# Acidic extracellular pH promotes prostate cancer bone metastasis by enhancing PC-3 stem cell characteristics, cell invasiveness and VEGF-induced vasculogenesis of BM-EPCs

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Received March 1, 2016; Accepted July 7, 2016

DOI: 10.3892/or.2016.4997

**Abstract.** Bone metastasis is a main cause of cancer-related mortality in patients with advanced prostate cancer. Emerging evidence suggests that the acidic extracellular microenvironment plays significant roles in the growth and metastasis of tumors. However, the effects of acidity on bone metastasis of PCa remain undefined. In the present study, PC-3 cells were cultured in acidic medium (AM; pH 6.5) or neutral medium (NM; pH 7.4), aiming to investigate the effects and possible mechanisms of acidic extracellular microenvironment in bone metastasis of PCa. Our results showed that AM can promote spheroid and colony formations, cell viability and expression of stem cell characteristic-related markers in PC-3 cells. Moreover, AM stimulates MMP-9 secretion and promotes invasiveness of PC-3 cells, and these effects can be inhibited by blocking of MMP-9. Furthermore, AM stimulates VEGF secretion of PC-3 and AM conditioned medium ($CM_{AM}$) promotes vasculogenesis of BM-EPCs by increasing cell viability, migration, tube formation, which involved activating the phosphorylation of VEGFR-2, Akt and P38, when pH of NM conditioned medium ($CM_{NM}$) was modulated the same as AM conditioned medium ($CM_{AM}$). Further studies have shown that $CM_{NM}$ induced vasculogenesis of BM-EPCs can be inhibited by the inhibition of VEGFR2 with DMH4. These findings suggest that acidic extracellular microenvironment may have the potential to modulate prostate cancer bone metastasis by enhancing PC-3 stem cell characteristics, cell invasiveness and VEGF-induced vasculogenesis of BM-EPCs. Improved anticancer strategies should be designed to selectively target acidic tumor microenvironment.

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*Key words:* acidic tumor microenvironment, prostate cancer, bone metastasis, cancer stem cells, endothelial progenitor cells, vasculogenesis

## Introduction

Bone metastasis is significantly related to cancer-related mortality, and is a common complication in patients with advanced prostate cancer. Once tumor cells metastasize to bone, there are no effective treatments for the affected patients and the prognosis is usually poor (1,2). Solid tumors are considered to live in a special pathophysiologic microenvironment different from normal tissues (3). Increasing evidence indicates that the unique tumor microenvironment are involved in tumor initiation, growth, progression, invasion and metastasis (4,5). Although the importance of acidic tumor microenvironment in sustaining tumor progression and metastasis is widely recognized, the underlying mechanisms are still poorly understood, and additional studies are urgently required to better understand the role of acidic tumor microenvironment in tumor metastasis.

Extracellular acidosis (low pH) due to hypoxia (6), excessive glycolysis (7), hyperexpression of carbonic anhydrase (8) and poor perfusion (9) is a tumor microenvironmental stressor. Extracellular pH (pHe) is significantly lower than neighboring normal tissues in many tumors and may decrease to a lower level with the enlargement of tumor volume (6,9). This pathological microenvironment promotes malignant progression and metastasis by activating several intracellular signaling pathways (10,11). In addition, acidic tumor microenvironment may blunt the effectiveness of antitumor therapy (12). Consequently, a more thorough understanding of the role of acidic tumor microenvironment in bone metastasis may help to develop more effective therapeutics.

Accumulating evidence suggests that cancer stem cells (CSCs), a small number of cancer cells with stem cell properties,

have the potential of unlimited proliferation, self-renewal and multipotent differentiation. CSCs have been reported to regulate cancer cells from the original tumor to a distant organ (13), although the underlying mechanisms remain unclear. Previous study showed that acidic pHe can promote a stem cell-like phenotype in glioma (14). However, whether acidic tumor microenvironment promotes prostate cancer bone metastasis by regulating the cancer stem cell characteristics of prostate cancer remains unclear.

Acidosis has also been reported to increase the production of proteinases and pro-angiogenic factors in tumor cells, which are generally believed to accelerate the invasion and metastasis of tumor cells (15,16). The matrix metalloproteinases (MMPs) secreted by the tumor and stroma cells may facilitate tumor growth, invasion and metastasis through degrading the basement membranes and extracellular matrix (ECM) (17). It is reported that the expression of MMP-9 was significantly higher in the PCa than that of the normal adjacent tissues, and there was significant correlation between MMP-9 expression and clinicopathological stage (18). Moreover, compared with normal prostatic glands, angiogenic factor vascular endothelial growth factor (VEGF) expression and microvessel density is significantly higher in the premalignant and malignant tissues (19). In addition, the expression of VEGF significantly correlates with metastasis of PCa (20). However, whether acidic stress can affect the cytokine expression in PCa and then affect the tumor invasiveness and new vasculature formation is still unknown.

Tumor-associated neovasculature is an important process for solid tumor progression and metastasis. Despite the significant progress that has been made in the application of antiangiogenic drugs for the treatment of cancer, the antiangiogenic therapy remains transient and with insufficient efficacy (21). Emerging evidence indicates that circulating bone marrow-derived endothelial progenitor cells (BM-EPCs) are recruited into the tumor microenvironment during tumor vasculogenesis and ultimately promote metastatic spreading through newly formed blood vessels (22,23). In addition, VEGF plays a more important role in the recruitment of BM-EPCs to tumor neovascularization sites and promote vasculogenesis of BM-EPC (24,25). Taken together, these studies suggest an important relationship between VEGF and BM-EPCs in the formation of tumor vessel. Although the positive role of acidic tumor microenvironmentin promoting tumor progression and metastasis has been extensively investigated, little is known as to its effect on BM-EPCS-mediated vasculogenesis. Understanding the roles of acidic microenvironment in tumor vessel formation is crucial for designing new anti-angiogenic drugs to treat solid malignancies.

In the present study, we investigated the effect of acidic extracellular microenvironment on PC-3 stem cell characteristics and cell invasiveness after incubation in acidic medium (AM) or neutral medium (NM). We isolated human bone marrow-derived EPCs to determine the effect of PC-3 $CM_{AM}$ on BM-EPCs functions. Additionally, we further investigated the mechanisms involved in PC-3 $CM_{AM}$-inducted vasculogenesis of BM-EPC.

## Materials and methods

*Cell culture and acidic/NM preparation*. Human prostate cancer cell line PC-3 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA), streptomycin (100 $\mu$g/ml) and penicillin (100 U/ml). PC-3 cells were incubated in a humidified incubator (21% $O_2$, 5% $CO_2$, 37˚C). The pH value of complete medium was measured by pH and adjusted to 6.5 AM or 7.4 NM with hydrochloric acid or sodium hydroxide.

*Conditioned medium preparation and enzyme-linked immunosorbent assay (ELISA)*. PC-3 cells were cultured in 150 $cm^2$ flasks in RPMI-1640 complete medium. Upon reaching 70-80% confluency, cells were washed twice with phosphate-buffered saline (PBS) and were then cultured in acidic (pH 6.5, 1% FBS) or neutral (pH 7.4, 1% FBS) medium for additional 48 h. Subsequently, AM CM ($CM_{AM}$) and NM CM ($CM_{NM}$) were harvested and filtered. The MMP-9 and VEGF level of CMs were detected by ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Thereafter, the CMs were stored at -80˚C for further use.

*Spheroid formation assay*. The spheroid formation assay was performed as we previously described (26,27). In brief, PC-3 cells were incubated in AM or NM for 48 h, then, single cell suspensions (400 cells/well) were added into 6-well non-adherent plates (Corning, Corning, NY, USA) and grown in serum-free spheroid formation media consisting of RPMI-1640 medium supplemented with 10 ng/ml of bFGF, 20 ng/ml of EGF and 2% B27 (all from Invitrogen and Sigma). After 2 weeks, spheroids (diameter >100 $\mu$m) were counted and captured under a light microscope (magnification, x100).

*Colony formation assay*. Briefly, after incubating in AM or NM for 48 h, PC-3 cell suspensions (300 cells/well) were plated in 65-mm Petri dishes and incubated for 15 days. Subsequently, the dishes were washed twice with PBS before being fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. The colonies (≥50 cells/colony) were counted.

*Cell viability and invasion assay*. After incubating in AM or NM for 48 h, PC-3 cell suspensions ($1x10^4$ cells/well) were plated into 96-well plates. After 24 h, cell viability was examined by a Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) following the manufacturer's instructions. Transwell system (Corning Costar, Acton, MA, USA) with polycarbonate filters (8-$\mu$m pore size; 6.5 mm diameter) was used for invasion assay. PC-3 cells were incubated in AM or NM for 48 h before experiments. Next, cells were resuspended in serum-free RPMI-1640 medium with or without anti-MMP-9 antibody (5 $\mu$g/ml) or general MMP inhibitor (GM6001; 15 $\mu$mol/l) (both from Chemicon, Temecula, CA, USA). Cell suspensions (200 $\mu$l) ($1x10^6$ cells/ml) were plated onto the upper chambers, and then 600 $\mu$l RPMI-1640 complete medium (10% FBS) was added to the lower chamber. After 48 h, the non-invading cells on the upper surface of upper chamber were wiped off by cotton swab. After that, the invading cells on the lower surface were washed twice with PBS before being fixed with 4% paraformaldehyde for 20 min, and then stained with 0.1% crystal violet for 15 min.

The invasive cells were counted and captured at a magnification of x100.

*Isolation and cultivation of BM-EPCs*. The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (approval no. 2008-55). BM-EPCs in our experiments were isolated from human bone marrow, which was extracted from lumbar vertebral body of patients (12 donors; age range, 42-63 years; mean age, 52.1 years) in the First Affiliated Hospital of Sun Yat-sen University. All the informed patients underwent lumbar fusion due to lumber degenerative diseases without tumor, hematological and metabolic diseases. This procedure of isolation and cultivation of BM-EPCs were performed as we previously described (28). BM-EPCs at passages 2-3 were used in the present study.

*BM-EPC cell viability and migration assay*. BM-EPCs ($1x10^4$ cells/well) were incubated in EGM-2 into 96-well plates for 24 h. Then, cells were exposed to NM, $CM_{AM}$ and $CM_{NM}$ (added 1% FBS to NM and CMs, and modulated pH to 7.4). After 36 h, cell viability was evaluated by a CCK-8 according to the manufacturer's instructions. Cell migration was also evaluated by Transwell system with polycarbonate filters (8-$\mu$m pore size, 6.5 mm diameter). First, BM-EPCs were resuspended in serum-free RPMI-1640 medium. Cell suspensions (200 $\mu$l) ($1x10^6$ cells/ml) were plated onto the upper chambers, and then 600 $\mu$l NM, $CM_{AM}$ and $CM_{NM}$ (added 1% FBS to NM and CMs, and modulated pH to 7.4) was added to the lower chamber. After 16 h, the non-migrating cells on the upper surface of upper chamber were wiped off by a cotton swab. The cells on the lower surface were washed twice with PBS before being fixed with 4% paraformaldehyde for 20 min, and then stained with 0.1% crystal violet for 15 min. The migrated cells were counted and captured at a magnification of x100.

*BM-EPC tube formation assay*. In Vitro Angiogenesis Assay kit (Chemicon) was used to evaluate the tube formation ability of BM-EPCs according to the manufacturer's instructions. Briefly, after thawing at 4˚C, Matrigel was added to 96-well plate, and then incubated at 37˚C for 30 min. Next, cells were re-suspended in NM, $CM_{AM}$ and $CM_{NM}$ (added 1% FBS to NM and CMs, and modulated pH to 7.4). Then cell suspensions ($1x10^4$ cells/well) were plated onto Matrigel-precoated 96-well plates and then incubated at 37˚C for 16-18 h. The capillary-like structures were examined and the number of closed network units was counted under a light microscope (magnification, x100).

*Western blot analysis*. Briefly, after washing twice with ice-cold PBS, cells were lysed with extraction buffer (Novagen, Merck, Darmstadt, Germany) for 30 min on ice. Next, the lysate was centrifuged (12,000 x g for 10 min at 4˚C), and protein concentration was measured with Bradford reagent (Bio-Rad, Hercules, CA, USA). Total proteins (30 $\mu$g) were separated on 10% SDS-polyacrylamide gels, and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% fat-free milk powder, transferred blots were incubated with primary antibodies: CD133, purchased from Miltenyi Biotech (Auburn, CA, USA); CD44, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Oct4, Klf4, GAPDH, phospho-VEGFR2, phospho-P38, phospho-AKT, VEGFR2, P38 and AKT, were purchased from Cell Signaling Technology (Beverly, MA, USA).

*Statistical analysis*. All experiments in the present study were performed in triplicate. All experimental data are shown as the mean ± standard deviation (SD). SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) was used for data analysis. Statistical differences between the groups were performed by Student's t-test. A P-value $<0.05$ was regarded as statistically significant.

## Results

*Acidic pHe promotes PC-3 stem cell characteristics*. To test whether acidic extracellular microenvironment promotes PC-3 cell metastasis is through targeting cancer stem cell-like characteristics, we first investigated whether acidic pHe regulates the expression of prostate CSC stemness-related markers in the PC-3 cell line. PC3 cells were incubated in AM (pH 6.5) or NM (pH 7.4) for 48 h, then the expression of CSC stemness-related markers (including CD133, CD44, Oct4 and Klf4) in PC-3 cells was examined by western blotting. As shown in Fig. 1C, CD44, Oct4 and KLF4 were significantly upregulated after 48 h incubation in AM (pH 6.5). CD133 protein expression also increased after incubation in AM (pH 6.5), although this effect was relatively less obvious. Results showed that acidic pHe may be involved in regulating the prostate stem cell characteristics. This was further confirmed by spheres and colony formation assays of PC-3 cells. Results showed the sizes and numbers of spheres in non-adherent culture significantly increased when cells were pre-treated with AM (Fig. 1A). In addition, colony forming efficiency and cell viability significantly increased when cells were pre-treated with AM (Fig. 1B). These results suggest that acidic extracellular microenvironment is involved in regulating the stemness of PC-3 cells.

*Acidic pHe induces MMP-9 and VEGF secretion*. It is now well confirmed that MMP-9 perform central roles in tumor progression and metastasis (29). In addition, VEGF has been reported to participate in the recruitment of BM-EPCs to tumor neovascularization sites and play critical roles in regulating vasculogenesis of BM-EPCs (25). Therefore, we estimated whether acidic stress can affect the cytokine expression in PC-3 cells. PC-3 cells were incubated in AM or NM for 48 h, then the CMs were harvested. The secretion of MMP-9 and VEGF in CMs was measured by ELISA. Compared with $CM_{NM}$, the expression of MMP-9 and VEGF is significantly higher in $CM_{AM}$ (Fig. 2). These results suggest that acidic tumor microenvironment may stimulate the secretion of many proteolytic enzymes such as MMP-9 and VEGF, which ultimately contribute to tumor invasion, metastasis and vasculogenesis.

*MMP-9 is involved in acid-induced invasion of PC-3 cells*. It is reported that upregulation of MMP-9 expression increase the capacity of invasion in prostate cancer cell line (30,31). Furthermore, the production of MMP-9 in $CM_{AM}$

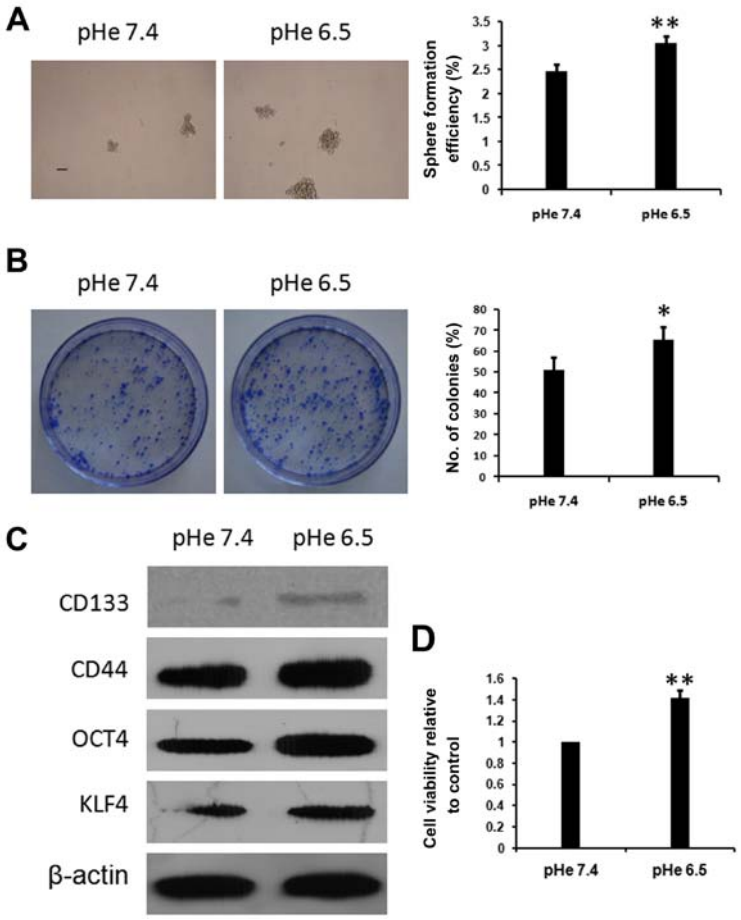


Figure 1. Acidic pHe (pHe 6.5) promotes PC-3 cancer stem cell characteristics. (A) Sphere formation assay of PC-3 cells pre-treated with AM or NM. (B) Colony forming assay of PC-3 cells pre-treated with AM or NM. (C) Protein levels of CD133, CD44, KLF4 and OCT4 were determined by western blot analysis. (D) Cell viability was measured by CCK-8. Data are expressed as the mean ± SD n=3; $^{*}$P<0.05, $^{**}$P<0.01 vs. control group (pre-treated with NM, pHe 7.4).

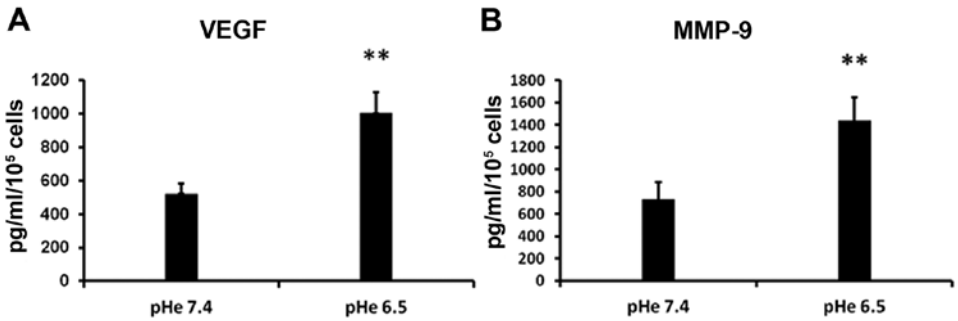


Figure 2. Acidic pHe induces MMP-9 and VEGF secretion. PC-3 cells were incubated in AM (pHe 6.5) or NM for 48 h, then the supernatants were harvested and analyzed by ELISA. (A) MMP9 secretion in PC-3 cells. (B) VEGF secretion in PC-3 cells; $^{**}$P<0.01 vs. control group (incubated in NM, pHe 7.4).

was higher than that in the $CM_{NM}$ (Fig. 2B). It seems that acidic extracellular may induce MMP-9 expression in PC-3 cells and then promote cell invasiveness. Therefore, in our experiments, anti-MMP-9 antibody and the general MMP inhibitor (GM6001) were used to further assess the role of MMP-9 in acid-regulated cell invasiveness. As expected, in the absence of anti-MMP-9 antibody or GM6001, PC-3 cells showed higher invasion activities after 48 h incubation in AM than that in the NM (Fig. 3A and C). Notably, the addition of anti-MMP-9 antibody or GM6001 significantly decreased the invasive ability of PC-3 cells, which were pre-treated in AM or NM for 48 h. However, in the presence of anti-MMP-9 antibody or GM6001, there was no significant differences in the invasive ability between cells pre-treated with AM and cells pre-treated with NM. Taken together, these results revealed that upregulation of MMP-9 in PC-3 cells is involved in the acidy-induced invasion of PC-3 cells.

*PC-3 $CM_{AM}$ promotes cell viability, migration and tube formation of BM-EPCs*. To observe the possible effects of PC-3 CM

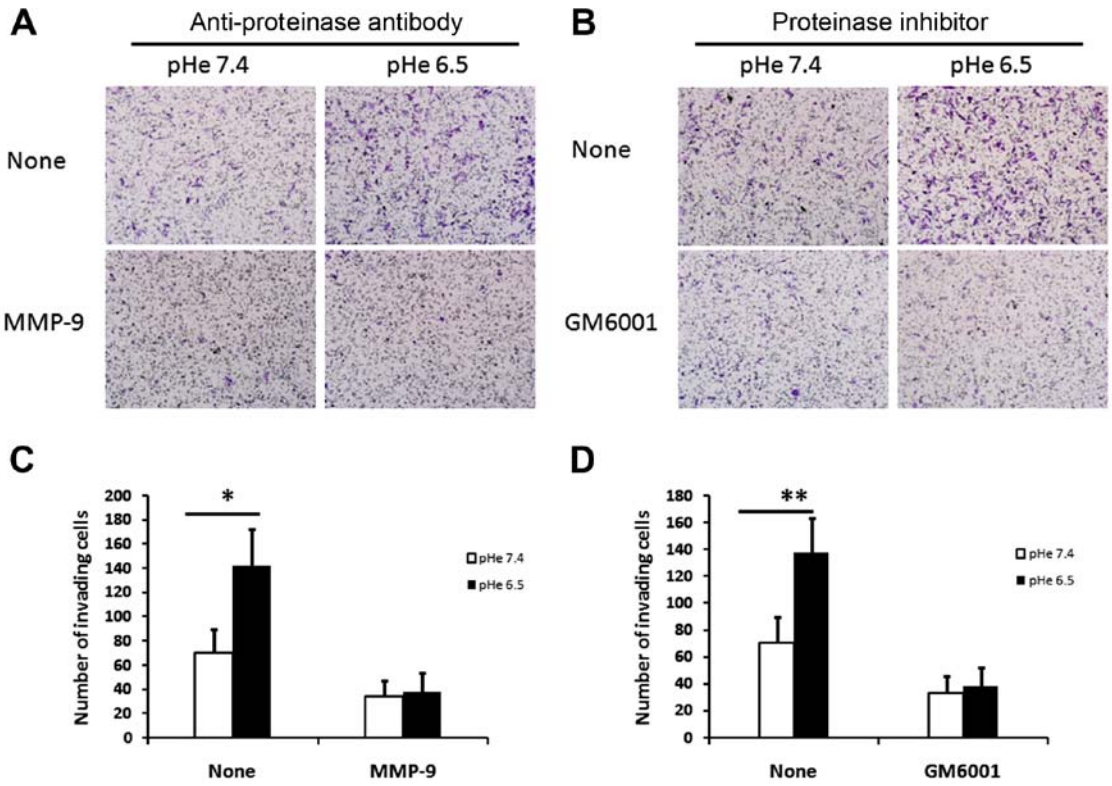


Figure 3. Acidic pHe (pHe 6.5) promotes PC-3 cell invasion by secretion of MMP-9 expression. Anti-MMP-9 antibody (5 Ag/ml) or general MMP inhibitor (GM6001; 15 $\mu$mol/l) were used to evaluate the effect of MMP-9 on the invasive ability of PC-3 cells pre-treated with AM or NM. (A) Representative images of invasion assay in PC-3 cells (pre-treated with AM or NM) in the absence/presence of anti-MMP-9 antibody (5 Ag/ml). (B) Representative images of invasion assay in PC-3 cells (pre-treated with AM or NM) in the absence/presence of GM6001 (15 $\mu$mol/l). (C) Quantitative analysis of invasion assay in PC-3 cells (pre-treated with AM or NM) in the absence/presence of anti-MMP-9 antibody (5 Ag/ml). (D) Quantitative analysis of invasion assay in PC-3 cells (pre-treated with AM or NM) in the absence/presence of GM6001 (15 $\mu$mol/l). Data are expressed as the mean ± SD n=5; $^{*}$P<0.05, $^{**}$P<0.01 vs. control group (pre-treated with NM, pHe 7.4).

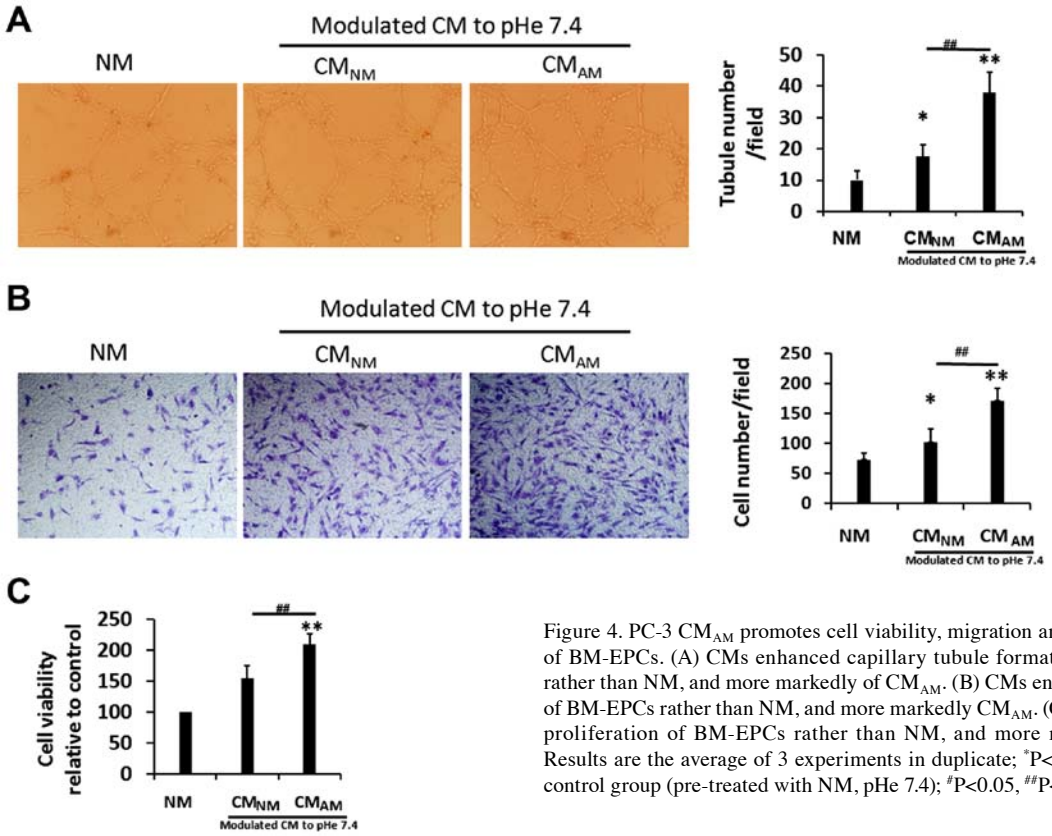


Figure 4. PC-3 $CM_{AM}$ promotes cell viability, migration and tube formation of BM-EPCs. (A) CMs enhanced capillary tubule formation of BM-EPCs rather than NM, and more markedly of $CM_{AM}$. (B) CMs enhanced migration of BM-EPCs rather than NM, and more markedly $CM_{AM}$. (C) CMs enhanced proliferation of BM-EPCs rather than NM, and more markedly $CM_{AM}$. Results are the average of 3 experiments in duplicate; $^{*}$P<0.05, $^{**}$P<0.01 vs. control group (pre-treated with NM, pHe 7.4); $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CM_{AM}$.

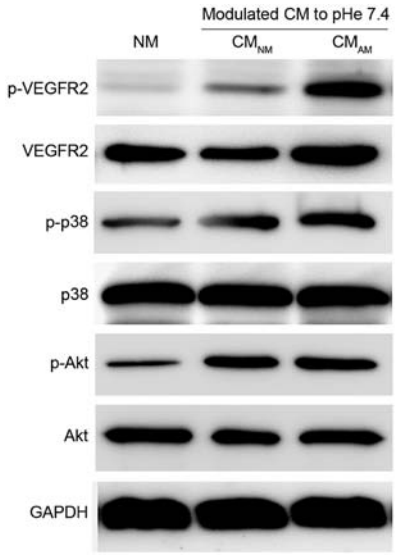


Figure 5. PC-3 $CM_{AM}$ induces the activation of VEGFR2-, Akt- and p38-phosphorylation of BM-EPCs. BM-EPCs were treated with NM, $CM_{AM}$, $CM_{NM}$ (added 1% FBS to NM and CMs, and modulated pH to 7.4) for 2 h, and then harvested. Protein expression of phospho-VEGFR2, VEGFR2, phospho-Akt, Akt, phospho-p38 and p38 was detected by western blot analysis. GAPDH was used as a loading control. Western blot data are representative of at least 3 independent experiments with similar results.

on the vasculogenesis of BM-EPC, we examined whether CMs or NM modulate cell viability, migration and tube formation of BM-EPCs. We found that $CM_{AM}$ and $CM_{NM}$ significantly enhanced cell viability and migration of BM-EPCs than NM, and the effects on cell viability and migration was more obvious in $CM_{AM}$ (Fig. 4B and C). Similarly, $CM_{AM}$ and $CM_{NM}$ promoted capillary tubule formation of BM-EPCs rather than NM, and more markedly in $CM_{AM}$ (Fig. 4A). These data suggest that PC-3 $CM_{AM}$ can promote vasculogenesis of BM-EPCs.

*PC-3 $CM_{AM}$ induces activation of VEGFR2-, Akt- and p38-phosphorylation*. VEGF/VEGFR signaling pathway has been confirmed to play central roles in pathological angiogenesis, and binding of VEGFR2 with VEGF can activate numerous downstream signal pathways, including Akt and p38, which sequentially promote endothelial cell growth, migration and tube formation (32-34). Therefore, we further evaluated whether these signaling pathways are involved in the PC-3 $CM_{AM}$-induced vasculogenesis of BM-EPCs. Western blotting showed the expression levels of phosphorylated VEGFR2, phosphorylated Akt and phosphorylated P38 was significantly increased in $CM_{AM}$ group compared with $CM_{NM}$ and NM groups (Fig. 5). PC-3 $CM_{AM}$ showed obvious promotion effect on phosphorylated AKT and P38. These data revealed that PC-3 $CM_{AM}$ promoted VEGFR2 signal through activation of AKT and p38, then induced vasculogenesis of BM-EPCs.

*$CM_{AM}$-induced vasculogenesis of BM-EPC is partly reduced by the inhibition of VEGFR2 with DMH4*. VEGF has been reported to play critical roles in regulating vasculogenesis of BM-EPCs (24,25). As we previously described, the production of VEGF in $CM_{AM}$ was higher than that in $CM_{NM}$ (Fig. 2A). Taken together, it suggests that VEGF may play an important role in CM-induced vasculogenesis of BM-EPCs. We further

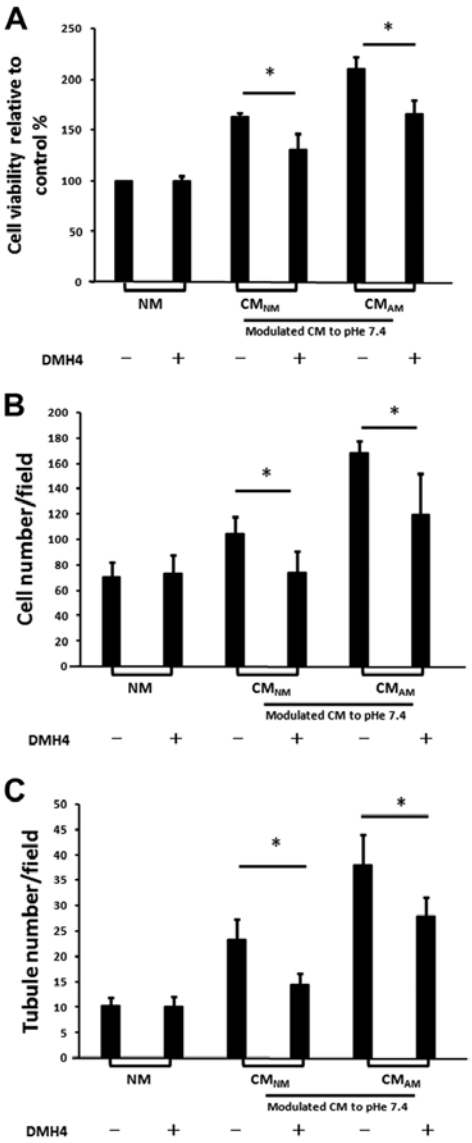


Figure 6. PC-3 $CM_{AM}$-induced BM-EPC-mediated vasculogenesis can be inhibited by the inhibition of VEGFR2 with DMH4. The inhibition of VEGFR2 with DMH4 was used to evaluate the effects of VEGF on vasculogenesis of BM-EPCs treated with NM or CMs. (A) Quantitative analysis of cell viability of BM-EPCs (treated with NM or CMs) in the absence/presence of the inhibition of VEGFR2 with DMH4. (B) Quantitative analysis of migration of BM-EPCs (treated with NM or CMs) in the absence/presence of the inhibition of VEGFR2 with DMH4. (C) Quantitative analysis of tube formation of BM-EPCs (treated with NM or CMs) in the absence/presence of the inhibition of VEGFR2 with DMH4. Data are expressed as mean ± SD n=3; $^{*}$P<0.05, $^{**}$P<0.01 vs. control group (pre-treated with NM, pHe 7.4).

assessed the effects of DMH4 on CM-induced vasculogenesis of BM-EPCs (Fig. 6). We found that the addition of DMH4 to the CMs reduced the cell viability, migration and tube formation of BM-EPCs. The results further confirm that acidic pHe may promote BM-EPCs-mediated vasculogenesis by stimulating the secretion of VEGF in PC-3 cells.

## Discussion

Many types of cancer are reported to be characterized by the presence of cancer stem cells (CSCs), residing in acidic microenvironment, and these rare cell subpopulations are

currently considered responsible for tumor initiation, maintenance and post-therapeutic recurrence. Additionally, CSCs are considered to be involved in bone metastasis of breast (35,36) and prostate cancer (37,38). Acidic tumor microenvironment seems to promote a stem cell-like phenotype in glioma (14). Although the importance of tumor microenvironment in tumor metastasis is recognized, whether acidic tumor microenvironment promotes prostate cancer bone metastasis by enhancing the cancer stem-like cell characteristics of prostate cancer remains unclear.

In the present study, the PC-3 cell line which derives from a metastatic bone of prostate cancer was used as a cell model to investigate the possible effect of acidic extracellular microenvironment on the bone metastasis in prostate cancer. We found that acidic pHe enhanced the PC-3 cell proliferation and tumor sphere formation. Furthermore, acidic pHe increased the expression of CSC stemness-related markers (including CD133, CD44, Oct4 and Klf4) in PC-3 cells. These findings demonstrate that acidic pHe positively regulate the stem cell characteristics of PC-3 cells. Importantly, these stem cell-like cancer cells are considered to be the initial factor in tumor progression and distant metastasis (39). Thus, our results revealed that acidic extracellular microenvironment may promote prostate cancer bone metastasis by enhancing the cancer stem cell characteristics. However, further studies are necessary to uncover the exact mechanism of how acidic pHe enhances PC-3 stem cell characteristics.

It is now well confirmed that the MMPs perform central roles in tumor progression and metastasis (29,40). In addition, MMPs are considered to be promising biomarkers in the diagnosis and prognosis of cancers (41). Given the critical roles that MMPs play in cancer progression and metastasis, MMP inhibitors have been developed to treat cancers (42). Previous studies showed that upregulation of MMP-9 expression increase the capacity of invasion and metastasis in a prostate cancer cell line (30). In addition, it is reported that the MMP-9 was significantly higher in PCa than that of normal adjacent prostate, and its expression closely correlated with clinicopathological stage (18). In the present study, we found that acidic pHe increased the secretion of MMP-9 as well as PC-3 invasiveness, and the capacity of cell invasiveness promoted by acidic pHe can be repressed by MMP-9 antibodies or MMP-9 inhibitors. The results of the present study indicated that acidic pHe may promote cell invasiveness through stimulating upregulation of MMP-9. Recently, it is reported that acidic microenvironment significantly enhances invasiveness of PC-3 cells by stimulating the secretion of cathepsin B (43). These observations are consistent with other reports that proteolytic enzymes are closely related to the invasion and metastasis of many tumors (44,45). Therefore, it seems that acidic tumor microenvironment may stimulate the secretion of many proteolytic enzymes such as MMP-9, which ultimately contributes to tumor invasion and metastasis.

Accumulative evidence illustrated that circulating BM-EPCs are recruited around the tumor by growth factors and chemokines and contribute to an important component of tumor microenvironment (22,23). Importantly, the crucial role of BM-EPCs in mediating the tumor-associated neovasculature has been deeply investigated (46,47). Furthermore, VEGF has been reported to participate in the recruitment of BM-EPCs to tumor neovascularization sites and play critical roles in regulating vasculogenesis of BM-EPCs (25).

VEGF/VEGFR signaling pathway has been confirmed to play central roles in pathological angiogenesis, and various anti-angiogenic drugs have been developed to fight cancer by blocking this pathway (25,48). Previous studies demonstrated that two types of VEGFR (VEGFR1 and VEGFR2) were expressed in BM-EPC (49). Circulating $VEGFR2^+$ BM-EPCs are proved to correlate with tumor metastasis (50). VEGFR2 can activate many downstream signal pathways, including Akt and p38, which sequentially promote endothelial cell growth, migration and tube formation (32,33). In our experiments, the phosphorylation of VEGFR2, Akt and p38 in BM-EPCs was increased by PC-3 CM and the phosphorylation level was more significant when PC-3 cells were cultured at pHe 6.5. This finding indicates that PC-3 $CM_{AM}$ promotes VEGF-induced vasculogenesis of BM-EPCs, which leads to activating the phosphorylation of VEGFR-2, Akt and P38.

In the present study, we found that PC-3 CMs enhanced proliferation and migration of BM-EPCs rather than NM, and the positive impacts on BM-EPCs were more significant when PC-3 cells were cultured at pHe 6.5. Similar impact was found in CM-induced tube formation of BM-EPCs. On the other hand, these positive roles of PC-3 CMs on BM-EPCs were partly reduced by DMH4. The results of the present study revealed that acidic pHe may promote BM-EPCs-mediated vasculogenesis by stimulating the secretion of VEGF in PC-3 cells.

In conclusion, acidic tumor microenvironment may have the potential to modulate the prostate cancer metastasized to bone by enhancing cancer stem cell characteristics, cell invasiveness and VEGF-induced vasculogenesis of BM-EPCs. Given the critical roles that acidic tumor microenvironment plays in tumor growth and metastasis, anticancer strategies should be designed to selectively target acidic tumor microenvironment.

## Acknowledgements

The present study was supported by a grant from the National Natural Science Foundation of China (no. 81402227), the National Basic Research Program of China [973 Program (2012CB619105)], and the Guangdong Natural Science Foundation (no. 2014A030310157).

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