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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)* Bone morphogenetic protein-2 (BMP-2), an osteoblastic growth and differentiation factor is found to inhibit estradiol-stimulated growth of estrogen receptor (ER) containing MCF7 human breast cancer cells as well as the ER negative but EGF-responsive growth of MDA MB 231 cells. The underlying mechanism for growth inhibition by BMP-2 involved inhibition in retinoblastoma protein (pRb) phosphorylation, which is essential for the cells to proliferate. We also demonstrated that BMP-2 inhibits mitogen activated protein kinase (MAPK) activity in these breast cancer cells. Since MAPK is stimulated by most of the cell proliferating mitogens, inhibition of MAPK activity will result in potent inhibition of cell proliferation. Thus BMP-2 is capable of negatively regulating two essential pathways of cell proliferation and could prove to be a good target for drug development in breast cancer cell growth, if it proves to do the same *in vivo*. In order to test the validity of our *in vitro* finding we developed a mouse model where we can study the effect of BMP-2 on breast tumor growth. In addition, since bone is the primary site of breast cancer cell metastasis, we developed an assay to quantitate osteolysis in our mouse model caused by breast tumor growth. Using RTPCR we found that breast tumor cells express low or undetectable levels of BMP receptors (BMPR). We have overexpressed BMPR and are in the process of overexpressing BMP-2 in breast cancer cells. We will test the transfected cells for growth and metastatic potential *in vivo*. The results will lay a background for testing BMP-2 as a therapeutic agent for inhibition of breast cancer cell growth and skeletal metastasis.

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INTRODUCTION:

The aim of our study is to identify novel therapeutic application for bone morphogenetic protein-2 (BMP-2) in breast cancer cell growth and skeletal metastasis. Earlier we have shown that BMP-2 inhibits estradiol-induced growth of MCF7 human breast cancer cells (Biochimica et Biopysica Acta, 1497: 186-196, 2000). In that study we have demonstrated the mechanism by which BMP-2 inhibits the growth of MCF7 cells. Breast tumors fall in two broad categories: estrogen receptor (ER) positive (growth of these cells are stimulated by estradiol) and ER negative (cell growth is not sensitive to estradiol). Following the work with ER positive MCF7 cells we have studied growth inhibition of ER negative MDA MB 231 human breast cancer cells by BMP-2 and the underlying mechanism (Biochem Biophysical Research Comm. 272: 705-711, 2000). We found that BMP-2 inhibits growth of both types of cell lines using similar mechanism. Breast tumors grow very aggressively in the primary site (breast) as well as the secondary sites (lung, bone etc.). Currently we are testing whether BMP-2 can inhibit breast tumor cell growth at the primary site and at the secondary bone site. We have developed and standardized an *in vivo* model for studying breast tumor cell growth and their bone metastasis in nude mice. This model will be invaluable for understanding the role of BMP-2 *in vivo* in inhibiting breast tumor growth and bone metastasis. The results will help to establish BMP-2 as a clinically important candidate for controlling breast cancer cell growth and osteolytic metastasis.

BODY:

Task 1. **To correlate the levels of BMP-2 and BMP-2 receptor expression in breast cancer cell lines and tissue samples with their bone metastasis status (months 1-12)**

- **Perform Northern as well as the RT-PCR analysis of BMP-2 and BMPR expression in different ER negative and ER positive cells and in different breast tumor tissue samples (months 1-6).**

- **Analyze the expression level of BMP-2 and BMPR different ER negative and ER positive cells and in different breast tumor tissue samples (months 6-7).**

In last report I provided data to show that the breast cancer cells express BMP receptors at a very low level whereas expression of BMP-2 was abundant in these cells. I am experiencing some delay in obtaining the breast cancer tissue samples. Upon receiving the samples I will analyze the expression of BMP-2 and its receptors in them.

- **Correlate the metastatic potential of the breast cancer cells using the animal model described above *by in vivo* study (months 6-12).**

- **Correlate metastatic property of the tissue samples with the expression levels of BMP-2 and BMPR in these samples (months 7-8).**

We have made substantial progress in establishing a bone metastatic model for breast cancer cells in mice. The models available for studying bone metastasis of breast cancer cells to date involved injection of breast cancer cells into left ventricle of mouse heart. Though extensively used by a few groups of researchers, we experienced severe practical drawbacks while using this model in our laboratory. Thus during the past year we were working to develop a mouse model to study the metastasis of breast cancer cells to bone. We have injected breast cancer cells into the mammary fat pad of female nude mice and followed the development of tumor and osteolytic metastasis in these mice. We have used both MDA MB231 (estrogen receptor [ER] negative) and MCF7 (ER positive) human breast cancer cells for this study. The tumor size obtained from a representative experiment is given in Table 1. Mice injected with both the cell lines developed osteolytic metastasis within 15 days following the injection. Fig. 1 shows the extent of osteolysis obtained from injection of these cell lines into mice. Table 2 shows the quantitation of the area of osteolysis at different days after injection in this newly developed mouse model of bone metastasis from breast cancer. As shown, this model is ideal for studying the tumor formation as well as the osteolytic metastasis from breast cancer cell injection. Using this model we will study the effect of BMP-2 on growth of breast cancer cell *in vivo*. We will also study the effect of BMP-2 on osteolytic metastasis driven by these

TABLE 1
Tumor Volume (mm^3)

Cell line		Day 15	Day 20	Day 25
MCF 7	R	24.7	32	40
	L	71	147	192
MDA MB 231	R	1529	2818	2588
	L	620	1256	1584

TABLE 1. Tumor volume for MCF7 and MDA MB 231 cells in nude mouse. Cells were injected in in right (R) and left (L) mammary fat pads of female nude mice and the tumor volumes were noted at days 15, 20 and 25.

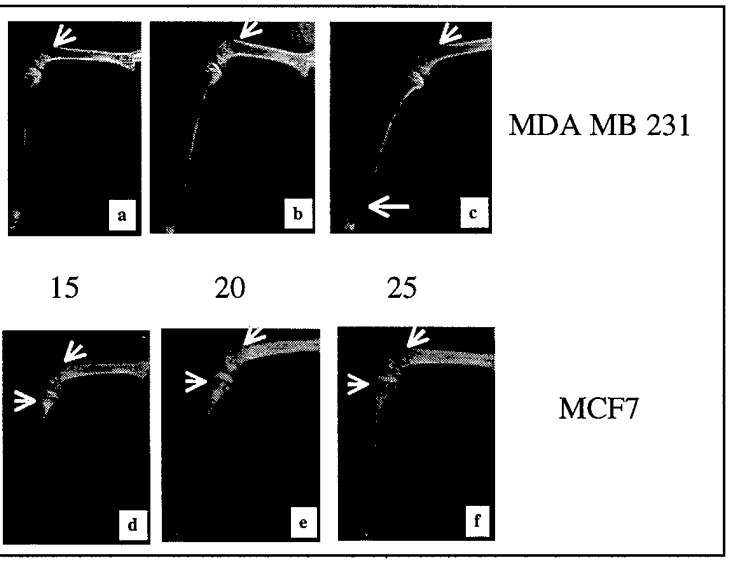

Fig 1. Osteolytic metastasis in nude mice injected with MDA MB 231 and MCF7 breast cancer cells. Mice injected with breast cancer cells (as described in Table 1) are X-rayed for detection of osteolytic metastasis at days 15 (a, d), 20 (b, e) and 25 (c, f) after the injection. Arrows show the position of visible osteolysis.

Table 2: *Area of osteolysis in nude mice injected with breast cancer cells. Osteolysis is quantitated from the X-ray films shown in Fig.1, using a computerized film reader. The area of osteolysis is expressed in the unit of mm^2.*

Cell Line	Day 15	Day 20	Day 25
MCF 7		0.080	0.633
MDA MB 231	0.123	0.125	0.177

breast cancer cells in the same mouse. We will attempt to collect the breast tumor tissue samples from Baylor college of Medicine at Houston. We will test a few of those cell lines in our in vivo animal model.

Task 2. **To prove the clinical importance of BMP-2 expression and bone metastasis of breast cancer cells by genetically engineering the BMP-2 status of breast cancer cells and study the metastatic phenotype of the altered cells using an *in vivo* model of metastasis (months 6-24).**

- **Genetical alteration of the BMP-2 protein and receptor status (in cells chosen based on the results of task 1) by using stable transfection of corresponding cDNAs. These experiments will be initiated simultaneously with some of the experiments described in the Task 1. This period will be necessary to establish all the clonal cell lines and analyze their BMP protein and receptor expression profiles and finally to test the chemotactic properties of these cells. As the cell lines will be available, they will be tested for their metastatic potential by the in vivo assay in the animal model described above (months 6 - 24).**

Since different breast cancer cell lines showed low abundance of BMP receptors IA and IB (BMPR IA and IB), we have attempted to overexpress these receptors in MDA MB 231 and MCF7 cell lines. We have stably transfected hemagglutinin (HA)-tagged BMPR IA and IB expression plasmids into both these cell lines and have isolated mass cultures of clones and individual clonal cell population. Currently we are analyzing the cell lines for expression of these receptors by Western blot analysis. Fig.2 shows a preliminary experiment in our attempt to analyze these cell lines using Western blotting with anti hemagglutinin antibody as a probe. The results show that both the clones and the mass cultures express the HA-tagged receptors. We will further analyze these cell populations and will characterize them by their growth kinetics in

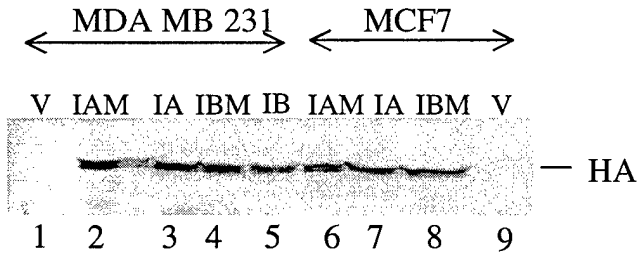

Fig.2. Analysis of Breast cancer cells expressing BMPR IA and IB. MDAMB231(lanes 1-5) and MCF7 (lanes 6-9) cells were stably transfected with HA-tagged IA and IB expression plasmids. Mass cultures (IAM, IBM) and clonal cultures (IA, IB) were analyzed for expression of receptors by Western analysis using HA antibody. Lanes 1 and 9 represents empty vector (V) transfected cells .

response to exogenous BMP-2. We will select a few clonal cell lines expressing high levels of BMPR IA or BMPR IB and will test them in our *in vivo* mouse model system for their growth potential and osteolytic metastasis.

Task 3. **To study differential effect of BMP-2 on ER-positive and ER-negative breast cancer cell growth and to investigate the underlying mechanism (months 6-18).**

- **Study effect of BMP-2 on ER-negative breast cancer cells *in vitro* by Flowcytometric analysis of cell cycle progression (months 6-8).**

Study the underlying mechanism of BMP-2 induced growth regulation of breast cancer cells. This part will include determination of the effect of BMP-2 on the MAPK pathway by enzymatic activity assay for MAPK, studying growth kinetics of breast cancer cells in the presence of MEK inhibitor and identification of other targets for BMP-2 action in these cells (months 9-24).

Last year we reported the mechanism by which BMP-2 inhibits estradiol-induced growth of MCF-7 cells (BBA 2000). Following that we tested the effect of BMP-2 on growth inhibition of estrogen receptor negative (ER –ve) MDA MB 231 human breast cancer cells. This work has been published in BBRC (BBRC 272: 705-711, 2000). Please see attached reprint in the Appendix, which gives the details of this part of the work. We found that BMP-2 inhibited EGF-induced growth of MDA MB 231 cells. We studied the mechanism of this growth inhibition and found that BMP-2 increased p21 expression, inhibited cyclin dependent kinase (cdk) activities and finally inhibited phosphorylation of pRB. This resulted in hypophosphorylated pRB which was inhibitory to cell cycle progression and thus leads to decreased rate of cell proliferation. These results were similar to our finding on the mechanism by which BMP-2 inhibits estradiol induced growth of MCF-7 cells. This shows that BMP-2 is capable of inhibiting breast cancer cell growth irrespective of their ER status. This makes BMP-2 a very potent growth inhibitory agent as it can inhibit both nuclear and cytoplasmic events regulating cell proliferation of different types of breast cancer cells.

Task 4. **To correlate the growth inhibitory effect of BMP-2 on ER-positive breast cancer cells *in vivo* (months 18-36).**

- **To study autocrine BMP-2 action (*in vitro*) : Analysis of effect of estradiol on growth kinetics of MCF-7 cells stably transfected with BMP-2 cDNA (months 18-24).**

Our earlier attempts to stably transfect MCF7 cells using a CMV promoter driven BMP-2 cDNA did not result in any clonal population of cells overexpressing BMP-2. Recently we have recloned BMP-2 cDNA in PCDNA3 expression vector (Stratagene) and have tested the expression of BMP-2 from this plasmid in transient transfection experiments. Currently we are in the process of stably transfecting MCF7 cells with the new BMP-2 expression plasmid.

- **To study autocrine BMP-2 action (*in vivo*) : Xenograft tumor formation assay of MCF-7 cells stably transfected with BMP-2 cDNA or vector alone (months 24-36).**

Towards this task we have standardized the *in vivo* model for studying the effect of BMP-2 overexpression in MCF7 cells in mice. Once we have isolated MCF7 cells overexpressing BMP-2 we will test them using our *in vivo* model of nude mice.

Key Research Accomplishments:

- Identification of BMP-2 as a potential *in vitro* growth inhibitor for the both ER positive and ER negative breast cancer cells.
- Understanding of the underlying mechanism of BMP-2-induced inhibition of breast cancer cell growth *in vitro*.
- Establishment of *in vivo* mouse model for studying breast cancer cell growth and skeletal metastasis.

Reportable Outcome:

- **Manuscript:** Bone morphogenetic Protein-2 blocks MDA-MB231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation. Nandini Ghosh-Choudhury, Kathleen Woodruff, Wenbo Qi, Anthony Celeste, Sherry L. Abboud and Goutam Ghosh Choudhury. Biochem Biophysical Research Comm. 272: 705-711, 2000.

- **Manuscript:** Bone morphogenetic Protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells. Nandini Ghosh-Choudhury, Goutam Ghosh-Choudhury, Anthony Celeste, Paramita M. Ghosh, Marissa Moyer, Sherry L. Abboud and Jeffrey Kreisberg. Biochimica et Biopysica Acta, 1497: 186-196, 2000.

Conclusions:

We have shown that bone morphogenetic protein-2 (BMP-2) can significantly inhibit the growth of breast cancer cells *in vitro*. We have studied this growth inhibitory role of BMP-2 in estrogen-responsive and non-responsive breast cancer cells and have found that it inhibits the growth of both the cell types with equal potency. In estradiol-responsive human breast cancer cells, BMP-2 can inhibit estradiol-induced growth of these cells. BMP-2 also inhibits EGF-induced growth of ER negative MDA MB 231 human breast cancer cells. We have also identified the mechanism by which BMP-2 inhibits the growth of these cells (Biochimica et Biopysica Acta, 1497: 186-196, 2000; Biochem Biophysical Research Comm. 272: 705-711, 2000). We have developed *in vivo* mice model system to test this *in vitro* phenomenon. If BMP-2 is found out to be as effective *in vivo*, then BMP-2 will prove to be a clinically important molecule for the breast cancer patients.

APPENDIX

Manuscripts

1. Bone morphogenetic Protein-2 blocks MDA-MB231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation. Nandini Ghosh-Choudhury, Kathleen Woodruff, Wenbo Qi, Anthony Celeste, Sherry L. Abboud and Goutam Ghosh Choudhury. Biochem Biophysical Research Comm. 272: 705-711, 2000.
2. Bone morphogenetic Protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells. Nandini Ghosh-Choudhury, Goutam Ghosh-Choudhury, Anthony Celeste, Paramita M. Ghosh, Marissa Moyer, Sherry L. Abboud and Jeffrey Kreisberg. Biochimica et Biopysica Acta, 1497: 186-196, 2000.

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Bone Morphogenetic Protein-2 Blocks MDA MB 231 Human Breast Cancer Cell Proliferation by Inhibiting Cyclin-Dependent Kinase-Mediated Retinoblastoma Protein Phosphorylation

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Bone morphogenetic protein-2 (BMP-2) has been shown to act as an antiproliferative agent for a number of different cell types. We show that BMP-2 dose-dependently inhibits growth of MDA MB 231 human breast cancer cells. Epidermal growth factor (EGF) stimulates DNA synthesis and entry of these cells into the S-phase. BMP-2 inhibits EGF-induced DNA synthesis by arresting them in G1 phase of the cell cycle. BMP-2 increases the level of cyclin kinase inhibitor p21. Furthermore, we show that exposure of MDA MB 231 cells to BMP-2 stimulates association of p21 with cyclin D1 and with cyclin E resulting in the inhibition of their associated kinase activities. Finally, BMP-2 treatment is found to cause hypophosphorylation of the retinoblastoma protein (pRb), a key regulator of cell cycle progression. Our data provide a mechanism for the antiproliferative effect of BMP-2 in the breast cancer cells. © 2000 Academic Press

Key Words: **BMP-2; pRb; p21; breast cancer cells.**

The growth of breast cancer cells is regulated by a variety of steroid hormones and growth factors. In the late stage of hormone-dependent tumor cell growth, the cells become hormone-independent. One such human breast tumor cell line, MDA MB 231 (MDA), derived from a metastasized human adenocarcinoma, is estrogen-independent (1). However, they maintain the responsiveness to growth factors, such as epidermal growth factor (EGF) (2). The proliferative signals generated in the cytosol integrate into the nucleus to activate the cyclins and cyclin-dependent kinases (CDKs) that regulate the cell cycle progression of breast tumor cells (3). Thus in the early G1 phase of cell cycle, the D type cyclin activates the CDK 4 while in the mid and late G1, cyclin E and CDK2 are activated. These kinases also remain activated during S-phase of cell cycle (4, 5). These kinases phosphorylate the retinoblastoma tumor suppressor retinoblastoma protein (pRb) to drive the cells through the cell cycle (6). However, the cyclin kinase inhibitor proteins, such as p21, interacts with the cyclins thus inhibiting the CDKs resulting in blockage of cell cycle (7–9). Many agents that inhibit cell proliferation regulate the expression and activity of this group of proteins (7).

Bone morphogenetic proteins are structurally similar to the TGF β super family (10). Bone morphogenetic protein-2 (BMP-2), a member of this large family of proteins, stimulates growth and differentiation of osteogenic and chondrogenic cells during bone remodeling and also plays an important role in embryogenesis (10–13). Similar to TGF β, BMPs exert their effect via specific type I and type II serine-threonine kinase receptors. Binding of BMP-2 to the type II receptor induces the oligomerization of the receptor complex resulting in phosphorylation of the type I receptor and recruitment of downstream signaling proteins Smad 1, Smad 5 and Smad 8 (14, 15). Among these, Smad 1 has been extensively studied as the target of BMPR signaling. Type I BMPR-phosphorylated Smad1 then heterodimerizes with Smad 4 and translocates to the nucleus to act as a transcription factor and induce genes that mediate the biological activity of BMP-2 (16).

BMP-2 has recently been reported to have a growth inhibitory effect on prostate cancer cells (17). BMP-2 also inhibits smooth muscle cell proliferation (18). We have recently shown that BMP-2 inhibits PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells by inhibiting mitogen activated protein

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kinase (MAPK) cascade (19, 20). A very high dose of BMP-2 inhibits soft agar growth of a variety of tumor samples including breast tumor (21). However the mechanism of inhibition of tumor cell proliferation by BMP-2 is not yet clear.

In this study we demonstrate that BMP-2 inhibits growth of MDA MB 231 human breast cancer cells in culture in the presence and in the absence of EGF. BMP-2 increases p21 cyclin kinase inhibitor in these cells and inhibits cyclin D1- and E-associated kinases. Furthermore BMP-2 inhibits pRb phosphorylation, which results in accumulation of cells in the G1 phase of cell cycle resulting in inhibition of DNA synthesis.

MATERIALS AND METHODS

Materials. Tissue culture reagents were purchased from Gibco/BRL (Rockville, MD). EGF was from R & D (Minneapolis, MN). Phospho pRb antibody was purchased from New England Biolabs. All other antibodies, GST-pRB and Protein A/G plus were obtained from Santa Cruz. Protein A–Sepharose CL 4B was purchased from Pharmacia. Histone H1 was purchased from Sigma. ECL reagent was purchased from Pierce laboratories. Recombinant BMP-2 was obtained from Genetics Institute.

Cell culture. MDA MB 231 cells were grown in IMEM with 5% fetal bovine serum. For experiments the cells were grown in complete medium for 48 h and serum deprived for 24 h before addition of 100 ng/ml EGF. EGF causes modest proliferative response in this isolate of MDA MB 231 cells. For cell cycle analysis, near confluent cells were used for 24 h serum-deprivation to arrest in G0/G1 phase before addition of EGF to release them. To detect the effect of EGF on MDA MB 231 cell proliferation, serum free medium was changed every 6 h.

Measurement of DNA synthesis. DNA synthesis was measured as ^{3}H-thymidine incorporation into trichloroacetic acid insoluble material as described previously (19, 20).

Flow cytometric analysis. Trypsinized MDA MB 231 cells were washed with PBS and fixed in 70% ethanol for 30 min at −20°C. The cells were then centrifuged at 1500 × g for 4 min, washed with PBS containing 1% BSA and resuspended in 150 μl PBS. For nuclear staining, the cells were treated with 50 μl of 1 mg/ml RNase A followed by 100 μl of 100 μg/ml propidium iodide and incubated at 4°C for 24 h. The cells were then analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) using 200 mW of light at 488 nm produced by an argon-ion laser. The fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

Immunoprecipitation and immunoblotting. The cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF and 0.1% aprotinin) for 30 min at 4°C. The cleared cell lysate was immunoprecipitated with required antibody essentially as described previously (19, 20). Immunoblotting of the immunoprecipitates or the cell lysate was also performed as described previously (19, 20, 22).

Cyclin D1- and E-associated kinase activity. The assay was performed according to the method of Gong et al (23). Briefly, cell lysates were immunoprecipitated with cyclin D1 or cyclin E antibody. The immunebeads were resuspended in kinase assay buffer (20 mM Tris-HCl, pH 7.5 and 4 mM $MgCl_2$). For cyclin D1-associated kinase assay a fragment of pRb containing the *in vivo* phosphorylation sites was used as substrate. For cyclin E-associated kinase activity, histone H1 was used as substrate. The reaction was carried out with 25 μM ATP containing 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction product was separated by SDS–polyacrylamide gel electrophoresis and the phosphorylated proteins were visualized by autoradiography.

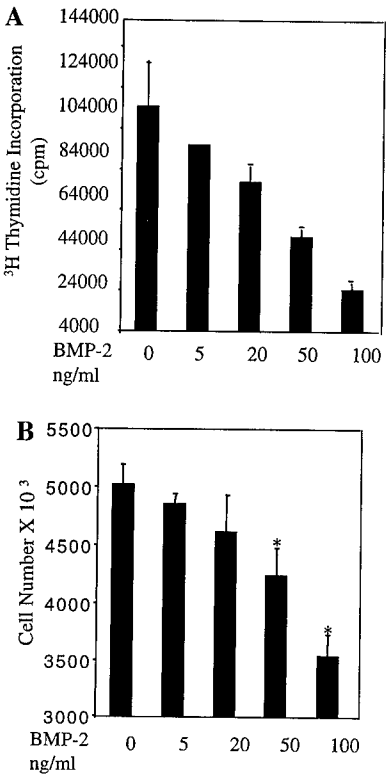

FIG. 1. (A) Effect of BMP-2 on DNA synthesis in MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with different concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20). (B) Effect of BMP-2 on MDA MB 231 cell proliferation. Subconfluent cultures of MDA MB 231 cells in triplicate dishes were incubated with different concentrations of BMP-2 for 24 h. Number of cells were counted for each treatment condition. Mean ± SE of three independent experiments. *$P < 0.05$ vs untreated cells.

RESULTS

BMP-2 inhibits MDA MB 231 cell proliferation. MDA MB 231 cells grow very aggressively in culture. BMP-2 has recently been shown to have an antiproliferative effect on certain cell lines, including primary mesangial cells and prostate cancer cells (17, 19, 20). We examined the effect of BMP-2 on DNA synthesis in MDA MB 231 cells. MDA MB 231 cells were incubated with increasing concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis in these cells. Figure 1A shows that BMP-2 inhibits DNA synthesis in a dose-dependent manner. Approximately 75% inhibition in DNA synthesis was observed at 100 ng/ml BMP-2. To

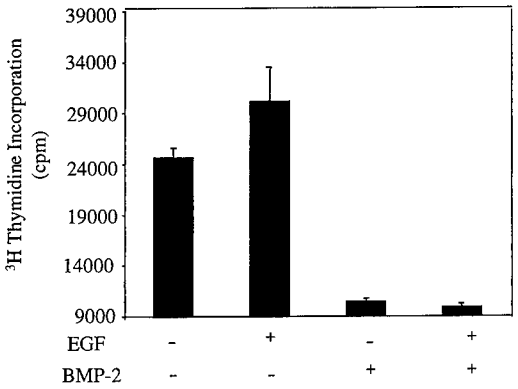

FIG. 2. Effect of BMP-2 on EGF-induced DNA synthesis. Serum-deprived MDA MB 231 cells were incubated with 100 ng/ml BMP-2 for 30 min followed by 100 ng/ml EGF for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20).

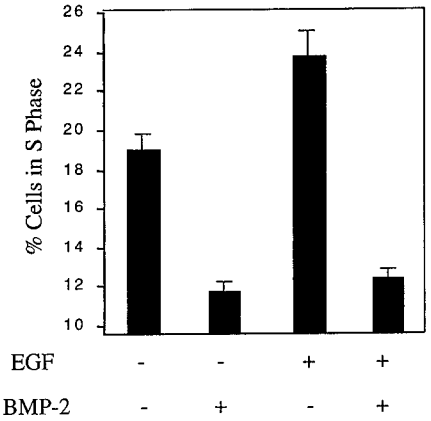

FIG. 3. S-phase analysis of EGF-induced MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with BMP-2 for 30 min and treated in the presence or absence of 100 ng/ml EGF for 24 h. Cells were then trypsinized and analyzed by flow cytometry as described under Materials and Methods. The percentage of cells in S-phase was plotted. Means of triplicate determinations are shown.

confirm this observed effect of BMP-2, we studied the effect of BMP-2 on MDA MB 231 cell proliferation in culture. Growing MDA MB 231 cells were treated with increasing concentrations of BMP-2. The cell number was determined. The data show that BMP-2 dose dependently inhibits MDA MB 231 cell growth in 24 h (Fig. 1B). As evident, 100 ng/ml BMP-2 inhibited proliferation of MDA MB 231 cells by 70% as compared to the untreated control. These data indicate that BMP-2 dose-dependently inhibits proliferation of MDA-MB-231 cells.

Inhibition of EGF-induced growth of MDA MB 231 cells by BMP-2. Activation of EGF receptor family is often associated with breast cancer cell growth (24). To test the effect of BMP-2 on EGF-induced DNA synthesis in MDA MB 231 cells, [^{3}H]thymidine incorporation was determined in the presence and absence of BMP-2. As shown in Fig. 2, EGF increases the DNA synthesis in MDA MB 231 cells by 20%. However, BMP-2 inhibits DNA synthesis by 58% in these cells in the presence of EGF. Since the increase in DNA synthesis by EGF was modest, we tested the effect of EGF on MDA MB 231 cell cycle progression using FACS analysis. Using this technique, EGF increased the number of cells in S phase by 31% (Fig. 3). These data indicate that the mitogenic effect of EGF on MDA MB 231 cells is due to increased progression of these cells from G1 to S phase. Incubation with BMP-2 shows a 58% decrease of cells in the S-phase (Fig. 3). This decrease in the S phase population is accompanied by an increase in cell number at the G1 phase of cell cycle (Table 1). These data indicate that BMP-2 inhibits EGF-induced MDA MB 231 cell proliferation by arresting them at the G1 phase of cell cycle.

BMP-2 induces CDK inhibitor p21 in MDA MB 231 cells. The key proteins that regulate cell cycle progression from G1 to S phase are cyclins D and E and the CDKs that are associated with these cyclins (4, 5). The presence of cyclin kinase inhibitor such as p21 causes cell cycle arrest in G1 by quenching cyclin D and E resulting in inhibition of their associated kinase activity (7–9). Since BMP-2 inhibits G1 to S phase transition (Fig. 3), we tested the effect of BMP-2 on p21 expression in the presence and absence of EGF by immunoblot analysis. As shown in Fig. 4, EGF does not have any significant effect on p21 protein level. However, BMP-2 increases the abundance of p21 in these cells in the presence (compare lane 4 with 3) and in the absence (compare lane 2 with 1) of EGF. These data provide the first evidence that BMP-2 may inhibit MDA MB 231 cell proliferation by increasing the level of p21.

Increased association of p21 with cyclin D1 and cyclin E in the presence of BMP-2. Activation of CDKs is an important step for cell cycle progression from G1 to S phase (3). p21, by associating with cyclins D1 and E, makes them unavailable for activating CDKs and thus

TABLE 1

Flow Cytometric Analysis of MDA MB 231 Cells for G1 and S Phase Quantitation

	% of cells in G1 phase	% of cells in S phase
Control	40.86	19.40
EGF	35.51	24.15
BMP-2	45.32	12.10
EGF + BMP-2	42.93	12.87

Note. Serum-starved MDA MB 231 cells were treated with 100 ng/ml EGF in the presence and absence of 100 ng/ml BMP-2. The cells were analyzed by flow cytometric technique for quantitation of cells present in G1 and S phase.

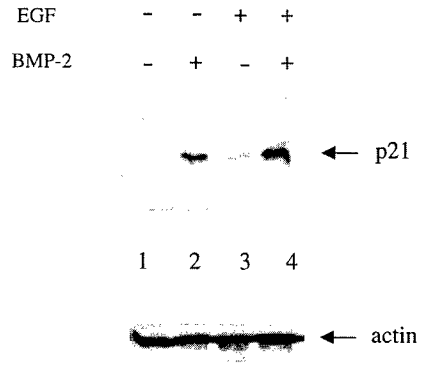

FIG. 4. Effect of BMP-2 on the cyclin kinase inhibitor p21 expression. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence and absence of BMP-2. Equal amounts of cell lysates were analyzed by immunoblotting with p21 antibody and the signal was developed by ECL as described under Materials and Methods. Arrow indicates the position of p21 protein (upper panel). Lower panel shows immunoblotting of same samples with anti-actin antibody.

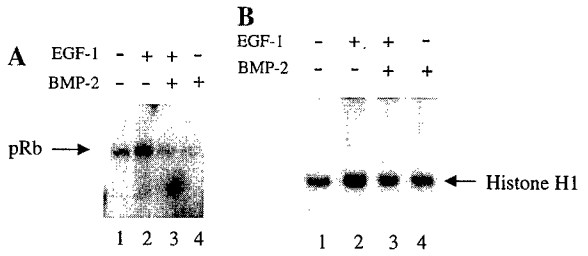

FIG. 6. Effect of BMP-2 on EGF-induced cyclin D1- and cyclin E-associated kinase activity. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence or absence of BMP-2. The lysates were immunoprecipitated with cyclin D1 (A) or cyclin E (B) antibodies. The immunoprecipitates were assayed for kinase activity in the presence of [γ^{32}P]ATP using pRb (A) or histone H1 (B) as substrates. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The arrow indicates the phosphorylated pRb (A) and histone H1 (B).

inhibits cell cycle progression to S phase (7–9). Since BMP-2 increased the level of p21 (Fig. 4), we investigated whether p21 associates with cyclin D1 and cyclin E in BMP-2-treated MDA MB 231 cells in the presence or absence of EGF. p21 was immunoprecipitated from cells treated with EGF and combination of BMP-2 and EGF. The immunoprecipitates were immunoblotted either with cyclin D1 (Fig. 5A) or with cyclin E (Fig. 5B) antibody. The results show that EGF does not have any significant effect on association of p21 with these two cyclins. In contrast, incubation of cells with BMP-2 results in increased association of p21 with cyclin D1 and cyclin E in the presence (compare lanes 3 with 1 in Figs. 5A and 5B) or in the absence (compare lanes 2 with 4) of EGF. These data indicate that increased association of p21 with cyclin D1 and E may inhibit the kinase activities associated with these cyclins.

BMP-2 treatment inhibits kinase activity associated with cyclin D1 and cyclin E. Cell cycle progression depends on phosphorylation of a number of regulatory

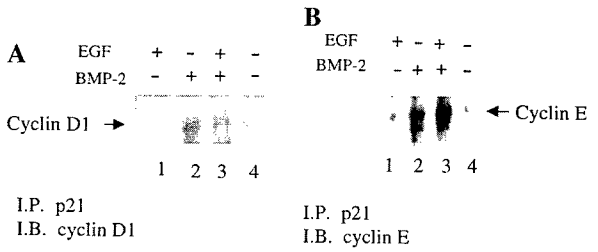

FIG. 5. Effect of BMP-2 on association of p21 with cyclin D1 and cyclin E. Equal amounts of cleared cell lysates from MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoprecipitated (I.P.) with anti-p21 antibody. The immunoprecipitates were analyzed by immunoblotting (I.B.) with anti-cyclin D1 antibody (A) and anti-cyclin E antibody (B). The arrows indicate the positions of cyclin D1 and cyclin E in A and B, respectively.

proteins by CDKs. In the G1 phase of cell cycle, cyclin D1-associated kinase is activated to initiate the cells to progress into S phase (4, 5). Since BMP-2 increases the association of cyclin kinase inhibitor p21 with cyclin D1 (Fig. 5A) and inhibits G1 to S phase progression in MDA MB 231 cells (Fig. 3), we investigated the effect of BMP-2 on cyclin D1-associated kinase activity. The lysates of MDA MB 231 cells treated with either EGF or EGF plus BMP-2 were immunoprecipitated with cyclin D1 antibody and assayed for associated kinase activity using pRb as substrate. As shown in Fig. 6A, EGF increases the pRb phosphorylation by cyclin D1-associated kinase (compare lane 2 with lane 1). BMP-2 inhibits EGF-induced pRb phosphorylation (compare lane 4 with lane 2). In mid G1 to S phase of cell cycle, cyclin E-associated kinase is activated (5, 25). Therefore, we immunoprecipitated cyclin E from lysates of MDA MB 231 cells and assayed for associated kinase activity using histone H1 as substrate. Figure 6B shows that EGF stimulates cyclin E-associated kinase activity (compare lane 2 with lane 1). However, BMP-2 inhibits EGF-induced histone H1 kinase activity (compare lane 4 with lane 2). During cell cycle progression, CDK2 remains activated in the S phase. Immunecomplex kinase assay of CDK2 immunoprecipitates showed increased CDK2 activity by EGF and BMP-2 inhibited EGF-induced CDK2 activity (data not shown). These data indicate that BMP-2 intercepts cyclin-dependent kinases to inhibit cell cycle progression of MDA MB 231 cells.

BMP-2 blocks pRb phosphorylation in MDA MB 231 cells. Activation of CDKs during mid G1 and S phase of cell cycle phosphorylate the retinoblastoma tumor suppressor protein pRb to drive the cells through cell cycle (6, 7). In MDA MB 231 cells the level of pRb phosphorylation was determined using a phospho-pRb antibody. An immunoblot analysis of lysates of cells treated with EGF alone and EGF plus BMP-2 is shown in Fig. 7. Lysates from control and

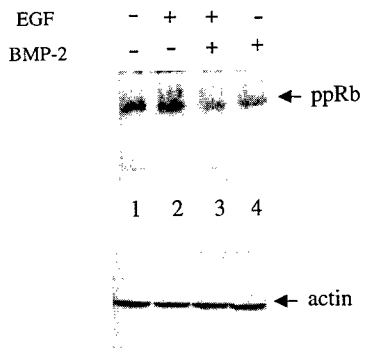

FIG. 7. Effect of BMP-2 on EGF-induced pRb phosphorylation. 50 μg cleared cell lysates of MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoblotted with an anti-phospho-pRb antibody. The arrow indicates phosphorylated pRb (ppRb).

EGF-treated cells show increased pRb phosphorylation (lanes 1 and 2). But in cells treated with BMP-2, the level of total phosphorylated pRb is significantly reduced (lane 3 and 4). One reason of increased phosphorylation in control MDA MB 231 cells may be due to their aggressive growth even in the absence of serum. These data provide the first evidence that BMP-2 inhibits pRb phosphorylation and indicate that this inhibition of pRb phosphorylation by cyclin-dependent kinases may result in attenuation of MDA MB 231 cell proliferation.

DISCUSSION

Our data in this study provide the first evidence that BMP-2 inhibits EGF-induced proliferation of MDA MB 231 cells by blocking cyclin dependent kinase activities and pRb phosphorylation. This inhibition may be due to BMP-2-induced increase in p21 cyclin kinase inhibitor level in these cells.

Breast cancer cells often metastasize to bone presumably because of a favorable growth-promoting environment provided by the bone where various growth factors are expressed in abundance (26). Our data show that one of the potent osteogenic factors, BMP-2, inhibits proliferation of MDA MB 231 cells in culture (Fig. 1). This observation was unexpected, as it was logical to predict that BMP-2, which is present in the microenvironment of bone, may not have any effect, or it may support growth of these cancer cells present in the microenvironment of bone. However, BMP-2 is present in bone matrix at a very low concentration (1–2 ng/g), whereas other growth factors such as insulin-like growth factor II (IGFII) is present at a concentration of 1500 ng/g (26, 27). Thus it is possible that the concentration of BMP-2 in the bone microenvironment may not be sufficient to inhibit the breast cancer cell growth. This may explain the requirement of a high concentration of BMP-2 to inhibit the proliferation of MDA MB 231 cells in culture (Fig. 1). Also we have shown previously that BMP-2 inhibits proliferation of primary mesangial cells at a very high concentration (19, 20). It should be noted that inhibition of prostate cancer cell proliferation is also achieved at a relatively high concentration (17).

EGF receptors in many breast cancer cells play an important role in the pathogenesis of tumor cell proliferation (24). Activation of EGFR stimulates its intrinsic tyrosine kinase activity and recruitment of cytosolic signaling proteins (28). The signals generated in the cytosol converge in the nucleus and activate cell cycle progression of breast tumor cells. EGF is a modest mitogen for MDA MB 231 breast cancer cells and we show slight induction of cell proliferation and G1 to S phase progression in these cells (Figs. 2 and 3). BMP-2 inhibited MDA MB 231 cell proliferation regardless of the presence of EGF (Figs. 1 and 2). Our results indicate that BMP-2 treatment arrests MDA cells at the G1 phase of cell cycle.

Nuclear targets of growth factor-mediated induction of cell proliferation are the cell cycle regulatory proteins (3–7). Cyclin D1 and E regulate the progression of cells in the G1 to S phase of cell cycle (5, 25). CDKs are regulated by cyclin kinase inhibitors (7, 8, 29). One of these proteins, p21, is a potent inhibitor of CDKs associated with cyclins D and E. It has been shown previously that p21 stimulates withdrawal from the cell cycle coupled to terminal differentiation (30). In breast carcinomas, increased expression of p21 was associated with relapse free survival (31). In addition to inhibition of CDKs, p21 inhibits DNA replication directly by binding to PCNA (32). In the present study we show that BMP-2 increases the level of p21 in MDA MB 231 breast tumor cells (Fig. 4). These data indicate that BMP-2-induced reduction in cell proliferation and S-phase entry may be due to the increased expression of p21 protein.

Increased cyclin D1-associated kinase activity is associated with increased proliferation of breast cancer cells (33). One of the mechanisms by which p21 blocks cell cycle progression is via interaction with cyclin D1 and cyclin E, subsequently resulting in inhibition of CDK activity (7–9). Our results show that BMP-2 increases the association of p21 with cyclin D1 and cyclin E in MDA MB 231 cells (Fig. 5) resulting in inhibition of their associated kinase activities (Fig. 6). Thus one of the mechanisms by which BMP-2 may inhibit MDA MB 231 cell proliferation is by inhibiting cyclin dependent kinases that are known to be activated in mid to late G1 and S phases of cell cycle.

During G1 phase of cell cycle the transcription factor E2F is associated with hypophosphorylated pRb. Cyclin dependent kinases phosphorylate pRb (34). pRb is also hyperphosphorylated in various breast cancer cells and tissues (35). Thus phosphorylated and inactivated pRb releases E2F transcrip-

tion factor which then activates transcription of a number of important genes necessary for cells to enter into S phase (34). In the present study we have shown that pRb is hyperphosphorylated in the presence of EGF in MDA MB 231 cells and BMP-2 causes reduction in the level of pRb phosphorylation in the presence and absence of EGF (Fig. 7). This observation provides one of the first mechanisms by which BMP-2 may inhibit MDA MB 231 breast cancer cell proliferation. Our findings may have important therapeutic implications in breast cancer treatment once the mechanism of action of BMP-2 has been characterized in more detail.

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

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Abstract

The biologic effects and mechanisms by which bone morphogenetic proteins (BMPs) function in breast cancer cells are not well defined. A member of this family of growth and differentiation factors, BMP-2, inhibited both basal and estradiol-induced growth of MCF-7 breast tumor cells in culture. Flow cytometric analysis showed that in the presence of BMP-2, 62% and 45% of estradiol-stimulated MCF-7 cells progressed to S-phase at 24 h and 48 h, respectively. Estradiol mediates growth of human breast cancer cells by stimulating cyclins and cyclin-dependent kinases (CDKs). BMP-2 significantly increased the level of the cyclin kinase inhibitor, p21, which in turn associated with and inactivated cyclin D1. BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity. Also estradiol-induced CDK2 activity was inhibited by BMP-2. This inhibition of CDK activity resulted in hypophosphorylation of retinoblastoma protein thus keeping it in its active form. These data provide the first evidence by which BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells.

Keywords: BMP-2; pRb; p21; Breast cancer cell

1. Introduction

17-β-Estradiol (estradiol) acts as a potent mitogen for breast epithelial cells and thus causes increased cell growth, both in vivo and in vitro. In estradiol-responsive human breast cancer cells like MCF-7, the hormone responsiveness is mediated by estrogen receptors (ERs) [1]. Activation of ER stimulates cyclin-dependent kinases (CDKs) to induce proliferation of MCF-7 cells. On the other hand, increased expression of cyclin kinase inhibitor p21 blocks CDK activity necessary for retinoblastoma protein (pRb) phosphorylation [2]. These results indicate that a concerted effect of different cell cycle proteins regulates cell cycle progression.

Bone morphogenetic proteins (BMPs), BMP 1–9, constitute a group of growth factors that are in-

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volved in ectopic bone formation [3]. They are produced as pro-mature forms, which are processed to active dimers of the mature region in a manner similar to the transforming growth factor β (TGFβ) [4]. Due to this similarity, BMPs are categorized as members of the TGFβ super family. In addition to other functions during embryonic development and limb formation, BMPs regulate chondrogenesis and osteogenesis [5–7]. Though BMPs have been detected in osteosarcomas and soft tissue carcinomas, the role of BMPs in breast cancer is still unclear. Like TGFβ receptors, multiple BMP receptors have recently been identified. They form two closely related groups known as type I and type II receptors, which contain multiple members. Both the receptor types have serine/threonine kinase activity in their cytoplasmic domains [8,9]. Three downstream target molecules for BMP-2 have recently been identified. These targets are Smad 1, Smad 5 and Smad 8. BMP-2 stimulates association of Smad 1 with the BMP receptor followed by phosphorylation of the Smad 1 C-terminus by the type I receptor [10,11]. Receptor-phosphorylated Smad 1 undergoes heterodimerization with the tumor suppressor protein Smad 4. This heterodimer then translocates to the nucleus and participates in transcription of genes [10,12].

Recently, Nakaoka et al. demonstrated that BMP-2 inhibits smooth muscle cell proliferation [13]. BMP-2 blocks serum and androgen-induced growth of human prostate cancer cells in culture [14]. We have recently shown that BMP-2 at a moderate dose blocked PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells without any effect on matrix gene expression [15,16]. Also a high dose of recombinant BMP-2 has recently been shown to inhibit soft agar growth of a variety of tumor samples including breast tumor [17]. However, the mechanism of BMP-2-mediated inhibition of tumor cell growth is not known. In this report, we demonstrate the inhibitory effect of BMP-2 on estradiol-induced MCF-7 human breast cancer cell proliferation in culture. BMP-2 increases the levels of cyclin kinase inhibitor p21 without any effect on estradiol-induced cyclin D1 expression. We also show that BMP-2 inhibits estradiol-induced cyclin D1-associated kinase and CDK2 activity with concomitant reduction of pRb phosphorylation. This is the first elucidation of the signaling mechanisms, involved in BMP-2-mediated inhibition of estradiol-induced breast cancer cell growth.

2. Materials and methods

Tissue culture materials were purchased from Gibco. Estradiol, phenyl methyl sulphonyl fluoride (PMSF), soybean inhibitor, leupeptin, myelin basic protein, propidium iodide and RNase A were obtained from Sigma. Histone H1 was purchased from Boehringer Mannheim. GST-pRb was obtained from Santa Cruz. Micro BCA reagent and enhanced chemiluminescence (ECL) kit were purchased from Pierce. Protein A-Sepharose CL 4B was purchased from Pharmacia. All antibodies were obtained from Santa Cruz. Recombinant BMP-2 was obtained from Genetics Institute.

MCF-7 breast cancer cells were obtained from Dr. Robert Klebe (Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio) and were routinely maintained in DMEM–F12 medium containing 10% new born calf serum. These MCF-7 cells are highly responsive to estradiol and tamoxifen. For experiments designed to test the mitogenic effect of estradiol, cells were grown in complete medium for 48 h to reach subconfluency and then placed in phenol red-free and serum-free DMEM for 48 h before addition of estradiol. Treatment with serum-free medium slows the growth of cells because they tend to arrest at G0/G1 phase. For cell cycle analysis near confluent cells were used for serum-deprivation to arrest in G0/G1 phase before addition of estradiol to release them.

2.1. Flow cytometric analysis

MCF-7 cells were trypsinized and washed with phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol for 30 min at −20°C, centrifuged at 1500×*g* for 4 min, washed with PBS containing 1% bovine serum albumin (BSA) and resuspended in 150 μl PBS. For nuclear staining with propidium iodide, the cells were treated with 50 μl of 1 mg/ml RNase A (Sigma) followed by 100 μl of 100 μg/ml propidium iodide. The cells were incubated at 4°C for 18–24 h before they were analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry

Systems, San Jose, CA). Cells were illuminated with 200 mW of light at 488 nm produced by an argon-ion laser and the fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20 000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

2.2. MTT (3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) assay for cell proliferation

Proliferation of MCF-7 cells in response to estradiol was determined using the MTT assay as described elsewhere [18]. In brief, 50 μl of 5 mg/ml MTT was added to the culture medium of growing cells (1 ml medium/well) and incubated for 4 h at 37°C in a humidified atmosphere with 5% CO_2. The medium was removed and 200 μl of DMSO was added to each well. The absorbance of the dissolved dye was measured at 540 nm.

2.3. Immunoprecipitation and immunoblotting

Immunoprecipitation was carried out according to methods described elsewhere, with minor modifications [16,19]. In brief, cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 50 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml PMSF, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μg/ml aprotinin) and cleared of cell debris by centrifugation at 4°C. Protein estimation was done in supernatant by a micro BCA assay. 200 μg protein was routinely precleared by incubating with 20 μl of swelled protein A-Sepharose beads for 1 h in the cold. The cleared supernatant was immunoprecipitated at 4°C for 18–24 h using 1 μg of antibody followed by addition of 20 μl swelled protein A-Sepharose beads for 1 h. The protein A-Sepharose beads containing the antigen–antibody complex were then gently washed three times with immunoprecipitation buffer before eluting the bound proteins in the sodium dodecyl sulfate (SDS)–polyacrylamide gel loading buffer.

Immunoblotting was performed essentially as previously described [20]. Briefly, the cleared cell lysates or immunoprecipitates were separated in SDS–polyacrylamide gels (12% or 7.5% depending on the protein sizes). The proteins were electrophoretically transferred onto Nytran membrane. Following the transfer, the proteins were incubated with blocking solution (50 mM Tris–HCl pH 7.4–150 mM NaCl–0.2% Tween 20 (TBST) containing 5% non-fat dry milk) for 1 h at room temperature, followed by overnight incubation in primary antibody solution prepared in TBST containing 1% BSA. The membrane was subsequently washed five times in TBST for 5 min each, before the horseradish peroxidase-conjugated secondary antibody was added in TBST for 1 h at room temperature. The membrane was finally washed in TBST, five times for 5 min each and the antigen–antibody complex was detected using an ECL kit (Pierce) as per manufacturer's recommendations.

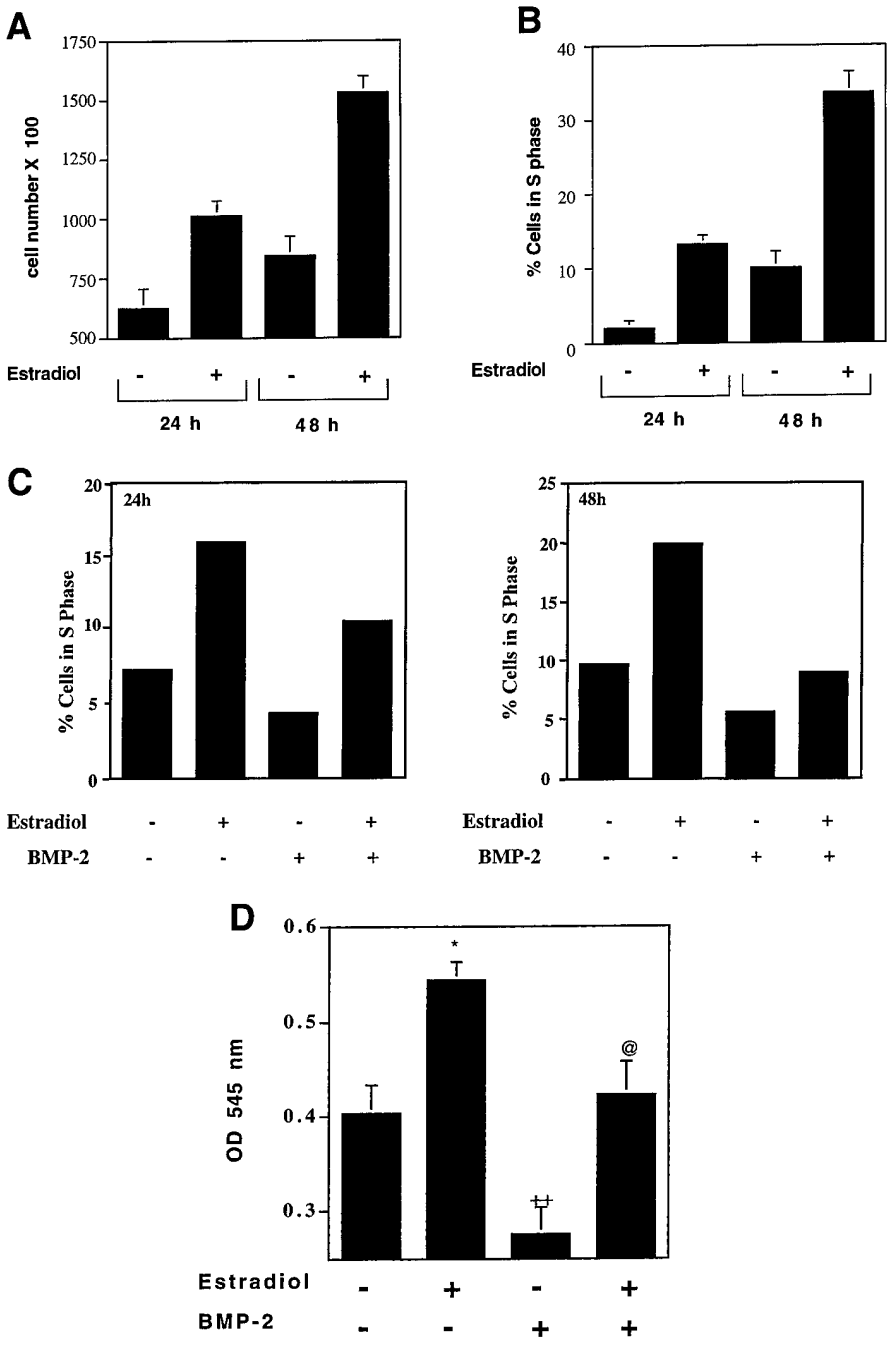
2.4. Cyclin D1-associated kinase and CDK2 assay

The assay was performed using the method of Gong et al. [21]. Briefly, cleared cell lysate was immunoprecipitated using antibody against cyclin D1 or CDK2 as described above. The immunecomplex beads were resuspended in kinase buffer (20 mM Tris–HCl pH 7.5 and 4 mM $MgCl_2$). To measure the cyclin D1-associated kinase activity, a fragment of pRb, that contains the in vivo phosphorylation sites, was used. For CDK2 activity, calf thymus histone H1 was used as a substrate. The reaction was carried out in the presence of 25 μM 'cold' ATP and 10 μCi [γ-^{32}P]ATP for 30 min at 37°C. The phosphorylated proteins were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. Phosphorylation was quantitated using a densitometric scan of the phosphorylated bands in autoradiogram.

3. Results

3.1. Growth inhibition of MCF-7 cells by BMP-2

Estradiol is a potent mitogen for ER positive human MCF-7 breast carcinoma cells [22]. To establish the optimal conditions to assess the effect of BMP-2 on MCF-7 cell proliferation, MCF-7 cells were treated with 1 nM 17-β-estradiol for 24 and 48 h,



respectively, and the cell number was counted. As expected, estradiol increased the cell number at each time point (Fig. 1A). To examine the effect of estradiol on cell cycle progression, MCF-7 cells were subjected to flow cytometry. At 24 and 48 h, a significantly higher percentage of cells were in S-phase in the presence of estradiol, as compared to unstimulated control cells (Fig. 1B). At 48 h, 10% of cells entered the S-phase even in the absence of estradiol. This may be due to incomplete quiescence of MCF-7 breast tumor cells. An alternative possibility may be accumulation of mitogens in the culture medium during 48 h of incubation in the serum-deprived medium.

To determine the effect of BMP-2 on cell cycle progression of MCF-7 cells stimulated by estradiol,

Fig. 1. Effect of estradiol and BMP-2 on MCF-7 cell proliferation. (A) Confluent layers of MCF-7 cells in 35 mm tissue culture dishes were serum-starved for 48 h and incubated with 1 nM estradiol for 24 and 48 h. Cells in each dish were counted. Increase in cell number was 1.6- and 1.8-fold, respectively, at 24 and 48 h. (B) Flow cytometric analysis of estradiol-induced MCF-7 cell proliferation. Serum-deprived MCF-7 cells were grown in the presence or absence of 1 nM estradiol for 24 and 48 h. Cells were then trypsinized and analyzed by flow cytometry as described in Section 2. The percentage of cells in S-phase was plotted. The percentage of cells in S-phase was increased by 6.5- and 3.7-fold at 24 and 48 h, respectively. Means of triplicate determinations are shown in A and B. (C) Quantitation of flow cytometric analysis of cells treated with BMP-2 and estradiol. MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol for 24 h (left panel) and 48 h (right panel) before subjecting them to flow cytometric analysis. The percentage of cells in S-phase was plotted for each condition. (D) Effect of BMP-2 on estradiol-induced MCF-7 cell proliferation. 48 h serum-deprived MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol. MTT assay was performed as an index of cell proliferation as described in Section 2. Results are means ± S.E.M. of three independent experiments. $*P < 0.05$ vs. untreated cells. $++P < 0.05$ vs. untreated control. $@P < 0.05$ vs. estradiol-treated cells.
←

cells were incubated with estradiol for 24 and 48 h, either in presence or absence of BMP-2. BMP-2 inhibited estradiol-induced S-phase progression of these cells. Quantitation of these results shows that only 62% and 45% of estradiol-treated MCF-7 cells entered S-phase at 24 and 48 h, respectively, in the presence of BMP-2 (Fig. 1C, left and right panels). BMP-2 alone also inhibited S-phase entry of control cells, by 41% and 43% at 24 and 48 h, respectively. To determine if the effect of BMP-2 on estradiol-induced cell cycle progression correlated with cell growth, MTT assays were performed. Fig. 1D shows that BMP-2 significantly inhibited estradiol-stimulated as well as basal MCF-7 cell proliferation. A photomicrograph of MCF-7 cells in the absence and presence of BMP-2 is shown in Fig. 2. As evident, treatment of these cells for 48 h with BMP-2 does not have any toxic effect. Taken together, these results indicate that BMP-2 inhibits estradiol-induced cell growth by preventing the entry of MCF-7 cells into S-phase.

3.2. *BMP-2 stimulates expression of cyclin kinase inhibitor, p21, in estradiol-treated MCF-7 cells*

Progression of the cell cycle is regulated by a series of CDKs [23]. These serine/threonine kinases are positively regulated by cyclins [24,25]. One of the G1 phase cyclins, cyclin D1, is overexpressed in more than 50% of human breast adenocarcinomas [26–28]. We studied the effect of estradiol on cyclin D1 expression. In accordance with the previous report [29], estradiol treatment of MCF-7 cells increased the level of cyclin D1 (Fig. 3A, compare lane 2 with lane 1). However, pretreatment of MCF-7 cells with BMP-2 had no significant effect on estradiol-induced expression of cyclin D1 (Fig. 3A, compare lane 4 with lane 2). These data indicate that the effect of BMP-2 on estradiol-induced MCF-7 cell proliferation is not caused by the modulation of cyclin D1 levels during cell cycle progression.

CDK activity is also regulated by cyclin kinase inhibitors [25]. One such protein, p21, is a universal

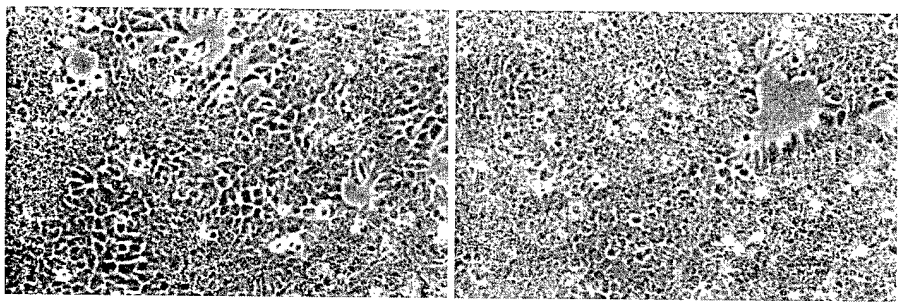


Fig. 2. Photomicrograph of MCF-7 cells in the presence and absence of BMP-2. Serum-deprived MCF-7 cells were incubated with BMP-2 for 48 h before taking the photograph. The phase contrast photomicrograph is shown.

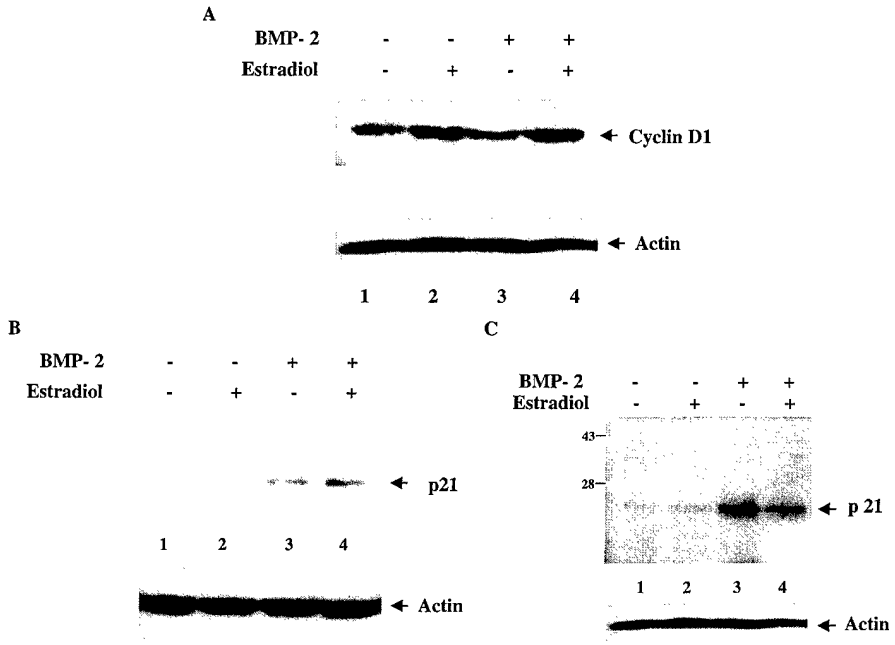


Fig. 3. (A) Effect of BMP-2 on estradiol-induced expression of cyclin D1 and p21. MCF-7 cells serum-deprived for 48 h were incubated with 1 nM estradiol in the presence and absence of 100 ng/ml BMP-2 for subsequent 48 h. Cleared cell lysates were analyzed by immunoblotting with cyclin D1. (B and C) Effect of BMP-2 on p21 expression. Serum-deprived MCF-7 cells were incubated with estradiol in the presence and absence of 100 ng/ml BMP-2 for 6 h (B) and 48 h (C), respectively. The lysates were immunoblotted with p21 antibody and the signal was developed by ECL. Lower panels show immunoblotting of the same lysates with anti-actin antibody to demonstrate equal loading of proteins in each lane.

inhibitor of CDKs, that interacts with multiple cyclin–CDK complexes. It thereby inhibits their kinase activity, which drives the cells through the cell cycle [2,30,31]. To understand the mechanism of BMP-2 inhibition of cell cycle progression, we studied its effect on p21 expression. MCF-7 cells were incubated with BMP-2 and estradiol for 6 and 48 h. At both time points, estradiol did not have any affect on p21 protein expression as determined by immunoblot analysis (Fig. 3B,C, compare lanes 2 with lanes 1 in both panels). In contrast, treatment of MCF-7 cells with BMP-2 alone significantly increased the level of p21 protein expression (Fig. 3B,C, compare lanes 3 with lanes 1). In the cells co-treated with BMP-2 and estradiol (lane 4), the level of p21 expression remained increased as compared to untreated and estradiol-treated cells. These data indicate that the inhibitory effect of BMP-2 on MCF-7 cell proliferation may partly be due to its effect on increased expression of p21.

3.3. *BMP-2 inhibits cyclin D1-associated kinase activity via p21*

For p21 to exert its inhibitory effect on cell cycle progression, it must associate with one of the cyclin–CDK complexes [24,25]. Since estradiol-induced increase in cyclin D1 levels resulted in cell progression, while BMP-2-induced increase in p21 levels caused G1 arrest, we analyzed the association of p21 with cyclin D1 under similar conditions. Lysates of serum-deprived MCF-7 cells treated with estradiol in the presence and absence of BMP-2 were immunoprecipitated with the antibody to p21, followed by immunoblotting with a cyclin D1 antibody. As shown in Fig. 4A, estradiol alone has no effect on association of cyclin D1 with p21. In contrast, p21 was found to be associated with cyclin D1 in cells treated with BMP-2 alone or in combination with estradiol (Fig. 4A, lanes 3 and 4). These data indicate that BMP-2 treatment causes an increased association of p21 with

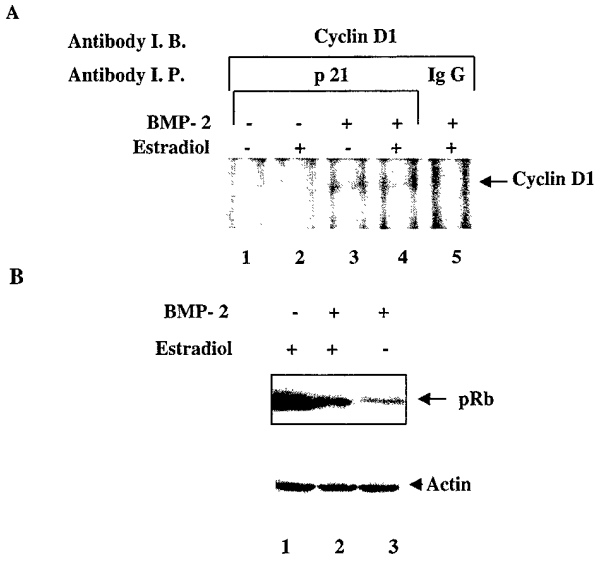


Fig. 4. (A) Effect of BMP-2 on association of p21 with cyclin D1. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 3B,C, were immunoprecipitated (I.P.) with either anti-p21 or control IgG. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 12% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-cyclin D1 antibody. Lanes 1–4 represent p21 immunoprecipitates. Lane 5 shows IgG immunoprecipitates. (B) Effect of BMP-2 on estradiol-induced cyclin D1-associated kinase activity. Serum-deprived MCF-7 cells were incubated with estradiol in the presence of BMP-2. The lysates were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for kinase activity in the presence of [γ-^{32}P]ATP using pRb as substrate. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows an immunoblot of same samples with actin antibody.

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cyclin D1 in MCF-7 cells which may result in the inhibition of cyclin D1-dependent kinase activity.

More recently, a role for p21 has been described as the assembly factor for cyclin D and CDK4/6 [32,33].

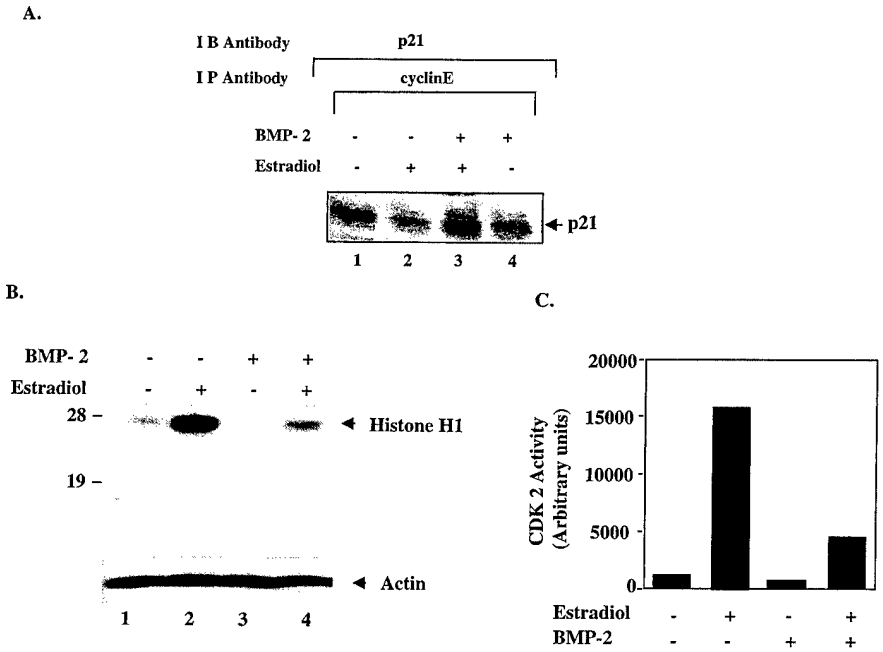

Fig. 5. (A) Effect of BMP-2 on association of p21 with cyclin E. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 4, were immunoprecipitated (I.P.) with anti-cyclin E. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 15% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-p21 antibody. Effect of BMP-2 on estradiol-induced CDK2 activity. (B) The cleared cell lysates from MCF-7 cells treated as described in Fig. 4 were immunoprecipitated with an anti-CDK2 antibody. The washed immunebeads were used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The labeled proteins were separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows immunoblotting of the same lysates with anti-actin antibody. (C) Quantitation of histone H1 phosphorylation. The radioactivity incorporated into histone H1 in (A) was measured by a densitometric scan as described in Section 2 and plotted as histogram.

Another role of p21 in cell cycle is its inhibitory effect on CDK activity. Increased expression of p21 has been shown to inhibit both cyclin D1 and cyclin E-associated kinases [2]. To test this, lysates of MCF-7 cells treated with BMP-2 and estradiol were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for D1-associated kinase activity using pRb as in vitro substrate. As shown in Fig. 4B, BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity (compare lane 2 with lane 1). These data indicate that the inhibitory effect of BMP-2 may involve reduced pRb phosphorylation by cyclin D1-associated kinase (see below).

3.4. *BMP-2 inhibits estradiol-induced CDK2 kinase activity and pRb phosphorylation*

In the late G1 phase of cell cycle progression, E-type cyclin regulates CDK activity which is necessary for cells to enter and proceed through the S-phase [34]. p21 has been shown to regulate cyclin E via physical association. Since the p21 level was increased by BMP-2 (Fig. 3B,C), we tested if recombinant BMP-2 regulates p21 association with cyclin E. Cyclin E immunoprecipitates from lysates of estradiol or BMP-2 plus estradiol-treated MCF-7 cells were immunoblotted with p21 antibody. The results show that BMP-2 stimulated increased association of p21 with cyclin E in the presence and absence of estradiol as compared to estradiol alone (Fig. 5A, compare lanes 3 and 4 with lane 2). During late G1 and S-phase, cyclin E regulates CDK2 activity. To understand the mechanism of regulation of CDK2 in MCF-7 breast cancer cells, we analyzed the kinase activity associated with CDK2 in cells treated with estradiol in the presence or absence of BMP-2. Cell lysates were immunoprecipitated with a CDK2 antibody. The immunebeads were then used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The data showed increased phosphorylation of histone H1 by CDK2 in cells treated with estradiol (Fig. 5B, compare lane 2 with 1). Estradiol-induced CDK2 activity was significantly inhibited by BMP-2 (Fig. 5B, compare lane 4 with lane 2). Quantitation of histone H1 phosphorylation showed 11-fold increase in CDK2 activity in the presence of estradiol (Fig. 5C), and BMP-2 inhibited 70% of estradiol-induced CDK2 activity (Fig. 5C). BMP-2 also partially inhibited the basal activity of CDK2 in MCF-7 cells. These data indicate that BMP-2-mediated inhibition of estradiol-induced MCF-7 proliferation may in part be due to its inhibitory effect on CDK2 activity.

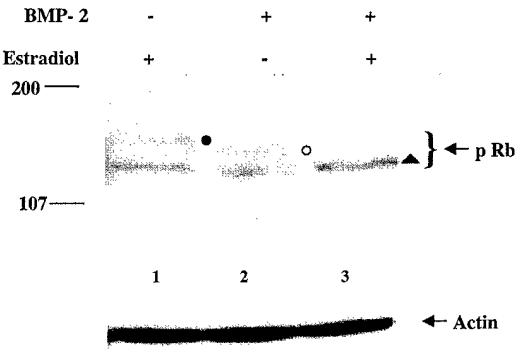

Fig. 6. Effect of BMP-2 on estradiol-induced pRb phosphorylation. The cleared cell lysates of MCF-7 cells, treated with estradiol in the presence and absence of BMP-2, were immunoblotted with an anti-pRb antibody. The migration of molecular weight markers (in kDa) is shown in the left margin. The filled circle shows hyperphosphorylated pRb in the highest phosphorylated form. The open circle shows pRb in intermediate phosphorylated form and the filled triangle shows pRb in hypophosphorylated form.

One of the targets of CDKs during cell cycle progression is pRb [24,35]. Hypophosphorylated pRb is active and inhibits cell cycle progression. Proliferative signals integrate into the nucleus to induce CDK-dependent phosphorylation of pRb rendering pRb inactive and resulting in DNA synthesis [35]. We showed in Fig. 4B that in in vitro kinase assay, pRb phosphorylation is reduced by BMP-2. To study the effect of BMP-2 on estradiol-induced pRb phosphorylation in MCF-7 cells, we analyzed lysates of MCF-7 cells by phosphorylation-dependent mobility shift assay. The degree of pRb phosphorylation is determined by its electrophoretic mobility, with hyperphosphorylated pRb forms migrating slower than the hypophosphorylated form in SDS gel. Lysates from MCF-7 cells, treated with estradiol or BMP-2 alone or with estradiol in the presence of BMP-2, were immunoblotted with an anti-pRb antibody. As shown in Fig. 6, estradiol caused hyperphosphorylation of pRb as indicated by the slower migration of this protein (lane 1, indicated by filled circle). Treatment of MCF-7 cells with BMP-2 alone resulted in the partial phosphorylation of pRb (lane 2, indicated

by open circle). In contrast, BMP-2 significantly inhibited hyperphosphorylation of pRb induced by estradiol and only hypophosphorylated pRb was the predominant form detected (Fig. 6, lane 3, indicated by filled triangle). These data indicate that the observed growth inhibitory effect of BMP-2 in estradiol-induced MCF-7 breast cancer cell proliferation could be caused by decreased CDK-dependent pRb phosphorylation.

4. Discussion

Our study demonstrates an inhibitory effect of BMP-2 on estradiol-induced MCF-7 breast cancer cell proliferation. BMP-2 stimulates increased expression of p21 cyclin kinase inhibitor. Consistent with this idea is our observation showing inhibition of estradiol-induced cyclin D1-associated kinase and CDK2 activity in response to BMP-2. Finally, we provide the first evidence that BMP-2 maintains the pRb tumor suppressor protein in a partially phosphorylated form.

Binding of estrogen to its receptor regulates a cohort of responsive genes that appears to regulate cell cycle progression. CDK4 and CDK6 form complexes with D-type cyclins during mid and late phases of G1, while CDK2 binds to cyclin E and D during late G1 [36–38]. One link between proliferative signals and cell cycle progression is provided by the induction of the secondary response genes, such as cyclin D1, following mitogenic stimulation [36]. In breast cancer, chromosome 11q13, which contains the cyclin D1 gene, has been shown to be amplified preferentially in ER positive tumors [39,40]. It has also been suggested that overexpression of cyclin D1 in MCF-7 cells causes them to proliferate in growth factor-deprived conditions [41]. In simvastatin or lovastatin-arrested MCF-7 human breast cancer cells, estrogen stimulates cell cycle entry by increasing cyclin D1 expression [42]. This effect of estrogen was due to transcriptional activation of the cyclin D1 gene by an estrogen-regulated response region present between the −944 bp of upstream sequences and the transcription start site of the cyclin D1 gene [42]. Cyclins, in association with CDKs and cyclin kinase inhibitors, control cell cycle progression through different phases of transitions and checkpoints. One of the cyclin kinase inhibitors, p21, has been shown to stimulate withdrawal from the cell cycle coupled to terminal differentiation [43]. Immunohistochemical analysis of breast carcinomas has shown that increased expression of p21 was associated with relapse-free survival [44]. p21 inhibits all the CDKs associated with cyclins A, D1 and E that are required for G1/S progression [31,45]. In addition to CDK inhibition, and thereby blocking cells from entering S-phase, p21 inhibits the DNA replication directly by binding to PCNA [46]. In the present study, we show that estradiol-induced S-phase entry of MCF-7 breast carcinoma cells is inhibited by the growth and differentiation factor BMP-2 (Fig. 1). Furthermore, our results demonstrate that BMP-2 causes increase in the levels of p21 protein as early as 6 h which sustains until 48 h (Fig. 3B,C). These data indicate that our observation of BMP-2-induced reduction in S-phase entry (Fig. 1C) and reduced proliferation (Fig. 1D) may be due to the increased expression of p21 protein (Fig. 3B,C). One of the mechanisms by which p21 blocks cells from entering S-phase is via interaction with cyclin D1 during G1 phase of the cell cycle, subsequently resulting in inhibition of CDK4 activity [2]. In the present study, we demonstrate association of p21 with cyclin D1 in the presence of BMP-2 (Fig. 4A). This may be the cause of reduced cyclin D1-associated kinase activity (Fig. 4B).

In addition to activation of cyclin D1/CDK4 during G1 phase, activation of cyclin E/CDK2 in late G1 is required for cells to progress through the cell cycle [38,47]. p21 inhibits both cyclin D1/CDK4 activity and cyclin E/CDK2 activity [2,30,48]. Treatment of MCF-7 breast cancer cells with estradiol stimulates cyclin D1-associated kinase (Fig. 4B) and CDK2 activity (Fig. 5B), which confirms the previous finding [47]. Pretreatment of cells with BMP-2, however, significantly blocked the estrogen-induced increase in both these kinase activities (Figs. 4B and 5B). Furthermore, BMP-2 increased the association of p21 with cyclin E (Fig. 5A). Our data for the first time demonstrate that BMP-2 targets the cell machinery at the level of CDKs. Thus one of the mechanisms by which BMP-2 inhibits MCF-7 cell proliferation is by inhibiting CDKs that are known to be activated in mid to late G1 and S-phases of cell cycle.

One of the targets of G1 CDKs is the tumor suppressor protein pRb [35]. This notion is established from various in vitro and in vivo studies. Cyclin D1/CDK4 complex can phosphorylate pRb in vitro [49]. The physiologic regulators that intercept CDK4/6 activity also block pRb phosphorylation. Similarly, overexpression of cyclin E in human osteosarcoma cells increases pRb phosphorylation [50]. pRb is also hyperphosphorylated in various breast cancer cells and tissues by cyclin E/CDK2 activity [51]. In tamoxifen-arrested MCF-7 cells, estradiol stimulates cyclin E/CDK2-dependent pRb phosphorylation [47]. We have also shown that treatment of serum-deprived MCF-7 cells with estradiol increased the level of hyperphosphorylated inactive pRb and that presence of BMP-2 during estradiol treatment caused reduction in the degree of pRb phosphorylation (Fig. 6). These observations describe one of the first mechanisms by which BMP-2 may inhibit MCF-7 breast cancer cell growth in culture.

In summary, we have demonstrated that BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells. This effect of BMP-2 appears to be mediated by inhibition of positive cell cycle regulatory proteins. Hyperproliferation of estrogen-responsive breast cancer cells is one of the major causes of tumor formation in early stages of breast cancer. Agents such as BMP-2 that inhibit estradiol-induced breast cancer cell proliferation may prove to be important therapeutic tools once their mechanisms of action are more thoroughly characterized.

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