Expression of FOXL2 in human normal pituitaries and pituitary adenomas

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Many transcription factors have important roles in the function and differentiation of the human pituitary adenomas. Forkhead box gene transcription factor L2, Foxl2, is expressed during mouse pituitary development and co-localizes with the expression of α-glycoprotein hormone subunit (αGSU). In addition, Foxl2 regulates expression of the αGSU gene (*Cga*) in cell culture. To elucidate the functional role of FOXL2 in the human pituitary, we examined the expression and localization of FOXL2 in normal human pituitaries and various types of pituitary adenomas. Human pituitary adenomas were obtained by trans-sphenoidal surgery from 67 patients. Three normal adult pituitaries were obtained from autopsies of non-endocrine cases. The localization of FOXL2 and pituitary hormones in these pituitary patients was examined by immunohistochemical staining and RT–PCR. Quantitative analysis of FOXL2 protein was performed by immunoblotting. FOXL2 was localized in the nuclei of ∼20% of normal pituitary cells that also co-expressed gonadotropins including follicule-stimulating hormone β (FSHβ), luteinizing hormone β (LHβ), and αGSU, whereas it was observed in minor proportion of thyroid-stimulating hormone (TSH)-producing cells, prolactin (PRL)-producing cells, and precursor of adrenocorticotropic hormone (ACTH)-producing cells. FOXL2 immunoreactivity was not detected in growth hormone (GH)-producing cells or S100-positive folliculo-stellate cells. In human pituitary adenomas, FOXL2 was expressed in the nuclei of the adenoma cells. FOXL2 was detected in 13 of 15 gonadotropin-subunit-producing adenoma (Gn-oma) cases and 8 of 11 null cell adenoma cases, but its incidence was reduced or not detected in the other types of adenomas. The results of this study suggest that FOXL2 contributes to the human-specific functional expression and the differentiation of gonadotroph cells and adenomas.
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Factors that are important for pituitary organogenesis exist as exogenous and endogenous signaling gradients. The pituitary gland develops from Rathke's pouch, and the pituitary primordium appears on embryonic day (E) 8.5 in mice. The hormone-producing cells of the pituitary gland appear initially as α-glycoprotein hormone subunit (αGSU) positive cells on E11 and, subsequently, differentiate into anterior pituitary hormone-producing cells.[1] The pituitary cell types can be classified into three cell lineages: the growth hormone (GH)-prolactin (PRL)-thyroid-stimulating hormone (TSH) (GH-PRL-TSH) cell lineage, the proopiomelanocortin (precursor of adrenocorticotropic hormone, ACTH) lineage, and the gonadotropin (luteinizing hormone/follicle-stimulating hormone; LH/FSH) lineage. Several transcription factors are known to regulate pituitary development, proliferation, and the functional differentiation of these lineages. *Pitx1*, *Pitx2*, *Lhx3*, *Lhx4*, and *Rpx/Hesx1* are expressed in early pituitary development, and are essential for normal pituitary organogenesis.[2–6] On the other hand, late functional transcription factors, including *Tpit*, *Gata2*, *Sf1*, *Egr1*, Estrogen receptor (*Esr1*), and *Zn-15*, regulate the functional differentiation of each type of hormone-producing cell.[7–12] Forkhead factors are a class of transcription factors that are important for diverse developmental processes. Deficiencies of individual forkhead genes may result in

deficiency of speech and language, diabetes, immunodeficiency, cleft palate, and eye development disorders.[13–16] One member of the forkhead family, *Foxl2*, is expressed in the eye, the ovary and the pituitary. In putative *Foxl2* knockout animals, eye lids are not formed completely, and the ovary does not mature and lack proliferating cells.[17] In humans, FOXL2 has been shown to be mutated in type I—blepharophimosis/ptosis/epicanthus inversus syndrome (BPES).[18] Type I BPES is also associated with eyelid abnormalities and premature ovarian failure (POF).

Kioussi *et al*[19] reported that *Foxl2*, also known as pituitary forkhead factor, is expressed in the developing pituitary gland. However, key questions remain concerning the onset of expression, the types of pituitary cells that express *Foxl2*, and its upstream regulators. Previously, we established the position of *Foxl2* in the genetic hierarchy of pituitary development. *Foxl2* is co-localized with αGSU only in quiescent cells of the mouse pituitary from E11.5 through adulthood and regulates the αGSU promoter (*Cga* promoter) in cell culture.[20]

In contrast to the studies of *Foxl2* in the development of mouse pituitary, there have been no extensive studies on FOXL2 expression in human normal pituitaries and in adenomas. This study aimed to elucidate whether the FOXL2 expression is related to functional differentiation in human pituitaries. Our present study deals with the localization of FOXL2 in human pituitaries and various types of adenomas. Our results suggest a novel role for FOXL2 in human pituitaries.

Materials and methods

Clinical Characteristics of Human Pituitary Specimens

Specimens of pituitary adenomas were obtained at the time of trans-sphenoidal surgery from 67 patients at the Nippon Medical School and its affiliated hospitals. Three normal pituitaries were obtained at autopsy from adult individuals without pituitary disorders. Pituitary adenomas were classified on the basis of clinical and biological findings, including the baseline hormone and dynamic testing. The clinical and endocrinologic features were as follows: 19 patients with GH-producing adenomas and symptoms of acromegaly; 9 PRL-producing adenomas; 8 ACTH-producing adenomas and typical Cushing's syndrome; 5 TSH-producing adenomas; 26 non-functioning adenomas that presented no clinical evidence of anterior pituitary hormone excess and did not show high serum concentrations of any of the anterior pituitary hormone except mild hyperprolactinemia (< 100 ng/ml) and included 11 null cell adenomas (hormone immunonegative) and 15 gonadotropin-subunit-producing adenomas. These adenomas were classified based on their clinical manifestation, biological function,

and morphology including immunohistochemistry and ultrastructure.

FOXL2 and Pituitary Hormones Immunohistochemistry

The pituitary glands from the patients were fixed overnight in 20% formalin at 4 °C. The fixed tissues were washed in PBS and dehydrated through an ethanol series and embedded in paraffin. Tissue sections of 2 μm thickness were prepared for immunohistochemistry. Slides were dewaxed and rehydrated before staining. For FOXL2 immunostaining, epitopes were exposed by autoclaving for 5 min in 10 mM citric acid (pH 6.0). Anti-FOXL2 antibody (abcam, Cambridge, UK) was used at a 1:200 dilution. Anti-PRL (NHPP, NIDDK, Bethesda, MD, USA), anti-human GH (DAKO cytomation, Denmark), and anti-human αGSU (NHPP) rabbit antibodies were used at 1:600, 1:400, and 1:100, respectively. Anti-human LHβ and FSHβ (Immunotech, Marseille, France), anti-human TSHβ (Advanced Immunochemical, Long Beach, CA, USA), and anti-human ACTH (DAKO Cytomation) monoclonal antibodies were used at 1:200, 1:100, and 1:200, respectively. Anti-cow S100 polyclonal antibody (DAKO Cytomation) was used at 1:100. Sections were incubated with these primary antibodies for 60 min at room temperature, followed by incubation in biotin-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Signals were amplified using the Avidin–biotin peroxidase complex method (Vector Laboratories, Burlingame, CA, USA) according to
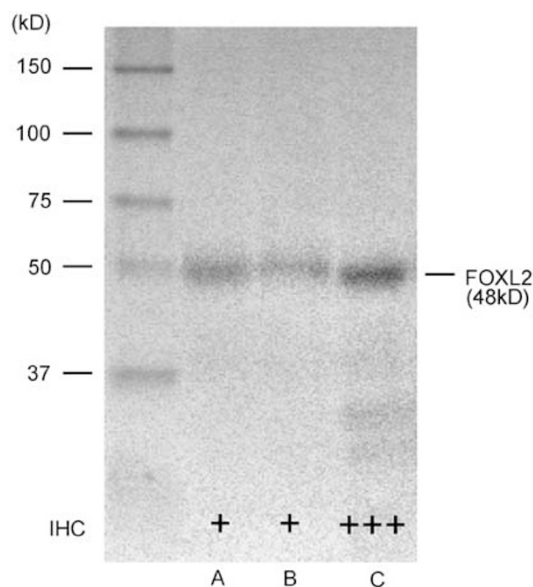

Figure 1 FOXL2 expression levels were correlated with FOXL2 immunoreactivities. The expression levels of FOXL2 protein was examined by immunoblotting. The quantities of FOXL2 protein were correlated with the frequencies of FOXL2 immunoreactivity in pituitary adenomas. IHC; the results of immunohistochemical analysis of FOXL2 expression from Table 2. (A) Gonadotropinoma case 9; (B) null cell adenoma case 8; (C) gonadotropinoma case 4.
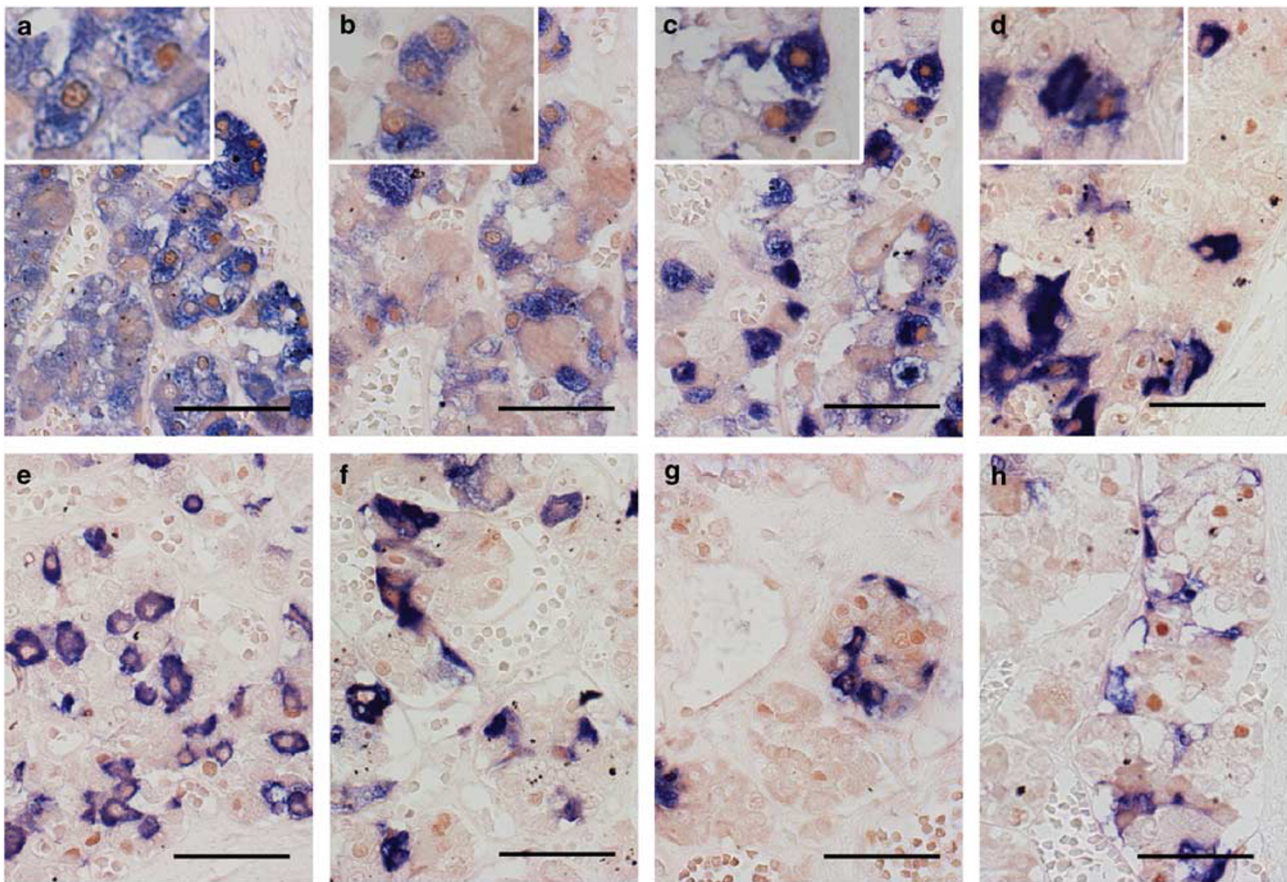
Figure 2 Expression of FOXL2 as shown by double-immunohistochemical staining in human normal pituitary glands. Expression of FOXL2 protein is observed in the nuclei of anterior pituitary cells as a brown color. The immunoreactivities of pituitary hormones and S100 protein are shown as a blue color. FOXL2 immunoreactivity was localized mainly in gonadotroph cells (**a**, αGSU; **b**, FSHβ; **c**, LHβ) and a small fraction of (**d**) TSHβ-positive cells (arrow heads). The immunoreactivity was observed rarely in (**e**) GH-producing cells, (**f**) PRL-producing cells, (**g**) ACTH-producing cells, and (**h**) S100-positive folliculo-stellate cells. Bar, 50 μm.

the manufacturer's instructions. Peroxidase activity was visualized with 3,3′-diaminobenzidine (DAKO Cytomation, Glostrup, Denmark), and the sections were lightly counterstained with methyl green. Immunohistochemical specificity of all the antibodies was confirmed by non-immune immunoglobulins or normal serum as negative controls.

Immunoblotting for FOXL2 Protein in Pituitary Adenomas

Tissue aliquots were snap frozen in liquid nitrogen for immunoblot analysis. Pituitary tumor tissue protein extracts were prepared in β-mercaptoethanol containing sample buffer and resolved under reducing conditions on 10% SDS–polyacrylamide gels using standard methods. Resolved proteins were transferred to nitrocellulose membranes and probed with antibodies against FOXL2 (1:2000; abcam) overnight at 4 °C. After washing, membranes were incubated with appropriate IgG-HRP conjugates, and immunoreactive protein bands were visualized with ECL plus (GE Healthcare UK, Buckinghamshire, UK). Total protein was normalized by tissue weight or

Ponceau S staining, and protein bands were quantified by densitometry (Video Densitometer model 620; Bio-Rad Laboratories, Hercules, CA, USA).

RT–PCR Analysis of FOXL2 mRNA Expression from FFPE Samples

All paraffin blocks were cut on the same microtome with single use disposable blades. Briefly, five 7 μm sections of stored formalin-fixed paraffin-embedded (FFPE) pituitary adenoma tissues were deparaffinized in xylene at 50 °C, followed by washes in ethanol. Total RNA was extracted by RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA). RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System RT–PCR kit (Invitrogen Life Technologies, CA, USA) after incubation with proteinase K. RNase inhibitor (RNaseOUT) and random primer were also from Invitrogen Life Technologies.

A normal pituitary cDNA was purchased from BioChain Institute (Hayward, CA, USA) as a FOXL2-positive control. PCR was performed using the AmpliTaq Gold PCR kit (Applied Biosystems, Foster

Table 1 The expression of FOXL2 protein in anterior pituitary-hormone-producing cells and S100 positive cells in human normal pituitaries (expressed as percentage)

	αGSU	FSHβ	LHβ	TSHβ	GH	PRL	ACTH	S100
FOXL2	60.50 ± 0.07	61.30 ± 3.22	64.80 ± 0.04	6.39 ± 0.01	ND	1.77 ± 0.00	3.83 ± 0.02	ND

ND, not detected.
The data are the mean ± s.d.

Table 2 Results of immunohistochemical analysis for anterior pituitary hormones and FOXL2 in 67 pituitary adenomas

Diagnosis	Case no.	Age/sex	Immunohistochemistry							
			GH	PRL	ACTH	TSHβ	LHβ	FSHβ	αGSU	FOXL2
Null cell adenomas	1	46/M	−	−	−	−	−	−	−	+++
	2	75/M	−	−	−	−	−	−	−	+++
	3	71/M	−	−	−	−	−	−	−	+++
	4	65/M	−	−	−	−	−	−	−	++
	5	45/F	−	−	−	−	−	−	−	+/−
	6	66/F	−	−	−	−	−	−	−	+/−
	7	56/M	−	−	−	−	−	−	−	+/−
	8	51/F	−	−	−	−	−	−	−	+
	9	45/F	−	−	−	−	−	−	−	−
	10	41/F	−	−	−	−	−	−	−	−
	11	52/F	−	−	−	−	−	−	−	−
Gonadotropin-subunit-positive adenomas	1	50/M	−	−	−	−	−	+	+	+++
	2	52/M	−	−	−	−	−	+	+	+++
	3	71/F	−	−	−	−	−	−	+	+++
	4	64/M	−	−	−	−	+	++	+	+++
	5	79/M	−	−	−	−	−	+	+	+++
	6	31/M	−	−	−	−	−	+	−	+++
	7	53/M	−	−	−	−	−	++	+	++
	8	56/F	−	−	−	−	−	+	+/−	++
	9	63/M	−	−	−	−	++	+++	+++	+
	10	51/F	−	−	−	−	−	+/−	+/−	+
	11	60/M	−	−	−	−	−	++	−	+
	12	61/F	−	−	−	−	+	++	−	+
	13	35/F	−	−	−	−	−	+	+	+/−
	14	39/F	−	−	−	−	−	+	+	−
	15	36/F	−	−	−	−	+	+	−	−
TSH-producing adenomas	1	40/M	−	−	−	+++	−	−	+++	+/−
	2	M	+	−	+	+++	+/−	−	+	+/−
	3	53/F	+	+	−	+++	−	−	++	−
	4	62/F	+	−	−	+	−	−	++	−
	5	57/F	−	−	−	+++	−	−	−	−
GH-producing adenomas	1	47/M	+++	++	++	+	−	−	++	+
	2	56/F	+++	+++	−	−	−	−	+/−	+/−
	3	57/F	+++	−	−	−	−	−	+++	−
	4	55/F	+++	+	−	−	−	−	+	−
	5	56/F	+++	+++	−	−	−	−	+++	−
	6	66/F	+++	+++	−	−	−	−	+++	−
	7	38/F	+++	++	−	−	−	−	+	−
	8	46/F	+++	++	−	−	−	−	+++	−
	9	52/F	+++	+	−	−	−	−	−	−
	10	58/M	++	+	−	−	−	−	++	−
	11	43/F	+++	+	−	−	−	−	−	−
	12	58/M	++	−	−	−	−	−	+	−
	13	60/F	+++	+	−	−	−	−	−	−
	14	28/F	+++	+	−	−	−	−	−	−
	15	41/F	++	++	−	−	−	−	+	−
	16	46/M	+++	+++	−	−	−	−	−	−
	17	22/M	+++	++	−	−	−	−	−	−
	18	70/F	+++	+++	−	−	−	−	−	−
	19	42/F	+++	−	−	−	−	−	−	−

Table 2 Continued

Diagnosis	Case no.	Age/sex	Immunohistochemistry							
			GH	PRL	ACTH	TSHβ	LHβ	FSHβ	αGSU	FOXL2
PRL-producing adenomas	1	30/F	+/−	+	−	−	−	−	−	+/−
	2	F	−	+	−	−	−	−	−	+/−
	3	25/F	−	++	−	−	−	−	−	−
	4	F	+/−	+++	−	−	−	−	−	−
	5	32/M	−	+++	−	−	−	−	−	−
	6	30/M	−	+++	−	−	−	−	−	−
	7	F	−	+++	−	−	−	−	−	−
	8	63/F	−	+++	−	−	−	−	−	−
	9	20/F	−	+++	−	−	−	−	−	−
ACTH-producing adenomas	1	32/M	−	−	+++	−	−	−	++	−
	2	F	−	−	++	−	−	−	+/−	−
	3	28/F	−	−	+++	−	−	−	−	−
	4	72/F	−	−	+++	−	−	−	−	−
	5	76/F	−	−	+++	−	−	−	−	−
	6	26/F	−	−	+++	−	−	−	−	−
	7	72/F	−	−	+++	−	−	−	−	−
	8	52/F	−	−	+++	−	−	−	−	−

Minus indicates negative; +/−, <5%; +, 5–20%; ++, 20–50%; +++, over 50% of adenoma cells; ND; not done.
FOXL2 immunoreactivity is estimated by frequency.

City, CA, USA) according to the manufacturer's instructions, and each specific primer was as follows: human *FOXL2* primers, 5′-GAGTTTTTGT TGGGCCTTCA-3′ and 5′-GAGGGTGAAACTTCCCC AAT-3′; human *βActin* primers, 5′-TCCTCAGCAGG ATTTTCACC-3′ and 5′-CATGTTGGCGTCAAACTT GT-3′. cDNA samples for *FOXL2* PCR were incubated for 37 cycles of PCR amplification on a Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany). The FOXL2 and β actin PCR products were detected as bands of 97 and 103 bp, respectively. The PCR products were analyzed by electrophoresis with 12% Acrylamide gel and visualized by ethidium bromide staining. The density of PCR products was measured by CS Analyzer 2.0 (ATTO & Rise Corporation, Tokyo, Japan).

Results

The specificity of the anti-FOXL2 antibody was confirmed by immunoblotting (Figure 1). The anti-FOXL2 antibody detected a single band at 48 kDa. The quantities of FOXL2 protein were correlated with the frequencies of FOXL2 immunoreactivity in pituitary adenomas (Table 2). The localization of FOXL2 was examined by immunodouble staining in three normal pituitaries. The immunoreactivity of FOXL2 protein was observed in the nuclei of the pituitary cells (Figures 2a–h). Table 1 summarizes the expression ratios (expressed as percentage) that were obtained by counting the FOXL2-positive cells among the anterior pituitary hormone-producing cells and S100 protein-positive folliculo-stellate cells. Approximately 20% of the anterior pituitary

cells were FOXL2 positive, and most of the FOXL2-positive cells were gonadotropin-subunit-producing cells. In other words, most of the FOXL2-positive cells were also FSHβ/LHβ/αGSU positive (Figures 2a–c), whereas FOXL2 immunoreactivity was scarcely observed in PRL- or ACTH-producing cells (Figures 2f and g). FOXL2 co-localized in many of gonadotropin-subunit-producing cells. However, FOXL2 was detected only in a minority of TSH-producing cells, which were also known as αGSU expressing cells (Figure 2d). FOXL2 immunoreactivity was not detected in GH-producing cells or S100-positive folliculostellate cells (Figures 2e and h).

In 67 human pituitary adenomas, FOXL2 immunoreactivity was demonstrated in the nuclei of the adenoma cells. The results of the immunohistochemical examination of the different types of adenoma are summarized in Table 2. FOXL2 was detected in 8 of 11 (72.7%) null cell adenoma cases (Figures 3aA and 3bA) and in 13 of 15 (86.7%) gonadotropin-subunit-producing adenoma cases (Gn-omas) (Figures 3aB and 3bG). These adenomas showed diffuse and strong nuclear reactivity for FOXL2 protein (Figures 3aA and B). However, in 2 of 5 TSH-producing adenomas (TSHomas), some weakly positive (+/−) cells for FOXL2 were observed (Figures 3aC and 3bC). FOXL2 was expressed very rarely in GH-producing adenomas (GHomas), PRL-producing adenomas (PRLomas) (Figures 3aD, E and 3bD, E). FOXL2 was not detected in ACTH-producing adenomas (Figures 3aF and bF).

The mRNA expression for FOXL2 was examined by RT–PCR in 30 human pituitary adenomas that were taken from the same FFPE samples analyzed by
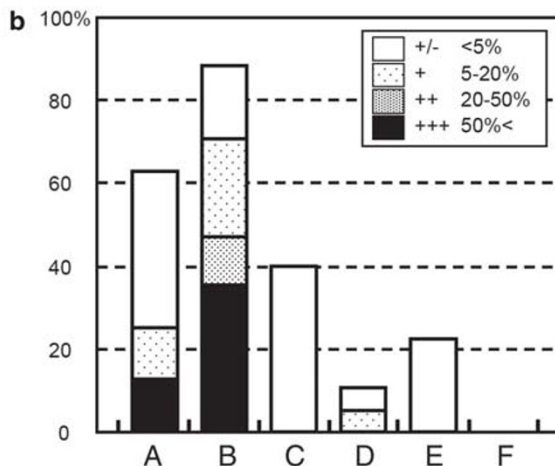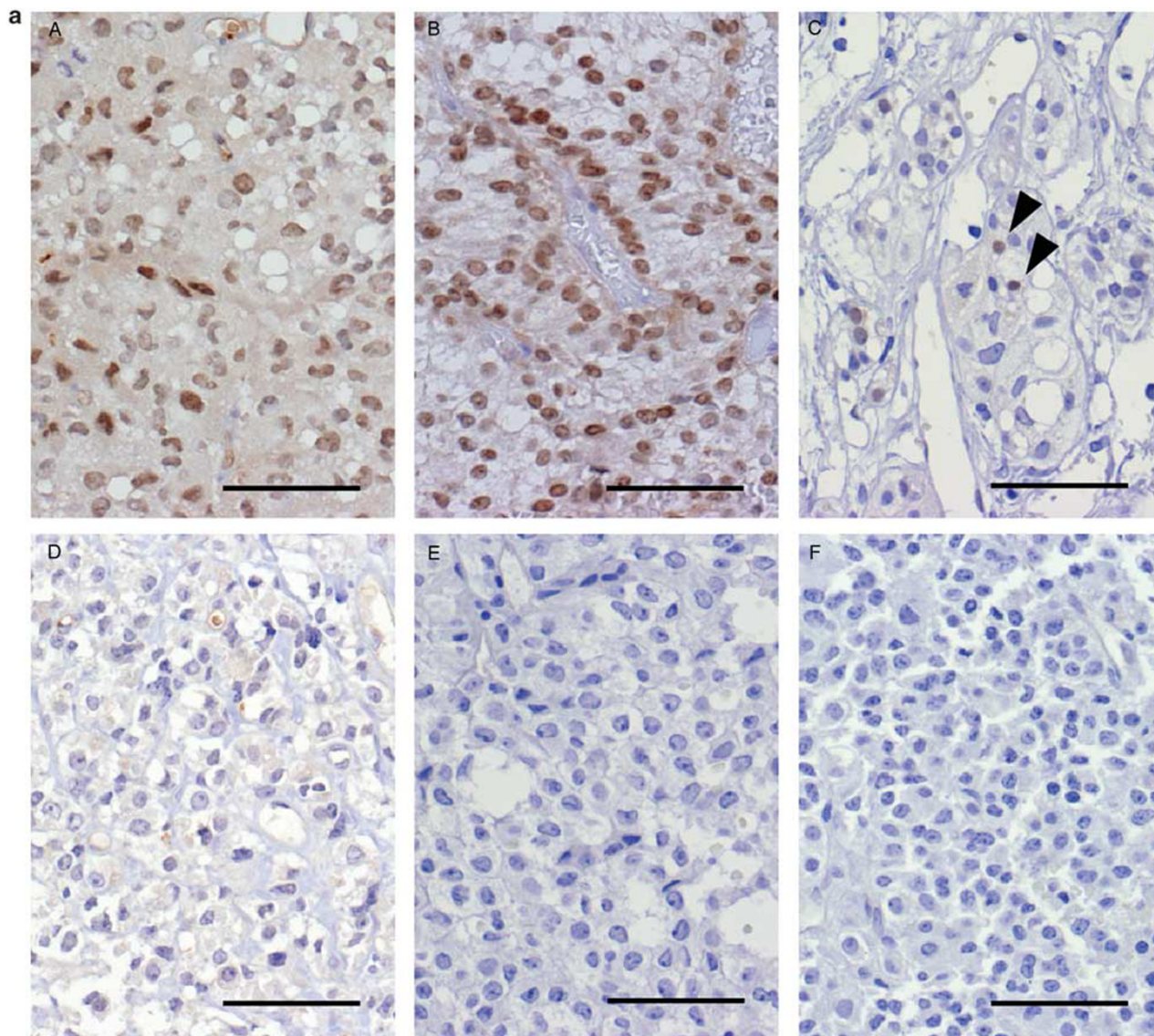
Figure 3 FOXL2 expression in pituitary adenomas. (**a**) FOXL2 immunoreactivity was examined in 67 human pituitary adenomas. Arrowhead, FOXL2-positive nuclei. (**b**) Proportions of cells displaying positive results for FOXL2 in the pituitary adenomas were compared. Null cell adenoma (A), gonadotropin-subunit-positive adenoma (B) have diffuse and strong nuclear reactivity for FOXL2 protein. FOXL2 immunoreactivity was observed in (A) null cell adenomas and (B) gonadotropin-subunit-positive adenomas. Some weakly positive (+/−) cells for FOXL2 were observed in (C) TSH-producing adenomas. FOXL2 was occasionally expressed in (D) GH-producing adenomas, (E) PRL-producing adenomas, but not in (F) ACTH-producing adenomas. Bar, 50 μm.
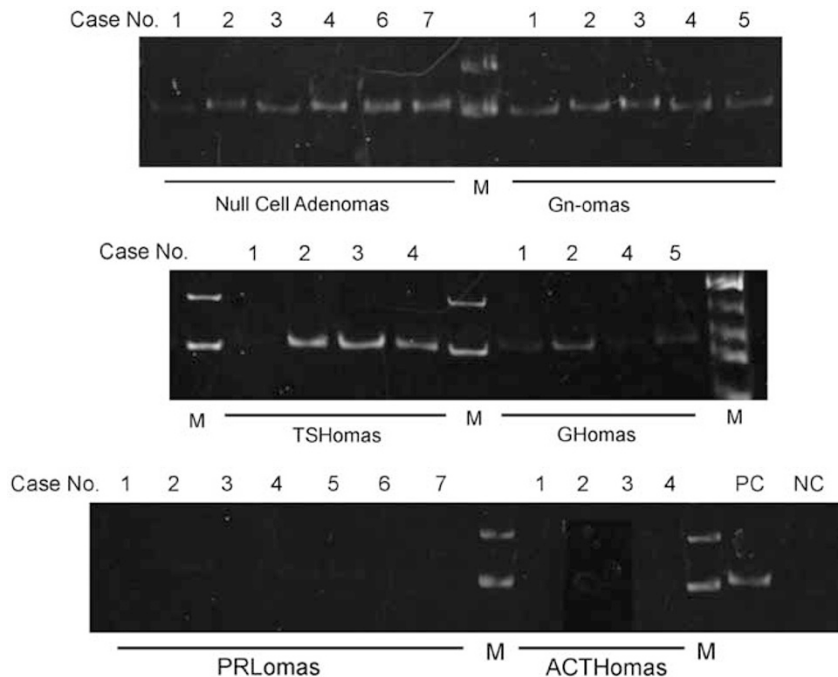
Figure 4 The expression of *FOXL2* mRNA was detected in pituitary adenomas using RT–PCR. M, marker; PC, cDNA from normal human pituitary as positive control; NC, sterile water as negative control.

IHC (Figure 4). FOXL2 mRNA was detected in all six null cell adenomas, all 5 Gn-omas, 3 of 4 TSHomas, and 3 of 4 GHomas, but was not detected in all 7 PRLomas or in all 4 ACTHomas.

Discussion

Several transcription-regulating proteins have been identified in the anterior pituitary and have been implicated as key elements in the determination of cell-specific phenotypes and the regulation of hormone gene expression. In pituitary adenomas, transcription factors also regulate various genes that have important roles in functional differentiation.[21,22] FOXL2, a Fox (forkhead box) gene transcription factor that maps to the BPES locus on chromosome 3q22–23, is expressed during embryonic development and participates in terminal differentiation. *FOXL2* is mutated in BPES, which is characterized by eyelid malformations, and POF.[18] Mutations in individuals with BPES type I create premature stop codons in *FOXL2*, presumably producing a truncated protein lacking the carboxyl terminus. In the mouse, expression is observed in the perioptic mesenchyme, developing eyelids, ovary, and pituitary. Foxl2 co-localizes with the glycoprotein hormone, αGSU, in quiescent cells of the mouse pituitary from E11.5 through adulthood, suggesting a possible role in suppressing cell proliferation and/or promoting cell differentiation.[20] In mouse and porcine pituitary cells, the role of Foxl2 is related to Activin A-regulated *Fshβ* transcription.[23]

Human FOXL2 is highly homologous to mouse Foxl2 with 90% sequence identity and 95% similarity at the protein level. Within the DNA-binding domain of FOXL2, there is 100% similarity at the protein level, suggesting similar promoter recognition between the two species.[18] Most human pituitary adenomas are monoclonal in origin, and various transcription factors, such as PIT1, NEUROD1, GATA2, MSX1, and PTX1, may participate in the differentiation and functional expression of pituitary adenomas.[24–29]

We report that FOXL2 is expressed mainly in gonadotropes of normal pituitaries, and is observed in Gn-omas and null cell adenomas, which are derived from gonadotroph (LH/FSH producing) cells.[30] Our findings suggest that FOXL2 has a role in differentiation toward gonadotropin (LHβ, FSHβ, αGSU)-producing cells in normal pituitaries. No staining was observed in GH cells or pure GHomass. A few cells are positive for FOXL2 in αSU-positive GHomas. This may indicate further subclassification of the GH cell population, GH + αGSU double-positive cells, and pure GH cells. This staining characteristic is observed in pituitary adenomas, but in a less strict manner. In pituitary adenomas, the results of RT-PCR suggests that *FOXL2* transcripts were not only detected in Gn-omas and null cell adenomas, but also in TSHomas and GHomas. It is considered that FOXL2 expression was not sufficient for functional differentiation toward TSHomas and GHomas (Figure 5). These observations in human pituitary glands indicate that FOXL2 may have a role in gonadotroph cell differentiation and its committed cell lineages (Figure 5). Interaction between SF1 and FOXL2 is a key issue in this respect and remains to be further investigated.
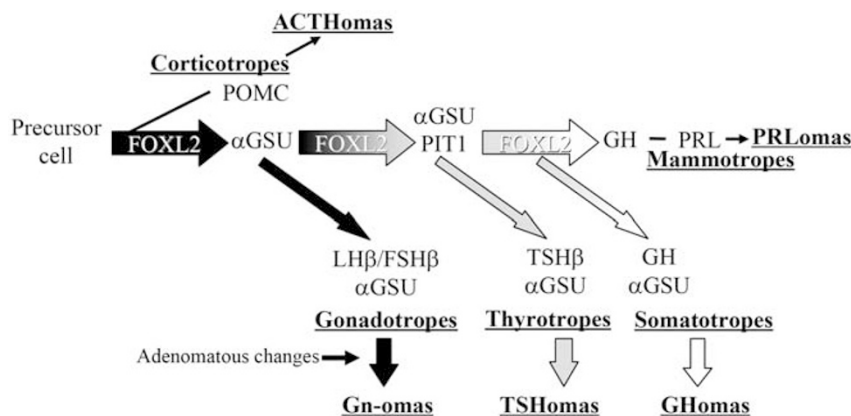
Figure 5 Schematic representation of human anterior pituitary differentiation predicted from the FOXL2 expression patterns in normal and adenomas. FOXL2 has its role in differentiation toward gonadotropin-producing cells in normal pituitaries, although it is not observed in GH cells or pure GHomas. A few FOXL2 positive cells are observed in a few cases of αSU-positive GHomas. FOXL2 transcripts are not only observed in Gn-omas and null cell adenomas, but also in TSHomas and GHomas. It is considered that FOXL2 expression was not enough for functional differentiation in TSHomas and GHomas. And its function may be more important to the differentiation of gonadotroph cell lineage than thyrotroph and somatotroph cell lineages.

In conclusion, FOXL2 expressing cells were confined mainly to gonadotropin-subunit-producing cells in human normal pituitaries. FOXL2 was detected in a small fraction of TSH-producing cells. FOXL2 was localized immunohistochemically in many of the gonadotroph cell adenomas. Our results suggest that FOXL2 contributes to the specific functional expression and differentiation in gonadotroph cells and in gonadotroph adenomas in humans.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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