IPO8 and FBLX10: New reference genes for gene expression studies in human adipose tissue.

Running head: Control genes in human adipose tissue

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ABSTRACT

Housekeeping genes frequently used in gene expression studies are highly regulated in human adipose tissue. To ensure a correct interpretation of results, it is critical to select appropriate reference genes. Subcutaneous and omental adipose tissue expression was analyzed from lean and obese subjects using whole genome cDNA microarrays to identify stably expressed genes and commercial Taqman Low Density Arrays (LDAs), with 16 common control genes. The best candidate gene from microarrays analysis was F-box and leucine-rich repeat protein-10 (FBXL10) (fold-change 10^{-3} $p<0.01$), an ubiquitous nucleolar protein evolutionarily conserved. Hypoxanthine-phosphoribosyl-transferase-1 (HPRT1) and Importin-8 (IPO8), were the best reference genes among the 16 genes in the LDAs with coefficients of variation (CV) of 4.51% and 4.55%, respectively. However, when the LDAs data were further analyzed by the geNORM and Normfinder softwares, IPO8, a nuclear protein mediating import of proteins, was the first and the third better reference gene, respectively. IPO8 and FBXL10 were further validated by Real-Time PCR in additional omental and subcutaneous fat samples and primary cultured preadipocytes. According to their CV, IPO8 resulted more suitable than FBXL10 in both adipose tissue depots and subcutaneous preadipocytes, while FBXL10 performed better than IPO8 in omental cultured preadipocytes, . Both genes expression levels did not change throughout adipogenesis.

Thus, we provide clear evidence that IPO8 and FBXL10 are good candidates to use as reference genes in gene expression studies in human omental and subcutaneous adipose tissues as well as differentiated primary preadipocytes.

INTRODUCTION

Endogenous or reference genes are those that are constitutively expressed in all tissues and have an essential role in the maintenance of the cellular function (1). In line with this role, it is generally assumed that their expression levels are similar in different conditions or cell types. However, it has been shown that genes usually considered as reference genes exhibit large variations in different tissues or experimental conditions (2-5).

Regarding the adipose tissue, a highly regulated and complex organ, the bibliography describes as housekeeping genes some that are regulated under hormonal stimulation or during adipogenic differentiation. That is the case of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB) or 18S ribosomal RNA (18S) (6-9). It seems fundamental to choose a good control gene in order to allow an adequate analysis of gene expression studies. When working with a single tissue or cell type, this is solved using a gene whose expression levels remain constant in those specific samples; however, in human adipose tissue, the expression of genes is different depending on their anatomical location (subcutaneous (SC) or omental (OM)). As OM adipose tissue differs considerably from the subcutaneous SC depot, physiological and gene expression differences have been reported between both localizations (10-12).

The aim of this study was to find the most suitable reference gene for gene expression studies in human adipose tissues and primary cultures of preadipocytes, using microarrays and statistical methods. For this purpose we evaluated the variation in gene expression level in SC and OM adipose samples from lean and

obese subjects using Taqman® Endogenous Control Arrays *Low Density Arrays* (LDA), which contain 16 control genes normally used in the literature as endogenous genes, and to compare the results with stable genes obtained from microarrays. Though microarrays are commonly applied to find genes with differential expression across experimental conditions, the data may also be used to identify stably expressed genes (5). Different stability algorithms have been used with microarray data, in order to select candidate genes based on the fold expression changes (13, 14), variance of expression (15) or integrative correlations (16).The statistical methods used in our study were geNORM, Normfinder and analysis of coefficient of variance (%CV).

In this study we show that IPO8 and FBLX10 are two previously unidentified genes, which are good control genes for expression studies involving human isolated differentiated preadipocytes and whole adipose tissue.

METHODS AND PROCEDURES

Subjects

Human adipose tissue was obtained from the OM and SC depots from 45 obese patients, 20 males and 25 females, undergoing bariatric surgery. Five of these patients were diabetic. OM and SC adipose tissue from 4 lean (3 males and 1 female) subjects were also obtained by Nissen fundoplication. The samples were immediately frozen and stored at -80º C until processed. Surgeries were performed at the Hospital Universitario La Paz (Madrid), Hospital Clínico S. Carlos (Madrid) and Clínica Universitaria de Navarra (Pamplona). Patients were classified as obese or lean according to their body mass index (BMI), calculated as the ratio between the weight in kilograms and the square of the height in meters. Obese patients were 40.4 ± 1.7 years old and showed a BMI of 49.7 ± 1.1 kg/m^2 (mean ± SEM) whereas lean subjects were 47.5 ± 7.1 years old and presented a BMI of 23.8 ± 0.3 kg/m^2. Basal glucose, triglycerides and total cholesterol concentrations in plasma were 114 ± 6 mg/dl, 139 ± 19 mg/dl and 182 ± 5 mg/dl in obese patients, respectively, while the respective concentrations of lean patients were 91 ± 1, 71 ± 12 and 197 ± 13 mg/dl. Although glucose and triglycerides were significantly different between both groups ($p<0.05$), variables were within the normal range. All subjects gave informed consent. The protocols were approved by the Ethical Committees of the Hospitals and our institution, and are in agreement with the European laws on biomedical research and with the principles of the Declaration of Helsinki.

Primary cultures of human omental preadipocytes.

Human adipose tissue was obtained from the abdominal OM and SC regions from 8 additional obese patients (7 women and 1 men) undergoing bariatric surgery. Patients with a mean age of 43.9 ± 4.1 yr, showed a BMI of 49.3 ± 2.2 kg/m^2, with no other pathologies. The protocol followed the ethical considerations as above.

Preadipocytes were isolated and differentiated under defined adipogenic conditions (17). After a 10 days differentiation period, cells were treated for 48 h with 10% depleted serum in presence or absence of insulin (3 nM), and treated (or not) with 2 nM triiodothyronine, rosiglitazone (1 μM), dexamethasone (100 nM), hydrocortisone (100 nM) or norepinephrine (5 μM**)** during the last 24 h. Cells were harvested at day 12 of differentiation. Depleted serum was obtained as follows: first, hypothyroid serum was obtained by depleting heat-inactivated fetal bovine serum of thyroid hormones with the anion exchange resin AG1-X8 as described (18). Depleted serum was obtained by treatment of hypothyroid serum with charcoal overnight (20 mg charcoal/ ml serum). This treatment further depleted serum from the remaining thyroid hormones and other hormones such as insulin and glucocorticoids**.** After harvesting the cells, total RNA was extracted.

In differentiation experiments, cells were just differentiated without any further treatment and harvested at 0, 6 and 12 days.

RNA extraction

Approximately 100 mg of each adipose sample was used to extract RNA with the RNeasy lipid tissue kit (QIAGEN, Madrid, Spain) following the protocol provided by

the manufacturer. RNA integrity and concentration were checked by the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Microarrays

"Human Genome Survey Microarray V2.0" from Applied Biosystems (Foster City, CA) contains 60-mer oligonucleotides specific for 32,878 probes corresponding to 29,098 genes. The data set includes genes from both public databases (RefSeqs, GenBank and Mammalian Gene Collection) and the Celera® Genomics database (Rockville, MD). Ten arrays were utilized for OM adipose tissue and 14 for SC adipose tissue from men and women. Total RNA samples obtained from 12 obese and 4 lean subjects were amplified into digoxigenin labelled cRNA using Applied Biosystem *Chemiluminiscent RT-ITV Labeling KIT (*Foster City, CA) according to the manufacturer´s directions. The amplified and labelled cRNA was injected into each microarray hybridization chamber and hybridized at 55 ºC for 16 h. Detection at 458 nm was performed with the *Chemiluminiscent Detection* kit (Applied Biosystems, Foster City, CA) and image acquisition was carried out using the 1700 *Chemiluminiscent Microarray Analyzer*.

Arrays data were read, pre-processed and filtered according to the platform manufacturer´s recommendation with the Applied Biosystems software (Foster City, CA). For Applied Biosystems Expression Arrays, an excellent signal detection is set as signal to noise (S/N)> 3 and quality flag< 5000. Thus, probes having a S/N ratio below 3 and a Flag value above 5,000 in all samples were discarded. After filtering, 14,564 genes in the OM depot and 19,183 genes in the SC depot from the original

29,098 genes were obtained. Before being compiled into an expression profile data matrix, all arrays were quantile-normalized to be comparable because this method ensures that the intensities have the same empirical distribution across arrays. Genes differentially expressed were selected by using the LIMMA package(19) from the Bioconductor project (http://www.bioconductor.org) (20) contained in the Spotfire Integromics software. The statistical analysis described above was performed by Integromics (Granada, Spain). Among the rest of the genes not diferentially expressed, we selected as stable those genes which in both depots showed a fold change < 0.01.

TaqMan® Low Density Endogenous Control Panel microfluidic cards

To validate the variation in gene expression levels of various commonly used endogenous controls, a TaqMan® Low Density Endogenous Control Panel was used (Applied Biosystems, Foster City, CA). This microfluidic card (or LDA) contains 16 human Taqman® Gene Expression Assays (Table 1). Each LDA has a 3 well replicate for each assay, and eight ports for loading of cDNA and Taqman Universal Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 100 μl. The 384-well micro fluid cards were run in the following PCR program: 50 °C for 2 min, 94 °C for 10 min and 40 cycles of 97 °C for 30 s followed by 60 °C for 1 min. To measure RNA quantity (Ct), samples were monitored by the Applied Bioystem 7900HT Fast Real-Time PCR System (Foster City, CA).

OM and SC adipose tissues from 4 obese and 4 lean subjects were used. Fifty nanograms of each RNA were reversed-transcribed with the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA) following the manufacturer´s

instructions. Each sample was loaded in one of the eight ports of the microfluidic cards, analyzed in triplicates obtaining an average Ct and the coefficient of variation (CV). This CV equals the standard deviation divided by the mean Ct (expressed as percentage) and was used to compare the variation degree among the 16 control genes. In addition to CV analysis, geNORM Visual Basic (21) and Normfinder applications for Microsoft Excel (15) were used to confirm results. Each of these methods generates a measure of reference gene stability, which can be used to rank the reference genes in order of stability. Both softwares generate a stability value of which a lower value indicates increased stability in gene expression.

Taqman RT-PCR

A further analysis was performed with conventional Taqman RT-PCR, using pre-designed Taqman Assays on demand (Applied Biosystems, Foster City, CA) for IPO8, 18S, FBXL10, leptin, interleukin 6 and adiponectin. For cDNA synthesis, 1 μg of total RNA was reverse transcribed in a 20 μl final volume using iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Real-time PCR reactions were performed on a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA).

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). Significance of differences between two groups is shown, and it was measured by Student`s t-test. Statistical analysis of microarrays and LDAs have been explained in the corresponding sections.

RESULTS

Microarray gene expression analysis

A whole genome microarray assay analysis from a group of human adipose tissue (OM and SC adipose tissue from 4 lean and 12 obese subjects) (unpublished results) identified genes with a moderate abundance, which were consistently expressed in all the samples across the study. Among them, those with a fold-change <0.01 in both, OM and SC depots were chosen. From 29,098 genes analyzed, 44% and 58% of the OM and SC genes, respectively, were expressed with a good signal quality. Among these, 42 genes were found in OM adipose tissue and 248 genes in SC fat tissue with a stable expression, exhibiting at least a p <0.01. After comparing stably expressed genes from both depots, only 2 genes were common to OM and SC localizations, FBXL10 and FTCD. FBXL10 is a nucleolar protein that belongs to the JHDM family (JmjC-domain-containing histone demethylase), while FCTD (Forminotransferase-cyclo deaminase) is a bifunctional enzyme, a metabolic intermediate between the catabolism of histidine and that of folate. Considering the features of both proteins, we proposed FBXL10 as the most adequate as a control gene to perform further analyses, because it is an ubiquitous protein that is conserved evolutively and regulates transcription of ribosomal genes (18S, 5S, 8 S and 28S), which are essential for the maintenance of cell functions.

Taqman Low Density Arrays

To look for possible endogenous controls for gene expression in human adipose tissue, we used predesigned Taqman low density arrays with a panel of 16 human genes that are commonly used as endogenous controls (cat#4367563, Applied Biosystems, Foster City, CA). The genes are shown in Table 1.

cDNA from 16 different samples of adipose tissue (4 OM from lean, 4 OM from obese, 4 SC from lean and 4 SC from obese individuals) were run down all channels of 2 identical microfluidic cards with 16 endogenous genes. The data obtained were virtually identical with a correlation coefficient of 0.989, which shows that variability of gene expression between microfluidic cards is negligible.

Two samples from obese patients of each depot were run in triplicates in the 4 channels of each microfluidic card with the 16 control genes and compared to another card, in which 2 samples from lean OM and SC tissue were loaded. Except for 18S with the highest expression, the other genes exhibited Cts ranging from 23 to 32 cycles. Mean Ct values for FBXL10 (the most stable gene from microarrays) in OM and SC adipose tissue samples were 31.34 ± 0.32 and 31.96 ± 0.34 respectively.

The data showed a low coefficient of variation (CV%) between wells. Data are shown in Table 1.

Taking OM and SC samples as a whole, the control genes with the most stable expression were HPRT1 and IPO8, whereas the genes with high variation in the expression levels were 18S, RPLP0, ACTB and GAPDH, which are widely used as reference genes. When analyzing individually OM and SC tissues, HPRT1 was not as suitable as IPO8 in OM tissue, thus we decided to perform a further evaluation of stability. For this purpose, we analyzed the results from the LDAs with two powerful previously published Microsoft Excel-based softwares: the geNORM and the NormFinder programs. Both are available freely on internet sites (http://medgen.ugent.be/genorm and http://www.mdl.dk) geNorm determines the most stable reference genes from a set of tested genes within a panel, and

calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of reference genes (21). From geNORM results (Figure 1, upper panel), HPRT1 resulted to have the third highest M value whereas the two genes with the lowest M value were PPIA and IPO8, confirming that IPO8 is very suitable as a reference gene in adipose tissue. When NormFinder program was used with the 16 reference genes (Figure 1, lower panel), RPLP0 and GAPDH resulted the first and second genes in the ranking, while IPO8 turned out to be the third more suitable gene. Considering the results from the 3 analysis used (%CV, Genorm and Normfinder), IPO was the most suitable gene because its rankings were consistent with the 3 methods: 1st (Genorm), 2nd (%CV) and 3rd (NormFinder), despite some discrepancies with other genes. Although GAPDH with NormFinder resulted the second best gene, we think it is not suitable because its %CV was 7.06% (Rank 13) and in addition, as said in the introduction section, it is well described as a gene whose expression varies in different conditions in adipocytes. In a similar way, RPLP0 was ranked as 1st by NormFinder and 2nd by Genorm, however, it was ranked the last but one gene (Rank 15) by the %CV method. In addition, there are concerns about using ribosomal protein genes as reference.

The following step was to check in 30 subjects using both adipose localizations, the stability of IPO8 (the most suitable gene from LDA) versus 18S (the most used in the literature), and versus FBXL10 (the most stable gene from our microarray data) by real time RT-PCR. When CVs were compared, the 18S showed a coefficient of variation of 17.1%, while the CV of FBXL10 and IPO8 were 3.61% and 3.04%, respectively.

In order to further evaluate the reference gene stability on a gene whose expression is well known to be elevated in obese patients, leptin was normalized to 18S, IPO8 or FBXL10 (Figure 2, panels A and B). Figure 2 shows that only normalization with IPO8, but not with 18S or FBXL10, reflected the normal increase in leptin expression in both omental and subcutaneous depots. Other genes such as adiponectin and interleukin 6 were studied in the same samples. Adiponectin expression in omental samples showed a tendency to decrease with all reference genes, but only was significant with 18S, and almost with IPO8 ($p=0.05$) (Figure 2, panel C). In SC adipose tissue, adiponectin showed the opposite pattern with the 3 reference genes, but changes only were significant with IPO8 (Figure 2, panel D). Interleukin 6 increased significantly in omental adipose tissue only when it was normalized with IPO8 (150-fold change) or FBXL10 (76-fold change) (Figure 2, panel E), while no changes were observed in SC samples with any of the reference genes (Figure 2, panel F).

To further investigate the role of IPO8 and FBXL10 as reference genes, we decided to check it in primary cultures of human OM and SC adipocytes submitted to different treatments, in absence or presence of insulin, triodothyronine, norepinephrine, dexamethasone, hydrocortisone and rosiglitazone. The expression of both FBXL10 and IPO8 was not changed by the different treatments. Although both genes presented a good CV in these cultured adipocytes, FBXL10 exhibited a slightly better and lower CV than IPO8 (0.85% vs 0.95%) in the adipocytes from the omental depot, while IPO8 showed better CV than FBXL10 in those adipocytes from SC origin (2.15% vs 3.05%, respectively).

We further analyzed the IPO8 and FBXL10 expression at different days of differentiation (0, 6 and 12 days) of OM and SC cultured preadipocytes to study if these transcripts are modulated through adipogenesis. When data from differentiation experiments were taken as a whole, the %CV were 3.0% and 3.2% for FBXL10 and IPO8 respectively in OM adipocytes. GAPDH was also measured showing a higher CV of 6.3%. In SC differentiated preadipocytes FBXL10, IPO8 and GAPDH showed a CV of 2.51%, 2.54% and 6.73% respectively. The levels of expression of the three genes obtained from each differentiation day in adipocytes from both depots appear in Figure 3. As seen, no variations of Ct were observed for FBXL10 and IPO8, in contrast to GAPDH that showed significant changes along differentiation. Due to the exponential nature of this technique, each cycle (Ct) difference implicates a two-fold change in the real mRNA levels. The lack of variations of FBXL10 and IPO8 transcripts throughout differentiation reinforces their use as reference genes in this type of cultures of adipose cells, in contrast to GAPDH mRNA changes.

Adipocyte differentiation was checked by visual inspection of the cells which accumulated lipids progressively and by measuring the mRNA levels of leptin and aP2, two well known markers for late adipogenesis. The relative fold-change of leptin mRNA increased from 1.0 ± 0.2 at day 0, to 1.8 ± 0.10 at day 6 and 11.9 ± 4.5 at day 12 ($p<0.05$ *vs* day 0) in the OM adipocytes, and from 1 ± 0.5 (day 0) to 2.1 ± 0.6 (day 6) and to 7.0 ± 0.4 (day 12, $p<0.05$ *vs* day 0) in adipocytes from SC origin. Changes at day 6 were not significant. The relative fold-change of aP2 was 1± 0.3, 2158.0 ± 349.7, 392.4 ± 119.3 at 0, 6 and 12 days in OM adipocytes, respectively, and 1± 0.35, 4799.5 ± 750.7 and 3283.1 ± 532.4 at the same times in SC cells. All

changes in the aP2 transcript were significant ($p<0.01$) when compared to their respective day 0.

DISCUSSION

A determinant factor in the analysis of gene expression by quantitative RT-PCR is the election of a good control gene to ensure an adequate normalization of the results in the quantification of the expression. Although the scientific literature normally refers to housekeeping genes as synonyms of reference or control genes, these terms should not be indistinctly used, as many housekeeping genes vary greatly with different experimental or pathophysiological conditions.

In fact, our work shows that genes commonly used in these types of studies vary significantly in both depots of adipose tissue in lean and obese individuals.

The adipose tissue is not considered any more as a mere deposit of fat, but an organ with an essential endocrine role. The molecular mechanisms causing the hyperplasia or hypertrophy of this tissue have not fully identified, thus studies on gene expression can help to better understand the processes leading to obesity.

ACTB, GAPDH and 18S are the most commonly used genes to correct loading in gene expression studies (2, 22, 23). However, the present study shows they are the least stable genes studied, considering both depots, from the 16 ones used in the commercial microfluidic cards, as their CV were above the 5% level. Although there are controversial results regarding the 18S in adipose tissue (8, 24) a drawback to its use as a control gene is the considerable imbalance between mRNA and rRNA fractions (25) with different polymerases involved in their transcription (26). Ribosomal expression is altered in some experimental conditions (27) and under certain biological factors and drugs (28, 29). In addition, 18S shows resistance to degradation as compared to mRNAs (21). As 18S has no introns, an additional error appears if DNAse treatment of the samples fails or is incomplete.

ACTB and GAPDH have been used extensively as single control genes (2). In adipose tissue ACTB is regulated by hypocaloric diets (30) while GAPDH is very sensitive to any perturbation in cellular homeostasis and experimental manipulation (2, 31, 32), as our results demonstrate in differentiation experiments.

RT-PCR, geNORM and Normfinder programs were used to find the most suitable gene of a panel of 16 human genes that are commonly used as endogenous controls both in OM and SC adipose tissues. The most suitable gene was IPO8, which resulted the best choice using the 3 methods. At present, there are some statistical methods like geNORM, NormFinder, or Global Pattern Recognition for the optimization of reference gene selection. These methods have been compared with similar results in some reports (13, 33) but not in others (15).

In this study, we also used our unpublished microarrays data to identify genes with low variation in expression levels in human adipose tissue. FBXL10 was the gene with the highest expression stability in OM and SC human adipose tissue. This gene codes for a nucleolar protein that represses transcription of ribosomal RNA genes (37). In addition to its low variation, FBXL10 has the characteristics of a housekeeping gene as it is an evolutionarily conserved and ubiquitously expressed member of the JHDM and it is essential for the maintenance of cellular function. When FBXL10 and IPO8 in adipose tissue were compared, both genes had a low CV, but IPO8 exhibited a better outcome.

The choice of IPO8 as reference gene in human OM and SC adipose samples was further supported when 18S, FBXL10 and IPO8 were used to normalize common adipocytic genes such as leptin, interleukin 6 and adiponectin. IPO8 belongs to the

importins beta family, nuclear proteins involved in the nucleolar transport. Very recently it has been published that IPO8 is required for cytoplasmic miRNA-guided gene silencing and is involved in loading Ago complexes into a variety of different mRNA targets, affecting the nuclear localization of Ago proteins (34). IPO8 has been proposed previously as a reference gene in the human motor cortex as it showed no differences in a study with different brain regions from chronic alcoholics and control subjects (35) and lung samples (36).

Both IPO8 and FBXL10 performed well when OM and SC primary differentiated preadipocytes were used under different treatments which can mimic some of the changes in the humans under distinct physiological and pharmacological status. Thus, in primary cultures of human OM adipocytes the CV of IPO8 was even better than in OM adipose tissue. Interestingly, FBXL10 showed a slightly better CV than IPO8 in omental differentiated preadipocytes in culture, but not in subcutaneous ones. It is noteworthy that neither IPO8 nor FBXL10 changed during the differentiation process reinforcing their role as control genes in these cells.

Considering that the use of reference genes for gene expression studies is an important topic, and the fact that tissue/protocols or even types of subjects can differ among laboratories, we suggest that this kind of experiments should be performed with more than one reference gene.

In conclusion, based on the evidence provided in the present study, we propose and recommend IPO8 and FBXL10 as appropriate reference genes, which have not been previously identified for this purpose, in expression studies of human adipose tissue and cultured preadipocytes.

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DISCLOSURE

The authors have nothing to disclose.

REFERENCES

1 Butte AJ, Dzau VJ, Glueck SB. Further defining housekeeping, or "maintenance," genes Focus on "A compendium of gene expression in normal human tissues". *Physiol Genomics* 2001;7:95-6.

2 Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. *Biotechniques* 2000;29:332-7.

3 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-93.

4 Kouadjo KE, Nishida Y, Cadrin-Girard JF, Yoshioka M, St-Amand J. Housekeeping and tissue-specific genes in mouse tissues. *BMC Genomics* 2007;8:127.

5 Lee PD, Sladek R, Greenwood CM, Hudson TJ. Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. *Genome Res* 2002;12:292-7.

6 Gabrielsson BG, Olofsson LE, Sjogren A, et al. Evaluation of reference genes for studies of gene expression in human adipose tissue. *Obes Res* 2005;13:649-52.

7 Gorzelniak K, Janke J, Engeli S, Sharma AM. Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Horm Metab Res* 2001;33:625-7.

8 Catalan V, Gomez-Ambrosi J, Rotellar F, et al. Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus. *Horm Metab Res* 2007;39:495-500.

9 Barroso I, Benito B, Garcia-Jimenez C, Hernandez A, Obregon MJ, Santisteban P. Norepinephrine, tri-iodothyronine and insulin upregulate

glyceraldehyde-3-phosphate dehydrogenase mRNA during Brown adipocyte differentiation. *Eur J Endocrinol* 1999;141:169-79.

10 Misra A, Garg A, Abate N, Peshock RM, Stray-Gundersen J, Grundy SM. Relationship of anterior and posterior subcutaneous abdominal fat to insulin sensitivity in nondiabetic men. *Obes Res* 1997;5:93-9.

11 Gesta S, Bluher M, Yamamoto Y, et al. Evidence for a role of developmental genes in the origin of obesity and body fat distribution. *Proc Natl Acad Sci U S A* 2006;103:6676-81.

12 Tran TT, Yamamoto Y, Gesta S, Kahn CR. Beneficial effects of subcutaneous fat transplantation on metabolism. *Cell Metab* 2008;7:410-20.

13 de Brouwer AP, van Bokhoven H, Kremer H. Comparison of 12 reference genes for normalization of gene expression levels in Epstein-Barr virus-transformed lymphoblastoid cell lines and fibroblasts. *Mol Diagn Ther* 2006;10:197-204.

14 Szabo A, Perou CM, Karaca M, et al. Statistical modeling for selecting housekeeper genes. *Genome Biol* 2004;5:R59.

15 Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;64:5245-50.

16 Saviozzi S, Cordero F, Lo Iacono M, Novello S, Scagliotti GV, Calogero RA. Selection of suitable reference genes for accurate normalization of gene expression profile studies in non-small cell lung cancer. *BMC Cancer* 2006;6:200.

17 Van Harmelen V, Rohrig K, Hauner H. Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. *Metabolism* 2004;53:632-7.

18 Samuels HH, Stanley F, Casanova J. Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* 1979;105:80-5.

19 Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 2005;21:2067-75.

20 Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5:R80.

21 Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.

22 Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* 2000;46:69-81.

23 Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002;29:23-39.

24 Jorgensen SM, Kleveland EJ, Grimholt U, Gjoen T. Validation of reference genes for real-time polymerase chain reaction studies in Atlantic salmon. *Mar Biotechnol (NY)* 2006;8:398-408.

25 Solanas M, Moral R, Escrich E. Unsuitability of using ribosomal RNA as loading control for Northern blot analyses related to the imbalance between messenger and ribosomal RNA content in rat mammary tumors. *Anal Biochem* 2001;288:99-102.

26 Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 2004;313:856-62.

27 Barbu V, Dautry F. Northern blot normalization with a 28S rRNA oligonucleotide probe. *Nucleic Acids Res* 1989;17:7115.

28 Spanakis E. Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. *Nucleic Acids Res* 1993;21:3809-19.

29 Johnson ML, Redmer DA, Reynolds LP. Quantification of lane-to-lane loading of poly(A) RNA using a biotinylated oligo(dT) probe and chemiluminescent detection. *Biotechniques* 1995;19:712-5.

30 Gabrielsson BG, Karlsson AC, Lonn M, et al. Molecular characterization of a local sulfonylurea system in human adipose tissue. *Mol Cell Biochem* 2004;258:65-71.

31 Ke LD, Chen Z, Yung WK. A reliability test of standard-based quantitative PCR: exogenous vs endogenous standards. *Mol Cell Probes* 2000;14:127-35.

32 Zhu G, Chang Y, Zuo J, et al. Fudenine, a C-terminal truncated rat homologue of mouse prominin, is blood glucose-regulated and can up-regulate the expression of GAPDH. *Biochem Biophys Res Commun* 2001;281:951-6.

33 Radonic A, Thulke S, Bae HG, Muller MA, Siegert W, Nitsche A. Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections. *Virol J* 2005;2:7.

34 Weinmann L, Hock J, Ivacevic T, et al. Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell* 2009;136:496-507.

35 Johansson S, Fuchs A, Okvist A, et al. Validation of endogenous controls for quantitative gene expression analysis: application on brain cortices of human chronic alcoholics. *Brain Res* 2007;1132:20-8.

36 Nguewa PA, Agorreta J, Blanco D, et al. Identification of Importin 8 (IPO8) as the most accurate reference gene for the clinicopathological analysis of lung specimens. *BMC Mol Biol* 2008;9:103.

37 Frescas D, Guardavaccaro D, Bassermann F, Koyama-Nasu R, Pagano M. JHDM1B/FBXL10 is a nucleolar protein that represses transcription of ribosomal RNA genes. *Nature* 2007;450:309-13.

FIGURES LEGENDS

Figure 1. Reference gene stability measurements. The GENORM and NORMFINDER softwares were used to calculate the most stable genes among the 16 genes included in the Taqman low density human endogenous control panel. RNA samples of omental and subcutaneous adipose tissue from lean and obese patients were used. The most stable genes are those with the lower values of stability.

Figure 2. Effects of normalization by 3 reference genes (18S, IPO8 and FBXL10) on leptin (A, B), adiponectin (C, D) and interleukin 6 (E, F) mRNA expression in omental and subcutaneous adipose tissue of obese (n=33) and lean subjects (n=4). Data are mean ± SEM. * $p< 0.05$

Figure 3. mRNA levels (expressed as mean Ct ± SEM) of FBXL10 (upper panel), IPO8 (middle panel) and GAPDH (lower panel) in omental and subcutaneous human preadipocytes at different days of differentiation (0, 6 and 12 days). *$p< 0.05$ vs its respective 0 day, #$p < 0.05$ vs its respective 6 day. Data are from 3 independent experiments from each depot.

TABLE 1. Ranking of 16 commonly used housekeeping genes in OM and SC adipose tissue samples from obese and lean subjects, based on their CV%.

	OM+SC				SC		OM	
SYMBOL	Mean Ct	SD	CV (%)	Rank	CV(%)	Rank	CV(%)	Rank
HPRT1	30.36	1.37	4.51	1	3.83	1	4.83	6
IPO8	28.74	1.31	4.55	2	4.72	2	4.57	5
HMBS	31.81	1.53	4.80	3	5.25	3	4.38	3
TFRC	28.87	1.39	4.81	4	5.32	4	4.46	4
POLR2A	27.97	1.37	4.90	5	6.13	7	3.71	1
YWHAZ	31.18	1.58	5.07	6	6.09	6	4.24	2
B2M	23.59	1.31	5.55	7	5.39	5	5.71	10
PKG	26.17	1.54	5.88	8	6.34	9	5.66	9
GUSB	29.07	1.72	5.91	9	6.56	10	5.56	8
PPIA	24.85	1.48	5.95	10	6.70	11	5.45	7
TBP	31.02	1.93	6.22	11	6.23	8	6.62	12
UBC	24.71	1.72	6.94	12	8.07	14	5.74	11
GADPH	26.10	1.84	7.06	13	7.59	13	6.90	13
ACTB	23.72	1.93	8.12	14	8.68	15	7.96	14
RPLP0	25.28	2.19	8.65	15	6.88	12	10.03	15
18S	13.42	1.84	13.70	16	15.65	16	11.52	16

OM, omental; SC, subcutaneous; CV, coefficient of variation; HPRT1, Hypoxanthine phosphoribosyl transferase 1; IPO8, importin 8; HMBS, Hidroxymethylbilane synthase; TFRC, Transferrin receptor; POLR2A, RNA Polymerase II; YWHAZ, Tyrosin 3-

monooxygenase/tryptophan-monooxygenase activation protein; B2M, Beta-2-microglobulin; PKG, Phosphoglycerate kinase; GUSB, beta-Glucuronidase; PPIA, Peptidylprolyl isomerase A; TBP, TATA binding protein; UBC, Ubiquitin C; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ACTB, beta-Actin; RPLP0, Ribosomal large P0; 18S, 18S Ribosomal RNA.