# Short-Term Pharmacological Suppression of the Hyperprolactinemia of Infertile hCG-Overproducing Female Mice Persistently Restores Their Fertility

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Female infertility is often associated with deregulation of hormonal networks, and hyperprolactinemia is one of the most common endocrine disorders of the hypothalamic-pituitary axis affecting the reproductive functions. We have shown previously that transgenic female mice overexpressing human chorionic gonadotropin $\beta$-subunit (hCG$\beta$+ mice), and producing elevated levels of bioactive LH/hCG, exhibit increased production of testosterone and progesterone, are overweight and infertile, and develop hyperprolactinemia associated with pituitary lactotrope adenomas in adult age. In the present study, we analyzed the influence of the hyperprolactinemia of hCG$\beta$+ females on their reproductive phenotype by treating them with the dopamine agonists, bromocriptine and cabergoline. Long-term bromocriptine treatment of adult mice was effective in the control of obesity, pituitary growth, and disturbances in the hormone profile, demonstrating that hyperprolactinemia was the main cause of the hCG$\beta$+ female phenotype. Interestingly, short-term treatment (1 wk) with cabergoline applied on 5-wk-old mice corrected hyperprolactinemia, hyperandrogenism, and hyperprogesteronemia, prevented pituitary overgrowth, normalized gonadal function, and recovered fertility of adult hCG$\beta$+ females after hormone-induced and natural ovulation. The same cabergoline treatment in the short term applied on 3-month-old hCG$\beta$+ females failed to recover their reproductive function. Hence, we demonstrated that the short-term cabergoline treatment applied at a critical early stage of the phenotype progression effectively prevented the hyperprolactinemia-associated reproductive dysfunction of hCG-overproducing females. **(*Endocrinology* 153: 5980–5992, 2012)**

Female infertility is often caused by hormonal imbalance, and it involves alterations in the pituitary-ovarian function and ovulation by integrated central and peripheral mechanisms. Prolactin is a pituitary hormone with a wide array of functions in different species, and it has a pivotal role in the reproduction-related physiological and pathophysiological conditions in mammals. It is well known that elevated circulating levels of prolactin cause infertility, and hyperprolactinemia belongs to the most common endocrine disorders of the hypothalamic-pituitary axis (1–3). In humans, hyperprolactinemia induces amenorrhea, anovulation, reduced libido, and orgasmic dysfunctions (3). These effects have mainly been associated with inhibition of the hypothalamic GnRH pulsatility, suppression of the preovulatory gonadotropin surge, and the consequent inhibition of gonadal function (4–6). Nevertheless, the experimental and clinical implications of a direct effect of prolactin on the ovarian function are still poorly understood.

The characterization of mutant mouse models has been useful for better understanding of the role of prolactin in

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Abbreviations: *E2f1*, E2F transcription factor 1; hCG, human chorionic gonadotropin; hCG$\beta$+cab, cabergoline-treated hCG$\beta$+ mice; PRLR$^{-/-}$, knockout mice for prolactin receptor; qRT-PCR, quantitative real-time RT-PCR; TG, transgenic; WT, wild type.

reproduction. Knockout mice for prolactin (7) and prolactin receptor ($PRLR^{-/-}$) (8) exhibit multiple reproductive disturbances and are completely infertile. $PRLR^{-/-}$ ovaries are normal, they do not present abnormal follicular development or ovulatory function, and the fertilization rates of these mice are comparable with wild-type (WT) animals. However, the cells of corpora lutea undergo apoptosis from d 1.5 after mating (9). These results highlight the importance of prolactin in the maintenance of murine corpora lutea and progesterone production during pregnancy (1, 10).

The main signal regulating prolactin secretion is the inhibitory action of dopamine. This hypothalamic neurotransmitter, acting through the dopamine $D_2$ receptor, suppresses the high intrinsic secretory activity of the pituitary lactotrophs, reduces prolactin gene expression, and activates several interacting intracellular signaling pathways that inhibit lactotroph proliferation (11). Accordingly, many dopamine agonists are used to treat hyperprolactinemia (12–17). Of them, bromocriptine has been used over the past 30 yr, but a considerable number of patients are resistant to this treatment, and it has multiple side effects (12, 13). Cabergoline provides an alternative often with better results (14–16), and the ovulatory cycle and fertility can be recovered after cabergoline treatment in patients with hyperprolactinemia due to macro- or microprolactinomas (17). In addition, cabergoline administration to female rats prevents embryo implantation due to a deficiency in progesterone production (18).

Although the impact of elevated prolactin on human fertility is well established, further experimental studies to unravel the mechanisms of this effect are still needed. Rodent models such as drug-induced chronic hyperprolactinemia in rats (19), pituitary-transplanted rats (20), or dopamine $D_2$ receptor-deficient mice (21) have not provided sufficient information about the reproductive consequences of these endocrine alterations. Consequently, additional animal models are still needed to better understand the mechanisms of hyperprolactinemia-related infertility.

Increased human chorionic gonadotropin (hCG)/LH action alters the endocrine balance and reproductive function in mice and humans of both sexes (22–25). Our previous studies have demonstrated that transgenic (TG) female mice overexpressing the hCG$\beta$-subunit (hCG$\beta$+ mice) are infertile due to several reproductive disturbances. These mice exhibit increased hCG levels and dramatically altered reproductive hormone profile, which includes elevated levels of prolactin, progesterone, and testosterone. Later in life, the hCG$\beta$+ mice develop pituitary prolactinomas and mammary gland tumors (23, 26, 27).

The aim of the present study was to analyze the influence of the hyperprolactinemic condition of the hCG$\beta$+ female mice on their infertility. To this end, we treated juvenile and adult hCG$\beta$+ females with the dopamine agonist cabergoline for 1 wk, and morphological and biochemical analyses, estrous cyclicity, and pregnancy success were monitored thereafter. We compared these effects with the morphological and biochemical changes after a long-term treatment with bromocriptine on adult females. We show here that hyperprolactinemia is the main cause for the reproductive defects of adult hCG$\beta$+ females, and it can be prevented by a short-term treatment with cabergoline at the beginning of the reproductive age.

## Materials and Methods

### Animals

All the experiments were performed in TG female mice overexpressing the hCG$\beta$ subunit under control of the human ubiquitin C promoter (hCG$\beta$+). Generation, housing, and genotyping of the hCG$\beta$+ TG mice have been previously described (23). The hCG$\beta$+ and WT mice were of the FVB/N genetic background. Mice were maintained under controlled conditions (12 h light, 12 h dark cycle, 22 C) and were allowed free access to laboratory chow and tap water. All experimental procedures were performed according to the National Institutes of Health Guidelines for Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (Buenos Aires, Argentina) and the University of Turku (Turku, Finland).

### Cabergoline treatment

Transgenic hCG$\beta$+ female mice of 5 or 12 wk of age were injected ip with 500 $\mu$g/kg of cabergoline (Laboratorios Beta S.A., Buenos Aires, Argentina) suspended in 0.25% methylcellulose as vehicle (28). The females received three injections of cabergoline every other day during 1 wk and further analyses started 2 wk after the end of the treatment (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The hCG$\beta$+ females used as controls were injected with vehicle only. Data from WT mice treated with cabergoline were not included because in a pilot study, we found no differences in biochemical and morphological parameters compared with nontreated WT mice.

### Bromocriptine treatment

Transgenic and WT females were treated with bromocriptine at the age of 2 months. Anesthesia was induced by 2% tribromoethanol (ip). Bromocriptine was administered using a commercial Bromocriptine mesylate pellet (5 mg/pellet, 90 d release, catalog no. NC-231; Innovative Research of America, Sarasota, FL). A small incision was made in the back skin of the mouse; a pellet was implanted under the skin and changed 80–90 d after the first operation. The incision was closed by one suture. Sham

animals underwent a similar operation. The treatment was finished at the age of 6 months.

### Fertility tests

The estrous cycle stages of WT, hCGβ+, and cabergoline-treated hCGβ+ mice (hCGβ+cab) were determined by daily cytological examination of vaginal smears for 21 consecutive days, starting at 35 d of age: predominantly cornified epithelium indicated the estrous stage, predominantly nucleated cells indicated the proestrous stage, both cornified and leukocytes indicated the metestrous stage, and predominant leukocytes indicated the diestrous stage (29). The duration of each cycle stage was also determined by calculating the percent of days in each stage from d 42 to 60.

To determine fertility, 2- or 3-month-old females were superovulated by ip injection of 7.5 IU pregnant mare's serum gonadotropin (PMSG; Novormon; SYNTEX S.A., Buenos Aires, Argentina), followed by 7.5 IU of hCG 48 h later (Gonacor; Ferring, Buenos Aires, Argentina). Females were mated individually with adult WT males immediately after the induction of ovulation. Mating index was determined by monitoring the presence of vaginal plugs the morning after mating. Fertility index was determined by verifying the birth of live pups 20–21 d after mating. The ability to nurse was analyzed by assessing the survival of offspring 96 h postpartum. Natural matings were continued until 6 months of age, and the numbers of litters and pups were recorded.

### Sample collection

Mice were weighed and killed at 2 or 6 months of age by $CO_2$ asphyxiation, and blood samples were obtained by cardiac puncture immediately thereafter. Serum samples were separated by centrifugation and stored at −20 C until hormone measurements. Pituitaries and ovaries were isolated, weighed, snap frozen, and stored at −70 C for RNA isolation or processed for histology.

### RNA isolation and gene expression assays

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. As previously described (30), 2 μg of RNA was treated with deoxyribonuclease I (Invitrogen) and reverse transcribed in a 20-μl reaction using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamers (Biodynamics, Seattle, WA). For quantitative real-time RT-PCR (qRT-PCR), primer sets were designed for the specific amplification of genes (Supplemental Table 1); cyclophilin A (*Ppia*) was used as internal control. Each sample was assayed in duplicate using 4 pmol of each primer, 1× SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and 2–20 ng of cDNA in a total volume of 13 μl. Amplification was carried out in an ABI PRISM 7500 sequence detection system (Applied Biosystems). For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (31) was applied. An expression ratio was determined for each sample by calculating the following: $(E_{target})^{\Delta Ct(target)}/(E_{Ppia})^{\Delta Ct(Ppia)}$, where E is the efficiency of the primer set, Ct is cycle threshold, and ΔCt = Ct(reference cDNA) − Ct(experimental cDNA). The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (nanograms of cDNA) per reaction *vs.* Ct value [$E = 10^{-(1/slope)}$]. The efficiencies of 2 ± 0.1 were considered optimal. The results were expressed relative to a reference sample (a WT sample chosen *ad random*).

### Hormone measurements

Serum prolactin and FSH concentration were measured by RIA, according to a method described previously (23, 30). The results were presented in terms of the mouse reference preparation AFP-6476C or the rat-FSH-RP-2 standard, provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The sensitivities of the assays were 200 ng/liter for prolactin and 800 ng/liter for FSH. The intra- and interassay coefficients of variation were 7 and 12%, respectively.

Serum estradiol levels were measured by immunofluorometric assay after diethyl ether extraction, using the estradiol Delfia kit (Perkin-Elmer-Wallac, Inc.. Turku, Finland). The sensitivity of the assay was 7 pmol/liter (23). The serum testosterone and progesterone levels were measured by conventional RIA after diethyl ether extraction, according to a method described previously (23, 30). The intra- and interassay coefficients of variation were less than 12%.

### Histological analysis

Ovaries were fixed overnight in 4% paraformaldehyde, dehydrated in ethanol, and embedded in paraffin wax. Sections of 5 μm in thickness were mounted on slides and stained with hematoxylin and eosin.

### Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed with one-way ANOVA followed by a Bonferroni's *post hoc* test to establish the level of significance. In those experiments in which the effects of two factors (genotype and treatment) were studied, the two-way ANOVA followed by Fisher's least significance difference *post hoc* test was performed. Data were transformed when required. A value of $P < 0.05$ was considered statistically significant.

## Results

### Effect of long-term treatment with bromocriptine on the phenotype of hCGβ+ female mice

The hCGβ+ and WT females were treated with the dopamine agonist bromocriptine from 2 to 6 months of age and analyzed at the end of the treatment (Table 1). As previously shown (23, 27), the body weights, the pituitary weights, and serum prolactin, progesterone, and testosterone concentrations of the hCGβ+ females were significantly increased as compared with WT mice, and bromocriptine treatment significantly reduced these parameters. Coincident with previous results (23), neither serum estradiol (WT: 96.8 ± 26.2; hCGβ+: 96.6 ± 13.6 pmol/liter; n = 5) nor FSH (WT: 4.7 ± 1.4; hCGβ+: 3.9 ± 0.5 ng/ml; n = 5) levels showed significant differences between the WT and hCGβ+ females at 6 months of age.

**TABLE 1.** Effects of long-term treatment with bromocriptine on WT and hCGβ+ females

| | WT | WT+Br | hCGβ+ | hCGβ+Br |
|---|---|---|---|---|
| Body weight (g) | 27.4 ± 0.8[a] | 23.5 ± 1.0[b] | 43.1 ± 1.8[c] | 29.3 ± 1.9[a] |
| Ovary weight (mg) | 6.10 ± 0.44[a] | 3.88 ± 0.27[b] | 7.73 ± 1.16[a] | 4.18 ± 0.30[b] |
| Pituitary weight (mg) | 2.50 ± 0.10[a] | 2.38 ± 0.13[a] | 18.79 ± 2.34[b] | 6.06 ± 0.32[c] |
| Prolactin (ng/ml) | 69.1 ± 13.6[a] | 40.6 ± 14.0[a] | 6549 ± 1517[b] | 167.1 ± 13.3[c] |
| Progesterone (nmol/liter) | 4.70 ± 1.02[a] | 6.46 ± 1.45[a] | 164.30 ± 38.46[b] | 21.53 ± 4.36[c] |
| Testosterone (nmol/liter) | 0.33 ± 0.02[a,c] | 0.27 ± 0.03[a] | 0.79 ± 0.09[b] | 0.45 ± 0.05[c] |

Mice were treated with bromocriptine (Br) pellets from 2 to 6 months of age and analyzed at 6 months of age. Data are presented as mean ±SEM (n = 6–17). Two-way ANOVA, followed by least significance difference *post hoc* test, was conducted. *Different superscript letters* indicate significant differences between the groups ($P$ at least $< 0.05$).

Consequently, serum levels of these hormones were not analyzed in the bromocriptine-treated groups. The body and ovary weights of WT mice were significantly reduced after treatment with bromocriptine. The pituitary weights and serum prolactin, progesterone, and testosterone levels of WT animals did not show significant responses to bromocriptine.

### Effect of short-term treatment with cabergoline on the estrous cycle of hCGβ+ females at early adulthood

To avoid any interference on the reproductive performance of the female mice by the continuous long-term treatment with dopamine agonists (18), hCGβ+ females were subjected to a 1-wk-long treatment with the potent agonist, cabergoline, which was applied to young females (5 wk of age) and analyzed thereafter. The estrous cycles of the WT, hCGβ+, and hCGβ+cab mice were examined from the beginning of the treatment until 2 months of age (Fig. 1A). The vaginal smears demonstrated that WT females presented with a normal estrous cycle. However, the hCGβ+ mice showed disrupted estrous cycles, with a continuous diestrous-type pattern from 6 wk of age onward, indicative of high progesterone secretion. In addition, the hCGβ+ mice spent a reduced number of days in estrus and an increased number in diestrus as compared with WT (Fig. 1B). Importantly, the 1-wk-long treatment of hCGβ+ mice with cabergoline applied at 5 wk of age normalized their estrous cycles at early adulthood, being similar to those observed in WT mice (Fig. 1, A and B).

### A short-term treatment with cabergoline reversed the phenotype of hCGβ+ females at early adulthood

The phenotype of WT, hCGβ+, and cabergoline-treated hCGβ+ females were analyzed in early adulthood (2 months of age), coincident with the age of the breeding tests (Fig. 2). At this age, the body weight of hCGβ+ females was significantly increased as compared with WT and was reduced in the hCGβ+cab group (Fig. 2A). The pituitary weights of the hCGβ+ females were significantly increased as compared with WT, and the treatment with cabergoline normalized them (Fig. 2B). The ovarian weights did not show significant differences in any group of mice studied (Fig. 2C). The cabergoline treatment did not affect the body, pituitary, or ovary weight of WT females (data not shown).

The serum levels of prolactin, progesterone and testosterone were significantly increased in the hCGβ+ females at 2 months of age, and cabergoline treatment significantly reduced these levels to values comparable with WT mice (Fig. 2, D–F). Serum estradiol levels did not show significant differences between WT and hCGβ+ females at 2 months of age (WT: 124.5 ± 33.5; hCGβ+: 95.3 ± 17.9 pmol/liter; n = 5); neither did serum FSH present significant differences at this age (WT: 5.4 ± 0.8; hCGβ+: 4.9 ± 0.4 ng/ml; n = 5). Consequently, serum levels of these hormones were not analyzed in the cabergoline-treated group.

Because this treatment induced significant changes in the hormone profile of hCGβ+ females, we further analyzed the effect of cabergoline on the pituitary function, in terms of the expression of genes involved in prolactin and gonadotropin subunit production by qRT-PCR. Due to the significant increase in the pituitary size of the hCGβ+ females, the gene expression of pituitary hormones was calculated as the total gene expression per gland by multiplying the relative expression with the pituitary weight. The gene expression of *Prl* and the prolactin regulatory element-binding protein *Preb* (32) showed an increase in hCGβ+ females, and there was a tendency for a lower expression after cabergoline treatment, but the difference did not reach statistical significance (Fig. 2, G and H). Pituitary *Fshb*, *Cga*, and *Gnrhr* expression levels (Fig. 2, I–K) did not differ between the groups. The relative expression levels for *Prl*, *Preb*, *Fshb*, *Cga*, and *Gnrhr* did not show significant differences between the groups (Supplemental Fig. 2).

The relative expression of *Lhcgr* and *Akr1c18* involved in ovarian steroidogenesis was assessed in 2-month-old WT, hCGβ+ and hCGβ+cab females. No changes in the

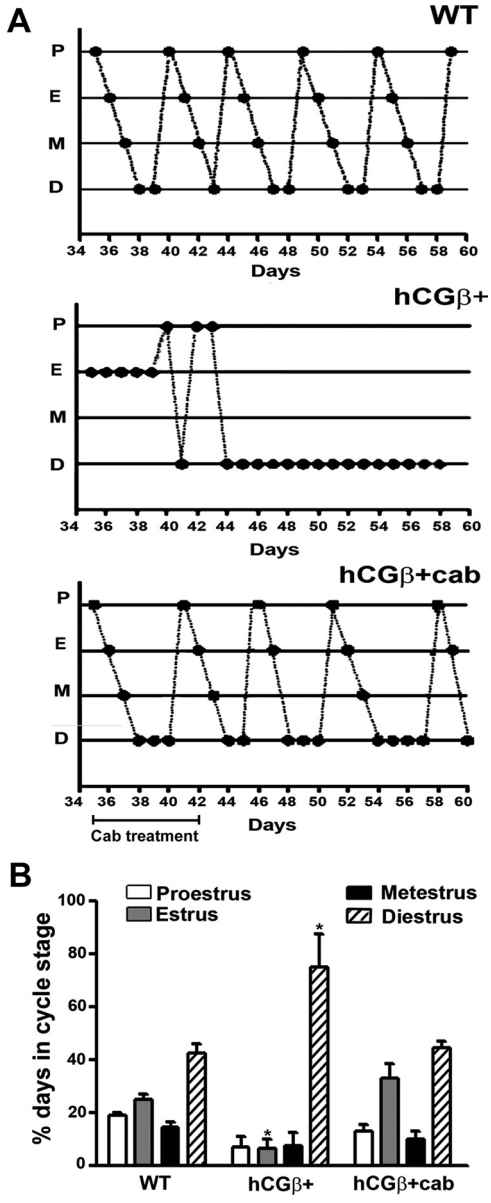


**FIG. 1.** Estrous cycle stages of WT, hCG$\beta$+, and hCG$\beta$+cab females. Cabergoline was injected into 5-wk-old-hCG$\beta$+ females for 1 wk. A, Representative estrous cycles of WT, hCG$\beta$+, and hCG$\beta$+cab female mice as examined daily from 35 to 60 d of age. B, Percentage of days in each cycle stage was analyzed from 42 to 60 d of age. Data are presented as mean ± SEM (n = 4–6). One-way ANOVA, followed by Bonferroni's *post hoc* test, was conducted. *, hCG$\beta$+ *vs.* WT, hCG$\beta$+cab, $P < 0.05$.

expression of *Lhcgr* were detected (Fig. 2L), whereas reduced expression of *Akr1c18*, encoding the progesterone-metabolizing enzyme 20$\alpha$-hydroxysteroid dehydrogenase, was apparent at this age (Fig. 2M). Cabergoline treatment partially restored the reduced expression of the latter gene at 2 months of age. The expression of *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, and *Star* in the 2-month-old hCG$\beta$+ ovary, analyzed both in mice with and without cabergoline treatment, did not show significant differences as compared with WT mice (data not shown).

Ovaries from WT, hCG$\beta$+, and hCG$\beta$+cab mice were analyzed histologically at 2 months of age for changes in their follicular development (Fig. 3A). WT ovaries included follicles at all stages and exhibited several corpora lutea in each section. The hCG$\beta$+ ovaries at 2 months of age showed multiple hemorrhagic cysts, large luteinized follicles, and luteinized areas in the interstitial tissue, indicative of premature luteinization. Treatment with cabergoline normalized the ovarian histology, as shown by follicles at various stages of maturation and by several corpora lutea without evidence of trapped oocytes within luteal tissue, indicating occurrence of ovulation (Fig. 3A).

## An early short-term treatment with cabergoline restored fertility of hCG$\beta$+ females

The females were subjected to fertility tests between 2 and 6 months of age. Fertility studies after ovulation induction demonstrated that 2-month-old hCG$\beta$+ females were unable to mate or become pregnant, whereas all WT female mice showed vaginal plug and gave birth to live pups (hCG$\beta$+: 0%; WT: 100%; n = 5–10; Table 2). On the other hand, 70% of ovulation-induced hCG$\beta$+cab females of 2 months of age mated with WT males. All the hCG$\beta$+cab females that showed vaginal plugs gave birth to live pups. Thereafter, the females were kept with males and monitored for the occurrence of natural pregnancies for the following 3 months. In this regard, 40% of the hCG$\beta$+cab females gave birth to a second litter without hormone induction 20–21 d after the first delivery (Table 2) and maintained fertility until the end of the experiment, whereas all control hCG$\beta$+ females remained infertile. Cabergoline-treated hCG$\beta$+ mothers showed good nursing performance because all their pups survived until weaning. Furthermore, all hCG$\beta$+cab females that became pregnant by natural ovulation delivered a similar number of pups per litter as WT females (Table 2).

We further analyzed whether the fertility of hCG$\beta$+ females treated with cabergoline at 12 wk of age could be equally efficiently rescued as that of mice treated at 5 wk of age. Twelve-week-old hCG$\beta$+ females were treated with cabergoline for 1 wk and superovulated 2 wk later. None of the hCG$\beta$+ females treated with cabergoline was able to mate in these conditions (Table 2).

## An early short-term treatment with cabergoline reversed the phenotype of hCG$\beta$+ females at late adulthood

To determine a possible age-dependent response of cabergoline to the reproductive physiology, 5-wk-old and 12-wk-old hCG$\beta$+ females were treated with cabergoline for 1 wk, and each analyzed at the end of the experiment, at the age of 6 months. The body weight of hCG$\beta$+ females

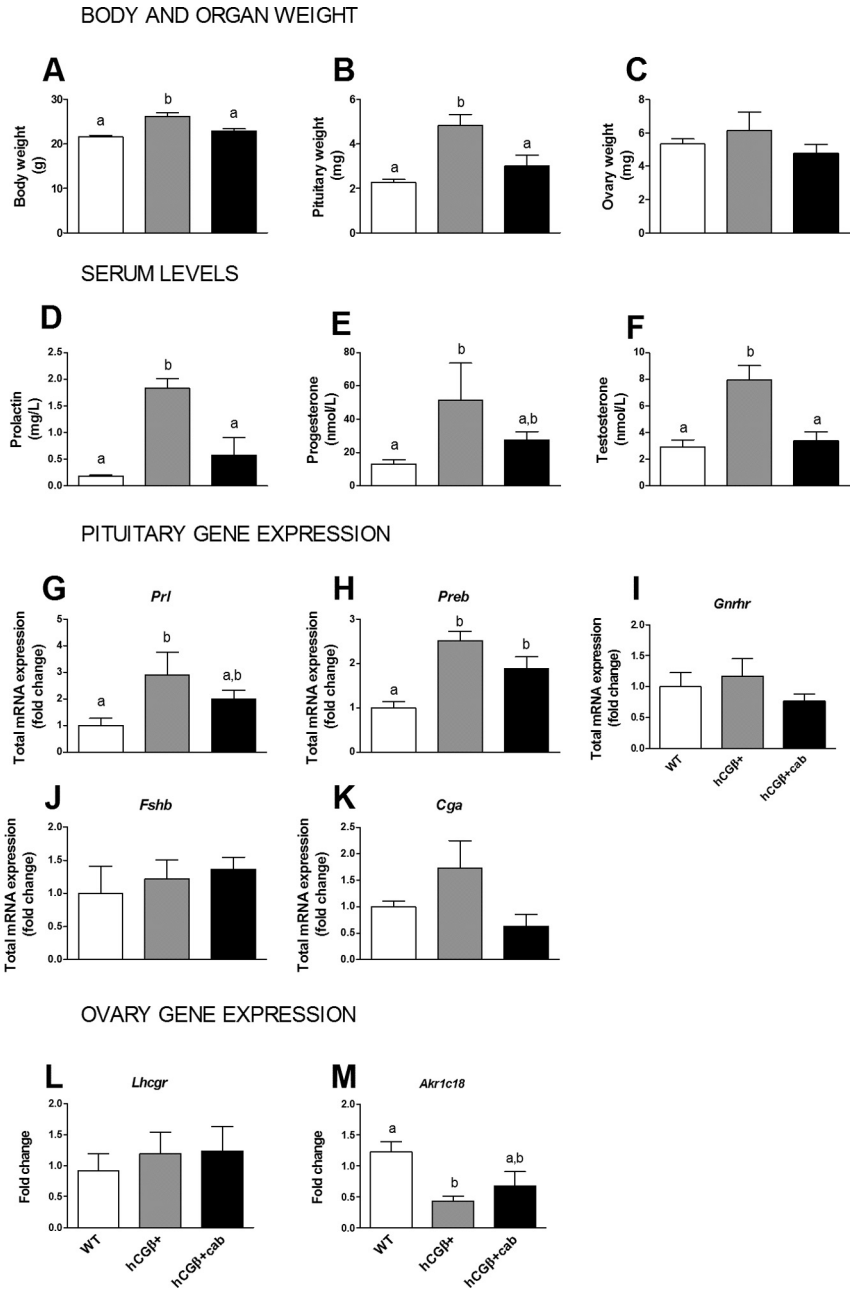


**FIG. 2.** Effect of short-term treatment with cabergoline on the phenotype of 2-month-old hCG$\beta$+ females. Cabergoline was injected into 5-wk-old hCG$\beta$+ females (hCG$\beta$+cab) for 1 wk, and the phenotype was analyzed at 2 months of age. Body (A), pituitary (B), and ovary (C) weight of WT, hCG$\beta$+, and hCG$\beta$+cab females (n = 4–8). Serum prolactin (D), progesterone (E), and testosterone (F) concentration (n = 4–8). The total mRNA expression analysis of *Prl* (G), *Preb* (H), *Gnrhr* (I), *Fshb* (J), and *Cga* (K) in pituitaries was carried out by qRT-PCR and calculated as the relative gene expression multiplied by the pituitary weight (n = 4). The relative mRNA expression of *Lhcgr* (L) and *Akr1c18* (M) in ovaries was carried out by qRT-PCR (n = 4). Data are presented as mean ± SEM. One-way ANOVA, followed by Bonferroni's *post hoc* test, was conducted. *Different letters* indicate a value of at least $P < 0.05$.

was significantly increased as compared with WT, and the treatment did not affect this change in either of the hCG$\beta$+cab groups (Fig. 4A). The pituitary weight (Fig. 4B) as well as serum prolactin, progesterone, and testosterone levels (Fig. 4, D–F) of the hCG$\beta$+ females were significantly increased as compared with WT and were reduced by the cabergoline treatment applied at 5 wk of age but not at 12 wk of age. The ovarian weight did not show significant differences in any group of mice studied (Fig. 4C). The total pituitary expression of *Prl* and *Preb* were highly increased in hCG$\beta$+ females at 6 months of age, and both were significantly reduced by cabergoline in the 5-wk-old hCG$\beta$+cab group but not in the 12-wk treated group (Fig. 4, G and H). The relative expression levels for *Prl* and *Preb* are shown in Supplemental Fig. 3.

We then determined the expression of genes involved in ovarian steroidogenesis. A significant increase in the expression of *Lhcgr* was detected in 6-month-old hCG$\beta$+ ovaries. This change was prevented when cabergoline was administered at 5 wk of age, whereas no changes were found when the same treatment was applied to 12-wk-old hCG$\beta$+ females (Fig. 4I). Conversely, the expression of *Akr1c18* was reduced in the hCG$\beta$+ ovaries, whereas cabergoline administered to 5-wk-old hCG$\beta$+ females normalized them to WT levels. In the females treated at the age of 12 wk, the *Akr1c18* expression remained as low as that measured in the TG group (Fig. 4J). The gene expression of *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, and *Star* in the 6-month-old hCG$\beta$+ ovary was not significantly different as compared with WT (data not shown).

As previously reported (23), at 6 months of age, massive ovarian luteinization with large luteomas and trapped oocytes were evident, indicative of failure of ovulation (Fig. 3B). The 6-month-old hCG$\beta$+ females subjected to a long-term bromocriptine treatment exhibited ovaries with decreased amount of luteinized areas and large cystic follicles. Treatment with cabergoline at 5 wk of age normalized the ovarian histology, as shown by follicles at various stages of maturation and by several corpora lutea without evidence of trapped oocytes within luteal tissue, indicating occurrence of ovulation. Ovaries

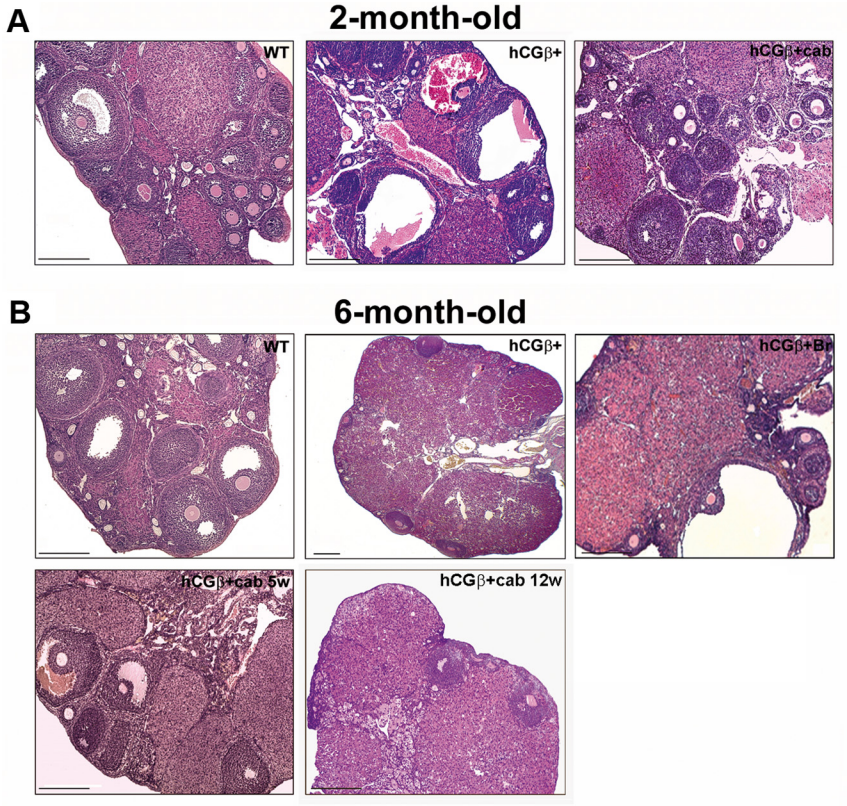


**FIG. 3.** Histological analysis of WT, hCG$\beta$+, cabergoline-treated hCG$\beta$+ (hCG$\beta$+cab), and bromocriptine-treated hCG$\beta$+ (hCG$\beta$+Br) ovaries. A, Cabergoline was injected into 5-wk-old hCG$\beta$+ females (hCG$\beta$+cab) for 1 wk and ovaries were analyzed at 2 months of age. Normal histology of WT ovaries showed all stages of follicular development; hCG$\beta$+ ovaries showed luteinized follicles and areas of luteinized cells in the interstitium and the presence of multiple cystic follicles; hCG$\beta$+cab females showed normalized structures of follicles and corpora lutea. B, Cabergoline was injected into 5-wk-old hCG$\beta$+ (hCG$\beta$+cab 5w) or 12-wk-old hCG$\beta$+ (hCG$\beta$+cab 12w) females for 1 wk and ovaries were analyzed at 6 months of age. WT ovaries appeared with normal structures; ovaries from hCG$\beta$+ females exhibited massive luteinization and few remnant follicles; hCG$\beta$+Br females showed large luteinized areas and cysts; hCG$\beta$+cab 5w ovaries showed normalized structures similar to WT; hCG$\beta$+cab 12w ovaries exhibited massive luteinization and few follicles. Representative sections were stained with hematoxylin-eosin. *Scale bar*, 250 $\mu$m.

from cabergoline treatment applied to hCG$\beta$+ females at 12 wk of age showed extensive luteinization with few follicles (Fig. 3B).

### Effect of short-term treatment with cabergoline on pituitary gene expression in hCG$\beta$+ females

To further understand the mechanism by which the short treatment with cabergoline administered to 5-wk-old hCG$\beta$+ females induced a persistent modulatory effect on the pituitary growth, we examined the expression of the proliferating markers *Pcna*, *Ccnd1*, and E2F transcription factor 1 (*E2f1*) and the cyclin-dependent kinase inhibitor *Cdkn2b* (33) on hCG$\beta$+ females (Fig. 5). At 2 months of age, the relative gene expression of *Pcna*, *Ccnd1*, and *E2f1* did not show significant differences between the different groups, thus suggesting a major contribution of nonproliferative processes to the pituitary weight gain at this age. The expression of *Cdkn2b* was significantly increased in the 2-month-old cabergoline-treated hCG$\beta$+ mice, as compared with nontreated hCG$\beta$+. The 6-month-old hCG$\beta$+ pituitaries showed a significant induction of the pituitary proliferating and cell cycle regulators, in association with the increased proliferative activity and pituitary tumor formation (27), further suggesting that these components of cell cycle control determine pituitary homeostasis. These effects were prevented by the early short-term treatment with cabergoline and maintained persistently inhibited thereafter (Fig. 5).

## Discussion

Many lines of evidence in humans and experimental models indicate that changes in the secretion or action of a single hormone are sufficient to affect the integrity of the hypothalamic-pituitary-gonadal axis and thus leading to infertility (22–27, 30, 34). We have shown previously that TG hCG$\beta$+ female mice, as a consequence of elevated levels of bioactive hCG, exhibit increased levels of testosterone and progesterone and develop hyperprolactinemia due to pituitary lactotrope adenomas in adult life (23, 27). These females are overweight, infertile, and anovulatory and have profound alterations in the reproductive endocrine axis (23). In the present study, we found that hyperprolactinemia was essential for the phenotypic defects of the hCG$\beta$+ females because most of them were reversed by treatment with dopamine agonists with proven efficacy in hyperprolactinemia (16, 17, 20, 35).

Prolactin-secreting adenomas are the most common type of pituitary tumors accounting for 30–40% of hyperprolactinemic infertility in women of reproductive age (13). Several authors have reported the normalization of prolactin secretion and shrinkage or disappearance of macro- or microprolactinomas in patients treated with bromocriptine or cabergoline, with recovery of fertility (17, 36, 37). In our study, long-term bromocriptine treatment between 2 and 6 months of age succeeded in the

**TABLE 2.** Fertility testing of WT, hCG$\beta$+, and hCG$\beta$+cab females

| | Mating Index[§] | Fertility Index[§] | Second litter[#] | Pups/female/litter‡ |
|---|---|---|---|---|
| WT | 10/10 | 10/10 | 9/10 | $7 \pm 0.5^a$ |
| hCG$\beta$+ | 0/5 | 0/5 | 0/5 | $0^b$ |
| hCG$\beta$+cab 5w | 7/10 | 7/10 | 4/10 | $5.3 \pm 0.6^a$ |
| hCG$\beta$+cab 12w | 0/4 | 0/4 | 0/4 | $0^b$ |

WT, hCG$\beta$+, and hCG$\beta$+cab females treated at 5 wk (hCG$\beta$+cab 5w) or 12-wk (hCG$\beta$+cab 12w) of age were superovulated and mated to WT males 2 wk later. One-way ANOVA, followed by Bonferroni's *post hoc* test, was conducted. *Different superscript letters* indicate significant differences between the groups ($P$ at least $< 0.05$).

§ Mating index was determined as the number of females with vaginal plug over the total of females used in each experiment. Data are presented as mean ±SEM (n = 7–19). Fertility index was determined as the number of females that delivered live pups over the total of females used in each experiment.

# Number of females that delivered a second litter through natural mating with a WT male, over the total of females used in each experiment.

‡ Number of pups per female per litter, recorded in conditions of natural mating during a 3-month breeding period.

control of obesity, pituitary growth and disturbances in the hormone profile of hCG$\beta$+ females (fertility was not tested). Interestingly, the other dopamine agonist cabergoline administered to 5-wk-old hCG$\beta$+ females only for 1 wk, corrected the hyperprolactinemia of the hCG$\beta$+ females, even in the long term, as measured at 2 and 6 months of age. We found concomitant reversal of pituitary overgrowth, normalization of gonadal function, and recovery of fertility of the treated hCG$\beta$+ females. In this regard, previous studies have emphasized the efficiency of cabergoline in normalization of prolactin levels and improvement of amenorrhea or anovulation in humans, with better results than with bromocriptine (16, 17). The pharmacological approach used herein provided evidence that many of the phenotypic characteristics of the hCG$\beta$+ females were normalized by an early short-term treatment with cabergoline, thus confirming the effectiveness of this drug in the control of hyperprolactinemia. The persistent (at least 5 months) effect of the short-term (1 wk) treatment was an unexpected finding, which depended on the timing because the same treatment administered at 12 wk (3 months) of age failed to rescue the hCG$\beta$+ phenotype. It appeared that the chronically elevated hCG secretion from early stages of sexual maturation induced persistent alterations on the pituitary-gonadal axis that could not be reversed by a short treatment with cabergoline once the dysfunctional phenotype was established in adulthood.

As also shown in the present study, the elevated levels of hCG produced several reproductive alterations, including suppression of pregnancy, anovulation, and estrous cycle defects. The occurrence of constant diestrus has been observed also in other experimental animals with elevated LH, prolactin, or progesterone levels (20, 38, 39). Because hyperprolactinemia is commonly associated with anovulation both in rodents and humans, the inhibition of prolactin hypersecretion by cabergoline was a logical approach to revert infertility in the hCG$\beta$+ mice. Cabergoline has demonstrated to be efficient and well tolerated, and no deleterious effects on mother or fetus have been observed in treatments of infertility in humans (13). Effectively, treatment of 5-wk-old female mice with cabergoline, even for a short duration, restored the normal cyclicity and pregnancy success with normal timing of parturition. In contrast, the short-term cabergoline treatment of 3-month-old hCG$\beta$+ females failed to rescue their reproductive function. This strongly suggests that the functional alterations of the hCG$\beta$+ ovary at an early age are critical for the reproductive disturbances in adulthood.

In rodents, prolactin is an essential luteotrophic agent by maintaining the corpus luteum function and progesterone production during early pregnancy or pseudopregnancy (40). The effect of prolactin in this process involves the stimulation of increased progesterone synthesis in response to pituitary LH by the up-regulation of *Lhcgr* expression in luteal cells and by the inhibition of the expression of *Akr1c18*, encoding the 20$\alpha$-hydroxysteroid dehydrogenase enzyme that converts progesterone into a biologically inactive 20$\alpha$-dihydroprogesterone (41–43). It has been shown that the administration of hCG to PRLR$^{-/-}$ mice stimulates their *Lhcgr* expression but is unable to restore fertility due to persistently high expression of *Akr1c18*, preventing thus the maintenance of sufficient progesterone levels to allow embryo implantation (44). Our results showed that 2-month-old hCG$\beta$+ females exhibited premature luteinization of the ovarian follicles and interstitial cells and the occurrence of hemorrhagic cysts. The abnormal ovarian structure of the 2-month-old hCG$\beta$+ females was accompanied by elevated concentrations of prolactin, which apparently induced down-regulation of ovarian *Akr1c18* mRNA expression (43). Consequently, although the *Lhcgr* expression was unchanged, the high hCG concentration was likely to induce the steroidogenic pathway, which, together with the reduced expression of *Akr1c18*, would

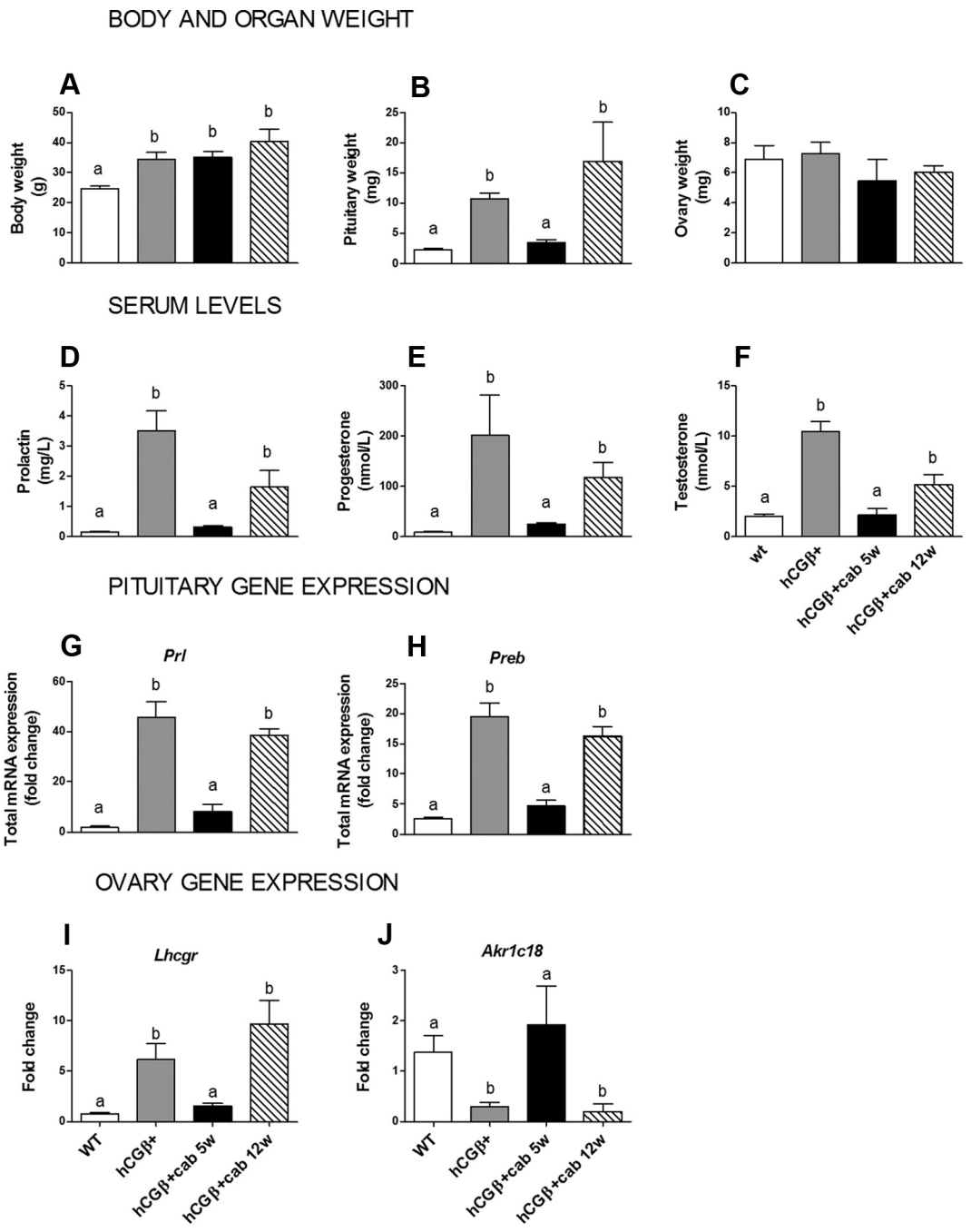


**FIG. 4.** Effect of short-term treatment with cabergoline on the phenotype of 6-month-old hCG$\beta$+ females. Cabergoline was injected to 5-wk-old hCG$\beta$+ females (hCG$\beta$+cab 5w) or 12-wk-old hCG$\beta$+ females (hCG$\beta$+cab 12w) for 1 wk, and the phenotype was analyzed at 6 months of age. Body (A), pituitary (B), and ovary (C) weight of WT, hCG$\beta$+, hCG$\beta$+cab 5w, and hCG$\beta$+cab 12w females (n = 4–8) are shown. Serum prolactin (D), progesterone (E), and testosterone (F) concentrations (n = 4–8) are shown. The total mRNA expression analysis of *Prl* (G) and *Preb* (H) in pituitaries was carried out by qRT-PCR and calculated as the relative gene expression multiplied by the pituitary weight (n = 4). The relative mRNA expression of *Lhcgr* (I) and *Akr1c18* (J) in ovaries was carried out by qRT-PCR (n = 4). Data are presented as mean ± SEM. One-way ANOVA, followed by Bonferroni's *post hoc* test, was conducted. *Different letters* indicate a value of at least $P < 0.05$.

explain the elevated levels of circulating progesterone of these mice.

Our results showed that the pituitary-gonadal function appeared disturbed already in 2-month-old hCG$\beta$+ females, and these alterations became more intense throughout life. The persistent stimulus of prolactin and hCG in 6-month-old hCG$\beta$+ females provoked massive ovarian luteinization, with the entrapment of oocytes within luteinized follicles as a clear evidence of ovulation failure. These morphological changes were accompanied by a significant increase of ovarian *Lhcgr* in concert with reduced *Akr1c18* expression, which resulted in elevated levels of

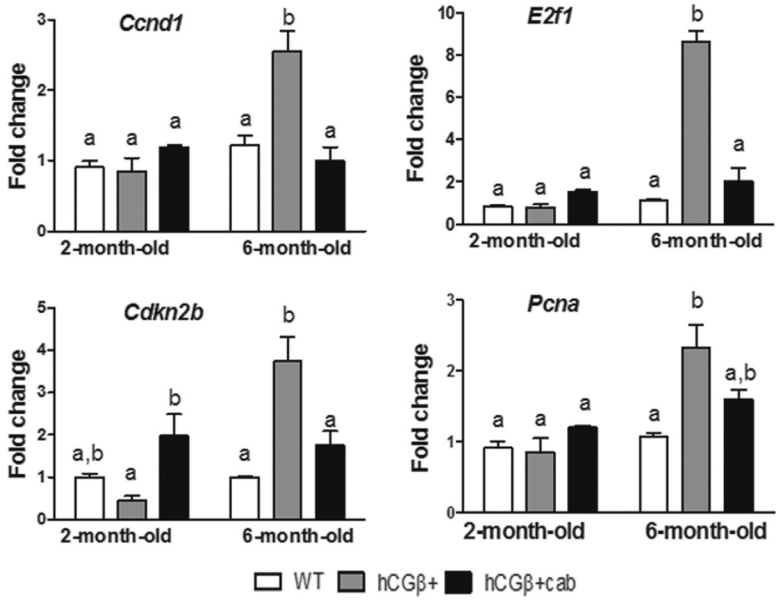


**FIG. 5.** Gene expression of pituitary proliferation markers in WT, hCG$\beta$+, and hCG$\beta$+cab females. Cabergoline was injected into 5-wk-old hCG$\beta$+ females (hCG$\beta$+cab) for 1 wk, and pituitaries were analyzed at 2 or 6 months of age. The mRNA expression analysis of *Ccnd1, E2f1*, *Cdkn2b*, and *Pcna* was carried out by qRT-PCR. Data are presented as mean ± SEM (n = 4). One-way ANOVA, followed by Bonferroni's *post hoc* test, was conducted. *Different letters* indicate a value of at least $P < 0.05$.

circulating progesterone. It is interesting that after a short-term cabergoline treatment at 5 wk of age, all alterations in the ovarian function of the hCG$\beta$+ mice were prevented, in parallel with the reduction of progesterone and testosterone synthesis. These findings remark the importance of prolactin in triggering the ovarian defects in this model. There are several studies showing that dopamine agonist therapy reduces the incidence of ovarian hyperstimulation syndrome in women at risk (45–47) and in hyperprolactinemic women with polycystic ovary syndrome undergoing assisted reproduction (48). This effect seems to be due to a deregulation of the dopaminergic tone and dopamine $D_2$ receptor signaling that affects the vascular permeability of the ovary (49, 50).

The influence of estradiol on prolactin secretion, lactotrope proliferation, and formation of prolactinomas is well known (51–53). In hCG$\beta$+ females, the development of prolactinomas is dependent on the ovarian function because ovariectomy prevents the hyperprolactinemia and pituitary adenoma formation, in the face of persistently elevated hCG production (23). Conspicuously, the estradiol levels in the hCG$\beta$+ mice are elevated only during a short period peripubertally, and thereafter they are indistinguishable from WT levels in later life (23). The molecular mechanisms by which the dopamine agonists act on the pituitary gland have long been recognized (11, 35, 54). Nevertheless, the mechanism to explain that a short treatment with cabergoline may persistently suppress the prolactin production and pituitary expansion, even several months after treatment deserves a special consideration.

In our previous report, we found that the elevated progesterone levels promote the growth of these estrogen-dependent tumors in hCG$\beta$+ mice through activation of the tumorigenic cyclin D1/cyclin-dependent kinase 4/retinoblastoma protein/E2F1 signaling cascade (27). We showed here that the gene expression of proliferating cell nuclear antigen (*Pcna*), cyclin D1 (*Ccnd1*), *E2f1*, and the cell cycle regulator *Cdkn2b* were not activated in 2-month-old hCG$\beta$+ pituitaries, suggesting a nonproliferative processes involved in the increased pituitary size at this age. In contrast, these regulator factors were suppressed in cabergoline-treated 6-month-old hCG$\beta$+ females and thus correlated with the blockade of pituitary expansion. In this regard, our results show that the cabergoline treatment was able to abolish the main proliferative stimulus responsible for the pituitary growth and tumor development. Based on our findings, we suggest that once the prolactin secretion was initially controlled by cabergoline, the massive luteinization of the ovary and the progesterone production were prevented. In this regard, lactotrope proliferation would be suppressed both by a direct action on the pituitary by increasing the dopaminergic tone and also by an indirect effect by reducing the progesterone-induced tumorigenic signaling pathways (27).

Different studies carried out in patients with prolactinomas have shown controversies about the influence of prolactin and dopamine agonist therapies on the body weight (55–58). In female rats, a direct relationship between prolactin and increased food intake and body weight was demonstrated, indicating that elevated prolactin regulates the energy balance, an effect that can be suppressed by bromocriptine (59, 60). A reduction in body weight gain was also shown in old $PRLR^{-/-}$ female mice (61). Our results showed that cabergoline treatment reduced the increased body weight of 2-month-old hCG$\beta$+ females. In addition, long-term treatment with bromocriptine was efficient in preventing obesity of 6-month-old hCG$\beta$+ females.

In summary, we have demonstrated that the primary cause of infertility in the hCG$\beta$+ mice is the elevated level of prolactin. Hyperandrogenism, hyperprogesteronemia,

acyclicity, and anovulation are all conditions triggered by prolactin deregulation that could be reversed by dopamine agonist treatment in the presence of persistently high hCG levels. We demonstrated that long-term bromocriptine treatment reversed the hyperprolactinemia of the hCG$\beta$+ females. Cabergoline administration for a short time at the beginning of the reproductive age proved effective as a preventive treatment for hyperprolactinemia-associated reproductive dysfunctions in these mice. It will be interesting to ascertain whether such a situation can also occur in certain reproductive pathologies in humans. In this respect, the hCG hypersecreting mouse model contributes to a better understanding of the interplay of LH and prolactin in the regulation of ovarian function. Even though the role of prolactin in the ovarian function has species-specific features, recent data suggest a possible contribution of prolactin for the ovarian function and the initializing of human pregnancy (62). It is possible that subtle changes in the secretion of prolactin are sufficient to affect the reproductive function also in humans.

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