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Growth Factors, Cytokines, Cell Cycle Molecules

Lack of Noggin Expression by Cancer Cells Is a Determinant of the Osteoblast Response in Bone Metastases

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Prostate and mammary cancer bone metastases can be osteoblastic or osteolytic, but the mechanisms determining these features are unclear. Bone morphogenetic and Wnt proteins are osteoinductive molecules. Their activity is modulated by antagonists such as noggin and dickkopf-1. Differential expression analysis of bone morphogenetic and Wnt protein antagonists in human prostate and mammary cancer cell lines showed that osteolytic cell lines constitutively express *in vitro* noggin and dickkopf-1 and at least one of the osteolytic cytokines parathyroid hormone-related protein, colony-stimulating factor-1, and interleukin-8. In contrast, osteoinductive cell lines express neither noggin nor dickkopf-1 nor osteolytic cytokines *in vitro*. The noggin differential expression profile observed *in vitro* was confirmed *in vivo* in prostate cancer cell lines xenografted into bone and in clinical samples of bone metastasis. Forced noggin expression in an osteoinductive prostate cancer cell line abolished the osteoblast response induced *in vivo* by its intraosseous xenografts. Basal bone resorption and tumor growth kinetics were marginally affected. Lack of noggin and possibly dickkopf-1 expression by cancer cells may be a relevant mechanism contributing to the osteoblast response in bone metastases. Concomitant lack of osteolytic cytokines may be permissive of this effect. Noggin is a candidate drug for the adjuvant therapy of bone metastasis. ***(Am J Pathol 2007, 170:160–175; DOI: 10.2353/ajpath.2007.051276)***

Prostate and mammary cancer are among the leading cancers diagnosed and the second leading cause of cancer death in men and women, respectively.[1] Both cancers show a high propensity to metastasize to bone. Whereas prostate cancer (CaP) elicits predominantly an osteoblast response resulting in osteosclerotic lesions, mammary cancer (CaM) triggers preferentially an osteoclast reaction with bone resorption and consequent osteolytic lesions.[2] Osteolytic and osteosclerotic lesions are prone to pathological fractures. A better understanding of the mechanism(s) determining the osteoclast and osteoblast response to cancer metastases is essential for the identification of therapeutic strategies for prevention of pathological bone fractures in cancer patients.

Several factors stimulating osteoblast proliferation and differentiation in a paracrine manner have been shown to be released by CaP and CaM cells in the bone microenvironment and have been postulated to mediate osteoblast response in bone metastasis.[3,4] Factors that modulate proliferation and differentiation can act directly on the osteoblast progenitors or indirectly by activation of factors involved in their generation.[4] Paradigmatic molecules regulating directly osteoblast generation are the bone morphogenetic proteins (BMPs).[5] BMPs were first identified by their ability to induce ectopic chondro-osteogenesis *in vivo*.[6] They play a crucial role in skeletal and joint morphogenesis, bone

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remodeling, and fracture repair by inducing proliferation, lineage determination, differentiation, and apoptosis in chondrocyte and osteoblast precursors.[7,8] Subsequently, they were shown to act as multifunctional regulators of embryonic patterning and organogenesis, tissue remodeling, and repair.[9,10] A crucial regulatory mechanism is the modulation of BMP signaling by a class of secreted molecules, BMP antagonists, functioning through direct association with BMPs, thereby sequestering BMPs in the extracellular space and preventing binding to cell surface receptors. BMP antagonists have been divided in three subfamilies: the differential screening-selected gene aberrant in neuroblastoma (DAN), the twisted gastrulation, and the chordin/noggin.[11]

Antagonism of BMP activity by noggin and chordin is critical for embryonic chondro-osteogenesis and joint formation.[12–14] Osteoblast-targeted overexpression of noggin[15,16] and gremlin[17] results in osteopenia attributable to impaired osteoblast recruitment and function, indicating that the extracellular control of the BMP concentration is also essential in adult life for maintaining the balance between bone resorption and bone formation during bone remodeling. Expression of BMP antagonists is BMP-dependent, indicating that a feedback mechanism maintaining an optimal ratio between BMPs and BMP antagonists is also necessary to achieve optimal bone mass.[18] Further evidence that antagonism with molecules directly involved in osteoblast generation, such as the Wnt protein family,[19–22] is critical for modulating bone mass has been given by the finding that the rare human disease sclerosteosis, characterized by excessive bone formation of the whole skeleton, is attributable to the loss of expression of the *SOST* gene,[23] encoding the Wnt antagonist sclerostin.[24,25] Conversely, bone-targeted overexpression of sclerostin in mice causes osteopenia.[26] Furthermore, it has recently been shown that dickkopf-1 (DKK-1), another antagonist of Wnt signaling, modulates the osteoblast reaction in osteolytic foci of multiple myeloma.[27]

We hypothesized that loss of BMP and Wnt antagonist expression in bone metastatic CaP and CaM cells would unmask the osteoinductive effects of BMPs and Wnts released by the cancer cells at the bone metastatic site. To test this hypothesis, we first evaluated the expression *in vitro* of extracellular BMP and Wnt antagonists, as well as the expression of osteoinductive and osteolytic cytokines, in a variety of CaP and CaM cell lines, which possess either osteolytic or osteoinductive potential *in vivo*. The pattern of expression was verified also *in vivo* for CaP cell lines xenografted into bone and in clinical samples of bone metastasis. We further investigated whether forced expression of the BMP antagonist noggin in an osteoinductive CaP cell line would abolish the osteoblast response in its experimental bone metastasis *in vivo*.

Materials and Methods

Animals

Male CB17 SCID and BALB/c nude mice were purchased from Charles River France (L'Arbresle, France) and housed in individual ventilated cages according to the Swiss guidelines for the care and use of laboratory animals. Mice were 7 weeks old when used for the intraosseous implantation of tumor cells. For surgical manipulation, mice were anesthetized as described previously.[28] Mice were sacrificed by CO_2 euthanasia at the end of the observation period or at first signs of distress.

Clinical Samples of Bone Metastasis

Three CaP and one CaM clinical specimens of bone metastasis were obtained by radiography-guided biopsy, with prior informed consent of the patient and approval by the ethics committee of the University of Heidelberg–Mannheim, Germany. The CaM bone metastasis was classified as osteolytic whereas the three CaP bone metastases were classified as osteoblastic according to radiographical and histological criteria by a certified radiologist and a certified pathologist.

Bone specimens were snap-frozen in liquid nitrogen immediately after surgical excision and embedded in precooled RNase-free water. Twelve-μm-thick cryosections were obtained with a tungsten carbide blade (C profile; Leica Microsystems, Bensheim, Germany) and the aid of the CryoJane tape-transfer system (CTS; Instrumedics Inc., Hackensack, NJ) in a cryomicrotome (CM 3050; Leica Microsystems) set at −24°C. Bone cryosections were transferred onto 4× adhesive-coated slides (Instrumedics) as described recently,[29] fixed in 70% ethanol, stained with Mayer's hematoxylin (Sigma Diagnostics Inc., St. Louis, MO) and eosin Y (Merck, Darmstadt, Germany), and dehydrated by increasing grades of ethanol followed by xylene.

Laser Capture Microdissection

The P.A.L.M. Robot Micro-Beam (P.A.L.M. Instruments, Bernried, Germany) was used to excise by laser capture microdissection ~10,000 pure cancer cells from each clinical specimen of bone metastasis. The selection of purely neoplastic cells was in each case verified by a certified pathologist.

Cell Lines and Cell Culture

We studied a range of human CaP and CaM cell lines, with different tumorigenic and metastatic potential, shown to induce either osteolytic or osteosclerotic bone metastases, as shown by others[28,30–35] and by us (A.W. and M.G.C., not shown) (Table 1). The osteolytic human CaP cell line PC-3 [American Type Culture Collection (ATCC)/LGC Promochem, Molsheim, France] and its isogenic clone PC-3M-Pro4, selected *in vivo* for enhanced metastatic potential[32] (kindly provided by Dr. I.J. Fidler, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX), were grown in Dulbecco's modified Eagle's medium. The osteoinductive, androgen-dependent and non-bone metastatic human prostate cancer cell line LNCaP, and its isogenic variants C4-2 and C4-2B, androgen-indepen-

Table 1. Cancer Cell Lines Used in This Study Listed According to Their Tissue Origin and Osteotropic Effects

	Prostate cancer	Mammary cancer
Osteolytic	PC-3[34]	MDA-MB-231[35]
	PC-3M-Pro4[32]	MDA-231B[28]
Osteoinductive	LNCaP[31]	T-47D[33]
	C4-2[30]	ZR-75-1[33]
	C4-2B[31]	

The osteolytic or the osteoinductive effect was confirmed in our laboratory for all the cell lines listed except for the T-47D cell line, which did not generate tumors after intraosseous implantation in tibiae of immunocompromised *nu/nu* mice.

dent and spontaneously metastasizing to bone after orthotopic implantation[30] (kindly provided by Dr. L. Chung, Winship Cancer Center, Emory University, Atlanta, GA) were grown in T-medium. The osteolytic human mammary cancer cell line MDA-MB-231 (ATCC) and its isogenic clone MDA-231B, selected after sequential passaging *in vivo* for bone-restricted metastatic potential[28] were grown in Dulbecco's modified Eagle's medium. The osteoinductive human mammary cancer cell lines T-47D and ZR-75-1 were purchased from ATCC and cultured in RPMI 1640 medium. The mouse osteoblast-like cell line KS483[36] was cultured routinely in phenol red-free minimum essential medium-α. All media were supplemented with 10% fetal bovine serum (BioWittaker, Verviers, Belgium). Cell lines were stimulated either with 10 ng/ml of recombinant human transforming growth factor (TGF)-β1 (R&D Systems Europe Ltd., Abingdon, UK) or 100 ng/ml of recombinant human BMP-2 (R&D Systems) or 100 ng/ml of recombinant human BMP-6 (kindly provided by Prof. S. Vukicevic, Genera, Croatia) for 6 or 24 hours.

Generation of Conditioned Media

Cells were seeded at the density of 1.25 to 2.5 $\times$ 10^4 cells/cm^2. After 1 day, the medium was replaced with serum-free medium, and the cells were cultured for a further 48 hours. The cell-conditioned media (CM) were centrifuged and stored in aliquots at -20°C for later use. The cell number was determined and, where necessary, serum-free medium was added to the CM to normalize for differences in cell density between samples.

Cell Proliferation Assay

Cells were seeded at the density of 10^4 cells/cm^2 and cultured for a total of 8 days. Cell proliferation was determined daily with a tetrazolium salt-based method (MTT assay, Cell Proliferation Kit I; Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's protocol.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA extraction from subconfluent cultures of the various cell lines and from intraosseous xenografts of parental, noggin-, and mock-transfected C4-2B/luc$^+$ cells, and of PC-3 and PC-3M-Pro4 cells, was performed with RNeasy (Qiagen, Hombrechtikon, Switzerland). Reverse transcription was performed with M-MLV-RT (Promega, Wallisellen, Switzerland) and random primers (Roche Diagnostics). Human-specific real-time PCR (TaqMan) primers and probes (Applied Biosystems, Rotkreuz, Switzerland) are listed in Table 2. Extraction of total RNA from pure CaP or CaM cells, laser-microdissected from clinical specimens of bone metastasis, was performed according to the TriSpin method[37] with several modifications, as previously described.[29] cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) and PCR amplified in Mx3000P (Stratagene, Amsterdam, The Netherlands) using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturers' protocols. Sense and anti-sense human-specific noggin primers used were 5′-TGTGCAAGCCGTCCAAGT-3′ and 5′-GAGCACTTGCACTCGGAAAT-3′.

Table 2. Real-Time Primers and Probes Used in This Study

Gene	Primers/probe*
PTHrP	Hs_00174969_m1
CSF-1	Hs_00174164_m1
RANKL	Hs_00243522_m1
OPG	Hs_00171068_m1
IL-8	Hs_00184979_m1
BMP-2	Hs_00154192_m1
BMP-3	Hs_00609638_m1
BMP-4	Hs_00181626_m1
BMP-6	Hs_00233470_m1
PDF (GDF-15)	Hs_00171132_m1
TGF-β1	Hs_00171257_m1
Noggin	Hs_00271352_s1
DAN	Hs_00185054_m1
Gremlin	Hs_00171951_m1
SOST	Hs_00228830_m1
DKK-1	Hs_00183740_m1
β-actin	Hs_99999903_m1
GAPDH	Hs_99999905_m1

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
*TaqMan gene expression assays were from Applied Biosystems.

Determination of Cytokine Protein Concentration

Colony stimulating factor-1 (CSF-1) concentration in serum-free CM was determined by the Quantikine human CSF-1 immunoassay (R&D Systems), parathyroid hormone-related protein (PTHrP) by a two-site immunoradiometric assay (Nichols Institute Diagnostics, Bad Vilbel, Germany), and endothelin-1 (ET-1) by enzyme-linked immunosorbent assay (Biomedica, Wien, Austria). Protein secreted into the medium was normalized to the cell number at the end of the culture period.

Construction of the Noggin Expression Vector

The full-length human *noggin* cDNA was excised from the plasmid pBSII SK+.hNG (kindly provided by Regeneron Pharmaceuticals, Inc., Tarrytown, NY) with *Kpn*I and *Not*I

and ligated into the pcDNA3.1/Hygro expression vector (Invitrogen) (*pcDNA3.1/Nog*).

Stably Transfected Clones

The C4-2B cell line was transfected with the luciferase (*luc*) expression vector pCMV*luc*[28] with SuperFect transfection reagent (Qiagen). A luc-positive clone was selected and transfected with *pcDNA3.1/Nog* to generate C4-2B-*Nog* clones and with the original plasmid pcDNA3.1/Hygro to generate C4-2B-mock clones.

Immunoblotting

Noggin expression in transfected C4-2B cells was determined in concentrated CM and in cell lysates prepared with radioimmunoprecipitation assay buffer supplemented with 1 mmol/L phenylmethyl sulfonyl fluoride (Sigma, Buchs, Switzerland). Protein concentration was determined by D_c protein assay (Bio-Rad, Reinach, Switzerland), and proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gels (30 μg of total protein per lane) and transferred on Hybond-P membranes (Amersham Biosciences, Otelfingen, Switzerland). Membranes were incubated with 40 ng/ml of a rat monoclonal antibody against the human native noggin protein[38] (RP57-16; kindly provided by Dr. A.N. Economides, Regeneron Pharmaceuticals, Inc.) and detected with an horseradish peroxidase-labeled anti-rat secondary antibody (1:1000; Amersham Biosciences). A mouse monoclonal anti-actin antibody (1:5000; Chemicon International, Juro Supply GmbH, Lucerne, Switzerland) was used as loading control. Immunoreactivity was visualized with the ECL Advanced chemiluminescence substrate (Amersham Biosciences) using the VersaDoc imaging system and QuantityOne imaging software (Bio-Rad).

Secretion of Biologically Active Noggin by Stably Transfected Clones

For osteoblast differentiation assays, KS483 cells were seeded at a density of $1.5 \times 10^4/cm^2$. At confluency (day 4), ascorbic acid (50 μg/ml; Merck Inc., Whitehouse Station, NJ) and CM of parental C4-2B, and of C4-2B-derived *Nog* and mock clones to a final concentration of 10% (v/v) or recombinant mouse noggin (R&D Systems) were added to the cultures. At day 7 alkaline phosphatase (ALP) activity in cell lysates was determined and corrected for the amount of DNA in the culture as previously described.[36]

Intraosseous Implantation of Parental, Noggin-, and Mock-Transfected C4-2B/luc$^+$ Cells, and of PC-3 and PC-3M-Pro4 Cells

The cells were inoculated into the marrow cavity of the left tibia at the density of 5 to $8 \times 10^5/10$ μl of phosphate-buffered saline, as previously described.[28] A group of five to seven animals was inoculated with each cell clone. A further group of five to seven animals, inoculated into the bone marrow cavity with phosphate-buffered saline alone (= sham), served as control. A first experiment with parental and three noggin- (*Nog17*, *19*, and *20*) and mock (mock1, 2, and 4)-transfected C4-2B/*luc*$^+$ cells was terminated after 8 weeks. A second experiment with parental and only one noggin (*Nog20*)- and one mock-transfected (mock4) C4-2B/*luc*$^+$ cells was terminated after 12 weeks. The experiments with PC-3 and PC-3M-Pro4 were terminated after 5 and 4 weeks, respectively.

Radiography

Radiographs of the mice were taken using X-Omat TL films (Kodak, Lausanne, Switzerland) as previously described.[28]

Whole Body Bioluminescent Imaging (BLI)

BLI was performed as previously described[28] at 2-week intervals.

Peripheral Quantitative Computed Tomography (pQCT)

Bone mineral density and content of the tibia site of the tumor xenografts were determined with a small animal pQCT scanner (XCT Research SA; Norland Stratec, Pforzheim, Germany) at the end of experimental periods indicated above. Measurements were performed at 2.5 mm and 3 mm distal from the reference point (cleft of the knee joint).

High-Resolution Micro-CT

At the end of the experimental period indicated above, representative tibiae from a mouse implanted with C4-2B/*luc*$^+$ cells and a sham-operated mouse were scanned with a micro-CT (Skyscan 1076; Gloor Instruments, Uster, Switzerland) at a voxel size resolution of 9 μm.

Histochemistry

The left tibia, site of intraosseous implantation of parental and stably *Nog*- and mock-transfected C4-2B/*luc*$^+$ cells, were fixed and processed for paraffin embedding. Four-μm serial sections were stained either with Mayer's hematoxylin and eosin Y or for tartrate-resistant acid phosphatase as described previously.[39] The number of osteoclasts/bone surface was determined using the Scion Image software (Scion, Frederick, MD). Osteoclasts were counted as multinucleated tartrate-resistant acid phosphatase-positive cells on at least 5-mm total bone surface adjacent to tumor, on three mid-sagittal sections for each individual bone specimen.

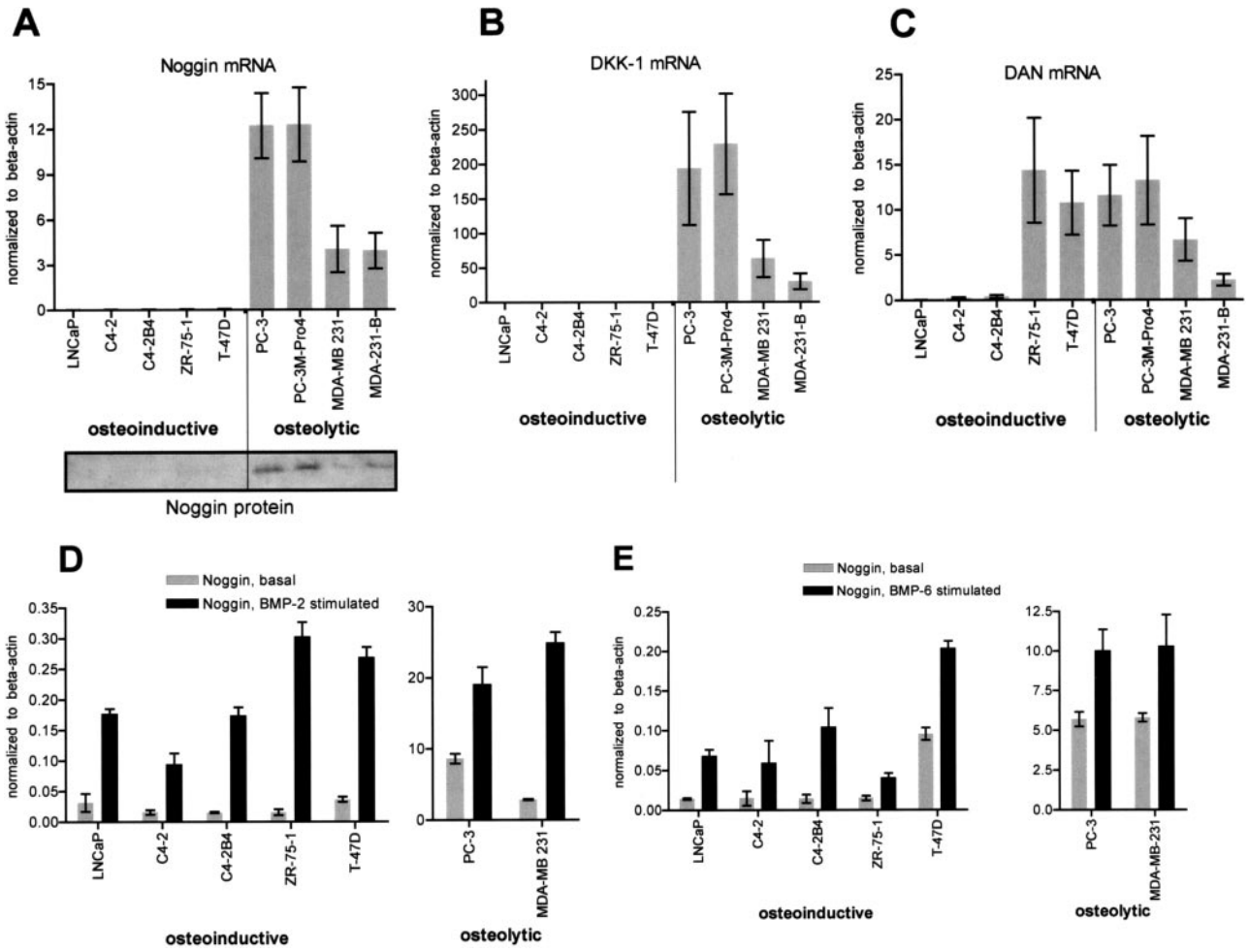

Figure 1. Expression of BMP and Wnt antagonists in osteoinductive and osteolytic cancer cell lines. **A:** Noggin mRNA and protein. **B:** DKK-1 mRNA. **C:** DAN mRNA. **D:** Stimulation of noggin mRNA expression by BMP-2. **E:** Stimulation of noggin mRNA expression by BMP-6. The mRNA levels (±SEM) were quantified by real-time RT-PCR relative to β-actin endogenous control ($n \geq 3$). Equal amounts of CM were immunoblotted with noggin antibody. Cells were stimulated with 100 ng/ml BMP-2 or BMP-6 for 24 hours.

Statistical Analysis

Rates of proliferation *in vitro* and growth *in vivo* were compared with the two-way analysis of variance test. The effect of cell-conditioned media on the ALP activity of KS483 cells and the bone architectural parameters obtained by pQCT were analyzed by the one-way analysis of variance. Osteoclast numbers were compared by the unpaired *t*-test.

Results

Expression of BMP and Wnt Antagonists in Osteolytic and Osteoinductive Cancer Cell Lines in Vitro

To test whether differential expression of BMP and/or Wnt antagonists may explain the osteoblastic or osteolytic response in bone metastases, we investigated noggin, DAN, gremlin, SOST, and DKK-1 mRNA expression *in vitro* in a set of CaP and CaM cell lines ordered according to their osteoinductive or osteolytic potential *in vivo* (Table 1). All of the osteolytic cell lines tested, either of CaP or CaM origin, expressed considerable amounts of noggin mRNA, the CaP cell lines expressing approximately threefold higher mRNA levels than the CaM cell lines. In contrast, all of the osteoinductive CaP and CaM cell lines expressed noggin mRNA at extremely low levels (Figure 1A), which were in average almost 400-fold lower than those expressed by the osteolytic cell lines. This differential noggin mRNA expression was also reflected in different levels of secreted protein (Figure 1A). An equivalent pattern of distribution between osteoinductive and osteolytic cell lines was evident for DKK-1 mRNA expression. Although osteoinductive cell lines indifferently showed complete lack of its expression, all of the osteolytic cell lines showed considerable amounts of DKK-1 expression, the CaP cell lines expressing approximately fourfold higher mRNA levels than the CaM cell lines (Figure 1B). All of the osteolytic cell lines and osteoinductive CaM cell lines expressed DAN mRNA, whereas the osteoinductive CaP cell lines expressed it at very low or undetectable levels (Figure 1C). SOST

mRNA expression was detectable at a very low level only in MDA-MB-231 cells, but it was absent in all remaining cell lines (not shown). Gremlin mRNA could not be detected in any of the cell lines tested (not shown).

Expression of BMP antagonists is primarily induced by BMPs, such as BMP-2 and BMP-6,[18] and noggin is induced by BMP-6 in the osteoinductive CaP cell line LNCaP.[40] Thus, we investigated whether relevant differences in noggin expression between osteoinductive and osteolytic cancer cell lines also persist after BMP induction. Either BMP-2 or BMP-6, at the concentration of 100 ng/ml, induced a 2- to 20-fold higher expression of noggin mRNA in both osteoinductive and osteolytic cell lines. However, the amount of noggin RNA induced by both BMPs was 100-fold higher in osteolytic cell lines compared with osteoinductive cell lines. Furthermore, the average expression level of noggin mRNA in the osteoinductive cell lines after BMP stimulation was 40-fold lower than the constitutive expression level in nonstimulated osteolytic cell lines (Figure 1, D and E). Thus, the amount of constitutive or BMP-induced noggin expression in osteoinductive cell lines was negligible when compared with osteolytic cell lines and may still not have been sufficient for antagonizing the BMP locally released *in vivo*. Nevertheless, the increase in noggin expression induced *in vitro* by BMP-2 and BMP-6 seems to exclude that the lack of constitutive noggin expression in the osteoinductive cell lines is the consequence of an impaired response to BMPs. TGF-β1 did not stimulate noggin expression in any of the tested cell lines (not shown). Collectively, the results above suggest a strong correlation between lack of noggin and DKK-1 expression *in vitro* and the osteoinductive potential *in vivo* of all the CaP and CaM cell lines examined in this study.

Expression of noggin in Pure CaP or CaM Cells, Laser-Microdissected from Clinical Specimens of Bone Metastasis

Noggin has been reported to be expressed in cells of the osteoblast lineage.[41,42] Thus, to avoid the interference by noggin expressed by the bone stromal component in human bone metastasis biopsies, we analyzed noggin expression in 100% pure cancer cells isolated from metastatic bone lesions by laser capture microdissection. Relative noggin expression, normalized to β-actin, was found to be 0.353 in an osteolytic CaM bone metastasis, and 0.182, 0.006, and 0.000093 in three osteoblastic CaP bone metastasis, respectively.

Expression of Members of the TGF-β Superfamily in Osteolytic and Osteoinductive Cell Lines in Vitro

BMPs are considered major players in the osteoblast response to cancer bone metastasis.[3,4] The osteoinductive cell lines, especially those of CaP origin, mainly expressed BMP-6 (Figure 2, A–D). In addition, the osteoinductive cell line T-47D expressed low levels of BMP-4 (Figure 2C). Conversely, osteolytic cell lines preferentially expressed BMP-2, -3, and -4, but very low amounts of BMP-6 (Figure 2, A–D). Discrete amounts of prostate-derived factor (PDF) were mainly expressed in the CaP cell lines, either osteoinductive or osteolytic (Figure 2E). Therefore, no pattern of BMP family member mRNA expression characteristic either of osteoinductive or of osteolytic cancer cell lines emerged from the cell lines analyzed in this study. In addition, expression of one or more members of the BMP family by the osteoinductive cell lines further suggested that their extremely low noggin expression is not attributable to lack of induction by endogenous BMP.

Other molecules considered responsible for the increased osteoblast response in osteosclerotic bone metastases are part of the TGF-β family.[4,43,44] Here we found a substantial mRNA expression of TGF-β_1 in all of the osteolytic, but not in the osteoinductive cell lines examined so far (Figure 2F). Thus, tumor cell-derived TGF-β_1 seems not to be related to the osteoinductive property of cancer cell lines.

Expression in Vitro *of Cytokines Influencing Bone Resorption*

The relevance of bone-resorbing cytokines such as PTHrP, receptor activator of nuclear factor-κB ligand (RANKL), and its decoy receptor osteoprotegerin (OPG) in osteolytic bone metastasis is well documented.[43,44] However, less attention has been devoted to a systematic investigation aiming at clarifying whether the absence of expression of bone-resorbing cytokines in cancer cells is a prerequisite for allowing full manifestation of their osteoinductive potential.

In the cell lines examined in this study, we found that only osteolytic cell lines expressed substantial amounts of PTHrP and CSF-1 mRNA and protein and interleukin (IL)-8 mRNA. Although osteolytic CaP cell lines expressed and secreted predominantly PTHrP and IL-8 (Figure 3, A and B; and Supplemental Figure 1, see *http://ajp.amjpathol.org*), osteolytic CaM cell lines expressed and secreted mostly CSF-1 (Figure 3, C and D). These results are in agreement with previous studies reporting the expression of PTHrP,[45,46] CSF-1,[47–49] and IL-8[50,51] in both CaP and CaM cell lines.

RANKL mRNA was expressed at a very low level or absent in all of the cell lines tested (Figure 3E). OPG mRNA was expressed at detectable levels by both CaM or CaP osteolytic cell lines and by the osteoinductive CaM cell line ZR-75-1 (Figure 3F). OPG mRNA expression in intraosseous xenografts of the osteolytic cell line PC-3 was approximately threefold higher than *in vitro*, but it was reduced by ~3.5-fold in intraosseous xenografts of the variant cell line PC-3M-Pro4. OPG mRNA was not detectable in intraosseous xenografts of the osteoinductive cell line C4-2B (not shown). Lack of RANKL expression by bone metastatic cancer cell lines has already been described.[52,53] This is consistent with the view that

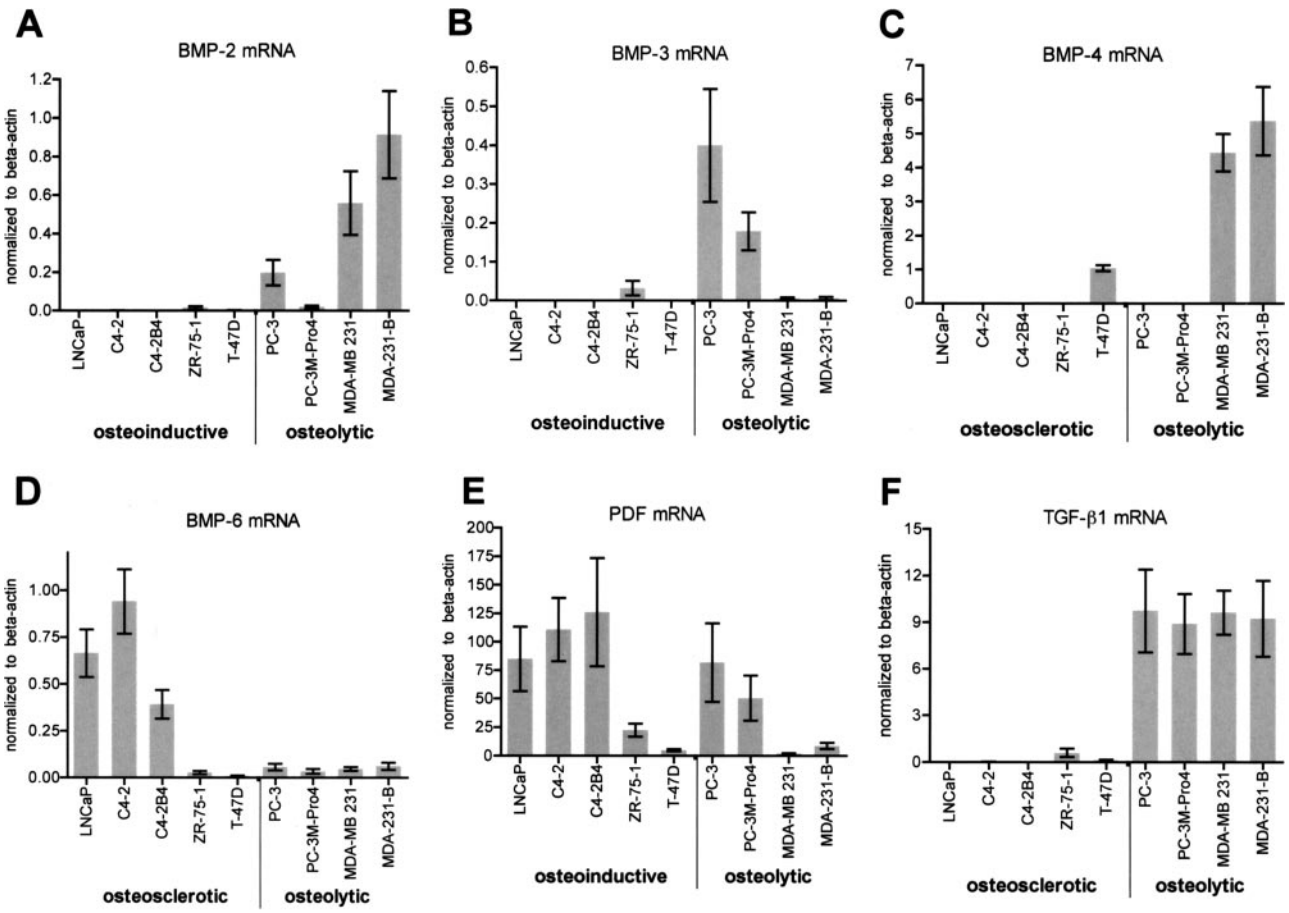

Figure 2. Expression of BMP, PDF, and TGF-β mRNAs in osteoinductive and osteolytic cancer cell lines. **A:** BMP-2. **B:** BMP-3. **C:** BMP-4. **D:** BMP-6. **E:** PDF. **F:** TGF-β. The mRNA levels ($\pm$SEM) were quantified by real-time RT-PCR relative to β-actin endogenous control ($n \geq 3$).

in osteolytic bone metastasis RANKL is osteoblast-derived and is induced by cancer cell-derived PTHrP.[44,53] Our finding showing OPG expression *in vitro* and *in vivo* prevalently in osteolytic, but not in osteoinductive, cancer cell lines is in agreement with previous reports.[52,53] Osteolytic cell lines exhibit high tumorigenic and metastatic potential and their OPG expression is consistent with the postulated role of OPG in protecting cancer cells from tumor necrosis factor-related-apoptosis inducing ligand (TRAIL)-induced apoptosis.[54] Taken together, the observations above, although limited to a small number of cancer cell lines, suggest that the osteolytic potential of cancer cell lines depends prevalently on their ability to stimulate osteoclast generation directly by secreting CSF-1 and IL-8, and, indirectly, via PTHrP-induced release of RANKL by bone stromal cells.

Growth Characteristics and Bone Effects in Vivo *of the C4-2B Cell Clone Stably Transfected with the pCMV-Luciferase Vector (C4-2B/luc$^+$ Cells)*

Based on the observation that low expression of noggin *in vitro* correlates with the osteoinductive potential *in vivo* of cancer cell lines, we tested whether forced noggin expression in an osteoinductive cell line, constitutively lacking noggin expression, could abolish the osteoblast response *in vivo*. We have previously reported on the sensitivity and reliability of noninvasive whole body BLI as a method to monitor and quantify the development and progression of bone metastasis in living animals.[28] To apply this method, we first established C4-2B cell clones with stable expression of the *luc* gene. One clone was selected based on high *luc* expression, gene expression profile of osteotropic cytokines *in vitro*, tumor take, and bone reaction after intraosseous implantation *in vivo* equivalent to those of the parental C4-2B cells (not shown). After implantation of C4-2B/*luc*$^+$ cells into the tibia marrow cavity of immunodeficient mice, tumor take was 100%, as illustrated in Figure 4A. Tumor growth was monitored and quantified as bioluminescent emission, which was clearly detectable in all animals from day 28 on (Figure 4B). Signal intensity further increased in the following 8 weeks. Radiographs taken 12 weeks after intratibial inoculation of C4-2B cells, at which time animals were sacrificed, showed an enlargement in the upper half of the bone shaft, with cortical thinning and mixed areas of osteoblastic and osteolytic reaction in the upper one third of the bone shaft (Figure 4C). In contrast, ra-

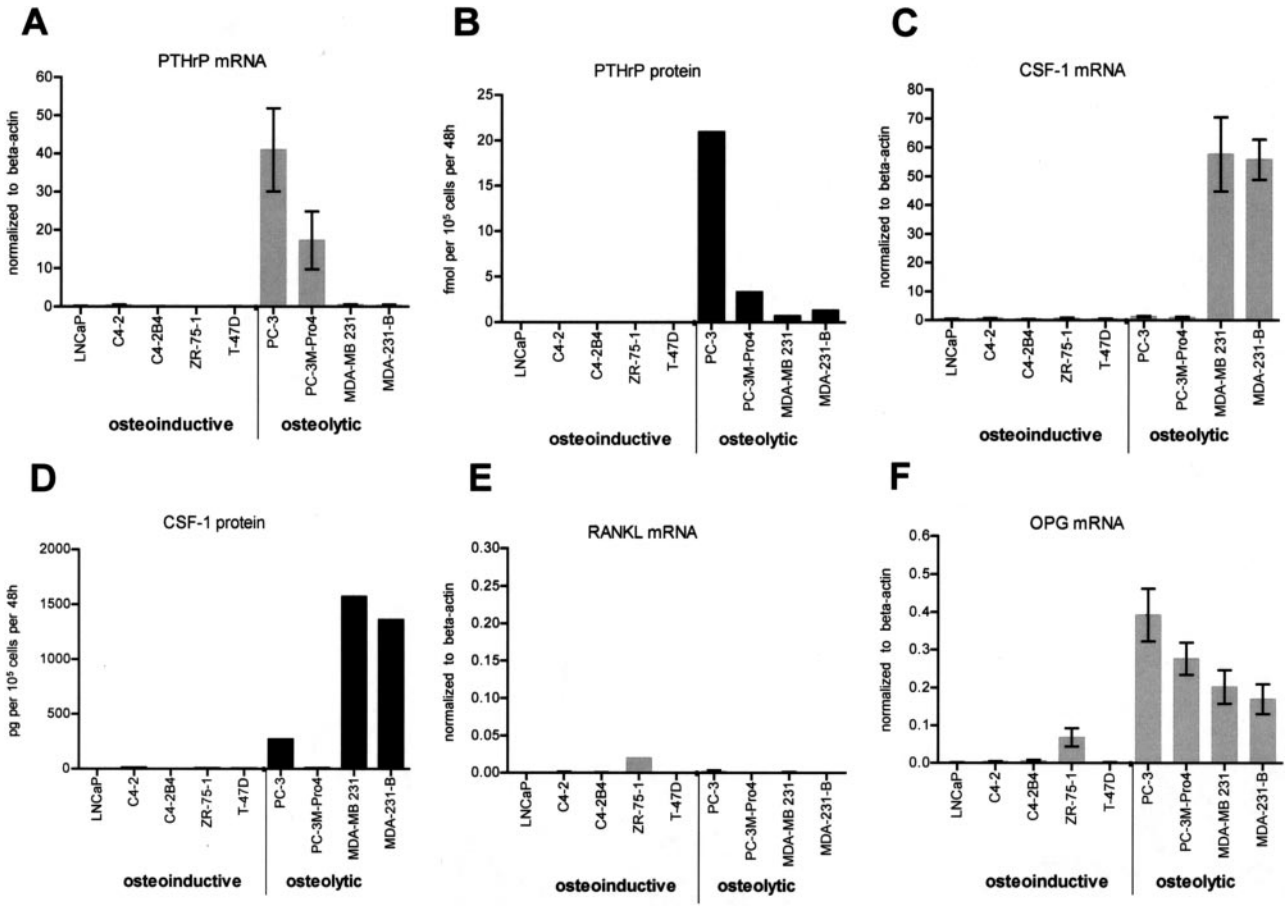

Figure 3. Expression of cytokines influencing bone resorption in osteoinductive and osteolytic cancer cell lines. **A:** PTHrP mRNA. **B:** PTHrP protein. **C:** CSF-1 mRNA. **D:** CSF-1 protein. **E:** RANKL mRNA. **F:** OPG mRNA. The mRNA levels (±SEM) were quantified by real-time RT-PCR relative to β-actin endogenous control ($n \geq 3$). PTHrP concentration (fmol per 10^5 cells) in culture supernatant was measured by a two-site immunoradiometric assay. CSF-1 concentration (pg per 10^5 cells) in culture supernatant was measured by immunoassay.

diographs taken 3 weeks after intratibial inoculation of PC-3 cells show only minor modifications of the bone shape and cortex but a well-defined area of osteolysis at the site of inoculation. *Ex vivo* micro-CT analysis of sham-operated and C4-2B-implanted bones demonstrates profound changes in the bone architecture of the tibia implanted with C4-2B cells, compared with the sham-operated tibia (Figure 4D). A marked enlargement of the bone shaft, associated with a thinning of the cortex, and an increased density of randomly oriented and thinner trabeculae were the principal modifications induced by the presence of tumor cells. The presence of an osteoblast reaction was confirmed by the histological examination showing thin and irregular bone trabeculae covered by active osteoblasts within bone marrow areas invaded by cancer cells (Figure 4E). These alterations are consistent with a mixed, osteosclerotic and osteolytic, reaction equivalent to that described for the C4-2 cell line, from which the C4-2B cells are derived.[31,55]

Synthesis and Secretion in Vitro *of Biologically Active Noggin by C4-2B/luc$^+$/Nog$^+$ Clones*

C4-2B/*luc*$^+$ cells were transfected with a noggin expression plasmid (*Nog*) or with the empty pcDNA3.1 vector (mock). Three noggin-expressing clones (*Nog17, Nog19*, and *Nog20*) and three mock clones (mock1, mock2, and mock4) were selected and tested for noggin protein expression (Figure 5A). No noggin was detected in the cell lysate of C4-2B/*luc*$^+$ cells, which is consistent with the lack of noggin secretion by parental C4-2B cells (Figure 1A). All three *Nog* clones show detectable noggin protein, both in the cell lysate and in the culture supernatant. *Nog19* and *Nog20* secreted almost equivalent amounts of noggin protein, whereas *Nog17* secreted a considerably lower amount, reflected by their noggin mRNA expression (not shown). In contrast, none of the three mock clones secreted detectable amounts of noggin (not shown).

The biological activity of noggin secreted by transfected C4-2B cells was verified in an osteoblast differentiation assay *in vitro*.[36] CM from all three *Nog* clones decreased basal ALP expression in KS483 osteoblast cells. *Nog19* and *Nog20* clones secreted approximately the same amount of biologically active noggin, whereas *Nog17* secreted lower amounts (Table 3). No significant effect on ALP expression was induced by CM from mock clones. The difference for the levels of biologically active noggin between the *Nog17* and *Nog19/Nog20* clones

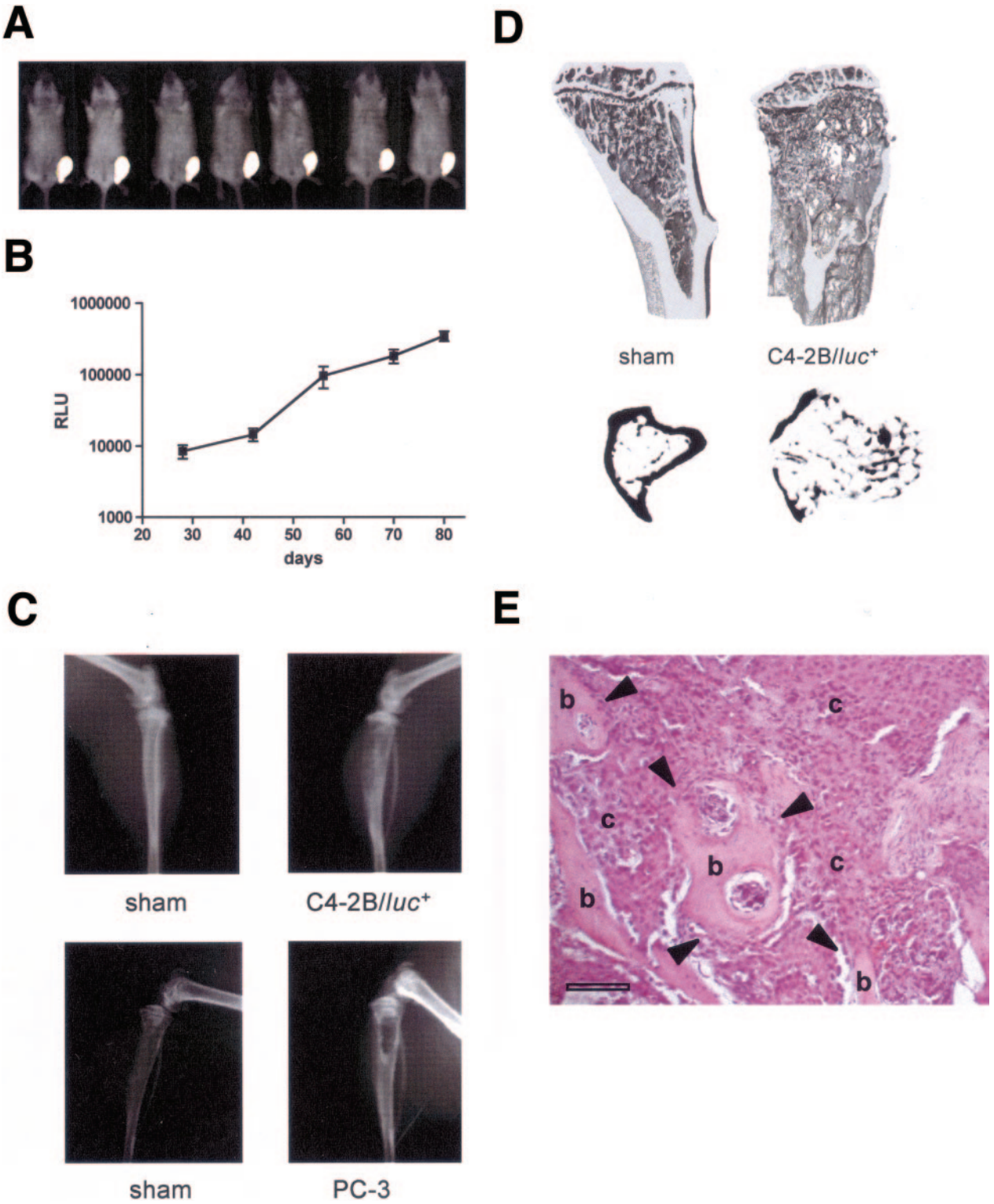

Figure 4. Growth characteristics and bone effects *in vivo* of C4-2B/*luc*$^+$ cells. **A:** Tumor take after intraosseous inoculation of the cells. Bioluminescent photon emission was detected at day 80. Signals are displayed as pseudo-color image at a 0- to 6-bit range on ventral projections of the mice (n = 7). **B:** Tumor growth *in vivo* of C4-2B/*luc*$^+$ cells. Bioluminescent signal (RLU ± SEM) emitted from the left tibia was quantified at days 28, 42, 72, and 80 after intraosseous implantation of the cells (n = 7 mice). **C:** Radiographical aspect of bone lesions induced by intraosseous inoculation of C4-2B/*luc*$^+$ and PC-3 cells. Radiographs of the left proximal tibia were taken 90 and 22 days after inoculation into the bone marrow cavity of C4-2B/*luc*$^+$ and PC-3 cells, respectively. Radiographs of the left tibia 90 and 22 days after sham operation are shown for comparison. **D:** Effect of intraosseous growth of C4-2B/*luc*$^+$ cells on the bone architecture. Three-dimensional reconstruction images and transversal sections at 2.17 mm from the articular surface of proximal tibiae, either sham-operated (left) or inoculated with C4-2B/*luc*$^+$ cells (right), obtained by micro-CT scan at day 80 after intraosseous inoculation. **E:** Histology of bone lesions induced by C4-2B/*luc*$^+$ cells. H&E-stained section of the proximal left tibia 90 days after the intraosseous inoculation of C4-2B/*luc*$^+$ cells. b, bone trabeculae; c, cancer cells. **Arrowheads** indicate bone-forming surfaces covered by active osteoblasts.

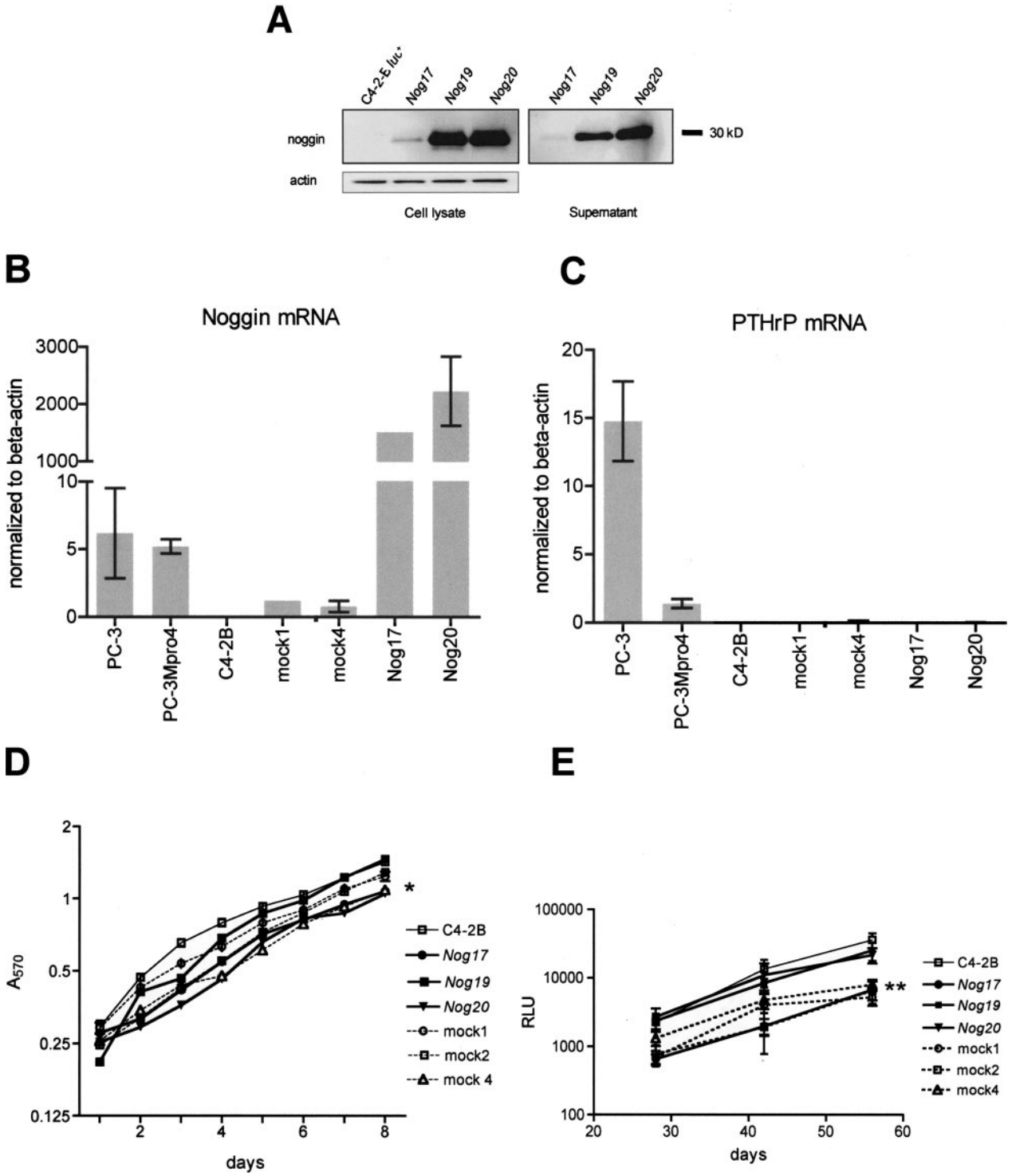

Figure 5. Characterization of *Nog*- and mock-transfected C4-2B/*luc*$^+$ cell clones and expression of noggin and PTHrP in intraosseous xenografts of CaP cell lines. **A:** Noggin synthesis and secretion by *Nog*-transfected C4-2B/*luc*$^+$ cells *in vitro*. Equal amounts of total protein (30 μg) of cell lysate or culture supernatants were immunoblotted with noggin or actin antibody. Noggin (**B**) and PTHrP (**C**) mRNA expression in intraosseous xenografts of *Nog*-, mock-transfected C4-2B/*luc*$^+$ cell clones, and parental C4-2B cells, and of PC-3 and PC-3M-Pro4 cells. The mRNA levels (±SEM) were quantified by real-time RT-PCR relative to β-actin endogenous control (n = 1 to 3). **D:** Proliferation *in vitro* of *Nog* and mock C4-2B/*luc*$^+$ cells was measured by a MTT assay for 8 days. *$P < 0.05$ between C4-2B/*luc*$^+$ and *Nog17*, *Nog20*, mock4 from day 2 (n = 3). **E:** Growth *in vivo* of *Nog* and mock C4-2B/*luc*$^+$ cells. Bioluminescent signal (RLU ± SEM) emitted from the left tibia was quantified at days 28, 42, and 56 after intraosseous implantation. **$P < 0.01$ between C4-2B/*luc*$^+$, *Nog19*, *Nog20*, and *Nog17*, mock1, mock2, and mock4 at day 56 (n = 5 to 6).

was considerably less pronounced than for the amounts of the immunoreactive, secreted protein (Figure 5A). Secretion by *Nog19/Nog20* clones of additional factors stimating ALP expression in KS483 cells, and thus interfering with the noggin inhibitory activity, may explain this discrepancy.

Table 3. Effect on ALP Expression in Osteoblast-Like Cells by Conditioned Medium of Parental C4-2B Cells, and of *Nog* and Mock-Transfected C4-2B Clones

Clone	ALP activity*	Noggin equivalent†
Parental C4-2B	72.9 ± 1.9	0
Nog17	60.2 ± 2.5‡	0.4 μg/ml
Nog19	43.2 ± 6.3‡	1 μg/ml
Nog20	38.2 ± 5.3‡	1.3 μg/ml
Mock1	72.3 ± 5.6	0
Mock2	68.9 ± 2.4	0
Mock4	71.4 ± 3.8	0

*ALP activity was measured 3 days after the addition of conditioned medium and corrected for the amount of DNA in each culture. Mean of six individual cultures ± SEM.

†Equivalent noggin concentration was estimated by comparison with the inhibition of ALP activity exerted by known amounts of recombinant noggin added to the culture. The concentration of 100 ng/ml of recombinant human noggin was exerting maximal inhibitory effect on ALP activity in KS483 cells.

‡$P < 0.05$ versus parental C4-2B cells and mock clones.

Noggin, DKK-1, and PTHrP Expression in Intraosseous Xenografts of Parental, Noggin-, and Mock-Transfected C4-2B/luc$^+$ Cells, and of PC-3 and PC-3M-Pro4 Cells

It is widely recognized that the reciprocal interactions between cancer cells and the bone microenvironment *in vivo* may modify the phenotype and the pattern of cytokines expressed by cancer cells *in vitro*.[35] To exclude this possibility, we analyzed noggin and PTHrP expression *in vivo* in intraosseous xenografts of either osteolytic or osteoinductive CaP cell lines. The osteolytic PC-3 and its derivative PC-3M-Pro4 cell line expressed both noggin and PTHrP, although to a lesser extent than *in vitro*. Instead, the osteoinductive C4-2B cell line, as well as the mock clones derived from the C4-2B/luc$^+$ cells did not express either noggin or PTHrP (Figure 5, B and C). Thus, the differential expression of noggin and PTHrP between osteolytic and osteoinductive CaP cell lines shown *in vitro* could be confirmed *in vivo*. Noggin mRNA expression at the end of the *in vivo* experiment was high in both the C4-2B cell clones *Nog17* and *Nog20* (Figure 5B), thus confirming persistence of noggin expression *in vivo*. Equally to the parental C4-2B cells, *Nog17/Nog20* and mock1/mock4 transfected clones did not express PTHrP (Figure 5C). DKK-1 mRNA expression *in vivo* was undetectable in parental and noggin- and mock-transfected C4-2B/luc$^+$ cells (not shown).

Growth Characteristics in Vitro *and* in Vivo *of C4-2B/luc$^+$/Nog$^+$ Clones*

The growth rate *in vitro* was equivalent for *Nog19*, mock1, and mock2 cell clones and the parental C4-2B/*luc*$^+$ cell line (Figure 5D). In contrast, *Nog17*, *Nog20*, and mock4 clones showed a slightly, but significantly ($P < 0.05$), lower growth than the parental C4-2B/*luc*$^+$ cell line. *Nog19* and *Nog20* cells showed an intraosseous growth rate and an end-point tumor burden, as assessed by whole body BLI monitoring in the living animals, equivalent to that of parental cells, whereas *Nog17* and all of the three mock-transfected clones grew to a lesser extent ($P < 0.01$ at day 56) (Figure 5E). Equivalent results were obtained in a second experiment in which the intraosseous growth rate of parental C4-2B/*luc*$^+$, *Nog20*, and mock4 cells was monitored for 80 days (not shown). This marginal effect on tumor growth could be partially attributable to a difference in the original number of tumor cells inoculated. Taken together, the BLI monitoring of tumor growth indicates that noggin forced expression has only a negligible effect on the intraosseous growth rate and tumor burden of C4-2B cells.

Effects on Bone in Vivo *of C4-2B/luc$^+$/Nog$^+$ Clones*

To evaluate the influence of forced noggin expression in C4-2B/*luc*$^+$on the bone response induced by this osteoinductive CaP cell line *in vivo*, we analyzed by pQCT the bone structure of tibiae inoculated with either parental or *Nog*- or mock-transfected cells. In a first experiment, pQCT analysis was performed 56 days after intraosseous implantation (Figure 6A). Tibiae inoculated with the parental C4-2B/*luc*$^+$, when compared with those sham-operated, showed an enlargement of the bone shaft associated with a thinning of the cortex and an increase in trabecular bone, as indicated by the increase in total bone area (TBA), trabecular bone area (TrBA), and content (TrBC) and by the decrease in the cortical bone area (CBA) and content (CBC). These modifications of the bone architecture, which are consistent with those already shown in Figure 4C, suggest that the osteoinductive effect induced by the C4-2B/*luc*$^+$ tumor is prevalently acting on the trabecular bone. The enlargement of the bone shaft associated with the thinning of the bone cortex may be a direct consequence of the altered bone remodeling and/or the effect of an attempt by the bone to compensate for the reduced mechanical competence of the tumor-induced trabecular bone.

The tibiae inoculated with the *Nog17* cells showed a significantly lower expression of the structural parameters of excess of bone formation, such as high TBA, TrBA, and TrBC, and of bone cortical thinning, such as low CBA and CBC, compared with the tibiae implanted with parental C4-2B/*luc*$^+$or mock1 cells. Most importantly, there was no difference in any of these parameters between sham-operated tibiae and those inoculated with the *Nog17* clone (Figure 6A). This effect was also evident for *Nog19* and *Nog20* clones (not shown). This result clearly indicates that forced noggin expression in C4-2B/*luc*$^+$ cells alone is sufficient to abolish their osteoinductive activity. The observation that *Nog20* expressed *in vivo* only twice as much noggin mRNA compared with the *Nog17* clone (Figure 5B) may explain why both these *Nog*-cell clones exerted equivalent bone effects. Noggin does not increase tumor growth rate and burden (see above, Figure 5E), in agreement with a previous report by others.[56] This excludes that an increase in tumor burden may be responsible for the normalization of TrBA and TrBC.

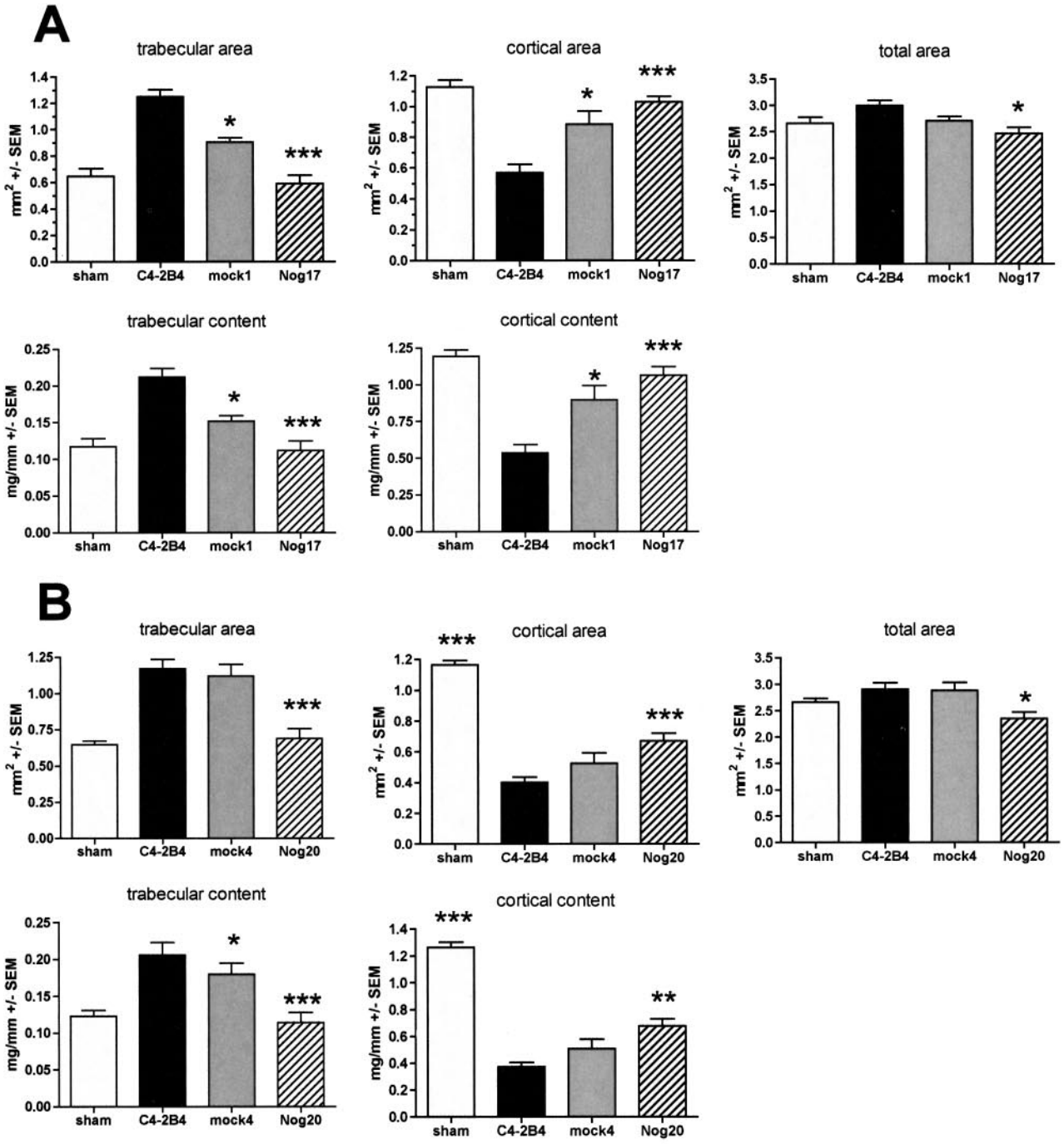

Figure 6. Effects of intraosseous *Nog*- and mock-transfected C4-2B/*luc*$^+$ cells on bone architectural parameters and osteoclast number. **A:** Area (mm^2 ± SEM) of trabecular (***$P < 0.001$ versus C4-2B/*luc*$^+$; *$P < 0.05$ versus *Nog17*, $P < 0.01$ versus C4-2B/*luc*$^+$), cortical (***$P < 0.001$ versus C4-2B/*luc*$^+$; *$P < 0.05$ versus C4-2B/*luc*$^+$), and total bone (*$P < 0.05$ versus C4-2B/*luc*$^+$), and mineral content (mg/mm ± SEM) of trabecular (***$P < 0.001$ versus C4-2B/*luc*$^+$; *$P < 0.05$ versus C4-2B/*luc*$^+$) and cortical bone (***$P < 0.001$ versus C4-2B/*luc*$^+$; *$P < 0.05$ versus C4-2B/*luc*$^+$) measured by pQCT in tibiae 56 days after inoculation into the bone marrow cavity of PBS (sham) or parental C4-2B/*luc*$^+$, mock1, and *Nog17* cells ($n = 5$ to 7). **B:** Area (mm^2 ± SEM) of trabecular bone (***$P < 0.001$ versus C4-2B/*luc*$^+$ and mock4), cortical bone (***$P < 0.001$ versus all; **$P < 0.01$ versus C4-2B/*luc*$^+$), and total bone (*$P < 0.05$ versus C4-2B/*luc*$^+$ and mock4), and mineral content (mg/mm ± SEM) of trabecular bone (***$P < 0.001$ versus C4-2B/*luc*$^+$; *$P < 0.05$ versus *Nog20*) and cortical bone (***$P < 0.001$ versus all; **$P < 0.01$ versus C4-2B/*luc*$^+$) measured by pQCT in tibiae 80 days after inoculation into the bone marrow cavity of PBS (sham) or parental C4-2B/*luc*$^+$, mock4, and *Nog20* cells ($n = 7$). **C:** Mean osteoclast (OCL) number (±SEM) per mm bone surface in histological sections of tibiae 56 days after inoculation into the bone marrow cavity of PBS (sham) or parental C4-2B/*luc*$^+$ cells, *Nog17/Nog20*, and mock1/mock4 clones (**$P < 0.01$ versus C4-2B/*luc*$^+$ and mock4, $P < 0.05$ versus sham and mock1; $n = 3$ to 5).

In the experiment above none of the tibia implanted with the three mock-transfected clones (mock1, 2, and 4) showed an extent of modifications of the pQCT parameters equivalent to those seen in the tibiae implanted with the parental cells. This is most probably attributable to the fact that none of the mock clones developed within 8 weeks an intraosseous tumor burden and, consequently, an osteoinductive effect equivalent to that of the parental or *Nog*-transfected C4-2B/*luc*$^+$ cells. Therefore, in a next experiment the period of observation was prolonged to 12 weeks after the intraosseous implantation of parental C4-2B/*luc*$^+$ cells, *Nog20*, and mock4 clones. At this time point pQCT analysis showed equivalent bone architectural modifications for both mock cell- and parental cell-implanted tibiae. Consistent with the previous experiment, implantation of the *Nog20* clone reverted the indices of excess of bone formation, such as high TrBA and TrBC, to the normal values of the sham-operated animals, as shown for the *Nog20* clone (Figure 6B), corroborating that forced expression of noggin blunts completely the osteoinductive effect of the parental C4-2B/*luc*$^+$ cells. In contrast to the previous experiment, CBA and CBC of bones inoculated with *Nog20* cells did not attain the values of the sham-operated animals, although significantly different from those inoculated with parental cells. This was also reflected by the smaller TBA of bones implanted with *Nog20* cells compared with all other bones. It is conceivable that the longer exposure to the noggin inhibitory activity on bone formation may have exerted a detrimental effect on the periosteal bone apposition responsible for the age-related cortical drift.[57]

Effects of C4-2B/luc$^+$/Nog$^+$ Clones on Osteoclasts

In the experiment in which intraosseous xenografts of parental C4-2B/*luc*$^+$ cells, *Nog* (*Nog17*, *19*, and *20*)-, and mock (mock1, 2, and 4)-transfected clones were allowed to grow for 56 days, histomorphometry shows a significantly lower number of osteoclasts in bones implanted with *Nog20* cells ($P < 0.01$) than in sham-operated bones and in bones xenografted with parental C4-2B/*luc*$^+$ cells or mock-transfected (mock1 and 4) clones. A tendency to a lower osteoclast number was also seen in tibiae inoculated with *Nog17* cells, secreting lower amounts of noggin (Figure 5A and Table 3) but not attaining statistical significance (Figure 6C). This result confirms previous reports suggesting that noggin possesses an anti-bone-resorbing activity *in vitro* and *in vivo*, which is mediated by an inhibitory effect on osteoclast generation.[42,58] Histomorphometry also showed that the osteoclast number in bones xenografted with parental C4-2B/*luc*$^+$ cells and mock-transfected (mock1 and 4) clones does not significantly differ from that of sham-operated bones. This strongly suggests that intraosseous growth of C4-2B/*luc*$^+$ cells does not alter basal bone resorption.

Discussion

Here we show that lack of expression *in vitro* of the BMP antagonist noggin, of the Wnt antagonist DKK-1, and of the bone-resorbing cytokines PTHrP, CSF-1, and IL-8 by human CaP and CaM cell lines correlates with their osteoinductive potential *in vivo*. In contrast, all osteolytic CaP and CaM cell lines constitutively express *in vitro* both noggin and DKK-1 and at least one of the bone-resorbing cytokines PTHrP, CSF-1, and IL-8. This observation, although limited to a relatively restricted number of representative cell lines, suggests that both inhibition of the osteoblast response and stimulation of osteoclast recruitment are necessary for the full expression of the osteolytic phenotype. Noggin, DKK-1, and PTHrP are also differentially expressed in intraosseous xenografts of osteolytic and osteoinductive CaP cell lines, indicating that in the latter their expression cannot be induced *in vivo* by the bone microenvironment. Forced expression of biologically active noggin in the CaP cell line C4-2B abolishes its osteoinductive activity *in vivo*, demonstrating that lack of noggin expression has a causal role in the pathogenesis of osteoblastic bone metastases.

The mechanism(s) determining the exaggerated osteoblast response in osteosclerotic bone metastases is poorly understood.[4,43,59] An increased secretion and/or activation of factors inducing osteoblast recruitment by the bone metastatic cancer cells is commonly believed to cause the excess of bone matrix deposition.[4] Our findings suggest a novel mechanism by which the lack of antagonist molecules to osteoinductive factors, rather than the excess of osteoinductive factors, contributes essentially to the osteoblast response in bone metastasis. A similar mechanism is at the origin of sclerosteosis, the sole example for a human congenital disease characterized by a generalized excess of bone formation.[23]

Several BMPs have been shown to be produced by CaP cell lines.[60,61] Here we show that at least one BMP species is expressed by all CaP and CaM cell lines independently of their osteoinductive or osteolytic potential. Noggin has been used as a tool to block BMP function because it is a specific inhibitor of BMP activity and does not seem to have actions independent of BMP binding. However, noggin possesses a rather promiscuous binding activity among various BMPs,[62] which precludes a precise definition of the BMP(s) prevalently involved in determining the osteoblast response. Nevertheless, a recent report has provided evidence for a primary role of BMP-6 in the osteoinductive activity of the CaP cell line LuCaP 23.1.[60] This study, showing that forced noggin expression abolished the osteoinductive activity *in vivo* of the CaP cell line C4-2B, expressing mainly BMP-6, further supports this view.

Absent or greatly reduced expression of bone resorbing cytokines in osteoinductive cancer cell lines has also been postulated to unbalance further the bone response in favor of an osteoblast reaction.[43,59] The lack of expression of bone-resorbing cytokines in osteoinductive CaM and CaP cell lines shown in the present study supports this view. To provide experimental evidence *in vivo* for this hypothesis, we are currently investigating the possibility of inducing osteolytic potential *in vivo* by forced CSF-1 expression in an osteoinductive CaP cell line.

It has been suggested that bone metastatic CaP cells induce an initial phase of bone resorption, which in turn

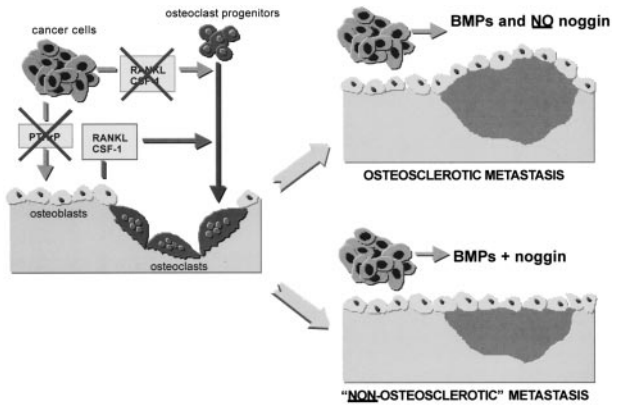

Figure 7. Model explaining the osteoinductive effect of metastatic cancer cells during bone remodeling and the inhibition of this effect by noggin. In physiological bone remodeling the amount of bone removed by osteoclast resorption, under the control of the osteoblast-derived osteoclastogenic cytokines CSF-1 and RANKL, is replaced by an equal amount of newly formed bone. This mechanism maintains the bone mass in perfect balance. In osteosclerotic bone metastasis cancer cells secrete factors, ie, BMPs, inducing osteoblast recruitment. If BMPs are not counterbalanced by their antagonists, ie, noggin, the increased number of newly recruited osteoblasts lay down an excess of bone matrix in the resorption lacuna. The absence of production by the cancer cells of factors stimulating osteoclast generation favors this imbalance.

promotes their growth and osteoinductive activity[59] and, conversely, that repression of their osteoinductive activity unmasks their original osteolytic potential.[63] In the present study repression of the osteoblast response by forced noggin expression *in vivo* reverses the bone structural parameters to normal control values, suggesting that there is a return to physiological bone remodeling and that C4-2B cells do not produce additional osteolytic activity. This is also substantiated by the facts that, at least *in vitro*, none of the osteoinductive cancer cell lines express the osteolytic cytokines PTHrP, CSF-1, and IL-8, and that in bones xenografted with C4-2B cells there is no increase in osteoclast number when compared with sham-operated bones. The normalization of bone parameters cannot be referred to an enhanced tumor volume because forced noggin expression does not increase tumor burden. In addition, noggin exerts an inhibitory effect on osteoclast generation, as shown here and by others,[16,42,58] which may counterbalance osteolytic effects, if any, induced by C4-2B cells. Therefore, we postulate a model in which the osteoblast response in bone metastasis is, at least partially, the result of the lack of noggin secretion by cancer cells and, consequently, of the unopposed effect of the excess of BMPs released locally by cancer cells themselves. This leads to an increased recruitment of osteoblasts laying down an excess of bone matrix at sites of physiological bone resorption, with a net increase in the bone mass adjacent to cancer cell deposits. Concomitant lack of production by cancer cells of factors stimulating osteoclast generation and activity may further favor this imbalance. In this model cancer cells do not need to induce an initial phase of bone resorption to promote their growth and osteoinductive activity, because the excess of bone formation is coupled to physiological bone resorption (Figure 7).

BMPs, either released during bone resorption or secreted in an autocrine manner, may contribute to the tumorigenic and invasive properties of bone metastatic cancer cells.[58,60] Recently, it has been shown that noggin counteracts these effects in the highly tumorigenic and osteolytic CaP cell line PC-3.[58] In the present investigation, we could not find a relevant effect on growth *in vitro* and *in vivo* by forced noggin expression in the osteoinductive CaP cell line C4-2B, possessing less tumorigenic and metastatic potential than the PC-3 cell line. Likewise, it has been reported that forced noggin expression has no impact on tumor growth induced *in vivo* by intraosseous inoculation of LAPC-9,[56] another osteoinductive CaP cell line. Furthermore, BMP-6 inhibition does not affect the growth *in vitro* of C4-2B or proliferation *in vitro* and subcutaneous growth of the osteoinductive CaP cell line LuCaP23.1.[60] However, BMP-6 inhibition reduces the size of intraosseous LuCaP23.1 tumors, suggesting that BMP-6 may indirectly affect tumor growth by modulating the bone microenvironment.[60] It is well recognized that osteoinductive cancer cell lines display less tumorigenic and metastatic potential than osteolytic cancer cell lines. It is also known that TGF-β has a dual role in cancer, acting both as a tumor suppressor and as a tumor promoter. This ambiguity depends on the accumulation of genetic and epigenetic alterations that progressively unbalance TGF-β signaling toward increased oncogenic effects.[64,65] An equivalent mechanism may explain the variable growth response to BMP stimulation and, conversely, to noggin inhibition among cancer cell lines with different oncogenic potential.

Whether lack of noggin expression is also relevant for determination of the osteosclerotic feature of bone metastasis in CaP and CaM patients has not yet been established. Noggin is expressed at very low levels in adult tissues in general and in the adult skeleton.[15] This has hampered our attempts to test by traditional methods of mRNA and protein detection *in situ* whether noggin is differentially expressed by cancer cells also in clinical samples of osteolytic and osteoblastic bone metastasis. On the other hand, noggin expression by the bone stromal component may interfere with its mRNA analysis by quantitative PCR when performed on bulk tissue samples of human bone metastasis. A preliminary analysis of noggin mRNA expression in a very limited sample number of pure cancer cells isolated by laser-capture microdissection of bone metastasis biopsies seems to confirm that noggin is expressed at lower levels in osteoblastic than osteolytic bone metastases. We are currently extending this analysis to a larger number of clinical specimens of bone metastasis.

A causal role for Wnt in the pathogenesis of osteosclerotic bone metastasis has been demonstrated. The canonical Wnt signaling is involved in determining bone mass.[20–22] The extracellular Wnt-antagonist DKK-1 secreted by myeloma cells is responsible for the suppression of the osteoblast activity in the osteolytic lesions that characterize this neoplastic disease.[27] Conversely, it has been demonstrated that lack of DKK-1 expression, and thus an unopposed osteoblast response to Wnt signaling, is responsible for the osteoinductive activity of bone met-

astatic CaP cells.[63] In addition, here we show that lack of DKK-1 expression correlates also with the osteoinductive potential of cell lines of CaM origin. Here we propose that, by a similar mechanism, lack of noggin expression by cancer cells may lead to an unopposed osteoblast response to BMPs and, therefore, concur to the pathogenesis of osteosclerotic bone metastasis of both CaP and CaM origin. Taken together, these considerations suggest that both BMP and Wnt signaling pathways play an essential role as osteoinductive factors in osteosclerotic metastases. However, the exact hierarchy of these signaling pathways in determining the osteoblast response is not yet defined. BMP and Wnt signaling clearly cooperate and co-regulate each other in promoting osteoblast differentiation. However, it is still controversial whether they operate in parallel or whether BMP activity is required downstream of a Wnt stimulus.[66] Our results and previous reports[56,63] show that both noggin- and DKK-1-mediated inhibition of the BMP and Wnt signaling, respectively, can repress the cancer-induced osteoblastic response *in vivo*. This supports the concept of a parallel cooperation between BMP and Wnt signaling in bone formation.

Bones affected by either osteolytic or osteosclerotic metastases are prone to pathological fractures. The dual inhibitory effect of noggin on the osteoblast and osteoclast reactions in bone metastatic lesions may result in a decreased incidence of pathological bone fractures. In addition, noggin may also exert a direct inhibitory effect on tumor growth.[67] Therefore, noggin could prove to be useful as an adjuvant drug in the therapy of bone metastasis.

Acknowledgments

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