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Vascular Endothelial Growth Factor Contributes to the Prostate Cancer-Induced Osteoblast Differentiation Mediated by Bone Morphogenetic Protein

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ABSTRACT

Human prostate cancer has a high predisposition to metastasize to bone, resulting in the formation of osteoblastic metastases. The mechanism through which prostate cancer cells promote osteoblastic lesions is undefined. Vascular endothelial growth factor (VEGF) has been implicated as a mediator of osteoblast activity. In the present study, we examined if prostate cancer cells promote osteoblastic activity through VEGF. We found that LNCaP and C4-2B prostate cancer cell lines and primary tumor and metastatic prostate cancer tissues from patients expressed VEGF. Bone morphogenetic proteins (BMPs), which are normally present in the bone environment, induced VEGF protein and mRNA expression in C4-2B cells. Furthermore, BMP-7 activated the VEGF promoter. Noggin, a BMP inhibitor, diminished VEGF protein expression and promoter activity in C4-2B cells. Conditioned media (CM) from C4-2B cells induced pro-osteoblastic activity (increased alkaline phosphatase, osteocalcin, and mineralization) in osteoblast cells. Both noggin alone and anti-VEGF antibody alone diminished C4-2B CM-induced pro-osteoblastic activity. Transfection of C4-2B cells with VEGF partially rescued the C4-2B CM-induced pro-osteoblastic activity from noggin inhibition. These observations indicate that BMPs promote osteosclerosis through VEGF in prostate cancer metastases. These results suggest a novel function for VEGF in skeletal metastases. Specifically, VEGF promotes osteoblastic lesion formation at prostate cancer bone metastatic sites.

INTRODUCTION

Advanced prostate cancer (CaP) often metastasizes to bone. At the metastatic bone site, CaP often induces bone formation, resulting in an overall appearance of osteosclerosis. The resulting bone is immature woven bone as opposed to organized mechanically sound lamellar bone. The metastatic lesions produce severe pain and predispose the bone to fracture. Thus, preventing progression of these metastases may help improve the quality of life of CaP patients. Accordingly, understanding the mechanisms that promote CaP-induced osteosclerosis may help identify targets to diminish the progression of these lesions.

CaP cells produce a variety of factors that have direct or indirect osteogenic properties (reviewed in Refs. 1–3). Some of these factors, such as bone morphogenetic proteins (BMPs; Refs. 4–6) and ET-1 (7) may directly stimulate differentiation of osteoblast precursors to mature mineral-producing osteoblasts. Other factors, such as parathyroid hormone-releasing protein, may work through inhibition of osteoblast apoptosis (8–10). Despite this gamut of putative mediators of CaP-induced osteosclerosis, there are no *in vivo* studies that unequivocally demonstrate their role in this process. However, one family of these molecules, the BMPs, is somewhat unique among osteogenic factors. Specifically, the BMPs are the only recognized factors that can initiate osteoblast differentiation from uncommitted progenitors, *in vitro,* as well as *in vivo* (11–16). This observation, along with reports that several BMPs are expressed in prostate tumors but at low or undetectable levels in normal prostate (5, 17, 18), suggest that BMPs contribute to the mechanism of CaP-mediated osteogenesis.

There are several lines of evidence that suggest angiogenesis is critical for bone formation by providing vascularization needed for bone-forming cells (19–21). Angiogenesis is mediated by several proangiogenic factors; however, there is evidence that one proangiogenic factor, vascular endothelial growth factor (VEGF), promotes bone production directly, *e.g.,* it has recently been demonstrated that VEGF stimulates chemotactic migration of primary human osteoblasts (22) and osteoblast differentiation (23, 24). Furthermore, VEGF expression is regulated by the osteoblastogenic BMPs (25, 26). The observations that VEGF promotes osteoblastic activity, in addition to several reports that demonstrate CaP patients express increased serum VEGF (reviewed in Ref. 27), led us to explore if VEGF contributes to CaP's ability to promote pro-osteoblastic activity.

MATERIALS AND METHODS

Recombinant Proteins. BMP-2, -4, -6, and -7 recombinant proteins were obtained from R&D Systems (Minneapolis, MN).

Cells and Tissues. The LNCaP cell-derived series of human CaP cell lines (reviewed in Ref. 28) closely mimics clinical CaP progression. Specifically, the tumors secrete prostate-specific antigen, progress from androgen dependence to androgen independence, and develop osteoblastic skeletal metastases. One of the cell lines, C4-2B, was isolated from metastatic CaP cell lesions found in the lumbar spine of an athymic murine host. LNCaP cells were maintained in RPMI 10 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Inc.). C4-2B cell line, an osteogenic derivative cell line of LNCaP, was maintained in T medium [80% DMEM (Life Technologies, Inc.), 20% F12 (Irving Scientific, Santa Ana, CA), 3 grams/liter $NaCO_3$, 100 units/liter penicillin G, 100 μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml transferrin, 0.25 μg/ml biotin, and 25 μg/ml adenine], supplemented with 5% FBS. The HOBIT cell line (generously provided by B. L. Riggs, Mayo Foundation, Rochester, MN) was established by transfecting normal adult human osteoblast-like cells with pSV3 neo, a plasmid encoding SV40 small and large-T antigen (29). HOBIT cells approximate the phenotype of mature osteoblasts, including: (*a*) 1,25-dihydroxy vitamin D-inducible expression of osteocalcin and alkaline phosphatase (ALP); (*b*) expression of α(I)procollagen, osteopontin 1a, transforming growth factor β, interleukin-1β, interleukin-6, androgen receptor, and estrogen receptor; and (*c*) production of a mineralizable matrix. HOBIT cells were maintained in F12/DMEM supplemented with 15% FBS. Because HOBIT cells do not mineralize well *in vivo,* we used MC3T3 cells, a murine preosteoblast cell line known for its ability to mineralize *in vitro* (30). MC3T3 cells were maintained in α-MEM and 20% FBS. To induce mineral production, cells were supplemented with 10 mM β-glycerophosphate (Sigma, St. Louis, MO) and 50 μg/ml L-ascorbic acid (Sigma) for the indicated time.

For VEGF protein quantification, tissues from autopsies were collected within several hours of the patient's death, immediately flash frozen in liquid nitrogen, and maintained at -80°C until used as described previously (31).

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Collection of tissues was approved by the University of Michigan Human Subjects Internal Review Board.

Obtaining Conditioned Media. Conditioned media (CM) was obtained from LNCaP or C4-2B cells by plating 5×10^6 cells in 10-cm tissue culture dishes for 12 h in T media with 10% FBS. The media was then changed to 10 ml of RPMI plus 0.5% FBS, and supernatants were collected 24 h later. To normalize for differences in cell density because of proliferation during the culture period, cells from each plate were collected, and total DNA content/plate was determined (spectrophotometric absorbance at 260 nm). CM was then normalized for DNA content between samples by adding RPMI.

Protein Isolation. Cells and tissues were homogenized in radioimmunoprecipitation assay buffer (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) with the addition of the protease inhibitors phenylmethylsulfonyl fluoride (10 μl/ml), aprotinin, and sodium orthovanadate (10 μl/ml) using a dounce homogenizer while maintaining temperature at 4°C. Bone metastases were first ground into fine chips with an orthopedic bone grinder before homogenization. The samples were then incubated on ice for 30 min, then transferred to microcentrifuge tubes, and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was removed and centrifuged again. The resulting supernatant fluid was used to measure total protein levels. All samples were then normalized to 1 mg of protein per milliliter with the addition of PBS.

Assays for Specific Proteins. ALP activity was measured in supernatants using a colorimetric assay based on the conversion of *P*-nitrophenylphosphate as directed by the manufacturer (ALP assay; Sigma). Intact osteocalcin was measured in supernatants by competitive EIA (Metra Osteocalcin; Quidel Corp., Santa Clara, CA), as recommended by the manufacturer. The sensitivity of this assay is 0.45 ng/ml. VEGF was measured using a commercially available human-specific ELISA (R&D Systems). The sensitivity of this assay is 5 pg/ml. VEGF was not detectable in media containing 10% FBS.

Mineralization Assays. To stain mineral, cultures were either fixed in 95% ethanol at 37°C for 5 min, rehydrated, then stained using von Kossa's method as described previously (32) or stained for alizarin red as described previously (32). Briefly, the media was removed, and the cells were air dried, fixed in 50% ethanol three times, and then stained with alizarin red (Sigma; 100 mg/ml in 0.01% NaOH) for 5 min. After a PBS wash, retained dye was extracted in a solution of 20% methanol and 10% acetic acid, and then the absorbance was measured at A^{450}.

Plasmids and Transfections. To create the VEGF promoter reporter construct pSV-VEGF(−2271/+382)-Luc, first the SV40 poly(A) signal was excised from pGL2-basic (Promega, Madison, WI) using *Xba*I and *Bam*HI, and ligated into the *Xba*I and *Bam*HI sites of pBluescript sk(-) (Stratagene), resulting in the plasmid designated pBSV. Then, the luciferase cDNA was excised from pGL2-basic using *Hind*III and *Bam*HI and ligated into *Hind*III and *Bam*HI sites of pBSV, resulting in the plasmid designated pBSVL. Then, genomic DNA (1 μg), purified from human lymphocytes was subjected to LA-PCR (Takara Shuzo Co., Ltd., Tokyo, Japan) using primers for VEGF. The forward primer was 5′-ACCTTGCTGGGTACCACCATGGAG-3′, and the reveres primer was 5′-GGACGAAAAGTTTCAGTGCGACGC-3′. PCR conditions were 94°C for 1 min once, then 94°C for 30 s, 65°C for 1 min, and 72°C for 4 min repeated for 30 cycles. The ~3-kb PCR product was cloned into the *Sma*I site of pBluescript and sequenced to confirm that it was the VEGF promoter. Then, the 2.6-kb *Hind*III fragment that encompassed 2271 bp upstream of the VEGF transcription initiation site and 382 bp downstream of the transcription initiation site was subcloned into *Hind*II site upstream of luciferase in pBSVL to give the plasmid designated pSVVEGF(−2271/+382)-Luc. The VEGF 165 expression vector, which contains the cDNA encoding the 165 VEGF isoform, has been described previously (33). Cells were transfected using liposome-mediated transfection (Superfect; Qiagen, Valencia, CA) as recommended by the manufacturer. Luciferase activity was measured and normalized between transfections using the dual luciferase assay (Promega).

Quantification of VEGF mRNA Expression. VEGF mRNA levels were determined using reverse-transcription PCR as we have described previously (34), with minor modifications. Briefly, total RNA was purified using TRIzol (Life Technologies, Inc.) and synthesized to cDNA using Ready-To-Go T-primed First-Strand Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The cDNA was amplified using PCR at 94°C for 15 s, 62°C for 30 s, and 72°C for 30 s for 30 or 25 cycles for VEGF and β-actin, respectively. The primers were VEGF forward 5′-CAGAAGGAGGAGGGCAGAAT-3′; VEGF reverse 5′-CACGTCTGCGGATCTTGTAC-3′; GAPDH forward 5′-TGAAGGTCG-GTGTGAACGGATTTGGTC-3′; and GAPDH reverse 5′-CATGTAGGC-CATGAGGTCCACCAC-3′. These primers give amplified products detectable on an agarose gel as single bands of 397 and 960 bp for VEGF and GAPDH, respectively. The band densities were quantified using Bioquant Imaging System (R&M Biometrics, Nashville, TN) and normalized for GAPDH levels.

Data Analysis. Statistical significance was determined for multivariate comparisons using ANOVA and Fisher's probable least significant difference for post hoc analysis. Student's *t* test was used for bivariate analyses. Statistical significance was determined as $P \leq 0.05$. Statistical calculations were performed using Statview software (Abacus Concepts, Berkeley, CA).

RESULTS

BMPs Promote VEGF Expression from CaP Cells. To detect and quantify VEGF expression in CaP, protein collected from different cell lines and frozen tissue samples, obtained from rapid autopsy of patients within several hours of their death as described previously (31), were subjected to ELISA. VEGF expression was ~60% higher in the metastatic C4-2B cell line compared with its nonmetastatic parental LNCaP cell line. Primary tumors and metastases expressed ~100% higher levels of VEGF than normal prostate (Fig. 1). However, there were no differences in VEGF expression among primary and metastatic tumors. These data demonstrate that VEGF expression is present and up-regulated in clinical CaP.

BMPs have been reported to regulate VEGF expression in several systems (25, 35). Thus, because BMPs are present in CaP and the bone environment, it follows that they may modulate VEGF expression in CaP cells. As described previously (5, 17, 18, 36, 37), we first confirmed that BMP-2, -4, -6, and -7 were present in CaP cells LNCaP and C4-2B (Western analysis; data not shown). Accordingly, the ability of BMPs to regulate VEGF expression in the CaP cells was determined. Several BMPs induced VEGF protein expression in a dose-dependent fashion in C4-2B cells (Fig. 2*A*). BMPs induced VEGF mRNA expression between 4- and 6-fold (Figs. 2, *B* and *C*). These findings demonstrate that BMPs regulate VEGF expression in CaP cells.

BMPs Activate the VEGF Promoter in C4-2B Cells through an Autocrine Mechanism. The observation that BMPs increased steady-state VEGF mRNA expression gave rise to the possibility that BMPs activate the VEGF promoter. To test this possibility, C4-2B cells were transfected with a VEGF promoter-driven luciferase ex-

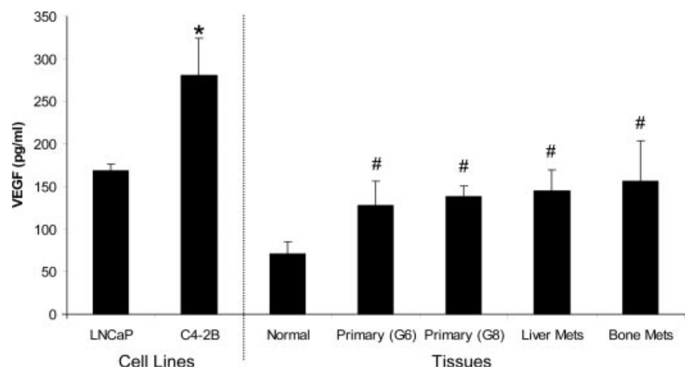

Fig. 1. Prostate cancer cells and tissues express vascular endothelial growth factor (VEGF). LNCaP or C4-2B cells (1×10^5 cells/well) were plated in 12-well plates in RPMI plus 10% fetal bovine serum. After 48 h, cell culture supernatants were collected and subjected to VEGF ELISA and normalized to total protein concentration in the supernatant. Prostate or prostate metastatic tissues were harvested from prostate cancer patients at prostatectomy or autopsy, respectively. Tissues were homogenized, the cell lysate was normalized to 1 mg of protein per milliliter, and supernatants and cell lysates were subjected to VEGF ELISA. Tissue type (number of samples): LNCaP (3); C4-2B (3); normal prostate (3); primary tumor Gleason 6 (4); primary tumor Gleason 8 (3); liver mets (5); and bone mets (3). Results are reported as mean ± SD. $*P < 0.001$ *versus* LNCaP; $^{\#}P < 0.01$ *versus* normal prostate.

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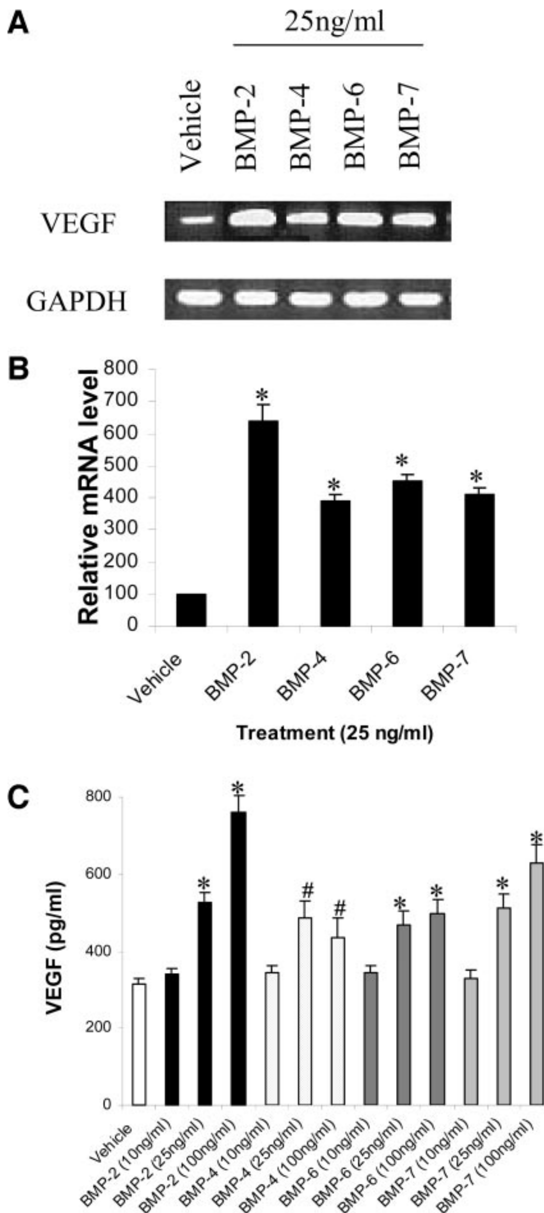

Fig. 2. Bone morphogenetic proteins (BMPs) induce VEGF mRNA and protein expression from C4-2B cells. In *A* and *B,* C4-2B cells (1×10^5 cells/well) were plated in 12-well plates in RPMI plus 10% FBS. The cells were treated, respectively, with 25 ng/ml BMP-2, -4, -6, and -7 or vehicle for 48 h, followed by reverse-transcription PCR. Each treatment was performed in triplicate. In *A,* a typical PCR gel is shown. In *B,* PCR bands subjected to densitometry and VEGF mRNA were normalized to GAPDH mRNA. Results are mean ± SD from three separate experiments. $*P < 0.01$ compared with vehicle. In *C,* C4-2B cells (1×10^5 cells/well) were plated in 12-well plates in RPMI plus 10% FBS. The cells were treated, respectively, with 0, 10, 25, and 100 ng/ml BMP-2, -4, -6, and -7 for 48 h. The VEGF protein content was assayed in culture supernatants by VEGF ELISA and normalized to total protein concentration in the supernatant. Each treatment was performed in triplicate. Results are reported as mean ± SD from three separate experiments. $^{\#}P < 0.05$ or $*P < 0.01$ compared with vehicle.

pression vector or empty control vector, and it was determined if exogenous BMPs induced the VEGF promoter. BMP-7 induced the VEGF promoter in a dose responsive fashion (Fig. 3*A*; similar results were observed for BMPs 2 and 6; data not shown). Because CaP cells secrete BMPs, it was next determined if BMP-induced VEGF mRNA expression occurs through autocrine activation of the VEGF promoter. Noggin, a broad BMP inhibitor, was added into the C4-2B cell cultures to determine whether inhibiting endogenous BMP activity modulated endogenous VEGF promoter activity and protein expression. Noggin decreased both the VEGF promoter and protein activity in a dose-dependent fashion (Fig. 3, *B* and *C*). These data demonstrate that C4-2B cells produce BMPs that activate VEGF expression in an autocrine fashion.

C4-2B Cells Activate Osteoblast Activity through BMPs and VEGF. C4-2B cells have an osteoblast-like phenotype and can promote osteoblastic activity from preosteoblast cells (32). On the basis of this and taken together with the observation that BMPs regulate VEGF expression in C4-2B cells and several recent reports that VEGF promotes bone formation (22–24, 38), it was next examined if VEGF contributes to C4-2B cells' ability to promote osteoblast activity. C4-2B CM induced ALP and osteocalcin secretion into the supernatant of the human osteoblast-like HOBIT cells (Fig. 4, *A* and *B*; compare vehicle *versus* CM only). Furthermore, C4-2B cell CM induced mineralization in the murine osteoblast precursor MC3T3 cells (Fig. 4*C*). Blocking BMPs with noggin diminished C4-2B CM-induced ALP and osteocalcin production and mineralization in HOBIT cells (Fig. 4). Blocking VEGF with anti-VEGF antibody inhibited

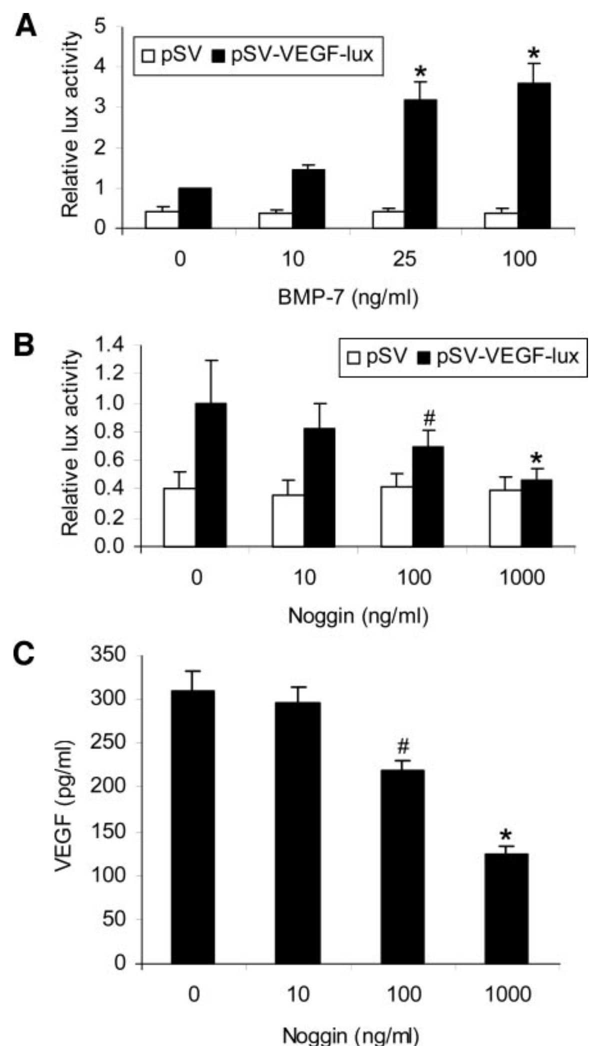

Fig. 3. BMPs activate the VEGF promoter in an autocrine fashion. In *A,* C4-2B cells were plated in 12-well plates in RPMI with 5% FBS, and after 24 h, they were transfected with pSV-VEGF(−2271/+392)-lux or the empty vector (pBSVL). After transfection (24 h), cells were treated with vehicle or BMP-7 at indicated levels. After the addition of BMP-7 (24 h), cells were harvested, and total cell lysates were subjected to assay for luciferase (*lux*) levels. Each treatment was performed in triplicate. $*P < 0.01$ *versus* 0 ng/ml. In *B,* C4-2B were transfected as described in *A,* then treated with noggin at the indicated concentrations. Lux was measured 24 h after the addition of noggin. $^{\#}P < 0.05$ or $*P < 0.01$ *versus* 0 ng/ml. In *C,* cells were treated as in *B,* and supernatants were collected at 48 h for VEGF ELISA. Supernatants were normalized for total protein levels. $^{\#}P < 0.05$ or $*P < 0.01$ *versus* 0 ng/ml.

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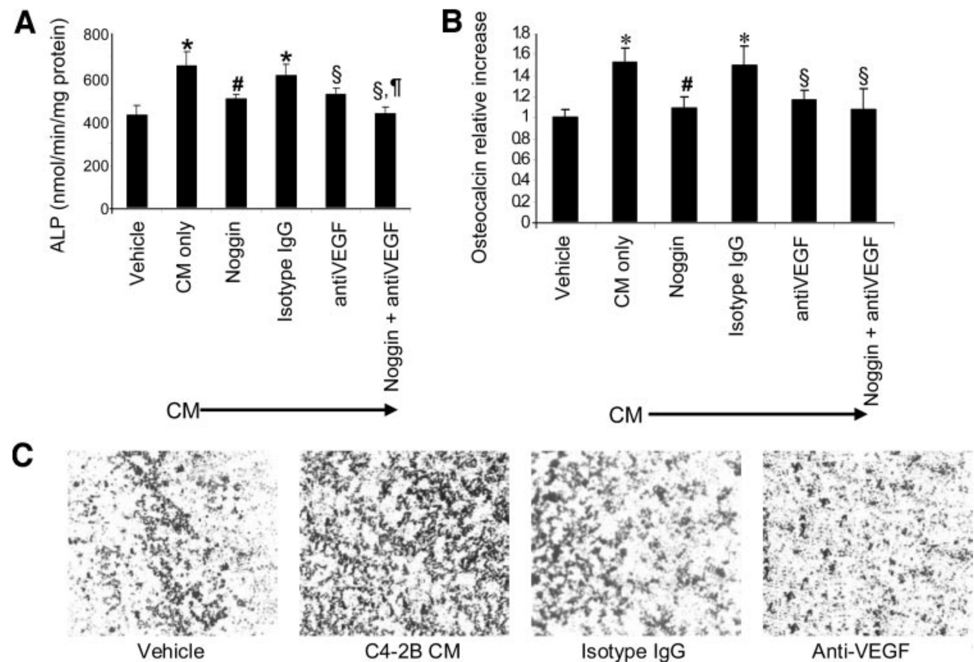

Fig. 4. VEGF contributes to C4-2B-mediated BMP activity. Human primary osteoblasts were plated in 12-well plates (1×10^5 cells/well) in DMEM/F-12 plus 10% FBS. After 1 week, the cells were treated as indicated by the *arrow* with conditioned media (*CM*; 50%) from C4-2B cell cultures, and the addition of noggin (1 μg/ml), IgG, or anti-VEGF (1 μg/ml) as indicated for 48 h and alkaline phosphatase (*ALP*; *A*) or osteocalcin (*B*) was then measured in culture supernatants. Additionally, MC3T3 cells (*C*) were plated as above, and when confluent, ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) were added to promote mineralization, cells were treated as indicated, and mineralization was identified using von Kossa staining 14 days later. Results are reported as mean ± SD of three experiments. $*P < 0.01$ compared with vehicle. $^{\#}P < 0.05$ compared with CM only. $^{\S}P < 0.05$ compared with isotype IgG or CM. $^{\P}P < 0.05$ compared with anti-VEGF.

C4-2B CM-induced ALP and osteocalcin production and mineralization in HOBIT cells at levels similar to inhibition of BMPs (Fig. 4; compare anti-VEGF *versus* CM only). These results demonstrate that C4-2B cells promote an osteoblastic phenotype through BMPs and VEGF. The combination of noggin and anti-VEGF had no effect compared with either agent alone (Fig. 4). These data indicate that BMPs and VEGF contribute to CaP pro-osteoblastic activity.

The ability of BMPs to induce VEGF expression in an autocrine fashion, combined with the observation that blocking BMPs inhibits CaP-induced osteoblastic activity, suggests that VEGF may contribute to BMPs' ability to promote CaP-induced osteoblastic activity. To test this possibility, we determined whether VEGF could rescue noggin-mediated inhibition of C4-2B CM's ability to promote osteoblastic activity. Accordingly, C4-2B cells were transiently transfected with a VEGF expression vector (or control vector), then treated with noggin or vehicle control. Overexpression of VEGF partially abrogated (between 40 and 60%) the noggin-mediated reduction of C4-2B's ability to induce ALP, osteocalcin, and mineralization (Fig. 5). These results, taken together with the observation that inhibition of VEGF diminishes C4-2B-mediated pro-osteoblastic activity in the presence of BMPs, demonstrates that C4-2B cells induce pro-osteoblastic activity through BMP-mediated regulation of VEGF.

DISCUSSION

The mechanisms through which CaP promotes osteosclerosis at metastatic sites are currently unknown. The data presented in this study demonstrate that VEGF's ability to promote bone formation may also play a role in CaP-induced osteosclerosis.

Our observation that VEGF is expressed in CaP tissues is consistent with previous publications in which VEGF was identified in formalin-fixed banked CaP tissues (39). Our results extend these studies by quantifying VEGF levels and identifying its expression in metastases isolated from the same individuals within hours of their death. The finding that there were no significant differences in VEGF expression among primary tumors and metastatic sites suggests that VEGF is up-regulated early in tumor development. This is consistent with the requirement of angiogenesis for growth of a primary tumor. Furthermore, that there were no differences in VEGF expression among the different metastatic sites indicates that the metastatic microenvironment does not influence VEGF expression. However, these results differ with results published previously that demonstrate VEGF expression is increased in breast cancer bone metastases compared with tumor in nonbone metastases in a murine model (40). Differences in tissue types (*i.e.,* breast *versus* CaP), biology of the metastatic tumor (*i.e.,* osteolytic in breast metastases *versus* osteoblastic in CaP metastases), and the fact that this study was performed on mice, as opposed to the use of human tissues in the current study, may account for these differences. Nonetheless, the observation that CaP-produced VEGF is expressed at bone metastatic sites demonstrates that it has the opportunity to interact with preosteoblasts and other bone-formative cells.

BMPs are well recognized to promote bone growth. Furthermore, several reports have documented the presence of BMPs in CaP tissues, including their metastases. These observations, in addition to several reports that BMPs regulated VEGF expression, led us to explore whether this occurred in CaP cells. Our experiments demonstrated that BMPs induce VEGF protein and mRNA expression through activation of the promoter. However, our data do not rule out that in addition to promoter activation, BMPs modify mRNA stability. These data are consistent with reports that BMPs stimulate angiogenesis through osteoblast-derived VEGF (26) and that BMPs induce VEGF expression in other systems, such as rat calvarial (25) and MC3T3 cells (35).

A well-recognized aspect of CaP metastases is their ability to promote osteosclerosis. Our observations that CM from C4-2B cells promotes osteoblastic activity is consistent with the osteosclerotic ability of CaP metastases and previous reports of pro-osteoblastic-inducing activity of CM from other CaP cell lines (6). The observation that inhibition of BMPs reduced the pro-osteoblastic activity of C4-2B CM suggested that they are important mediators of C4-2B cells' pro-osteoblastic effects. Because we had identified that BMPs regulate VEGF expression in these cells and blocking VEGF reduced C4-2B cells' pro-osteoblastic effect similar to that of blocking BMPs, it followed that VEGF may act downstream of BMPs in CaP cells to promote pro-osteoblastic activity. This possibility was further supported by the combined observations that blocking VEGF activity

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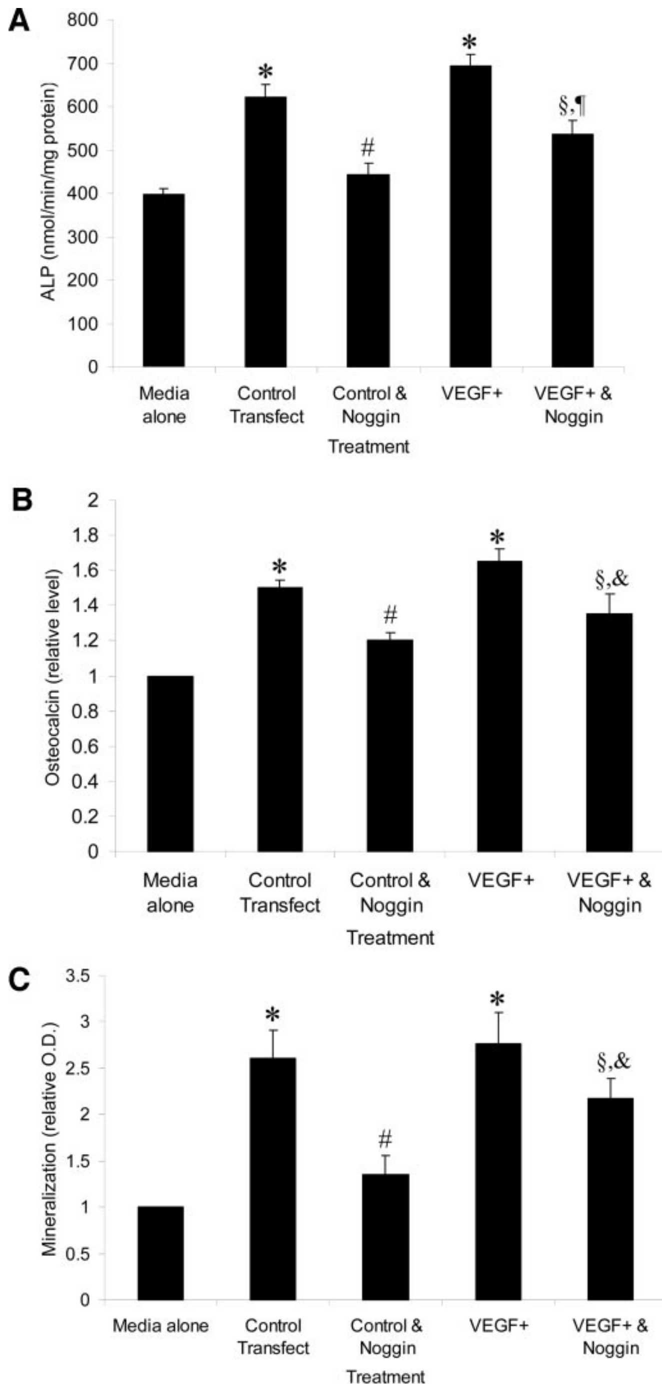

Fig. 5. VEGF induces osteoblastic activity independent of BMPs. C4-2B cells were transfected with the VEGF189 expression vector (*VEGF+*) or control empty vector, and after 48 h, CM was collected, and noggin (2 μg/ml) or vehicle was added to the CM. In *A* and *B*, human primary osteoblasts were plated in 12-well plates (1×10^5 cells/well) in DMEM/F-12 plus 10% FBS. After 1 week, the cells were treated as indicated with CM from empty vector-transfected or VEGF 189-transfected C4-2B cells (50 volume for volume) containing either noggin (1 μg/ml final concentration) or vehicle as indicated for 48 h, and alkaline phosphatase (*ALP*; *A*) or osteocalcin (*B*) was measured in culture supernatants. In *C*, MC3T3 cells were plated as above, when confluent, ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) were added to promote mineralization in the presence of CM with noggin as described above. Mineralization was then quantified by measuring alizarin red retention in the cultures 14 days later. Results are shown as mean ± SD from two experiments. $*P < 0.01$ *versus* media alone; $^{\#}P < 0.01$ *versus* control transfect; $^{\S}P < 0.01$ *versus* media alone; $^{¶}P < 0.01$ *versus* VEGF; $^{\&}P < 0.05$ *versus* VEGF.

alone partially inhibited C4-2B CM-induced osteoblastic activity (showing the presence of BMPs alone only partially contribute to this activity) and that overexpression of VEGF partially abrogated the decrease of pro-osteoblastic activity that occurred on blocking BMPs.

These studies have several limitations, including the *in vitro* nature of the experiments. Thus, one cannot clearly conclude that the pro-osteoblastic activity occurring *in vitro* can be translated to *in vivo* effects. Furthermore, noggin blocks several BMPs; thus, it is not clear if any specific BMP plays a critical role in CaP-induced osteoblastic activity or whether several BMPs are responsible for this activity. Finally, measurement of VEGF levels in CaP tissue samples may be confounded by noncancer cells in the samples.

In summary, in addition to VEGF's well recognized ability to promote angiogenesis, our results indicate a novel function for VEGF in the development of CaP cells' skeletal metastases, namely, the ability to induce osteoblast differentiation. These results suggest that VEGF contributes to the CaP-induced osteoblast differentiation mediated by BMPs.

REFERENCES

1. Boyce, B. F., Yoneda, T., and Guise, T. A. Factors regulating the growth of metastatic cancer in bone. Endocr. Relat. Cancer, *6:* 333–347, 1999.
2. Deftos, L. J. Prostate carcinoma: production of bioactive factors. Cancer (Phila.), *88:* 3002–3008, 2000.
3. Yoneda, T. Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur. J. Cancer, *34:* 240–245, 1998.
4. Autzen, P., Robson, C. N., Bjartell, A., Malcolm, A. J., Johnson, M. I., Neal, D. E., and Hamdy, F. C. Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br. J. Cancer, *78:* 1219–1223, 1998.
5. Harris, S. E., Harris, M. A., Mahy, P., Wozney, J., Feng, J. Q., and Mundy, G. R. Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate, *24:* 204–211, 1994.
6. Hullinger, T. G., Taichman, R. S., Linseman, D. A., and Somerman, M. J. Secretory products from PC-3 and MCF-7 tumor cell lines upregulate osteopontin in MC3T3-E1 cells. J. Cell. Biochem., *78:* 607–616, 2000.
7. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A., and Simons, J. W. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat. Med., *1:* 944–949, 1995.
8. Chen, H. L., Demiralp, B., Schneider, A., Koh, A. J., Silve, C., Wang, C. Y., and McCauley, L. K. Parathyroid hormone and parathyroid hormone-related protein exert both pro- and anti-apoptotic effects in mesenchymal cells. J. Biol. Chem., *277:* 19374–19381, 2002.
9. Karaplis, A. C., and Vautour, L. Parathyroid hormone-related peptide and the parathyroid hormone/parathyroid hormone-related peptide receptor in skeletal development. Curr. Opin. Nephrol. Hypertens., *6:* 308–313, 1997.
10. Cornish, J., Callon, K. E., Lin, C., Xiao, C., Moseley, J. M., and Reid, I. R. Stimulation of osteoblast proliferation by C-terminal fragments of parathyroid hormone-related protein. J. Bone Miner. Res., *14:* 915–922, 1999.
11. Thies, R. S., Bauduy, M., Ashton, B. A., Kurtzberg, L., Wozney, J. M., and Rosen, V. Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20–17 stromal cells. Endocrinology, *130:* 1318–1324, 1992.
12. Asahina, I., Sampath, T. K., and Hauschka, P. V. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp. Cell Res., *222:* 38–47, 1996.
13. Ahrens, M., Ankenbauer, T., Schroder, D., Hollnagel, A., Mayer, H., and Gross, G. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol., *12:* 871–880, 1993.
14. Ji, X., Chen, D., Xu, C., Harris, S. E., Mundy, G. R., and Yoneda, T. Patterns of gene expression associated with BMP-2-induced osteoblast and adipocyte differentiation of mesenchymal progenitor cell 3T3-F442A. J. Bone Miner. Metab., *18:* 132–139, 2000.
15. Gori, F., Thomas, T., Hicok, K. C., Spelsberg, T. C., and Riggs, B. L. Differentiation of human marrow stromal precursor cells: bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J. Bone Miner. Res., *14:* 1522–1535, 1999.
16. Abe, E., Yamamoto, M., Taguchi, Y., Lecka-Czernik, B., O'Brien, C. A., Economides, A. N., Stahl, N., Jilka, R. L., and Manolagas, S. C. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J. Bone Miner. Res., *15:* 663–673, 2000.
17. Bentley, H., Hamdy, F. C., Hart, K. A., Seid, J. M., Williams, J. L., Johnstone, D., and Russell, R. G. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. Br. J. Cancer, *66:* 1159–1163, 1992.
18. Weber, K. L., Bolander, M. E., Rock, M. G., Pritchard, D., and Sarkar, G. Evidence for the upregulation of osteogenic protein-1 mRNA expression in musculoskeletal neoplasms. J. Orthop. Res., *16:* 8–14, 1998.
19. Streeten, E. A., and Brandi, M. L. Biology of bone endothelial cells. Bone Miner., *10:* 85–94, 1990.
20. Probst, A., and Spiegel, H. U. Cellular mechanisms of bone repair. J. Investig. Surg., *10:* 77–86, 1997.
21. Bouletreau, P. J., Warren, S. M., Spector, J. A., Steinbrech, D. S., Mehrara, B. J., and Longaker, M. T. Factors in the fracture microenvironment induce primary osteoblast angiogenic cytokine production. Plast. Reconstr. Surg., *110:* 139–148, 2002.

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22. Mayr-Wohlfart, U., Waltenberger, J., Hausser, H., Kessler, S., Gunther, K. P., Dehio, C., Puhl, W., and Brenner, R. E. Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. Bone, *30:* 472–477, 2002.
23. Midy, V., and Plouet, J. Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. Biochem. Biophys. Res. Commun., *199:* 380–386, 1994.
24. Street, J., Bao, M., deGuzman, L., Bunting, S., Peale, F. V., Jr., Ferrara, N., Steinmetz, H., Hoeffel, J., Cleland, J. L., Daugherty, A., van Bruggen, N., Redmond, H. P., Carano, R. A., and Filvaroff, E. H. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc. Natl. Acad. Sci. USA, *99:* 9656–9661, 2002.
25. Yeh, L. C., and Lee, J. C. Osteogenic protein-1 increases gene expression of vascular endothelial growth factor in primary cultures of fetal rat calvaria cells. Mol. Cell. Endocrinol., *153:* 113–124, 1999.
26. Deckers, M. M., van Bezooijen, R. L., van der Horst, G., Hoogendam, J., van Der Bent, C., Papapoulos, S. E., and Lowik, C. W. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology, *143:* 1545–1553, 2002.
27. Turner, K., and Jones, A. Vascular endothelial growth factor in prostate cancer. Urology, *56:* 183, 2000.
28. Thalmann, G. N. N., Sikes, R. A., Wu, T. T., Degeorges, A., Chang, S. M., Ozen, M., Pathak, S., and Chung, L. W. LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate, *44:* 91–103, 101, 144, 2000.
29. Keeting, P. E., Scott, R. E., Colvard, D. S., Anderson, M. A., Oursler, M. J., Spelsberg, T. C., and Rigg, B. L. Development and characterization of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV40 large T antigen. J. Bone Miner. Res., *7:* 127–136, 1992.
30. Franceschi, R. T., and Iyer, B. S. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J. Bone Miner. Res., *7:* 235–246, 1992.
31. Rubin, M. A., Putzi, M., Mucci, N., Smith, D. C., Wojno, K., Korenchuk, S., and Pienta, K. J. Rapid ("warm") autopsy study for procurement of metastatic prostate cancer. Clin. Cancer Res., *6:* 1038–1045, 2000.
32. Lin, D. L., Tarnowski, C. P., Zhang, J., Dai, J., Rohn, E., Patel, A. H., Morris, M. D., and Keller, E. T. Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes *in vitro*. Prostate, *47:* 212–221, 2001.
33. Cheng, S. Y., Nagane, M., Huang, H. S., and Cavenee, W. K. Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189. Proc. Natl. Acad. Sci. USA, *94:* 12081–12087, 1997.
34. Fujita, H., Koshida, K., Keller, E. T., Takahashi, Y., Yoshimito, T., Namiki, M., and Mizokami, A. Cyclooxygenase-2 promotes prostate cancer progression. Prostate, *53:* 232–240, 2002.
35. Kozawa, O., Matsuno, H., and Uematsu, T. Involvement of p70 S6 kinase in bone morphogenetic protein signaling: vascular endothelial growth factor synthesis by bone morphogenetic protein-4 in osteoblasts. J. Cell. Biochem., *81:* 430–436, 2001.
36. Barnes, J., Anthony, C. T., Wall, N., and Steiner, M. S. Bone morphogenetic protein-6 expression in normal and malignant prostate. World J. Urol., *13:* 337–343, 1995.
37. Hamdy, F. C., Autzen, P., Robinson, M. C., Horne, C. H., Neal, D. E., and Robson, C. N. Immunolocalization and messenger RNA expression of bone morphogenetic protein-6 in human benign and malignant prostatic tissue. Cancer Res., *57:* 4427–4431, 1997.
38. Peng, H., Wright, V., Usas, A., Gearhart, B., Shen, H. C., Cummins, J., and Huard, J. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. J. Clin. Investig., *110:* 751–759, 2002.
39. Ferrer, F. A., Miller, L. J., Andrawis, R. I., Kurtzman, S. H., Albertsen, P. C., Laudone, V. P., and Kreutzer, D. L. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: *in situ* and *in vitro* expression of VEGF by human prostate cancer cells. J. Urol., *157:* 2329–2333, 1997.
40. van der Pluijm, G., Sijmons, B., Vloedgraven, H., Deckers, M., Papapoulos, S., and Lowik, C. Monitoring metastatic behavior of human tumor cells in mice with species-specific polymerase chain reaction: elevated expression of angiogenesis and bone resorption stimulators by breast cancer in bone metastases. J. Bone Miner. Res., *16:* 1077–1091, 2001.

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