.

In the present study, we first examined the expression of OLFM4 in cervical cancer tissues with metastasis

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and found that the expression of OLFM4 was significantly reduced in metastatic cancerous tissues compared to the adjacent noncancerous tissues. Then the role of OLFM4 in regulating epithelial–mesenchymal transition (EMT), migration, and invasion of cervical cancer cells was explored. Finally, we investigated the mechanisms of OLFM4 in regulating cervical cancer metastasis by enhancing OLFM4 expression. The results revealed that augmentation of OLFM4 in cervical cancer cells inhibits its cell migrative and invasive abilities by targeting the mTOR signaling pathway. Our findings not only illuminate the mechanism of cervical cancer metastasis but also identify OLFM4 as a molecular marker for advanced cervical cancer treatment and prognosis.

MATERIALS AND METHODS

Cell Culture and Phosphatidic Acid (PA) Treatment

Human cervical cancer cells CaSki and HeLa were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The CaSki cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin. The HeLa cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin. The cells were maintained in humidified incubator at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Cells were treated with PA (Taize Inc., Beijing, P.R. China) at a final concentration of 100 μM.

Patients

We obtained primary tumors with metastasis and adjacent normal tissues from 27 patients who underwent radical operation of cervical carcinoma at Jinan Women and Children's Health Hospital (Jinan, P.R. China) in 2015–2016. Tissue samples were stored at −196°C in liquid nitrogen freezers. Pathologic diagnosis of all the patients was verified by pathologists in Jinan Women and Children's Health Hospital. Signed informed consent was obtained from all patients, and the study was confirmed by the Institutional Review Board at Shandong University.

Plasmids and Transfection

The full-length coding sequence of OLFM4 was cloned into pcDNA3.1 vector (Clontech, Palo Alto, CA, USA). X-tremeGENE HP DNA Transfection reagent (Roche, Indianapolis, IN, USA) was used to transfect the plasmids into indicated cells. The transfection procedures were based on the manufacturer's protocol.

Reverse Transcription and qRT-PCR

Total RNA was extracted from the tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. First-strand cDNA synthesis was performed on 1 μg of RNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit and random hexamer primer. Quantitative real-time polymerase chain reaction (qRT-PCR) was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl on a 7900 Real-Time PCR System (Applied Biosystems). The sequences of the primer pairs are as follows: OLFM4 forward primer 5′-CTGCCAGACACCACCTTTCC-3′ and reverse primer 5′-CTCGAAGTCCAGTTCAGTGTAAG-3′; GAPDH forward primer 5′-GCCGCATCTTCTTTTGCGTCGC-3′ and reverse primer 5′-TCCCGTTCTCAGCCTTGACGGT-3′. The expression level of target mRNA was calculated by the Ct method and normalized by human GAPDH expression level. We performed all assays in triplicate and presented the data as mean ± SD.

Western Blot and Antibodies

Cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 10 mM NaF) supplemented with protease inhibitors (Roche). The whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were probed with specific primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies. Bound proteins were visualized with ECL (Millipore) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). Antibodies against OLFM4, E-cadherin, vimentin, and β-actin were from Cell Signaling Technology (Danvers, MA, USA).

Wound Healing Assay

Cells were seeded and cultured overnight in a six-well plate, and the monolayer cells were wounded by scratching with sterile plastic 200-μl micropipette tips. Cells were washed three times with PBS and cultured for 24 h in serum-free medium. Images were taken by phase-contrast microscopy at 0 h and 24 h after wounding. The assays were independently performed in triplicate.

Migration and Invasion Assay

Cell migration and invasion assays were conducted using Transwell migration chambers (8-μm pore size; Millipore) according to the manufacturer's instructions. The cells were suspended and seeded into the upper chambers with or without Matrigel in serum-free medium. The lower chambers were filled with 600 μl of complete growth medium. The cells were incubated at 37°C in a 24-well plate for 48 h. The unmigrated cells on the upper surface of the membrane were removed using cotton swabs, and the cells attached to the lower chamber membrane were stained with crystal violet (0.1%, w/v in 20 nM

4-morpholinepropanesulfonic acid) for 20 min. For each experiment, five fields were observed for counting cell numbers, and three independent assays were repeated.

Immunofluorescent Staining

Cells were seeded on culture slides (Costar, Manassas, VA, USA) and cultured overnight. The cells were fixed with 4% paraformaldehyde in PBS and permeabilized using 0.5% Triton X-100. After the cells were blocked for 30 min in 10% BSA (Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated with primary antibodies and further incubated with FITC-conjugated secondary antibodies (Invitrogen). Following three additional washes, the slides were stained with 4-,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min for nuclei visualization and then examined using an Olympus BX51 microscope.

MTT Assay

Cells were plated in 96-well plates at about 3,000 cells per well in sextuplicate 24 h after transfection. Cell proliferation was monitored at desired time using MTT (Promega, Madison, WI, USA). In brief, the MTT assay was performed by adding 20 μl of MTT (5 mg/ml) for 4 h. Then the results were quantitated spectrophotometrically at 570 nm. The experiments were repeated three times.

Soft Agar Assay

The soft agar assay was performed to investigate the anchorage-independent growth of the test cells. The cells (1×10^3) were suspended in 1 ml of culture medium containing 0.35% agar (USB Corporation, Cleveland, OH, USA) and seeded onto a base layer of 1 ml of 0.75% agar bed in six-well plates. Visible colonies were counted after 3 weeks. The experiments were repeated three times in triplicate.

Statistical Analysis

Data were described as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA, USA) and SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences between groups were assessed using Student's *t*-test, and a value of $p < 0.05$ was considered statistically significant.

RESULTS

OLFM4 Expression Is Downregulated in Human Metastatic Cervical Cancer Tissues

To investigate the clinical significance of OLFM4 in advanced cervical cancer, qRT-PCR and Western blot were performed to detect the expression of OLFM4 in human metastatic cervical cancer tissues and the adjacent noncancerous tissues. qRT-PCR analysis showed that OLFM4 mRNA levels were lower in 13 of 18 (72%) metastatic cervical cancer tissues compared to the corresponding adjacent noncancerous tissues (Fig. 1A). The mean value of OLFM4 mRNA levels in the cancer tissues was significantly lower than that in the normal tissues ($p < 0.01$) (Fig. 1B). Consistent with the qRT-PCR results, Western blot analysis showed the protein levels of OLFM4 in seven of nine (77.8%) metastatic cervical cancer tissues were also evidently decreased in comparison with the corresponding adjacent noncancerous tissues (Fig. 1C). The mean value of OLFM4 protein levels in the cancer tissues was significantly lower than that in the normal tissues ($p < 0.05$) (Fig. 1D). These results demonstrate that OLFM4 expression is downregulated at both mRNA and protein levels in human metastatic cervical cancer tissues.

OLFM4 Represses EMT in Cervical Cancer Cells

To further understand the biological roles of OLFM4 in cervical cancer progression, we transfected OLFM4 expression plasmids into HeLa and CaSki cells. Western blot was performed to confirm the expression of OLFM4 in these cells (Fig. 2A). Accompanied with the ectopic expression of OLFM4, HeLa cells lost mesenchymal characteristics to some extent and exhibited epithelial morphology, compared to the control cells (Fig. 2B). Consistent with this observation, Western blot analysis of molecular markers associated with epithelial and mesenchymal phenotypes revealed gains of epithelial marker (E-cadherin) and losses of mesenchymal marker (vimentin) in OLFM4 overexpression cell lines (Fig. 2C). Influence of OLFM4 on EMT was further confirmed by immunofluorescence staining (Fig. 2D and E). These results clearly indicate that overexpression of OLFM4 in cervical cancer cells suppresses EMT.

OLFM4 Inhibits Migration and Invasion of Cervical Cancer Cells

EMT properties are essential for tumor cells to migrate from adjacent tissues and seed new tumors in distant sites. We next examined whether OLFM4 could regulate the migratory and invasive capacities of cervical cancer cells. The effect of OLFM4 on cell migration was first examined by wound healing assay. The result showed that ectopic expression of OLFM4 markedly restrained migration of the HeLa and CaSki cells compared with their corresponding control cells (Fig. 3A and C). This result was further confirmed by Boyden's chamber assay (Fig. 3B and D). Moreover, HeLa and CaSki cells transfected with OLFM4 expression plasmids showed impaired invasive abilities in Matrigel invasion assay (Fig. 3B and D). Taken together, these results indicate that overexpression of OLFM4 inhibits migratory and invasive capacity of cervical cancer cells.

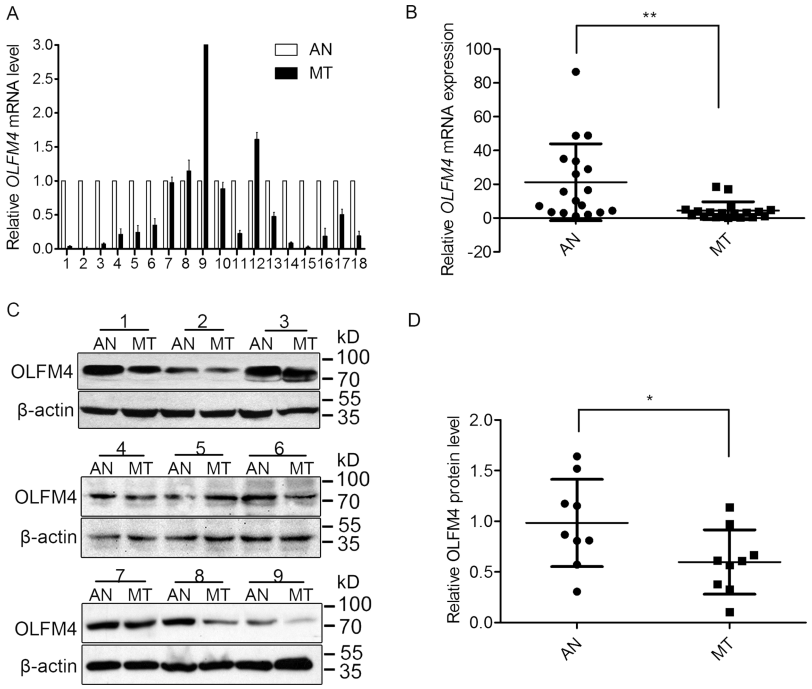

Figure 1. Olfactomedin 4 (OLFM4) expression is decreased in human metastatic cervical cancer tissues. (A) The mRNA of OLFM4 was detected by quantitative real-time polymerase chain reaction (qRT-PCR) in human metastatic cervical cancer tissues and the adjacent noncancerous tissues. (B) OLFM4 mRNA expression levels in human metastatic cervical cancer tissues and the adjacent noncancerous tissues ($n = 18$) are shown as scatter diagram. Data are mean ± SD. (C) The protein of OLFM4 was detected by Western blot in human metastatic cervical cancer tissues and the adjacent noncancerous tissues. (D) Relative OLFM4 protein expression levels in human metastatic cervical cancer tissues and the adjacent noncancerous tissues ($n = 9$) are shown as scatter diagram. Data are mean ± SD. AN, tumor adjacent nontumorous tissue; MT, metastatic cervical cancer tissue. $^*p < 0.05$, $^{**}p < 0.01$ based on Student's *t*-test.

OLFM4 Inhibits Growth of Cervical Cancer Cells

We then investigated the effect of OLFM4 on cell growth by MTT assay. Forced expression of OLFM4 in both HeLa and CaSki cells led to a significant decrease in cell proliferation compared with their respective controls (Fig. 4A). Further, we examined whether OLFM4 overexpression influenced anchorage-independent cell growth by soft agar colony formation assay. Numbers of colonies formed by HeLa transfected with OLFM4 expression plasmids were significantly lower than those transfected with vector (Fig. 4B). These data indicate that OLFM4 can inhibit proliferation of cervical cancer cells.

OLFM4 Inhibits mTOR Signal Pathway and Activation of mTOR Signaling Recovers the Migratory and Invasive Capacity of Cervical Cancer Cells

As the mTOR activity has been reported to participate in the regulation of cancer cell mobility and metastasis[10–12], the influence of OLFM4 on mTOR signaling was subsequently investigated. Western blot analysis revealed that phosphorylation of mTOR was significantly decreased by the overexpression of OLFM4 both in HeLa and CaSki cells, while the total level of mTOR remained unchanged (Fig. 5A). This result indicates the involvement of OLFM4 in regulating mTOR activity in cervical cancer cells. Furthermore, to determine whether the suppressive role of OLFM4 on cell mobility could be abrogated by aberrant mTOR signal activation, HeLa and CaSki cells transfected with OLFM4 expression plasmids were treated with mTOR activator (PA), simultaneously. Then Boyden chamber assay and Matrigel chamber assay were performed. As expected, PA treatment dramatically promoted the migratory and invasive capacity of HeLa and CaSki cells transfected with OLFM4 plasmid (Fig. 5B and C). These results illustrate that OLFM4 could inhibit migration and invasion via interfering mTOR pathway in cervical cancer cells.

DISCUSSION

The expression pattern of OLFM4 has been widely investigated in many types of human tumors, especially in gastrointestinal tumors[13–15]. It is believed to play a crucial role in tumor development and progression by regulating diverse cellular processes, including cell

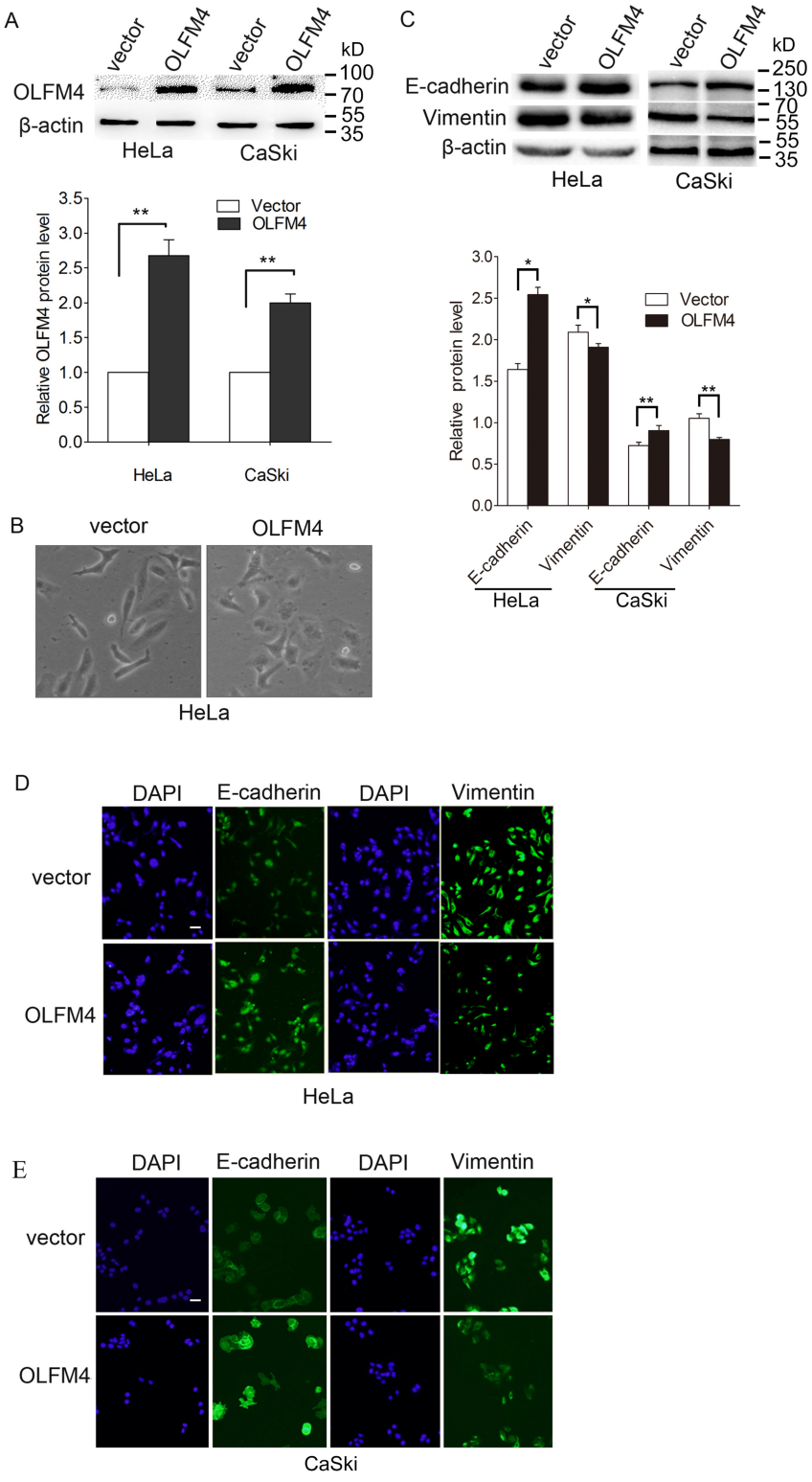

Figure 2. OLFM4 represses epithelial–mesenchymal transition (EMT) in cervical cancer cells. (A) The protein of OLFM4 was verified by Western blot in HeLa and CaSki cells with ectopic OLFM4 expression. Data are mean ± SD. (B) Phase-contrast microscopic images of HeLa–OLFM4 cells. (C) Expression of epithelial and mesenchymal markers was analyzed in HeLa and CaSki cells by Western blot. Data are mean ± SD. (D, E) Expression of epithelial and mesenchymal markers was analyzed in HeLa (D) and CaSki (E) cells by immunofluorescence staining. Scale bars: 20 μm (D and E). $*p<0.05$, $**p<0.01$ based on Student's *t*-test.

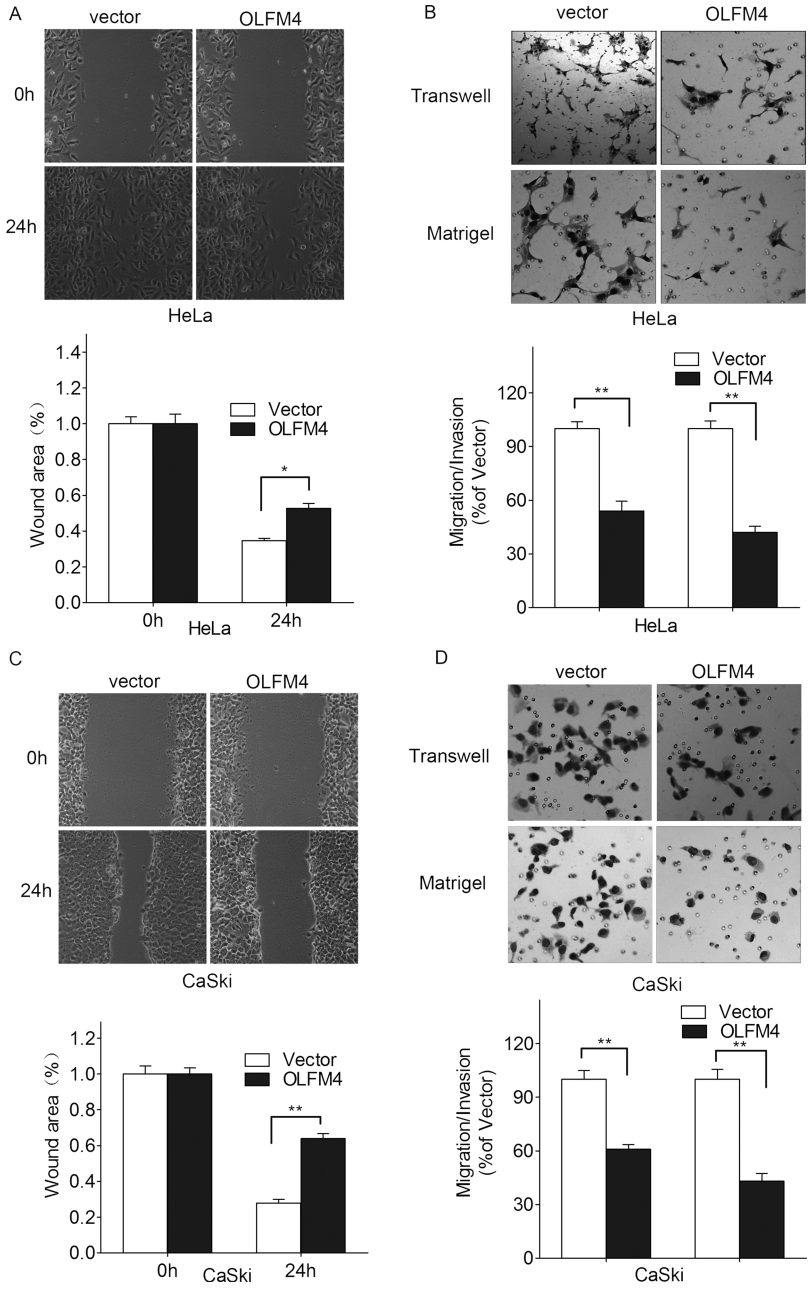

Figure 3. OLFM4 inhibits migration and invasion of cervical cancer cells. HeLa and CaSki cells transfected with OLFM4 expression plasmids or control vector were subjected to wound healing assay (A and C), Boyden's chamber assay (B and D, top), and Matrigel invasion assays (B and D, bottom). Data are mean ± SD. *$p < 0.05$, **$p < 0.01$ based on Student's *t*-test.

cycle progression, cell proliferation, cell apoptosis, cell adhesion, and migration[16–18]. However, the function of OLFM4 in cervical cancer is rarely reported. Yu et al.[9] reported that OLFM4 expression is increased in invasive squamous cell carcinomas (ISCCs) but decreased in poorly differentiated ISCCs compared with well-differentiated ISCCs, suggesting the contribution of OLFM4 in cervical tumorigenesis, whereas whether OLFM4 plays a role in cervical cancer metastasis remains unknown.

In this article, we delineated for the first time the mechanistic role of OLFM4 in regulating cervical cancer metastasis. We found that the expression of OLFM4 is decreased significantly in cervical cancer with metastasis. Forced expression of OLFM4 was then mediated by

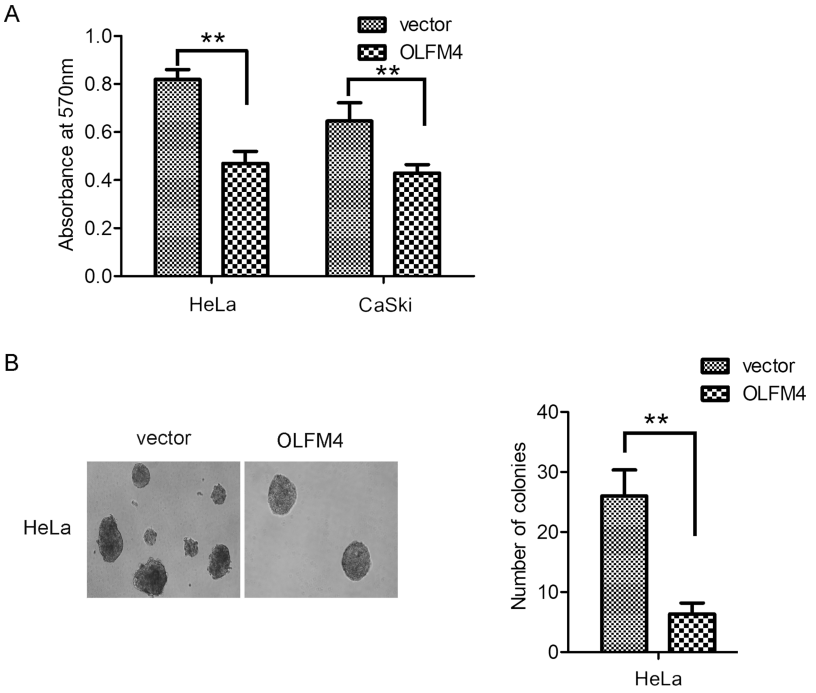

Figure 4. OLFM4 inhibits growth of cervical cancer cells. (A) MTT was used to analyze the growth of HeLa and CaSki cells transfected with OLFM4 expression plasmids or control vector. (B) Soft agar assay was performed to examine the anchorage-independent growth of HeLa cells transfected with OLFM4 expression plasmids or control vector. Data are mean ± SD. $^{**}p < 0.01$ based on Student's *t*-test.

transfecting OLFM4 expression plasmids into cervical cancer cells HeLa and CaSki, transiently. The increased expression of OLFM4 in these cells was verified by Western blot, which indirectly confirmed that the expression plasmids could be introduced into the indicated cells efficiently. Functionally, OLFM4 overexpression inhibited EMT, migration, and invasion of cervical cancer cells. Other studies have also demonstrated that OLFM4 serves to facilitate cell adhesion of NIH3T3 and HEK293 cells[19] and inhibit migration and invasion of prostate and gastric cancer cells[20,21]. Consistent with these previous reports, our results highlight a pivotal role for OLFM4 in cervical cancer metastasis.

Metastasis is an essential factor that contributes to the poor prognosis of cervical cancer patients[22]. Increasing evidence revealed that the process of EMT may be a prerequisite for cancer cell metastasis by endowing cells with migratory and invasive properties[23,24]. Our current experimental findings uncover the connection between OLFM4 and EMT in cervical cancer cells, which was previously unknown.

It is well known that OLFM4 is a secreted glycoprotein, and plasma levels of OLFM4 are tightly associated with tumor progression, lymph node invasion, and metastases. However, to date, little is known about how extracellular OLFM4 exerts its function in tumor initiation and progression[25,26]. In this study, forced expression of OLFM4 in cervical cancer cells significantly inhibited cell growth, migration, and invasion. Whether secreted OLFM4 plays a role in these events needs further studies.

A recent study disclosed elevated mTOR activity in promoting cell EMT, motility, and metastasis[27]. In dissecting the molecular mechanisms of the cell motility and invasiveness regulation by OLFM4, we proposed that mTOR signaling activity may be inhibited by augmented expression of OLFM4. Not surprisingly, subsequent study showed that OLFM4 overexpression led to marked inhibition of the mTOR pathway. More importantly, functional analysis further confirmed that reactivation of the mTOR pathway significantly weakened the inhibitory effects of OLFM4 on cell mobility and invasiveness.

In conclusion, our study sheds light on a pivotal role of OLFM4 in inhibiting EMT and metastasis, at least partly by regulating the mTOR signaling pathway in cervical cancer. Our findings suggest that OLFM4 may serve as a potential molecular marker for cervical treatment and prognosis.

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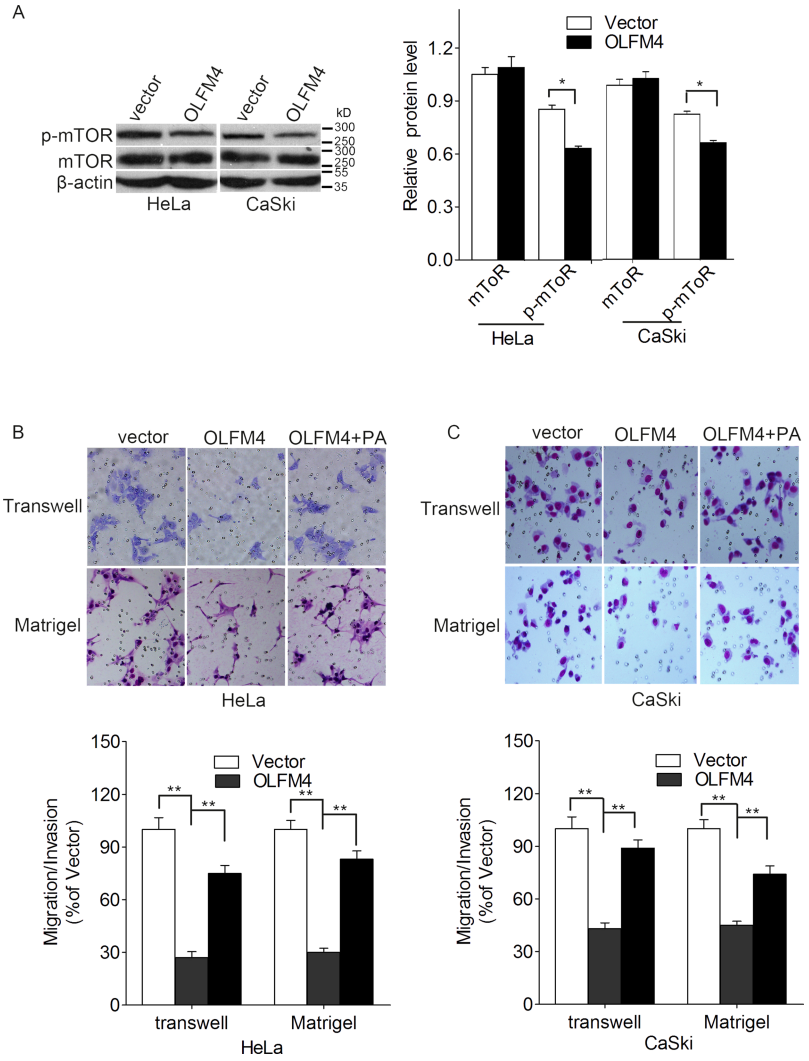

Figure 5. OLFM4 inhibits mTOR signal pathway and reactivation of mTOR signaling recovers the migratory and invasive capacity of cervical cancer cells. (A) Phosphorylated and total mTOR levels of HeLa–OLFM4 and CaSki–OLFM4 cells or their vector control cells were analyzed by Western blot. (B, C) The indicated cells treated with or without phosphatidic acid (PA) were subjected to Transwell migration (top) and Matrigel invasion assays (bottom). Data are mean ± SD. *$p<0.05$, **$p<0.01$ based on Student's *t*-test.

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