Life Sciences 221 (2019) 267–273

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Serum promotes vasculogenic mimicry through the EphA2/VE-cadherin/AKT pathway in PC-3 human prostate cancer cells

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ARTICLE INFO

Keywords:
Serum
Vasculogenic mimicry
EphA2
VE-cadherin
AKT
PC-3 cells

ABSTRACT

Aims: Serum is widely used for *in vitro* cell culture of eukaryotic cells. Although serum is well known to affect various biological activities in cancer cells, its effect in vasculogenic mimicry (VM) is not yet fully defined. Thus, this study investigated the role of serum in VM in human prostate cancer (PCa) PC-3 cells.

Main methods: Invasion assay and 3D culture VM tube formation assay are performed. VM-related molecules are checked by western blot and reverse transcriptase-polymerase chain reaction. Nuclear twist is detected by confocal after twist-FITC/DAPI double staining.

Key findings: Serum dramatically induced not only invasion but also VM. Serum increased the phosphorylation of erythropoietin-producing hepatocellular A2 (EphA2) without affecting EphA2 expression. Both the protein and mRNA expression levels of vascular endothelial cadherin (VE-cadherin) are up-regulated by serum. Twist expression was increased in the nucleus by serum. Serum activated AKT through phosphorylation, despite the unchanged AKT expression. Serum caused an increase in matrix metalloproteinase-2 (MMP-2) and laminin subunit 5 gamma-2 (LAMC2) protein expressions. Wortmannin, a phosphoinositide-3-kinase inhibitor, significantly decreased serum-induced invasion and VM.

Significance: These results demonstrated that serum activates EphA2 and up-regulates twist/VE-cadherin, which in turn activate AKT that up-regulates MMP-2 and LAMC2, thereby inducing the invasion and VM of human PCa PC-3 cells.

1. Introduction

Blood supply that serves oxygen and nutrients is indispensable for survival in mammalian cells [1]. In addition, tumor growth beyond 2–3 mm in diameter and metastasis ultimately require a rich blood supply through new blood vessels formation by endothelial cells (EC) referred to as “angiogenesis” [1,2]. Angiogenesis is a promising and attractive target for successful treatment of cancer patients. However, in animal model, anti-angiogenic therapy delayed in tumor growth during the initial treatment period, but the tumor regrew gradually, indicating resistance to VEGF inhibition [3]. In addition, there was little or no beneficial effect in some cancer patients treated with anti-angiogenic drugs [4–6]. These reports suggested that the failure of anti-angiogenic therapy may be due to sufficient blood supply through alternative vessel formation. In 1999, it was reported that aggressive melanoma cells themselves but non-aggressive cells without EC formed matrix-rich and vascular-like networks called vasculogenic mimicry (VM) [7–9]. The VM vessels is distinguished from the EC vessels through CD31 (an EC marker)-periodic acid-schiff (PAS, staining basement membrane) double staining. The VM vessel is CD31-negative and PAS-positive due to matrix-rich networks but the EC vessel is CD31-positive and PAS-positive or only CD31-positive [10,11]. Anti-VEGF therapy had no effect on the inhibition of tumor growth in VM-competent tumor-bearing mice but not in VM-incompetent tumor-bearing mice [3]. Patients with VM-positive cancers had a significantly lower 5-year survival rate than those with VM-negative cancers. VM is closely correlated with tumor metastasis and poor prognosis in cancer patients [12].

Prostate cancer (PCa) is the most frequently diagnosed cancer and is the second cause of cancer-related deaths in North American men [13,14]. Despite treating with traditional radiation or chemotherapy strategies, treatment efficiency is still low in PCa patients. Thus, it is

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https://doi.org/10.1016/j.lfs.2019.02.043
Received 31 December 2018; Received in revised form 14 February 2019; Accepted 20 February 2019
Available online 21 February 2019
0024-3205/

necessary that the development of new strategies based on predictive targets or biomarkers that were fully addressed. Clinical study showed that VM was mainly observed in the high-risk PCa patients and was positively correlated with Gleason score, pathological stage, lymph node metastasis and distant metastasis [13,15]. Also, overall survival (OS) and disease-free survival (DFS) were significantly poorer in VM-positive patients than VM-negative patients, suggesting that as an adverse predictor for OS and DFS, VM is an independent marker of poor prognosis in PCa patients [15]. Thus, VM can be a promising and attractive target for cancer therapy strategy in PCa patients.

Serum contains growth factors, hormones, amino acids, proteins, lipids, carbohydrates, vitamins and other compounds, and is used for the *in vitro* cell culture of eukaryotic cells. Fetal bovine serum (FBS) is the most widely applicable serum-supplement due to having more growth factors and low level of γ-globulins, facilitating cell growth [16]. Cells treated with FBS cause a significantly greater increase in proliferation than cells treated with horse serum [17]. Serum is popularly used as a chemoattractant for the migration and invasion of various types of cancer cells [18–20]. Henry et al. [21] reported that the migration of keratinocytes is obvious greater in the presence of serum compared with plasma. Because serum contains some migration-promoting factor but not plasma. Although serum has various biological activities, its effect in VM process by cancer cells is not yet well defined. Thus, this study investigated the role of serum in VM formation in human PCa PC-3 cells.

2. Materials and methods

2.1. Cell culture

Human prostate cancer cell line, PC-3 was purchased form Korean Cell Line Bank (KCLB, Seoul), and was cultured in RPMI 1640 (Welgene, Daegu) with 10% fetal bovine serum (FBS, Welgene, Daegu) and 1% antibiotics (Welgene, Daegu) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Trasnwell invasion assay

Cell invasion assay was performed using a Transwell® cell culture insert with 8 μm pores (Corning Inc., NY). Each of insert was coated with a 1:20 dilution of Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) and allowed to polymerize at 37 °C for 2 h. Cells (1×10^5 cell/well/400 μl) were seeded into the inserts and various concentrations of serum (0, 1, 2 and 5%) in RPMI 1640 were added in the lower wells. For inhibitor assay, wortmannin (Millipore, Burlington, MA) was loaded in the inserts with or without serum. After 24 h incubation at 37 °C, cells were fixed and stained with Diff quick solution (Sysmex, japan). The cells on the inserts were wiped off with a cotton swab and then dried in the air. Then, the invaded cells were photographed under an inverted light microscope Ts2_PH (Nikon, Japan) at 200× magnification and counted.

2.3. 3D culture VM tube formation assay

Cells (3.2×10^5) were seeded on a 24-well plate, which was coated with 100 μl of matrigel at 37 °C for 1 h and treated with various concentrations of serum. For inhibitor assay, cells were treated with wortmannin with or without serum. After 16 h incubation at 37 °C, images were photographed under an inverted light microscope Ts2_PH (Nikon, Japan) at 40× magnification and the number of formed VM structures was counted.

2.4. Western blot analysis

Cells (3.7×10^5) were seeded on a 6-well plate and treated with various concentrations of serum at 37 °C for 24 h. The cells were digested in RIPA buffer containing inhibitors for phosphatases and proteases (Thermo Scientific, Rockford, IL). Total protein lysates (20 μg) were separated by 8 or 10% SDS-PAGE gel and transferred onto a nitrocellulose transfer membrane (Pall Corporation, Port Washington, NY) for 110 min at 300 mA. After blocking with 5% nonfat skim milk or 5% bovine serum albumin (BSA) for 1 h at RT, the membrane was probed with specific primary antibodies (Table 1) at 4 °C overnight and then specific secondary antibodies (Table 1) for 2 h at RT. Proteins were detected using an ECL detection kit (GE Healthcare) and quantified using an ImageJ 1.40 g software (National Institute of Health, Bethesda, MD).

Table 1
Antibodies used in this study.

Antibody	Company	Dilution	Product no.
β-actin	Sigma-Aldrich	1:20,000	A5316
pAKT	CST	1:2000	4060
AKT	CST	1:2000	4691
MMP-2	Abcam	1:1000	ab86607
LAMC2	Abcam	1:1000	ab96327
EphA2	CST	1:1000	6997
pEphA2	CST	1:1000	6347
VE-cadherin	Abgent	1:1000	AP2724a
Twist	Abcam	1:1000	ab50887
Goat anti-rabbit IgG-HRP	CST	1:5000	7074P2
Goat anti-mouse IgG-HRP	Bio-Rad	1:5000	STAR120P

CST, Cell Signaling Technology (Beverly, MA); Sigma-Aldrich (St Louis, MO); Abcam (Cambrige, UK); Abgent (San Diego, CA).

2.5. Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells (3.7×10^5) were seeded on a 6-well plate and treated with various concentrations of serum at 37 °C for 24 h. Total RNA was extracted from the cells using a TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA samples were reverse transcribed and synthesized as cDNA using Oligo dT primers (Bioneer Corporation, Daejeon) and M-MLV reverse transcriptase (Promega, Madison, WI). PCR was carried out using EconoTaq PLUS GREEN 2× Master mix (Lucigen, Middleton, WI) with specific primers (Table 2). The PCR products were identified on 2% agarose gels and quantified using an ImageJ 1.40 g software (National Institute of Health).

2.6. Immunofluorescence assay

Cells (1.2×10^5) were seeded in an 8 well-chamber slide and treated with 2% of serum at 37 °C for 24 h. The cells were fixed with 3.7% formaldehyde for 10 min, kept stable in 0.2% Triton-X in PBS for 10 min and put a blocking buffer (5% BSA in PBS) for 1 h at room temperature (RT). After washing three time with PBS, the cells were incubated with Twist antibody (Abcam, ab50887, 1:50) overnight at 4 °C and FITC-conjugated secondary antibody (Millipore, 12–506, 1:100) for 1 h at RT. After washing three time with PBS, the nuclei were stained with DAPI (1 μg/ml in PBS) for 5 min at RT. The slides were mounted in 30% glycerol and images were obtained by a FLUOVIEW FV10i confocal microscope (Olympus, Tokyo, Japan) at 600×

Table 2
Primers used in this study.

mRNA	Primer sequences	Size	Annealing temperature
β-actin	S: GAGAAGATGACCCAGATCATGT AS: ACTCCATGCCCAGGAAGGAAGG	463	60
VE-cadherin	S: GCACCAGTTTGGCCAATATA AS: GGGTTTTTGCATAATAAGCAGG	149	60

magnification.

2.7. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis between four groups was calculated by one-way ANOVA followed by Tukey's studentized range test using a GraphPad Prism software (GraphPad Software Inc.). Means with different alphabets are significant different between groups. Statistical analysis between two groups was calculated by Student's *t*-test using a Sigma plot software (Systat Software Inc.) with $p < 0.05$ as considered statistically significant.

3. Results

3.1. Serum promotes the formation of VM in PC-3 cells

Serum is well known to facilitate cell motility such as migration and invasion [21,22]. Also, VM is closely associated with motility of cancer cells [23,24]. Transwell invasion assay was performed to investigate whether serum promotes the invasive ability of PC-3 cells. Cells were seeded into matrigel-coated inserts and serum was added to induce cell motility in the lower wells. As expected, serum strikingly induced cell invasion in a dose-dependent manner, which was significantly increased by 6.3-, 8.9- and 10.4-fold at 1, 2 and 5% of serum compared with untreated control, respectively (Fig. 1A and B). These results demonstrated that serum induces the invasion of PC-3 cells.

To evaluate whether serum induces the VM formation of PC-3 cells, 3D culture VM formation assay was carried out in serum-treated cells for 16 h on a matrigel-coated well plate. Serum dramatically increased the formation of complete tubular channels by 1.7-, 2.1- and 2.2-fold at

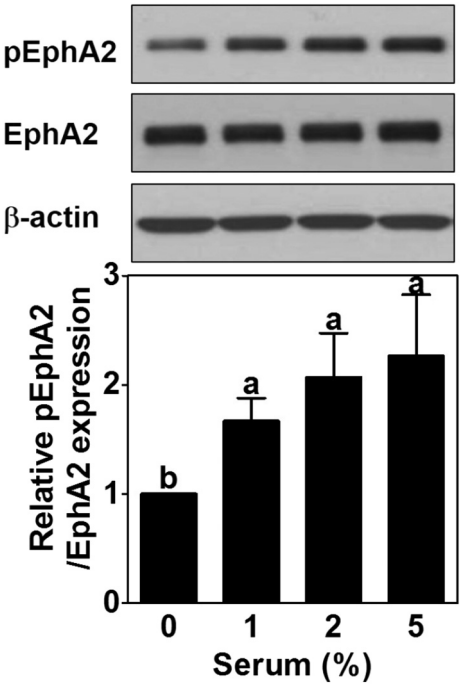

Fig. 2. Serum activates EphA2 in PC-3 cells. Cells were treated with various concentrations of serum for 24 h. Protein lysates were detected by western blot using the indicated antibodies. β-actin is a loading control. Data are presented as mean ± SD and were statistically calculated by one-way ANOVA followed by Tukey's studentized range. Means with different alphabets are significant different between groups.

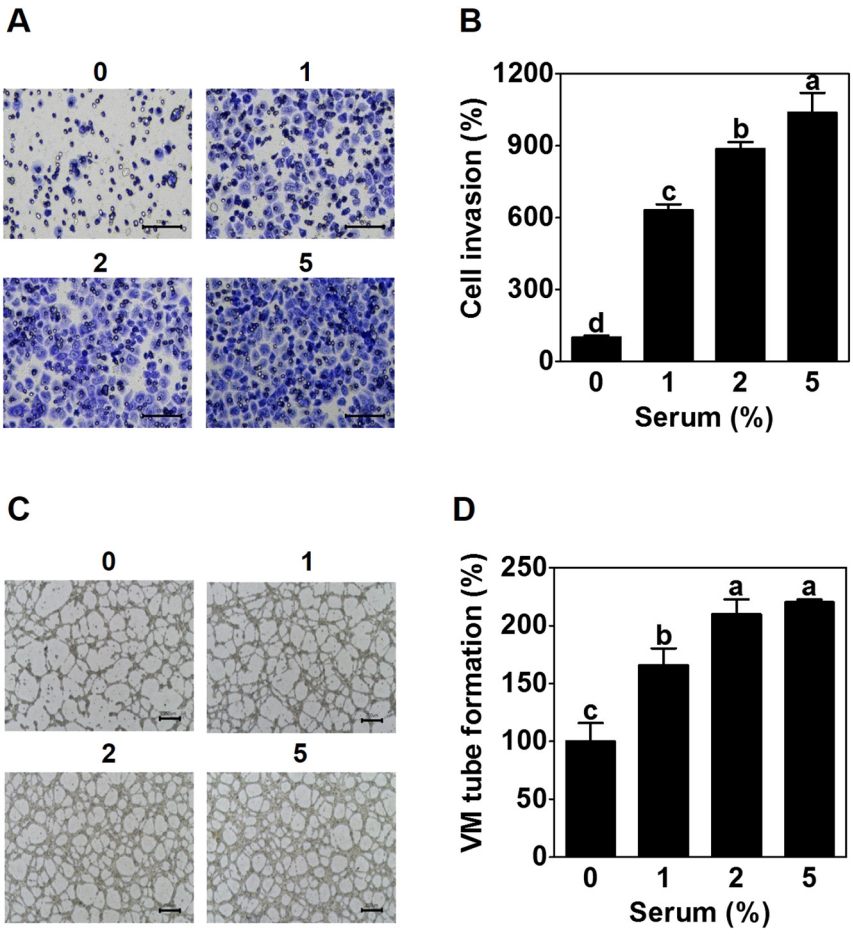

Fig. 1. Serum promotes the formation of VM in PC-3 cells. (A) Invasion assay was performed using a matrigel-coated transwell plate. After 24 h incubation with serum as a chemoattractant, cells were stained, and images were photographed under an inverted light microscope at 200× magnification. Scale bar = 100 μm. (B) The number of invaded cells was counted. (C) Cells were treated with various concentrations of serum (0, 1, 2, 5%) on a matrigel-coated well plate. After 16 h incubation, images were photographed under an inverted light microscope at 40× magnification. Scale bar = 250 μm. (D) The number of formed VM structures was counted. Data are presented as mean ± SD and were statistically calculated by one-way ANOVA followed by Tukey's studentized range. Means with different alphabets are significant different between groups.

1, 2 and 5% compared with untreated control, respectively (Fig. 1C and D). These results indicated that serum promotes the VM of PC-3 cells.

3.2. Serum activates EphA2 in PC-3 cells

To explore whether serum regulates the activation of erythropoietin-producing hepatocellular A2 (EphA2) to induce VM, PC-3 cells were treated with serum and then the expressions of phospho-EphA2 and EphA2 were determined by western blot. Serum significantly increased the phosphorylation level of EphA2 compared with untreated control regardless of serum concentrations, whereas there was no effect of serum on EphA2 expression between four groups. (Fig. 2). These results indicated that serum activates EphA2 in PC-3 cells.

3.3. Serum up-regulates VE-cadherin expression through increasing the nuclear twist expression in PC-3 cells

To demonstrate whether serum regulates the vascular endothelial cadherin (VE-cadherin) expression to induce VM, PC-3 cells were treated with serum and then the protein level of VE-cadherin was checked by western blot. Serum effectively up-regulated VE-cadherin expression compared with untreated control in a dose-dependent manner (Fig. 3A). To define whether VE-cadherin mRNA level contributes to the up-regulation of VE-cadherin protein level, RT-PCR was conducted after serum treatment. Consistent with protein level, serum except at 1% effectively increased VE-cadherin mRNA expression compared with untreated control in a dose-dependent manner (Fig. 3B). These results indicated that serum regulates VE-cadherin expression at the transcriptional level in PC-3 cells.

To identify whether twist is involved in the upregulation of VE-cadherin expression by serum, western blot and immunofluorescence assay were performed after serum treatment. As shown in Fig. 3C, serum except at 1% dramatically increased twist protein level compared with untreated control in a dose-dependent manner. Immunofluorescence staining showed that serum enhanced twist expression compared with untreated control in the nucleus (Fig. 3D). Taken together, these results verified that serum causes an increase in the nuclear twist expression, thereby up-regulating VE-cadherin expression in PC-3 cells.

3.4. Serum activates AKT and up-regulates MMP-2 and LAMC2 expressions in PC-3 cells

To evaluate the involvement of AKT in serum-induced VM, western blot was conducted after serum treatment. The phosphorylation of AKT was strikingly increased by all concentrations of serum compared with untreated control in a dose-dependent manner, despite the unchanged

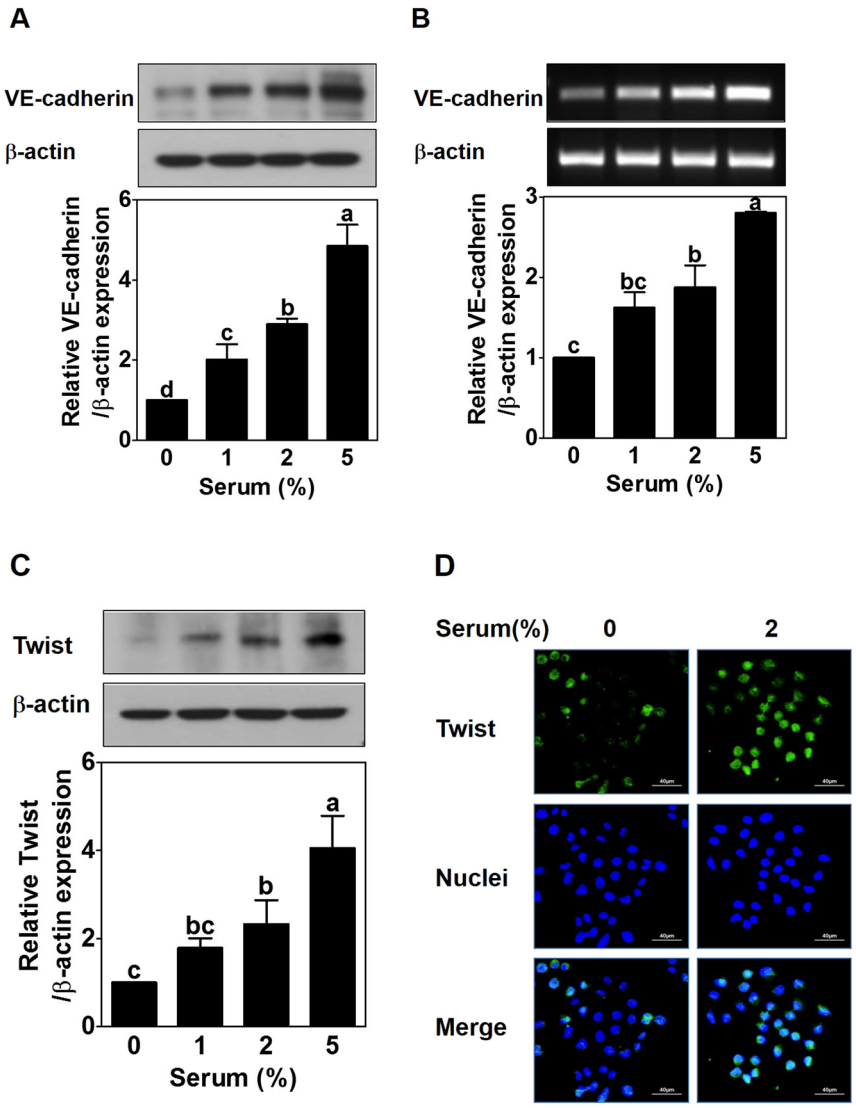

Fig. 3. Serum up-regulates VE-cadherin expression through increasing the nuclear twist expression in PC-3 cells. Cells were treated with various concentrations of serum for 24 h. (A) Protein lysates were detected by western blot using the VE-cadherin antibody. (B) mRNA level was analyzed by RT-PCR using the indicated primers. (C) Protein lysates were detected by western blot using the twist antibody. β-actin is a loading control. Data are presented as mean ± SD and were statistically calculated by one-way ANOVA followed by Tukey's studentized range. Means with different alphabets are significant different between groups. (D) Serum-treated cells for 24 h were fixed, permeabilized and blocked. After incubated with Twist antibody followed by FITC-conjugated secondary antibody and counterstained with DAPI (nuclei). Images were obtained by a confocal microscope at 600× magnification. Scale bar = 40 μm.

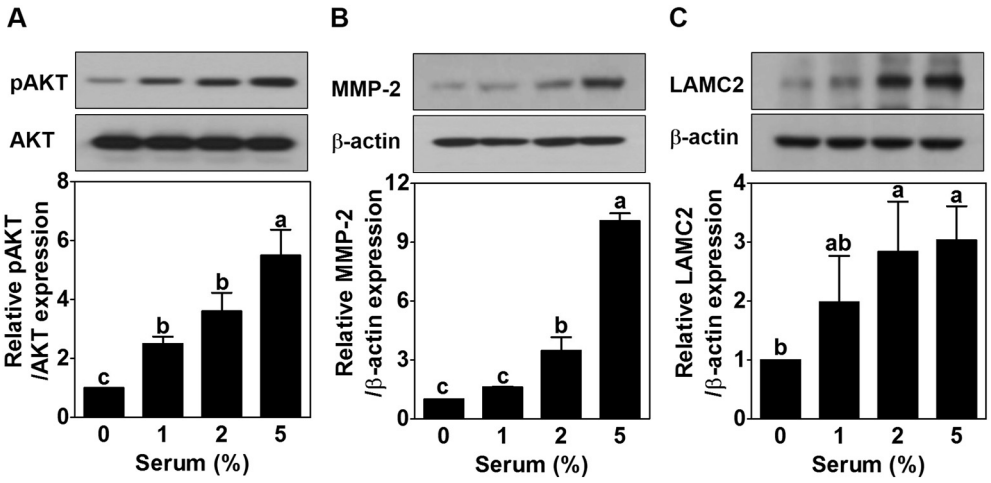

Fig. 4. Serum activates AKT and up-regulates MMP-2 and LAMC2 expressions in PC-3 cells. Cells were treated with various concentrations of serum for 24 h. Protein lysates were detected by western blot using the phospho-AKT, AKT (A), MMP-2 (B) and LAMC2 (C) antibodies. β-actin is a loading control. Data are presented as mean ± SD and were statistically calculated by one-way ANOVA followed by Tukey's studentized range. Means with different alphabets are significant different between groups.

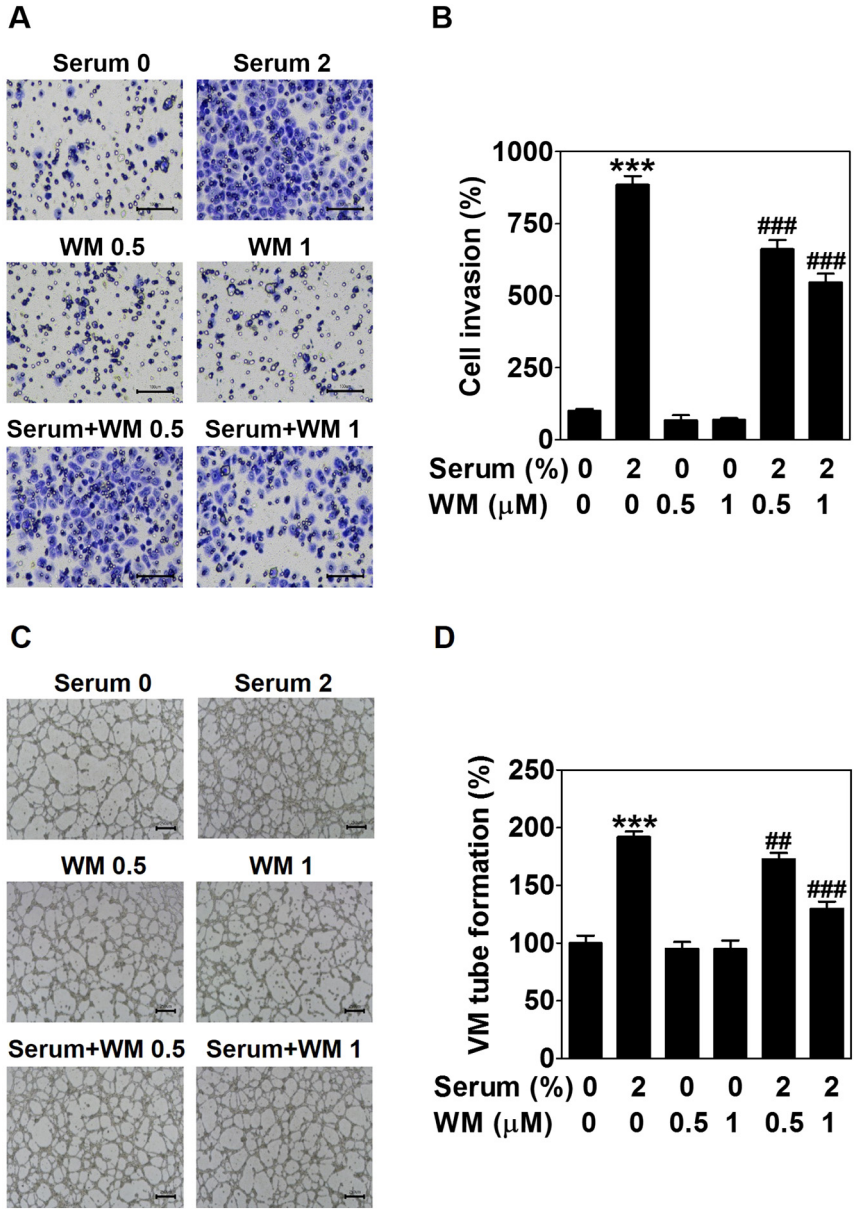

Fig. 5. Serum promotes the formation of VM through activating the AKT pathway in PC-3 cells. (A) Invasion assay was performed using a matrigel-coated transwell plate. After 24 h incubation with serum as a chemoattractant in the presence or absence of wortmannin (WM), cells were stained, and images were photographed under an inverted light microscope at 200× magnification. Scale bar = 100 μm. (B) The number of invaded cells was counted. (C) Cells were treated with serum in the presence or absence of wortmannin (WM) on a matrigel-coated well plate. After 16 h incubation, images were photographed under an inverted light microscope at 40× magnification. Scale bar = 250 μm. (D) The number of formed VM structures was counted. Data are presented as mean ± SD and were statistically calculated by Student's *t*-test. *** $p < 0.001$ *vs.* untreated control. ## $p < 0.01$ and ### $p < 0.001$ *vs.* serum-treated control.

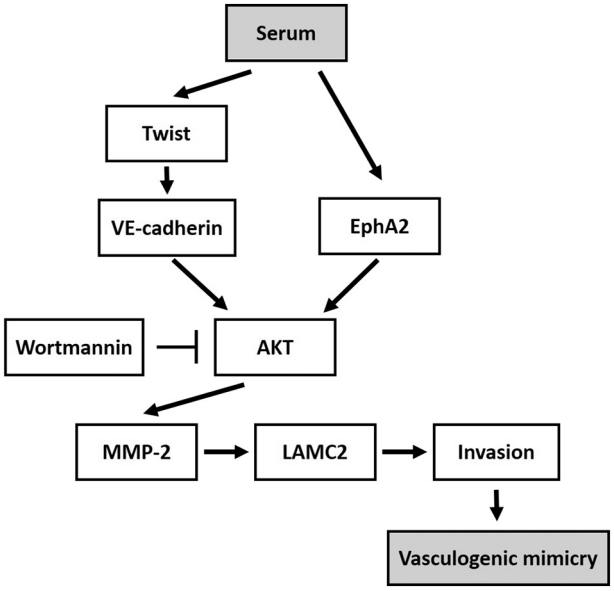

Fig. 6. Proposed molecular mechanisms for the serum-induced vasculogenic mimicry of human PCa PC-3 cells.

total AKT expression (Fig. 4A). These results indicated that AKT is activated by serum. In addition, serum except at 1% up-regulated the expressions of matrix metalloproteinase-2 (MMP-2) and laminin subunit 5 gamma-2 (LAMC2) in a dose-dependent manner (Fig. 4B and C). Taken together, these results indicated that the AKT/MMP-2/LAMC2 signaling pathway is engaged in VM process induced by serum.

3.5. Serum promotes the formation of VM through activating the AKT pathway in PC-3 cells

To further detect whether the AKT pathway is essential part for VM formation by serum, transwell invasion assay and 3D culture VM formation assay were used after serum treatment in the presence or absence of wortmannin (WM), a phosphoinositide-3-kinase (PI3K) inhibitor. After incubation, invasion was obviously induced in serum only treated cells compared with untreated cells, which was significantly reduced by WM in a dose-dependent manner (Fig. 5A and B). Consistent with invasion results, serum led to an evident increase in VM formation compared with control, which was also suppressed by WM in a dose-dependent manner (Fig. 5C and D). WM itself had no effect on invasion (Fig. 5A and B) and VM formation (Fig. 5C and D). These results suggested that the AKT pathway is required to induce VM by serum.

4. Discussion

As a serum-supplement, FBS is most widely used for the *in vitro* cell culture of eukaryotic cells [16]. Although serum is reported to affect invasion, migration, proliferation and cell growth in several types of cancer [17–20], the involvement of serum in VM formation is not yet fully elucidated. Thus, this study investigated whether and how serum affects VM formation in human PCa PC-3 cells.

VM is the *de novo* formation of vascular-like networks by aggressive and metastatic cancer cells without EC and is observed in various types of cancer cells including PCa [7–9]. VM is resistant to anti-angiogenic therapies that inhibit vessel formation by EC [25]. In addition, VM expression is closely linked with pathological stage, invasion and metastasis, leading to poor prognosis including shorter OS and 5-year survival in cancer patients [12,13,15,23]. Thus, VM is a novel target for cancer therapy strategy. As expected, serum dramatically elevated the invasive ability of PC-3 cells by transwell invasion assay (Fig. 1A and B). PC-3 cells formed the vascular-like tubular structure after serum treatment by 3D culture VM formation assay (Fig. 1C and D). These results demonstrated that serum can promote the formation of VM in PCa cells.

EphA2 is a receptor tyrosine kinase that plays a key role in invasion, metastasis and VM formation [13,26,27]. Cell invasion was highly induced by EphA2 overexpression in prostate cancer cells [27]. Also, EphA2 expression and phosphorylation levels in PCa were significantly correlated with VM *in vitro* and *in vivo* [13]. Serum activated EphA2 by phosphorylation in PC-3 cells (Fig. 2), indicating that it may be involved in serum-induced invasion (Fig. 1A and B) and VM formation (Fig. 1C and D).

VE-cadherin is an adhesion molecule exclusively expressed in EC and is essential for proper vascular development through controlling EC behavior [28,29]. Interestingly, it is overexpressed in aggressive cancer cells but not non-aggressive cancer cells [24,25]. Knockout of VE-cadherin expression in aggressive melanoma cells suppressed VM formation [30,31]. Serum increased VE-cadherin at both protein (Fig. 3A) and mRNA levels (Fig. 3B) in PC-3 cells. As a transcription factor, twist regulates VE-cadherin expression through direct binding to VE-cadherin promoter, promoting VM formation [23,32,33]. Twist was overexpressed in the nucleus in VM-positive human hepatocellular carcinoma [33]. Serum treatment caused an increase in nuclear twist expression (Fig. 3C and D). Taken together, these results indicated that serum upregulates VE-cadherin expression through elevating nuclear twist expression.

EphA2 and VE-cadherin are co-localized in cell-cell junctions, activating the PI3K/AKT pathway [24]. As a downstream of EphA2 and VE-cadherin, AKT participates in VM process by regulating MMP-2 and LAMC2 [24,31,34,35]. Serum activated AKT by phosphorylation without affecting AKT expression (Fig. 4A). MMP-2 is emerging as a key player in angiogenesis, invasion and metastasis by the breakdown of basement membrane and extracellular matrix (ECM) [36–38]. LAMC2 is upregulated in most cancers and is engaged in tumor invasion and metastasis. Patients with high LAMC2 expression have a shorter OS than those with low LAMC2 expression [39]. Cancer with VM-competent strongly expresses MMP-2 and ECM component including LAMC2 [34]. Serum upregulated the protein levels of MMP-2 and LAMC2 in PC-3 cells (Fig. 4B and C). After WM, a PI3K inhibitor that inhibits the phosphorylation of AKT [40], treatment, serum-induce invasion (Fig. 5A and B) and VM (Fig. 5C and D) were effectively inhibited. These results indicated that serum activates the AKT pathway, stimulating MMP-2 and LAMC2 expressions, which mediate the invasion and VM formation of PC-3 cells.

5. Conclusion

The results from this study in human PCa PC-3 cells are summarized in Fig. 6. This study demonstrated that serum induces the invasion and VM of PC-3 cells. Serum activates EphA2 and increases VE-cadherin expression through nuclear twist expression. As a downstream of EphA2/VE-cadherin, AKT is activated by serum treatment, thereby upregulating the protein expressions of MMP-2 and LAMC2. Serum-induced invasion and VM formation are suppressed by inhibiting the AKT pathway. Thus, serum promotes VM by regulating EphA2/VE-cadherin/AKT pathway in human PCa PC-3 cells. These results serve new insight into how serum affects VM process. However, more research into the molecular mechanisms of VM is required for development as a prognostic and predictive marker in PCa.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by Basic Science Research Program

through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07040506).

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