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Fibroblast growth factor-2 in hyperplastic pituitaries of D2R knockout female mice

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Cristina C, Díaz-Torga G, Góngora A, Guida MC, Perez-Millán MI, Baldi A, Becu-Villalobos D. Fibroblast growth factor-2 in hyperplastic pituitaries of D2R knockout female mice. *Am J Physiol Endocrinol Metab* 293: E1341–E1351, 2007. First published September 11, 2007; doi:10.1152/ajpendo.00260.2007.—Dopamine D2 receptor (D2R) knockout (KO) female mice develop chronic hyperprolactinemia and pituitary hyperplasia. Our objective was to study the expression of the mitogen fibroblast growth factor (FGF2) and its receptor, FGFR1, comparatively in pituitaries from KO and wild-type (WT) female mice. We also evaluated FGF2 subcellular localization and FGF2 effects on pituitary function. FGF2-induced prolactin release showed a similar response pattern in both genotypes, even though basal and FGF2-stimulated release was higher in KO. FGF2 stimulated pituitary cellular proliferation (MTS assay and [^{3}H]thymidine incorporation), with no differences between genotypes. FGF2 concentration (measured by ELISA) in whole pituitaries or cultured cells was lower in KO ($P < 0.00001$ and 0.00014). Immunofluorescence histochemistry showed less FGF2 in pituitaries from KO females and revealed a distinct FGF2 localization pattern between genotypes, being predominantly nuclear in KO and cytosolic in WT pituitaries. Finally, FGF2 could not be detected in the conditioned media from pituitary cultures of both genotypes. FGFR1 levels (Western blot and immunohistochemistry) were higher in pituitaries of KO. Basal concentration of phosphorylated ERKs was lower in KO cells ($P = 0.018$). However, when stimulated with FGF2, a significantly higher increment of ERK phosphorylation was evidenced in KO cells ($P \leq 0.02$). We conclude that disruption of the D2R caused an overall decrease in pituitary FGF2 levels, with an increased distribution in the nucleus, and increased FGFR1 levels. These results are important in the search for reliable prognostic indicators for patients with pituitary dopamine-resistant prolactinomas, which will make tumor-specific therapy possible.

dopaminergic D2 receptor; fibroblast growth factor receptor-1; extracellular signal-regulated kinase phosphorylation; prolactin; immunohistochemistry

DOPAMINE (DA), acting on lactotroph dopaminergic D2 receptors (D2Rs) in the pituitary, inhibits prolactin synthesis and secretion as well as cell proliferation. Accordingly, we have demonstrated that D2R knockout (KO) mice develop hyperprolactinemia, lactotroph hyperplasia, and pituitary adenomas (3, 31) with many areas of extravasated red blood cells or peliosis. Pituitary tumor development confirms the importance of the neurotransmitter DA in the regulation of lactotroph function. DA, however, is not the only factor involved in the proliferation of lactotrophs.

Previous studies have indicated that several angiogenic growth factors and receptors might be important, both for tumor-associated angiogenesis and by acting as autocrine or paracrine growth factors on tumor cells (25). We have recently demonstrated that pituitary vascular endothelial growth factor A (VEGF-A) is overexpressed in D2R KO female mice and suggested its participation in intrahypophyseal endothelial cell proliferation, a process that may increase the availability of different growth factors and mitogens to the gland (8). Many growth factors are not only produced but also exert their effects in the pituitary, and it is well known that they are implicated in pituitary tumorigenesis in different experimental models.

Among them, basic fibroblast growth factor-2 (basic FGF, or FGF2) was originally isolated from the bovine pituitary and has a pleiotropic activity affecting both vasculature and parenchyma cell proliferation and differentiation (24). FGF2 belongs to a large family of heparin-binding growth factors comprising at least 22 structurally related members (44). FGF2 expression is complex; at least four FGF2 isoforms (18, 22, 22.5, and 24 kDa) in humans and three (18, 21, and 22 kDa) in mouse are synthesized through alternative translation initiation from CUG codons (16). The 18-kDa isoform is predominantly cytoplasmic but can also be found in the extracellular matrix, while the higher-molecular-weight isoforms are localized in nuclei and ribosomes (16, 47). Recently, a 34-kDa isoform was reported with the most upstream CUG codon among all FGF2 forms (2). None of the isoforms has a typical secretory signal sequence, but alternative pathways have been described for their export from the cell (16).

The biological effects of FGF2 are mediated through four high-affinity transmembrane receptors (FGFR1–FGFR4) that have intrinsic tyrosine kinase activity (41). They can be found on a wide variety of cell membrane surfaces including endothelial cells, where FGF2 exerts its proangiogenic functions. In the present work, we decided to focus on FGFR1, as it was found in the normal human pituitary as well as in pituitary adenomas (1), and its mRNA was described in the rat neural and anterior lobe (21). Furthermore, FGFR1 has been proposed as a candidate marker of pituitary tumors together with FGF2 and pituitary tumor transforming gene (PTTG) (55); indeed, the FGF2 receptor FGFR1 was found to be highly expressed in pituitary tumors compared with the normal gland (38). Furthermore, significantly increased FGFR1 mRNA expression was described in functioning tumors that invaded the sphenoid bone compared with those that did not, thus raising

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the possibility of using the FGFR1 as a molecular marker of tumor biological behavior (38). On the other hand, it has also been determined that cytoplasmic FGFR1 immunoreactivity was inversely correlated with maximum pituitary tumor diameter (18).

FGF2 participates in pituitary development and proliferation and regulates hormone synthesis and secretion, affecting prolactin and thyroid-stimulating hormone production (34). It is mainly produced by folliculostellate cells (15), although somatotrophs and gonadotrophs have also been reported to be sources of this growth factor (37, 50).

The 18-kDa FGF2 isoform is highly expressed in the normal human pituitary, while pituitary adenomas produce predominantly the 24-kDa form (36). FGF2 participates in estradiol-mediated pituitary hyperplasia under both physiological and pharmacological conditions (26, 27, 50). It is also expressed by human pituitary adenoma cells in vitro, and high levels of serum FGF2 were found in patients bearing pituitary tumors, declining following surgical adenomectomy (13).

The regulation of pituitary FGF2 by the D2R has not been reported yet, even though the D2R and FGF2 have been shown to participate in pituitary hyperplasia. In other tissues, the D2R upregulates FGF2 expression. For example, it has been reported that DA acting via D2R stimulates the expression of FGF2 in some areas of the rat brain such as prefrontal cortex, striatum, and hippocampus (19, 48) and in striatal astrocytes (35). Additionally, during dopaminergic neuron degeneration, as in Parkinson's disease, there is FGF2 depletion and a retention of FGFR1 immunoreactivity in substantia nigra neurons (56). In the present work, we used pituitaries from D2R KO and wild-type (WT) female mice to study the expression of FGF2 and FGFR1. We also evaluated FGF2 subcellular localization in relation to the development of pituitary hyperplasia. Furthermore, we analyzed the participation of FGF2 in prolactin secretion, cell proliferation, and phosphorylation of extracellular signal-regulated kinases (ERKs) comparatively in pituitaries from WT and D2R KO female mice.

MATERIALS AND METHODS

Animals

D2R KO mice, official strain designation B6;129S2-*Drd2*^{tm1low} by the Induced Mutant Resource at The Jackson Laboratory (Bar Harbor, ME), generated by targeted mutagenesis of the D2R gene in embryonic stem cells (3, 31), were used. The original F_2 hybrid strain (129S2/Sv × C57BL/6J) containing the mutated D2R allele was backcrossed for at least eight generations to WT C57BL/6J mice. Mutant and WT mice were generally the product of heterozygote crossings, and, in all cases, sibling controls were used. Female mice were housed in groups of four or five with mixed genotypes in an air-conditioned room with lights-on at 0700 and lights-off at 1900. They had free access to laboratory chow and tap water. WT, heterozygous, and KO mice were identified by PCR of genomic DNA, as previously described (12). Animals were used at 8–10 mo, and pituitaries from KO females were hyperplastic at this moment. All experimental procedures were reviewed and approved by the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires [in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health (A-5072-01)].

Blood was collected by decapitation. Sera were kept at −20°C until radioimmunoassays were performed. Pituitaries were excised for Western blot analysis, described below (see *Western Blot*).

Reagents

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO).

Radioimmunoassay

Prolactin was measured by radioimmunoassay (RIA) using mouse-specific reagents provided by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) National Hormone and Pituitary Program (NHPP) (Dr. A. F. Parlow, NHPP, Torrance, CA).

Assays were performed using 10 μl of serum in duplicate or the adequate quantity of diluted medium from cultured cells. Results are expressed in terms of mouse prolactin reference preparation 3 (RP3). Intra- and interassay coefficients of variation were 7.2 and 12.8%, respectively.

Western Blot

Anterior pituitaries were homogenized in 80 μl of ice-cold buffer containing 60 mM Tris·HCl, 1 mM EDTA (pH 6.8), and a mix of protease inhibitors (PMSF, TPCK, TAME, ZPCK, and TLCK) in a handheld microtissue homogenizer. The homogenate was then centrifuged at 800 *g* for 5 min at 4°C. An aliquot of supernatant was taken to quantify proteins by the Lowry method. Thirty micrograms of proteins in 10 μl of buffer were mixed with 10 μl of 2× sample buffer (60 mM Tris·HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 50 mM DTT, pH 6.8). Samples were sonicated for 20 s, heated for 5 min at 95°C, and subjected to 12% SDS-PAGE. The gel was then blotted onto a nitrocellulose membrane (Bio-Rad, Buenos Aires, Argentina), blocked with 3% nonfat milk in PBS-0.05% Tween, and probed with the corresponding primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. Polyclonal rabbit FGFR1 antibody (Flg C-15, sc-121, 1:800; Santa Cruz Biotechnology, Santa Cruz, CA) was used. This antibody recognizes the carboxy terminus of the Flg receptor. Monoclonal mouse actin antibody (actin Ab-1 kit, 1:10,000; Oncogene, Research Products, Calbiochem-Novabiochem International) was employed to quantify the actin content. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham, Aylesbury, UK). Band intensities were quantified using ImageQuant software.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed to quantify FGF2 content in pituitary homogenates and pituitary cell culture lysates and supernatants of WT and KO female mice. Preliminary studies demonstrated that pituitary homogenates needed an extraction step with heparin-Sepharose beads (Pharmacia Biotech) to purify the growth factor from the sample. Forty-five microliters of each sample containing 200 μg of protein were mixed with 80 μl of 10 mM Tris·HCl and 50 μl of heparin-Sepharose and left mixing overnight at 4°C. Beads were washed twice with 0.6 M NaCl-10 mM Tris·HCl, and, finally, FGF2 was eluted off the heparin-Sepharose pellet with 2 M NaCl-10 mM Tris·HCl. The FGF2 assay was carried out using the Quantikine FGF Basic Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's specifications. The minimum detectable dose of FGF2 is 3 pg/ml with this kit, and it has no cross-reactivity with related peptides.

Immunohistochemistry

FGFR1 immunostaining was performed on pituitaries following routine protocols, as previously described (45). Pituitaries were fixed in 4% formol and embedded in paraffin. Paraffin sections were cut in a microtome, every 4 μm, and mounted onto glass slides. Sections were deparaffinized, rehydrated, washed in PBS, and treated with 3% H_2O_2 in PBS for 20 min at room temperature to block endogenous peroxidase. The slides were preincubated in 3% nonfat milk in PBS

for 1 h to block nonspecific binding sites, and a 1:100 solution of the first antibody (Flg C-15, sc-121; Santa Cruz Biotechnology) was then added to the sections. After incubation overnight at 4°C, slides were washed with PBS and incubated at room temperature for 1 h with a biotin-labeled second antibody against rabbit IgG and then with a preformed ABC complex for 30 min (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Subsequently, slides were immersed in a 0.05% 3,3′-diaminobenzidine solution in 0.1 M Tris buffer, pH 7.2, containing 0.01% H_2O_2. After brown color developed, slides were removed, the reaction was stopped by immersion in PBS, and sections were counterstained with methyl green, dehydrated, and coverslipped with Permount. Immunoreactive cells were visualized with the aid of an Axiostar Plus Zeiss microscope equipped with a Canon PowerShot G6 digital camera at a magnification of ×100. The intensity of FGFR1 immunoreactivity was quantified by computerized image analysis (Optimas, Bioscan), as already described (22). Four animals of each genotype were used, and a minimum of four to five pituitary sections obtained at different levels of each gland were used for quantitation. Results were pooled for the individual animals. To visualize localization of FGFR1 in relation to gonadotropes, after the diaminobenzidine reaction was stopped, slides were incubated in preheated 10 mM Na citrate buffer, pH 6, for 10 min in a microwave oven at maximum power. Slides were allowed to return to room temperature in the citrate buffer, washed with PBS, and incubated with rabbit anti-rat luteinizing hormone (LH) (NHPP, NIDDK, Torrance, CA) followed by a biotinylated anti-rabbit IgG (Vector Laboratories) and then a Vector SG staining kit (Vector Laboratories).

Indirect immunofluorescence was applied to detect cells expressing FGF2 of D2R KO and WT female mice pituitaries on paraffin-embedded sections, previously fixed in 4% formol. Antigen retrieval with 20 μg/ml proteinase K was performed on tissue sections followed by incubation for 2 h with 3% nonfat milk blocking buffer. A goat polyclonal FGF2 antibody that recognizes high and low isoforms of FGF2 of human, mouse, and rat origin (dilution 1:100, Santa Cruz Biotechnology) was used, and, finally, sections were incubated with FITC rabbit anti-goat IgG (dilution 1:100; Zymed Laboratories, San Francisco, CA). Sections were counterstained with propidium iodide and mounted in Vectashield (Vector Laboratories) to prevent fading of immunofluorescence. Sections were examined on a Nikon C1 Plan Apo ×60/1.4 oil confocal laser scanning system. The excitation wavelength was 488 nm for FITC-induced fluorescence. Specificity studies carried out by omitting primary antisera or by preabsorbing the primary antisera with homologous antigen excess showed a complete absence of the fluorescent signal.

FGF2 immunoreactivity in the nucleus was recorded as the FGF2 nuclear index, which was calculated as the percentage of pituitary cells with FGF2 immunoreactivity in the nuclei in relation to total pituitary cells in each section determined by the propidium iodide-stained nuclei.

Double-Labeling Immunofluorescence and Confocal Laser Microscopy

Double-labeling immunofluorescence was applied to specifically identify the cell type(s) expressing FGFR1. Double immunostaining was performed on paraffin-embedded sections of D2R KO and WT mice. We combined rabbit anti-FGFR1 (Santa Cruz Biotechnology) antibody with a goat polyclonal antibody against prolactin (Santa Cruz Biotechnologies) or monkey anti-rat growth hormone (GH) (NHPP, NIDDK). After a rinsing in PBS, the double-stained sections were incubated at room temperature for 2 h with FITC donkey anti-rabbit IgG (Santa Cruz Biotechnology) for FGFR1 and Texas Red-X-conjugated donkey anti-goat IgG (Santa Cruz Biotechnologies) and goat anti-monkey IgG (Santa Cruz Biotechnologies) for prolactin and GH, respectively. After a rinsing in PBS, the sections were mounted in Vectashield (Vector Laboratories) to prevent fading of the immunofluorescence stain. Sections were examined on a C1 Plan Apo ×60/1.4 oil confocal laser scanning system (Nikon, Tokyo, Japan). The excitation wavelength was 488 nm for FITC and 543 nm for Texas Red-X-induced fluorescence. Specificity studies were carried out by omitting primary antisera or by preabsorbing the primary antisera with homologous antigen excess; all showed the absence of the fluorescent signal.

Cell Dispersion and Culture

Cell culture was performed as previously described (8). Anterior pituitaries from 8- to 10-mo-old female WT and KO mice were weighed and placed in chambers containing freshly prepared Krebs-Ringer bicarbonate buffer without Ca^{2+} or Mg^{2+}. Buffer contained 14 mM glucose, 1% BSA, 2% MEM amino acids, 1% MEM vitamins (Life Technologies, Buenos Aires, Argentina), and 2 mM glutamine; was previously gassed for 15 min with 95% O_2 and 5% CO_2; and was adjusted to pH 7.35–7.40. Buffer was filtered through a 0.45 μm-pore diameter membrane (Nalgene). Pituitaries were washed three times with Krebs-Ringer bicarbonate buffer and then cut into 1-mm pieces. Fragments were washed and incubated in the same buffer containing 0.5% trypsin for 30 min at 37°C in 95% O_2 and 5% CO_2, followed by an additional 2 min with 50 μl of deoxyribonuclease I (1 mg/ml; Worthington Biochemical, Lakewood, NJ). Digestion was ended by adding 1 mg/ml lima bean trypsin inhibitor. Fragments were dissociated to single cells by gentle passage through Pasteur pipette. The resulting suspension was filtered through nylon gauze (160-μm pore size) and centrifuged for 10 min at 1,000 *g*. Before centrifugation, an aliquot of cellular suspension was taken to quantify pituitary cell yield with the use of a Neubauer chamber. Viability of cells, determined by Trypan blue exclusion, was always >90%. Cells were cultured for 5 days in DMEM, 10% horse serum, and 2.5% FBS (Gibco, Buenos Aires, Argentina). As previously described, cultured cells from WT animals had a higher percentage of GH cells and a lower percentage of prolactin cells compared with those from KO animals, which nevertheless maintained secretory responses with the exception of dopamine response (8, 20). Cells were then washed and stimulated with 1 or 10 ng/ml recombinant human FGF2 for 48 h in DMEM-F-12+2.2 g/l $NaHCO_3$ (BIC) 0.5% BSA medium without serum.

After culturing of 320,000 cells in 24-well plates as described above, 800 μl of conditioned media (CM) were collected and used in human umbilical cord vein endothelial cell (HUVEC) proliferation assays and for FGF2 determination by ELISA.

ERK Phosphorylation Assay

Cells were grown in 24-well plates (250,000 cells/well) as described above in *Cell Dispersion and Culture*. On the fifth day of culture, medium was washed, and cells were treated with or without 50 ng/ml FGF2 for 5, 10, and 15 min. They were then lysed in 1× sample buffer (30 mM Tris·HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 25 mM DTT, pH 6.8), sonicated for 20 s, heated for 5 min at 95°C, and subjected to 12% SDS-PAGE. Mouse monoclonal anti-phospho-ERK antibody (1:800, sc-7383, pERK E-4) and rabbit polyclonal ERK1 total antibody (1:700, sc-94, ERK1 K-23) were purchased from Santa Cruz Biotechnology. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham, Aylesbury, UK). For repeated immunoblotting, membranes were incubated in stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM mercaptoethanol, pH 6.7) for 40 min at 50°C and reprobed. Band intensities were quantified using the ImageQuant software.

Cell Proliferation Assays

[^{3}H]thymidine incorporation. Culture procedure was the same as described above. [^{3}H]thymidine (0.2 μCi/well, 87.7 Ci/mmol; NEN Life Science Products, Boston, MA) was added to cultures (P96, 35,000 cells/well). After 24 h of incubation, medium was discarded and the cells were removed and lysed by treatment with 0.05% trypsin

and 0.02% EDTA in deionized water. The reaction was stopped 20 min later by filtering under vacuum through GF/C Whatman filters using the Nunc Cell Harvester 8. After five washes with deionized water, the filters were placed in plastic vials with 3 ml of scintillation solution, and radioactivity was counted in a Beckman counter. Each experiment was repeated six times.

MTS proliferation assay. Proliferation of anterior pituitary cells was also colorimetrically determined at 490 nm using a commercial proliferation assay kit (CellTiter 96, Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). After incubation of 35,000 cells/well in a 96-well plate with various concentrations of FGF2 for 48 h, cells were incubated with 333 mg/l 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 25 M phenazine methosulfate solution for 0.5, 1, and 2 h at 37°C in a humidified 5% CO_2 atmosphere. The absorbance of soluble formazan produced by cellular reduction of MTS was measured at 490 nm using an ELISA reader (Merck Sensident Scan). In each experiment, four to six mice of each genotype were used; experiments were repeated four times, and each had quadruplicate samples.

Endothelial Cell Culture and Proliferation Assay

HUVECs were isolated from the umbilical cord by enzymatic digestion with collagenase as previously described (8). Cells were cultured in T75 flasks in M199 supplemented with 20% FBS, growth factor mix [2 ng/ml FGF2, 10 ng/ml VEGF, 5 ng/ml epidermal growth factor (EGF)], and 50 μg/ml gentamycin and maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air. The culture medium was changed every 72 h, and HUVEC confluent cultures were washed twice with PBS, released with 0.05% (wt/vol) trypsin and 5 mM EDTA, and subcultured. Cell proliferation studies were carried out using endothelial cells at passages four to eight.

The proliferation of HUVEC was measured by [^{3}H]thymidine incorporation. HUVECs were harvested with trypsin-EDTA and suspended in M199 (supplemented with 20% FBS and 50 μg/ml gentamycin) at a density of 25,000 cells/ml and were then seeded into a 96-well plate (100 μl/well: 2,500 cells/well) and incubated for 2 h for attachment. Then, 50 μl of the same medium with FGF2 (final concentration 2 ng/ml), VEGF-A (final concentration 10 ng/ml), EGF (1 ng/ml), or CM collected from pituitary cell cultures were added alone or with 2.5 μg/ml polyclonal antibody against FGF2 or VEGF-A. Cells were incubated for 24 h before addition of 5 μCi/ml [^{3}H]thymidine. After 48 h of incubation, the assay was ended by adding 50 μl of guanidine HCl, and the cells were lysed by a freezing-defrost cycle. The DNA was harvested in Whatman GF/C filters by use of an eight-well harvester (Cell Harvester 8, Nunc), and 1 ml of scintillation solution (OptiPhase Hifase 3) was added. The [^{3}H]thymidine incorporation was measured by use of a liquid scintillation counter.

Statistical Analyses

Results are expressed as means ± SE. FGF2 and FGFR1 expression, [^{3}H]thymidine uptake, FGF2 nuclear index in immunohistochemistry, and basal mitogen-activated protein kinase (MAPK) expression were analyzed by Student's *t*-test. MTS proliferation assay, prolactin release in vitro, and the effect of CM on proliferation of HUVEC were analyzed by two-way ANOVA for independent measures for the effects of genotype and treatment. ERK phosphorylation induced by FGF2 at different periods was analyzed by two-way ANOVA for repeated measures. In all cases, if P of interaction was found to be significant, individual means were compared by Tukey's honest significant difference test; if interaction was not significant, groups of means were analyzed by the same test. In culture experiments (prolactin secretion or proliferation assays), N refers to the number of independent experiments; in each experiment, at least four WT pituitaries and three KO pituitaries were used, and treatments were assayed in quadruplicate wells in each experiment. $P < 0.05$ was considered significant.

RESULTS

We measured prolactin secretion induced by 1 and 10 ng/ml FGF2 comparatively in pituitary cells from WT and KO mice and found that KO cells secreted higher levels of this hormone both basally and in response to FGF2 (Fig. 1, $P < 0.0001$ and 0.01 for the effects of genotype and 10 ng/ml FGF2 treatment, respectively). Nevertheless, the percent increment of prolactin over basal levels showed a similar pattern in both genotypes (interaction $P = 0.83$).

We tested whether FGF2 had a differential effect on pituitary cell proliferation in vitro by comparing genotypes. This was determined using the MTS proliferation assay and [^{3}H]thymidine uptake. As previously described (8), basal proliferation was lower in cells from KO animals {MTS (optical density): 0.49 ± 0.02 and 0.23 ± 0.01, WT vs. KO, $P < 0.02$; and in [^{3}H]thymidine uptake (cpm): 1,090 ± 55 and 590 ± 050, WT vs. KO, $P < 0.01$; Fig. 2, *A–C*}. In the MTS assay, we found that FGF2 stimulated pituitary cell proliferation only at the high dose (10 ng/ml), and there were no differences between genotypes (Fig. 2, *A* and *B*, P interaction = 0.82, FGF 10 ng/ml: $P < 0.01$). As shown in Fig. 2*C*, similar results were obtained using [^{3}H]thymidine incorporation ($P < 0.005$ for the effect of 10 ng/ml FGF2 in both genotypes).

The concentration of FGF2 in WT and KO pituitaries was measured by ELISA. Pituitary homogenate extracts from KO mice, purified by heparin-Sepharose treatment, exhibited a lower FGF2 content compared with those from WT mice (Fig. 3*A*, normalized to μg of protein, $P < 0.00002$). FGF2 concentration in pituitary cells cultured for 5 days was also lower in KO mice compared with their WT counterparts (Fig. 3*B*, $P < 0.0002$).

In accordance, immunofluorescence histochemistry showed less FGF2 immunoreactivity in pituitaries from KO females, but, interestingly, it also revealed a distinct FGF2 localization

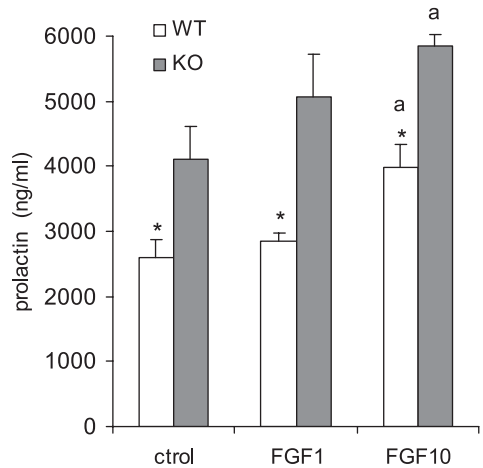

Fig. 1. Prolactin release in cultured pituitary cells from wild-type (WT) and knockout (KO) female mice; 35,000 cells were seeded, and after 5 days they were stimulated with buffer (control; ctrol) or fibroblast growth factor-2 (FGF2) (1 and 10 ng/ml; FGF1 and FGF10, respectively) for 48 h. Results are expressed in ng/ml. *$P < 0.05$ vs. KO. [a]$P < 0.05$ vs. respective control for both genotypes. $N = 4$ experiments; in each experiment, 4 WT pituitaries and 3 KO pituitaries were used.

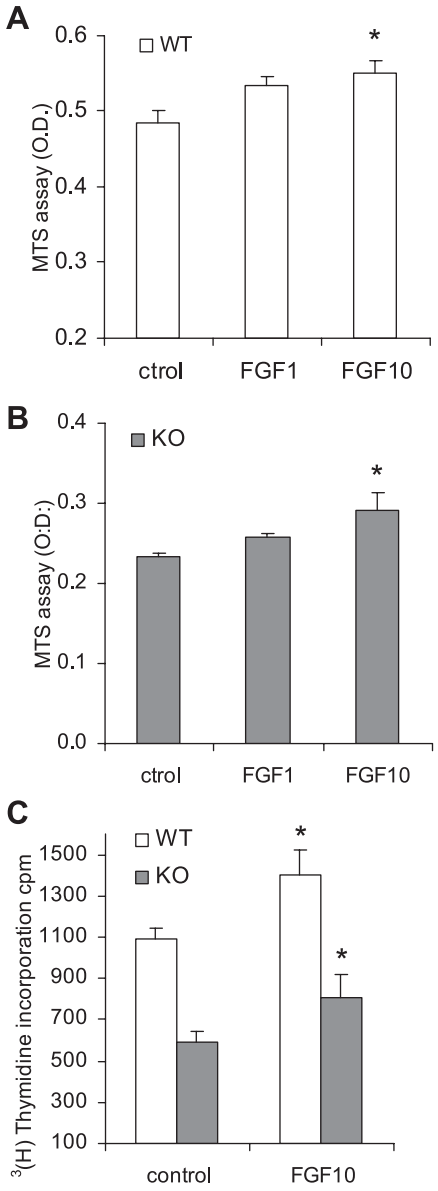

Fig. 2. *A* and *B*: MTS proliferation assay in cultured pituitary cells from WT and KO female mice; 35,000 cells were seeded/well, and after 5 days they were stimulated with buffer (control) or FGF2 (1 and 10 ng/ml; FGF1 and FGF10, respectively) for 48 h. Results are expressed as the average optical density (OD) for each treatment assayed in quadruplicate. *$P < 0.05$ vs. control. $N = 4$ for each group. *C*: effect of 10 ng/ml FGF2 on [^{3}H]thymidine incorporation in cultured pituitary cells ($N = 6$ and 6 experiments for WT and KO, respectively). Results are expressed as average cpm. *$P < 0.05$ vs. respective control.

pattern between genotypes, being predominantly nuclear in KO and cytosolic in WT sections (Fig. 4*A*). Nuclear indexes of FGF2 immunopositivity were 32.4 ± 5.1% in KO and 24.3 ± 2.3% in WT ($P < 0.05$).

We assayed the levels of the FGFR1 in pituitary homogenates by Western blot and immunohistochemistry. Western blot results showed that there were higher levels of FGFR1 in pituitaries of KO compared with WT mice (Fig. 5*A*, $P < 0.05$). Immunohistochemistry confirmed these data (Fig. 4*B*), and we were also able to determine that FGFR1 localized mainly in the cytoplasm of pituitary cells from both genotypes (Fig. 4*B*). Double staining characterized the positivity for FGFR1 in KO pituitaries, mainly in cells containing cytoplasmic reactivity for prolactin (Fig. 4, *C–E*); nevertheless, we also found localization in a low proportion of GH and LH cells (not shown). Therefore, the expression of these receptors is not completely cell type specific.

It has been shown that FGF2 regulates cell proliferation by activating ERK1 and -2 phosphorylation in different cell types. We evaluated the action of 50 ng/ml FGF2 for 5, 10, 15, and 30 min on ERK phosphorylation in pituitary cells in vitro. First, and to our surprise, we observed a lower basal concentration of pERK in KO cells compared with their WT counterparts (Fig. 6*A*, $P < 0.02$). However, when stimulated with FGF2, a significantly higher increment of ERK phosphorylation was evidenced in KO cells at 5 and 10 min ($P \leq 0.02$, Fig. 6, *B* and *C*).

Because FGF2 has strong angiogenic properties in many tissues, we tested the capability of CM from KO and WT pituitary cells to enhance [^{3}H]thymidine incorporation by HUVECs. The specificity of the antibody used to block FGF2 action at the endothelial receptor is shown (see Fig. 7*B*). Anti-FGF2 antibody blocked the effect of synthetic FGF2 but not of VEGF or EGF, and, furthermore, anti-VEGF blocked VEGF but not FGF2 or EGF action. The pituitary supernatants increased DNA synthesis of HUVECs, and this effect was partially abrogated by a specific FGF2 antibody in both genotypes to the same extent (Fig. 7*A*). However, [^{3}H]thymidine incorporation in BIC buffer was also significantly decreased by the FGF2 antibody, suggesting that the effect observed could be due to FGF2 of HUVEC and not of CM origin (interaction $P = 0.79$, effect of treatment $P < 0.0001$, and effect of antibody pretreatment $P < 0.05$). In fact, when we measured FGF2 content of HUVECs in the presence of CM from pitu-

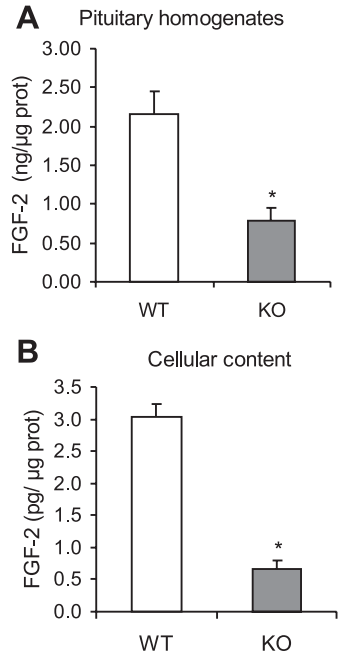

Fig. 3. Basal FGF2 concentration in pituitary homogenates (*A*) and cellular lysates (*B*) from WT and KO female mice as determined by ELISA. Prot, protein. *$P < 0.05$ vs. WT. $N = 9$ and 10 animals for homogenates for WT and KO, respectively; $N = 3$ and 3 cell cultures for cellular lysates for WT and KO, respectively.

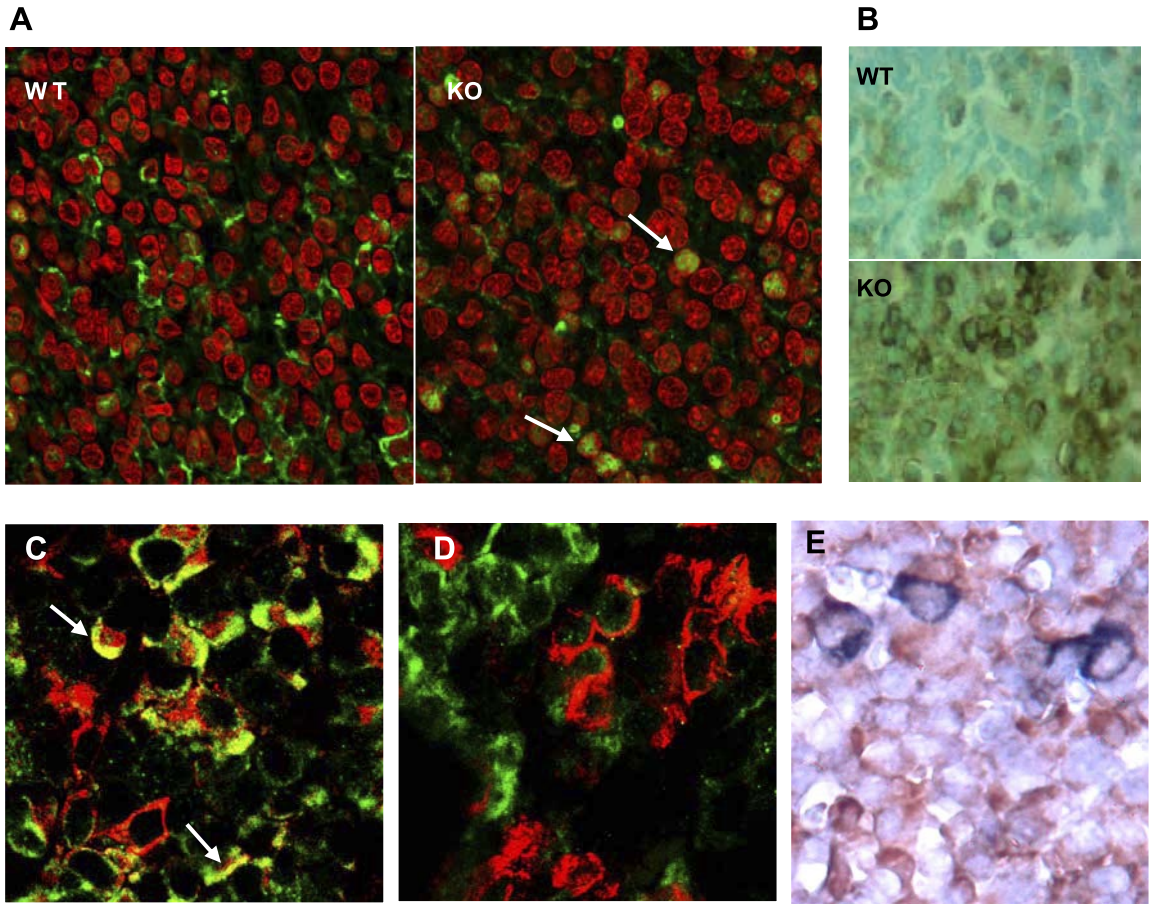

Fig. 4. *A*: representative immunofluorescent staining for FGF2 in pituitaries of WT and KO female mice. FGF2 is visualized in the green immunofluorescent channel. Cells are counterstained with propidium iodide to mark nuclei (red channel). Arrows are FGF2-stained nuclei. *B*: representative immunohistochemistry of fibroblast growth factor receptor-1 (FGFR1; brown) in pituitaries from WT (*top*) and KO (*bottom*) female mice. Cells were counterstained with methyl green. *C* and *D*: double-label immunofluorescent staining combined with confocal laser microscopy in the pituitary of dopaminergic D2 receptor (D2R) KO mice. FGFR1 is visualized in the green immunofluorescent channel and the pituitary hormones [prolactin (*C*) or growth hormone (GH; *D*)] in the red immunofluorescent channel. Arrows indicate double-stained pituitary cells. Objective lens magnification, ×60/1.4 oil. *E*: double immunohistochemistry of FGFR1 (brown) and luteinizing hormone (blue) in a KO pituitary. Cells were counterstained with hematoxylin. *A–E*: each image is representative of staining patterns seen in at least 3 independently examined mice pituitaries.

itary cells of both genotypes or BIC, we found similar levels ($P = 0.39$, Fig. 7*C*). And, not surprisingly, no detectable FGF2 levels were found in the culture media of pituitary cells from both genotypes, as this growth factor is not a secreted protein, and probably the concentration in the CM was under the detection limit of the ELISA method employed.

DISCUSSION

In the present work, we demonstrate that disruption of D2R modifies FGF2 and FGFR1 expression in the pituitary. FGF2 concentration was reduced in hyperplastic pituitaries of D2R KO mice, whereas FGFR1 expression was increased. FGF2 induced an increased pERK phosphorylation in cells from KO pituitaries, but this effect could not be related to increased proliferation in this experimental model. Since FGF2 subcellular localization was different between genotypes (nuclear in KO and cytoplasmic in WT), it might act in an intracrine way in these tumors independently of pERK activation.

FGF2 participates in the development and function of numerous organs as well as in angiogenic processes in normal and tumoral tissues (5). It was first identified and characterized from the bovine pituitary (23), and, of all organs tested, pituitary glands have the highest concentration (24). FGF2 specifically increases the secretory activity of pituitary cells (4, 6, 40) and has been proposed as one of the candidates involved in cell proliferation in estrogen-induced pituitary hyperplasia (27, 50). Moreover, FGF2 and VEGF are considered the classical endogenous angiogenic factors, and FGF2 is recognized as an important factor in tumor formation in the anterior pituitary (24, 36). In a previous work (12), we described an increase in VEGF expression in the pituitaries of D2R KO mice, and VEGF has important effects on FGF2 expression in different tissues (46). Furthermore, an association between FGF2 expression and PTTG (57), the oncogenic protein discovered in a cell line of pituitary tumor, has been described, and we previously found no increase in PTTG expression in pituitaries of D2R KO mice (9). Therefore, our aim was to establish the relation of pituitary FGF2 in the D2R KO mouse to lactotrope proliferation, prolactin secretion, and angiogenesis of the gland.

The D2R female KO mouse is an excellent model to study prolactinoma development. Female KO mice develop lac-

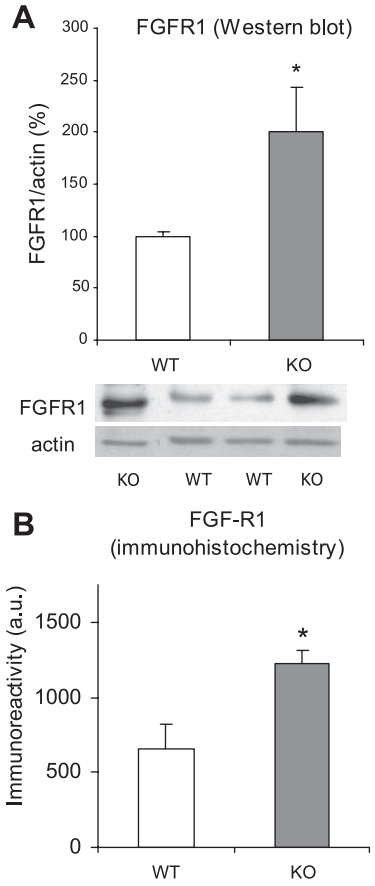

Fig. 5. *A*: comparative pituitary FGFR1 content (evaluated by Western blot) in WT and KO female mice. For each sample, arbitrary units (AU) of band intensities of FGFR1 were related to band intensities of the respective actin and compared with those of WT females considered 100% in each series of experiments.*$P < 0.05$ vs. WT. $N = 9$ and 10 animals, respectively. *Bottom*: representative Western Blot of FGFR1 and corresponding actin in pituitary homogenates from WT and KO animals. *B*: quantification of FGFR1 content by immunohistochemistry in WT and KO pituitaries; $N = 3$ and 3 animals, respectively, for each genotype. *$P < 0.05$ vs. WT.

totroph hyperplasia (31) followed by lactotroph tumor formation (3). In a previous work, we demonstrated that pituitary glands of female D2R KO mice had markedly increased numbers of cells containing prolactin (10). These lactotrophs were hyperstimulated with rapid turnover of prolactin and limited storage capacity.

Our present results indicate that exogenous FGF2 stimulated not only prolactin secretion but also the proliferation of pituitary endocrine cells and that the effect was similar between genotypes. As previously described (8), basal proliferation index of WT pituitary cells in primary culture was higher than in KOs, an effect likely due to the acute loss of dopamine inhibition; consistent with this interpretation, differences in proliferation rate between genotypes were evident only in the first hour after plating. Nevertheless, low FGF2 expression in KO cells might also account for lower proliferation rate. On the other hand, increased proliferation in vivo may be dependent on a cohort of growth factors available by the angiogenic process that is increased in KO and not in WT mice.

We found that the pituitary concentration of endogenous FGF2 in homogenates and cellular lysates was lower in KO mice. The result was unexpected, as pituitary FGF2 has been reported to be increased in estradiol-induced hyperplasia in rats (27, 50). However, we must consider that KO female mice have low estrogen levels because of hyperprolactinemia (31, 49). On the other hand, it has been shown that, in the central nervous system, activation of D2Rs increases FGF2 expression in rat prefrontal cortex, hippocampus, and striatum (19). Therefore, the lack of pituitary D2R activation in the KO mice might lead to inappropriate FGF2 expression. Furthermore, it has been shown that the pituitary tumor transforming gene (pttg) enhances FGF2 expression (57), and parallel increases in pttg and FGF2 expression were found in murine models of pituitary tumors (26) and human pituitary adenomas (29, 38). In turn, FGF2 induces pttg, determining vascular cell proliferation. Interestingly, PTTG is overexpressed in estrogen-induced pituitary hyperplasia (27), but it is reduced in the pituitaries from D2R KO mice (9). Therefore, the low FGF2 levels found in our

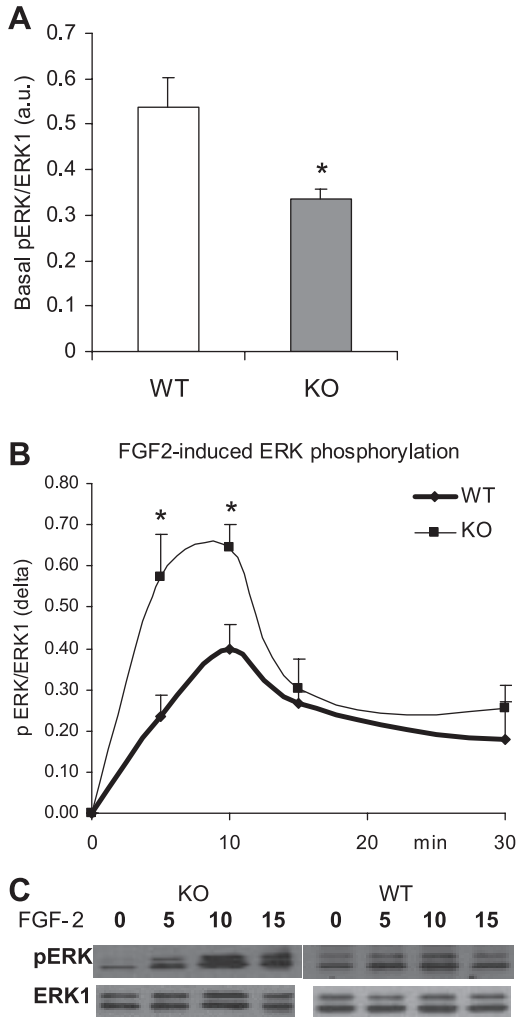

Fig. 6. *A*: pERK related to total ERK1 expression (AU = densitometric quantitation of pERK divided by densitometric quantitation of ERK1) in cultured pituitary cells in basal conditions in both genotypes. $N = 5$ animals for each genotype. *$P < 0.05$. *B*: kinetics of FGF2-induced phosphorylation of ERKs. Pituitary cells in culture were treated with 50 ng/ml FGF2 for different time periods (increment of pERK/ERK1 at each time point in relation to respective basal levels). *$P < 0.05$ vs. WT. $N = 5$ animals for each time and genotype. *C*: representative Western blot of pERK and ERK1 in pituitary cell lysates from WT and KO female mice.

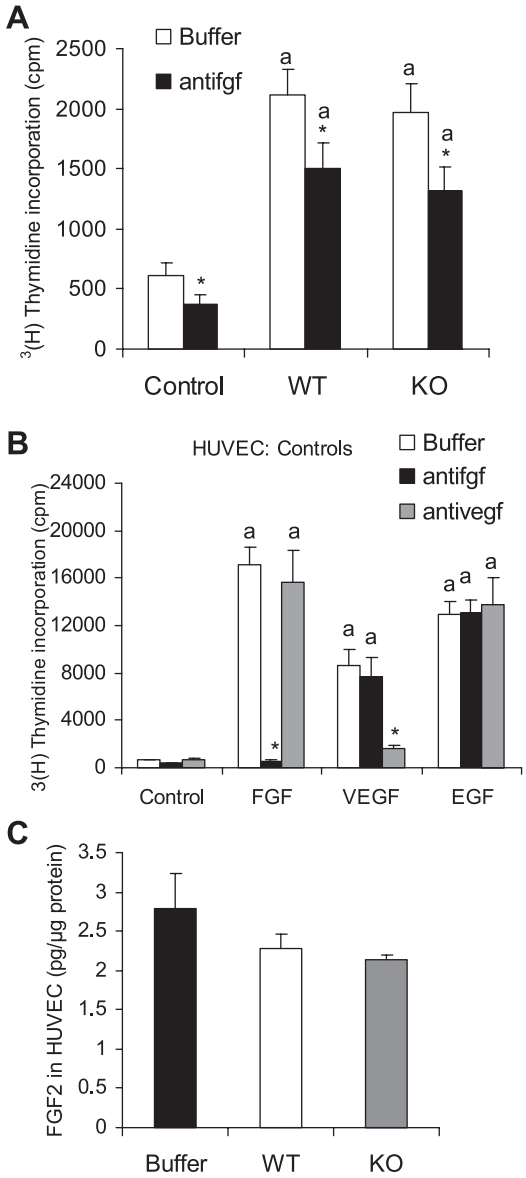

Fig. 7. Effect of conditioned media (CM) obtained from pituitary cell cultures of KO or WT female mice on human umbilical vein endothelial cell (HUVEC) proliferation. *A*: HUVECs were grown to confluence and then exposed to CM or serum-free DMEM (control) in the absence (buffer) or presence of anti-FGF2 (200 ng/ml). Proliferation was estimated as the incorporation of [^{3}H]thymidine into DNA, and results are expressed in cpm. $N = 4$ cell cultures. $^aP < 0.05$ vs. control. $^*P < 0.05$ vs. respective buffer. *B*: internal controls of HUVEC proliferation, murine VEGF-A (10 ng/ml), FGF2 (2 ng/ml), or epidermal growth factor (EGF; 1 ng/ml) in the presence or absence of anti-FGF2 (200 ng/ml) or anti-VEGF. $N = 5$. *C*: effect of buffer and WT or KO CM on HUVEC FGF2 expression as determined by ELISA (50,000 endothelial cells/well, $N = 3$).

experimental model may also be related to low PTTG expression.

We must also keep in mind that FGF2 is present in the basement membrane, and some components of the pituitary basement membrane, such as laminin, decrease in the tumorigenic process of the D2R KO mouse (33). This reduction in FGF2 could reflect a more general reduction of the basement membrane by action of matrix metalloproteinases, which exhibit high activity in all types of human pituitary adenomas. These metalloproteinases, secreted by pituitary cells, could release growth factors from the extracellular matrix that, in turn, may control pituitary cell proliferation and hormone secretion. Therefore, laminin and extracellular matrix-bound FGF2 might be released in the process of extracellular matrix remodeling and participate in proliferation and angiogenesis in an early step of tumor formation. Remodeling of the existing extracellular matrix and diminished cell adhesiveness have been linked to pituitary tumorigenesis in transgenic mice with overexpression of a truncated FGFR4 isoform (14). Finally, it was noted that FGF2 levels in human pituitary tumors were lower than those in the normal hypophysis (51).

Immunohistochemical results showed that there was a different localization pattern of FGF2 between genotypes. Whereas in KO mice pituitaries, there was an important nuclear FGF2 staining, in WT glands, it was principally cytoplasmic. It is possible that different FGF2 subcellular localization could be associated in our model with different molecular weight isoforms, as previously reported in pituitaries of estrogenized male rats (43) and in COS-1 cells transfected with single/multiple FGF2 forms (16). These studies showed that high-molecular-weight FGF2 isoforms are mainly found in the cellular nuclei and the 18-kDa isoform in the cytoplasm. This can be explained on the basis of the FGF2 DNA sequence, as there is a nuclear localization sequence upstream of the AUG initiation codon, and this region is only encountered in the larger forms of FGF2 (5). It is interesting to note that, in nontumorous human pituitary glands, the FGF2 immunostaining appeared almost exclusively in the cytoplasm (37), and low-molecular-weight isoforms were found, whereas, in human pituitary adenomas, the 24-kDa isoform was predominant (36). In another study (18), it was shown that recurrent pituitary adenomas had significantly larger FGF2 nuclear indexes, and FGF nuclear index also correlated significantly with the maximum tumor diameters and the invasiveness to the cavernous sinuses. Furthermore, D2R activation in astrocytic cultures resulted in enhanced FGF2 immunoreactivity in the cytosol and not in the nucleus, indicating that the D2R might participate in differential FGF2 cellular localization (35). These results are in accordance with those we describe in the pituitary of D2R KO mice, in which FGF2 expression is reduced, and nuclear localization is enhanced.

The differential localization pattern observed for this growth factor in the D2R KO and WT pituitaries may be related to critical effects of FGF2 in lactotrope differentiation, proliferation, or secretion. Despite its lower expression levels in adult KO pituitaries, FGF2 could contribute to the early development and maintenance of hyperplasia in these mice and participate in the increased pituitary proliferation observed in vivo (8). In this regard, it has been demonstrated in other experimental models that nuclear accumulation of FGF2 correlated with proliferation of subconfluent normal astrocytes and was constitutively present in nuclei of continuously proliferating glioma cells independently of cell density (30).

FGF2 exerts its action through four high-affinity transmembrane receptors (FGFR1–FGFR4) that have intrinsic tyrosine kinase activity (41). It was previously published that human pituitary tumors express abnormal isoforms of all the receptors, with a cytoplasmic localization (1). In rodents, FGFR1 has been related to FGF2 action in the pituitary (21). We found that FGFR1 was increased in KO pituitaries. This finding could be

related to the low expression of FGF2 found, which would be insufficient to downregulate the expression of its receptor, as previously described in other systems (7, 42). In normal human pituitaries, FGFR1 is localized in cells containing cytoplasmic reactivity for all pituitary hormones (1); in the present experiments, we found that FGFR1 in KO pituitaries localized mainly in lactotropes, which is consistent with its prolactin-releasing activity. Nevertheless, FGFR1 was also found in some gonadotropes and somatotropes.

The augmented levels of FGFR1 in KO adenohypophyses may explain the enhanced FGF2-induced ERK phosphorylation observed in this genotype. ERKs have been reported to be involved in FGF2 as well as in numerous growth factor-evoked proliferation in different systems (39, 54). ERKs belong to a family of protein serine/threonine kinases that are believed to function as integrators of mitogenic signals originated from several distinct classes of cell surface receptors, mainly tyrosine kinase receptors. In response to an extracellular stimulus, their activated forms, $p42^{mapk}$ (pERK2) and $p44^{mapk}$ (pERK1), are generated by phosphorylation of specific threonine and tyrosine residues.

However, interestingly, in lactotrophs stimulation of D2R leads to the activation of ERKs (p44/42) of the MAPK pathway, and this process correlates with an inhibition of lactotroph proliferation both in vitro and in vivo (28). Consequently, in D2R KO pituitaries lacking D2R signaling, we found lower basal levels of phosphorylated ERKs, an event that might be associated with the increment of the pituitary proliferation observed in vivo. In in vitro studies, we have shown that FGF2 was capable not only of increasing ERK phosphorylation but also of inducing proliferation in KO pituitary cells; therefore, we could hypothesize that, in this model, FGF2 might exert mitogenic actions activating other pathways different from ERKs at the pituitary level.

We also wished to determine whether pituitary FGF2 could increase the proliferation of endothelial cells (this process being mandatory for angiogenesis). Our data from HUVEC experiments suggest that pituitary-derived FGF2 would not participate in WT or in D2R KO mice in pathological angiogenesis of the gland, since, even though it is synthesized by endocrine cells, the CM from these cells does not modify endothelial cell growth, as it is not secreted. This coincides with the fact that FGF2 does not have a signal sequence and, therefore, is not a secreted protein (44). Nevertheless, it remains to be elucidated whether pituitary FGF2 could affect the proliferation, survival, or permeability of the pituitary endothelial cells in a juxtacrine or paracrine way in vivo when released from the basement membrane. But how is FGF2, a protein lacking a signal peptide, secreted into extracellular space? It was observed that the 18-kDa form of FGF2 can be translocated through the plasma membrane and quantitatively exported in an energy-dependent manner (17). Therefore, FGF2 may act in an autocrine or paracrine manner, as shown in estrogen-induced pituitary hyperplasia (53). In this regard, the low-molecular-weight FGF2, which is mainly cytosolic, correlates with proliferation in the pituitary (43). However, different subcellular localization of FGF2 has also been linked to different mechanisms of action of the growth factor. It has been shown that nuclear FGF2 may act in an intracrine manner, through intracellular pathways independent of cell surface receptors, and regulate proliferation, differentiation, and survival of cells (11). Our finding of increased nuclear accumulation of FGF2 may elucidate an action of the growth factor in hyperplastic cells that is independent of ERK phosphorylation (32).

We believe that the increased vascularity and peliosis observed in KO pituitaries are dependent on increased VEGF, as we have demonstrated previously (8). FGF2 has not been associated with peliosis or increased permeability and generally gives rise to more mature and stronger capillaries (46). On the other hand, studies of HUVEC proliferation suggest an independence between VEGF and FGF2 in this animal model of pituitary hyperplasia. CM of KO and WT pituitaries, which contain VEGF (8), did not modify FGF2 production by endothelial cells. Furthermore, it may be inferred that the VEGF and FGF2 interdependency observed in different experimental models (46) does not occur in D2R KO hyperplasia, as we found high VEGF and low FGF2 levels.

In conclusion, we describe differential distribution and concentration of FGF2 in the pituitary of D2R WT and KO female mice. Regulation of subcellular localization may be a mechanism for controlling the biological activities of certain proteins. Therefore, the pattern described might indicate different biological roles for FGF2 in both genotypes, and, moreover, this growth factor could induce proliferation of KO cells in vivo according to the increased availability of receptors and despite the lower levels found within the pituitary. We believe FGF2 may be an autocrine/paracrine effector of prolactin secretion and pituitary growth, but it would not participate as an angiogenic factor, at least in KO pituitaries. Moreover, given the importance of vascular phenotype for the progression of tumors (52), we speculate that the benign course and the low rate of prolactinoma growth might be consistent with the low levels of FGF2 found in KO pituitaries.

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REFERENCES

1. **Abbass SA, Asa SL, Ezzat S.** Altered expression of fibroblast growth factor receptors in human pituitary adenomas. *J Clin Endocrinol Metab* 82: 1160–1166, 1997.
2. **Arnaud E, Touriol C, Boutonnet C, Gensac MC, Vagner S, Prats H, Prats AC.** A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol Cell Biol* 19: 505–514, 1999.
3. **Asa SL, Kelly MA, Grandy DK, Low MJ.** Pituitary lactotroph adenomas develop after prolonged lactotroph hyperplasia in dopamine D2 receptor-deficient mice. *Endocrinology* 140: 5348–5355, 1999.
4. **Baird A, Mormede P, Ying SY, Wehrenberg WB, Ueno N, Ling N, Guillemin R.** A nonmitogenic pituitary function of fibroblast growth factor: regulation of thyrotropin and prolactin secretion. *Proc Natl Acad Sci USA* 82: 5545–5549, 1985.
5. **Bikfalvi A, Klein S, Pintucci G, Rifkin DB.** Biological roles of fibroblast growth factor-2. *Endocr Rev* 18: 26–45, 1997.
6. **Black EG, Logan A, Davis JR, Sheppard MC.** Basic fibroblast growth factor affects DNA synthesis and cell function and activates multiple signalling pathways in rat thyroid FRTL-5 and pituitary GH3 cells. *J Endocrinol* 127: 39–46, 1990.

7. **Choi J, Ko MK, Kay EP.** Subcellular localization of the expressed 18 kDa FGF-2 isoform in corneal endothelial cells. *Mol Vis* 6: 222–231, 2000.
8. **Cristina C, Diaz-Torga G, Baldi A, Gongora A, Rubinstein M, Low MJ, Becu-Villalobos D.** Increased pituitary vascular endothelial growth factor-A in dopaminergic D2 receptor knockout female mice. *Endocrinology* 146: 2952–2962, 2005.
9. **Cristina C, Diaz-Torga GS, Goya RG, Kakar SS, Perez-Millan MI, Passos VQ, Gianella-Neto D, Bronstein MD, Becu-Villalobos D.** PTTG expression in different experimental and human prolactinomas in relation to dopaminergic control of lactotropes. *Mol Cancer* 6: 4, 2007.
10. **Cristina C, García-Tornadú I, Diaz-Torga G, Rubinstein M, Low MJ, Becu-Villalobos D.** The dopaminergic D2 receptor knockout mouse: an animal model of prolactinoma. *Front Horm Res* 35: 50–63, 2006.
11. **Delrieu I.** The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Lett* 468: 6–10, 2000.
12. **Diaz-Torga G, Feierstein C, Libertun C, Gelman D, Kelly MA, Low MJ, Rubinstein M, Becu-Villalobos D.** Disruption of the D2 dopamine receptor alters GH and IGF-I secretion and causes dwarfism in male mice. *Endocrinology* 143: 1270–1279, 2002.
13. **Ezzat S, Smyth HS, Ramyar L, Asa SL.** Heterogeneous in vivo and in vitro expression of basic fibroblast growth factor by human pituitary adenomas. *J Clin Endocrinol Metab* 80: 878–884, 1995.
14. **Ezzat S, Zheng L, Asa SL.** Pituitary tumor-derived fibroblast growth factor receptor 4 isoform disrupts neural cell-adhesion molecule/N-cadherin signaling to diminish cell adhesiveness: a mechanism underlying pituitary neoplasia. *Mol Endocrinol* 18: 2543–2552, 2004.
15. **Ferrara N, Schweigerer L, Neufeld G, Mitchell R, Gospodarowicz D.** Pituitary follicular cells produce basic fibroblast growth factor. *Proc Natl Acad Sci USA* 84: 5773–5777, 1987.
16. **Florkiewicz RZ, Baird A, Gonzalez AM.** Multiple forms of bFGF: differential nuclear and cell surface localization. *Growth Factors* 4: 265–275, 1991.
17. **Florkiewicz RZ, Majack RA, Buechler RD, Florkiewicz E.** Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. *J Cell Physiol* 162: 388–399, 1995.
18. **Fukui S, Otani N, Nawashiro H, Yano A, Nomura N, Miyazawa T, Ohnuki A, Tsuzuki N, Katoh H, Ishihara S, Shima K.** Subcellular localization of basic fibroblast growth factor and fibroblast growth factor receptor 1 in pituitary adenomas. *Brain Tumor Pathol* 19: 23–29, 2002.
19. **Fumagalli F, Bedogni F, Maragnoli ME, Gennarelli M, Perez J, Racagni G, Riva MA.** Dopaminergic D2 receptor activation modulates FGF-2 gene expression in rat prefrontal cortex and hippocampus. *J Neurosci Res* 74: 74–80, 2003.
20. **Garcia-Tornadu I, Rubinstein M, Gaylinn BD, Hill D, Arany E, Low MJ, Diaz-Torga G, Becu-Villalobos D.** GH in the dwarf dopaminergic D2 receptor knockout mouse: somatotrope population, GH release, and responsiveness to GH-releasing factors and somatostatin. *J Endocrinol* 190: 611–619, 2006.
21. **Gonzalez AM, Logan A, Ying W, Lappi DA, Berry M, Baird A.** Fibroblast growth factor in the hypothalamic-pituitary axis: differential expression of fibroblast growth factor-2 and a high affinity receptor. *Endocrinology* 134: 2289–2297, 1994.
22. **Gonzalez Iglesias A, Diaz-Torga G, Piroli G, Achaval-Zaia R, De Nicola AF, Libertun C, Becu-Villalobos D.** Bromocriptine restores Angiotensin II response in pituitary hyperplasia. *Mol Cell Endocrinol* 165: 67–74, 2000.
23. **Gospodarowicz D, Cheng J, Lui GM, Baird A, Bohlen P.** Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc Natl Acad Sci USA* 81: 6963–6967, 1984.
24. **Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G.** Characterization and biological functions of fibroblast growth factors. *Endocr Rev* 8: 95–114, 1987.
25. **Graeven U, Fiedler W, Karpinski S, Ergun S, Kilic N, Rodeck U, Schmiegel W, Hossfeld DK.** Melanoma-associated expression of vascular endothelial growth factor and its receptors FLT-1 and KDR. *J Cancer Res Clin Oncol* 125: 621–629, 1999.
26. **Heaney AP, Fernando M, Melmed S.** Functional role of estrogen in pituitary tumor pathogenesis. *J Clin Invest* 109: 277–283, 2002.
27. **Heaney AP, Horwitz GA, Wang Z, Singson R, Melmed S.** Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. *Nat Med* 5: 1317–1321, 1999.
28. **Iaccarino C, Samad TA, Mathis C, Kercret H, Picetti R, Borrelli E.** Control of lactotrop proliferation by dopamine: essential role of signaling through D2 receptors and ERKs. *Proc Natl Acad Sci USA* 99: 14530–14535, 2002.
29. **Ishikawa H, Heaney AP, Yu R, Horwitz GA, Melmed S.** Human pituitary tumor-transforming gene induces angiogenesis. *J Clin Endocrinol Metab* 86: 867–874, 2001.
30. **Joy A, Moffett J, Neary K, Mordechai E, Stachowiak EK, Coons S, Rankin-Shapiro J, Florkiewicz RZ, Stachowiak MK.** Nuclear accumulation of FGF-2 is associated with proliferation of human astrocytes and glioma cells. *Oncogene* 14: 171–183, 1997.
31. **Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, Ben-Jonathan N, Grandy DK, Low MJ.** Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron* 19: 103–113, 1997.
32. **Keresztes M, Boonstra J.** Import(ance) of growth factors in(to) the nucleus. *J Cell Biol* 145: 421–424, 1999.
33. **Kuchenbauer F, Theodoropoulou M, Hopfner U, Stalla J, Renner U, Tonn JC, Low MJ, Arzt E, Stalla GK, Paez-Pereda M.** Laminin inhibits lactotroph proliferation and is reduced in early prolactinoma development. *Mol Cell Endocrinol* 207: 13–20, 2003.
34. **Larson GH, Koos RD, Sortino MA, Wise PM.** Acute effect of basic fibroblast growth factor on secretion of prolactin as assessed by the reverse hemolytic plaque assay. *Endocrinology* 126: 927–932, 1990.
35. **Li A, Guo H, Luo X, Sheng J, Yang S, Yin Y, Zhou J, Zhou J.** Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons. *FASEB J* 20: 1263–1265, 2006.
36. **Li Y, Koga M, Kasayama S, Matsumoto K, Arita N, Hayakawa T, Sato B.** Identification and characterization of high molecular weight forms of basic fibroblast growth factor in human pituitary adenomas. *J Clin Endocrinol Metab* 75: 1436–1441, 1992.
37. **Marin F, Boya J.** Immunocytochemical localization of basic fibroblast growth factor in the human pituitary gland. *Neuroendocrinology* 62: 523–529, 1995.
38. **McCabe CJ, Khaira JS, Boelaert K, Heaney AP, Tannahill LA, Hussain S, Mitchell R, Olliff J, Sheppard MC, Franklyn JA, Gittoes NJ.** Expression of pituitary tumour transforming gene (PTTG) and fibroblast growth factor-2 (FGF-2) in human pituitary adenomas: relationships to clinical tumour behaviour. *Clin Endocrinol (Oxf)* 58: 141–150, 2003.
39. **Meloche S, Seuwen K, Pages G, Pouyssegur J.** Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol Endocrinol* 6: 845–854, 1992.
40. **Mormede P, Baird A.** Estrogens, cyclic adenosine 3′,5′-monophosphate, and phorbol esters modulate the prolactin response of GH3 cells to basic fibroblast growth factor. *Endocrinology* 122: 2265–2271, 1988.
41. **Moscatelli D.** High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J Cell Physiol* 131: 123–130, 1987.
42. **Moscatelli D.** Autocrine downregulation of fibroblast growth factor receptors in F9 teratocarcinoma cells. *J Cell Physiol* 160: 555–562, 1994.
43. **Mukdsi JH, De Paul AL, Petiti JP, Gutierrez S, Aoki A, Torres AI.** Pattern of FGF-2 isoform expression correlated with its biological action in experimental prolactinomas. *Acta Neuropathol (Berl)* 112: 491–501, 2006.
44. **Okada-Ban M, Thiery JP, Jouanneau J.** Fibroblast growth factor-2. *Int J Biochem Cell Biol* 32: 263–267, 2000.
45. **Piroli G, Torres A, Grillo C, Lux-Lantos V, Aoki A, De Nicola AF.** Mechanisms in progestin antagonism of pituitary tumorigenesis. *J Steroid Biochem Mol Biol* 64: 59–67, 1998.
46. **Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M.** Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* 16: 159–178, 2005.
47. **Renko M, Quarto N, Morimoto T, Rifkin DB.** Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J Cell Physiol* 144: 108–114, 1990.
48. **Roceri M, Molteni R, Fumagalli F, Racagni G, Gennarelli M, Corsini G, Maggio R, Riva M.** Stimulatory role of dopamine on fibroblast growth factor-2 expression in rat striatum. *J Neurochem* 76: 990–997, 2001.

49. **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.
50. **Schechter J, Weiner R.** Changes in basic fibroblast growth factor coincident with estradiol-induced hyperplasia of the anterior pituitaries of Fischer 344 and Sprague-Dawley rats. *Endocrinology* 129: 2400–2408, 1991.
51. **Silverlight JJ, Prysor-Jones RA, Jenkins JS.** Basic fibroblast growth factor in human pituitary tumors. *Clin Endocrinol (Oxf)* 32: 669–676, 1990.
52. **Straume O, Akslen LA.** Importance of vascular phenotype by basic fibroblast growth factor, and influence of the angiogenic factors basic fibroblast growth factor/fibroblast growth factor receptor-1 and ephrin-A1/EphA2 on melanoma progression. *Am J Pathol* 160: 1009–1019, 2002.
53. **Strauss O, Mergler S, Wiederholt M.** Regulation of L-type calcium channels by protein tyrosine kinase and protein kinase C in cultured rat and human retinal pigment epithelial cells. *FASEB J* 11: 859–867, 1997.
54. **Sulpice E, Bryckaert M, Lacour J, Contreres JO, Tobelem G.** Platelet factor 4 inhibits FGF2-induced endothelial cell proliferation via the extracellular signal-regulated kinase pathway but not by the phosphatidylinositol 3-kinase pathway. *Blood* 100: 3087–3094, 2002.
55. **Tfelt-Hansen J, Kanuparthi D, Chattopadhyay N.** The emerging role of pituitary tumor transforming gene in tumorigenesis. *Clin Med Res* 4: 130–137, 2006.
56. **Tooyama I, Kawamata T, Walker D, Yamada T, Hanai K, Kimura H, Iwane M, Igarashi K, McGeer EG, McGeer PL.** Loss of basic fibroblast growth factor in substantia nigra neurons in Parkinson's disease. *Neurology* 43: 372–376, 1993.
57. **Zhang X, Horwitz GA, Prezant TR, Valentini A, Nakashima M, Bronstein MD, Melmed S.** Structure, expression, and function of human pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 13: 156–166, 1999.