Peroxisome proliferator-activated receptor γ ligands inhibit VEGF-mediated vasculogenic mimicry of prostate cancer through the AKT signaling pathway

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Abstract. Vasculogenic mimicry (VM) describes functional vascular channels composed only of tumor cells and its presence predicts a poor prognosis for patients with prostate cancer. The present study demonstrated that prostate cancer PC-3 cells were able to form a patterned matrix or tubular VM in 3D cultures *in vitro* and rosiglitazone (RSG), the ligand of peroxisome proliferator-activated receptor γ (PPARγ) and effectively inhibited the formation of VM structures in a dose- and PPARγ-dependent manner. In addition, RSG significantly inhibited prostate cancer cell migration and invasion. The inhibitory effect of RSG on VM formation could be at least partially explained by an RSG-driven downregulation of vascular endothelial growth factor (VEGF) levels and phosphorylation of AKT, which is known to be important in VM. Furthermore, the present study highlighted that VEGF and the phosphoinositide 3-kinase/AKT pathway exert a positive feedback regulation in the process of VM formation. These findings reveal new therapeutic potential for PPARγ ligands in anti-cancer therapy.

Introduction

The development and growth of a tumor requires a sufficient blood supply. It is a widely accepted paradigm that tumor vasculature is mostly generated through angiogenesis, the process of new vessel growth from pre-existing vessels (1). However, tumors do not only rely on host blood vessels for nourishment; they are also able to form their own vasculature (2). In 1999, Maniotis *et al* (3) reported that highly aggressive uveal melanomas are able to form vessels using tumor cells instead of endothelial cells and this phenomenon of tumor vascularization has been named vasculogenic mimicry (VM). VM has been described in prostate cancer and certain other types of highly aggressive tumors, and is associated with tumor cell migration and invasion (4-7). Several key molecules, including vascular endothelial (VE)-cadherin, matrix metalloproteinases (MMPs), laminin-5γ2 chains and CD147 have been implicated in VM (8,9). Several studies have demonstrated that vascular endothelial growth factor (VEGF) is important in the formation of VM and suggested that VEGF→ephrin type-A receptor (EphA2)→MMPs→VM is the main pathway for VM formation (10). Although VM has been demonstrated to occur in prostate cancer, a degree of which correlates with a poor clinical outcome (11), the precise molecular events underlying the process of VM in prostate cancer have remained to be fully elucidated.

Peroxisome proliferator-activated receptor (PPAR) is a member of the family of nuclear receptor transcription factors. PPARγ is expressed in numerous different tissues and regulates lipid metabolism, glucose homoeostasis and inflammation (12). PPARγ is the molecular target of the thiazolidinedione (TZD) class of antidiabetic drugs. The activation of PPARγ by TZDs and other ligands have been demonstrated to inhibit proliferation and invasion, and to induce apoptosis and cell cycle arrest in prostate and other cancer cells through PPARγ-dependent and -independent pathways (13-15). Certain studies demonstrated that TZDs are able to induce the inhibition of angiogenesis in cancer and human umbilical vein endothelial cells (16,17). By contrast, several studies suggested that PPARγ activation may enhance tumor angiogenesis (18). In summary, the molecular mechanisms underlying the anti-cancer effects of TZD remain elusive.

Previous studies have demonstrated that the activation of PPARγ affects angiogenesis and VEGF production by tumor cells. In addition, VEGF is crucial in tumor angiogenesis and VM formation (10). Thus, the present study hypothesized that VM formation may be a potential target of PPARγ ligands. The present study investigated the effect of rosiglitazone (RSG), a ligand of PPARγ, on the formation of VM in PC-3 cells and

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the underlying mechanisms *in vitro*. To the best of our knowledge, the present study was the first to provide evidence that exposure of PC-3 cells to RSG induced decreased migration, invasion and formation of VM. The inhibitory effect of RSG on VM formation may at least partially be explained by an RSG-driven downregulation of VEGF and the phosphorylation of AKT.

Materials and methods

Cell culture. The human prostate cancer cell line PC-3 was maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), $1x10^5$ U/l of penicillin and streptomycin (HyClone, Logan, UT, USA) at 37˚C in an incubator with 5% CO_2.

Three-dimensional cultures. Matrigel® (BD Biosciences, San Jose, CA, USA) was dropped onto glass coverslips and allowed to polymerize for 1 h at 37˚C. The cells were then seeded on top of the gels at a high density and allowed to incubate. The addition of conditioned media containing 10% FBS was performed by the pretreatment and the continuous treatment regimes during the 48 h incubation period in three-dimensional cultures (19). Fresh media containing drugs was added every 24 h. For quantization of VM networks, the number of tube connections per field (magnification, x40) was counted. The Student's t-test was performed on at least five fields for each variable collected from three different experiments.

Cell migration assay. Cell migration was evaluated using an *in vitro* wound-healing assay. PC-3 cells were plated in a six-well plate and allowed to grow to confluence. The monolayer culture was then scrape-wounded with a sterile micropipette tip to create a denuded zone (gap) of constant width. The cells were exposed to various concentrations of RSG, 24 h after removing the cellular debris with phosphate-buffered saline (PBS). The speed of wound closure was monitored by measuring the ratio of the distance to that at 0 h. Each experiment was performed in triplicate and the results are presented as the mean ± standard error.

Cell invasion assay. The invasive potential was quantified using a Matrigel-coated transwell system, as previously described (20). The chamber (Corning Inc., Corning, NY, USA), which contained an 8-μm pore size polycarbonate membrane filter, was coated with Matrigel® and inserted into a 24 well culture plate. RSG was pre-incubated with PC-3 cells at various concentrations, respectively, for 24 h prior to seeding. The cell suspension (200 μl) was added into the upper chamber in serum-free medium and 500 μl of medium, which was supplemented with 20% FBS, was added into the lower chamber. Following 48 h incubation, the cells on the upper surface of the filters were completely removed by wiping with cotton swabs. Then cells that invaded the lower surface of the membrane were fixed with methanol and stained with crystal violet. Each experiment was conducted in triplicate. The cells were counted in five fields in each well by light microscopy. The cells were assessed for their relative invasion ability as percentages of the untreated controls.

Western blot analysis. Western blot analysis was performed to assess the protein levels of VE-cadherin and the expression and phosphorylation of signal transduction molecules, including AKT. Following being treated with RSG (Sigma-Aldrich, St. Louis, MO, USA), recombinant human (rh) VEGF165 (R&D Systems, Minneapolis, MN, USA), LY294002 (Cell Signaling Technology, Danvers, MA, USA) or GW9662 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), PC-3 cells were washed twice with PBS and treated with extraction buffer (50 mM of Tris-Cl; pH 7.5; 150 mM of NaCl, 0.1% SDS, 1% NP-40 and 0.5% deoxycholic acid). The cell extractions were collected and centrifuged at 10,000 x g for 10 min at 4˚C, and the supernatants were collected as cell lysates. The cell lysates were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were inhibited with 5% (w/v) bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 and then blotted with primary antibody. Subsequently, the membranes were incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G; Wuhan Boster Biological Technology, Ltd, Wuhan, Hubei, China). The immune-detected proteins were then visualized by enhanced chemiluminescence. The untreated cells served as a negative controls and β-actin was used as a control for equal loading. The antibodies against VE-cadherin, AKT, p-AKT (Ser473) and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA).

ELISA assay. Conditioned medium was prepared as mentioned above and VEGF concentrations in the medium were determined using a commercial Human VEGF Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. The absorbance at 450 nm was measured and corrected using the 540 nm reading on an ELX-800 uv microplate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA).

quantitative polymerase chain reaction (qPCR) assay. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed. qPCR was accomplished with TransStart Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China) using a Bio-Rad iQ5 Sequence Detection System (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: one cycle at 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The results were normalized to those obtained with GAPDH. Each sample was assayed in triplicate and the experiment was repeated twice. The CT value was measured and $2^{-\Delta CT}$ ($\Delta CT = CT - CT_{GAPDH}$) was defined as the quantity of the amplified fragment. The primer sequences are listed in Table I.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was analyzed by one-way analysis of variance. If significance was observed, the Dunnett's post-hoc

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCATGAGAAGTATGACAACAGCC	GGGTGCTAAGCAGTTGGTG
VE-cadherin	GACGCCCGGCCTTCCCTCTA	TCGTGGTCCGCCTCGTCCTT
VEGF	TTACGGTCTGTGTCCAGTGTA	TTCTCTGTTATGTTGCCAGCC

VEGF, vascular endothelial growth factor; VE-cadherin, vascular endothelial cadherin.

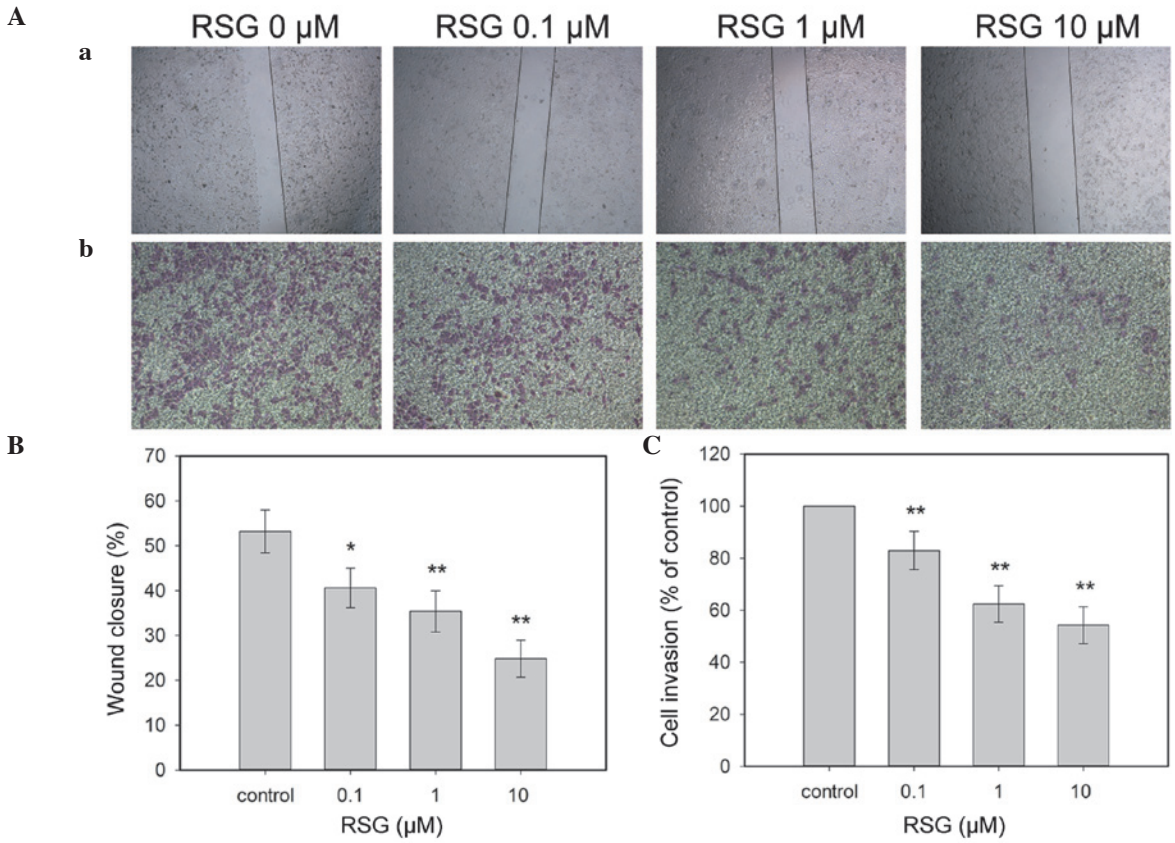

Figure 1. Effects of RSG on the migration and invasion of PC-3 cells. (Aa) Images of the cells which migrated to the wounded region were captured (magnification, x40); (Ab) images of the invaded cells were captured (magnification, x100). (B) Wound area of the cell cultures was quantified in four fields in each treatment and the data were calculated from three independent experiments. (C) Invaded PC-3 cells were counted in five random fields in each treatment and data were calculated from three independent experiments. *P<0.05, **P<0.01, compared with the untreated control. RSG, rosiglitazone.

test was used to determine the difference between treatment groups and the untreated group. P<0.05 was considered to indicate a statistically significant difference.

Results

RSG inhibits the migration and invasion of PC-3 cells. VM is associated with cell migration and invasion. To examine the effect of RSG on PC-3 cells, an *in vitro* wound healing assay was performed. Since TZDs activate PPARα and PPARδ receptors at concentrations >10 μM (21), TZDs were used at concentrations of 10 μM or less. Following incubation with different concentrations of RSG for 24 h, the migration of PC-3 cells to the denuded zone was suppressed in a dose-dependent manner (Fig. 1Aa and 1B). These results revealed that RSG significantly inhibited the motility of PC-3 cells.

Using a chamber invasion assay, it was revealed that RSG suppressed the invasion of PC-3 cells across the Matrigel-coated filter in a dose-dependent manner. Treatment with 0.1, 1 and 10 μM of RSG inhibited 17.01, 27.62 and 35.74% of cell invasion, respectively (Fig. 1Ab and 1C). The results indicated that RSG markedly inhibited the invasion of PC-3 cells.

RSG prevents the development of VM in a PPARγ-dependent manner. In order to elucidate the VM capability of PC-3 cells, a well-established *in vitro* model of VM formation was utilized. PC-3 cells were able to form patterned matrix VM or typical pipe-like VM networks within the Matrigel medium within 48 h.

Next, the present study investigated the effect of RSG on VM formed by PC-3 cells. The *in vitro* tube formation assay demonstrated that RSG effectively inhibited the formation

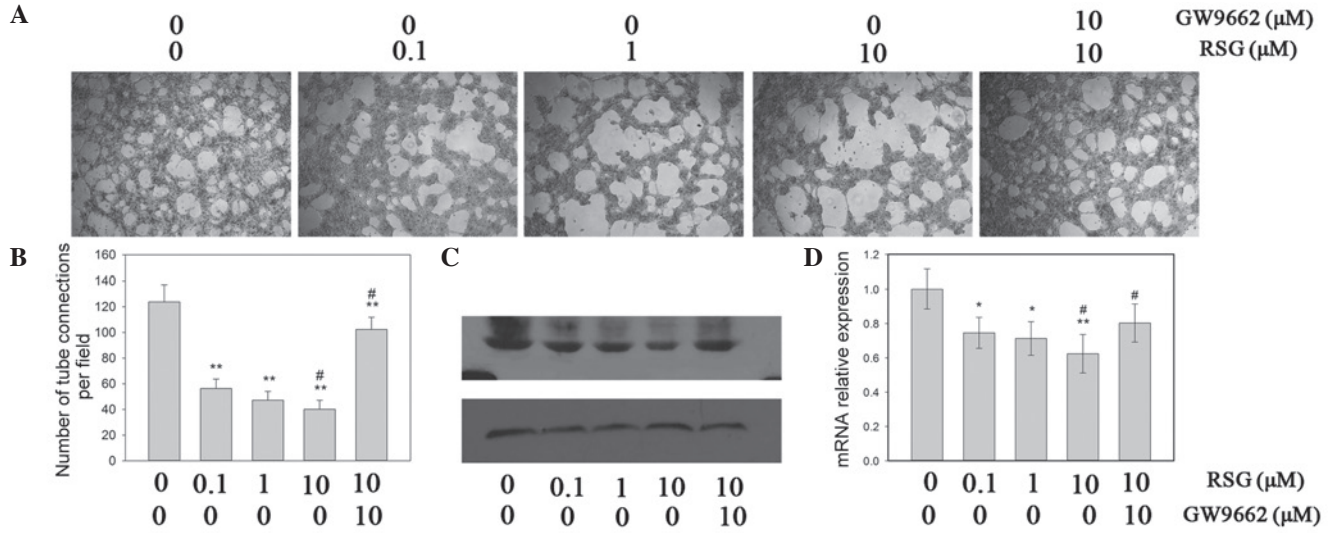

Figure 2. Effect of RSG on the VM formation of PC-3 cells. (A) PC-3 cells were incubated with Matrigel for 48 h in the absence or presence of RSG at the concentrations indicated. Images of the formation of tube-like structures were captured (magnification, x40). (B) Typical pipe-like structures of the cell cultures were quantified in four fields in each treatment and data were calculated from three independent experiments. (C) Protein and (D) mRNA expression levels of VE-cadherin were reduced in RSG-treated PC-3 cells compared with those without treatment. *P<0.05, **P<0.01, compared with the untreated control. $^{\#}$P<0.05, GW9662 pretreatment group versus 10 μM RSG treatment group. RSG, rosiglitazone; VM, vasculogenic mimicry; VE-cadherin, vascular endothelial cadherin.

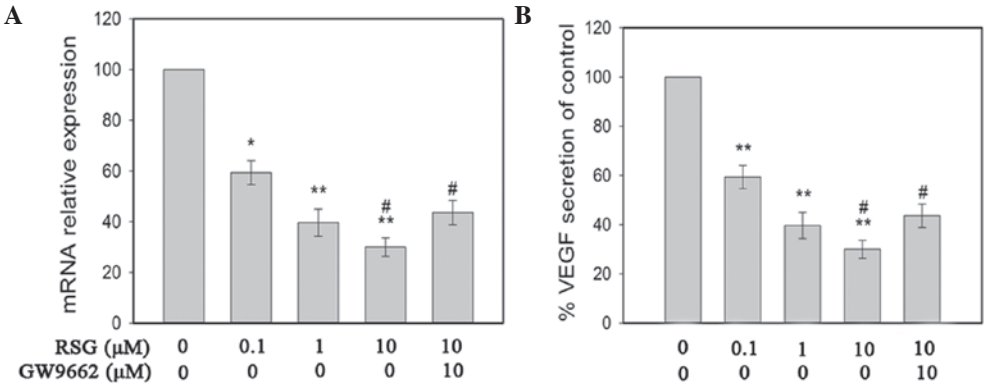

Figure 3. Effect of RSG on VEGF expression and secretion in PC-3 cells. PC-3 cells were treated with various concentrations of RSG (0, 0.1, 1 or 10 μM) for 48 h prior to harvest. Normal culture was used as the negative control. (A) Total RNA was extracted and the expression of VEGF was measured by Quantitative polymerase chain reaction. The data represent the results from three independent experiments. (B) VEGF secretion levels (expressed as the percentage of the control) in PC-3 cells conditioned media following 48 h of treatment with RSG. *P<0.05, **P<0.01, compared with the untreated control. $^{\#}$P<0.05, GW9662 pretreatment group versus 10 μM RSG treatment group. RSG, rosiglitazone; VEGF, vascular endothelial growth factor.

of capillary-like structures in a dose-dependent manner (Fig. 2A and B). Western blot analysis of whole-cell lysates from these experimental samples was conducted to assess the expression of VM-associated markers, including VE-cadherin. The results demonstrated that RSG significantly decreased the expression of VE-cadherin at the mRNA and protein levels (P<0.05; Fig. 2C and D).

Although RSG was able to affect the VM process and the expression of VE-cadherin, the mechanisms underlying these events remain to be elucidated. To confirm whether these effects were dependent upon the activation of PPARγ, GW9662, a PPARγ antagonist, was used to inhibit the function of PPARγ in prostate cancer cells. The results demonstrated that GW9662 inhibited RSG-induced reduction of VM formation. Furthermore, the RSG-induced downregulation of VE-cadherin in PC-3 cells was attenuated upon addition of GW9662 (Fig. 2). Taken together, these data suggested that RSG inhibited the VM formation of PC-3 cells in a PPARγ-dependent manner.

Effect of RSG on the expression of VEGF. Since prostate cancer cells induce angiogenesis through the expression of angiogenic factors, including VEGF, the present study evaluated whether VEGF stimulates the formation of VM in PC-3 cells, utilizing an *in vitro* tube formation assay. Quantification of the tubule number demonstrated that rhVEGF165 effectively promoted the formation of capillary-like structures (Fig. 5A and B). Furthermore, RSG inhibited the expression of VEGF in PC-3 cells. The secretion of VEGF protein was reduced to 59.38, 39.64 and 29.99% in PC-3 cells in the presence of RSG as compared with the control (Fig. 3A). Similar results were observed while detecting the VEGF mRNA levels by qPCR (Fig. 3B). Furthermore, the RSG-induced downregulation of VEGF in PC-3 cells was attenuated upon addition of GW9662

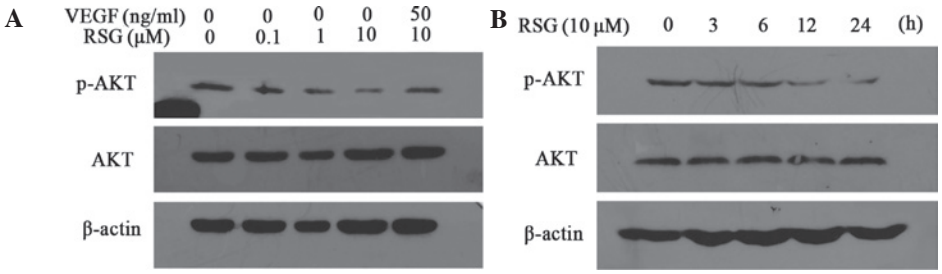

Figure 4. Effects of RSG on the phosphorylation of AKT. PC-3 cells were treated with (A) various doses of RSG for 24 h, in the absence or presence of rhVEGF165 (50 ng/ml) or (B) 10 μM RSG for 3, 6, 12, 18 and 24 h. The phosphorylation of AKT was determined by SDS-PAGE and western blotting. β-actin was used as a loading control. RSG, rosiglitazone; rhVEGF165, recombinant human vascular endothelial growth factor 165; VEGF, vascular endothelial growth factor.

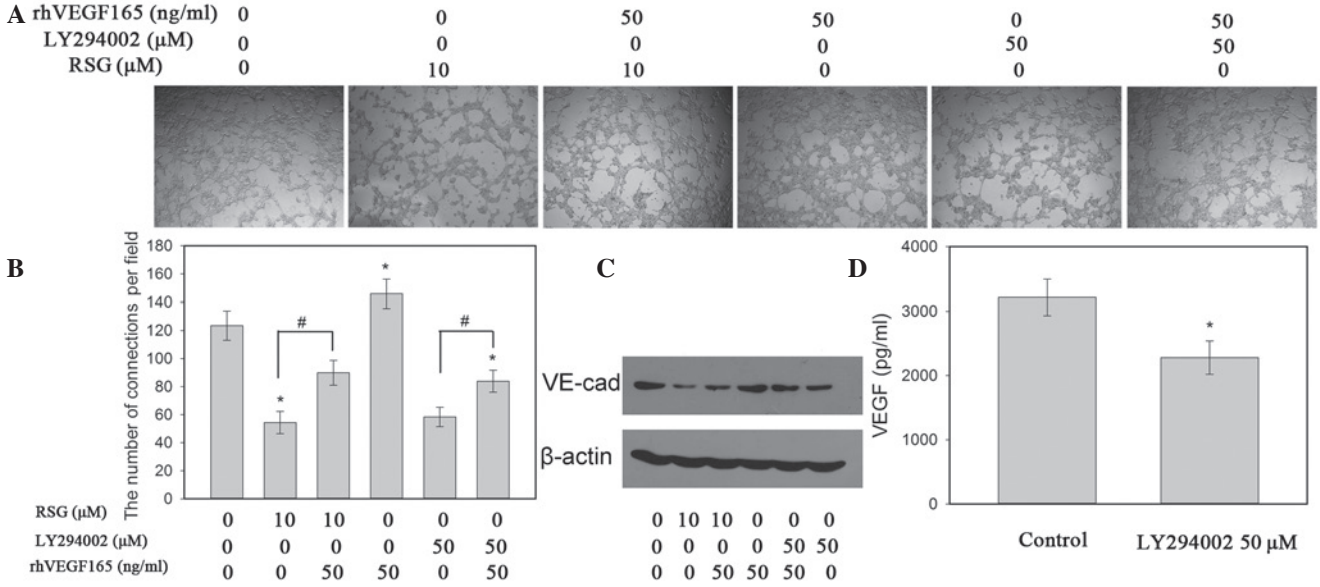

Figure 5. Mechanism of the anti-VM formation in the presence of RSG. (A) Assessment of VM formation upon exposure to RSG (10 μM), LY294002 (50 μM) or rhVEGF165 (50 ng/ml) for 48 h in PC-3 cells. Images of the formation of tube-like structures were captured (magnification, x40). (B) Typical pipe-like structures of the cell cultures were quantified in four fields in each treatment and data were calculated from three independent experiments. (C) Effects of RSG, LY294002 and VEGF on the expression of VE-cadherin at the protein level. (D) Effects of LY294002 on the secretion of VEGF were determined by antibody sandwich ELISA. Data are expressed as the mean ± standard deviation. *P<0.05, compared with the untreated control. #P<0.05, compared with another. VM, vasculogenic mimicry; RSG, rosiglitazone; rhVEGF165, recombinant human vascular endothelial growth factor 165.

(Fig. 3). The present study also demonstrated that the addition of rhVEGF165 partly inhibited RSG-induced downregulation of VM formation and VE-cadherin expression (Fig. 5A and C). These data suggest that the inhibition VM of PC-3 cells by RSG may partly occur through suppressing VEGF expression.

RSG modulates the expression of VEGF and VM via the AKT pathway. Several studies have indicated that signaling proteins, including PI3K and AKT, are involved in the regulation of the expression of MMPs and the promotion of metastasis (22). To understand the signaling pathways responsible for VEGF production and VM formation, the activation of AKT was examined. As expected, RSG downregulated the phosphorylation of AKT, the active form of PI3K/AKT, in a dose- and time-dependent manner (Fig. 4), which indicated that AKT may also be involved in the inhibition of VM formation in PC-3 cells.

To further investigate whether the inhibition of VM formation and VE-cadherin expression proceeded through the inhibition of the PI3K-AKT signaling pathway, PC-3 cells were treated with a PI3K inhibitor (LY294002; 20 μM) for 48 h. This disturbance of the AKT pathway resulted in the downregulation of VEGF secretion in PC-3 cells (Fig. 5D), which is consistent with the results of other studies (23). In addition, treatment with LY294002 significantly reduced cell VM formation and VE-cadherin expression (Fig. 5A and C), suggesting that the inhibition of VM formation and VEGF expression by RSG partly occurred through suppressing the PI3K pathway. Of note, the addition of rhVEGF165 eradicated the inhibitory effect of RSG on the phosphorylation of AKT (Fig. 4A). These data suggest that the inactivation of the PI3K/AKT pathway by RSG may be mediated by the inhibition of VEGF in an autocrine manner.

Discussion

Globally, 903,500 new cases of prostate cancer were estimated to occur, second only to lung cancer in males, and 258,400 mortalities from prostate cancer were estimated for 2011 (24). The majority of patients with localized early stage disease do

not develop metastases and do not succumb to prostate cancer. However, among patients with castration-resistant metastatic prostate cancer, >80% of patients develop bone metastasis, which is the most common site of metastases in this group (25). Therefore, understanding the molecular mechanisms of prostate cancer invasiveness and angiogenesis are of significant value to the prostate cancer field.

Targeting the blood supply by inhibiting the formation of blood vessels may lead to tumor growth arrest. Numerous angiogenesis inhibitors have been therapeutically used in preclinical and clinical settings (26). VEGF receptor tyrosine kinase inhibitors and a VEGF-neutralizing antibodies have been clinically validated to target VEGF or its receptors as an anticancer treatment. However, amongst other limitations of angiogenesis inhibitors, tubule formation was not inhibited. For instance, treatment with angiogenesis inhibitors did not inhibit tube formation of aggressive uveal and cutaneous melanoma cells *in vitro* (27). Of note, recent findings indicated that anti-angiogenesis treatment may elicit the malignant progression of various types of tumor (28). Furthermore, the inhibition of endothelial angiogenesis may unintentionally promote VM formation in tumors (29), as recently evidenced by a study demonstrating increased VM channels in tumor-bearing mice receiving short-term treatment of the anti-VEGF monoclonal antibody bevacizumab (30). It has been demonstrated that several drugs were able to inhibit VM (31,32). However, at present, data on the effect of anti-angiogenesis treatment on VM network formation remain inconsistent. The inhibition of endothelial cell migration by PPARγ ligands has been described, bolstering the anti-angiogenic activity of PPAR ligands (17,33). An earlier study has demonstrated that the activation of PPARγ by TZDs inhibits angiogenesis and neovascularization *in vitro* and *in vivo* and inhibits the release of VEGF from smooth muscle cells. The present study revealed that RSG was able to inhibit VM formation of the highly aggressive prostate cancer cell line PC-3 *in vitro* in a dose- and PPARγ-dependent manner. The results demonstrated that RSG significantly decreased the expression of VM-associated markers, including VE-cadherin, at the mRNA and protein levels, in line with the results of functional studies. RSG also reduced the migration and invasion capacity of PC-3 cells. Therefore, PPARγ agonists may be further used to unravel new biological mechanisms that drive VM, angiogenesis and tumor progression.

VEGF and its receptors appear to contribute to VM formation in certain types of cancer (10,34). However, VEGF did not demonstrate any effect on VM formation of Ewing sarcoma cells and hepatocellular carcinoma cell lines (35,36), and the effect of VEGF on the VM formation of prostate cancer cells remains to be elucidated. The present study demonstrated that rhVEGF165 increased the VM formation of PC-3 cells. Notably, it was demonstrated that VEGF was downregulated at the mRNA and protein secretion levels upon RSG treatment. These effects were dose dependent and the concentrations used in the present study were consistent with those reported by others (17). However, another study reported that TZDs enhanced VEGF expression in non-small cell lung cancer cells (37). The variation of outcome from those studies may be due to differences in culture and experimental conditions, the type and site of carcinoma or a combination of these factors.

Besides the functional study, elucidation of the mechanisms is crucial for understanding RSG as a potential anticancer agent. PI3K and AKT are important for the signaling of angiogenic growth factors that are closely associated with cancer cell migration, proliferation, angiogenesis and VM (38,39). Although LY294002 is useful for conducting preclinical studies on PI3K inhibition, its poor solubility and narrow therapeutic index preclude its use in humans. It has been reported that the PPARγ agonist was able to decrease the phosphorylation of AKT (33,40). The present study used the PPARγ agonist, RSG, to stimulate PC-3 cells. Our results demonstrated that RSG downregulated the phosphorylation of AKT in a dose- and time-dependent manner. In order to examine this possibility, the PI3K inhibitor LY294002 was used to inhibit these pathways to establish a link with VM formation. The data demonstrated that the disturbance of the AKT pathway resulted in the inhibition of the formation of VM in PC-3 cells and the downregulation of VE-cadherin and VEGF expression in PC-3 cells. Therefore, the present study suggested that RSG inhibited VM formation of PC-3 cells, which may be partly through suppressing PI3K-associated pathways. In addition, the stimulation of the cells with rhVEGF165 in the culture medium attenuated the inhibitory effect of RSG on the AKT pathway and cancer phenotypes. Previous studies demonstrated that the PI3K/AKT pathway may be activated by growth factors or hypoxia and are able to promote tumor angiogenesis through the enhanced expression of hypoxia-inducible factor 1 and VEGF (41). In addition, in the present study and in other studies (42,43), suppression of these pathways by a PI3K inhibitor markedly reduced VEGF protein levels. Thus, the present study concluded that the PI3K/AKT pathway and VEGF may generate a positive autocrine loop. These findings associated with the dual inhibition of the VEGF/PI3K/AKT cascade and the PI3K/AKT/VEGF cascade by RSG treatment in prostate cancer cells provided insights into the molecular mechanisms of the anti-tumor effects of RSG and support its potential clinical application.

In conclusion, the present study indicated that RSG effectively inhibited VM formation of PC-3 cells *in vitro* by inhibiting VEGF gene expression and phosphorylation of AKT. The present study may provide preliminary evidence for further elucidating the mechanisms underlying VM inhibition by TZDs and its mode of action. RSG was used as a proof-of-principle to determine the efficacy of PPARγ agonists in the inhibition of VM formation.

Acknowledgements

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