Comprehensive Human Adipose Tissue mRNA and MicroRNA Endogenous Control Selection for Quantitative Real-Time-PCR Normalization

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The accurate quantification of cellular and tissue mRNA and microRNA content is reliant upon the selection of stable endogenous control transcripts for normalizing quantitative real-time-PCR (qRT-PCR) data. Using the combination of unbiased and informed approaches and a wide range of human adipose tissues and cells, we sought to identify invariant control transcripts for mRNA and microRNA. A total of 26 mRNA transcript candidates were selected from the literature. MicroRNA candidates were selected from a microRNA-microarray (Agilent, *n* = 22 tissues), and together with candidates from the literature resulted in 14 different microRNAs. The variability of these mRNA and microRNA transcripts were then tested in a large (*n* = 180) collection of a variety of human adipose tissues and cell samples. Phosphoglycerate kinase-1 (*PGK1*) and peptidylprolyl isomerase A (*PPIA*) were identified as the most stable mRNAs across all tissues and panels. MiR-103 was overall the most stable microRNA transcript across all biological backgrounds. Several proposed and commonly used normalization transcripts were found to be highly variable. We then tested the effect on expression of two established adipocyte-related transcripts (fatty acid binding protein 4 (*FABP4*) and microRNA-145 (*miR-145*)), either normalized to the optimal or a commonly used controls transcript. This test clearly indicated that spurious results could arise from using less stable control transcripts for mRNA and microRNA qRT-PCR.

Obesity (2010) doi:10.1038/oby.2010.257

Reliable quantification of coding messenger RNAs (mRNA), and more recently, small non-coding regulatory microRNA (miRNA) is key to the understanding of cell and tissue function. Normally, this relies on using quantitative real-time PCR amplification (qRT-PCR) of a candidate gene transcript normalized to invariant endogenous control transcripts (1), sometimes referred to as a "housekeeping gene". However, such control transcripts may differ between tissues and cell types, or experimental situations, and there is need to define each system in advance (2,3). This requirement is often overlooked.

Adipose tissue is composed of different cell types (adipocytes of various size and stages of differentiation, vascular/endothelial cells, inflammatory cells) the proportions of which may differ between fat depots. Among the adipocytes there appears to be distinct developmental patterns between tissue depots (4) as well as distinct functional properties (5). Transcriptional profiles within each depot can also be influenced by gender, metabolic state, or even the method of biopsy-taking (6). Therefore, the selection of endogenous control genes for both mRNA and miRNA needs to be carefully considered to not only reflect stable transcripts between the different conditions under investigation but also between differing proportions of cell types found with and between depots. Only a few studies have considered this methodological issue regarding mRNA, and there is no data on a formal evaluation of optimal miRNA control transcripts in adipose tissue. For mRNA, the results are unfortunately conflicting. The three studies exploring the optimal choice of mRNA control transcripts have taken different approaches to identify and evaluate control transcripts. Both Gabrielsson *et al.* (7) and Hurtado Del Pozo *et al.* (8) took an unbiased approach (microarray) to identify novel candidates but differed in their findings, presumably due to differing samples sizes and the origin of tissues studied. However, both report large ribosomal protein PO (*RPLPO*) among the most stable and *18s* as variable. Catalán *et al.* (9), suggested that the *18s* ribosomal RNA as the most suitable mRNA control transcript, but the authors arrived at this conclusion without using an overall variability algorithm.

Here, we make use of a large and diverse collection of human adipose tissues together with human adipocytes and make a

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Received 23 June 2010; accepted 21 September 2010; advance online publication 14 October 2010. doi:10.1038/oby.2010.257

Table 1 Details of the adipose tissue and adipocyte samples used for mRNA and microRNA qRT-PCR reactions

Panel	Sample	Groups	Biopsy method	Extraction method
Oxford Biobank (10)	80	Supcomparisons: lean/obese, gender, tissue site	Needle biopsy	MirVana column
Rosiglitazone study (11)	35	Day 0 (n = 12), day 1 (n = 12), day 14 (n = 11)	Needle biopsy	MirVana column
Adipocyte cell culture (13)	33	3 Palmitate concentrations (n = 11 each group)	—	Tri-reagent total RNA
Omental vs. sub-cut	18	Omental (n = 9), sub-cut (n = 9) paired samples	Surgical	Tri-reagent total RNA
Lipoma vs. sub-cut	14	Lipoma (n = 7), sub-cut (n = 7) paired samples	Surgical	MirVana column

qRT-PCR, quantitative real-time PCR.

formal evaluation of mRNA and miRNA candidates. We use unbiased array methods to identify miRNA candidates but we also consider reported transcripts (mRNA and miRNA) and all possible candidates are evaluated together. The objective is to generate an invariant and robust set of endogenous control transcripts for mRNA and miRNA that can be used in human applications comparing different adipose tissues and adipocytes.

METHODS AND PROCEDURES

Tissue panels

To assess the transcript variability in a wide panel of samples we put together a set of 180 samples selected to encompass a broad range of adipose tissue origins and experimental conditions (Table 1). Human fat depots were represented by omental, abdominal subcutaneous, and gluteal tissue. The effect of obesity was considered: lean (BMI <25 kg/m^2) vs. obese (BMI >30 kg/m^2), with equal gender representation. Growth pattern and stimulation of adipogenesis was represented by including surgically removed lipomas vs. normal adjacent adipose tissue and samples taken before and after 14 days of systemic rosiglitazone treatment (4 mg BD) (11). Methodological issues like biopsy retrieval method (needle vs. surgical) and RNA extraction method (Tri-reagent vs. column) were also included. Finally we prepared differentiated adipocytes from preadipocytes isolated from the stromovascular fraction of subcutaneous biopsies (Table 1). Needle biopsy samples were taken under local anesthesia using a 12-gauge needle and immediately frozen in liquid nitrogen. Surgical biopsies were taken during elective surgery and immediately frozen. Preadipocytes were differentiated and exposed to either 0 µm, 50 µm, or 200 µm palmitate (13). All biopsies and cells were homogenized in Tri-reagent (cat. no. AM9738, Ambion, Austin, TX) and RNA was extracted with either a standard Tri-reagent protocol or using Ambion MirVana columns (cat. no. AM1561, Ambion).

miRNA microarray study

Of the 80 human subcutaneous adipose tissue biopsy samples selected from the Oxford Biobank (10) for this study (see Table 1) a subset of 22 were selected for a miRNA microarray endogenous control pilot study. This subset was evenly split between lean and obese subjects and comprised 14 abdominal tissues of which eight had paired gluteal samples. MiRNA arrays were run with the Agilent Human miRNA Microarray Kit V2 (Agilent Technologies, Santa Clara, CA) following manufacturers standard protocol with 400 ng total RNA and data was log transformed and Quantile normalized in the R statistical environment.

Transcript selection, qRT-PCR, and analysis

To select miRNA targets from the array study we excluded those falling below or near background expression (excluding 543 of the 723 miRNAs on the Agilent chip). Next, if the coefficient of variation across the 22 samples was >3% and/or if the transcript was not found within the Applied Biosystems Megaplex RT primer pool A (cat. no. 4399966; Applied Biosystems, Carlsbad, CA) they were excluded (n = 132). From the remaining 48, nine were selected (Figure 1b) for a variety of reasons: previously reported in other tissues, high expression, access to a pilot microarray profile in the rosiglitazone study (see ref. 11). We then considered miRNAs (*miR-25* and *let-7a*) and other small RNA candidates from the literature (*RNU6B*, *RNU44*, and *RNU44*) if they also met the above criteria within the microarray dataset (12) and (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_044972.pdf). The mRNA endogenous control candidates were selected from a combination of: commonly used ubiquitous endogenous controls, those found within the Applied Biosystems endogenous control panel (cat. no. 4367563) and from candidates proposed in the literature as stable in adipose tissue (7–9). The final set of mRNA and miRNA transcripts selected is found in Figure 1.

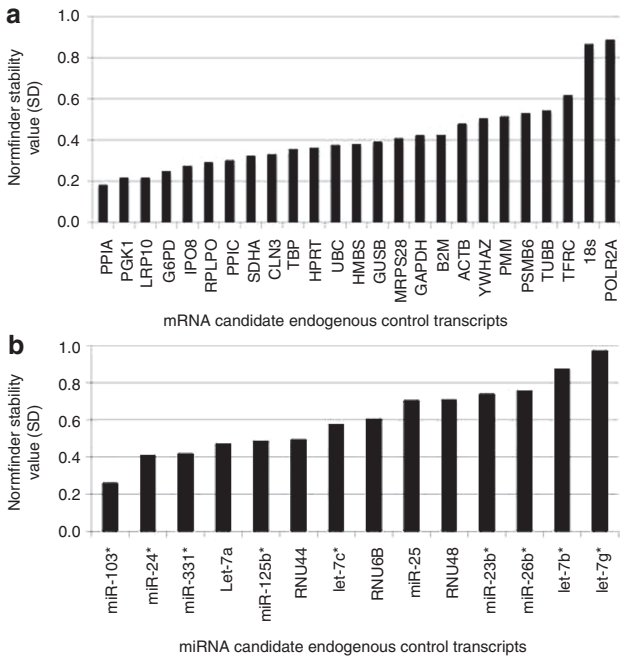

Figure 1 The Normfinder stability values for mRNA endogenous control transcript candidates (**a**) and microRNA endogenous control transcripts (**b**) for the combined expression data from all adipose tissue panels ("All Adipose Tissue Samples" column in **Supplementary Table S3b,d** online), the most stable transcripts having the smallest stability values. The Normfinder data for all the comparisons made, including those presented here can be found in **Supplementary Table S3b,d** online. *Denotes that the microRNA was detected in the microarray profile study.

The test of transcript variability between the 180 samples was made using qRT-PCR reactions for both mRNA and miRNA. For mRNA first strand cDNA synthesis 500 ng of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat. no. 4368813). MiRNA was reverse transcribed using the Megaplex RT Human Primer Pool A with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, cat. nos. 4399966 and

4366597, respectively) using 400 ng total RNA. qRT-PCR reactions were carried out in triplicate using a 1/50 dilution of each cDNA with Applied Biosystems assays (**Supplementary Tables S1** and **S2** online) and TaqMan Universal PCR Master Mix (Applied Biosystems, cat. no. 4326614). Reactions were run in 384 well plates in a 6 µl final volume and run on an Applied Biosystems 7900HT machine. Expression values were calculated by the ΔC_t transformation method (14):

$$\Delta C_t = \text{Efficiency}^{\text{calibrator } C_t - \text{sample } C_t},$$

using the 1/100 dilution from a standard curve generated from a pool of all cDNAs as the calibrator for all samples.

Determination of transcript expression variability

To select suitable endogenous controls between groups of samples one must consider the circular argument of how to select stable normalizing transcripts without first normalizing the candidates. To address this, two established methods that get around the circular argument by looking at data variability alone were compared to quantify transcript variability. GeNorm (2) uses a pairwise comparison in a reiterative process to identify the most stable pair of transcripts, and Normfinder (3) generates model-based estimates of expression variability based on observed standard deviations. Both algorithms were implemented in the GENEX software (http://www.multid.se/).

To model the impact of choosing different mRNA and miRNA normalizations we selected fatty acid binding protein 4 (*FABP4*) (15) and microRNA-145 (*miR-145*) (16) because of their reported regulation in adipose tissue and normalized the qRT-PCR results to either the most stable transcripts (as defined by Normfinder) or commonly used, but variable transcripts, for both the mRNA and miRNA data. This test was performed in the $n = 80$ panel from the Oxford Biobank (**Table 1**).

RESULTS

Candidate transcript selection

A total of 26 mRNA and 14 small non-coding RNA endogenous control candidates were selected (**Figure 1**). The small RNAs included 11 miRNAs together with the commonly used snoRNAs, RNU6b, RNU44, and RNU48. The details of these transcripts as well as the qRT-PCR performance can be found in **Supplementary Tables S1** and **S2** online. Expression levels of the different transcripts can be approximated from the "Average C_ts" column (high C_t equals a low expression and *vice versa*).

Transcript variability analysis

There was a high degree of concordance between GeNorm and Normfinder in the mRNA data ($r = 0.97$), but there was some discrepancy at either extreme end of the distribution. As Normfinder considers all data together in a single model rather than excluding the most variable transcripts in each iteration step, it shows a slightly greater and more informative spread of data. The summary data of the Normfinder results is presented in **Figure 1** and the equivalent GeNorm figure can be found in **Supplementary Figure S1** online. A breakdown of all variability analysis results is detailed in **Supplementary Table S3** online.

mRNA endogenous control selection

Overall, the two most stable mRNA transcripts were peptidylprolyl isomerase A (*PPIA*) then phosphoglycerate kinase-1 (*PGK1*; **Figure 1a**), being the top two for the larger Oxford Biobank panel and all combined dataset comparisons (**Supplementary Table S3** online). The only tissue/cell type that differed was the lipoma samples in which *PPIA* and *PGK1* were ranked 5 and 7. However, when the average fold change across the remaining 24 candidate genes was normalized for either the mean of *PPIA* and *PGK1* or the mean of *UBC* and *CLN3*, this seemed to have no effect (0.986 ± 0.073 normalized to *CLN3-UBC* and 0.984 ± 0.076 normalized to *PPIA-PGK1*). The commonly used *18s* RNA was by far the most variable in all panels except the primary culture cells where it was found to be the most stable with *PGK1* and *PPIA* ranking second and

Table 2 The consequences of normalization factor on the variable transcripts *FABP4* and *miR-145*

	FABP4 to *PPIA*[a,d]	*FABP4* to *PPIA* and *PGK1*[b,d]	*FABP4* to *ACTB*[c,d]	*FABP4* to GAPDH[c,d]	*FABP4* to *18s*[c,d]
Abdominal vs. gluteal ($n = 40/40$)	1.07 ($P = 0.3$)	1.02 ($P = 0.6$)	1.06 ($P = 0.3$)	0.98 ($P = 0.6$)	1.36 ($P < 0.001$)
Male vs. female ($n = 40/40$)	1.1 ($P = 0.2$)	1.08 ($P = 0.4$)	1.33 ($P = 0.04$)	1.16 ($P = 0.07$)	1.1 ($P = 0.6$)
Lean vs. obese ($n = 40/40$)	0.77 ($P < 0.001$)	0.79 ($P < 0.001$)	0.7 ($P < 0.001$)	0.73 ($P < 0.001$)	0.98 ($P = 0.7$)
	MiR-145 to *miR-103*[a,d]	*MiR-145* to *miR-24*, *103* and *331*[b,d]	*MiR-145* to *RNU6B*[c,d]	*MiR-145* to *RNU48*[c,d]	*MiR-145* to *RNU44*[c,d]
Abdominal vs. gluteal ($n = 40/40$)	0.90 ($P = 0.31$)	0.92 ($P = 0.59$)	0.83 ($P < 0.01$)	0.91 ($P = 0.59$)	0.92 ($P = 0.36$)
Male vs. female ($n = 40/40$)	1.26 ($P < 0.01$)	1.25 ($P = 0.015$)	1.04 ($P = 0.68$)	1.23 ($P = 0.10$)	1.08 ($P = 0.29$)
Lean vs. obese ($n = 40/40$)	0.69 ($P < 0.001$)	0.66 ($P < 0.001$)	0.66 ($P < 0.001$)	0.59 ($P < 0.001$)	0.65 ($P < 0.001$)

ACTB, actin, β; *FABP4*, fatty acid binding protein 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *miR-145*, microRNA-145; *PGK1*, phosphoglycerate kinase-1; *PPIA*, peptidylprolyl isomerase A.

[a]The variable mRNA transcript *FABP4* or miRNA transcript *miR-145* normalized to the most stable endogenous control transcript (as defined by Normfinder); [b]the variable mRNA transcript *FABP4* or miRNA transcript *miR-145* normalized to the most stable set of endogenous control transcripts (as defined by Normfinder); [c]the variable mRNA transcript *FABP4* or miRNA transcript *miR-145* normalized to the less stable but commonly used endogenous control transcripts; [d]analysis was performed on the Oxford Biobank panel ($n = 80$, **Table 1**).

Expression values are fold changes of the normalized expression values for each comparison (*P* value).

sixth, respectively. The transcripts *LRP10*, *IPO8*, and *CLN3*, previously identified as candidate endogenous controls in adipose tissue (7,8) were ranked third, fifth, and ninth in the overall analysis.

miRNA endogenous control selection

The concordance between GeNorm and Normfinder for the miRNA data was good, but lower than for mRNA ($r = 0.77$). However, among the 14 small RNAs selected, *miR-103* was the overall single most stable transcript (**Figure 1b**) being in the top five for all tissue/cell comparisons and in the top two for most of them. This was followed by *miR-24* and *miR-331*. Members of the *Let7* family as well as *miR-125b* were also ranked highly in some panels (**Supplementary Table S3** online).

The consequences of endogenous control choice

Modeling the effect of using the most stable endogenous control transcript vs. less stable options is shown in **Table 2**. Normalization of *FABP4* to *ACTB*, *GAPDH* or *18s* resulted in significant shifts in the observed transcript abundance, whereas comparing the use of *PPIA* alone or the average of *PPIA* and *PGK1* made little difference. Normalizing to *18s* caused a gain of significance between fat depots and a loss of significance between BMI groups. Normalization of *FABP4* to *ACTB* or *GAPDH* resulted in a gain of significance for gender. Normalizing *miR-145* to the commonly used snoRNAs *RNU6b*, *RNU48*, or *RNU44* instead of *miR-103* also resulted in presumably spurious disappearance and emergence of positive or negative effects for *miR-145* when comparing gender or fat depot.

DISCUSSION

We have used the combination of unbiased and informed approaches to select stable endogenous control mRNA and miRNA transcripts and tested the overall variation in an exceptionally large and varied collection of adipose tissues and adipocytes. For miRNA, this has not been done before in adipose tissue and the identification of *miR-103* through an unbiased approach is therefore helpful. For mRNA, the results agrees with the general view that no single endogenous control is likely to remain stable across different biological backgrounds (17), however, the two mRNA (*PPIA* and *PGK1*) were consistently found as among the most stable transcripts across fat depot origin, gender, BMI, and isolated adipocytes. It has been proposed that normalization to more than one stable transcript creates robust data sets (2,17). Although our modeling data does not identify such an advantage for the combination of *PPIA* and *PGK1* compared with *PPIA* alone, the safe recommendation would be that both these transcripts should be part of such a pool if that approach is taken. In addition, with the specific example of adipocyte cell culture, *18s* could also be considered a suitable candidate. When the same approach was taken for miRNA there was no obvious difference using the combination of *miR-103* and two additional stable transcripts compared with *miR-103* alone. Although, due to its relatively low expression (high CT in **Supplementary Table S2** online) combination with the higher expressed miR-24 and/or miR-331 should be considered.

It was recently reported that *miR-103* is downregulated in *ob/ob* mice adipose tissue and upregulated during murine adipogenesis (18). Whether these observations depended on the miRNA normalization (*snoRNA202*) or imply a species difference remains unknown.

Modeling the relative *FABP4* and *miR-145* transcript abundance depending on the choice of normalization strategy gave disturbing results as it was clear that choosing less stable, but often used, normalization transcripts both removed significant differences in relative *miR-145* and *FABP4* abundance as well as generated, presumably spurious, significant differences. This underlines the issue of appropriately choosing stable transcript for normalization of miRNA and mRNA abundance in adipose tissue.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

ACKNOWLEDGMENT

This study was partially funded by EU FP7 LipidomicNet (#202272). A.L.G. is a MRC New Investigator (81696).

DISCLOSURE

The authors declared no conflict of interest.

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