

Molecular regulation of cervical cancer growth and invasion by VEGFa

Baohuan Chen · Chunxiao Zhang · Pingping Dong · Yuanying Guo · Nan Mu

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Abstract Although anti-vascular endothelial growth factor a (VEGFa) treatment has been well applied in cervical cancer therapy, the underlying molecular basis has not been precisely identified. Here, we examined the levels of VEGFa on the tumor growth and invasion in four commonly used human cervical cancer cell lines. We found that overexpression of VEGFa in these lines increased the tumor growth and invasiveness, while inhibition of VEGFa decreased the tumor growth and invasiveness. To figure out the involved signaling pathways, we applied specific inhibitors for ERK/MAPK, JNK, and PI3K/Akt signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines and found that only inhibition of PI3K/Akt signal transduction abolished VEGFa-induced increases in cell growth and invasiveness. Inhibition of Akt downstream mTor signaling similarly inhibited cell growth and invasion in VEGFa-overexpressing cervical cancer cells, suggesting that VEGFa may activate PI3K/Akt, and subsequently its downstream mTor signaling pathway, to promote cervical cancer cell growth and invasion. Furthermore, the effects of VEGFa-induced activation of mTor signaling cascades appeared to promote cancer cell growth through cyclinD1 and CDK4 activation and promote cancer cell invasion through MMP2 and MMP3. Taken together, our data suggest that anti-VEGFa treatment in cervical

cancer may inhibit both tumor cell growth and invasion through PI3k/Akt/mTor signaling pathway.

Keywords Cervical cancer · Vascular endothelial growth factor a · PI3k · Akt · mTor · Cancer growth and invasion

Introduction

Cervical cancer is the fourth most common cause of cancer as well as the fourth most common cause of death from cancer in women. Many patients experience recurrence after the optimal treatment [1–3]. Therefore, there is a compelling requirement for improving our understanding of the precise molecular mechanism controlling the tumorigenesis, growth, and invasion of cervical cancer.

Although knowledge accumulates on the molecular mechanisms underlying carcinogenesis in the cervix, the exact signal transduction cascades remain undefined. Angiogenesis plays an essential role in invasion and metastasis of various cancer, including cervical cancer [4–6]. Cancer angiogenesis is potential for advanced tumor growth, by provision of oxygen and nutrients, and a path for its metastasis, in which vascular endothelial growth factor (VEGF) is the most important signal protein for stimulating vasculogenesis and angiogenesis [7, 8].

There are six secreted proteins in the VEGF family: VEGFa, VEGFb, VEGFc, VEGFd, VEGFe, and placental growth factor, among which VEGFa is the most potential signal protein for stimulation of vasculogenesis and angiogenesis during development, regeneration, and carcinogenesis [8–11]. In the past years, the promising results of bevacizumab in therapeutic trials for cervical cancer have highlighted VEGFa pathway as an attractive therapeutic target [1–3].

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in

Baohuan Chen and Chunxiao Zhang equally contributed to this paper.

B. Chen · C. Zhang · P. Dong (✉)
Department of Gynecology, Yantaishan Hospital, 91 Jiefang Road,
Yantai 264001, China
e-mail: pingping_dong14@163.com

Y. Guo
School of Public Health, Jilin University, Changchun, China

N. Mu
Department of Gynecology and Obstetrics, Yuhuangding Hospital,
Yantai, China

embryonic development, cell regeneration, tissue remodeling, and tumorigenesis [12, 13]. Based on historical assessment of the substrate specificity of the MMPs and on their cellular localization, four groups of MMPs have been defined, including the collagenases, the gelatinases, the stromelysins, and the membrane-type MMPs. MMP1, MMP8, MMP13, and MMP18 are from the collagenase group and are capable of degrading triple-helical fibrillar collagens. The main substrates of the gelatinases are type IV collagen and gelatin, which distinguished these enzymes from others. MMP2 and MMP9 are major members in this group. The stromelysins display inability of cleaving the triple-helical fibrillar collagens, and the three members of this group are MMP3, MMP10, and MMP11. All six membrane-type MMPs (MMP14, MMP15, MMP16, MMP17, MMP24, and MMP25) have a furin cleavage site in the propeptide, which is a feature also shared by MMP11. However, it is believed that these groups are arbitrary in that a number of MMPs have not been included in any of the traditional groups [12, 13]. Overexpression of MMPs has been reported to facilitate metastatic spread of different cancer cells and appears to be important molecules to directly promote cancer metastasis [14, 15].

Cancer cell growth is controlled by various cell cycle activators and inhibitors. Cell cycle activators include two classes of regulatory molecules, cyclins (e.g., cyclinB, cyclinD1, cyclinE) and cyclin-dependent kinases (CDKs, e.g., CDK4 and CDK6), which determine the progress of a cell cycle [16–19]. Cyclins have no catalytic activity and CDKs are inactive in the absence of a partner cyclin. When activated by a bound cyclin, CDKs undergo phosphorylation to activate or inactivate target proteins to orchestrate an entry into the next phase of the cell cycle. CDKs are constitutively expressed in cells whereas cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals [16–19]. Two families of genes, the *cip/kip* (CDK interacting protein/kinase inhibitory protein) family and the inhibitor of kinase 4/alternative reading frame (*INK4a/ARF*) family, are responsible for prevention of the cell cycle progression. The *cip/kip* family includes the genes *p21*, *p27*, and *p57*. They may suspend cell cycle in G1 phase, by binding to, and inactivating, cyclin-CDK complexes [16–20]. *p21* is activated by *p53*, while *p27* is activated by transforming growth factor beta.

Here, we examined the levels of VEGFa on the tumor growth and invasion in four commonly used human cervical cancer cell lines. We found that overexpression of VEGFa in these lines increased the tumor growth and invasiveness, while inhibition of VEGFa decreased the tumor growth and invasiveness. To figure out the involved signaling pathways, we applied specific inhibitors for ERK/MAPK, JNK, and PI3K/Akt signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines and found that only

inhibition of PI3K/Akt signal transduction abolished VEGFa-induced increases in cell growth and invasiveness. Inhibition of Akt downstream mTor signaling similarly inhibited cell growth and invasion in VEGFa-overexpressing cervical cancer cells, suggesting that VEGFa may activate PI3K/Akt, and subsequently its downstream mTor signaling pathway, to promote cervical cancer cell growth and invasion. Furthermore, the effects of VEGFa-induced activation of mTor signaling cascades appeared to promote cancer cell growth through cyclinD1 and CDK4 activation and promote cancer cell invasion through MMP2 and MMP3. Taken together, our data suggest that anti-VEGFa treatment in cervical cancer may inhibit both tumor cell growth and invasion through PI3k/Akt/mTor signaling pathway.

Materials and methods

Cell lines, transfection, and pathway inhibitors

Four most commonly studied human cervical cancer cell lines Caski, C33A, Siha, and HeLa were all purchased from ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Caski, C33A, Siha, and HeLa cells were transfected with a VEGFa-overexpressing plasmid [21] or a small short hairpin interfering RNA for VEGFa (shVEGFa) [7], as has been previously described [22]. Briefly, the plasmid construct was generated by subcloning PCR-amplified full-length human VEGFa cDNA or shVEGFa sequence (5'-TGTGAATGCAGACCAAAGA-3') into pcDNA3.1-EGFP. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. Since the plasmid construct also contains a gene-encoding GFP under the control of a CMV promoter, it allows purification of GFP-positive transfected cells by flow cytometry. LY294002, SP600125, PD98059, and rapamycin were all purchased from Sigma (USA).

Cell proliferation assay

For cell proliferation assay, pretreated cells were seeded into 96-well plate at 5,000 cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, USA), according to the instruction from the manufacturer.

Transwell matrix penetration assay

A total of 5×10^5 cells were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA, USA) and incubated at 37 °C for 22 h. The

cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for 10 random 100X fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed, and the data are presented as mean±standard deviation (SD).

ELISA assay

The concentration of VEGFa in the conditioned medium from cultured cells was determined by a VEGFa ELISA kit (RayBiotech, Norcross, GA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition medium was added to a well coated with VEGFa monoclonal antibody, and then immunosorbent by biotinylated monoclonal antihuman VEGFa antibody at room temperature for 2 h. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid, and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

RT-qPCR

RNA was extracted from the cultured cells with RNeasy kit (Qiagen, Hilden, Germany) and then used as templates for reverse transcription for cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed in duplicate with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were normalized against beta-actin and then were compared with the control.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction. All values are depicted as mean±SD from five individuals and are considered significant if $p < 0.05$.

Results

Generation of cervical cancer cell lines with adapted VEGFa levels

In the past years, the promising results of bevacizumab in therapeutic trials for cervical cancer have highlighted VEGF signaling pathway as an attractive therapeutic target. However, the precise molecular mechanism remains not completely identified. Here, we aimed to examine the effects

of VEGFa on the tumor growth and invasion and tried to understand the exact regulation pathways. We selected four commonly used human cervical cancer lines for our study. Caski, C33A, Siha, and Hela cells were all transfected with either a VEGFa-overexpressing plasmid, or a small shVEGFa, resulting in four VEGFa-overexpressing lines and four VEGFa-knockdown lines, respectively. The VEGFa overexpression or knockdown in these lines was assured by examining mRNA by RT-qPCR (Fig. 1a) and by examining secreted proteins in the conditioned media by ELISA (Fig. 1b).

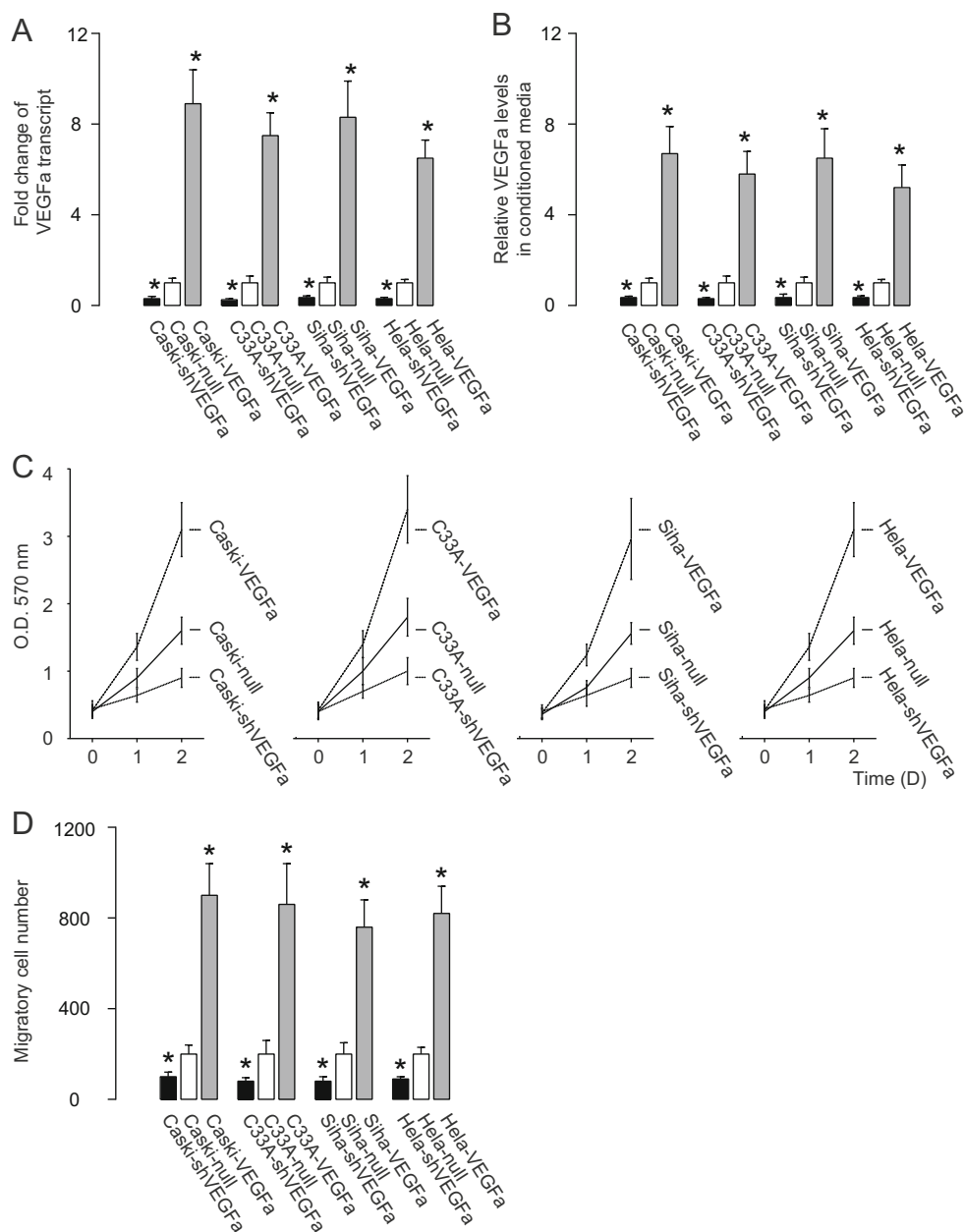
VEGFa levels affected growth and invasiveness of the cervical cancer cells

We found that overexpression of VEGFa in all cervical cancer cell lines significantly increased the tumor growth in an MTT assay, while inhibition of VEGFa decreased the tumor growth (Fig. 1c). These data suggest that VEGFa may play an essential role in the growth of cervical cancer cells. Similarly, we found that overexpression of VEGFa in all cervical cancer lines significantly increased the tumor invasiveness in a transwell matrix penetration assay, while inhibition of VEGFa decreased the tumor invasiveness (Fig. 1d). These data suggest that VEGFa may play an essential role in the invasion of cervical cancer cells.

VEGFa regulated growth of the cervical cancer cells through PI3k/Akt/mTor pathway

To figure out the signaling pathways through which VEGFa promoted tumor growth, we applied specific inhibitors for extracellular-related kinase/mitogen-activated protein kinase (ERK/MAPK), specific Jun N-terminal kinase (JNK), and phosphatidylinositol 3-kinase (PI3k)/protein kinase B (Akt) signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines. We found that only use of a specific PI3k/Akt signaling pathway inhibitor, LY294002 (20 $\mu\text{mol/l}$), but not use of either a specific ERK/MAPK signaling pathway inhibitor, PD98059 (10 $\mu\text{mol/l}$), or a specific JNK pathway inhibitor, SP600125 (10 $\mu\text{mol/l}$), substantially abolished the effect of overexpression of VEGFa on the increase in tumor growth (Fig. 2a). Moreover, application of a specific mTor inhibitor, rapamycin (100 ng/ml), similarly inhibited the cell growth in VEGFa-overexpressing cervical cancer cells, suggesting that VEGFa may activate PI3K/Akt, and subsequently its downstream mTor signaling pathway, to promote cervical cancer cell growth (Fig. 2a). We then examined the effects of VEGFa-induced activation of mTor signaling on cell cycle activators cyclinB, cyclinD1, cyclinE, CDK4, and CDK6 and cell cycle inhibitor p27. We found that cyclinD1 and CDK4 seemed to be activated by VEGFa downstream signaling (Fig. 2b).

Fig. 1 VEGFa levels affected growth and invasiveness of the cervical cancer cells. **a, b** Generation of cervical cancer cell lines with adapted VEGFa levels. We selected four commonly used human cervical cancer lines, Caski, C33A, Siha, and Hela cells, and transfected them with either a VEGFa-overexpressing plasmid or shVEGFa, resulting in four VEGFa-overexpressing lines and four VEGFa-knockdown lines, respectively. The VEGFa overexpression or knockdown in these lines was assured by examining mRNA by RT-qPCR (**a**) and by examining secreted proteins in the conditioned media by ELISA (**b**). **c** Overexpression of VEGFa in all cervical cancer lines significantly increased the tumor growth in an MTT assay, while inhibition of VEGFa decreased the tumor growth. **d** Overexpression of VEGFa in all cervical cancer lines significantly increased the tumor invasiveness in a transwell matrix penetration assay, while inhibition of VEGFa decreased the tumor invasiveness. * $p < 0.05$

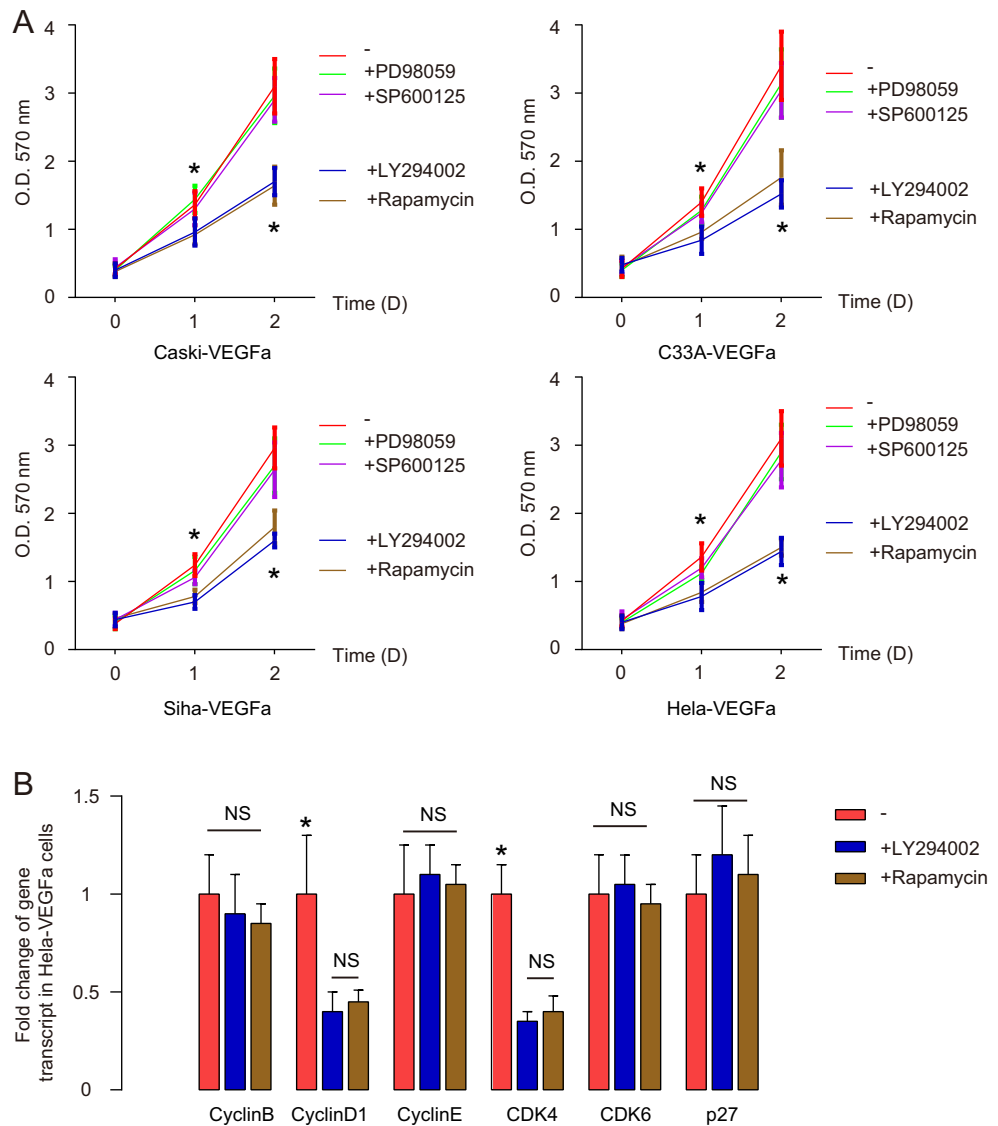


VEGFa regulated invasiveness of the cervical cancer cells through PI3k/Akt/mTor pathway

To figure out the signaling pathways through which VEGFa promoted tumor invasion, we also applied specific inhibitors for ERK/MAPK, JNK, and PI3k/Akt signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines. Interestingly, we also found that only use of LY294002 (20 $\mu\text{mol/l}$), but not use of either PD98059 (10 $\mu\text{mol/l}$), or SP600125 (10 $\mu\text{mol/l}$), substantially abolished the effect of overexpression of VEGFa on the increase in tumor invasiveness (Fig. 3a). Moreover, application of rapamycin (100 ng/ml) similarly inhibited the cell invasion in VEGFa-overexpressing cervical cancer cells, suggesting that VEGFa

may activate PI3K/Akt, and subsequently its downstream mTor signaling pathway, to promote cervical cancer cell invasion (Fig. 3a). Thus, VEGFa appeared to regulate both cell growth and invasion of cervical cancer through PI3K/Akt/mTor signaling pathway. We then examined the effects of VEGFa-induced activation of mTor signaling on the expression of the MMPs (MMP2, MMP3, MMP7, MMP9, MMP13, and MMP26) that had been detected in cervical tissues and cancers before. We found that MMP2 and MMP3 seemed to be regulated by VEGFa (Fig. 3b) suggesting that VEGFa may regulate MMP2 and MMP3, and consequently cervical cancer cell invasion, through PI3K/Akt/mTor signaling pathway. Our findings were thus summarized in a schematic (Fig. 4).

Fig. 2 VEGFa regulated growth of the cervical cancer cells through PI3k/Akt/mTor pathway. **a, b** To figure out the signaling pathways through which VEGFa promoted tumor growth, we applied specific inhibitors for ERK/MAPK, JNK, PI3k/Akt, and mTor signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines. **a** Tumor growth was examined in an MTT assay. **b** RT-qPCR on cell cycle activators and inhibitor: cyclinB, cyclinD1, cyclinE, CDK4, CDK6, and p27. LY294002 is a specific inhibitor for Akt phosphorylation. Rapamycin is a specific inhibitor for mTor. PD98059 is a specific inhibitor for ERK1/2 phosphorylation. SP600125 is a specific inhibitor for JNK. * $p < 0.05$. NS nonsignificant



Discussion

Poor prognosis and treatment for cervical cancer result largely from inadequate understanding of its molecular tumorigenesis. Angiogenesis is one of the most important process by which cervical cancer grows and invades. Tumor cells not only secrete angiogenetic molecules like VEGFa to increase vessel permeability and promote endothelial cell proliferation and survival, but also secrete MMPs to degrade extracellular matrix. All these events are critical for cancer angiogenesis and metastasis. Although there are six secreted proteins in the VEGF family—VEGFa, VEGFb, VEGFc, VEGFd, VEGFe, and placental growth factor—VEGFa has been found to have the most potential effect on stimulation of vasculogenesis and angiogenesis during development, regeneration, and carcinogenesis [8–11]. In the past years, the promising results of bevacizumab in therapeutic trials for cervical cancer have

highlighted VEGFa pathway as an attractive therapeutic target [1–3].

MMPs are well-known for their importance in the breakdown of extracellular matrix in embryonic development, cell regeneration, tissue remodeling, and tumorigenesis [12, 13]. Some studies have demonstrated that overexpression of MMPs may facilitate metastatic spread of different cancer cells to promote cancer metastasis, and furthermore, MMPs levels seem to be regulated by VEGFa in some cancers [14, 15]. Cancer cell growth is directly controlled by various cell cycle activators and inhibitors. Their regulation by VEGFa, however, is rarely reported [16–19].

Here, we examined the levels of VEGFa on the tumor growth and invasion in four commonly used human cervical cancer cell lines, to exclude a cell line-dependent possibility in data interpretation. We found that overexpression of VEGFa in these lines increased the tumor growth and invasiveness,

Fig. 3 VEGFa regulated invasiveness of the cervical cancer cells through PI3k/Akt/mTor pathway. **a, b** To figure out the signaling pathways through which VEGFa promoted tumor invasion, we applied specific inhibitors for ERK/MAPK, JNK, PI3k/Akt, and mTor signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines. **a** Tumor growth was examined in an MTT assay. **b** RT-qPCR on MMPs (MMP2, MMP3, MMP7, MMP9, MMP13, and MMP26). * $p < 0.05$. NS nonsignificant

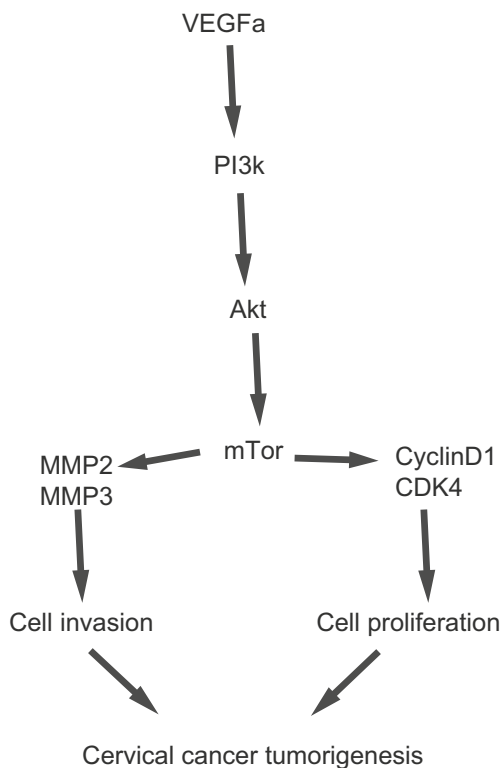
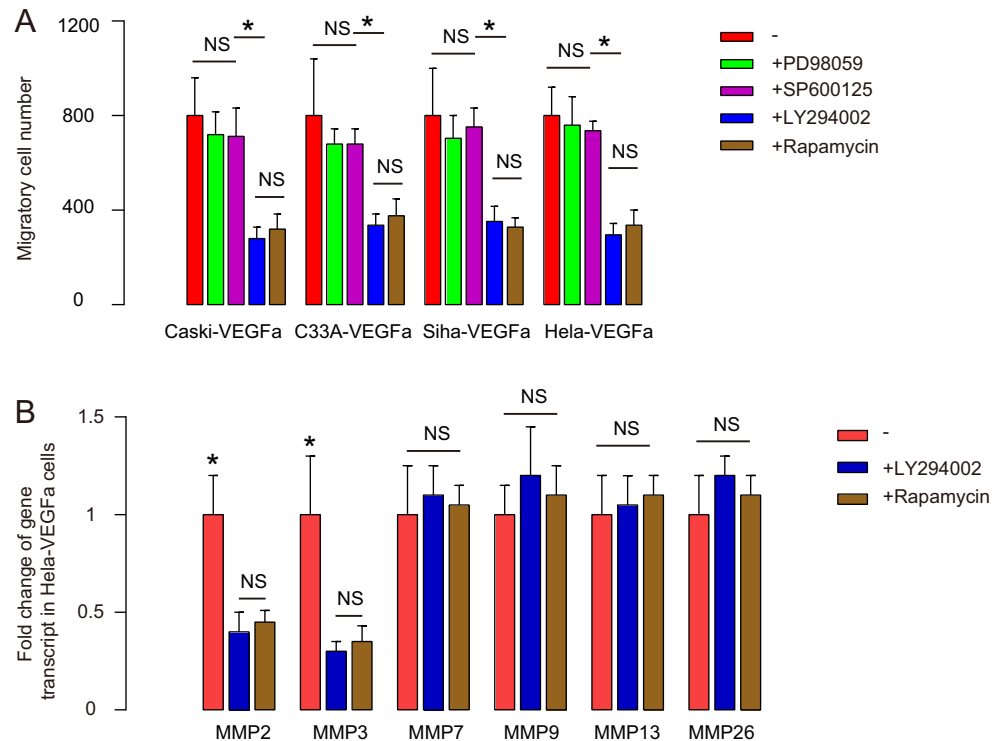


Fig. 4 Schematic of the model. VEGFa activates PI3K/Akt/mTor signaling in cervical cancer, which subsequently activates cyclinD1 and CDK4 to increase cell growth and activates MMP2 and MMP3 to augment cell invasion

while inhibition of VEGFa decreased the tumor growth and invasiveness. Thus, VEGFa appeared to play a pivotal role in cervical cancer tumorigenesis and appeared to be an essential trophic factor. These data are consistent with previous findings and the effective bevacizumab treatment for cervical cancer, highlighting VEGFa as a potential target for cervical cancer therapy.

To figure out the involved signaling pathways, we applied specific inhibitors for ERK/MAPK, JNK, and PI3K/Akt signaling pathways, respectively, to VEGFa-overexpressing cervical cancer lines and found that only inhibition of PI3K/Akt signal transduction abolished VEGFa-induced increases in cell growth and invasiveness. Inhibition of Akt downstream mTor signaling similarly inhibited cell growth and invasion in VEGFa-overexpressing cervical cancer cells, suggesting that VEGFa may activate PI3K/Akt, and subsequently its downstream mTor signaling pathway, to promote cervical cancer cell growth and invasion. Furthermore, the effects of VEGFa-induced activation of mTor signaling cascades appeared to promote cancer cell growth through cyclinD1 and CDK4 activation and promote cancer cell invasion through MMP2 and MMP3. Taken together, our data suggest that anti-VEGFa treatment in cervical cancer may inhibit both tumor cell growth and invasion through PI3k/Akt/mTor signaling pathway.

In the current study, we have dissected the molecular mechanism underlying the angiogenesis, growth, and invasiveness of cervical cancer. Further delineation of the precise molecular mechanism that mediates the regulation of MMP2,

MMP3, cyclinD1, and CDK4 by mTor signaling may substantially improve our understanding of the controls for the growth and metastasis of cervical cancer. Our study also shed light on the exact mechanism of the current anti-VEGFa cancer therapy.

Conflicts of interest None

References

- Delli Carpini J, Karam AK, Montgomery L. Vascular endothelial growth factor and its relationship to the prognosis and treatment of breast, ovarian, and cervical cancer. *Angiogenesis*. 2010;13:43–58.
- del Campo JM, Prat A, Gil-Moreno A, Perez J, Parera M. Update on novel therapeutic agents for cervical cancer. *Gynecol Oncol*. 2008;110:S72–6.
- Zagouri F, Sergeantanis TN, Chrysikos D, Filipits M, Bartsch R. Molecularly targeted therapies in cervical cancer. A systematic review. *Gynecol Oncol*. 2012;126:291–303.
- Kim JG. Molecular targeted therapy for advanced gastric cancer. *Korean J Intern Med*. 2013;28:149–55.
- Ilson DH. Angiogenesis in gastric cancer: hitting the target? *Lancet*. 2014;383:4–6.
- Scartozzi M, Giampieri R, Loretelli C, Bittoni A, Mandolesi A, Faloppi L, et al. Tumor angiogenesis genotyping and efficacy of first-line chemotherapy in metastatic gastric cancer patients. *Pharmacogenomics*. 2013;14:1991–8.
- Xiao X, Prasad K, Guo P, El-Gohary Y, Fischbach S, Wiersch J, et al. Pancreatic duct cells as a source of VEGF in mice. *Diabetologia*. 2014;57:991–1000.
- Bagri A, Kouros-Mehr H, Leong KG, Plowman GD. Use of anti-vegf adjuvant therapy in cancer: challenges and rationale. *Trends Mol Med*. 2010;16:122–32.
- Xiao X, Guo P, Chen Z, El-Gohary Y, Wiersch J, Gaffar I, et al. Hypoglycemia reduces vascular endothelial growth factor a production by pancreatic beta cells as a regulator of beta cell mass. *J Biol Chem*. 2013;288:8636–46.
- Ferrara N. Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol*. 2009;29:789–91.
- Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298–307.
- Davidson B, Reich R, Risberg B, Nesland JM. The biological role and regulation of matrix metalloproteinases (mmp) in cancer. *Arkh Patol*. 2002;64:47–53.
- Rhee JS, Coussens LM. Reckoning mmp function: Implications for cancer development. *Trends Cell Biol*. 2002;12:209–11.
- Mitra A, Chakrabarti J, Chattopadhyay N, Chatterjee A. Membrane-associated mmp-2 in human cervical cancer. *J Environ Pathol Toxicol Oncol Off Organ Int Soc Environ Toxicol Cancer*. 2003;22:93–100.
- Thompson EW, Yu M, Bueno J, Jin L, Maiti SN, Palao-Marco FL, et al. Collagen induced mmp-2 activation in human breast cancer. *Breast Cancer Res Treat*. 1994;31:357–70.
- Harashima H, Dissmeyer N, Schnittger A. Cell cycle control across the eukaryotic kingdom. *Trends Cell Biol*. 2013;23:345–56.
- Diaz-Moralli S, Tarrado-Castellarnau M, Miranda A, Cascante M. Targeting cell cycle regulation in cancer therapy. *Pharmacol Ther*. 2013;138:255–71.
- Bertoli C, Skotheim JM, de Bruin RA. Control of cell cycle transcription during g1 and s phases. *Nat Rev Mol Cell Biol*. 2013;14:518–28.
- Lim S, Kaldis P. Cdks, cyclins and ckis: roles beyond cell cycle regulation. *Development*. 2013;140:3079–93.
- Xiao X, Gaffar I, Guo P, Wiersch J, Fischbach S, Peirish L, et al. M2 macrophages promote beta-cell proliferation by up-regulation of smad7. *Proc Natl Acad Sci U S A*. 2014;111:E1211–20.
- Wang YQ, Guo X, Qiu MH, Feng XY, Sun FY. Vegf overexpression enhances striatal neurogenesis in brain of adult rat after a transient middle cerebral artery occlusion. *J Neurosci Res*. 2007;85:73–82.
- Biggs 3rd WH, Meisenhelder J, Hunter T, Cavenee WK, Arden KC. Protein kinase b/akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor fkhrl. *Proc Natl Acad Sci U S A*. 1999;96:7421–6.