

Bone Morphogenetic Protein (BMP)-6 Signaling and BMP Antagonist Noggin in Prostate Cancer

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ABSTRACT

It has been proposed that the osteoblastic nature of prostate cancer skeletal metastases is due in part to elevated activity of bone morphogenetic proteins (BMPs). BMPs are osteoinductive morphogens, and elevated expression of BMP-6 correlates with skeletal metastases of prostate cancer. In this study, we investigated the expression levels of BMPs and their modulators in prostate, using microarray analysis of cell cultures and gene expression. Addition of exogenous BMP-6 to DU-145 prostate cancer cell cultures inhibited their growth by up-regulation of several cyclin-dependent kinase inhibitors such as p21/CIP, p18, and p19. Expression of noggin, a BMP antagonist, was significantly up-regulated by BMP-6 by microarray analysis and was confirmed by quantitative reverse transcription-polymerase chain reaction and at the protein level. Noggin protein was present in prostate biopsies and localized to the epithelial components of prostate by immunohistochemistry. Recombinant noggin inhibited the function of BMP-6, suggesting a negative feedback regulation of BMP activity and indicating a strategy for the development of a novel therapeutic target in the treatment of painful osteosclerotic bone metastases of prostate cancer.

INTRODUCTION

Prostate cancer was diagnosed in more than 220,000 Americans last year, and nearly 30,000 Americans died of this disease. Excruciating bone pain, fractures, and death caused by prostate cancer are invariably the result of metastases, the majority of which are osteoblastic and osteosclerotic to bone (1–5). Whereas the pathological events are well established, the molecular events involved in prostate cancer metastasis are only beginning to be understood. The bone morphogenetic protein (BMP) family of proteins was originally identified by the ability of BMPs to induce new bone formation when implanted in ectopic subcutaneous sites in rats (6–8). BMPs initiate new bone formation by recruiting progenitor stem cells and initiating their growth and differentiation into bone in a sequence of events reminiscent of the steps seen in bone formation during embryogenesis (8). BMPs are active at low concentrations (10–50 ng/ml), although the exact determination of BMP concentrations required to elicit a response *in vivo* is complicated by the fact that BMPs are often bound to the extracellular matrix rather than soluble. At the mRNA level, it has been shown that expression of BMP-7, but not BMP-4, is androgen dependent in mouse (9) and human prostates (10).

The profound effects that BMPs have on cell fate and determination necessitate a tight regulation of BMP activities. There are more than a dozen different human BMPs, each of which has its own subset of activities and unique expression patterns. This regulation can occur at different levels of the BMP signaling cascade, either extracellularly, at the cell membrane, or within the cell. A family of extracellular BMP

antagonists has recently been discovered. These are secreted proteins that bind to BMPs and reduce their bioavailability for interactions with the BMP receptors. Extracellular BMP antagonists include noggin, follistatin, sclerostatin, chordin, DCR, BMPMER, cerberus, gremlin, DAN, and others (refs. 11–16; reviewed in ref. 17). There are several type I and type II receptors that bind to BMPs with different affinities. BMP activity is also regulated at the cell membrane level by receptor antagonists such as BAMBI (18), which acts as a kinase-deficient receptor. Intracellularly, the regulation of BMP activity at the signal transduction level is even more complex. There are inhibitory Smads (Smad-6 and Smad-7), as well as inhibitors of inhibitory Smads (AMSH and Arkadia). There are proteins that can target either receptors or Smads for degradation (Smurf, SANE, and CHIP) and phosphatases that reduce receptor phosphorylation (GADD34-PP1c). A host of receptor and Smad binding proteins can modulate the nuclear translocation of Smad complexes (EID and DLX) or alter Smad binding to the receptors (XIAP, BRAM, FKBP12, and others). Within the nucleus, transcriptional coactivators and corepressors modulate the transcription of BMP response genes (*YY1*, *Ski*, *Sno*, *ECSIT*, *Tob*, *CBP/P300*, and many more). It is the combined effect of this large symphony of BMP signaling molecules that will ultimately determine cellular response to BMP.

BMP-6 expression is increased in prostate adenocarcinoma, and several studies have shown a correlation between elevated BMP-6 and osteoblastic metastases (19–23). At present, there is still much to be learned about the function of BMPs and their antagonists in prostate cancers. In this study, we investigated the expression levels of BMPs, their antagonists, and molecules associated with BMP activity. In particular, we investigated the expression of BMP-6 and the BMP antagonist noggin in prostate cancer cell lines, with special focus on reciprocal feedback regulation and proliferation.

MATERIALS AND METHODS

Cell Culture. DU-145 and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in the recommended media (Invitrogen, Carlsbad, CA). Fetal bovine serum (FBS) and charcoal-stripped FBS were purchased from Hyclone (South Logan, UT). Cells were seeded in media supplemented with 10% FBS at a density of 200,000 cells per well in 6-well dishes (approximately 20,000 cells/cm²) and allowed to attach overnight. The culture medium was replaced with media supplemented with 1% charcoal-stripped FBS for 24 hours, and then BMP-6, BMP-7, noggin, and steroid hormones (in media supplemented with 1% charcoal-stripped FBS) were added for the indicated times. Phenol red was not a component of any media used. Recombinant noggin was a generous gift from Dr. Aris Economides (Regeneron Pharmaceuticals, Tarrytown, NY). BMP-6 and BMP-7 were generous gifts from Dr. T. K. Sampath (Creative Biomolecules, Hopkinton, MA). Unless noted, all other reagents were purchased from Invitrogen.

Quantitative Reverse Transcription-Polymerase Chain Reaction. DNA-free total RNA was prepared with RNeasy mini-columns, using the on-column DNase digestion step (Qiagen, Valencia, CA). Quantitative reverse transcription-polymerase chain reaction (RT-PCR; TaqMan) primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA), and their sequences (in 5' to 3' direction) were as follows: BMP6Fwd, CCGTGTAGTATGGGCCTCAGA; BMP6Rev, TCACAACCCAC-AGATTGCTAGTG; BMP6Probe, CGTGATGTCAAATTCAGCCAGCCTT; NogginFwd, CCTGGTGGACCTCATCGAA; NogginRev, CAGCGTCTCGT-

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TCAGATCCTT; NogginProbe, ACCCAGACCCTATCTTTGACCCAGG; GremlinFwd, GCAAAACCCAGCCGCTTA; GremlinRev, GGTGATGATGGTGC GACTGT; GremlinProbe, CAGACCATCCACGAGGAAGGCTGC; CerberusFwd, ACCATGAGGAAGCTGAGGAGAA; CerberusRev, GCAGG-GCTGGTGGCTACA; and CerberusProbe, AGATCTGTTTGTGCGAGTGC-CACACC. Real-time quantitative RT-PCR was done using a Sequence Detector 7700 and the EZ-RT-PCR reagents from Applied Biosystems. Expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase, and the fold change was calculated using the formula $2^{\Delta\Delta Ct}$ as described by Applied Biosystems. Each RT-PCR was done in triplicate, and the results shown are representative of at least two repeated experiments.

Western Blotting. Human prostate cancer biopsies were obtained from Dr. Ralph de Vere White (Department of Urology, University of California Davis Medical Center) and homogenized in reducing SDS-PAGE sample loading buffer that contained 8 mol/L urea. After centrifugation for 5 minutes at 12,000 \times g, the supernatants were boiled for 5 minutes and then electrophoresed on precast 4–12% gradient gels (Invitrogen). Cultures of 200,000 cells were lysed in 100 μ l of 1 \times SDS-PAGE sample buffer, and 40 μ l were loaded into each lane of a SDS-PAGE precast cell (corresponding to 80,000 cells per lane for even protein loading), which was then processed as described above. After transfer to Immobilon-P (Millipore, Bedford, MA), the membranes were blocked with 2% bovine serum albumin and then incubated with the anti-noggin (Regeneron Pharmaceuticals) and anti-BMP-6 antibodies at 2 to 10 μ g/ml and anti-p21 (BD Biosciences, San Jose, CA) and anti-phospho-Smad (Cell Signaling, Beverly, MA) at the dilutions recommended by the manufacturers. Detection was done by either horseradish peroxidase-conjugated goat antirabbit secondary antibodies and enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) or alkaline phosphatase-conjugated goat antibodies and Enhanced ChemiFluorescence and quantified on a Storm-860 scanner (Amersham Biosciences).

Immunohistochemistry. A slide array of 80 characterized, paraffin-embedded 2-mm human prostate biopsies (Imgenex, San Diego, CA) was characterized by Imgenex using the Gleason grading scale. The slide array was incubated with 25 μ g/ml mouse anti-noggin monoclonal antibody using the VectaStain ABC-AP kit according to the manufacturer's protocols (Vector Laboratories, Burlingame, CA).

Cell Proliferation Assay. Cells were seeded in media supplemented with 10% FBS at a density of 1.5×10^5 cells per well in 6-well dishes and allowed to attach overnight. The culture medium was replaced with media supplemented with 10% charcoal-stripped FBS for 24 hours, and then BMP-6 and dihydrotestosterone [DHT (in media supplemented with 10% charcoal-stripped FBS)] were added for a period of 7 days. Media were replaced and supplemented with BMP-6 and DHT every 2 to 3 days. Cells were lysed by the addition of 500 μ l 0.5% SDS solution, and DNA was isolated using the phenol/chloroform method. The relative DNA concentration was obtained using the PicoGreen assay (Molecular Probes, Eugene, OR) and quantified on a Storm-860 scanner.

Microarray Analysis. DU-145 cells were seeded in media supplemented with 10% FBS at a density of 3×10^6 cells per 150-cm² flask (approximately 20,000 cells per cm²) and allowed to attach overnight. The culture medium was replaced with media supplemented with 1% charcoal-stripped FBS for 24 hours, and then BMP-6 was added in media supplemented with 1% charcoal-stripped FBS. DNA-free total RNA was prepared using RNEasy mini-columns, using the on-column DNase digestion step. RNA samples were processed for analysis on Affymetrix U133 2.0 Plus arrays by the Gene Expression Resource core facility at University of California Davis Medical Center. These arrays cover the entire human genome and include more than 47,000 gene transcripts.

Public Databases. Twenty-seven normal and 52 cancerous prostate samples were processed on Affymetrix U95A microarrays by Singh *et al.* (24), and the data were made publicly available.¹ We analyzed these files with dChip 1.3² to determine the presence or absence of each gene listed and whether there was differential regulation in tumor *versus* normal biopsies. SAGE and elec-

tronic Northern expression data, when available, were taken from the Cancer Genome Anatomy Project databases.³

RESULTS

It is the combined effect of the many BMP signaling and regulatory proteins that ultimately determines cellular response to the presence of exogenous BMP. We decided on a simple experimental model consisting of DU-145 prostate cancer cells cultured in the presence or absence of exogenously added BMP-6. We used this model to determine which of the BMP signaling genes were (a) present in the cells and (b) affected by the addition of BMP-6. Microarray experiments were performed with RNA harvested from BMP-6-treated and control DU-145 cells. These experiments confirmed the presence of BMP-6 mRNA, as well as the presence of the type I and type II BMP receptors and Smads required for BMP signal transduction. The various components of the BMP signaling system expressed by DU-145 cells are presented in Table 1.

We next extended these findings beyond the culture model and asked which of these BMP signaling genes are present in prostate using publicly available cancer microarrays and serial analysis of gene expression (SAGE) data (Table 1). In the genes detected, there is considerable agreement between the prostate biopsies and the DU-145 cell line. However, comparison of extended BMP family and related signaling molecules showed that many genes that were present in the DU-145 cell line were not detectable in RNA from the prostate biopsies.

We were particularly interested in the genes that negatively regulate BMP signaling because elevated BMP-6 expression correlated with bone metastasis of prostate cancer. Noggin, Smad-6, Smad-7, and BAMBI are negative regulators of BMP signaling whose expression was increased on BMP-6 treatment of DU-145 cells. BAMBI was also up-regulated in cancerous prostates. The regulation of noggin by BMP-6 will be presented in greater detail below.

BMP-6 and Noggin Are Present in Prostate. To confirm the presence of BMP-6 and noggin in prostate at the protein level, immunoblots of prostate cancer homogenate were probed with antibodies against human BMP-6 and noggin. Both of these proteins were expressed in the homogenized prostate cancer biopsies (Fig. 1A, Lanes 1 and 2, respectively).

Immunohistochemistry was used to examine the distribution of noggin in normal and cancerous human prostates. All prostate biopsies reacted positively with the anti-noggin antibody, and Fig. 1B shows four representative images. Staining in normal prostate was observed primarily in epithelial cells, with little or no staining in the stromal cells. In high-grade cancers, noggin protein was localized to areas dense with carcinoma cells. In samples with mixed areas of connective tissue and carcinoma, noggin was localized to the carcinoma cells, and there was little or no staining in the connective tissue. Using this technique, we were not able to demonstrate a difference in noggin staining intensity among normal epithelium and low-grade and high-grade cancer cells.

BMP-6 Induces Noggin Expression. Recombinant human BMP-6 protein added to cultures of DU-145 and LNCaP prostate cancer cell lines for 48 hours resulted in a dose-dependent increase in noggin mRNA, as shown in Fig. 2A. This confirms the previous observations made in rat osteoblasts (25) that noggin can be induced by BMP-6 in certain cell types. The mRNA levels of two additional extracellular BMP antagonists, gremlin and cerberus, were examined in the same samples and showed no significant changes in the presence of BMP-6 protein (Fig. 2C). In LNCaP prostate cancer cell culture, the BMP-

¹ <http://www.broad.mit.edu/cgi-bin/cancer/datasets.cgi>.

² www.dchip.org.

³ <http://cgap.nci.nih.gov/>.

Table 1 *BMP signaling molecules present in prostate*

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
BMP antagonists				
extracellular				
231798_at	Noggin	Up		<2
209763_at	Chordin-like	Absent	Absent	
211248_s_at	Chordin variant 3	Absent		
221674_s_at	Chordin variant 1	Absent	Absent	
203592_s_at	Follistatin-like 3	Present no change	Present no change	
207345_at	Follistatin isoform FST317 precursor	Present no change		
204948_s_at	Follistatin isoform FST344 precursor	Absent		
223557_s_at	Transmembrane protein with EGF-like and two follistatin-like domains 2	Absent		
1564511_a_at	Weakly similar to follistatin-related protein precursor	Absent		
221378_at	CER1 cerberus 1 homolog	Absent		<2
220794_at	Related to DAC and cerberus, =prdc	Absent		<2
224811_at	Sclerostin	Present no change		<2
213456_at	Sclerostin-domain-containing-1 = ectodin	Absent	Absent	
218469_at	CKTSF1B1,DRM; IHG-2; GREMLIN	Absent	Absent	
201621_at	DAN; NBL1; NO3; MGC8972; D1S1733E	Present no change	Down	Down
241986_at	BMPER	Absent		
201894_s_at	Decorin	Present no change		Down
BMP/TGF-β family members				
205574_x_at	BMP-1, isoform 1	Present no change	Absent	Up
206725_x_at	BMP-1, isoform 2	Absent		
202701_at	BMP-1, isoform 3	Absent		
207595_s_at	BMP-1, isoform 4	Present no change		
205289_at	BMP-2	Present no change	Absent	<2
205290_s_at	BMP-2	Absent		
208244_at	BMP-3	Absent	Absent	
206159_at	BMP-3B = GDF10	Absent	Absent	<2
211518_s_at	BMP-4	Absent	Absent	Down
205430_at	BMP-5	Absent	Absent	<2
205431_s_at	BMP-5	Absent	Absent	<2
206176_at	BMP-6	Present no change	Absent	<2
241141_at	BMP-6	Absent	Absent	<2
209590_at	BMP-7	Present no change	Absent	<2
211260_at	BMP-7	Absent	Absent	<2
211259_s_at	BMP-7	Absent	Absent	<2
207866_at	BMP-8	Absent	Absent	<2
207865_s_at	BMP-8	Present no change	Absent	
221136_at	BMP-9 = GDF-2	Absent		<2
208292_at	BMP-10	Absent	Absent	<2
226234_at	BMP-11 = GDF-11	Present no change	Absent	<2
226232_at	BMP-11 = GDF-11	Absent		
216860_s_at	BMP-11 = GDF-11	Present no change		
216854_at	BMP-11 = GDF-11	Absent		
206614_at	BMP-14 = GDF-5 = CDMP-1	Absent	Absent	<2
1555665_at	BMP-14 = GDF-5	Absent		
221332_at	BMP-15	Absent		<2
216514_at	BMPY	Present no change		
216369_at	BMPY	Absent		
206397_x_at	GDF1	Absent	Absent	
229448_at	GDF1	Absent		
220053_at	GDF-3	Absent		<2
207145_at	MSTN myostatin = GDF-8	Absent	Absent	Up
221314_at	GDF-9	Present no change		<2
221576_at	PLAB = prostate differentiation factor = GDF-15	Absent	Up	
229868_s_at	PLAB = prostate differentiation factor = GDF-15	Absent		
221577_x_at	PLAB = prostate differentiation factor = GDF-15	Present no change		
BMP related molecules				
216047_x_at	SEZ6L, has BMP domains	Absent	Absent	<2
204926_at	Activin A = INHBA	Absent	Absent	<2
210141_s_at	INH A = Inhibin A	Present no change	Absent	<2
210511_s_at	INHBA	Absent	Absent	<2
205258_at	INHBB	Absent	Present no change	<3
207687_at	INHBC = Inhibin β C	Present no change	Absent	<2
207688_s_at	INHBC = Inhibin β C	Present no change	Absent	
210587_at	INHBE = Inhibin β E	Present no change		<2
209591_s_at	TGF-B1	Down		
203084_at	TGF-B1	Absent	Present no change	4-7
220406_at	TGF-B2	Absent	Absent	<4
220407_s_at	TGF-B2	Absent		
209908_s_at	TGF-B2	Absent		
209909_s_at	TGF-B2	Absent		
209747_at	TGF-B3	Absent	Absent	Down
206012_at	TGF-B4 = EBAF = LEFTY	Absent	Absent	<2
221373_x_at	PSPN = persephin	Absent		<2
210683_at	NRTN = neuriturin	Absent	Present no change	<2
230916_at	Nodal	Absent		

Table 1 *Continued*

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
206268_at	LEFTB	Absent	Absent	
221359_at	GDNF	Absent		<2
206516_at	AMH = MIS	Absent	Absent	<2
BMP receptors				
203935_at	ALK-2, Genecard ACVR1	Present no change	Present no change	2-3
204832_s_at	BMPRI1A	Present no change		
213578_at	BMPRI1A = ALK-3	Present no change	Present no change	<2
210523_at	BMPRI1B = ALK-6	Absent		
208223_s_at	BMPRI1B = ALK-6	Present no change	Absent	<2
209920_at	BMPRI2	Present no change	Absent	4-7
210214_s_at	BMPRI2	Absent		
205327_s_at	ACVR2 = activin A receptor type II	Present no change	Absent	<2
220028_at	ACVR2B	Present no change	Absent	
208218_s_at	ACVR1B = ALK-4	Absent	Absent	2-3
208222_at	ACVR1B = ALK-4	Absent		
208219_at	ACVR1B = ALK-4	Absent		
206943_at	TGFBR1 = ALK-5	35% Present no change	Absent	
1552519_s_at	ACVR1C = ALK-7	Absent		
205209_at	Alk-4 = activin A receptor type IB	Absent		
204731_at	TGFBR3 = betaglycan	Present no change	Down	<2
226625_at	TGFBR3 = betaglycan	Present no change		
208944_at	TGFBR2	Present no change	Down	Down
207334_s_at	TGFBR2	Present no change		
210838_s_at	ACVRL1	Absent	Absent	<2
BMP/TGF-β receptor interacting proteins/ downstream kinases				
208756_at	EIF3S2 = TGF β receptor interactin protein	Present no change	Unchanged	32-63
211536_x_at	MAP3K7 = TAK1	Present no change	Present no change	<2
211537_x_at	MAP3K7 = TAK1	Present no change		
206853_s_at	MAP3K7	Present no change		
206854_s_at	MAP3K7	Present no change		
218486_at	TIEG2	Present no change	Absent	2-3
216262_s_at	TGIF2	Present no change	Absent	
205210_at	TRAP-1	Absent	Absent	
228586_at	Endoglin = ENG	Absent		
201808_s_at	Endoglin = ENG	Absent	Present no change	<2
201809_s_at	Endoglin = ENG	Absent		
1563874_at	TRAG = WDR7	Absent	Present no change	2-3, up?
207644_at	FOXH1 = FAST1	Absent	Absent	
207530_s_at	CDKN2B	Absent	Absent	<2
221211_s_at	C21orf7	Present no change		<2
225557_at	AXUD1 = <i>Tgf-β</i> induced apoptosis protein	Present no change		4-7
202039_at	TIAF1 = <i>Tgf-β</i> induced anti-apoptotic factor 1	Present no change	Absent	4-7
201506_at	TGFBI = BIGH3 = <i>Tgf-β</i> induced 1	Up	Present no change	Up
209622_at	STK16 = TGF- β stimulated factor 1	Present no change	Absent	4-7
224938_at	SGK1	Present no change	Absent	<2
224939_at	SGK1	Present no change		
201739_at	SGK1	Present no change		
214044_at	RYR2	Absent		<2
207557_s_at	RYR2	Absent		
203901_at	TAB1 = MAP3K7IP1	Present no change	Absent	<2
BMP signal transduction				
210993_s_at	SMAD-1 = MADR1	Present no change	Present no change	<2
203075_at	SMAD-2	Present no change	Present no change	<2-6
203077_s_at	SMAD-2	Present no change		
203076_s_at	SMAD-2 = MADR2	Present no change		
205396_at	SMAD-3	Present no change	Present no change	<2-3
205398_s_at	SMAD-3	Present no change		
205397_x_at	SMAD-3 = JV15-2	Absent		
202527_s_at	SMAD-4	Present no change	Present no change	<2-9, down slightly
212347_x_at	SMAD-4	Present no change		
205187_at	SMAD-5	Present no change	Absent	<2
205188_s_at	SMAD-5	Present no change		
209887_at	SMAD-6	Absent		
209886_s_at	SMAD-6	Up		
213565_s_at	SMAD-6	Absent		
207069_s_at	SMAD-6	Up	Absent	<2-3
204790_at	SMAD-7	Up	Present, Down, $P = 0.036$	<2-3
206320_s_at	SMAD-9, =SMAD8A, SMAD8B, SMA9_Human	Absent	Absent	<2
220263_at	DAMS (Smad in the antisense direction)	Absent		<2
SMAD interacting proteins				
212668_at	SMURF-1	Absent	Absent	8-15
215458_s_at	SMURF-1	Present no change		
230820_at	SMURF-2	Up	Absent	<2-3, up slightly
205596_s_at	SMURF-2	U		
219409_at	SNIP = Smad nuclear interacting protein	Down		<2
203603_s_at	SIP-1 = ZFHX1B = Smad interacting protein	Down	Absent	2-7, down
204893_s_at	MADHIP = SARA	Present no change	Present no change	<2
208446_s_at	MADHIP = SARA variant 2	Present no change		

Table 1 *Continued*

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
203313_s_at	TGIF = TGIF_HUMAN	Present no change	Present no change	<2-7 up slightly
218724_s_at	TGIF2 = TGIF2_HUMAN	Present no change		<2
202160_at	CREBBP	Present no change	Present no change	8-15 to <2 down
211808_s_at	CREBBP	Absent		
218604_at	MAN1 = human homolog of SANE??	Present no change		
225426_at	MAN1 = human homolog of SANE??	Present no change		
225429_at	MAN1 = human homolog of SANE??	Present no change		
224886_at	CHIP = STUB1	Present no change		26-31
227625_s_at	CHIP = STUB1	Present no change		
233049_x_at	CHIP = STUB1	Present no change		
217934_x_at	CHIP = STUB1	Present no change		
202014_at	GADD34	Present no change	Present no change	<2
37028_at	GADD34	Present no change		
235629_at	DLX1 Distal-less homeobox 1	Absent		
1560100_at	DLX1 Distal-less homeobox 1	Absent		
211698_at	EID-1, =CRI1 genecard	Present no change	Present no change	32-63 to 16-32 down
1569080_at	Arkadia	Absent		<2
202811_at	AMSH	Present no change	Present no change	2-7, down slightly
231891_at	AMSH-2	Absent		<2
227607_at	AMSH-2	Present no change		
227606_s_at	AMSH-2	Present no change		
203304_at	NMA = Bambi	Up	Up	Up <2 to 8-15
218225_at	Ecsit = SITPEC	Present no change		Up <2 to 4-7
225498_at	TOB1	Present no change	Present no change	Up <2 to 16-31
228834_at	TOB1	Present no change		
204270_at	SKI	Present no change		
229265_at	SKI	Present no change	Absent	<2
213755_s_at	SKI	Absent		
215889_at	SNO = SKIL	Absent	Absent	<2
232379_at	SNO = SKIL	Absent		
200047_s_at	YY1	Present no change	Present no change	4-7
213494_s_at	YY1	Present no change		
201902_s_at	YY1	Absent		
205471_s_at	Dach1	Absent	Absent	Down slightly
205472_s_at	Dach1	Absent		

Abbreviation: TGF, transforming growth factor.

6-induced increase of noggin mRNA was not dependent on the presence of androgens (data not shown). The induction of noggin mRNA was observed in both the LNCaP (androgen receptor-positive) and DU-145 (androgen receptor-negative) cell lines, providing further evidence that this is an androgen-independent event (Fig. 2A).

The increased noggin production on BMP-6 treatment was also confirmed at the protein level. The addition of 100 ng/ml BMP-6 to DU-145 cell culture for 48 hours resulted in the expression of detectable amounts of noggin in the cells, whereas noggin protein was not detectable in cells cultured in the absence of BMP-6 (Fig. 1A, *Lanes 3 and 4*). Noggin protein was detected in the cells and their associated extracellular matrix, but not in the cell culture supernatant.

Noggin Inhibits BMP-6 Activity. Having shown that BMP-6 reproducibly up-regulates noggin at the mRNA and protein levels, we designed experiments to determine whether noggin could act as an antagonist to BMP-6. Previously, it had been shown that noggin inhibits the activity of BMP-2, -4, and -7, but the effect of noggin on BMP-6 had not been demonstrated. Increasing amounts of noggin were added to cells cultured in the presence of 100 ng/ml BMP-6 for 48 hours, and quantitative RT-PCR was used to measure the up-regulation of noggin mRNA. Noggin protein at higher doses (1-3 μ g/ml) reduced BMP-6 activity by 50% to 100% in both LNCaP and DU-145 cells. As a control, in the absence of BMP-6 protein, noggin protein had no discernible effect (Fig. 2C).

BMP-6 Activates Negative Feedback Mechanisms. Because elevated BMP-6 expression in prostate correlates with poor patient prognosis, we were interested in studying the expression of negative regulators of BMP activity. As described above, the profound effects of BMPs on cells and tissues have given rise to many levels of mechanisms to regulate these effects, including negative feedback loops that are activated by BMP-6. Table 2 is a summary of microarray

data showing that BMP-6 activates several negative feedback mechanisms when added to DU-145 cell culture. Inhibitory Smad-6 and Smad-7 are up-regulated and function to block BMP signal transduction by interfering with the activation of receptor Smads (Smads 1, 5, and 8; ref. 26). Smurf-2 is up-regulated by BMP-6 and functions by increasing the degradation rate of receptor Smads, thus attenuating BMP signaling (27). BAMBI was up-regulated by BMP-6 and acts as a kinase-deficient type I receptor unable to activate Smads (18). These proteins are examples of negative feedback regulation that reduce or weaken BMP signaling.

However, a few regulatory proteins were affected by BMP-6 that further activate the BMP signaling pathway. SIP-1, a Smad-binding protein and transcriptional repressor of E-cadherin, was down-regulated by BMP-6. E-cadherin has been proposed as a tumor suppressor gene, and its expression is frequently decreased in prostate cancers. SNIP-1 was also down-regulated by BMP-6 treatment and has been shown to suppress the transforming growth factor β signaling pathway (28).

Again, we wanted to extend these findings beyond the DU-145 cell culture model and ask which of these BMP signaling genes are involved in prostate cancer. To a large extent, the DU-145 cancer cell model system agrees with both the SAGE and cancer *versus* normal prostate microarray data, although the data from the DU-145 cell arrays (Affymetrix 133 2.0 Plus chips) are much more complete than the chips used by Singh *et al.* (24) (Affymetrix U95A chips) and also more sensitive than the SAGE technology. The cancer *versus* normal prostate comparisons reveal information about BMP regulation beyond what is possible with DU-145 cell culture microarrays. For example, DAN, an extracellular BMP antagonist protein, was down-regulated in prostate cancers compared with normal prostate both on microarray and SAGE data sets and was present but not affected by

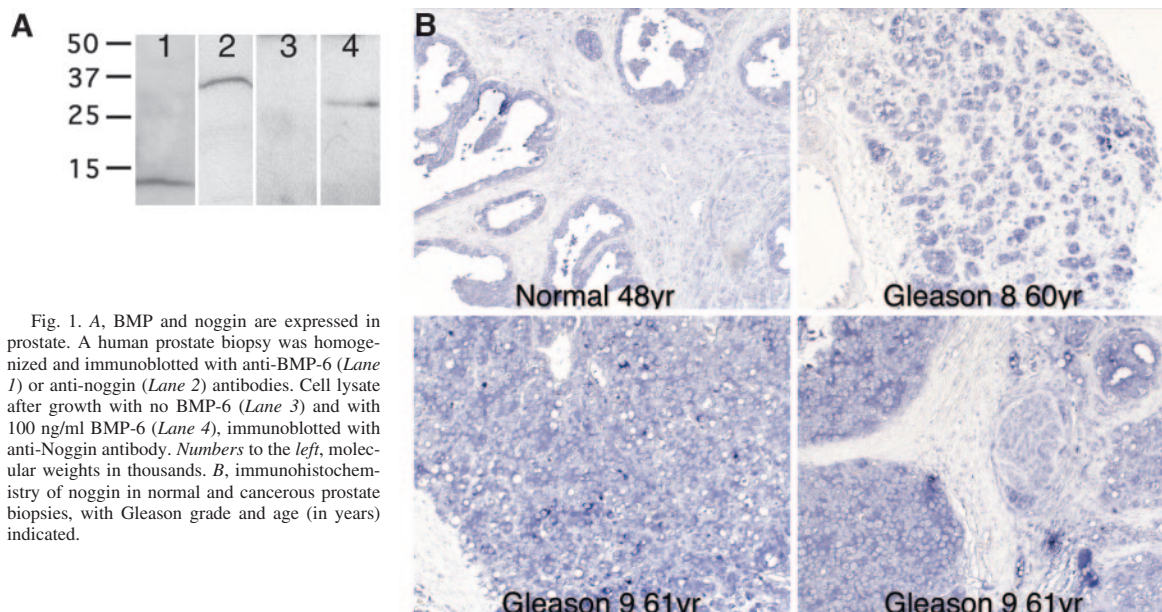


Fig. 1. A, BMP and noggin are expressed in prostate. A human prostate biopsy was homogenized and immunoblotted with anti-BMP-6 (Lane 1) or anti-noggin (Lane 2) antibodies. Cell lysate after growth with no BMP-6 (Lane 3) and with 100 ng/ml BMP-6 (Lane 4), immunoblotted with anti-Noggin antibody. Numbers to the left, molecular weights in thousands. B, immunohistochemistry of noggin in normal and cancerous prostate biopsies, with Gleason grade and age (in years) indicated.

BMP-6 in DU-145 cell culture. These comparisons are presented in Table 2.

In summary, we have demonstrated that BMP-6 induces noggin expression in prostate cancer cell culture at the mRNA and protein levels. Recombinant noggin protein inhibits this activity of BMP-6 in a prostate cancer cell culture system. In addition to noggin, BMP-6 activates several other negative (and some positive) feedback mechanisms that may attenuate BMP signal transduction. The cancer cell model data are in agreement with data derived from comparing normal and cancerous prostate.

BMP-6 Induces Smad Phosphorylation. Many studies have documented the function of BMPs in the context of bone development and embryogenesis. However, the function of BMP-6 in the adult prostate remains unknown. To extend the documented correlation

between elevated BMP-6 expression and tumor metastases, we wanted to (a) examine the BMP-6 signal transduction pathway, (b) identify some of the downstream targets, and (c) characterize the effects of BMP-6 on the biology of prostate cancer cells.

The first few steps in BMP signal transduction involve binding of BMP to its receptors, which are activated and subsequently phosphorylate Smads. These pathways are often disrupted in cancer cells (29). To test whether Smads are phosphorylated on BMP-6 treatment of DU-145 and LNCaP cells, immunoblots were performed on cell lysates and probed with antibodies that specifically recognize only phosphorylated Smad-1, -5, and -8. BMP-6 treatment induced Smad phosphorylation within 30 minutes, and elevated levels of phosphorylated Smads continued to be detected to at least 96 hours of BMP-6 treatment, as shown in Fig. 3A. All samples were compared with

Fig. 2. BMP-6 induces noggin, a BMP antagonist, and reduces proliferation. A, BMP-6 was added to DU-145 (□) and LNCaP (■) prostate cancer cell cultures for 48 hours, in the presence of 1% charcoal-stripped serum and 100 ng/ml DHT. Fold increase of noggin mRNA was measured by quantitative RT-PCR. B, increasing amounts of recombinant noggin were added to DU-145 cells for 48 hours in the presence of 100 ng/ml BMP-6 and 100 ng/ml DHT. BMP-6 activity was measured as increased noggin mRNA by quantitative RT-PCR. C, recombinant BMP-6 or noggin was added to cultures of DU-145 cells for 48 hours, and fold increase of noggin, gremlin, and cerberus mRNA was measured by quantitative RT-PCR. D, cells (2×10^5) were cultured for 7 days in a base medium of RPMI 1640 with 10% charcoal-stripped FBS and with the addition of either 100 ng/ml BMP-6, 5 nmol/L DHT, or both BMP-6 and DHT. DNA content measurement was done using PicoGreen.

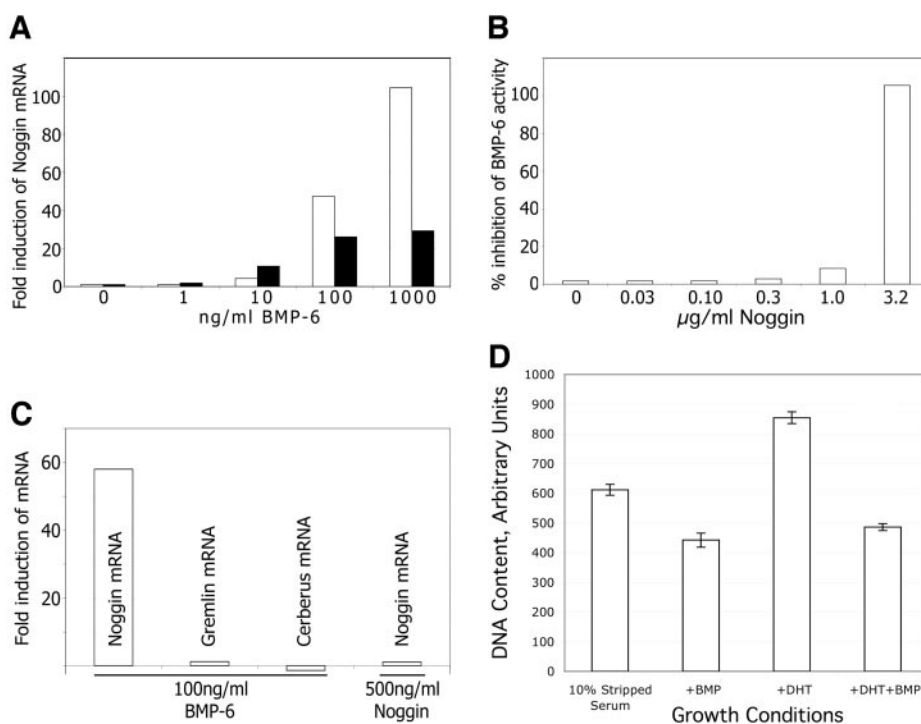


Table 2. BMP signaling molecules affected by BMP-6 in prostate cancer

Affymetrix probe ID	BMP related protein	Significant change with BMP treatment DU-145 cell line	U-95A chip publicly available prostate data (27 normal vs. 52 cancer)	SAGE in prostate (tags per 200,000)
231798_at	Noggin	Up	N/D	<2
201621_at	DAN = NBL	Present no change	Down	Down
221576_at	PLAB = prostate differentiation factor = GDF-15	Absent	Up	N/D
205258_at	INHBB	Absent	Present no change	<3
209591_s_at	TGF-B1	Down	Present no change	4-7
209747_at	TGF-B3	Absent	Down	Down
210683_at	NRTN = neurturin	Absent	Present no change	<2
204731_at	TGFBR3 = betaglycan	Present no change	Down	<2
208944_at	TGFBR2	Present no change	Down	Down
203304_at	NMA = Bambi	Up	Up	Up
228586_at	Endoglin = ENG	Absent	Present no change	<2
1563874_at	TRAG = WDR7	Absent	Present no change	2-3
201506_at	TGFBI = BIGH3 = TGF- β induced 1	Up	Present no change	Up
207069_s_at	SMAD-6	Up	Absent	<2-3
204790_at	SMAD-7	Up	Down? $P = 0.036$	<2-3
230820_at	SMURF-2	Up	Absent	<2-3
219409_at	SNIP = smad nuclear interacting protein	Down	N/D	<2
203603_s_at	SIP-1 = ZFH1B = Smad interacting protein	Down	Absent	Down

Abbreviation: TGF, transforming growth factor; N/D, no data.

untreated cells cultured for the same length of time under otherwise identical conditions. The amount of unphosphorylated Smad-1 protein remained essentially identical between BMP-6-treated and control cells. Thus, the Smad phosphorylation pathway is intact in these cells and used for BMP signal transduction in response to exogenous BMP-6.

BMP-6 Inhibits Cell Proliferation. Although microarray analysis has provided insight into how cells respond to BMP-6 at the molecular level, the response at the cellular level is still unknown. One component of malignant prostate cancer is uncontrolled growth, and another component is androgen independence. Experiments were performed to determine whether BMP-6 affects the proliferation rate of androgen-dependent LNCaP cells. BMP-6 inhibited LNCaP cell proliferation after 1 week of cell culture in media supplemented with 10% charcoal-stripped (androgen-free) serum, as shown in Fig. 2D. Two lines of evidence indicated that this growth-inhibitory effect is not dependent on the presence of androgens. First, the effect was seen with or without addition of DHT to the medium. Second, the growth-inhibitory effect of BMP-6 was also seen in androgen-independent DU-145 cells (data not shown).

BMP-6 Induces p21/CIP Expression. The next set of experiments were done to gain insight into how BMP-6 may control cell proliferation. Microarray data showed that BMP-6 treatment caused a strong up-regulation of p21/CIP mRNA within 6 hours. P21/CIP is a cell cycle control protein shown to mediate BMP-dependent growth reduction in other cell types (30) and to be induced by BMP-2 and -4 in prostate (31).

Treatment with BMP-6 caused an increase of cell cycle control protein p21/CIP, which was detected at the mRNA level in DU-145 cells and confirmed at the protein level in both DU-145 and LNCaP cells. LNCaP cells expressed a higher basal level of p21 protein than DU-145 cells, and in both cell lines, there was a time-dependent increase of p21/CIP protein with BMP-6 treatment, as shown in Fig. 3B. P21/CIP protein levels were detectably increased after 90 minutes of BMP-6 treatment and remained elevated at least until 96 hours after BMP-6 treatment compared with cells cultured for the same length of time without BMP-6 in otherwise identical media. Thus we show evidence that the decreased proliferation of BMP-6-treated cells may be due in part to increased levels of p21/CIP protein.

A number of additional cell cycle control proteins were regulated at the mRNA level by BMP-6 treatment in DU-145 cells, as shown in Table 3. Most of these changes are consistent with reduced proliferation as a result of BMP-6 treatment. Cyclin-dependent kinase inhib-

itors p21/CIP, p18, and p19 were up-regulated, and cyclin-dependent kinases were down-regulated.

BMP-6 Induces Expression of Id Proteins. Id proteins are inhibitors of basic helix-loop-helix transcription factors that are under the control of BMP pathways in other cell types (32) and have been identified as markers of prostate cancer progression (33-35). We found that BMP-6 dramatically increased the expression of Id-1, Id-2, and Id-3 in the microarray, as shown in Table 3.

DISCUSSION

The misregulation of BMP and BMP signaling pathways in prostate cancer is becoming increasingly apparent. BMP-6 was studied in this report due to the demonstrated correlation of increased prostatic BMP-6 expression and cancer severity. However, recent publications also show reduced expression of BMP-2 and Smads 4 and 8 in prostate cancers (29), and one report shows that BMP-5 is up-regulated in benign prostatic hyperplasia (36).

In this study, a comprehensive list of genes involved in generating and regulating the activities of BMPs was compiled. We tested the expression of these genes in DU-145 cells and analyzed how their

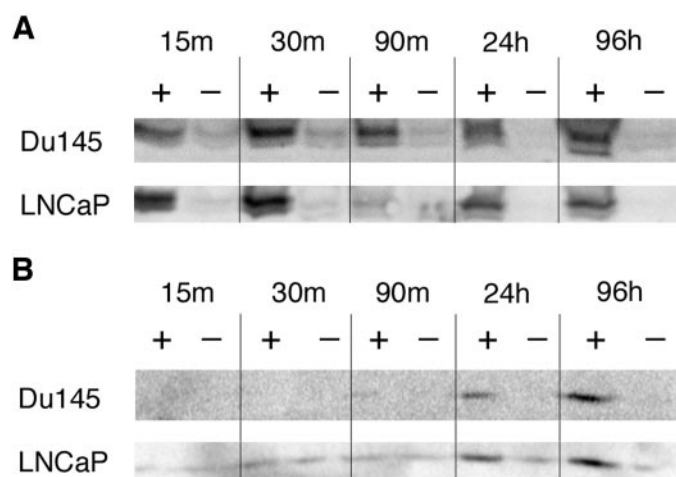


Fig. 3. BMP-6 increases Smad phosphorylation and p21 expression. Immunoblot of DU-145 and LNCaP cell lysates after treatment with 100 ng/ml BMP-6 for 15, 30, and 90 minutes; 24 hours; and 96 hours. Lysates from treated and untreated cells are at equivalent total protein in each sample. Primary antibody was anti-phospho-Smad in A; the membrane was stripped and reprobbed with anti-p21 in B.

Table 3 Cell cycle and Id proteins regulated by BMP-6

Probe set	Gene	Accession no.	t Test
Up-regulated by BMP-6 treatment in DU-145 cells			
202284_s_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	0.0000
211792_s_at	Cyclin-dependent kinase inhibitor 2C (p18)	U17074	0.0468
210240_s_at	Cyclin-dependent kinase inhibitor 2D (p19)	U20498	0.0032
208501_at	Growth factor independent 1B (potential regulator of CDKN1A, translocated in CML)	NM_004188	0.0097
223394_at	CDK4-binding protein p34SEI1	BC002670	0.0138
210721_s_at	p21(CDKN1A)-activated kinase 7	AB040812	0.0489
208937_s_at	Inhibitor of DNA binding 1	D13889	0.0000
201565_s_at	Inhibitor of DNA binding 2	NM_002166	0.0000
207826_s_at	Inhibitor of DNA binding 3	NM_002167	0.0000
Down-regulated by BMP-6 treatment in DU-145 cells			
203154_s_at	p21(CDKN1A)-activated kinase 4	NM_005884	0.026
239470_at	p21(CDKN1A)-activated kinase 6	AI668644	0.028
1555310_a_at	p21(CDKN1A)-activated kinase 6	BC035596	0.037
209112_at	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	BC001971	0.009
219534_x_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	NM_000076	0.027
216894_x_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	D64137	0.022
213348_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	N33167	0.035
203468_at	Cyclin-dependent kinase (CDC2-like) 10	NM_003674	0.015

NOTE. Accession numbers refer to GenBank and RefSeq entries (<http://www.ncbi.nlm.nih.gov/>).

expression is regulated by BMP-6. To relate these cell culture observations to whole prostates, we searched publicly available data from National Center for Biotechnology Information and Massachusetts Institute of Technology, and we found that the cell culture model in many ways mimics what occurs in the prostate. Of the top 50 genes with high expression in tumor and the top 50 genes with high expression in normal prostate identified by Singh *et al.* (24), 13 are either up- or down-regulated by BMP-6 in DU-145 cell culture. Comparisons between cell culture and whole prostate microarray data, although useful to extend the validity of cell culture observations, must also be viewed critically because of the many cell types contributing to RNA from whole prostates.

Special attention was paid to antagonists of BMP activity, especially noggin. Prostate cancer cells were shown to express BMP-6 and noggin at the mRNA and protein levels. Noggin was localized predominantly in normal epithelial and carcinoma cells in prostate tissue biopsies. Surprisingly, it was not detected in cell culture unless the cells were stimulated with BMP-6. Together with the observation that noggin was not detectable in the cell culture supernatant, this may indicate that there is very limited diffusion of the protein away from the cells in which it is produced. It will also be interesting to study whether the BMP-6 produced by prostate carcinoma can diffuse past the noggin bound to the heparan sulfate proteoglycan in the extracellular matrix (37, 38). Because little is known about the relative binding affinities of noggin to the various BMP family members, it remains to be determined whether noggin specifically inhibits BMP-6 availability in prostate epithelium or whether it functions as a general antagonist of all BMPs.

BMP-6 inhibited the proliferation of DU-145 and LNCaP cells. We demonstrated that BMP-6 causes changes in the expression of cell cycle control proteins. Specifically, BMP-6 caused strong up-regulation of p21/CIP, which may explain in part the reduced proliferation seen in BMP-6-treated cells. This effect was not androgen dependent because BMP-6 reduced proliferation and increased p21/CIP expression in both DU-145 and LNCaP cells, and in the presence and absence of DHT. DU-145 cells, in addition to being androgen independent, have a missense mutation in the p53 gene (39). This indicates that BMP-6 induces p21/CIP protein and mRNA expression in a p53-independent manner.

The antiproliferative response of prostate cancer cells to BMP-6 seems counterintuitive, given the established correlation between aggressive cancer and elevated BMP-6 expression. However, the BMP-

6-induced increase in Id protein expression may explain in part the increased resistance to apoptosis (40) and the correlation between BMP overexpression and cancer progression. In prostate cancers metastatic to bone, the effect of the BMP-6 in the surrounding bone environment may be related to the osteosclerotic nature of the metastatic lesions.

The findings that BMP-6 up-regulated noggin expression at mRNA and protein levels and, conversely, that noggin inhibited BMP-6 function have potential therapeutic implications for noggin in ameliorating prostate bone metastases and attendant pain and morbidity. The challenge for the future is to dissect BMP-6 signaling in prostate cancer, both in the prostate cancer *in situ* and in the bone microenvironment in the immediate vicinity of the metastases.

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REFERENCES

- Huggins C, Hodges CV. Studies on prostate cancer. I. The effect of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1941;1:293-7.
- Mundy GR, Yoneda T. Facilitation and suppression of bone metastasis. *Clin Orthop* 1995;312:34-44.
- Orr FW, Sanchez-Sweetman OH, Kostenuik P, Singh G. Tumor-bone interactions in skeletal metastases. *Clin Orthop* 1995;312:19-33.
- Galasko CSB. Diagnosis of skeletal metastases and assessment of response to treatment. *Clin Orthop* 1995;312:64-75.
- Nelson JB, Hedican SP, George DJ, et al. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* 1995;1:944-9.
- Reddi AH, Huggins C. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. *Proc Natl Acad Sci USA* 1972;69:1601-5.
- Urist MR. Bone: formation by autoinduction. *Science (Wash DC)* 1965;150:893-9.
- Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotechnol* 1998;16:247-52.
- Thomas R, Anderson WA, Raman V, Reddi AH. Androgen-dependent gene expression of bone morphogenetic protein 7 in mouse prostate. *Prostate* 1998;37:236-45.
- Masuda H, Fukabori Y, Nakano K, Shimizu N, Yamanaka H. Expression of bone morphogenetic protein-7 (bmp-7) in human prostate. *Prostate* 2004;59:101-6.
- Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM. The xenopus dorsalizing factor gremlin identifies a novel family of secreted proteins that antagonize bmp activities. *Mol Cell* 1998;1:673-83.
- Larrain J, Bachiller D, Lu B, et al. Bmp-binding modules in chordin: a model for signaling regulation in the extracellular space. *Development (Camb)* 2000;127:821-30.
- Merino R, Rodriguez-Leon J, Macias D, et al. The bmp antagonist gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development (Camb)* 1999;126:5515-22.

14. Pearce JJ, Penny G, Rossant J. A mouse cerberus/dan-related gene family. *Dev Biol* 1999;209:98–110.
15. Topol L, Bardot B, Zhang Q, et al. Biosynthesis, post-translational modification, and functional characterization of drm/gremlin. *J Biol Chem* 2000;275:8785–93.
16. McMahon R, Murphy M, Clarkson M, et al. Igh-2, a mesangial cell gene induced by high glucose, is human gremlin. Regulation by extracellular glucose concentration, cyclic mechanical strain, and transforming growth factor beta1. *J Biol Chem* 2000;275:9901–4.
17. Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev* 2003;24:218–35.
18. Sekiya T, Adachi S, Kohu K, et al. Identification of bmp and activin membrane-bound inhibitor (bambi), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. *J Biol Chem* 2004;279:6840–6.
19. Bentley H, Hamdy FC, Hart KA, et al. Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. *Br J Cancer* 1992;66:1159–63.
20. Hamdy FC, Autzen P, Robinson MC, et al. Immunolocalization and mRNA expression of bone morphogenetic protein-6 in human benign and malignant prostatic tissue. *Cancer Res* 1997;57:4427–31.
21. Autzen P, Robson CN, Bjartell A, et al. Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. *Br J Cancer* 1998;78:1219–23.
22. De Pinieux G, Flam T, Zerbib M, et al. Bone sialoprotein, bone morphogenetic protein 6 and thymidine phosphorylase expression in localized human prostatic adenocarcinoma as predictors of clinical outcome: a clinicopathological and immunohistochemical study of 43 cases. *J Urol* 2001;166:1924–30.
23. Thomas BG, Hamdy FC. Bone morphogenetic protein-6: potential mediator of osteoblastic metastases in prostate cancer. *Prostate Cancer Prostatic Dis* 2000;3:283–5.
24. Singh D, Febbo P, Ross K, et al. Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* 2002;1:203–9.
25. Gazzerro E, Gangji V, Canalis E. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *J Clin Invest* 1998;102:2106–14.
26. Miyazono K, Suzuki H, Imamura T. Regulation of tgf-beta signaling and its roles in progression of tumors. *Cancer Sci* 2003;94:230–4.
27. Arora K, Warrior R. A new smurf in the village. *Dev Cell* 2001;1:441–2.
28. Kim RH, Wang D, Tsang M, et al. A novel smad nuclear interacting protein, snip1, suppresses p300-dependent tgf-beta signal transduction. *Genes Dev* 2000;14:1605–16.
29. Horvath LG, Henshall SM, Kench JG, et al. Loss of bmp2, smad8, and smad4 expression in prostate cancer progression. *Prostate* 2004;59:234–42.
30. Wong GA, Tang V, El-Sabeawy F, Weiss RH. Bmp-2 inhibits proliferation of human aortic smooth muscle cells via p21cip1/waf1. *Am J Physiol Endocrinol Metab* 2003;284:E972–9.
31. Brubaker KD, Corey E, Brown LG, Vessella RL. Bone morphogenetic protein signaling in prostate cancer cell lines. *J Cell Biochem* 2004;91:151–60.
32. Kowanzet M, Valcourt U, Bergstrom R, Heldin C, Moustakas A. Id2 and id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. *Mol Cell Biol* 2004;24:4241–54.
33. Coppe JP, Itahana Y, Moore DH, Bennington JL, Desprez P. Id-1 and Id-2 proteins as molecular markers for human prostate cancer progression. *Clin Cancer Res* 2004;10:2044–51.
34. Ouyang XS, Wang X, Lee DT, Tsao SW, Wong Y. Overexpression of Id-1 in prostate cancer. *J Urol* 2002;167:2598–602.
35. Chetcuty A, Margan S, Mann S, et al. Identification of differentially expressed genes in organ-confined prostate cancer by gene expression array. *Prostate* 2001;47:132–40.
36. Luo J, Dunn T, Ewing C, et al. Gene expression signature of benign prostatic hyperplasia revealed by cDNA microarray analysis. *Prostate* 2002;51:189–200.
37. Paine-Saunders S, Viviano BL, Economides AN, Saunders S. Heparan sulfate proteoglycans retain noggin at the cell surface: a potential mechanism for shaping bone morphogenetic protein gradients. *J Biol Chem* 2002;277:2089–96.
38. Reddi AH. Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and dan. *Arthritis Res* 2001;3:1–5.
39. Isaacs W, Carter B, Ewing C. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 1991;51:4716–20.
40. Ling MT, Wang X, Ouyang XS, et al. Id-1 expression promotes cell survival through activation of nf-kappab signaling pathway in prostate cancer cells. *Oncogene* 2003;22:4498–508.