

# Functional role of estrogen in pituitary tumor pathogenesis

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Pituitary hyperplasia and lactotroph replication are induced by estrogen. The product of the pituitary tumor transforming gene (*PTTG*) exhibits in vitro and in vivo transforming activity and induces basic bFGF secretion, thereby modulating pituitary angiogenesis and tumor formation. We demonstrated previously that pituitary pttg is induced by estrogen and bFGF, the latter being expressed in a concordant fashion with pttg in experimental and human pituitary adenomas. We now elucidate the role of estrogen in paracrine regulation of pituitary tumorigenesis by PTTG. Coincident with the circulating rat estradiol surge and maximal pituitary proliferation, pituitary pttg mRNA, bFGF, and VEGF expression increased approximately threefold during proestrus and estrus. Osmotic mini-pump coinfusion of estrogen and antiestrogen abrogated estrogen-induced pituitary pttg expression in vivo, suppressed serum PRL concentrations by 88%, and attenuated prolactin-secreting pituitary tumor growth by 41% in rats. Antiestrogen treatment of primary human pituitary tumor cultures reduced PTTG expression approximately 65%. Pituitary pttg, bFGF, and VEGF are cyclically expressed during the rat estrus cycle, concordantly with estrogen levels. Because antiestrogens reduced PTTG expression in human pituitary tumors in vitro and suppressed experimental tumor growth in vivo, concomitantly with reduced PRL secretion, these results indicate a role for selective antiestrogens in treating pituitary tumors.

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## Introduction

Pituitary tumor transforming gene (*PTTG*), originally isolated from GH4 pituitary cells, is tumorigenic in vivo (1, 2) and is the mammalian homologue of a *Xenopus* securin inhibitor of chromatid separation (3). As a securin protein, PTTG is expressed in a cell-cycle dependent manner, peaking during the G2/M phase (4). Cells overexpressing PTTG exhibit increased bFGF secretion (2, 5), and conditioned medium derived from these stable transfectants induced in vitro and in vivo angiogenesis (6).

The human *PTTG* family consists of at least four homologous genes, of which *PTTG<sub>1</sub>* is located on chromosome 5q33 (7), and is expressed at low levels in several normal human tissues and abundantly expressed in neoplasms, including pituitary tumors, where highest expression was observed in prolactin-secreting (PRL-secreting) tumors (8–10). We recently demonstrated that pituitary pttg is regulated by estrogen and involved in early pituitary lactotroph transformation and onset of angiogenesis (11).

Because estrogens promote lactotroph proliferation, we elucidated the role of the estrogen receptor in paracrine regulation of pituitary tumorigenesis by PTTG. We show here that pituitary pttg, bFGF, and VEGF expression are regulated during the rat estrus cycle, with maximal expression occurring coincidentally with peak serum estradiol levels and proliferating cytoplasmic nuclear antigen (PCNA) expression. Furthermore, selective antiestrogen

treatment blocks estrogen-induced pituitary pttg expression in vitro and in vivo and inhibits lactotroph tumor growth in vivo. Estrogen-regulated PTTG, bFGF, and VEGF expression may therefore play a role in the early molecular events leading to development of pituitary transformation and prolactinoma development.

## Methods

**Animals.** Ovariectomized Fischer 344 rats (140–150 g; Harlan Sprague Dawley Inc., Indianapolis, Indiana, USA) were housed in a controlled environment (light on, 0500–1900 hours, 24°C) with free access to food and water, and their use was approved by the Institutional Animal Care and Use Committee. Subcutaneously implanted osmotic pumps (OP) (100  $\mu$ l, ALZET), containing 17- $\beta$ -estradiol (1,000 ng), 4-hydroxytamoxifen (860  $\mu$ g), ICI-182780 (500  $\mu$ g), or raloxifene (480  $\mu$ g) in 90% polyethylene glycol (PEG)/5% ethanol/5% DMSO solution were employed to infuse estrogen and/or antiestrogen for 48-hour intervals.

For study of pttg expression during the rat estrus cycle, daily vaginal smears were collected from twenty, 4-day cycling, virgin female Long-Evans rats (12 weeks of age), housed as above, to determine their estrus cycle patterns. Only animals that exhibited at least three consecutive 4-day cycles were considered to be regularly cyclic and used in subsequent experiments. Rats were euthanized in the early afternoon (1400 hours) by CO<sub>2</sub> inhalation, pituitary tissues immersed in liquid N<sub>2</sub> and stored at –80°C

for RNA and/or protein extraction, and serum collected for estradiol and progesterone assay (12).

Rat pituitary GH3 cells in DMEM (~500,000) were inoculated subcutaneously in twenty 2-month-old female Wistar-Furth rats. By 6 weeks, visible subcutaneous tumors developed, and animals were randomized to receive mini-OP infusion with vehicle (90% PEG/0.1% DMSO/9.9% DMEM) or the pure antiestrogen ICI-182780 (0.5 µg per day). Rats were euthanized by CO<sub>2</sub> inhalation, and tumor volume calculated according to the formula, in centimeters:  $(3.14 \times \text{length} \times \text{width} \times \text{depth})/6$ .

**Cell culture.** GH3 rat pituitary (PRL- and growth hormone-secreting [PRL- and GH-secreting]) cells were cultured in Hams F12 medium supplemented with 15% horse serum (HS) and 2.5% FCS and antibiotics at 37°C in 5% CO<sub>2</sub>. Subconfluent GH3 cells were maintained for 3 days in phenol red-free F12 with 15% HS and 2.5% FCS, which had been pretreated with dextran-coated charcoal before treatment with diethylstilbestrol (DES; 10<sup>-8</sup> and 10<sup>-10</sup> M), 2-OH estradiol (10<sup>-6</sup> M), and/or ICI-182780 (Tocris Cookson Inc., Ballwin, Missouri, USA) (10<sup>-6</sup> M). Serum estradiol assay confirmed significant removal of estrogens (34 pg/ml pretreatment, 10 pg/ml after charcoal stripping).

**Patients and tissues.** Samples of 14 surgically resected invasive pituitary macroadenomas (six gonadotroph, five nonfunctioning, one GH-secreting, one PRL-secreting, and one ACTH-secreting) were obtained from 26 consecutive unselected patients after surgical resection, in accordance with institutional guidelines. For primary human pituitary cultures, pituitary tumor tissue, freshly obtained at surgery, was minced mechanically and digested for 45 minutes at 37°C with 0.35% collagenase and 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, Missouri, USA) in 10 ml DMEM. Cell suspensions were filtered and resuspended for 24 hours in low-glucose DMEM containing 10% FBS, 2 mmol/l glutamine, and antibiotics, before treatment with vehicle or ICI-182780, 10<sup>-5</sup> to 10<sup>-6</sup> M, for 48 hours. Sufficient protein was obtained from eight cases (three gonadotroph, five nonfunctioning) for Western blot analysis.

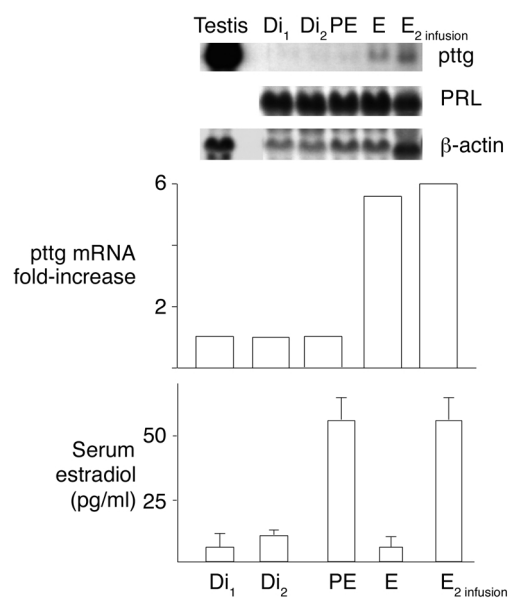
**Northern blot analysis.** Total RNA was extracted from cell cultures and from excised tissues (after tissue homogenization) with TRIzol (Life Technologies Inc., Gaithersburg, Maryland, USA). RNA derived from rat testes served as a positive control for PTTG expression. Electrophoresed RNA was transferred to Hybond-N nylon membranes (Amersham International, Amersham, United Kingdom), cross-linked, prehybridized (1 hour), and hybridized (2 hours) at 68°C with rat or human PTTG cDNA in the presence of 100 µg/ml salmon sperm DNA (Stratagene, La Jolla, California, USA). A 900-bp PTTG cDNA fragment spanning the entire coding region was labeled with [ $\alpha$ -<sup>32</sup>P] dCTP using Klenow enzyme (Life Technologies Inc.). Posthybridization washes were followed by air drying and autoradiography. PTTG<sub>1</sub> mRNA expression was quantitated using scanning densitometry and normalized to either  $\beta$ -actin or 18S ribosomal RNA expression.

**Western blot analysis.** Proteins were prepared from pituitary tissues and cells using RIPA buffer (100 mM NaCl, 0.1% Triton X-100, and 50 mM Tris-HCl, pH 8.3) containing a cocktail of enzyme inhibitors (1 mM phenylmethylsulfonylfluoride, 2 µg/ml aprotinin, and 200 µg/ml leupeptin) and denatured (2 minutes, 100°C) in loading buffer. Protein concentration was determined by the Bradford assay using BSA as a standard. Soluble proteins (50 µg) were separated by electrophoresis in 12% SDS-PAGE gels, transferred to PVDF membranes (Amersham International), incubated in 5% non-fat milk in PBS-0.05% Tween solution, followed by incubation with Ab's to bFGF (1:2,000), VEGF (1:2,000) (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and PTTG (1: 5000), for 24 hours at 4°C, or PCNA (Dako Corp., Carpinteria, California, USA) for 2 hours at room temperature. After washing in PBS-0.05% Tween (six times for 10 minutes), blots were incubated with appropriate horseradish peroxidase-conjugated (HRP-conjugated) anti-IgGs for 1 hour at room temperature. After further washes, antigen-Ab complexes were visualized by the enhanced chemiluminescence (ECL) detection system on Hyperfilm ECL. PTTG expression was quantitated using scanning densitometry and normalized to  $\beta$ -actin expression.

**Statistical analysis.** Results are expressed as mean plus or minus SEM values, and statistical analysis was performed using nonparametric *t* test or ANOVA with Bonferroni's multiple comparison test, taking *P* values less than 0.05 as significant.

## Results

**PTTG is regulated by estrogen in vivo.** Because we had demonstrated previously that estrogen induces pttg mRNA in rat pituitary GH3 cells in vitro, we sought to determine the role of estrogen in pituitary pttg regulation in vivo. Coincident with the proestrus serum estradiol surge ( $56 \pm 4$  pg/ml) in 4-day cycling virgin Long-Evans rats, pttg mRNA expression in pituitary-derived tissue extracts increased approximately sixfold during estrus (Figure 1). Because PTTG regulates bFGF expression (2), we examined pituitary bFGF and VEGF expression by Western blot analysis. Estrogen-mediated increase in pituitary pttg mRNA expression during estrus was associated with an approximately 3.4-fold increase in pituitary bFGF and an approximately 2.2-fold increase in VEGF expression, respectively (Figure 2). Increased bFGF and VEGF was also evident in proestrus and preceded increased pituitary pttg mRNA expression by approximately 12 hours, suggesting that additional PTTG-independent estrogen-mediated mechanisms are also involved in bFGF and VEGF induction. PTTG exhibits cell-cycle dependent expression (4, 13), peaking during G2/M, and may be a marker of tumor invasiveness (8, 9). The relationship of pituitary proliferation and PTTG expression was therefore examined by testing pituitary PCNA expression. PCNA expression (Figure 2) peaked (approximately twofold) concordantly with maximal



**Figure 1**  
PTTG is regulated by estrogen in vivo. Representative Northern blot analysis and pttg mRNA quantitation of pituitary tissue extracts derived from twenty 4-day cycling virgin female Long-Evans rats and serum estradiol levels measured at 1400 hours.  $Di_1$ , diestrus 1;  $Di_2$ , diestrus 2; PE, proestrus; E, estrus;  $E_2$ , estrogen-treatment. Rat testis served as a positive control. pttg, PRL, and  $\beta$ -actin (internal control). Each lane represents results of five pituitary glands.

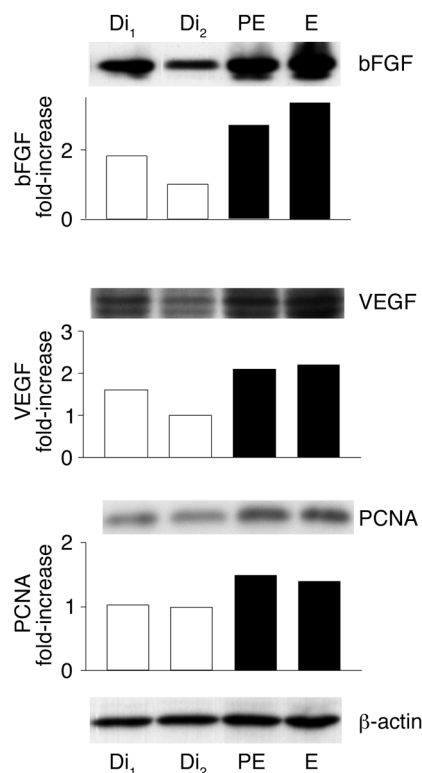
PTTG expression, further indicating that cyclical PTTG expression during the rat estrus cycle is associated with estrogen-mediated pituitary proliferation.

Since naturally occurring catechol-metabolites of estradiol may mediate pituitary estrogenic actions (14, 15), we next examined the effects of  $10^{-6}$  M 2-hydroxy-estradiol (2-OHE<sub>2</sub>) treatment on rat GH3 cells (Figure 3). Catecholestrogen treatment led to an approximately threefold increase in pituitary pttg mRNA expression ( $P < 0.01$ ), similar to the magnitude of pttg mRNA induction following DES treatment ( $P < 0.01$ ). Furthermore, both DES- and 2-OHE<sub>2</sub>-mediated pttg mRNA induction were almost completely abrogated by coadministration of the specific antiestrogen, ICI-182780 ( $10^{-5}$  M,  $P < 0.001$ ), confirming that the catechol-estrogen effects on pituitary PTTG expression are mediated through the estrogen receptor.

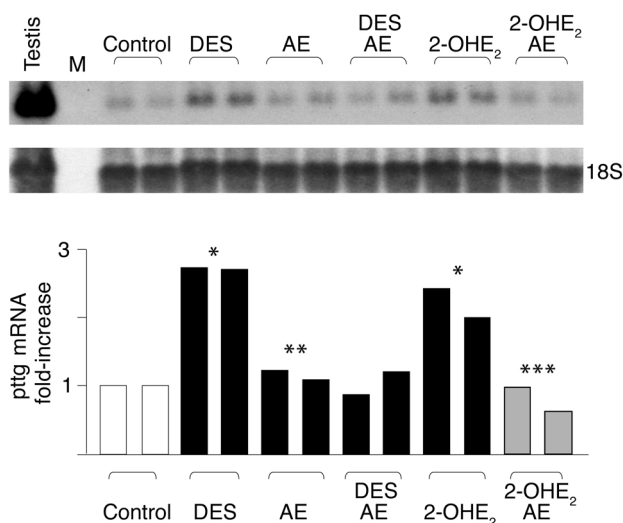
In view of the observed in vivo increase in estrogen-mediated pituitary PTTG expression and increased pituitary proliferation, we tested whether antiestrogen administration may block estrogen-mediated pituitary PTTG expression and PTTG-mediated pituitary actions. Mini-OP infusion of estrogen (1  $\mu$ g) for 48 hours resulted in an approximately 2.1-fold increase in pituitary pttg mRNA expression. Coinfusion of estrogen (1,000 ng) and one of these three antiestrogens, 4-hydroxytamoxifen (860  $\mu$ g), raloxifene (480  $\mu$ g), and ICI-182780 (500  $\mu$ g) for 48 hours (Figure 4), or pretreatment of animals with progesterone (2 mg) for 48 hours (data not shown), abrogated estrogen-induced

pituitary pttg expression in vivo, supporting the rationale for use of antiestrogens as inhibitors of pituitary PTTG-mediated actions (Figure 4).

**Selective antiestrogen treatment inhibits pituitary tumor growth in vivo.** To examine the role of selective antiestrogens in inhibiting pituitary tumor growth, the pure estrogen receptor (ER) antagonist, ICI-182780 (0.5  $\mu$ g per day), was then infused into Wistar-Furth rats harboring subcutaneous implanted PRL- and GH-secreting pituitary tumors. Six weeks after GH3 cell inoculation, all animals developed large tumors and were randomly assigned to receive vehicle or mini-OP infusion of ICI-182780 (0.5  $\mu$ g per day). Before treatment, baseline serum PRL (vehicle,  $365 \pm 26$ ; ICI,  $345 \pm 76$   $\mu$ g/ $\mu$ l,  $P = NS$ ), and tumor volume (vehicle,  $372 \pm 38.3$ ; ICI,  $329 \pm 79.7$  mm<sup>3</sup>,  $P = NS$ ) were similar in animals receiving either mini-OP anti-estrogen infusion or vehicle (Figure 5). Tumor growth continued unabated in untreated animals, necessitating their euthanization due to tumor burden at 9 weeks. Administration of the pure ER antagonist, ICI-182780 (0.5  $\mu$ g per day) suppressed serum PRL concentrations by 88% (vehicle,  $25980 \pm 6603$ ; ICI,  $3110 \pm 2091$  ng/ml,  $P < 0.001$ ) and attenuated tumor growth by 41% (tumor volume: vehicle,  $473 \pm 30$ ; ICI,  $329 \pm 80$  mm<sup>3</sup>,  $P = 0.03$ ) (Figure 5).



**Figure 2**  
Cyclical pituitary bFGF and VEGF in rat estrus cycle. Western blot immunodetection and quantitation of bFGF, VEGF, PCNA, and  $\beta$ -actin (internal control) immunoreactivity in pituitary tissue extracts derived from 4-day cycling virgin female Long-Evans rats, sacrificed at 1400 hours.  $Di_1$ , diestrus 1;  $Di_2$ , diestrus 2; PE, proestrus; E, estrus. Each lane represents mean results of five pituitary glands.



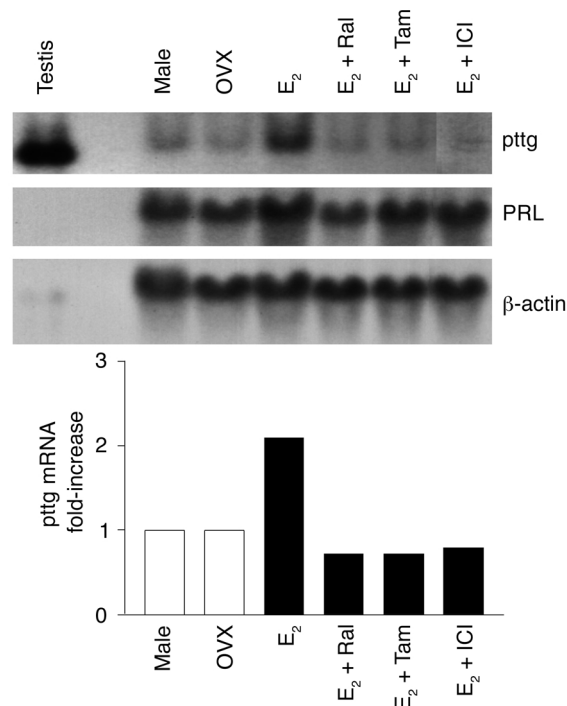
**Figure 3**

Regulation of pttg- and PRL-mRNA in vitro by DES and 2-OHE<sub>2</sub>. GH3 cells were incubated (3 days) in medium containing serum pre-treated with charcoal-stripped serum before addition of DES (10<sup>-8</sup> M) or 2-OHE<sub>2</sub> (10<sup>-6</sup> M) with/without the antiestrogen (ICI-182780 10<sup>-6</sup> M) (AE) for 48 hours. pttg mRNA expression relative to 18S (internal control) is depicted in lower panel. M, marker. Rat testis served as a positive control. Experiments were performed twice in triplicate wells for each data point. Each lane represents the mean of three dishes. ANOVA = 0.0003. Bonferroni multiple comparisons: \**P* < 0.01 compared with control; \*\**P* < 0.001 compared with DES; and \*\*\**P* < 0.001 compared with 2-OHE<sub>2</sub>.

*Antiestrogens inhibit PTTG expression in primary pituitary tumor cultures in vitro.* In view of our previous findings of enhanced PTTG mRNA and bFGF expression in human and animal pituitary tumor models, and because human pituitary tumors express ER (16), we treated primary pituitary tumor cultures from 14 consecutive surgically resected invasive pituitary macroadenomas (six gonadotroph, five nonfunctioning, one GH-secreting, one PRL-secreting, and one ACTH-secreting) with the antiestrogen ICI-182780 (10<sup>-5</sup>–10<sup>-6</sup> M for 48 hours) in vitro (Figure 6) to examine antiestrogen effects on PTTG expression. Insufficient total RNA obtainable from human adenomas did not permit Northern blot analyses, but sufficient protein was obtained from eight tumors (three gonadotroph, five nonfunctioning) for Western blot analysis. ICI-182780 treatment of primary human pituitary tumor cultures reduced PTTG expression in four of these eight pituitary tumors (mean ± SEM PTTG decrease, 64% ± 9%, range 20–100%), further extending the findings in the animal tumor model. In three of these cases, decreased PTTG expression was associated with reduced PCNA expression (mean ± SEM PCNA decrease, 47% ± 9%, range 34–74%), in keeping with reduced proliferation rates. Decreased bFGF (mean ± SEM bFGF decrease, 37% ± 12%, range 25–50%) and Bcl-2 (mean ± SEM Bcl-2 decrease, 49% ± 16, range 33–65%) expression was observed in two of four antiestrogen-responsive tumors.

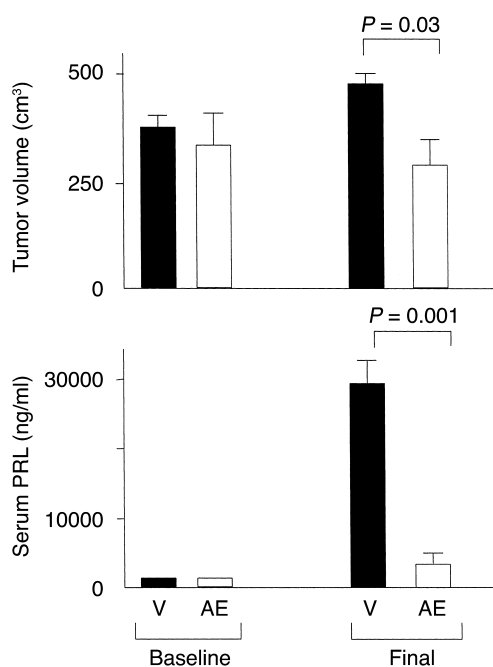
## Discussion

Female rat pituitary cell proliferation fluctuates considerably, with highest activity occurring during estrus and lowest cell growth during diestrus (17). Double-immunostaining studies with 5-bromo-2'-deoxyuridine (BrDU) and PCNA demonstrated that lactotrophs are the highest proportion of proliferating pituitary cells and that cell renewal rates double in the female compared with the male rat pituitary. Using a variety of proliferative assays, several studies have shown increased BrDU incorporation (17, 18), increased PCNA expression (18) and increased polyamine synthesis (19) during the late proestrus and estrus phases of the rat estrus cycle. Estrus cycle-related pituitary proliferation is largely confined to lactotrophs (18), little BrDU incorporation is seen in other anterior pituitary cell types (20), and no significant changes in pituitary GH mRNA content occurs during the estrus cycle (21). We observed increased pituitary PCNA expression in association with increased pituitary PTTG mRNA, consistent with increased pituitary proliferative activity during the estrus phase. During proestrus, rising 17-β-estradiol concentrations induce synchronous expression of ER, progesterone receptor (PR), and Ki-67 in estrogen-responsive tissues (22). In early diestrus, 17-β-estradiol levels fall and progesterone levels plateau. Progesterone administration to rats before estrogen



**Figure 4**

Estrogen-induced pituitary PTTG is abrogated by antiestrogens in vivo. Northern blot and quantitative analysis of pituitary tissue extracts derived following mini-OP 17-β-estradiol infusion (1,000 ng for 48 hours) (E<sub>2</sub>), with/without coinfusion of the antiestrogens, raloxifene (480 μg) (Ral); 4-hydroxytamoxifen (860 μg) (Tam), or ICI-182780 (500 μg) (ICI) for 48 hours in ovariectomized (OVX) F344 rats and vehicle-administered ovariectomized controls. *n* = 4 animals in each group. pttg, PRL, and β-actin (internal control).



**Figure 5**

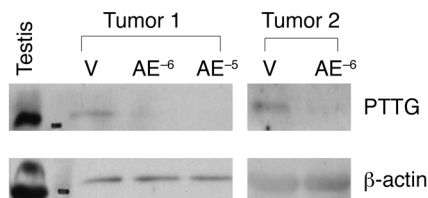
Selective antiestrogen treatment inhibits pituitary tumor growth in vivo. Pretreatment (baseline) and post-treatment tumor volumes and serum PR levels following mini-OP infusion of vehicle or antiestrogen ICI-182780 (0.5 µg per day) was infused in 20 female Wistar-Furth rats harboring subcutaneous pituitary tumors. All animals developed tumors. Each bar represents mean ± SEM for ten animals per group. \* $P = 0.03$ ; \*\*  $P < 0.001$ .

administration blocked estrogen-mediated increases in pituitary pttg mRNA expression. A striking feature of the observed cyclical pituitary pttg mRNA expression is the rapidity of its induction and disappearance, being largely restricted to the estrus phase. Many hormones, growth factors, and cytokines are cyclically expressed during the estrus cycle and contribute to rapid pituitary cyclical lactotroph proliferative activity. Progesterone downregulates ER expression and may thereby limit estrogen-mediated pituitary pttg mRNA induction, providing a potential mechanism for the rapid decrease in pituitary pttg mRNA during diestrus (23).

In humans, combined exogenous estrogen/progestin administration in the form of oral contraceptives or postmenopausal hormone replacement therapy does not appear to initiate pituitary tumor development or to increase individual susceptibility to prolactinoma development (24, 25). Although not all authors agree, several lines of investigation support a role for estrogens in the development/progression of lactotroph tumors. In vitro estrogen administration stimulates PRL secretion (26, 27), PRL-secreting tumors are more common in females (28), and in vivo estrogen administration induces lactotroph tumors in humans and in animal models (29). Pituitary lactotroph hyperplasia occurs in physiological states of estrogen excess, during estrus in several species and in human pregnancy (18). Pituitary tumor growth occurs in approximately 23% of women harboring macro-

prolactinomas during pregnancy, requiring surgery or reinstitution of dopamine agonist therapy (30). Furthermore, although pituitaries derived from men with prostate cancer treated with high-dose estrogen did not show increased lactotroph proliferation (31), several cases of lactotroph adenoma development have been reported in male-female transexuals given pharmacological doses of unopposed estrogen, emphasizing the powerful proliferative effects of unopposed estrogen on pituitary cells (32–34). Furthermore, ER expression is highest in PRL-secreting pituitary tumors derived from both male as well as female patients, as compared with other pituitary tumor types (35), and indeed may correlate with pituitary tumor size (35, 36). A role for estrogen in other pituitary tumor types is less clear, but the ER is expressed in all pituitary tumor subtypes (15, 35–39) and represents an attractive therapeutic target in pituitary tumors (40). In addition, although antiestrogen administration induces lactotroph apoptosis in vitro (41) and prevents lactotroph tumor development in in vivo animal models (42), previous studies have not examined the role of antiestrogens in inhibition or regression of pituitary tumor growth. ICI-182780, is a nonselective pure ER antagonist, which has potential advantages over tamoxifen-derivatives that have partial agonist actions in the pituitary (43). In addition, ICI-182780 appears to downregulate ER expression and block ER-mediated gene transcription (44). Tamoxifen has been administered to a patient with an aggressive nonfunctioning pituitary tumor, although it is unclear whether this tumor expressed ER (45).

We have demonstrated abundant and concordant PTTG and bFGF expression in human pituitary tumors (11). During early-stage lactotroph tumor development in a rat pituitary tumor model, we observed estrogen-mediated increased pituitary pttg, bFGF, and VEGF expression, which was coincident with pituitary angiogenesis (11). We now show that pituitary pttg, bFGF, and VEGF are cyclically expressed during the rat estrus cycle, and that selective antiestrogen administration abrogates estrogen-stimulated pituitary pttg expression in vivo. Previous studies have demonstrated that tamoxifen administration prevents occurrence of subcutaneously inoculated pituitary tumors in animals (41).



**Figure 6**

Antiestrogens inhibit PTTG expression in human pituitary tumor cultures in vitro. Representative Western blot immunodetection of PTTG in two primary pituitary gonadotroph (Luteinizing hormone immunopositive) tumor cultures following vehicle (V) or antiestrogen treatment (AE) (ICI-182780,  $10^{-6}$  M for 48 hours). β-actin immunoblotting confirmed equivalent protein loading (50 µg). Rat testes served as a positive control.

However, because patients usually present with already established and actively growing pituitary tumors, the approach we have taken here, in commencing antiestrogen treatment in animals with already established pituitary tumors, more closely resembles the clinical therapeutic context. Administration of the pure antiestrogen, ICI-182780, suppressed experimental pituitary tumor growth in vivo (~41% decrease) concomitant with reduced PRL secretion (~88% decrease), underpinning the powerful growth-inhibitory effect of ER blockade in lactotroph pituitary tumors. Furthermore, ICI-182780 reduced PTTG expression in human pituitary tumors in vitro, supporting a role for selective antiestrogen treatment in human pituitary tumors. This inhibitory effect was not confined to lactotroph tumors and was seen in various secretory and some non-functioning pituitary tumor subtypes, in keeping with previous studies identifying ER expression in all pituitary tumor subtypes (15, 35–39).

As in other tissues, multiple targets and complex interactions mediate estrogenic proliferative effects (46, 47) and include growth factor induction, direct *cis*-mediated actions on cell-cycle regulatory genes, and inhibition of apoptosis by induction of antiapoptotic proteins, such as Bcl-2 (48). We demonstrated previously concordant pituitary PTTG and bFGF induction in experimental animal and human pituitary tumors (11), and high Bcl-2 expression has been described in pituitary tumors (49). Therefore, we examined bFGF and Bcl-2 expression in the ICI-responsive pituitary tumors. Decreased bFGF and Bcl-2 expression was observed in two of these four tumors, providing a potential mechanism for antiestrogen-mediated pituitary tumor growth inhibition.

Although high-dose estrogen administration to ovariectomized female rats leads to increased pituitary PTTG- and PRL-mRNA expression, we did not observe a concomitant change in pituitary PRL mRNA expression during the rat estrus cycle. One possible explanation for this is that pituitary PRL mRNA expression during the rat estrus cycle exhibits an episodic temporal expression pattern. The first peak is noted at approximately 1400 hours on proestrus day, the second at approximately 0400 hours on estrus, and the third at approximately 2200 hours on estrus (50). Because we euthanized animals at 1400 hours on the day of estrus, the rapid transitory peak in pituitary PRL mRNA expression may have been missed.

Catechol-estrogen formation by 2-hydroxylation is a major pathway of estrogen metabolism in the pituitary (51, 52). 2-OHE<sub>2</sub> combines dose-related estrogen agonistic and antagonistic properties, whereas 2-OHE<sub>1</sub> has predominantly antagonistic activities (14). 2-OHE<sub>2</sub> treatment caused an approximately threefold increase in pituitary pttg mRNA. Estrogen 2-hydroxylase activity increases dramatically and specifically in rat brain during proestrus (51, 53, 54), supporting our hypothesis that 2-OHE<sub>2</sub> mediates increased pituitary PTTG expression in the estrus cycle. Furthermore, our

results, showing abrogation of 2-OHE<sub>2</sub>-mediated PTTG induction with ICI-182780, support previous studies indicating that catechol-estrogen actions are mediated via the ER (15).

The regulation of pituitary PRL mRNA expression during the rat estrus cycle is complex and involves direct effects of estrogen and catechol metabolites, on PRL transcription, indirect activation of transcription followed by rapid and abundant PRL secretion, and mammotrope proliferation, respectively (50). Estrogen regulates pituitary PTTG mRNA expression, and we showed previously that PTTG overexpression leads to increased bFGF expression and that bFGF stimulates PTTG mRNA expression. bFGF treatment increases PRL transcription and secretion in vitro (55), and targeted bFGF overexpression induces lactotroph hyperplasia (56). These studies indicate that increased pituitary bFGF expression precedes increased pituitary PTTG mRNA expression, which in turn will lead to further increases in pituitary bFGF. It is possible that estrogen-mediated increases in PTTG and bFGF cooperatively serve to coordinate the complex temporal secretion of pituitary PRL.

The results show here that pituitary PTTG and bFGF are cyclically regulated by estrogen, peaking concomitantly with increased lactotroph proliferation during estrus. As a securin protein, PTTG expression alters during the normal cell-cycle, peaking at G2/M before mitosis. It is then rapidly degraded, because sustained PTTG expression dysregulates sister chromatid separation and results in cell aneuploidy (13). Failure to degrade PTTG in a timely manner may render pituitary cells susceptible to aneuploidy and subsequent transformation. We hypothesize that estrogen-mediated amplified and/or sustained pituitary PTTG expression, during normal physiological cyclical pituitary proliferation, predisposes the lactotroph to transformation and provides novel insight into factors promoting pituitary lactotroph tumor development.

The lack of adequate longer-term functional differentiated human pituitary tumor culture material makes comprehensive analysis in individual cases difficult, and much insight into pituitary tumor pathogenesis derives from in vitro and/or in vivo animal studies. It is not possible from this study to confirm that the ICI-182780-mediated tumor inhibition observed in animal models would occur in human pituitary tumors, but the observed decrease in human pituitary tumor PTTG expression by the antiestrogen supports the in vivo animal studies, showing antiestrogen-mediated inhibition of pituitary tumor proliferation. Therefore, given that most pituitary tumor subtypes express ER, the results support the use of selective antiestrogen management of pituitary tumors.

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