Published in final edited form as:
Pituitary. 2015 February ; 18(1): 31–41. doi:10.1007/s11102-014-0553-1.

Expression of the pituitary stem/progenitor marker GFRα2 in human pituitary adenomas and normal pituitary

Nestoras Mathioudakis[1], **Ram Sundaresh**[2], **Alexandra Larsen**[2], **William Ruff**[2], **Jennifer Schiller**[2], **Hugo Guerrero Cázares**[2], **Peter Burger**[3], **Roberto Salvatori**[1], and **Alfredo Quiñones-Hinojosa**[2]

[1]Division of Endocrinology, Diabetes, & and Metabolism Johns Hopkins University School of Medicine 1830 E. Monument Street, Suite 333 Baltimore, Maryland 21287 [2]Department of Neurological Surgery Johns Hopkins University School of Medicine 4940 Eastern Ave 301 Mason Lord Drive, Suite 2100 Baltimore, MD 21224 Office: (410) 550-3367 Fax: (410) 550-0748
[3]Department of Pathology Johns Hopkins University School of Medicine Baltimore, Maryland

Abstract

Purpose—Recent studies suggest that adult pituitary stem cells may play a role in pituitary tumorigenesis. We sought to explore whether the Glial cell-line derived neurotrophic factor receptor alpha 2 (GFRα2), a recently described pituitary stem/progenitor marker, might be differentially expressed in pituitary adenomas versus normal pituitary.

Methods—The expression of GFRα2 and other members of the GFR receptor family (GFRα1, α3, α4) were analyzed using RT-PCR, western blot, and immunohistochemistry in 39 pituitary adenomas, 14 normal pituitary glands obtained at autopsy, and cDNA from 3 normal pituitaries obtained commercially.

Results—GFRα2 mRNA was ~2.6 fold under-expressed in functioning adenomas (P <0.01) and ~3.5 fold over-expressed in non-functioning adenomas (NFAs) (P <0.05) compared to normal pituitary. Among NFAs, *GFRα2* was significantly over-expressed (~5-fold) in the gonadotropinoma subtype only (P<0.05). GFRα2 protein expression appeared to be higher in most NFAs, although there was heterogeneity in protein expression in this group. GFRα2 protein expression appeared consistently lower in functioning adenomas by IHC and western blot. In normal pituitary, GFRα2 was localized in Rathke's remnant, the putative pituitary stem cell niche, and in corticotropes.

Conclusion—Our results suggest that the pituitary stem cell marker GFRα2 is under-expressed in functioning adenomas and over-expressed in NFAs, specifically gonadotropinomas. Further studies are required to elucidate whether over-expression of GFRα2 in gonadotropinomas might play a role in pituitary tumorigenesis.

INTRODUCTION

In recent years, several lines of evidence have converged to establish the presence of a stem/progenitor niche within the adult pituitary gland [1]. This stem cell niche is localized in the marginal zone (MZ), the cell layer containing remnants of Rathke's pouch, and is

characterized by the expression of various embryonic and pituitary-specific transcription factors, cell adhesion proteins, and neuronal-related markers [2]. Cells expressing these stem cell markers have been isolated and induced to differentiate into each lineage of the anterior pituitary [2]. Since normal developmental pathways are often deregulated in neoplasms, there has been recent interest in the relationship between adult pituitary stem cells and tumorigenesis [3]. In mouse models, over-expression of the WNT/β-catenin pathway leads to the development of tumors resembling the human adamantinomatous craniopharyngioma [4], while conditional deletion of the retinoblastoma tumor suppressor results in silent corticotrope adenomas from progenitor cells expressing the embryonic transcription factor Pax7 [5]. There is one study that suggests the presence of stem-like cells in two human pituitary adenomas [6].

Based on the above-mentioned premises, we sought to study expression of a recently described pituitary stem/progenitor cell marker, Glial cell-line derived neurotrophic factor receptor alpha 2 (GFRα2), in human pituitary adenomas and normal human pituitary[7]. This receptor belongs to a family of four RET co-receptors (GFRα1-α4) involved in central and peripheral nervous system development [8]. In rodents and humans, cells co-expressing GFRα2 and the pituitary-specific transcription factor Prophet of Pit-1 (PROP-1) are localized at the marginal zone, and in rodents they were shown to display pluripotency and self-renewal capacity [7]. GFRα1 and RET have previously been studied in pituitary adenomas and were found to be expressed predominantly in GH-secreting adenomas, while GFRα3 was recently shown to be expressed in a minority of adamantinomatous craniopharyngiomas [9, 10]. To the best of our knowledge, the expression of GFRα2 has not been systematically studied in pituitary adenomas. We hypothesized that GFRα2 might be over-expressed in some clinically non-functioning pituitary adenomas (NFAs) because at least a minority of these tumors are hormonally silent and lack cell-type specific differentiation [11]. We used intraoperative-obtained human pituitary tumor tissue from clinically non-functioning and hormone-secreting pituitary adenomas, as well as normal pituitary tissue obtained from autopsy and a commercial source.

METHODS

Tumors Samples and Controls

All samples were obtained under Johns Hopkins University IRB approval, with written informed consent obtained prior to surgery. During the period of study (2010–2012), 43 pituitary adenomas were obtained at the time of surgery and were flash frozen for RNA and protein. After extraction, sufficient RNA quantity was available for 39 adenomas. All samples represented independent tumors (i.e. no repeat surgeries) from different individuals not suspected of having a familial pituitary adenoma syndrome. Limitations of sample size meant that some tumors had sufficient material for RNA analysis but not for protein analysis.

A total of 17 normal pituitary glands were used as controls. Fourteen pituitary glands were obtained at autopsy from individuals with no history or gross evidence of pituitary disease. Additionally, cDNA from 3 normal pituitaries was purchased commercially from BioChain, Inc. (Newark, CA) as external controls. These external controls were used to verify the

quality of RNA obtained from our autopsy samples, since the post-mortem interval was generally much shorter in the commercially obtained samples (mean ± SD: 5 ± 1 hrs) compared to the internally obtained samples (mean ± SD: 27 ± 11 hrs). Details related to autopsy specimens and their use in the study are provided in Supplemental Table 1. For RT-PCR experiments, the mean age at death for autopsy subjects (mean ± SD: 63 ± 3 years) was higher than the mean age at surgery for the study subjects (mean ± SD: 48 ± 15 years) ($p < 0.01$). There was an even gender distribution in both study subjects and controls.

Tumor Classification

Clinico-pathologic classification of pituitary adenomas was based on a combination of clinical and biochemical data, histology, immunostaining of pituitary hormones, and, in some cases, RT-PCR of lineage-specific transcription factors (Table 1) [12–15]. Histopathologic review was independently conducted by a neuropathologist (P.B.) who was blinded to the clinical diagnosis, and the clinical diagnosis was made by the treating endocrinologist. In cases of clinically NFA where hormone staining was completely absent or the staining pattern did not align clearly with an adenoma subtype (n=9), RT-PCR of tumor tissue for lineage-specific transcription factors was used to confirm the cytological differentiation of the tumor [16–18]. The following genes were used to determine cytological differentiation: steroidogenic factor 1 (SF1), GATA-2, LHβ, FSHβ, alpha-glycoprotein subunit (αGSU), and estrogen receptor 1 (ESR1) indicated gonadotrope origin; TBX19 and POMC indicated corticotrope origin; thyrotroph embryonic factor (TEF) and TSHβ indicated thyrotrope origin; prolactin (PRL) indicated lactotrope origin; growth hormone (GH) indicated somatotrope origin. In cases of NFAs where combined IHC and RT-PCR data did not indicate a clear cytological differentiation, tumors were deemed “silent unclassified.” Although some tumors displayed hormone staining and transcription factor patterns potentially indicative of a plurihormonal cell type (i.e. silent subtype 3 adenoma), we could not exclude the possibility that this was the result of trapped non-tumoral cells. We considered tumors with hormone staining and transcription factor expression across multiple pituitary lineages to be “unclassified.”

RT-qPCR

RNA was extracted from all samples using Qiagen’s RNeasy Mini kit (Valencia, CA) following manufacturer’s recommendations, and genomic DNA contamination was eliminated using Invitrogen’s DNA free system (Grand Island, NY). One μg of total RNA was reverse transcribed using Invitrogen’s SuperScript III first strand synthesis system. All primers were used at final concentration of 5 μmol. All RT-qPCR reactions were heat denatured for 10 minutes at 95 °C, followed by 30 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, 70 °C for 30 seconds, and a final extension step at 72 °C for 10 minutes. Water and cDNA in the absence of reverse transcriptase were used as negative controls. The primers used are shown in Supplemental Table 2.

For quantitative RT-PCR, we used Applied Biosystems SYBR Green PCR master mix (Carlsbad, CA) at a final volume of 15 μl for all reactions. 0.2 μl of diluted cDNA was used per reaction, performed in triplicate, and analyzed using Applied Biosystems 7300 Real-Time PCR system. The double delta Ct method was used to determine the level of

expression as normalized to reference gene GAPDH and is expressed as fold change relative to a purchased external pituitary control (Autopsy Sample 1). This sample was chosen as the comparator because of the short post-mortem interval (and consequently lower potential risk of RNA degradation) and close approximation in age of the deceased and mean age of study subjects. For qualitative RT-PCR, platinum PCR Supermix from Invitrogen was used at a final volume of 20 μl.

Immunohistochemical Detection

Hormone immunostains used for diagnostic purposes were performed by the Immunopathology Laboratory in the Department of Pathology using enzymatic DAB reaction. IHC staining of GFRα2 alone or in combination with other antigens was performed using immunofluorescence (38 tumor samples studied). The following panel of antibodies was used at 1:100 dilution: mouse anti-ACTH (Santa Cruz, SC-69648), rabbit anti-ACTH (Abcam, AB74976), goat anti-GH (Santa Cruz, SC-10365), mouse anti-GH (Santa Cruz, SC-51602), goat anti-PRL (Santa Cruz, SC-7805), rabbit anti-PRL (Spring Bioscience, E17490), goat anti-TSHβ (Santa Cruz, SC-7815), rabbit anti-TSHβ (Abcam, AB64378), goat anti-FSHβ (Santa Cruz, SC-7797), rabbit anti-FSHβ (Abcam, AB8746), mouse anti-LHβ (Abcam, AB15226), rabbit anti-LHβ (Abcam AB76902), mouse anti-S100β (Millipore, MAB079-1), rabbit anti-α-GSU (Abcam, AB92738), mouse anti-α-GSU (Novus Biologicals, NB-100-62265), mouse anti-GFAP (Millipore, MAB3402). The following antibodies were used at 1:50 dilution: goat anti-neurturin (Abcam, AB63949), goat anti-Ret (Santa Cruz, SC-1290), rabbit anti-Ret (Santa Cruz, SC-167), mouse anti-Ret (Abcam, AB1840). Rabbit anti-GFRα2 (QED Bioscience, QED1135) was used at 1:50 dilution for IHC. Each commercial antibody was verified to have reactivity against the human protein of interest. Omission of primary antibody was used in all experiments as a negative control and no staining was observed. The following panel of secondary antibodies was used at 1:500 dilution: Donkey anti-rabbit 594 (Invitrogen, A21207), Donkey anti-goat 488 (Invitrogen, A11055), and Donkey anti-mouse 488 (Invitrogen, A21202).

Formalin-fixed paraffin embedded tissues were deparaffinized and rehydrated then immersed in 0.1% Sudan Black B (Sigma, St. Louis, MO) in 70% Ethanol for 20 minutes to minimize autofluorescence [19]. Antigen retrieval was performed using sodium citrate buffer in 95 °C (10 mM, 0.05% Tween-20, pH 6.0) at 95 °C for 20 minutes. PBS was used to wash stains and dilute antibodies when GFRα2 was stained alone. For every primary antibody and pituitary sample, the tissue was stained with and without primary antibody and omission of the primary antibody resulted in no labeling. In addition, when tissue slices obtained from the same pituitary were treated with serially diluted primary antibody concentrations and identical fixation conditions, staining went from dense to light to imperceptible. For co-immunostaining of cell surface and cytoplasmic antigens, a mild detergent (0.02% Tween-20 in PBS) was used in the antibody diluent to permit penetration of antibodies and detection of cytoplasmic antigens without compromising the staining of cell surface antigens. Primary antibodies were incubated overnight followed by secondary antibodies for one hour in the dark at room temperature and 15 minutes of DAPI nuclear counterstaining. A blocking step with either donkey or goat serum was used between the two

antigen detection cascades. Images of GFRα2 immunostaining were acquired at the same exposure time.

Western Blot Analysis

Twenty g of protein were loaded onto a 4–12% SDS-polyacrylamide gel. Proteins were transferred onto a PVDF membrane and probed with monoclonal mouse anti-human GFRα2 antibody (R&D Systems, Minneapolis, MN) at 1:500 dilution. Twenty-one tumor samples had sufficient protein concentration to permit loading of 20 g per well for analysis by Western blot.

Statistical Analysis

GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Two-tailed t-test (Mann-Whitney test) was used for comparison of means, two-sided chi square test with Fisher's exact test was used for comparison of proportions, and one-way Analysis of Variance (ANOVA) was used in multiple group comparisons with Bonferroni's post-hoc test.

RESULTS

Gene expression profile of GFRα2 in pituitary adenomas

GFRα2 was significantly under-expressed in functioning pituitary adenomas and over-expressed in NFAs relative to normal pituitary (Figure 1). In addition, NFAs showed significantly higher expression of GFRα2 relative to all functioning adenomas combined, the majority of which were GH-secreting adenomas. The normal pituitary samples had similar expression of GFRα2 compared to the reference sample. Since GFRα2 gene expression was significantly higher in the NFAs, we explored whether there were differences in its expression among the subtypes of NFAs. We found that GFRα2 was significantly over-expressed (4.6 fold, p-value = 0.01) in gonadotropinomas, but not in the other silent adenoma subtypes. The gene expression profiles of the other members of the GFRα family (α1, α3, α4) are shown in Supplemental Figure 1.

Gene expression of the GFRα2 ligand neurturin and co-receptor RET in pituitary adenomas

At the gene level, the GFRα2 co-receptor, RET, was under-expressed in NFAs compared to controls (3.3 vs. 12.7 fold, p-value <0.01), while the GFRα2 ligand, Neurturin, had slightly higher expression relative to controls (0.4 vs. 0.2 fold, p-value <0.05).

Localization of GFRα2 immunostaining in normal pituitary

In the normal pituitary, GFRα2 immunostaining was observed most intensely in the marginal zone at the interface of the posterior and anterior pituitary (Figure 2a). The majority of cells within Rathke's remnant cysts stained positively for GFRα2. Co-immunostaining of GFRα2 with each of the anterior pituitary hormones showed GFRα2 immunoreactivity most strongly in corticotropes. GFRα2 staining was seen in clusters

surrounding gonadotropes but there was not clear staining of GFRα2 seen within this cell type (Figure 2b).

Weak co-immunostaining of GFRα2 with its co-receptor RET and stronger co-immunostaining with its ligand neurturin was observed in the normal pituitary (Figure 3a). We also investigated whether GFRα2 might be expressed in folliculostellate cells of the anterior pituitary. These hormonally-null cells, which form a functional neural network within the gland, have been proposed to have progenitor properties [20]. We did not find significant GFRα2 expression in folliculostellate cells, with only rare co-staining of GFRα2 and the folliculostellate cell markers S100 or GFAP being observed (Figure 3b).

Immunohistochemical staining of GFRα2 in pituitary adenomas

Immunohistochemical staining of GFRα2 among pituitary tumors revealed that gonadotropinomas showed the most intense and diffuse staining, with scattered cells positive for both GFRα2 and gonadotrope hormones (Figure 4A-D). Within an individual gonadotropinoma, there was heterogeneity in the pattern of GFRα2 staining. In some areas, the number of GFRα2 positive cells exceeded the number of hormone-positive cells (Figure 4A-B), while in other areas, the opposite pattern was observed (Figure 4C-D). In two ACTH-secreting adenomas, one tumor showed focal areas of GFRα2/ACTH co-localization (Figure 4E), while the other tumor showed both focal and diffuse GFRα2 staining (Figure 4F-H). GFRα2 immunostaining was absent in tumors of the Pit-1 lineage (PRL, GH, and TSH-secreting adenomas) (Figure 4I-L).

Correlation of GFRα2 protein and mRNA expression

Consistent with the mRNA results, western blot suggested lower GFRα2 expression in functioning adenomas relative to normal pituitary (Figure 5). Although several NFAs appeared to have higher protein expression of GFRα2 by western blot, GFRα2 mRNA and protein expression did not directly correlate for all tumors. Among tumors that were analyzed by both RT-PCR and Western Blot, a concordant pattern of expression was observed in 11 of 20 (55%). Among the 9 discordant cases, 7/9 (78%) showed over-expression by RT-PCR and appeared to be under-expressed relative to control by Western Blot, while 2/9 (22%) showed under-expression by RT-PCR and higher protein expression. Among the functioning adenomas, 6/7 (86%) had concordant mRNA and protein patterns, while only 5/12 (41%) of the NFAs showed concordance.

DISCUSSION

Cancer stem cells have long been reported in many primary brain malignancies [21–23], but whether stem cells exist in benign tumors, such as pituitary adenomas, remains uncertain [3]. Historically, cancer stem cells have been isolated experimentally from solid tumors (i.e. with flow-activated cell cytometry) using markers specific for normal stem cells of the same organ [24]. In the normal adult pituitary gland, candidate stem cells have been shown to express the embryonic stem cell markers Sox2 and Sox9, the neuronal marker Nestin, and co-expression of GFRα2/Prop1 [2]. While this study was not designed to demonstrate the existence of stem cells in pituitary adenomas, the comprehensive gene expression profile of

a stem cell marker in pituitary adenomas provides preliminary evidence that progenitor/stem cells could be involved in adenoma cytogenesis.

In this study, we demonstrate that GFRα2 is under-expressed at the gene and protein level in functioning pituitary tumors, particularly those of the Pit-1 lineage (GH, PRL, and TSH). This finding indirectly supports the candidacy of GFRα2 as a pituitary stem-related marker, since most of the functioning pituitary tumors we studied are derived from cells of the Pit-1 lineage, the most terminally differentiated cells in pituitary development. By IHC, we did not observe any staining of GFRα2 in tumors of the Pit-1 lineage (GH, PRL, TSHβ) and there was negligible protein expression by western blot in these tumor types.

Contrary to the other functioning pituitary adenoma types, GFRα2 mRNA was not under-expressed in ACTH-secreting adenomas, and there was diffuse and focal immunostaining of GFRα2 seen in these tumors. The unique gene and protein expression pattern of ACTH-secreting tumors among the functioning adenomas is not unexpected, as ACTH-secreting cells arise from an earlier precursor cell during pituitary differentiation [25]. Furthermore, the pattern of GFRα2 expression in ACTH tumors was in line with our observations in normal pituitary tissue, where GFRα2 stained most intensely with corticotropes. In this respect, our findings differ from those of Garcia-Lavandeira *et al*, who first established GFRα2 as a stem cell marker within the pituitary gland. In their study, GFRα2 was restricted mainly to Rathke's remnant, with some scattered staining observed within the AP [7]. While we observed a similar pattern of staining in the marginal zone (the pituitary stem cell niche), we also observed GFRα2 staining in corticotropes and adjacent to (but not within) gonadotrope cell clusters in the AP bordering the MZ. The reasons for this difference are not readily apparent, but it deserves mention that the observation of GFRα2 expression within a differentiated pituitary cell type casts some doubt on the candidacy of this receptor as an independent pluripotent stem cell marker. While we did not explore this directly, it is plausible that GFRα2-positive/PROP-1-positive cells (as shown by Garcia-Lavandeira *et al*) are restricted to the MZ and maintain pluripotency, while GFRα2-positive/PROP-1 negative cells at the border of the MZ represent more committed progenitor cells.

Contrary to the functioning adenomas, GFRα2 mRNA was over-expressed in NFAs. This heterogeneous group of pituitary tumors has no clinical evidence of hormone hypersecretion, and therefore adenomas belonging to this group can also be described as silent [16]. NFAs are comprised predominantly of gonadotropinomas, although adenomas derived from any pituitary lineage can be hormonally silent. We had initially hypothesized that GFRα2 would be over-expressed in the NFAs because at least a minority of these tumors are hormonally null and lack cell-type specific differentiation. In fact, after careful cytologic characterization of the silent adenomas by PCR, we were unable to identify any cases of true null cell adenomas; consistent with current understanding, most of the NFAs were found to belong to the gonadotropin lineage [16]. Since we included all types of adenomas and classified the silent adenomas according to their cytological differentiation, we were able to show that GFRα2 mRNA is specifically over-expressed in gonadotropinomas. It does not appear that GFRα2 is playing a role in the "silencing" of adenomas *per se*, since we only observed higher expression in the gonadotropinomas, but

not other types of silent adenomas. Rather, this finding suggests that GFRα2 may play a role in modulation or differentiation of gonadotrope cells.

Although we observed statistically significant over-expression of GFRα2 in NFAs by RT-PCR, this did not always correlate with the protein expression observed by IHC and western blot. Generally, western blot and IHC suggested higher protein expression in NFAs compared to the other adenoma types, however some NFAs with high mRNA expression did not appear to have significant protein expression by either method of detection. Similarly, some NFAs that appeared to have high protein expression had very low gene expression. Discordant gene and protein expression is not uncommon in malignancies [26–28]. It has been shown that different cell types within a tumor can display variable concordance in expression at the gene and protein level [29, 28] It is possible that different pituitary tumor cell types may have variable degradation rates of mRNA and protein or differences in post-translational modifications. The higher concordance between mRNA and protein in the functioning adenomas compared to the NFAs may be a reflection of greater homogeneity with respect to cytologic differentiation among functioning adenomas compared to non-functioning adenomas. While gonadotropinomas showed variable expression of LHβ, FSHβ, and αGSU (potentially reflecting differing stages of differentiation), most of the functioning adenomas showed pure expression of a single hormone. Ultimately, since it is the protein concentration that determines cellular function, the overexpression of GFRα2 mRNA in NFAs can be interpreted only as correlative rather than causative, and further studies would be needed to determine whether GFRα2 exerts any effect on gonadotrope cell growth or differentiation.

By inference from our findings in both normal and tumor tissue, it appears that GFRα2 is present in corticotrope and gonadotrope cell types. Since corticotrope and gonadotrope differentiation arise from a common Rathke's pouch progenitor, it is conceivable that GFRα2 is a marker of a progenitor cell common to these lineages [30–34, 25, 35]. Interestingly, the finding of GFRα2 immunostaining most strongly in ACTH cells in the normal pituitary and in cells adjacent to α-GSU cell clusters follows the expected differentiation pattern in which ACTH and α-GSU appear contemporaneously [30, 36]. In the adult pituitary gland, LH-producing gonadotrope cells form a network alongside the pre-established corticotrope network and the two lineages maintain direct contact throughout adulthood [37]. Furthermore, it has recently been demonstrated that some silent corticotropinomas share characteristics of gonadotropinomas, including cytological differentiation markers and ultrastructural features [38]. Given these observations, an alternative role for GFRα2 might be to facilitate "cross-talk" between gonadotrope and corticotrope cells.

GFRα2 signaling occurs via homodimerization of the co-receptor RET and initiation of downstream intracellular pathways [8]. Although its co-receptor RET and ligand neurturin showed co-immunoreactivity with GFRα2 in the normal pituitary, we did not find parallel gene expression patterns in pituitary adenomas. Consistent with a previous study, RET was under-expressed in NFAs [9]. Expression of the GFRα2 ligand neurturin was slightly higher in NFAs relative to controls, but this difference was marginal and the expression was still lower than that of the reference control sample. While further *in vitro* studies would be

required to elucidate the intracellular signaling pathways involved in pituitary tumors, these findings suggest the possibility of a RET-independent GFRα signaling mechanism, which has been described [8].

Given the role of folliculostellate (FS) cells in pituitary paracrine modulation and their putative stem cell role, we explored whether GFRα2 might be expressed in FS cells of the normal pituitary. We found that only rare numbers of GFRα2 positive cells expressed FS cell markers, particularly S100β. FS cells are not usually found in abundance in pituitary adenomas, making it unlikely that trophic stimulation of gonadotropes via GFRα2 signaling is a significant driver of tumor growth [17]. However, it is worth mentioning that Garcia-Lavandeira *et al* noted that half of the GFRα2/Prop1 stem cells in the rodent pituitary expressed S100β, suggesting that these stem cells could give rise to both hormone-producing and neuron-like cells [7]. Those authors posit that these stem cells could thereby play a role in controlling the physiological demands on the pituitary gland (i.e. puberty, lactation, growth, etc.) throughout the lifetime of the organism [7].

A main strength of our study is the representation of each adenoma type, with a relatively large sample size of NFAs and GH adenomas. Inclusion of the rare thyrotropinoma allowed us to infer that GFRα2 is not implicated in tumors of the Pit-1 lineage. In addition, we had a large sample of control pituitary glands, with validation of our internal autopsy samples using commercially obtained controls. We comprehensively classified the NFAs using RT-PCR of transcription factors in some cases to determine the cytological differentiation. A limitation of our study is the small sample number of ACTH and TSH tumors, but this reflects the rarity of these tumors and the generally small size of these tumors, which can make it difficult to obtain workable amount of material in some cases. In addition, although we were able to study protein expression by IHC for all tumors, we did not have sufficient samples to analyze protein expression by Western blot for all tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors wish to acknowledge Mr. Moses Chappell in the Department of Pathology for his assistance in obtaining pituitary autopsy specimens, and Ms. Leslie Reynolds in the Department of Pathology for her assistance in obtaining pituitary tumor slides. This work was supported by NIH T32 Grant #5T32DK007751-15 (NM) and NIH R01 NS070024 (AQH).

REFERENCES

1. Castinetti F, Davis SW, Brue T, Camper SA. Pituitary stem cell update and potential implications for treating hypopituitarism. Endocrine reviews. 2011; 32(4):453–471. [PubMed: 21493869]
2. Florio T. Adult pituitary stem cells: from pituitary plasticity to adenoma development. Neuroendocrinology. 94(4):265–277. [PubMed: 22116388]
3. Rostad S. Pituitary adenoma pathogenesis: an update. Curr Opin Endocrinol Diabetes Obes. 2012; 19(4):322–327. [PubMed: 22617628]
4. Gaston-Massuet C, Andoniadou CL, Signore M, Jayakody SA, Charolidi N, Kyeyune R, Vernay B, Jacques TS, Taketo MM, Le Tissier P, Dattani MT, Martinez-Barbera JP. Increased Wingless (Wnt)

signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans. Proc Natl Acad Sci U S A. 108(28):11482–11487. [PubMed: 21636786]

5. Hosoyama T, Nishijo K, Garcia MM, Schaffer BS, Ohshima-Hosoyama S, Prajapati SI, Davis MD, Grant WF, Scheithauer BW, Marks DL, Rubin BP, Keller C. A Postnatal Pax7 Progenitor Gives Rise to Pituitary Adenomas. Genes Cancer. 1(4):388–402. [PubMed: 20811506]
6. Xu Q, Yuan X, Tunici P, Liu G, Fan X, Xu M, Hu J, Hwang JY, Farkas DL, Black KL, Yu JS. Isolation of tumour stem-like cells from benign tumours. Br J Cancer. 2009; 101(2):303–311. [PubMed: 19568241]
7. Garcia-Lavandeira M, Quereda V, Flores I, Saez C, Diaz-Rodriguez E, Japon MA, Ryan AK, Blasco MA, Dieguez C, Malumbres M, Alvarez CV. A GRFa2/Prop1/stem (GPS) cell niche in the pituitary. PLoS One. 2009; 4(3):e4815. [PubMed: 19283075]
8. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 2002; 3(5):383–394. [PubMed: 11988777]
9. Japon MA, Urbano AG, Saez C, Segura DI, Cerro AL, Dieguez C, Alvarez CV. Glial-derived neurotropic factor and RET gene expression in normal human anterior pituitary cell types and in pituitary tumors. J Clin Endocrinol Metab. 2002; 87(4):1879–1884. [PubMed: 11932334]
10. Garcia-Lavandeira M, Saez C, Diaz-Rodriguez E, Perez-Romero S, Senra A, Dieguez C, Japon MA, Alvarez CV. Craniopharyngiomas express embryonic stem cell markers (SOX2, OCT4, KLF4, and SOX9) as pituitary stem cells but do not coexpress RET/GFRA3 receptors. J Clin Endocrinol Metab. 97(1):E80–E87. [PubMed: 22031517]
11. Mete O, Asa SL. Clinicopathological correlations in pituitary adenomas. Brain Pathol. 2012; 22(4): 443–453. [PubMed: 22697380]
12. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM, Montori VM. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2008; 93(5):1526–1540. [PubMed: 18334580]
13. Dichek HL, Nieman LK, Oldfield EH, Pass HI, Malley JD, Cutler GB Jr. A comparison of the standard high dose dexamethasone suppression test and the overnight 8-mg dexamethasone suppression test for the differential diagnosis of adrenocorticotropin-dependent Cushing's syndrome. J Clin Endocrinol Metab. 1994; 78(2):418–422. [PubMed: 8106630]
14. Katznelson L, Atkinson JL, Cook DM, Ezzat SZ, Hamrahian AH, Miller KK. American Association of Clinical Endocrinologists medical guidelines for clinical practice for the diagnosis and treatment of acromegaly--2011 update. Endocr Pract. 2011; 17(Suppl 4):1–44. [PubMed: 21846616]
15. Melmed S, Casanueva FF, Hoffman AR, Kleinberg DL, Montori VM, Schlechte JA, Wass JA. Diagnosis and treatment of hyperprolactinemia: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96(2):273–288. [PubMed: 21296991]
16. Greenman Y, Stern N. Non-functioning pituitary adenomas. Best Pract Res Clin Endocrinol Metab. 2009; 23(5):625–638. [PubMed: 19945027]
17. Asa, SL. AFIP Atlas of Tumor Pathology. 4 ed.. Vol. 15. Washington, DC: American Registry of Pathology; 2011. Tumors of the Pituitary Gland.
18. DeLellis RA, LR.; Heitz, PU.; Eng, C. 2004 World Health Organization classification of tumours: Tumours of endocrine organs. Lyons: IARC; 2004.
19. Oliveira VC, Carrara RC, Simoes DL, Saggioro FP, Carlotti CG Jr, Covas DT, Neder L. Sudan Black B treatment reduces autofluorescence and improves resolution of in situ hybridization specific fluorescent signals of brain sections. Histol Histopathol. 2010; 25(8):1017–1024. [PubMed: 20552552]
20. Lepore DA, Roeszler K, Wagner J, Ross SA, Bauer K, Thomas PQ. Identification and enrichment of colony-forming cells from the adult murine pituitary. Exp Cell Res. 2005; 308(1):166–176. [PubMed: 15916758]
21. Guerrero-Cazares H, Attenello FJ, Noiman L, Quinones-Hinojosa A. Stem cells in gliomas. Handb Clin Neurol. 2012; 104:63–73. [PubMed: 22230436]
22. Hsu W, Mohyeldin A, Shah SR, Gokaslan ZL, Quinones-Hinojosa A. Role of cancer stem cells in spine tumors: review of current literature. Neurosurgery. 2012; 71(1):117–125. [PubMed: 22418583]

23. Chesler DA, Berger MS, Quinones-Hinojosa A. The potential origin of glioblastoma initiating cells. Front Biosci (Schol Ed). 2012; 4:190–205. [PubMed: 22202053]
24. Chen K, Huang YH, Chen JL. Understanding and targeting cancer stem cells: therapeutic implications and challenges. Acta Pharmacol Sin. 2013
25. Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC, Dattani MT. Genetic regulation of pituitary gland development in human and mouse. Endocrine reviews. 2009; 30(7):790–829. [PubMed: 19837867]
26. Kordek R, Potemski P, Kusinska R, Pluciennik E, Bednarek A. Basal keratin expression in breast cancer by quantification of mRNA and by immunohistochemistry. J Exp Clin Cancer Res. 2010; 29:39. [PubMed: 20426817]
27. Nordengren J, Casslen B, Gustavsson B, Einarsdottir M, Willen R. Discordant expression of mRNA and protein for urokinase and tissue plasminogen activators (u-PA, t-PA) in endometrial carcinoma. Int J Cancer. 1998; 79(2):195–201. [PubMed: 9583736]
28. Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG. Discordant protein and mRNA expression in lung adenocarcinomas. Mol Cell Proteomics. 2002; 1(4):304–313. [PubMed: 12096112]
29. Pascal LE, True LD, Campbell DS, Deutsch EW, Risk M, Coleman IM, Eichner LJ, Nelson PS, Liu AY. Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. BMC Genomics. 2008; 9:246. [PubMed: 18501003]
30. Osamura RY, Kajiya H, Takei M, Egashira N, Tobita M, Takekoshi S, Teramoto A. Pathology of the human pituitary adenomas. Histochem Cell Biol. 2008; 130(3):495–507. [PubMed: 18688636]
31. Asa SL, Ezzat S. The cytogenesis and pathogenesis of pituitary adenomas. Endocrine reviews. 1998; 19(6):798–827. [PubMed: 9861546]
32. Pulichino AM, Vallette-Kasic S, Tsai JP, Couture C, Gauthier Y, Drouin J. Tpit determines alternate fates during pituitary cell differentiation. Genes Dev. 2003; 17(6):738–747. [PubMed: 12651892]
33. Drouin, J. Pituitary Development. Melmed, S., editor. London: The Pituitary. Academic Press; 2012.
34. Nolan LA, Levy A. A population of non-luteinising hormone/non-adrenocorticotrophic hormone-positive cells in the male rat anterior pituitary responds mitotically to both gonadectomy and adrenalectomy. J Neuroendocrinol. 2006; 18(9):655–661. [PubMed: 16879164]
35. Vankelecom H, Chen J. Pituitary stem cells: Where do we stand? . Molecular and cellular endocrinology. 2013
36. Dubois PM, el Amraoui A, Heritier AG. Development and differentiation of pituitary cells. Microsc Res Tech. 1997; 39(2):98–113. [PubMed: 9361262]
37. Mollard P, Hodson DJ, Lafont C, Rizzoti K, Drouin J. A tridimensional view of pituitary development and function. Trends Endocrinol Metab. 2012; 23(6):261–269. [PubMed: 22436593]
38. Cooper O, Ben-Shlomo A, Bonert V, Bannykh S, Mirocha J, Melmed S. Silent corticogonadotroph adenomas: clinical and cellular characteristics and long-term outcomes. Horm Cancer. 2010; 1(2): 80–92. [PubMed: 20717480]

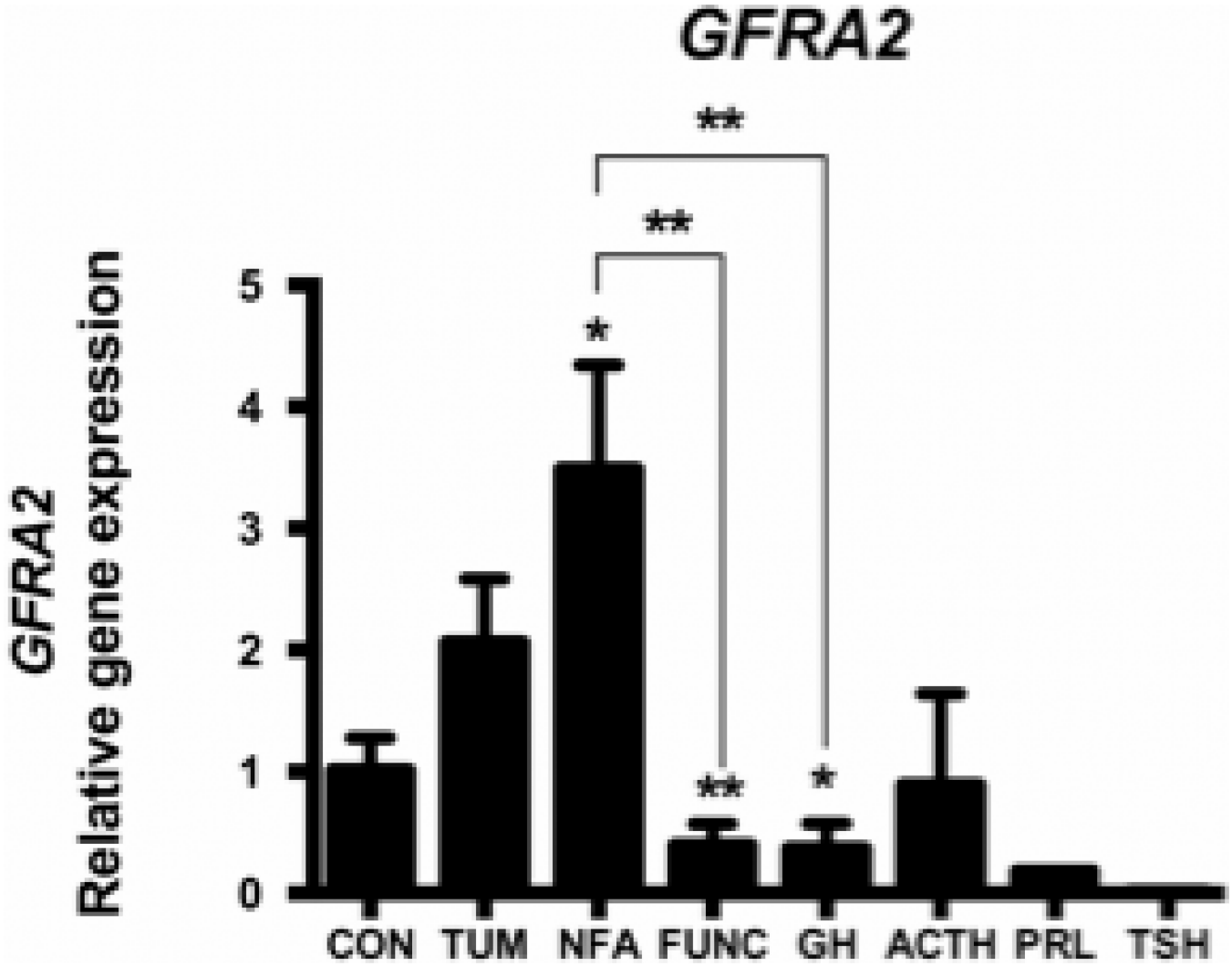

Figure 1.

Real time PCR Analysis of GFRα2 gene expression in normal pituitary (CON, n=13), all pituitary tumors (TUM, n=39), non-functioning adenomas (NFA, n=21), functioning adenomas (FUNC, n= 18), GH-secreting adenomas (GH, n=14), ACTH-secreting adenomas (ACTH, n=2), prolactin-secreting adenoma (PRL, n=1), and TSH-secreting adenoma (TSH, n=1). Data are presented as mean transcript fold changes relative to one normal control sample and normalized to GAPDH, with each bar representing the standard error of the mean (SEM). *$p<0.05$, **$p<0.01$ (relative to control when indicated above error bars or between groups as specified by brackets). GFRα2 is over-expressed in NFAs (relative to normal pituitary, functioning adenomas, and GH adenomas).

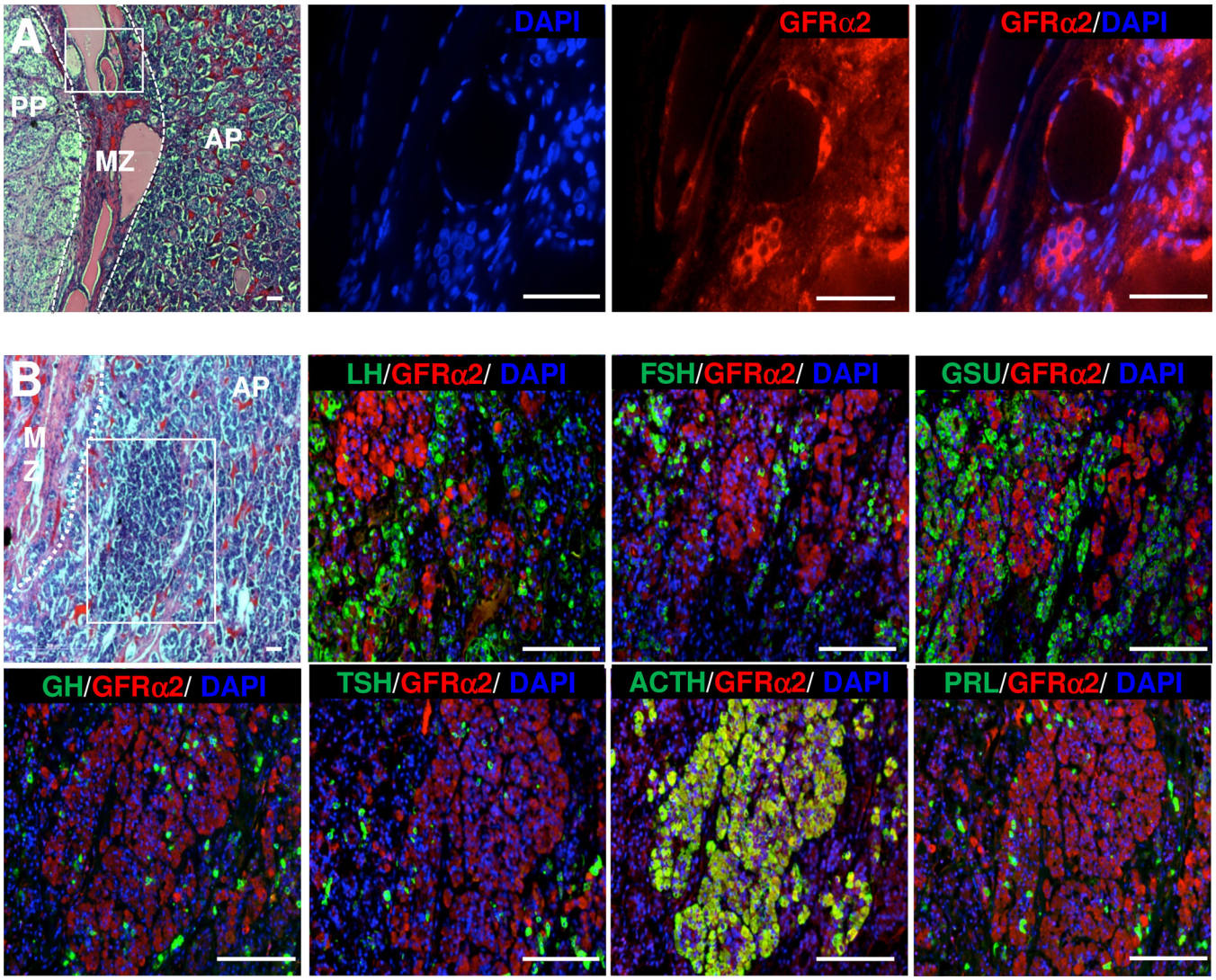

Figure 2.

A. Top left panel is H&E stain at 5x magnification showing the marginal zone (MZ) at the interface of the anterior (AP) and posterior pituitary (PP). White box delineates a typical Rathke's remnant cyst, shown at higher magnification (40x) in the immunofluorescence panels. Immunofluorescence panels show DAPI nuclear staining, GFRα2 staining, and merged DAPI/GFR 2 staining, showing that GFRα2 staining is seen in the Rathke's remnant cysts. **B.** Top left panel is H&E stain at 5x magnification of the AP at the border of the MZ, with white box outlining a basophilic cell cluster shown in the immunofluorescence panels at higher magnification (40x). Co-immunostaining of each of the AP hormones (green) is shown with GFRα2 (red), with co-localized staining appearing yellow/orange. The immunostains were performed on 5 μm consecutive sections of tissue for comparison of co-localization of GFRα2 and hormones at a single location. GFRα2 co-localizes nearly 100% with ACTH. There is GFRα2 staining adjacent to cell clusters of α-GSU, LHβ, and FSHβ positive cells (gonadotrope lineage), but there does not appear to be co-localization of GFRα2 with gonadotrope cells. GH, TSH, and PRL cells were scattered throughout the

GFRα2 positive cell clusters, but no co-localization was seen with these hormones and GFRα2. White bar represents 200 μm.

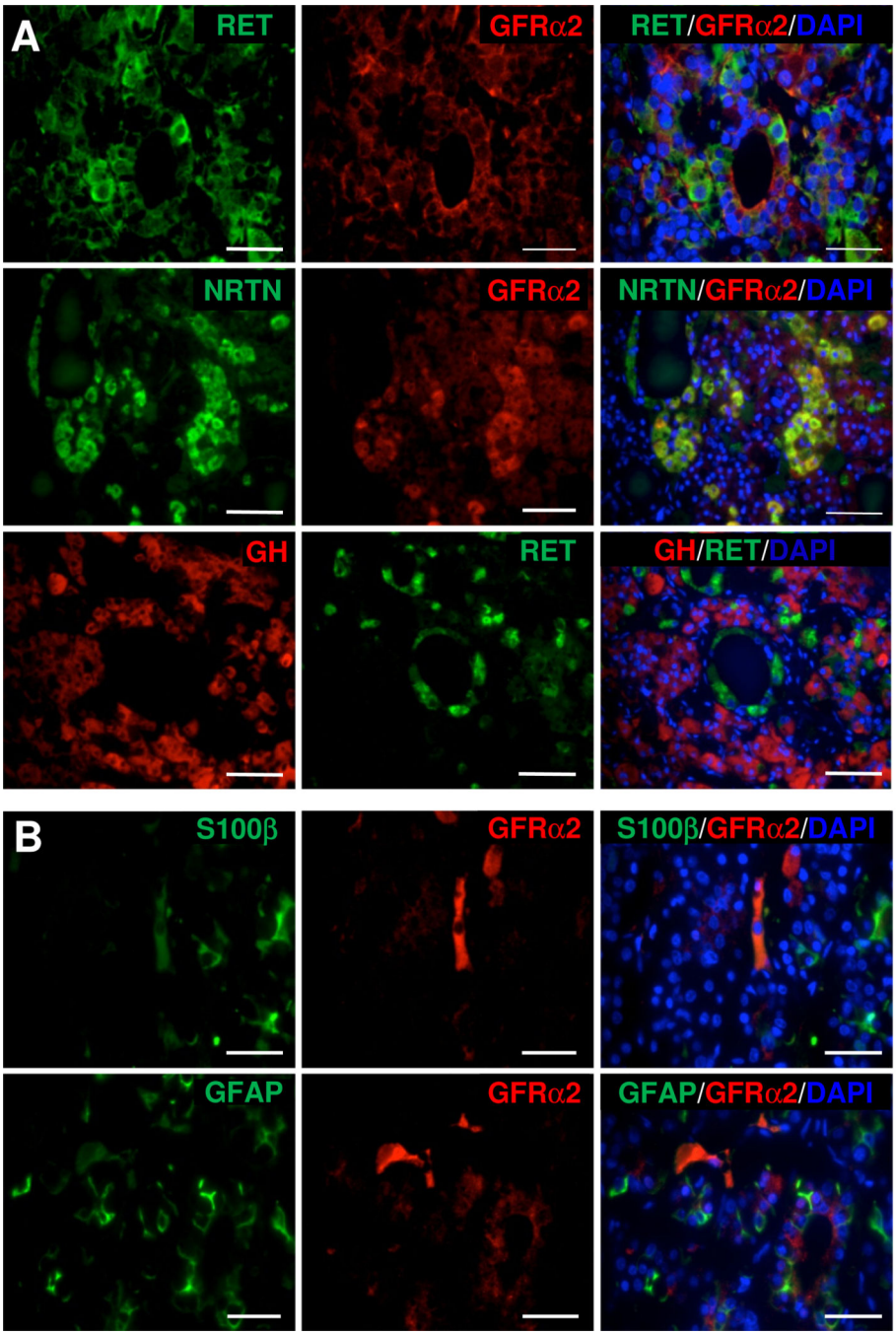

Figure 3.

A. Co-immunostaining of GFRα2 with its ligand (RET) and receptor neurturin (NRTN) in the normal anterior pituitary (AP). Top row: Co-immunostaining of RET (green) and GFRα2 (red), shows that GFRα2 and RET generally co-localize, although some strongly positive RET cells do not show significant staining for GFRα2. Middle row: Co-immunostaining of NRTN (neurturin) and GFRα2, shows strong co-localization of the ligand and receptor. Bottom row: Co-immunostaining of GH (red) and RET (green) in the normal AP, showed staining in distinct cell populations. **B.** Co-immunostaining of GFRα2

and the folliculostellate cell markers S100β and GFAP (green) and GFRα2 (red) in the normal AP, showing that a weakly positive S100β cell co-stains for GFRα2, while there was no significant co-localization of GFAP and GFRα2. White bar represents 100 μm.

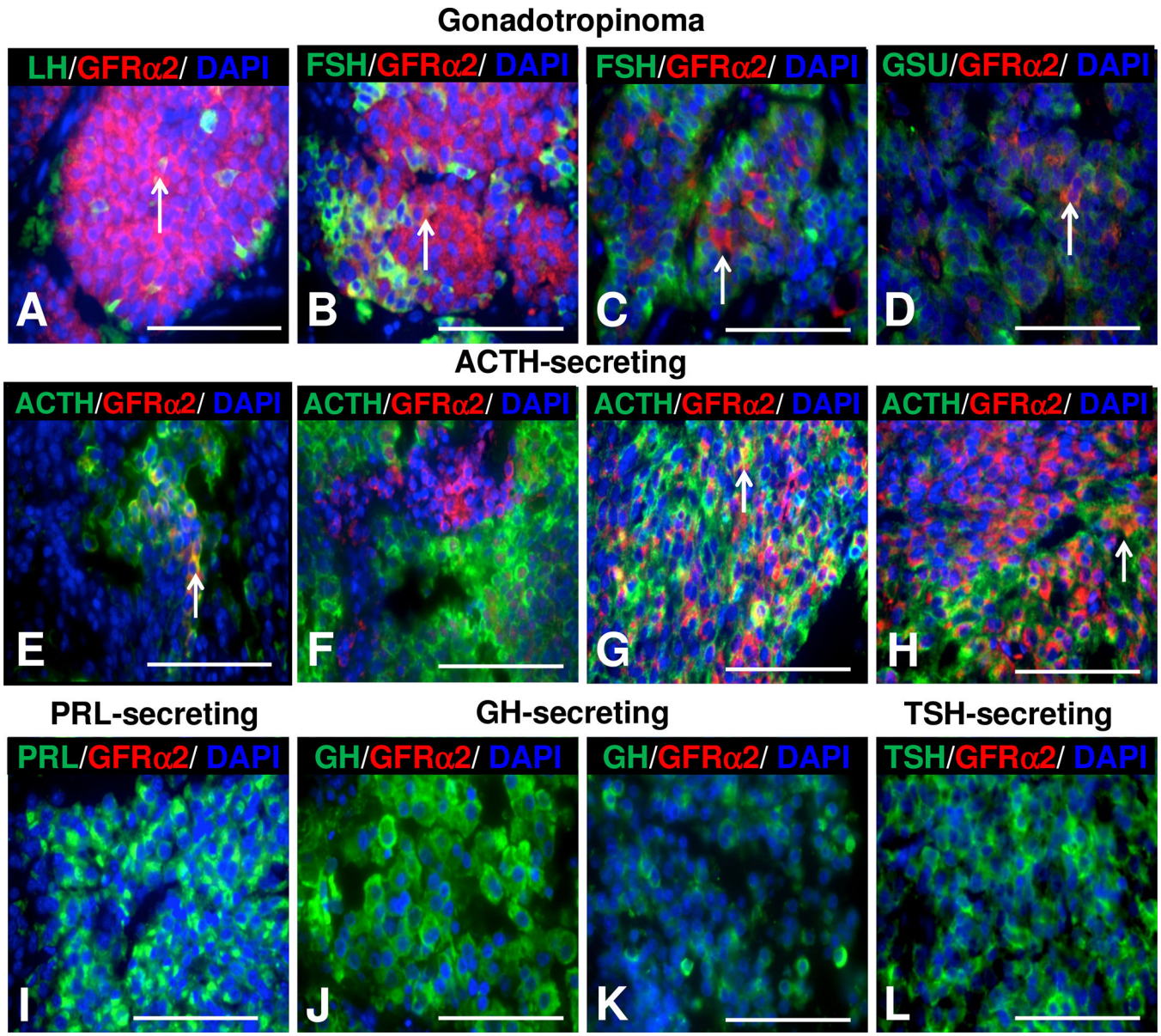

Figure 4.

Double immunostaining of GFRα2 (red) and relevant pituitary hormones (green) in representative pituitary adenomas. Co-localization appears yellow/orange and is indicated by white arrows. White bar represents 100 μm. **A-D**) show staining of gonadotrope hormones in a gonadotropinoma. Diffuse cell surface staining of GFRα2 is seen with scattered co-localization of GFRα2 and gonadotrope hormones in characteristic pseudorosettes. **E-H**) show staining of two ACTH-secreting adenomas (E&F=one tumor; G&H = second tumor). Co-immunostaining (white arrows) of GFRα2 and ACTH was focal in one tumor (E) and diffuse in the other (G&H). **I-L**) show absent GFRα2 immunostaining in tumors of the Pit-1 lineage: PRL-secreting adenoma, two GH-secreting adenomas, and a TSH-secreting adenoma.

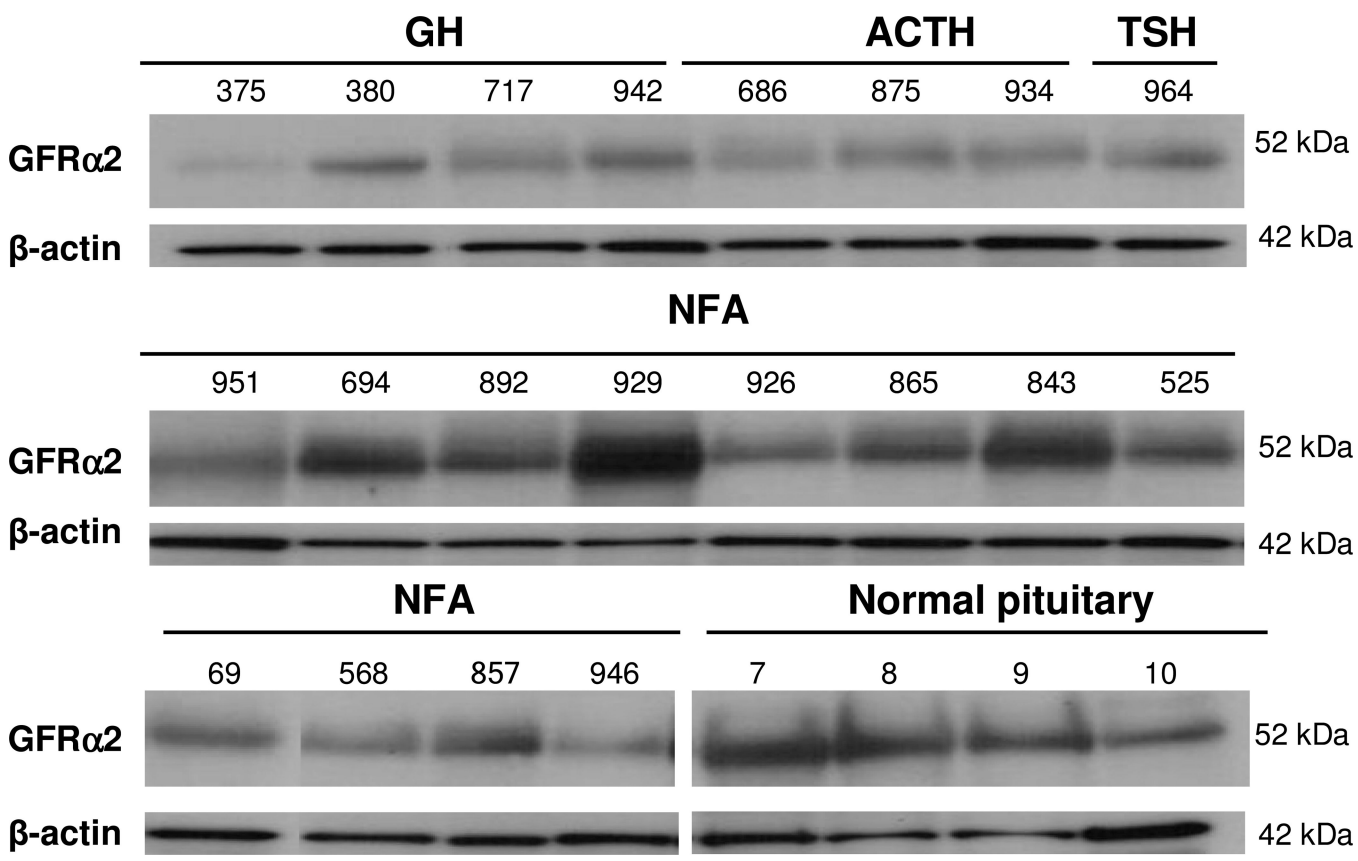

Figure 5.

Western Blot analysis of GFRα2 protein expression in 20 pituitary adenomas and 4 normal pituitary controls. Numbers indicate tumor sample. β-actin shown as loading control. Relative to normal pituitary, functioning adenomas (GH, ACTH, TSH) appear to have lower GFRα2 protein expression, while most NFAs show equal or greater protein expression.

Pituitary. Author manuscript; available in PMC 2016 February 01.

Table 1

Tumor #	Max. tumor diameter (cm)	Age, sex	Clinical Diagnosis	Hormone staining (IHC)	Pituitary Transcription Factor Expression (RT-PCR)	Clinicopathologic Classification
926	5.1	50, M	NFA	LH, FSH, GSU		Gn
568	2.1	46, M	NFA	FSH, GSU		Gn
946	3.3	59, F	NFA	FSH, GSU		Gn
892	4.0	74, F	NFA	Negative	FSH, LH	Gn
952	1.9	52, M	NFA	Negative	FSH, LH	Gn
945	3.7	29, M	NFA	FSH, GSU	SF-1, GATA-2, FSH, LH, aGSU	Gn
865	3.4	47, M	NFA	GSU	FSH, LH	Gn
857	5.1	46, F	NFA	FSH, GSU		Gn
928	1.3	41, F	NFA	GSU	SF-1, FSH, LH	Gn
843	2.6	70, M	NFA	Weak PRL	LH, TEF	Gn
649	1.5	67, F	NFA	GH, PRL		Silent GH/PRL
831	1.9	38, M	NFA	GH, PRL		Silent GH/PRL
525	2.9	68, F	NFA	GH, PRL		Silent GH/PRL
868	2.1	35, F	NFA	PRL		Silent PRL
683	3.6	50, M	NFA	FSH, GSU, PRL, GH		Silent plurihormonal
929	1.9	47, F	NFA	FSH, GSU, PRL, GH		Silent plurihormonal
694	3.0	67, M	NFA	FSH, GSU, PRL, GH		Silent plurihormonal
699	4.4	51, M	NFA	GSU, PRL, GH*	SF-1, FSH, LH, GH	Silent unclassified
721	2.8	56, M	NFA	Negative	SF-1, Tpit, TEF	Silent unclassified
951	4.8	52, F	NFA	GSU, PRL	GSU, Tpit	Silent unclassified
850	1.8	72, F	NFA	ND		NFA**
851	1.3	57, F	GH	GH		GH
380	2.8	68, M	GH	GH		GH
881	2.8	50, M	GH	GH, PRL		GH
880	0.5	37, F	GH	GH, PRL		GH

Tumor #	Max. tumor diameter (cm)	Age, sex	Clinical Diagnosis	Hormone staining (IHC)	Pituitary Transcription Factor Expression (RT-PCR)	Clinicopathologic Classification
711	0.5	45, M	GH	Negative		GH
717	1.8	51, M	GH	GH, PRL, GSU		GH
716	1.1	44, M	GH	GH, GSU, PRL		GH
907	1.9	34, M	GH	GH, PRL, GSU, TSH		GH
726	3.5	35, M	GH	GH, PRL, GSU		GH
375	2.0	43, F	GH	GH, GSU		GH
522	2.2	43, F	GH	GH, PRL		GH
942	1.9	60, F	GH	GH, LH		GH
492	1.5	50, F	GH	GH, PRL, GSU		GH
801	2.1	17, F	GH	GH, GSU		GH
875	0.8	20, F	ACTH	ACTH		ACTH
686	2.5	18, M	ACTH	Negative		ACTH
696	0.8	26, F	PRL	PRL		PRL
964	1.9	61, F	TSH	TSH, GSU		TSH

* incomplete hormonal staining (FSH and TSH staining not performed);

** hormone staining not available, so tumor classification was based on clinical diagnosis. GSU = alpha glycoprotein subunit.