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Strain Difference in Regulation of Pituitary Tumor Transforming Gene (*PTTG*) in Estrogen-induced Pituitary Tumorigenesis in Rats

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Recently a novel oncogene, *PTTG* (pituitary tumor transforming gene) was isolated from a rat pituitary tumor cell line whose expression is apparently correlated with pituitary tumorigenesis. In the rat, estradiol (E_2) is known to induce anterior pituitary hyperplasia. The effects of E_2, however, vary greatly among rat strains. Therefore we examined the expression of *PTTG* and its regulation by E_2 in F344, Wistar, Brown-Norway and Donryu rats. Four-week-old females were ovariectomized and a pellet containing 10 mg of E_2 was given s.c. Total RNA was isolated from the pituitary gland and *PTTG* mRNA was measured with a competitive RT-PCR technique. The F344 strain was the most susceptible to E_2 induction of pituitary tumorigenesis, followed by Wistar and Brown-Norway, while no increase in pituitary weight was noted in Donryu rats. *PTTG* mRNA in the gland was induced by E_2 within 48–72 h in F344 and Wistar, but not in Brown-Norway or Donryu strains. These data suggest that *PTTG* expression may at least in part be responsible for strain differences in E_2-induced pituitary tumorigenesis.

Key words: Pituitary tumorigenesis — *PTTG* — Estrogen regulation — Strain difference — Competitive RT-PCR

The oncogene *PTTG* (pituitary tumor transforming gene) was first isolated from rat GH (growth hormone) tumor cell lines by differential mRNA display.[1)] Induction of *PTTG* alone results in transformation in NIH 3T3 cells, which become tumorigenic *in vivo*. Human *PTTG* was found to be expressed at only low levels in normal pituitary tissues but highly in secreting and non-secreting pituitary tumors as well as in a variety of tumor cell lines, including lung carcinoma, melanoma, leukemia, lymphoma and HeLa examples.[2–4)] More recent investigations have revealed that the gene is identical to securin, a sister chromatid separation inhibitor, whose overexpression may have an impact on cancer development by increasing genetic instability.[5)]

Chronic treatment of rats with estradiol (E_2) is known to result in the development of anterior pituitary tumors, which are initially benign masses and hormone-dependent, but may subsequently become autonomous. A study with F344 rats showed that *PTTG* mRNA in the pituitary gland increases in this process and that E_2 may regulate the expression.[6)] However, susceptibility to E_2 induction of pituitary tumors is highly strain-dependent.[7, 8)] The F344 strain is the most widely studied and the most sensitive to estrogen. Other rat strains including ACI, Wistar-Furth and Copenhagen have been reported to display estrogen-dependent growth and tumor induction.[9–11)] On the other hand, the Brown-Norway, Holtzman and Sprague Dawley strains appear to be insensitive or resistant.[7, 12)] When susceptible strains were treated with E_2, the size of the pituitary gland started to increase immediately and steadily expanded without any evident focal growth.[9)] The designation of tumor is given on the basis of the size of the gland and the abnormal appearance of the cells, which are highly correlated.[13)] Thus, the E_2-induced weight increase in the gland well represents the E_2-dependent tumorigenicity. The hypothalamus is not essential for the E_2 stimulation of pituitary growth, based on experiments involving transplantation of the pituitary gland.[14)] Several investigations have indicated the contribution of multiple genetic loci to this susceptibility.[7, 15)] To examine whether expression of *PTTG* and its regulation by E_2 might be involved in E_2-induced pituitary tumorigenesis, strain differences were assessed in the present study.

MATERIALS AND METHODS

Animals Female F344, SD, Donryu and Brown-Norway rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa). They were maintained with free access to basal diet and tap water. All animals were surgically ovariectomized upon receipt and implanted with pellets containing 10 mg of E_2 subcutaneously as described previously.[16)] For the time-course study, F344 rats were treated with E_2 for 4, 8 and 13 weeks in the long term experiment (5 animals, each point), and for 12, 24, 48 and 96 h for the short term (4 animals, each point). For the comparison study among four strains, they were treated with E_2 for 72 h for the short term effects and for 4 weeks for the long term effect (5 animals, each strain and treat-

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ment). Animals were sacrificed under ether anesthesia. The pituitary gland and the uterus of each rat were weighed and immediately frozen in liquid nitrogen and stored at −80°C. Blood samples were collected from the abdominal artery and separated sera were stored at −20°C until assayed. All experiments were conducted under the guidelines of 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University.'

Total RNA extraction and reverse transcription Total RNAs were prepared from pituitary tissues with Isogen (Wakojunyaku, Osaka), a premixed RNA isolation reagent based on the acid guanidium thiocyanate-phenol-chloroform extraction method, following the supplied protocol. Aliquots were treated with 1 U of RQ1 DNase (Promega, Madison, WI) in 20 μl buffer containing 10 U of RNase inhibitor, RNasin (Promega) and 1 μg of RNA was reverse-transcribed with 200 U of MMLV-RT (Life Technologies, Rockville, MD) and 50 ng of oligo-dT primer in 12 μl of buffer containing 1 m*M* dNTP, 100 m*M* Tris HCl (pH 8.3), 150 m*M* KCl, 6 m*M* $MgCl_2$, 60 m*M* dithiothreitol and 5 U/μl RNasin with incubation at 37°C for 60 min.

PCR primers r*PTTG*-f1 and r*PTTG*-r with the sequences 5′-ATGGCTACTCTGATCTTTGT (1−20) and 5′-TTAA-ATATCTGCATCGTAAC (581−600) were employed for r*PTTG* detection by PCR with the expected amplified size of 600 bp. For construction of the competitor, r*PTTG*-f2 and r*PTTG*-f2f1 (r*PTTG*-f2 linking to r*PTTG*-f1) with the sequences of 5′-GTTACTGAAAAGCCAGTGAA (199−218) and 5′-ATGGCTACTCTGATCTTTGTGTTAC-TGAAAAG were used. Primer sequences and conditions for G3PDH (glycerol-3-phosphate dehydrogenase) were chosen according to Weir *et al.*[17] (the expected size of the amplified fragment was 534 bp).

Construction of competitor DNA fragments PCR amplifications were carried out with 20 pmol each of primers in a total volume of 50 μl containing 1.25 U of Ex-*Taq* DNase polymerase (TaKaRa Shuzo Co., Otsu), 0.2 m*M* dNTP and the supplied buffer. Each amplification was performed with 30 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, ending with 72°C for 5 min. PCR products were purified with agarose gel electrophoresis followed by cleaning with a GFX Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). First, a 600 bp fragment was amplified by PCR with r*PTTG*-f1 and r*PTTG*-r from rat pituitary tumor cDNA. The DNA sequence was confirmed with an ABI PRISM 310 capillary sequencer (Applied Biosystems, Foster City, CA). Using this fragment as a template, the next PCR amplification with primers r*PTTG*-f2 and r*PTTG*-r was performed and the resulting 401 bp PCR fragment was gel-purified. Then the third PCR amplification was carried out from the 401 bp fragment with r*PTTG*-f1f2 and r*PTTG*-r to obtain a 421 bp competitor DNA for r*PTTG*.

Competitive PCR Sample cDNA (equivalent to 0.2 μg of total RNA) and various amounts (0−15 fg) of competitor DNA were co-amplified by PCR with Ex-*Taq* using the r*PTTG*-f1 and r*PTTG*-r primer set. The amplification conditions were as described above in a final volume of 20 μl. Each PCR product was electrophoretically separated on a 1.5% agarose gel containing ethidium bromide at 0.2 μg/ml. The image was digitized with a video capturing device, PrintGraph (Atto Co., Tokyo) and intensities of the blots were quantified using Scion Image software (Scion Corp., Frederick, MD). The log ratio of the blot intensities of sample cDNA over the competitor in each lane was plotted against amounts of the competitor. Quantity of r*PTTG* mRNAs was determined where the ratio was equal to 1.[18]

Prolactin (PRL) radioimmunoassay Serum PRL was measured by radioimmunoassay with NIADDK reagents following the recommended protocol, the details of which have been reported previously.[19]

E_2 radioimmunoassay Serum E_2 was measured with a radioimmunoassay kit for rat serum E_2, purchased from Immunotech, Inc. (Marseille, Cedex, France).

Statistical analysis Statistical comparisons were made using Student's *t* test.

RESULTS

Increase in pituitary weight and *PTTG* mRNA expression due to E_2 in ovariectomized female F344 rats Significant increases in pituitary weight were noted one week after the implantation of a high-dose E_2 pellet. The increase continued linearly for 13 weeks. RT-PCR detection of *PTTG* mRNA indicated elevated expression of the gene in the pituitary tumor induced by E_2 (Fig. 1).

Strain differences in E_2-induced pituitary weight increase Absolute weights of the pituitary gland varied among the four different strains. In 5-week-old control groups, the smallest size of 4.4±0.2 mg was noted in Brown-Norway rats, while the average size was 13.0 mg in the Wistar strain. Four-week treatment with E_2 increased pituitary weights in a strain-dependent manner (Table I). The pituitary glands of F344 rats showed the highest sensitivity to E_2 in terms of weight increase. On the other hand, no weight increase in the gland was noted in Donryu rats. There were no differences in serum E_2 levels among the four strains of estrogenized rats, while the hormone was undetectable in ovariectomized animals.

***PTTG* mRNA levels in different strains of estrogenized rats** Data for *PTTG* mRNA levels quantified by competitive RT-PCR in ovariectomized and estrogenized rats of the four different strains are summarized in Table II. The *PTTG* levels in ovariectomized rats varied among the strains. When the rats were treated with E_2 for 4 weeks, the level was significantly increased only in F344 rats.

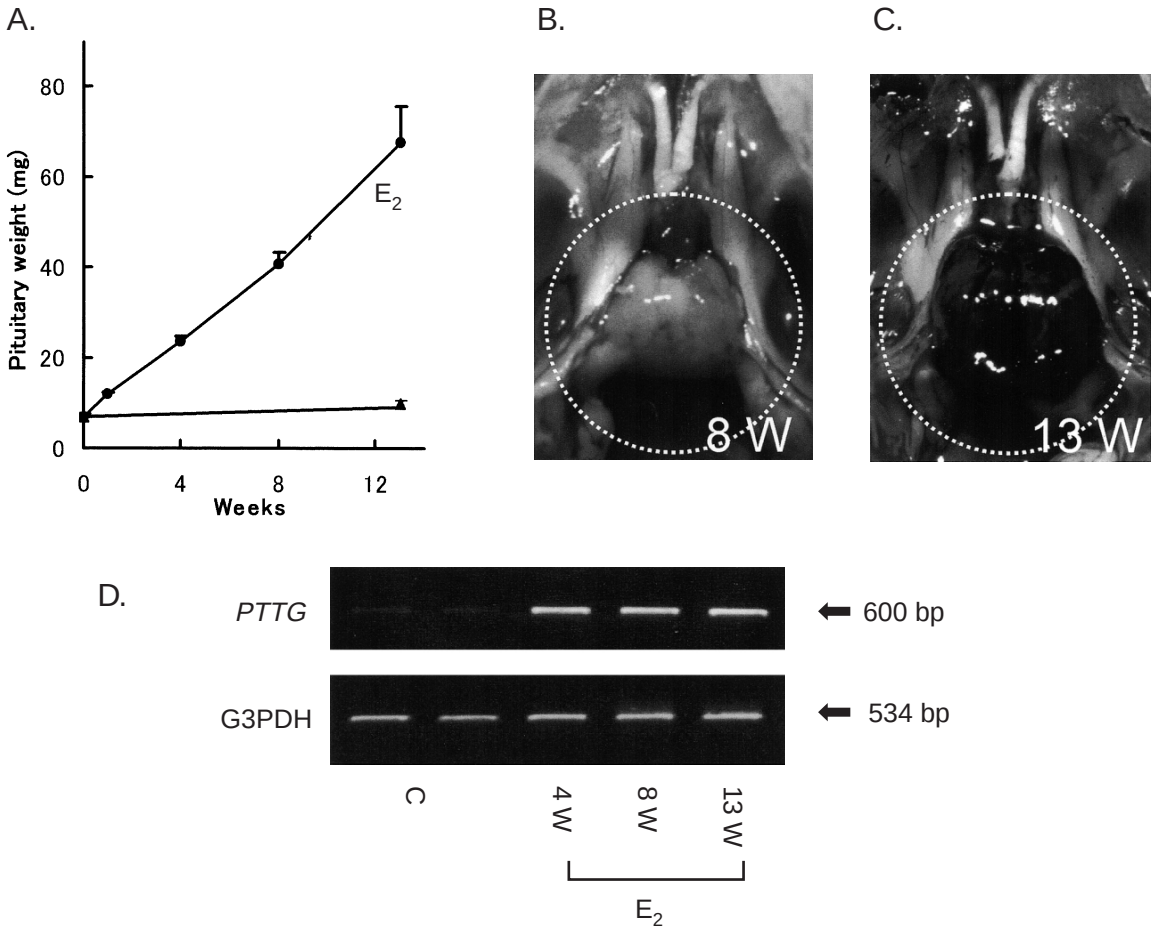

Fig. 1. Induction of pituitary tumors in ovariectomized female F344 rats treated with a 10 mg E_2 pellet. A. Increase in pituitary weights (n=5, each point). B, C. E_2 induced pituitary tumors at weeks 8 and 13. D. Expression of *PTTG* mRNA in the pituitary gland, detected by semi-quantitative RT-PCR.

Table I. Increase in Pituitary Weight by 4 Weeks of E_2 Treatment in Different Strains of Rats

Strain		Body weight (g)	Pituitary weight (mg)	Increase (fold)
F344	Ov[a)]	145±4.3[b)]	7.5±0.1	
	Ov+E_2	128±11.8	23.7±1.1**	3.16
Wistar	Ov	208±4.3	13.0±2.1	
	Ov+E_2	192±11.8	28.7±3.2**	2.21
Brown-Norway	Ov	146±3.2	4.4±0.2	
	Ov+E_2	124±0.1	6.8±0.6**	1.55
Donryu	Ov	215±9.0	10.7±1.0	
	Ov+E_2	166±5.8	11.2±1.0	1.05

a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E_2 was given when they were 5 weeks old.
b) Mean±SEM (n=5).
** Significant difference from the respective ovariectomized group value (P<0.01).

Early increase in *PTTG* mRNA in the pituitary gland in estrogenized F344 rats Semi-quantitative RT-PCR indicated *PTTG* mRNA to be significantly increased 48 h after implantation of a pellet containing 10 mg of E_2 (Fig. 2). Measurement confirmed rapid increase in serum E_2 despite the s.c. administration route as a pellet.

Early changes in uterus weights and serum PRL levels in response to E_2 Significant increases in uterus weights were noted in estrogenized groups of all of the strains at 72 h after E_2 administration. PRL levels, however, differed among strains, increasing sharply in the F344 and Wistar cases, but not significantly changing in Donryu rats (Table III).

Early changes in *PTTG* mRNA due to E_2 in the four strains of rats Data for *PTTG* mRNA levels in the different strains of rat treated with E_2 in pellets for three days, quantified by the competitive RT-PCR technique, are sum-

marized in Table IV. Significant induction of mRNA of *PTTG* was noted in F344 and Wistar rats.

DISCUSSION

In the rat, chronic treatment with E_2 is known to induce anterior pituitary hyperplasia and results in tumors which are initially benign and hormone-dependent, but may subsequently become autonomous. The effects of the hormone, however, vary greatly among rat strains.[7, 8] Our data clearly demonstrated *PTTG* in the pituitary gland to be regulated by E_2 differently in each stain, with possible involvement in the strain differences in E_2-induced tumorigenesis.

Table II. *PTTG* mRNA Levels in the Pituitary Gland in Different Strains of Rats Treated with E_2 for 4 Weeks

Strain		*PTTG* mRNA (fg/mg total RNA)	Increase (fold)
F344	Ov[a)]	6.5±0.5[b)]	
	Ov+E_2	24±1.1**	3.7
Wistar	Ov	1.8±0.5	
	Ov+E_2	3.0±3.2	1.7
Brown-Norway	Ov	5.9±0.9	
	Ov+E_2	6.5±0.6	1.1
Donryu	Ov	2.7±0.9	
	Ov+E_2	3.0±1.0	1.1

a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E_2 was given when they were 5 weeks old.
b) Mean±SEM (n=5).
** Significant difference from the respective ovariectomized group value (P<0.01).

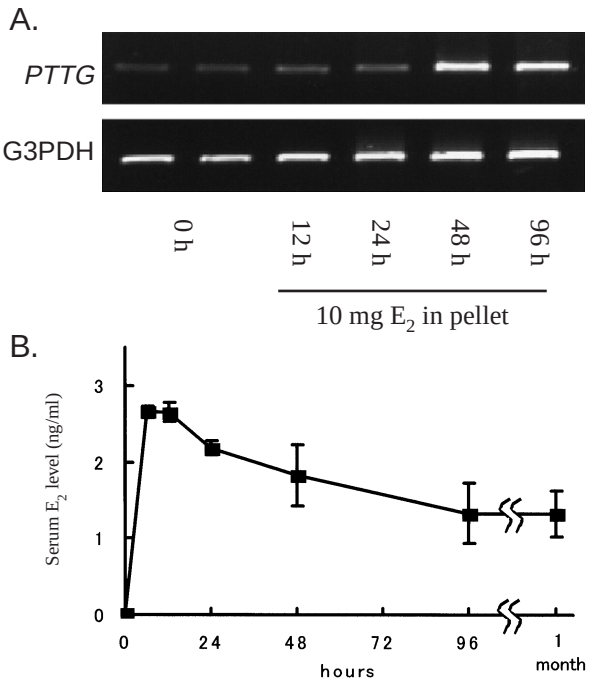

Fig. 2. Expression of *PTTG* mRNA in the pituitary gland (A) in ovariectomized female F344 rats treated with E_2 for various time periods and their serum E_2 levels (B, n=4, each point).

Table III. Body, Pituitary, Uterus Weights and Serum E_2 and PRL Levels in Different Strains of Rats Treated with E_2 for 72 h

Strain		Body weight (g)	Pituitary weight (mg)	Uterus weight (mg)	Serum E_2 (ng/ml)	Serum PRL (ng/ml)
F344	Ov[a)]	129±4.2[b)]	7.9±1.4	30±3.8	n.d.[c)]	23±2.5
	Ov+E_2	100±4.2**	8.7±1.6	394±99.0	885±351**	179±51.0*
Wistar	Ov	212±5.2	10.8±0.6	73±12.9	n.d.	59±15.0
	Ov+E_2	145±9.3**	12.4±1.2	361±16.7**	634±11.1*	227±96.1*
Brown-Norway	Ov	135±0.9	6.6±0.7	47±2.3	n.d.	21±2.0
	Ov+E_2	100±3.4**	5.8±0.3	617±209.0	1005±204**	90±21.5*
Donryu	Ov	187±1.7	8.9±0.3	81±3.5	n.d.	33±3.5
	Ov+E_2	157±1.1**	10.4±0.6	1125±26.7**	576±106*	36±8.4

a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E_2 was given when they were 5 weeks old.
b) Mean±SEM (n=5).
c) n.d.: not detected.
*, ** Significant difference from the respective ovariectomized group value (* P<0.05, ** P<0.01).

Table IV. Induction of *PTTG* mRNA in the Pituitary Glands of Different Strains of Rats Treated with E_2 for 72 h

Strain		*PTTG* mRNA (fg/mg total RNA)	Increase (fold)
F344	Ov[a)]	8.4±1.0[b)]	
	Ov+E_2	24±4.2*	2.9
Wistar	Ov	3.6±1.0	
	Ov+E_2	12.8±2.2*	3.6
Brown-Norway	Ov	11.8±1.8	
	Ov+E_2	15.8±1.8	1.3
Donryu	Ov	5.4±1.8	
	Ov+E_2	6.0±2.0	1.1

a) Animals were ovariectomized at 4 weeks of age and a pellet containing 10 mg of E_2 was given when they were 5 weeks old.
b) Mean±SEM (n=5).
* Significant difference from the respective ovariectomized group value (P<0.01).

PTTG was first isolated in rat growth hormone-secreting pituitary cell lines by the mRNA differential display method.[1, 3)] Two pituitary tumor-specific mRNAs were found to be overexpressed; one was the insulin-induced growth response protein and the other was the new gene, *PTTG*, with a cDNA encoding a 199-amino-acid, 25-kDa protein. Northern analysis in normal adult rats indicated that only testis contained significant amounts of *PTTG* mRNA, which was a truncated mRNA of approximately 1 kb in contrast to the 1.3 kb mRNA expressed in pituitary tumor cells. When a fibroblast cell line, NIH3T3, was transfected with a *PTTG* expression vector, it exhibited colony formation, anchorage-independent growth in soft agar and development of tumors in nude mice. Subsequent investigations revealed the human homologue of *PTTG* to exist as a 202-amino-acid protein.[2)] Human *PTTG* was found to be highly expressed in leukemias, lung carcinomas, lymphomas and HeLa cell lines as well as in pituitary adenomas. The protein contains both proline and basic amino acid-rich domains potentially involved in cancer-related signal transduction, and has been reported to be identical to a vertebrate sister-chromatid separation inhibitor (serurin), whose increased expression may result in genetic instability with chromosome gain or loss.[5)]

When pituitary tumors are induced by E_2, clear transitions between hyperplasia, adenoma and carcinoma are not apparent despite differences in cellular atypia. The size of the pituitary gland steadily increases without any evident focal growth. It is typical for pituitary adenoma to be detected as a hemorrhagic tumor, an example being shown in Fig. 1. The designation of tumor is given on the basis of the size of the gland and the abnormal appearance of the cells. Since the two are highly correlated,[13)] E_2-induced weight increase in the pituitary gland was taken to represent E_2-responsive tumorigenicity in the present study. Satoh *et al.*[20)] demonstrated pituitary glands to develop adenomas in all F344 rats treated with E_2 for only 7 weeks, and the adenomas progressed to carcinomas featuring local invasion or metastasis within 13 weeks after the start of hormone exposure.

Susceptibility to E_2-induced pituitary tumor is highly strain-dependent.[7)] We used four different strains which are commercially available. F344 is known to be particularly susceptible, while Donryu rats are very resistant as far as one month of E_2 treatment is concerned. Interestingly, the normal sizes of the pituitary also differ among strains, being considerably smaller in the Brown-Norway case. The determined basal levels of *PTTG* mRNA also varied among strains, although they did not correlate with the normal pituitary weights.

Heaney *et al.*[6)] reported that *PTTG* mRNA in the pituitary gland increases early after E_2 administration in F344 rats, in accordance with our data. While a period of time necessary to induce *PTTG* was within 24–48 h here, being slightly longer than the 12–24 h of the previous report, the increase in *PTTG* was definitely prior to development of pituitary hyperplasia. Whatever the interval for initiating the transcription was, the gene might not be under direct control of E_2. We compared *PTTG* induction by E_2 after 72 h in the different strains, this time point being chosen on the basis of our time-course study, and demonstrated the initial rise to be dependent on strain and correlated with susceptibility to pituitary tumorigenesis. It is well known that E_2 stimulates PRL synthesis predominantly through activation of transcription.[21–23)] Serum PRL levels became extremely high in both F344 and Wistar rats when they were treated with E_2 for 72 h, while, interestingly, only a moderate increase was noted in the Brown-Norway strain and no induction in Donryu rats. However, the classical investigation by Wiklund and Gorski,[24)] who compared pituitary tumor susceptibility between F344 and Holtzman strains, indicated that the lack of tumor formation in the Holtzman rat pituitary is not due to a general unresponsiveness to E_2. They found increased DNA synthesis during the first 2–4 days of E_2 treatment and then a return to normal afterwards. On the other hand, elevation of PRL was seen as a primary response to E_2 which was sustained on prolonged E_2 treatment. This seems not to be the case in the Donryu strain which showed virtually no elevation in serum PRL level at 72 h after estrogenization in the present study. The comparison between ACI and SD rats by Stone *et al.*,[12)] however, gave findings consistent with our results, PRL induction by E_2 in ACI being much higher than that in SD rats on day 2 of diethylstilbestrol (DES) treatment. It should be pointed out that the so-called general responsiveness to E_2 in the pituitary gland has not been quantitatively assessed in a thorough manner. Serum E_2 levels were high in all four strains in the present

study, indicating no difference in the E_2 metabolism. The genomic structure of rat *PTTG* has been characterized and at least a 745 bp 5′ flanking sequence was revealed to be required for transcriptional activation.[25] Transfection experiments with a rat *PTTG*-5′-flanking luciferase reporter in a rat pituitary cell line, GH3, showed that E_2 could significantly induce transcription through the promoter, albeit the effect was very weak.[6] Since there is no evidence to indicate a direct relationship between promoter sequence and E_2 receptors, activation by E_2 may be indirect, although the response clearly requires E_2 receptors according to our recent results (unpublished). Although evidence for involvement of *PTTG* in pituitary tumorigenesis appears to be convincing, other genes may also contribute to strain differences. Ying[26] found that p53 and rb mRNAs were increased in response to DES treatment in SD, but not in F344 rats. C-fos has also been reported to be regulated by E_2 in a specific manner in the pituitary gland of F344 rats.[27]

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