Decreased Expression of Bone Morphogenetic Protein (BMP) Receptor Type II Correlates with Insensitivity to BMP-6 in Human Renal Cell Carcinoma Cells

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ABSTRACT

Purpose: **Bone morphogenetic proteins (BMPs) are members of a family of pleiotropic growth factors that play a critical role during renal development as well as maintaining kidney homeostasis. In the present study, we investigated the potential role of BMP receptors (BMPRs) in renal cell carcinoma (RCC) cells.**

Experimental Design: **Immunohistochemistry was used to investigate the expression of BMPRs in human RCC tissues. As an *in vitro* model of RCC, three cell lines were used: 112, 117, and 181. Northern blot, immunoblot, and reverse transcription-PCR were used to study the expression of BMPRs in the cell lines. Finally, cells were transfected using LipofectAMINE.**

Results: **Normal human kidney tissues express the three BMPRs: types RIA, RIB, and RII. In contrast, human RCC cells frequently exhibit a loss of expression of BMP-RII. In tissue culture, BMP-6 inhibits in a dose-dependent manner the proliferation of 112 cells but not of 117 and 181 cells. Assays for BMPRs demonstrated that 117 and 181 cells express low levels of BMP-RII RNA. When these two BMP-6 resistant cell lines were infected with the adenovirus containing the constitutively active form of BMP-RIA or -RIB in combination with a BMP-6-responsive luciferase reporter construct, luciferase activity increased. Finally, when these cell lines were transfected with BMP-RII, BMP-6-sensitivity was restored.**

Conclusions: **These results demonstrate that human RCC tissues frequently have decreased levels of expression of BMP-RII and that the human RCC cell lines 117 and 181 are resistant to the growth-inhibitory effect of BMP-6 because they have decreased levels of expression of BMP-RII.**

INTRODUCTION

In the United States, RCC[5] afflicts ~27,000 patients and claims ~11,000 lives annually (1). It is the tenth most common cancer and constitutes 3% of all adult malignancies (1). The incidence of RCC has steadily increased from 1935 to 1989 (2). Surgical resection of the primary tumor is the mainstay of treatment. However, 25–30% of patients present with metastatic disease at the time of diagnosis (3), and 30% of patients with clinically localized disease relapse after radical nephrectomy (4). The prognosis for patients with metastasis is bleak with a 5-year survival rate of less than 10% for stage IV disease (5). A number of clinical trials have demonstrated that RCC is resistant to chemotherapy (6), and immunotherapy with interleukin-2 (IL-2) and/or IFN-α results in a partial or complete response in 10–20% of patients (7). To optimize the currently available treatments and to develop novel therapeutic approaches, a better understanding of molecular mechanisms of renal cell carcinogenesis is necessary.

BMPs, the largest subfamily of the TGF-β superfamily, are pleiotropic growth factors that were originally isolated from the bone as proteins that induce bone and cartilage formation (8, 9). Since the initial isolation, it has been demonstrated that BMPs are critical during mammalian development, cellular chemotaxis, and cellular differentiation (reviewed in Ref. 10). Although investigations of BMP knockout mice suggest that individual BMPs function independently, the specific role(s) of each type of BMPs remains to be elucidated. Of note, BMP signaling follows the paradigm established by TGF-β signaling. As with TGF-β, BMPs signal through an interaction with a heteromeric complex of BMP receptors type I and II (BMP-RI and -RII, respectively). Ligand binding results in cross-phosphorylation of type I receptor by type II; type I receptor, in turn, propagates BMP signaling (11). Currently, three type I receptors (Act-RI, BMP-RIA, and BMPR-IB) and three type II receptors (Act-RII, Act-RIIB, and BMP-RII) have been identified. *In vitro* experiments have shown that all members of BMPs that have been investigated and belong to the TGF-β superfamily bind to

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[5] The abbreviations used are: RCC, renal cell carcinoma; BMP, bone morphogenetic protein; BMPR (or BMP-R), BMP receptor; TGF-β, transforming growth factor-β; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; BMP-RIA-CA, constitutively active BMP-RIA; BMP-RIB-CA, constitutively active BMP-RIB.

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BMP-RII in combination with BMP-RIA or -RIB (12). In contrast, Act-RII/-RIIB and Act-RI does not bind BMP-4 (13). Therefore, BMP-RII, -RIA, and -RIB may be considered BMP-specific receptors.

It has been hypothesized that BMPs are critical for normal renal development based on the following line of evidence. First, BMP-7 knockout mice display a gradual cessation of nephrogenesis in association with a reduction in branching of the ureteric bud and the loss of metanephric mesenchyme via apoptosis (14). Second, Miyazaki *et al.* (15) showed in BMP-4 heterozygous mice that BMP-4 inhibits the ectopic budding of the wolffian duct or the ureteric bud while promoting the elongation of the branching ureter within the metanephros. Lastly, BMP-2 has been shown to inhibit renal collecting tubule formation and, thus, has been suggested to play an important role during the renal branching morphogenesis (16). With regard to BMPRs in the kidney, it has been demonstrated that both BMP-RIA and -RIB are present in the developing renal collecting system (17) and that BMP-RII is preferentially localized to the renal cortex of the rat kidney (18).

Because these observations suggest that BMPs and BMPRs are important factors during normal renal development and homeostasis, we investigated the expression of BMP-RIA, -RIB, and -RII in human RCC tissues and the effect of BMP-6 in association with the expression of BMPRs in three previously characterized human RCC cell lines. We report that human RCC tissues frequently have a loss of expression of BMP-RII and that the decreased levels of expression of BMP-RII are associated with insensitivity to the growth-inhibitory effect of BMP-6 in human RCC cells.

MATERIALS AND METHODS

Patients and Tissues. After obtaining the appropriate approval from the institutional review board, formalin-fixed and paraffin-embedded tissue specimens of 30 histopathologically diagnosed RCCs and 5 samples of benign kidney were obtained from the archives of the Department of Urology, College of Medicine, Yonsei University (Seoul, Korea). Tumor specimens were divided according to the Fuhrman system of nuclear grading. Sections at 4-μm thickness were made and were kept at room temperature until use. One of the sequential sections from each specimen was stained with H&E to verify the histopathological diagnosis.

Immunohistochemistry. All three primary antibodies were purchased from R&D Systems (Minneapolis, MN; BMP-RIA, Cat. no. AF346; BMP-RIB, Cat. no. AF505; BMP-RII, Cat. no. 811). According to the manufacturer's product sheet, all three antibodies were generated in goats immunized with purified recombinant human BMPRs.

Tissue sections were deparaffinized in Xyless and rehydrated with PBS. Endogenous peroxidase activity was inactivated by incubation in 0.3% H_2O_2 for 10 min. After a preincubation with 2% normal serum to block nonspecific sites, the sections were incubated with primary antibodies in a humidified chamber for 18 h at 4°C. All three primary antibodies were used at a concentration of 2 μg/ml. Antigenic binding sites were visualized with a serial incubation with biotinylated secondary antibody, followed by the avidin-biotin-horseradish peroxidase complex, and diaminobenzidine tetrahydrochloride before counterstaining with Gill's hematoxylin. Negative control sections were processed in an identical manner by substitution of primary antibody with a normal IgG fraction. All negative control sections showed no color reactions.

A specimen was classified as negative if the staining level was comparable with that of the negative control slide. All negative cases were confirmed with at least two independent staining experiments. In addition, all stainings were reviewed independently by at least two investigators (I. Y. K., D-H. L.).

Cell Culture and Mitogenic Assay. The establishment and characterization of the three human RCC cell lines, 112, 117, and 181, have been described previously (19). All of the cells used in this study were from the 28th through the 35th passages. Cells were routinely maintained in DMEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Human BMP-6 (R&D Systems, Minneapolis, MN) was diluted to 2 μg/ml and added to culture medium at preselected concentrations.

For cell counts, cells were plated at 20,000/well in 24-well culture plates in DMEM supplemented with 10% FBS and were allowed to adhere for 24 h. Then the cultures were washed two times with PBS, and the cells from previously selected wells were counted to determine the plating efficiency. Cells in the remaining wells were cultured for 4 days in DMEM supplemented with 1% FBS containing BMP-6 at 0, 10, 100, and 500 ng/ml. The medium was changed at day 2. After removing the medium, cells were trypsinized with 0.5 ml of 0.05% trypsin and were counted using a hemocytometer.

RNA Isolation and Northern Blot Analysis. Cells were harvested and total RNA was isolated using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's protocol. Once isolated, 15 μg of total RNA were separated by electrophoresis in 1% formaldehyde-agarose gel and were transferred to nylon membrane (Zeta-Probe GT membrane; Bio-Rad Laboratories, Hercules, CA). The membranes were subsequently rinsed in 2× SSC [1× SSC = 0.15 M NaCl and 0.015 M Na Citrate, (pH 7.0)] and were cross-linked using UV light. Prehybridization was performed in 50% formamide, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% SDS (w/v), and 250 μg/ml heat-denatured salmon sperm DNA for 2 h at 42°C. Hybridization was performed overnight at 42°C in the prehybridization solution containing probe labeled with [^{32}P]dCTP using a random oligonucleotide priming kit (Prime It; Stratagene, La Jolla, CA). The membranes were washed sequentially in 2× SSC-0.1% SDS for 15 min, 0.5× SSC-0.1% SDS for 15 min, and 0.1× SSC-0.1% SDS for 30 min. Autoradiography was carried out at −70°C.

Immunoblot Analysis. Cells were harvested, placed in sample buffer (0.0625 M Trizma base, 2% SDS, and 5% 2-mercaptoethanol), and boiled for 5 min. Electrophoresis was carried out using 50 μg of total protein in each lane. After electrophoresis, protein was transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad). After the transfer, the membranes were incubated in blocking buffer TBST (5% nonfat dry milk, Tris-buffered saline, and 0.1% Tween) for 1 h. Subsequently, the membranes were incubated with appropriate antibodies at a dilution of 1:500 overnight at room temperature. All primary antibodies were purchased from R&D Systems (Minneapolis,

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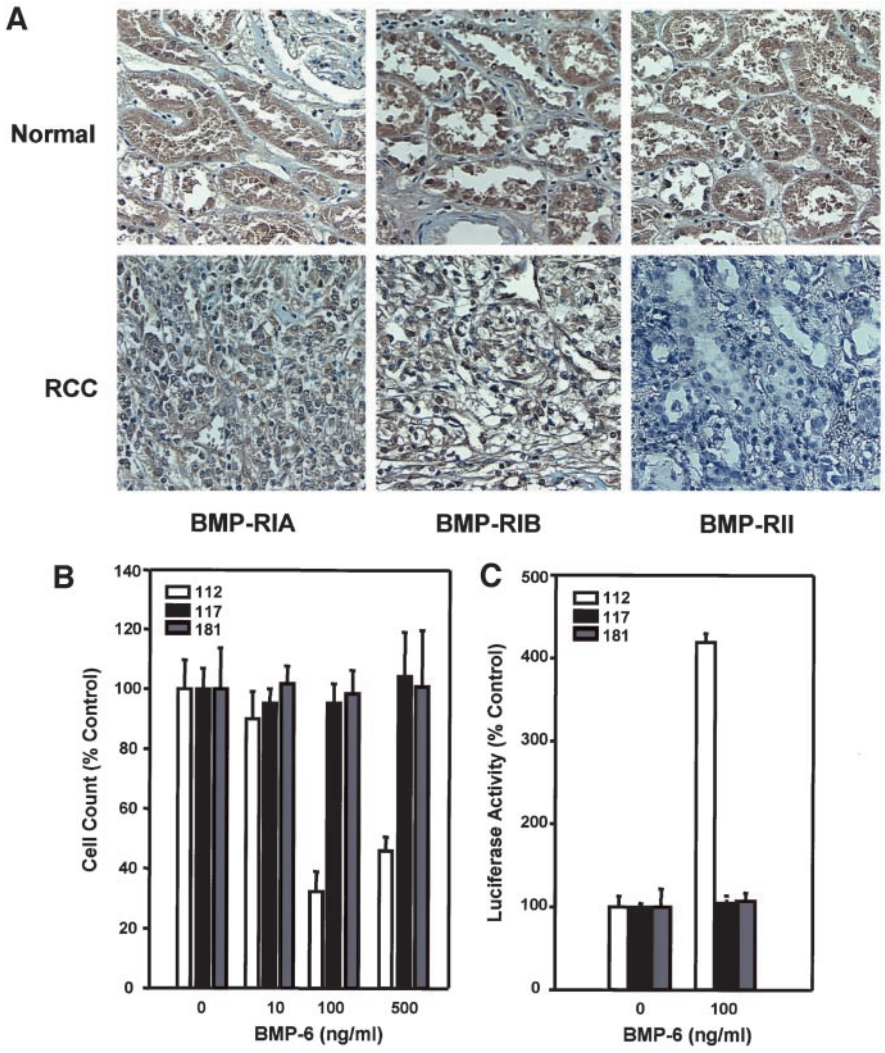

Fig. 1 *A*, expression of BMPRs in human RCC tissues (case 28 from Table 1). *B*, effect of BMP-6 on proliferation of human RCC cells. Cells were treated with BMP-6 as described in the "Materials and Methods" section. *C*, effect of BMP-6 on gene transcriptional activity. Of the three cell lines investigated, only RCC 112 was sensitive to BMP-6 in a dose-dependent manner.

MN). After washing with TBST, the membranes were incubated in the presence of appropriate secondary antibody at a dilution of 1:3000 for 2 h. After washing several times with TBST, immunoreactive bands were visualized by enhanced chemiluminescence.

RT-PCR. Total RNA was reverse-transcribed using Superscript (Life Technologies, Inc.) and random hexamer using the following conditions: 42°C for 50 min and 70°C for 15 min. Subsequently, PCR amplification was performed as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles followed by a 10-min incubation at 72°C. The following primers were used: BMP-RII, 5′-tcagatatatggcaccagaagtg, 3′-gtggagaggctgtgacacttg; BMP-RIA, 5′-aatggagtaaccttagcaccagag, 3′-agctgagtccaggaacctgtac; BMP-RIB, 5′-ggttgcctgtggtcacttctgg, 3′-tagtctgtgattaggtacaactgg. To visualize the PCR products, the samples were subjected to electrophoresis in 1% agarose gel followed by staining with ethidium bromide. The authenticity of the products was confirmed by sequencing.

Transient Transfection and Luciferase Activity Assay. Cells were seeded in 6-well plates at 100,000/well. After allowing the cells to adhere, they were transiently transfected with pSBE4, the plasmid containing the luciferase reporter gene under the control of 4 tandem repeats of smad-binding element (SBE), with or without BMP-RII using Lipofectin according to the manufacturer's directions (Life Technologies, Inc.). Briefly, 1 μg of pSBE4 with or without BMP-RII and 12 μl of Lipofectin were added with 1 ml of transfection medium (Opti-mem, Life Technologies, Inc.) to each well and were incubated for 24 h. Subsequently, fresh medium was added, and the cells were incubated for an additional 24 h. Finally, 100 ng/ml of BMP-6 was added, and the cultures were maintained for an additional 16 h. The extent of the promoter activity of pSBE4 was assayed by measuring luciferase activity using a commercial luciferase assay kit (Enhanced Luciferase Assay Kit; Analytical Luminescence Laboratory, San Diego, CA).

Adenovirus Infection. Recombinant adenoviruses were kindly provided by Dr. Kohei Miyazono (Department of Biochemistry, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan). High-titered stocks of recombinant viruses were grown in 293T cells and were purified. Infection of recombinant adenoviruses was performed at a multiplicity of infection (MOI) of $<8 \times 10^2$ plaque-forming units/cell.

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Statistical Analysis. All of the numerical data are expressed as mean ± SE of triplicate observations. Differences of means among different treatments were compared by χ^2. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Expression of BMPRs in Human RCC Tissues. Initially, the levels of expression of BMP-RIA, -RIB, and -RII were investigated in human RCC tissues. The results, shown in Fig. 1*A*, demonstrated that the three BMPRs are expressed predominantly by the tubular epithelial cells in the normal kidney. In RCC cells, the levels of expression of BMP-RIA and -RIB were similar to that of the normal renal tubular cells. In contrast, the level of expression of BMP-RII was significantly decreased in RCC cells (Table 1).

Effect of BMP-6 in Human RCC Cells. To determine the effect of the loss of BMP signaling in human RCC cells, we subsequently investigated the role of BMP-6 in three human RCC cell lines. Initially, cell numbers were determined after treatment with increasing doses of BMP-6 for 4 days. The result, shown in Fig. 1*B*, demonstrate that BMP-6 inhibits the proliferation of 112 but not of 117 and 181 cells in a dose-dependent manner. At 100 ng/ml of BMP-6, the number of 112 cells was less than 40% of that of the control. Because the activation of a BMP-responsive promoter that is present in the plasmid pSBE4 has been reported to reflect the gene-transcriptional activity of BMPs (20), the three RCC cell lines were transiently transfected with pSBE4. After the transfection, the cells were treated with 100 ng/ml BMP-6 for 16 h. Fig. 1*C* shows that the BMP-6 treatment resulted in a 4-fold induction of luciferase activity in 112 cells. As with the result of the proliferation assay, BMP-6 treatment did not alter significantly the levels of luciferase activity in 117 and 181 cells.

Table 1 Expression of BMP-RIA, -RIB, and -RII in benign and malignant human kidney tissues

Case no.	Fuhrman grade	BMP-RIA	BMP-RIB	BMP-RII
1	Benign	+	+	+
2	Benign	+	+	+
3	Benign	+	+	+
4	Benign	+	+	+
5	Benign	+	+	+
6	1	+	+	+
7	1	+	+	−
8	1	+	+	+
9	1	+	−	+
10	1	−	+	+
11	1	+	+	+
12	2	+	+	−
13	2	+	+	+
14	2	+	−	−
15	2	−	+	+
16	2	+	+	+
17	2	+	+	−
18	2	+	+	−
19	3	+	+	−
20	3	+	+	−
21	3	+	+	−
22	3	+	+	+
23	3	+	+	+
24	3	−	−	−
25	3	+	+	−
26	3	+	+	−
27	4	−	−	−
28	4	+	+	−
29	4	+	+	−
30	4	+	+	−
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32	4	−	+	−
33	4	+	−	+
34	4	+	+	−
35	4	+	+	−

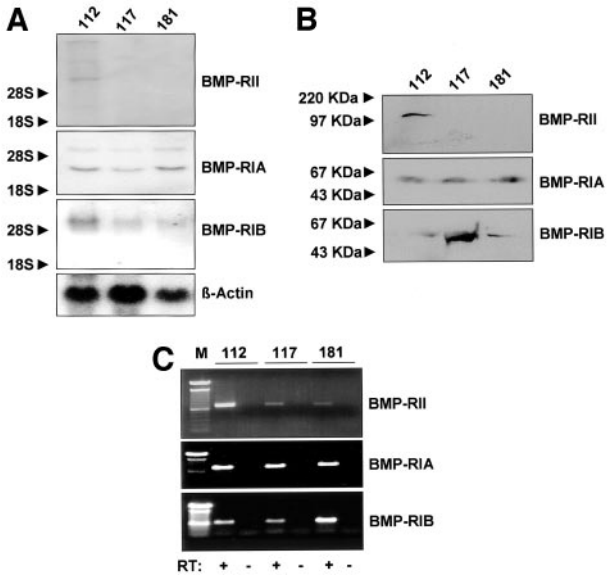

Fig. 2 Expression of BMPRs in human RCC cells. *A*, Northern blot analysis. *B*, immunoblot analysis. *C*, RT-PCR. When compared with the BMP-6-sensitive cell line 112, RCC 117 and 181 cells exhibited decreased expression of BMP-RII. *KDa*, molecular weight (M_r) in thousands.

Expression of BMPRs in Human RCC Cells. To determine the mechanism of loss of sensitivity to BMP-6 in 117 and 181 cells, Northern blot analysis for BMP-RII, -RIA, and -RIB was carried out (Fig. 2*A*). As reported previously, there were two- and three-splice variants for BMP-RIA and -RII, respectively. In the BMP-6-sensitive 112 cells, all three BMPRs were detected. In contrast, both 117 and 181 cells had undetectable levels of expression of BMP-RII. To substantiate the results of the Northern blot analysis, we subsequently performed immunoblot analysis (Fig. 2*B*). As expected, 117 and 181 cells did not express detectable levels of expression of BMP-RII. To enhance the level of sensitivity of detection for BMP-RII in 117 and 181 cells, RT-PCR was carried out. As shown in Fig. 3*C*, the two BMP-6-insensitive cell lines were positive for BMP-RII. These results, taken together, suggest that 117 and 181 cells express low levels of BMPR-II that is detectable only by RT-PCR.

Because a loss of one allele at the genomic DNA level may lead to a gene-dosage effect of BMP-RII in RCC cells, Southern blot analysis was performed. The results did not demonstrate

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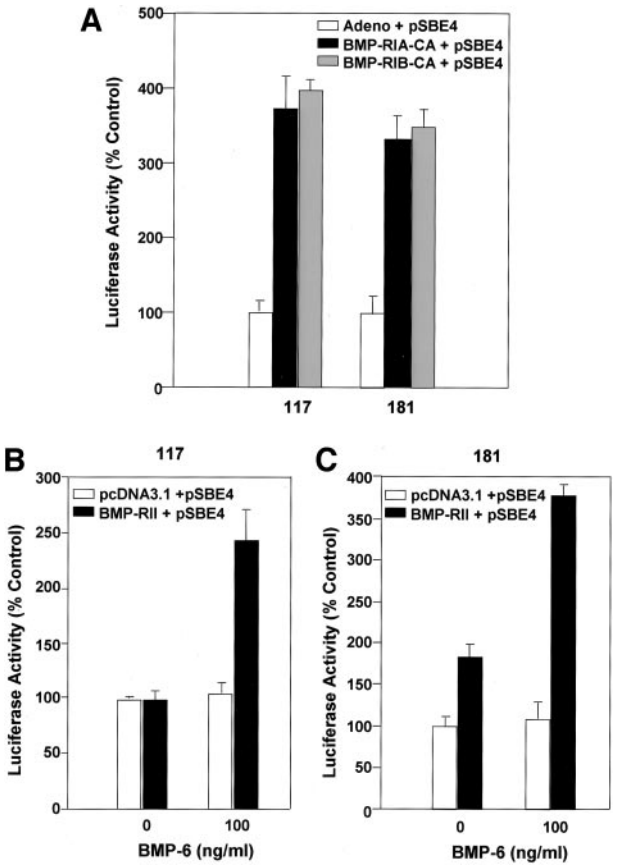

Fig. 3 *A*, effect of BMP-RIA-CA and BMP-RIB-CA, respectively, on gene transcriptional activity in the BMP-6-resistant cell lines. Adenovirus containing either BMP-RIA-CA or BMP-RIB-CA were infected into the BMP-6-resistant cell lines as described in the "Materials and Methods" section. Both BMP-RIA-CA and BMP-RIB-CA increased the level of gene transcriptional activity in the two BMP-6-resistant cell lines. These results suggest that the level of BMP-6-signaling defect in these cells is at the receptor level. *B*, effect of BMP-RII transfection on gene transcriptional activity in 117 and 181 cells. Transfection with BMP-RII increased the level of luciferase activity in both cell lines after treatment with BMP-6 at 100 ng/ml. These results suggest that the insensitivity to BMP-6 is due to a decreased level of expression of BMP-RII in 117 and 181 cells. Adeno, adenovirus carrying β-galactosidase.

any gross genetic abnormalities in 117 and 181 cells (data not shown).

The Level of Signaling Defect in Human RCC Cells. To determine the level of BMP signaling defect in the two human RCC cell lines, 117 and 181, adenovirus containing BMP-RIA-CA or BMP-RIB-CA was used. After the infection with either adenovirus, the cells were transiently transfected with pSBE4, and luciferase activity was measured. As shown in Fig. 3*A*, the levels of luciferase activity in 117 and 181 cells increased significantly after the infection with both BMP-RIA-CA and BMP-RIB-CA. These results demonstrate that the signaling molecules for gene transcriptional activity of BMPs that are down-stream of BMPRs are intact and that the level of BMP-signaling defect is at the receptor level in both 117 and 181 cells.

Restoration of BMP Signaling in Human RCC Cells by BMP-RII. To show that the decreased levels of expression of BMP-RII is the mechanism responsible for the resistance to BMP-6, 117 and 181 cells were transiently cotransfected with BMP-RII and pSBE4. After the treatment with 100 ng/ml BMP-6, luciferase activity was again measured. The results, shown in Fig. 3*B*, demonstrate that transfection with BMP-RII resulted in a significant level of induction of luciferase activity in the two BMP-6-resistant cell lines. Interestingly, the level of luciferase activity increased in 181 cells in the absence of BMP-6 after the transfection with BMP-RII, which suggests the endogenous production of BMPs in 181 cells.

DISCUSSION

Results of the present study demonstrate a significant loss of expression of BMP-RII in human RCC tissues. In addition, a correlation between the sensitivity to BMP-6 and the expression of its cognate receptor BMP-RII in three human RCC cell lines (112, 117, and 181) was observed. Specifically, it was demonstrated that the BMP-6-sensitive cell line 112 expresses BMP-RII, -RIA, and -RIB. On the other hand, 117 and 181 cells were insensitive to BMP-6 and expressed decreased levels of BMP-RII RNA and protein. Infection with an adenovirus containing either BMP-RIA-CA or BMP-RIB-CA demonstrated that the level of BMP-6-signaling defect is at the receptor level in both 117 and 181 cells. Finally, when 117 and 181 cells were transfected with BMP-RII, BMP-6 sensitivity was restored. These observations, taken together, provide a valuable insight regarding the potential role of BMPs and BMPRs in human RCC cells.

BMPs were originally given the name because they were isolated from the bone. However, recently published works clearly demonstrate that this group of growth factors is important for normal renal development and homeostasis. For example, BMP-7 knockout mice have bilateral renal agenesis, and BMP-2 and -4 null mice exhibit abnormal renal development and branching morphogenesis (14–16). In the present study, it was demonstrated that the proliferation of the human RCC cell line 112 is inhibited by BMP-6 in a dose-dependent manner. This observed inhibitory effect of BMP-6, consistent with the general characteristics of TGF-β superfamily members, supports the notion that BMPs are differentiating factors in the kidney. The effect of the loss of sensitivity to BMP-6 on tumorigenic potential in RCC cells remains unclear at present. Nevertheless, because BMPs likely function in the kidney as regulators of homeostasis and prevent abnormal proliferation of renal tubules, the loss of sensitivity to BMPs in RCC cells may be necessary to escape the normal physiological constraint on cellular proliferation and to achieve the malignant phenotype. Further work is necessary to verify this concept.

In agreement with the paradigm of TGF-β and its receptors expression during carcinogenesis, results of the present study indicate that the decreased expression of BMPRs may be a potential mechanism for acquiring resistance to the growth-inhibitory effect of BMPs. Specifically, the two BMP-resistant RCC cell lines, 117 and 181, exhibited decreased levels of expression of BMP-RII when compared with the BMP-6-sensitive cell line, 112. Furthermore, BMP-6 signaling was restored with the transient transfection of BMP-RII. The mechanism

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underlying the decreased level of expression of BMP-RII is not clear at present. One possibility is the loss of one allele at the genomic DNA level, thus, resulting in a gene-dosage effect. However, Southern blot analysis did not demonstrate any gross genetic abnormalities (data not shown). Alternatively, transcriptional repression and/or decreased mRNA stability may be the responsible mechanism.

As with TGF-β, BMP signaling requires both type I and type II receptors (11). Thus, the effect of the loss of expression of BMP-RI on tumorigenic potential should be similar to that of BMP-RII. In the present study, only the abnormal expression of BMP-RII was demonstrated in human RCC cells. Nevertheless, it is likely that the loss of expression of BMP-RI will also lead to an altered response to BMP treatment. Experiments are under way to test this hypothesis.

In conclusion, results of the present study demonstrated that BMP-6 is a potent inhibitor of growth in human RCC cells. In addition, some RCC cells gain resistance to the growth-inhibitory effect of BMP-6 through abnormal expressions of BMP-RII. Because BMP is present in abundant amount in the kidney, the loss of sensitivity to BMPs may signify a critical point during the development and progression of RCC. In the future, the mechanism for the loss of BMPRs expression in RCC cells will be investigated.

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