Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone

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Metastatic disease is the major cause of cancer deaths, and recurrent tumors at distant organs are a critical issue. However, how metastatic tumor cells become dormant and how and why tumors recur in target organs are not well understood. In this study, we demonstrate that BMP7 (bone morphogenetic protein 7) secreted from bone stromal cells induces senescence in prostate cancer stem-like cells (CSCs) by activating p38 mitogen-activated protein kinase and increasing expression of the cell cycle inhibitor, p21, and the metastasis suppressor gene, NDRG1 (N-myc downstream-regulated gene 1). This effect of BMP7 depended on BMPR2 (BMP receptor 2), and BMPR2 expression inversely correlated with recurrence and bone metastasis in prostate cancer patients. Importantly, this BMP7-induced senescence in CSCs was reversible upon withdrawal of BMP7. Furthermore, treatment of mice with BMP7 significantly suppressed the growth of CSCs in bone, whereas the withdrawal of BMP7 restarted growth of these cells. These results suggest that the BMP7–BMPR2–p38–NDRG1 axis plays a critical role in dormancy and recurrence of prostate CSCs in bone and suggest a potential therapeutic utility of BMP7 for recurrent metastatic disease.

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Abbreviations used: AR, androgen receptor; BLI, bioluminescence imaging; CM, conditioned medium; CSC, cancer stem-like cell; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; hBMSC, human BM-derived MSC; MAPK, mitogen-activated protein kinase; MSC, mesenchymal stem cell; qRT-PCR, quantitative RT-PCR; SA-β-gal, senescence-associated β-galactosidase; shRNA, short hairpin RNA.

Despite significant improvement in recent therapeutic technologies, >90% of the cancer deaths are still attributed to metastatic disease (Peinado et al., 2008). In the case of prostate and breast cancers, 20–50% patients who have localized cancer and have been "successfully" treated with surgery eventually experience recurrent disease after many years (Karrison et al., 1999; Weckermann et al., 2001; Pfitzenmaier et al., 2006). How metastatic tumor cells become dormant in the distant organs is virtually unknown. However, because recurrent disease is almost always fatal, it is of paramount importance to elucidate the underlying molecular mechanism of dormancy and recurrence to identify novel therapeutic targets. The dormant state of metastatic cell is thought to be controlled by both genetic changes in tumor cell and microenvironment of the metastasized organs (Aguirre-Ghiso, 2007). According to the recent tumor stem cell theory, metastatic cells must have stem-like characteristics such as abilities of self-renewal and differentiation in addition to their invasive capability (Pantel and Alix-Panabières, 2007; Polyak and Weinberg, 2009). Therefore, only a fraction of primary tumor cells is able to establish colonization at the distant organ and also become dormant. However, because dormant lesions in most cases consist of a solitary or small number of tumor cells, they are clinically undetectable, which significantly hampers the progress of research in this field. This dormant state is thought to be maintained by virtue of the balance of local microenvironment such as stroma–tumor cell interaction and secreted growth factors and pro- and antiangiogenic factors, as well as local immune system (Derynck et al., 2001; Bhowmick et al., 2004; Aguirre-Ghiso, 2007). The most critical question is to identify such key players

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that modulate the tumor cell dormancy and to dissect the responsive signaling pathways.

Recently, others demonstrated, through a series of elegant experiments, that the signaling cascade that is controlled by the balance of two prominent pathways, p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), is the key determining factor for tumor cell dormancy (Aguirre-Ghiso et al., 2001, 2003, 2004; Aguirre-Ghiso, 2007). p38 signaling is also known to be involved in the regulation of cell cycle arrest and plays a crucial role in the induction of senescence in response to a variety of stress, including oncogenic stress (Bulavin and Fornace, 2004; Bulavin et al., 2004; Dasari et al., 2006; Tront et al., 2006; Han and Sun, 2007; Sun et al., 2007; Wagner and Nebreda, 2009). A high ratio of ERK/p38 expression was indeed observed in metastatic lesions in an animal model of ovarian cancer, which supports the notion that the balance of ERK and p38 determines the fate of disseminated tumor cells whether to proliferate or stay in dormant state at the distant organ (Aguirre-Ghiso et al., 2004). However, extracellular factors in the microenvironment, either cell–cell interaction or secretory factors, that regulate p38 signaling and modulate the concomitant tumor cell dormancy and recurrence are yet to be determined.

In this study, we found that one of the TGF-β family members, BMP7 (bone morphogenetic protein 7), which is secreted from bone stromal cells, induces senescence through activation of p38 MAPK, cell cycle inhibitor, p21, and the tumor metastasis suppressor gene, *NDRG1* (*N-myc downstream-regulated gene 1*) in prostate cancer stem-like cells (CSCs). Importantly, this BMP7-induced senescence was found to be reversible, indicating that BMP7 plays a critical role in tumor dormancy and recurrence. Our results also suggest that targeting BMP7 signaling may become effective therapeutics to prevent recurrence of metastatic prostate cancer.

RESULTS

A secretory factor from bone stromal cells suppresses the growth of prostate cancer cells

Bone stromal cells are considered to play a critical role in generating a niche for metastasized tumor cells by secreting various growth-inhibitory as well as growth-promoting factors in the microenvironment, which may contribute to tumor dormancy and recurrence (McAllister et al., 2008; Zhang et al., 2009). In an attempt to identify such factors, we first cultured PC3 mm, a metastatic prostate cancer cell line, in the presence of the conditioned medium (CM) of human BM stromal cells (HS5). We found that the CM of HS5 significantly promoted the expression of the key cell cycle inhibitors, p21 and p27 (Fig. 1 A). The up-regulation of p21 by the CM of HS5 was also observed in a panel of prostate cancer cell lines (Fig. 1 B).

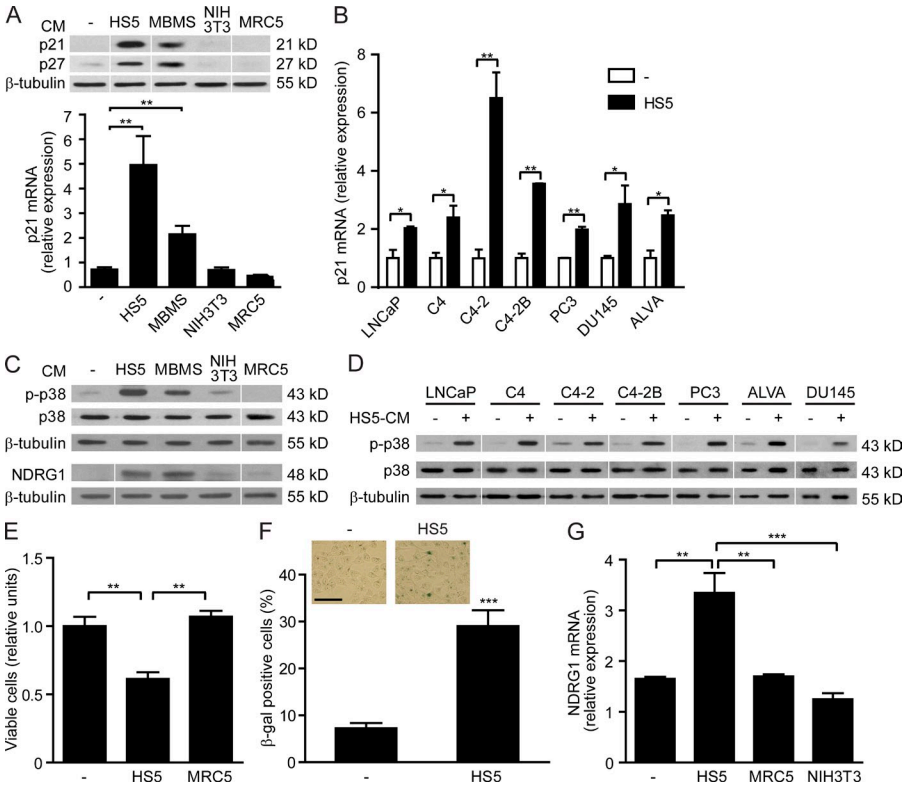

Figure 1. CM of bone stromal cells induces senescence in prostate cancer cells. (A) The prostate cancer cell line PC3 mm was cultured in the presence of CM from human bone stromal cells (HS5), mouse BM stroma (MBMS), mouse embryonic fibroblasts (NIH3T3), or human lung fibroblasts (MRC5) or serum-free medium (−). Expression of p21, p27, and β-tubulin was examined by qRT-PCR ($n = 3$) and Western blot. (B) The prostate tumor cell lines LNCaP, C4, C4-2, C4-2B, PC3, DU145, and ALVA were cultured in the presence of CM of HS5 or serum-free medium (−), and the expression of p21 was examined by qRT-PCR ($n = 3$). (C) PC3 mm was cultured in the presence of CM of HS5, MBMS, NIH3T3, or MRC5 or serum-free medium (−), and the activation of p38 (phosphorylated p38, p-p38; total p38, p38) and the expression of NDRG1 and β-tubulin were examined by Western blot. (D) LNCaP, C4, C4-2, C4-2B, PC3, ALVA, and DU145 were cultured in the presence or absence of CM of HS5, and the expression of p-p38, p38, and β-tubulin was examined by Western blot. (E) PC3 mm was cultured in the presence or absence of CM of HS5 or MRC5 for 48 h, and cell viability was measured by the MTT assay ($n = 3$). (F) PC3 mm was cultured in the presence or absence of HS5-CM for 48 h, and SA–β-gal staining (blue-green) was performed ($n = 4$). The inset shows representative images of three independent experiments. Bar, 100 µm. (G) PC3 mm was cultured in the presence of CM of HS5, MRC5, or NIH3T3 or serum-free medium (−), and the expression of *NDRG1* was measured by qRT-PCR ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed three times independently, and representative data are shown. Results are shown as mean ± SEM.

Moreover, p38 MAPK, which has been shown to be a key regulator of tumor cell dormancy (Aguirre-Ghiso, 2007), was strongly activated in prostate cancer cell lines in the presence of the CM of bone stromal cells (Fig. 1, C and D). In fact, we found that the CM of HS5 significantly suppressed the growth of prostate cancer cell lines (Fig. 1 E and not depicted) and induced senescence-associated β-galactosidase (SA–β-gal) activity (Fig. 1 F) but not apoptosis of PC3 mm cells (not depicted), suggesting that the bone stromal cells secrete a growth-inhibitory factor. Interestingly, we also found that the CM of bone stromal cells significantly up-regulated the metastasis suppressor gene, NDRG1, which was previously shown to suppress tumor metastasis in prostate, breast, and colon cancers (Fig. 1, C and G; Guan et al., 2000; Bandyopadhyay et al., 2003, 2004). It should be noted that we previously showed a significant inverse correlation between NDRG1 expression and bone metastasis in prostate cancer patients (Bandyopadhyay et al., 2003). These results strongly suggest that a secretory factor of bone stromal cells induces cellular senescence by activating p38, NDRG1, and cell cycle inhibitors in tumor cells.

BMP7 up-regulates a metastasis suppressor gene, NDRG1, through activation of p38

The next critical question is the identity of the secretory factor from bone stromal cell. Because TGF-β (Mundy, 2002; Massagué, 2008) and TGF-β family members, BMPs (Celeste et al., 1990; Mundy, 2002) and FGF2 (fibroblast growth factor 2; Johnson et al., 1998; Mundy, 2002; Korah et al., 2004), are all known to exist in the bone microenvironment, we first examined the effect of a selective inhibitor of TGF-β type 1 receptor, SB431542, and a general BMP inhibitor, Noggin, on the activation of NDRG1 and p38. The addition of Noggin to HS5-CM in our assay system significantly blocked the ability of CM to activate NDRG1 and p38, whereas SB431542 did not affect the CM-induced p38 activation, and recombinant human TGF-β failed to activate NDRG1 (Fig. 2 A and not depicted).

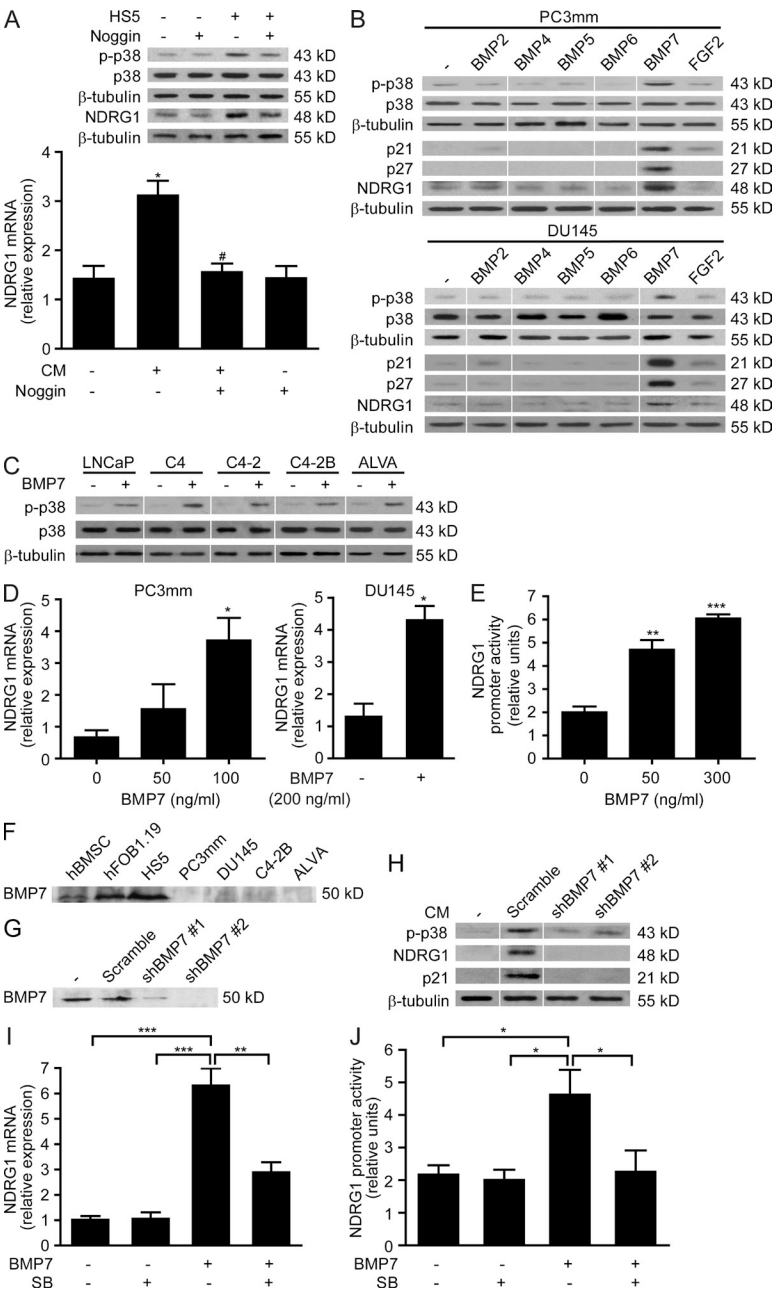

Figure 2. BMP7 up-regulates NDRG1 through activation of p38. (A) PC3 mm cells were grown in the presence of HS5-CM with 3 µg/ml BMP inhibitor Noggin (+) or vehicle (−), and the expression of NDRG1, p-p38, p38, and β-tubulin was examined by Western blot and qRT-PCR ($n = 3$; *, $P < 0.05$ vs. first bar; #, $P < 0.05$ vs. second bar). (B) The effect of BMP2, BMP4, BMP5, BMP6, BMP7, and FGF2 on the expression of p-p38, p38, p21, p27, *NDRG1*, and β-tubulin in PC3 mm and DU145 cells was examined by Western blot. (C) LNCaP, C4, C4-2, C4-2B, and ALVA were cultured with or without 200 ng/ml BMP7, and the expression of p-p38, p38, and β-tubulin was examined by Western blot. (D) The effect of the indicated concentrations of BMP7 on NDRG1 expression in PC3 mm and DU145 was examined by qRT-PCR ($n = 3$; *, $P < 0.05$). (E) The effect of the indicated concentrations of BMP7 on the expression of NDRG1 in PC3 mm was examined by *NDRG1* reporter assay ($n = 3$; **, $P < 0.01$; ***, $P < 0.001$). (F and G) Western blot for BMP7 in the CM from bone stromal cells (hBMSC, hFOB1.19, and HS5) and prostate cancer cells (PC3 mm, DU145, C4-2B, and ALVA; F) or CM from HS5 that had either scrambled shRNA (scramble) or shRNA for *BMP7* (shBMP7; G). (H) PC3 mm cells were cultured with the CM from HS5/scramble or HS5/shBMP7, and the expression of p-p38, NDRG1, p21, and β-tubulin was examined by Western blot. (I and J) PC3 mm cells were treated with or without 200 ng/ml BMP7 in the presence or absence of 10 µM of the p38 inhibitor SB203580 (SB), and the expression of *NDRG1* was examined by qRT-PCR (I) and by *NDRG1* reporter assay (J; $n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All experiments were performed three times independently, and representative data are shown. Results are shown as mean ± SEM.

We then directly tested the effect of purified BMPs as well as FGF2 using both PC3 mm and DU145 cells and found that only BMP7 among these factors was able to activate all of p38, NDRG1, p21, and p27 (Fig. 2 B). The activation of p38 was also observed in a panel of prostate cancer cell lines (Fig. 2 C). Moreover, BMP7 significantly up-regulated the *NDRG1* messenger RNA expression and promoter activity in a dose-dependent manner (Fig. 2, D and E). Furthermore, our results of Western blot analysis demonstrate that BMP7 is expressed at a much higher level in HS5 as well as other bone stromal cells including human BM-derived mesenchymal stem cells (MSCs [hBMSCs]) and human osteoblast cells (hFOB1.19) than in the prostate cancer cells (Fig. 2 F). These results suggest that the major sources of BMP7 at the metastatic sites are various bone stromal cells rather than cancer cells per se. To further verify that BMP7 secreted in CM was responsible for the activation of these molecules, we silenced the *BMP7* gene in HS5 by short hairpin RNA (shRNA; Fig. 2 G). Indeed, CM of BMP7–knocked down HS5 failed to activate p38, NDRG1, and p21, whereas CM of control HS5 strongly stimulated all three proteins (Fig. 2 H). We also found that the activation of *NDRG1* by BMP7 was significantly blocked by the p38 inhibitor, SB203580, suggesting that BMP7 up-regulates NDRG1 through activation of the p38 pathway (Fig. 2, I and J).

BMP7 induces reversible senescence through activation of p38, p21, and NDRG1

BMP7 was originally identified as an osteoinductive factor from bone (Celeste et al., 1990; Ozkaynak et al., 1990), and it has been shown to be involved in normal bone morphogenesis and to play an important role in bone regeneration (Dudley et al., 1995; Luo et al., 1995; Friedlaender et al., 2001; Ristiniemi et al., 2007). To further assess the growth inhibitory effect of BMP7 on tumor cells, we measured SA–β-gal activity in prostate cancer cells and found that BMP7 indeed induced growth arrest with features of cellular senescence and p21 expression (Fig. 3, A–C). Although this activation of p21 by BMP7 was not affected by dominant-negative Smad4 (Fig. 3 D), it was significantly suppressed by the p38 inhibitor (Fig. 3 E), suggesting that BMP7 up-regulates p21 through a non-Smad pathway. Furthermore, knockdown of p21 in PC3 mm cells abrogated BMP7-induced NDRG1 expression, whereas the status of phospho-p38 was not affected by p21 knockdown (Fig. 3 F), implying that BMP7 up-regulates NDRG1 through the activation of p38 and p21. Therefore, these results strongly suggest that the BMP7–p38–p21–NDRG1 axis plays a key role in tumor cell dormancy in the bone.

To gain further insight into BMP7-mediated cell growth arrest, we incubated PC3 mm cells with BMP7 for 8 d followed by withdrawal of this factor. BMP7 significantly suppressed the cell growth; however, cells regained the ability to grow upon withdrawal of BMP7 (Fig. 4 A). We also examined the effect of BMP7 withdrawal on Erk activity. In this experiment, epidermal growth factor (EGF) was used as an activator of Erk. We found that the phosphorylation of Erk was greatly suppressed in the presence of BMP7, whereas p38 phosphorylation was strongly enhanced (Fig. 4 B). Interestingly, withdrawal of BMP7 restored the Erk phosphorylation, whereas it reversed the phosphorylation of p38, suggesting that the p38/Erk ratio plays an important role in determining whether the tumor cells stay in dormant state or proliferate. Furthermore, we tested this reversibility of the BMP7 effect on senescence by SA–β-gal staining. PC3 mm cells were cultured with BMP7 for 48 h, and then they were incubated another 48 h either with (+/+) or without (+/−) BMP7. We found that BMP7 treatment (+/+) significantly increased senescent cells compared with

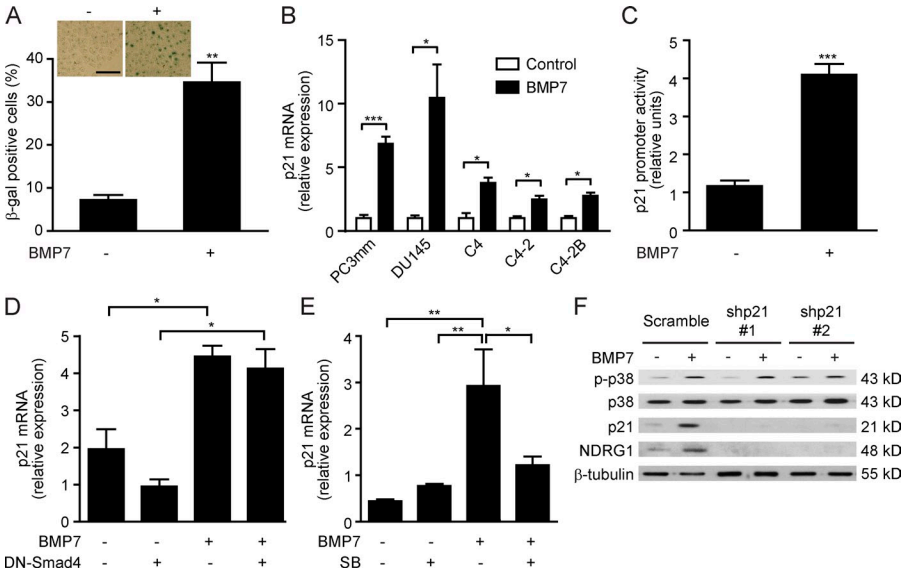

Figure 3. BMP7 up-regulates NDRG1 by activating p38 and p21. (A) Effect of 200 ng/ml BMP7 on senescence in PC3 mm was examined by SA-β-gal staining ($n = 4$). −, control vehicle. The inset shows representative images of three independent experiments. Bar, 100 µm. (B and C) Effect of 200 ng/ml BMP7 on p21 was measured by qRT-PCR and p21 reporter assay in PC3 mm (B and C) and DU145, C4, C4-2, and C4-2B cells (B; $n = 3$). Control, control vehicle. (D) PC3 mm cells were transiently transfected with dominant-negative Smad4 (DN-Smad4) or empty vector and then cultured with or without BMP7 for 48 h. Expression of p21 was assessed by qRT-PCR ($n = 3$). (E) PC3 mm cells were treated with or without 200 ng/ml BMP7 and/or 10 µM SB203580 (SB), and the expression of p21 was examined by qRT-PCR ($n = 3$). (F) PC3 mm cells that had either scrambled shRNA (scramble) or shRNA for p21 (shp21) were treated with or without BMP7, and the expression of p-p38, p38, p21, NDRG1, and β-tubulin was examined by Western blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All experiments were performed three times independently, and representative data are shown. Results are shown as mean ± SEM.

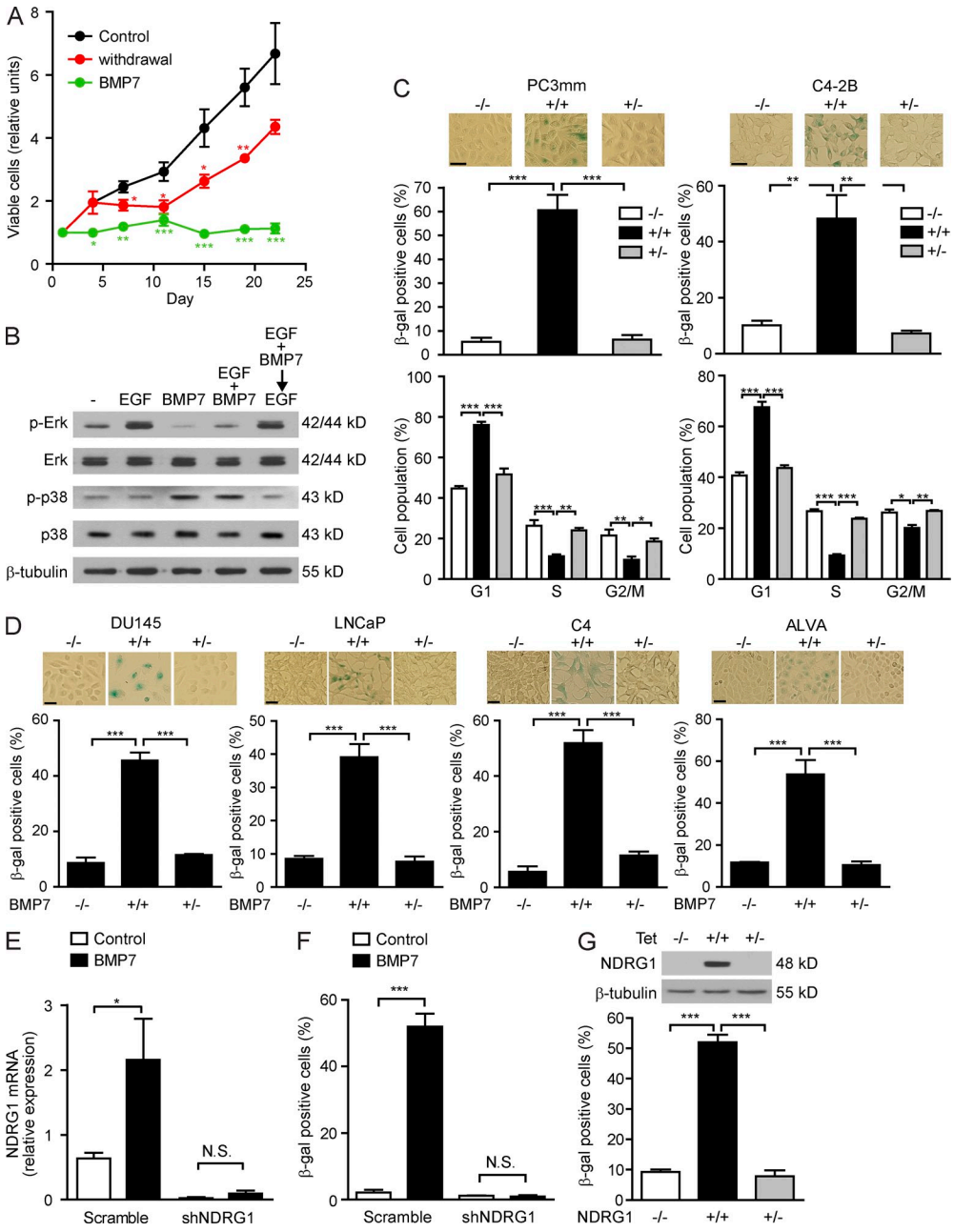

Figure 4. BMP7 induces reversible senescence. (A) Effect of BMP7 on the growth of PC3 mm cells was measured by the MTT assay. Cells were treated with 200 ng/ml BMP7 continuously (green), with BMP7 between day 4 and 11 followed by BMP7 withdrawal (red), or with control vehicle (black; $n = 5$). (B) PC3 mm cells were cultured with 10 ng/ml EGF or 200 ng/ml BMP7 alone, or EGF with BMP7, or EGF with BMP7 followed by BMP7 withdrawal, and the expression of p-Erk, Erk, p-p38, p38, and β-tubulin was examined by Western blot. (C) Effect of BMP7 on growth arrest was examined by SA–β-gal staining (top) and cell cycle analysis (bottom). PC3 mm cells (left) and C4-2B cells (right) were cultured with (+/+) or without (−/−) BMP7 for 96 h. +/− cells were treated with BMP7 for 48 h followed by withdrawal of BMP7 and further incubation for 48 h ($n = 3$). (D) Effect of BMP7 on senescence was examined by SA–β-gal staining. DU145, LNCaP, C4, and ALVA were cultured with (+/+) or without (−/−) BMP7 for 96 h. +/− cells were treated with BMP7 for 48 h followed by withdrawal of BMP7 and further incubation for 48 h ($n = 3$). (C and D) The insets show representative images of three independent experiments. Bars, 50 µm. (E and F) PC3 mm cells that had either scrambled shRNA (scramble) or shRNA for *NDRG1* (shNDRG1) were cultured with BMP7 or control vehicle, and the knockdown of NDRG1 was confirmed by qRT-PCR ($n = 3$; E). These cells were stained for SA–β-gal activity ($n = 4$; F). (G) Effect of NDRG1 on senescence was examined by SA–β-gal staining (bottom). PC3 mm cells stably expressing Tet-NDRG1 were cultured with (+/+) or without (−/−) tetracycline for 96- or 48-h treatment followed by 48-h withdrawal of tetracycline (+/−; $n = 4$). The expression of NDRG1 and β-tubulin was examined by Western blot (top). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The experiment in A was performed twice, experiments in B–G were performed three times independently, and representative data are shown. Results are shown as mean ± SEM.

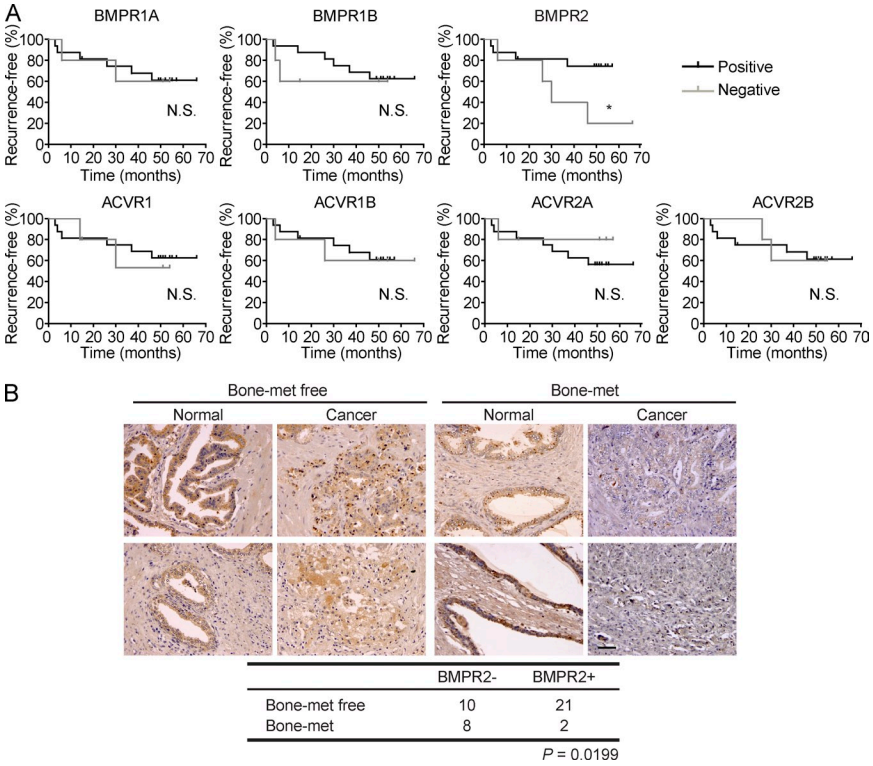

Figure 5. The expression of BMPR2 receptor is inversely correlated with recurrence and bone metastasis of prostate cancer patients. (A) The relation between recurrence-free survival and the expression (positive or negative) of the indicated BMP receptors was analyzed using an existing cohort data of prostate cancer patients (n = 21; Glinsky et al., 2004). *, P = 0.0485 (Log-rank test). (B, top) Representative images of immunohistochemical staining for BMPR2 on prostate cancer patient samples with or without bone metastasis. Bar, 50 µm. (bottom) The correlation between BMPR2 expression and bone metastasis (met) status was evaluated by Fisher's exact test.

control (−/−) cells that were cultured in the absence of BMP7 throughout the period (Fig. 4 C, left). To our surprise, withdrawal of BMP7 (+/−) significantly reduced the senescent cells to the control level, suggesting that BMP7-induced senescence is reversible. In support of this observation, we performed cell cycle analysis and found that the BMP7 treatment (+/+) significantly increased the fraction of cells in the G_1 phase compared with the control (−/−), whereas withdrawal of BMP7 (+/−) significantly decreased the cell population in the G_1 state, suggesting that BMP7 induced G_1 arrest in PC3 mm cells. We also observed the reversible senescence phenotype upon withdrawal of BMP7 in other prostate cancer cell lines (Fig. 4, C [right] and D). Among the cell lines we tested in these experiments, PC3 mm and DU145 are androgen receptor (AR) negative, whereas LNCaP, C4, C4-2B, and ALVA are AR positive. Interestingly, the reversible senescence phenotype was observed in both AR-negative and -positive cells, suggesting that the effect of BMP7 on recurrent growth is independent of the AR status of the cells. We then examined whether NDRG1, a downstream effector of BMP7 signaling, also plays a role in the induction of senescence in prostate cancer cells. When *NDRG1* expression was knocked down by shRNA in PC3 mm (Fig. 4 E), the cells failed to enter senescence even in the presence of BMP7 (Fig. 4 F). In contrast, up-regulation of NDRG1 by adding tetracycline for a total of 96 h (+/+) to the PC3 mm cell line, which had the tetracycline-inducible NDRG1, significantly induced senescence; however, the 48-h withdrawal of tetracycline (+/−) after 48-h incubation significantly reversed this effect (Fig. 4 G). These results suggest that BMP7 induces reversible senescence in prostate cancer cells and that this effect is indeed mediated by NDRG1.

BMP receptor, BMPR2, has an inverse correlation with bone metastasis and recurrence

The results of our in vitro experiments suggest that BMP7-induced reversible senescence contributes to tumor dormancy and recurrence in bone environment. Therefore, we examined the possibility that the status of BMP receptors in patients is a determining factor in bone metastasis and recurrence of prostate cancer. To test this hypothesis, we first analyzed the expression profile of the BMP receptors in prostate cancer patients and their clinical status using the existing database (http://www-genome.wi.mit.edu/cancer/; Glinsky et al., 2004). Among these receptors, the expression of *BMPR2* had significant positive correlation with the recurrence-free survival of patients (Fig. 5 A). To further investigate the role of BMPR2 in prostate cancer and its clinical relevance to bone metastasis, we performed immunohistochemical analysis for BMPR2 with a prostate tissue array that contains 41 primary prostate cancer samples. Consistently, there was a significant inverse correlation between BMPR2 expression and bone metastasis of prostate cancer patients (Fig. 5 B), suggesting that BMPR2 plays a critical role in tumor dormancy and recurrence in bone metastasis. To directly address this question, we first examined the status of BMPR2 expression in our experimental metastasis model in vivo. We isolated cancer cells from micrometastases in tibiae of mice after implanting PC3 mm cells through intracardiac injection. We then examined the expression of BMPR2 in these cells by Western blot and found that a comparable level of BMPR2 expression to the parental cells was maintained in these cells (not depicted). Next, we asked whether knockdown of BMPR2 can abrogate the BMP7-induced dormancy in bone metastasis. To this end, mice were inoculated with PC3 mm cells that had either shRNA for *BMPR2* (shBMPR2) or scrambled shRNA

(control; Fig. 6 A) by intracardiac injection followed by BMP7 treatment. The progression of bone metastasis was monitored by bioluminescence imaging (BLI) using stably expressed luciferase reporter. We found that the BMP7 treatment significantly delayed the onset of bone metastasis of the control group; however, the knockdown of BMPR2 significantly attenuated the effect of BMP7 (Fig. 6 B). These results suggest that BMPR2 plays a direct role in BMP7-induced suppression of bone metastasis. We also confirmed that BMPR2 knockdown disrupted BMP7 signaling in vitro. As shown in Fig. 6 C, BMPR2 deficiency strongly abrogated activation of p38, NDRG1, and p21 by BMP7. Furthermore, by using a cell line with tetracycline-inducible shBMPR2, we found that the knockdown of BMPR2 significantly decreased the senescent cells even in the presence of BMP7 (Tet+/+, BMP7), whereas the withdrawal of tetracycline (i.e., the recovery of BMPR2 expression) ameliorated the effect of BMP7 on the induction of senescence (Tet+/−, BMP7; Fig. 6 D). Collectively, these results suggest that BMPR2 is a critical mediator of BMP7 signaling for tumor cell dormancy in bone metastasis.

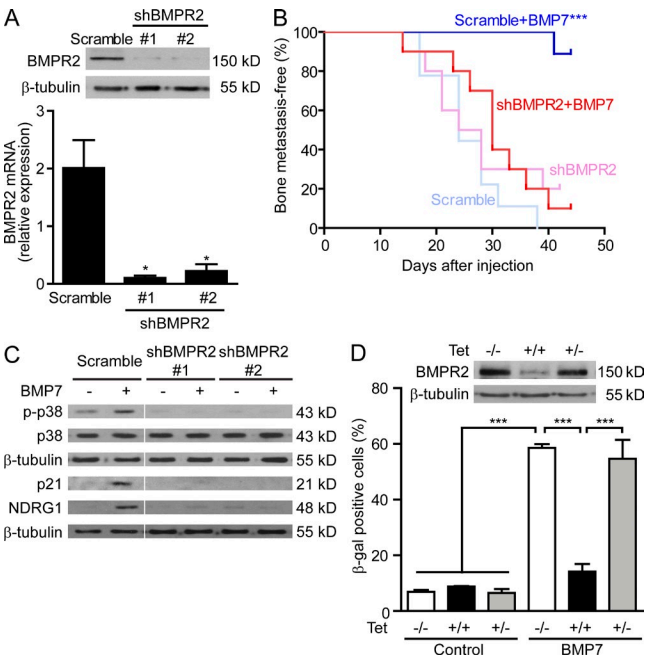

Figure 6. BMPR2 mediates BMP7-induced reversible senescence. (A) The expression of BMPR2 and β-tubulin in PC3 mm that had either scrambled shRNA (scramble) or shRNA for *BMPR2* (shBMPR2) was examined by Western blot (top) and qRT-PCR (bottom; $n = 3$). (B) Kaplan-Meier analysis for bone metastasis–free survival of mice after intracardiac injection of PC3 mm cells that carried either scrambled shRNA (scramble; $n = 9$) or shRNA for *BMPR2* (shBMPR2; $n = 10$) followed by treatment with vehicle or BMP7. Scramble versus Scramble + BMP7: ***, $P < 0.0001$; shBMPR2 versus shBMPR2 + BMP7: NS; Scramble + BMP7 versus shBMPR2 + BMP7: ***, $P = 0.0002$ by Log-rank test. (C) PC3 mm/scramble cells and PC3 mm/shBMPR2 cells were treated with or without BMP7, and the expression of p-p38, p38, p21, NDRG1, and β-tubulin was examined by Western blot. (D) The PC3 mm cells stably expressing Tet-shBMPR2 were cultured with (+) or without (−) induction of shBMPR2 in the presence or absence of BMP7, followed by assaying SA-β-gal. −/−, no induction; +/+, continuous induction; +/−, 48-h induction followed by 48-h withdrawal of tetracycline ($n = 3$). ***, $P < 0.001$. The expression of BMPR2 and β-tubulin was examined by Western blot (inset). Experiments in A, C, and D were performed three times, the experiment in B was performed twice independently, and representative data are shown. Results are shown as mean ± SEM.

BMP7 suppresses the growth of CSCs

According to the cancer stem cell theory, a dormant and recurrent tumor cell must retain stem cell–like properties (Pantel and Alix-Panabières, 2007; Polyak and Weinberg, 2009). Therefore, we intended to examine the effect of BMP7 on CSCs. We first isolated the CSC population ($CD24^-/CD44^+/CD133^+$) from PC3 mm 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 ($CD24^+/CD44^-/CD133^-$; Fig. 7 A). We also found that BMP7 was able to activate p38, NDRG1, and p21 (Fig. 7, B–D) and that the induction of *NDRG1* and p21 was mediated by p38 (Fig. 7, C and D) in the CSCs. 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 (Fig. 7 E). Furthermore, we found that BMP7 was also able to induce senescence in CSCs (Fig. 7 F, +/+) with concomitant activation of *NDRG1* and p21 (Fig. 7, G and H, +/+). Of note, the BMP7-mediated induction of senescence as well as the activation of *NDRG1* and p21 was reversed after withdrawal of BMP7 (Fig. 7, F–H, +/−). Similarly, up-regulation of NDRG1 in CSCs significantly suppressed the sphere formation and induced senescence, whereas de-induction of NDRG1 by withdrawal of tetracycline reversed this effect (Fig. 7, I and J). These results suggest that BMP7 was also able to induce reversible senescence in CSCs through activation of p38, p21, and NDRG1.

BMP7 suppresses the growth of CSCs in a reversible manner in vivo

Our results strongly support a notion that BMP7 secreted from bone stromal cells plays a role in tumor dormancy by suppressing the growth of CSCs in the bone environment. To determine whether the BMP7 from bone stromal cells indeed induces tumor growth arrest and senescence in vivo, we next used an animal model of bone metastasis. CSCs that were labeled with luciferase were isolated and 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 (Fig. 8 A). 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 coinjecting 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 (Fig. 8 B). 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. It is noted that BMP7 is currently FDA approved for healing bone fractures after surgery without having apparent adverse effect (Friedlaender et al., 2001; Ristiniemi et al., 2007). To directly address the possibility of using BMP7 for therapy of bone metastatic disease, 100 µg/kg BMP7 was administrated daily i.v. after intracardiac injection of CSCs from PC3 mm or C4-2B cells to the mice. As shown in Fig. 8 C, BMP7 treatment significantly suppressed the bone metastasis compared with the control. Importantly, withdrawal of BMP7 treatment significantly abrogated its suppressive effect and induced recurrent metastatic growth in the bones. To ensure the responsiveness of tumors derived from CSCs to BMP7, we also examined the effect of BMP7 on bone derivatives that were isolated from mice tibiae and found that BMP7 was indeed able to induce reversible senescence in these bone derivatives (Fig. 8 D). These results suggest that BMP7 suppresses bone metastasis by inducing senescence in disseminated CSCs in the bone.

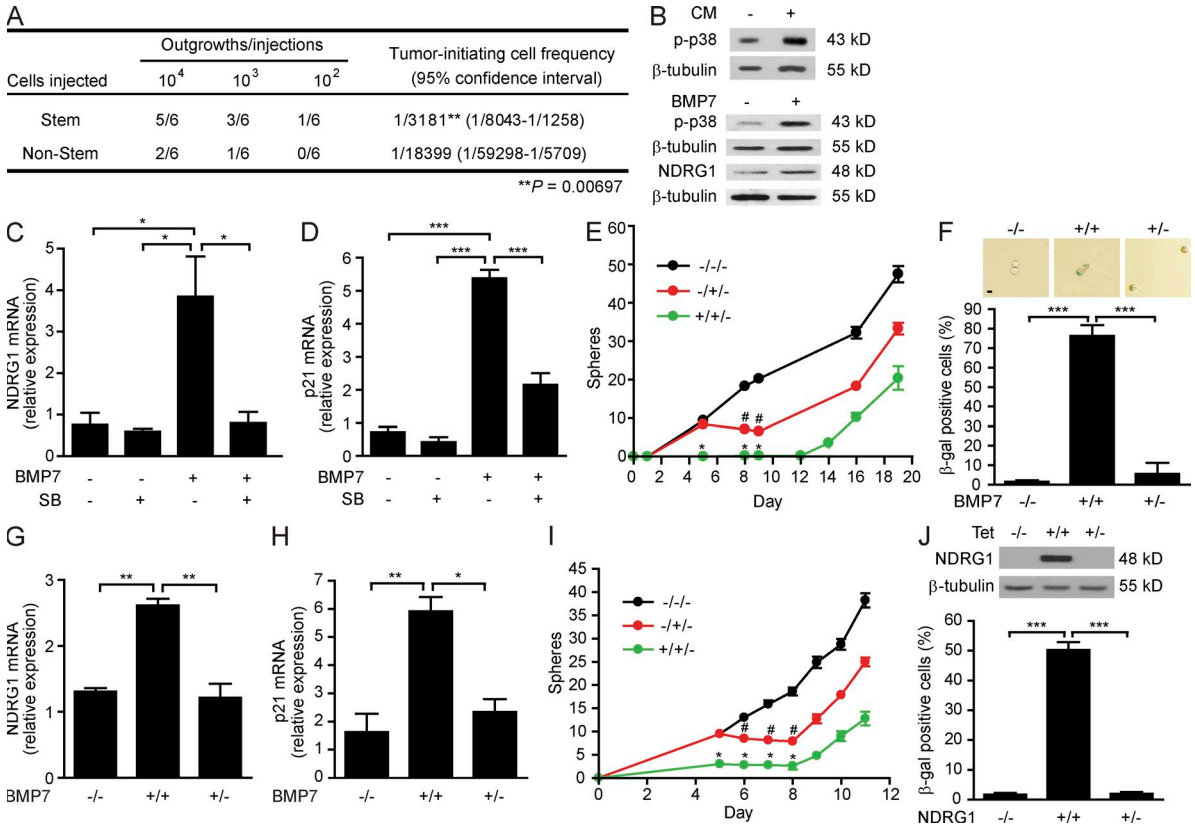

Figure 7. BMP7 induces reversible senescence in CSCs through activation of p38, p21, and NDRG1. (A) CSCs isolated from PC3 mm were injected subcutaneously into nude mice, and the growth of tumors was monitored by BLI. (B) The CSCs were treated with the HS5-CM or BMP7, and the expression of p-p38, NDRG1, 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) was 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 d, treated with BMP7 for 4 d, and no treatment thereafter; +/+/−, treatment for 9 d 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 ($n = 6$). ***, $P < 0.001$. Bar, 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 in E for CSCs from PC3 mm/Tet-NDRG1 with (+) or without (−) induction of NDRG1, followed by assaying sphere formation. −/−/−, no induction throughout; −/+/−, no induction for 5 d, induction for 3 d, and no induction thereafter; +/+/−, induction for 8 d and no treatment thereafter ($n = 5$). *, $P < 0.001$ versus −/−/−; #, $P < 0.001$ versus −/−/−. (J) A similar experiment was performed as in F for CSCs from PC3 mm/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 (top). The experiment in A was performed twice, experiments in B–J were performed three times independently, and representative data are shown. Results are shown as mean ± SEM.

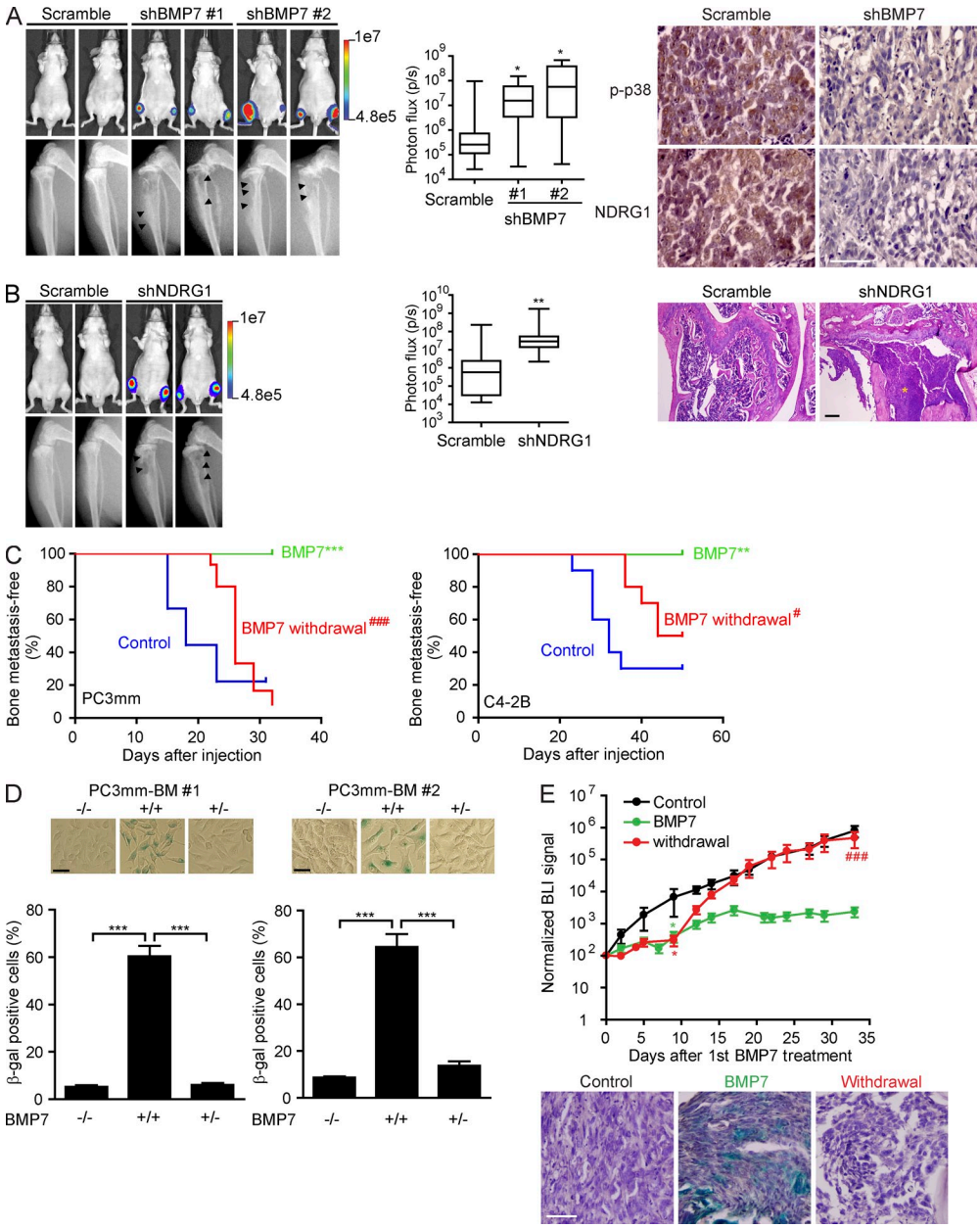

Figure 8. BMP7 suppresses tumor growth, but withdrawal induces tumor recurrence of CSCs in vivo. (A) CSCs isolated from PC3 mm were coinjected into tibiae of nude mice with control (scramble; $n = 11$) or BMP7–knocked down (shBMP7 #1, $n = 14$; #2, $n = 10$) HS5 cells. (left) BLI and x-ray radiography of bone lesions from representative mice in each group 4 wk after inoculation. Osteolytic lesions are indicated by arrowheads. (middle) Normalized BLI signals after 4 wk. *, $P < 0.05$ by Mann-Whitney test. (right) Representative images of immunohistochemical staining of tumors in the tibiae for p-p38 and NDRG1. (B) CSCs isolated from PC3 mm/shNDRG1 ($n = 12$) or PC3 mm/scramble ($n = 12$) were coinjected with HS5 into tibiae of nude mice. (left) BLI and x-ray radiography of representative mice in each group 4 wk after inoculation. Osteolytic lesions are indicated by arrowheads. (middle) BLI after 4 wk. **, $P < 0.01$ by Mann-Whitney test. (right) H&E staining of the tibiae from representative mice 4 wk after inoculation. The asterisk indicates a tumor. (C) Kaplan-Meier bone metastasis–free survival curve of mice after intracardiac injection with CSCs isolated from PC3 mm (left) or C4-2B (right) followed by treatment with vehicle or BMP7. Control, control vehicle treatment (PC3 mm, $n = 9$; C4-2B, $n = 10$); BMP7, continuous treatment with BMP7 (PC3 mm, $n = 15$; C4-2B, $n = 10$); BMP7 withdrawal, BMP7 treatment (2 wk for PC3 mm and 3 wk for C4-2B) followed by withdrawal of BMP7 (PC3 mm, $n = 9$; C4-2B, $n = 10$). ***, $P = 0.0008$; and **, $P = 0.0012$ versus control; and ###, $P < 0.0001$; and #, $P = 0.0289$ versus BMP7 by Log-rank test. (D) Effect of BMP7 on senescence was examined by SA-β-gal staining. The bone derivatives of PC3 mm (PC3 mm–BM) were cultured with (+/+) or without (−/−) BMP7 for 96 h. +/− cells were treated with BMP7 for 48 h followed by withdrawal of BMP7 and further incubation for 48 h ($n = 3$). ***, $P < 0.001$. (E, top) After injection of CSCs into mouse tibiae, BMP7 was administered daily i.v. Control, control vehicle treatment ($n = 13$); BMP7, continuous treatment with BMP7 ($n = 10$); withdrawal, 10-d treatment followed by withdrawal of BMP7 ($n = 10$). *, $P < 0.05$ versus control; ###, $P < 0.001$ versus BMP7 treatment. (bottom) Representative SA-β-gal staining (blue-green) of tumors in the tibiae of mice at the end point. Experiments in A–C and E were performed twice, the experiment in D was performed three times independently, and representative data are shown. Results are shown as mean ± SEM. Bars: (A, D, and E) 50 µm; (B) 200 µm.

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 with control (Fig. 8 E), 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 features of cellular senescence during BMP7 treatment (Fig. 8 E, BMP7), whereas SA–β-gal staining was strongly decreased after withdrawal of BMP7 (Fig. 8 E, 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.

DISCUSSION

Recurrent disease is the most daunting issue for cancer patients because virtually no treatment option is available and it is almost always fatal. Clinically, tumor dormancy is defined as asymptomatic or having minimum residual disease after treatment (Aguirre-Ghiso, 2007). The disseminated cancer cells often undergo dormancy for years to decades before recurrence; however, little is known about the underlying molecular mechanism of the pathological process, although several theories have been proposed, including balance of immunosurveillance, lack of angiogenesis, and induction of quiescence of tumor cells by microenvironmental factors (Aguirre-Ghiso, 2007; Steeg and Theodorescu, 2008). In this study, we have demonstrated that BMP7 secreted from bone stromal cells is capable of inducing senescence to the CSCs through activation of p38, p21, and NDRG1. Importantly, this senescence is reversible and the CSCs regain the ability to proliferate immediately upon withdrawal of BMP7 both in vitro culture and in our animal models. Consistent with these data, the expression of a BMP7 receptor, BMPR2, was found to have significant correlation with recurrence-free survival and significant inverse correlation with bone metastasis of prostate cancer patients. Therefore, our results strongly suggest that BMP7 plays a critical role in tumor dormancy and recurrence, particularly in bone metastasis.

BMP7 belongs to the TGF-β family, and it is involved in various functions of normal development including bone morphogenesis (Dudley et al., 1995; Friedlaender et al., 2001; Chen et al., 2004; Ristiniemi et al., 2007). Several lines of recent evidence suggest that BMP7 contributes to the inhibition of migration and invasion of cancer cells and this may be because of the ability of BMP7 to counteract epithelial–mesenchymal transition (EMT), which is a hallmark of the aggressive tumor phenotype (Zeisberg et al., 2003; Buijs et al., 2007a; Ye et al., 2007). We also found that BMP7 indeed up-regulated the expression of epithelial genes such as E-cadherin, whereas it suppressed mesenchymal genes such as fibronectin and vimentin in PC3 mm cells (unpublished data). In this context, it is noteworthy that EMT induced by Twist1 and/or Twist2 was shown to lead human epithelial cells to escape from oncogene-induced premature senescence (Ansieau et al., 2008). Similarly, another transcription factor Zeb1-mediated EMT has also been suggested to suppress cellular senescence (Liu et al., 2008). Therefore, this ability of BMP7 to counteract EMT may partly contribute to the BMP7-induced reversible senescence of the CSCs. Although BMP7 is expressed in a variety of normal tissues, aberrant expression of BMP7 in various epithelial tumors has also been reported (Mehler et al., 1997; Bobinac et al., 2005; Hruska et al., 2005; Buijs et al., 2007b). The results of expression analysis for BMP7 in prostate and breast cancers are contradictory depending on the reports, which suggests intricate roles of this gene in tumor progression, similar to the role of TGF-β in tumorigenesis (Massagué, 2008). However, it has recently been reported that BMP7 expression in primary human prostate cancer tissue was strongly down-regulated compared with normal prostate epithelium and that ectopic expression of BMP7 in, or exogenous addition of BMP7 to, prostate cancer cell lines significantly suppressed metastatic potential in vivo (Buijs et al., 2007a; Morrisey 2010). Therefore, it is plausible that although BMP7 is necessary for the maintenance of normal differentiation of prostate gland tissue, the loss of expression of this gene promotes EMT and metastatic ability of CSCs. Importantly, our study showed that BMP7 is expressed at a much higher level in the bone stromal cells than in the prostate cancer cells. When the tumor cells reach the distant organs, BMP7 secreted in the microenvironment may again block EMT and promote differentiation of the tumor cells to become senescent and dormant. In addition, our results indicate that BMP7 also induced NDRG1, which was originally identified as a differentiation-related gene. However, questions still remain about what causes the change in BMP7 expression in clinical settings that allows the outgrowth of metastasis. One possible cause is aging as reported that the expression of BMP7 has been observed to be dramatically reduced in human articular cartilage during aging (Chubinskaya et al., 2002). Another possibility is that BMP7 expression is affected by androgen status, which is also associated with aging. The expression of *BMP7* messenger RNA was indeed shown to be increased by androgen and decreased after orchidectomy (Thomas et al., 1998). Therefore, androgen ablation therapy as well as age-related decline in androgens may cause the change in expression of BMP7 that allows the recurrent growth of dormant cancer cells.

BMP7 has been shown to induce bone formation in animal models and enhance bone repair in clinical studies (Sampath et al., 1992; Ripamonti et al., 1996; Friedlaender et al., 2001; Ristiniemi et al., 2007). Our in vivo studies showed that BMP7 treatment inhibited the tumor-induced osteolysis, suggesting that osteoinductive function of BMP7 may also contribute to the tumor suppression in the bone by counteracting the osteolytic activity of cancer cells. The processes of bone resorption and bone formation are almost always coupled, and both resorption and formation have been shown to be activated in most bone metastases (Mundy, 2002). Although bone metastases of prostate cancer are frequently osteoblastic, 20–60% of patients have metastases that are osteolytic or mixed osteolytic and osteoblastic (Niell et al., 1983;

Shimazaki et al., 1992; Cheville et al., 2002). Furthermore, it has been reported that the course of bone lesions showed a tendency to change from the osteolytic to osteoblastic type and relapse was often accompanied by an increase in the osteolytic type lesion (Shimazaki et al., 1992), suggesting that osteolysis plays an important role in bone metastases of prostate cancer. Interestingly, androgen ablation, which is a standard treatment for prostate cancer, has been shown to increase osteoclastic bone resorption and bone loss (Smith et al., 2001, 2005; Guise et al., 2006). This excess fracture risk associated with hormone therapy may be related to the development of bone metastases of prostate cancer.

Cellular senescence is generally viewed as an irreversible process and considered to serve as a barrier against tumorigenesis and metastasis; however, in this study, we have shown that BMP7 reversibly induced senescence and blocked proliferation of CSCs. The reversibility of senescence has been shown in several normal cells. Sage et al. (2003) previously reported that acute loss of retinoblastoma tumor suppressor reversed senescence in mouse embryonic fibroblasts, and they also showed that Ras oncogene–induced senescence was reversed by the loss of retinoblastoma. In addition, inactivation of tumor suppressor, p53, was also shown to reverse replicative senescence in mouse embryonic fibroblasts and human fibroblasts (Beauséjour et al., 2003; Dirac and Bernards, 2003). More recently, reactive oxygen species–induced senescence in primary human fibroblast was found to be reversed by knockdown of PLA2R (phospholipase A2 receptor; Augert et al., 2009). To our knowledge, our result is the first study to show the reversibility of senescence in CSCs, and we are proposing that BMP7-induced reversible senescence is at least a part of the underlying mechanism of tumor cell dormancy and recurrence. Therefore, it is conceivable that loss of BMP7 receptors may serve as one of the mechanisms for metastatic cells to escape from dormancy and promote metastatic recurrence. We indeed found that expression of BMPR2, one of the BMP7 receptors, was significantly correlated with recurrence-free survival and inversely correlated with bone metastasis of prostate cancer patients. We have also shown that when BMPR2 was knocked down in prostate cancer cells, they became irresponsive to activation of p38 signaling and to concomitant induction of senescence in vitro. In support of our hypothesis, BMPR2 expression was also found to be frequently lost in aggressive bladder and colon cancers (Kim et al., 2004; Kodach et al., 2008a,b). Therefore, the BMP7-BMPR2 signaling is considered to play a critical role in metastatic tumor growth and dormancy.

Because the self-renewal of CSCs requires an appropriate niche at the metastasized site that provides specific growth-promoting factors, recurrent growth of such dormant cells is likely to be triggered by stem cell–related pathways such as Wnt, Notch, and Hedgehog (Visvader and Lindeman, 2008; Rosen and Jordan, 2009). Understanding the signaling pathway of BMP7-induced dormancy is an intriguing question, and elucidating the cross talk mechanism with recurrent signaling is clinically an important issue. BMPs exert their biological function through both Smad-dependent and -independent pathways (Derynck and Zhang, 2003; Miyazono et al., 2010). In this study, we have shown that BMP7 induced senescence via a non-Smad pathway by activating p38 MAPK and p21. p38 is a bifunctional cell signaling molecule, and it is reported to both suppress and promote tumor cell proliferation in a context-dependent manner (Wagner and Nebreda, 2009). Interestingly, Ventura et al. (2007) recently reported that p38 was capable of coordinating self-renewal and differentiation of the normal lung stem and progenitor cells and that inactivation of p38 led to abnormal proliferation of lung epithelial cells followed by hypersensitivity to Kras-mediated tumor induction, suggesting an important tumor-suppressive role of p38 at an early stage of tumorigenesis. It should also be noted that p38-activating kinases, MKK4 and MKK6, have been shown to suppress metastasis of ovarian cancer, suggesting a diverse function of p38 in tumor progression (Hickson et al., 2006). Recently, several lines of evidence suggest that the balance of p38 and ERK is a critical factor to determine the state of dormancy and proliferation in several types of tumor cells (Aguirre-Ghiso et al., 2003, 2004). p38 was indeed shown to be able to arrest cell cycle by up-regulating the key negative cell cycle regulator, p21, which is also activated during cellular senescence (Han and Sun, 2007; Abbas and Dutta, 2009). In contrast, ERK is a major positive cell cycle regulator that is responsive to many extracellular mitotic stimuli by regulating G_0–G_1–S phase transition (Hoshino et al., 1999). How the balance of p38/ERK is controlled and what downstream signaling coordinates the direction to dormancy or recurrence are not yet clearly defined; however, our data indicate that the level of BMP7 expression in the metastatic microenvironment or status of BMP receptors of tumors plays a pivotal role in modulating p38 activation and in determining the fate of CSCs for dormancy and recurrence.

NDRG1 expression has been shown to be significantly down-regulated in a variety of carcinomas, including prostate, breast, colon, pancreas, and esophageal squamous cell carcinomas (van Belzen et al., 1997; Bandyopadhyay et al., 2003, 2004; Ando et al., 2006; Maruyama et al., 2006), and importantly, the expression of NDRG1 was shown to be inversely correlated with bone metastasis of prostate cancer patients (Bandyopadhyay et al., 2003). NDRG1 exerts both tumor-suppressor and metastasis-suppressor activities in a cell context–dependent fashion, and we indeed previously showed that NDRG1 was capable of directly suppressing metastasis without affecting the primary tumor growth in an animal model of prostate cancer (Bandyopadhyay et al., 2003). In this study, we demonstrated that NDRG1 was able to induce reversible cellular senescence in a p38- and p21-dependent manner in CSCs. Our results also indicate that the up-regulation of NDRG1 by BMP7 is mediated by p21. p21 has been shown to directly bind to c-Myc, E2F1, and STAT3 and inhibit their transcriptional activity (Kitaura et al., 2000; Abbas and Dutta, 2009). Importantly, both c-Myc and N-Myc have been shown to suppress NDRG1 expression (Li and Kretzner, 2003; Ellen et al., 2008). Therefore, NDRG1 activation by

BMP7 is likely to be mediated by inhibition of c-Myc by p21, although this possibility needs to be further tested. These results suggest that BMP7 induces cellular senescence in CSCs by activating NDRG1 via the p38 signaling pathway followed by mobilizing the intrinsic transcriptional network, which eventually leads to tumor dormancy.

The ultimate goal of treating patients with dormant tumors is to eradicate the residual cancer cells that are believed to be the cancer stem cells; however, confining such cells in the state of perpetual dormancy is another rational approach. Considering that the BMP7–p38–NDRG1 axis induces growth arrest of prostate CSCs, as we reported in this study, and given that BMP7 is already FDA approved for clinical use to heal bone fractures, BMP7 or a small molecule that mimics its function may serve as a potential antirecurrence agent to treat cancer patients with metastatic disease.

MATERIALS AND METHODS

Cell culture. PC3 mm was provided by I.J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX). DU145, HS5, NIH3T3, MRC5, and hFOB1.19 were obtained from the American Type Culture Collection. ALVA41 was provided by W. Rosner (Columbia University, New York, NY). LNCaP, C4, C4-2, and C4-2B were obtained from the University of Texas MD Anderson Cancer Center. The PC3 mm/Tet-NDRG1 cell line was established as previously described (Bandyopadhyay et al., 2006). hBMSCs were isolated from human BM (Lonza) by depletion of red blood cells followed by enrichment of plastic adherent cells. The MSC population was characterized by flow cytometry for expression of negative (CD34 and CD45) and positive (CD44 and CD29) surface markers, and their multipotent potential was confirmed by the capacity to differentiate into adipogenic and osteogenic lineages. hBMSCs were maintained in minimum essential medium with 20% fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin. hFOB1.19 was cultured in 1:1 DME medium/Ham's F12 medium without phenol red supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin at 34°C, and it was differentiated into mature phenotype at 39°C for 24 h. Other cells were grown in RPMI 1640 medium with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin, and they were grown at 37°C in a 5% CO_2 atmosphere. For bioluminescent tracking, cell lines were lentivirally transduced with firefly luciferase. shRNA-expressing lentiviral plasmids carrying puromycin selection markers for BMP7, p21, NDRG1, and BMPR2 were obtained from Thermo Fisher Scientific. The tetracycline-inducible shRNA knockdown was achieved using the Tet-pLKO system (Addgene plasmid 21915; Wiederschain et al., 2009) by targeting the sequence 5′-GCCTATGGAGTGAAATTATTTCTCGAGAAATAATTTCACTCCATAGGC-3′ for *BMPR2*. Recombinant human BMP4, BMP5, BMP6, and BMP7 were purchased from ProSpec. Recombinant human BMP2 and FGF2 were obtained from GenWay Biotech, Inc. Recombinant human Noggin was purchased from R&D Systems. Recombinant human TGF-β was obtained from Pathtech. SB203580 and SB431542 were purchased from Sigma-Aldrich. The dominant-negative mutant of Smad4 was a gift from M.P. Decaestecker (Vanderbilt University, Nashville, TN).

Isolation of CSCs. CSCs were isolated by magnetic bead sorting using a MACS Separator (Miltenyi Biotec). PC3 mm or C4-2B cells were incubated with specific antibodies as follows: anti-CD24-biotin (STEMCELL Technologies), anti-CD44-APC (BioLegend), and anti-CD133-biotin (Miltenyi Biotec). $CD24^-/CD44^+/CD133^+$ cells were then enriched by using a MACS magnet and MS columns (Miltenyi Biotec). All MACS procedures were performed according to the manufacturer's instructions.

Western blot. The cells were lysed and analyzed by immunoblotting using antibodies specific for the following proteins: total and phospho-p38, p21, p27 (Cell Signaling Technology), NDRG1 (gift from T. Commes, Université de Montpellier 2, Montpellier, France), BMP7, BMPR2 (R&D Systems), and β-tubulin (Millipore).

Sphere-forming assay. CSCs were plated (500 cells/ml) in ultra-low attachment plates (Corning) with DME/F12 supplemented with 2% B27 (Invitrogen), 20 ng/ml EGF (Sigma-Aldrich), and 4 µg/ml insulin (Sigma-Aldrich). They were then incubated with or without 200 ng/ml BMP7 or tetracycline as described in the legend for Fig. 7 (E and I). The number of prostaspheres was counted, and data were represented as the means ± SEM.

Reporter assay. The promoter plasmids of pGL3-NDRG1 (promoter region −1,433 to 97) and p21 (WWP-luciferase; a gift from B. Vogelstain, Howard Hughes Medical Institute, Baltimore, MD) were used. They were transfected to PC3 mm using Lipofectamine, and cells were cultured with or without BMP7 or CM for 48 h and subjected to reporter assay. Luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega) and Luminometer (Berthold Detection Systems). For each experiment, the Renilla expression plasmid, phRG-TK (Promega), was cotransfected as an internal control, and the promoter activities were normalized accordingly.

Quantitative RT-PCR (qRT-PCR) analysis. Total RNA was isolated from the cells and reverse transcribed. The cDNA was then amplified with a pair of forward and reverse primers for the following genes: *NDRG1* (5′-CGCTGAGGTGAAGCCTTTGG-3′ and 5′-GGTTCATGCCGATGTCATGG-3′), *p21* (5′-GGAAGACCATGTGGACCTGTC-3′ and 5′-CGGATTAGGGCTTCCTCTTGG-3′), *BMP7* (5′-GATCTCTTCCTGCTCGACAG-3′ and 5′-CAACTTGGGGTTGATGCTCTG-3′), *BMPR2* (5′-CCAAGAGTGTCACTATGAAG-3′ and 5′-TGAATGAGGTGGACTGAGTG-3′), and β-actin (5′-TGAGACCTTCAACACCCCAGCCATG-3′ and 5′-CGTAGATGGGCACAGTGTGGGTG-3′). The thermal cycling conditions composed of an initial denaturation step at 95°C for 1 min followed by 35 cycles of PCR using the following profile: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s.

In situ apoptosis assay. PC3 mm cells were cultured in the presence of CM of HS5 or MCF7 for 48 h and fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton-X 100/0.1% sodium citrate at 4°C. The cells were then washed extensively, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling assay was performed using the In Situ Cell Death Detection kit/TMR Red (Roche). The reaction was stopped after 1 h, and the number of apoptotic cells in each well was counted under an SP5 spectral laser-scanning confocal microscope (Leica).

Animal experiments. All animal work was performed in accordance with a protocol approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine. Athymic nude mice (Harlan) with the ages of 4–5 and 7–8 wk were used for tumorigenic ability assay and other xenograft experiments, respectively. For tumorigenic ability assay, 10^2, 10^3, or 10^4 cells were injected subcutaneously into the mice, and primary tumor growth was monitored weekly by BLI. For intraosseous inoculation, 2×10^4 CSCs with or without 2×10^4 bone stromal cells were injected into the tibiae of the mice. For recurrence assay, 100 µg/kg BMP7 or vehicle alone was subsequently injected daily into the tail vein. For metastasis experiments, 10^5 CSCs isolated from PC3 mm cells, 10^6 CSCs isolated from C4-2B cells, or 5×10^5 PC3 mm/shBMPR2 or PC3 mm/scramble cells were injected into the left cardiac ventricle of the mice. To obtain bone derivatives of PC3 mm CSCs in micrometastases, 10^5 GFP/luciferase-expressing cells were injected into the left cardiac ventricle of the mice. 4 wk after the injection, metastasized cells in tibiae were flashed with a 26-guage needle, and GFP-positive cells were sorted. Several independent bone

derivatives were isolated and named as PC3 mm–BM. The progression of cell growth and development of metastases were monitored by BLI. At the experimental endpoint, mice were sacrificed, and x-ray images of the tibial bone were also taken using a Faxitron instrument (model MX-20; Faxitron Bioptics, LLC).

Histological analysis. All work was performed in accordance with the Springfield Committee for Research Involving Human Subjects. A prostate cancer tissue microarray was obtained from US Biomax, Inc. The tissue microarray slide contained 41 samples from prostate cancer patients for whom clinical data of their bone metastasis status had been collected. Formaldehyde-fixed and paraffin-embedded human prostate tissue specimens were obtained from the surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan) and Iwate Medical School (Iwate, Japan). The sections were baked at 60°C for 1 h, deparaffinized in xylene, and rehydrated. Hind limb long bones of nude mice that were injected with cancer cells were excised and fixed in 10% neutral-buffered formalin. The bone was decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histological services were provided by American HistoLabs, Inc. Immunohistochemical staining of histological sections was performed according to the previously published protocol (Bandyopadhyay et al., 2006) using the EnVision plus System (Dako) and antibodies specific to the following proteins: BMPR2 (Abcam), phospho-p38 (Cell Signaling Technology), and NDRG1 (gift from T. Commes).

SA–β-gal staining. Cells were treated with or without BMP7 or tetracycline as indicated in the figure legends, and SA–β-gal assay was performed using the SA–β-gal staining kit (Cell Signaling Technology) according to the manufacturer's instruction. For staining of CSCs, single cells were obtained by enzymatic digestion of the spheres after each treatment and then washed with PBS before being stained using the SA–β-gal staining kit. For staining of tibiae, samples were fixed in 10% neutral-buffered formalin, decalcified in 10% EDTA for 1 wk, and then washed in PBS before being stained using the SA–β-gal staining kit. After staining, tissues were embedded in paraffin and subsequently counterstained with H&E.

Proliferation assay. Cells were seeded into 96-well plates with 2,000 cells/well in regular growth medium. The cells were then cultured overnight to settle and adhere followed by treating them with conditioned media for 48 h. Cell viability was measured by the MTT assay according to the manufacturer's recommendations (Roche).

Cell cycle analysis. Cells were treated with or without BMP7 as indicated in the figure legends, and they were trypsinized, washed twice in PBS, and fixed in 70% ice-cold ethanol. The fixed cells were washed twice in PBS, treated with 50 μg/ml propidium iodide and 250 μg/ml RNase A for 30 min at 37°C, and analyzed by flow cytometry using the C6 flow cytometer (Accuri Cytometers) and FCS Express software (De Novo Software).

Statistical analysis. Results were reported as mean ± SEM. For in vitro experiments, the Student's *t* test or one-way analysis of variance was applied. For in vivo experiments, group comparisons were performed using the nonparametric Mann-Whitney test or unpaired Student's *t* test. Kaplan-Meier curve comparison was performed with the log-lank test. Tumorigenicity and statistical significance were evaluated with the ELDA software (Extreme Limiting Dilution Analysis; http://bioinf.wehi.edu.au/software/elda/index.html).

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REFERENCES

Abbas, T., and A. Dutta. 2009. p21 in cancer: intricate networks and multiple activities. *Nat. Rev. Cancer.* 9:400–414. http://dx.doi.org/10.1038/nrc2657

Aguirre-Ghiso, J.A. 2007. Models, mechanisms and clinical evidence for cancer dormancy. *Nat. Rev. Cancer.* 7:834–846. http://dx.doi.org/10.1038/nrc2256

Aguirre-Ghiso, J.A., D. Liu, A. Mignatti, K. Kovalski, and L. Ossowski. 2001. Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol. Biol. Cell.* 12:863–879.

Aguirre-Ghiso, J.A., Y. Estrada, D. Liu, and L. Ossowski. 2003. ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). *Cancer Res.* 63:1684–1695.

Aguirre-Ghiso, J.A., L. Ossowski, and S.K. Rosenbaum. 2004. Green fluorescent protein tagging of extracellular signal-regulated kinase and p38 pathways reveals novel dynamics of pathway activation during primary and metastatic growth. *Cancer Res.* 64:7336–7345. http://dx.doi.org/10.1158/0008-5472.CAN-04-0113

Ando, T., H. Ishiguro, M. Kimura, A. Mitsui, H. Kurehara, N. Sugito, K. Tomoda, R. Mori, N. Takashima, R. Ogawa, et al. 2006. Decreased expression of NDRG1 is correlated with tumor progression and poor prognosis in patients with esophageal squamous cell carcinoma. *Dis. Esophagus.* 19:454–458. http://dx.doi.org/10.1111/j.1442-2050.2006.00618.x

Ansieau, S., J. Bastid, A. Doreau, A.-P. Morel, B.P. Bouchet, C. Thomas, F. Fauvet, I. Puisieux, C. Doglioni, S. Piccinin, et al. 2008. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell.* 14:79–89. http://dx.doi.org/10.1016/j.ccr.2008.06.005

Augert, A., C. Payré, Y. de Launoit, J. Gil, G. Lambeau, and D. Bernard. 2009. The M-type receptor PLA2R regulates senescence through the p53 pathway. *EMBO Rep.* 10:271–277. http://dx.doi.org/10.1038/embor.2008.255

Bandyopadhyay, S., S.K. Pai, S.C. Gross, S. Hirota, S. Hosobe, K. Miura, K. Saito, T. Commes, S. Hayashi, M. Watabe, and K. Watabe. 2003. The Drg-1 gene suppresses tumor metastasis in prostate cancer. *Cancer Res.* 63:1731–1736.

Bandyopadhyay, S., S.K. Pai, S. Hirota, S. Hosobe, Y. Takano, K. Saito, D. Piquemal, T. Commes, M. Watabe, S.C. Gross, et al. 2004. Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression. *Oncogene.* 23:5675–5681. http://dx.doi.org/10.1038/sj.onc.1207734

Bandyopadhyay, S., Y. Wang, R. Zhan, S.K. Pai, M. Watabe, M. Iiizumi, E. Furuta, S. Mohinta, W. Liu, S. Hirota, et al. 2006. The tumor metastasis suppressor gene Drg-1 down-regulates the expression of activating transcription factor 3 in prostate cancer. *Cancer Res.* 66:11983–11990. http://dx.doi.org/10.1158/0008-5472.CAN-06-0943

Beauséjour, C.M., A. Krtolica, F. Galimi, M. Narita, S.W. Lowe, P. Yaswen, and J. Campisi. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* 22:4212–4222. http://dx.doi.org/10.1093/emboj/cdg417

Bhowmick, N.A., E.G. Neilson, and H.L. Moses. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature.* 432:332–337. http://dx.doi.org/10.1038/nature03096

Bobinac, D., I. Marić, S. Zorić, J. Spanjol, G. Dordević, E. Mustać, and Z. Fuckar. 2005. Expression of bone morphogenetic proteins in human metastatic prostate and breast cancer. *Croat. Med. J.* 46:389–396.

Buijs, J.T., C.A. Rentsch, G. van der Horst, P.G. van Overveld, A. Wetterwald, R. Schwaninger, N.V. Henriquez, P. Ten Dijke, F. Borovecki, R. Markwalder, et al. 2007a. BMP7, a putative regulator of epithelial homeostasis in the human prostate, is a potent inhibitor of prostate cancer bone metastasis in vivo. *Am. J. Pathol.* 171:1047–1057.

Buijs, J.T., N.V. Henriquez, P.G.M. van Overveld, G. van der Horst, I. Que, R. Schwaninger, C. Rentsch, P. Ten Dijke, A.-M. Cleton-Jansen, K. Driouch, et al. 2007b. Bone morphogenetic protein 7 in the development and treatment of bone metastases from breast cancer. *Cancer Res.* 67:8742–8751. http://dx.doi.org/10.1158/0008-5472.CAN-06-2490

Bulavin, D.V., and A.J. Fornace. 2004. p38 MAP kinase's emerging role as a tumor suppressor. *In* Advances in Cancer Research, Vol. 92. G.F. Vande Woude, and G. Klein, editors. Elsevier Academic Press, San Diego. 95–118.

Bulavin, D.V., C. Phillips, B. Nannenga, O. Timofeev, L.A. Donehower, C.W. Anderson, E. Appella, and A.J. Fornace Jr. 2004. Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. *Nat. Genet.* 36:343–350. http://dx.doi.org/10.1038/ng1317

Celeste, A.J., J.A. Iannazzi, R.C. Taylor, R.M. Hewick, V. Rosen, E.A. Wang, and J.M. Wozney. 1990. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA.* 87:9843–9847. http://dx.doi.org/10.1073/pnas.87.24.9843

Chen, D., M. Zhao, and G.R. Mundy. 2004. Bone morphogenetic proteins. *Growth Factors.* 22:233–241. http://dx.doi.org/10.1080/08977190412331279890

Cheville, J.C., D. Tindall, C. Boelter, R. Jenkins, C.M. Lohse, V.S. Pankratz, T.J. Sebo, B. Davis, and M.L. Blute. 2002. Metastatic prostate carcinoma to bone: clinical and pathologic features associated with cancer-specific survival. *Cancer.* 95:1028–1036. http://dx.doi.org/10.1002/cncr.10788

Chubinskaya, S., B. Kumar, C. Merrihew, K. Heretis, D.C. Rueger, and K.E. Kuettner. 2002. Age-related changes in cartilage endogenous osteogenic protein-1 (OP-1). *Biochim. Biophys. Acta.* 1588:126–134.

Dasari, A., J.N. Bartholomew, D. Volonte, and F. Galbiati. 2006. Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements. *Cancer Res.* 66:10805–10814. http://dx.doi.org/10.1158/0008-5472.CAN-06-1236

Derynck, R., and Y.E. Zhang. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature.* 425:577–584. http://dx.doi.org/10.1038/nature02006

Derynck, R., R.J. Akhurst, and A. Balmain. 2001. TGF-β signaling in tumor suppression and cancer progression. *Nat. Genet.* 29:117–129. http://dx.doi.org/10.1038/ng1001-117

Dirac, A.M.G., and R. Bernards. 2003. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J. Biol. Chem.* 278:11731–11734. http://dx.doi.org/10.1074/jbc.C300023200

Dudley, A.T., K.M. Lyons, and E.J. Robertson. 1995. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* 9:2795–2807. http://dx.doi.org/10.1101/gad.9.22.2795

Ellen, T.P., Q. Ke, P. Zhang, and M. Costa. 2008. NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states. *Carcinogenesis.* 29:2–8. http://dx.doi.org/10.1093/carcin/bgm200

Friedlaender, G.E., C.R. Perry, J.D. Cole, S.D. Cook, G. Cierny, G.F. Muschler, G.A. Zych, J.H. Calhoun, A.J. LaForte, and S. Yin. 2001. Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. *J. Bone Joint Surg. Am.* 83-A:S151–S158.

Glinsky, G.V., A.B. Glinskii, A.J. Stephenson, R.M. Hoffman, and W.L. Gerald. 2004. Gene expression profiling predicts clinical outcome of prostate cancer. *J. Clin. Invest.* 113:913–923.

Guan, R.J., H.L. Ford, Y. Fu, Y. Li, L.M. Shaw, and A.B. Pardee. 2000. Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. *Cancer Res.* 60:749–755.

Guise, T.A., K.S. Mohammad, G. Clines, E.G. Stebbins, D.H. Wong, L.S. Higgins, R. Vessella, E. Corey, S. Padalecki, L. Suva, and J.M. Chirgwin. 2006. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. *Clin. Cancer Res.* 12:6213s–6216s. http://dx.doi.org/10.1158/1078-0432.CCR-06-1007

Han, J., and P. Sun. 2007. The pathways to tumor suppression via route p38. *Trends Biochem. Sci.* 32:364–371. http://dx.doi.org/10.1016/j.tibs.2007.06.007

Hickson, J.A., D. Huo, D.J. Vander Griend, A. Lin, C.W. Rinker-Schaeffer, and S.D. Yamada. 2006. The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. *Cancer Res.* 66:2264–2270. http://dx.doi.org/10.1158/0008-5472.CAN-05-3676

Hoshino, R., Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-i, H. Wada, J. Fujimoto, and M. Kohno. 1999. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene.* 18:813–822. http://dx.doi.org/10.1038/sj.onc.1202367

Hruska, K.A., S. Mathew, and G. Saab. 2005. Bone morphogenetic proteins in vascular calcification. *Circ. Res.* 97:105–114. http://dx.doi.org/10.1161/01.RES.00000175571.53833.6c

Johnson, M.R., C. Valentine, C. Basilico, and A. Mansukhani. 1998. FGF signaling activates STAT1 and p21 and inhibits the estrogen response and proliferation of MCF-7 cells. *Oncogene.* 16:2647–2656. http://dx.doi.org/10.1038/sj.onc.1201789

Karrison, T.G., D.J. Ferguson, and P. Meier. 1999. Dormancy of mammary carcinoma after mastectomy. *J. Natl. Cancer Inst.* 91:80–85. http://dx.doi.org/10.1093/jnci/91.1.80

Kim, I.Y., D.-H. Lee, D.K. Lee, W.J. Kim, M.M. Kim, R.A. Morton, S.P. Lerner, and S.J. Kim. 2004. Restoration of bone morphogenetic protein receptor type II expression leads to a decreased rate of tumor growth in bladder transitional cell carcinoma cell line TSU-Pr1. *Cancer Res.* 64:7355–7360. http://dx.doi.org/10.1158/0008-5472.CAN-04-0154

Kitaura, H., M. Shinshi, Y. Uchikoshi, T. Ono, S.M. Iguchi-Ariga, and H. Ariga. 2000. Reciprocal regulation via protein-protein interaction between c-Myc and p21(cip1/waf1/sdi1) in DNA replication and transcription. *J. Biol. Chem.* 275:10477–10483. http://dx.doi.org/10.1074/jbc.275.14.10477

Kodach, L.L., S.A. Bleuming, A.R. Musler, M.P. Peppelenbosch, D.W. Hommes, G.R. van den Brink, C.J.M. van Noesel, G.J.A. Offerhaus, and J.C.H. Hardwick. 2008a. The bone morphogenetic protein pathway is active in human colon adenomas and inactivated in colorectal cancer. *Cancer.* 112:300–306. http://dx.doi.org/10.1002/cncr.23160

Kodach, L.L., E. Wiercinska, N.F.C.C. de Miranda, S.A. Bleuming, A.R. Musler, M.P. Peppelenbosch, E. Dekker, G.R. van den Brink, C.J.M. van Noesel, H. Morreau, et al. 2008b. The bone morphogenetic protein pathway is inactivated in the majority of sporadic colorectal cancers. *Gastroenterology.* 134:1332–1341. http://dx.doi.org/10.1053/j.gastro.2008.02.059

Korah, R., M. Boots, and R. Wieder. 2004. Integrin α5β1 promotes survival of growth-arrested breast cancer cells: an in vitro paradigm for breast cancer dormancy in bone marrow. *Cancer Res.* 64:4514–4522. http://dx.doi.org/10.1158/0008-5472.CAN-03-3853

Li, J., and L. Kretzner. 2003. The growth-inhibitory Ndrg1 gene is a Myc negative target in human neuroblastomas and other cell types with overexpressed N- or c-myc. *Mol. Cell. Biochem.* 250:91–105. http://dx.doi.org/10.1023/A:1024918328162

Liu, Y., S. El-Naggar, D.S. Darling, Y. Higashi, and D.C. Dean. 2008. Zeb1 links epithelial-mesenchymal transition and cellular senescence. *Development.* 135:579–588. http://dx.doi.org/10.1242/dev.007047

Luo, G., C. Hofmann, A.L. Bronckers, M. Sohocki, A. Bradley, and G. Karsenty. 1995. BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* 9:2808–2820. http://dx.doi.org/10.1101/gad.9.22.2808

Maruyama, Y., M. Ono, A. Kawahara, T. Yokoyama, Y. Basaki, M. Kage, S. Aoyagi, H. Kinoshita, and M. Kuwano. 2006. Tumor growth suppression in pancreatic cancer by a putative metastasis suppressor gene Cap43/NDRG1/Drg-1 through modulation of angiogenesis. *Cancer Res.* 66:6233–6242. http://dx.doi.org/10.1158/0008-5472.CAN-06-0183

Massagué, J. 2008. TGFbeta in Cancer. *Cell.* 134:215–230. http://dx.doi.org/10.1016/j.cell.2008.07.001

McAllister, S.S., A.M. Gifford, A.L. Greiner, S.P. Kelleher, M.P. Saelzler, T.A. Ince, F. Reinhardt, L.N. Harris, B.L. Hylander, E.A. Repasky, and R.A. Weinberg. 2008. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell.* 133:994–1005. http://dx.doi.org/10.1016/j.cell.2008.04.045

Mehler, M.F., P.C. Mabie, D. Zhang, and J.A. Kessler. 1997. Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* 20:309–317. http://dx.doi.org/10.1016/S0166-2236(96)01046-6

Miyazono, K., Y. Kamiya, and M. Morikawa. 2010. Bone morphogenetic protein receptors and signal transduction. *J. Biochem.* 147:35–51. http://dx.doi.org/10.1093/jb/mvp148

Morrissey, C., L.G. Brown, T.E. Pitts, R.L. Vessella, and E. Corey. 2010. Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment. *Neoplasia.* 12:192–205.

Mundy, G.R. 2002. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer.* 2:584–593. http://dx.doi.org/10.1038/nrc867

Niell, H.B., G.M. Palmieri, C.L. Neely, T.A. Maxwell, S.C. Hopkins, and M.S. Soloway. 1983. Total, dialyzable, and nondialyzable postabsorptive hydroxyproline. Values in patients with cancer. *Arch. Intern. Med.* 143:1925–1927. http://dx.doi.org/10.1001/archinte.143.10.1925

Ozkaynak, E., D.C. Rueger, E.A. Drier, C. Corbett, R.J. Ridge, T.K. Sampath, and H. Oppermann. 1990. OP-1 cDNA encodes an osteogenic protein in the TGF-beta family. *EMBO J.* 9:2085–2093.

Pantel, K., and C. Alix-Panabières. 2007. The clinical significance of circulating tumor cells. *Nat. Clin. Pract. Oncol.* 4:62–63. http://dx.doi.org/10.1038/ncponc0737

Peinado, H., S. Rafii, and D. Lyden. 2008. Inflammation joins the "niche". *Cancer Cell.* 14:347–349. http://dx.doi.org/10.1016/j.ccr.2008.10.012

Pfitzenmaier, J., W.J. Ellis, E.W. Arfman, S. Hawley, P.O. McLaughlin, P.H. Lange, and R.L. Vessella. 2006. Telomerase activity in disseminated prostate cancer cells. *BJU Int.* 97:1309–1313. http://dx.doi.org/10.1111/j.1464-410X.2006.06194.x

Polyak, K., and R.A. Weinberg. 2009. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat. Rev. Cancer.* 9:265–273. http://dx.doi.org/10.1038/nrc2620

Ripamonti, U., B. Van Den Heever, T.K. Sampath, M.M. Tucker, D.C. Rueger, and A.H. Reddi. 1996. Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1 (hOP-1, bone morphogenetic protein-7). *Growth Factors.* 13:273–289. http://dx.doi.org/10.3109/08977199609003228

Ristiniemi, J., T. Flinkkilä, P. Hyvönen, M. Lakovaara, H. Pakarinen, and P. Jalovaara. 2007. RhBMP-7 accelerates the healing in distal tibial fractures treated by external fixation. *J. Bone Joint Surg. Br.* 89-B:265–272. http://dx.doi.org/10.1302/0301-620X.89B2.18230

Rosen, J.M., and C.T. Jordan. 2009. The increasing complexity of the cancer stem cell paradigm. *Science.* 324:1670–1673. http://dx.doi.org/10.1126/science.1171837

Sage, J., A.L. Miller, P.A. Pérez-Mancera, J.M. Wysocki, and T. Jacks. 2003. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature.* 424:223–228. http://dx.doi.org/10.1038/nature01764

Sampath, T.K., J.C. Maliakal, P.V. Hauschka, W.K. Jones, H. Sasak, R.F. Tucker, K.H. White, J.E. Coughlin, M.M. Tucker, R.H. Pang, et al. 1992. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J. Biol. Chem.* 267:20352–20362.

Shimazaki, J., T. Higa, S. Akimoto, M. Masai, and S. Isaka. 1992. Clinical course of bone metastasis from prostatic cancer following endocrine therapy: examination with bone x-ray. *Adv. Exp. Med. Biol.* 324:269–275. http://dx.doi.org/10.1007/978-1-4615-3398-6_29

Smith, M.R., F.J. McGovern, A.L. Zietman, M.A. Fallon, D.L. Hayden, D.A. Schoenfeld, P.W. Kantoff, and J.S. Finkelstein. 2001. Pamidronate to prevent bone loss during androgen-deprivation therapy for prostate cancer. *N. Engl. J. Med.* 345:948–955. http://dx.doi.org/10.1056/NEJMoa010845

Smith, M.R., W.C. Lee, J. Brandman, Q. Wang, M. Botteman, and C.L. Pashos. 2005. Gonadotropin-releasing hormone agonists and fracture risk: a claims-based cohort study of men with nonmetastatic prostate cancer. *J. Clin. Oncol.* 23:7897–7903. http://dx.doi.org/10.1200/JCO.2004.00.6908

Steeg, P.S., and D. Theodorescu. 2008. Metastasis: a therapeutic target for cancer. *Nat. Clin. Pract. Oncol.* 5:206–219. http://dx.doi.org/10.1038/ncponc1066

Sun, P., N. Yoshizuka, L. New, B.A. Moser, Y. Li, R. Liao, C. Xie, J. Chen, Q. Deng, M. Yamout, et al. 2007. PRAK is essential for ras-induced senescence and tumor suppression. *Cell.* 128:295–308. http://dx.doi.org/10.1016/j.cell.2006.11.050

Thomas, R., W.A. Anderson, V. Raman, and A.H. Reddi. 1998. Androgen-dependent gene expression of bone morphogenetic protein 7 in mouse prostate. *Prostate.* 37:236–245. http://dx.doi.org/10.1002/(SICI)1097-0045(19981201)37:4<236::AID-PROS5>3.0.CO;2-C

Tront, J.S., B. Hoffman, and D.A. Liebermann. 2006. Gadd45a suppresses Ras-driven mammary tumorigenesis by activation of c-Jun NH2-terminal kinase and p38 stress signaling resulting in apoptosis and senescence. *Cancer Res.* 66:8448–8454. http://dx.doi.org/10.1158/0008-5472.CAN-06-2013

van Belzen, N., W.N. Dinjens, M.P. Diesveld, N.A. Groen, A.C. van der Made, Y. Nozawa, R. Vlietstra, J. Trapman, and F.T. Bosman. 1997. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. *Lab. Invest.* 77:85–92.

Ventura, J.J., S. Tenbaum, E. Perdiguero, M. Huth, C. Guerra, M. Barbacid, M. Pasparakis, and A.R. Nebreda. 2007. p38α MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nat. Genet.* 39:750–758. http://dx.doi.org/10.1038/ng2037

Visvader, J.E., and G.J. Lindeman. 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer.* 8:755–768. http://dx.doi.org/10.1038/nrc2499

Wagner, E.F., and A.R. Nebreda. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat. Rev. Cancer.* 9:537–549. http://dx.doi.org/10.1038/nrc2694

Weckermann, D., P. Müller, F. Wawroschek, R. Harzmann, G. Riethmüller, and G. Schlimok. 2001. Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. *J. Urol.* 166:699–703. http://dx.doi.org/10.1016/S0022-5347(05)66046-6

Wiederschain, D., S. Wee, L. Chen, A. Loo, G. Yang, A. Huang, Y. Chen, G. Caponigro, Y.M. Yao, C. Lengauer, et al. 2009. Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle.* 8:498–504. http://dx.doi.org/10.4161/cc.8.3.7701

Ye, L., J.M. Lewis-Russell, H. Kynaston, and W.G. Jiang. 2007. Endogenous bone morphogenetic protein-7 controls the motility of prostate cancer cells through regulation of bone morphogenetic protein antagonists. *J. Urol.* 178:1086–1091. http://dx.doi.org/10.1016/j.juro.2007.05.003

Zeisberg, M., J.-i. Hanai, H. Sugimoto, T. Mammoto, D. Charytan, F. Strutz, and R. Kalluri. 2003. BMP-7 counteracts TGF-β1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat. Med.* 9:964–968. http://dx.doi.org/10.1038/nm888

Zhang, X.H.F., Q. Wang, W. Gerald, C.A. Hudis, L. Norton, M. Smid, J.A. Foekens, and J. Massagué. 2009. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell.* 16:67–78. http://dx.doi.org/10.1016/j.ccr.2009.05.017