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VEGF111b, a new member of VEGFxxxb isoforms and induced by mitomycin C, inhibits angiogenesis

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ABSTRACT

Vascular endothelial growth factor (VEGF-A) stimulating angiogenesis is required for tumor growth and progression. The conventional VEGF-A isoforms have been considered as pro-angiogenic factors. Another family of VEGF-A isoforms generated by alternative splicing, termed VEGFxxxb isoforms, has anti-angiogenic property, exemplified by VEGF165b. Here, we identify a new number of VEGFxxx family-VEGF111b induced by mitomycin C, although not detected in mitomycin C-unexposed ovarian cancer cells. SKOV3 cells were transfected with $pcDNA_{3.1}$ empty vector, $pcDNA_{3.1}$-VEGF111b or $pcDNA_{3.1}$-VEGF165b to collect conditioned mediums respectively. VEGF111b overexpression inhibits proliferation, migration and tube formation of endothelial cell by inhibiting VEGF-R2 phosphorylation and its downstream signaling, similar to VEGF165b but slightly lower than VEGF165b. The anti-angiogenic property depends on the six amino acids of exon 8b of the VEGFxxxb isoforms. Our results show that VEGF111b is a novel potent anti-angiogenic agent that can target the VEGF-R2 and its signaling pathway to inhibit ovarian tumor growth.

1. Introduction

Angiogenesis plays a key role in tumor growth and progression [1]. A principal angiogenic promoter that stimulates the migration of endothelial cells, sprouting of blood vessels, and generation of new vessels from existing vascular endothelium in tumors is the vascular endothelial growth factor (VEGF-A) [2,3]. Anti-angiogenic therapy targeting VEGF-A is becoming an additional therapeutic strategy to surgery, chemotherapy and radiotherapy, which has attracted more attention.

The human VEGF-A gene has been assigned to chromosome 6p21.3. It contains 8 exons, separated by seven introns, and its coding region spans approximately 14 kb [4]. Alternative splicing of full-length VEGF pre-mRNA gives rise to two known families of protein isoforms that differ by only six amino acids at their C-terminal end (Fig. 1). The conventional VEGFxxx isoforms, where xxx refers to the number of amino acids, are formed by the proximal splice site (PSS) selection in exon 8 (termed exon 8a) and differentially splicing in exons 5, 6 or 7 [5]. The six amino acids encoded by exon 8a are CDKPRR (Fig. 1). The major isoforms of VEGFxxx family, demonstrated to be pro-angiogenic, are VEGF165, VEGF189, VEGF121, VEGF145, VEGF183, VEGF206 and VEGF111 [6]. However, another sister family of VEGF isoforms, generically referred to as VEGFxxxb isoforms, are formed by distal splice site (DSS) selection 66 bp downstream of the PSS site in exon 8 (termed exon 8b) [7–9]. Exon 8b encodes a unique amino acids sequence SLTRKD (Fig. 1). In VEGFxxxb family, VEGF165b, VEGF121b, VEGF145b and VEGF183b have been identified in succession and demonstrated to be anti-angiogenic [5]. The first verified and widely reported VEGFxxxb family member is VEGF165b, which has been clearly shown to inhibit endothelial cell growth and migration in vitro and angiogenesis in tumor and non-tumor-related angiogenesis [7,10,11].

Abbreviations: FBS, fetal bovine serum; VEGF, vascular endothelial growth factor; PSS, proximal splice site; DSS, istal splice site; VEGF-R, VEGF receptor; HRP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; RT-PCR, reverse transcriptase-PCR.

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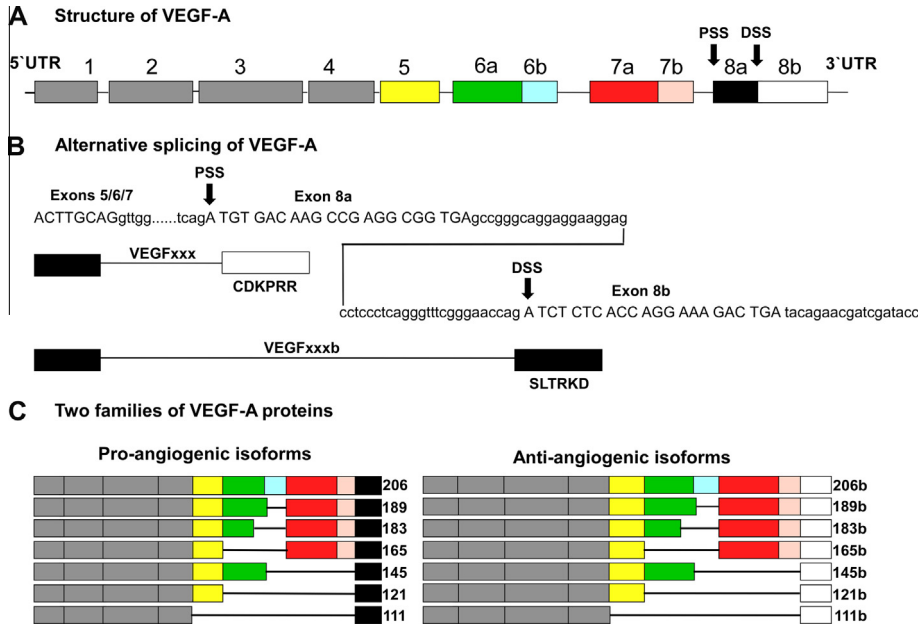

Fig. 1. Structure of VEGF-A gene and alternative splicing of VEGF-A generate VEGFxxx and VEGFxxxb isoforms. (A) VEGF-A contains eight exons. Proximal splice site (PSS) selection in the terminal exon 8 generates the pro-angiogenic VEGFxxx family, whereas distal splice site (DSS) selection results in the anti-angiogenic VEGFxxxb family. (B) Alternative splicing of the C-terminal end leads to the possibility of two sister families of VEGF-A isoforms: VEGFxxx and VEGFxxxb, differing only in last six amino acids (CDKPRR or SLTRKD).

Although a large number of evidences on the expression and property of VEGF165b have already been published, there is very little evidence on other VEGFxxxb family members, and their existence and property is still unknown. In 2007, Mineur reported a new VEGFxxx family member, VEGF111, and demonstrated that it could only be induced in the condition of genotoxic agents, such as camptothecin, mimosin, mitomycin C and UV-B [12]. The VEGF111 coding sequence consists of exons 1–4 and 8a. DSS in exon 8 has stronger splicing advantages than PSS [13]. Whether VEGF111b exits and plays a role in anti-angiogenic effect has never been demonstrated. Thus we speculate the presence of VEGF111b. Therefore, in this study, we detected and discovered a new member of VEGFxxxb family, VEGF111b, under the induction of mitomycin C. We constructed eukaryotic expression vector of VEGF111b for sequencing, prepared VEGF111b polyclonal antibody, and finally confirmed the hypothesis that VEGF111b also show anti-angiogenic properties.

2. Materials and methods

2.1. Reagents and antibodies

Mitomycin C was obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Anti-VEGF-R1, and anti-VEGF-R2 were purchased from Beyotime (Jiangsu, China). All other primary antibodies were purchased from Abcam (Cambridge, TX, USA). Horseradish peroxidase (HRP)-labeled anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz (Dallas, TX, USA).

2.2. Cell lines

Human umbilical vein endothelial cells (HUVECs) were extracted from umbilical cords from caesarean sections (The General Hospital of the People's Liberation Army, Beijing, China). The study protocol was approved by the local ethics committee. The cells were cultured in Endothelial Cell Medium (ECM, Science) consisting of 5% foetal bovine serum (FBS), 1% endothelial cell growth supplement and 1% penicillin and streptomycin solution. Human ovarian cancer cells SKOV3 were obtained from the Chinese Academy of Medical Sciences and cultured in Roswell Park Memorial Institute-1640 culture (RPMI-1640, HyClone), supplemented with 10% FBS (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. RT-PCR analysis

SKOV3 cells were treated with 100 μg/ml mitomycin C for 24 h, then total RNA was extracted using Trizol reagent (Invitrogen, USA). Complementary DNA was made using oligo dT primer (TransGen, Beijing) by the manufacturer. According to alternative splicing of VEGF-A, the VEGF111b mRNA is composed of exons 1–4 and 8b. We designed forward primer of VEGF111b in exon 4, and reverse primer in the junction of exon fourth and 8b. GAPDH was amplified as an internal control. Primers sequences are listed as follows: VEGF111b 5′-CCACTGAGGAGTCCAACATCA-3′ (forward); 5′-AATGCAGATGTGACAAGCCGAG-3′ (reverse). VEGF165b 5′-GAGATGAGCTTCCTACAGCAC-3′ (forward); 5′-TTAAGCTTTCAGTCTTTCCTGGTGAGAGATCTGCA-3′ (reverse). GAPDH 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward); 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (reverse). PCR products were separated and visualised using 4% agarose/ethidium bromide gel.

2.4. Production of polyclonal antibody VEGF111b

Synthetic peptide fragments of the 8 amino acids CRSLTRKD in the C-terminal sequence of VEGF111b were coupled to KLH serving as carrier molecules and then used to immunize two male New Zealand long ear rabbits. The animals received subcutaneous injections of 0.5 ml peptide-KLH conjugates in Freund's Complete Adjuvant every 2 weeks. A week after the last immunization ear vein blood was taken for enzyme linked immunosorbent assay (ELISA) titer analysis. When the titers reached to the requirement, serum was collected to purify VEGF111b polyclonal antibody by ammonium sulfate precipitation.

2.5. Collection of the conditioned medium

Full-length VEGF111b or VEGF165b (generated by RT-PCR from SKOV3 cells) were cloned into the expression vector $pcDNA_{3.1}$ according to standard methodology. SKOV3 cells were plated into 6-well plate and transiently transfected with 4 μg/well $pcDNA_{3.1}$ empty vector, $pcDNA_{3.1}$-VEGF111b or $pcDNA_{3.1}$-VEGF165b for 24 h, using lipofectamine™ 2000 (Invitrogen) as per manufacturer's instructions and then incubated in ECM with 1% BSA for 24 h followed by collection of the conditioned medium (CM). VEGF165b CM was used as positive controls, and the CM of SKOV3 cells transfected with the empty vector was used as a negative control. The CM of SKOV3 cells with no transfection was also a negative control. The medium was spun down at 3000 rpm, 15 min, and the supernatant was collected and stored at −80 °C. CM was concentrated 30 times and then analysed by Western blot with cell lysate together to confirm expression levels of VEGF isoforms using VEGF165b monoclonal antibody or VEGF111b polyclonal antibody.

2.6. Proliferation assay

HUVECs (4×10^3/well) were seeded into 96-well plate. Following day, cells were treated with control medium, empty vector CM, VEGF111b CM or VEGF165b CM, respectively, for 24 or 48 h. Cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays. After incubation, the cells were stained with 20 μl of 5 mg/ml MTT (Ameresco, USA) and lysed in 100 μl DMSO each well. The absorbance was read on a plate reader (model 550, BioRad, USA) at 570 nm. The average values were determined from quadruplicate readings, and the experiment was repeated in triplicate.

2.7. Wound-healing migration assay

HUVECs were seeded into 24-well plate and grown to 90–95% confluence. Wound was created by scraping with a sterile pipette tip. The wounded cells were washed three times with PBS and covered with control medium or CMs, respectively. The cells were allowed to migrate for 16 h. At 0 h, 8 h, and 16 h after scratching, images were taken under the inverted microscope (Nikon, Japan). The percentage of the wound healing was calculated as (the width of wound at 0 h – the width of wound at 8 or 16 h)/the width of wound at 0 h in 10 random high power fields.

2.8. Transwell migration assay

Quantitative cell migration assay was performed using a modified Boyden chamber (Minicell, Millipore, USA) with 8.0 μm pore polycarbonate filter inserts in 24-well plate. The lower chamber was filled with 0.6 ml of ECM containing 5% FBS. HUVECs were plated into the upper chamber at 1×10^5 cells/well with control medium or CMs and incubated at 37 °C for 12 h to allow cells to migrate. After incubation, cells on the upper side of the filter were removed by a cotton swab, and the migrated cells on the lower side of the filter were fixed with methanol, stained with crystal violet and counted with an inverted microscope (Nikon, Japan). Migration was quantified by counting the number of stained cells in 10 random high power fields.

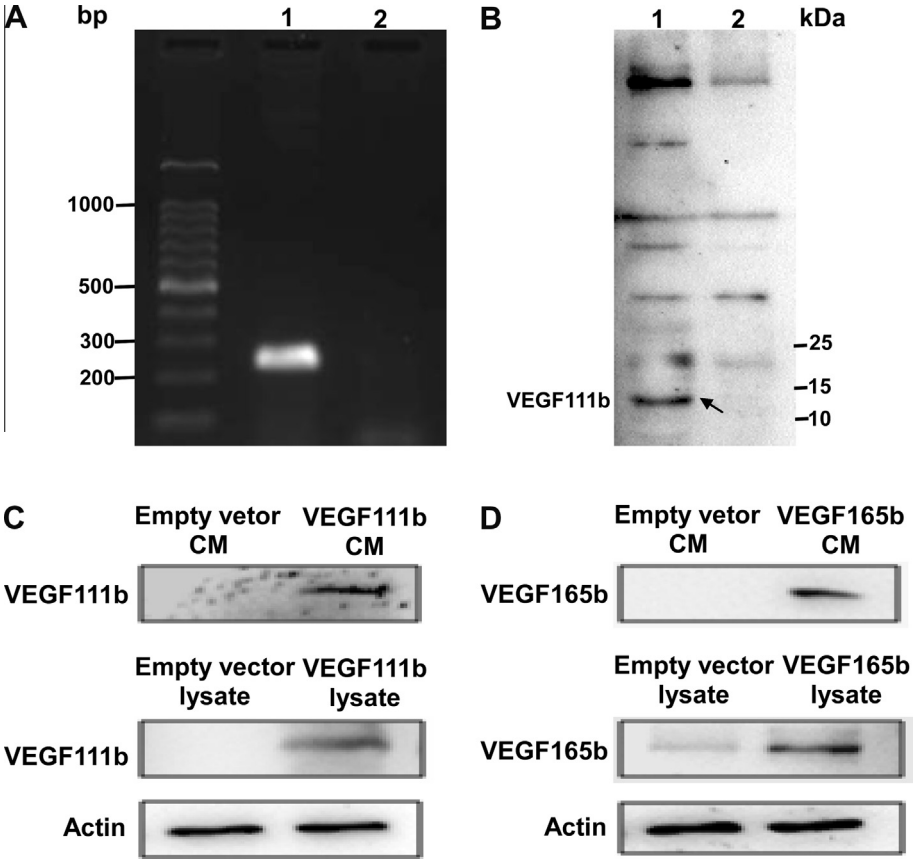

Fig. 2. Mitomycin C induces mRNA and protein expression of VEGF111b. (A and B) RT-PCR and Western blot to detect VEGF111b. VEGF111b mRNA was observed in SKOV3 cells after treating with 100 μg/ml mitomycin C (A, lane 1), and VEGF111b protein (arrow heads) was detected in SKOV3 cells after treating with 100 μg/ml mitomycin C by using VEGF111b polyclonal antibody (B, lane 1), but not observed in mitomycin C-unexposed SKOV3 cells (A, lane 2; B, lane 2). (C and D) Empty vector, $pcDNA_{3.1}$-VEGF111b and $pcDNA_{3.1}$-VEGF165b were transfected into SKOV3 cells. VEGF111b (C) and VEGF165b (D) were both found in the conditioned medium and lysate on the respective transfected SKOV3 cells.

2.9. Tube formation assay

Tube formation assay was performed by following the published protocol [14]. Briefly, Basement Membrane Matrix (BD Matrigel™) was thawed at 4 °C overnight. 96-well plate was coated with matrix and incubated at 37 °C for 1 h to allow matrix to polymerize. HUVECs were mixed with control medium or CMs and added to the top of the gel and incubated at 37 °C. The formation of the capillary-like tubes was observed after 3 h, and the pictures were captured with inverted microscope (Nikon, Japan).

2.10. Western blot

HUVECs were seeded into 6-well plate and then treated with control medium or CMs for 30 min. The cells were lysed using cell lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.1% SDS, 1% Triton X-100). Equivalent amounts of cell lysates and concentrated CMs were separated by SDS–PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked in 5% skimmed milk for 2 h and then incubated with respective primary antibody over night at 4 °C followed by the incubation of the appropriate HRP-conjugated secondary antibody for 1.5 h at room temperature. The signal was detected with SuperSignal West Pico substrate (Thermo scientific, Rockford, IL, USA).

2.11. Statistical analysis

All values were expressed as the mean ± SEM. The data were analyzed using Student's *t* test, or one-way ANOVA test. *P* value of <0.05 was considered statistically significant. GraphPad Prism (GraphPad Software Inc., San Diego, California, USA) was used for these analyses.

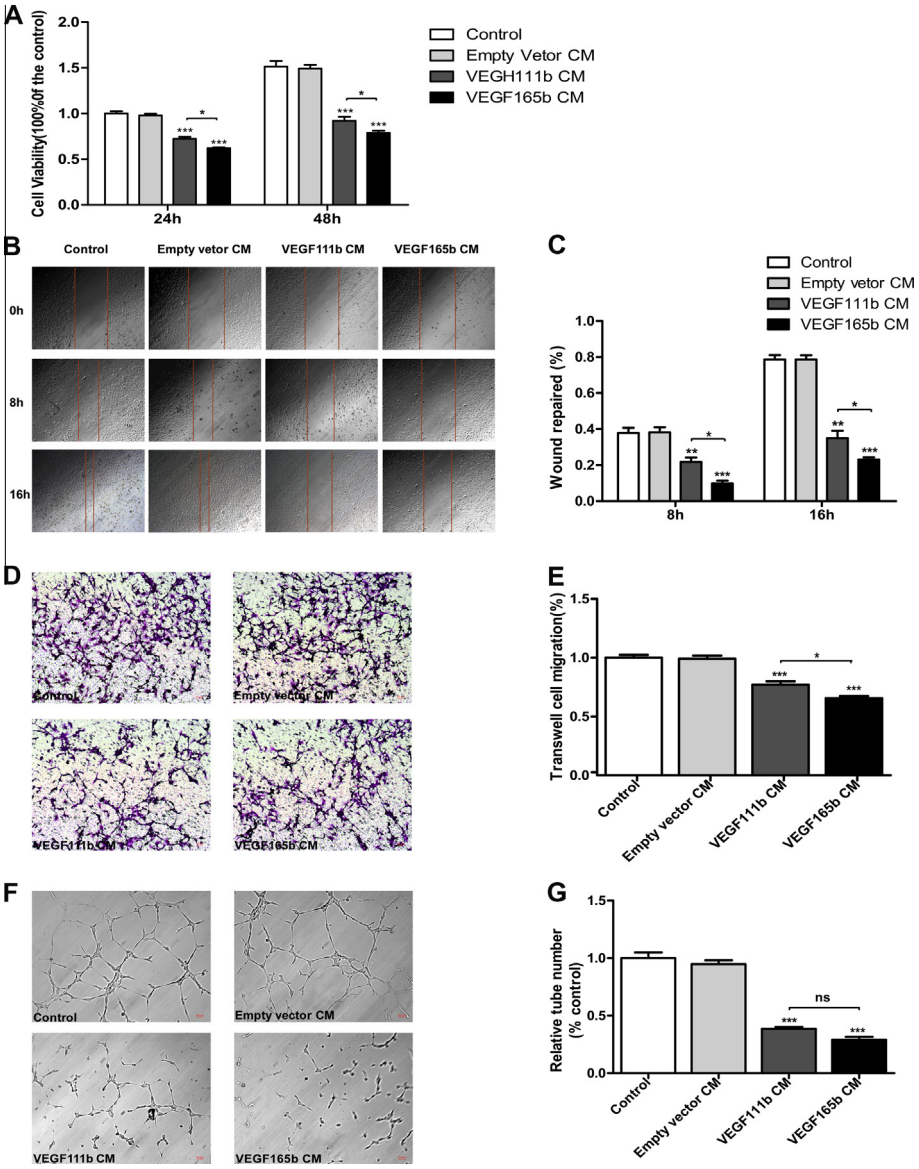

Fig. 3. VEGF111b overexpression inhibits endothelial cell proliferation, migration and tube formation. (A) MTT assay on the proliferation of HUVECs treated with control medium, empty vetor CM, VEGF111b or VEGF165b, respectively, for 24 h and 48 h. Cell viability was expressed as percentage in comparison with empty vector CM (*$P < 0.05$; ***$P < 0.001$). (B and C) Wound healing assay on the migration of HUVECs treated with control medium or CMs at 0 h, 8 h, and 16 h. The percentage of the wound repaired was calculated as (the width of wound at 0 h – the width of wound at 8 h/16 h)/the width of wound at 0 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (D and E) Transwell assay on the migration of HUVECs treated with control medium or CMs for 12 h. Quantification of the migration ability was expressed as the number of migrating cells at 100× magnification (*$P < 0.05$; ***$P < 0.001$). (F and G) Tube formation assay was used to evaluate the angiogenesis potential. Capillary morphogenesis was quantified by measuring the number of tubes by counting 9 random fields/sample at 100× magnification (***$P < 0.001$; ns, no significance). Error bars show the mean ± SEM of the triplicates from 3 independent experiments.

3. Results

3.1. VEGF111b expression could be induced by mitomycin C in SKOV3 cells

According to alternative splicing of VEGFxxx and VEGFxxxb families, We designed VEGF111b specific primers and observed VEGF111b mRNA production (Fig. 1A, lane 1) by using RT-PCR in SKOV3 cells after treatment with 100 μg/ml mitomycin C. Sequence analysis of VEGF111b were shown in Supplementary Fig. S1 and demonstrated that VEGF111b is composed of exons 1–4 and 8b. This product was not observed in mitomycin C-unexposed cells (Fig. 1A, lane 2). Theoretically this new splice variant encodes a 111 amino acids-long human VEGF molecule. The junction between exons 4 and 8b is specific for VEGF111b in VEGFxxxb family, and the only difference of VEGF111b and VEGF111 is PSS or DSS selection in the terminal exon 8. By using the specific reverse primer sitting astride in exons 4 and 8b, VEGF111b isoform was specifically amplified by using RT-PCR in SKOV3 cells.

To determine whether VEGF111b protein was induced in SKOV3 cells after treating with 100 μg/ml mitomycin C, we prepared a polyclonal antibody specific to the eight amino acids at the C-terminal end of VEGF111b. VEGF111b protein was detected by Western blot in SKOV3 cells after treating with mitomycin C (Fig. 2B, lane 1). However, VEGF111b was not detected in mitomycin C-unexposed cells (Fig. 2B, lane 2).

3.2. VEGF111b overexpression inhibits endothelial cell proliferation, migration and tube formation

Eukaryotic expressing vector $pcDNA_{3.1}$-VEGF111b, $pcDNA_{3.1}$-VEGF165b and empty vector were transfected into SKOV3 cells, then CMs and lysates were collected to determine whether VEGF111b can be secreted from SKOV3 cells. Western blot showed that VEGF111b protein was detected in the CMs and lysates, indicating that VEGF111b could be freely secreted (Fig. 2C and D), in contrast to cells transfected with empty vector plasmid, in which VEGF111b was not detected in the medium or lysate.

To determine whether VEGF111b secreted from SKOV3 cells was active on HUVECs, CM from SKOV3 with VEGF111b overexpression was used to study proliferation and migration of HUVECs. Firstly, VEGF111b CM inhibited proliferation of HUVECs with a 26 ± 10%, and 38 ± 7% decrease in proliferation at 24 h and 48 h, respectively (vs empty vector CM, $P < 0.001$; Fig. 3A). VEGF111b inhibited the proliferation of HUVECs in a time-dependent manner. Moreover, the inhibition level of VEGF111b was less than that of VEGF165b ($P < 0.05$; Fig. 3A). Second, we analyzed migration ability by using wound-healing and transwell assays. It was found that HUVECs migration ability was inhibited by VEGF111b. Wound healing assay showed that HUVECs migration ability was significantly inhibited by VEGF111b (vs empty vector CM, $P < 0.01$; Fig. 3B and C). Transwell assay also showed than HUVECs migration ability in VEGF111b CM was reduced by 22 ± 11% (vs empty vector CM, $P < 0.001$; Fig. 3D and E). Moreover, VEGF111b was less than VEGF165b in the level of migration inhibition ($P < 0.05$; Fig. 3B–E). Third, we used endothelial cell tube formation assay to indicate the angiogenesis potential. VEGF111b and VEGF165b showed significant anti-tube formation activity so that tube formation was observably reduced (vs empty vector CM, $P < 0.001$; Fig. 3F and G). Tube formation (Fig. 3C) was reduced by 56 ± 10% and 65 ± 13% respectively, compared with empty vector CM. There was no significant difference between VEGF111b and VEGF165b.

3.3. VEGF111b inhibits angiogenesis through inhibiting VEGF-R2/PI3K/Akt and VEGF-R2/ERK1/2 phosphorylation

To determine whether VEGF111b signaling was mediated through VEGF-R2, we investigated the levels of total and phosphorylated VEGF-R2 (p-VEGF-R2) in HUVECs after treatment with control medium or CMs for 4 h. Total protein levels of VEGF-R2 were not significantly changed. By contrast, p-VEGF-R2 (Y1175) were decreased after treatment with VEGF111b CM and VEGF165b CM, and the phosphorylation level of the VEGF-R2 induced by VEGF165b CM was lower than the that induced by VEGF111b (Fig. 4A). However, we did not detect any phosphorylation levels change of the p-VEGF-R1. A detailed analysis of downstream signaling pathways was performed to detect the levels of phosphorylated PI3K, Akt, and p44/42 MAPK (ERK1/2). Expressions of p-PI3K, p-Akt, and p-ERK1/2 were also inhibited after VEGF111b CM and VEGF165b CM treatment. Moreover, phosphorylation level of p-PI3K, p-Akt, and p-ERK1/2 induced by VEGF165b CM was lower than the activation induced by VEGF111b (Fig. 4B).

4. Discussion

Here, we list new data for the first time presenting existence of VEGF111b as a different VEGFxxxb family member. Similar to VEGF111 [12], the mRNA and protein expression of VEGF111b are induced in the human ovarian cancer cells by genotoxic agents such as mitomycin C. We failed to detect VEGF111b without mitomycin C treatment. Thus, the expression of VEGF111b similarly depends upon the treatment of mitomycin C inducing

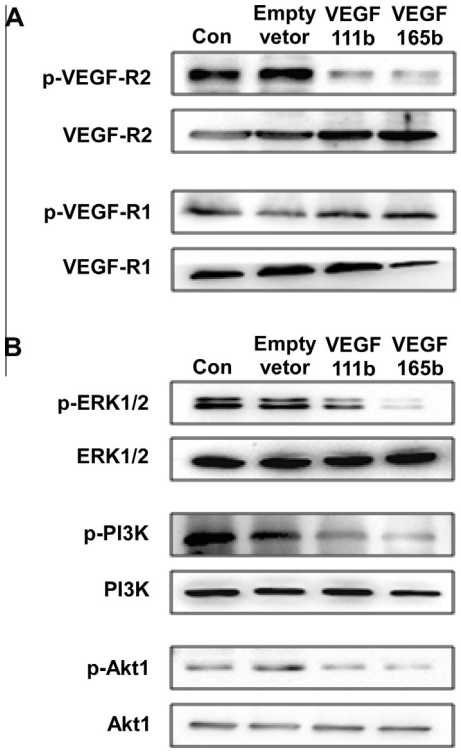

Fig. 4. VEGF111b reduces VEGFR-2 phosphorylation and its downstream signaling. HUVECs treated with control medium or CMs, respectively, for 30 min. (A) Total and phosphorylated VEGF-R2 were measured by Western blotting. p-VEGF-R2 was reduced by VEGF111b CM and VEGF165b CM. (B) Downstream signaling proteins of VEGF-R2 were measured by Western blotting. p-PI3K, p-Akt, and p-ERK1/2 were reduced by VEGF111b CM and VEGF165b CM, correspondingly. Actin served as loading control.

double-strand breaks. Its expression in other types of cultured cells, or induced by other genotoxic agents will be addressed in further studies.

Since VEGF165b was firstly reported [7], which was generated by exon 8 C-terminal distal splicing, leading to a six amino acids substitution (CDKPRR to SLTRKD). Gene sequences analysis of VEGF165b reveals receptor (VEGF-R1 and VEGF-R2)-binding domain exist in exons 3 and 4. Exon 8a has been now identified to be essential for neuropilin-1 (NRP-1) and heparin binding [15,16]. The VEGF165b isoform that lacks this moiety is unable to bind NRP-1, resulting in reduced VEGFR-2 tyrosine phosphorylation and downstream signaling [17,18]. Afterwards, VEGF121b, VEGF145b and VEGF189b were gradually discovered [19–21]. It showed that there was a whole sister family of VEGFxxxb isoforms and confirmed that they have similar anti-angiogenic properties [6]. In agreement with these observations, we have found that VEGF111b could also inhibit angiogenesis. VEGF111b inhibits proliferation, migration and tube formation of endothelial cell, which is slightly less efficient than VEGF165b. This may be related to the lack of exons 5–7 in VEGF111b which affect the location of the receptor-binding interfaces in the dimeric molecule.VEGF111b, a new splicing isoform of VEGF-A consisting of exons 1–4 and 8b, has binding domain of VEGF-R1 and VEGF-R2 that are located in exons 3 and 4. In virtue of exon 8b generated by C-terminal distal splicing to replace 8a, similar to VEGF165b, VEGF111b lacks the binding sites of NRP-1 and heparin. Although we have shown that VEGF111b might bind to conventional VEGF-R2 with the results of reducing VEGFR-2 tyrosine phosphorylation and accordingly downstream signaling, further work is needed to clarify the mechanism differences between VEGF111b and its corresponding isoform VEGF111. Research into mechanistic differences between VEGF165b and VEGF165 could give us some tips. VEGF165b cannot fully bind the VEGFR-2/NRP-1 complex [18,22], leading to a partial rotation of the intracellular domain of VEGFR-2 [23]. Maybe, VEGF111b have similar receptor binding mechanism, resulting in reduced phosphorylation of intracellular tyrosine residue 1175 on VEGFR-2 and a weaker and transient phosphorylation of downstream PI3K/Akt and ERK1/2.

In the research of VEGFxxx family, plasmin could cleave VEGF between Arg110 and Ala111 to generate VEGF110 fragment, and matrix metalloproteinases, such as MMP-3, MMP-7 and MMP-9, could cleave VEGF to generate VEGF113 fragment [24]. VEGF110 and VEGF113 both have strong pro-angiogenic activity. However, the cleavage sites of the plasmin and MMPs also exist in the amino acid sequences of VEGF165b and VEGF121b [25]. As a result of these enzymes, VEGF121b and VEGF165b not only lost SLTRKD sequence which is necessary to inhibit angiogenesis, but generate VEGF110 and VEGF113 fragments which can promote the angiogenesis activity. Compared with VEGF121b and VEGF165b, VEGF111b has no cleavage sites for the plasmin and MMPs. Thus, the biological stability of VEGF111b may be much higher than that of VEGF165b and VEGF121b. In summary, the inhibitory properties of VEGF111b on the process of in vitro angiogenesis and its remarkable resistance to proteolysis make it a beneficial alternative candidate of tumor angiogenesis inhibitors for therapeutic use in ovarian tumors. Further studies will be required to elucidate the mechanism of different alternative splicing of VEGF-A for favoring DSS selection in exon 8 to express more anti-angiogenic isoforms.

Author contributions

G.F. and L.X.L. conceived and designed the study. G.F. and J.K. carried out the experimental work and wrote the manuscript. M.S. and B.P. interpreted the data. Y.Y.Q. and L.M.Z. conceived and designed the study and wrote the manuscript.

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Disclosures

The authors disclosed no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.144.

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