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IDENTIFICATION OF BONE MORPHOGENETIC PROTEINS AND THEIR RECEPTORS IN HUMAN BREAST CANCER CELL LINES: IMPORTANCE OF BMP2

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The most frequent site of breast cancer metastasis is bone suggesting that some breast cancers express proteins that facilitate this process. We evaluated whether a highly metastatic breast cancer cell line, MDA-MB-231, and a less metastatic breast cancer cell line, MCF-7, contain bone morphogenetic proteins (BMP). Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrated that MDA and MCF-7 cells contain mRNAs for BMP receptors IA, IB and II. RT-PCR indicated the presence of mRNAs for BMPs 2 and 3 but not 4 and 7 in breast cells. Using a RT-PCR strategy with molecular beacons, we found that the mRNA for BMP2 in MDA cells was decreased by 75% after a sublethal dose of radiation. An ELISA using an antibody specific for BMP2 demonstrated that BMP2 protein was reduced after radiation of MDA cells. The mRNA for BMP2 was expressed to a lesser extent in MCF-7 cells than MDA cells and was not altered after radiation treatment of MCF-7 cells as demonstrated by molecular beacon RT-PCR. Recombinant human BMP2 decreased the proliferation of MDA cells to a greater extent than MCF-7 cells. These results expand the number of tissues that contain BMPs and demonstrate the effect of this signalling pathway of the growth state of these tissues.

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The most frequent site of metastasis of breast cancer is bone.[1,2] Paget's theory of metastasis (seed and soil) suggests that the migration of breast cancer cells to bone may result from adhesion or growth properties of breast cells for bone. An important signalling pathway in bone is bone morphogenetic proteins (BMP) and their receptors (BMPR).[3,4] From a therapeutic perspective, some BMPs can induce cartilage and bone formation in vivo.

BMPs and their receptors are members of the transforming growth factor β (TGF-β) superfamily.[3,4] Three bone morphogenetic protein receptors have been identified: two type I receptors (IA and IB) and one type II receptor. There are 15 BMPs reported and BMPs 2, 4 and 7 bind to BMPRs. Ligand binding and signal transduction of BMPs are regulated by a heteromeric complex of one of the type I receptors plus the type II receptor. TGF-β and the BMPs activate the SMAD family of transcription factors, which mediate the expression of some gene products.[5] TGF-β and the BMPs utilize common and unique SMAD activators and repressors for gene expression, suggesting cross-talk between these pathways.

Recent evidence suggests that the expression of the BMPs may not be restricted to bone tissues. Numerous studies have demonstrated the importance of the BMPs in various aspects of vertebrate body patterning.[6] In addition, the mRNAs for BMPRs and BMPs 2, 4 and 7 are expressed in prostate cancer cells.[7] Recombinant human BMP2 decreased prostate cell proliferation in presence of media containing serum or androgen.[8] This effect may be related to an increased expression of the mRNA for BMPRIB in prostate cells with androgen treatment. In foetal and postnatal mammary gland, the mRNAs for BMPs 2 and 4 have been detected.[9]

To the best of our knowledge, it has not yet been reported whether BMPs are expressed and have functional consequences in human breast cancer cells. Furthermore, since radiation therapy is commonly used for the treatment of breast cancer it will be

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TABLE 1. Relative mRNA expression of BMPs in two human breast cancer cell lines

	MDA cells		MCF-7 cells	
	− rad	+ rad	− rad	+ rad
BMPR1A	+ +	+ +	+	+
BMPR1B	+	+	+	+
BMPRII	+ +	+ +	+	+
BMP2	+ + +	+ (↓75%)	+ (weak)	+
BMP3	+ +	+ +	+	+
BMP4	−	−	−	−
BMP5	+	+	+	+
BMP6	+	+ (↓50%)	+	+
BMP7	−	−	−	−

The + represent the apparent concentration of the various BMPs and BMPRs based on semiquantitative RT-PCR. Note that we have not detected the presence of BMP4 and 7 despite repeated attempts. Also, the only mRNA that appears to be significantly altered following radiation exposure is BMP2 (see beacon results actual values.)

important to determine if the expression of BMPs is regulated by radiation in breast cancer cells. Our results demonstrate the presence of BMPRs and BMPs in human breast cancer cells. Sublethal radiation decreased BMP2 in MDA-MB-231 cells. Recombinant human BMP2 slowed the proliferation of breast cancer cells.

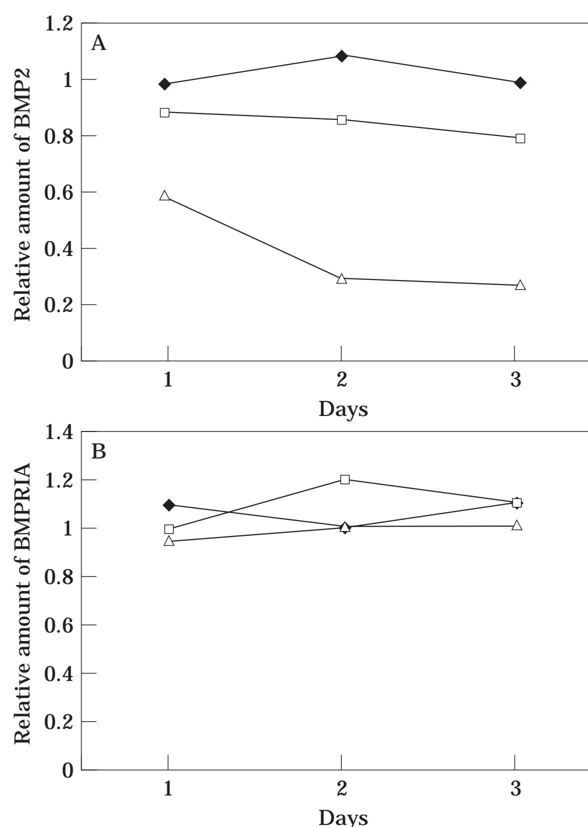

Figure 1. RT-PCR-effect of radiation on BMPRs, BMPs.

Graphs showing the relative amount of BMP2 (A) and BMPRIA (B) in MDA cells in the presence and absence of radiation for various times. The amount of BMP2 or BMPRI1 is relative to the amount of GAPDH. (◆), control; (□), 25R; (△), 500R.

RESULTS

We used semiquantitative RT-PCR to identify if the mRNAs for BMPs and their receptors were present in MDA and MCF-7 cells. RT-PCR identified the mRNAs for BMPRs IA, IB and II in MDA and MCF-7 cells (Table 1 for comprehensive listing). Using GAPDH as an internal reference, the amount of mRNA for the BMPRs appeared to be more abundant in MDA cells than in MCF-7 cells. RT-PCR also demonstrated the presence of mRNAs for BMPs 2, 3, 5 and 6 in both cell lines. The amount of BMP2 mRNA was greater in MDA cells than MCF-7 cells. The mRNA for BMP3 was low in both cell lines. The mRNAs for BMPs 4 and 7 were not detected in the breast cell lines (Table 1).

Next, we determined whether a subtherapeutic dose of radiation of MDA and MCF-7 cells would alter the mRNA expression of the BMPs in MDA and MCF-7 cells. Usng GAPDH as an internal reference in semiquantitative RT-PCR, the mRNA for BMP2 was decreased by irradiation of MDA cells (Fig. 1A). At 2–3 days post-irradiation (500 rads), BMP2 was decreased by approximately 75%. A lower dose of radiation also decreased the apparent amount of BMP2 (see 25 rads, Fig. 1A). None of other BMPs (except BMP6) or BMPRs mRNAs appeared to be substantially changed after radiation (for the results for BMPRIA, see Fig. 1B).

The use of semiquantitative RT-PCR has technical drawbacks. First, densitometry (or radiography) makes the quantitation of the PCF products on agarose gels. Second, the linear phase of the PCR reaction (and the PCR product) is determined by densitometry. Thus, the reaction for the internal reference standard GAPDH (24 cycles) must be conducted separately from the reaction for the BMPs (30 cycles) in order for the PCR products produced to be in the linear phase of the reaction. Third, artifact bands produced during the PCR reaction can hamper separation and quantitation of the PCR product. In order to develop a more direct and quantitative assay for measuring the amounts of PCR products we have developed a molecular beacon RT-PCR assay.

Molecular beacons are single-stranded oligonucleotide probes that become fluorescent when they bind to complementary DNA[8] (Fig. 2). Beacons possess a stem-loop structure where a fluorophore is linked to one end of the oligonucleotide and a quencher is linked to other end (Fig. 2). below the T_m

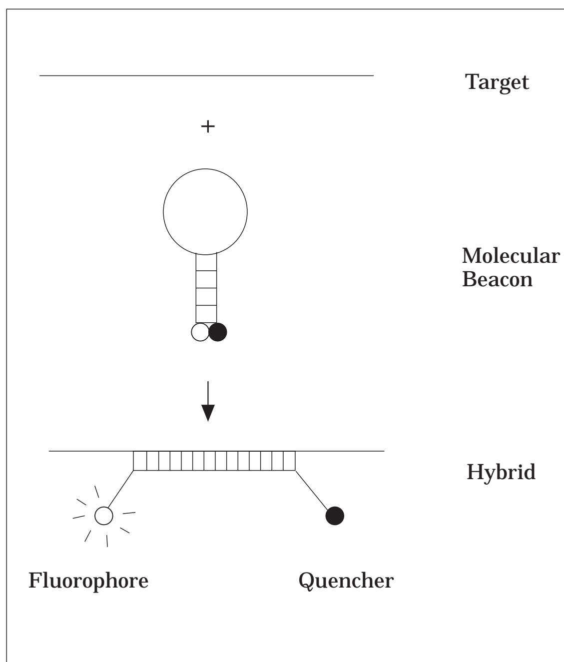

Figure 2. Structure and operation of molecular beacon.

Below the melting temperature, these molecules are non-fluorescent since the stem loop maintains the quencher close to the fluorophore. Above the melting temperature or in the presence of target, the beacon hybridizes to the target and the fluorophore is released from the quencher resulting in fluorescence.

or in the absence of target DNA, the beacon does not emit fluorescence since the hairpin stem keeps the fluorphore close to the quencher. Above the T_m or in the presence of the target DNA, the fluorphore is separated from the quencher and fluorescence is emitted. The advantages of using molecular beacons in RT-PCR are several. First, the fluorescence is measured in real-time in a spectrofluorometric thermal cycler and eliminates the need for agarose gels. Second, the beacons only bind to a perfectly complementary target sequence. Third, the amplification of PCR products can be directly measured by monitoring the fluorescence of the beacon as a function of the number of PCR cycles. Fourth, fluorescence has a greater linear range than ethidium bromide or ^{32}P-radiation, enabling more accurate quantitation of the PCR product. Fifth, multiple beacons can be used in a PCR reaction since the cycler can measure fluorescence of several beacons simultaneously. For example, a beacon specific for GAPDH (containing fluorescein) and a beacon for BMP2 (containing tetramethylrhodamine) can be used in a multiplex reaction to measure an internal reference standard and a specific mRNA.

Using a spectrofluorometric thermal cycler, the molecular beacon for GAPDH or BMP2 did not exhibit fluorescence at 50°C consistent with the beacon forming a stem-loop structure below its T_m. At 72°C, the beacons exhibited fluorescence since the T_m for the stem was lower than the extension temperature used in the PCR reaction. The beacon containing a 16-bp sequence specific for GAPDH demonstrated an increase in fluorescence with increasing cycle number in a PCR reaction with GAPDH primers (data not shown). Analysis of the PCR product on an agarose gel demonstrated a band that co-migrated with authentic GAPDH (data not shown). Similar to the GADPH beacon, the beacon specific for BMP2 exhibited fluorescence in the presence of BMP2 produced during a PCR reaction containing BMP2 primers (data not shown). The BMP2 produced in he beacon reaction co-migrated with authentic BMP2 on an agarose gel (data not shown).

We performed a PCR reaction containing PCR primers for GAPDH and BMP2 and with the beacons for GAPDH and BMP2. Using GAPDH as an internal reference, the amount of BMP2 produced in MDA cells was approximately 3-fold greater than the amount of BMP2 in MCF-7 cells (Fig. 3A). In MDA cells, radiation (500 rads) decreased the mRNA for BMP2 by 75% after 2 days (Fig. 3B). The mRNA for BMP2 in MCF-7 cells was not significantly altered by exposure to radiation (data not shown for beacon).

We used an BMP2 ELISA to determine the concentrations of BMP2 in MDA and MCF-7 cells in the presence or absence of radiation. As expected from the RT-PCR results, the concentration of BMP2 protein from MDA cells was significantly decreased by approximately 75% with 500 rads after 48 h (Table 2). The concentration of BMP2 from MCF-7 cells was lower than from MDA cells and was not significantly affected by radiation.

We measured the effect of recombinant human BMP2 on human breast cancer cell proliferation using the MTT assay. BMP2 50 and 100 ng/ml) significantly slowed the proliferation of MDA cells during a 5-day study (Table 3). In MDA cells, the effect of BMP2 (50 ng/ml) was slightly more effective than the effect of 0.5 ng/ml of TGF-β1. In MCF-7 cells, only BMP2 (100 ng/ml) significantly reduced proliferation (a decrease of 20% relative to the control) (data not shown).

DISCUSSION

Determining the factors and signalling pathways that mediates the effects of radiation will be important for designing new therapeutic interventions. The most frequent site of breast cancer metastasis is bone suggesting that some breast cancers express proteins that facilitate this process. We have evaluated whether a highly metastatic breast cancer cell line, MDA-MB-231, and a less metastatic breast cancer cell line,

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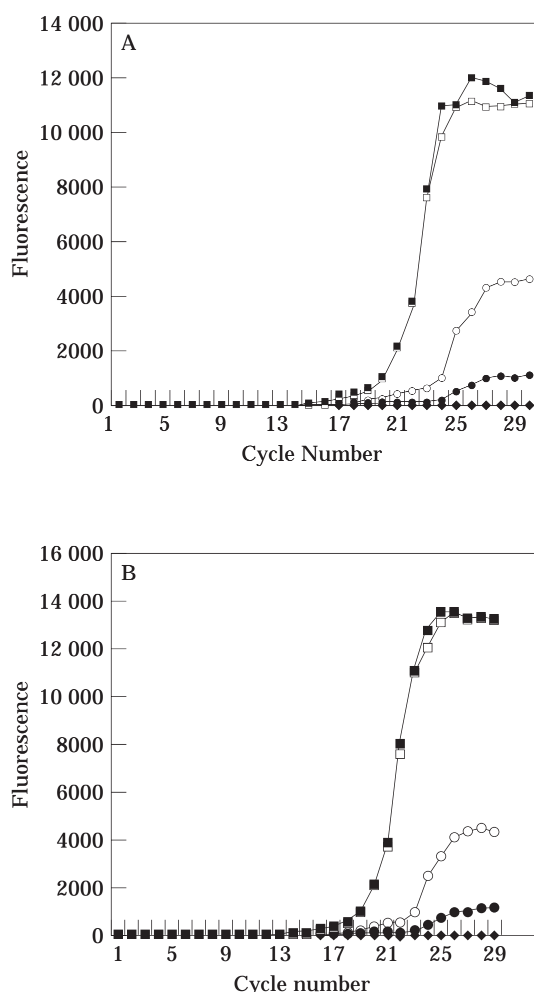

Figure 3. Results of molecular beacon RT-PCR.

First-strand cDNA synthesis was used in an RT-PCR reaction containing GAPDH and BMP2 beacons in a fluorescence thermal cycler. (A) The amounts of GAPDH and BMP2 from MDA and MCF-7 cells in the absence of radiation. (B) The amounts of GAPDH and BMP2 from MDA cells in the presence or absence of radiation (500 rads for 48 h). (◆), control; (□), GAPDH (MDA cells); (■) GAPDH (MCF-7 cells); (○), BMP2 (MDA cells); (●), BMP2 (MCF-7 cells).

MCF-7, contain bone morphogenetic proteins. MDA and MCF-7 cells possess mRNAs for BMPRs IA, IB and II and BMPs 2 and 3. The breast cell lines did not express the mRNAs for BMP4 and 7. In general, the mRNAs for the BMPRs and the BMPs were greater in MDA cells than in MCF-7 cells using semiquantitative RT-PCR. Using a RT-PCR strategy with molecular beacons we showed that the mRNA for BMP2 in MDA cells was decreased by 75% after a sublethal dose of radiation. An ELISA with an antibody for BMP2 demonstrated a decrease in BMP2 protein in MDA cells after radiation.

BMP2 decreased the proliferation of the breast cell lines but had greater inhibitory activity on MDA cells than on MCF-7 cells. Previous studies demon-

TABLE 2. Results of ELISA for BMP2 in human breast cancer cells

Cell Type	Time (h)	BMP2 (pg/ml)
MDA, 0R	0	341 ± 35
MDA, 0R	48	317 ± 30
MDA, 500R	0	378 ± 42
MDA, 500R	48	110 ± 13*
MCF-7, 0R	0	82 ± 10
MCF-7, 0R	48	72 ± 8
MCF-7, 500R	0	90 ± 8
MCF-7, 500R	48	73 ± 9

MDA or MCF-7 cells in serum-free media were irradiated at 0 or 500 rad and conditioned media collected after 0 and 48 h. BMP2 in the media was determined as described under Materials and Methods. The amount of BMP2 was normalized by the relative cell number (using the MTT assay) and expressed as the mean ± SE of two or three independent measurements. *Indicates that MDA cells after 500 rad (48 h) expressed significantly less BMP2 protein compared to MDA cells in the absence of radiation at 0 or 48 h or MDA cells after 500 rad at 0 h (Microsoft Excel, ANOVA).

TABLE 3. Effect of recombinant human BMP2 on human breast cancer cell proliferation after 5 days

MDA cells	Absorbance (Day 0)	Absorbance (Day 5)
Control	0.52 ± 0.05	1.25 ± 0.11
+0.5 ng/ml TGF-β		0.76 ± 0.08*
+2.5 ng/ml TGF-β		0.09 ± 0.08*
+5.0 ng/ml TGF-β		0.00 ± 0.0*
+5.0 ng/ml BMP2		1.10 ± 0.09
+50 ng/ml BMP2		0.65 ± 0.07*
+100 ng/ml BMP2		0.44 ± 0.06*

Cells (1000/well) were incubated in media containing 0.1% serum with vehicle, 0.5 ng/ml TGF-β1 or 100 ng/ml BMP2 for a total of 5 days. The media and the various factors were changed after 3 days. The relative cell number was measured using the MTT assay as described in the Methods. The data are the mean of two independent experiments with three replicates. *Indicates that the growth of MDA cells was significantly lower than the growth of the control cells (Microsoft Excel, Anova).

strated the presence of the TGF-β signalling pathway in breast cells and its importance in maintaining the growth state of the cells.[11,12] Our results demonstrate the presence of another subfamily of TGF-β factors and receptors in breast cancer cells. One theory of malignant conversion of normal to cancer breast cells involves the loss (or a decrease) in the functioning of the TGF-β pathway. Some reports have shown that breast cells less responsive to TGF-β express fewer type II receptors (TGF-β RII) than cells more responsive to TGF-β. Some MCF-7 cells have low amounts of TGF-β RII, providing an explanation for the lack of inhibitory activity by TGF-β on MCF-7 cells in culture.[13] The MDA cells in culture appear to respond to TGF-β and contain both TGF-β RI and II. Interestingly, using semiquantitative RT-PCR the mRNAs for the BMPR and BMPs (especially BMP2) were present in greater quantities in MDA cells compared to MCF-7 cells. This observation is consistent with our findings that the proliferation of MDA cells is affected to a greater extent than the proliferation of MCF-7 cells by

recombinant BMP2. The explanation for the greater response of MDA compared to MCF-7 cells is probably related to the apparently greater concentration of BMPRs in MDA compared to MCF-7 cells as determined by semiquantitative RT-PCR. The growth inhibitory effects of BMP2 have been previously observed with various types of tumour cells from patients including human breast cancer cells.[14] However, these studies did not address of whether the responses produced by BMP2 correlated the presence or absence of BMPRs.

One successful treatment for breast cancer is radiation therapy. Our results herein demonstrate that BMP2 in MDA cells is decreased by 75% after radiation. Initially, we hypothesized that BMP2 might be stimulatory for breast cancer growth providing an explanation for its downregulation following radiation treatment. However, the observation that BMP2 decreases the proliferation of human breast cancer cells (and prostate cells) suggests that it may play a role in repressing the activity of a growth inhibitory pathway, such as TGF-β. In prostate cells, the inhibitory effect of BMP2 was correlated with the increased expression of the mRNA for BMPR IB by androgen. Further experiments will be necessary to discern the exact mechanism(s) for the inhibition of cancer cell growth by BMP2.

We have demonstrated that human breast cancer cells contain BMPs and that radiation substantially decreases BMP2. Also, BMP2 inhibits the proliferation of breast cancer cells. These results expand the number of tissues that contain BMPs and demonstrate the effect of this signalling pathway of the growth state of these tissues. Further characterization of the effect of the BMP pathway on the growth and metastasis of breast cancer cells is warranted.

MATERIALS AND METHODS

Materials

All cell culture media and supplies were obtained from GIBCO BRL (Gaithersburg, MD) except where indicated. Purified human recombinant TGF-β1 was purchased from R & D systems (Minneapolis, MN). The primers for RT-PCR were synthesized by Research Genetics (Huntsville, AL). The molecular beacons were synthesized by Midland Certified Reagent Company (Midland, TX). The recombinant human BMP2 and the BMP2 antibody were generously provided by Genetics Institute (Cambridge, MA).

Cell culture

Human breast cancer cell lines MDA-MB-231 (oestrogen receptor negative) and MCF-7 (oestrogen receptor positive) were originally obtained from ATCC. The MCF-7 and MDA cell lines were grown in DMEM (Phenol-red free) with 10% fetal calf serum (HyClone Laboratories Inc., Logan, UT) and 1 μg/ml gentamycin. Working cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and the media was changed every 2 or 3 days. For radiation experiments, cells (2×10^4) were seeded into 10-cm^2 plates (Becton Dickson, Lincoln Park, NJ) at least 24 h before the assay. Prior to radiation, cells were washed twice in medium with serum-free media, and placed into 2 ml of serum-free media. Radiation was produced from a Philips X-ray machine at 160 kV, 10 mA, 50 cmtsd and a 0.5 mm A1 filter was used. For preparation of conditioned medium, the medium was collected in siliconized tubes, spun at $1000 \times g$ for 3 min to remove cell debris and then flash-frozen at −70°C.

RT-PCF assay

RNA was extracted from the cells using TRIZOL (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by incubation with 0.25 μg of oligo dT (Gibco BRL, Gaithersburg, MD) at 70°C for 5 min, followed by 4°C for 5 min and then incubated with reverse transcriptase buffer [10 mM $MgCl_2$, 50 mM KCl, 50 mM Tris-HCl, pH 8.3, 10 mM DTT, 0.5 mM spermidine (Promega Corp., Madison, WI)], dNTPs at 2 mM (Pharmacia Biotech, Piscataway, NJ) and 16 U of AMV reverse transcriptase (Promega Corp., Madison, WI) for 42°C for 1 h. Aliquots of first-strand cDNA (1 μl) were used in PCR reactions containing 5 pmol of 5′ and 3′ primers, PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin [Fischer Scientific, Pittsburgh, PA)], dNTPs at 0.2 mM (Pharmacia Biotech, Piscataway, NJ) and 0.2 U of Taq polymerase (Fischer Scientific, Pittsburgh, PA). The expected sizes of the PCR products and the primer sequences used are as follows:

GAPDH, 451 bp, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 3′-TCC ACC ACC CTG TTG CTG TA-5′;
BMPRIA, 1401 bp, 5′-GCA TAA CTA ATG GAC ATT GCT-3′-GGT AGC CTC CTC TTT GAG AT-5′;
BMPR-IB, 634 bp, 5′-GCA GCA CAG ACG GAT ATT GT-3′ and 3′TCA CAA CTA CTC CGT ACT TT-5′;
BMPRII, 694 bp, 5′-ACG GGA GAG AAG ACG AGC CT-3′ and 3′-GCT TGG GAG AGA ACT AGA TC-5′;
BMP2, 671 bp, 5′-TCA TAA AAC CTG CAA CAG CCA ACT CG-3′ and 3′-CAC CCA CAG CGA TCA TGT CG-5′;
BMP3, 623 bp, 5′-AGG TCT CTG AAC ACA TGC TG-3′ and 3′-GGT GTC CCT GTA AGC TTG AT-5′;
BMP4, 397 bp, 5′-GAC CTA TGG AGC CAT TCC GTA-3′ and 3′-TCA GGG ATG CTG CTG AGG TT-5′; and
BMP7, 276 bp, 5′-CAG CCT GCA AGA TAG CCA TT-3′ and 3′-GAG CAG GAA GAG ATC CGA TT-5′.

For the GAPDH primer set, the reaction profile was an initial denaturation at 94°C for 2 min and then 24 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by a final extension at 72°C for 5 min. For the BMP3, 4 and 7 primer sets the reaction profile was an initial denaturation at 94°C for 2 min and then 30 cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 2 min followed by a final extension at 72°C for 10 min. For the BMPRIA, BMPRIB, BMPRII and BMP2 primer sets the reaction profile will be an initial denaturation step at 95°C for 3 min followed by 30 cycles

each at 95°C for 3 min followed by 30 cycles each at 95°C for 45 s, 60°C for 1 min and 72° C for 90 s followed by a final extension at 72°C for 10 min. The number of cycles utilized for each set was determined to be within the linear range of amplification of the particular product. PCR reactions were performed using a MJ Research Peltier thermal cycler (Watertown, MA). Reaction products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Semi-quantitation of the ethidium bromide stained bands was performed using the IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA). For example, the expression of the BMP2 was based on comparison with the internal reference, GAPDH. Several independent experiments indicated that the housekeeping genes GAPDH and β-actin were not changed after radiation exposure (data not shown).

For molecular beacon RT-PCR, molecular beacons[10] were designed and produced for GADPH and BMP2. The sequences for beacons were identified by entering the sequences within the PCR products into the Overgo Script program (John McPherson, Genome Sequencing Center, Washington University, St Louis, MO). Further refinements in the sequences for the beacons were done by identifying the T_m. The sequence of the stem was performed using the 80% percent G:C rule.[7] A DNA folding program to estimate the stability of the hairpin stem (available at *http://www.ibc.wustl.edu/~zuker/dna/form1.shtml*) was used.

The GAPDH beacon was

5′-[6 flourescein (FA)] GGT CGA GAG GCA GGG ATG ATG CGA CC (DABC)-3′ and
BMP2 beacon was
5′-[tetramethylrhodamine (TMR)] GGA CGC CAC CTG CTT GCA TTC CGT CC (DABC)-3′.

The beacons were purified using anion-exchange HPLC and the composition was analysed by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. PCR reactions (50 μl) contained both the GAPDH and BMP2 primer sets, units of Amplitaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ), 200 μM of the dNTPs, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0). The first-strand cDNA synthesis was used to initiate each reaction. The thermal cycling program consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at 50°C, and 30 s at 72°C on a spectrofluoremetric thermal cycler (Applied Biosystems Prism 7700). Fluorescence was monitored during the 50°C annealing steps.

MTT assay

For the proliferation assay with recombinant human BMP2 or TGF-β, MDA or MCF-7 cells were seeded into 96-well plates (appr. 1000 cells/well) at least 24 h prior to the start of the experiment. The cells were rinsed with media containing 0.1% serum and incubated in media containing 0.1% serum for 8 h. The cells were then incubated with fresh media containing 0.1% serum alone or with 5, 50, and 100 ng/ml BMP2 or 0.5, 2.5 and 5 ng/ml TGF-β. The media and the various factors were changed after 3 days. For the determination of relative cell number the medium was removed and the cells were rinsed twice with serum-free medium and incubated with 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO) suspended in DMEM for 2 h at 37°C.[8,9] The MTT medium was removed and the cells were quickly resuspended in dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO). The final solution was measured at an absorbance of 540 nm in a spectrophotometer (Spectronic 1001, Bausch & Lomb). The assays were repeated in at least two independent experiments with three replicates.

BMP2 ELISA

The amount of BMP2 in the conditioned media was determined with a BMP2 enzyme-linked immunosorbent assay using anti-BMP2 monoclonal antibody (hAb2/5.10.24, Genetics Institute, Cambridge, MA). The optical densities were measured in a Bio-Tek Microplate Autoreader (Winooski, VT). The concentration of BMP2 in the conditioned media was obtained by comparing the optical densities with recombinant, human BMP2. The values for BMP2 in the conditioned media was normalized for relative number of cells using values obtained in the MTT assay. Each assay was repeated in at least two independent experiments with three replicates.

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REFERENCES

1. Coleman RE, Ruben RD The clinical course of bone metastases from breast cancer. Br J Cancer 55:61–66.
2. Yoneda T, Sasaki A, Mundy GR (1994) Osteolytic bone metastasis in breast cancer. Breast Cancer Res Treat 32:73–84.
3. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534.
4. Wozney JM, Rosen V (1998) Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop 346:26–37.
5. Zhou S, Buckhaults P, Zawel L, Bunz F, Riggins G, Dai JI, Kern SE, Kinzler KW, Vogelstein B (1998) Targeted deletion of SMAD4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. Proc Natl Acad Sci USA 95:2412–2416.
6. Thomsen GH (1997) Antagonism within and around the organizer BMP inhibitors in vertebrate body patterning. Trends Genet 13:209–211.
7. Harris SE, Harris MA, Mahy P, Wozney J, Feng JQ, Mundy GR (1994) Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate 24:204–211.

8. Ide H, Yoshida T, Matsumoto N, Aoki K, Osada Y, Sugimura T, Terada M (1997) Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. Cancer Res 57:5022–5027.

9. Phippard DJ, Weber-Hall SJ, Sharpe PT, Naylor MS, Jayatalake H, Maas R, Woo I, Roberts-Clark D, Francis-West PH, Liu YH, Maxson R, Hill RE, Dale TC (1996) Regulation of MSX-1, MSX-2, BMP-2 and BMP-4 during fetal and postnatal mammary gland development. Dev 122:2729–2737.

10. Tyagi S, Kramer FR (1996) Molecular beacons probes that fluoresce upon hybridization. Nature Biotechnol 14:303–308.

11. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB (1987) Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48:417–428.

12. Arrick BA, Korc M, Derynck R (1990) Differential regulation of expression of three transforming growth factor beta species in human breast cancer cell lines by estradiol. Cancer Res 50:299–303.

13. Chen C, Wang X-F, Sun L (1997) Expression of transforming growth factor beta (TGFbeta) Type III receptor restores autocrine TGFbetal activity in human breast cancer MCF-7 cells. J Biol Chem 272:12 862–12 867.

14. Soda H, Raymond E, Sharma S, Lawrence R, Cerna C, Gomez L, Timony GA, Von Hoff DD, Izbicka E (1998) Antiproliferative effects of recombinant human bone morphogenetic protein-2 on human tumor colony-forming units. Anti-Cancer Drugs 9:327–331.