



HAL
open science

A novel aspect of imprinting: higher maternal expression of $G\alpha s$ in human lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue, and heart

Stefanie Klenke, Winfried Siffert, Ulrich H. Frey

► To cite this version:

Stefanie Klenke, Winfried Siffert, Ulrich H. Frey. A novel aspect of imprinting: higher maternal expression of $G\alpha s$ in human lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue, and heart. *Molecular and Cellular Endocrinology*, 2011, 341 (1-2), pp.63. <10.1016/j.mce.2011.05.032>. <hal-00719877>

HAL Id: hal-00719877

<https://hal.science/hal-00719877v1>

Submitted on 22 Jul 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

A novel aspect of *GNAS* imprinting: higher maternal expression of *Gαs* in human lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue, and heart

Stefanie Klenke, Winfried Siffert, Ulrich H. Frey

PII: S0303-7207(11)00284-X
DOI: [10.1016/j.mce.2011.05.032](https://doi.org/10.1016/j.mce.2011.05.032)
Reference: MCE 7865

To appear in: *Molecular and Cellular Endocrinology Molecular and Cellular Endocrinology*

Received Date: 5 January 2011
Revised Date: 11 May 2011
Accepted Date: 11 May 2011

Please cite this article as: Klenke, S., Siffert, W., Frey, U.H., A novel aspect of *GNAS* imprinting: higher maternal expression of *Gαs* in human lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue, and heart, *Molecular and Cellular Endocrinology Molecular and Cellular Endocrinology* (2011), doi: [10.1016/j.mce.2011.05.032](https://doi.org/10.1016/j.mce.2011.05.032)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



A novel aspect of *GNAS* imprinting: higher maternal expression of *Gαs* in human lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue, and heart

Stefanie Klenke¹, Winfried Siffert¹, Ulrich H. Frey^{1,2}

¹Institut für Pharmakogenetik, Universität Duisburg-Essen and Universitätsklinikum Essen, Hufelandstr. 55, 45122 Essen, Germany; ²Klinik für Anästhesiologie und Intensivmedizin, Universität Duisburg-Essen and Universitätsklinikum Essen, Hufelandstr. 55, 45122 Essen, Germany

Corresponding author and responsible for reprint requests: Ulrich Frey, Klinik für Anästhesiologie und Intensivmedizin, Universität Duisburg-Essen and Universitätsklinikum Essen, Hufelandstr. 55, 45122 Essen, Germany; Phone: +49 201 723 1401; Fax: +49 201 723 5949, Email: ulrich.frey@uk-essen.de

Abstract

The human *GNAS* gene is imprinted in a tissue-specific manner, being expressed primarily from the maternal allele in pituitary, thyroid, renal proximal tubules and gonads, but is supposed to be biallelically expressed with an equal allelic expression in most other tissues. We analysed allelic expression of *Gas* using Pyrosequencing. By genotyping the *GNAS* T393C polymorphism we quantified mRNA transcripts in lymphoblasts (Ly, n=11), peripheral blood mononuclear cells (PBMC, n=18), mammary adipose tissue (MAT, n=23) and heart tissue (HT, n=44). Allelic expression analysis revealed an unequal allelic expression (ratio maternal/total*100 \pm SEM: 55.7 \pm 1% (95%CI 53.4 % - 58.1 %) in Ly, 56.1 \pm 0.8 (95%CI 54.5 % - 57.7 %) in PBMC, 54.5 \pm 0.8 % (95%CI 53 % - 56.1 %) in MAT and 54.1 \pm 0.6% (95% CI 53 % - 55.3 %) in HT). Maternal ratio differed significantly from the mean (p<0.0001). This phenomenon may be a general feature existing in all tissues.

Key terms: alleles, genomic imprinting, *GNAS*, G protein alpha s, Pyrosequencing

1. Introduction

The human *GNAS* gene is located on chromosome 20q13.2-13.3 and expression of *GNAS* products is regulated both by imprinting and alternative splicing (Weinstein et al., 2007). Usage of alternative promoters and first exons that splice onto a common exon (exon 2) results in multiple gene products. The ubiquitously expressed G-protein α -subunit *G α s* is generated by the most downstream promoter and mediates stimulation of adenylyl cyclase and the production of the second-messenger cyclic AMP (cAMP) via a number of receptor types, e.g. β_1 - and β_2 -adrenoreceptors. Cyclic AMP subsequently activates protein kinase A (PKA) which catalyzes the phosphorylation of various proteins important for cellular signaling.

The *G α s* protein consists of 13 exons and there exist four splice variants differing by inclusion (long form) or exclusion (short form) of exon 3, with both forms being differentially expressed in various tissues (Frey et al., 2005). *G α s* is imprinted in a tissue-specific manner, being expressed primarily from the maternal allele in pituitary, thyroid, renal proximal tubules, gonads and the paraventricular nucleus of the hypothalamus, but is believed to be biallelically expressed with an equal contribution of the maternal and paternal allele in most tissues (Weinstein et al., 2001; Germain-Lee et al., 2002; Mantovani et al., 2002; Liu et al., 2003; Chen et al., 2009; Yu et al., 1998).

Heterozygous inactivating *GNAS* mutations result in Albright hereditary osteodystrophy (AHO) with maternal inheritance of AHO also leading to multihormone resistance [pseudohypoparathyroidism type 1a (PHP1A)] and paternal inheritance leading to AHO alone (pseudopseudohypoparathyroidism) (Yu et al., 1998; Hayward et al., 2001; Germain-Lee et al., 2002; Mantovani et al., 2002; Liu et al., 2003; Weinstein et al., 2006). Classically, the obesity in AHO is described as occurring similarly in both conditions (ALBRIGHT et al., 1952). However, there is now evidence that the development of obesity only occurs with maternal *G α s* mutations in PHP1A (Long et al., 2007). The underlining mechanisms for the development of obesity are not yet fully understood, but it seems that adipose tissue is unlikely involved in this parent-of-origin effect, since studies have not given any evidence for *G α s* imprinting in adipose tissue in humans (Mantovani et al., 2004; Germain-Lee et al., 2005) and heterozygous adipose-specific *G α s* deficiency in mice results in no obvious phenotype (Chen et al., 2010). However, one study revealed a reduced lipolytic responsiveness to epinephrine in adipocytes

from patients with PHP1A (Carel et al., 1999) and one suggested a preferential maternal expression of *Gas* in adipose tissue (Yu et al., 1998). A recent study with *Gas* brain-specific knock-out mice has shown that central-nervous system specific disruption of *Gas* is sufficient to produce obesity, which underlines that *Gas* imprinting within parts of the central nervous system is the most likely explanation for obesity occurring in PHP1A (Chen et al., 2009).

Although *GNAS* is imprinted in a tissue-specific manner, the promoter is not methylated (Kozasa et al., 1988; Liu et al., 2005). Therefore, further studies have focused on the identification of imprinted control regions of *GNAS*. Promoters and first exons for transcripts encoding NESP55, Nespas, XLas, and 1A (also known as A/B) are methylated on either the maternal or the paternal allele (Figure 1). NESP55 contains a paternally methylated promoter and is exclusively maternally expressed (Hayward et al., 1998; Li et al., 2000). In contrast, XLas is expressed only paternally due to methylation of the maternal promoter (Hayward et al., 1998). An additional promoter generates paternally expressed antisense transcripts (Nespas) that cross the NESP55 exon (Li et al., 2000). The exon 1A promoter region is located just upstream of *Gas* exon 1 and contains a methylated region on the maternal allele and is thought to be a primary imprint control region, since its methylation is established during oogenesis and maintained through development (Liu et al., 2000b). Furthermore, studies in mice have shown that paternal deletion of the exon 1A region results in reversal of *Gas* imprinting with biallelic expression of *Gas* (Williamson et al., 2004; Liu et al., 2005).

In line with these findings, in pseudohypoparathyroidism type 1B (PHPIB), a form of renal parathyroid hormone resistance, maternal –specific methylation of the exon 1A is absent (Jan de Beur et al., 2003; Liu et al., 2000a). However, exact imprinting mechanisms are not yet fully understood.

Among the possible explanations are the reciprocal regulation of the exon 1A and *Gas* promoter on the paternal allele, the presence of boundary elements that insulate the *Gas* promoter or a silencer element, which can bind a tissue-specific repressor and which can be inhibited by methylation (Weinstein et al., 2001; Liu et al., 2000a; Weinstein, 2001). These possible explanations are based on the assumptions of an equal allelic expression of *Gas* in non-hormone target tissues and imprinting in hormone target tissues in PHP1A and involve a tissue-specific factor.

Investigations concerning imprinting and allelic expression analysis in the past were primarily focused on hormone-specific tissues, which contribute to the hormone resistant phenotype in PHP1A. To this, allelic expression analysis was mostly performed by restriction analysis following evaluation by an imaging densitometer (Mantovani et al., 2002; Mantovani et al., 2004; Zheng et al., 2001). This method has the disadvantage that allele-specific expression cannot be quantified accurately and subtle differences may remain unrecognized. We have recently established a method based on Pyrosequencing to quantify allele-specific transcript expression in a highly accurate manner (Sun et al., 2005). The Pyrosequencing technique is based on the detection of released pyrophosphate (PPi) during DNA synthesis (Ronaghi et al., 1996). An enzymatic mixture consisting of sulphurylase and luciferase converts PPi to ATP, which then drives the conversion of luciferin to oxyluciferin, yielding detectable light emission, seen as one peak, which is proportional to the amount of incorporated nucleotides (Ronaghi et al., 1998; Ahmadian et al., 2000).

The aim of the present study was, therefore, the investigation and quantification of *Gas* allelic expression using pyrosequencing in different tissues which are thought not to be imprinted since they do not contribute to the hormone resistant phenotype in PHP1A.

2. Material and methods

2.1 Cells and tissue samples

B lymphoblast cell lines: Human Epstein-Barr virus immortalized lymphoblasts from 24 individuals were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (PAA, Pasching, Austria) at 37°C in a humidified atmosphere of 5% CO₂ in air, as previously described (Siffert et al., 1995). One day before RNA preparation, lymphoblasts were subcultured at a density of 5 -10 x 10⁵/ml into fresh medium and a total of 5-10 x 10⁶ cells were used for RNA preparation. To exclude that immortalization of lymphoblasts with Epstein-Barr virus affects the imprinting status, we also used peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) from 44 healthy blood donors were isolated by Ficoll–Hypaque (Pharmacia, Erlangen, Germany) density centrifugation. In all, 1 to 2x10⁸ PBMCs were used for RNA preparation. **Heart tissue:** Adult human right atrial specimens were

obtained from 94 patients undergoing cardiac surgery as described (Frey et al., 2008b). Adipose tissue: 49 mammary adipose tissue samples were obtained from women undergoing elective mammary reduction surgery (Hauner et al., 2002). All adipose tissue samples were immediately snap frozen in liquid nitrogen during the surgical procedure. Written informed consent was obtained from all subjects.

RNA was prepared using the RNeasy Kit obtained from Qiagen (Hilden, Germany) according to the manufacturer's instructions. RNA from mammary adipose tissue was prepared using a modified protocol. Briefly, up to 500 mg adipose tissue was mixed with 600 μ l RLT lysis buffer (Qiagen, Hilden, Germany) and mechanically homogenized for 1 min followed by centrifugation at 13.000 rpm for 1 min. The supernatant was saved followed by addition of 70% ethanol. The pellet was resuspended in 300 μ l RLT buffer and centrifuged for 1 min at 13.000 rpm. The supernatants from both centrifugation steps were combined, loaded onto a RNeasy column and processed following the manufacturer's instructions. RNA samples were treated with DNase I.

All tissue and cell line samples were genotyped for the *GNAS* T393C polymorphism as described below. In total, we obtained 96 samples which were heterozygous for the polymorphism (lymphoblasts (Ly), n=11, peripheral blood mononuclear cells (PBMC), n=18, mammary adipose tissue (MAT), n=23 and heart tissue (HT), n=44). With those 96 heterozygous samples we measured *GNAS* exon 1 C allele frequency in cDNA (Figure 3B, 3C and 6C), paternal exon 1A expression (Figure 5C) and maternal exon 1 expression (Figure 7).

Tissue and cell line samples were also genotyped for the *MLH1* A655G polymorphism (rs1799977). In total, we obtained 88 tissue and cell line samples (Ly, n=9, RBMC, n=22, MAT, n=10 and HT, n=47) which were heterozygous for the polymorphism and used for measuring A allele frequency in cDNA.

2.2 PCR, RT – PCR and allele-specific expression

DNA was extracted from blood or tissue by standard techniques. Determination of the *GNAS* T393C polymorphism was performed by PCR using the primers 5'- TGTGGCCGCCATGAGCAA-3' and 5'-

TAAGGCCACACAAGTCGGGGT-3'. The 145 nt PCR fragment was then digested with *FokI* (NEB, Beverly, USA) and *GNAS* T393C genotypes were determined by gel electrophoresis.

To determine allele-specific expression, first-strand cDNA was synthesized from ~1 µg of total cellular RNA with oligo-dT-primers (Roche, Mannheim, Germany) by using M-MLV reverse transcriptase as recommended by the manufacturer (Invitrogen, Karlsruhe, Germany). The resulting cDNA was diluted 1:10 and 3 µl of the dilution was used for PCR.

We recently established a technique for allele-specific quantification using Pyrosequencing (Sun et al., 2005). For *GNAS* allelic expression analysis, we used primers 5'-CCATGGGCTGCCTCGGGAACA-3' (exon 1) and biotinylated 5'-CGGGAGGGAAGTCAAAGTC-3' (exon 5) to amplify a 435 bp (long form) or a 390 bp (short form) fragment from cDNA derived from the different tissues which were heterozygous for the T393C polymorphism using a slowdown PCR technique including 5% DMSO (Frey et al., 2008a). The PCR product was then subjected to pyrosequencing of the T393C polymorphism using the primer 5'-CAGTTCAGAGTGGACTACAT-3'. The allelic ratios were calculated using the Pyrosequencing software (Figure 2). To determine the paternal allele in heterozygous samples, the samples were amplified with primers 5'-GCCTTGCGTGTGAGTGCACCT-3' (exon 1A) and biotinylated 5'-CGGGAGGGAAGTCAAAGTC-3' (exon 5) to amplify a 324 bp fragment with the sense primer annealing in exon 1A followed by pyrosequencing of the T393C polymorphism using the primer 5'-CAGTTCAGAGTGGACTACAT-3'.

For calculating allelic ratios of *GNAS* in genomic DNA we used sense primer 5'-GCCATGAGCAACCTGGTG-3' (exon 5) and biotinylated 5'-CGGGAGGGAAGTCAAAGTC-3' (exon 5) resulting in a 109 bp product followed by pyrosequencing of the T393C polymorphism using the primer 5'-CAGTTCAGAGTGGACTACAT-3'.

For calculating *MLH1* allelic expression in genomic DNA and cDNA we used sense primer 5'-AGGAGAGACAGTAGC-3' and biotinylated antisense primer 5'-CGACTAACAGCATT-3' to amplify a 87 bp product which was then subjected to pyrosequencing of the A655G polymorphism using the sequencing primer 5'-GGACAATATTCGCTCC-3'.

2.3 Statistical methods

All data are expressed as means \pm standard error of the mean (S.E.M.). Continuous variables were compared using unpaired Student's t-test or analysis of variance (ANOVA).

For allele quantification using calibration curves, calibration samples for the *GNAS* T393C polymorphism were prepared by mixing homozygous DNA samples at different ratios. PCR from the mixture was carried out with primers as described above. Calibration curves, represented by the plots of the measured *versus* the expected relative amounts of a given allele, were generated using linear regression analysis.

For heterozygous *GNAS* T393C samples we compared C allele ratios of DNAs. Ratios were determined using the Pyrosequencing software resulting in a mean fractional C allele frequency in DNA samples of 49.6%. The value of 49.6% is due to the measurement of the pyrosequencing technology. We used one sample t-test to determine whether maternal cDNA expression differs from the hypothetical value of 50.

Data were regarded significantly different if the affiliated p-value was below 0.05.

3. Results

3.1 Establishment of a method for allele-specific quantification of *GNAS* transcripts by Pyrosequencing

We first investigated whether the peak heights determined by the Pyrosequencing software were actually proportional to the concentration of a specific allele. We used different ratios of DNA samples from human blood donors who were homozygous for the *GNAS* T393C polymorphism (CC and TT genotypes). PCR was carried out and the products were subjected to Pyrosequencing. The height of the C-peak increased with increasing CC/TT ratio in the DNA mixture. The measured peak heights were plotted against known CC/TT DNA ratios and subjected to linear regression analysis. We found an excellent linear correlation between the concentration of the "C" allele and measured C allele peak heights ($y = 0.16 + 0.97x$, $r^2 = 0.98$, $p < 0.001$, Figure 3A). Therefore, this method is suitable to quantify allele-specific transcripts of *GNAS*.

3.2 Comparison of C allele ratios of DNAs and cDNAs

For heterozygous *GNAS* T393C samples, we compared C allele ratios of DNAs and cDNAs. cDNA allele ratios were determined in the 96 tissue and cell line samples (lymphoblasts (Ly), n=11, peripheral blood mononuclear cells (RBMC), n=18, mammary adipose tissue (MAT), n=23 and heart tissue (HT), n=44). Ratios were determined using the Pyrosequencing software resulting in a mean fractional C allele frequency in DNA samples of 49.6 %, the 95 % confidence interval spanning 1.1 % (95 % CI 49.1 % - 50.2 %). Expression from the C allele in cDNA samples showed a higher variation with a mean fractional C allele frequency of 48.8 % and a 95 % confidence interval spanning 2.4 % (95 % CI 47.6 % - 50 %, Figure 3B).

To investigate whether variation in C allele frequency in cDNA occurs in all of our tissues samples, we analyzed the data separately for lymphoblasts, peripheral blood mononuclear cells, mammary adipose tissue and heart tissue (Figure 3C). We observed in each tissue a variation in C allele frequency (lymphoblasts: mean C allele frequency 49.6 % (95% CI 44.9 % - 54.2 %), peripheral blood mononuclear cells: mean C allele frequency 49.6 % (95%CI 46.1 % - 53.1 %), mammary adipose tissue: mean C allele frequency 49.3 % (95%CI 46.8 % - 51.8 %) and heart tissue: mean C allele frequency 48 % (95% CI 46.4 % - 49.6 %).

Finally, we determined whether another non-imprinted gene will result in a cDNA allelic expression, which is in the range of DNA allelic expression. We chose the mismatch repair gene *MLH1* and the common A655G polymorphisms for allelic expression analysis, since these exchange has already been used for allelic expression studies with pyrosequencing (Kwok et al., 2010).

For heterozygous *MLH1* A655G samples, we compared A allele ratios of DNAs and cDNAs. cDNA allele ratios were determined in 88 tissue and cell line samples (lymphoblasts, n=9, peripheral blood mononuclear cells, n=22, mammary adipose tissue, n=10 and heart tissue, n=47). We observed a mean fractional A allele frequency in DNA samples of 50 %, the 95 % confidence interval spanning 0.8 % (95 % CI 49.6 % - 50.4 %). Expression from the A allele in cDNA samples showed a similar variation with a mean fractional A allele frequency of 49.9 % and a 95 % confidence interval spanning 0.8 % (95 % CI 49.5 % - 50.3 %, Figure 4). No differences between different cell line and tissue samples were found (data not shown).

3.3 Quantification of allelic variation for the *GNAS* T393C polymorphism in different tissues and cell lines

The evaluation of parental origin of transcription of the *Gas* gene was carried out on samples heterozygous for the *GNAS* T393C polymorphism. The evaluation of parental origin of *Gas* transcripts was established by amplification of exon 1A and determination of T393C genotype and allelic expression analysis by Pyrosequencing. Representative pyrograms for paternal C and T alleles are shown in Figures 5A and Figure 5B. As expected, we found an almost 100% imprinting of exon 1A in the 96 tissue and cell line samples (ratio paternal/total*100 \pm SEM: 98 \pm 0.3 %, 95% CI 97.4 % - 98.6 %, Figure 5C).

We next investigated whether unequal allelic expression might be responsible for the variation in C allele ratios of cDNA. When both alleles in a heterozygous sample were equally expressed, we expected a pyrogram as shown in Figure 2 (height of the T = 1/3 of height of the C). However, we observed different pyrograms and two representative pyrograms are shown in Figures 6A and Figure 6B. If in heterozygous sample the T allele was inherited from the father, the height of the T was less than the expected 1/3 of the height of the C allele (Figure 6A), so the maternally *Gas* expression from the C allele was increased. When the C allele was transmitted from the father, the height of the C decreased and the height of the T allele increased, so that the T allele was higher than the expected 1/3 of the height of the C allele (Figure 6B). So again, maternally derived *Gas* expression was increased. Therefore, we concluded that there is an unequal allelic expression, with predominant expression from the maternal allele over the paternal allele.

We next investigated whether we could confirm these findings in all of our tissue and cell line samples (lymphoblasts (n=11), peripheral blood mononuclear cells (n=18), mammary adipose (n=23) and heart (n=44) tissue) and determined mean C allele frequency from the maternal and the paternal allele (Figure 6C). Mean C allele frequency from the maternal allele was above 50% in all tissues examined (lymphoblasts: 54.8%, peripheral blood mononuclear cells: 55.7%, mammary adipose tissue: 53.4% and heart tissue: 52.1%), whereas mean C allele frequency from the paternal allele was below 50% in all tissues examined (lymphoblasts: 43.2%, peripheral blood mononuclear cells: 43.5% mammary adipose tissue: 44% and heart tissue: 43.9%). Maternal C allele frequency differed significantly from

paternal C allele frequency in lymphoblasts ($p=0.0004$), peripheral blood mononuclear cells ($p<0.0001$) mammary adipose tissue ($p<0.0001$) and heart tissue ($p<0.0001$).

Finally, we calculated exon 1 percentage maternal expression/all in lymphoblasts ($n=11$), peripheral blood mononuclear cells ($n=18$), mammary adipose ($n=23$) and heart ($n=44$) tissue. We observed a maternal over paternal expression. The least expressed allele was always the same as that represented in exon 1A transcripts. In lymphoblasts, the ratio maternal/total*100 \pm SEM was 55.7 ± 1 % (95% CI 53.4 % - 58.1 %), in peripheral blood mononuclear cells 56.1 ± 0.8 % (95% CI 54.5 % - 57.7 %), in mammary adipose tissue 54.5 ± 0.8 % (95%CI 53 % - 56.1 %) and in heart tissue 54.1 ± 0.6 % (95% CI 53 % - 55.3 %). Maternal allele ratio differed significantly from the mean of 50% ($p<0.0001$ for all tissues; $p=0.0003$ in lymphoblasts, $p<0.0001$ in peripheral blood mononuclear cells $p<0.0001$ in mammary adipose tissue and $p<0.0001$ in heart tissue). Only three tissues (two heart tissues and one peripheral blood mononuclear cell sample) of the 96 investigated samples showed a maternal ratio of below 50 % (49.4 %, 48.1% and 49.9%; Fig. 7).

4. Discussion

Here we demonstrate for the first time that *Gas* shows an unequal allelic expression with maternal over paternal expression in lymphoblasts, peripheral blood mononuclear cells, mammary adipose and heart tissue. *Gas* is known to be imprinted in a tissue-specific manner being primarily expressed from the maternal allele in pituitary, thyroid, renal proximal tubules, gonads, and the paraventricular nucleus of the hypothalamus, all tissues contributing to hormone resistance in pseudohypoparathyroidism type 1A (PHP1A) (Weinstein et al., 2001; Germain-Lee et al., 2002; Mantovani et al., 2002; Liu et al., 2003; Chen et al., 2009; Yu et al., 1998). *Gas* is biallelically expressed in most tissues and the maternal and paternal allele are supposed to contribute equally to *Gas* expression (Weinstein et al., 2007; Yu et al., 1998; Germain-Lee et al., 2005; Mantovani et al., 2004; Crane et al., 2009). However, most of these studies showing a biallelic expression equally distributed upon both alleles have not focused on detection of subtle differences between maternal and paternal expression.

We used the high sensitive Pyrosequencing method for allele expression analysis. Pyrosequencing was originally developed for the detection of single nucleotide polymorphisms, but is also used for assessing SNP allelic frequencies in DNA mixtures (Gruber et al., 2002; Wasson et al., 2002) and for detection of allelic imbalance in gene expression (Wang and Elbein, 2007). Using this technology, we found a higher variation in C allele ratios of the *GNAS* T393C polymorphism in cDNA compared to human genomic DNA, while we found a comparable A allele ratio of the *MLH1* A655G polymorphism in DNA and cDNA. This finding gave rise to the hypothesis of unequal allelic *Gas* expression in tissues which are thought not to be imprinted. Therefore, we evaluated parental origin of *Gas* in samples which were heterozygous for the *GNAS* T393C polymorphism by amplification of exon 1A and determination of T393C genotype. We found a monoallelic paternal expression of exon 1A, which is known to be imprinted and only expressed from the paternal allele (Liu et al., 2000b; Ishikawa et al., 1990; Liu et al., 2000a). To this, imprinting status in the samples was confirmed. Allelic expression analysis of exon 1 transcripts by Pyrosequencing revealed that the least expressed allele was always the same as that represented in exon 1A transcripts. Therefore, in lymphoblasts, peripheral blood mononuclear cells, mammary adipose and heart tissue we found an unequal allelic expression with maternal expression over paternal expression (ratio maternal/total*100 \pm SEM: 55.7 \pm 1 % in lymphoblasts, 56.1 \pm 0.8 % in peripheral blood mononuclear cells, 54.5 \pm 0.8 % in mammary adipose tissue and 54.1 \pm 0.6 % in heart tissue).

Imprinting generally results in the expression of either the paternal or the maternal allele and corresponding silencing of the other allele (Bartolomei and Tilghman, 1997; Wood and Oakey, 2006), but partial imprinting in which the two parental alleles are expressed to different degrees has also been described (Morison et al., 2005; Wolf et al., 2008). In a study by Mantovani et al. a *Gas* ratio maternal/all of 75.7 % for thyroid tissues, of 68.8 % for granulosa cell samples, of 92.3 % for normal pituitaries and of 87.8 % for adenomatous pituitary samples was found (Mantovani et al., 2002), all tissues being imprinted and contributing to hormone resistance in PHP1A. In the same study an equal expression of *Gas* from the maternal and the paternal allele in adrenal and lymphoblasts was found (Mantovani et al., 2002). Imprinting of *Gas* in the thyroid gland could be confirmed by Liu et al., showing that the majority of *Gas* mRNA transcripts was derived from the maternal allele (72%).

Another study demonstrated an equal allelic expression of *Gas* in human bone and adipose tissue, although the ratio maternal/all was 53.2 %, which was, however, not statistically significant (Mantovani et al., 2004). To investigate at which time point imprinting of *Gas* occurs in embryonic development, allelic expression of *Gas* in human primordial germ cells was determined. In embryonic germ cells, a ratio maternal/all of 52.6 % was found, which was not statistically significant ($p=0.07$). Therefore, a biallelic expression with occurrence of *Gas* imprinting 11 weeks after fertilization was assumed (Crane et al., 2009). Taken together, some of these studies found a maternal expression of higher than 50 % in tissues, which are thought not to be imprinted, however, as the allelic expression analysis was not statistically significant. Determination of allelic expression analysis in these studies was performed by RT-PCR followed by restriction analysis and evaluation by densitometry. Here we could show that using Pyrosequencing we were able to identify unequal allelic expression with maternal over paternal expression. Therefore our data are not in contrast with the previous published studies, but rather confirm these studies showing a statistically significantly higher maternal over paternal expression in non hormone-target tissues.

Our data further support the hypothesis that adipose tissue is unlikely involved in parent-of-origin effects which lead to obesity in PHP1A. We found similar results in mammary adipose tissue, heart tissue, lymphoblasts and peripheral blood mononuclear cells. While heart tissue, lymphoblasts and peripheral blood mononuclear cells do not contribute to the hormone resistant phenotype in PHP1A (ALBRIGHT et al., 1952; Long et al., 2007; Mantovani et al., 2004; Germain-Lee et al., 2005; Chen et al., 2010), our data are in line with the study by Chen et al., showing that central-nervous system specific disruption of *Gas* is sufficient to produce obesity (Chen et al., 2009). However, it has to be mentioned, that allelic expression analysis was performed in adipocytes isolated from human mammary tissue. Since it is known that different adipose tissue depots (omental, subcutaneous, mammary tissue) differ in their lipolytic responsiveness and sensitivity to hormones (Richelsen et al., 1991), we can not necessarily transfer our observations to other adipose tissue depots.

The relevance of our findings remains to be resolved. However, our findings may partly explain the molecular and clinical overlap between PHP1A and PHP1B, which has been reported in recent studies (de Nanclares et al., 2007; Mariot et al., 2008; Unluturk et al., 2008; Mantovani et al., 2010). Whereas

PHPIA is caused by heterozygous loss of function mutations inherited from the mother in exons 1-13, PHPIB is caused by disruption of long-range imprinting control elements in *GNAS*. Typically is the loss of imprinting at the exon 1A differentially methylated region, with consequent presumed decreased *Gas* expression in tissues where this protein is imprinted. Recently, four independent studies found methylation defects similar to those observed in PHPIB in patients which haven been clinically diagnosed with PHPIA, some of those patients presenting with mild features of AHO (de Nanclares et al., 2007; Mariot et al., 2008; Unluturk et al., 2008; Mantovani et al., 2010). Furthermore, an overlap between PHPIA and PHPIB was found in terms of *Gas* activity, since a reduced *Gas* activity in erythrocyte membranes of patients with PHPIB compared to healthy controls was observed. Interestingly, this effect was significantly lower in patients with some features of AHO (Zazo et al., 2011). Therefore, it might be supposed that *Gas* expression is subject to allelic bias in a larger number of tissues that currently known.

However, further studies are required to clarify these findings and exact mechanisms of *Gas* imprinting.

In conclusion, our results provide the first evidence that *Gas* expression shows allelic bias, with higher maternal than paternal expression, even in tissues in which heterozygous inactivating *Gas* mutations do not seem to cause parent-of-origin specific hormone resistance.

Declaration of interest: There is no conflict of interest that could inappropriately influence, or be perceived to influence, the work

Submission declaration: The work has not been published previously, is not under consideration for publication elsewhere, publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.

Funding: This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Reference List

- Ahmadian,A., Gharizadeh,B., Gustafsson,A.C., Sterky,F., Nyren,P., Uhlen,M., and Lundeberg,J. (2000). Single-nucleotide polymorphism analysis by pyrosequencing. *Anal. Biochem.* 280, 103-110.
- ALBRIGHT,F., FORBES,A.P., and HENNEMAN,P.H. (1952). Pseudo-pseudohypoparathyroidism. *Trans. Assoc. Am. Physicians* 65, 337-350.
- Bartolomei,M.S. and Tilghman,S.M. (1997). Genomic imprinting in mammals. *Annu. Rev. Genet.* 31, 493-525.
- Carel,J.C., Le,S.C., Condamine,L., Mallet,E., Chaussain,J.L., Adnot,P., Garabedian,M., and Bougneres,P. (1999). Resistance to the lipolytic action of epinephrine: a new feature of protein Gs deficiency. *J. Clin. Endocrinol. Metab* 84, 4127-4131.
- Chen,M., Chen,H., Nguyen,A., Gupta,D., Wang,J., Lai,E.W., Pacak,K., Gavrilova,O., Quon,M.J., and Weinstein,L.S. (2010). G(s)alpha deficiency in adipose tissue leads to a lean phenotype with divergent effects on cold tolerance and diet-induced thermogenesis. *Cell Metab* 11, 320-330.
- Chen,M., Wang,J., Dickerson,K.E., Kelleher,J., Xie,T., Gupta,D., Lai,E.W., Pacak,K., Gavrilova,O., and Weinstein,L.S. (2009). Central nervous system imprinting of the G protein G(s)alpha and its role in metabolic regulation. *Cell Metab* 9, 548-555.
- Crane,J.L., Shamblott,M.J., Axelman,J., Hsu,S., Levine,M.A., and Germain-Lee,E.L. (2009). Imprinting Status of Galpha(s), NESP55, and XLalphas in cell cultures derived from human embryonic germ cells: GNAS imprinting in human embryonic germ cells. *Clin. Transl. Sci.* 2, 355-360.
- de Nanclares,G.P., Fernandez-Rebollo,E., Santin,I., Garcia-Cuartero,B., Gaztambide,S., Menendez,E., Morales,M.J., Pombo,M., Bilbao,J.R., Barros,F., Zazo,N., Ahrens,W., Juppner,H., Hiort,O., Castano,L., and Bastepe,M. (2007). Epigenetic defects of GNAS in patients with pseudohypoparathyroidism and mild features of Albright's hereditary osteodystrophy. *J. Clin. Endocrinol. Metab* 92, 2370-2373.
- Frey,U.H., Bachmann,H.S., Peters,J., and Siffert,W. (2008a). PCR-amplification of GC-rich regions: 'slowdown PCR'. *Nat. Protoc.* 3, 1312-1317.
- Frey,U.H., Lieb,W., Erdmann,J., Savidou,D., Heusch,G., Leineweber,K., Jakob,H., Hense,H.W., Lowel,H., Brockmeyer,N.H., Schunkert,H., and Siffert,W. (2008b). Characterization of the GNAQ promoter and association of increased Gq expression with cardiac hypertrophy in humans. *Eur. Heart J.* 29, 888-897.
- Frey,U.H., Nuckel,H., Dobrev,D., Manthey,I., Sandalcioglu,I.E., Eisenhardt,A., Worm,K., Hauner,H., and Siffert,W. (2005). Quantification of G protein Gaalphas subunit splice variants in different human tissues and cells using pyrosequencing. *Gene Expr.* 12, 69-81.
- Germain-Lee,E.L., Ding,C.L., Deng,Z., Crane,J.L., Saji,M., Ringel,M.D., and Levine,M.A. (2002). Paternal imprinting of Galpha(s) in the human thyroid as the basis of TSH resistance in pseudohypoparathyroidism type 1a. *Biochem. Biophys. Res. Commun.* 296, 67-72.
- Germain-Lee,E.L., Schwindinger,W., Crane,J.L., Zewdu,R., Zweifel,L.S., Wand,G., Huso,D.L., Saji,M., Ringel,M.D., and Levine,M.A. (2005). A mouse model of albright hereditary osteodystrophy generated by targeted disruption of exon 1 of the Gnas gene. *Endocrinology* 146, 4697-4709.
- Gruber,J.D., Colligan,P.B., and Wolford,J.K. (2002). Estimation of single nucleotide polymorphism allele frequency in DNA pools by using Pyrosequencing. *Hum. Genet.* 110, 395-401.

- Hauner,H., Rohrig,K., and Siffert,W. (2002). Effects of the G-protein beta3 subunit 825T allele on adipogenesis and lipolysis in cultured human preadipocytes and adipocytes. *Horm. Metab Res.* 34, 475-480.
- Hayward,B.E., Barlier,A., Korbonits,M., Grossman,A.B., Jacquet,P., Enjalbert,A., and Bonthron,D.T. (2001). Imprinting of the G(s)alpha gene GNAS1 in the pathogenesis of acromegaly. *J. Clin. Invest* 107, R31-R36.
- Hayward,B.E., Moran,V., Strain,L., and Bonthron,D.T. (1998). Bidirectional imprinting of a single gene: GNAS1 encodes maternally, paternally, and biallelically derived proteins. *Proc. Natl. Acad. Sci. U. S. A* 95, 15475-15480.
- Ishikawa,Y., Bianchi,C., Nadal-Ginard,B., and Homcy,C.J. (1990). Alternative promoter and 5' exon generate a novel Gs alpha mRNA. *J. Biol. Chem.* 265, 8458-8462.
- Jan de Beur,S., Ding,C., Germain-Lee,E., Cho,J., Maret,A., and Levine,M.A. (2003). Discordance between genetic and epigenetic defects in pseudohypoparathyroidism type 1b revealed by inconsistent loss of maternal imprinting at GNAS1. *Am. J. Hum. Genet.* 73, 314-322.
- Kozasa,T., Itoh,H., Tsukamoto,T., and Kaziro,Y. (1988). Isolation and characterization of the human Gs alpha gene. *Proc. Natl. Acad. Sci. U. S. A* 85, 2081-2085.
- Kwok,C.T., Ward,R.L., Hawkins,N.J., and Hitchins,M.P. (2010). Detection of allelic imbalance in MLH1 expression by pyrosequencing serves as a tool for the identification of germline defects in Lynch syndrome. *Fam. Cancer* 9, 345-356.
- Li,T., Vu,T.H., Zeng,Z.L., Nguyen,B.T., Hayward,B.E., Bonthron,D.T., Hu,J.F., and Hoffman,A.R. (2000). Tissue-specific expression of antisense and sense transcripts at the imprinted Gnas locus. *Genomics* 69, 295-304.
- Liu,J., Chen,M., Deng,C., Bourc'his,D., Nealon,J.G., Erlichman,B., Bestor,T.H., and Weinstein,L.S. (2005). Identification of the control region for tissue-specific imprinting of the stimulatory G protein alpha-subunit. *Proc. Natl. Acad. Sci. U. S. A* 102, 5513-5518.
- Liu,J., Erlichman,B., and Weinstein,L.S. (2003). The stimulatory G protein alpha-subunit Gs alpha is imprinted in human thyroid glands: implications for thyroid function in pseudohypoparathyroidism types 1A and 1B. *J. Clin. Endocrinol. Metab* 88, 4336-4341.
- Liu,J., Litman,D., Rosenberg,M.J., Yu,S., Biesecker,L.G., and Weinstein,L.S. (2000a). A GNAS1 imprinting defect in pseudohypoparathyroidism type IB. *J. Clin. Invest* 106, 1167-1174.
- Liu,J., Yu,S., Litman,D., Chen,W., and Weinstein,L.S. (2000b). Identification of a methylation imprint mark within the mouse Gnas locus. *Mol. Cell Biol.* 20, 5808-5817.
- Long,D.N., McGuire,S., Levine,M.A., Weinstein,L.S., and Germain-Lee,E.L. (2007). Body mass index differences in pseudohypoparathyroidism type 1a versus pseudopseudohypoparathyroidism may implicate paternal imprinting of Galpha(s) in the development of human obesity. *J. Clin. Endocrinol. Metab* 92, 1073-1079.
- Mantovani,G., Ballare,E., Giammona,E., Beck-Peccoz,P., and Spada,A. (2002). The galpha gene: predominant maternal origin of transcription in human thyroid gland and gonads. *J. Clin. Endocrinol. Metab* 87, 4736-4740.
- Mantovani,G., Bondioni,S., Locatelli,M., Pedroni,C., Lania,A.G., Ferrante,E., Filopanti,M., Beck-Peccoz,P., and Spada,A. (2004). Biallelic expression of the Gsalpha gene in human bone and adipose tissue. *J. Clin. Endocrinol. Metab* 89, 6316-6319.

- Mantovani,G., de,S.L., Barbieri,A.M., Elli,F.M., Bollati,V., Vaira,V., Labarile,P., Bondioni,S., Peverelli,E., Lania,A.G., Beck-Peccoz,P., and Spada,A. (2010). Pseudohypoparathyroidism and GNAS epigenetic defects: clinical evaluation of albright hereditary osteodystrophy and molecular analysis in 40 patients. *J. Clin. Endocrinol. Metab* 95, 651-658.
- Mariot,V., Maupetit-Mehouas,S., Sinding,C., Kottler,M.L., and Linglart,A. (2008). A maternal epimutation of GNAS leads to Albright osteodystrophy and parathyroid hormone resistance. *J. Clin. Endocrinol. Metab* 93, 661-665.
- Morison,I.M., Ramsay,J.P., and Spencer,H.G. (2005). A census of mammalian imprinting. *Trends Genet.* 21, 457-465.
- Richelsen,B., Pedersen,S.B., Moller-Pedersen,T., and Bak,J.F. (1991). Regional differences in triglyceride breakdown in human adipose tissue: effects of catecholamines, insulin, and prostaglandin E2. *Metabolism* 40, 990-996.
- Ronaghi,M., Karamohamed,S., Pettersson,B., Uhlen,M., and Nyren,P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242, 84-89.
- Ronaghi,M., Uhlen,M., and Nyren,P. (1998). A sequencing method based on real-time pyrophosphate. *Science* 281, 363, 365.
- Siffert,W., Roskopf,D., Moritz,A., Wieland,T., Kaldenberg-Stasch,S., Kettler,N., Hartung,K., Beckmann,S., and Jakobs,K.H. (1995). Enhanced G protein activation in immortalized lymphoblasts from patients with essential hypertension. *J. Clin. Invest* 96, 759-766.
- Sun,A., Ge,J., Siffert,W., and Frey,U.H. (2005). Quantification of allele-specific G-protein beta3 subunit mRNA transcripts in different human cells and tissues by Pyrosequencing. *Eur. J. Hum. Genet.* 13, 361-369.
- Unluturk,U., Harmanci,A., Babaoglu,M., Yasar,U., Varli,K., Bastepe,M., and Bayraktar,M. (2008). Molecular diagnosis and clinical characterization of pseudohypoparathyroidism type-Ib in a patient with mild Albright's hereditary osteodystrophy-like features, epileptic seizures, and defective renal handling of uric acid. *Am. J. Med. Sci.* 336, 84-90.
- Wang,H. and Elbein,S.C. (2007). Detection of allelic imbalance in gene expression using pyrosequencing. *Methods Mol. Biol.* 373, 157-176.
- Wasson,J., Skolnick,G., Love-Gregory,L., and Permutt,M.A. (2002). Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by pyrosequencing technology. *Biotechniques* 32, 1144-6, 1148, 1150.
- Weinstein,L.S. (2001). The stimulatory G protein alpha-subunit gene: mutations and imprinting lead to complex phenotypes. *J. Clin. Endocrinol. Metab* 86, 4622-4626.
- Weinstein,L.S., Chen,M., Xie,T., and Liu,J. (2006). Genetic diseases associated with heterotrimeric G proteins. *Trends Pharmacol. Sci.* 27, 260-266.
- Weinstein,L.S., Xie,T., Zhang,Q.H., and Chen,M. (2007). Studies of the regulation and function of the Gs alpha gene Gnas using gene targeting technology. *Pharmacol. Ther.* 115, 271-291.
- Weinstein,L.S., Yu,S., Warner,D.R., and Liu,J. (2001). Endocrine manifestations of stimulatory G protein alpha-subunit mutations and the role of genomic imprinting. *Endocr. Rev.* 22, 675-705.
- Williamson,C.M., Ball,S.T., Nottingham,W.T., Skinner,J.A., Plagge,A., Turner,M.D., Powles,N., Hough,T., Papworth,D., Fraser,W.D., Maconochie,M., and Peters,J. (2004). A cis-acting control region is required exclusively for the tissue-specific imprinting of Gnas. *Nat. Genet.* 36, 894-899.

Wolf, J.B., Cheverud, J.M., Roseman, C., and Hager, R. (2008). Genome-wide analysis reveals a complex pattern of genomic imprinting in mice. *PLoS. Genet.* 4, e1000091.

Wood, A.J. and Oakey, R.J. (2006). Genomic imprinting in mammals: emerging themes and established theories. *PLoS. Genet.* 2, e147.

Yu, S., Yu, D., Lee, E., Eckhaus, M., Lee, R., Corria, Z., Accili, D., Westphal, H., and Weinstein, L.S. (1998). Variable and tissue-specific hormone resistance in heterotrimeric Gs protein alpha-subunit (G α) knockout mice is due to tissue-specific imprinting of the g α gene. *Proc. Natl. Acad. Sci. U. S. A* 95, 8715-8720.

Zazo, C., Thiele, S., Martin, C., Fernandez-Rebollo, E., Martinez-Indart, L., Werner, R., Garin, I., Hiort, O., and Perez de, N.G. (2011). G α activity is reduced in erythrocyte membranes of patients with pseudohypoparathyroidism due to epigenetic alterations at the GNAS locus. *J. Bone Miner. Res.*

Zheng, H., Radeva, G., McCann, J.A., Hendy, G.N., and Goodyer, C.G. (2001). G α s transcripts are biallelically expressed in the human kidney cortex: implications for pseudohypoparathyroidism type 1b. *J. Clin. Endocrinol. Metab* 86, 4627-4629.

ACCEPTED MANUSCRIPT

Legends

Figure 1:

Organization of the human *GNAS* locus. The general organization of *GNAS* maternal (above) and paternal (below) alleles are shown (not drawn to scale). Four alternative exons splice into exon 2 and generate four different transcripts (NESP55, XL α s, a noncoding gene product from exon 1A and Gas). Arrows indicate transcription start sites. Also marked is the T393C polymorphism located in exon 5.

Figure 2:

Expected Pyrosequencing result by amplification of exon 1 obtained from cDNAs which were heterozygous for the *GNAS* T393C polymorphism. Peak heights were quantified using the Pyrosequencing software.

PCR products were amplified with PCR primers located in exon 1 (Sense Primer) and exon 5 (Antisense Primer) as indicated with arrows. We then used the pyrosequencing technique to determine allelic expression. We used the sequencing primer 5'-CAGTTCAGAGTGGACTACAT-3' in exon 5, which has its 3'-end directly at the T393C polymorphism and the nucleotide dispensation order (G-T-C-G-T-G-A-G-T). The first nucleotide (G) does not result in light emission, since these G will not be synthesized to the cDNA-strand, because it does not conform to the corresponding *GNAS* exon 5 sequence. The second nucleotide presents a "T". Since we used samples heterozygous for the T393C polymorphism this nucleotide leads to light emission, however, peak height has half height compared to homozygous T allele carrier. The third nucleotide is a "C". This will lead to a peak, which is three times higher than the peak of the nucleotide 2, since light emission will release from the heterozygous "C" and the following homozygous C in the *GNAS* exon 5 sequence. The incorporated G at position 4 does not conform to the *GNAS* exon 5 sequence, and does therefore not result in light emission. The next incorporated nucleotides (5-9) conform to the further *GNAS* exon 5 sequence and therefore lead to light emission classically observed in homozygous samples.

Shown is the expected Pyrogram when both alleles (C and T) were equally expressed: height of the T = 1/3 of height of the C.

Figure 3:

(A) Validation of Pyrosequencing using the *GNAS* T393C polymorphism for allele-specific transcript quantification. Genomic DNA mixtures with known CC/TT ratios of *GNAS* T393C homozygous samples were subjected to Pyrosequencing and the calibration curve for the *GNAS* T393C polymorphism was calculated. Measured C alleles were determined from the C peak ratio from repeated Pyrosequencing analysis. A linear relationship between measured and expected ratios could be confirmed over the whole range investigated ($y = 0.16 + 0.97x$, $r^2 = 0.98$, $p < 0.001$).

(B) Comparison of C allele ratios of DNAs and cDNAs from heterozygous samples of the *GNAS* T393C polymorphism. Mean C allelic ratios in DNA samples were 49.6 % (95% CI 49.1 % - 50.2 %) which is marked with the dotted line and due to the measurement of the pyrosequencing technology. C allelic ratios in cDNA samples showed a distinct variation (mean 48.8 %, 95% CI 47.6 %- 50 %), which gave rise to an unequal allelic expression. cDNA was obtained from the 96 cell line and tissue samples (lymphoblasts, n=11, peripheral blood mononuclear cells, n=18, mammary adipose tissue, n=23 and heart tissue, n=44).

(C) Comparison of C allele ratios of cDNAs from lymphoblasts (n=11), peripheral blood mononuclear cells (n=18), mammary adipose tissue (n=23) and heart tissue (n=44). We observed an unequal allelic expression in each tissue sample examined (lymphoblasts: mean C allele frequency 49.6 % (95%CI 44.9 % - 54.2 %), peripheral blood mononuclear cells: mean C allele frequency 49.6 % (95%CI 46.1 % - 53.1 %), mammary adipose tissue: mean C allele frequency 49.3 % (95%CI 46.8 % - 51.8 %) and heart tissue: mean C allele frequency 48 % (95% CI 46.4 % - 49.6 %).

Abbreviations: Ly, lymphoblasts; PBMC, peripheral blood mononuclear cells; MAT, mammary adipose tissue; HT, heart tissue

Figure 4:

Comparison of A allele ratios of DNAs and cDNAs from heterozygous samples of the *MLH1* A655G polymorphism. Mean A allelic ratios in DNA samples were 50 % (95% CI 49.6 % - 50.4 %) which is marked with the dotted line. A allelic ratios in cDNA samples showed a similar variation (mean 49.9 %, 95% CI 49.5 %- 50.3 %). cDNA was obtained from 88 cell line and tissue samples (lymphoblasts,

n=9, peripheral blood mononuclear cells, n=22, mammary adipose tissue, n=10 and heart tissue, n=47).

Figure 5: Observed Pyrosequencing results by amplification of exon 1A obtained from cDNAs which were heterozygous for the *GNAS* T393C polymorphism. Peak heights were quantified using the Pyrosequencing software. PCR products were amplified with PCR primers located in exon 1A (Sense Primer) and exon 5 (Antisense Primer) as indicated. “E” corresponds to enzyme and “S” to substrate. Both components are dispensed before nucleotide are incorporated to the reaction. “G”, “C”, “A” and “T” correspond to the nucleotide guanine, cytosine, adenosine and thymine.

(A) Since the exon 1A is only expressed from the paternal allele, the pyrograms show a 100% allelic expression from the paternal C allele. The first incorporated “T” corresponding to the T393C polymorphism does not lead to light emission, since a 100% expression from paternal C is comparable to homozygous T393C CC genotypes.

(B) Observed pyrogram when the T allele is inherited from the father: 100% allelic expression from the paternal T allele. The first incorporated “T” and “C” evoke an equal peak height, since a 100% expression from the paternal T is comparable to homozygous T393C TT genotypes and therefore equal to the next incorporated, homozygous “C”.

(C) Maternal imprinting of exon 1A in the 96 tissues (lymphoblasts (Ly), n=11, peripheral blood mononuclear cells (PBMC), n=18, mammary adipose tissue (MAT), n=23 and heart tissue (HT), n=44) investigated. As expected, we found a monoallelic paternal expression of exon 1A, which is methylated on the maternal allele (ratio paternal/total \pm SEM: 98 ± 0.3 %, 95 % CI 97.4 % - 98.6 %).

Figure 6:

Observed Pyrosequencing results by amplification of exon 1 obtained from cDNAs which were heterozygous for the *GNAS* T393C polymorphism. Peak heights were quantified using the Pyrosequencing software.

(A) Shown is a representative observed pyrogram when the T allele was inherited from the father. As explained in the figure legend to figure 2, we expected that the height of the T would be 1/3 of the

height of the C. However, we observed that the height of the T was less than 1/3 of the height of the C allele. Therefore, the expression from the maternal C allele was increased.

(B) Shown is a representative observed pyrogram when the C allele was inherited from the father. The height of the T was higher than the expected 1/3 of the height of the C allele. Therefore, expression from the maternal T allele was increased.

“E” corresponds to enzyme and “S” to substrate. Both components are dispensed before nucleotide are incorporated to the reaction.

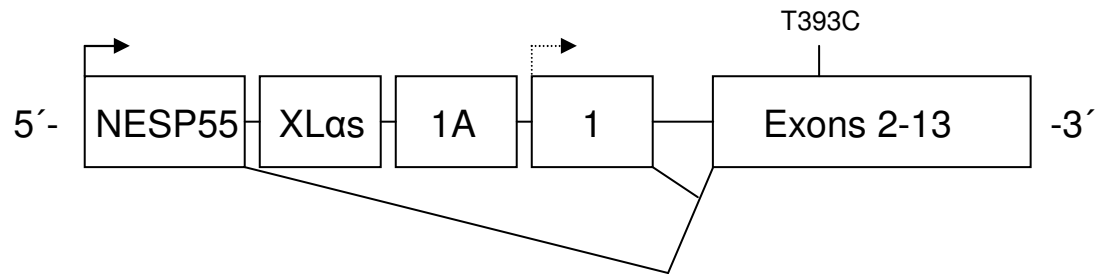
(C) Comparison of C allele ratios of lymphoblasts (n=11), peripheral blood mononuclear cells (n=18), mammary adipose tissue (n=23) and heart tissue (n=44) divided by C allelic expression from the maternal or the paternal allele. Mean C allele frequency from the maternal allele was above 50% in all tissues examined (lymphoblasts (Ly): 54.8%, peripheral blood mononuclear cells (PBMC): 55.7%, mammary adipose tissue (MAT): 53.4% and heart tissue (HT): 52.1%) and mean C allele frequency from the paternal allele was below 50% in all tissues examined (lymphoblasts: 43.2%, peripheral blood mononuclear cells: 43.5%, mammary adipose tissue: 44% and heart tissue: 43.9%). Maternal C allele frequency differed significantly from paternal C allele frequency in lymphoblasts (p=0.0004), peripheral blood mononuclear cells (p<0.0001), mammary adipose tissue (p<0.0001) and heart tissue (p<0.0001).

Figure 7:

Differential allelic expression in 11 lymphoblasts (Ly), 18 peripheral blood mononuclear cells (PBMC), 23 mammary adipose tissues (MAT) and 44 heart tissues (HT) of exon 1. We found an unequal allelic expression with maternal over paternal expression in lymphoblasts (ratio maternal/total*100 ± SEM: 55.7 ± 1 %, 95 % CI 53.4 % - 58.1 %), peripheral blood mononuclear cells (56.1 ± 0.8, 95% CI 54.5 % - 57.4 %), mammary adipose tissue (54.5 ± 0.8 %, 95% CI 53 % - 56.1 %) and heart tissue (54.1 ± 0.6 %, 95 % CI 53 % -55.3 %). The least expressed allele was always the same represented in exon 1A transcripts. The dotted line is set to the hypothetical mean of 50%.

Figure 1

Maternal allele



Paternal allele

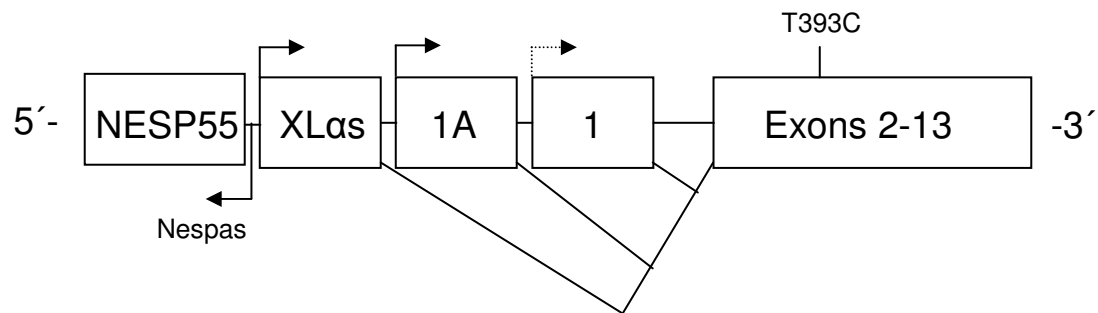


Figure 2

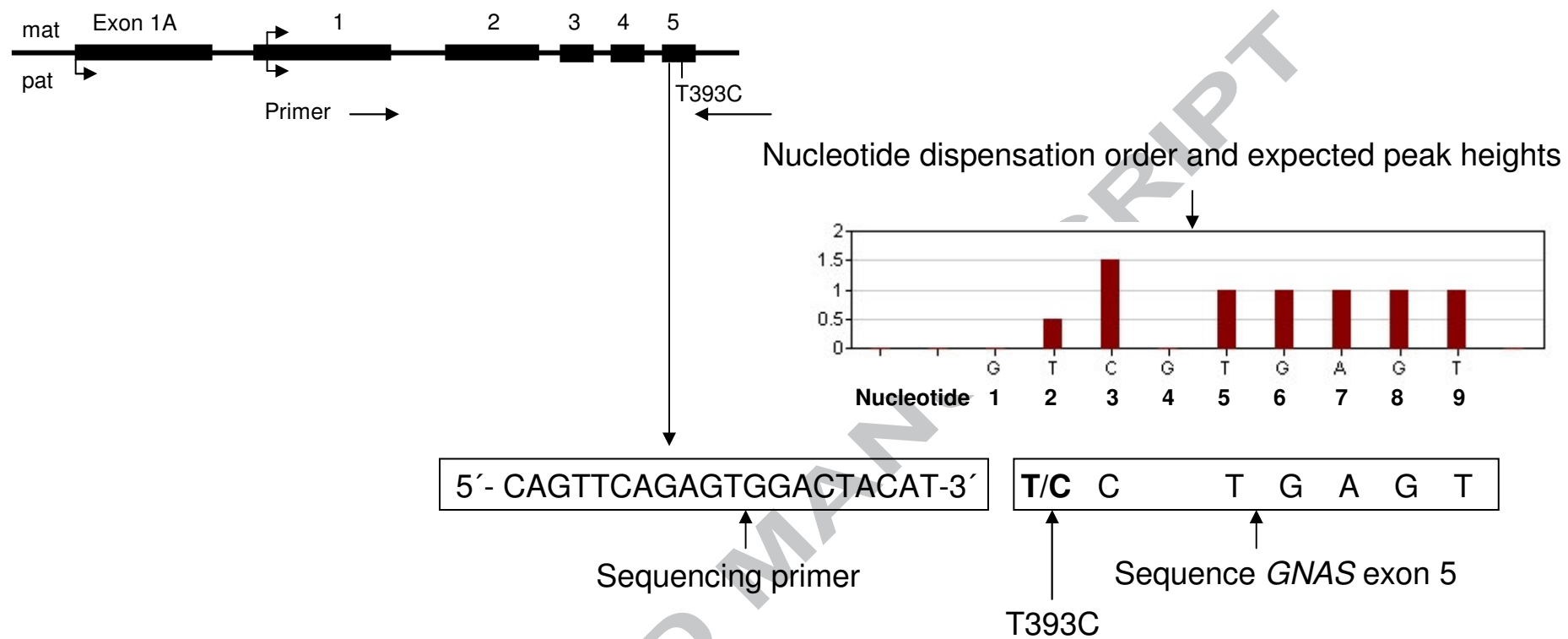


Figure 3

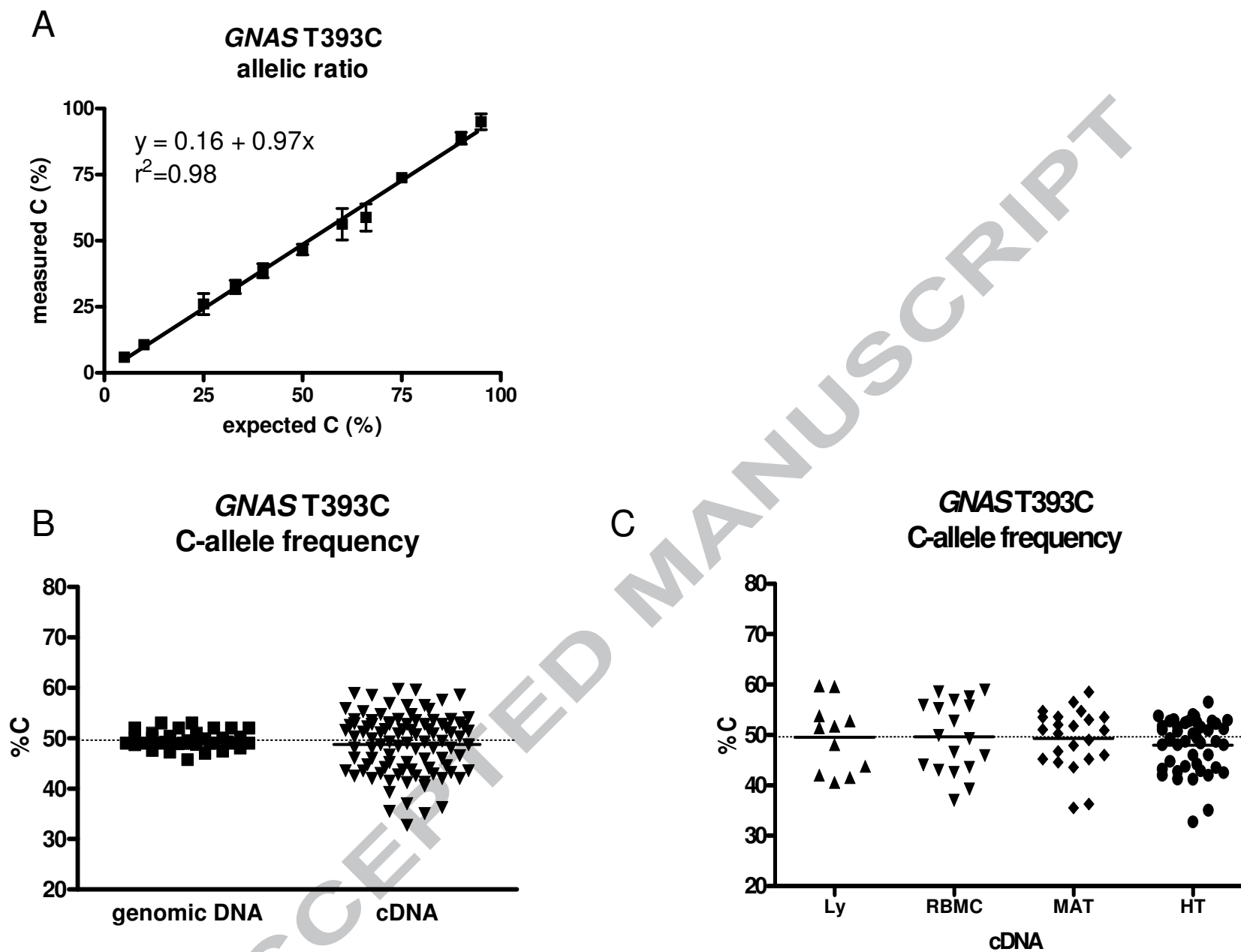


Figure 4

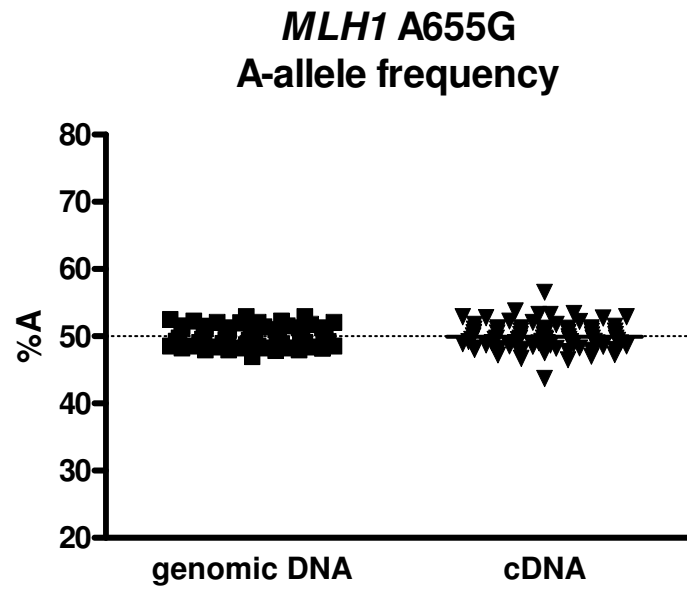


Figure 5

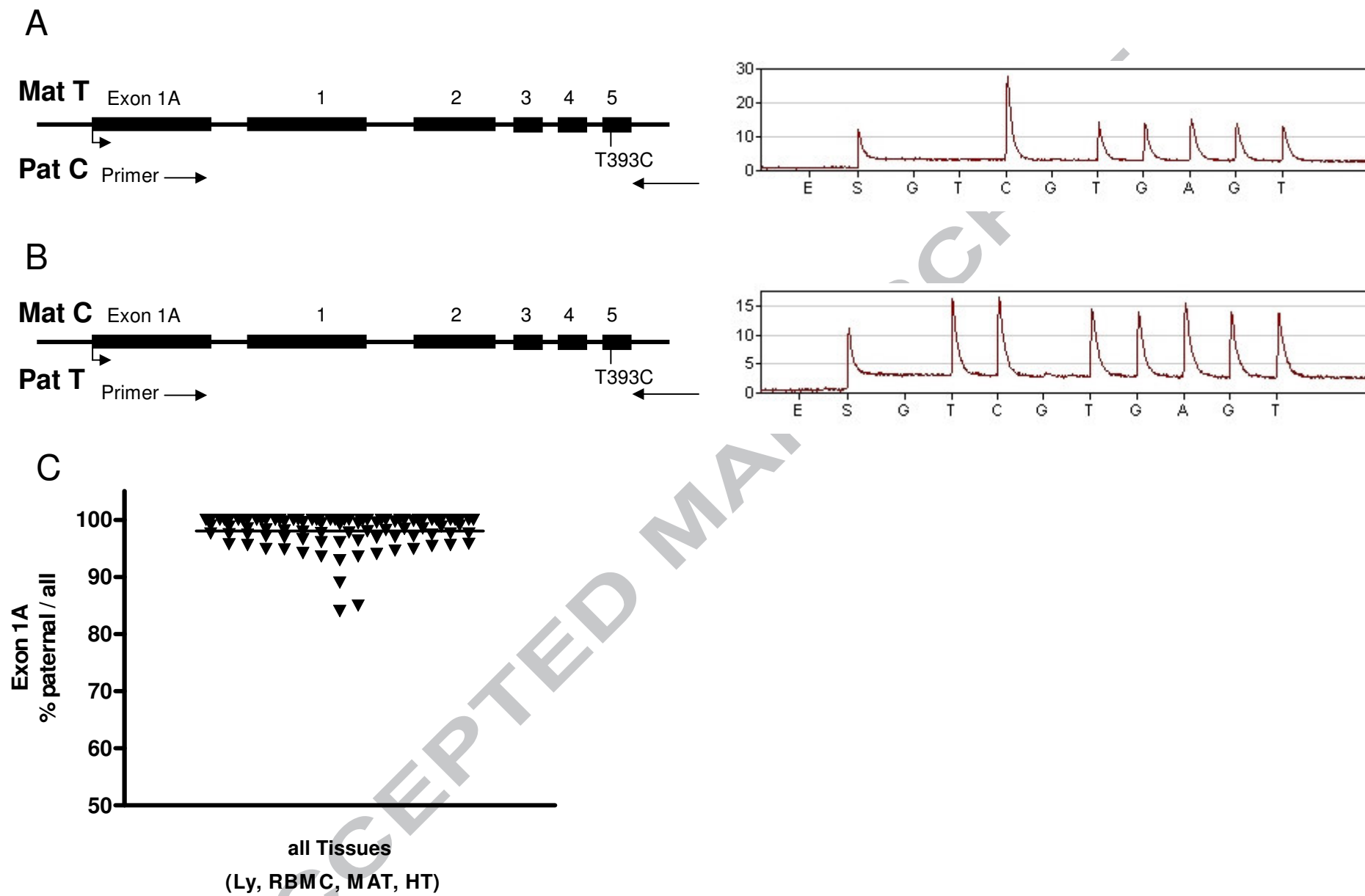


Figure 6

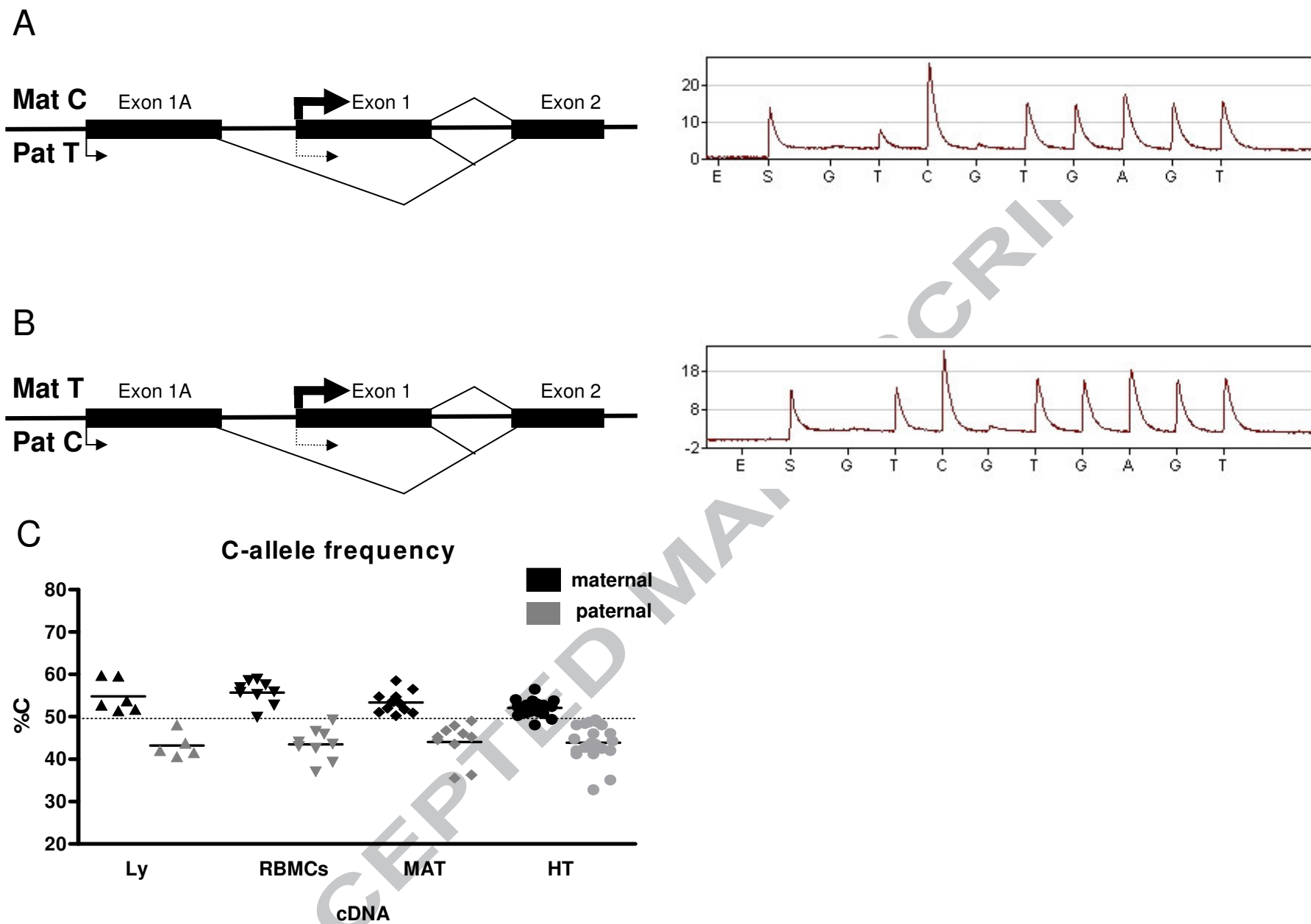
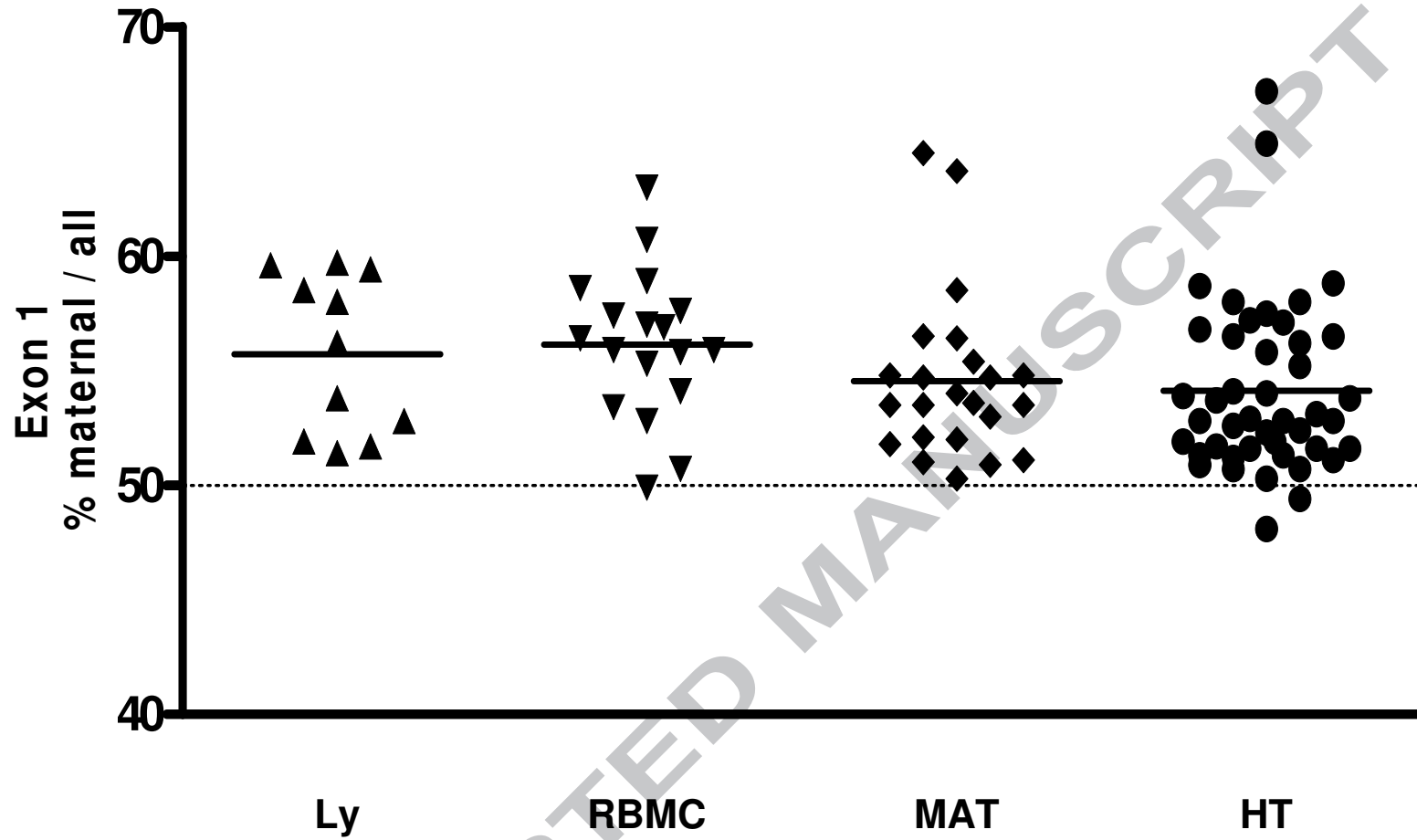


Figure 7



Highlights:

- The human *GNAS* gene is imprinted in a tissue-specific manner
- Exact mechanisms of *Gas* imprinting are not yet fully understood
- We analysed allelic expression using the highly sensitive Pyrosequencing technology
- We found an unequal allelic expression in different non-imprinted tissues
- This phenomenon may be a general feature existing in all tissues

ACCEPTED MANUSCRIPT