Role of Smad1 and Smad4 proteins in the induction of p21^{WAF1,Cip1} during bone morphogenetic protein-induced growth arrest in human breast cancer cells

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Abstract

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor- β family of cytokines. The recent observation that BMPs can inhibit breast cancer cell proliferation *in vitro* suggests that BMPs or the BMP pathway may hold promise as therapeutic targets for the control of breast tumor growth in women. Better to understand the mechanism of BMP-induced growth arrest we examined the effect of BMP-2 and mediators of BMP-2 action on cell proliferation and p21^{Cip1} expression in breast cancer cell lines. We show here that BMP-2 potently inhibited the proliferation of breast cancer cell lines that express both Smad1 and Smad4 (CAMA-1, MCF7, MDA-MB-231, T-47D, ZR-75-1), but not that of cells that only express Smad1 (MDA-MB-468). Growth inhibition correlated with up-regulation of p21 mRNA and protein levels. Up-regulation of p21 was resistant to

cycloheximide but not to actinomycin D, suggesting that it occurred at the transcriptional level. Using p21 promoter-luciferase reporter constructs we mapped the BMP-responsive region of the p21 promoter to within 211 base pairs of the transcription start site. Induction of p21 promoter activity was rapid and coincided with up-regulation of p21 mRNA and protein levels. p21 promoter activity required both Smad1 and Smad4 and was induced by either BMP-2 or constitutively active type I BMP receptors. Moreover, the C-terminal SSVS region of Smad1 was necessary for activation of the p21 promoter by BMP-2. Taken together, these results indicate that the mechanism of BMP-induced p21 promoter activation involves BMP receptors and BMP Smads.

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Introduction

Breast cancer is the second most frequent cause of cancer-related deaths in North American women (Greenlee *et al.* 2001). The proliferation of human breast cancer cells is regulated by steroid hormones and growth factors. Estrogens are well known to stimulate the proliferation of estrogen receptor-expressing breast cancer cells and blockade of estrogen action remains the cornerstone of the endocrine therapy of breast cancer in women.

The bone morphogenetic proteins (BMPs) are members of a larger family of growth factors that includes activins and transforming growth factor beta (TGF- β). BMPs, activins and TGF- β are structurally related and employ similar signal transduction pathways that involve the Smad proteins and transmembrane receptors (BMP receptors, activin receptors and TGF- β receptors) (Hu *et al.* 1998, Massague 1998, Attisano & Wrana 2000, Massague *et al.* 2000, Miyazono 2000). BMP signaling is initiated by the association between BMPs such as BMP-2 and the membrane-associated type I and type II BMP receptors.

Upon activation of the receptors by the cytokine, the type I receptor phosphorylates specific intracellular proteins named Smads. The Smads phosphorylated by the type I BMP-receptors are Smad1, Smad5 and Smad8. Once phosphorylated, these receptor-associated Smads associate with the product of the tumor suppressor gene *Smad4*. The resulting hetero-oligomer is then translocated to the nucleus to modulate the expression of BMP-responsive genes, presumably by interacting with BMP response elements in the promoters of BMP target genes such as *Smad6*, *Xvent2*, *Xvent-2B* or *JunB* (Jonk *et al.* 1998, Johnson *et al.* 1999, Hata *et al.* 2000, Henningfeld *et al.* 2000, Ishida *et al.* 2000).

The role of BMPs in development and endochondral bone formation has been well characterized but their role in breast cancer remains poorly understood (Groeneveld & Burger 2000). However, it has been shown that BMP-2 mRNA is expressed in the mammary gland and that some human breast cancer cell lines secrete BMP-2 (Phippard *et al.* 1996, Arnold *et al.* 1999). We first observed that Smad1 mRNA, which mediates the BMP-2 signal to the

nucleus, was expressed in breast cancer cell lines. Moreover, it has recently been shown that BMP-2 inhibits the growth of MCF7 and MDA-MB-231 cells and up-regulates p21 protein (Ghosh-Choudhury *et al.* 2000a,b). These observations led us to investigate the mechanism of BMP-2-induced inhibition of breast cancer cell proliferation. We show here that BMP-2 is a potent inhibitor of MCF7, ZR-75-1, T-47D, CAMA-1 and MDA-MB-231 breast cancer cell proliferation *in vitro*. A more detailed examination of BMP-2-induced growth arrest in MCF7 and T-47D cells demonstrated that BMP-2 directly up-regulates p21 mRNA and protein levels. Finally, we show that co-transfected Smad1, Smad4 and BMP-2, or constitutively active BMP-2 type I receptors (ALK3QD and ALK6QD), act synergistically to induce p21 promoter activity in human breast cancer cells.

Materials and Methods

Cells, hormones and antihormones

The cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA, USA). For routine culture, T-47D, CAMA-1 and ZR-75-1 cells were grown in phenol red-free RPMI 1640 whereas MCF7 cells were grown in DMEF12 medium. The medium was supplemented with 1 nM 17 β -estradiol (E₂), 2 mM L-glutamine, 100 IU penicillin/ml, 50 μ g streptomycin/ml and 5% (v/v) fetal bovine serum (FBS). MDA-MB-231 and MDA-MB-468 cells were cultured in MEM supplemented with non-essential amino acids, 100 IU penicillin/ml, 50 μ g streptomycin/ml and 5% (v/v) fetal bovine serum. The culture medium was changed every 2 to 3 days. 17 β -Estradiol was purchased from Steraloids (Wilton, NH, USA). Recombinant human BMP-2 (rhBMP-2) was kindly provided by Dr Bich Nguyen (Genetics Institute Inc., Cambridge, MA, USA). BMP-2 was resuspended in PBS buffer supplemented with 0.1% (w/v) BSA as carrier protein.

Cell proliferation assays

Cell proliferation assays with rhBMP-2 were conducted in 24-well plates. MCF7, T-47D, CAMA-1 and ZR-75-1 cells were plated at 1.5×10^4 cells/well and cultured for 3 days in medium containing 5% dextran-coated charcoal-treated FBS (DCC-FBS). The medium was then replaced with fresh medium alone, fresh medium containing E₂ or BMP-2, or fresh medium containing E₂ and 1 to 100 ng rhBMP-2/ml as indicated in the figure legends. The culture medium was changed every 2 to 3 days. Cell proliferation was evaluated on day 10 by measuring the DNA content of each well as previously described (Fiszer-Szafarz *et al.* 1981, Simard *et al.* 1997). The same amount of ethanol or BMP-2 vehicle (PBS-BSA

0.1%) was added to each well. MDA-MB-231 and MDA-MB-468 cells were plated at 10^4 cells/well for two days in their respective media. The medium was then replaced with medium containing rhBMP-2 (50 ng/ml) or BMP-2 vehicle (PBS-BSA 0.1%). DNA content was measured on day 5 (MDA-MB-231) or day 7 (MDA-MB-468).

p21 regulation experiments

To assess the effect of BMP-2 on p21 mRNA and protein expression, MCF7 and T-47D cells were cultured in their respective media containing 5% (v/v) DCC-FBS and 1 nM E₂ for 3 days. The medium was then changed and BMP-2 (50 ng/ml) or BMP-2 vehicle (PBS-BSA 0.1%) were added for the indicated time periods. At each time point, the cells were harvested with viokase, counted and washed twice with PBS. Cells were lysed in Tri-Reagent (Molecular Research Co., Cincinnati, OH, USA) for mRNA studies. For protein extraction, the cells were resuspended in protein lysis buffer (Triton X-100 1% (v/v), 20 mM Hepes, 10% glycerol (v/v), 1.5 mM MgCl₂, 150 mM NaCl, 1 mM EDTA, Complete Protease Inhibitors (Roche Molecular Biochemicals, Mannheim, Germany), 50 mM NaF and 1 mM Na₃VO₄), kept on ice for 30 min, and then centrifuged for 15 min. The supernatant was then collected.

Plasmids

The plasmids containing Smad cDNA fragments that were used as probes for Northern blots and in transfection assays were constructed as follows: the Smad1 cDNA was amplified from LNCaP cell RNA by RT-PCR using *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and oligonucleotide primers corresponding to nucleotides 1–25 and 1398–1373 of the Smad1 coding sequence. The primers contained restriction sites for subcloning the Smad1 PCR products into the BamHI and XbaI sites of pcDNA3-FLAG to generate pcDNA3 FLAG-Smad1. pcDNA3-FLAG was generated by subcloning annealed oligonucleotides encoding the FLAG epitope (MDYKD DDDKL) into the HindIII and BamHI sites of pcDNA3 (Invitrogen, Burlington, Ontario, Canada). The Smad1 cDNA was amplified by PCR using *Pfu* polymerase (Stratagene) and oligonucleotide primers corresponding to nucleotides 1–35 and 1398–1373 of the Smad1 coding sequence. The 5' oligonucleotide used for the construction of hemagglutinin epitope (HA)-tagged Smad1 expression plasmids contained sequences encoding the HA to allow expression of Smad1 with an N-terminal epitope tag. The HA-Smad1 expression vector containing serine to alanine changes at positions 462, 463 and 465 (HA-Smad1 AAVA) was constructed using the following 3' oligonucleotide: 5'-gacgactctagattatgctactgctgcaataggattatg-3'. The PCR product was then subcloned into pcDNA3.

AU1-epitope-tagged Smad4 was subcloned in pcDNA3 as previously described (Pouliot & Labrie 1999). Full-length p21 cDNA was cloned in pBSKS by RT-PCR using *Pfu* polymerase and oligonucleotide primers corresponding to nucleotides 1–20 and 492–470 of the p21 coding sequence. The pCMV5 ALK3Q233D and pCMV5 ALK6Q203D constructs were generated by site-directed mutagenesis of plasmids expressing the wild-type receptors (kindly provided by Dr Liliana Attisano, University of Toronto, Ontario, Canada) using the Quickchange Site-directed Mutagenesis kit (Stratagene). Constructs were sequenced using the Sequenase Version 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, OH, USA). The pGL3–2300/+16 p21 promoter luciferase reporter construct was generously provided by Dr Bert Vogelstein (Johns Hopkins Oncology Center, MD, USA). The pGL3–211/+16 p21 promoter construct was generated by removing the 2.1 kb KpnI/PstI fragment from pGL3–2300/+16. The p21–60/+16 p21 promoter construct was generated by removing the 2.3 kb NheI-SmaI fragment from pGL3–2300/+16. The –2300(Δ–215/–61)/+16 p21 promoter construct was generated by removing the 154-bp PstI-ApaI fragment from pGL3–2300/+16. The –211(Δ–124/–60)/+16 p21 promoter construct was generated by removing the 64-bp SmaI fragment from pGL3–211/+16.

Northern blot analysis

To detect Smad1 or p21 mRNAs, fragments encompassing the entire coding region of Smad1 or p21 were excised from the constructs described above and gel purified. [α - 32 P]-dCTP-labeled cDNA probes were prepared using the random-priming method (DECAprime II; Ambion, Austin, TX, USA). Northern blot analysis was performed as previously described (Blais *et al.* 1998, Pouliot & Labrie 1999).

Immunoblotting

Protein concentrations were measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (15 μ g per lane) were separated on 12% SDS polyacrylamide gels and electroblotted to 0.2 μ m nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The blots were probed separately with antibodies against p21 (PharMingen International, San Diego, CA, USA, #65961A), p27 (Transduction Laboratories, Lexington, KY, USA, #K25020) and α -tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, #sc-8035) as indicated in the corresponding figures, followed by detection using the Renaissance Western Blot Chemiluminescence Reagent Plus (Pierce Chemicals, Rockford, IL, USA). Each antibody recognized a single polypeptide of the expected size. Protein levels were quantified by scanning densitometry using the BioImage Visage 110S

and the WBA software (Genomic Solutions, Ann Arbor, MI, USA). Protein abundance is expressed as protein to α -tubulin ratios.

Transfections and luciferase assays

T-47D cells were plated at a density of 1.5×10^5 cells per well in 12-well plates. Eighteen to twenty-four hours after plating, T-47D cells were transfected with p21-Luc constructs (250 ng), Smad expression plasmids (125 ng), pcDNA3, and/or plasmids expressing constitutively active activin receptor-like kinase (ALK) receptors (125 ng), and pRL-null control vector (125 ng), using Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). Equal amounts of DNA were added to each well by adding pcDNA3 vector as necessary. The transfected cells were then cultured for 24 h and BMP-2 (50 ng/ml or as indicated in the figures) or an equivalent volume of BMP-2 vehicle (PBS-BSA 0.1%) was added. Twenty hours later, the cells were lysed and the luciferase activities were measured using the Dual Luciferase Assay kit (Promega, Madison, WI, USA) and a Berthold MicroLumat Plus LB96V luminometer. For experiments using constitutively active ALK receptors, transfected cells were harvested 48 h after transfection after which cells were lysed and luciferase activities were measured. p21 promoter-driven-firefly luciferase activity was normalized for renilla luciferase. Data are expressed as relative luciferase activity (firefly luciferase activity divided by renilla luciferase activity; means $\pm$ standard deviation (s.d.) of a duplicate from one representative experiment).

Results

The BMP-Smad, Smad1, is expressed in human breast cancer cell lines

To determine if BMP-2 may have a role in the control of breast cancer cell proliferation, we examined if the BMP pathway-associated Smad, Smad1, was expressed in human breast cancer cell lines. As shown in Fig. 1, Northern blot analysis revealed that Smad1 mRNA is expressed in the four estrogen-sensitive (MCF7, ZR-75-1, T-47D and CAMA-1) and two estrogen-insensitive (MDA-MB-231 and MDA-MB-468) breast cancer cell lines studied. We previously reported that Smad4 was expressed in 4 out of 5 breast cancer cell lines reported here (MCF7, ZR-75-1, T-47D and MDA-MB-231), thereby demonstrating that the proteins that mediate the BMP-2 signal to the nucleus are expressed in human breast cancer cell lines (Pouliot & Labrie 1999).

BMP-2 is a potent inhibitor of breast cancer cell proliferation

To determine if BMPs can modulate the proliferation of human breast cancer cells, we tested the effect of recombinant human BMP-2 (rhBMP-2) on the estrogen-induced growth of MCF7, CAMA-1, ZR-75-1 and

Figure 1 Smad1 is expressed in human breast cancer cell lines. Total RNA samples (10 µg per lane) from MCF7, ZR-75-1, T-47D, CAMA-1, MDA-MB-231, MDA-MB-468 cells were size-separated on a 1% agarose/2.2 M formaldehyde gel, immobilized on nylon membranes, and hybridized to a radiolabeled Smad1 cDNA probe.

T-47D cells. The human breast cancer cells were grown for 10 days in medium containing the potent natural estrogen 17 β -estradiol (E_2 , 1 nM), alone or in combination with 1 to 100 ng rhBMP-2/ml (Fig. 2A). Cell proliferation was assessed by measuring cellular DNA content, which is a reliable indication of cell number (Simard *et al.* 1997). Estradiol alone stimulated cell proliferation as reflected by 1.4- (ZR-75-1) to 14.2-fold (MCF7) increases in DNA content. On the other hand, E_2 -induced growth of all estrogen-responsive human breast cancer cell lines was strongly inhibited by rhBMP-2 (Fig. 2A). BMP-2 produced a complete inhibition of the estradiol-induced growth of estrogen-sensitive breast cancer cells at a dose of 100 ng/ml. Moreover, BMP-2 alone did not stimulate the proliferation of any cell line indicating that BMP-2 is a pure inhibitor of all estrogen-sensitive human breast cancer cell lines studied.

We also examined the effect of BMP-2 on the growth of estrogen-insensitive breast cancer cells (Fig. 2B). Treatment of MDA-MB-231 cells with BMP-2 (50 ng/ml) inhibited their proliferation by 46% whereas MDA-MB-468 cells were unaffected by BMP-2 treatment. This was not unexpected since MDA-MB-468 cells lack the *Smad4* gene (Schutte 1999). However, other genetic factors could account for the lack of growth inhibition by BMP-2 in MDA-MB-468 cells.

BMP-2 up-regulates p21 mRNA and protein levels in human breast cancer cell lines

Cyclin-dependent kinase inhibitors (CKIs) are involved in growth arrest induced by many growth inhibitors including TGF- β . CKIs are small proteins that bind to cyclin-dependent kinases (CDKs) to inhibit their activity. They are well known to cause a G1-phase arrest when over-

expressed in mammalian cells (Sherr & Roberts 1995, Pestell *et al.* 1999, Sherr & Roberts 1999).

Given the inhibitory effect of BMP-2 on the growth of breast cancer cells, we examined the effect of BMP-2 on CKI protein levels during the G1-phase arrest. p21 and p27 proteins are expressed in all the breast cancer cell lines we studied that are sensitive to BMP-2 (data not shown, Gray-Bablin *et al.* 1997). We therefore examined the effect of BMP-2 on p21 and p27 protein levels. As shown in Fig. 3A, BMP-2 (50 ng/ml) up-regulated p21 protein levels in MCF7 and T-47D breast cancer cells. In fact, treatment of MCF7 and T-47D cells with BMP-2 caused 4.2- and 6.6-fold increases, respectively, in p21 protein levels (Fig. 3B). This effect is specific to p21 since BMP-2 did not affect p27 protein levels.

To ascertain if the effect of BMP-2 on p21 protein levels was a rapid phenomenon, we conducted a time-course experiment where we examined the levels of p21 protein and mRNA in MCF7 and T-47D cells. As shown in Fig. 4A, the induction of p21 protein was very rapid since we observed that p21 protein levels increased 5.5 times in MCF7 and 2.5 times in T-47D cells after only 6 h of treatment with BMP-2. BMP-2 treatment resulted in similar increases in p21 mRNA levels. As shown in Fig. 4B, after only 6 h of treatment with BMP-2, p21 mRNA levels increased 3.6 times in T-47D cells and 3.0 times in MCF7 cells. The induction of p21 mRNA was sustained for at least 24 h of treatment in both cell lines.

To determine whether the induction of p21 mRNA requires protein synthesis, T-47D cells were treated with BMP-2 in combination with the protein synthesis inhibitor cycloheximide for 2 h. As shown in the Northern blot of Fig. 5, p21 mRNA levels were induced by BMP-2 even in the presence of cycloheximide, indicating that the BMP-2-induced increase in p21 mRNA is independent of new protein synthesis. In contrast to cycloheximide, the RNA synthesis inhibitor actinomycin D abolished the induction of p21 mRNA by BMP-2, demonstrating that the induction of p21 mRNA by BMP-2 requires transcription.

Induction of p21 promoter activity by Smad proteins

The expression of BMP-responsive genes is thought to be mediated by receptor-associated Smads (Smad1, 5 and 8) acting in concert with Smad4 and possibly other transcription factors. We therefore postulated that Smad1 and Smad4 could be involved in the up-regulation of p21 and examined the effect of Smad1 and Smad4 on p21 promoter activity in transient expression experiments in T-47D cells. The p21 promoter constructs used in these experiments are illustrated in Fig. 6A. We first tested the effect of Smad1 and Smad4 on a 'full-length' promoter construct containing 2.3 kb of p21 promoter DNA (–2300/+16) cloned upstream of the luciferase reporter gene. As shown in Fig. 6A, in the absence of co-transfected Smad1 and

Figure 2 BMP-2 is a potent inhibitor of breast cancer cell proliferation. (A) The effect of recombinant human BMP-2 (rhBMP-2) on breast cancer cell proliferation was tested in triplicate as described in Materials and Methods. For estrogen-sensitive cell lines, the cells were cultured for 10 days in 5% DCC-FBS medium containing E₂ (1 nM) alone or in combination with 1, 10, 50, 100 ng rhBMP-2/ml. (B) MDA-MB-231 and MDA-MB-468 cells were cultured in their respective media for 2 days. The medium was then refreshed and 50 ng/ml rhBMP-2 were added for 5 (MDA-MB-231) or 7 (MDA-MB-468) days. These results are representative of at least 3 independent experiments.

Smad4, BMP-2 alone caused an 81% increase in $-2300/+16$ p21 promoter activity. However, when T-47D cells were transfected with plasmids expressing Smad1 and Smad4 in the presence of BMP-2, we observed an average 3.7-fold increase in $-2300/+16$ p21 promoter activity. These results show that Smad1 and Smad4, when over-expressed, can mediate the BMP-2 signal to the p21 promoter.

A series of p21 promoter constructs were then made to further define the BMP-responsive region of the p21

promoter. Deletion of nucleotides -2300 to -212 relative to the transcription initiation site resulted in a promoter construct ($-211/+16$) that displayed approximately half the basal activity of the $-2300/+16$ construct, but that remained responsive to BMP-2. As shown in Fig. 6A, BMP-2 increased $-211/+16$ p21 promoter activity approximately 8.4 times (mean of six independent experiments) in the presence of co-expressed Smad1 and Smad4. On the other hand, deletion of all nucleotides upstream of -60 produced a p21 promoter construct with

Figure 3 BMP-2 up-regulates p21 protein levels in MCF7 and T-47D breast cancer cells. (A) MCF7 and T-47D cells were cultured for 3 days in medium containing 5% DCC-FBS and E₂ (1 nM). The medium was then refreshed and rhBMP-2 (50 ng/ml) was added to T-47D and MCF7 cells for 24 and 48 h respectively. These results are representative of 3 independent experiments. (B) p21 and p27 protein levels after BMP-2 treatment. Protein abundance is expressed as fold change relative to control (mean ± S.D. of 3 experiments).

approximately 8% the basal activity of the -2300/+16 construct, and completely abrogated the induction by BMP-2. In fact, the activity of the -60/+16 p21 promoter was similar to that of the promoter-less pGL3-Basic plasmid. These results suggested that elements important for basal and BMP-induced activity are contained between nucleotides -211 and -60 of the p21 promoter. This hypothesis was confirmed by deleting nucleotides between -215 and -61 of the full-length p21 promoter. The resulting promoter construct, -2300Δ(-215/-61)/+16, had approximately 13% the basal activity of -2300/+16 and was unresponsive to BMP-2 and Smads. To further map the BMP-2 responsive region, we deleted nucleotides -124 to -60 of the

-211/+16 construct. This region contains many binding sites for the Sp1 transcription factor which is involved in the induction of p21 by extracellular signals, including TGF-β and Smad3 (Datto *et al.* 1995a, Moustakas & Kardassis 1998, Owen *et al.* 1998, Billon *et al.* 1999, Sowa *et al.* 1999, Zhang *et al.* 1999, Lu *et al.* 2000). Deletion of the region between nucleotides -124 and -60 of the -211/+16 construct completely abrogated induction by BMP-2.

The experiments described above were performed using Smad1 and Smad4 in combination since previous reports have shown that these two Smads act synergistically on promoters to mediate the BMP-2 signal (Johnson *et al.* 1999, Hata *et al.* 2000). To confirm that Smad1 and Smad4 exert a synergistic stimulatory effect on p21 promoter activity, the -211/+16 p21 promoter construct was co-transfected with plasmids expressing Smad1 and Smad4, alone and in combination, in T-47D cells. As shown in Fig. 6B, BMP-2 alone caused a 40% increase in -211/+16 p21 promoter activity. Transfection of Smad1 or Smad4 alone did not increase p21 promoter activity in the absence of BMP-2 but weakly stimulated -211/+16 promoter activity in the presence of BMP-2 (2.4- and 1.6-fold respectively). However, co-transfection of Smad1 and Smad4 increased -211/+16 promoter activity by 80% in the absence of BMP-2 whereas a 5.3-fold increase was observed in the presence of BMP-2. Note that the magnitude of the synergistic effect of Smad1 and Smad4 was related to the dose of BMP-2 used (Fig. 6C). Treatment of T-47D cells transfected with the p21 promoter with 1, 10, 50 or 100 ng/ml BMP-2 alone caused 1.9-, 3.3-, 3.8-, and 4.1-fold increases in p21 promoter activity respectively. Similarly, co-transfection of Smad1 and Smad4 into T-47D cells treated with 0, 1, 10, 50 or 100 ng/ml BMP-2 induced p21-Luc activity in a dose-dependent manner by 3.9-, 6.3-, 13.3-, 16.4 and 16-fold.

Since induction of p21 mRNA occurs rapidly following BMP-2 treatment, we next examined if the induction of the p21 promoter by Smad1 and Smad4 was a rapid phenomenon. BMP-2 was added to cells that had been transfected with the -211/+16 p21 promoter alone or with Smad1 and Smad4 expression plasmids 24 h earlier. Because the basal activity of the p21 promoter increased slightly over time, relative luciferase activity at each time point is reported as the fold induction relative to the corresponding control (cells transfected with empty vector in the absence of BMP-2 for each time point). As shown in Fig. 6D, the induction of the p21 promoter by BMP-2 is very rapid since we detected a 2.9-fold induction of the relative luciferase activity in the Smad1/4 transfected group treated with BMP-2 at the earliest time point studied i.e. 3 h post treatment.

Activation of Smad1 by type I BMP receptors requires the phosphorylation of the C-terminal serine residues located at positions 462, 463 and 465 of Smad1

Figure 4 Time-course of the induction of p21 protein and mRNA by BMP-2 in MCF7 and T-47D breast cancer cells. MCF7 and T-47D cells were cultured for 3 days in medium containing 5% DCC-FBS and E₂ (1 nM). The medium was then refreshed and rhBMP-2 (50 ng/ml) was added for the indicated periods. Protein and mRNA were analyzed as described in Materials and Methods. (A) Western blot analysis of the p21 protein. (B) Northern blot analysis of p21 mRNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control.

(Kretzschmar *et al.* 1997). Replacing these three serine residues by alanine residues abrogates the ability of Smad1 to be activated by BMP-2 and, therefore, to activate transcription. To confirm that BMP-2-induced p21 promoter activation involves phosphorylation of Smad1, we tested the ability of a non-phosphorylatable mutant of Smad1 (Smad1 AAVA) to mediate p21 promoter activation in response to BMP-2.

As shown in Fig. 7A, expression of Smad1 alone or in combination with Smad4 in the presence of BMP-2 led to 2·8- and 11·3-fold increases, respectively, in -211/+16 p21 promoter activity in the presence of BMP-2. However, BMP-2-induced up-regulation of p21 promoter activity was abrogated when a plasmid expressing Smad1 AAVA was transfected alone or in combination with a Smad4-expressing plasmid. These results confirm that the C-terminal serine residues of Smad1 are necessary for Smad1 to mediate induction of the p21 promoter in response to BMP-2. Moreover, because the C-terminal

Figure 5 Protein synthesis is not necessary for the BMP-2-induced up-regulation of p21 mRNA in human breast cancer cells. T-47D cells were cultured for 3 days in medium containing 5% DCC-FBS and E₂ (1 nM). The medium was then refreshed and rhBMP-2 (10 ng/ml) was added in the presence of cycloheximide (CHX; 10 µg/ml) or actinomycin D (Act.D; 1 µg/ml) for 2 h. The Northern blots were performed as indicated in Materials and Methods. These results are representative of 3 independent experiments. GAPDH was used as a loading control.

Figure 6 Smad1, Smad4 and BMP-2 act synergistically to activate the p21 promoter in human breast cancer cells. (A) Schematic representation of the p21 promoter constructs and fold activation ($\pm$ s.d.) by BMP-2, Smad1 and Smad4. 'n' is the number of independent experiments. Cells were transfected with the indicated p21 promoter luciferase-reporter plasmids or Smad vectors and pRL-null renilla control vector as described in Materials and Methods. p21 promoter-driven firefly luciferase activity was normalized for renilla luciferase. (B) Smad vectors were transfected alone or in combination as described in Materials and Methods together with the -211/+16 p21 promoter construct. This experiment is representative of 3 independent experiments carried out in duplicate. (C) T-47D cells were transfected with the -211/+16 p21 promoter construct and pcDNA3 or Smad1 and Smad4 expression plasmids. The cells were then cultured for 24 h after which they received 1 to 100 ng/ml BMP-2 for 18 h. This experiment is representative of 3 independent experiments carried out in duplicate. (D) Time-course experiment of the induction of the p21 promoter by Smad1, Smad4 and BMP-2. Twenty-four hours post transfection, T-47D cells were treated with 50 ng/ml BMP-2 and harvested at the indicated times. Data are expressed as fold change relative to the control at each time point. These results are representative of two independent experiments carried out in duplicate.

serine residues of Smad1 are substrates for serine/threonine kinase receptors, these results suggest that endogenous BMP receptors are involved in mediating BMP-2-induced activation of p21 promoter constructs in transient expression experiments.

To confirm this hypothesis, we tested the effect of constitutively active type I BMP receptors on p21 promoter activity in transient expression assays. Plasmids expressing a constitutively active mutant of BMPR-1A/ALK3 (ALK3Q233D) or BMPR-1B/ALK6

Figure 7 Constitutively active ALK3 and ALK6 activate the p21 promoter. (A) C-terminal serines 462, 463 and 465 of Smad1 are necessary for transactivation of the p21 promoter by BMP-2. Cells were transfected with the -211/+16 p21 promoter luciferase-reporter plasmid or Smad expression vectors as described in Materials and Methods. After 24 h, the medium was refreshed and BMP-2 (100 ng/ml) or an equivalent volume of BMP-2 dissolution buffer was added for 20 h after which luciferase activities were measured. This experiment is representative of 3 independent experiments carried out in duplicate. (B) T-47D and (C) MDA-MB-231 cells were transfected with the -211/+16 p21 promoter luciferase-reporter plasmid and the indicated Smad expression vectors and/or a constitutively active form of ALK3 (ALK3Q233D) or ALK6 (ALK6Q203D). The transfected cells were harvested 48 h after transfection. p21 promoter-driven firefly luciferase activity was normalized for renilla luciferase. These experiments are representative of 3 independent experiments carried out in duplicate.

(ALK6Q203D) were cotransfected with the -211/+16 p21 promoter construct into T-47D cells, alone or in combination with plasmids expressing Smad4 and either Smad1 or Smad1 AAVA. As shown in Fig. 7B, expression of ALK3Q233D or ALK6Q203D alone (without cotransfected Smad expression plasmids) resulted in 2.3- to 3.5-fold increases in p21 promoter activity. On the other hand, coexpression of either ALK3Q233D or ALK6Q203D with both Smad1 and Smad4 resulted in 18.4- to 25.4-fold increases in p21 promoter activity. The stimulatory effect of constitutively active BMP receptors was abrogated when Smad1 AAVA was expressed in place of Smad1. Constitutively active type I BMP receptors exerted a similar stimulatory effect on p21 promoter activity in MDA-MB-231 cells, thereby confirming that this effect is not restricted to a single breast cancer cell line (Fig. 7C). These results provide

evidence that constitutively active type I BMP receptors can mimic the effect of BMP-2 on p21 promoter activity.

Discussion

The results presented in this manuscript extend previous observations on the role of bone morphogenetic proteins in the control of breast cancer cell proliferation and provide new insight into the mechanism of BMP-induced growth arrest in breast cancer cells.

Bone morphogenetic proteins are members of the TGF- β superfamily of cytokines and, like TGF- β , employ similar signal transduction pathways. While the role of TGF- β in the control of breast cancer cell proliferation has been extensively studied, considerably less is known about

the role of BMPs. Following the original observation of Liu *et al.* (1998) that BMP-2 and BMP-4 inhibit DNA synthesis in estrogen insensitive Hs578T cells, we have extended this observation to four estrogen-sensitive cell lines namely MCF7, CAMA-1, T-47D and ZR-75-1. The finding that BMP-2 can counteract the stimulatory effect of the most potent natural mitogen, estradiol, in these cells indicates that BMP-2 exerts a dominant growth inhibitory effect. Based on this observation it is tempting to speculate that the BMP pathway could potentially serve as a therapeutic target to achieve inhibition of breast tumor growth in women.

It has also been reported that BMP-2 is secreted by MDA-MB-231 and MCF7 cells but little is known of the intracellular mediators of BMP action in breast cancer cell lines (Arnold *et al.* 1999). In an earlier publication, we reported that Smad4 mRNA is expressed in four of the six cell lines tested here (Pouliot & Labrie 1999). CAMA-1 cells were not tested whereas MDA-MB-468 cells do not express Smad4 due to a deletion of the gene (Schutte 1999). In the present report we show that all six breast cancer cell lines tested express Smad1 mRNA. Smad4 is the co-Smad whereas Smad1 is one of three Smad proteins (Smad1, 5 and 8) that have been identified as mediators of BMP signalling (Massague & Wotton 2000). On the basis of this information, cells that express both Smad1 and Smad4 would be expected to respond in some manner to BMP unless other unknown genetic changes have occurred in these cells. In fact, five of the six breast cancer cell lines we tested were growth-inhibited by BMP-2. Only the Smad4-deficient MDA-MB-468 cells were resistant to the growth suppressive effect of BMP-2. While other genetic factors could account for the lack of growth inhibition by BMP-2 in MDA-MB-468 cells, these results suggest that Smad4 is required for BMP-induced growth arrest. Moreover, these data indicate that BMP-2 is likely to play an important role as an autocrine regulator of breast cancer cell proliferation. It will be of great interest to identify the factors that control BMP production and action in breast cancer cells.

To better understand the mechanism by which BMP-2 inhibits the growth of human breast cancer cells, we examined the effect of BMP-2 on the expression of CKIs in two of the five BMP-responsive cell lines, MCF7 and T-47D. Several CKIs, namely p15, p21 and p27, have been implicated in growth arrest induced by TGF- β (Hannon & Beach 1994, Datto *et al.* 1995a,b, Reynisdottir *et al.* 1995, Reynisdottir & Massague 1997). We focussed our attention on p21 and p27 because p21 and p27 are expressed in all of the BMP-responsive breast cancer cell lines we tested whereas the p15 gene is deleted in MDA-MB-231 cells (Gray-Bablin *et al.* 1997). We found that BMP-2 up-regulates p21 protein levels in both MCF7 and T-47D cells. This is in agreement with previous reports that have implicated p21 in BMP-induced growth arrest in hybridoma B, breast cancer and myeloma cells

(Ishisaki *et al.* 1999, Ghosh-Choudhury *et al.* 2000a,b, Kawamura *et al.* 2000). However, the mechanism of induction of p21 by BMP-2 has not been demonstrated. We demonstrate here that BMP-2 induces p21 at the mRNA level. In addition, we found that this effect is rapid, direct and mainly occurs at the transcriptional level since induction of p21 is sensitive to actinomycin D and resistant to cycloheximide. Contrary to p21, the levels of p27 protein were unaffected by BMP-2 treatment. Our results also demonstrate that p15 and p16 are not essential for the growth inhibition of breast cancer cells by BMP-2 because p15 is not expressed in MDA-MB-231 cells whereas p16 is not expressed in MCF7, ZR-75-1, T-47D and MDA-MB-231 cells, all of which are growth-inhibited by BMP-2 (Gray-Bablin *et al.* 1997).

We conducted a series of experiments using p21 promoter constructs to characterize further the mechanism of p21 up-regulation by BMP-2. In transient expression assays, BMP-2, Smad1 and Smad4 proteins activated the p21 promoter within three hours, supporting our hypothesis that induction of p21 occurs at the transcriptional level and does not require new protein synthesis. The rapid induction of the p21 promoter by Smad1/4 proteins is in agreement with previous studies that have shown that Smad1 is translocated to the nucleus only 30 min after BMP-2 treatment (Liu *et al.* 1996, Kretzschmar *et al.* 1997).

Deletion studies of the p21 promoter mapped the BMP-responsive region of the promoter to nucleotides located between positions -215 and -61 relative to the transcription initiation site. While the promoter elements that mediate the BMP response remain to be more precisely defined, the experiments we performed using a BMP-responsive p21 promoter (-211/+16) allowed us to confirm that Smad1 and Smad4 can transactivate the p21 promoter in response to BMP-2. The data also demonstrate that the C-terminal serine residues of Smad1 are necessary for BMP-2-induced activation of the p21 promoter in transient expression assays. These results suggest strongly that the induction of p21 promoter activity by BMP-2 is mediated by BMP receptors initiating BMP signal transduction by phosphorylating and activating Smad1. In fact, constitutively active type IA and type IB BMP receptors mimicked the effect of BMP-2. Based on these results, we propose that the signal transduction pathway involved in BMP-2-induced p21 promoter activation is in agreement with the general model proposed for signal transduction by Smad proteins.

Transcriptional regulation by Smad proteins can be achieved via various mechanisms involving Smad proteins acting alone or in concert with other transcription factors, most likely via direct interactions (Massague *et al.* 2000). This implies that a number of different regulatory elements may be involved in or required for activation of the p21 promoter by BMP-2. In fact, several putative Smad binding elements (5'-GNCT-3') are located between

positions -214 and -130 of the promoter. In addition, TGF- β and Smad3 response elements identified respectively by Datto *et al.* (1995a), Pardali *et al.* (2000) and Moustakas and Kardassis (1998) are located between positions -124 and -60. Since deletion of the region containing Sp1 binding sites located between positions -124 and -60 of the p21 promoter abrogated the response to BMP-2, we believe that Sp1 may be involved in the activation of the p21 promoter by BMP-2. This last hypothesis is reinforced by the very recently published papers that show that Smad4 could interact with Sp1 to regulate the p15, COL1A2, p21, and PAI-1 promoters (Datta *et al.* 2000, Feng *et al.* 2000, Pardali *et al.* 2000, Poncelet & Schnaper 2001). Since Smad1 is known to interact with Smad4, a complex involving Smad1, Smad4 and Sp1 could therefore explain the effects observed on the p21 promoter. A more detailed approach involving mutagenesis of discrete promoter elements will be required to define the exact mechanism of inhibition of cell growth by BMP-2.

While our work was in progress, Ghosh-Choudhury *et al.* (2000a,b) also reported that BMP-2 inhibits the proliferation of MCF7 and MDA-MB-231 cells and induces p21, thereby confirming our observations. In addition, Ghosh-Choudhury *et al.* reported that BMP-2 promotes the association of p21 with cyclin E, that it inhibits cyclin E-associated kinase activity, and that BMP-2 inhibits the phosphorylation of the retinoblastoma (pRb) protein. According to our results and those of Ghosh-Choudhury *et al.*, we can now propose a model for the inhibition of the growth of estrogen-sensitive human breast cancer cell lines. This model involves the activation of BMP receptors by BMP-2 which would then activate the Smad1 protein. Smad1 would then functionally cooperate with Smad4 to activate the transcription of the p21 promoter. Increased levels of the p21 protein would then bind and inhibit the cyclin E-cdk2 complex and arrest breast cancer cells in the G1 phase of the cell cycle through hypophosphorylation of the pRb protein.

In conclusion, this study shows that estrogen-sensitive human breast cancer cell lines are strongly inhibited by BMP-2 independently of their sensitivity to TGF- β . Moreover, we show that this growth inhibition involves the direct induction of the CKI p21 at the transcriptional level. Finally, we show that Smad1, Smad4, as well as ALK3 and ALK6, can mediate BMP-2-induced p21 promoter activity. These results provide new insight into the mechanisms by which the BMP-2 pathway inhibits the growth of human breast cancer cells.

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