Published in final edited form as:

Bone. 2017 October ; 103: 177–187. doi:10.1016/j.bone.2017.07.001.

Mice maintain predominantly maternal *Gnas* expression throughout life in brown fat tissue (BAT), but not other tissues

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Abstract

The murine *Gnas* (human *GNAS*) locus gives rise to *Gnas* and different splice variants thereof. The *Gnas* promoter is not methylated thus allowing biallelic expression in most tissues. In contrast, the alternative first *Gnas*/*GNAS* exons and their promoters undergo parent specific methylation, which limits transcription to the non-methylated allele. Pseudohypoparathyroidism type Ia (PHP1A) or type Ib (PHP1B) are caused by heterozygous maternal *GNAS* mutations suggesting that little or no *Gnas* is derived in some tissues from the non-mutated paternal *GNAS* thereby causing hormonal resistance. Previous data had indicated that *Gnas* is mainly derived from the maternal *Gnas* allele in brown adipose tissue (BAT) of newborn mice, yet it is biallelically expressed in adult BAT. This suggested that paternal *Gnas* expression is regulated by an unknown factor(s) that varies considerably with age. To extend these findings, we now used a strain-specific SNP in *Gnas* exon 11 (rs13460569) for evaluation of parent-specific *Gnas* expression through the densitometric quantification of *Ban*II-digested RT-PCR products and digital droplet PCR (ddPCR). At all investigated ages, *Gnas* transcripts were derived in BAT predominantly from the maternal *Gnas* allele, while kidney and liver showed largely biallelic *Gnas* expression. Only low or undetectable levels of other paternally *Gnas*-derived transcripts were observed, making it unlikely that these are involved in regulating paternal *Gnas* expression. Our findings suggest that a cis-acting factor could be implicated in reducing paternal *Gnas* expression in BAT and presumably in proximal renal tubules, thereby causing PTH-resistance if the maternal *GNAS*/*Gnas* allele is mutated.

Keywords

Stimulatory G protein; Imprinting; *GNAS*; Brown adipose tissue

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Disclosure summary

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2017.07.001>.

1. Introduction

The human *GNAS* complex on chromosome 20q13.3 is one of few genetic loci that undergo differential methylation, thus allowing transcription of some mRNAs from only one parental allele [1–6]. *Gnas*, the murine ortholog of *GNAS*, is located on mouse chromosome 2. It shows an organization similar to that of the human homolog and undergoes indistinguishable epigenetic modifications (Fig. 1). Exons 1–12 (1–13 in human) encode the α -subunit of the stimulatory heterotrimeric guanosine 5' triphosphate-binding (GTP-binding) protein (G α s). This ubiquitously expressed signaling protein couples hormonal stimulation of various G protein-coupled receptors (GPCR) to adenylate cyclase, which results in the generation of intracellular cAMP; subsequent activation of protein kinase A (PKA) regulates the functions of proteins further down-stream.

Through the use of different first exons and their promoters, several alternative mRNA variants are generated from *Gnas* that are spliced onto exons 2–12 (2–13 in human). These additional mRNAs encode XL α s (XL α s in human), an extra-large form of G α s [7], Nesp (NESP55 in human), a neuroendocrine secretory protein with incompletely defined biological importance [8,9], and an 1A transcript that may lead to the generation of an amino-terminally truncated G α s [10]. Furthermore, the *Gnas* locus gives rise to AS, a non-coding antisense RNA [11, 12] and an alternative open-reading frame within the XL exon that encodes ALEX, which may antagonize the actions of XL α s [13].

The promoters for the alternative first exons, i.e. XL, 1A, and AS are methylated on the maternal *Gnas* allele, while the promoter for exon Nesp is methylated on the paternal allele; consequently transcription initiated at these exons occurs only at the non-methylated paternal and maternal allele, respectively [1–6]. In addition to these epigenetic marks at the differentially methylated regions (DMR) within *Gnas* (*GNAS*), transcriptional regulation at this locus is further complicated by the fact that the non-methylated exon 1 promoter, which gives rise to G α s, is not equally active for both parental alleles in all tissues. Thus, G α s transcripts are thought to be derived from both parental alleles in most tissues, including distal renal tubules, liver, and bone; in contrast, predominantly maternal G α s expression occurs in proximal renal tubules, thyroid, and pituitary [1,2,14,15], and possibly other organs yet to be identified. Using wild-type mice and animals lacking *Gnas* exon E1 on maternal allele (E1^{mat}−), previous studies had suggested that G α s transcripts are derived in brown adipose tissue (BAT) predominantly from the maternal *Gnas* allele, at least during the immediate post-natal period. In older mice, G α s transcripts appeared to be derived from both parental alleles [15–17]. The converse was observed for G α s transcription in the proximal renal tubules; namely biallelic expression early in life, yet slightly >60% maternal expression by two weeks of life [15]. These findings in wild-type and E1^{mat}− mice, which are consistent with the delayed onset of PTH-resistance in humans affected by either pseudohypoparathyroidism type Ia (PHP1A) or pseudohypoparathyroidism type Ib (PHP1B) [15,18,19] indicated that the factors regulating G α s transcription are not limited to epigenetic mechanisms. In fact, it was suggested that the underlying regulatory events might involve an unknown factor(s), which could be abundantly expressed in tissues such as proximal renal tubules, BAT, and possibly other cell types, where it reduces paternal G α s transcription (“silencer” hypothesis [5,15–17]). Alternatively, such a factor could be required

for the efficient paternal expression of this widely expressed signaling protein (“enhancer” hypothesis); however, this putative regulatory protein may be found only at insufficient amounts in tissues with predominantly maternal *Gas* expression.

Based on available data [15–17], it seemed feasible to identify the as yet unknown *cis* acting factor that limits, age-dependently, paternal *Gas* expression in proximal renal tubules and BAT through RNA sequencing approaches. However, laser capture-based isolation of suitable renal cells for extraction of sufficient RNA quantities is challenging. We therefore considered analyzing BAT-derived RNA using mice of different ages after establishing which ages would provide the largest difference in paternal *Gas* transcription.

2. Materials and methods

2.1. Mice

Three different strains of wild-type mice were investigated, namely 129/Sv (129), C57BL/6 (C57), and CD1 (Jackson Laboratory, Bar Harbor, ME, USA). Animals were maintained at the MGH animal facility according to NIH institutional guidelines (12 h light/dark cycle; free access to water and food). 129 and C57 mice were mated to generate C57/129 and 129/C57 offspring, respectively (convention for matings: female × male). Matings between female mice carrying a floxed *Gnas* exon 1 allele (CD1-E1^{fl/fl}) [17] and wild-type CD57 males, i.e. CD1-E1^{fl/mat}/C57^{pat} animals were also used. Mice carrying a floxed *Gnas* exon 1 allele are in the CD1 background, but carry a homozygous ‘C’ nucleotide due to the introduction of the floxed cassette from the 129 background. Mice carrying a targeted deletion of *Gnas* exon 1 (E1^{mat-/-}) were kindly provided by Dr. Lee Weinstein, NIH [17]. Mice with deletion of this exon on the maternal (E1^{mat-/-}) or the paternal allele (E1^{pat-/-}) were maintained on the FVB background. No statistically significant differences were observed for males and females regarding the parental contribution to *Gas* mRNA or other *Gnas*-derived transcripts; results from both genders were therefore combined.

2.2. RNA isolation, RT-PCR and quantitative RT-PCR (qRT-PCR)

Interscapular BAT, liver, whole kidney, gonadal white adipose tissue (gWAT), heart, and brain were studied from both genders. Animals were sacrificed by an isoflurane overdose, and tissues were removed immediately and snap frozen in liquid nitrogen. Total RNA was isolated using TRIzol (ThermoFisher, Waltham, MA, USA) followed by silica column purification according to the manufacturer's instructions (Ambion Pure Link, Invitrogen, Waltham, MA, USA). After DNAase treatment (42 °C, 2 min), 1 µg RNA was reverse transcribed (37 °C, 15 min) using a combination of oligo-dT and random hexamer primers (TAKARA, Shiga, Japan) followed by inactivation of reverse transcriptase (85 °C, 5 s). Expression of *Nesp*, *Xl*, *1A*, and *Gas* transcripts and primer specificity was analyzed by PCR using transcript specific forward primers (*Nesp* (f1); *Xl* (f2); *1A* (f3); *Gas* (f4)) and a common reverse primer derived from *Gnas* exon 12 (r2) (see Fig. 1); primer sequences are provided in Supplemental Table 1. All cDNAs were amplified using the same PCR cycling profile; denaturation (95 °C, 5 min), 30 cycles of denaturation (94 °C, 30 s) for *Gas* and 40 cycles for the other transcripts, annealing (65 °C, 30 s), and extension (72 °C, 60 s), additional 7-minute extension during the last cycle.

Transcript abundances were determined using the same cDNA-specific forward primers as above (f1 through f4) combined with a common reverse primer derived from *Gnas* exon 2 (r1). cDNA was synthesized from 1 µg of total RNA, as described above; qRT-PCR was performed using FastStart Universal SYBER Green Master (Rox) (Roche, IN, USA); mRNA levels were calculated using the comparative Ct method and expressed as arbitrary units relative to the β 2-microglobulin (β 2M) housekeeping gene or as percentage of transcript levels encountered on mouse day P0 (normalized to 100%). Average C_t value for β 2M mRNA amplification in BAT was 20 ± 0.3 (mean $\pm$ SE) for all analyzed time points (P0 through P120; $n = 3$ for each time point). Kidney, liver, and gWAT consistently had similar C_t values at different investigated time points, namely 20.0 ± 0.25 , 18.5 ± 0.3 , and 18.3 ± 0.12 , respectively ($n = 3$; averaged for all analyzed time points). For quantification in $E1^{mat-}$ and $E1^{pat-}$ mice, a qRT-PCR kit (QIAGEN) was used with TaqMan probes for *Gas* and β -actin as house-keeping control (Mm00530548_m1 for *Gas* and Mm00607939_s1 for β -actin from Applied Biosystems).

2.3. Endonuclease digestion and densitometric analyses

Gnas exon 11 carries in the 129 mice the nucleotide cytosine (C) at position c.1009 from the start codon (rs13460569), while the C57 and CD1 strains carry the nucleotide guanine (G). To determine the parental contribution to overall *Gas* synthesis, RT-PCR products from mice heterozygous for this SNP were incubated (37 °C, 6 h) with the endonuclease *Ban*II (5' ...GRGCY^C...3') (New England BioLabs, Beverly, MA, USA). The resulting DNA fragments (band a: undigested DNA band; bands b and c: if cleaved by *Ban*II) were separated on a 2% agarose gel (Lonza, Rockland, ME, USA); densitometric quantification was performed on each of the three EtBr-stained bands using an α -INNOTECH FLOURCHEM-SP imager (Quansys, Logan, Utah, USA). Percent allelic contribution was determined by calculating the sum of the quantified DNA amounts in bands a, b, and c, and then dividing either a or b plus c by the sum of all three bands.

2.4. Digital droplet PCR (ddPCR)

The parental contribution to *Gas* transcription was assessed also by ddPCR [20] taking advantage of the same C/G polymorphism in *Gnas* exon 11. The forward primer for this approach (f5) anneals to the 3' end of exon 10, while the reverse primer (r3) anneals to sequences derived from exons 11 and 12 to generate an amplicon of 106 bp (see Fig. 1). Two probes were designed; one probe specific for the allele containing the C nucleotide that was labeled with FAM (5'-/56-FAM/CCCAGCCC $\underline{C}$ GGAGAGGACCCA/3IABkFQ/-3') and one probe specific for the allele containing the G nucleotide labeled with HEX (5'-/56-HEX/CCGAGCCC $\underline{G}$ GGAGAGGACCCAC/3IABkFQ/-3'); both probes furthermore contained Iowa Black Dark Quenchers (3IABkFQ) at the 3' end (Integrated DNA Technologies, Coralville, IA, USA). All DNA templates were initially diluted to 10 ng/µl in H₂O. Reactions (30 ngDNA template/reaction) were then set up using Supermix for Probes (BioRad, Hercules, CA, USA) before generating droplets on a BioRad automatic droplet generator. Subsequent PCR was performed on an Eppendorf eppgradient S machine (Eppendorf, Hamburg, Germany) (cycling protocol: 95 °C, 10 min; followed by 40 cycles: 94 °C, 30 s; 60 °C, 60 s; 72 °C, 20 s; heat-inactivation: 98 °C, 10 min; hold at 12 °C). These conditions were chosen after performing an annealing temperature gradient to optimize the

PCR conditions and to ensure that amplimers from the different mouse strains were equally represented when using the selected cycling conditions. Samples were analyzed on a BioRad QX200 sample reader and data were calculated using Quantasoft software (BioRad, Hercules, CA, USA). Overloaded samples were re-analyzed after adjusting the starting template concentration (calculated from previous results) to yield 750–1000 positive droplets per reaction, which allowed complete separation of both probes (Supplemental Fig. 1).

To further validate the ddPCR approach, genomic DNA (10 ng/μl) from matings between different wild-type strains was amplified using primers annealing to nucleotide sequences in introns 11 and 12 (f6) and (r4) respectively, thus spanning the entire exon 11 (size 364 bp). FAM and HEX probes, as well as PCR amplification conditions, are described above. These experiments revealed that both parental alleles are equally amplified from genomic DNA (Supplemental Fig. 2A–D).

2.5. Western blot analysis

Isolated tissues were homogenized and lysed in a Tris-buffered solution containing 150mMNaCl and 1% Triton X-100with a proteinase inhibitor cocktail (Roche, IN, USA). A total of 10 μg protein lysates were separated on a 10% SDS-PAGE before blotting onto a PVDF membrane (Millipore, MA, USA). After blocking with 5% non-fat dry milk (NFDm) in Tris-buffered saline with 0.1% Tween (0.1% TBST), membranes were incubated with a rabbit anti-mouse Gαs polyclonal antibody. A monoclonal rabbit antibody against GAPDH was used for quantification of Gαs protein concentration (see Supplemental Table 2). Densitometric quantification of Gαs protein relative to GAPDH house-keeping protein was performed using ImageJ software, as previously described [21].

2.6. Statistical analysis

All data are represented as mean ± SEM. Statistical significance for the different *Gnas* transcripts and various time points was determined by two-tailed Student's *t*-test and one-way analysis of variance (ANOVA). In order to assess a possible age- and time-dependent effect of the parental contribution to total Gαs expression throughout life, linear regression analyses using Pearson correlation coefficient were calculated and represented by *r* and *p* values, respectively. Differences were considered significant for *p* < 0.05. Statistical analysis was performed using SAS software.

3. Results

Previous data using tissues from wild-type mice and animals with ablation of *Gnas* exon 1 on the maternal allele had indicated that paternal Gαs expression is much lower in BAT from neonatal than from adult mice [15–17]. To confirm these findings in mice with two wild-type alleles and to determine in BAT whether the post-natal changes in paternal Gαs expression could be related to changes in expression of the other paternally-derived transcripts, we assessed the transcript levels of Gαs, XI, and 1A by RT-PCR, and compared the findings to those in kidney and liver.

Experiments with 129^{mat}/C57^{pat} offspring showed that *Gαs* is the most abundantly expressed transcript in the three investigated tissues, namely BAT, kidney, and liver; however, the absolute mRNA levels varied considerably (Fig. 2A–C). The highest *Gαs* mRNA concentration was observed in BAT on day P0, while the level of this transcript was almost 10-fold lower in total kidney RNA, and almost 100-fold lower in liver. By day P20, *Gαs* message levels had declined by 6-fold in BAT; smaller changes were observed in kidney, liver, and gWAT. All analyzed tissues showed a decline in *Gαs* transcript levels by P20, which then remained unchanged throughout the later time points (P30 through P120; Supplemental Fig. 3A–D). At this age, the *Gαs* message level in gWAT was as low as in liver on day P0, and it decreased only moderately at the subsequent time points. To further validate the results in BAT, *Gαs* protein levels were estimated by Western blot analysis at different time points; protein levels were normalized to P10, as the earliest available time point. Densitometric analysis of the protein bands revealed a moderate drop of *Gαs* level after day P20 with lowest levels at P120, which is similar to the decline observed from RNA levels (Supplemental Fig. 3E, Supplemental Table 1).

In wild-type mice, levels of the alternatively spliced *Gnas* transcripts were much lower than those encoding *Gαs*. Thus, the 1A transcript levels in BAT were much lower than those encoding *Gαs* at P0 ($6.4 \pm 0.6\%$ of *Gαs*) and these declined further by day P20 ($3.5 \pm 0.4\%$ of *Gαs*). *Xl* and *Nesp* transcripts in BAT were even lower at birth and virtually absent at the two later time points ($<1\%$ of *Gαs*) (see Fig. 2 and Supplemental Fig. 3). Analysis of kidney and liver revealed only low amounts of 1A transcripts on day P0; no or extremely low 1A, *Xl*, and *Nesp* message levels were detected in these two tissues and in gWAT.

In order to understand whether these observations are strain-specific, we analyzed mice from different backgrounds. Overall similar results were obtained in other mixed background (CD1-C57), when evaluating offspring of matings between female mice carrying a floxed *Gnas* exon 1 allele (CD1-E1^{fl/fl}) [17] and wild-type CD57 males, i.e. CD1-E1^{flmat}/C57^{pat} animals (data not shown).

To assess whether *Gαs* is derived predominantly from either the maternal or the paternal *Gnas* allele, cDNA from BAT, kidney, liver, and gWAT of 129^{mat}/C57^{pat} offspring was amplified with *Gαs*-specific primers (see Fig. 1; primers f4 and r2). The PCR products were then incubated with the endonuclease *Ban*II, which cuts the 129-derived amplicon at nucleotide c.1009, but not the PCR product derived from C57 mice (Fig. 3A). After gel electrophoresis and EtBr staining, densitometric analysis revealed that $20 \pm 0.2\%$ of the *Gαs* transcripts were derived in BAT on day P0 from the paternal allele, while $80 \pm 0.2\%$ were from the maternal allele (Fig. 3B). The paternal *Gαs* contribution increased with age, reaching $38 \pm 2.9\%$ by day P120. In kidney, liver, and gWAT, no major changes in paternal contribution were observed between days P0 and P120 (Fig. 3C–E). Statistical analyses, performed by calculating the Pearson correlation coefficient, showed an age-dependent reduction of the maternal contribution to total *Gαs* expression in BAT ($r = -0.8$, $p < 0.01$). No change in parent-specific *Gαs* expression was observed in kidney and liver; consequently regression lines for both tissues revealed no statistical significance. gWAT showed a significant Pearson correlation coefficient only when including the data for day 120. Similar parental contributions to total *Gαs* expression were obtained for the different

investigated tissues when the mating scheme was reversed to generate C57^{mat}/129^{pat} offspring (data not shown).

To confirm these semi-quantitative findings through an independent, quantitative approach, total RNA from BAT, kidney, liver, and gWAT of 129^{mat}/C57^{pat} offspring was analyzed for *Gαs* expression by ddPCR using probes that detect either cytosine or guanine at c.1009, i.e. the SNP in *Gnas* exon 11 (Fig. 4A). The ddPCR experiments revealed $21 \pm 0.7\%$ paternal and $79 \pm 0.7\%$ maternal *Gαs* expression in BAT on day P0. By day P120, the paternal *Gαs* contribution had increased to $30 \pm 3.2\%$, while maternal *Gαs* expression was reduced to $70 \pm 3.2\%$ (Fig. 4B). In kidney, liver, and gWAT (Fig. 4C–E), ddPCR revealed across all investigated time points a significantly higher paternal *Gαs* contribution than observed in BAT. Interestingly though, compared to the densitometry analyses of the *Ban*II-digested PCR products, ddPCR analysis of *Gnas*-derived transcripts in liver show a slightly higher maternal contribution, similar to the results seen in kidney. Statistical analysis of the ddPCR findings using Pearson correlation coefficient revealed an age-dependent effect for the parental contribution in BAT, namely a progressive reduction of the maternal contribution to total *Gαs* transcription ($r = -0.74$, $p < 0.001$). However, analysis by either densitometry or ddPCR showed no age-dependent change for the relative parental contribution to total *Gαs* expression in kidney, liver, and gWAT. Overall, similar ddPCR results were obtained when analyzing RNA from C57^{mat}/129^{pat} and from CD1-E1^{fl-mat}/C57^{pat} offspring (data not shown).

Total *Gαs* transcript levels at P30, as determined by qRT-PCR, were highest in BAT and brain, followed by heart, kidney, gWAT, and liver (Fig. 5).

To determine the prenatal mRNA levels of *Gαs* and the other three *Gnas*-derived transcripts, total RNA was obtained on E16.5, E17.5, and E18.5 from BAT, kidney, and liver from 129^{mat}/C57^{pat} fetuses (Fig. 6A–C). As for post-natal BAT, *Gαs* transcripts were considerably more abundant than 1A, XI, and Nesp; levels of all four transcripts were much lower in fetal kidney and liver (Supplemental Fig. 4). The relative contribution of the paternal allele to *Gαs* mRNA levels declined from E16.5 to P0. Thus, incubation of *Gαs*-derived amplicons (E1 through E12) with *Ban*II revealed that the paternal *Gαs* contribution declined from $34 \pm 0.7\%$ on day E16.5 to $24 \pm 0.7\%$ on E18.5, which is similar to the decline observed using ddPCR (Fig. 7A). In kidney and liver on the other hand, paternal *Gαs* transcription remained unchanged at the three time points analyzed (Fig. 7B, C).

To determine whether the absence of *Gαs* transcript on either the maternal or the paternal allele affects *Ucp1* expression and thus potentially thermogenesis, total RNA from BAT of newborn E1^{mat-} and E1^{pat-} mice was isolated and analyzed. Compared to wild-type littermates, E1^{mat-} mice showed much reduced *Gαs* transcript levels ($100 \pm 9\%$ vs $19.7 \pm 1.7\%$), while this mRNA was less reduced in E1^{pat-} mice ($100 \pm 6.6\%$ vs $78.2 \pm 3.5\%$) (Fig. 8A and B). Reduced *Gαs* levels in BAT were associated with a dramatic reduction of *Ucp1* transcript levels, but only in E1^{mat-} mice (wild-type: $98 \pm 9\%$; E1^{mat-}: $39 \pm 9\%$).

4. Discussion

Resistance to one or several hormones is well established in patients affected by PHP1A or PHP1B [6,22,23]. The resulting biochemical changes are caused in PHP1A by point mutations, deletions, or insertions involving those maternal *GNAS* exons that encode Gαs, while the different forms of PHP1B are associated with a loss-of-methylation (LOM) at the maternal *GNAS* exon A/B alone or LOM at two additional differentially methylated regions [6,24]. These epigenetic *GNAS* changes reduce Gαs expression from the maternal allele through unknown mechanisms thus leading to little or no production of Gαs protein from this parental allele [6].

The maternal *GNAS* mutations that cause PHP1A or PHP1B do not lead to biochemical changes immediately after birth. For example, evidence for PTH-resistance in PHP1B does not develop until after infancy and clinical manifestations of hypocalcemia usually do not become apparent until the second decade of life [15,18,19,25]. Furthermore, some PHP1B patients with documented maternally inherited *STX16* mutations develop only mild or no hormonal resistance at all [26]. This suggested that a lack of methylation imprints on the maternal *GNAS* allele does not always lead to reduced Gαs expression in the proximal renal tubules. Furthermore, some patients present with hypothyroidism well before PTH-resistance becomes clinically evident [27,28]. Thus although all PHP1B patients lack methylation of *GNAS* exon A/B on both alleles, reduced Gαs transcription can vary age-dependently from tissue to tissue.

To explain the variability in timing and tissue-specificity of hormonal resistance in PHP1A, and particularly in PHP1B patients, the presence of a “silencer” or “enhancer” has been hypothesized that contributes importantly to the regulation of Gαs expression from the paternal allele [5, 15–17]. Consistent with this hypothesis, studies in murine PHP models have indicated that Gαs is expressed preferentially from the maternal allele in select tissues, such as proximal renal tubules and newborn BAT [15–17]. In addition, analysis of mice lacking the maternal *Gnas* exon 1 (*E1^{mat-}*) in BAT or in proximal renal tubules, showed that parent-specific Gαs expression changes over time [15–17]. However, no longitudinal studies had been performed to confirm these findings in wild-type animals.

To study the age-dependent changes in Gαs expression, we assayed parent-specific Gαs transcript levels in different tissues through two independent approaches, namely a semi-quantitative assessment of *Ban*II-digested RT-PCR products and ddPCR for quantitative allele-specific measurements. Both approaches took advantage of a strain-specific SNP in *Gnas* exon 11. While endonuclease digestion allowed the allele-specific evaluation of each *Gnas*-derived parental transcript, ddPCR required the generation of smaller amplicons, thus making it impossible to differentiate between paternally expressed Gαs, XI, and 1A. However, 1A and particularly XI transcript levels were much lower in all investigated tissues and at all investigated ages (see Fig. 2; Supplemental Fig. 3) making it unlikely that ddPCR provided misleading results regarding the parental contribution to Gαs expression.

Using both techniques, we showed that the maternal allele contributes in BAT considerably more to total Gαs expression than the paternal allele. A significantly higher maternal Gαs

contribution was furthermore revealed by ddPCR in kidney, liver, and gWAT across the different post-natal time points. These data are consistent with the conclusion that a putative “silencer” is found at variable levels in different tissues.

It is conceivable that ddPCR preferentially amplified cDNA derived from the maternal *Gnas* allele. However, analysis of genomic DNA from 129/C57 offspring revealed an indistinguishable contribution of the allele comprising either G or C (exon 11 polymorphism), thus making it likely that both parental alleles are amplified equally well. Furthermore, analysis of total RNA from tissues of C57/129 and 129/C57 offspring, i.e. animals in which the nucleotide variant was located either on the paternal or the maternal allele, yielded indistinguishable results. Our assay to assess the parent-specific *Gαs* transcription thus appear to be reliable, especially for tissues with less abundant *Gαs* expression, in which semi-quantitative analysis like endonuclease digestion and densitometry analysis are not sufficiently sensitive. Even in tissues with reduced *Gαs* expression, such as liver and kidney, the maternal *Gnas* allele contributed more to total *Gαs* expression; this is consistent with previous pyrosequence analyses to assess the c.T393C polymorphism in several human tissues, including lymphoblasts, peripheral blood mononuclear cells, mammary adipose, and heart tissue [29].

Predominantly maternal *Gαs* expression was previously observed for neonatal BAT [15–17], which appeared to become biallelic later in life [17]. For these studies, parental *Gαs* contribution was determined by comparing mRNA levels of wild-type (WT) and *Gnas* exon 1-ablated mice ($E1^{mat-}$); thus paternal *Gαs* expression in these $E1^{mat-}$ mice could have been overestimated because of a compensatory increase in transcription from the non-manipulated paternal *Gnas* allele. Although our studies confirmed that *Gαs* in BAT is preferentially derived from the maternal allele before and after delivery, we showed that paternal contribution to total *Gαs* transcription increased only to approximately 30% by day P120 (see Fig. 4). This small increase in paternal *Gαs* contribution in adult BAT may be a reflection of gradual invasion of white fat, blood vessels and thus blood cells, i.e. cells in which both parental *Gnas* alleles contribute similar amounts of *Gαs*mRNA [30]. Thus, contamination with non-BAT tissue could have contributed to the apparent increase in paternally-derived *Gαs* mRNA in BAT from older animals. On the other hand, this approximately 10% up-regulation of the paternal contribution could also represent a time-dependent relaxation of the silencing mechanism on the paternal allele that may coincide with the decline in 1A transcription.

Restricting *Gαs* expression largely to the maternal allele in BAT may have developed during evolution to limit the levels of uncoupling protein 1 (UCP1), which is generated through a cAMP-dependent mechanism [31]. In fact, BAT from newborn $E1^{mat-}$ mice showed a more prominent reduction in *Gαs* transcript levels than BAT from $E1^{pat-}$ mice predicting that cAMP formation is much more reduced when this tissue is derived from animals lacking maternal *Gαs* expression. Based on our findings and studies previously reported by others [17], the contribution of the paternal *Gnas* allele to total *Gαs* levels in BAT is limited. However, only BAT from $E1^{mat-}$ mice revealed reduced *Ucp1* mRNA levels, suggesting that the impact of paternally-derived *Gαs* on this transcript is not readily detectable. Limiting *Gαs* expression to a single *Gnas* allele in BAT would be expected to reduce energy

expenditure by uncoupling lipid oxidation from ATP generation [32]. This finding emphasizes the biological importance of the process that silences *Gαs* transcription from the paternal allele, which is likely to prevent excessive heat generation and to preserve nutrients for vital functions. In PHP1A, maternal *GNAS* mutations eliminate the predominant source of *Gαs* protein in BAT, which could explain the reduced resting energy expenditure and increased visceral fat accumulation, observed in pediatric [33,34], but not in adult patients affected by this disorder [35]. It is therefore likely that additional mechanisms contribute to obesity observed in PHP1A patients [36], including reduced lipolysis stimulated by the sympathetic nervous system [37] and *Gαq*-/ $\alpha 11$ -dependent regulation of food intake [38].

In summary, using two independent approaches, we showed that the maternal contribution to total *Gαs* expression is considerably higher in BAT, while the other investigated murine tissues revealed much smaller, albeit significant differences for both parental alleles, at least when assessed by ddPCR. Unlike BAT, transcription of *Gαs* from the maternal and paternal *Gnas* allele appears to remain largely unchanged over time in other tissues. To study the age-dependent changes in *Gαs* mRNA expression, we analyzed the transcript levels in various tissues, namely BAT, kidney, liver and gWAT. In these tissues, *Gαs* mRNA levels were highest at birth and abruptly decline between P10 and P20. A similar dramatic decrease was also seen in *Gαs* protein levels thus confirming our mRNA findings (see Suppl. Fig. 3E). *Gαs* mRNA levels were >100-fold higher than those encoding *Xla*s making it unlikely that active transcription from this alternative first exons and its promoter affects *Gαs* expression from the paternal *Gnas* allele. Likewise, *Gαs* transcripts levels were 10- to 20-fold higher than the 1A transcript levels making a direct competition between the promoters for these two transcripts unlikely. However, the statistically significant age-dependent increase in paternal *Gαs* expression coincides with a major decline in 1A transcripts, which is derived only from the paternal allele. 1A transcripts could thus have a role in regulating *Gαs* expression, which would be consistent with the observation that a 2.3 kb deletion from the paternal allele comprising exon 1A and the adjacent nucleotide sequences results in a major increase in *Gαs* expression [39]. Alternatively, a “silencer” interacts with this region thereby reducing paternal *Gαs* expression thus causing hormonal resistance if maternal *GNAS*/*Gnas* mutations abolish maternal *Gαs* protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (RO1 DK46718-20 to H.J.) and (RO1 AR064231 to M.L.W.).

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Fig. 1.

Schematic depiction of the paternal and the maternal *Gnas* allele. *Gnas* is encoded by exon E1 (red box) and exons 2–12 (gray boxes) that are also shared by the other sense transcripts that use alternative first exons and their promoters, namely exon 1A (green box), exon XI (blue box), and exon Nesp (two pink boxes). For the antisense transcript only the first exon is shown (AS-1; gray box). Parent-specific methylation of the four differentially methylated regions (DMR) is indicated by **. The mRNAs derived from the *Gnas* locus are shown by vertical lines linked to arrows and the approximate locations of the different primers that were used for qRT-PCR (arrows) and ddPCR (arrowheads) are shown with codes underneath. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Relative expression of four *Gnas* transcripts in different tissues at post-natal days P0 (left panels), P10 (middle panels), and P20 (right panels); BAT (A), kidney (B), and liver (C). Female 129/Sv (129) wild-type mice were mated with wild-type C57BL/6 (C57) males to generate 129^{mat}/C57^{pat} offspring. Tissues were harvested for extraction of total RNA, which was amplified by qRT-PCR using forward primers specific for either *Gnas* exons Nesp, XI, 1A, or $G_{\alpha S}$, and the same reverse primer located in *Gnas* exon 2. Expression of each *Gnas* transcript was normalized for the message levels of the house-keeping gene β 2-microglobulin (β 2M) (upper graphs in panels A–C) or as percentage of $G_{\alpha S}$ expression for

the respective time points (lower graphs in panels A–C). On day P0, *Gαs* mRNA concentration was 12.16 ± 1.03 arbitrary units in BAT; 1.5 ± 0.28 and 0.15 ± 0.01 arbitrary units in kidney and liver respectively. For each for the different post-natal days, absolute and relative expression is presented as mean $\pm$ SEM ($n = 3$). Note the different scales for the y-axis. Data for kidney, liver, and gWAT for the additional time points are provided in Suppl. 3. **, $p < 0.01$.

Fig. 3.

Parent-specific *Gas* expression in BAT, kidney, liver, and gWAT using endonuclease digestion and subsequent densitometry quantification of the DNA bands after agarose gel electrophoresis. Panel A: schematic representation of the exon structure of mouse *Gas* mRNA; E1, derived from *Gnas* exon 1 and 2–12 derived from *Gnas* exons 2–12. The C57BL/6 (C57) allele carries a guanine at position c.1009, while the 129/Sv (129) strain carries a cytosine. Total RNA from the four different tissues of 129^{mat}/C57^{pat} offspring was amplified using the forward primer f4 (exon E1) and the reverse primer r2 (exon 12) provides a 1090 bp amplicon (a), which is cleaved in bands of 896 bp (b) and 194 bp (c),

respectively, upon incubation with *Ban*II. % parental contribution in BAT (panel B), kidney (panel C), liver (panel D), gWAT (panel E). White columns represent paternal *Gαs* transcripts; black columns represent maternal *Gαs* transcripts. % parental contribution are presented as mean ± SEM and derived for each time point from 3 animals from 3 independent matings. In panel B, parental contribution at P0 is different compared to all time points (P10–P120). *r* values represent Pearson correlation Coefficient. **, *p* < 0.01; NS, not statistically significant.

Fig. 4.

Digital droplet PCR (ddPCR) to quantitatively determine the parental contribution of $G\alpha_s$ expression in BAT, kidney, liver, and gWAT from crosses between wild-type 129/Sv females (homozygous for C) in *Gnas* exon 11) and wild-type C57BL/6 males (homozygous for G). Panel A: schematic representation of the primers and probes used. % parental contribution in BAT (panel B), kidney (panel C), liver (panel D), and gWAT (panel E). White columns represent paternal $G\alpha_s$ transcript levels; black columns represent maternal $G\alpha_s$ transcript levels. Kidney, liver, and gWAT showed paternal contributions of $38.9 \pm 0.47\%$, $37.8 \pm 0.43\%$, and $44.1 \pm 0.4\%$, respectively, of total $G\alpha_s$ mRNA at various time points.

Compared to ddPCR results, densitometry analysis of the *Ban*II-digested bands revealed for the same time points a slightly higher paternal contribution in kidney (*Ban*II incubation: $44.7 \pm 0.6\%$, ddPCR: $38.9 \pm 0.5\%$) and liver (*Ban*II incubation: $48.0 \pm 0.8\%$; ddPCR: $37.8 \pm 0.4\%$). % parental contribution are presented as mean $\pm$ SEM and derived for each time point from 3 animals from 3 independent matings. r values represent Pearson correlation Coefficient. *, $p < 0.05$; NS, not statistically significant.

Fig. 5. *Gnas* mRNA expression levels at P30 in different tissues ($n = 3$) from matings between wild-type female 129/Sv (129) and wild-type C57BL/6 (C57) males. Data for absolute expression corrected by $\beta 2M$ are presented as mean $\pm$ SEM and derived for each tissue from 3 animals, each from 3 different matings.

Fig. 6.

Gnas-derived transcripts in BAT from embryos at day E16.5 (panel A), E17.5 (panel B), and E18.5 (panel C). Expression of each *Gnas* transcript was normalized for the message levels of the house-keeping gene β 2-microglobulin (β 2M) and expressed as absolute values (upper graphs in each panel) or as percentage of $G\alpha s$ expression for the respective time points (lower graphs in each panel). $G\alpha s$ expression increased from 6.7 ± 0.4 (arbitrary unit) on E16.5 to 16.3 ± 2.0 (arbitrary unit) on E18.5. For each pre-natal day, absolute and relative expression is presented as mean $\pm$ SEM and derived from 4 animals from 3 different matings. **, $p < 0.01$.

Fig. 7.

Parental contribution of *Gas* transcription determined by endonuclease digestion (upper panel) and ddPCR (lower panel). PCR amplification and subsequent analyses by densitometry or ddPCR using BAT (panel A), kidney (panel B), and liver (panel C) from embryos at day E16.5, E17.5, and E18.5, respectively, which were compared to data obtained from RNA of newborn animals (P0). *Gas* parental contribution in BAT declined (*Ban*II incubation: $34 \pm 0.7\%$ on E16.5 to $24 \pm 0.7\%$ on E18.5; ddPCR: $31 \pm 0.2\%$ on E16.5 to $24 \pm 0.06\%$ on E18.5), while the parent-specific *Gas* transcription remained unchanged (*Ban*II incubation: $44.1 \pm 1.9\%$ and $42.1 \pm 1.5\%$; ddPCR: $38.8 \pm 0.18\%$ and $38.1 \pm 0.4\%$ on E16.5 and E18.5, respectively). White columns represent paternal *Gas* transcript levels; black columns represent maternal *Gas* transcript levels. *, $p < 0.05$; **, $p < 0.01$.

Fig. 8.

G α s and Ucp1 mRNA expression in BAT from newborn E1^{mat-} (panel A) and E1^{pat-} mice (panel B). G α s levels were measured using TaqMan and were normalized for the expression of β -actin, and expressed as percentage of the wild-type littermates (lower panel A and B). Ucp1 levels were normalized for expression of β 2-microglobulin (β 2M) and expressed as percentage of the wild-type littermates (upper panels A and B). White columns represent E1^{mat-} and E1^{pat-} transcript levels; black columns represent wild-type littermates transcript

levels. Each column is presented as mean $\pm$ SEM derived from BAT of 3 different animals.
*, $p < 0.05$.