Differential expression of neurogenins and NeuroD1 in human pituitary tumours

A Fratticci[1], F A Grieco[1], C Spilioti[1], F Giangaspero[2,3], L Ventura[4], V Esposito[2,5], M Piccirilli[5], A Santoro[5], A Gulino[2,3], G Cantore[2], E Alesse[1] and **M L Jaffrain-Rea[1,2,6]**

[1]Department of Experimental Medicine, University of L'Aquila, Via Vetoio, Coppito 2 - 67100 L'Aquila (AQ), Italy

[2]Neuromed Institute, IRCCS, Pozzilli, Via Atinense 18, 86077 Pozzilli (IS), Italy

[3]Department of Experimental Medicine and Pathology, University 'La Sapienza', Policlinico Umberto 1°, Viale dell'Università, 00161 Rome (RM), Italy

[4]Pathology, S Salvatore Hospital, L'Aquila, Coppito, 67 100 L'Aquila (AQ), Italy

[5]Departments of Neurological Sciences, Policlinico Umberto 1°, Via Regina Margherita, 00161 Roma (RM), Italy

[6]Fondazione 'Carlo Ferri', Via E. Riva 42, 00015 Monterotondo (RM), Italy

(Correspondence should be addressed to M L Jaffrain-Rea; Email: jaffrain.ml@libero.it)

(A Fratticci and F A Grieco contributed equally to this work)

Abstract

Basic helix-loop-helix (bHLH) transcription factors are involved in neuroendocrine cell growth and differentiation. Though NeuroD1 is viewed as corticotroph specific, its overexpression in non-corticotroph pituitary adenomas (PAs) may reflect the activation of molecular pathways involving other bHLH factors, like neurogenins. To search for neurogenin–NeuroD1 molecular pathways in the human normal and tumoural pituitary. Fifty-one PAs – 22 clinically non-secreting (CNS) and 29 secreting respectively – and normal human pituitaries (NP) were studied for NeuroD1 and neurogenins (*Ngn1*, *Ngn2* and *Ngn3*) gene expression by RT-PCR and quantitative real-time RT-PCR (qRT-PCR). Immunohistochemistry for Ngn2/3 was performed in some cases. NeuroD1, Ngn2, Ngn3 and Ngn1 were observed in up to 84·3, 76·5, 30·4 and 9·1% of PA respectively, only NeuroD1 and Ngn2 being frequently overexpressed when compared with NP. Whereas NeuroD1 expression was higher in corticotroph and CNS adenomas ($P=0·0001$ versus Pit-1-dependent PA), Ngn2 expression was higher in secreting PA, especially in Pit-1-dependent PA ($P=0·007$ and $P=0·0006$ versus CNS respectively). Pit-1-dependent PA which received pre-operative pharmacological treatment expressed higher Ngn2 levels than untreated cases ($P=0·025$). Nuclear Ngn2 was observed in NP and in most PA, especially ACTH- and GH-secreting adenomas. Nuclear Ngn3 was observed in a minority of secreting PA. Ngn2 is normally expressed in the anterior pituitary and frequently expressed in PA, but does not account for NeuroD1 overexpression where present. Owing to their low and inconstant expression, the biological significance of Ngn1/3 in the adult pituitary is uncertain.

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Introduction

Basic helix-loop-helix (bHLH) transcription factors play a fundamental role in the ontogenesis of highly differentiated cells, especially during neurogenesis, myogenesis and haemopoiesis (Massari & Murre 2000), and proneural bHLH are involved in the development of neuroendocrine cells (Lanigan *et al.* 1998, Edlund 1999, Ma *et al.* 1999, Ito *et al.* 2000). Basic HLH proteins can act early during ontogenesis, as determinating factors which recruit cells to a specific progenitor pool, or later as final differentiating factors. Though bHLH factors have been observed in the anterior adult pituitary (Jackson *et al.* 1993), studies on the transcriptional control of pituitary development have mainly focused on homeodomain factors acting since early embryogenesis, and factors essential for cell-specific differentiation like *Pit1* in prolactin/growth hormone/thyrotrophin (PRL/GH/TSH)-secreting cells and Tpit/Tbx19 in corticotrophs respectively (Dasen & Rosenfeld 2001, Lamolet *et al.* 2001). The possible role of bHLH proteins has been progressively emerging during the last decade, essentially involving members of the proneural family NeuroD1/Math/neurogenins. NeuroD1 is currently viewed as a corticotroph-specific factor, which plays a dispensable role in corticotroph differentiation and collaborates with Pitx1 and Tpit to regulate proopiomelanocortin gene expression (Poulin *et al.* 1997, 2000, Lamolet *et al.* 2004). Though neurogenins (Ngn1/NeuroD3, Ngn2 and Ngn3) induce NeuroD1 expression in the nervous system/neural crest (Ngn1 and Ngn2; Ma *et al.* 1999) and in gastroenteropancreatic neuroendocrine cells (Ngn3; Huang *et al.* 2000), only Ngn2 has been observed in the developing pituitary yet, though apparently unrelated to corticotroph differentiation

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(Lamolet *et al.* 2004). Finally, while the human homologue of *Drosophila* achaete–scute ASH1 has been identified in the normal adult pituitary (Ferretti *et al.* 2003), there is recent evidence that the corresponding zebrafish homologue *ascl1* plays a fundamental role in pituitary development (Pogoda *et al.* 2006). Thus, there is increasing interest in elucidating the role of bHLH factors in pituitary development, function and diseases.

A body of literature has been dedicated to the possible role of bHLH proteins in tumorigenesis over the last 15 years (Semenza 2002, Ivanova *et al.* 2005, Kwok *et al.* 2005), but few data on proneural bHLH expression are available. NeuroD1, ASH1 and Ngn1/NeuroD3 can be expressed by primary neuroectodermal tumours, with Ngn1 expression associating with a metastatic potential in medulloblastomas (Rostomily *et al.* 1997). In contrast, overexpression of NeuroD1 or Ngn1 induces differentiation of neuroblastoma cells (Cho *et al.* 2001, Kim *et al.* 2002). In human pituitary tumours, NeuroD1 is expressed by corticotroph adenomas and also by most non-secreting adenomas (Oyama *et al.* 2001, Ferretti *et al.* 2003), with a possible overexpression in this latter group (Ferretti *et al.* 2003), suggesting a possible activation of some bHLH molecular pathway in these tumours. The expression of neurogenins in PA has not been studied yet.

The aim of this study was to look for neurogenins as putative members of the bHLH proteins identified in pituitary tumours (Jackson *et al.* 1993), to analyse possible neurogenins–NeuroD1 molecular pathways in pituitary tumour cells and to evaluate their possible prognostic value if present.

Material and Methods

Patients and samples

Surgical biopsies of 51 pituitary adenomas (PAs) were collected at the time of surgery, most of them in an *RNAlater* solution (Qiagen), and successively stored at −80 °C until processing. Four normal adult pituitary glands (seven fragments) were collected within 48-h post-mortem in patients who died from non-endocrine diseases and snap-frozen – in three cases, half pituitary was formalin-fixed and paraffin-embedded for histological purposes. The study was approved by the local ethical committee. PA included 22 clinically non-secreting (CNS) and 29 secreting adenomas (13 GH-, 7 PRL-, 7 adrenocorticotrophin (ACTH)- and 2 TSH-secreting adenomas respectively). Tumour volume and invasiveness were systematically recorded on the basis of neuroradiological criteria and intra-operative findings, and classified according to Wilson's criteria (Wilson *et al.* 1984). Most were macroadenomas ($n=47$), 30 were invasive and 11 were recurrent respectively. Among patients with secreting adenomas, 12 had received pre-operative pharmacological therapy consisting of somatostatin analogues ($n=8$, including six GH-secreting and two TSH-secreting PAs respectively) or dopamine agonist therapy ($n=3$, two PRL-secreting and one GH-secreting respectively). PAs were routinely characterised by immunohistochemistry for hormone secretion, using polyclonal anti-PRL, anti-GH, anti-follicle-stimulating hormone (anti-FSH), anti-luteinizing hormone (anti-LH), anti-ACTH antibodies (Orthodiagnostic Systems, Raritan, NJ, USA) and the streptavidin–biotin peroxidase method (Dako Cytomation, Milan, Italy). CNS adenomas were then classified into null cell ($n=14$), FSH/LH-secreting ($n=5$), and silent secreting adenomas ($n=2$, one silent ACTH-secreting and one silent GH-secreting adenomas respectively); immunohistochemical data were missing in one case. In most cases, the index of cell proliferation was evaluated by immunohistochemical determination of the Ki-67 antigen using the MIB-1 monoclonal antibody (DBA Italia SpA, Milan, Italy), as previously described (Jaffrain-Rea *et al.* 2002). A dopamine agonist-treated PRL-secreting pituitary carcinoma and its dural metastasis, two additional untreated GH-secreting adenomas and five adamantinomatous craniopharyngiomas were available for immunohistochemistry only.

RT-PCR analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen s.p.A) according to the manufacturer's instructions. In order to remove any contamination by genomic DNA, DNase treatment was performed as an additional step before RNA elution and repeated before each retrotranscription step. RNA quality was equally preserved in snap-frozen and RNA*later*-treated samples. First-strand cDNA synthesis was performed using 1 μg total RNA in the presence of 50 U MuLV reverse transcriptase and random hexamers (Applied Biosystems, distributed by Applera Italia s.r.l, Monza, Italy). Briefly, RT-PCR amplification of target genes was performed using 150 ng equivalent RNA, in the presence of 0·5 pM specific sense and antisense primers, 0·3 mM dNTPs, 1·5 mM $MgCl_2$ and 2·5 U Gold Taq polymerase (Applied Biosystems) in a final volume of 50 μl, for a total of 35 cycles for pituitary factors and up to 45 cycles for bHLH factors respectively. Dimethyl sulfoxide (DMSO; 5%) was added to the reaction mix for RT-PCR amplification of the *Ngn1* gene. The pituitary factors Pit-1 and Tpit were used as cell-specific markers in order to exclude significant contamination of tumour samples by normal pituitary tissue. Primers for NeuroD1 and Pit-1 have been synthesised according to the previously reported sequences (Pellegrini *et al.* 1994, Ferretti *et al.* 2003 respectively), whereas primers specific for Ngn1, Ngn2 and Ngn3, and Tpit have been designed on the relative Genbank sequences (Table 1).

Real-time PCR analysis

The expression of bHLH transcripts was further studied by quantitative real-time RT-PCR (qRT-PCR) based on a Taqman methodology, using an ABI Prism 7700 Sequence Detection System (Applied biosystems, Applera Italia, Monza, Italy), and compared with β-actin expression. Ready-to-use gene expression assays were purchased from Applied Biosystems,

Table 1 Primer sequences used for conventional RT-PCR analysis

Gene; Genbank access	Primers	Oligonucleotide sequence	Annealing (T °C)	PCR fragment length (bp)
Tpit	Forward	AGA ATG GCA GAC GGA TGT T	54	670
NM_005149	Reverse	GTC CTC GGA GAC CCG AAT		
Ngn1	Forward	CTT GAG ACC TGC ATC TCC GAC	62	678
BC028226	Reverse	GCG TTG TGT GGA GCA AGT CTT		
Ngn2	Forward	GGT CTG GTA CAC GAT TGC AAA C	60	416
AF303002	Reverse	GCT GTT GGT GCA ACT CCA CGT		
Ngn3	Forward	TCC AAG TGA CCC GTG AGA CG	57	417
AJ133776	Reverse	TCC AGC GCG TAC AAG CTGT		

with the following identification numbers: Hs_00159598 (NeuroD1), Hs_00246211 (Ngn1), Hs_00702774 (Ngn2), Hs_01875204 (Ngn3) and Hs_99999903 (β-actin). Each reaction was performed in a final volume of 20 µl with 80 ng equivalent RNA, 1 µl primers/fluorescent oligoprobe mix and 10 µl UNG-universal mastermix (Applied Biosystems); for each bHLH factor assay, a β-actin assay was run on the same batch of cDNA. The thermal cycling conditions included 2 min at 50 °C, 10 min of denaturation at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed at least in duplicate.

Immunohistochemistry

Tissue sections were dewaxed in xylene and rehydrated through a descending ethanol series. Antigen retrieval was obtained by microwave boiling in citrate buffer (pH 6·0) for 5, 5 and 3 min. Rabbit polyclonal antibodies directed against Ngn2 and Ngn3 were purchased from Chemicon (distributed by DBA Italia s.r.l, Segrate, Italy). Immunohistochemistry was performed using Ngn2 and Ngn3 at a dilution of 1:200 and 1:50 respectively, with a multilink biotinylated antibody and the avidin–biotin peroxidase system according to manufacturer's instructions ($LSAB^+$ kit, Dako Cytomation), introducing a blocking step for endogenous avidin and biotin activity. Co-localisation studies for Ngn2 and pituitary hormones were performed using pre-diluted mouse monoclonal antibodies for pituitary hormones (Dako Cytomation). For this purpose, a double step immuno-histochemical study using the $LSAB^+$ kit (Marx *et al.* 1999) was designed to first detect cytoplasmic hormone using DAB as a chromogen, followed by a second step for the detection of nuclear Ngn2/3 using Novored as a chromogen (Vector, DBA Italia s.r.l). Blocking of endogenous peroxidase and avidin–biotin activities were performed at each step.

Statistical analysis

All data are expressed in mean ± S.E.M., and statistical analyses were performed using a Statview 5.01 software (SAS Institute, Cary, NC, USA) for PC. For clarity of exposition, PRL-, GH- and TSH-secreting adenomas, which show many similarities in bHLH expression profile, were grouped together as Pit-1 dependent, by opposition to the Pit-1-independent CNS and corticotroph PA. Because of the non-normal distribution observed for most parameters, non-parametric tests were used to compare or correlate continuous values (Mann–Whitney and Spearman tests respectively). Distribution of nominal values was compared by the χ^2 test. The level of significance was set at $P<0{\cdot}05$.

Results

bHLH *gene expression in the normal pituitary gland and in PAs*

General results Table 2 shows *bHLH* gene expression in normal pituitary (NP) samples and in PA according to RT-PCR and qRT-PCR respectively, and examples of RT-PCR experiments are shown in Fig. 1. Differences between RT-PCR and qRT-PCR results reflect the higher sensitivity

Table 2 Expression of NeuroD1 and neurogenin (Ngn) transcripts in the normal human pituitary and in pituitary adenomas

	NeuroD1		Ngn1		Ngn2		Ngn3	
	RT-PCR	qRT-PCR	RT-PCR	qRT-PCR	RT-PCR	qRT-PCR	RT-PCR	qRT-PCR
Normal pituitaries	7/7 (100%)	7/7 (100%)	0/7 (0%)	1/7 (14·0%)	4/7 (57·1%)	7/7 (100%)	1/7 (14·0%)	4/7 (57·1%)
Pituitary adenomas	41/51 (80·4%)	43/51 (84·3%)	2/44 (4·5%)	4/44 (9·1%)	21/51 (41·2%)	40/51 (78·4%)	11/51 (21·6%)	14/46 (30·4%)

RT-PCR, conventional RT-PCR; qRT-PCR, quantitative real-time RT-PCR.

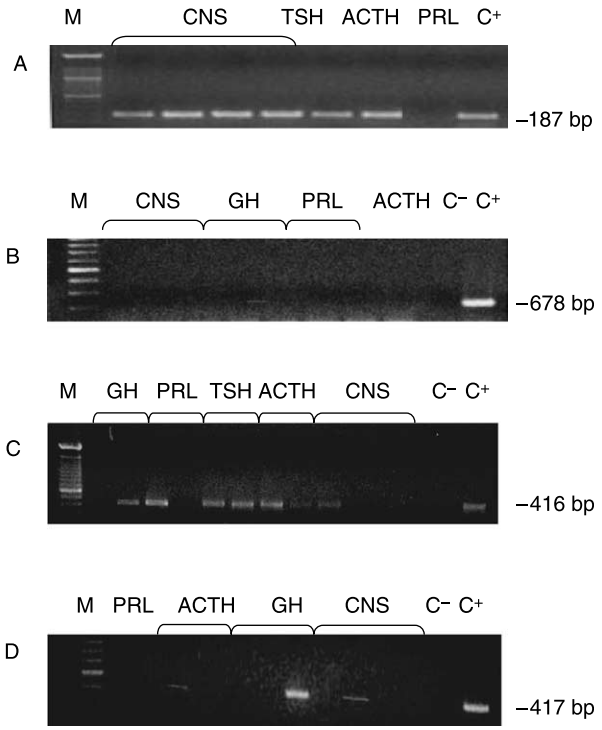

Figure 1 RT-PCR for bHLH transcription factors in pituitary adenomas. Shown are representative examples of RT-PCR for NeuroD1 (A), Ngn1 (B), Ngn2 (C) and Ngn3 (D) respectively. Data were obtained after 45 cycles of amplification and electrophoresis on a 1·8% agarose gel. M, molecular weight marker; C^-, negative controls; C^+, positive controls; the phenotype of pituitary adenoma samples is indicated for each experiment.

of the latter methodology in samples expressing low transcript levels. Overall, NeuroD1 was observed in all NP and up to 84·3% of PA respectively. Ngn2 was the most commonly expressed member of the Ngn family, being present in up to 4/6 NP and 76·5% of PA respectively, whereas Ngn3 and Ngn1 were detected in up to 30·4 and 9·1% of PA respectively.

A preliminary study of β-actin transcripts was performed before the analysis of qRT-PCR data, indicating similar C_T levels in NP and PA (22·15 ± 0·30 vs 22·70 ± 0·40 respectively, $P=0·86$). PA tended to overexpress NeuroD1 and Ngn2 as compared with NP, though this was not statistically significant, due to the marked variability observed in PA ($P=0·25$ for NeuroD1 and $P=0·35$ for Ngn2 respectively). Overall, Ngn2 and NeuroD1 transcripts were inversely correlated ($P=0·015$); accordingly, NeuroD1 expression was lower in Ngn2-expressing PA ($P=0·023$). Where present, Ngn3 and Ngn1 were expressed at very low levels – 100- to 1000-fold less than NeuroD1 or Ngn2.

Analysis of qRT-PCR data according to PA phenotype Analysis of individual levels of NeuroD1, Ngn2 and Ngn3 transcripts revealed a differential expression of *bHLH* genes according to PA phenotype (Fig. 2). Overexpression of NeuroD1 could be observed in CNS and corticotroph PA, with very low levels of expression in Pit-1-dependent PA where detected. Accordingly, NeuroD1 expression was significantly higher in Pit-1-independent than in Pit-1-dependent PA ($P=0·001$), especially when considering CNS adenomas only ($P=0·0008$ versus Pit-1-dependent PA). In contrast, overexpression of Ngn2 could be observed in Pit-1-dependent and corticotroph PA, with very low levels in CNS adenomas where detected. Accordingly, Ngn2 expression was significantly higher in secreting than in CNS PA ($P=0·007$), especially when considering Pit-1-dependent PA only ($P=0·0006$ versus CNS). Among CNS, only 1/5 gonadotroph adenoma expressed detectable levels of Ngn2. The Ngn2/NeuroD1 ratio was ≥ 1 in most Pit-1-dependent but only in a minority of Pit-1-independent PA expressing both factors (11/15 Pit-1 dependent, 1/11 CNS and 1/7 ACTH-secreting respectively, $\chi^2=13·3$, $P=0·001$).

Ngn3 tended to be preferentially expressed by secreting compared with CNS adenomas ($P=0·09$), especially by Pit-1-dependent PA ($P=0·02$ versus CNS adenomas), but overexpression was exceptional. In all cases, the Ngn3/NeuroD1 ratio was remarkably low ($<0·01$). Very low levels of Ngn1 were detected in three secreting and one non-secreting PA respectively.

Analysis of qRT-PCR data according to other tumour characteristics Overall, no relationship was found between bHLH expression and any parameter of tumour aggressiveness such as tumour volume, invasiveness or Ki-67 index of cell proliferation (data not shown). Because of the differential expression of *bHLH* genes, CNS and Pit-1-dependent PA were then analysed as distinct subgroups. In CNS adenomas, NeuroD1 expression tended to be higher in invasive tumours ($P=0·058$ versus non-invasive), but no significant variation in either NeuroD1 or Ngn2 expression was found according to tumour volume or Ki-67 index (data not shown). In Pit-1-dependent PA, Ngn2 expression was unrelated to tumour volume or invasiveness, but tended to be lower in tumours expressing a Ki-67 index $\geq 3\%$ ($P=0·12$) and was significantly higher in those which received pre-operative pharmacological treatment compared with those which did not ($P=0·025$). Despite the limited number of samples, similar trends were observed for prolactinomas treated with dopamine agonists and for somatotroph adenomas treated with somatostatin analogues compared with their untreated counterparts ($P=0·07$ and $P=0·12$ respectively). Ngn1 was expressed by two highly invasive and recurrent PA (one ACTH and one null cell respectively), and by two intrasellar-secreting adenomas treated with somatostatin analogues pre-operatively (one GH-secreting and one TSH-secreting respectively).

Expression of neurogenin proteins in the normal pituitary and in pituitary tumours Where present, Ngn2 immunopositivity was clearly restricted to the nucleus.

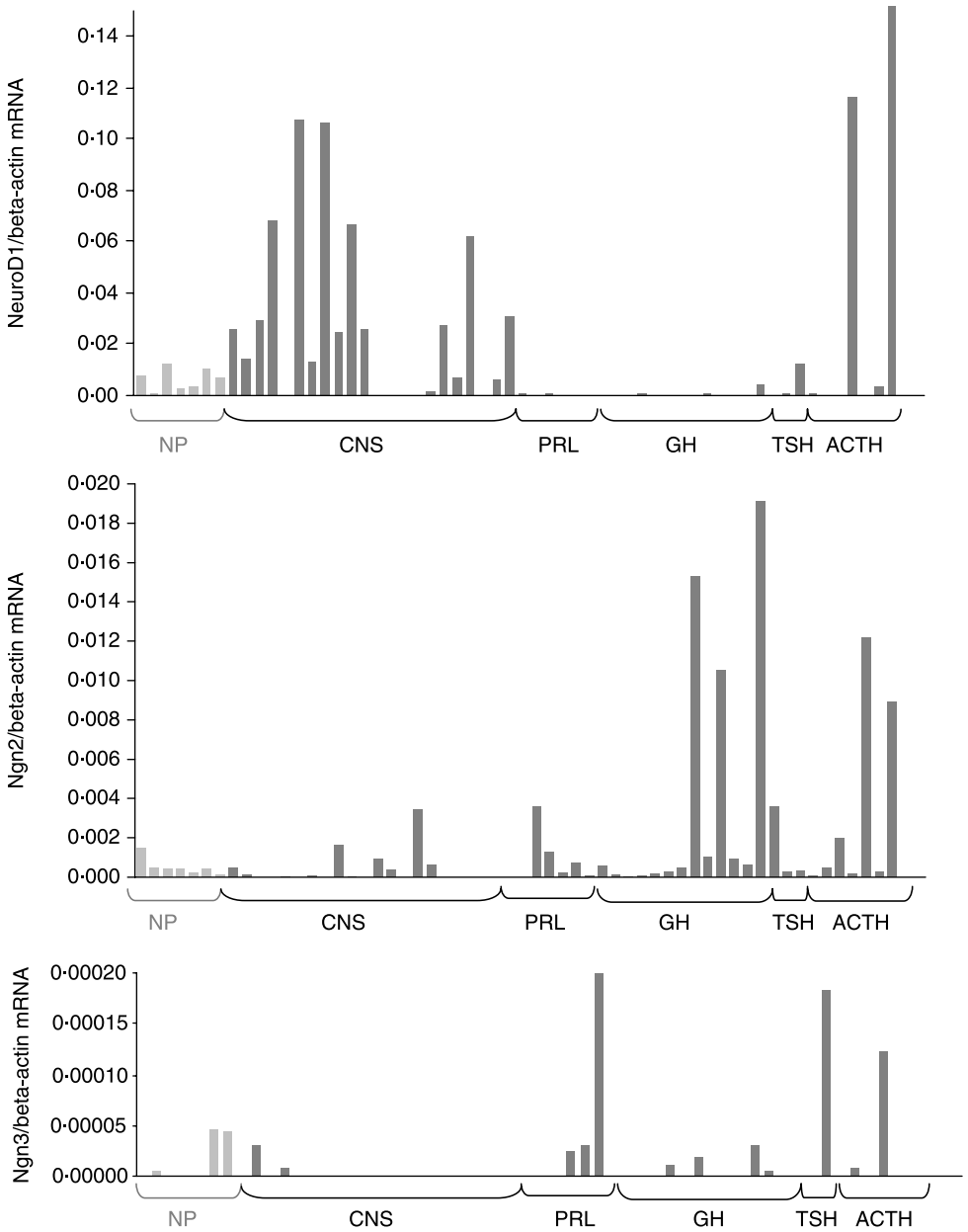

Figure 2 NeuroD1, Ngn2 and Ngn3 expression in the normal pituitary (NP) and in pituitary adenomas (PAs) according to qRT-PCR. Shown are individual levels of each factor observed in NP and in PA classified according to functional phenotype. Note the different scale used to show the very low levels of Ngn3 expression.

Data obtained in the NP are shown in Fig. 3. Ngn2 was observed in scattered cells of the adenohypophysis, in most cells of the pars intermedia, but not in the neurohypophysis. Co-localisation experiments showed Ngn2 expression in most secreting cells, with a predominance of corticotrophs and somatotrophs and the possible exception of gonadotrophs where no clear positivity could be documented. Ngn2 immunostaining was stronger in most pituitary tumours, including PA, the pituitary carcinoma and some craniopharyngiomas (Fig. 4A–E). Data obtained in 29 PA are summarised in Table 3. Ngn2 expression was diffuse in all ACTH- and in most GH- and PRL-secreting PA and focal in the two TSH-secreting and in most CNS PA where present. In somatotroph adenomas, Ngn2 immunopositivity was stronger in treated than in untreated cases. The pituitary PRL-secreting carcinoma and the corresponding dural

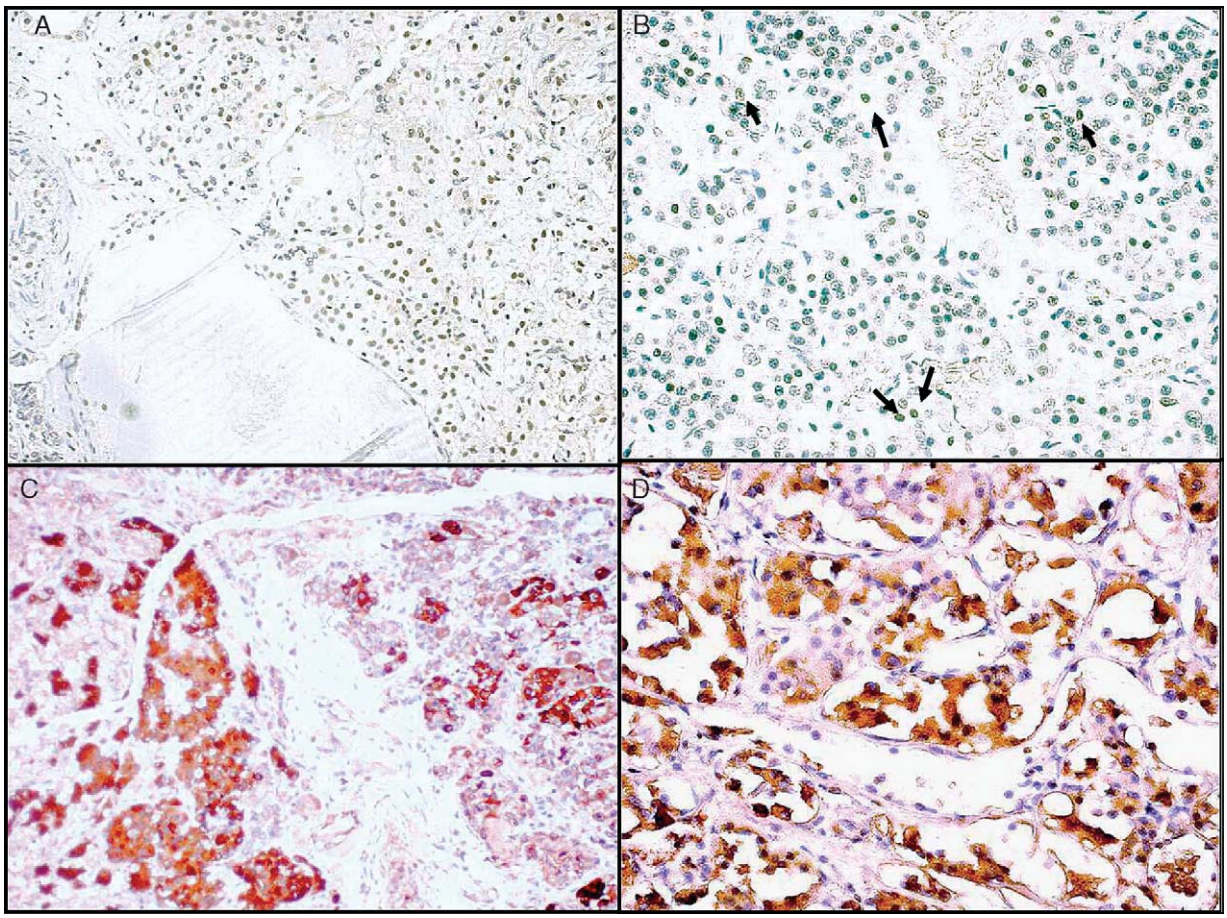

Figure 3 Expression of Ngn2 protein in the normal human pituitary. (A) A number of Ngn2+ nuclei are observed in the pars intermedia; (B) scattered nuclei with moderate staining for Ngn2 are observed in the anterior pituitary (examples are indicated by an arrow); (C) nuclear Ngn2 staining in ACTH cells (from left to right: pars intermedia, anterior pituitary); (D) nuclear Ngn2 staining in some GH-secreting cells. Magnification 20× (A and B) and 40× (C and D).

metastasis showed diffuse Ngn2 expression. Ngn2 immunostaining was also observed in the epithelial cells of craniopharyngiomas.

No nuclear Ngn3 protein could be detected in the NP, and a moderate positivity was observed in two treated GH-secreting out of the 11 PAs studied by immunohistochemistry (Fig. 4F), despite 10/11 expressed Ngn3 transcripts (two ACTH-, three CNS, three GH-, one PRL- and one TSH-secreting PAs respectively). The weak cytoplasmic positivity observed in most NP and PA was considered as likely artefactual, since it was present regardless of Ngn3 transcripts. The PRL-secreting PA and the corresponding dural metastasis were negative for Ngn3 immunostaining.

Discussion

This study shows for the first time that members of the neurogenin family can be expressed by normal and tumourous pituitaries, with a clear predominance of Ngn2. While extending our previous findings in such tissues (Ferretti *et al.* 2003), it reveals an unexpected inverse relationship between Ngn2 and NeuroD1 in PA, reflecting a differential expression according to tumour phenotype.

The NeuroD1 expression profile observed in PA is consistent with previous reports (Oyama *et al.* 2001, Ferretti *et al.* 2003) and additional considerations can be made at the light of qRT-PCR data. Overexpression of NeuroD1 was more frequent in CNS than in corticotroph adenomas. As a corticotroph marker, a consistent overexpression could be expected in this latter subgroup, suggesting some degree of NeuroD1 downregulation in corticotroph adenomas, as reported in a neuroblastoma cell line (Chae *et al.* 2004). In CNS adenomas, the trend toward a higher expression of NeuroD1 in invasive tumours may be indicative of a developmental expression preceding corticotroph differentiation, with tumoural reactivation as a result of a relative dedifferentiation process (Ferretti *et al.* 2003). Alternatively, extracellular signals may induce NeuroD1 in these tumours.

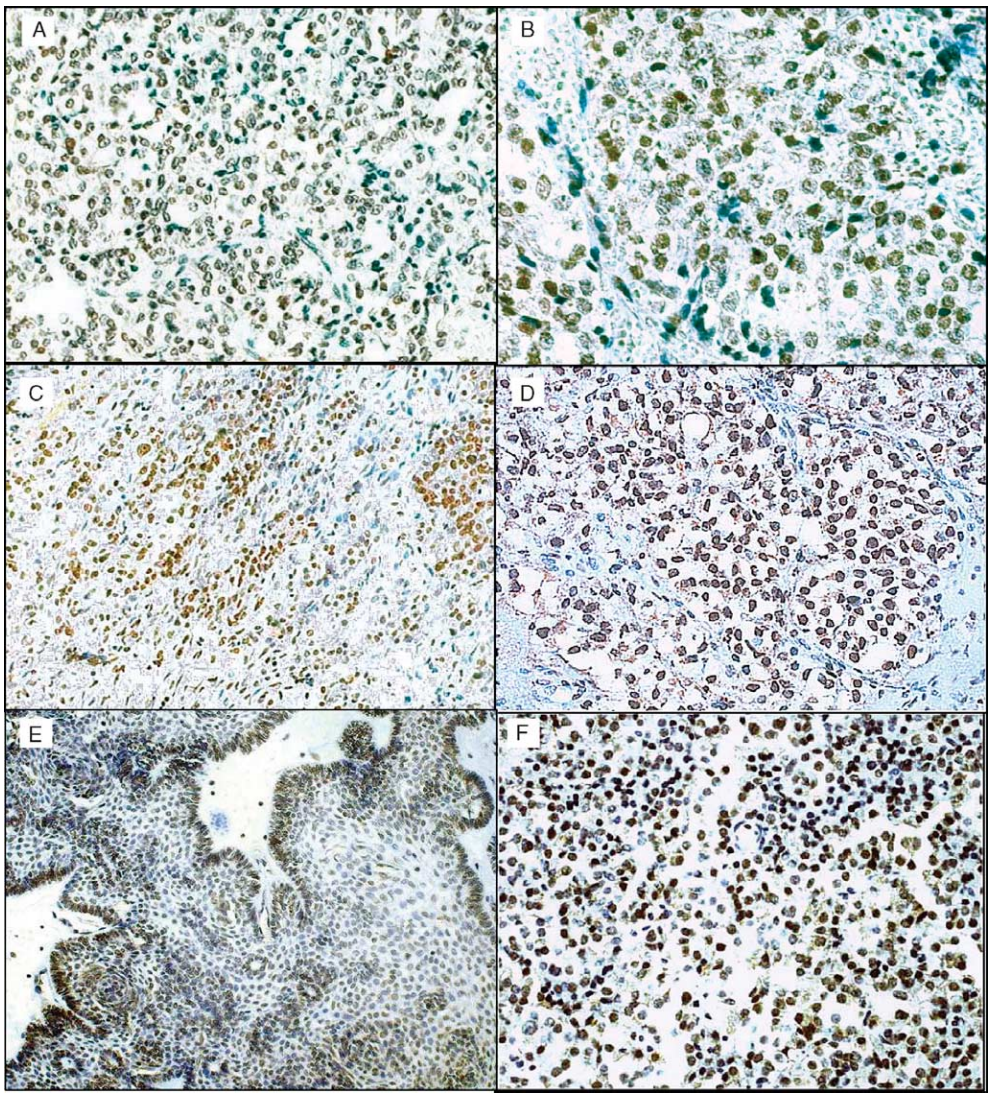

Figure 4 Expression of Ngn proteins in human pituitary tumours. (A–E) Nuclear immunostaining for Ngn2 (A) a GH-secreting, (B) an ACTH-secreting and (C) a TSH-secreting pituitary adenomas respectively, in a dural metastasis from a PRL-secreting pituitary carcinoma (D) and in an adamantinous craniopharyngioma (E). (F) Immunostaining for Ngn3 in a GH-secreting adenoma. Magnification 20× (A, C and E) and 40× (B, D and F).

Finally, the low levels of NeuroD1 expression observed in Pit-1-dependent PA can explain previously discordant reports, based on differently sensitive methods (Oyama *et al.* 2001, Ferretti *et al.* 2003).

Ngn2 was detected in the NP, with a frequent over-expression in PA but significant variations according to tumour phenotype. Similar data were found at a protein level, with a stronger immunostaining in most PA and a nuclear localisation suggestive of biological activity. Ngn2 was expressed by corticotrophs of the pars intermedia and scattered cells of the adenohypophysis. Though no restricted phenotype specificity was observed, nuclear staining was more evident in somatotrophs and corticotrophs but unclear in gonadotrophs. A preferential expression of Ngn2 by secreting PA was found at both transcriptional and protein levels, with a diffuse immunopositivity in corticotrophinomas, somatotrophinomas and prolactinomas. Ngn2 immunostaining was focal in TSH-secreting adenomas and in some CNS adenomas, suggesting expression by a subset of adenomatous cells. This raises the issue of the possible role of Ngn2 in the pituitary. During mice ontogenesis, Ngn2 appears at the end of Rathke's pouch differentiation and turns off a few days later, and seems unrelated to corticotroph differentiation (Lamolet *et al.* 2004). The expression of Ngn2 by the normal adult pituitary is suggestive of a dual effect during ontogenesis, once as a precocious factor – data

Table 3 Immunohistochemical detection of neurogenin 2 (Ngn2) in pituitary adenomas

							IHC‡	
	Age/sex	**Clinical presentation***	**Adenoma†**	**Hormone**	**Ki-67%**	**Ngn2 mRNA§**	Positivity	Staining
Case								
1	44/F	Cushing	M	ACTH	1·0	+	++	+/++
2	58/F	Cushing	m	ACTH	nd	+	++	+/++
3	61/F	Cushing	m	ACTH	1·0	+	++	+
4	26/F	Cushing	M-inv-ESS	ACTH	5·0	+	++	+
5	49/F	Cushing	M-SSE	ACTH	3·0	+	++	+
6	66/F	CNS-subclinical hypercortisolism	M-inv-SSE	ACTH-silent	10·0	++	+++	+/++
7	51/F	CNS	M-inv-SSE	FSH/LH	0·5	+	Focal (+)	+/−
8	68/F	CNS	M-inv (R)	Null cell	0·5	−	−	−
9	60/M	CNS	M-SSE	Null cell	0·0	+	−	−
10	67/M	CNS	M-SSE	Null cell	nd	+	Focal (+)	+/−
11	16/M	CNS	Giant	Null cell	4·0	++	Focal (++)	+
12	64/M	CNS	Giant (R)	Null cell	0·5	+	−	−
13	38/M	CNS	M-SSE	Null cell	3·0	+	Focal (+)	+/++
14	57/M	CNS	M-inv-SSE	Null cell	1·0	+	+	+
15	17/F	CNS	M-SSE	GH-silent	3·0	++	++	+/++
16	33/F	Acromegaly^{T}	M-inv	GH	1·0	+	+++	++
17	39/F	Acromegaly^{T}	M	GH/PRL	nd	+	++	+/++
18	61/F	Acromegaly^{T}	m	GH/multi H	0·0	+	+++	+/++
19	53/F	Acromegaly^{T}	m	GH	*nd*	++	+++	++
20	26/F	Acromegaly	M-inv-SSE	GH/PRL	6·0	+	+	+
21	43/M	Acromegaly	M	GH/PRL	3·0	+	+	+
22	30/M	Acromegaly	M-SSE	GH	8·0	nd	+	+
23	12/M	Gigantism	M	GH/PRL	15·0	nd	+/−	+
24	48/M	Prolactinoma	M-inv-SSE	PRL	nd	+	+/−	+/−
25	58/F	Prolactinoma^{T}	M-inv	PRL	1·0	++	+	+
26	45/F	Prolactinoma	Giant	PRL	7·0	+	++	+/−
27	43/F	Prolactinoma^{T}	M-inv-SSE	PRL	5·0	+	+	+/−
28	48/M	Hyperthyroidism^{T}	M	TSH	0·5	+	Focal (+++)	++
29	28/F	Previous hyperthyroidism (thyroidectomy)^{T}	M	TSH/multiH	0·5	++	Focal (+++)	++

*T, pre-operative pharmacological treatment; †m, microadenoma; M, macroadenoma; inv, invasive; SSE, suprasellar extension; (R), recurrent; nd, not done; ‡according to qRT-PCR; data are quoted as follows: −, negative; +, in the normal pituitary range (mean ±2 S.D.); ++, overexpression (>2 S.D.); §IHC was quoted as follows: positivity, +/− <10%; +10–30%; ++30–60%; +++ >60% of adenoma cells; and intensity, +/− faint; +moderate; ++ intense respectively.

obtained in craniopharyngiomas arising from Rathke's pouch may support this hypothesis – and thereafter during final cell differentiation, possibly contributing to maintain a differentiated secreting phenotype. Other precocious developmental factors – *Pitx* factors, *Hesx*1, PROP-1, have already been observed in normal adult and adenomatous pituitaries (Nakamura *et al.* 1999, Pellegrini-Bouiller *et al.* 1999, Mantovani *et al.* 2006). The preferential expression of Ngn2 in secreting PA appears to be related to tumour phenotype rather than to tumorigenesis itself for a series of reasons: (1) Ngn2 expression is unrelated to tumour volume or aggressiveness; (2) its lower expression in CNS adenomas may reflect the common gonadotroph origin of these tumours (Gittoes 1998) – indeed – the lowest expression was found in gonadotroph adenomas; (3) the highest levels of Ngn2 in CNS were observed in two silent secreting CNS and (4) the higher expression of Ngn2 in Pit-1-dependent PA which received pre-operative pharmacological therapy suggests that the control of mammosomatotroph cell proliferation is associated with Ngn2 expression–accordingly, Ngn2 expression tended to be lower in Pit-1-dependent PA expressing a high Ki-67 index. This point would deserve further investigation.

Ngn3 transcripts were observed at very low levels in some NP samples and in about 30% of PA, with a preferential expression in secreting PA. Nuclear Ngn3 protein was found in a minority of GH-secreting PA. Thus, though all but one Ngn3-positive adenoma also expressed NeuroD1, the Ngn3–NeuroD1 pathway does not seem to play a significant role in pituitary tumours. Its possible role in pituitary ontogenesis is even more incertain, since no Ngn3 protein was observed in the developing mouse pituitary (Lamolet *et al.* 2004). At the moment, neuroendocrine cells clearly dependent on Ngn3 for NeuroD1 induction during development are limited to

the gastroenteropancreatic tract, which is of endodermal origin (Gradwohl *et al.* 2000). However, no overexpression of Ngn3/NeuroD1 has been observed in gastrointestinal carcinoid tumours (Nakakura *et al.* 2005).

Very low levels of Ngn1 transcripts were detected in $<10\%$ of normal and PA samples. Ngn1 has a preferential neural expression (Cau *et al.* 2002, Ross *et al.* 2003) and no Ngn1 protein was observed in the normal developing mouse (Lamolet *et al.* 2004). Thus, despite NeuroD1 was also expressed by Ngn1-expressing adenomas, the Ngn1–NeuroD1 pathway in neuroendocrine cells appears to be limited to a subset of neural crest-derived cells (Greenwood *et al.* 1999, Ma *et al.* 1999) and is unlikely to play a significant role in the pituitary.

In conclusion, Ngn2 is normally expressed in the human adult pituitary and can be overexpressed by secreting PA. Ngn2 is unlikely to account for NeuroD1 overexpression, which is prevalently observed in non-secreting PA. Though we cannot exclude downregulation of Ngn2 by NeuroD1 in this latter case, it is likely that both factors are differentially regulated in pituitary tumour cells. Possible links between Ngn2 expression and the control of cell proliferation by pharmacological treatment of secreting PA would deserve further investigation.

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