Supplementary Data

Materials and methods

Hormone measurements

Serum E2 and P4 were measured after extraction with diethyl ether by a fluoroimmunoassays (FIA), using commercial Delfia progesterone and estradiol kits (Perkin Elmer-Wallac, Turku, Finland) according to the manufacturer's instructions.

Immunohistochemistry

The following antibodies were used in immunohistochemistry: monoclonal rat anti-mouse Ki-67 (1/200) (DakoCytomation, Glostrup, Denmark), monoclonal rabbit anti-cleaved caspase 3 (1/100) (Cell Signaling Technology, Danvers, MA, USA), monoclonal mouse anti-human progesterone receptor (1/100) (DakoCytomation), polyclonal rabbit anti-mouse laminin (1/200) (Chemicon International, Temecula, CA, USA), polyclonal rabbit anti-mouse ERα (1/1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-human ACTH (1/5000), rabbit anti-mouse PRL (1/5000) and guinea-pig anti-rat GH (1/5000), the latter three kindly donated by Dr A F Parlow (NIH). For PRL and Ki-67 double immunohistochemistry, Ki-67 was first labelled with diaminobenzidine (DAB) as substrate for horse radish peroxidase (HRP). After extensive washing with PBS, peroxidase was inactivated by using 3 % H_2O_2 followed by staining for PRL. Staining was visualized using VIP (Vector Laboratories Inc., Burlingame, CA, USA) producing red/purple color as substrate for HRP.

Western hybridizations

Cells were lysed on ice in buffer containing 0.5% Triton X-100, 150mM NaCl, 25mM KCl, 25mM Tris, pH 7.4, 1mM $CaCl_2$ plus 1mM sodium orthovanadate (Calbiochem) and protease inhibitor cocktail (Mini-complete protease inhibitor, Roche). Proteins from GH3 cells were separated by SDS-PAGE on a 4–12% (w/v) Bis-Tris gel (Invitrogen) (final loading concentration of 25mg total protein, determined using the Bradford assay method (BioRad)) and transferred to a nitrocellulose membrane (Invitrogen). The membranes were incubated overnight at 4°C in either rabbit anti-human pERK (Cell Signaling Technology) or pRB (Abcam), 1/1000 in TBS–T containing 5% (w/v) BSA. Membranes were incubated with goat antirabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, UK), 1/2000 (pERK) or 1/2500 (pRB) in TBS–T containing 5% (w/v) BSA (pERK and pRB). Proteins were visualized by incubating with ECL detection reagents (Amersham Biosciences) and exposed onto Hyperfilm ECL. To confirm integrity of protein transfer and equal loading, membranes were stripped and re-probed for β-actin using a mouse monoclonal β-actin antibody (Abcam) at 1/5000.

Immunofluorescence

GH3 cells were plated onto coverslips in a 24-well plate, at a density of 50 000 cells/well. The following day the cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Non-specific binding was blocked by 1h incubation in blocking buffer (3% BSA (w/v), 0.05% (v/v) Tween 20 in PBS). The cells were incubated overnight at 4°C with primary progesterone receptor antibody. On day two, the cells were incubated for 1h at room temperature with fluorescent secondary antibody (1/500) before a 5 min incubation in the dark with 4,6-diamidino-2-phenylindole (1/5000). Coverslips were mounted onto the slides with the use of Vectorshield (Vector Laboratories Inc., UK) and all sections were stored at 4°C in the dark until visualization.

Quantitative RT-PCR

Quantitative (q) RT-PCR was performed, and the primer pairs were used, as described in Supplementary Table 1. Standards and samples were run in triplicates or duplicates, and at least three individual samples were analyzed per experimental group. Results were calculated using the standard curve method with serial dilutions of standards, and the level of cytoplasmic *β-actin* (*Actb*) mRNA was used to normalize the expression of the other mRNA species. Two other housekeeping genes (*Eif4h*, *Rpl19*) were also measured to confirm the stable expression of *Actb*. All genes were first analyzed from 6-month-old WT and TG mice using at least 4 individual samples per group, and these preliminary results were used to select genes for more detailed

analyses. Supplementary Table 1 also presents the differences of CT-values of the genes studied, and of the housekeeping gene *Actb* in WT mice. Although the efficacy of the PCR-reaction was not similar for each gene, the difference of the CT-values provides information of the actual abundance of gene expression in the different experimental groups. For evaluation of total pituitary hormone expression, *Actb* adjusted relative expression of pituitary hormones was multiplied by weights of the glands.

Culture of GH3 cells

The dexamethasone (Sigma-Aldrich) used in the cultures was dissolved in dimethyl sulfoxide to a concentration of 1mM, and further diluted in medium to the required concentrations. Other details are as in the main text.

Results

Timing of development of prolactinomas

The pituitary weights of the TG females were clearly higher than those of the WT controls, and they kept increasing with age in the TG females, reaching up to 150 mg at 12 months (Supplementary Fig. 1A). Pituitary tumors of this size caused deformations of the skull and elevated intracranial pressure, with consequent rapid weight loss and ataxia. Serum PRL levels (Supplementary Fig. 1B) correlated with the pituitary weights and marked elevations were seen in TG females reaching up to 27 mg/L at the age of 12 months. These results were in line with our previous observations (Rulli *et al.* 2002).

Histological analyses

Histological evaluation of the pituitary glands at 4 months of age revealed that the weight-gain of the TG pituitary glands was due to enlarged anterior lobes. There were already signs of increased vascularization, but the cellular architecture remained rather normal. Some mitotic figures were observed, and some large vacuoled cells revealed signs of secretory activity at this age (Supplementary Fig. 2). Immunostaining showed increased number of PRL positive cells in lateral parts of the anterior pituitary glands (Supplementary Fig. 2). Laminin immunostaining, a negative marker of pituitary adenomas (Kuchenbauer *et al.* 2003), showed almost normal staining pattern in most of the samples at 4 months (Supplementary Fig. 2). At this age, GH and ACTH positive cells were present also in the TG pituitary glands, and all visible abnormalities were located in the most lateral areas of the glands, whereas the middle portions of the glands were still occupied by almost normal looking tissue without Ki-67 positive cells or strong CCND1 nuclear staining and with completely normal looking laminin pattern. In addition, numerous PRL negative cells were seen in the middle parts of the glands (Supplementary Fig. 2).

At the age of 6 months, the anterior pituitary glands of the TG females were clearly enlarged, presenting with increased numbers of mitotic figures, extravasated blood cells, and atypical cellular architecture with large vacuolized cells with prominent nuclei indicating high secretory activity (Supplementary Fig. 2). Also the vasculature was more prominent than at 4 months. Small PRL positive nodules of morphologically identical cells covered most of the lateral lobes of the anterior pituitary gland, with only few GH positive cells. ACTH positive cells were rarely seen in the lateral regions of the glands at 6 months, and laminin immunostaining was now absent in most nodules, indicating a transition from hyperplastic to adenomatous pituitary growth between 4–6 months. Normal looking tissue with only few CCND1 positive cells (see Fig. 3) and normal laminin staining pattern (Supplementary Fig. 2) still remained in the middle portion of the glands and lining the adenomatous nodules. The normal looking tissue showed normal laminin staining pattern, and also the reticulin fiber staining (not shown) appeared normal, but the number of Ki-67 positive cells in these areas (see Fig. 1) was clearly reduced.

At the age of 12 months, clearly visible marcoscopic nodules of adenomas were seen in the TG pituitary glands (Supplementary Fig. 2). These nodules were surrounded by a thin pseudocapsule consisting of cells of the adjacent tissue being compressed by the adenomatous nodule. The cells in the nodules were vacuolized with prominent nuclei and large numbers of mitotic figures. The nodules were formed only by PRL positive cells; GH positive cells lined the macroscopic nodules, neither were ACTH positive cells observed in the nodules. At this age, all normal pituitary tissue had disappeared.

Histological and immunohistological data of the hormone antagonist and hormone replacement

experiments

The same immunostainings as with the intact WT and TG mice at 4, 6 and 12 months (Supplementary Fig. 2) were done for histological samples of the hormone antagonist and replacement treatments (not shown). In brief the findings correlated closely with pituitary weights of the different groups, as a crude index of intensity of the adenomatous growth.

Hormone levels, uterine weights and pituitary E2 and P4 receptor expression

E2 replacement yielded higher serum concentrations than measured in WT and TG females (WT: 37 ± 6 pmol/L; TG: 59 ± 7 pmol/L, vs. E2-treatments: 210 ± 30 pmol/L). As shown previously (Rulli *et al.* 2002), P4 levels were highly upregulated in the TG females, as compared with any other treatment groups, and P4 replacement returned the post-Gx levels back to those of intact TG mice (Supplementary Fig. 3A,B). The somewhat elevated P4 levels in the bromoctripine and mifepristone treated TG females did not differ significantly from WT controls.

Uterine weights of the TG mice were higher than those of the WT mice, and except for bromocriptine, all treatments decreased them in both groups of mice (Supplementary Fig. 3C).

In line with their elevated E2 levels, in comparison to the WT and TG mice, *Pgr* expression by qRT-PCR of the E2 treated animals was elevated (Supplementary Fig. 3D), but not affected by P4. The combination E2 + P4 treatment did not induce further elevation of *Pgr* expression over that caused by E2 alone. At protein level, by immunohistochemistry, most of the WT and TG pituitary cells in all groups were ESR1 and PGR positive (Supplementary Fig. 3E).

qRT-PCR analyses

Pituitary gene expression was analyzed as total pituitary mRNA content (i.e. housekeeping gene-adjusted relative pituitary hormone gene expression x pituitary weight). Supplementary Table 1 presents relative expression levels, TG vs. WT, of selected genes with putative role in the pituitary tumorigenesis. Responses of the anterior pituitary hormone genes to the hormone antagonist and hormone treatments (Table 1) are presented in Supplementary Fig. 4. In line with the hormone measurements and immunohistochemical analyses, it was found that especially the level of *Prl* expression was dramatically increased (Supplementary Fig. 4A – note logarithmic scale). Also E2 induced the expected increase in *Prl* expression, as demonstrated by the low level of this message in Gx in females, and elevation upon E2 treatment (Supplementary Fig. 4B), in line with the serum levels of PRL. Likewise, *Gh* expression was increased in the intact TG pituitaries, and gonadectomy (Gx) inhibited the increase, but the expression did not respond to E2 (Supplementary Figs. 4C,D). The *GH* increase in the TG mice could be due to the elevated *HMGA2* expression (Fig. 4), because transgenic mice overexpressing this gene have been shown to develop mixed PRL/GH pituitary tumors (Fedele *et al.* 2002). *Tshb* was slightly upregulated in TG females and by E2 in the hormone replacement experiment, but did not show marked responses to other hormonal manipulations (Supplementary Figs. 4E,F). *Fshb* expression showed the expected increase after Gx, and the responses to hormonal manipulations were variable (Supplementary Fig. 4G,H). *Pomc* expression showed relatively minor differences between the treatment groups (Supplementary Figs. 4I,J). Taken together, these expression patterns indicated that only the lactotroph cells undergo substantial activation in the hCGβTG mice.

Additional data on experiments with GH3 cells

P4 alone had no effect on GH3 cell proliferation, neither did it enhance the proliferation effect of the maximally stimulating E2 concentration of 1nmol/L (Supplementary Fig. 5A). When P4 was replaced with dexamethasone (Supplementary Fig. 5B), no additive effect on cell proliferation stimulated by E2 was observed, indicating that the P4 effect is not due to activation of glucocorticoid receptors. Both *Esr1*, *Esr2* and *Pgr* (a and b isoforms) were expressed in GH3 cells, but neither *CYP17* nor *CYP19*, as demonstrated by RT-PCR (Supplementary Fig. 5C). Hence, the P4 effect can be mediated in GH3 cells through the cognate nuclear receptor, but not through conversion of P4 to E2. At protein level, immunofluorescence staining clearly demonstrated the presence of PGR in the CH3 cells (Supplementary Fig. 5D). The pERK antibody showed a significant increase in p42 in cells co-treated with 0.1–1000nM P4 and 100pM E2 (Supplementary Fig. 5E). Also pRB phosphorylation (as a sign of RB inactivation) was significantly increased in cells treated with 1–1000nM P4 in combination with 100pM E2.

References

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