RESEARCH ARTICLE

Adiponectin inhibits VEGF-A in prostate cancer cells

Qiruo Gao · Junhua Zheng · Xudong Yao · Bo Peng

Abstract A role of adiponectin in tumorigenesis has recently been appreciated. Although plasma adiponectin levels in subjects with prostate cancer have been found to be significantly lower than in subjects with benign prostatic hyperplasia or in normal healthy controls, the underlying molecular mechanisms remain unknown. Here, we not only detected significant decreases in plasma adiponectin levels in prostate cancer patients, but also showed significant decreases in adiponectin receptor I (AdipoR1) levels in the resected prostate cancer specimen. Prostate cancer cell lines examined in the current study had all lower levels of adiponectin and AdipoR1, compared to normal healthy prostate tissue. Moreover, overexpression of adiponectin in prostate cancer cells decreased production of vascular endothelial growth factor A (VEGF-A), while adiponectin depletion increased VEGF-A. Furthermore, adiponectin seemed to activate AMPK/TSC2 to inhibit mTor-mediated activation of VEGF-A. Taken together, our data suggest that adiponectin may play an essential role in suppressing growth of prostate cancer cells through inhibition of VEGF-A-mediated cancer neovascularization.

Keywords Prostate cancer · Adiponectin · Vascular endothelial growth factor A (VEGF-A) · AMPK · TSC2 · mTor

Q. Gao · J. Zheng (✉) · X. Yao · B. Peng
Department of Urology, Shanghai 10th People's Hospital, Tongji University School of Medicine, 301 Yanchangzhong Road, Shanghai 200072, China
e-mail: morphee@163.com

J. Zheng
e-mail: junhua_zhang14@163.com

Introduction

The prostate is the gland for production of semen fluid. Prostate cancer is a common malignant tumor and frequently occurs among aged men. Although prostate cancers typically grow slowly, aggressive prostate cancers capable of invasion also occur, and the most common sites for prostate cancer metastasis are the skeleton system and lymph nodes [1–4]. Blood test for prostate-specific antigen (PSA) is generally used in prostate cancer screening, and abnormal results require further ultrasound, MRI, or biopsy examinations. The therapeutic methods for prostate cancer often depend on the severity and the stage of the cancer, including watchful waiting, surgery, hormone therapy, radiation therapy, chemotherapy, or combined [1–4]. Since current treatments are not sufficient to provide satisfactory therapy for all subjects, especially the aggressive ones with distal metastasis, further knowledge on the prostate cancer growth and metastasis is highly needed [1–7].

The initiation of new blood vessels through angiogenesis and neovascularization promotes tumor growth and invasion [8–12]. Tumor cells release soluble angiogenic factors to enhance new vessel formation and restructure of existing vessels to allow tumors to outgrow [8–12]. Th angiogenic switch involves an adaptation of the balance between pro-angiogenic and anti-angiogenic factors [8–12]. The most potent pro-angiogenic factor is vascular endothelial growth factor A (VEGF-A) [13–17], which has also been shown to play a critical role in the cancer vessel formation in prostate cancer and has been used as a therapeutic target [8–12].

Adiponectin is an adipose tissue-derived hormone and is expressed nearly exclusively in adipose tissue. Adiponectin has important biological functions against diabetes, atherosclerosis, inflammation, and cell replication [18–23]. Adiponectin has two receptors, adiponectin receptor I

(AdipoR1) and adiponectin receptor II (AdipoR2) [24], which distribute differently in human tissue. Specifically, AdipoR1 is most abundant in skeletal muscle but is also present in endothelial cells and other tissues. AdipoR2, however, is predominantly expressed in the liver [25].

Recently, the anti-carcinogenic effects of adiponectin have been appreciated and are supposed to result from modulation in the signaling pathways controlling cell proliferation and apoptosis [18–23]. Association of adiponectin with the risk of different types of cancers [18–23, 26] has been reported. However, the underlying mechanisms are far from being elucidated. Moreover, a relationship between adiponectin and VEGF-A, especially in prostate cancer, has not been studied.

In the current study, we not only detected significant decreases in plasma adiponectin levels in prostate cancer patients, but also showed significant decreases in AdipoR1 levels in the resected prostate cancer specimen. All of the prostate cancer cell lines examined in the current study had reduced adiponectin and AdipoR1 levels, compared to normal healthy prostate tissue. Moreover, overexpression of adiponectin in prostate cancer cells decreased production of VEGF-A, while adiponectin depletion increased VEGF-A. Furthermore, adiponectin seemed to activate AMPK/TSC2 to inhibit mTor-mediated activation of VEGF-A.

Materials and methods

Patient tissue specimens

Resected specimens were taken from 15 subjects with tissues from prostate cancer (PC), 23 subjects with tissues from benign prostatic hyperplasia (BP), and 22 subjects with tissues from normal healthy prostate (NH). All specimens had been histologically and clinically diagnosed at the Department of Urology, Shanghai 10th People's Hospital of Tongji University School of Medicine from 2009 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Radioimmunoassay (RIA)

The concentration of adiponectin in the plasma from patients was measured by radioimmunoassay using a human adiponectin RIA Kit (Linco Research, St. Louis, MO, USA). The blood samples were taken 3 days before surgery. After 12 h of fasting, the peripheral venous blood sample was taken at 8 a.m. of the day, put into EDTA-rinsed tubes and centrifuged for 20 min at 2000 rpm to separate the plasma. None of

the patients were taking any other drugs that could have affected the plasma adiponectin levels.

Cell lines

PC3, DU-145, CA-HPV-10, and MDA-PCa-2b are four commonly used human prostate cancer lines, all were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA).

Cell transfection

Prostate cancer cells were transfected either with an adiponectin-overexpressing plasmid (Adipo), or with a small short hairpin interfering RNA for adiponectin (shAdipo; sequence 5′-GGACAACGACUAUCUGCUAUU-3′), or with a control plasmid expressing a scrambled sequence (Null). Briefly, we used a pEGFP-C1 plasmid (Clontech, Mountain View, CA, USA) in this study. The transgenes were Adipo, shAdipo, or Null (as a control) under the control of a CMV promoter. The Adipo construct was amplified by PCR with EcoRI-restriction-endonuclease-forward and NheI-restriction-endonuclease-reverse primers, using the human liver cDNA as a template. The construct was then subcloned into the EcoRI and NheI sites of the pEGFP-C1 plasmid. Sequencing was performed to confirm the correct orientation of the final plasmids. Transfection was performed by Lipofectamine 2000 reagent (Invitrogen).

ELISA assay

The concentration of VEGF-A in the conditioned media from cultured cells was determined by a human VEGF-A ELISA kit (Raybio, 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 VEGF-A polyclonal antibody, and then immunosorbented by biotinylated monoclonal anti-human VEGF-A 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.

Western blot

Protein was extracted from the cultured cells or patients' specimen by RIPA buffer (Sigma, St Louis, USA) for Western blot. The supernatants were collected after centrifugation at

12,000×g at 4 °C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-Rad, China), and whole lysates were mixed with 4× SDS loading buffer (125 mmol/l Tris–HCl, 4 % SDS, 20 % glycerol, 100 mmol/l DTT, and 0.2 % bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western blot are anti-adiponectin, anti-AdipoR1, anti-AdipoR2, anti-phosphorylated AMPK (pAMPK), anti-TSC2, anti-phosphorylated mTor (pmTor), anti-VEGF-A, and α-tubulin (all from Cell Signaling, LA, USA). Speci-matched, HRP-conjugated antibodies (Jackson Labs, LA, USA) are used. Images shown in the figure were representative from five repeats in one group.

HUVEC transwell collagen gel assay

Human umbilical vein endothelial cells (HUVEC) were grown in M-200 supplemented medium with low serum growth supplement (Invitrogen). HUVEC endothelial cells were embedded in a collagen gel and plated on a 24-well culture plate. The plate was kept at 37 °C in a CO_2 incubator for 10–15 min to make the collagen polymerize, and then the same number of prostate cancer cells (with adiponectin modification) was added to the transwell and put onto the plate.

The plate with transwell was returned to the CO_2 incubator. Media was replenished every day, and culture images were taken after 3 days.

Statistical analysis

All statistical analyses were carried out using the SPSS 19.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction. All values are depicted as mean±standard deviation from five individuals and are considered significant if $p < 0.05$.

Results

Plasma adiponectin levels in prostate cancer were significantly lower than normal prostate tissue

A role of adiponectin in tumorigenesis has recently been appreciated. We analyzed plasma adiponectin levels in subjects with prostate cancer (PC) and found that they were significantly lower than in subjects with benign prostatic hyperplasia (BP) or in normal healthy controls (NH) (Fig. 1a). Moreover, we also analyzed adiponectin receptors in the resected PC, BP, and NH tissues by Western blot. We found that while there were undetectable levels of adiponectin receptor II (AdipoR2) in any prostate tissues, significantly lower levels of adiponectin receptor I (AdipoR1) were detected in the PC tissue, compared to BP and NH, by quantification (Fig. 1b) and by representative images (Fig. 1c). Liver (LV) was used as a positive control, specifically for AdipoR2. Thus, adiponectin signaling was inhibited in prostate cancer.

Fig. 1 Plasma adiponectin levels in prostate cancer were significantly lower than normal prostate tissue. a Plasma adiponectin levels in subjects with prostate cancer (PC) were compared with subjects with benign prostatic hyperplasia (BP) or in normal healthy controls (NH). b–c The adiponectin receptor I (AdipoR1) and II (AdipoR2) in the resected PC, BP, and NH tissues were analyzed by Western blot, shown by quantification (b), and by representative images (c). Liver (LV) was used as a positive control specifically for AdipoR2. *$p < 0.05$
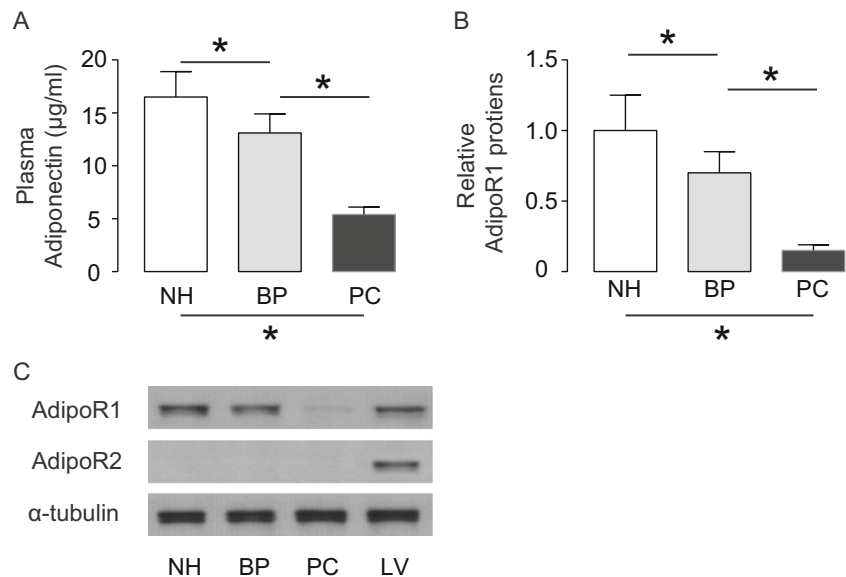
Low levels of adiponectin and AdipoR1 were detected in prostate cancer cell lines

Then, we examined adiponectin and AdipoR1 levels in four commonly used prostate cancer cell lines, PC3, DU-145, CA-HPV-10, and MDA-PCa-2b, all with an epithelial cell origin. We found that all these prostate cancer cells expressed significantly lower levels of adiponectin and AdipoR1, compared to normal prostate tissue, by representative images (Fig. 2a) and by quantification (Fig. 2b, c). These data suggest that inhibition of adiponectin signaling may be a general phenomenon in prostate cancer.

Adiponectin inhibited production of VEGF-A by prostate cancer cells

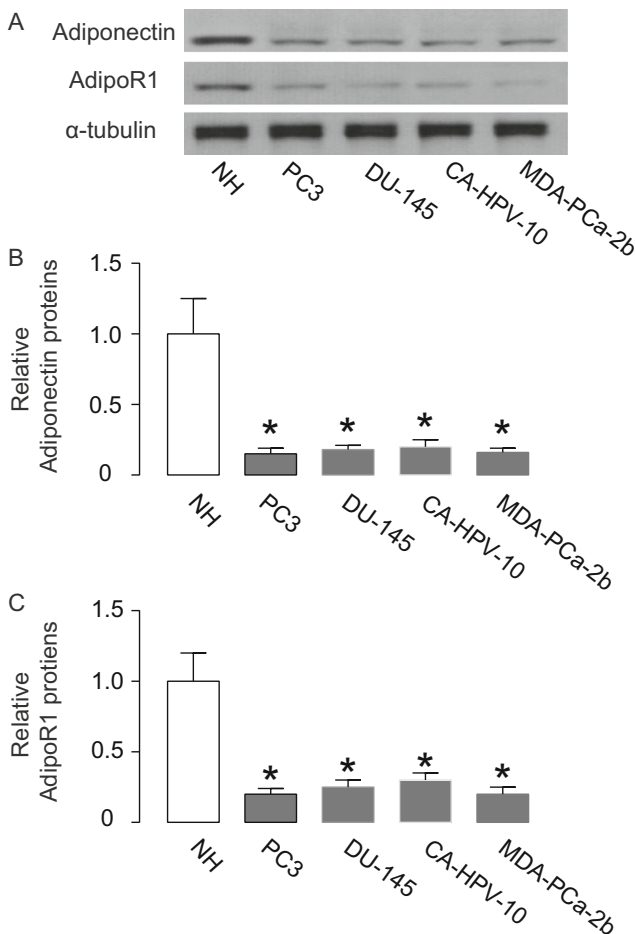Adiponectin is well known for its potential of phosphorylating and activating AMP kinase (AMPK), which subsequently

Fig. 2 Reduction of adiponectin was detected in all examined prostate cancer cell lines. Adiponectin and AdipoR1 levels were examined in four commonly used prostate cancer cell lines, PC3, DU-145, CA-HPV-10, and MDA-PCa-2b, all with an epithelial cell origin, by Western blot, shown, shown by representative images (**a**), and by quantification (**b**–**c**). *NH* normal healthy controls. *$p < 0.05$

inhibits mTor in a TSC2-dependent manner in different cell types [18–23, 27, 28]. Activation of mTor induces neovascularization [29, 30]. Thus, we hypothesize that adiponectin may activate AMPK/TSC2 to suppress mTor to inhibit production of angiogenic factors, e.g., VEGF-A.

To prove it, we either overexpressed adiponectin or inhibited it in PC3 cells, which were confirmed by analyzing adiponectin levels in these adiponectin-modified PC3 cells (Fig. 3a). We found that overexpression of adiponectin in PC3 cells significantly decreased the production of VEGF-A, while adiponectin depletion significantly increased VEGF-A (Fig. 3b). In a HUVEC tube formation assay, we found that HUVECs substantially increased their tube formation when co-cultured with adiponectin-depleted PC3 cells, and substantially decreased their tube formation when co-cultured with adiponectin-overexpressing PC3 cells (Fig. 3c, d).

Overexpression of adiponectin increased phosphorylated AMPK and TSC2 and decreased phosphorylated mTor and VEGF-A. Depletion of adiponectin decreased phosphorylated AMPK and TSC2 and increased phosphorylated mTor and VEGF-A (Fig. 4).

Taken together, these data suggest that adiponectin may play an essential role in suppressing growth of prostate cancer cells through inhibition of VEGF-A-mediated cancer neovascularization. The effects of adiponectin may be conducted through its activation on AMPK/TSC2-mediated mTor inhibition (Fig. 5).

Discussion

Prostate cancer appears to be an increasingly important public health problem in an aging population. Since development of prostate cancer often co-occurs in the subjects of insulin resistance with obesity, decreased adiponectin levels have thus been associated with development of prostate cancer, besides obesity and insulin resistance.

Of note, a recent study has reported significantly lower plasma adiponectin levels in subjects with prostate cancer than in subjects with benign prostatic hyperplasia or in normal healthy controls. Moreover, plasma adiponectin levels were inversely associated with the grade and stage of prostate cancer [26]. These data highly suggest that adiponectin may play a role in suppressing growth and invasion of prostate cancer.

Several signaling molecules are known to mediate adiponectin-induced metabolic effects [31]. These pathways are AMPK signaling pathway, nuclear factor-kB (NF-kB) signaling pathway, c-Jun NH2-terminal kinase (JNK) signaling pathway, signal transducer and activator of transcription 3 (STAT3) signaling pathway, peroxisome proliferators activated receptor (PPAR)-a signaling pathway, and p38 mitogen-

Fig. 3 Adiponectin inhibited production of VEGF-A by prostate cancer cells. **a–b** We either overexpressed adiponectin or inhibited it in PC3 cells, and examined **a** adiponectin levels by Western blot or **b** VEGF-A levels in the conditioned media by ELISA in these PC3 cells (PC3-Adipo, PC3-Null, PC3-shAdipo). **c–d** In a HUVEC tube formation assay, we found that HUVECs substantially increased their tube formation when co-cultured with adiponectin-depleted PC3 cells, and substantially decreased their tube formation when co-cultured with adiponectin-overexpressing PC3 cells, shown **c** by representative images and **d** by quantification. *$p < 0.05$
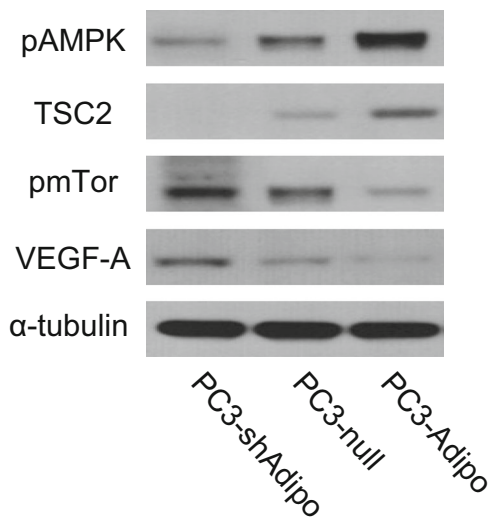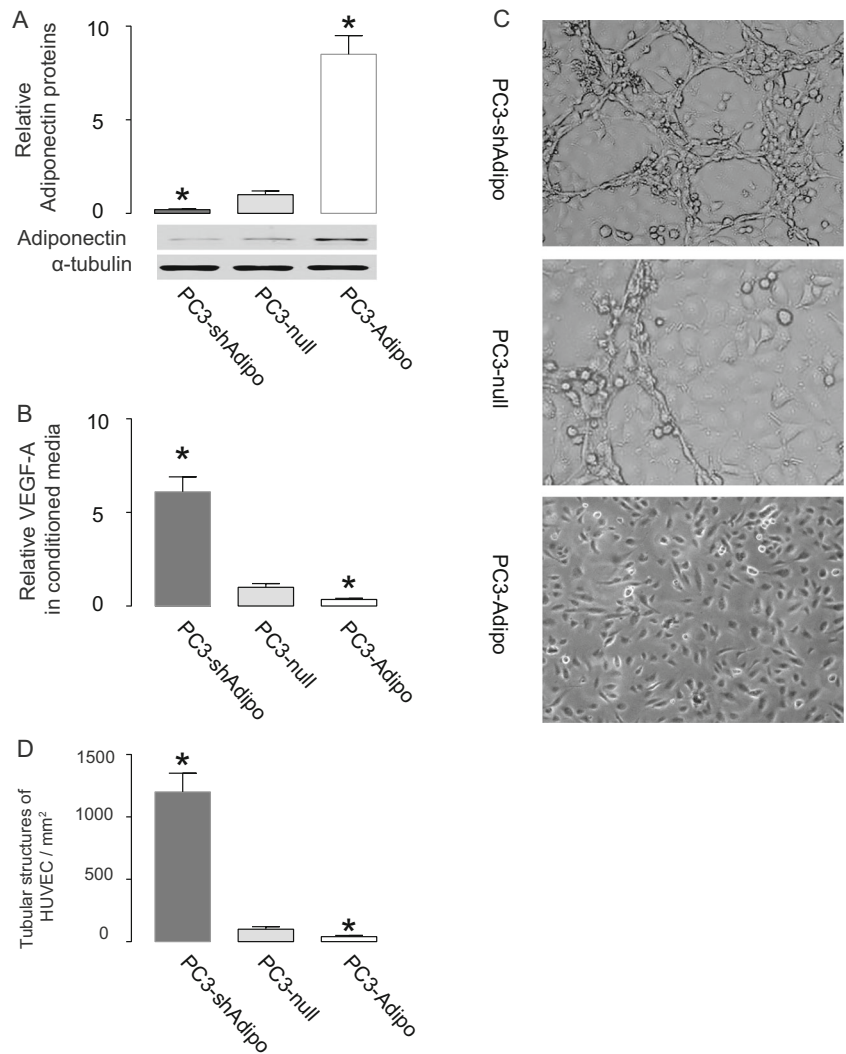

Fig. 4 Overexpression of adiponectin increased phosphorylated AMPK and TSC2 and decreased phosphorylated mTor. Phosphorylated AMPK, TSC2, phosphorylated mTor, and VEGF-A were examined by Western blot in the adiponectin-modified PC3 cells
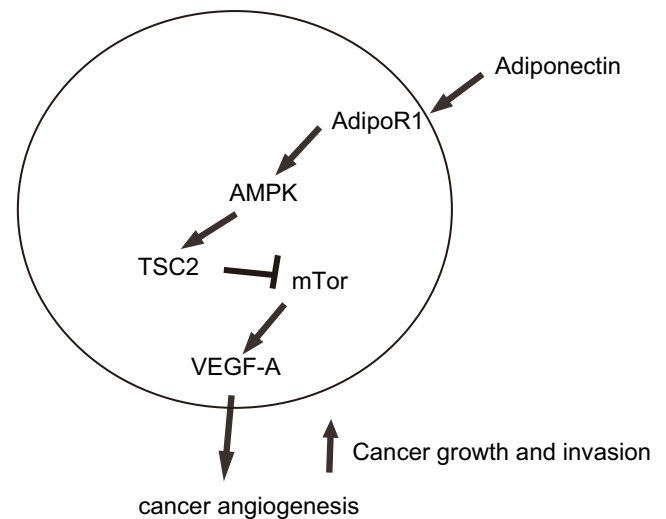
Fig. 5 Schematic of the model. Adiponectin plays an essential role in suppressing growth of prostate cancer cells through inhibition of VEGF-A-mediated cancer neovascularization. The effects of adiponectin may be conducted through its activation on AMPK/TSC2-mediated mTor inhibition

activated protein (MAP) kinase signaling pathway [31]. Among all these pathways, AMPK signaling is best characterized and has been shown to be activated potentially by adiponectin binding to its receptor [31]. Since AMPK directly regulates mTor, which controls vascularization, we thus hypothesize that adiponectin may activate AMPK/TSC2 to suppress mTor to inhibit production of angiogenic factors, e.g., VEGF-A.

We proved our hypothesis in the current study by confirming this regulation pathway. Moreover, data from our HUVEC tube formation assay demonstrate that the regulation of VEGF-A by adiponectin should be functional and directly related to vessel formation. Since cancer vascularization is critical for cancer invasion and metastasis, our data are consistent with clinical findings that have revealed a correlation of plasma adiponectin levels and prostate cancer grade.

Taken together, all these data suggest that adiponectin may play an essential role in suppressing growth of prostate cancer cells through inhibition of VEGF-A-mediated cancer neovascularization. The effects of adiponectin appear to be conducted through its activation on AMPK/TSC2-mediated mTor inhibition. Our work, thus, highlights adiponectin as a promising therapeutic target for the control and treatment of prostate cancer.

Conflicts of interest None.

References

1. Saylor PJ. Prostate cancer: the androgen receptor remains front and centre. Nat Rev Clin Oncol. 2013;10:126–8.

2. Alva A, Hussain M. The changing natural history of metastatic prostate cancer. Cancer J. 2013;19:19–24.

3. Beltran H, Rubin MA. New strategies in prostate cancer: translating genomics into the clinic. Clin Cancer Res. 2013;19:517–23.

4. Xin L. Cells of origin for cancer: an updated view from prostate cancer. Oncogene. 2013;32:3655–63.

5. Huang S, Liao Q, Li L, Xin D. PTTG1 inhibits SMAD3 in prostate cancer cells to promote their proliferation. Tumour Biol. 2014;35: 6265–70.

6. Xia Q, Li C, Bian P, Wang J, Dong S. Targeting SMAD3 for inhibiting prostate cancer metastasis. Tumour Biol. 2014;35:8537–41.

7. Zhang Q, Hong B, Wu S, Niu T. Inhibition of prostatic cancer growth by ginsenoside Rh2. Tumour Biol. 2014.

8. Antonarakis ES, Carducci MA. Targeting angiogenesis for the treatment of prostate cancer. Expert Opin Ther Targets. 2012;16:365–76.

9. Kluetz PG, Figg WD, Dahut WL. Angiogenesis inhibitors in the treatment of prostate cancer. Expert Opin Pharmacother. 2010;11: 233–47.

10. Aragon-Ching JB, Dahut WL. VEGF inhibitors and prostate cancer therapy. Curr Mol Pharmacol. 2009;2:161–8.

11. Delongchamps NB, Peyromaure M. The role of vascular endothelial growth factor in kidney and prostate cancer. Can J Urol. 2007;14: 3669–77.

12. Delongchamps NB, Peyromaure M, Dinh-Xuan AT. Role of vascular endothelial growth factor in prostate cancer. Urology. 2006;68:244–8.

13. Ferrara N. Vascular endothelial growth factor. Arterioscler Thromb Vasc Biol. 2009;29:789–91.

14. Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol Dis. 2007;38:258–68.

15. Nieves BJ, D'Amore PA, Bryan BA. The function of vascular endothelial growth factor. Biofactors. 2009;35:332–7.

16. Xiao X, Prasadan 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.

17. 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.

18. Allott EH, Masko EM, Freedland SJ. Obesity and prostate cancer: weighing the evidence. Eur Urol. 2013;63:800–9.

19. Perrier S, Jarde T. Adiponectin, an anti-carcinogenic hormone? A systematic review on breast, colorectal, liver and prostate cancer. Curr Med Chem. 2012;19:5501–12.

20. Buschemeyer 3rd WC, Freedland SJ. Obesity and prostate cancer: epidemiology and clinical implications. Eur Urol. 2007;52:331–43.

21. Mistry T, Digby JE, Desai KM, Randeva HS. Obesity and prostate cancer: a role for adipokines. Eur Urol. 2007;52:46–53.

22. O'Malley RL, Taneja SS. Obesity and prostate cancer. Can J Urol. 2006;13 Suppl 2:11–7.

23. Baillargeon J, Rose DP. Obesity, adipokines, and prostate cancer (review). Int J Oncol. 2006;28:737–45.

24. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature. 2003;423:762–9.

25. Goldstein BJ, Scalia R. Adiponectin: a novel adipokine linking adipocytes and vascular function. J Clin Endocrinol Metab. 2004;89: 2563–8.

26. Barb D, Neuwirth A, Mantzoros CS, Balk SP. Adiponectin signals in prostate cancer cells through Akt to activate the mammalian target of rapamycin pathway. Endocr Relat Cancer. 2007;14:995–1005.

27. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115:577–90.

28. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002;4:648–57.

29. Geissler EK, Schlitt HJ, Thomas G. mTOR, cancer and transplantation. Am J Transplant. 2008;8:2212–8.

30. Lee DF, Hung MC. All roads lead to mTOR: integrating inflammation and tumor angiogenesis. Cell Cycle. 2007;6:3011–4.

31. Vansaun MN. Molecular pathways: adiponectin and leptin signaling in cancer. Clin Cancer Res. 2013;19:1926–32.