Bone Morphogenetic Proteins 2 and 5 Are Down-regulated in Adrenocortical Carcinoma and Modulate Adrenal Cell Proliferation and Steroidogenesis

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Abstract

Bone morphogenetic proteins (BMP) have been shown to affect tumorigenesis in a variety of tumors. Quantitative PCR analysis revealed down-regulation of BMP2 and BMP5 in tissue samples from adrenocortical carcinoma and adrenocortical tumor cell lines compared with normal adrenal glands. Integrity of BMP-dependent pathways in these cell lines could be shown by activation of the Smad1/5/8 pathway with subsequent increase of ID protein expression upon incubation with BMP2 or BMP5. On a functional level, BMP treatment resulted in inhibition of cell proliferation and viability in a dose- and time-dependent manner. This growth inhibitory effect was associated with BMP-dependent reduction of AKT phosphorylation under baseline conditions and under insulin-like growth factor costimulation. Furthermore, steroidogenic function, including melanocortin-2 receptor and steroidogenic enzyme expressions, was profoundly reduced. In vitro demethylation treatment and overexpression of GATA6 resulted in reactivation of BMP-dependent pathways with concomitant modulation of steroidogenesis. Taken together, we show that loss of expression of members of the BMP family of ligands is a common finding in adrenocortical tumors and we provide evidence that BMP-dependent pathways are likely to be involved in the modulation of the malignant and functional phenotype of adrenocortical cancer cells. [Cancer Res 2009;69(14):5784–92]

Introduction

Adrenocortical carcinoma (ACC) are rare tumors with an estimated prevalence between 4 and 12 per million and an overall poor prognosis that has not significantly improved over the last decades (1). Surgery remains the mainstay for tumor treatment with a curative approach in patients with early diagnosis. Advanced tumor stages, however, are commonly identified during initial diagnostic work-up, which require systemic treatment modalities. Medical therapy is limited to common cytotoxic agents, which are usually given in combination with the adrenolytic substance mitotane (o,p'-DDD). Unfortunately, these treatment protocols are associated with substantial toxic side effects (2). As for many other tumor entities, specific molecular targeting with kinase inhibitors or monoclonal antibodies holds the promise of therapies with favorable efficacy and tolerability also for patients with adrenocortical cancer (3, 4). Nevertheless, these strategies require detailed knowledge of molecular pathways involved in adrenocortical tumorigenesis, which are only partially available to date (5).

Various growth factors and cytokines, including insulin-like growth factor (IGF), transforming growth factor-α, transforming growth factor-β (TGF-β), vascular endothelial growth factor, and interleukins, have been shown to regulate cellular growth and function of normal adult and fetal adrenals (6). Similarly, the presence of a functional bone morphogenetic protein (BMP) pathway in adrenocortical cells has been previously reported and involvement of BMP6 on mineralocorticoid secretion has been proposed (7, 8). BMPs, which belong to the TGF-β superfamily, have been originally identified by their capacity to induce endochondral bone formation (9) but have since been recognized to play a much more widespread role, particularly in the regulation of cell growth, apoptosis, and differentiation (reviewed in ref. 10). In addition, in a number of cancer entities, BMPs have been shown to act as autocrine or paracrine modifiers of tumor growth and function (11).

BMPs signal through serine/threonine kinase receptors, which are composed of type I and type II subtypes. Upon ligand binding, signal transduction by phosphorylation of receptor-regulated Smad proteins is initiated, which in turn heterodimerize with the common partner Smad-4 and translocate into the nucleus. This complex binds to specific DNA sequences and activates or represses BMP target genes, e.g., ID (inhibitor of DNA binding proteins), depending on the recruitment of further cofactors. BMPs are under tight regulation through proteins with antagonistic properties including extracellular noggin and follistatin and intracellular inhibitory Smads, among others (reviewed in refs. 10, 12).

In the current study, we aimed to assess the potential role of BMPs on adrenocortical tumorigenesis. We therefore determined the expression of various BMPs and their receptors in human ACC and performed in vitro studies to define the functional relevance of the observed alterations of in vivo expression profiles. Altogether, we show that both BMP2 and BMP5 inhibit proliferation and modulate steroidogenesis of human adrenocortical tumor cells in vitro. These functional findings, which can be modified by pharmacologic demethylation and overexpression of GATA6, might, thus, present a potential important regulator of ACC biology.

Materials and Methods

Human adrenocortical tissues and primary cell culture. Diagnosis and endocrine work-up of patients with adrenal masses was defined following standard clinical, biochemical, imaging, and histologic criteria. All
patients gave written informed consent, and the study was approved by the local ethical committee of the University Clinic Freiburg and Munich. For RNA analysis, tissues were immediately frozen after surgery and kept at −80°C until further handling. Primary cell culture was performed as described earlier (13).

Cell culture. ACC cell lines NCIh295 (European Collection of Cell Cultures) and SW13 were cultured in DMEM/F12 (Life Technologies), containing 2.5% NuSerum IV (BD Bioscience) plus supplements (14). Before treating the cells with recombinant human BMP2/5 or noggin (R&D Systems), cells were precultured in DMEM/F12, containing 0.3% NuSerum (Low NuSerum), prestarved in serum-free medium (SFM) for proliferation studies or cultured in 2.0% UltroserG medium (Pall) for aldosterone measurements.

RNA extraction and quantitative PCR analysis. RNA was extracted, purified with the TURBO DNA-free kit (Ambion), and cDNA generated as described earlier (15). Quantitative real-time PCR (qPCR) amplifications were performed using SYBRGreen technique (15) with the following primers: BMP2 and BMPR 1a/1b/II (16); BMP4/5/6 (7); 3

\[\text{hSD}_{-}\text{HSD}_F: \text{CTAGT-TACGCCCTCTTCTG}, \text{3} \text{hSD}_{-}\text{HSD}_R: \text{AATGTCTCCTTCAAGTACAGT}, \text{product: 285 bp, T}_{\text{A}}: 58°C; \text{CYP17}_F: \text{TGGCCCCATCTATTCTGTTC}, \text{CYP17}_R: \text{GGCCAGCATATCACACAATG}, \text{472 bp, T}_{\text{A}}: 60°C; \text{melanocortin-2 receptor (MC2-R; ref. 17). Quantification was adjusted using the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT}_F: \text{TGCTGACCTGCTGGATTACA}, \text{HPRT}_R: \text{CCTGACCAAGGAAAGCAAAG}, \text{230 bp, T}_{\text{A}}: 58°C) and \beta\text{-actin (}\text{\beta\text{-actin}_F: TCATGAAGTGTACGTGGACATCC,}\text{\beta\text{-actin}_R: TCCAGTACCGGTGCTGGACCC},\text{product: 230 bp, T}_{\text{A}}: 58°C) and \beta\text{-actin (}\text{\beta\text{-actin}_F: TCATGAAGTGTACGTGGACATCC,}\text{\beta\text{-actin}_R: TCCAGTACCGGTGCTGGACCC},\text{product: 230 bp, T}_{\text{A}}: 58°C).}

Figure 1. Expression analysis of members of the BMP pathway in ACC and adrenocortical tumor cell lines (SW13 and NCIh295) reveal lower expression of BMP2 and BMP5 and reduced but retained BMPR expression (A) compared with NAG. Accordingly, BMP5 and pSMAD1/5/8 immunoreactivity is significantly lower in ACC samples compared with NAG (B). Treatment of adrenocortical tumor cells with BMP2 or BMP5 shows integrity of BMP-dependent pathways by phosphorylation of SMAD 1/5/8 (immunoblots, C) and up-regulation of ID1 and ID3 expression in a dose-dependent manner (D). NCIh295 cells and primary adrenocortical tumor cells (D, right) were precultured and treated with either rhBMP2, BMP5 alone, or in combination with the antagonist noggin (C, right), respectively. Values for qPCR were normalized by \beta\text{-actin levels. Inserts in immunohistochemical images display negative staining controls. Bars, 50 μm.}
β-actin: R: CTTAGAAGCATTTGGCTGGAGAGTG. 286 bp, Tm: 56°C. For ID1 and ID3 detection, Quantitect Primer Assays and Quantitect SYBR Green Mix were purchased from Qiagen and quantified according to the manual (Qiagen).

Morphologic examination and immunohistochemistry. Immunohistochemistry of paraffin-embedded tissues was performed, as described earlier (15). For antigen retrieval, slides were boiled in 10 mmol/L sodium citrate buffer (pH 6.0) and incubated with the following primary antibodies: BMP5 (1:100, LifeSpan Biosciences) and p-SMAD1/5/8 (1:80, Cell Signalling Technology). Morphologic examination was performed with a standard light microscope.

Proliferation assay. For proliferation and cell viability studies, cells were plated in quadruplicates at a density of 25,000 per well in a 96-well-plate in SFM and allowed to adhere overnight. Cells were then treated with BMP2 (30 ng/mL), BMP5 (200 ng/mL), or in combination with human recombinant IGF-1 (kindly provided by Ipsen Pharma) at indicated concentrations for 96 h. Medium and substances were renewed every 24 h. For time-dependent proliferation studies, cells were seeded at a density of 150,000 per well in a 24-well-plate (n = 3) and treated with BMP2 (30 ng/mL) or vehicle for the indicated time points. For cell proliferation assay, 5-bromo-2′-deoxyuridine (BrdUrd) was added to the cells, whereas, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT) was used to test cell viability as described (14).

Protein extraction and blot analysis. Protein extraction and immunoblots were performed as described (18). Phosphorylated Smad1/5/8 (1:500), phosphorylated AKT (pAKT: 1:5,000), total AKT (1:5,000, all from Cell Signaling Technology, Inc.), and β-actin antibody (1:5000; Sigma–Aldrich Corp.) were used as primary antibodies. Quantification was achieved by the measurement of the pixels and adjacent analyses using the imaging program ImageJ (NIH).

Measurement of steroid hormone levels. NCIh295 cells were preincubated to 80% confluence in 24-well plates. After 24-h pretreatment, fresh SFM containing BMP2 or BMP5, alone or in combination with forskolin, was added to the cells. The supernatant of each well was collected after a period of 48 h, and accumulated levels of hormones were measured by the appropriate assay. Cortisol was measured by an electrochemiluminescence immunoassay (Elecys, Roche) and DHEA-S using a chemiluminescent enzyme immunoassay (Immulite2000, Diagnostic Products Corporation). For aldosterone determination, NCIh295 cells were incubated for 48 h with BMP2 and BMP5, respectively, and aldosterone was measured using a nonisotopic immunoassay for time-resolved fluorescence measurement as described earlier (19).

Transfection and luciferase transporter assay. Cells were preincubated for 24 to 48 h, and transfections were performed overnight (for MC2-R study; Metafectene, Biontex Laboratories) or for 4 h (TransFast Transfection Reagent, Promega). The next day, cells were treated with BMP2/5 alone or in combination with forskolin (10 μmol/L) or norggin (2 μg/mL) for MC2-R promoter studies and with 9cis-retinoic acid (9cis-RA; 5 μmol/L) or phorbol 12-myristate 13-aceta (PMA; 10 nmol/L; Sigma–Aldrich Corp.), respectively, for BMP2 promoter studies. After 48 h, cells were lysed for RNA extraction and further qPCR analysis or for luciferase measurement (Luciferase-Reporter-Assay, Promega). Firefly Renilla (pRL-TK; Promega) was thereby used for normalization. Experiments were done with a full-length MC2-R-Luc promoter construct, several deletion constructs, and a MC2-R construct with mutated steroidogenic factor-1 (SF-1) binding sides (14, 20). BMP expression studies were performed using either a GATA6 expression vector (kindly provided by Dr. Markku Heinikimo, University of Helsinki) alone or in combination with a BMP2 promoter construct (kindly provided by Dr. Di Chen, University of Rochester).

DNA extraction, methylation analysis, and demethylation treatment. All fresh DNA was isolated with the following primary antibodies: Wizard genomic DNA Purification kit (Promega) following the manufacturer’s protocol for tissue samples. The EpiTect Bisulftite kit (Qiagen) was used for bisulfite modification according to the manual. Methylation-specific PCR (MSP) for BMP2 promoter studies were performed, as described previously (21, 22). For MSP Locus_1 of the BMP2 promoter region, the following primer pairs were used: methylated, 5′-TTTTTGTAGATGAGGGTGG (sense), TAACTCTAAAAACCACTACCTT (antisense); methylated, GTAAGGGCGTGTTATAGCGTC (sense), CATCGCGAGGTAAAT (antisense). Locus_2 was amplified according to Wen and colleagues (23). For demethylation studies, cells were treated daily with 5-aza-2′-deoxycytidine (5-AZA; Sigma–Aldrich) for 96 h (5 or 10 μmol/L). Subsequently, 1 μmol/L trichostatin A (TSA; Sigma–Aldrich Corp.), as described (24), or norggin (3 μmol/L) was added for additionally 48 h. After treatment, cells were washed with PBS, harvested, and RNA and DNA isolated.

Statistical analysis. All results are expressed as mean ± SE and, if not indicated otherwise, calculated from data generated from at least three independent experiments. P values of <0.05 were considered to be significant and are either indicated in the figures by * for comparison between different treatments or by ** for comparison within each treatment group. Controls [normal adrenal gland (NAG) or untreated cells] are set in general as 100%. GraphPad Prism (GraphPad Software, Inc.) was used for Student’s t test analysis, as well as for calculation of the Spearman correlation coefficient.

Results

Expression analysis of members of the BMP pathway in ACC and NCH295 cells. To define potential candidates for further functional studies, we analyzed the expression levels of different BMPs and their receptors in human ACCs compared with NAGs by qPCR analysis. Detailed quantification was performed for BMP2 and BMP5, as well as for the BMP receptor subunits, including type Ia (BMPR1a), type Ib (BMPR1b), and type II (BMPR2). Notably, mRNA expression of BMP2 (56.1 ± 7.2% versus 100.0 ± 10.7%, P < 0.01) and BMP5 (2.6 ± 0.6% versus 100.0 ± 21.1%, P = 0.0001; Fig. 1A) was significantly down-regulated in ACCs compared with NAG (Fig. 1A). These findings were recapitulated in NCH295 (BMP2, 44.7 ± 10.6%; P = 0.06; BMP5, 0.04 ± 0.04%, P = 0.02 versus NAG) and SW13 adrenocortical cell lines (BMP2, 26 ± 21.3%; P = 0.05; BMP5, 0.08 ± 0.03%, P = not significant versus NAG; Fig. 1A). Similarly, expression levels for BMPR subunits were lower but clearly detectable by qPCR in all ACC samples and with some variations in NCH295 and SW13 cells (Fig. 1A, bottom).

In accordance with the expression analyses, BMP5 immunoreactivity, which was detectable in the normal adrenal cortex, was absent in all investigated ACC samples (n = 4 versus n = 4 NAG, exemplified in Fig. 1B, top). These findings were further substantiated by the determination of p-SMAD1/5/8 expression, which was lower in adrenal cancer samples compared with NAG (Fig. 1B, bottom).

BMP-dependent pathways are intact and inducible in adrenocortical tumor cells. To ensure the integrity of BMP-dependent pathways in adrenocortical tumor cells, NCH295 and SW13 cells were incubated with increasing doses of recombinant human BMP2 and BMP5, respectively, which showed a dose-dependent BMP-induced phosphorylation of downstream SMAD1/5/8 proteins (Fig. 1C, middle left; Supplementary Fig. S1A). These effects could be blocked by coinucation with the BMP-specific antagonist noggin (Fig. 1C, right). Furthermore, both BMP2 and BMP5 increased ID1 and ID3 expression in a dose- and time-dependent manner (Fig. 1D, middle left; Supplementary Fig. S1B; data not shown). Likewise, BMP2-dependent up-regulation of ID3 was readily detectable also in primary cultures of human ACC cells (Fig. 1D, right).

Effects of BMP2 and BMP5 treatment on cell proliferation and cell viability. As BMPs have been reported to play an important role in the regulation of cellular proliferation in several tumor entities, we investigated the influence of BMPs on adrenocortical tumor cell growth. BrdUrd assay revealed a
BMP2- and BMP5-dependent inhibition of cell proliferation in NCIh295R and SW13 cells (BMP2, 61.9 ± 1.7% versus baseline, P < 0.001; BMP5, 65.5 ± 5.3% versus baseline, P < 0.001; Fig. 2A, left) and primary adrenocortical cancer cells (BMP2, 61.9 ± 1.7% versus baseline, P < 0.001; BMP5, 65.5 ± 5.3% versus baseline, P < 0.001; Fig. 2A, right; Supplementary Fig. S1C), which could be further detailed in time course experiments (Fig. 2B, right; data not shown). Likewise, both BMP2 and BMP5 also exerted inhibitory effects on cell viability, as assessed by MTT assays in NCIh295 (Fig. 2B and C, left) and in primary ACC cultures (Fig. 2B and C, right).

As IGF-I receptor–dependent pathways transduce the most potent mitogenic stimuli for adrenocortical tumorigenesis (25, 26), we further evaluated effects of BMP on cell growth under IGF-I–stimulated conditions. Whereas IGF-I resulted in a significant increase in cell viability, these effects were inhibited by coinubation with BMP2 (Fig. 2B) and BMP5 (Fig. 2C), respectively, in a dose-dependent manner in NCIh295 cells [IGF-I, 120.0 ± 3.2% versus IGF-I + BMP2 (50 ng/mL), 103.1 ± 3.4%, P < 0.01 and versus IGF-I + BMP5 (100 ng/mL), 79.9 ± 5.7%, P < 0.001] and in primary adrenocortical tumor cells [IGF-I, 142.1 ± 2.6% versus IGF-I + BMP2 (50 ng/mL), 110.3 ± 1.4%, P < 0.001; IGF-I, 161.0 ± 3.3% versus IGF-I + BMP5 (150 ng/mL), 146.6 ± 1.8%, P < 0.01].

Taken together, these results provide ample evidence for growth inhibitory effects of both BMP2 and BMP5 on adrenocortical tumor cells.
**BMP2 and BMP5 reduce IGF-I–induced AKT protein phosphorylation.** An increasing body of evidence suggests that BMP can also signal through SMAD-independent pathways (10, 27). Thus, we further evaluated potential cross-signaling of BMPs with IGFs, which represent crucial adrenocortical mitogens (3), and activators of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. As expected, time course experiments showed IGF-I–dependent up-regulation of pAKT in NCIh295 cells, which was detectable already after the minimal incubation period of 15 minutes (data not shown) and was sustained up to 20 hours (Fig. 3A and B). Notably, at all time points, AKT phosphorylation was reduced by BMP2 or BMP5 treatment under both baseline and IGF-I–stimulated conditions when compared with total AKT. As AKT phosphorylation represents a downstream mediator of IGF-dependent pathways, these findings provide evidence for a BMP-induced decrease of active IGF signaling.

Figure 3. BMP2 and BMP5 reduce baseline and IGF-I–induced AKT protein phosphorylation. Cells were treated with BMP2 (50 ng/mL) or BMP5 (150 ng/mL) alone or in combination with IGF-I (100 ng/mL) for 1, 3, 10, and 20 h. Proteins were analyzed by immunoblotting for pAKT and total AKT (tAKT, A) and quantified (B) for the different time points under baseline conditions and under IGF-I treatment.

Figure 4. Forskolin-induced steroidogenic hormone production is reduced by BMP2 and BMP5 in NCIh295 cells. After incubation for 48 h with BMP2 (A) or BMP5 (B) alone or in combination with forskolin, supernatants of each experiment were subjected to the appropriate hormone assays.
Forskolin-induced steroidogenic hormone production is reduced by BMP2 and BMP5 in NCIh295 cells. In contrast to BMP6, which has been reported to stimulate aldosterone secretion in adrenocortical cells (7), both BMP2 (Fig. 4A) and BMP5 (Fig. 4B) significantly reduced forskolin-induced secretion of aldosterone (BMP2, factor 2, \(P < 0.001\); BMP5, factor 1.5, \(P = 0.01\)) and cortisol (BMP2, factor 2.6, \(P < 0.001\); BMP5, factor 1.9, \(P = 0.01\)) levels, whereas DHEA-sulfate levels were significantly reduced only by BMP2 treatment. In contrast, no significant changes in hormone levels were detectable under baseline conditions with the applied BMP treatment protocols.

BMP2 and BMP5 down-regulate expression of steroidogenic enzymes and the adrenocorticotropic hormone receptor in NCIh295 cells. To further detail BMP-dependent effects on steroidogenesis, we evaluated the regulation of the key determinants of adrenocortical steroidogenesis, including cytochrome P450 17-a-hydroxylase (CYP17), cytochrome P450 3-hydroxysteroid dehydrogenase (3\(\beta\)HSD), and the adrenocorticotropic hormone receptor (MC2-R). In accordance with the hormonal findings, qPCR revealed BMP2- and BMP5-dependent down-regulation of CYP17 in a dose- and time-dependent manner (Fig. 5A, left and middle; data not shown). Furthermore, primary ACC cells were analyzed under baseline conditions and upon forskolin stimulation, and in both cases, CYP17 expression was significantly diminished by BMP2 (BMP2, 44.6 ± 1.9% versus baseline, 100.0 ± 3.2%, \(P < 0.0001\); forskolin + BMP2, 315.0 ± 5.8% versus forskolin, 571.8 ± 29.5%, \(P < 0.001\); Fig. 5A, right). Similarly, 3\(\beta\)HSD and MC2-R expression in NCIh295 cells could be decreased by 62% and 45% for BMP2 treatment and by 25% and 44% for BMP5 treatment, respectively, at baseline (Fig. 5B; data not shown) and more robustly under forskolin-stimulated conditions (3\(\beta\)HSD: forskolin, 505 ± 22% versus forskolin + BMP2, 657 ± 14%, \(P < 0.0001\); versus forskolin + BMP5, 498 ± 49% \(P < 0.0001\), data not shown; MC2-R: forskolin, 1154 ± 9.3% versus forskolin + BMP2, 199 ± 6%, \(P < 0.0001\); versus forskolin + BMP5, 125 ± 8.8%, \(P < 0.0001\), Fig. 5B, left and middle). Moreover, coincubation of BMP5 with noggin was able to block BMP5-induced inhibition of MC2-R expression in NCIh295 cells (Fig. 5B, right).

BMP5-dependent reduction of endogenous MC2-R expression could be recapitulated in luciferase assays using a human full-length promoter construct [BMP5 (100 ng/mL), 65.9 ± 0.7% versus baseline, 100.0 ± 3.7%, \(P < 0.001\); Fig. 5C, left]]. Interestingly, this BMP5-induced suppression in promoter activity was still present in deletion constructs, in which all SF-1 binding sites had been removed by site-directed mutagenesis (Fig. 5C, middle), and also in 5' deletion constructs down to a fragment size of ~64 bp (Fig. 5C, right). Values for qPCR were normalized by HPRT levels.

Taken together, these findings show that BMP2 and BMP5 are able to down-regulate genes involved in steroidogenesis in adrenocortical tumor cells, thus, modulating their steroidogenic capacity.
BMP2 can be reconstituted in adrenocortical tumor cells by GATA6, retinoid acid, and phorbol ester treatment. Promoter sequence analysis and functional studies have identified a number of transcription factors, including Sox9, nuclear factor-kappa B (NF-kB), and GATA transcription factors, to be involved in transcriptional regulation of BMP2 and BMP5 in nonadrenal cells (28, 29). Furthermore, retinoic acid compounds and the phorbol ester PMA have been reported to stimulate BMP2 (30). Upon screening of ACC samples for altered expression pattern of these transcription factors, significant lower expression for Sox9 (ACC, 18.4 ± 1.5% versus NAG, 100.0 ± 8.7%; P < 0.0001; data not shown) and GATA6 (ACC, 29.1 ± 7.2% versus NAG, 100.0 ± 13.7%; P < 0.0001) were found in ACC samples, as well as in NCIh295 and SW13 cell lines (Fig. 6A, left). Interestingly, overexpression of GATA6, as well as treatment with 9cis-RA and PMA, was able to induce endogenous BMP5 expression (GATA6, 3100 ± 1667%, P < 0.0001; 9cis-RA, 432.8 ± 276.6%, P = not significant; PMA, 2414 ± 1016%, P < 0.0001 versus baseline, 100.0 ± 14.7%; Fig. 6A, middle). Similarly, GATA6 overexpression and incubation with 9cis-RA and PMA also stimulated BMP2 promoter activity, which was further amplified by cotreatment with GATA6 overexpression (9cis-RA + GATA6, +65% versus 9cis-RA alone; P < 0.01; PMA + GATA6, +596%; P < 0.001 versus PMA alone; Fig. 6A, right).

Demethylation of the BMP2 promoter region is associated with modulation of BMP-dependent pathways in adrenocortical tumor cells. Gene silencing by hypermethylation represents an epigenetic phenomenon that has been postulated as a possible mechanism resulting in the loss of BMP expression in gastric and colorectal cancer cells (21, 23, 31). Thus, MSP was used to study methylation of the BMP2 promoter region in adrenocortical tissues. However, analysis of ACC samples revealed the presence of both methylated (M) and unmethylated (U) alleles at two different CpG islands within the BMP2 promoter region, which was indistinguishable from those of NAG (Supplementary Fig. S2B), indicating that hypermethylation of these CpG islands seem not to have major effect on the loss of BMP2 expression in vivo.

Figure 6. GATA6 overexpression and pharmacologic demethylation induce reactivation of BMP-dependent pathways in adrenocortical NCIh295 cells. Expression analysis of GATA6 in ACC and adrenocortical tumor cell lines show lower expression levels (A, left) compared with NAG. Overexpression of GATA6, as well as incubation with 9cis-RA and PMA, is able to up-regulate endogenous BMP5 (A, middle) and BMP2 promoter activity in NCIh295 cells, whereas this effect is further amplified by concomitant GATA6 overexpression (A, right). Demethylation treatment with 5-AZA is accompanied by lower BMP2 promoter methylation as evident by MSP (B, left), increase in endogenous BMP2 expression (B, right), up-regulation of ID3 expression (C, left), and down-regulation of CYP17 (C, right), as assessed by qPCR. ID3 up-regulation can be antagonized by cotreatment with noggin (C, left).
Independent from the pathophysiologic cause of BMP down-regulation in ACC, pharmacologic demethylation of CpG islands can be used to modify gene silencing in a given tumor. In accordance with this notion, treatment of NCIh295 cells with the demethylation agent 5-AZA resulted in a dose-dependent demethylation (by 30% for the 5 μmol/L 5-AZA incubation and by 41% for the 10 μmol/L 5-AZA incubation) of the BMP2 promoter region (Fig. 6B, left). As an indication of the functional relevance of this demethylation treatment, qPCR analysis detected up-regulation of endogenous BMP2 expression levels (5-AZA: 5 μmol/L, 226.9 ± 32.3%; P < 0.01; 10 μmol/L, 232.6 ± 32.5% versus baseline, 100.0 ± 2.8%, P < 0.001; Fig. 6B, right), as well as reactivation of BMP-dependent pathways after 5-AZA treatment with up-regulation of ID3 expression levels (5 μmol/L, 409.0 ± 57.1% versus baseline, 100.0 ± 3.2%, P < 0.0001; Fig. 6C, left). Whereas the histone deacetylase inhibitor TSA alone failed to activate ID3 up-regulation (104.3 ± 15.9% versus baseline, 100.0 ± 3.2%, P = 0.72), it further increased 5-AZA–dependent induction of ID3 expression. These demethylation-dependent effects on ID3 expression could be abolished by cotreatment with the specific BMP antagonist noggin (Fig. 6C, left). Furthermore, 5-AZA–induced demethylation was accompanied by down-regulation of CYP17 expression levels (Fig. 6C, right), which could be further intensified by cotreatment with TSA (29.9 ± 4.7% versus 5-AZA alone, 60.1 ± 3.8%, P < 0.001), whereas TSA treatment alone resulted in a considerable up-regulation of CYP17 mRNA levels, most likely through chromatin stabilization rather than BMP-dependent mechanisms. Taken together, these results are in line with the concept that pharmacologic demethylation, including that of the BMP2 promoter region, is able to modulate BMP-dependent pathways in adrenocortical tumor cells.

**Discussion**

In the present study, we show down-regulation or loss of expression of BMP2 and BMP5 in ACC samples compared with NAGs. In line with these findings, lower staining intensity of pSMAD1/5/8 and reduced expression of three of the four ID proteins (ID1, ID3, and ID4; data not shown) in ACC tissues provide indirect evidence for functional significance of these in vivo results. Despite of this aberrant expression pattern, BMP-dependent pathways remain intact in adrenocortical tumor cells, as suggested by BMP-induced Smaad1/5/8 phosphorylation and up-regulation of ID1/3 in NCIh295 and SW13 cell lines and primary adrenocortical tumor cells.

Although there is ample evidence that BMPs have a significant effect as modulators of tumor properties, the potential effects of individual BMPs on a specific tumor entity is largely unpredictable. The expression levels of individual members of BMPs not only vary among tumor tissues, but each BMP also differs in its main attributes and functional effect, depending on the cell type and tumor stage, as well as on environmental factors (32, 33). Herein, we show that BMPs inhibit adrenocortical tumor growth as treatment with recombinant BMP2 and BMP5 resulted in a dose- and time-dependent reduction of adrenocortical proliferation and viability. Interestingly, the p38/AKT pathway, which mediates IGF-dependent actions on proliferation, apoptosis, and cell survival in the adrenal cortex (34, 35) is counteracted by BMP as evident by inhibition of Akt phosphorylation under baseline and IGF-1–induced conditions. Thus, these data are in line with the concept that BMP-induced modulation of IGF-mediated signaling could contribute to the observed growth inhibitory effects of BMPs.

Moreover, we present evidence that BMP2 and BMP5 also suppress steroidogenesis in adrenal cancer cells. Adding recombinant BMP2 or BMP5 to adrenocortical tumor cells diminished MC2-R and steroidogenic enzyme expressions in a dose- and time-dependent manner, which was associated with reduced mineralocorticoid, glucocorticoid, and androgen output. SF-1 represents one of the major regulator of steroidogenic enzyme and MC2-R gene expression (36). However, promoter experiments including 5′ deletion constructs and constructs with mutations of SF-1–responsive elements did not alter BMP-dependent down-regulation of MC2-R promoter activity. Interestingly, in this short promoter interval, two consensus sequences for E-box elements are present that could potentially be targeted by ID proteins through inhibition of stimulating basic helix-loop-helix factors (37). In accordance with this concept, only recently, the basic helix-loop-helix factor Bmal1 has been shown to be required for circadian glucocorticoid secretion through E-box elements on the Star promoter (38–40). Thus, whereas SF-1–dependent mechanisms seem not to be necessary for the observed BMP-induced inhibition of MC2-R expression, the inhibitory effects on genes involved in adrenal steroidogenesis could be a direct consequence of BMP-dependent up-regulation of ID protein levels. As dysregulation of steroid production leading to hypersecretion and functional autonomy is a common finding in ACC patients (1), it is possible that the observed down-regulation of BMPs in ACC samples could represent one of the modulating factors contributing to this clinical phenotype.

A variety of transcription factors, including Sox9, NF-κB, and GATA transcription factors, have been implicated in the regulation of BMP promoter activity in extra-adrenal tissues (28, 29), and a number of pharmacologic agents including retinoid acids and protein kinase C activators (such as phorbol ester PMA) have been described to modulate BMP expression (30). In agreement with findings from the literature (41), we detected significantly lower levels of GATA6 in ACC samples compared with NAG. Interestingly, overexpression of GATA6 in NCIh295 cells was able to reconstitute BMP5 expression and induce BMP2 promoter activity. Moreover, as treatment with 9cis-retinoid acid and PMA resulted in further increase of BMP2 promoter activity, these findings point toward a potential approach to restore endogenous BMP levels in ACC cells.

Another general mechanism for down-regulation of gene expression in tumors comprises epigenetic events including hypermethylation of promoter regions located in CpG islands (42, 43). In contrast to methylation-associated loss of BMP expression, which has been described for prostate cancer and gastric carcinomas (21, 44), we did not identify methylation patterns in CpG islands of the BMP2 promoter to be associated with the observed loss of gene expression in vivo. However, upon treatment of NCIh295 cells with the demethylation agent 5-AZA, BMP2 promoter methylation was reduced in a dose-dependent manner, which was accompanied by reactivation of endogenous BMP2 expression and activation of downstream pathways as evident by up-regulation of the ID3 protein. Whereas the addition of noggin abolished demethylation-induced ID up-regulation, CYP17 expression was down-regulated upon demethylation treatment. Although these findings could be interpreted as an effect of the reactivated BMP pathway, 5-AZA is a rather unspecific agent that certainly affects the expression of several other genes in this in vitro system. Thus, involvement of other pathways that ultimately result in increased ID3 expression and down-regulation of CYP17 cannot be excluded.

Despite its unspecific mode of action, 5-AZA–dependent treatment protocols for MDS and chronic myelomonocytic leukemia have been associated with a rather restricted profile of documented side effects (45). The clinical and molecular profiles of...
individual tumors differ underlining the difficulties for a tailored therapy fitting each patient (46). Thus, therapies that alter global mechanisms such as chromatin remodeling and epigenetic silencing might present a promising approach as an anticancer treatment strategy. Whether demethylation-based therapies can individualize tumors differ underlining the difficulties for a tailored cancer phenotype of adrenocortical cancer in a clinical meaningful manner remains, however, to be determined in future studies in appropriate in vivo models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

Bone Morphogenetic Proteins 2 and 5 Are Down-regulated in Adrenocortical Carcinoma and Modulate Adrenal Cell Proliferation and Steroidogenesis

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