Development of cystic glandular hyperplasia of the endometrium in Mullerian inhibitory substance type II receptor–pituitary tumor transforming gene transgenic mice

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Abstract

The pituitary tumor transforming gene (*PTTG*)/securin is an oncogene that is involved in cell cycle regulation and sister chromatid separation. *PTTG* is highly expressed in various tumors including ovarian tumors, suggesting that *PTTG* may play a role in ovarian tumorigenesis. Overexpression of *PTTG* resulted in induction of cellular transformation *in vitro* and tumor formation in nude mice. To ascertain *PTTG* function in ovarian tumorigenesis, we generated a transgenic mouse model of *PTTG* by cloning *PTTG* cDNA downstream of Mullerian inhibitory substance type II receptor gene promoter (*MISIIR*) in order to target the ovarian surface epithelium. By screening of transgenic animals, we identified five founders (four males and one female). Using the four male founders, we developed four transgenic lines. *PTTG* expression was increased in ovarian surface epithelium,

ovarian granulosa cells, as well as in the pituitary gland. Transgenic females did not develop any visible ovarian tumors at 8–10 months of age; however, there was an overall increase in the corpus luteum mass in transgenic ovary, suggesting increased luteinization. These changes were associated with an increase in serum LH and testosterone levels. In addition, there was a generalized hypertrophy of the myometrium of *MISIIR*–*PTTG* transgenic uteri with cystic glandular and hyperplasia of the endometrium. Based on these results, we conclude that the overexpression of *PTTG* may be required to initiate precancerous conditions but is not sufficient to induce ovarian tumorigenesis and may require another partner to initiate cellular transformation.

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Introduction

Even though ovarian cancer ranks fifth in prevalence among gynecological malignancies, it is the second deadliest (Auersperg *et al.* 2002). The primary reason for this fatalistic outcome is the absence of symptoms in the early stage of the disease. Unlike colon and cervical cancer, ovarian cancer has no identifiable precancerous lesions that can be used for screening (Zhang *et al.* 2004). In fact, screening by transvaginal ultrasonography and RIA for CA-125 (Hamilton *et al.* 2003, Hellstrom *et al.* 2003) show low specificity and sensitivity that make the two methods unreliable for detecting the initial stages of the disease. Consequently, the 5-year survival rate is <30% (Schwartz *et al.* 2002). The histological classification of ovarian cancer is complex. Common ovarian tumors include epithelial, germ cell, and non-stromal sex cord. Among these ovarian cancer subtypes, epithelial ovarian cancer is the most fatal and the most common, accounting for 90% of all ovarian cancers (Vanderhyden *et al.* 2003). In order

to understand the etiology of ovarian cancer, several animal models have been developed (Connolly *et al.* 2003, Garson *et al.* 2003, Liu *et al.* 2004); however, none of these was specifically targeted to epithelial cells. The major impediment for the development of mouse model of ovarian epithelial cancer is the lack of a specific promoter and oncogene that can direct gene expression to epithelial cells that lead to transformation of these cells. Recently, Connolly *et al.* (2003) used 5' upstream region of the Mullerian inhibitory substance type II receptor (*MISIIR*) gene to derive tissue-specific expression of SV40 tag specifically to the epithelium of the female reproductive tract, including the ovarian surface epithelium (OSE). In their studies, 50% of female transgenic mice developed bilateral ovarian tumors accompanied by ascites and peritoneal implants. Female *MISIIR* tag transgenic mice rapidly develop tumors and are nearly always infertile and die at an early age. Therefore, this ovarian tumor model has limitation in understanding the biological basis of ovarian cancer initiation and progression.

To understand the human oncogenesis, we searched for oncogenes and cloned and characterized pituitary tumor transforming gene (*PTTG*) from human testis (Kakar & Jennes 1999). Our initial studies were based on the work of Pei & Melmed (1997), who cloned PTTG from rat pituitary tumor cell line GH4. Cloning of PTTG from various ovarian tumors revealed sequence identity with that from testis, suggesting no mutation in PTTG sequence in ovarian tumors (Puri *et al.* 2001). PTTG was found to be multidomain and multifunctional protein. One of its major functions reported is the inhibition of sister chromatid separation during cell cycle (Zou *et al.* 1999). The binding of PTTG to separase blocks sister chromatid separation until metaphase, where PTTG becomes target for ubiquitination by anaphase promoting complex (APC), thus releasing separase. The release of separase allows sister chromatid separation. Expression of PTTG is cell cycle dependent; it peaks at the G2/M phase and undergoes complete degradation at anaphase (Ramos-Morales *et al.* 2000).

Numerous studies have demonstrated that human PTTG displays distinct pattern of expression. In normal human tissues, PTTG expression is very restricted, with high levels in the testis and low levels in the thymus, colon, and small intestine (Pei & Melmed 1997, Zhang *et al.* 1999). In contrast, PTTG is highly expressed in a variety of human primary tumors as well as tumor cell lines including carcinomas of the ovary, lung, testis, kidney, colon, thyroid, pituitary, liver, breast, prostate, melanoma, leukemia, and lymphoma (Zhang *et al.* 1999, Puri *et al.* 2001, Yu *et al.* 2003, Kim *et al.* 2005, Rehfeld *et al.* 2006), suggesting that PTTG may be involved in tumorigenesis of many organs. Furthermore, the expression levels of PTTG correlate with increased tumor invasiveness in human pituitary tumors with hormone overproduction (Saez *et al.* 1999) and degree of malignancy, pathogenesis and/or progression of colorectal, breast, and thyroid tumors (Heaney *et al.* 2000, Solbach *et al.* 2004, Kim *et al.* 2006). PTTG has been identified as one of the signature genes among eight genes associated with tumor metastasis that is up-regulated in human primary solid tumors (Ramaswamy *et al.* 2003). A relationship between the survival rate and the level of expression of PTTG in esophageal cancer has been reported (Shibata *et al.* 2002).

Overexpression of PTTG in NIH3T3 and HEK293 cells was found to increase cell proliferation and colony formation in soft agar and to promote tumor development in nude mice (Kakar & Jennes 1999, Zhang *et al.* 1999, Hamid *et al.* 2005). In addition, PTTG overexpression was found to increase the secretion and the expression of basic fibroblast growth factor (bFGF; Ishikawa *et al.* 2001, Hamid *et al.* 2005), vascular endothelial growth factor (VEGF; McCabe *et al.* 2002, Hamid *et al.* 2005), interleukin-8 (IL-8; Hamid *et al.* 2005), MMP-2 (Malik & Kakar 2006) and to activate the expression of c-myc (Pei 2001). To define the role of PTTG in cell proliferation and tumorigenesis, Melmed and his group showed that mice lacking PTTG (*PTTG*−/−) displayed aberrant cell cycle progression, premature sister chromatid separation, and chromosomal instability (Wang *et al.* 2001).

Furthermore, male *PTTG*−/− mice developed testicular and splenic hypoplasia and thymic hyperplasia. In subsequent studies, these investigators showed that *PTTG*−/− mice exhibited impaired β -cell proliferation and developed type I diabetes (Wang *et al.* 2003). While 80% of Rb heterozygous mice develop pituitary tumors, crossing of *PTTG* (−/−) mice with Rb (−/+) decreased the prevalence of pituitary tumor development to 30% (Chesnokova *et al.* 2005), suggesting a critical role of PTTG in pituitary tumorigenesis. This hypothesis is further supported by Abbud *et al.* (2005) who, using the α -GSU gene promoter to target PTTG expression to the pituitary gland, showed development of pituitary adenomas, prostate hyperplasia, and an increase in serum luteinizing hormone (LH) and testosterone levels in α -GSU-PTTG male transgenic mice. Taken together, these data strongly suggest that PTTG plays crucial role in tumorigenesis. In order to elucidate its function in ovarian epithelial cancer, we directed PTTG expression to ovarian surface epithelium using the *MISIIR* gene promoter. Our data shows that PTTG is capable of inducing initial cellular transformation; however, it is not sufficient for complete tumor development and may require another partner to achieve ovarian tumorigenesis.

Materials and Methods

Construction of *MISIIR*–*PTTG* transgene plasmid

PTTG cDNA was amplified using the specific primers sense 5′-TCG AAT TCG ACC TGC AAT AAT CCA GAA T-3′ and antisense 5′-TCA GAATTC CAC CAA ACT CTG AAG CAT T-3′ from human testis as described previously (Kakar & Jennes 1999). The amplified product was then digested with *Eco*R1 and subcloned into pBluescript plasmid containing insulin promoter and β -globin gene (Generous gift from Dr Paul Epstein, University of Louisville). The insulin promoter was replaced by *MISIIR* gene promoter (kindly provided by Dr Denise Connolly, Fox Chase Cancer Center, Philadelphia, PA, USA). The recombinant clones were isolated and sequenced to confirm the authenticity of the sequence and orientation. Activation of the *MISIIR* promoter to derive the expression of *PTTG* gene in epithelial tumor cells was confirmed by transfecting the *MISIIR*–*PTTG* plasmid into ovarian epithelial tumor cell line A2780 as described previously (Kakar & Jennes 1999).

Generation and screening of transgenic mice

Generation of transgenic mice was achieved by injection of *MISIIR*–*PTTG* cDNA into pronuclei injection. Briefly, transgene (*MISIIR*–*PTTG*) was excised using *Bam*HI and *Xho*I restriction enzymes from the recombinant plasmid, purified, and injected into male pronuclei from hybrid CD2F1 (DBA/2XBalb/C) mice. Injection of the transgene was carried out by the Transgenic Core Facility, University of

Louisville, supervised by Dr Paul Epstein. Viable oocytes were then transferred into CD2F1 pseudo pregnant females. Genotyping of the transgene was performed using tail DNA using PCR kit from Sigma using the specific primers: sense 5'-CCC TTT CTC TGC CTG TTT CC-3' (A) and antisense 5'-GCT TTA ACA GTC TTC TCA GT-3' (B) (Fig. 1) that specifically amplify transgene sequence. The PCR conditions were 94 °C for 5 min, 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and repeated for five cycles followed by 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles.

Animal housing

Animals were housed in a conventional facility with a 12 h light:12 h darkness cycle. All animals were treated in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by Animal Use and Care Committee at the University of Louisville.

Southern blot hybridization

Southern blot hybridization was carried out to confirm the positive founders. Livers from transgenic F1 population were digested overnight at 55 °C in TNES buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS) containing proteinase K (10 mg/ml). After incubation, sodium chloride was added to each sample to the final concentration of 6 M followed by vigorous mixing for 15 s. Samples were centrifuged at 12 000–14 000 *g* for 5 min at room temperature. Supernatants were transferred to a new tubes and one volume of cold 95% ethanol was added to each sample. Precipitated DNA was spooled and washed with 70% ethanol and allowed to air dry for 5–10 min at room temperature. The DNA pellet was resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). For negative control, we used mouse genomic DNA (Promega). For Southern blot hybridization, 10 µg genomic DNA was digested overnight by EcoRI restriction enzyme and separated on a 0.8% agarose gel. The gel was denatured for 45 min (0.5 M NaOH, 1.5 M NaCl), neutralized for 30 min (0.5 M Tris, 1.5 M NaCl, pH 7.5),

and then transferred to Nytron membrane (GE HealthCare Biosciences, Piscataway, NJ, USA) overnight in 20× SSC buffer. PTTG labeled probe was generated by restriction of PTTG cDNA from pcDNA-PTTG plasmid (Kakar & Jennes 1999) using EcoRI and BamHI restriction enzymes, followed by labeling with [α -³²P]dCTP using random hexamer labeling kit (Promega). Membranes were u.v. cross-linked and prehybridized for 30 min with ExpressHyb solution (Clontech Laboratories) followed by incubation with hybridization solution containing the labeled probe for 1 h at 65 °C. Membranes were washed for 15 min with 2× SSC/0.1% SDS thrice at room temperature followed by one wash in 0.2× SSC/0.1% SDS at 55 °C for 20 min. Membranes were dried and exposed to X-ray film overnight at –80 °C.

RNA isolation and analysis of gene expression by RT-PCR

To verify the expression of the PTTG in mouse tissues, total RNA from ovary, uterus, and testis from transgenic and non-wild-type mice were isolated as described previously (Kakar & Jennes 1999) using Trizol reagent. One microgram of RNA was used to synthesize cDNA using iScript cDNA synthesis Kit (Bio-Rad Laboratories). For PCR amplification, 2 µl first-strand cDNA were used as a template in a 50 µl PCR containing 5 µl 10× PCR buffer, 4 µl dNTP (2.5 mM each of dATG, dCTP, dGTP, and dTTP), 0.25 µl EX Taq polymerase (Takara), and 200 nM of each primer. The primers used were: sense 5'-TGG ATC CTG AGA ACT TCA GGG T-3' (C) and antisense 5'-GCT TTA ACA GTC TTC TCA GT-3' (B) (Fig. 1). For control, GAPDH primers used were: forward 5'-TGA TGA CAT CAA GAA GGT GGT-3' and GAPDH reverse 5'-TCC TTG GAG GCC ATG TGG GCC-3'. PCR conditions were: initial denaturing at 95 °C for 5 min, 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s for 30 cycles with final extension at 72 °C for 7 min. To determine the expression of PTTG mRNA in human ovarian tumor cell line (A2780) and mouse pituitary gonadotrope cell line (LbT2), we used specific primers in PCR for human PTTG forward primer 5'-GACCTGCAATAATCCAGAAT-3' and reverse primer 5'-GCTTTAACAGTCTTCTCAGT-3'; mouse PTTG forward primer 5'-GGCATCTAAGGATGGGTGA-3' and reverse primer 5'-TTCGGCAACACTCTGTTGACTG-3'.

Figure 1 Generation of MISIIR-PTTG transgene. Transgenic construct used in the generation of the MISIIR-PTTG transgenic mice. PTTG cDNA was cloned within β -globin gene downstream of MISIIR gene promoter.

The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

Histological analyses and immunohistochemistry

For histopathological analysis, tissues were fixed in 10% buffered formalin overnight and embedded in paraffin using standard technique. Five micrometer paraffin sections were cut and stained with H&E by the Pathology Core Research Laboratory, University of Louisville and evaluated by human and animal pathologists (Dr Martin and Dr Proctor respectively). For immunohistochemical analysis, sections were deparaffinized in xylene and dehydrated in a graded series of alcohol. Antigen retrieval was conducted by boiling the slides in 10 mM sodium citrate (pH 6.0) at 95 °C for 30 min and cooled to room temperature over a period of 30 min. Intrinsic peroxidase activity was blocked by incubation with 0.3% H₂O₂ for 10 min. Non specific binding sites were blocked with goat normal serum (1.5%) for 30 min followed by incubation with PTTG antiserum or PCNA monoclonal antibody clone Ab-16 (NeoMarkers) overnight in a humidified chamber at 4 °C. Dilutions of PTTG antiserum and PCNA (mouse monoclonal) antibody were 1:1500 and 1:200 respectively. For PTTG protein detection, slides were washed with PBS and then treated with biotin-labeled secondary antibody (Vector laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Slides were stained with 3,3'-diaminobenzidine (Sigma), washed with PBS, counterstained with hematoxylin, dehydrated, treated with xylene, and mounted. Detection of proliferating cell nuclear antigen (PCNA) was accomplished by using the mouse on mouse peroxidase kit (Vector laboratories Inc.) according to the manufacturer's instructions. Primary antibody was eliminated and goat or mouse serum was used for negative controls and analyzed in parallel. Immunohistochemical images were acquired on Nikon Eclipse E400 and ACT-1.1 imaging software (Huntley, IL, USA). Measurement of the ovary sections and quantification of immunostaining of PTTG expression in the ovarian surface epithelium were performed using Metamorph Imaging Software (version 6.2 Universal Imaging, Downingtown, PA, USA).

Western blot analysis

Western blot analysis for PTTG protein was performed as described previously (Kakar *et al.* 2001). A2780 and LβT2 cell lines were cultured in complete growth media to 80–90% confluence. Cells were washed and lysed with lysis buffer (10 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA). Protein concentration was assayed by Bradford method (Bio-Rad Laboratories). Forty micrograms of protein from each sample were separated on a 15% SDS polyacrylamide gel and transferred to nitrocellulose membranes (GE Healthcare Biosciences). Membrane was blocked overnight with 5% non-fat milk/Tween (20 mM Tris–HCl, 150 mM NaCl, 0.1%

Tween-20, pH 7.6; tris buffered saline with Tween (TBST) and was incubated with human PTTG antiserum diluted at 1:1500, 1: 3000, and 1: 5000 for 1 h at room temperature followed by washing thrice with TBST and incubation with horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody diluted at 1:5000. The immune complexes formed were detected by enhanced chemiluminescence (ECL) reagents (GE HealthCare Biosciences).

Measurements of serum hormone levels

Transgenic and wild-type mice were killed and the blood was collected from the dorsal vein. Serum follicle-stimulating hormone (FSH) and LH levels were measured by Rat FSH and LH enzymeimmunoassay (EIA) kits (GE HealthCare Biosciences) according to the manufacturer's instructions. The sensitivity of the assays was 6.25–400 and 0.4–100 ng/ml for FSH and LH respectively. Estradiol was assayed using Roche E170 immunoassay analyzer (electrochemiluminescent detection) at the University of Louisville Hospital laboratory. Sensitivity of the assay for estradiol (E2) was 5–4300 pg/ml. Progesterone (P4) was assayed using two different systems (Roche E170 immunoassay analyzer and Cayman Chemicals, Ann Arbor, MI, USA, EIA kit). Sensitivity for P4 was 7.8–1000 pg/ml. Testosterone levels were analyzed by EIA kit (Cayman Chemicals). Sensitivity for testosterone assay was 3.4–400 pg/ml. Serum LH levels in males were measured by Rat LH RIA kit (GE Healthcare Biosciences) according to the manufacturer's protocol. All measurements were performed in duplicate except for E2 and for some samples for testosterone due to shortage of sample volume.

Figure 2 Identification of transgenic founders. (A) PCR analysis to identify positive founders. DNA isolated from F0 tails was used for genotyping. A 1.2 Kb product was detected in four males (0313, 0711, 0416, and 1211) and one female (0725). (B) Southern blot analysis was used to confirm the presence of the transgene in the F1 generation. Genomic DNA isolated from liver was digested by EcoRI at 37 °C. The digested product was resolved on a 0.8% agarose gel and transferred to a nylon membrane. ³²P-labeled PTTG cDNA was used as a probe. A 700 bp band was detected in F1 samples from the four founders. Mouse genomic DNA was used as a negative control.

Statistical analysis

SigmaStat for windows was used for Student's *t*-test to determine the level of statistical significance. The level of statistical significance was set at $P < 0.05$.

Results

Generation of *MISIIR-PTTG* transgene mice

To define the role of PTTG in ovarian tumorigenesis and to generate a transgenic model that can be used to study biology of ovarian epithelial cancer, we selected *MISIIR* gene promoter to drive PTTG expression to the surface epithelium. PTTG cDNA was cloned downstream of *MISIIR* gene promoter (Fig. 1). To test the promoter activity to derive the expression of PTTG, we transfected ovarian epithelial carcinoma cells (A2780) with *MISIIR-PTTG* plasmid cDNA. Western blot analysis of the protein prepared from the cells transfected with *MISIIR-PTTG* plasmid showed a high level of expression of PTTG protein when compared with cells transfected with *MISIIR* plasmid (data not shown), indicating the expression of *PTTG* gene under the control of *MISIIR* gene promoter. The transgene construct containing the *MISIIR-PTTG* gene promoter and PTTG cDNA sequence was excised from the vector using *SacI* and *XhoI* restriction endonucleases, purified, and injected into male pronuclei of fertilized oocytes from hybrid CD2F1 (DBA/2Balb/C) mice as described in Materials and Methods.

Screening of 140 F0 by PCR analysis using tail DNA resulted in identification of five positive founders (four males and one female; Fig. 2A). The four male founders were bred with wild-type Friend Virus B-Type (FVB) females to generate four transgenic lines (0313, 0416, 0711, and 1211; Table 1). All transgenic male founders were fertile and produced a comparable size litter to wild-type, except for 1211 founder which showed a comparatively smaller size litter (Table 1). Positive male and female transgenic mice from the same line were bred to produce F2 generation (Table 2). Southern blot hybridization analysis was used to confirm the penetration of transgene to the F1 generation in all transgenic lines. A PCR product of 700 bp was detected in all samples tested, but was absent in the negative control (Fig. 2B), suggesting the presence

Table 2 Breeding summary for F2 generation

	F1 generation	F2 total number of litter	Number of positive litter in F2
Founder			
0313	3 males/5 females	42	34
0711	2 males/3 females	21	16
1211	2 males/2 females	11	06

of transgene in the genome. Transgene female mice from F1 population appeared to be healthy and normal; however, transgenic females from F2 generation showed very aggressive behavior which resulted in the death of some mice, and in many cases, mice had to be separated and maintained in separate cages. The reasons of such aggressive behavior remain undetermined.

PTTG expression in *MISIIR-PTTG* transgenic mice

Transgenic and age-matched wild-type animals were killed at 8–10 months of age. Tissue distribution of transgene expression was examined using RT-PCR analysis of RNA samples isolated from the ovary, uterus, testis, stomach, and

Figure 3 Transgene expression in ovaries and testes: (A) RT-PCR analysis of transgene expression in RNA isolated from ovaries of 0313 and 0711 lines and wild-type was performed as described in Material and Methods. The product was visualized by ethidium bromide staining. Samples were denatured, neutralized, and transferred overnight to nylon membranes. 32 P-labeled PTTG cDNA was used as a probe, and the bands were visualized by exposing the membranes overnight to X-ray film at -80°C . (B) Transgene expression was detected by RT-PCR in the testes of two founders (0313 and 0416) as indicated by a band of 400 bp. RNA from FVB testes was used as a negative control. No expression was detected in the stomach or kidney tissue from transgenic animals.

Table 1 Breeding summary of transgenic founders

Founder	Number of breeding	Total number of litter	Number of positive litter in F1
0313	08 (WT)	43	19
0711	10 (WT)	82	17
0416	09 (WT)	37	09
1211	10 (WT)	29	10
0725	None	None	None

WT, Wild-type.

kidney. Four females each from the 0313 and 0711 lines were examined for transgene expression in the ovary. Using specific primers (C and B Fig. 1), a product of 400 bp was identified in 50% of transgenic females and absent in wild-type mice (Fig. 3A). On the other hand, transgene expression was not detected in the uterus of either transgenic or wild-type animals (data not shown). Furthermore, transgene expression was detected in the testes of transgenic founders and absent in male FVB testes (Fig. 3B). No expression was detected in kidney or stomach tissue from transgenic animals (Fig. 3B).

To address the affinity of the human PTTG antibody raised by us (Kakar *et al.* 2001) for mouse PTTG protein, we tested human PTTG antibody on two cell lines: human ovarian epithelial tumor cell line (A2780) and mouse pituitary gonadotrope tumor cell line (LβT2). Initially, we determined the expression of PTTG mRNA in both cell lines using RT-PCR and specific primers as described in Materials and Methods. Both the cell lines showed high levels of expression of corresponding PTTG mRNA (Fig. 4A). To determine the cross-reactivity of human PTTG antibody to mouse PTTG protein, we performed western blot analysis as described previously (Kakar *et al.* 2001). Briefly, 40 µg protein from each cell line were separated on a 15% agarose gel and subjected

to western blot analysis. We used three dilutions of human PTTG antibody (1:1500, 1:3000, and 1:5000). As shown in Fig. 4B, an intense immunoreactive product was detected in A2780 cell line using all the three dilutions of human PTTG antibody. In contrast, no immunoreactive or a very weak immunoreactive product (at dilution of 1:1500) was detected in LβT2 cell line (Fig. 4B), suggesting no or weak cross-reactivity of human PTTG antibody with mouse PTTG protein.

Immunostaining of ovarian tissue using a PTTG antiserum (Kakar *et al.* 2001) showed an increase in cytoplasmic staining of PTTG protein in MISIR-PTTG transgenic ovarian epithelium when compared with wild-type (Fig. 4C, compare a and b) and was absent in negative control (Fig. 4C and c). This was further confirmed by measuring the average intensity of PTTG expression in the ovarian epithelium. Using Metamorph software as described in Materials and Methods, the average intensity was significantly increased by 40% in transgenic females when compared with wild-type (Fig. 4D). PTTG protein was also detected in granulosa cells in transgenic animals (Fig. 5, compare a and c with b). In contrast, fewer granulosa cells were positive for PTTG staining in wild-type ovaries (which could be non-specific binding), and no PTTG staining was detected in

Figure 4 Expression of PTTG in human ovarian epithelial tumor cell line (A2780) and mouse gonadotrope tumor cell line (LβT2). Localization of PTTG to the OSE by immunohistochemical analysis: (A) PCR analysis showed similar amount of PTTG mRNA expression from both A2780 and LβT2 cell lines. (B) Western blot analysis of PTTG protein expression from A2780 and LβT2 cells. Human PTTG antibody recognized PTTG protein from A2780 cell line at three different concentration (a = 1:1500, b = 1:3000, c = 1:5000) but not from LβT2 cell line. (C) PTTG protein in ovarian epithelium was detected by immunostaining for PTTG (brown) and counterstained with hematoxylin (blue). An increase in PTTG expression was observed in transgenic epithelium when compared with wild-type (compare a and b) indicated by the black arrows. No staining was observed in sections in which primary antibody was omitted (c). (D) Average intensity of immunostaining for PTTG in the OSE from transgenic ($n=5$) and wild-type mice ($n=3$). Three fields were used for each section. Note that the higher the intensity, the lower the value, $P<0.05$. All magnification from a to c is 40×.

Figure 5 PTTG expression in granulosa cells. PTTG protein in granulosa (GC) cells from transgenic female mice and wild-type. Paraffin embedded tissues were stained with PTTG anti-serum as described in Material and Methods. An increase in the number of granulosa cells expressing PTTG detected in MISIIR-PTTG transgenic ovaries and absent in thecal cells (TC) (compare transgenic a and c with wild-type b). Arrow indicates PTTG-positive granulosa cells. No staining was detected in slides where primary antibody was omitted (d). All magnification from a to d is 40 $\times$.

negative control (Fig. 5d). No PTTG immunostaining was observed in the corpus luteum of either transgenic or wild-type ovaries (data not shown).

Activation of *MISIIR* gene promoter in pituitary has been reported (Teixeira *et al.* 1999). Therefore, we determined the expression of *PTTG* transgene in anterior pituitary. As shown in Fig. 6, an overall increase in PTTG protein expression was detected in transgenic when compared with wild-type pituitary (compare a and b). Histopathological analysis of the pituitary gland did not reveal any abnormality (data not shown). Due to unavailability of specific monoclonal antibody for each hormone for specific cell type in anterior

Figure 6 PTTG expression in the anterior pituitary gland. Immunohistochemical analysis of PTTG expression in the pituitary gland. An overall increase in PTTG expression was detected in the pituitary of transgenic female mice (A) when compared with wild-type (B), magnification is 40 $\times$.

pituitary, identification of cell type in anterior pituitary that expresses *PTTG* transgene protein remains unknown.

Histopathology of MISIIR-PTTG transgenic mice

To assess the consequence of PTTG expression on phenotype, female mice from 0313 ($n=9$) and 0711 ($n=10$) lines were compared with eight wild-type FVB females. At least three successive sections were examined to confirm the differences. Overall, females from both F1 and F2 generations presented similar phenotypes; therefore, the data from F1 and F2 from the same line were pooled. Gross examination of transgenic ovary did not show any visible tumor. Histological examination of ovarian sections from transgenic mice revealed developing follicles. However, primary follicles were rarely noted in transgenic ovaries. Furthermore, transgenic ovaries displayed an overall increase in corpus luteum (CL) mass (Fig. 7, compare a and b) that resulted in a significant increase in ovary size (area in μm^2 in wild-type ovaries, 3.6 ± 1.98 , $n=5$; area for 0313 transgenic line ovaries, 6.8 ± 2.1 , $n=5$, $P<0.03$; and area for 0711 transgenic line ovaries, 6.65 ± 2 , $n=5$, $P<0.03$; Fig. 7c).

Histopathological analysis of the transgenic uteri revealed cystic glandular hyperplasia, which is a common phenotype associated with sustained levels of estrogen on the uterus (Tang *et al.* 1984). These glands were cystically dilated and were filled with fluid (Fig. 8, compare a and b with e and f). In some cases, these glands occupied the entire endometrium and displaced the stroma. There was no evidence of cycling such as scarring, hemosiderin laden macrophages, or stromal histiocytes which were prevalent in the wild-type uterus (Fig. 8, compare b and f). Furthermore, both of the luminal and glandular epithelium displayed an increase in mitotic bodies when compared with wild-type as indicated by PCNA staining (Fig. 8 compare c and d with g). Intense PCNA staining is indicative of higher proliferating cells in transgenic compared with wild-type (Baptist *et al.* 1993).

Two male founders developed abnormal growth in the testicular area at 6 months of age (Fig. 9A, a and b). Pathological examination revealed a plasmasarcoma in the preputial gland in founder 0313 (Fig. 9A and c). All transgenic males were killed at 8–10 months of age and none of them showed morphological changes in anterior pituitary or development of testicular tumor. However, there was obstruction of the urinary bladder in 60% of males examined (Fig. 9B a and b). The bladders were filled with white debris and microscopic examination indicated the presence of leukocytes (+++), RBC (+++), a few uric acid crystals, and bacteria.

Hormone analysis

Given that PTTG expression was targeted to two hormone-producing tissues (ovary and pituitary), we examined the hormone profile in transgenic females by measuring serum LH, FSH, E2, P4, and testosterone levels (Table 3). At 8–10 months of age, transgenic females displayed elevated serum

Figure 7 Histological analysis of MISIIR-PTTG transgenic ovary. Paraffin embedded ovarian sections from MISIIR-PTTG or wild-type animals were stained with H&E. An increase in the corpus luteum mass was observed in transgenic females when compared with wild-type (compare a and b). Original magnification of both panels; 4 $\times$. The size of the ovaries in μm^2 from transgenic and wild-type animals was determined by measuring the length and the width of the histological sections at 4 $\times$ magnification using Metamorph Software version 6.2. A significant increase in the transgenic ovary area was detected in both lines, $P < 0.03$ (c).

LH levels when compared with wild-type (Fig. 10A). Two animals in this group showed substantially higher values (13 and 37 ng/ml respectively) and were not included in our analysis. A significant increase in testosterone levels was observed in transgenic when compared with wild-type animals (Fig. 10B). In contrast, no significant difference in serum FSH levels was observed between transgenic and wild-type animals (data not shown). Serum E2 levels were 1.5-fold higher in MISIIR-PTTG transgenic females when compared with wild-type (Table 3), whereas no difference in P4 levels was observed between transgenic and wild-type (Table 3). Measurement of serum LH and testosterone levels in males

showed a significant increase in LH levels (twofold) and testosterone levels (5.3-fold) in 0313 line when compared with wild-type (Table 3). Due to shortage of serum samples, we were unable to analysis for serum LH and testosterone levels in 0711 line.

Discussion

PTTG is highly expressed in most of the tumors including ovarian tumors. To demonstrate the oncogenic function of PTTG in the development of ovarian epithelial cancer and its

MISIIR-PTTG

Wild-type

Figure 8 Histological analysis of MISIIR-PTTG transgenic uterus. Cystic glandular hyperplasia in the uterine cavity of MISIIR-PTTG female mice. The uteri from MISIIR-PTTG transgenic mice (a and b) had large cysts and multiple small fluid-filled glandular cysts (e and f). Scar formation and hemosiderin laden granules (asterisk in f) were evident in wild-type but absent in transgenic uterus. Both wild-type and transgenic uteri were PCNA positive; however, the latter showed multiple mitotic cells as indicated by dark homogeneous stained cells in both luminal and glandular epithelial cells. However PCNA staining was more intense in transgenic mice compared with wild-type indicative of higher proliferative cells. (compare transgenic c and d with wild-type g). Negative control sections in which primary antibody were omitted did not display any PCNA staining (h). Magnification in a and e: 10 $\times$ and b, c, d, f, g, and h: 40 $\times$.

Figure 9 Phenotype changes in MISIIR-PTTG male founders (A) Growth of the preputial gland in one of the founders displaying tumor of soft tissue (a and b). H&E staining of preputial gland showing tumor of soft tissue plasmasarcoma (c). (B) Enlargement of urinary bladder in MISIIR-PTTG transgenic male which was not observed in wild-type (a and b). Bladder was filled with white matter (white arrow).

Figure 10 Serum LH and testosterone levels: serum concentration of LH (A) and testosterone (B) were determined in 8- to 10-month-old transgenic and wild-type female mice using EIA kit as described in Material and Methods, $P < 0.05$.

progression, we generated transgenic mice expressing PTTG under the control of MISIR promoter. Using this approach, we were successful in targeting the PTTG transgene to the ovary and pituitary but were unable to detect transgene expression in the uterus. These results are in agreement with previous reports that MISIR promoter is active in the ovary (Teixeira *et al.* 1999) and consistent with Connolly *et al.* (2003) who failed to detect MISIR expression in the uterus. There was no visible tumor in any of the tissues targeted by MISIR gene promoter in transgenic females. In spite of the high level of PTTG expression in the ovarian epithelium in transgenic animals (Fig. 4), we did not detect any evidence of tumor development. The lack of phenotypic change in the epithelium may be because ovarian surface epithelium (OSE) is much more difficult to transform and another oncogene may be required to achieve transformation. This hypothesis is based on the results from Orsulic *et al.* (2002), who showed induction of ovarian tumors in p53-deficient animals by adding two of the oncogenes *c-myc*, *K-ras*, and *Akt*. Consistent with these results, Dinulescu *et al.* (2005) failed to develop ovarian tumors on expression of oncogenic K-ras or conditioned PTEN deletion within OSE cells. However, a combination of two mutations in the ovary led to the induction of invasive and widely metastatic endometrioid ovarian adenocarcinomas with complete penetration within 7 weeks, suggesting that the cooperation of at least two genes is required to transform ovarian epithelial cells to tumors. Another possible reason for the lack of tumor development could be due to limited level of expression of PTTG in the

epithelium that is not high enough to cause transformation of OSE. Finally, we cannot exclude the possibility that PTTG expression in the OSE produced changes within the genotype, but were not sufficient to change the phenotype. Therefore, further study is required to determine changes in the gene expression within the ovarian surface epithelium in PTTG transgenic females.

Pathologically, PTTG transgenic ovaries share some properties with hCG β and LH β -CTP transgenic mice (Risma *et al.* 1995, Rulli *et al.* 2002), such as elevated serum LH and testosterone levels and an increase in CL mass. Development of uterine cystic glandular hyperplasia in MISIR-PTTG transgenic animals (Fig. 7) is consistent with the hCG β transgenic uterus phenotype. Even though we observed a marginal increase in serum E2 levels in transgenic animals when compared with wild-type females, development of cystic glandular hyperplasia in the endometrium is an E2-induced effect (Tang *et al.* 1984), suggesting that E2 serum levels may have been elevated in transgenic females at some point prior to kill. In fact, a temporal change in E2 levels was observed in hCG β transgenic females (Rulli *et al.* 2002). In spite of increased serum E2 at early age in hCG β transgenic females, E2 returned to normal by age 2–6 months.

Overexpression of PTTG is thought to influence cell proliferation and cell survival (Wang *et al.* 2003, Chesnokova *et al.* 2005). Using the MISIR gene promoter, we targeted PTTG to granulosa cells during the proliferative phase (Hirobe *et al.* 1994, Baarends *et al.* 1995, McGee *et al.*

Table 3 Hormone profile in wild-type and Mullerian inhibitory substance type II receptor (MISIR)-PTTG transgenic animals

Wild-type	Mean $\pm$ S.E.M.	MISIR-PTTG	Mean $\pm$ S.E.M.
LH (female)	0.6 ng/ml $\pm$ 0.13; n=4	0313 line	1.5 ng/ml $\pm$ 0.4; n=6
LH (male)	1.09 ng/ml $\pm$ 0.06; n=4	0313 line	2.1 ng/ml $\pm$ 0.01; n=9
Testosterone (female)	65 pg/ml $\pm$ 17.7; n=8	0313 line	377.7 pg/ml $\pm$ 103.8; n=8
Testosterone (male)	335.6 pg/ml $\pm$ 18.5; n=5	0313 line	1773 pg/ml $\pm$ 19.8; n=9
Estradiol (female)	7.4 pg/ml $\pm$ 1.2; n=3	0313 line	11 pg/ml $\pm$ 0.9; n=6
Progesterone (female)	7 ng/ml $\pm$ 1.3; n=8	0313 line	7.1 ng/ml $\pm$ 1.4; n=6

2001). During the proliferative phase, a dominant follicle is selected and E2 is produced. Increased levels of E2 suppress the development of other follicles by inhibiting FSH secretion from the pituitary gland, thus allowing fewer follicles to continue to ovulation. However, the continuous rise in circulating E2 from developing follicles will stimulate the pituitary gland to produce LH, causing the LH surge that induces ovulation. Since MISIR promoter targets PTTG expression to granulosa cells during proliferative phase, we hypothesize that PTTG may promote follicle survival, enhancing folliculogenesis and decreasing follicular apoptosis. This may result in an increase in the overall E2 levels. This can explain the development of cystic glandular hyperplasia of the endometrium observed in PTTG transgenic female mice. Whether that translates in increased ovulation in transgenic animals remains unclear. Unlike LH β -CTP and hCG β models, PTTG transgenic females were fertile at least up to 3 months of age and produced average size litter. However, histological analysis of the uterus at 8–10 months did not show any evidence of cycling, suggesting that the reproductive cycle was altered in these animals at later stage.

Immunohistochemical analysis of the anterior pituitary from the transgenic females demonstrated an overall increase in PTTG expression in the anterior pituitary of transgenic mice (Fig. 6). Teixeira *et al.* (1999) reported the presence of MISIR gene expression in the anterior pituitary. This suggests that PTTG expression in the pituitary could be a direct effect of MISIR promoter activity. If indeed E2 was elevated, then we may anticipate that E2 had influenced PTTG levels in the pituitary (Heaney *et al.* 1999, 2002). However, our human PTTG antibody did not cross-react with murine PTTG protein; therefore, increase in PTTG expression observed in anterior pituitary is result of activation of MISIR gene promoter in anterior pituitary. Possibility of increase in endogenous PTTG expression as a result of increase in E2 levels is not ruled out. Recently, Abbud *et al.* (2005) reported an increase in expression of PTTG in anterior pituitary cells in male transgenic animals using α -GSU promoter, leading to increase in serum LH levels. Based on these results, an increase in serum LH levels in female transgenic animals observed in our studies could be result of increased PTTG expression in the anterior pituitary. Consistent with hCG β and LH β -CTP mice models (Risma *et al.* 1995, Rulli *et al.* 2002), MISIR–PTTG transgenic females showed a significant increase in serum testosterone levels which may be contributing to the aggressive behavior observed in PTTG transgenic females. Unlike hCG β and LH β -CTP mice model, PTTG transgenic females did not show elevated P4 levels despite the massive luteinization. This difference may be explained by the fact that serum LH in PTTG transgenic animals was increased by approximately threefold (Table 3), while both hCG β and LH β -CTP models LH levels were elevated by 5- and 40-fold respectively. On the other hand, serum testosterone levels in each model were increased by sixfold. One may conclude that there is an enhanced synthesis of testosterone in PTTG transgenic females even with the moderate elevation in LH levels. Since P4 is an

early precursor in steroid biosynthesis, we hypothesize that enhanced conversion of P4 to testosterone is masking any potential changes in P4 levels. Although testosterone primarily produced in thecal cells by the action of type 5,17- β -HSD (Luu-The *et al.* 2001), the expression of this enzyme in the corpus luteum (Pelletier *et al.* 1999) suggests that it may influence testosterone production in the luteal phase. Despite the sex difference, α -GSU transgenic males also showed elevated serum LH and testosterone, suggesting that PTTG expression influences the synthesis of both hormones.

MISIR–PTTG transgenic male mice presented with enlarged urinary bladder (Fog-10). The cause of the enlargement of bladder in MISIR–PTTG males remains unknown. However, bladder obstruction is associated with prostate hyperplasia as observed in α -GSU–PTTG and mitogen activated protein kinase T (MAPKT) models (Majumder *et al.* 2003). Therefore, enlargement of bladder in male transgenic animals might be due to elevated levels of serum LH and testosterone. MISIR expression has been reported in prostate gland by Teixeira *et al.* (1999), suggesting that direct expression of PTTG in the prostate may also contribute to this phenotype.

In summary, using MISIR gene promoter, we targeted PTTG expression to three different tissues, ovarian surface epithelium, granulosa cells, and pituitary. None of these tissues developed any visible tumor. PTTG transgenic females presented with increased ovary size, hyperplasia of the endometrium, and increased serum LH and testosterone levels. These data suggest that PTTG is capable of inducing initial transformation but is not sufficient for tumorigenesis, and may require a hormonal trigger or cooperation of other gene(s), such as inactivation of p53, to initiate tumorigenesis and to promote tumor growth.

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