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Paternal imprinting of $G\alpha_s$ in the human thyroid as the basis of TSH resistance in pseudohypoparathyroidism type 1a

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Abstract

Albright hereditary osteodystrophy (AHO) is characterized by multiple somatic defects secondary to mutations in the *GNAS1* gene. AHO patients with mutations on maternally inherited alleles are resistant to multiple hormones (e.g., PTH, TSH), a variant termed pseudohypoparathyroidism (PHP) type 1a, due to presumed tissue-specific paternal imprinting of the α chain of G_s as demonstrated in murine renal proximal tubule and fat cells. Studies in human tissues thus far revealed imprinting only in pituitary. Because mild hypothyroidism due to TSH resistance occurs in most PHP type 1a patients, we investigated whether $G\alpha_s$ is imprinted in thyroid. Examination of eight normal thyroids demonstrated significantly greater expression from the maternal *GNAS1* allele, with paternal $G\alpha_s$ transcripts accounting for only 25.9–40.4%. Expression of NESP55, $XL\alpha_s$, and 1A was uniallelic. We conclude that $G\alpha_s$ is incompletely imprinted in the thyroid, which provides an explanation for mild TSH resistance in PHP type 1a.

Keywords: Imprinting; GNAS1; G proteins; Genetics; Hormones; Pseudohypoparathyroidism; Albright hereditary osteodystrophy

Albright hereditary osteodystrophy (AHO) is a genetic disorder characterized by somatic defects that include short stature, obesity, delayed puberty, subcutaneous ossifications, and brachydactyly [1]. It is caused by heterozygous inactivating mutations in the *GNAS1* gene that lead to reduced expression or function of the α chain of G_s, the heterotrimeric G protein that couples heptahelical receptors to stimulation of adenylyl cyclase (reviewed in [2,3]). AHO patients with *GNAS1* mutations on maternally inherited alleles [4] also manifest resistance to multiple hormones such as PTH, TSH, gonadotropins, and glucagon [2], a condition termed pseudohypoparathyroidism type 1a (PHP type 1a). By contrast, AHO patients with *GNAS1* mutations on paternally inherited alleles have only the phenotypic features of AHO without hormonal resistance, a condition termed pseudopseudohypoparathyroidism (pseudoPHP) [5]. This inheritance pattern, derived from analysis of published pedigrees, implicated genomic imprinting of the *GNAS1* gene as a possible regulatory mechanism [4,6].

The *GNAS1* locus located at chromosome 20q13.2 and the syntenic mouse *Gnas* locus at distal chromosome 2 exhibit a very complex pattern of genomic imprinting. The *GNAS1* gene consists of 13 exons that encode $G\alpha_s$. Upstream of exon 1 of *GNAS1* are three alternative first exons [7–9] that each splice onto exons 2–13 to create novel transcripts: (1) $XL\alpha_s$ that is expressed only from the paternal allele [7,8] and encodes a signaling protein that stimulates adenylyl cyclase but lacks a known receptor [10,11]; (2) NESP55 that is expressed only from the maternal allele and encodes a secretory protein [7,8,12,13]; and (3) the exon 1A (associated first exon) transcript that is derived from the paternal allele and does not encode a known protein [9,14,15]. Each of these alternative first exons is

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imprinted, and the promoter regions contain differentially methylated regions (DMR) that are methylated on the non-expressed allele [7,8,14,16].

Paternal imprinting of *GNAS1* provides an explanation for the non-Mendelian inheritance pattern of PHP type 1a and pseudoPHP. However, experimental evidence for imprinting of $G\alpha_s$ in human tissues is lacking, as patients with PHP type 1a and pseudoPHP have a similar 50% reduction in $G\alpha_s$ expression in all tissues examined [2,17]. In virtually all human tissues examined thus far, $G\alpha_s$ expression has been found to be biallelic [7,8,18,19]. This biallelic expression is consistent with the apparent absence of DMR within/near the promoter region of $G\alpha_s$ [16,20]. However, recent evidence has shown that the maternal $G\alpha_s$ allele is preferentially expressed in the human pituitary, suggesting "relaxed" paternal imprinting in this tissue [21].

Murine models of AHO were developed through disruption of a single *Gnas* gene in murine embryonic stem cells [22,23], and have revealed an apparent tissue-specific pattern of $G\alpha_s$ imprinting. Analysis of heterozygous *Gnas*+/− knockout mice has demonstrated that $G\alpha_s$ expression is derived primarily from the maternal allele in imprinted cells, such as the renal proximal tubule and fat cells, but is derived from both alleles in other tissues examined [22,24,25]. Thus, mice that inherit a defective maternal *Gnas* allele have markedly reduced levels of $G\alpha_s$ protein in paternally imprinted tissues. In contrast, $G\alpha_s$ levels are 50% of normal in non-imprinted tissues of *Gnas*+/− mice whether they have inherited a defective maternal or paternal *Gnas* allele, as both alleles are active in these tissues. Because mild TSH resistance occurs in most patients with PHP type 1a [26], we sought to determine whether $G\alpha_s$ expression is paternally imprinted in the thyroid. Here, we demonstrate that $G\alpha_s$ expression derives predominantly from the maternal *GNAS1* allele in the human thyroid. These results are consistent with incomplete paternal imprinting of $G\alpha_s$, and provide an explanation for mild TSH resistance in patients with PHP type 1a.

Materials and methods

Thyroid tissue was obtained from 16 subjects who underwent subtotal thyroidectomy for excision of a thyroid nodule. Histological examination confirmed that thyroid sections used in this study consisted of normal tissue and contained at least 90% follicular epithelial cells. The protocol was approved by the Institutional Review Boards of Washington Hospital Center and The Johns Hopkins Hospital. Total RNA was isolated using the Trizol reagent according to the manufacturer's instructions (Gibco/BRL). First strand cDNA was synthesized from 1 μg of total RNA using Superscript II (Gibco-BRL) reverse transcriptase and random hexamers according to the manufacturer's recommendations. We amplified the transcript-specific PCR products that contained the *GNAS1* exon 5 *Fok*I polymorphism [27] using a common reverse primer corresponding to nucleotide sequences in exon 6 and specific forward primers corresponding to nucleotide sequences in the first exons for NESP55, $XL\alpha_s$, exon 1A, and $G\alpha_s$. These primers and the PCR conditions are described in Table 1. To optimize the likelihood of distinguishing differences between levels of maternally derived and paternally derived transcripts, PCR was performed using a number of cycles that yielded products within the linear range of amplification (data not shown). After PCR, 15 μl samples were electrophoresed through 6% acrylamide gels and after staining with ethidium bromide, appropriate-sized DNA bands were isolated and purified. DNA fragments were sequenced directly with the USB Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, OH). Quantification of band intensity was performed with the PhosphorImager system (Bio-Rad). To control for the variability in sample loading between the lanes, eight bands surrounding the polymorphic band were quantified (for both the T and C lanes). The mean value for each was calculated and the background density subtracted such that these lanes could be normalized to one another and adjusted for this loading variability. For the maternal and paternal alleles (determined from the NESP55 and $XL\alpha_s$ sequences, respectively) the densities were adjusted for this normalization. The relative expression of the maternal and paternal alleles was then calculated as a percentage of the normalized values for each of the two alleles. Results are presented as the means ± SEM, and measurements were compared with the unpaired Student's t test. p values smaller than 0.05 were considered significant. The relative expression of the paternal allele was calculated as the proportion of the paternal allele out of the sum of the normalized values for both alleles.

Results

We isolated RNA from normal regions of thyroids that had been removed at surgery from individuals with thyroid nodules. We then performed RT-PCR using an upstream primer specific for each of the four alternative first exons of *GNAS1* that correspond to transcripts for NESP55, $XL\alpha_s$, 1A, and $G\alpha_s$. A common downstream primer was within exon 6 of *GNAS1* to genotype alleles using a highly variable single nucleotide polymorphism in codon 131 (T/C) of exon 5 (Fig. 1). Eight of 16 subjects were heterozygous for this polymorphism and were therefore informative. In each case expression was uniallelic for NESP55 (maternal), $XL\alpha_s$ (paternal), and 1A (paternal) (data not shown), as is consistent with other studies [7,8,14].

RT-PCR analysis of $G\alpha_s$ mRNA revealed expression of both maternal and paternal alleles in these samples. However, further analysis of $G\alpha_s$ transcripts revealed that the intensity of the radiolabeled bands corresponding to the T and C nucleotides was not equivalent, indicating that expression of the maternal and paternal alleles was not equal (Fig. 2). In all eight cases there was greater expression of the maternal $G\alpha_s$ allele, which coincided with uniallelic expression of NESP55 transcripts. Expression of the maternal allele compared to the paternal allele was 0.713 ± 0.025 versus 0.309 ± 0.017 ($p < 0.0001$; Fig. 3). The range of expression of the paternal allele was 25.9–40.4% (Fig. 4). The preferential expression of the maternal allele was consistent in all eight samples studied, and was similar whether the maternal genotype was T (two cases) or C (six cases).

Table 1
RT-PCR of *GNAS1* transcripts

Transcript and primers	PCR conditions	Product size (bp)
NESP55 F 5′-GTCACTAATGGAGGACGCCGT-3′ R 5′-TTCGTAGCAGGCACGCACTCCTTCAACCTC-3′	94 °C × 3 min; 94 °C × 30 s; 55 °C × 30 s; 72 °C × 1 min; ×39 cycles; 72 °C × 5 min	400
XLα_s F 5′-GGATGCCTCCGCTGGTTTGAGCATCGGG-3′ R 5′-TTCGTAGCAGGCACGCACTCCTTCAACCTC-3′	94 °C × 3 min; 94 °C × 30 s; 55 °C × 30 s; 72 °C × 1 min; ×39 cycles; 72 °C × 5 min	818
Exon 1A F 5′-GGACACTCAGTCGCGTCGGCAC-3′ R 5′-TTCGTAGCAGGCACGCACTCCTTCAACCTC-3′	95 °C × 3 min; 94 °C × 30 s; 60 °C × 3 min; ×35 cycles; 68 °C × 5 min	510
Gα_s F 5′-ATGGGCTGCCTCGGGAACAGT-3′ R 5′-TTCGTAGCAGGCACGCACTCCTTCAACCTC-3′	95 °C × 3 min; 94 °C × 30 s; 66 °C × 2 min; ×31 cycles; 68 °C × 5 min	482

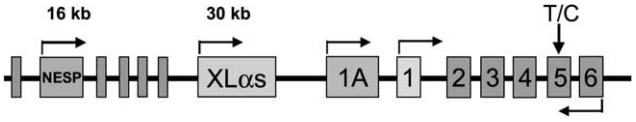

Fig. 1. The *GNAS1* gene complex. The *GNAS1* gene complex consists of 13 exons that encode the signaling protein Gα_s. Upstream of exon 1 are three alternative exons, labeled exon 1A, XLα_s, and NESP55. These three alternative first exons are spliced to exons 2–13 to produce unique transcripts. NESP55 is transcribed exclusively from the maternal allele; XLα_s and exon 1A are transcribed exclusively from the paternal allele. RT-PCR using an upstream primer specific for each of the four alternative first exons of *GNAS1* and a common downstream primer within exon 6 of *GNAS1* enabled us to genotype the alleles using a highly variable single nucleotide polymorphism in codon 131 (T/C) of exon 5.

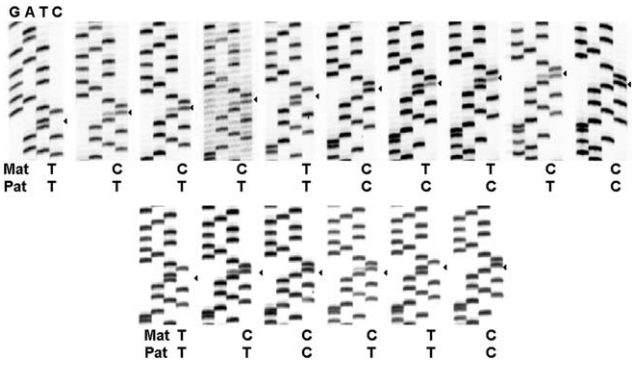

Fig. 2. Sequence analysis of the product of RT-PCR of Gα_s exon 5 derived from thyroid RNA from 16 subjects. Eight of the 16 subjects were heterozygous for the polymorphism in codon 131 (T/C). The intensity of the radiolabeled bands corresponding to the T and C nucleotides for each of these eight subjects was not equivalent, thereby indicating unequal expression of the two alleles.

Discussion

Expression of Gα_s is biallelic in most tissues [7,8,18], but recent studies show that preferential expression of the maternal allele occurs in the human pituitary [21]

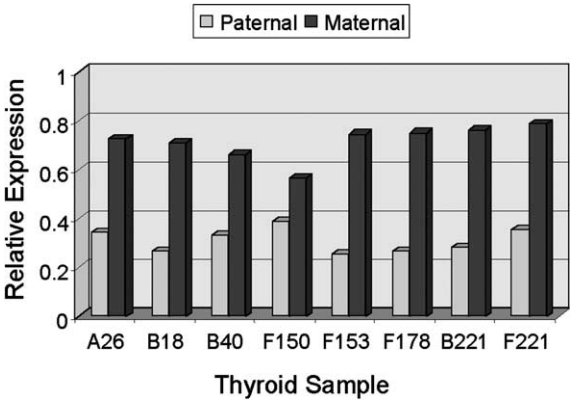

Fig. 3. Relative expression of Gα_s alleles in normal human thyroid. Quantification of band intensity of the polymorphic alleles was performed with the Phosphorimager system (Bio-Rad) and corrected for variability of sample loading between lanes. In all eight subjects heterozygous for the exon 5 polymorphism there was significantly greater expression of the maternal allele compared to the paternal allele; the mean ± SEM was calculated for the maternal and paternal alleles (0.713 ± 0.025 versus 0.309 ± 0.017).

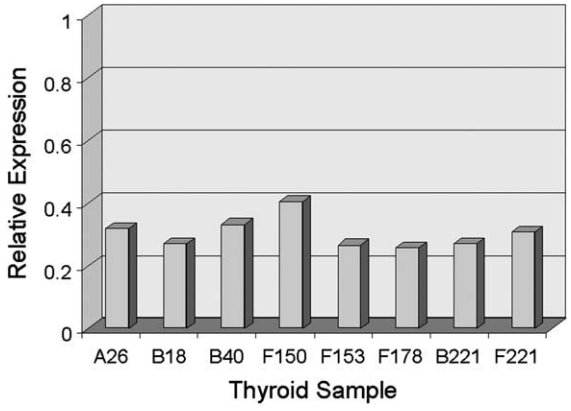

Fig. 4. Relative expression of paternal Gα_s alleles in normal human thyroid. The relative expression of the paternal allele was calculated as the proportion of the paternal allele out of the sum of the normalized values for both alleles. The range of expression of the paternal allele was 25.9–40.4%.

and in murine renal proximal tubule cells and adipocytes [22]. The tissue-specific paternal imprinting found in murine renal proximal tubule cells leads to PTH resistance in *Gnas*+/− mice that have inherited the defective *Gnas* allele maternally [22], and a similar mechanism has been proposed to explain PTH resistance in humans with PHP type 1a. Because most patients with PHP type 1a also have mild TSH resistance, we investigated the possibility that $G\alpha_s$ is paternally imprinted in the human thyroid. We report here that there is incomplete (i.e., "relaxed") paternal imprinting of *GNAS1* in the human thyroid, with preferential expression of the maternal allele compared to the paternal allele. The proportion of paternal $G\alpha_s$ transcripts ranged from 25.9% to 40.4% in the thyroid samples we analyzed.

The incomplete paternal imprinting of $G\alpha_s$ in the thyroid is consistent with previous observations of $G\alpha_s$ expression in the human pituitary as well as in murine renal proximal tubule cells [22]. Although the majority of expression of $G\alpha_s$ in these tissues derives from the maternal allele, there is some expression from the paternal allele. In the four adult pituitary samples reported by Hayward et al. [21] the proportion of paternal $G\alpha_s$ transcripts was as high as 14%. The expression of $G\alpha_s$ mRNA in renal proximal tubule cells of *Gnas* knockout mice that had inherited a defective *Gnas* allele maternally was $38 \pm 4\%$ normal and represents transcription derived from the normal (but presumably imprinted) paternal allele [22]. By contrast, $G\alpha_s$ expression was $89 \pm 9\%$ normal in mice that inherited a defective *Gnas* allele paternally [22]. Therefore, although there appears to be tissue-specific repression of transcription from the paternal $G\alpha_s$ allele, this effect is incomplete.

The partial imprinting of the paternal $G\alpha_s$ allele in the human thyroid could explain the development of mild TSH resistance seen in almost all patients with PHP type 1a [26]. In PHP type 1a, TSH levels are commonly elevated at birth and often precede the development of elevated levels of PTH (and PTH resistance) by several years [28–30]. Typically TSH levels are only mildly elevated, and serum levels of thyroid hormone are normal or only slightly reduced. PHP type 1a patients do not have circulating anti-thyroid antibodies, and do not develop a goiter despite elevated serum levels of TSH [26], a finding consistent with impaired responsiveness to TSH due to decreased expression of $G\alpha_s$ protein. A previous study of a single patient with PHP type 1a with mild primary hypothyroidism demonstrated reduced, but not absent, levels of $G\alpha_s$ protein in plasma membranes prepared from thyroid tissue [31]. Basal as well as sodium fluoride-stimulated adenylyl cyclase activities in these membranes were normal, indicating that the number of $G\alpha_s$ molecules in these membranes, although reduced by comparison to normal, was still sufficient when directly activated to stimulate adenylyl cyclase maximally. By contrast, 100 nM TSH stimulated adenylyl cyclase activity 2.7-fold in membranes from the patient, whereas a 10-fold increase in activity was detected in membranes from normal subjects. These results are consistent with the current modes of stoichiometry and compartmentalization in G protein-coupled receptor signaling [32]. In the case of the β-adrenergic receptor-coupled signal cascade, the ratio of β-adrenergic receptor-G_s-adenylyl cyclase is approximately 1:100:3, and receptor activation of G_s appears to be the critical factor for amplification of signaling [33–35]. Although this model would predict that either receptor or adenylyl cyclase, but not G_s, would set the limit on the maximal efficiency of the system, this analysis fails to consider the effect of potential compartmentalization of signaling molecules in the plasma membrane. Thus, our current results, taken in the context of previous observations that demonstrated reduced TSH-stimulated adenylyl cyclase in thyroid membranes from a patient with PHP type 1a [31], argue that in PHP type 1a an insufficient number of $G\alpha_s$ molecules are physically associated with TSH receptors to assure normal TSH signaling.

In the mouse, paternal imprinting of $G\alpha_s$ occurs in the proximal renal tubules, which accounts for the development of PTH resistance in *Gnas*+/− mice inheriting the mutated maternal allele and normal PTH responsiveness in *Gnas*+/− mice inheriting the mutated paternal allele [22]. Although these results provide an explanation for the development of PTH resistance in human subjects with PHP type 1a, studies of *GNAS1* expression in human fetal tissues, including kidney cortex, have failed to demonstrate paternal imprinting of $G\alpha_s$ [19]. Rather than discard the notion that imprinting, with reduced expression of $G\alpha_s$, accounts for hormone resistance in PHP type 1a, we believe our present results may offer an alternative explanation. Our results show biallelic expression of *GNAS1* in the thyroid, but with preferential maternal expression rather than complete repression of paternal allelic transcription. Similar results have been demonstrated in GH-secreting tumors and normal pituitaries [21] and in the renal cortex of *Gnas*+/− mice [22]. Thus, it is likely that the relatively heterogeneous population of cells examined, including not only proximal renal tubular areas but also condensing mesenchyme and developing nephrons, as well as the number of amplification cycles and non-quantitative technique used to analyze PCR products, may have accounted for the failure to detect preferential expression of the maternal allele in previous studies of fetal human kidney cortex [19]. In addition, fetal human kidney cortex may be quite different from the postnatal kidney cortex.

The absence of typical features of an imprinted transcription unit, as well as the incomplete repression of paternal allele transcription, suggest that $G\alpha_s$ transcription is regulated by a secondary mechanism such as

the insulator model that has been proposed to explain reciprocal expression of the H19 and IGF2 genes [36]. Further evidence in support of this model comes from recent studies of patients with PHP type 1b that associate loss of methylation of the exon 1A DMR with reduced expression of $G\alpha_s$ in the renal cortex [14,15,20].

In conclusion, our results demonstrate relaxed paternal imprinting of $G\alpha_s$ in human thyroid. Although the precise molecular mechanism for the temporal and spatial paternal imprinting of $G\alpha_s$ is not completely defined, cell-specific paternal imprinting provides an explanation for the unusual pattern of inheritance and distribution of hormone resistance in PHP type 1a.

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