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14. ABSTRACT

Bone is the most common metastatic site for prostate cancer. The growth of the tumor cells in the bone is generally slow and they often become dormant until an appropriate microenviroment is established for their re-growth. The recent stem cell theory predicts that the metastatic cells are a small population of stem-like cells in the primary tumor. However, the precise mechanism of dormancy is virtually unknown, and identifying the responsible factors and understanding their underlining mechanism are crucial for developing a novel therapeutic approach. Our preliminary data indicate that (i) bone morphogenetic protein 7 (BMP7) which is secreted from bone marrow stromal cells is able to induce senescence to prostate tumor cell and (ii) this induction is mediated by activation of the tumor metastasis suppressor gene, N-myc downstream regulated gene 1 (NDRG1). These results strongly suggest that the BMP7-NDRG1axis plays a critical role in dormancy of prostate tumor cells in the bone. The overall goal of this proposal is to elucidate the mechanism of BMP7-induced dormancy in tumor stem cells and explore a possibility of using BMP7 as an anti-metastatic drug for prostate cancer.

15. SUBJECT TERMS
Prostate cancer, dormancy, BMP7, tumor stem cell, bone metastasis

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INTRODUCTION

Bone is the most common metastatic site for prostate cancer which affects approximately 70% of patients with advanced disease (1). Despite this clinical importance, the exact molecular mechanism of the bone-specific metastasis has not been clearly defined. The growth of the tumor cells in the bone is generally slow and they often become dormant until an appropriate microenviroment is established for their re-growth. According to the recent cancer stem cell theory, which still remains a hypotheses, recurrent tumor must arise from a dormant tumor stem cell (2). However, the precise mechanism of dormancy is virtually unknown, and identifying the responsible factors and understanding the underlining mechanism are crucial for developing a novel therapeutic approach. Our recent preliminary data suggest that BMP7 (bone morphogenetic protein 7) which is secreted from bone stromal cells is able to induce senescence to prostate tumor cells and that this induction is mediated by activation of the tumor metastasis suppressor gene, NDRG1 (N-myc downstream regulated gene 1). Therefore, we hypothesize that a prostatic tumor stem cell becomes dormant in the bone through the BMP7-mediated activation of p38 and NDRG1.

BODY

Task 1. To clarify the signaling pathway of BMP7-induced senescence in tumor stem cell.

(a) Examine whether BMP7 blocks Wnt pathway by activating p38-NDRG1 axis.
(b) Test whether BMP7 blocks Wnt pathway by increasing the binding of NDRG1 to LRP6.
(c) Examine whether the BMP7-induced senescence is dependent on Wnt signaling.
(d) Examine whether BMP7-induced MET (mesenchimal-epithelial-transition) is dependent on Wnt signal and whether it is prerequisite for senescence.

To accomplish Task 1 (a)-(c), we first examined the effect of BMP7 and Wnt on the growth of prostate cancer cells. As shown in Figure 1, BMP7 significantly suppressed the growth, while Wnt3a significantly promoted the prostate cancer cell growth. Furthermore, up-regulation of NDRG1 counteracted the effect of Wnt3a on the cancer cell growth, suggesting that the balance of BMP and Wnt signalings may determine whether the tumor cells stay in growth arrest or proliferate. Next, we isolated cancer stem cells (CSCs, CD24-/CD44+/CD133+) from PC3mm cells that were labeled with the luciferase gene. Our result of serial dilution assay for tumor initiating ability in animals indicates that the CSC population was significantly more tumorigenic than non-stem cells ($CD24^{+}$/$CD44^{-}$/$CD133^{-}$) (Figure 2A). We also found that BMP7 was able to activate p38, NDRG1 and p21 (Figure 2B-2D) in CSCs, and that the induction of NDRG1 and p21 was mediated by p38 (Figure 2C and 2D). In addition, BMP7 significantly inhibited the sphere forming ability of CSCs; however, these cells regained the growth ability after withdrawal of BMP7 from the medium (Figure 2E). Furthermore, BMP7 was also able to induce senescence in CSCs (Figure 2F; +/+) with concomitant activation of NDRG1 and p21 (Figure 2G; +/+ and 2H; +/+). Of note, the BMP7-mediated induction of senescence as well as the activation of NDRG1 and p21 was reversed after withdrawal of BMP7 (Figure 2F; +/-, 2G; +/-, and 2H; +/-). Similarly, up-regulation of NDRG1 in CSCs by using PC3mm cell line which had tetracycline-inducible

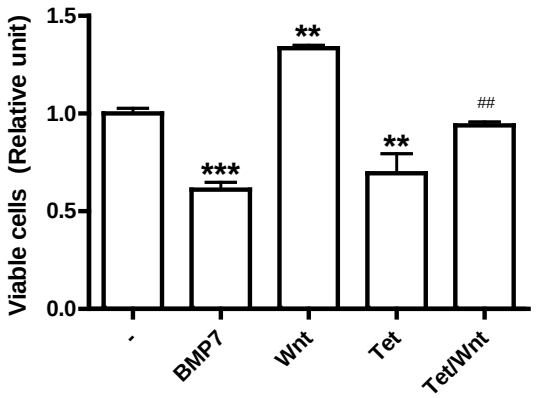

Figure 1. NDRG1 counteracts the effect of Wnt on cancer cell growth. PC3mm/Tet-NDRG1 cells were cultured for 96 h with or without BMP7, Wnt3a or tetracycline, and the cell viability was measured by MTS assay. **; P<0.01, ***; P<0.001 versus -, ##; P<0.01 versus Wnt. *n* = 3.

NDRG1 significantly suppressed the sphere formation and induced senescence, while de-induction of NDRG1 by withdrawal of tetracycline reversed this effect (Figure 2I and 2J). These results suggest that BMP7 was able to induce reversible senescence in CSCs through activation of p38, p21 and NDRG1.

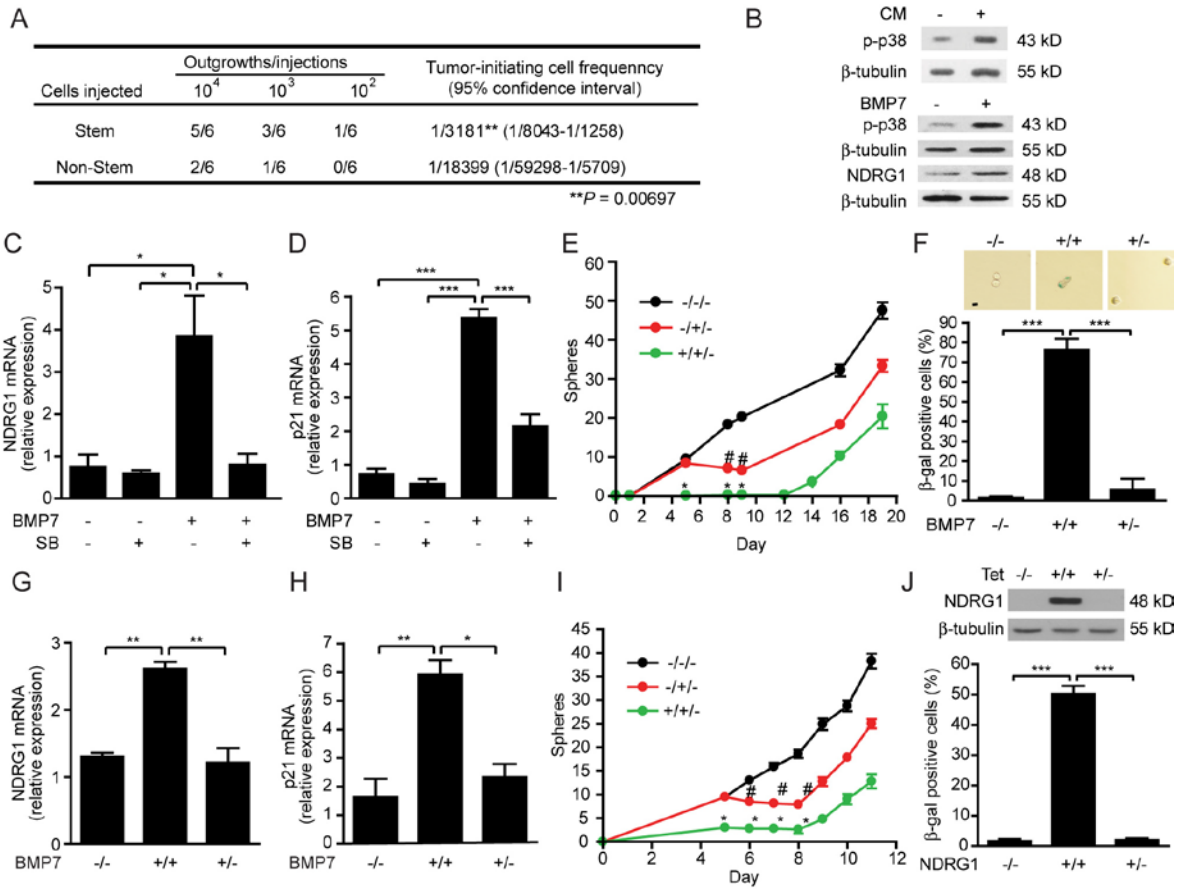

Figure 2. BMP7 induces reversible senescence in cacner stem-like cells (CSCs) through activation of p38, p21 and NDRG1. (A) CSCs isolated from PC3mm were injected subcutaneously into nude mice, and the growth of tumor was monitored by bioluminescence imaging (BLI). (B) The CSCs were treated with the HS5 (bone stromal cells)-CM or BMP7, and the expression of p-p38, NDRG1 and β-Tubulin was examined by Western blot. (C and D) The CSCs were treated with or without BMP7 and/or SB203580 (SB), and the expression of NDRG1 (C) and p21 (D) were examined by qRT-PCR. n = 3, *; P<0.05, ***; P<0.001. (E) Effect of BMP7 on the sphere forming ability of CSCs was measured. (-/-/-): no treatment throughout. (-/+/-): no treatment for 5 days, treated with BMP7 for 4 days, and no treatment thereafter. (+/+/-): treatment for 9 days, and no treatment thereafter (n = 5, *; P<0.001 versus (-/-/-), #; P<0.001 versus (-/-/-)). (F) CSCs were cultured in the presence (+/+), absence (-/-) or withdrawal after treatment (+/-) of BMP7, and SA-β-gal staining was performed. n = 6, ***; P<0.001. Scale bar represents 10 μm. (G and H) The CSCs were treated with BMP7, and the expression of NDRG1 (G) and p21 (H) was measured by qRT-PCR. (-/-): no treatment control, (+/+): continuous treatment with BMP7 for 96 h, (+/-): 48 h-treatment followed by 48 h-withdrawal of BMP7. n = 3, *; P<0.05, **; P<0.01. (I) A similar experiment was performed as (E) for CSCs from PC3mm/Tet-NDRG1 with (+) or without (-) induction of NDRG1, followed by assaying sphere formation. (-/-/-): no induction throughout. (-/+/-): no induction for 5 days, induction for 3 days, and no induction thereafter. (+/+/-): induction for 8 days, and no treatment thereafter (n = 5, *; P<0.001 versus (-/-/-), #; P<0.001 versus (-/-/-)). (J) A similar experiment was performed as (F) for CSCs from PC3mm/Tet-NDRG1. They were cultured with (+/+) or without (-/-) induction or withdrawal after induction of NDRG1 (+/-), and SA-β-gal staining was performed (n = 8, ***; P<0.001). The expression of NDRG1 and β-Tubulin was examined by Western Blot (upper panel).

To accomplish Task 1 (d), we examined whether BMP7 activates MET program in prostate cancer cells. We found that BMP7 significantly reduced vimentin (mesenchymal marker) expression while it significantly augmented E-cadherin (epithelial marker) expression (Figure 3A, B). Moreover, up-regulation of NDRG1 induced similar MET effect (Figure 3C, D), suggesting that BMP7-NDRG1 axis induced MET in prostate cancer cells.
Therefore, we now have identified that BMP7-p38-NDRG1 axis plays critical roles in inducing senescence and MET in CSCs.

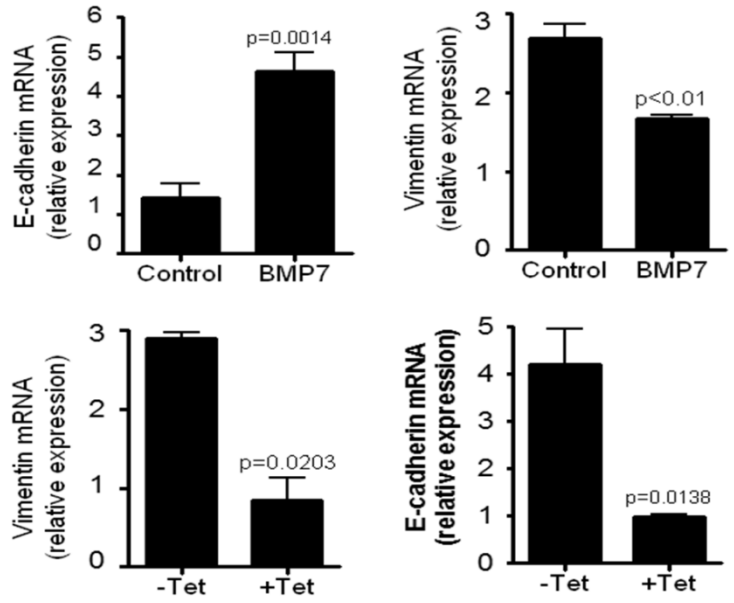

Figure 3. BMP7 induces MET. (A and B) PC3mm cells were cultured with BMP7 or control vehicle for 48 h and the expression of vimentin (A) and E-cadherin (B) were examined by qRT-PCR. (C and D) PC3mm/Tet-NDRG1 cells were cultured with or without tetracycline for 48 h and the expression of vimentin (C) and E-cadherin (D) were examined by qRT-PCR.

To accomplish Task 1, we also examined the effect of Wnt pathway on BMP7-induced senescence, and cancer stem cell population. We found that BMP7significantly suppressed the Wnt 3-mediated self-renewal of CSCs (Figure 4 A, B). In addition, BMP7 was able to induce senescence in cancer cells even in the presence of Wnt ligand (Figure 4 C).

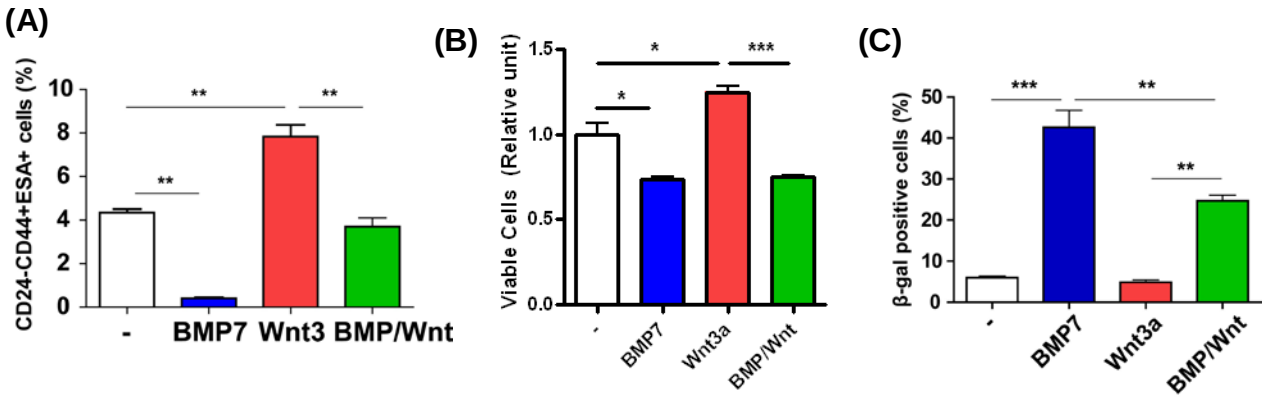

Figure 2. BMP7 suppresses Wnt-induced CSCs growth. PC3mm cells were treated with BMP7 and recombinant Wnt3a for 48h, and the CSC population (A), cell proliferation (B) and senescence (C) were measured by FACS, MTS and SA-β-gal staining.

Task 2. To examine the effect of BMP7 on tumor stem cell *in vivo*
(a) Co-inject tumor stem cell and bone marrow stromal cell into nude mouse using calcium-phosphate scaffold and examine the effect of BMP7 on the tumor growth.

(b) Examine the effect of BMP7 on dormancy by injecting tumor stem cell via intracardiac followed by directly injecting BMP7 intravenously.

To accomplish Task 2, we isolated tumor stem cells from PC3mm labeled with luciferase gene and these cells were seeded onto CaP scaffolds with HS5 (bone stromal cells), and then these constructs were implanted into the upper back of nude mice. Recombinant human BMP7 was peritumorally injected daily after implantation. Tumor growth was monitored weekly by bioluminescent imaging. We found that BMP7 treatment significantly suppressed the tumor growth (Figure 4A and B). To further examine the effect of BMP7 on the tumor growth in bone, CSCs were injected into tibiae of mice with HS5 cells that had either scrambled shRNA (control) or shRNA for BMP7 (shBMP7). We found that the control HS5 was able to suppress tumor growth and activated p38 and NDRG1; however, HS5 with shBMP7 significantly abrogated such an effect on CSCs and failed to suppress tumor-induced osteolysis in the tibiae (Figure 4C). These results suggest that BMP7 secreted from the bone stromal cells is indeed capable of inducing growth arrest of CSCs through activation of p38 and NDRG1 in the bone environment. We also assessed the effect of NDRG1 in the suppression of tumor growth by co-injecting HS5 and CSCs in which NDRG1 expression was knocked down by shRNA (shNDRG1), into mice tibiae. We found that knockdown of NDRG1 in CSCs enabled these cells to grow significantly greater than that of control CSCs even when they were co-injected with HS5 (Figure 4D). These results indicate that the BMP7-NDRG1 axis plays a critical role in the growth suppression of metastasized prostate tumor cells in bone, and also strongly suggest the potential therapeutic utility of BMP7 for metastatic disease.

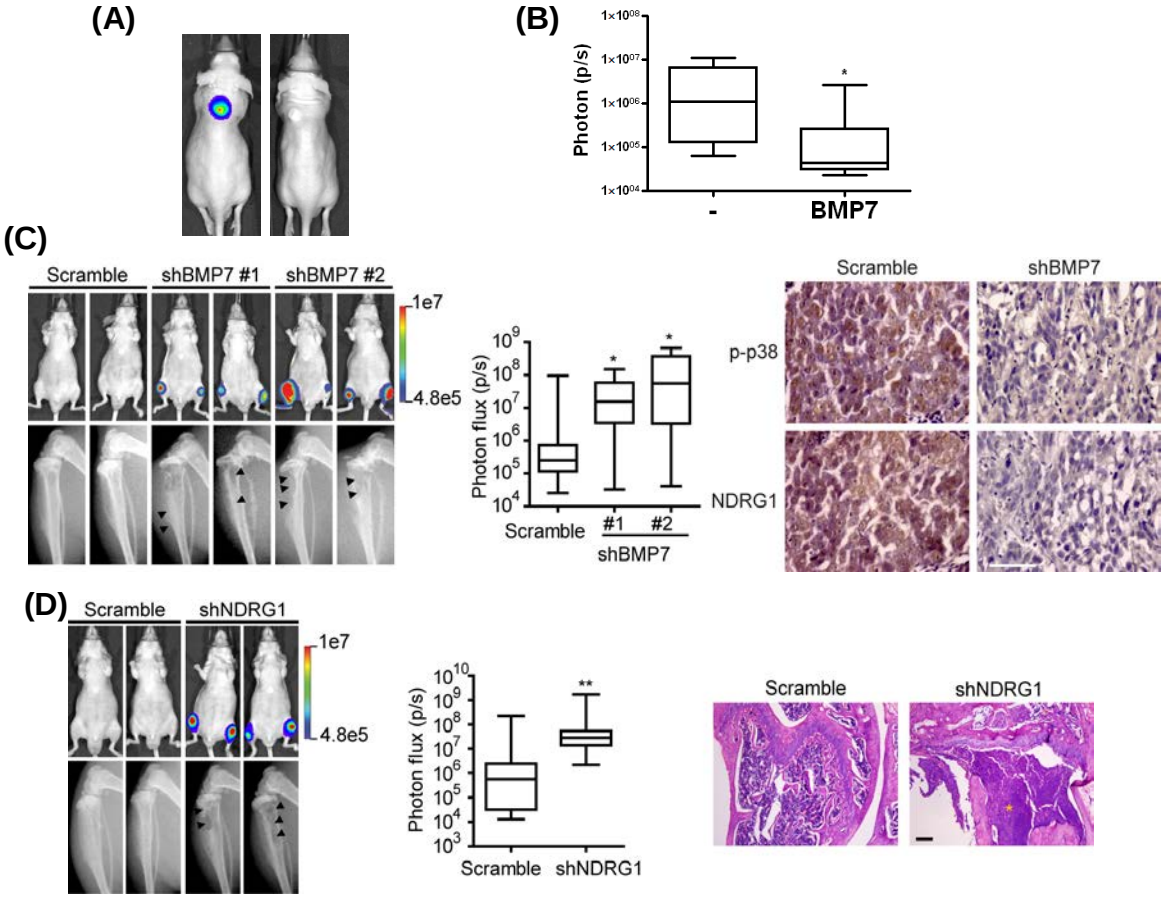

Figure 4. BMP7 suppresses tumor growth of CSCs *in vivo*. (A and B) CSCs from PC3mm were seeded onto CaP scaffolds with HS5 and these constructs were implanted into nude mice, and then BMP7 was administrated daily. BLI of representative mice in each group six weeks after implantation (A). Normalized BLI signals after six weeks (B). -: control vehicle, *n*=6, BMP7: 100 g/kg BMP7 treatment, *n*=10. *; *P*=0.0225 by Mann-Whitney test. (C) CSCs isolated from PC3mm were co-injected into tibiae of nude mice with control (scramble, *n* = 11) or BMP7-knocked down (shBMP7, #1: *n* = 14, #2: *n* = 10) HS5 cells. Left panel: bioluminescence imaging (BLI) and x-ray radiography of bone lesions from representative mice in each group four weeks after inoculation. Osteolytic lesions are indicated by arrow heads. Center panel: Normalized BLI signals after four weeks. *; *P*<0.05 by Mann-Whitney test. Right panel: Representative images of immunohistochemical staining of tumors in the tibiae for p-p38 and NDRG1. Scale bar represents 50 μm. (D) CSCs isolated from PC3mm/shNDRG1 (*n* = 12) or PC3mm/scramble (*n* = 12) were co-injected with HS5 into tibiae of nude mice. Left panel: BLI and x-ray radiography of representative mice in each group four weeks after inoculation. Osteolytic lesions are indicated by arrow heads. Center panel: BLI after four weeks. **; *P*<0.01, by Mann-Whitney test. Right panel: H&E staining of the tibiae from representative mice four weeks after inoculation. Asterisk indicates tumor. Scale bar represents 200 μm.

To directly address the possibility of using BMP7 for therapy of bone metastatic disease, 100 μg/kg of BMP7 was administrated daily through i.v. after intracardiac injection of CSCs from PC3mm or C4-2B cells to the mice. As shown in Figure 5A, BMP7 treatment significantly suppressed the bone metastasis compared to the control. Importantly, withdrawal of BMP7 treatment significantly abrogated its suppressive effect and induced recurrent metastatic growth in the bones. These results suggest that BMP7 suppresses bone metastasis by inducing senescence in disseminated CSCs in the bone. To further verify the inhibitory effect of BMP7 in the bone, CSCs were inoculated directly into mouse tibiae followed by BMP7 treatment. We found that tumor growth in tibiae was indeed significantly suppressed by BMP7 treatment compared to control (Figure 5B), suggesting that direct injection of BMP7 can block the growth of CSCs in the bone. Moreover, withdrawal of BMP7 treatment led the tumor to regain the ability to further proliferate in the bone. Of note, the tumor cells in tibiae exhibited feature of cellular senescence during BMP7 treatment (Figure 5B: BMP7), whereas SA-β-gal staining was strongly decreased after withdrawal of BMP7 (Figure 5B: Withdrawal). These results again suggest that BMP7 plays a critical role in balancing the dormancy and recurrence of CSCs in bone metastasis of prostate cancer.

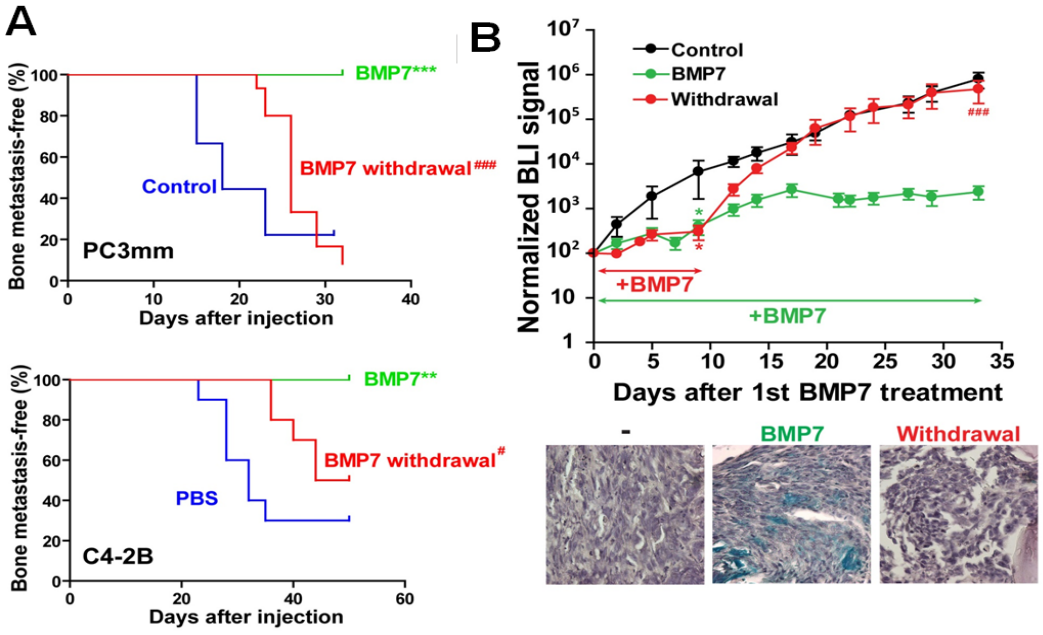

Figure 5. BMP7 suppresses tumor growth but withdrawal induces tumor recurrence of CSCs *in vivo.* (A) Kaplan-Meier bone metastasis-free survival curve of mice after intracardiac injection with CSCs isolated from PC3mm (upper panel) or C4-2B (lower panel) followed by treatment with vehicle or BMP7. Control: control vehicle treatment (PC3mm: n = 9, C4-2B: n = 10), BMP7: continuous treatment with BMP7 (PC3mm: n = 15, C4-2B: n = 10), BMP7 withdrawal: BMP7-treatment (2 weeks for PC3mm and 3 weeks for C4-2B) followed by withdrawal of BMP7 (PC3mm: n = 9, C4-2B: n = 10).***; P=0.0008, **; P=0.0012 versus control, ###; P<0.0001, #; P=0.0289 versus BMP7 by Log-rank test. (B) Upper panel: After injection of CSCs into mouse tibiae, BMP7 was administered daily through i.v. (●): control vehicle treatment, n = 13. (●): continuous treatment with BMP7, n = 10. (●):10-day-treatment followed by withdrawal of BMP7, n = 10. *; P<0.05 versus control (●), ###; P<0.001 versus BMP7 treatment (●). Lower panel: Representative SA-β-gal staining of tumors in the tibiae of mice at the end point. Data represent the mean ± SEM of at least two independent experiments.

Task 3. To examine whether serum level of BMP7 and Wnt ligand correlates with bone metastasis in patients with prostate cancer

To accomplish Task 3, we first analyzed the BMP7 and Wnt3a expression in benign prostate tissue from healthy donors and prostate cancer tissues from patients using an existing data base. We found that BMP7 was significantly down-regulated in prostate cancer tissues compared to benign tissues. On the other hand, Wnt3a was significantly up-regulated in the prostate cancer tissues (Figure 6).

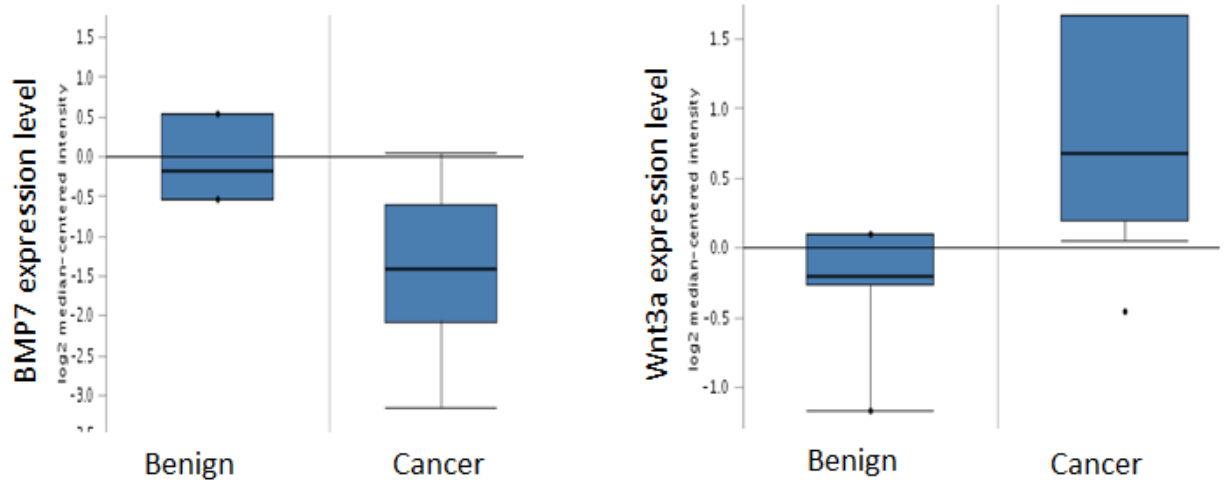

Figure 6. Expression of BMP7 and Wnt3a in clinical samples. The expression levels of BMP7 and Wnt3a were examined in Oncomine data base.

KEY RESEARCH ACCOMPLISHMENTS

1. We found that NDRG1 counteracts the growth promoting effect on prostate cancer cells by Wnt.
2. We found that BMP7 induces senescence by activating p38, p21 and NDRG1 in CSCs.
3. We found that BMP7-induced senescence is reversible.

4. We found that BMP7 induces MET in prostate cancer cells.
5. We found that BMP7 significantly suppresses the growth of CSCs on CaP scaffold model.
6. BMP7 suppresses tumor growth but withdrawal induces tumor recurrence of CSCs in vivo

REPORTABLE OUTCOMES

Peer reviewed publications

Aya Kobayashi, Hiroshi Okuda, Fei Xing, Puspa R. Pandey, Misako Watabe, Shigeru Hirota, Sudha K. Pai, Wen Liu, Koji Fukuda, Christopher Chambers, Andrew Wilber and Kounosuke Watabe "Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone" J Exp Med. 2012 Mar 12;209(3):639.

Abstract/presentation

1. Aya Kobayashi, Hiroshi Okuda, Puspa R. Pandey, Misako Watabe, Sudha K. Pai, Shigeru Hirota, Fei Xing, Wen Liu, Bo Xia, Kounosuke Watabe
   Poster Presentation: "BMP7 regulates dormancy and recurrence of prostate cancer stem cell in bone"
   Joint MRS-AACR Conference: Metastasis and the Tumor Microenvironment, 2010, Philadelphia, PA
2. Aya Kobayashi, Hiroshi Okuda, Puspa Pandey, Misako Watabe, Sudha K. Pai, Fei Xing, Shigeru Hirota, Wen Liu, and Kounosuke Watabe
   Poster Presentation: "Dormancy and recurrence of prostate cancer stem cell are regulated by bone morphogenetic protein 7 in bone"
   Department of Defense (DOD) Prostate Cancer Research Program (PCRP) Innovative Minds in Prostate Cancer Today (IMPaCT) Conference, 2011, Orlando, FL

Employment

Fei Xing, Ph.D. (postdoctoral fellow) has been partly supported by the current grant from May 2012

CONCLUSIONS

We have shown that BMP7 secreted from bone stromal cells induces senescence through activation of p38, p21 and NDRG1. We have also shown that this senescence is reversible and the CSCs regain the ability to proliferate upon withdrawal of BMP7. Therefore, we are now in a strong position to pursue the rest of tasks. In the next fiscal year, we will address two important points. First, we will clarify whether BMP7 blocks the Wnt pathway and whether the Wnt pathway interferes with the BMP7-induces senescence and MET. Secondly, we will compare the expression level of BMP7 and Wnt ligand in sera of prostate cancer petients with or without metastatic disease. The results of this research will provide us with strong rationale for using BMP7 as an anti-recurrence agent to treat prostate cancer patients with metastatic disease. Because the previous recipient of this fellowship (Dr. Aya Kobayashi) left the lab and went back to her own country, Dr. Fei Xing assumed this grant from May 2012. However, Dr. Fei and his mentor (Dr. Watabe) recently moved to University of Mississipppi Medical Center on Aug. 1. Accordingly, the progress of this project was somewhat delayed and we requested 6 months extension of the followship. The request is currently in pending.

When it is approved, Dr. Xing is planning to complete the rest of the project.

So what?

Despite the significant improvement in recent therapeutic technologies, metastatic disease is still the ultimate cause of death in prostate cancer patients (3). Therefore, understanding the underlying mechanisms of dormancy and recurrence in bone metastasis is of paramount interest for developing a effective approach to treat and prevent recurrent prostate cancer. We believe that elucidation of such mechanisms will provide a paradigm shift in this research field and eventually lead to the development of a novel therapeutic approach to this devastating disease.

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