Sex Differences in the Pituitary Transforming Growth Factor-β1 System: Studies in a Model of Resistant Prolactinomas

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Dopamine and estradiol interact in the regulation of lactotroph cell proliferation and prolactin secretion. Ablation of the dopamine D2 receptor gene (*Drd2*$^{-/-}$) in mice leads to a sexually dimorphic phenotype of hyperprolactinemia and pituitary hyperplasia, which is stronger in females. TGF-β1 is a known inhibitor of lactotroph proliferation. TGF-β1 is regulated by dopamine and estradiol, and it is usually down-regulated in prolactinoma experimental models. To understand the role of TGF-β1 in the gender-specific development of prolactinomas in *Drd2*$^{-/-}$ mice, we compared the expression of different components of the pituitary TGF-β1 system, including active cytokine content, latent TGF-β–binding protein isoforms, and possible local TGF-β1 activators, in males and females in this model. Furthermore, we evaluated the effects of dopamine and estradiol administration to elucidate their role in TGF-β1 system regulation. The expression of active TGF-β1, latent TGF-β–binding protein isoforms, and several putative TGF-β1 activators evaluated was higher in male than in female mouse pituitary glands. However, *Drd2*$^{-/-}$ female mice were more sensitive to the decrease in active TGF-β1 content, as reflected by the down-regulation of TGF-β1 target genes. Estrogen and dopamine caused differential regulation of several components of the TGF-β1 system. In particular, we found sex- and genotype- dependent regulation of active TGF-β1 content and a similar expression pattern for 2 of the putative TGF-β1 activators, thrombospondin-1 and kallikrein-1, suggesting that these proteins could mediate TGF-β1 activation elicited by dopamine and estradiol. Our results indicate that (1) the loss of dopaminergic tone affects the pituitary TGF-β1 system more strongly in females than in males, (2) males express higher levels of pituitary TGF-β1 system components including active cytokine, and (3) estradiol negatively controls most of the components of the system. Because TGF-β1 inhibits lactotroph proliferation, we propose that the higher levels of the TGF-β1 system in males could protect or delay the development of prolactinomas in *Drd2*$^{-/-}$ male mice. **(*Endocrinology* 154: 4192–4205, 2013)**

A growing number of studies demonstrate that the prevalence, incidence, and severity of diseases depend on physiological, hormonal, and genetic differences between sexes and that these differences might affect the responses to therapies (1). Currently, consideration of these sex differences in the pharmacokinetics and pharmacodynamics of drugs is thought to be essential for improving therapeutic efficacy while minimizing adverse events (2). It is well known that gonadal hormones are the earliest and direct cause of sex differences.

The prolactin (PRL)–secreting adenomas (ie, prolactinomas) usually present sexual differences in their incidence, tumor size, and behavior. Microprolactinomas occur more frequently in women than in men, particularly

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Abbreviations: Drd2, dopamine D2 receptor; HSD, honestly significant difference; KLK1, kallikrein 1; LAP, latency-associated peptide; KO, knockout; LTBP, latent TGF-β–binding protein; MMP, matrix metalloproteases; PAI-1, plasminogen-activator inhibitor; PRL, prolactin; qPCR, quantitative real-time PCR; TβRII, TGF-β type II receptor; TIDA, tuberoinfundibular dopaminergic system; TMEPAI, transmembrane prostate androgen-induced protein; TSP1, thrombospondin-1; WT, wild-type.

between the second and third decades of life (fertile period), when the tumor ratio between the sexes is estimated to be 10:1. Interestingly this sexual difference disappears after the fifth decade of life, when serum estradiol decreases in women, and from that age onward the frequency of prolactinomas is similar in both sexes (3).

Dopamine and estradiol are the main physiological regulators of lactotroph function, exerting opposing actions, inhibitory and stimulatory, respectively, on prolactin secretion and cell proliferation (4). Dopamine, through the dopamine D2 receptor (Drd2), expressed in lactotrophs, inhibits PRL synthesis and release as well as lactotroph proliferation (5, 6). On the other hand, estradiol induces PRL secretion, not only by stimulating PRL gene expression in lactotrophs but also by modifying lactotrophic responses to other inhibitory and stimulatory factors (7). In addition, estradiol stimulates lactotroph function indirectly by decreasing hypothalamic dopamine production (7).

Deregulation in the control of lactotroph proliferation can lead to the development of prolactinomas. Prolactinomas are the most prevalent type of pituitary tumor in humans and usually remain responsive to dopamine inhibition, with dopaminergic agonists being the first choice of therapy. However, about 10% to 15% of patients exhibit resistance to dopaminergic drugs, and, currently, there are no alternative therapies. Finding alternative mechanisms to inhibit lactotroph function might provide new therapies for these resistant tumors.

An excellent model to study Drd2-resistant prolactinomas is the transgenic mouse lacking functional Drd2 receptors (*Drd2*$^{-/-}$; knockout [KO]). *Drd2*$^{-/-}$ mice display chronic hyperprolactinemia and pituitary lactotroph hyperplasia (8, 9). Interestingly, the loss of Drd2 causes a more pronounced effect in females than in males (8, 10). At 8 months of life, the lack of dopaminergic control increases serum PRL levels by 15-fold in females and only 7-fold in males. Moreover, females develop lactotroph hyperplasia from 6 month onward, whereas age-matched Drd2-deficient males have no morphological adenohypophysial lesions (11). However, both males and females develop pituitary lactotroph adenomas at 17 to 20 months of age.

Besides dopamine and estradiol, many other growth factors, hypothalamic releasing factors, and neurotransmitters regulate lactotroph function (for a review, see Ref. 7). Among them, TGF-β1 represents a key candidate for mediating the effects of dopamine and estradiol on lactotroph physiology. TGF-β1 and its receptor, TGF-β type II receptor (TβRII), are expressed in lactotrophs, and this cytokine inhibits lactotroph cell proliferation and PRL secretion by an autocrine/paracrine mechanism (12–14).

Estradiol has been shown to decrease TGF-β1 synthesis and release and lactotroph TβRII expression (12, 15). On the other hand, dopamine, acting on Drd2s, up-regulates TGF-β1 expression (16). Interestingly, it was suggested that TGF-β1 partly mediates dopamine inhibitory action on lactotrophs (12, 17). Therefore, because dopamine and estradiol are the most important regulators of lactotroph function, and they both regulate pituitary TGF-β1 availability, we postulate that the TGF-β1 system is a target of the estradiol-dopamine interaction in the regulation of PRL secretion and lactotroph proliferation. Moreover, sex differences in the pituitary TGF-β1 system could explain sex differences in the control of lactotroph function.

TGF-βs are synthesized as larger precursor molecules that contain a propeptide called latency-associated peptide (LAP). LAP is processed by furin-like enzymes within the trans-Golgi but remains associated with the mature cytokine by noncovalent interactions (18). In addition, while still in the endoplasmic reticulum, the LAP-TGF-β complex is linked by covalent association to the latent TGF-β–binding protein (LTBP). The large latent TGF-β complex is secreted and incorporated through LTBP interaction as a component of the extracellular matrix, which acts as a cytokine reservoir. Within the large latent TGF-β complex, TGF-β remains latent and must undergo a highly regulated activation process by which TGF-β is released from LAP to bind to its receptor (18, 19). Therefore, latent TGF-β activation is a crucial event in governing cytokine biological activity.

Several latent TGF-β1 activators have been described, including proteases, thrombospondin-1 (TSP1), integrins αvβ6 and αvβ8, and reactive oxygen species, among others. However, their individual biological importance in releasing TGF-β1 from its latent complex and their local regulation in different tissues are still not fully understood (18, 20, 21).

Neither the regulation of the components of the pituitary TGF-β1 system nor the local TGF-β1 activation has been studied previously, and, therefore, the pituitary TGF-β1 activators are still not known. Because TGF-β1 inhibits lactotroph function and TGF-β1 was found to be down-regulated in 2 different experimental models of prolactinomas (14, 15, 22), studying the regulation of the pituitary TGF-β1system might be useful to identify novel targets for alternative treatments in resistant prolactinomas.

The aim of the present study was to characterize the components of the pituitary TGF-β1 system, the sex differences, and their modulation by dopamine and estrogen. We also evaluated the impact of the loss of dopaminergic control in these components, comparing wild-type (WT) and *Drd2*$^{-/-}$ mice.

Materials and Methods

Female and male *Drd2*$^{-/-}$ mice (official strain designation B6.129S2-*Drd2*^{tm1Low}/J by the Induced Mutant Resource at The Jackson Laboratory), generated by targeted mutagenesis of the *Drd2* gene in embryonic stem cells, were used (9, 11). The original F2 hybrid strain (129S2/Sv × C57BL/6J), containing the mutated *Drd2* allele, was backcrossed for 8 generations to WT C57BL/6J mice. Both isoforms of Drd2 (D2L and D2S) are nonfunctional in mutant mice. Mutant and WT mice were generally the product of heterozygous crossings, and, in all cases, sibling controls were used. Mice were housed in groups of 4 or 5 with mixed genotypes in an air-conditioned room with lights on at 7:00 AM and off at 7:00 PM. Animals had free access to laboratory chow and tap water. *Drd2*$^{+/+}$ (WT), heterozygous, and *Drd2*$^{-/-}$ mice were identified by PCR of genomic DNA as described previously (8). Animals were used at 8 months of age, at which time the pituitary glands from *Drd2*$^{-/-}$ females were hyperplastic (pituitary weight, 2.43 ± 0.08 vs 4.56 ± 0.22 g, WT vs *Drd2*$^{-/-}$, respectively; $P < .001$). Female mice were randomly cycling during the experiments. All experimental procedures were reviewed and approved by the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires (Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, A#5072–01).

In vivo experiments

WT female and male mice were injected IP with saline solution (control group), the Drd2 antagonist sulpiride (10 mg/kg; IVAX Laboratories), or the Drd2 agonist cabergoline (2 mg/kg; Beta Laboratories). Animals were killed by decapitation after 30 minutes (short-term) or 24 hours (long-term) of treatment.

Another set of *Drd2*$^{-/-}$ and *Drd2*$^{+/+}$ mice were injected with estradiol valerate (0.2 mg/kg SC, Progynon Depot; Schering) or castor oil (control group) and killed 24 hours later. After every treatment, trunk blood was collected, and anterior pituitaries were removed. Sera were kept at −20°C until RIAs were performed. Pituitaries were excised as described below for Western blot, quantitative real-time PCR (qPCR), or ELISA assays.

qPCR

Pituitaries from different experimental groups were collected in RNA*later* (Ambion). Total RNA was extracted from

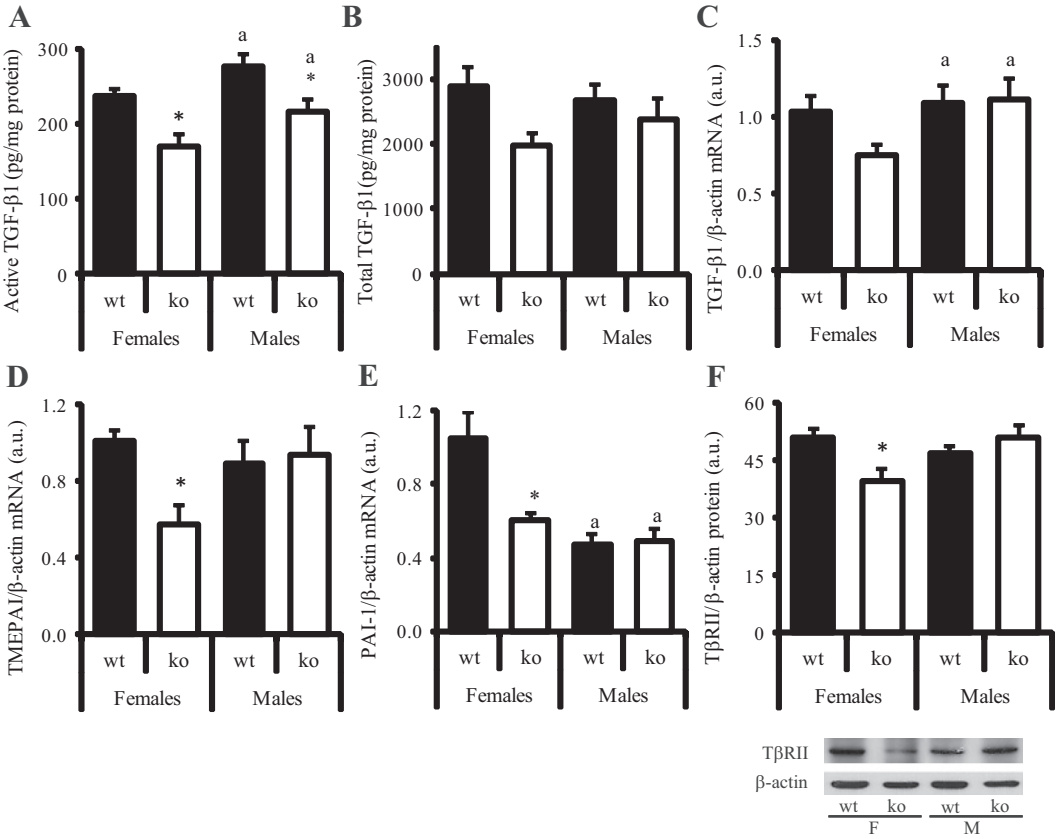

Figure 1. Sex differences in active and total TGF-β1 content in pituitaries of WT and *Drd2*$^{-/-}$ (KO) mice. A, Active TGF-β1 was measured by ELISA in pituitary samples (n = 10–15/group). Significant differences among genotypes (*, $P < .0001$) and sexes (a, $P = .004$) were found. B, Total TGF-β1 content was measured by ELISA after sample acidification with 1 N HCl (n = 8–13/group). C–E, mRNA transcripts indicated were amplified with specific primers by qPCR and normalized to β-actin. Results are expressed relative to those for WT females (n = 4–9/group). C, TGF-β1 mRNA levels. a, $P = .007$ vs genotype-matched females. D, TMEPAI mRNA expression. Genotype × sex interaction, $P = .035$. a, $P = .037$ vs WT females. E, PAI-1 mRNA levels. Genotype × sex interaction, $P = .038$. a, $P = .001$ vs genotype-matched females. Differences among genotypes were observed only in females (*, $P = .042$ vs WT females). F, TβRII protein expression relative to that of β-actin was evaluated by Western blot in pituitary samples and quantified by densitometry (n = 5–7/group). Representative immunoreactive bands are shown. Genotype × sex interaction, $P = .005$. *, $P = .014$ vs WT females. a, $P = .029$ vs KO female. Statistical analysis: 2-way ANOVA followed by a Tukey post hoc test when the interaction between effects was significant. a.u., arbitrary units; F, female; M, male.

the tissue using an RNeasy Protect Mini Kit (Qiagen). RT was performed using 750 ng of total RNA, and the resulting cDNA was used for qPCR analysis. qPCRs were performed using specific primers and the QuantiFast SYBR Green PCR kit (Qiagen) on an iCycler Thermal Cycler (Bio-Rad). Target gene expression was quantified by comparing the threshold cycle (C_T) with that of β-actin by using the comparative C_T method ($\Delta\Delta C_T$). The primer sequences used for qPCR can be found in Supplemental Table 1.

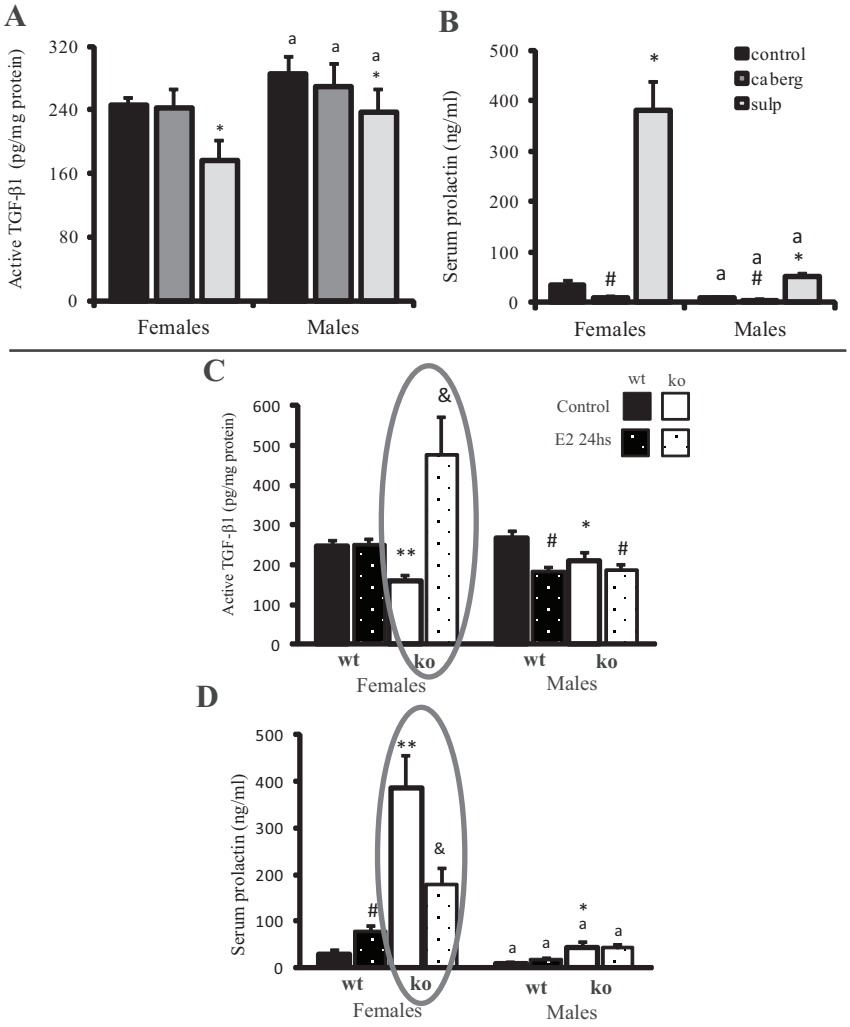

Figure 2. Sex differences in active TGF-β1 regulation by dopamine and estradiol in vivo. A, Active TGF-β1 content (picograms per milligram of protein) measured by ELISA in pituitary extracts of WT male and female mice after a 30-minute in vivo treatment with the Drd2 agonist, cabergoline (caberg, 2 mg/kg IP), the Drd2 antagonist, sulpiride (sulp, 10 mg/kg IP), or saline (control) (n = 5–10/group). *, $P = .03$ vs sex-matched control; a, $P = .03$ vs treatment-matched females. B, Serum PRL concentration in the experimental animals shown in A, measured by RIA. #, $P = .042$; *, $P = .0001$ vs sex-matched controls; a, $P = .008$ vs treatment-matched females. C, active TGF-β1 content measured by ELISA in pituitary extracts of male and female mice after 24 hours of treatment with estradiol valerate (E2 24hs, 0.2 mg/kg SC) or castor oil (control, SC). Males and females showed a differential response to E2 treatment (factorial ANOVA, interaction of genotype × treatment × sex, $P = .005$). Posterior analysis by 2-way ANOVA in each sex showed the following: in females, interaction of genotype × treatment, $P < .0001$; **, $P = .001$ vs WT control; &, $P = .0001$ vs KO control (n = 7–11); and in males, interaction of genotype × treatment, $P = .03$; #, $P = .0002$ vs genotype matched control; *, $P = .003$ vs WT control (n = 7–11). D, Serum PRL values of experimental animals used in panel C, measured by RIA. The triple interaction was not significant. a, $P < .0001$ vs genotype and treatment-matched females. Interaction of genotype × treatment, $P = .0006$. In females, #, $P = .006$ vs WT control; **, $P = .0001$ vs WT control; &, $P = .0001$ vs KO control. In males, *, $P = .003$ vs WT control. Statistical analysis: factorial ANOVA to evaluate the effects of sex, genotype, and treatment. To simplify the analysis, we next evaluated the effect of estradiol valerate treatment and genotype separately in males and females by 2-way ANOVA followed by a Tukey post hoc test. Gray circles highlight the inverse correlation among active cytokine and serum PRL, found only in KO females.

Detection of total and active TGF-β1

ELISAs were performed to quantify active or total TGF-β1 content in pituitary homogenates of WT and *Drd2*$^{-/-}$ female and male mice with a TGF-β1 Emax ImmunoAssay System (Promega).

Pituitaries were excised and homogenized, as described for Western blotting, and the homogenates were centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant protein contents were measured with a Qubit fluorometer and a Quant-iT Protein Assay Kit (Invitrogen).

TGF-β1 is expressed as picograms per milligram of protein. The minimum detectable dose of biologically active TGF-β1 was 32 pg/mL with less than 3% cross-reactivity with TGF-β2 and TGF-β3 at 10 ng/mL.

To assay total TGF-β1, samples were acidified to pH 2.6 by adding 1 N HCl for 20 minutes at room temperature, followed by neutralization with 1 N NaOH to pH 7.6.

The details of the procedures for Western blotting, RIA, and gelatin zymography are described in the Supplemental Materials and Methods.

Statistical analyses

Results are expressed as means ± SEM. Comparisons among sexes and genotypes were evaluated by a 2-way ANOVA followed by a Tukey honestly significant difference (HSD) test when the interaction between effects was significant. Dopaminergic drug treatments were compared by 1-way ANOVA followed by a Tukey HSD test. Active TGF-β1 content and serum PRL values (see Figure 2, C and D) were analyzed by 3-way ANOVA to test the interaction between sex, genotype, and treatment effects. Further analysis was performed separately in each sex by 2-way ANOVA. The effect of estradiol treatment on mRNA expression was compared by 2-way ANOVA followed by a Tukey HSD test separately for each sex.

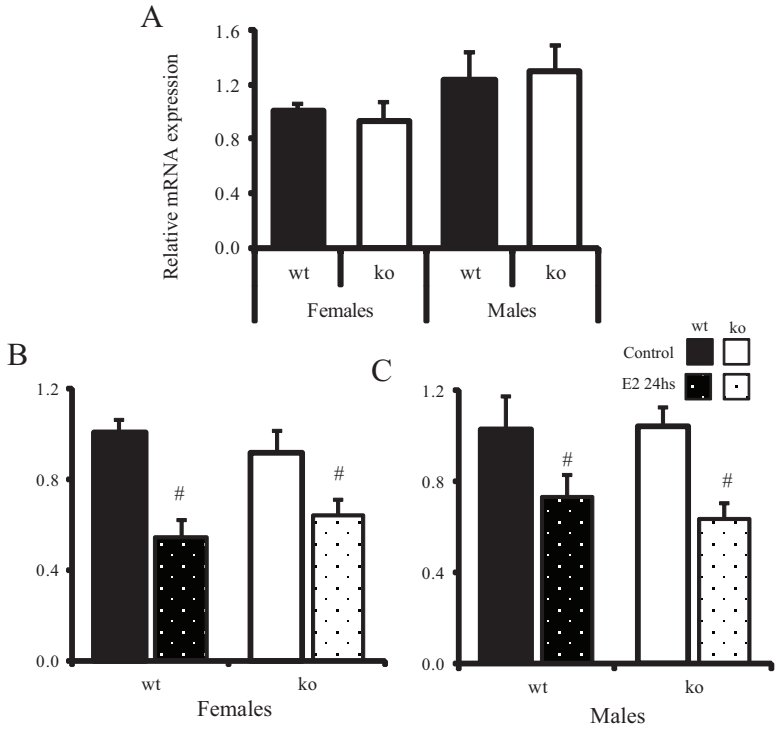

Figure 3. Furin expression and regulation in the pituitary glands of WT and *Drd2*$^{-/-}$ mice. Furin mRNA expression was detected by qPCR and normalized to that of β-actin. A, Comparative mRNA levels among sexes and genotypes, relative to those of WT females (n = 6–7). B and C, Effect of an in vivo treatment with estradiol valerate (E2 24hs, 0.2 mg/kg SC) or castor oil (control SC) after 24 hours in females (n = 4–7, B) and males (n = 5, C). Results are expressed relative to the WT control group of each sex. #, $P = .0006$ vs genotype-matched control in females (B); #, $P = .0027$ vs genotype-matched control in males (C). Statistical analysis: 2-way ANOVA.

Results

Sex differences in active and total TGF-β1 content in pituitaries from *Drd2*$^{+/+}$ and *Drd2*$^{-/-}$ mice

Because the dopaminergic tone at the pituitary level is lower in males than in females (23) and dopamine controls TGF-β1 activity (14), we first sought to evaluate sex differences in local active and total TGF-β1 and the impact of the absence of functional Drd2s in both sexes. We found that male pituitaries contained higher levels of active cytokine ($P = .004$) (Figure 1A) and mRNA levels ($P = .007$) (Figure 1C) than female pituitaries, despite the fact that there are equal amounts of total TGF-β1 in both sexes (Figure 1B). The loss of dopaminergic control in *Drd2*$^{-/-}$ mice caused a decrease in active pituitary TGF-β1 levels in both sexes ($P < .0001$) (Figure 1A). To evaluate whether the lower basal levels of active TGF-β1 in Drd2 KO pituitaries affect the biological activity of the cytokine, we measured the expression of 2 TGF-β1-inducible genes: the androgen-induced transmembrane protein (transmembrane prostate androgen-induced protein [TMEPAI]) (24, 25) and the plasminogen-activator inhibitor 1 (PAI-1) (26). TMEPAI and PAI-1 mRNA expression were downregulated only in female *Drd2*$^{-/-}$ compared with WT pituitaries (Figure 1, D and E, respectively), although decreased levels of active TGF-β1 were found in *Drd2*$^{-/-}$ mice of both sexes.

The lower levels of TMEPAI and PAI-1 mRNA expression in female KO pituitaries were consistent with the lower TβRII expression that we described earlier (14). On the other hand, no genotype differences were found in TβRII expression in males (Figure 1F), in accordance with the unaltered expression of TGF-β1 target genes, suggesting that intact cytokine activity is maintained despite the lower TGF-β1 active levels in KO males.

Sex differences in dopaminergic and estrogenic regulation of pituitary active TGF-β1

We next explored the effect of a dopaminergic Drd2 agonist (cabergoline) and an antagonist (sulpiride) on pituitary active TGF-β1 content in WT mice. We found that a 30-minute treatment with sulpiride (10 mg/kg body weight IP) decreased active TGF-β1 in pituitaries in both sexes (Figure 2A), whereas no effect of cabergoline (2 mg/kg body weight IP) was observed. Serum PRL was measured by RIA as a control of the dopaminergic drug effect (Figure 2B). As expected, serum PRL levels were stimulated by sulpiride ($P = .0001$) and decreased by cabergoline ($P = .042$) in both sexes.

We have previously reported that the estradiol effect on pituitary active TGF-β1 depends on the presence of an intact dopaminergic control in female mice (14). Interestingly, a 24-h treatment with estradiol valerate (0.1 mg/kg) elicited a differential response on active TGF-β1 content, which was dependent on sex and genotype (interaction of genotype × treatment × sex effect, $P = .005$) (Figure 2C). Estradiol treatment caused a decrease in pituitary active TGF-β1 content in WT males, whereas WT females showed no response to the steroid. On the other hand, when dopaminergic control is lost (KO pituitaries), no effect of estradiol was found in KO males, whereas a strong increase in active TGF-β1 was observed in pituitaries from Drd2 KO females ($P < .0001$).

A differential estradiol effect on serum PRL between the sexes was also observed: no differences were found in

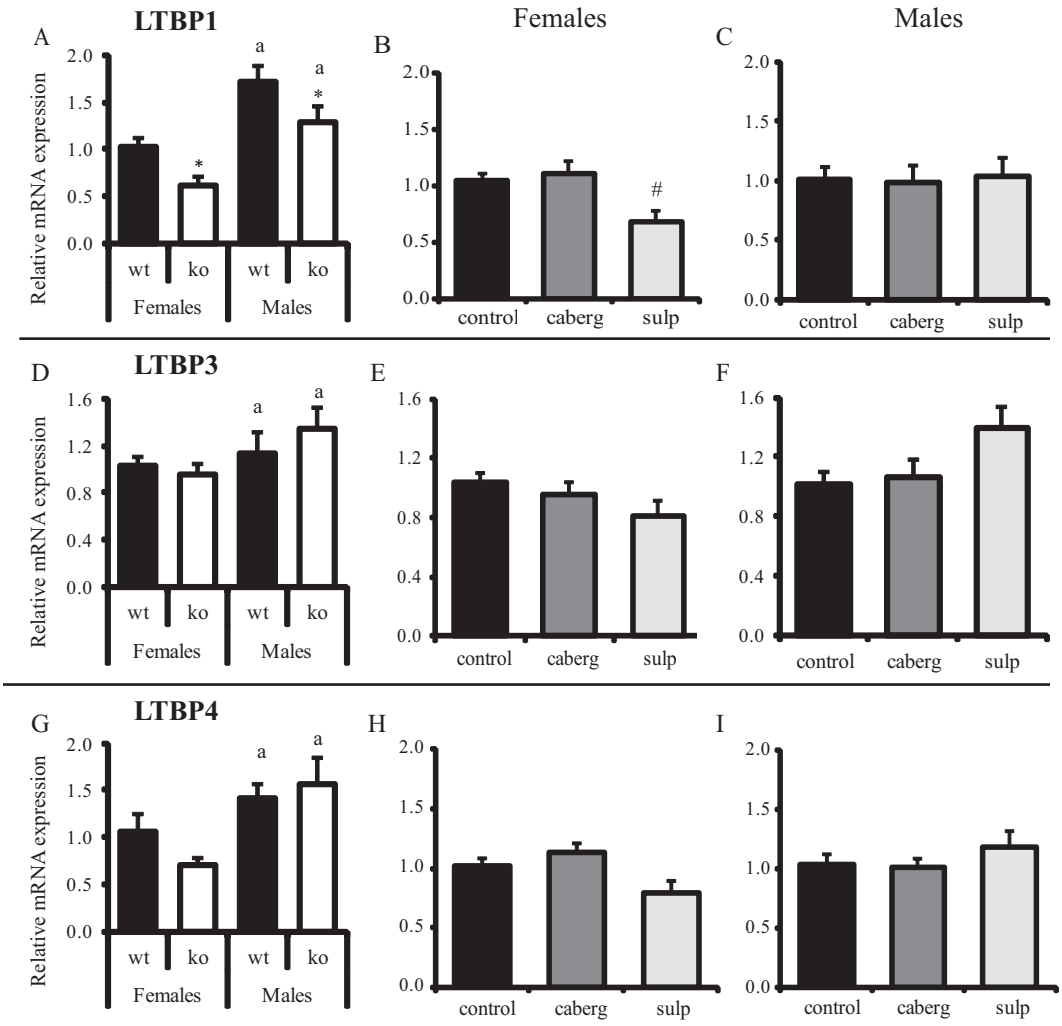

Figure 4. LTBP expression in the pituitary glands of WT and *Drd2*$^{-/-}$ mice. LTBP1, LTBP3, and LTBP4 mRNA expression levels were evaluated by qPCR and normalized to that of β-actin. Each isoform expression was compared among genders and genotypes (A, D, and G). Dopaminergic regulation of LTBPs expression was evaluated by in vivo treatment with cabergoline (caberg, 2 mg/kg IP), sulpiride (sulp, 10 mg/kg IP) or saline (control, IP) for 24 hours in WT female (B, E, and H) and male mice (C, F, and I). Statistical analysis: 2-way ANOVA for sex and genotype comparison and 1-way ANOVA for dopaminergic drug effect. The interaction between effects was not significant. *, sex-matched differences among genotypes; a, genotype-matched differences among sexes; #, treatment vs control differences. A, *, $P < .0001$; a, $P = .0036$. B, #, $P = .024$. D, a, $P = .007$. G, a, $P = .032$. n = 5–8 animals/group.

males, regardless of the genotype, whereas WT and KO females responded in opposite ways, showing a strong interaction between dopamine and estradiol on PRL regulation. Inverse correlation between pituitary active TGF-β1 content and PRL serum was observed only in KO females, as we described previously (14).

Characterization of other components of the pituitary TGF-β1 system

Furin expression

The LAP-TGF-β precursor molecule is processed by furin-like enzymes within the trans-Golgi. Because other protein convertases, such as PC1 and PC2, have been shown to be regulated by dopamine through Drd2 (27), we next studied the expression of furin in pituitary homogenates to evaluate whether alterations in this first step in TGF-β processing could explain sex and genotype differences found in synthesis, storage, and activity of the cytokine. Furin mRNA expression was similar in pituitaries form both sexes and genotypes (Figure 3A). Accordingly, we did not find any effect of a 24-h treatment with cabergoline or sulpiride in WT mice (data not shown). However, a 24-hour estradiol treatment caused a downregulation of furin mRNA expression regardless of genotype in both females (Figure 3B) ($P = .0006$) and males (Figure 3C) ($P = .0027$).

Pituitary LTBP isoform characterization

Among the 4 LTBP isoforms described (1, 2, 3, and 4), only LTBPs 1, 3, and 4 complex to the TGF-β1 propeptide

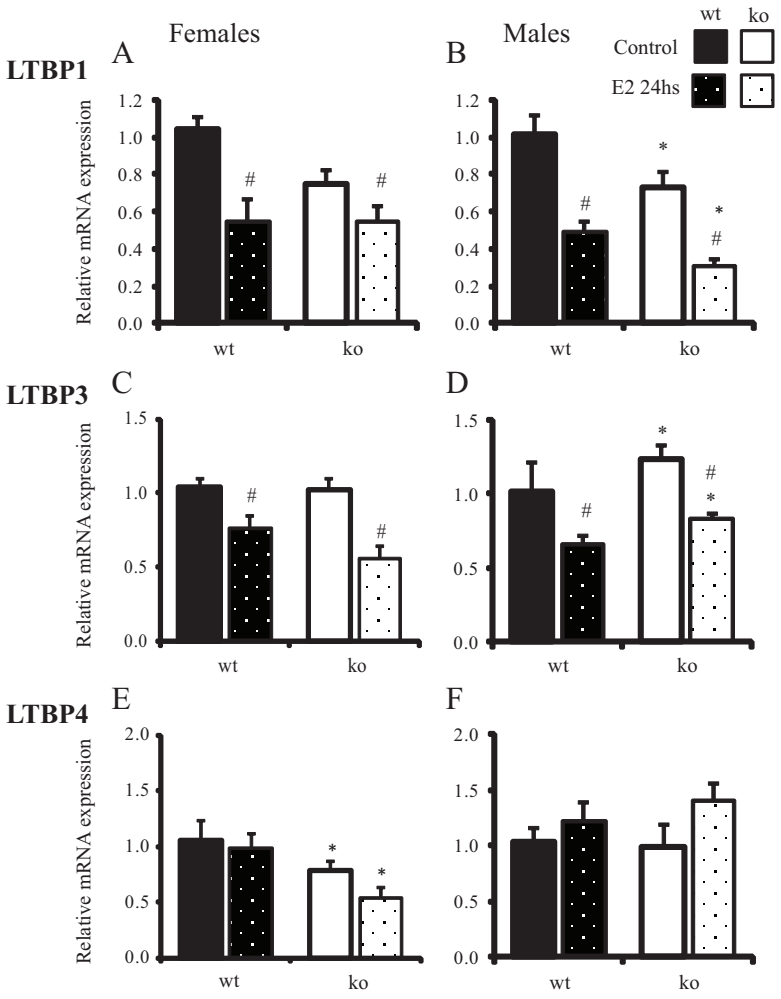

Figure 5. Estrogenic regulation of LTBP mRNA expression. WT and *Drd2*$^{-/-}$ female and male mice were in vivo treated with estradiol valerate (E2 24hs, 0.2 mg/kg SC) or castor oil (control SC) for 24 hours, and each LTBP mRNA expression was detected by qPCR as indicated. Statistical analysis: 2-way ANOVA. The interaction between effects was not significant. *, treatment-matched differences among genotypes; #, genotype-matched differences among treatment with estrogen valerate. A, #, $P < .0001$. B, *, $P = .038$; #, $P = .002$. C, #, $P = .001$. D, *, $P = .047$; #, $P = .0006$. E, *, $P = .01$. n = 4–8 animals/group.

(19). We recently described the expression of LTBP isoforms in normal and tumoral pituitaries in rats (22). However, at that time, there were no data reflecting LTBP synthesis and regulation in the pituitary. Therefore, we extended our observations to quantify LTBP levels in WT and *Drd2*$^{-/-}$ mice of both sexes and their regulation by dopamine and estradiol.

We found that pituitary expression of LTBP1, LTBP3, and LTBP4 mRNA was higher in males than in females (Figure 4). Only LTBP1 was reduced in *Drd2*$^{-/-}$ pituitaries of both sexes ($P < .0001$), suggesting a dopaminergic regulation of its synthesis. However, an in vivo treatment with the Drd2 antagonist sulpiride in WT mice decreased LTBP1 mRNA expression only in females (Figure 4B). LTBP3 and LTBP4 isoform expression was not regulated by sulpiride or cabergoline in either sex. After 24 hours, estradiol treatment significantly decreased mRNA levels of LTBP1 and LTBP3 in both sexes and genotypes (Figure 5 A–D).

Local TGF-β1 activators

TGF-β1 activators in the pituitary have not yet been described. The fact that we found decreased content of active TGF-β1 in male and female *Drd2*$^{-/-}$ pituitaries, with no differences in total cytokine levels, led us to hypothesize that one or more of the local activators could be altered in KO pituitaries. Furthermore, the regulation we found of active TGF-β1 levels by estradiol and dopamine suggests that these hormones must be modulating the action of local activators. To identify putative TGF-β1 activators in the pituitary, we studied the expression and regulation of several molecules with the ability to release mature TGF-β1 from its latent complexes, which were previously described in the literature (18).

Matrix metalloproteases (MMPs). MMP2 and MMP9 activity was evaluated by gelatin zymography. We could only detect the inactive zymogens, pro-MMP2 and pro-MMP9, suggesting very low or absent basal activity of pituitary gelatinases (see Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://end.endojournals.org). We found that both pro-MMP2 and pro-MMP9 were decreased in female and male *Drd2*$^{-/-}$ pituitaries with respect to those in the WT (Supplemental Figure 1). Males showed lower pro-MMP2 levels than females, whereas no differences were observed in pro-MMP9 levels between sexes. MMP2 mRNA expression, measured by qPCR was decreased in the pituitaries of both female and male KO mice (Figure 6A). Contrary to what we observed by zymography, males showed higher expression of MMP2 mRNA than females.

Pro-MMP2 activation occurs at the cell surface and is mediated primarily by MT-MMPs, a subfamily of transmembrane domain MMPs, including MT1-MMP. MT1-MMP not only is necessary for MMP2 activation but also participates in the activation of TGF-β1 (28–30). We

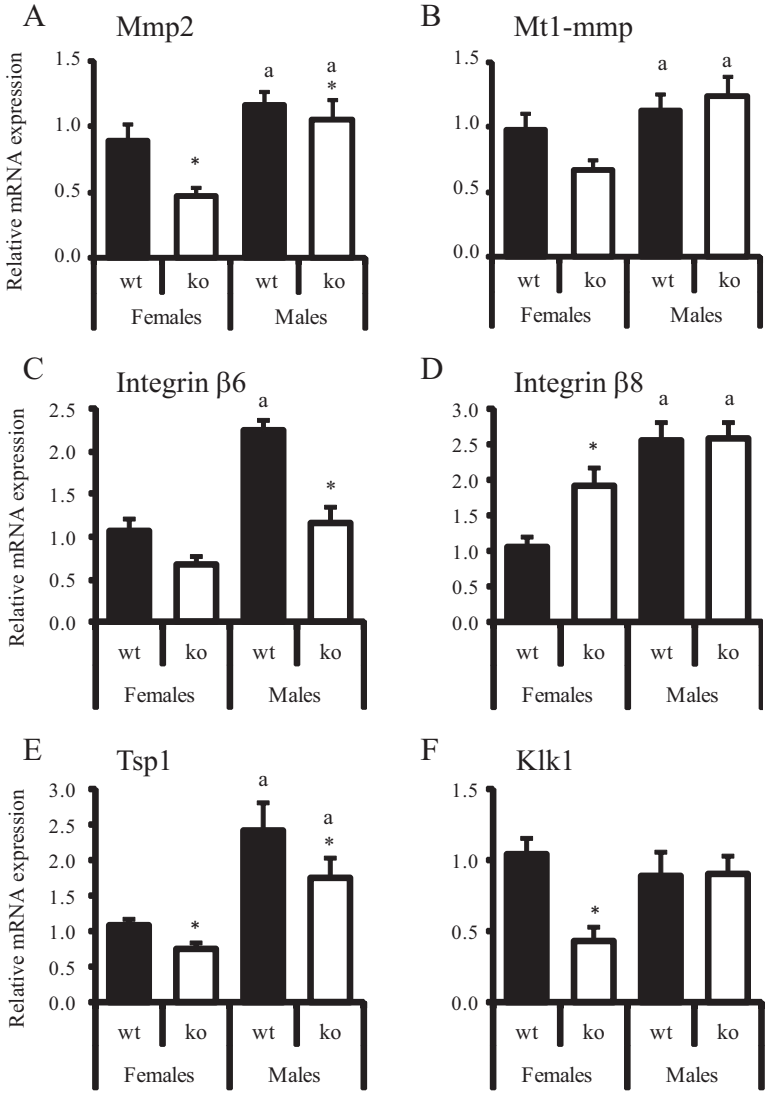

Figure 6. Expression of possible TGF-β1 activators in the pituitary gland of WT and *Drd2*$^{-/-}$ mice. mRNA expression of each of the transcripts indicated was evaluated by qPCR. The comparison between sexes and genotypes was analyzed by 2-way ANOVA. A, *, $P = .025$ vs sex-matched WT; a, $P = .0008$ vs genotype-matched females. B, a, $P = .01$ vs genotype-matched females. C, Sex × genotype interaction, $P = .014$. *, $P < .0002$ vs the rest of the groups; a, $P = .0005$ vs WT females. D, Sex × genotype interaction, $P = .06$. *, $P = .047$ vs WT females. a, $P = .0005$ vs WT females. E. *, $P = .025$ vs gender-matched WT; a, $P < .0001$ vs genotype-matched females. F, Sex × genotype interaction, $P = .025$. *, $P = .013$ vs WT female. n = 4–9/group.

found higher levels of MT1-MMP mRNA in males than in females (Figure 6B). Differences between genotypes or interactions between effects were not found by 2-way ANOVA.

Integrins. The activation of TGF-β1 mediated by integrins occurs by their interaction with the amino acid sequence RGD present in LAP. Among the large number of integrins that recognize this RGD sequence, the integrins $\alpha v\beta 6$ and $\alpha v\beta 8$ efficiently bind and activate the latent TGF-β1 (30–32). We found different expression patterns of integrins $\alpha v\beta 6$ and $\alpha v\beta 8$ in male and female pituitaries (Figure 6, C and D). Integrin $\beta 6$ and $\beta 8$ mRNA expression was higher in the pituitaries of WT males than in those of WT females. Both integrins displayed differential regulation by dopamine. In the case of integrin $\beta 6$, although male KO pituitaries presented lower mRNA levels, dopaminergic regulation was not observed after 24 hours of treatment with cabergoline or sulpiride in either sex (Figure 7, A and B). On the contrary, integrin $\beta 8$ expression was strongly dependent on negative dopaminergic control in females (Figure 7C). Integrin $\beta 6$ and $\beta 8$ mRNA expression was inhibited by estrogen, regardless of the genotype and sex (Figure 8, A–C). Integrin $\beta 8$ was the only potential TGF-β1 activator evaluated that was overexpressed in female hyperplastic *Drd2*$^{-/-}$ pituitaries.

TSP1 and kallikrein-1 (KLK1). TSP1 is one of the major TGF-β1 activators described in multiple tissues. We found that males expressed higher TSP1 mRNA levels than females (Figure 6E), and *Drd2*$^{-/-}$ mice of both sexes showed lower TSP1 pituitary expression than their WT counterparts ($P = .025$). Interestingly, only females showed a positive dopaminergic regulation evidenced by the inhibitory effect of in vivo sulpiride treatment on TSP1 expression (Figure 7E). Contrary to what was found with the previous activators evaluated, treatment with estradiol (24 hours) caused an increase in TSP1 levels in both male and female pituitaries in both genotypes (Figure 8E).

Kallikreins comprise a family of serine proteases of similar structure and are found in various tissues and biological fluids involved in the specific processing of propeptide bioactive precursors into their products. Among them, KLK1 was described in rat pituitary more than 10 years ago (33). KLK1 showed a marked sexual difference, with higher expression in females than in males, and it was detected by immunohistochemistry in PRL-secreting cells (34). Previous studies showed dopaminergic and estrogenic regulation of KLK1 in the pituitaries of female rats

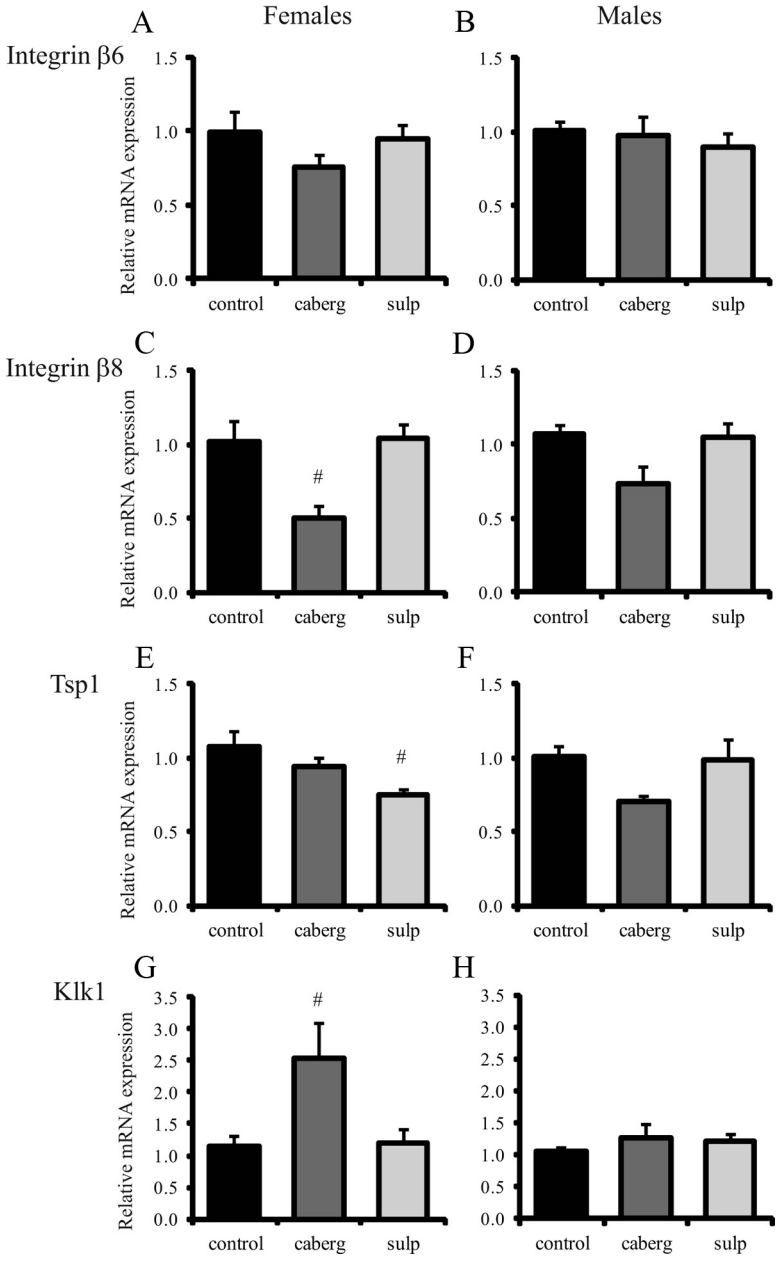

Figure 7. Dopaminergic regulation of possible TGF-β1 activators. mRNA expression of each of the indicated activators was evaluated after in vivo treatment with cabergoline (caberg, 2 mg/kg IP), sulpiride (sulp, 10 mg/kg IP), or saline (control, IP) for 24 hours in WT female (left column) and male mice (right column). Statistical analysis: 1-way ANOVA followed by a Tukey post hoc test. C, #, $P = .039$ vs control. E, #, $P = .041$ vs control. G, #, $P = .008$ vs control. n = 4–9/group.

(33, 35, 36), but the local function of this protease is still not known.

We observed KLK1 expression in mouse pituitaries, and no sex differences were observed (Figure 6F). KLK1 expression was regulated by dopamine in female but not male pituitaries. We found lower KLK1 expression in female KO pituitaries, and, accordingly, the Drd2 agonist cabergoline caused a marked increase in KLK1 expression in WT females (Figure 7G). In vivo treatment with estrogen caused a remarkable induction of pituitary KLK1 in males in both genotypes ($P < .0001$) (Figure 8H). However, in females this marked effect was observed only in the absence of the dopaminergic control ($P = .0001$) (Figure 8G).

Discussion

In the present results, we describe sex differences in the pituitary TGF-β1 system and its local regulation by dopamine and estradiol. We have exhaustively characterized several components involved in each of the steps of TGF-β1 regulation, including its synthesis, processing, assembly and storage, activation, and biological function. We found that the pituitary TGF-β1 system is profoundly affected by estradiol and dopamine stimulation, and this may have an impact on the regulation of PRL secretion by lactotroph cells.

We demonstrated that male mice have higher levels of pituitary active cytokine than females, without alterations in the total TGF-β1 levels. In accordance with the higher active TGF-β1 content in males, we also found increased expression of several other components of the system, including LTBPs and possible local activators, compared with those in females. Because we found that estrogen treatment negatively regulates most of these components, we hypothesize that higher levels of active TGF-β1 in males could be related to their lower circulating estrogen levels.

On the other hand, the impact of the chronic loss of dopaminergic tone on the pituitary TGF-β1 system was stronger in females. Even though both *Drd2*$^{-/-}$ males and females present lower pituitary active TGF-β1 content than WT mice, only females showed evidence of decreased TGF-β1 activity, evaluated by the down-regulation of TGF-β1 target genes and TβRII expression.

Sex differences in dopaminergic tone and the estradiol effect at the pituitary level were described decades ago. In

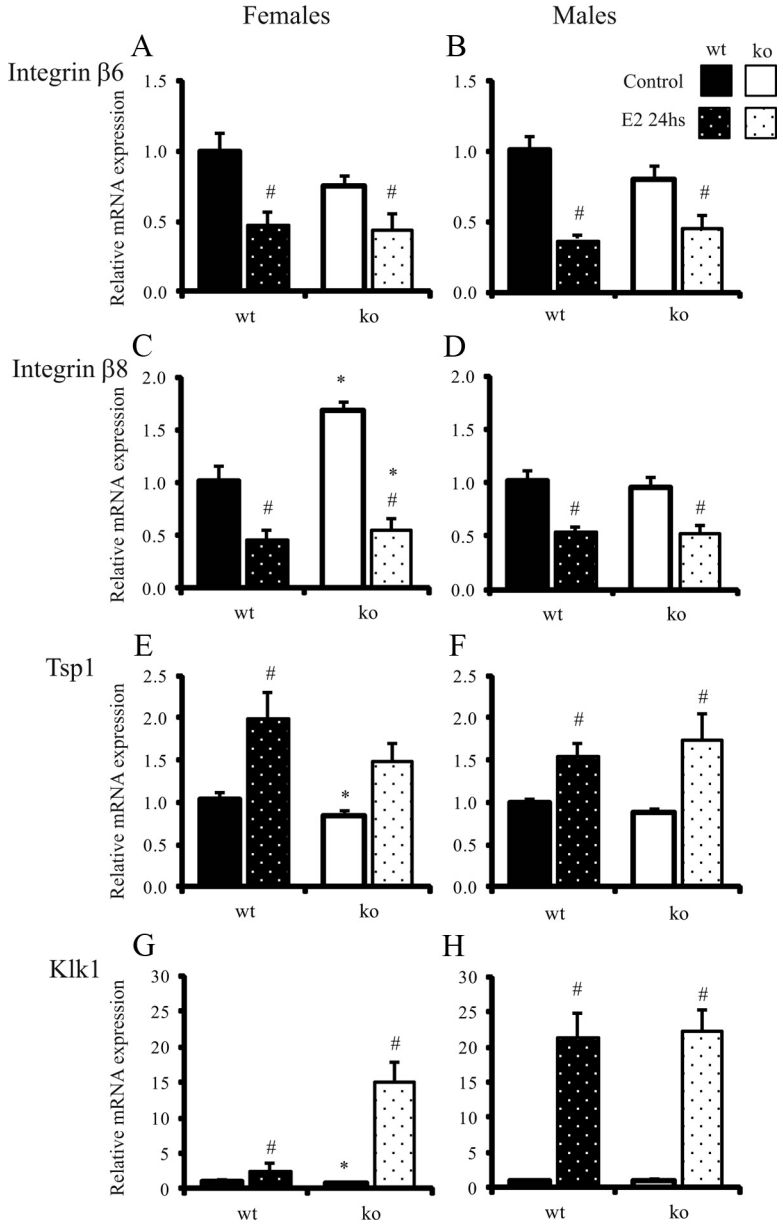

Figure 8. Estrogenic regulation of possible TGF-β1 activators in the pituitary gland. mRNA expression was analyzed after 24 hours in vivo treatment with estradiol valerate (E2 24hs, 0.2 mg/kg SC) or castor oil (control, SC) in WT and *Drd2*$^{-/-}$ female mice (left column) and in WT and *Drd2*$^{-/-}$ male mice (right column). Statistical analysis: 2-way ANOVA, *, treatment-matched differences among genotypes; #, genotype-matched differences among estradiol valerate treatment. A, #, $P = .006$. B, #, $P < .0001$. C, *, $P = .05$; #, $P = .0002$. D, #, $P = .0001$. E, *, $P = .046$; #, $P < .0001$. F, #, $P < .0001$. G, Interaction between genotype and sex effects was significant, $P < .0001$. *, $P = .0087$ vs WT control; #. P= vs control KO. J, #, $P < .0001$ vs genotype-matched control.

1981, Gudelsky and Porter (23) found that the concentration of dopamine in the median eminence of cycling female rats during diestrus is approximately 7 times higher than stalk plasma dopamine in intact male rats. Dopaminergic neurons of the tuberoinfundibular dopaminergic system (TIDA) are considered to be the major physiological regulators of PRL secretion. Basal TIDA activity and responsiveness to PRL is 5 times higher in females than in males (7). In agreement with these data, our group and others noted that the loss of dopamine action through Drd2 disruption has a more profound effect on pituitary function in female than in male mice (8, 10). Therefore, the stronger dopaminergic tone on female pituitaries could explain the more marked impact of the loss of Drd2 on the pituitary TGF-β1 system in females than in males. In accordance with the dopaminergic control of active cytokine activity, we found that a short-term treatment with sulpiride decreased active TGF-β1 levels in WT mice, whereas cabergoline treatment was not able to further increase TGF-β1 levels. The continuous dopaminergic tone on the pituitary could be exerting its maximum stimulatory effect on cytokine activation, impeding further action by cabergoline. However, cabergoline inhibited PRL secretion suggesting that part of its action on Drd2 inhibition of PRL release is independent of TGF-β1.

Interestingly, serum PRL values were inversely correlated with active TGF-β1 levels only in females, suggesting a relation between local active cytokine, Drd2 impairment, and PRL levels in female mice.

Estradiol effect on active TGF-β1 and PRL secretion appears to be sex-specific and depends on the presence of dopaminergic tone

The most surprising effect on active cytokine was that elicited by estradiol treatment. We found that estradiol treatment caused a strong increment in active TGF-β1 levels in *Drd2*$^{-/-}$ females, whereas no effect was found in the pituitaries of WT female mice.

We previously demonstrated that both estradiol and dopamine increase pituitary TGF-β1 activity. Acute treatment with estradiol, which potentially increases active TGF-β1 in the pituitary, has a concomitant central action on TIDA neurons, decreasing the dopaminergic tone (7,

37), and this event could potentially counteract the releasing effect of estradiol on cytokine activity in WT females. On the other hand, in KO female mice, the decrease in the dopaminergic tone induced by estradiol is not detected by the *Drd2*$^{-/-}$ pituitaries. The final action is a strong increase in TGF-β1 activity due to the direct action of estradiol in the pituitary. We postulate that this increase in local active cytokine induces a concomitant decrease in serum PRL levels in *Drd2*$^{-/-}$ females, outstripping the positive effect of estradiol.

It is important to consider the possibility that pituitaries from KO females present hyperstimulated lactotrophs with rapid turnover of PRL but limited storage capacity (9). In this situation, it is easier to recognize an inhibitory effect (TGF-β1) than a stimulatory effect (estradiol). In contrast, WT lactotrophs contain large numbers of secretory granules because of the continuous inhibitory action of dopamine. Then the stimulatory effect of estradiol on PRL release (direct on lactotrophs or mediated by inhibition of dopaminergic tone) is rapidly recognized, even without changes in active cytokine expression.

Finally, different responses on the impact of estradiol found in males could be related to sexual differences described previously in lactotroph sensitivity to estradiol and different sensitivity of the TIDA to estradiol (38).

All of the present data support the hypothesis that there is a crucial relationship between sex steroids and dopamine tone in regulating lactotroph function. Their effects are interdependent, further indicating that the balance between estradiol and dopamine is an important factor in the regulation of pituitary TGF-β1 function.

It is worth noting that, because neither dopamine nor estradiol is capable of activating TGF-β1 directly, their regulatory role on active cytokine levels might occur by the modulation of local activators or components of the latent TGF-β complex assembly. Supporting this possibility, we also found sex differences and regulation by dopamine and estradiol of the different LTBP isoforms and many of the local activators studied.

Pituitary LTBP characterization

LTBPs are important mediators of TGF-β extracellular availability and participate in proper latent TGF-β activation (19). Data from LTPB-null mice indicate that each LTBP isoform may have unique functions that are not redundant (for review, see Ref. 19). Our results show for the first time that LTBP1, LTBP3, and LTBP4 are expressed in mouse pituitaries of both sexes and that LTBP isoforms are differentially regulated by dopamine and estradiol. Only LTBP1 showed decreased levels in *Drd2*$^{-/-}$ pituitaries in both sexes, suggesting dopaminergic control of this protein. However, 24 hours of sulpiride treatment inhibited LTBP1 mRNA levels only in females. Lower levels of LTBP1 in both sexes in *Drd2*$^{-/-}$ pituitaries could be related to the lower levels of active TGF-β1 found in this genotype. Accordingly, TGF-β1 has been noted to enhance its own expression as well as LTBP1 levels in both normal and transformed human lung fibroblasts (39). Moreover, reduced TGF-β1 activation correlated with decreased production and secretion of LTBP has been described in several transformed cells (40, 41).

On the other hand, estradiol inhibited pituitary LTBP1 and LTBP3 expression in both sexes and genotypes. Therefore, the increased levels of these LTBPs in male pituitaries could be related to lower levels of estrogen in this sex.

Characterization of possible pituitary TGF-β1 activators

The regulation of TGF-β1 activation in the pituitary has not been studied previously, and, therefore, local activators are not known. Some of the activators described are present in the pituitary and are candidates for regulation by estradiol: TSP-1, MMP9, and MMP2 among others (42–45). As a first approach, we studied the mRNA expression of some of the activators described previously in other tissues and their regulation by estrogen and dopamine. The overall analysis of our results yields 2 patterns:

1. Male pituitaries presented higher levels of expression of most activators that female pituitaries. This was the case for MMP2, MT1-MMP, integrins αvβ6 and αvβ8, and TSP1. The increased expression of these pituitary TGF-β1 activators could be associated with the higher levels of active cytokine found in males.
2. The impact of the absence of Drd2 on the expression of the activators was more pronounced in female pituitaries. The loss of dopaminergic control decreased the expression of most of the local TGF-β1 activators studied only in females (*Drd2*$^{-/-}$) with respect to that of their WT counterparts). This was the case for MMP2, MMP9, MT1-MMP, TSP1, and KLK1, and the decreases in these molecules were consistent with the decreases in the content of active TGF-β1 in female KO pituitaries. In contrast, males showed no differences between genotypes for these activators, except for TSP1. None of the activators studied exhibited dopaminergic regulation by sulpiride or cabergoline in males.

But which of all the putative activators studied could be mediating the effect of estradiol and dopamine on active TGF-β1? Among the molecules studied, only KLK1 and

TSP1 presented patterns of regulation that were similar to those described previously for active TGF-β1 in females (Figure 2, A and C). KLK1 and TSP1 responded to positive dopaminergic control and had increased expression after estrogen stimulation, as observed in active TGF-β1 in female KO pituitaries. These findings identify these activators as the candidates for involvement in the activation of TGF-β1 in the pituitary, mediating the action of dopamine and estradiol on cytokine activity.

The role of TSP1 as a TGF-β1 activator is well documented and TSP1 represents one of the few molecules whose action has been demonstrated both in vitro and in vivo (46). The TSP1 decrease in the pituitary of rats treated chronically with estrogen was described previously (42). We have now demonstrated that TSP1 levels are also decreased in a second model of experimental prolactinomas, the *Drd2*$^{-/-}$ female mice.

However, we found an unexpected up-regulation of TSP1 after estradiol treatment that was contrary to previous data in rats chronically treated with estrogen. One possible explanation for that difference is the different times of treatment (24 hours in mice [acute effect] vs 4 weeks in rats [chronic effect]). Not only estrogen but also dopamine appears to stimulate TSP1 expression. Accordingly, *Drd2*$^{-/-}$ pituitaries of both sexes presented lower TSP1 levels, and we found that treatment with sulpiride inhibited the pituitary expression of TSP1.

There are studies in the recent literature showing direct activation of TGF-β1 by kallikreins. It has been demonstrated in vitro and ex vivo that KLK1, 2, and 5 interact with LAP or with LTBP1, causing the release of active cytokine (47, 48). In our female mouse model we found clear positive dopaminergic control of KLK1, demonstrated not only by the lower expression in female KO pituitaries but also by the stimulation caused by the treatment with the agonist cabergoline. We also demonstrated a strong positive response of this protease to estrogen in both sexes. Whereas in males the estrogen response was similar in both genotypes, in females the increase in KLK1 mRNA levels was only manifested in the absence of dopaminergic control (*Drd2*$^{-/-}$ pituitaries). This remarkable increment of pituitary KLK1 in response to estradiol (23-fold) in KO females was consistent with the marked activation of TGF-β1 induced by estrogen, evidenced only in this group (Figure 2C).

Our results suggest a possible role of pituitary KLK1 as a local TGF-β1 activator, mediating the effects of dopamine and estradiol on the activation of this potent cytokine. However, further studies are needed to effectively demonstrate that KLK1 is activating TGF-β1 within the pituitary gland.

To summarize, we found sex differences in the regulation of the TGF-β1 system. These alterations might account for sex differences found in lactotroph function and regulation and could explain differences found in the sex incidence of prolactinoma development in this model. In males, the increased levels of most of the pituitary TGF-β1 system components, including active cytokine, probably related to the lower circulating estrogen levels, could protect pituitaries from excessive lactotroph proliferation and prolactinoma development. Interestingly, in humans, sex differences found in the incidence of prolactinoma development disappears after the fifth decade of life, when serum estradiol decreases in women, and from that age onward the frequency of prolactinomas is similar in both sexes (3). On the other hand, the pituitary TGF-β1 system was deeply affected in females by the loss of dopaminergic regulation, and this could also account for the sex differences in prolactinoma development in *Drd2*$^{-/-}$ mice.

About 10% to 15% of patients harboring prolactinomas exhibit resistance to dopaminergic drugs, and to date, there have been no alternative therapies. As an intermediate of dopamine inhibition on lactotroph function, TGF-β1 could be a target in the treatment of resistant prolactinomas. Increasing local active cytokine levels could be an effective treatment for these tumors. Moreover, we recently demonstrated in rats with estradiol-induced prolactinomas that treatment with synthetic TSP1 analogs increases local active TGF-β1, which could be involved in the reduction of serum PRL levels and prolactinoma growth parameters (23).

Therefore, advanced knowledge of the pituitary TGF-β1system regulation is an important tool for future clinical studies.

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