

# BMP-2 inhibits tumor growth of human renal cell carcinoma and induces bone formation

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Bone morphogenetic protein-2 (BMP-2), a member of the transforming growth factor superfamily, has been shown to have inhibitory effect on many tumor types. However, the effect of BMP-2 on human renal cell carcinoma (RCC) is still unknown. We previously showed that BMP-2 inhibits tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cells. Our study investigates the effect of BMP-2 on human RCC using ACHN and Caki-2 cell lines. Three types of BMP receptors were found to be expressed in ACHN and Caki-2 cells. *In vitro*, BMP-2 was found to inhibit the growth of ACHN and Caki-2 cells. The antiproliferative effect seems to be due to cell cycle arrest in the G1 phase, which was revealed by flow cytometry analysis. Using reverse transcriptase polymerase chain reaction analysis, we demonstrated BMP-2 upregulated osteogenic markers *Runx-2* and *Collagen Type I* gene expression in ACHN and Caki-2 cells. Treatment of ACHN and Caki-2 cells with BMP-2 induced a rapid phosphorylation of Smad1/5/8. *In vivo*, all animals receiving low number of ACHN ( $1 \times 10^4$ ) and Caki-2 ( $5 \times 10^4$ ) cells treated with 30  $\mu\text{g}$  of BMP-2 per animal showed limited tumor growth with significant bone formation, whereas untreated cells developed large tumor masses without bone formation in immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice. These results suggest that BMP-2 inhibits growth of RCC as well as causes induction of osseous bone formation. Further research is needed to determine the relationship between inhibition of cell proliferation and bone induction.

Renal cell carcinoma (RCC) is the eighth most common cancer and highly lethal, accounting for about 13,120 deaths in the United States in 2011.<sup>1</sup> The incidence continues to increase by 2% worldwide yearly. The major cause of death from RCC is metastasis that is resistant to therapy.<sup>2</sup> RCC most commonly metastasizes to lung, bone, liver and brain. Approximately 35% of patients with metastases have skeletal complications causing pathological fractures, nerve compression syndromes, severe pain and hypercalcemia.<sup>3</sup> Although radiation therapy is often used as adjuvant treatment for bone lesions, it is often ineffective in slowing disease progression in the skeleton or indefinitely relieving pain. Bone metastases in RCC mainly cause destructive osteolytic lesions in bone and lead to marked morbidity.<sup>4</sup> Therefore, to more

effectively control this disease and to improve patient survival rate, there is an urgent need to develop new treatment options for preventing RCC tumor cell growth.

Bone morphogenetic protein-2 (BMP-2) has the capability to induce the entire cascade of cartilage and bone formation *in vivo*.<sup>5</sup> Besides inducing bone formation, BMP-2 has also been shown to play important roles in the regulation of various cellular processes, including cell differentiation, proliferation, morphogenesis, cellular survival and apoptosis.<sup>6,7</sup> Similar to transforming growth factor (TGF)- $\beta$ , BMP-2 exerts its effect *via* specific Type 1 and Type 2 transmembrane serine/threonine kinase receptors (BMPR). Binding of BMP-2 to the Type 2 receptor induces oligomerization of the receptor complex, leading to phosphorylation of the Type 1 receptor and recruitment of downstream signaling Sma- and Mad-related proteins (Smads). As a result, Smad1, Smad5 and Smad8 are activated by BMP-2 receptors. Phosphorylated Smad1/5/8 forms a complex with Smad4 and then translocates to the nucleus to modulate the transcription of a variety of target genes that mediate the biological activity of BMP-2.<sup>8,9</sup>

More recently, BMP-2 has been demonstrated to play different roles on cancer cells dependent on the tissue type and environment. BMP-2 has been shown to stimulate the growth of pancreatic carcinoma, lung carcinoma and prostate cancer cells in the absence of androgen.<sup>10-12</sup> On the other hand, BMP-2 clearly inhibits the growth of tumor cells of many origins including breast cancer,<sup>13,14</sup> myeloma,<sup>15</sup> gastric cancer,<sup>16</sup> colon cancer<sup>17</sup> and prostate cancer.<sup>12,18</sup> Our previous

**Key words:** BMP-2, renal cell carcinoma, Smad, osteogenic differentiation, metastasis

**Grant sponsor:** National Institutes of Health; **Grant number:** R01 AR 056649; **Grant sponsor:** Department of Neurosurgery, University of Michigan

**DOI:** 10.1002/ijc.27444

**History:** Received 12 Sep 2011; Accepted 5 Jan 2012; Online 24 Jan 2012

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study also showed that BMP-2 inhibits the tumorigenicity of cancer stem cells in the human osteosarcoma OS99-1 cells.<sup>19</sup> To date, there are no published studies on the impact of recombinant human BMP-2 (rhBMP-2) on human RCC. rhBMP-2 is currently approved by the United States Food and Drug Administration to promote certain spinal fusions and is commercially available for clinical application for bone repair. We hypothesized that rhBMP-2 might be used for RCC bone metastasis to facilitate bone growth after surgical resection if rhBMP-2 could exert an inhibitory effect on the growth of human RCC.

## Material and Methods

### Cell culture

Human renal carcinoma cell lines ACHN and Caki-2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium/F12 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

### Cell proliferation assay

To investigate the effects of BMP-2 on cell growth, ACHN and Caki-2 cells were inoculated at  $5 \times 10^3$  cells per well in 96-well culture plates. The following day, the culture medium was changed to 1% serum-containing medium, and the cells were grown for 24 hr. Cells were then treated with 10, 100 or 300 ng/ml of rhBMP-2 (GenScript Corporation, Piscataway, NJ) or vehicle control for 24, 48 and 72 hr. Cell growth was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI) as described previously.<sup>19</sup>

### Cell cycle analysis

For cell cycle analysis by flow cytometry, ACHN and Caki-2 cells were inoculated in six-well culture plates. The following day, the culture medium was changed to 1% serum-containing medium, and the cells were grown for 24 hr. Cells treated with 300 ng/ml of BMP-2 or vehicle control for 48 hr were then fixed with 70% ethanol for 20 min on ice. Cells were suspended in PBS containing 50 µg/ml of propidium iodide (Invitrogen, Grand Island, NY) and 100 µg/ml of RNase (Invitrogen) to stain nuclear DNA for 30 min at room temperature. DNA content was analyzed using flow cytometry (Becton Dickinson, San Jose, CA). Data were analyzed for DNA histograms using BD CellQuest software (Becton Dickinson).

### Flow-cytometric analysis of apoptosis

To determine whether BMP-2 could induce apoptosis, ACHN and Caki-2 cells were inoculated in six-well culture plates. The following day, the culture medium was changed to 1% serum-containing medium, and the cells were grown for 24 hr. Cells were then treated with 300 ng/ml of BMP-2 or vehicle control for 48 hr. Annexin V-cy3-labeled Apoptosis Detection Kit

(Invitrogen) was used for the detection of apoptotic cells by flow cytometry as described previously.<sup>20</sup> Data were analyzed using CellQuest software (Becton Dickinson).

### Semiquantitative reverse transcriptase polymerase chain reaction

To test the expression of BMP receptors in ACHN and Caki-2 cells, semiquantitative polymerase chain reaction (PCR) was performed as described previously.<sup>19</sup> The PCR primers included BMPRIA (forward, 5'-AAT GGAGTAACCTTAG-CACCAGAG-3'; reverse, 5'-AGCTGAGTCCAGGAACCTGTAC-3'), BMPR1B (forward, 5'-GGTTGCCTGTGGTCACTTCTGG-3'; reverse, 5'-TAGTCTGTG ATTAGGTACAACCTG G-3'), BMPR2 (forward, 5'-TCAGATATATGGCACCAGAA GTG-3'; reverse, 5'-GTGGAGAGGCTGGTGACACTTG-3') and β-actin (forward, 5'-GCGGGAAAT CGTGCGTGACA TT-3'; reverse, 5'-GGCAGATGGTTCGTTTGGCTGAATA-3'). MCF-7 cell line was used as positive controls as described previously.<sup>21</sup>

### Quantitative real-time polymerase chain reaction

ACHN and Caki-2 cells were inoculated in 10-cm culture dishes. The following day, the culture medium was changed to 1% serum-containing medium, and the cells were grown for 24 hr. Cells were then treated with 300 ng/ml of BMP-2 or vehicle control for the time indicated. Quantitative real-time PCR (qRT-PCR) of osteogenic markers *Runx-2* (Hs00231692\_m1) and *Collagen Type I* (Hs00164004\_m1), *BMPRIA* (Hs01034913\_g1), *BMPR1B* (Hs00176144-m1), *BMPR2* (Hs00176148-m1) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression were conducted using Eppendorf Mastercycler Realplex Detection System (Eppendorf, Germany), as we previously described.<sup>19</sup> Real-time quantitative primers were designed and purchased from Applied Biosystems. A positive standard curve for each primer was obtained using serially diluted cDNA sample mixture. The quantity of gene expression was calculated using standard samples and normalized with *GAPDH*.

### Immunocytochemical staining

Immunocytochemistry was performed using three antibodies: BMPRIA, BMPR1B and BMPR2 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Immunofluorescent staining was performed as we previously reported.<sup>22,23</sup> Digital images were captured using an Olympus microscope (Olympus America, Milville, NY).

### Western blot analysis

Cells treated with BMP-2 (300 ng/ml) or vehicle control were lysed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Pittsburgh, PA) supplemented with protease inhibitor (Promega), phenylmethanesulfonyl fluoride (Sigma, Brooklyn, NY) and PhosStop (Roche, Basel, Switzerland). The protein concentrations were then measured using bicinchoninic acid (BCA) assay (Thermo Scientific). Next, the

protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto hybond-C pure nitrocellulose membrane (Amersham, Piscataway, NJ). Membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 with 5% nonfat dry milk, and then incubated with antiphospho-Smad1/5/8 or anti-GAPDH (Cell Signaling Technology, Danvers, MA) overnight. After washing with TBS with Tween 20, the secondary antibodies were added. Finally, the proteins were visualized with the enhanced chemiluminescence (ECL) chemiluminescence system (Amersham).

### ***In vivo* cotreatment experiments**

Our previous results indicated that 30  $\mu\text{g}$  of BMP-2 could inhibit the tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cells in immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) (NOD/SCID) mice<sup>19</sup>; therefore, 30  $\mu\text{g}$  of BMP-2 was used to examine the tumor inhibitory effect of BMP-2 on ACHN and Caki-2 cells. BMP-2 (30  $\mu\text{g}$ ) was coated with Affi-Gel blue beads (Bio-Rad Laboratories, Philadelphia, PA) at 37°C for 1 hr as described previously.<sup>24</sup> Different numbers of ACHN and Caki-2 cells were suspended in 100  $\mu\text{l}$  of Affi-Gel blue beads, cells treated with the same volume of vehicle were used as a control and then cells with BMP-2 or vehicle control mixed with Matrigel (1:1 volume; BD Biosciences, San Jose, CA) were subcutaneously injected into right and left lower abdominal area of NOD/SCID mice (Harlan Laboratories, Indianapolis, IN). Tumor growth was monitored weekly for 12 weeks. Tumors formed were removed, and a portion of each tumor was processed for histological analysis. All animal studies were performed according to protocol approved by the Institutional Animal Care and Use Committee of the University of Michigan.

### ***In vivo* assessment of bone formation**

After the mice were killed, radiographs were obtained using the Faxitron X-ray (Faxitron X-ray Corp., Lincolnshire, IL). For microcomputed tomography (micro-CT) analysis, specimens were scanned on a micro-CT scanner (EVS Corporation, Memphis, TN). GEMS MicroView software (GE Healthcare Biosciences, Piscataway, NJ) was used to make a three-dimensional reconstruction from the set of scans. For histomorphometry, specimens were stained for hematoxylin and eosin (H&E) staining and Masson's trichrome staining to show Collagen Type I protein in the newly formed bone. Undecalcified sections were stained with von-Kossa staining to identify the calcification during osteogenesis in the tumor.

### **Statistical analysis**

Data were expressed as the mean  $\pm$  standard deviation. Statistically significant differences were determined using SPSS 11 software (SPSS, Chicago, IL). A *p*-value of  $<0.05$  was considered statistically significant.

## **Results**

### **Expression of BMP receptors in ACHN and Caki-2 cells**

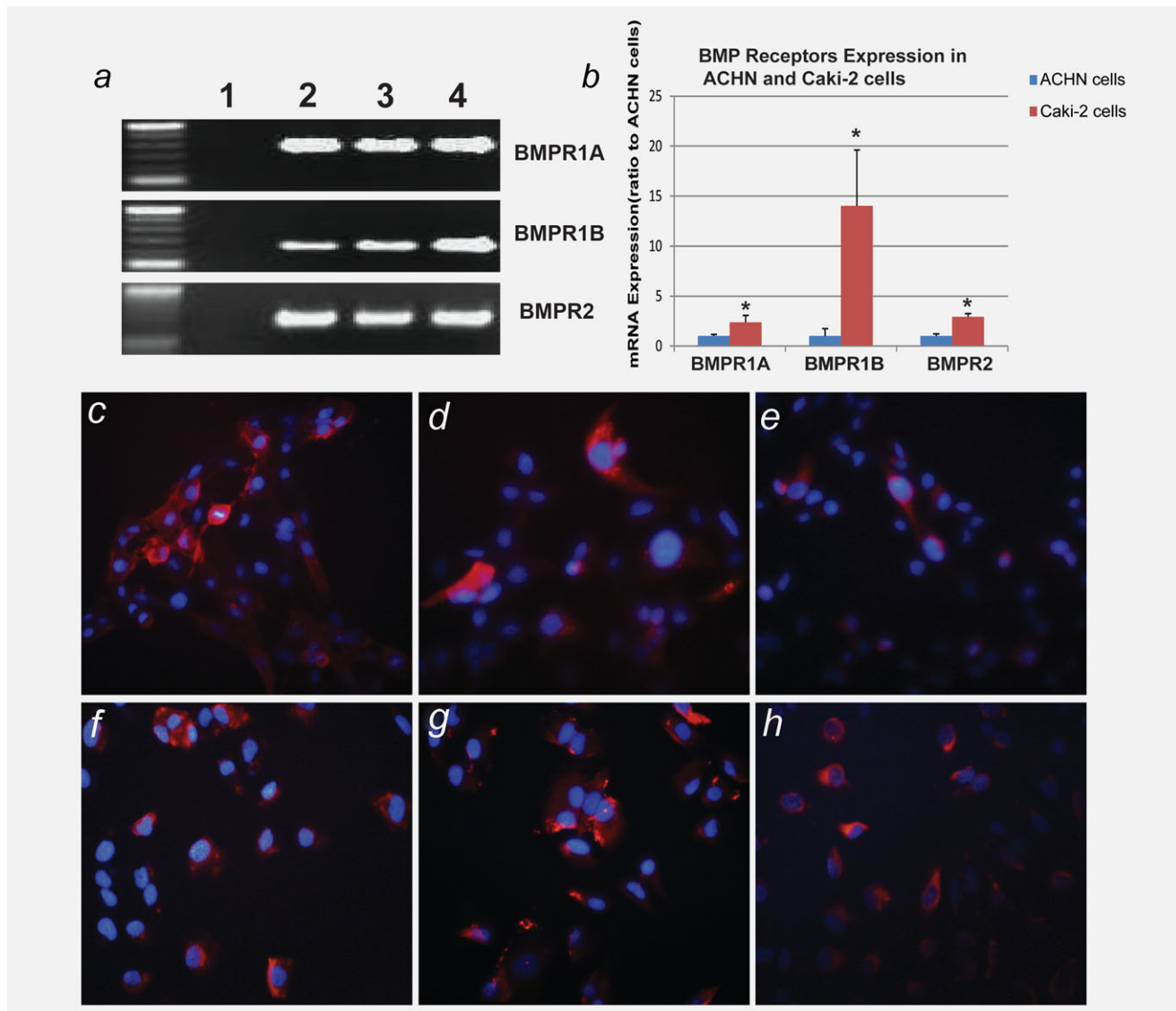
BMP-2 induces a physiological response through the activation of receptors specific for the BMP-2. Intracellular activation begins with BMP-2 binding to Type 2 receptor, which leads to the binding of this complex to either Type 1A or 1B receptor and then phosphorylates the Smad transcription factors.<sup>9</sup> To determine whether the BMP-2 receptors are expressed in ACHN and Caki-2 cells, we first sought to investigate the mRNA expression of BMPRI1A, BMPRI1B and BMPRI2. PCR analysis revealed that BMPRI1A, BMPRI1B and BMPRI2 were expressed in ACHN and Caki-2 cells as shown in Figure 1a, and real-time PCR demonstrated that BMP receptors are more expressed in Caki-2 cells than in ACHN cells (Fig. 1b). All three types of BMP receptors were further demonstrated to be present in ACHN (Figs. 1c–1e) and Caki-2 (Figs. 1f–1h) cells by immunofluorescent staining. These data suggest that human RCC cells have the potential capability of being activated by the BMP-2 ligand.

### **BMP-2 inhibits the proliferation of ACHN and Caki-2 cells**

The proliferation activity of ACHN and Caki-2 cells was examined by MTS assay after BMP-2 (0, 10, 100 or 300 ng/ml) treatment for 24, 48 and 72 hr. As shown in Figure 2, the proliferation of cells treated with 300 ng/ml of BMP-2 was significantly inhibited at 24 hr and tended to be more significant at 48 and 72 hr. Treatment of cells with 10 ng/ml at the three time points induced a stimulation of cell growth to a lesser but significant extent, whereas ACHN and Caki-2 cells demonstrated modest but significant decreases in growth when treated with 100 ng/ml of BMP-2 at the three time points (Figs. 2a and 2b). As 300 ng/ml of BMP-2 significantly produced an inhibitory effect in cell growth in ACHN and Caki-2 cells, we chose this dose for the following experiments *in vitro*.

### **BMP-2 induces G1 cell cycle arrest but has no effect on apoptosis in ACHN and Caki-2 cells**

A possible explanation for the observed growth inhibition of ACHN and Caki-2 cells in response to BMP-2 is either apoptosis or inhibition of new DNA synthesis. We next examined the effects of BMP-2 on ACHN and Caki-2 cell cycle progression and apoptosis by flow cytometry. ACHN (Fig. 3a) and Caki-2 (Fig. 3b) cells incubated with 300 ng/ml of BMP-2 for 48 hr showed approximately 30 and 37% decreases in the S-phase, respectively, compared to controls. The decrease in the S-phase population was accompanied by an increase in the cell number at the G1 phase of the cell cycle. ACHN and Caki-2 cells incubated with 300 ng/ml of BMP-2 for 48 hr showed approximately 15 and 7% increases in the G1 phase, respectively, compared to controls. Because apoptosis may be one of the consequences of cell cycle arrest, we then examined whether BMP-2 induces apoptosis in ACHN and Caki-2 cells using Annexin V staining, which



**Figure 1.** Expression of BMP receptors in ACHN and Caki-2 cells. (a) Messenger RNA transcripts for BMP receptors. Lane 1: negative control; Lane 2: ACHN cells; Lane 3: Caki-2 cells; Lane 4: MCF-7 cells (positive control). (b) Relative quantitative mRNA expression of BMPR1A, BMPR1B and BMPR2 genes in ACHN and Caki-2 cells. Gene expression levels were normalized to  $\beta$ -actin. BMP receptors are more expressed in Caki-2 cells than in ACHN cells (\* $p < 0.05$ ). Representative immunofluorescent staining of BMPR1A, BMPR1B and BMPR2 in ACHN (c–e) and Caki-2 (f–h) cells. The nuclei counterstained with 4',6-diamidino-2 phenylindole (DAPI; blue areas). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

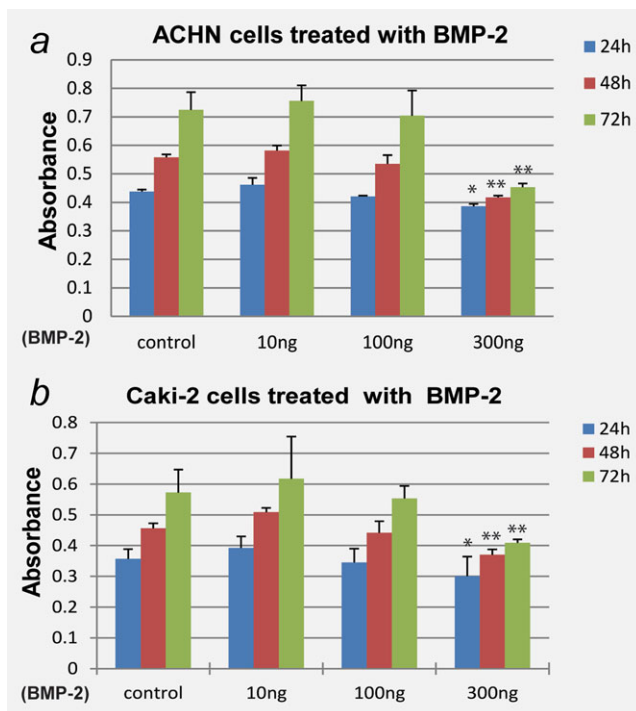
detects early apoptosis by binding to membrane phospholipid translocated from the inner to the outer leaflet of the plasma membrane during apoptosis.<sup>25</sup> Our results indicated that no cell apoptosis was shown by annexin V staining at 48 hr after treatment in ACHN and Caki-2 cells (Fig. 3c). These data suggest that BMP-2 inhibits ACHN and Caki-2 cell proliferation by arresting them at the G1 phase of the cell cycle.

#### **BMP-2 upregulates expression of osteogenic markers in ACHN and Caki-2 cells**

BMP-2 has been shown to upregulate osteogenic marker in human prostate cancer cells and osteosarcoma cells.<sup>18,19</sup> We

therefore sought to determine whether BMP-2 upregulates the transcription of osteogenic markers *Runx-2* and *Collagen Type I* in human RCC cells. ACHN and Caki-2 cells treated with BMP-2 at 300 ng/ml were assayed for *Runx-2* and *Collagen Type I* mRNA level by qRT-PCR. Cells treated with the same volume of vehicle were used as a control. As shown in Figure 4a, the increased *Runx-2* in ACHN cells was significantly detected as early as the first 30 min of stimulation and sustained for 8 hr of BMP-2 stimulation and then showed increased trend until 48 hr after BMP-2 treatment, whereas *Collagen Type I* in ACHN cells exhibited a continuous increase as early as the first 30 min of stimulation and had a





**Figure 2.** BMP-2 inhibits the proliferation of ACHN and Caki-2 cells. ACHN and Caki-2 cells were inoculated in 96-well plates at  $5 \times 10^3$  cells per well. The following day, the culture medium was changed to 1% serum-containing medium, and then the cells were grown for 24 hr. Cells were treated with 10, 100 or 300 ng/ml of BMP-2 or vehicle control for 24, 48 and 72 hr. Growth of ACHN cells (a) and Caki-2 cells (b) was significantly inhibited by the addition of 300 ng/ml of BMP-2 for 24, 48 and 72 hr (\* $p < 0.05$ ; \*\* $p < 0.001$ ). The results are representative of six independent experiments. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

significant increase at 1 hr of BMP-2 stimulation and had an approximately 15-fold increase at 48 hr after BMP-2 treatment (Fig. 4b). Figure 4c shows that *Runx-2* in Caki-2 cells continuously increased starting 30 min and had a significant increase at 1 hr of BMP-2 stimulation and sustained the increase 48 hr after BMP-2 treatment, whereas the increased *Collagen Type I* in Caki-2 cells was significantly detected as early as 2 hr and had an approximately threefold increase 48 hr after BMP-2 treatment (Fig. 4d). These data revealed that osteogenic markers *Runx-2* and *Collagen Type I* were upregulated by BMP-2 in ACHN and Caki-2 cells.

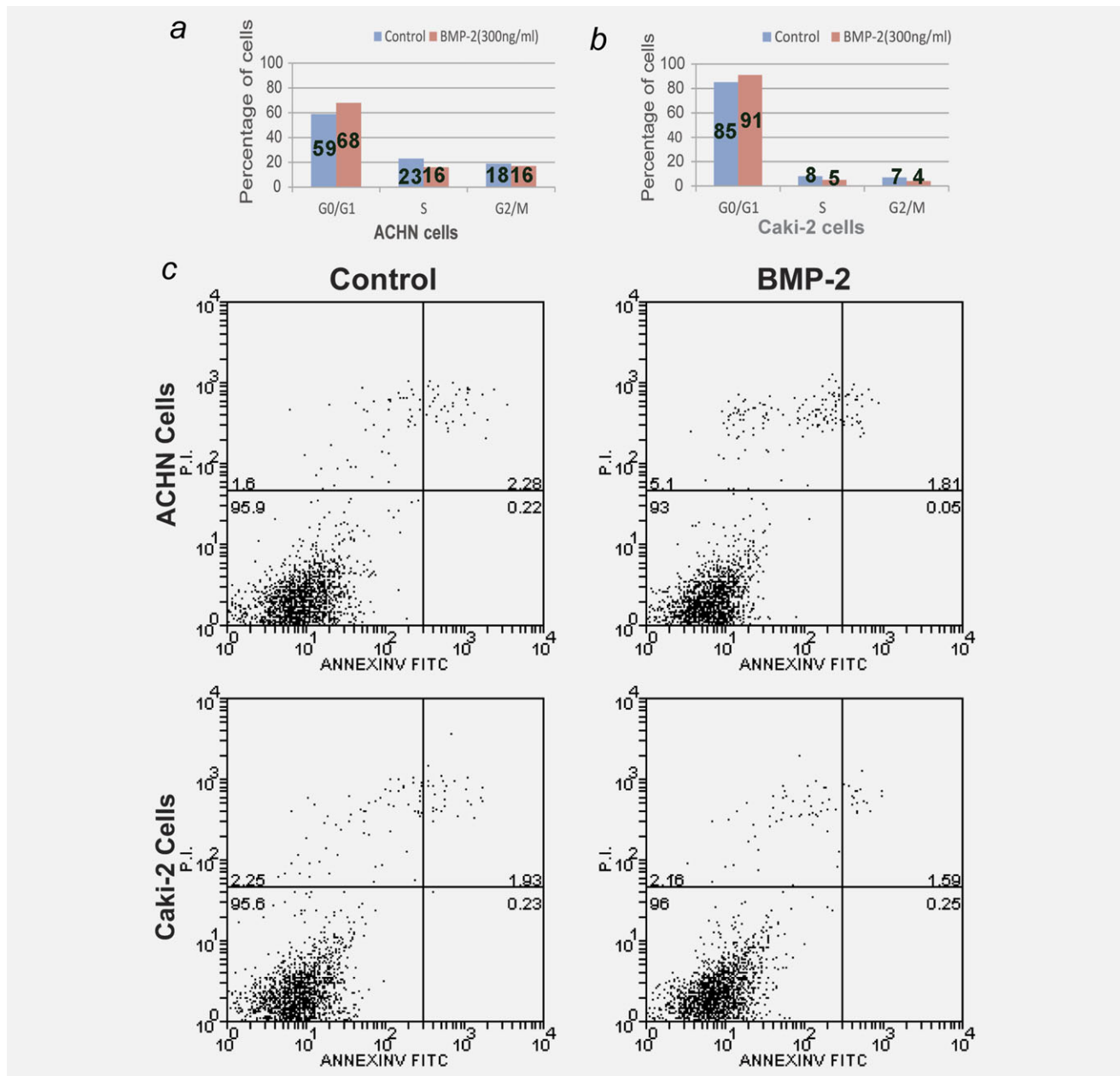
#### BMP-2 activates Smad1/5/8 in ACHN and Caki-2 cells

To determine whether BMP-2 signaling pathways were functional in these two cell lines, we stimulated cells with BMP-2 and examined the phosphorylation and nuclear translocation of Smad1/5/8, because BMP-2 is thought to predominantly act through the activation of these transcription factors.<sup>26</sup> The ability of BMP-2 to phosphorylate Smad1/5/8 was first detected by Western blot analysis using an antibody that spe-

cifically recognizes the phosphorylated forms after cells were treated with BMP-2 or vehicle control. As shown in Figure 5a, phosphorylation of Smad1/5/8 in ACHN and Caki-2 cells was induced as early as 5 min of treatment with 300 ng/ml of BMP-2 and remained elevated after 48 hr of treatment. Localization of phosphorylated Smad1/5/8 was also examined by immunofluorescent staining in cultured ACHN and Caki-2 cells. Consistent with the results obtained by Western blot analysis, phosphorylated Smad1/5/8 was clearly shown in the nuclei of ACHN and Caki-2 cells treated with BMP-2 at 300 ng/ml for 30, 60 and 90 min, respectively (Fig. 5b). These results revealed that BMP receptors are functional and BMP-2 can induce a classical Smad signaling pathway in ACHN and Caki-2 cells.

#### BMP-2 induces bone formation in ACHN and Caki-2 cells in vivo

On the basis of the previous findings, we reasoned that if BMP-2 induces osteogenic marker expression, it might induce bone formation *in vivo*. Therefore, we next injected ACHN and Caki-2 cells with BMP-2 or vehicle control subcutaneously into NOD/SCID mice. Injection of 30  $\mu$ g of BMP-2 per animal was chosen based on our prior work on human osteosarcoma cells, in which BMP-2 inhibits the tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cell line *in vivo*.<sup>19</sup> Experiments were performed as previously described.<sup>27</sup> At 12 weeks after BMP-2 treatment, the animals were euthanized and the bone formations were evaluated. No bone formation was found in eight mice with injection of  $1 \times 10^6$  ACHN and Caki-2 cells with BMP-2 treatment, and conversely, the cells treated with 30  $\mu$ g BMP-2 per animal generated bigger tumors in NOD/SCID mice than cells without BMP-2 counterpart (data not shown). When as many as  $1 \times 10^5$  ACHN and Caki-2 cells with BMP-2 treatment were injected, no bone formations but smaller tumors were observed in eight mice. All four animals receiving  $1 \times 10^4$  ACHN cells treated with 30  $\mu$ g of BMP-2 per animal exhibited retarded tumor growth with significant bone formation, whereas untreated cells exhibited tumor growth without bone formation in NOD/SCID mice (Figs. 6a and 6b). For Caki-2 cells, bone formations were found in all four mice with the injection of  $5 \times 10^4$  cells with BMP-2 treatment. The resulting bone ossicles were evaluated by radiograph and micro-CT. The dense bony ossicles were clearly visualized by radiograph and micro-CT in the ACHN and Caki-2 cells with BMP-2 treatment (Fig. 6c), whereas no bony ossicles were found in the cells without BMP-2 counterpart (data not shown). Subsequent histopathological analyses with H&E, Masson's trichrome and Von Kossa staining confirmed that bone formation induced by ACHN and Caki-2 cells with BMP-2 treatment. As shown in Figure 6d, the sections with Masson's trichrome staining showed blue collagen in the newly formed bone, whereas the sections stained with von-Kossa staining clearly showed calcified bone matrix. These results suggest that



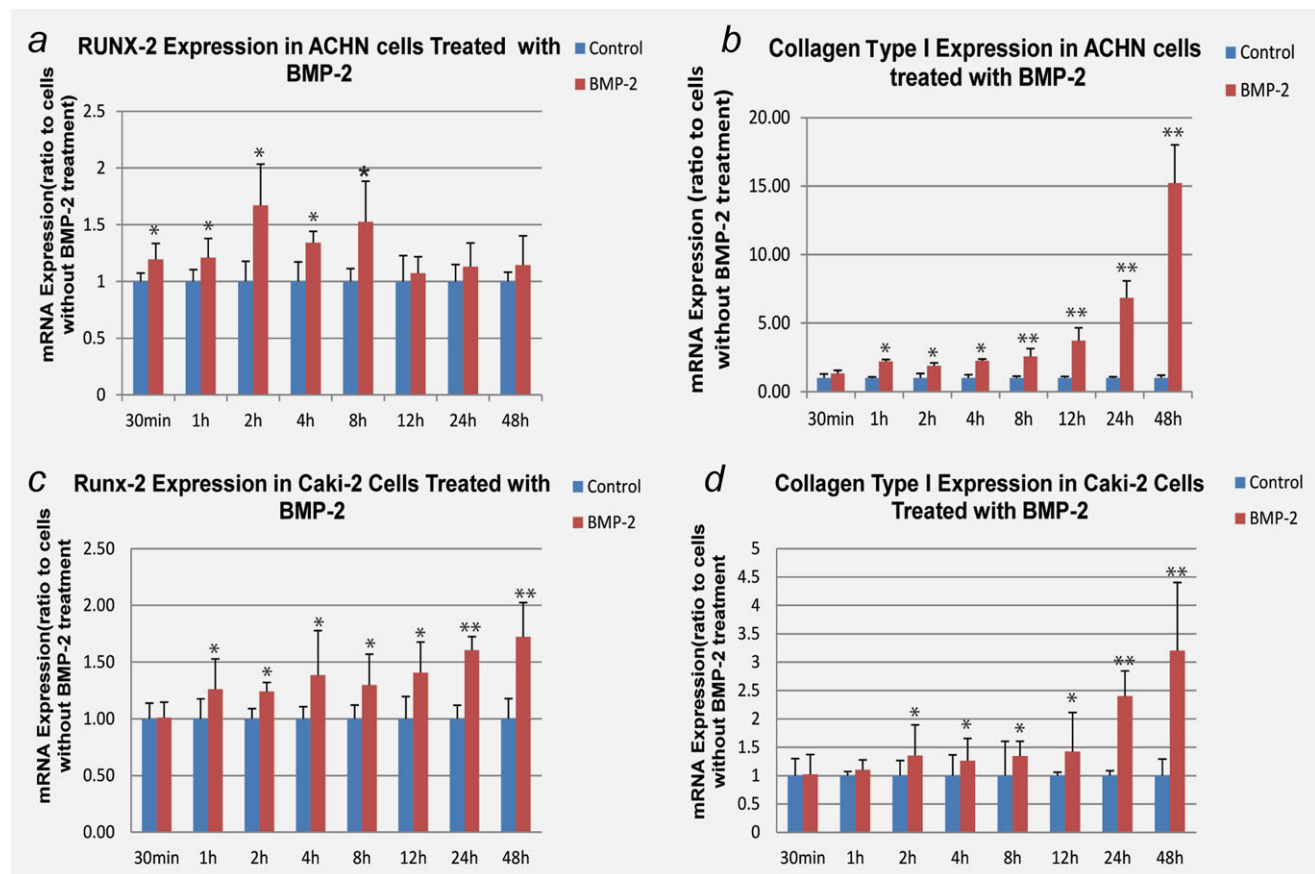
**Figure 3.** BMP-2 induces G1 cell cycle arrest but has no effect on apoptosis in ACHN and Caki-2 cells. ACHN and Caki-2 cells treated with 300 ng/ml of BMP-2 or vehicle control for 48 hr were analyzed for cell cycle by flow cytometry. ACHN (a) and Caki-2 (b) cells exhibited approximately 30 and 37% decreases in the S-phase, respectively, compared to controls. (c) ACHN and Caki-2 cells treated with 300 ng/ml of BMP-2 or vehicle control for 48 hr were stained with FITC-conjugated Annexin V in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. The lower right quadrant indicates the percentage of early apoptotic cells. No more apoptotic cells were found in ACHN and Caki-2 cells with BMP-2 treatment than those without BMP-2 treatment. The results are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

BMP-2 inhibited tumor growth by inducing bone formation in ACHN and Caki-2 cells *in vivo*.

### Discussion

RCC is the most common malignant tumor arising from the kidney and it accounts for more than 30,000 new cases yearly in the United States.<sup>28</sup> 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.<sup>29</sup> Metastatic RCC is a highly fatal disease. The tumor is chemoresistant to currently available therapies. The metastatic bone lesions are destructive and relatively resistant to local treatment with radiation. Pathological fractures of bone containing metastases are frequently treated by surgeons because metastatic bone tumors are more common than primary malignant bone tumors.<sup>30</sup>



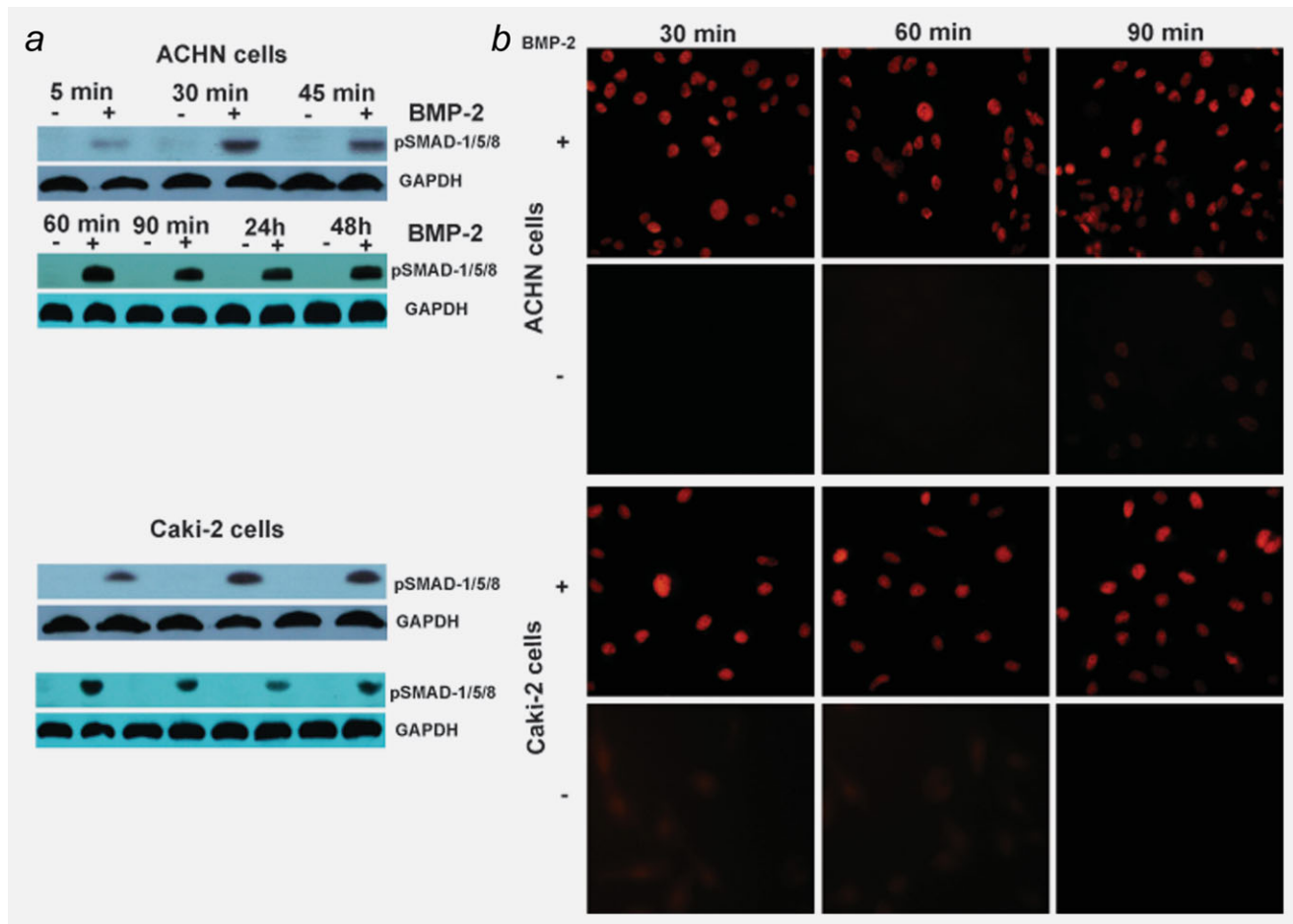
**Figure 4.** BMP-2 upregulates expression of osteogenic markers in ACHN and Caki-2 cells. Relative quantitative mRNA expression of *Runx-2* and *Collagen Type I* genes in ACHN and Caki-2 cells treated with 300 ng/ml of BMP-2 for the time indicated. Gene expression levels were normalized to *GAPDH*. *Runx-2* in ACHN and Caki-2 cells was significantly detected as early as 30 min and 1 hr after BMP-2 treatment, respectively (a and c), whereas *Collagen Type I* had a significant increase in ACHN and Caki-2 cells as early as 1 and 2 hr of BMP-2 treatment, respectively (b and d; \* $p < 0.05$ ; \*\* $p < 0.001$ ). Each experiment was performed three times; representative examples are shown. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

BMP-2, a member of the TGF- $\beta$  superfamily, was originally investigated for its ability to induce the entire cascade of cartilage and bone formation *in vivo*.<sup>5</sup> More recently, BMP-2 has been shown to produce a complex set of effects in cancers. BMP-2 appears to have an inhibitory effect on many tumors including breast cancer, myeloma, gastric cancer, colon cancer and prostate cancer.<sup>12–18</sup> Treatment of human osteosarcoma-derived tumor-initiating cells with BMP-2 has also been observed to inhibit cell proliferation, induction of differentiation and importantly reduce the ability to form tumors in immunodeficient mice.<sup>19</sup> Conversely, BMP-2 has also been found to stimulate the growth of pancreatic carcinomas, lung carcinoma and prostate cancer.<sup>10–12</sup> However, no evidence on the effect of BMP-2 on human RCC cells has been reported.

In our study, we sought to examine the effect of BMP-2 on human RCC using human RCC ACHN and Caki-2 cell lines. It has been reported that BMP signaling for the growth and differentiation of normal or neoplastic cells is dependent on its receptors.<sup>31</sup> There are currently three characterized BMP receptors: BMPRI1A, BMPRI1B and BMPRI1R2. Activation of the

BMP receptor complex initiates intracellular signaling transduction.<sup>9</sup> Using regular PCR and immunostaining, we demonstrated that all three types of BMP receptors were expressed in ACHN and Caki-2 cells, suggesting that BMP-2 could bind to its receptors and activate cell signaling to affect RCC cell activities. Furthermore, we observed that BMP-2 had a significant inhibitory effect on ACHN and Caki-2 cell proliferation at a concentration of 300 ng/ml compared to much lower concentrations. This result is consistent with previous studies that have shown an inhibitory effect of BMP-2 on cancer cell growth including prostate cancer, breast cancer, myeloma, gastric cancer, colon cancer and osteosarcoma.<sup>12–18</sup> A possible explanation for the growth inhibition of ACHN and Caki-2 cells in response to BMP-2 is either apoptosis or inhibition of new DNA synthesis. Our results indicated that BMP-2 did not induce subsequent cell apoptosis, but instead induced cell cycle arrest in the G1 phase, consistent with results reported for gastric cancer cell lines and prostate cancer cell lines.<sup>16,18</sup>

It has been reported that BMP-2 induces the differentiation of uncommitted cells along with osteoblastic pathways in several cell types including mesenchymal stem cells,



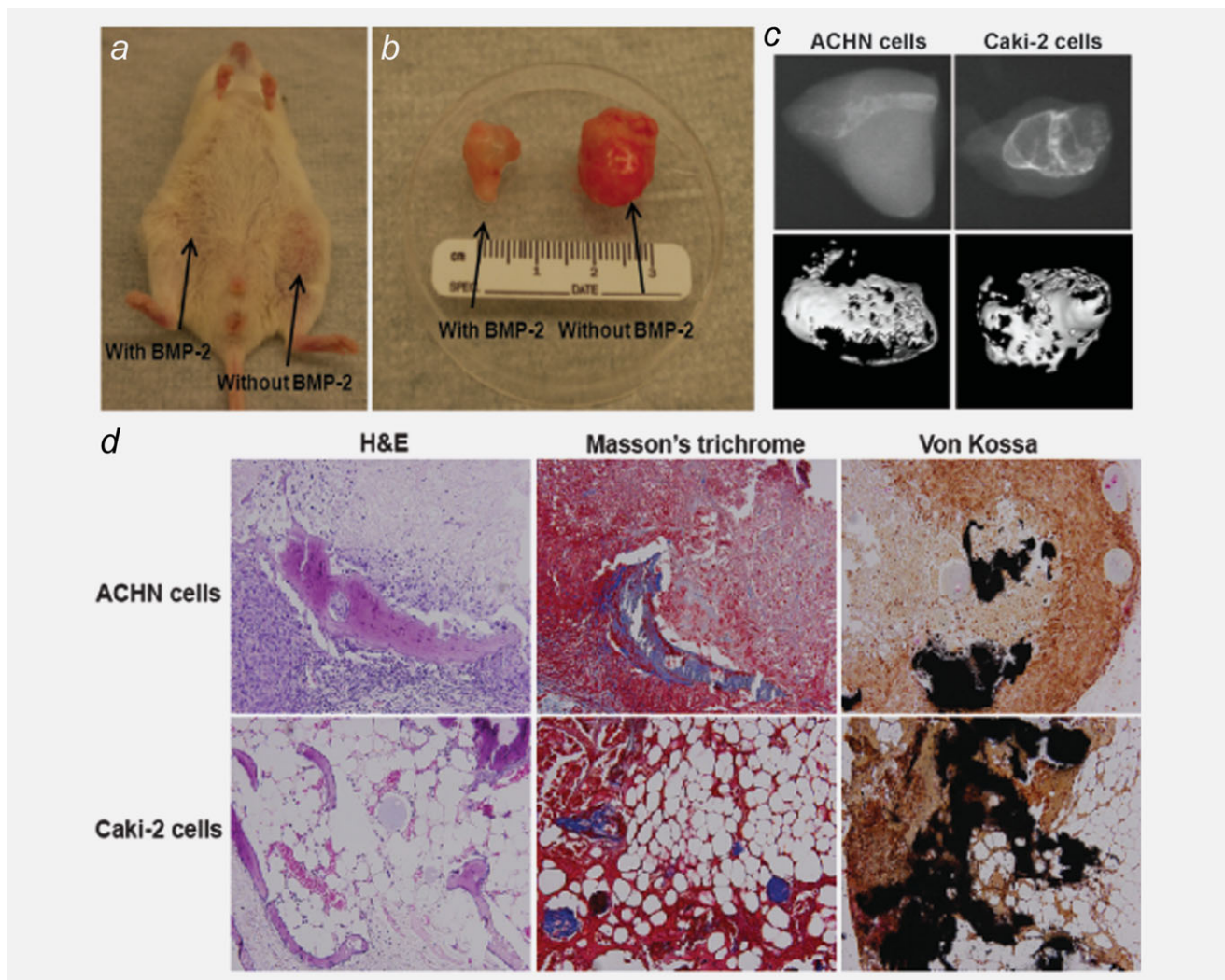
**Figure 5.** BMP-2 activates Smad1/5/8 in ACHN and Caki-2 cells. Phosphorylated Smad1/5/8 in ACHN and Caki-2 cells treated with 300 ng/ml of BMP-2 or vehicle control was detected either by Western blot analysis or by using immunofluorescent staining. Phosphorylation of Smad1/5/8 in ACHN and Caki-2 cells was induced as early as 5 min and remained elevated after 48 hr of treatment (a). Phosphorylated Smad1/5/8 was clearly shown in the nuclei of ACHN and Caki-2 cells treated with BMP-2 for 30, 60 and 90 min (b). Equally loaded protein amounts were demonstrated by detection of GAPDH. Each experiment was performed three times; representative examples are shown. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

myoblast cells and malignant fibrous histiocytoma cells.<sup>32–34</sup> Runx-2 is a transcriptional factor known to regulate the expression of bone-related factors in osteoblasts and thus induce osteoblastic differentiation.<sup>35</sup> Collagen Type I presents by far the most predominant collagen in vertebrate organisms and composes 85–90% of the total bone matrix.<sup>36</sup> Runx-2 and Collagen Type I are known to be upregulated by BMP-2 in human prostate cancer cells and osteosarcoma cells.<sup>18,19</sup> Therefore, using qRT-PCR, we found that *Runx-2* was significantly upregulated in ACHN and Caki-2 cells treated with BMP-2 at a concentration of 300 ng/ml as early as 30 min and 1 hr after BMP-2 stimulation, respectively, compared to untreated controls. *Collagen Type I* was significantly enhanced in ACHN and Caki-2 cells as early as 1 and 2 hr after BMP-2 stimulation, respectively. These genes and their transcripts are functional differentiation markers of osteoblastic phenotype.<sup>37</sup> Thus, our findings suggest that BMP-2 might induce human RCC cells to express an osteoblastic phenotype and thus activate osteogenic differentiation to form bone.

BMP-2 is thought to predominantly exert their effect *via* binding to two types of receptors, leading to downstream transduction of the BMP signal through phosphorylation of specific intracellular proteins called Smads.<sup>8</sup> To further explore whether or not BMP-2 signaling pathways were functional in these two cell lines, the phosphorylation and nuclear translocation of Smad1/5/8 were detected by Western blot analysis and immunostaining using an antibody that specifically recognizes the phosphorylated Smad1/5/8. Our results indicated that phosphorylation of Smad1/5/8 in ACHN and Caki-2 cells was induced as early as 5 min of treatment with 300 ng/ml of BMP-2 and remained elevated after 48 hr of treatment. The elevation of Smad activation at the 5-min time point, before *Runx-2* and *Collagen Type I* were significantly upregulated at 30 min and 1 hr, suggests that transcription regulation of bone-related osteogenic markers *Runx-2* and *Collagen Type I* gene expression by BMP-2 was mediated *via* the Smads transducer.

Because *in vitro* analysis does not always reflect exactly the *in vivo* situation, mouse experiments approximating *in*





**Figure 6.** BMP-2 induces bone formation in ACHN and Caki-2 cells *in vivo*. (a and b) Representative tumor growth of ACHN cells without BMP-2 treatment at the injection site in a NOD/SCID mouse. Smaller tumor formation was seen at the injection site with BMP-2 treatment counterpart. (c) Representative bone formations generated from ACHN and Caki-2 cells with BMP-2 treatment were clearly demonstrated by radiograph and micro-CT measurements of bony ossicles. Images are representative of  $n = 4$  animals per group. (d) Representative bone nodule formations were further confirmed by H&E staining (left), Masson's trichrome staining showing blue collagen (middle) and Von Kossa staining showing calcified bone matrix (right). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

*in vivo* conditions are essential to confirm cell culture results and to assess the potential use of BMP-2 clinically. In our study, we found that all animals receiving low numbers of ACHN ( $1 \times 10^4$ ) and Caki-2 ( $5 \times 10^4$ ) cells treated with 30  $\mu\text{g}$  of BMP-2 per animal resulted in retarded tumor growth with significant bone formation, whereas untreated cells developed large tumor masses without bone formation in NOD/SCID mice. The bone formation was further confirmed by radiograph and micro-CT and histopathological analysis of sections from bony tissues formed by ACHN and Caki-2 cells treated with BMP-2. Thus, exposure to BMP-2 induced bone formation with resultant limited tumor growth. This result is consistent with previous studies of primary cancers that showed ossification of tumor areas as a result of BMP-2.<sup>38–40</sup> In addition, Fong *et al.*<sup>41</sup> reported that viral vec-

tor-induced expression of BMP-2 in a breast cancer cell line and a colon cancer cell line induces calcification of tumors to arrest tumor growth. However, we also found that large number of ACHN and Caki-2 ( $1 \times 10^6$ ) cells treated with 30  $\mu\text{g}$  of BMP-2 per animal induced a stimulation of tumor growth, suggesting that the inhibitory effect of BMP-2 on tumor growth may be related to tumor cell number. Therefore, the effect of rhBMP-2 on cancer cell growth may depend not only on the particular cell type, activity of BMP receptors and growth environment but may also depend on the rhBMP-2 dose used relative to the cancer cell density.

Although our report is the first to provide data indicating that BMP-2 has an inhibitory effect on human RCC ACHN and Caki-2 cells by inducing bone formation, the use of two cell lines provides limited evidence. Further research using

more cell lines and primary tumors is therefore necessary to confirm the findings of our study.

In conclusion, BMP-2 appears to cause cell cycle arrest in the G1 phase of ACHN and Caki-2 cells. In addition, BMP-2 upregulates osteogenic markers Runx-2 and Collagen Type I gene

expression in ACHN and Caki-2 cells, and this osteoinductive effect may be mediated by Smad signaling pathway. Our findings provide evidence on the potential therapeutic effectiveness of BMP-2 on RCC. Further studies to delineate the relationship between tumor growth inhibition and osteoinduction are needed.

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