0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(1):247–256

doi: 10.1210/en.2005-0958

Bone Morphogenetic Protein-4 Inhibits Corticotroph Tumor Cells: Involvement in the Retinoic Acid Inhibitory Action

Damiana Giacomini,* Marcelo Páez-Pereda,* Marily Theodoropoulou, Marta Labeur, Damian Refojo, Juan Gerez, Alberto Chervin, Silvia Berner, Marco Losa, Michael Buchfelder, Ulrich Renner, Günter K. Stalla, and Eduardo Arzt

Laboratorio de Fisiología y Biología Molecular (D.G., D.R., J.G., E.A.), Departamento de Fisiología, Biología Molecular y Celular, Facultad Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina; Max-Planck Institute of Psychiatry (M.P.-P., M.T., M.L., U.R., G.K.S.), 80804 Munich, Germany; Hospital Santa Lucía (A.C., S.B.), 1232 Buenos Aires, Argentina; Department of Neurosurgery (M.L.), Ospedale San Raffaele Instituto di Ricovero e Cura a Carattere Scientifico, 20132 Milan, Italy; Department of Neurosurgery (M.B.), University of Gottingen Medical School, 37075 Gottingen, Germany; and Affectis Pharmaceuticals (M.P.-P.), 80804 Munich, Germany

The molecular mechanisms governing the pathogenesis of ACTH-secreting pituitary adenomas are still obscure. Furthermore, the pharmacological treatment of these tumors is limited. In this study, we report that bone morphogenetic protein-4 (BMP-4) is expressed in the corticotrophs of human normal adenohypophysis and its expression is reduced in corticotrophinomas obtained from Cushing's patients compared with the normal pituitary. BMP-4 treatment of AtT-20 mouse corticotrophinoma cells has an inhibitory effect on ACTH secretion and cell proliferation. AtT-20 cells stably transfected with a dominant-negative form of the BMP-4 signal cotransducer Smad-4 or the BMP-4 inhibitor noggin have increased tumorigenicity in nude mice, showing that BMP-4 has an inhibitory role on corticotroph tumorigenesis *in vivo*. Because the activation of the retinoic acid receptor has an inhibitory action on Cushing's disease progression, we analyzed the putative interaction of these two pathways. Indeed, retinoic acid induces both BMP-4 transcription and expression and its antiproliferative action is blocked in Smad-4dn- and noggin-transfected Att-20 cells that do not respond to BMP-4. Therefore, retinoic acid induces BMP-4, which participates in the antiproliferative effects of retinoic acid. This new mechanism is a potential target for therapeutic approaches for Cushing's disease. (*Endocrinology* 147: 247–256, 2006)

CORTICOTROPH ADENOMAS are usually small microadenomas at the time of diagnosis, accounting for about 10–15% of all clinically recognized pituitary adenomas (1–3). ACTH-secreting pituitary tumors generate a glucocorticoid hypersecretion disorder, called Cushing's disease, which produces adrenal hyperplasia, abnormal fat deposition, thinning of the skin, hypertension, osteoporosis, diabetes, and psychological disturbances (4–6). Monoclonal expansion of transformed corticotroph cells of the anterior pituitary is one of the mechanisms that leads to ACTH-secreting pituitary tumors development (7), although pluriclonality has also been suggested (8). Although transsphenoidal surgical removal of ACTH-secreting pituitary microadenomas (<1 cm diameter) is an effective approach with an overall cure rate of about 70%, it is only effective in 30% of macroadenomas (>1 cm diameter) (9–11). Moreover, some inhibitors of ACTH production have been described but they are not effective in Cushing's disease treatment (12).

Much work has been done to find out not only the molecular pathogenetic mechanism of ACTH-secreting pituitary tumors development but also an effective drug therapy for these pituitary tumors. In a previous study, we demonstrated that retinoic acid treatment reduces proopiomelanocortin (POMC) gene transcription and ACTH production by inhibiting the AP-1 and Nur77/Nurr1 transcriptional activities in pituitary ACTH-secreting tumor cells (13). Expression in ACTH-secreting AtT-20 cells of the orphan receptor COUP-TFI, a negative regulator of the retinoic acid response pathway (14), blocked the inhibitory action of retinoic acid (13). *In vivo* experiments in nude mice have shown that retinoic acid administration completely blocks corticotroph tumor growth and reverses the endocrine alterations and symptoms of Cushing's disease (13). All these data support the notion that retinoic acid might be an interesting drug for Cushing's disease treatment (13). Although the inhibitory mechanism of retinoic acid on ACTH biosynthesis at the level of AP-1 and Nur77/Nurr1 transcriptional activities and POMC transcription in ACTH-secreting tumor cells has been demonstrated, the inhibitory mechanism over cell proliferation is still not clear. Furthermore, the related receptor, peroxisome proliferator-activated receptor-γ has also been shown as an important target for drug therapies for Cushing's disease. Peroxisome proliferator-activated receptor-γ-activating ligands, induce cell-cycle arrest and apoptosis in corticotrophinoma cells, induce tumor growth arrest *in vivo*, and inhibit ACTH and corticosterone secretion from tumoral cells (15).

First Published Online September 29, 2005

* D.G. and M.P.-P. have contributed equally.

Abbreviations: BMP-4, Bone morphogenetic protein-4; FCS, fetal calf serum; POMC, proopiomelanocortin; PRL, prolactin.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Several hypothalamic factors and hormones as well as growth factors such as TGF-β, platelet-derived growth factor, and cytokines are involved in the molecular and cellular mechanisms that lead to pituitary tumor development (2, 5, 16–18). Especially estrogens and members of the TGF-β family are involved in the control of lactotroph cell proliferation and promote prolactinomas formation (19–23). Bone morphogenetic protein-4 (BMP-4), a member of the TGF-β family that plays a central role during pituitary organogenesis (24, 25), is over-expressed in different prolactinomas models including dopamine receptor 2 knockout ($D2R^{-/-}$) mice, Fisher rats treated with estradiol and in human tumors, compared with normal tissue and other pituitary adenoma types. Furthermore, GH3 rat lactosomatotrophinoma cells stably transfected with plasmids that express proteins that block BMP-4 action had reduced tumorigenicity in nude mice, providing evidence that the BMP-4 stimulatory pathway plays a role in prolactinoma development *in vivo* (26).

BMPs, as well as other members of the TGF-β family, mediate their effects by binding to a family of membrane tyrosine kinases receptors and subsequently activating a family of receptor substrates, the Smad proteins, which act as transcription factors in the nucleus (27–29). The Smad-4 protein functions as a signal cotransducer for the different Smad proteins and is required for active transcriptional complexes to assemble. The resulting Smad complex is then free to move into the nucleus and competent to bind Smad-binding elements in association with transcriptional coactivators or corepressors. Proteins such as noggin, gremlin, and caronte bind BMP proteins and prevent their association with signaling tyrosine kinase receptors, thus controlling the extracellular activating phase of BMP pathway (27–29).

Taking into consideration the crucial role of BMP-4 during pituitary organogenesis and prolactinoma tumorigenesis, we decided to study its potential action in corticotrophinoma growth. In this study, we report the differential BMP-4 expression in normal and adenomatous corticotrophs and its inhibitory action on corticotrophinoma cell proliferation. Furthermore, we show that BMP-4 mediates the antiproliferative action of retinoic acid in these cells. These results point to a novel mechanism by which BMP-4 and retinoic acid may prevent Cushing's disease development.

Materials and Methods

Cell culture

AtT-20 pituitary corticotroph tumor cells were obtained from the American Type Culture Collection (Rockville, MD) (30). AtT-20 cells were cultured in DMEM (pH 7.3) supplemented with 10% fetal calf serum (FCS), 2.2 g/liter $NaHCO_3$, 10 mM HEPES, and 2 mM glutamine until they were confluent under 5% CO_2 atmosphere at 37 C.

Cells were treated with 10, 50, 100, or 200 ng/ml BMP-4, 0.001–1 μM noggin, 10 ng/ml TGF-β (all three from R&D Systems, Minneapolis, MN), or 10 or 100 nM all-*trans* retinoic acid (Sigma, St. Louis, MO) that was dissolved in ethanol-DMSO as a 10 mM stock solution and handled in the dark. The diluting mixture was used in all the *in vitro* experiments as control.

Patients and tissues

Patients with pituitary adenomas and clinical symptoms of Cushing's disease were diagnosed by hormonal testing and magnetic resonance imaging as previously described (31). All tumors were benign and graded according to the modified Hardy's classification (32). Tumor tissue was obtained by transsphenoidal surgery and was transported in sterile medium. Samples from five human pituitary glands were obtained from autopsy cases without any evidence of endocrinological disease, with postmortem delay between 8 and 12 h. The tissues were snap-frozen and stored at −80 C. For immunohistochemistry, 8-μm sections of shock-frozen ACTH-secreting tumor or normal tissue were thaw-mounted onto SuperFrost Plus slides (Menzel-Glaser, Hamburg, Germany), fixed in 4% paraformaldehyde in PBS, and stored in 96% ethanol at 4 C until used.

Cell proliferation and viability

A WST-1 assay (Roche Molecular Biochemicals, Basel, Switzerland) was used to measure cell proliferation and cell viability following the manufacturer's instructions as previously described (33). The method was validated by total cell count. Acridine orange-ethidium bromide staining was used to rule out toxic effects.

Plasmids

The Smad-4 dominant-negative mutant (Smad-4dn) expression vector consists of a truncated DPC-4 (Smad-4) cDNA (1–514) fused to FLAG expressed under the control of the cytomegalovirus promoter (34). The noggin expression vector contains 1 kb of the mouse noggin cDNA driven by the EF1α promoter (35). The mouse BMP-4 1A promoter contains the −2372/+258 fragment from the 5′ flanking region and includes all the necessary sequences for the expression and regulation of BMP-4 in pituitary cell cultures (36). COUP-TFI expression vector contains the 1.5-kb segment of the mouse COUP-TFI cDNA under the control of a cytomegalovirus promoter, and COUP-TFI control vector contains an incomplete segment of the mouse COUP-TFI cDNA that codifies for a truncated inactive protein (37). The human c-*myc* promoter construction contains the −2329/+510 fragment from the 5′ flanking region upstream from the luciferase gene of the pGL3-Basic vector (38).

AtT-20 stable clones

AtT-20 cells were stably transfected with noggin or Smad-4dn expression vectors or the corresponding control vector using lipofectamine as described below, and after 48 h, were selected in culture medium with 600 ng/ml G418 (Invitrogen, Eggenstein, Germany). The selected AtT-20 noggin and AtT-20 Smad-4dn clones were tested for noggin and Smad-4dn expression by Northern blot and Western blot, respectively as described below. Three additional stable clones for each construct had similar results as the one that is shown.

Transfection and reporter assays

Cell transfection was performed with lipofectamine using standard procedures, as previously described (39). After plating in six-well plates, the cells were transfected for 6 h in OPTIMEM (Invitrogen) using 8 μl of lipofectamine (Invitrogen) per well and 1 μg total plasmid DNA. Cells were then washed and left overnight in serum-free DMEM. The following day, cells were washed and treated for 6 or 24 h in serum-free DMEM with the indicated compounds. An additional 200 ng of the RSV-β-gal construction was cotransfected in all the experiments to correct for variations in transfection efficiency as previously described (39). Luciferase activity was measured with a Junior luminometer (Berthold, Bad Wildbad, Germany). Results are ratios of luciferase and β-galactosidase activity. Protein content was measured by a Bradford assay as a further control for the reproducibility of the sampling procedure.

Western blot analysis

Western blot was performed as previously described (26). Briefly AtT-20 cells were washed once with PBS (pH 7.0), and then cell lysates were prepared in standard cracking buffer and boiled for 3 min. Equal levels of protein (30 μg) were electrophoresed by 12% SDS-PAGE. Proteins were blotted onto nitrocellulose western blotting membranes (Sigma) using standard procedures and anti-BMP-4 (1:1,000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-GADPH (1:50,000) (Santa Cruz Biotechnology Inc.), or anti-FLAG antibody (Sigma) were added. The

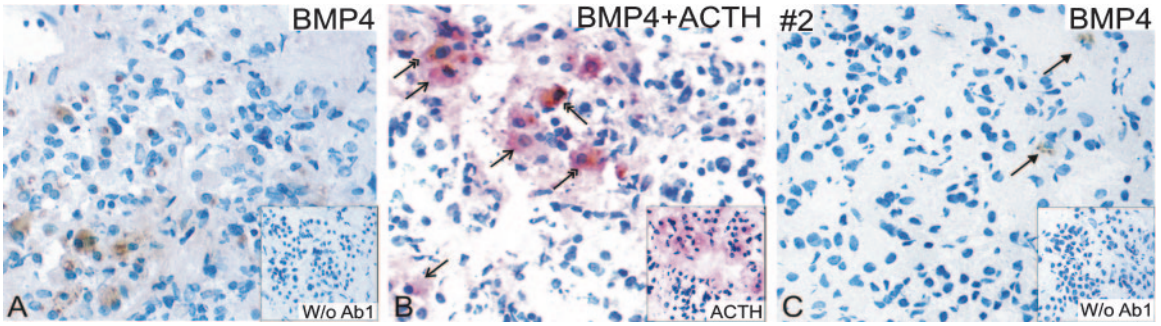

FIG. 1. BMP-4 immunoreactivity in human normal and adenomatous pituitary. A, Normal human pituitary tissue slides were prepared, and BMP-4 immunoreactivity was detected by immunohistochemistry with a goat anti-BMP-4 antibody (1:100), as described in *Materials and Methods*. Cytoplasmatic immunostaining for BMP-4 is shown; BMP-4 was detected in clusters of endocrine cells in normal human anterior pituitary gland. One of five human tissue slides with similar results is shown. B, Specific colocalization of BMP-4 and ACTH is shown. BMP-4 with antibody (1:100) and ACTH with antibody (1:800) immunoreactivity was detected by immunohistochemistry as described in *Materials and Methods*. BMP-4 is visualized with diaminobenzidine (*brown*) and ACTH with vector red (*red*). *Single arrowheads*, ACTH-secreting cells; *double arrow*, cells immunoreactive for both BMP-4 and ACTH. One of five human tissue slides with similar results is shown. C, Corticotrophinomas tissue slides were prepared and BMP-4 immunoreactivity was detected by immunohistochemistry as described in *Materials and Methods*. Cytoplasmatic BMP-4 staining is shown in a representative corticotrophinoma (no. 2 from Table 1) of a total of 15 ACTH tumor preparations. *Filled arrows*, BMP-4 immunopositive cells. A–C, Corresponding negative control without primary antibody for BMP-4 (with or without ACTH antibody) is inserted at the *right corner* of the picture.

primary antibodies were detected with HRP-conjugated antigoat IgG secondary antibodies (Santa Cruz Biotechnology Inc.).

Northern blot analysis

Total RNA extraction and Northern blot analysis were performed as described previously (31). Briefly, total RNA was isolated by the guanidine isothiocyanate phenol-chloroform extraction method, denatured with glyoxal, electrophoresed, and transferred to a nylon membrane. Filters were prehybridized for 1 h at 60 C and then the probe was added and hybridized for 12 h. Blots were washed at increasing salt and temperature stringency and then exposed to Kodak XAR5 film at −70 C with intensifying screens for 6 d. A 0.5-kb noggin cDNA fragment (35) and a 6.6-kb *Eco*RI fragment of a 28s and part of the 18s mouse cDNA (31) were labeled with a random-priming kit using [α-^{32}P]dCTP (specific activity, 2–4 $\times$ 10^8 cpm/μg). The loading control with the fragment of 18s cDNA as probe was performed in each blot.

Immunohistochemistry

To detect BMP-4 immunoreactivity in normal pituitary and in corticotrophinomas, a goat polyclonal BMP-4 antibody (1:100) (Santa Cruz Biotechnology Inc.) was used in combination with antigoat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complex (Vector Laboratories). Immunoreactivity was visualized with diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. ACTH was detected with a monoclonal antibody (1:800) (Dako Diagnostika, Hamburg, Germany) in combination with an antimouse IgG (Sigma) and mouse alkaline phosphatase-anti-alkaline phosphatase (Sigma), using vector red (Vector Laboratories). Specific antibodies for prolactin (PRL), FSH, LH, GH, and TSH were also used (1:500) (all from Immunotech, Karlsruhe, Germany). Negative controls were performed by omitting the primary antibody.

In vivo tumor formation

ACTH-secreting experimental tumors were formed by injecting AtT-20 control, AtT20 noggin, or AtT20 Smad-4dn clone cells into a subdermal pouch in BALB/cAnN Crl-nu BR nude mice as previously described (26, 30). To evaluate BMP-4 role on tumor formation, suboptimal amounts of clone cells were injected in nude mice to distinguish differences in tumor growth. All the cells were controlled for viability. Tumor formation was monitored every 2 d during 3 wk. The tumor volume was measured as previously described (26). Briefly a vernier was used to measure the short and long axis of the tumor and the following formula was used: tumoral volume = [(short axis)^2 $\times$ long axis $\times$ 0.4]. Blood samples were collected by heart puncture from halothane-anesthetized animals. Histological examination was performed by hematoxylin-eosin staining of shock-frozen tissues. All animal experiments were performed in accordance with institutional regulations of animal care.

Hormone measurements

ACTH was determined by radioimmunoassay as previously described (31, 33).

Statistical analysis

Statistics were performed using one-factor ANOVA in combination with Scheffé's test.

Results

BMP-4 expression in pituitary corticotroph tumors

In the normal human adenohypophysis, BMP-4 was present in 40% of endocrine cells, which were mostly found in clusters and rarely scattered (Fig. 1A). Double immunohistochemistry revealed that 30% of the somatotroph, corticotroph, and thyrotroph population were immunopositive for BMP-4 (Fig. 1B). On the other hand, PRL and FSH/LH immunopositive cells rarely stained for BMP-4. Immunohistochemistry in 15 corticotrophinomas revealed variable BMP-4 expression (Fig. 1C and Table 1). Seven of 15 cases had very low BMP-4 immunoreactivity and five of 15 cases did not express BMP-4. Only three of 15 cases had a number of BMP-4-expressing cells higher than 10%. No correlation with tumor grade was observed. The overall expression in corticotrophinomas was lower than in prolactinomas, as we have already described (26). An overall reduced BMP-4 expression is observed in Cushing's disease adenomas compared with normal corticotroph cells (Fig. 1 and Table 1). Based on this differential BMP-4 expression pattern between normal and tumor corticotroph cells, we further studied a possible role of BMP-4 in corticotroph tumor function and growth.

TABLE 1. BMP-4 immunoreactivity in corticotrophinomas

No.	Diagnosis	Grade	IHC	Percentage of cells ip for BMP-4
NP1	Normal pituitary			30
NP2	Normal pituitary			50
NP3	Normal pituitary			40
NP4	Normal pituitary			40
NP5	Normal pituitary			50
C1	CUSH	II	ACTH	<1
C2	CUSH	II	ACTH	1
C3	CUSH	II	ACTH	80
C4	CUSH	II	ACTH	1
C5	CUSH	II	ACTH	0
C6	CUSH	III	ACTH	0
C7	CUSH	III	ACTH	70
C8	CUSH	III	ACTH	0
C9	CUSH	II	ACTH	10
C10	CUSH	I	ACTH	1
C11	CUSH	II	ACTH	<1
C12	CUSH	I	ACTH	2
C13	CUSH	II	ACTH	0
C14	CUSH	I	ACTH	1
C15	CUSH	II	ACTH	0

Normal pituitary and corticotrophinomas (CUSH) tissue slides were prepared and BMP-4 and ACTH immunoreactivity was detected by immunohistochemistry (IHC) as described in *Materials and Methods*. Grade, Classification stage of corticotrophinoma; ip, immunopositive.

BMP-4 inhibits murine corticotroph tumor cells ACTH-secretion and cell proliferation

We examined the effect of BMP-4 on ACTH secretion by AtT-20 corticotroph tumor cells. BMP-4 treatment inhibited both the endogenous and forskolin-stimulated ACTH induction in a dose-dependent manner (Fig. 2A). The specific BMP-4 antagonist noggin blocked dose dependently this inhibitory effect (Fig. 2B). The inhibitory effect of BMP-4 on ACTH production was not affected by dexamethasone, because cotreatment with both factors did not shown an increment in BMP-4 inhibitory effect (Fig. 2C).

To further establish the actions of BMP-4 on corticotroph cells, we studied cell proliferation. We observed that BMP-4 inhibited AtT-20 cell proliferation after 3 d of BMP-4 treatment, and the effect is still observed after 5 d (Fig. 3A). On the contrary, TGF-β treatment did not affect cell proliferation (Fig. 3A). Similar results were obtained by total cell count, and no cell death was observed by acridine orange-ethidium staining, ruling out toxic effects (data not shown).

The c-*myc* oncogene is a critical regulator of cell cycle progression and it was recently described that BMP-4 induces c-*myc* expression in GH3 lactosomatotrophinoma cells and in human prolactinomas (26). Thus, we determined whether BMP-4 exerts its antiproliferative action in AtT-20 cells by altering c-*myc* activity. Indeed, as shown in Fig. 3B, BMP-4 inhibited c-*myc* promoter activity.

BMP-4 effects on murine corticotrophs tumor cells in vivo

Smad-4 protein functions as a signal cotransducer for the different TGF-β superfamily factors and is necessary for active transcriptional complexes to assemble. Preventing dimerization of active complexes can block BMP-4 signaling, and BMP-4 extracellular binding can be prevented by noggin, which is a BMP-4-specific antagonist.

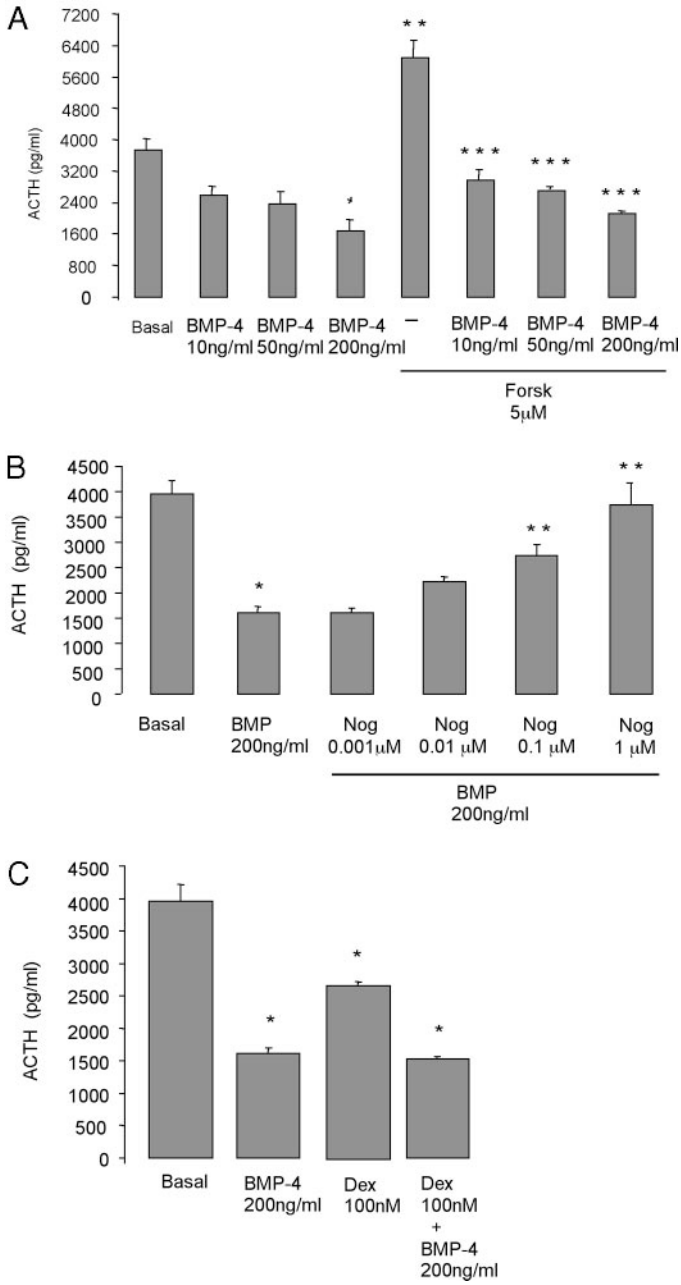

FIG. 2. BMP-4 inhibits ACTH production. A, AtT-20 cells were treated for 24 h with BMP-4, Forskolin (Forsk), or their combinations as indicated. *, $P < 0.05$ and **, $P < 0.001$ with respect to basal; ***, $P < 0.001$ with respect to forskolin, ANOVA with Scheffé's test. B, AtT-20 cells were treated for 24 h with BMP-4 alone and in combination with noggin (Nog). *, $P < 0.01$ with respect to basal; **, $P < 0.001$ with respect to 200 ng/ml BMP-4, ANOVA with Scheffé's test. C, AtT-20 cells were treated for 24 h with BMP-4, dexamethasone (Dex), or their combination as indicated. *, $P < 0.001$ with respect to basal, ANOVA with Scheffé's test. A–C, ACTH was measured in the supernatants by radioimmunoassay, and the average of four wells per treatment and SEs from one representative experiment of three with similar results are shown.

To test whether BMP-4 antiproliferative effect on AtT-20 cells also affects the ability of ACTH-secreting cells to promote tumors *in vivo*, we produced AtT-20 clones stably transfected with the Smad-4dn form or noggin to block BMP-4

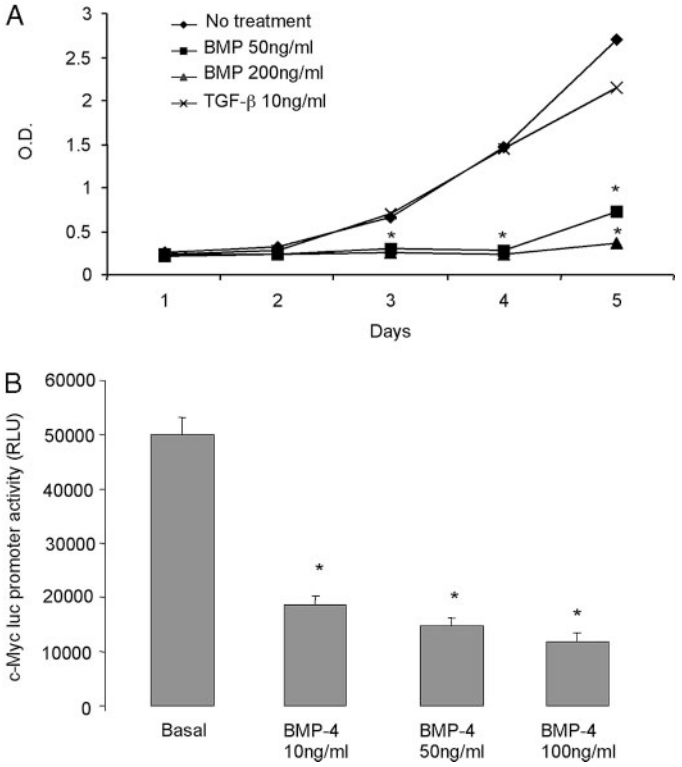

FIG. 3. BMP-4 inhibits AtT-20 cell proliferation. A, AtT-20 cells were seeded at 5000 cells per well in 96-well plates with 10% FCS. After attachment, cells were washed twice with PBS and incubated in FCS-free medium for the indicated period of time. Cells were treated with BMP-4 or TGF-β, and cell proliferation was measured each day during 5 d using the WST-1 assay. Average of four wells per treatment and SEs from one representative experiment of three with similar results are shown. *, $P < 0.001$ with respect to nontreated cells (◆), ANOVA with Scheffé's test. B, AtT-20 cells were transfected with c-Myc-LUC (1 μg) reporter vector and treated with BMP-4. An additional 200 ng of the RSV-β-gal construction was cotransfected in all the experiments to correct for variations in transfection efficiency. After 24-h treatment, luciferase activity was measured and normalized as described in *Materials and Methods*. Values indicating the mean ± SE of luciferase to β-galactosidase ratio of one representative experiment of three with similar results are shown. *, $P < 0.001$ with respect to basal, ANOVA with Scheffé's test.

signaling (Fig. 4, A and B, *inset*). Expression of Smad-4dn or noggin blocked the antiproliferative effect of BMP-4 (Fig. 4, A and B). Taking into account the BMP-4 inhibitory action *in vitro*, we hypothesized that blocking BMP-4 inhibitory growth control would result in enhanced tumor growth. Thus, to observe differences in tumor growth, nude mice were injected with a suboptimal concentration of AtT-20 cells stably transfected with an empty vector, Smad-4dn, or with noggin. When a suboptimal concentration of AtT-20 cells stably transfected with the empty vector (control cells) were injected into nude mice no tumors, were observed even after 8 wk (Fig. 5, A and B). In contrast, after 2 wk, tumors developed in all the mice injected with AtT-20 Smad-4dn or AtT-20 noggin clone cells (Fig. 5, A and B). Moreover, the phenotype of the three mice groups was different; whereas the control group did not show any change, the Smad-4dn and noggin groups presented a characteristic Cushing's disease appearance with noticeably thinner skin and sc fat tissue increase.

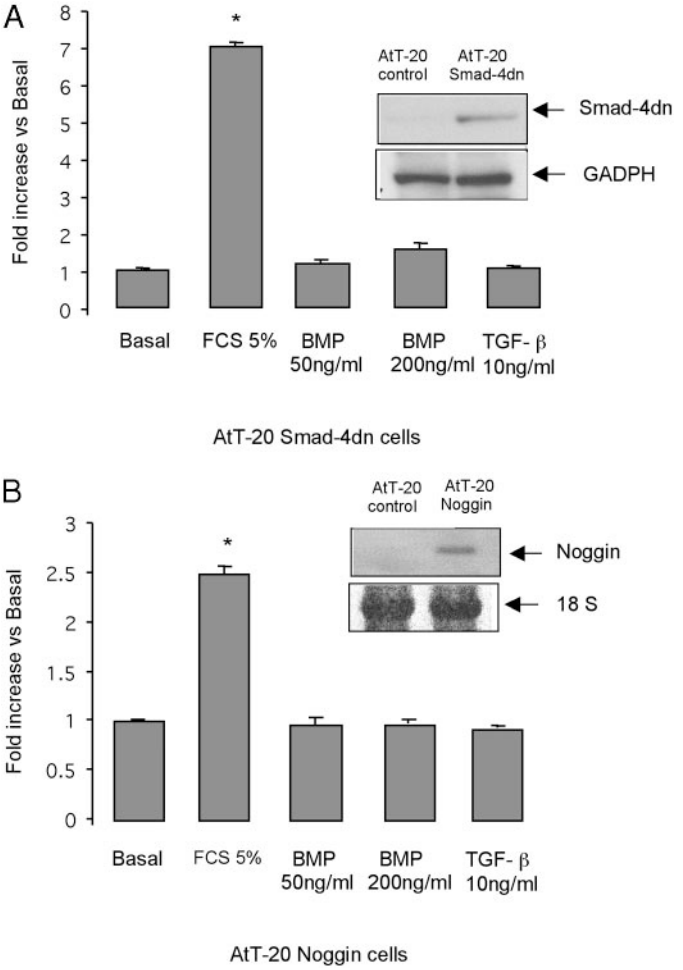

FIG. 4. Establishment and characterization of AtT-20 Smad-4dn and AtT-20 noggin stable clones. A, AtT-20 Smad-4dn cells were seeded at 5000 cells per well in 96-well plates with 10% FCS. After attachment, cells were washed twice with PBS and incubated in FCS-free medium. Cells were treated for 72 h with BMP-4, TGF-β, or 5% FCS and proliferation was measured by WST-1 assay. *, $P < 0.001$ with respect to basal, ANOVA with Scheffé's test. *Inset*, Smad-4dn (FLAG) expression was checked by Western blot against a FLAG epitope contained in the AtT-20 cells transfected with a expression vector for Smad-4dn compared with empty vector (control), as described in *Materials and Methods*. The band corresponding to Smad-4dn-Flag protein was detected by an anti-FLAG antibody (1:5,000) and is indicated by an *arrow*. Equal loading was assessed by GAPDH detection using a monoclonal GAPDH antibody (1:50,000). B, AtT-20 noggin cells were seeded and treated as described in A. *, $P < 0.001$ with respect to basal, ANOVA with Scheffé's test. *Inset*, Noggin expression was checked by Northern blot in the AtT-20 cells transfected with a expression vector for noggin compared with empty vector (control), as described in *Materials and Methods*. The band corresponding to noggin (1.4 kb) was detected with a specific probe and is indicated by an *arrow*. Equal loading was assessed by 18s RNA detection using a specific 18s cDNA. A and B, Values over the basal value are express as folds increase *vs.* basal (basal = 1). Average of four wells per treatment and SEs from one representative experiment of three with similar results are shown.

Retinoic acid inhibits corticotroph cells by inducing BMP-4

Given the reduced expression of BMP-4 and its inhibitory action in tumor corticotroph growth, we studied its putative involvement in retinoic acid antiproliferative action. We an-

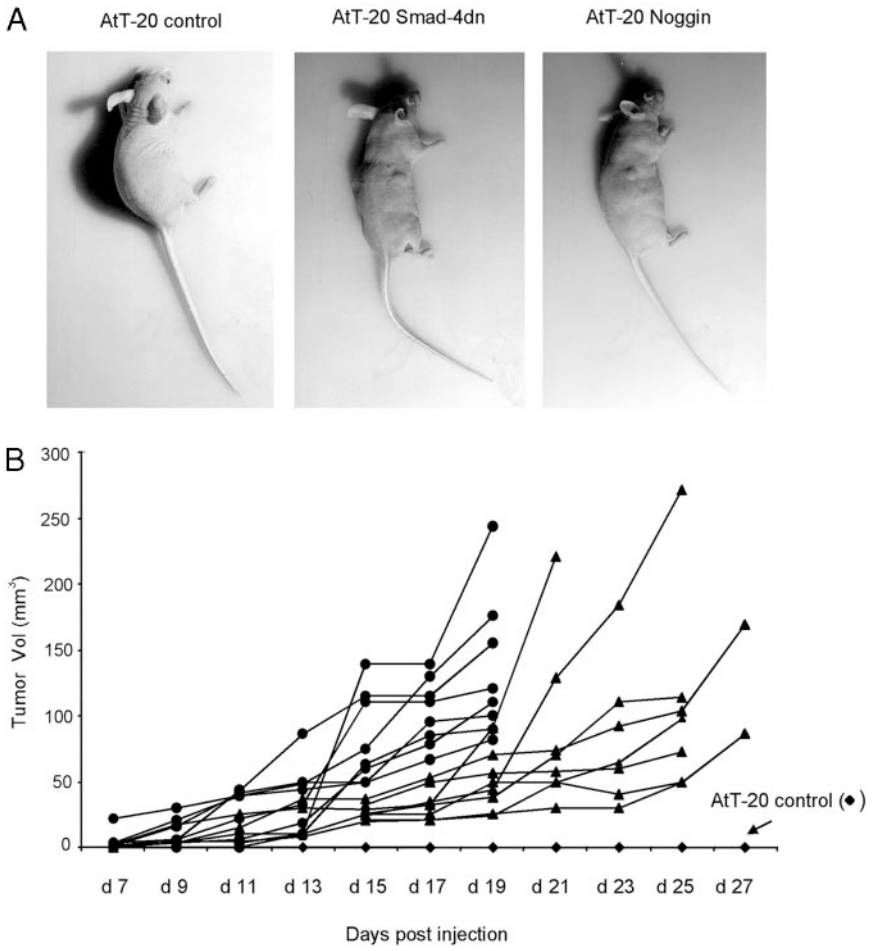

FIG. 5. BMP-4 blocks corticotroph tumor growth *in vivo*. A, Nude mice were injected sc with 1×10^6 AtT-20-control, AtT-20-Smad-4dn, or AtT-20-noggin cells. Seven days after injection, tumors formed by AtT-20-Smad-4dn and AtT-20-noggin cells were observed, compared with animals injected with AtT-20-control cells that did not develop tumors. Tumor growth is shown in a representative, from a total of eight, nude mouse of each group. B, Tumor growth was monitored by measuring tumor volume as described in *Materials and Methods*. The development of tumors in AtT-20-Smad-4dn and AtT-20-noggin animals started to be apparent at d 7 after injection, whereas AtT-20-control showed no tumor formation. Results are from two independent experiments each including four animals injected with each cell line as shown in A. ●, AtT-20 noggin cells; ▲, AtT-20 Smad-4dn; and ♦, AtT-20-vector cells.

alyzed whether retinoic acid induces BMP-4 in AtT-20 cells. After 24 h of treatment with 10–100 nM retinoic acid, an increase in BMP-4 promoter transcriptional activity was observed (4-fold induction) (Fig. 6A). An almost complete inhibition of the BMP-4 promoter responsiveness to retinoic acid was observed when cotransfecting the BMP-4-LUC promoter construct with a COUP-TFI expression vector (Fig. 6A). Moreover, the expression of COUP-TFI enhances the transcription of BMP-4 (3-fold induction) (Fig. 6A). To control the ability of BMP-4 promoter to be further activated in the presence of COUP-TFI, we treated the cells with cAMP and observed the induction of BMP-4 promoter (Fig. 6A). We further evaluated BMP-4 expression by Western blot. Retinoic acid increased BMP-4 protein levels and this stimulatory effect diminished after 24 h of treatment (Fig. 6B).

To test the hypothesis that the inhibitory effect of retinoic acid in AtT-20 cell proliferation could be mediated by BMP-4, we examined whether BMP-4 signaling is necessary for retinoic acid effects. In both AtT-20 Smad-4dn and AtT-20 noggin cells that do not respond to BMP-4, retinoic acid treatment was not able to inhibit cell proliferation (Fig. 7, A and B).

AtT-20 control cells responded to both BMP-4 and retinoic acid treatment. However, combined administration of the two compounds did not result in a further antiproliferative effect, supporting the notion that BMP-4 and retinoic acid might interact (Fig. 7C).

Discussion

The pathogenic mechanisms as well as the treatment of Cushing's disease remains still an open question. In this study, we demonstrate for the first time that BMP-4 expression is reduced in corticotrophinoma cells and that BMP-4 regulates not only corticotroph cell physiology but also inhibits its tumorigenic potential. This inhibitory effect of BMP-4 constitutes a novel mechanism for pituitary corticotroph pathogenesis. We also found that retinoic acid, used as a pharmacological agent in different types of cancer (40–42) and which inhibits corticotrophinoma cells growth (13), may act through BMP-4 expression in corticotroph cells. Therefore, BMP-4 induction by retinoic acid may be an interesting mechanistic target for retinoic acid as a therapeutic option for Cushing's disease treatment.

We demonstrate in this study that BMP-4 inhibits both the endogenous ACTH production and forskolin-induced ACTH stimulation in a dose-dependent manner, and this inhibitory effect was blocked by noggin, which is a specific antagonist of BMP-4. These results are in agreement with recently published data showing that BMP-4 has an inhibitory effect on the transcriptional activity of POMC promoter (43). BMP-4 inhibits not only AtT-20 cell proliferation but also c-*myc* promoter transcriptional activity. We have demonstrated that, in lactosomatotrophinoma GH3 cell line and

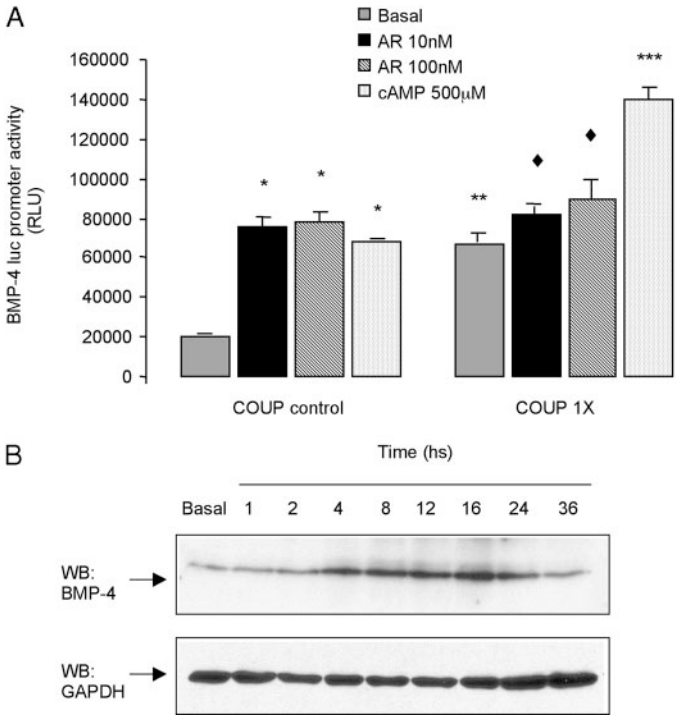

FIG. 6. Retinoic acid induces BMP-4 transcription and protein expression. A, AtT-20 cells were cotransfected with BMP-4-LUC (500 ng) and COUP-TFI (500 ng) expression vector or COUP-TFI control vector (500 ng) and treated with retinoic acid (RA) and cAMP. An additional 200 ng of the RSV-β-gal construct was cotransfected in all the experiments to correct for variations in transfection efficiency. After 24-h treatment, luciferase activity was measured and normalized as described in *Materials and Methods*. Values indicating the mean ± SE of luciferase to β-galactosidase ratio of one representative experiment of three with similar results are shown. *, $P < 0.001$ with respect to basal COUP-TFI control; **, $P < 0.001$ with respect to basal COUP-TFI control; ♦, not significant with respect to basal COUP-TFI, ANOVA with Scheffé's test. B, AtT-20 cells were treated with 100 nM retinoic acid for the indicated times. After treatment, cells were lysed and the protein extracts were analyzed by Western blot for BMP-4 expression using a polyclonal BMP-4 antibody (1:1,000) as described in *Materials and Methods*. Equal loading was assessed by GAPDH detection using a monoclonal GAPDH antibody (1:50,000). One representative of three independent experiments with similar results is shown.

human prolactinomas in which BMP-4 has a promoting role during tumor development, BMP-4 induces c-*myc* expression. This differential effect on c-*myc* depending on the cell lineage can be explained by the association of Smad-1 (BMP-4 signaling transducer protein) with different cofactors presented in each cell lineage. For example in GH3 cells BMP-4 stimulation triggers Smad-1/ER physical interaction that may have stimulatory effects over transforming gene promoters as c-*myc*. In corticotrophs, Smad-1 may possibly be interacting with other proteins resulting in negative effects over c-*myc* expression. Based on these results and the different studies that place TGF-β family as important regulating factors in pituitary tumor development (23, 26), we studied a possible role for BMP-4 in corticotroph cells tumorigenicity *in vivo*. At low cell concentration doses AtT-20 cells present no tumorigenic capacity when injected in nude mice. In contrast, AtT-20 Smad-4dn, and AtT-20 noggin clones in which the BMP-4 signaling pathway is blocked developed visible tumors starting at 1 wk after injection as well as symptoms of Cushing's disease. Therefore, inhibition of the BMP-4 signaling results in an increase of the AtT-20 cells tumorigenic potential, demonstrating an inhibitory role of BMP-4 in the development of corticotroph tumors *in vivo*.

BMP-2 and −4 play a central role during the initial steps of the anterior pituitary development (25, 44–46). BMP-4 is required during the first stage of pituitary organogenesis for the proliferation of the Rathke's pouch placode, which gives rise to Pit-1 lineage cells, which includes the PRL-secreting cells, among others. Moreover, overexpression of noggin or a dominant-negative BMP-receptor (BMPR1A) in the anterior pituitary leads to the arrest of the development of the pit-1-expressing lineage. During the second pituitary organogenesis stage an inhibition of BMP-2 ventrodorsal gradient by an FGF8 dorsoventral gradient, leads to corticotroph differentiation (25, 44–46). Overexpression of FGF8 in the developmental pituitary results in Pit-1 lineage cells absence and corticotroph enhanced differentiation, suggesting that it is necessary to inhibit BMP signaling for normal corticotroph development (47). We have shown that BMP-4 has a strong stimulatory effect on prolactinoma cell proliferation (26). Moreover the activin/BMP system has also been described in the development of human pituitary gonadotropinomas and nonfunctioning adenomas (48). In the present study, we show BMP-4 expression in 40% of endocrine cells in the normal adenohypophysis and most of the BMP-4 immunopositive cells were somatotrophs and corticotrophs but not lactotrophs. On the other hand, in pituitary adenomas, BMP-4 is expressed at low variable levels in corticotrophinoma cells but at high levels in prolactinoma cells. Thus BMP-4, a corticotroph growth inhibitor, is expressed in the normal pituitary but is reduced in corticotroph tumors. In analogy to the situation during pituitary organogenesis, this distinctive expression may allow corticotroph tumoral growth promotion. BMP-4 not only is crucial during pituitary organogenesis but has an opposite role in the different pituitary lineages. BMP-4 promotes pituitary prolactinoma pathogenesis through a Smad/estrogen receptor cross-talk but inhibits corticotroph pathogenesis and Cushing's disease. It is not clear at this stage, which is the trigger for BMP-4 action (enhancement of proliferation in prolactinomas and loss of inhibition control in Cushing's corticotroph) in adult pituitary tumor cells. Thus, BMP-4 acts as a regulator no only of pituitary cells undergoing organogenesis differentiation, but also of transformed, not normal, adult pituitary cells, and it has a pituitary linage cell specificity during the tumorigenic process.

Retinoic acid treatment in AtT-20 corticotroph cells induces BMP-4 transcription and BMP-4 expression. Similar actions of retinoic acid have been described in human and murine oesteoblastic cell lines (49). Inhibiting retinoic acid signaling with COUP-TFI (14, 50–52) blocks the stimulatory effect of retinoic acid on BMP-4, although the promoter may still be stimulated by other pathways. These results are in agreement with previous ones that establish that COUP-TFI can block retinoic acid inhibitory effects on POMC-LUC reporter activity. Also COUP-TFI expression only in normal corticotroph cells but not in pituitary Cushing tumors might

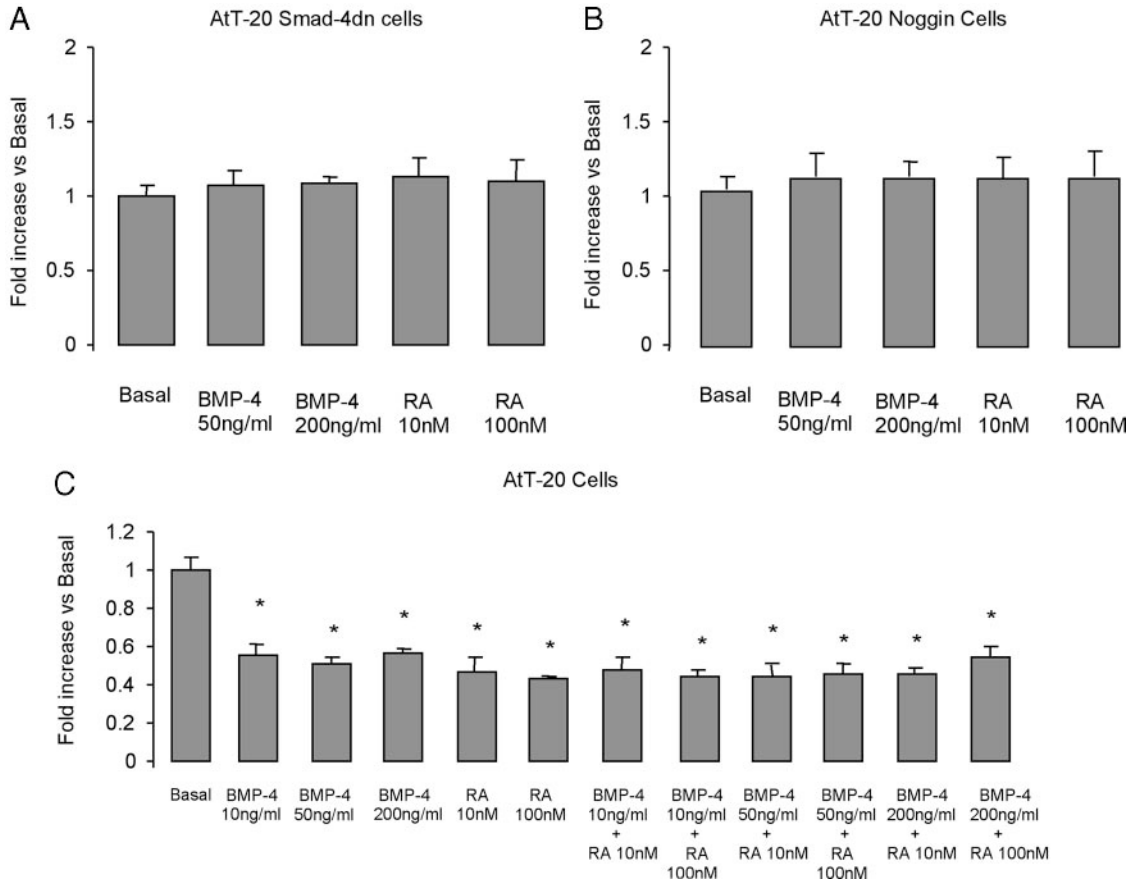

FIG. 7. Smad-4dn and noggin block the inhibitory effect of retinoic acid. AtT-20 Smad-4dn (A), AtT-20 noggin (B), and AtT-20 cells (C) were seeded at 5000 cells per well in 96-well plates with 10% FCS. After attachment, cells were washed twice with PBS and incubated in FCS-free medium. Cells were treated with BMP-4, retinoic acid, or their combinations as indicated, and cell proliferation was measured after 6 d using the WST-1 assay. Values over the basal value are express as folds increase *vs.* basal (basal = 1). Average of four wells per treatment and SEs from one representative experiment of three with similar results are shown. *, $P < 0.001$ with respect to basal, ANOVA with Scheffé's test.

be the explanation for the effects of retinoic acid in transformed but not in normal corticotroph cells (13). Moreover, we show that overexpression of COUP-TFI enhances, by a mechanism independent of retinoic acid, the transcription of BMP-4 in AtT-20 cells, suggesting that the differential expression of BMP-4 in normal but not tumor cells may be under the control of COUP-TFI, as it has been described in an osteoblast model (36).

Both BMP-4 and retinoic acid inhibit AtT-20 cell proliferation, but there was no further inhibition in the presence of both factors, even at low doses, suggesting that they may share an overlapping mechanism (which is saturated at low doses). BMP and steroid receptor cross-talk takes place in several cells. A cross-talk mechanism between BMP-4 and estrogens that regulates cell proliferation has been described and a physical interaction between steroid receptors and TGF-β-induced Smad proteins has been recently shown (26, 53, 54). Several studies have also established a different interaction between retinoic acid and BMPs (55–57). In embryonal carcinoma cells, retinoic acid controls BMP-2 and BMP-4 regulation (58–60), and the combination of retinoic acid and BMP-2 or BMP-4 synergistically induces apoptosis of these cells inhibiting neurons and glial cells but leading to smooth muscle cell differentiation (61, 62). To investigate whether retinoic acid transduces its antiproliferative effect through BMP-4 induction, its effect was tested in cells in which the BMP-4 pathway was blocked. In AtT-20 noggin and AtT-20 Smad-4dn cells, the inhibitory action of retinoic acid is abolished, showing that, besides the possibility of a further cross-talk with BMP-4 signaling pathway, retinoic acid is acting through the induction of BMP-4 expression, which in turn has a central role in preventing corticotroph cell proliferation and tumor cell growth.

The present study highlights a role of BMP-4 in the inhibition of corticotrophinoma cell growth and suggests a novel mechanism for the pathogenesis of Cushing's-associated pituitary adenomas. Furthermore, the fact that BMP-4 is induced by and mediates some of the effects of retinoic acid highlights a novel mechanism of retinoic acid action in corticotrophinoma cells. The knowledge of the cross-talk between retinoic acid and BMP-4 may be a useful tool for the design of new—based on retinoic acid—therapeutic approaches for Cushing's disease treatment.

Acknowledgments

We thank J. Massagué for the Smad-4dn expression vector, K. Monzen for the noggin expression vector, M. Tsai for the COUP-TFI expression vector, S. E. Harris for the mouse BMP-4 luciferase reporter

vector, and K. Mitsuyasu for the human c-Myc luciferase reporter vector. We also thank Esther Jelen for technical assistance.

Received July 28, 2005. Accepted September 17, 2005.

Address all correspondence and requests for reprints to: Dr. E. Arzt, Laboratorio de Fisiología y Biología Molecular, Departemento de Fisiología, Biología Molecular y Celular, Facultad Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellon II, 1428 Buenos Aires, Argentina. E-mail: earzt@fbmc.fcen.uba.ar.

This work was supported by grants from the University of Buenos Aires, the Argentine National Research Council (CONICET), and Agencia Nacional de Promoción Científica y Tecnológica-Argentina.

D.G., D.R., and E.A. are members of the Instituto de Fisiología, Biología Molecular y Neuro Ciencias-Argentine National Research Council (CONICET).

References

1. **Asa SL, Ezzat S** 1998 The cytogenesis and pathogenesis of pituitary adenomas. Endocr Rev 19:798–827
2. **Yu R, Melmed S** 2001 Oncogene activation in pituitary tumors. Brain Pathol 11:328–341
3. **Kovacs K, Horvath E, Vidal S** 2001 Classification of pituitary adenomas. J Neurooncol 54:121–127
4. **Ross EJ, Linch DC** 1982 Cushing's syndrome—killing disease: discriminatory value of signs and symptoms aiding early diagnosis. Lancet 2:646–649
5. **Dahia PL, Grossman AB** 1999 The molecular pathogenesis of corticotroph tumors. Endocr Rev 20:136–155
6. **Wajchenberg BL, Mendonca BB, Liberman B, Pereira MA, Carneiro PC, Wakamatsu A, Kirschner MA** 1994 Ectopic adrenocorticotropic hormone syndrome. Endocr Rev 15:752–787
7. **Alexander JM, Biller BM, Bikkal H, Zervas NT, Arnold A, Klibanski A** 1990 Clinically nonfunctioning pituitary tumors are monoclonal in origin. J Clin Invest 86:336–340
8. **Clayton RN, Farrell WE** 2001 Clonality of pituitary tumours: more complicated than initially envisaged? Brain Pathol 11:313–327
9. **Mampalam TJ, Tyrrell JB, Wilson CB** 1988 Transsphenoidal microsurgery for Cushing disease. A report of 216 cases. Ann Intern Med 109:487–493
10. **Miller JW, Crapo L** 1993 The medical treatment of Cushing's syndrome. Endocr Rev 14:443–458
11. **Colao A, Di Sarno A, Marzullo P, Di Somma C, Cerbone G, Landi ML, Faggiano A, Merola B, Lombardi G** 2000 New medical approaches in pituitary adenomas. Horm Res 53(Suppl 3):76–87
12. **Engler D, Redei E, Kola I** 1999 The corticotropin-release inhibitory factor hypothesis: a review of the evidence for the existence of inhibitory as well as stimulatory hypophysiotropic regulation of adrenocorticotropin secretion and biosynthesis. Endocr Rev 20:460–500
13. **Paez-Pereda M, Kovalovsky D, Hopfner U, Theodoropoulou M, Pagotto U, Uhl E, Losa M, Stalla J, Grubler Y, Missale C, Arzt E, Stalla GK** 2001 Retinoic acid prevents experimental Cushing syndrome. J Clin Invest 108:1123–1131
14. **Tran P, Zhang XK, Salbert G, Hermann T, Lehmann JM, Pfahl M** 1992 COUP orphan receptors are negative regulators of retinoic acid response pathways. Mol Cell Biol 12:4666–4676
15. **Heaney AP, Fernando M, Yong WH, Melmed S** 2002 Functional PPAR-γ receptor is a novel therapeutic target for ACTH-secreting pituitary adenomas. Nat Med 8:1281–1287
16. **Ray D, Melmed S** 1997 Pituitary cytokine and growth factor expression and action. Endocr Rev 18:206–228
17. **Arzt E** 2001 gp130 cytokine signaling in the pituitary gland: a paradigm for cytokine-neuro-endocrine pathways. J Clin Invest 108:1729–1733
18. **Arzt E, Pereda MP, Castro CP, Pagotto U, Renner U, Stalla GK** 1999 Pathophysiological role of the cytokine network in the anterior pituitary gland. Front Neuroendocrinol 20:71–95
19. **Zafar M, Ezzat S, Ramyar L, Pan N, Smyth HS, Asa SL** 1995 Cell-specific expression of estrogen receptor in the human pituitary and its adenomas. J Clin Endocrinol Metab 80:3621–3627
20. **Lieberman ME, Maurer RA, Claude P, Wiklund J, Wertz N, Gorski J** 1981 Regulation of pituitary growth and prolactin gene expression by estrogen. Adv Exp Med Biol 138:151–163
21. **Friend KE, Chiou YK, Lopes MB, Laws Jr ER, Hughes KM, Shupnik MA** 1994 Estrogen receptor expression in human pituitary: correlation with immunohistochemistry in normal tissue, and immunohistochemistry and morphology in macroadenomas. J Clin Endocrinol Metab 78:1497–1504
22. **Chaidarun SS, Eggo MC, Stewart PM, Barber PC, Sheppard MC** 1994 Role of growth factors and estrogen as modulators of growth, differentiation, and expression of gonadotropin subunit genes in primary cultured sheep pituitary cells. Endocrinology 134:935–944
23. **Hentges S, Sarkar DK** 2001 Transforming growth factor-β regulation of estradiol-induced prolactinomas. Front Neuroendocrinol 22:340–363
24. **Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, Westphal H** 1997 Multistep control of pituitary organogenesis. Science 278:1809–1812
25. **Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP, Rosenfeld MG** 1998 Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev 12:1691–1704
26. **Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK, Arzt E** 2003 Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. Proc Natl Acad Sci USA 100:1034–1039
27. **Shi Y, Massague J** 2003 Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113:685–700
28. **Massague J** 2000 How cells read TGF-β signals. Nat Rev Mol Cell Biol 1:169–178
29. **Massague J, Chen YG** 2000 Controlling TGF-β signaling. Genes Dev 14:627–644
30. **Leung CK, Paterson JA, Imai Y, Shiu RP** 1982 Transplantation of ACTH-secreting pituitary tumor cells in athymic nude mice. Virchows Arch A Pathol Anat Histol 396:303–312
31. **Arzt E, Stelzer G, Renner U, Lange M, Muller OA, Stalla GK** 1992 Interleukin-2 and interleukin-2 receptor expression in human corticotrophic adenoma and murine pituitary cell cultures. J Clin Invest 90:1944–1951
32. **Bates AS, Farrell WE, Bicknell EJ, McNicol AM, Talbot AJ, Broome JC, Perrett CW, Thakker RV, Clayton RN** 1997 Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker. J Clin Endocrinol Metab 82:818–824
33. **Paez Pereda M, Ledda MF, Goldberg V, Chervin A, Carrizo G, Molina H, Muller A, Renner U, Podhajcer O, Arzt E, Stalla GK** 2000 High levels of matrix metalloproteinases regulate proliferation and hormone secretion in pituitary cells. J Clin Endocrinol Metab 85:263–269
34. **Hata A, Lagna G, Massague J, Hemmati-Brivanlou A** 1998 Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev 12:186–197
35. **Monzen K, Shiojima I, Hiroi Y, Kudoh S, Oka T, Takimoto E, Hayashi D, Hosoda T, Habara-Ohkubo A, Nakaoka T, Fujita T, Yazaki Y, Komuro I** 1999 Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. Mol Cell Biol 19:7096–7105
36. **Feng JQ, Chen D, Cooney AJ, Tsai MJ, Harris MA, Tsai SY, Feng M, Mundy GR, Harris SE** 1995 The mouse bone morphogenetic protein-4 gene. Analysis of promoter utilization in fetal rat calvarial osteoblasts and regulation by COUP-TFI orphan receptor. J Biol Chem 270:28364–28373
37. **Pipaon C, Tsai SY, Tsai MJ** 1999 COUP-TF upregulates NGFI-A gene expression through an Sp1 binding site. Mol Cell Biol 19:2734–2745
38. **Takeshita T, Arita T, Higuchi M, Asao H, Endo K, Kuroda H, Tanaka N, Murata K, Ishii N, Sugamura K** 1997 STAM, signal transducing adaptor molecule, is associated with Janus kinases and involved in signaling for cell growth and c-*myc* induction. Immunity 6:449–457
39. **Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E** 2002 Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638–1651
40. **Kelloff GJ** 2000 Perspectives on cancer chemoprevention research and drug development. Adv Cancer Res 78:199–334
41. **Kurie JM** 1999 The biologic basis for the use of retinoids in cancer prevention and treatment. Curr Opin Oncol 11:497–502
42. **Lotan R** 1996 Retinoids in cancer chemoprevention. FASEB J 10:1031–1039
43. **Nudi M, Ouimette JF, Drouin J** 2005 Bone morphogenic protein (Smad)-mediated repression of proopiomelanocortin transcription by interference with Pitx/Tpit activity. Mol Endocrinol 19:1329–1342
44. **Dasen JS, Rosenfeld MG** 1999 Signaling mechanisms in pituitary morphogenesis and cell fate determination. Curr Opin Cell Biol 11:669–677
45. **Dasen JS, Rosenfeld MG** 2001 Signaling and transcriptional mechanisms in pituitary development. Annu Rev Neurosci 24:327–355
46. **Kioussi C, Carriere C, Rosenfeld MG** 1999 A model for the development of the hypothalamic-pituitary axis: transcribing the hypophysis. Mech Dev 81:23–35
47. **Ericson J, Norlin S, Jessell TM, Edlund T** 1998 Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 125:1005–1015
48. **Takeda M, Otsuka F, Suzuki J, Kishida M, Ogura T, Tamiya T, Makino H** 2003 Involvement of activin/BMP system in development of human pituitary gonadotropinomas and nonfunctioning adenomas. Biochem Biophys Res Commun 306:812–818
49. **Helvering LM, Sharp RL, Ou X, Geiser AG** 2000 Regulation of the promoters for the human bone morphogenetic protein 2 and 4 genes. Gene 256:123–138
50. **Kliewer SA, Umesono K, Heyman RA, Mangelsdorf DJ, Dyck JA, Evans RM** 1992 Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc Natl Acad Sci USA 89:1448–1452
51. **Wu Q, Li Y, Liu R, Agadir A, Lee MO, Liu Y, Zhang X** 1997 Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan

receptors nur77 and COUP-TF and their heterodimerization. EMBO J 16:1656–1669
52. **Beland M, Lohnes D** 2005 Chicken ovalbumin upstream promoter-transcription factor members repress retinoic acid-induced Cdx1 expression. J Biol Chem 280:13858–13862
53. **Kang HY, Huang KE, Chang SY, Ma WL, Lin WJ, Chang C** 2002 Differential modulation of androgen receptor-mediated transactivation by Smad3 and tumor suppressor Smad4. J Biol Chem 277:43749–43756
54. **Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F** 2001 Cross-talk between transforming growth factor-β and estrogen receptor signaling through Smad3. J Biol Chem 276:42908–42914
55. **Sumantran VN, Brederlau A, Funa K** 2003 BMP-6 and retinoic acid synergistically differentiate the IMR-32 human neuroblastoma cells. Anticancer Res 23:1297–1303
56. **Skillington J, Choy L, Derynck R** 2002 Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol 159:135–146
57. **Li X, Schwarz EM, Zuscik MJ, Rosier RN, Ionescu AM, Puzas JE, Drissi H, Sheu TJ, O'Keefe RJ** 2003 Retinoic acid stimulates chondrocyte differentiation and enhances bone morphogenetic protein effects through induction of Smad1 and Smad5. Endocrinology 144:2514–2523
58. **Rogers MB, Rosen V, Wozney JM, Gudas LJ** 1992 Bone morphogenetic proteins-2 and -4 are involved in the retinoic acid-induced differentiation of embryonal carcinoma cells. Mol Biol Cell 3:189–196
59. **Rogers MB** 1996 Receptor-selective retinoids implicate retinoic acid receptor α and γ in the regulation of bmp-2 and bmp-4 in F9 embryonal carcinoma cells. Cell Growth Differ 7:115–122
60. **Glozak MA, Rogers MB** 1996 Specific induction of apoptosis in P19 embryonal carcinoma cells by retinoic acid and BMP2 or BMP4. Dev Biol 179:458–470
61. **Glozak MA, Rogers MB** 2001 Retinoic acid- and bone morphogenetic protein 4-induced apoptosis in P19 embryonal carcinoma cells requires p27. Exp Cell Res 268:128–138
62. **Glozak MA, Rogers MB** 1998 BMP4- and RA-induced apoptosis is mediated through the activation of retinoic acid receptor α and γ in P19 embryonal carcinoma cells. Exp Cell Res 242:165–173

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Erratum

The rOGED database referenced in the article "Development and Application of a Rat Ovarian Gene Expression Database" by Misung Jo, Mary C. Gieske, Charles E. Payne, Sarah E. Wheeler-Price, Joseph B. Gieske, Ignatius V. Ignatius, Thomas E. Curry, Jr., and CheMyong Ko (*Endocrinology* **145:**5384–5396, 2004) has moved to a new location. The old address, http://web5.mccs.uky.edu/kolab/rogedendo.aspx, is no longer valid. The new address is http://app.mc.uky.edu/kolab/rogedendo.aspx.