Expression of guanylyl cyclase-B (GC-B/*NPR2*) receptors in normal human fetal pituitaries and human pituitary adenomas implicates a role for C-type natriuretic peptide

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Abstract

C-type natriuretic peptide (CNP/*Nppc*) is expressed at high levels in the anterior pituitary of rats and mice and activates guanylyl cyclase B receptors (GC-B/*Npr2*) to regulate hormone secretion. Mutations in *NPR2/Npr2* can cause achondroplasia, GH deficiency, and female infertility, yet the normal expression profile within the anterior pituitary remains to be established in humans. The current study examined the expression profile and transcriptional regulation of *NPR2* and GC-B protein in normal human fetal pituitaries, normal adult pituitaries, and human pituitary adenomas using RT-PCR and immunohistochemistry. Transcriptional regulation of human *NPR2* promoter constructs was characterized in anterior pituitary cell lines of gonadotroph, somatolactotroph, and corticotroph origin. *NPR2* was detected in all human fetal and adult pituitary samples regardless of age or sex, as well as in all adenoma samples examined regardless of tumor origin. GC-B immunoreactivity was variable in normal pituitary, gonadotrophinomas, and somatotrophinomas. Maximal transcriptional regulation of the *NPR2* promoter mapped to a region within −214 bp upstream of the start site in all anterior pituitary cell lines examined. Electrophoretic mobility shift assays revealed that this region contains Sp1/Sp3 response elements. These data are the first to show *NPR2* expression in normal human fetal and adult pituitaries and adenomatous pituitary tissue and suggest a role for these receptors in both pituitary development and oncogenesis, introducing a new target to manipulate these processes in pituitary adenomas.

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Introduction

The particulate guanylyl cyclase (GC) receptors mediate the effects of natriuretic peptides, heat-stable enterotoxins, odor, and peptidase activity in a broad range of tissues by increasing the levels of cyclic guanosine $3',5'$-monophosphate (cGMP). GC-A and GC-B are probably the best characterized receptors in this family and act as specific receptors for atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) respectively. GC-B is expressed throughout the body but is present in many endocrine tissues and is widely expressed throughout the CNS (for review see Fowkes & McArdle (2000) and Potter *et al*. (2006)). Mice lacking the *Npr2* gene, which encodes GC-B, are dwarf due to severe deficiency in the process of endochondral ossification and exhibit early lethality (Tamura *et al*. 2004). Intriguingly, these mice are also GH deficient, implicating a potential pituitary phenotype. Mutations in the gene (natriuretic peptide precursor C (*Nppc*)) encoding CNP essentially phenocopy those in *Npr2* (Chusho *et al*. 2001), but to date, no mutations have been reported in the human *NPPC* gene. Instead, translocations have been shown to cause overexpression of *NPPC*, resulting in skeletal overgrowth and deformities (Bocciardi *et al*. 2007, Moncla *et al*. 2007). However, the role of GC-B receptors, or of CNP, in the anterior pituitary remains poorly understood.

Our previous molecular and pharmacological studies performed on well-established rodent anterior pituitary cell lines (Fowkes *et al*. 1999, 2000, Thompson *et al*. 2009) have shown that GC-B signaling is the major pathway used by CNP in most anterior pituitary cell types. Despite this, there is a paucity of data describing the expression of GC-B in human pituitaries and pituitary adenomas. In the current study, we have examined the mRNA expression of *NPR2* in a range of human pituitary adenomas and normal human fetal and adult pituitaries, determined the transcriptional regulation of the human GC-B promoter in anterior pituitary cell lines, and established the localization of GC-B protein expression by immunohistochemistry in a range of human pituitary adenomas.

Materials and methods

Cell culture

αT3-1, LβT2, GH3, and AtT-20 cells were cultured as described previously (Fowkes *et al*. 2000, Thompson *et al*. 2009) using DMEM (Sigma–Aldrich) containing 10% FCS, penicillin, and streptomycin.

Mouse primary pituitary cell culture

Pituitaries were removed from 8-week-old random cycling female C57/BL6 mice (20 per experiment) and subjected to enzymatic dispersion as described previously (McArdle & Poch 1992, Thompson *et al*. 2009). Dispersed cells were then cultured overnight in 24-well plates before transfection with the indicated *NPR2* reporter gene constructs.

RNA extraction from human pituitary tumors and fetal pituitaries

Human pituitary adenomas were obtained from the Thomas Willis Brain Bank (project ethics reference 07/H0606/85), Department of Neuropathology, John Radcliffe Hospital, Oxford, UK. Tissue was collected immediately at the postoperative stage, snap frozen, and stored at $-80\,°C$ before extracting total RNA using the Trizol method followed by RNA cleanup and DNase treatment, as described previously (Thompson *et al*. 2009). Normal human fetal pituitary RNA samples were obtained from fetuses collected following medical termination of pregnancy (between 14 and 18 weeks of gestation), subject to local ethical approval (by the Lothian Paediatrics/Reproductive Medicine Research Ethics Subcommittee) as described previously (Pope *et al*. 2006). Normal pituitary samples from patients were obtained by autopsy within 6 h of death (University Clinical Centre, Belgrade, Serbia), as described previously (Butz *et al*. 2010). These individuals had no evidence of any endocrine disease. All biometric data relating to human samples are summarized in Tables 1, 2 and 3.

RT-PCR analyses of NPR2 and NPPC expression in human pituitary tissue

Following generation of the first-strand cDNA (Applied Biosystems, Paisley, UK), PCR was performed using components from the Qiagen *Taq* Core kit and Thermo-Start DNA polymerase and buffer kit. The following primer sets were used: *NPR2*: F, AACGGGCGCA-TTGTGTATATCTGCGGC and R, TTATCACAG-GATGGGTCGTCCAAGTCA; *NR5A1*: F, GCCAGG-AGTTCGTCTGTCTC and R, TCAATGAGAA-GGTTGTTGCG; *POU1F1*: F, TGGAGTGATGG-CAGGCAGTTTAA and R, TTTCACCGGTTTTTC-TCTCTGCCT; and *NPPC*: F, CTGCTCACGC-TGCTCTCC and R, CCTTCTTGTTGGCTCCTTTG. All PCR was performed between 30 and 35 cycles before analysis of products using 1.6% (w/v) agarose gel electrophoresis.

Table 1 Biometric data and adenoma diagnosis (by IHC) of archived patient samples

Tumor	Age	Sex	Diagnosis (IHC)
1	49	M	Gonadotroph
2	70	M	Gonadotroph
3	49	M	Gonadotroph
4	66	M	Gonadotroph
5	71	M	Gonadotroph
6	74	M	Gonadotroph
7	68	M	Gonadotroph
8	31	M	Gonadotroph
9	60	F	Gonadotroph
10	68	F	Gonadotroph
11	65	F	Gonadotroph
12	65	F	Somatotroph/lactotroph
13	26	F	Somatotroph
14	36	F	Somatotroph/lactotroph
15	58	F	Somatotroph
16	51	M	Somatotroph
17	39	F	Null
18	45	M	Null
19	67	F	Corticotroph
20	48	M	Corticotroph

Immunohistochemistry for GC-B

Human gonadotroph and somatotroph adenoma paraffin-fixed samples were sectioned to 4 μm for analysis. Paraffin embedding was removed with Histoclear before subjecting the sections to antigen retrieval and immuno-histochemistry for GC-B using a rabbit polyclonal antibody (Abcam, Cambridge, UK) according to the standard protocols described previously (Suliman *et al.* 2007). All slides were immersed in hematoxylin counterstain, washed, dehydrated, and then mounted on distrene-plasticizer-xylene before examination using a Leica microscope.

Bioinformatic analyses of transcription factor response elements in the human NPR2 promoter

The human *NPR2* promoter sequence (accession number AY528561) was analyzed using two online databases, PATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) and WWW Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/index.html).

Plasmids and transient transfection studies

The reporter constructs encoding the human *NPR2* promoter have been described previously (Rahmutula *et al.* 2004). All constructs were verified for orientation and correct sequence by restriction endonuclease digests. Large-scale preparation and purification of plasmids was performed by alkaline lysis and resin purification (Qiagen Ltd). For transfections, 3×10^5 cells/well were plated in 24-well plates and transfected using Lipofectin (Invitrogen) with 1.25 μg/well of each reporter construct along with 0.5 μg/well BosGal, and protein extracts were assayed for reporter gene activity as described previously (Thompson *et al.* 2009). The data shown were normalized for protein content using a Nanodrop (ND1000, Thermo Scientific, Loughborough, UK).

Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear protein extracts were prepared from 1×10^6 GH3 or LβT2 cells using a modification of a method described previously (Schreiber *et al.* 1989, Thompson *et al.* 2009). Probes were created by filling in the $5'$-AGCT overhangs of the annealed *NPR2* oligo-nucleotides with Klenow polymerase, and EMSAs were performed as described previously (Thompson *et al.* 2009). The following primer sets were used: *NPR2-PROX*: F, *AGCT*CAGCGGGGGAGGGGCGG-GGGCGGGCGGCCT and R, *AGCT*AGGCCGCCCG-CCCCCGCCCCTCCCCCGCTG; *NPR2-MID*: F, *AG-CT*CGCCGGAGGGTGGGGCCGGGA and R, *AG-CT*TCCCGGCCCCACCCTCCGGCG; *NPR2-DIST*: F, *AGCT*GCGCCCGGGTGGGGGGTGGGG and R, *AGCT*CCCCACCCCCCACCCGGGCGC.

For gel shifts, 2 μl Sp1 or Sp3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the reaction to identify the components of the specific protein complexes, and these samples were incubated on ice for 60 min. The reactions were then incubated for 15 min at 30 °C in the presence of 1 ng probe. Complexes were electrophoresed on a 5% native acrylamide gel, dried, and visualized by autoradiography.

Statistical analysis and data presentation

For reporter gene activities in pituitary cell lines, the data shown were normalized and pooled to represent the mean ± s.e.m. of at least four independent

Table 2 Biometric data of human fetal pituitary samples

Sample	Age at termination (weeks/days)	Sex
1	16w/3d	M
2	14w/4d	F
3	18w/6d	F
4	16w/6d	M
5	14w/6d	M
6	19w/4d	F
7	15w	F
8	19w/3d	F

Table 3 Biometric data of normal adult pituitary samples

Sample	Age	Sex	Cause of death
1	37	M	Road traffic accident
2	19	F	Road traffic accident
3	72	M	Myocardial infarction
4	68	M	Cerebral hemorrhage

experiments, each performed in triplicate. The reporter gene assays in primary mouse pituitary cells represent the mean $\pm$ S.E.M. of four individual transfections. Numerical data were subjected to ANOVA and were followed, where appropriate, by Bonferonni's multiple comparisons test, accepting $P < 0.05$ as significant, using inbuilt equations in GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

Expression of NPR2 and NPPC in normal human fetal and adult pituitaries

Having recently established that mouse pituitaries and gonadotroph cell lines of different developmental origin expressed most components of an intact natriuretic peptide system (Thompson *et al.* 2009), we initially examined a series of normal human fetal pituitary samples for the expression of *NPR2* and *NPPC* transcripts. RNA was extracted from eight normal human fetal pituitaries and four normal adult pituitaries. As shown in Fig. 1A, *NPR2* and *NPPC* transcripts were detected in all fetal pituitary samples, regardless of age or sex. Expression of *POU1F1* (encoding Pit-1) and *NR5A1* (encoding SF-1) was also detected in all fetal samples. Similarly, *NPR2* and *POU1F1* transcripts were also detected in all the normal adult pituitary samples (Fig. 1B). Interestingly, *NPPC* transcripts were detected in 3/4 normal pituitary samples, and *NR5A1* being detected in 2/4 normal pituitary samples. Collectively, these data suggest that *NPR2* and *NPPC* are expressed in both developing and adult human anterior pituitary tissues.
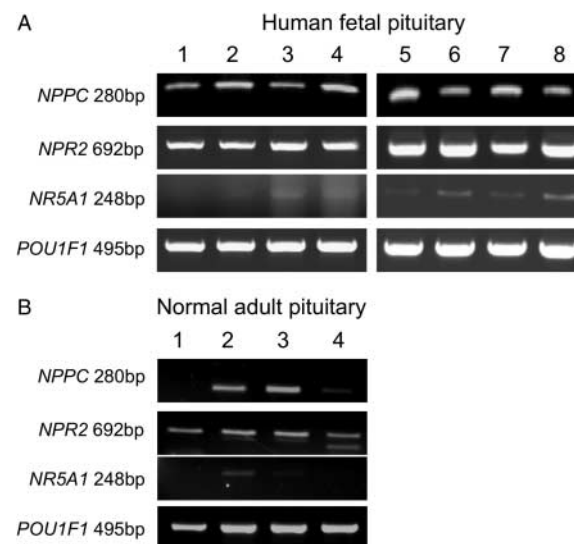
Expression of NPR2 in human pituitary adenomas

We broadened our investigations to determine the expression of *NPPC* and *NPR2* in human pituitary adenomas. RT-PCR was performed on RNA samples extracted from a range of tumors. As shown in Fig. 2A, all pituitary tumors of gonadotroph origin expressed *NPR2*, and for those in which sufficient cDNA remained, *NPPC* transcripts were also detected (tumors 3–8). As expected, all these tumors expressed

the gonadotroph-specific marker, *NR5A1*. Interestingly, *POU1F1* transcripts were detected in the majority of these gonadotroph origin tumors, albeit at variable intensity. Identical RT-PCR screens were performed on RNA extracted from somatotrophinomas (Fig. 2B) as well as null cell adenomas, silent ACTH tumors, and a normal pituitary sample (Fig. 2C). Again, all samples examined expressed *NPR2* transcripts, and where sufficient cDNA remained, *NPPC* transcripts were also detected (tumors 12–15). Expression of *NR5A1* and *POU1F1* was absent in the two corticotroph tumors, whereas both null cell adenomas expressed *POU1F1*, but only one expressed *NR5A1*. In keeping with previous studies, some somatotrophinomas expressed *NR5A1* transcripts (Aylwin *et al.* 2001). These studies represent the first description of *NPR2* and *NPPC* transcripts in a range of human pituitary adenomas.

Detection of GC-B immunoreactivity in human pituitary adenomas

Having established that *NPR2* is expressed in all pituitary adenoma samples examined and in human fetal pituitaries, we next examined whether GC-B protein could be detected in tissue from some of the adenoma samples analyzed previously.

Figure 1 (A and B) Expression of *NPR2* and *NPPC* in normal human pituitary fetal pituitaries and normal adult pituitaries. (A) Total RNA was extracted from human fetal pituitary samples collected from embryos terminated between 12 and 20 weeks. Thirty-cycle RT-PCR was performed for *NPPC*, *NPR2*, *NR5A1*, and *POU1F1* expression. (B) Total RNA was extracted from four normal, cadaveric, adult pituitaries. Thirty-five-cycle RT-PCR was performed for *NPPC*, *NPR2*, *NR5A1*, and *POU1F1* expression.
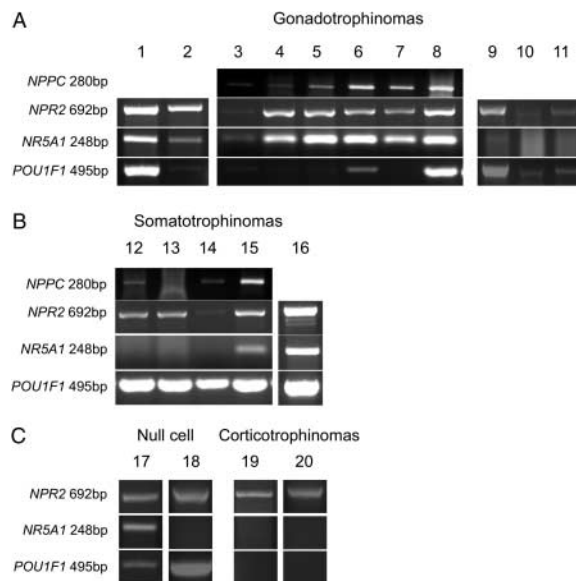
Figure 2 (A, B and C) Expression of *NPR2* and *NPPC* in a range of human pituitary adenomas. RT-PCR was performed over 30 cycles using *NPR2*- and *NPPC*-specific primers in gonadotrophinomas (A), somatotrophinomas (B), or other pituitary tumor types (C). As a control, the expression of the gonadotroph-specific transcription factor *NR5A1*, as well as *POU1F1*, was also performed.

Immunohistochemistry was performed for GC-B immunoreactivity (GC-B-ir) in a range of sections taken from some of the gonadotrophinoma and somatotrophinoma samples characterized for the molecular expression analyses. Initial optimization studies were performed on tissues known to express GC-B protein, and specific GC-B-ir was detected in mouse Purkinje cells of the cerebellum and posterior pituitary tissue (Fig. 3A), as described previously (Herman *et al*. 1996, Müller *et al*. 2009). Normal adult human pituitary tissue also revealed specific GC-B-ir (Fig. 3B). These optimized conditions were used on the human pituitary adenoma samples, and, GC-B-ir was detected in the gonadotrophinomas (Fig. 3C) and somatotrophinomas (Fig. 3D) examined, although the intensity of the apparent GC-B expression was variable. These data suggest that GC-B protein is also expressed in human pituitary adenomas and normal human pituitary tissue.

Transcriptional regulation of the human NPR2 promoter in anterior pituitary cell lines

The expression profile of *NPR2* in both normal and adenomatous human pituitary tissue, regardless of cellular origin, suggested that the control of *NPR2* expression in all anterior pituitary cell types might share a common mechanism. To examine whether cell-type-specific regulation of the *NPR2* promoter could occur, we transiently transfected well-established pituitary tumor cell lines representative of gonadotrophs (αT3-1 and LβT2), somatolactotrophs (GH3), and corticotrophs (AtT-20) with a series of human *NPR2* promoter deletion constructs, as described previously (Rahmutula *et al*. 2004). Promoter activity was determined after 24 h by reporter gene assays.

Gonadotroph cell lines

The promoter was active in both early- (αT3-1, *Cga* expressing) and later-stage (LβT2, *Cga*, *Lhb*, and *Fshb* expressing) gonadotrophs (see Fig. 4), and in both cell lines, the most proximal −214 bp of the human *NPR2* promoter was sufficient to drive maximal reporter gene activity (to 7.9 ± 1.8-fold ($P < 0.001$) and 3.2 ± 0.2-fold ($P < 0.05$) in αT3-1 and LβT2 cells respectively). Furthermore, in LβT2 cells, but not in αT3-1 cells, the −645 construct also showed maximal activity (3.6 ± 0.8-fold, $P < 0.01$), suggesting that additional transcriptional activity is switched on during the process of gonadotroph development.

Somatolactotroph and corticotroph cell lines

As shown in Fig. 4, the *NPR2* reporter gene constructs were active in both GH3 (*Gh* and *Prl* expressing) and AtT-20 (*Pomc* expressing) cells, with the −214 construct driving maximal transcriptional activity (to 7.8 ± 1.3-fold ($P < 0.001$) and 2.9 ± 0.5-fold ($P < 0.01$), in GH3 and AtT-20 cells respectively). Finally, to determine whether the most active *NPR2* promoter constructs could also function in non-immortalized cells, similar transfections were performed in cultures of mouse pituitary cells. As shown in Fig. 4E, the −214 and −441 constructs showed significant transcriptional activity compared with pGL3LUC (to 13.5 ± 3.1-fold (*$P < 0.05$) and 15.3 ± 2.8-fold (**$P < 0.01$) respectively). Collectively, these data suggest that sequences residing within this region regulate basal expression of *NPR2* and that this is common to all anterior pituitary cell types.

### Molecular characterization of the proximal human *NPR2* promoter

The reporter gene assay data suggested that −214 bp of the proximal human *NPR2* promoter was sufficient for maximal basal promoter activity (see Fig. 4A, B, C, D and E). We further characterized the region between −135 and −214, initially through a bioinformatic screen to establish the presence of relevant
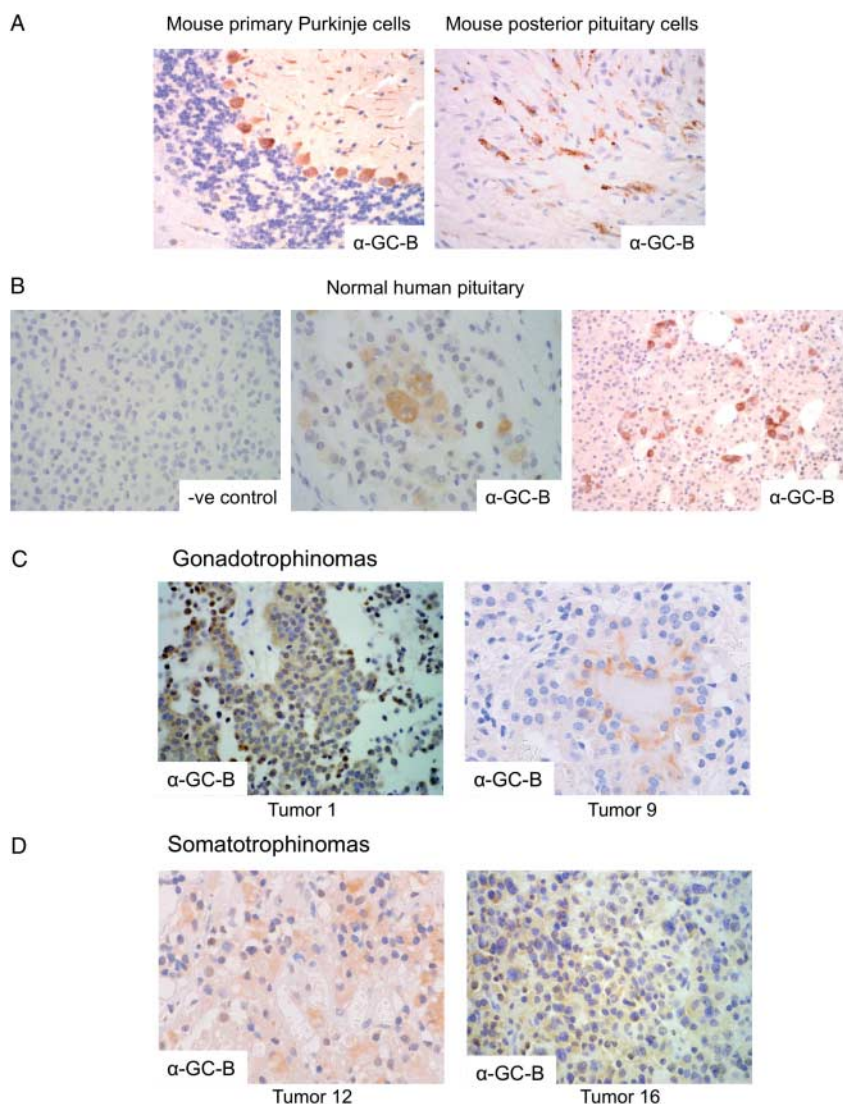
Figure 3 (A, B, C and D) Detection of GC-B immunoreactivity (GC-B-ir) in human pituitary gonadotrophinomas and somatotrophinomas. (A) Optimization images of GC-B-ir in mouse primary Purkinje cells (left) and posterior pituitary cells (right). (B) GC-B-ir in two independent normal human pituitary samples. The negative control slide was generated without incubation with primary antibody. (C and D) GC-B-ir in sections of gonadotrophinomas (tumors 1, 9) and somatotrophinomas (tumors 12, 16). The brown staining indicates GC-B-ir, and the blue/purple is hematoxylin counterstain. All images were taken at either 20× or 40× magnification.

transcription factor binding sites. As no cell-type-specific promoter activity had been detected (Fig. 4), we were particularly interested in ubiquitously expressed transcription factors that have previously been implicated in the transcriptional control of the *NPR2* promoter, namely members of the specificity protein family (Rahmutula *et al.* 2004). As shown in Fig. 5A, numerous consensus Sp1/Sp3 binding sites were found in this proximal promoter region. We subdivided these GC-rich 'hotspots' into proximal (*prox*), middle (*mid*), and distal (*dist*) regions and generated ^{32}P-labeled probes to perform EMSA

analyses. As shown in Fig. 5B, all the three *NPR2* probes (*prox*, *mid*, and *dist*) formed complexes with nuclear extracts from GH3 and LβT2 cells. Each of these regions was capable of competing for protein complexes, as excess unlabeled *prox*, *mid*, and *dist* annealed oligonucleotides successfully competed for ^{32}P-labeled *prox* probe (Fig. 5C). However, it was clear from these initial EMSAs that the *prox* and *mid* regions of the *NPR2* promoter yielded the more intense complex formation. Therefore, subsequent supershift experiments were performed using these two probes only. As shown in Fig. 5D and E, the presence of Sp1
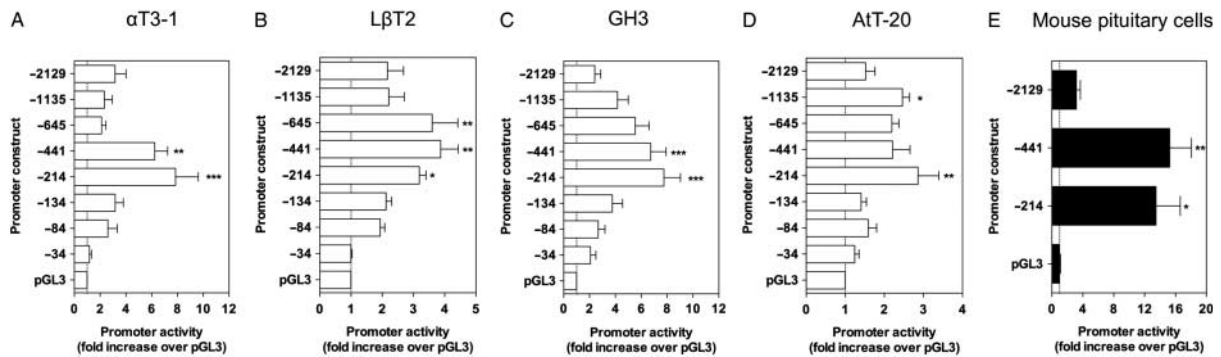
Figure 4 (A, B, C, D and E) Transcriptional regulation of the human *NPR2* promoter in rodent anterior pituitary tumor cell lines and mouse primary pituitary cells. A series of deletion constructs encoding the human *NPR2* promoter were transiently transfected into (A) αT3-1, (B) LβT2 (gonadotrophs), (C) GH3 (somatolactotrophs), and (D) AtT-20 (corticotrophs) and left for 24 h. The data shown are pooled from at least four independent experiments, each performed in triplicate and expressed as mean ± s.e.m., normalized as fold increase over promoterless control (pGL3LUC). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from pGL3LUC. (E) Similar transfections were performed using dispersed primary mouse pituitary cells. The data shown are pooled from four individual transfections and expressed as mean ± s.e.m., normalized as fold increase over promoterless control (pGL3LUC). *$P < 0.05$, **$P < 0.01$, significantly different from pGL3LUC.

and Sp3 antibodies in the incubation reaction resulted in either a reduction and/or supershift of complexes formed by the interaction of nuclear extracts with *prox* and *mid* probes. Collectively, these data reveal that Sp1 and Sp3 proteins, acting either as homo- or hetero-dimers, form complexes with the human *NPR2* promoter that may regulate basal gene transcription in pituitary cells.

Discussion

Many previous studies have established the presence of the CNP and its receptor, GC-B, in rat, mouse, and sheep pituitaries (Komatsu *et al*. 1991, Konrad *et al*. 1992, Minamino *et al*. 1993, Yandle *et al*. 1993, McArdle *et al*. 1994, Herman *et al*. 1996, Thompson *et al*. 2009), yet the expression of GC-B in human pituitary tissue has not been reported. We show for the first time that *NPR2* expression is found in normal human fetal pituitary samples, ranging from 12 to 20 weeks of gestation, suggesting that natriuretic peptide signaling is required during pituitary development in humans. In addition, the presence of *NPR2* transcripts in normal adult pituitary tissue would support a role for GC-B signaling in later life as well. Furthermore, using a selection of human pituitary adenomas, *NPR2* expression was detected at both the mRNA and protein level, regardless of the cellular origin of the tumor.

Our present studies, combining RT-PCR, reporter gene assays, and immunohistochemistry, convincingly show that *NPR2* is expressed in a broad range of both normal, developing fetal and adult pituitaries and human pituitary adenomas of different cellular origin. The presence of *NPR2* transcripts in tumors of multiple

anterior pituitary cell lineages again supports our previous studies that showed potent CNP-stimulated cGMP accumulation occurs in immortalized anterior pituitary cell lines of all origins (Fowkes *et al*. 2000, Thompson *et al*. 2009). It is clear that natriuretic peptide signaling is likely to be responsible for significant production of cGMP in the human anterior pituitary. Whether gonadotroph cells are the major source of CNP-stimulated cGMP in the pituitary, as we have shown in rat and mouse pituitaries and cell lines (Fowkes *et al*. 2000, Thompson *et al*. 2009), remains to be established.

The detection of *NPPC* transcripts are the first to document the developmental expression in human pituitaries and suggest that, along with *NPR2*, CNP is involved in the local regulation of the developing anterior pituitary gland. Furthermore, we were also able to detect *NPPC* transcripts in gonadotrophinoma and somatotrophinoma samples and in three of the four normal human pituitary samples (Figs 1 and 2A, B). The relatively variable expression of the *NPPC* transcripts might indicate an mRNA stability issue of the *NPPC* gene in archival tissue, and in this regard, highly conserved 3′-untranslated regions have been identified in the *nppc* gene of several elasmobranch species that are associated with mRNA stability and translational efficiency (Hyodo *et al*. 2006). However, it is also possible that *NPPC* is expressed in a cell-type-specific manner in the pituitary as we, and others, have reported previously in rodents (McArdle *et al*. 1993, Thompson *et al*. 2009), and the variable expression in the current study is merely a reflection of the relative contribution of those cell types in each of the tumor or normal pituitary samples examined.
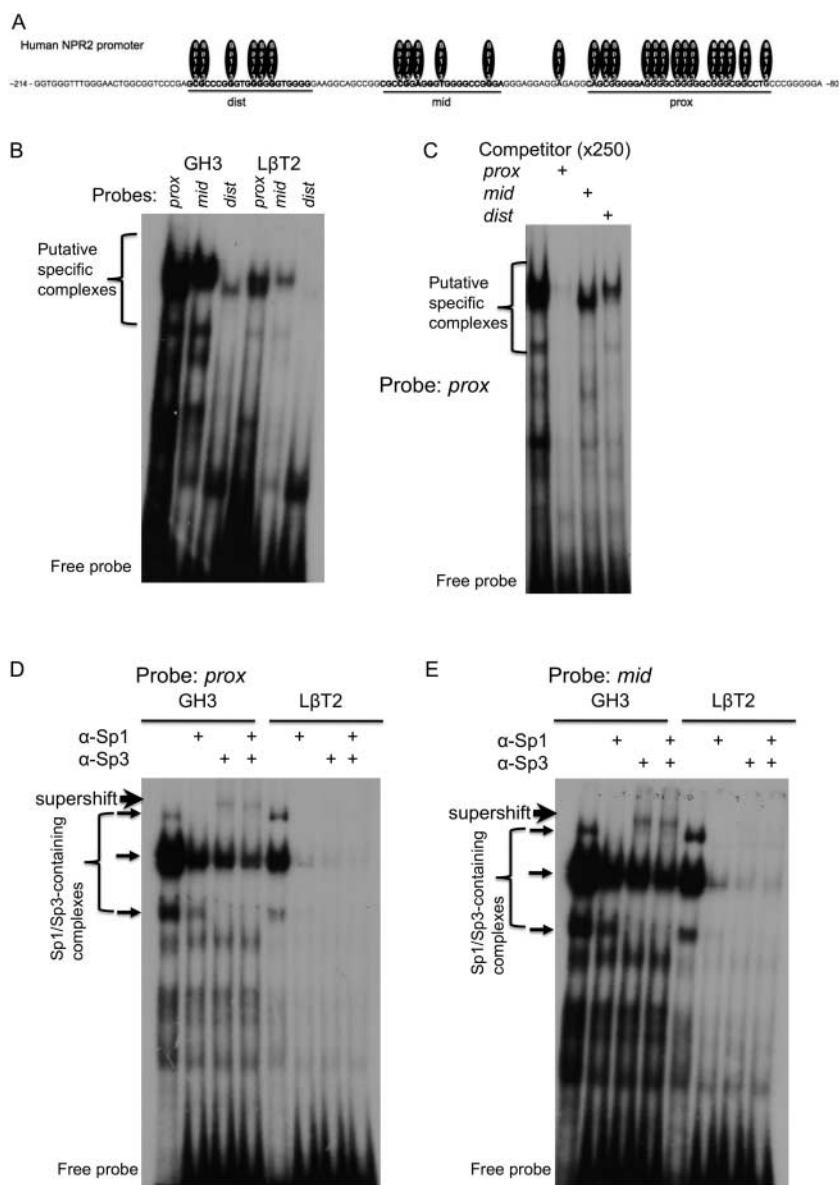
Figure 5 (A, B, C, D and E) Molecular characterization of the proximal human *NPR2* promoter. (A) Bioinformatic screening of the human *NPR2* promoter revealed the presence of numerous, consensus Sp1 and Sp3 binding throughout the proximal 214 bp of the promoter. Three areas of this region were identified for further characterization by EMSA analysis (prox, mid, and dist). (B) EMSA analyses using nuclear extracts from GH3 and LβT2 cells, and three probes encoding the prox, mid, and dist regions of the NPR2 promoter. (C) Competitor assay using excess unlabeled prox, mid, and dist oligonucleotides to compete specific binding to the prox probe, in the presence of GH3 nuclear extracts. (D and E) Supershift assays, using Sp1 and Sp3 antibodies to identify the putative specific protein complexes detected by EMSA in the presence of either the prox (D) or mid (E) probes. The data shown are representative of at least two such experiments.

The factors regulating the expression of *NPR2* are still not fully defined. Initial cloning and molecular characterization studies on the human *NPR2* promoter in rat aortic smooth muscle cells uncovered a requirement of Sp1 factors in controlling *NPR2* transcription (Rahmutula *et al*. 2004). Our current studies reveal that an *NPR2* promoter construct containing only 214 bp of the proximal promoter is sufficient to drive maximal promoter activity, in keeping with the previous pattern of promoter activity reported in rat aortic smooth muscle cells (Rahmutula *et al*. 2004). There are numerous Sp1/Sp3 response elements within this region of the human promoter (see Fig. 5A), so it is likely that Sp1 family proteins are

also major regulators of *NPR2* expression in pituitary tissue. Sp1 is ubiquitously expressed and has been implicated in the regulation of gonadotroph-specific genes (Kaiser *et al*. 2000, Weck *et al*. 2000, Bachir *et al*. 2003, Thompson *et al*. 2009). As the profile of *NPR2* transcriptional activity remained comparable in all cell lines investigated in our study, this suggests that any potential role of Sp1 in the control of *NPR2* expression is common to all pituitary cell lineages and is unlikely to contribute to any cell-type-specific expression of *NPR2*. Our detailed EMSA analyses, using nuclear protein extracts from GH3 and LβT2 cells, revealed complexes indicative of Sp1/Sp3 homo- and heterodimers, strongly implicating these transcription factors in the basal regulation of *NPR2* transcription. However, while the use of pituitary tumor-derived cell lines is informative in demonstrating that endogenous Sp1/Sp3 proteins can bind to the proximal *NPR2* promoter, further investigations are required to establish whether these GC-rich elements of the *NPR2* promoter are important *in vivo*. Nevertheless, previous transcriptional studies on the *NPR2* promoter in rat aortic smooth muscle cells showed that mutation of these putative Sp1/Sp3 binding sites results in up to 90% inhibition of basal promoter activity, whereas overexpression of exogenous Sp1 or Sp3 protein can activate *NPR2* promoter activity (Rahmutula *et al*. 2004), clearly supporting a role for Sp1/Sp3 proteins in the expression of human GC-B receptor.

While we acknowledge that Sp1 family proteins are ubiquitous, it is interesting to note that Sp1 is involved in the transcriptional regulation of other members of the natriuretic peptide/GC/cGMP signaling pathway. Both the promoters for ANP (*NPPA*) and the GC-A receptor (*NPR1*) have been shown to be regulated by Sp1 (Liang *et al*. 1999, Kumar *et al*. 2010, Hu *et al*. 2011), and we, and others, have indicated a role for Sp1/Sp3 in controlling *Nppc* transcription in pituitary cell lines (Ohta *et al*. 1993, Thompson *et al*. 2009). The other major regulator of cGMP signaling is the gaseous neurotransmitter, nitric oxide (NO), and both neuronal and endothelial forms of the NO-synthesizing enzyme, NO synthase (*NOS1* and *NOS3*, respectively), are transcriptionally controlled by Sp1 (Kleinert *et al*. 1998, Bachir *et al*. 2003). Finally, the promoters for the cGMP-selective phosphodiesterase 5 enzyme genes (*PDE5A1* and *PDE5A2*) have also been shown to contain Sp1 response elements (Yanaka *et al*. 1998, Kotera *et al*. 1999). Collectively, it appears that transcriptional control of proteins involved in cGMP signaling is heavily influenced by Sp1 family proteins.

While the predominant regulatory region involved in controlling *NPR2* transcription appears to reside within the most proximal 214 bp, our current findings demonstrate some differential regulation of the human *NPR2* promoter in gonadotroph cell lines. The −645 bp promoter was also maximally active in LβT2 cells, whereas activity of this construct in the developmentally immature αT3-1 cells was not significant. *In silico* analysis of this region of the human NPR2 promoter reveals numerous putative response elements for nuclear hormone receptors (e.g. for glucocorticoids, estrogen, and retinoic acid). It remains to be established whether responsiveness to these hormones is enhanced in the more mature gonadotroph to subsequently alter *NPR2* transcription.

Expression of natriuretic peptide receptors has previously been shown in tumors of other cellular origin. The GC-A receptor, encoded by *NPR1/Npr1*, is highly expressed in prostate cancer cells (Wang *et al*. 2011), and natriuretic peptides have been demonstrated to act as anticancer therapies to treat pancreatic, breast, and lung carcinomas implanted within athymic mice (Vesely 2009). Our demonstration that pituitary tumor tissue also expresses *NPR2*/GC-B raises the intriguing possibility that these receptors might be useful in generating novel therapies, although pituitary adenomas are usually slow growing and rarely malignant (Melmed 2003). It remains to be established whether GC-B receptors in human pituitary tissue can act as secretagogue modulators, although previous studies suggest that both CNP and NO are capable of stimulating GH secretion from rodent pituitaries (Shimekake *et al*. 1994), human fetal pituitaries, and human pituitary adenomas (Rubinek *et al*. 2005).

While there are some reports on pituitary roles for natriuretic peptide signaling, such as increasing GH secretion (Hartt *et al*. 1995, Shimekake *et al*. 1994, Eckert *et al*. 2003) or inhibiting ACTH secretion (Guild & Cramb 1999), these effects may well be cell-type specific and are not known to be recapitulated in humans. Genetically manipulated mouse models of disrupted *Nppc* or *Npr2* also point to a pituitary role for CNP and GC-B (Chusho *et al*. 2001, Tamura *et al*. 2004), given the profound growth and reproductive disruption to these animals, yet these intriguing possibilities remain to be determined within a pituitary-specific context. Our current observations of *NPR2* expression in all endocrine cell types of the human anterior pituitary support previous suggestions that CNP acts as an autocrine or paracrine regulator in the pituitary, but the nature of this paracrinicity is unknown (Denef 2008). Although the presence of a pituitary natriuretic peptide system in other vertebrate species suggests that cGMP signaling is important for normal pituitary function, understanding of whether a

similar system is present in human pituitaries is very limited. Before our current observations of pituitary *NPPC* and *NPR2* expression, previous studies on humans suggest that normal pituitary tissue expresses *NPPB* (encoding BNP), *PRKG2* (encoding protein kinase G II), and *PDE11A* (encoding phosphodiesterase 11A) (Gerbes *et al*. 1994, Fawcett *et al*. 2000, Zhan & Desiderio 2004). The presence of both *PKG2* and *PDE11A* in human pituitaries indicates potential cGMP-target proteins, which might regulate the effects of CNP/GC-B signaling. PDE11A acts as a dual-specificity cyclic nucleotide phosphodiesterase that causes cAMP and cGMP hydrolysis (Francis *et al*. 2011) and, intriguingly, mutations within *PRD11A* are associated with several endocrine neoplasias (Horvath *et al*. 2006, 2009). It is, therefore, feasible that a pituitary natriuretic peptide system in humans might serve to regulate cyclic nucleotide-regulated hormone secretion. Alternatively, as CNP and GC-B are major local regulators of the cardiovasculature, it is tempting to speculate that *NPR2* expression is contributing to the local dynamic control of pituitary blood flow, in both normal and adenomatous tissue, given the highly vascularized properties of the anterior pituitary (Turner *et al*. 2000). It is clear that understanding the physiological relevance of our expression studies within human pituitary tissue remains to be established.

Declaration of interest

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