Imprinting of the $G_s\alpha$ gene *GNAS1* in the pathogenesis of acromegaly

Rapid Publication

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Approximately 40% of growth hormone–secreting pituitary adenomas have somatic mutations in the *GNAS1* gene (the so-called *gsp* oncogene). These mutations at codon 201 or codon 227 constitutively activate the α subunit of the adenylate cyclase–stimulating G protein G_s. *GNAS1* is subject to a complex pattern of genomic imprinting, its various promoters directing the production of maternally, paternally, and biallelically derived gene products. Transcripts encoding $G_s\alpha$ are biallelically derived in most human tissues. Despite this, we show here that in 21 out of 22 *gsp*-positive somatotroph adenomas, the mutation had occurred on the maternal allele. To investigate the reason for this allelic bias, we also analyzed *GNAS1* imprinting in the normal adult pituitary and found that $G_s\alpha$ is monoallelically expressed from the maternal allele in this tissue. We further show that this monoallelic expression of $G_s\alpha$ is frequently relaxed in somatotroph tumors, both in those that have *gsp* mutations and in those that do not. These findings imply a possible role for loss of $G_s\alpha$ imprinting during pituitary somatotroph tumorigenesis and also suggest that $G_s\alpha$ imprinting is regulated separately from that of the other *GNAS1* products, NESP55 and XLαs, imprinting of which is retained in these tumors.

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Introduction

The *GNAS1* gene on chromosome 20q13 encodes the α subunit of the heterotrimeric guanosine 5′triphosphate–binding (GTP-binding) protein, G_s. G_s couples hormonal stimulation of various cell-surface receptors to the activation of adenylate cyclase. A number of endocrine syndromes are caused by heterozygous mutations that affect $G_s\alpha$. McCune-Albright syndrome (polyostotic fibrous dysplasia, skin hyperpigmentation, and autonomous endocrine hyperfunction) results from somatic mosaicism for a codon 201 mutation, which constitutively activates $G_s\alpha$ by impairing its intrinsic GTPase activity (1). Codon 201 mutations are also found in some endocrine tumors (2) (see below).

In contrast, germline mutations that inactivate $G_s\alpha$ result in the autosomal dominant syndrome Albright's hereditary osteodystrophy (AHO; characterized by short stature, brachydactyly, and subcutaneous calcification) (3, 4). However, when such mutations are maternally inherited, in addition to AHO, resistance to the peripheral action of several hormones, notably parathyroid hormone (PTH), results (pseudohypoparathyroidism type Ia; PHP-Ia). It was this parental origin effect in AHO/PHP that first suggested that *GNAS1* might be an imprinted gene (5).

Despite this indirect evidence for $G_s\alpha$ imprinting, in both AHO and PHP-Ia the activity of $G_s\alpha$ in erythrocytes is 50% of normal (6, 7), and RNA studies of several human fetal tissues showed biallelic *GNAS1* expression (8). It is therefore believed that $G_s\alpha$ is monoallelically expressed only in a few sites, particularly the proximal renal tubule, the main PTH target tissue. This would be consistent with the fact that in PHP-Ia patients, renal cAMP response to PTH is absent or severely impaired (9), suggesting target cell $G_s\alpha$ levels much less than the 50% seen in erythrocytes.

Molecular studies have shown that both human *GNAS1* and murine *Gnas* are indeed imprinted, but in a highly complex way. In addition to $G_s\alpha$, *GNAS1* encodes two other proteins, extra-large α_s (XLαs) and 55-kDa neuroendocrine secretory protein (NESP55). All three mRNA species include exons 2–13, spliced in each case to a distinct first exon. NESP55 transcripts are maternal, while XLαs transcripts, initiating 14 kb further downstream, are paternal (10, 11). A spliced, paternally expressed, noncoding antisense transcript arises approximately 2.5 kb upstream of XLαs and traverses the NESP55 exon (12). $G_s\alpha$ transcripts, initiating at exon 1, approximately 37 kb downstream of XLαs, are biallelic. Finally, a fourth sense promoter, approximately 2 kb upstream of exon 1, directs expression of paternal transcripts that are probably not translated, since they contain an alternative first exon (1A or A/B) lacking an initiation codon (13–15).

In humans, monoallelic expression of $G_s\alpha$ transcripts has not been demonstrated. In the mouse, though, $G_s\alpha$ does appear to be predominantly maternal in origin in renal cortex and in brown and white fat. In these tissues, $G_s\alpha$ protein and mRNA levels are much lower in mice with a het-

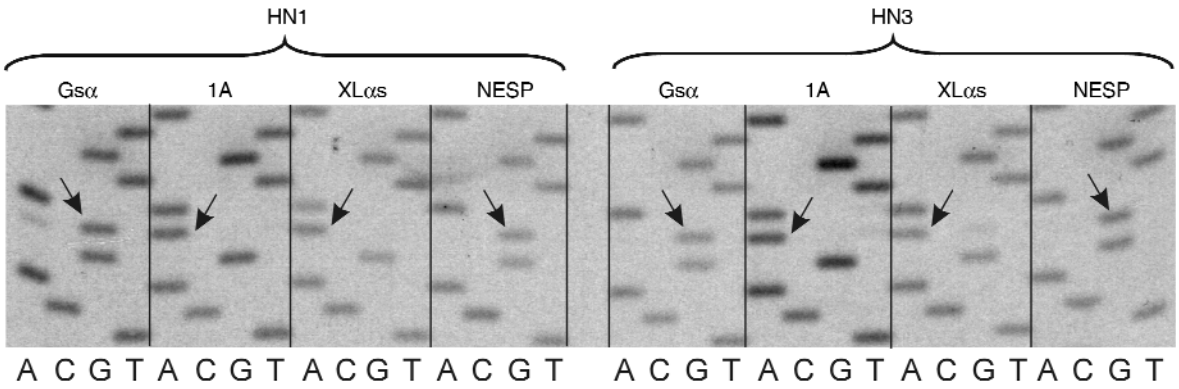

Figure 1
Imprinting of *GNAS1* transcripts in normal human pituitary. Sequences are shown for each of the four *GNAS1* transcript types ($G_s\alpha$, exon 1A, XLαs, and NESP55) across the site of the exon 5 A/G polymorphism (reverse strand). Results from two normal pituitaries (HN1 and HN3) are shown. In each case the polymorphic residue is indicated by an arrow. The $G_s\alpha$ transcripts are exclusively (HN3) or predominantly (HN1) maternal in origin, i.e., derived from the NESP55-expressing chromosome.

erozygous knockout mutation of the maternal *Gnas* allele, compared with those with a paternal knockout (16).

While $G_s\alpha$ is ubiquitous, NESP55 and XLαs are highly expressed only in certain neurosecretory tissues, including pituitary (17, 18). XLαs, like $G_s\alpha$, can bind G βγ subunits and activate adenylate cyclase (17, 19), but a hormone receptor able to activate XLαs has not been identified. The function of NESP55 is unclear; it was originally suggested to be the proteolytic precursor for certain bioactive peptides (20).

Approximately 40% of pituitary somatotroph adenomas contain heterozygous mutations in *GNAS1* that constitutively activate $G_s\alpha$ by substitutions at arginine 201 (R201) or glutamine 227 (Q227) (2, 21–23). Less frequently, the same mutations occur in thyroid tumors (24), Leydig cell tumors of the ovary and testis (25), nonsomatotroph (corticotroph or nonfunctioning) pituitary adenomas, pheochromocytomas, and parathyroid adenomas (26, 27). In Japan only 4–10% of somatotroph tumors have *GNAS1* mutations (28, 29). The role of activated *GNAS1* in neoplasia has earned it an alternative title — the *gsp* oncogene. In somatotroph tumors lacking a *gsp* oncogene, the factors involved in tumorigenesis are largely unknown (30).

Methods

Pituitary adenomas were obtained at transsphenoidal surgery. They were classified using clinical and biochemical findings and immunocytochemical and morphological data, including absence of contamination with normal pituitary (31). Normal human pituitary autopsy specimens were used as controls. Somatotroph adenomas were tested for *gsp* mutations as described (32). From the group of *gsp*$^-$ somatotroph tumors and nonfunctioning adenomas, only those heterozygous at the exon 5 polymorphism were selected (see Results). Institutional approval was received from the Ethics Committees of the University of Aix-Marseilles and St. Bartholomew's Hospital, and informed consent was received from each patient.

Total RNA was isolated using the SV kit (Promega Corp., Madison, Wisconsin, USA) or Tri-Reagent (Sigma Chemical Co., St. Louis, Missouri, USA); cDNA was made from 2 μg of total RNA using random-primed reverse transcription as described previously (31, 33).

RT-PCR used a "hot start" followed by six cycles of 94°C for 30 seconds, 66°C for 30 seconds (–0.5°C per cycle), 72°C for 90 seconds, and then 34 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 90 seconds. Reactions used a common downstream primer GNAS10R and one or other of the exon-specific upstream primers as follows: NESP5, dTCGGAATCTGACCACGAGCA; XL1F, dGGATGCCTCCGCTGGTTTCAG; GNASEXA, dGCCTTGCGTGTGAGTGCACCT; GNAS1F, dCCATGGGCTGCCTCGGGAACA; GNAS10R, dCACGAAGATGATGGCAGTCAC.

Sequencing was performed using a ThermoSequenase cycle sequencing kit and ^{33}P-dideoxynucleotides (Amersham Pharmacia Biotech, Uppsala, Sweden). Sequencing across the codon 131 polymorphism was primed with oligonucleotide dCCTTGGCATGCTCATAGAATTC and across codons 201 and 227 with dCCTGGACAAGATCGACGTGA. Quantification of band intensity was done using either the PhosphorImager system (Bio-Rad Laboratories Inc., Hercules, California, USA) or the ChemiImager 4400 system (Alpha Innotech Corp., San Leandro, California, USA).

Results

$G_s\alpha$-encoding transcripts are monoallelically expressed in the normal pituitary. We identified four adult pituitaries heterozygous for the exon 5 polymorphism (at codon 131 of $G_s\alpha$). RT-PCRs were performed, each using the same downstream primer in exon 10 paired with a primer specific for one of the four alternative first exons. These reactions selectively amplify *GNAS1* transcripts specific for one of the three protein-coding mRNAs (NESP55, XLαs, or $G_s\alpha$) or the noncoding 1A transcript.

Figure 1 shows the sequence across the polymorphic site of cDNAs from two of these samples. NESP55 and XLαs are expressed from opposite alleles, as shown previously in a range of

human fetal tissues, in all of which NESP55 is maternally and XLαs paternally derived (11). Exon 1A transcripts were shown recently to be paternally expressed in blood cells of two adults (15). We found similar paternal-specific expression of 1A transcripts in a range of first-trimester fetal tissues (data not shown). Figure 1 shows that the same is also true of adult pituitary.

Examining $G_s\alpha$ transcripts, however, we found that in contrast to the biallelic expression seen in all human tissues examined previously, in the pituitary these transcripts are monoallelic. In each case, the expressed allele is the same as that represented in NESP55 transcripts. XLαs and exon 1A transcripts, in contrast, contain only the opposite allele. These results suggest that in the pituitary, $G_s\alpha$ transcripts, like NESP55, are derived from the maternal allele. The same result was obtained in all four adult pituitary cDNA samples; the maximum proportion of paternal $G_s\alpha$ transcript in any of these samples was 14%.

Somatic activating mutations in somatotroph adenomas occur on the maternal allele. Codons R201 and Q227, the sites of the *gsp* mutations, lie in exons 8 and 9 and hence are present in the XLαs and NESP55, as well as the $G_s\alpha$, transcripts. Examination of maternal NESP55 and paternal XLαs transcripts for the presence of the *gsp* mutation should therefore permit the inference of which parental allele carries the mutation.

We examined 22 *gsp*$^+$ adenomas in this way, including examples with mutations at codons 201 and 227, and also different mutations at each codon. From each tumor, we generated three RT-PCR products (specific for $G_s\alpha$, XLαs, or NESP55) and sequenced across the *gsp* mutation site (Figure 2a). In 21 cases (15 R201C, one R201S, one R201H, three Q227L, and one Q227R) the *gsp* mutation was present in the NESP55 but not the XLαs transcript. This suggests that in somatotroph adenomas, oncogenic *gsp* mutations virtually always occur on the maternal allele.

In a single adenoma, the mutation (R201H) was found in XLαs (and 1A) transcripts, but not in NESP55 transcripts, suggesting it had occurred on the paternal allele. This mutation creates a *Nla*III restriction site. *Nla*III digestion of the RT-PCR products was performed to verify the sequencing result, confirming the presence of the mutation on the XLαs but not the NESP55 transcript (not shown). The other R201H adenoma, however, had the mutation on the maternal allele (also verified by *Nla*III digests), indicating that there is nothing peculiar to the R201H mutation that requires it to occur on the paternal chromosome.

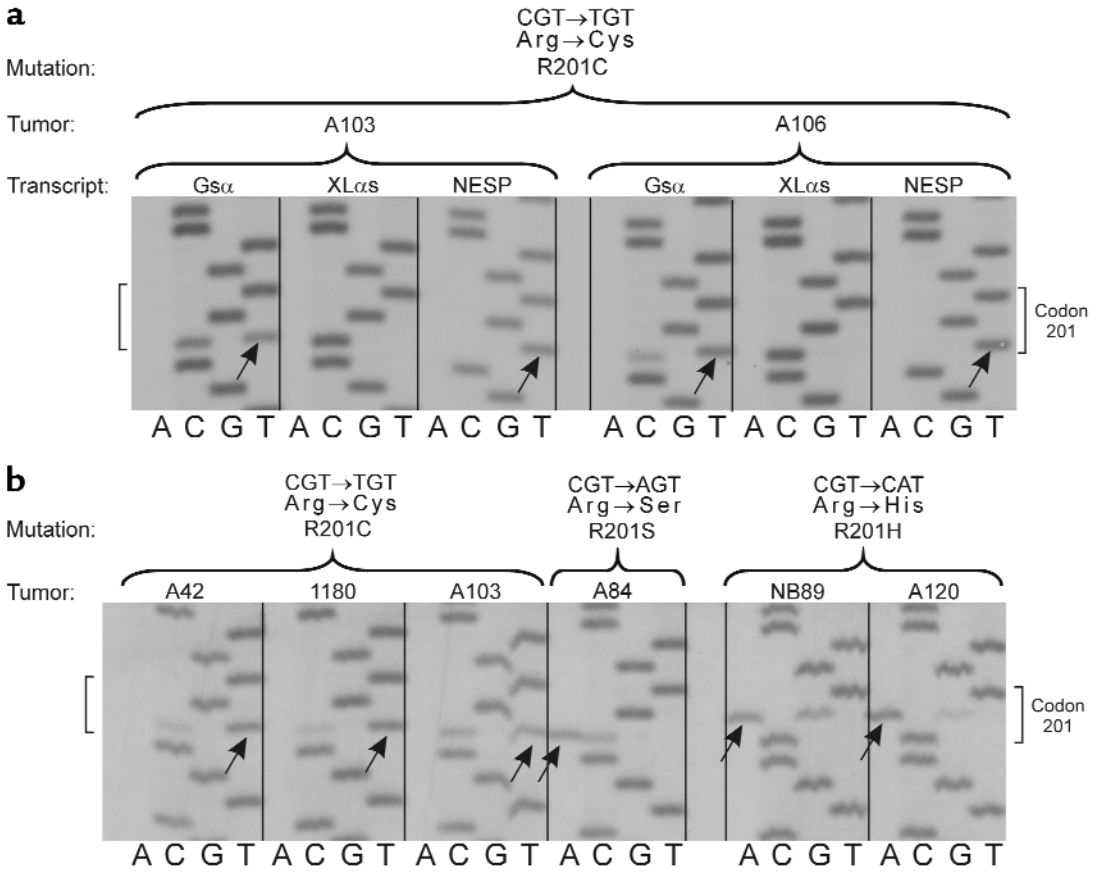

Figure 2

(**a**) Sequences across codon 201 of the three protein-coding *GNAS1* transcripts ($G_s\alpha$, XLαs, and NESP55) in two R201C *gsp*$^+$ adenomas (A103, A106). In both examples, the normal allele CGT is expressed from the paternal (XLαs) chromosome, while only the mutated allele TGT (arrow) is represented in the maternal NESP55 transcripts. (**b**) Sequence across codon 201 (normally CGT) of the $G_s\alpha$ transcript in six *gsp*$^+$ adenomas, illustrating the variation in relative expression level of the two $G_s\alpha$ alleles. All these adenomas carry the *gsp* mutation on the maternal allele, apart from NB89, which has the *gsp* mutation on the paternal allele. In each case, the mutant allele is indicated by an arrow.

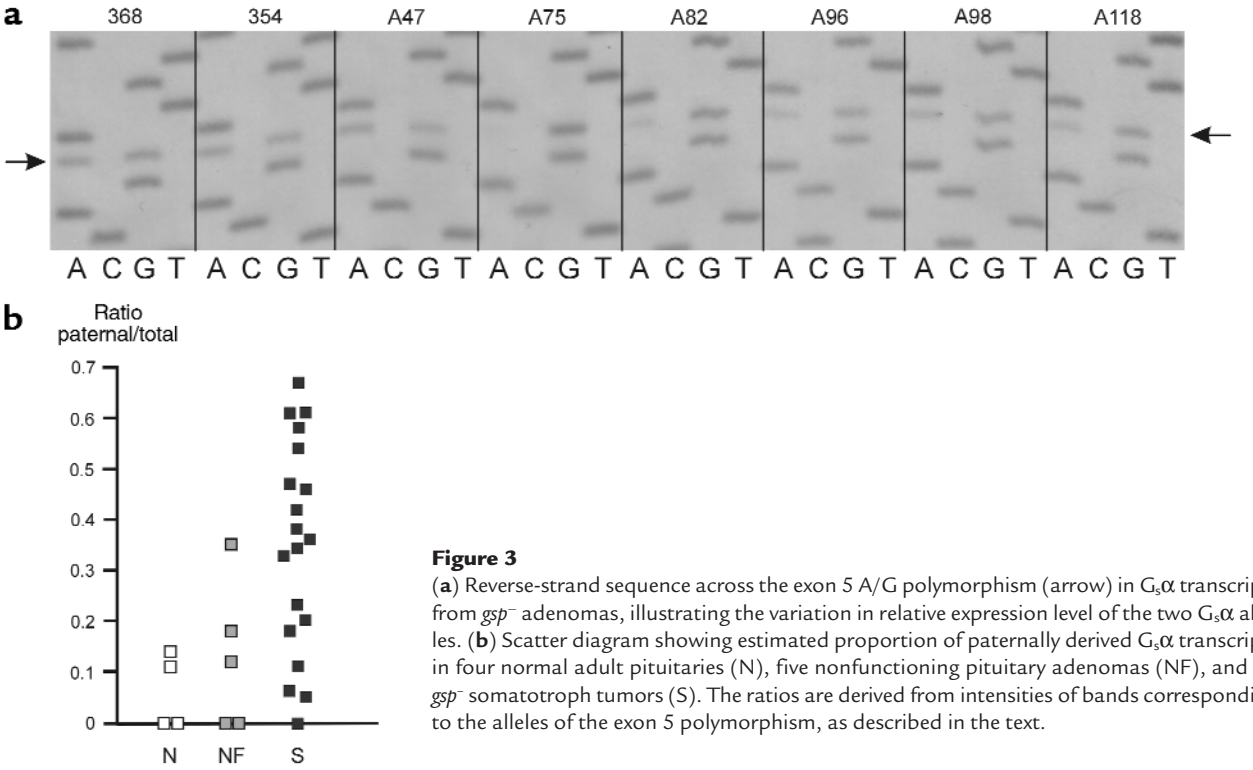

Figure 3
(**a**) Reverse-strand sequence across the exon 5 A/G polymorphism (arrow) in $G_s\alpha$ transcripts from *gsp*⁻ adenomas, illustrating the variation in relative expression level of the two $G_s\alpha$ alleles. (**b**) Scatter diagram showing estimated proportion of paternally derived $G_s\alpha$ transcripts in four normal adult pituitaries (N), five nonfunctioning pituitary adenomas (NF), and 19 *gsp*⁻ somatotroph tumors (S). The ratios are derived from intensities of bands corresponding to the alleles of the exon 5 polymorphism, as described in the text.

Dysregulated imprinting of $G_s\alpha$ transcripts in somatotroph tumors. In *gsp*⁺ pituitary tumors, the mutant is usually more abundant than the normal transcript (34). Previously, this has been attributed to a proliferative advantage from high expression of a dominant oncogene. However, a simpler explanation is that *gsp* mutations occur on the more highly expressed maternal $G_s\alpha$ allele. Figure 2b shows that among *gsp*⁺ tumors the ratio of mutant to normal $G_s\alpha$ transcript is quite variable. However, none of our 22 adenomas expressed solely mutant $G_s\alpha$, even although most showed a bias in favor of the mutant $G_s\alpha$ transcript. This implies that although in most tumors transcription of $G_s\alpha$ from the maternal chromosome remains predominant, there is relaxation of the monoallelic expression pattern, at least in some. We addressed this point further in the context of *gsp*⁻ somatotroph adenomas.

Sixty percent of somatotroph adenomas are *gsp* negative (*gsp*⁻), and the genetic events involved in their development are largely unknown. In several types of tumor relaxation of *IGF2/H19* imprinting occurs (35–37). We therefore wondered whether altered *GNAS1* imprinting might be a factor in the pathogenesis of *gsp*⁻ somatotroph tumors. We examined 19 *gsp*⁻ adenomas that were heterozygous at the exon 5 polymorphism. Figure 3a shows typical examples of the $G_s\alpha$ transcript sequences. The allelic contributions to $G_s\alpha$ expression vary considerably, ranging from exclusively maternal (e.g., A75) to approximately equal (e.g., 368).

A semiquantitative index of this variability was derived. To control for uneven sample loading, we normalized the intensity of the polymorphic band to that of four neighboring bands in the same lane and then calculated the paternal contribution as a proportion of the sum of the normalized values for the two alleles. (The maternal or paternal allele assignment was based on analysis of NESP55 and XLαs transcripts, as before.) In addition to 19 *gsp*⁻ somatotroph tumors, for comparison we analyzed the four heterozygous normal adult pituitaries and five nonfunctioning pituitary adenomas.

Although there is wide variation among the *gsp*⁻ somatotroph tumors, a substantial number shows a much greater proportion of paternally derived $G_s\alpha$ than is seen in either the normal samples or the nonfunctioning tumors (Figure 3b). In the normal pituitaries, $G_s\alpha$ expression was either exclusively from or very strongly biased toward the maternal allele. In contrast, in almost two-thirds of *gsp*⁻ adenomas, $G_s\alpha$ expression was approximately biallelic (paternal proportion 0.3–0.7).

As judged from codon 201 or 227 analysis, $G_s\alpha$ expression was also biallelic in several of the 22 *gsp*⁺ adenomas (e.g., Figure 2b, tumors A103, A84, NB89). Thus there appears to be relaxation of $G_s\alpha$ imprinting both in *gsp*⁺ and *gsp*⁻ somatotroph tumors. This loss of imprinting may therefore be a secondary feature of adenoma progression. We have shown previously that the *GNAS1* transcript levels in *gsp*⁻ somatotroph adenomas are more variable than in *gsp*⁺ adenomas (38). It is possible that part of that variability could be due to relaxation of $G_s\alpha$ imprinting control, resulting in an increase in $G_s\alpha$ transcript.

Discussion

We have found that in normal human pituitary, unlike most tissues, $G_s\alpha$ is monoallelically derived. Furthermore, this imprinted expression pattern is relaxed in a substantial proportion of growth hormone–secreting (GH-secreting) adenomas. In contrast, the opposing monoallelic expression patterns of

NESP55 and XLαs are not altered in the GH-secreting tumors. This dissociation between imprinting in the exon 1 region and the far upstream NESP55/XLαs region is consistent with the fact that germline imprinting mutations in PHP-Ib patients can also have discordant effects on the two regions (15).

The five hormone-secreting cell types of the anterior pituitary derive from a common progenitor (39, 40). Cell type–specific hormone production can be observed by week 12 of human development (41). Since we observed predominantly monoallelic $G_s\alpha$ expression in whole adult pituitary, it is likely that all regions of the pituitary express $G_s\alpha$ monoallelically and therefore that this imprinted expression pattern is established in an early common precursor cell. However, biallelic expression of $G_s\alpha$ in a minority cell population of normal pituitary might not be detectable by our analysis, so it remains theoretically possible that $G_s\alpha$ expression is biallelic in the normal somatotroph. We feel this is unlikely, though, given the extreme allelic bias in origin of *gsp* mutations.

Given the role of $G_s\alpha$ in mediating the pituitary response to GHRH (42), its monoallelic expression is somewhat paradoxical. One might predict that PHP-Ia patients, who carry maternal null *GNAS1* mutations, would have no functional $G_s\alpha$ in the pituitary. Although GH deficiency has been reported in PHP-Ia (43), it is not typical. Possibly, therefore, when an inactive *GNAS1* allele is inherited maternally, compensatory expression of the paternal $G_s\alpha$ allele occurs sufficient to allow normal pituitary function. This seems possible, given our observation of a small, variable proportion of paternally derived $G_s\alpha$ transcript in two of the normal pituitaries analyzed. There may be some polymorphic or temporal variation in the degree of imprinting of $G_s\alpha$, but larger numbers of normal pituitaries need to be examined to assess this point.

Like others, we find that in gsp^+ tumors the mutant is often overexpressed relative to the normal allele. This might indicate a need for high-level *gsp* expression to allow tumor progression, but we find that this inequality in *gsp* and normal transcript levels is not invariable and is therefore unlikely to be required for tumor development. It is more likely that the higher *gsp* expression simply reflects the fact that the mutations occur on the maternal allele, which is normally expressed at a higher level in the pituitary. Presumably, a *gsp* mutation on a paternal allele would be silent in most cases.

Nonetheless, all gsp^+ tumors do express some $G_s\alpha$ (often considerably) from the (nonmutated) paternal allele. This could reflect either an absolute need for some normal $G_s\alpha$ function within the cell or a secondary relaxation of imprinting as part of the tumorigenic process. In support of the latter interpretation is our finding that a similar relaxation of $G_s\alpha$ imprinting is also seen at least as frequently in gsp^- tumors. Therefore, for gsp^+ tumors we favor a model in which the monoallelic origin of $G_s\alpha$ in normal pituitary dictates that the initial *gsp* mutation must occur on a maternal allele in order to have a phenotypic effect, but that during subsequent tumor growth this monoallelic expression pattern is lost. As a caveat, it is not known whether the gsp^+ and normal mRNAs are equally stable. If gsp^+ transcripts were less stable, then the steady-state preponderance of maternal (mutated) over paternal (normal) mRNAs would be less pronounced than in normal cells. Nonetheless, such a mechanism would not easily explain the variability in maternal/paternal ratio shown in Figure 2b and also cannot explain the apparent relaxation of imprinting in gsp^- tumors.

Acknowledgments

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