Research Article

Bone Morphogenic Proteins Are Overexpressed in Malignant Melanoma and Promote Cell Invasion and Migration

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Abstract

Malignant melanoma cells are known to have altered expression of growth factors compared with normal human melanocytes. These changes probably favor tumor growth and progression and influence the tumor environment. The induction of transforming growth factor β1 (TGF-β1), TGF-β2, and TGF-β3 expression in malignant melanoma has been reported before, whereas the expression of related bone morphogenic protein (BMP) molecules has not been analyzed in melanomas until now. Here, we show that BMP4 and BMP7 are up-regulated in nine melanoma cell lines, whereas BMP2 is overexpressed in only two of the analyzed cell lines. Immunohistochemistry of primary and metastatic melanoma also shows increased BMP4 and BMP7 expression compared with nevi. Promoter studies reveal that expression is controlled at the transcriptional level. The transcription factor Ets-1 was identified as a positive regulator for BMP4 expression. In order to determine the functional relevance of BMP expression in malignant melanoma, chordin-expressing cell clones and antisense BMP4 cell clones were generated. The clones in which BMP4 activity and expression are reduced show no changes in proliferation or in attachment-independent growth when compared with controls. However, a strong reduction of migratory and invasive properties was observed in these cells, suggesting that BMP4 promotes melanoma cell invasion and migration and therefore has an important role in the progression of malignant melanoma. (Cancer Res 2005; 65(2): 448-56)

Introduction

Bone morphogenic protein (BMP) is a member of the transforming growth factor β (TGF-β) superfamily whose expression has been linked to several aspects of embryonic development, including the establishment of the basic embryonic body plan, morphogenesis of organs, regulation of cell proliferation, differentiation, apoptosis, and chemotaxis (1). Like other members of the TGF-β superfamily, BMPs elicit their cellular effects via specific type I and II serine/threonine receptors. The activated BMP type I receptor phosphorylates specific receptor-regulated Smad proteins, which can then assemble into heteromeric complexes with the common partner Smad4 (2). Heteromeric Smad complexes efficiently translocate into the nucleus, where they regulate the transcription of target genes. In clear contrast to normal cells, carcinoma cells derived from several organs (e.g., breast, colon) express TGF-β but are resistant to its growth inhibitory effects (3, 4). Therefore, it has been proposed that TGF-β may act as a tumor promoter in advanced stages of tumor progression. In malignant melanoma, expression of the three TGF-β isoforms positively correlates to tumor progression both *in vitro* and *in vivo* (5–8).

Besides TGF-β, BMP mRNAs have been shown to be expressed in a variety of human carcinoma cell lines (9–12). However, their levels of expression in malignant melanoma have not been analyzed. Furthermore, despite their significant morphogenetic activities during embryogenesis, a biological role for BMP2, BMP4, and BMP7 in human melanoma has not been evaluated. The purpose of this study was to determine the expression levels of BMP2, BMP4, and BMP7 in human malignant melanoma and to investigate whether BMPs have a biological role in tumor development.

Materials and Methods

Cell Culture. The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK Mel 28, SK Mel 3, and HTZ19d and B16 were described previously (13). The cell lines Mel Ei, Mel Wei, Mel Ho, and Mel Juso were derived from a primary cutaneous melanoma; Mel Im, Mel Ju, SK Mel 28, SK Mel 3, and HTZ19d were derived from metastases of malignant melanomas; and B16 was derived from primary melanoma in mice. Cells were maintained in DMEM supplemented with penicillin (400 units/mL), streptomycine (50 μg/mL), L-glutamine (300 μg/mL), and 10% FCS (Sigma, Deisenhofen, Germany) and split at a 1:5 ratio every 3 days. Urotsa cells (14) were maintained in RPMI 1640, penicillin (400 units/mL), streptomycine (50 μg/mL), L-glutamine (300 μg/mL), and 5% FCS. Cell proliferation was determined using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay (Roche, Mannheim, Germany).

Stable Transfection of Melanoma Cells with Chordin and asBMP4. A panel of Mel Im cell clones expressing chordin was established by stable transfection with sense expression plasmids (generous gift from Theresa E. Gratsch and K. Sue O'Shea). For the generation of asBMP4 cells, a 670-bp fragment of the BMP4 coding region was cloned in antisense orientation into the pCMX-PL1 vector and Mel Im cells were stably transfected. Plasmids were cotransfected with pcDNA3 (Invitrogen, NV Leek, The Netherlands), containing the selectable marker for neomycin resistance. Controls received pcDNA3 alone. Transfections were done using Lipofectamine Plus (Invitrogen, Grouingen, The Netherlands). One day after transfection, cells were placed into selection medium containing 50 μg/mL G418 (Sigma). After 25 days of selection, individual G418-resistant colonies were subcloned.

Immunohistochemistry. Paraffin-embedded preparations of normal skin, nevi, primary, and metastases of malignant melanomas were screened for BMP4 and BMP7 protein expression by the avidin-biotin complex method (DAKO-LSAB2-Kit, DAKO, Hamburg, Germany). The tissues were deparaffinated, rehydrated and incubated with primary polyclonal BMP4 (1:50, Novocastra Laboratories Ltd., Newcastle upon Tyne, England) or BMP7-antibody (1:50, Santa Cruz, Santa Cruz, CA) over night at 4°C. The secondary antibody supplied with the kit was incubated

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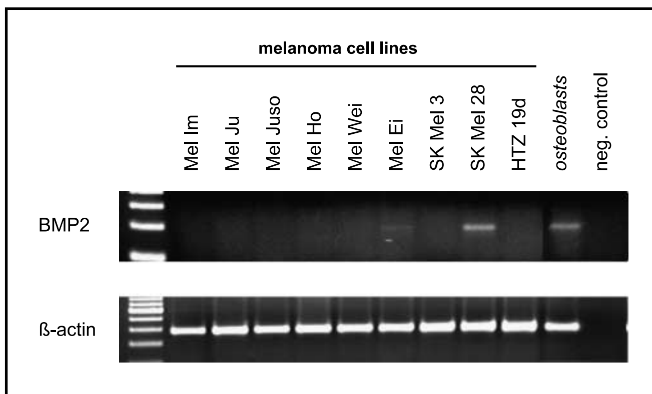

Figure 1. Expression of BMP2 in malignant melanoma. The expression of BMP2 mRNA was analyzed by RT-PCR in the melanoma cell lines Mel Im, Mel Ju, Mel Juso, Mel Ho, Mel Wei, Mel Ei, SK Mel 3, SK Mel 28, and HTZ19d. Mel Ei and SK Mel 28 express BMP2 at detectable levels. Osteoblast mRNA was used as a positive control and β-actin RT-PCR was done to assess the quality of the cDNA and to ensure equal amounts of cDNA used in the PCR reaction.

for 30 minutes at room temperature. Antibody binding was visualized using AEC-solution (for LSAB2-Kit). Finally, the tissues were counter-stained by hemalaun.

RNA Isolation and Reverse Transcription. Total cellular RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany) and cDNAs were generated by reverse transcriptase reaction done in 20 μL reaction volume containing 2 μg of total cellular RNA, 4 μL of 5 × first strand buffer (Invitrogen), 2 μL of 0.1 mol/L DTT, 1 μL of dN_6-primer (10 mmol/L), 1 μL of deoxynucleotide triphosphates (10 mmol/L), and DEPC water. The reaction mixture was incubated for 10 minutes at 70°C, 200 units of Superscript II reverse transcriptase (Invitrogen) were added and RNAs were transcribed for 1 hour at 37°C. Reverse transcriptase was inactivated at 70°C for 10 minutes and the RNA was degraded by digestion with 1 μL RNase A (10 mg/mL) at 37°C for 30 minutes.

Expression Analysis. Reverse transcription-PCR (RT-PCR) analysis of BMP2, BMP4, and BMP7 was done using specific primers for BMP2: 5′-GACACTGAGACGCTGTTCC-3′ and 5′-CCATGGTCGACCTT-TAGG-3′ (201-bp fragment), BMP4: 5′-GCCGGAGGGCCAAGCGTAGCCC-TAAG-3′ and 5′-CTGCCTGATCTCAGCGGCACCCACATC-3′ (351-bp fragment), BMP7: 5′-GCCAGCCTGCAAGATAGCCATTTCC-3′ and 5′-GAG-CACCTGATAAACGCTGATCCGG-3′ (215-bp fragment), chordin: 5′-CGCAG-CAATCTAGATCCACA-3′ and 5′-GCACCCTGCCAAATGAGA-3′ (446-bp fragment). The PCR reaction was done in a 50-μL reaction volume containing 5 μL 10 × Taq-buffer, 1 μL of cDNA, 1 μL of each primer (20 mmol/L), 0.5 μL of deoxynucleotide triphosphates (10 mmol/L), 0.5 units of Taq polymerase, and 41 μL of water. The amplification reactions were done by 33 cycles of 1 minute at 94°C, 1 minute at 62°C, and a final extension step at 72°C for 1.5 minutes. The PCR products were resolved on 1.5% agarose gels.

Analysis of BMP4 Expression by Quantitative PCR. Quantitative real time-PCR was done on a LightCycler (Roche). cDNA template (2 μL), 2 μL 25 mmol/L $MgCl_2$, 0.5 μL (20 mmol/L) of forward and reverse primers, and 2 μL of SybrGreen LightCycler Mix in a total of 20 μL were applied to the following PCR program: 30 seconds 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 seconds, 3 seconds 68°C, 5 seconds 72°C, 81°C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and checking the PCR products on 1.8% agarose gels.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation assay was done using a ChIP Assay Kit (Upstate, Lake Placid, NY). Cells (1 × 10^6) were seeded into a T25 culture flask, cultured for 24 hours, fixed with 1% formaldehyde and lysed in SDS lysis buffer. DNA was sheared using a sonicator (Bandelin, Berlin, Germany). Immunoprecipitation was done using 8 μL of anti Ets-1 antibody (Geneka, Montreal, Canada) or 8 μL of anti β-actin antibody (25 μg/mL) as an irrelevant antibody, respectively. The precipitated DNA fragments were analyzed by PCR reaction using the following primers: BMP4 promoter: 5′-GTGGGGTTTGGTGGGTTTG-3′ and 5′-GGATAGAGAGGCTTCCTTGAGC-3′ (175-bp fragment), BMP4 1.site mut: 5′-AATCATCAGTTTGGGCAGCAG-3′ and 5′-CGCCCACATCCTCCCATC-3′ (263-bp fragment), and BMP4 2.site mut: 5′-GGAGGCGATTAAGGGAGGAG-3′ and 5′-GGATGCCGAACTCACCTAGC-3′ (178-bp fragment). For the PCR reaction, the protocol used was 10 minutes denaturing at 95°C, 34 cycles of 1 minute denaturing at 95°C, 1 minute annealing at 64°C, 1 minute amplification at 72°C, and a final extension for 10 minutes at 72°C.

Protein Analysis *In vitro* (Western Blotting). Conditioned cell culture supernatant (1 × 10^6 cells in a T25 flask for 48 hours) was denatured at 94°C for 10 minutes after the addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and the proteins were subsequently separated on NuPage-SDS-gels (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), the membranes were blocked in 5% dry milk/TBS (100 mmol/L Tris/ HCl, 150 mmol/L NaCl) + 0.05% Tween 20 for 1 hour and incubated with 0.2 μg/mL of polyclonal goat anti-chordin antibody (R&D, Richmond, CA) or 0.2 μg/mL of polyclonal goat anti-BMP4 antibody (R&D) overnight at 4°C. A 1:5,000 dilution of rabbit anti-goat-Alkaline Phosphatase (Zymed Laboratories, San Francisco, CA) was used as secondary antibody. Staining was done using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma). Equal loading of the cell culture supernatants was verified by detecting the amounts of the secreted molecule MIA by ELISA (Roche).

Transfection Experiments. BMP2, BMP4, or BMP7 promoter reporter constructs were generated by cloning a murine gene fragment of 2,225, 1,618, 2,255 bp, respectively, into the reporter gene plasmid pGL3 basic (Invitrogen). Mutagenesis was done by site-directed mutagenesis (Clontech, Palo Alto, CA). Cells (2 × 10^5 per well) were seeded into 6-well plates and transfected with 0.5 μg of reporter constructs using Lipofectamine Plus (Invitrogen). Cotransfection was done using an antisense Ets-1 construct in pcDNA3 (15) or an AP-2α expression plasmid (16). Twenty-four hours after transfection the cells were lysed and the luciferase activity measured. To normalize transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega, Mannheim, Germany) was cotransfected and renilla luciferase activity measured by a luminometric assay (Promega). All transfections experiments were repeated thrice.

Invasion Assay. Invasion assays were done using Boyden Chambers containing polycarbonate filters with 8-μm pore size (Costar, Bodenheim, Germany), essentially as described previously (13). Filters were coated with Matrigel (diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany).

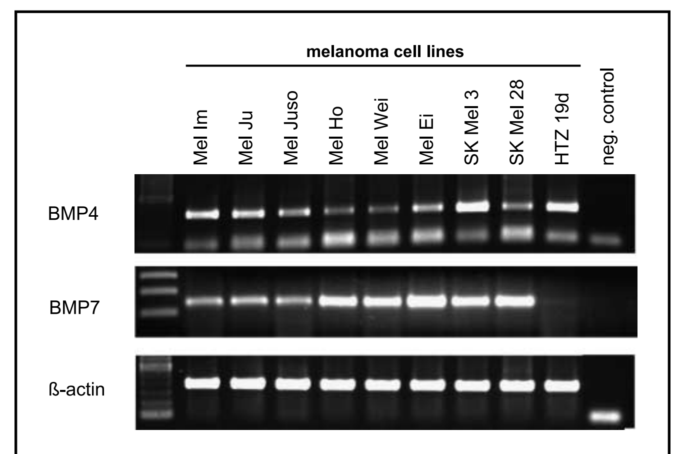

Figure 2. Expression of BMP4 and BMP7 in malignant melanoma. The expression of BMP4 and BMP7 was assessed by RT-PCR in nine melanoma cell lines. All cell lines tested express BMP4 and BMP7, save HTZ19d which do not express detectable amounts of BMP-7. β-Actin control was done as in Fig. 1.

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The lower compartment was filled with fibroblast-conditioned medium, used as a chemoattractant. Melanoma cells were harvested by trypsinization for 2 minutes, resuspended in DMEM without FCS at a density of 2×10^5 cells/mL and placed in the upper compartment of the chamber. After incubation at 37°C for 4 hours, the filters were collected and the cells adhering to the lower surface fixed, stained and counted.

Anchorage-Independent Growth Assay. Cells were seeded into 6-well plates in DMEM, 0.36% agar (Sigma), supplemented with 10% FCS on top of a 0.72% agar bed in similar medium. The cultures were incubated for 14 days and the colonies were measured and photographed. Colony size was measured using a Carl Zeiss microscope and the K300 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). For each cell clone, the diameter of 20 colonies was determined and statistics was done.

Statistical Analysis. Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's paired *t* test. $P < 0.05$ was considered statistically significant. All calculations were done using the GraphPad Prism software (GraphPad software, Inc, San Diego, CA).

Results

Several groups including our own have reported the overexpression of TGF-β1, TGF-β2, and TGF-β3 in malignant melanoma (6–8). Here, we were interested in screening the levels of expression of BMPs focussing on BMP2, BMP4, and BMP7.

Analysis of BMP2, BMP4, and BMP7 Expression in Malignant Melanoma. We first analyzed the expression of BMP2, BMP4, and BMP7 in melanoma cell lines by using RT-PCR. Expression of BMP2 was only detectable in two of the analyzed cell lines, SK Mel 28 and Mel Ei (Fig. 1), whereas BMP4 was expressed in all cell lines and BMP7 in all but one (HTZ19d, Fig. 2). These results were validated by real-time PCR, which confirmed a strong expression of BMP4 and a moderate expression of BMP7 in the melanoma cell lines (data not shown).

These *in vitro* results were extended to an *in vivo* setting by comparing the expression levels of BMP4 and BMP7 in melanoma tissues by immunostaining sections of nevi, primary melanoma, and metastasis of malignant melanoma (Table 1). These results confirmed that BMPs are up-regulated during transformation as nevi showed at best weak staining of BMP4 and BMP7, whereas strong staining of BMP4 and BMP7 was detected in primary melanoma (Fig. 3). Metastasis of malignant melanoma had moderate BMP4 expression levels in all tumor cells and very strong BMP7 expression in approximately half of the tumor cells.

Table 1. BMP4 and BMP7 expression in nevi, primary melanoma, and metastasis of malignant melanoma

Expression level	Nevi (total = 11 sections)	Primary melanoma (total = 5 sections)	Metastasis of malignant melanoma (total = 4 sections)
BMP4			
No	2		
Weak	8		
Moderate	1		4
Strong		5	
BMP7			
No	1		
Weak	9		
Moderate	1		
Strong		5	4

NOTE: Immunohistochemistry analysis of nevi sections revealed no or only weak expression of BMP4 and BMP7. Primary melanomas showed strong expression for both proteins, whereas metastases of malignant melanoma showed moderate BMP4 expression and strong BMP7 expression.

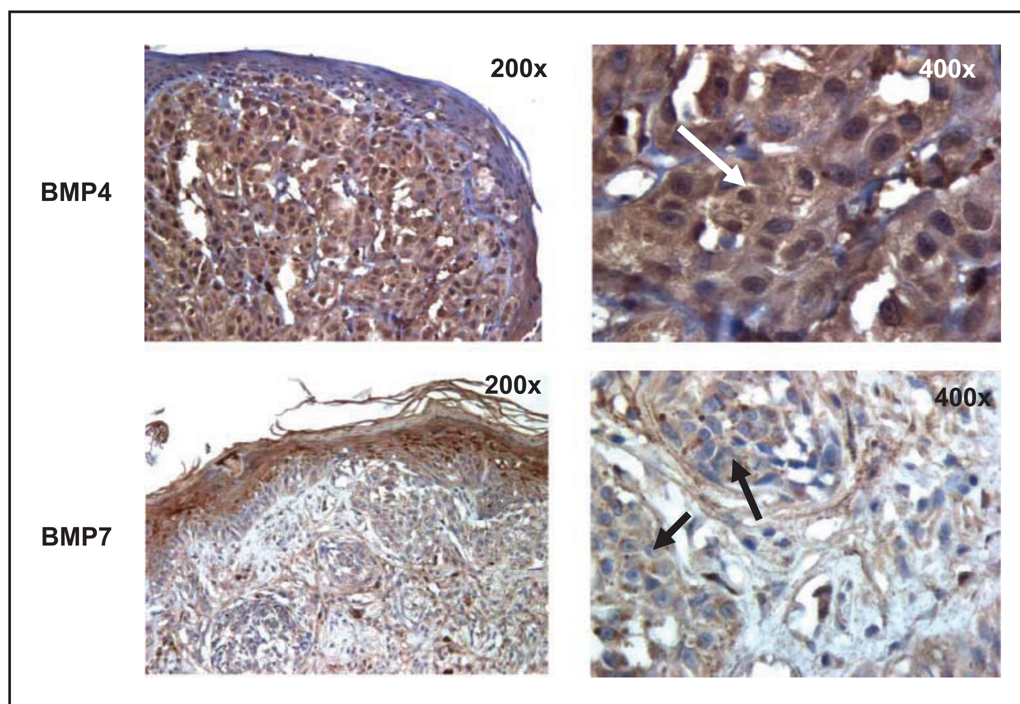

Figure 3. BMP4 and BMP7 expression in tissue sections of primary melanoma. BMP4 and BMP7 protein expression were analyzed by immunohistochemistry in nevi (11 sections), primary melanoma (5 sections), and metastasis of malignant melanoma (4 sections). Strong expression of both proteins was detected in primary melanoma as shown in the figure. *Arrow,* tumor nests in the 400× magnification.

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Table 2. BMP4, BMP7, Ets-1, and AP-2 expression in nevi, primary melanoma, and metastasis of malignant melanoma

	Nevi	Primary melanoma	Metastasis of malignant melanoma
BMP4	weak	strong	expression weaker than PM
BMP7	weak	strong	single cells strong
Ets-1	weak	strong	strong
AP-2	strong	weak	no expression [Baldi et al. (JCB 2001), Bar-Eli M. (Pigment Cell Res 2001)]

NOTE: Expression levels of BMP4, BMP7, Ets-1, and AP-2 were compared at different stages of melanoma development and progression. BMP4, BMP7, and Ets-1 are up-regulated in melanoma compared to nevi, whereas AP-2 expression is decreased in primary melanoma and lost in metastasis of malignant melanoma.

Ets-1 and AP-2 as Possible Regulators of BMP Expression. In order to analyze the mechanisms of regulation of BMP gene expression in melanoma cells, we subcloned the promoter regions of the murine *bmp2*, *bmp4*, and *bmp7* genes into a reporter vector. The murine and human *bmp* promoters are highly homologous within the regions analyzed. Transcriptional activity of these promoters was assessed in five human cell lines and one murine melanoma cell line to ensure that the activation of the murine promoter constructs is consistent between murine and human melanoma cell lines. As shown in Fig. 4*A*, the *bmp4* promoter was strongly activated in all the tested melanoma cell lines, with levels ranging from 30- to 45-fold over control, except in the HTZ19d cells. The *bmp7* promoter was also active in these cells, though at lower levels (2- to 11-fold). The promoter of the *bmp2* gene exhibited barely detectable activity or none at all. The levels of promoter activation in murine melanoma cells (B16) were similar to the human melanoma cells (SK Mel 28, Mel Im, Mel Ho, Mel Ei, and HTZ19d) for all promoter constructs.

Sequence analysis of the *bmp4* promoter region revealed the presence of putative binding sites for several transcription factors,

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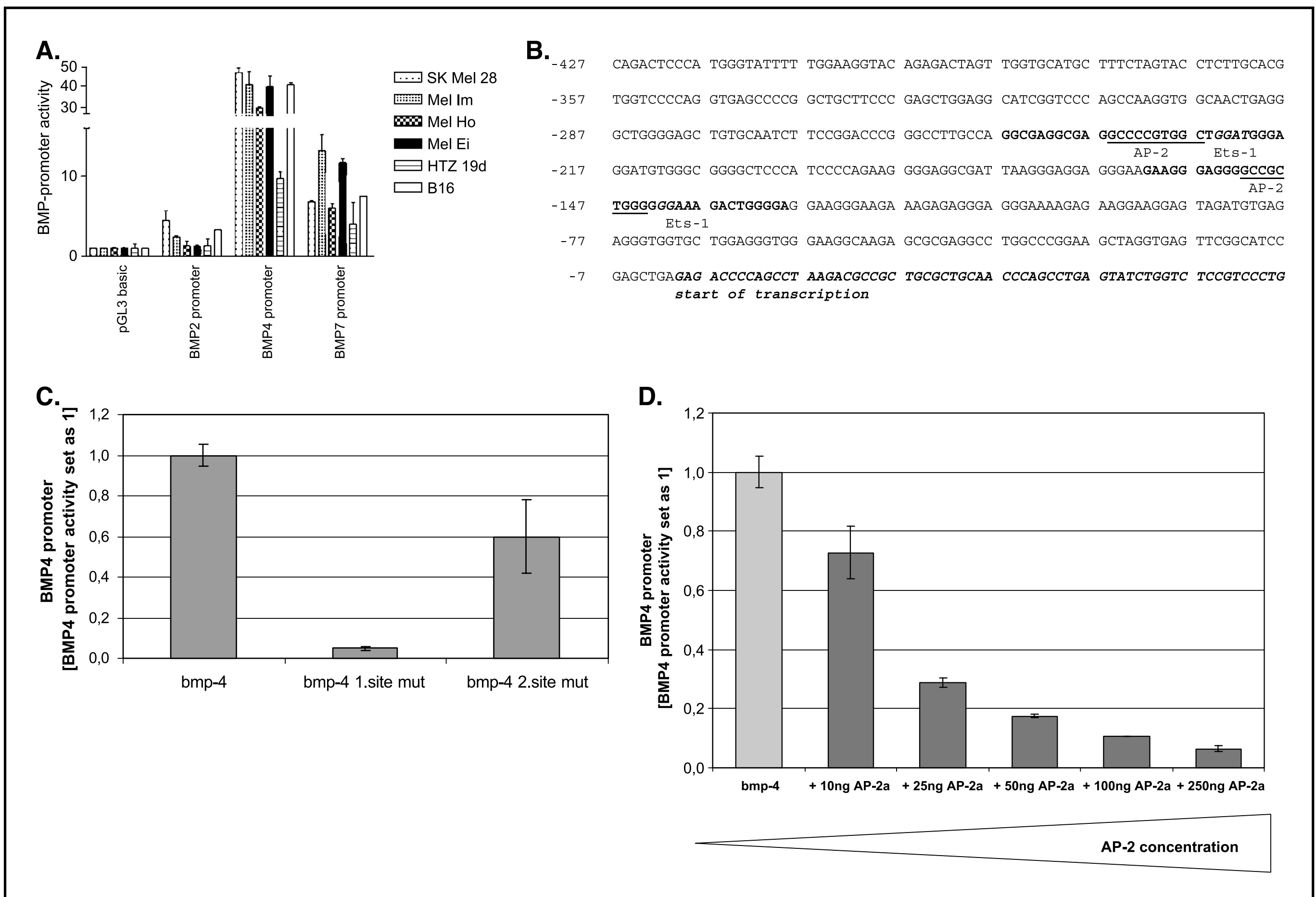

Figure 4. Analysis of *bmp2*, *bmp4*, and *bmp7* promoter activity. *A,* murine promoter regions of the *bmp2*, *bmp4*, and *bmp7* genes were cloned into pGL3-basic reporter vector, and promoter activity was estimated after transfection of the human melanoma cell lines SK Mel 28, Mel Im, Mel Ho, Mel Ei, and HTZ19d and the murine cell line B16 as a control. *B,* sequence analysis of the promoter region of *bmp4* reveals the presence of putative binding sites for AP-2 (*underlined*) and Ets-1 (*red*). *Regions in bold,* mutated to generate the constructs BMP4 1.site mut and 2.site mut. The transcription start site is indicated. *C,* directed mutation of AP-2/Ets-1 sites either abolished (bmp-4 1.site mut) or greatly reduced (bmp-4 2. site mut) transactivation of the promoter in melanoma cells. *D,* transfection of increasing amounts of an AP-2α expression plasmid together with the *bmp4* promoter construct into the melanoma cell line Mel Im induced a significant dose-dependent repression of the promoter activity.

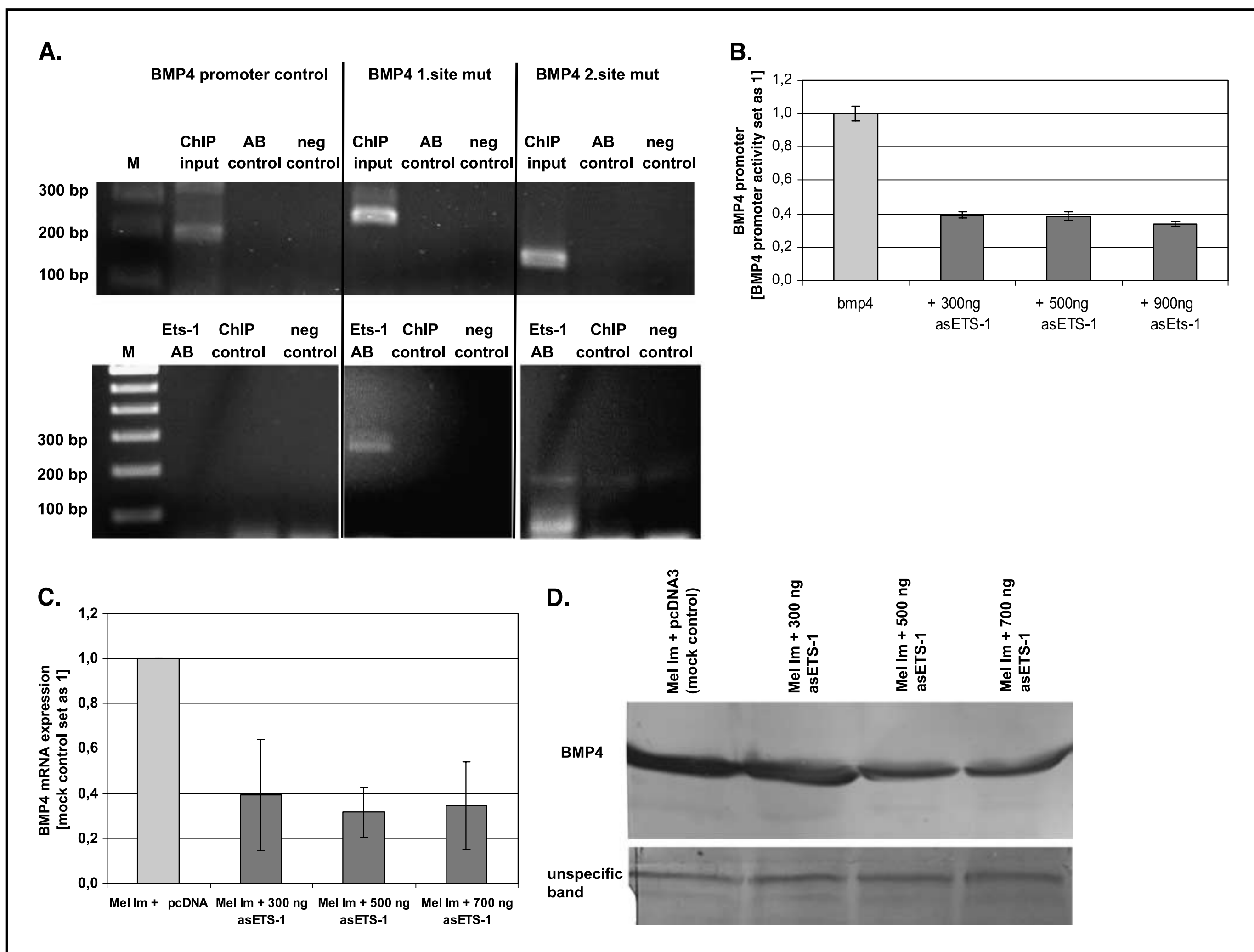

Figure 5. Regulation of the *bmp4* promoter by Ets-1. *A,* chromatin immunopreciptiation (*ChIP*) of genomic DNA extracted from B16 melanoma cells using an anti-Ets-1 antibody (*Ets-1 AB*) was performed. Precipitated DNA fragments were analyzed by PCR using specific primers flanking the potential Ets-1 binding sites (BMP4 1.site mut and BMP4 2.site mut). As a control, primers designed for a promoter region located downstream of the Ets-1 binding site (BMP4 promoter control) were further used. To verify specificity ChIP was performed without antibody (*ChIP control*) and using an irrelevant antibody (*AB control*). As a positive control for the PCR reaction, genomic DNA of the melanoma cell line B16 prepared for ChIP (*ChIP input*) was used. Water served as a negative control for the PCR reaction (*neg control*). *B,* transient transfection of melanoma Mel Im cells with Ets-1 antisense oligonucleotides reduces Ets-1 activity on the BMP4 reporter vector. *C,* Mel Im cells were transiently transfected with increasing amounts of an antisense Ets-1 expression vector and BMP4 transcript levels analyzed by real-time RT-PCR analysis. *D,* Western blot analysis of BMP4 expression levels in cells treated as in C. A nonspecific band is shown as a loading control. Furthermore, the expression level of the secreted molecule MIA in the cell culture supernatant was measured (Mel Im, 20.01 ng/mL; Mel Im + 300 ng asETS-1, 38.59 ng/mL; Mel Im + 500 ng asETS-1, 33.49 ng/mL; Mel Im + 700 ng asETS-1, 31.49 ng/mL) by ELISA.

including AP-2 and Ets-1 (Fig. 4*B*). Since we have shown previously that Ets-1 was up-regulated in melanomas (15), we assessed the importance of each of the AP-2/Ets-1 binding sites by inactivating them and assaying transcriptional activation of the promoter. Site-directed mutation of the first AP-2/Ets-1(−237) binding site resulted in a dramatic loss of promoter activity (Fig. 4*C*), whereas mutation of the second AP-2/Ets-1(−152) binding site resulted in a 40% reduction of activity, suggesting that both sites are important for expression of the *bmp4* gene. However, a single mutation within the AP-2/Ets-1(−237) binding site that abolishes AP-2 binding but does not affect Ets-1 binding did not alter the reporter gene activity compared with the unmutated control (data not shown). Because AP-2 is lost in malignant melanoma (refs. 17–19; Table 2), we overexpressed AP-2 and investigated its effects on the *bmp4* reporter vector in Mel Im melanoma cells. Overexpression of AP-2 resulted in a dose-dependent repression of promoter activity (Fig. 4*D*), suggesting that AP-2 has a repressing function on BMP4 expression and that the loss of AP-2 in malignant melanoma could be responsible in part for the overexpression of BMP4.

We then analyzed the contribution of Ets-1 to the transactivation of the *bmp4* promoter. By using chromatin immunoprecipitation, we could show that Ets-1 is bound to the region containing the two AP-2/Ets-1 sites of the *bmp4* promoter (Fig. 5*A*), and is therefore likely to be an endogenous activator of BMP4 expression. We als confirmed that Ets-1 is strongly up-regulated in malignant melanoma cells (ref. 15; Table 2).

We chose to down-regulate the expression of Ets-1 by using an antisense approach in order to assess Ets-1 function on the *bmp4* gene. Transfection of the melanoma cell line Mel Im with Ets-1 antisense expression plasmids resulted in a significant

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down-regulation of *bmp4* promoter activity (Fig. 5*B*) and of the levels of expression of the endogenous transcript (Fig. 5*C*). Consequently, the protein was also expressed at lower levels in the Ets-1 antisense transfected cells (Fig. 5*D*). On the other hand, transfection of an Ets-1 expression plasmid in Urotsa cells, which normally express Ets-1 at low levels, induced an increase in *bmp4* promoter activity of up to 2.5-fold. Taken together, these data show that Ets-1 is an endogenous activator of the *bmp4* gene in melanoma cells.

Down-regulation of BMP Activity in Malignant Melanoma Cells. Chordin, a protein which is normally not expressed by melanoma cells, interacts with BMPs and inhibits their activity by sequestering BMP ligands in the extracellular space and preventing interactions with their membrane receptors (20, 21). Because chordin is not expressed by the melanoma cell lines used here (Fig. 6*A*), we generated melanoma cells which stably overexpress chordin, as shown by RT-PCR and Western blot analysis (Fig. 6*A* and *B*). In addition, we also produced stable BMP4 antisense cell clones to specifically inhibit BMP4 expression. BMP4 expression levels in these clones were significantly decreased as shown by quantitative RT- PCR (Fig. 6*C*). The influence of chordin on BMPs secreted by melanoma cells as well as the reduction of BMP4 expression by antisense BMP4 transfection were assessed by assaying the activity of a BMP responsive element. In both situations, the activity of the BMP-responsive element was decreased (Fig. 7*A*). Two mock transfected cell clones, three of the chordin expressing cell clones (CHRD1, CHRD5, and CHRD9) and three BMP4 antisense cell clones (asBMP4 7, asBMP4 8, and asBMP4 9) were chosen for further functional analysis. Cell proliferation was not significantly different among all cell clones (pcDNA3 1, 100% ± 21.4%; pcDNA3 2, 87.0% ± 11.7%; CHRD1, 86.8% ± 16.9%; CHRD5, 74.0% ± 10.4%; CHRD9, 83.1% ± 3.4%; asBMP4 7, 100.7% ± 0.8%; asBMP4 8, 107.0% ± 4.2%; asBMP4 9, 99.4% ± 12.0%). When cells were grown in anchorage-independent conditions, there were no differences in colony formation (Fig. 7*B*) as all cell clones gave rise to similar colonies (pcDNA3 1, 100% ± 9.1%; pcDNA3 2, 101.3% ± 9.6%; CHRD1, 102.7% ± 19.6%; CHRD5, 87.6% ± 11.5%; CHRD9, 97.2% ± 1fs 7.6%; asBMP4 7, 91.1% ± 14.1%; asBMP4 8, 94.2% ± 9.3%; asBMP4 9, 95.4% ± 10.9%). On the other hand, the invasive properties of the melanoma cells were clearly affected by the overexpression of chordin or BMP4 antisense RNA as shown by the significant inhibition of invasion in chordin transfected as well as in BMP4 antisense cell clones when compared with controls (***, $P < 0.001$; **, $P < 0.01$; Fig. 7*C*).

Discussion

BMPs are multifunctional proteins that regulate the fate of different cell types, including mesenchymal and endothelial cells. BMPs are important regulators of cell development and differentiation of various organs and are also involved in tumor formation and progression, which arise from changes in the fate of highly differentiated cells in healthy tissues. It has been shown that BMP2 transcripts are expressed in various carcinoma cell lines from gastric, ovarian, prostate, pancreatic, and breast origin (10, 12, 22). The highly homologous BMP4 and BMP7 transcripts are also expressed in human carcinoma cell lines (23–25). Until now, the role of BMPs in malignant melanomas had not been addressed. Therefore, we studied expression and the role of BMPs in melanoma cell lines.

In contrast to other types of cancer, BMP2 expression is not prominent in melanomas, as it was only detected in two out of

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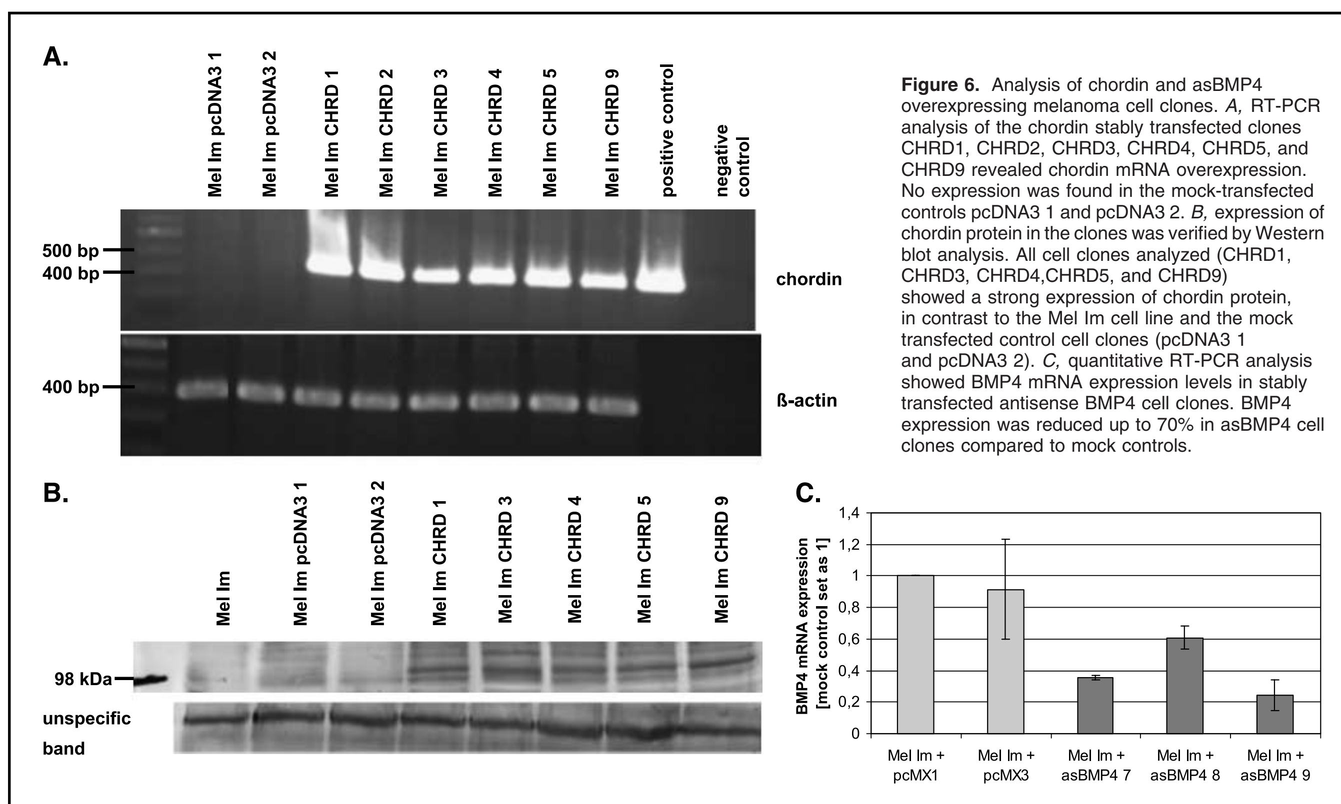

Figure 6. Analysis of chordin and asBMP4 overexpressing melanoma cell clones. *A,* RT-PCR analysis of the chordin stably transfected clones CHRD1, CHRD2, CHRD3, CHRD4, CHRD5, and CHRD9 revealed chordin mRNA overexpression. No expression was found in the mock-transfected controls pcDNA3 1 and pcDNA3 2. *B,* expression of chordin protein in the clones was verified by Western blot analysis. All cell clones analyzed (CHRD1, CHRD3, CHRD4,CHRD5, and CHRD9) showed a strong expression of chordin protein, in contrast to the Mel Im cell line and the mock transfected control cell clones (pcDNA3 1 and pcDNA3 2). *C,* quantitative RT-PCR analysis showed BMP4 mRNA expression levels in stably transfected antisense BMP4 cell clones. BMP4 expression was reduced up to 70% in asBMP4 cell clones compared to mock controls.

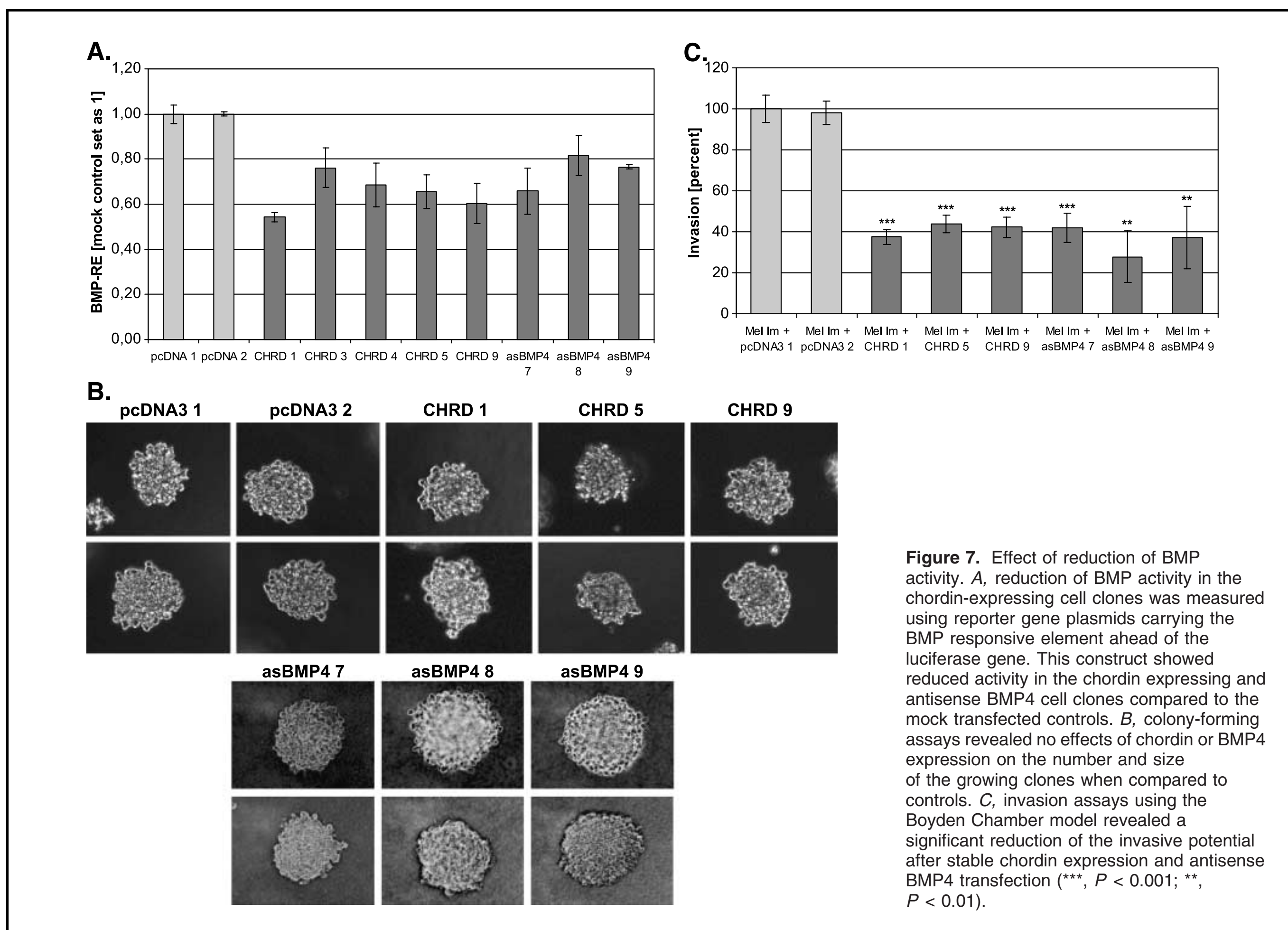

Figure 7. Effect of reduction of BMP activity. *A,* reduction of BMP activity in the chordin-expressing cell clones was measured using reporter gene plasmids carrying the BMP responsive element ahead of the luciferase gene. This construct showed reduced activity in the chordin expressing and antisense BMP4 cell clones compared to the mock transfected controls. *B,* colony-forming assays revealed no effects of chordin or BMP4 expression on the number and size of the growing clones when compared to controls. *C,* invasion assays using the Boyden Chamber model revealed a significant reduction of the invasive potential after stable chordin expression and antisense BMP4 transfection (***, $P < 0.001$; **, $P < 0.01$).

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nine analyzed cell lines. On the other hand, BMP4 and BMP7 are expressed by all the analyzed cell lines, with notably larger expression levels of BMP4.

Promoter studies using the promoter regions of the *bmp2*, *bmp4*, and *bmp7* genes revealed that expression of these molecules is controlled at the transcriptional level. A strong activity of the *bmp4* promoter construct and a moderate activity of the *bmp7* promoter construct were found in all melanoma cell lines analyzed, whereas almost no activity of the *bmp2* promoter construct was detectable. These experiments are in agreement with the observations that BMP4 and BMP7 are more highly expressed in these cells. Subsequent studies concentrating on the strongly active *bmp4* promoter revealed negative regulation of promoter activity by AP-2 and positive regulation by Ets-1. Putative AP-2 and Ets-1 binding sites within the *bmp4* promoter region were already identified by Ebara et al. (26) and Shore et al. (27) but were not functionally described. Here, we show that the putative binding sites are recognized by AP-2 and Ets-1 and that AP-2 has a repressing effect on *bmp4* promoter activity. A repressing activity of AP-2 was previously shown for the regulation of MCAM or protease-activated receptor 1 (17, 28). These observations are particularly noteworthy since AP-2 expression is known to be lost or strongly down-regulated in malignant melanoma (17–19). Taken together with our data, these results strongly suggest that the down-regulation of AP-2 in melanoma cells enables the up-regulation of the *bmp4* gene in these cells. Furthermore, we have recently shown that Ets-1 is overexpressed and active in melanoma (15), and we show here that Ets-1 is a direct regulator of the endogenous *bmp4* gene, having the opposite effect of AP-2 on an adjacent binding site.

BMPs have been shown to stimulate the migration of noncancerous human cells (29–32) and to induce the migration of neural crest cells (33), from which melanocytes are derived. Since migration is important for the ability of a tumor to invade and metastasize, we examined whether the endogenous expression of BMPs was important for tumor invasion *in vitro*. Down-regulation of BMP activity induced no changes in proliferation or in the ability of the cells to grow in an attachment-independent manner. However, general inhibition of BMP activity or specific down-regulation of BMP4 induced a strong reduction of the invasive potential, suggesting that BMP expression, including that of BMP4, plays a specific role in melanoma cell invasion and metastasis. In agreement with our results, recent studies have described a role for BMP2 in the promotion of tumor cell

migration and invasion and in the stimulation of tumor growth in lung carcinomas *in vivo* (34), suggesting a prominent role for BMPs in the tumor process.

Although several studies have shown that BMPs inhibit cell proliferation *in vitro* (11, 35, 36), these effects on proliferation may depend on the presence of other cytokines. Indeed, BMP2 has been shown to enhance proliferation during early wound healing (32) and also to stimulate the proliferation of growth plate chondrocytes. In contrast, in melanoma cells we could not detect any effect of BMP2 on proliferation.

The effects of BMPs on cell growth may therefore vary depending on the cell type. As the biological function of BMPs comes under greater scrutiny, it is becoming clear that their activities are not only associated with the formation of bone or cartilage; BMP2 and BMP4 also induce the migration and differentiation of precursor cells that are not involved in bone or cartilage formation. The biological activity of BMPs may depend not only on the particular cell types present, but may vary depending on the presence of other modulators.

Interestingly, a study published recently by Hoffman et al. (37) suggested that BMP expression is regulated by fibroblast growth factor (FGF). They could show that BMP7 and BMP4 expression was up-regulated via FGFR1 in submandibular gland development. This correlation was also observed in the FGFR1-deficient mouse, where altered expression of BMP2 and BMP4 was detected (38), and in several other studies (39–41). FGFR1 and basic FGF are known to be expressed in a high percentage of malignant melanomas (42). Furthermore, it is known that basic FGF increases cell survival and growth by an autocrine loop (43) and that basic FGF promotes the progression of melanocytes to melanoma (44, 45). Additionally, it was shown that expression of dominant negative FGFR1 results in down-regulation of melanoma growth (46) and that antisense targeting of basic fibroblast growth factor or FGFR1 causes both a block of tumor growth *in vivo* (47) and in differentiation of melanoma cells *in vitro* (48). Subsequent analysis of the interaction between BMP and FGF signaling in malignant melanoma must be done to understand the molecular details of this pathway.

Future studies will most likely define even a broader role of the BMPs in postnatal tissues. Consequently, further studies are needed to better define the autocrine effects of BMPs on human tumorigenesis.

In summary, our studies show that BMPs are strongly expressed in malignant melanoma cells as well as in human tissue of primary melanoma and metastasis of malignant melanoma. These results suggest that BMPs have an important biological function in human malignant melanomas.

Acknowledgments

Received 3/25/2004; revised 9/30/2004; accepted 11/11/2004.

Grant support: Deutsche Forschungsgemeinschaft (A-K. Bosserhoff).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. J. Johnson (University of Munich, Germany) for providing melanoma cell lines; Theresa E. Gratsch and K. Sue O'Shea (University of Michigan Medical School) for providing the chordin expression vector; Steven Dooley (RWTH Aachen University Hospital, Germany) for providing the BMP-RE construct; and Jacqueline Schlegel, Susanne Wallner, and Sabine Troppmann for excellent technical assistance.

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References

1. Hogan BL. Bone morphogenetic proteins in development. Curr Opin Genet Dev 1996;6:432–8.
2. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-β superfamily signalling. Genes Cells 2002;7:1191–204.
3. Teicher BA. Malignant cells, directors of the malignant process: role of transforming growth factor-β. Cancer Metastasis Rev 2001;20:133–43.
4. Gold LI. The role for transforming growth factor-β (TGF-β) in human cancer. Crit Rev Oncog 1999;10:303–60.
5. Albino AP, Davis BM, Nanus DM. Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. Cancer Res 1991;51:4815–20.
6. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-β2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. Am J Pathol 1994;145:97–104.
7. Van Belle P, Rodeck U, Nuamah I, Halpern AC, Elder DE. Melanoma-associated expression of transforming growth factor-β isoforms. Am J Pathol 1996;148:1887–94.
8. Schmid P, Itin P, Rufli T. *In situ* analysis of transforming growth factor-βs (TGF-β1, TGF-β2, TGF-β3), and TGF-β type II receptor expression in malignant melanoma. Carcinogenesis 1995;16:1499–503.
9. Gobbi G, Sangiorgi L, Lenzi L, et al. Seven BMPs and all their receptors are simultaneously expressed in osteosarcoma cells. Int J Oncol 2002;20:143–7.
10. Hatakeyama S, Ohara-Nemoto Y, Kyakumoto S, Satoh M. Expression of bone morphogenetic protein in human adenocarcinoma cell line. Biochem Biophys Res Commun 1993;190:695–701.
11. Ide H, Yoshida T, Matsumoto N, et al. Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. Cancer Res 1997;57:5022–27.
12. Kleeff J, Maruyama H, Ishiwata T, et al. Bone morphogenetic protein 2 exerts diverse effects on cell growth *in vitro* and is expressed in human pancreatic cancer *in vivo*. Gastroenterology 1999;116:1202–16.
13. Jacob K, Wach F, Holzapfel U, et al. *In vitro* modulation of human melanoma cell invasion and proliferation by all-*trans*-retinoic acid. Melanoma Res 1998;8:211–9.
14. Petzoldt JL, Leigh IM, Duffy PG, Sexton C, Masters JR. Immortalisation of human urothelial cells. Urol Res 1995;23:377–80.
15. Rothhammer T, Hahne JC, Florin A, et al. The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. Cell Mol Life Sci 2004;61:118–28.
16. Poser I, Dominguez D, de Herreros AG, et al. Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor snail. J Biol Chem 2001;276:24661–6.
17. Tellez C, McCarty M, Ruiz M, Bar-Eli M. Loss of activator protein-2α results in overexpression of protease-activated receptor-1 and correlates with the malignant phenotype of human melanoma. J Biol Chem 2003;278:46632–42.
18. Karjalainen JM, Kellokoski JK, Eskelinen MJ, Alhava EM, Kosma VM. Downregulation of transcription factor AP-2 predicts poor survival in stage I cutaneous malignant melanoma. J Clin Oncol 1998;16:3584–91.
19. Huang S, Jean D, Luca M, Tainsky MA, Bar-Eli M. Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. EMBO J 1998;17:4358–69.
20. Piccolo S, Sasai Y, Lu B, De Robertis EM. Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 1996;86:589–98.
21. Millet C, Lemaire P, Orsetti B, Guglielmi P, Francois V. The human chordin gene encodes several differentially expressed spliced variants with distinct BMP opposing activities. Mech Dev 2001;106:85–96.
22. Kiyozuka Y, Nakagawa H, Senzaki H, et al. Bone morphogenetic protein-2 and type IV collagen expression in psammoma body forming ovarian cancer. Anticancer Res 2001;21:1723–30.
23. Hatakeyama S, Gao YH, Ohara-Nemoto Y, Kataoka H, Satoh M. Expression of bone morphogenetic proteins of human neoplastic epithelial cells. Biochem Mol Biol Int 1997;42:497–505.
24. Sulzbacher I, Birner P, Trieb K, Pichlbauer E, Lang S. The expression of bone morphogenetic proteins in osteosarcoma and its relevance as a prognostic parameter. J Clin Pathol 2002;55:381–5.
25. Grcevic D, Marusic A, Grahovac B, Jaksic B, Kusec R. Expression of bone morphogenetic proteins in acute promyelocytic leukemia before and after combined all *trans*-retinoic acid and cytotoxic treatment. Leuk Res 2003;27:731–8.
26. Ebara S, Kawasaki S, Nakamura I, et al. Transcriptional regulation of the mBMP-4 gene through an E-box in the 5′-flanking promoter region involving USF. Biochem Biophys Res Commun 1997;240:136–41.
27. Shore EM, Xu M, Shah PB, et al. The human bone morphogenetic protein 4 (BMP-4) gene: molecular structure and transcriptional regulation. Calcif Tissue Int 1998;63:221–9.
28. Nyormoi O, Bar-Eli M. Transcriptional regulation of metastasis-related genes in human melanoma. Clin Exp Metastasis 2003;20:251–63.
29. Willette RN, Gu JL, Lysko PG, et al. BMP-2 gene expression and effects on human vascular smooth muscle cells. J Vasc Res 1999;36:120–5.
30. Cunningham NS, Paralkar V, Reddi AH. Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor β1 mRNA expression. Proc Natl Acad Sci U S A 1992;89:11740–4.

31. Fiedler J, Roderer G, Gunther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. J Cell Biochem 2002;87:305–12.
32. King GN, Hughes FJ. Bone morphogenetic protein-2 stimulates cell recruitment and cementogenesis during early wound healing. J Clin Periodontol 2001;28:465–75.
33. Dorsky RI, Moon RT, Raible DW. Environmental signals and cell fate specification in premigratory neural crest. BioEssays 2000;22:708–16.
34. Langenfeld EM, Calvano SE, Abou-Nukta F, et al. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. Carcinogenesis 2003;24:1445–54.
35. Hjertner O, Hjorth-Hansen H, Borset M, et al. Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. Blood 2001;97:516–22.
36. Andrews PW, Damjanov I, Berends J, et al. Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). Lab Invest 1994;71:243–51.
37. Hoffman MP, Kidder BL, Steinberg ZL, et al. Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis *in vitro* through BMP- and FGF-dependent mechanisms. Development 2002;129:5767–78.
38. Dell'Era P, Ronca R, Coco L, et al. Fibroblast growth factor receptor-1 is essential for *in vitro* cardiomyocyte development. Circ Res 2003;93:414–20.
39. Hyatt BA, Shangguan X, Shannon JM. BMP4 modulates fibroblast growth factor-mediated induction of proximal and distal lung differentiation in mouse embryonic tracheal epithelium in mesenchyme-free culture. Dev Dyn 2002;225:153–65.
40. Lillien L, Raphael H. BMP and FGF regulate the development of EGF-responsive neural progenitor cells. Development 2000;127:4993–5005.
41. Ericson J, Norlin S, Jessell TM, Edlund T. Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 1998;125:1005–15.
42. Xerri L, Battyani Z, Grob JJ, et al. Expression of FGF1 and FGFR1 in human melanoma tissues. Melanoma Res 1996;6:223–30.
43. Shan S, Robson ND, Cao Y, et al. Responses of vascular endothelial cells to angiogenic signaling are important for tumor cell survival. FASEB J 2004;18:326–8.
44. Nesbit M, Nesbit HK, Bennett J, et al. Basic fibroblast growth factor induces a transformed phenotype in normal human melanocytes. Oncogene 1999;18:6469–76.
45. Meier F, Caroli U, Satyamoorthy K, et al. Fibroblast growth factor-2 but not Mel-CAM and/or β3 integrin promotes progression of melanocytes to melanoma. Exp Dermatol 2003;12:296–306.
46. Yayon A, Ma YS, Safran M, Klagsbrun M, Halaban R. Suppression of autocrine cell proliferation and tumorigenesis of human melanoma cells and fibroblast growth factor transformed fibroblasts by a kinase-deficient FGF receptor 1: evidence for the involvement of Src-family kinases. Oncogene 1997;14:2999–3009.
47. Wang Y, Becker D. Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth. Nat Med 1997;3:887–93.
48. Becker D, Lee PL, Rodeck U, Herlyn M. Inhibition of the fibroblast growth factor receptor 1 (FGFR-1) gene in human melanocytes and malignant melanomas leads to inhibition of proliferation and signs indicative of differentiation. Oncogene 1992;7:2303–13.

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