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Prolactin receptor antagonism in mouse anterior pituitary: effects on cell turnover and prolactin receptor expression

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[1]*Instituto de Investigaciones en Reproducción, Universidad de Buenos Aires, Buenos Aires, Argentina; and* [2]*Institut National de la Santé et de la Recherche Médicale, U845, Centre de Recherche "Croissance et Signalisation", Equipe "Physiopathologie des hormones PRL/GH", and Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, site Necker, F-75015, Paris, France*

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Ferraris J, Boutillon F, Bernadet M, Seilicovich A, Goffin V, Pisera D. Prolactin receptor antagonism in mouse anterior pituitary: effects on cell turnover and prolactin receptor expression. *Am J Physiol Endocrinol Metab* 302: E356–E364, 2012. First published November 15, 2011; doi:10.1152/ajpendo.00333.2011.—Since anterior pituitary expresses prolactin receptors, prolactin secreted by lactotropes could exert autocrine or paracrine actions on anterior pituitary cells. In fact, it has been observed that prolactin inhibits its own expression by lactotropes. Our hypothesis is that prolactin participates in the control of anterior pituitary cell turnover. In the present study, we explored the action of prolactin on proliferation and apoptosis of anterior pituitary cells and its effect on the expression of the prolactin receptor. To determine the activity of endogenous prolactin, we evaluated the effect of the competitive prolactin receptor antagonist Δ1–9-G129R-hPRL in vivo, using transgenic mice that constitutively and systemically express this antagonist. The weight of the pituitary gland and the anterior pituitary proliferation index, determined by BrdU incorporation, were higher in transgenic mice expressing the antagonist than in wild-type littermates. In addition, blockade of prolactin receptor in vitro by Δ1–9-G129R-hPRL increased proliferation and inhibited apoptosis of somatolactotrope GH3 cells and of primary cultures of male rat anterior pituitary cells, including lactotropes. These results suggest that prolactin acts as an autocrine/paracrine antiproliferative and proapoptotic factor in the anterior pituitary gland. In addition, anterior pituitary expression of the long isoform of the prolactin receptor, measured by real-time PCR, increased about 10-fold in transgenic mice expressing the prolactin receptor antagonist, whereas only a modest increase in the S3 short-isoform expression was observed. These results suggest that endogenous prolactin may regulate its own biological actions in the anterior pituitary by inhibiting the expression of the long isoform of the prolactin receptor. In conclusion, our observations suggest that prolactin is involved in the maintenance of physiological cell renewal in the anterior pituitary. Alterations in this physiological role of prolactin could contribute to pituitary tumor development.

apoptosis; proliferation; Δ1–9-G129R-hPRL; prolactin receptor isoforms

ANTERIOR PITUITARY EXPRESSES PROLACTIN RECEPTORS (PRLR) (20, 26), and hence, prolactin (PRL) secreted by lactotropes could exert autocrine or paracrine actions on anterior pituitary cells. Although PRL inhibits its own secretion mainly by increasing dopaminergic activity at the hypothalamus, a short-loop regulation of PRL secretion at the lactotrope level has been proposed (6, 13). In fact, it has been observed that PRL inhibits its own expression in lactotropes (9).

PRL acts as a proliferative factor in some tissues, such as mammary gland, prostate, and lymphoid cells (11), but exerts proapoptotic actions in others, such as luteal cells (12) and hair follicles (10). Although older studies using in vitro cell cultures suggested that PRL could act as a growth factor for anterior pituitary cells (15, 17), more recent investigations involving genetically modified mouse models actually achieved opposite conclusions because lack of PRL activity in PRL-knockout mice ($PRL^{-/-}$) and PRLR knockout-mice ($PRLR^{-/-}$) was shown to lead to pituitary hyperplasia and tumor development (8, 27). Also, it has been shown that exogenous PRL decreases in vitro lactotrope proliferation in D2R-knockout mice ($D2R^{-/-}$) (27), suggesting that PRL may regulate the size of the population of anterior pituitary cells, particularly lactotropes. However, the physiological role of endogenous PRL in anterior pituitary cell renewal remains unclear.

Various isoforms of PRLR have been reported, referred to as long, short, and, in humans, intermediate PRLR. These isoforms are produced by alternative splicing and differ in the length of their intracellular domain, which determines the characteristics of their signaling. The long isoform of the PRLR ($PRLR_{long}$) leads to activation of different pathways, such as Jak2/STAT, MAPK, Src (11), or phosphatidylinositol 3-kinase (PI3K)/Akt (22), whereas the short isoform of the PRLR ($PRLR_{short}$) phosphorylates Jak2 and activates MAPK and PI3K pathways without recruiting STAT proteins (5, 11). PRLR isoforms not only homodimerize but also heterodimerize, and it has been proposed that $PRLR_{short}$ can act as a dominant negative of $PRLR_{long}$ activation (5). Thus, the relative amount of different PRLR isoforms expressed in a tissue may explain different PRL actions (6). In this respect, PRL modulates its activity in target cells by inducing the internalization and degradation of its own receptor (29) and by regulating the expression of PRLR isoforms (7, 28, 30).

The evaluation of PRL actions in anterior pituitary has been hampered by the fact that the hormone is constantly present in the system, which can lead to misinterpretation of experimental data obtained by the addition of exogenous PRL (27). In addition, various isoforms of PRL have been proposed to exert the opposite effect on pituitary cells, further complicating the picture (17). Therefore, to determine the actual effects of secreted PRL in anterior pituitary, it is necessary to inhibit its activity. The development of a pure PRLR antagonist provided a new tool for the study of the role of PRL functions (4, 13). Δ1–9-G129-hPRL is a competitive antagonist that forms a

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nonfunctional complex with PRLR. It has been assayed in several in vitro systems (13, 18) and was demonstrated recently to inhibit in vivo actions of transgenic PRL expressed specifically in prostate tissue, thereby preventing the development of prostate tumors (25).

In the present study, we evaluated the effect of endogenous PRL on cell renewal and expression of PRLR isoforms in the anterior pituitary gland. We used transgenic mice systemically expressing Δ1–9-G129R-hPRL as a tool to evaluate the physiological role of endogenous PRL in vivo and Δ1–9-G129R-hPRL to antagonize the effects of PRL released by anterior pituitary cells in vitro.

MATERIALS AND METHODS

Drugs

All drugs, media, and supplements were obtained from Sigma (St. Louis, MO) except for fetal bovine serum (Natocor), amphotericin B, essential amino acids, and gentamicin (Invitrogen, Carlsbad, CA), deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), anti-BrdU (BD Bioscience, San Jose, CA), anti-mouse FITC- and anti-guinea pig rhodamine-conjugated secondary antibodies (Chemicon International, Temecula, CA), guinea pig anti-rat PRL antiserum (Dr. A. Parlow, National Hormone and Pituitary Program, Torrance, CA), and the materials indicated below. Recombinant Δ1–9-G129R-hPRL was obtained as previously described (4).

Animals

This study was approved by the Comité Régional d'Éthique pour l'Expérimentation Animale, Ile-de-France, Université Paris Descartes, and the Institutional Animal Care and Use Committee of the School of Medicine, University of Buenos Aires. Mice and rats were housed in controlled conditions of light (12:12-h light-dark cycles) and temperature (20–25°C), and fed ad libitum. Male Δ1–9-G129R-hPRL transgenic (TG) mice systemically expressing Δ1–9-G129R-hPRL were generated on a BALB/c-J background as described previously (25). Wild-type (WT) male littermates were used as control mice. Five- to 7-mo-old mice were injected with BrdU (50 mg/kg ip) and euthanized 24 h later by cervical dislocation. Pituitaries were removed within minutes, weighed, fixed, and processed for immunohistochemistry. In some experiments, after neurointermediate lobe removal, anterior pituitaries were processed for RNA extraction. In other experiments, adult male Wistar rats (230–250 g) were used. Anterior pituitary glands were removed within minutes after decapitation and processed for primary culture.

Culture of GH3 Cells

GH3 somatolactotrope cells were grown as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 μl/ml MEM amino acids, 2 mM glutamine, 5.6 μg/ml amphotericin B, and 25 μg/ml gentamicin and 10% fetal calf serum treated previously with 0.025% dextran-0.25% charcoal to remove steroids (FCS). One hundred thousand cells were seeded onto coverslides in 24-well tissue culture plates for BrdU incorporation and TUNEL studies. For BrdU incorporation assay, cells were incubated in the same medium containing 10% FCS for 24 h and then incubated in the same medium containing PRLR antagonist Δ1–9-G129R-hPRL (5 μg/ml) for 8 h. Six hours before fixation, BrdU (200 μM) was added to culture media. For apoptosis and cell cycle evaluation, 1.5×10^5 cells·ml^{-1}·well^{-1} were incubated for 24 h in supplemented DMEM without FCS and then incubated in the same medium containing Δ1–9-G129R-hPRL (5 μg/ml) for 8 h.

Primary Culture of Anterior Pituitary Cells

A pool of anterior pituitary cells from four male rats was used for each culture. Anterior pituitary glands were washed with DMEM with 3 mg/ml bovine serum albumin (DMEM-BSA). Then, anterior pituitaries were cut into small fragments and dispersed enzymatically by successive incubations in DMEM-BSA containing 0.75% trypsin, 10% FCS, and 45 U/ml deoxyribonuclease type I (DNAse). Finally, the cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without Ca^{2+} and Mg^{2+}. Dispersed cells were washed and resuspended in DMEM with 10% FCS. Cell viability as assessed by trypan blue exclusion was >90%. Cells were seeded onto coverslides in 24-well tissue culture plates for BrdU incorporation detection or TUNEL assay. Cells were seeded and treated as described for GH3 cells.

Detection of BrdU Incorporation and TUNEL Assay

Pituitaries from WT and TG mice were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 4 h and embedded in paraffin. Sections (4 μm) were deparaffinized in xylene and rehydrated in graded ethanol. For in vitro studies, after the incubation period, cells were fixed in 4% PFA for 10 min.

Tissue sections or cells were permeabilized by microwave irradiation. For BrdU incorporation assay in vivo, pituitary sections were incubated with anti-BrdU diluted in nuclease solution (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol and then incubated with FITC-conjugated anti-mouse secondary antibody (1:200). For BrdU incorporation assay in GH3 cells and cultured anterior pituitary cells, after permeabilization, coverslides were incubated for 30 min with DNAse (100 U/ml) at 37°C to break DNA strands and to allow anti-BrdU antibody to interact with incorporated BrdU. Then, cells were blocked with 10% donkey serum in PBS-0.2% Triton X-100 for 90 min and incubated with mouse anti-BrdU antibody (1:200) overnight. Then, slides were incubated with a rhodamine-conjugated anti-mouse secondary antibody (1:75). For lactotrope detection in anterior pituitary primary cultures, after incubation with 10% normal donkey serum in PBS-0.2% Triton for 90 min, cells were incubated for 1 h with guinea pig rat prolactin antiserum (1:1,500) followed by 1 h of incubation with rhodamine-conjugated anti-guinea pig secondary antibody (1:200). Control slides were incubated with buffer instead of primary antibodies.

For TUNEL assay, DNA strand breaks were labeled with digoxigenin-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase (0.18 U/μl) according to the manufacturer's protocol. After incubation with 10% sheep serum in PBS for 90 min, sections or cells were incubated with antidigoxygenin-FITC antibody (1:10) to detect incorporation of nucleotides into the 3′-OH end of damaged DNA. In anterior pituitary primary cultures, before the incubation with antidigoxygenin-FITC antibody, cells were blocked with 10% donkey serum and 10% sheep serum in PBS for 90 min and incubated for 60 min with guinea pig anti-rat prolactin antiserum (1:1,500). Then, cells were incubated with antidigoxygenin-FITC antibody

Table 1. *Sequence of primers used for real-time PCR*

Primer	Sequence
PRLR ECD forward	5′-ATAAAAGGATTTGATACTCATCTGCTAGAG-3′
PRLR ICD long reverse	5′-TGTCATCCACTTCCAAGAACTCC-3′
PRLR ICD S1 reverse	5′-CATAAAAACTCAGTTGTTGGAATCTTCA-3′
PRLR ICD S2 reverse	5′-GGAAAAAGACATGGCAGAAACC-3′
PRLR ICD S3 reverse	5′-AGTTCCCCTTCATTGTCCAGTTT-3′
Cyclophilin forward	5′-CAGGTCCTGGCATCTTGTCC-3′
Cyclophilin reverse	5′-TTGCTGGTCTTGCCATTCCT-3′

PRLR, prolactin receptor; ECD, extracellular domain; ICD, intracellular domain.

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Table 2. *L-PRLR is predominant in male anterior pituitary*

	WT, %	TG, %
L-PRLR	92.23	99.13
S1-PRLR	ND	ND
S2-PRLR	4.29	0.32
S3-PRLR	3.47	0.54
Total	100.00	100.00

WT, wild type; TG, transgenic; L-PRLR, long isoform of PRLR; S1-, S2-, and S3-PRLR, short isoforms of PRLR; ND, not determined. PRLR expression was determined by real-time PCR using specific primers for the S1, S2, and S3 isoforms. Relative expression of each isoform is shown as %total PRLR expression in WT and TG mice.

(1:10) and rhodamine-conjugated anti-guinea pig secondary antibody (1:200) in the same buffer.

Coverslides and sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenylindoledihydrochloride (DAPI) for DNA staining and visualized in a fluorescent light microscope (Axiophot; Carl Zeiss, Jena, Germany).

In tissue sections, the percentage of BrdU-positive cells was calculated as (BrdU-positive cells/total anterior pituitary cells) × 100, where total cells were DAPI-positive nuclei counted in four random slices for each animal. Since the number of TUNEL-positive cells in sections from in vivo studies was very low, the percentage of apoptotic cells was expressed as the number of TUNEL-positive cells/field, as described previously (21). Cells from the neurointermediate lobe were excluded from the count. For in vitro studies, the percentage of apoptotic (TUNEL-positive) or proliferating (BrdU-positive) cells was calculated as (TUNEL-positive or BrdU-positive cells/total cells) × 100. The percentage of apoptotic (TUNEL-positive) or proliferating (BrdU-positive) lactotropes was calculated as (TUNEL-positive or BrdU positive prolactin-immunoreactive cells/total prolactin-immunoreactive cells) × 100. The percentage of lactotropes determined by immunofluorescence represented ~20% of the population of anterior pituitary cells.

Flow Cytometry

GH3 cells were harvested with 0.05% trypsin-EDTA. Cells were washed with PBS, resuspended in 300 μl of PBS-0.1% BSA, and fixed with 5 ml of 70% ice-cold methanol by gentle vortexing for 30 min at −20°C. Then, 5 ml of PBS-0.1% BSA was added to each tube. After centrifugation, DNA was stained with propidium iodide (PI; 50 μg/ml) in PBS containing ribonuclease (10 μg/ml) for 20 min at 37°C. Fluorescence intensity of ≥6,000 gated cells/tube was analyzed by flow cytometry using a FACScan (Becton Dickinson). Cells with a PI staining intensity lower than the G_0/G_1 peak were considered hypodiploid. Analysis of DNA content and determination of Sub G_0/G_1 (hypodiploid cells), G_0/G_1, S, and G_2/M-phases of cell cycle were performed using WinMDI 98 and Cylcherd 1.2 softwares.

RNA Extraction and Real-Time PCR

Anterior pituitaries were extracted, washed in RNAeasy Solution (Qiagen, Santa Clarita, CA), and immediately frozen in liquid nitrogen. RNA was extracted using the RNAeasy Micro Kit (Qiagen), following the manufacturer's protocol. Briefly, frozen anterior pituitaries were homogenized using a cold mortar and pestle and placed in 350 μl of RTL lysis buffer (Qiagen) containing β-mercaptoethanol, rehomogenized with a needle, and centrifuged. The supernatant was washed with 70% ethanol, centrifuged, and transferred onto an RNAeasy spin column (Qiagen). RNA was collected with 14 μl RNAse-free water and stored at −80°C. Reverse transcription was performed using SuperScript II Reverse Transcriptase according to the manufacturer's protocol. One-hundred twenty-five nanograms of total RNA was reverse transcribed. After incubation for 5 min at 65°C with 1 μl of oligo(dT) and 1 μl of dNTP Mix, RNA samples were incubated for 2 min at 42°C with a mix containing DTT and RNAse Out (Invitrogen). After addition of 1 μl of SuperScript II RT (Invit-

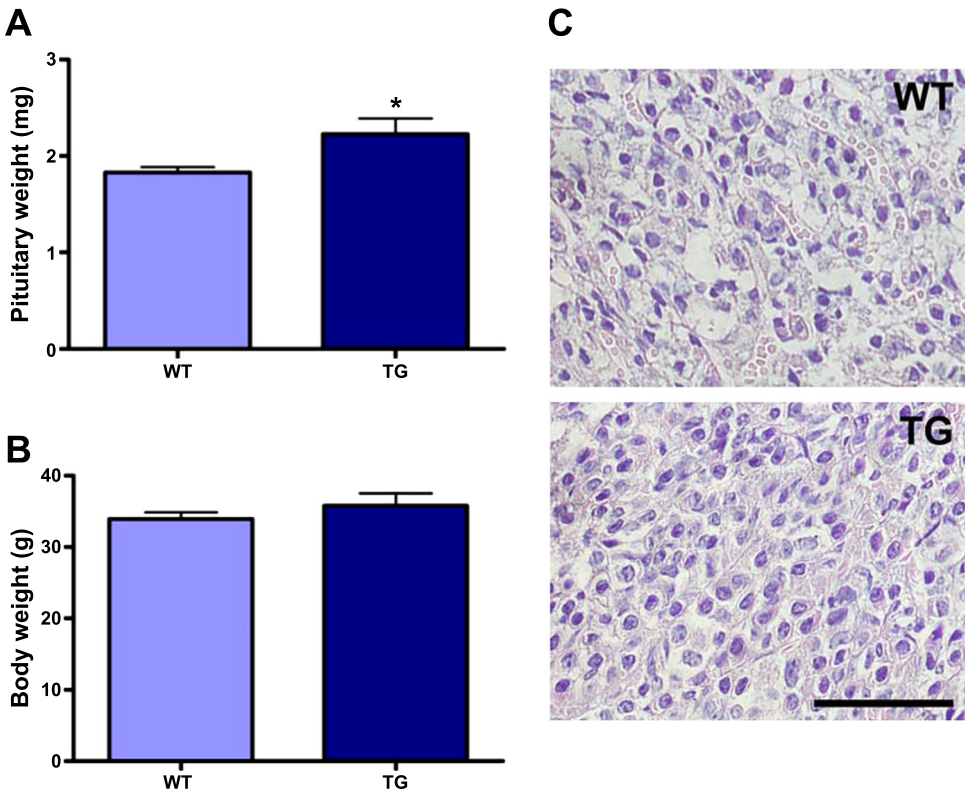

Fig. 1. Pituitary weight increases in transgenic (TG) mice expressing Δ1–9-G129R-hPRL. Each column represents pituitary weight (*A*) and body weight (*B*) (means ± SE) of 7–12 animals/group. *$P < 0.05$ vs. wild type (WT), Student *t*-test. *C*: representative images of anterior pituitary sections from WT and TG mice stained with hematoxylin-eosin. Scale bar, 50 μm.

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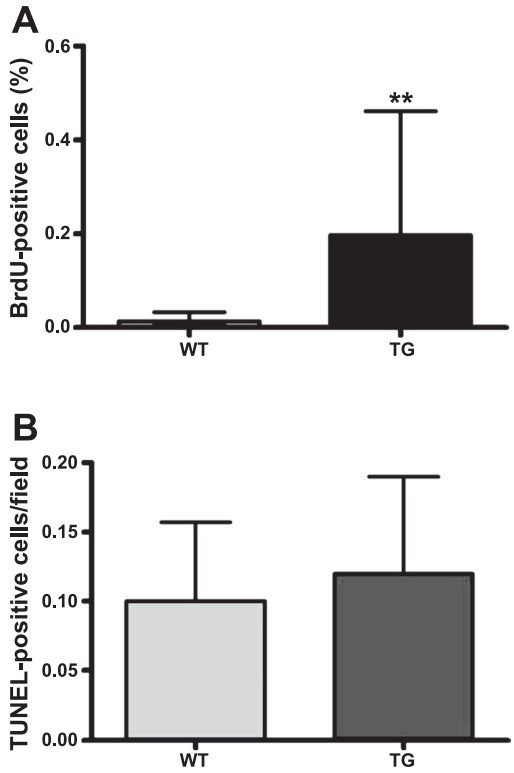

Fig. 2. Prolactin receptor (PRLR) antagonist Δ1–9-G129R-hPRL increases anterior pituitary cell proliferation in vivo. WT and TG mice expressing Δ1–9-G129R-hPRL were injected with bromodeoxyuridine (BrdU; 50 mg/kg) and euthanized 24 h later. *A*: each column represents the percentage ± confidence interval (CI; 95%) of BrdU-positive cells ($n \geq 10{,}000$ cells/animal, 4–6 animals/group). $^{**}P < 0.01$ vs. WT, χ^2 test. *B*: each column represents the mean ± SE of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells/field ($n = 6–8$ animals/group).

rogen), samples were incubated for 50 min at 42°C, and the reaction was heat-inactivated (70°C for 15 min). Finally, samples were treated with RNAse H (Invitrogen) to remove any possible RNA complementary to cDNA.

For real-time PCR, forward primers directed to mouse PRLR common extracellular domain and reverse primers directed specifically to intracellular domains of mouse $PRLR_{long}$, S1, S2, or S3 $PRLR_{short}$ isoforms and forward and reverse primers directed to cyclophilin were used (Table 1). All primers were designed using Primer 3 software (Steve Rozen) and obtained from Eurogentec (Angers, France).

Real-time PCR was performed using Applied Biosystems 7300 Real-Time PCR System. For each reaction, 25 μl of solution containing 5 μl of cDNA, 20 μM forward and reverse primers, and 12.5 μl of Power SyberGreen PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used. All reactions were performed in duplicate. Negative controls included amplification of RNA (without reverse transcription) and water. Amplification was initiated by a 2-min preincubation at 50°C followed by 40 cycles at 95°C for 30 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 30 s, terminating at 95°C for the last 15 s (melting).

Expression levels were normalized to mouse cyclophilin expression, performed in parallel as endogenous control. Real-time PCR data were analyzed by calculating the $2^{-\Delta C_T}$ value for each experimental unit, with C_T being the cycle threshold number and ΔC_T being the difference between the C_T values for each PRLR isoform and cyclophilin. In Table 2, the percentage of expression of each PRLR isoform was calculated relative to the expression of total PRLR in WT or TG mice. In Fig. 7, data for each PRLR isoform mRNA were expressed relative to PRLR expression in WT mice.

Statistical Analysis

In vivo experiments. BrdU-positive cells were counted in four randomly selected slices from each animal. Results were expressed as the percentage ± 95% confidence interval (CI) of BrdU-positive cells. Differences between proportions were analyzed by χ^2 (21). TUNEL-positive anterior pituitary cells were counted on 10–20 fields (×400) in anterior pituitary sections from each mouse. The mean of TUNEL-positive cells per field from each mouse was considered an individual value and analyzed by Student's *t*-test (21). Pituitary weight, body weight, and real-time PCR data were expressed as means ± SE and analyzed by Student's *t*-test. Differences were considered significant if $P < 0.05$. Statistical analysis was performed using GraphPad Prism 4 software.

In vitro experiments. The number of TUNEL-positive and BrdU-positive GH3 cells, total anterior pituitary cells, and lactotropes

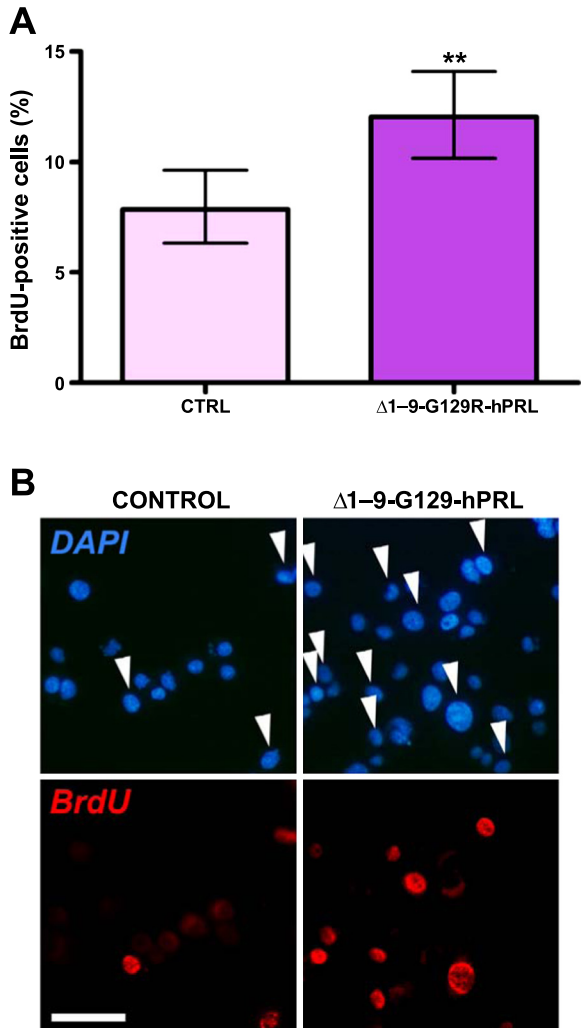

Fig. 3. PRLR blockade by Δ1–9-G129R-hPRL induces proliferation of GH3 cells. GH3 cells were incubated with or without Δ1–9-G129R-hPRL (5 μg/ml) for 8 h. *A*: proliferation rate was determined by BrdU incorporation and fluorescence microscopy. Each column represents the percentage ± CI (95%) of BrdU-positive GH3 cells ($n \geq 1{,}000$ cells/group). $^{**}P < 0.01$ vs. respective control (CTRL) without Δ1–9-G129R-hPRL, χ^2 test. *B*: representative images of GH3 cells showing immunoreactivity for BrdU (red) counterstained with 4,6-diamidino-2-phenylindoledihydrochloride (DAPI; blue). Arrowheads indicate BrdU-positive cells. Scale bar, 50 μm.

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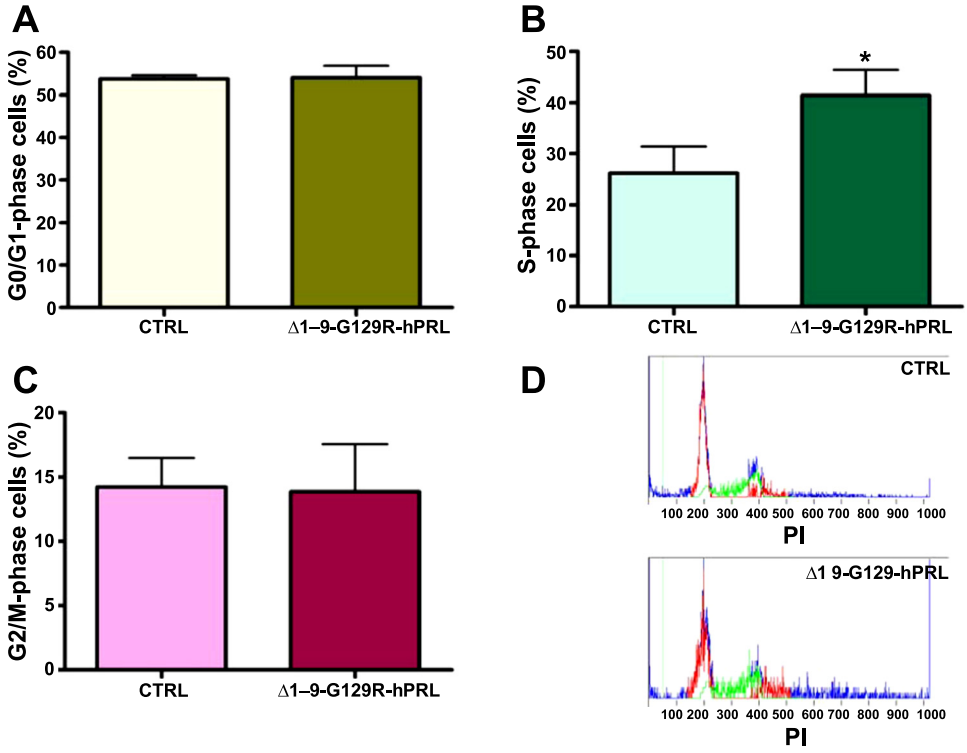

Fig. 4. PRLR antagonism increases the percentage of GH3 cells in S phase of the cell cycle. GH3 cells were incubated with or without PRLR antagonist Δ1–9-G129R-hPRL (5 μg/ml) for 8 h. *A–D*: cell cycle was analyzed by flow cytometry using propidium iodide (PI). Each column represents the percentage ± SE of G_0/G_1 phase (*A*), S phase (*B*), and G_2/M phase cells (*C*) ($n \geq 5$ wells/group). *$P < 0.05$ vs. respective control without Δ1–9-G129R-PRL, Student *t*-test. *D*: representative histograms of DNA content of GH3 cells incubated in the presence or absence of the PRLR antagonist.

was determined in duplicate slides from independent experiments. Results were expressed as the percentage ± 95% CI of TUNEL-positive or BrdU-positive cells or TUNEL-positive or BrdU-positive lactotropes of the total number of cells counted in each specific condition. Differences between proportions were analyzed by χ^2 (24).

The percentage of hypodiploid cells or cells in the S, G_2/M, or G_0/G_1 phases of the cell cycle was expressed as means ± SE of five

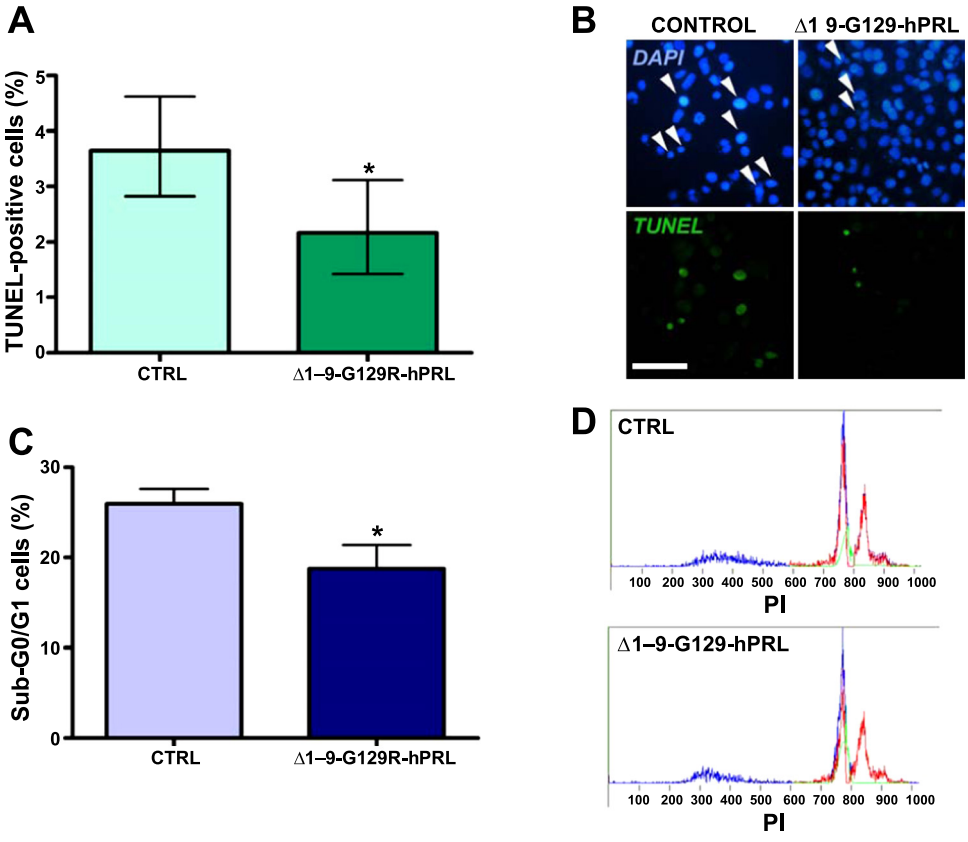

Fig. 5. PRLR antagonism decreases GH3 cell apoptotic rate. GH3 cells were incubated with or without PRLR antagonist Δ1–9-G129R-hPRL (5 μg/ml) for 8 h. *A*: each column represents the percentage ± CI (95%) of TUNEL-positive cells ($n \geq 1{,}000$ cells/group). *$P < 0.05$ vs. respective control without Δ1–9-G129R-hPRL, χ^2 test. *B*: representative images of GH3 cells showing DNA fragmentation determined by the TUNEL method (green). Arrowheads indicate apoptotic cells. Scale bar, 50 μm. *C*: %hypodiploid cells was determined by flow cytometry using PI. Each column represents the mean ± SE of the percentage of sub-G_1 cells ($n = 5$–6 wells/group). *$P < 0.05$ vs. respective control without Δ1–9-G129R-PRL, Student *t*-test. *D*: representative histograms of fluorescence intensity of DNA content of GH3 cells incubated in the presence or absence of the PRLR antagonist.

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to six wells per group and analyzed by Student's *t*-test. Differences were considered significant if $P < 0.05$. Statistical analysis was performed using GraphPad Prism 4 software.

RESULTS

Anterior Pituitary Cell Turnover in the Presence of PRLR Antagonist

To study the action of endogenous PRL in anterior pituitary cell renewal, we evaluated the effect of PRLR antagonist Δ1–9-G129R-hPRL in vivo, using transgenic mice that constitutively and systemically express this antagonist (25). WT and TG mice were injected with BrdU and euthanized 24 h later. Rates of apoptosis and mitosis in the anterior pituitary were assessed. Although body weight remained unchanged, the weight of the pituitary gland in TG mice was 25% higher than in WT mice (Fig. 1, *A* and *B*). Microscopic observation of tissue sections suggested that this increase was due to anterior pituitary gland hyperplasia (Fig. 1*C*). In fact, the anterior pituitary proliferation index (%BrdU-positive cells, detected by immunofluorescence) was significantly higher in TG mice than in WT mice (Fig. 2*A*). We observed no changes in apoptotic rate in pituitaries from TG mice (Fig. 2*B*). The increase in cell proliferation resulting from inhibition of PRLR signaling by the presence of Δ1–9-G129R-hPRL supports an antiproliferative role of endogenous PRL in the anterior pituitary gland.

Effect of PRLR Antagonism on Proliferation and Apoptosis of GH3 Somatolactotrope Cell Line and Anterior Pituitary Cells in Culture

Transgenic mice expressing the PRLR antagonist Δ1–9-G129R-hPRL were reported to have normal PRL serum levels (25), suggesting no profound alteration of the dopaminergic tone. Consequently, we hypothesized that the increased cell proliferation observed in anterior pituitaries of these mice was more likely to reflect a direct effect of PRLR antagonism than a reduction of the inhibitory dopaminergic tone. To explore this hypothesis, we investigated the effect of the PRLR antagonist on GH3 cells, a somatolactotrope cell line that constitutively releases PRL (19). To inhibit activation of PRLR by

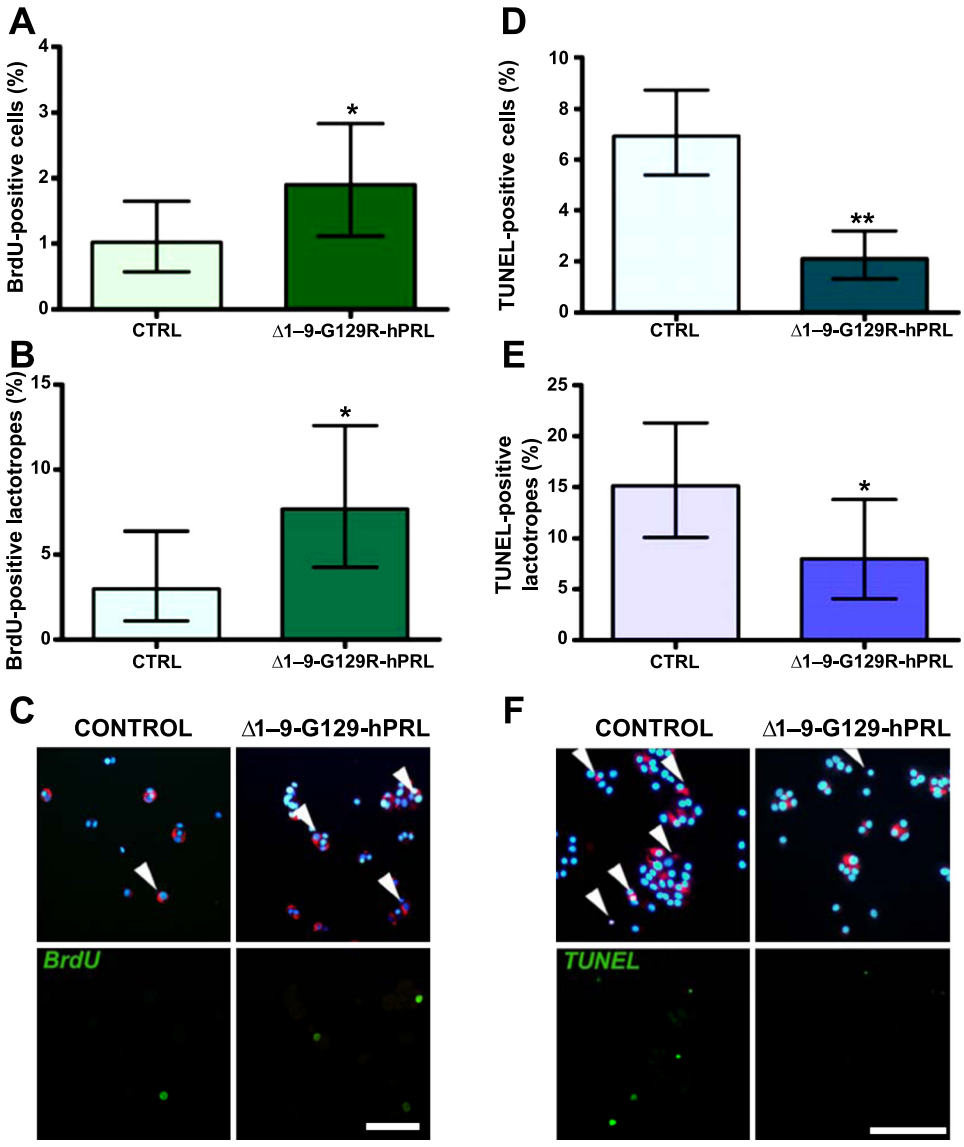

Fig. 6. PRLR blockade by Δ1–9-G129R-hPRL induces proliferation and decreases apoptosis of total anterior pituitary cells and lactotropes in culture. Primary cultures of anterior pituitary cells were incubated with or without Δ1–9-G129R-hPRL (5 μg/ml) for 8 h. *A* and *B*: proliferation rate was determined by BrdU incorporation and fluorescence microscopy. Lactotropes were identified by immunocytochemistry. Each column represents the percentage ± CI (95%) of BrdU-positive total anterior pituitary cells ($n \geq 1{,}200$ cells/group; *A*) and lactotropes ($n \geq 200$ cells/group; *B*). $^*P < 0.05$ vs. respective control without Δ1–9-G129R-hPRL, χ^2 test. *C*: representative images of anterior pituitary cells and lactotropes (red) showing immunoreactivity for BrdU (green) counterstained with DAPI (blue). Arrowheads indicate BrdU-positive cells. Scale bar, 50 μm. *D* and *E*: apoptosis rate was determined by TUNEL assay and fluorescence microscopy. Lactotrpes were identified by immunocytochemistry. Each column represents the percentage ± CI (95%) of TUNEL-positive total anterior pituitary cells ($n \geq 900$ cells/group; *D*) and lactotropes ($n \geq 200$ cells/group; *E*). $^*P < 0.05$ vs. respective control without Δ1–9-G129R-hPRL, χ^2 test. $^{**}P < 0.01$. *F*: representative images of anterior pituitary cells and lactotropes (red) TUNEL-positive (green) counterstained with DAPI (blue). Arrowheads indicate TUNEL-positive cells. Scale bar, 50 μm.

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endogenous PRL secreted into the culture medium, GH3 cells were incubated with or without Δ1–9-G129R-PRL (5 μg/ml) for 8 h. Six hours before fixation, BrdU was added to the culture medium. The percentage of BrdU-positive cells (detected by immunofluorescence) increased under treatment with the PRLR antagonist (Fig. 3). In other experiments, we evaluated the effect of Δ1–9-G129R-hPRL on GH3 cell cycle progression using flow cytometry. The percentage of cells in the S phase increased when PRL effect was antagonized by Δ1–9-G129R-hPRL, whereas the proportion of cells in the other phases of the cell cycle was not modified by the antagonist (Fig. 4). We also studied the effect of the PRLR antagonist on the apoptosis of GH3 cells. Cells were incubated with or without Δ1–9-G129R-hPRL for 8 h. Apoptosis was evaluated by TUNEL method and flow cytometry (as %cells with hypodiploid DNA content). We observed a reduction in apoptosis when GH3 cells were incubated in the presence of the PRLR antagonist (Fig. 5).

The effect of endogenous prolactin on anterior pituitary cell renewal was also confirmed using primary cultures. The blockade of PRLR by Δ1–9-G129R-hPRL (5 μg/ml, 8 h) induced both an increase in BrdU-positive (Fig. 6*A*) and a reduction in TUNEL-positive anterior pituitary cells (Fig. 6*D*). PRL immunostaining (Fig. 6, *C* and *F*) revealed that lactotropes were affected by PRLR signaling blockade. Also, a subpopulation of PRL-negative cells was responsive to PRLR antagonist. Taken together, these results suggest that PRL released into the medium acts as a proapoptotic and antiproliferative factor in anterior pituitary cells, including lactotropes.

Effect of PRLR Antagonism on the Expression of PRLR Isoforms in the Anterior Pituitary

Anterior pituitaries from WT and TG mice expressing Δ1–9-G129R-hPRL were harvested and processed for RNA extraction. Quantitative RT-PCR was performed using specific primers for long or short (S1, S2, and S3) PRLR isoforms. The main isoform expressed in the anterior pituitary from WT or TG mice was $PRLR_{long}$. The expression of S1 $PRLR_{short}$ was not detected in the anterior pituitary of any genotype (Table 2). The presence of the PRLR antagonist modified the expression of $PRLR_{long}$ and S3 $PRLR_{short}$ isoforms. $PRLR_{long}$ expression increased ~10-fold in the presence of the PRLR antagonist, whereas S3 $PRLR_{short}$ expression increased only modestly (Fig. 7). The S2 and S3 $PRLR_{short}$ isoforms combined represent ~8% of total PRLR expression in WT mice, whereas this proportion decreased to <1% in the presence of the PRLR antagonist (Table 2). Taken together, these results suggest that PRL inhibits the expression of the $PRLR_{long}$ isoform in the anterior pituitary.

DISCUSSION

In the present report, we show that antagonizing PRLR expressed in pituitary cells using a pure PRLR antagonist devoid of residual agonistic activity leads to increased cell proliferation and decreased apoptosis. This study strongly suggests that endogenous PRL naturally secreted by anterior pituitary cells exerts autocrine/paracrine antiproliferative and proapoptotic effects. It was suggested that lactotrope hyperplasia and pituitary tumor development observed in $D2R^{-/-}$ mice could be due to the high circulating PRL levels observed in these animals. Hence, PRL would act as an autocrine proliferative factor for anterior pituitary cells, particularly lactotropes (15, 17, 26). Contrary to this hypothesis, observations in $PRL^{-/-}$ mice indicated that PRL is not necessary for the onset or progression of pituitary hyperplasia and that the lack of the dopamine inhibitory signal is the primary factor inducing abnormal lactotrope growth in these knockout mice (8). However, the fact that double-knockout mice lacking both PRLR and D2R exhibited further increased pituitary weight compared with single knockouts argued for a dopamine-independent control of pituitary growth by PRL (27).

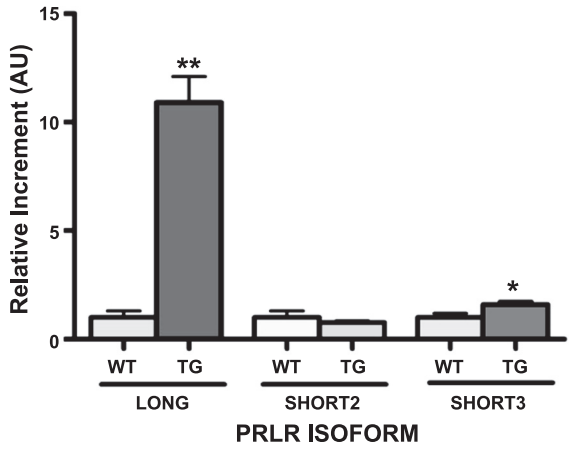

Fig. 7. PRLR expression increases in the presence of Δ1–9-G129R-hPRL. Expression of PRLR isoforms in anterior pituitary gland from WT and TG mice expressing Δ1–9-G129R-hPRL. Real-time RT-PCR was performed using specific primers for the PRLR long and short (S1, S2, S3) isoforms. Each column represents the relative increment ± SE of the long isoform of the PRLR ($PRLR_{long}$), S2 short isoform of the PRLR ($PRLR_{short}$), or S3 $PRLR_{short}$ isoform expression compared with each isoform in WT mice (n = 5–6 animals/group). $^{**}P < 0.01$ and $^{*}P < 0.05$, Student t-test. AU, arbitrary units.

We observed that the inhibition of PRLR activation in Δ1–9-G129R-hPRL TG mice increased anterior pituitary cell proliferation about 15-fold. Dopaminergic control of lactotrope function involves inhibition of lactotrope proliferation (2) and induction of apoptosis through D2 receptor activation (23). It could be speculated that functional inhibition of hypothalamic PRLR in these TG mice could decrease dopamine release to the portal system, leading to a reduction in dopaminergic inhibitory activity at the pituitary level. However, contrary to what was reported for $PRLR^{-/-}$ mice, 6-mo-old Δ1–9-G129R-hPRL mice present no clear increase in serum PRL levels (25), suggesting that the dopaminergic tone is not altered profoundly in these mice. An incomplete blockade of PRLR at the hypothalamic level in male TG mice may lead to the absence of significant variations in serum PRL at the age studied. Accordingly, studies involving primary cultures of rat hypothalamic cells showed that a small population of cells positive for the dopamine-synthesizing enzyme tyrosine hydroxylase was resistant to very high concentrations of PRLR antagonist (18). Hence, the inhibition by the antagonist of a direct action of PRL at the pituitary level could explain the effects reported in this study for TG mice.

In fact, our in vitro studies show direct effects of the PRLR antagonist on GH3 cells as well as on normal anterior pituitary cells and lactotropes, increasing proliferation and reducing apoptosis. These observations suggest that pituitary PRL reg-

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ulates anterior pituitary cell turnover via autocrine/paracrine mechanisms. Although we observed no change in apoptosis of anterior pituitaries from Δ1–9-G129R-hPRL TG mice, our in vitro experiments indicate that PRL exerts a proapoptotic action on anterior pituitary cells, suggesting that this effect contributes to the role of PRL in anterior pituitary cell renewal. The failure to detect in vivo increased apoptosis in the presence of PRLR antagonist could be due to the low apoptotic rate of pituitary gland and the rapid removal of apoptotic bodies by neighboring cells (21). Alternatively, because Δ1–9-G129R-hPRL acts in a competitive manner to prevent PRL from binding its receptor, the antagonist/agonist molar ratio existing inside the pituitary of TG mice may be limiting regarding the inhibitory effect that could be achieved by the antagonist. Taken together, these observations suggest that the increase in pituitary weight observed when PRLR signaling is reduced in these TG mice would be a consequence of the decrease of antiproliferative and proapoptotic actions exerted by pituitary PRL in anterior pituitary cells. In this respect, direct comparison of pituitary hypertrophy in our mice vs. age-matched PRLR-knockout (in which PRLR signaling is totally abolished) could not be made since Schuff et al. (27) reported data for animals of 1 yr of age and older. Although paracrine or indirect effects involving other PRL-regulated factors as proposed previously (27) cannot be completely ruled out, the response of lactotropes to the inhibition of PRLR signaling supports the hypothesis that this hormone acts primarily as an autocrine factor on lactotropes. In addition, PRL also affects the turnover of other anterior pituitary cell types.

The ultimate effects of PRL on its target tissues depend on the type of PRLR isoforms that are expressed, since they display different abilities to trigger intracellular signaling pathways. Our study indicates that both long and short PRLR isoforms are present in anterior pituitary from male mice and that the isoform expressed mainly is the long form of the PRLR, as Nagano and Kelly (20) reported in female rat pituitary. PRL has been shown to inhibit its own action by various mechanisms, including reduction of its own expression (9), negative regulation of intracellular signaling (1), and PRLR internalization (29). In the presence of the PRLR antagonist, we noticed an increase of $PRLR_{long}$ mRNA expression, suggesting that PRL is able to downregulate its own activity in the anterior pituitary gland by reducing PRLR expression, as shown previously in other tissues (7).

In female rats, the anterior pituitary cell turnover that takes place at each estrous cycle is a highly regulated process in which several factors were demonstrated to participate (32). A peak of proliferation occurs at estrus, whereas the highest rate of apoptosis is observed at proestrus (14, 31). PRL plasma levels are relatively constant along the estrous cycle, except during proestrus, when an abrupt increase in its secretion occurs as a result of high circulating estrogen levels (11). Because the peak of PRL occurs when the rate of apoptosis is the highest, the proapoptotic and antiproliferative effects of PRL observed in the present study could be involved in the control of the cyclic changes in anterior pituitary cell renewal that occurs in each estrous cycle in females. The role and mechanisms of action of PRL in regulating the plasticity of anterior pituitary gland in female rodents is under investigation by our group.

In conclusion, the present study indicates that PRL could be involved in the maintenance of physiological cell turnover in the anterior pituitary and suggests that alterations in PRL function could contribute to pituitary tumor development. Because prolactinomas are benign PRL-hypersecreting tumors, one question that arises from this work is to which extent antiproliferative and proapoptotic properties of PRL are maintained in prolactinomas and possibly contribute to their phenotype. Further investigations are needed to elucidate the role of the antiproliferative and proapoptotic actions of PRL and PRLR signaling in anterior pituitary tumorigenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.F., V.G., and D.P. did the conception and design of the research; J.F., F.B., and M.B. performed the experiments; J.F. and D.P. analyzed the data; J.F., A.S., V.G., and D.P. interpreted the results of the experiments; J.F. and D.P. prepared the figures; J.F., A.S., V.G., and D.P. drafted the manuscript; J.F., A.S., V.G., and D.P. edited and revised the manuscript; J.F., F.B., M.B., A.S., V.G., and D.P. approved the final version of the manuscript.

REFERENCES

1. **Bachelot A, Binart N.** Reproductive role of prolactin. *Reproduction* 133: 361–369, 2007.
2. **Ben-Jonathan N, Hnasko R.** Dopamine as a prolactin (PRL) inhibitor. *Endocr Rev* 22: 724–763, 2001.
3. **Ben-Jonathan N, Oliver C, Weiner HJ, Mical RS, Porter JC.** Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. *Endocrinology* 100: 452–458, 1977.
4. **Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA, Goffin V.** Development of pure prolactin receptor antagonists. *J Biol Chem* 278: 35988–35999, 2003.
5. **Binart N, Bachelot A, Bouilly J.** Impact of prolactin receptor isoforms on reproduction. *Trends Endocrinol Metab* 21: 362–368, 2010.
6. **Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA.** Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 19: 225–268, 1998.
7. **Bowen JM, Telleria CM, Towns R, Keyes PL.** Downregulation of long-form prolactin receptor mRNA during prolactin-induced luteal regression. *Eur J Endocrinol* 143: 285–292, 2000.
8. **Cruz-Soto ME, Scheiber MD, Gregerson KA, Boivin GP, Horseman ND.** Pituitary tumorigenesis in prolactin gene-disrupted mice. *Endocrinology* 143: 4429–4436, 2002.
9. **Devost D, Boutin JM.** Autoregulation of the rat prolactin gene in lactotrophs. *Mol Cell Endocrinol* 158: 99–109, 1999.
10. **Foitzik K, Krause K, Conrad F, Nakamura M, Funk W, Paus R.** Human scalp hair follicles are both a target and a source of prolactin, which serves as an autocrine and/or paracrine promoter of apoptosis-driven hair follicle regression. *Am J Pathol* 168: 748–756, 2006.
11. **Freeman ME, Kanyicska B, Lerant A, Nagy G.** Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 80: 1523–1631, 2000.

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12. **Gaytán F, Bellido C, Morales C, Sánchez-Criado JE.** Cyclic changes in the responsiveness of regressing corpora lutea to the luteolytic effects of prolactin in rats. *Reproduction* 122: 411–417, 2001.
13. **Goffin V, Bernichtein S, Touraine P, Kelly PA.** Development and potential clinical uses of human prolactin receptor antagonists. *Endocr Rev* 26: 400–422, 2005.
14. **Hashi A, Mazawa S, Kato J, Arita J.** Pentobarbital anesthesia during the proestrous afternoon blocks lactotroph proliferation occurring on estrus in female rats. *Endocrinology* 136: 4665–4671, 1995.
15. **Hosojima H, Wyche JH.** Prolactin control of growth and prolactin autoregulation in cultured human pituitary cells. *Horm Res* 21: 240–245, 1985.
17. **Krown KA, Wang YF, Ho TW, Kelly PA, Walker AM.** Prolactin isoform 2 as an autocrine growth factor for GH3 cells. *Endocrinology* 131: 595–602, 1992.
18. **Ma FY, Anderson GM, Gunn TD, Goffin V, Grattan DR, Bunn SJ.** Prolactin specifically activates signal transducer and activator of transcription 5b in neuroendocrine dopaminergic neurons. *Endocrinology* 146: 5112–5119, 2005.
19. **Melmed S, Carlson HE, Briggs J, Hershman JM.** Autofeedback of prolactin in cultured prolactin-secreting pituitary cells. *Horm Res* 12: 340–344, 1980.
20. **Nagano M, Kelly PA.** Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. *J Biol Chem* 269: 13337–13345, 1994.
21. **Pisera D, Candolfi M, Navarra S, Ferraris J, Zaldivar V, Jaita G, Castro MG, Seilicovich A.** Estrogens sensitize anterior pituitary gland to apoptosis. *Am J Physiol Endocrinol Metab* 287: E767–E771, 2004.
22. **Pujianto DA, Curry BJ, Aitken RJ.** Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* 151: 1269–1279, 2010.
23. **Radl DB, Ferraris J, Boti V, Seilicovich A, Sarkar DK, Pisera D.** Dopamine-induced apoptosis of lactotropes is mediated by the short isoform of D2 receptor. *PLoS One* 6: e18097, 2011.
24. **Radl DB, Zarate S, Jaita G, Ferraris J, Zaldivar V, Eijo G, Seilicovich A, Pisera D.** Apoptosis of lactotrophs induced by D2 receptor activation is estrogen dependent. *Neuroendocrinology* 88: 43–52, 2008.
25. **Rouet V, Bogorad RL, Kayser C, Kessal K, Genestie C, Bardier A, Grattan DR, Kelder B, Kopchick JJ, Kelly PA, Goffin V.** Local prolactin is a target to prevent expansion of basal/stem cells in prostate tumors. *Proc Natl Acad Sci USA* 107: 15199–15204, 2010.
26. **Saiardi A, Bozzi Y, Baik JH, Borrelli E.** Antiproliferative role of dopamine: loss of D2 receptors causes hormonal dysfunction and pituitary hyperplasia. *Neuron* 19: 115–126, 1997.
27. **Schuff KG, Hentges ST, Kelly MA, Binart N, Kelly PA, Iuvone PM, Asa SL, Low MJ.** Lack of prolactin receptor signaling in mice results in lactotroph proliferation and prolactinomas by dopamine-dependent and -independent mechanisms. *J Clin Invest* 110: 973–981, 2002.
28. **Shao R, Nutu M, Weijdegård B, Egecioglu E, Fernandez-Rodriguez J, Tallet E, Goffin V, Ling C, Billig H.** Differences in prolactin receptor (PRLR) in mouse and human fallopian tubes: evidence for multiple regulatory mechanisms controlling PRLR isoform expression in mice. *Biol Reprod* 79: 748–757, 2008.
29. **Swaminathan G, Varghese B, Thangavel C, Carbone CJ, Plotnikov A, Kumar KG, Jablonski EM, Clevenger CV, Goffin V, Deng L, Frank SJ, Fuchs SY.** Prolactin stimulates ubiquitination, initial internalization, and degradation of its receptor via catalytic activation of Janus kinase 2. *J Endocrinol* 196: R1–R7, 2008.
30. **Tan D, Walker AM.** Short form 1b human prolactin receptor down-regulates expression of the long form. *J Mol Endocrinol* 44: 187–194, 2010.
31. **Yin P, Arita J.** Proestrous surge of gonadotropin-releasing hormone secretion inhibits apoptosis of anterior pituitary cells in cycling female rats. *Neuroendocrinology* 76: 272–282, 2002.
32. **Zárate S, Zaldivar V, Jaita G, Magri L, Radl D, Pisera D, Seilicovich A.** Role of estrogens in anterior pituitary gland remodeling during the estrous cycle. *Front Horm Res* 38: 25–31, 2010.

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