

The Gs α Gene: Predominant Maternal Origin of Transcription in Human Thyroid Gland and Gonads

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Mutations in the guanine nucleotide binding α -subunit 1 gene (GNAS1) cause Albright's hereditary osteodystrophy, and the parent of transmission determines variable phenotypic expression of the disease. This has suggested that GNAS1 may be under tissue-specific imprinting control, although studies so far available have failed to clearly define the pattern of GNAS1 expression in humans. To establish if GNAS1 is imprinted in human endocrine tissues, we selected 14 thyroid, 10 granulosa cell, 13 pituitary (3 normal glands, 7 GH-secreting adenomas, and 3 nonfunctioning adenomas), 3 adrenal, and 11 lymphocyte samples shown to be heterozygous for a known polymorphism in exon 5. RNA from these tissues was analyzed by RT-PCR, and expression from both parental alleles was evaluated by enzymatic digestion and subsequent quantification of the resulting fragments. The parental origin of Gs α was

assessed by evaluating neuroendocrine secretory protein 55 and extra large α s-like protein transcripts, which have been shown to be monoallelically and parent-specifically expressed from the maternal and paternal allele, respectively. By this approach, the great majority of thyroid ($n = 12$), ovarian ($n = 7$), and pituitary ($n = 11$) samples showed an almost exclusive or significantly predominant expression of the maternal allele over the paternal one, whereas in lymphocyte and adrenal samples both alleles were equally expressed. Our results provide evidence for a predominant maternal origin of GNAS1 transcripts in different human adult endocrine tissues, particularly thyroid, ovary, and pituitary, and strongly suggest that this mechanism may play a crucial role in the determination of the phenotypic expression of Albright's hereditary osteodystrophy. (*J Clin Endocrinol Metab* 87: 4736–4740, 2002)

GENOMIC IMPRINTING IS an epigenetic phenomenon affecting a small number of genes by which one allele (maternal or paternal) undergoes, either during the embryogenesis or in the postnatal period, a partial or total loss of expression (1). In humans, imprinting plays a role in the pathogenesis of various disorders, such as Angelman, Beckwith-Wiedeman, and Prader-Willi syndromes. More recently, Liu *et al.* (2) identified in patients affected with pseudohypoparathyroidism (PHP) type Ib (3) an imprinting defect of guanine nucleotide binding α -subunit 1 gene (GNAS1), which is likely to be involved in the pathogenesis of the disease.

GNAS1 maps on human chromosome 20q13 and on mouse distal chromosome 2. Both regions have been recently shown to be under complex imprinting control (4–8). In fact, by using alternative promoters and first exons splicing into a common site in exon 2, the GNAS1 locus gives rise not only to the Gs α gene, but to at least three other gene products, *i.e.* the extra large α s-like protein (XL α s) (9), the neuroendocrine secretory protein 55 (NESP55) (10) and a nontranslated transcript deriving from Exon 1A (11) (Fig. 1). A fourth antisense transcript is believed to play a role in regulating NESP55 expression (12). The XL α s, antisense, and 1A transcripts are expressed only from the paternal allele, whereas NESP55 is expressed specifically from the maternal allele.

Heterozygous loss of function mutations in GNAS1 in-

herited from the mother lead to PHP type Ia, in which Albright's hereditary osteodystrophy (AHO), a disorder characterized by a constellation of physical features, is associated to end organ resistance to the action of different hormones, primarily PTH, TSH, and gonadotropins. Interestingly, when the same mutations are inherited from the father, patients show the physical abnormalities of AHO, without any evidence of hormone resistance (pseudo-pseudohypoparathyroidism, PPHP) (13, 14).

Although the pattern of inheritance of PHP Ia is consistent with imprinting of GNAS1 paternal allele, different expression studies on RNA from various human fetal tissues have failed to demonstrate monoallelic expression of this gene (15, 16). Consistent with the view that genomic imprinting may be a phenomenon restricted to selective tissues and evolving in postnatal life, monoallelic expression of the Gs α gene has been recently shown in the human adult pituitary, although this gland is not classically included among the target organs resistant to hormone action in AHO (17–19). The aim of this study was to investigate Gs α allelic expression and parental origin in human adult endocrine tissues that are differentially involved in the determination of PHP Ia phenotype.

Materials and Methods

Tissues

The study included 25 normal perinodular thyroid tissues obtained from patients undergoing surgery for multinodular goiter or toxic adenoma; 22 granulosa cell specimens (from ovarian follicular fluid) from patients undergoing *in vitro* fertilization because of idiopathic infertility; 27 pituitary tissues, including 6 normal glands obtained at autopsy, 13 GH-secreting and 7 nonfunctioning pituitary adenomas surgically removed from patients with pituitary adenomas; 6 normal perinodular

Abbreviations: AHO, Albright's hereditary osteodystrophy; GNAS1, guanine nucleotide binding α -subunit 1 gene; NESP55, neuroendocrine secretory protein 55; PHP, pseudohypoparathyroidism; PPHP, pseudo-pseudohypoparathyroidism; XL α s, extra large α s-like protein.

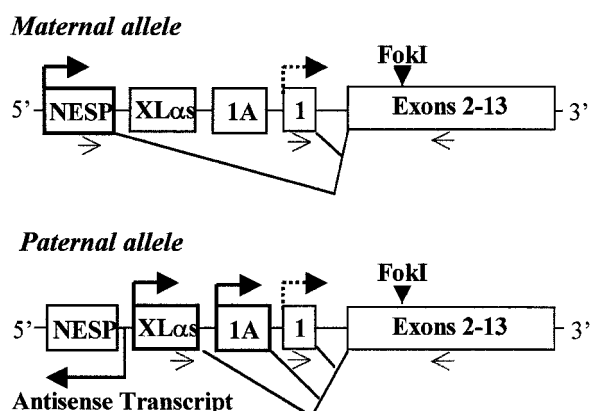


FIG. 1. Genomic organization of human GNAS locus. The figure shows four alternative first exons that splice into exon 2, generating four different transcripts: Gs α , NESP55, XLas, and a noncoding gene product from exon 1A. Both the maternal and paternal alleles are shown: arrows designate transcription start sites. An additional antisense transcript has been recently demonstrated to be expressed only from the paternal allele. The arrowhead marks the polymorphic FokI site in exon 5; gray arrows indicate RT-PCR primers.

adrenal glands obtained from patients undergoing surgery for benign cortical adenomas, and 20 blood samples from normal subjects.

Informed consent was obtained in all cases, and prior project approval was given by the local ethic committee.

PCR, RT-PCR, and digestions

DNA and RNA were extracted from all samples with standard methods. Exon 5 (GenBank accession no. M21741 J03647) was amplified from genomic DNA by PCR using the primers and under the conditions described previously (sense: 5'-ATGAAAGCAGTACTCCTAACTGA-3'; antisense: 5'-TGGATGCTCCTGCCCATGTG-3') (20). The amplified products were then screened by digestion for a silent T to C polymorphism (Ile 131, nucleotides 297–299) that creates an FokI site (1 h at 37°C, with 1 U of enzyme). Heterozygosity for this polymorphism was then confirmed by direct sequencing (ABI-PRISM 310, PE Applied Biosystems, Foster City, CA).

To examine allele-specific gene expression, 5 μ g total RNA were reverse transcribed (Promega Corp., Madison WI) and then subjected to PCR (28 cycles at 94/58/72°C for 45/45/45 sec) using a common downstream primer (5'-CCTTGGCATGCTCATAGAATTC-3') (5) located in exon 6 and 3 exon 1-specific upstream primers amplifying Gas (GenBank accession no. M21139 J03647, nucleotides 789–807), XLas (GenBank accession no. AJ 251760, nucleotides 16000–16021), and NESP55 (GenBank accession no. AJ 251760, nucleotides 1432–1451) genes, respectively (Gas: 5'-CCATGGGCTGCCTCGGGAACA-3'; XLas: 5'-CGCAGTAAGCTCATCGACAAAG-3'; NESP55: 5'-AGCCCGAGGACAAAGATCCA-3'). RT-PCR products were digested with FokI as for genomic DNA and visualized on 2% Nusieve-1% agarose gels. Bands from Gs α RT-PCR were finally evaluated by an imaging densitometer (GS-700, Bio-Rad Laboratories, Inc., Richmond, CA), and the maternal contribution was calculated as a percentage of the sum of the normalized values for the two alleles.

Somatotroph adenomas were also screened for somatic mutations at codon 201 or 227 of the Gas gene (the *gsp* oncogene) as previously described (21).

Results

The evaluation of parental origin of transcription of the Gs α gene was carried out on samples found to be heterozygous for the polymorphism in exon 5, that is 14 thyroid, 10 granulosa cell, 13 pituitary (3 normal glands, 7 GH-secreting adenomas, 3 of which were *gsp*+, and 3 nonfunctioning adenomas), 3 adrenal, and 11 lymphocyte samples. The pa-

rental origin of Gs α transcripts was established by FokI digestion and direct sequencing of NESP55 and XLas transcripts. Both analyses showed the expected monoallelic expression from opposite alleles. In particular, when a NESP-derived transcript showed to carry the polymorphism, *i.e.* the correspondent band was digested by FokI, the XLas-derived transcript from the same sample always displayed a wild-type sequence and was not digested by the same enzyme. In contrast, Gs α -derived transcripts were always biallelically expressed but, with the exception of two samples, all thyroid tissues showed a significant predominant expression of the maternal allele over the paternal one (Figs. 2A and 4), *i.e.* the most expressed allele was always the same represented in NESP55 transcripts (ratio maternal/total $\times 100 \pm$ SD: $75.7 \pm 13.4\%$). In particular, in five samples we observed a striking predominance of the maternally derived transcript ($89.6 \pm 4.4\%$), whereas in the other seven this phenomenon was seen at a lesser but still significant extent ($72.7 \pm 4.3\%$). Moreover, in two patients we could compare Gs α allelic expression between thyroid gland and lymphocytes from peripheral blood. As shown in Fig. 2B, the predominance of the maternal allele observed in the thyroid was absent in lymphocyte samples.

Seven of 10 granulosa cell samples showed a less striking but still significant predominance of the maternal allele ($68.8 \pm 6.7\%$) (Figs. 3 and 4).

A striking predominance of the maternal allele was observed in both normal and adenomatous pituitary tissues. In particular, all the normal pituitaries ($n = 3$) displayed an almost exclusive Gs α expression from the maternal allele ($92.3 \pm 3\%$), with a maximum 11% contribution from the paternal one. With the exception of two adenomas (one *gsp*-GH-secreting and one nonfunctioning tumor) in which the two alleles were equally expressed, a similar pattern of expression was demonstrated in the adenomatous samples ($87.8 \pm 7.8\%$), with no difference observed between nonfunctioning and GH-secreting adenomas (either *gsp*+ or *gsp*-) (Figs. 2C and 4).

In all adrenal and lymphocyte samples, both the paternal and the maternal alleles were expressed at the same extent (Fig. 4).

Discussion

Our study first demonstrates that, in specific endocrine tissues, GNAS1 transcription mainly derives from the maternal allele. In particular, a predominant, though not exclusive, maternal origin of Gs α was observed in adult human thyroid, ovarian cells and pituitary, whereas in the adrenal both the paternal and maternal alleles were equally expressed. The cell specificity of GNAS1 imprinting was strongly supported by the experiments showing a similar maternal and paternal contribution to GNAS1 expression in lymphocytes obtained from subjects in whom the removed thyroid only expressed the maternally derived GNAS1. In tissues mainly expressing the maternal allele, the predominance of the maternally derived transcript occurred at a variable extent in different samples, being almost exclusive in the majority of pituitary samples, largely predominant in most thyroid samples and less striking, but still significantly

FIG. 2. *GNAS1* expression patterns in different human tissue samples derived from RT-PCR of mRNA. In each figure, *arrows* indicate undigested and digested bands respectively. Double bands observed in all samples are due to alternative splicing of exon 3, characteristic of all *GNAS1* transcripts. A, Expression of *Gsα*, *NESP55*, and *XLαs* genes after 1 h digestion with *FokI* in four different thyroid (T) samples heterozygous for the polymorphism in exon 5. With the exception of sample T5, which shows a comparable intensity of bands from the two alleles, the others display a striking predominance of one allele over the other. In all cases, study of *NESP55* and *XLαs* expression indicated the maternal allele to be the predominant one. Uncut (U) and cut (C) controls are also shown in the figure. B, Comparison of *Gsα* expression in thyroid (T) and lymphocytes (L) from two samples: the predominance of maternal origin of transcription observed in thyroid samples appear to be lost in lymphocyte ones, thus providing evidence for tissue-specific imprinting of this gene. C, Analysis of allelic expression of *GNAS1* transcripts in human pituitary glands: N, normal pituitary; NA, nonfunctioning adenoma; *gsp*⁺ and *gsp*[−], GH-secreting adenoma positive and negative, respectively, for mutations of the *gsp* oncogene.

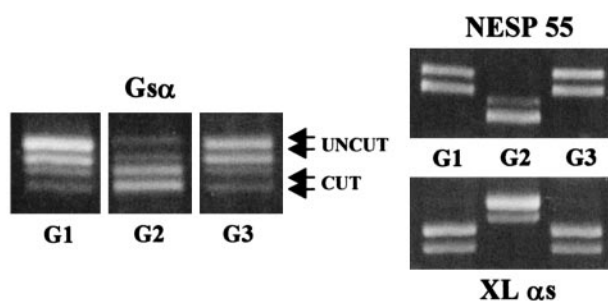
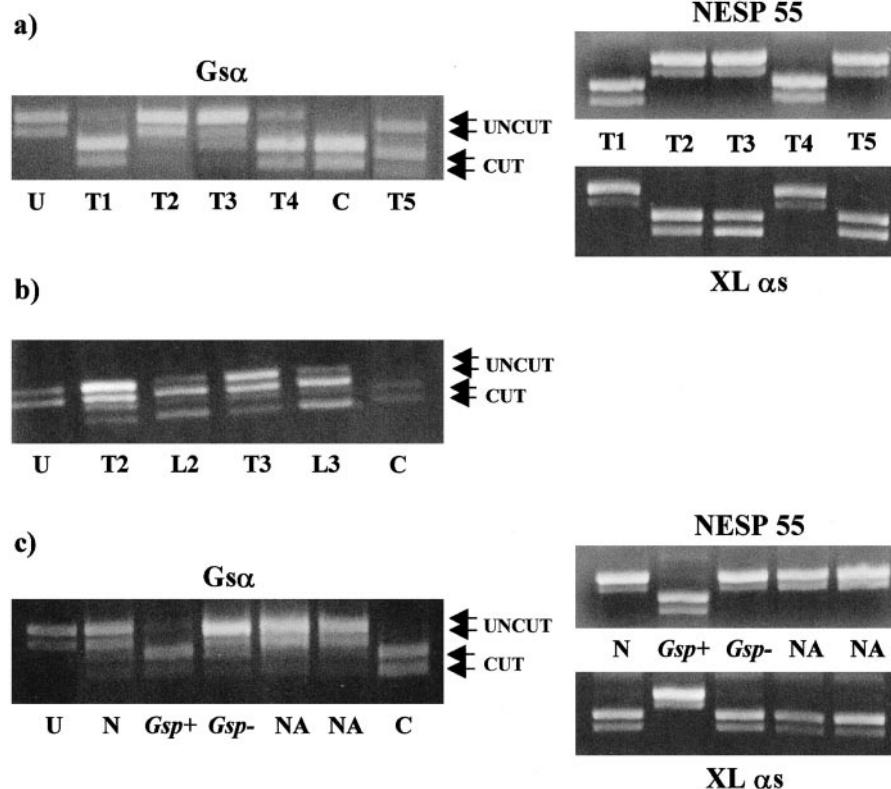


FIG. 3. Expression of *GNAS1*-derived transcripts (*Gsα*, *NESP55*, and *XLαs*) in granulosa cell extracts (G1, G2, and G3); as observed in thyroid and pituitary samples, the maternal allele resulted to be more expressed respect to the paternal one. However, densitometric evaluation of these bands demonstrated a higher contribution from the paternal allele compared with the one seen in the pituitary and the thyroid (see *Results* for more details).

higher than the paternally derived transcript, in granulosa cells. This variable pattern of *GNAS1* imprinting is consistent with the incomplete transcriptional repression of other imprinted genes that have been previously observed in individual tissues (22–24), indicating that imprinting is not necessarily an all-or-nothing phenomenon. Mechanisms underlying these findings include either biallelic expression by a subset of cells within a tissue or an only partial silencing of one allele in all cells of such tissues. Moreover, the difference observed among different samples, particularly among granulosa cell preparations, might also be, at least partially, attributed to the different degree of contamination of tissue samples by the peripheral blood, which biallelically expressed *GNAS1*.

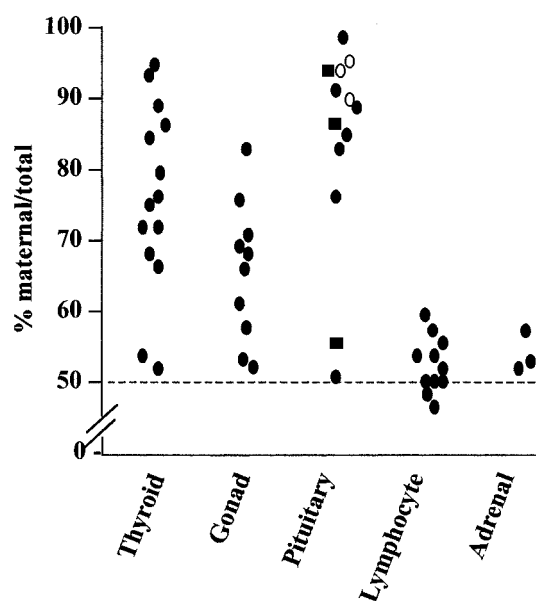


FIG. 4. The diagram shows the estimated percentage of maternally derived transcripts in 14 thyroid glands, 10 gonads (granulosa cells), 12 pituitary glands (*open circles*, normal ones; *filled circles*, somatotroph adenomas; *filled squares*, nonfunctioning adenomas), 11 lymphocyte samples, and 3 adrenal glands.

The data reported here strongly support the hypothesis that imprinting of the *Gsα* gene is the potential mechanism responsible for occurrence of variable resistance to hormone action in patients with *GNAS1* mutations. First, the predominant, though not exclusive, maternal origin of *Gsα* is consistent with the pattern of inheritance of PHP Ia. Indeed,

mutations in GNAS1 inherited from the mother lead to PHP type Ia, in which AHO is associated with multiple hormone resistance, whereas the same mutations inherited from the father lead to PPHP in which AHO is not accompanied by any evidence of hormone resistance (13, 14). Although imprinting of GNAS1 has been proposed to explain the occurrence of PHP Ia and PPHP in patients with AHO (14), expression studies carried out up to now in various human fetal tissues have failed to demonstrate monoallelic expression of this gene (15, 16). The recent report of GNAS1 biallelic expression in human kidney cortex (16) is only apparently in contrast with our findings. In fact, Zheng *et al.* (16) carried out their study on fetal tissues, and it has been demonstrated that imprinting can be a process beginning and evolving in post-natal life (25). This hypothesis is also supported by the observation that PHP Ia patients do not show any sign of hormone resistance at birth, but they generally develop hypocalcemia, hypothyroidism, and hypogonadism over childhood or adolescence. Moreover, the kidney cortex was studied as a whole, whereas data from mice indicate imprinting of GNAS1 as a cell-specific phenomenon, limited to the proximal renal tubule, *i.e.* the main PTH target tissue.

Secondly, imprinting of GNAS1 paternal allele was present in selective tissues, such as the thyroid and the gonad, the two endocrine tissues that, besides the kidney, are mostly affected in PHP Ia patients. The observation of a different extent of maternal contribution to GNAS1 expression in thyroid and gonadal samples may explain why hypothyroidism is present in the totality of patients with PHP Ia, whereas hypogonadism is demonstrated in a lower percentage of cases, though it is possible that the slight differences observed in this still restricted number of samples may not have a clinical impact. In agreement with the notion that, with the exception of a single report (26), patients with PHP Ia show a normal responsiveness to ACTH, both the paternal and maternal allele was equally expressed in the adrenal gland, thus justifying the occurrence of resistance to some (PTH, TSH, and gonadotropins), but not all hormones that activate Gs-coupled pathways in patients with PHP Ia.

The findings of a striking predominance of the maternal allele in normal pituitary glands confirms the recent report by Hayward *et al.* (17). These data are in contrast with what observed in PHP Ia patients, who do not display, with the exception of anecdotal case reports (27–29), any sign of GH deficiency, as it would be expected by the coupling of the GHRH receptor to the cAMP-dependent pathway via Gs protein. However, the observation that GHRH is also effective in triggering intracellular calcium increase in somatotrophs could partially explain the preserved GH secretion in these patients (30). Alternatively, it is also possible to hypothesize a compensatory expression of the paternal allele in the presence of a defective GNAS1 transcription, though it remains unclear why such compensation does not occur in the kidney, the thyroid or the gonad.

Finally, in contrast with the report by Hayward *et al.* (17) of a partial loss of imprinting in somatotroph adenomas, our data from both nonfunctioning and GH-secreting adenomas (either carrying mutations of the *gsp* oncogene or not) are similar to those observed in normal glands. Based on the present study and given the relatively low number of cases

reported in the literature, additional studies are required to confirm a role for GNAS1 imprinting in the pathogenesis of acromegaly.

In conclusion, our study demonstrates a predominant maternal origin of GNAS1 transcripts in different human adult endocrine tissues, particularly in the thyroid and the gonad. These data strongly support the hypothesis that imprinting of the GNAS1 gene is the potential mechanism responsible for occurrence of variable resistance to hormone action in patients with AHO.

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References

- Bartolomei MS, Tilgham SM 1997 Genomic imprinting in mammals. *Annu Rev Genet* 31:493–525
- Liu J, Litman D, Rosenberg MJ, Yu S, Biesecker LG, Weinstein LS 2000 A GNAS1 imprinting defect in pseudohypoparathyroidism type 1b. *J Clin Invest* 106:1167–1174
- Bastepe M, Juppner H 2000 Pseudohypoparathyroidism. New insights into an old disease. *Endocrinol Metab Clin North Am* 29:569–589
- Yu S, Yu D, Lee E, Eckhaus M, Lee R, Corria Z, Accili D, Westphal H, Weinstein LS 1998 Variable and tissue-specific hormone resistance in heterotrimeric Gs protein α -subunit (Gs α) knockout mice is due to tissue-specific imprinting of the Gs α gene. *Proc Natl Acad Sci USA* 95:8715–8720
- Hayward BE, Kamiya M, Strain L, Moran V, Campbell R, Hayashizaki Y, Bonthron DT 1998 The human GNAS1 gene is imprinted and encodes distinct paternally and biallelically expressed G proteins. *Proc Natl Acad Sci USA* 95:10038–10043
- Hayward BE, Moran V, Strain L, Bonthron DT 1998 Bidirectional imprinting of a single gene: GNAS1 encodes maternally, paternally and biallelically derived proteins. *Proc Natl Acad Sci USA* 95:15475–15480
- Peters J, Wroe SE, Wells CA, Miller HJ, Bodle D, Beechey CV, Williamson CM, Kelsey G 1999 A cluster of oppositely imprinted transcripts at the *Gnas* locus in the distal imprinting region of mouse chromosome 2. *Proc Natl Acad Sci USA* 96:3830–3835
- Liu J, Yu S, Litman D, Chen W, Weinstein LS 2000 Identification of a methylation imprint mark within the mouse *Gnas* locus. *Mol Cell Biol* 20:5808–5817
- Kehlenbach RH, Matthey J, Huttner WB 1994 XLas is a new type of G protein. *Nature* 372:804–809
- Ischia R, Lovisetti-Scamihorn P, Hogue-Angeletti R, Wolkersdorfer M, Winkler H, Fischer-Colbrie R 1997 Molecular cloning and characterization of NESP55, a novel chromogranin-like precursor of a peptide with 5-HT_{1B} receptor antagonist activity. *J Biol Chem* 272:11657–11662
- Ishikawa YC, Bianchi B, Nadal-Ginard, Homey CJ 1990 Alternative promoter and 5' exon generate a novel Gs α mRNA. *J Biol Chem* 265:8458–8462
- Hayward B, Bonthron DT 2000 An imprinted antisense transcript at the human GNAS1 locus. *Hum Mol Genet* 9:835–841
- Nakamoto JM, Sandstrom AT, Brickman AS, Christenson RA, Van Dop C 1998 Pseudohypoparathyroidism type 1a from maternal but not paternal transmission of a Gs α gene mutation. *Am J Med Genet* 77:261–267
- Weinstein LS, Yu S 1999 The role of genomic imprinting of Gs α in the pathogenesis of Albright's hereditary osteodystrophy. *Trends Endocrinol Metab* 10:81–85
- Campbell R, Gosdem CM, Bonthron DT 1994 Parental origin of transcription from the human GNAS1 gene. *J Med Genet* 31:607–614
- Zheng H, Radeva G, McCann JA, Hendy GN, Goodyer CG 2001 Gas transcripts are biallelically expressed in the human kidney cortex: implications for pseudohypoparathyroidism type 1b. *J Clin Endocrinol Metab* 86:4627–4629
- Hayward BE, Barlier A, Korbonits M, Grossman AB, Jacquet P, Enjalbert A, Bonthron DT 2001 Imprinting of the Gs gene GNAS1 in the pathogenesis of acromegaly. *J Clin Invest* 107:R31–R36
- Weinstein LS 2001 The stimulatory G protein α -subunit gene: mutations and imprinting lead to complex phenotypes. *J Clin Endocrinol Metab* 86:4622–4626
- Weinstein LS, Yu S, Ecelbarger CA 2000 Variable imprinting of the hetero-

- trimeric G protein $Gs\alpha$ -subunit within different segments of the nephron. *Am J Physiol* 278:F507–F514
20. Mantovani G, Romoli R, Weber G, Brunelli V, De Menis E, Beccio S, Beck-Peccoz P, Spada A 2000 Mutational analysis of *GNAS1* in patients with pseudohypoparathyroidism: identification of two novel mutations. *J Clin Endocrinol Metab* 85:4243–4248
 21. Ballare E, Mantovani S, Lania A, Di Blasio AM, Vallar L, Spada A 1998 Activating mutations of the *Gs α* gene are associated with low levels of *Gs* α protein in growth hormone-secreting tumors. *J Clin Endocrinol Metab* 83:4386–4390
 22. Mutter GL, Stewart CL, Chaponot ML, Pomponio RJ 1993 Oppositely imprinted genes *H19* and insulin-like growth factor II are co-expressed in human androgenetic trophoblast. *Am J Hum Genet* 53:1092–1102
 23. Mohandas TK, Stern HJ, Meeker CA, Passage MB, Muller U, Page DC, Yen PH, Shapiro LJ 1990 Steroid sulfatase gene in XX males. *Am J Hum Genet* 46:369–376
 24. Muller CR, Migl B, Traupe H, Ropers HH 1980 X-linked steroid sulfatase: evidence for different gene-dosage in males and females. *Hum Genet* 54:197–199
 25. Gould TD, Pfeifer K 1998 Imprinting of mouse *Kvlqt 1* is developmentally regulated. *Hum Mol Genet* 7:483–487
 26. Tsai Ks, Chang CC, Wu DJ, Huang TS, Tsai IH, Chen FW 1989 Deficient erythrocyte membrane $Gs\alpha$ activity and resistance to trophic hormones of multiple endocrine organs in two cases of pseudohypoparathyroidism. *Taiwan Yi Xue Hui Zaz Hi* 88:450–455
 27. Shima M, Nose O, Shimizu K, Seino Y, Yabuuchi H, Saito T 1988 Multiple associated endocrine abnormalities in a patient with pseudohypoparathyroidism type Ia. *Eur J Pediatr* 147:536–538
 28. Scott DC, Hung W 1995 Pseudohypoparathyroidism type Ia and growth hormone deficiency in two siblings. *J Pediatr Endocrinol Metab* 8:205–207
 29. Kaji M, Umeda K, Ashida M, Tajima T 2001 A case of pseudohypoparathyroidism type Ia complicated with growth hormone deficiency: recovery of growth hormone secretion after vitamin D therapy. *Eur J Pediatr* 160:679–681
 30. Schofl C, Sandow J, Knepel W 1987 GRF elevates cytosolic free calcium concentration in rat anterior pituitary cells. *Am J Physiol* 253:E591–E594