Sex differences in the development of prolactinoma in mice overexpressing hCGβ: role of TGFβ1

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Abstract

Female transgenic mice that overexpress the human chorionic gonadotrophin β subunit (hCGβ+) develop prolactinomas, whereas hCGβ+ males do not. The high levels of circulating hCG induce massive luteinization in the ovary of hCGβ+ females, and progesterone becomes the primary steroid hormone produced, but estradiol remains at physiological level. The involvement of high levels of progesterone in lactotroph proliferation is not clearly understood; hence, the pathogenesis of prolactinomas in hCGβ+ females remains unclear. TGFβ1 is an inhibitor of lactotroph function, and the reduced TGFβ1 activity found in prolactinomas has been proposed to be involved in tumor development. The aim of the present work was to study the role of TGFβ1 in the gender-specific development of prolactinomas in hCGβ+ mice. We compared the expression of different components of the pituitary TGFβ1 system in males and females in this model. We found reduced TGFβ1 levels, reduced expression of TGFβ1 target genes, TGFβ1 receptors, *Ltbp1*, *Smad4* and *Smad7* in hCGβ+ female pituitaries. However, no differences were found between the transgenic and wild-type male pituitaries. We postulate that decreased pituitary TGFβ1 activity in hCGβ+ females is involved in the development of prolactinomas. In fact, we demonstrated that an in vivo treatment carried out for increasing pituitary TGFβ1 activity, was successful in reducing the prolactinoma development, and the hyperprolactinemia in hCGβ+ females. Moreover, the stronger TGFβ1 system found in males could protect them from excessive lactotroph proliferation. Sex differences in the regulation of the pituitary TGFβ1 system could explain gender differences in the incidence of prolactinoma.

Key Words
- pituitary
- prolactinoma
- hCGβ
- TGFβ1

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Introduction

The human chorionic gonadotrophin (hCG), secreted normally by the placenta, is a member of the family of glycoprotein hormones (with the pituitary hormones LH, FSH and TSH) that share structural similarities. They are heterodimers of two non-covalently associated subunits: the common α- and the hormone-specific β-subunit. Individually, each subunit has not known biological activity (Gharib *et al.* 1990).

The hCGβ and the LHβ subunits have 83% homology, but hCGβ contains four additional O-linked glycosylation sites in a C-terminal extension of 24 amino acids, that confers to hCGβ a longer circulatory half-life and higher biopotency as compared to LH (Gharib *et al.* 1990). Both LH and hCG interact with the same LH/hCG receptor, which belongs to the large family of G-protein-coupled receptors, promoting ovarian steroidogenesis and ovulation in females and testicular androgen production in males.

Elevated gonadotrophin secretion can be observed in female or male infertility and upon ovarian tumorigenesis. Genetically modified mouse models have provided fundamental tools to study disorders arising from the loss- or the gain-of-function of gonadotrophins in reproductive pathologies (reviewed in Jonas *et al.* 2014).

Phenotypes of the transgenic mouse model overexpressing the hCGβ subunit (hCGβ+ mice) present gender differences. Although hCGβ+ males are fertile, with normal spermatogenesis and sperm quality despite reduced testis size and serum FSH (Rulli *et al.* 2003), females present precocious puberty, infertility, enhanced ovarian steroidogenesis and abnormal uterine structure (Rulli *et al.* 2002). Adult females develop mammary tumors with characteristics of adenocarcinoma at the age of 9–10 months. On the other hand, females, but not males, also develop prolactinomas. The pituitary enlargement becomes evident from the age of 2 months, and progress to adenoma by the age of 8–10 months, concomitant with severe hyperprolactinemia (Rulli *et al.* 2002).

Factors that trigger the prolactinoma development in hCGβ+ females are not fully elucidated. One of the most studied stimuli involved in prolactinoma development is the circulating estradiol level. In hCGβ+ females, the stimulation of the immature ovary by hCG induces transient high levels of estradiol with a 3- to 4-fold increase at one month of age. However, thereafter, the continuous high levels of circulating hCG induce massive luteinization, and progesterone becomes the predominant steroid hormone produced. Adult hCGβ+ female mice present with a 50- to 100-fold excess of progesterone, 3- to 6-fold-increase in testosterone but, interestingly, physiological levels of estradiol (Rulli *et al.* 2002). As the involvement of high levels of progesterone or testosterone in lactotroph proliferation is not clearly understood, new investigations are needed to clarify the pathogenesis of prolactinomas in hCGβ+ female mice.

The main regulators of lactotroph functions are dopamine (DA) and estradiol. They interact in the regulation of cell proliferation and prolactin (PRL) secretion (Maurer 1982, Ben Jonathan & Hnasko 2001). DA inhibits lactotroph proliferation and PRL synthesis and release, acting through the dopamine D2 receptor (Drd2) expressed in lactotrophs (Ben-Jonathan 1985, Missale *et al.* 1998). Estradiol stimulates *PRL* gene transcription modifying lactotrophic responses to other stimulatory or inhibitory factors and indirectly decreases DA production from the hypothalamus (Freeman *et al.* 2000). In addition, other hypothalamic or pituitary factors contribute to the regulation of lactotroph function (reviewed in Freeman *et al.* 2000). Among them, the locally produced transforming growth factor beta 1 (TGFβ1) is known for inhibiting lactotroph proliferation and PRL secretion (Sarkar *et al.* 1992, 1998, Recouvreux *et al.* 2011).

The TGFβ biology is complex, and its activity is highly regulated due to its powerful effects on embryogenesis, development and tissue homeostasis (Heldin *et al.* 2009, Galvin-Burgess *et al.* 2013, Itoh *et al.* 2014).

In the pituitary, DA and estradiol, the main regulators of lactotroph function, regulate TGFβ1 synthesis and activation, and the expression of several components of the TGFβ1 system (Recouvreux *et al.* 2011, 2013). DA increases the TGFβ1 expression in lactotrophs (Sarkar *et al.* 2005), whereas estradiol decreases cytokine synthesis and release and type 2 TGFβ receptor (TβR2) expression (Sarkar *et al.* 1992, Pastorcic *et al.* 1995). Moreover, it was previously demonstrated that TGFβ1 mediates, at least in part, the DA and estradiol effect on lactotroph functions.

The importance of TGFβ1 in inhibiting lactotroph function is clearly demonstrated by the fact that in human prolactinomas, as well as in animal models of prolactinomas, the TGFβ1 expression and activity were found reduced, suggesting its involvement in the tumor development (Pastorcic *et al.* 1995, Recouvreux *et al.* 2011, 2012, 2016).

The aim of the present work was to study the involvement of TGFβ1 system in the development of prolactinoma in hCGβ+ mice, and in the gender differences observed in the appearance of this phenotype.

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Materials and methods

Animals

All studies were performed in transgenic mice from both sexes, overexpressing the hCGβ subunit under the control of human ubiquitin C promoter (hCGβ+). In all cases, wild-type (WT) littermates were used as controls. These mice, with FVB/N background, were genotyped as previously described (Rulli *et al.* 2002). Animals were kept in a temperature-controlled room with lights on at 07:00 h and off at 19:00 h and were given free access to laboratory chow and tap water.

All experimental procedures were performed according to the NIH Guidelines for Care and Use of Experimental Animals (Division of Animal Welfare, Office for Protection of Research Risks, National Institutes of Health, A#5072-01) and were approved by the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (IBYME-CONICET).

Animals were used at 6 months of age, at which age the pituitaries from hCGβ+ females were hyperplastic. Trunk blood was collected after decapitation, and anterior pituitaries were weighed after removal. Serum samples were separated by centrifugation and stored at −20°C for biochemical analyses. Anterior pituitaries of different experimental groups were stored in TRIzol (Invitrogen) or ice-cold buffer containing a mix of proteases inhibitors at −70°C for posterior RNA isolation and ELISA assays, respectively.

Radioimmunoassay (RIA)

Serum prolactin levels were measured by RIA using mouse-specific reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases and National Hormone and Pituitary Program (Dr A F Parlow, NHPP, Torrance, CA). Assays were performed using 10 µL serum in duplicate. Results are expressed in nanograms per milliliter. The inter- and intra-assay coefficients of variation were 6.9% and 11.6%, respectively.

RNA isolation and analysis of gene expression

Anterior pituitaries and hypothalami were collected and processed in TRIzol Reagent (Invitrogen), and total RNA was isolated according to manufacturer's protocol. One microgram of RNA was reverse-transcribed in a 20 µL reaction volume using MMLV-RT (Promega) and random primers (Biodynamics). The resulting cDNA was used for quantitative real-time PCR analysis (qPCR). qPCRs were performed using specific-designed primers and the Fast Start Universal SYBR Green Master Rox (Roche) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Differences in the cDNA target gene expression were quantified by comparing the threshold cycle (CT) with that of *CyclophilinB* using the comparative CT method (ΔΔCT). The primer sequences used for qPCR are shown in Table 1.

Detection of total and active TGFβ1

ELISAs were performed to quantify total or active TGFβ1 concentration in pituitary homogenates using the TGFβ1 DuoSet ELISA development system (DY1679, R&D Systems), following the manufacturer's instructions.

Anterior pituitaries of different groups were collected and homogenized in 100 µL of ice-cold buffer containing 100 mM Tris, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, pH 7.4 and a mix of proteases inhibitors

Table 1 Primer sequences for qPCR.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
CyclophilinB	GACCCTCCGTGGCCAACGAT	ACGACTCGTCCTACAGATTCATCTC
TβR2	AATTCCCAGCTTCTGGCTCA	GTGCTGTGAGAGACGCGGGCTTC
Tmepai	TGTCCTCGGAAGGATGCCTCTGG	CAGCGAGTCGGTCAGTGGGC
Klf14	CAGCTCGTCTGGCTCCAA	AGGCACTCGGCAGCGAA
Alk5	TCCAAACCACAGAGTAGGCAC	TCATGGATTCCACCAATAGAACA
Alk1	GCGTTGACCAGCAGACACCC	CAGTGCGGTGAGGCGAGCAG
Smad4	CACCCGCCAAGTAATCGCGCA	CTGGCCGGCTGACTTGTGGAAG
Smad7	AACCCCGAGCTGGTGTGCTG	TGGACAGCCTGCAGTTGGTTTGA
Ltbp1	AGCACCATCACCTCTGCTCT	CAGACACTGCTGCTCCAA
Th	CCAGAGAGGACAAGGTTCCC	ATACGCCTGGTCAGAGAAGC
Drd2	ATCGTCTCGTTCTACGTGCC	GTTGCCCTTGAGTGGTGTC
Tgfβ3	TGAGCTCTTCCAGATACTTCGAC	GGATGCTGATTTCCAGACCCA
Bmp4	TTGCAGCTTTCTAGAGGTCCC	TCAGCATTCGGTTACCAGGAA

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(phenylmethylsulfonyl fluoride, tosyl phenylalanyl chloromethyl ketone, tert-Amyl methyl ether, N-carbobenzyloxy-L-phenylalanine chloromethyl ketone and tosyl-L-lysyl chloromethyl ketone), using a hand-held micro-tissue homogenizer. The homogenates were centrifuged at 8900 ***g*** for 10 min at 4°C. The supernatant was collected, and protein concentration was measured by Lowry method. Equal protein amounts were loaded per well. TGFβ1 concentration was expressed as picograms per milligrams of protein. To assay total TGFβ1, samples were previously acidified to pH 2.6 by adding 1 M HCl for 20 min at room temperature, followed by neutralization with 1 M NaOH to pH 7.6.

Determination of DA concentration in hypothalamic tissue by HPLC

Whole hypothalami, including the preoptic area, were excised considering an area limited anteriorly by the optic chiasma and laterally by the hypothalamic fissures. The posterior limit was a plane passing in the limit with the mammillary bodies, excluding them. The in-depth limit was the subthalamic sulcus. Tissues were kept at −70°C until used.

HPLC-coupled electrochemical detection of DA was performed using a Varian 5000 liquid chromatograph coupled to an electrochemical detector (BAS LC-4C). Hypothalamic tissues were weighed, homogenized and deproteinized in 0.2 N perchloric acid (1/20). Homogenates were centrifuged. The supernatants were injected (50 µL) onto a 12.5 cm × 4 mm Nova-Pak C18 (Waters) reverse phase column. Mobile phase for DA determinations contained $NaH_2PO_4.H_2O$ 0.076 M, EDTA 0.99 mM, PICB8 5.24 mL/L and 6% methanol. The electrode potential was fixed at 0.7 V. Peak heights were measured by Peak Simple Chromatography Data System (Model 302 Six Channel USB). Values were quantified based on standard curves using the same software. DA concentration was determined based on tissue wet weight.

Experimental protocol for ABT-898 treatment

Four-month-old hCGβ+ female mice were divided into two groups and treated with 100 mg/kg ABT-898 (i.p.) or vehicle (5% dextrose, control group). The drugs were injected three times per week during three weeks. Twenty-four hours after the last injection, animals were killed, and trunk blood was collected for serum PRL determination by RIA. Pituitaries were carefully excised and weighed after removing the neurohypophysis. Anterior pituitaries were used for TGFβ1 ELISA assay and for pituitary PRL concentration measurement by RIA. Female WT siblings were used as control.

Drug ABT-898 peptide was generously supplied by Abbott Laboratories.

Statistical analysis

Results are expressed as mean ± S.E.M. Two-way ANOVA was performed, as the effects of two factors (genotype and gender) were evaluated, followed by Bonferroni's *post hoc* test. Dopamine concentration measurements were evaluated by Student's *t* test. The *in vivo* treatment results were evaluated by one-way ANOVA followed by Tukey's honestly significant difference test. $P < 0.05$ was considered significant. Data were transformed when required.

Results

Sex differences in pituitary weight and prolactin secretion in WT and hCGβ+ mice

As it was previously shown (Rulli *et al.* 2002, Ahtiainen *et al.* 2010, Ratner *et al.* 2012), hCGβ+ female mice developed prolactinomas, presenting a markedly increase in pituitary weight compared to their WT counterparts at 6 months of age (Fig. 1A, $P < 0.0001$). Nevertheless, no differences among genotypes were found in the pituitary weight in males, instead of both sexes hCGβ+ mice presenting with elevated levels of bioactive

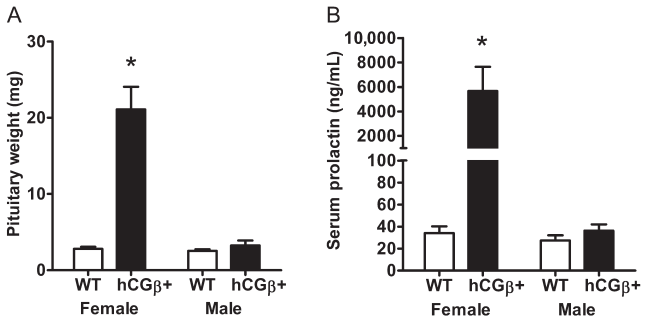

Figure 1
Sex differences in pituitary weight and prolactin secretion in WT and hCGβ+ mice. (A) Pituitary weight (mg) of 6-month-old mice ($n = 11–12$/group). Two-way ANOVA: genotype × sex interaction ($P = 0.0002$). Bonferroni's *post hoc* test: Differences among genotypes were observed only in females (*$P < 0.0001$ vs wt female). (B) Serum prolactin levels were measured by RIA ($n = 11–12$/group). Genotype × sex interaction ($P < 0.0001$). Significant differences among genotypes were observed only in females (*$P < 0.0001$ vs wt female).

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LH/hCG (males 21 ± 4, females 99 ± 20 IU/L; Rulli *et al.* 2002, 2003).

In correlation to the pituitary weight, serum prolactin levels were also found significantly increased in hCGβ+ female mice compared to their WT counterparts (Fig. 1B, $P<0.0001$), whereas no differences were found among genotypes in males.

Sex differences in active and total TGFβ1 concentration in pituitaries from WT and hCGβ+ mice

We next evaluated whether sex differences found in pituitary weight and prolactin secretion from hCGβ+ mice could be related to alterations in the local TGFβ1 system.

When we measured pituitary TGFβ1 concentration by ELISA, we found higher levels of both total and active TGFβ1 in male pituitaries compared to female mice (Fig. 2A, $P<0.0001$; Fig. 2B, $P<0.0001$). A lower concentration of both total (Fig. 2A, $P<0.001$) and active (Fig. 2B, $P<0.01$) TGFβ1 was found in hCGβ+ female mice when compared to their WT siblings. On the other hand, no differences among genotypes were found in cytokine content in pituitaries from males.

TGFβ1 biological activity

To evaluate whether the lower levels of active TGFβ1 found in pituitaries from hCGβ+ female mice affect the biological activity of the cytokine, we measured the expression of two known TGFβ1 target genes: the Krüppel-like factor 14 (*Klf14*) (Truty *et al.* 2009) and the transmembrane androgen-induced protein (*Tmepai*) (Brunschwig *et al.* 2003, Levy & Hill 2005). *Klf14* and *Tmepai* mRNA expression were found higher in male mice compared to that in females (Fig. 3A and B, respectively, $P<0.0001$), according to the sex differences observed in pituitary-active TGFβ1 content. Both *Klf14* and *Tmepai* mRNA expression were found decreased in female hCGβ+ compared to their WT counterparts (Fig. 3A and B, $P<0.001$). This finding is in agreement with the marked decrease in total and active TGFβ1 content observed in hCGβ+ female pituitaries (Fig. 2).

TGFβ1 receptors

The expression of the type 1 (*Alk1* and *Alk5*) and type 2 (*TβR2*) TGFβ receptors was found increased in male pituitaries compared to that in females (Fig. 4, $P<0.0001$). No genotype differences were found in males, but in females, the mRNA expression of *TβR2* (Fig. 4A, $P<0.001$) and *Alk5* (Fig. 4B, $P<0.001$) was found reduced in hCGβ+ compared to their WT counterparts. On the other hand, no differences among genotypes were found in *Alk1* expression in female pituitaries (Fig. 4C).

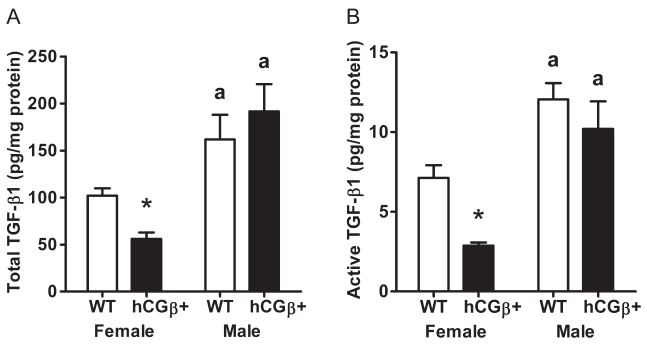

Figure 2
Active and total TGFβ1 concentration in pituitaries from WT and hCGβ+ mice. Active and total TGFβ1 content was measured by ELISA in pituitary homogenates ($n=8$–9/group). (A) Total TGFβ1: Two-way ANOVA, genotype × sex interaction ($P=0.0054$). Bonferroni's *post hoc* test was conducted as the interaction between effects was significant. Differences among gender: *a*, $P<0.0001$. Differences among genotypes were observed only in females (*$P<0.001$ vs wt female). (B) Active TGFβ1 concentration: Two-way ANOVA, genotype × sex interaction ($P=0.0274$). Bonferroni's *post hoc* test: Sex differences: *a*, $P<0.0001$. Significant differences among genotypes were observed only in females (*$P<0.01$).

Other components of TGFβ1 system

When we measured the mRNA expression of *Smad4* and *Smad7*, we also observed gender and sex differences. Both

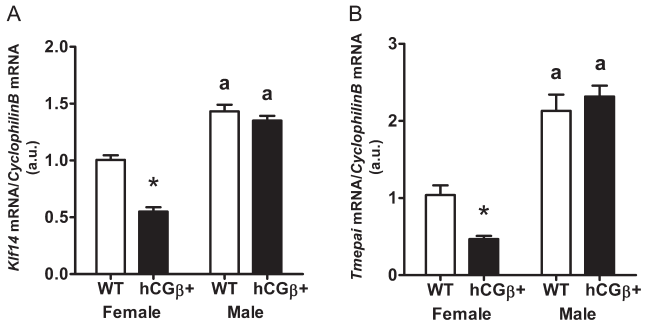

Figure 3
Sex and genotype differences in TGFβ1 biological activity. mRNA transcripts were amplified with specific primers by qPCR and normalized to *CyclophilinB*. Results are expressed relative to those for WT females ($n=7$–11/group) and analyzed by two-way ANOVA. Bonferroni's *post hoc* test was conducted as the interaction between effects was significant. (A) *Klf14* mRNA expression: genotype × sex interaction ($P=0.0036$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were observed only in females (*$P<0.001$ vs wt female). (B) *Tmepai* mRNA expression. Genotype × sex interaction: $P=0.0003$. Sex differences: *a*, $P<0.0001$. Differences among genotypes were observed only in females (*$P<0.001$ vs wt females).

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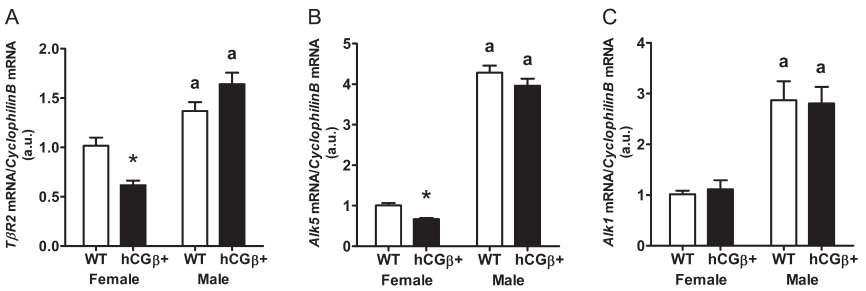

Figure 4
Sex differences in TGFβ1 receptors expression. mRNA transcripts were amplified with specific primers by qPCR and normalized to *CyclophilinB*. Results are expressed relative to those WT females ($n=7$–12/group) and analyzed by two-way ANOVA. Bonferroni's *post hoc* test was conducted as the interaction between effects was significant. (A) *TβR2* mRNA expression: genotype × sex interaction ($P=0.0003$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were observed only in females (*$P<0.001$ vs wt female). (B) *Alk5* mRNA expression: genotype × sex interaction $P=0.0004$. Sex differences: *a*, $P<0.0001$. Differences among genotypes were observed only in females (*$P<0.001$ vs wt female). (C) *Alk1* mRNA expression. Genotype × sex interaction was no significant. Sex differences: *a*, $P<0.0001$.

Smads presented higher expression in male pituitaries compared to females ($P<0.05$), but without differences among genotypes. On the other hand, *Smad4* and *Smad7* mRNA expression were found decreased in pituitaries from female hCGβ+ compared to their WT counterparts (*Smad4* Fig. 5A; *Smad7* Fig. 5B; $P<0.001$).

On the other hand, latent TGFβ-binding protein 1 (*Ltbp1*) expression was also found increased in male pituitaries when compared to females (Fig. 5C, $P<0.0001$). Reduced *Ltbp1* expression was found in hCGβ+ female pituitaries compared to their WT counterparts ($P<0.01$), whereas no differences among genotypes were found in males.

Dopaminergic system

As it was demonstrated that DA increases TGFβ1 expression in lactotrophs (Sarkar *et al.* 2005), as well as the pituitary TGFβ1 activity (Recouvreux *et al.* 2011), we were interested in evaluating whether a decreased dopaminergic tone could be involved in the decreased pituitary TGFβ1 activity. For this purpose, we measured the tyrosine hydroxylase (TH) expression in hypothalamic homogenates. We found decreased *Th* mRNA in males compared to females (Fig. 6A, $P<0.0001$), but without genotype differences. On the other hand, *Th* expression was found reduced in hCGβ+ females compared to their WT siblings ($P<0.001$). This result could imply a lower dopaminergic tone reaching the pituitary in this group. We next measured the pituitary expression of *Drd2* in all groups. As shown in Fig. 6B, the *Drd2* expression was found higher in male pituitaries compared to females, in accordance with lower dopaminergic tone in this sex ($P<0.0001$), without differences among genotypes. But in females, the *Drd2* expression was found significantly increased in hCGβ+ group compared to their WT siblings ($P<0.001$), as expected when the dopaminergic tone is reduced. Finally, to know whether genotype differences

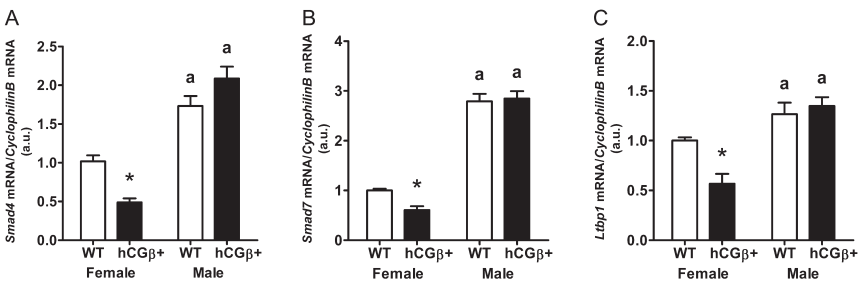

Figure 5
Sex and genotype differences in other components of TGFβ1 system. mRNA transcripts were amplified with specific primers by qPCR and normalized to *CyclophilinB*. Results are expressed relative to those WT females ($n=5$–12/group) and analyzed by two-way ANOVA. Bonferroni's *post hoc* test was conducted when the interaction between effects was significant. (A) *Smad4* mRNA expression: genotype × sex interaction ($P=0.0027$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were found only in females (*$P<0.05$ vs wt female). (B) *Smad7* mRNA expression. Genotype × sex interaction: $P=0.0137$. Sex differences: *a*, $P<0.0001$. Differences among genotypes were found only in females (*$P<0.001$ vs wt female). (C) *Ltbp1* mRNA expression: genotype × sex interaction ($P=0.01$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were found only in females (*$P<0.01$ vs wt female).

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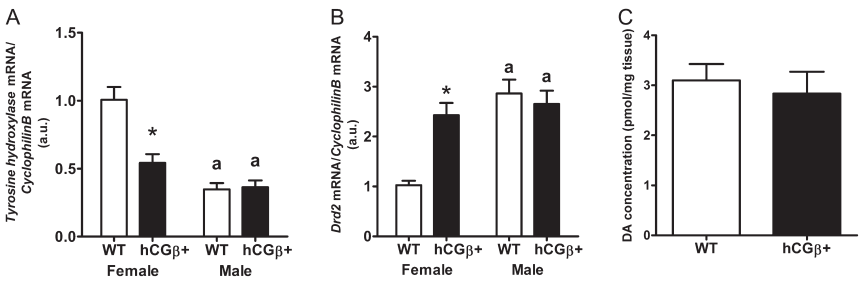

Figure 6

Sex and genotype differences in dopaminergic system. (A and B) mRNA transcripts were amplified with specific primers by qPCR and normalized to *CyclophilinB*. Results are expressed relative to those WT females ($n=5$–7/group) and analyzed by two-way ANOVA. Bonferroni's *post hoc* test was conducted when the interaction between effects was significant. (A) *Th* mRNA expression: genotype × sex interaction ($P=0.0021$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were found only in females (*$P<0.001$ vs wt female). (B) Dopamine D2 receptor gene (*Drd2*) mRNA expression. Genotype × sex interaction ($P=0.0021$). Sex differences: *a*, $P<0.0001$. Differences among genotypes were found only in females (*$P<0.001$ vs wt female). (C) DA concentration in female hypothalami performed by HPLC. Results are expressed relative to WT females ($n=6$/group). Student's *t* test was performed ($P=0.6334$), no differences were found between groups.

found in hypothalamic *Th* expression in females were reflected in DA concentration, we evaluated the hypothalamic DA concentration by HPLC in WT and hCGβ+ females. As it could be observed in Fig. 6C, we did not find differences among groups.

Thrombospondin 1 (TSP-1) synthetic analogue ABT-898 normalizes pituitary-active TGFβ1 levels in hCGβ+ females

TSP-1 is a large multifunctional glycoprotein, component of the extracellular matrix, involved in multiple biological processes such as angiogenesis, apoptosis and activation of TGFβ1 (Lawler 2002). ABT-898 (Abbott Laboratories) is a TSP-1 synthetic analogue that mimics TSP-1 antiangiogenic action (Haviv *et al.* 2005). We have previously studied the effect of ABT-898 on experimental prolactinomas induced by chronic diethylstilbestrol (DES) treatment in female rats (Recouvreux *et al.* 2012). We demonstrated that an *in vivo* ABT-898 treatment markedly enhanced pituitary-active TGFβ1 concentration in the tumors, counteracted the increase in pituitary size and reduced the serum prolactin levels, as well as pituitary proliferation rate induced by DES.

To demonstrate the involvement of the reduced pituitary TGFβ1 activity in prolactinoma development in hCGβ+ female mice, we next conducted the ABT– *in vivo* treatment in our experimental model. We found that ABT-898 treatment in fact recovered the pituitary-active TGFβ1 concentration (Fig. 7A). In accordance, pituitary weight was reduced in the ABT-treated hCGβ+ group (Fig. 7B, $P<0.05$), as well as the serum prolactin levels (Fig. 7C, $P<0.01$). As females used in this experiment were younger (4–5 months) than the previously used mice (Fig. 1B, 6–7 months), the hyperprolactinemia observed in hCGβ+ females was not as high as previously observed, since serum PRL levels increase exponentially from 3 months onwards. On the other hand, Fig. 7D shows higher PRL concentration in WT pituitary homogenates, due to the normal inhibitory effect of dopamine and TGFβ1 on hormone secretion. But the pituitary PRL concentration decreased in prolactinomas from hCGβ+ control female because this inhibition was lost ($P<0.001$). Interestingly, as ABT treatment enhanced biological TGFβ1 activity, the inhibition on hormone secretion was recovered ($P<0.05$).

Other members of TGFβ family

Finally, as other members of the TGFβ growth factor family, such as TGFβ3 and BMP4, have also been involved in prolactinoma development, we next measured the mRNA expression of these factors in our experimental model. As Fig. 8A shows, we did not find genotype differences in pituitary *Tgfβ3* mRNA levels in females. However, we found a gender difference: decreased *Tgfβ3* mRNA levels were found in male pituitaries related to females ($P=0.0009$).

On the other hand, when we evaluated pituitary *Bmp4* mRNA levels, it was found significantly reduced in hCGβ+ female pituitaries compared to WT littermates (Fig. 8B, $P<0.001$). Interestingly, we found an important gender difference in pituitary *Bmp4* expression: *Bmp4* levels were significantly reduced in male pituitaries compared to females ($P<0.0001$).

These results show that neither BMP4 nor TGFβ3 are involved in prolactinoma development in hCGβ+ females.

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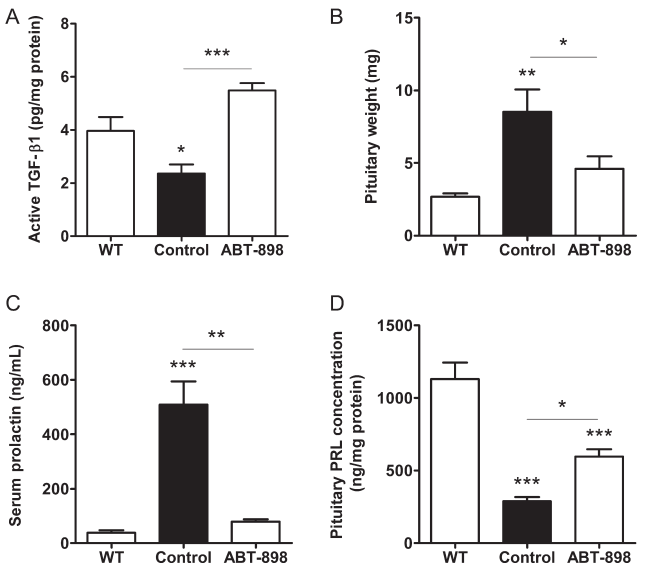

Figure 7

Thrombospondin 1 (TSP-1) synthetic analog ABT-898 normalizes pituitary active TGF-β1 level. (A) Active TGFβ1 concentration was measured by ELISA in pituitary homogenates ($n=5$–7/group). One-way ANOVA followed by Tukey's test was performed ($P=0.0007$). hCGβ+ control pituitaries showed lower active cytokine concentration levels compared to WT females (*$P<0.05$). ABT treatment increased active TGFβ1 levels in hCGβ+ pituitaries (ABT-898) compared to control hCGβ+ females (***$P<0.001$). (B) Pituitary weight (mg, $n=5$–7/group). One-way ANOVA was performed followed by Tukey's test ($P=0.0054$). hCGβ+ control females showed higher pituitary weight compared to WT (**$P<0.01$). hCGβ+ ABT-898-treated female mice showed decreased pituitary weight compared to hCGβ+ control group (*$P<0.05$). (C) Serum prolactin levels measured by RIA ($n=5$–7/group). One-way ANOVA was performed followed by Tukey's test ($P<0.0001$). hCGβ+ control females showed higher serum PRL levels compared to WT (***$P<0.001$). hCGβ+ ABT-898-treated female hCGβ+ mice showed decreased serum PRL levels compared to hCGβ+ control female group (**$P<0.01$). (D) Pituitary PRL concentration measured by RIA in pituitary homogenates ($n=5$–7/group). One-way ANOVA was performed followed by Tukey's test ($P=0.0004$). hCGβ+ control females as well as hCGβ+ ABT-898-treated females showed lower pituitary PRL concentration levels compared to WT (***$P<0.001$). However, hCGβ+ ABT-898-treated group partially recover pituitary PRL content, showing increased pituitary PRL concentration levels compared to hCGβ+ control group (*$P<0.05$).

Discussion

In the present study, we describe alterations and sex differences in the pituitary TGFβ1 system of transgenic hCGβ+ mice. Active and total cytokine levels, TGFβ1 biological activity as well as the expression of *Ltbp1*, *TβR2*, *Alk5*, *Smad4* and *Smad7* were found decreased in tumoral hCGβ+ female pituitaries when compared with WT siblings.

On the other hand, we found that male pituitaries presented higher levels of active and total cytokine than females. In accordance, we also found the expression of several other components of the system increased, including *Ltbp1*, *TβR2*, *Alk5*, *Alk1*, *Smad4* and *Smad7*, and TGFβ1 target genes (*Tmepai* and *Klf14*) in male pituitaries when compared to females.

TGFβ1 being an important inhibitory factor of lactotroph function, we postulate that: 1- decreased TGFβ1 activity found in pituitaries from hCGβ+ females is involved in their development of prolactinomas; 2- the higher expression of TGFβ1 system found in male pituitaries could protect this sex from the prolactinoma development, even in the presence of high levels of hCG.

To demonstrate the involvement of the reduced pituitary TGFβ1 activity in prolactinoma development in hCGβ+ female mice, we conducted an *in vivo* treatment to recover the pituitary-active TGFβ1 concentration. In fact, we found that ABT-898 treatment was successful in restoring pituitary TGFβ1 activity and, in accordance, pituitary weight, as well as the serum prolactin levels, were reduced in the ABT-treated hCGβ+ group.

As mentioned before, the hyperstimulation of the immature ovary by constant high hCG levels, induces a marked alteration in ovarian steroid production at early stage of sexual maturation in hCGβ+ females and induces precocious puberty, and production of high levels of estradiol, testosterone and progesterone during the first month of age. Subsequently, the persistent high hCG levels induces a massive luteinization and a constant increase in serum progesterone. By 6 months of age, females, but not males, present with large prolactinomas and marked hyperprolactinemia (Rulli *et al.* 2002, Ratner *et al.* 2012). Even though the effect of chronically elevated levels of estradiol is well known to induce experimental prolactinomas (Heaney *et al.* 1999, 2002), adult hCGβ+ females present normal estradiol levels. In a previous work, amplifying effect of progesterone was demonstrated on the growth of these estrogen-dependent tumors in hCGβ+ females (Ahtiainen *et al.* 2010). However, males exposed to high serum levels of hCG and androgens do not develop pituitary tumors (Ahtiainen *et al.* 2005). Thus, additional factors must be involved in the development of prolactinomas in hCGβ+ females.

The elevated estrogens level presented by hCGβ+ females at early age was proposed to partially explain the occurrence of prolactinoma in adulthood (Rulli *et al.* 2002, Ratner *et al.* 2012). In fact, an ovariectomy at 6 weeks of age totally abolished the pituitary gland enlargement and the hyperprolactinemia. Perhaps, this fact could initiate the process of transformation that was later accompanied by the effect of other factors, including high progesterone levels. In this regard, it was described that progesterone not only acts at the pituitary level but

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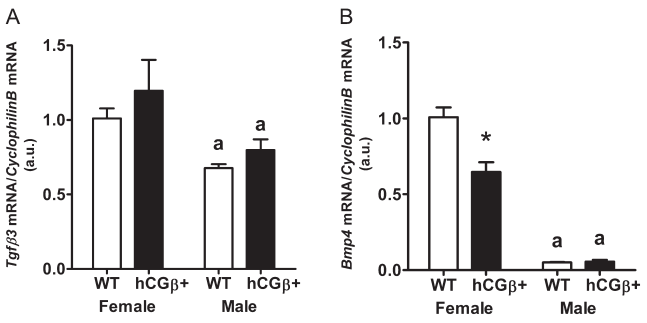

Figure 8

Other members of TGFβ family. mRNA transcripts were amplified with specific primers by qPCR and normalized to *CyclophilinB*. Results are expressed relative to those WT females ($n = 5–7$/group) and analyzed by two-way ANOVA. Bonferroni's *post hoc* test was conducted when the interaction between effects was significant. (A) *Tgfβ3* mRNA expression: genotype × sex interaction ($P = 0.9966$). Sex differences: *a*, $P < 0.0009$. No differences among genotypes were found. (B) *Bmp4* mRNA expression. Genotype × sex interaction: $P = 0.0055$. Sex differences: *a*, $P < 0.0001$. Differences among genotypes were found only in females (*$P < 0.001$ vs wt female).

also in the hypothalamus, where it suppresses messenger ribonucleic acid levels in the arcuate nucleus (Arbogast & Voogt 1993, 1994).

In fact, the high levels of progesterone present in hCGβ+ female could be involved in the decreased expression of hypothalamic *Th* we found in this group. Because TH activity is the main critical factor that controls DA synthesis, its decreased activity could influence the DA levels reaching the pituitary. In accordance, the *Drd2* expression was found increased in pituitaries from hCGβ+ female compared to their WT siblings, and it could be reflecting a lower dopaminergic tone in this group.

As DA, acting through the Drd2, upregulates TGFβ1 and *TβR2* expression in lactotrophs (Sarkar *et al.* 2005), as well as the local cytokine activity (Recouvreux *et al.* 2011), the reduced levels of active and total TGFβ1, as well as *TβR2* expression we found in tumoral pituitaries from hCGβ+ female, could reflect a lower dopaminergic tone in hCGβ+ females. However, no differences were found in hypothalamic DA concentration, measured by HPLC, among WT and hCGβ+ females. The pressure exercised on the median eminence by the prolactinoma in hCGβ+ females could prevent the release and transport of DA and other hypothalamic factors toward the pituitary. Nevertheless, with the present results, we cannot assure that the lower TGFβ1 activity found in hCGβ+ female pituitaries is a consequence of lower dopaminergic tone reaching the pituitaries in this group, and this deserves future studies.

On the other hand, in males, lower hypothalamic *Th* and higher pituitary *Drd2* expression were observed, according to lower dopaminergic tone in this sex compared to females (Gudelsky & Porter 1981, Freeman *et al.* 2000), but interestingly, the hCGβ overexpression did not induce alterations in males.

The reduced expression of the other components of TGFβ1 system we observed in hCGβ+ female pituitaries could be related to the decreased TGFβ1 biological activity found in this group. In this regard, it was described that the cytokine enhances its own expression as well as *Ltbp1* levels in several normal and transformed cells (Taipale *et al.* 1996, Weikkolainen *et al.* 2003). Moreover, a dose-related increase in *Ltbp1* production was demonstrated in response to treatment with TGFβ1 (Dallas *et al.* 1994, Koli & Keski-Oja 1995). *Smad7* transcription is also regulated by TGFβ1 through direct binding of Smad3 and Smad4 to the *Smad7* promoter (Nagarajan *et al.* 1999, Stopa *et al.* 2000). On the contrary, we did not observe genotype differences in the pituitary TGFβ1 system in males. Moreover, all the components evaluated were found increased in male pituitaries, compared to WT females, and this factor could protect them from tumor development.

Other members of the TGFβ growth factor family, such as TGFβ3 and BMP4, have also been shown to play a role in prolactinoma development. Because *Tgfβ3* is synthetized by lactotrophs, we could expect increased pituitary *Tgfβ3* expression reflecting the increase in the proportion of lactotrophs in hCGβ+ female pituitaries. However, we did not find significant genotype differences. As pituitary *Tgfβ3* mRNA levels are regulated by estradiol (Hentges *et al.* 2000), the lack of genotype differences could be a consequence of normal and physiological levels of estradiol present in hCGβ+ females (Rulli *et al.* 2002). On the other hand, the decreased *Tgfβ3* mRNA levels found in male pituitaries could be the result of lower serum estradiol level in this sex. Hence, with this result, we demonstrated that the prolactinoma development in hCGβ+ females does not depend on pituitary *Tgfβ3* expression.

Regarding pituitary BMP4 expression, it has been previously demonstrated, by immunohistochemistry, that it is principally confined to the somatotroph, corticotroph and thyrotroph cell populations, and rarely detectable in lactotroph cells in normal pituitary (Giacomini *et al.* 2006). However, BMP4 protein was found overexpressed in several experimental models of prolactinomas, and even in human prolactinomas compared with normal pituitaries (Paez-Pereda *et al.* 2003). However, and in contrast to these previous findings, other group found BMP4 overexpression only in a low proportion of the human prolactinoma assayed, finding reduced BMP4 expression in the others (Yacqub-Usman *et al.* 2012).

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When we assayed *Bmp4* mRNA expression in the hCGβ+ mouse model, we found it significantly reduced in hCGβ+ female pituitaries compared with their WT siblings. On the other hand, we found a sharp gender difference. Male pituitaries express greatly reduced levels of *Bmp4* without differences among genotypes. It would therefore be worth finding out the causes and consequences of (a) the gender differences found and (b) the decreased *Bmp4* mRNA expression found in hCGβ+ female pituitaries. With the present results, we could assure that the prolactinoma development in hCGβ+ female does not depend on *Bmp4* overexpression.

Gender differences in prolactinoma incidence and behavior have been previously described. Women present higher prevalence of prolactinomas during the fertile period (20–50 years), when the tumor ratio between the sexes is estimated to be 10:1. But after the fifth decade of life, when serum estradiol decreases, this sexual difference disappears and the frequency is similar between sexes. The higher levels of serum estradiol have been proposed to be involved in the higher incidence of prolactinomas in fertile women (Colao *et al.* 2003, Gillam *et al.* 2006).

Interestingly, we have previously demonstrated that estradiol negatively controls most of the components of the TGFβ1 system (Recouvreux *et al.* 2013). In fact, we here described sex differences in the pituitary TGFβ1 system in this model, as we had previously observed in another well-characterized model of prolactinoma, the transgenic knockout mice lacking functional dopamine receptor type 2 (Drd2$^{-/-}$).

In summary, we postulate that the reduced TGFβ1 activity we found in hCGβ+ female pituitaries is involved in the development of female prolactinomas. We demonstrated that by enhancing the pituitary TGFβ1 activity, we succeeded in reducing the tumor growth, decreasing the hyperprolactinemia and recovering the inhibition of hormone secretion in hCGβ+ females.

Even though the high serum progesterone levels could amplify the effect of normal estradiol, acting directly on lactotroph proliferation, it also induced a decrease in hypothalamic TH, and it could decrease the dopamine levels reaching the pituitary. The influence of these factors merits future studies.

On the other hand, the sex differences observed in regulation of the pituitary TGFβ1 system could explain the gender differences found in the incidence of prolactinoma development: the stronger TGFβ1 system found in male pituitaries could protect them from excessive lactotroph proliferation.

Finally, prolactinomas are the most prevalent type of hormone-secreting pituitary tumors in humans, and generally respond well to the therapies with dopamine agonists. However, for patients exhibiting resistance to these drugs, alternative treatments are desired. Our results place the synthetic TSP-1 analogue as potential alternative or complementary therapy in current treatments against prolactinomas, especially in those that are resistant to dopaminergic drugs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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